Propofol Neurotoxicity Is Mediated by p75 Neurotrophin Receptor Activation

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ABSTRACT

Background: Propofol exposure to neurons during synaptogenesis results in apoptosis, leading to cognitive dysfunction in adulthood. Previous work from our laboratory showed that isoflurane neurotoxicity occurs through p75 neurotrophin receptor ($p75^{NTR}$) and subsequent cytoskeleton depolymerization. Given that isoflurane and propofol both suppress neuronal activity, we hypothesized that propofol also induces apoptosis in developing neurons through $p75^{NTR}$.

Methods: Days *in vitro* 5–7 neurons were exposed to propofol (3 μM) for 6 h and apoptosis was assessed by cleaved caspase-3 (Cl-Csp3) immunoblot and immunofluorescence microscopy. Primary neurons from $p75^{NTR-/-}$ mice or wild-type neurons were treated with propofol, with or without pretreatment with TAT-Pep5 (10 μM, 15 min), a specific $p75^{NTR}$ inhibitor. $P75^{NTR-/-}$ neurons were transfected for 72 h with a lentiviral vector containing the synapsin-driven $p75^{NTR}$ gene (Syn-$p75^{NTR}$) or control vector (Syn–green fluorescent protein) before propofol. To confirm our *in vitro* findings, wild-type mice and $p75^{NTR-/-}$ mice (PND5) were pretreated with either TAT-Pep5 or TAT-ctrl followed by propofol for 6 h.

Results: Neurons exposed to propofol showed a significant increase in Cl-Csp3, an effect attenuated by TAT-Pep5 and hydroxyfasudil. Apoptosis was significantly attenuated in $p75^{NTR-/-}$ neurons. In $p75^{NTR-/-}$ neurons transfected with Syn-$p75^{NTR}$, propofol significantly increased Cl-Csp3 in comparison with Syn–green fluorescent protein–transfected $p75^{NTR-/-}$ neurons. Wild-type mice exposed to propofol exhibited increased Cl-Csp3 in the hippocampus, an effect attenuated by TAT-Pep5. By contrast, propofol did not induce apoptosis in $p75^{NTR-/-}$ mice.

Conclusion: These results demonstrate that propofol induces apoptosis in developing neurons *in vivo* and *in vitro* and implicate a role for $p75^{NTR}$ and the downstream effector RhoA kinase.

What We Already Know about This Topic

- Isoflurane leads to reductions in the activity-dependent processing of brain-derived neurotrophic factor (proBDNF) to the survival-promoting mature form BDNF in immature brain
- The authors hypothesized that this mechanism applies to other general anesthetics that reduce neuronal activity

What This Article Tells Us That Is New

- Propofol induced apoptosis in immature mouse neurons both *in vitro* and *in vivo* through alterations in BDNF signaling
- This provides a potential therapeutic target for prevention of developmental neurotoxicity

DURING synaptogenesis, on postnatal day 5–7, anesthetics lead to neurodegeneration.[1–3] Many anesthetics cause neurotoxicity, which include midazolam and nitrous oxide, isoflurane, sevoflurane, propofol, thiopental, and ketamine.[4–11] In addition, isoflurane does not induce neuronal apoptosis on postnatal day 15, but does alter synaptic plasticity; these changes persist for at least 4 weeks postexposure.[12] Of significant concern is that neonatal exposure to anesthetics results in neurocognitive and behavioral abnormalities during adolescence and adulthood.[2,3,13,14] Although the mechanism by which this toxicity occurs is not

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clear, γ-aminobutyric acid ($GABA_A$) agonism and N-methyl-D-aspartate receptor antagonism play a central role.

Recently we demonstrated that proBDNF-$p75^{NTR}$ signaling mediates isoflurane neurotoxicity in developing neurons *in vivo* and *in vitro*. Brain-derived neurotrophic factor (BDNF) is important to both prosurvival and proapoptotic signaling pathways. BDNF is stored as a proneurotrophin (proBDNF) within synaptic vesicles and is proteolytically cleaved to mature BDNF (mBDNF) in the synaptic cleft by plasmin, a protease activated by tissue plasminogen activator (tPA).[15–18] Prosurvival signaling is triggered by mBDNF agonism of tropomyosin receptor kinase B, which leads to neurite outgrowth and synapse maturation and stabilization.[15,17,19] In contrast, noncleaved proBDNF binds to the p75 neurotrophin receptor ($p75^{NTR}$) and activates RhoA, a small GTPase that regulates actin cytoskeleton polymerization resulting in inhibition of axonal elongation, growth cone collapse, and apoptosis.[16,20–22] Neuronal stimulation is important in this process because proBDNF is constitutively secreted while tPA release is regulated; without neuronal depolarization, conversion of proBDNF to mBDNF may be blunted, which then leads to preferential signaling through $p75^{NTR}$. Upon neuronal excitation, tPA release results in plasmin production and subsequent generation of mBDNF-tropomyosin receptor kinase B activation, leading to neuronal survival, neurite sprouting, and synaptogenesis.[15–17,22]

Head *et al.* showed that isoflurane induces apoptosis in DIV5 neurons, a finding that did not occur in DIV14 or DIV21 neurons.[22] This effect was attenuated by TAT-Pep5, a $p75^{NTR}$ intracellular domain inhibitor, suggesting a role for $p75^{NTR}$ in isoflurane-mediated neurotoxicity. Apoptosis was also attenuated by pretreatment with tPA or plasmin, suggesting that isoflurane-mediated neurotoxicity is caused in part by suppressing tPA release and preventing proBDNF conversion to mBDNF, thus leading to preferential $p75^{NTR}$ signaling, RhoA activation, actin cytoskeleton depolymerization, and subsequent neuronal apoptosis. Lemkuil *et al.* extended these findings by showing that isoflurane exposure increases $p75^{NTR}$-RhoA activation in parallel with apoptosis, and that inhibition of RhoA activation or cytoskeleton stabilization attenuates the isoflurane-mediated neurotoxic effects.[23]

Although these data support the premise that proBDNF-$p75^{NTR}$ signaling plays a significant role in neonatal neurotoxicity, a number of questions remain. If this mechanism is important to anesthetic neurotoxicity, then it should also be involved in toxicity mediated by other anesthetics that activate $GABA_A$ receptor (*e.g.*, propofol). In the studies of Head *et al.* and Lemkuil *et al.*, $p75^{NTR}$ inhibition was achieved pharmacologically. In addition to concerns about nonspecific effects of pharmacologic agents, complete suppression of RhoA activation was not achieved; residual RhoA activity may have influenced the results.[24] To address these concerns, we investigated the role of $p75^{NTR}$-RhoA- RhoA kinase (ROCK) pathway in propofol-induced neurotoxicity.

Materials and Methods

Preparation of Neuronal Cell Cultures

All studies performed on animals were approved by Veteran Affairs San Diego Institutional Animal Care and Use Committee (San Diego, California) and conform to the guidelines of Public Health Service Policy on Human Care and Use of Laboratory Animals.

Neonatal mouse neurons (BALB/c; The Jackson Laboratory, Bar Harbor, ME) were isolated using a papain dissociation kit (Worthington Biochemical, Lakewood, NJ) as previously described.[22] $p75^{NTR}$ knockout BALBc mice were provided by Don Pizzo, Ph.D. (Project Scientist, University of California, San Diego, Department of Pathology), at the Veterans Affairs San Diego Healthcare System (originally generated by Dr. Kuo-Fen Lee[25]). Neurons were isolated from 1- or 2-day-old pups (postnatal day 1 or 2) and grown in culture for 4 to 7 days *in vitro*. Neurons were cultured in Neuobasal A media supplemented with B27 (2%), 250 mM GLUTMax1, and penicillin/streptomycin (1%). Neurons were cultured on poly-D-lysine/laminin (2 g/cm^2) coated plates or coverslips at 37°C in 5% CO_2 for 4–7 days before experiments. Lentiviral (LV) vectors driven by neuronal specific synapsin promoters expressing $p75^{NTR}$ or green fluorescent protein (GFP) (LV-syn-$p75^{NTR}$ and LV-syn-GFP, respectively) were generated by and obtained from Atsushi Miyanohara, Ph.D. (Assistant Professor, Gene Therapy Program, University of California, San Diego, La Jolla, California), at the University of California, San Diego Viral Vector Core. Cleaved-caspase 3 (Cl-Csp3) (Cell Signaling, Danvers, MA) and drebrin (Abcam, Cambridge, MA) were used to detect apoptosis and the F-actin cytoskeleton, respectively, *via* immunoblot or immunofluorescence deconvolution or confocal microscopy. Cl-Csp3 and drebrin were normalized to the nuclear stain 4′,6-diamidino-2-phenylindole (Molecular Probes/Invitrogen, Carlsbad, CA). Cl-Csp3 immunoblots were quantified by densitometry and normalized to total caspase-3 (T-Csp3). $p75^{NTR}$ antibody was obtained from Abcam. The cell-permeable peptide, TAT-Pep5 [H-YGRKKRRQRRR-CFFRGGFFNHNPRYC-OH], which blocks the intracellular association of $p75^{NTR}$ with RhoGDI, thus blocking its ability to activate RhoA and hydroxyfasudil (HA 1100 hydrochloride), were purchased from CalBiochem (Gibbstown, NJ) and Tocris (Ellsville MO), respectively.

Anesthetic Neurotoxicity Model

In vitro, primary neuronal cultures were placed within an incubator and exposed to propofol 3 μM for 6 h in a gas mixture of 5% CO_2, 21% O_2, balance nitrogen at a flow rate of 2 l/min. The temperature in the incubator was maintained at 37°C. Neurons were harvested for analysis 2 h postexposure. *In vivo*, neonatal mice on postnatal day 5 were given a single intraperitoneal injection of propofol (100 mg/kg). Mice were sacrificed 6 h postexposure and brains were prepared by perfusion fixation for immunofluorescence confocal microscopy. Mice were administered TAT-Pep5 intraperitoneal (10 μM) 15 min before propofol injections. All

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mice were kept in room air and on a warming pad maintained at 37°C.

Lentiviral Vector Transfection

Primary neuronal cultures were transfected with a lentiviral vector that expresses p75NTR driven by a neuronal specific synapsin-promoter (LV-syn-p75NTR) for 72 h, and then exposed to propofol. A lentiviral vector that expresses GFP driven by a neuronal specific synapsin-promoter (LV-syn-GFP) served as control.

Protein Extraction and Western Blot Analysis

Proteins in cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% acrylamide gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroelution. Membranes were blocked in 20 mM phosphate-buffered saline Tween (1%) containing 4% bovine serum albumin and incubated with primary antibody overnight at 4°C as previously described.[22,23] Primary antibodies were visualized using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech, Santa Cruz, CA) and chemo luminescent reagent (Amersham Pharmacia Biotech, Piscataway, NJ). All displayed bands are expected to migrate to the appropriate size and were determined by comparison with molecular weight standards. We performed an oversaturation analysis with the UVP Imaging software. Red pixilation is assigned to the bands as an indicator of oversaturation. Image J (National Institutes of Health, Bethesda, MD) was used for densitometric analysis of immunoblots with normalization of cleaved caspase-3 to total caspase-3.

Immunofluorescence Confocal Microscopy

Neurons were prepared for immunofluorescence microscopy as previously described.[22,23] Primary neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, incubated with 100 mM glycine (pH 7.4) for 10 min to quench aldehyde groups, permeabilized in buffered Triton X-100 (0.1%) for 10 min, blocked with 1% bovine serum albumin/PBS/Tween (0.05%) for 20 min, and then incubated with primary antibodies in 1% bovine serum albumin/PBS/Tween (0.05%) for 24–48 h at 4°C. Excess antibody was removed by washing with PBS/Tween (0.1%) for 15 min followed by incubation with fluorescein isothiocyanate or Alexa-conjugated secondary antibody (1:250) for 1 h. To remove excess secondary antibody, tissue or cells were washed six times at 5-min intervals with PBS/Tween (0.1%) and incubated for 20 min with the nuclear stain 4′,6-diamidino-2-phenylindole (1:5,000) diluted in phosphate-buffered saline. Cells were washed for 10 min with phosphate-buffered saline and mounted in gelvatol for microscopic imaging. Confocal images were captured with an Olympus confocal microscope system (Applied Precision, Inc., Issaquah, WA.) that included a Photometrics CCD (Photometrics, Tucson, AZ) mounted on a Nikon TE-200 (Nikon, Melville, NY) inverted epi-fluorescence microscope. Between 30

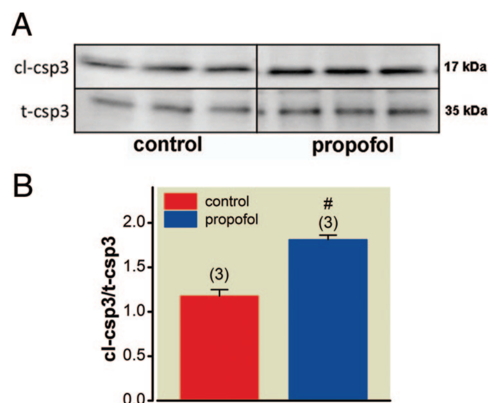

Fig. 1. Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Neurons were then exposed to propofol 3.0 μM for 6 h in 5% CO_2 in air. Apoptosis was evaluated after propofol exposure by cleaved caspase-3 immunoblot. (*A*) Immunoblot analysis shows an increase in apoptosis marker cleaved caspase-3, with propofol exposure. (*B*) Quantitation of the data are represented in the graph (n = 3; # P = 0.002). Sample size is indicated above the error bars ± SEM. These data demonstrate that, *in vitro*, primary neurons from neonatal rodents exposed to propofol exhibit increased apoptosis. Cl-Csp3 = cleaved caspase-3; T-Csp3 = total caspase-3.

and 80 optical sections spaced by approximately 0.1–0.3 μm were captured. Exposure times were set such that the camera response was in the linear range for each fluorophore. Maximal projection volume views or single optical sections were visualized. Pixels were assessed quantitatively by CoLocalizer Pro 1.0 software (Colocalization Research Software, Japan and Switzerland). Statistical analysis was performed using Prism 4 (GraphPad Software, La Jolla, CA).

Apoptosis Quantification

The cleaved caspase-3 pixels (red, Alexa 594) were normalized to nuclear stained pixels (blue, 405).[22,23] Cleaved caspase-3 is an executioner and marker of apoptosis. Ten visual fields at 40× magnification were counted per experimental condition.

Cytoskeletal Depolymerization Quantification

The drebrin pixels (green, Alexa 488) were normalized to nuclear stained pixels (blue, 405).[22,23] Drebrin is a filamentous F-actin binding protein that stabilizes the actin cytoskeleton within neuritic processes. Pixel values were obtained after subtracting background through normalized threshold values in Colocalizer Pro as previously described.[26,27] A reduction in neuritic processses is indicated by decreased drebrin protein expression. Sample size (n) equals number of neurons counted per experimental condition.

Statistical Analysis

All parametric data were analyzed by either two-tailed unpaired *t* tests (fig. 1–5) or by one-way ANOVA with Bonferroni correction (fig. 6). Significance was set at $P < 0.05$.

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Statistical analysis was performed using Prism 4 (GraphPad Software). Sample size (n) represents the amount of times the experiments were repeated on separate neuronal cell culture preparations derived from 12–20 postnatal day 1–3 pups.

Results

Propofol Exposure Increases Apoptosis in Primary Mouse Neurons (Days *In Vitro* 5–7)

Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Propofol (3 μM, 6 h) exposure resulted in a significantly increased (n = 3; $P = 0.002$) expression of Cl-Csp3 compared with control (fig. 1).

Propofol Exposure Decreases Neuritic Processes in Primary Mouse Neurons (Days *In Vitro* 5–7)

Based on our previous data on isoflurane-mediated neurotoxicity, a key mechanistic tenet of injury is preferential activation of $p75^{NTR}$ signaling pathway leading to actin cytoskeleton destabilization and subsequent apoptosis.[22,23] Because we hypothesize that propofol mediates neuronal apoptosis through a similar mechanism to that of isoflurane, we investigated the effects of propofol exposure on formation of neuritic processes as measured by drebrin, a neuronal F-actin binding protein and marker of dendritic filopodial spines.[22,23] Primary neurons were isolated from neonatal rodent pup brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Propofol (3 μM, 6 h) exposure resulted in a significantly decreased (n = 5; $P = 0.008$) expression of drebrin compared with control (fig. 2).

TAT-Pep5 Attenuates Propofol-induced Cleaved Caspase-3 Activation in Primary Mouse Neurons (Days *In Vitro* 5–7)

Previous work from our laboratory has demonstrated that isoflurane-mediated neurotoxicity in developing primary neurons (days *in vitro* 5–7) is mediated through neuronal suppression and subsequent proBDNF activation of $p75^{NTR}$.[22,23] Moreover, using TAT-Pep5, we showed that signaling from $p75^{NTR}$ to ρ was mediating by isoflurane neurotoxicity.[23] We tested whether propofol exposure to primary neurons (days *in vitro* 5–7) also causes cellular injury in a pattern similar to isoflurane (*i.e.*, propofol induces neuronal apoptosis through $p75^{NTR}$ activation). TAT-Pep5 (10 μM, 15 min) treatment of primary mouse neurons (days *in vitro* 5–7) before propofol exposure (3 μM, 6 h) significantly attenuated Cl-Csp3 expression (n = 3; $P = 0.046$) compared with propofol exposure without TAT-Pep5 pretreatment (fig. 3).

Hydroxyfasudil Attenuates Propofol-induced Cleaved Caspase-3 Activation in Primary Mouse Neurons (Days *In Vitro* 5–7)

RhoA is known to regulate actin cytoskeleton dynamics in neurons and cause growth cone collapse, leading to apoptosis.[28–30] RhoA is activated by $p75^{NTR}$ and mediates its effects through downstream activation of ROCK.[31] Because ROCK is indirectly activated by $p75^{NTR}$ and inhibition of $p75^{NTR}$ with TAT-Pep5 attenuates propofol-mediated apoptosis in developing neurons (days *in vitro* 5–7), we hypothesized that inhibition of ROCK would attenuate propofol-mediated apoptosis. Primary mouse neurons (days *in vitro* 5–7) were pretreated with a ROCK inhibitor, hydroxyfasudil (10 μM, 15 min), before propofol exposure (3 μM, 6 h); hydroxyfasudil significantly attenuated Cl-Csp3 activation (n = 3; $P = 0.007$) compared with propofol exposure (with hydroxyfasudil vehicle) in the absence of hydroxyfasudil pretreatment (fig. 4).

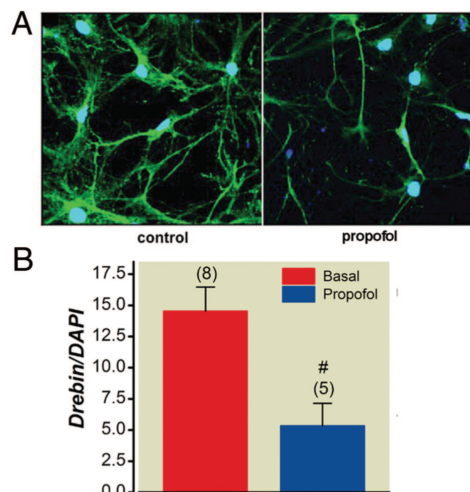

Fig. 2. Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. On day *in vitro* 7 neurons were exposed to propofol 3.0 μM for 6 h in 5% CO_2 in air. Neuritic processes were evaluated after propofol exposure by drebrin immunofluorescence microscopy. Nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole). (*A*) Immunofluorescence microscopy shows a decrease in drebrin in neurons exposed to propofol *versus* control. (*B*) Quantitation of the data are represented in the graph (n = 5; # $P = 0.008$). Sample size is indicated above the error bars ± SEM. These data demonstrate that, *in vitro*, primary neurons from neonatal rodents exposed to propofol exhibit a reduction in neuritic processes. DAPI = 4′,6-diamidino-2-phenylindole.

Primary Neurons Transfected with a Lentiviral Vector that Expresses $p75^{NTR}$ Driven by a Neuronal-specific Synapsin Promoter (LV-syn-$p75^{NTR}$) Increases $p75^{NTR}$ Expression

Because our results from figures 3 and 4 demonstrate that inhibition of $p75^{NTR}$ signaling attenuates propofol-mediated apoptosis in developing primary neurons, we hypothesized that primary neurons from $p75^{NTR}$ knockout ($p75^{NTR-/-}$) mice would be less susceptible to propofol-mediated apoptosis, and that reexpression of $p75^{NTR}$ in $p75^{NTR-/-}$ neurons would reestablish anesthetic vulnerability. Primary neurons were isolated from $p75^{NTR-/-}$ mice at

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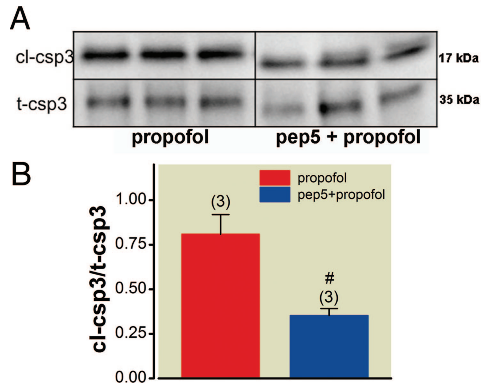

Fig. 3. Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Neurons were pretreated with TAT-Pep5 (10 μM; 15 min), a $p75^{NTR}$ intracellular domain antagonist, before propofol exposure. After pretreatment, neurons were exposed to propofol 3.0 μM for 6 h in 5% CO_2 in air. Apoptosis was evaluated after propofol exposure by cleaved caspase-3 immunoblot. (*A*) Immunoblot analysis shows a decrease in apoptosis marker, cleaved caspase-3, with TAT-Pep5 pretreatment. (*B*) Quantitation of the data are represented in the graph (n = 3; # P = 0.046). Sample size is indicated above the error bars ± SEM. Cl-Csp3 = cleaved caspase-3; T-Csp3 = total caspase-3.

postnatal day 1–3 and grown *in vitro* for 5–7 days. On day 4 *in vitro*, neurons were transfected with a lentiviral vector that expresses $p75^{NTR}$ driven by a neuron-specific synapsin promoter (LV-syn-$p75^{NTR}$) or GFP control vector (LV-syn-GFP) for 72 h. Immunoblot analysis demonstrated a dose-dependent increase in $p75^{NTR}$ protein expression after transfection with LV-syn-$p75^{NTR}$ (figs. 5A and B). On day 7 *in vitro*, neurons were exposed to propofol (3 μM, 6 h) and subjected to immunofluroscence confocal microscopy. Fixed neurons were incubated with antibodies to drebrin, and Cl-Csp3, and the nuclear marker 4′,6-diamidino-2-phenylindole. Propofol administration to $p75^{NTR-/-}$ neurons (as confirmed by polymerase chain reaction, fig. 5C) on day 7 *in vitro* transfected with LV-syn-GFP did not result in neuronal apoptosis as indicated by Cl-Csp3 immunofluorescence (fig. 5Di), suggesting that $p75^{NTR}$ is necessary for propofol-induced neurotoxicity. By contrast, propofol administration to $p75^{NTR-/-}$ neurons on day 7 *in vitro* transfected with LV-syn-$p75^{NTR}$ significantly increased (n = 10; P = 0.0002) levels of Cl-Csp3 (fig. 5Dii).

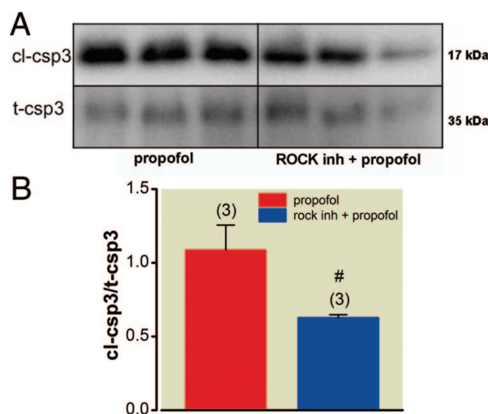

Fig. 4. Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Neurons were pretreated with hydroxyfasudil (10 μM; 15 min), a ρ kinase (ROCK) inhibitor, before propofol exposure. After pretreatment, neurons were exposed to propofol 3.0 μM for 6 h in 5% CO_2 in air. Apoptosis was evaluated after propofol exposure by cleaved caspase-3 immunoblot. (*A*) Immunoblot analysis shows a decrease in apoptosis marker, cleaved caspase-3, with hydroxyfasudil pretreatment. (*B*) Quantitation of the data are represented in the graph (n = 3; # P = 0.007). Sample size is indicated above the error bars ± SEM. Cl-Csp3 = cleaved caspase-3; ROCK inh = hydroxyfasudil; T-Csp3 = total caspase-3.

TAT-Pep5 Attenuates Propofol-mediated Apoptosis in the Hippocampus of Wild-type Mice In Vivo

To further confirm our *in vitro* findings, wild type mice were pretreated with either TAT-Pep5 (10 μM) or TAT-ctrl (10 μM) intraperitoneal followed by exposure to propofol with for 6 h *in vivo*. In addition, $p75^{NTR-/-}$ mice were exposed to propofol for 6 h. Wild-type mice exhibited a significant increase in Cl-Csp3 in the dentate gyrus ($P < 0.0001$), CA3 ($P < 0.0001$), and CA1 ($P < 0.001$) regions of the hippocampus, compared with intralipid-treated controls (n = 4 or 5, fig. 6A). TAT-Pep5 significantly attenuated propofol-mediated apoptosis ($P < 0.001$, dentate gyrus; $P < 0.001$, CA3; $P < 0.001$, dentate gyrus), suggesting that activation of ρ by $p75^{NTR}$ is involved in propofol neurotoxicity. There was no observed apoptosis in propofol-treated $p75^{NTR-/-}$ mice (n = 4, fig. 6B), suggesting that $p75^{NTR}$ expression mediates propofol-mediated neuronal cell death *in vivo*.

Discussion

Anesthetic agents inhibit neuronal activity in part by potentiating $GABA_A$ receptors, inhibiting N-methyl-D-aspartate channels, or activating two-pore potassium channels.[32] We have recently shown that reduced neuronal activity during the critical period of synaptogenesis leads to neuronal apoptosis by preferential signaling of proBDNF *via* $p75^{NTR}$.[22,23] The mechanism by which $p75^{NTR}$ activation leads to neurodegeneration is mediated in part through RhoA activation, actin cytoskeleton destabilization, and subsequent apoptosis.[23] Specifically, these findings pertained to studies conducted with isoflurane. Previous work has shown that propofol exposure during the neonatal period leads to a similar pattern of injury as seen with isoflurane.[33] Because propofol and isoflurane depress neuronal activity and are $GABA_A$ receptor agonists, the present study was conducted to determine whether the mechanisms involved in isoflurane-mediated neurotoxicity are also the key mediators in propofol-induced neuronal cell death. The present study is the first to demonstrate that propofol exposure to developing neurons induces apoptosis through the $p75^{NTR}$-RhoA-ROCK pathway.

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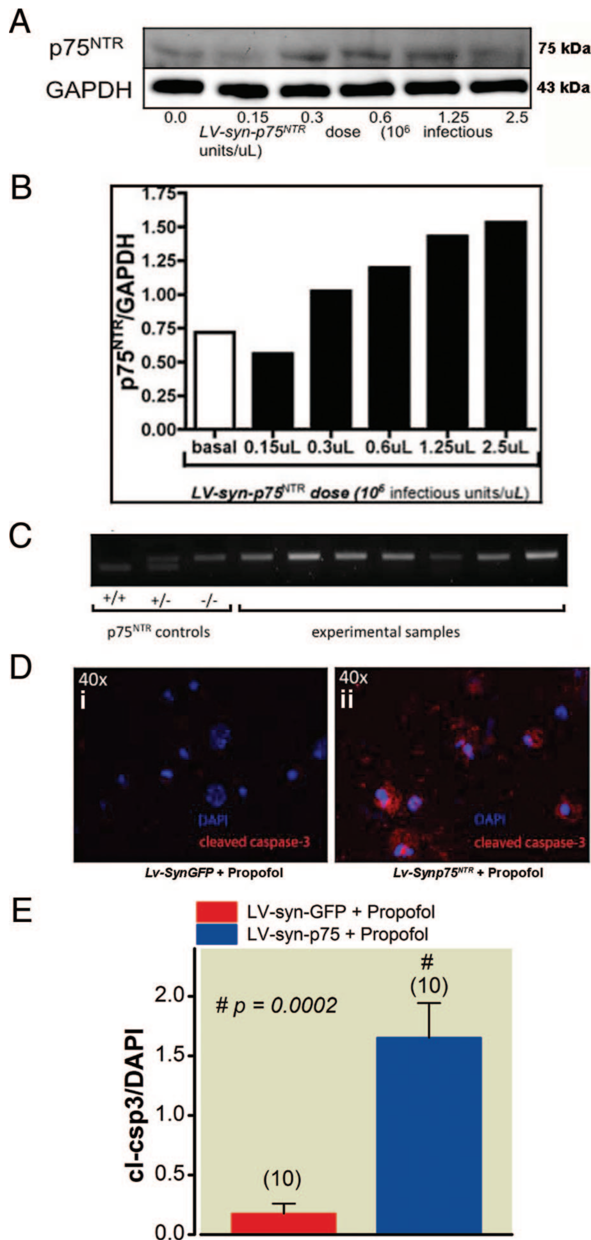

Fig. 5. Primary neurons were isolated from neonatal rodent brains at postnatal day 1–3 and grown *in vitro* for 5–7 days. Neurons were then transfected for 72 h with increasing doses of a lentiviral (LV) vector that expresses $p75^{NTR}$ driven by a neuronal-specific synapsin promoter (LV-syn-$p75^{NTR}$). (*A*) Immunoblot analysis shows an increase in $p75^{NTR}$ expression with increasing doses of LV-syn-$p75^{NTR}$. (*B*) Quantitation of the data are represented in the graph. These data demonstrate that, *in vitro*, primary neurons from neonatal rodents transfected for 72 h with LV-syn-$p75^{NTR}$ exhibits a dose-dependent increase in $p75^{NTR}$ expression. (*C*) Polymerase chain reactor confirmed that rodents used for primary neuronal cultures were $p75^{NTR}$ knockout genotype (-/-). On day *in vitro* 4, $p75^{NTR}$ knockout neurons were transfected with LV-syn-$p75^{NTR}$ for 72 h. LV-syn-GFP served as a control. On day *in vitro* 7 neurons were exposed to propofol 3.0 μM for 6 h and apoptosis was evaluated by cleaved caspase-3 immunofluorescence microscopy. (*D*) Immunofluorescence microscopy shows an increase in cleaved caspase-3 in $p75^{NTR}$ knockout neurons transfected with LV-syn-$p75^{NTR}$ and exposed to propofol *versus* LV-syn-GFP transfected neurons exposed to propofol. (*E*) Quantitation of the data are represented in the graph (n = 10; *P* = 0.0002). Sample size is indicated above the error bars ± SEM. These data demonstrate that, *in vitro*, reexpression of $p75^{NTR}$ in $p75^{NTR}$ knockout neurons reestablishes propofol-mediated apoptosis. Cl-Csp3 = cleaved caspase-3; DAPI = 4′,6-diamidino-2-phenylindole; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GFP = green fluorescent protein; LV-syn-$p75^{NTR}$ = a neuronal-specific synapsin promoter.

Exposure of developing neurons (days *in vitro* 5–7) to propofol results in decreased dendritic spines and increased apoptosis, and both these effects were attenuated by either $p75^{NTR}$ or ROCK inhibition. Apoptosis was not observed in $p75^{NTR-/-}$ neurons *in vitro* or $p75^{NTR-/-}$ mice *in vivo*. Moreover, reexpression of $p75^{NTR}$ in $p75^{NTR-/-}$ neurons using a neuron-specific promoter (synapsin) reestablished the neurotoxic effects from propofol. These results strongly suggest a role for $p75^{NTR}$ in mediating anesthetic-induced neuronal apoptosis. While our data are in support of proBDNF-$p75^{NTR}$ signaling being involved in propofol-mediated neurotoxicity, it still remains to be answered whether this is the sole or dominant mechanism. Other studies have reported that $GABA_A$ activation contributes to anesthetic mediated neurotoxicity in immature neurons, and that this toxicity might be mediated by GABA mediated excitation in neonatal neurons.[34,35] However, our results show that propofol exposure does not induce apoptosis in $p75^{NTR-/-}$ neurons even though $GABA_A$ activation would be expected. This suggests that $GABA_A$ signaling may be disrupted in $p75^{NTR-/-}$ neurons, or that $GABA_A$ activation *per se* does not play a major role in propofol-mediated neurotoxicity. Although we did not directly measure $GABA_A$ activation, unpublished findings from our group show GABA protein expression in $p75^{NTR-/-}$ is similar to wild-type, and that onset of, recovery from, and sensitivity to propofol anesthesia in $p75^{NTR-/-}$ mice pups parallel that in wild-type mice pups. Despite clinical evidence of deep anesthesia, neuronal apoptosis was not observed in the $p75^{NTR-/-}$. Other work from our laboratory demonstrated that treatment of day *in vitro* 4 neurons with 4-aminopyridine, an agent that induces synaptic release of neurotransmitters, prevented anesthetic-mediated neurotoxicity. Given that propofol and isoflurane are modulators of the GABA channel and increase Cl^- current significantly only in the presence of GABA, one would have expected an increase in neurotoxicity with synaptic release of GABA. To the contrary, in our *in vitro* model, 4-aminopyridine reduced death in neurons exposed to anesthetic. In addition, in our original paper in which we put forward the proBDNF-$p75^{NTR}$ hypothesis, we measured tPA levels with and without anesthetic administration *in vitro* and found that tPA levels were substantially reduced by isoflurane. If GABA-mediated excitation had occurred, we would have expected increased tPA

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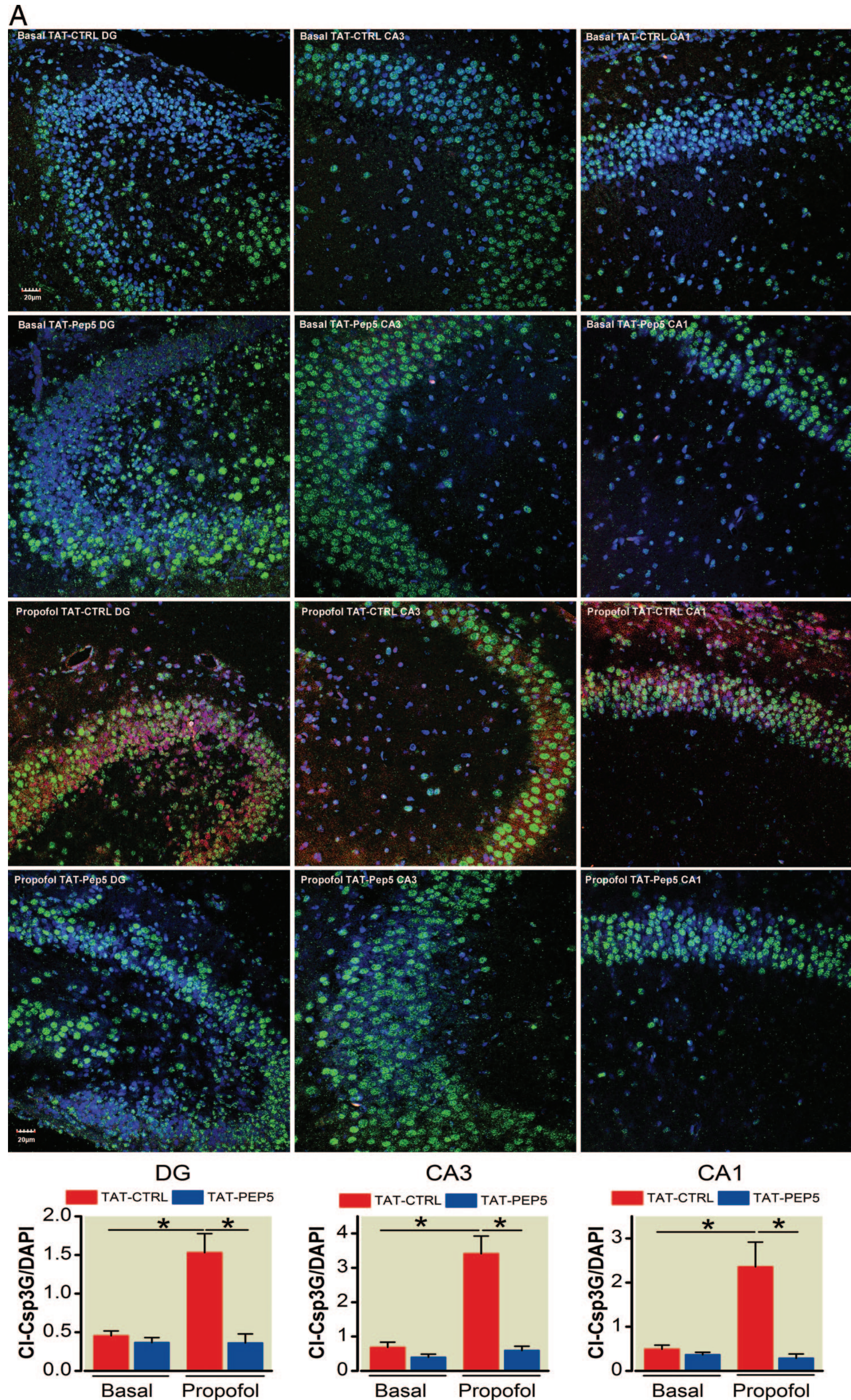

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Fig. 6. Postnatal day 5 wild-type or p75^{NTR} knockout mice were given an intraperitoneal (intraperitoneal) injection of propofol (100 mg/kg) or intralipid for 6 h and apoptosis was evaluated by cleaved caspase-3 immunofluorescence. (*A*) Dentate gyrus, CA3, and CA1 regions of the hippocampus are indicated on image. Basal TAT-CTRL, Basal TAT-Pep5, propofol TAT-CTRL, and propofol TAT-Pep5 are indicated on the image. Immunofluorescence microscopic analysis shows that wild-type mice exhibited a significant increase in cleaved caspase-3 in the dentate gyrus (* $P = 0.002$), CA3 (* $P = 0.008$), and CA1 (* $P = 0.007$) regions of the hippocampus compared with intralipid-treated controls (n = 4 or 5). TAT-Pep5 (10 μM, 15 min) significantly attenuated

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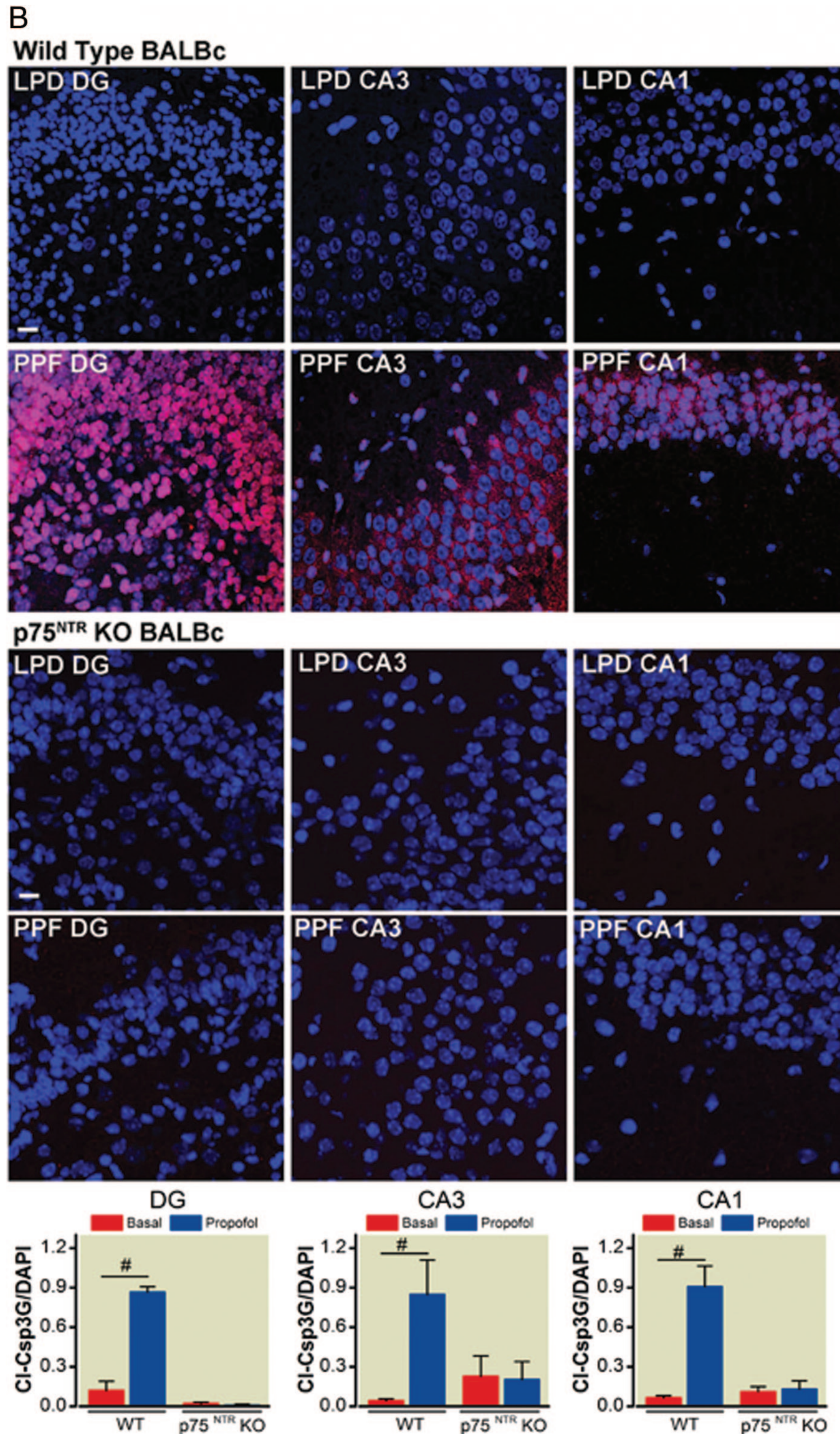

Fig. 6. (Continued) propofol-mediated apoptosis (* $P = 0.005$, CA1; * $P = 0.002$, CA3; * $P = 0.004$, dentate gyrus). (*B*) Additional immunofluorescence microscopic analysis shows that p75^{NTR} knockout mice exhibited no increase in cleaved caspase-3 in the dentate gyrus, CA3, or CA1 following propofol exposure compared to wild-type (# $P = 0.0008$, dentate gyrus; # $P = 0.03$, CA3; # $P = 0.002$, CA1). Quantitation of the data are represented in the graphs. Sample size is indicated above the error bars ± SEM. *Scale bar* = 20 μm. Cl-Csp3 = cleaved caspase-3; DAPI = 4′,6-diamidino-2-phenylindole; DG = dentate gyrus; KO = knockout.

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levels; what we observed was in fact the opposite. In aggregate, it is our belief that our data support the premise that a reduction in neuronal activity is one of the causes of anesthetic neurotoxicity; confirmation of this awaits results of electrophysiologic studies. Therefore, a reasonable argument can be made that in our *in vitro* and *in vivo* model system, propofol-mediated neuronal death does not appear to be primarily mediated by $GABA_A$ activation but rather though activation of p75^{NTR}.

Anesthetic agents have pleiotrophic effects and it is therefore unsurprising that a number of other mechanisms by which they induce toxicity may be operative. Straiko *et al.* have shown that treatment of postnatal day 5 mice with lithium counteracted propofol- and ketamine-mediated suppression of extracellular-regulated kinase phosphorylation and subsequent neurapoptosis.[36] Lithium has also been shown to be an inhibitor of GSK3β[37]; given the salutary effect of lithium, it is possible that GSK3β may also contribute to anesthetic neurotoxicity. Work by Wang *et al.* showed that antisense knockdown of N-methyl-D-aspartate receptor subunit NR1 or NR2A, but not NR2B, reduced phencyclidine-induced neurapoptosis.[38] These authors suggested that blockade of N-methyl-D-aspartate receptors leads to an upregulation of N-methyl-D-aspartate receptor in neurons. These neurons may subsequently be more vulnerable to excitotoxic injury. A more recent finding demonstrated that ketamine exposure results in aberrant cell cycle reentry of neurons and subsequent apoptosis in the developing rat brain.[39] What remains to be defined is whether anesthetic neurotoxicity is a function of a dominant mechanism or a combination of mechanisms. In addition, the possibility that the underlying mechanisms of toxicity might be dependent upon the class of anesthetic agents (predominantly $GABA_A$ agonists or N-methyl-D-aspartate receptor antagonists) remains to be explored.

Actin cytoskeleton organization and dynamics play a critical role for development and maturation of neurons.[40] RhoA and its effector ρ-associated kinase, ROCK, are key mediators of actin rearrangement and formation. RhoA is a small GTPase that is activated by p75^{NTR} signaling; RhoA activation leads to actin cytoskeleton depolymerization and subsequent apoptosis.[28–31,41–46] The RhoA effects are through activation of ROCK, a serine/threonine kinase.[47,48] In the present study pretreatment with a RhoA-signaling pathway inhibitor before propofol exposure significantly attenuated apoptosis. This strongly supports proBDNF-p75^{NTR} signaling as a common mechanism of isoflurane and propofol-mediated neurotoxicity.[22,23] A limitation to the present study is that we have not investigated the effects of anesthetics on other small GTPases, such as Rac1. Rac1 is a small GTPase known to be promote dendritic spine development through activation of N-methyl-D-aspartate receptors.[49,50] Anesthetics antagonize N-methyl-D-aspartate receptors and therefore may promote actin cytoskeleton depolymerization through decreased Rac1 activity. This may contribute to the decrease in neuritic processes seen with propofol exposure in neonatal neurons (fig. 2), a notion worthy of further investigation. Although we investigated RhoA and actin depolymerization specifically in neurons, others have shown that anesthetics also affect RhoA/myosin light-chain-signaling cascade and lead to alterations in actin organization in astroglia, a finding that may shed light on how anesthetics affect the interplay between astroglia and neurons during development or following injury.[51]

Conclusion

Collectively, these results demonstrate that propofol induces apoptosis in developing neurons during the critical period of synaptogenesis and mechanistically links activation of p75^{NTR} and the downstream effector ROCK in this neurotoxic process. As such, the results provide a mechanistic framework upon which novel therapeutic approaches for the prevention of anesthetic neurotoxicity can be developed.

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