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 (p75NTR) and subsequent cytoskeleton depolymerization. Given that isoflurane and propofol both suppress neuronal activity, we hypothesized that propofol also induces apoptosis in developing neurons through p75NTR.

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 p75NTR−/− mice or wild-type neurons were treated with propofol, with or without pretreatment with TAT-Pep5 (10 μM, 15 min), a specific p75NTR inhibitor. p75NTR−/− neurons were transfected for 72 h with a lentiviral vector containing the synapsin-driven p75NTR gene (Syn-p75NTR) or control vector (Syn– green fluorescent protein) before propofol. To confirm our in vitro findings, wild-type mice and p75NTR−/− 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 p75NTR−/− neurons. In p75NTR−/− neurons transfected with Syn-p75NTR, propofol significantly increased Cl-Csp3 in comparison with Syn– green fluorescent protein– transfected p75NTR−/− mice. 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 p75NTR−/− mice.

Conclusion: These results demonstrate that propofol induces apoptosis in developing neurons in vivo and in vitro and implicate a role for p75NTR 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...
clear, γ-aminobutyric acid (GABA_A) agonism and N-methyl-D-aspartate receptor antagonism play a central role.

Recently we demonstrated that proBDNF-p75NTR 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 (p75NTR) 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 p75NTR. 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 p75NTR intracellular domain inhibitor, suggesting a role for p75NTR 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 p75NTR signaling. RhoA activation, actin cytoskeleton depolymerization, and subsequent neuronal apoptosis. Lemkuil et al. extended these findings by showing that isoflurane exposure increases p75NTR-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-p75NTR 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 acti-"
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 electrodession. 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 (0.05%) for 15 min followed by incubation with fluo- rescin 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 (0.01%) 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 (Applied Precision, Inc., Issaquah, WA) that included a Photometrics CCD (Photomet- rics, Tucson, AZ) mounted on a Nikon TE-200 (Nikon, Melville, NY) inverted epi-fluorescence microscope. Between 30 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 cytoskel- eton 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 processes is indicated by decreased dre- brin 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 Bonfer- roni correction (fig. 6). Significance was set at P < 0.05.
Propofol Exposure Decreases Neuritic Processes 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 Increases Apoptosis 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 p75NTR 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 p75NTR. \(^ {22,23}\) Moreover, using TAT-Pep5, we showed that signaling from p75NTR to RhoA was mediated 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 p75NTR 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 p75NTR and mediates its effects through downstream activation of ROCK. \(^ {31}\) Because ROCK is indirectly activated by p75NTR and inhibition of p75NTR 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% CO2 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 p75NTR Driven by a Neuronal-specific Synapsin Promoter (LV-syn-p75NTR) Increases p75NTR Expression

Because our results from figures 3 and 4 demonstrate that inhibition of p75NTR signaling attenuates propofol-mediated apoptosis in developing primary neurons, we hypothesized that primary neurons from p75NTR knockout (p75NTR/−/−) mice would be less susceptible to propofol-mediated apoptosis, and that reexpression of p75NTR in p75NTR/−/− neurons would reestablish anesthetic vulnerability. Primary neurons were isolated from p75NTR/−/− mice at
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 p75NTR driven by a neuron-specific synapsin promoter (LV-syn-p75NTR) or GFP control vector (LV-syn-GFP) for 72 h. Immunoblot analysis demonstrated a dose-dependent increase in p75NTR protein expression after transfection with LV-syn-p75NTR (figs. 5A and B). On day 7 in vitro, neurons were exposed to propofol (3 µM, 6 h) and subjected to immunofluorescence confocal microscopy. Fixed neurons were incubated with antibodies to drebrin, and Cl-Casp3, and the nuclear marker 4',6-diamidino-2-phenylindole. Propofol administration to p75NTR−/− 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-Casp3 immunofluorescence (fig. 5Dii), suggesting that p75NTR is necessary for propofol-induced neurotoxicity. By contrast, propofol administration to p75NTR−/− neurons on day 7 in vitro transfected with LV-syn-p75NTR significantly increased (n = 10; P = 0.0002) levels of Cl-Casp3 (fig. 5Dii).

**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. We have recently shown that reduced neuronal activity during the critical period of synaptogenesis leads to neuronal apoptosis by preferential signaling of proBDNF via p75NTR. The mechanism by which p75NTR activation leads to neurodegeneration is mediated in part through RhoA activation, actin cytoskeleton destabilization, and subsequent apoptosis. 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. 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 p75NTR-RhoA-ROCK pathway.

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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 p75NTR intracellular domain antagonist, before propofol exposure. After pretreatment, neurons were exposed to propofol 3.0 µM for 6 h in 5% CO2 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 = 0.046). Sample size is indicated above the error bars ± SEM. Cl-Casp3 = cleaved caspase-3; T-Casp3 = total caspase-3.

**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 p7 kinase (ROCK) inhibitor, before propofol exposure. After pretreatment, neurons were exposed to propofol 3.0 µM for 6 h in 5% CO2 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-Casp3 = cleaved caspase-3; ROCK inh = hydroxyfasudil; T-Casp3 = total caspase-3.

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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 p75NTR or ROCK inhibition. Apoptosis was not observed in p75NTR−/− neurons in vitro or p75NTR−/− mice in vivo.

Moreover, reexpression of p75NTR in p75NTR−/− neurons using a neuronal-specific promoter (synapsin) reestablished the neurotoxic effects from propofol. These results strongly suggest a role for p75NTR in mediating anesthetic-induced neuronal apoptosis. While our data are in support of proBDNF-p75NTR 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 activation contributes to anesthetic mediated neurotoxicity in immature neurons, and that this toxicity might be mediated by GABA mediated excitation in neonatal neurons. However, our results show that propofol exposure does not induce apoptosis in p75NTR−/− neurons even though GABA activation would be expected. This suggests that GABA signaling may be disrupted in p75NTR−/− neurons, or that GABA activation per se does not play a major role in propofol-mediated neurotoxicity. Although we did not directly measure GABA activation, unpublished findings from our group show GABA protein expression in p75NTR−/− is similar to wild-type, and that onset of, recovery from, and sensitivity to propofol anesthesia in p75NTR−/− mice parallel that in wild-type mice pups. Despite clinical evidence of deep anesthesia, neuronal apoptosis was not observed in the p75NTR−/−. 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-p75NTR hypothesis, we measured tPA levels with and without anesthetic administration in vitro and found that tPA levels were substantially reduced by isoflurane. If GABAA activation per se does not play a major role in propofol-meditated neurotoxicity, one would have expected an increase in neurotoxicity with synaptic release of GABA.

Moreover, reexpression of p75NTR in p75NTR knockout neurons transfected with LV-syn-p75NTR or 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 p75NTR in p75NTR knockout neurons reestablishes propofol-mediated apoptosis. Cl-Casp3 = cleaved caspase-3; DAPI = 4′,6-diamidino-2-phenylindole; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GFP = green fluorescent protein; LV-syn-p75NTR = a neuronal-specific synapsin promoter.

**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 p75NTR driven by a neuronal-specific synapsin promoter (LV-syn-p75NTR). (A) Immunoblot analysis shows an increase in p75NTR expression with increasing doses of LV-syn-p75NTR. (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-p75NTR exhibits a dose-dependent increase in p75NTR expression. (C) Polymerase chain reaction confirmed that rodents used for primary neuronal cultures were p75NTR knockout genotype (−/−). On day in vitro 4, p75NTR knockout neurons were transfected with LV-syn-p75NTR 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 p75NTR knockout neurons transfected with LV-syn-p75NTR 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 p75NTR in p75NTR knockout neurons reestablishes propofol-mediated apoptosis.

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Postnatal day 5 wild-type or p75NTR knockout mice were given an 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 the 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...
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 \( \pm \) SEM. Scale bar = 20 \( \mu \)m. Cl-Csp3 = cleaved caspase-3; DAPI = 4',6-diamidino-2-phenylindole; DG = dentate gyrus; KO = knockout.
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 pleiotropic 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 GSK3^β; given the salutary effect of lithium, it is possible that GSK3^β 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 p-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 pre-treatment 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 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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