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Inhibition of p75 Neurotrophin Receptor Attenuates Isoflurane-mediated Neuronal Apoptosis in the Neonatal Central Nervous System

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Background: **Exposure to anesthetics during synaptogenesis results in apoptosis and subsequent cognitive dysfunction in adulthood. Probrain-derived neurotrophic factor (proBDNF) is involved in synaptogenesis and can induce neuronal apoptosis *via* p75 neurotrophic receptors ($p75^{NTR}$). proBDNF is cleaved into mature BDNF (mBDNF) by plasmin, a protease converted from plasminogen by tissue plasminogen activator (tPA) that is released with neuronal activity; mBDNF supports survival and stabilizes synapses through tropomyosin receptor kinase B. The authors hypothesized that anesthetics suppress tPA release from neurons, enhance $p75^{NTR}$ signaling, and reduce synapses, resulting in apoptosis.**

Methods: **Primary neurons (DIV5) and postnatal day 5-7 (PND5-7) mice were exposed to isoflurane (1.4%, 4 h) in 5% CO_2, 95% air. Apoptosis was assessed by cleaved caspase-3 (Cl-Csp3) immunoblot and immunofluorescence microscopy. Dendritic spine changes were evaluated with the neuronal spine marker, drebrin. Changes in synapses in PND5-7 mouse hippocampi were assessed by electron microscopy. Primary neurons were exposed to tPA, plasmin, or pharmacologic inhibitors of $p75^{NTR}$ (Fc-$p75^{NTR}$ or TAT-Pep5) 15 min before isoflurane. TAT-Pep5 was administered by intraperitoneal injection to PND5-7 mice 15 min before isoflurane.**

Results: **Exposure of neurons *in vitro* (DIV5) to isoflurane decreased tPA in the culture medium, reduced drebrin expression (marker of dendritic filopodial spines), and enhanced Cl-Csp3. tPA, plasmin, or TAT-Pep5 stabilized dendritic filopodial spines and decreased Cl-Csp3 in neurons. TAT-Pep5 blocked isoflurane-mediated increase in Cl-Csp3 and reduced synapses in PND5-7 mouse hippocampi.**

Conclusion: **tPA, plasmin, or $p75^{NTR}$ inhibition blocked isoflurane-mediated reduction in dendritic filopodial spines and neuronal apoptosis *in vitro*. Isoflurane reduced synapses and enhanced Cl-Csp3 in the hippocampus of PND5-7 mice, the latter effect being mitigated by $p75^{NTR}$ inhibition *in vivo*. These data support the hypothesis that isoflurane neurotoxicity in the developing rodent brain is mediated by reduced synaptic tPA release and enhanced proBDNF/$p75^{NTR}$-mediated apoptosis.**

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A CONVENTIONAL premise of general anesthesia is that anesthetics produce a nontoxic and reversible state of unconsciousness. However, recent evidence has indicated that exposure of neonatal animals to anesthetics triggers widespread neurodegeneration, leading to persistent memory and learning abnormalities during adulthood.[1–3] Specifically, exposure of postnatal day 5–10 (PND5-10) rat pups to a combination of isoflurane, nitrous oxide, and midazolam resulted in substantial neurodegeneration in the hippocampus and neocortex[1] as well as impaired electrophysiologic and behavioral function in the hippocampus weeks to months after exposure.[1,3] Such neurotoxicity has now been demonstrated for ketamine, midazolam,[4] diazepam, pentobarbital,[5] thiopental,[3] nitrous oxide,[1] and propofol.[3,6–8] The pattern of neonatal anesthetic neurotoxicity is similar to that produced by ethanol, exposure to which *in utero* is associated with the development of fetal alcohol syndrome. These observations have raised concerns about the potentially adverse impact of general anesthesia in the human fetus, neonate, and infant.[3,9]

A key element of anesthetic neurotoxicity is that the brain is most susceptible to injury during the period of synaptogenesis.[10] An important signaling mechanism that contributes to synaptogenesis and to consolidation and maturation of synapses is the neurotrophin brain-derived neurotrophic factor (BDNF).[11] BDNF has dual function in that it can enhance neuronal survival or cause neuronal apoptosis through activity at either the tropomyosin receptor kinase B (TrkB) or neurotrophic receptor ($p75^{NTR}$), respectively.[12–14] BDNF is secreted from synaptic vesicles as a proneurotrophin (proBDNF) that is proteolytically cleaved in the synaptic cleft by plasmin to a mature form (mBDNF).[15] Plasminogen, the precursor to plasmin, is cleaved by tissue plasminogen activator (tPA), a protease released from presynaptic vesicles.[16] Mature BDNF (mBDNF) preferentially binds to TrkB receptors to promote neu-

ronal survival and synaptogenesis.[15] In the absence of tPA, proBDNF is uncleaved and binds with high affinity to $p75^{NTR}$, resulting in reduced synaptogenesis, withdrawal of dendritic spines, and neuronal apoptosis.[15] Thus tPA can serve to control which effect of BDNF is predominant.[17]

The mechanisms by which anesthetics mediate neurotoxicity are not clear. What is common among the anesthetics that have proven neurotoxicity in experimental studies is a profound suppression of neuronal activity; this has the potential to adversely affect the tPA-plasminogen-plasmin system. The release of tPA is activity-dependent,[18] and it is therefore conceivable that anesthetics might suppress neuronal activity, reduce tPA release, and enhance proBDNF signaling *via* the $p75^{NTR}$, thereby leading to loss of dendritic filopodial spines and synapses and subsequent neuronal apoptosis. The current study, using *in vitro* neuronal cultures and *in vivo* mouse pups, was conducted to test this hypothesis.

Materials and Methods

Preparation of Neuronal Cell Cultures

All studies performed on animals were approved by the Veteran Affairs San Diego Institutional Animal Care and Use Committee and conform to relevant National Institutes of Health guidelines.

Neonatal mouse neurons (The Jackson Laboratory, Bar Harbor, ME) were isolated using a papain dissociation kit (Worthington Biochemical, Lakewood, NJ) as previously described.[19] Mix cortical and hippocampal neurons were isolated from 1-day-old pups (PND1) and grown in culture for 5 to 21 days *in vitro* (DIV). Neurons were cultured in Neuobasal A media supplemented with B27 (2%), 250 mM GLUTMax1, and penicillin/streptomycin (1%). Cells were cultured on poly-D-lysine/laminin (2 $\mu g/cm^2$)–coated plates or coverslips at 37°C in 5% CO_2 for 5, 14, or 21 days before experiments. Cleaved-caspase 3 (Cell Signaling, Danvers, MA) and/or caspase-activated DNase (CAD)–positive cells (Santa Cruz Biotech, Santa Cruz, CA) were used to determine apoptosis *via* immunoblot and/or immunofluorescence and deconvolution microscopy. Antibodies to phospho-JNK and GAPDH were obtained from Cell Signaling and Imgenex (San Diego, CA), respectively. Cell death was normalized to total caspase-3 or the neuronal marker doublecortin (Abcam, Cambridge, MA) or NeuN (Chemicon/Millipore, Billerica, MA) of cells imaged. TAT-Pep5 was purchased from CalBiochem (Gibbstown, NJ).

Anesthetic Neurotoxicity Model

In Vitro. Neurons were placed in a plexiglass chamber within an incubator and exposed to 1.4% isoflurane delivered from a calibrated vaporizer in a gas mixture of 5% CO_2, 21% O_2, balance nitrogen gas at a flow rate of 2 l/min. The concentration of isoflurane was continuously monitored by a Datex Capnomac (DRE Medical, Inc., Louisville, KY).

In Vivo. Animals were exposed to isoflurane (1.4%; air flow served as the carrier) at a flow rate of 2 l/min for 4 h. Body temperature was monitored with an 8 mm, 22-gauge temperature probe (Mon-a-therm 6510; Mallinckrodt Medical, Inc., St. Louis, MO) that was affixed to the dorsal neck. The temperature in the incubator was maintained at 37°C; this results in core body temperature in mouse pups in the range of 36.5–37.5°C (data not shown).

Protein Extraction and Western Blot Analysis

Proteins in cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 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 (PBS) Tween (1%) containing 4% bovine serum albumin (BSA) and incubated with primary antibody overnight at 4°C as previously described.[20] Primary antibodies were visualized using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech) and ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ). All displayed bands are expected to migrate to the appropriate size and were determined by comparison to molecular weight standards (Santa Cruz Biotech). The amount of protein per fraction was determined using a dye-binding protein assay (Bio-Rad, Hercules, CA). Image J was used for densitometric analysis of immunoblots.‖

Tissue Plasminogen Activator ELISA

Neurons were exposed to isoflurane for 4 h, and media from both control and isofluorane-treated neurons were frozen at −80°C before enzyme-linked immunosorbent assay (ELISA) (Innovative Research, Novi, MI). A 96-well plate was precoated with biotinylated plasminogen activator inhibitor-1 (PAI-1) for 30 min before three washes with wash buffer (provided by the supplier). tPA standard (0.05–10 ng/ml) and unknown samples (*i.e.*, media from control and isofluorane-exposed neurons) were added to the plate for 30 min, washed three times, and incubated with anti-tPA primary antibody for 30 min, followed by anti-rabbit horseradish peroxidase–conjugated secondary antibody for an additional 30 min. After three additional washes, tetramethylbenzidine substrate was added to the wells for 10 min, and the reaction was

‖ Image J: Image processing and analysis in Java. Available at: http://rsb.info. nih.gov/ij/. Accessed December 19, 2008.

quenched with 1 M H_2SO_4 and read at 450 nm on a spectrophotometer (TECAN Infinite M200, San Jose, CA).

Immunofluorescence and Deconvolution Microscopy

Neurons were prepared for immunofluorescence microscopy as previously described.[21] The following antibodies were used for immunofluorescence: cleaved caspase 3, drebrin, doublecortin (Abcam), and NeuN (Chemicon/Millipore). Tissue and cells were fixed with 4% paraformaldehyde in PBS 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% BSA/PBS/Tween (0.05%) for 20 min, and then incubated with primary antibodies (1:100) in 1% BSA/PBS/Tween (0.05%) for 24–48 h at 4°C. Excess antibody was removed by incubation with PBS/Tween (0.1%) for 15 min, and the samples were incubated with fluorescein isothiocyanate (FITC) or Alexa-conjugated secondary antibody (1:250) for 1 h. To remove excess secondary antibody, cells were washed six times at 5-min intervals with PBS/Tween (0.1%) and incubated for 20 min with the nuclear stain Dapi (1:5000) diluted in PBS. Cells were then washed for 10 min with PBS and mounted in gelvatol for microscopic imaging. Deconvolution images were obtained as described[21] and captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA). The system includes a Photometrics CCD (Tuscon, AZ) mounted on a Nikon TE-200 (Melville, NY) inverted epi-fluorescence microscope. Between 30 and 80 optical sections spaced by approximately 0.1–0.3 μm were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. Lenses included 100× (NA 1.4), 60× (NA 1.4), and 40× (NA 1.3). The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc.) on a Silicon Graphics Octane workstation. Image analysis was performed with Data Inspector program in SoftWorx. Maximal projection volume views or single optical sections were visualized. Colocalization of pixels was assessed quantitatively by CoLocalizer Pro 1.0 software (Tokyo, Japan). Overlap coefficient according to Manders was used to determine the degree of colocalization on whole cells or membrane regions of interest after subtracting background through normalized threshold values.[22] The values were defined as 0 to 1, with 1 implying that 100% of both components overlap with the other part of the image. Statistical analysis was performed using Prism 4 (GraphPad Software, Inc., La Jolla, CA).

Dendritic Spine Quantification

The drebrin pixels (green) were normalized to doublecortin pixels (red). Because dendritic filopodial spines are nearly completely devoid of intermediate filaments and microtubules (as indicated by doublecortin staining)[23] and only contain components of the actin cytoskeleton (as indicated by drebrin staining), a neuronal microtubule marker (doublecortin) was used to normalize any changes in spinal actin cytoskeletal expression. Drebrin is a filamentous (F-)actin binding protein that stabilizes the actin cytoskeleton within dendritic filopodial spines.[23,24] A reduction in dendritic filopodial spines is indicated by decreased drebrin protein expression. Approximately 15–20 neurons were counted per experiment (4–6 experiments total).

$p75^{NTR}$-small Interference RNA

Neurons were incubated with small interfering ribonucleic acid (siRNA) (Ambion, Inc., Foster City, CA; 200 pmol; sense: CAGCUGCAAGCAGAACAAGtt; antisense: CUUGUUCUGCUUGCAGCUGtt) using Lipofectamine 2000 transfection reagent (Invitrogen) for 72 h and then exposed to isoflurane. Scrambled siRNA served as control.

Histologic Preparation

Under deep pentobarbital anesthesia, a midline thoracotomy was performed and the descending thoracic aorta was occluded. A 20-gauge needle was inserted into the left ventricle, and the animal was perfused transcardially with 20 ml of heparinized saline followed by 20 ml of 4% buffered formalin. The right atrium was incised to permit free flow of perfusion fluid. The brain was removed and postfixed for 24–48 h in fixative. The brain was sectioned into 4-mm blocks, placed in tissue holders, and dehydrated in graded concentrations of ethanol in a tissue processor (Autotechnicon; Technicon Instruments, Tarrytown, NY). The tissue holders were then transferred into a paraffin bath in an oven at 37°C under vacuum suction. The paraffin blocks were separated from tissue holders and mounted in a Histostat 820S microtome (Reichert Scientific Instruments, Depew, NY); 5- to 10-μm sections were then cut, placed on glass slides, and incubated overnight at 37°C. The tissue sections were then prepared for immunohistochemistry.

Electron Microscopy

Brains were transcardially perfusion fixed with standard Karnovsky's fix, 4% paraformaldehyde, 1% gluteraldehyde, 0.1 M cacodylate buffer with 5 mM $CaCl_2$. PND5-7 animals were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer, and 5 mM $CaCl_2$ to prevent tissue artifacts. Hippocampi were dissected from whole brains after 24 h, and 400-μm vibratome slices were prepared and refixed for an additional 24 h. Brains were blocked (*i.e.*, dissected) to include hippocampal areas: one hemisphere for sagittal orientation, and one hemisphere for coronal. Blocks were refixed for an additional 24 h before postfixation with 1% OsO_4 in 0.1 M cacodylate buffer, *en bloc* stained

with uranyl acetate, and embedded with flat orientation to locate appropriate hippocampal regions of interest. Each block was thick sectioned, stained with toludine blue, and retrimmed to isolate hippocampal areas before preparation of grids. Grids (70-nm sections) were stained with uranyl acetate and lead nitrate for contrast and observed on the JEOL 1200 EX-II electron microscope (JEOL, Tokyo, Japan) equipped with a digital camera system. Twenty-five random low-magnification micrographs of the stratum radiatum were obtained from each specimen. Micrographs were analyzed for the quantity of synapses and for synapse abnormalities (reduction or changes in synapse and dendritic filopidal spine morphology, *i.e.*, degradation of cytoskeletal architecture). The dendritic profiles were characterized by abundant organelles such as mitochondria and endoplasmic reticulum and frequent contacts from vesicle-filled axon terminals. Spine synapses were identified by an electron-dense region presynaptically associated with vesicles and that lacked cellular organelles or contained a spine aparatus (as indicated by cytoskeletal architecture) postsynpatically as described previously.[25–27] Dendritic filopodial spines were also identified as dendritic extensions rich in F-actin and possessing postsynaptic densities (PSD) at their surfaces as previously described.[28,29] Approximately 25 electron micrographs (3350 μm^2) per animal were analyzed in a blinded fashion for total synapse number per area (synapse #/3350 μm^2).

Statistical Analysis

All parametric data were analyzed by unpaired *t* tests or ANOVA Bonferroni multiple comparison as appropriate; *post hoc* comparisons were made by Student-Neuman-Keuls tests. Significance was set at $P < 0.05$. Statistical analysis was performed using Prism 4 (GraphPad Software, Inc.).

Results

Isoflurane Induces Apoptosis in Div5 but not Div14 or Div21 Neurons

Cortical and hippocampal neurons were isolated on PND1 and were grown in culture up to 5, 14, and 21 days (days *in vitro* - DIV5, DIV14, and DIV21). Isoflurane (1.4%, 4 h) exposure increased cleaved caspase-3 (Cl-Csp3) relative to total caspase-3 (T-Csp3) in DIV5 neurons (fig. 1A), but not in DIV14 (n = 3, $P = 0.22$), or DIV21 (n = 3, $P = 0.68$) (fig. 1B) neurons. These data are consistent with the age-dependent neurotoxicity of isoflurane that has been demonstrated previously.[1,9,30]

tPA and Plasmin, but Not Metallomatrix Proteinase-7 or Metallomatrix Proteinase-9, Mitigate Isoflurane-mediated Neuronal Apoptosis

tPA is a critical modulator of BDNF.[31] tPA release is activity-dependent,[18] and anesthetics suppress neuronal activity; we therefore hypothesized that isoflurane would lower tPA release. DIV5 primary neurons were exposed to isoflurane (1.4%, 4 h), and tPA levels in the culture medium were measured by ELISA. Neurons exposed to isoflurane had less tPA (n = 6, $P = 0.0013$) in the media compared to control (fig. 2A). Addition of 0.03–3 μg/ml tPA to the medium dose-dependently attenuated isoflurane-mediated increase in Cl-Csp3 at 2 h (fig. 2B) after exposure. Exogenous application of matrix metalloproteinase-7, which can potentially cleave proBDNF to mBDNF, and matrix metalloproteinase-9, which cleaves proNGF to mNGF, did not reduce isoflurane-mediated neuronal apoptosis (fig. 2C), whereas 3 μg/ml tPA reduced (n = 3, $P = 0.02$) isoflurane-mediated (n = 3, $P = 0.02$) apoptosis. In addition, tPA application increased levels of phosphorylated Akt (n = 3, $P = 0.002$) (fig. 2D), a downstream signaling component of TrkB activation. Application of plasmin also attenuated isoflurane-mediated neuronal apoptosis (n = 3, $P = 0.004$); the neuroprotective effects of both plasmin and tPA were blocked by the serine protease inhibitor, aprotinin (fig. 2E).

Isoflurane Treatment Decreases Dendritic Filopodial Spines in Neonatal Primary Neurons

Neonatal primary neurons (DIV5) were exposed to isoflurane (1.4%, 4 h) and stained for dendritic filopodia (*i.e.*, immature filopodial spines) using a neuronal F-actin marker, drebrin, and the dendritic shaft/neuronal microtubule marker, doublecortin. Basally, drebrin staining revealed thin protrusions (termed dendritic filopodia) common to immature neurons[24] (fig. 3A). Exposure of DIV5 neurons to isoflurane significantly reduced (n = 6, $P = 0.01$) the number of dendritic filopodial spines. Treatment with the proteases, plasmin (n = 6, $P = 0.02$) or tPA (n = 5, $P = 0.04$), significantly attenuated the isoflurane-induced loss of neuronal dendritic filopodia (fig. 3B). Quantitation is expressed as drebrin pixels (green) normalized to doublecortin immunofluorescence (red pixels) along the dendrites only (soma immunofluorescence was not included) (fig. 3C). In addition, Western blot images demonstrate that addition of exogenous tPA significantly mitigated (n = 3, $P = 0.006$) the isoflurane-mediated (n = 3, $P = 0.005$) reduction in drebrin protein expression (fig. 3D).

Pharmacological Inhibition of the $p75^{NTR}$ or siRNA-mediated Knockdown Attenuates Isoflurane-induced Apoptosis

If isoflurane increases proBDNF signaling *via* the $p75^{NTR}$, then knockdown or inhibition of this receptor should reduce toxicity related to this pathway. Primary neurons (DIV5) were treated with the $p75^{NTR}$ inhibitors (Fc-$p75^{NTR}$, a chimera expressing only the extracellular domain of the $p75^{NTR}$, or TAT-Pep5, an intracellular inhibitor of $p75^{NTR}$) or siRNA for $p75^{NTR}$ before isoflurane exposure (1.4%, 4 h). Preincubation with Fc-$p75^{NTR}$

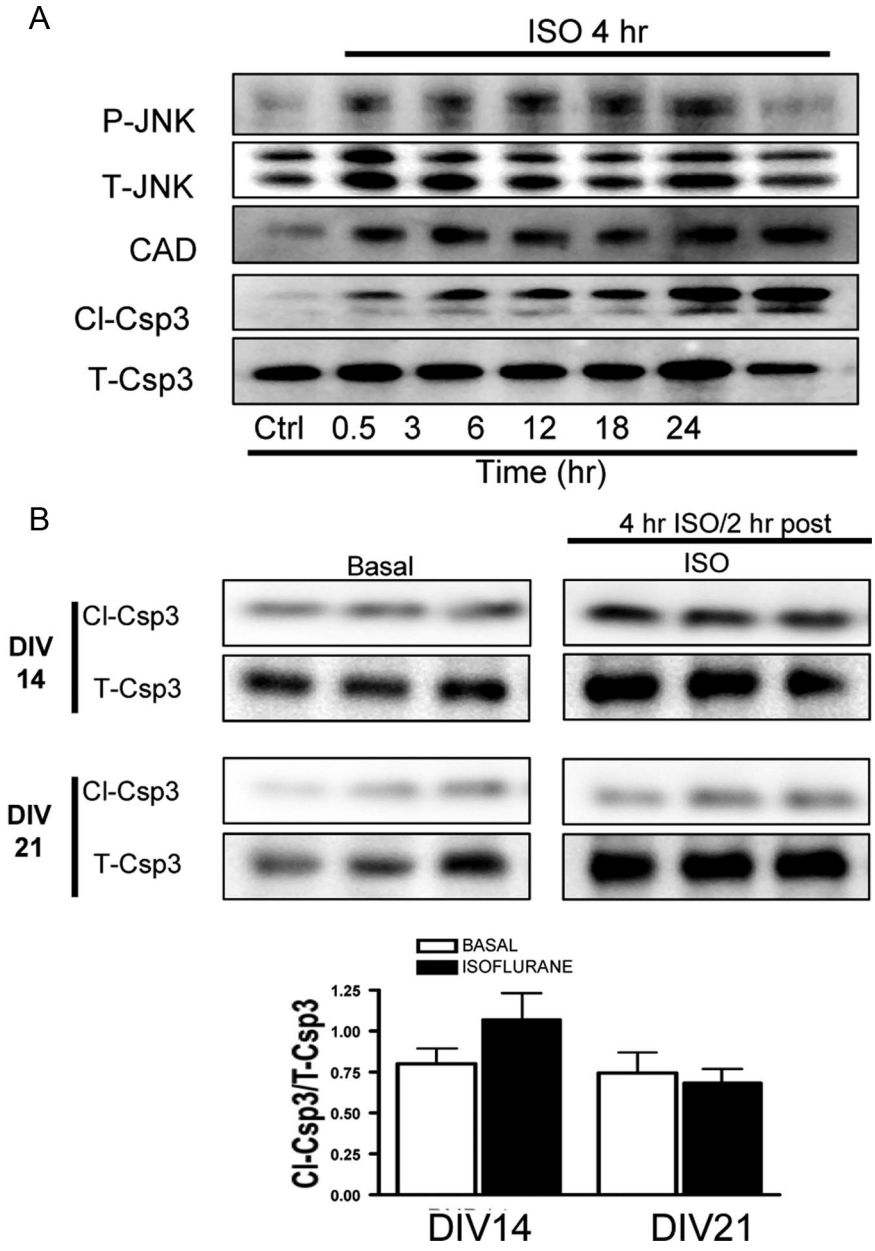

Fig. 1. Isoflurane (Iso) exposure increases cell death in neurons 5 days *in vitro* (DIV5) but not in neurons 14 and 21 days *in vitro* (DIV14 and DIV21, respectively). Primary neurons (DIV5) were exposed to 1.4% isoflurane for 4 h, and neurons were lysed at various time intervals after exposure. The proapoptotic proteins, phosphorylated c-Jun N-terminal kinase (P-JNK), caspase activated DNase (CAD), and cleaved caspase-3 (Cl-Csp3), were enhanced at 0.5, 3, 6, 12, 18, and 24 h (with the exception of P-JNK, which returned to basal levels before 24 h) after isoflurane exposure as shown in *A*. Primary neonatal neurons (DIV14 and DIV21) were exposed to isofluorane, and representative immunoblots are shown in *B*. Isoflurane slightly increased (not significantly) apoptosis in DIV14 ($P = 0.2191$, n = 3), but it had no effect on DIV21 ($P = 0.6821$, n = 3) neonatal neurons. In contrast to DIV5 neurons, these findings indicate that DIV14 and DIV21 neurons are not vulnerable to isoflurane-induced apoptosis. *Error bars* = SEM. T-Csp3 = total caspase 3.

(1.35 μg/ml) (fig. 4A, n = 3, $P = 0.001$ *vs.* basal, $P = 0.003$ *vs.* isoflurane) or TAT-conjugated Pep5 (10 μM) (fig. 4B, n = 3, $P = 0.007$ *vs.* basal, $P = 0.006$ *vs.* isoflurane) significantly attenuated isoflurane-mediated neuronal apoptosis. $p75^{NTR}$-siRNA resulted in significant knockdown (approximately 62%; n = 3, $P = 0.005$) of the neurotrophin receptor after 72 h and attenuated isoflurane-mediated apoptosis (n = 3, $P = 0.001$ *vs.* basal-scrambled control [SC], $P = 0.001$ *vs.* isoflurane-scrambled control) compared to SC neurons (fig. 4C).

We next tested whether TAT-Pep5 was efficacious in reducing anesthetic-induced neurotoxicity and loss of synapses in PND5 mice *in vivo*. Neonatal C57BL/6 mice (PND5-7) were given TAT-Pep5 (intraperitoneal injection; 10 μM) 15 min before isoflurane exposure (1.4%, 4 h). Isoflurane significantly enhanced cleaved caspase 3 (Cl-Csp3) in the CA1 (n = 6, $P = 0.003$) and CA3 (n = 7, $P = 0.007$) of the hippocampus in PND5 mice (fig. 4D) 2 h after exposure. TAT-Pep5 significantly attenuated isoflurane-mediated increase in Cl-Csp3 in both the CA1 (n = 7, $P = 0.007$) and CA3 (n = 7, $P = 0.007$) regions. In contrast, isoflurane exposure did not enhance Cl-Csp3 in PND21 mice (fig. 4E), indicating that the brains of mice at this age are less vulnerable to isoflurane-induced neurotoxicity.

Isoflurane Reduces Synapse Number in the Hippocampus of PND5-7 Mouse Pups

Synapse number in the hippocampus (stratum radiatum; mossy fiber to CA3 and Shaeffer collaterals from CA3 to CA1) of neonatal mouse pups (PND5-7) 2 h after isoflurane exposure (1.4%, 4 h) was quantitated by elec-

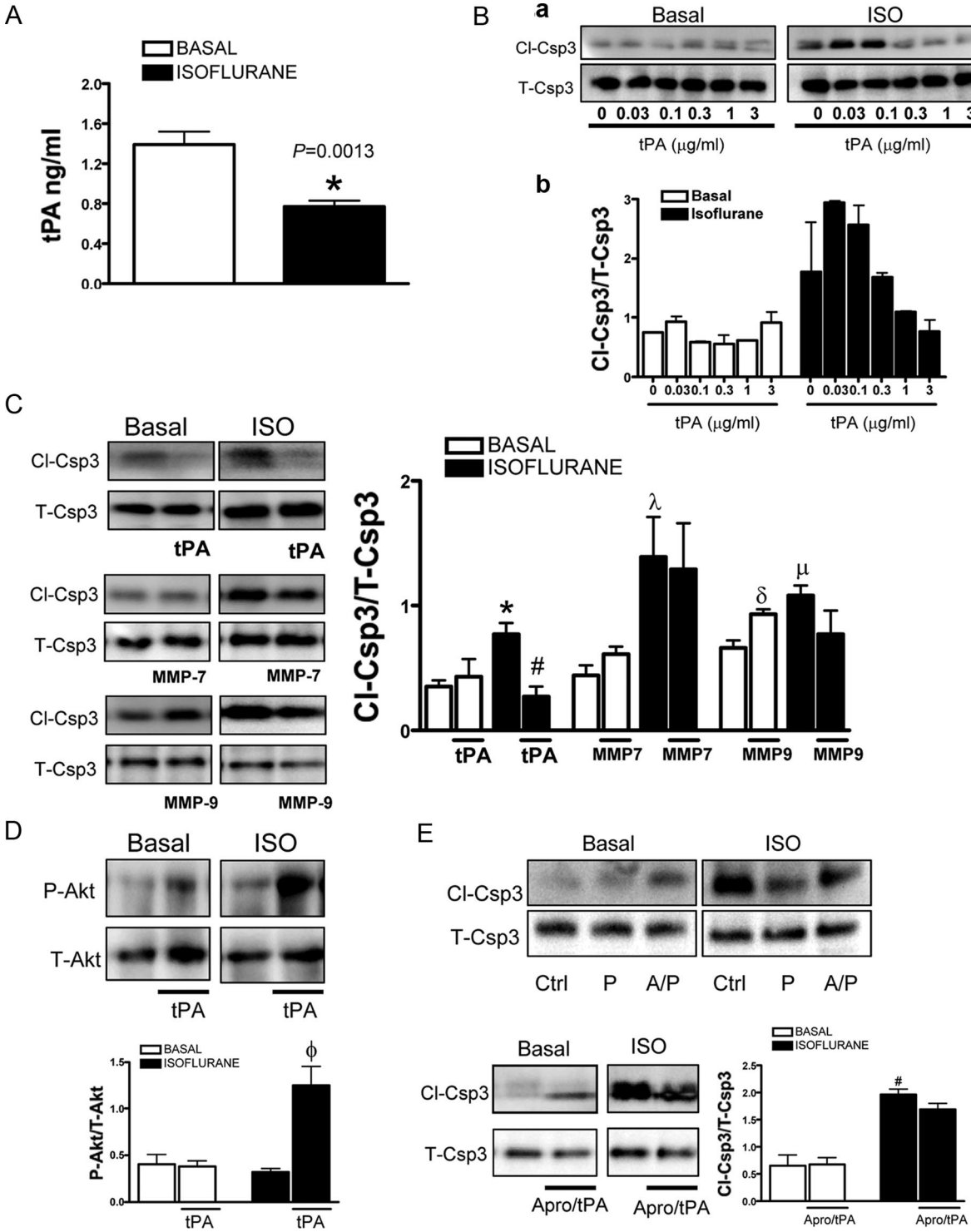

Fig. 2. Tissue plasminogen activator (tPA) decreases isoflurane-induced cell death in primary neurons 5 days *in vitro* (DIV5). Primary neurons were exposed to 1.4% isoflurane for 4 h, and the media were frozen 2 h after exposure at −80°C before tPA ELISA. (*A*) The media from neurons exposed to isoflurane (1.4%, 4 h; 2 h post) contained significantly (n = 6, * P =0.001) less tPA compared with media from nonexposed cells (approximately 45% decrease; 1.40 ng/ml BASAL *vs*. 0.77 ng/ml isoflurane). (*B*) Neurons were pretreated with increasing doses of tPA (0.03 to 3 μg/ml) before isoflurane (1.4%, 4 h; 2 h post), and neuronal cell death was assessed 2 h after exposure with the apoptotic marker, cleaved caspase 3 (Cl-Csp3). (*Ba* and *Bb*) Increasing doses of tPA decreased isoflurane-induced Cl-Csp3 with a maximum decrease at 3 μg/ml. (*C*) tPA (3 μg/ml) (n = 3, # P = 0.02), but not matrix metalloproteinases (MMP)-7 (2 μg/ml) or MMP-9 (1 μg/ml), significantly decreased isoflurane-induced Cl-Csp3. MMP-9 significantly elevated Cl-Csp3 under basal conditions (P = 0.01). * Isoflurane alone *versus* control alone (n = 3, P = 0.01). λ Isoflurane *versus* control (n = 3, P = 0.01). μ Isoflurane *versus* control (n = 3, P = 0.01). δ MMP-9 basal *versus* basal alone (n = 3, P = 0.02). (*D*) tPA significantly (n = 3, φ P = 0.002) enhanced the prosurvival kinase phosphorylated Akt (P-Akt) even in the presence of isoflurane. (*E*) Application of the serine protease inhibitor, aprotinin (Apo) (3 μg/ml), blocked the protective effect of 3 μg/ml plasmin (P) (*upper blots*) and tPA (*lower blots*) (n = 3, # P = 0.004). *Error bars* = SEM. T-Csp3 = total caspase 3.

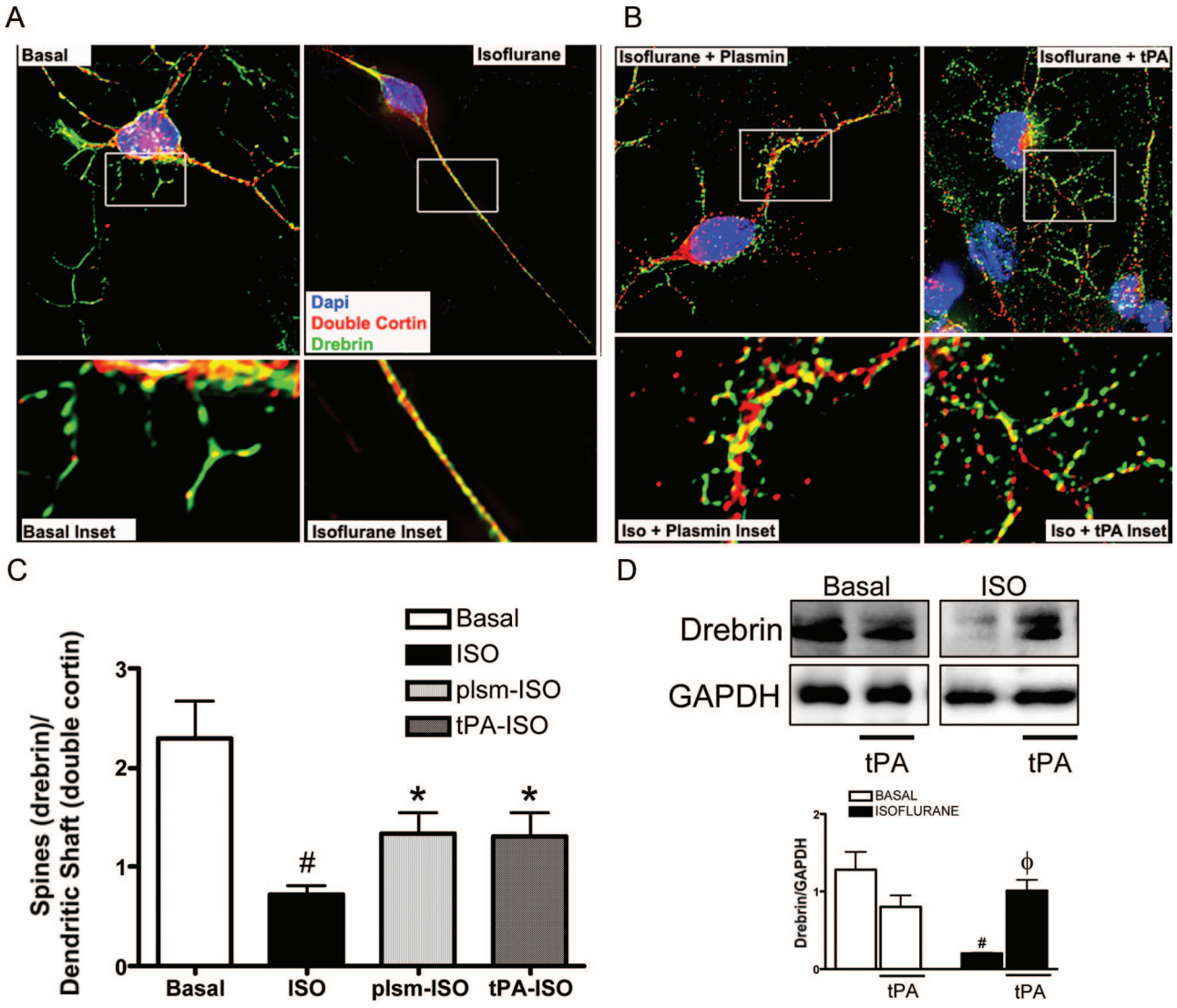

Fig. 3. Isoflurane (Iso) treatment decreases dendritic filopodia (immature filopodial spines) in neonatal primary neurons as assessed by neuronal F-actin (drebrin) immunofluorescence microscopy. Neonatal primary neurons (5 days *in vitro* [DIV5]) were exposed to isoflurane (1.4%, 4 h) and stained for the dendritic spine/neuronal F-actin marker, drebrin, and the dendritic shaft/neuronal microtubule marker, doublecortin, 2 h after exposure. (*A*) Drebrin staining along dendritic shafts was significantly (n = 6, # P = 0.01) decreased after isoflurane treatment. (*B*) Treatment with the proteases, plasmin (plsm) (3 μg/ml) (n = 6, * P = 0.02) or tissue plasminogen activator (tPA) (3 μg/ml) (n = 5, * P = 0.04) significantly attenuated the isoflurane-induced loss of neuronal dendritic filopodial spines. (*C*) Quantitation is expressed as drebrin (*green pixels*) normalized to doublecortin immunofluorescence (*red pixels*) along the dendrites only (no soma immunofluorescence was included). (*D*) Isoflurane significantly (n = 3, # P = 0.006) decreased drebrin protein expression; tPA significantly (n = 3, ϕ P < 0.005) mitigated isoflurane-mediated reduction in drebrin (*top panel*, representative blots; *lower graph*, densitometry). These data demonstrate that isoflurane reduced the number of dendritic filopodia and drebrin protein expression in developing neurons. The *white box* in the isoflurane image lists the color codes for dapi, drebrin, and doublecortin. *Scale bar* = 10 μm; *error bars* = SEM.

tron microscopy. Synapses with postsynaptic densities and presynaptic vesicles are clearly visible in representative control images (fig. 5Aa). By contrast, there was a significant reduction in total synapses in PND5-7 pups exposed to isoflurane (fig. 5Ab; n = 6, P = 0.01). This reduction in synapses was significantly attenuated by pretreatment with TAT-Pep5 (fig. 5Ac; n = 5, P = 0.01). Higher magnification (6600×) electron microscopy images for control (fig. 5Ba), isoflurane (fig. 5Bb), and TAT-Pep5 (fig. 5Bc) are shown in figure 5B. Quantitation of data is represented by the graph in figure 5C. These findings extend our results from *in vitro* studies demonstrating that (1) PND5-7 animals are vulnerable to anesthetic-induced neurotoxicity and (2) these neurotoxic effects from isoflurane can be mitigated by TAT-Pep5 *in vivo*.

Discussion

The results of the current study are consistent with the neurotoxicity of isoflurane that has been demonstrated previously.[1–3] Primary neurons (DIV5) and mouse pups

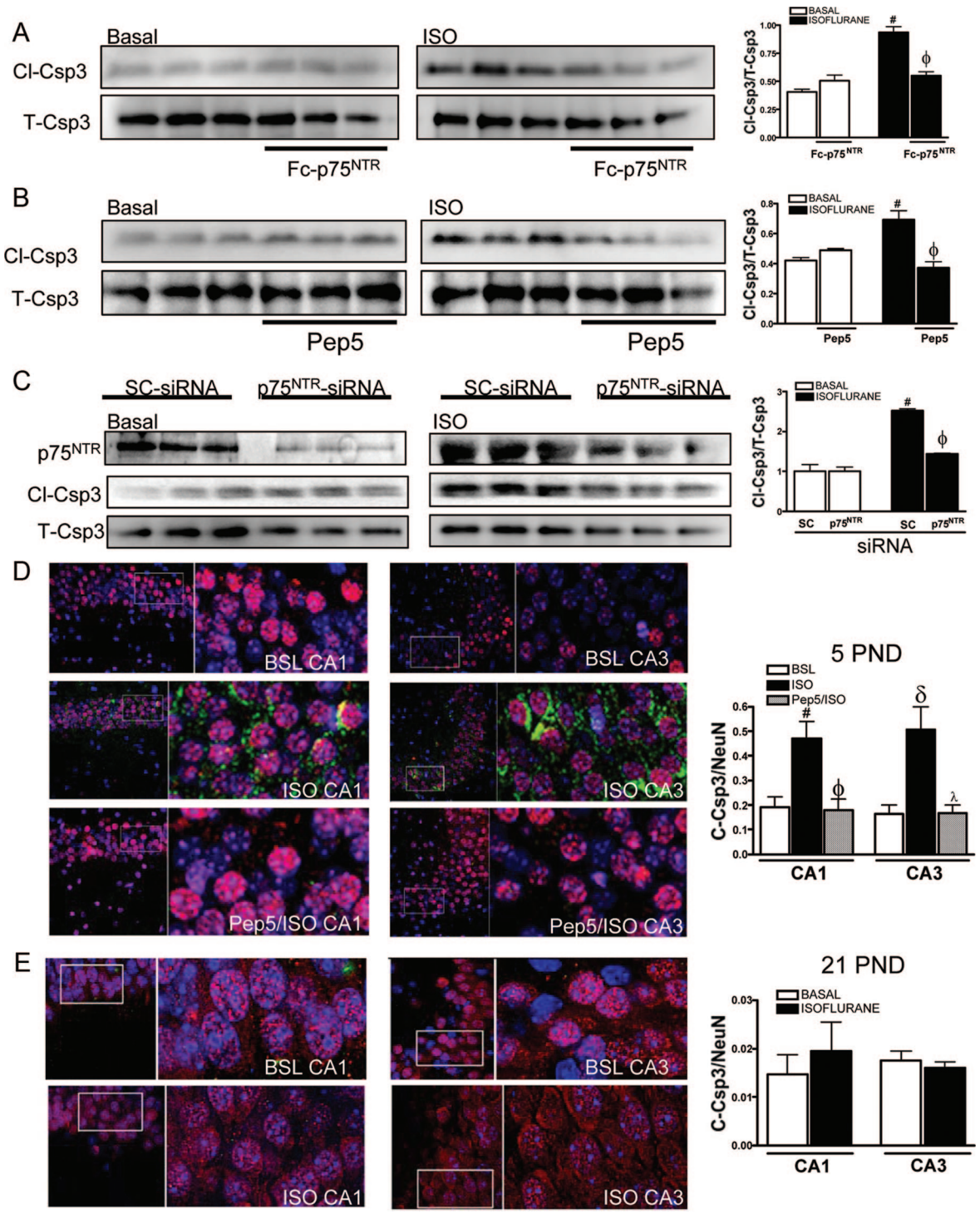

Fig. 4. Pharmacological inhibition of the p75 neurotrophin receptor (p75^{NTR}) or small interfering ribonucleic acid (siRNA)–mediated knockdown attenuates isoflurane (ISO)-induced apoptosis in DIV5 neonatal neurons. Primary neurons (5 days *in vitro* [DIV5]) were treated with the p75^{NTR} inhibitors (Fc-p75^{NTR} or TAT-Pep5) or siRNA for p75^{NTR} before isoflurane exposure (1.4%, 4 h) (*left panels*, representative immunoblots; *right panels*, densitometry). Preincubation with (*A*) Fc-p75^{NTR} (neutralizing antibody to BDNF) (1.35 μg/ml; n = 3, # *P* = 0.001 *vs.* basal, ϕ *P* = 0.003 *vs.* isoflurane) or (*B*) TAT-conjugated Pep5 (10 μM; n = 3, # *P* = 0.007 *vs.* basal, ϕ *P* = 0.006 *vs.* isoflurane) significantly attenuated isoflurane-mediated neuronal apoptosis. (*C*) p75^{NTR}-siRNA resulted in significant knockdown of the neurotrophin receptor (approximately 62% protein reduction; n = 3, *P* = 0.005) after 72 h and significantly attenuated isoflurane-mediated apoptosis (n = 3, # *P* = 0.001 *vs.* basal scrambled control; n = 3, ϕ *P* = 0.001 *vs.* isoflurane scrambled control). (*D*) Neonatal C57BL/6 mice were given TAT-Pep5 intraperitoneal (10 μM) 15 min before isoflurane exposure (1.4%, 4 h). Isoflurane significantly enhanced cleaved caspase 3 (Cl-Csp3) in the CA1 (n = 6, # *P* = 0.003) and CA3 (n = 7, δ *P* = 0.007) of the hippocampus in postnatal day (PND) 5–7 mice *in vivo*. TAT-Pep5 significantly attenuated isoflurane-mediated increases in Cl-Csp3 in both CA1 (n = 7, ϕ *P* = 0.003) and CA3 (n = 7, λ *P* = 0.007) regions. (*E*) In contrast, isoflurane exposure did not significantly enhance Cl-Csp3 in PND21 mice (n = 4). *Error bars* = SEM. CA = *Cornu Ammonis* (ram's horn); Pep5 = p75^{NTR} inhibitor; SC = scrambled control.

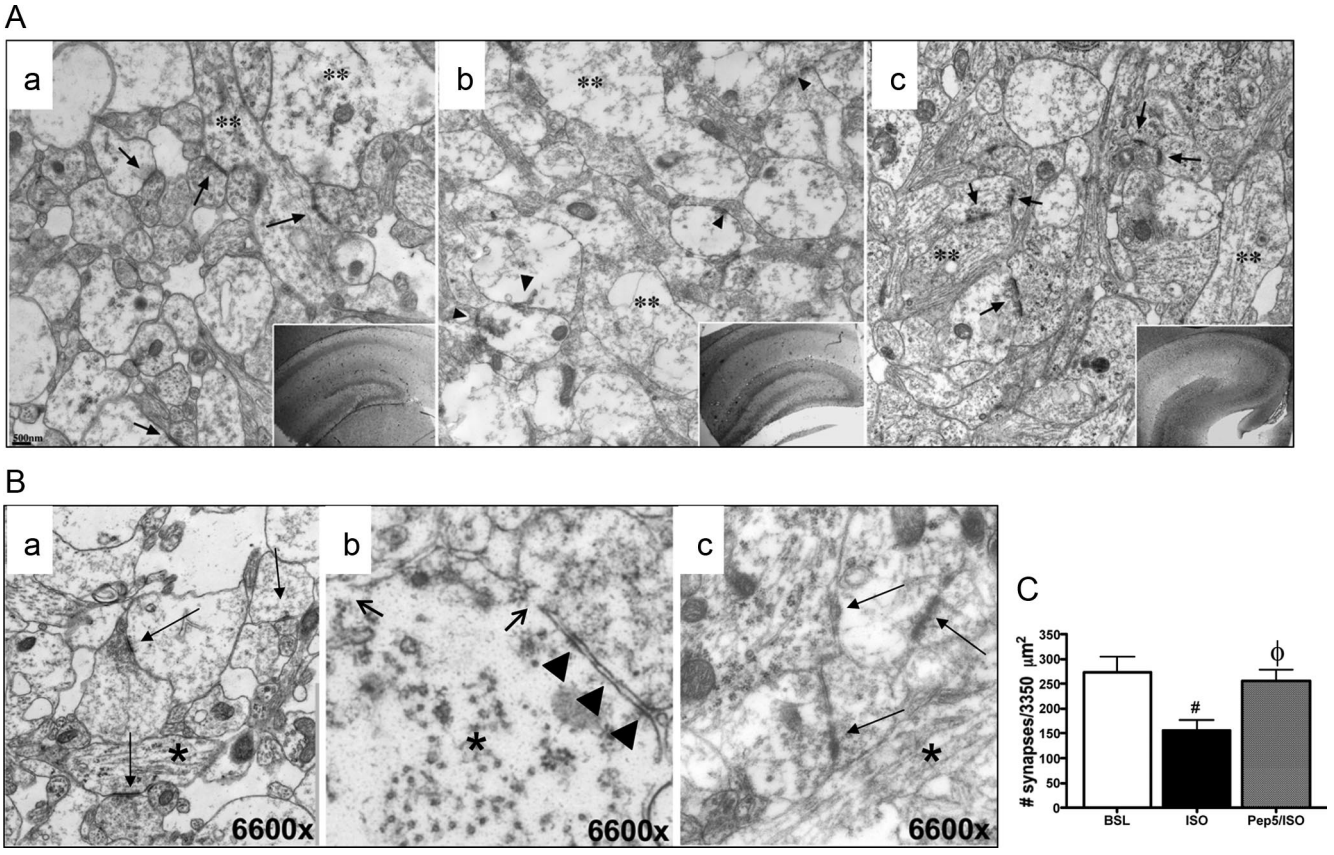

Fig. 5. TAT-Pep5, a p75^{NTR} inhibitor, attenuates isoflurane-mediated loss of synapses in the CA3 and CA1 of 5-day-old C57BL/6 mice *in vivo*. (*A*) Electron microscopy shows that isoflurane (*b*) significantly (n = 6, # *P* = 0.01) decreased the number of synapses (*arrowheads*) in the hippocampus compared to basal (*arrows*) (*a*, n = 4) in postnatal day (PND) 5 mice. TAT-Pep5 (*c*) significantly (n = 5, ϕ *P* = 0.01) attenuated isoflurane-mediated decrease in hippocampal synapses (*c, arrows*). ** = dendritic shafts/spine heads. *Scale bar* = 500 nm. Insets are light microscopic images of representative surveyed hippocampi (magnification, 5×). (*B*) Higher magnification image shows disintegration of postsynaptic densities (*arrowheads*) and membrane disruption (*open arrows*) after isoflurane exposure (*b*). * = Intact (*a* and *c*) and disrupted (*b*) cytoskeleton. Quantitation of the data is represented by the graph in panel *C*. *Scale bar* = 500 nm; *Error bars* = SEM.

(PND5-7) were vulnerable to injury during the period of synaptogenesis, whereas the toxicity was not apparent at the age of 14 and 21 days DIV and *in vivo*. *In vitro*, isoflurane exposure reduced tPA release, and the administration of either tPA or plasmin reduced neurotoxicity. Reduction of p75^{NTR} signaling, either with siRNA-mediated knockdown of p75^{NTR} or with pharmacologic inhibition with Fc-p75^{NTR} or TAT-Pep5, abolished isoflurane injury. *In vivo*, isofluorane-mediated injury to the CA1 and CA3 sectors of the hippocampus was apparent. Administration of TAT-Pep5 before isoflurane exposure abolished injury. In aggregate, the results suggest that isoflurane neurotoxicity is mediated at least in part by a reduction of tPA release and preferential signaling of proBDNF *via* the p75^{NTR}.

There is a convincing body of evidence to indicate that the brain is vulnerable to anesthetic neurotoxicity during synaptogenesis.[10] In the developing brain, synaptic connections with appropriate target neurons are essential for neuronal survival because neurons are dependent on trophic support from their targets.[32–34] Loss of synaptic connection leads to apoptosis. An important element contributing to synaptogenesis and to consolidation and maturation of synapses is the neurotrophin BDNF.[11] Neurotrophins are synthesized as proneurotrophins (proNTs), which subsequently undergo proteolytic cleavage to yield the biologically active mature neurotrophins.[35] mBDNF preferentially bind to the TrkB receptor to promote neuronal survival and synaptogenesis.[13] proBDNF serves as a ligand for the p75^{NTR} and initiates neuronal apoptosis.[12] Up to 60% of the secreted neurotrophins are in the pro-form.[15] Several proteases, including plasmin and matrix metalloproteinases, proteolytically cleave proNTs; however, the most significant protease is plasmin.[12,13] Plasmin exists as plasminogen, and the key enzyme that cleaves plasminogen is tPA, which is secreted from presynaptic terminals, and its secretion is activity-dependent.[16–18]

Our data show that isoflurane reduces the amount of tPA present in culture media of 5-day-old primary neu-

rons. tPA is released from neurons in an activity-dependent manner,[16–18] and this reduction in tPA may be due to neuronal suppression by isoflurane. By contrast, proBDNF undergoes both activity-dependent and constitutive release.[15] Therefore, by suppressing neuronal activity during the key period of synaptogenesis, anesthetics might reduce tPA release and subsequent plasmin activation, thereby enhancing proBDNF signaling *via* $p75^{NTR}$. Increase in the activation of JNK, a downstream consequence of $p75^{NTR}$ activity,[36] with isoflurane exposure supports this premise. The consequence of this is neuronal death, either due to a failure of synaptic stabilization and consolidation (with the subsequent failure of affected neurons to obtain target derived trophic factor support) or $p75^{NTR}$-mediated apoptosis. Previous application of tPA led to the activation of Akt (P-Akt), a downstream kinase of TrkB signaling critical to neuronal survival,[37] attenuated anesthetic-induced neurotoxicity as well as the loss of synaptic filopodial spines in developing neurons. The present studies used both cellular and animal models to demonstrate the novel involvement of tPA-plasmin-proBDNF-$p75^{NTR}$ signaling in anesthetic neurotoxicity.

In addition to its proteolytic function, tPA has a number of nonproteolytic activities. tPA binds to the low-density lipoprotein receptor (LRP-1) and triggers signaling cascades that lead to phosphorylation of ERK1/2[38] and Akt[39]; this effect of tPA is independent of its proteolytic activity. Both ERK1/2[40] and Akt[41] have antiapoptotic activity. The LRP-1 receptor is expressed in neurons and glia.[39] tPA can also bind to and directly activate the urokinase-like plasmingoen activator receptor and trigger intracellular signaling.[42] The possibility that the reduction in anesthetic neurotoxicity might be a function of the nonproteolytic effects of tPA (activation of survival signaling) exists. Aprotinin is a serine protease inhibitor of the proteolytic activity of tPA.[12,43] Data showing that aprotinin abrogated the protective efficacy of tPA suggests, in our model systems, that the salutary effect of tPA is mediated by its proteolytic activity and not by a receptor-mediated effect. Additional unpublished findings from our laboratory indicate that pretreatment of neurons (DIV5) with 4-aminopyridine, a potassium channel blocker that serves to enhance vesicular release from presynaptic terminals,[44] reduces isoflurane-mediated cell death in a dose-dependent manner, suggesting that enhanced neuronal activity can alleviate the neurotoxic effects from isoflurane. Furthermore, plasmin administration reduced neurotoxicity, indicating that the proteolytic activity (presumably cleavage of proBDNF to mBDNF) is required for prevention of anesthetic neurotoxicity.

Although plasmin is the major protease that cleaves proNTs, other enzymes such as matrix metalloproteinases can also cleave proNTs.[12,13] Matrix metalloproteinases-9 cleaves proNGF to yield mNGF, and matrix metalloproteinases-7 can similarly generate mBDNF. To determine whether these enzymes play a role in the prevention of isoflurane neurotoxicity, we added matrix metalloproteinases-7 or matrix metalloproteinases-9 to neurons exposed to isoflurane. The failure of either of these matrix metalloproteinases to reduce isoflurane toxicity indicates that these enzymes do not cleave proBDNF to mBDNF in our model systems, thus supporting the premise that it is tPA and plasmin that have neuroprotective efficacy.

In addition to reducing mBDNF-TrkB signaling (*i.e.*, decreased Akt phosphorylation), isoflurane increased $p75^{NTR}$ signaling. $p75^{NTR}$ modulates synaptic transmission, axonal elongation, and growth cone collapse, and it initiates neuronal apoptosis.[14,45–47] In $p75^{NTR-/-}$ mice, dendritic complexity, which is increased substantially, can be reduced by $p75^{NTR}$ overexpression,[48] indicating a negative modulatory effect of $p75^{NTR}$ on dendritic spine development. Fc-$p75^{NTR}$ is a chimera that expresses extracellular domain (amino acids 1–250).[49,50] This domain serves to bind and scavenge BDNF and therefore potently reduces $p75^{NTR}$ signaling. Our data, which show that inhibition of $p75^{NTR}$ with TAT-Pep5, Fc-$p75^{NTR}$, or targeted siRNA reduces anesthetic-mediated neuronal apoptosis, indicate that anesthesia results in $p75^{NTR}$-dependent neuronal apoptosis in the neonatal brain, perhaps by proBDNF agonism at $p75^{NTR}$. Furthermore, TAT-Pep5 attenuation of anesthesia-induced neurotoxicity suggests that the neurodegenerative effects from anesthetics may be in part due to alterations to the actin cytoskeleton. The question that remains is whether isoflurane neurotoxicity is due to the loss of mBDNF-TrkB signaling, increased proBDNF-$p75^{NTR}$ signaling or a combination of both.

In the development of therapeutic options to prevent anesthetic neurotoxicity, a central concern is whether these interventions are directly toxic themselves. In our studies, we investigated the potential of the protective effect of $p75^{NTR}$ inhibition against anesthetic neurotoxicity. Reduction of $p75^{NTR}$ activity by extracellular inhibition with Fc-$p75^{NTR}$, by intracellular inhibition with TAT-Pep5, or by knockdown of $p75^{NTR}$ with siRNA was clearly reduced by isofluorane-mediated injury. Importantly, though, the interventions did not directly injure neurons, indicated by the fact that Cl-Csp3 immunoblot (fig. 4, A–C) was similar in the nonanesthetized control and $p75^{NTR}$ inhibition groups. A second concern is that protective agents might inhibit developmental neuroapoptosis. This is a normal physiologic process in the developing brain that is essential for the removal of excess neurons; inhibition of apoptosis therefore might produce the undesirable effect of preventing this normal clearance of unneeded neurons. Our data clearly show that, although $p75^{NTR}$ inhibition was effective in reducing isoflurane neurotoxicity, the basal level of apoptosis

(Cl-Csp3 immunoblot) was not different in the nonanesthetized control and $p75^{NTR}$ inhibition groups (fig. 4, A–C). These data indicate that short-term inhibition of $p75^{NTR}$ activity does not have direct toxicity and does not inhibit normal developmental neuroapoptosis. It should be noted that injury was evaluated only in the hippocampus and not in other structures that are injured by anesthesia, such as the cortex and thalamus. Whether $p75^{NTR}$ inhibition adversely affects the cortex and thalamus is not currently clear. In addition, the effect of long-term inhibition of $p75^{NTR}$ during synaptogenesis needs further investigation.

In the majority of anesthetic neurotoxicity studies focus on neuroapoptosis. However, the current investigation indicates that the injury is not restricted to neuronal death; it also includes the loss of dendritic filopodial spines and synapses. The quantity of dendritic filopodial spines was dramatically reduced after isoflurane exposure both *in vitro* and *in vivo*. Most of the excitatory synapses are located on dendritic filopodial spines.[51] The loss of filopodial spines would therefore significantly reduce excitatory transmission and should lead to loss of synapses. Indeed, electron microscopy of the stratum radiatum in the hippocampus (mossy fiber-CA3 and CA3-CA1 connections) demonstrated a reduction in the number of synapses as well as ablation of existing synapses compared to nonanesthetized control state (fig. 5). However, a limitation to the latter EM findings is the absence of measurements of presynaptic profile diameter, active zone length, or number of synaptic vesicles across conditions in addition to alterations in organelle and dendritic shaft volume. More thorough EM analysis in future studies may yield a comprehensive morphologic profile of changes that occur with isoflurane and other anesthetics during synaptogenesis. The current results are consistent with those reported by Nikizad *et al.*[52]; a general anesthetic dose of midazolam led to a significant, albeit transient, reduction in the quantity of proteins associated with synapses (*e.g.*, synaptophysin, synaptobrevin, SNAP-25). Of interest is the observation that the reduction in synapse number in the mossy fiber to CA3 connections (approximately 50%) was considerably greater than the extent of apoptosis in either the CA3 sector or the dentate gyrus. The relationship between synapse loss and neuronal apoptosis will require further investigation.

A recent finding suggests that proBDNF undergoes proteolysis intracellularly and is released in the mature form.[53] These data, which are in contradiction to those reported previously by Lu *et al.*,[31] raise the question of the relevance of proBDNF to isoflurane neurotoxicity, at least within the framework that has been proposed herein. In support of our central premise, our data (not shown) indicate that proBDNF, when measured with an antibody that is specific for the proBDNF form, is present in the neuronal lysates. When tPA or plasmin are administered, the level of proBDNF is reduced. Moreover, isoflurane exposure does not affect proBDNF levels. Collectively, these data indicate that neurons exposed to isoflurane are also exposed to proBDNF and that tPA and plasmin, which mitigate isoflurane toxicity, reduce proBDNF.

Our study has limitations. We were unable to measure proBDNF levels in the culture media of control and isofluorane-exposed neurons. The most sensitive ELISA kit currently available does not have the requisite sensitivity to detect the very small quantities of proBDNF that might be present in the culture medium. In the absence of proBDNF measurements, the argument for a central role of proBDNF in isofluorane-mediated neuroapoptosis is weakened. However, a key piece of evidence that supports our hypothesis is the effect of Fc-$p75^{NTR}$, a peptide that binds proBDNF[49,50] and reduces $p75^{NTR}$ signaling. This peptide strongly reduced isoflurane toxicity, and its effect was comparable to tPA, plasmin, TAT-Pep5, and siRNA against $p75^{NTR}$. In aggregate, these data strongly suggest that proBDNF-$p75^{NTR}$ signaling plays an important role in isoflurane toxicity. A second limitation is that the means by which isoflurane reduced tPA release are not clear. tPA release is activity-dependent, and isoflurane potently reduces neuronal activity. While there are convincing data to demonstrate the neuronal suppression effect of isoflurane *in vivo*, such assurance cannot be provided for *in vitro* neuronal cultures due to the lack of measurable electrical activity data in cultured neurons. Nonetheless, data showing a decrease in tPA in the culture media after isoflurane exposure does *suggest* a reduction in neuronal activity.[16,17] Finally, the sample sizes in most of the *in vitro* studies are small. This increases the possibility of an α error. However, the consistency of the data between the *in vitro* neuronal cultures and the *in vivo* studies (where sample sizes were larger) argues against this possibility.

Conclusions

In summary, our findings support our hypothesis that anesthetic-induced neurotoxicity in the neonatal mouse central nervous system is a function of decreased synaptic tPA release, reduced mBDNF signaling *via* TrkB, and enhanced proBDNF signaling *via* $p75^{NTR}$ (fig. 6). The results strongly indicate that TAT-Pep5 or other interventions that limit $p75^{NTR}$ signaling or enhance TrkB signaling, may serve as potential therapeutic approaches for the prevention of anesthetic-induced neurotoxicity in the developing central nervous system.

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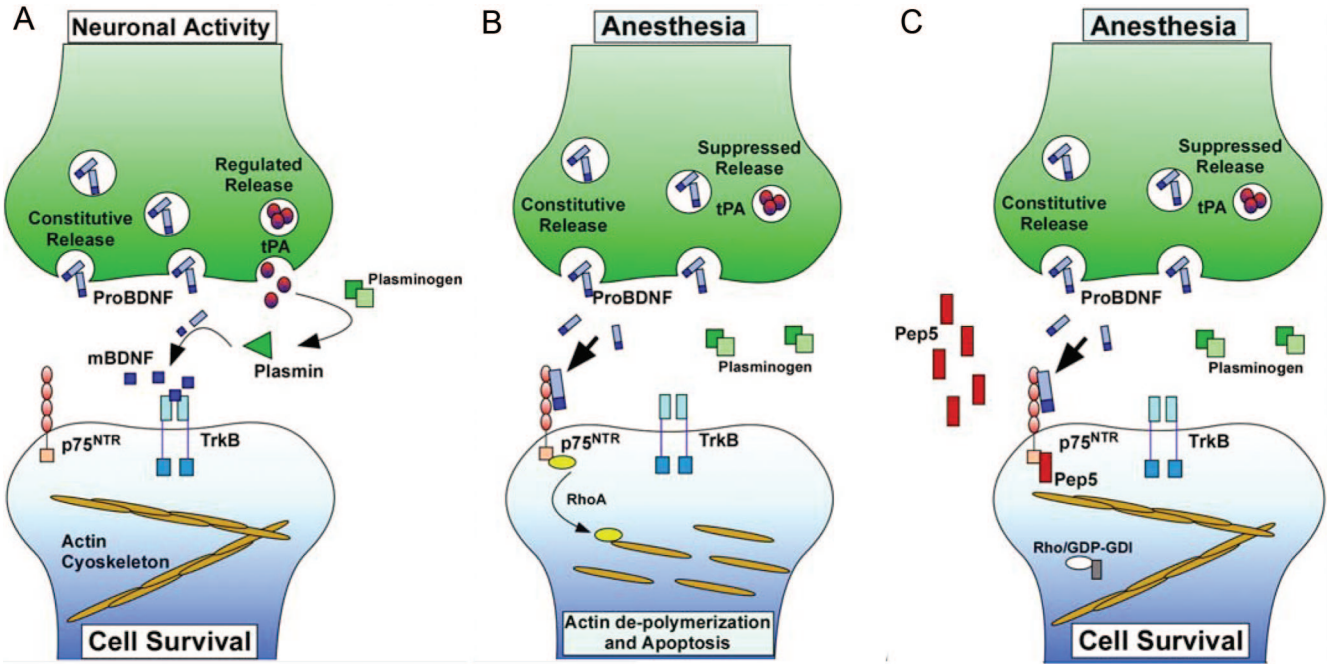

Fig. 6. Schematic demonstrating the ability TAT-Pep5 to block the neurotoxic effects of anesthetics on the developing neurons. (*A*) Neurotrophin brain-derived neurotrophic factor (BDNF) is converted from proBDNF to mature BDNF (mBDNF) by plasmin in the synaptic cleft. Tissue plasminogen activator (tPA), which converts plasminogen to plasmin, is released from presynaptic vesicles upon depolarization. mBDNF promotes neuronal survival through TrkB receptors; proBDNF induces apoptosis through $p75^{NTR}$. (*B*) Exposure of developing neurons (5 days *in vitro* [DIV5] cells and postnatal day 5-7 [5-7PND] pups) to isoflurane results in reduced tPA release into the synaptic cleft, elevated proBDNF, and enhanced activation of p75 neurotrophin receptor ($p75^{NTR}$), leading to apoptosis. (*C*) TAT-Pep5 binds $p75^{NTR}$ intracellularly and blocks ρ-guanosine diphosphate (GDP) (inactive form) from binding to the $p75^{NTR}$. When rho-GDP binds $p75^{NTR}$, it exchanges GDP for guanosine triphosphate (GTP) and becomes activated RhoA, resulting in depolymerization of the actin cytoskeleton, leading to apoptosis. TAT-Pep5 prevents the $p75^{NTR}$-mediated activation of RhoA and subsequent apoptosis in developing neurons exposed to anesthetics.

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