



Published in final edited form as:

Anesthesiology. 2009 April ; 110(4): 813–825. doi:10.1097/ALN.0b013e31819b602b.

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

Brian P. Head, Ph.D. [Postdoctoral Fellow]¹, Hemal H. Patel, Ph.D. [Assistant Professor]¹, Ingrid R. Niesman, M.S. [Research Assistant]¹, John C. Drummond, M.D. [Professor]^{1,2}, David M. Roth, Ph.D., M.D. [Professor]^{1,2}, and Piyush M. Patel, M.D. [Professor]^{1,2}

¹ Department of Anesthesiology, University of California, San Diego, La Jolla, California 92093

² VA San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, USA

Abstract

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 tPA that is released with neuronal activity; mBDNF supports survival and stabilizes synapses through TrkB receptors. We hypothesized that anesthetics suppresses tPA release from neurons, enhances p75^{NTR} signaling, reduces 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% CO₂, 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 prior to isoflurane. TAT-Pep5 was administered by intraperitoneal injection to PND5-7 mice 15 min prior to 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.

Corresponding author: Piyush M. Patel, University of California, San Diego, Department of Anesthesiology, VA Medical Center 125, 3350 La Jolla Village Drive, San Diego, CA 92161-5085 ppatel@ucsd.edu, Phone: 858-552-8585 x6927; Fax: 858-534-0104.

Summary Statement. Isoflurane induced neuroapoptosis, reduction in filopodial spines and reduction in synapse number was attenuated by p75 neurotrophin receptor inhibition in the hippocampus of post-natal day 5-7 mice.

Introduction

A conventional premise of general anesthesia is that anesthetics produce a non-toxic 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 adulthood¹⁻³. Specifically, exposure of post-natal day 5-10 (PND5-10) rat pups to a combination of isoflurane, nitrous oxide and midazolam resulted in substantial neurodegeneration in the hippocampus and neocortex¹ 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, midazolam⁴, diazepam, pentobarbital⁵, thiopental³, N₂O¹ 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 synaptogenesis¹⁰. An important signaling mechanism that contributes to synaptogenesis, as well as consolidation and maturation of synapses, is the neurotrophin BDNF (brain derived neurotrophic factor)¹¹. BDNF has dual function in that it can enhance neuronal survival or cause neuronal apoptosis through activity at either the TrkB (tropomyosin receptor kinase B) or p75^{NTR} (neurotrophic receptor), respectively¹²⁻¹⁴. 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 pre-synaptic vesicles¹⁶. mBDNF preferentially binds to TrkB receptors to promote neuronal survival and synaptogenesis¹⁵. 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 apoptosis¹⁵. Thus tPA can serve to control which effect of BDNF is predominant¹⁷.

The mechanisms by which anesthetics mediate neurotoxicity are not clear. What is common amongst the anesthetics that have proven neurotoxicity in experimental studies is a profound suppression of neuronal activity; this has the potential to adversely impact the tPA-plasminogen-plasmin system. The release of tPA is activity dependent¹⁸ 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 present 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 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 described¹⁹. 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 Neurobasal A media supplemented with B27 (2%), 250 mM GLUTMax1, and penicillin/streptomycin (1%). Cells were cultured on poly-D-lysine/laminin (2 µg/cm²) coated plates or coverslips at 37°C in 5% CO₂ for 5, 14, or 21 days prior to 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 plexi-glass chamber within an incubator and exposed to 1.4% isoflurane, delivered from a calibrated vaporizer, in a gas mixture of 5% CO₂, 21% O₂, 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 4h. Body temperature was monitored with a 8 mm-22 gauge temperature probe (Mon-a-therm model 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 (SDS)-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 (PBS) Tween (1%) containing 4% bovine serum albumin (BSA) and incubated with primary antibody overnight at 4°C as previously described²⁰. 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.
1

Tissue plasminogen activator enzyme-linked immunosorbent assay (ELISA)

Neurons were exposed to isoflurane for 4 h and media from both control and isoflurane treated neurons were frozen at -80°C prior to enzyme-linked immunosorbent assay (ELISA) (Innovative Research, Novi, MI). A 96 well plate was pre-coated with biotinylated PAI-1 (plasminogen activator inhibitor-1) for 30 min followed by 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 isoflurane 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. Following three additional washes, tetramethylbenzidine substrate was added to the wells for 10 min and the reaction was quenched with 1 M H₂SO₄ and read at 450 nm on a spectrophotometer (TECAN Infinite M200, San Jose, CA).

¹free download <http://rsb.info.nih.gov/ij/>. (last accessed 12/19/2008)

Immunofluorescence and Deconvolution Microscopy

Neurons were prepared for immunofluorescence microscopy as previously described²¹. Antibodies used for immunofluorescence were the following: 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 described²¹ 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 ~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 \times (NA 1.4), 60 \times (NA 1.4) and 40 \times (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 were assessed quantitatively by CoLocalizer Pro 1.0 software. 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 values²². 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) (see review by Calabrese et al.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 which 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.

Histological 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 post-fixed 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). 5-10 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% glutaraldehyde, 0.1 M cacodylate buffer with 5 mM CaCl₂. PND5-7 animals were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer and 5 mM CaCl₂ to prevent tissue artifacts. Hippocampi were dissected from whole brains after 24 h and 400 µm vibratome slices prepared and re-fixed 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 re-fixed for an additional 24 h followed by post-fixation with 1% OsO₄ 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 toluidine blue, and re-trimmed to isolate hippocampal areas prior to preparation of grids. Grids (70 nm sections) were stained with uranyl acetate and lead nitrate for contrast and observed on the electron microscope [JEOL 1200 EX-II (Tokyo, Japan)] equipped with a digital camera system. 25 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 filopodial 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 associated with vesicles presynaptically and that lacked cellular organelles or contained a spine apparatus (as indicated by cytoskeletal architecture) postsynaptically as described previously²⁵⁻²⁷. 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 µm²) per animal were analyzed in a blinded fashion for total synapse number per area (synapse #/3350 µm²).

Statistical Analysis

All parametric data were analyzed by unpaired t-tests or ANOVA Bonferroni's 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 (C1-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 matrilin-1 or matrilin-2, mitigate isoflurane-mediated neuronal apoptosis

tPA is a critical modulator of BDNF³¹. Since tPA release is activity dependent¹⁸, and anesthetics suppress neuronal activity, we 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 tPA, from 0.03 to 3 µg/ml, to the medium, dose dependently attenuated isoflurane-mediated increase in C1-Csp3 at 2 h (fig. 2B) post 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), while tPA (3 µg/ml) 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, φp=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 neurons²⁴ (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, φp=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} prior to isoflurane exposure (1.4%, 4 h). Pre-incubation with Fc-p75^{NTR} (1.35 µg/ml) (fig. 4A, n=3, #p=0.001 vs basal, φp=0.003 vs isoflurane) or TAT conjugated Pep5 (10 µM) (fig. 4B, n=3, #p=0.007 vs basal, φp=0.006 vs isoflurane) significantly attenuated isoflurane-mediated neuronal apoptosis. p75^{NTR}-siRNA resulted in significant knockdown (~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, φp=0.001 vs isoflurane-scrambled control) compared to scrambled control (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 prior to isoflurane exposure (1.4%, 4 h). Isoflurane significantly enhanced cleaved caspase 3 (C1-Csp3) in the CA1 (n=6, #p=0.003) and CA3 (n=7, δp=0.007) of the hippocampus in PND5 mice (fig. 4D) 2h post exposure. TAT-Pep5 significantly attenuated isoflurane-mediated increase in C1-Csp3 in both the CA1 (n=7, φp=0.007) and CA3 (n=7, λp=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 electron microscopy. Synapses, with post-synaptic densities and presynaptic vesicles, are clearly visible in representative control images (fig. 5A-a). By contrast, there was a significant reduction in total synapses in PND5-7 pups exposed to isoflurane (fig. 5A-b; $n=6$, $\#p=0.01$). This reduction in synapses was significantly attenuated by pre-treatment with TAT-Pep5 (fig. 5A-c; $n=5$, $\phi p=0.01$). Higher magnification (6600 \times) EM images for control (fig. 5B-a), isoflurane (fig. 5B-b), and TAT-Pep5 (fig. 5B-c) are shown in fig. 5B. Quantitation of data is represented by the graph fig. 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 present study are consistent with the neurotoxicity of isoflurane that has been demonstrated previously¹⁻³. Primary neurons (DIV5) and mouse pups (PND5-7) were vulnerable to injury during the period of synaptogenesis whereas the toxicity was not apparent at the age of 14 (DIV) 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*, isoflurane mediated injury to the CA1 and CA3 sectors of the hippocampus was apparent. Administration of TAT-Pep5 prior to 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 that indicates that the brain is vulnerable to anesthetic neurotoxicity during synaptogenesis¹⁰. In the developing brain, synaptic connections with appropriate target neurons are essential for neuronal survival as neurons are dependent upon trophic support from their targets³²⁻³⁴. Loss of synaptic connection leads to apoptosis. An important element contributing to synaptogenesis, as well as consolidation and maturation of synapses is the neurotrophin BDNF¹¹. Neurotrophins are synthesized as pro-neurotrophins (proNTs), which subsequently undergo proteolytic cleavage to yield the biologically active mature neurotrophins³⁵. The mature form of BDNF (mBDNF) preferentially bind to the TrkB receptor to promote neuronal survival and synaptogenesis¹³. proBDNF serves as a ligand for the p75^{NTR} and initiates neuronal apoptosis¹². Up to 60% of the secreted neurotrophins are in the pro-form¹⁵. 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 tissue plasminogen activator (tPA). tPA is secreted from pre-synaptic terminals, and its secretion is activity-dependent¹⁶⁻¹⁸.

Our data show that isoflurane reduces the amount of tPA present in culture media of 5 day old primary neurons. tPA is released from neurons in an activity dependent manner¹⁶⁻¹⁸ and this reduction in tPA may be due to neuronal suppression by isoflurane. By contrast, proBDNF undergoes both activity dependent and constitutive release¹⁵. 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} activity³⁶, 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. Prior application of tPA led to the activation of Akt (P-Akt), a downstream kinase of TrkB signaling critical to neuronal survival³⁷, attenuated anesthetic induced neurotoxicity as well as the loss of synaptic filopodial spines in developing neurons. The present studies utilized 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 non-proteolytic activities. tPA binds to the low density lipoprotein receptor (LRP-1) and triggers signaling cascades that lead to phosphorylation of ERK1/2³⁸ and Akt³⁹; this effect of tPA is independent of its proteolytic activity. Both ERK1/2⁴⁰ and Akt⁴¹ have anti-apoptotic activity. The LRP-1 receptor is expressed in neurons and glia³⁹. tPA also can bind to and directly activate the urokinase-like plasminogen activator receptor, or uPAR, and trigger intracellular signaling⁴². The possibility that the reduction in anesthetic neurotoxicity might be a function of the non-proteolytic effects of tPA (activation of survival signaling) exists. Aprotinin is a serine protease inhibitor of the proteolytic activity of tPA^{12,43}. Data which show 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 pre-treatment of neurons (DIV5) with 4-aminopyridine, a potassium channel blocker which serves to enhance vesicular release from pre-synaptic terminals⁴⁴, 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, growth cone collapse, and initiates neuronal apoptosis^{14,45-47}. In p75^{NTR}^{-/-} mice, dendritic complexity, which is increased substantially, can be reduced by p75^{NTR} overexpression⁴⁸, 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, either by extracellular inhibition with Fc-p75^{NTR}, intracellular inhibition with TAT-Pep5, or by knockdown of p75^{NTR} with siRNA, was clearly reduced isoflurane mediated injury. Importantly, though, the interventions did not directly injure neurons as indicated by the fact that Cl-Csp3 immunoblot (fig. 4A-C) was similar in the non-anesthetized 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, while p75^{NTR} inhibition was effective in reducing isoflurane neurotoxicity, the basal level of apoptosis (Cl-Csp3 immunoblot) was not different in the non-anesthetized control and p75^{NTR} inhibition groups (fig. 4A-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 presently clear. In addition the effect of long-term inhibition of p75^{NTR} during synaptogenesis needs further investigation.

In the majority of studies of anesthetic neurotoxicity, the focus has been on neuroapoptosis. However, the present investigation indicates that the injury is not restricted to neuronal death but 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*. The majority of excitatory synapses are located on dendritic filopodial spines⁵¹. The loss of filopodial spines therefore would 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 ablation of existing synapses compared to non-anesthetized control state (fig. 5). However, a limitation to the latter EM findings is the absence of measurements of pre-synaptic profile diameter, active zone length, or number of synaptic vesicles across conditions in addition to alterations in organelle and dendritic shaft volume. More in depth EM analysis in future studies may yield a comprehensive morphologic profile of changes that occur with isoflurane and other anesthetics during synaptogenesis. The present 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 that are 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 (~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 form⁵³. 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 isoflurane 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 isoflurane 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 following isoflurane exposure does suggest a reduction in neuronal activity^{16,17}. Finally, in most of the *in vitro* studies, the sample sizes 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 CNS is a function 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 CNS.

Acknowledgments

We are grateful for the assistance from the University of California San Diego Cancer Center Digital Imaging Shared Resource, in particular James Feramisco, Ph.D. (Professor of Medicine, University of California, San Diego, La Jolla, California), Kersi Pestonjamas, Ph.D. (Junior Faculty, University of California, San Diego, La Jolla, California), Steve McMullen, B.A., J.D. (technician, University of California, San Diego, La Jolla, California). We are also grateful for the technical support from Yue Hu, B.S. (technician, University of California, San Diego, La Jolla, California), Michael Kidd, B.S. (technician, University of California, San Diego, La Jolla, California), Blake Chin-Lee, B.S. (technician, University of California, San Diego, La Jolla, California), and Stephanie Cipta, B.S. (technician, University of California, San Diego, La Jolla, California). In some cases, three-dimensional perspective views were made at the VisLab in the San Diego Supercomputer Center using National Partnership for Advanced Computational Infrastructure Scalable Visualization Tools.

Funding. This work is supported by National Institutes of Health, Bethesda, Maryland, RO1 NS 047570 (P. M. Patel), National Institutes of Health, Bethesda, Maryland, RO1 HL091071-01 (H. H. Patel), and Merit Award from the Department of Veterans Affairs (D. M. Roth).

References

1. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003;23:876–82. [PubMed: 12574416]
2. Jevtovic-Todorovic V, Wozniak DF, Benshoff ND, Olney JW. A comparative evaluation of the neurotoxic properties of ketamine and nitrous oxide. *Brain Res* 2001;895:264–7. [PubMed: 11259788]
3. Fredriksson A, Ponten E, Gordh T, Eriksson P. Neonatal Exposure to a Combination of N-Methyl-d-aspartate and gamma-Aminobutyric Acid Type A Receptor Anesthetic Agents Potentiates Apoptotic

- Neurodegeneration and Persistent Behavioral Deficits. *Anesthesiology* 2007;107:427–36. [PubMed: 17721245]
4. Young C, Jevtovic-Todorovic V, Qin YQ, Tenkova T, Wang H, Labruyere J, Olney JW. Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. *Br J Pharmacol* 2005;146:189–97. [PubMed: 15997239]
 5. Olney JW, Ishimaru MJ, Bittigau P, Ikonomidou C. Ethanol-induced apoptotic neurodegeneration in the developing brain. *Apoptosis* 2000;5:515–21. [PubMed: 11303910]
 6. Honegger P, Matthieu JM. Selective toxicity of the general anesthetic propofol for GABAergic neurons in rat brain cell cultures. *J Neurosci Res* 1996;45:631–6. [PubMed: 8875327]
 7. Spahr-Schopfer I, Vutskits L, Toni N, Buchs PA, Parisi L, Muller D. Differential neurotoxic effects of propofol on dissociated cortical cells and organotypic hippocampal cultures. *Anesthesiology* 2000;92:1408–17. [PubMed: 10781288]
 8. Vutskits L, Gascon E, Tassonyi E, Kiss JZ. Clinically relevant concentrations of propofol but not midazolam alter in vitro dendritic development of isolated gamma-aminobutyric acid-positive interneurons. *Anesthesiology* 2005;102:970–6. [PubMed: 15851884]
 9. Wise-Faberowski L, Zhang H, Ing R, Pearlstein RD, Warner DS. Isoflurane-induced neuronal degeneration: an evaluation in organotypic hippocampal slice cultures. *Anesth Analg* 2005;101:651–7. [PubMed: 16115969]
 10. Viberg H, Ponten E, Eriksson P, Gordh T, Fredriksson A. Neonatal ketamine exposure results in changes in biochemical substrates of neuronal growth and synaptogenesis, and alters adult behavior irreversibly. *Toxicology* 2008;249:153–9. [PubMed: 18550250]
 11. Luikart BW, Nef S, Virmani T, Lush ME, Liu Y, Kavalali ET, Parada LF. TrkB has a cell-autonomous role in the establishment of hippocampal Schaffer collateral synapses. *J Neurosci* 2005;25:3774–86. [PubMed: 15829629]
 12. Lee R, Kerami P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. *Science* 2001;294:1945–8. [PubMed: 11729324]
 13. Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* 2004;306:487–91. [PubMed: 15486301]
 14. Lu LX, Yon JH, Carter LB, Jevtovic-Todorovic V. General anesthesia activates BDNF-dependent neuroapoptosis in the developing rat brain. *Apoptosis* 2006;11:1603–15. [PubMed: 16738805]
 15. Lu B. Pro-region of neurotrophins: role in synaptic modulation. *Neuron* 2003;39:735–8. [PubMed: 12948441]
 16. Baranes D, Lederfein D, Huang YY, Chen M, Bailey CH, Kandel ER. Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. *Neuron* 1998;21:813–25. [PubMed: 9808467]
 17. Lochner JE, Spangler E, Chavarha M, Jacobs C, McAllister K, Schuttner LC, Scalettar BA. Efficient copackaging and cotransport yields postsynaptic colocalization of neuromodulators associated with synaptic plasticity. *Dev Neurobiol* 2008;68:1243–56. [PubMed: 18563704]
 18. Gualandris A, Jones TE, Strickland S, Tsirka SE. Membrane depolarization induces calcium-dependent secretion of tissue plasminogen activator. *J Neurosci* 1996;16:2220–5. [PubMed: 8601802]
 19. Huettner JE, Baughman RW. Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci* 1986;6:3044–60. [PubMed: 3760948]
 20. Akram A, Christoffel D, Rocher AB, Bouras C, Kovari E, Perl DP, Morrison JH, Herrmann FR, Haroutunian V, Giannakopoulos P, Hof PR. Stereologic estimates of total spinophilin-immunoreactive spine number in area 9 and the CA1 field: Relationship with the progression of Alzheimer's disease. *Neurobiol Aging* 2008;29:1296–307. [PubMed: 17420070]
 21. Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA. G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *J Biol Chem* 2005;280:31036–44. [PubMed: 15961389]
 22. Zinchuk O, Fukushima A, Hangstefer E. Dynamics of PAF-induced conjunctivitis reveals differential expression of PAF receptor by macrophages and eosinophils in the rat. *Cell Tissue Res* 2004;317:265–77. [PubMed: 15290234]

23. Calabrese B, Wilson MS, Halpain S. Development and regulation of dendritic spine synapses. *Physiology (Bethesda)* 2006;21:38–47. [PubMed: 16443821]
24. Sekino Y, Kojima N, Shirao T. Role of actin cytoskeleton in dendritic spine morphogenesis. *Neurochem Int* 2007;51:92–104. [PubMed: 17590478]
25. Bamji SX, Shimazu K, Kimes N, Huelsken J, Birchmeier W, Lu B, Reichardt LF. Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. *Neuron* 2003;40:719–31. [PubMed: 14622577]
26. Bouwman J, Maia AS, Camoletto PG, Posthuma G, Roubos EW, Oorschot VM, Klumperman J, Verhage M. Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. *Neuroscience* 2004;126:115–26. [PubMed: 15145078]
27. Elia LP, Yamamoto M, Zang K, Reichardt LF. p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. *Neuron* 2006;51:43–56. [PubMed: 16815331]
28. Hara Y, Pickel VM. Dendritic distributions of dopamine D1 receptors in the rat nucleus accumbens are synergistically affected by startle-evoking auditory stimulation and apomorphine. *Neuroscience* 2007;146:1593–605. [PubMed: 17490822]
29. Medvedev NI, Rodriguez-Arellano JJ, Popov VI, Davies HA, Tigaret CM, Schoepfer R, Stewart MG. The glutamate receptor 2 subunit controls post-synaptic density complexity and spine shape in the dentate gyrus. *Eur J Neurosci* 2008;27:315–25. [PubMed: 18215230]
30. Jevtovic-Todorovic V, Beals J, Benshoff N, Olney JW. Prolonged exposure to inhalational anesthetic nitrous oxide kills neurons in adult rat brain. *Neuroscience* 2003;122:609–16. [PubMed: 14622904]
31. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. *Nat Rev Neurosci* 2005;6:603–14. [PubMed: 16062169]
32. Clarke PG. Neuronal death during development in the isthmo-optic nucleus of the chick: sustaining role of afferents from the tectum. *J Comp Neurol* 1985;234:365–79. [PubMed: 3988990]
33. Oppenheim RW. Cell death during development of the nervous system. *Annu Rev Neurosci* 1991;14:453–501. [PubMed: 2031577]
34. Vicario-Abejon C, Collin C, McKay RD, Segal M. Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J Neurosci* 1998;18:7256–71. [PubMed: 9736647]
35. Edwards RH, Selby MJ, Garcia PD, Rutter WJ. Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. *J Biol Chem* 1988;263:6810–5. [PubMed: 3360808]
36. Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EB, Said F, Bonni A, Barker PA. Apoptosis induced by p75NTR overexpression requires Jun kinase-dependent phosphorylation of Bad. *J Neurosci* 2003;23:11373–81. [PubMed: 14673001]
37. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 1997;275:661–5. [PubMed: 9005851]
38. Hu K, Lin L, Tan X, Yang J, Bu G, Mars WM, Liu Y. tPA protects renal interstitial fibroblasts and myofibroblasts from apoptosis. *J Am Soc Nephrol* 2008;19:503–14. [PubMed: 18199803]
39. An J, Zhang C, Polavarapu R, Zhang X, Zhang X, Yepes M. Tissue-type plasminogen activator and the low-density lipoprotein receptor-related protein induce Akt phosphorylation in the ischemic brain. *Blood* 2008;112:2787–94. [PubMed: 18628488]
40. Hetman M, Xia Z. Signaling pathways mediating anti-apoptotic action of neurotrophins. *Acta Neurobiol Exp (Wars)* 2000;60:531–45. [PubMed: 11200182]
41. Chong ZZ, Li F, Maiese K. Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. *Histol Histopathol* 2005;20:299–315. [PubMed: 15578447]
42. Maupas-Schwalm F, Auge N, Robinet C, Cambus JP, Parsons SJ, Salvayre R, Negre-Salvayre A. The sphingomyelin/ceramide pathway is involved in ERK1/2 phosphorylation, cell proliferation, and uPAR overexpression induced by tissue-type plasminogen activator. *Faseb J* 2004;18:1398–400. [PubMed: 15231724]
43. Kang HM, Kalnoski MH, Frederick M, Chandler WL. The kinetics of plasmin inhibition by aprotinin in vivo. *Thromb Res* 2005;115:327–40. [PubMed: 15668193]

44. Baldwin ML, Cammarota M, Sim AT, Rostas JA. Src family tyrosine kinases differentially modulate exocytosis from rat brain nerve terminals. *Neurochem Int* 2006;49:80–6. [PubMed: 16500731]
45. Dechant G, Barde YA. The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 2002;5:1131–6. [PubMed: 12404007]
46. Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev* 2000;14:2919–37. [PubMed: 11114882]
47. Ciruela F, Canela L, Burgueno J, Soriguera A, Cabello N, Canela EI, Casado V, Cortes A, Mallol J, Woods AS, Ferre S, Lluís C, Franco R. Heptaspanning membrane receptors and cytoskeletal/scaffolding proteins: focus on adenosine, dopamine, and metabotropic glutamate receptor function. *J Mol Neurosci* 2005;26:277–92. [PubMed: 16012201]
48. Zagrebelsky M, Holz A, Dechant G, Barde YA, Bonhoeffer T, Korte M. The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons. *J Neurosci* 2005;25:9989–99. [PubMed: 16251447]
49. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M. Expression and structure of the human NGF receptor. *Cell* 1986;47:545–54. [PubMed: 3022937]
50. Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD. The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J Cell Biol* 1998;140:911–23. [PubMed: 9472042]
51. Tada T, Sheng M. Molecular mechanisms of dendritic spine morphogenesis. *Curr Opin Neurobiol* 2006;16:95–101. [PubMed: 16361095]
52. Nikizad H, Yon JH, Carter LB, Jevtovic-Todorovic V. Early exposure to general anesthesia causes significant neuronal deletion in the developing rat brain. *Ann N Y Acad Sci* 2007;1122:69–82. [PubMed: 18077565]
53. Matsumoto T, Rauskolb S, Polack M, Klose J, Kolbeck R, Korte M, Barde YA. Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. *Nat Neurosci* 2008;11:131–3. [PubMed: 18204444]

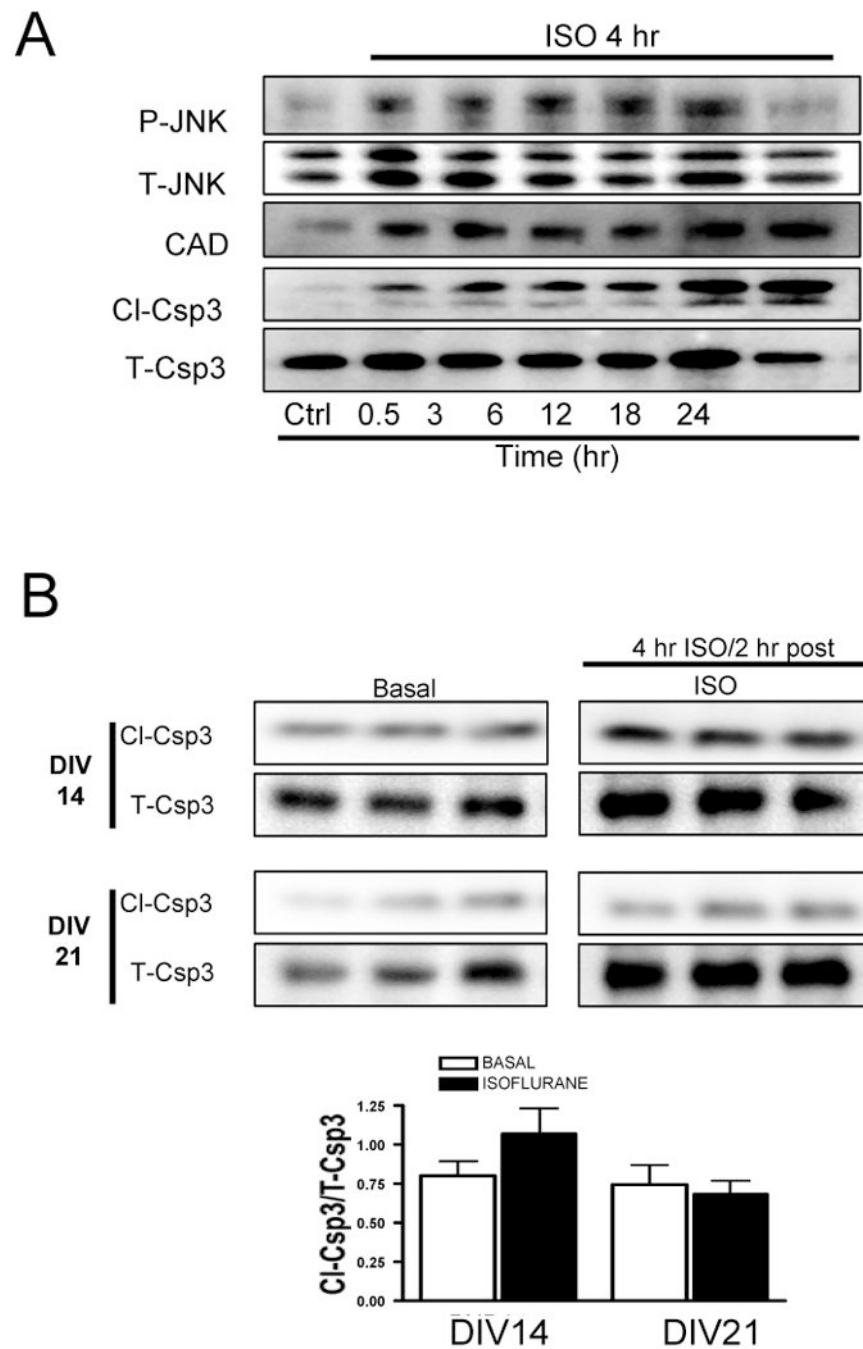


Fig. 1. Isoflurane exposure increases cell death in DIV5, but not DIV14 or DIV21 *in vitro*. Primary neurons (5 days in vitro - DIV) were exposed to 1.4% isoflurane for 4 h and neurons were lysed at various time intervals post exposure. The pro-apoptotic proteins, P-JNK, CAD, and cleaved caspase-3 (Cl-Csp3), were enhanced at 0.5, 3, 6, 12, 18, and 24 h (with the exception of P-JNK which returned to basal levels prior to 24 hr) post isoflurane exposure as shown in 1A. Primary neonatal neurons (DIV14 and DIV21) were exposed to isoflurane and representative immunoblots are shown in Fig 1B. Isoflurane slightly increased (not significant) apoptosis in DIV14 ($p=0.2191$, $n=3$) but 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, standard error of the mean (s.e.m.).

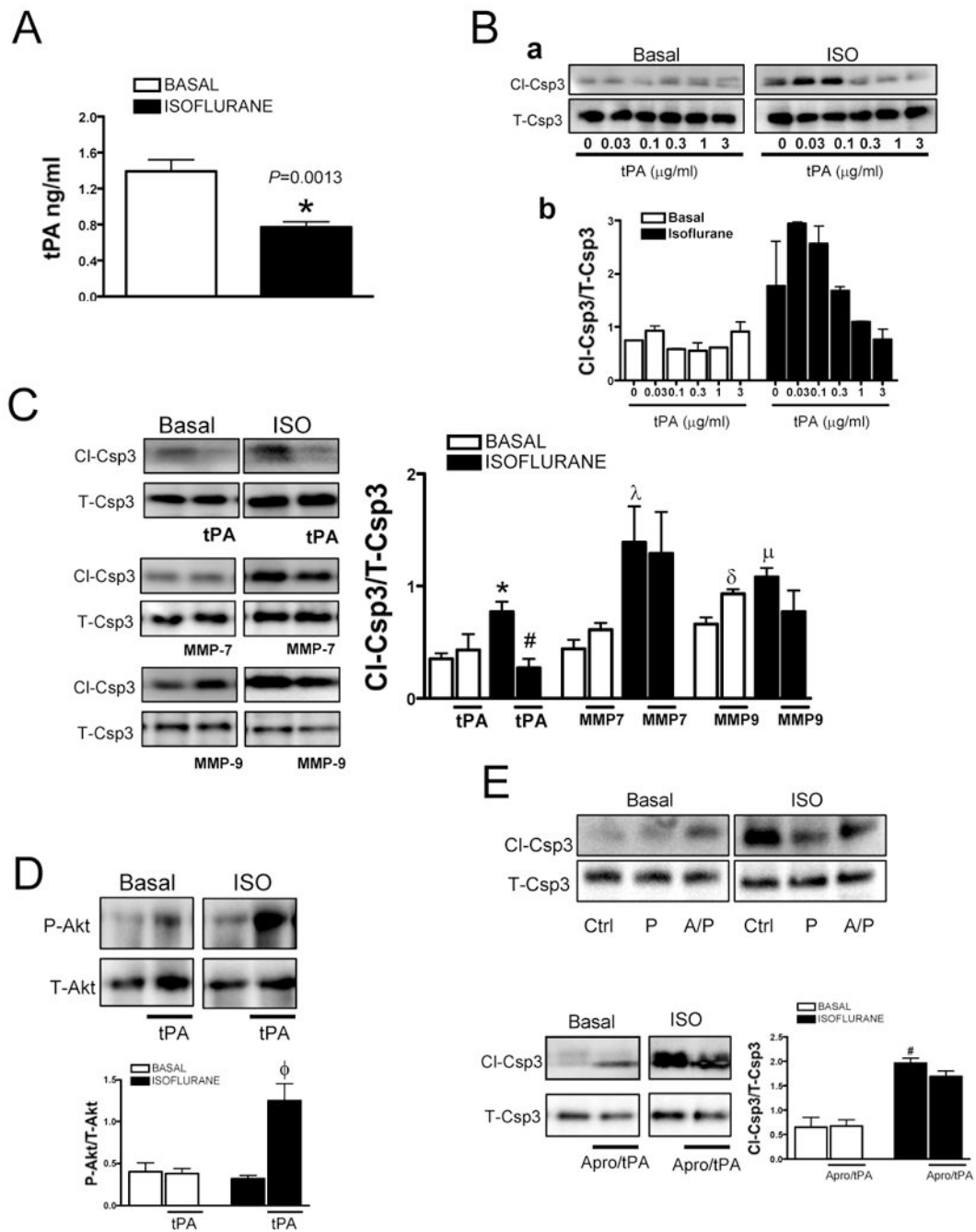


Fig. 2. Tissue plasminogen activator (tPA) decreases isoflurane-induced cell death in DIV5 primary neurons. Primary neurons (5 days *in vitro* - DIV) were exposed to 1.4% isoflurane for 4 h and the media was frozen 2 h post exposure at -80°C prior to tPA ELISA. The media from neurons exposed to isoflurane (4 h - 1.4%; 2 h post) contained significantly (*n=6, *p*=0.001) less tPA compared to media from non-exposed cells (~45% decrease; 1.40 ng/ml-BASAL vs 0.77 ng/ml-isoflurane) as shown in 2A. Neurons were pre-treated with increasing doses of tPA (0.03 to 3 μg/ml) prior to isoflurane (4 h - 1.4%; 2 h post) and neuronal cell death was assessed 2 h post-exposure with the apoptotic marker, cleaved caspase 3 (CI-Csp3) (fig. 2B). Increasing

doses of tPA decreased isoflurane-induced Cl-Csp3 with a maximum decrease at 3 $\mu\text{g/ml}$ as shown in 2Ba,b. tPA (3 $\mu\text{g/ml}$) ($n=3$, $\#p=0.02$), but not matrix metalloproteinases-7 (2 $\mu\text{g/ml}$) or matrix metalloproteinases-9 (1 $\mu\text{g/ml}$), significantly decreased isoflurane-induced Cl-Csp3 as shown in 2C. Matrix metalloproteinases-9 significantly elevated Cl-Csp3 under basal conditions ($p=0.01$). tPA significantly ($n=3$, $\phi p=0.002$) enhanced the pro-survival kinase P-Akt even in the presence of isoflurane as shown in 2D. Application of the serine protease inhibitor, aprotinin (3 $\mu\text{g/ml}$), blocked the protective effect of plasmin (3 $\mu\text{g/ml}$), (upper blots) and tPA (lower blots) ($n=3$, $\#p=0.004$) as shown in 2E. Error bars, standard error of the mean (s.e.m.).

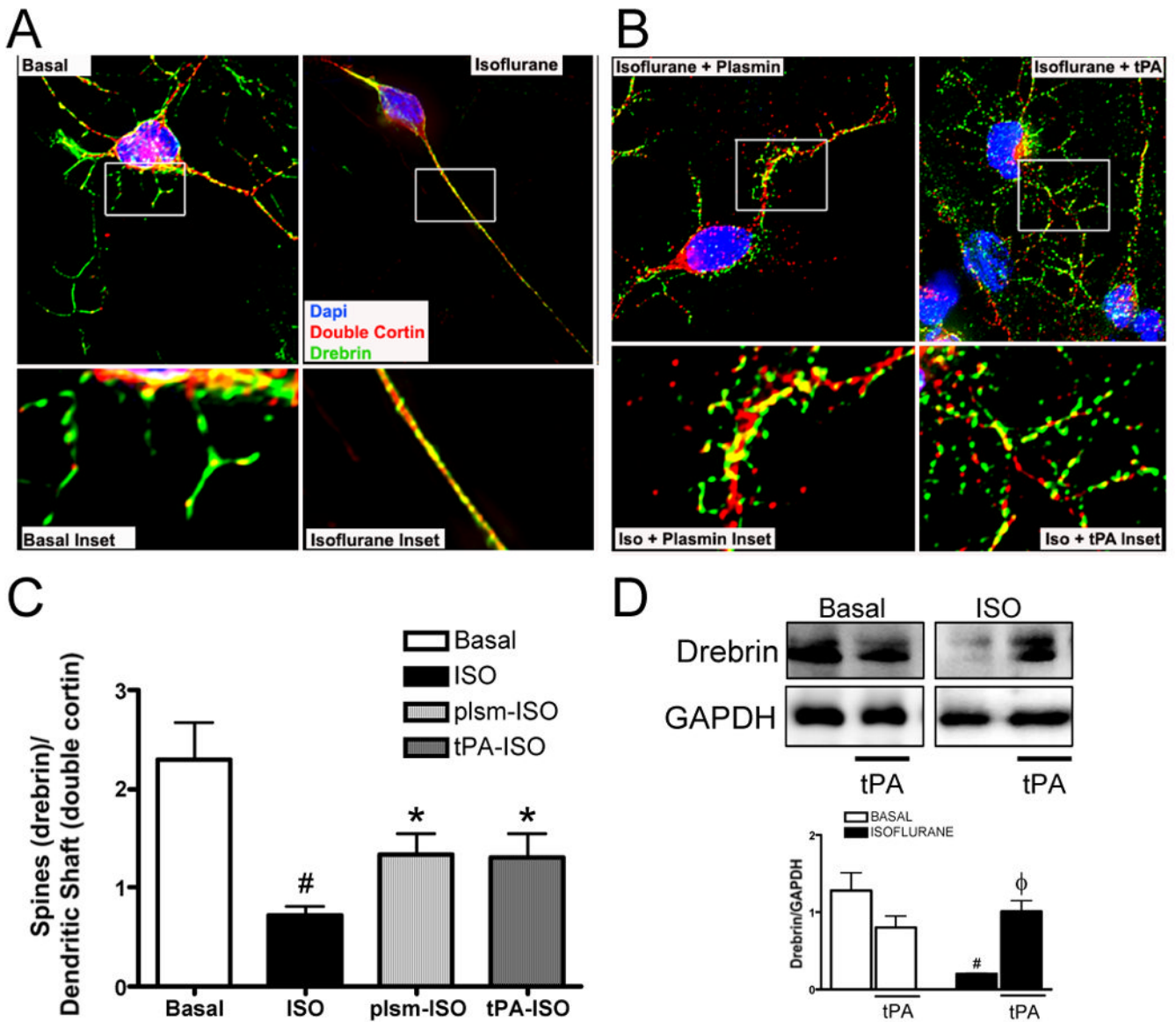


Fig. 3. Isoflurane treatment decreases dendritic filopodia (immature filopodial spines) in neonatal primary neurons as assessed by neuronal F-actin (drebrin) immunofluorescence microscopy. Neonatal primary neurons (5 days *in vitro* – DIV) were exposed to isoflurane (4 h - 1.4%) and stained for the dendritic spine/neuronal F-actin marker, drebrin, and the dendritic shaft/neuronal microtubule marker, doublecortin, 2 h post exposure. Drebrin staining along dendritic shafts was significantly ($n=6$, $\#p=0.01$) decreased following isoflurane treatment as shown in 3A. Treatment with the proteases, plasmin (3 $\mu\text{g/ml}$) ($n=6$, $*p=0.02$) or tPA (3 $\mu\text{g/ml}$) ($n=5$, $*p=0.04$) significantly attenuated the isoflurane-induced loss of neuronal dendritic filopodial spines as shown in 3B. Quantitation is expressed as drebrin (green pixels) normalized to doublecortin immunofluorescence (red pixels) along the dendrites only (no soma immunofluorescence was included) as shown in 3C. Isoflurane significantly ($n=3$, $\#p=0.006$) decreased drebrin protein expression; tPA significantly ($n=3$, $\phi p<0.005$) mitigated isoflurane-mediated reduction in drebrin (top panel: representative blots; lower graph: densitometry) as shown in 3D. 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, standard error of the mean (s.e.m.).

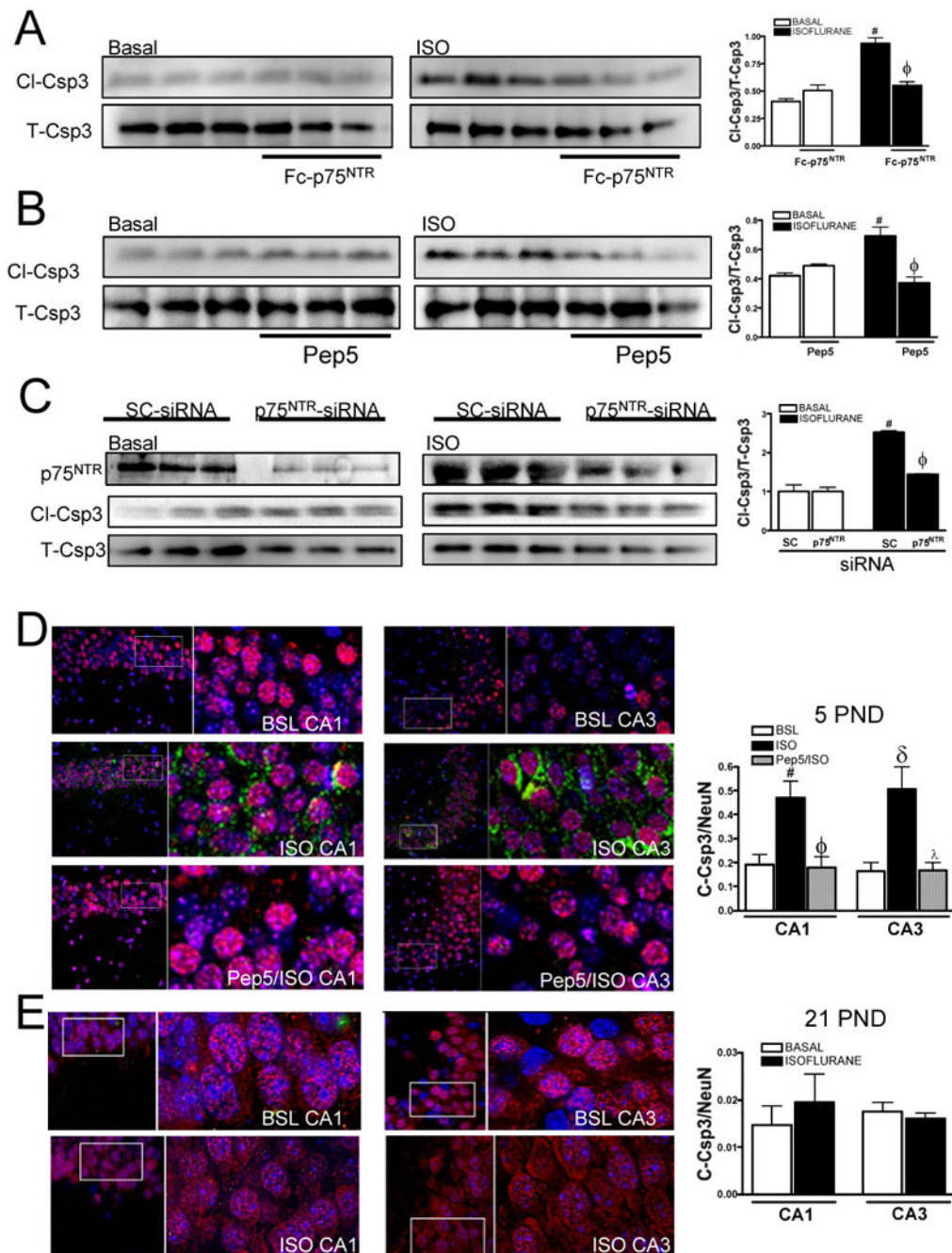


Fig. 4. Pharmacological inhibition of the p75^{NTR} or siRNA-mediated knockdown attenuates isoflurane-induced apoptosis in DIV5 neonatal neurons. Primary neurons (5 days *in vitro* – DIV) were treated with the p75^{NTR} inhibitors (Fc-p75^{NTR} or TAT-Pep5) or siRNA for p75^{NTR} prior to isoflurane exposure (4 h - 1.4%) (left panels: representative immunoblots; right panels: densitometry). Pre-incubation with Fc-p75^{NTR} (1.35 μ g/ml; n=3, #p=0.001 vs basal, ϕ p=0.003 vs isoflurane) or TAT conjugated Pep5 (10 μ M; n=3, #p=0.007 vs basal, ϕ p=0.006 vs isoflurane) significantly attenuated isoflurane-mediated neuronal apoptosis as shown in 4A and 4B respectively. p75^{NTR}-siRNA resulted in significant knockdown of the

neurotrophin receptor (~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; $n=3$, $\phi p=0.001$ vs isoflurane scrambled control) as shown in 4C. Neonatal C57BL/6 mice were given TAT-Pep5 IP (10 μM) 15 min prior to isoflurane exposure (4 h - 1.4%) as shown in 4D. Isoflurane significantly enhanced cleaved caspase 3 (Cl-Csp3) in the CA1 ($n=6$, $\#p=0.003$) and CA3 ($n=7$, $\delta p=0.007$) of the hippocampus in PND5-7 mice *in vivo*. TAT-Pep5 significantly attenuated isoflurane-mediated increases in Cl-Csp3 in both CA1 ($n=7$, $\phi p=0.003$) and CA3 ($n=7$, $\lambda p=0.007$) regions. In contrast, isoflurane exposure did not significantly enhance Cl-Csp3 in PND21 mice ($n=4$) as represented in 4E. Error bars, standard error of the mean (s.e.m.).

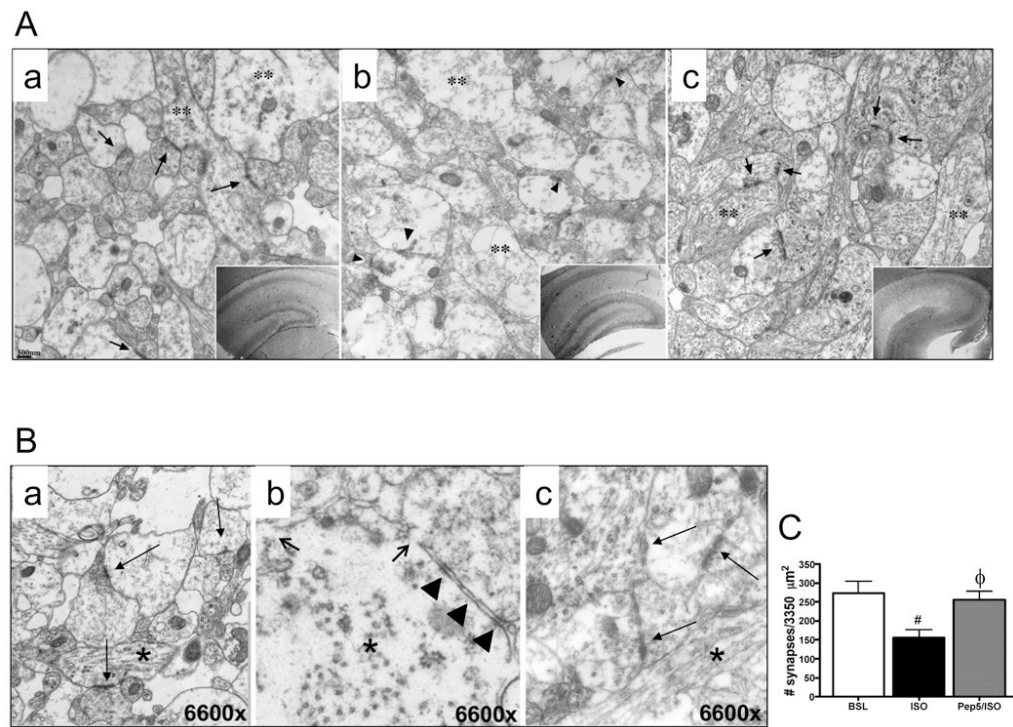


Fig. 5. TAT-Pep5, a p75^{NTR} inhibitor, attenuates isoflurane-mediated loss of synapses in the CA3 and CA1 of 5-day-old C57BL/6 mice *in vivo*. Fig 5A: Electron microscopy shows that isoflurane (b) significantly ($n=6$, $\#p=0.01$) decreased the number of synapses (arrowheads) in the hippocampus compared to basal (arrows) (a, $n=4$) in PND5 mice. TAT-Pep5 (c) significantly ($n=5$, $\phi p=0.01$) attenuated isoflurane-mediated decrease in hippocampal synapses (c-arrows). Double asterisks (**) represent dendritic shafts/spine heads. Scale bar, 500 nm. Insets are light microscopic images of representative surveyed hippocampi (Mag, 5 \times). Fig 5B: Higher magnification image shows disintegration of post-synaptic densities (arrowheads) and membrane disruption (open arrows) following isoflurane exposure (b). Intact (a and c) and disrupted (b) cytoskeleton are indicated by a single asterisk (*). Quantitation of the data is represented by the graph in 5C. Scale bar, 500 nm. Error bars, standard error of the mean (s.e.m.).

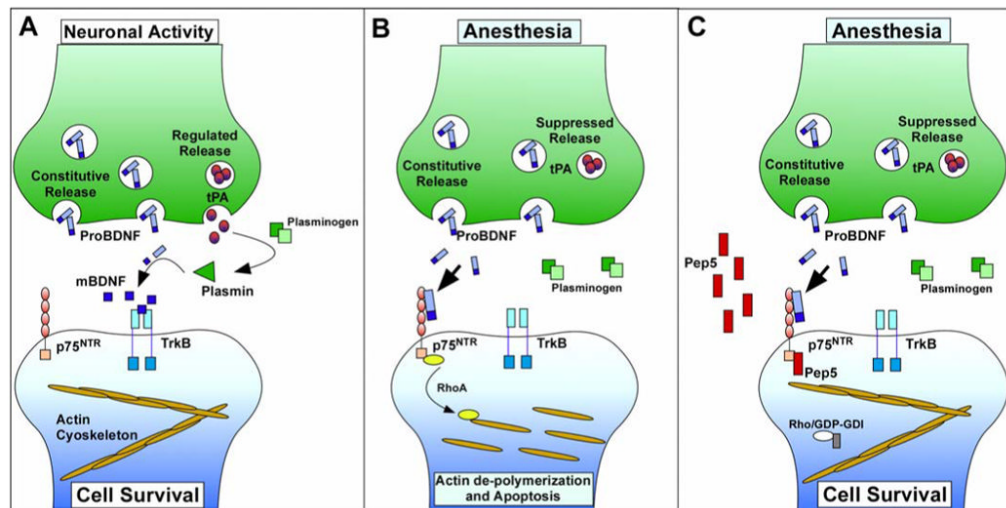


Fig. 6.

Schematic demonstrating the ability TAT-Pep5 to block the neurotoxic effects of anesthetics on the developing neurons. Fig. 6a: Neurotrophin BDNF is converted from proBDNF to mature (m)BDNF by plasmin in the synaptic cleft. Tissue plasminogen activator (tPA), which converts plasminogen to plasmin, is released from pre-synaptic vesicles upon depolarization. mBDNF promotes neuronal survival through TrkB receptors; proBDNF induces apoptosis through p75^{NTR}. Fig. 6b: Exposure of developing neurons (DIV5 cells and 5-7 PND 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. Fig. 6c: TAT-Pep5 binds p75^{NTR} intracellularly and blocks Rho-GDP (inactive form) from binding to the p75^{NTR}. When Rho-GDP binds p75^{NTR}, it exchanges GDP for 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.