Astrocytes Protect against Isoflurane Neurotoxicity by Buffering pro-brain–derived Neurotrophic Factor

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ABSTRACT

Background: Isoflurane induces cell death in neurons undergoing synaptogenesis *via* increased production of pro-brain–derived neurotrophic factor (proBDNF) and activation of postsynaptic p75 neurotrophin receptor ($p75^{NTR}$). Astrocytes express $p75^{NTR}$, but their role in neuronal $p75^{NTR}$-mediated cell death remains unclear. The authors investigated whether astrocytes have the capacity to buffer increases in proBDNF and protect against isoflurane/$p75^{NTR}$ neurotoxicity.

Methods: Cell death was assessed in day *in vitro* (DIV) 7 mouse primary neuronal cultures alone or in co-culture with age-matched or DIV 21 astrocytes with propidium iodide 24 h after 1 h exposure to 2% isoflurane or recombinant proBDNF. Astrocyte-targeted knockdown of $p75^{NTR}$ in co-culture was achieved with small-interfering RNA and astrocyte-specific transfection reagent and verified with immunofluorescence microscopy. proBDNF levels were assessed by enzyme-linked immunosorbent assay. Each experiment used six to eight replicate cultures/condition and was repeated at least three times.

Results: Exposure to isoflurane significantly ($P < 0.05$) increased neuronal cell death in primary neuronal cultures (1.5 ± 0.7 fold, mean ± SD) but not in co-culture with DIV 7 (1.0 ± 0.5 fold) or DIV 21 astrocytes (1.2 ± 1.2 fold). Exogenous proBDNF dose dependently induced neuronal cell death in both primary neuronal and co-cultures, an effect enhanced by astrocyte $p75^{NTR}$ inhibition. Astrocyte-targeted $p75^{NTR}$ knockdown in co-cultures increased media proBDNF (1.2 ± 0.1 fold) and augmented isoflurane-induced neuronal cell death (3.8 ± 3.1 fold).

Conclusions: The presence of astrocytes provides protection to growing neurons by buffering increased levels of proBDNF induced by isoflurane. These findings may hold clinical significance for the neonatal and injured brain where increased levels of proBDNF impair neurogenesis. **(Anesthesiology 2015; 123:810-9)**

ANESTHETIC neurotoxicity, characterized in animal models by widespread induction of neuronal cell death, disruption in synapse formation and stabilization, and impairment of neurocognitive development, appears to occur primarily during the period of active synaptogenesis.[1] A primary mechanism contributing to anesthetic neurotoxicity during this period has been attributed to the modulation of brain-derived neurotrophic factor (BDNF),[2,3] a central regulator of neurogenesis,[4] synaptogenesis,[5] and neurotransmission.[6] In addition to playing a critical role in normal brain development,[7] BDNF signaling is instrumental to learning and long-term memory consolidation[8,9] and repair of the brain and spinal cord after injury in the adult.[10,11] Whether BDNF induces prodeath or prosurvival signaling in postsynaptic neurons is determined by the relative balance of the proneurotrophin form of BDNF (proBDNF) and the proteolytically cleaved mature form. Postsynaptic signaling of mature BDNF promotes neurite formation and stabilization of existing synapses *via* activation of tropomyosin receptor kinase B, whereas proBDNF signaling induces postsynaptic neuronal death *via* activation of p75 neurotrophin receptor ($p75^{NTR}$, or low-affinity nerve growth factor receptor). Anesthetic neurotoxicity occurs *via* decreased proteolytic cleavage of proBDNF,[2] leading to augmentation of postsynaptic proBDNF/$p75^{NTR}$ binding, resulting in disruption of synaptogenesis and induction of postsynaptic neuronal death. This observation has generated concern about potential effects of volatile anesthetic exposure on brain development in neonates and the possibility of neurocognitive sequelae but may also theoretically impact synaptogenesis and neurogenesis in the normal and/or injured adult brain.

What We Already Know about This Topic

- General anesthetics produce neurotoxicity in immature neurons in part through altered signaling by the brain-derived neurotrophic factor pathway
- The role of astrocytes in modulating anesthetic neurotoxicity is unknown

What This Article Tells Us That Is New

- Using cultured mouse neurons and astroglia, co-culture with astrocytes reduced the neurotoxicity of isoflurane by buffering increases in pro-brain–derived neurotrophic factor
- Modulation of brain-derived neurotrophic factor signaling or astrocyte function is a potential approach to prevent anesthetic neurotoxicity

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Growth, maintenance, and repair of the neuronal network, in both neonates and adults, are coordinated by resident astrocytes,[12,13] specialized glia that are the most abundant cells in the human brain. In addition to their role in neuronal housekeeping and protection, astrocytes have been shown to play a significant role in neurotransmission, such that the association between astrocyte processes and neuronal synapses has been coined the "tripartite synapse."[13] Astrocytes modulate synaptic transmission *via* glutamate uptake[14] and are central to synapse formation and stabilization.[15,16] An individual astrocyte may contact up to 100,000 neurons[17] serving to integrate signals within the neuronal network. Moreover, astrocytes themselves communicate with adjacent astrocytes *via* intercellular gap junctions to function as a coordinated syncytium,[18] providing an additional astrocyte-dependent layer of neuronal regulation. More recently, astrocytes have also been shown to express $p75^{NTR}$, which can bind and internalize proBDNF, removing it from the extracellular space.[19] However, the role of astrocytes in BDNF-mediated neuronal signaling has not been defined, and whether astrocytes have the capacity to functionally buffer increases in pro-BDNF and protect adjacent neurons from proBDNF/$p75^{NTR}$-mediated cell death has not been investigated. In the current study, we used primary neuronal, astrocyte, and neuronal-astrocyte co-cultures to test the hypothesis that astrocytes mitigate increases in proBDNF *via* astrocyte-dependent $p75^{NTR}$ binding, resulting in a reduction in BDNF/$p75^{NTR}$-mediated neuronal cell death.

Materials and Methods

Animal Protocols

All experiments were performed according to the protocols approved by the Stanford University Animal Care and Use Committee (Stanford, California) and followed the National Institutes of Health guidelines for animal welfare.

Cell Cultures

Relatively pure astrocyte cultures were prepared from postnatal day 1 to 3 Swiss Webster mice.[20] In brief, after euthanasia, brains were removed in a sterile field and cortices were freed of meninges in ice-cold Eagle's minimal essential medium (Gibco, USA) and incubated in 0.05% trypsin/EDTA (Life Technologies, USA) for 30 min at 37°C followed by mechanical dissociation. Neocortical cells were plated at a density of two hemispheres per 10 ml Dulbecco's modified Eagle medium (Gibco) with 10% equine serum (ES, HyClone; GE Healthcare Life Sciences, USA), 10% fetal bovine serum (FBS, Hyclone), and 10 ng/ml epidermal growth factor (Sigma Chemicals, USA).

Relatively pure neuronal cultures were prepared from cortices of embryonic day 15 or 16 mice.[20] Cortices were collected in ice-cold Eagle's minimal essential medium, digested with 0.05% trypsin/EDTA for 15 min at 37°C, mechanically dissociated, and then plated in Dulbecco's modified Eagle's medium containing 26 mM $NaHCO_3$ (Sigma Chemicals), 24 mM glucose (Sigma Chemicals), 5% FBS, and 5% ES. On day *in vitro* 2 (DIV 2), medium was exchanged with glial-conditioned medium, which has same composition as neuronal plating medium but lacking FBS, incubated with DIV 14 primary glial cultures for 7 days, filtered, and stored at −20°C until use. Before use, conditioned medium was supplemented with B-27 (2%; Gibco) and cytosine arabinoside (3 μM; Sigma-Aldrich, USA), the later added to inhibit glial proliferation.

For co-culture experiments, two culture conditions were used. Method 1: Neurons from a single dissection were plated on near-confluent astrocyte cultures (DIV 14).[21] Method 2: To assess whether age-matched astrocytes also protect neurons from isoflurane toxicity, co-cultures were prepared from a single neocortical dissection in a similar manner to neuronal cultures but increasing the serum to 7.5% each of FBS and ES, adding 5 ng/ml epidermal growth factor and omitting B-27 and cytosine arabinoside. Neuronal, astrocyte, and neuronal-astrocyte co-cultures were maintained in a 37°C humidified incubator with 5% CO_2 in room air atmosphere.

Cultures were characterized by quantitating the cell types immunohistochemically using neuronal, astrocyte, and microglial markers (fig. 1, A and B). Microglial content in our astrocyte cultures ranged from 0 to 4.6% (average 1.5 ± 1.3%) and no detectable neurons. Our neuronal culture microglial content ranged from 0.4 to 1.5% (average 1.1 ± 0.7%) and less than 1% astrocytes, whereas neuronal-astrocyte co-cultures ranged from 0.5 to 3.1% (average 1.5 ± 1.0%) microglial content. The relative maturity of our astrocytes was assessed using markers for immature (vimentin) and mature (glial fibrillary acidic protein [GFAP]) astrocytes and showed a decline in the ratio of vimentin to GFAP with DIV (fig. 1, C and D).

Experimental Protocols

Primary neuronal and mixed neuronal-astrocyte co-cultures at neuronal DIV 7 were exposed for 1 h to either 2% isoflurane (assessed with a Datex 245 Airway Gas Monitor; Datex Corp., USA) in premixed carrier gas (5% CO_2, 21% O_2, and balance nitrogen) or to carrier gas alone. Gas flow was maintained at a rate of 2 l/min in a sealed, humidified incubator at 37°C. Neurons were assessed for cell death 24 h after isoflurane exposure. In additional experiments, isoflurane-induced neuronal cell death was assessed in primary neuronal cultures after application of conditioned medium from neuronal-astrocyte co-cultures, in co-cultures after astrocyte-targeted knockdown of $p75^{NTR}$, and in co-cultures with and without the astrocyte–glutamate transporter inhibitor dihydrokainic acid (Tocris Bioscience, United Kingdom). Finally, neuronal cell death was assessed in neuronal, astrocyte, and neuronal-astrocyte co-cultures 24 h after application of recombinant proBDNF (1 to 100 pg/ml; R+D Systems, USA) plus protease inhibitor (diluted to 1X; G-Biosciences, USA), with and without the $p75^{NTR}$

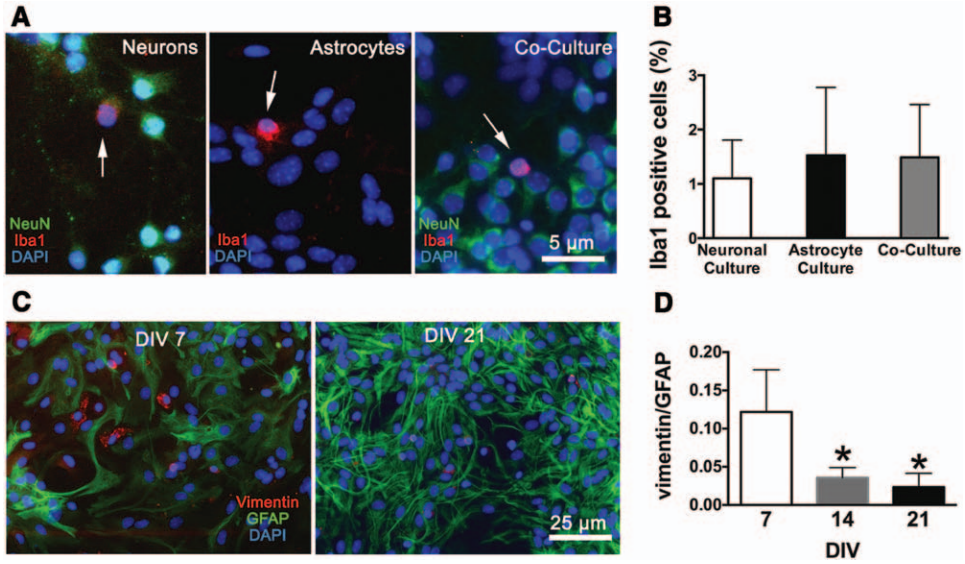

Fig. 1. Characterization of the three types of cultures. (*A*) Immunofluorescence staining of neuronal cultures, astrocyte cultures, and co-cultures with the microglial marker Iba1 (*red*), costained with the neuronal marker neuronal nuclei (NeuN) (*green*), and the nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI) (*blue*). *Arrows* identify microglia. (*B*) Quantitation of microglia in the cell cultures. (*C*) Immunofluorescence staining of astrocyte cultures at day *in vitro* (DIV) 7 and 21 with the immature astrocyte marker vimentin (*red*), the mature astrocyte marker glial fibrillary acidic protein (GFAP) (*green*), and DAPI (*blue*). (*D*) The ratio of vimentin to GFAP at 7, 14, and 21 DIV. Mean ± SD, n = 6 cultures per group, two fields averaged per culture; **P* < 0.05 *versus* DIV 7.

inhibitor transactivating transcriptional activator peptide 5 (TAT-Pep5, 10 μM; EMD Millipore, USA).

Assays for Neuronal Cell Death

Neuronal viability in primary neuronal cultures was assessed after staining with Hoechst 33342 (5 μM; Sigma Chemicals) and propidium iodide (PI, 5 μM; Sigma Chemicals). PI stains dead cells, whereas Hoechst is a cell-permeant nucleic acid stain that labels nuclei of both live and dead cells. PI-positive cells were manually counted by a blinded investigator, whereas numbers of Hoechst-positive cells were calculated using an automated macro (Image J, v1.49b; National Institutes of Health, USA). PI-positive and Hoechst-positive cells were counted in three microscopic fields per well at ×200 magnification. The number of PI-positive cells was expressed as a percent of the total number of cells. In co-cultures, neurons were differentiated from astrocytes using fluorescence immunocytochemistry with antibody specific for neuronal nuclei (NeuN) (described in further detail below in "Fluorescence Immunocytochemistry"). Dead neurons in co-cultures were identified as double positive for PI and NeuN and expressed as a percent of the total number of NeuN-positive cells. Immunofluorescence was visualized with an epifluorescence microscope (Zeiss Axiovert 200M; Carl Zeiss AG, Germany) at ×200 magnification.[22]

Astrocyte-targeted p75^{NTR} Knockdown

p75^{NTR} messenger RNA (mRNA) and protein expression were assessed in primary astrocyte cultures by reverse transcription quantitative polymerase chain reaction (PCR) and fluorescence immunocytochemistry (below), respectively, 24 h after transfection with small-interfering RNA (siRNA) against p75^{NTR} (30 pmol per well, Silencer Select cat. no. 4390771; Life Technologies) or negative mismatch control (cat. no. AM4615; Life Technologies). Transfection was achieved using Lipofectamine-2000 (Life Technologies) according to the manufacturer's instructions. Transfection with Lipofectamine-2000 reagents has been demonstrated[23] to preferentially target astrocytes in neuronal-astrocyte co-cultures. In the current study, we verified astrocyte-specific targeting in co-cultures using a reporter plasmid (30 pmol per well pDS-Red2-N1; Clontech Laboratories, USA), assessed with fluorescence immunocytochemistry 24 h after transfection. p75^{NTR} protein expression was then assessed in neuronal-astrocyte co-cultures 24 h after transfection with p75^{NTR} siRNA (30 pmol per well).

Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry was performed on cell cultures in 24-well plates.[22] In brief, cultures were fixed in 4% paraformaldehyde for 30 min at room temperature and nonspecific binding was blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 h. Cells were then incubated with primary antibody to the neuronal marker NeuN (1:500, cat. no. mab377; EMD Millipore), the astrocyte markers GFAP (1:500, cat. no. ab7260; Abcam, USA) and vimentin (1:500 cat. no. ab8978; Abcam), and/or the microglial marker Iba1 (1:500, cat. no. ab178680; Abcam) overnight at 4°C. Cells were washed and subsequently incubated with Alexa Fluor 488-nm-conjugated or 594-nm-conjugated secondary antibody (1:500; Invitrogen, USA) for 1 h. Cells were counterstained with 4′,6′-diamidino-2-phenylindole (0.5 μg/ml; Sigma-Aldrich), a cell-permeant nuclear dye, for total cell count.

Real Time-quantitative PCR

Total RNA was isolated with TRIzol® (Life Technologies) from cultures 24 h after transfection. Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit for total RNA (Life Technologies).[24] Predesigned primer/probes for PCR were obtained from Life Technologies for mouse $p75^{NTR}$ (#Mm01309638) and glyceraldehyde 3-phosphate dehydrogenase (#Mm99999915) mRNA. Real time-quantitative PCR was conducted using the TaqMan® Assay Kit (Life Technologies).[24] Measurements for $p75^{NTR}$ mRNA were normalized to within-sample glyceraldehyde 3-phosphate dehydrogenase (ΔCt), and comparisons were calculated as the inverse log of the $\Delta\Delta$Ct from the mean of the control treatment group.[25]

Assay for proBDNF in Media

Concentrations of proBDNF in culture media were assessed before and immediately after isoflurane exposure, with and without transfection with $p75^{NTR}$ siRNA 24 h before, by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions (cat. no. BEK-2217-2P; Biosensis, Australia). Absorbance at 450 nm was measured *via* microplate reader (Versamax; Molecular Devices, USA).

Statistical Analysis

All cultures were inspected microscopically before randomly assigned to experimental treatment, and data collection and analysis were performed blinded to treatment group. No data were excluded from inclusion in the final analysis. Sample sizes were determined from previous experience using these cell cultures.[21] All data reported are representative of at least three independent experiments with n = 6 to 8 cultures per treatment, using two to three images per culture averaged. proBDNF ELISA data are representative of at least three independent experiments, with n = 12 cultures per treatment. All data are reported as means ± SD. For comparisons between two groups, statistical difference was determined by unpaired, two-tailed Student *t* test using Prism 6.0d software (GraphPad, USA). For comparisons between multiple groups, a one-way ANOVA with Bonferroni-corrected *post hoc* comparison of all means to the control group (for proBDNF and dihydrokainate dose–response curves) or *post hoc* comparison of all means to all other means (for proBDNF ELISA measurements) using Prism 6.0d software. In all analyses, a *P* value of less than 0.05 was considered significant.

Results

Isoflurane Induces Neuronal Cell Death in Neuronal Cultures but Not in Neuronal-astrocyte Co-cultures

A 1-h exposure to 2% isoflurane induced a significant increase in cell death (PI-positive cells) in primary neuronal cultures (fig. 2, A and B). However, DIV 7 neurons growing in the presence of either DIV 21 astrocytes (fig. 2, C and D) or age-matched astrocytes (fig. 2, E and F) were protected from the effect of the same isoflurane exposure. This finding demonstrates that the observed protection from isoflurane neurotoxicity afforded by astrocytes occurred independently of the relative maturity of the astrocytes from DIV 7 to 21.

Next, to test whether this effect was due to a secreted factor specific to neuronal-astrocyte co-cultures, the normal glia-conditioned media used in neuronal cultures were replaced with freshly harvested co-cultures conditioned media 1 h before isoflurane exposure. Media replacement did not result in any significant protection of neurons from isoflurane toxicity (fig. 3, A and B), suggesting that the protective effect of astrocytes was dependent on the physical presence of astrocytes. No differences in the total numbers of neurons were observed between treatment groups (data not shown).

Astrocyte $p75^{NTR}$ Knockdown in Co-culture Results in Increased Neuronal Cell Death from Isoflurane

$p75^{NTR}$ protein and mRNA expression in primary astrocyte cultures was significantly reduced 24 h after transfection with $p75^{NTR}$ siRNA relative to mismatch control sequence using the transfection agent Lipofectamine-2000 (fig. 4, A–C). Co-cultures transfected with reporter plasmid using Lipofectamine-2000 resulted in preferential targeting of astrocytes, with less than 2% transfected cells identified as neurons. Co-cultures transfected with $p75^{NTR}$ siRNA using this method also showed a significant decrease in $p75^{NTR}$ protein expression (fig. 4, D and E), however, to a smaller degree relative to transfection in primary astrocyte culture alone (fig 4B), consistent with preferential knockdown in astrocytes. Transfection with $p75^{NTR}$ siRNA 24 h before isoflurane exposure resulted in a significant increase in neuronal cell death (fig. 5, A and B) relative to control transfection (fig. 5, C and D), suggesting that astrocyte $p75^{NTR}$ expression contributed to neuronal protection. Treatment with either $p75^{NTR}$ siRNA or isoflurane alone resulted in a modest increase in proBDNF levels in co-culture media (fig. 5E). However, combined treatment with both $p75^{NTR}$ siRNA and isoflurane resulted in a further increase in proBDNF levels *versus* either treatment alone (fig. 5E bar furthest right). Together with the observation that only combined treatment resulted in increased neuronal cell death (fig. 5, A and B), this finding suggests that the reduction in astrocyte $p75^{NTR}$ with siRNA critically reduced the buffering capacity of astrocytes to isoflurane-induced increases in proBDNF. No differences in the total numbers of neurons were observed between treatment groups (data not shown).

Isoflurane Neurotoxicity in Neuronal-astrocyte Co-cultures Is Mediated by proBDNF/$p75^{NTR}$

Application of increasing levels of proBDNF (coadministered with protease inhibitor to prevent conversion to mature BDNF), beginning with physiologic levels measured in media, resulted in a progressive increase in neuronal cell death in both primary neuronal (fig. 6A) and neuronal-astrocyte

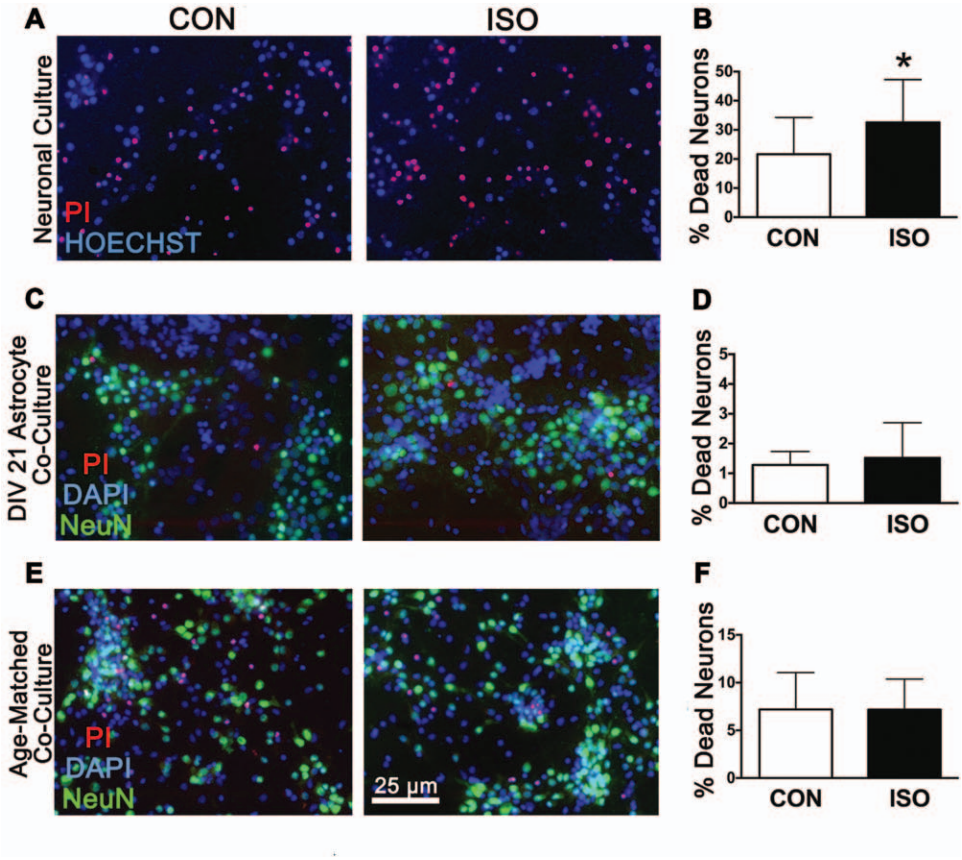

Fig. 2. Isoflurane exposure in primary neuronal and neuronal-astrocyte co-cultures. (*A* and *B*) Fluorescent staining of dead neurons with propidium iodide (PI, *red*) and live neurons with Hoechst (*blue*) in day *in vitro* (DIV) 7 neuronal cultures 24 h after a 1-h exposure to 2% isoflurane (ISO) or exposure to carrier gas exposure alone (CON). (*C–F*) co-cultures of neurons with DIV 21 or DIV 7 (age matched) astrocytes were immunostained for the neuronal marker neuronal nuclei (NeuN) (*green*) and stained with 4′,6′-diamidino-2-phenylindole (DAPI) and PI. Dead neurons are identified as triple positive for PI, NeuN, and DAPI (*blue*), live neurons double positive for NeuN and DAPI. Neuronal death does not increase after isoflurane exposure when astrocytes are present. (*E* and *F*) In age-matched co-cultures, baseline (CON) levels of neuronal cell death are higher than in co-culture with mature astrocytes (*D*), but lower than in neuronal cultures (*B*). Graphs are representative of data from three independent experiments each with n = 6 cultures per treatment, three fields averaged per culture; mean ± SD, **P* < 0.05 *versus* control.

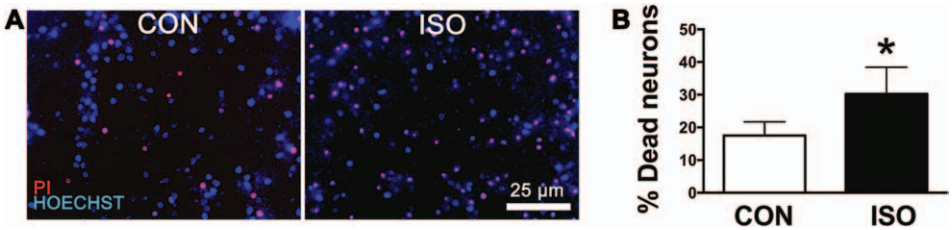

Fig. 3. Isoflurane exposure of neuronal cultures after replacement of media with conditioned media from co-cultures. (*A*) Micrographs of neuronal cultures stained with propidium iodide (PI, *red*) and Hoechst (*blue*) to identify dead and live neurons. (*B*) Quantitation of percent dead neurons. Graph is representative of data from three independent experiments each with n = 6 cultures per treatment, three fields averaged per culture; mean ± SD, **P* < 0.05 *versus* control. CON = control carrier gas treatment; ISO = 1 h 2% isoflurane treatment.

co-cultures (fig. 6B). In co-cultures, pretreatment with $p75^{NTR}$ siRNA augmented neuronal cell death from 100 pg/ml proBDNF, whereas pretreatment with the intracellular $p75^{NTR}$ inhibitor TAT-Pep5 blocked the neurotoxic effect from the same proBDNF dose (fig. 6B). Thus, similar to the observations in neuronal cultures,[2,3] proBDNF-induced cell death of neurons in co-culture is mediated by $p75^{NTR}$ signaling and mitigated by $p75^{NTR}$ expression on astrocytes. Interestingly, addition of the same concentrations of proBDNF to primary astrocyte cultures did not induce cell death (fig. 6C), providing evidence for a functional difference between astrocytes and neurons in response to binding

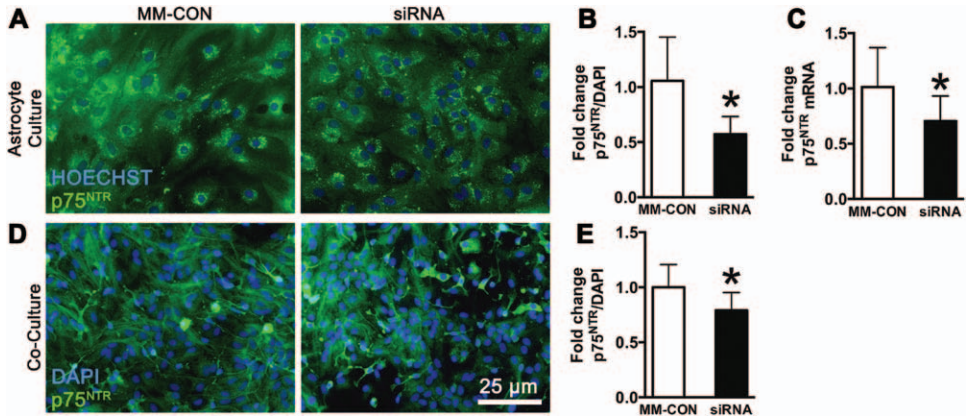

Fig. 4. Targeting astrocytes in neuronal-astrocyte co-culture for knockdown of p75 neurotrophin receptor ($p75^{NTR}$). (*A*) Fluorescence immunohistochemical staining of primary astrocyte cultures demonstrates easily identifiable levels of cellular expression of $p75^{NTR}$ (*green*) that is quantified and normalized to total cell number with 4′,6′-diamidino-2-phenylindole (DAPI) (*blue*). Relative to transfection with mismatch-control sequence (MM-CON), transfection with small-interfering RNA (siRNA) to $p75^{NTR}$ results in significant reduction in both $p75^{NTR}$ immunofluorescence (*B*) and messenger RNA (mRNA) expression (*C*) in astrocytes. (*D*) Co-cultures stained for $p75^{NTR}$ and colabeled with the nuclear stain DAPI show both neuronal and astrocyte staining. (*E*) Relative to transfection with MM-CON, transfection of co-cultures with $p75^{NTR}$ siRNA results in a significant reduction in expression levels of $p75^{NTR}$, but to a lower degree than that observed in astrocyte cultures (*B*), consistent with astrocyte-targeted $p75^{NTR}$ knockdown. Graphs are representative of data from three independent experiments each with n = 8 cultures per treatment; mean ± SD, $^*P < 0.05$ *versus* control.

of proBDNF to $p75^{NTR}$. Because glutamatergic excitotoxicity has been proposed as a potential mechanism contributing to anesthetic neurotoxicity,[26] we investigated whether astrocyte-mediated protection from isoflurane neurotoxicity was mediated by astrocytic glutamate uptake. Addition of the astrocyte–glutamate transporter inhibitor dihydrokainic acid did not result in any effect on isoflurane-mediated cell death (fig. 6D), suggesting that glutamate buffering does not contribute to astrocyte-mediated protection from isoflurane neurotoxicity in these co-cultures. No differences in the total numbers of neurons or astrocytes were observed between treatment groups (data not shown).

Discussion

This study is the first to demonstrate that astrocytes protect neurons from isoflurane toxicity and that this protection occurs *via* reduced availability of proBDNF to bind to neuronal $p75^{NTR}$ (fig. 7). Previous finding[2] suggested that isoflurane neurotoxicity in primary neuronal cultures is limited to the period of active synaptogenesis (DIV 4 to 7) and decreases after approximately at DIV 10. Relatively few studies have investigated the role astrocytes play in the development of brain injury from isoflurane exposure. Lunardi *et al.*[27] demonstrated that prolonged (24 h) exposure to high-dose (3%) isoflurane impaired growth of DIV 4 but not DIV 15 astrocytes. Culley *et al.*[28] observed that isoflurane exposure to astrocytes at DIV 16 did not affect astrocyte survival or the ability to support synaptogenesis. Most recently, Ryu *et al.*[29] demonstrated that isoflurane preexposure of astrocytes at DIV 10 impaired axonal growth in developing co-cultures but did not contribute to astrocyte cell death. In the current study, DIV 7 neurons were protected from isoflurane exposure whether in the presence of age-matched (DIV 7) or DIV 21 astrocytes. These observations demonstrate that astrocyte-mediated protection is present in relatively immature astrocytes and is maintained in more mature astrocytes.

Significantly more baseline neuronal injury was observed in neurons cultured alone than in neurons cultured with age-matched astrocytes, and the least injury was seen in neurons cultured on mature astrocytes. Neither conditioned media from astrocyte cultures nor conditioned media from co-cultures provided protection from isoflurane neurotoxicity. This suggests that (1) concentrations of additional secreted factors resulting from neuron–astrocyte cross-talk were either too low or otherwise did not contribute to the neuroprotective effect observed in this study; and (2) the physical presence of astrocytes is necessary for neuroprotection from isoflurane toxicity.

Astrocyte-mediated neuroprotection that requires astrocytes in close proximity is shown in the current model (fig. 7) of synaptic BDNF signaling. proBDNF is released from the presynaptic terminal into the synapse and likely has a limited period of activity before conversion to mature BDNF by plasmin. Debate exists as to the physiologic relevance of proBDNF to *in vivo* signaling.[30] Bergami *et al.*[19] previously demonstrated that binding of proBDNF to $p75^{NTR}$ results in endocytosis and removal of proBDNF from the synaptic cleft. In this study, we observed a significant increase in proBDNF levels in media from co-cultures with astrocyte-targeted knockdown of $p75^{NTR}$ after isoflurane exposure. This suggests that extrasynaptic levels of proBDNF increased secondary to reduced uptake mediated by astrocyte $p75^{NTR}$.

Ryu *et al.* recently reported reduced levels of mature BDNF in media from co-cultures of neurons with astrocytes

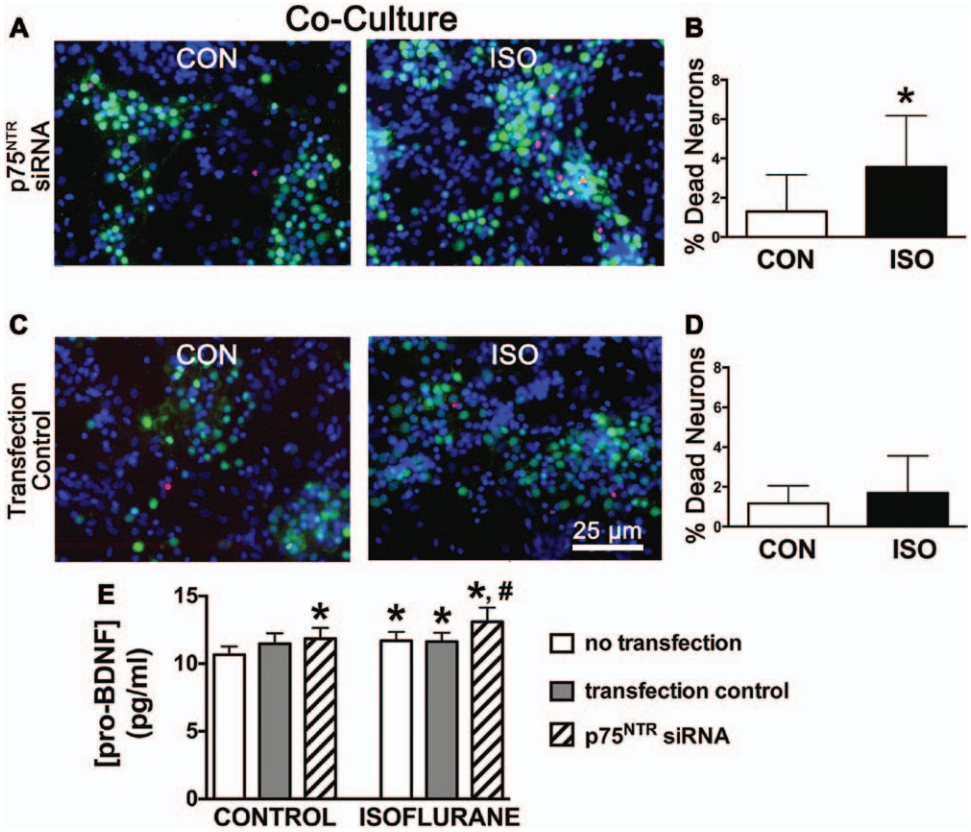

Fig. 5. Isoflurane exposure in neuronal-astrocyte co-cultures after astrocyte-targeted knockdown of p75 neurotrophin receptor ($p75^{NTR}$). (*A* and *B*) Co-cultures transfected with small-interfering RNA (siRNA) against the pro-brain–derived neurotrophic factor (proBDNF) receptor $p75^{NTR}$ demonstrate low control (CON) levels of dead neurons, identified as both propidium iodide positive (*red*) and neuronal nuclei (NeuN) positive (*green*); 4′,6′-diamidino-2-phenylindole (*blue*) stains all cell nuclei. Isoflurane exposure significantly increased neuronal cell death in siRNA-transfected co-cultures, relative to control gas exposure alone but failed to increase neuronal death in cultures transfected with mismatch-control (MM-CON) (*C* and *D*). Graphs represent three independent experiments with n = 6 cultures per experiment. (*E*) Levels of proBDNF in media measured by enzyme-linked immunosorbent assay increased in co-cultures treated with either $p75^{NTR}$ siRNA or isoflurane exposure alone. Coapplication of $p75^{NTR}$ siRNA and isoflurane resulted in a significantly greater increase in proBDNF than either treatment alone after isoflurane exposure. Graph is representative of three independent experiments with n = 12 cultures per treatment, each culture measured in duplicate; mean ± SD, **P* < 0.05 *versus* no treatment; #*P* < 0.05 *versus* all other conditions. ISO = 1 h 2% isoflurane treatment.

subjected to isoflurane *preexposure*, suggesting an alteration in BDNF signaling; however, whether this was due to alterations in proBDNF levels or to changes in plasmin activity was not determined. In the current study, application of increasing levels of recombinant proBDNF resulted in a significant corresponding increase in neuronal cell death in both neuronal cultures and neuronal-astrocyte co-cultures. Interestingly, the lowest toxic dose (2.5 pg/ml) in both culture models approximated the increase in proBDNF levels we observed in media from co-cultures subjected to astrocyte-targeted $p75^{NTR}$ knockdown plus isoflurane exposure, a treatment that increased the neuronal cell death.

At a dose of 100 pg/ml proBDNF, neuronal cell death in co-cultures increased substantially, likely representing saturation of astrocytic proBDNF uptake. This was exacerbated by $p75^{NTR}$ knockdown and reversed by inhibition of $p75^{NTR}$ signaling, verifying a proBDNF/$p75^{NTR}$ mechanism of neuronal cell death, possibly *via* rat sarcoma virus homolog family member A (RhoA)–mediated induction of proapoptotic pathways.[3] Notably, proBDNF did not kill astrocytes indicating that proBDNF binding to $p75^{NTR}$ has different effects on cell survival between astrocytes and neurons under these conditions. This is supported by previous observations[27] that although prolonged exposure of immature astrocytes to high concentrations of isoflurane impaired astrocyte maturation and morphological development, it paradoxically *reduced* the activation of rat sarcoma virus homolog family member A. Moreover, activation of $p75^{NTR}$ by the lower-affinity ligand nerve growth factor failed to induce cell death of cultured hippocampal astrocytes.[31] Future investigations delineating $p75^{NTR}$ cell signaling pathways in astrocytes may yield further insight into their potential to coordinate synaptic transmission.

Astrocytes regulate synaptogenesis, synaptic function, and synapse elimination through modulation of glutamatergic signaling and *via* contact-dependent signals.[15,16] Astrocytes have been shown to protect neurons from

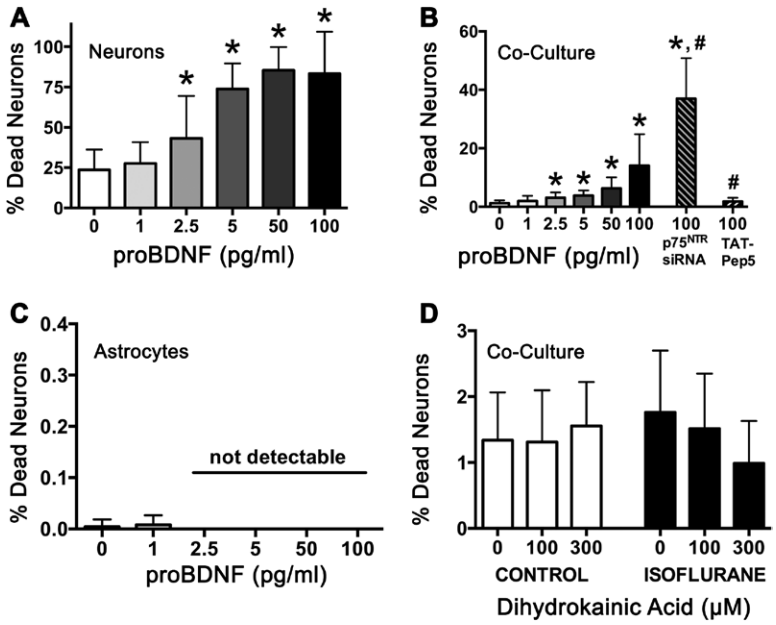

Fig. 6. pro-Brain–derived neurotrophic factor (proBDNF)/p75 neurotrophin receptor ($p75^{NTR}$) signaling mediates neuronal cell death in neuronal-astrocyte co-cultures. (*A*) Application of increasing doses of recombinant proBDNF plus protease inhibitor induces neuronal cell death in primary neuronal cultures. (*B*) In neuronal-astrocyte co-cultures, application of proBDNF also results in neuronal cell death, but much higher doses are required relative to neuronal cultures. Coapplication of $p75^{NTR}$ small-interfering RNA (siRNA) exacerbated neuronal cell death, whereas $p75^{NTR}$ intracellular signaling inhibitor Tat-Pep5 reversed this effect, indicating that proBDNF binding to $p75^{NTR}$ on astrocytes and proBDNF signaling are involved. (*C*) In astrocyte cultures, application of proBDNF had no effect on cell survival. (*D*) In neuronal-astrocyte co-cultures, pretreatment with the astrocyte–glutamate transporter inhibitor dihydrokainate had no effect on neuronal cell death subsequent to a 1 h exposure to 2% isoflurane suggesting glutamate uptake does not contribute to protection. All graphs represent n = 6 cultures per treatment, three fields averaged per culture, mean ± SD. *$P < 0.05$ *versus* no added proBDNF, #$P < 0.05$ *versus* 100 pg/ml proBDNF alone. TAT-pep5 = transactivating transcriptional activator peptide 5.

glutamate-mediated excitotoxicity during pathophysiologic stresses such as stroke,[32] traumatic brain injury,[33] and spinal cord injury.[34] The results from the current study demonstrate that astrocytic glutamate uptake did not contribute to astrocyte-mediated protection from isoflurane, instead suggesting that modulation of postsynaptic proBDNF/$p75^{NTR}$ signaling was responsible for astrocyte-mediated neuroprotection. Functional modulation of BDNF signaling by astrocytes may be relevant in the adult hippocampus where BDNF signaling is central to normal learning and memory formation.[8,9] The results from the current study suggest that astrocytes may provide a novel cellular mechanism to manipulate neuronal BDNF signaling.

Although BDNF is a known regulator of neonatal brain development coordinating prodeath and prosurvival signaling, several lines of evidence also support a role for BDNF signaling in recovery from injury in the adult brain after stroke,[35] traumatic brain injury,[36] and spinal cord injury.[37] However, therapies targeting manipulation of BDNF signaling are limited by the short plasma half-life of BDNF[38] and relatively poor blood–brain barrier penetrance.[39] Future studies using *in vivo* models of central nervous system injury in adult animals are needed to investigate the effects of targeted overexpression of astrocyte $p75^{NTR}$ and inhibition of neuronal $p75^{NTR}$ as potential targets for the development of new therapies to encourage recovery.

Limitations

Caution should be exercised in extrapolating these *in vitro* results to the clinical setting. For example, despite evidence from the current study demonstrating protection of DIV 7 neurons from isoflurane toxicity by DIV 7 astrocytes, previous study[1] has demonstrated isoflurane-induced neuronal cell death *in vivo* at postnatal day 7. DIV-7-cultured brain cells are unlikely to fully recapitulate the phenotypes of cells developing *in vivo*, and in particular, the ratio of astrocytes to neurons in culture is likely higher than seen *in vivo* at this age and may contribute to the difference between these observations.

Differences between *in vivo* and *in vitro* observations may also result from loss of the anatomic organization of the intact brain and loss of physiologic contributions from other cell types that are absent in culture. Effects of isoflurane on brain perfusion may contribute to *in vivo* effects and are also absent in culture. Although microglia are present throughout the brain, in our isolated cell cultures, they are present at low levels (on average < 2%), suggesting that in these *in vitro* observations they are unlikely to contribute measurably to the protection seen, even though they may express $p75^{NTR}$.

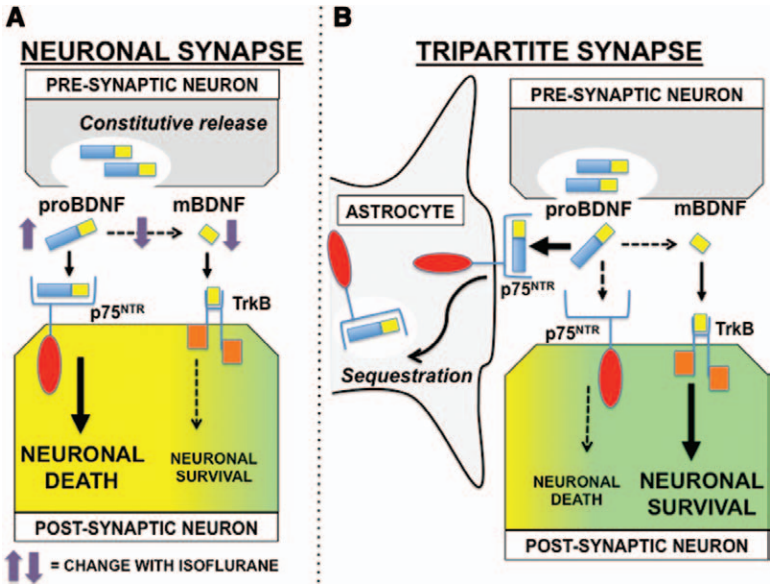

Fig. 7. A model of astrocyte-mediated protection from isoflurane neurotoxicity. (*A*) In the neuronal synapse, pro-brain–derived neurotrophic factor (proBDNF) is released constitutively from the presynaptic neuron and undergoes proteolytic cleavage by plasmin to the mature form (mBDNF). Binding of proBDNF to neuronal postsynaptic p75 neurotrophin receptors ($p75^{NTR}$) initiates actin destabilization and neuronal cell death, whereas binding of mBDNF to tyrosine-related kinase B (TrKB) receptors promotes cytoskeletal stabilization and neuronal cell survival. Isoflurane exposure decreases plasmin activity, leading to an increase in synaptic proBDNF and a decrease in mBDNF, resulting in postsynaptic neuronal cell death. (*B*) The tripartite synapse includes the contribution of astrocytes to synaptic homeostasis. $p75^{NTR}$ expressed on astrocytes serves to sequester proBDNF from the synaptic cleft. This function effectively buffers the isoflurane-induced increase in neuronal proBDNF/$p75^{NTR}$ binding, thereby promoting postsynaptic neuronal survival.

Finally, it is interesting to note that astrocytes in the human brain are larger, more complex, and greater in number relative to neurons than in the rodent brain.[40] This suggests that human brains may inherently posses a greater capacity for neuroprotection from anesthetic toxicity *versus* rodent brains and that bolstering astrocyte defense may be a potential therapeutic strategy.

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Competing Interests

The authors declare no competing interests.

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