Neuroprotective Effects of Dexmedetomidine against Glutamate Agonist-induced Neuronal Cell Death Are Related to Increased Astrocyte Brain-derived Neurotrophic Factor Expression

Vincent Degos, M.D., Ph.D.,* Tifenn Le charpentier, M.S.,† Vibol Chhor, M.D.,‡ Olivier Brissaud, M.D., Ph.D.,§ Sophie Lebon, Ph.D.,|| Leslie Schwendimann, M.S.,# Nathalie Bednareck, M.D., Ph.D.,§ Sandrine Passemard, M.D., Ph.D.,§ Jean Mantz, M.D., Ph.D.,** Pierre Gressens, M.D., Ph.D.††

ABSTRACT

Background: Brain-derived neurotrophic factor (BDNF) plays a prominent role in neuroprotection against perinatal brain injury. Dexmedetomidine, a selective agonist of α_2-adrenergic receptors, also provides neuroprotection against glutamate-induced damage. Because adrenergic receptor agonists can modulate BDNF expression, our goal was to examine whether dexmedetomidine's neuroprotective effects are mediated by BDNF modulation in mouse perinatal brain injury.

Methods: The protective effects against glutamate-induced injury of BDNF and dexmedetomidine alone or in combination with either a neutralizing BDNF antibody or an inhibitor of the extracellular signal-regulated kinase pathway (PD098059) were compared in perinatal ibotenate-induced cortical lesions (n = 10–20 pups/groups) and in mouse neuronal cultures (300 μM of ibotenate for 6 h). The effect of dexmedetomidine on BDNF expression was examined *in vivo* and *in vitro* with cortical neuronal and astrocyte isolated cultures.

Results: Both BDNF and dexmedetomidine produced a significant neuroprotective effect *in vivo* and *in vitro*. Dexmedetomidine enhanced *Bdnf4* and *Bdnf5* transcription and BDNF protein cortical expression *in vivo*. Dexmedetomidine also enhanced *Bdnf4* and *Bdnf5* transcription and increased BDNF media concentration in isolated astrocyte cultures but not in neuronal cultures. Dexmedetomidine's protective effect was inhibited with BDNF antibody (mean lesion size ± SD: 577 ± 148 μm *vs.* 1028 ± 213 μm, n = 14–20, $P < 0.001$)

What We Already Know about This Topic

- Both brain-derived neurotrophic factor and dexmedetomidine are neuroprotective
- α2 Adrenergic receptor agonists modulate the expression of brain-derived neurotrophic factor, but its role in neuroprotection remains unknown

What This Article Tells Us That Is New

- Using *in vitro* and *in vivo* mouse models of perinatal brain injury, dexmedetomidine's neuroprotective effects were shown to involve modulation of brain-derived neurotrophic factor expression
- This effect of dexmedetomidine required activation of an extracellular signal-regulated kinase–dependent pathway probably involving the synthesis of brain-derived neurotrophic factor in astrocytes, consistent with an indirect astrocyte-dependent neuroprotective mechanism

*Associate Researcher, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, PremUP, Paris, France, and AP-HP, Groupe Pitie Salpetrière, Department of Anesthesia and Critical Care, Paris, France. † Research Assistant, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and PremUP, Paris, France. ‡ PhD Student, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and PremUP, Paris, France. § Assistant Professor, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and PremUP, Paris, France. || Bioengineer Researcher, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and PremUP, Paris, France. # Research Assistant, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and PremUP, Paris, France. ** Professor, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, and AP-HP, Beaujon Hospital, Department of Anesthesia and Critical Care, Clichy, France. †† Professor, Inserm, U676, Paris, France, Université Paris 7, Faculté de Médecine Denis Diderot, Paris, France, PremUP, Paris, France, and Institute for Reproductive and Developmental Biology, Imperial College, Hammersmith Campus, London, United Kingdom.

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Address correspondence to Dr. Degos: Inserm U676, 48, bvd Serrrurier, Paris France. degosv@gmail.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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and PD098059 *in vivo* but not in isolated neuron cultures. Finally, PD098059 inhibited the increased release of BDNF induced by dexmedetomidine in astrocyte cultures.

Conclusion: These results suggest that dexmedetomidine increased astrocyte expression of BDNF through an extracellular signal-regulated kinase-dependent pathway, inducing subsequent neuroprotective effects.

GLUTAMATE-induced toxicity, also known as excitotoxicity, is a leading cause of neuronal cell death in the human brain, especially in perinatal brain injury. Antagonists of the ionotropic N-methyl-D-aspartic Acid (NMDA) glutamate receptor are potent neuroprotective agents in several animal models of perinatal brain lesions.[1] However, NMDA receptors play key roles in successive steps of brain development, including proliferation, migration, survival, and differentiation of neurons.[2] Therefore, blocking glutamate receptors at specific neurodevelopmental stages might adversely affect brain development.

Histopathologic and behavioral data[3] show that several drugs primarily used for sedation exacerbate developmental neuronal cell death.[4] These drugs include NMDA receptor blockers (*e.g.*, volatile anesthetics including nitrous oxide and ketamine) and drugs that potentiate γ-aminobutyric acid–ergic transmission (*e.g.*, IV and volatile anesthetics).[4] These deleterious effects in rodent models are attributed to disturbances in growth factors that result in activation of apoptosis.[5] Growth factors that have anti-apoptotic properties, such as insulin-like growth factor, nerve growth factor, and brain-derived neurotrophic factor (BDNF), can prevent hypoxic or excitotoxic neuronal death in animal models of perinatal damage.[6]

Dexmedetomidine is a potent and highly selective agonist of α_2-adrenergic receptors with a broad spectrum of effects on the human brain, including clinical sedation, anesthetic-sparing effects, and analgesia.[7,8] Recent experimental work indicates that dexmedetomidine exhibits long-term effects on the brain, including neuroprotection against excitotoxic damage through α_2-adrenergic receptors.[9] Our group has shown that dexmedetomidine reduces the severity of excitotoxic brain injury in the perinatal period in mice.[10,11] We have shown that dexmedetomidine preconditions brain tissue against ischemic cell death and caspase-3 expression in hippocampal slices subjected to oxygen glucose deprivation.[12] In addition, dexmedetomidine exerts synergistic neuroprotective effects with xenon that have been observed 30 days after ischemic injury in rodents.[13] Some of dexmedetomidine's neuroprotective properties proceed via activation of signaling cascades, including the extracellular signal regulated kinase (ERK) pathway.[12] Interestingly, other adrenergic receptor agonists have been described to increase BDNF expression in nonneuronal cells such as astrocytes.[14]

As disturbances in growth factors such as BDNF can affect neuroprotective strategies, the aim of this study was to characterize dexmedetomidine's effects on BDNF release in brain excitotoxic models. We hypothesized that dexmedetomidine has an indirect neuroprotective effect through modulation of BDNF expression.

Materials and Methods

Animals

Experiments on rodents were carried out in compliance with appropriate European Community Commission guidelines (86/609), and our institutional review board approved the protocol (approval number A751901, Paris, France). Swiss mice pups of both sexes and time-pregnant Swiss mice were handled according to institutional guidelines of Institut National de la Santé et de la Recherche Médicale (Paris, France). Rodents had unrestricted access to food and water and were kept at 22°C with a normal light cycle (12 h/12 h). Two complete litters were excluded because the two mothers ate several postnatal-day 5 (P5) pups just after human manipulation for intraperitoneal injection. Otherwise, pups did not experience unexpected lethality in the study and were euthanized with decapitation according to our local guidelines.

In Vivo *Pharmacologic Reagents*

Ibotenate (Tocris, Bristol, United Kingdom), a glutamate analog, activates both NMDA and group I metabotropic receptors but not α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate and kainate receptors. Ibotenate was diluted in phosphate-buffered saline (PBS) containing 0.02% acetic acid at 5 μg/μl. Dexmedetomidine (α_2-adrenergic receptor agonist, Orion Pharmos, Turku, Finland) was diluted in PBS and administrated with an intraperitoneal injection at 3 μg/kg diluted in 5 μl. The control group received the same volume of PBS intraperitoneal (5 μl).

Brain-derived neurotrophic factor (Alomone Labs, Jerusalem, Israel) was diluted in PBS and administered at 10 μg/kg intraperitoneal 30 min before ibotenate. Neutralizing BDNF antibody (BDNFab, 50 ng/μl, AB1779SP, Millipore, Molsheim, France) and PD098059 (a direct inhibitor of ERK1/2 phosphorylation; 0.3 mg/kg corresponding to 0.6 μg/μl, Tocris) were diluted in PBS and simultaneously administrated with ibotenate via intracranial injection (same final dilution, 5 μg/μl).

In Vivo *Ibotenate-Induced Excitotoxic Brain Lesions*

Five microliter of dexmedetomidine or 5 μl of PBS was intraperitoneally injected in P5 pups. Sixty minutes later (M60), excitotoxic brain lesions were induced via intracranial injection of ibotenate. As previously described,[10,15] pups were anesthetized using 1% isoflurane for less than 4 min and maintained under a warming lamp. Two boluses of 1 μl of ibotenate were injected into the neopallial parenchyma. After the injections, the pups were allowed to recover and returned to their dams. The pups' body temperatures were measured from the intraperitoneal injection of dexmedetomidine until 60 min after

the ibotenate intracranial injection (M120). We measured the emission of infrared radiations from the skin surface of the interscapular region using an infrared camera (model Thermovision A20; FLIR Systems, Boston, MA) according to a method previously developed in newborn rodents.[16,17]

Determination of Lesion Size

Animals were sacrificed 5 days after intracerebral injection (P10) by decapitation. Their brains were removed and post-fixed in 4% formol for 5 days at room temperature, then dehydrated in alcohol, and embedded in paraffin. As previously described,[15] coronal serial sections of 18 μm thickness were cut and stained with cresyl violet (Sigma, Lyon, France). Sectioning permitted an accurate and reproducible determination of the maximal sagittal fronto-occipital diameter of the lesion, which was equal to the number of sections where the lesion was present, multiplied by 18 μm representing an index of its volume.

Primary Isolated Neuronal Cultures

Cultured neurons were derived from the cerebral cortex of embryonic 14.5-day (E14.5) Swiss mice (Janvier, Paris, France).[18] Cells were seeded in 12-well culture plates (350×10^3 cells per well) pre-coated with 15 μg/ml poly-DL-ornithine (Sigma, France) or 96-well culture plates (7×10^4 cells per well) pre-coated with 30 μg/ml poly-DL-ornithine. Purity of the neuron cell culture was 97.7% (not shown), confirming our previous study. To test the impact of serum concentration on dexmedetomidine's neuroprotective effects, increasing concentrations of fetal calf serum were tested (0.4% and 4%).

Primary Isolated Astrocyte Cultures

Primary mixed glial cell cultures were prepared from the cortices of newborn Swiss mice (P0 to P1). After dissection of the cortices and removal of the meninges, tissues were chopped into small pieces and subsequently mechanically dissociated. Microglial cells were isolated from primary mixed glial cultures on day *in vitro* (DIV) 14 by shaking vigorously. After removing the microglial cell medium, the plates were treated with 5 min of chemical dissociation with 0.25% trypsin (Gibco, France), and the cells were suspended in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum (Gibco, France) with 0.01% of penicillin-streptomycin.

In Vitro Pharmacologic Reagents

Ibotenate (Tocris) diluted in PBS was administrated to neuronal culture at DIV 12 at 300 μM for 6 h. BDNF (Alomone Labs) was diluted in PBS and administrated at 0.5, 2.5, and 10 μM, 30 min before ibotenate. Dexmedetomidine (Orion Pharmos) was diluted in PBS and administrated at 0.3, 1, and 10 μM 30 min before ibotenate. Neutralizing BDNFab (AB1779SP, Millipore) was diluted in PBS and administrated at 10 μM 30 min before dexmedetomidine or BDNF. Yohimbine, an α_2-adrenoreceptor antagonist (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France), was diluted in PBS and administrated at 10 μM 30 min before dexmedetomidine. PD098059 (a direct inhibitor of ERK1/2 phosphorylation; Sigma-Aldrich) was diluted in PBS and administrated at 1 and 10 μM 30 min before dexmedetomidine.

Quantification of Neuronal and Astrocyte Survival

Neuronal and astrocyte cell survival was quantified 3 h after treatment with ibotenate using the colorimetric CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. In this assay, the tetrazolium compound is bioreduced by cells into a formazan product that is soluble in tissue culture medium. In brief, 20 μl of the tetrazolium compound was added to each well of a 96-well-plate containing 7×10^4 neuronal cells per well after DIV 12 and DIV 7. Formazan absorbance was measured 1 h later at 490 nm using the Beckman Coulter Paradigm™ Detection Platform (Beckman, Fullerton, CA).

RNA Extraction and Quantification of Gene Expression

Dexmedetomidine was administrated at 1 μM 60 min before RNA extraction for the neuronal and astrocyte cultures. Total RNA from primary neuronal and astrocyte cell cultures was extracted 1 h after dexmedetomidine treatment, and from cortical hemispheric regions (sample of 5 mm diameter around the injection) 4 h after dexmedetomidine treatment by the RNeasy mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the iScript™ kit (Bio-Rad, Paris, France). To amplify *Bdnf1, Bdnf2, Bdnf3,* and *Bdnf4* isoforms specifically, we designed the appropriate specific sets of sense and antisense primers using Primer 3 and M-fold software (table 1). Concerning *Bdnf5*, we designed specific sense and antisense primers corresponding to the sequence shared by the four *Bdnf* isoforms. Quantitative polymerase chain reaction was then performed on a CFX96 (Bio-Rad) with the Sybr Green SuperMix (Bio-Rad). Relative expressions of the different *Bdnf* transcripts were compared to the expression of the *Gapdh* gene.

BDNF Expression Quantification

BDNF protein concentrations were compared on brain cortical samples after normalization for the total protein concentration of each sample (4 h after dexmedetomidine intraperitoneal injection corresponding to 3 h after ibotenate intracranial injection) and on astrocyte culture media, using the mouse BDNF ELISA kit (KA033, Abnova, Taipei, Taiwan). Yohimbine (10 μM) and PD098059 (10 μM) were administrated *in vitro* 30 min before dexmedetomidine (1 μM) and 24 h before the BDNF quantification.

Table 1. Primers Used for qRT-PCR for bdnf mRNA Variants 1–5

	Sense Primers	Antisense Primers	Product Length	NCBI Reference
bdnf1	AGGACAGCAAAGCCACAATGT	CCTTCATGCAACCGAAGTATG	92 pb	NM_007540.4
bdnf2	GGGCTGGAGAGAGAGTCAGA	CGCTAGGAAGCCAACTTCAG	76 pb	NM_001048139.1
bdnf3	AGCCCAGTTCCACCAG	CATGCAACCGAAGTATGAAAT	75 pb	NM_001048141.1
bdnf4	AGCAGCTGCCTTGATGTTTAC	ATGCAACCGAAGTATGAAATA	112 pb	NM_001048142.1
bdnf5	ACTCCACTGCCCATGATGTA	TGAACAAATGCTGGTCTTTCC	99 pb	Common part of the 4 isoforms
gapdh	GGCCTTCCGTGTTCCTAC	TGTCATCATATCTGGCAGGTT	79 pb	XM_001473623.1

BDNF = brain-derived neurotrophic factor; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; mRNA = messenger ribonucleic acid; NCBI = National Center for Biotechnology Information; qRT-PCR = Quantitative reverse transcriptase polymerase chain reaction.

Phospho-ERK1/2 Quantification

Phospho-ERK1/2 was quantified in brain cortical samples after normalization for the total protein concentration of each sample and astrocyte cultures using a specific ELISA Kit for the phosphorylated form of ERK1/2 at two positions: ERK1 (T202/Y204) and ERK2 (T185/Y187) (R&D, Minneapolis, MN).

Statistical Analysis

Data were analyzed with the Student *t* test or one-way ANOVA after the normality of the data was assessed using the Shapiro–Wilk W test and the equality of the variances using the F test. The one-way ANOVA analyses were followed by *post hoc* analysis using multiple-comparison tests (two-sided Bonferroni). For the cortical BDNF expression quantification, a two-way ANOVA was performed including the effect of the intraperitoneal injection (dexmedetomidine *vs.* PBS), the effect of the intracranial injection (ibotenate *vs.* PBS), and their interaction. *In vivo* experiments were performed with 11–22 animals per condition from three different series of pups. *In vitro* experiments were performed with 12–24 wells per condition from at least three different series of cell cultures. Relative transcript gene expression and ELISA protein quantifications were established with more than five different samples from each group from two different cell cultures. BDNF and phospho-ERK 1/2 ELISA quantification were established twice with five different samples from each group from two different cell cultures. Data are expressed and represented as mean ± SD, and two-sided *P*-values were considered significant when $p < 0.05$. Statistics were performed with Graph-Pad Prism version 5 software.

Results

BDNF *and Dexmedetomidine Exert Neuroprotective Effects* In Vivo *and* In Vitro *against Ibotenate-Induced Neurotoxicity*

To investigate the protective effect of BDNF itself, the lesion size of mice pups previously treated with intraperitoneal injections of BDNF was compared to the lesion size of control pups after ibotenate-induced neurotoxicity. BDNF injections decreased lesion size by approximately 30% (Fig. 1, A and B, n = 12–18, $P < 0.01$). In primary neuronal cell cultures of ibotenate-induced neurotoxicity which generated 76 ± 13% of neuronal death in DIV 12 neurons (data not shown), BDNF also exerted a significant, dose-dependent, direct neuroprotective effect (n = 24, Fig. 1C). Interestingly, in the same primary neuronal cell cultures without any excitotoxic stress, BDNF was still able to increase cell viability (data not shown), which demonstrates the anti-apoptotic effect of BDNF at this stage.

When the pups were treated with intraperitoneal injections of dexmedetomidine 1 h before the ibotenate-induced lesion, the lesion size was statistically decreased (822 ± 200 μm, *vs.* 577 ± 148 μm, n = 11–14, $P < 0.01$, Fig. 1D). Body temperatures of pups treated by dexmedetomidine were not different from those of controls (data not shown). *In vitro*, dexmedetomidine also presented a direct neuroprotective effect at DIV 12 (n = 16–24, Fig. 1E). Dexmedetomidine's protective effect was still significant at DIV 7 (n = 22–24, Fig. 1F) and when the neuronal media was supplemented with 0.4% and 4% of fetal calf serum (n = 24, Fig. 1G). Dexmedetomidine's direct neuroprotective effect was inhibited with yohimbine, an α_2-adrenergic receptor antagonist (n=16–24, Fig. 1H), suggesting that neuronal α_2-adrenergic receptor activation was involved in dexmedetomidine's direct protective effect. In addition, a high dose of dexmedetomidine (10 μM) was not neuroprotective (Fig. 1E), and dexmedetomidine did not exert any anti-apoptotic effect in the primary neuronal cell culture without any excitotoxic stress (data not shown).

Dexmedetomidine Enhances BDNF *Expression in Astrocyte but Not Neuron Cultures*

The transcript splice variant expression of *bdnf* was then compared 3 h after the ibotenate-induced lesions (Fig. 2A), corresponding to 4 h after dexmedetomidine or PBS intraperitoneal injections. The *bdnf4* expression was increased after dexmedetomidine injections (150 ± 40%, n = 5, $p < 0.05$). The *bdnf5* expression that corresponded to the average expression of *bdnf* splice variants was also increased (142 ± 27%, n = 5, $P < 0.05$) in the cortical lesion. We confirmed with BDNF protein quantification that

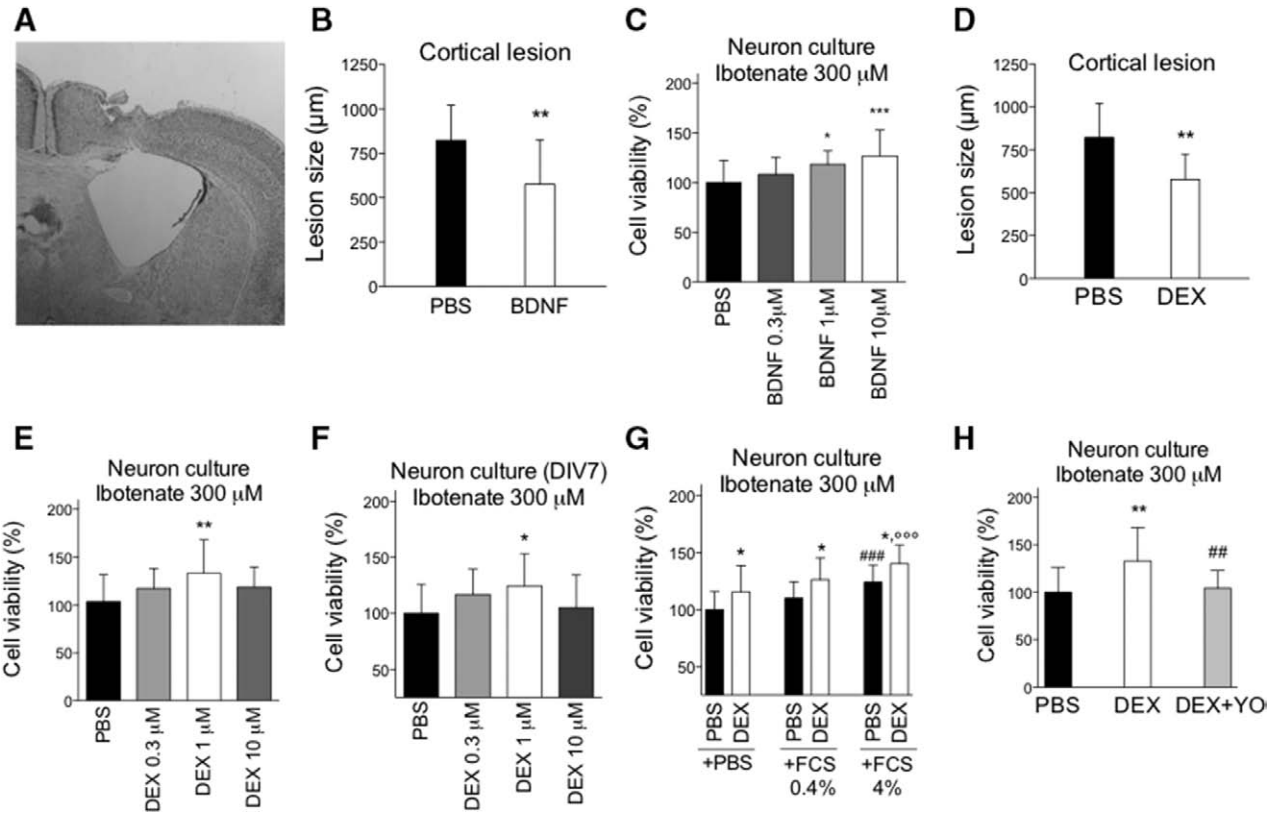

Fig. 1. Brain-derived neurotrophic factor (BDNF) and dexmedetomidine protective effects in glutamate agonist-induced *in vivo* and *in vitro* models. (*A*) Picture showing the excitotoxic ibotenate-induced lesions. Ibotenate (10 µg in 2µl) was injected intracerebrally to 5-day-old mouse pups. The pups were sacrificed on postnatal day 10 and their brains were embedded in paraffin before sectioning. (*B*) Effect of BDNF in excitotoxic challenge to mouse pups with ibotenate-induced lesions. $^{**}P < 0.01$ *versus* phosphate-buffered saline (PBS). (*C*) Effect of increasing concentrations of BDNF (PBS, 0.3, 1, and 10 µM) in excitotoxic challenge (ibotenate 300 µM) to primary cortical isolated neuronal cultures on day *in vitro* (DIV) 12 with ibotenate-induced toxicity. $^{*}P < 0.05$ and $^{***}P < 0.001$ *versus* PBS by ANOVA with *post hoc* analysis. (*D*) Effect of intraperitoneal injection of dexmedetomidine (DEX, 3 µg/kg) 1 h before excitotoxic challenge to pups mice with ibotenate-induced lesions. $^{**}P < 0.01$ *versus* PBS. (*E–F*) Effect of increasing concentrations of dexmedetomidine (none, 0.3, 1, and 10 µM) in excitotoxic challenge (ibotenate 300 µM) to primary cortical isolated neuronal cultures at DIV 12 (*E*) and at DIV 7 (*F*) with ibotenate-induced cell death. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* PBS by ANOVA with *post hoc* analysis. (*G*) Effect of increasing concentrations of fetal calf serum (PBS, 0.4% and 4%) on dexmedetomidine's neuroprotective effect in excitotoxic challenge (ibotenate 300 µM). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* PBS. (*H*) Effect of yohimbine (10 µM) in dexmedetomidine's protective effect in excitotoxic challenge (ibotenate 300 µM). $^{**}P < 0.01$ *versus* PBS, ## $P < 0.01$ *versus* DEX 1 µM by ANOVA with *post hoc* analysis. The *bars* represent the mean length of the lesion along the sagittal fronto-occipital axis ± SD for *in vivo* experiments, and the cell viability ratio ± SD, corresponding to the percent of the controls (ibotenate 300 µM defined as 100% of cell viability) for *in vitro* experiments. FCS = fetal calf serum; YO = yohimbine.

intraperitoneal injection of dexmedetomidine increased BDNF cortical concentration after PBS and ibotenate intracranial injection (n = 5, Fig. 2B).

The same *bdnf* primers were tested in primary isolated neuronal and astrocyte cell cultures 1 h after 1 µM of dexmedetomidine. Interestingly, dexmedetomidine did not promote *bdnf4* and *bdnf5* expression in isolated neuron cultures (n = 5–6, Fig. 2C) but increased *bdnf4* and *bdnf5* expression (139 ± 9%, $P = 0.003$ and 124 ± 10%, $P = 0.04$, respectively, with n=5) in isolated astrocyte cultures (Fig. 2D). Although dexmedetomidine and ibotenate treatment did not affect astrocyte cell viability (Fig. 2E), BDNF concentration in the astrocyte culture media was significantly increased after dexmedetomidine (n = 12, $P < 0.01$, Fig. 2F). Yohimbine was able to inhibit the increase in BDNF induced by dexmedetomidine (n = 12, Fig. 2F), which indicates that dexmedetomidine induced BDNF release through α_2-adrenergic receptors.

Neuroprotective Effect of Dexmedetomidine Is Related to BDNF *and ERK Pathway* In Vivo *but Not* In Vitro

In the same pup model of excitotoxic lesions, intracranial coadministration of neutralizing BDNFab inhibited dexmedetomidine's protective effect (n = 14–20, $P < 0.001$, Fig. 3A). Lesion sizes were not affected by intracranial administration of BDNFab itself (n = 12, Fig. 3A). However, BDNFab did not inhibit dexmedetomidine's protective effect in neuronal cultures (131 ± 40%, n = 16–24, $P < 0.05$,

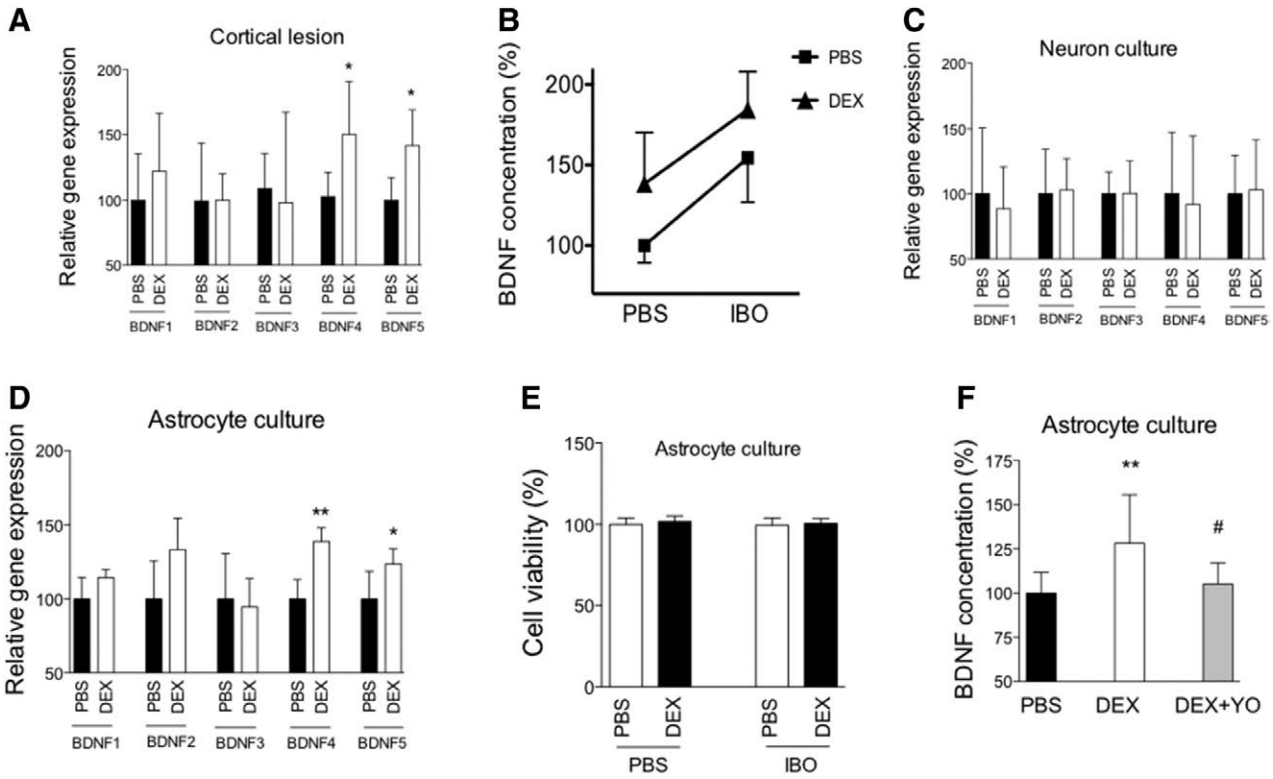

Fig. 2. Modulation of brain-derived neurotrophic factor (BDNF) expression by dexmedetomidine. (*A*) Effect of dexmedetomidine (DEX, 3 μg/kg) *versus* phosphate-buffered saline (PBS) on mRNA *bdnf* normalized relative expression in cortical ibotenate (IBO)-induced lesions. (*B*) Effect of 1 h of DEX (1 μM) *versus* PBS prior to IBO intracranial injection *versus* PBS on relative BDNF concentration. Two-way ANOVA analyses showed that IBO and DEX injections were both significant predictors without interactions ($P < 0.001$ and $P = 0.007$, respectively and $P = 0.72$ for the interaction term). (*C*) Effect of 1 h of DEX (1 μM) *versus* PBS on mRNA *bdnf* normalized relative expression in isolated neuron culture. The *bars* represent the normalized relative expression ± SD of each gene of interest (GOI) corresponding to the ratio between each gene expression and *gapdh*. (*D*) Effect of 1 h of DEX (1 μM) *versus* PBS prior to 300 μM of IBO *versus* PBS on astrocyte cell viability. (*E*) Effect of 1 h of DEX (1 μM) *versus* PBS on mRNA *bdnf* normalized relative expression in isolated astrocyte culture. $^{**}P < 0.01$, $^{*}P < 0.05$ *versus* PBS. (*F*) Effect of 24 h of DEX (1 μM) and the association of DEX with yohimbine (DEX+YO 10 μM) on BDNF protein relative concentration in the media of astrocyte culture. $^{**}P < 0.01$ *versus* PBS, #$P < 0.05$ *versus* DEX 1 μM by ANOVA with *post hoc* analysis. YO = yohimbine.

Fig. 3B). Although BDNFab had no major effect on cell viability on its own (Fig. 3B), BDNFab inhibited the BDNF protective effect (not shown).

As we previously demonstrated using a hippocampal slice model that the ERK1/2 pathway was involved in dexmedetomidine's protective effect,[12] we hypothesized that dexmedetomidine's protective effect would be dependent on the neuronal ERK1/2 pathway. We first confirmed with intracranial coadministration of PD098059 and ibotenate that the inhibition of ERK1/2 phosphorylation blocked dexmedetomidine's *in vivo* protective effect ($p < 0.001$) without having any significant effect itself (n=11–22, Fig. 4A). Results in neuronal cell cultures showed that dexmedetomidine neuroprotective's effect was not affected by PD098059. Indeed, dexmedetomidine was still protective against ibotenate-induced neurotoxicity in the presence of increasing concentrations of ERK inhibitors (n = 16–24, Fig. 4B), indicating that the neuroprotective effect of dexmedetomidine was not directly related to the neuronal ERK pathway. The role of the ERK pathway in dexmedetomidine's protective effect *in vivo,* but not *in vitro*, suggests that nonneuronal cells play a role in dexmedetomidine's protective effect through ERK pathway. We further found that dexmedetomidine increased phospho-ERK1/2 concentration in astrocyte cultures (n = 5, Fig. 4C) as well as in the cortical lesion (data not shown), and that PD098059 was able to inhibit dexmedetomidine's increase of Phospho-*ERK1/2* (n = 5, Fig. 4C). Finally, we demonstrated that dexmedetomidine-induced BDNF increase was inhibited by PD098059 in astrocyte cultures (n = 12, Fig. 4D). Altogether, our data suggested that *in vivo* dexmedetomidine-induced neuroprotection is also related to dexmedetomidine-induced activation of *ERK1/2* and BDNF release by astrocytes (Fig. 5).

Discussion

The main finding of this study is that dexmedetomidine exhibits neuroprotective effects against neonatal glutamate-induced injury via an increase in BDNF expression. We

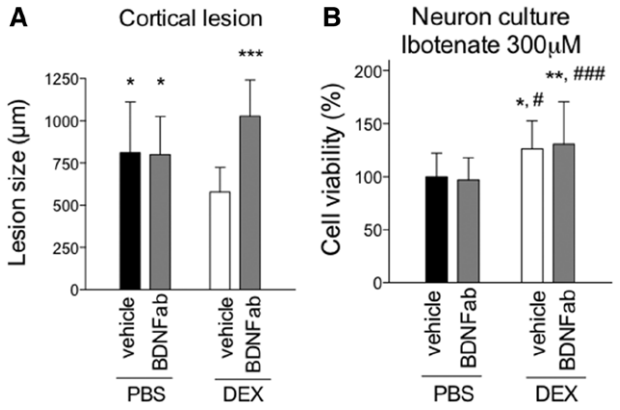

Fig. 3. Effects of brain-derived neurotrophic factor antibody (BDNFab) on dexmedetomidine's protective effects. (*A*) Effect of the intracranial injection of neutralized BDNFab in glutamate agonist challenge to mouse pups with ibotenate-induced lesions with and without dexmedetomidine protection. Statistically significant differences between experimental groups are shown by ANOVA with *post hoc* analysis: **P* < 0.05, ****P* < 0.001 *versus* dexmedetomidine+vehicle group. (*B*) Effect of BDNFab in excitotoxic challenge to primary cortical isolated neuron cultures with ibotenate-induced toxicity with and without dexmedetomidine coadministration. Statistically significant differences between experimental groups are shown by ANOVA with *post hoc* analysis: **P* < 0.05, ***P* < 0.01 *versus* ibotenate alone (PBS+vehicle) and #*P* < 0.05, ###*P* < 0.001 *versus* ibotenate+BDNFab (PBS+BDNFab). The *bars* represent the mean length of the lesion along the sagittal fronto-occipital axis ± SD for *in vivo* and the cell viability ratio ± SD, corresponding to the percent of the controls (defined as 100% cell viability) for *in vitro* experiments. DEX = dexmedetomidine; PBS = phosphate-buffered saline.

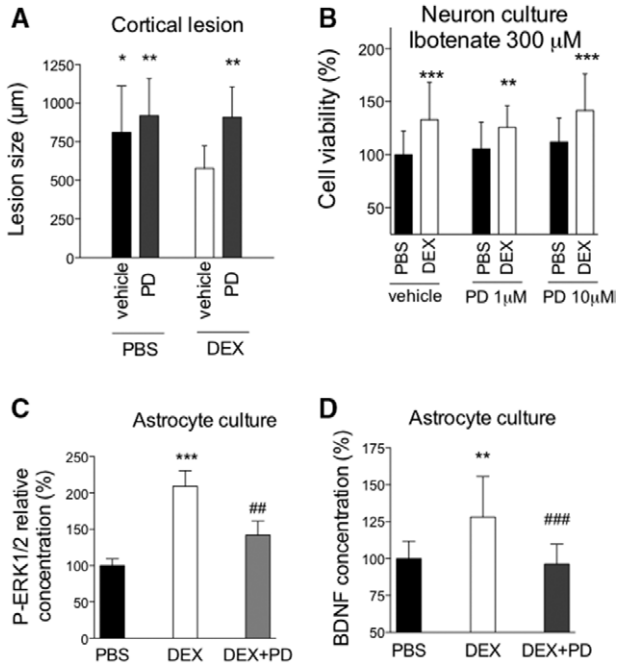

Fig. 4. Effects of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation on dexmedetomidine's protective effects. (*A*) Effect of the inhibition of ERK1/2 phosphorylation with PD098059 (PD) in glutamate agonist challenge to pups mice with ibotenate-induced lesions with and without dexmedetomidine protection. Statistically significant differences between experimental groups are shown by ANOVA with *post hoc* analysis: **P* < 0.05, ***P* < 0.01 *versus* dexmedetomidine+vehicle group. (*B*) Effect of PD in glutamate agonist challenge to primary cortical isolated neuronal cultures with ibotenate-induced toxicity with and without dexmedetomidine coadministration. Statistically significant differences between control and treatment conditions are shown: ****P* < 0.001, ***P* < 0.01 *versus* phosphate-buffered saline (PBS) for each PD doses. (*C*) Phospho-ERK1/2 quantification in astrocytes cultures in control culture (PBS), after dexmedetomidine (DEX, 1 µM) and dexmedetomidine with PD098059 (DEX+PD) administration. ****P* < 0.01 *versus* PBS, ##*P* < 0.01 *versus* DEX by ANOVA with *post hoc* analysis. (*D*) Effect of 24 h of dexmedetomidine (DEX, 1 µM) and the association of dexmedetomidine with PD (DEX+PD) on BDNF protein normalized concentration in the media of astrocyte culture. ***P* < 0.01 *versus* PBS, ###*P* < 0.001 *versus* DEX by ANOVA with *post hoc* analysis. The *bars* represent the mean length of the lesion along the sagittal fronto-occipital axis ± SD for *in vivo* and the cell viability ratio ± SD, corresponding to the percent of the controls (defined as 100% cell viability) for *in vitro* experiments. PD = PD098059 (ERK1/2 phosphorylation inhibitor).

further demonstrate that astrocytes, but not neurons, are involved in dexmedetomidine-induced BDNF expression and that astrocyte expression of BDNF and dexmedetomidine neuroprotective effects are both related to the *ERK1/2* pathway.

Dexmedetomidine Enhances BDNF *Expression in Astrocytes*

Several lines of evidence have shown that dexmedetomidine improves neuronal survival in adult excitotoxic lesions[19,20] as well as in rodent neonates.[10,11] One of the best known actions of dexmedetomidine in the brain is presynaptic inhibition of noradrenaline release and cell firing in noradrenergic neurons, but only a minor fraction of α_2-adrenoceptors appear to be presynaptic.[9] This study showed that dexmedetomidine presented some direct and concentration-dependent neuroprotective effects in neuronal ibotenate-induced neurotoxicity. This protection was affected by yohimbine, an α_2-adrenergic inhibitor. Because dexmedetomidine is a potent and highly selective agonist of α_2-adrenergic receptors, this direct neuroprotective effect may be neuronal α_2-adrenergic receptor–dependent. As Talke and Bickler[21] showed, dexmedetomidine can modulate the release of glutamate in hypoxic hippocampal rodent slices, suggesting that glutamate-induced excitotoxicity can play an important role in dexmedetomidine's neuroprotection.

Interestingly, we also showed *in vivo* that dexmedetomidine stimulated *bdnf* expression and that neutralizing BDNF antibodies inhibit some of dexmedetomidine's neuroprotective effect. These results suggest that dexmedetomidine presents a strong protective effect against excitotoxic damage and

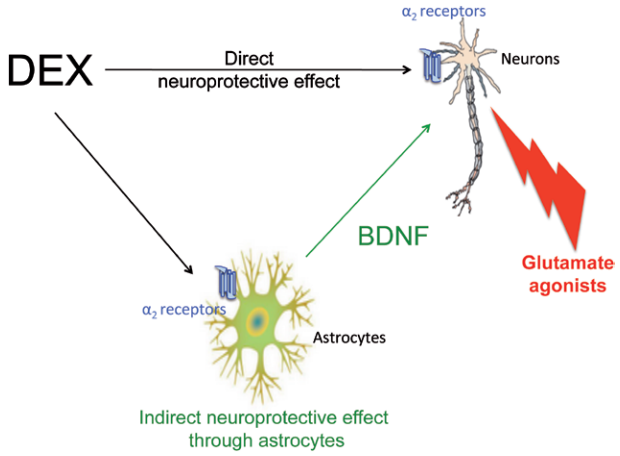

Fig. 5. Schematic representation of the direct and indirect neuroprotective effect of dexmedetomidine. In the context of excitotoxicity, dexmedetomidine presented a direct neuroprotective effect that was brain-derived neurotrophic factor (BDNF) independent. Through astrocyte's α_2-adrenergic receptors, dexmedetomidine was able to activate extracellular signal-regulated kinase (ERK) pathway and subsequently increase the BDNF release of astrocyte, suggesting an indirect neuroprotective effect of dexmedetomidine through astrocytes. DEX = dexmedetomidine.

that dexmedetomidine's protection is also related to BDNF expression. Interestingly, neuronal *bdnf* expression was not affected by dexmedetomidine, and neutralizing BDNF did not affect dexmedetomidine's direct neuroprotective effects. Because dexmedetomidine increased *bdnf* expression and BDNF release in astrocytes, these data suggest that dexmedetomidine increased astrocyte expression of BDNF, which induced a subsequent astrocyte-dependent neuroprotective effect.

Increasing evidence has shown dynamic interactions between neurovascular cells, including endothelial cells, astrocytes, microglia, and neurons in excitototoxic lesions.[22,23] Astrocyte cells provide metabolites, shuttle ions, and water and scavenge reactive oxygen species and reactive metals. They modulate inflammation, participate in the clearance of cell debris, and produce neurotrophic factors.[24] Thus, astrocytes are key to protecting against excitotoxicity induced by a wide range of factors. Recent data support the hypothesis that dexmedetomidine exerts its protective effects via a direct effect on astrocyte α_2-adrenergic receptors.[11,25] In the murine cerebral cortex, α_2-adrenergic receptors are expressed in astrocytes and oligodendrocytes.[26] In this study, we demonstrated for the first time that dexmedetomidine stimulates BDNF expression in cultured astrocytes and in cortical lesions. Critically, BDNF regulates neuronal function by promoting survival, enhancing synaptic plasticity via neurotrophic tyrosine kinase receptor type 2 (TrkB).[27] However, the release of the precursor of BDNF[28] into the extracellular space has also been shown. Interestingly, the precursor of BDNF is described to promote cell death and attenuate synaptic transmission[29] through the activation of low-affinity nerve growth factor receptor (p75). Equilibrium between BDNF (18 KDa) and the precursor of BDNF (35 KDa) is a key issue for the neuronal release of BDNF,[30] and it could also be important for the release of BDNF from astrocytes.

Involvement of ERK1/2 Pathway

BDNF/TrkB-stimulated intracellular signaling is critical for morphogenesis, neuronal survival, and neuroplasticity. It is well known that binding of BDNF to TrkB activates various intracellular signaling pathways, including ERK, phospholipase C, and phosphoinositide 3-kinase pathways. The ERK pathway plays an important role during brain development, neuron proliferation, and apoptosis.[31] Interestingly, the ERK pathway is not only involved in neuronal stability but also in astrocytes' response. Indeed, various stimuli—such as neurotransmitters, growth factors, glutamate, and oxidative stress—cause astrocytic responses through phosphokinase C activation and ERK pathways.[31]

Our group previously showed that the ERK pathway plays a key role in dexmedetomidine neuroprotection in hippocampal slices,[12,32,33] although the specific brain cells directly involved in this protective phenotype was unknown. In this study, we found that the ERK pathway was involved in the *in vivo* protective effect of dexmedetomidine but not in the neuronal protective effect *in vitro*. These data suggest that dexmedetomidine-induced ERK activation is mainly protective by nonneuronal cell activation. Recently, it has been shown that FK506 and cyclosporine A can increase ERK1/2 expression in the nuclear fraction of astrocytes[33] and also promote *bdnf* expression. Our study suggested for the first time a role of the ERK1/2 pathway for BDNF release by astrocytes after dexmedetomidine stimulation. Adrenergic receptor activation has already been shown to increase BDNF expression in astrocytes,[14] and here, we confirmed it with dexmedetomidine's activation and yohimbine's inhibitive effects.

Methodological Strengths and Limitations

We used a pharmacologic and *in vitro* approach to investigate the cellular mechanisms involved in dexmedetomidine's neuroprotective effects. The clinical relevance of the choice of drug concentrations can be an issue with an *in vitro* model. In neuronal cultures, the dexmedetomidine-induced neuroprotective effect is concentration dependent, as has already been shown with other models.[10] Here, we checked that this effect was not dependent on the age of the neurons (DIV 7 and DIV 12) or the serum concentration. Interestingly, dexmedetomidine presents a U-shaped protective response, with a ceiling effect observed for the highest dose (10 μM). The high-dose toxicity of dexmedetomidine has already been described in studies of permanent occlusion of the middle cerebral artery in rats. In humans, plasma concentrations up to 1 μM can be reached after an IV administration, which supports the physiologic relevance of our choice of

dexmedetomidine concentrations in this study.[34] Moreover, in this study, we found a strong discordance between our *in vitro* and *in vivo* experiments, which gave us the opportunity to look at nonneuronal cells such as astrocytes. These findings point to the fact that the brain neurovascular unit should be studied as a global set and that neuronal phenotypes can be highly modulated by other proximal cells.

We used a well-characterized murine model of ibotenate-induced excitotoxic brain injury in this study. It displays remarkable histopathologic similarities to human neuronal injuries described after hypoxia in human neonates.[15] With this model, the initial intracerebral injection of ibotenate directly activates local NMDA and metabotropic group 1 receptors, triggering a secondary release of glutamate as well as many neurotoxic compounds such as free radicals, cytokines, and catecholamines. However, this model is not able to mimic other clinical conditions such as ischemic stroke or hypoxia precisely. Moreover, it is now clear that some conditions like systemic inflammation can exacerbate acute brain lesions, and thus modify brain response to neuroprotective drugs.[15]

Even if *in vitro* BDNF concentration were increased in astrocyte culture and that the *bdnf* expression occurred before the neuronal cell death in our model, we cannot completely exclude that the increased expression of *bdnf in vivo* could be due to the protected brain tissue by dexmetedomidine's direct effect. To answer the issue of the relative roles of neurons and astrocytes activated by dexmedetomidine in the context of ibotenate-induced brain lesions, tools such as conditional knockout mice specifically for α_2 receptors in neuron under neuron's promoter or in astrocyte under astrocyte promoter would be relevant. Because dexmedetomidine improves neuronal survival in adult excitotoxic lesions[19,20] as well as in rodent neonates,[10,11] the increased *bdnf* expression could be related to the protected brain tissue itself. This hypothesis cannot be refuted with our model, but several arguments suggest that dexmetedomidine can act on *bdnf* expression itself: (1) the selective increase in *bdnf4* and not the other variants, (2) the very early expression of *bdnf* as opposed to the time course of ibotenate-induced brain lesion, and (3) the increased *bdnf* expression in astrocytes without any cell death.

Taken together, our findings support that dexmedetomidine stimulates BDNF expression in astrocytes. But there are caveats to our findings that require further research. We did not quantify the precursor of BDNF/BDNF ratio, neither did we identify all soluble factors that are released from astrocytes stimulated by dexmedetomidine. Astrocytes can release many growth factors besides BDNF, such as glial cell-derived neurotrophic factor,[35] vascular endothelial growth factor,[36] and epidermal growth factor,[36] all of which can promote neuronal survival. Therefore, it will be interesting in future studies to carefully dissect the network of interacting factors that could be stimulated by dexmedetomidine and to determine whether the release of proBDNF/BDNF is also dependent on the ERK pathway.

Moreover, BDNF has already been shown to increase NMDA receptor currents,[37] indicating that BDNF could somehow increase neurotoxicity. Thus, the time-dependant effects of dexmedetomidine and BDNF release need further studies.

Excessive glutamate release leads to excitotoxicity, which has an important role in many brain disorders, including trauma and ischemic brain injuries.[38–40] Recently, using a similar model of glutamate agonist intracranial injections, Dawson *et al.*[41,42] described that glutamate excitotoxic effects were mediated through influx of calcium through NMDA receptors, leading to generation of poly-(adenosine diphosphate ribose) polymer, that kills cells through apoptosis inducing factor. This form of cell death has recently been designated parthanatos, to distinguish it from apoptosis, autophagy, and necrosis. How dexmedetomidine and dexmedetomidine-induced BDNF release can interact with glutamate agonist-induced cell death, and if this protective effect is specific for perinatal brain injuries will need further studies.

In conclusion, our data suggested that increased BDNF expression in the brain after dexmedetomidine stimulation is mediated by astrocytes, and that this astrocyte-dependent neuroprotective process is ERK related. These results offer an original working hypothesis on potential targets for dexmedetomidine neuroprotection.

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