

# Dexmedetomidine Prevents Excessive $\gamma$ -Aminobutyric Acid Type A Receptor Function after Anesthesia

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## ABSTRACT

**Background:** Postoperative delirium is associated with poor long-term outcomes and increased mortality. General anesthetic drugs may contribute to delirium because they increase cell-surface expression and function of  $\alpha 5$  subunit-containing  $\gamma$ -aminobutyric acid type A receptors, an effect that persists long after the drugs have been eliminated. Dexmedetomidine, an  $\alpha 2$  adrenergic receptor agonist, prevents delirium in patients and reduces cognitive deficits in animals. Thus, it was postulated that dexmedetomidine prevents excessive function of  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptors.

**Methods:** Injectable (etomidate) and inhaled (sevoflurane) anesthetic drugs were studied using cultured murine hippocampal neurons, cultured murine and human cortical astrocytes, and *ex vivo* murine hippocampal slices.  $\gamma$ -Aminobutyric acid type A receptor function and cell-signaling pathways were studied using electrophysiologic and biochemical methods. Memory and problem-solving behaviors were also studied.

**Results:** The etomidate-induced sustained increase in  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptor cell-surface expression was reduced by dexmedetomidine (mean  $\pm$  SD, etomidate:  $146.4 \pm 51.6\%$  vs. etomidate + dexmedetomidine:  $118.4 \pm 39.1\%$  of control,  $n = 8$  each). Dexmedetomidine also reduced the persistent increase in tonic inhibitory current in hippocampal neurons (etomidate:  $1.44 \pm 0.33$  pA/pF,  $n = 10$ ; etomidate + dexmedetomidine:  $1.01 \pm 0.45$  pA/pF,  $n = 9$ ). Similarly, dexmedetomidine prevented a sevoflurane-induced increase in the tonic current. Dexmedetomidine stimulated astrocytes to release brain-derived neurotrophic factor, which acted as a paracrine factor to reduce excessive  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptor function in neurons. Finally, dexmedetomidine attenuated memory and problem-solving deficits after anesthesia.

**Conclusions:** Dexmedetomidine prevented excessive  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptor function after anesthesia. This novel  $\alpha 2$  adrenergic receptor- and brain-derived neurotrophic factor-dependent pathway may be targeted to prevent delirium. (ANESTHESIOLOGY 2018; 129:477-89)

THE availability of safe, modern general anesthetic drugs has ensured that most patients now survive surgery and are discharged from the hospital. However, many patients experience cognitive deficits such as delirium in the first few days after surgery.<sup>1,2</sup> Delirium is associated with a loss of independence, a failure to return to work, a substantial increase in healthcare costs and death.<sup>1-3</sup> Given that more than 312 million patients undergo anesthesia and surgery each year,<sup>4</sup> it is essential to develop strategies to reduce postoperative delirium. Although nonpharmacologic interventions are somewhat effective, pharmacologic prevention and treatments are needed.<sup>1</sup>

The pathophysiology underlying postoperative delirium is complex and multifactorial; however, anesthetic drugs are likely a contributing factor. We have previously shown that even a single, brief exposure to a commonly used general anesthetic drug can trigger a sustained increase in cell-surface expression and function of extrasynaptic  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptors containing the  $\alpha 5$  subunit

### What We Already Know about This Topic

- Dexmedetomidine is effective in reducing delirium in the postoperative period and in the intensive care unit in patients and prevents memory deficits in experimental animals subjected to anesthesia. The mechanism by which dexmedetomidine preserves cognition is not clear.
- In experimental models, even a single anesthetic exposure leads to a sustained increase in the expression of cell-surface  $\gamma$ -aminobutyric acid type A receptors that contain  $\alpha 5$  subunits. Whether dexmedetomidine reduces this increase and preserves cognition was evaluated.

### What This Article Tells Us That Is New

- Dexmedetomidine, by an  $\alpha 2$  receptor-mediated release of brain-derived neurotrophic factor from astrocytes, reduced neuronal  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptor expression and prevented cognitive deficits after anesthesia.
- The data suggest that suppression of anesthesia-induced sustained increase in  $\alpha 5$   $\gamma$ -aminobutyric acid type A receptor expression may be a potential therapeutic target for the prevention of postoperative and intensive care unit delirium.

This article is featured in "This Month in Anesthesiology," page 1A. This article has a video abstract. D.-S.W., K.K., and G.L. contributed equally to this article.

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( $\alpha 5$  GABA<sub>A</sub> receptors) in hippocampal neurons.<sup>5</sup> These changes persist long after the anesthetic drugs have been eliminated from the body. To date, no effective strategy has been identified to prevent excessive  $\alpha 5$  GABA<sub>A</sub> receptor cell-surface expression after general anesthesia.

The  $\alpha 2$  adrenergic receptor agonist dexmedetomidine lowers the incidence and duration of delirium in patients who have undergone cardiac and noncardiac surgery.<sup>6–8</sup> Dexmedetomidine also reduces delirium in patients who have been sedated for days in intensive care units.<sup>9</sup> Studies of laboratory animals have confirmed that dexmedetomidine can prevent cognitive deficits after anesthesia and surgery.<sup>10–13</sup> Here, we investigated the cellular and molecular mechanisms that contribute to the cognition-sparing properties of dexmedetomidine. We postulated that dexmedetomidine reduces the excessive function of  $\alpha 5$  GABA<sub>A</sub> receptors in neurons that persists after general anesthesia and thereby attenuates postanesthetic cognitive deficits.

## Materials and Methods

### Experimental Animals

Experiments were approved by the Animal Care Committee of the University of Toronto. All studies were performed in accordance with guidelines from the Canadian Council on Animal Care. The mice were located in a pathogen-free facility (25 ± 1°C) on a 14-h light/10-h dark cycle (lights on 6:00 AM to 8:00 PM). Timed-pregnant CD1 mice were used to prepare the cell cultures (Charles River, USA). Male C57BL/6 mice aged 8 to 9 weeks (Charles River) were used for behavioral studies, which were performed between 10:00 AM and 5:00 PM. Only male mice were selected for the behavioral studies because the estrous cycle influences the expression of extrasynaptic GABA<sub>A</sub> receptors.<sup>14</sup> Each mouse was assigned a random number that was generated by Microsoft (USA) Excel 2016. The assigned numbers were sorted from the smallest to the largest, and then the mice were allocated to different treatment groups. Researchers were blinded to the treatment groups for all behavioral experiments and the studies using *ex vivo* brain slices.

### Anesthesia

The mice were treated with dexmedetomidine (25 µg/kg, intraperitoneally), which was followed immediately by etomidate (20 mg/kg, intraperitoneally). Normal saline

(0.9% w/v of NaCl, intraperitoneally) and propylene glycol (35% v/v in normal saline) were used as vehicle controls for dexmedetomidine and etomidate, respectively. To prevent hypoxia, each mouse was anesthetized in an air-tight acrylic chamber that was flushed with oxygen and medical air (70% air, 30% O<sub>2</sub>) delivered at a flow of 1 l/min, as described previously.<sup>5</sup> Temperature in the chamber was maintained at 35°C with a heating blanket to prevent hypothermia. A total of 44 mice were treated with dexmedetomidine and etomidate. Four of these mice died 30 to 50 min after treatment. None of the mice treated with etomidate alone (n = 42) or dexmedetomidine alone (n = 43) died.

### Cell-surface Biotinylation

An anti-GABA<sub>A</sub> receptor antibody for  $\alpha 5$  (PhosphoSolutions, USA), an anti- $\beta$ -actin antibody (GenScript Biotech Corporation, USA), and an anti-Na<sup>+</sup>/K<sup>+</sup> ATPase antibody (Developmental Studies Hybridoma Bank, USA) were used to measure cell-surface expression of  $\alpha 5$  GABA<sub>A</sub> receptors, as previously described.<sup>5</sup>

### Primary Cell Culture

Primary cultures of hippocampal and cortical neurons were prepared from mice (embryonic days 16 to 18).<sup>15,16</sup> For the astrocyte cultures, cortical explants were isolated from mouse embryos, and cell cultures were grown to confluence in Dulbecco's modified Eagle's medium and 10% fetal bovine serum for 4 weeks.<sup>17–19</sup> The cells were then enzymatically dissociated with 0.05% trypsin–EDTA (Life Technologies, USA). The astrocyte-containing cell suspension was placed over hippocampal neurons that were cultured for 7 days. Cocultures were maintained for 7 to 10 days before recording from the neurons.

### Cultured Astrocytes from Human Optic Nerve

Human astrocytes were isolated from optic nerve heads obtained from deceased donors. Such astrocytes grow well in primary cell culture and closely resemble cortical astrocytes. Their functional properties have been characterized.<sup>20,21</sup> After the fifth passage, the human astrocytes were plated and grown to confluence in a monolayer.

### Whole-cell Voltage-clamp Recordings

Conventional whole-cell voltage-clamp recordings were performed from cultured hippocampal and cortical neurons.<sup>5,16</sup> At the time of recordings, the neurons become polarized, have extensive axonal and dendritic arbors, and form numerous, functional synapses. Thus, they resemble mature neurons *in vivo*.<sup>22–24</sup> To record the tonic GABA<sub>A</sub> receptor-mediated current, GABA (0.5 µM) was added to the extracellular solution. This GABA concentration was selected because it is similar to the extracellular concentration of GABA that occurs *in vivo*.<sup>25–27</sup> The competitive GABA<sub>A</sub> receptor antagonist bicuculline (20 µM) was used to measure the amplitude of the tonic current.<sup>5,16,28</sup> Previous studies showed that the

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tonic current in hippocampal neurons is generated primarily by  $\alpha 5$  GABA<sub>A</sub> receptors.<sup>5,29</sup> Current was recorded at a holding potential of  $-60$  mV at room temperature.

### Western Blotting

Astrocytes were treated with dexmedetomidine ( $0.1 \mu\text{M}$ ) or vehicle ( $1 \times$  phosphate-buffered saline) for 1 h. The culture medium was then exchanged with fresh medium without fetal bovine serum. After 2 h, the medium was collected and then lysed in a cold lysis buffer that contained phosphatase and a protease inhibitor cocktail (Sigma–Aldrich, USA). After centrifugation at 10,000 relative centrifugal force for 10 min, the protein concentration was measured with a bicinchoninic acid kit (Thermo Scientific Pierce Protein Biology, USA). Proteins were separated by electrophoresis with sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Primary antibodies to detect phosphorylated extracellular signal–regulated kinase (1:1,000, Cell Signaling, USA), extracellular signal–regulated kinase (1:1,000, Cell Signaling), and the corresponding secondary antibody (1:4,000, Cell Signaling) were used, and the membrane was developed with the ECL kit (PerkinElmer, Canada). An antibody against  $\beta$ -actin (1:4,000, Santa Cruz Biotechnology, USA) was used as an internal control.

### Real-time Polymerase Chain Reaction Detection of Brain-derived Neurotrophic Factor Messenger RNA

Mouse astrocytes were maintained in culture for 4 weeks. The cells were treated with dexmedetomidine ( $0.1 \mu\text{M}$ ) or vehicle ( $1 \times$  phosphate-buffered saline) for 1 h. The culture medium was exchanged with fresh medium that lacked fetal bovine serum and were incubated for 2 h. Astrocytes were then lysed with a TRIzol reagent (Thermo Fisher Scientific, USA) to extract the total RNA. The first-strand cDNA was generated from total RNA with iScript reverse transcription supermix for quantitative real-time polymerase chain reaction (Bio-Rad Laboratories, Canada). Primer probe sets for mouse brain-derived neurotrophic factor were purchased from Bio-Rad Laboratories (Unique Assay ID: qMmuCED0050333, UniGene: Mm.1442). Polymerase chain reaction amplification was done using SsoAdvanced Universal SYBR Green Supermix reagent (Bio-Rad Laboratories). The level of messenger RNA (mRNA) was measured with a CFX96 real-time polymerase chain reaction detection system (Bio-Rad Laboratories) and normalized to  $\beta$ -actin RNA transcript levels. The comparative  $2^{-\Delta\Delta\text{CT}}$  method was used for relative quantification.

### Enzyme-linked Immunosorbent Assay Analysis of Brain-derived Neurotrophic Factor

Mouse astrocytes were cultured in 96-well plates for 4 weeks and then treated with dexmedetomidine ( $0.01$  to  $10 \mu\text{M}$ ) or vehicle control for 1 h. Six wells were used for each drug concentration. The culture medium was then changed to  $50 \mu\text{l}$  of fresh medium that lacked fetal bovine serum for 2 h. The

conditioned medium was collected and analyzed for brain-derived neurotrophic factor with an enzyme-linked immunosorbent assay kit (lot No. 381264701; BosterBio, USA). Briefly,  $200 \mu\text{l}$  of medium was loaded in each well of a 96-well plate and then coated with mouse monoclonal antibody against brain-derived neurotrophic factor. A brain-derived neurotrophic factor-specific biotin-conjugated secondary antibody was then added, followed by the avidin–biotin–peroxidase complex enzyme and substrate incubation. The absorbance of each sample was measured at  $450$  nm using microplate reader (Synergy Mx, BioTek, USA). A standard curve was created by using the brain-derived neurotrophic factor standard, and then the concentration of brain-derived neurotrophic factor was measured.

### Novel Object Recognition to Test Memory

The novel object recognition assay was performed as previously described.<sup>5</sup> An opaque cylinder-shaped arena that measured  $28.5$  cm in diameter and  $29$  cm in height was housed in a dimly lit room. Each mouse was habituated to the chamber for 5 min 24 h before testing. The mouse was trained and tested 24 h after drug treatment. During the training phase, the mouse was allowed to explore two identical “sample” objects for 5 min. Interaction time with the objects was defined when the mouse was sniffing, licking, or touching the object with the nose. The time when the mouse was approaching, climbing, or sitting on the object was excluded.

### Puzzle Box to Test Problem-solving

Executive function was assessed using a puzzle box,<sup>30</sup> which consisted of an illuminated start box ( $58 \times 28 \times 27.5 \text{ cm}^3$ ) and a dark, enclosed goal box ( $14 \times 28 \times 27.5 \text{ cm}^3$ ). A doorway and an underpass that connected the start box with the goal box allowed the animals to move between the two compartments. The mice were tested during a 3-day period, and the level of difficulty increased sequentially over day 1 (trials 1 to 3), day 2 (trials 4 to 6), and day 3 (trials 7 to 8). To assess problem-solving abilities, the task was made increasingly more difficult by modifying the entrance to the goal box. On day 1, trial 1, the goal box was accessible through both an open doorway and an open underpass. On day 1, trial 2, the doorway was closed, and the goal box was accessible only through the underpass. Trial 3 was conducted 2 min after trial 2 under the same conditions to assess an ability to recall escape strategies. Trials 4 to 6 were conducted on day 2. Trial 4 was performed under the same conditions as trial 2. During trial 5, the task was rendered more difficult by closing the door and obstructing the underpass with corncob bedding. This condition required that the mouse burrow through the bedding to gain access to the goal box. Trial 6 was performed 2 min after trial 5 under the same conditions. Trials 7 to 8 were conducted on day 3. Trial 7 was performed under the same conditions as trial 5. During trial 8, the difficulty was increased by obstructing the underpass with a cardboard plug, which the mouse had to remove to gain access to the goal box. During

the 2-min intertrial intervals, the mouse was taken out of the puzzle box and placed in a cage that was separate from their home cage. Performance was video-recorded, and the time required for each animal to enter the goal box, which was defined as having all four paws in the goal compartment, was measured. A maximum finish time of 5 min was assigned if the mouse failed to reach the goal box during the trial.

### Drugs and Chemicals

Tetrodotoxin and brain-derived neurotrophic factor were purchased from Alomone Labs (Israel). 6-Cyano-7-nitroquinoxaline-2,3-dione, (2R)-amino-5-phosphonovaleric, and bicuculline were obtained from Abcam (United Kingdom). GABA, etomidate, clonidine, yohimbine, and PD98059 were obtained from Sigma–Aldrich. Dexmedetomidine was obtained from Santa Cruz Biotechnology, sevoflurane was obtained from AbbVie Inc. (Canada), and ANA12 was from Cedarlane (Canada).

### Statistical Analysis

The data are presented as means  $\pm$  SD, together with the 95% CI of the mean. An unpaired Student's *t* test was used to compare two groups, where appropriate. For comparing three or more groups, the one-way ANOVA or two-way ANOVA followed by the Tukey's multiple comparisons *post hoc* test was used. The normality of datasets was tested with the D'Agostino–Pearson omnibus test ( $n \geq 8$ ) or the Kolmogorov–Smirnov test ( $n < 8$ ). To test for homogeneity of variances, the Brown–Forsythe test and an F test was used for the ANOVA and unpaired Student's *t* test, respectively. When assumptions were not met, the nonparametric Mann–Whitney U test was performed instead of the unpaired Student's *t* test. Because there is a high degree of variation for puzzle box data, the normality assumptions and homogeneity of variances for the log<sub>10</sub>-transformed data were confirmed with the D'Agostino–Pearson omnibus test and the Brown–Forsythe test, respectively. The Kaplan–Meier survival analyses of puzzle box data were compared with the log-rank pairwise comparison test, and right censoring was applied at the cut-off time of 5 min. A linear mixed model was employed for statistical analysis of cell-surface and total expression of the GABA<sub>A</sub> receptor  $\alpha 5$  subunit using IBM SPSS Statistics for Windows, version 23 (IBM Corp., USA). All other analyses were performed using GraphPad Prism 6.01 (GraphPad Software Inc., USA). A two-tailed hypothesis test was used, and statistical significance was set at  $P < 0.05$ . No statistical power calculation was conducted before the study, and the sample size was based on our previous experience with this experimental design. During peer review, the sample size for real-time polymerase chain reaction detection of brain-derived neurotrophic factor mRNA was increased, but no adjustments were made for interim analyses. No outliers were excluded from the statistical analyses.

## Results

### Dexmedetomidine Reduces Cell-surface Expression and Function of $\alpha 5$ GABA<sub>A</sub> Receptors after General Anesthesia

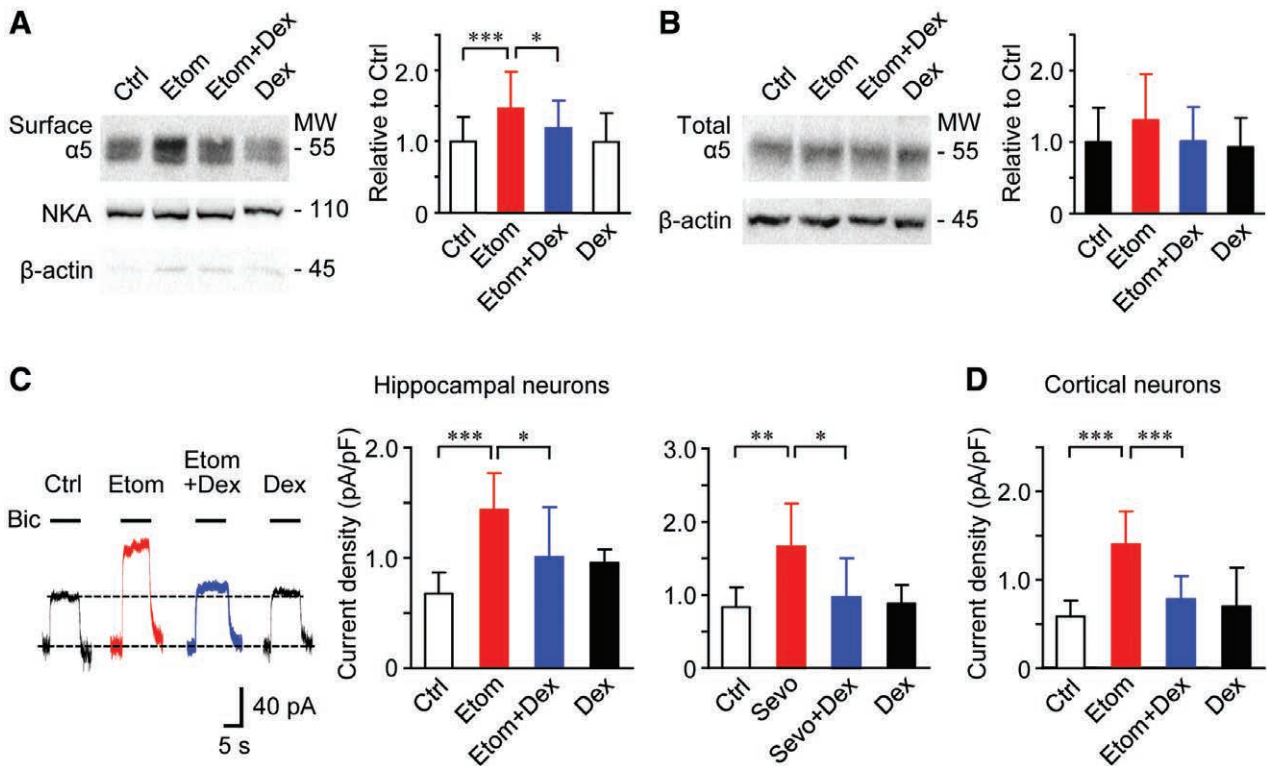
We first asked whether dexmedetomidine reduced excessive cell-surface expression of  $\alpha 5$  GABA<sub>A</sub> receptors in mice that were treated with an anesthetic drug. Etomidate was selected for these proof-of-concept studies because it has no active metabolites, it is relatively selective for GABA<sub>A</sub> receptors, it is rapidly eliminated *in vivo*, and it causes minimal depressant effects on cardiac function and respiration. Etomidate is also a structural analog of the “next generation” of injectable anesthetic drugs.<sup>31,32</sup>

The mice were treated with dexmedetomidine (25  $\mu$ g/kg, intraperitoneally) followed by etomidate (20 mg/kg, intraperitoneally). Twenty-four hours later, the mice were euthanized, and *ex vivo* hippocampal slices were processed for biotinylation analyses of surface proteins. Etomidate triggered a sustained increase in the cell-surface expression of  $\alpha 5$  GABA<sub>A</sub> receptors (146.4  $\pm$  51.6% of control, 95% CI 107.4 to 185.6%;  $n = 8$  each; fig. 1A) as shown previously.<sup>5</sup> Cotreatment with dexmedetomidine reduced this increase (118.4  $\pm$  39.1% of control, 95% CI 89.1 to 147.6%;  $n = 8$ ). The total expression of  $\alpha 5$  subunit protein was not significantly changed by either etomidate or dexmedetomidine (fig. 1B).

To determine whether dexmedetomidine prevented the increase in  $\alpha 5$  GABA<sub>A</sub> receptor function that persists after the anesthetic has been eliminated, a tonic inhibitory current was recorded from hippocampal neurons that were cocultured with cortical astrocytes. The design of these studies was based on our previous work showing that astrocytes were required to generate the anesthetic-induced persistent increase in tonic current in neurons.<sup>5</sup> Cocultures of neurons and astrocytes were treated with etomidate (1  $\mu$ M) and/or dexmedetomidine (0.1  $\mu$ M) for 1 h and then washed, and 24 h later, the tonic current was recorded. Etomidate alone increased the amplitude of the tonic current, whereas no increase was observed in cultures cotreated with etomidate and dexmedetomidine (control: 0.68  $\pm$  0.19 pA/pF, 95% CI 0.48 to 0.88,  $n = 6$ ; etomidate: 1.44  $\pm$  0.33 pA/pF, 95% CI 1.20 to 1.67,  $n = 10$ ; etomidate + dexmedetomidine: 1.01  $\pm$  0.45 pA/pF, 95% CI 0.66 to 1.36,  $n = 9$ ; fig. 1C).

We next asked whether the most commonly used inhaled general anesthetic, sevoflurane, also increased the tonic current in hippocampal neurons and, if so, whether dexmedetomidine prevented this increase. Treating the cocultures with a clinically relevant concentration of sevoflurane (266  $\mu$ M) increased the amplitude of the tonic current, and this increase was prevented by cotreatment with dexmedetomidine (fig. 1C).

Cognitive deficits after anesthesia also involve the function of the cortex. Thus, we studied whether etomidate triggered a persistent increase in tonic current in cortical neurons. Cortical neurons that were cocultured with astrocytes were pretreated with etomidate, as described above. The amplitude of the tonic current measured 24 h after treatment was increased. This increase did not occur in cortical neurons that were cotreated with etomidate and dexmedetomidine (fig. 1D).



**Fig. 1.** Dexmedetomidine prevents the sustained increase in extrasynaptic  $\gamma$ -aminobutyric acid type A ( $GABA_A$ ) receptor function induced by general anesthetics. (A, B) Representative Western blots and summarized data of the cell-surface (A) and total (B) expression of  $\alpha 5$  subunits.  $n = 8$ . A linear mixed model:  $F_{(3,21)} = 9.1$ ,  $P = 0.0005$  for (A) and  $F_{(3,21)} = 1.9$ ,  $P = 0.16$  for (B). Molecular weight (MW) is shown as kDa. (C) Representative recordings of tonic current generated by  $GABA_A$  receptors. The summarized data show that dexmedetomidine (Dex, 0.1  $\mu M$ ) prevented the sustained increase in a tonic current induced by etomidate (Etom, 1  $\mu M$ ) and sevoflurane (Sevo, 266  $\mu M$ ) in hippocampal neurons cocultured with astrocytes. (Middle)  $n = 6, 10, 9, 9$  (left to right); one-way ANOVA,  $F_{(3,30)} = 8.3$ ,  $P = 0.0004$ , Tukey's multiple comparisons test. (Right)  $n = 10, 9, 7, 9$  (left to right); one-way ANOVA,  $F_{(3,31)} = 7.5$ ,  $P = 0.0007$ , Tukey's multiple comparisons test. (D) Quantified data show similar effects of dexmedetomidine (0.1  $\mu M$ ) in cortical neurons cocultured with astrocytes.  $n = 7, 14, 11, 11$  (left to right); one-way ANOVA,  $F_{(3,39)} = 13.3$ ,  $P < 0.0001$ , Tukey's multiple comparisons test. The data are means  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Bic = bicuculline; Ctrl = control; NKA = Na/K-ATPase.

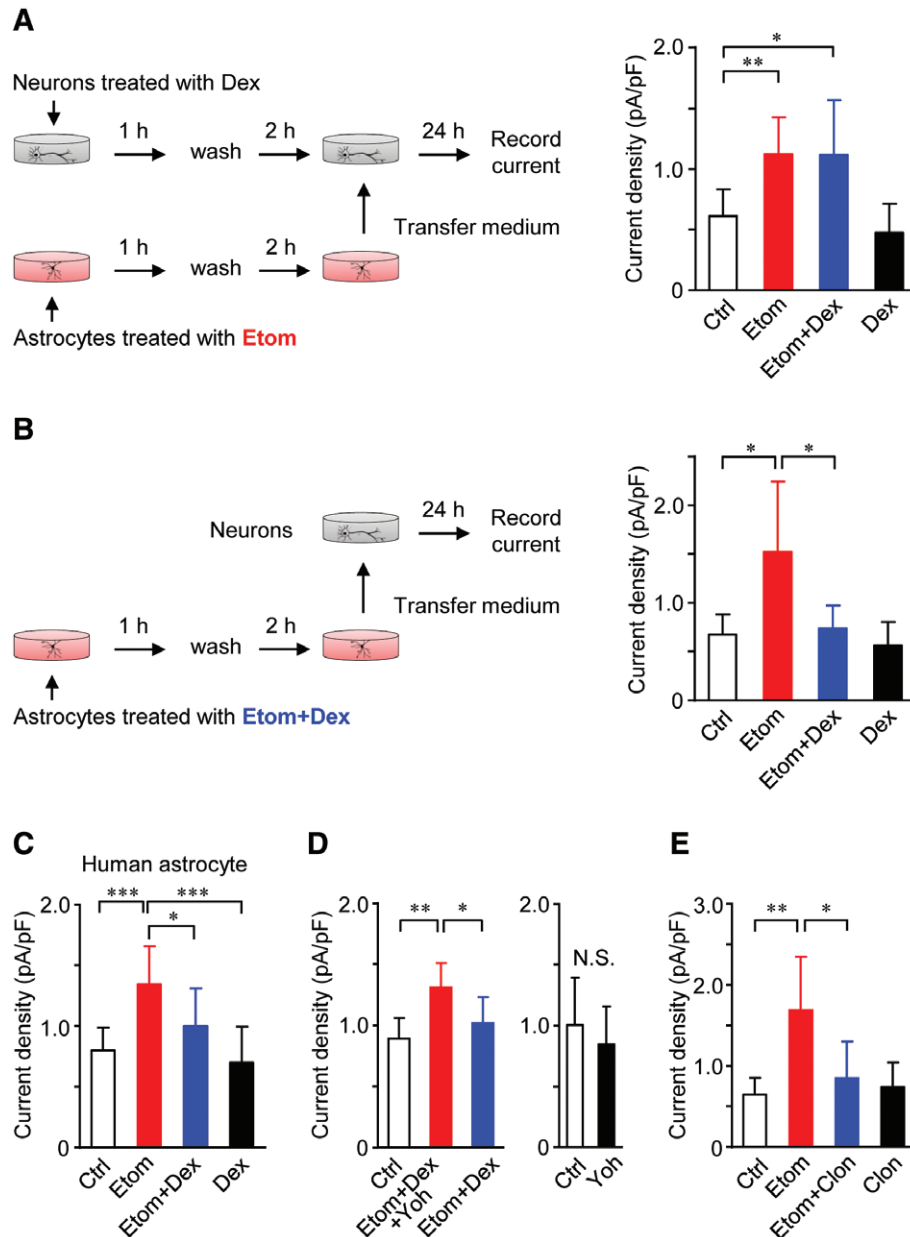
### Dexmedetomidine Targets $\alpha 2$ Adrenergic Receptors in Astrocytes

Dexmedetomidine prevented the anesthetic-induced increase in tonic current in neurons by acting either: (1) directly on the neurons or (2) indirectly on the astrocytes to release factors that regulate  $GABA_A$  receptors in neurons. To distinguish between these two potential mechanisms, astrocytes and hippocampal neurons were cultured separately. First, the astrocytes alone were treated with etomidate (1  $\mu M$ ), and the neurons were treated with dexmedetomidine (0.1  $\mu M$ ). After 1 h, the cells were washed with drug-free medium, and 2 h later, the conditioned medium from the astrocytes was transferred to the dexmedetomidine-treated neurons. Neurons were incubated for 24 h, and then the tonic current was recorded (fig. 2A). Under these conditions, dexmedetomidine did not prevent the etomidate-induced increase in current. Thus, dexmedetomidine treatment of astrocytes was necessary to prevent the anesthetic-induced increase in tonic current in neurons.

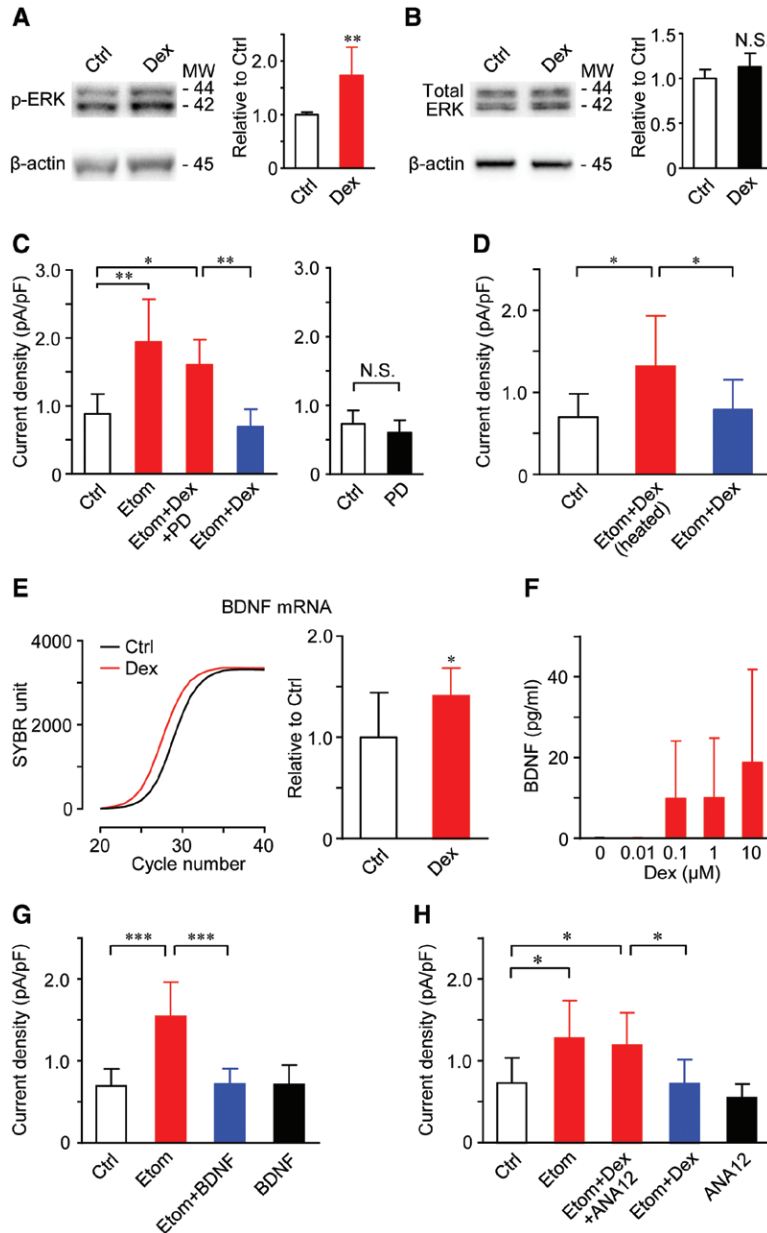
To determine whether dexmedetomidine treatment of astrocytes was sufficient to reduce the tonic current in

neurons, astrocytes alone were treated with etomidate (1  $\mu M$ ) or dexmedetomidine (0.1  $\mu M$ ) plus etomidate for 1 h, washed, and incubated for 2 h. The conditioned medium was then transferred to hippocampal neurons, and 24 h later, the tonic current was recorded in the neurons (fig. 2B). Medium from etomidate-treated, but not vehicle-treated, astrocytes increased the amplitude of the tonic current. Importantly, medium from astrocytes treated with both dexmedetomidine and etomidate yielded no such increase. Thus, dexmedetomidine treatment of astrocytes was sufficient to prevent the etomidate-induced persistent increase in tonic current.

Astrocytes from humans and mice share many phenotypic properties; however, their transcriptome, structural, and functional features can differ.<sup>33</sup> Therefore, we studied human astrocytes obtained from optic nerve heads to determine whether they also released soluble factors that increased the tonic current in mouse hippocampal neurons and, if so, whether treatment of human astrocytes with dexmedetomidine could attenuate such an increase.



**Fig. 2.** Dexmedetomidine activation of  $\alpha_2$  adrenergic receptors in astrocytes prevents the etomidate-induced increase in tonic current. (A) Schematic diagrams and summarized data show that astrocytes are necessary for the preventative effects of dexmedetomidine (Dex). When neurons alone were treated with dexmedetomidine (0.1  $\mu\text{M}$ ), dexmedetomidine did not prevent etomidate (Etom)-induced sustained increase in tonic current.  $n = 8, 12, 7, 7$  (left to right); one-way ANOVA,  $F_{(3,30)} = 9.6$ ,  $P = 0.0001$ ; Tukey's multiple comparisons test. (B) Schematic diagrams and summarized data show that treatment of astrocytes was sufficient for dexmedetomidine effects. When astrocytes were treated with both dexmedetomidine (0.1  $\mu\text{M}$ ) and etomidate (1  $\mu\text{M}$ ), the etomidate-induced sustained increase in tonic current was abolished.  $n = 6, 7, 5, 6$  (left to right); one-way ANOVA,  $F_{(3,20)} = 6.7$ ,  $P = 0.003$ ; Tukey's multiple comparisons test. Due to the relatively small sample size, a Brown-Forsythe test showed inequality of group variances. Because all other datasets obtained using a similar experimental design showed equal variances, we performed one-way ANOVA. (C) Human astrocytes were also sufficient for the preventative effects of dexmedetomidine. When human astrocytes were treated with both dexmedetomidine (0.1  $\mu\text{M}$ ) and etomidate (1  $\mu\text{M}$ ), the etomidate-induced sustained increase in tonic current was abolished.  $n = 13, 13, 9, 6$  (left to right); one-way ANOVA,  $F_{(3,37)} = 11.4$ ,  $P < 0.0001$ ; Tukey's multiple comparisons test. (D) The  $\alpha_2$  adrenergic receptor antagonist yohimbine (Yoh, 1  $\mu\text{M}$ ) blocked dexmedetomidine attenuation of sustained increase in tonic current. (Left)  $n = 6$ ; one-way ANOVA,  $F_{(2,15)} = 7.5$ ,  $P = 0.006$ ; Tukey's multiple comparisons test. (Right)  $n = 6$ ; unpaired Student's  $t$  test,  $P = 0.46$ . (E) The  $\alpha_2$  adrenergic receptor agonist clonidine (Clon, 1  $\mu\text{M}$ ) mimicked dexmedetomidine effects.  $n = 6, 6, 6, 7$  (left to right); one-way ANOVA,  $F_{(3,21)} = 7.5$ ,  $P = 0.0013$ ; Tukey's multiple comparisons test. The data are means  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Ctrl = control; N.S. = not significant.



**Fig. 3.** Dexmedetomidine acts through phosphorylated extracellular signal-regulated kinase- and brain-derived neurotrophic factor (BDNF)-dependent pathways. (A, B) Representative Western blots and summarized data of the phosphorylated extracellular signal-regulated kinase (A) and the total extracellular signal-regulated kinase (B).  $\beta$ -Actin was used as a loading control. (A)  $n = 6$ ;  $P = 0.002$ ; nonparametric Mann-Whitney U test. (B)  $n = 4$ ; unpaired Student's  $t$  test. The molecular weight (MW) is shown as kDa. (C) Extracellular signal-regulated kinase inhibitor PD98059 (PD, 50  $\mu$ M) blocked dexmedetomidine attenuation of etomidate-induced increase in tonic current. Treating astrocytes with PD98059 alone had no effect on the tonic current. (Left)  $n = 5, 6, 6, 7$  (left to right); one-way ANOVA,  $F_{(3,20)} = 12.6, P < 0.0001$ ; Tukey's multiple comparisons test. (Right)  $n = 5, 6$  (left to right); unpaired Student's  $t$  test,  $P = 0.29$ . (D) Conditioned medium from astrocytes treated with dexmedetomidine (0.1  $\mu$ M) could prevent the etomidate-induced increase in tonic current in neurons. Heating the conditioned medium abolished this effect.  $n = 8, 11, 12$  (left to right); one-way ANOVA,  $F_{(2,28)} = 5.6, P = 0.009$ ; Tukey's multiple comparisons test. (E) Representative curves and summarized data show that the level of brain-derived neurotrophic factor messenger RNA was increased in the conditioned medium from astrocytes treated with dexmedetomidine (0.1  $\mu$ M).  $n = 8, *P = 0.042$ , unpaired Student's  $t$  test. (F) Brain-derived neurotrophic factor in medium from astrocytes treated with dexmedetomidine (0.1 to 10  $\mu$ M) was measured with enzyme-linked immunosorbent assay. Levels were detected only when astrocytes were treated with dexmedetomidine at concentrations greater than 0.01  $\mu$ M.  $n = 5$ . (G) Brain-derived neurotrophic factor mimicked dexmedetomidine and prevented etomidate-induced increase in tonic current.  $n = 8, 7, 8, 6$  (left to right); one-way ANOVA,  $F_{(3,25)} = 16.6, P < 0.0001$ ; Tukey's multiple comparisons test. (H) Brain-derived neurotrophic factor receptor (tropomyosin receptor kinase B [TrkB]) antagonist ANA 12 blocked the effects of dexmedetomidine.  $n = 8, 11, 12, 9, 7$  (left to right); one-way ANOVA,  $F_{(4,42)} = 7.7, P < 0.0001$ ; Tukey's multiple comparisons test. The data are means  $\pm$  SD. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . Ctrl = control; Dex = dexmedetomidine; Etom = etomidate; N.S. = not significant.

Cultured human astrocytes were treated with etomidate and dexmedetomidine for 1 h and then washed. The conditioned medium was transferred to the cultured neurons 2 h later, and the tonic current was recorded 24 h later (fig. 2B). The conditioned medium from the etomidate-treated human astrocytes increased the tonic current; cotreatment with dexmedetomidine prevented this increase (fig. 2C).

Dexmedetomidine is a high-affinity  $\alpha_2$  adrenergic receptor agonist. To determine whether activation of  $\alpha_2$  adrenergic receptor was necessary for the protective effects of dexmedetomidine, the antagonist yohimbine (1  $\mu\text{M}$ ) was coapplied with dexmedetomidine (0.1  $\mu\text{M}$ ). Yohimbine blocked the dexmedetomidine attenuation of etomidate-induced increase in tonic current (fig. 2D). In addition, the less potent  $\alpha_2$  adrenergic receptor agonist clonidine (1  $\mu\text{M}$ ) mimicked the effect of dexmedetomidine, because it prevented the etomidate-induced increase in tonic current in hippocampal neurons (fig. 2E). Collectively, these results suggest that dexmedetomidine activates  $\alpha_2$  adrenergic receptors in astrocytes to reverse the etomidate-induced persistent increase in tonic current in neurons.

### **Dexmedetomidine Acts through Phosphorylated Extracellular Signal-regulated Kinase- and Brain-derived Neurotrophic Factor-dependent Pathways**

Activation of  $\alpha_2$  adrenergic receptors in astrocytes stimulates multiple G protein-coupled receptor signaling pathways, including those involving extracellular signal-regulated protein kinases 1 and 2.<sup>34,35</sup> We reasoned that the increase in phosphorylated extracellular signal-regulated kinase could contribute to dexmedetomidine reduction of the tonic current in neurons. To investigate whether dexmedetomidine increased levels of phosphorylated extracellular signal-regulated kinase in astrocytes, cultured murine astrocytes were pretreated with dexmedetomidine (0.1  $\mu\text{M}$ , for 1 h) and then washed, and 2 h later, the cells were processed for Western blotting analysis. The results showed that dexmedetomidine increased phosphorylated extracellular signal-regulated kinase but not total extracellular signal-regulated kinase (fig. 3, A and B).

To determine whether the dexmedetomidine-induced increase in phosphorylated extracellular signal-regulated kinase was involved in the signaling pathway that prevented the etomidate-induced increase in tonic current in hippocampal neurons, cultured astrocytes were cotreated with dexmedetomidine and PD98059 (50  $\mu\text{M}$ ), a compound that inhibits the production of phosphorylated extracellular signal-regulated kinase. Dexmedetomidine failed to prevent the etomidate-induced increase in tonic current in neurons (fig. 3C), suggesting that dexmedetomidine acted *via* a phosphorylated extracellular signal-regulated kinase 1/2-dependent pathway.

The results show that dexmedetomidine stimulates astrocytes to release one or more soluble factors that act in a paracrine manner to prevent the etomidate-induced increase in tonic current in neurons. These factor(s) were heat-sensitive

because heating the conditioned medium from dexmedetomidine-treated astrocytes to 99.9°C for 5 min prevented the supernatant from triggering the etomidate-induced increase in tonic current in neurons (fig. 3D).

Dexmedetomidine stimulation of phosphorylated extracellular signal-regulated kinase could lead to an increase in the expression of genes that encode for neurotrophic factors such as brain-derived neurotrophic factor, which is known to modulate GABA<sub>A</sub> receptor activity.<sup>34–36</sup> Indeed, treating the astrocytes with dexmedetomidine (0.1  $\mu\text{M}$ ) increased the expression of brain-derived neurotrophic factor mRNA (fig. 3E). Dexmedetomidine also increased brain-derived neurotrophic factor protein levels (fig. 3F).

To test whether brain-derived neurotrophic factor was sufficient to mimic the effects of the unidentified soluble factor, exogenous brain-derived neurotrophic factor (200 pg/ml) was applied directly to the neurons. Brain-derived neurotrophic factor prevented the etomidate-induced increase in tonic current (fig. 3G). Because brain-derived neurotrophic factor is known to act on tropomyosin receptor kinase B (TrkB) receptors, we next tested whether treating the hippocampal neurons with an antagonist for TrkB receptors (ANA 12, 0.01  $\mu\text{g/ml}$ ) abolished dexmedetomidine reduction of the etomidate-enhanced tonic current (fig. 3H). ANA 12 prevented the effect of dexmedetomidine, suggesting that dexmedetomidine stimulates astrocytes to release brain-derived neurotrophic factor, which in turn acted on hippocampal neurons to reduce the etomidate-induced increase in tonic current.

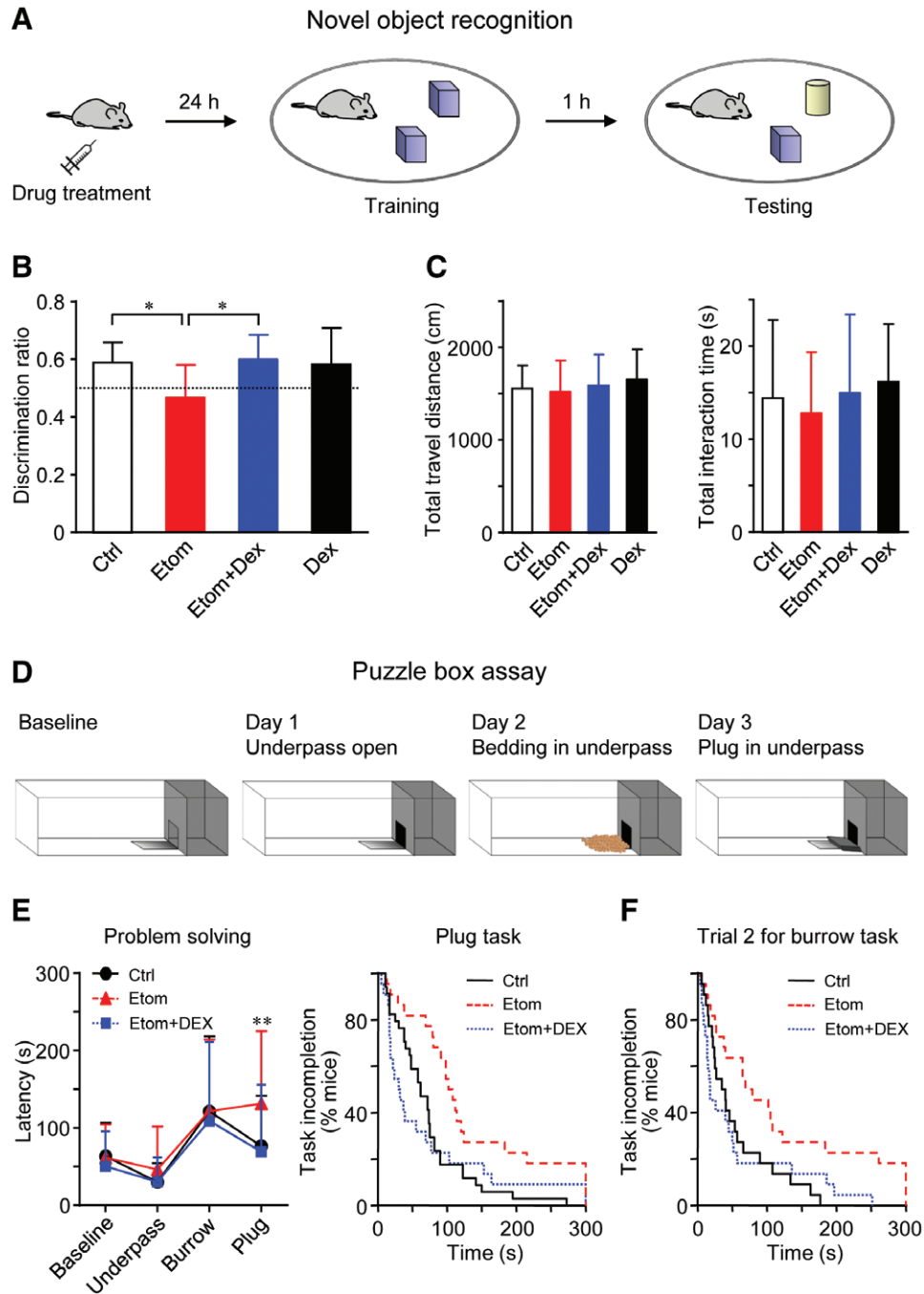
### **Dexmedetomidine Prevents Postanesthetic Cognitive Deficits**

Finally, we asked whether dexmedetomidine prevented the behavioral deficits in memory and problem-solving that are observed *in vivo* after the anesthetic drug has been eliminated from the body. The novel object recognition task was selected and performed first because this test has been shown to be sensitive to an increase in  $\alpha_5$  GABA<sub>A</sub> receptor activity.<sup>5</sup>

The mice were treated with dexmedetomidine (25  $\mu\text{g/kg}$ , intraperitoneally) or vehicle then etomidate (20 mg/kg, intraperitoneally) and 24 h later were studied using the novel object recognition task.<sup>30</sup> Notably, dexmedetomidine markedly prolonged the duration of etomidate-induced anesthesia. Specifically, dexmedetomidine alone did not cause the loss of righting reflex, but cotreatment of dexmedetomidine plus etomidate prolonged the duration of the loss of righting reflex by 32% (etomidate:  $26.8 \pm 7.9$  min *vs.* etomidate + dexmedetomidine:  $35.3 \pm 7.6$  min,  $n = 22$ ; unpaired Student's *t* test,  $P = 0.0007$ ). These results are consistent with the anesthetic-potentiating effects of dexmedetomidine observed in patients.<sup>37</sup>

The novel object recognition task measures the ability of mice to distinguish a novel object from a familiar object (fig. 4A). During the training phase, the interaction time of the mice was similar for the two objects (data not shown).





**Fig. 4.** Dexmedetomidine prevents persistent cognitive deficits after general anesthesia. (A) A timeline illustrates the experimental design. (B) Memory performance on the novel object recognition task.  $n = 10, 12, 10, 13$  (left to right); one-way ANOVA,  $F_{(3,41)} = 4.1$ ,  $P = 0.01$ . \* =  $P < 0.05$ , Tukey's multiple comparisons test. The discrimination ratio for each treatment group is significantly different from the theoretical mean of 0.5 except for the etomidate group (Ctrl:  $P = 0.003$ , Etom:  $P = 0.35$ , Etom+Dex:  $P = 0.005$ , Dex:  $P = 0.037$ ; one-sample  $t$  test). (C) Total travel distance (one-way ANOVA,  $F_{(3,41)} = 0.39$ ,  $P = 0.76$ ) and the total interaction time (one-way ANOVA,  $F_{(3,41)} = 0.45$ ,  $P = 0.72$ ) from the same cohort as shown in B. (D) Diagrams show the timeline and various problem-solving tasks for the puzzle box assay. (E, left) After treatment with etomidate, mice exhibited a longer latency to enter the goal box for the plug task and dexmedetomidine reduced the latency.  $n = 22$ . Two-way ANOVA, effect of tasks:  $F_{(3,252)} = 21.0$ ,  $P < 0.0001$ ; effect of treatment:  $F_{(2,252)} = 6.3$ ,  $P = 0.002$ ; effect of interaction:  $F_{(6,252)} = 0.82$ ,  $P = 0.56$ . \*\* =  $P < 0.01$  compared with etomidate + dexmedetomidine, Tukey's multiple comparisons test. (Right) Kaplan–Meier survival plots further illustrate the effects of etomidate and dexmedetomidine on the plug task. Log-rank pairwise comparison:  $P = 0.016$  for Etom versus Ctrl,  $P = 0.022$  for Etom versus Etom + DEX, and  $P = 0.77$  for Etom + DEX versus Ctrl. (F) Similarly, etomidate increased the latency for the mice to complete the task and dexmedetomidine abolished the increase. Log-rank pairwise comparison:  $P = 0.016$  for Etom versus Ctrl,  $P = 0.009$  for Etom versus Etom + DEX, and  $P = 0.96$  for Etom + DEX versus Ctrl. The data are means  $\pm$  SD. Ctrl = control; Dex or DEX = dexmedetomidine; Etom = etomidate.

During the testing phase, the mice that were treated with etomidate failed to distinguish the novel object, as shown by the low discrimination ratio (*i.e.*, the time spent exploring the novel object relative to time spent exploring both objects; fig. 4B). Dexmedetomidine markedly attenuated the etomidate-induced deficits in memory performance. The cognition-sparing effect of dexmedetomidine could not be attributed to differences in locomotion or overall exploratory drive (fig. 4C).

Next, we used the puzzle box assay to investigate: (1) whether a single dose of etomidate caused deficits in problem-solving and, if so, (2) whether dexmedetomidine prevented such deficits. The mouse was placed in a well lit environment that offered access to the preferred, darkened “goal” box. Admission to the goal box was made progressively more difficult over a series of trials that spanned over 3 days (fig. 4D).

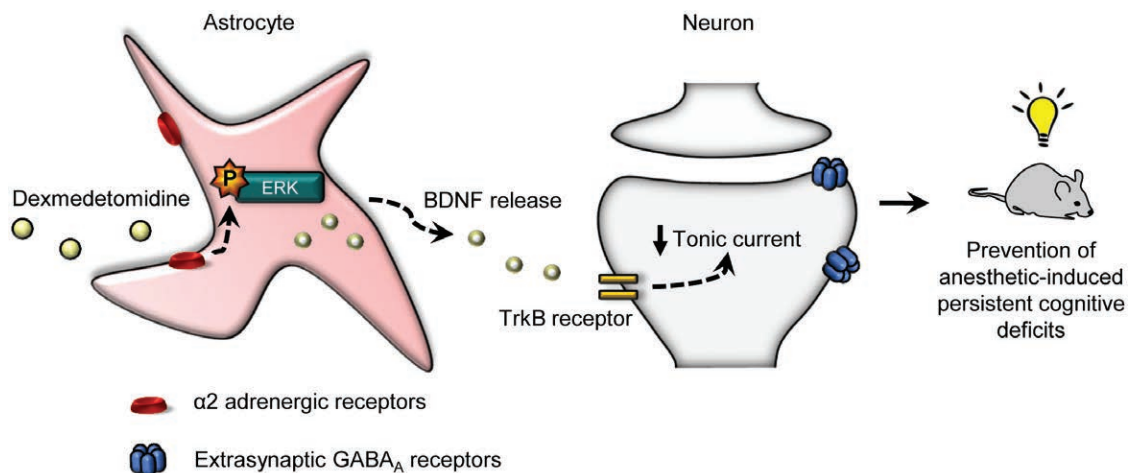
Performance of simpler tasks, including the underpass and burrowing tasks, did not differ between treatment groups (fig. 4E). Etomidate caused a deficit for the more difficult plug task, which was performed on day 3 as evidenced by an increase in the latency to complete the task (control:  $76.4 \pm 65.5$  s, 95% CI 47.4 to 105.4,  $n = 22$ ; etomidate:  $131.4 \pm 93.8$  s, 95% CI 89.8 to 173.0,  $n = 22$ ;  $P = 0.08$ ; but  $P = 0.016$  for log-rank pairwise comparison). This deficit was prevented in mice cotreated with dexmedetomidine because the latency was significantly reduced to  $69.1 \pm 86.6$  s (95% CI 30.7 to 107.5,  $n = 22$ ). Etomidate also prolonged the latency to complete the second burrow task on day 2, which was performed 2 min after the first burrow task (fig. 4F). These results suggest that etomidate impaired problem-solving at least in part by reducing short-term memory and that cotreatment with dexmedetomidine prevented this impairment.

## Discussion

The goal of this study was to use a reverse translational approach to identify the cellular and molecular mechanisms that contribute to the cognition-sparing properties of dexmedetomidine. The results show that dexmedetomidine activates  $\alpha_2$  adrenergic receptors in astrocytes and stimulates the release of brain-derived neurotrophic factor, which acts as a paracrine factor to prevent excessive cell-surface expression and function of  $\alpha_5$  GABA<sub>A</sub> receptors in neurons. Behavioral studies confirmed that cotreatment with dexmedetomidine attenuates the postanesthetic deficits in memory and problem-solving (fig. 5). Therefore, administration of dexmedetomidine is a plausible pharmacologic strategy to reduce excessive cell-surface expression of  $\alpha_5$  GABA<sub>A</sub> receptors in neurons after general anesthesia.

Several converging lines of evidence indicate that  $\alpha_2$  adrenergic receptors in astrocytes, but not those in neurons, mediate the effects of dexmedetomidine. First,  $\alpha_2$  adrenergic receptors are abundantly expressed in cortical astrocytes.<sup>38</sup> Second, our current studies of cultured astrocytes and neurons show that dexmedetomidine treatment of isolated astrocytes is both necessary and sufficient to prevent the etomidate-induced increase in tonic current in neurons. Finally, the  $\alpha_2$  adrenergic receptor agonist clonidine mimics the effects of dexmedetomidine, whereas the antagonist yohimbine abolishes these effects.

Our results show that dexmedetomidine activation of  $\alpha_2$  adrenergic receptors in astrocytes increases the level of phosphorylated extracellular signal-regulated kinase 1/2. These kinases can translocate from the cytosol to the nucleus where they activate transcription factors that promote gene expression.<sup>39,40</sup> Such changes could lead to an increase in brain-derived neurotrophic factor mRNA levels and subsequent release of brain-derived neurotrophic factor. These findings are also supported by previous studies showing an increase in



**Fig. 5.** Dexmedetomidine reduces the excessive function of extrasynaptic  $\alpha_5$   $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptors in neurons after general anesthesia and thereby prevents delirium. Dexmedetomidine activates  $\alpha_2$  adrenergic receptors expressed in astrocytes and increases the level of phosphorylated extracellular signal-regulated kinase (p-ERK). These events stimulate the release of brain-derived neurotrophic factor (BDNF), which acts as a paracrine factor to stimulate tropomyosin receptor kinase B (TrkB) receptors expressed in neurons. Activation of TrkB receptors reduces cell-surface expression of extrasynaptic  $\alpha_5$  GABA<sub>A</sub> receptors in neurons and thereby prevents deficits in memory and problem-solving after general anesthesia.

brain-derived neurotrophic factor in association with dexmedetomidine.<sup>35,41</sup> Furthermore, exogenous brain-derived neurotrophic factor mimics the effects of dexmedetomidine in that it reduces the etomidate-induced increase in tonic current in neurons. In contrast, inhibition of TrkB receptors blocks the effects of dexmedetomidine. Collectively, these data support a model in which dexmedetomidine stimulates the release of brain-derived neurotrophic factor from astrocytes, and the brain-derived neurotrophic factor acts on hippocampal neurons to prevent a persistent increase in tonic current (fig. 5).

The results of this study are important for several reasons. First, dexmedetomidine alone did not cause the loss of the righting reflex, but it did prolong the duration of etomidate-induced anesthesia by 32%. Despite prolonging the hypnotic properties of etomidate, dexmedetomidine had cognition-restoring effects in mice. Second, the data provide a plausible model to account for the cognition-sparing properties of dexmedetomidine. The results identify both  $\alpha 2$  adrenergic receptors in astrocytes and  $\alpha 5$  GABA<sub>A</sub> receptors in neurons as potential therapeutic targets for treatments of postoperative delirium. Dexmedetomidine has limited clinical utility because of potential adverse cardiorespiratory effects and should only be administered by a skilled care provider in a highly monitored environment. Other  $\alpha 2$  adrenergic receptor agonists may mimic the ability of dexmedetomidine to prevent postanesthetic cognitive deficits. For example, clonidine can be administered orally for sedation<sup>42</sup> and may serve as an alternative in some patients. In addition, new drugs that target the expression and/or function of extrasynaptic  $\alpha 5$  GABA<sub>A</sub> receptors may offer additional prevention and treatment options. Finally, exogenous brain-derived neurotrophic factor prevents excessive GABA<sub>A</sub> receptor function and may be useful for patients who fail to tolerate  $\alpha 2$  adrenergic receptor agonists.<sup>6</sup> Interestingly, treatment with brain-derived neurotrophic factor was shown to be safe in a study of patients with amyotrophic lateral sclerosis.<sup>43</sup>

Our study has several limitations. First, dexmedetomidine could trigger the release of additional paracrine factors that have not been identified yet regulate extrasynaptic GABA<sub>A</sub> receptor activity. Second, we did not compare the effects of dexmedetomidine on pro-brain-derived neurotrophic factor *versus* brain-derived neurotrophic factor levels. Astrocytes release pro-brain-derived neurotrophic factor, a 32-kDa precursor protein that often has effects opposite to those of brain-derived neurotrophic factor.<sup>44</sup> For example, pro-brain-derived neurotrophic factor promotes cell death through activation of a low-affinity nerve growth factor receptor (p75),<sup>45</sup> whereas the 14-kDa protein brain-derived neurotrophic factor, promotes cell survival *via* activation of TrkB receptors.<sup>46</sup> The equilibrium between pro-brain-derived neurotrophic factor and brain-derived neurotrophic factor may be a determinant in the protective effects of dexmedetomidine. In addition, the effects of other commonly used anesthetic drugs, such as propofol or sedative benzodiazepines, were not studied but are of interest for future studies.

In summary, this study supports a model in which dexmedetomidine prevents postanesthetic cognitive deficits by reducing cell-surface expression and function of  $\alpha 5$  GABA<sub>A</sub> receptors in neurons through the release of brain-derived neurotrophic factor from astrocytes. The implications of these results extend well beyond the perioperative context, given that increased  $\alpha 5$  GABA<sub>A</sub> receptor function has been associated with several neurologic disorders including stroke, Alzheimer disease, depression, and inflammation-induced brain injury.<sup>16,47–49</sup> Dexmedetomidine and related drugs that target similar signaling pathway may help to prevent and treat these disorders.

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## Competing Interests

The authors declare no competing interests.

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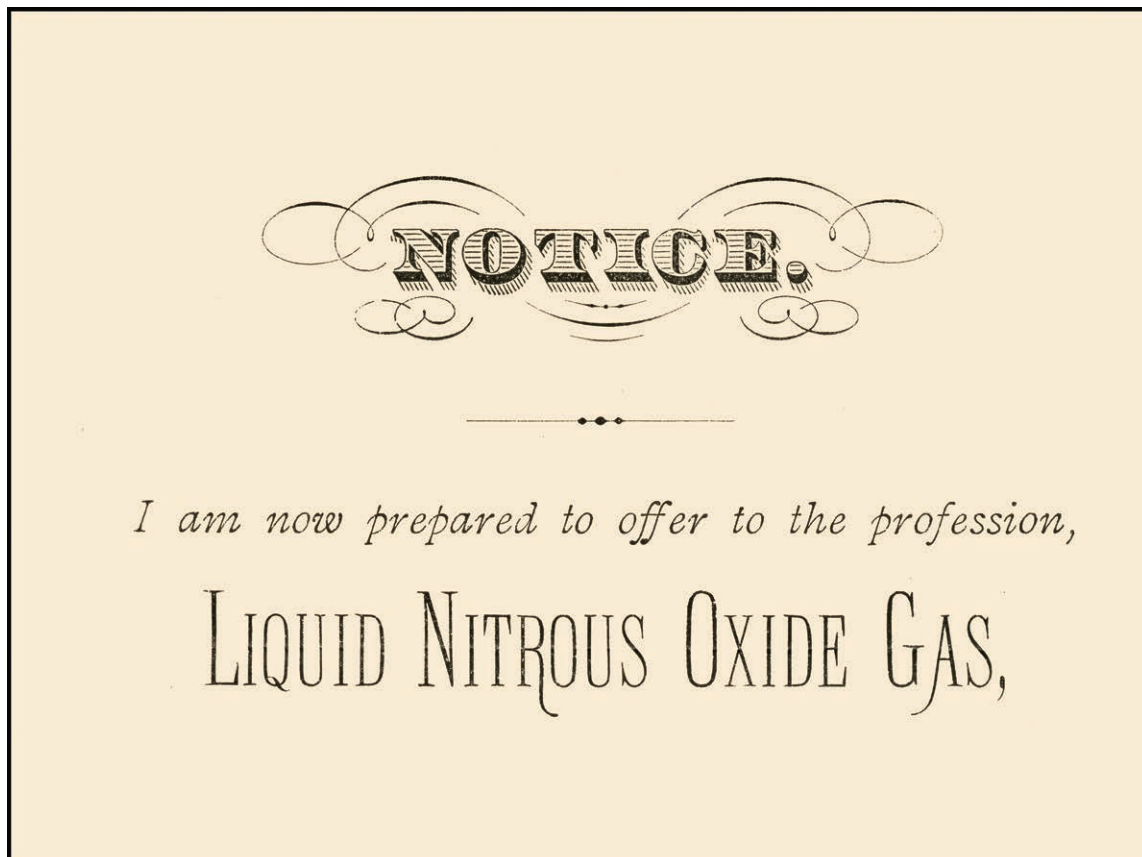
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### J. K. P. Pine Collars New York's Upstate Market on Nitrous Oxide Cylinders



From the Wood Library-Museum's Ben Z. Swanson Collection, this notice (above) was extracted from a broadside published by James Knox Polk Pine (1841 to 1919; banker, philanthropist, and collar manufacturer) of Troy, New York. An astonishing commercial success at both patenting and producing shirt collars, J. K. P. Pine started a side business of selling 100-gallon cylinders of nitrous oxide for \$16 or refilling them for \$6. Situated about 150 miles north of Manhattan on New York's eastern border with Vermont and Massachusetts, J. K. P. Pine's Troy could "save the [two-day] delay of sending your Cylinder to New York to be re-filled." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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