

# Dexmedetomidine Prevents Cognitive Decline by Enhancing Resolution of High Mobility Group Box 1 Protein–induced Inflammation through a Vagomimetic Action in Mice

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## ABSTRACT

**Background:** Inflammation initiated by damage-associated molecular patterns has been implicated for the cognitive decline associated with surgical trauma and serious illness. We determined whether resolution of inflammation mediates dexmedetomidine-induced reduction of damage-associated molecular pattern–induced cognitive decline.

**Methods:** Cognitive decline (assessed by trace fear conditioning) was induced with high molecular group box 1 protein, a damage-associated molecular pattern, in mice that also received blockers of neural (vagal) and humoral inflammation-resolving pathways. Systemic and neuroinflammation was assessed by proinflammatory cytokines.

**Results:** Damage-associated molecular pattern–induced cognitive decline and inflammation (mean  $\pm$  SD) was reversed by dexmedetomidine (trace fear conditioning:  $58.77 \pm 8.69\%$  vs.  $41.45 \pm 7.64\%$ ,  $P < 0.0001$ ; plasma interleukin [IL]-1 $\beta$ :  $7.0 \pm 2.2$  pg/ml vs.  $49.8 \pm 6.0$  pg/ml,  $P < 0.0001$ ; plasma IL-6:  $3.2 \pm 1.6$  pg/ml vs.  $19.5 \pm 1.7$  pg/ml,  $P < 0.0001$ ; hippocampal IL-1 $\beta$ :  $4.1 \pm 3.0$  pg/mg vs.  $41.6 \pm 8.0$  pg/mg,  $P < 0.0001$ ; hippocampal IL-6:  $3.4 \pm 1.3$  pg/mg vs.  $16.2 \pm 2.7$  pg/mg,  $P < 0.0001$ ). Reversal by dexmedetomidine was prevented by blockade of vagomimetic imidazoline and  $\alpha_7$  nicotinic acetylcholine receptors but not by  $\alpha_2$  adrenoceptor blockade. Netrin-1, the orchestrator of inflammation–resolution, was upregulated (fold-change) by dexmedetomidine (lung:  $1.5 \pm 0.1$  vs.  $0.7 \pm 0.1$ ,  $P < 0.0001$ ; spleen:  $1.5 \pm 0.2$  vs.  $0.6 \pm 0.2$ ,  $P < 0.0001$ ), resulting in upregulation of proresolving (lipoxin-A $_4$ :  $1.7 \pm 0.2$  vs.  $0.9 \pm 0.2$ ,  $P < 0.0001$ ) and downregulation of proinflammatory (leukotriene-B $_4$ :  $1.0 \pm 0.2$  vs.  $3.0 \pm 0.3$ ,  $P < 0.0001$ ) humoral mediators that was prevented by  $\alpha_7$  nicotinic acetylcholine receptor blockade.

**Conclusions:** Dexmedetomidine resolves inflammation through vagomimetic (neural) and humoral pathways, thereby preventing damage-associated molecular pattern–mediated cognitive decline. (**ANESTHESIOLOGY 2018; 128:921–31**)

OVER the last decade we have generated several lines of evidence that implicate surgery-initiated systemic inflammation and neuroinflammation in the development of postoperative cognitive decline both in preclinical<sup>1–3</sup> and clinical settings.<sup>4</sup> The engagement of the innate immune system, which triggers the inflammatory response, resulting in postoperative cognitive decline, is due to the damage-associated molecular pattern, high molecular group box 1 protein (HMGB1), which is passively released from traumatized tissue.<sup>5,6</sup>

While postoperative cognitive decline was first reported in the setting of general anesthesia,<sup>7</sup> no difference has been reported in the incidence of postoperative cognitive decline in patients randomized to receive general *versus* regional anesthetic techniques.<sup>8</sup> These previous studies may have been underpowered, and larger trials are progressing to understand whether regional or general anesthesia is less likely to result in postoperative cognitive decline (Regional versus General Anesthesia for Promoting Independence after Hip Fracture [REGAIN] Trial; NCT02507505).<sup>9</sup> Notwithstanding the outcome of the REGAIN trial, many patients will require either sedatives and/or general anesthesia combined with a regional technique.

### What We Already Know about This Topic

- Inflammation has been associated with cognitive disorders following surgery and medical illness
- Preclinical evidence suggests a role for high molecular group box 1 protein in cognitive disorders after an inflammatory insult

### What This Article Tells Us That Is New

- In a preclinical model, dexmedetomidine prevented cognitive deficits resulting from administration of high molecular group box 1 protein *via* both vagomimetic and humoral pathways
- The results are consistent with the notion that the cognitive deficits noted after surgery or medical illness may be prevented by the administration of dexmedetomidine

While some comparative studies have suggested that the frequency and/or severity of postoperative cognitive decline may be affected by the choice and dose of the anesthetic/sedative agent that is used for the surgical procedure,<sup>10</sup> and that exposure to deeper anesthetic stages results in a higher incidence of delirium,<sup>11</sup> larger, appropriately powered studies are needed to avoid a type 2 statistical error, as was suspected in the Hip Fracture in Elderly Patients study (NCT01199276).<sup>12</sup>

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It is notable that animal cohorts receiving anesthesia and analgesia alone did not differ from the control group (no anesthesia/analgesia, no surgery).<sup>1,2</sup> Ethical considerations prevented us from using a “surgery only” (*i.e.*, without anesthesia/analgesia) cohort to which we could compare the neuroinflammatory and cognitive effects provoked by the addition of anesthetic and analgesic drugs. Now that we have demonstrated that systemically administered HMGB1 reproduces the surgical phenotype with high fidelity,<sup>5</sup> we are ethically able to study a surrogate of the “surgery only” cohort without use of anesthesia/hypnotics to which we can compare the effects of adding these drugs.

To determine whether anesthetic/hypnotics have a modulating effect on the surgical phenotype, we have chosen to first study dexmedetomidine because it reduces cognitive decline<sup>13,14</sup> and inflammation,<sup>15</sup> including that associated with acute neurologic injury,<sup>16</sup> by an as-yet-undetermined mechanism. Because of the challenge that there is “questionable biologic plausibility” for the beneficial effects that were noted postoperatively with dexmedetomidine,<sup>14</sup> we addressed the hypothesis that the ameliorative effect of dexmedetomidine on cognitive decline is due to resolution of inflammation through neural and humoral processes.

## Materials and Methods

### Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco (AN 167062) and conformed to National Institutes of Health (Bethesda, Maryland) guidelines. Twelve- to 14-week-old wild-type male C57BL/6J mice (Jackson Laboratory, USA) were used for this study. All animals were fed standard rodent food and water and were housed (five mice per cage) in a controlled environment with 12-h light/dark cycles. Mice were tagged and randomly allocated to each group before any treatment or procedure. Researchers were blinded to the group assignment, which was revealed only after completing the analysis. Mice did not experience unexpected lethality in the study, and animals were euthanized according to our institutional animal care and use committee guidelines.

### Drug Administration

Recombinant HMGB1 (R&D Systems, USA) was dissolved in phosphate-buffered saline and administered intraperitoneally 50  $\mu\text{g}/\text{kg}$ , a dose that we had previously reported to produce a similar inflammatory and cognitive response as that seen after surgery.<sup>5</sup>

Dexmedetomidine (Sigma-Aldrich, USA) was dissolved in 0.9% sterile saline and administered 50  $\mu\text{g}/\text{kg}$  intraperitoneally every 2 h for three doses immediately after HMGB1 (fig. 1, A and B) or at 20  $\mu\text{g}/\text{kg}$  intraperitoneally for four doses in the surgical model (fig. 1, C and D). These doses were selected to simulate perioperative sedation either with

(fig. 1, C and D) or without (fig. 1, A and B) isoflurane anesthesia.

Yohimbine (Sigma-Aldrich) was dissolved in 0.9% sterile saline, and 1.5 mg/kg was administered intraperitoneally, a dose that effectively blocks  $\alpha_2$  adrenoceptor-mediated responses.<sup>17</sup> Atipamezole (Sigma-Aldrich) was dissolved in 5% dimethyl sulfoxide in saline, and 3 mg/kg was administered intraperitoneally, a dose that effectively blocks both imidazole receptor and  $\alpha_2$  adrenoceptor-mediated responses. Methyllycaconitine (Sigma-Aldrich) was dissolved in 0.9% sterile saline and 4 mg/kg was administered intraperitoneally, a dose that blocks  $\alpha_7$  nicotinic acetylcholine receptor-mediated response.<sup>2</sup>

### Aseptic Surgical Trauma

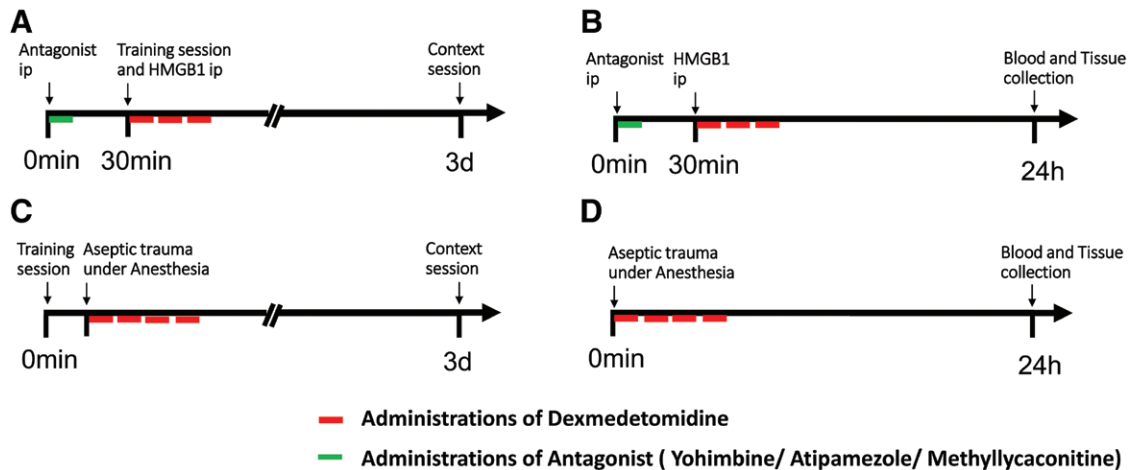
Under aseptic conditions, groups of mice were subjected to an open tibia fracture of the left hind paw with an intramedullary fixation as previously described.<sup>5</sup> Briefly, mice received general anesthesia with 2% isoflurane, and analgesia was achieved with buprenorphine 0.1 mg/kg administered subcutaneously, immediately after anesthetic induction. Warming pads and temperature-controlled lights were used to maintain body temperature. The entire procedure from the induction of anesthesia to the end of surgery lasted  $12 \pm 5$  min.

### Cognitive Testing

Trace fear conditioning was used to assess learning and memory in rodents as previously described.<sup>2,3</sup> Briefly, mice are trained to associate a conditional stimulus, such as a tone, with an aversive, unconditional stimulus, such as a foot shock. Aversive memory is associated with freezing behavior when the rodent is reexposed to the same context. The behavioral study was conducted using a conditioning chamber (Med Associates Inc., USA) and an unconditional stimulus (two periods of 2-s foot shock of 0.75 mA). Behavior was captured with an infrared video camera (Video Freeze; Med Associates Inc.). Mice underwent a context test 72 h after training, during which no tones or foot shocks were delivered. Lack of movement, indicating freezing behavior, was analyzed by the software of the video recordings. With this model, perturbations of the hippocampus that are associated with memory impairment result in disruption of recall of the fear responses to the presentation of the same context, resulting in a reduction in freezing behavior.<sup>5</sup>

### Blood and Tissue Sample Harvesting

Twenty-four hours after the specific intervention, blood was collected transcardially after thoracotomy under isoflurane anesthesia and placed in heparin-coated syringes. After collection of blood, mice were immediately perfused with saline, and the hippocampus, brain, lung, and spleen were then rapidly extracted and stored at  $-80^\circ\text{C}$  for further testing. After centrifugation of the blood sample at 3,400 rotations per minute for 10 min at  $4^\circ\text{C}$ , plasma was collected and stored at  $-80^\circ\text{C}$  until these were assayed.



**Fig. 1.** Study design. (A) Mice were randomly allocated to 10 groups ( $n = 15/\text{group}$ ) and were pretreated intraperitoneally (ip) with antagonists (yohimbine/atipamezole/methyllycaconitine). Thirty minutes later, mice were trained in the trace fear conditioning paradigm. After the training session, high mobility group box 1 protein (HMGB1) or vehicle (phosphate-buffered saline) was administered ip. Dexmedetomidine was administered every 2h, for a total of three times. Seventy-two hours after HMGB1, testing was performed in the trace fear conditioning. (B) Mice were randomly allocated to 10 groups ( $n = 8/\text{group}$ ) and were pretreated ip with antagonists (yohimbine/atipamezole/methyllycaconitine), and 30 min later HMGB1 was administered. Dexmedetomidine was administered every 2h for a total of three times. Blood and tissue were collected 24h later. (C) Mice were randomly allocated to three groups ( $n = 15/\text{group}$ ): control (vehicle only), surgery/anesthesia, and surgery/anesthesia + dexmedetomidine. Mice were trained in the trace fear conditioning paradigm. After the training session, animals were anesthetized with isoflurane and subjected to aseptic trauma. Dexmedetomidine was administered and the mice were tested in the trace fear conditioning three days later. (D) Mice were randomly allocated to three groups ( $n = 5\text{--}6/\text{group}$ ): control (vehicle only), surgery/anesthesia, and surgery/anesthesia + dexmedetomidine. Mice were anesthetized with isoflurane and subjected to aseptic trauma. Dexmedetomidine was administered and blood and tissue were collected 24h later.

### Circulating Cytokines

Plasma interleukin (IL)-6 and IL-1 $\beta$  were quantified using commercially available enzyme-linked immunosorbent kits, according to the manufacturer's instructions (R&D Systems, USA).

### Hippocampal Inflammatory Markers

Two different techniques were used to assess hippocampal inflammation. For experiments described in figure 1B, the hippocampus was homogenized and sonicated in cell lysis buffer (Cell Signaling Technology, USA) plus protease inhibitor (Halt Protease Inhibitor Single-Use Cocktail, Thermo Fisher Scientific, USA) and phenylmethanesulfonyl fluoride (Cell Signaling Technology). Protein concentration was assayed with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). IL-6 and IL-1 $\beta$  were measured using commercially available enzyme-linked immunosorbent kits, according to the manufacturer's instructions (R&D Systems). For experiments described in figure 1D, after mice were perfused with saline, the hippocampus was rapidly extracted, placed in RNAlater solution (Qiagen, The Netherlands), and stored at 4°C overnight. Total RNA was extracted using RNeasy Lipid Tissue Kit (Qiagen). Extracted RNA was treated with recombinant DNase I by using a RNase-Free Dnase set (Qiagen). Messenger RNA (mRNA) concentrations were determined with a ND-1000 Spectrophotometer (NanoDrop; Thermo Fisher Scientific), and mRNA was

reverse-transcribed to complementary DNA with a High Capacity RNA to-cDNA Kit (Applied Biosystems, USA). TaqMan Fast Advanced Master Mix (Applied Biosystems) and specific gene-expression assays were used for quantitative polymerase chain reaction, actin- $\beta$  (NM\_007393.1), and IL-6 (Mm00446190\_m1). Quantitative polymerase chain reaction was performed using StepOnePlus (Applied Biosystems). Each sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle  $\Delta\Delta\text{Ct}$  and normalized to  $\beta$ -actin. Results are expressed as fold increases relative to controls.

### Measurement of Circulating Leukotriene B<sub>4</sub> and Lipoxin A<sub>4</sub>

Plasma leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and lipoxin A<sub>4</sub> (LXA<sub>4</sub>) were quantified using commercially available enzyme-linked immunosorbent kits, according to the manufacturer's instructions (Biomatik USA, LLC, USA). Results are expressed as fold-change compared with that measured in control mice that did not receive any intervention.

### Measurement of Netrin-1 (in the Lung and Spleen) and Albumin (in the Brain)

Tissues were homogenized with RIPA Lysis Buffer (Cell Signaling Technology) plus protease inhibitor (Halt Protease Inhibitor Single-Use Cocktail, Thermo Fisher Scientific) and phenylmethanesulfonyl fluoride (Cell Signaling Technology) and sonicated. Protein concentration was

assayed with Pierce BCA Protein Assay kit (Thermo Prod). For immunoblotting, the buffer for the samples was prepared by adding 950  $\mu$ l of 2 $\times$  Laemmli Sample Buffer to 50  $\mu$ l of 2-mercaptoethanol (Bio-Rad, USA). The protein samples were mixed in a 1:1 ratio with the sample buffer. After boiling for 5 min, 20  $\mu$ g of protein was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the analytes were transferred onto nitrocellulose transfer membranes. After the membranes were incubated with blocking buffer (LICOR Biosciences, USA) for 1 h at room temperature, these were incubated with primary antibodies at 1:1,000 dilution overnight at 4°C. The primary antibodies that were used for immunoblotting were rabbit monoclonal antibodies directed against murine netrin-1 (ab126729, Abcam, USA) and murine albumin (ab207327, Abcam). For the loading control, a rabbit monoclonal antibody directed at murine glyceraldehyde-3-phosphate dehydrogenase (ab181602, Abcam) was used. After washing four times with Tween buffered saline containing 0.1% Tween, membranes were incubated with a 1:10,000 dilution of IRDye 680RD- or 800RD-labeled goat antirabbit antibody (LICOR Biosciences) for 1 h at room temperature. Membranes were washed three times with TBS containing 0.1% Tween and once with TBS, and images were captured and quantified using a LI-COR Imager (LICOR Biosciences).

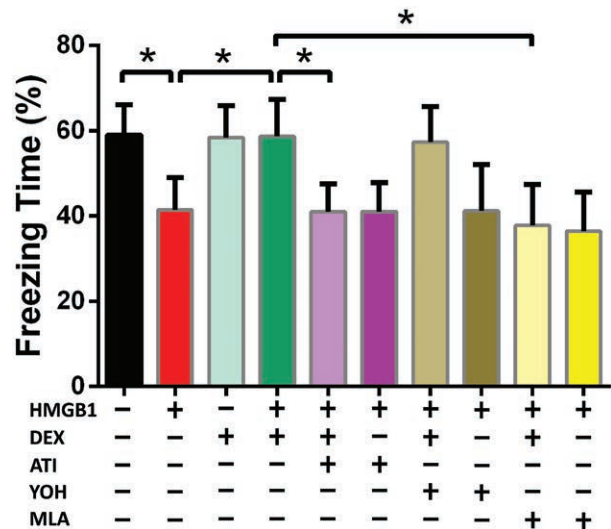
### Statistical Analysis

All data in this study were analyzed using Prism 6.0 (GraphPad Software, USA) and were expressed as mean  $\pm$  SD. Statistical comparison was performed by a one-way ANOVA followed by Tukey test for *post hoc* analysis. Significance was set at *P* value less than 0.05. No statistical power calculation was conducted before our study, and the sample size selected was based on our previous experience using this design.<sup>3,18</sup>

## Results

### Dexmedetomidine Prevents Cognitive Decline Induced by HMGB1 in an Imidazoline- and $\alpha_7$ Nicotinic Acetylcholine Receptor-dependent Mechanism

Consistent with our previous report,<sup>5</sup> HMGB1 significantly decreased freezing time (%) compared to the control group ( $41.45 \pm 7.64\%$  vs.  $58.94 \pm 7.22\%$ ;  $P < 0.0001$ ; fig. 2). Administration of dexmedetomidine prevented HMGB1-induced cognitive decline ( $58.77 \pm 8.69\%$  vs.  $41.45 \pm 7.64\%$ ;  $P < 0.0001$ ). In contrast to the lack of effect by an  $\alpha_2$  adrenoceptor-blocking dose of yohimbine, atipamezole, which has blocking activity at both the  $\alpha_2$  adrenergic receptor and the imidazoline receptor, prevented dexmedetomidine-induced reversal of HMGB1-mediated cognitive decline ( $40.87 \pm 6.60\%$  vs.  $58.77 \pm 8.69\%$ ;  $P < 0.0001$ ). The reversal effect by dexmedetomidine of HMGB1-induced cognitive decline was prevented by the  $\alpha_7$  nicotinic acetylcholine

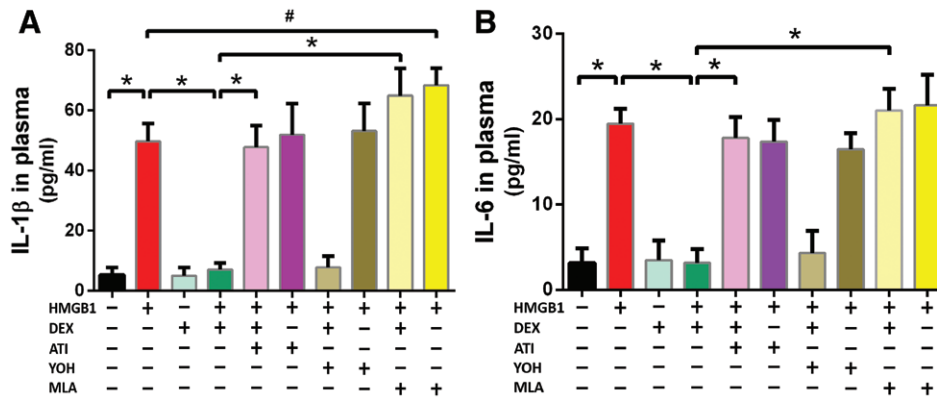


**Fig. 2.** Dexmedetomidine prevents high mobility group box 1 protein (HMGB1)-induced decrement in freezing behavior in an atipamezole- and methyllycaconitine-sensitive manner. Ten groups of randomly assigned mice ( $n = 15$ /group) were administered antagonists (methyllycaconitine, atipamezole, yohimbine) before HMGB1 and subjected to trace fear conditioning training with and without dexmedetomidine exposure. Testing for freezing behavior in the trace fear conditioning context was undertaken 72 h later. Freezing time data are expressed as means  $\pm$  SD and were analyzed by one-way ANOVA and Tukey *post hoc* test. \* $P < 0.0001$  for comparisons shown.

receptor antagonist, methyllycaconitine ( $37.79 \pm 9.51\%$  vs.  $58.77 \pm 8.69\%$ ;  $P < 0.0001$ ).

### Dexmedetomidine Prevents HMGB1-induced Systemic Inflammation through an Imidazoline- and $\alpha_7$ Nicotinic Acetylcholine Receptor-dependent Mechanism

Twenty-four hours after HMGB1 administration, plasma IL-1 $\beta$  (fig. 3A) and IL-6 (fig. 3B) were significantly increased ninefold (IL-1 $\beta$ :  $49.8 \pm 6.0$  pg/ml vs.  $5.2 \pm 2.5$  pg/ml,  $P < 0.0001$ ) and sixfold (IL-6:  $19.5 \pm 1.7$  pg/ml vs.  $3.1 \pm 1.7$  pg/ml,  $P < 0.0001$ ), respectively. Exposure to dexmedetomidine reduced the plasma concentration of both proinflammatory cytokines to normal levels (IL-1 $\beta$ :  $7.0 \pm 2.2$  pg/ml vs.  $49.8 \pm 6.0$  pg/ml,  $P < 0.0001$ ; IL-6:  $3.2 \pm 1.6$  pg/ml vs.  $19.5 \pm 1.7$  pg/ml,  $P < 0.0001$ ). Among the  $\alpha_2$  adrenoceptor antagonists, only atipamezole, which also has activity at the imidazole receptor, abolished the antiinflammatory response of dexmedetomidine (IL-1 $\beta$ :  $47.8 \pm 7.2$  pg/ml vs.  $7.0 \pm 2.2$  pg/ml,  $P < 0.0001$ ). While methyllycaconitine, the  $\alpha_7$  nicotinic acetylcholine receptor antagonist, also prevented inhibition by dexmedetomidine of the peripheral inflammatory response to HMGB1 (IL-1 $\beta$ :  $65.0 \pm 9.0$  pg/ml vs.  $7.0 \pm 2.2$  pg/ml,  $P < 0.0001$ ), it is notable that methyllycaconitine, alone, significantly enhanced the inflammatory response to HMGB1 (IL-1 $\beta$ :  $68.4 \pm 5.8$  pg/ml vs.  $49.8 \pm 6.0$  pg/ml,  $P = 0.006$ ).



**Fig. 3.** Dexmedetomidine prevents high mobility group box 1 protein (HMGB1)-induced peripheral inflammation in an atipamezole- and methyllycaconitine-sensitive manner. Ten groups of randomly assigned mice ( $n = 8/\text{group}$ ) were administered antagonists (methyllycaconitine, atipamezole, yohimbine) before HMGB1 in the presence or absence of dexmedetomidine. Twenty-four hours after HMGB1, mice were euthanized, and the blood was harvested and assayed by enzyme-linked immunosorbent assay for circulating interleukin (IL)-1 $\beta$  (A) and IL-6 (B). Data are expressed as means  $\pm$  SD and analyzed by one-way ANOVA and Tukey *post hoc* test. \* $P < 0.0001$ , # $P < 0.01$  for comparisons shown.

### Dexmedetomidine Prevents HMGB1-induced Hippocampal Inflammation through an Imidazoline- and $\alpha_7$ Nicotinic Acetylcholine Receptor-dependent Mechanism

At 24 h after HMGB1, hippocampal IL-1 $\beta$  (fig. 4A) and IL-6 (fig. 4B) were significantly increased eightfold ( $41.6 \pm 8.0$  pg/mg *vs.*  $5.5 \pm 2.0$  pg/mg,  $P < 0.0001$ ) and sixfold ( $16.2 \pm 2.7$  pg/mg *vs.*  $2.7 \pm 1.5$  pg/mg,  $P < 0.0001$ ), respectively. Exposure to dexmedetomidine reduced the hippocampal concentration of both proinflammatory cytokines to normal levels for IL-1 $\beta$  ( $4.1 \pm 3.0$  pg/mg *vs.*  $41.6 \pm 8.0$  pg/mg,  $P < 0.0001$ ) and for IL-6 ( $3.4 \pm 1.3$  pg/mg *vs.*  $16.2 \pm 2.7$  pg/mg,  $P < 0.0001$ ). Among the  $\alpha_2$  adrenoceptor antagonists, only atipamezole abolished dexmedetomidine's antiinflammatory response of IL-1 $\beta$  ( $43.7 \pm 7.7$  pg/mg *vs.*  $4.1 \pm 3.0$  pg/mg,  $P < 0.0001$ ) and IL-6 ( $14.4 \pm 2.8$  pg/mg *vs.*  $3.4 \pm 1.3$  pg/mg,  $P < 0.0001$ ). Methyllycaconitine, the  $\alpha_7$  nicotinic acetylcholine receptor antagonist, also prevented inhibition by dexmedetomidine of the HMGB1-induced peripheral inflammatory response as reflected by IL-1 $\beta$  ( $45.8 \pm 10.1$  pg/mg *vs.*  $4.1 \pm 3.0$  pg/mg,  $P < 0.0001$ ) and IL-6 ( $16.5 \pm 2.9$  pg/mg *vs.*  $3.4 \pm 1.3$  pg/mg,  $P < 0.0001$ ). Unlike systemic inflammation (fig. 3), methyllycaconitine did not enhance the hippocampal inflammatory response to HMGB1 (fig. 4).

### Dexmedetomidine Reverses HMGB1-induced Downregulation of Netrin-1 Expression in an $\alpha_7$ Nicotinic Acetylcholine Receptor-sensitive Manner

Accompanying the sterile inflammation induced by HMGB1 (figs. 3 and 4), netrin-1 expression is significantly decreased in the lung ( $0.7 \pm 0.1$  *vs.*  $1.0 \pm 0.2$ ,  $P = 0.0173$ , fig. 5A) and spleen ( $0.6 \pm 0.2$  *vs.*  $1.0 \pm 0.2$ ,  $P = 0.0220$ , fig. 5B), organs that are vagally innervated. Exposure to dexmedetomidine reverses HMGB1-induced netrin-1 downregulation in both organs ( $1.5 \pm 0.1$  *vs.*  $0.7 \pm 0.1$ ,  $P < 0.0001$ , fig. 5A;  $1.4 \pm 0.2$  *vs.*  $0.7 \pm 0.2$ ,  $P < 0.0001$ , fig. 5B), and pretreatment with

methyllycaconitine, the  $\alpha_7$  nicotinic acetylcholine receptor antagonist, prevented reversal of netrin expression by dexmedetomidine ( $0.7 \pm 0.1$  *vs.*  $1.5 \pm 0.1$ ,  $P < 0.0001$ , fig. 5A;  $0.6 \pm 0.2$  *vs.*  $1.4 \pm 0.2$ ,  $P < 0.0001$ , fig. 5B).

### Dexmedetomidine Reverses HMGB1-induced Changes in the Expression of Circulating LTB $_4$ and LXA $_4$ in an $\alpha_7$ Nicotinic Acetylcholine Receptor-sensitive Mechanism

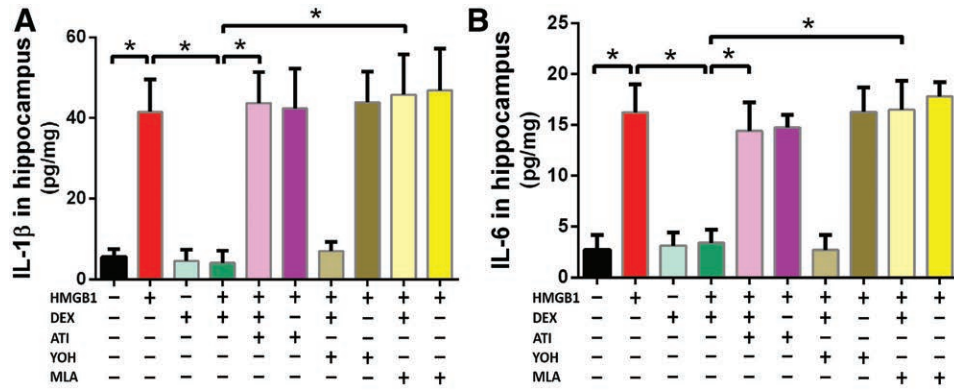
Accompanying the sterile inflammation induced by HMGB1 (figs. 3 and 4) and the downregulation of netrin-1 expression (fig. 5), there is a threefold upregulation in the relative expression of LTB $_4$ , the proinflammatory lipid mediator ( $2.7 \pm 0.4$  *vs.*  $1.0 \pm 0.2$ ,  $P < 0.0001$ , fig. 6A). Dexmedetomidine reverses HMGB1-induced upregulation of LTB $_4$  ( $1.0 \pm 0.2$  *vs.*  $2.7 \pm 0.4$ ,  $P < 0.0001$ , fig. 6A), and this reversal is prevented by methyllycaconitine ( $3.0 \pm 0.3$  *vs.*  $1.0 \pm 0.2$ ,  $P < 0.0001$ , fig. 6A). Conversely, dexmedetomidine upregulated the expression of LXA $_4$  ( $1.7 \pm 0.2$  *vs.*  $1.2 \pm 0.2$ ,  $P < 0.0001$ , fig. 6B), the specific proresolving mediator, an effect that was reversed by methyllycaconitine ( $0.9 \pm 0.2$  *vs.*  $1.7 \pm 0.2$ ,  $P < 0.0001$ , fig. 6B).

### Dexmedetomidine Reverses HMGB1-induced Leakage of the Blood-Brain Barrier

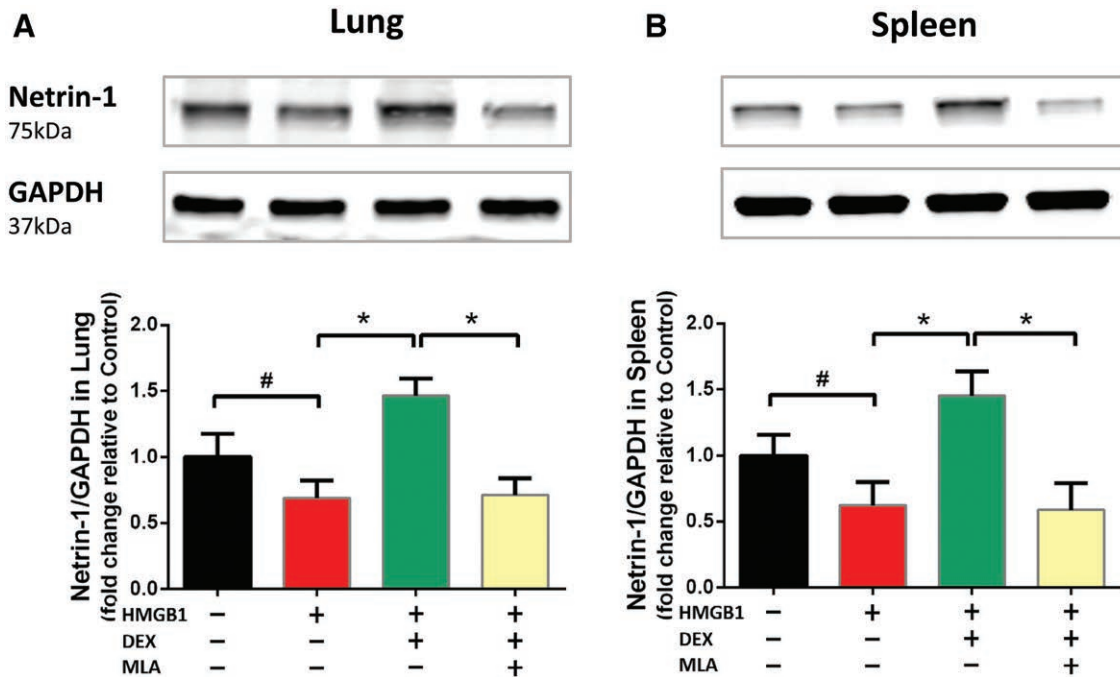
Accompanying the HMGB1-induced inflammation (figs. 3 and 4), the blood-brain barrier is disrupted, as evidenced by a significant upregulation of albumin expression in the brain assessed by immunoblotting. The upregulation in brain albumin expression ( $1.0 \pm 0.2$  *vs.*  $1.7 \pm 0.3$ ,  $P = 0.0019$ ) is suppressed by dexmedetomidine ( $1.7 \pm 0.3$  *vs.*  $1.0 \pm 0.2$ ,  $P = 0.002$ ; fig. 7).

### Dexmedetomidine Reverses Surgery-induced Cognitive Decline and Inflammation

Because isoflurane had no effect on HMGB1-induced cognitive decline (data not shown), the effect of dexmedetomidine



**Fig. 4.** Dexmedetomidine prevents high mobility group box 1 protein (HMGB1)–induced hippocampal inflammation in an atipamezole- and methyllycaconitine-sensitive manner. Ten groups of randomly assigned mice (n = 8/group) were administered antagonists (methyllycaconitine, atipamezole, yohimbine) before HMGB1 in the presence or absence of dexmedetomidine. Twenty-four hours after HMGB1, mice were euthanized, and the hippocampus was harvested and assayed by ELISA for interleukin (IL)-1β (A) and IL-6 (B). Data are expressed as means ± SD and were analyzed by one-way ANOVA and Tukey *post hoc* test. \*P < 0.0001 for comparisons shown.



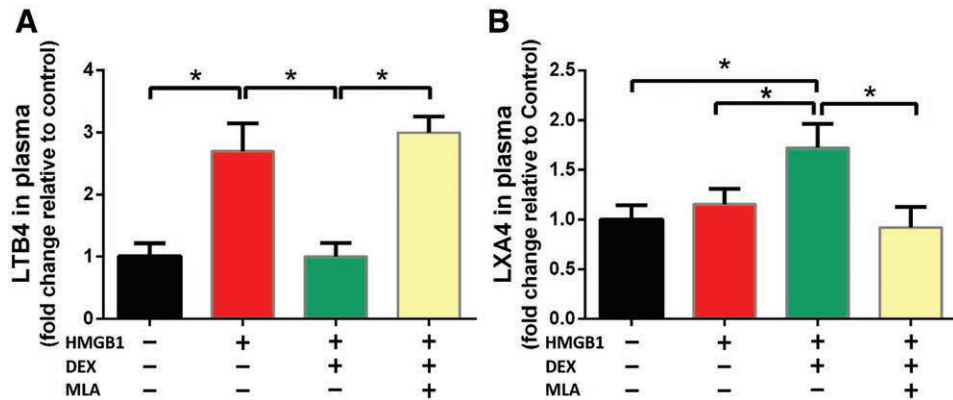
**Fig. 5.** Dexmedetomidine prevents high mobility group box 1 protein (HMGB1)–induced downregulation of netrin-1 expression in the lung (A) and spleen (B) in an  $\alpha_7$  nicotinic acetylcholine receptor–dependent manner. Four groups of randomly assigned mice (n = 5/group) were administered saline vehicle (control), HMGB1 alone, HMGB1+ dexmedetomidine, or HMGB1 + dexmedetomidine + methyllycaconitine. Twenty-four hours later, mice were euthanized, and lung (A) and spleen (B) were harvested for expression of netrin-1 by immunoblotting. Data are expressed as means ± SD fold-change relative to control and were analyzed by one-way ANOVA and Tukey *post hoc* test. # P < 0.05 and \*P < 0.0001 for comparisons shown. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

on surgery plus isoflurane-induced cognitive decline was investigated. Surgery-induced cognitive decline was reversed by dexmedetomidine ( $35.91 \pm 5.03\%$  vs.  $54.62 \pm 8.82\%$ ,  $P < 0.0001$ , fig. 8A). Twenty-four hours after surgery, we observed a significant decrease in circulating IL-6 ( $83.2 \pm 60.2$  pg/ml vs.  $30.1 \pm 13.7$  pg/ml,  $P = 0.013$ , fig. 8B). The change of hippocampal mRNA expression of IL-6 after surgery was reversed by dexmedetomidine ( $8.6 \pm 1.3$  vs.  $10.7 \pm 1.0$ ,  $P = 0.012$ , fig. 8C).

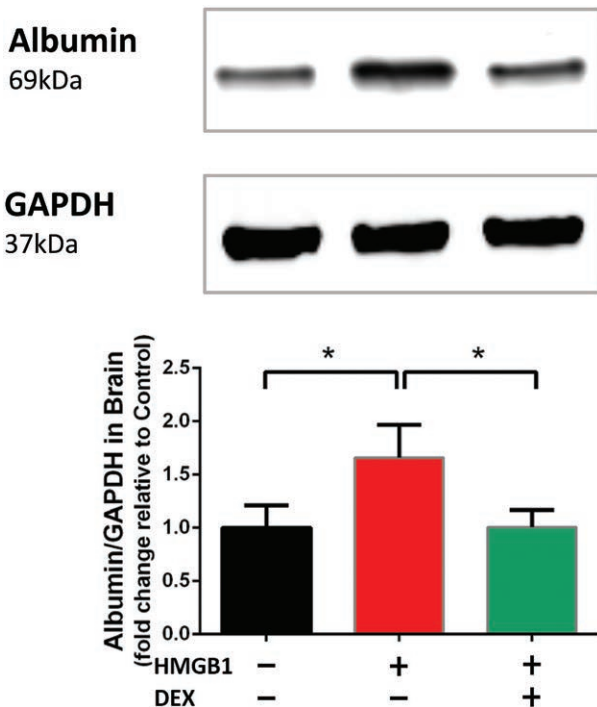
## Discussion

### Recapitulation of Main Findings

Using HMGB1 to generate a surrogate of the surgical phenotype without the confounding influence of general anesthesia, dexmedetomidine reversed HMGB1-induced cognitive decline (fig. 2), as well as systemic (fig. 3) and hippocampal (fig. 4) inflammation. In each case, reversal by dexmedetomidine of the HMGB1-induced surgical phenotype



**Fig. 6.** Dexmedetomidine downregulates the circulating proinflammatory mediator leukotriene B<sub>4</sub> (LTB<sub>4</sub>; A) and upregulates the circulating proresolving mediator lipoxin A<sub>4</sub> (LXA<sub>4</sub>; B). Four groups of randomly assigned mice (n = 8/group) were administered saline vehicle (control), high mobility group box 1 protein (HMGB1) alone, HMGB1+ dexmedetomidine, or HMGB1 + dexmedetomidine + methyllycaconitine. Twenty-four hours later, mice were euthanized and the blood was harvested and assayed by ELISA for plasma LTB<sub>4</sub> (A) and LXA<sub>4</sub> (B). Data are expressed as means ± SD fold-change relative to control and were analyzed by one-way ANOVA and Tukey *post hoc* test. \*P < 0.0001 for comparisons shown.



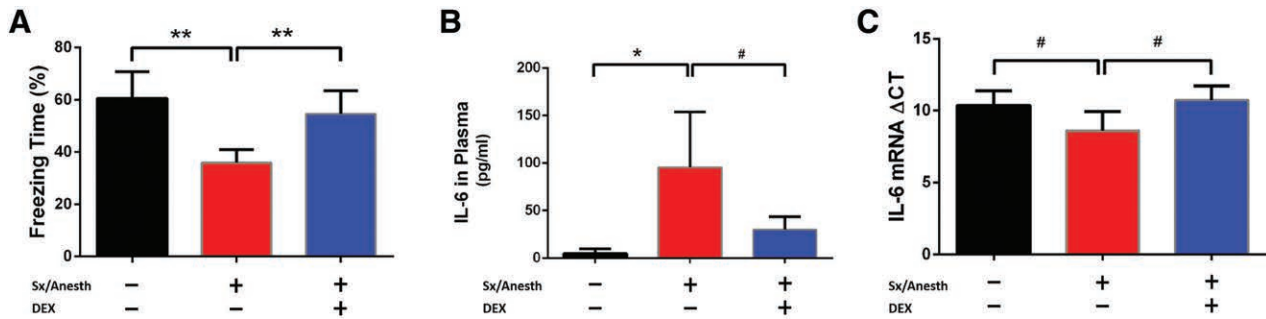
**Fig. 7.** Dexmedetomidine reverses high mobility group box 1 protein (HMGB1)-induced leakage of blood-brain barrier. Three groups of randomly assigned mice (n = 5/group) were treated with vehicle (control), HMGB1, or HMGB1 + dexmedetomidine. Twenty-four hours after treatment, mice were euthanized, and the brains were harvested for immunoblotting of albumin expression. Data are expressed as means ± SD relative to control and were analyzed by one-way ANOVA and Tukey *post hoc* test. \*P < 0.01 for comparisons shown. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

was blocked by atipamezole (figs. 2–4), an α<sub>2</sub> adrenoceptor antagonist that also has activity at the imidazole receptor,<sup>19</sup> but not by yohimbine, an α<sub>2</sub> adrenoceptor antagonist that has no activity at the imidazole receptor. Reversal by

dexmedetomidine of the surgical phenotype was also blocked by methyllycaconitine (figs. 2–4), the α<sub>7</sub>nAChR antagonist. Dexmedetomidine reversed the HMGB1-induced downregulation of netrin-1 in the vagally innervated lung (fig. 5A) and spleen (fig. 5B); reversal by dexmedetomidine was attenuated by antagonism of signaling at the vagal termini by α<sub>7</sub> nicotinic acetylcholine receptor blockade with methyllycaconitine. Upregulation of LTB<sub>4</sub>, the proinflammatory humoral response to HMGB1, was prevented by dexmedetomidine (fig. 6A), while the proresolving mediator, LXA<sub>4</sub>, was upregulated by dexmedetomidine (fig. 6B). In each case, the effects of dexmedetomidine were negated by pretreatment with methyllycaconitine, the α<sub>7</sub> nicotinic acetylcholine receptor blocker (fig. 6). Disruption of the blood-brain barrier by HMGB1 was reversed by dexmedetomidine (fig. 7). Finally, perioperative exposure to dexmedetomidine prevented surgery-induced cognitive decline and peripheral and hippocampal inflammation (fig. 8).

**Justification for the Use of HMGB1 to Produce the Surgical Phenotype**

HMGB1 regulates transcription of nuclear factor kappa-light-chain-enhancer of activated B cells,<sup>20</sup> establishing it as an extracellular orchestrator of the systemic inflammatory response.<sup>21</sup> Previously, we showed the causal role of HMGB1 in mediating postoperative cognitive decline after passive release from traumatized tissues.<sup>5</sup> After binding to pattern recognition receptors on circulating immunocytes, HMGB1 initiates the innate immune response to aseptic surgical trauma. Exogenously administered HMGB1 reproduces the surgical phenotype if myeloid-derived circulating monocytes are present,<sup>5</sup> a feature also noted after surgery. Other cardinal features of the surgical phenotype, including peripheral (fig. 3) and neuroinflammation (fig. 4) and disruption of the blood-brain barrier (fig. 7), are reproduced after HMGB1 administration.<sup>2</sup>



**Fig. 8.** Dexmedetomidine reverses surgery-induced cognitive decline (A) and peripheral (B) and neuroinflammation (C). Three groups of randomly assigned mice ( $n = 15/\text{group}$ ) were treated with (1) vehicle, (2) tibia fracture during anesthesia (Sx/Anesth) + vehicle, and (3) Sx/Anesth + dexmedetomidine before training in the trace fear conditioning paradigm (A). Testing for freezing behavior in the trace fear conditioning context was undertaken 72 h later. Freezing time data are expressed as means  $\pm$  SD and were analyzed by one-way ANOVA and Tukey *post hoc* test. Three groups of randomly assigned mice ( $n = 6/\text{group}$ ) were treated with (1) vehicle, (2) tibia fracture during anesthesia (Sx/Anesth) + vehicle, and (3) Sx/Anesth + dexmedetomidine. Mice were euthanized at 24 h, and blood and brain were harvested. Plasma interleukin (IL)-6 was assayed by enzyme-linked immunosorbent assay (B), and hippocampal IL-6 was assayed by quantitative polymerase chain reaction (C). The means  $\pm$  SD of the mean for expression of IL-6 protein (B) and mRNA (C) were analyzed by one-way ANOVA and Tukey *post hoc* test. # $P < 0.05$ ; \* $P < 0.01$ ; \*\* $P < 0.0001$ .

### Use of Antagonists with Which to Probe the Mechanism for Dexmedetomidine's Effects in Reversing the Surgical Phenotype

$\alpha_2$  Adrenoceptor agonists such as dexmedetomidine and clonidine have an imidazole ring structure facilitating binding to and activation of the imidazoline receptor.<sup>22</sup> Because atipamezole, which has antagonist activity at the imidazoline receptor,<sup>20</sup> was able to block dexmedetomidine's reversal of the surgical phenotype while yohimbine, which has no activity at the imidazoline receptor, was ineffective, we invoke an action mediated by the imidazoline receptor for the reversal of dexmedetomidine's effect. An important property of imidazoline receptor agonists is its negative chronotropic property,<sup>23</sup> which is prevented by vagotomy.<sup>24</sup> As enhancement of vagal activity by  $\alpha_2$  agonists with an imidazole ring has been well documented<sup>25</sup> and, as the bradycardic effect of  $\alpha_2$  agonists with an imidazole ring structure is unrelated to its binding affinity to  $\alpha_2$  adrenoceptors,<sup>19</sup> we propose that this imidazoline receptor-mediated vagomimetic action may be the mechanism whereby dexmedetomidine reverses the surgical phenotype.

Increase in vagal activity has been shown to be an important mechanism for resolving inflammation.<sup>26</sup> The  $\alpha_7$  nicotinic acetylcholine receptor transduces the inflammation-resolving vagomimetic effect.<sup>27</sup> A supporting finding that the enhanced vagal activity is the likely explanation for dexmedetomidine's reversal of the surgical phenotype is provided by the fact that  $\alpha_7$  nicotinic acetylcholine receptor blockade by methyllycaconitine eliminated the ameliorative effect produced by dexmedetomidine (figs. 2–4).

### Role of Vagal Stimulation on Neural- and Humoral-mediated Resolution of Inflammation

Neural<sup>28</sup> and humoral<sup>23</sup> pathways have been implicated in the resolution of acute inflammation. Neural mechanisms

involve efferent vagal fibers in which release of acetylcholine activates  $\alpha_7$  nicotinic acetylcholine receptor on immunocompetent cells,<sup>29</sup> thereby inhibiting NF- $\kappa$ B and downregulating synthesis of proinflammatory cytokines.<sup>30</sup> For the humoral pathway, biotransformation of free fatty acids (arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid) elaborates specific proresolving lipid mediators.<sup>31</sup> During synthesis of eicosanoid hormones, there are juncture points at which a precursor, such as leukotriene  $A_4$ , can elaborate two different products ( $LTB_4$  and  $LXA_4$ ) with diametrically opposite effects, namely, proinflammation and proresolution, respectively.<sup>32</sup> Dexmedetomidine appears to shunt the biotransformation pathway to the synthesis of  $LXA_4$  at the expense of  $LTB_4$  (fig. 6).

Humoral and neural pathways have been recently linked to vagally mediated expression of netrin-1.<sup>33</sup> Netrin-1, originally identified as a neuronal guidance protein, also limits inflammation.<sup>34</sup> Interestingly, netrin-1 expression is downregulated in vagally innervated lung and spleen after HMGB1 administration, an effect that is reversed by dexmedetomidine (fig. 5). A similar downregulation of netrin-1 expression has been noted in other inflammation-inducing experimental settings.<sup>33</sup>

### Integrating Results into Existing Molecular Mechanism Model for Postoperative Cognitive Decline

Previously, we had shown that trauma-induced release of HMGB1 engages circulating monocytes<sup>5</sup> to stimulate the synthesis of proinflammatory cytokines that are capable of disrupting the blood–brain barrier,<sup>2</sup> enabling the passage of bone marrow–derived monocytes to enter into the brain, attracted by upregulation of the chemokine MCP-1 by microglia in the hippocampus.<sup>3</sup> Within the hippocampus, the bone marrow–derived monocytes activate microglia, resulting in release of proinflammatory cytokines that disrupt



long-term potentiation,<sup>35</sup> the neurobiologic correlate of learning and memory. Within 7 days, the inflammation usually resolves, as does postoperative cognitive decline, except in vulnerable animals, in which inflammation and cognitive decline can both be exaggerated<sup>3</sup> and more persistent.<sup>18</sup>

Dexmedetomidine appears to reverse postoperative cognitive decline by enhancing the inflammation-resolving pathways. Dexmedetomidine stimulates the vagus through imidazoline receptor activation, resulting in inhibition of the NF- $\kappa$ B–dependent synthesis of proinflammatory cytokines (figs. 3 and 4) through activation of  $\alpha_7$  nicotinic acetylcholine receptor (figs. 3 and 4). Netrin-1 is downregulated by HMGB1 through a vagal pathway, and this is reversed by dexmedetomidine (fig. 5), resulting in a change in the elaboration of specific proresolving mediators (increase in LXA<sub>4</sub> and decrease in LTB<sub>4</sub>) that resolves inflammation (fig. 6).

## Caveats

### Methyllycaconitine-induced Upregulation of Circulating Proinflammatory Cytokines

Methyllycaconitine was used in a dose that blocks the  $\alpha_7$  nicotinic acetylcholine receptor–mediated inhibition of synthesis of proinflammatory cytokines.<sup>2</sup> However, at this dose there was an increase in HMGB1-induced proinflammatory cytokines (fig. 3), and it may be argued that the reversal of the anti-inflammatory effect of dexmedetomidine could be due to the nonspecific enhancement of proinflammatory cytokines independent of any specific action that dexmedetomidine exerts on the  $\alpha_7$  nicotinic acetylcholine receptor through vagal stimulation. It is notable that in the hippocampus, where cognitive decline is produced, there was not a similar methyllycaconitine-induced enhancement of proinflammatory cytokines.

### Doses of Dexmedetomidine Used in HMGB1- and Surgery-induced Cognitive Decline

In the HMGB1-induced surrogate of the surgical phenotype, we used a dexmedetomidine dose of 50  $\mu$ g/kg, while in the trauma-induced model, a dexmedetomidine dose of 20  $\mu$ g/kg was used. The reason that these doses differ was to prevent a significant increase in the sedative effect of dexmedetomidine in the presence of isoflurane<sup>36</sup> that may increase postoperative cognitive decline.<sup>11</sup> The lower dexmedetomidine dose in the surgical model (fig. 8) was as effective as the higher dose in the HMGB1 model (figs. 2–4) at reversing cognitive decline and inflammation.

### Relevance of Findings in Young Mice to Vulnerable Animal Models

In the current study, we only tested the efficacy and putative mechanisms whereby dexmedetomidine prevents inflammation and cognitive decline in young mice. Whether the observed cognitive decline–reducing properties of dexmedetomidine occurs in vulnerable models (including aging, obesity,<sup>3</sup> and metabolic syndrome<sup>18</sup>) remains to be determined.

### Relevance of Preclinical Mechanistic Findings in Light of Dexmedetomidine's Ability to Reverse the Surgical Phenotype in Clinical Studies

Sedation with dexmedetomidine reduces the likelihood of delirium in the intensive care unit when compared to benzodiazepines<sup>13,37</sup> and when dexmedetomidine was administered during the first postoperative night to elderly surgical patients.<sup>14</sup> Because dexmedetomidine changes the activity in neuronal pathways in a similar manner to those altered during natural sleep<sup>38</sup> and produces similar electroencephalographic changes to those seen during natural sleep,<sup>39</sup> and because both sleep deprivation<sup>40</sup> and sleep fragmentation<sup>41</sup> induce neuroinflammation and cognitive decline, we had conjectured that the cognition-enhancing effects of dexmedetomidine were due to its unique sedative profile. However, as dexmedetomidine-induced sedation is mediated by the  $\alpha_{2A}$  adrenoceptors<sup>42</sup> that are antagonized by yohimbine,<sup>43</sup> our finding that reversal by dexmedetomidine of the surgical phenotype is insensitive to yohimbine challenges that explanation.

We recognize that the reversal of postoperative cognitive decline by dexmedetomidine is not universal. Recently, Deiner *et al.*<sup>44</sup> reported that dexmedetomidine lacked efficacy in preventing the onset of postoperative delirium.<sup>44</sup> Further studies are needed to define the patient subgroups that may be resistant to the cognitive decline–reducing properties of dexmedetomidine.

### Future Applications

These preclinical studies were performed in mice that do not exhibit exaggerated and persistent cognitive decline. The effectiveness of dexmedetomidine will need to be established in reagents that have abnormalities in their inflammation-resolving pathways including in advanced age with its “inflammaging” processes<sup>45</sup> and the metabolic syndrome with its precocious aging phenotype.<sup>46</sup>

While we have already shown that dexmedetomidine is effective at decreasing delirium in mechanically ventilated medical/surgical intensive care patients<sup>13</sup> as well as in non-ventilated surgical patients,<sup>14</sup> it will be important to demonstrate that wound healing and the ability to combat infection are not jeopardized by a dexmedetomidine intervention that perturbs the innate immune system. Regarding infection, it is notable that survival is enhanced by dexmedetomidine in a preclinical model of sepsis,<sup>47</sup> a finding that was also noted in a *post hoc* analysis of the Maximizing Efficacy of Targeted Sedation and Reducing Neurological Dysfunction (MENDS) trial.<sup>48</sup> However, dexmedetomidine was not shown to improve outcome in a recently reported trial of septic patients; this question is being further addressed in the MENDS II trial (NCT01739933) comparing outcomes in septic patients sedated with dexmedetomidine *versus* propofol. Whether the existing role of dexmedetomidine as a perioperative and procedural sedative agent can be supplemented by indications in which resolution of inflammation requires bolstering will need to be studied further. Furthermore, if the

activation of the imidazoline receptor and subsequent vagal outflow are confirmed to be the reason for dexmedetomidine's antiinflammatory properties, it may be possible to reproduce these effects with a selective imidazoline receptor agonist and avoid  $\alpha_2$  adrenoceptor properties such as sedation.

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## Competing Interests

Dr. Maze is a coinventor on a patent for the use of dexmedetomidine for sedation. Between 1987 and 1991, Dr. Maze's laboratory at Stanford University (Stanford, California) received \$250,000 for the assignment of the patent to Farnos (Espoo, Finland), the company that synthesized dexmedetomidine. Between 1995 and 2008, Dr. Maze was intermittently paid as a consultant by Orion-Farnos (Espoo, Finland), Abbott Labs (Chicago, Illinois), and Hospira (Lake Forest, Illinois) for advising on the pivotal phase III clinical trials, approval of the new drug application, and subsequent marketing of the product. Dr. Maze has not received any payments for at least the last 5 yr. Dr. Maze has not and will not receive royalty payments for sales of dexmedetomidine. The other authors declare no competing interests.

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