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Dexmedetomidine ameliorates perioperative neurocognitive disorders by suppressing monocyte-derived macrophages in mice with preexisting traumatic brain injury

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NK: This author helped by performing animal surgery, behavioral testing, immunohistochemistry and immunoblotting.
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YY: This author helped by performing behavioral testing and immunohistochemistry.
ST: This author helped by performing behavioral testing.
All authors conceived and designed the study and were involved in analyzing and interpreting data and drafting and revising the text, and read and approved the final manuscript.

Abstract

Background: Traumatic brain injury (TBI) initiates immune responses involving infiltration of monocyte-derived macrophages (MDMs) in the injured brain tissue. These MDMs play a key role in perioperative neurocognitive disorders (PND). We tested the hypothesis that pre-anesthetic treatment with dexmedetomidine (DEX) could suppress infiltration of MDMs into the hippocampus of TBI model mice, ameliorating PND.

Methods: We first performed bone marrow transplantation from green fluorescent protein-transgenic mice to C57BL/6 mice to identify MDMs. We used only male mice for homogeneity. Four weeks after transplantation, a controlled cortical impact model of TBI was created using recipient mice. Four weeks after TBI, mice received pretreatment with DEX prior to general anesthesia (GA). Mice performed the Barnes maze test (8–12 mice/group) 2 weeks after GA and were euthanized for immunohistochemistry (4–5 mice/group) or immunoblotting (7 mice/group) 4 weeks after GA.

Results: In Barnes maze tests, TBI model mice showed longer primary latency (mean difference, 76.5 [95% confidence interval 41.4–111.6], $P<.0001$ vs. Naïve), primary path length (431.2 [98.5–763.9], $P=.001$ vs. Naïve), and more primary errors (5.7 [0.62–10.7], $P=.017$ vs. Naïve) than Naïve mice on experimental day 3. Expression of MDMs in the hippocampus was significantly increased in TBI mice compared to Naïve mice (2.1 [0.6–3.7], $P=.003$ vs. Naïve). Expression of MCP1-positive areas in the hippocampus was significantly increased in TBI mice compared to Naïve mice (0.38 [0.09–0.68], $P=.007$ vs. Naïve). Immunoblotting indicated significantly increased expression of interleukin-1β in the hippocampus in TBI mice compared to Naïve mice (1.59 [0.08–3.1], $P=.035$ vs. Naïve). In contrast, TBI mice pretreated with DEX were rescued from these changes and showed no significant difference from naïve mice. Yohimbine, an $\alpha 2$ receptor antagonist, mitigated the effects of DEX (primary latency: 68.3 [36.5–100.1], $P<.0001$ vs. TBI-DEX; primary path length: 414.9 [120.0–709.9] $P=.0002$ vs. DEX; primary errors: 6.6 [2.1–11.2], $P=.0005$ vs. TBI-DEX, expression of MDMs: 2.9 [1.4–4.4], P=.0001 vs. TBI-DEX; expression of MCP1: 0.4 [0.05–0.67], P=.017 vs. TBI-DEX; expression of interleukin-1β: 1.8 [0.34–3.35], P = 0.01 vs. TBI-DEX).

Conclusions: Pre-anesthetic treatment with DEX suppressed infiltration of MDMs in the hippocampus and ameliorated PND in TBI model mice. Pre-anesthetic treatment with DEX appears to suppress infiltration of MDMs in the hippocampus and may lead to new treatments for PND in patients with a history of TBI.

Key Points

- **Question:** Does pre-anesthetic treatment with dexmedetomidine suppress infiltration of monocyte-derived macrophages into the hippocampus of traumatic brain injury model mice, ameliorating perioperative cognitive disorders?
- **Findings:** Pre-anesthetic treatment with dexmedetomidine suppressed infiltration of

monocyte-derived macrophages in the hippocampus and ameliorated perioperative neurocognitive disorders in traumatic brain injury model mice.

- **Meaning:** Pre-anesthetic treatment with dexmedetomidine may lead to new treatments for perioperative neurocognitive disorders in patients with a history of traumatic brain injury.

Glossary of Terms

α_2-AR = α_2-adrenergic receptor; **ANOVA** = analysis of variance; **BBB** = blood-brain barrier; **BMT** = bone marrow transplantation; **BT** = behavioral test; **CCI** = controlled cortical impact injury; **CCR2** = C-C chemokine receptor type 2; **CNS** = central nervous system; **DAPI** = 4',6-diamidino-2-phenylindole dihydrochloride solution; **DEX** = dexmedetomidine; **EGFP** = enhanced green-fluorescent protein; **GA** = general anesthesia; **GAPDH** = glyceraldehyde-3-phosphate dehydrogenase; **GFAP** = glial fibrillary acidic protein; **GFP** = green fluorescent protein; **IB** = immunoblotting; **IHC** = immunohistochemistry; **IL-1β** = interleukin 1β; **MCP-1** = monocyte chemotactic protein-1; **MDMs** = monocyte-derived macrophages; **PBS** = phosphate-buffered saline; **PND** = perioperative neurocognitive disorders; **SD** = standard deviation; **TBI** = traumatic brain injury; **TBS-T** = Tris buffered saline with Tween®-20; **Veh** = vehicle; **WB** = Western blotting; **YOH** = yohimbine.

Introduction

Traumatic brain injury (TBI) is a leading cause of disability and mortality, particularly in a young and healthy population. Survivors suffer from worse cognitive function and poorer quality of life after injury.[1] Permanent cognitive deficits induced by TBI not only restrict the productivity of victims in daily life, but also impose substantial socioeconomic burdens on their families and communities.[2,3]

Perioperative neurocognitive disorder (PND) is the most common complication, occurring after surgery.[4,5,6] Whereas PND mostly resolves in weeks to months, patients with pre-existing vulnerabilities such as Alzheimer's disease, cerebrovascular disease and TBI may suffer irreversible, permanent PND.[5,6,7,8] Nevertheless, whether anesthetics may amplify PND in the presence of pre-existing vulnerabilities has not been clarified.

Microglia are an important part of the immune response to damage to the central nervous system (CNS). Microglia are classified into two types: resident microglia, which are innate immune-related glial cells of CNS; and monocyte-derived macrophages (MDMs), which infiltrate into the brain from the blood via opening of the blood-brain barrier (BBB) during brain injury and CNS diseases.[6,9] Inflammation caused by TBI allows BBB extravasation of various immune cells, including MDMs, resulting in neuroinflammation.[10,11] MDMs recruit and accumulate into the injured brain tissue via activation of monocyte chemotactic protein-1 and C-C chemokine receptor type 2 (MCP1-CCR2) signaling, contributing to inflammation even during nonpathological periods and chronic cognitive deficits.[11,12] Surgery and anesthetic agents also trigger inflammation and lead to lowering of the

BBB.[4-6] MDMs initiate hippocampal neuroinflammation and development of PND.[4,6,13,14]
Dexmedetomidine (DEX), a highly selective α_2-adrenergic receptor (α_2-AR) agonist, is currently approved for sedating patients under various clinical situations. A previous clinical study revealed that DEX does not affect cerebral blood flow in patients with TBI.[15] In fact, DEX pretreatment reportedly exerts neuroprotective effects, alleviating isoflurane-induced neurocognitive deficits in rodent models.[16,17] DEX has been shown to improve neurological outcomes both by inhibition of microglia and by modulation of neuroinflammation via α_2-ARs after spinal cord injury in rats.[18] However, the relationship between infiltration of MDMs into the hippocampus and the effect of pretreatment with DEX on PND in an animal model with a history of TBI has not yet been clarified. We hypothesized that accumulation of MDMs in the hippocampus could be a major contributor to PND in a mouse model of TBI. In the present study, we investigated whether pretreatment with DEX could suppress the infiltration of MDMs in the hippocampus and ameliorate PND in a mouse model of pre-existing TBI.

Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sapporo Medical University (approval no. 16-092) and conformed to National Institutes of Health guidelines. This manuscript adheres to the applicable ARRIVE guidelines. Experiments were conducted using male C57BL/6 mice and male C57BL/6-Tg (CAG-EGFP) mice (Japan SLC, Hamamatsu, Japan) between 8 and 12 weeks old, weighing 20–25 g at the start of the experiments. Mice of a single sex were chosen for homogeneity with our previous study[19], which would facilitate comparisons as needed. The mice were housed in our institutional animal care facility in a temperature-controlled room (22–24° C) under a 12-h light/12-h dark cycle with unlimited access to food and water. Mice were randomly allocated to six groups, consisting of three control groups (Transplant, Naïve and Sham) and three experimental groups (TBI, TBI-DEX and TBI-DEX-YOH). All animals underwent bone marrow transplantation (BMT). The Transplant group underwent BMT alone, with no anesthesia or surgery (Figure 1A). At 4 weeks after BMT, the Naïve group underwent only induction of anesthesia without surgery, the Sham group underwent induction of anesthesia and craniotomy, but no TBI, and the TBI, TBI-DEX and TBI-DEX-YOH groups underwent TBI (Figure 1A). Next, at 8 weeks after BMT, the Naïve, Sham, TBI, TBI-DEX and TBI-DEX-YOH groups received general anesthesia (GA). Prior to GA, the TBI-DEX group received pretreatment with DEX and the TBI-DEX-YOH group received injection of yohimbine (YOH) and DEX (Figure 1B). All mice underwent cognitive behavior testing 10 weeks after BMT. Two weeks after behavioral testing, all mice other than the Transplant group were deeply anesthetized and euthanized by rapid decapitation, and their brain tissues were harvested for immunohistochemistry and immunoblotting (Figure 1A).

BMT

BMT was performed with C57BL/6 mice as the recipients, for identification of MDMs. We isolated bone marrow cells from the tibias and femurs of C57BL/6-Tg(CAG-EGFP) mice that expressed green fluorescent protein (GFP). Bone marrow cells ($4–6 \times 10^6$) were injected into the tail veins of recipient mice, which also received busulfan (Otsuka Pharmaceutical, Tokyo, Japan) (20 mg/kg/day for 5 consecutive days) intraperitoneally prior to BMT for myelosuppression. Four weeks after BMT, the number of GFP-positive cells in peripheral blood was counted using a FACS Aria II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The chimeric ratio was calculated using the following formula: number of GFP-positive cells/number of monocytes × 100. Mice with a chimeric ratio <85.0% were excluded from the experiments. The chimeric ratio of transplanted mice was 96.8 ± 3.0% in the present study.

Surgery

Four weeks after BMT, the TBI, TBI-DEX and TBI-DEX-YOH groups were subjected to controlled

cortical impact injury (CCI), as described in our previous study.[19] Mice were fixed in a stereotactic frame under isoflurane anesthesia (5% for 30 s for induction, followed by 1.5% mixed with oxygen at 1 L/min as maintenance) and received buprenorphine intraperitoneally (0.05 mg/kg). A portable drill was then used to perform craniotomy over the right hemisphere to generate a burr hole. The bone flap was carefully removed and a 3-mm-diameter piston was centered on the dura at approximately +0.5 to -2.5 mm from the bregma and 3 mm lateral to the sagittal suture. The CCI model used an electromagnetic impactor (Impact One, myNeuroLab.com, Richmond, IL) (contact velocity; 3.0 m/s, deformation; 1.0 mm). Finally, the skin incision was sutured after injury induction. Sham mice underwent only craniotomy without CCI. Sham, TBI, TBI-DEX and TBI-DEX-YOH groups received buprenorphine (0.05 mg/kg) subcutaneously for postoperative analgesia at the end of surgery.

Pre-treatment with DEX and GA

Four weeks after TBI induction, TBI-DEX and TBI-DEX-YOH group mice received pre-treatment with intraperitoneal DEX (50 μg/kg), and Naïve, Sham and TBI mice received vehicle injection (the same volume of 0.9% sterile saline) 30 min prior to exposure to 2.0% isoflurane with oxygen at 1 L/min for 30 min (Figure 1B). In addition, TBI-DEX-YOH group mice were administered an α_2-AR antagonist YOH, (1.5 mg/kg) (Sigma-Aldrich, St. Louis, MO), 30 min prior to pretreatment with DEX. In all animals, core temperature was measured and maintained at 37 $\pm$ 0.5° C using a heating light during GA.

Behavioral tests for PND

Ten weeks after BMT, all mice were tested to assess the acquisition of spatial memory using the Barnes maze paradigm. This maze consists of a circular table 112 cm in diameter, with 20 holes each 50 cm from the center of the maze. A variety of visual cues are provided in the testing area throughout the task process. The assay was performed as 3 trials per day for 3 consecutive days (Figure 2A). The escape box location was moved each day after completion of the 3 trials. Mice were placed in an opaque box at the center of the maze for 15 s. The trial recording began with removal of the container. According to a previous study,[20] each trial lasted for 180 s or until the mouse entered the escape box. Any mouse that did not reach the escape box within the allotted time was guided to the escape box. The maze and escape box were cleaned with ethanol between each trial. Mice were tracked, recorded and analyzed using the LimeLight™ video tracking system (version 4.1.12; Actimetrics, Wilmette, IL). According to previous reports,[20-22] primary latency (time taken to first locate the escape box), primary path length (distance traveled to first locate the escape box), and primary errors (number of times the mouse placed its nose in a hole that did not lead to the escape box before finding the escape box) were assessed and mean primary latency, primary path length, and primary errors of all trials performed on each day were analyzed.

Immunohistochemistry

At 4 weeks after GA, animals were anesthetized with sodium pentobarbital intraperitoneally (50 mg/kg) and euthanized by transcardiac perfusion with phosphate-buffered saline followed by 4% paraformaldehyde, and brain tissue samples were extracted. Brains were post-fixed in paraformaldehyde overnight and stored in 30% sucrose, then cut into serial 20-μm coronal sections in a cryostat, and collected as free-floating sections. Every fourth section through the hippocampus, which included CA1, CA2, CA3 and the dentate gyrus, was incubated with primary antibodies for ionized calcium-binding adapter molecule 1 (Iba-1) (1:500; Wako Pure Chemical Industries, Osaka, Japan), glial fibrillary acidic protein (GFAP) (1:400; EMD Millipore, Billerica, MA), MCP-1 (1:500; Abcam, Cambridge, UK), alpha-2A adrenoreceptor (α_{2A}-AR) (1:500; Sigma-Aldrich, St. Louis, MO), alpha-2B adrenoreceptor (α_{2B}-AR) (1:500; PA5-49610, Thermo Fisher Scientific, Waltham, MA), alpha-2C adrenoreceptor (α_{2C}-AR) (1:500; Thermo Fisher Scientific), nischarin, as a functional imidazoline-1 receptor (1:500; Proteintech, Rosemont, IL), and interleukin 1 beta (IL-1β) (1:500; Abcam) overnight at 4° C. Sections were then incubated for 2 h with Alexa Fluor 594-conjugated secondary antibody (Invitrogen, Carlsbad, CA) or Alexa Fluor 647-conjugated secondary antibody (Millipore, Burlington, MA) at a dilution of 1:500. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride solution (1:1000; Dojindo Laboratories, Kumamoto, Japan), and the resultant images were observed using confocal laser microscopy (A1; Nikon, Tokyo, Japan). The bilateral hippocampal area and areas positive for GFP, Iba-1, GFAP and MCP-1 were measured using ImageJ software (National Institutes of Health, Bethesda, MD). Areas showing positive staining are presented as mean percentages of hippocampal volume (positive area divided by total hippocampal volume).

Immunoblotting

At 4 weeks after GA, mice were euthanized by decapitation under deep anesthesia induced with sodium pentobarbital i.p. (50 mg/kg). Hippocampal tissue (50-100 mg) was dissected on ice and homogenized at 4° C with carbonate lysis buffer (500 mM sodium carbonate, pH 11.0) containing a protease and phosphatase inhibitor. After homogenization, samples were sonicated and nuclear components were removed by centrifugation at 1000×*g* for 10 minutes, and protein concentration was quantified by Bradford assay and normalized to 2.0 μg/μl for later use. Equal amounts of protein were loaded. Electrophoresis was performed on samples using 4-12% acrylamide gels (Bolt™ Bis-Tris Plus; Thermo Fisher Scientific) and transferred to polyvinylidene fluoride membranes (Millipore Sigma, Burlington, MA) by electroelution. Membranes were blocked in blocking solution (20 mM Tris-buffered saline with Tween 20 (TBS-T) (0.1%) containing 3% bovine serum albumin), then incubated with primary antibody to IL-1β (1:500; Cell Signaling Technology, Beverly, MA) overnight at 4° C. After washes with TBS-T, the membrane was incubated with a species-specific infrared-dye-labeled secondary antibody for 1 h at room temperature and washed with TBS-T. All protein expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(Cell Signaling Technology, Danvers, MA). Densitometry of the different bands was further quantified using ImageJ software.

Statistical analysis

Statistical analysis was performed with Prism 7 software (GraphPad, La Jolla, CA). Normality of the distribution of data was assessed using the Shapiro-Wilk test. For within-group analysis of behavioral testing, repeated-measures two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc analysis was used. For within-group analysis of hippocampal volume of Iba-1/GFP, GFAP, and MCP-1 and immunoblot of IL-1β, ordinary one-way ANOVA followed by Tukey's multiple comparison post hoc analysis was used. All tests were two-tailed with values of $P < 0.05$ considered as significant and each P value was adjusted to account for multiple comparisons. Data are presented as mean difference and standard deviation (SD). The sample size 'n' represents the number of animals for each group. The investigators performing the behavioral and immunohistochemical tests were blinded to mouse groupings, and the code was broken only for analysis.

The primary outcome of the present study was primary latency on experimental day 3 in the Barnes maze paradigm. Sample size was calculated using G*power 3.1 (Heinrich-Heine-University, Dusseldorf, Germany). A total of 60 mice were calculated to be required to detect a 40-s mean difference and SD of 35 s in escape latency (power 80%, $\alpha < .05$ [2-tailed]). We defined the secondary outcome as the expression of GFP-positive areas in the hippocampus in immunohistochemical analysis. Basis on our pilot study results and a previous study,[5] we estimated that a sample of 5 animals per group would be necessary to demonstrate a 50% decrease in GFP-positive areas in the hippocampus (with $\alpha = 0.05$ and $1-\beta = 0.8$). No exclusions for outliers were made in the present study. None of the variables had missing data, and no outliers appeared in the present study. All results were examined with the animal as the unit of analysis.

Results

Pretreatment with DEX ameliorated spatial memory impairment after GA

Specifics of each sample were as follows: Transplant mice, n=12; Naïve mice, n=8; Sham mice, n=12; TBI mice, n=8; TBI-DEX mice, n=9; and TBI-DEX-YOH mice, n=12. In the Barnes maze tests, no spatial memory impairment was observed in any control groups (Transplant, Naïve, and Sham) on experimental day 3 (Figure S1). TBI mice showed longer primary latency on experimental day 3 (177 ± 11 s vs. 100 ± 66 s, $P < .0001$ vs. Naïve) (Figure 2B). TBI-DEX mice showed improved primary latency (98 ± 68 s vs. 177 ± 11 s, $P < .0001$ vs. TBI) (Figure 2B). In TBI-DEX-YOH mice, primary latency was significantly extended (167 ± 32 s vs. 98 ± 68 s, $P < .0001$ vs. TBI-DEX) (Figure 2B). TBI mice showed a longer primary path length and more primary errors (primary path length: 665 ± 384 cm vs. 234 ± 157 cm, $P < .001$ vs. Naïve, Figure S1B; primary errors: 8.9 ± 5.3 errors vs. 3.2 ± 2.2 errors, $P = .017$ vs. Naïve, Figure S1C). TBI-DEX mice showed amelioration of both primary path length and primary errors (primary path length: 264 ± 212 cm vs. 665 ± 384 cm, $P = .0025$ vs. TBI, Figure 2C; primary errors: 2.8 ± 2.7 errors vs. 8.9 ± 5.3 errors, $P = .0052$ vs. TBI, Figure 2D). TBI-DEX-YOH mice displayed counteraction of DEX treatment (primary path length: 673 ± 320 cm vs. 264 ± 212 cm, $P = .0002$ vs. TBI-DEX, Figure 2C; primary errors: 9.5 ± 6.0 errors vs. 2.9 ± 2.8 errors, $P = .0005$ vs. TBI-DEX, Figure 2D).

Pretreatment with DEX suppressed hippocampal accumulation of MDMs after GA

Accumulated GFP-positive cells in TBI mice showed a ramified shape and were positive staining with Iba-1 (Figure 3A). This result indicated that $GFP^{+}Iba1^{+}$ cells were MDMs and $GFP^{-}Iba1^{+}$ cells were resident microglia. To examine whether isoflurane exposure induced the accumulation of MDMs, resident microglia and astrocytes in the hippocampus, expressions of GFP, Iba-1, or GFAP-positivity were measured bilaterally in the hippocampus. Expression of GFP-positive areas in the ipsilateral hippocampus was significantly increased in TBI mice compared to that in Naïve mice (2.6 ± 1.3% vs. 0.5 ± 0.4%, $P = .003$; Figure 3B, C). This increase was reversed in TBI-DEX mice (0.5 ± 0.4%, $P > .999$ vs. Naïve). However, expression of GFP-positive areas in the ipsilateral hippocampus was significantly increased in TBI-DEX-YOH mice (3.4 ± 1.1% vs. 0.5 ± 0.4%, $P = .0001$ vs. TBI-DEX). As in the ipsilateral hippocampus, expression of GFP-positive areas in the contralateral hippocampus was significantly increased in TBI mice and TBI-DEX-YOH mice (TBI: 1.0 ± 0.4% vs. 0.4 ± 0.3%, $P = .012$ vs. Naïve; TBI-DEX-YOH: 1.1 ± 0.2% vs. 0.3 ± 0.2, $P = .0009$ vs. TBI-DEX, Figure 3B, C). TBI-DEX mice showed no significant difference in expression of GFP-positive areas (0.3 ± 0.2%, $P = .970$ vs. Naïve). No significant differences in expressions of Iba-1- or GFAP-positive areas bilaterally in the hippocampus were seen among the five groups (Iba-1: ipsilateral, $P = 0.933$; contralateral, $P > 0.999$, Figure 3D; GFAP: ipsilateral, $P = 0.988$; contralateral, $P = 0.760$, Figure 3F).

Expression of MCP-1 on MDMs in the hippocampus

MCP-1 is one of the key chemokines that regulate the infiltration of CCR2-expressing MDMs into the CNS from the bloodstream.[23] Since previous studies have reported that the MCP-1/CCR2 axis was the main signaling pathway for infiltration of MDMs,[4] we investigated whether MCP1-CCR2 signaling was involved in the accumulation of MDMs in the hippocampus of TBI mice at 4 weeks after GA. Increased expression of MCP1 in the hippocampus was confirmed by immunohistochemistry (Figure 4A, C). An MCP1-positive reaction was double-stained for GFP-positive MDMs in the hippocampus (Figure 4B). Expression of MCP1-positive areas in the hippocampus was significantly increased in TBI mice compared to Naïve mice (0.5 ± 0.2% vs. 0.1 ± 0.1%; $P = .006$ vs. Naïve, Figure 4C). This increased expression was counteracted in TBI-DEX mice (0.04 ± 0.01%, P = .920 vs. Naïve). However, expression of MCP1-positive areas was significantly increased in TBI-DEX-YOH mice (0.4 ± 0.1% vs. 0.04 ± 0.01%, $P = .017$ vs. TBI-DEX) (Figure 4C).

Presence and expression of α_2-AR and IL-1β in the hippocampus of TBI mice

To investigate whether DEX suppressed the accumulation of MDMs in the hippocampus via a direct effect on α_2-AR in the hippocampus, localization of α_2-AR in the hippocampus of TBI mice was evaluated immunohistochemically. All subtypes of α_2-AR, including alpha$_2$A-AR, alpha$_2$B-AR and alpha$_2$C-AR, existed on MDMs in the hippocampus of TBI mice (Figure 5A). These results suggested that DEX suppressed accumulation of MDMs in the hippocampus via direct effects on α2-ARs on the MDMs themselves. To investigate the protein expression of inflammatory cytokines that induce PND, ipsilateral hippocampal homogenates were obtained. These hippocampal homogenates from TBI mice expressed higher level of IL-1β protein compared to Naïve mice (3.5 ± 1.5 vs. 1.9 ± 0.6, $P = .035$ vs. Naïve, Figure 5B,C). TBI-DEX mice did not show high expression of IL-1β protein (2.0 ± 0.6, $P > .999$ vs. Naïve). However, hippocampal homogenates from TBI-DEX-YOH mice demonstrated higher IL-1β protein expression (3.8 ± 1.0 vs. 2.0 ± 0.6, $P = .016$ vs. TBI-DEX).

Discussion

We demonstrated that accumulation of MDMs in the hippocampus via MCP-1/CCR2 signaling caused deterioration of isoflurane-induced PND in mice with pre-existing TBI. Notably, we also demonstrated that administration of DEX prior to isoflurane exposure suppressed infiltration of MDMs and ameliorated PND.

Previous studies have reported that surgical trauma under isoflurane exposure disrupted the integrity of the blood brain barrier (BBB), induced infiltration of MDMs in the hippocampus and caused PND.[4,13] However, the interactions of PND with surgery or anesthetic have in fact remained unknown.[5] Despite minimal detrimental effects caused by the anesthetic itself, pre-existing TBI might be amplified by exposure to isoflurane, allowing infiltration of MDMs. Actually, in the present study, the three control groups (Transplant, Naïve, Sham) showed no significant differences in any analyses. Besides, TBI groups revealed that the exposure to isoflurane itself induced PND and infiltration of MDMs. These results indicate that with pre-existing vulnerability, the anesthesia itself can strongly influence on outcomes.

Unlike previous studies which have been limited to the acute phase of PND in the absence of pre-existing vulnerabilities,[4,13] the present investigation focused on reversing PND following a history of TBI. Several studies have observed that TBI induces both immediate and delayed increases in permeability of BBB.[10,11,23-25] Although the amount of MDMs peaked at several hours following injury, MDMs could maintain strong signaling capability to recruit and easily accumulate in the injured region even during the nonpathological period, contributing to chronic cognitive dysfunction.[10,11] Such findings strongly support our new results that pre-existing TBI is a crucial risk factor for lasting PND via the accumulation of MDMs in the hippocampus.

DEX have shown neuroprotective effects in the acute phase of TBI clinically in patients[26] and also in experimental rodent models.[2,17,27,28] Furthermore, several randomized clinical trials have demonstrated that perioperative administration of DEX ameliorated PND in elderly patients with vulnerable cognitive function.[29-31] Although multiple studies have elucidated the benefits of DEX in the acute phase of TBI or the perioperative period, no clinical or basic studies focusing on the effects of DEX on PND in the chronic phase of TBI have been reported. The novelty of the present study was that DEX attenuated PND even when administered within an chronic time window suffering after TBI, because optimal treatment timing restricted to the acute phase may be unfeasible in many clinical settings. Our results could expand the existing neuroprotective indication for DEX, which are currently limited only to the acute phase of TBI, to the chronic phase.

In the present study, we demonstrated that pre-treatment with DEX reduced MCP1 expression in the hippocampus. MCP1 is a potent chemoattractant for circulating blood monocytes via binding to its receptor, CCR2.[32] Previous studies have shown that upregulated MCP1 expression in the hippocampus is associated with infiltration of MDMs in the hippocampus and deteriorated neurological function in both TBI mice[10-12] and PND mice.[13,14] In addition, knock-out of CCR2 to inhibit the infiltration of MDMs in the brain has been reported to prevent neurological deficits.[10,11,13]

Our findings are consistent with previous reports, in which MCP1-CCR2 signaling was strongly associated with accumulation of MDMs and neurocognitive deficits in the hippocampus. However, as yet, little is known regarding the influence of DEX on MCP1 expression on MDMs. DEX was previously reported to be a potent suppressor of MCP-1 in lipopolysaccharide-induced astrocytes, as the main component of glial cells, via the action of α_2-ARs.[33] We therefore investigated the presence of α_2-ARs on MDMs to clarify the interaction between the effect of DEX and MCP-1 expression on MDMs. We demonstrated that all subtypes of α_2-ARs exist on MDMs in the hippocampus of TBI mice. Further, yohimbine, as an α_2-AR antagonist, counteracted the effects of DEX in TBI mice, as indicated by the results of all analyses, including MCP-1 expression. These results suggested that DEX attenuated MCP1 expression on MDMs via α_2-ARs on MDMs in the hippocampus.

In PND, numerous inflammatory factors and immune cells from the peripheral blood infiltrate the brain, causing damage to brain tissue and leading to cognitive deficits.[3,5,34] Previous studies have reported that inflammatory factors, including IL-1β, increase postoperatively in the hippocampus of animals.[10,14,34,35] Furthermore, high concentrations of IL-1β produced by MCP-1-CCR2 signaling in the hippocampus might affect the plasticity of synapses and disrupt long-term potentiation in the hippocampus, resulting in impaired learning and memory.[36] Our results, which are consistent with previous reports, suggested that IL-1β production by MDMs stimulated the development of PND.

Important limitations to the current study need to be recognized. First, we only used male mice. As sex is an important variable for PND,[5,37,38] our findings need to be validated in female mice. Second, we investigated the effect of anesthesia for PND without surgery. The relationships between PND and surgery or anesthetic itself remain unclear.[5] A future study using an anesthesia/surgery model is needed to deepen the knowledge of PND. Third, we used a single volatile anesthetic, isoflurane. Previous studies have reported that volatile anesthetics modulate outcomes.[8,39] Future studies will need to investigate the interaction of anesthetics with TBI. In addition, we did not include mice treated using DEX alone. Further investigation is warranted to clarify the effects of DEX on MDMs and cognitive function. Moreover, the dose of DEX was selected according to previous studies.[17,40] Additional studies examining the optimal dose and safety of DEX therapy are warranted. Last, we did not investigate the polarization of MDMs. Some researchers have pointed out that DEX may modulate directly microglial polarization via α_2-ARs on the surface of MDMs. [15,34] Future studies investigating the effects of DEX on polarization of MDMs could offer insights into the precise mechanisms.

In conclusion, these results reveal that isoflurane exposure itself leads to PND through infiltration of MDMs in the hippocampus via MCP-1-CCR2 signaling with pre-existing TBI. In the presence of a pre-existing vulnerability, anesthetic itself detrimentally affects perioperative cognitive ability. Notably, pre-treatment with DEX suppressed infiltration of MDMs in the hippocampus and ameliorated PND in TBI mice within an chronic time window from brain injury experiences.

The present results significantly extend the accumulated knowledge on how pre-anesthetic

treatment with DEX and MDMs could provide novel targets for new therapeutics for PND with pre-existing vulnerability. We hope that these findings will accelerate translational research to improve perioperative outcomes for patients with a history of TBI.

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Figure legends

Figure 1. Schematic illustration of the experimental design of the study. A) The study design included BMT, induction of TBI, administration of GA, behavioral tests, IHC, and IB. B) Experimental protocol for anesthetic pretreatment with DEX. BMT, bone marrow transplantation; DEX, dexmedetomidine; GA, general anesthesia; IB, immunoblotting; IHC,

immunohistochemistry; TBI, traumatic brain injury; YOH, yohimbine.

Figure 2. Pre-treatment with DEX ameliorated spatial memory impairment after GA in a mouse model of TBI. Specifics of each sample are as followed: TBI mice, n=8; TBI-DEX mice, n=9; TBI-DEX-YOH mice, n=12. A) The averages of primary latency, primary path length, and primary errors for all trials performed on each day were analyzed. B) TBI-DEX mice show improved primary latency than TBI mice on experimental day 3. Data are expressed as means ± SD. ***$P < .0001$ vs. TBI mice on experimental day 3. TBI-DEX-YOH mice show a longer primary latency than TBI-DEX mice on experimental day 3.$^{\dagger\dagger\dagger}P < .0001$ vs. TBI-DEX mice on day 3. (C) TBI-DEX mice show amelioration of primary path length than TBI mice on experimental day 3. Data are expressed as means ± SD. **P=.0025 vs. TBI on experimental day 3. TBI-DEX-YOH mice show longer primary path length than TBI-DEX mice on experimental day 3. $^{\dagger\dagger\dagger}P = .0002$ vs. TBI-DEX mice on day 3. (D) TBI-DEX mice show amelioration of primary errors than TBI mice on experimental day 3. Data are expressed as means ± SD. **$P = .0052$ vs. TBI. TBI-DEX-YOH mice show more primary errors than TBI-DEX mice on experimental day 3. $^{\dagger\dagger}P = .0005$ vs. TBI-DEX mice on day 3. DEX, dexmedetomidine; GA, general anesthesia; SD, standard deviation; TBI, traumatic brain injury; YOH, yohimbine.

Figure 3. Pre-treatment with DEX suppresses the accumulation of MDMs in the hippocampus. A) Overlap of GFP-positive cells (green) and Iba-1 positive areas (red). Scale bars, 20 μm (magnified images). B) Immunofluorescence confocal microscopy for GFP-positive cells stained with Iba-1 in the ipsilateral hippocampus. Scale bars, 100 μm (magnified images). C) Expression of GFP-positive areas in the hippocampus in the five groups. Data are expressed as mean ± SD (n = 5 mice/group). **$P = .017$ vs. Naïve mice in the ipsilateral hippocampus. $^{\dagger\dagger\dagger}P = .0001$ vs. TBI-DEX mice in the ipsilateral hippocampus. *$P = .012$ vs. Naïve mice in the contralateral hippocampus. $^{\dagger\dagger}P = .0009$ vs. TBI-DEX mice in the contralateral hippocampus. D) Expression of Iba-1 positive areas in the hippocampus in the five groups. Data are expressed as mean ± SD (n = 4 mice/group). E) Immunofluorescence confocal microscopy for GFAP-positive cells in the hippocampus. Scale bars, 100 μm (magnified images). F) Expression of GFAP-positive areas in the hippocampus in the five groups. Data are expressed as mean ± SD (n = 4 mice/group). DAPI, 4',6-diamidino-2-phenylindole dihydrochloride solution; DEX, dexmedetomidine; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; SD, standard deviation; TBI, traumatic brain injury; YOH, yohimbine.

Figure 4. Expression of MCP-1 on MDMs in the hippocampus. A) Immunofluorescence confocal microscopy for GFP-positive cells stained with MCP1 in the hippocampus. Scale bars, 100 μm (magnified images). B) Overlap of GFP-positive cells (green) with MCP1 (red). Scale bars, 20 μm (magnified images). C) Expression of MCP1-positive areas in the hippocampus in the five groups.

Data are expressed as means ± SD (n = 4 or 5 mice/group). $^{**}P = .006$ vs. Naïve mice. $^{\dagger}P = .017$ vs. TBI-DEX mice. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride solution; DEX, dexmedetomidine; GFP, green fluorescent protein; MCP-1, monocyte chemotactic protein-1; SD, standard deviation; TBI, traumatic brain injury; YOH, yohimbine.

Figure 5. Presence and expression of α2-ARs and IL-1β in the hippocampus of TBI mice. A) Representative images of immunofluorescence staining for α_2A-AR, α_2B-AR and α_2C-AR. Scale bars, 20 μm (magnified images). B) Immunoblot of IL-1β and GAPDH in ipsilateral hippocampal homogenates. C) Quantification of data in C. Data are expressed as means ± SD (n = 7 mice/group). $^{*}P = 0.035$ vs. Naïve mice. $^{\dagger}P = 0.016$ vs. TBI-DEX mice. AR, antibody; DEX dexmedetomidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IL-1β, interleukin 1β; SD, standard deviation; TBI, traumatic brain injury; YOH, yohimbine.

Figure S1. Pre-treatment with DEX ameliorated spatial memory impairment after GA in a mouse model of TBI. Specifics of each sample are as followed: Transplant mice, n=12; Naïve mice, n=8; Sham mice, n=12; TBI mice, n=8; TBI-DEX mice, n=9; TBI-DEX-YOH mice, n=12). A) The averages of primary latency, primary path length, and primary errors for all trials performed on each day were analyzed. B) TBI and TBI-DEX-YOH mice show a longer primary latency than Naïve mice on experimental day 3. Data are expressed as means ± SD. $^{***}P < 0.001$ vs. Naïve mice on day 3. $^{\dagger\dagger\dagger}P < 0.001$ vs. Naïve mice on day 3. (C) TBI and TBI-DEX-YOH mice show longer primary path length than Naïve mice on experimental day 3. Data are expressed as means ± SD. $^{***}P < 0.001$ vs. Naïve mice on day 3. $^{\dagger\dagger\dagger}P < 0.001$ vs. Naïve mice on day 3. (D) TBI and TBI-DEX-YOH mice show more primary errors than Naïve mice on experimental day 3. Data are expressed as means ± SD. $^{*}P = 0.017$ vs. Naïve mice on day 3. $^{\dagger\dagger}P = 0.003$ vs. Naïve mice on day 3. DEX, dexmedetomidine; GA, general anesthesia; SD, standard deviation; TBI, traumatic brain injury; YOH, yohimbine.