Impact of Preoperative Environmental Enrichment on Prevention of Development of Cognitive Impairment following Abdominal Surgery in a Rat Model

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ABSTRACT

Background: Sustained neuroinflammation may contribute to the pathogenesis of postoperative cognitive dysfunction (POCD). Here, the authors evaluated the preventive effect of preoperative environmental enrichment (PEE) on the development of neuroinflammation and concomitant POCD in a rat abdominal surgery model.

Methods: Young and aged rats were assigned to one of four groups using a 2 × 2 experimental design: PEE *versus* sedentary condition for 14 days, by abdominal surgery *versus* anesthesia alone (n = 8 in each group). After a 7-day postsurgical recovery period, cognitive function was assessed using a novel object recognition test, followed by measurement of hippocampal levels of proinflammatory cytokines. Under identical conditions, microglia were isolated from the hippocampus for assessment of cytokine response to lipopolysaccharide.

Results: In the sedentary group, aged, but not young, rats receiving surgery showed memory deficits (novel object preference during testing phase of 54.6 ± 7.8% *vs.* 76.9 ± 11.3% in nonsurgery group, $P < 0.05$) and increased hippocampal levels of cytokines compared with nonsurgical rats. PEE had no effects on novel object preference in nonsurgery animals (78.6 ± 10.7%), whereas it attenuated surgery-induced impairment of novel object preference (70.9 ± 15.0%, $P < 0.05$ *vs.* sedentary/surgery group) as well as increase of cytokine levels in hippocampus. Furthermore, upon *ex vivo* stimulation with lipopolysaccharide, cytokines release from hippocampal microglia isolated from aged rats before intervention was significantly higher in comparison with young rats. PEE resulted in reduction of these age-related microglial phenotypic changes.

Conclusions: PEE could prevent the development of neuroinflammation and related POCD in aged rats by reversion of a proinflammatory phenotype of hippocampal microglia. **(Anesthesiology 2015; 123:160-70)**

POSTOPERATIVE cognitive dysfunction (POCD) is one of the common complications in geriatric patients.[1–3] POCD has been shown to be associated with long-term disability, higher healthcare costs, and even increased mortality,[4] whereas its specific underlying mechanisms still remain largely unknown. More recently, several studies have demonstrated that neuroinflammation in the hippocampus, a region important to cognition and highly vulnerable to aging, is most likely to be involved in the pathogenesis of POCD.[5–8] Furthermore, preclinical evidence has shown that microglia in a normal aged brain are shifted toward the inflammatory phenotype, known as "microglial priming."[9,10] Primed microglia can trigger an exaggerated release of proinflammatory cytokines, which contribute to prolonged neuroinflammation following a peripheral immune challenge. In particular, proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), released from microglia within the hippocampus

What We Already Know about This Topic

- Anesthesia and surgery are associated with postoperative cognitive dysfunction (POCD), especially in aged subjects.
- Environmental enrichment has been shown to attenuate the adverse effects of anesthetics in the developing brain. Whether such enrichment mitigates POCD in aged subjects is not known.
- The impact of preoperative environmental enrichment on POCD was investigated in young and aged rats subjected to anesthesia and laparotomy.

What This Article Tells Us That Is New

- Anesthesia and surgery were associated with memory deficits, microglial activation, and elaboration of inflammatory cytokines in aged, but not young, animals.
- Preoperative environmental enrichment attenuated cognitive deficits and cytokine production in the brain.
- The data suggest that preoperative environmental enrichment can mitigate the adverse effects of anesthesia and surgery on postoperative cognitive function.

Corresponding article on page 7. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

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are well characterized to play a pathogenic role in cognitive disorders in neurodegenerative diseases.[6,11,12] Based on these findings, we hypothesized that age-related microglial priming in the hippocampus plays a critical role in the development of POCD in the elderly population.

There is considerable evidence that cognitive interventions, such as physical activity (PA) and cognitive activity (CA), have positive effects on age-related cognitive changes as well as early-stage dementia in humans.[13–15] In addition, animal models mimicking these interventions, in which rodents were exposed to enriched environment, showed improvement in cognitive performance.[16,17] Although the mechanism of these benefits has been debated, both interventions are reported to have common positive effects on microglial number, proliferation, and phenotype in the brain.[18–20] Furthermore, the effectiveness of PA or CA intervention alone is limited, whereas combined intervention showed greater improvement of memory performance in rats.[21] Therefore, we further hypothesized that preoperative environmental enrichment (PEE), a housing of animals in an enriched environment including a combination of PA and CA task, could prevent the development of POCD *via* restoration of the proinflammatory phenotype in aged microglia.

We tested these hypotheses by first investigating, in young and aged rats, the effects of PEE on the development of POCD using a novel object recognition task after abdominal surgery (laparotomy and small intestinal manipulation). Furthermore, in order to determine the contribution of microglial phenotype change in the hippocampus, we next examined the effects of PEE on microglia responses to a proinflammatory stimulus in *ex vivo* preparations.

Materials and Methods

Animals and Experimental Group

All procedures performed on animals were approved by the Institutional Animal Care and Use Committee of the Kochi Medical School, Kochi, Japan.

Aged (24 to 25 months) male Wistar rats were used in two sets of experiments according to the schema presented in figure 1. Experiment 1 was designed for assessment of *in vivo* cognition and measurement of hippocampal cytokines, and rats were randomly assigned to one of four groups using a 2×2 experimental design: PEE *versus* sedentary condition, by abdominal surgery *versus* anesthesia and analgesia alone; each group consisted of eight animals. In experiment 2, microglia in the hippocampus were isolated for *ex vivo* analysis at baseline, after PEE or sedentary condition, and after abdominal surgery following PEE or sedentary condition. Five rats were used per group at each time point. Throughout the experiment, all rats were housed in pairs with a 12-h light/dark cycle, and food and water were provided *ad libitum*.

To test whether these experimental models are specific for aged animals, we further replicated the same experiment using young rats (2 to 3 months).

Open-field Test

After the random distribution of the groups, all rats were screened for spontaneous locomotor activity, anxiety, and

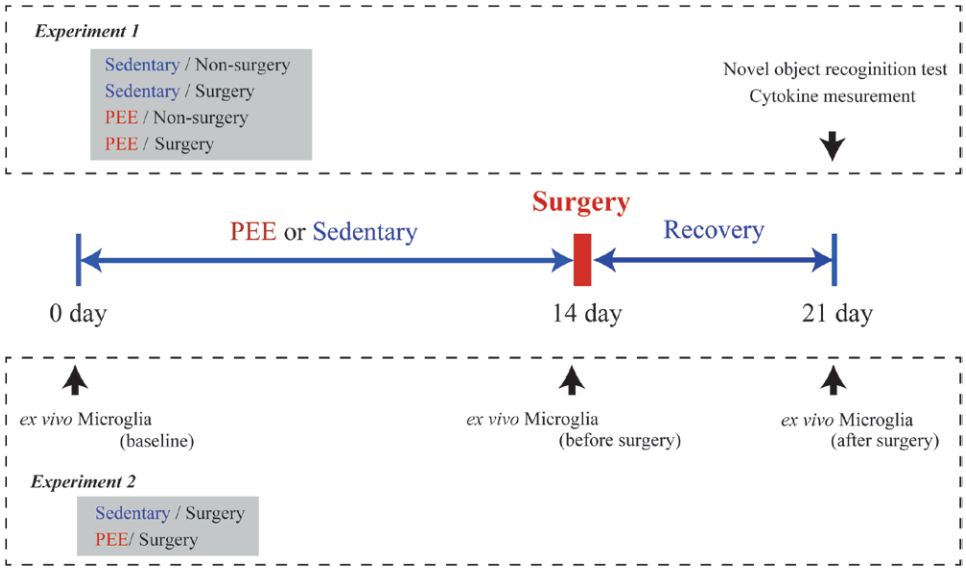

Fig. 1. Experimental protocol and time course. All rats were exposed to preoperative environmental enrichment (PEE) or sedentary condition for 14 days following surgery (laparotomy and small intestinal manipulation) or nonsurgery and allowed 7 days of recovery. Experiment 1: Cognitive testing followed by quantification of hippocampal cytokines 7 days after surgery. Experiment 2: *Ex vivo* measurement of proinflammatory cytokine production from primary microglia isolated from the hippocampus before intervention (baseline), after intervention, and 7 days after surgery. All of these experimental protocols were first carried out in aged rats, followed by young rats.

adaptivity to the given task environment 7 days before the start of experiments by a repeated open-field test according to our previous study.[22] Briefly, rats were exposed to the same transparent Plexiglas open field (base, 45 cm × 45 cm; height, 35 cm) for three consecutive 5-min trials, with an interval of 30 min between each trial. Spontaneous activity was recorded by using an infrared photo-beam detection system (Model LE 8810; Panlab, S.L., Spain). The activity counts at the third exposure were compared with those after the first exposure.

PEE Protocol

The environmental enrichment paradigm used in this study is designed to enhance both CA and PA based on previous reports in rodents.[21] After concluding the baseline open-field tests, the rats in the PEE group were given a 24-h alternating condition of paired housing in modified Hebb–Williams mazes (CA; 100 cm ×100 cm × 30 cm) and individual access to a running wheel (PA; 36-cm diameter) over a period of 14 days. In order to expedite exploration, the pattern of the Hebb–Williams maze was changed randomly during each daily session, and food rewards were scattered throughout the maze. The control rats were confined to a cage with no maze or running wheel.

Anesthesia and Surgery

Anesthesia was induced in an induction chamber flushed with 2 to 3% isoflurane and oxygen at 2 L/min until recumbent, after which the animal was placed on a nose cone connected to a vaporizer to maintain isoflurane (1.5 to 2.0%) in oxygen at 0.5 L/min during the procedure. Isoflurane vaporizer was calibrated with a Riken Fi-21 Gas Indicator (Riken Kiki CO., Japan) before anesthetic procedure. The pedal withdrawal and palpebral reflexes were used as indicators of adequate anesthetic depth. The fur on the surgical site was shaved and then cleaned with chlorhexidine. Surgical group rats underwent a 2-cm midline incision as an abdominal surgery model. During the surgery, approximately 10 cm of the small intestine was exteriorized from the peritoneal cavity, covered with moist gauze, and then manipulated with fingers for 3 min. Afterward, the muscle and skin were repaired separately with 5-0 Vicryl sutures (polyglactin 910; Ethicon, Inc., U.S.A.). Tissue adhesive glue was also used to ensure closure of the skin incision. The wound infiltration with 0.2% ropivacaine (300 μl) was used for postoperative analgesia, since we previously confirm its effectiveness for the postlaparotomy pain in rats without affecting cognition.[22] The precise surgery duration was fixed at 10 min for each procedure. Body temperature was monitored by a rectal probe and maintained at 36.9° ± 0.4°C by a heating lamp throughout the entire procedure. Nonsurgical control rats were only anesthetized, shaved, and given analgesia (ropivacaine) in the same manner as experimental rats. Mean arterial pressure was measured by tail-cuff plethysmography (BP-98A; Softron, Japan), and arterial oxygen saturation and pulse rate were measured noninvasively by using the MouseOX Plus (Starr LifeSciences Corp., USA), during isoflurane anesthesia. Following the completion of surgery or the sham procedure, the rats recovered in an incubator, maintained at 35°C for 30 min. They were then returned to their home cages. After a 7-day postsurgical recovery period, cognitive function was assessed using a novel object recognition test.

Novel Object Recognition Task

Hippocampus-dependent cognitive function was assessed by a novel object recognition test that has been previously described and modified.[23] Briefly, each rat was individually habituated to the test chamber in the absence of objects for 5 min on three consecutive days (from postoperative day 4 to 6). Lack of innate side preference was confirmed during this period. The experimental apparatus consisted of a Plexiglas open-field box with an open top and was cleaned with 70% ethanol and water between subjects. Two different types of objects used in the current study were 10 cm in diameter × 7 cm high blue plastic bowls and 10 cm red plastic flower-shaped objects. The objects were secured to the floor to prevent rats from displacing them. In pilot studies, both young (n = 5) and aged (n = 5) rats could easily discriminate the objects and displayed no preferences when two different objects were presented in the open field. On the day of testing, each rat was allowed to freely explore the open-field arena containing two identical objects (either bowls or flower objects) for 5 min (familiarization phase). After a 1-h retention interval, the rat was returned to the experimental chamber with a new set of objects containing one identical and one novel object (bowl or flower object). The rat was again allowed to explore the objects for 5 min (testing phase). Which object was novel and the left/right position of the novel object was counterbalanced in order to prevent potential bias due to preferences for particular locations or objects.

All testing was conducted during the dark phase of the light/dark cycle in a dimly lit room and videorecorded. The animal's behavior was monitored by an overhead video camera (HandyCam HDR-CX560, Sony, Japan) connected to a computer equipped with Ethovision tracking software (Noldus Information Technology, The Netherlands), and object interaction was scored manually by an experimenter blinded to the study group. Object exploration was defined as time spent sniffing the object when the rat's nose was in contact with the object and/or within 1 cm from the object. Recognition memory was expressed as a novel object preference ratio that was calculated as the ratio of time spent exploring either of the two objects during the familiarization phase or the novel object during the test phase over the total time spent exploring both objects. After the completion of the cognitive testing, all rats were sacrificed by cervical decapitation under terminal anesthesia with pentobarbital (80 mg/kg body weight, intraperitoneal) and then exsanguinated by transcardiac perfusion with ice-cold standard phosphate-buffered

saline. The hippocampus was quickly dissected and was homogenized with a polytron homogenizer (Kinematica Inc., Switzerland) in ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, pH = 7.4) containing protease inhibitors cocktail (P8340, Sigma-Aldrich, USA). The homogenates were centrifuged (11,000*g*, 20 min, 4°C), and the supernatants were aliquoted and frozen at −80°C until required for enzyme-linked immunosorbent assay (ELISA).

Acute Isolation of Microglia from Hippocampus

In another experiment, under identical experimental conditions (experiment 2), microglia were acutely isolated from the hippocampus as previously described with some modification.[24] Briefly, rats were exsanguinated by transcardiac perfusion with ice-cold standard phosphate-buffered saline following terminal anesthesia with pentobarbital. Whole hippocampi were rapidly harvested in Dulbecco's Modified Eagle's medium (DMEM) containing 4.5 g glucose and 25 mM HEPES with 10% fetal bovine serum. The brain samples were minced into pieces with a razor blade and digested with 0.1% trypsin and Dispase II (3.6 U/ml; Roche, BD Bioscience, USA) for 1 h at 37°C with shaking (100 strokes/min). Resulting homogenates were centrifuged at 600*g* for 10 min at 4°C. Supernatants were removed and cell pellets were resuspended in 4 ml of 70% isotonic Percoll and overlaid with equal volumes of 37% and 30% isotonic Percoll. The gradient was centrifuged at 2,000*g* for 20 min. Microglia were collected from the interphase between the 70% and 37% Percoll layers and resuspended in DMEM culture media containing 10% fetal bovine serum. Purity of cultures was greater than 95% as verified by immunocytochemistry using antibodies to CD68 to identify microglia. Microglia cells were plated at a density of 10^4 cells/100 μl in a 24-well dish in DMEM containing 10% fetal bovine serum. Before cell treatment, medium was replaced with fresh serum-free medium followed by stimulation with lipopolysaccharide at a concentration of 0.1, 1, 10, or 100 ng/ml or media alone for 24 h at 37°C, 5% CO_2. At the end of the incubation, the medium was collected and stored at −20°C for subsequent analysis by ELISA.

Enzyme-linked Immunosorbent Assay

Commercially available ELISA kits for measuring rat IL-1β (ER2IL1B, Thermo Scientific, USA) and TNF-α (438207, Biolegend, USA) were used according to the manufacturers' instructions. Absorbance was read using an ELISA microplate reader (ThermoMax; Molecular Devices, USA). The interassay coefficients of variation for IL-1β and TNF-α were 7.6% and 7.9%, respectively, and the intraassay coefficients of variation for IL-1β and TNF-α were 3.3% and 4.1%, respectively.

Statistical Analysis

Since these behavioral experiments were an exploratory investigation, we did not conduct an *a priori* sample size calculation. However, we cannot rule out that the small sample size in these behavioral studies introduced a positive bias. Therefore, we replicated the novel object recognition testing under the same conditions.

All data were expressed as the mean ± SD. Differences between the study groups were compared with the Kruskal–Wallis test and differences between individual groups with the Wilcoxon–Mann–Whitney test with Bonferroni correction. Correlations between variables were analyzed by Spearman correlation test. Difference between age groups and dose–response effects were evaluated using a two-way ANOVA to identify main effects and if necessary were followed by pairwise comparison using Bonferroni test. All data were analyzed using the statistical software SAS (version 9.3; SAS Institute Inc., USA) and SPSS (version 11; SPSS Inc., USA). *P* value less than 0.05 was considered statistically significant.

Results

Before the beginning of the experiments, all animals were subjected to a repeated open-field test to screen for baseline locomotor, anxiety, and adaptive function. With exposure to the same open field for three repeated trials, similar levels of habituation were shown in all experimental groups; the locomotor activity at the third trial was decreased to 37 to 49% of that at the first trial (data not shown). Furthermore, there were no differences in total locomotor counts during trials among the groups. These findings indicated that baseline locomotor, anxiety, and adaptive function among groups may be comparable. In addition, no differences in arterial oxygen saturation level, pulse rate, and body temperature were observed among groups during the period of anesthesia (table 1), and all rats recovered from anesthesia and surgery uneventfully.

Novel Object Recognition Performance

Seven days after surgery, the effects of PEE on hippocampal-mediated working memory was assessed by a novel object recognition task. During the training phase, there was no biased exploratory preference for either one of the two objects among all groups in both young (fig. 2A; $P = 0.28$) and aged (fig. 2B; $P = 0.37$) rats. In addition, total exploration time during the training phase did not differ within each age group, implying that task motivation and ability during testing were comparable within each age group (table 2; young, $P = 0.93$; aged, $P = 0.91$). The total exploration times in young group tended to be shorter than those in aged group, but the difference was not of statistical significance in two-way ANOVA (main effect for group, $F_{(1, 56)} = 2.70$; $P = 0.11$).

During the testing phase, in the young and aged groups, the sedentary/nonsurgical rats spent more time exploring the novel object than the familiar object (novel object preference of 80.9 ± 8.7% in young group and 76.9 ± 11.3% in aged group). These results indicate the successful recognition

Table 1. Physiological Parameters during Isoflurane Anesthesia

	Mean Arterial Pressure (mmHg)		Pulse Rate (beats/min)		Oxygen Saturation (%)		Body Temperature (°C)	
Group	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
Young								
Sedentary/nonsurgery	103.8 ± 10.5	101.7 ± 11.7	385.8 ± 22.1	375.3 ± 30.0	98.8 ± 1.7	98.6 ± 1.3	37.0 ± 0.3	36.9 ± 0.6
Sedentary/laparotomy	99.8 ± 12.5	98.8 ± 8.1	370.6 ± 24.1	384.1 ± 32.1	98.3 ± 1.6	98.5 ± 1.2	36.9 ± 0.3	36.9 ± 0.3
PEE/nonsurgery	104.5 ± 11.4	104.3 ± 9.1	378.1 ± 28.0	380.9 ± 28.6	98.9 ± 1.5	98.1 ± 1.5	36.8 ± 0.3	36.8 ± 0.3
PEE/laparotomy	101.6 ± 9.9	99.7 ± 9.0	382.4 ± 30.9	375.1 ± 34.8	99.0 ± 1.1	97.6 ± 1.8	36.8 ± 0.2	36.7 ± 0.4
Aged								
Sedentary/nonsurgery	106.0 ± 13.9	103.1 ± 11.8	377.6 ± 34.9	372.8 ± 15.6	98.3 ± 1.9	98.3 ± 1.4	36.8 ± 0.4	36.8 ± 0.4
Sedentary/laparotomy	101.1 ± 11.4	99.6 ± 13.1	376.4 ± 21.3	382.4 ± 24.1	99.1 ± 1.4	98.5 ± 1.4	36.8 ± 0.3	36.9 ± 0.3
PEE/nonsurgery	103.9 ± 9.0	104.3 ± 7.9	368.1 ± 34.8	377.0 ± 20.2	98.1 ± 1.8	98.1 ± 1.9	36.9 ± 0.3	37.0 ± 0.3
PEE/laparotomy	104.4 ± 8.8	102.0 ± 8.3	391.4 ± 43.4	368.3 ± 33.8	98.4 ± 1.5	98.6 ± 1.4	36.8 ± 0.5	36.9 ± 0.4

Each parameter was recorded at time 1—after induction of anesthesia, before procedure and at time 2—immediately after procedure, before termination of anesthesia. Data were expressed as the mean ± SD. Each group consisted of eight animals.

PEE = preoperative environmental enrichment.

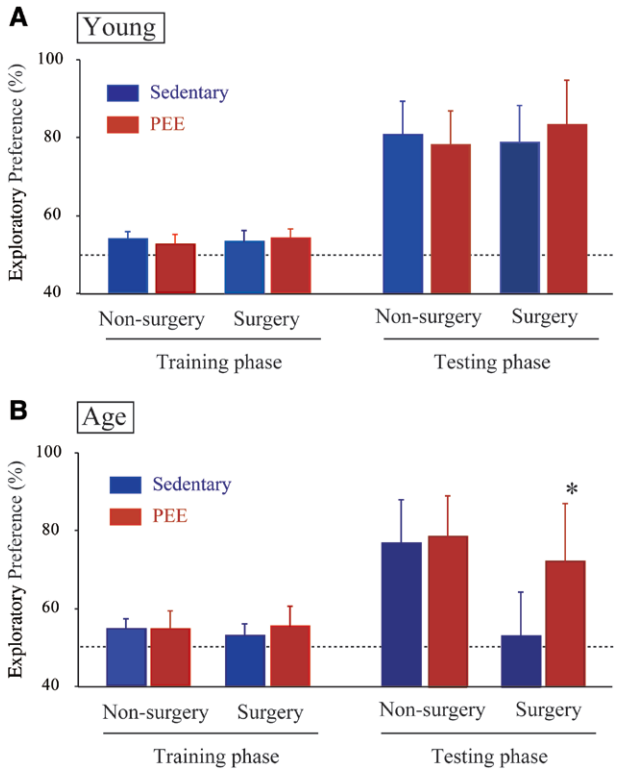

Fig. 2. Effects of preoperative environmental enrichment (PEE) on cognitive function assessed by a novel object recognition test in young (*A*) and aged (*B*) rats. Percentage of preference between two objects in the training phase and testing phase of the novel object recognition test performed 7 days after nonsurgery or surgery in sedentary or PEE rats is shown. Each *vertical bar* represents the mean ± SD (n = 8 in each group). *$P < 0.05$ *versus* sedentary/surgery group, Kruskal–Wallis ANOVA followed by Wilcoxon–Mann–Whitney test with Bonferroni correction.

memory comparably in young and aged rats ($P = 0.94$). In the young rats, neither PEE nor surgical intervention showed any significant influence on novel object recognition

Table 2. Total Exploration Time during the Training Phase of Novel Object Recognition Test

	Sedentary		PEE	
	Nonsurgery	Surgery	Nonsurgery	Surgery
Young	50.3 ± 12.1	46.0 ± 14.6	50.8 ± 9.9	52.9 ± 7.5
Aged	43.4 ± 10.9	40.9 ± 10.4	41.8 ± 10.4	42.1 ± 7.1

Total time spent exploring the two objects in each group is expressed as mean ± SD in seconds. Each group consisted of eight animals.

PEE = preoperative environmental enrichment.

($P = 0.77$). On the other hand, the sedentary/surgical rats in the aged group exhibited significantly impaired novel object recognition performance (novel object preference of 54.6 ± 7.8%, $P < 0.05$ *vs.* sedentary/nonsurgery group). However, such impairment was not observed in the PEE/surgery group (novel object preference of 70.9 ± 15.0% *vs.* 78.6 ± 10.7% in PEE/nonsurgery group; $P = 0.33$). More importantly, the novel object preference in the PEE/surgery group was significantly higher than that in the sedentary/surgery group ($P < 0.05$).

Identical findings were obtained in replicated studies (see Supplemental Digital Content 1, http://links.lww.com/ALN/B148). This may strengthen the reliability of the original results.

Levels of Hippocampal Cytokines after Recognition Memory Testing

Following behavioral testing, the levels of TNF-α and IL-1β in the hippocampus were measured. For the young rats, the average levels of hippocampal TNF-α and IL-1β were comparable among all groups (fig. 3A, $P = 0.81$ and fig. 3B, $P = 0.73$). In addition, both levels of TNF-α and IL-1β in nonsurgery group were comparable between young and aged rats. On the other hand, for the aged rats with preoperative sedentary condition, both cytokines were significantly higher in the surgery group than in the nonsurgery group

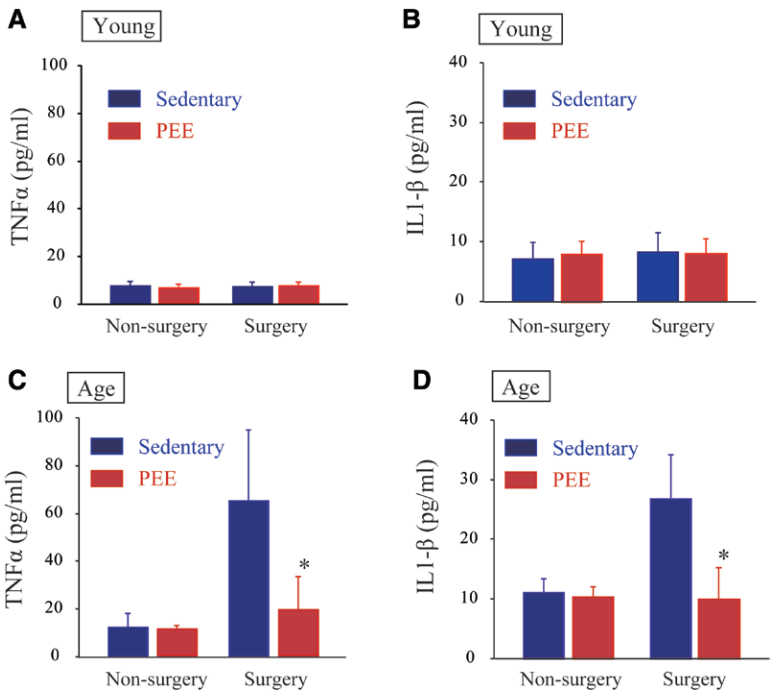

Fig. 3. The levels of proinflammatory cytokines in the hippocampus. The average levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in young (*A* and *B*) and aged (*C* and *D*) rats in each group are shown. Each *vertical bar* represents the mean ± SD (n = 8 in each group). *$P < 0.05$ *versus* sedentary/surgery group, Kruskal–Wallis ANOVA followed by Wilcoxon–Mann–Whitney test with Bonferroni correction. PEE = preoperative environmental enrichment.

(fig. 3C, $P < 0.01$ and fig. 3D, $P < 0.01$). However, these increases in cytokines after surgery were not found in the aged rats subjected to PEE (fig. 3C, $P = 0.49$ and fig. 3D, $P = 0.51$ *vs.* PEE/nonsurgery group). In addition, plasma levels of both cytokines in either young or aged rats were comparable among the groups (data not shown), suggesting that increased inflammatory cytokines in an aged hippocampus 7 days after surgery may not be associated with exaggerated inflammatory response in the periphery.

Taking all the aged rat groups together, novel object recognition performance in the testing phase was inversely correlated with the hippocampal levels of both TNF-α (fig. 4A, n = 32; $R^2 = -0.849$; $P < 0.01$) and IL-1β (fig. 4B, n = 32; $R^2 = -0.792$; $P < 0.01$). This relationship suggests that neuroinflammation in the hippocampus may play a pivotal role in cognitive deficits after surgery in aged rats.

Ex Vivo *Immunosensitivity of Hippocampal Microglia for Lipopolysaccharide*

Lipopolysaccharide has been shown to stimulate cytokine production from rodent and human microglia. Therefore, we next examined TNF-α and IL-1β release of microglia isolated from aged hippocampi compared with young hippocampi following stimulation with different concentrations of lipopolysaccharide (0 to 100 ng/ml). As shown in figure 5A, the lipopolysaccharide-induced increase in TNF-α was greater in the microglia of aged rats than young rats (main effect for group, $F_{(1, 40)} = 336.17$; $P < 0.01$). The increase in IL-1β caused by lipopolysaccharide was also greater in the microglia of aged rats (fig. 5B; main effect for group, $F_{(1, 40)} = 27.68$; $P < 0.01$). These results indicate, consistent with other reports, that normal aging may prime microglia for an exaggerated responsiveness to proinflammatory stimuli.

To determine the time course of cytokine secretion of the microglia isolated from young and aged microglia, the levels of TNF-α and IL-1β were measured 12, 24, and 48 h after addition of lipopolysaccharide (10 ng/ml). At each time point postinjection, both TNF-α and IL-1β were higher in the microglia of aged hippocampi compared with young hippocampi at each time point (fig. 5, C and D). These results demonstrate that the differences in hippocampal cytokine levels after exposure to lipopolysaccharide were dependent on age, but not the timing of sample collection.

Effects of PEE on Age-related Changes in Microglial Sensitivity to Lipopolysaccharide

To investigate whether PEE could influence microglial immunosensitivity, we measured the lipopolysaccharide sensitivity of hippocampal microglia isolated from sedentary or PEE rats both after PEE and surgery (fig. 1). In all experimental conditions, there was no difference between PEE and sedentary rats in basal levels of TNF-α and IL-1β. In young microglia, the lipopolysaccharide-induced increase in TNF-α was comparable between the sedentary and PEE groups after the intervention period (fig. 6A; main effect for group, $F_{(1, 40)} = 1.50$; $P = 0.23$) as well as after surgery (fig. 6B; main effect for group, $F_{(1, 40)} = 1.81$; $P = 0.19$). On the other hand, in aged microglia, the lipopolysaccharide-induced

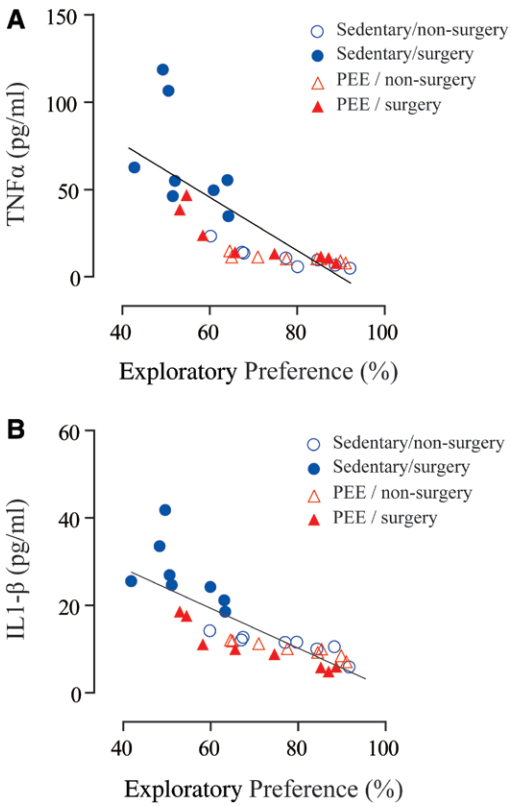

Fig. 4. The relationship between the levels of proinflammatory cytokines and cognitive function after surgery. Correlation of the levels of either tumor necrosis factor (TNF)-α (*A*) or interleukin (IL)-1β (*B*) in the hippocampus of aged rats with each donor rat's novel object recognition performance at the testing phase, showing an inverse relationship. Data points are classified into four experimental groups (n = 8 in each group). PEE = preoperative environmental enrichment.

increase in TNF-α was markedly decreased in the PEE group compared with that of the sedentary group (fig. 6C; main effect for group, $F_{(1, 40)} = 54.84$; $P < 0.01$). This reduction of immunosensitivity after PEE was also observed after surgery (fig. 6D; main effect for group, $F_{(1, 40)} = 40.04$; $P < 0.01$). With respect to IL-1β levels, a two-way ANOVA revealed similar trends of main effects of group to those observed for TNF-α in both young (fig. 7A; main effect for group, $F_{(1, 40)} = 0.01$; $P = 0.91$; fig. 7B; main effect for group, $F_{(1, 40)} = 0.77$; $P = 0.39$) and aged (fig. 7C; main effect for group, $F_{(1, 40)} = 25.23$; $P < 0.01$; fig. 7D; main effect for group, $F_{(1, 40)} = 31.26$; $P < 0.01$) microglia.

Discussion

In an *in vivo* setting, our results demonstrated that the aged, but not young, rats in the sedentary group developed cognitive impairment and an increase in hippocampal proinflammatory cytokines following abdominal surgery. Notably, it could be avoided by PEE for 14 days before surgery. In addition, the results in the *ex vivo* experiment showed that subsequent to peripheral immune stimulation with lipopolysaccharide, the release of proinflammatory cytokines was higher in microglia isolated from aged hippocampi compared with young hippocampi. However, PEE could reduce this exaggerated cytokine response. These findings, taken together, imply that PEE could prevent the development of POCD *via* reversion of the age-related proinflammatory phenotype of microglia in the hippocampus.

Peripheral innate immune activation has been shown to activate microglia within the central nervous system, resulting in developing various forms of cognitive impairment, including POCD.[5–8,25,26] As the primary source of proinflammatory cytokines, activated microglia are key mediators of neuroinflammation.[9,10] Previous preclinical data have shown that several proinflammatory cytokines, especially TNF-α and IL-1β, inhibit the hippocampal long-term potentiation, a synaptic mechanism of learning and memory formation.[11,12] Furthermore, these cytokines can decrease the hippocampal brain-derived neurotrophic factor levels, leading to reduced neural plasticity and subsequent cognitive impairment.[8] In the current study, our results further demonstrate that cognitive performance 7 days after surgery was inversely correlated with the levels of both TNF-α and IL-1β in the hippocampus (fig. 4). These findings affirm that surgery-induced neuroinflammation characterized by increased proinflammatory cytokines derived from activated microglia is one of the crucial mechanisms of the development of POCD. Therefore, strategies aimed at inhibiting microglial activation as well as the inflammatory processes secondary to tissue injury could represent a therapeutic target for POCD.

Although several risk factors have been identified, advanced age has been most consistently associated with the pathogenesis of POCD.[1–3] In agreement with these observations, our *in vivo* experiments showed that surgery-induced hippocampal neuroinflammation and related memory defects were observed in aged rats but not in young rats (figs. 2 and 3). Furthermore, aged microglia, compared with those that are young, isolated from the hippocampus showed a greater production of proinflammatory cytokines in response to immune stimulation with lipopolysaccharide inflammatory challenge (fig. 5). This age-related phenotype change is consistent with the phenomenon known as "microglial priming."[9,10] Emerging evidence suggests that the priming of microglia is implicated in the cognitive decline associated with aging.[9,10] These findings together imply that age-related microglia priming could make elderly surgical patients more susceptible to the development of POCD.

Numerous clinical observational and preclinical studies have found that PA and CA are both associated with improved cognitive performance and lower future dementia risk.[13–17] Several studies have also shown that behavioral interventions that combine PA and CA may have more global effects than either PA or CA alone.[21,27] To our knowledge, although this is the first study demonstrating the prophylactic efficacy of PEE, a combination of PA and

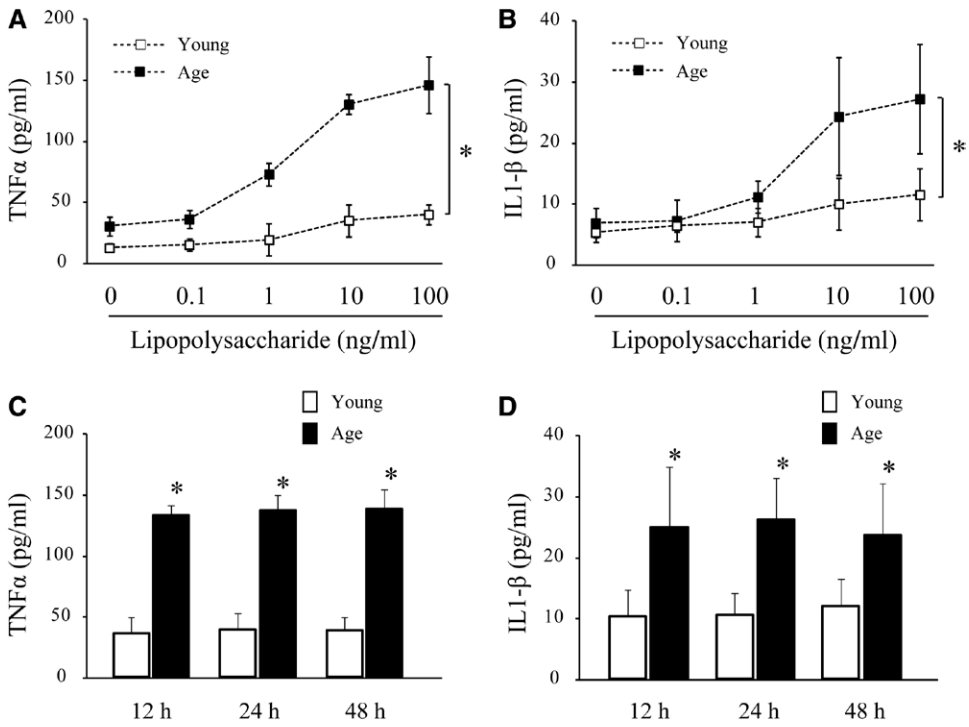

Fig. 5. Effects of *ex vivo* stimulation with lipopolysaccharide on the cultured microglia. Hippocampal microglia were isolated from either young or aged rats before surgery (baseline). Primary microglia were stimulated with 0.1, 1, 10, or 100 ng/ml or media alone and levels of tumor necrosis factor (TNF)-α (*A*) and interleukin (IL)-1β (*B*) were determined from supernatants collected 24 h later. Each *vertical bar* represents the mean ± SD (n = 5 in each group). **P* < 0.05, two-way ANOVA. In separate experiments, microglia were exposed to 10 ng/ml lipopolysaccharide and supernatants were collected at 12, 24, and 48 h after lipopolysaccharide addition and assayed for TNF-α (*C*) and IL-1β (*D*). Each *vertical bar* represents the mean ± SD (n = 8 in each group). **P* < 0.05 *versus* young group, two-way ANOVA followed by Bonferroni posttests.

CA task, on the development of POCD, beneficial effects of PA/CA intervention with enriched environment on cognitive function have been found in a rodent model of vascular dementia, traumatic brain injury, and Alzheimer disease.[28–30] Although the precise underlying mechanisms remain debated, mechanical studies have shown that exposure to enriched environment could produce a broad-spectrum cognitive-enhancing effect against brain aging: that is, enhanced neurogenesis, growth factors, neurotransmitters, and possibly synaptic plasticity.[13–17] In addition, the environmental enrichment have been shown to have an effect on microglial markers, morphology, and proliferation, especially in the hippocampus, which may be associated with cognitive improvement.[18,19,31] Our results further indicated that PEE could attenuate the age-related exaggerated proinflammatory cytokine response of hippocampal microglia (figs. 6 and 7). Therefore, PEE may provide the opportunity to reduce the cognitive vulnerability associated with microglial priming, which could potentially protect elderly patients from the development of POCD.

It has been reported that PA, in and of itself, may have antiinflammatory effects within the aged brain.[32] In this context, early and aggressive mobilization is currently recommended for critical care patients to reduce the incidence and duration of delirium, an acute form of cognitive impairment.[33] However, it might be difficult, especially for elderly patients, to comply with an early rehabilitation program due to postoperative pain or physical and functional restrictions. On the other hand, since most patients scheduled for elective surgery have a waiting time before admission, PEE can be widely applied without any limitations. Our results further demonstrated that PEE induced mitigation of age-related microglial phenotypic changes even 7 days after abdominal surgery. Recent time-course analysis using a rat abdominal surgery model revealed that hippocampal neuroinflammation and related microglial activation were found at 7 days after surgery, which resolved to normal levels by 14 days after surgery.[8] Therefore, the effects of PEE may persist long enough to encompass the critical period of POCD development.

There are some limitations in the current study. First, the behavioral experiments in young and aged groups were conducted with similar protocol but at different times as described in the Materials and Methods, which may lead to an additional bias in the direct comparison between these two groups. Second, in this study, we used isoflurane-anesthetized rats without surgery as control and demonstrated a critical role of surgery-induced neuroinflammation in the development of POCD. However, previous studies also show that isoflurane anesthesia *per se* could alter cognitive

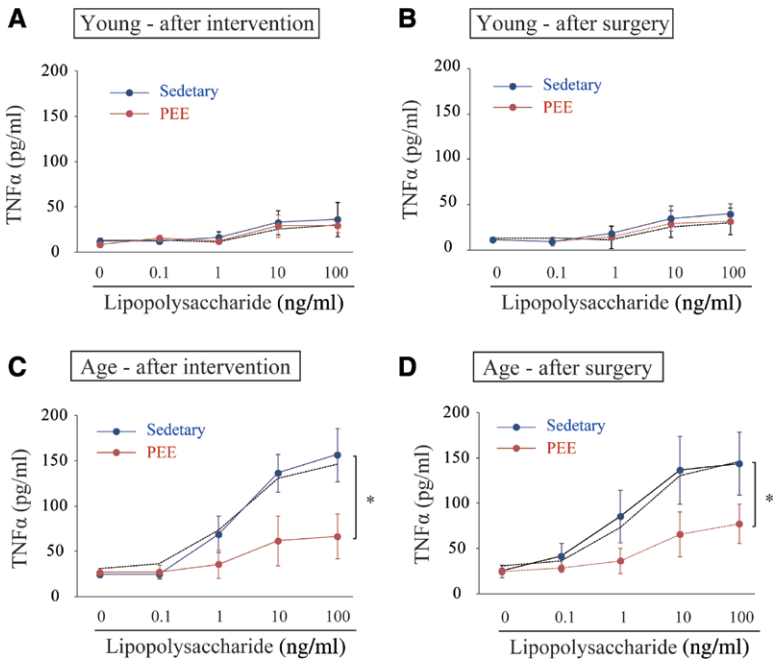

Fig. 6. Concentration–response effects of *ex vivo* stimulation with lipopolysaccharide on the production of tumor necrosis factor (TNF)-α in cultured microglia. Hippocampal microglia were isolated from young (*A* and *B*) and aged (*C* and *D*) rats after either sedentary or preoperative environmental enrichment (PEE) and after surgery. Cultured microglia were stimulated with 0.1, 1, 10, or 100 ng/ml or media alone and levels of TNF-α were determined from supernatants collected 24 h later. Each *vertical bar* represents the mean ± SD (n = 5 in each group). To visually show the changes after intervention or surgery from baseline, the baseline response presented in figure 5A was added to the figure of each corresponding group as a dashed line. **P* < 0.05 *versus* sedentary group, two-way ANOVA.

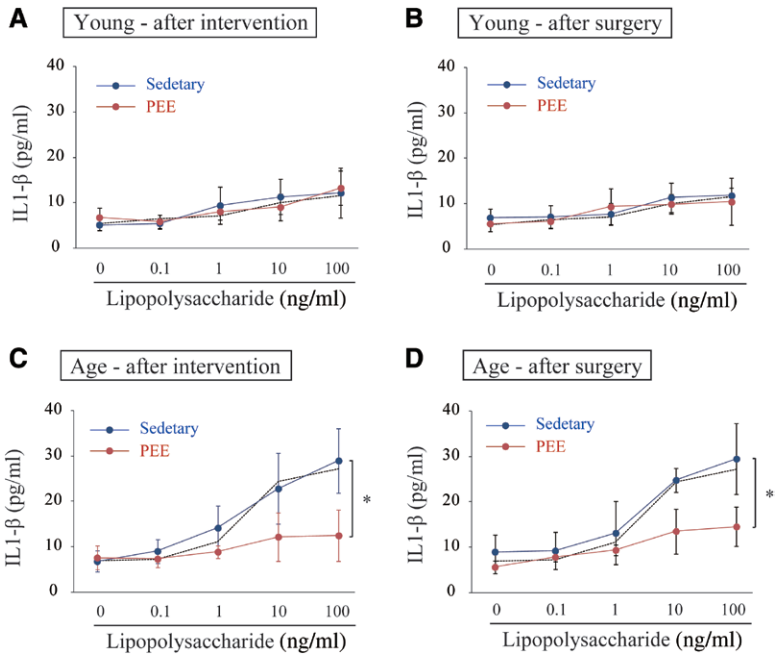

Fig. 7. Concentration–response effects of *ex vivo* stimulation with lipopolysaccharide on the production of interleukin (IL)-1β in cultured microglia. Hippocampal microglia were isolated from young (*A* and *B*) and aged (*C* and *D*) rats after either sedentary or preoperative environmental enrichment (PEE) and after surgery. Cultured microglia were stimulated with 0.1, 1, 10, or 100 ng/ml or media alone and levels of IL-1β were determined from supernatants collected 24 h later. Each *vertical bar* represents the mean ± SD (n = 5 in each group). To visually show the changes after intervention or surgery from baseline, the baseline response presented in figure 5B was added to the figure of each corresponding group as a dashed line. **P* < 0.05 *versus* sedentary group, two-way ANOVA.

function in nonsurgical animals.[34,35] To assess the potential contribution of anesthetics, an absolute control group of animals that receive neither anesthesia nor surgery should be included in the future research. Third, previous studies demonstrate higher baseline levels of TNF-α and IL-1β in the aged when compared with the young brain,[36] whereas this could not be concluded in our experiment. It may be that the sensitivity of ELISA was not enough to detect these differences. Fourth, although not addressed in the current study, morphological and immunophenotypic changes in microglia in the hippocampal subregions are revealed to be associated with age-related cognitive decline.[9] Therefore, the histopathologic analysis should also be conducted to extend more understanding of the mechanisms underlying our findings.

With a rapidly aging population worldwide, there will be an increasing number of elderly patients undergoing major surgery.[37] Nevertheless, advanced age itself has been reported to be independently associated with increased perioperative mortality and morbidity.[38] Therefore, high-quality care for geriatric surgical patients should be required to optimize surgical outcomes.[37] In particular, it has been reported that preoperative health status can influence postoperative physical functional decline.[39,40] Hence, several strategies have emerged to optimize physiological function during the presurgical period, aiming to minimize the risk of postoperative adverse consequences.[41] These interventions may be more beneficial and more effective in a vulnerable population like geriatric surgical patients. Our findings in the current study extend this concept to cognitive function by demonstrating in aged rats the preventive effect of PEE on the development of POCD. Nevertheless, a number of important translational questions remain such as what are the best types, durations, and intensities of cognitive intervention for the prevention of the development of POCD. Therefore, future studies are needed to answer these topics before our findings can be translated into clinical practice.

In conclusion, our results indicate that PEE could reduce the exaggerated microglial proinflammatory response to immune challenge in aged hippocampi, which may protect against the development of neuroinflammation and related memory deficits after abdominal surgery in aged rats. These findings imply that age-related phenotype changes in hippocampal microglia, as a major determinant of cognitive vulnerability to aging, may be a potentially modifiable risk factor for preventing POCD during the preadmission period.

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Competing Interests

The authors declare no competing interests.

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