

Original Research

Ramelteon Ameliorates Isoflurane-Induced Hyperactivity and Social-Recognition Impairment in Aged Mice

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Abstract

Background: Ramelteon, a melatonin receptor agonist, has been reported to alleviate postoperative delirium (POD), although its efficacy remains controversial. The mechanisms of ramelteon's effects are unclear and few animal studies have addressed POD-related behavioral and molecular changes. We investigated the specific postoperative behavioral and molecular changes that result from ramelteon pretreatment. **Methods:** Ramelteon (0.03 or 0.3 mg/kg, p.o.) was given to mice once a day for 7 consecutive days before abdominal laparotomy under 2 h of isoflurane anesthesia. Postoperative locomotor activity was monitored for 7 days using s.c.-implanted Nano-tag devices in the dorsal region of aged mice (70–80 weeks). One day after surgery, a social interaction test was administered that used a habituation-discrimination paradigm to evaluate social recognition, specifically the ability to distinguish a novel aged intruder from a familiar young intruder after exposure to the latter. Working memory and related cognitive functions were evaluated using the Y maze and novel-object recognition tests. Cytokine levels and microglial activation in the prefrontal cortex and hippocampus were analyzed by western blotting 24 h post-surgery. **Results:** Isoflurane anesthesia for 2 h did not impair spontaneous alternation in the Y maze or performance in the novel-object recognition test. However, it induced prolonged hyperactivity and a decrease in social-recognition performance. Pretreatment with ramelteon at a dose of 0.3 mg/kg, but not 0.03 mg/kg, attenuated postoperative hyperactivity and preserved normal social recognition. Furthermore, ramelteon significantly reduced isoflurane-induced elevation of interleukin-1 β in the prefrontal cortex but not in the hippocampus. **Conclusion:** Isoflurane anesthesia combined with abdominal surgery was associated with prolonged hyperactivity and impaired social recognition, although other cognitive domains such as working memory appeared to remain unaffected. Ramelteon appeared to alleviate these behavioral and neuroinflammatory changes, suggesting its potential for preventing certain postoperative neurobehavioral alterations.

Keywords: aged mice; hyperactivity; isoflurane anesthesia; ramelteon; social recognition deficit

1. Introduction

In 2008, approximately 230 million individuals worldwide were reported to be undergoing surgeries requiring general anesthesia each year [1]. Among these, an estimated 26% experience transient neurobehavioral disturbances within the first week after surgery, and about 10% continue to show such symptoms even 3 mo later [2]. These postoperative disturbances are particularly prevalent in older adults, with incidence rates reported to reach up to 53% [3]. One of the clinically recognized conditions in this spectrum of symptoms is postoperative delirium (POD), which is characterized as an acute and fluctuating disturbance in mental status, typically occurring from the immediate postoperative period up to 5 days after surgery [4]. Clinically, POD is classified into hyperactive, hypoactive, and mixed subtypes [5], and patients often exhibit a range of symptoms, including cognitive impairment, disorganized thinking, anxiety, and sleep disturbances [6,7]. The incidence of such conditions varies depending on factors such as age, type of surgery (e.g., abdominal, orthopedic, urological, and thoracic), and surgical setting (elective, emer-

gency, or urgent), with reported rates ranging between 10% and 50% [8,9]. Recent studies have focused on the impact of anesthetic agents, methods of administration, and duration of exposure as potential contributors to postoperative neurobehavioral changes [10–12]. These conditions are associated with increased postoperative morbidity, prolonged hospitalization, poor functional recovery, and reduced long-term survival [13]. Despite their clinical significance, the underlying mechanisms remain poorly understood. Currently, pharmacological treatments are limited, primarily due to insufficient knowledge of the pathophysiology of these postoperative neurobehavioral alterations [14].

A more comprehensive understanding of the pharmacological actions of anesthetics may hold significant clinical relevance for the prevention and treatment of postoperative neurobehavioral disturbances [12]. Accordingly, there is growing interest in developing effective therapeutic strategies based on a better understanding of the underlying mechanisms of such conditions. At present, oral antipsychotic drugs are recommended as the first-line phar-



macological treatment for POD [15]. However, none have demonstrated robust therapeutic efficacy [16]. Surgery under isoflurane anesthesia has been shown to induce various neurobehavioral impairments in mouse models, including hippocampal and striatal dysfunction associated with sleep fragmentation [17]. Repeated anesthesia and surgery do not impair learning and memory in middle-aged mice [18], but their effects in aged animals remain unclear, particularly in relation to region-specific neuroinflammatory responses and associated behavioral changes [19]. Although many studies have focused on cognitive and memory impairments, research addressing persistent postoperative hyperactivity in rodent models remains limited, and the mechanisms underlying this phenomenon are largely unknown. One previous study reported that isoflurane anesthesia induced hyperlocomotion only during the first 10 min after the cessation of a 20-min exposure [20], but that finding was based on a single study using young mice, and the behavioral assessments were restricted to the immediate post-anesthesia period. Recent evidence has indicated that general anesthesia, with or without surgery, may contribute to the development of postoperative neurocognitive disturbances, particularly in aged individuals, with neuroinflammation considered to be a potential contributing factor [21]. In aged mice, surgical trauma combined with anesthesia can disrupt the blood–brain barrier and activate microglia, leading to elevated levels of proinflammatory cytokines, such as IL-1 β and IL-6, in the hippocampus [22]. Although such changes do not necessarily impair learning and memory performance in middle-aged or aged mice, they may still affect other behavioral domains [19,23]. Those findings underscore the vulnerability of the aged brain to anesthesia-related neuroinflammatory responses and highlight the importance of using brain-region-specific and age-sensitive behavioral paradigms to assess postoperative cognitive decline.

Recent studies have shown that ramelteon—a melatonin type 1 (MT₁) and type2 (MT₂) receptor agonist—may improve certain behavioral and cognitive functions in mice [24,25]. Moreover, melatonin has been suggested to alleviate impairments induced by long-term exposure to isoflurane by improving circadian-rhythm resynchronization in aged mice [26,27]. In clinical settings, ramelteon has shown potential therapeutic effects in patients with delirium [28–30]. In contrast, some reports have claimed that ramelteon does not ameliorate delirium symptoms in the postoperative period [31–34]. These inconsistent findings may be attributable to the currently unclear mechanisms underlying its potential neuroprotective effects, which have led to a lack of consensus on the optimal timing of its administration, as well as to considerable variability in patients' surgical backgrounds. In light of the above, the present study investigated whether a combination of 2 h of isoflurane anesthesia and abdominal surgery induced postoperative changes in locomotor activity, social recognition, and

working memory in aged male mice (70–80 weeks old). We also examined whether pretreatment with ramelteon mitigated these changes. Furthermore, to explore potential molecular mechanisms underlying the observed behavioral alterations, we assessed neuroinflammatory responses in the prefrontal cortex and hippocampus, focusing on cytokines such as IL-1 β and IL-6, as well as microglial activation.

2. Materials and Methods

2.1 Animals and Ethical Considerations

We purchased 40 male C57BL/6J mice aged 32 weeks, 15 aged 48 weeks, 40 aged 54 weeks, and 15 aged 71 weeks from Charles River Laboratories (Yokohama, Japan). All mice were used in the experiment only after reaching at least 70 weeks of age. In addition, 35 mice were reared at the animal facility of Ozu University by maintaining C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) at 6 weeks of age until they reached 70 weeks. The mice were housed in a controlled environment (25 $\pm$ 2 °C, 12-hour light/dark [08:00–20:00 h on] cycle), with unrestricted access to food (#CE-2, CLEA Japan) and water, in groups of 4–5 per cage. After the experiments, the mice were euthanized using a combination of anesthetics: intraperitoneal administration of medetomidine hydrochloride (0.3 mg/kg, Cat. No. 135-17473, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), butorphanol tartrate (5.0 mg/kg, Cat. No. 021-19001, FUJIFILM Wako Pure Chemical Corp.), and midazolam (4.0 mg/kg, Cat. No. 1124401A1060, Sandoz Group AG, Basel, Switzerland), followed by cervical dislocation, after the behavioral tests.

2.2 Chemicals and Antibodies

Ramelteon (Lot. No. RMT-322010-J1) was provided by Sanyo Chemical Laboratories Co., Ltd. (Osaka, Japan). Isoflurane was purchased from Sigma-Aldrich (#792632, St. Louis, MO, USA). The anti-mouse monoclonal antibody against IL-1 β (1:1000, Cat. No. 12242) was obtained from Cell Signaling Technology (Danvers, MA, USA). Both the anti-rabbit polyclonal antibody against ionized calcium-binding adapter molecule 1 (IBA-1, 1:2000, Cat. No. 10904-1-AP) and the anti-mouse monoclonal antibody against β -actin (1:2000, Cat. No. HRP-60008) were obtained from Proteintech (Rosemont, IL, USA). The anti-mouse monoclonal antibody against IL-6 (1:2000, Cat. No. 554400) was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.3 Anesthesia and Surgical Procedures

The mice were randomly assigned to one of the following groups: the 10-min isoflurane anesthesia with surgery (sham control) group, the 2-h isoflurane anesthesia with surgery group, or the ramelteon-treated 2-h isoflurane anesthesia with surgery group (0.03, or 0.3 mg/kg). For the surgical procedure, mice were initially anesthetized

with 3% isoflurane in 100% air at a flow rate of 2 L/min for 30 min to perform a simple laparotomy. This was followed by maintenance anesthesia with 1.5% isoflurane administered in a transparent anesthetic chamber for up to 2 h, with additional isoflurane provided as needed. A longitudinal midline incision was made through the skin, abdominal muscles, and peritoneum, extending from the xiphoid process to 0.5 cm proximal to the pubic symphysis. A segment of the small intestine was gently exteriorized using forceps and held for 1 min. The incision was then closed in layers using 5-0 Vicryl sutures. At the end of the procedure, eutectic mixture of local anesthetics (EMLA) cream (2.5% lidocaine and 2.5% prilocaine, Sato Pharmaceutical Co., Ltd, Tokyo, Japan) was applied to the incision sites. To alleviate incision-related pain, the cream was reapplied every 12 h until behavioral testing at 24 h post-surgery or for 48 h in mice used for locomotor-activity assessment. The temperature of the anesthetic chamber was maintained using a TCM-01 system (Brain Science Idea Co., Ltd., Osaka, Japan), with rectal temperature controlled at 37 ± 0.5 °C during anesthesia and surgery. After recovery from anesthesia, each mouse was returned to a home cage with *ad libitum* access to food and water. To prevent interference with the wound by cage mates, mice were housed individually until experimental use.

2.4 Nano-Tag Implantation and Measurement of Locomotor Activity

Based on a previous report that found the mean duration of delirium to be less than 9 days (4.0 ± 5.1 days; $n = 144$) [35], we measured the locomotor activity for at least 7 days after the 2-h isoflurane anesthesia with surgery. For mice designated for 7-day locomotor activity recording, a Nano-tag device (Kissei Comtec Co., Ltd., Nagano, Japan), which records spontaneous activity, was surgically implanted subcutaneously on the dorsal side of the body. Sham control mice were anesthetized with 3% isoflurane for both induction and mock abdominal surgery, with the total isoflurane exposure limited to under 10 min, including the time required for Nano-tag implantation. To prevent wound dehiscence, the incision site was secured at two points using a surgical needle holder and a Reflex 7 mm wound clip applier (RS-9250; CellPoint Scientific, Inc., Gaithersburg, MD, USA). To prevent interference with the wound by cage mates, mice were housed individually until experimental use. Mice subjected to locomotor-activity monitoring were housed individually throughout the entire 7-day recording period. Locomotor activity was recorded in 4-min bins based on movement frequency using the Nano-tag device, and data collected by the Nano-tag were transferred to a contactless smart card reader (FeliCa, ISO/IEC 18092, Sony Corporation, Tokyo, Japan). After surgery, these mice were housed under a 12-h light–dark cycle for 10 days. Locomotor activity was recorded every 5 min over a continuous 10-day period and analyzed using Nano-tag

Viewer software (version 1.1.3; Kissei Comtec Co., Ltd., Matsumoto, Japan) [36].

2.5 Ramelteon Administration

Melatonin is secreted at night in humans, and stimulation of melatonin receptors has been reported to be effective in promoting sleep [37]. The active phase of mice differs from that of humans by approximately 12 h; they are nocturnal and primarily active during the dark phase. In rodents, melatonin secretion has been reported to occur during this active period at night [38]. Therefore, in the present study, male C57BL/6J mice aged 70–80 weeks received an intraperitoneal administration of either vehicle (corn oil; Cat. No. 23-0320, Sigma-Aldrich) or ramelteon at a dose of 0.03 or 0.3 mg/kg, dissolved in corn oil immediately before use [39], once daily at 1800 h (2 h before the onset of the dark phase), for 7 consecutive days prior to surgery. For cognitive function tests, administration continued until the day before the test (i.e., the day of surgery). Additionally, the effects of ramelteon on activity levels after isoflurane anesthesia were assessed by administering it once daily at 1800 h for 7 days after isoflurane anesthesia.

2.6 Behavioral Tests

At 70 weeks of age, the mice were randomly assigned to one of the following behavioral-testing groups: the 10-min isoflurane anesthesia with surgery (sham control) group, the 2-h isoflurane anesthesia with surgery group, or the ramelteon-treated 2-h isoflurane anesthesia with surgery group (0.03 or 0.3 mg/kg). Two investigators, blind to the group assignments, performed all behavioral tests. We recorded all behavioral tests using a web camera installed with the ANY-maze software (version 6.35; Muromachi Kikai Co., Ltd., Tokyo, Japan) to collect and analyze the data for all behavioral tests.

2.6.1 Social Interaction Test

The social interaction test was performed 24 h after isoflurane anesthesia and abdominal surgery. The test mouse was initially placed in an acrylic box (30 cm × 30 cm × 30 cm) with all four walls covered in white paper and allowed to explore for 5 min to habituate to the test environment. After habituation, an unfamiliar young (6-week-old) male C57BL/6J mouse (Stranger 1), with no prior contact with the test mouse, was placed inside a 300 mL beaker positioned in the corner (zone 1) of the test box. During the 12-min session, the test mouse was exposed to the young mouse over four consecutive 3-min bins. As the 5th test, the young mouse was replaced with an aged mouse (70–75 weeks old) using the same beaker. The time spent, the distance traveled, and the number of entries into zone 1 were recorded by a web camera and analyzed using ANY-maze software. The habituation-discrimination paradigm [40,41] was used to assess habituation and discrimination scores. The time spent and distance traveled in zone 1 during the

fourth 3-min bin (4th test) were divided by those recorded during the first 3-min bin (1st test). The discrimination index was calculated as the ratio of the time spent, distance traveled, and number of entries into zone 1 during the final 3-min bin (5th test) to those recorded during the fourth time bin.

2.6.2 Y-maze Test

A gray polyvinyl chlorid Y maze (YM-40M, Brain Science Idea Co., Ltd., Osaka, Japan) with three arms, each measuring 50 mm (W) × 400 mm (D) × 120 mm (H), was placed on the floor, and the ambient illumination at floor level was adjusted to 40 lux. All Y-maze tests were conducted under controlled lighting conditions. A 30-lux light source was placed behind the right arm of the Y maze and remained constant throughout all sessions [42]. To avoid direct illumination of the maze, the light was angled away from the apparatus. This configuration provided a consistent external spatial cue without introducing excessive visual stress to the animals. Each mouse was placed midway along the start arm (Arm A), facing the center of the Y, and allowed to explore for 8 min after a 2-min habituation period. The sequence of entries into each arm was recorded using a webcam integrated with behavioral tracking software (ANY-maze, version 5.3). Spontaneous alternation performance is considered an index of active retrograde working memory [43]. The percentage of spontaneous alternation was calculated as the number of triads (entries into three different arms in succession, without returning to a previously visited arm) relative to the number of alternation opportunities, using the following formula: $[\text{Number of spontaneous alternations} / (\text{Total number of arm entries} - 2)] \times 100$. The total number of entries into each of the three arms, the total distance traveled, and the total time spent in each arm (A, B, and C) were recorded. Mice that did not show a significant bias in the time spent among the arms were selected for the evaluation of spatial working memory based on their spontaneous alternation behavior. The Y-maze test was conducted 24 h after isoflurane anesthesia. After each session, the apparatus was cleaned with 70% ethanol (Cat. No. 14034-71, KANTO CHEMICAL Co. INC., Tokyo, Japan) to eliminate olfactory cues.

2.6.3 Novel-Object Recognition Test

As the evaluation in the current study had to be conducted 24 h after isoflurane anesthesia with surgery, the object-recognition test was performed using a modified version of the method described by Leger *et al.* [44]. An acrylic box (internal dimensions: 300 mm [W] × 300 mm [D] × 300 mm [H]) was used, with its outer surfaces covered in white paper. The illumination inside the box was set to 30 lux. On the test day, each mouse was first placed in the box for 5 min to habituate to the environment. One h later, the mouse was reintroduced into the same box for a 5-min free exploration session. Two identical objects, ap-

proximately 5 cm in size and made of red plastic with different shapes, were placed in the center of the box, spaced 10 cm apart. After 5 min, one of the identical objects was replaced with a novel object—a white cylindrical rubber object measuring 5 cm in diameter and 4.5 cm in height—and the mouse was again allowed to explore freely for 5 min. To evaluate novel-object recognition, the number of entries and total distance traveled within each object zone were measured by ANY-maze software. During the second 5-min session (with two identical objects), mice with no strong preference for either object were selected for analysis. Recognition was quantified by calculating the ratio of approaches to one familiar object over the other (F/F) and the ratio of approaches to the novel object relative to the familiar object (N/F) during the third 5-min session. To minimize the influence of object placement bias, the final measure was the normalized preference ratio, calculated as N/F divided by F/F (i.e., $N/F / F/F$). The novel-object recognition test was conducted right after the Y-maze test, which was carried out 24 h after isoflurane anesthesia. After each trial, the apparatus was cleaned with 70% ethanol to remove olfactory cues.

2.7 Western Blotting

After euthanizing the mice, the prefrontal cortex and hippocampus were harvested for Western Blot analysis. Mouse prefrontal cortex and hippocampal tissues were homogenized using a Dounce homogenizer (Cat. No. 357544, SANSYO Co., Ltd. Tokyo, Japan), and the mixture was centrifuged at 500 × g for 5 min at 4 °C to isolate the total fraction, excluding the nuclear fraction. The membrane and cytosolic fractions, excluding the nuclear fraction, were extracted using a commercially available LysoPur Nuclear Extractor Kit (Cat. No. 295-73901, FUJIFILM Wako Chemicals). Phosphatase and protease inhibitors were added to the nuclear fraction buffers before hippocampal homogenization (cOmplete for phosphatase inhibitors: Cat. No. 4693159001, Merck Millipore, MA, USA) and protease inhibitors (PhosSTOP: Cat. No. 4906837001, Merck Millipore). Western Blotting was performed as previously described [45]. Briefly, protein extracts were mixed with a sample buffer containing 50 mM Tris-HCl (pH 7.6, Cat. A0321, Tokyo Chemical Industry Co., Ltd. (TCI)), 2% SDS (Cat. No. 02873-75, NACALAI TESQUE, INC., Kyoto, Japan), 10% glycerol (Cat. No. S0373, TCI), 10 mM dithiothreitol (Cat. No. 048-29224, FUJIFILM Wako Chemicals), and 0.2% bromophenol blue (Cat. No. 021-02911, FUJIFILM Wako Chemicals), and the solution was boiled at 94 °C for 5 min. The samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8%–10% gels (8%–10% acrylamide (Cat. No. 01080-00, KANTO CHEMICAL Co. INC.)/bis (N,N'-methylenebisacrylamide, Cat. No. M0506, TCI), 1× WIDE RANGE Gel Preparation Buffer for PAGE (Cat. 07831-94, NACALAI TESQUE,

INC.), 0.1% ammonium persulfate (Cat. No. 01307-30, KANTO CHEMICAL Co. INC.), 0.06% N,N,N',N'-Tetramethylethylenediamine (TEMED, Cat. No. T0147, TCI) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated for 1 h with PVDF Blocking Reagent for Can Get Signal (Cat. No. NYPBR01, Toyobo, Osaka, Japan). For antigen retrieval, Can Get Signal Solution 1 and Solution 2 (Cat. No. NKB-101, Toyobo) were mixed with the primary and secondary antibodies, respectively. Blots were detected using Western BLoT Ultra-Sensitive HRP Substrate (Takara Bio, Inc., Shiga, Japan) and visualized using the ChemiDoc Touch Imaging System (Model 1708370, Bio-Rad Laboratories, Inc., Tokyo, Japan). Protein bands were analyzed with ImageJ software (Version 1.53t; Wayne Rasband (National Institutes of Health (NIH)), Bethesda, MD, USA).

2.8 Statistical Analysis

All statistical analyses were performed using Bell-Curve software (version 3.20; Social Survey Research Information Co., Ltd., Tokyo, Japan) [46]. A two-way repeated measures analysis of variance (ANOVA; isoflurane $\times$ time) was conducted to analyze locomotor activity levels in mice over 7 days, comparing the sham treatment (control) with 2-h isoflurane anesthesia + laparotomy. A three-way repeated measures ANOVA (isoflurane $\times$ drug $\times$ time) was performed to assess the effect of ramelteon on locomotor activity over 7 days. For each time point, a one-way ANOVA was applied, with Tukey's honestly significant difference (HSD) test used for post hoc comparisons if significant differences were found between groups. One-way ANOVA was performed for each of the following experiments: Y-maze test, novel object recognition test, social interaction test, and Western blot analysis. When significant differences were detected, Tukey's HSD test was used for post hoc pairwise comparisons. Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance on the graphs is indicated by symbols as follows: * or # for $p < 0.05$, and ** or ## for $p < 0.01$, with different symbols used to distinguish between different pairwise comparisons.

3. Results

3.1 Effect of 2-h Isoflurane Exposure and Ramelteon Treatment on Locomotor Activity Over 7 Days After Surgery

Because abnormal increases or decreases in locomotor activity are commonly observed after surgery [5], and the mean duration of postoperative behavioral disturbances has been reported to be less than 9 days (Fig. 1A, 4.0 ± 5.1 days; $n = 144$) [35], we measured locomotor activity over a 7-day period after a 2-h isoflurane exposure combined with laparotomy. Three-way repeated measures ANOVA (isoflurane $\times$ time $\times$ ramelteon) revealed no significant 3-way interaction on locomotor activity ($F_{65,489} = 1.059$, $p = 0.354$;

Fig. 1B,C and Table 1-1,1-2; Table 1-1: days 0.5–3.5 and Table 1-2: days 4–7. Actograms of individual mice from all experimental groups are shown in **Supplementary Fig. 1A–F**). However, both 2-h isoflurane exposure combined with laparotomy and ramelteon pretreatment significantly affected locomotor activity (2-h isoflurane: $F_{1,29} = 45.33$, $p < 0.01$; ramelteon: $F_{2,29} = 3.673$, $p < 0.05$; Fig. 1C and Table 1-1,1-2). From the third to the seventh dark phase, the interaction between 2-h isoflurane combined with laparotomy and ramelteon pretreatment significantly affected locomotor activity, with significant differences observed only during the second and seventh light phases (Fig. 1B,C and Table 1-1,1-2). Tukey HSD post hoc tests revealed that locomotor activity in the 2-h isoflurane exposure combined with laparotomy group treated with vehicle was significantly higher than in the sham-treated vehicle group during the third to seventh dark phases (Fig. 1B,C and Table 1-1,1-2). Additionally, ramelteon 0.3 mg/kg significantly reduced locomotor activity from the third to the seventh dark phases, with activity levels similar to those of the sham control treated with vehicle group during these time points (Fig. 1B,C and Table 1-1,1-2). These findings indicate that 2-h isoflurane exposure combined with laparotomy induced sustained hyperactivity during the dark (active) phase after surgery, and that this effect can be mitigated by ramelteon pretreatment at 0.3 mg/kg. In the second light phase, a non-active phase for mice, both ramelteon pretreatments (0.03 and 0.3 mg/kg) in the sham control group produced significantly less activity than there was in vehicle-treated sham control mice. This suggests that ramelteon may promote sleep during the early postoperative phases.

3.2 Effect of 2-h Isoflurane Exposure and Ramelteon on Social Interaction

Since pretreatment with ramelteon at 0.3 mg/kg attenuated the prolonged hyperactivity observed after 2-h isoflurane anesthesia and surgery, we further examined the effects of ramelteon pretreatment on the effect on social recognition and interaction 24 h after 2-h isoflurane exposure combined with laparotomy, as well as the effect of ramelteon pretreatment (0.3 mg/kg) on social recognition after 2-h isoflurane anesthesia combined with laparotomy. Three-way repeated measures ANOVA revealed a significant interaction effect among isoflurane, ramelteon, and time on social interaction (as measured by the number of entries into the zone in the presence of the young mouse; Fig. 2A) ($F_{41,49} = 7.24$, $p < 0.05$; Fig. 2C). A two-way ANOVA (isoflurane $\times$ ramelteon), followed by Tukey's HSD post hoc test, showed that the number of entries into the zone of a young mouse 24 h post-surgery at the fourth 3-min bin in mice exposed to 2-h isoflurane with laparotomy was significantly higher than that of either sham control mice or 2-h isoflurane exposure with laparotomy and pretreatment of ramelteon 0.3 mg/kg (fourth 3-min bin: $F_{3,95} = 3.65$, $p < 0.05$; Tukey's HSD: 2-hour isoflurane com-

Table 1-1. Statistics for locomotor activity in a 12-hour interval after 2-hour isoflurane exposure (Day 0.5–3.5).

Day	Phase	F (5, 95)	p value	Group 1	Group 2	z value	p value	F value
0.5	Dark	0.2925	0.9160	Sham	Sham + R 0.3 mg/kg	0.8040	0.9658	*
					Sham + R 0.03 mg/kg	1.9150	0.3975	
					2 h ISO	2.2200	0.2369	
					2 h ISO + R 0.3 mg/kg	0.6697	0.9847	
					2 h ISO + R 0.03 mg/kg	3.1952	0.0225	
				2 h ISO	2 h ISO + R 0.3 mg/kg	1.4939	0.6670	
					2 h ISO + R 0.03 mg/kg	0.9397	0.9344	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	2.4337	0.1542	
1	Light	1.663	0.1511	Sham	Sham + R 0.3 mg/kg	5.9218	$p < 0.001$	**
					Sham + R 0.03 mg/kg	3.7415	0.0041	**
					2 h ISO	2.8278	0.0612	
					2 h ISO + R 0.3 mg/kg	1.5982	0.5997	
					2 h ISO + R 0.03 mg/kg	2.6757	0.0891	
				2 h ISO	2 h ISO + R 0.3 mg/kg	1.1849	0.8417	
					2 h ISO + R 0.03 mg/kg	0.1466	1.0000	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	1.0383	0.9029	
1.5	Dark	2.118	0.0699	Sham	Sham + R 0.3 mg/kg	2.5666	0.1150	
					Sham + R 0.03 mg/kg	0.7297	0.9775	
					2 h ISO	6.9969	$p < 0.001$	
					2 h ISO + R 0.3 mg/kg	0.4604	0.9973	
					2 h ISO + R 0.03 mg/kg	7.3409	$p < 0.001$	
				2 h ISO	2 h ISO + R 0.3 mg/kg	6.2988	$p < 0.001$	
					2 h ISO + R 0.03 mg/kg	0.3314	0.9994	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	6.6302	$p < 0.001$	
2	Light	2.462	0.0383	Sham	Sham + R 0.3 mg/kg	7.3290	$p < 0.001$	**
					Sham + R 0.03 mg/kg	6.1490	$p < 0.001$	**
					2 h ISO	2.9509	0.0444	*
					2 h ISO + R 0.3 mg/kg	0.1795	1.0000	
					2 h ISO + R 0.03 mg/kg	2.4826	0.1387	
				2 h ISO	2 h ISO + R 0.3 mg/kg	2.6705	0.0902	
					2 h ISO + R 0.03 mg/kg	0.4512	0.9976	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	2.2194	0.2372	
2.5	Dark	4.100	0.0021	Sham	Sham + R 0.3 mg/kg	0.7631	0.9727	
					Sham + R 0.03 mg/kg	2.2293	0.2327	
					2 h ISO	11.3613	$p < 0.001$	
					2 h ISO + R 0.3 mg/kg	0.4305	0.9980	
					2 h ISO + R 0.03 mg/kg	10.3745	$p < 0.001$	
				2 h ISO	2 h ISO + R 0.3 mg/kg	11.3628	$p < 0.001$	
					2 h ISO + R 0.03 mg/kg	0.9509	0.9312	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	10.4119	$p < 0.001$	
3	Light	1.794	0.1215	Sham	Sham + R 0.3 mg/kg	4.6530	$p < 0.001$	**
					Sham + R 0.03 mg/kg	3.3345	0.0149	*
					2 h ISO	3.7046	0.0046	**
					2 h ISO + R 0.3 mg/kg	1.5435	0.6352	
					2 h ISO + R 0.03 mg/kg	4.7767	$p < 0.001$	
				2 h ISO	2 h ISO + R 0.3 mg/kg	2.0825	0.3035	
					2 h ISO + R 0.03 mg/kg	1.0331	0.9047	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	3.1156	0.0282	

Table 1-1. Continued.

Day	Phase	F (5, 95)	<i>p</i> value	Group 1	Group 2	z value	<i>p</i> value	
3.5	Dark	4.440	0.0011	Sham	Sham + R 0.3 mg/kg	0.3643	0.9991	
					Sham + R 0.03 mg/kg	0.3181	0.9995	
					2 h ISO	12.5594	<i>p</i> < 0.001	**
					2 h ISO + R 0.3 mg/kg	1.5879	0.6064	
					2 h ISO + R 0.03 mg/kg	10.1619	<i>p</i> < 0.001	**
				2 h ISO	2 h ISO + R 0.3 mg/kg	10.5724	<i>p</i> < 0.001	##
					2 h ISO + R 0.03 mg/kg	2.3103	0.1989	
					2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	8.2621	<i>p</i> < 0.001

** $p < 0.01$, * $p < 0.05$ vs. Sham; ## $p < 0.01$ vs. 2 h ISO, \$\$ $p < 0.01$ vs. 2 h ISO + R 0.3 mg/kg. ISO, isoflurane; R, ramelteon.

Table 1-2. Statistics for locomotor activity in a 12-hour interval after 2-hour isoflurane exposure (Day 4–7).

Day	Phase	F (5, 95)	p value	Group 1	Group 2	z value	p value	
4	Light	1.608	0.1654	Sham	Sham + R 0.3 mg/kg	3.4815	0.0095	**
					Sham + R 0.03 mg/kg	1.1122	0.8741	
					2 h ISO	5.4155	$p < 0.001$	**
					2 h ISO + R 0.3 mg/kg	1.8384	0.4444	
					2 h ISO + R 0.03 mg/kg	5.2916	$p < 0.001$	**
				2 h ISO	2 h ISO + R 0.3 mg/kg	3.4470	0.0106	*
					2 h ISO + R 0.03 mg/kg	0.1194	1.0000	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	3.3276	0.0152	*
4.5	Dark	2.434	0.0402	Sham	Sham + R 0.3 mg/kg	3.9452	0.0020	**
					Sham + R 0.03 mg/kg	0.4191	0.9983	
					2 h ISO	8.5581	$p < 0.001$	**
					2 h ISO + R 0.3 mg/kg	1.1150	0.8730	
					2 h ISO + R 0.03 mg/kg	3.7327	0.0042	**
				2 h ISO	2 h ISO + R 0.3 mg/kg	9.3211	$p < 0.001$	##
					2 h ISO + R 0.03 mg/kg	4.6498	$p < 0.001$	##
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	4.6713	$p < 0.001$	\$\$
5	Light	1.771	0.1262	Sham	Sham + R 0.3 mg/kg	3.8557	0.0028	**
					Sham + R 0.03 mg/kg	2.1535	0.2678	
					2 h ISO	4.9593	$p < 0.001$	**
					2 h ISO + R 0.3 mg/kg	1.2485	0.8102	
					2 h ISO + R 0.03 mg/kg	5.4375	$p < 0.001$	**
				2 h ISO	2 h ISO + R 0.3 mg/kg	3.5758	0.0070	**
					2 h ISO + R 0.03 mg/kg	0.4608	0.9973	
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	4.0366	0.0015	**
5.5	Dark	5.550	$p < 0.001$	Sham	Sham + R 0.3 mg/kg	4.5769	$p < 0.001$	**
					Sham + R 0.03 mg/kg	2.4600	0.1457	
					2 h ISO	13.6269	$p < 0.001$	**
					2 h ISO + R 0.3 mg/kg	0.7124	0.9798	
					2 h ISO + R 0.03 mg/kg	4.3981	$p < 0.001$	**
				2 h ISO	2 h ISO + R 0.3 mg/kg	13.8177	$p < 0.001$	##
					2 h ISO + R 0.03 mg/kg	8.8931	$p < 0.001$	##
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	4.9246	$p < 0.001$	\$\$
6	Light	1.433	0.2195	Sham	Sham + R 0.3 mg/kg	3.8471	0.0029	**
					Sham + R 0.03 mg/kg	2.0172	0.3386	
					2 h ISO	2.9833	0.0407	*
					2 h ISO + R 0.3 mg/kg	0.1438	1.0000	
					2 h ISO + R 0.03 mg/kg	5.4158	$p < 0.001$	**

Table 1-2. Continued.

Day	Phase	F (5, 95)	<i>p</i> value	Group 1	Group 2	z value	<i>p</i> value					
6.5	Dark	2.404	0.0424	2 h ISO	2 h ISO + R 0.3 mg/kg	3.0133	0.0375	*				
					2 h ISO + R 0.03 mg/kg	2.3440	0.1858					
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	5.3573	<i>p</i> < 0.001	**				
				Sham	Sham + R 0.3 mg/kg	4.6122	<i>p</i> < 0.001	**				
					Sham + R 0.03 mg/kg	1.2940	0.7860					
					2 h ISO	5.6154	<i>p</i> < 0.001	**				
					2 h ISO + R 0.3 mg/kg	0.4443	0.9977					
					2 h ISO + R 0.03 mg/kg	7.3083	<i>p</i> < 0.001	**				
				2 h ISO	2 h ISO + R 0.3 mg/kg	5.8393	<i>p</i> < 0.001	##				
					2 h ISO + R 0.03 mg/kg	1.6313	0.5780					
				2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	7.4706	<i>p</i> < 0.001	\$\$				
				7	Light	2.376	0.0445	Sham	Sham + R 0.3 mg/kg	4.6657	<i>p</i> < 0.001	**
									Sham + R 0.03 mg/kg	2.4917	0.1360	
									2 h ISO	3.4721	0.0098	**
2 h ISO + R 0.3 mg/kg	0.8710	0.9520										
2 h ISO + R 0.03 mg/kg	7.4996	<i>p</i> < 0.001	**									
2 h ISO	2 h ISO + R 0.3 mg/kg	2.5065	0.1316									
	2 h ISO + R 0.03 mg/kg	3.8810	0.0026					##				
2 h ISO + R 0.3 mg/kg	2 h ISO + R 0.03 mg/kg	6.3875	<i>p</i> < 0.001					\$\$				

** $p < 0.01$, * $p < 0.05$ vs. Sham; ## $p < 0.01$ vs. 2 h ISO, \$\$ $p < 0.01$ vs. 2 h ISO + R 0.3 mg/kg.

bined + vehicle treatment, $p < 0.05$ vs. sham + vehicle; $p < 0.05$ vs. 2-h isoflurane + laparotomy + ramelteon 0.3 mg/kg; Fig. 2B,C). These results suggest that 2-h isoflurane with laparotomy impaired the familiarization process with the young mouse, as indicated by a lack of habituation by the fourth 3-min bin. However, ramelteon treatment (0.3 mg/kg) improved the ability of aged mice to become familiar with young mice, which was impaired by 2-hour isoflurane anesthesia with laparotomy in aged mice. Mice are known to actively explore a newly introduced intruder mouse. Over time, as the mice become habituated, the number of entries into the zone of the intruder mouse gradually decreases, reflecting habituation [40,41]. Therefore, we used the ratio of the number of entries into the zone of the young intruder mouse during the fourth 3-min bin to that during the first 3-min bin (4th/1st) as a habituation score, with lower values indicating greater habituation.

A two-way ANOVA (isoflurane $\times$ ramelteon) revealed that the interaction between isoflurane and ramelteon significantly affected the habituation level, as indicated by the number of entries into the zone where the young mouse was presented ($F_{1,29} = 4.31$, $p < 0.05$; Fig. 2D). Tukey's HSD post hoc test indicated that pretreatment with ramelteon 0.3 mg/kg did not affect the impairment in the ability to habituate to the young mouse, which was observed in mouse group of the 2-h isoflurane exposure with laparotomy and vehicle treatment ($p = 0.77$; Fig. 2D). However, pretreatment with ramelteon 0.3 mg/kg prevented impairment in the ability to discriminate the newly introduced older mouse from the previously encoun-

tered familiar young mouse in the fifth 3-min bin, which was observed in vehicle-treated mice exposed to 2-h isoflurane with laparotomy ($F_{1,29} = 6.77$, $p < 0.01$; Tukey HSD: 2-h isoflurane + laparotomy + vehicle: $p < 0.05$ vs. sham + vehicle; 2-h isoflurane + laparotomy + ramelteon 0.3 mg/kg: $p < 0.05$ vs. 2-h isoflurane + laparotomy + vehicle; Fig. 2E). Furthermore, a decrease in sniffing time across repeated trials has been suggested to reflect recognition of the mouse as familiar [40,41]. In our study, after repeated presentations, a novel (aged) mouse was introduced, and increased investigation of the novel mouse reflected social novelty preference. We used the ratio of the time spent in the intruder zone during the fourth 3-min bin to that during the first 3-min bin (4th/1st) as a habituation score, with lower values generally indicating greater habituation. A two-way repeated measures ANOVA revealed a significant interaction between isoflurane and ramelteon on time spent in the zone where the young mice were presented across the first to fourth 3-min bins ($F_{4,149} = 3.57$, $p < 0.01$; Fig. 2B,F). Tukey's HSD post hoc test following the one-way ANOVA indicated that in the 2-h-isoflurane with laparotomy group, the time spent in the zone with the young mouse was significantly longer than that of both the sham control group and the 2-h-isoflurane with laparotomy group and pretreatment of ramelteon 0.3 mg/kg ($F_{3,95} = 3.76$, $p < 0.05$; Tukey's HSD: 2-h isoflurane + laparotomy + vehicle, $p < 0.05$ vs. sham + vehicle; $p < 0.05$ vs. 2-h isoflurane + laparotomy ramelteon 0.3 mg/kg; Fig. 2F).

Finally, we measured the habituation/discrimination scores as described above. However, there was no signifi-

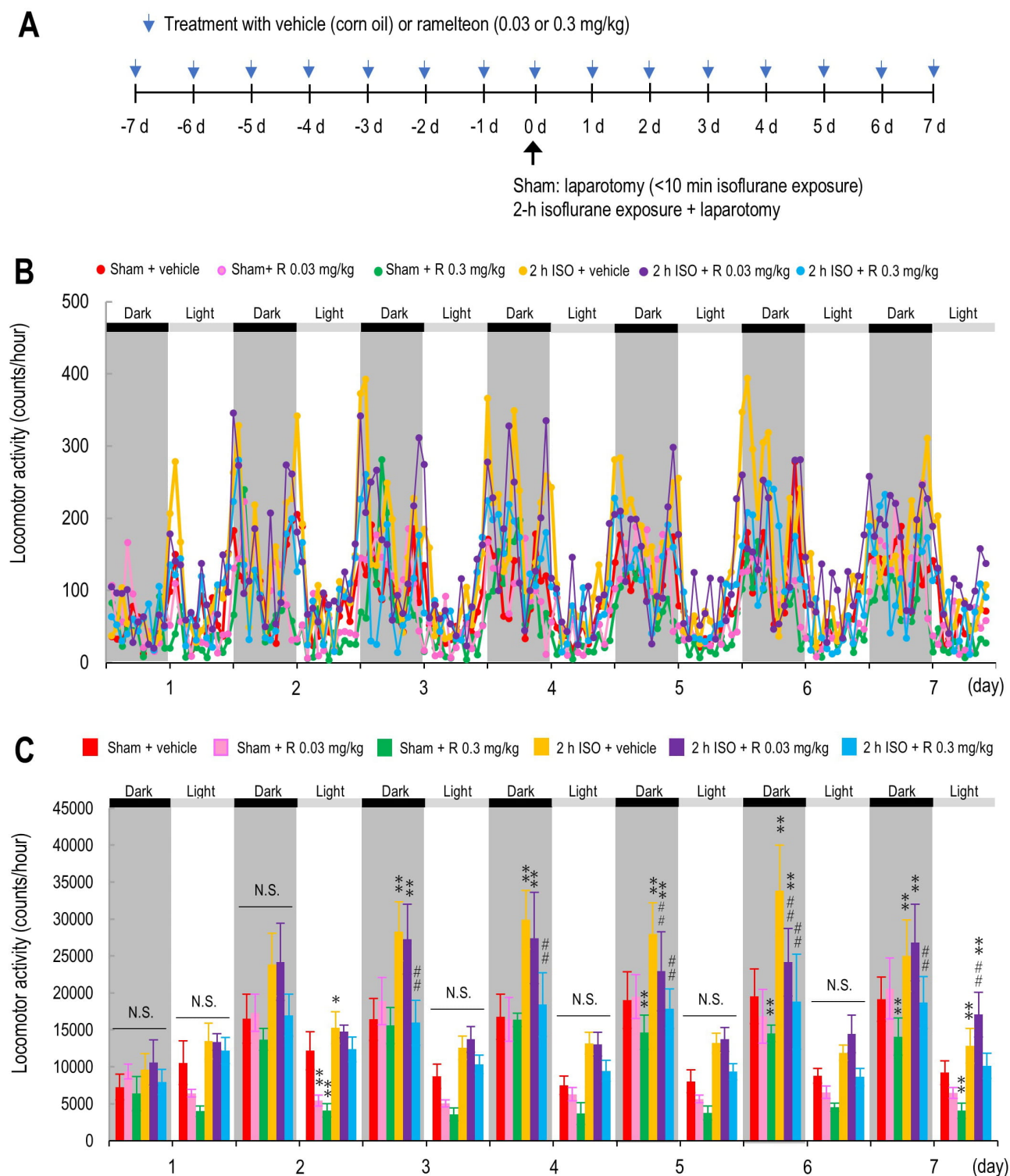


Fig. 1. Gross locomotor activity averaged in 2-hour intervals from Day 1 to Day 7. (A) Schematic diagram of the experimental design. (B,C) Locomotor activity levels (B) and daily averaged gross locomotor activity (C) from Day 1 to Day 7 in the following groups: vehicle-treated sham group (red; $n = 7$), ramelteon 0.03 mg/kg-treated sham group (pink; $n = 5$), ramelteon 0.3 mg/kg-treated sham group (light green; $n = 5$), vehicle-treated 2-hour isoflurane exposure group (yellow; $n = 6$), ramelteon 0.03 mg/kg-treated 2-hour isoflurane group (purple; $n = 6$), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane group (light blue; $n = 6$). Horizontal dark gray bars indicate the dark phase; horizontal light gray bars indicate the light phase of the day. $p < 0.01$ (**) or $p < 0.05$ (*) vs. vehicle-treated sham group; $p < 0.01$ (##) vs. vehicle-treated 2-hour isoflurane group. N.S. indicates no significant difference.

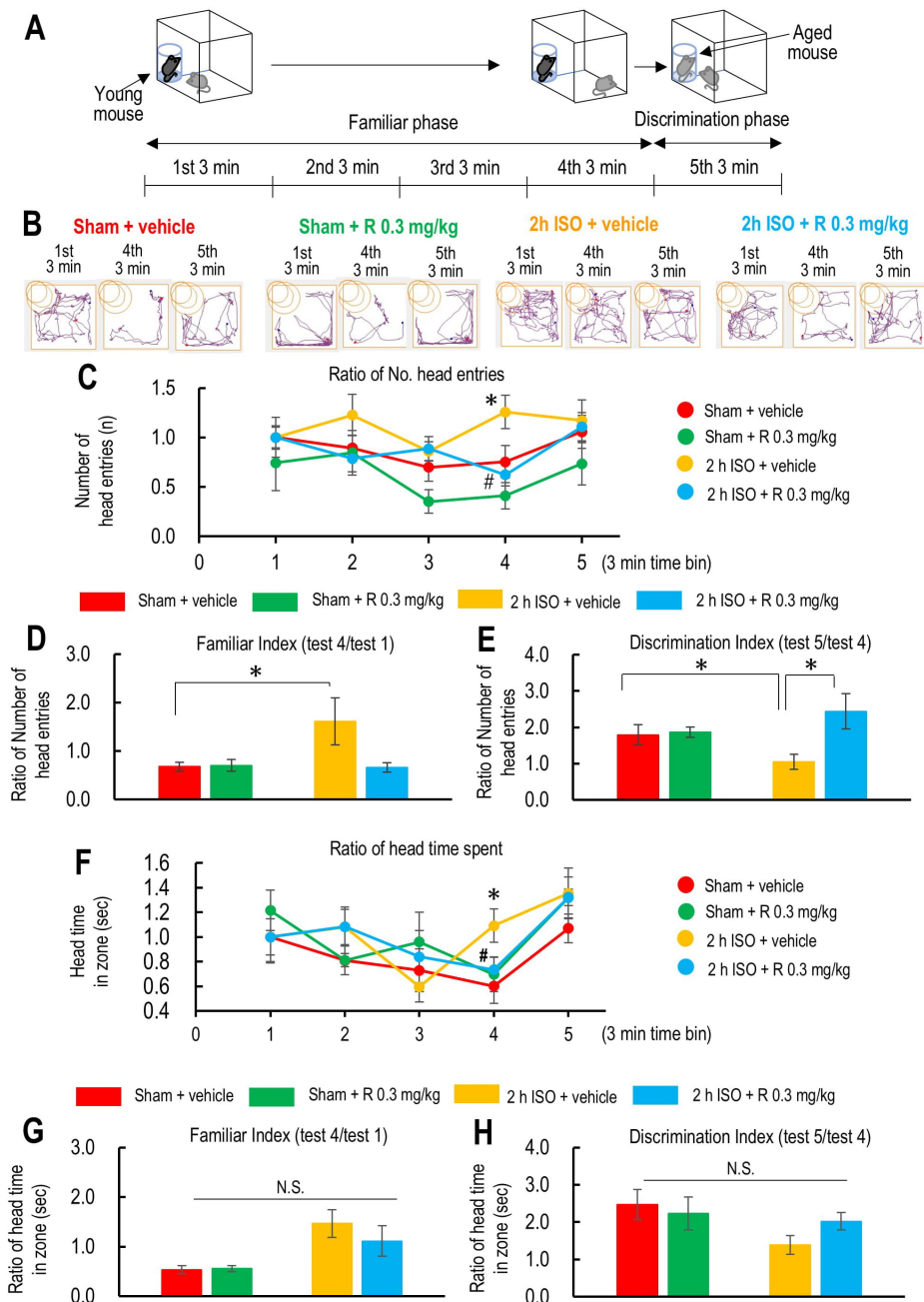


Fig. 2. The effect of ramelteon on the social interaction test in aged mice after 2-hour isoflurane exposure and laparotomy. (A) Schematic drawings represent the protocol for the social interaction test. (B) Movement trajectories of the initial and final 3 minutes of approach to the young mouse and the trajectory of the mouse approaching the novel-aged mouse. (C) Time course of the ratio of the total number of mouse head entries into the zone where the young mouse is present in the beaker placed at the corner of the test box in the vehicle-treated sham group (red; $n = 8$), ramelteon 0.3 mg/kg-treated sham group (light green; $n = 5$), vehicle-treated 2-hour isoflurane exposure group (yellow; $n = 9$), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane exposure group (light blue; $n = 8$). (D) Ratio of the number of mouse head entries into the zone where the young mouse is present during test 4 against test 1 (test 4/test 1) as a familiar score. (E) Ratio of the number of mouse head entries into the zone where the aged mouse is present during test 5 (test 4/test 1) against that into the zone where the young mouse is present during test 4 as a discrimination score. (F) Time course of the ratio of the time spent in the zone where the young mouse is present in the beaker placed at the corner of the test box. (G) Ratio of the time spent in the zone where the young mouse is present during test 4 against test 1 (test 4/test 1) as a familiar score. (H) Ratio of the time spent in the zone where the aged mouse is present during test 5 (test 4/test 1) against that in the zone where the young mouse is present during test 4 as a discrimination score. $p < 0.05$ (*) vs. vehicle-treated sham group. $p < 0.05$ (#) vs. vehicle-treated 2-hour isoflurane exposure group. N.S. indicates no significant difference.

cant interaction between isoflurane and ramelteon on time spent in the zone with the young mouse, as measured by the habituation score ($F_{1,29} < 1.0$; Fig. 2G). Additionally, the discrimination score was not significantly affected by the interaction between isoflurane and ramelteon ($F_{1,29} = 1.503$, $p = 0.231$; Fig. 2H). These findings suggest that while prolonged isoflurane exposure may have disrupted some aspects of social familiarization and novelty recognition, the effects were selective and not uniformly observed across all behavioral parameters. Pretreatment with ramelteon at 0.3 mg/kg may offer partial protection against certain social behavioral alterations induced by prolonged isoflurane anesthesia.

3.3 Effect of 2-h Isoflurane Exposure and Ramelteon Pretreatment on Spatial Working Memory

To assess whether the 2-h exposure to isoflurane combined with laparotomy alone, or in combination with pretreatment with ramelteon at 0.3 mg/kg, affects hippocampus-dependent spatial working memory, aged mice were subjected to the Y-maze test 24 h after surgery. The test was conducted over a 10-min period, excluding the initial 2-min habituation phase; the remaining 8-min exploration phase was used to assess spatial working memory. A one-way ANOVA revealed that neither prolonged isoflurane anesthesia nor ramelteon pretreatment resulted in differences in spontaneous alternation behavior from that of the vehicle-pretreated sham group ($F_{3,28} = 1.57$, $p = 0.223$; Fig. 3A). Total distance traveled was also unaffected by either treatment ($F_{3,28} < 1.0$; Fig. 3B). Furthermore, there was no significant bias in the distance traveled among the three arms ($F_{2,80} < 1.0$; Fig. 3C). Similarly, neither the time spent in each arm ($F_{2,80} < 1.0$, $p = 0.481$) nor the number of entries into each arm ($F_{2,80} = 1.540$, $p = 0.221$) differed between the isoflurane-exposed and ramelteon-pretreated groups (Fig. 3D,E). These findings suggest that 2-h isoflurane exposure with laparotomy did not induce detectable impairments in spatial working memory under the present conditions, and that ramelteon pretreatment did not exert measurable effects on this task.

3.4 Effect of 2-h Isoflurane Exposure and Ramelteon Pretreatment on Novel-Object Recognition Test

Next, we investigated whether non-spatial recognition memory was influenced by 2-h isoflurane exposure with laparotomy and pretreatment of ramelteon at a dose of 0.3 mg/kg in aged mice after surgery. One-way ANOVA indicated that neither 2-h isoflurane exposure nor ramelteon pretreatment (0.3 mg/kg) affected locomotor activity during the open field test ($F_{3,25} < 1.0$; Fig. 4A). Additionally, neither 2-h isoflurane exposure nor ramelteon pretreatment (0.3 mg/kg) affected performance in the novel-object recognition test (one-way ANOVA: $F_{3,25} = 1.709$, $p = 0.196$; Fig. 4B). All groups equally approached the two identical objects, but the number of entries into the zone locating the

novel object was significantly higher than that for the familiar object in each group: sham + vehicle ($F_{3,27} = 12.27$, $p < 0.01$; Tukey's HSD: novel object, $p < 0.01$ vs. familiar object), sham + ramelteon 0.3 mg/kg ($F_{3,19} = 5.461$, $p < 0.01$; Tukey's HSD: novel object, $p < 0.05$ vs. familiar object), 2-h isoflurane + vehicle ($F_{3,27} = 17.515$, $p < 0.01$; Tukey's HSD: novel object, $p < 0.01$ vs. familiar object), and 2-h isoflurane + ramelteon 0.3 mg/kg ($F_{3,23} = 7.360$, $p < 0.01$; Tukey's HSD: novel object, $p < 0.01$ vs. familiar object) (Fig. 4C–F). These findings suggest that neither 2-h isoflurane exposure nor ramelteon pretreatment impaired non-spatial recognition memory under the present experimental conditions.

3.5 Effect of 2-h Isoflurane Exposure and Ramelteon Pretreatment on Cytokines and Microglial Activation in the Prefrontal Cortex and Hippocampus

Finally, we investigated the effect of 2-h isoflurane exposure with laparotomy and ramelteon pretreatment on cytokines and microglial activation in the prefrontal cortex and hippocampus. Since ramelteon at 0.3 mg/kg did not affect social recognition or working memory in the vehicle-treated sham control mice, we examined its effects only in the 2-h isoflurane anesthesia with laparotomy group by comparing protein levels of cytokines, markers of microglial activation, and brain-derived neurotrophic factor (BDNF), a marker of synaptic plasticity, among the vehicle-treated sham group, the vehicle-treated isoflurane group, and the ramelteon-treated isoflurane anesthesia with laparotomy group. IL-1 β protein levels in the prefrontal cortex were significantly higher in 2-h isoflurane-exposed mice than in the vehicle-treated sham control group. However, this increase in IL-1 β was prevented by pretreatment with ramelteon (0.3 mg/kg) in the prefrontal cortex (Fig. 5A), whereas IL-1 β levels in the hippocampus (Fig. 5B) were not affected by 2-h isoflurane exposure (prefrontal cortex: one-way ANOVA, $F_{2,14} = 4.548$, $p < 0.05$; sham + vehicle: $p < 0.05$ vs. 2-h isoflurane; $p = 0.8678$, vs. 2-h isoflurane + ramelteon 0.3 mg/kg; Fig. 5C: one-way ANOVA, $F_{2,20} < 1.0$; Fig. 5D). The original western blotting (WB) figures of IL-1 β and IL-6 for Fig. 5A,B are provided in the **Supplementary Figs. 2,3**. The original WB figures of β -Actin for Fig. 5A,B are provided in the **Supplementary Fig. 4**. IL-6 protein levels were unaffected by 2-h isoflurane exposure in both the prefrontal cortex and hippocampus 24 h after surgery (prefrontal cortex: $F_{2,8} < 1.0$; hippocampus: $F_{2,11} < 1.0$; Fig. 5E,F).

We then examined whether microglial activation was induced by 2-h isoflurane exposure with laparotomy by assessing the IBA-1 protein levels in the prefrontal cortex and hippocampus. IBA-1 protein levels were not affected by 2-h isoflurane exposure, suggesting that significant microglial activation was not observed in either region (IBA-1 in prefrontal cortex: $F_{2,17} = 1.808$, $p = 0.120$; IBA-1 in hippocampus: $F_{2,8} < 1.0$; Fig. 5G–J). The origi-

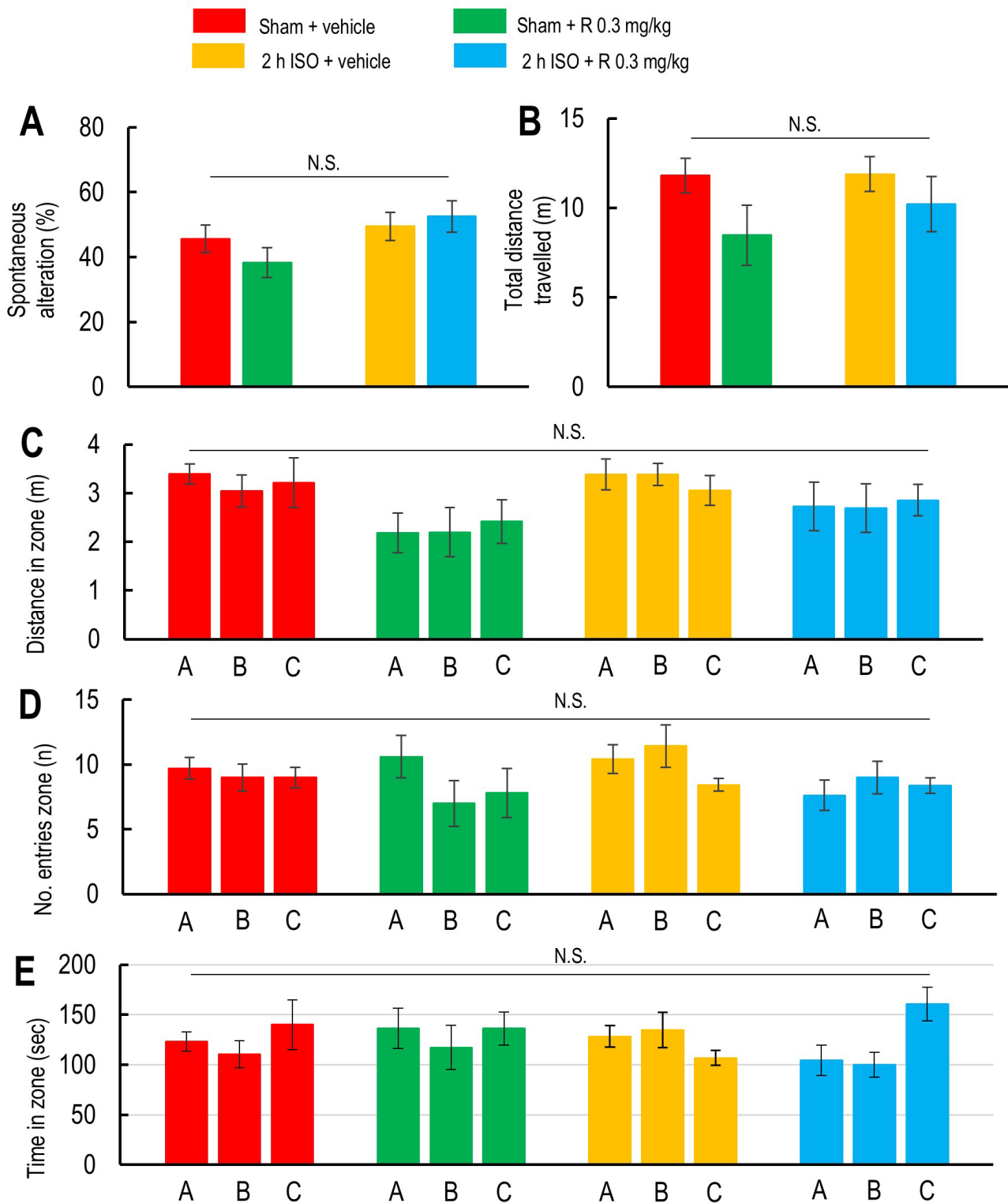


Fig. 3. Effects of 2-hour isoflurane exposure with laparotomy and ramelteon pretreatment on working memory assessed by Y-maze spontaneous alternation. (A,B) Spontaneous alternation percentage (A) and the total distance traveled during the test (B) in the vehicle-treated sham group (red; $n = 7$), ramelteon 0.3 mg/kg-treated sham group (light green; $n = 5$), vehicle-treated 2-hour isoflurane exposure group (yellow; $n = 7$), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane exposure group (light blue; $n = 8$). Total distance traveled in each arm (C), total number of entries into each arm (D), and total time spent in each arm (E) were analyzed to assess cognitive performance and general activity in the vehicle-treated sham group (red), ramelteon 0.3 mg/kg-treated sham group (light green), vehicle-treated 2-hour isoflurane exposure group (yellow), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane exposure group (light blue). N.S. indicates no significant difference.

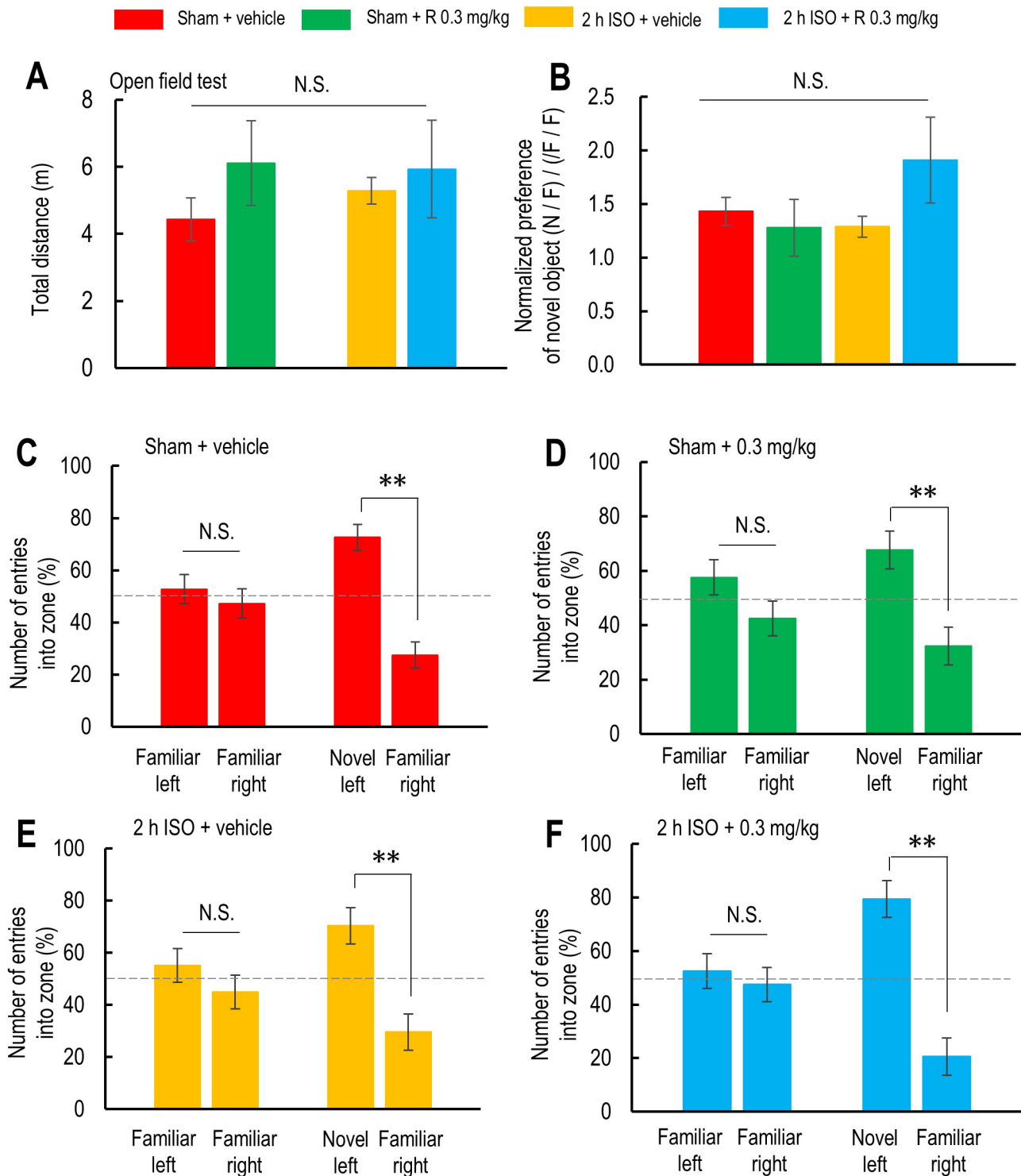


Fig. 4. Effects of 2-hour isoflurane exposure with laparotomy and ramelteon pretreatment on performance in the novel object recognition test. (A,B) Total distance traveled during the test (A) and normalized preference for the novel object (B) in the vehicle-treated sham group (red; $n = 7$), ramelteon 0.3 mg/kg-treated sham group (light green; $n = 5$), vehicle-treated 2-hour isoflurane exposure group (yellow; $n = 7$), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane exposure group (light blue; $n = 6$). (C–F) Number of entries into the object zones during the first 5 minutes (with two identical objects) and the last 5 minutes (with one familiar and one novel object) in the vehicle-treated sham group (C), ramelteon 0.3 mg/kg-treated sham group (D), vehicle-treated 2-hour isoflurane exposure group (E), and ramelteon 0.3 mg/kg-treated 2-hour isoflurane exposure group (F). $p < 0.01$ (**). N.S. indicates no significant difference.

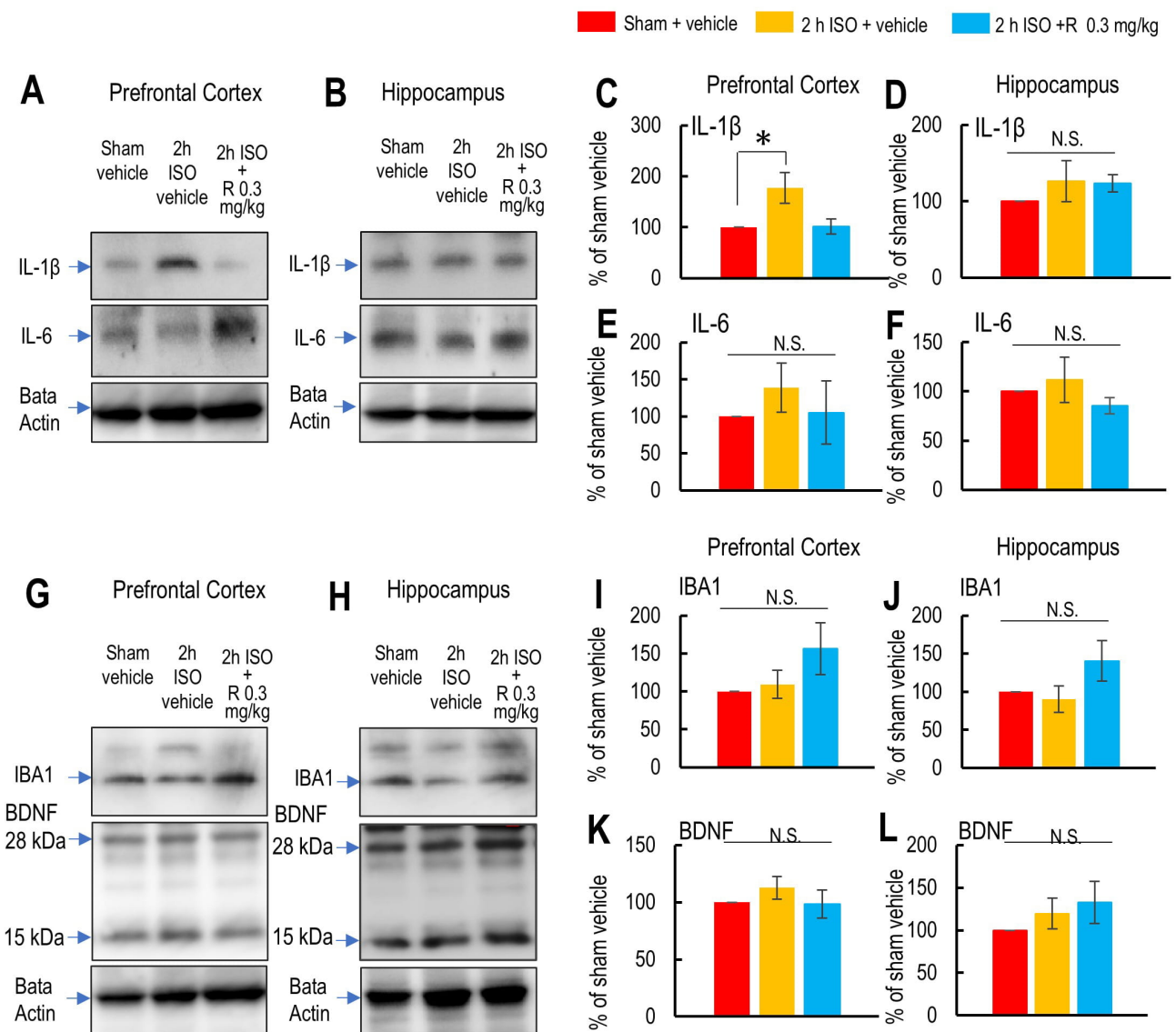


Fig. 5. Effects of 2-hour isoflurane exposure with laparotomy and ramelteon pretreatment on protein levels of cytokines, activated microglia, and brain-derived neurotrophic factor (BDNF) in the prefrontal cortex and hippocampus. (A,B) Representative western blot images showing IL-1 β (upper) and IL-6 (lower) in the prefrontal cortex (A) and hippocampus (B). (C,D) Relative protein levels of IL-1 β in the prefrontal cortex (C) and hippocampus (D) in the 2-hour isoflurane exposure group and the ramelteon (0.3 mg/kg)-treated 2-hour isoflurane exposure group, expressed as a percentage of the levels in the vehicle-treated sham group ($n = 5$ for cortex; $n = 6$ for hippocampus). (E,F) Relative protein levels of IL-6 in the prefrontal cortex ($n = 3$) (E) and hippocampus ($n = 4$) (F). (G,H) Representative western blot images showing IBA-1 (upper) and BDNF (lower) in the prefrontal cortex (G) and hippocampus (H). (I,J) Relative IBA-1 protein levels in the prefrontal cortex ($n = 6$) (I) and hippocampus ($n = 3$) (J). (K,L) Representative western blot images showing BDNF in the prefrontal cortex ($n = 4$) (K) and hippocampus ($n = 6$) (L) compared to the vehicle-treated sham group. $p < 0.05$ (*). N.S. indicates no significant difference.

nal WB figures for Fig. 5G,H are provided in the **Supplementary Figs. 5,6**). These results suggest that IBA-1 expression may not reliably indicate microglial activation, as morphological changes in microglia appeared to correlate more strongly with IL-1 β expression [47].

Additionally, we assessed whether the levels of BDNF, a key protein involved in synaptic plasticity, were affected by 2-h isoflurane exposure. BDNF levels were

not altered by 2-h isoflurane exposure in either the prefrontal cortex or hippocampus (BDNF in prefrontal cortex: $F_{2,11} < 1.0$; BDNF in hippocampus: $F_{2,20} < 1.0$; Fig. 5G,H,K,L). These results suggest that 2-h isoflurane exposure did not influence synaptic plasticity. In summary, 2-h isoflurane exposure specifically increased IL-1 β levels in the prefrontal cortex, but it did not significantly affect the hippocampus or other markers of microglial activation,

such as IBA-1. Furthermore, synaptic plasticity, as indicated by BDNF levels, was not affected by isoflurane exposure in our model using aged mice.

4. Discussion

4.1 A Model of Postoperative Hyperactivity Induced by 2-h Isoflurane Exposure in Aged Mice

Postoperative memory impairment and cognitive dysfunction have been observed in various rodent models, including aged mice, with several underlying neuronal mechanisms proposed to account for these impairments, such as neuroinflammation, synaptic dysfunction, and disturbances in neurotransmitter systems [17,18,48]. However, the mice used in the present study demonstrated prolonged hyperactivity after isoflurane exposure, which may represent a phenomenon related to, but distinct from, the clinical features of POD. POD can manifest in at least two forms: a hypoactive form, characterized by drowsiness and reduced motor activity, and a hyperactive form, characterized by agitation, aggression, restlessness, and the potential for self-harm or harm to others. Both forms are associated with core features of delirium, including disturbances in attention, awareness, and cognition [49]. Postoperative hyperactive delirium, a specific subtype of POD, affects approximately 15% of older patients undergoing non-cardiac surgeries, such as hip-fracture repair, under regional anesthesia [50]. POD is often observed starting from postoperative Day 1 and can persist for up to 1 week [51]. Although many studies have reported cognitive dysfunction after inhalational anesthesia in mice and rats, no studies to date have documented sustained hyperactivity over several days post-surgery in rodent models, despite numerous clinical reports on POD and hyperactivity [49,52]. Intracranial surgery in mice under isoflurane anesthesia has been reported to increase locomotor activity during the initial peak of the dark phase; however, locomotor activity usually decreases from postoperative Day 2 [53]. Additionally, volatile anesthetics have been shown to induce hyperactivity during the induction phase but produce full anesthesia at higher concentrations [54,55]. However, these phenomena are not considered to represent POD. In our study, a 2-h exposure to isoflurane significantly increased locomotor activity for over 7 days after surgery, particularly during the dark phase, which corresponds to the active period in mice. Notably, this prolonged hyperactivity was not observed in younger mice (6–10 weeks old; **Supplementary Fig. 7**) after the same isoflurane exposure. These findings suggest that our model may represent a specific form of postoperative hyperactivity in aged mice, but further studies are needed to clarify its direct relevance to POD.

4.2 Social-Recognition Deficit and Protein Level of IL-1 β in the Prefrontal Cortex of Aged Mice

In the present study, we found that social recognition, as assessed by the social-interaction test, was im-

paired in mice after 2-h isoflurane exposure with laparotomy, but not in mice that underwent a sham laparotomy. The prefrontal cortex has been suggested to play a key role in regulating social interactions, including social approach and recognition [56,57]. Reduced excitatory synaptic transmission in the pyramidal neurons of the supragranular layer of the prefrontal cortex has been shown to lead to impaired social interaction [58]. Reciprocal connections between the thalamic area and the prefrontal cortex also play an important role in social recognition by encoding social information [57]. Moreover, N-methyl-D-aspartic acid (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate glutamate receptors in the prefrontal cortex have been implicated in social-recognition memory [59], and synaptic plasticity in this region is crucial for cognitive behaviors [60,61]. Therefore, elevated neuronal excitability in pyramidal neurons of the supragranular layer of the prefrontal cortex contributes to social recognition and memory.

In the present study, 2-h isoflurane exposure increased the protein level of IL-1 β in the prefrontal cortex, which is consistent with a previous report indicating that surgery increases the IL-1 β level in the prefrontal cortex [62]. IL-1 β reduces the magnitude of long-term potentiation in the rat frontal cortex [63], as well as in the hippocampus of young animals [64,65]. Therefore, the increased IL-1 β level after 2 h of isoflurane exposure may impair social recognition by inhibiting the induction of synaptic plasticity in the prefrontal cortex, as observed in the present study. Although our findings demonstrated a region-specific increase in IL-1 β expression in the prefrontal cortex alongside impaired social recognition, these results remain correlational and do not establish a direct causal relationship. Further studies employing targeted pharmacological interventions, such as IL-1 receptor antagonists or microglial inhibitors, are needed to elucidate the mechanistic role of neuroinflammatory signaling in postoperative cognitive dysfunction.

4.3 Hippocampus-Dependent Cognitive Functions and Hippocampal Cytokine Levels Were not Affected by a 2-h Isoflurane Exposure Combined With Laparotomy

Although many studies have reported hippocampus-dependent memory impairments and increased cytokine levels in the hippocampus after long-term isoflurane exposure [66–71], the current study found no changes in hippocampal cytokine levels, including IL-1 β and IL-6, after 2 h of isoflurane exposure. Some studies have reported that long-term exposure to isoflurane (5 to 6 h) can induce cognitive dysfunction by disrupting the circadian rhythm in mice [26,27]. In addition, 2-h isoflurane anesthesia has also been demonstrated to impair the working memory in rats and mice [66–68]. Notably, some of these studies assessed cognitive function 3 to 7 days after surgery, whereas the current study evaluated cognitive function 24 h postoperatively. In contrast to these reports, another study suggested

that hippocampus-dependent cognitive functions may be impaired within 9 h after a 2-h isoflurane exposure, but not at 24 h after surgery [72]. Given that more than two-thirds of patients develop delirium on postoperative Day 0 or Day 1 [73], we evaluated cognitive function in aged mice 24 h after a 2-h isoflurane exposure. In the present study, aged mice subjected to isoflurane anesthesia and abdominal surgery exhibited preserved performance in both the spontaneous alternation Y-maze task and the novel-object recognition task. These findings suggest that the cognitive domains assessed by these tasks—spatial working memory and object recognition—were not significantly impaired in our experimental paradigm. In the present study, we examined the impact of isoflurane anesthesia and surgery using two working-memory tasks: the spontaneous alternation Y maze, which involves the hippocampus, and the novel-object recognition task with a short retention interval, which primarily reflects hippocampus-independent memory processes. The Y-maze spontaneous-alternation task, although partially reliant on hippocampal integrity, is also influenced by activity in the prefrontal cortex and basal forebrain [42]. Similarly, the short-retention novel-object-recognition-test protocol used in our study (5-min delay) is primarily mediated by the perirhinal cortex rather than the hippocampus [74,75]. Thus, the absence of cognitive deficits in these tasks did not rule out the possibility of subtle impairments in hippocampus-dependent processes, which may have only been detectable using more selective paradigms such as the long-delay novel-object recognition test or spatial navigation tests.

Our molecular analyses revealed a region-specific neuroinflammatory response, characterized by significantly increased IL-1 β levels, in the prefrontal cortex but not in the hippocampus. This pattern aligned with our behavioral findings: social-habituation behavior—a process strongly associated with the function of the prefrontal cortex and amygdala—was impaired, whereas recognition memory and working memory performance remained intact. Previous studies have focused on the role of the prefrontal cortex and its susceptibility to neuroinflammation in mediating social cognition [76]. Additionally, the nucleus reuniens of the thalamus, which connects the prefrontal cortex and hippocampus, may contribute to the coordination of memory-related processes between these regions [77,78]. Our results suggest that localized neuroinflammation in the prefrontal cortex may have selectively impaired social cognitive functions, while sparing hippocampus- and perirhinal-cortex-mediated tasks under the current experimental protocol. We should note that the cognitive tests used in this study may not fully capture all aspects of hippocampus-dependent memory; further research using more specific and sensitive tests is required. Future studies incorporating hippocampus-specific tasks and circuit-level manipulations will be valuable to further dissect the region-specific contributions to postoperative cognitive alterations in aged

animals. Moreover, the lack of a control group receiving isoflurane anesthesia alone limited our ability to distinguish the effects of anesthesia from those of surgery, and this limitation should be acknowledged. Achieving a reliable incidence of cognitive dysfunction induced by isoflurane anesthesia in rodents may require more carefully standardized experimental conditions to better understand the mechanisms of POD in the future.

4.4 Possibility of a Preventive Effect of Ramelteon Pretreatment on the Onset of Postoperative Cognitive Dysfunction

In the current study, we found that pretreatment with ramelteon for 7 days before to 2-h isoflurane exposure prevented hyperlocomotor activity for 7 days, social recognition deficits, and an increase of IL-1 β protein levels in the prefrontal cortex of aged mice 24 h after surgery; in addition, hippocampus-dependent learning and memory, as well as cytokine levels in the hippocampus, were not affected. As previously reported, pretreatment with melatonin for 7 days prevented cognitive dysfunction in mice on Day 3 after exposure to 5- to 6-h isoflurane by modulating the sleep-wake cycle [26,27]. Significant alterations in circadian rhythm were not observed in the current study. Hyperlocomotor activity was not induced by 6-h isoflurane exposure in 2-month-old C57BL/6J mice [27], which is consistent with our observation in 6-week-old C57BL/6J mice (**Supplementary Fig. 2**). Therefore, the hyperlocomotor activity observed for 7 days after isoflurane exposure appears to be specific to aged mice. We administered ramelteon once daily, 1 h before the onset of the dark phase (melatonin is secreted in the dark phase) which is the active period for mice [38]. From these results, we inferred that ramelteon reduced locomotor activity during the dark phase. In young mice, ramelteon 0.3 mg/kg reduced locomotor activity during the light phase only, the inactive period for rodents, for the first 2 days. In aged mice, however, the same dose only affected locomotor activity during the dark phase throughout the 7 days after 2-h isoflurane exposure. These results suggest that ramelteon pretreatment (0.3 mg/kg) may not have simply reduced locomotor activity in general but rather modulated rest during the inactive phase in young mice and mitigated hyperlocomotor activity during active periods in aged mice. In clinical studies using double-blind, randomized, placebo-controlled trials, the effects of ramelteon on POD have yielded controversial results [28,29,31–34]. Therefore, the efficacy of the melatonin receptor agonist ramelteon for treating delirium has not yet been conclusively established.

Additional strategies targeting different mechanisms of POD should include controlling the depth of sedation during anesthesia administration. In line with the controversial results of ramelteon treatment, due to the multifaceted and heterogeneous nature of POD, replication of

the full spectrum of its clinical manifestations in a single animal model remains challenging. Likewise, it is unlikely that a single pharmacological intervention can comprehensively ameliorate all associated symptoms. Therefore, it is essential for future research to delineate which specific aspects of POD are represented by each animal model and to identify targeted pharmacological strategies that can selectively mitigate those symptoms. Such an approach would facilitate a more nuanced understanding of POD pathophysiology and support the development of tailored therapeutic interventions. In line with this, accumulating pharmacological evidence suggests that the neuroprotective and anti-inflammatory effects of melatonin are primarily mediated by MT₁ and MT₂ receptors, which are expressed in POD-relevant brain regions such as the prefrontal cortex and hippocampus. For instance, luzindole, a non-selective MT₁/MT₂ antagonist, has been widely used to investigate receptor-mediated effects of melatonin in the central nervous system [79]. Similarly, the selective MT₂ antagonist *cis*-4-Phenyl-2-propionamidotetralin (4P-PDOT) has been shown to block melatonin-induced modulation of neuroinflammation and cognition in rodents [80]. Although our study did not include MT-receptor antagonists, the observed protective effects of ramelteon—especially its suppression of IL-1 β in the prefrontal cortex and rescue of social recognition—may reflect its receptor-specific actions. Future studies incorporating MT₁/MT₂ antagonists will be essential to delineate the receptor-level mechanisms underlying the therapeutic effects of ramelteon in postoperative neurocognitive dysfunction.

4.5 Study Limitations

Repeated exposure to isoflurane has been investigated in animal models because single exposures often did not produce robust or reproducible cognitive impairments in rodents [81–83]. However, the results of these studies have been inconsistent, particularly regarding the extent of cognitive deficits caused by repeated anesthetic exposure in aged animals. Although some studies reported no impairments in spatial memory or psychomotor performance in aged mice [19,82,83], others suggested potential adverse effects on cognitive functions depending on factors such as age, duration of exposure, and interval of exposure. For example, repeated exposures to isoflurane even facilitated spatial learning in young adult mice [81]. Those findings suggested that increasing the frequency or duration of anesthetic exposure does not necessarily exacerbate cognitive dysfunction. Furthermore, our current design incorporates surgical stress combined with a single isoflurane exposure in aged mice, which may better replicate the clinical conditions that produce postoperative cognitive dysfunction. Thus, although repeated-exposure models may offer additional insights, a single exposure, combined with surgery, remains a relevant and valid approach to studying postoperative neurobehavioral changes. Nevertheless, future stud-

ies that include repeated exposures would be valuable for further exploring the effects of anesthetic duration and frequency on postoperative cognitive dysfunction.

Another limitation is that the present study did not include an anesthesia-only control group. Our paradigm was designed to reflect realistic perioperative conditions, in which general anesthesia is typically administered for surgical procedures as is the case in many studies using mice [18,72,84–86]. However, it is important to consider the potential impact of prolonged anesthesia alone. Previous studies have shown that anesthesia alone does not consistently result in cognitive or behavioral impairments [85,87]. On the other hand, it has also been reported that isoflurane anesthesia indeed alters the brain metabolites of mice immediately after anesthesia [88]. Those findings suggest that the behavioral changes observed in our study may have stemmed primarily from the combination of surgical stress and prolonged anesthesia. Nonetheless, including an anesthesia-only group in future studies would help clarify the individual contribution of anesthesia to postoperative neurobehavioral outcomes, particularly in aged brains.

5. Conclusion

A 2-h isoflurane anesthesia exposure, combined with abdominal surgery, induced prolonged hyperactivity and impaired social recognition in aged mice, without impairing working memory functions; both of these cognitive processes involve not only the hippocampus but also other brain regions such as the perirhinal cortex. Pretreatment with ramelteon 0.3 mg/kg prevented these deficits and suppressed IL-1 β elevation in the prefrontal cortex. These findings suggest that our model reflects certain aspects of postoperative behavioral disturbances, particularly hyperactivity and mild neuroinflammatory changes in aged animals. Ramelteon may alleviate postoperative neurobehavioral impairments by modulating prefrontal inflammation, which supports its potential as a therapeutic agent for POD. However, considering the heterogeneity of POD in clinical settings, caution is warranted in generalizing these results. No single animal model can recapitulate all its symptoms, nor is it likely that a single drug can address all its manifestations. Future research should clarify which symptoms each model captures, and should identify targeted strategies for mitigating specific behavioral and molecular alterations.

Abbreviations

ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; IBA, ionized calcium-binding adapter molecule 1; IL, interleukin; ISO, isoflurane; MT, melatonin; NIH, National Institutes of Health; N.S., not significant; 4P-PDOT, *cis*-4-Phenyl-2-propionamidotetralin; POD, postoperative delirium; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean;

TEMED, N,N,N',N'-Tetramethylethylenediamine; Tukey HSD test, Tukey honestly significant difference test; WB, western blotting.

Availability of Data and Materials

Data are available from the corresponding author upon a reasonable request and with the permission of KS.

Author Contributions

ET, MA, RM, and KS performed experiments and acquired and analyzed all data. KS designed the experiments, performed the experiments, and analyzed all the data. KS conceived and designed the experiments and wrote the first draft of the manuscript. ET, MA, RM, and KS analyzed and interpreted all data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Animal Care Committee of Ohu University (Approval Nos. 2022-34, 2023-14, and 2024-29). All animal procedures were conducted in accordance with the guidelines of the Animal Care Committee of Ohu University. The three Rs principles (Replacement, Reduction, and Refinement) were implemented throughout the present study by adopting the principles of laboratory animal care to minimize distress and utilizing the minimum number of required animals for all experiments. This study was carried out in compliance with the ARRIVE guidelines. We strictly adhered to the principles of laboratory animal care to minimize animal stress and use the minimum number of animals necessary for the experiments.

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Conflict of Interest

The authors declare the following financial and personal relationships that could be perceived as potential competing interests: KS has received research funding from Lead Chemical Co., Ltd. All other authors declare no conflicts of interest. No employees of Lead Chemical Co., Ltd. were involved in data analysis, peer review selection, or any editorial decisions related to this manuscript.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/JIN42403>.

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