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A modified mouse model of perioperative neurocognitive disorders exacerbated by sleep fragmentation

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Abstract: Aging is one of the greatest risk factors for postoperative cognitive dysfunction (POCD), also known as perioperative neurocognitive disorder (PND). Animal models of PND are usually induced in mice over 18 months of age, which imposes expensive economic and time costs for PND-related studies. Sleep disorders, including sleep fragmentation, are reported to aggravate memory impairment in neurocognitive-related diseases such as Alzheimer's disease (AD). Therefore, the aim of the present study was to explore whether a PND model could be constructed in younger mice with the help of fragmented sleep. We found that fragmented sleep followed by laparotomy under isoflurane anesthesia could stably induce PND in 15-month-old mice. To determine whether the neurocognitive decline in this model could be salvaged by clinical treatments, we administered repetitive transcranial magnetic stimulation (rTMS) to the model mice before anesthesia and surgery. We found that 10 days of high-frequency rTMS (HF-rTMS) could improve spatial learning and memory deficits in this modified PND model. We are the first to successfully construct a PND model in younger mice,which is more economical, that can be used as an alternative model for future PND studies.
Key words: anesthesia, animal model, perioperative neurocognitive disorder, sleep, surgery

Introduction

Postoperative cognitive dysfunction (POCD), also known as perioperative neurocognitive disorder (PND), is one of the most common serious complications after anesthesia and surgery in elderly patients. PND is classified as early postoperative delirium (POD) or long-term POCD and clinically manifests as perioperative confusion, anxiety, personality changes, and memory impairment [1–3]. Aging, surgical stress, and anesthesia are regarded as the most important risk factors for PND. Animal models of PND are usually constructed by using mice over 18 months of age, which imposes expensive economic and time costs for PND-related studies [4, 5]. Therefore, a younger PND model is needed to meet research needs. Aging is also correlated with sleep disorders especially fragmented sleep, which has been shown in previous studies to be characterized by increased wakefulness after sleep, increased arousals, and decreased quantity and quality of "deep" sleep stages in the elderly [6–8]. Unlike sleep deprivation, sleep fragmentation (SF) does not necessarily affect total sleep time, but it does reduce the total time spent in "deep" sleep [9]. It has been noted that older patients suffer more

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sleep fragmentation after hospital admission due to noise, pain, lighting, anxiety, and medical screening, while it is not clear whether preoperative sleep fragmentation will increases vulnerability to PND in older patients, advancing the age to PND susceptibility [10, 11]. An interesting perioperative issue is whether it is possible to create a younger PND model with exacerbation of a sleep disorder.

There are no effective methods of preventing or treating PND yet. Repetitive transcranial magnetic stimulation (rTMS) is a safe, inexpensive, noninvasive method of extracranial stimulation used worldwide to treat neurological or psychiatric disorders and to improve or maintain cognitive function in healthy older adults [12–14]. We therefore considered whether rTMS plays a protective role in the "pathological" setting for PND patients with preoperative SF as it does in patients with other cognitive-related disorders or healthy individuals. Electrical stimulation can excite or inhibit neuronal activity in the brain, and by adjusting its stimulation parameters, such as frequency and intensity, it can play different roles in neuromodulation [15, 16]. Different TMS frequencies have different effects on cognition in animal experiments. High-frequency rTMS (HF-rTMS) improves spatial learning and memory impairment in aged mice, and the effect of 5 Hz is more significant than 25 Hz [17].

Therefore, the present study aimed to investigate whether a PND model could be created in younger mice through the exacerbation of a sleep disorder and if so, whether rTMS before surgery could improve the neurocognitive functions of PND mice.

Materials and Methods

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Tongji University (Shanghai, China, TJBH07922101), and all procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Aged (18 months), middle-aged (15 and 12 months), and young male C57BL/6J mice (6 months and 6 weeks) were purchased from the Laboratory Animal Management Department of the Shanghai Institute of Family Planning Science. All mice were habituated for 1 week before receiving any intervention and housed (3 to 5 mice) in a controlled environment with an ambient temperature of 22 ± 1°C, 30 to 70% humidity, a 12-h light (ZT0-ZT12)/12-h dark (ZT12-ZT24) cycle, and access to food and water *ad libitum*.

PND model

Mice were subjected to experimental laparotomy under isoflurane anesthesia to construct PND models as previously reported, with some modifications [18]. Briefly, mice were anesthetized by inhalation of 2.0% isoflurane (oxygen flow rate 0.5 l/min), and the mid-abdomen was shaved and disinfected. A 2.5-cm-long longitudinal incision was then made along the midline of the abdomen, and the skin and fascia were incised sequentially in layers to separate the right and left rectus abdominis muscles. A segment of the ileum innervated by a mesenteric artery was then gently retracted with ophthalmic forceps and placed on the surface of sterile gauze moistened with warm saline at 37°C, which was used to cover the exposed intestinal segment. After 10 min, the gauze was removed, and the exposed bowel segment was returned to the abdominal cavity to avoid postoperative intestinal obstruction. The abdomen was closed, and the rectus abdominis fascia and skin were closed in layers with absorbable sutures and disinfected. Lidocaine cream was applied to complete the postoperative analgesia. The entire procedure took 30 min.

SF model

Referring to a previous report, mice were placed in a sleep deprivation apparatus [19] (cylindrical container 30 cm in diameter and 35 cm in height with a rotating bar at the bottom) and subjected to a regular light (ZT0-12)/ dark (ZT12-24) cycle with free access to food and water. During the 72 h from ZT8 to ZT80, the rotating bar was rotated along the bottom of the cage every 120 s; in other words, the mice were allowed periods of 120 s of consecutive sleep continuity, interrupted by 10 s, and were aroused 30 times/h. This is the frequency of sleep interruption typically observed in sleep apnea and reported as a novel animal model of SF [9].

rTMS treatment

To administer rTMS to the mice, the mice were placed vertically in a plastic immobilizer, the cylindrical shape of which temporarily inhibited the movement of the mice and exposed the top of the head without causing injury [20]. The mice breathed normally under rTMS stimulation without significant struggling. The rTMS was delivered by a magnetic stimulator (CCY-II, Wuhan Yiruide Medical Equipment, Wuhan, China) connected to a round coil (diameter: 6.5 cm) [21]. The heads of the mice were pressed against the center of the coil. The rTMS was performed between 9 a.m. and 12 a.m. for 10 consecutive days. On each day, the mice received 10 sessions

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of rTMS treatment with inter-session interval of 30 s. In each session, 100 burst trains of 5 Hz stimulation were delivered, with the magnetic stimulation intensity set at 0.84 T [17]. The sham and control mice underwent the same procedures, including restraint and being exposed to the noise from the magnetic stimulator, except that they were not placed under the coil.

Experimental design

Experiment 1: To observe the trend of PND susceptibility in mice at different ages, 6-week-, 6-month-, 12-month-, 15-month-, and 18-month-old male C57BL/6J mice were subjected to surgery with isoflurane anesthesia, and neurocognitive function was consecutively assessed from postoperative day 3 to 10 (Fig. 1A).

Experiment 2: To observe the effect of SF on PND susceptibility, mice of appropriate age according to the results of Experiment 1 were selected to produce SF 72 h before surgery, followed by neurocognitive assessment from postoperative day 3 to 10 (Fig. 1B).

Experiment 3: To explore whether rTMS has an ameliorative effect on postoperative cognitive abilities in PND mice with preoperative SF, PND-susceptible mice were selected for rTMS treatment on 10 consecutive days before surgery, and neurocognitive assessments were performed from postoperative days 3 to 10 (Fig. 1C).

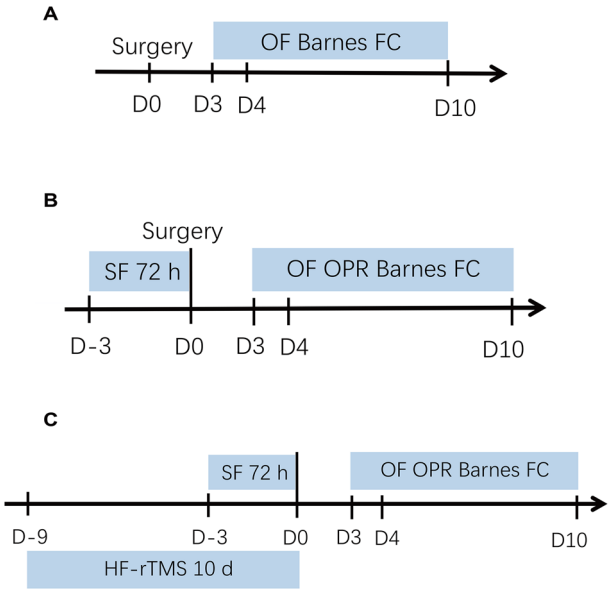

Fig. 1. Timelines of the experiments. (A) The timeline of laparotomy surgical modeling and behavioral testing of neurocognitive function, including the object-place recognition test (OPR), the open field test (OF), Barnes maze test (Barnes), and fear conditioning test (FC) in experiment 1. (B) The timeline of preoperative sleep fragmentation modeling and behavioral testing of neurocognitive function in experiment 2. (C) The timeline of preoperative sleep fragmentation modeling for HF-rTMS treatment and behavioral tests of neurocognitive function in experiment.

Neurocognitive function assessment

Object-place recognition: The object-place recognition test (OPR) was used to assess hippocampal-dependent spatial memory capacity according to a previous protocol, with some modifications [22]. The test was performed in a square opaque plastic box (40 × 40 × 35 cm) with visual cues hanging on three of the walls and a camera set up right above the box and connected to Animal Tracking System (Smart 3.0, Harvard Apparatus, Holiston, USA). Mice were subjected to object position coding and recall on the third day after modeling and habituation the night before. The OPR test consisted of habituation, training, and recall phases. For the habituation phase, mice were removed from their home cages, placed in the middle of an open field, and allowed to explored freely for 10 min, followed by placing them back into their home cages (the results from the habituation phase can be used as the results of an open field (OF) test used to assess the mobility and anxiety of mice). For the training phase, two identical objects were placed in two different quadrants of the open field, and the mice were then placed in the center of the open field and allowed to explore for 10 min 1 day after the habituation test. For the recall phase, one of the objects was moved to a new quadrant, and the mice were allowed to explore the same field for 5 min at 2 h after the training phase. Sniffing or touching the object at close range was considered valid exploration. The percentages of time spent exploring novel and old locations of objects were compared to assess recognition memory. The recognition memory index (%) was calculated as follows: (time spent exploring the new location − time spent in exploring the old location)/total exploration time × 100%.

Barnes maze test: The Barnes maze test was performed as described previously [5]. The Barnes maze comprised a circular open platform (approximately 90 cm in diameter) with 20 equally spaced holes (one of which was connected to a dark room called the escape box) that was located in a quiet area, artificially divided into four quadrants, and surrounded by a dark curtain, with four simple shapes (square, circle, triangle and star) as markers and a camera set up directly above the platform and connected to Smart Animal Tracking System 3.0. The experiment consisted of a training phase (from postoperative day 3 to 7) and a testing phase (on postoperative day 8) on a total of 6 consecutive days. Before each test, mice were allowed to habituate to the test room for 1 h. Odor cues were removed between sessions by wiping with 75% ethanol after each test. During the training phase,

bright light (200 W) and white noise (85 dB) were used as aversive stimuli. On the first day of training (postoperative day 3), the mice were placed in a clear glass enclosure in the center of the platform for 3 min, guided to the escape box, and allowed to stay there for 1 min. On the subsequent 4 days of the training phase (postoperative day 4 to 7), a total of 15 trials, comprising the 3 trials on postoperative day 4 and another 4 trials per day for the next 3 days (4 min per trial, 15 min apart), were performed. The white noise was turned off when the mice reached the escape box, and the mice were then allowed to remain in the escape box for 1 min and subsequently returned to their cages. During the testing phase, the escape box was removed, and the mice were then allowed to move freely for 2 min under the same aversive stimuli. An increase in escape latency, increase in the number of incorrect holes explored, and decrease in target quadrant time in the Barnes maze indicate that mice suffer from cognitive impairment. The target quadrant time ratio (%) was calculated as follows: time spent in the target quadrant during the test session / 120 s × 100).

Fear conditioning test: Mice were subjected to a contextual fear conditioning paradigm using the Ugo Basile Fear Conditioning System (Ugo Basile Srl, Gemonio, Italy), with slight modifications [5, 23], The fear conditioning test consisted of habituation (on postoperative day 8), training (on postoperative day 9), and testing phases (on postoperative day 10). On the habituation day, the mice were allowed to move freely in the conditioning chamber (17 cm × 17 cm × 25 cm) for 10 min. On the training day, the mice were received a total of five foot-shocks current, 0.7 mA for 2 s; foot shock interval, 35–60 s). On the testing day (24 h after training), the mice were tested for 5 min for contextual memory retrieval in the absence of aversive stimuli. The chamber was cleaned before and after each session with 75% ethanol. Freezing behavior was quantified using the ANY-maze Software (Stoelting Co., Wood Dale, IL, USA). Animals were considered to be in a state of freezing if no movement was detected within 2 s. The freezing ratio (%) was calculated as follows: freezing time/300 s × 100.

Immunofluorescence staining

On day 2 (48 h) following experimental laparotomy under isoflurane anesthesia to induce POCD, mice were anaesthetized and perfused with 0.9% saline, followed by 4% paraformaldehyde. The hippocampus was then collected and post-fixed with paraformaldehyde overnight at 4°C, followed by dehydration with 30% sucrose at 4°C for 72 h and freezing in Tissue Tek OCT (Sakura, Torrance, USA). Brain coronal sections (300 μm) were prepared using a vibratome (CM1950, Leica Biosystems, Nussloch, Germany) and stored at −20°C until immunofluorescence staining. The sections were permeabilized using 0.5% Triton X-100 and blocked with 5% goat serum for 90 min at room temperature. Incubation with chicken polyclonal GFAP (1:500, catalog no. ab254083, Abcam, Cambridge, UK) was Performed overnight at 4°C, and the sections were then washed in PBS. Next, the sections were incubated with goat anti-chicken IgG conjugated with Alexa Fluor 488 (1:500, code 103-545-155, Jackson ImmunoResearch, West Grove, PA, USA) in the dark for 90 min at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; catalog number 62248, Thermo Fisher Scientific, Waltham, MA, USA). Images were then obtained using a confocal laser fluorescence microscope (FV3000, Olympus, Tokyo, Japan). For each mouse, four random fields were selected from each section (hippocampal CA1 region) of each sample. The fluorescence intensity of astrocytes in the imaged field was quantified using the ImageJ software.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 9.1 software. For comparisons between pairs of groups, the Mann-Whitney test and unpaired Student *t*-test were used. To compare neurocognitive abilities between different ages, one-way ANOVA with a post hoc Dunnett's test was used. For spatial learning (primary latency), a two-way ANOVA was used to determine the statistical significance of differences between groups at different time points by Sidak's multiple comparisons. $P<0.05$ was considered to indicate statistical significance.

Results

Age-dependent effects of PND on hippocampal function

To elucidate whether SF increases hippocampal vulnerability to postoperative cognitive dysfunction, we first constructed a reliable PND model. Among the various PND models evaluated, the model based on experimental laparotomy was the most reproducible [5]. In the PND model, age is critical. In this study, to determine the appropriate age at which hippocampal-related cognitive decline can be induced in the PND model, mice were subjected to experimental laparotomy at different ages (6 weeks, 6 months, 12 months, 15 months, and 18 months), followed by a series of comprehensive behavioral tests of neurocognitive function from postoperative day 3 to day 10 (open field test, Barnes maze test, and

Table 1. Age-dependent hippocampal damage after laparotomy

Mean ± SEM	6 w	6 mo	12 mo	15 mo	18 mo	
Average speed (m/s)	0.074 ± 0.006	0.076 ± 0.007	0.068 ± 0.007	0.06 ± 0.008	0.063 ± 0.006	*P*=0.4843
Barnes maze training primary latency (s) D1	113.2 ± 15.1	146.9 ± 13.05	143.2 ± 10.69	139.5 ± 10.77	154.9 ± 8.404	*P*=0.4047
Barnes maze training primary latency (s) D2	61.34 ± 5.449	70.28 ± 11.9	74.39 ± 9.878	85.1 ± 10.52	106.7 ± 12.42	
Barnes maze training primary latency (s) D3	30.51 ± 2.474	35.07 ± 5.382	37.25 ± 4.717	56.85 ± 9.453	85.28 ± 11.13	
Barnes maze training primary latency (s) D4	26.15 ± 2.715	34.76 ± 2.749	37.76 ± 4.714	51.74 ± 8.261	51.73 ± 5.682	
Time in the target quadrant (%)	52.49 ± 4.296	48.17 ± 3.486	44.14 ± 2.322	40.63 ± 2.172	33.93 ± 3.978	*P*=0.005**
Freezing time ratio (%)	67.59 ± 5.325	65.63 ± 6.22	60.16 ± 5.777	56.55 ± 6.143	31.2 ± 4.197	*P*=0.0006***

***P*<0.01, ****P*<0.001.

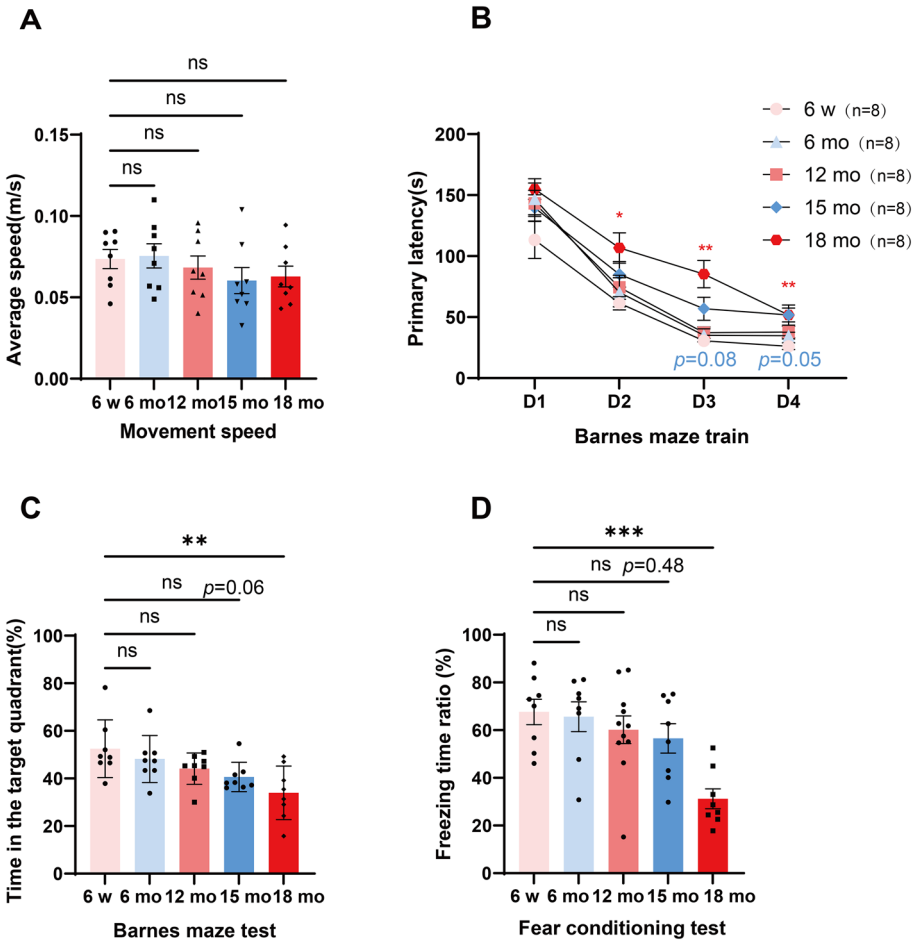

Fig. 2. Age-dependent hippocampal damage after laparotomy. (A) Locomotor activity was measured by OF (one-way ANOVA, *P*=0.4843). (B) Spatial learning ability was measured by the latency during the training phase in the Barnes maze test (Sidak's multiple comparisons test; *P*=0.131, *P*=0.031, *P*=0.006**, and *P*=0.009** for D1-D4, respectively, for 6w vs. 18m; *P*=0.168, *P*=0.248, *P*=0.08, and *P*=0.05 for D1-D4, respectively, for 6w vs. 15w). (C) Spatial memory performance was measured by the time in the target quadrant during the testing phase in the Barnes maze test (one-way ANOVA, *P*=0.005**; post hoc Dunnett's test, *P*=0.774, *P*=0.2531, *P*= 0.0578, and *P*= 0.0015** for 6w vs. 6m, 6w vs. 12m, 6w vs. 15m, and 6w vs. 18m, respectively). (D) Freezing time ratio on the fear conditioning test (FC) testing day (one-way ANOVA, *P*=0.0006***; post hoc Dunnett's test, *P*= 0.9976, *P*=0.733, *P*=0.4827, and *P*=0.0004*** for 6w vs. 6m, 6w vs. 12m, 6w vs. 15m, and 6w vs. 18m, respectively). Data are presented as the mean ± SE, with n=8 per group.

fear conditioning test) (Table 1).

Our results indicated no differences in locomotor abilities after laparotomy in mice that were 6 weeks to 18 months of age, as indicated by the velocity in the open field test (Fig. 2A). However, the Barnes maze and fear conditioning tests indicated that mice of different ages showed significant differences. In the Barnes maze test, postoperative spatial learning ability was similar be-

tween middle-age (12 and 15 months) and young (6 weeks and 6 months) mice but was significantly impaired in aged mice (18 month), as indicated by significantly prolonged latency during the training phase ($P<0.05$, Fig. 2B). Moreover, postoperative impairment of spatial memory performance was also observed in 18-month-old mice, as indicted by less time spent in the target quadrant compared with young and middle-aged mice in the testing phase ($P<0.01$, Fig. 2C). In the fear conditioning test, postoperative fear memory was also similar between middle-age and young mice but was significantly impaired in aged mice, as indicated by a significantly decreased freezing time during the testing phase ($P<0.001$, Fig. 2D). Noteworthily, the 15-month-old mice showed a trend toward decreased results in the Barnes maze and fear conditioning tests (Figs. 2B–D), although the decreases did not reach the level of statistical significance, indicating that 15 months might represent an age threshold for postoperative learning and memory impairment in mice.

Preoperative SF increased susceptibility to PND

To clarify the potential effects of SF on PND, 15-month-old mice (threshold age according to Experiment 1) were exposed to 3 consecutive days of SF, which has been proven to reliably interfere with the sleep rhythms of mice [24], and then underwent surgery and anesthesia as previously described. Our results revealed that 15-month-old mice with preoperative SF showed cognitive decline and impaired spatial memory (Table 2), as indicated by less time spent in the new location and a decreased difference recognition index in the OPR test compared with the mice that only received surgery or SF (Fig. 3C). Besides, impaired postoperative spatial learning, as indicated by significantly prolonged latency on training days 1 and 2 (Fig. 3D), and impaired memory, as indicated by less time spent in the target quadrant (Fig. 3E), more errors in explorations (Fig. 3F), and prolonged latency of the first successful attempt to reach the target hole (Fig. 3G), in the testing phase, were also observed in 15-month-old mice with preoperative SF in the Barnes maze test compared with the mice that only received surgery or SF. Moreover, postoperative fear memory was also significantly impaired, as indicated by a decreased freezing time in 15-month-old mice with preoperative SF in the conditioned fear test, compared with the mice that only received surgery or SF (Fig. 3H). To rule out the possibility that this decrease in spatial learning and memory was due to motor disability, velocities were assessed by using the open field test in all groups. Our results showed that surgery alone or surgery plus preoperative SF did not impair motor function in mice (Fig. 3A), and no significant anxiety-like behavior was observed in any groups when comparing the time spent in the central area (Fig. 3B). To test whether preoperative fragmented sleep could further advance the age of susceptibility to PND to 12 months of age in mice, we performed the same tests described above with 12-month-old mice instead of 15-month-old mice, and the results showed that spatial memory impairment could not be induced in 12-month-old mice. (Supplementary Fig. 1).

To observe the similarities in the mechanisms of cognitive decline and spatial memory impairment caused by preoperatively imposed SF in 15-month-old mice and the PND model in 18-month-old mice, we measured changes in the immunoreactivity of GFAP in the hippocampus to assess the response status of astrocytes. Activation of astrocytes is one of the main pathological manifestations of neuroinflammation, and previous studies have confirmed that astrocytes play a crucial role in the development and progression of PND [25–27]. Our results showed that GFAP immunoreactivity and mean fluorescence intensity were markedly increased after experimental laparotomy under isoflurane anesthesia, with longer, thicker, and dense connections between

Table 2. Preoperative sleep fragmentation (SF) increased perioperative neurocognitive disorder (PND)-induced hippocampal injury

Mean ± SEM	No Surgery-No SF (n=8)	No Surgery-SF (n=8)	Surgery-No SF (n=8)	Surgery-SF (n=8)	No Surgery-No SF vs. No Surgery-SF	No Surgery-No SF vs. Surgery-No SF	Surgery-No SF vs. Surgery-SF
Average speed (m/s)	0.053 ± 0.003	0.047 ± 0.003	0.048 ± 0.002	0.045 ± 0.002	*P*=0.1908	*P*=0.2115	*P*=0.4797
Center ratio (%)	16.65 ± 1.956	13.49 ± 2.535	13.26 ± 1.735	13.53 ± 1.639	*P*=0.3393	*P*=0.2150	*P*=0.9099
Difference index	0.454 ± 0.05	0.403 ± 0.053	0.289 ± 0.081	–0.009 ± 0.049	*P*=0.4872	*P*=0.1046	*P*=0.007**
Barnes maze training primary latency (s) D1	125.5 ± 7.703	137.5 ± 13.18	131.4 ± 15.53	163.7 ± 7.623	*P*=0.5373		
Barnes maze training primary latency (s) D2	50.50 ± 6.509	63.60 ± 6.793	70.86 ± 10.93	86.16 ± 8.178			
Barnes maze training primary latency (s) D3	47.23 ± 4.059	59.19 ± 11.52	52.37 ± 9.235	39.77 ± 7.326			
Barnes maze training primary latency (s) D4	36.31 ± 3.568	35.11 ± 6.359	39.77 ± 7.326	58.93 ± 11.07			
Time in the target quadrant (%)	43.37 ± 1.831	43.26 ± 3.244	40.19 ± 2.311	31.63 ± 1.655	*P*=0.9766	*P*=0.2994	*P*=0.0093**
First time to the target hole (s)	18.30 ± 3.231	18.85 ± 2.474	23.92 ± 3.579	35.22 ± 3.320	*P*=0.8956	*P*=0.2635	*P*=0.0364*
No. of exploratory errors	2 ± 0.267	2.75 ± 0.366	3.125 ± 0.3981	6.375 ± 1.194	*P*=0.1717	*P*=0.0656	*P*=0.0191*
Freezing time ratio (%)	57.87 ± 4.94	59.51 ± 4.038	56.55 ± 6.143	36.18 ± 6.235	*P*=0.8012	*P*=0.8689	*P*=0.0355*

*$P<0.05$, **$P<0.01$.

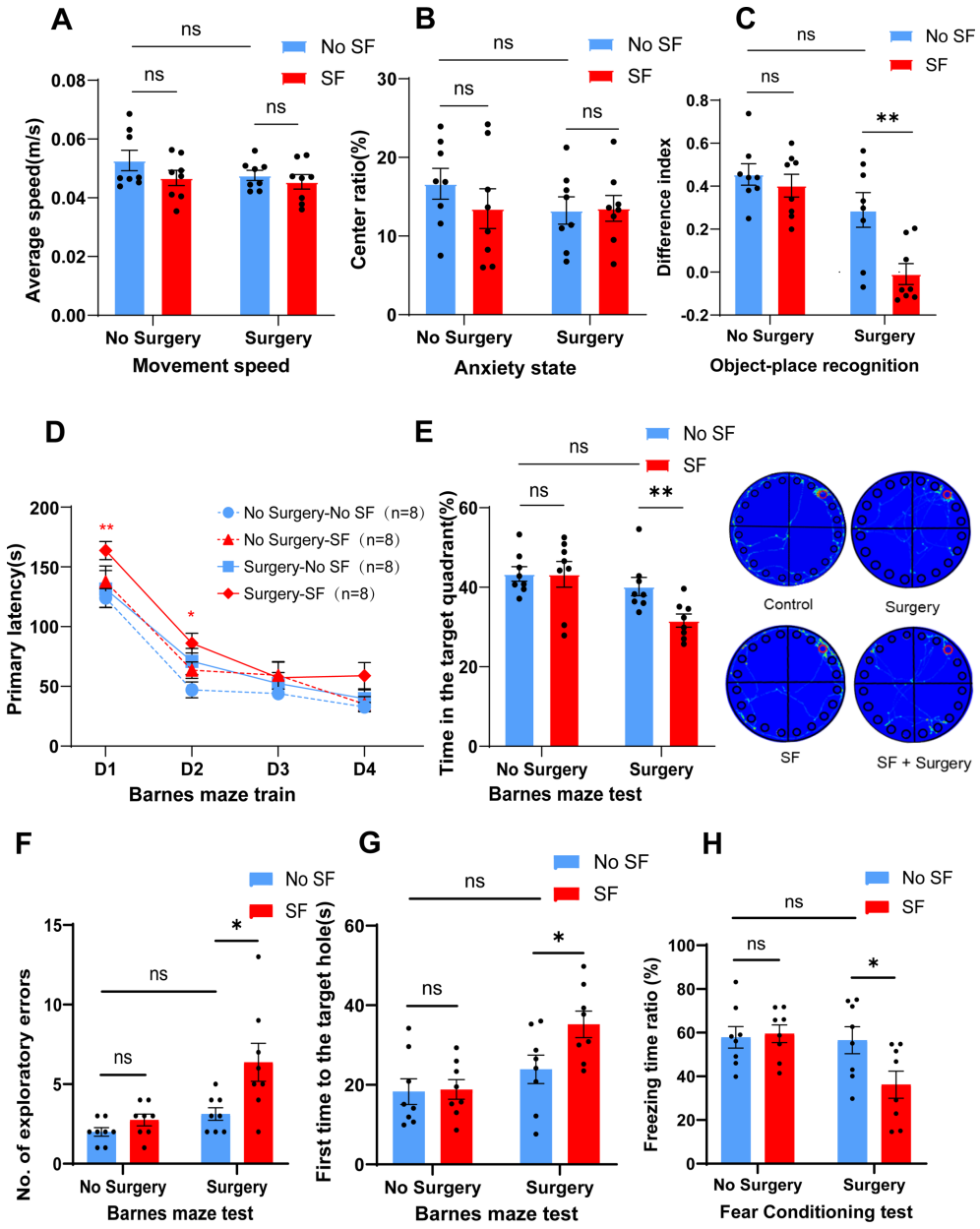

Fig. 3. Preoperative sleep fragmentation (SF) increased perioperative neurocognitive disorder (PND)-induced hippocampal injury. A) Locomotor activity was measured by movement speed in the open field test (OF) (unpaired Student's *t*-test; P=0.1908, P=0.2115, and P=0.4797 for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively). (B) Anxiety state was measured by center ratio in the OF (unpaired Student's *t*-test; P=0.3393, P=0.215, and P=0.9099 for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively). (C) Spatial memory performance was measured by the difference index related to the time spent in the new location in the new position recognition test (OPR) (unpaired Student's *t*-test; P=0.4872, P=0.1046, and P=0.007** for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively). (D) Spatial learning ability was measured by the latency during the training phase in the Barnes maze test (P=0.5437, P<0.0001****, and P=0.059 for interaction, time, and treatment by two-way ANOVA, respectively; P=0.5437, P<0.0001****, and P=0.059 for interaction, time, and treatment by two-way ANOVA, respectively; P=0.0091**, P=0.0112*, P=0.8056, and P=0.1948 for No Surgery-No SF vs. Surgery-SF in D1–D4 by post hoc Dunnett's test, respectively). (E) Spatial memory was measured by the time spent in the target quadrant (unpaired Student's *t*-test; P=0.9766, P=0.2994, and P=0.0093** for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively). (F, G) The errors in explorations (Mann-Whitney test; P=0.1717, P=0.0656, and P=0.0191* for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively) and latency of the first successful attempt to the target hole in the testing phase of the Barnes maze test (unpaired Student's *t*-test; P=0.8956, P=0.2635, and P=0.0364* for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, Surgery-No SF vs. Surgery-SF, respectively). (H) Fear memory was measured by the freezing time ratio on the fear conditioning test (FC) testing day (unpaired Student's *t*-test; P=0.8012, P=0.8689, and P=0.0355* for No Surgery-No SF vs. No Surgery-SF, No Surgery-No SF vs. Surgery-No SF, and Surgery-No SF vs. Surgery-SF, respectively). All data are presented as the mean ± SE, with n=8 per group.

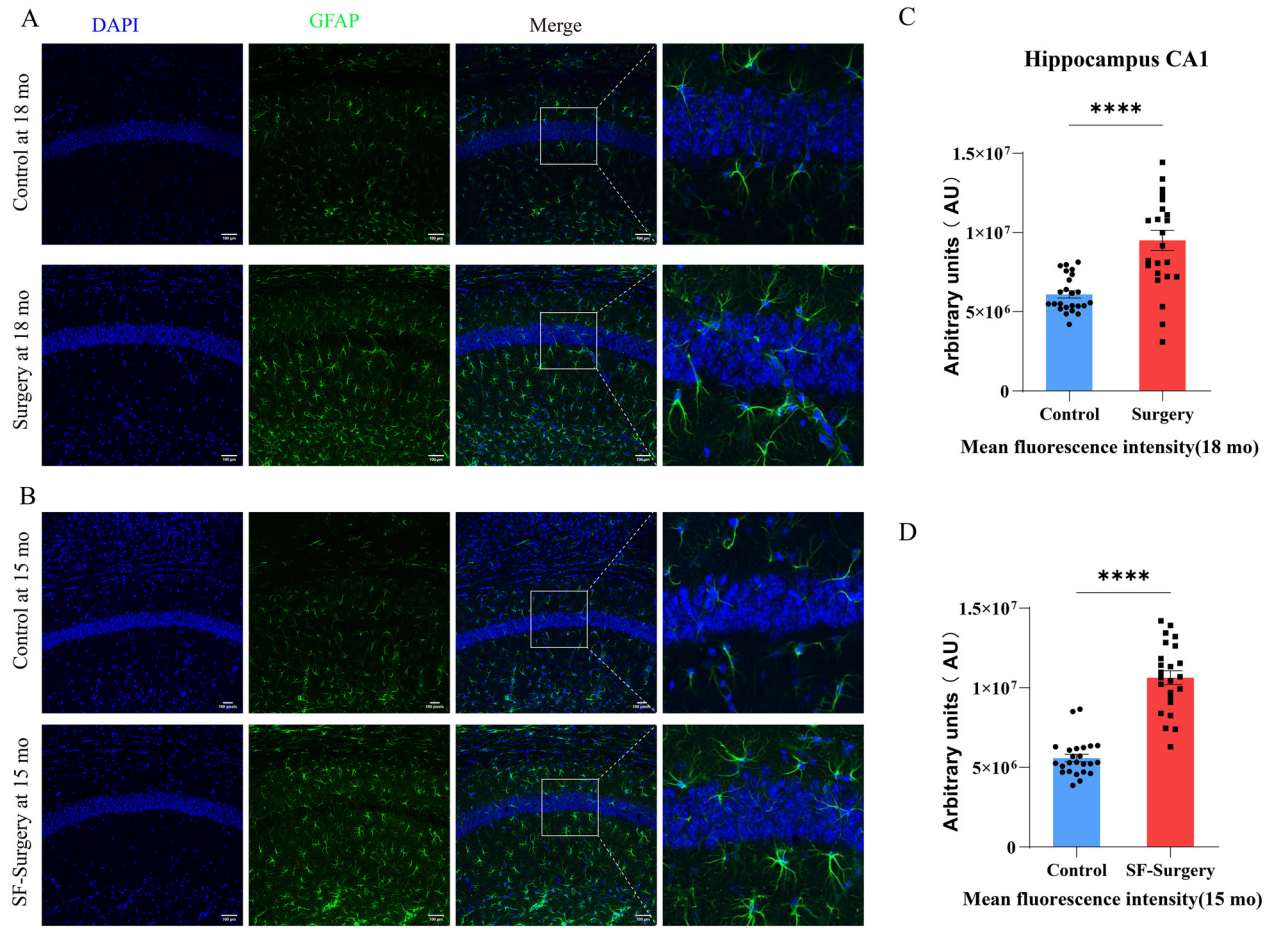

Fig. 4. Immunofluorescence staining of the region of CA1 in the hippocampus on the day after experimental laparotomy. (A) GFAP immunoreactivity in the CA1 region for 18-month-old mice. (B) GFAP immunoreactivity for 15-month-old mice. (C) Mean fluorescence intensity in the region of CA1 in the hippocampus for 18-month-old mice (unpaired Student's *t*-test, $P<0.0001$**** for No Surgery/sleep fragmentation (SF) vs. Surgery,). (D) Mean fluorescence intensity in the region of CA1 in the hippocampus for 15-month-old mice (unpaired Student's *t*-test, $P<0.0001$**** for No Surgery/SF vs. Surgery-SF,). DAPI, nuclear marker (blue fluorescence); GFAP, astrocyte marker (green fluorescence). Scale bar: 100 μm. All data are presented as the mean ± SEM for each group (n=6 per group, 4 views each).

neurons in the CA1 region in 18-month-old mice compared with controls (Figs. 4A and C). Similarly, the same degrees of significant increase in immunoreactivity and mean fluorescence intensity of GFAP were found in the 15-month-old mice compared with the control group (Figs. 4B and D). Astrocyte-driven neuroinflammation, which is a major neuropathological cause of PND [28], was present in a previous model of PND at 18 months of age. On the other hand, immunofluorescence staining results showed that astrocyte activation and increased neuroinflammation were also present in a 15-month-old mouse model of increased PND susceptibility with preoperative SF, suggesting that this mechanism reliably advances the age of susceptibility to PND from 18 to 15 months.

Preoperative rTMS prevented PND

In order to observe whether rTMS had a protective effect on the neurocognitive function in PND mice, 15-month-old PND mice were randomly assigned to the following 4 groups: sham without rTMS (No SF/Surgery-No rTMS), sham with rTMS (No SF/Surgery-rTMS), preoperative sleep fragmentation without rTMS (SF-Surgery-No rTMS), and preoperative sleep fragmentation model with rTMS (SF-Surgery-rTMS) (Table 3). Our results showed that rTMS effectively improved the cognitive decline and impaired spatial memory indicated by more time spent in the new location and an increased recognition index in the OPR test compared with the mice that did not receive rTMS (Fig. 5C). Furthermore, improved postoperative spatial learning, as indicated by significantly decreased latency on training day 2 (Fig. 5D), and memory, as indicated by more time spent in the target quadrant (Fig. 5E), fewer errors in explorations (Fig. 5F), and decreased latency of the first successful attempt to the target quadrant (Fig. 5G), were

Table 3. Effect of repetitive transcranial magnetic stimulation (rTMS) on neurocognitive function in perioperative neurocognitive disorder (PND) mice

Mean ± SEM	No Surgery/SF-No TMS (n=8)	No Surgery/SF-TMS (n=8)	Surgery-SF-No TMS (n=8)	Surgery-SF-TMS (n=8)	No Surgery/SF-No TMS vs. No Surgery/SF-TMS	No Surgery/SF-No TMS vs. Surgery-SF-No TMS	Surgery-SF-No TMS vs. Surgery-SF-TMS
Average speed (m/s)	0.042 ± 0.003	0.043 ± 0.004	0.051 ± 0.003	0.042 ± 0.002	*P*=0.8752	*P*=0.088	*P*=0.0504
Center ratio(%)	16.57 ± 2.802	15.29 ± 2.637	11.95 ± 2.387	10.39 ± 1.99	*P*=0.7453	*P*=0.2306	*P*=0.6235
Difference index	0.426 ± 0.068	0.48 ± 0.084	0.076 ± 0.064	0.32 ± 0.06	*P*=0.6285	*P*=0.0021**	*P*=0.0143*
Barnes maze training primary latency (s) D1	133.5 ± 9.61	114.1 ± 6.258	146 ± 9.936	142.1 ± 7.275	*P*=0.001**		
Barnes maze training primary latency (s) D2	63.87 ± 7.356	48.75 ± 5.205	99.67 ± 11.32	50.62 ± 3.835			
Barnes maze training primary latency (s) D3	40.52 ± 2.88	35.57 ± 3.223	62.32 ± 11.74	31.31 ± 3.928			
Barnes maze training primary latency (s) D4	32.33 ± 3.979	39.86 ± 5.478	32.78 ± 3.958	30.77 ± 3.711			
Time in the target quadrant (%)	52.84 ± 2.797	47.12 ± 3.534	35.53 ± 3.378	47.9 ± 3.518	*P*=0.2254	*P*=0.0015**	*P*=0.0237*
First time to the target hole (s)	3.125 ± 1.06	3.625 ± 0.865	9.375 ± 1.546	3.75 ± 0.881	*P*=0.7201	0.0049**	*P*=0.0069**
No. of exploratory errors	14.1 ± 2.249	19.8 ± 1.976	39.14 ± 4.897	21.43 ± 3.089	*P*=0.078	*P*=0.0004***	*P*=0.0085**
Freezing time ratio (%)	70.66 ± 6.553	75.18 ± 6.927	38.61 ± 9.519	64.24 ± 5.088	*P*=0.643	*P*=0.0149*	*P*=0.0324*

*P<0.05, **P<0.01, ***P<0.001.

also observed in the Barnes maze test in the testing phase in mice receiving rTMS compared with those that did not receive rTMS. Moreover, postoperative fear memory was also significantly improved, as indicated by the increased freezing time in the conditioned fear test in mice receiving rTMS compared with those that did not receive rTMS (Fig. 5H). Similarly, no motor dysfunction was observed in any groups of mice (Fig. 5A), and no significant protective role of rTMS on anti-anxiety-like behavior was observed compared with the mice that did not receive rTMS (Fig. 5B).

Discussion

In this study, our findings suggested that preoperative sleep fragmentation increased the vulnerability 15-month-old mice to PND, which leads to the development of a novel modified model of PND with exacerbation of SF. We also demonstrated and highlighted that rTMS could be an effective and promising treatment for improving spatial learning and memory dysfunction and preventing the cognitive decline induced by SF and surgery.

Surgical stress, anesthesia, and age are independent risk factors for PND, with older age being the greatest risk factor for PND development [29]. However, global aging is gradually accelerating (https://population.un.org/wpp/), and the increase in the elderly population will bring about a number of health and medical problems, such as PND and sleep disorders [6, 7]. It has been noted that elderly patients suffer from more fragmented sleep after hospitalization [10, 30]. Sleep disorders, including fragmented sleep, increase the risk of PND [11]. In previous reports, the incidences of PND at 1 week and 3 months after surgery in patients over 60 years of age were 25.8–41.4% and 9.9–12.7%, respectively [1, 2]. Therefore, a large majority of PND mice models are constructed in rodents older than 18-months of age [4, 5]. However, the high cost and time-consuming rearing of elderly animals are not ideal for conducting experiments, which has resulted in many constraints on PND-related studies. Our results for mice that underwent laparotomy surgery under isoflurane inhalation anesthesia at 6 weeks to 18 months of age revealed that only the18-month-old mice successfully presented symptoms of neurocognitive impairment. Since a meta-analysis has shown that sleep disorders, including fragmented sleep, increase the risk of perioperative cognitive impairment by 4.59-fold [11], we designed further experiments to verify whether preoperative sleep fragmentation in mice could advance the age of PND. In this study, the sleep fragmentation protocol used in the model was optimized according to McAlpine *et al.* [19]. Unlike the effect of complete sleep deprivation, this protocol mimics the chronic, deep sleep disorders seen in obstructive sleep apnea and other clinical disorders with sleep fragmentation and more accurately reflects clinical symptoms. The complete sleep cycle in mice is 150 s, and the sleep deprivation apparatus used in this study had a rotating bar that rotated once at 120-s intervals to interrupt the sleep cycle [9]. Under this model, the ratio of timespent awake increased, total rapid eye movement sleep (REMS) time decreased, and continuous non-rapid eye movement sleep (NREM) time decreased, but total NREMS time did not change significantly [19]. The results suggest that performing laparotomy under isoflurane anesthesia after 72 h of sleep fragmentation advances the age of onset of PND from 18 to 15 months of age, successfully establishing a younger mouse model of PND.

Object-place memory depends on the hippocampus for encoding, consolidation, and retrieval [31, 32] and is well monitored for hippocampus-related cognitive impairment. The acquisition phase and acquisition probe

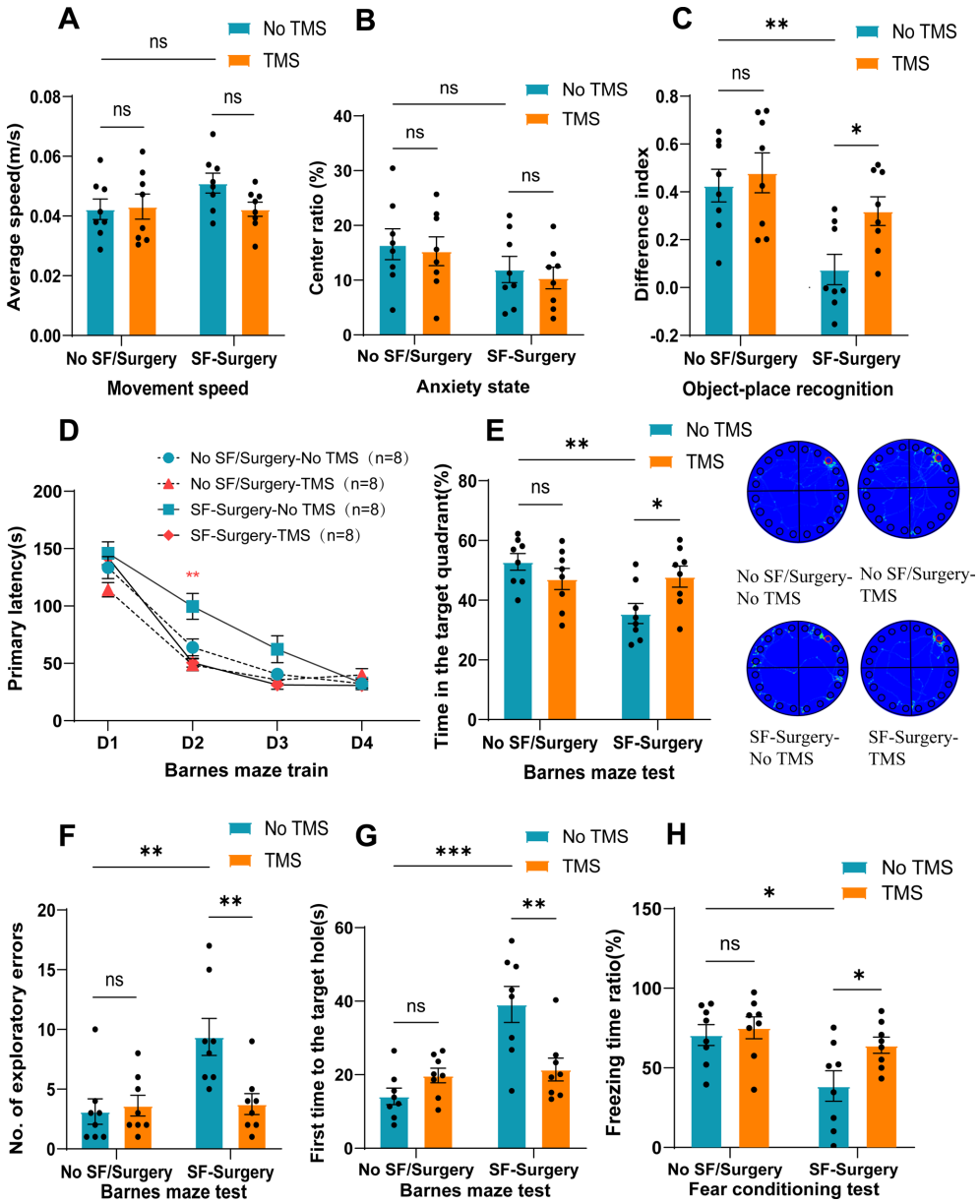

Fig. 5. Effect of repetitive transcranial magnetic stimulation (rTMS) on neurocognitive function in perioperative neurocognitive disorder (PND) mice. (A) Locomotor activity was measured by movement speed in the open field test (OF) (unpaired Student's *t*-test; P=0.8752, P=0.088, and P=0.0504 for No Surgery/sleep fragmentation (SF)-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). (B) Anxiety state was measured by center ratio in the OF (unpaired Student's *t*-test; P=0.7453, P=0.2306, and P=0.6235 for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). (C) Spatial memory performance was measured by the difference index related to the time spent in the new location in OPR (unpaired Student's *t*-test; P=0.6285, P=0.0021**, and P=0.0143* for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). (D) Spatial learning ability was measured by the latency during the training phase in the Barnes maze test (post hoc Dunnett's test; P=0.9786, P=0.0076**, P=0.0835, and P=0.966 for D1–D4 for Surgery-SF-No TMS vs. Surgery-SF-TMS). (E) Spatial memory was measured by the time spent in the target quadrant (unpaired Student's *t*-test, P=0.2254, P=0.0015**, and P=0.0237* for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). (F, G) Errors in explorations (unpaired Student's *t*-test; P=0.7201, P=0.0049**, and P=0.0069** for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively) and latency of the first successful attempt to the target hole in the testing phase in the Barnes maze test (unpaired Student's *t*-test; P=0.078, P=0.0004***, and P=0.0085** for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). (H) Fear memory was measured by freezing time ratio on the fear conditioning test (FC) testing day (unpaired Student's *t*-test; P=0.643, P=0.0149*, and P=0.0324* for No Surgery/SF-No TMS vs. No Surgery/SF-TMS, No Surgery/SF-No TMS vs. Surgery-SF-No TMS, and Surgery-SF-No TMS vs. Surgery-SF-TMS, respectively). All data are presented as the mean ± SE, with n=8 per group.

trial in the Barnes maze test allow an evaluation of spatial learning and spatial memory retrieval (retention). The results of this test are believed to be associated with hippocampus function [33, 34]. Fear memory is the best-studied form of memory. It has been thoroughly studied over the last 60 years, mainly using two classical conditioning procedures (contextual fear conditioning and fear conditioning to tones). Fear memory is formed in the hippocampus (contextual conditioning and inhibitory avoidance) and is therefore an accurate and efficient tool for the assessment of hippocampal impairment associated with PND. All of the above neurocognitive tests demonstrated that preoperative sleep fragmentation caused hippocampal-related impairment of learning and memory function, advancing the age of susceptibility to PND from 18 to 15 months of age in mice. These results suggest that sleep fragmentation may be a key risk factor for increased vulnerability to PND. Neuroinflammation is the general pathogenic mechanism of PND [28], and brain mast cells are thought to be the "first responders" to neuroinflammation, with increased mast cell numbers observed after surgery, followed by astrocyte activation, inflammatory factor production, and subsequent cognitive deficits [35]. Given that previous studies have shown that surgery increases astrocyte activation and leads to cognitive deficits in a rat model of PND [26], we further examined whether the increased susceptibility to PND due to sleep fragmentation exacerbates neuroinflammation of PND mice by examining astrocyte activation. The results showed that SF exposure prior to experimental laparotomy under isoflurane anesthesia in 15-month-old mice produced a degree of neuroinflammation similar to that in the 18-month-old mouse PND model. Most previous PND models were modeled using older animals over 18 months of age, which are expensive and not readily available. The results of this study provide a new animal model of PND at 15 months of age that shows hippocampus-related cognitive impairment via a mechanism similar to that of a previous PND model in 18-month-old mice associated with both astrocyte activation and increased neuroinflammation. In other words, PND can be induced in 15-month-old mice as long as they are subjected to sleep fragmentation before surgery and is consistent with a common clinical phenomenon.

Pharmacological treatments are used to treat cognitive decline in the general population with limited effectiveness. These drugs include some cholinesterase inhibitors (Aricept, Exelon, Razadyne) and the NMDA receptor antagonist memantine (Namenda) [29]. We could not find any reports on the use of these drugs to reduce PND in the perioperative period. The effectiveness of HF-rTMS treatment has been reported by a series of clinical studies. In a meta-analysis that included 9 studies with a total of 369 patients, it was shown that rTMS appears to improve global cognitive function and memory in patients with MCI and may be accepted well and have mild adverse effects. In 7 studies that included a total of 94 patients with mild to moderate AD, HF-rTMS (>1.0 Hz), but not low-frequency stimulation (≤1.0 Hz), was found to have a significant effect on improving cognition in patients with AD. In addition, in a study of 66 healthy individuals, HF-rTMS was found to alleviate cognitive impairment associated with 24-h sleep deprivation (SD), improve hypothalamic-pituitary-adrenal axis overactivity, reduce frontal blood activation induced by SD, and reduce the depletion of plasma pro-BDNF [14, 36–38]. In AD mice, HF-rTMS can reduce Aβ deposition by promoting Aβ clearance and reducing Aβ production, effectively reducing long-term memory loss in the 5xFAD mouse model [21] and enhancing learning and memory in senescence-accelerated-prone mouse 8 (SAMP8) mice [39]. It has beneficial effects on Parkinson's disease (PD) model mice and naturally aging mice with neuroplasticity and improves cognitive deficits [40], and it modulates cognition and mood in stroke patients [41, 42]. Additionally, similar to clinical reports, 7 sessions of HF-rTMS also improved cognitive function in sleep deprivation model animals [20]. However, no reports have shown its effects in PND models. The HF-rTMS programming used in previous animal studies was modified for use in the present study. The couse of treatment usually comprises HF-rTMS treatment sessions over a period of 7 days, but the period range from 7 days to 8 weeks [43, 44], 7 days is usually a course of treatment. Considering the clinical practical implications, we adopted a 10-day course before surgery (7 days of pretreatment + 3 days of fragmented sleep) and showed that this HF-rTMS treatment protocol had beneficial effects on the postoperative learning memory ability of a new 15-month-old PND model. However, unlike that in the report by Ma *et al.* [17], this treatment protocol did not show cognitive improvement in healthy 15-month-old mice, probably due to a shorter treatment session, later behavioral testing, and different behavioral paradigm chosen.

A limitation of this study isthe lack of exploration of other pathogenic mechanisms for the new PND model in 15-month-old mice with the help of SF, despite confirmation that preoperative SF increases the susceptibility to PND and that both the new PND model in 15-month-old mice and the old PND model in 18-month-old mice are associated with astrocyte activation and neuroinflammation. Therefore, whether both models

share exactly the same mechanisms deserves further investigation.

In conclusion, we are the first to successfully construct a younger model of PND in 15-month-old mice with the help of sleep fragmentation followed by laparotomy under isoflurane anesthesia, which makes PND-related studies more economical, that can be used as an alternative model for future PND studies.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form (at www.icmje.org/coi_disclosure.pdf) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Authors' Contributions

Wu T conceived the study, designed experiments, interpreted and analyzed the data, and wrote the manuscript. Li M contributed to the animal experiments. Tian L, Cong P, and Huang X contributed to the experimental design. Wu H and Zhang Q contributed to revision of the manuscript. Xiong L and Zhang H conceived and designed experiments, supervised the work, and edited the manuscript. All authors have read and approved this manuscript prior to submission.

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Data Availability

The authors declare that all data supporting the findings of this study are available within the article and from a corresponding author upon reasonable request.

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