

1 **Title page**

2 **Disruption of the GABAergic System Contributes to the Development of**
3 **Perioperative Neurocognitive Disorders after Anesthesia and Surgery in Aged**
4 **Mice**

5
6 **Running title:** Disruption of the GABAergic system leading to PND

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1 **Abstract**

2 **Aims:** Perioperative neurocognitive disorders (PND) are associated with cognitive
3 impairment in the preoperative or postoperative period, and neuroinflammation is
4 thought to be the most important mechanisms especially during the postoperative
5 period. The GABAergic system is easily disrupted by neuroinflammation. This study
6 investigated the impact of the GABAergic system on PND after anesthesia and surgery.

7 **Methods:** An animal model of laparotomy with inhalation anesthesia in 16-month old
8 mice was addressed. Effects of the GABAergic system were assessed using biochemical
9 analysis. Pharmacological blocking of $\alpha 5$ GABA_ARs or P38 mitogen-activated protein
10 kinase (MAPK) was applied to investigate the effect of the GABAergic system.

11 **Results:** After laparotomy, the hippocampus-dependent memory and long-term
12 potentiation were impaired, the levels of IL-6, IL-1 β and TNF- α upregulated in the
13 hippocampus, the concentration of GABA decreased, and the protein levels of the
14 surface $\alpha 5$ GABA_ARs up-regulated. Pharmacological blocking of $\alpha 5$ GABA_ARs with
15 L655,708 alleviated laparotomy induced cognitive deficits. A further study found that
16 the P38 MAPK signaling pathway was involved and pharmacological blocking with
17 SB203,580 alleviated memory dysfunction.

18 **Conclusions:** Anesthesia and surgery caused neuroinflammation in the hippocampus,
19 which consequently disrupted the GABAergic system, increased the expressions of
20 surface $\alpha 5$ GABA_ARs especially through the P38 MAPK signaling pathway, and
21 eventually led to hippocampus-dependent memory dysfunctions.

22 **Keywords**

23 neuroinflammation, perioperative neurocognitive disorders, GABAergic system,
24 $\alpha 5$ GABA_A receptors, mitogen-activated protein kinase

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1 **1. Introduction**

2 Perioperative neurocognitive disorders (PND), a general term for cognitive
3 impairment identified during the preoperative or postoperative period, are known to
4 negatively affect multiple cognitive domains such as memory, attention, and
5 concentration after anesthesia and surgery¹⁻³. At the point of discharge, the incidence of
6 PND is 25% to 40% among the elderly⁴ and significantly affects patients' outcomes and
7 increases mortality, especially in aging patients⁵.

8 Neuroinflammation is a common factor contributing to cognitive deficits especially
9 the hippocampus-dependent memory impairment after anesthesia and surgery⁵⁻⁹.
10 Neuroinflammation is also a dynamic, multi-stage physiological response, mainly
11 manifesting as the activation of natural immune cells in the central nervous system,
12 accompanied by the release of a variety of pro-inflammatory factors that ultimately lead
13 to changes of homeostasis in the central microenvironment¹⁰. However, the exact
14 mechanism underlying how neuroinflammation causes memory deficits is not well
15 understood and there are no treatments that are available to effectively reverse or
16 prevent memory deficits after anesthesia and surgery¹¹. Therefore, it is necessary to
17 explore the down-stream mediators of neuroinflammation that induce memory deficits.

18 Changes in multiple neurotransmitter receptors have been demonstrated to be
19 associated with memory deficits^{12,13}. The GABAergic system also participates in the
20 processes of learning, memory, and synaptic plasticity¹⁴. GABA type A receptors
21 (GABA_ARs) comprise different subunits, and different combinations of GABA_ARs
22 have shown different localization and distinct physiological and pharmacological
23 characteristics¹⁵. In particular, the α 5-subunit-containing subtype of GABA_ARs
24 (α 5GABA_ARs), which makes up 20-25% of the hippocampal GABA_ARs¹⁵, are
25 specifically localized to extrasynaptic regions of hippocampal pyramidal neurons and
26 are mainly involved in mediating tonic inhibition, as well as being implicated in
27 processing memory^{16,17}. Furthermore, the increase of α 5GABA_ARs activity causes
28 profound memory blockade. Parallely, a reduction in the expression or functions of the
29 α 5GABA_ARs improves certain memory performance^{14,18}. Here we hypothesized that
30 anesthesia and surgery will cause neuroinflammation in the hippocampus, targeting the
31 GABAergic system, especially the α 5GABA_ARs pathway, affecting LTP and resulting
32 in hippocampus-dependent memory deficits.

33 **2. Materials and methods**

34 **2.1 Animals**

35 A total of 183 female c57BL/6J mice (16-month old) were purchased from the
36 Experimental Animal Center of Tongji Medical College, Huazhong University of
37 Science and Technology. All animals were housed five per cage in maintained
38 temperature of 22±1°C with a 12hour light/dark cycle with free access to food and water.
39 All procedures were in accordance with the Guidelines of the National Institutes of
40 Health Guide for the Care and Use of Laboratory Animals.

41 **2.2 Groups and Laparotomy surgery**

42 The laparotomy model was established as previously described with minor
43 improvements³. Mice were inducted with 3% isoflurane and maintained with 1.3%

1 isoflurane. Then an incision about 1.0cm was made at the site 0.5cm below the right
2 rib. The small intestine of about 10cm was exposed onto a sterile gauze for 15min and
3 then returned back into the abdominal cavity. The muscle and skin were closed with 4-
4 0 sutures, respectively. Lidocaine cream was applied at the incision site to reduce
5 postoperative pain. For the anesthesia group, mice only received anesthesia as described
6 above while for the control group, mice were given oxygen in the induction box with
7 free movement.

8 **2.3 Novel object recognition test (NORT)**

9 The operator was blinded to the experiment and handled the mice for 1 minute a day,
10 for a total of 6 days before the test. Then mice were put into the box to accommodate
11 to the condition for 5 minutes. In the training stage, two identical rectangular blocks
12 were placed on the same side of the box, and the mice were allowed to explore for 5
13 minutes. Exploratory behaviors included sniffing, licking, and climbing on pieces of
14 wood. In the testing stage, a rectangular block was replaced by a cylinder, and mice
15 were placed into the box to explore for another 5 minutes. The learning and memory
16 ability were evaluated by the discrimination ratio which is represented by $C/(A+C)$,
17 where C is the time spent exploring the novel object, A is the time spent exploring the
18 familiar object, and A+C is the total time spent exploring the two objects. In addition,
19 the mice were screened when the total exploring time was less than 5s or they explored
20 only one of the objects during the training phase.

21 **2.4 Fear condition test (FCT)**

22 Fear condition tests were performed as previously reported³. Briefly, after mice
23 accommodated to the condition, one tone-foot-shock pairing was given (tone, 30s,
24 70dB, 1kHz; foot-shock, 2s, 0.5mA, a 30s interval after the shock). Then they were
25 given another shock pairing (three pairings in total). 24 hours after the training session,
26 the mice were put back into the same test chamber to assess the contextual fear
27 conditioning. Two hours later, the tone fear conditioning was assessed. Mice were
28 placed into a novel chamber that changed the environment and the same tone was
29 delivered for 3 minutes. Freezing behavior was defined as the absence of all visible
30 movement except for respiration.

31 **2.5 Nuclear magnetic resonance (NMR)**

32 Brain tissues for NMR analysis were performed as previously conducted¹⁹ and
33 briefly described as following. In order to avoid the impact of post-mortem changes,
34 mice were deeply anesthetized with 4% isoflurane and then microwaved using a
35 domestic microwave oven (0.75kw, 15s). After that, brain tissue was taken, weighed
36 and quickly frozen to -80°C.

37 HCl/methanol (200μL, 0.1M) and 60% ethanol (vol/vol, 400μL) were added into the
38 EP tubes and homogenized with Tissuelyser for 90s at a frequency of 20Hz (Tissuelyser
39 II, QIAGEN, Germany). The mixture was centrifuged for 15 minutes at 12,000r and
40 the supernatant was collected into a 5ml EP tube. The substance was extracted twice
41 with 800μL 60% ethanol. All the supernatants were collected and desiccated with the
42 centrifugal drying apparatus (Thermo Scientific 2010, Germany), and the dried product
43 was collected for further NMR studies.

44 The phosphate buffer solution [PBS, pH = 7.2, 60μL, 120mg/L 3-(Trimethylsilyl)]

1 propionic-2, 2, 3, 3, d4 acid sodium salt (TSP, 269913-1G, Sigma-Aldrich) in D2O] and
2 the double distilled water (540 μ L) were added into the 5ml EP tubes to dissolve the
3 dried product and TSP was set as the internal standard. The solution was shaken evenly
4 with a high-speed vortex until the precipitates were dissolved, and the mixture
5 centrifuged at 12,000r for 10 minutes. The supernatant (530 μ L) was then collected and
6 transferred to a 5 mm NMR tube for 1H NMR analysis.

7 NMR spectra testing were performed at 298 K on a BrukerAvance III 600 MHz NMR
8 spectrometer equipped with an inverse cryogenic probe (BrukerBiospin, Germany).
9 The 1H NMR spectra were acquired with a standard WATERGATE pulse sequence,
10 and processed in the commercial software TOPSPIN and NMRSpec, as well as a home-
11 made tool based on a MATLAB code.

12 **2.6 MSD multi-spot assay**

13 The hippocampus was homogenized and centrifuged at 12,000r for 15 minutes at
14 4°C. The supernatants were collected and the levels of IL-6, IL-1 β and TNF- α were
15 detected using commercially available proinflammatory panel 1 (mouse) kits (Meso
16 Scale Discovery (MSD®, Gaithersburg, MD, USA))²⁰. The procedures were performed
17 according to the manufacturer's instructions, and the concentrations of IL-6, IL-1 β and
18 TNF- α are presented as pg/ml⁸.

19 **2.7 Electrophysiology in vitro**

20 Mice were deeply anesthetized with pentobarbital sodium (50mg/kg, *i.p.*) and then
21 decapitated. The brain was quickly removed and placed into an ice-cold oxygenated
22 (95% O₂ and 5% CO₂) high-sucrose solution that contained (in mM): 213sucrose, 3KCl,
23 1NaH₂PO₄, 0.5CaCl₂, 5MgCl₂, 26NaHCO₃ and 10glucose. Hippocampal slices (300-
24 320 μ m) were prepared as described previously²¹⁻²³. The slices were transferred to a
25 holding chamber containing ACSF consisting of (in mM): 124NaCl, 26NaHCO₃, 3KCl,
26 1.2MgCl₂·6H₂O, 1.25NaH₂PO₄·2H₂O, 10C₆H₁₂O₆ and 2CaCl₂ at PH 7.4, 305mOsm.
27 The slices were allowed to recover at 31.5°C for 30 minutes and then at room
28 temperature (RT) for at least 1 hour.

29 Acute slices were transferred to the recording chamber, and the long-term
30 potentiation (LTP) of evoked field postsynaptic potentials (fPSPs) was recorded from
31 the stratum radiatum in CA1 following electrical stimulation of the Schaffer collateral
32 pathway. After the stable baseline of at least 30 minutes, high-frequency stimulation
33 (HFS, 100Hz, 50 pulse, four trains at 20s interval) was used to induce LTP and then
34 recorded for another 60 minutes.

35 **2.8 Western blot**

36 Hippocampal protein samples were prepared as previously described²⁴ and were
37 separated using 10% SDS-PAGE and subsequently transferred to polyvinylidene
38 fluoride membranes (Millipore, Billerica, MA, USA) for electroblotting. The
39 membranes were blocked with 5% BSA in TBST (0.1%) for 2 hours at RT, incubated
40 with primary antibody overnight at 4°C, and then incubated with horseradish
41 peroxidase (HRP)-conjugated secondary antibodies for 2 hours at RT. The antibodies
42 used in this study include rabbit anti- α 5GABA_A receptors, anti-GAT-3 (1:500-1000,
43 Alomone labs, Germany), rabbit anti-GAD65 (1:1000, Abcam, Cambridge, UK), rabbit
44 anti-P38, p-P38, ERK1/2, p-ERK1/2, JNK1/2, p-JNK1/2 (1:1000-2000, Cell Signaling

1 Technology, MA, USA), mouse anti-GAPDH HRP-conjugated goat-anti-mouse IgG or
2 anti-rabbit IgG(1:1000-5000, Promoter, Wuhan, China). The protein bands were
3 visualized using chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo
4 Scientific) and measured using a computerized image analysis system (ChemiDoc
5 XRS+, BIO-RAD, CA, USA).

6 **2.9 Immunofluorescence**

7 Brain slices for immunofluorescence were prepared as previously reported²⁴. The
8 sections were blocked with 10% donkey serum and 0.3% Triton 1 hour at RT. Then the
9 sections were incubated overnight at 4°C with mouse anti-Iba1 antibody (1:300, Wako,
10 Japan). After washing with PBS, the sections were incubated with Alexa Fluor 488-
11 labeled donkey anti-rabbit secondary antibody (1:200, Invitrogen, Carlsbad, CA) at
12 37°C for 2 hours. Images were captured using a laser scanning confocal microscope
13 (FV1000, Olympus, Tokyo, Japan).

14 **2.10 Quantitative Real-Time PCR (RT-PCR)**

15 Total RNA and cDNA from the hippocampus were prepared as outlined before³.
16 Quantitative real-time PCR was performed on the ABI7900 (Illumina, USA) with
17 SYBR Green Master Mix kit (TAKARA, Japan). The conditions for the PCR reaction
18 were as following: Incubated at 50°C for 2 minutes and then at 95°C for 10 minutes
19 and then followed by 40 cycles at 95°C for 30s and 60°C for 30s. The sequences of
20 specific primers are summarized in table1.

21 **2.11 Statistical analysis**

22 All results are presented as mean ± SEM. An unpaired Student's T-test was used to
23 compare two groups. For three groups, One-way ANOVA followed by Bonferroni post
24 hoc test was applied. Two-way ANOVA was used to analyze NORT and FCT after using
25 L655,708 or SB203,580. GraphPad Prism 7.0 was used for all analyses and $p < 0.05$ was
26 considered statistically significant in this study.

27 **3. Results**

28 **3.1 Hippocampus-dependent memory and LTP were impaired after anesthesia and** 29 **surgery in aged mice.**

30
31 In the NORT, no difference was found in the total time spent on identical objects
32 among the three groups during the training stage ($F_{(2,30)}=1.07, p=0.35$; Figure1B). In
33 the testing phase, mice spent more time on the novel object than on the familiar object
34 in the control and anesthesia treated groups ($F_{(2,40)}=147.7, p<0.001$; Figure1C).
35 However, the time spent on the novel and familiar objects did not differ in the
36 laparotomy mice. Further analysis of the discrimination ratio revealed that there was a
37 distinct difference among the three groups. And the discrimination ratio in the control
38 and anesthesia groups was greater than that in the laparotomy group ($F_{(2,30)}=32.21,$
39 $p<0.001$; Figure1D). In the FCT, no statistical difference was found in tone freezing
40 time which was the hippocampus-independent memory ($F_{(2,30)}=1.29, p=0.29$; Figure1E).
41 However, there was a significant difference in the context freezing time among the three
42 groups ($F_{(2,30)}=15.97, p<0.01$; Figure1F). In this study, mice in the laparotomy group
43 spent less freezing time than those in the control group, and there was no difference
44 between the control and anesthesia groups (Figure1F). Next, we assessed whether the

1 hippocampal LTP was impaired after laparotomy. There was a remarkable increase in
2 the amplitude of fPSP (% of baseline) in the control and anesthesia slices after HFS
3 ($F_{(2,18)}=54.46$, $p<0.001$; Figure1G). The amplitude was increased from $103.8\% \pm 2.6\%$
4 to $164.1\% \pm 15.2\%$ in slices from the control mice and $100\% \pm 0.7\%$ to $156.5\% \pm 7.8\%$ in
5 the anesthesia slices. In contrast, LTP was impaired and increased slightly from
6 $103\% \pm 2.4\%$ to $103.3\% \pm 11.7\%$ in the laparotomy slices (Figure1G). These results
7 demonstrate that deficits of hippocampus-dependent memory and impairment of LTP
8 were caused by anesthesia and surgery rather than by anesthesia alone.

9 **3.2 Hippocampal neuroinflammation was observed after anesthesia and surgery** 10 **in aged mice.**

11 Compared with the control and anesthesia mice, the morphology of microglia in the
12 laparotomy mice was clearly changed and manifested mainly as hypertrophy in the cell
13 body in the CA1, CA3 and DG regions of the hippocampus (Figure2A). Next, we
14 examined cytokine expressions of IL-1 β , IL-6 and TNF- α in the hippocampus. The
15 MSD results showed that IL-1 β and IL-6 were obviously up-regulated ($F_{(2,6)}=7.05$,
16 $p=0.03$; Figure2B; $F_{(2,6)}=13.42$, $p=0.006$; Figure2C) in the laparotomy group, but the
17 expression of TNF- α was increased both in the anesthesia and laparotomy groups
18 ($F_{(2,6)}=12.7$, $p=0.007$; Figure2D). These results demonstrate that anesthesia and surgery
19 could cause severe inflammatory response in the hippocampus.

20 **3.3 Hippocampal GABAergic system was disrupted and surface $\alpha 5$ GABA_ARs** 21 **were selectively involved after anesthesia and surgery in aged mice.**

22 Next, we examined the changes in levels of neurotransmitters after anesthesia and
23 surgery in the hippocampus and used absolute concentrations to compare the
24 differences among the three groups. The NMR results showed no difference in the
25 levels of glutamate among the three groups ($F_{(2,24)}=0.11$, $p=0.90$; Figure3A), while the
26 levels of GABA were clearly decreased in the laparotomy group ($F_{(2,24)}=4.43$, $p=0.02$;
27 Figure3B). The raw data of the average and deviation of these two transmitters are
28 presented (Figure3C). Next, we examined the transcription levels of $\alpha 5$, $\alpha 1$ and $\beta 3$
29 subunits, at 1 day, 3 days, 7 days and 10 days after laparotomy using quantitative RT-
30 PCR. There was no significant difference at any time point of $\alpha 1$ ($F_{(8,18)}=1.49$, $p=0.23$;
31 Figure3D) and $\beta 3$ ($F_{(8,18)}=2.05$, $p=0.09$; Figure3E) subunits levels. While the $\alpha 5$ subunit
32 level was increased at 1 day and continued to increase at 3 days, 7 days and 10 days
33 after laparotomy ($F_{(8,18)}=13.85$, $p<0.0001$; Figure3F). Then, we detected the protein
34 levels of GAT-3, GAD65 and surface $\alpha 5$ GABA_ARs using western blot. The results
35 showed that the expressions of GAT-3 and GAD65 were evidently decreased after
36 laparotomy ($F_{(2,9)}=10.82$, $p=0.004$; Figure3G; $F_{(2,9)}=11.73$, $p=0.003$; Figure3H), which
37 signified that the synthesis of GABA was reduced. At the same time, the levels of
38 surface $\alpha 5$ GABA_ARs were upregulated in the laparotomy mice ($F_{(2,12)}=6.56$, $p=0.01$;
39 Figure3I). These results demonstrate that anesthesia and surgery could disrupt the
40 GABAergic system in the hippocampus and selectively increase expressions of surface
41 $\alpha 5$ GABA_ARs.

42 **3.4 Pharmacological blockade of $\alpha 5$ GABA_ARs with L655,708 could reverse** 43 **anesthesia and surgery induced hippocampus-dependent memory deficits in aged** 44 **mice.**

1 To further investigate the role of $\alpha 5\text{GABA}_A$ Rs after anesthesia and surgery in
2 inducing learning and memory deficits, the specific inverse agonist L655,708 was used
3 to reduce the affinity for GABA by acting upon the $\alpha 5\text{GABA}_A$ Rs. In the NORT, no
4 significant difference was found in the total time spent on identical sample objects
5 during the training stage after using L655,708 ($F_{(2,14)}=0.003$, $p=0.99$; Figure4B).
6 However, the time spent exploring the novel object and the discrimination ratio were
7 prominently increased in the laparotomy group after administering L655,708
8 ($F_{(6,42)}=14.34$, $p<0.001$; Figure4C; $F_{(2,14)}=8.06$, $p=0.005$; Figure4D). In the FCT, no
9 difference was found in the freezing time to the tone ($F_{(2,14)}=0.03$, $p=0.97$; Figure4E).
10 The percentage of context freezing time was increased in the laparotomy mice after
11 administering L655,708 ($F_{(2,14)}=29.82$, $p<0.001$; Figure4F). In addition, the amplitude
12 of fPSPs in the laparotomy mice was increased from $103.8\%\pm 4.3\%$ to $146.4\%\pm 4.9\%$
13 after the application of L655,708 ($t=6.47$, $p<0.001$; Figure4I), and there was no
14 difference between the control and anesthesia groups ($t=0.11$, $p=0.92$; Figure4G; $t=1.02$,
15 $p=0.33$; Figure4H). These results indicate that blocking $\alpha 5\text{GABA}_A$ Rs with L655,708
16 could reverse anesthesia and surgery induced hippocampus-dependent memory deficits.

17 **3.5 P38 MAPK signaling pathway was specifically activated after anesthesia and** 18 **surgery in aged mice.**

19 To explore the potential signaling pathway of the cellular response to inflammatory
20 stimuli, the expressions of MAPK signaling pathways including P38, p-P38, JNK1/2,
21 p-JNK1/2, ERK1/2 and p-ERK1/2 proteins were evaluated using western blot. The
22 expression of p-P38 was obviously up-regulated in the laparotomy group ($F_{(2,9)}=1.45$,
23 $p=0.28$; Figure5C). No statistical difference was observed in the expression of P38,
24 ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 ($F_{(2,9)}=2.83$, $p=0.12$; Figure5A; $F_{(2,9)}=0.03$,
25 $p=0.97$; figure5B). These results indicate that the P38 MAPK signaling pathway was
26 specially activated in the hippocampus after anesthesia and surgery in aged mice.

27 **3.6 Pharmacological blockade of the P38 MAPK signaling pathway with** 28 **SB203,580 could reverse anesthesia and surgery induced hippocampus-dependent** 29 **memory deficits in aged mice.**

30 SB203,580 is the selective inhibitor of the P38 MAPK signaling pathway. Therefore,
31 we used SB203,580 to further investigate the role of the P38 MAPK signaling pathway
32 in inducing learning and memory deficits after anesthesia and surgery. In the NORT, no
33 difference was found in the total time spent exploring identical sample objects among
34 the three groups after using SB203,580 ($F_{(2,14)}=0.01$, $p=0.99$ C; Figure6B). However,
35 the time spent at the novel object and the discrimination ratio were prominently
36 increased in the laparotomy group after administering SB203,580 ($F_{(6,42)}=28.08$,
37 $p<0.001$; Figure6C; $F_{(2,14)}=166$, $p<0.001$; Figure6D). In the FCT, no statistical
38 difference was found in the freezing time to the tone ($F_{(2,14)}=0.09$, $p=0.91$; Figure6E),
39 while the percentage of context freezing time was increased in the laparotomy group
40 after administering SB203,580 ($F_{(2,14)}=6.03$, $p=0.01$; Figure6F). At the same time, a
41 qualitative decrease in p-P38 and surface $\alpha 5\text{GABA}_A$ Rs expressions was observed in
42 the laparotomy mice after using SB203,580 ($F_{(2,6)}=10.38$, $p=0.01$; Figure6I; $F_{(2,6)}=35.4$,
43 $p=0.005$; Figure6J), but there was no difference shown in the expressions of p-ERK1/2
44 and p-JNK1/2 ($F_{(2,6)}=1.11$, $p=0.39$; Figure6G; $F_{(2,6)}=3.87$, $p=0.08$ Figure6H). In

1 hippocampal slices, the amplitude of fPSPs in the laparotomy mice was increased from
2 100.7%±2.4% to 147.1%±3.1% after the application of SB203,580 ($t=11.79$, $p<0.0001$;
3 Figure6M), yet there was no difference between the control and anesthesia groups
4 ($t=0.32$, $p=0.75$; Figure6K; $t=0.01$, $p=0.99$; Figure6L). These results illustrate that
5 blocking the P38 MAPK signaling pathway could reverse anesthesia and surgery
6 induced hippocampus-dependent memory deficits possibly by preventing the
7 trafficking of $\alpha 5$ GABA_ARs.

9 **4. Discussion**

10 PND are mainly experienced as memory deficits by elderly people which seriously
11 affects their quality of life, but the pathophysiology of the dysfunction remains unclear.
12 In the current study, we found that anesthesia and surgery caused robust
13 neuroinflammation in the hippocampus, which in turn disrupted the GABAergic system,
14 especially by targeting surface $\alpha 5$ GABA_ARs traffic through activating the P38 MAPK
15 signaling pathway which eventually led to hippocampus-dependent memory deficits.

16 Numerous studies have shown that neuroinflammation is the main reason for PND^{9,25}.
17 Systemic inflammation caused by surgery could induce neuroinflammation, mainly
18 through destroying the permeability of the blood-brain barrier²⁶⁻²⁸, hence, promoting
19 the activation of local microglia. Activated microglia cells subsequently release more
20 inflammatory cytokines^{9,25,29-31}. In our research, the levels of IL-1 β , IL-6 and TNF- α in
21 the hippocampus were up-regulated and microglia clearly activated after anesthesia and
22 surgery. The results indicate that the hippocampus suffered significant inflammation
23 after laparotomy under isoflurane anesthesia. However, TNF- α was also increased after
24 anesthesia without surgery, but no activation of microglia was found in the
25 hippocampus. It suggests that isoflurane anesthesia alone could not induce harmful
26 inflammation in the hippocampus, which is in line with Wang et al. and Kawano et al.'s
27 findings^{32,33}. Callaway et al. and Crosby et al. demonstrated that exposure to
28 sevoflurane or isoflurane anesthesia alone had no impact on learning and memory in
29 the rodent^{34,35}. Jennifer et al. also reported that learning task performance showed no
30 significant changes after exposure to anesthesia alone in adult populations³⁶. In brief,
31 hippocampal neuroinflammation caused by anesthesia and surgery was much more
32 serious in aged mice than that caused by anesthesia alone. The degree of severity of
33 hippocampal neuroinflammation could be closely related to the memory loss after
34 anesthesia and surgery.

35 In the central nervous system, the GABAergic system contributes to controlling the
36 excitability of neuronal networks. However, the functions of the GABAergic system
37 are easily affected by inflammation, including GABAergic neuronal density, GABA
38 and its synthetic machinery and GABA receptors. Qiu, et al reported that hippocampal
39 Parvalbumin interneurons contributed to cognitive dysfunction in aged mice³⁷. Here,
40 we found that the concentration of GABA in the hippocampus was decreased after
41 anesthesia and surgery. At the same time, the protein expressions of GAT-3 and
42 GAD65³⁸ were decreased after anesthesia and surgery. Dysfunction of GAT-3 is related
43 to several neurological diseases, such as Alzheimer's disease³⁹. Other studies showed
44 that GAD65 is associated with GABAergic synaptic transmission and plasticity, and

1 that the reduction in GAD65 contributed to neuropsychiatric disorders in mice⁴⁰. Here
2 we found that transcription of the $\alpha 5$ subunit and the levels of surface $\alpha 5$ GABA_ARs
3 were increased after anesthesia and surgery. Sustained increase in $\alpha 5$ GABA_ARs activity
4 disrupted memory and synaptic plasticity⁴¹. Pharmacologically blocking $\alpha 5$ GABA_ARs
5 with L655,708 reversed anesthesia and surgery and induced hippocampus-dependent
6 memory deficits and LTP. Inhibition or elimination of $\alpha 5$ GABA_ARs improved the
7 Morris water maze performance and fear conditioning in mice⁴². However, Gao et al
8 suggested that prophylactic use of L655,708 does not prevent isoflurane-induced
9 memory deficits in aged mice⁴³. One reason could be that they used a different animal
10 model. They took an animal model which only received inhalation anesthesia, without
11 surgery whereas in our study, the animal received both inhalation anesthesia and surgery.
12 The pathophysiology process could therefore, be different between these two animal
13 models. The other reason could be that L655,708 was administrated prophylactically in
14 their study, but post anesthesia and surgery in ours.

15 Upregulation of surface $\alpha 5$ GABA_ARs are primarily associated with activation of the
16 P38 MAPK signaling pathway, and the signaling pathway is known to be an important
17 regulator of GABA_ARs trafficking⁴⁴. Cytokines, that induce activation of the P38
18 MAPK signaling pathway, are widely reported in some other inflammation models⁴⁵.
19 In our study, we tested three typical pathways of MAPK and found that the protein level
20 of p-P38 selectively increased. Pharmacological blocking of the P38 MAPK signaling
21 pathway with SB203,580 reversed anesthesia and surgery induced hippocampus-
22 dependent memory deficits, and reduced the levels of p-P38 and surface $\alpha 5$ GABA_ARs,
23 which is consistent with results of Orser et al.

24 There are several limitations in our study. Firstly, we did not explore the changes of
25 tonic inhibitory currents regulated by $\alpha 5$ GABA_ARs to investigate the effect of
26 $\alpha 5$ GABA_ARs on postsynaptic functions. Secondly, since the gene knockout technology
27 can effectively distinguish the functions of different subunits, we could have used
28 knockout mice to further verify the functions of $\alpha 5$ GABA_ARs. Lastly, some studies
29 have demonstrated that postoperative pain is also a factor influencing the cognitive
30 behavior. Post-surgery pain could not be totally avoided in this study and deserves
31 further investigation.

32 In summary, our study revealed that hippocampus-dependent memory was disrupted
33 by anesthesia and surgery rather than by anesthesia alone. Anesthesia and surgery
34 caused neuroinflammation in the hippocampus, which consequently disrupted the
35 GABAergic system, increased the expressions of surface $\alpha 5$ GABA_ARs especially
36 through activating the P38 MAPK signaling pathway, which eventually led to
37 dysfunctions of hippocampus-dependent memory. Therefore, our research may provide
38 a new viewpoint for exploring the mechanisms of PND, while $\alpha 5$ GABA_ARs may serve
39 as a potential target for preventing or treating PND.

40 41 **Acknowledgments**

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1 **Conflicts of interest**

2 The authors declare no competing interests.

4 **References**

- 5 1. Evered L, Silbert B, Knopman DS, et al. Recommendations for the nomenclature of
6 cognitive change associated with anaesthesia and surgery-2018. *Br J Anaesth.*
7 2018;121(5):1005-1012.
- 8 2. Gao L, Taha R, Gauvin D, Othmen LB, Wang Y, Blaise G. Postoperative cognitive
9 dysfunction after cardiac surgery. *Chest.* 2005;128(5):3664-3670.
- 10 3. Xiao JY, Xiong BR, Zhang W, et al. PGE2-EP3 signaling exacerbates hippocampus-
11 dependent cognitive impairment after laparotomy by reducing expression levels of
12 hippocampal synaptic plasticity-related proteins in aged mice. *CNS Neurosci Ther.* 2018.
- 13 4. Daiello LA, Racine AM, Yun Gou R, et al. Postoperative Delirium and Postoperative
14 Cognitive Dysfunction: Overlap and Divergence. *Anesthesiology.* 2019;131(3):477-491.
- 15 5. Barrientos RM, Hein AM, Frank MG, Watkins LR, Maier SF. Intracisternal interleukin-1
16 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory
17 response in aged rats. *The Journal of neuroscience : the official journal of the Society for*
18 *Neuroscience.* 2012;32(42):14641-14648.
- 19 6. Skvarc DR, Berk M, Byrne LK, et al. Post-Operative Cognitive Dysfunction: An exploration
20 of the inflammatory hypothesis and novel therapies. *Neurosci Biobehav Rev.* 2018;84:116-
21 133.
- 22 7. Femenia T, Gimenez-Cassina A, Codeluppi S, et al. Disrupted Neuroglial Metabolic
23 Coupling after Peripheral Surgery. *The Journal of neuroscience : the official journal of the*
24 *Society for Neuroscience.* 2018;38(2):452-464.
- 25 8. Sparkman NL, Buchanan JB, Heyen JR, Chen J, Beverly JL, Johnson RW. Interleukin-6
26 facilitates lipopolysaccharide-induced disruption in working memory and expression of
27 other proinflammatory cytokines in hippocampal neuronal cell layers. *The Journal of*
28 *neuroscience : the official journal of the Society for Neuroscience.* 2006;26(42):10709-
29 10716.
- 30 9. Rosi S, Ramirez-Amaya V, Vazdarjanova A, Worley PF, Barnes CA, Wenk GL.
31 Neuroinflammation alters the hippocampal pattern of behaviorally induced Arc
32 expression. *The Journal of neuroscience : the official journal of the Society for*
33 *Neuroscience.* 2005;25(3):723-731.
- 34 10. Bright F, Werry EL, Dobson-Stone C, et al. Neuroinflammation in frontotemporal dementia.
35 *Nat Rev Neurol.* 2019;15(9):540-555.
- 36 11. Terrando N, Eriksson LI, Ryu JK, et al. Resolving postoperative neuroinflammation and
37 cognitive decline. *Ann Neurol.* 2011;70(6):986-995.
- 38 12. Stellwagen D, Beattie EC, Seo JY, Malenka RC. Differential regulation of AMPA receptor
39 and GABA receptor trafficking by tumor necrosis factor-alpha. *The Journal of*
40 *neuroscience : the official journal of the Society for Neuroscience.* 2005;25(12):3219-3228.
- 41 13. Viviani B, Bartesaghi S, Gardoni F, et al. Interleukin-1beta enhances NMDA receptor-
42 mediated intracellular calcium increase through activation of the Src family of kinases. *The*
43 *Journal of neuroscience : the official journal of the Society for Neuroscience.*
44 2003;23(25):8692-8700.

- 1 14. Collinson N, Kuenzi FM, Jarolimek W, et al. Enhanced learning and memory and altered
2 GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA
3 receptor. *J Neurosci*. 2002;22(13):5572-5580.
- 4 15. Papatheodoropoulos C, Koniaris E. alpha5GABAA receptors regulate hippocampal sharp
5 wave-ripple activity in vitro. *Neuropharmacology*. 2011;60(4):662-673.
- 6 16. Rudolph U, Mohler H. Analysis of GABAA receptor function and dissection of the
7 pharmacology of benzodiazepines and general anesthetics through mouse genetics.
8 *Annu Rev Pharmacol Toxicol*. 2004;44:475-498.
- 9 17. Rodgers FC, Zarnowska ED, Laha KT, et al. Etomidate Impairs Long-Term Potentiation In
10 Vitro by Targeting alpha5-Subunit Containing GABAA Receptors on Nonpyramidal Cells.
11 *The Journal of neuroscience : the official journal of the Society for Neuroscience*.
12 2015;35(26):9707-9716.
- 13 18. Lecker I, Yin Y, Wang DS, Orser BA. Potentiation of GABAA receptor activity by volatile
14 anaesthetics is reduced by alpha5GABAA receptor-preferring inverse agonists. *Br J*
15 *Anaesth*. 2013;110 Suppl 1:i73-81.
- 16 19. Liu T, He Z, Tian X, et al. Specific patterns of spinal metabolites underlying alpha-Me-5-
17 HT-evoked pruritus compared with histamine and capsaicin assessed by proton nuclear
18 magnetic resonance spectroscopy. *Biochim Biophys Acta*. 2017;1863(6):1222-1230.
- 19 20. Ahluwalia B, Magnusson MK, Isaksson S, Larsson F, Ohman L. Effects of Aloe barbadensis
20 Mill. extract (AVH200(R)) on human blood T cell activity in vitro. *J Ethnopharmacol*.
21 2016;179:301-309.
- 22 21. Yang X, Yao C, Tian T, et al. A novel mechanism of memory loss in Alzheimer's disease
23 mice via the degeneration of entorhinal-CA1 synapses. *Mol Psychiatry*. 2018;23(2):199-
24 210.
- 25 22. Shu S, Zhu H, Tang N, et al. Selective Degeneration of Entorhinal-CA1 Synapses in
26 Alzheimer's Disease via Activation of DAPK1. *The Journal of neuroscience : the official*
27 *journal of the Society for Neuroscience*. 2016;36(42):10843-10852.
- 28 23. Pei L, Wang S, Jin H, et al. A Novel Mechanism of Spine Damages in Stroke via DAPK1 and
29 Tau. *Cereb Cortex*. 2015;25(11):4559-4571.
- 30 24. Song Z, Xiong B, Zheng H, et al. STAT1 as a downstream mediator of ERK signaling
31 contributes to bone cancer pain by regulating MHC II expression in spinal microglia. *Brain,*
32 *behavior, and immunity*. 2017;60:161-173.
- 33 25. Cunningham C, Champion S, Lunnon K, et al. Systemic inflammation induces acute
34 behavioral and cognitive changes and accelerates neurodegenerative disease. *Biol*
35 *Psychiatry*. 2009;65(4):304-312.
- 36 26. Bowman GL, Dayon L, Kirkland R, et al. Blood-brain barrier breakdown,
37 neuroinflammation, and cognitive decline in older adults. *Alzheimers Dement*.
38 2018;14(12):1640-1650.
- 39 27. Nation DA, Sweeney MD, Montagne A, et al. Blood-brain barrier breakdown is an early
40 biomarker of human cognitive dysfunction. *Nat Med*. 2019;25(2):270-276.
- 41 28. van de Haar HJ, Burgmans S, Jansen JF, et al. Blood-Brain Barrier Leakage in Patients with
42 Early Alzheimer Disease. *Radiology*. 2017;282(2):615.
- 43 29. Czerniawski J, Guzowski JF. Acute neuroinflammation impairs context discrimination
44 memory and disrupts pattern separation processes in hippocampus. *The Journal of*

- 1 *neuroscience : the official journal of the Society for Neuroscience*. 2014;34(37):12470-
2 12480.
- 3 30. Skelly DT, Griffin EW, Murray CL, et al. Acute transient cognitive dysfunction and acute
4 brain injury induced by systemic inflammation occur by dissociable IL-1-dependent
5 mechanisms. *Mol Psychiatry*. 2018.
- 6 31. Williamson LL, Sholar PW, Mistry RS, Smith SH, Bilbo SD. Microglia and memory:
7 modulation by early-life infection. *The Journal of neuroscience : the official journal of the*
8 *Society for Neuroscience*. 2011;31(43):15511-15521.
- 9 32. Kawano T, Yamanaka D, Aoyama B, et al. Involvement of acute neuroinflammation in
10 postoperative delirium-like cognitive deficits in rats. *J Anesth*. 2018;32(4):506-517.
- 11 33. Wang HL, Ma RH, Fang H, Xue ZG, Liao QW. Impaired Spatial Learning Memory after
12 Isoflurane Anesthesia or Appendectomy in Aged Mice is Associated with Microglia
13 Activation. *J Cell Death*. 2015;8:9-19.
- 14 34. Callaway JK, Jones NC, Royse AG, Royse CF. Sevoflurane anesthesia does not impair
15 acquisition learning or memory in the Morris water maze in young adult and aged rats.
16 *Anesthesiology*. 2012;117(5):1091-1101.
- 17 35. Crosby C, Culley DJ, Baxter MG, Yukhananov R, Crosby G. Spatial Memory Performance 2
18 Weeks After General Anesthesia in Adult Rats. *Anesthesia & Analgesia*. 2005;101(5):1389-
19 1392.
- 20 36. Walters JL, Zhang X, Talpos JC, et al. Sevoflurane exposure has minimal effect on cognitive
21 function and does not alter microglial activation in adult monkeys. *Neurotoxicology*.
22 2019;71:159-167.
- 23 37. Qiu L-L, Luo D, Zhang H, et al. Nox-2-Mediated Phenotype Loss of Hippocampal
24 Parvalbumin Interneurons Might Contribute to Postoperative Cognitive Decline in Aging
25 Mice. *Frontiers in Aging Neuroscience*. 2016;8.
- 26 38. Crowley T, Cryan JF, Downer EJ, O'Leary OF. Inhibiting neuroinflammation: The role and
27 therapeutic potential of GABA in neuro-immune interactions. *Brain, behavior, and*
28 *immunity*. 2016;54:260-277.
- 29 39. Chazalon M, Paredes-Rodriguez E, Morin S, et al. GAT-3 Dysfunction Generates Tonic
30 Inhibition in External Globus Pallidus Neurons in Parkinsonian Rodents. *Cell Rep*.
31 2018;23(6):1678-1690.
- 32 40. Lange MD, Jungling K, Paulukat L, et al. Glutamic acid decarboxylase 65: a link between
33 GABAergic synaptic plasticity in the lateral amygdala and conditioned fear generalization.
34 *Neuropsychopharmacology : official publication of the American College of*
35 *Neuropsychopharmacology*. 2014;39(9):2211-2220.
- 36 41. Zurek AA, Yu J, Wang DS, et al. Sustained increase in alpha5GABAA receptor function
37 impairs memory after anesthesia. *J Clin Invest*. 2014;124(12):5437-5441.
- 38 42. Soto PL, Ator NA, Rallapalli SK, et al. Allosteric modulation of GABA(A) receptor
39 subtypes:effects on visual recognition and visuospatial working memory in rhesus
40 monkeys [corrected]. *Neuropsychopharmacology : official publication of the American*
41 *College of Neuropsychopharmacology*. 2013;38(11):2315-2325.
- 42 43. Gao T, Liu Y, Zhao Z, et al. L-655,708 Does not Prevent Isoflurane-induced Memory
43 Deficits in Old Mice. *Transl Neurosci*. 2019;10:180-186.
- 44 44. Li Z, Liu P, Zhang H, et al. Role of GABAB receptors and p38MAPK/NF-kappaB pathway

1 in paclitaxel-induced apoptosis of hippocampal neurons. *Pharm Biol.* 2017;55(1):2188-
2 2195.
3 45. Zhang XY, Xu ZP, Wang W, et al. Vitamin C alleviates LPS-induced cognitive impairment
4 in mice by suppressing neuroinflammation and oxidative stress. *Int Immunopharmacol.*
5 2018;65:438-447.

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32 **Figure legends**

33 **Figure1. Behavioral tests and hippocampal LTP in aged mice.** (A) Illustration of the
34 experimental processes. 16-month old female mice were randomly divided into 3
35 groups (Control, Anesthesia, Laparotomy). Behavioral tests were conducted from 8
36 days to 11 days after anesthesia or laparotomy. Samples were taken for LTP, MSD and
37 NMR 7 days after anesthesia or laparotomy. (B-D) In the NORT, the total time spent
38 with two same objects was similar among the three groups. In the laparotomy group,
39 the mice spent less time on the novel object and presented lower discrimination ratio
40 compared with the other two groups. (n=11) (E-F) In the FCT, the mice in the
41 laparotomy group showed lower freezing time to the context, and there was no
42 difference in the tone freezing time. (n=11) (G) Hippocampal LTP was impaired in the
43 laparotomy mice. (n=7) Data are presented as mean \pm SEM. ** p <0.01, *** p <0.001,
44 ### p <0.001.

45
46 **Figure2. The morphology of microglia and the levels of inflammatory cytokines in**

1 **the hippocampus.** (A) Microglia was activated in the CA1, CA3 and DG regions in
2 the laparotomy mice. The white arrow points to the activated microglia. (B-D) The
3 levels of IL-1 β , IL-6 and TNF- α in the laparotomy mice was up-regulated and TNF- α
4 was also increased in the anesthesia mice. (n=3) Data are presented as mean \pm SEM.
5 * p <0.05, ** p <0.01.

6
7 **Figure3. The expressions of neurotransmitters and different subunits of**
8 **GABA_ARs.** (A-B) The expression of GABA was decreased in the laparotomy mice and
9 no difference was found about glutamate. (n=9) (C) The different average spectra of
10 selected metabolites (GABA and glutamate). (D-F) The mRNA level of α 5 subunit was
11 up-regulated at 1 day and continued to 10 days after laparotomy. No difference was
12 found about the α 1 and β 3 subunits. (n=3) (G-I) The expressions of GAT-3 and GAD65
13 were decreased and the levels of surface α 5GABA_ARs were increased in the laparotomy
14 mice. (n=4) Data are presented as mean \pm SEM. * p <0.05, ** p <0.01.

15
16 **Figure4. L655,708 could reverse anesthesia and surgery induced learning and**
17 **memory deficits in aged mice.** (A) The diagram shows the process of the experiment.
18 The time points of L655,708 (0.5mg/kg, *i.p.*) or vehicle administered are marked by the
19 red arrow. Samples were taken at the end of the experiment. (B-D) In the NORT, the
20 time spent with objects was similar among the three groups, while the time spent with
21 a novel object and the discrimination ratio were increased in the laparotomy mice after
22 using L655,708. (n=8) (E-F) In the FCT, there was no difference in the tone freezing
23 time after using L655,708. However, the freezing scores for memory of context was
24 increased in the laparotomy mice after using L655,708. (n=8) (G-I) The amplitude of
25 fPSPs in the laparotomy group was increased after using L655,708, while there was no
26 difference in the control and anesthesia mice. (n=7) Data are presented as mean \pm SEM.
27 ** p <0.01, *** p <0.001, ### p <0.001.

28
29 **Figure5. The protein levels of MAPK signaling pathway in the hippocampus.** (A-
30 C) The protein level of p-P38 was increased after laparotomy compared to the control
31 and anesthesia groups, and there was no difference in the expressions of P38, JNK1/2,
32 p-JNK1/2, ERK1/2 and p-ERK1/2. (n=4) Data are presented as mean \pm SEM. ** p <0.01.

33
34 **Figure6. SB203,580 could reverse anesthesia and surgery induced learning and**
35 **memory deficits in aged mice.** (A) the diagram shows the process of the experiment.
36 The time points of SB203,580 (10mg/kg *i.p.*) or vehicle administered are marked by
37 the red arrow. Samples were taken at the end of the experiment. (B-D) In the NORT,
38 the time spent with objects was similar among the three groups, while the time spent
39 with the novel object and the discrimination ratio were increased in the laparotomy
40 mice after using SB203,580. (n=8) (E-F) In the FCT, the context freezing time was
41 increased in the laparotomy mice after using SB203,580, and there was no difference
42 in the tone freezing time. (n=8) (G-J) The protein levels of p-P38 and surface
43 α 5GABA_ARs were decreased in the laparotomy mice after using SB203,580, and no
44 difference was found in the expressions of p-JNK1/2 and p-ERK1/2. (n=4) (K-M) The

1 amplitude of fPSPs in the laparotomy mice was increased after using SB203,580, and
2 there was no difference in the control and anesthesia mice. (n=7). Data are presented as
3 mean \pm SEM. * p <0.05, ** p <0.01, *** p <0.001, ### p <0.001, **** p <0.0001.
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