International Immunopharmacology 70 (2019) 446–458

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Two-hit model of postintensive care syndrome induced by lipopolysaccharide challenge and subsequent chronic unpredictable stress in mice

Mingjie Mao[a,1], Shuming Li[b,1], Manman Zong[a], Lili Qiu[b], Jiaojiao Yang[b], Jiangyan Xia[b], Jianjun Yang[c,*], Muhuo Ji[b,*]

[a] Department of Anesthesiology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China
[b] Department of Anesthesiology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, China
[c] Department of Anesthesiology, First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

ARTICLE INFO

Keywords:
Animal model
Sepsis
Behavioral outcomes
Stressor
Fluoxetine

ABSTRACT

Postintensive care syndrome (PICS) is defined as a new or worsening impairment in cognition, mental health, and physical function after critical illness. However, there is still a lack of a clinically relevant animal model. Thus, development of a PICS model is essential for understanding the mechanism underlying PICS and screening treatment methods for this neuropsychiatric disorder. The purpose of this study was to establish a clinically relevant PICS model based on the two-hit concept, in which lipopolysaccharide (LPS, 3 mg/kg) injection was served as the first hit and subsequent modified chronic unpredictable stress as the second hit. In order to pharmacologically verify the proposed model of PICS, we studied the effectiveness of fluoxetine to reverse the behavioral and molecular abnormalities in this model. In the present study, body- and adrenal weight changes proved our model was effective, as reflected by body weight loss, increased adrenals weight, and a significantly increased level of plasma corticosterone. Moreover, our PICS model displayed reproducible anxiety- and depression like behavior and cognitive impairments. Neurobiological investigations revealed a significant up-regulation of the microglial marker CD68 and pro-inflammatory cytokine IL-6 in the hippocampus of stressed mice. Notably, chronic treatment with fluoxetine for three weeks reversed most of the affected parameters. In summary, we believe that we have developed a new model of PICS that is clinically relevant, which could advance the mechanism research and the development of therapeutic strategies.

1. Introduction

Postintensive care syndrome (PICS) is defined as a new or worsening impairment in cognition, mental health, and functional disability after critical illness [1–3]. It has been reported that 25 to 78% of Intensive Care Unit (ICU) survivors experience negative neurocognitive consequences, which are characterized by cognitive impairments (e.g., impaired executive function, memory, and attention), mental health morbidities (acute stress disorder, anxiety, depression, and post-traumatic stress disorder), and physical impairments [2]. Among specific populations, such as patients with acute respiratory distress syndrome, the prevalence of neurocognitive impairments is even greater, and may be as high as 78% at hospital discharge, 46% at 1 year, and 25% at 6 years, respectively [4]. As more patients survive critical illness, preventing or treating PICS has become one major public health issue. Unfortunately, current therapeutic interventions are relatively ineffective and the development of novel treatments is hampered by the lack of a well-characterized animal model.

Severe sepsis is the most common noncardiac cause of critical illness admitted to ICU and contributes to significant long-term neurobehavioral abnormalities [1]. Similar changes in cognitive and behavioral domains can be described in animal models of sepsis-survival, suggesting that such approaches may be useful in understanding mechanisms and factors that shape post-septic neurobehavioral outcomes [5–8]. Various attempts have been made to replicate sepsis-induced long-term neurocognitive impairments using animal models. At present, the most commonly used animal models are induced by lipopolysaccharide (LPS) challenge or cecal ligation and puncture (CLP) [5–8].

* Corresponding authors.
E-mail addresses: yjyangjj@126.com (J. Yang), jimuhuo2009@sina.com (M. Ji).
[1] These two authors have contributed equally to this manuscript.

https://doi.org/10.1016/j.intimp.2019.03.012
Received 22 January 2019; Received in revised form 27 February 2019; Accepted 6 March 2019
Available online 08 March 2019

These preclinical animal models do provide evidence whereby sepsis exerts detrimental effects on the central nervous system. However, it should be noted that critically ill patients in the ICU not only have primary etiologies such as sepsis but also face tremendous physical and psychological stressors, including social isolation, chemical and physical restraints, sleep disturbances due to changes in biological rhythm induced by noise and light on, and other environmental factors [10,11]. These environmental stressors have profound effects on brain function, both structurally and functionally. Our recent study suggests that stress has even more negative impact on cognitive dysfunction in comparison to immune challenge stimulated by LPS injection alone [6], highlighting the important role of stress on subsequent neurobehavioral performance.

Therefore, the present study aims to establish a clinically relevant animal model of PICS based on the two-hit concept, in which LPS injection was served as the first hit and subsequent chronic unpredictable stress as the second hit. In order to pharmacologically verify the proposed model of PICS, we investigated the effectiveness of fluoxetine to reverse the behavioral and molecular abnormalities in this new animal model of PICS.

2. Materials and methods

2.1. Animals

All experiments were carried out on male C57BL/6 mice, which were between 12 and 14 weeks old at the start of each experiment. Experimental protocols and monitoring for suffering were treated according to the National Institute of Health Guidelines on the use of laboratory animal and with approval of the Animal Care and Use Committee of Zhongda Hospital, Medical School, Southeast University, Nanjing, China. Animals were maintained on a reversed 12:12-h light/dark cycle and had ad libitum access to water and mouse standard diet. Mice were allowed to acclimate to the new surroundings for 2 weeks before initiation of any experimental procedure. All behavioral tests were carried out between 9:00 and 12:00 AM on the designated day of experiment.

2.2. Drug treatments

LPS (*Escherichia coli* endotoxin 0111: B4, Sigma, Lot # 064M4125V, Shanghai, China) was dissolved in pyrogen-free 0.9% isotonic saline and intraperitoneally (*i.p.*) injected at a dose of 3 mg/kg at a volume of 5.0 ml/kg. All injections were prepared freshly on the treatment day and injections for all subjects were given from 8:00 to 9:00 AM to control for circadian effects. To test whether fluoxetine treatment can reverse the behavioral abnormities in this new model of PICS, 20 mg/kg fluoxetine (Tocris Bioscience, Bristol, UK) was given in the drinking water 1 week after LPS injection until the end of the behavioral tests.

2.3. Modified stress model

The stress model is a modified version of the chronic unpredictable stress protocol originally designed for mice. To be clinically relevant, we selected stressors that are frequently encountered in the critically ill patients living in the ICU. Briefly, animals were housed singly and exposed to four of the following stressors daily in a random order for 21 days, including physical restraint for 6 h (a stainless mesh that allowed for a close fit to mice), cage tilt 45 °C overnight (12 h), food/water deprivation overnight, odor overnight, wet bedding overnight, lights-off for 3 h during the daylight phase, noise overnight, and light on overnight (Table 1). Animals in the control group were housed in groups of 4–6 in standard environment and received no stressors.

2.4. Survival rate and body weight measurement

Following LPS challenge and stressors, animals were individually assessed in terms of mortality and weight daily at 11:00 AM during the stress period. At the end of the experiment, adrenal was collected and then weighted.

2.5. Behavioral experiments

At the end of the chronic stress period, a battery of well-established behavioral tests was used to assess behavioral alterations as we previously described [6,9]. All behavioral studies were performed between 13:00 and 17:00 PM under dim lighting conditions. Mice were transported and left for habituation to the testing room for 2 h before each behavioral testing. Each animal underwent no more than three tests, with less stressful tests (open field, novel object recognition test, and sucrose preference test) preceding more stressful tests (elevated plus maze, fear conditioning test, and forced swim test). All animals underwent only one aversive test. All the behavior of mice was recorded using a video camera (Shanghai Softmaze Information Technology Co. Ltd., Shanghai, China).

2.6. Open field test

Mice were placed individually in the center of the plastic chamber (50 cm × 50 cm × 40 cm) and were allowed to freely explore the arena for 5 min. The total distance traveled and time spent in the center of the open field arena were recorded. Between each trial, the mouse was removed while the arena was cleaned with 70% ethanol solution.

2.7. Novel object recognition test

The object recognition task was performed in a square wooden open-field apparatus (40 × 40 × 40 cm). One day before the test, the mice were habituated to the experimental arena for 10 min without any objects. In the first (training) trial, two familiar objects were presented. The second trial (testing) with one familiar object and one novel object present in the respective zones of the open field, with 60-min intervals between trials, during which the animals were placed back to their home cages. The time spent with each object was recorded, and the cognitive outcomes were determined by the “preference index” for the second trial, which was calculated using the following formula: preference index = Time spent in novel object zone / (time spent in familiar object zone + time spent in novel object zone).

2.8. Elevated plus maze

The elevated plus maze was conducted in accordance with the protocol as we previously described [12]. The maze consisted of a central area of diameter 10 cm, from which four arms extended of length 45 cm, width 5 cm. Two arms were open without walls, while the other two were enclosed by high walls (50 cm). Entrance to an arm was counted where all four of an animals' paws were within the arm. Animals underwent one five-minute testing session each.

2.9. Sucrose preference test

Anhedonia was measured by preference for a sucrose solution over water, using a two-bottle free choice method. Briefly, each mouse was presented simultaneously with two bottles, one with 1% sucrose solution and the other containing tap water. Mice were then given a free choice between either tap water or 1% sucrose in tap water solution for 24 h. After 12 h, the position of the two bottles was switched to control for a side preference in drinking behavior. Twenty-four hours later, the bottles were then weighed to measure how much liquid was consumed. Sucrose preference was calculated as sucrose consumption / (sucrose

Table 1
Modified unpredictable chronic stress paradigm.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	C, D, G, H	A, C, F, G	B, D, F, H	A, B, C, G	B, D, E, F	A, D, F, H	B, C, F, G
Week 2	A, B, E, H	A, C, E, G	B, C, D, G	A, B, D, H	C, E, F, G	A, E, G, H	A, E, F, G
Week 3	B, C, F, G	B, E, G, H	A, D, F, G	A, C, G, H	A, B, D, E	B, C, D, H	C, D, F, H

A, light on overnight (12 h); B, physical restraint for 6 h; C, cage tilt 45° for 12 h; D, lights-off for 3 h during the daylight phase; E, wet bedding overnight; F, odor overnight; G, noise in the room for 12 h; H, food and water deprivation overnight.

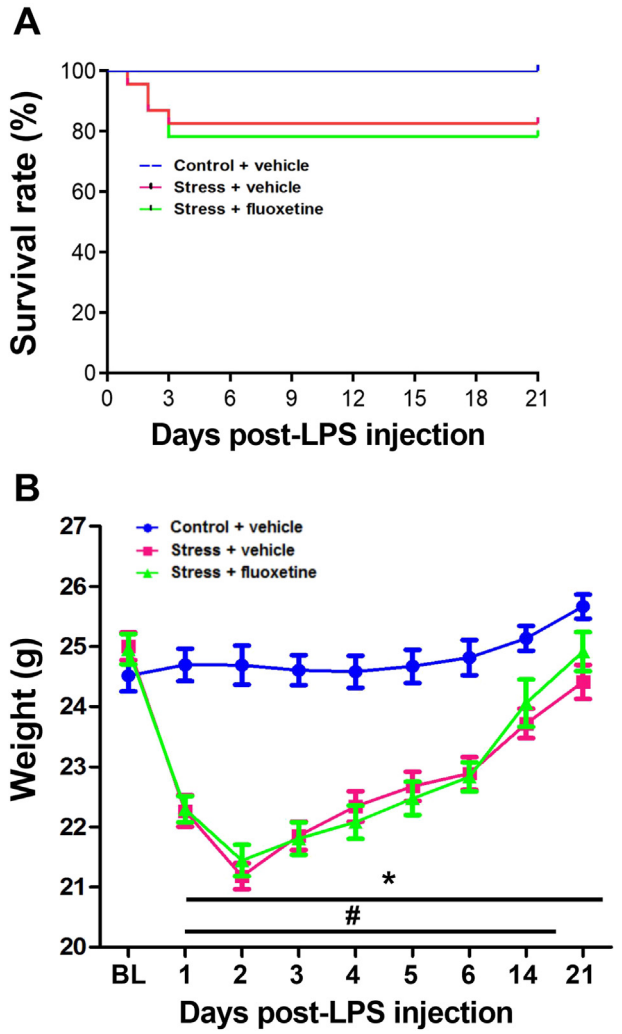

Fig. 1. Survival rate and body weight changes. (A) No animals died in the control group. The survival rate was 82.609% in the stress group and 78.261% in the stress + fluoxetine group (n = 20–23). (B) Stress caused a significant decrease in body weight compared to pre-challenge values. In addition, stress induced significant body weight loss throughout the experimental period when compared with control mice (n = 20–23).*$p < 0.05$ vs control group; #$p < 0.05$ vs baseline level. BL, baseline. LPS, lipopolysaccharide.

consumption + water consumption) × 100%.

2.10. Fear conditioning test

Mice were placed into the conditioning chamber (32 cm × 25 cm × 25 cm), with a stainless-steel shock grid floor. The mice were allowed to explore for 3 min for habituation, and then a 30 s, 80 dB, 1 kHz tone (CS) was delivered, followed by a 2 s, 0.75 mA foot shock (US) through stainless steel bars by a constant current generator. The contextual memory was tested 24 h after the training. The animals were placed back to the original training chamber to monitor the freezing behavior, which was defined as an absence of any movements for > 3 s. The cued fear memory was tested 2 h later in a novel environment with a continuous 3 min training tone presentation to monitor freezing behavior.

2.11. Forced swim test

This test measures depressive-like behavior with immobility taken as the dependent measure of behavioral despair. Mice were placed singly in a 4 l clear plexiglass beaker (15 cm diameter 30 cm height) filled with water (20–24 °C) for 6 min, with the immobility scored in the final four min only. Time spent immobile (absence of movement except leg kicks to stay afloat) is then used as a measure of behavioral despair and helplessness, a rodent analogue of depressive-like behavior.

2.12. Immunofluorescence

Under deep isoflurane anesthesia, mice were perfused transcardially with normal saline, followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min. Brains were harvested and postfixed in 4% PFA for 6 h and then with 30% sucrose for 24 h at 4 °C. Brains were freeze-mounted in optimal cutting temperature embedding medium, cut into 25-μm-thick sections using a cryostat, and mounted on slides. Slices were blocked with 3% bovine serum albumin for 1 h at room temperature. The sections were then incubated with a rat anti-ionized calcium-binding adaptor molecule-1 (IBA1) (1:500; Wako, Japan), CD68 (1:500; BIO-RAD, USA), and anti-glial fibrillary acidic protein (GFAP, 1:1000, Sigma, USA) antibody overnight at 4 °C, followed by 1 h incubation with the secondary antibodies (Cy3-conjugated donkey anti-rat IgG (1:300; Santa Cruz Biotechnology, Dallas, TX)) at room temperature. After washing in PBS, sections were counterstained with 4′, 6-diamidino-2-pheny-lindole (DAPI) for 15 min at room temperature and mounted on glass slides and coverslipped with fluorescence mounting medium. Photomicrographs of sections were taken using a digital camera connected to an Olympus BX-51 light microscope under constant light intensity. A total of three independent microscopic fields in each section were randomly acquired in the hippocampus. Images were captured under high-power magnification (400×) (Olympus, FV1000, Japan) in every eighth section. The intensity of IBA1, CD68, and GFAP positive cells was quantified with Image J software (U.S. National Institutes of Health, Bethesda, MD, USA).

2.13. Enzyme-linked immunosorbent assay (ELISA) analysis

Mice were killed by an *i.p.* injection of 2% sodium pentobarbitone (60 mg/kg) and then the chest cavity and the heart were opened and blood was collected in a pre-chilled syringe. After centrifugation of the blood homogenates, supernatants were collected and immediately stored at −80 °C. The concentrations of corticosterone in supernatants were analyzed by using ELISA kits (Senbeijia, Nanjing, China) according to the manufacturer's instructions. The amount of corticosterone was expressed in nanograms per milliliter of plasma. The prefrontal cortex (PFC) and hippocampus were collected, then separated, and placed in a homogenizer. Homogenates were centrifuged at 5000 *g*

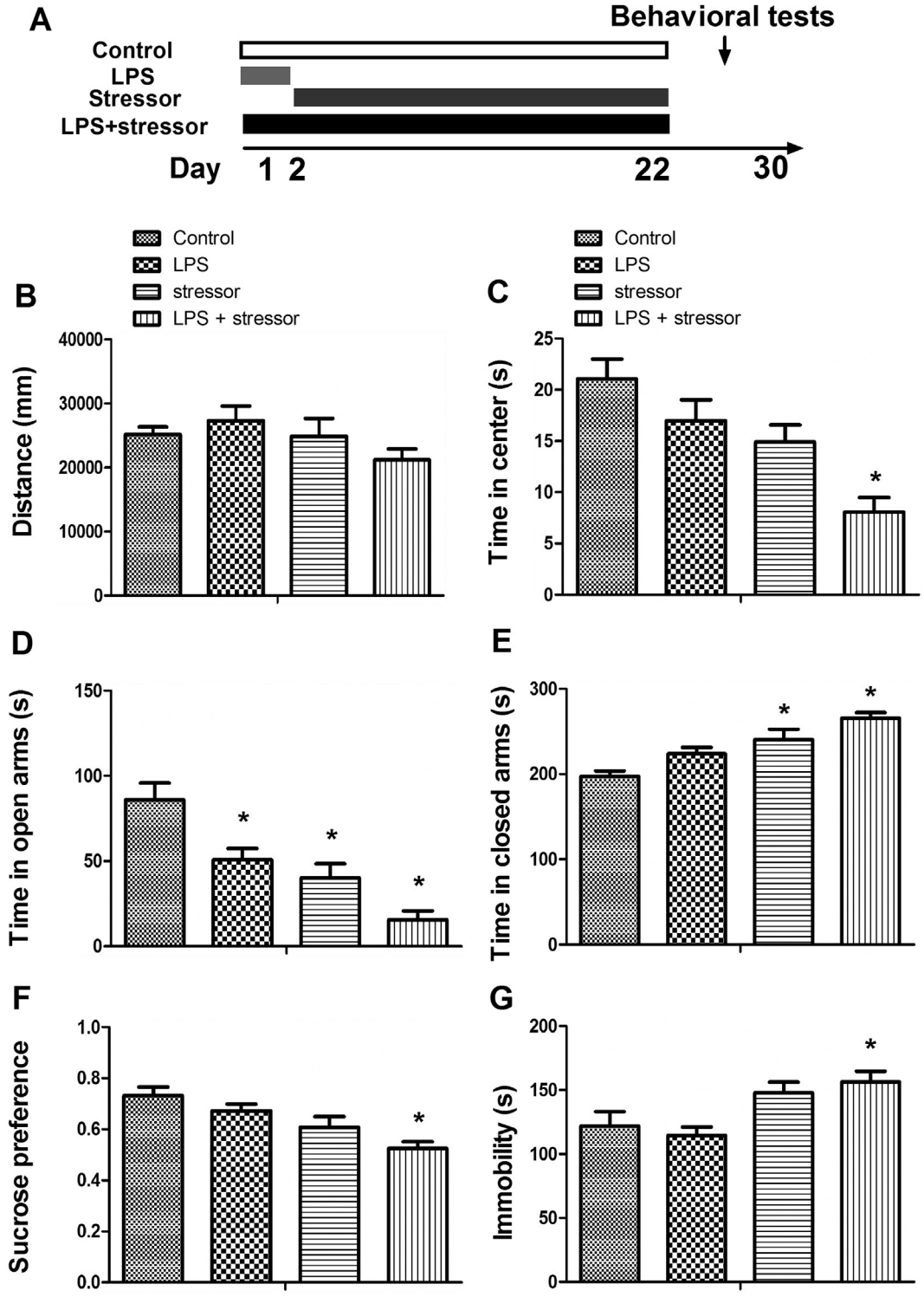

Fig. 2. Effects of LPS and stressor on anxiety- and depression like behavior. (A) Schematic timeline of the experimental procedure. (B) LPS and/or stressor had no effect on distance traveled in the open field test. (C) LPS + stressor significantly decreased the time spent in the center in the open field test. (D) LPS and/or stressor decreased the time in the open arms. (E) Stressor or LPS + stressor had increased time in the closed arms in the elevated plus maze test. (F–G) Effects of LPS and stressor on behavioral performance in the sucrose preference test and forced swim test (n = 14–15). *$p < 0.05$ vs control group. LPS, lipopolysaccharide.

for 10 min at 4 °C. The levels of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10, brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) were determined with ELISA kits (JianCheng Biotechnology, Nanjing, China) according to the manufacturer's instructions.

2.14. Statistical analysis

Statistical analyses were done using the Statistical Package for Social Sciences (SPSS version 22.0; SPSS Inc., IBM). Data are presented as mean ± standard error of the mean (S.E.M.). The data were carefully screened for normality and homogeneity of variance. Weight gain

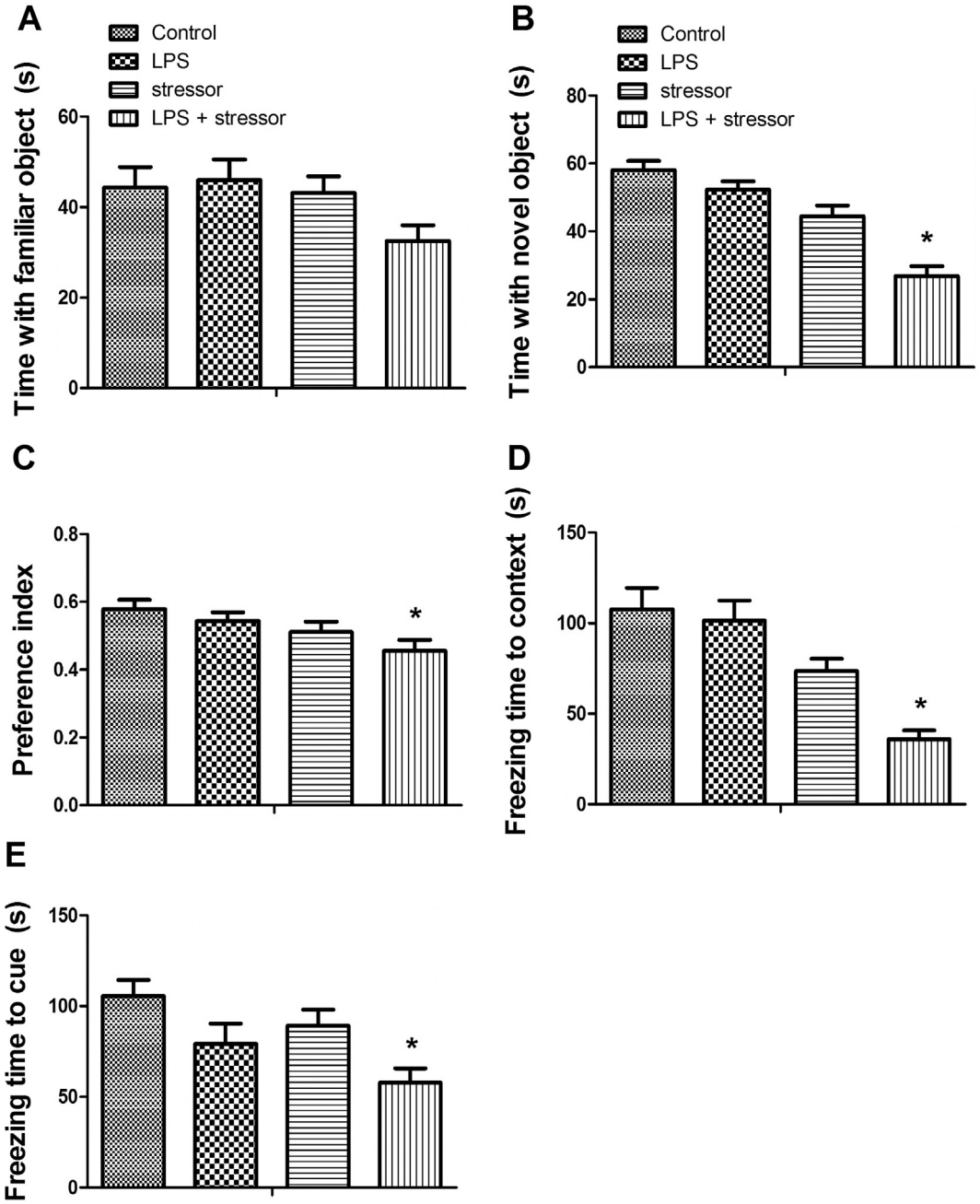

Fig. 3. Effects of LPS and stressor on cognitive performance. (A–C) Effects of LPS and stressor on cognitive performance in the novel object recognition test. LPS + stressor group had significant decreased time with novel object. (D–E) Effects of LPS and stressor on behavioral performance in the fear conditioning test. LPS + stressor group had significant decreased freezing time to the context and cue in the fear conditioning test (n = 14–15). *$p < 0.05$ vs control group. LPS, lipopolysaccharide.

was analyzed by two-way analysis of variance (ANOVA) for repeated measures, followed by the Bonferroni post hoc test. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's test. When ANOVA's assumptions were not fulfilled, we used the Kruskal–Wallis test and Mann-Whitney *U* test for multiple comparisons. The survival rate was estimated by Kaplan–Meier method and compared by the log-rank test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Survival rate and body weight changes

As shown in Fig. 1A, no animals died in the control group. The survival rate was 82.609% in the stress group and 78.261% in the stress + fluoxetine group. Body weight change is a commonly used physical parameter to assess the efficacy of stress exposure. At the beginning of the experiment, there was no difference in body weight among the control, stress, and stress + fluoxetine groups. Stress caused a significant decrease in body weight compared to pre-challenge values. Moreover, stress induced significant body weight loss throughout the experimental period when compared with control mice. There was a

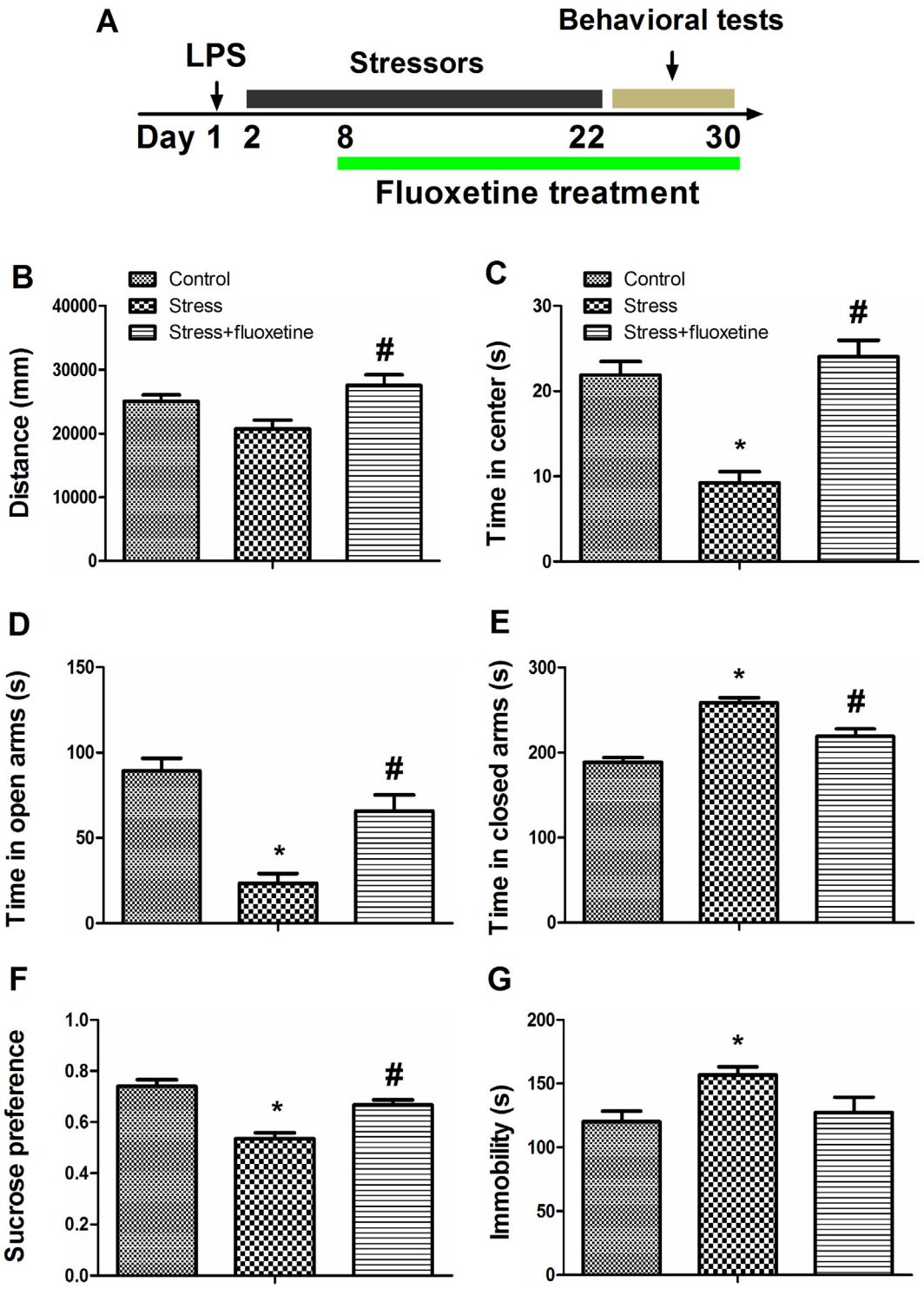

Fig. 4. Effects of stress and fluoxetine on anxiety- and depression like behavior. (A) Schematic timeline of the experimental procedure. (B–C) Effects of stress and fluoxetine on behavioral performance in the open field test. Stress (LPS + stressor) group had significantly decreased time in the center of the open field arena, which can be reversed by the fluoxetine treatment. (D–E) Effects of stress and fluoxetine on behavioral performance in the elevated plus maze test. Stress (LPS + stressor) group had significantly decreased time in the open arms, which can be reversed by the fluoxetine treatment. (F–G) Effects of stress and fluoxetine on behavioral performance in the sucrose preference test and forced swim test. Stress (LPS + stressor) group had significantly decreased sucrose preference, which can be reversed by the fluoxetine treatment (n = 18–20). *$p < 0.05$ vs control group; #$p < 0.05$ vs stress group. LPS, lipopolysaccharide.

trend toward an increased body weight at the end of the stress protocol in the stress + fluoxetine group as compared with the stress group.

3.2. Behavioral evaluations

At the end of the stress period, a battery of well-established behavioral tests was used to assess behavioral alterations. In the first set of experiment, we compared behavioral alterations among the control,

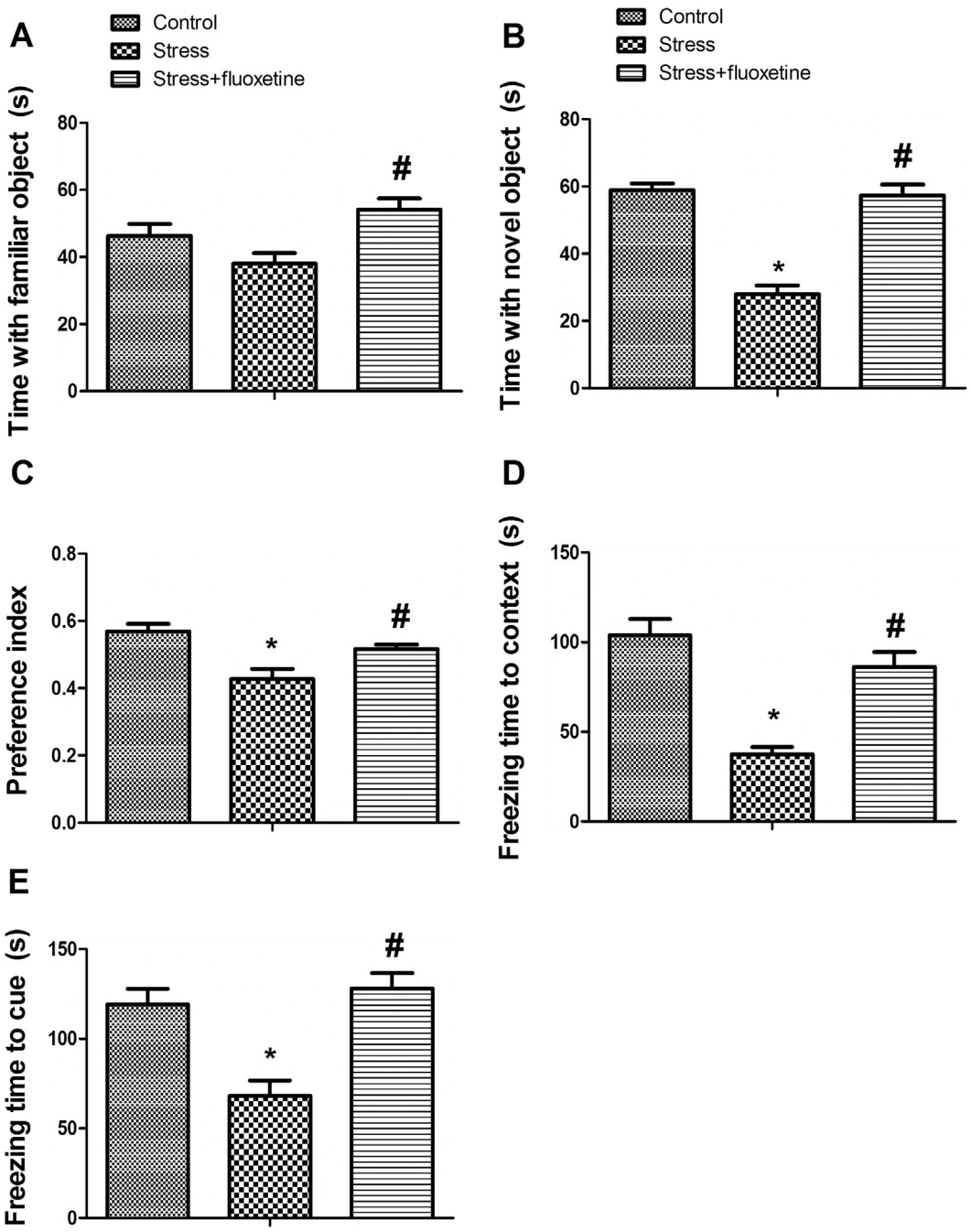

Fig. 5. Effects of stress and fluoxetine on cognitive performance. (A–C) Effects of stress and fluoxetine on cognitive performance in the novel object recognition test. Stress (LPS + stressor) group had significantly decreased time with the novel object, which can be reversed by the fluoxetine treatment. (D–E) Effects of stress and fluoxetine on behavioral performance in the fear conditioning test. Stress (LPS + stressor) group had significantly decreased freezing time to the context and cue in the fear conditioning tests, which can be reversed by the fluoxetine treatment (n = 18–20). *$p < 0.05$ vs control group; #$p < 0.05$ vs stress group.

LPS, stressor, and LPS + stressor (stress group) groups. As shown in Figs. 2 and 3, LPS or stressor exposure alone resulted in some behavioral abnormalities. However, mice in the stress (LPS + stressor) group displayed reproducible anxiety- and depression like behavior and cognitive impairments. Accordingly, we selected the two-hit stress protocol to establish the PICS model.

The open field test was performed to investigate whether stress influence locomotor activity and anxiety-like behavior. Although stress did not affect the total distance traveled (Fig. 4B), it significantly decreased time in the center of the arena as compared with the control mice ($F_{(2, 54)} = 24.852$, $P < 0.001$, Fig. 4C). In the elevated plus maze test, the time spent in the open arms decreased significantly ($F_{(2, 54)} = 26.309$, $P < 0.001$, Fig. 4D) while it increased in the closed arms ($F_{(2, 54)} = 19.734$, $P < 0.001$, Fig. 4E) in the stress group compared with the control group. These results suggested that stress induced anxiety-like behavior, which was prevented by chronic fluoxetine treatment.

In the sucrose preference test, stressed mice exhibit significantly less preference for sucrose than controls ($F_{(2, 54)} = 20.701$, $P < 0.001$, Fig. 4F). However, chronic fluoxetine treatment significantly reversed this parameter. In addition, mice in the stress group showed more immobility in the forced swim test as compared with the control group ($F_{(2, 54)} = 20.701$, $P < 0.001$, Fig. 4G). Intriguingly, chronic fluoxetine treatment was not able to completely reverse immobility in the

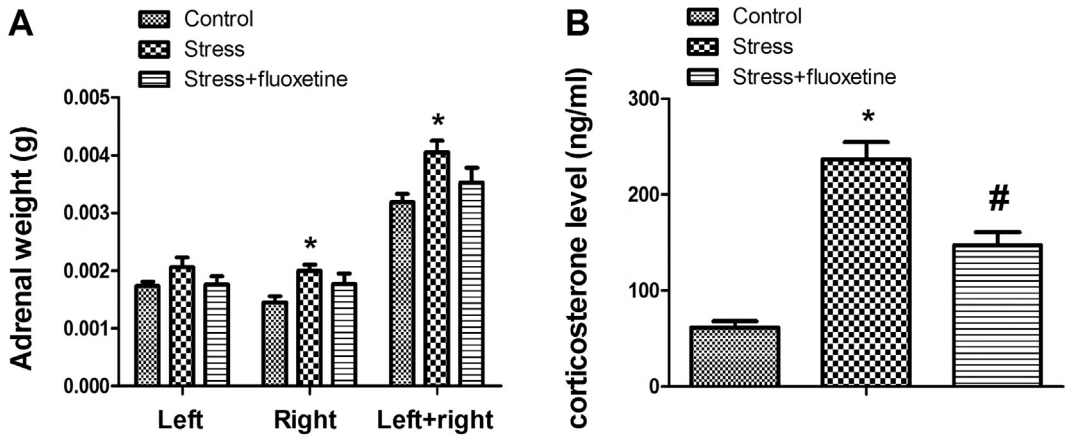

Fig. 6. Effects of stress and fluoxetine on adrenal gland weight and plasma level of corticosterone. (A) Effects of stress and fluoxetine on adrenal gland weight. Stress (LPS + stressor) group had significantly increased adrenal gland weight, which was not completely reversed by fluoxetine treatment (n = 12). (B) Effects of stress and fluoxetine on plasma level of corticosterone. Stress (LPS + stressor) group had significantly increased plasma level of corticosterone, which was reversed by fluoxetine treatment (n = 12). *$p < 0.05$ vs control group; #$p < 0.05$ vs stress group.

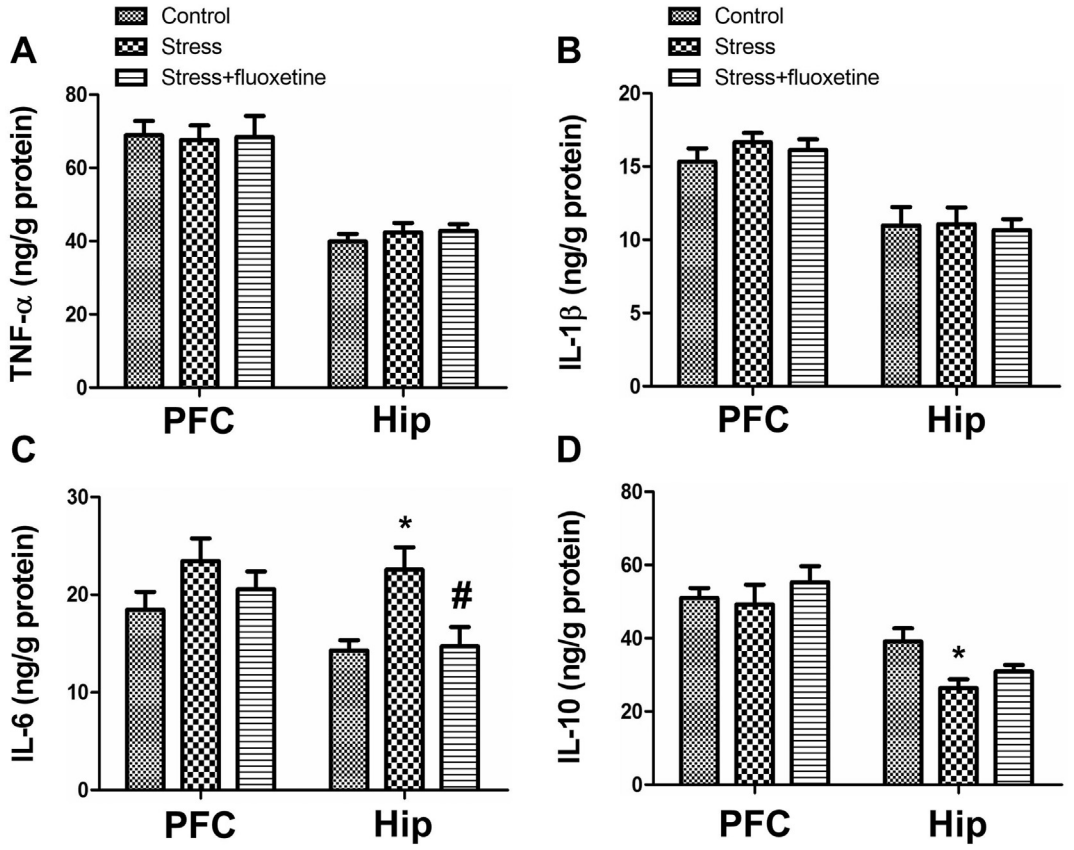

Fig. 7. Effects of stress and fluoxetine on brain TNF-α, IL-1β, IL-6, and IL-10 levels. Stress (LPS + stressor) group had significantly increased hippocampal level of IL-6, which was reversed by fluoxetine treatment (n = 8). *$p < 0.05$ vs control group; #$p < 0.05$ vs stress group. PFC, prefrontal cortex; Hip, hippocampus.

stress + fluoxetine group.

The novel object recognition test was used to evaluate working memory. Stress decreased their exploration time with the novel object ($F_{(2, 54)} = 44.355$, $P < 0.001$, Fig. 5B) and the discrimination index ($F_{(2, 54)} = 9.864$, $P < 0.001$, Fig. 5C) compared with the control group, suggesting stress induced working memory impairment. In addition, we performed the fear conditioning test to evaluate whether stress impaired contextual fear memory. As reflected in Fig. 5, stress significantly decreased the freezing time in the contextual fear conditioning test as compared with the control group ($F_{(2, 54)} = 21.698$, $P < 0.001$, Fig. 5D). Also, stress significantly decreased the post-tone freezing time in the auditory-cued fear test when compared with the control group ($F_{(2, 54)} = 14.12$, $P < 0.001$, Fig. 5E). Of note, most of these cognitive impairments could be ameliorated by chronic fluoxetine treatment.

3.3. Adrenal gland weight and plasma level of corticosterone changes

Adrenal gland weight is an effective indicator of stress level. As shown in Fig. 6, stressed mice had a significant increase on adrenal

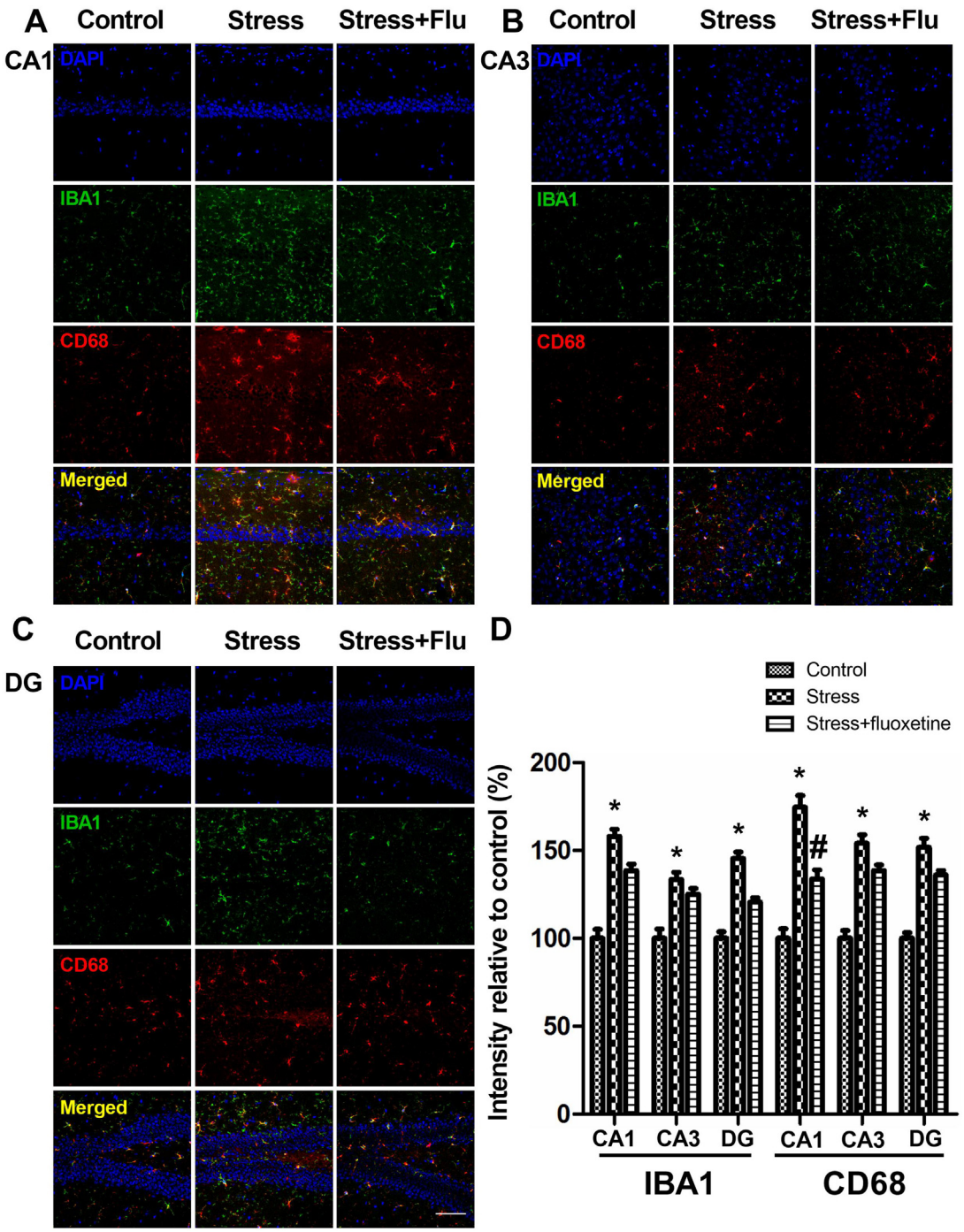

Fig. 8. Double-immunofluorescence staining to detect co-localization of IBA1 and CD68 in the hippocampus. (A–C) Representative images of IBA1 (green) and CD68 (red) in the CA1, CA3, and DG regions. (D) Quantification of IBA1 and CD68 intensity. Data are shown as mean ± SEM (n = 6), *$P < 0.05$ vs control group; #$p < 0.05$ vs stress group, scale bar = 50 μm. Flu, fluoxetine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

weight ($F_{(2, 33)} = 4.555$, $P = 0.018$). In addition, there was a significantly increased corticosterone level in the stress group ($F_{(2, 33)} = 42.86$, $P < 0.001$). However, chronic fluoxetine treatment decreased plasma level of corticosterone but not the adrenal weight compared with the stress group.

3.4. Inflammatory mediator changes

As shown in Fig. 7, IL-6 level was significantly increased in the hippocampus in the stress group as compared with the control group ($F_{(2, 21)} = 6.347$, $P = 0.007$), which was prevented by fluoxetine treatment. Although anti-inflammatory IL-10 level was significantly decreased in the stress group than that in the control group, it was not reversed by fluoxetine treatment. However, there was no difference in brain TNF-α and IL-1β levels among groups.

3.5. IBA1 and CD68 changes

The intensities of IBA1 and CD68 in the hippocampus increased significantly in the stress group as compared with the control group

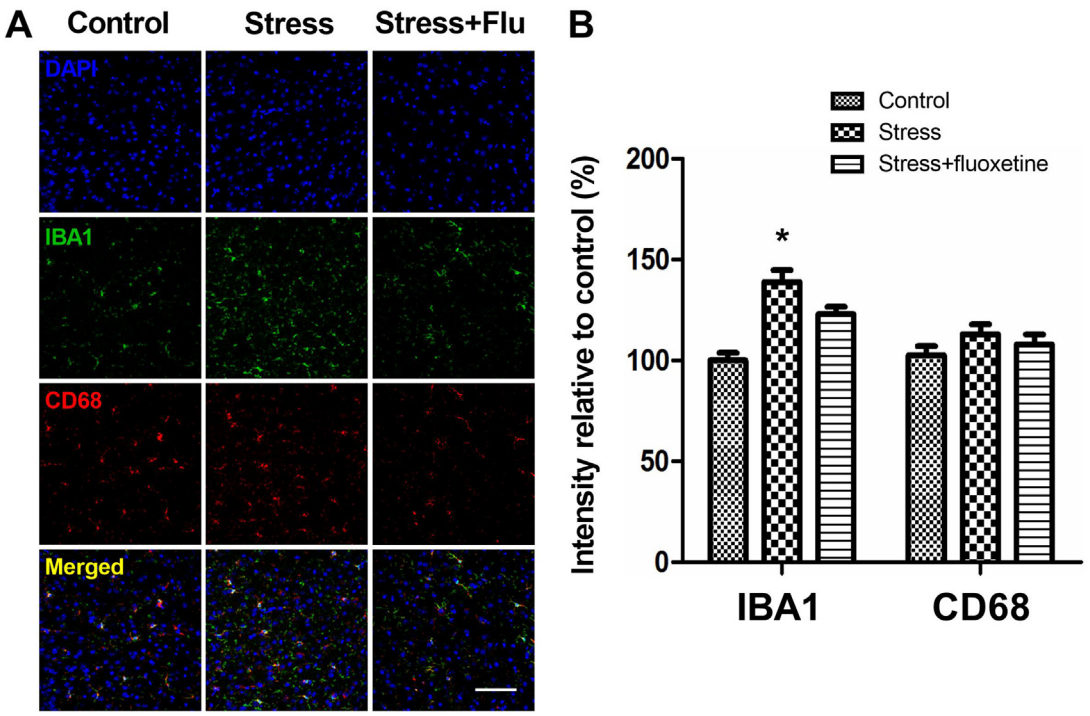

Fig. 9. Double-immunofluorescence staining to detect co-localization of IBA1 and CD68 in the PFC. (A–C) Representative images of IBA1 (green) and CD68 (red) in the PFC. (D) Quantification of IBA1 and CD68 intensity. Data are shown as mean ± SEM (n = 6), *$P < 0.05$ vs control group, scale bar = 50 μm. Flu, fluoxetine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 8). However, chronic treatment with fluoxetine only reversed the intensity of CD68 in the CA1 region of the hippocampus. In the PFC, stress increased the intensity of IBA1 when compared with control mice (Fig. 9), which was not reversed by fluoxetine treatment. However, stress and fluoxetine treatment had no effect on CD68 expression in the PFC.

3.6. GFAP changes

The intensity of GFAP in the hippocampus was significantly increased in the stress group as compared with the control group (Fig. 10), which was not reversed by fluoxetine treatment. However, stress and fluoxetine treatment did not significantly affect GFAP expression in the PFC (Fig. 11).

3.7. Neurotrophic factors changes

Stress significantly decreased hippocampal BDNF level as compared with the control group ($F_{(2,\ 21)} = 5.307$, $P = 0.014$, Fig. 12A). Of note, chronic treatment with fluoxetine reversed hippocampal BDNF level as compared with the stress group. However, stress and fluoxetine did not significantly affect GDNF expression in the PFC and hippocampus (Fig. 12B).

4. Discussion

The present study aims to establish a reliable and clinically relevant PICS model based on the two-hit concept, which is of major clinical importance due to the relevant interactions of these entities. Through this model, we found that stressed mice displayed reproducible anxiety- and depression like behavior and cognitive impairments, which were accompanied by a substantial increase in inflammatory mediator and an activation of glial cells in the hippocampus. Notably, chronic treatment with fluoxetine for three weeks normalized most of the behavioral and molecular abnormalities in stressed mice. Therefore, we believe our model may be used to study the pathophysiology of PICS and in evaluation of pharmaceutical therapies.

Advances in critical care medicine have led to improved survival rates among those patients admitted to the ICU. Consequently, PICS has become one major public health issue and thus has significant health and socio-economic implications [13]. However, investigations of the impact of neurological dysfunction have been relatively neglected relative to other organ systems. It has now become increasingly aware of the cognitive consequences of critically ill patients within or after discharge from the ICU [1–3]. Severe sepsis is the most common non-cardiac cause of critical illness admitted to ICU and survivors of severe sepsis frequently experience substantial long-term psychiatric symptoms after hospital discharge [1–3]. Indeed, it has been demonstrated that patients in medical and surgical ICUs are at increased risk for long-term cognitive impairment [14], while approximately one third of ICU survivors experience anxiety symptoms that are persistent during their first year of recovery [15]. Also, clinically important depressive symptoms occurred in approximately one-third of ICU survivors and were persistent through 12-month follow-up [16]. However, translational research into PICS is hampered by the lack of a clinically relevant animal model. Therefore, development of a new and clinically relevant animal model is essential for understanding the mechanism underlying PICS and screening treatment methods.

Through several years of research, several animal models of sepsis-induced adverse neurobehavioral outcomes have been created. Currently, most studies have used bolus injection of LPS or CLP to study both the short- and long-term neurobehavioral consequences [5–9]. However, the majority of studies mainly focus on the effects of primary disease such as sepsis per se, they do not evaluate other various exogenous stressors that have been frequently encountered in the ICU, including social isolation, chemical and physical restraints, and sleep disturbances due to changes in biological rhythm induced by noise and light on, etc. [10,11]. As more research into the neurodevelopmental model occurred, it became apparent that such a single stress (one hit) model was unable to mimic the clinical features of PICS. Indeed, there is

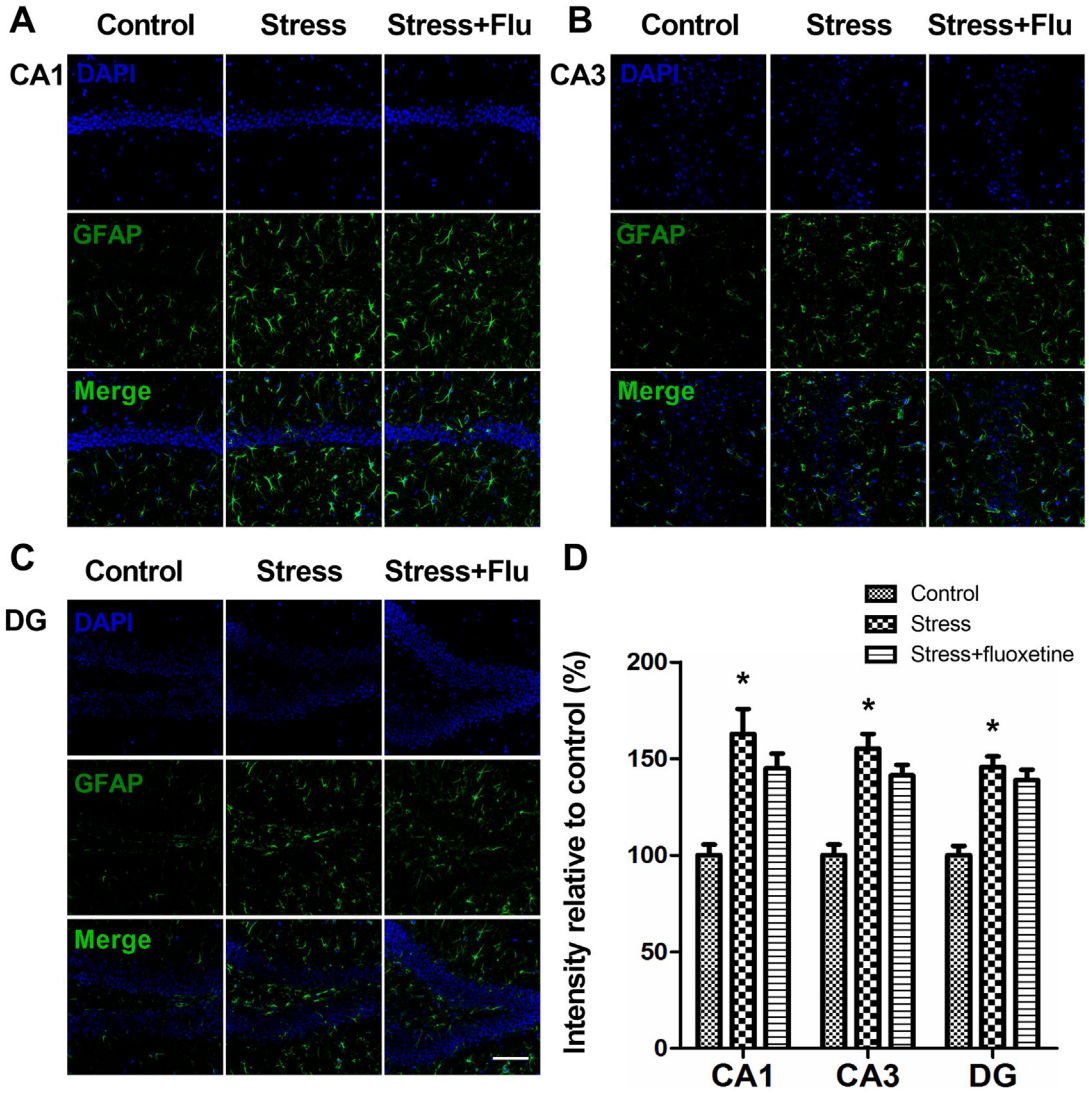

Fig. 10. Immunofluorescence staining to detect GFAP in the hippocampus. (A–C) Representative images of GFAP in the CA1, CA3, and DG regions of the hippocampus. (D) Quantification of GFAP intensity. Data are shown as mean ± SEM (n = 6), $^{*}P < 0.05$ vs control group, scale bar = 50 μm. Flu, fluoxetine.

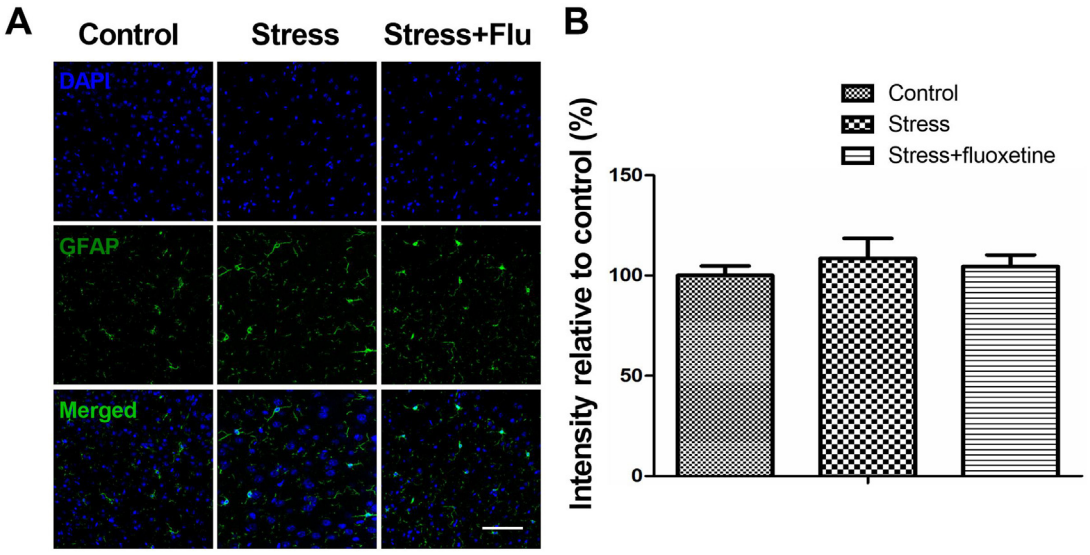

Fig. 11. Immunofluorescence staining to detect GFAP in the PFC. (A) Representative images of GFAP in the PFC. (B) Quantification of GFAP intensity. Data are shown as mean ± SEM (n = 6), scale bar = 50 μm. Flu, fluoxetine.

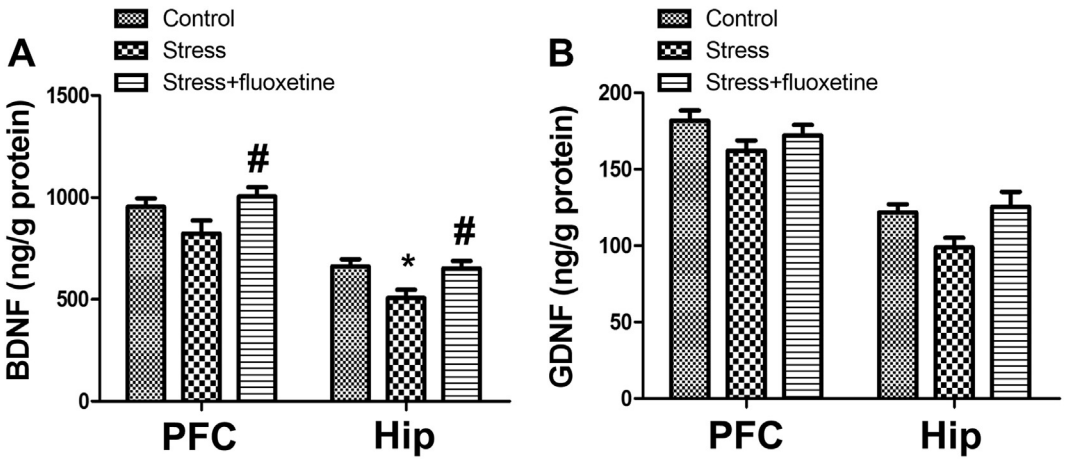

Fig. 12. Effects of stress and fluoxetine on brain neurotrophic factors. Stress (LPS + stressor) group had significantly decreased hippocampal level of BDNF, which was reversed by fluoxetine treatment. *$p < 0.05$ vs control group; #$p < 0.05$ vs stress group. PFC, prefrontal cortex; Hip, hippocampus.

now accumulating evidence suggesting a profound role of exogenous stressors on cognitive dysfunction [6,17,18]. Therefore, it is necessary to incorporate these stressors into the new animal model to better understand the neurobehavioral consequences and mechanism underlying PICS.

In the present study, we aimed to establish a clinically relevant animal model of PICS based on the two-hit concept, in which LPS injection was served as the first hit and subsequent chronic unpredictable stress as the second hit. In our model, LPS injection was used as the first hit because severe sepsis is the most common noncardiac cause of critical illness admitted to ICU [1]. Previous study has demonstrated that LPS-induced sepsis in mice is followed by long-lasting increases in depressive- and anxiety like behaviors but not signs of cognitive impairment [5]. By contrast, it is reported that post-septic encephalopathy in humans is characterized by both cognitive and affective impairments [19]. Our study also showed that LPS injection or chronic modified unpredictable stress alone induced some neurobehavioral abnormalities, which suggests that a single stress model was not sufficient to induce clinical features of PICS. This might also explain the discrepancy between animal and human results reported previously [5,19]. In fort with this hypothesis, it has been demonstrated that stressor exposure and immunogenic challenges can synergistically increase behavioral, endocrine, and neuroinflammatory responses [17]. It is well known that the critically ill patients experience tremendous physical and psychological stressors, most notably are sleep disruption due to changes in biological rhythm induced by noise and light pollution, social isolation, chemical and physical restraints, and other key environmental factors. Indeed, excessive noise in the ICU environment is thought to be a significant factor in sleep disturbance and that this in turn can result in increased morbidity [20]. It is reported that physical restraint is highly prevalent in the critically ill patients, with up to 75% of mechanically ventilated adults having physical restraints applied at least once during their ICU admission [21]. Accordingly, we subjected mice to modified chronic unpredictable stressors to serve as the second hit. Specifically, we designed this animal model by presenting randomly four per day in isolated mice, in an intermittent and unpredictable fashion, mimicking the variability of stressors encountered in the ICU. By using this paradigm, we have shown that the two-hit mouse model displays reproducible anxiety- and depression like behavior and cognitive impairment without markedly affecting general exploratory behavior. Notably, treatment with fluoxetine for three weeks reversed most of the neurobehavioral abnormalities, which suggests that our current model is effective.

The second major finding of this study is that stress increased adrenal weight and induced a long-lasting increase in corticosterone level. Substantial evidence has suggested that stressful life events might trigger mood disorders by over-activating hypothalamic-pituitary-adrenal (HPA) axis [23,24]. Increased levels of cortisol and the size of pituitary and adrenal glands are often, although not always, found in major depression [24]. Thus, the increased corticosterone values resembling the HPA axis activity also proved the effectiveness of our stress protocol. In addition, our stress model induced significantly enhanced inflammatory response, as reflected by increased expression of CD68 in the hippocampus. This is further confirmed by higher level of hippocampal IL-6 in the stressed mice. It has been demonstrated that inflammation signifies one key mechanism that is actively investigated to obtain insight into the pathophysiology of neuropsychiatric disorders [25–27]. Specifically, IL-6 has been studied as an inflammatory cytokine responsible for the anhedonic consequences of stress [28]. Thus, these neuro–immune–endocrine findings further underscore the usefulness of our new model of PICS. In this study, we also found that stress caused significantly decreased level of BDNF in the hippocampus. It has been previously demonstrated that neurotrophic factors, particularly BDNF, is a critical mediator of the activity-dependent plasticity and its abnormality is associated with many neuropsychiatric disorders [29]. As expected, we found that chronic treatment with fluoxetine for three weeks reversed most of the affected parameters. This is consistent with recent studies demonstrating that the current antidepressants including fluoxetine, are able to prevent depression-like behavior and alterations in proinflammatory cytokines in animal model of depression [29,30]. Indeed, antidepressant drugs rapidly activate TrkB signaling and gradually increase BDNF expression, and the behavioral effects of antidepressants are mediated by and dependent on BDNF signaling through TrkB at least in rodents [31,32]. Therefore, the efficacy of fluoxetine in reversing neurobehavioral abnormalities may be attributed to the effects of fluoxetine by normalizing HPA axis dysfunction and enhancing neurotrophic profiles [33]. Surprisingly, we found that chronic fluoxetine treatment only reversed microglia but not astrocyte activation, which can explain why chronic fluoxetine treatment did not completely reverse all the neurobehavioral abnormalities in our study.

Our study has several limitations that have to be acknowledged. Firstly, clinical sepsis arises mainly as a result of the activity of various bacterial products, which stimulates and establishes the inflammatory process; therefore, it is very difficult to simulate this syndrome in laboratory research. Although some of the differences between animal models and humans preclude direct extrapolation from animal studies to the patient, animal studies are essential to provide a necessary tool to understand the pathogenesis and treatment of PICS. Secondly, we did not include all of the stressors relevant to ICU in our model. Although it is not possible to develop a full animal model of PICS, future studies should consider these risk factors. Thirdly, the long-term neurobehavioral outcomes and the effectiveness of fluoxetine to reverse the

behavioral and molecular abnormalities in this new animal model of PICS warrant further investigation.

In summary, our neurobiological and behavioral investigations provided compelling evidence that the two-hit concept including LPS injection and subsequent chronic unpredictable stress can induce marked molecular and cellular changes leading to anxiety- and depression like behavior and cognitive impairments. Although our current model should be considered to be a modified version of the chronic unpredictable stress paradigm designed for critically ill patients living in the ICU, we believe that our model will serve as a paradigm for investigating the complex pathophysiology, therapeutic strategies, and treatment of PICS.

Authors' contributions

MJM and MMZ carried out the behavioral study and drafted the manuscript. SML and LLQ performed the immunoassays and enzyme-linked immunosorbent assay. JJY and JYX participated in the design of the study and performed the statistical analysis. MHJ and JJY designed the study. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was supported by the grants from the National Natural Science Foundation of China (Nos., 81600950, 81771156, 81772126).

References

[1] P.P. Pandharipande, T.D. Girard, J.C. Jackson, A. Morandi, J.L. Thompson, B.T. Pun, N.E. Brummel, C.G. Hughes, E.E. Vasilevskis, A.K. Shintani, K.G. Moons, S.K. Geevarghese, A. Canonico, R.O. Hopkins, G.R. Bernard, R.S. Dittus, E.W. Ely, BRAIN-ICU Study Investigators, Long-term cognitive impairment after critical illness, N. Engl. J. Med. 369 (14) (2013) 1306–1316.

[2] J.C. Jackson, P.P. Pandharipande, T.D. Girard, N.E. Brummel, J.L. Thompson, C.G. Hughes, B.T. Pun, E.E. Vasilevskis, A. Morandi, A.K. Shintani, R.O. Hopkins, G.R. Bernard, R.S. Dittus, E.W. Ely, Bringing to light the risk factors and incidence of neuropsychological dysfunction in ICU survivors (BRAIN-ICU) study investigators. Depression, post-traumatic stress disorder, and functional disability in survivors of critical illness in the BRAIN-ICU study: a longitudinal cohort study, Lancet Respir. Med. 2 (5) (2014) 369–379.

[3] J.C. Jackson, E.W. Ely, M.C. Morey, V.M. Anderson, L.B. Denne, J. Clune, C.S. Siebert, K.R. Archer, R. Torres, D. Janz, E. Schiro, J. Jones, A.K. Shintani, B. Levine, B.T. Pun, J. Thompson, N.E. Brummel, H. Hoenig, Cognitive and physical rehabilitation of intensive care unit survivors: results of the RETURN randomized controlled pilot investigation, Crit. Care Med. 40 (4) (2012) 1088–1097.

[4] M.E. Mikkelsen, J.D. Christie, P.N. Lanken, R.C. Biester, B.T. Thompson, S.L. Bellamy, A.R. Localio, E. Demissie, R.O. Hopkins, D.C. Angus, The adult respiratory distress syndrome cognitive outcomes study: long-term neuropsychological function in survivors of acute lung injury, Am. J. Respir. Crit. Care Med. 185 (12) (2012) 1307–1315.

[5] S.T. Anderson, S. Commins, P.N. Moynagh, A.N. Coogan, Lipopolysaccharide-induced sepsis induces long-lasting affective changes in the mouse, Brain Behav. Immun. 43 (2015) 98–109.

[6] M.H. Ji, H. Tang, D. Luo, L.L. Qiu, M. Jia, H.M. Yuan, S.W. Feng, J.J. Yang, Environmental conditions differentially affect neurobehavioral outcomes in a mouse model of sepsis-associated encephalopathy, Oncotarget 8 (47) (2017) 82376–82389.

[7] L. Yang, M. Wang, Y.Y. Guo, T. Sun, Y.J. Li, Q. Yang, K. Zhang, S.B. Liu, M.G. Zhao, Y.M. Wu, Systemic inflammation induces anxiety disorder through CXCL12/CXCR4 pathway, Brain Behav. Immun. 56 (2016) 352–362.

[8] L. Tuon, C.M. Comim, F. Petronilho, T. Barichello, I. Izquierdo, J. Quevedo, F. Dal-Pizzol, Time-dependent behavioral recovery after sepsis in rats, Intensive Care Med. 34 (9) (2008) 1724–1731.

[9] M.H. Ji, L.L. Qiu, H. Tang, L.S. Ju, X.R. Sun, H. Zhang, M. Jia, Z.Y. Zuo, J.C. Shen, J.J. Yang, Sepsis-induced selective parvalbumin interneuron phenotype loss and cognitive impairments may be mediated by NADPH oxidase 2 activation in mice, J. Neuroinflammation 12 (2015) 182.

[10] S. McKinley, L.M. Aitken, J.A. Alison, M. King, G. Leslie, E. Burmeister, D. Elliott, Sleep and other factors associated with mental health and psychological distress after intensive care for critical illness, Intensive Care Med. 38 (4) (2012) 627–633.

[11] D.M. Wade, D.C. Howell, J.A. Weinman, R.J. Hardy, M.G. Mythen, C.R. Brewin, S. Borja-Boluda, C.F. Matejowsky, R.A. Raine, Investigating risk factors for psychological morbidity three months after intensive care: a prospective cohort study, Crit. Care 16 (5) (2012) R192.

[12] J. Xia, Z. Lu, S. Feng, J. Yang, M. Ji, Different effects of immune stimulation on chronic unpredictable mild stress-induced anxiety- and depression-like behaviors depending on timing of stimulation, Int. Immunopharmacol. 58 (2018) 48–56.

[13] Y. Kondo, R. Fuke, T. Hifumi, J. Hatakeyama, T. Takei, K. Yamakawa, S. Inoue, O. Nishida, Early rehabilitation for the prevention of postintensive care syndrome in critically ill patients: a study protocol for a systematic review and meta-analysis, BMJ Open 7 (3) (2017) e013828.

[14] T.J. Iwashyna, E.W. Ely, D.M. Smith, K.M. Langa, Long-term cognitive impairment and functional disability among survivors of severe sepsis, JAMA 304 (16) (2010) 1787–1794.

[15] S. Nikayin, A. Rabiee, M.D. Hashem, M. Huang, O.J. Bienvenu, A.E. Turnbull, D.M. Needham, Anxiety symptoms in survivors of critical illness: a systematic review and meta-analysis, Gen. Hosp. Psychiatry 43 (2016) 23–29.

[16] A. Rabiee, S. Nikayin, M.D. Hashem, M. Huang, V.D. Dinglas, O.J. Bienvenu, A.E. Turnbull, D.M. Needham, Depressive symptoms after critical illness: a systematic review and meta-analysis, Crit. Care Med. 44 (9) (2016) 1744–1753.

[17] J. Gibb, F. Al-Yawer, H. Anisman, Synergistic and antagonistic actions of acute or chronic social stressors and an endotoxin challenge vary over time following the challenge, Brain Behav. Immun. 28 (2013) 149–158.

[18] M.L. Mitchell, F. Coyer, S. Kean, R. Stone, J. Murfield, T. Dwan, Patient, family-centred care interventions within the adult ICU setting: an integrative review, Aust. Crit. Care 29 (4) (2016) 179–193.

[19] C.N. Widmann, M.T. Heneka, Long-term cerebral consequences of sepsis, Lancet Neurol. 13 (6) (2014) 630–636.

[20] S.J. Jaiswal, S. Garcia, R.L. Owens, Sound and light levels are similarly disruptive in ICU and non-ICU wards, J. Hosp. Med. 12 (10) (2017) 798–804.

[21] L. Rose, C. Dale, O.M. Smith, L. Burry, G. Enright, D. Fergusson, S. Sinha, L. Wiesenfeld, T. Sinuff, S. Mehta, A mixed-methods systematic review protocol to examine the use of physical restraint with critically ill adults and strategies for minimizing their use, Syst. Rev. 5 (1) (2016) 194.

[23] M. Banasr, G.W. Valentine, X.Y. Li, S.L. Gourley, J.R. Taylor, R.S. Duman, Chronic unpredictable stress decreases cell proliferation in the cerebral cortex of the adult rat, Biol. Psychiatry 62 (5) (2007) 496–504.

[24] K. Wingenfeld, O.T. Wolf, HPA axis alterations in mental disorders: impact on memory and its relevance for therapeutic interventions, CNS Neurosci. Ther. 17 (6) (2011) 714–722.

[25] F.A. Radtke, G. Chapman, J. Hall, Y.A. Syed, Modulating neuroinflammation to treat neuropsychiatric disorders, Biomed. Res. Int. 2017 (2017) 5071786.

[26] D.J. Stein, M.F. Vasconcelos, L. Albrechet-Souza, K.M.M. Ceresér, R.M.M. de Almeida, Microglial over-activation by social defeat stress contributes to anxiety- and depressive-like behaviors, Front. Behav. Neurosci. 11 (2017) 207.

[27] N. Yue, H. Huang, X. Zhu, Q. Han, Y. Wang, B. Li, Q. Liu, G. Wu, Y. Zhang, J. Yu, Activation of P2X7 receptor and NLRP3 inflammasome assembly in hippocampal glial cells mediates chronic stress-induced depressive-like behaviors, J. Neuroinflammation 14 (1) (2017) 102.

[28] J.J. Yang, N. Wang, C. Yang, J.Y. Shi, H.Y. Yu, K. Hashimoto, Serum interleukin-6 is a predictive biomarker for ketamine's antidepressant effect in treatment-resistant patients with major depression, Biol. Psychiatry 77 (3) (2015) e19–e20.

[29] C. Kraus, E. Castrén, S. Kasper, R. Lanzenberger, Serotonin and neuroplasticity - links between molecular, functional and structural pathophysiology in depression, Neurosci. Biobehav. Rev. 77 (2017) 317–326.

[30] S.T. Anderson, S. Commins, P. Moynagh, A.N. Coogan, Chronic fluoxetine treatment attenuates post-septic affective changes in the mouse, Behav. Brain Res. 297 (2016) 112–115.

[31] S. Ghosal, E. Bang, W. Yue, B.D. Hare, A.E. Lepack, M.J. Girgenti, R.S. Duman, Activity-dependent brain-derived neurotrophic factor release is required for the rapid antidepressant actions of scopolamine, Biol. Psychiatry 83 (1) (2018) 29–37.

[32] M. Ma, Q. Ren, C. Yang, J.C. Zhang, W. Yao, C. Dong, Y. Ohgi, T. Futamura, K. Hashimoto, Adjunctive treatment of brexpiprazole with fluoxetine shows a rapid antidepressant effect in social defeat stress model: role of BDNF-TrkB signaling, Sci. Rep. 6 (2016) 39209.

[33] C.M. Pariante, A.H. Miller, Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment, Biol. Psychiatry 49 (5) (2001) 391–404.