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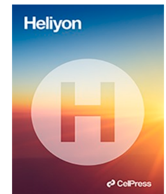

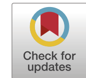

Prior exposure to stress exacerbates neuroinflammation and causes long-term behavior changes in sepsis

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A B S T R A C T

Background: Neuroinflammation can occur during sepsis and is now regarded as the main mechanism underlying various related central nervous system (CNS) disorders. Another well-known factor causing neuroinflammation is psychological stress. In the current study, we examined the effects of prior exposure to stress on sepsis-induced neuroinflammation and CNS symptoms.
Experimental procedure: Balb/c mice were subjected to wet bedding stress for 2 days, then lipopolysaccharide (LPS) was intraperitoneally administered. For examining the neuroinflammation, the expression of proinflammatory cytokines and NF-κB activity in the brain was analyzed by RT-PCR and ELISA-based assay. Additionally, immunohistochemical study using Iba-1 was performed. Finally, behavior tests were examined one month after LPS treatment.
Result and conclusion: Stress exposure induced the upregulation of IL-1β, IL-6 and TNFα mRNA in the cerebral cortex 4 h after LPS administration. Suggesting an underlying mechanism, LPS-induced NF-κB activation was significantly upregulated with stress in the brain. Histologically, microglia in the cerebral cortex were reactive and became more abundant with stress, while these effects were further increased with LPS injection. Behavioral analysis conducted showed a significant increase of anxiety-like behaviors in the stressed mice. These results suggest that prior exposure to stress exacerbates neuroinflammation during sepsis and induces long-term behavior changes.

1. Introduction

Due to recent medical progress, the short-term survival rate of severe sepsis patients has improved significantly. However, their long-term prognosis remains poor, with a five-year survival rate as low as 18% [1]. Furthermore, as much as half of septic patients treated in intensive care unit present neurological symptoms that include anxiety and depression after recovery from the acute phase [2]. These neurological symptoms often persist for a long period of time, which could hinder the patient's rehabilitation, and have an adverse effect on the prognosis [3,4]. In terms of underlying mechanism, the involvement of neuroinflammation has been reported [5], but it is not fully elucidated.

Additionally, psychological stress is well-known to cause mental health problems such as anxiety, depression, and personality disorders [6,7]. Recent studies have shown that neuroinflammation is deeply involved in the pathogenesis of stress induced psychiatric disorders [8,9]. Specifically, mental stress causes microglial reactivity in the brain and increases their release of various inflammatory mediators, including proinflammatory cytokines which adversely affect neurons and cause long-lasting CNS symptoms like depression [10].

In this study, we investigated the outcomes of stress exposure on sepsis-induced neuroinflammation. Older age, compromised immune system, longer hospital stays and invasive devices, but also psychological stress, are common risk factors for sepsis [11,12]. It

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is easily conceivable that septic patients would suffer psychiatric stresses even before the onset of disease. In fact, stress itself is considered as a potential cofactor in the pathogenesis of infectious diseases, supporting its association with sepsis [13].

Additionally, surgical procedures can cause systemic inflammatory response as in sepsis [14]. Neuroinflammation is induced by systemic inflammation after the surgery and can be the cause of neurological complications that include postoperative cognitive dysfunction, delirium and depression [15,16]. Before surgery, patients also often suffer from anxiety, fear, and sleep disorders, and commonly experience psychological stress [17,18]. These stressors can be exacerbating factors for neuroinflammation and have the potential of affecting postsurgical clinical courses.

In the current study, we adopted a 48-h wet bedding paradigm, and lipopolysaccharide (LPS) intraperitoneal injection or cecal ligation and puncture (CLP) as a sepsis model. Wet bedding is usually performed as part of the chronic unpredictable mild stress (CUMS) paradigm, which is commonly used to model in the search for antidepressant drugs for major depression disorder in rodents [19]. According to a recent report, wet bedding alone can exaggerate neuroinflammation in experimental autoimmune encephalomyelitis (EAE) model mice [20]. We investigated how this relatively short-term stress exposure molecularly, histologically, and behaviorally affects neuroinflammation after the onset of sepsis.

2. Materials and Methods

2.1. Animals

This study (Permit Number: MedKyo19315) was approved by the Animal Research Committee of Kyoto University (Kyoto, Japan). All experiments were performed according to the institutional and National Institutes of Health (NIH) guidelines for the care and the use of animals. All mice (Balb/c, 8–11 weeks old, male) were purchased from Japan SLC Inc. (Shizuoka, Japan). Food and water were provided *ad libitum*, and the mice were maintained under controlled environmental conditions (24 °C, 14-h light/10-h dark cycle ordinally, or 12-h light/12-h dark within the period of behavioral experiments). Considering the effect on mice circadian rhythm, one week of habituation time was adopted when light/dark cycle was changed [21]. Mice are fed standard chow diet (Oriental Yeast Co., Ltd. Tokyo, Japan), and were housed three per cage in a ventilated rack.

2.2. Animal stress model and induction of sepsis

Mice were exposed to prior stress, wet bedding, as previously reported with minor modification [20]. Briefly, the mice were kept in the wet paper-chip bedding (700 mL water in a cage) for 2 days. The cage dimensions were 504 cm^2 floor area and 160 mm height. The upper layer of the chip was set to get wet with water. After the stress paradigm, intraperitoneal injection with LPS (5 mg/kg, *E. coli* 055: B5, SB203580, Sigma-Aldrich, Missouri) was performed. The amount of LPS (5 mg/kg) was previously determined [22]. LPS was dissolved into saline at the concentration of 0.5 mg/mL, and the same amount of saline was intraperitoneally administrated for the control group. CLP was performed as previously described [23]. Under isoflurane anesthesia, a 1 cm midline incision was made to expose the cecum. The cecum was partially ligated 1 cm from the end with 1-0 silk suture and punctured for once with an 18-gage needle. After gently compressing the cecum and verifying the fecal leakage through the needle hole, the ligated cecum was returned to the abdominal cavity, and the incisions were closed. Sham operated animals underwent an identical procedure, but the cecum was not ligated or punctured. Throughout the operations, the body temperature of mice was maintained between 36.5 °C and 37.5 °C, and all efforts were made to minimize suffering. Mice of the stress only group were transferred to the normal cage after the stress paradigm, and sacrificed at the time indicated. Specimens were removed 4 or 24 h after LPS administration, 4 h after CLP and nephrectomy for PCR assay. For NF-κB investigation and blood IL-6 concentration measurement, specimens were removed 1.5 h after LPS administration. At the time point of specimen collection, mice were euthanized by isoflurane inhalation followed by cervical dislocation or decapitation.

2.3. Behavioral tests

Eight-week-old Balb/c male mice were divided into two groups, which were exposed or not to the stress paradigm. After the Barnes maze (BM) test training, mice from the stress group were exposed to two days of wet bedding. All the mice were administered LPS 24 h before the spatial probe test. During behavioral testing, mice were housed three per cage and were maintained under controlled environmental conditions (24 °C, 12-h light/12-h dark cycles) with food and water provided *ad libitum*. All prepared mice completed all scheduled experiments. The timeline of behavior tests was as follows;

3 weeks for BM test training.
Wet bedding for 48 h.
Day 0; LPS treatment (mice were 11-week-old at that time).
Day 1 and 8; BM probe test and re-training.
Day 14–16; Fear Conditioning (Day 14; Conditioning trial, Day 15; Context trial, Day 16; Cued trial).
Day 23; Light/Dark transition.
Day 29; Y-maze.
Day 37; Forced swimming test.

2.3.1. Barnes maze test (BM)

The BM measures long-term spatial memory and learning abilities. The circular maze (O′ Hara & CO., Ltd., Tokyo, Japan) presents 12 holes on the circumference and an escape box is placed under one of the holes (target hole; TH). The mice were habituated to the maze one day before the acquisition phase. During the training phase (2 weeks, 16 trials), the mice were trained to escape into the TH. In the probe trial, the escape box was removed and the mice explored the maze freely for 180 s. The maze was rotated between mice and disinfected with hypochlorous water to remove any remaining olfactory cues. The TimeBCM software (O′ Hara & CO.) was used to analyze the number of TH entries and the number of incorrect holes checks.

2.3.2. Y-maze (YM)

In the YM tests, mice were individually placed in the center of a YM apparatus (O′ Hara & CO.) and were allowed to move freely and explore the three arms of the YM for 10 min. The total numbers of arm entries (A) and numbers of three consecutive arm entries (B) were counted. The percentage (%) of alternation was calculated using the following formula: alternation (%) = $B/(A - 2) \times 100$.

2.3.3. Fear conditioning (FC)

In the conditioning test (day 1), each mouse was placed in a white box with a stainless-steel bar on the floor. During the test, three sets of 30-s tone stimulation (55 dB, white noise) and 2-s foot electric shock (0.3 mA) were administered. In the context test (day 2), each mouse was placed in the same box as on day 1 for 5 min to measure context-dependent freezing. In the cued test (day 3), the mice were placed in a new chamber for 6 min and given the same sound stimulation as on day 1 for the remaining 3 min. Freezing when exposed to the unconditioned context and to the sound stimulus was measured. The TimeFZ4 software (O′ Hara & CO.) was used for analysis.

2.3.4. Light/Dark transition (LD)

For this test, home cages were divided into two sections of equal size using a partition with a door. One chamber was brightly illuminated using white diodes (390 lux), whereas the other chamber was kept dark (2 lux). The mice were placed into the dark side and the door was opened automatically for 3 s after the mouse was detected by the infrared camera. The mice were allowed to move freely between the two chambers with the door open for 10 min. Using an automatic analysis system, TimeLD4 (O′ Hara & CO.), we videotaped the behavior of the mice and calculated the distance traveled in the light *versus* dark rooms, then calculated the ratio of time spent in each room.

2.3.5. Forced swimming test (FST)

The mice were individually placed in a transparent cylindrical container filled with hypochlorite water (7.5 cm deep, 25 ± 0.5 °C). Hypochlorite water (HSP corporation, Okayama, Japan) was changed between animals to remove odors. Concentration of hypochlorite was 50 ppm and considered to be sufficiently low according to the past report [24]. Mice behavior was recorded with a CCD camera over a 10 min as previously described [25,26]. The Time TS4 software (O′ Hara & CO.) was used for video recording and analysis, in which the duration of immobility was measured to provide a depressive behavioral score. Images were captured at a rate of one frame per second. For each pair of successive frames, the area (pixels) within which the mouse moved was measured. The Time TS4 is a program to recognize a black object as a mouse and white as background, and if there is no change in the black area of 80 pixels per second, it is calculated as immobile. The Time TS4 is a modified version of image FZ, and its detailed specification was described in the past report [27].

2.4. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was purified from the bilateral frontal cerebral cortex and right inferior lobe of lung using Nucleospin® RNA II Kits (Macherey-Nagel, Duren, Germany) in accordance with the manufacturer's instructions. First-strand cDNA synthesis and qRT-PCR were performed using a One Step TB Green PrimeScript™ RT-PCR Kit II (Takara Bio, Shiga, Japan). Subsequently, qRT-PCR assays were conducted using a Thermal Cycler Dice Real-Time System II TP 900 (Takara Bio). The primers for mouse 18S rRNA and IL-6 genes were purchased from Qiagen (Valencia, CA; Catalog Numbers: 18S mouse, QT02448075; IL-6 mouse, QT00098875). The primers targeting IL-1β and TNFα were purchased from Invitrogen (CA, USA) and the primer targeting IL-1α was purchased from FASMAC (Kanagawa, Japan). The primer sequences utilized were the following: IL-1α 5′-AAGACAAGCCTGTGTTGCTGAAGG-3′ (forward) and 5′-TCCCAGAAGAAAATGAGGTCGGTC-3′ (reverse); IL-1β 5′-CGACAAAATACCTGTGGCCT-3′ (forward) and 5′-TTCTTTGGGTATTGCTTGGG-3′ (reverse); TNFα 5′-TCGTAGCAAACCACCAAGTG-3′ (forward) and 5′-CCTTGAAGAGAACCTGGGAGT-3′ (reverse). The delta CT method was used to calculate the relative fold change of targeted genes. For each target mRNA, the fold changes in expression were calculated relative to those of 18S rRNA, and the value of treated group was normalized that of each control group.

2.5. Nuclear protein preparation and trans-AM assays

Nuclear extracts were purified from the whole brain and right lung using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). The activation of nuclear factor-kappa B (NF-κB) was quantified using an ELISA-based assay kit (*Trans*-AM®; Active Motif). Briefly, nuclear extracts (20 μg) were incubated in 96-well plates coated with oligonucleotides containing the NF-κB consensus site (5′-GGGACTTTCC-3′) binding element. After incubation, NF-κB antibody, HRP-conjugated antibody, and chromogenic reagent were

sequentially added, and NF-κB activity was determined according to the absorbance at 450 nm using a spectrophotometer. Data are presented as the fold change in absorbance value compared to the control group.

2.6. Surgical procedures

Unilateral nephrectomy was performed as previously described with minor modification [28]. Briefly, under isoflurane anesthesia, mice were placed in prone position and the left kidney was removed. Sham operated animals underwent an identical procedure, except that the kidney was only exposed. Throughout the operation, body temperature of the mice was maintained between 36.5 °C and 37.5 °C and all efforts were made to minimize suffering. In the stress and nephrectomy group, surgery was performed one day after the 48-h wet bedding stress. Mice of the stress-only group were transferred to the normal cage after the stress paradigm and sacrificed at the same time with other groups. At the time point of specimen collection, mice were euthanized by isoflurane inhalation followed by cervical dislocation.

2.7. Enzyme-linked immunosorbent assays (ELISA) for plasma corticosterone and IL-6

Blood was collected by puncturing the heart under isoflurane anesthesia and separated by centrifugation for 20 min at 1200×*g* and 4 °C. Concentrations of corticosterone and IL-6 were respectively measured using AssayMax Corticosterone ELISA (AssayPro LLC, St. Charles, Missouri, USA) and IL-6 ELISA (Abcam plc, Cambridge, UK) kits.

2.8. Immunohistochemistry

Under isoflurane anesthesia, mice were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Skulls were carefully removed and whole brains were harvested. Brains were then post-fixed in 4% PFA for 48 h, embedded in paraffin blocks, and sectioned coronally and ventrally at 5-μm of thickness starting at 1 mm anterior to Bregma. The brain sections were placed on glass slides, deparaffinized, and dried at 58 °C for 24 h. Immunohistochemistry for the microglial marker rabbit *anti*-Iba-1 (ab107159, Abcam) was performed. Briefly, the glass slides were microwaved for 20 min to activate the target antigen, and then Iba-1 antibody diluted to 1:2000 was added overnight at 4 °C. The slides were next incubated with biotinylated second antibody diluted to 1:300 in PBS for 40 min, followed by washes in PBS (6 times, 5 min). Avidin-biotin-peroxidase complex (ABC) (ABC-Elite, Vector Laboratories, CA) at a dilution of 1:100 in BSA was added for 50 min. After washing in PBS, a colorimetric reaction was performed using diaminobenzidine and the cellular nuclei were counterstained using hematoxylin. The images taken with a microscope (40× objective) were inverted in black and white, and threshold values were adjusted to mark Iba-1-stained areas with ImageJ software (NIH, MD). Thresholds were kept constant across all the images, while images from three randomly captured for each animal in the cortex and hippocampus were used for analysis.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.3.1 (GraphPad Software Inc., CA), and a p value of <0.05 was

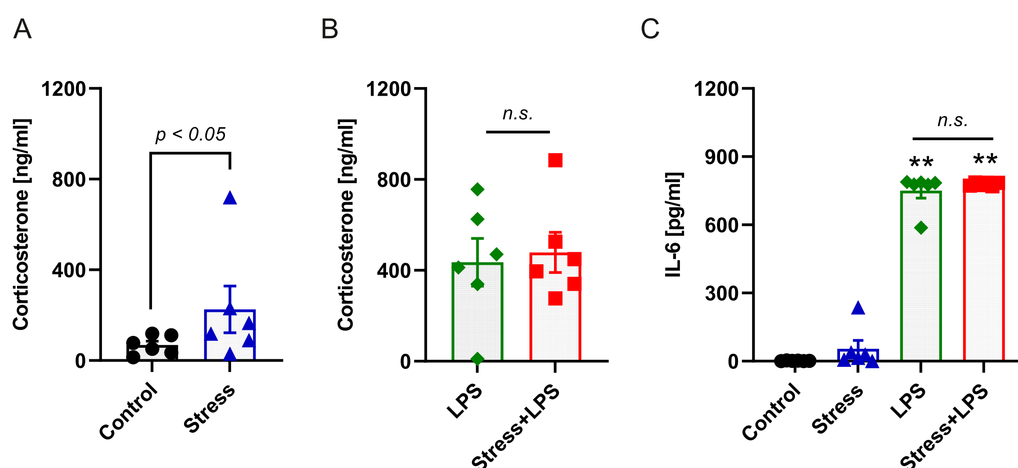


Fig. 1. Plasma corticosterone and IL-6 levels upon wet bedding stress and LPS administration. Mice were exposed to wet bedding stress for two days and plasma corticosterone levels (ng/mL) were determined by enzyme immunoassay (ELISA) (n = 6) (A). Plasma corticosterone (B) and IL-6 levels (pg/mL) (C) at 1.5 h after LPS injection were analyzed by ELISA (n = 6). Data is presented as means ± S.E.M; Statistical analysis was performed with Mann–Whitney U-tests (A, B) or two-way ANOVA followed by Holm-Sidak's test (C) (main effects; LPS: F = 860.7, $p < 0.0001$, stress: F = 3.128, $p =$ 0.14, interaction, F = 0.2352, $p = 0.65$). N.S., not significant, *$p < 0.01$ compared to control; N.S., not significant.

considered as statistically significant. All data were presented as means ± standard error of the mean (S.E.M). Differences between the two groups were analyzed with unpaired Student's t-tests, while comparison among multiple groups, one-way or two-way ANOVA with pairwise multiple comparison tests (Holm-Sidak's methods) and two-way ANOVA repeated measures were applied. Oher wise, if appropriate, Mann–Whitney U-tests were used.

3. Result

3.1. Prior stress exposure exacerbates LPS-induced neuroinflammation

To examine the effect of stress, we employed wet bedding as a stress paradigm. First, we measured plasma corticosterone levels to confirm that exposure to wet bedding for 48 h induces endocrine responses. As shown in Fig. 1A, the plasma corticosterone concentration increased from 68.1 ± 11.0 ng/mL to 225.7 ± 66.3 ng/mL ($p < 0.05$, Mann–Whitney), suggesting that wet bedding can mediate activation of the hypothalamic-pituitary-adrenal axis. We also investigated the effect of wet bedding stress in LPS treated mice

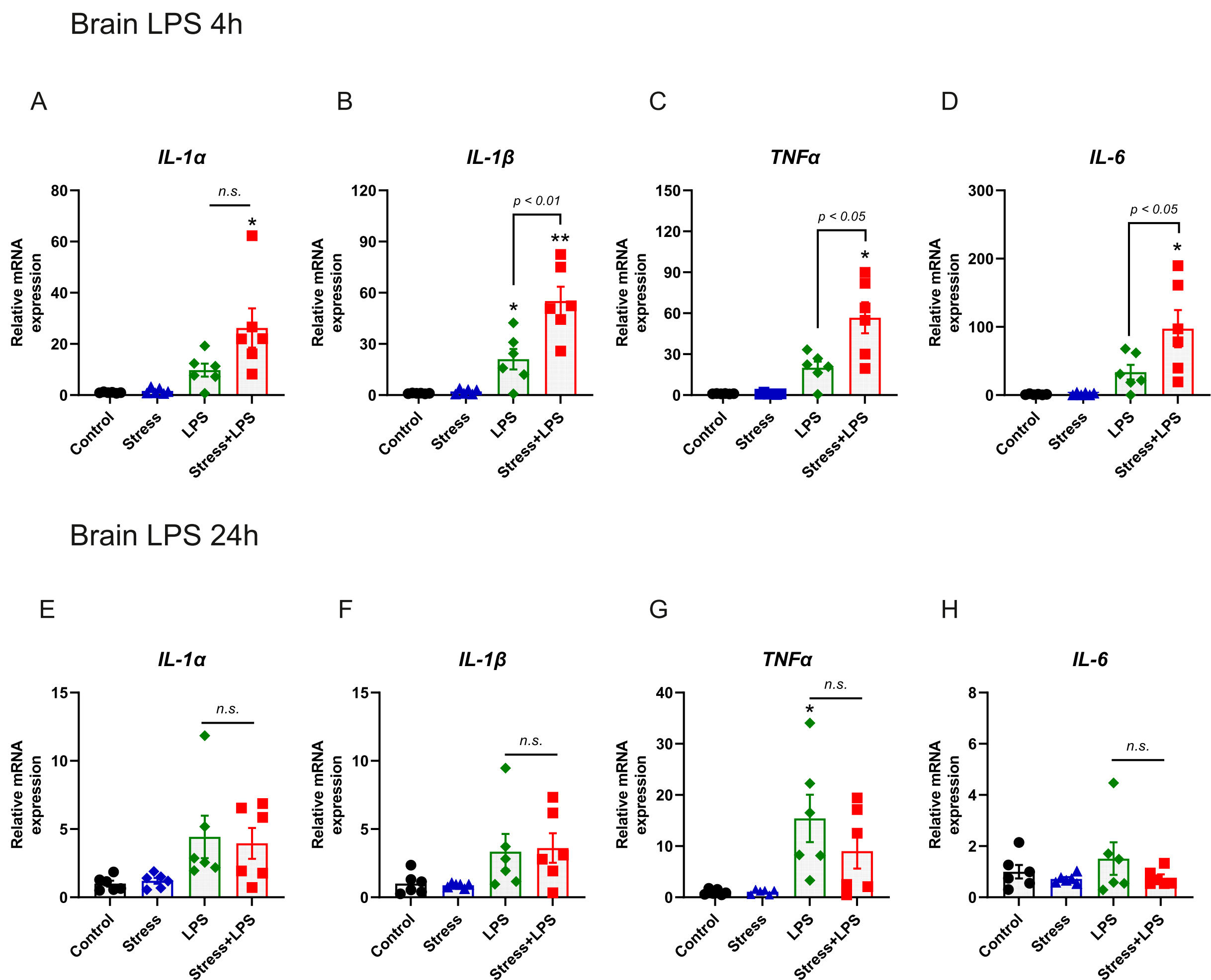


Fig. 2. Effects of prior stress on LPS-induced proinflammatory cytokines in the brain. Mice were exposed to wet bedding stress for two days followed by intraperitoneal injection of LPS (5 mg/kg). Real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure the bilateral frontal cerebral mRNA expression levels of IL-1α (A, E), IL-1β (B, F), TNFα (C, G) and IL-6 (D, H), after four (A–D) or 24 (E–H) h of LPS treatment (n = 6). All data are presented as means ± S.E.M; Statistical analyses were performed using two-way ANOVA followed by Holm-Sidak's test. (A) IL-1α (main effect; LPS: $F = 14.20$, $p < 0.013$, stress: $F = 6.147$, $p = 0.06$, interaction: $F = 4.914$, $p = 0.08$), (B) IL-1β (main effects; LPS: $F = 34.32$, $p = 0.002$, stress: $F = 27.08$, $p = 0.004$, interaction: $F = 23.37$, $p = 0.05$), (C) TNFα (main effects; LPS: $F = 28.63$, $p = 0.003$, stress: $F = 13.26$, $p = 0.015$, interaction: $F = 13.35$, $p = 0.015$), (D) IL-6 (main effects; LPS: $F = 13.03$, $p = 0.015$, stress: $F = 9.893$, $p = 0.026$, interaction: $F = 9.703$, $p = 0.026$), (E) IL-1α (main effects; LPS: $F = 7.745$, $p = 0.04$, stress: $F = 0.0264$, $p = 0.88$, interaction: $F = 0.172$, $p = 0.70$) , (F) IL-1β (main effects; LPS: $F = 7.166$, $p = 0.04$, stress: $F = 0.01335$, $p = 0.91$, interaction: $F = 0.049$, $p = 0.833$), (G) TNFα (main effects; LPS: $F = 9.170$, $p = 0.03$, stress: $F = 3.399$, $p = 0.12$, interaction: $F = 3.432$, $p = 0.12$), (H) IL-6 (main effects; LPS: $F = 0.539$, $p = 0.50$, stress: $F = 2.814$, $p = 0.1543$, interaction: $F = 0.291$, $p = 0.613$). $*p < 0.05$, $**p < 0.01$ compared to control; N.S., not significant.

to find that stress did not have the additive effect (Fig. 1B). Next, to confirm the effect of LPS, plasma IL-6 concentration was measured, which revealed that intraperitoneal treatment with LPS (5 mg/kg) elevated IL-6 significantly, although wet bedding stress did not have an additive effect (main effects; LPS: $p < 0.0001$, stress: $p = 0.14$, interaction: $p = 0.65$, two-way ANOVA) (Fig. 1C). Then we investigated the effect of prior exposure to stress on LPS-induced neuroinflammation using qRT−PCR. This analysis showed that stress significantly increased LPS-induced levels of proinflammatory cytokines, including IL-1β, TNFα and IL-6 (Fig. 2A–D) in the cerebral cortex after 4 h of LPS administration (main effects; LPS, stress and their interaction: $p < 0.05$, two-way ANOVA), but there was no effect of stress after 24 h (two-way ANOVA) (Fig. 2E–H). The same study was performed with CLP mice as another representative model of sepsis. Proinflammatory cytokines, IL-1β and IL-6 expression after 4 h of CLP operation was significantly upregulated ($p < 0.05$, one-way ANOVA with Holm-Sidak's test) with prestress exposure (Fig. 3A–C). Contrary to the brain, in the lung, LPS-induced proinflammatory cytokine expression did not increase with stress (one-way or two-way ANOVA) and was instead suppressed for IL-6 ($p < 0.05$, Holm-Sidak's test) (Fig. 4A–H). To provide insights into the mechanisms underlying this proinflammatory cytokine regulation, we used an ELISA-based kit to assess NF-κB activity, which is implicated in proinflammatory molecule induction among various organs. This experiment showed that stress exposure exacerbates NF-κB transcriptional activity only in the brain ($p < 0.01$, one-way ANOVA with Holm-Sidak's test) (Fig. 5A and B).

3.2. *Effect of prior exposure to stress and subsequent LPS treatment on microglia*

Microglia are considered to play a central role in neuroinflammation. To evaluate the possible changes of microglia after wet bedding stress and subsequent LPS treatment, we quantified the immunoreactivity against Iba-1, a macrophage and microglia-specific marker. In the cerebral cortex, reactive microglia displaying hypertrophic cell bodies and thickened protrusions were observed in the stress group, while these morphological changes were further augmented with LPS (main effects; LPS, stress: $p < 0.05$, interaction: $p = 0.1473$, two-way ANOVA) (Fig. 6A and B). By contrast, stress did not increase Iba-1-positive area in the hippocampus (main effects; LPS: $p < 0.05$, stress: $p = 0.285$, interaction: $p = 0.105$, two-way ANOVA) (Fig. 6C).

3.3. *Effect of prior stress exposure on surgery-induced neuroinflammation*

Before surgery, patients often present with anxiety and sleep disorders, among other symptoms related to stress. To investigate how prior stress affects surgery-induced neuroinflammation, we performed a unilateral nephrectomy. Our findings indicate that nephrectomy significantly induced proinflammatory cytokine expression in the brain ($p < 0.05$, one-way ANOVA) but prior stress exposure did not affect these results (Holm-Sidak's test) (Fig. 7A–C).

3.4. *Prior stress exposure increases anxiety-like behaviors in a sepsis mouse model*

To clarify how stress induced neuroinflammation affects the behavior of septic mice, we lastly performed a comprehensive behavioral analysis, represented schematically in Fig. 8A. As the lethality of CLP mice were relatively high and more than half died

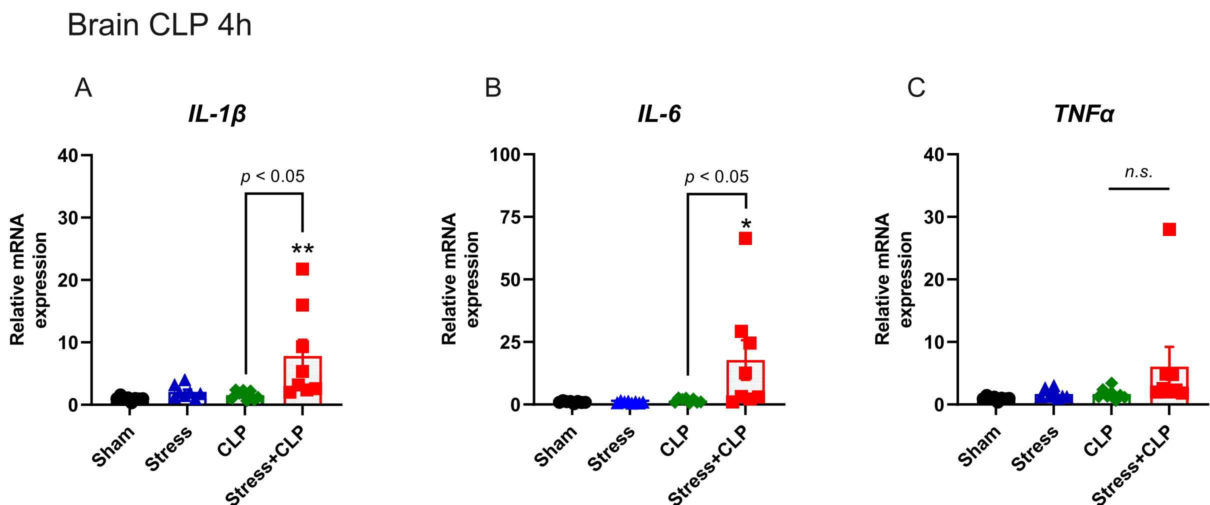


Fig. 3. Effects of prior stress on cecal ligation and puncture (CLP)-induced proinflammatory cytokines in the brain. Mice were exposed to wet bedding stress for two days, then the bilateral frontal cerebral cortex was removed after 4 h of cecal ligation and puncture (CLP). The control group was subjected to laparotomy only (sham). Real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure the mRNA expression levels of IL-1β (A), IL-6 (B) and TNFα (C) (n = 8). All data are presented as means ± S.E.M; Statistical analyses were performed using one-way ANOVA, followed by Holm-Sidak's test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control; N.S., not significant.

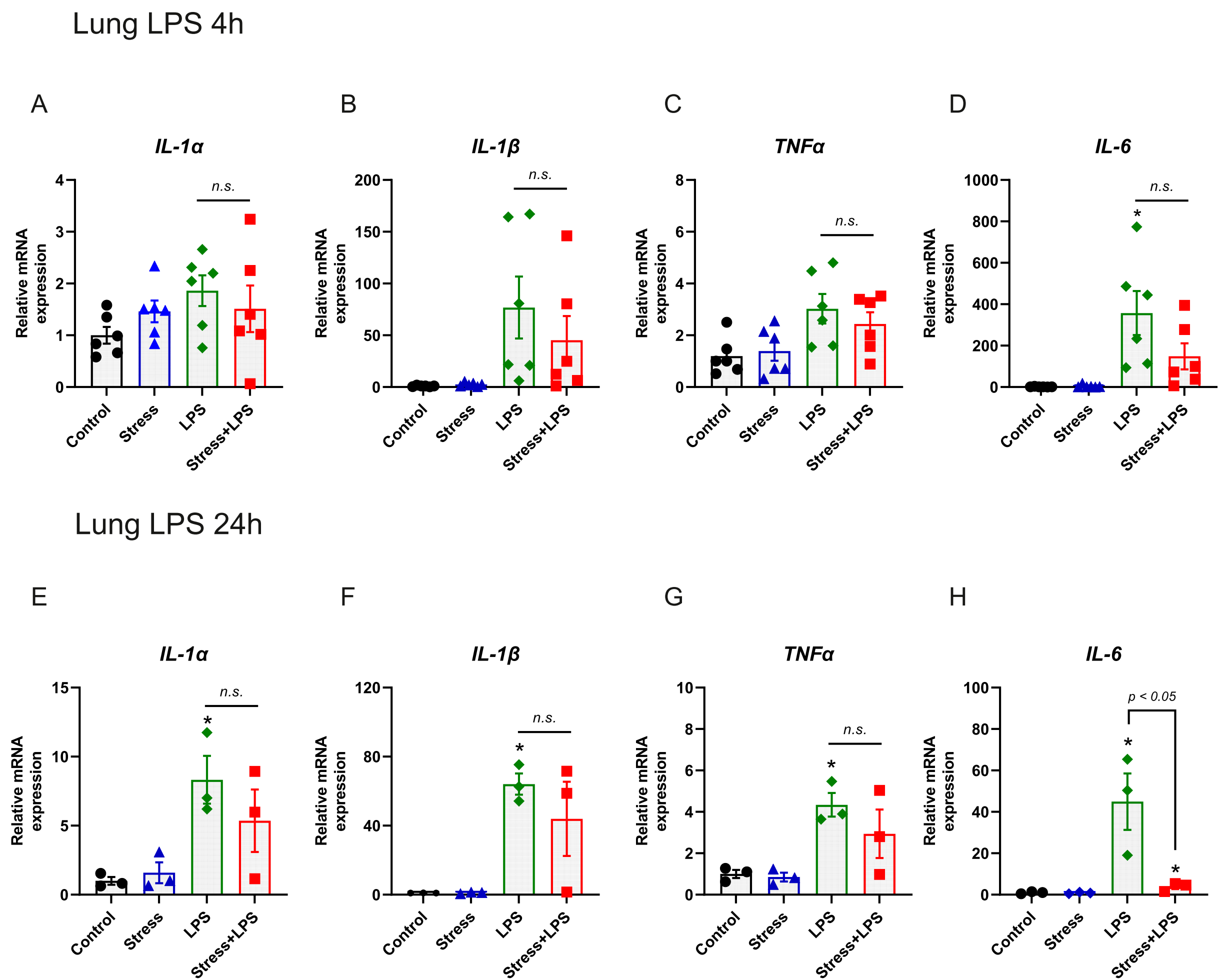


Fig. 4. Effects of prior stress on LPS-induced proinflammatory cytokines in the lung. The effects of wet bedding stress on LPS-induced proinflammatory cytokines were measured in lung samples. The mice were exposed to wet bedding stress for two days, then the right inferior lobe of the lung was resected after four (A–D) or 24 (E–H) h of LPS treatment (5 mg/kg). Real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure the mRNA expression levels of IL-1α (A, E), IL-1β (B, F), TNFα (C, G) and IL-6 (D, H) (A–D: n = 6, E–H: n = 3). Statistical analysis was performed one-way or two-way ANOVA with Holm-Sidak's test. All data are presented as means ± S.E.M; N.S., not significant, *$p < 0.05$ compared to control; N.S., not significant.

within a month, LPS mice were adopted as a model. The mice were treated with LPS, with or without prior stress exposure. The stress group was exposed to two days of wet bedding before LPS administration. The BM probe test, which assesses long-term spatial memory, showed no significant change with stress (unpaired t) (Fig. 8B). In addition, the YM test showed no significant differences between groups (unpaired t) (Fig. 8C), suggesting that short- and long-term memory were not affected by stress. To evaluate anxiety-like behaviors, the FC and LD tests were next conducted. Stressed mice showed longer freezing time in the FC conditioning trial (main effects; LPS, stress and their interaction: $p < 0.05$, two-way repeated-measures of ANOVA) (Fig. 8D). In context and cued trial, a tendency to freeze longer in stress group was observed over time, but the difference was not statistically significant (two-way repeated-measures of ANOVA) (Fig. 8D). In addition, stressed mice stayed longer in the dark box in the LD test ($p < 0.05$, unpaired t) (Fig. 8E). We lastly performed the FS test. The immobile time, a measure of depressive-like behavior, was tended to be prolonged in the stress group ($p < 0.05$, unpaired t) (Fig. 8F).

4. Discussion

Septic patients sometimes present with CNS symptoms that include depression and anxiety after recovering from the acute phase of infection, which generally reduces their lifelong prognosis [3,29]. While the CNS was once considered to be isolated from the systemic inflammatory response during sepsis by the blood-brain barrier (BBB) [30,31], recent studies have shown that the CNS has an inflammatory response, which is termed neuroinflammation, from the very early stages of sepsis [31,32]. Specifically, this response involves the reactivity of microglia, which are responsible for innate immunity in the CNS, followed by the infiltration of bone marrow

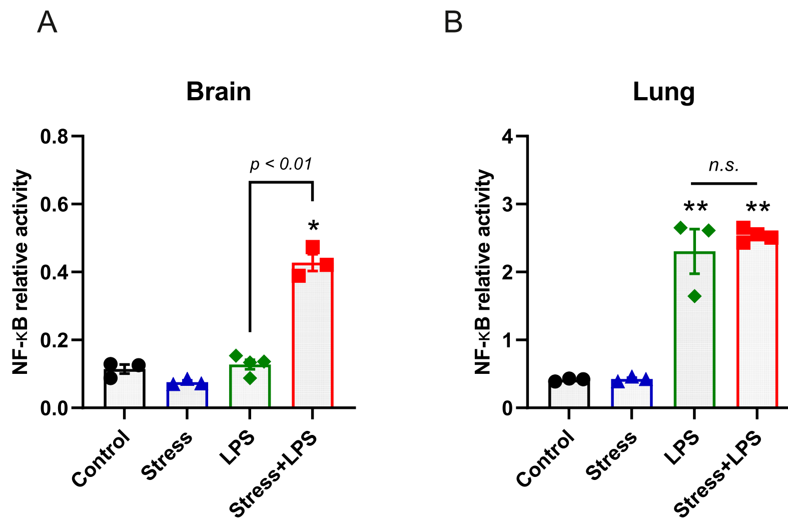


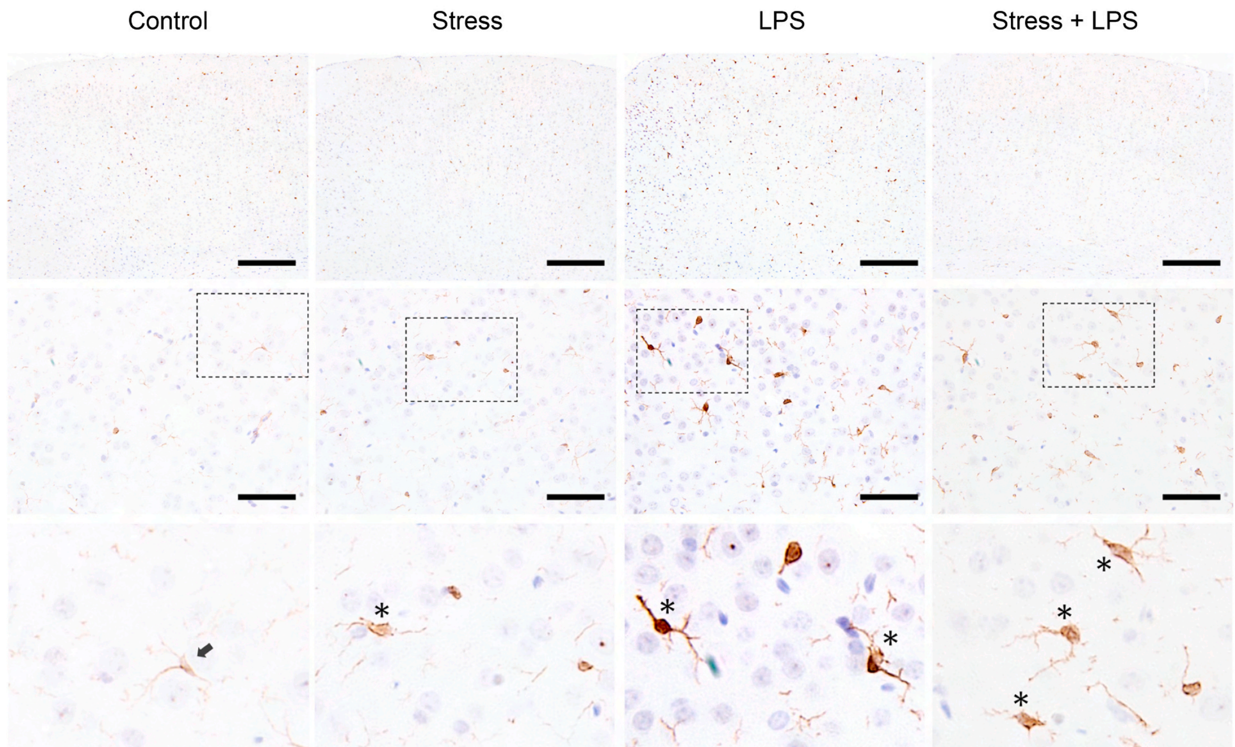
Fig. 5. Effects of prior stress on NF-κB activity in the brain and lung of LPS-treated mice. Nuclear factor-kappa B (NF-κB) transcriptional activity was measured in nucleoprotein samples prepared from the brain and lung. Mice were exposed to wet bedding stress for two days, then the brain (A) and lung (B) were removed after 1.5 h of LPS treatment (5 mg/kg). The NF-κB transcriptional activity was measured using an ELISA-based kit (n = 3–4 animals). All data are presented as means ± S.E.M.; Statistical analysis with one-way ANOVA followed by Holm-Sidak's test (A) and (B). $*p < 0.01$ compared to control; N.S., not significant.

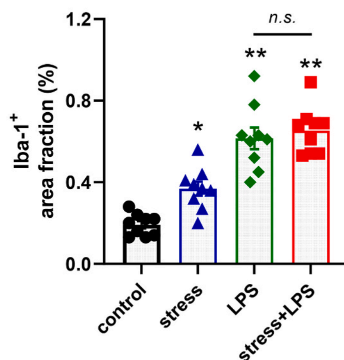
(BM)-derived inflammatory cells. These cells synergistically produce proinflammatory mediators such as cytokines, prostaglandins, and nitric oxide, which can damage neurons and cause various CNS disorders [33,34].

In the current study, we found that prior exposure to stress induced using wet bedding exacerbated neuroinflammation and significantly increased the anxiety-like behaviors of LPS-treated mice. The stress paradigm drastically increased LPS-induced expression of proinflammatory cytokines including IL-1β, IL-6 and TNFα in the cerebral cortex. Various factors including inflammation, aging and stress can transform microglia phenotypically, notably resulting in an elevated expression of major histocompatibility complex (MHC) II and Toll-like receptor [35,36], in addition to morphological changes characterized by de-ramified processes, a spherical cell body, and fragmented cytoplasm [37,38]. These microglial alterations are considered to reflect a "primed" state in which there is an excessive response to subsequent inflammatory stimuli [37,39]. In the current study, the 48-h stress load caused a morphological change of microglia into a reactive form. Considering microglia as the main source of proinflammatory cytokines in CNS [39,40], the exacerbated expression of proinflammatory cytokines measured after LPS treatment is expected to mainly derive from the microglia primed by prior stress exposure. On the other hand, we cannot exclude the possibility that BM-derived inflammatory cells infiltrated the brain and contributed to modulating neuroinflammation. Previous findings revealed that BM-derived cells take five to 10 days to invade the CNS after the onset of sepsis [40,41]. However, BM-derived inflammatory cells could infiltrate the CNS in hours when exposed to stress in advance [42], therefore more precise study including flow cytometry is necessary to clarify this point.

Contrary to the brain, in the lung, stress exposure did not augment the expression of proinflammatory cytokines. The administration of LPS was shown to induce the migration of neutrophils to the lung and upregulate their release of proinflammatory cytokines [43]. However, stress also stimulates the sympathetic-adrenal medulla system [44], while the adrenaline and noradrenaline produced by these reactions can reduce the production of proinflammatory cytokines from neutrophil [45,46]. Therefore, we hypothesize that the inflammatory reaction of neutrophils was inhibited by stress, resulting in a suppressed production of inflammatory cytokines upon LPS stimulation. In any case, the response of brain and lung to prior stress exposure was almost opposite, suggesting that the inflammatory response varies greatly between organs.

The CNS symptoms during sepsis are various [2]. Delirium and impaired consciousness are dominant in the acute phase [47], but cognitive decline as well as depression can become serious clinical problems in the long-term [48]. In our study, prior exposure to stress resulted in increased anxious and depressive behaviors in mice one month after LPS injection. Stress itself is a well-known factor causing depression [7]. The precise underlying mechanism is still controversial, but evidence showing that stress induced neuroinflammation is critical for depression are accumulating [49,50]. In addition, increased microglial expression of inflammatory cytokines including IL-1α and TNFα in the prefrontal cortex is required for depression in a social defeat model [51]. In our study, wet bedding stress increased the expression of TNFα in the cerebral cortex, suggesting that the depressive symptoms were caused by a similar mechanism. However, the proinflammatory cytokine upregulation pattern observed among the cerebral cortex in our study differed from what was reported in the social defeat model, in which cytokine increases prefrontal cortex specifically [51,52]. In previous report, proinflammatory cytokine induction occurred throughout the brain in a sepsis model [53], but how the different affected areas determine particular symptoms remains unclear. Therefore, further investigation is required to define the precise mechanism by which prior exposure to stress induces anxiety-like behaviors.





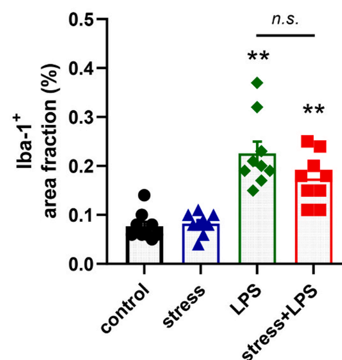


Fig. 6. Effects of wet bedding stress on microglia in the brain of LPS-treated mice. The stress groups (stress or stress + LPS group) were exposed to wet bedding stress for two days. The brain was removed after 4 h of LPS treatment (5 mg/kg). The brain was harvested and processed for Iba-1 staining. Representative images were chosen from different experimental groups. (A) The upper panel shows a low-magnification image. The middle panel shows a magnified view of the cortex. The bottom panel shows a high-magnification image, representing the morphology of microglia. Black arrow indicates surveillant form of microglia comprising a small cell body and long branched processes, while the asterisk indicates a reactive microglia showing a large cell body with thickened branches. Scale bar: 200 μm at upper panel, 50 μm at middle panel. Quantitative pathology scores were determined by calculating the Iba-1-positive area fraction described in Materials and Methods. Nine randomly chosen fields were analyzed for each group (n = 3 animals). All data are presented as means ± S.E.M; (B) Cerebral cortex (main effects; LPS: $F = 159.8$, $p < 0.0001$, stress: $F = 5.346$, $p = 0.0495$, interaction: $F = 2.574$, $p = 0.1473$) (C) Hippocampus (main effects: LPS, $F = 113.5$, $p < 0.0001$, stress: $F = 1.313$, $p = 0.285$, interaction: F = 3,341, $p = 0.105$). All data are presented as means ± S.E.M; Statistical analysis with two-way ANOVA followed by Holm Sidak's test. *$p < 0.05$, **$p < 0.01$ compared to control; N.S., not significant.

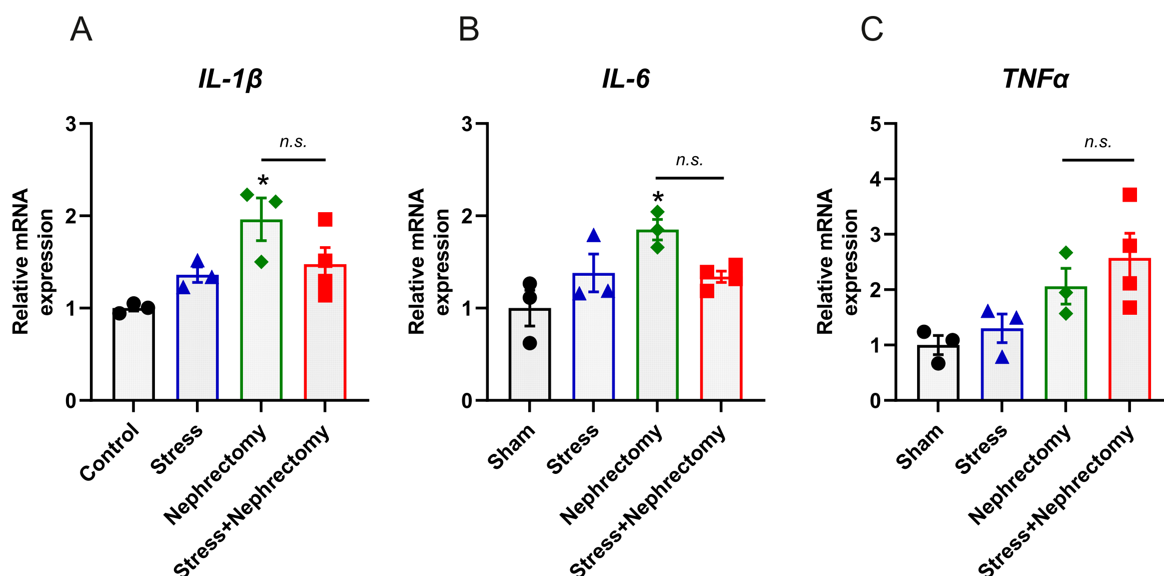
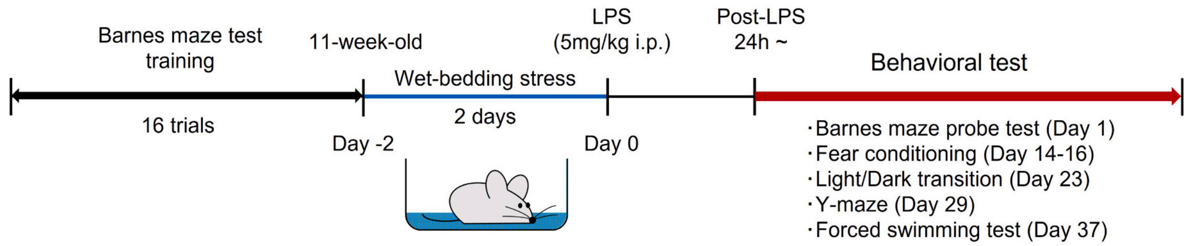


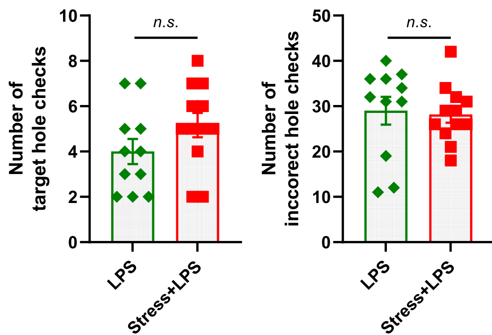
Fig. 7. Effect of prior stress on the brain proinflammatory cytokines in mice exposed to surgery. The effects of wet bedding stress pretreatment on surgical operation-induced proinflammatory cytokines were measured in samples prepared from the bilateral frontal cerebral cortex. Mice were exposed to wet bedding stress for two days, then the bilateral frontal cortex was removed after 4 h of unilateral nephrectomy. The control group was subjected to laparotomy only (sham). Real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure the mRNA expression levels of IL-1β (A), IL-6 (B) and TNFα (C) (n = 3–4). Statistical analysis was performed with one-way ANOVA followed by Holm-Sidak's test. All data are presented as means ± S.E.M; $*p < 0.05$ compared to control; N.S., not significant.

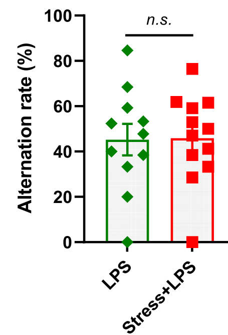
Stress is a known risk factor for sepsis [13], while patients with sepsis are likely to be stressed before its onset. However, because the onset of sepsis is unpredictable, medical interventions against stress before sepsis onset are considered unpractical. Additionally, it is very common for patients to experience psychological stress before surgery [54]. In this study, we investigated how stress affects neuroinflammation after nephrectomy and found that stress had a limited effect. However, the degree of neuroinflammation induced by nephrectomy was mild compared to LPS treatment, and this procedure may not have been sufficient to induce enough neuroinflammation. During surgery, substances released from damaged tissue (DAMPs) are thought to activate cells of the innate immune system, causing an inflammatory response similar to sepsis [55,56]. In a previous report, tibial fracture models resulted in a higher degree of neuroinflammation [57], indicating that more invasive surgical models may have yielded different results. In addition, the timing of surgery might have influenced the result. In the current study, we performed nephrectomy 24 h after the wet-bedding exposure, but past study reported that sleep disturbance stress just before the surgery significantly exaggerated neuroinflammation [57]. Therefore, there is a possibility that the time setting of experiments could have affected the result.

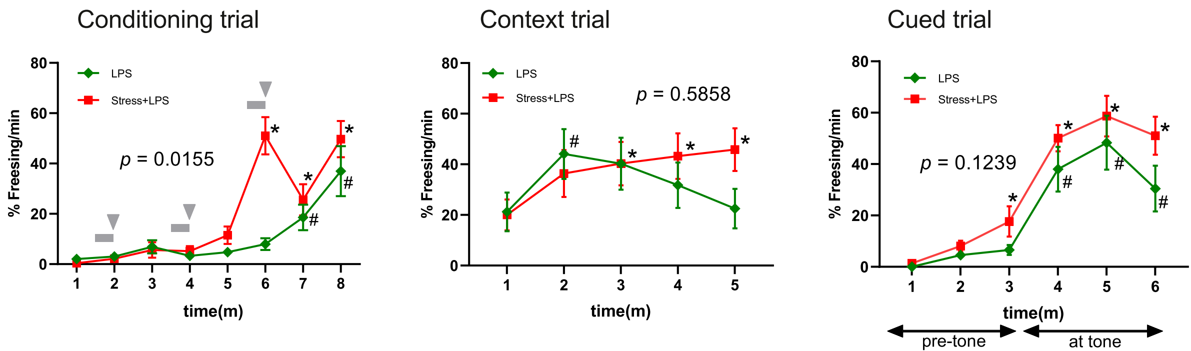
A limitation of this study is that only one type of stress paradigm, wet bedding for 48 h, was used. There are many types of stress models performed in animals. Wet bedding for 48 h was adopted in the current study because it is usually performed as part of the CUMS paradigm, which is commonly used to model depressive-like behavior in rodents. This paradigm comprises systemic and repeated exposure to varying unpredictable and uncontrollable stressors lasting days or weeks [58]. In the current study, we found an exacerbation of anxiety-like behaviors in mice one month later in behavior experiments, without changes in short-term and long-term cognitive memory abilities. In CUMS, beyond the exacerbation of depressive-like behavior, cognitive decline was also reported [59], suggesting that performing a complete CUMS paradigm could have resulted in different outcomes. In fact, the recent study adopting high-fat diet with complete CUMS showed that spatial memory synergistically deteriorated as well as anxiety-like behaviors increased [60]. Furthermore, the psychological stress that septic patients experience is also diverse, making it difficult to model their situation using a single model. In the future, it will be necessary to examine in more detail the types of stress causing particular behavioral changes after sepsis. Another limitation is that lack of control group in behavior experiments. We compared two group, LPS and LPS with stress, and did not examine mice without LPS treatment. The reason why we did not set control group is that increasing the number of groups would compromise the overall accuracy of the experiment by the drastic increase of experiments required to complete the set of behavioral experiments. Therefore, although we showed prestress exposure caused the one-month after behavior change in the LPS treated mice, strictly speaking, it is not known whether prestress exposure exacerbates LPS-induced depression-like symptoms.

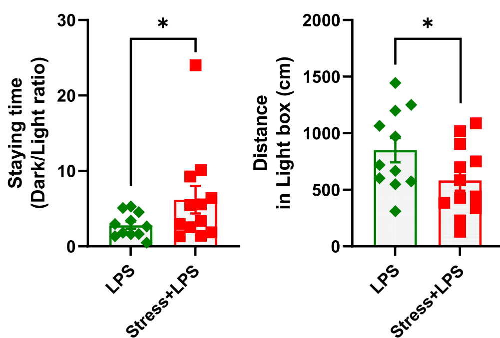
In conclusion, our findings reveal that prior exposure to stress exacerbates neuroinflammation after LPS administration and increases anxiety-like behaviors in sepsis model mice. Our results provide new insights into the implication of psychological stress in the progression of sepsis-induced CNS symptoms. The onset of CNS symptoms can have a serious impact on the long-term prognosis of septic patients, but it remains unclear which patients are at high risks. Our study suggests that prior exposure to stress may exacerbate CNS symptoms. Therefore, in cases where it is possible to predict the onset of systemic inflammation, acting on stress may improve the











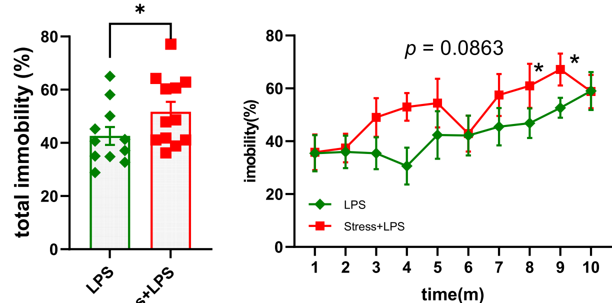


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Fig. 8. Long-term effects of stress on behavioral performance in post-septic mice. Behavioral analysis was performed following the illustrated protocol (A). All the mice experienced LPS-induced sepsis. The prestress group was exposed to two days of wet bedding stress before LPS administration. (B) The BM probe test, which assesses long-term spatial memory, showed no significant change with prior stress. (C) In the YM test, the total number of arm entries and number of three consecutive arm entries were determined and the alternation rate was calculated. (D) The FC test was performed with a three-day schedule consisting of the conditioning, context, and cued test. In each stage, the freezing time was measured. A tone was presented for 30 s (gray bars) followed by a 2-s foot shock (gray arrowheads) in conditioning trial. (E) In the LD test, the staying time ratio (Dark/Light) was analyzed. (F) In the FST, the immobility time was evaluated. Number of animals in each group is 11 and 12. All the data are presented as means ± S.E.M; Statistical analyses were performed using unpaired *t*-test except (D) and (F). $^*p < 0.05$ compared to LPS; N.S., not significant. Data in (D) and (F) were analyzed by two-way repeated measures ANOVA, followed by Holm-Sidak's test. The *p*-values indicate stress effect. (D) Conditioning trial (main effects; time: $F = 27.10$, $p < 0.0001$, stress: $F = 6.939$, $p = 0.0155$, interaction: $F = 6.390$, $p < 0.0001$), Context trial (time: $F = 3.471$, $p = 0.0113$, stress: $F = 0.3063$, $p = 0.5858$, interaction: $F = 1.948$, $p = 0.11$), Cued trial (main effects; time: $F = 45.38$, $p < 0.0001$, stress: $F = 2.568$, $p = 0.1239$, interaction: $F = 1.048$, $p = 0.3937$). (F) FST (main effects; time: $F = 3.895$, $p = 0.0001$, stress: $F = 3.238$, $p = 0.0863$, interaction: $F = 0.7977$, $p = 0.6188$). All data are presented as means ± S.E.M; In (D) and (F), $^*p < 0.05$ compared to time point 1 (Stress + LPS), $\#p < 0.05$ compared to time point 1 (LPS). BM: Barnes maze; YM: Y-maze; FC: Fear conditioning; LD: Light/Dark transition; FST: Forced swimming test.

prognosis of the patients.

Author contribution statement

Mariko Miyao; Tomoharu Tanaka: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Akiko Hirotsu; Kenichiro Tatsumi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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