

RESEARCH ARTICLE

Chronic unpredictable stress exacerbates surgery-induced sickness behavior and neuroinflammatory responses via glucocorticoids secretion in adult rats

Na Wang, Hong Ma, Zhe Li, Yalei Gao, Xuezhao Cao*, Yanhua Jiang, Yongjian Zhou, Sidan Liu

Department of Anesthesiology, The First Hospital of China Medical University, Shenyang, Liaoning, China

* xuezhacao2011@hotmail.com



OPEN ACCESS

Citation: Wang N, Ma H, Li Z, Gao Y, Cao X, Jiang Y, et al. (2017) Chronic unpredictable stress exacerbates surgery-induced sickness behavior and neuroinflammatory responses via glucocorticoids secretion in adult rats. PLoS ONE 12(8): e0183077. <https://doi.org/10.1371/journal.pone.0183077>

Editor: Cristoforo Scavone, Universidade de Sao Paulo, BRAZIL

Received: January 17, 2017

Accepted: July 28, 2017

Published: August 14, 2017

Copyright: © 2017 Wang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by the National Natural Science Foundation of China (No. 81300938). The role of the funder in this study: design.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Accumulated evidence indicates that stress sensitizes neuroinflammatory responses to a subsequent peripheral immune challenge. The present study investigated whether chronic unpredictable stress (CUS) aggravated surgery-induced sickness behavior and neuroinflammatory processes via glucocorticoids secretion in the adult brain.

Methods

Sprague-Dawley adult male rats (12–14 weeks old) were exposed to 14-day CUS and then subjected to partial hepatectomy 24 h after the last stress session. The rats were pretreated with an antagonist of the glucocorticoids (GCs) receptor RU486 (30 mg/kg, i.p.) 1 h prior to stress exposure. The behavioral changes were evaluated with open field test and elevated plus-maze test. The hippocampal cytokines interleukin (IL)-1 β and IL-6 were measured on postoperative days 1, 3 and 7. Ionized calcium binding adaptor protein (Iba)-1, microglial M2 phenotype marker Arg1, brain derived neurotrophic factor (BDNF) and CD200 were also examined at each time point.

Results

CUS exacerbated surgery-induced sickness behavior. Exposure to CUS alone failed to alter the levels of pro-inflammatory cytokines in the brain. However, CUS exaggerated surgery-induced pro-inflammatory cytokines expression (e.g. IL-1 β and IL-6) and upregulated the levels of Iba-1 on postoperative days 1 and 3. An additional significant decreased BDNF, CD200 and a lower level of Arg1 were also observed in the stressed rats following surgical procedure. Pretreatment with RU486 blunted the potentiating effects of CUS on surgery-induced sickness behavior and neuroinflammatory responses.

Conclusion

Chronic unpredictable stress enhanced surgery-induced sickness behavior and neuroinflammatory responses. Stress-induced GCs played a pivotal role in enhancing surgery-induced neuroinflammatory processes by modulation of microglia functions.

Introduction

Postoperative cognitive dysfunction (POCD), characterized by the progressive deterioration of intellectual/cognitive function, is a major complication following various surgical procedures [1]. Mounting evidence indicates that POCD is associated with surgery-induced neuroinflammatory processes, which may influence neuronal functioning either directly or through modulation of intraneuronal pathways, such as brain derived neurotrophic factor (BDNF) mediated pathway [2, 3]. High levels of neuroinflammatory cytokines, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α play a pivotal role in surgery-induced cognitive deficits [1, 4].

Acute and chronic stress sensitized or primed neuroinflammatory responses to both peripheral and central immunologic challenges [5, 6]. For example, chronic unpredictable stress (CUS) potentiated LPS-induced pro-inflammatory mediators (e.g., IL-1 β , inducible nitric oxide synthase, and TNF- α) in frontal cortex and hippocampus of rats [7]. Interestingly, treatment with exogenous glucocorticoids (GCs) is sufficient to replicate the phenomenon of stress-induced priming of neuroinflammatory responses to peripheral immune challenges [8]. Furthermore, pretreatment with glucocorticoid receptors (GR) antagonist RU486 blunted the potentiating effects of stress on nuclear factor kappa B (NF- κ B) expression [8, 9].

Surgical trauma caused sickness behavior and triggered neuroinflammatory responses in the brain of rats [1, 4, 10]. Psychological stress is common prior to the major surgery. It was reported to affect 60–80% of surgical patients [11]. The main objective of this study was to investigate whether CUS aggravated surgery-induced sickness behavior and neuroinflammatory responses in the adult rats. We also explored whether stress and the consequent increase of circulating GCs modulated the immunophenotype of microglia, thereby sensitizing neuroinflammatory responses to the subsequent surgical challenge.

Methods

Animals

Sprague-Dawley adult male rats (12–14 weeks old) were randomly divided into a total of six groups: control group (n = 30), CUS group (n = 36), RU486 group (n = 30), surgery group (n = 30), CUS+surgery group (n = 30), and RU486+CUS+surgery group (n = 30). All animals were housed in groups of four per cage except the day of surgery and had free access to food and water. Colony conditions were maintained at 25°C on a 12-h light/dark cycle (lights on at 07:00 A.M.). All rats were adapted to their environment for a minimum of 7 days before the experiments. The control rats stayed in their home cage. Partial hepatectomy was performed under general anesthesia (3% isoflurane in O₂ at 0.6 L/min) in the surgery group. Briefly, the liver was exposed through a 1–2 cm midline abdominal incision. The left lateral lobes of the liver (approximately corresponding to 30% of the organ) were excised. The wound was then infiltrated with 0.25% bupivacaine, and closed by sterile suture. To limit variability, all surgeries were performed by the same person. Animals in RU486 and RU486+CUS+surgery groups were intraperitoneally injected with a daily dose of RU486 (30 mg/kg, dissolved in DMSO) 1 h prior to stress exposure. All procedures were conducted in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by China Medical University Animal Care and Use Committee (No: IACUC-2017001).

Experimental procedure

Animals received 14-day CUS or CUS with RU486 injection. Twenty-four hours after the last stress session, the rats were subjected to partial hepatectomy under general anesthesia. The

body weight was measured every two days during the 14-day CUS. The behavioral changes were evaluated with relatively low-stress methods—open field test and elevated plus-maze test on postoperative days 1, 3 and 7. The rats were euthanized and the hippocampi were harvested for biochemical analyses after the behavioral test. The remaining rats were transcardially perfused with ice-cold saline (0.9%) for 3 min, followed by 4% paraformaldehyde for immunohistochemistry at each time point. The cardiac blood was collected on postoperative days 1, 3 and 7 in the morning (09.00 h).

Chronic unpredictable stress

Chronic variate stress was adapted from other models of variate stress with modifications [12]. Individual stressors and length of time applied each day are listed in Table 1. In all stress experiments, the stressed rats were exposed to the same order of stressful stimuli. Stress was applied at a different time of each day to minimize predictability. Restraint was carried out by placing the animal in a 21×6 cm plastic tube and adjusting it with a plaster tape on the outside, so that the animal could not move. Forced swimming was carried out by placing the animal in a glass tank with 25 cm of water depth at 23 ± 2°C. The control rats were manipulated every day for 10 min in the home cage to control for nonspecific handling effects.

Open-field test

The open-field observation cage consisted of a square wooden arena (100 × 100 × 50 cm) with its inside walls covered in black. It was divided into 25 equal squares, including peripheral area (16 around squares) and central area (9 middle squares). The rats were placed individually into the center of the area and allowed to explore freely. The test was conducted in a quiet room in the morning (8:00–12:00 A.M.). The animals were monitored for 5 minutes with a video tracking software (Smart, San Diego Instruments, San Diego, CA, USA). Spontaneous locomotor activity was assessed by the total amount of distance traveled in the chamber. The time in the central area was taken as measures of anxiety and exploratory behavior.

Elevated plus-maze test

The elevated plus-maze is an apparatus widely used to evaluate the level of anxiety in rodents. The level of anxiety is measured by variables such as the percentage of time spent and the

Table 1. Schedule of stressors used during the chronic variate stress treatment.

Day (Time)	Stressor	Time
1 (3:00 P.M.)	restraint	60 min
2 (9:00 A.M.)	forced swimming	15 min
3 (3:00 P.M.)	cold isolation	90 min
4 (5:00 P.M.)	lights on	overnight
5 (8:00 A.M.)	forced swimming	5 min
6 (4:00 P.M.)	water and food deprivation	overnight
7 (2:00 P.M.)	restraint	120 min
8 (3:00 P.M.)	lights off	120 min
9 (9:00 A.M.)	forced swimming	5 min
10(7:00 P.M.)	lights on	overnight
11(2:00 P.M.)	cold isolation	90 min
12(9:00 A.M.)	restraint	60 min
13(6:00 P.M.)	water and food deprivation	overnight
14(8:00 A.M.)	restraint	60 min

<https://doi.org/10.1371/journal.pone.0183077.t001>

number of entries in the open arms. Low percentage of time spent and the number of entries in the open arms indicate anxiety. The elevated plus-maze consisted of two open arms (50 cm×10 cm), two enclosed arms (50 cm×10 cm) and a central platform (10 cm×10 cm) made of black plexiglas. The closed arms were surrounded by a 50 cm wall, the open ones presented 0.5 cm edges in order to maximize open-arm entries. The apparatus was elevated at a height of 50 cm above the floor. Environmental temperature was maintained equal to the temperature measured in the housing room. The rats were individually placed at the centre of the maze, facing an open arm and allowed to freely explore the entire maze for 5 min on postoperative days 1, 3 and 7. After each observation, the elevated plus-maze was cleaned with ethyl alcohol (10%) to remove cues left from the preceding subject. The time spent on the open and closed arms and the number of entries made into each arm were recorded using a video camera (Sony, DCR-SX44E).

Plasma GCs concentration

Concentrations of GCs in plasma were quantified by using an enzyme-linked immunoassay (ELISA). In brief, cardiac blood was collected by cardiac puncture into EDTA coated syringes and centrifuged (3000 rpm for 10 min at 4°C). Plasma was collected and stored frozen (-80°C) until assaying. GCs titers were assessed by using a competitive enzyme immunoassay kit, following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western blot analysis

Hippocampus tissues were collected and homogenized in ice-cold lysis buffer for 30 minutes. The homogenate was centrifuged (12000 g for 10 min 4°C) and the quantity of protein in the supernatants was determined using a BCA protein assay kit (Fdbio science, China). Equal amounts of protein were loaded per well on a 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes, which were then blocked with 5% skim milk at room temperature for 2 h. Membranes were then incubated using the following antibodies: rabbit monoclonal anti-IL-1 β (1:2000; Abcam, Cambridge, UK), mouse polyclonal anti-IL-6 (1:2500; Abcam, Cambridge, UK), rabbit polyclonal anti-BDNF (1:2000; Abcam, Cambridge, UK) overnight at 4°C. The membranes were then incubated in appropriate secondary antibodies diluted in TBST for 2 h at room temperature. Chemiluminescence detection was performed using an ECL Western Blotting kit (cat. # 170–5060; Bio-Rad Laboratories, Inc.). Relative expression levels of protein were normalized by the ratio of target protein (IL-1 β , IL-6 and BDNF) to β -actin.

Real-time PCR

Total RNA from the different treatment conditions was extracted from the whole hippocampus with TRIzol reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA (1.0 μ g) using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan). Two microliters of cDNA were used to perform quantitative real-time PCR. The following primers were used to amplify the mRNA: CD200: 5'-CTGCACACAACCTGCATCCTT-3' (forward) and 5'-GGGCTTTGCTGTAAGTGACC-3' (reverse); Arg1: 5'-GCAGAGACCCAGAAGAATGG-3' (forward) and 5'-CACGATGTCCTTGGCAGATA-3' (reverse); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GGGGCTCTCTGCTCCTCCCTG-3' (forward) and 5'-AGGCGTCCGATACGGCCAAA-3' (reverse). GAPDH was adopted as an internal control, which were obtained from Sangon Biotech. The reverse transcription reaction was carried out under the following conditions: 95 °C for 10 min (initial denaturation), followed by 40 cycles of

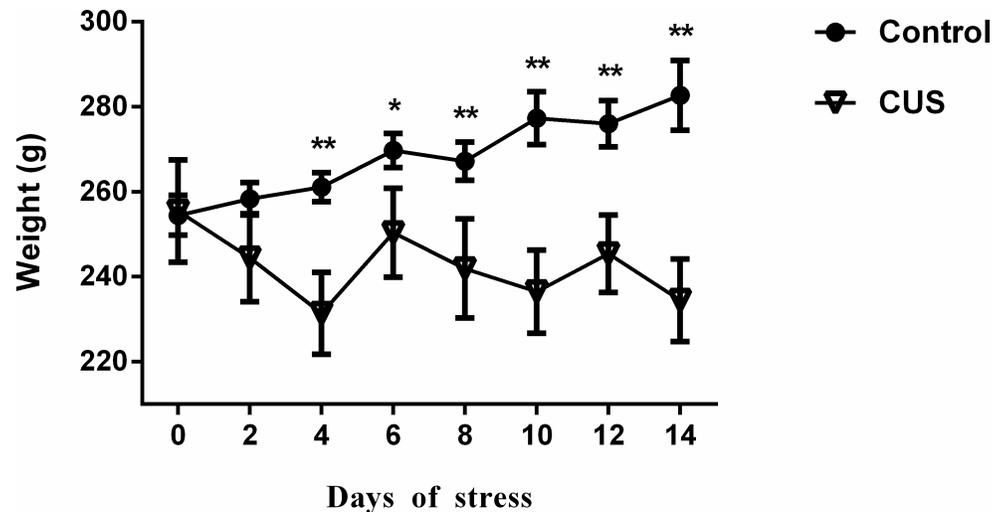


Fig 1. The stressed rats showed decline in body weight over 14 consecutive days of chronic unpredictable stress compared with the controls. The results are represented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ versus the day-matched control group.

<https://doi.org/10.1371/journal.pone.0183077.g001>

95 °C for 20 sec, 62 °C for 30 sec and 72 °C for 30 sec (amplification). The relative gene expression was determined by calculating the expression ratio of the gene of interest to GAPDH. The relative expression of mRNA was quantified using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

The sections (5 μ m) were pretreated with 3% H₂O₂ for 20 min and then incubated with the specific primary antibody overnight at 4 °C as follows: goat anti-ionized calcium-binding adaptor protein-1 (Iba1) (1:300; Abcam, Cambridge, UK). An appropriate antibody was applied for 60 min at room temperature. After thoroughly washed, the reaction products were visualized using the DAB method. The sections were counterstained with hematoxylin, dehydrated, and mounted. Control samples were run in parallel omitting the primary antibody. The integrated optical density (IOD) of positively stained area was analyzed at 200 \times magnification in CA1 region with image analysis software (Image-Pro Plus 6.0).

Statistical analysis

All data are presented as the mean \pm SEM. Statistical comparisons were subjected to a multivariate analysis of variance (ANOVA) in which stress, surgery and intervention were dependent variables. Bonferroni's test was employed when ANOVA showed significance. A p -value < 0.05 was considered to be statistically significant.

Results

Chronic unpredictable stress decreased the bodyweight of stressed rats

CUS exerted a negative effect on the bodyweight gain (Fig 1). While the controls maintained their weight through the protocol, the stressed animals lost 8.15% of weight ($p < 0.001$) during the 14-day CUS.

Chronic unpredictable stress exacerbated surgery-induced spontaneous locomotor activity impairment and increased the levels of anxiety

Two-way ANOVA of the total distance and the time in the central area in the open field test revealed significant effects of CUS ($p = 0.010$ and $p = 0.003$, respectively), surgery ($p < 0.001$ and $p < 0.001$, respectively) and CUS×surgery interaction ($p = 0.022$ and $p = 0.017$, respectively). There was no significant effect of the CUS on the total distance in the stressed animals compared with the controls 48 h post-stress ($p = 0.794$). The total distance was shorter in the surgery group compared to that in the control group on postoperative day 1 ($p = 0.028$). CUS produced an additive effect on the total distance in the surgical animals on postoperative days 1 and 3 ($p < 0.001$ and $p = 0.019$, respectively) (Fig 2A). Similarly, the time in the central area in the surgical rats was significantly shorter compared with the controls on postoperative day 1 ($p < 0.001$). Significant difference of the time in the central area was also observed between the surgery group and the CUS+surgery group on the postoperative days 1 and 3 ($p = 0.021$ and $p = 0.003$, respectively) (Fig 2B). When pretreated with RU486, the distance moved was similar between the surgery group and the RU486+CUS+surgery group. A similar pattern was observed for the time in the central area. RU486 alone did not depress locomotor activity and alter anxiety levels.

Analysis of the percentage of time spent and the number of entries in the open arms in the elevated plus-maze test revealed significant effects of CUS ($p = 0.021$ and $p < 0.001$, respectively), surgery ($p < 0.001$ and $p < 0.001$, respectively), and CUS+surgery interaction ($p = 0.031$ and $p = 0.011$, respectively). Compare to the controls, surgical trauma significantly decreased the percentage of time spent ($p < 0.001$ and $p < 0.001$, respectively) and the number of entries ($p < 0.001$ and $p = 0.002$, respectively) in the open arms on postoperative days 1 and 3 (Fig 3A and 3B). CUS or RU486 alone failed to significantly affect the percentage of time spent and the number of entries in the open arms. However, CUS produced an additional decrease in the open arm exploration in the surgical rats on postoperative day 1 ($p = 0.003$ and $p = 0.036$, respectively). When pretreated with RU486, the percentage of time spent ($p = 0.992$ and $p = 0.989$, respectively) and the number of entries ($p = 0.726$ and $p = 0.998$, respectively) in the open arms was similar between the surgery group and the RU486+CUS+surgery group on postoperative days 1 and 3 (Fig 3A and 3B).

Chronic unpredictable stress exaggerated surgery-induced neuroinflammatory responses in the hippocampus

Surgical trauma significantly increased the levels of IL-1 β and IL-6 on postoperative days 1 ($p < 0.001$ and $p = 0.033$, respectively) and 3 ($p = 0.03$ and $p = 0.011$, respectively). In the

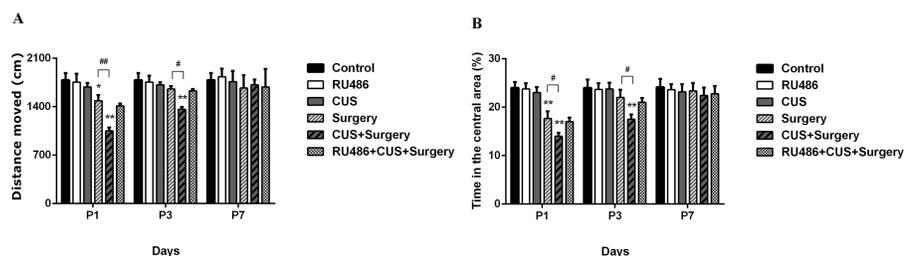


Fig 2. Chronic unpredictable stress exacerbated surgery-induced sickness behavior in the open field test. (A) Total distance moved in the chamber. (B) The time in the central area. The results are represented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ versus the control group; # $p < 0.05$, ## $p < 0.001$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g002>

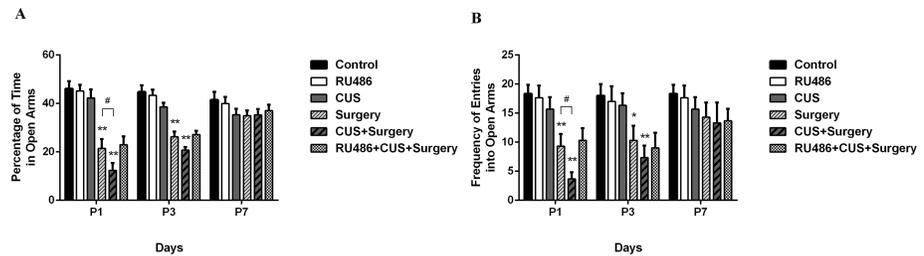


Fig 3. Surgical trauma significantly decreased open arms exploration in the elevated plus-maze test on postoperative days 1 and 3. Chronic unpredictable stress enhanced surgery-induced sickness behavior on postoperative day 1. (A) Percentage of time in open arms. (B) Frequency of entries into open arms. The results are represented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ versus the control group; # $p < 0.05$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g003>

absence of surgical challenge, CUS failed to alter the levels of pro-inflammatory cytokines. However, CUS amplified surgery-induced pro-inflammatory cytokine IL-1 β expression on postoperative day 3 ($p = 0.002$), and IL-1 β expression returned to baseline on day 7 ($p = 0.248$) compared with the day-matched surgery group (Fig 4). RU486 alone failed to significantly alter hippocampal pro-inflammatory cytokine levels when compared to the naive controls at any time point. However, administered with RU486 (30 mg/kg) prior to CUS and surgery, the levels of IL-1 β were similar in the RU486+CUS+surgery group and the surgery group. A similar pattern was observed for IL-6 protein (Fig 5). This indicated that RU486 substantially blunted the potentiating effects of CUS on surgery-induced pro-inflammatory processes in the hippocampus.

Chronic unpredictable stress upregulated surgery-induced microglial Iba-1 expression and reduced the mRNA levels of M2 phenotype marker Arg1

Compared to the naive controls, CUS failed to increase the expression of Iba-1 48 h post-stress. The levels of Iba-1 were significantly upregulated compared with the controls on postoperative days 1 ($p = 0.037$) and 3 ($p = 0.002$), and returned to baseline on day 7 ($p = 0.209$). Higher levels of Iba-1 were observed in the animals of CUS+surgery group compared with those of the surgery group on postoperative day 3 ($p = 0.027$). Pretreatment with RU486 blocked the effects of CUS on surgery-induced Iba-1 upregulation (Fig 6).

CUS failed to downregulate the levels of microglial M2 phenotype marker Arg1 in the stressed rats compared with the controls. The gene levels of Arg1 were significantly decreased following surgical challenge on postoperative day 1 ($p < 0.001$). CUS produced an additional reduction of Arg1 expression in the CUS+surgery group compared with the surgery group on postoperative days 1 and 3 ($p < 0.001$ and $p < 0.001$, respectively). Treatment with RU486 partially blunted the potentiating effects of CUS on surgery-induced downregulation of Arg1 expression in the hippocampus (Fig 7).

Chronic unpredictable stress and surgical trauma decreased the levels of CD200 mRNA in the hippocampus

Compared to the controls, CD200 mRNA expression was downregulated in the CUS group at 48 and 96 h post-stress ($p < 0.001$ and $p < 0.001$, respectively). The levels of CD200 mRNA was significantly decreased on postoperative days 1 and 3 ($p < 0.001$ and $p < 0.001$, respectively), and improved on day 7 ($p = 0.128$). An additional significant decrease in hippocampal

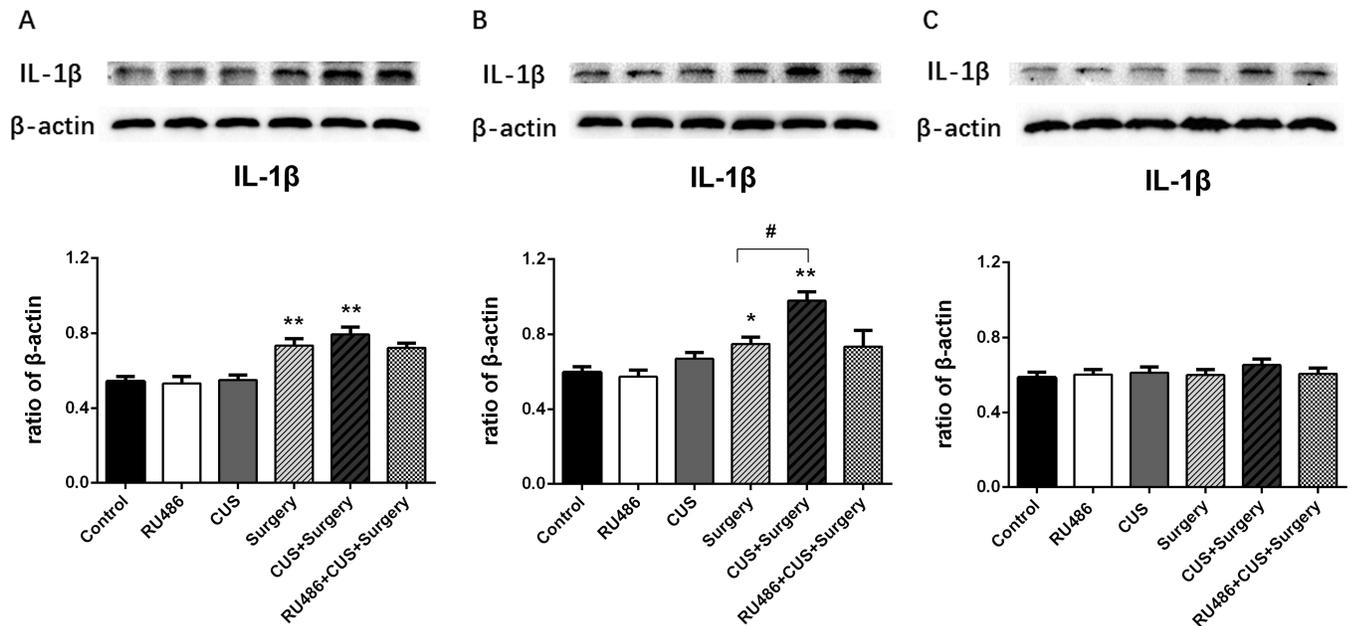


Fig 4. Compared with the surgery group, chronic unpredictable stress (CUS) amplified surgery-induced hippocampal IL-1β expression on postoperative day 3. Pretreatment with RU486 significantly blunted the potentiating effects of CUS on surgery-induced IL-1β levels in the brains of adult rats. (A) Postoperative day 1. (B) Postoperative day 3. (C) Postoperative day 7. The results are represented as the mean ± SEM. **p* < 0.05, ***p* < 0.001 versus the control group; #*p* < 0.05 versus the surgery group.

<https://doi.org/10.1371/journal.pone.0183077.g004>

CD200 mRNA expression was observed when partial hepatectomy was performed in the stressed animals on postoperative days 1 (*p* = 0.007) and 3 (*p* < 0.001). Administration of

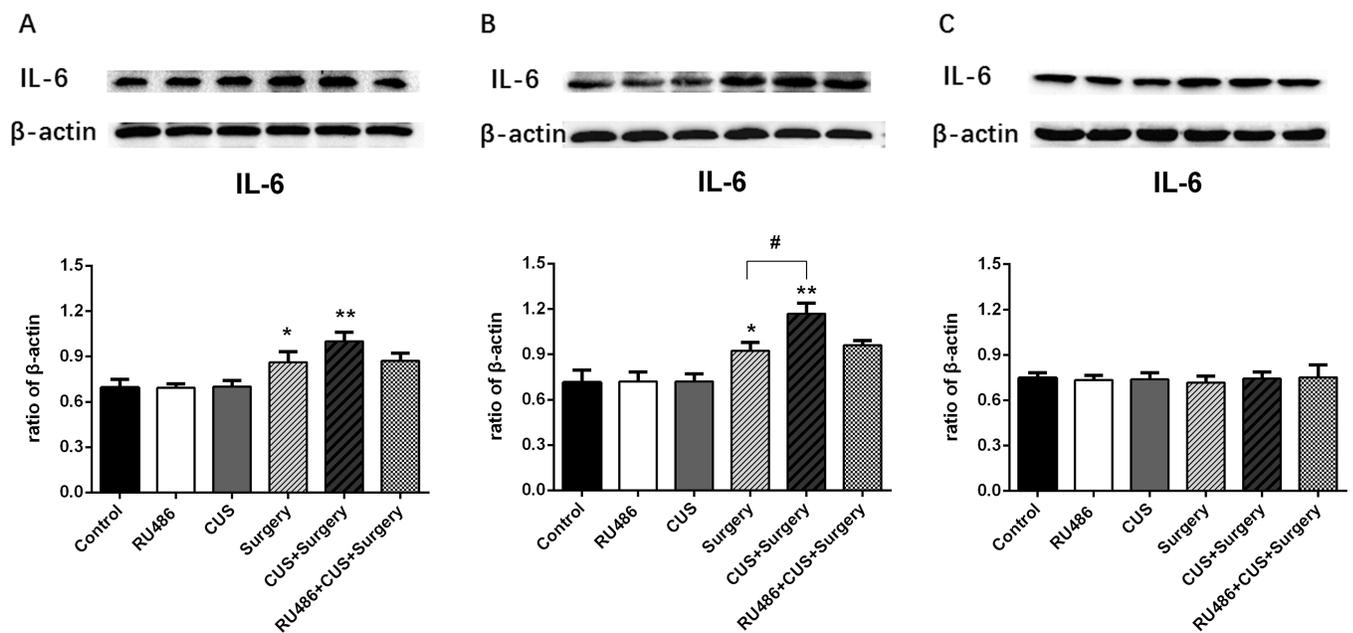


Fig 5. Chronic unpredictable stress (CUS) aggravated surgery-induced hippocampal IL-6 expression on postoperative day 3. Pretreatment with RU486 significantly blunted the potentiating effects of CUS on surgery-induced IL-6 levels in the brains of adult rats. (A) Postoperative day 1. (B) Postoperative day 3. (C) Postoperative day 7. The results are represented as the mean ± SEM. **p* < 0.05, ***p* < 0.001 versus the control group; #*p* < 0.05 versus the surgery group.

<https://doi.org/10.1371/journal.pone.0183077.g005>

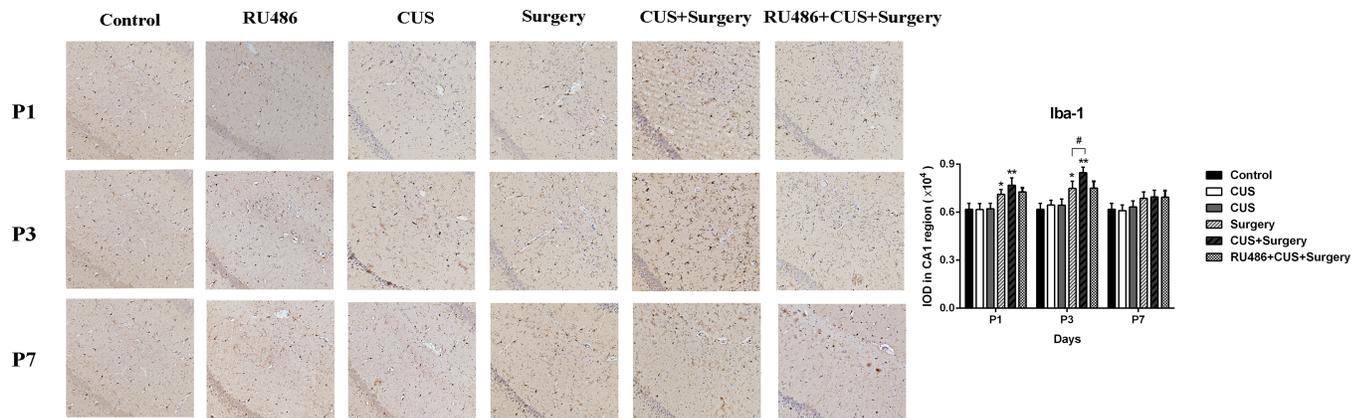


Fig 6. Chronic unpredictable stress (CUS) upregulated surgery-induced microglial Iba-1 expression on postoperative day 3 and the Iba-1 expression improved on day 7 compared with that of the surgery group. Pretreatment with RU486 significantly blunted the potentiating effects of CUS on surgery-induced Iba-1 levels on postoperative day 3 in the adult rats. The results are represented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ versus the control group, # $p < 0.05$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g006>

RU486 attenuated the potentiating effects of CUS on surgery-induced downregulation of CD200 expression (Fig 8).

The combination of chronic unpredictable stress and surgical trauma reduced the levels of BDNF in the brain

Surgical trauma significantly decreased the levels of BDNF compared with the controls on postoperative day 1 ($p < 0.001$), while CUS alone failed to downregulate BDNF expression in the stressed rats 48 h post-stress (Fig 9A). Furthermore, the lower levels of BDNF were

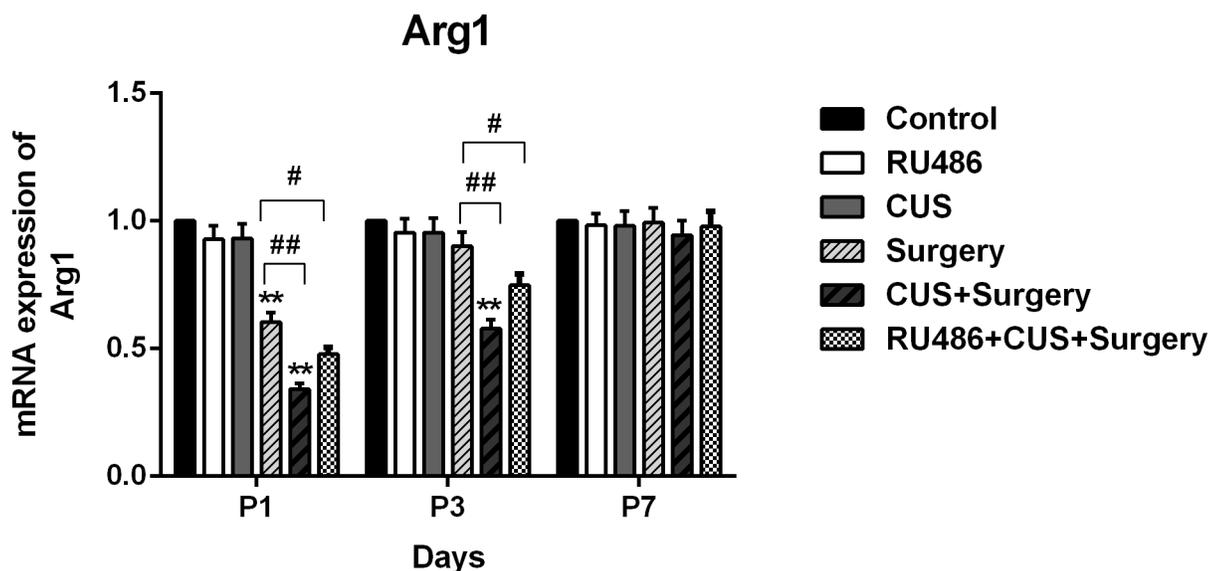


Fig 7. Compared with the control group, the mRNA levels of M2 phenotype marker Arg1 were significantly downregulated on postoperative day 1 in both surgery group and CUS+surgery group and recovered to basal level on day 7. An additional significant decrease in hippocampal Arg1 expression was observed in CUS+surgery group compared to the surgery group on postoperative days 1 and 3. Treatment with RU486 partially blunted the effects of CUS on surgery-induced downregulation of Arg1 expression. The results are expressed as the mean \pm SEM. ** $p < 0.001$ versus the control group; # $p < 0.05$, ## $p < 0.001$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g007>

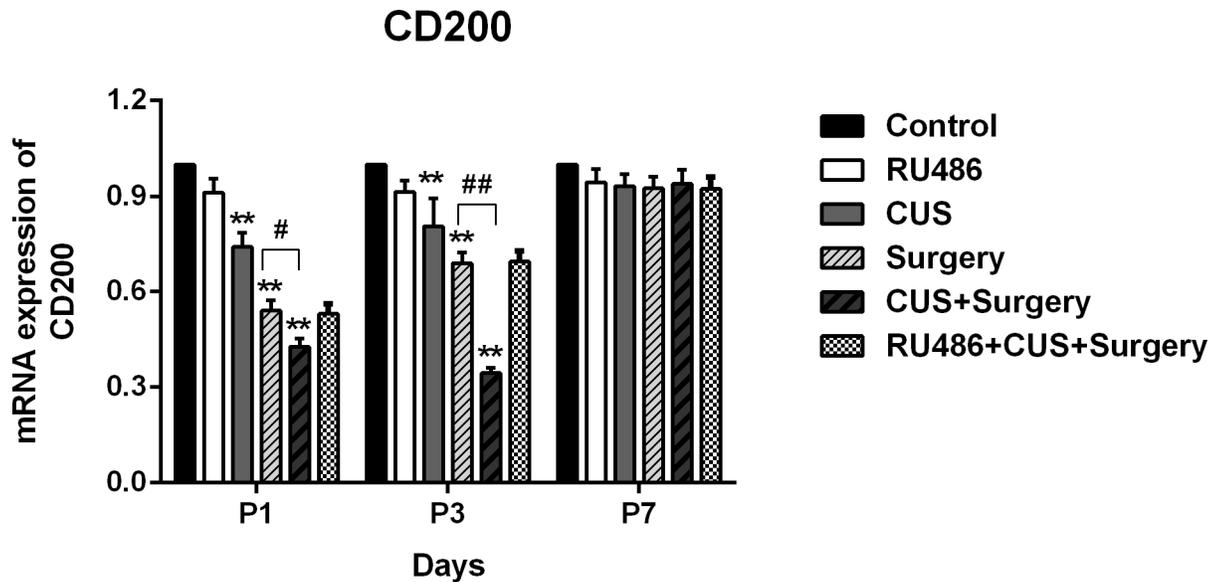


Fig 8. Hippocampal CD200 mRNA was downregulated on postoperative days 1, 3 and improved on day 7 in the surgical rats. CUS had an additional significant decrease in hippocampal CD200 mRNA expression on postoperative days 1 and 3. Administration of RU486 attenuated the effects of stress on surgery-induced downregulation of CD200 expression. The results are represented as the mean \pm SEM. ** $p < 0.001$ versus the control group; # $p < 0.05$, ## $p < 0.001$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g008>

observed in the CUS+surgery group compared with the surgery group on postoperative day 1 ($p = 0.002$) (Fig 9A). Pretreatment of RU486 blunted these priming effects induced by CUS (Fig 9A).

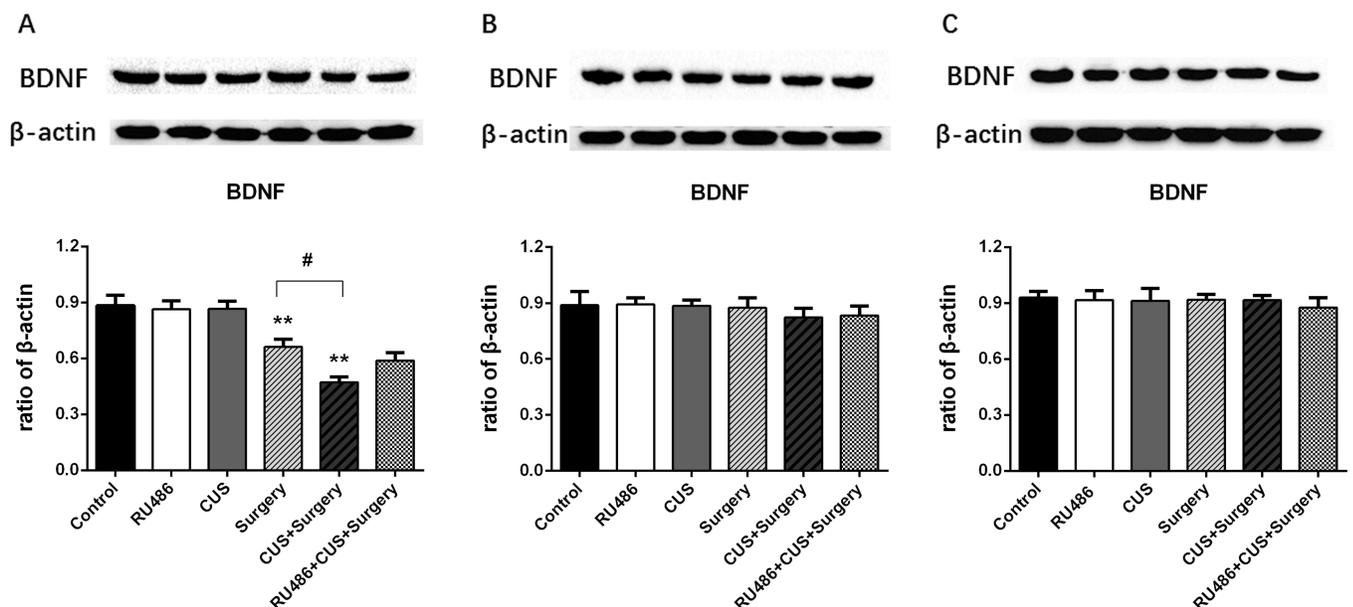


Fig 9. The combination of chronic unpredictable stress (CUS) and surgical trauma reduced the levels of BDNF in the brain on postoperative day 1. Pretreatment with RU486 significantly blunted the potentiating effects of CUS on surgery-induced BDNF levels in the brains of adult rats. (A) Postoperative day 1. (B) Postoperative day 3. (C) Postoperative day 7. The results are represented as the mean \pm SEM. ** $p < 0.001$ versus the control group; # $p < 0.05$ versus the surgery group.

<https://doi.org/10.1371/journal.pone.0183077.g009>

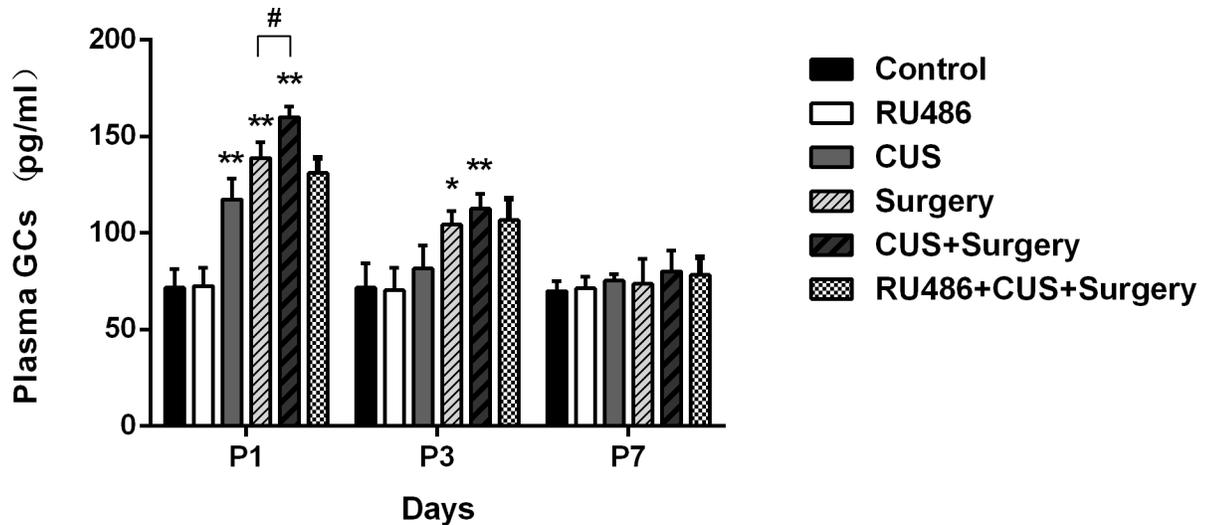


Fig 10. Surgical trauma significantly increased the levels of glucocorticoids (GCs) on postoperative days 1 and 3 compared with the controls. Chronic unpredictable stress enhanced surgery-induced the increased levels of GCs on postoperative day 1. The results are represented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ versus the control group, # $p < 0.05$ versus the surgery group. P1, P3, and P7: postoperative days 1, 3, and 7, respectively.

<https://doi.org/10.1371/journal.pone.0183077.g010>

Chronic unpredictable stress and surgical trauma increased the levels of plasma GCs

ELISA analysis showed that exposure to CUS resulted in a significant increase in plasma GCs compared to the non-stressed rats 48 h ($p < 0.001$) after the last session of stress and the levels of GCs returned to baseline 96 h post-stress (Fig 10). Surgical trauma significantly increased the levels of plasma GCs on postoperative days 1 and 3 ($p < 0.001$ and $p = 0.002$, respectively) and returned to baseline on day 7 compared with the controls. CUS enhanced the increased levels of GCs on postoperative day 1 ($p < 0.001$) (Fig 10).

Discussion

Accumulating evidence has shown that both acute and chronic stress potentiated the sickness response to LPS administered 24 h post-stress [13–15]. In the present study, surgical trauma impaired spontaneous locomotor activities and increased the levels of anxiety in the adult rats. CUS exacerbated surgery-induced sickness behavior. These effects were blocked by pretreatment with RU486, indicating that GCs, at least partly, is involved in the stress-induced sensitization of behavior changes following surgical challenge. Importantly, these changes were accompanied by microglial activation and neuroinflammatory responses in the brain, which might mediate sickness behavior.

The severity of the surgery influences the magnitude of the immune response and has been shown to correlate with the degree of postoperative inflammation and sickness behavior [16, 17]. Previous work has shown that the incidence of POCD in elderly patients after minor surgery (primarily laparoscopy) was significantly lower than those after cardiac and noncardiac major surgery suggesting that extent of surgery contributes to postoperative brain dysfunction [18]. A study by Hempenius et al also indicates that the severity of the surgical procedure is independent risk factors for postoperative delirium in elderly patients undergoing elective surgery [19]. Additionally, Rosczyk et al have demonstrated that locomotor activity is not depressed in both adult and aged mice following sham operation-minor abdominal surgery,

which reveals that the decrease in locomotion is not due to minor surgery procedure [20]. It is likely that a more “major” surgery—partial hepatectomy would induce a state of neuroinflammation that could result in sickness behavior.

Pro-inflammatory cytokines inhibit hippocampal neuronal functions, including long-term potentiation (LTP) and dendritic branching, which are involved in memory formation and maintenance [21]. Stressors can induce two different types of inflammatory responses in the brain. The first is a rapid, short duration (several hours) increase in inflammatory mediators [22]. Brain levels of pro-inflammatory cytokines are elevated immediately after a moderate duration (2 h) of stress and persist for 4–6 h [23]. The second is a slower developing, longer lasting (days) sensitization (or “priming”) of neuroinflammatory responses to subsequently occurring infectious/pathogenic stimuli or stressors (i.e. delayed challenge) [13]. Acute and chronic stress has been found to sensitize neuroinflammatory responses to both peripheral and central immunologic challenges [13, 24, 25]. CUS alone failed to modulate the expression of pro-inflammatory cytokines 48 h post-stress. However, consistent with a growing body of evidence, this study demonstrated the priming effects of CUS in neuroinflammatory processes [26]. CUS amplified surgery-induced neuroinflammatory responses in the brain. Additionally, Barrientos et al demonstrated a single intracisternal administration of IL-1 receptor antagonist (IL-1RA) at the time of surgery was sufficient to block both the behavioral deficit and the neuroinflammatory response. Injecting the same dose of IL-1RA peripherally failed to have a protective effect [27]. These data provided strong support for the specific role of central, not peripheral, IL-1 β in behavior deficits.

Microglia are primary immune cells and major source of pro-inflammatory cytokines in the central nervous system (CNS) [28, 29]. Espinosa-Oliva et al found that microglia were neuroimmune substrates of peripheral stress-induced GCs action, which in turn “prime” microglia to over-respond to subsequent challenge in the aged brain [24]. Notably, hippocampal microglia isolated 24 h post-stress also exhibited a potentiated neuroinflammatory response to LPS *ex vivo*, suggesting that central microglia instead of peripheral immunologic substrate(s) were sensitized or primed. In this study, CUS sensitized microglia to pro-inflammatory immunophenotype. When further stimulated in this state through a peripheral surgical challenge, microglia overexpressed pro-inflammatory cytokines (e.g., IL-1 β and IL-6). These data suggest that stress-induced microglial priming contributes to neuroinflammatory responses to subsequent surgical challenge.

On the basis of gene expression profiles, activated microglia may be categorized into two opposite types: M1 phenotype and M2 phenotype [30]. M1 represents a detrimental state of microglia, characterized by high expression of pro-inflammatory cytokines (e.g. IL-1 β , IL-6 and TNF- α). Conversely, M2 phenotype may reverse the neuron loss, repair neural networks and enhance the production of anti-inflammatory cytokines (e.g. IL-10) and neurotrophic mediators (e.g. BDNF) [30]. M1 and M2 polarization states of microglia play an important role in controlling the balance between pro-inflammatory and anti-inflammatory conditions. This study demonstrated that CUS exaggerated surgery-induced neuroinflammatory responses and exacerbated sickness behavior by inhibiting the function of M2-polarized microglia. CUS mediated the shift of the neuroimmune microenvironment toward a microglial M1 phenotype.

CD200 expressed on the surface of neurons is thought to maintain microglial cells in a quiescent state through interactions with its receptor CD200R [31]. The CNS parenchyma shows high levels of CD200 as well as very low levels of basal MHC-II and Iba-1, indicating the quiescent immunophenotype of the normal brain microenvironment [32]. The present study demonstrated that CUS downregulated hippocampal CD200 expression at 48 and 96 h post-stress. The effect of CUS on CD200 suggests that stress-induced sensitization of microglia may be mediated in part by attenuation of neuronal control of microglia.

GCs appear to play a pivotal role in CUS-induced potentiation of neuroinflammatory processes [9, 33]. GCs modulate innate immune signaling pathways (e.g., Toll-like receptors and inflammasome formation) that are pivotal to generating a pro-inflammatory immune response [34]. In this study, treatment with GCs receptor antagonist RU486 blocked CUS-induced priming of hippocampus pro-inflammatory responses and microglial activation following surgical procedure. These data suggest that elevated GCs may mediate CUS-induced sensitization of neuroinflammatory processes triggered by surgical trauma.

Human studies provide direct evidence of cognitive impairment following high levels of GCs. A 4-day treatment with dexamethasone produced a selective impairment of declarative memory [35]. Cushing's syndrome is associated with cognitive impairment and hippocampal atrophy that is reversible following the lowering of cortisol levels after treatment [36]. Consistent with previous research, this study demonstrated that surgical trauma significantly increased the levels of serum GCs, which related to sickness behavior [37]. Thus, a body of knowledge suggests that GCs, at least partly, involves behavior deficits in a variety of settings.

Increased levels of GCs are almost universally considered to be anti-inflammatory [38]. However, the results in this study appeared contradictory. Stress-induced GCs failed to suppress neuroinflammatory responses following surgical procedure. Of particular relevance to the present study, the timing of stress exposure relative to an immune challenge was a critical parameter in determining the outcome. Frank et al found that GCs treatment prior to LPS potentiated the neuroinflammatory response, whereas GCs treatment after LPS blunted neuroinflammatory responses to LPS. Notably, Barnum et al showed that CUS as well as a chronic psychological stress blunted the neuroinflammatory response to LPS [39]. It is important to note that LPS was administered two weeks after the last stress. Whereas consistent with other studies, partial hepatectomy was performed 24 h post-stress in this study [7]. The timing of immunologic challenge relative to stressor offset may account for these discrepant findings.

GCs bind two different receptors: high-affinity mineralocorticoid receptor (MR) and the lower-affinity GR in the CNS. MR is heavily occupied basally and becomes saturated by GCs levels in the mild stress range, whereas GR is heavily occupied only after major stressors [8]. Because MR and GR signaling can have different transcriptional effects, basal and high-stress GCs levels can have divergent, even opposite effects [40]. Pretreatment with RU486 blocked CUS-induced microglia activation and neuroinflammatory responses in the brain, which indicated that GCs and GR involved in the deleterious effects of CUS.

BDNF is an important regulator of synaptic transmission and LTP in the brain, which is associated with learning and memory formation [41, 42]. Administered neural injections of a BDNF antibody exacerbated cognitive deficits assessed by a Morris water maze [43]. Contrarily, exogenous BDNF improved the cognitive performance [44]. A growing body of evidence suggests that severe stress can suppress BDNF signaling, impair synaptic activity and increase susceptibility to affective disorders, resulting in neuronal atrophy and cognitive impairment [45]. Several evidence indicate that chronic stress and low level of BDNF are the major components of sickness behavior [46]. In this study, surgical trauma in combination with CUS inhibited BDNF expression in the brain, which was accompanied with sickness behavior. Surgery-induced behavioral deficits were mediated, in part, by downregulation of hippocampal BDNF expression.

Stress affects neuroimmune system functions both directly and indirectly. Previous research has indicated that chronic stress induces inflammatory responses, cognitive impairments and regulates microglial activity in the brain region [47, 48]. However, in this study, CUS alone failed to exacerbate sickness behavior, modulate the expression of pro-inflammatory cytokines and alter microglial Iba-1 and Arg 1 expression 48 h post-stress. Contradictory results may be considered to be due to different treatment protocols, such as animals (adult vs

aged rats), stress variables [e.g., duration (14 vs 40 days) and frequency] and time point of evaluation (48 vs 2 hours) [7]. Furthermore, Bian et al provides evidence that CUS-induced impairments in spatial learning and memory and CUS-induced changes in glia cells could be reversible [49].

This study provides converging evidence that CUS reinforces the effect of surgical trauma challenges in a synergistic manner, creating an exaggerated sickness behavior and neuroinflammatory responses and inhibiting BDNF expression in this rodent model. Stress-induced microglial activation contributes to the sensitization of pro-inflammatory responses. GCs play a pivotal role in enhancing stress-induced neuroinflammatory responses by modulation of microglia functions.

Supporting information

S1 Checklist. NC3Rs ARRIVE guidelines checklist.
(PDF)

S1 Dataset. Relevant data underlying the findings described in manuscript.
(XLS)

Author Contributions

Data curation: Na Wang.

Formal analysis: Na Wang, Hong Ma, Zhe Li, Yalei Gao.

Funding acquisition: Xuezhao Cao.

Investigation: Hong Ma, Yalei Gao, Xuezhao Cao.

Methodology: Zhe Li, Yalei Gao.

Project administration: Na Wang, Zhe Li, Yalei Gao, Yanhua Jiang, Yongjian Zhou, Sidan Liu.

Supervision: Hong Ma, Xuezhao Cao.

Writing – original draft: Xuezhao Cao.

Writing – review & editing: Hong Ma, Xuezhao Cao.

References

1. Cao XZ, Ma H, Wang JK, Liu F, Wu BY, Tian AY, et al. Postoperative cognitive deficits and neuroinflammation in the hippocampus triggered by surgical trauma are exacerbated in aged rats. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010; 34(8):1426–32. <https://doi.org/10.1016/j.pnpbp.2010.07.027> PMID: 20691747
2. Zhang Z, Li X, Li F, An L. Berberine alleviates postoperative cognitive dysfunction by suppressing neuroinflammation in aged mice. *Int Immunopharmacol*. 2016; 38:426–33. <https://doi.org/10.1016/j.intimp.2016.06.031> PMID: 27376853
3. Hovens IB, Schoemaker RG, van der Zee EA, Absalom AR, Heineman E, van Leeuwen BL. Postoperative cognitive dysfunction: Involvement of neuroinflammation and neuronal functioning. *Brain Behav Immun*. 2014; 38:202–10. <https://doi.org/10.1016/j.bbi.2014.02.002> PMID: 24517920
4. Ma Y, Cheng Q, Wang E, Li L, Zhang X. Inhibiting tumor necrosis factor- α signaling attenuates postoperative cognitive dysfunction in aged rats. *Mol Med Rep*. 2015; 12(2):3095–100. <https://doi.org/10.3892/mmr.2015.3744> PMID: 25955232
5. Wang Y, Cao X, Ma H, Tan W, Zhang L, Li Z, et al. Prior stressor exposure delays the recovery of surgery-induced cognitive impairment and prolongs neuroinflammation in aged rats. *Brain Res*. 2016; 1648:380–6. <https://doi.org/10.1016/j.brainres.2016.07.045> PMID: 27487302

6. Fonken LK, Weber MD, Daut RA, Kitt MM, Frank MG, Watkins LR, et al. Stress-induced neuroinflammatory priming is time of day dependent. *Psychoneuroendocrinology*. 2016; 66:82–90. <https://doi.org/10.1016/j.psyneuen.2016.01.006> PMID: 26799851
7. Munhoz CD, Lepsch LB, Kawamoto EM, Malta MB, Lima Lde S, Avellar MC, et al. Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal cortex and hippocampus via glucocorticoid secretion. *J Neurosci*. 2006; 26(14):3813–20. <https://doi.org/10.1523/JNEUROSCI.4398-05.2006> PMID: 16597735
8. Munhoz CD, Sorrells SF, Caso JR, Scavone C, Sapolsky RM. Glucocorticoids exacerbate lipopolysaccharide-induced signaling in the frontal cortex and hippocampus in a dose-dependent manner. *J Neurosci*. 2010; 30(41):13690–8. <https://doi.org/10.1523/JNEUROSCI.0303-09.2010> PMID: 20943909
9. Frank MG, Hershman SA, Weber MD, Watkins LR, Maier SF. Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. *Psychoneuroendocrinology*. 2014; 40:191–200. <https://doi.org/10.1016/j.psyneuen.2013.11.006> PMID: 24485491
10. Wang HL, Liu H, Xue ZG, Liao QW, Fang H. Minocycline attenuates post-operative cognitive impairment in aged mice by inhibiting microglia activation. *J Cell Mol Med*. 2016; 20(9):1632–9. <https://doi.org/10.1111/jcmm.12854> PMID: 27061744
11. Shevde K, Panagopoulos G. A survey of 800 patients' knowledge, attitudes, and concerns regarding anesthesia. *Anesthesia and analgesia*. 1991; 73(2):190–8. PMID: 1854034
12. Gamaro GD, Manoli LP, Torres IL, Silveira R, Dalmaz C. Effects of chronic variate stress on feeding behavior and on monoamine levels in different rat brain structures. *Neurochem Int*. 2003; 42(2):107–14. PMID: 12421590
13. Johnson JD, O'Connor KA, Hansen MK, Watkins LR, Maier SF. Effects of prior stress on LPS-induced cytokine and sickness responses. *Am J Physiol Regul Integr Comp Physiol*. 2003; 284(2):R422–32. <https://doi.org/10.1152/ajpregu.00230.2002> PMID: 12399247
14. Hains LE, Loram LC, Taylor FR, Strand KA, Wieseler JL, Barrientos RM, et al. Prior laparotomy or corticosterone potentiates lipopolysaccharide-induced fever and sickness behaviors. *J Neuroimmunol*. 2011; 239:53–60. <https://doi.org/10.1016/j.jneuroim.2011.08.011> PMID: 21907418
15. Biesmans S, Matthews LJ, Bouwknecht JA, De Haes P, Hellings N, Meert TF, et al. Systematic Analysis of the Cytokine and Anhedonia Response to Peripheral Lipopolysaccharide Administration in Rats. *Biomed Res Int*. 2016; 2016:9085273. <https://doi.org/10.1155/2016/9085273> PMID: 27504457
16. Huang TJ, Hsu RW, Li YY, Cheng CC. Less systemic cytokine response in patients following microendoscopic versus open lumbar discectomy. *J Orthop Res*. 2005; 23(2):406–11. <https://doi.org/10.1016/j.orthres.2004.08.010> PMID: 15734255
17. Nelson CJ, Lysle DT. Severity, time, and beta-adrenergic receptor involvement in surgery-induced immune alterations. *J Surg Res*. 1998; 80(2):115–22. <https://doi.org/10.1006/jsre.1998.5429> PMID: 9878301
18. Canet J, Raeder J, Rasmussen LS, Enlund M, Kuipers HM, Hanning CD, et al. Cognitive dysfunction after minor surgery in the elderly. *Acta Anaesthesiol Scand*. 2003; 47(10):1204–10. PMID: 14616316
19. Hempenius L, Slaets JP, van Asselt DZ, Schukking J, de Bock GH, Wiggers T, et al. Interventions to prevent postoperative delirium in elderly cancer patients should be targeted at those undergoing non-superficial surgery with special attention to the cognitive impaired patients. *Eur J Surg Oncol*. 2015; 41(1):28–33. <https://doi.org/10.1016/j.ejso.2014.04.006> PMID: 24857381
20. Rosczyk HA, Sparkman NL, Johnson RW. Neuroinflammation and cognitive function in aged mice following minor surgery. *Exp Gerontol*. 2008; 43(9):840–6. <https://doi.org/10.1016/j.exger.2008.06.004> PMID: 18602982
21. Vasconcelos AR, Yshii LM, Viel TA, Buck HS, Mattson MP, Scavone C, et al. Intermittent fasting attenuates lipopolysaccharide-induced neuroinflammation and memory impairment. *J Neuroinflammation*. 2014; 11:85. <https://doi.org/10.1186/1742-2094-11-85> PMID: 24886300
22. Nguyen KT, Deak T, Owens SM, Kohno T, Fleshner M, Watkins LR, et al. Exposure to acute stress induces brain interleukin-1beta protein in the rat. *J Neurosci*. 1998; 18(6):2239–46. PMID: 9482808
23. O'Connor KA, Johnson JD, Hansen MK, Wieseler Frank JL, Maksimova E, Watkins LR, et al. Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Res*. 2003; 991:123–32. PMID: 14575884
24. Espinosa-Oliva AM, de Pablos RM, Villaran RF, Arguelles S, Venero JL, Machado A, et al. Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. *Neurobiol Aging*. 2011; 32(1):85–102. <https://doi.org/10.1016/j.neurobiolaging.2009.01.012> PMID: 19286276
25. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, et al. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *J Neurosci*. 2011; 31(17):6277–88. <https://doi.org/10.1523/JNEUROSCI.0450-11.2011> PMID: 21525267

26. Sorrells SF, Sapolsky RM. An inflammatory review of glucocorticoid actions in the CNS. *Brain Behav Immun.* 2007; 21(3):259–72. <https://doi.org/10.1016/j.bbi.2006.11.00> PMID: 17194565
27. Barrientos RM, Hein AM, Frank MG, Watkins LR, Maier SF. Intracisternal interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. *J Neurosci.* 2012; 32(42):14641–8. <https://doi.org/10.1523/JNEUROSCI.2173-12.2012> PMID: 23077050
28. Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *FASEB J.* 2005; 19(10):1329–31. <https://doi.org/10.1096/fj.05-3776fje> PMID: 15919760
29. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. *Trends Immunol.* 2006; 27(1):24–31. <https://doi.org/10.1016/j.it.2005.11.006> PMID: 16316783
30. Olah M, Biber K, Vinet J, Boddeke HW. Microglia phenotype diversity. *CNS Neurol Disord Drug Targets.* 2011; 10(1):108–18. PMID: 21143141
31. Varnum MM, Kiyota T, Ingraham KL, Ikezu S, Ikezu T. The anti-inflammatory glycoprotein, CD200, restores neurogenesis and enhances amyloid phagocytosis in a mouse model of Alzheimer's disease. *Neurobiol Aging.* 2015; 36(11):2995–3007. <https://doi.org/10.1016/j.neurobiolaging.2015.07.027> PMID: 26315370
32. Wong WT. Microglial aging in the healthy CNS: phenotypes, drivers, and rejuvenation. *Front Cell Neurosci.* 2013; 7:22. <https://doi.org/10.3389/fncel.2013.00022> PMID: 23493481
33. Sun R, Zhao Z, Feng J, Bo J, Rong H, Lei Y, et al. Glucocorticoid-Potentiated Spinal Microglia Activation Contributes to Preoperative Anxiety-Induced Postoperative Hyperalgesia. *Mol Neurobiol.* 2016. [Epub ahead of print]
34. Frank MG, Watkins LR, Maier SF. Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun.* 2013; 33:1–6. <https://doi.org/10.1016/j.bbi.2013.02.004> PMID: 23459026
35. Newcomer JW, Craft S, Hershey T, Askins K, Bardgett ME. Glucocorticoid-induced impairment in declarative memory performance in adult humans. *J Neurosci.* 1994; 14(4):2047–53. PMID: 8198631
36. Starkman MN, Giordani B, Gebarski SS, Berent S, Schork MA, Schteingart DE. Decrease in cortisol reverses human hippocampal atrophy following treatment of Cushing's disease. *Biol psychiatry.* 1999; 46(12):1595–602. PMID: 10624540
37. Rasmussen LS, O'Brien JT, Silverstein JH, Johnson TW, Siersma VD, Canet J, et al. Is peri-operative cortisol secretion related to post-operative cognitive dysfunction? *Acta Anaesthesiol Scand.* 2005; 49(9):1225–31. <https://doi.org/10.1111/j.1399-6576.2005.00791.x> PMID: 16146456
38. Boumpas DT, Chrousos GP, Wilder RL, Cupps TR, Balow JE. Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. *Ann Intern Med.* 1993; 119(12):198–208. PMID: 8239251
39. Barnum CJ, Pace TW, Hu F, Neigh GN, Tansey MG. Psychological stress in adolescent and adult mice increases neuroinflammation and attenuates the response to LPS challenge. *J Neuroinflammation.* 2012; 9:9. <https://doi.org/10.1186/1742-2094-9-9> PMID: 22248083
40. Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The stressed CNS: when glucocorticoids aggravate inflammation. *Neuron.* 2009; 64(1):33–9. <https://doi.org/10.1016/j.neuron.2009.09.032> PMID: 19840546
41. Cortese GP, Barrientos RM, Maier SF, Patterson SL. Aging and a peripheral immune challenge interact to reduce mature brain-derived neurotrophic factor and activation of TrkB, PLCgamma1, and ERK in hippocampal synaptoneurosomes. *J Neurosci.* 2011; 31(11):4274–9. <https://doi.org/10.1523/JNEUROSCI.5818-10.2011> PMID: 21411668
42. Yirmiya R, Goshen I. Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav Immun.* 2011; 25(2):181–213. <https://doi.org/10.1016/j.bbi.2010.10.015> PMID: 20970492
43. Mu JS, Li WP, Yao ZB, Zhou XF. Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Res.* 1999; 835(2):259–65. PMID: 10415381
44. Cirulli F, Berry A, Chiarotti F, Alleva E. Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. *Hippocampus.* 2004; 14(7):802–7. <https://doi.org/10.1002/hipo.10220> PMID: 15382250
45. Ninan I. Synaptic regulation of affective behaviors; role of BDNF. *Neuropharmacology.* 2014; 76:684–95. <https://doi.org/10.1016/j.neuropharm.2013.04.011> PMID: 23747574
46. Han J, Wang DS, Liu SB, Zhao MG. Cytisine, a Partial Agonist of alpha4beta2 Nicotinic Acetylcholine Receptors, Reduced Unpredictable Chronic Mild Stress-Induced Depression-Like Behaviors. *Biomol Ther (Seoul).* 2016; 24(3):291–7.

47. Milior G, Lecours C, Samson L, Bisht K, Poggini S, Pagani F, et al. Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress. *Brain Behav Immun*. 2016; 55:114–25. <https://doi.org/10.1016/j.bbi.2015.07.024> PMID: 26231972
48. Kreisel T, Frank MG, Licht T, Reshef R, Ben-Menachem-Zidon O, Baratta MV, et al. Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Mol Psychiatry*. 2014; 19(6):699–709. <https://doi.org/10.1038/mp.2013.155> PMID: 24342992
49. Bian Y, Pan Z, Hou Z, Huang C, Li W, Zhao B. Learning, memory, and glial cell changes following recovery from chronic unpredictable stress. *Brain Res Bull*. 2012; 88(5):471–6. <https://doi.org/10.1016/j.brainresbull.2012.04.008> PMID: 22537595