Brief pup separation during lactation confers resilience in behavioural deficits induced by chronic restraint stress in postpartum C57BL/6J dams

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Background: The postpartum period is a complex time for females that affects health recovery. Stress during this period is one of the main risk factors for depression. Therefore, preventing stress-induced depression in the postpartum period is of great importance. Pup separation (PS) is a natural paradigm of postpartum care; however, the effect of different PS protocols during lactation on stress-induced depressive behaviours in dams is unknown. **Methods:** Lactating C57BL/6J mice were subjected to no pup separation (NPS), brief PS (15 min/day, PS15) or long PS (180 min/day, PS180) from postpartum day 1 to postpartum day 21 and were then subjected to chronic restraint stress (CRS) for 21 days. Behavioural tests, specifically the open field test (OFT), elevated plus maze (EPM) test and tail suspension test (TST), were performed. The expression of mRNA and protein in the hippocampus and microbiota composition were also assessed. **Results:** We observed CRS-induced anxiety- and depression-like behaviours in NPS dams. In addition, in NPS dams, microglial activation and the levels of NOD-like receptor pyrin domain containing 3, caspase-1 and interleukin-1β were increased, whereas expression levels of collapsing response mediator protein 2 (CRMP2) and α-tubulin were decreased. However, immobility time in the TST was lower in PS15+CRS dams than in NPS+CRS dams, and time spent in the centre during the OFT and in the open arms during the EPM test was higher in PS15+CRS dams, indicating resilience. Expression of hippocampal biomarkers of neuroinflammation was inhibited and levels of CRMP2-mediated neuroplasticity were increased in PS15+CRS dams. Notably, we observed taxonomic changes in the cecal microbiota across different PS groups, as well as relationships between gut microbiota composition and some biomarkers of hippocampal neuroinflammation and neuroplasticity. **Limitations:** The sample size for gut microbiota analysis in this study was small. **Conclusion:** Collectively, the results of this study confirm that brief PS confers stress resilience in CRS-induced behavioural deficits and reverses hippocampal neuroinflammation–neuroplasticity injury and gut microbiota imbalance.

Introduction

Major depressive disorder is a common mental health disorder characterized by a high incidence among women.[1] The World Health Organization has reported that depression seriously endangers women's health and will become the world's most common disease by 2030.[2] As an important component of positive psychology, resilience has become a hot topic in research on neuropsychiatric diseases.[3] Chronic stress is one of the most important risk and maintenance factors for mental illness, but many people show positive adaptations to adversity and do not exhibit serious mental illness; this phenomenon is called resilience.[4]

The postpartum period is complex and sometimes traumatic for females. Many infants are separated from their mothers after birth and cannot receive breastfeeding from their mothers or have skin-to-skin contact with them because of problems such as prematurity, neonatal illness and maternal illness, a phenomenon known as "maternal separation" or, in animals, "pup separation" (PS). Postnatal PS is an important environmental factor for mothers during lactation, affecting the mother's hormone secretion and promoting postpartum depression and anxiety. Studies have shown that high secretion of prolactin and oxytocin and low secretion of antidiuretic hormones and androgen during lactation can prevent postpartum anxiety and depression, thereby optimizing the stress response, whereas chronic inhibition of estrogen and progesterone caused by lactation may have antidepressant and anxiolytic effects.[5] However, long interruptions in mother–infant skin-to-skin contact

may disrupt the mother's normal secretion of hormones and induce changes in adult neurogenesis throughout the hippocampus,[6] making mothers vulnerable to environmental risk factors and mental disorders. There exist few recent relevant studies, and more research is needed to explore the influence on mothers of changes in environmental factors during lactation. This knowledge can help with the development of personalized plans for women during lactation, enhance their stress resistance and reduce the occurrence of depression.

Neuroinflammation is involved in the underlying pathologic mechanisms of depression. The hippocampus is one of the brain regions that experiences the most damage during stress-related emotion disorders.[7] The microglia, as resident immune cells, are among the most important inflammatory cells in the central nervous system. Active microglial cells in the hippocampus can promote the activation of a series of regional inflammatory responses,[8] including those involving NOD-like receptor pyrin domain containing 3 (NLRP3), interleukin-1β (IL-1β) and interleukin-18 (IL-18).[9–11] In an animal model of depression, emerging evidence indicates that neuroinflammation can damage hippocampal neuroplasticity; for example, hippocampal volume decreases by 4%–6%, and neuronal axons and dendritic trees retreat.[12] During pregnancy, inflammatory states range from higher levels at implantation and during childbirth to lower levels in mid-pregnancy.[13] Collapsing response mediator protein 2 (CRMP2) has been recently considered a novel microtubule-associated protein,[14] which confers neuroprotective effects by modulating hippocampal neuroplasticity.[15] In adulthood, the hippocampus exhibits a high level of plasticity and has receptors for steroid and peptide hormones that change throughout pregnancy. Many of these hormones cause neuroplastic changes in the hippocampus throughout late pregnancy and the early postpartum period.[16]

The proteomic results of a clinical study showed that CRMP2 is associated with the stress-induced microbiota–gut–inflammasome–brain axis, which is a novel research topic.[17] The microbiota–gut–brain axis consists of a communication network that controls and integrates gut and brain function and that seems to be a central modulator of health and disease. Given the unique inflammatory and immune changes that occur during pregnancy, the composition of the maternal gut microbiome evolves as pregnancy advances.[18] Microbiome disorders can influence the development of pathologic mechanisms in major depressive disorder.[19] A recent study in NLRP3-deficient mice confirmed the involvement of the microbiota–gut–brain axis in depression and showed a close connection between the NLRP3 pathway and gut microbiota.[20] Using anti-inflammatory drugs has been shown to change the variety and abundance of beneficial bacteria.[21]

The PS phenomenon is a natural paradigm of postpartum care. Clinical studies indicate that poor maternal and child contact care during the first year after birth makes mothers more vulnerable to depression.[22,23] Previous rodent studies showed that after a long duration of PS, the maternal care of dams was significantly impaired, but the

dams showed different degrees of anxiety- and depression-like behaviours and memory alterations.[24,25] However, the effect of different PS protocols on stress-induced depressive behaviours in dams is unknown, and their neurobiologic mechanism remains to be elucidated.

We hypothesized that different PS protocols during lactation would differentially affect stress vulnerability or resilience to stress re-exposure after the perinatal period in dams. Therefore, in this study, a suite of different PS protocols was developed and applied in mice, and the animals were then exposed to chronic restraint stress (CRS) to investigate the behavioural phenotypes in dams. To further explore the neurobiologic mechanism underlying this phenomenon, we then investigated microglial activation, some biomarkers of neuroinflammation and neuroplasticity in the hippocampus, and the composition of gut microbiota.

Methods

Animals

Pregnant C57BL/6J mice weighing 26–30 g were provided by the animal facility at the Renmin Hospital of Wuhan University. Female mice were mated at 8 weeks to males who were also 8 weeks old; each female was mated with a different male. When female mice were pregnant, they were individually housed under standard conditions (temperature 22 °C ± 1 °C; humidity 55% ± 5%) on a 12-hour light/dark cycle (lights on from 6 am to 6 pm) with free access to food and water. The NIH-41 standard diet for mice was provided by Medicience. To eliminate influencing factors, if cannibalism was observed or any pup death occurred after the females gave birth, we removed the female mice involved from the experiment. The number of pups per female mouse ranged from 5 to 7.

The experimental work was conducted in accordance with the Regulations of Experimental Animal Administration, issued by the State Committee of Science and Technology of the People's Republic of China, with the approval of the Ethics Committee in Renmin Hospital of Wuhan University.

Experimental design

Our study consisted of 2 experiments, as shown in Figure 1. In experiment 1, after delivery, each dam was randomly assigned to either of 2 groups: the control group (n = 8) or the CRS group (n = 8). The dams in these 2 groups were not separated from their pups. After weaning, mice in the CRS group were placed in well-ventilated 50 mL polystyrene tubes for 6 hours (8 am to 2 pm) per day for 21 consecutive days.

In experiment 2, after delivery, each dam was randomly assigned to 1 of 3 groups: the NPS+CRS group (no PS [NPS], n = 10), the PS15+CRS group (PS 15 min/d, n = 10) or the PS180+CRS group (PS 180 min/d, n = 10). Dams were subjected to the assigned PS protocols from postpartum day 1 to 21. Then, after weaning, all mice in the 3 groups underwent CRS as described above.
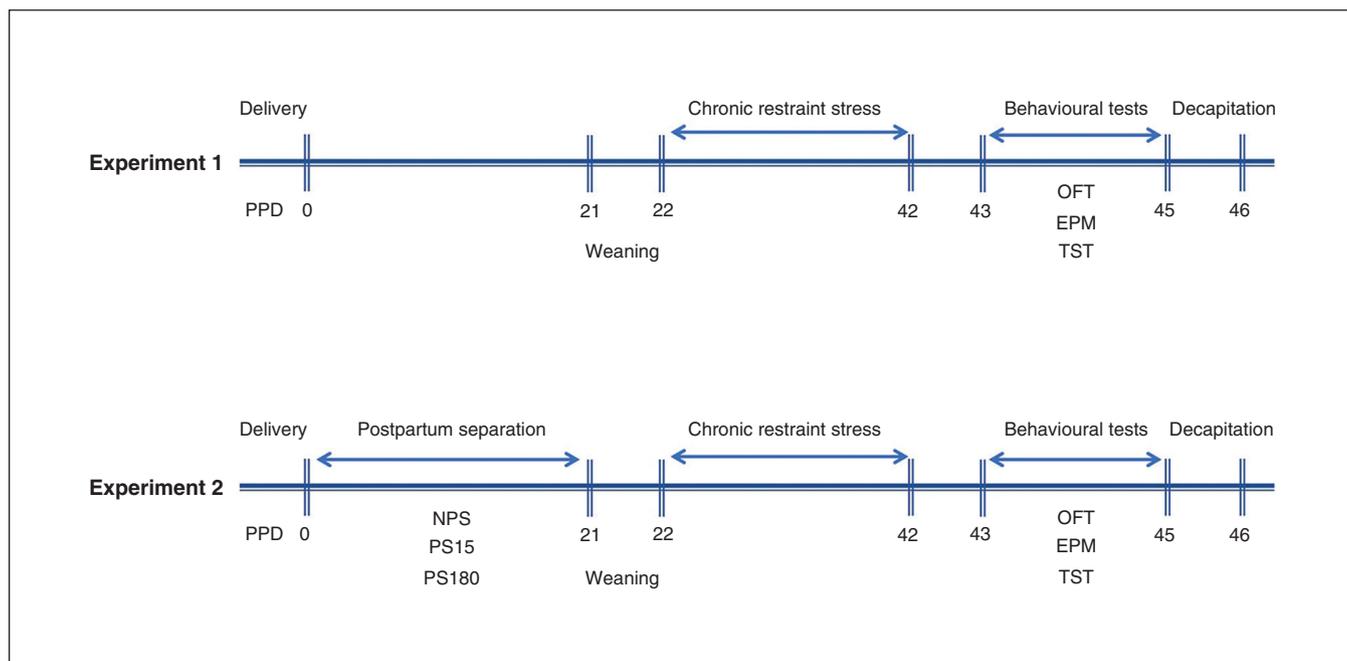
Fig. 1: Experimental procedures. Experiment 1: Half of the dams that did not undergo pup separation (PS) during lactation were subjected to chronic restraint stress. Experiment 2: Dams were subjected to various PS protocols during lactation and then exposed to chronic restraint stress after weaning. Note: Intervals along the horizontal rule showing time after delivery are not to scale. EPM = elevated plus maze; NPS = no pup separation; OFT = open field test; PPD = postpartum day; TST = tail suspension test.

Protocol for PS

In accordance with previous research, the PS procedure was performed for 15 minutes per day in the PS15 group (brief PS) and for 180 minutes per day in the PS180 group (long PS) over the period from postpartum day 1 to postpartum day 21. Each dam was first removed from the nest to a separate cage, and the pups, remaining in their original cage, were placed in an incubator (maintained at a temperature of 31 °C ± 1 °C). After the separation period, the dam was returned to the home cage and was left undisturbed. After weaning, the dams were fed in cages together, and dams in the same group were placed in the same cages, with each cage containing no more than 5 mice. After weaning, the dams in experiment 1 were divided among 2 cages. The dams in experiment 2 were divided among 3 cages.

Behavioural testing

The open field test (OFT) was performed on postpartum day 43, which was the day after the end of CRS. The open field chamber was made of transparent plastic (50 cm × 50 cm). The chamber was divided into 9 square areas, and a 25 cm × 25 cm square in the centre was defined as the centre zone. Individual mice were gently placed in the central area of the cage, and their activities were recorded for 5 minutes with an overhead video-tracking system (Ethovision XT 11.5, Noldus). The floor and walls of the apparatus were thoroughly cleaned with 75% alcohol after each trial.

The elevated plus maze (EPM) test was conducted on postpartum day 44. The EPM platform consisted of 2 open arms (35 cm × 5 cm) perpendicular to 2 closed arms (35 cm × 5 cm) of the same size, with a small central square (5 cm × 5 cm) between the arms. The maze was raised 50 cm above the floor. Each animal was placed at the centre of the maze, facing an open arm. The total number of entries into an open arm and the time spent in the open arms during the 5-minute period were recorded with the same video-tracking system. The apparatus was cleaned with 75% ethanol after each trial.

The tail suspension test (TST) was performed on postpartum day 45. Mice were suspended 40 cm above the floor by adhesive tape on the tip of the tail in a dark room. We manually recorded the total amount of time each mouse was immobile over a period of 6 minutes. The tester was blinded to group assignments.

Tissue collection and analysis

The day after completion of the behavioural tests, the dams were anesthetized with 1% pentobarbital (35 mg/kg). After decapitation, the brain and cecal content of each dam were immediately removed, rapidly frozen on dry ice and stored at −80 °C. The hippocampus was removed with microscissors for real-time quantitative polymerase chain reaction (PCR) and Western blot analysis.

Half of the mice in each group were randomly assigned to the PCR test group, and the whole hippocampus of each mouse was mixed for this purpose. TRIzol extraction reagent (no. 15596026, Invitrogen) was used to extract total

RNA. The RNA concentration and purity were determined by a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific). Then, the total RNA (2 μg) was reverse transcribed into cDNA using a PrimeScript RT Kit (RR820A, Takara). The reaction mixture was added to the RNA solution and incubated at 42 °C for 1 hour, heated at 70 °C for 5 minutes and cooled at 48 °C. Real-time PCR was performed using SYBR Master Mix on a Connect Real-Time PCR platform (Bio-Rad). The reaction was carried out in a GeneAmp 9700 PCR thermal cycler (ABI) under the following conditions: 95 °C for 30 seconds, 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds, 95 °C for 10 seconds and 60 °C for 5 seconds. Each sample in the PCR test was analyzed in triplicate, and an average was calculated for analysis. The resulting Cq values were calculated using CFX Manager Software version 3.1 (Bio-Rad). The real-time PCR primer sequences are presented in Appendix 1, Supplemental Figure 1, available at www.jpn.ca/lookup/doi/10.1503/jpn.220079/tab-related-content. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Cq values as an internal control.

Half of the mice in each group were randomly assigned to Western blot analysis, and the whole hippocampus of each mouse was used for this purpose. The total amount of protein was measured by the bicinchoninic acid method (Beyotime). Proteins (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gels and transferred electrophoretically to polyvinylidene fluoride membranes with the TGX Stain-Free FastCast acrylamide kit (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in tris-buffered saline 0.1% Tween-20 (TBST) for 1 hour and incubated overnight at 4 °C with the following primary antibodies (all from Abcam) in Bond Primary Antibody Diluent: rabbit anti-Iba1 (ab178846, dilution 1:1000), rabbit anti-NLRP3 (ab263899, dilution 1:1000), rabbit anti-caspase-1 (ab179515, dilution 1:1000), rabbit anti-IL-18 (ab191860, dilution 1:1000), rabbit anti-IL-1β (ab234437, dilution 1:1000), rabbit anti-CRMP2 (ab129082, dilution 1:20 000), mouse anti-α-tubulin (ab7291, dilution 1:5000) and rabbit anti-GAPDH (ab181602, dilution 1:1000). After 3 washes in TBST, the membranes were incubated with secondary antibodies horseradish peroxidase–labelled goat anti-rabbit IgG, dilution 1:5000 and goat anti-mouse IgG, dilution 1:10 000; Abcam) at room temperature for 1 hour. For each sample, Western blotting was performed in triplicate, and the average calculated for analysis. The proteins were detected by the ChemiDoc Touch Image System (Bio-Rad), and the results were standardized to the GAPDH band at 37 kDa as an internal control with Bio-Rad software.

Cecum sample collection and processing of sequencing data

In experiment 2, we randomly selected 1 dam from each cage for cecal sampling. We extracted microbial community genomic DNA using the TIANamp Stool DNA kit (Tiangen), according to the manufacturer's instructions. The DNA extract was assessed on 1% agarose gel, and DNA concentration and purity were determined with the Nano-Drop 2000 UV–Vis spectrophotometer. The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified using the primer pairs 338F (5'-ACTCCTACGGGAGGCAG-CAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') on the GeneAmp 9700 PCR thermal cycler. The PCR mixtures contained 4 μL of 5× TransStart FastPfu buffer, 2 μL of 2.5 mM deoxynucleotide triphosphates, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of TransStart FastPfu DNA polymerase, 10 ng of template DNA and double-distilled H_2O up to 20 μL. These PCR experiments were performed in triplicate. The PCR product was extracted from a 2% agarose gel, purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences) according to the manufacturer's instructions and quantified using a Quantus fluorometer (Promega).

Purified amplicons were pooled in equimolar amounts, and paired-end sequencing was performed on a MiSeq PE300 platform/NovaSeq PE250 platform (Illumina) according to the standard protocols developed by Wefind Biotechnology Co., Ltd. The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp preprocessor, version 0.20.0 and merged using FLASH (Fast Length Adjustment of Short Reads), version 1.2.7,[26] according to the following criteria. (1) The 300-bp reads were truncated at any site receiving an average quality score < 20 over a 50-bp sliding window, and the truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded. (2) Only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was 0.2. Reads that could not be assembled were discarded. (3) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, with exact barcode matching and 2 nucleotide mismatches during primer matching. Operational taxonomic units with a 97% similarity cut-off were clustered using UPARSE, version 7.1,[27] and chimeric sequences were identified and removed. The taxonomy of each representative operational taxonomic unit sequence was analyzed by RDP Classifier, version 2.2,[28] against the 16S rRNA database (SILVA, version 138) using a confidence threshold of 0.7.

Statistical analysis

The results are presented as the means ± standard errors of the means. We used SPSS software, version 22 (IBM Corp.) and Prism software, version 8.0 for Windows (GraphPad Software, Inc.) for the analysis. We used the Kolmogorov–Smirnov normality test and the variance homogeneity test to ensure that normality and variance heterogeneity assumptions were met. Statistical comparisons were performed using t tests or 1-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The Pearson correlation analysis was also conducted. Results were considered statistically significant if the p value was less than 0.05.

Results

Chronic restraint stress induced anxiety- and depression-like behaviours in NPS dams

To investigate the effect of CRS on anxiety- and depression-like behaviours in female mice, we performed behavioural tests — the OFT, EPM test and TST — in dams that did not undergo PS during lactation. In the OFT, the frequency of entering the centre and time spent in the centre were significantly lower in the CRS group than in the control group (frequency of entering the centre: $t_{14} = 8.457$, $p < 0.001$, Figure 2A; time spent in the centre: $t_{14} = 7.952$, $p < 0.001$, Figure 2B). Similarly, in the EPM test, the CRS group exhibited significantly lower frequency of entering the open arms and time spent in the open arms than the control group (frequency of entering the open arms: $t_{14} = 2.919$, $p = 0.011$, Figure 2C; time spent in the open arms: $t_{14} = 6.714$, $p < 0.001$, Figure 2D). As shown in Figure 2E, mice in the CRS group showed more immobility time in the TST than control mice ($t_{14} = 9.253$, $p < 0.001$).

Chronic restraint stress activated neuroinflammation and damage to neuroplasticity in NPS dams

We further investigated neuroinflammation biomarkers and the levels of some cytokines involved in hippocampal neuroplasticity to explore whether CRS activated neuroinflammation and damaged neuroplasticity in NPS dams with CRS-induced anxiety- and depression-like behaviours. As shown in Figure 3A, the mRNA expression level of Iba1, an important biomarker of microglial activation, was elevated in mice with CRS-induced behavioural deficits ($t_6 = 4.956$, $p = 0.003$). Mice that experienced CRS also exhibited significant elevation in mRNA expression levels of NLRP3 ($t_6 = 7.135$, $p < 0.001$,
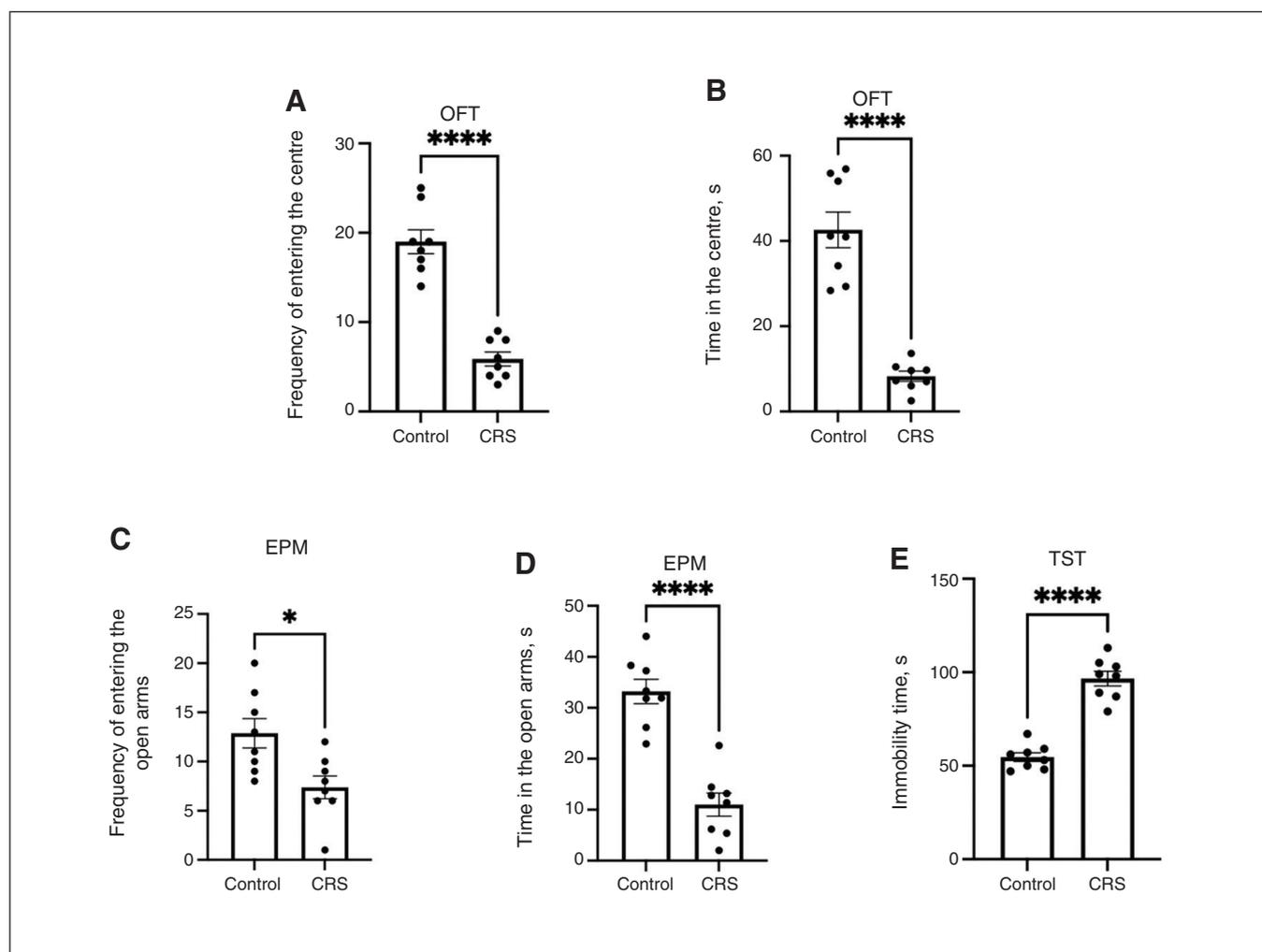
Fig. 2: Behavioural deficits induced by chronic restraint stress (CRS) in dams not exposed to pup separation. (A) Frequency of entering the centre and (B) time spent in the centre during the open-field test (OFT). (C) Frequency of entering the open arms and (D) time spent in the open arms during the elevated plus maze (EPM) test. (E) Immobility time during the tail suspension test (TST). Each bar represents the mean ± standard error of the mean, with $n = 8$ animals per group. Two groups (control and CRS) were analyzed using a t test (*$p < 0.05$, ****$p < 0.0001$).
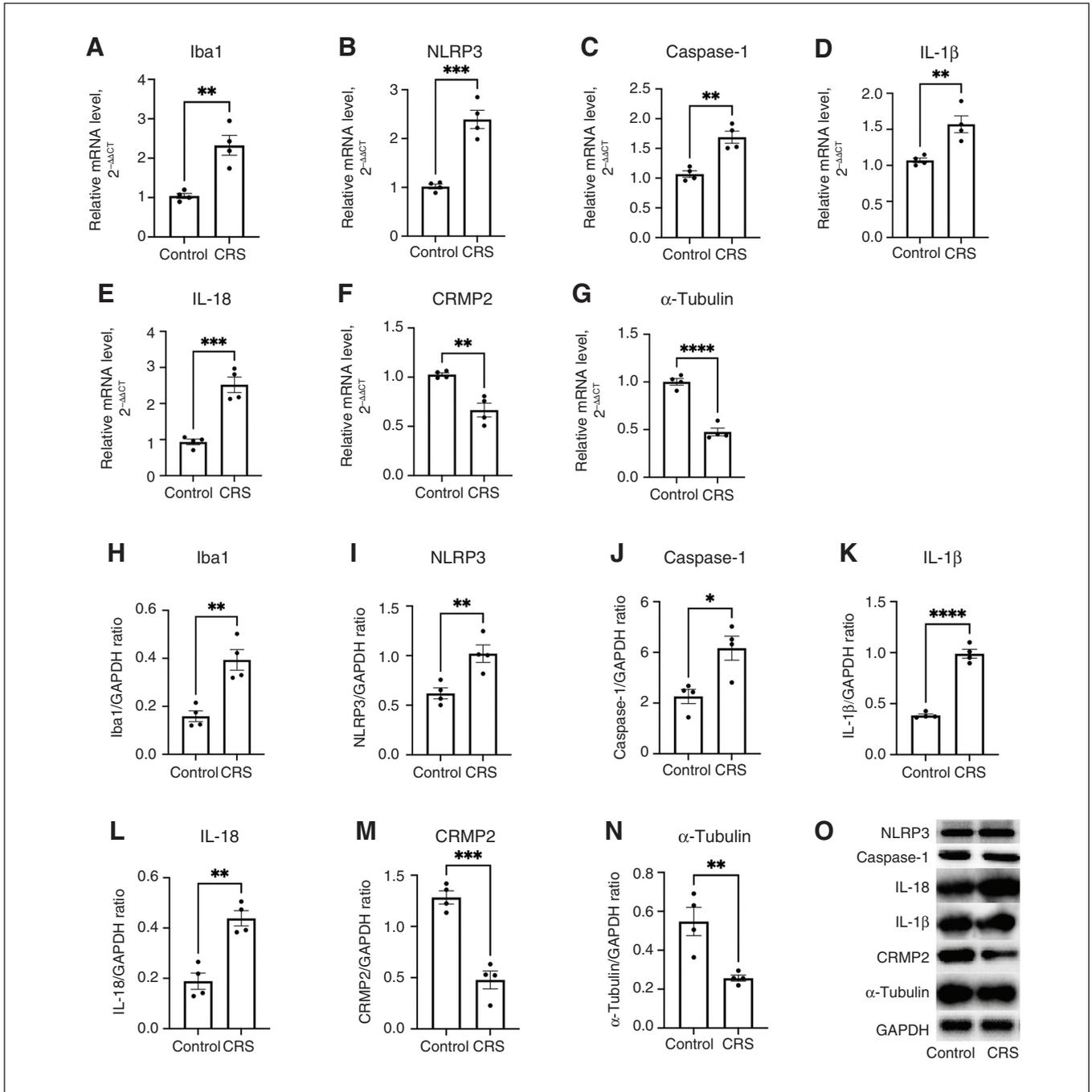
Fig. 3: Hippocampal mRNA and protein levels of neuroinflammatory and neuroplasticity biomarkers in dams with no pup separation. Real-time quantitative polymerase chain reaction analysis of relative mRNA levels for (A) Iba1, (B) NOD-like receptor pyrin domain containing 3 (NLRP3), (C) caspase-1, (D) interleukin-1β (IL-1β) and (E) interleukin-18 (IL-18); levels of (F) collapsing response mediator protein 2 (CRMP2) and (G) α-tubulin mRNA; Western blot analysis of protein expression of (H) Iba1, (I) NLRP3, (J) caspase-1, (K) IL-1β, (L) IL-18, (M) CRMP2 and (N) α-tubulin; and (O) densitometry analysis of the bands. Each bar represents the mean ± standard error of the mean, with $n = 4$ animals per group. Two groups (control and chronic restraint stress [CRS]) were analyzed using a t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Figure 3B), caspase-1 ($t_6 = 5.342$, $p = 0.002$, Figure 3C), IL-1β ($t_6 = 4.119$, $p = 0.006$, Figure 3D) and IL-18 ($t_6 = 6.899$, $p < 0.001$, Figure 3E). However, the levels of CRMP2 and α-tubulin, which are typical biomarkers of neuroplasticity, were signifi-

cantly lower in the CRS group (CRMP2: $t_6 = 4.987$, $p = 0.003$, Figure 3F; α-tubulin: $t_6 = 9.889$, $p < 0.001$, Figure 3G).

As shown in Figure 3H, Iba1 protein levels were also elevated in mice subjected to the CRS procedure ($t_6 = 4.844$, $p = $
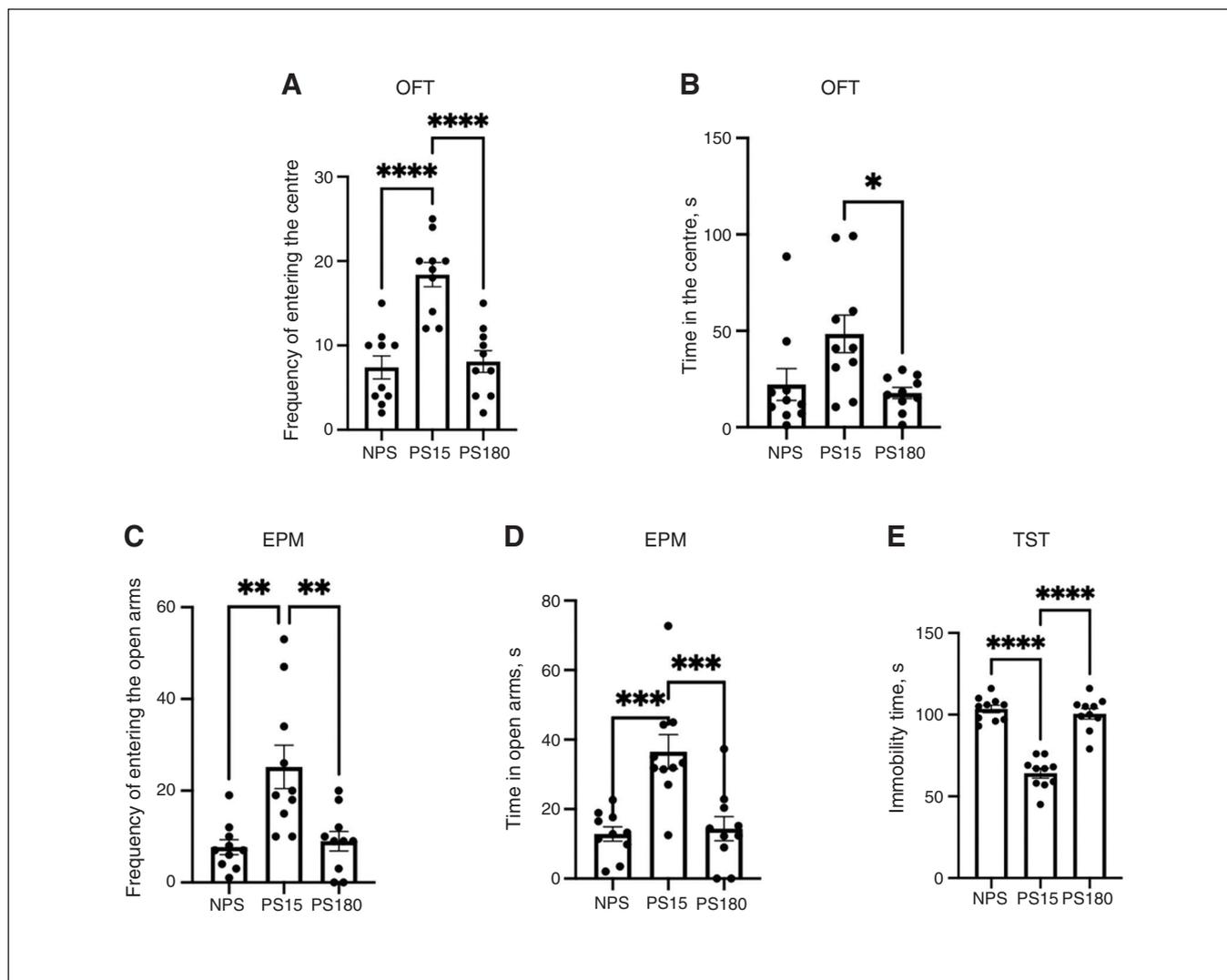
Fig. 4: Behavioural deficits induced by chronic restraint stress in dams exposed to brief pup separation during lactation, which conferred stress resilience. (A) Frequency of entering the centre and (B) time spent in the centre during the open-field test (OFT). (C) Frequency of entering the open arms and (D) time spent in the open arms during the elevated plus maze (EPM) test. (E) Immobility time during the tail suspension test (TST). Each bar represents the mean ± standard error of the mean, with n = 10 animals per group. Three groups were analyzed using analysis of variance followed by post hoc analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). NPS = no pup separation; PS15 = pup separation for 15 minutes; PS180 = pup separation for 180 minutes.

0.003). The protein expression levels of NLRP3, caspase-1, IL-1β and IL-18 were also significantly higher in the CRS group than in the control group (NLRP3: $t_6 = 3.859$, $p = 0.008$, Figure 3I; caspase-1: $t_6 = 3.457$, $p = 0.014$, Figure 3J; IL-1β: $t_6 = 13.17$, $p < 0.001$, Figure 3K; IL-18: $t_6 = 5.643$, $p = 0.001$, Figure 3L). The protein expression levels of CRMP2 and α-tubulin were significantly lower in the CRS group (CRMP2: $t_6 = 7.448$, $p < 0.001$, Figure 3M; α-tubulin: $t_6 = 3.929$, $p = 0.008$, Figure 3N).

Brief PS promoted resilience to CRS-induced anxiety- and depression-like behaviours in dams

In experiment 2, we explored the effects of various PS protocols on CRS-induced behavioural deficits in dams. In the OFT, there were significant differences in the frequency of entering the centre ($F_{2,27} = 20.41$, $p < 0.001$) and time spent in the centre ($F_{2,27} = 4.797$, $p = 0.0165$) among dams that underwent different PS protocols. Using post hoc analysis, we found that dams in the PS15+CRS group showed higher frequency of entering the centre than those in the NPS+CRS and PS180+CRS groups (NPS+CRS v. PS15+CRS, $p < 0.001$; PS180+CRS v. PS15+CRS, $p < 0.001$; Figure 4A). The PS15+CRS dams spent more time in the centre than the PS180+CRS dams ($p = 0.020$, Figure 4B), but there was no significant difference between the PS15+CRS and NPS+CRS groups. Additionally, no significant differences were observed between PS180+CRS and NPS+CRS dams in the frequency of entering the centre and time spent in the centre.

In the EPM test, there were significant differences in the frequency of entering the open arms ($F_{2,27}$ = 9.665, $p < 0.001$) and time spent in the open arms ($F_{2,27}$ = 12.95, $p < 0.001$) among dams that underwent different PS protocols. Using post hoc analysis, we found that dams in the PS15+CRS group showed higher frequency of entering the open arms than those in the NPS+CRS and PS180+CRS groups (Figure 4C). The PS15+CRS dams spent more time in the open arms than the NPS+CRS and PS180+CRS dams (Figure 4D). However, there were no significant differences in the frequency of entering the open arms or the time spent in the open arms between PS180+CRS and NPS+CRS dams.

During the TST, dams that underwent different PS protocols exhibited significant differences in immobility time ($F_{2,27}$ = 12.95, $p < 0.001$). In the post hoc analysis, the PS15+CRS dams exhibited significantly less immobility time than the NPS+CRS and PS180+CRS dams (Figure 4E). The PS180+CRS and NPS+CRS dams exhibited no differences in immobility time.

Brief PS inhibited hippocampal neuroinflammation in dams exposed to CRS

We explored whether different PS protocols affected microglial activation and the levels of some inflammatory cytokines in the hippocampus after CRS. The ANOVA revealed significant differences in the mRNA expression levels of Iba1, NLRP3, caspase-1, IL-1β and IL-18 among dams that underwent different PS protocols (Iba1: $F_{2,12}$ = 56.8, $p < 0.001$; NLRP3: $F_{2,12}$ = 122.1, $p < 0.001$; caspase-1: $F_{2,12}$ = 32.9, $p < 0.001$; IL-1β: $F_{2,12}$ = 37.5, $p < 0.001$; IL-18: $F_{2,12}$ = 23.29, $p < 0.001$). The post hoc analysis showed that the expression level of Iba1 mRNA was significantly lower in the PS15+CRS group than in the NPS+CRS and PS180+CRS groups, but there was no difference between the PS180+CRS and NPS+CRS groups (Figure 5A). The mRNA analysis also showed lower expression levels of NLRP3 (Figure 5B), caspase-1 (Figure 5C), IL-1β (Figure 5D) and IL-18 (Figure 5E) in PS15+CRS dams than in PS180+CRS and NPS+CRS dams, but there were no differences between PS180+CRS and NPS+CRS dams.

The ANOVA showed significant differences in the protein levels of Iba1, NLRP3, caspase-1, IL-18 and IL-1β among dams that underwent different PS protocols (Iba1: $F_{2,12}$ = 5.136, $p = 0.024$; NLRP3: $F_{2,12}$ = 4.983, $p = 0.027$; caspase-1: $F_{2,12}$ = 4.161, $p = 0.042$; IL-1β: $F_{2,12}$ = 5.412, $p = 0.021$; IL-18: $F_{2,12}$ = 4.747, $p = 0.030$). The post hoc analysis showed that Iba1 protein levels were significantly lower in PS15+CRS dams than in NPS+CRS and PS180+CRS dams (Figure 5H). Western blot analysis showed lower protein levels of NLRP3 (Figure 5I) and IL-18 (Figure 5L) in PS15+CRS dams than in PS180+CRS dams, but no differences between PS15+CRS and NPS+CRS dams or between NPS+CRS and PS180+CRS dams. In addition, the protein levels of caspase-1 (Figure 5J) and IL-1β (Figure 5K) were lower in PS15+CRS dams than in NPS+CRS dams, but no differences were found between PS15+CRS and PS180+CRS dams or between NPS+CRS and PS180+CRS dams.

Hippocampal neuroplastic injury was inhibited in dams exposed to brief PS and CRS

We also measured the mRNA expression levels of markers associated with neuroplasticity. The ANOVA revealed significant differences in CRMP2 and α-tubulin levels among dams in the 3 groups (CRMP2: $F_{2,12}$ = 38.89, $p < 0.001$; α-tubulin: $F_{2,12}$ = 61.53, $p < 0.001$). Post hoc analysis indicated that the mRNA levels of CRMP2 (Figure 5F) and α-tubulin (Figure 5G) were significantly higher in PS15+CRS dams than in the other groups. There were no differences between PS180+CRS and NPS+CRS dams.

In addition, ANOVA showed significant differences in CRMP2 and α-tubulin protein levels among dams in the 3 groups (CRMP2: $F_{2,12}$ = 5.301, $p = 0.022$; α-tubulin: $F_{2,12}$ = 6.253, $p = 0.014$). Post hoc analysis indicated that the expression levels of both CRMP2 (Figure 5M) and α-tubulin (Figure 5N) were higher in PS15+CRS dams than in PS180+CRS dams, but there were no differences between NPS+CRS and PS15+CRS dams, nor were there any differences between PS180+CRS and NPS+CRS dams.

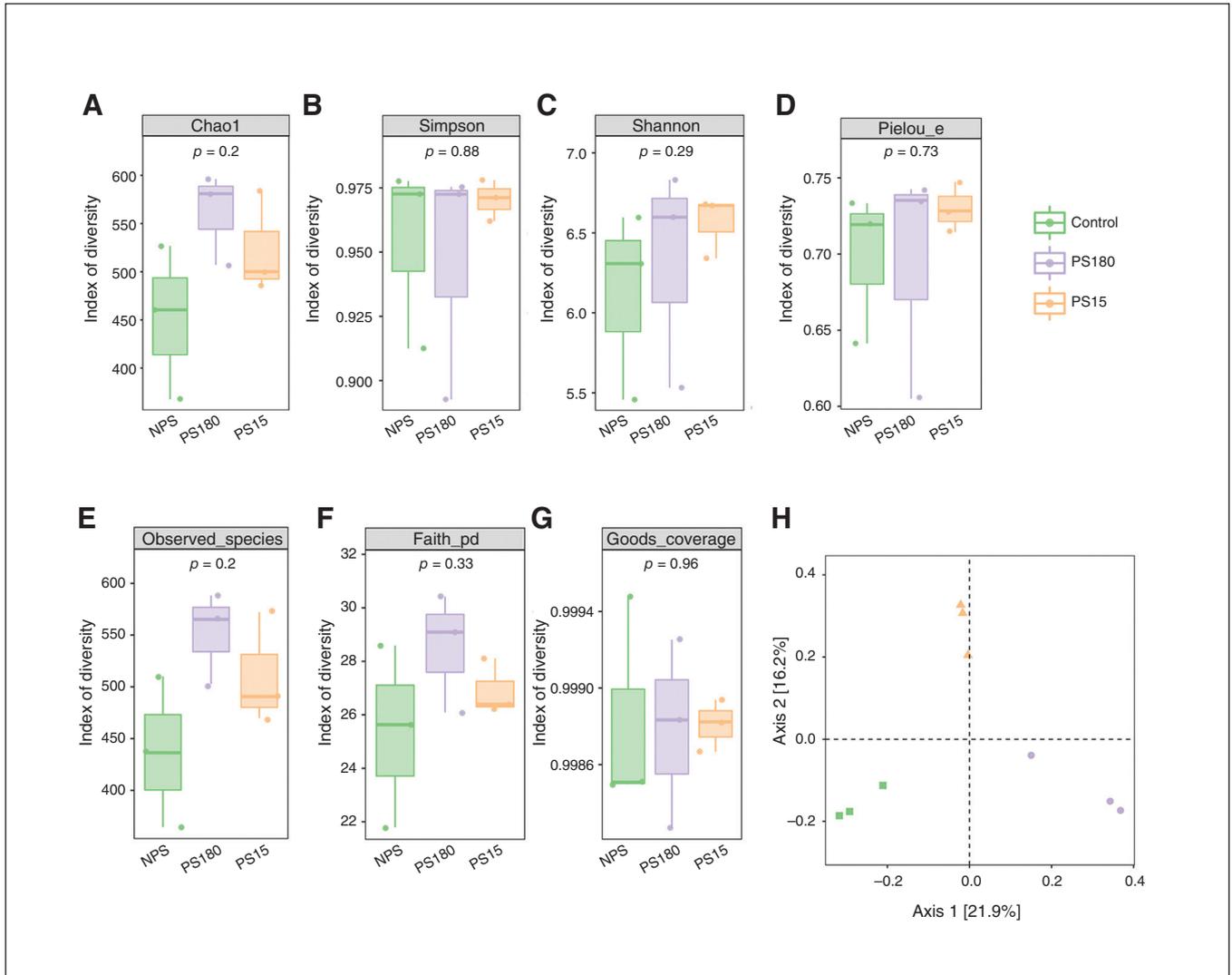*Compositional analysis of cecal microbiota in dams that underwent various PS protocols*

Our analysis of various indices of alpha diversity (Chao1, Simpson, Shannon, Pielou e [where "e" refers to evenness], observed species, Faith pd [where "pd" refers to phylogenetic diversity] and Good's coverage) in the cecal microbiota showed no significant differences (Figure 6A–6G). We also performed a beta diversity analysis, consisting of principal coordinates analysis to show the distribution of microbes in the intestinal microbiota, followed by permutational multivariate ANOVA. The results showed intergroup differences in intestinal distribution among the 3 groups ($F = 2.4488$, $p = 0.008$, Figure 6H), indicating that the composition of the cecal microbiota changed after the various PS processes.

We also investigated taxonomic changes. Analysis of the murine cecal microbiota showed a lower level of Actinobacteria and a higher level of Proteobacteria in PS180+CRS dams than in NPS+CRS dams (Actinobacteria: $F = 9.141$, $p = 0.015$, post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.014$, Figure 7A; Proteobacteria: $F = 6.415$, $p = 0.032$, post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.041$, Figure 7B). At the family level, as shown in Figure 7C, Erysipelotrichaceae abundance was significantly lower in the PS180+CRS dams than in the NPS+CRS or PS15+CRS dams ($F = 14.93$, $p = 0.005$; post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.004$; post hoc comparison of PS15+CRS and PS180+CRS, $p = 0.025$).

Additionally, at the genus level, the relative abundance of *Allobaculum* was significantly lower in PS180+CRS dams than in NPS+CRS and PS15+CRS dams ($F = 16.81$, $p = 0.004$; post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.003$; post hoc comparison of PS15+CRS and PS180+CRS, $p = 0.022$; Figure 7D). *Bifidobacterium* abundance was significantly higher in NPS+CRS dams than in PS15+CRS and
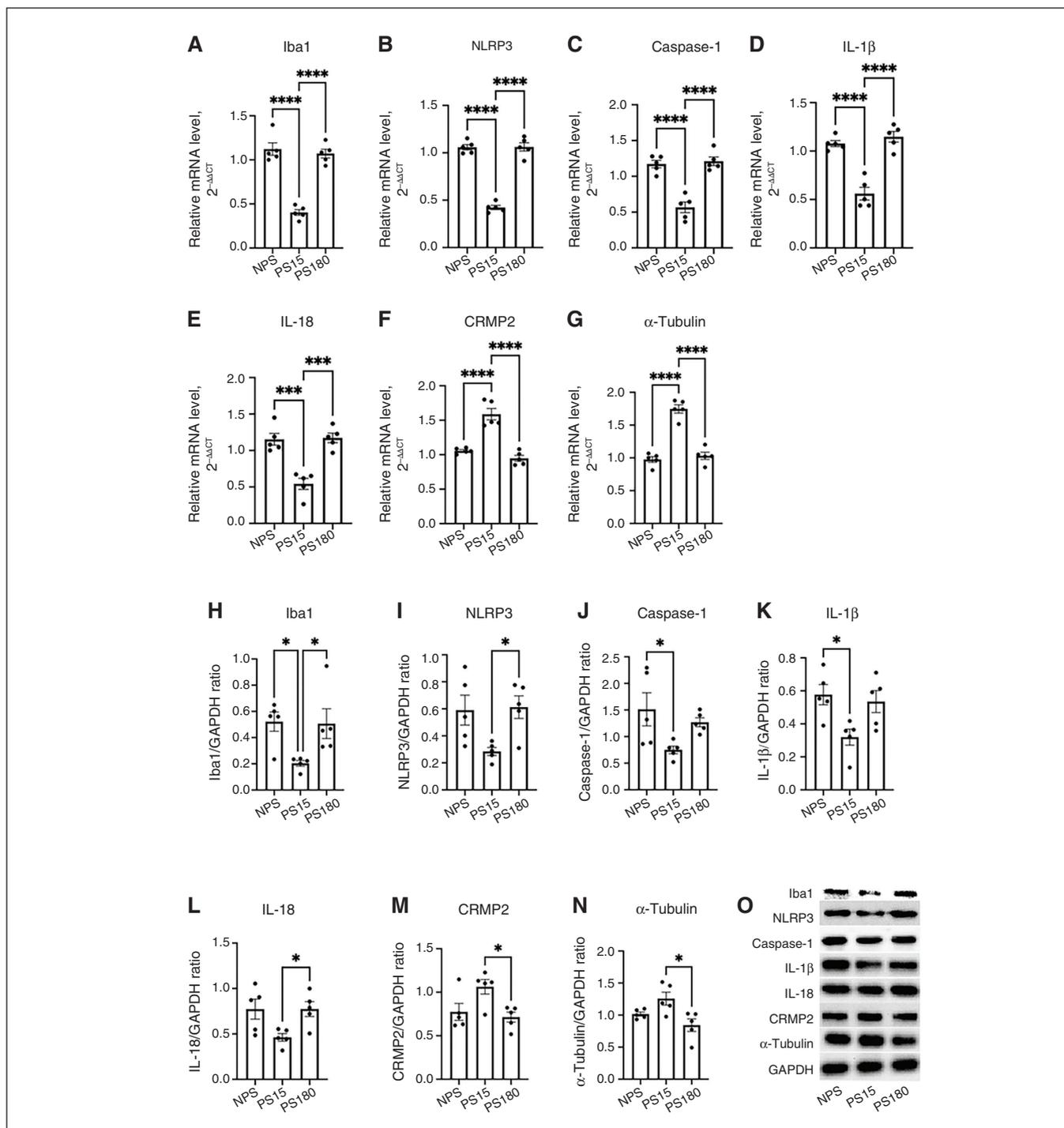
Fig. 5: Hippocampal mRNA and protein levels of neuroinflammatory and neuroplasticity biomarkers in dams subjected to chronic restraint stress (CRS) with various pup separation protocols. Real-time quantitative polymerase chain reaction analysis of relative mRNA levels for (A) Iba1, (B) NOD-like receptor pyrin domain containing 3 (NLRP3), (C) caspase-1, (D) interleukin-1β (IL-1β) and (E) interleukin-18 (IL-18); levels of (F) collapsing response mediator protein 2 (CRMP2) and (G) α-tubulin mRNA; Western blot analysis of protein expression of (H) Iba1, (I) BLRP3, (J) caspase-1, (K) IL-1β, (L) IL-18, (M) CRMP2 and (N) α-tubulin; and (O) densitometry analysis of the bands. Experiments with the group that underwent no pup separation and chronic restraint stress (NPS+CRS) and the group that underwent pup separation for 15 minutes followed by chronic restraint stress (PS15+CRS) were concluded. Each bar represents the mean ± standard error of the mean, with $n = 5$ animals per group. All 3 groups were analyzed using analysis of variance followed by a post hoc analysis (*$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$). NPS = no pup separation; PS15 = pup separation for 15 minutes; PS180 = pup separation for 180 minutes. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
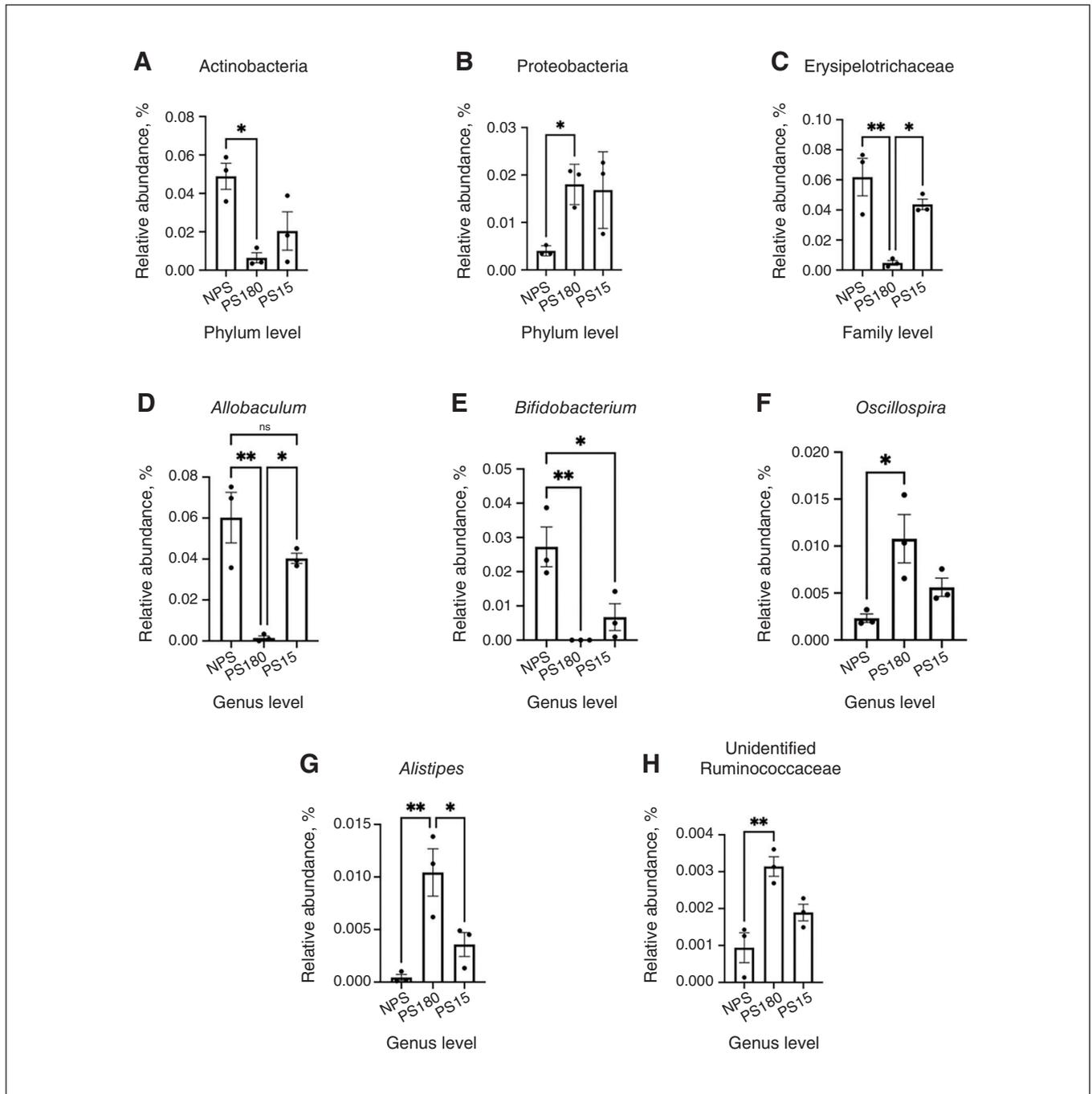
Fig. 6: Analysis of cecal microbiota composition in dams subjected to various pup separation protocols with chronic restraint stress: (A) Chao1, (B) Simpson, (C) Shannon, (D) Pielou e, (E) observed species, (F) Faith pd and (G) Good's coverage of alpha diversity analysis. (H) Principal coordinate analysis of beta diversity analysis. $n = 3$ animals per group. NPS = no pup separation; PS15 = pup separation for 15 minutes; PS180 = pup separation for 180 minutes.

PS180+CRS dams ($F = 12.27$, $p = 0.008$; post hoc comparison of NPS+CRS and PS15+CRS, $p = 0.027$; post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.008$; Figure 7E). Although *Oscillospira* abundance was significantly higher in PS180+CRS dams than in NPS+CRS dams ($F = 7.015$, $p = 0.027$; post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.023$; Figure 7F), *Alistipes* abundance was significantly higher in PS180+CRS dams than in both NPS+CRS and PS15+CRS dams ($F = 12.19$, $p = 0.008$; post hoc comparison of NPS+CRS and PS180+CRS, $p = 0.007$; post hoc comparison of PS15+CRS and PS180+CRS, $p = 0.037$; Figure 7G).

In the analysis of species abundance clustering, it was clear that the main microbiota differed among dams with different PS protocols, with *Akkermansia* dominating in the cecal microbiota of dams that underwent brief PS (Figure 8).

Relativity analysis of gut microbiota composition and hippocampal neuroinflammation and neuroplasticity

To explore the relations between microbiota composition and levels of some biomarkers of hippocampal neuroinflammation and neuroplasticity, we selected the first 20 bacteria at the family and genus levels in the compositional analysis for further correlation analysis. We performed correlation analysis and false discovery rate multiple-comparison correction for the expression levels of proteins in the intestinal microbiota and inflammatory and plasticity molecules. The results showed that among CRS dams that underwent different PS protocols, hippocampal levels of Iba1 (Actinobacteria: $r = 0.758$, $p = 0.018$; Proteobacteria: $r = -0.794$, $p = 0.011$), CRMP2 (Proteobacteria:

Fig. 7: Analysis of cecal microbiota composition in dams subjected to various pup separation protocols with chronic restraint stress. (A and B) Microbial distribution at the phylum level. (C) Microbial distribution at the family level. (D–H) Microbial distribution at the genus level. Each bar represents the mean ± standard error of the mean, with $n = 3$ animals per group. All 3 groups were analyzed using analysis of variance followed by a post hoc analysis (*$p < 0.05$, **$p < 0.01$). NPS = no pup separation; PS15 = pup separation for 15 minutes; PS180 = pup separation for 180 minutes.

$r = 0.735$, $p = 0.024$; Deferribacteres: $r = 0.892$, $p = 0.001$) and α-tubulin (Deferribacteres: $r = 0.813$, $p = 0.008$) were correlated with the relative abundance of some microbes of the gut microbiota at the phylum level of microbial distribution (Appendix 1, Supplemental Figure 2). Results at the family (Appendix 1, Supplemental Figure 3) and genus (Appendix 1, Supplemental Figure 4) levels of microbial distribution further supported the relations between gut microbiota composition and the activation of neuroinflammation and neuroplasticity impairment in the hippocampus.
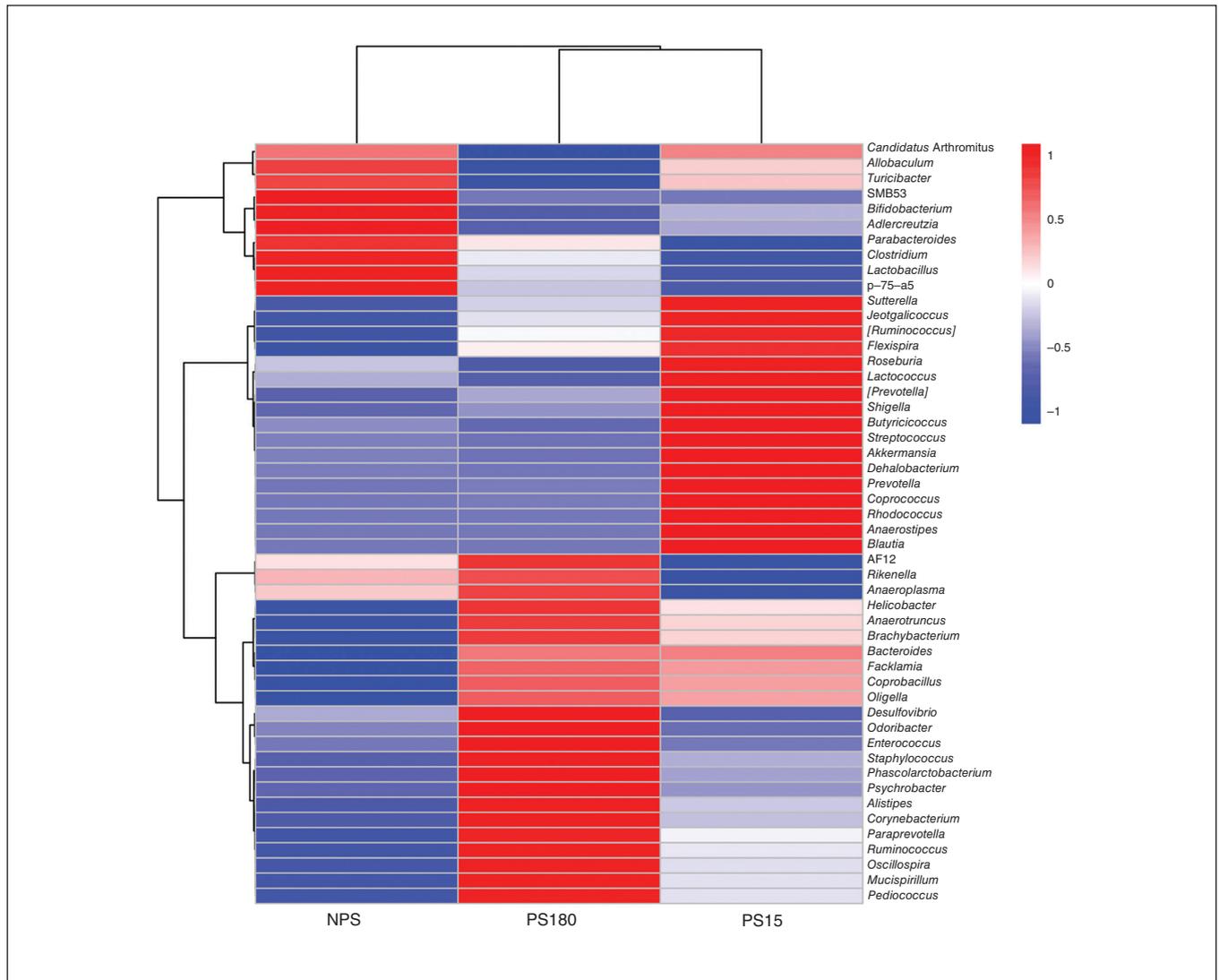
Fig. 8: Analysis of species abundance clustering in a heat map. The *y* axis shows species annotation information, the *x* axis represents sample information, and the clustering tree on the left of the figure is a species clustering tree. The cluster tree above is the cluster tree between the sample groups. *n* = 3 animals per group. NPS = no pup separation; PS15 = pup separation for 15 minutes; PS180 = pup separation for 180 minutes.

Discussion

In this study, we explored the effect of various PS protocols during lactation on dams with CRS-induced anxiety- and depression-like behaviours, along with potential mechanisms of neuroinflammation, neuroplasticity and changes in the gut microbiota. We first determined that CRS induced anxiety- and depression-like behaviours in dams that did not undergo the PS procedure, with these behaviours being associated with microglial activation; upregulated levels of expression of NLRP3, caspase-1, IL-18 and IL-1β; and downregulated levels of expression of CRMP2 and α-tubulin. However, the brief PS procedure promoted resilience to the behavioural deficits induced by CRS in dams. Unlike the situation in NPS+CRS dams, microglial activation and increased expression of some biomarkers of neuroinflammation (including NLRP3, caspase-1, IL-18 and IL-1β) were inhibited in PS15+CRS dams, and brief PS increased the expression levels of CRMP2 and α-tubulin. More importantly, the different PS protocols induced significant changes in the composition of the microbiota in dams.

Application of CRS is a widely used model in rodents and has been shown to induce depressive behaviour in male and nonparous female animals.[29,30] We explored the behavioural responsiveness of dams to CRS using the OFT, EPM test and TST. The OFT is widely used to evaluate locomotor activity and anxiety- and depression-like behaviours in rodents. Animals naturally prefer dark areas; as such, decreased locomotion and thigmotaxis to the brightly lit open areas and staying close to the walls of the field represent increased

anxiety- and depression-like behaviours.[31] A rodent with an aversion to open space will stay longer in the closed arm. The EPM test is based on the same phenomenon and is widely used to assess anxiety-like behaviours.[32] Immobility during the TST is due to an inability or a reluctance to exert effort, which indicates despair in rodents and is often used to assess depression-like behaviours.[33] In our study, time spent in the centre or open arms and the frequency of entering the centre or open arms during the OFT and EPM test, respectively, were significantly lower, whereas immobility in the TST was greater, in dams exposed to CRS. These results indicate significant behavioural deficits in the NPS dams that underwent CRS. Recent studies have documented anxiety- and depression-like behaviours in rodents induced by CRS,[29,30] consistent with our results.

The application of PS involves the same procedure as maternal separation, but it has different priorities. Previous studies focused on the effects of maternal separation on off-spring.[34,35] Given the CRS-induced behavioural deficits observed in NPS dams, our study further explored the effect of various postpartum PS procedures on the stress response behaviours of dams following CRS applied after weaning. In previous studies, long-duration PS altered hormone levels and activation of the hypothalamic–pituitary–adrenal axis and potentiated depression-like states in female rats;[36] prolonged PS also impaired both short- and long-term memory and increased anxiety-like behaviours in rat dams,[37] damaging their postpartum mental health. However, those previous studies did not use a second stressor after the various PS protocols. In our study, we subjected dams to secondary stress after exposure to PS. We observed that mice experiencing a long duration of PS and those that did not experience any PS both exhibited lower presence in the centre or open arms and lower frequency of entering the centre or open arms during the OFT and EPM test, respectively, and increased immobility time during the TST, all of which suggest significant anxiety- and depression-like behaviours, but compared with NPS dams, the degree of anxiety- and depression-like symptoms in dams after a long duration of PS was not elevated. This finding indicates that a long duration of PS during lactation may increase the sensitivity of dams to additional stress but may not affect the degree of anxiety and depression.

Notably, in our study, the PS15 dams, which were exposed to CRS after weaning, showed stress resilience behaviours. Specifically, PS15+CRS dams exhibited more time in the centre or open arms and higher frequency of entering the centre or open arms in the OFT and EPM test, respectively, and lower immobility time during the TST than the PS180+CRS dams and NPS+CRS dams, which indicated a resilience phenomenon. In recent years, the effect of resilience in preventing depression has attracted increasing attention.[38] In our previous study,[39] dams that underwent brief PS showed resistance to the emergence of anxiety- and depression-like behaviours caused by injection of lipopolysaccharide, which is consistent with the current results. In contrast to the significant decrease in interactions with offspring, such as pup retrieval and pup licking,[37,40] observed in dams subjected to PS of long duration, many studies have shown a significant increase in the amount of interaction between dams and pups after brief PS,[41] and this positive interaction improved the postpartum mental health of mothers during lactation.[42] This finding suggests that a brief interruption of mother–infant contact is beneficial for mothers' postpartum mental health during lactation and provides a new concept for the prevention of depression. However, there are few studies on the mechanism of stress resilience caused by brief PS.

Neuroinflammation is involved in the pathology of stress-induced depressive behaviours.[43–45] Application of CRS has been shown to induce persistent low-grade inflammation in rodents,[46] and many studies have found that these effects of CRS can be blocked by anti-inflammatory and antidepressant drugs.[47,48] In the current study, we found that CRS significantly upregulated microglial activation and the expression of NLRP3, caspase-1, IL-1β and IL-18 in the hippocampus of dams that did not undergo PS, which indicates that hippocampal neuroinflammation was increased by CRS. Microglia are key immune cells in the brain. Microglial activation can accelerate the release of some inflammatory factors, including IL-18 and IL-1β, which play neurotoxic roles in depression.[8] The compound NLRP3 is an intracellular multiprotein complex that links the perception of danger to the proteolytic activation of proinflammatory cytokines.[49] When NLRP3 is activated, it activates caspase-1 and promotes the conversion of pro-IL-1β into bioactive IL-1β, which in turn causes an inflammatory response.[50] Caspase-1 can increase the secretion of IL-1β and IL-18, produce corresponding mature cytokines and aggravate a series of inflammatory reactions.[51] The interleukin IL-18 is a member of the cytokine interleukin family and is widely expressed in activated microglia, astrocytes and neuronal cells. Some studies have shown that the level of IL-18 is significantly increased in patients with depression.[52] The NLRP3 inflammatory body cleaves precursors into mature IL-18 through increased caspase-1 activity, thus activating downstream transduction pathways to produce some inflammatory factors, which attack the brain. In addition, the increase in IL-18 release caused by NLRP3 activation is closely related to microglial activation.[53]

The activation of neuroinflammation and the release of some inflammatory factors can aggravate brain injury, especially through their effects on neuroplasticity. Our study showed significant downregulation of CRMP2 and α-tubulin in CRS dams, which represented damage to neuroplasticity in these animals. α-Tubulin is one of the main components of the microtubule system, and CRMP2 interacts directly with α-tubulin to modulate its function.[54,55] The protein CRMP2 is also related to the mechanisms of repair and regeneration that occur in adult brain neurons.[56] Our previous studies confirmed that CRMP2 was involved in the chronic unpredictable mild stress animal model, accompanied by differential regulation of DNA methylation at the CRMP2 promoter region.[57] This protein also interacts with α-tubulin, and regulation of CRMP2 activity influences neuronal plasticity and neurite length,[58] which further shows that CRMP2 affects microtubule plasticity. Phosphorylation of CRMP2 reduces its rate of binding to α-tubulin[59,60] and affects the polymerization or depolymerization of microtubule dimers in axonal growth cones.

In this study, we found that brief PS inhibited the activation of hippocampal neuroinflammation and neuroplasticity impairment in dams exposed to CRS after weaning. Specifically, microglial activation and the expression of some inflammatory cytokines, including NLRP3, caspase-1, IL-18 and IL-1β, in the hippocampus were decreased in PS15+CRS dams, and the PS15 protocol also increased the expression of CRMP2 and α-tubulin. These findings suggest a possible neurobiologic mechanism for resilience to stress exposure after brief PS during lactation. The mechanism of this resilience may be closely related to the neuroimmune hypothesis regarding PS-associated adversity.[61] A hypothesis that involves the effects of neuroinflammation on vulnerability to depression has been gradually accepted by researchers. The functional heterogeneity of microglia provides biologic support for the phenotypic difference in maternal behaviours. Microglia can remove harmful substances, improve neuroplasticity in the brain[62] and produce antidepressant effects by regulating autophagy.[63] A prospective study showed that long-chain n-3 polyunsaturated fatty acids reduced the risk of depression in puerperal women,[64] and further research showed that long-chain n-3 polyunsaturated fatty acids effectively inhibit neuroinflammation by suppressing microglial activation.[65]

A gene–environment interaction model for depression[66] indicates that genetic susceptibility may play an important role in the pathogenesis of depression, mainly by regulating inflammation in the brain. A recent study[67] showed that PS alters transcriptomic patterns, potentially resulting in neurobiologic changes; these changes have not yet been shown to lead to differences in behaviour but may provide conditions for various responses to repeated stress. Lactating rodents are sensitive to stress.[68] After dams are subjected to mild stress, such as brief PS, timely compensation enables the animals to better adapt to subsequent stress, thus promoting their postpartum mental health. These findings may represent the mechanism by which separation from pups changes the activity of the inflammasome in dams exposed to various PS protocols.

Neuroinflammatory factors can affect morphology and microtubule plasticity. However, in this study, the brief PS protocol administered during the lactation period inhibited neuroplastic injury in the hippocampus in dams that experienced CRS after weaning. Neuroprotection mediated by CRMP2 is associated with resilience. Deletion of this compound from the brain has been previously shown to affect locomotor activity in mice.[69] Brain-specific CRMP2 deletion leads to developmental deficits in neurons; enlargement of the ventricles; and impairment of social behaviour, locomotor activity, learning and memory.[70] A previous study confirmed that CRMP2-mediated neuroprotection and microtubule dynamics are increased in a resilience model of acute depression-like behaviours.[71] Some studies[72] have suggested a pathway that may interlink the activity of the serotonin 4 receptor (5-HT4R) with CRMP2 expression and dephosphorylation through elevated mRNA expression of brain-derived neurotrophic factor (BDNF) via 5-HT4R. Upregulated expression of neurotrophic factors, including BDNF, inhibits the phosphorylation of CRMP2 protein and increases CRMP2 levels, which promotes the growth of axons and dendrites.

By assessing neuroinflammation and neuroplasticity, we further studied changes in the gut microbiota of dams subjected to various PS protocols and CRS to explore the underlying mechanism of resilience. Intriguingly, we found a lower level of Actinobacteria and a higher level of Proteobacteria in dams subjected to long PS, and the relative abundance of *Bifidobacterium* was also lower in dams subjected to long PS. In previous studies, *Bifidobacterium* treatment prevented depression-like behaviour in mice; however, in our study, *Bifidobacterium* was lower in dams subjected to PS, a potential example of PS having an effect, but not the effects of different durations of PS on chronic stress during the postpartum period.[73] In clinical research, the abundance ratio of Actinobacteria to Proteobacteria was significantly decreased in patients with major depression,[74] and a decrease in *Bifidobacterium* abundance was commonly observed in patients with depression.[75] In our study, Actinobacteria levels were lower and Proteobacteria levels were higher in dams subjected to PS (relative to animals with no PS) because our study on gut microbiota did not include dams not subjected to CRS, and the results may be a potential effect of PS itself. In addition, in this study, abundances of Erysipelotrichaceae, *Akkermansia* and *Allobaculum* showed significant differences among dams that underwent different PS procedures. Erysipelotrichaceae organisms have been found to play an important role in promoting the prevention of diseases, such as diabetic nephropathy.[76] *Akkermansia* plays a beneficial role in glucose metabolism,[77,78] and *Allobaculum* is harmful to glucose and lipid metabolism and promotes the occurrence of diabetes.[79] However, the changes in Erysipelotrichaceae, *Akkermansia* and *Allobaculum* abundance under different PS processes and how they affect the occurrence of mental illness need further research.

A complex bidirectional reflex network between the central nervous system and the gastrointestinal tract has been confirmed. Signals from the gastrointestinal tract are transmitted to the brain through the neural network, affecting the emotional state of the brain and its ability to respond to stress. In turn, regulation of the brain can cause changes in gastrointestinal movement, secretion and immune function.[80] The relativity analysis in this study indicated that inflammasome disorder and neuroplasticity may play important roles in regulating the relation between stress and gut microbiota composition. Recent studies of *Akkermansia* have shown obvious beneficial effects in terms of resisting intestinal inflammation and repairing damage to intestinal barriers.[81,82] The heat map of microbial distribution in our study clearly showed that *Akkermansia* dominated in the cecal microbiota of mice subjected to brief PS, which provides the possibility of resistance to inflammatory activation. The abundance of *Alistipes* has also been found to be significantly increased in patients with inflammatory bowel disease.[83] In our experiment, *Alistipes* abundance in dams subjected to long PS was significantly increased, which further suggests the role of the microbiota in intestinal inflammation. Recently, some animal

studies have found that this 2-way communication along the microbiota–gut–brain axis can be mediated by the immune system in pups subjected to maternal separation.[84–86] In addition, the tight connection between the NLRP3 pathway and the gut microbiota has been demonstrated, and using anti-inflammatory drugs can change the variety and abundance of beneficial bacteria to prevent and treat depression.[87,88]

However, it was surprising that the dams subjected to long PS (PS180 group) did not differ from the controls in any measurement other than microbiota composition. Previous studies have shown that changes in the intestinal microbiota are closely related to activation of the intestinal immune system caused by changes in multiorgan metabolic inflammation.[89] In this study, the composition of intestinal microbiota in dams that underwent long PS (in contrast to those that did not undergo PS) changed after re-exposure to stress, which suggested that the degree of activation of the intestinal immune system may have differed between dams that underwent long PS and those that did not undergo PS. In this study, the measurement of immune indices mainly focused on the hippocampal tissue of the brain, and an analysis of the activation of hippocampal immunity showed no difference between the dams that underwent long PS and those that did not undergo PS. This finding indicates the likelihood that other mechanisms occurring in the brain prevented over-activation of brain immunity; the underlying mechanisms should be studied in the future.

Limitations

This study had one main limitation. The sample size for analysis of gut microbiota was small, which may have caused false-positive results. Therefore, future studies with larger sample sizes should be conducted for further exploration.

Conclusion

This study showed that CRS induced anxiety- and depression-like behaviours in dams that remained with their pups during lactation. More specifically, CRS activated hippocampal microglia, increased the expression levels of inflammatory cytokines, including NLRP3, caspase-1, IL-1β and IL-18, and damaged hippocampal neuroplasticity in dams. However, brief PS reversed the CRS-induced anxiety- and depression-like behaviours in dams, which indicates resilience. Activation of neuroinflammation and neuroplasticity impairment in the hippocampus were inhibited in dams subjected to brief PS and CRS. In addition, taxonomic shifts in the cecal microbiota were observed in dams subjected to long PS and CRS. Interestingly, we observed relations between gut microbiota composition and some biomarkers of hippocampal neuroinflammation and neuroplasticity in dams exposed to different PS protocols. Our study provides further evidence for the beneficial effect of brief PS and the potential microbiota–gut–brain axis mechanism. Intestinal microbiologic intervention in mothers experiencing long PS may be a new strategy for preventing postpartum depression.

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