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Research report

The effect of chronic stress on behaviors, inflammation and lymphocyte subtypes in male and female rats

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ABSTRACT

Excessively released proinflammatory mediators from activated macrophages and lymphocytes may contribute to the etiology of depression. However, the relationship between lymphocytes and depression is not fully understood. Although women have higher depression risk than men, sex/gender differences in psychoneuroimmunological mechanisms are still unclear. To explore these two questions, chronic unpredictable mild stress (CUMS) was used to evaluate the changes in behaviors, inflammation and lymphocyte subtypes in adult male and female Wistar rats. Results show that CUMS increased anhedonia and anxiety-like behaviors, along with increased serum corticosterone, hippocampal pro-inflammatory factors, CD11b, IFN-γ, IL-6 and IL-17, but decreased CD4, CD25, CD4/CD8 ratio, GFAP, 5-hydroxytryptamine (5-HT) and NE concentrations, regardless of sex. There was no positive correlation between sucrose preference and blood CD4/CD8 ratio, but a positive correlation between sucrose preference and spleen CD25, sucrose preference and neurotransmitters (NE and 5-HT), spleen CD25 and serum TGF-β1/IL-6 ratio were found, regardless of sex. Females presented higher basal locomotion, blood CD4, CD4/CD8 ratio, serum corticosteroid and IL-6 concentrations, but lower hippocampal norepinephrine (NE) than males. Although CUMS didn't induce significant sex differences, females presented more changes in CD4 and CD8 lymphocytes than male rats. CUMS caused abnormalities in corticosteroid, lymphocytes, cytokines and neurotransmitters, which might be the precursors for inducing depression-like behaviors in both sexes.

1. Introduction

Epidemiological studies have reported that women are more vulnerable to stress and it's effects on mental health compared to men [1], and are twice as likely as men to suffer from depression [2]. Given the apparent sex difference in depression, it is unfortunate that most pre-clinical animal experiments have been carried out on male rodents [3]. Thus, it is important to explore sex/gender differences in depression, which may provide better understanding and treatment for this disease in males and females.

Chronic stress is often considered as an inducer of depression [4,5]. Consistent with the hypothalamic-pituitary-adrenal (HPA) axis and macrophage/T-lymphocyte hypothesis of depression, chronic stress damages the feedback of HPA axis and induces an abnormal increase of corticosteroid, which desensitizes glucocorticoid receptors located on lymphocytes and causes lymphocyte dysfunction [6,7]. In the past three decades, growing evidence indicates that activated macrophages and T lymphocytes-induced inflammatory responses may trigger the onset and development of depression [6,8] (Fig. 1). Meta-analyses indicate clinical depression is associated with significant alterations in toxic CD8 cells, helper CD4 and related subtypes, such as pro-inflammatory Th1 and Th17, as well as anti-inflammatory Th2 and Treg [9–11]. Chronic stress activates peripheral lymphocyte subtypes and central microglia to continuously secrete pro-inflammatory Th1/M1 cytokines interferon-γ (IFN-γ) and reduce anti-inflammatory Th2/M2 cytokines IL-4 [12,13]. Furthermore, CD25 Treg lymphocytes derived from CD4 cells can

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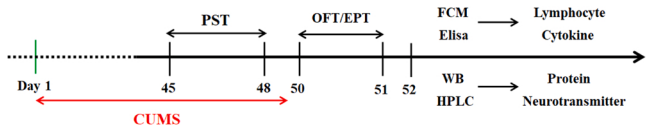

Fig. 1. Timeline of the study. CUMS: chronic unpredictable mild stress, PST: sucrose preference test, OFT: open-field test, EPT: elevated plus maze test, FCM: flow cytometry, WB: Western blots, HPLC: high performance liquid chromatography.

suppress the development of pro-inflammatory factors and are beneficial to the treatment of major depression [14]. More importantly, in clinical trials, decreased pro-inflammatory IL-1β and IL-6 cytokines, and increased CD25 Tregs and CD4/CD8 ratio are markers of major depression recovery [15,16]. However, some researchers report that chronic stress fails to induce the changes in blood CD4 and CD8 in female mice [17], whereas a reduction of spleen CD4 lymphocyte, and some increases in serum IL-1β and IL-6, as well as hippocampal IL-6 and IL-4, are found in male mice [18]. The fact that women are more likely to suffer from depression than men may be related to their greater sensitivity to stress and inflammatory stimuli [19,20]. However, chronic stress-induced changes in lymphocyte subtypes, as well as the relationship between lymphocytes and inflammatory factors in different sexes are still unclear.

In animal models, chronic unpredictable mild stress (CUMS) is used as a valid model of depression [4,5]. CUMS can directly activate M1-type microglia to produce pro-inflammatory mediators [21], which impairs astrocyte and neuronal function, thereby reducing neurotrophin levels, monoamine neurotransmitter concentration and neurogenesis in the hippocampus, a key brain region implicated in mood and cognition [22,23]. Furthermore, astrocytes can present neuroprotective and neuroinflammatory functions which are dependent on the microenvironment and polarizations of M1- and M2-type microglia [24]. For example, pro-inflammatory factors down-regulate the astrocyte marker glial fibrillary acidic protein (GFAP) and neurotrophin (BDNF) expression, and this can be reversed with TNF-α inhibitor infliximab treatment [25]. Our previous studies, in addition to those of others, have also demonstrated that the M1-type microglia inhibitor, minocycline, can reverse CUMS-induced depression-like behaviors by balancing the pro- and anti-inflammatory response of microglia, and subsequently restoring astrocyte neurotrophic and neurotransmitter function [12,26]. Moreover, when compared to males, females were more vulnerable to inflammation-induced mood and behavioral changes [27], with more decreased hippocampal serotonergic activity after CUMS [28].

Even though CUMS induces some changes in lymphocyte subtypes, glia phenotypes, inflammation and neurotransmitter function, the sex/gender differences in these changes, the relationship among behaviors, lymphocyte subtypes, inflammation and neurotransmitters in depression still remain unclear. Moreover, the hippocampus, controlling cognition and emotion, is a highly stress-sensitive brain region, which regulates behavioral and neuroendocrine responses in depression [29,30]. Therefore, the current study used a CUMS model of depression for 49 days to determine if there are differences between male and female rats in depression-like behaviors, blood and splenic lymphocytes, serum and hippocampal inflammatory levels, and hippocampal glia cells and neurotransmitter's function. It was expected the sex would play a significant role in CUMS effects on behaviors, inflammatory measures and lymphocytes.

2. Materials and methods

2.1. Animals

Forty adult male and female Wistar rats (8 weeks of age, Changsha Tianqin Biotechnology Co., Ltd) were housed two per cage with a 12 h light/dark cycle (lights on at 7:00 and off at 19:00) at the Guangdong Ocean University animal facility (SYXK (Yue) 2014–0053). The room temperature was set at 23 ± 1 ℃, humidity 50 ± 10%. Animals had standard rodent chow and water available ad libitum. Prior to the test procedure, rats were acclimated to the laboratory for seven days. The experiments were conducted in a blinded fashion and were approved by the Institutional Bioethics Committee (Guangdong Ocean University, China, IACUC-20170325–012). The experimental procedures were conducted in full compliance with the National Institutes of Health Guide for the Care and Use of the Laboratory Animals.

Table 1
Summary of the CUMS protocol.

Stressors	Experimental days
Cold exposure at 4 °C for 1 h	2, 8, 22, 30, 37, 44, 49
Swim stress at 18 °C for 10 min	3, 10, 20, 26, 34, 41, 46
Restraint for 1 h	1, 9, 19, 27, 33, 45, 49
Light overnight exposure	1, 11, 18, 28, 35, 40, 48
Mouse cage overnight exposure	5, 11, 14, 19, 22, 35, 41
Isolation overnight	5, 20, 24, 29, 38, 43, 48,
Cage tilting at 45° overnight	6, 13, 16, 26, 29, 36, 44
Stroboscopic light overnight 1/s	2, 10, 15, 25, 33, 37, 42
Crowding overnight (five rats per cage)	7, 13, 18, 21, 27, 34, 42
Mouse odor overnight exposure	6, 12, 17, 25, 32, 40, 46
Food deprivation overnight	4, 8, 21, 28, 31, 39, 47
Water deprivation overnight	3, 9, 16, 24, 30, 38, 47
Light off at the 3-h cycle	7, 14, 17, 23, 32, 39, 45
Wet bedding overnight	4, 12, 15, 23, 31, 36, 43

2.2. Experimental procedure

Twenty male and twenty female rats were divided equally into 4 experimental groups (n = 10 in each group) as a) male control (Male + CT), b) male stress (Male + CUMS), c) female control (Female + CT), d) female stress (Female + CUMS). Fig. 1 summarizes the general study design. Briefly, stress group rats were subjected to CUMS for 49 days and control group rats no stress. During day 45–48, the sucrose preference test (SPT) was performed. During days 50–51 the open-field test (OFT) and elevated plus maze test (EPT) were conducted at 7:30–13:30. Rats were euthanized after one day's rest.

2.3. Stress exposure paradigms

The CUMS protocol was carried out as previously described [12]. Briefly, 14 different stressors were applied (2 stressors per day) in a random order over a period of 49 days (Table 1).

2.4. Sucrose preference test

Anhedonia behavior was measured by the sucrose preference test as previously described [31]. On day 1, rats were offered two bottles of 1% (w/v) sucrose to consume at 19:00 for 24 h. On day 2, rats were offered one bottle of 1% sucrose and one bottle of fresh water at 19:00 for 24 h. On day 3, food and water deprivation occurred at 19:00 for 24 h. On day 4, rats were offered one bottle of 1% sucrose and one bottle of fresh water at 19:00, and then the consumption of sucrose and fresh water after 1 h was weighed. Sucrose preference (SP) was calculated according to the following formula, SP = sucrose intake / (sucrose intake + water intake) × 100%.

2.5. Elevated plus maze test

Anxiety-related behavior was measured with the elevated plus maze test as described previously [31]. Briefly, rats were placed on the central platform facing the same open arm and anxiety-related behaviors were recorded by SuperMaze behavior analysis system (Shanghai Xinruan Information Technology Co., Ltd, Shanghai, China) for 5 min by two

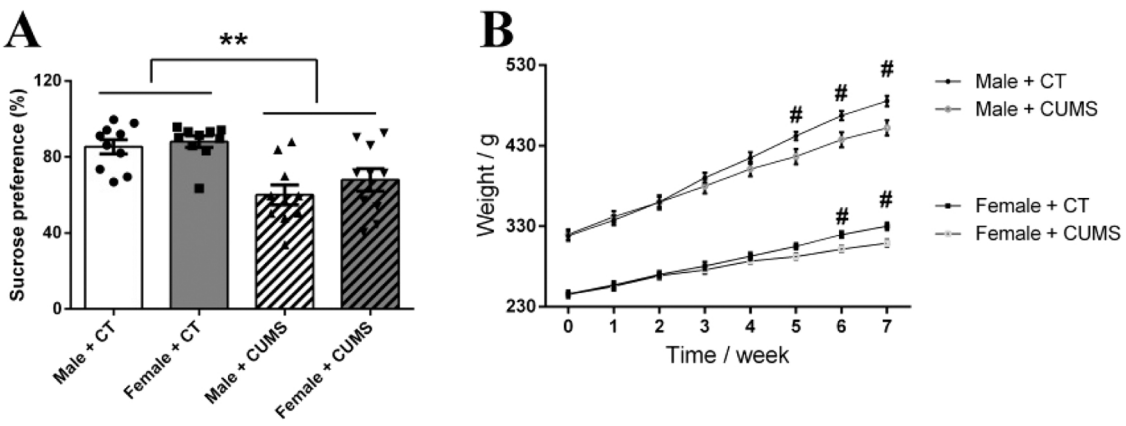

Fig. 2. CUMS decreased weight and sucrose consumption in both sexes. A) Sucrose preference. B) Body weight over seven weeks. All data are expressed as the mean ± SEM (N = 10 per group). $^{**}P < 0.01$ CUMS main effect; *post-hoc test*: $^{\#}P < 0.05$ CUMS vs. control.

highly trained observers blinded to the groups. Before the test, rats were acclimatized to the room for 1 h. After the test, the arena was cleaned with 0.5% EtOH. The number of open and closed arms entries and the time in open and closed arms, as well the number and time ratio of close/open, were quantified.

2.6. Open field test

Exploration and anxiety-related behaviors were assessed with the open-field test as described previously [32]. Briefly, a 60 W white light bulb was positioned 100 cm above the center of the cylindrical apparatus with white inner wall (diameter 1 m, height 40 cm). A rat was placed with it's head facing towards the same wall of the apparatus and the behavioral activity was recorded using the SuperMaze behavior analysis system (Shanghai Xinruan Information Technology Co., Ltd, Shanghai, China) for 5 min by two highly trained observers blinded to the groups. Before the test, rats were acclimatized to the room for 1 h. After the test the arena was cleaned with 0.5% EtOH. The number of entries into the center and the time spent in the central zone, as well as total locomotion in the open-field box, were recorded.

2.7. Collection of samples and assays

One day after behavioral testing, the animals were rapidly decapitated. Immediately after decapitation, blood samples from the trunk (200 μL per rat) were into 500 μL heparin anticoagulant tubes (MB1908, Meilunbio, China) for flow cytometry. Serum samples were centrifuged at 1800 rpm for 10 min. Serum was collected and stored at − 80 °C. Following brain extraction, hippocampus were quickly dissected on ice, frozen in liquid nitrogen, and stored at − 80 ℃ until whole hippocampus homogenization for subsequent ELISA and Western blot assays. The commercial rat ELISA kits were used to determine cytokines IL-6 (YT-0190R1), TGF-β1 (YT-0181R1) (Jiangsu Yu Tong Biotechnology Co., Ltd, China) and CORT (DG-70084D), IL-17 (DG-20148D), IFN-γ (DG-90958D), IL-10 (DG-70038D) (Beijing Dong Ge Biotechnology Co., Ltd, China) in both serum and hippocampal supernatant according to the manufacturer protocols.

2.8. Quantitation of T-lymphocyte subtypes with flow cytometry

The blood and spleen were harvested after the rats were euthanized. Lymphocytes were immediately extracted from the spleen by Lymphocyte Separation Medium (Tianjin Haoyang biological products Technology Co., Ltd, China). The single Lymphocyte suspension ($2 *10^6$/100 μL) and 100 μL fresh anticoagulant blood was surface stained with CD3-APC (1920424, eBioscience), CD4-FITC (4321795, eBioscience), CD8-PerCP/Cy5.5 (1998249, eBioscience) and CD25-PE (4330019, eBioscience) for 30 min at room temperature in the dark. 2 mL erythrocyte lysis buffer (Zhejiang Bozhen Biotechnology Co., Ltd, China) was added into blood samples to lyse erythrocytes. After washing with phosphate-buffered saline (PBS) buffer, the samples were detected and analyzed by CytoFLEX (Beckman, China).

2.9. Western blots

According to manufacturer's instructions, the total protein of the hippocampus was extracted with a commercial kit (BCA kit, Beijing dingguo changsheng Co., Ltd, China). Subsequently, 30–50 μg protein was loaded onto a 10% polyacrylamide gel, and then transferred to PVDF (Millipore, China) membrane. After incubation with western blot blocking solution reagent (Millipore, China) at room temperature for 2 h, membranes were incubated with primary antibody overnight at 4 ℃. After washing, the membranes were further incubated with secondary antibody for 2 h, then washed and detection was done using enhanced chemiluminescence (Millipore, China). The bands were scanned and analyzed using a chemiluminescence system (Tanon 5200, Shanghai, China). The antibodies for CD11b (ab75476, 160 kDa, 1:800) and BDNF (ab108319, 15 kDa, 1:2000) were purchased from Abcam Trading Company Ltd (Shanghai, China) and origin antibodies for GFAP (sc33673, 50 kDa, 1:400) and β-Actin (sc47778, 43 kDa, 1:400) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All target proteins were quantified by normalizing them to β-actin re-probed on the same membrane and then calculated as a percentage of the male control group.

2.10. . HPLC analysis of neurotransmitters and metabolites

Hippocampal tissues were homogenized in 0.60 M ice-cold perchloric acid containing 50 mM Na_2EDTA with 100 ng isoproterenol as an internal standard. The homogenates were centrifuged twice at 14,000 rpm/min for 15 min at 4 ℃. Supernatants were filtrated with 0.45 μm membrane and transferred to new tubes. The pH of supernatants was adjusted to 3.8 with 1 M sodium acetate, and then supernatants were stored at − 80 ℃ until use. For the HPLC analysis, 10 μL of the pH-adjusted supernatant was injected into an HPLC system with fluorescence detection (Agilent, Santa Clara, CA, USA). The neurotransmitters in the samples were separated by a C18 reverse-phase column (4.5 *150 mm) (Agilent, Santa Clara, CA, USA) with a mobile phase containing sodium acetate and citric acid. The mobile phase was prepared as follows: 0.1 M sodium acetate was mixed 0.1 M citric acid in a 10:9 ratio, and adjusted to PH 3.5 (0.1 M sodium citric buffer), mixed with methanol in a ratio of 85:15 and then supplemented with sodium octane sulfonate (100 mg/L), Na_2EDTA (5 mg/mL).

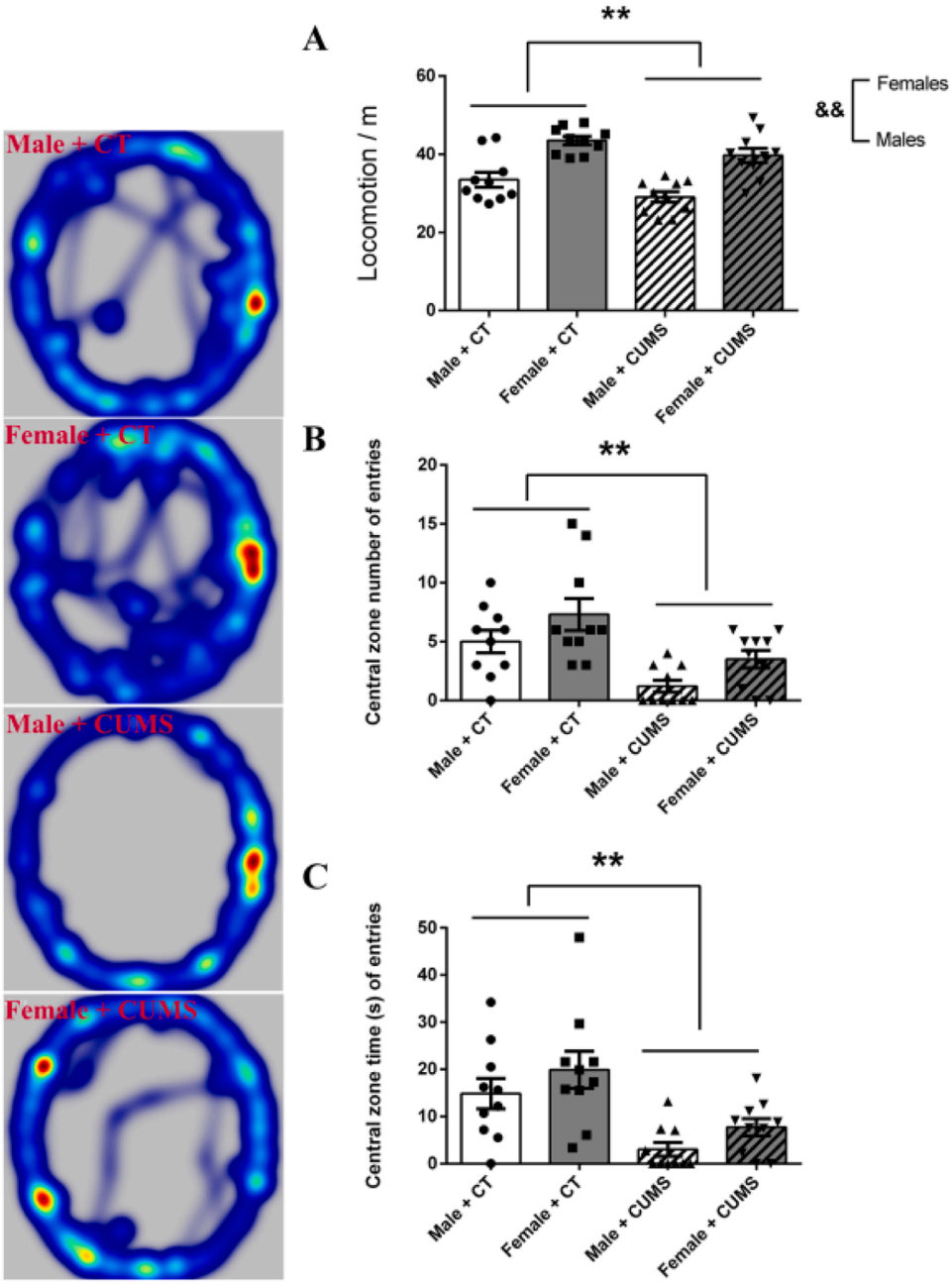

Fig. 3. The different behavioral changes of male and female rats in the open field test. A) The locomotion in the open field maze. B) The number of entries into the central zone. C) The time spent in the central zone. On the left is the track of rats in the open field box. All value expressed as the mean ± SEM (N = 10 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ CUMS main effect, $^{\&\&}P < 0.05$, $^{\&\&}P < 0.01$ sex main effect.

2.11. Statistical analysis

All data were expressed as mean ± SEM and analyzed by SPSS 20.0 software (IBM SPSS Statistics software, SPSS Inc. Chicago). Parametric data were measured by two-way analysis of variance (ANOVA, factors CUMS (stress/control) and sex (male/female)). Non-parametric data were analyzed using Kruskal-Wallis test, followed by a Bonferroni-corrected *post-hoc* Wilcoxon-Mann-Whitney U-test for significant data. Significance was set at $P < 0.05$ in all tests. A non-parametric Spearman rank correlation test was used to analyze linear correlations.

3. Results

3.1. CUMS induced anhedonia and decreased body weight, regardless of sex

There was a significant main effect of CUMS on sucrose consumption ($F_{1,36}$ =24.219, $P < 0.01$) with CUMS significantly reducing sucrose consumption in both male and female rats (Fig. 2A), regardless of sex. Repeated ANOVA showed a significant interaction between group and time (weeks) on body weight ($F_{7, 36} = 15.775$, $P < 0.01$). The *post-hoc test* revealed that CUMS significantly decreased the body weight after five weeks in both sexes (Fig. 2B).

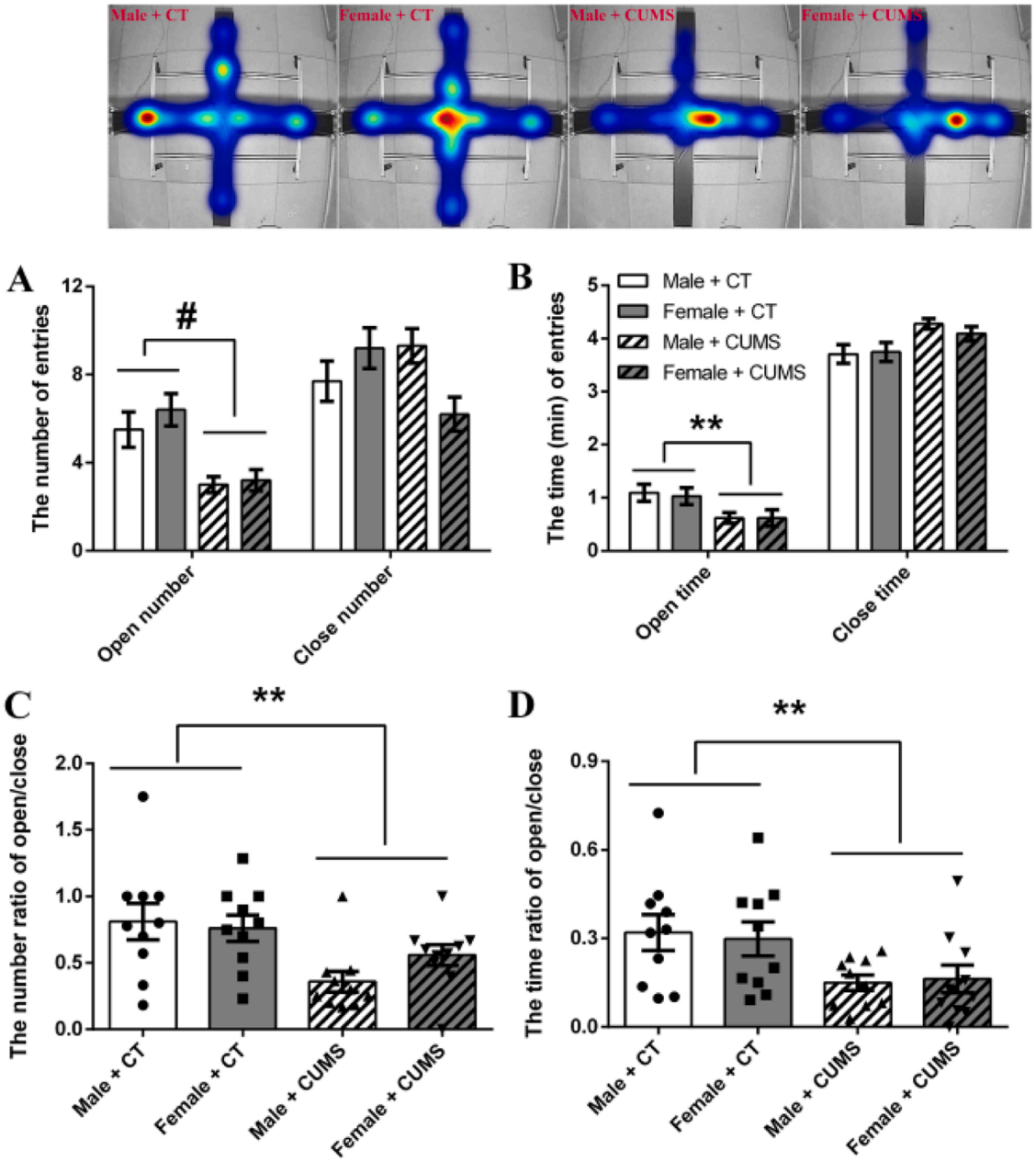

Fig. 4. The anxiety-like behaviors of male and female rats in the elevated plus maze. A) The number of entries into the open and close arms. B) The time spent in the open and close arms. C) The ratio of open to closed arm entries. D) The ratio of time spent in the open over the closed arm. On the top is the track of rats in the elevated plus maze. All values are expressed as the mean ± SEM (N = 10 per group). $^{**}P < 0.01$ CUMS main effect; *post-hoc test*: $^{\#}P < 0.05$ CUMS vs. control.

3.2. CUMS and sex effects on locomotion in the open field test

There were significant main effects of CUMS ($F_{1,36}$ =6.782, $P < 0.05$) and sex ($F_{1,36}$ =43.26, $P < 0.01$) on locomotion, but no interaction effect (Fig. 3A). CUMS significantly decreased locomotion in both sexes with females having increased locomotion compared to males. After CUMS, the locomotion was reduced by 13.1% in males and by 8.7% in females (Fig. 3A). Further, there was a significant main effect of CUMS on the number of central entries ($F_{1,36}$ =16.235, $P < 0.01$) and the time in the center of the test ($F_{1,36}$ =18.363, $P < 0.01$), regardless of sex and in the absence of an interaction effect (Fig. 3B, C). CUMS significantly decreased the number of entries and time spent in the central zone in both sexes.

3.3. CUMS significantly induced anxiety-like behavior, regardless of sex

There was a significant main effect of CUMS on the time in the open arm ($F_{1,36}$ =7.662, $P < 0.01$), the ratio of entries in open over closed arms($F_{1,36}$ =9.202, $P < 0.01$) and the ratio of time spent in the open over closed arms ($F_{1,36}$ =8.155, $P < 0.01$) (Fig. 4B, C, D). CUMS significantly decreased the time spent in the open arms, the number of entries into the open arm (H=11.842, $P < 0.01$) and the ratio of open to closed arm entries in both males and females.

3.4. CUMS and sex significantly affect serum corticosterone level

Kruskal-Wallis test indicated that there was a significant changes in serum corticosterone (CORT) concentration (H=17.463, $P < 0.01$). The post hoc *test* revealed serum corticosterone concentration at sacrifice was significantly higher in females than males in the control situation

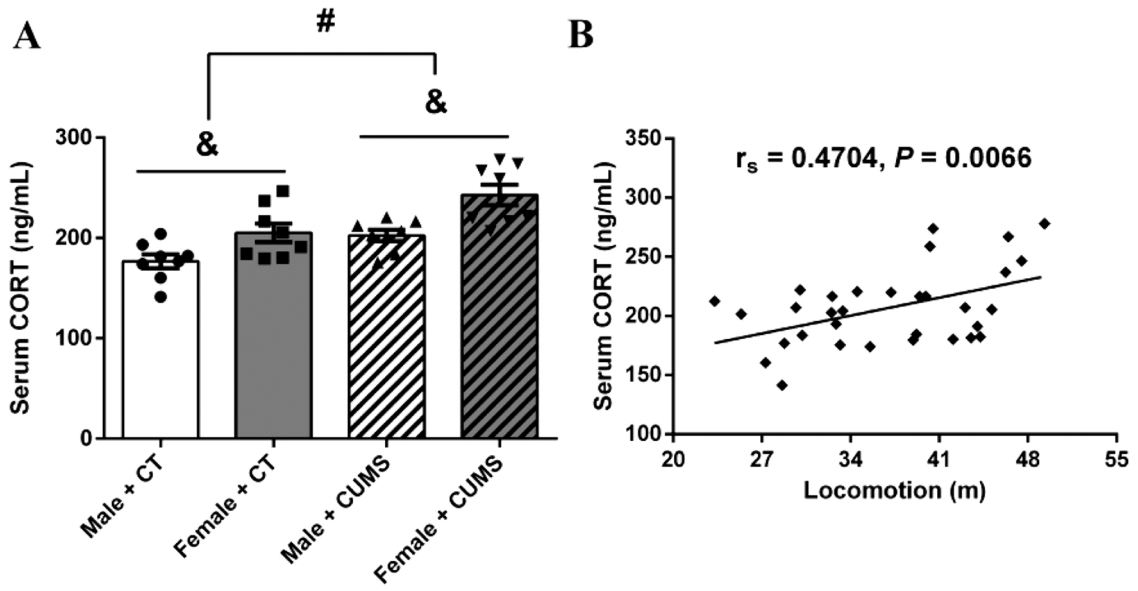

Fig. 5. CUMS increased serum CORT concentration both in females and males. A) Serum CORT concentration. B) Positive correlation between locomotion and serum CORT, sexes combined. All data are expressed as the mean ± SEM (N = 8 per group). *post-hoc test*: $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ females vs. males, $^{\#}P < 0.05$ CUMS vs. control.

($P < 0.05$). Stressed animals had a significant increase in serum concentration of CORT at the time of sacrifice, regardless of sex ($P < 0.05$). Compared to control animals of the same sex, CORT concentration at the time of sacrifice was increased by 14.5% in male and 18.3% in female rats exposed to CUMS (Fig. 5A). Moreover, the sex difference in locomotion behavior was reported by a previous study, in which increased locomotion was suppressed by corticosterone synthesis inhibitor metyrapone [33]. Hence, the correlation between corticosterone and locomotion was analyzed, and found a significant positive correlation between locomotion and serum CORT levels at sacrifice (r = 0.4704, $P < 0.01$), regardless of sex (Fig. 5B).

3.5. CUMS and sex effects on peripheral T-lymphocyte subtypes

There was a significant interaction effect between CUMS and sex in the percentage of lymphocyte CD4 ($F_{1,20}$ =5.983, $P < 0.05$), CD8 ($F_{1,20}$ =4.473, $P < 0.05$) and the ratio of CD4/CD8 ($F_{1,20}$ =6.143, $P < 0.05$) in the blood (Fig. 6A, B, D). The post hoc *tests* revealed that lower blood CD8% ($P < 0.05$) but higher CD4% ($P < 0.05$) and CD4/CD8 ratio ($P < 0.05$) were evident in female but not males in the control condition. Furthermore, stressed female rats showed a larger difference than stressed males when compared to control animals of the same sex. CD4 and CD4/CD8 ratio was decreased by 0.6% and 5.2% in male and 9.4% and 34.4% in female rats, but CD8 was increased by 2.8% in male and 36.2% in female rats after exposed to CUMS (Fig. 6B, D). There was also a significant main effect of CUMS, with stress significantly decreased spleen CD25 lymphocyte percentage in both sexes ($F_{1,20}$ =14.151, $P < 0.01$) (Fig. 6C). Moreover, there was a significant positive correlation between spleen CD25% and sucrose preference (r = 0.6723, $P < 0.01$) (Fig. 6F), but no correlations between blood CD4/8 ratio and sucrose preference (r = 0.342, $P = 0.1018$) (Fig. 6E), regardless of sex.

3.6. CUMS and sex effects on serum pro- and anti-inflammatory factors

CUMS significantly elevated the concentration of IL-6 ($F_{1,28}$ =5.575, $P < 0.05$) and IL-17 ($F_{1,28}$ =38.572, $P < 0.01$) and decreased IL-10 ($F_{1,28}$ =24.756, $P < 0.01$) and TGF-β1/IL-6 ratio ($F_{1,28}$ =22.577, $P < 0.01$) in serum, regardless of sex (Fig. 7A, B, D, E). There was a significant effect of sex on serum levels of IL-6 ($F_{1,28}$ =16.32, $P < 0.01$) with females showing higher serum IL-6 concentrations than males (Fig. 7B). After CUMS exposure, male IL-6 levels were increased 22.5% than control males, and female IL-6 levels were 20.3% higher than control females (Fig. 7B). Kruskal-Wallis tests indicated that there were significant effects in serum IFN-γ (H=12.815, $P < 0.01$) and IFN-γ/IL-10 ratio (H=19.647, $P < 0.01$). The post hoc test revealed that CUMS significantly increased serum IFN-γ ($P < 0.01$) and IFN-γ/IL-10 ratio ($P < 0.01$) in both sexes (Fig. 7A, C). Furthermore, this study found a positive correlation between CD25 and sucrose preference. A previous study has demonstrated that CD25 Treg lymphocytes was promoted by TGF-β, but inhibited by IL-6 [57]. In this case, there was a significant positive correlation between spleen CD25 and the ratio of TGF-β1/IL-6 in serum (r = 0.4596, $P < 0.05$) regardless of sex (Fig. 7F), which indicates a closed relationship between lymphocytes and inflammation in depression.

3.7. CUMS changed markers of microglia (CD11b) and astrocyte (GFAP) expression in the hippocampus, regardless of sex

CUMS significantly decreased the expression of hippocampal GFAP ($F_{1,20}$ =41.751, $P < 0.01$) in both sexes (Fig. 8B) and there was no main effect of sex or an interaction effect. Kruskal-Wallis test indicated that there was a significant changes in hippocampal microglia CD11b expression (H=12.847, $P < 0.01$). The post hoc test revealed CUMS significantly increased microglial protein expression of CD11b in both sexes (Fig. 8A).

3.8. CUMS resulted in an imbalance between hippocampal pro- and anti-inflammatory factors, regardless of sex

CUMS increased the concentrations of hippocampal IFN-γ ($F_{1,28}$ =15.163, $P < 0.01$) and IL-17 ($F_{1,28}$ =25.769, $P < 0.01$) as well IFN-γ/IL-10 ratio ($F_{1,28}$ =19.777, $P < 0.01$) regardless of sex (Fig. 9A, C, D). There were no other main effects or interactions. Kruskal-Wallis tests indicated that there were significant changes in concentrations of hippocampal IL-6 (H=23.395, $P < 0.01$) and TGF-β1 (H=23.178, $P < 0.01$). The post hoc test revealed that CUMS significantly increased the concentration of IL-6 while reducing TGF-β1 concentration in the hippocampus (Fig. 9B), regardless of sex.

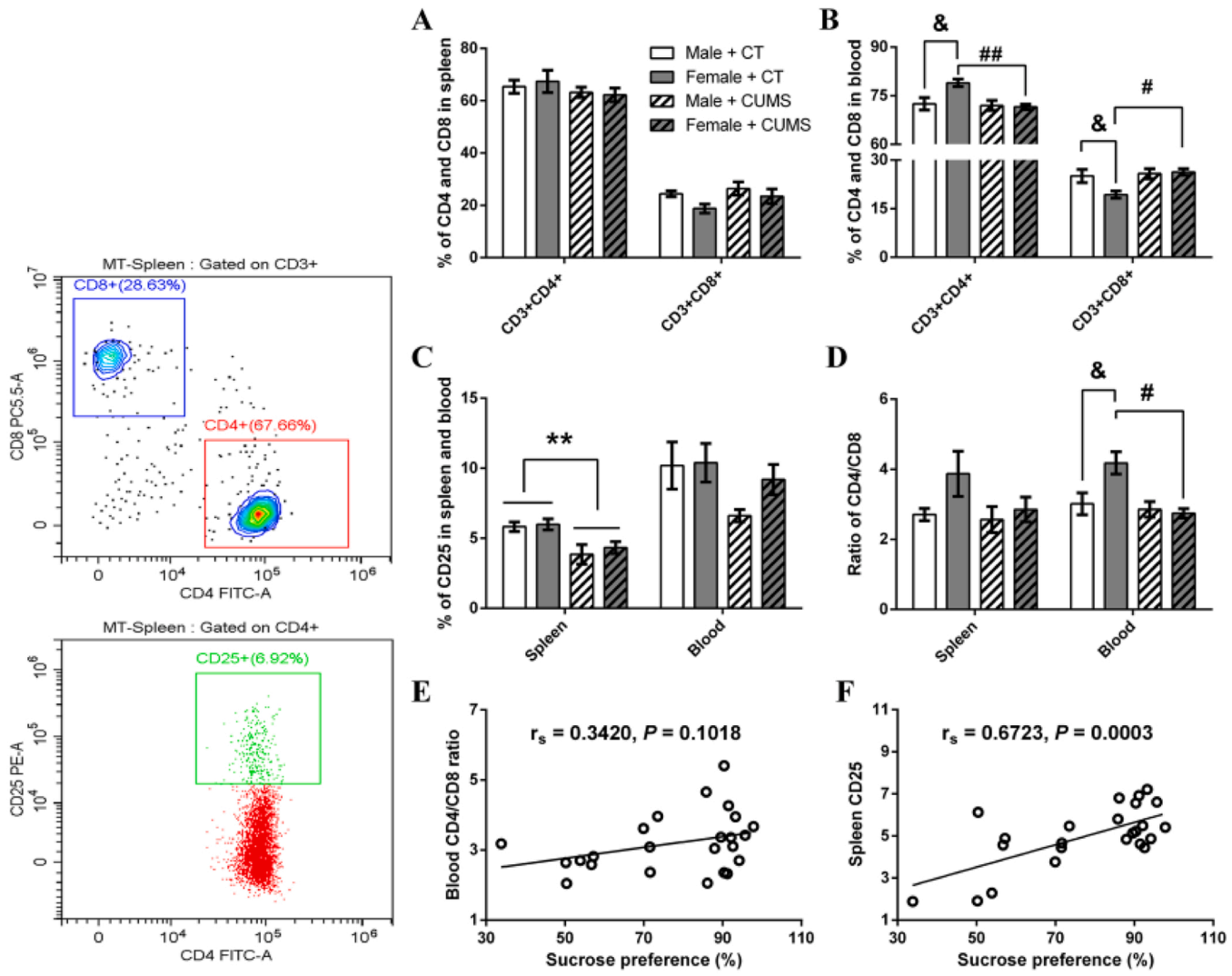

Fig. 6. Male and female lymphocyte subtypes in blood and spleen after CUMS. A) The percentage of CD4 and CD8 in spleen. B) The percentage of CD4 and CD8 in blood. C) The percentage of CD25 in spleen and blood. D) The ratio of CD4 and CD8 in spleen and blood. E) Correlation between sucrose preference and blood CD4/8 ratio, F) Correlation between spleen CD25 and sucrose preference, sexes combined. All data are expressed as the mean ± SEM (N = 6 per group). $^{**}P < 0.01$ CUMS main effect; *post-hoc test*: $^{\#\#}P < 0.01$ CUMS vs. control; $^{\&}P < 0.05$ Females vs. males.

3.9. CUMS and sex effects on hippocampal neurotransmitter

There was a significant main effect of CUMS ($F_{1,20}$ =30.466, $P < 0.01$) and sex ($F_{1,20}$ =28.884, $P < 0.01$) on hippocampal NE concentration without interaction effect (Fig. 10A). CUMS resulted in a reduction of hippocampal NE concentration by 21.6% in males and 20.2% in females compared to controls of the same sex (Fig. 10A). Additionally, CUMS significantly reduced hippocampal 5-HT ($F_{1,20}$ =20.586, $P < 0.01$) concentration (Fig. 10B), regardless of sex and there was no interaction effect. Moreover, positive correlations between sucrose preference and hippocampal NE (r = 0.4195, $P < 0.05$) (Fig. 10C), and hippocampal 5-HT (r = 0.5943, $P < 0.01$) was found (Fig. 10D). Fig. 11.

4. Discussion

In this study, sex differences and underlying neuroimmunological mechanisms in CUMS-induced depression using rat models was investigated. CUMS significantly induced depressive behaviors, increased serum corticosteroids and systemic pro-inflammatory response, decreased hippocampal neurotransmitter levels and peripheral lymphocyte function in both sexes. In unstressed animals sex differences were evident with females having increased levels of locomotion, serum CD4/CD8 ratio, serum CORT levels and serum IL-6 levels, as well as decreased hippocampal NE concentration compared to males. Although there were no significant sex differences, females presented more changes in CD4 and CD8 lymphocytes than males after chronic stress.

4.1. CUMS-induced changes in behaviors and serum corticosterone concentration

According to the HPA axis hypothesis of depression, excess release of glucocorticoids is one of the chief causes for mood disorders [34], and normalizing this by using a corticosteroid receptor antagonist could improve depression-like behaviors in stressed rats [35]. With regards to the sex differences, the present study found higher basal corticosteroid concentration and higher locomotor behaviors (evidenced by open field test) in females compared to males. A previous study has reported that running exercise significantly elevated locomotion in voluntary wheel running test, which was suppressed by corticosterone synthesis inhibitor metyrapone [33]. In the same lines, higher basal corticosteroid may induce higher locomotion, particularly in females. A positive correlation between locomotion and serum corticosterone concentration was found in the current study confirming that an increase in serum corticosterone

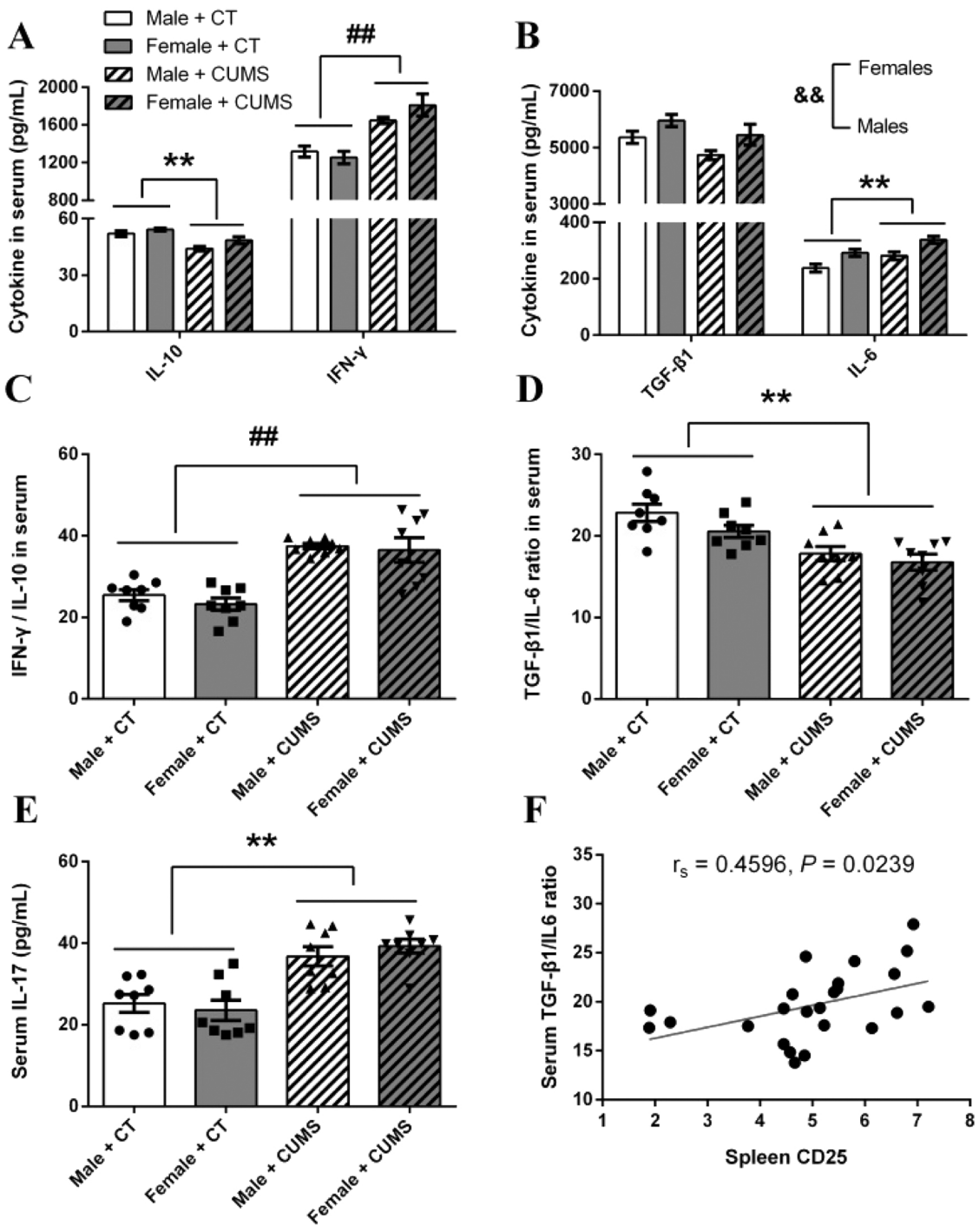

Fig. 7. Serum cytokines after CUMS in male and female rats. A) IL-10 and IFN-γ levels. B) IL-6 and TGF-β1 levels. C) The ratio of IFN-γ/IL-10. D) The ratio of TGF-β1 IL-6. E) IL-17. F) Positive correlation between spleen CD25 levels and serum TGF-β1/IL-6 ratio, sexes combined. All data are expressed as the mean ± SE (N = 8 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ CUMS main effect, $^{\&\&}P < 0.01$ sex main effect; *post-hoc test*: $^{\#\#}P < 0.01$ CUMS vs. control.

concentration may link with the higher locomotor activity. However, it was also observed that, male and female corticosterones were increased by 14.5% and 18.3% respectively, with decrease in locomotion by 13.1% in males and 8.7% in females after stress. This meant that CUMS significantly increased corticosterone concentration and reduced locomotion without sex differences. Previous research reported higher corticosterone levels in female rats than male rats after CUMS [36]. The differences may be due to the duration, degree or the type of stress. With regards to the status of other depression-like behaviors, the present study found CUMS induced anhedonia and anxiety in both sexes without sex differences, which was in line with our previous findings [31,37].

4.2. CUMS-induced changes in peripheral lymphocyte function

A chronic abnormal increase in corticosteroid concentration can desensitize glucocorticoid receptors located on macrophages/lymphocytes, which affects the release of pro-inflammatory factors and lymphocyte function [6,7,38]. Stress promotes the differentiation of naive CD4 T cells to Th1 and Th17 cells, and increases peripheral Th1/Th2 and IL-17/Treg cells ratio [39,40]. CD8 T cells also can

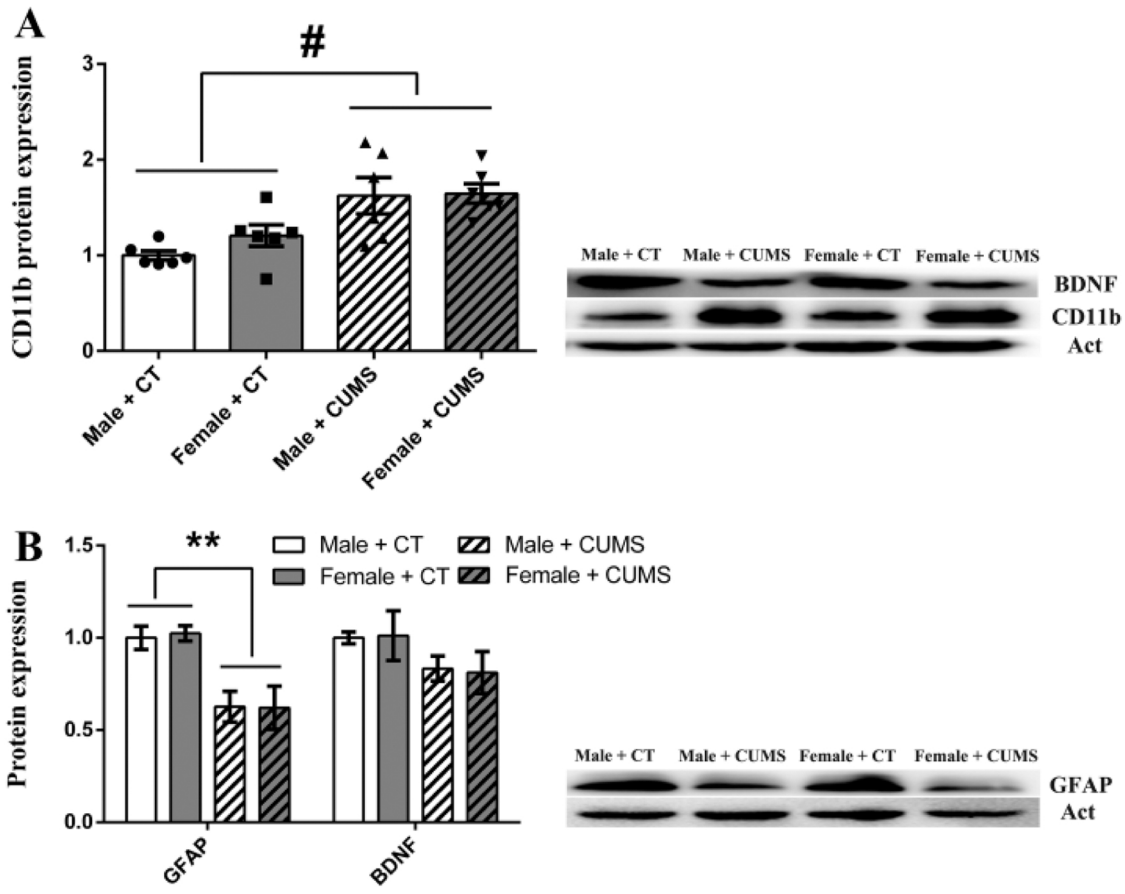

Fig. 8. Hippocampal glia cell expression after CUMS in male and female rats. A) CD11b expression in the hippocampus. B) GFAP and BDNF expression in the hippocampus. All values are expressed as the mean ± SEM (N = 6 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ CUMS vs. control. $^{**}P < 0.01$ CUMS main effect; *post-hoc test*: $^{\#}P < 0.05$ CUMS vs. control.

promote the pro-inflammatory response in stressed mice [41]. Increased pro-inflammatory IFN-γ/IL-4 (Th1/Th2) ratio and IL-17 (Th17) cytokines was found in stressed mice [40] and depressed patients [42,43]. Women show a longer and stronger immune/inflammatory response than men under an infection, tissue injury or stress [44–46]. In previous study, healthy women exhibited higher CD3 and CD4 T lymphocytes than healthy men in the blood [47]. Pro-inflammatory factors originating from T lymphocytes, such as IL-6, IL-12, IL-17, etc., are observed to be higher in females than males after inflammatory or stress stimulation [46,48]. With regards to the sex differences in lymphocyte subtypes, the present study found lower CD8 but higher CD4 and ratio of CD4/CD8 in female than male rats in the control condition. Interestingly, CUMS reversed this, by significantly increasing CD8 and decreasing CD4 and CD4/CD8 ratio in the blood of female rats. These sex differences remained with females having higher difference compared to males in the control group and when compared to control animals of the same sex. These may confirm that females have a stronger cell-mediated immunity dominated inflammatory response during infection or stress exposure [44,49,50]. Additionally, different studies reported no changes in blood CD4 and CD8 in female mice [17], while decreased blood CD4 and CD4/CD8 ratio, but increased CD8 cells, as well as serum pro-inflammatory TNF-α, IL-6 and IFN-γ concentrations were found in male rats after CUMS [51]. The reason for the variance between our observations and others may due to the CUMS paradigm used, or species differences. Importantly, our findings resemble clinical data that women have a stronger immune response than men in CD3 and CD4 lymphocytes and CD4/CD8 ratio in both healthy people [52] and depressive patients [53].

To further explore the mechanism of CD4 dysfunction in depression, our study measured CD4 subtype CD25 Treg lymphocyte, where a decline of anti-inflammatory spleen CD25 Tregs after CUMS exposure was observed in both sexes. Although no positive correlation between sucrose preference and blood CD4/CD8 ratio was found in the present study, clinical investigations have reported a strong negative correlation between the Beck Depression Inventory (BDI) and percentages of CD3, CD4 and CD4/CD8 ratio [54]. The reason for the different findings may be due to the CUMS paradigm, species differences, sample number limit and different types of depression-like behavior (such as helplessness) tested. Moreover, a positive correlation between depression-like behavior (sucrose preference) and CD4 lymphocyte subtypes (spleen CD25) were found in the present study. This is in line with clinical data where patients diagnosed with major depression have lower blood CD25 Treg cells [55]. Chronic stress significantly reduced CD25 Treg and induced depression-like behaviors, and these abnormal depression-like behaviors can also be found in CD25 Treg cell depleted mice [56]. The reduction of blood CD25 Treg in major depression can be recovered by antidepressant, imipramine [55,57]. Additionally, a positive correlation has been found between spleen CD25 and serum TGF-β1/IL-6 ratio in the present study. This is consistent with previous literature, where anti-inflammatory TGF-β can promote and anti-inflammatory IL-6 inhibits Treg differentiation and production [58]. Translation of total T lymphocytes to $Rag1^{-/-}$ mouse can significantly improve lipopolysaccharide induced inflammatory response and depression-like behaviors [59].

Overall, our data showed that females have a stronger basal immune background of CD4, CD8 lymphocytes and CD4/CD8 ratio. CUMS resulted in a larger decrease from basal CD4, CD4/CD8 ratio, but larger increase from basal CD8 in female than male rats. Although our study

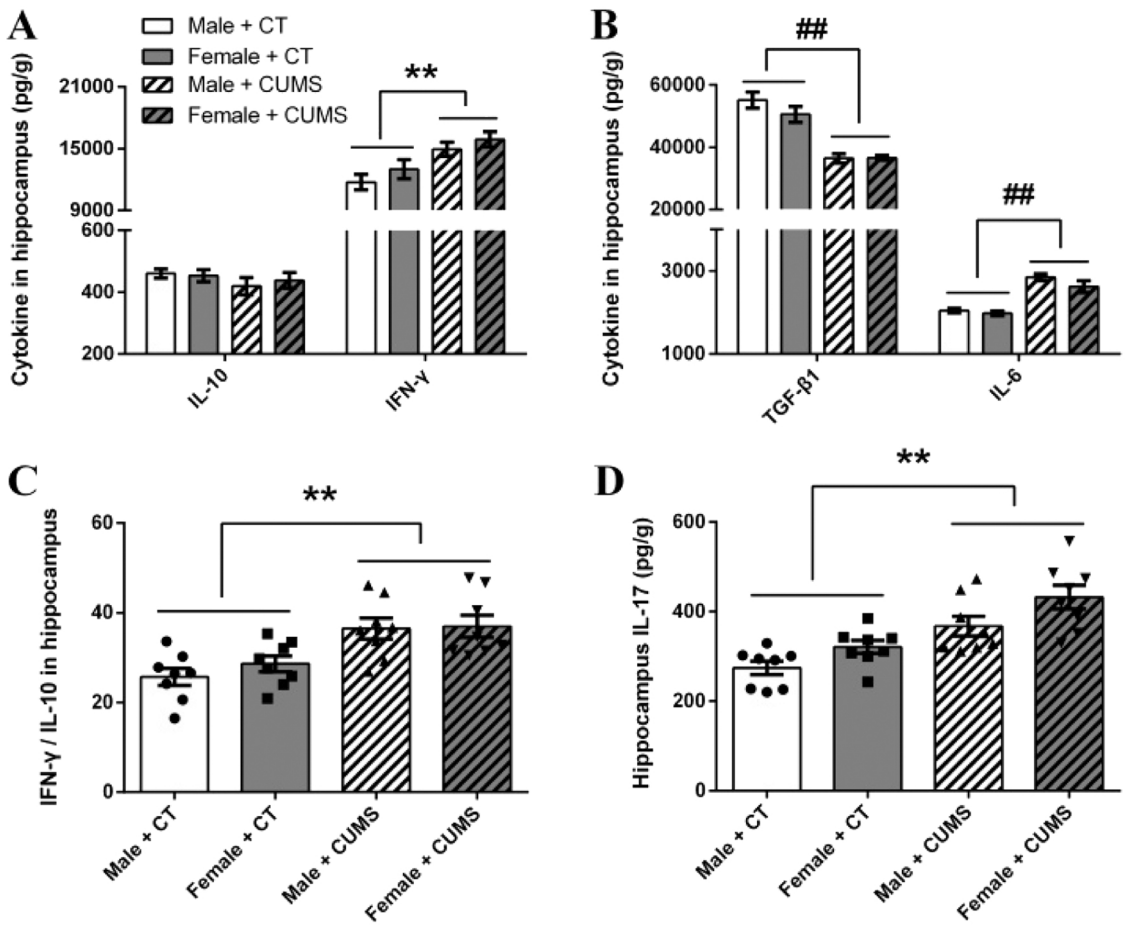

Fig. 9. Hippocampal cytokines after CUMS in male and female rats. A) IL-10 and IFN-γ. B) IL-6 and TGF-β1. C) The ratio of IFN-γ/IL-10. D) IL-17. All values are expressed as the mean ± SEM (N = 8 per group). $^{**}P < 0.01$ CUMS main effect; *post-hoc test*: $^{\#\#}P < 0.01$ CUMS vs. control.

did not find sex differences in depression-like behaviors (anhedonia), the lymphocyte subtype change may still play an important role in a higher clinical risk of depression in women.

4.3. CUMS-induced changes in peripheral cytokines

The monocyte-T-lymphocyte hypothesis of depression postulates that activated T lymphocyte subtypes-triggered inflammatory responses may induce the onset and development of depression [6,8]. With regards to the sex difference, the present study showed there was a higher serum IL-6 concentration in female than male rats in control situation. Increased plasma IL-6 concentration has been considered as a key factor for diagnosis of major depression, as well as the outcome of a therapeutic response [60]. Higher IL-6 pro-inflammatory background may indicate increased depression risk in women. Even though there was an increase in serum IL-6 concentration in both sexes after CUMS exposure, the change within the same sex was not significant, which may be one reason why there was no sex differences in anhedonia. Other studies have also demonstrated that CUMS markedly increased serum and hippocampal IL-6 concentrations in male mice [61–63]. The IL-6 levels in the medial prefrontal cortex (mPFC) was elevated only in males but not in females after stress though females had higher basal IL-6 levels [64]. However, clinical studies have reported that women with major depression show higher plasma IL-6 concentration than men [65], but no change in plasma IL-6 has also been observed in patients with major depression [66]. This rise in plasma IL-6 concentration can be reversed by antidepressant paroxetine treatment in major depression, in both sexes [67]. These varying findings may result from the severity and duration of the illness, or antidepressant treatment effects. Overall, although no sex differences were recorded after CUMS in our models, females having a higher basal IL-6 concentration might still play an important role in inflammation-related diseases. Present CUMS protocol (duration, degree or types of stress) and other depression-like behaviors (helplessness) should also be considered in the future studies, as gender differences of plasma IL-6 concentration have been found in patients with major depression [65].

With regards to the status of other pro- and anti-inflammatory factors, the present study found that CUMS significantly decreased serum anti-inflammatory IL-10, but increased pro-inflammatory IFN-γ, IL-17 and IFN-γ/IL-10 ratio without sex differences. These findings are in line with our previous results that CUMS can damage the balance between pro- and anti-inflammatory factors, which were observed in depressed patients [31,37].

4.4. CUMS-induced changes in hippocampal glia cells and neuroinflammation

In the central nervous system, CUMS exposure affected glia activity and the concentrations of cytokines in the hippocampus, as evidenced by decreased anti-inflammatory M2 factors IL-10 and GFAP, but increased pro-inflammatory M1 factors IFN-γ, IL-6, IL-17 and MI/M2 ratio IFN-γ/IL-10, without sex differences. These findings are in line with our previous studies that CUMS induced imbalance between pro-/anti-inflammatory factors, and decreased astrocyte neuroprotective function, including suppression of the expression of GFAP and BDNF [31,37]. Depression-like behaviors are significantly reversed by the anti-inflammatory drug minocycline through suppression of inflammatory responses both in males and females [31,68]. These suggest that the

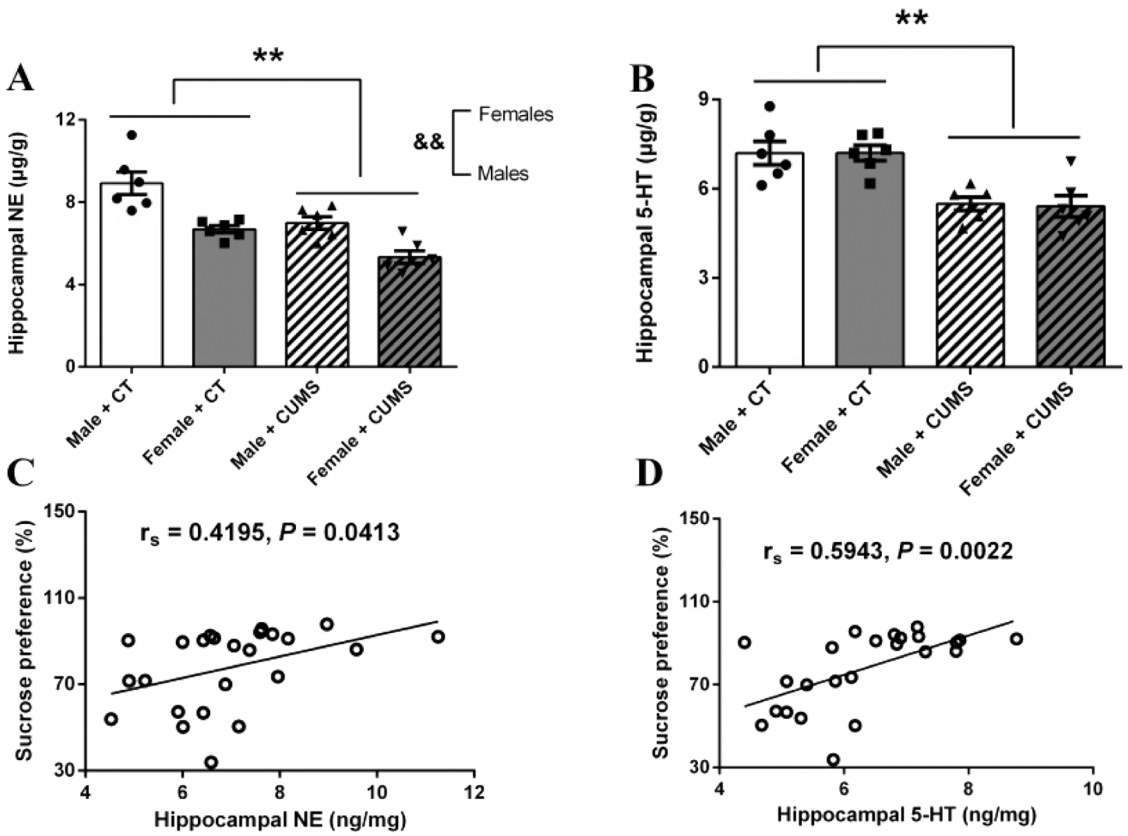

Fig. 10. Concentration of NE and 5HT in the hippocampus of male and female rats. A) Hippocampal NE concentration. B) Hippocampal 5-HT concentration. C) Correlation between hippocampal NE and sucrose preference, sexes combined. D) Correlation between hippocampal 5-HT and sucrose preference, sexes combined. All data are expressed as the mean ± SEM (N = 6 per group). $^{**}P < 0.01$ CUMS main effect; $^{\&\&}P < 0.01$ sex main effect.

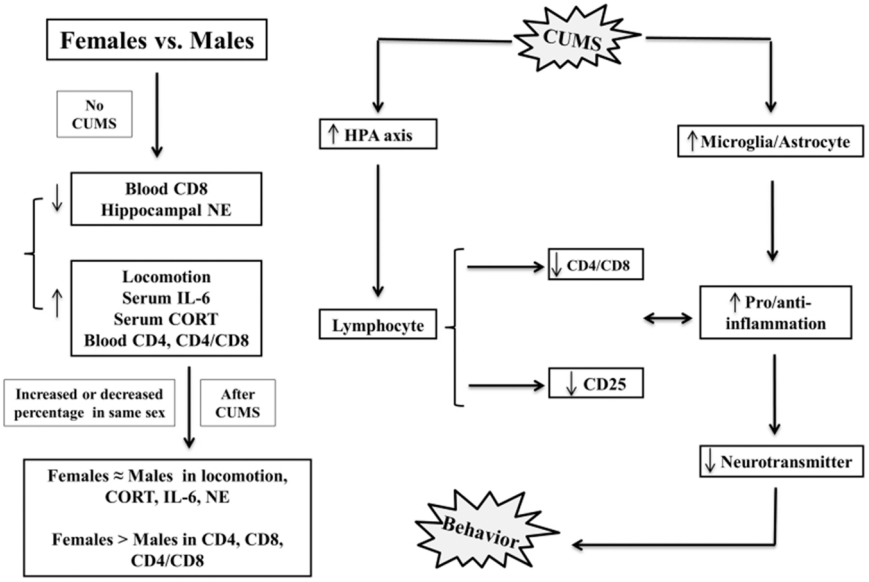

Fig. 11. Potential schematic and result's summary of the study. CUMS: chronic unpredictable mild stress, HPA: hypothalamic pituitary adrenocortical axis. "↑" represent increase, "↓" represent decrease. "≈ "represent similar increased or decreased percentage when compared to control animals of the same sex. "> "represent larger increased or decreased percentage when compared to control animals of the same sex.

balance between pro- and anti-inflammatory responses contribute to alleviate depression-like behaviors.

4.5. CUMS-induced changes in hippocampal neurotransmitter concentration

Numerous studies have demonstrated that activated microglia result in monoamine neurotransmitter deficiency by triggering inflammatory

signals, releasing excess of pro-inflammatory factors, or decreasing BDNF through suppression of astrocyte functions, thereby causing depression [69,70]. Indeed, the monoamine deficits have been extensively studied to explain various clinical phenotypes in psychiatric and neurologic diseases [71–73]. Accordingly, most antidepressant treatments target NE, 5HT synthesis pathways and their receptors [74,75]. With regards to the sex differences, the present study showed a lower basal hippocampal NE concentration in females when compared to male rats in control situation. As mentioned above, alterations in NE systems have been reported in depression and antidepressant drugs that increase NE levels are used in the treatment of depression [76,77]. In the present study, after CUMS exposure, a decrease in hippocampal NE concentration was found in both females and males, but statistically no significant sex differences were observed for this change in decreased percentage. This in line with our previous study that CUMS decreases NE concentrations in the hippocampus [37]. A previous study found lower levels of hippocampal NE in stressed female rats than stressed males [64]. Previous study has shown that serum norepinephrine decrease in female stressed mice was greater than that observed in male stressed mice, while the serum 5-HT drop in male stressed mice was greater than that in female mice [78]. The reason for the difference between our findings and others might due to the CUMS paradigm, the duration used, or age and species of the animal used. With regards to other neurotransmitters, in the present study, CUMS also decreased hippocampal 5-HT concentration without sex differences, which is in line with decreased hippocampal 5-HT and NE in our previous research [37]. A significant positive correlation between sucrose preference and hippocampal neurotransmitters (NE and 5-HT) was also found, regardless of sex. These findings thus support the hypothesis that the deficiency of monoamine neurotransmitters induced depression-like behaviors after CUMS exposure, but likely does not explain sex difference in depression susceptibly.

5. Limitations and future directions

The data from the current study is not sufficient to provide clear evidence that stress could promote depressive-like behaviors in females when compared to males. Future studies should try to standardize CUMS paradigm, keep view the duration and types of stress, animal species and age, as well as estrous cycle (females are more susceptible to stress and emotional). Moreover, other animal models of depression, such as olfactory bulbectomy, LPS or IL-1β inflammatory challenge model, social defeat, should be considered, because each model has its advantages and disadvantages. As well, helplessness (using forced swimming or tail suspension test), which is one of the core symptoms of depression, should be evaluated in the future. Additionally, specific brain areas should be studied, such as prefrontal cortex, amygdala, and hypothalamus. The role of lymphocyte and inflammatory response in the CNS should further be investigated by using transgenic mice or rodents with related factor treatments in different sexes.

6. Conclusion

The present study showed that CUMS significantly induced depression-like behaviors, which are related to the abnormalities in stress hormone, lymphocytes, inflammatory factors and monoamine neurotransmitters. More importantly, the present study found that several important parameters significantly differed in non-stressed females, such as higher locomotion, blood CD4/CD8 ratio, serum corticosterone and IL-6, but lower hippocampal NE concentration than male rats. Even though CUMS failed to induce the sex differences, the original abnormalities in several depression-related factors may still play important role in female depression vulnerability. Nevertheless, the study results may be used as a reference to standardize depression models and also choose a suitable treatment for depression in different genders.

CRediT authorship contribution statement

CS designed the experiments and edited the manuscript. CZ and BPL performed the experiments and analyzed the data. CZ drafted the paper. JLP, HS and UKK modified the paper and assisted with statistical analysis. All authors have read, discussed, and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Data Availability

Data will be made available on request.

Acknowledgments

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