



## Minocycline inhibits microglial activation and alleviates depressive-like behaviors in male adolescent mice subjected to maternal separation

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### ABSTRACT

Exposure to early adversity increases vulnerability to psychiatric disorders in later life. Microglia-mediated inflammation has been linked to psychopathology, so such inflammation may be a target for treating depression. Using a model of depression involving adolescent male C57BL/6J mice subjected to maternal separation, we explored whether using minocycline to mitigate inflammation can alleviate depression-like behaviors. Between postnatal days 1 and 14, male mice were separated from their mothers for 3 h per day. Minocycline (20 mg/kg) was administered intraperitoneally once daily for 2 weeks starting one week after weaning. Then the male mice were subjected to a second stress for 2 weeks. Results from the sucrose preference test, forced swimming test, and open field test showed that maternal separation did not obviously alter behavior of the male mice, but it did increase the risk of depression-like behaviors following a second stress. This increased risk disappeared if minocycline was given preemptively before the second stress. Maternal separation and second stress up-regulated pro-inflammatory markers and down-regulated anti-inflammatory markers in the hippocampus, and they activated microglia and promoted pro-inflammatory transitions in microglia. All these effects were reversed by minocycline. These changes in inflammatory processes correlated with changes in neurogenesis and BDNF expression in the hippocampus. Our results in this mouse model suggest the potential of minocycline for treating psychiatric disorders induced by early adversity.

### 1. Introduction

Major depression is a primary cause of human suffering and disability. According to the latest report from the World Health Organization, more than 300 million people live with depression (Mousavi et al., 2017). Research in humans and animals has shown that early life adversity can lead to many negative consequences, including significant mental disorders (Lehmann et al., 1999; Pena et al., 2017; Zhang et al., 2016). Mounting evidence indicates that early life stress affects brain development and increases lifelong risk of neuropsychological disorders such as depression, anxiety, and memory problems (Gracia-Rubio et al., 2016; Harrison et al., 2014; Kember et al., 2012). Most research about the effects of early stress on later development has focused on the hypothalamic-pituitary-adrenal axis response (Goodwill et al., 2018; Kokras et al., 2012; McIlwrick et al., 2016). Recent

investigations suggest that individuals exposed to early adversity can exhibit depression- and anxiety-like behaviors that are associated with immune system disturbances, which in turn may increase risk of inflammation-related disorders later in life (Danese and Lewis, 2017; Gracia-Rubio et al., 2016; Roque et al., 2016).

Microglia are the resident innate immune cells within the central nervous system. In addition to their immune functions, microglia play critical roles in the patterning and wiring of the brain in development (Matcovitch-Natan et al., 2016; Zhang et al., 2017). The early postnatal period is an important stage for microglial development and migration (Bilbo and Schwarz, 2009; Harry and Kraft, 2012). Adverse changes in early life can alter microglial responses to environmental challenges in later life (Calcina et al., 2016; Johnson and Kaffman, 2018). Maternal separation of rodent offspring from the mother, which has been used extensively to model the effects of early adversity (Lehmann et al.,

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1999), causes abnormal development of microglia in offspring (Gong et al., 2018; Réus et al., 2018). It causes the branches of hippocampal microglia to shorten and thicken, and the cell bodies to enlarge (Delpech et al., 2016). All these changes lead to increased secretion of proinflammatory cytokines in the brain (Burke et al., 2014; Cattaneo et al., 2015; Roque et al., 2016). The resulting over-activated inflammatory responses induce abnormal neural proliferation, differentiation, migration, and survival in the hippocampus, which are associated with pathological changes such as depression (Borsini et al., 2015; You et al., 2011). These considerations suggest that modulating microglial activation may help reduce the risk of mood disorders (Zhang et al., 2018).

Minocycline, a derivative of tetracycline that can cross the blood-brain barrier, is widely used to inhibit microglia (Kobayashi et al., 2013; Moller et al., 2016; Tikka et al., 2001). In fact, minocycline exerts neuroprotective activity and may be useful for treating psychotic disorders (Miyaoaka et al., 2012). In a mouse model of depression, minocycline attenuates microglia-mediated inflammation and prevents the development of depression-like symptoms (Kreisel et al., 2014; O'Connor et al., 2009; Riazi et al., 2015). In our previous study, minocycline promoted neurogenesis and ameliorated cognitive deficits in prepubertal offspring whose mothers had been exposed to stress during pregnancy (Zhao et al., 2015).

In this study, we investigated whether the ability of minocycline to inhibit microglial activation and protect neurons would allow it to mitigate depression-like behavior in male mice subjected to maternal separation. In light of evidence that maternal separation alone may not significantly alter offspring behavior (Peña et al., 2017) but instead may increase vulnerability to a second stress, we also examined the effects of minocycline when applied before a second stress. We found that preemptive intervention with minocycline reversed the production of proinflammatory molecules and depression-like symptoms induced by maternal separation and second stress.

## 2. Material and methods

### 2.1. Animals

Adult male and female C57BL/6J mice 2 months old and weighing 20–25 g were purchased from the Experimental Animal Center of Sichuan Provincial People's Hospital (Chengdu, China). They were maintained at  $23.5 \pm 1.5$  °C and humidity of  $60 \pm 10\%$  on a 12-h circadian cycle with lights on at 7:00 a.m. and free access to food and water. A single male mouse was housed with two females for breeding. After mating, pregnant females were housed individually. For each litter, the date of birth was defined as postnatal day (PD) 0. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Electronic Science and Technology of China.

### 2.2. Maternal separation and second stress

All mice were randomly assigned to a control or maternal separation group. In the latter group, pups were separated from their mothers for 3 h per day on PD 1–14 by removing the mothers from the nest. Temperature of pups huddled in the nest ranged from 28 to 35 °C, depending on the presence of the dam and age. The separated mothers were kept in the same room as the pups. In the control group, pups remained with mothers until weaning on PD 21. Only male pups were used in subsequent experiments.

For the second stress, pups were placed in a 50-ml tube with breathing holes from 10:00 a.m. until noon (2 h) each day. Pilot studies showed that this restraint stress provided subliminal stimulation that could induce significant behavioral changes in male pups subjected to maternal separation but not in control pups.

### 2.3. Minocycline treatment

After weaning, male pups were randomly assigned to receive once daily (10:00 a.m.) intraperitoneal injections of saline or minocycline (20 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) that had been diluted with saline to 2.5 mg/ml. These injections were given for 2 weeks. The dosage of minocycline was based on previous studies showing that doses ranging from 20 to 50 mg/kg/day effectively attenuated microglial activation (Hinwood et al., 2012; Raghavendra et al., 2003).

### 2.4. Behavioral tests

#### 2.4.1. Sugar preference test (SPT)

The SPT test was performed as described (Zhao et al., 2014). Before the test, individually housed mice were habituated for 72 h to consume 1% sucrose solution. The animals were deprived of food and water for 12 h, then provided with containers of 1% sucrose and water, which were randomly placed on either the left or right side of the cage. Each test involved a single 2-h session. Water bottles were weighed before and after the test to evaluate the consumption of sucrose and water. Sugar preference (%) was calculated as

$$\text{Sugar preference (\%)} = \frac{\text{sugar consumption (g)}}{[\text{sugar consumption (g)} + \text{water consumption (g)}]} \times 100\%.$$

#### 2.4.2. Forced swimming test (FST)

The FST was conducted as described (Porsolt et al., 1977) with a slight modification. Each mouse was individually placed in a 1000-mL glass cylinder of height 21 cm and diameter 12 cm containing water ( $23 \pm 1$  °C) to a depth of 12 cm. Animals were left inside the cylinder for 5 min to adapt. At 24 h later, mice were again placed in the cylinder for 6 min and were monitored continuously using a computer-assisted video camera system (FST-100 Forced Swimming Analysis System, Techman Soft, China). Data from the final 4 min of the test were used to determine time spent moving and time spent motionless. During this test, the cylinder was maintained in a quiet, confined environment to prevent external disturbances.

The results on the SPT and FST were used as described (Pena et al., 2017) to determine whether the animal exhibited depression-like behavior. Depression-like behavior was defined as the combination of (a) sucrose preference < the mean of all control mice and (b) immobility time during the FST > the mean of all control mice. Then the percent of tests on which each mouse met depression-like criteria was calculated.

#### 2.4.3. Locomotor activity test

Locomotor activity was examined using a 36-point infrared ray passive sensor system (ZZ-6, Techman Soft, Chengdu, China). Each mouse was placed into a chamber of the instrument and allowed to accommodate to the environment for 1 min. Then, locomotion activity was recorded automatically as the number of times the animal moved during 10 min.

#### 2.4.4. Open field test

This test measured movement and anxiety-like behavior. The open field consisted of a white acrylic box (50 × 50 × 30 cm) placed on top of a high table under dim lighting of illumination intensity 25 lx (Chourbaji et al., 2005). A virtual square (25 × 25 cm) in the floor area was set as the unobstructed central area. Each mouse was placed in the open field to acclimate to the environment for 5 min. Then, movements were recorded automatically for 5 min using a computer-assisted video tracking system (OFT-100 Open Field Activity Experiment System, Techman Soft). The system calculated the total distance traveled and time spent in the inner 25-cm area.

## 2.5. Immunofluorescence

Microglial morphology and neuronal proliferation in mouse brain were assessed using immunofluorescence. Pubertal male pups were injected intraperitoneally with bromodeoxyuridine (BrdU; 50 mg/kg, Sigma, USA) at 8:00 a.m. once daily for three days. At 2 h after the last injection, mice were anesthetized with sodium pentobarbital and perfused transcardially with saline, followed by a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were extracted and post-fixed in 4% paraformaldehyde for 48 h, then in 30% sucrose for 24 h. Coronal sections (35  $\mu$ m thick) were cut using a freezing microtome (CM1520; Leica Microsystems, Germany) and collected in PBS containing 0.03% sodium azide. Sets of 7 sequential slices were placed into each well of a 12-well plate, which was stored at 4 °C.

Sections were permeabilized for 15 min with Triton X-100 (0.5% in PBS), incubated in 1 M HCl for 30 min at 25–30 °C, washed twice in 0.1 M borate buffer (pH 8.5) for 10 min each time, blocked in 10% donkey serum for 2 h, and incubated overnight at 4 °C with the following primary antibodies: mouse anti-BrdU (1:400; Cell Signaling Technology, Japan), mouse anti-IL6 (1:100; Abcam, USA), and rabbit anti-Arg1 (1:100; Cell Signaling Technology). Then sections were washed three times in PBS for 5 min each time, and incubated for 1 h with the following secondary antibodies (1:400; Jackson ImmunoResearch, USA): Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated donkey anti-rabbit, and Alexa Fluor 594-conjugated donkey anti-goat. Sections were again washed three times with PBS, then stained for 5 min with DAPI (1:10,000; Roche, Switzerland) to label nuclei.

Sections to be stained for doublecortin (DCX) and Iba1 were processed in the same way, except that the steps involving HCl and borate buffer were omitted. The primary antibodies were goat anti-DCX (1:400; Santa Cruz, USA), and goat anti-Iba1 (1:400; Abcam).

Stained sections were then imaged using a fluorescence microscope (Olympus BX51, Tokyo, Japan) with the same settings in all experiments, and images were analyzed using Image J version 1.8.0 J (National Institutes of Health, Bethesda, MD, USA). All image analyses were conducted in a blinded manner: the analyst was unaware of how the animal was treated prior to tissue sectioning. To quantify proliferating cells, coronal sections containing a portion of the hippocampus and stained with antibodies against BrdU and DCX were counted for the number of positive cells. The number in each section was multiplied by the number of sections to estimate the total number per dentate gyrus (DG). Sections stained against Iba1 were evaluated in terms of microglial morphology as well as number and length of microglial processes in the entire DG. These measurements were made using the *skeleton analysis* tool in Image J (Young and Morrison, 2018). Iba1-positive microglia were also categorized (Walker et al., 2014; Wu et al., 2014) as being *ramified*, with rod-shaped cell bodies and fine, ramified processes (resting microglia); *hypertrophied*, with small cell bodies and long, thin processes (activated microglia); or *bushy*, with large somas and short thick processes, giving an overall rounded amoeboid morphology (activated microglia).

## 2.6. Real-time PCR

Animals were exsanguinated and perfused with saline, then hippocampi were quickly removed. Tissue was quickly placed into sterile tubes on ice. Total RNA was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturer's protocol and suspended in 30  $\mu$ l of nuclease-free water. An aliquot (5  $\mu$ g) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Takara Biomedical Technology, Japan), and cDNA was stored at –20 °C. Aliquots of cDNA were amplified in quantitative PCR reactions to determine levels of the following mRNAs: fractalkine receptor (CX3CR1), inducible nitric oxide synthase (iNOS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (INF- $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), tumor growth factor- $\beta$

(TGF- $\beta$ ), interleukin-4 (IL-4), interleukin-1 $\alpha$  (IL-1 $\alpha$ ), chitinase 3-like protein 3 (YM1), arginase 1 (Arg1), brain-derived neurotrophic factor (BDNF), and doublecortinDCX. All PCR reactions were conducted on a Bio-Rad CFX 96 cyclor (Hercules, USA) under the following conditions: 95 °C for 10 min, followed by 38 cycles at 95 °C for 3 s and at the annealing temperature for 30 s. The threshold amplification cycle number was determined for each reaction within the linear phase of the amplification plot, and relative mRNA levels were determined using the  $2^{-\Delta\Delta Ct}$  method. Values were normalized to those of the housekeeping gene  $\beta$ -actin.

The following primer pairs were used to amplify each mRNA:  *$\beta$ -actin*, 5'-GTA AAG ACC TCT ATG CCA ACA-3' and 5'-GGA CTC ATC GTA CTC CTG CT-3'; *CX3CR1*, 5'-CTG TTA TTT GGG CGA CAT TG-3' and 5'-AAC AGA TTT CCC ACC AGA CC-3'; *iNOS*, 5'-GTT CTC AGC CCA ACA ATA CAA-3' and 5'-GTG GAC GGG TCG ATG TCA C-3'; *TNF- $\alpha$* , 5'-CTG TAG CCC ACG TCG TAG C-3' and 5'-TTG AGA TCC ATG CCG TTG-3'; *INF- $\gamma$* , 5'-CGG CAC AGT CAT TGA AAG CCT A-3' and 5'-GTT GCT GAT GGC CTG ATT GTC-3'; *IL-1 $\beta$* , 5'-CTT CAG GCA GGC AGT ATC ACT CAT-3' and 5'-TCT AAT GGG AAC GTC ACA CAC CAG-3'; *IL-6*, 5'-TAG TCC TTC CTA CCC CAA TTT CC-3' and 5'-TTG GTC CTT AGC CAC TCC TTC-3'; *TGF- $\beta$* , 5'-GAC CGC AAC AAC GCC ATC TA-3' and 5'-GGC GTA TCA GTG GGG GTC AG-3'; *IL-4*, 5'-TCT CGA ATG TAC CAG GAG CCA TATC-3' and 5'-AGC ACC TTG GAA GCC CTA CAG A-3'; *IL-1 $\alpha$* , 5'-CGA AGA CTA CAG TTC TGC CAT T-3' and 5'-GAC GTT TCA GAG GTT CTC AGA G-3'; *YM1*, 5'-CAT TCA GTC AGT TAT CAG ATT CC-3' and 5'-AGT GAG TAG CAG CCT TGG-3'; *Arg1*, 5'-GCT CAG GTG AAT CGG CCT TT-3' and 5'-TGG CTT GCG AGA CGT AGA C-3'; *BDNF*, 5'-CGG CCG CCA TGA AAG AAG TA-3' and 5'-AGA CCT CTC GAA CCT GCC CT-3'; and *DCX*, 5'-GCG CCG CAG CAA GTC T-3' and 5'-TTG AGA GCT GAC TGC TGG AAG TT-3'.

## 2.7. Western blot analysis

Animals were exsanguinated and perfused with saline as described above. Total protein was extracted from hippocampal tissues using RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using a BCA kit (Beyotime Institute of Biotechnology) following the manufacturer's guidelines. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and electro-blotted onto nitrocellulose membranes (Millipore). Membranes were blocked with 5% skim milk for 2 h and incubated overnight at 4 °C with rabbit primary antibody (1:1000) against IL-6 (Abcam), IL-1 $\beta$  (Cell Signaling Technology), TGF- $\beta$  (Abcam), or IL-4 (Cell Signaling Technology). The membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000; Beyotime) and analyzed using the ECL-Plus kit (Millipore). Densitometry of protein bands was performed using ImageJ.

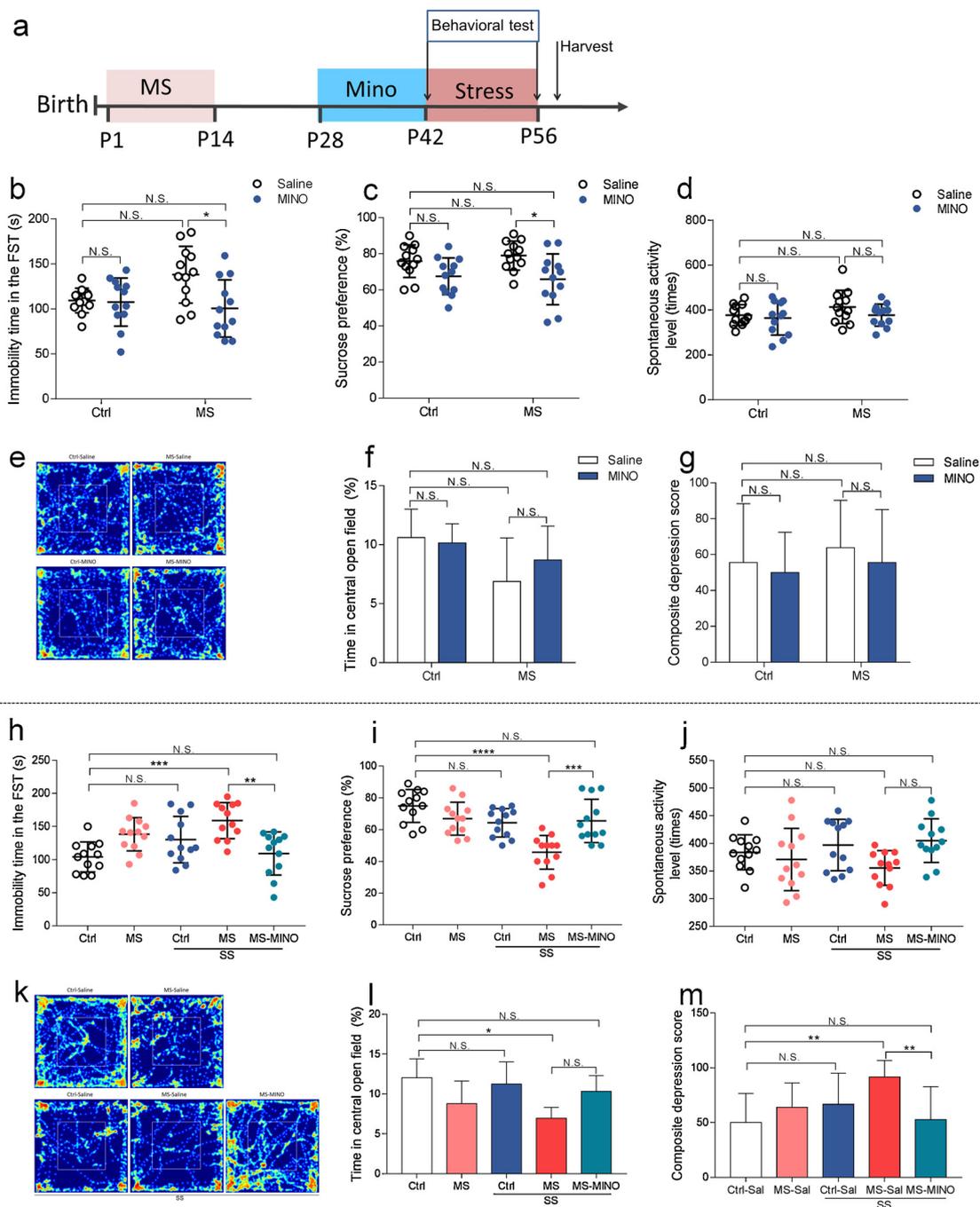
## 2.8. Statistical analysis

Data were expressed as mean  $\pm$  SEM and analyzed using SPSS for Windows® version 17.0 (IBM, Chicago, USA). Differences between groups were assessed for significance using one- and two-way analysis of variance, followed by Bonferroni's multiple comparisons test as the multiple-comparisons *post hoc* test. Differences associated with  $P < 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Preemptive treatment with minocycline reduces the susceptibility to depression-like behaviors in male adolescent mice subjected to maternal separation

The experimental paradigm is presented in Fig. 1a. Depression-like behaviors in males were evaluated on PD 42 in the SPT, FST, and OFT.

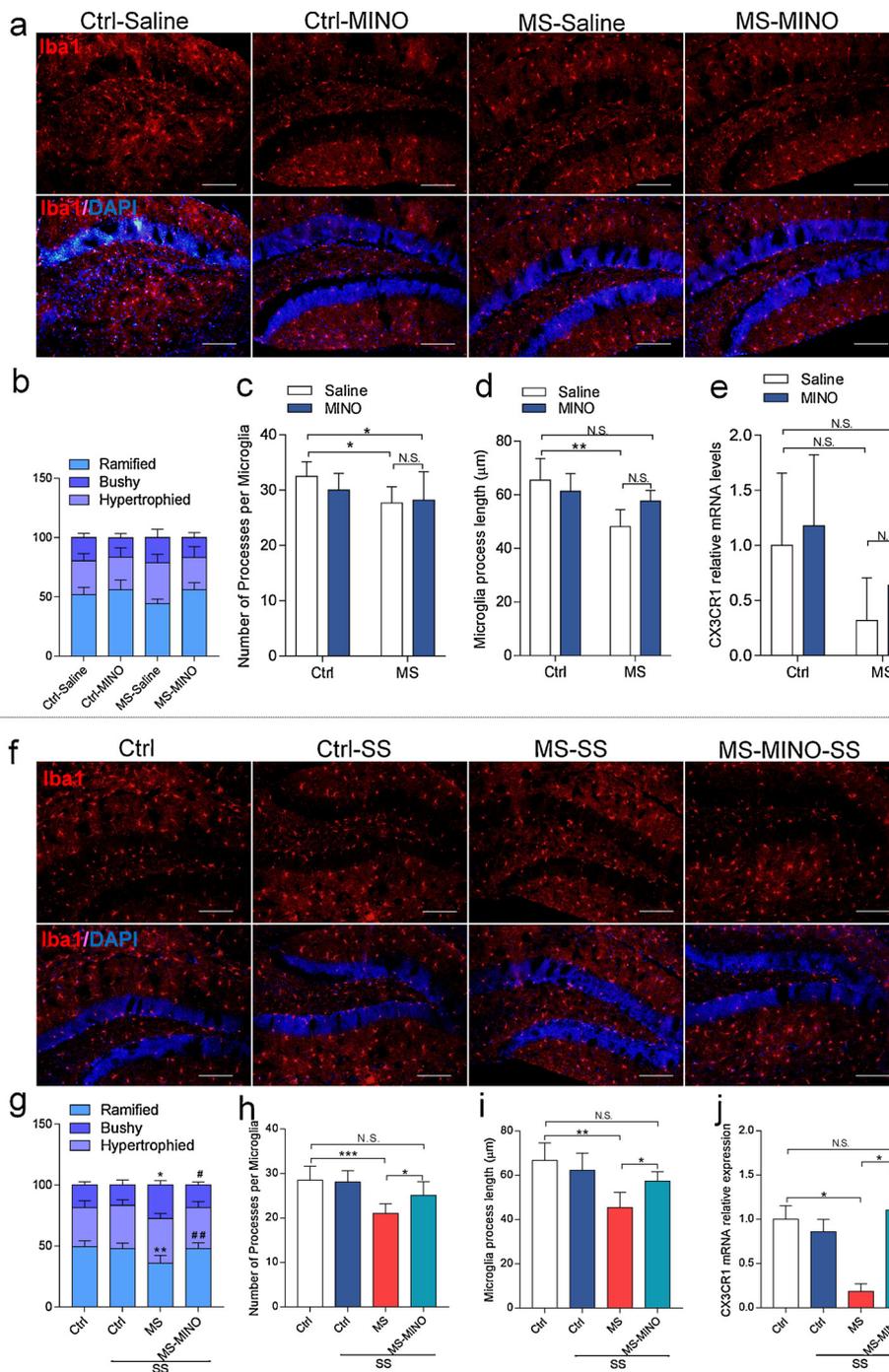


**Fig. 1. Effects of maternal separation and second stress on depression-like behaviors in male adolescent mice.** a. Schematic diagram of maternal separation (MS), minocycline treatment, second stress (SS) and behavioral tests. b-d. Measurement on postnatal day (PD) 42 of immobility time during forced swimming (b), sucrose preference (c), and number of movements in a locomotor activity test (d). e. Heat maps showing exploration in the open field test. f. Total exploration time in the center of the open field. g. Composite depression score based on forced swimming test and sucrose preference test on PD 42. h-j. Forced swimming (h), sucrose preference (i), and number of movements in the locomotor activity test (j) on PD 56. k. Heat maps showing exploration in the open field test. l. Total exploration time in the center of the open field. m. Composite depression score on PD 56. Data are mean ± SEM (n = 12 mice per group), and statistical analysis is shown in Table 1. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005, \*\*\*\* P < 0.001; N.S. = not significant.

Maternal-separated mice tended to spend longer time immobile than control mice in the FST, and 2-week minocycline treatment shortened the immobility time of maternal-separated mice (Fig. 1b). The SPT and OFT showed no significant differences between maternal-separated or control mice in terms of the number of spontaneous movements (Fig. 1c-1d and e-1f). The two groups of animals showed a similar composite depression score based on SPT and FST (Fig. 1g).

To examine whether minocycline treatment could have a sustained positive effect on depression-like behaviors later in life, male adolescent

mice treated with saline or minocycline were subjected to a second stress 2 weeks later. The two groups did not differ significantly in spontaneous movement levels (Fig. 1g). Saline-treated mice subjected to maternal separation and second stress showed significantly longer immobility in the FST and significantly lower sucrose consumption in the SPT than control animals, and these differences were not significant after minocycline treatment (Fig. 1h-1i). Saline-treated mice showed higher composite depression score, and this increase was nearly eliminated by minocycline treatment (Fig. 1m). Saline-treated animals



**Fig. 2.** Alteration of microglial phenotype in the hippocampus of maternal-separated male adolescent mice after second stress. **a.** Representative images of microglia in the DG of control (not maternal-separated) mice treated with saline (Ctrl-saline) or minocycline (Ctrl-minocycline) and maternal-separated mice treated with saline (MS-saline) or minocycline (MS-minocycline). (n = 5 per condition). Scale bar, 100  $\mu$ m. **b.** Distribution of microglial phenotypes on postnatal day (PD) 42. **c–d.** Quantitative analysis of process number (c) and length (d) per microglial cell in the DG on PD 42. **e.** Relative mRNA level of microglial marker CX3CR1 (n = 8). **f.** Representative images of microglia in DG on PD 56 (n = 5). Scale bar, 100  $\mu$ m. **g.** Microglial phenotypes in the different groups. **h–i.** Quantitative analysis of process number (h) and length (i) per microglial cell in the DG on PD 56. **j.** Relative mRNA level of microglial marker CX3CR1 (n = 8). Data are mean  $\pm$  SEM and are analyzed statistically in Table 2. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005 vs. Ctrl group; # P < 0.05, ## P < 0.01 vs. MS-saline group; N.S. = not significant.

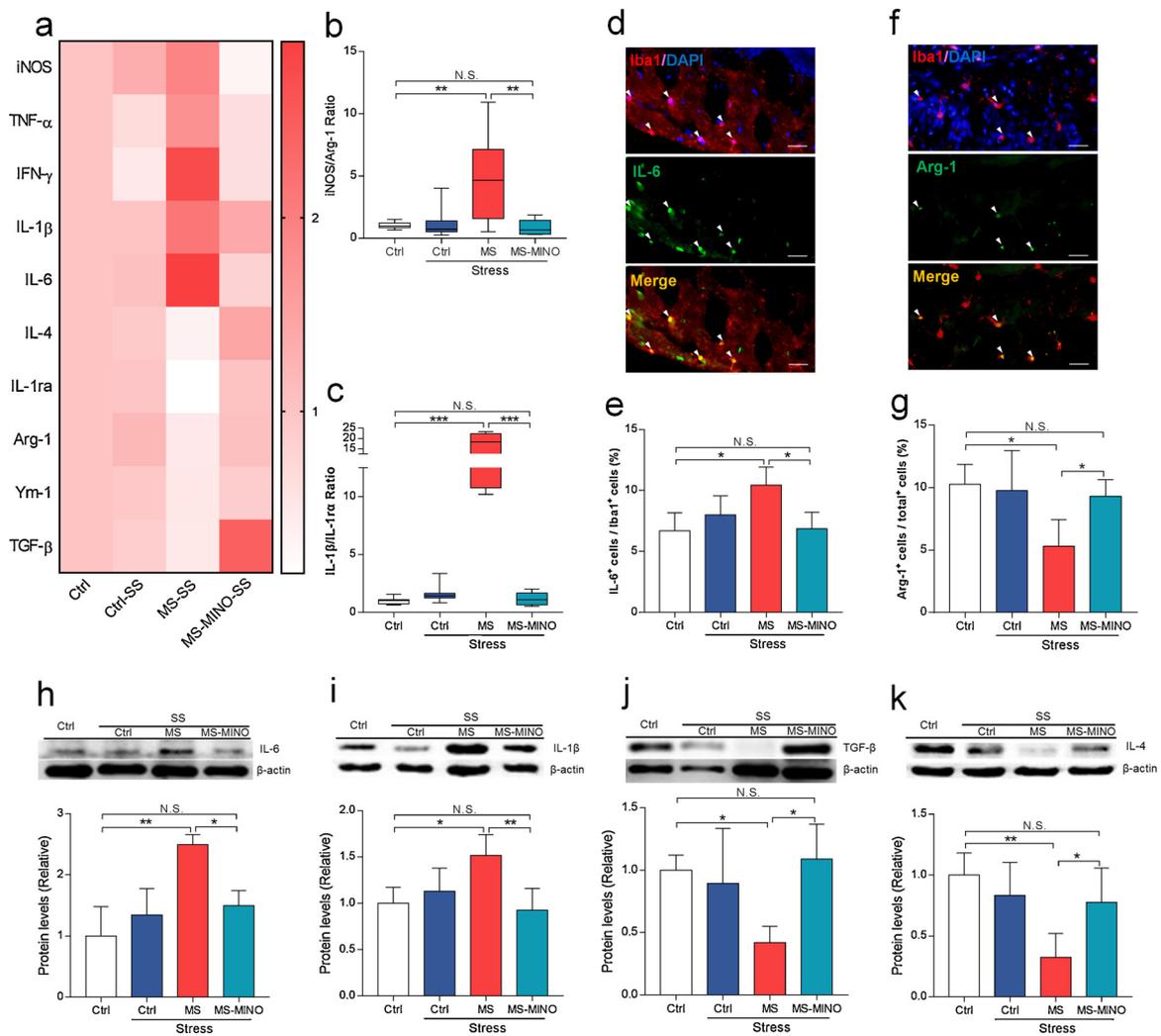
spent less time exploring the center of the open field than minocycline-treated animals (Fig. 1k–l).

### 3.2. Minocycline inhibits activation of hippocampal microglia induced by maternal separation and second stress

Microglial morphology was assessed based on anti-Iba1 immunofluorescence (Fig. 2a), while microglial phenotype was assessed as resting (ramified) or activated (hypertrophic, bushy). Maternal-separated mice tended towards slightly higher numbers of activated microglia than control mice, and this increase was reduced after minocycline administration (Fig. 2b). The average number and length of microglial processes in the DG was smaller in maternal-separated mice than in control mice, and minocycline was associated with longer

processes (Fig. 2c and d). Expression of the microglial marker CX3CR1 in hippocampus tended to be lower in maternal-separated mice than in control mice, and minocycline increased this expression (Fig. 2e).

Following second stress, maternal-separated mice showed a lower proportion of resting microglia and higher proportion of activated microglia than control animals. Minocycline decreased the proportion of activated microglia (Fig. 2g). After second stress, maternal-separated mice showed significantly less branching than control animals, while minocycline led to more branching in maternal-separated mice (Fig. 2h). Microglial processes were shorter in maternal-separated mice than in control mice, and minocycline attenuated this difference (Fig. 2i). CX3CR1 expression in the hippocampus was lower in maternal-separated mice than in control animals, and minocycline increased this expression (Fig. 2j).



**Fig. 3.** Effects of minocycline on cytokine expression in the hippocampus. **a.** Heat map of mRNA expression of microglial phenotype markers in hippocampus ( $n = 7$ ). **b–c.** Ratios of pro- to anti-inflammatory mediators in hippocampus: iNOS/Arg1 and IL-1 $\beta$ /IL-1r. **d–g.** Immunofluorescence staining of pro-inflammatory marker IL-6 (green) (d) and anti-inflammatory marker Arg1 (green) (f) located in Iba1 $^{+}$  (red) cells ( $n = 5$ ). Scale bar, 50  $\mu$ m. The ratio of IL-6 $^{+}$  Iba1 $^{+}$  cells to Iba1 $^{+}$  cells (e) or ratio of Arg1 $^{+}$  Iba1 $^{+}$  cells to Iba1 $^{+}$  cells (g) in hippocampus. White arrows indicate cells double-labeled with Iba1 as well as IL-6 or Arg1. Data are mean  $\pm$  SEM. **h–k.** Representative western blots and quantitation of pro-inflammatory cytokines IL-6 (h) and IL-1 $\beta$  (i) as well as anti-inflammatory cytokines TGF- $\beta$  (j) and IL-4 (k) on PD 56 ( $n = 5$ ). Data are analyzed statistically in Table 3. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ ; N.S. = not significant (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

### 3.3. Minocycline mitigates cytokine expression in hippocampus induced by maternal separation and second stress

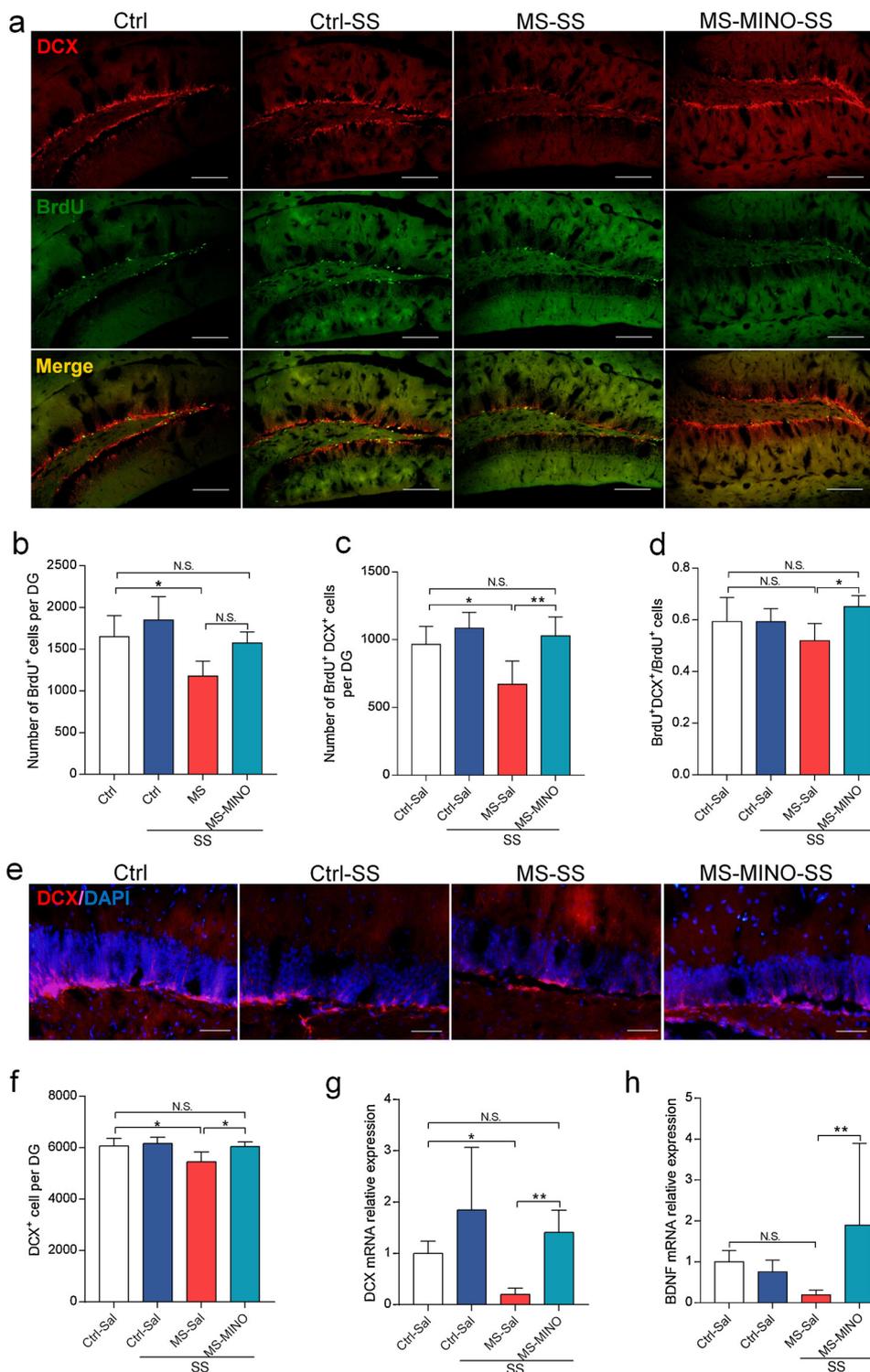
Second stress up-regulated several pro-inflammatory markers in the hippocampus (iNOS, TNF- $\alpha$ , IFN- $\gamma$ , IL-6, and IL-1 $\beta$ ) and down-regulated several anti-inflammatory markers (IL-4, TGF- $\beta$ , IL-1ra, IL-1 $\alpha$ , Ym-1, Arg1). Preemptive treatment with minocycline reversed these effects (Fig. 3a). These effects were confirmed based on calculation of ratios of pro- to anti-inflammatory mediators (IL-1 $\beta$ /IL-1r, iNOS/Arg1; Fig. 3b and c). Double-staining experiments showed that the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine Arg1 co-localized with microglial cells (Fig. 3d and f). The ratio of IL-6 $^{+}$  Iba1 $^{+}$  cells to Iba1 $^{+}$  cells was used to evaluate pro-inflammatory microglial activation, and the ratio of Arg1 $^{+}$  Iba1 $^{+}$  cells to Iba1 $^{+}$  cells was used to evaluate anti-inflammatory microglial activation. Minocycline attenuated second stress-induced increase in the (IL-6 $^{+}$  Iba1 $^{+}$ )/Iba1 $^{+}$  ratio, and it increased the (Arg1 $^{+}$  Iba1 $^{+}$ )/Iba1 $^{+}$  ratio (Fig. 3e and g). Second stress led to significantly higher levels of the pro-inflammatory cytokines IL-6 and IL-1 $\beta$  and significantly lower levels of the anti-inflammatory cytokines TGF- $\beta$  and IL-4 (Fig. 3h–k), and these effects were reversed by

minocycline.

### 3.4. Minocycline ameliorates hippocampal neurogenesis deficits induced by maternal separation and second stress and enhances BDNF expression

Neurogenesis in the hippocampus was assessed by labeling cells with the proliferative cell marker BrdU as well as the newborn neuron-specific marker DCX (Fig. 4a). Second stress led to markedly fewer proliferative cells in the DG of maternal-separated mice than in control animals, and minocycline did not reverse this effect (Fig. 4b). Second stress led to significantly more BrdU $^{+}$  DCX $^{+}$  cells in the DG in the maternal-separated animals than control animals, and minocycline further increased this number in maternal-separated mice (Fig. 4c). The ratio of BrdU $^{+}$  DCX $^{+}$  cells to BrdU $^{+}$  cells was used to assess the level of differentiation of neonatal neurons. Maternal-separated mice showed a greater level of differentiation after second stress if they were treated with minocycline than if they were treated with saline (Fig. 4d).

Minocycline led to significant up-regulation of DCX in the hippocampus following second stress (Fig. 4g). Second stress down-regulated BDNF in the hippocampus, and this effect was attenuated by



**Fig. 4. Neurogenesis in hippocampus of male adolescent mice after maternal separation and second stress.** a. Representative images of cells in hippocampal DG labelled with DCX (red) or BrdU (green) (n = 5). Scale bar, 100  $\mu$ m. b–d. Quantitation of BrdU<sup>+</sup> cells (b), BrdU<sup>+</sup> DCX<sup>+</sup> cells (c), and the ratio of BrdU<sup>+</sup> DCX<sup>+</sup> to BrdU<sup>+</sup> cells (d). e. Magnification of cells in the DG stained with DAPI (blue) and DCX (red) (n = 5). Scale bar, 20  $\mu$ m. f–h. Quantitation of DCX<sup>+</sup> cells (f) and relative levels of mRNAs encoding DCX (g) and BDNF (h) in hippocampus on PD 56 (n = 6). Data are mean  $\pm$  SEM and are analyzed statistically in Table 4. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005; N.S. = not significant (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

minocycline (Fig. 4h).

#### 4. Discussion

Maternal separation early in life contributes to psychiatric illnesses such as depression and anxiety in offspring (Gracia-Rubio et al., 2016; Harrison and Baune, 2014; Uchida et al., 2010). Our results show that when maternally-separated male mice were exposed to a second stress, elevated levels of pro-inflammatory cytokines impaired neurogenesis and were linked to depression-like behaviors. Minocycline inhibited

these microglia-mediated neuroinflammatory processes and stress-induced abnormal behavior.

Environmental factors subsequent to early traumatic experiences have been considered to be important triggers for behavioral abnormalities and psychiatric disorders. In this study we used a “two hit” stress model in mice and assessed behaviors using SPT and FST. The combination of maternal separation and second stress caused depression-like behaviors. Our results are consistent with the idea that maternal separation combined with second stress, but not maternal separation alone, lead to obvious behavioral deficits in adolescent offspring

(Knuesel et al., 2014). It is possible that the "first hit" of maternal separation renders the individual more vulnerable to stress induced by a "second hit" (Meyer, 2014). Although animals placed under chronic stress are widely used to study the neurobiology of depression, one of caveats of these models is that behavioral alterations of animals are their coping strategies to repeated stress exposures (Sadler and Bailey, 2016), which may also have contributed to our results.

In the present study, microglia in the hippocampus of maternal-separated male mice showed a reduced number of processes and shorter branches, indicating microglial activation (Delpech et al., 2016; Roque et al., 2016). In fact, this activated phenotype was further promoted by second stress. Microglia are the primary inflammatory mediator in the central nervous system and the main source of cytokines (Hanisch, 2002; Kim and Joh, 2006). Second stress significantly reduced levels of mRNA encoding CX3CR1, which regulates inflammatory activity by modulating communication between neurons and microglia in the central nervous system (Marin and Kipnis, 2017; Paolicelli et al., 2011; Rogers et al., 2011). At the same time it shifted the balance between pro- and anti-inflammatory cytokines in the hippocampus in favor of pro-inflammatory markers. Given that neuroinflammatory processes correlate with behavioral changes (Zhao et al., 2019), our findings suggest that early maternal separation in mice may contribute to microglia-mediated vulnerability to social stress challenges in the adulthood (Roque et al., 2016).

Minocycline inhibits inflammation in the central nervous system and thereby protects neurons, with minimal side effects (Clemens et al., 2018; Regen et al., 2016). In the present study, minocycline treatment led to microglia with smaller cell bodies and more ramified processes, and it reversed the down-regulation of CX3CR1 induced by maternal separation. Minocycline also rebalanced relative expression of the pro-inflammatory cytokines IFN- $\gamma$ , IL-6, and IL-1 $\beta$  as well as expression of the anti-inflammatory cytokine IL-4. The results suggest that using minocycline preemptively to inhibit microglial activation can decrease the susceptibility to stress. To the best of our knowledge, the present study shows that minocycline can inhibit inflammation-mediated neurotoxicity and ameliorate abnormal behavior in an animal model of early life stress.

Early life stress has lasting effects on adult neurogenesis and increases vulnerability to psychopathology in later life (Korosi et al., 2012; Naninck et al., 2015). Microglia in the hippocampus contribute to neurogenesis by clearing apoptotic cellular debris and providing neurotrophic support, and inflammatory events sustained of neural precursor cells niches in the postnatal brain of offspring, which contribute to neurodevelopmental disorders (Hammond et al., 2018; Ma et al., 2016; Paolicelli et al., 2011). In our study, maternal separation and second stress were associated with reductions in the total number of DCX<sup>+</sup> cells and the ratio of BrdU<sup>+</sup> to DCX<sup>+</sup> cells in adolescent male mice. This suggests that maternal separation and second stress cause inflammatory activation of the microglia, ultimately reducing neurogenesis in the hippocampal DG. Minocycline reversed this inhibition of neurogenesis by inhibiting inflammatory responses (Clemens et al., 2018; Regen et al., 2015). Our results are consistent with the idea that reversing stress-induced impairment of microglia-mediated neurogenesis can rescue depression-like behaviors in two-hit mice. However, this conclusion remains quite preliminary because we administered minocycline systemically, and the drug has pleiotropic effects on the peripheral and central nervous systems. Further work should clarify whether and how minocycline is responsible for the observed improvement in behavior in our twice-stressed animals.

The influence of early negative life events, such as maternal neglect, can last until adulthood and even throughout life. Stress initiates microglia-mediated inflammatory processes in the postnatal brain and renders it more vulnerable to negative effects of a second stress during adolescence. Two-hit of maternal separation combination with second stress may then impair neurogenesis and contribute to behavioral disorders. The intervention strategy of targeting microglia with

minocycline appears to be able to mitigate early adversity-induced behavioral outcomes. Further study should clarify whether minocycline exerts these effects by acting on microglia or by modulating other processes.

### Conflict of interest

The authors declare that they have no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.psyneuen.2019.04.021>.

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