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# Emotional Impairments and Neuroinflammation are Induced in Male Mice Invulnerable to Repeated Social Defeat Stress

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**Abstract—Prolonged stress triggers neuroinflammation, which plays a significant role in the development of depression; however, stressed people do not always suffer from depression because of individual differences in stress vulnerability. Negative cognitive bias (NCB) toward pessimistic judgment often underlies depressive episodes. However, a relationship between stress vulnerability, neuroinflammation, and NCB remains elusive. In addition, an animal model with all the traits would be a powerful tool for studying the etiology of depression and its therapeutic approaches. Accordingly, this study evaluated the effect of stress vulnerability on neuroinflammation and depression-related behaviors, including NCB in males, using a modified version of repeated social defeat stress (mRSDS) paradigm, a validated animal model of psychosocial stress. Exposure to mRSDS, consisting of 5 min of social defeat by unfamiliar CD-1 aggressor mice for five consecutive days, caused NCB, which co-occurred with depressive- and anxiety-like behaviors, and neuroinflammation in male BALB/c mice. Treatment with minocycline, an antibiotic with anti-inflammatory property, blocked mRSDS-induced depressive-like behaviors and neuroinflammation, but not NCB, indicating the limited effect of an anti-inflammatory intervention. In addition, marked differences were found in neuroinflammatory profiles and hippocampal gene expression patterns between resilient and unstressed mice, as well as between susceptible and resilient mice. Therefore, mice resilient to mRSDS are indeed not intact. Our findings provide insights into the unique features of the mRSDS model in male BALB/c mice, which could be used to investigate the etiological mechanisms underlying depression as well as bridge the gap in the relationship between stress vulnerability, neuroinflammation, and NCB in males.** 

**Key words: social defeat stress, negative cognitive bias, depression, neuroinflammation, microglia.**

## INTRODUCTION

Stress does not necessarily result in detrimental consequences, and it has beneficial effects on immune responses, brain function, and even stress sensitivity *per se* in certain circumstances. For instance, short-term moderate stress could enhance cellular immunity (Dhabhar et al., 2000, 2010) and induce initial acquisition of immune memory (Dhabhar and Viswanathan, 2005) and extinction of fear memory (Kirby et al., 2013). Moderate stressful events in life could also lead to stress resiliency to aversive experiences in later life in animals (Biggio et al., 2014; Santarelli et al., 2017) and humans (Chaby et al., 2013; Hsiao et al., 2016). Although prolonged exposure to stressful events has been demonstrated to induce emotional distress, consequently exerting immunosuppressive effects (Glaser and Kiecolt-Glaser, 2005) and causing psychiatric disorders, including anxiety and depression (Hettema et al., 2006; Kendler et al., 1999), some people (i.e., those with resilience)

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*Abbreviations:* ACFC, ambiguous-cue fear conditioning; ANOVA, analysis of variance; ATP, adenosine triphosphate; CS, conditioned stimuli; DG, dentate gyrus; DMEM, Dulbecco's modified Eagle's medium; FST, forced swim test; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; ICAM-1, intercellular adhesion molecule 1; LD, light–dark; LPS, lipopolysaccharide; Mino, minocycline; mRSDS, modified version of repeated social defeat stress; NCB, negative cognitive bias; NG2, polydendrocytes; NPY, neuropeptide Y; PBS, phosphate-buffered saline; SEM, standard error of the mean; SI, social interaction; TNF-α, tumor necrosis factor-α.

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can successfully cope with a range of adversity in their life even under the prolonged stressful conditions (Johnson et al., 2017), probably because of individual differences in stress vulnerability. To date, a variety of experimental animal stress models of emotional disturbance resulting from long-lasting exposure to stress, such as chronic mild stress (Willner, 2017; Willner et al., 1992), chronic restraint stress (McLaughlin et al., 2007), and maternal separation (Tractenberg et al., 2016), have been established in rodents. Notably, repeated social defeat stress (RSDS) (also called chronic SDS) paradigm, a validated animal model of psychosocial stress, has been widely used to investigate individual differences in stress vulnerability (Chou et al., 2014; Golden et al., 2011; Gomez-Lazaro et al., 2011; Hodes et al., 2014; Krishnan, 2014). In this paradigm, RSDS-exposed mice are often grouped into susceptible and resilient mice based on social avoidance behavior, which is a feature of depressive symptoms. However, studies have shown that resilient mice, as well as susceptible mice, exhibit anxiety-like behaviors, polydipsia, and high levels of corticosterone under swim stress (Krishnan et al., 2007), and resilient mice, rather than susceptible mice, display enhanced fear responses and deficits in fear extinction (Meduri et al., 2013). These findings would make it complex to interpret the resiliency and stress vulnerability in rodents.

Recently, it has been demonstrated that stress triggers neuroinflammation (Couch et al., 2013; Kreisel et al., 2014; Yirmiya et al., 2015), which is potentially associated with the pathology of psychiatric disorders, including depression (Muller, 2014) and anxiety (Salim et al., 2012). Several types of glial cells, including microglia (Yirmiya et al., 2015), astrocytes (Koo and Duman, 2009; Leng et al., 2018; Salmina et al., 2015), and polydendrocytes (NG2) cells (Nakano et al., 2017), have been well recognized to mediate the neuroinflammatory process in the brain. RSDS or interferon-alpha-induced neuroinflammation (McKim et al., 2016a,b; Wachholz et al., 2016) is prevented by the classical tricyclic antidepressant imipramine or an antibiotic with M1 (proinflammatory)-type microglial deactivation minocycline (Mino) (Kobayashi et al., 2013), consequently leading to recovery from depression-like behaviors (Ramirez et al., 2015; Zheng et al., 2015). These findings raise the possibility that suppression of neuroinflammation could be a therapeutic strategy for depression.

In addition, depressive episodes are concomitant with negative cognitive bias (NCB) toward pessimistic judgments (Beck, 2008; Erickson et al., 2005; Mendl et al., 2009), a significant characteristic of depression that is a negatively deformative interpretation (i.e., negative thinking) against ambiguous aversive situation (Chan et al., 2008; Dearing and Gotlib, 2009). Accumulating evidence has revealed that NCB universally occurs in a range of animals such as mice (Boleij et al., 2012; Crestani et al., 1999; Klemenhagen et al., 2006; Tsetsenis et al., 2007), rats (Enkel et al., 2010; Henningsen et al., 2009; Papciak et al., 2013; Rygula et al., 2013), chicks (Salmeto et al., 2011), and honeybees (Bateson et al., 2011), as well as humans, under particular conditions (e.g., RSDS or chronic mild stress, congenital helplessness, and genetic manipulation). NCB in depression could also contribute to the duration and severity of depressive episodes (Teasdale, 1983). Intriguingly, a few studies have demonstrated that chronic treatment with fluoxetine, a selective serotonin reuptake inhibitor, has a limited role in reducing NCB in congenital helpless rats (Anderson et al., 2013), and environmental enrichment can induce positive cognitive bias in rats (Brydges et al., 2011). However, it remains challenging to improve NCB in depression, and it is somewhat uncertain whether NCB is associated with stress vulnerability and neuroinflammation.

In this study, we hypothesized that there is a close relationship between stress vulnerability, NCB, and neuroinflammation induced by RSDS. Accordingly, using a stress hypersensitive-prone BALB/c mouse strain (Jacobson and Cryan, 2007), we examined whether RSDS-induced stress vulnerability affects behavioral functions (i.e., depression- and anxiety-like behaviors and NCB) and neuroinflammation, and investigated the effects of anti-inflammatory intervention by Mino treatment on RSDS-elicited changes.

## EXPERIMENTAL PROCEDURES

### Animals

Male BALB/c (7 weeks of age) and CD-1 (retired breeders) mice were purchased from Japan SLC (Hamamatsu, Japan). All animals were allowed to acclimate for at least 1 week after arrival. BALB/c mice were group-housed (four mice/cage), and CD-1 mice were singly housed during acclimation in a controlled environment (temperature, 23 ± 2 °C; humidity, 55% ± 10%; 12-h light/dark cycle with lights on at 08:00) with food (CE-2, CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. All cages (22.5 × 33.8 × 14 cm, CLEA Japan, Inc.) were provided with wood bedding material (Japan Laboratory Animals, Inc., Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan. Every effort was made to minimize the number of animals used and their suffering.

### Modified version of repeated social defeat stress (mRSDS)

This stress paradigm was performed as previously described (Golden et al., 2011; Ito et al., 2017) with some modifications. Briefly, testing BALB/c mice encountered unfamiliar resident CD-1 aggressor mice in their home cage for 5 min daily over five consecutive days (days 1–5, Fig. 1A). The CD-1 mice were screened and designated as aggressors only if their attack latencies were shorter than 60 s in 2–3 consecutive days prior to this stress paradigm. After 5 min of confrontation, testing mice were housed in pairs separated by a clear perforated Plexiglas divider for 24 h with free access to food and

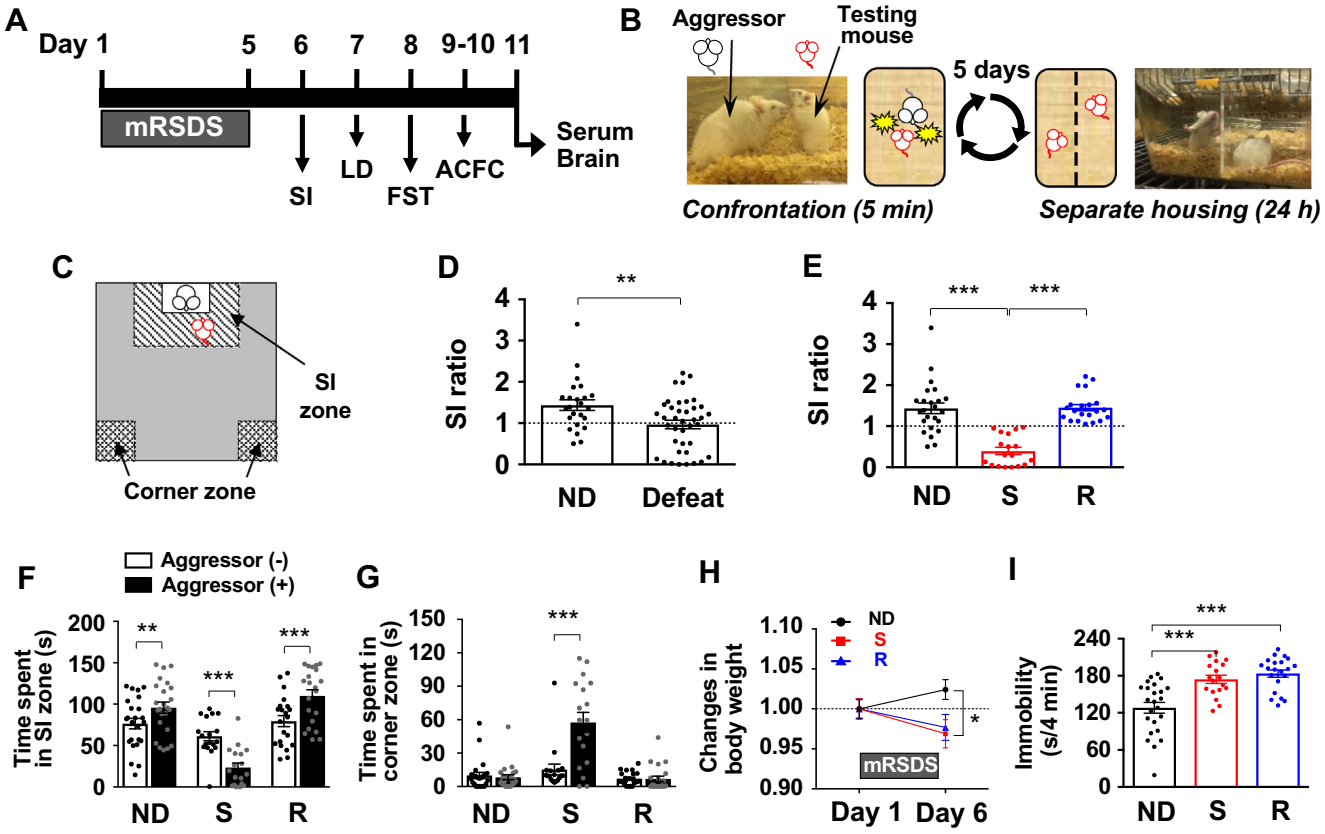


**Fig. 1.** mRSDS induces depression-like behaviors in BALB/c mice. **(A)** A representative schematic diagram of the experimental schedule. **(B)** A schematic procedure for mRSDS. **(C)** A schematic diagram showing a SI zone and corner zones. SI ratio **(D, E)** and time spent in SI **(F)** and corner zone **(G)** in the presence or absence of an aggressor mouse during the SI. **(H)** Changes in body weight, calculated by dividing the body weight on day 6 by that on day 1. **(I)** Duration of immobility in the FST. Data are represented as mean ± SEM ($n$ = 18–24 per group). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001. ND, no defeat; R, resilient; S, susceptible.

water (Fig. 1B). On each testing day, testing mice were defeated by novel aggressor mice to avoid acclimation to familiar aggressors. Undefeated control mice were handled every day, housed in pairs, separated by the perforated divider in cages, and rotated daily in a manner similar to defeated mice, but they were never exposed to aggressors. This stress paradigm was carried out between 14:00 and 16:00.

### Behavioral tests

In all behavioral tests from day 6 through day 10, mice were transported to the testing room and allowed to habituate to the room for approximately 60 min prior to testing. For minimizing the interference of each behavioral test, behavioral tests were performed in a manner to gradually increase stress intensities (days 6–10, Fig. 1A). Stress exposure and subsequent behavioral tests were independently performed in duplicate and combined to represent total number of animals per group.

### Social interaction test

Social interaction test was performed as previously described (Golden et al., 2011; Ito et al., 2017). On day 6 (Fig. 1C), each mouse was introduced into an opaque gray open field box (40 × 40 × 40 cm) with an empty perforated Plexiglas enclosure (7 × 10 × 40 cm) located in the social interaction (SI) zone (13.5 × 24.0 cm) at one end of the box and was allowed to explore freely for 150 s (the first trial). The mouse was then removed from the box and placed back into a holding cage for roughly 1 min. In the second trial, the mouse was re-introduced into the box with an unfamiliar aggressor mouse and was allowed to explore again for 150 s. Time spent in the SI zone and/or corner zone and total distance moved during each trial were recorded by a video tracking system (EthoVision 3.0; Noldus, Wageningen, Netherlands). The SI ratio was calculated by dividing the time spent in the SI zone when the aggressor mouse was present by the time spent in the SI zone when the aggressor mouse was absent. Conventionally, mice with a SI ratio of less than 1 were regarded as susceptible mice; in contrast, mice with a SI ratio more than 1 were regarded as resilient mice (Krishnan et al., 2007; Golden et al., 2011). This test was carried out between 12:00 and 17:00.

### Light–dark (LD) test

LD test was performed on Day 7 in a 2-compartment shuttle box (27 × 44 × 18.7 cm) as previously described (Kinsey et al., 2007) with some modifications. One

compartment (light side) with opaque white walls was open (approximately 300 lux), whereas the other compartment (dark side) with opaque black walls was closed (approximately 10 lux); both sides were equipped with the infrared sensor detection system (Supermex; Muromachi Kikai Co., Ltd., Tokyo, Japan). Mice were introduced into the position near the wall in the light side, facing away from the dark side, and were then allowed to explore freely for 5 min. Transitions of the testing mouse between the two compartments were monitored by a videotape recorder and infrared sensors. Total duration in the light side and latency to enter and frequency into the light side were manually scored, and locomotion in both sides was analyzed using CompACT AMS software (Muromachi Kikai Co., Ltd.). This test was carried out between 15:00 and 17:00.

### Forced swim test (FST)

FST was performed on Day 8 as previously described with some modifications (Krishnan et al., 2007). Mice were placed individually into a 5-L beaker containing 4 L of tap water (23 ± 1 °C) for 6 min. A mouse was considered immobile when it ceased struggling and remained floating motionless, with only the movements necessary to keep its head above water. All behaviors were videotaped, and the duration of immobility during the last 4 min of the FST was scored. This test was carried out between 15:00 and 17:00.

### Ambiguous-cue fear conditioning

Ambiguous-cue fear conditioning was carried out using two conditioning chambers equipped to deliver a scrambled foot shock through the grid floor, each of which was localized in a soundproof wooden box, as previously described (Crestani et al., 1999; Tsetsenis et al., 2007) with the following modifications. On the training day (Fig. 2A), mice were placed into the conditioning chamber for 8 min (light off at 40 s and on at 460 s). During the training, a tone cue (3 kHz, 20 s) was presented five times at 100, 180, 260, 340, and 420 s. Mice also received aversive foot shocks (0.7 mA, 1 s), which were terminated with each tone cue. After training, mice were returned to their home cages. On the testing day (Fig. 2B), mice were placed into the chamber with a different visual, olfactory, and tactile context (a white plastic floor, plastic round walls, and 2% vanilla scent) for 3 min. During testing, mice were habituated in the chamber lit for 30 s, and two conditioned stimuli (CS) were then presented with a 30-s interval: one dark for 60 s and one dark paired with tone (3 kHz) for 60 s. In the training session, the percentage of time spent freezing was quantified within every 20 s for 8 min using a video-based system with a motion detection algorithm (FreezeFrame 4, Actimetrics, Wilmette, IL, USA). In the testing session, the percentage of time spent freezing was also quantified during 30-s habituation (baseline) and the last 30 s of each CS presentation. In this paradigm, ‘‘dark” is a partial predictor (i.e., partial cue), and ‘‘dark-tone pairing” is a perfect one of the foot shocks (i.e., perfect cue). In this way, foot shock is sometimes received when the partial cue is presented, whereas the foot shock is always received when the perfect cue is presented (Crestani et al., 1999). Thus, mice are regarded to have NCB when no difference in freezing responses are found between partial and perfect cue presentation. Each session was carried out between 12:00 and 17:00.

### Brain perfusion

On day 11, under deep inhaled anesthesia with isoflurane (Pfizer, Tokyo, Japan), mice were transcardially perfused with cold phosphate-buffered saline (PBS), followed by a cold 4% paraformaldehyde solution (Wako Pure Chemical Industries, Osaka, Japan). Brains were collected and postfixed in a 4% paraformaldehyde solution at 4 °C overnight and then stored in 0.02% $NaN_3$/PBS at 4 °C until brain sectioning.

### Immunostaining

Serial coronal sections (50-μm thick) were obtained throughout the hippocampus using a vibratome (Technical Products International, St. Louis, MO, USA). Staining was performed in 24-well plates on free-floating sections. After incubation with 3% $H_2O_2$/80% methanol for 40 min at room temperature (RT), free-floating sections were incubated for 1 h at RT in a blocking buffer [1% bovine serum albumin (BSA; Wako Pure Chemical Industries) in PBS containing 0.3% Triton X-100 (PBS-T)], followed by incubation with primary antibodies in the blocking buffer at 4 °C. After rinsed in PBS-T, sections were incubated at RT with secondary antibodies. The antibodies used in immunostaining are listed in Table S1. Particularly, in NG2 staining, sections were incubated in an antigen retrieval solution (HistoVT One, Nacalai Tesque, Kyoto, Japan) for 15 min at 90 °C before the blocking step. Sections were then rinsed in PBS-T, incubated for 1 h at RT with the ABC kit (Vector Laboratories), and visualized with Vector DAB (Vector Laboratories). Sections were mounted on silane-coated slides, dried, counterstained with 0.05% toluidine blue (Sigma, St. Louis, MO, USA), dehydrated, and coverslipped. Images were captured using a light microscope (Olympus BX-41, Olympus Corporation, Tokyo, Japan). For quantitative image analyses, Iba1-, NG2-, and NPY-positive cells were counted using ImageJ software on every fourth section throughout the hippocampus of a brain hemisphere (bregma −1.5 mm to −3.4 mm). GFAP- and ICAM-1-positive staining were assessed as the average percent areas in the molecular layer and dentate gyrus (DG), respectively, on every fourth section throughout the hippocampus of a brain hemisphere.

### Assays for serum corticosterone and adiponectin

On the next day after behavioral tests, blood samples were collected from the iliofemoral artery under deep anesthesia with isoflurane. The blood samples were centrifuged at 6000 rpm for 1 min at 4 °C, and sera were stored at −80 °C until assayed. Commercial ELISA kits were used to assess serum levels of corticosterone

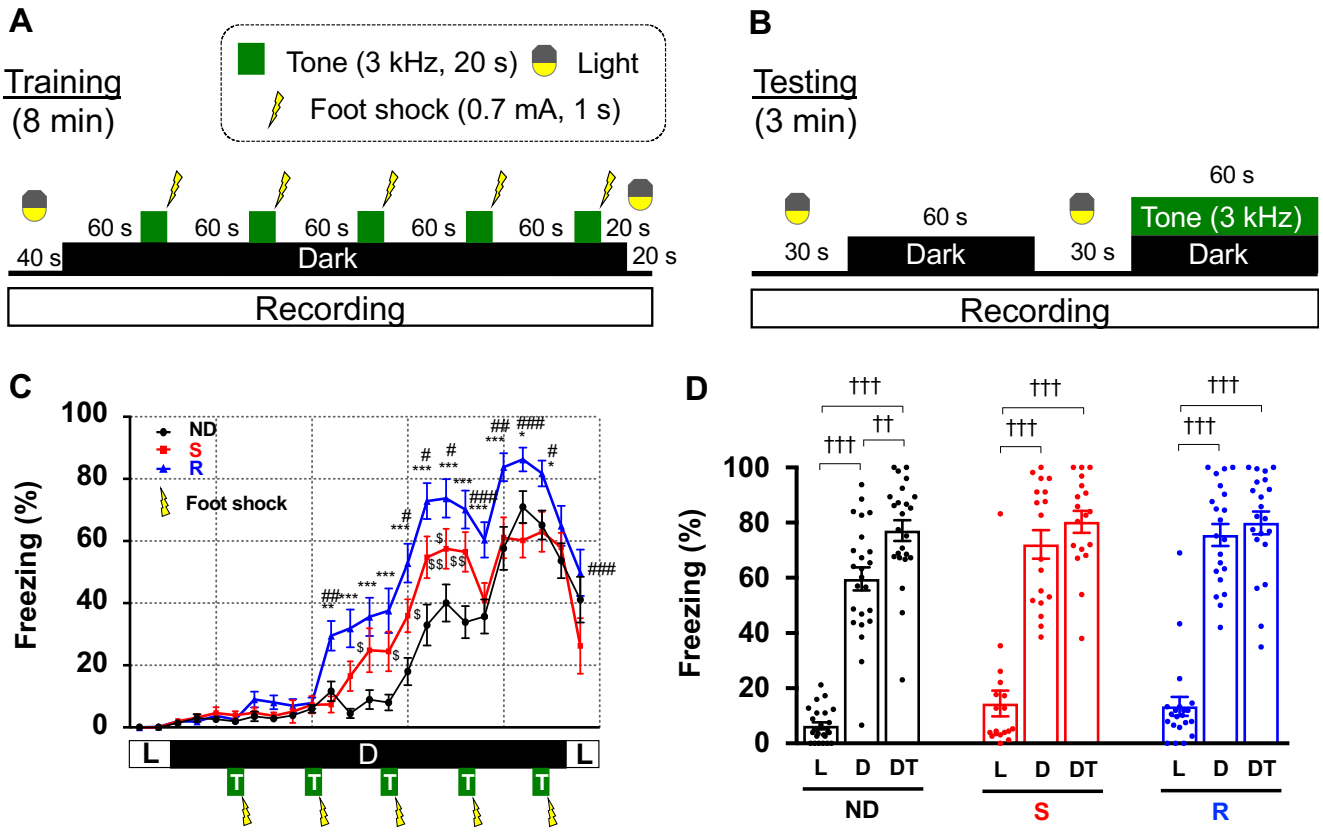


**Fig. 2.** mRSDS triggers negative cognitive bias in BALB/c mice. Schematic diagrams of the training session for 8 min **(A)** and the testing session for 3 min **(B)** in the ACFC. **(C)** A transition of percentage of freezing within 20-s bins for 8 min in the training of ACFC. **(D)** Percentage of freezing during 30-s lit habituation, the last 30-s dark, and the last 30-s dark/tone pairing. Data are represented as mean ± SEM ($n$ = 18–24 per group). R vs. ND: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; S vs. R: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; S vs. ND: $^{\$}p < 0.05$, $^{\$\$}p < 0.01$; $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$. D, dark; DT, dark and tone; L, light; ND, no defeat; R, resilient; S, susceptible; T, tone.

(Assaypro, St. Charles, MO, USA) and adiponectin (ALPCO, Salem, NH, USA) according to the manufacturer's instructions.

### Microglia isolation

On days 7–9, microglia were isolated from the adult testing mouse whole brain, except the cerebellum, as described previously (Ito et al., 2017; Lee and Tansey, 2013; Singh et al., 2014) with some modifications. Briefly, following decapitation, the whole brain, except the cerebellum, was readily extracted and chopped finely with a fine sharp scissor in ice-cold serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma) containing papain (20 U/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA), DNase I (2 mg/ml, Sigma), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The brain pieces prepared were incubated in a water bath at 37 °C for 20 min. Enzymatic digestion with papain was terminated by adding ice-cold DMEM/F12 containing 20% horse serum (Invitrogen) and 1% penicillin/streptomycin. The brain pieces were further triturated by gently pipetting and were then passed through a 100-μm cell strainer (Greiner Bio-one, Tokyo, Japan) to remove cell debris and undigested tissue pieces. The filtered cell suspension was centrifuged at 1000×$g$ for 5 min at 4 °C, and the supernatant was decanted. The cell pellet was then re-suspended by slow pipetting with 30% isotonic Percoll (GE Healthcare, Tokyo, Japan) in Hank's balanced salt solution without calcium and magnesium (Sigma) and centrifuged at 700×$g$ for 10 min at 4 °C. After the supernatant was aspirated, the cell pellet was re-suspended by pipetting with a lysis buffer (150 mM $NH_4Cl$, 0.24 mM $NaHCO_3$, 0.068 mM EDTA in distilled water, pH 7.4) to remove red blood cells, and the cell suspension was then centrifuged at 1000×$g$ for 5 min at 4 °C. This process was repeated twice to eliminate the remaining dead cells, red blood cells, and Percoll. The cell pellet was re-suspended in DMEM/F12 containing 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin, and the cell suspension was filtered through an 11-μm nylon mesh (Merck Millipore, Billerica, MA, USA). The harvested cells were counted using a handheld automated cell counter (Scepter 2.0, Merck Millipore).

### Ex vivo microglial stimulation assay with LPS and/or ATP

After microglia were plated at a density of $5 \times 10^4$ cells/well in 96-well plates (Fig. 5H) for 40 min, they were stimulated with lipopolysaccharide (LPS; serotype O111: B4; final concentration, 0.1 μg/ml; Sigma) or PBS for 17 h in a 5% $CO_2$ incubator at 37 °C. Supernatants were collected and stored at −80 °C until assayed for

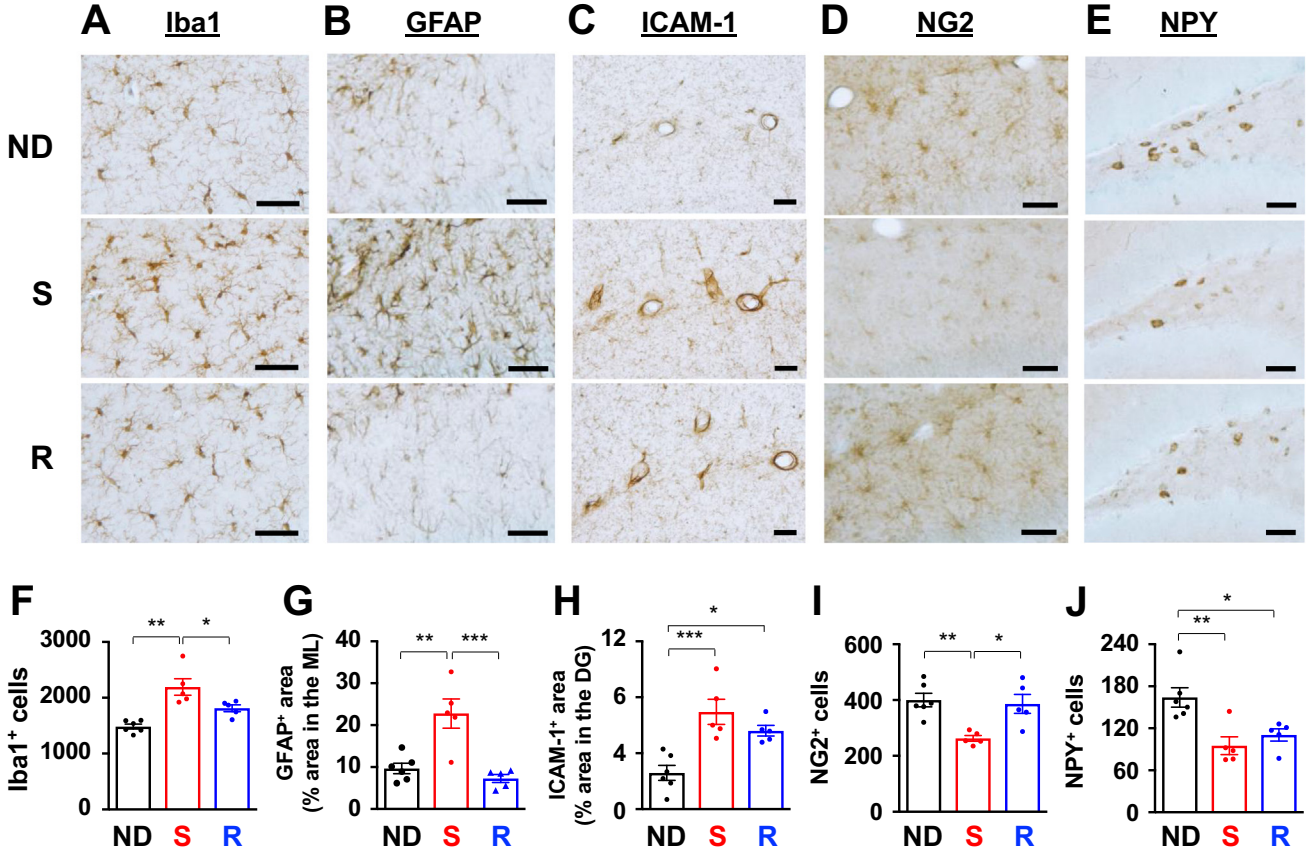


**Fig. 3.** Distinct neuroinflammatory profiles of susceptible and resilient mice. **(A-E)** Representative photomicrograph of positive cells and area for Iba1 **(A)**, GFAP **(B)**, ICAM-1 **(C)**, NG2 **(D)**, and NPY **(E)** in the dentate gyrus (DG) of the hippocampus. Scale bar = 50 μm. **(F–J)** The number of Iba1 **(F)**, NG2 **(I)**, and NPY **(J)**-positive cells in the DG. The percent positive area for GFAP **(G)** and NG2 **(H)** labeling in the molecular layer of the DG. Data are represented as mean ± SEM ($n$ = 5–6 per group). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001. ND, no defeat; R, resilient; S, susceptible.

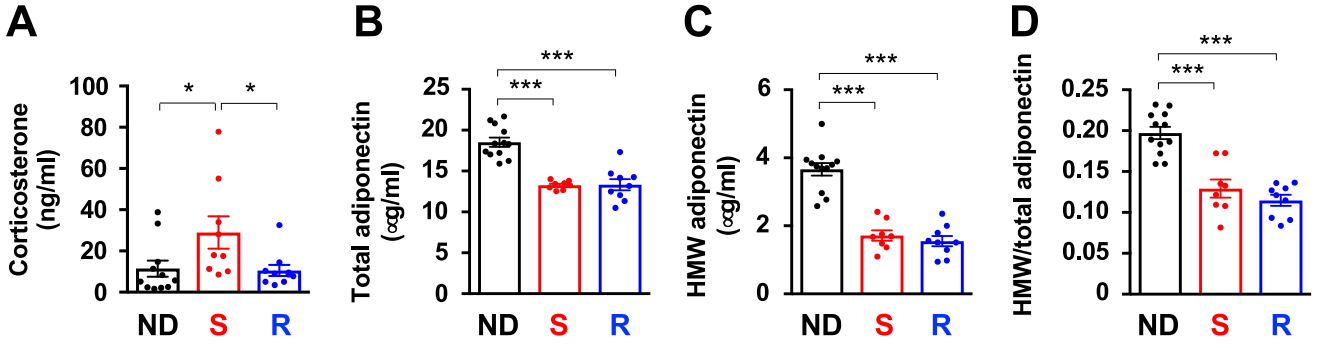


**Fig. 4.** Different biochemical serum profiles in susceptible and resilient mice. Serum levels of corticosterone **(A)** and total **(B)** and HMW adiponectin **(C)**. **(D)** A ratio of total and HMW adiponectin. Data are represented as mean ± SEM ($n$ = 8–12 per group). *$p$ < 0.05, ***$p$ < 0.001. ND, no defeat; R, resilient; S, susceptible.

IL-6 and TNF-α levels. In another experiment, adenosine triphosphate (ATP; final concentration, 0.1 mM; Sigma) or PBS was added 2 h after LPS stimulation, and 30 mins later, supernatants were collected and stored at −80 °C until assayed for IL-1β levels. In the experiments for the blockade effect of Mino (Fig. S2), Mino (Sigma; final concentration at 0–50 μg/ml) or PBS was added 40 min after microglial plating, and after incubation for 30 min, microglial cells were stimulated with LPS or PBS for 17 h. ATP (final concentration, 0.1 or 0.5 mM) or PBS was also added 3.5 h after LPS stimulation. After final stimulation with LPS for 17 h or ATP for 30 mins, supernatants were collected and stored at −80 °C until assayed for IL-6 and IL-1β levels. The remaining cells were incubated with 10% Alamar Blue (Thermo Fisher Scientific, Waltham, MA, USA) in DMEM/F12 for 90 min at 37 °C and 5% $CO_2$, and cell viability was measured using fluoroscopy with an Infinite M200 microplate

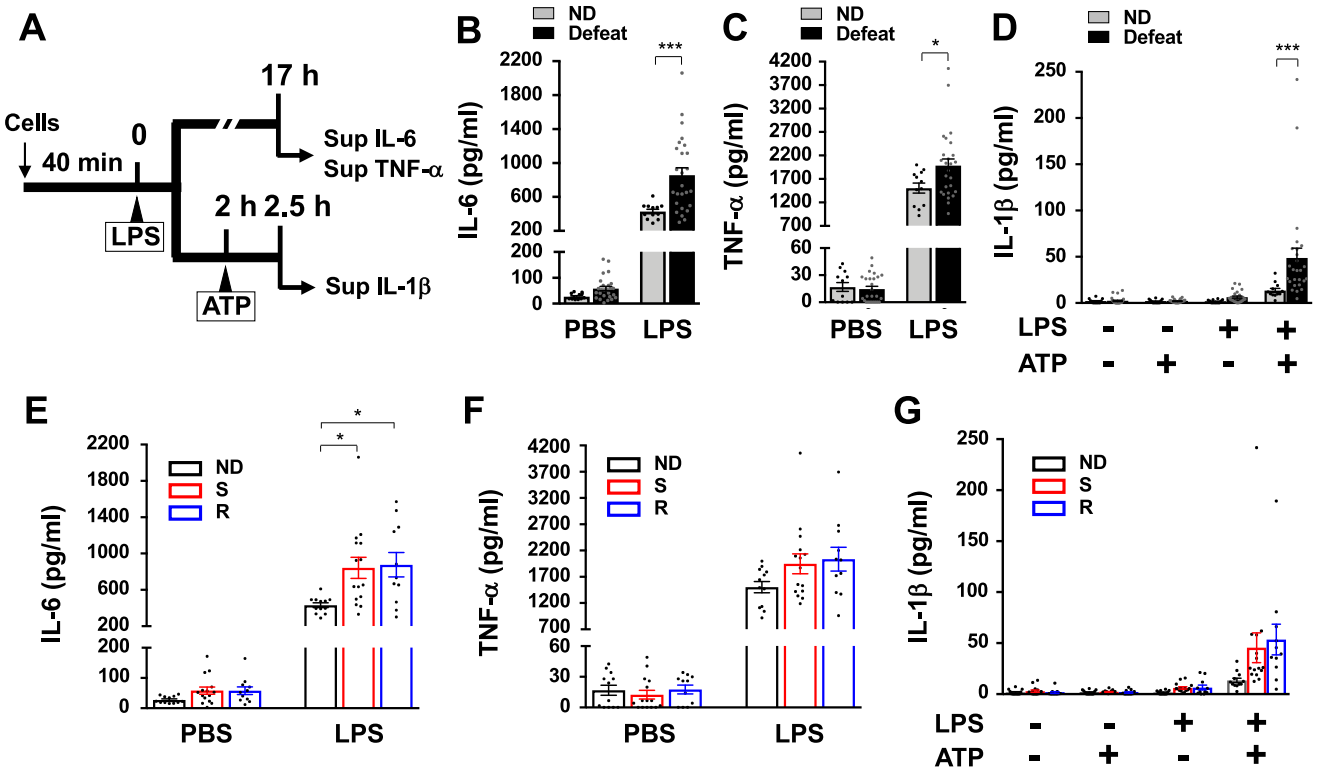


**Fig. 5.** Microglial response to immune stimulation in susceptible mice is similar to that in resilient mice. **(A)** A schematic diagram of the experimental schedule for *ex vivo* microglial stimulation assay with LPS and/or ATP. IL-6 **(B)** and TNF-α **(C)** levels of the supernatants in the absence or presence of LPS (non-defeated vs. defeated mice). **(D)** IL-1β levels of the supernatants in the absence or presence of LPS and/or ATP (non-defeated vs. defeated mice). **(E-G)** IL-6 **(E)** and TNF-α **(F)**, and IL-1β **(G)** levels of the supernatants in non-defeated, susceptible, and resilient mice. Data are represented as mean ± SEM ($n$ = 11–15 per group). *$p$ < 0.05, ***$p$ < 0.001. ND, no defeat; R, resilient; S, susceptible.

reader (Tecan Group Ltd., Männedorf, Switzerland) (excitation and emission wavelength at 544 and 590 nm, respectively).

### Assays for IL-6, TNF-α, and IL-1β

Cytokine levels in the supernatants were detected using commercially available ELISA kits (BD OptEIA™ ELISA set for IL-6 and IL-1β, BD Biosciences, San Diego, CA, USA; Mouse DuoSet ELISA for TNF-α, R&D systems) according to the manufacturer's instructions.

### Mino treatment

Mino (50 mg/kg, *i.p.*; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) or PBS was administered for 6 days under stressed or non-stressed conditions. In the stress paradigm, Mino treatment was initiated 1 day prior to the onset of mRSDS, and was terminated on the last day of mRSDS (Fig. 6A). The dose was chosen based on previous studies (Henry et al., 2008; Borre et al., 2012; Zheng et al., 2015).

### Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM), and analyzed using Prism7.0 (GraphPad Software, San Diego, CA, USA). Differences between two groups were compared by unpaired or paired two-tailed $t$ test. Comparisons between three or more groups were examined using one-way analysis of variance (ANOVA), or two-way repeated measures ANOVA, followed by Bonferroni's post hoc test. In all cases, statistical significance was set at $P < 0.05$.

## RESULTS

### mRSDS induces depression-like behaviors and NCB in BALB/c mice

The social interaction (SI) ratio was significantly lower in socially defeated mice than in undefeated control mice (Fig. 1D; unpaired two-tailed $t$ test; $t = 2.85$, df = 61, $p = 0.006$). Based on criteria for stress-vulnerable phenotypes in the SI test (Golden et al., 2011; Krishnan et al., 2007), defeated mice were classified into two cohorts, i.e., susceptible and resilient mice, which have SI ratios of less and more than 1, respectively [Fig. 1E; one-way ANOVA; $F_{(2,60)} = 30.46$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$]. Undefeated control mice and resilient mice spent longer time, whereas susceptible mice spent a much shorter time in the SI zone in the presence than in the absence of an aggressor (Fig. 1F; paired two-tailed $t$ test; undefeated: $t = 3.159$, df = 23, $p = 0.0044$; susceptible: $t = 5.207$, df = 17, $p < 0.0001$; resilient: $t = 8.2$, df = 20, $p < 0.0001$). Correspondingly, susceptible mice spent longer time in the corner zones in the presence than in the absence of an aggressor (Fig. 1G; paired two-tailed $t$ test; undefeated:

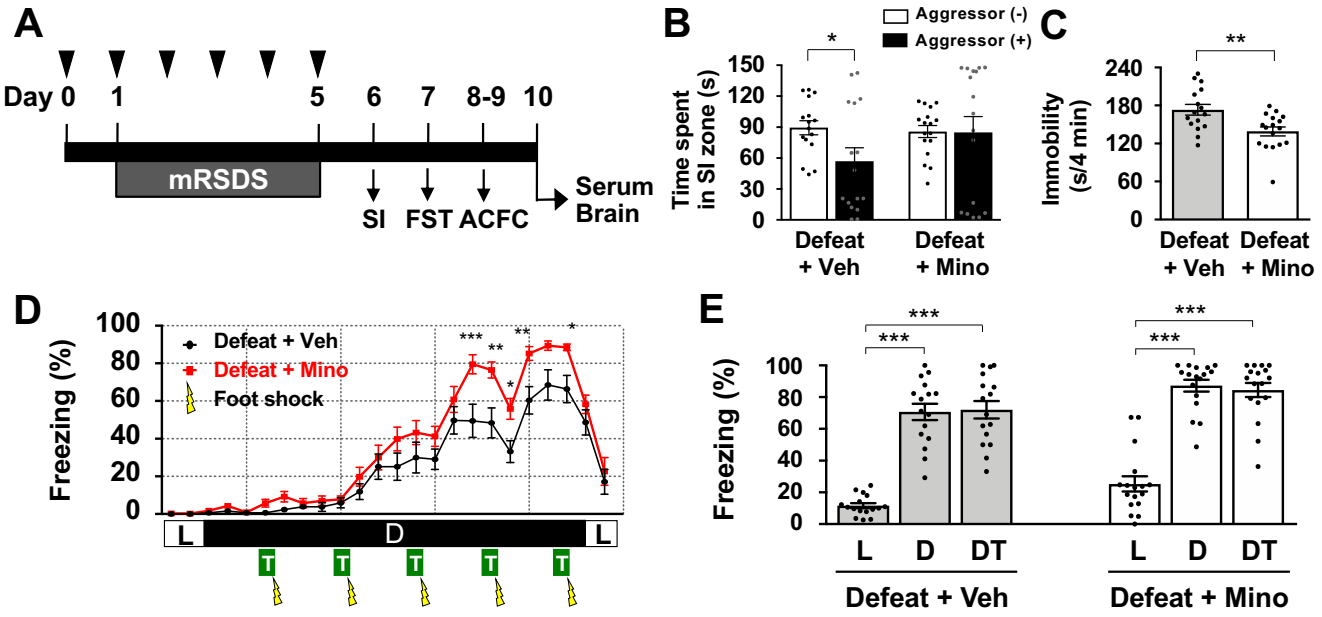


**Fig. 6.** Minocycline reverses mRSDS-induced depressive-like behaviors without affecting negative cognitive bias in mice. **(A)** A representative schematic diagram of the experimental schedule. Arrowheads show minocycline (Mino) injection. **(B)** Effects of Mino on mRSDS-induced reduction of time spent in the SI zone in the SI test. **(C)** Effects of Mino on mRSDS-induced increase in immobility in the FST. **(D)** A transition of percentage of freezing within 20-s bins for 8 min in the training of ACFC in Mino or vehicle-treated mice subjected to mRSDS. **(E)** Percentage of freezing during 30-s lit habituation, the last 30-s dark, and the last 30-s dark/tone pairing in Mino or vehicle-treated mice subjected to mRSDS. Data are represented as mean ± SEM ($n$ = 16–17 per group). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001. D, dark; DT, dark and tone; L, light; Mino, minocycline; T, tone; Veh, vehicle.

$t$ = 0.9196, df = 23, $p$ = 0.3673; susceptible: $t$ = 4.994, df = 17, $p$ < 0.0001; resilient: $t$ = 0.1101, df = 20, $p$ = 0.9134). A significant loss in body weight was observed in susceptible mice, but not in resilient mice, compared with undefeated control mice on day 6 [Fig. 1H; one-way ANOVA; $F_{(2,60)}$ = 4.048, $p$ = 0.0224; Bonferroni's post hoc test, $p$ < 0.05]. In the LD test on day 7, only susceptible mice tended to have reduced duration in and frequency into the light side and have a long latency from dark to light side [Table S2; One-way ANOVA; Duration in light side, $F_{(2,60)}$ = 2.642, $p$ = 0.0795; Latency, $F_{(2,60)}$ = 3.116, $p$ = 0.0516; Frequency into light side, $F_{(2,60)}$ = 2.956, $p$ = 0.0596; Bonferroni's post hoc test, $p$ < 0.1]; however, no difference in locomotion in either the dark or the light side was found between groups. Such a failure of anxiety induction by mRSDS was inconsistent with the findings in previous studies, which have demonstrated an anxiety-like phenotype in mRSDS-exposed mice (Kinsey et al., 2007; Krishnan et al., 2007). Given that the BALB/c mouse used in this study is perceived as an anxiety-prone strain (Jacobson and Cryan, 2007), we examined whether residential environment (Fig. S1B) without mRSDS *per se* affects anxiety-like behaviors and locomotion in their home cages. Mice in separate housing employed in this study showed significant increases in the distance moved during the open field test (Fig. S1C; unpaired two-tailed $t$ test; $t$ = 3.162, df = 9, $p$ = 0.0115) and in the latency into light side during the LD test (Fig. S1D; unpaired two-tailed $t$ test; $t$ = 2.698, df = 9, $p$ = 0.0245) compared with those in group housing. In addition, higher cumulative locomotor activity in home cages was found in separate housing mice than in group housing mice during both light and dark phases [Fig. S1E, F; one-way ANOVA; days 3–5, $F_{(3,16)}$ = 26.38, $p$ < 0.0001; days 8–10, $F_{(3,16)}$ = 30.36, $p$ < 0.0001]; however, average body temperature in home cages was lower in separate housing mice than in group housing mice [Fig. S1E, F; one-way ANOVA; days 3–5, $F_{(3,16)}$ = 16.36, $p$ < 0.0001; days 8–10, $F_{(3,16)}$ = 11.51, $p$ = 0.0003]. The high activity of separate housing mice in the open field box and home cage would indicate a lack of calmness, presumably resulting from anxiety. Thus, the findings suggest that separate housing, along with a slight imbalance of heat production, leads to elevated basal levels of anxiety in mice, including non-stressed control mice. In the FST on day 8, the duration of immobility was significantly increased in both susceptible and resilient mice compared with undefeated control mice [Fig. 1I; one-way ANOVA; $F_{(2,60)}$ = 17.13, $p$ < 0.0001; Bonferroni's post hoc test, $p$ < 0.001].

At the end of the battery of behavioral tests, using the ACFC paradigm, we examined whether mRSDS-exposed mice exhibit NCB (Beck, 2008; Mendl et al., 2009) in response to ambiguous aversive stimuli. In the training session of ACFC on day 9, mice showed increased freezing responses over trials with tone-shock pairings [Fig. 2C; two-way repeated measures ANOVA; interaction: $F_{(46,1380)}$ = 4.075, $p$ < 0.0001; time: $F_{(23,1380)}$ = 124.2, $p$ < 0.0001; group: $F_{(2,60)}$ = 14.82, $p$ < 0.0001]. Increased freezing responses were observed in susceptible and resilient mice compared with undefeated control mice, and remarkably, the freezing response was higher in resilient mice than in susceptible

mice. In the testing session of ACFC on day 10, undefeated control mice showed higher levels of freezing responses during conditioned stimuli (partial and perfect cues) than the baseline (light cue) [Fig. 2D; one-way ANOVA; $F_{(2,69)} = 124.1$, $p < 0.0001$], and a significant difference in the freezing response was observed between partial and perfect cues in undefeated control mice ($p < 0.01$). Although susceptible and resilient mice showed significant increases in freezing responses during both cues [Fig. 2D; one-way ANOVA; susceptible: $F_{(2,51)} = 59.76$, $p < 0.0001$; resilient: $F_{(2,60)} = 93.45$, $p < 0.0001$], no difference in freezing responses was found between partial and perfect cues in either susceptible or resilient mice. These results suggest that defeated mice show NCB in response to ambiguous partial cue.

### Distinct neuroinflammatory profile in susceptible and resilient mice

The serum and perfused brain were collected on day 11. Immunohistochemical analysis was performed for neuroinflammatory markers in the brain. The number of ionized calcium binding adaptor molecule 1 (Iba1)-positive microglial cells in the DG in the hippocampus was significantly higher in susceptible mice than in resilient and undefeated control mice [Fig. 3A, F; one-way ANOVA; $F_{(2,13)} = 15.59$, $p = 0.0004$; Bonferroni's post hoc test, $p < 0.05$ (susceptible vs. resilient), $p < 0.01$ (susceptible vs. undefeated)]. Hippocampal glial fibrillary acidic protein (GFAP)-positive astrocytes were significantly increased in susceptible mice than in resilient and undefeated control mice [Fig. 3B, G; one-way ANOVA, $F_{(2,13)} = 15.05$, $p = 0.0004$; Bonferroni's post hoc test, $p < 0.01$ (susceptible vs. undefeated), $p < 0.001$ (susceptible vs. resilient)], and the expression levels of intercellular adhesion molecule 1 (ICAM-1) were significantly higher in the DG in susceptible and resilient mice than in undefeated control mice [Fig. 3C, H; one-way ANOVA; $F_{(2,13)} = 12.89$, $p = 0.0008$; Bonferroni's post hoc test, $p < 0.05$ (resilient vs. undefeated), $p < 0.001$ (susceptible vs. undefeated)]. The number of NG2-positive cells was significantly lower in the DG in susceptible mice than in resilient and undefeated control mice [Fig. 3D, I; one-way ANOVA; $F_{(2,13)} = 9.056$, $p = 0.0034$; Bonferroni's post hoc test, $p < 0.05$ (susceptible vs. resilient), $p < 0.01$ (susceptible vs. undefeated)], whereas the number of neuropeptide Y (NPY)-positive cells was significantly reduced in the hilus of DG in susceptible and resilient mice compared with undefeated control mice [Fig. 3E, J; one-way ANOVA; $F_{(2,13)} = 9.055$, $p = 0.0034$; Bonferroni's post hoc test, $p < 0.05$ (resilient vs. undefeated), $p < 0.01$ (susceptible vs. undefeated)].

Serum corticosterone levels were significantly elevated in susceptible mice compared with resilient and undefeated control mice [Fig. 4A; one-way ANOVA; $F_{(2,27)} = 4.111$, $p = 0.0276$; Bonferroni's post hoc test, $p < 0.05$]. However, serum levels of total adiponectin [Fig. 4B; one-way ANOVA; $F_{(2,26)} = 32.45$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$] and HMW adiponectin [Fig. 4C; one-way ANOVA; $F_{(2,26)} = 50.26$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$] were significantly decreased in susceptible and resilient mice compared with undefeated control mice. Additionally, the ratio of serum HMW adiponectin to total adiponectin, which is an index for predicting insulin resistance and future development of metabolic syndrome (Hara et al., 2006), was significantly reduced in both susceptible and resilient mice compared with undefeated control mice [Fig. 4D; one-way ANOVA; $F_{(2,26)} = 30.26$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$].

Further, we examined whether mRSDS exacerbates neuroinflammation using ex vivo microglial stimulation assay with LPS and/or ATP (Fig. 5A). IL-6 released from microglia derived from susceptible and resilient mice [Fig. 5E; one-way ANOVA; $F_{(2,35)} = 5.328$, $p = 0.0095$; Bonferroni's post hoc test, $p < 0.05$], as well as defeated mice (Fig. 5B; one-way ANOVA; $F_{(3,72)} = 48.62$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$), was significantly elevated by LPS challenge compared with that from undefeated control mice. In contrast, no difference in TNF-$\alpha$ levels was found in the microglial culture media among groups, regardless of LPS stimulation [Fig. 5F; one-way ANOVA; PBS: $F_{(2,35)} = 0.3998$, $p = 0.6734$; LPS: $F_{(2,35)} = 2.312$, $p = 0.114$], although a significant difference in the TNF-$\alpha$ level was found between defeated and undefeated mice (Fig. 5C; one-way ANOVA; $F_{(3,72)} = 105$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.05$). Further, ATP activates the IL-1$\beta$-converting enzyme, caspase-1, via its purinergic $P2X_7$ receptor on microglia, resulting in potentiation of LPS-induced IL-1$\beta$ release (Facci et al., 2014; Sanz and Virgilio, 2000); therefore, we tested the effects of ATP on LPS-primed IL-1$\beta$ release from microglia. A significantly higher IL-1$\beta$ release following the short-term LPS and ATP challenges was observed in defeated mice than in undefeated mice [Fig. 5D; one-way ANOVA; $F_{(7,144)} = 12.86$, $p < 0.0001$; Bonferroni's post hoc test, $p < 0.001$], but susceptible and resilient mice showed a trend toward a gradual increase in IL-1$\beta$ release after short-term LPS and ATP challenges compared with undefeated control mice [Fig. 5G; one-way ANOVA; $F_{(2,35)} = 2.664$, $p = 0.0837$].

## MINO REVERSES MRSDS-INDUCED DEPRESSIVE-LIKE BEHAVIORS AND NEUROINFLAMMATION BUT HAS NO EFFECTS ON NCB.

Next, we determined whether Mino, an antibiotic with anti-inflammatory activity (Garrido-Mesa et al., 2013; Henry et al., 2008) (Fig. S2), ameliorates mRSDS-induced behavioral disturbances and neuroinflammation *in vivo* (Fig. 6A). In the SI test on day 6, the time spent in the SI zone in the presence of an aggressor was significantly shorter than that in the absence of aggressor in defeated mice, and the effects were suppressed by Mino treatment (Fig. 6B; paired two-tailed $t$ test; defeat + vehicle: $t = 2.514$, df = 15, $p = 0.0238$; defeat + Mino: $t = 0.0602$, df = 16, $p = 0.9528$). In the FST on day 7, Mino treatment significantly led to shorter immobility time than did vehicle treatment in defeated mice (Fig. 6C;

unpaired two-tailed $t$ test; $t = 3.11$, df = 31, $p = 0.004$). However, Mino had no effects on behavioral performances in either SI or FST in undefeated mice (Fig. S3). In the training session of ACFC on day 8, defeated mice showed increased freezing responses over trials, and surprisingly, the freezing response of Mino-treated mice was higher than that of vehicle-treated mice [Fig. 6D; two-way repeated measures ANOVA; interaction: $F_{(23,713)} = 2.315$, $p = 0.0005$; time: $F_{(23,713)} = 78.99$, $p < 0.0001$; group: $F_{(1,31)} = 11.5$, $p = 0.0019$]. However, in the testing session of ACFC on day 9, mRSDS-induced negative bias in an ambiguous partial cue presented was not ameliorated by Mino treatment [Fig. 6E; one-way ANOVA; defeat + vehicle: $F_{(2,45)} = 59.92$, $p < 0.0001$; defeat + Mino: $F_{(2,48)} = 64.38$, $p < 0.0001$].

In addition, Mino reversed mRSDS-induced changes in immunoreactivity of Iba1 (Fig. 7A; unpaired two-tailed $t$ test; $t = 4.429$, df = 14, $p = 0.0006$), GFAP (Fig. 7B; unpaired two-tailed $t$ test; $t = 5.685$, df = 14, $p < 0.0001$), ICAM-1 (Fig. 7C; unpaired two-tailed $t$ test; $t = 8.412$, df = 14, $p < 0.0001$), NG2 (Fig. 7D; unpaired two-tailed $t$ test; $t = 5.554$, df = 14, $p < 0.0001$), and NPY (Fig. 7E; unpaired two-tailed $t$ test; $t = 4.189$, df = 14, $p = 0.0009$) in the brain. However, serum levels of corticosterone (Fig. 7F; unpaired two-tailed $t$ test; $t = 0.5835$, df = 30, $p = 0.5639$) and total adiponectin (Fig. 7G; unpaired two-tailed $t$ test; $t = 0.3203$, df = 31, $p = 0.7509$) and HMW adiponectin (Fig. 7H; unpaired two-tailed $t$ test; $t = 1.093$, df = 31, $p = 0.2828$) were not altered by Mino treatment.

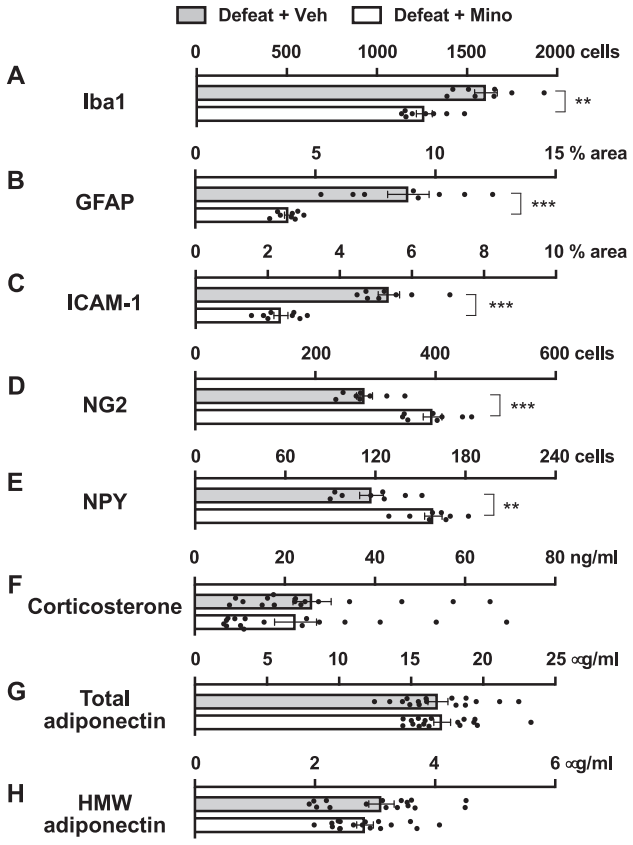


**Fig. 7.** Minocycline reverses mRSDS-elicited neuroinflammation in mice. The number of Iba1 **(A)**, NG2 **(D)**, and NPY **(E)**-positive cells; the percent positive area for GFAP **(B)** and ICAM-1 **(C)** labeling in the DG; and serum levels of corticosterone **(F)** and adiponectin **(G, H)** in minocycline or vehicle-treated mice subjected to mRSDS. Data are represented as mean ± SEM **((A–E)**, $n = 8$ per group; **(F)**, $n = 16$ per group; **(G, H)**, $n = 16$–17 per group). $^{**}p < 0.01$, $^{***}p < 0.001$. Mino, minocycline; Veh, vehicle.

## DISCUSSION

In this study, we extensively illustrated the unique characteristics of the mRSDS model in BALB/c mice. Exposure to mRSDS induced NCB as well as depressive-like behaviors, and hippocampal neuroinflammation regardless of stress vulnerability. Thus, mice resilient to mRSDS are no longer regarded as intact emotionally and neurobiologically. The present finding strongly highlights the heretofore underappreciated negative effects of stress on the resilient subpopulation. Anti-inflammatory intervention also prevented mRSDS-induced depressive-like behaviors and neuroinflammation, but not NCB. These results indicate that suppression of neuroinflammation does not always ameliorate the stress-induced behavioral deficits and might not be sufficient to improve negative-biased cognition.

Our findings demonstrated that compared with the traditional RSDS paradigm (Krishnan et al., 2007; Golden et al., 2011), relatively short-term stress exposure triggered robust depressive-like behaviors with body weight loss in BALB/c mice, and the mice could be classified into two groups based on stress vulnerability in the SI test. The induction of depressive-like behaviors by the short-term stress could be attributed to the intrinsic characteristic of the inbred BALB/c mouse strain, which has higher stress sensitivity and inherent anxiety-prone traits than other C57BL/6J strains (Jacobson and Cryan, 2007; Brinks et al., 2007; Savignac et al., 2011a,b; Abe-Higuchi et al., 2016). Apathy- and NCB-related behaviors were found in both susceptible and resilient mice when compared with undefeated control mice. These observations are in part consistent with the findings in a previous study, which showed that chronic psychosocial stress-induced NCB resulted from increased negative and decreased positive responses to the ambiguous cue in rats (Papciak et al., 2013). Considering our results along with this finding, we speculate that the mRSDS-resistant phenotype, as characterized by the SI test, might be no longer intact, although mice with the mRSDS-resistant phenotype might have higher abilities to successfully cope with stress than those with the stress-sensitive phenotype (Feder et al., 2009; Wood and Bhatnagar, 2015; Wood et al., 2015). Interestingly, it should be noted that acquisition of fear memory was established earlier and more intensively in the resilient mice than in susceptible and undefeated control mice; however, retrieval of fear memory in resilient mice was comparable with that in susceptible mice. It has been also demonstrated that resilience to social defeat stress leads to enhanced fear

expression and impaired fear extinction (Meduri et al., 2013), and the findings are supported by those in our study, which implicates enhanced fear acquisition and impaired fear discrimination in an ambiguous situation in resilient mice. Therefore, resilient mice might be vulnerable to maladaptive fear responses, although they likely have a specific coping strategy in response to social stress. Intriguingly, one study indicates that a transient increase in corticosterone before fear conditioning is likely to facilitate fear extinction in BALB/c mice (Brinks et al., 2009). Fear reactivity observed in susceptible mice, which had elevated serum corticosterone levels, in our study appears to be contradictory with this finding, and a significant difference in the duration of corticosterone exposure could be used to explain the discrepancy partially. Therefore, further studies are needed to assess how long-lasting heightened serum corticosterone levels following stress exposure affect fear memory and NCB in BALB/c mice.

Furthermore, the BALB/c mouse is known to be a strain prone to fear memory overgeneralization and shows strong fear responses to both context and cue stimuli in the fear conditioning (Brinks et al., 2008, 2009). In our study, impaired fear discrimination in defeated mice under an ambiguous condition may be in part due to the fear memory overgeneralization, although undefeated mice were able to discriminate the ambiguous aversive stimuli. Accumulating evidence has shown that chronic stress promotes fear memory overgeneralization and sensitizes the response to negative information in the amygdala (Dillon and Pizzagalli, 2018), a brain region known to mediate fear-related behavior, and the findings could account for the cognitive bias toward negative information in depressed individuals (Erickson et al., 2005). The amygdala, in which BALB/c mice show reduced benzodiazepine binding and expression (Chapouthier et al., 1991; Hode et al., 2000), is necessary for cued fear conditioning in rodents (Phillips and LeDoux, 1992) and for memorizing negative stimuli in depressed individuals (Hamilton and Gotlib, 2008); therefore, mRSDS-induced NCB in BALB/c mice might be associated with the amygdala's hyperactivity.

Noteworthy findings have demonstrated that the hippocampus plays an essential role in the fear response to ambiguous aversive stimuli (Tsetsenis et al., 2007), and the dorsal hippocampus contributes to consolidation and generalization of fear memory (Zelikowsky et al., 2014; Lynch et al., 2017; Stern et al., 2017). Therefore, we next focused on the hippocampus to unveil the mechanisms underlying behavioral variations induced by mRSDS. Neuroinflammation is a hot topic in the pathophysiological and therapeutic field of depression (Dantzer et al., 2011; Haapakoski et al., 2015). In the brain, activated microglia (Yirmiya et al., 2015) and astrocytes (Salmina et al., 2015) are directly associated with neuroinflammation. In addition, ICAM-1 expression, which is induced by stress and inflammatory signals on the brain microvascular endothelial cells, is responsible for monocyte trafficking to the brain and has an indirect link to the facilitation of neuroinflammation (Weber et al., 2016). Moreover, NG2 (Gao et al., 2010; Birey et al., 2015; Nakano et al., 2017) and NPY (Redrobe et al., 2002; Malva et al., 2012) are known to have anti-inflammatory and antidepressant-like effects. In this study, we found that susceptible and resilient mice had distinct profiles of neuroinflammation in the hippocampus. In susceptible mice, immunohistochemical analyses showed consistent changes in the proinflammatory profile (i.e., increased microglia and astrocytes, increased adhesion molecule ICAM-1 expression, and decreased NG2 glial cells and NPY interneurons) in the hippocampus. In addition, functional analysis using ex vivo microglial assay revealed enhanced proinflammatory priming in susceptible mice. Corticosterone and adiponectin have been of considerable interest as pro- and anti-inflammatory-related mediators, respectively (Ouchi et al., 1999; Smyth et al., 2004; MacPherson et al., 2005; Frank et al., 2010; Chabry et al., 2015; Nicolas et al., 2015). Our findings showing elevated serum corticosterone levels in susceptible mice, but not in resilient mice, are supported by those in several studies, which demonstrate a divergent expression pattern of the glucocorticoid receptor, to which corticosterone binds, in the hippocampus, hypothalamus, and amygdala according to stress vulnerability (Brinks et al., 2007; Han et al., 2017). Notably, adiponectin, which can pass through blood–brain barrier (Ebinuma et al., 2007; Kubota et al., 2007), is reduced by RSDS exposure and has an antidepressant-like activity (Liu et al., 2012). Thus, decreased serum adiponectin levels may underlie the depressive-like behaviors shown in our study. Moreover, we found significant differences in hippocampal gene expression related to cytokine, chemokine, and neurotransmitter signaling between susceptible and resilient mice (Table S3). The findings on global gene expression profiling in the hippocampus may provide additional information for stress vulnerability and neuroinflammation. Further studies are needed to clarify how these genes affect behaviors and neuroinflammation. Together with the behavioral findings mentioned above, these findings further highlight that resilient mice are not equivalent to undefeated control mice as well as susceptible mice (Meduri et al., 2013).

Suppression of neuroinflammation has been suggested to be a therapeutic strategy for depression. We verified that pretreatment with Mino attenuated mRSDS-induced depressive-like behaviors and concomitant proinflammatory activation in the brain, further supporting the speculation that anti-inflammatory interventions have beneficial effects on disrupted emotional behaviors including depression and anxiety following prolonged interferon-alpha treatment (Zheng et al., 2015), LPS injection (Henry et al., 2008), chronic restraint stress (Hinwood et al., 2013; Liu et al., 2018), social isolation stress (Wang et al., 2017), and chronic unpredictable stress (Kreisel et al., 2014; Zhang et al., 2019) in rodents. In line with the findings, a recent meta-analysis of clinical trials has also demonstrated that Mino exhibits an antidepressant effect with good tolerability in humans (Rosenblat and McIntyre, 2018). Interestingly, NCB and altered serum levels of corticosterone and adiponectin failed to be recovered by the Mino treatment. The dissociation between behavioral and

biochemical data following Mino treatment suggests a limited efficacy for the anti-inflammatory intervention. Several studies have demonstrated that Mino treatment prevents RSDS-induced impairment in spatial memory in mice (McKim et al., 2016a,b) and IFN-α-induced deficit in fear memory extinction in rats (Bi et al., 2016). Moreover, in some clinical trials, Mino add-on therapy has likely improved negative cognitive symptoms in early-phase schizophrenia (Levkovitz et al., 2010; Chaudhry et al., 2012). Hence, Mino may have differential effects depending on the types of memory and pathological states with NCB. However, a prospective study has shown that fluoxetine reduced NCB in rats (Anderson et al., 2013), but the paradigm used in this study is different from that in our study. Given the present findings together with anti-neuroinflammatory effects of some antidepressants such as imipramine (Ramirez and Sheridan, 2016), fluoxetine (Lu et al., 2017; Song et al., 2018), and paroxetine (Liu et al., 2014; Fujimori et al., 2015), further studies using our paradigm are needed to examine the effect of such antidepressants, and the findings would help explain the behavioral dissociation observed in our study.

This study has several limitations. First, this study only focused on the hippocampus as a brain region that is vulnerable to stress; however, involvement of the amygdala (a brain region that is responsible for fear memory) in behaviors, stress vulnerability, and neuroinflammation is still unclear. Therefore, further studies are necessary for investigating the role of the amygdala in stress vulnerability, neuroinflammation, and NCB. Second, it remains unknown whether stress-induced neuroinflammation is detrimental for neurons *per se,* as well as neural circuits and plasticity, which may affect some behavioral outcomes in our study. Therefore, further studies on neuronal damage and neural plasticity would help to interpret the hippocampal gene expression profile as well as stress vulnerability in this study. Third, this study focused on whether anti-neuroinflammatory intervention such as Mino treatment improved behavioral disturbances and neuroinflammatory profiles in defeated mice. Although we conformed effects of Mino to the minimum necessary in undefeated mice, further studies using undefeated mice would be useful for precise interpretation of its effects on defeated mice. Fourth, the hippocampal region analyzed in this study was the dorsal hippocampus. In the remarkable study reported by Pearson-Leary et al. (2017), a significant increase of neuroinflammation was shown in the ventral hippocampus, but not dorsal hippocampus, after chronic social defeat stress in rats. The discrepancy between this finding and our results in neuroinflammatory profiles of the dorsal hippocampus may be attributed to the differences in strain and stress paradigm. In the future, detailed studies on the neuroinflammatory profiles in the ventral hippocampus of BALB/c mice would contribute to better understanding of the relation between stress vulnerability and neuroinflammation. Moreover, numerous studies have reported that emotional and/or memory impairments are linked to neuroinflammation in a rat model of social defeat stress (Patki et al., 2013; Wood et al., 2015; Kopschina Feltes et al., 2019). Therefore, social defeat research in both mice and rats would be helpful to elucidate the mechanisms underlying stress vulnerability and neuroinflammation. Fifth, behavioral tests were sequentially conducted in one cohort while minimizing the impact of each behavioral test. However, it would be more appropriate to examine separate cohorts for avoiding the impact of sequential behavior tests and subsequently investigating the underlying mechanisms more properly. Further study would be needed to address the potential impact of sequential tests in the future. Last, we found that NCB showed a trend similar to despair behaviors in the FST, but not social avoidant behaviors in the SI test, suggesting a close relationship between NCB and lack of motivation. However, an association of NCB with other depressive symptoms, such as anhedonia, sense of fatigue, and sleep disturbance, remains unclear and thus warrants further investigations.

Collectively, this study demonstrated that mRSDS induced a negative cognition concomitant with depressive- and anxiety-like behavioral patterns, as well as neuroinflammation, in BALB/c mice. Systemic anti-inflammatory intervention also prevented mRSDS-induced depressive-like behaviors and neuroinflammation, but not NCB, probably indicating that suppression of neuroinflammation does not always abrogate stress-triggered overall behavioral deficits and is not sufficient to improve NCB. Moreover, we found characteristic differences in neuroinflammatory profiles and hippocampal gene expression patterns not only between susceptible and resilient mice but also between resilient and unstressed normal control mice, suggesting that mice resilient to mRSDS should not be regarded as intact. The findings provide additional insights into the unique features of the mRSDS model in BALB/c mice. Future comprehensive studies using the mRSDS model in BALB/c mice are needed to understand the pathophysiological mechanisms underlying psychiatric disorders, such as depression, and the findings would aid in the development of promising novel therapeutic approaches.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2020.07.023.

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