# Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain

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**Background:** Persistent anxiety-like symptoms may have an inflammatory-related pathophysiology. Our previous work using repeated social defeat (RSD) in mice showed that recruitment of peripheral myeloid cells to the brain is required for the development of anxiety. Here, we aimed to determine if 1) RSD promotes prolonged anxiety through redistribution of myeloid cells and 2) prior exposure to RSD sensitizes the neuroimmune axis to secondary subthreshold stress.

**Methods:** Mice were subjected to RSD and several immune and behavioral parameters were determined .5, 8, or 24 days later. In follow-up studies, control and RSD mice were subjected to subthreshold stress at 24 days.

**Results:** Repeated social defeat-induced macrophage recruitment to the brain corresponded with development and maintenance of anxiety-like behavior 8 days after RSD, but neither remained at 24 days. Nonetheless, social avoidance and an elevated neuroinflammatory profile were maintained at 24 days. Subthreshold social defeat in RSD-sensitized mice increased peripheral macrophage trafficking to the brain that promoted re-establishment of anxiety. Moreover, subthreshold social defeat increased social avoidance in RSD-sensitized mice compared with naïve mice. Stress-induced monocyte trafficking was linked to redistribution of myeloid progenitor cells in the spleen. Splenectomy before subthreshold stress attenuated macrophage recruitment to the brain and prevented anxiety-like behavior in RSD-sensitized mice.

**Conclusions:** These data indicate that monocyte trafficking from the spleen to the brain contributes re-establishment of anxiety in stress-sensitized mice. These findings show that neuroinflammatory mechanisms promote mood disturbances following stress-sensitization and outline novel neuroimmune interactions that underlie recurring anxiety disorders such as posttraumatic stress disorder.

**Key Words:** Anxiety, microglia, monocytes, neuroinflammation, PTSD, stress

Psychological stress contributes to the development of depression and anxiety disorders (1,2). The etiology of mood disorders is diverse (3,4), but stress-induced immune dysregulation promotes neuroinflammation underlying anxiety- and depressive-like behaviors (5–8). Indeed, increased neuroinflammatory signaling following stress augments neuroplasticity and leads to neuronal adaptations underlying anxiety and depressive symptoms (9–12). Thus, stress-induced neuroinflammation can contribute to the development of chronic mood disorders, such as posttraumatic stress disorder. While many have postulated that anxiety disorders have an inflammatory-related pathophysiology (13,14), no studies have examined the role of neuroinflammation in prolonged and recurrent anxiety.

Clinically relevant models of stress, including repeated social defeat (RSD), cause immune dysregulation, neuroinflammation, and anxiety-like behavior (15–18). For example, RSD significantly increased myeloid ($CD11b^+$) cells in the blood and spleen that maintain an inflammatory (i.e., primed) and glucocorticoid-insensitive phenotype (19–22). These findings are mirrored in clinical studies of stress where isolated mononuclear cells from stressed individuals have an enhanced inflammatory profile and have impaired glucocorticoid regulation. Redistribution of primed myeloid cells generates peripheral and central inflammation that has deleterious effects on behavior. For instance, RSD-induced anxiety-like behavior was associated with microglia activation, macrophage recruitment to the brain, and elevated proinflammatory cytokine production (16,18). Moreover, our recent findings indicate that peripheral myeloid cell trafficking to the brain was instrumental in development of RSD-induced anxiety. Indeed, repeated exposure to social defeat increased circulating monocytes and brain macrophages, which corresponded with development of anxiety. In addition, mice lacking the chemokine receptors, CCR2 and $CX_3CR1$, had limited macrophage recruitment to the brain and did not develop anxiety-like behavior after RSD (15). Thus, we aimed to 1) determine how long neuroinflammatory responses and anxiety-like behavior persisted following RSD; and 2) determine if subthreshold stress re-established myeloid cell trafficking and anxiety in RSD-sensitized mice.

Here, we show that RSD activated the neuroimmune axis to promote anxiety and key peripheral and central components of the neuroimmune axis remain sensitized. For instance, increased microglia activation and increased myeloid progenitors in the spleen persisted after RSD. These components were sensitized because subthreshold stress (i.e., acute social defeat) caused robust inflammation in RSD-sensitized mice (24 days) with increased peripheral monocyte/macrophage trafficking in the brain and elevated proinflammatory cytokine expression by microglia/macrophages. In addition, subthreshold stress in RSD-sensitized mice re-established anxiety and reinforced social avoidance. Splenectomy

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in RSD-sensitized mice before subthreshold stress disrupted monocyte release/trafficking to the brain and blocked the re-establishment of anxiety.

## Methods and Materials

### Animals

Male C57BL/6 (6–8 weeks) and CD-1 (12 months) mice were obtained from Charles River Laboratories (Wilmington, Massachusetts). C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated social defeat was performed as described in Supplement 1 and in previous reports (15,16,23). In brief, an intruder male CD-1 mouse was introduced into home cages of male C57BL/6 mice (three per cage) for 2 hours on 6 consecutive nights. Acute social defeat consisted of a single 2-hour exposure to the intruder mouse. Control mice (CON) were left undisturbed until sacrificed.

Anxiety-like behavior in the open field was determined as previously described (15). Mice were placed individually into the Plexiglas test apparatus (40 × 40 × 25 cm) and activity was recorded for 5 minutes. Time spent in the center and latency to enter the center of the open field were determined using an automated system (AccuScan Instruments, Columbus, Ohio).

Social avoidance was determined as previously described (24,25). In the empty trial, an experimental mouse was placed into the arena with an empty wire mesh cage and activity was recorded for 2.5 minutes. In the social trial, an unfamiliar CD-1 mouse was placed in the wire mesh cage and the experimental mouse was placed in the arena and activity was recorded for 2.5 minutes. Activity was video-recorded and analyzed using Noldus EthoVision Software (Leesburg, Virginia).

Green fluorescent protein $(GFP)^+$ bone marrow (BM)-Chimera mice were generated as described in Supplement 1 and in previous reports (15). In brief, mice were injected twice with low-dose busulfan, and 48 hours later $GFP^+$ bone marrow was transferred into recipients by tail vein injection. Recipient mice were undisturbed for 4 weeks to allow engraftment. This protocol resulted in approximately 45% $GFP^+$ cells in the bone marrow and 50% $GFP^+$ cells in the circulation 4 weeks after reconstitution (Figure S1 in Supplement 1).

Brain microglia enrichment and isolation of blood, bone marrow, and spleen cells were completed as described in Supplement 1. As previously described (16), microglia were enriched from brain homogenates by Percoll centrifugation in differential Percoll gradients.

Flow cytometric analysis of antigen expression was completed as described in Supplement 1 and in previous reports (15,16,23).

Interleukin-6 (IL-6) was determined from plasma using the BD OptEIA Mouse IL-6 enzyme-linked immunosorbent assay according to the manufacturer's instructions (BD Biosciences, San Jose, California). Absorbance was read at 450 nm using a Spectramax $Plus^{384}$ plate reader (Molecular Devices, Sunnyvale, California). The assay was sensitive to 10 ng/mL IL-6 and intra-assay coefficients of variation were less than 10%.

Immunohistochemistry was completed as described in Supplement 1 and in previous reports (15). In brief, fixed brain tissue was fluorescently labeled with rabbit anti-mouse ionized calcium binding adaptor molecule-1 (Iba-1) (Wako Chemicals, Richmond, Virginia) and rat anti-mouse Ly6C (Abcam, Cambridge, Massachusetts). Sections were incubated with appropriate conjugated secondary antibody. Immunofluorescence was visualized and images were captured using an epifluorescent Leica DM5000B microscope and Leica DFC300 FX camera (Buffalo Grove, Illinois).

Histological quantification of microglia and $GFP^+$ macrophages was determined as described in Supplement 1 and as previously reported (15,26,27). Iba-1 proportional area was reported as the average percentage area in the positive threshold for all representative images. $GFP^+$ cells in brain sections were categorized as perivascular or parenchymal based on morphology, Iba-1 co-localization, and spatial relationship to $Ly6C^+$ blood vessels.

RNA isolation and real-time polymerase chain reaction were completed as described in Supplement 1. In brief, messenger RNA (mRNA) was transcribed to complementary DNA and was amplified by real-time polymerase chain reaction and normalized based on reference complementary DNA (glyceraldehyde 3-phosphate dehydrogenase). Data were analyzed with comparative threshold cycle method (28).

Splenectomy surgery was performed 8 days after RSD as described in Supplement 1. Two weeks after splenectomy, mice were exposed to acute social defeat.

### Statistical Analysis

Data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) software (Cary, North Carolina). Observations greater than three interquartile ranges from the first and third quartile were excluded from analyses. Significant main effects and interactions were determined using one- (stress, day) or two- (stress × treatment) way analysis of variance using the general linear model procedures of SAS. Differences between group means were evaluated with *t* test using the least significant difference procedure of SAS.

## Results

### RSD-Induced Anxiety-like Behavior Was Resolved by 24 Days but Social Avoidance Was Maintained

To examine how long RSD-induced behavioral and immune alterations persisted, mice were subjected to repeated social defeat and anxiety-like behavior in the open-field and social avoidance were determined .5, 8, or 24 days later. In control mice, baseline behavior was not significantly different at the .5-, 8-, or 24-day time points, so these data were combined and presented as a single CON group. In addition, this experimental design was cross-sectional, so an individual mouse was tested once for each behavioral paradigm.

In the first experiment, RSD increased latency to enter the center of the open-field at .5 and 8 days compared with control mice ($p < .04$ for each; Figure 1A) and increased time spent in the center at .5 days ($p < .04$; Figure 1B). Nonetheless, indications of RSD-induced anxiety in the open-field were resolved by 24 days. In the second experiment, social avoidance was determined using a two-trial social interaction paradigm with an empty trial followed by a social trial (24,25). Representative activity traces of social interaction are shown from CON and RSD mice (24 days) for empty and social trials (Figure 1C). There were no differences between groups in time spent in the interaction zone during the empty trial (Figure 1D). During the social trial, however, time spent in the interaction zone was significantly decreased .5 ($p < .02$), 8 ($p = .08$), and 24 ($p < .02$) days after RSD (Figure 1D). In addition, mice subjected to RSD spent more time in the corners during the social trial at .5 and 24 days after RSD ($p < .02$ for each; Figure 1D). Overall, reductions in social interaction persisted