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

***Supplemental Information***

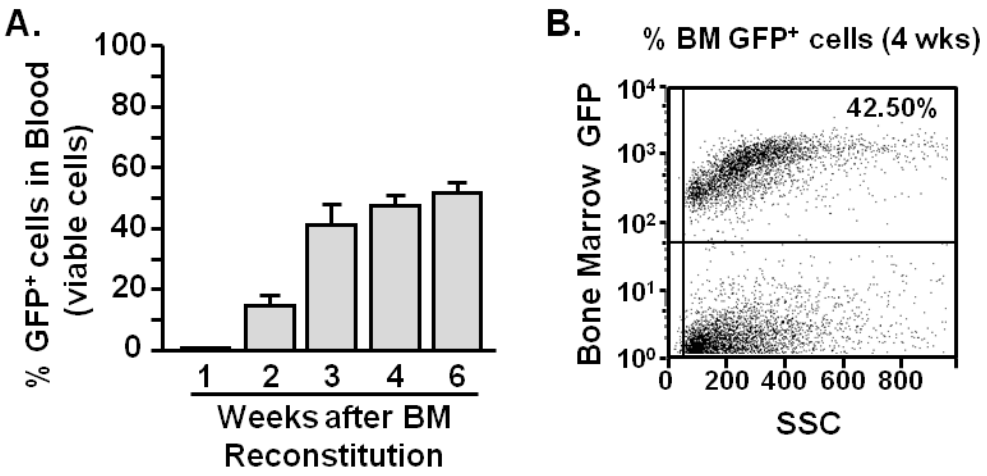


**Figure S1.** Busulfan and transfer of $GFP^+$ bone marrow (BM) progenitor cells resulted in partial reconstitution of bone marrow and blood. Male C57BL/6 mice were injected with busulfan (30 mg/kg) for two consecutive days and 48 hours later $GFP^+$ bone marrow progenitor cells were transferred via tail vein injection. Blood was collected once every week for six weeks. (**A**) Percentage of viable $GFP^+$ cells in the blood of recipient mice weeks after reconstitution. (**B**) In a related experiment, bone marrow samples were collected 4 weeks after reconstitution and the percentage of $GFP^+$ cells were determined. A representative dot plot of GFP/side scatter (SSC) in the bone marrow is shown.

## Supplemental Methods

### Animals

Male C57BL/6 (6-8 weeks) and CD-1 (12 months) mice were obtained from Charles River Laboratories. C57BL/6-Tg(CAG-EGFP)131/leysopJ were purchased from Jackson Laboratories. Mice were housed in 11.5"x 7.5"x 6" polypropylene cages under a 12 h light-dark cycle with ad libitum access to water and rodent chow. All procedures were in accordance with the NIH 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 (RSD)

An aggressive intruder male CD-1 mouse (retired breeder) was introduced into cages of established male cohorts (3 per cage) of C57BL/6 mice for six consecutive nights between 17:00 and 19:00 (2 h). During each cycle, submissive behavior including upright posture, fleeing, and crouching were observed to ensure that the resident mice showed subordinate behavior. If the intruder did not initiate a defeat within 5-10 minutes or was defeated by any of the resident mice then a new intruder was introduced. At the end of the 2 h period, the intruder was removed and the residents were left undisturbed until the following day when the paradigm was repeated. Different intruders were used on consecutive nights. The health status of the mice was carefully examined throughout the paradigm. Mice that were injured or moribund were removed from the study. Consistent with previous studies using RSD, less than 5% of mice met the early removal criteria (1, 2). Control mice were left undisturbed in their home cages until sacrificed.

### $GFP^+$ Bone Marrow-Chimera

To establish chimerism, recipient C57BL/6 male mice (6 weeks old) were injected intraperitoneal once daily for two consecutive days with busulfan in a 1:1 solution of DMSO and deionized $H_2O$ (30 mg/kg/100 µl). This dose of busulfan resulted in partial bone marrow ablation and no morbidity. Donor bone marrow-derived cells were isolated from the femur, passed through a 70 µm cell strainer, and total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter, Inc., Indianapolis, IN). Forty-eight hours later $1x10^6$ BM-derived cells that ubiquitously express green fluorescent protein (GFP)

(C57BL/6-Tg$^{(CAG-EGFP)}$131/leysopJ) were transferred via tail-vein injection to recipient mice (100 µl). Recipient mice were left undisturbed for 4 weeks to allow engraftment. The busulfan ablation protocol and reconstitution with bone marrow progenitors from GFP$^+$ mice consistently produced approximately 45% GFP$^+$ cells in the bone marrow and 50% GFP$^+$ cells in the circulation four weeks after reconstitution (Figure S1) (1).

**Isolation of Brain CD11b$^+$ Cells**

CD11b$^+$ cells were isolated from whole brain homogenates. The brain was collected, homogenized and passed through a 70 µm cell strainer. Homogenates were centrifuged at 600 x *g* for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll (GE-healthcare, Uppsala, Sweden). A discontinuous Percoll density gradient was layered as follows: 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 min at 2000 x *g* and cells were collected from the interface between the 70% and 50% Percoll layers. These cells were referred to as enriched brain CD11b$^+$ cells based on previous studies demonstrating that viable cells isolated by Percoll density gradient yields greater than 90% CD11b$^+$ cells (1-3).

**Flow Cytometry**

For enriched brain CD11b$^+$ cells, blood leukocytes, and bone marrow cells the Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience). Cells were washed and then incubated with the appropriate antibodies (CD45, CD11b, CD34; eBioscience), and Ly6C (BD Biosciences) for 1 h at 4$^o$C. Cells were washed and re-suspended in FACS buffer for analysis. Non-specific binding was assessed by using isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCaliber four-color cytometer (BD Biosciences).

**RNA Isolation and Real Time Polymerase Chain Reaction (PCR)**

RNA was collected from enriched brain CD11b$^+$ cells using USB PrepEase RNA spin kit (Affymetrix, Santa Clara, CA). RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed using the Applied Biosystems (Foster, CA) Assay-on-Demand Gene Expression protocol. In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (e.g., IL-1b, CCL2,

$CX_3CL1$) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ or TAMRA). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems, Foster, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference from GAPDH (4).

**Immunohistochemistry**

Brains were collected from mice after transcardial perfusion with sterile PBS (PBS, pH 7.4 w/ EDTA) and 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and incubated in 20% sucrose for an additional 24 h. Fixed brains were frozen with isopentane (-78ºC) and sectioned (20 µm) using a Microm HM550 cryostat. Brain regions were identified by reference markers in accordance with the stereotaxic mouse brain atlas. To label for Iba-1 (ionized calcium binding adapter molecule-1), sections were mounted on slides or placed free-floating in cryoprotectant until staining. Next, sections were washed in PBS with 1% BSA, blocked with 2% normal goat serum, and incubated with a rabbit anti-mouse Iba-1 antibody (Wako Chemicals, Richmond, VA). To label for Ly6C, sections were washed in PBS with 1% BSA, blocked with 5% normal goat serum, and incubated with a rat anti-mouse Ly6C antibody (Abcam, Cambridge, MA). Next, sections were washed in PBS with 1% BSA and incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor 594: Iba-1; DyLight 350: Ly6C). Sections were mounted on slides and cover-slipped with Fluoromount (Beckman Coulter, Inc., Fullerton, CA) and stored at -20°C. Fluorescent sections were visualized using an epi-fluorescent Leica DM5000B microscope. Images were captured using a Leica DFC300 FX camera and imaging software.

**Histology Quantification**

To quantify the phenotypic changes of microglia, digital image analysis of Iba-1 labeling was performed (5). Representative images (6-8 bilateral) were taken from each brain region at 20× magnification. A threshold for positive staining was determined for each image and was processed by densitometric scanning of the threshold targets using ImageJ software. Proportional area was reported as the average percentage area in the positive threshold for all representative

pictures. $GFP^+$ cells in the brain were quantified and classified as previously described (1, 6). In brief, $GFP^+$ cells that had a rod, elongated, or round/amoeboid morphology and co-localized with $Ly6C^+$ blood vessels were classified as perivascular. $GFP^+$ cells that had distinct processes, exhibited ramified or stellate morphology, and did not co-localize with $Ly6C^+$ blood vessels were classified as parenchymal. More than 95% of parenchymal $GFP^+$ cells co-localized with Iba-1 staining.

**Splenectomy**

Eight days after RSD, mice were anesthetized with isoflurane and the spleen was removed through a small incision. Splenic nerves and vasculature were cauterized. Incisions were closed with sutures and surgical staples. Surgeries were performed in sterile conditions and tools were sterilized with hot bead sterilizer (Fine Science Tools). To relieve pain, mice received subcutaneous injection of buprenorphine (0.05 mg/ml) immediately after surgery and 24 hours later. Two weeks after splenectomy mice were exposed to acute social defeat.

**Supplemental References**

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