The Influence of Microglial Elimination and Repopulation on Stress-Sensitization Induced by Repeated Social Defeat

Supplemental Information

Supplemental Methods:

Mice: Male C57BL/6 (6-8 weeks old) and male CD-1 (12 months, retired breeders) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA), and allowed to acclimate to their surroundings for 7-10 days prior to experiments. Resident C57BL/6 mice were housed in cohorts of three and aggressor CD-1 mice were individually housed. Transgenic CX3CR1^{CreER}/ROSA26-STOP^{flfl}-tdTomato were generated by breeding Cx3cr1^{CreER} (stock #021160) and Ai9 ROSA26-STOP^{flfl}-tdTomato (stock #007909) purchased from Jackson Laboratories. All mice were housed in 11.5”x 7.5”x 6” polypropylene cages. Rooms were maintained at 21°C under a 12-h light-dark cycle with *ad libitum* access to water and rodent chow. All procedures were in accordance with the National Institutes of Health Guidelines and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat (RSD) and Stress-Sensitization (SS): Mice were subjected to social stress (RSD) as previously described (1). In brief, an aggressive male intruder CD-1 mouse was introduced into cages of established male cohorts (3 per cage) of C57BL/6 mice for 2 hours between 17:00 and 19:00 for six consecutive nights. During each cycle, submissive behavior (e.g., upright posture, fleeing, and crouching) was observed to ensure defeat of the resident mice. A new intruder was introduced if he did not initiate an attack on the resident mice within the first 5-10 minutes or if he was defeated by any of the resident mice. 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. To avoid habituation, different intruders were used on consecutive nights. The health status of the mice was carefully examined throughout all experiments. Mice that were injured or moribund were removed from the study. Consistent with previous studies using RSD (2-5), less than 5% of mice met the early removal criteria. Control mice (CON) were left undisturbed in their home cages.

To study the sensitizing effects of RSD, mice were either exposed to control (naïve) or RSD (stress-sensitized [SS]) conditions. 24 days later, naïve and SS mice were exposed to one cycle of social defeat, as previously described (4, 6). All behavior and biological measures were obtained 14 h after the final cycle. This time point was selected because sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal axis (HPA) activation returns to baseline by 14 hours after the final cycle (7).

Plexxikon Oral Administration: PLX5622 was provided by Plexxikon Inc. (Berkley, CA) and formulated in standard AIN-76A rodent chow by Research Diets at a concentration of 1200 mg/kg. Control diet consisted of the standard AIN-76A rodent chow. Mice were provided *ad libitum* access to PLX5622 or control diet for 14 days to deplete microglia prior to exposure to RSD.

RNA-sequencing of FAC-sorted Microglia: Microglia were enriched using Percoll separation, labeled with anti-CD11b and CD45 antibodies, and FAC-sorted. Cells were lysed immediately and stored at -80C. RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) according to manufacturer's protocols. RNAseq libraries were prepared using the Ovation SoLo RNAseq System with AnyDeplete rRNA to remove rRNA and other abundant

transcripts according to the manufacturer's recommendation (Nugen, San Carlos, CA). RNAseq libraries were sequenced on an Illumina NextSeq 500 sequencing instrument according to manufacturer's protocols (Illumina, San Diego, CA). Sequences were aligned to the mm10 mouse reference genome using STAR Aligner (8). Factors of unwanted variance were controlled with RUVseq (9) and normalization and differential expression was determined using DESeq2 in R (10). Genes with $p < 0.05$ and fold changes over 1.5 (log2FC > 0.585) were considered differentially expressed. Differentially expressed genes were used for Ingenuity Pathway Analysis of significant upstream regulators (Qiagen, Hilden, Germany) (11).

Tamoxifen Injections: Tamoxifen (Sigma-Aldrich) was administered as a solution in corn oil beginning on PND 21. Mice received 20mg/kg tamoxifen per day for 4 consecutive days by intraperitoneal injection (i.p.) with approximately 50µl tamoxifen solution as described in (12).

Isolation of Cells from Bone Marrow, Spleen, and Blood: Tissues were collected immediately following CO_2 asphyxiation. Whole blood was collected with EDTA-lined syringes by cardiac puncture and red blood cells were lysed. Bone marrow was collected from the femur and flushed out with ice-cold HBSS. Tissue samples were washed with HBSS, filtered through a 70-µm nylon cell strainer, and the total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter, Brea, CA).

Flow Cytometry: $CD11b^+$ cells were isolated from brain homogenates as reported (1). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience; catalog number 553142). Cells were washed and then incubated with the appropriate antibodies (CD45, CD11b, CD115, eBioscience; Ly6C, BD Biosciences) for 1 h at 4°C. Cells were washed and then re-suspended in FACS buffer for analysis. Cell numbers were estimated using counting beads (BD

Biosciences). Non-specific binding was assessed using isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCalibur 9-color 3 laser cytometer (BD Biosciences, Franklin Lakes, NJ). Data were analyzed using FlowJo software (Tree Star) and positive labeling for each antibody was determined based on isotype labeled controls.

RNA Isolation and Real-Time PCR: A 1 mm coronal brain section that includes the cortex, hippocampus, striatum, and hypothalamus was removed and immediately flash frozen in liquid nitrogen. RNA was isolated using tri-reagent/isopropanol precipitation and RNA concentration was determined by NanoPhotometry (Implen, Munich, Germany). RNA (1.2µg) was reverse transcribed to cDNA using an RT-RETROscript kit (Ambion, ThermoFisher, Waltham, MA). For Percoll-enriched microglia, the PrepEase kit (USB, CA) was used to isolate RNA according to the manufacturer's instructions. Real-time quantitative PCR was performed using the Applied Biosystems Assay-on-Demand Gene Expression protocol. Experimental cDNA was amplified by real-time PCR where a target cDNA and reference cDNA (glceraldehyde-3-phosphate dehydrogenase [GAPDH]) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (FAM) and a 3' quencher dye (non-fluorescent quencher). Florescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems). Relative gene expression was analyzed using the ΔΔCT method normalized to average ΔCT of appropriate control group within each experiment and results are expressed as fold difference from GAPDH.

Ex Vivo Stimulation of Enriched CD11b$^+$ Cells from Brain: Microglia were isolated from whole-brain homogenates as previously described (1). In brief, brains were homogenized in 5 mL sterile PBS in a glass homogenizer, transferred to a sterile 15 mL tube, and pelleted at 600 x *g* for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic

Percoll (GE-Healthcare). 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* with low acceleration and break. Myelin debris was removed and microglia were collected from the interphase between the 70 and 50% Percoll layers. Microglia were then washed in PBS and pelleted at 600 x *g* for 6 min. Following isolation, microglia were immediately lysed for RNA isolation using the PrepEase kit (USB, CA) or resuspended in media (sterile high glucose DMEM (Gibco, 11995-065) + 10% FBS that was passed through a 0.2 Millipore filter) for *ex vivo* treatment. Microglia concentration was determined by trypan blue exclusion, adjusted to a density of 50,000 cells/100μL, and plated in a 96-well v-bottom plate. To assess microglia cytokine responsiveness, microglia were challenged *ex vivo* with 10μL of LPS at a concentration of 100ng/mL or media for 4 h at 37^0C, 5% CO_2. Following *ex vivo* manipulations, plates were centrifuged at 600 x *g* for 6 min to pellet cells and the supernatant was aspirated off. Cells were washed with 100μL PBS and centrifuged at 600 x *g* for 6 min. Cell lysis and cDNA synthesis was performed using the PrepEase kit (USB, CA) according to manufacturer's instructions.

Immunohistochemistry: Mice were transcardially perfused with cold saline followed by cold 4% PFA. Brains were post fixed in 4% PFA at 4°C for 24 hours followed by 30% sucrose for 48 hours. Fixed brains were frozen with isopentane (−78°C) and sectioned (20 μm) using a Microm HM550 cryostat (Thermofisher, Dublin, Ohio, USA). Brain regions were classified based on reference markers used in the stereotaxic mouse brain atlas (13). Sections were placed free-floating in cryoprotectant until staining. Sections were directly examined for YFP and tdTomato fluorescence by fluorescent microscopy. For Iba-1, c-Fos, and pCREB analysis, sections were washed in PBS with 1% bovine serum albumin (BSA), blocked with 5% normal donkey serum and 0.1% TritonX and incubated with the appropriate primary antibody (Iba-1, Wako Chemicals,

Richmond, VA cat# 019-19741; cFos, Cell Signaling, Danvers, MA cat#2250; pCREB, Cell Signaling, cat# 9198). Primary incubations were completed overnight at 4°C. Sections were then washed in PBS and incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor 488). Sections were mounted on slides, cover-slipped with Fluoromount G (Beckman Coulter, Inc., Pasadena, CA), and stored at -20°C. Fluorescent images were taken with a Zeiss 510 Meta confocal microscope.

Behavioral Analyses: *Anxiety-like behavior* in the open field was determined as described (1, 14) . The open-field test was used to assess anxiety-like behavior in these studies because our previous work shows that it is a robust and reproducible behavioral test in the context of social defeat (1, 2). Furthermore, measures of thigmotaxis in the open-field test demonstrate high degrees of validity for modeling anxiety-like behaviors (reviewed by (15)). For the open-field test, mice were placed in the corner of the test apparatus (40 × 40 × 25 cm Plexiglas box) and activity was recorded for 5 min. Mice with anxiety-like behavior enter the center of the open-field slower and spend less time in the open-field. Behavior was recorded an analyzed using an automated system (VersaMax, AccuScan Instruments, Omnitech Electronics Inc., Columbus, OH). *Social exploratory behavior* was determined as previously described (16) with a few modifications. In brief, experimental mice were introduced individually into the home cage of a novel juvenile (3 weeks old), who was in a protective cage, for a ten minute period. Behavior was videotaped and the cumulative amount of time the experimental mouse engaged in social investigation was determined. Social exploration tests were conducted during the light phase (between 0900h and 1700h) of the photoperiod. Behavior was scored by trained observers who were blind to the experimental treatments. Baseline social behavior was measured immediately before experimental treatment (time 0). Social behavior was determined as the amount of time

that the experimental subject spent actively investigating (e.g., anogenital sniffing or trailing) the juvenile. Results are expressed as percent decrease in social behavior compared to respective baseline measures.

Statistical Analysis: All data are expressed as treatment means ± standard error of the mean (SEM). Individual data points more than two standard deviations above and below the mean were counted as outliers, and were excluded in the subsequent analyses. To determine significant main effects and interactions between main factors, data were analyzed using one way, two way, or three way ANOVA using the General Linear Model procedures of SPSS statistical software (IBM). In the event of a main effect of experimental treatment, differences between group-means were evaluated by an F-protected t-test. *Post hoc* analyses (Fisher's LSD) are graphically presented in figures. Threshold for statistical significance was set at $p < 0.05$.

Supplemental References

1. Wohleb, E. S., Powell, N. D., Godbout, J. P., and Sheridan, J. F. (2013) Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33, 13820-13833
2. Wohleb, E. S., McKim, D. B., Shea, D. T., Powell, N. D., Tarr, A. J., Sheridan, J. F., and Godbout, J. P. (2014) Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. *Biol Psychiatry*
3. McKim, D. B., Niraula, A., Tarr, A. J., Wohleb, E. S., and Sheridan, J. F. (2016) Neuroinflammatory Dynamics Underlie Memory Impairments after Repeated Social Defeat. 36, 2590-2604
4. McKim, D. B., Patterson, J. M., Wohleb, E. S., Jarrett, B. L., Reader, B. F., Godbout, J. P., and Sheridan, J. F. (2015) Sympathetic Release of Splenic Monocytes Promotes Recurring Anxiety Following Repeated Social Defeat. *Biological psychiatry*
5. Ramirez, K., Shea, D. T., McKim, D. B., Reader, B. F., and Sheridan, J. F. (2015) Imipramine attenuates neuroinflammatory signaling and reverses stress-induced social avoidance. *Brain, behavior, and immunity* 46, 212-220
6. Wohleb, E. S., McKim, D. B., Shea, D. T., Powell, N. D., Tarr, A. J., Sheridan, J. F., and Godbout, J. P. (2014) Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. *Biological psychiatry* 75, 970-981
7. Hanke, M. L., Powell, N. D., Stiner, L. M., Bailey, M. T., and Sheridan, J. F. (2012) β-adrenergic blockade decreases the immunomodulatory effects of social disruption stress. *Brain Behav Immun*
8. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15-21
9. Risso, D., Ngai, J., Speed, T. P., and Dudoit, S. (2014) Normalization of RNA-seq data using factor analysis of control genes or samples. *Nature biotechnology* 32, 896-902
10. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15, 550
11. Kramer, A., Green, J., Pollard, J., Jr., and Tugendreich, S. (2014) Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* 30, 523-530
12. Sohal, D. S., Nghiem, M., Crackower, M. A., Witt, S. A., Kimball, T. R., Tymitz, K. M., Penninger, J. M., and Molkentin, J. D. (2001) Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circulation research* 89, 20-25
13. Paxinos, G., and Franklin, K. (2008) The mouse brain in stereotaxic coordinates. 3rd edition.
14. Wohleb, E. S., McKim, D. B., Shea, D. T., Powell, N. D., Tarr, A. J., Sheridan, J. F., and Godbout, J. P. (2014) Re-establishment of anxiety in stress-sensitized mice is caused by monocyte trafficking from the spleen to the brain. *Biological psychiatry* 75, 970-981
15. Prut, L., and Belzung, C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European journal of pharmacology* 463, 3-33
16. Corona, A. W., Huang, Y., O'Connor, J. C., Dantzer, R., Kelley, K. W., Popovich, P. G., and Godbout, J. P. (2010) Fractalkine receptor (CX3CR1) deficiency sensitizes mice to the behavioral changes induced by lipopolysaccharide. *J Neuroinflammation* 7, 93