IL-1 Receptor-1 on Vglut2+ neurons in the hippocampus is critical for neuronal and behavioral sensitization after repeated social stress

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ABSTRACT

Myriad findings connect stress and inflammation to mood disorders. Social defeat in mice promotes the convergence of neuronal, central inflammatory (microglia), and peripheral immune (monocytes) pathways causing anxiety, social avoidance, and “stress-sensitization.” Stress-sensitization results in augmented inflammation and the recurrence of anxiety after re-exposure to social stress. Different cell compartments, including neurons, may be uniquely sensitized by social defeat-induced interleukin-1 (IL-1) signaling. Therefore, the aim of this study was to determine if glutamatergic neuronal IL-1 receptor signaling was essential in promoting stress-sensitization after social defeat. Here, wild-type (IL-1R1+/−) mice and mice with IL-1 receptor-1 deleted selectively in glutamatergic neurons (Vglut2−/−IL-1R1−/−) were stress-sensitized by social defeat (6-cycles) and then exposed to acute defeat (1-cycle) at day 30. Acute defeat-induced neuronal activation (∆FosB and phospho-CREB) in the hippocampus of stress-sensitized mice was dependent on neuronal IL-1R1. Moreover, acute defeat-induced social withdrawal and working memory impairment in stress-sensitized mice were also dependent on neuronal IL-1R1. To address region and time dependency, an AAV2-IL-1 receptor antagonist construct was administered into the hippocampus after sensitization, but prior to acute defeat at day 30. Although stress-sensitized mice had increased hippocampal pCREB and decreased working memory after stress re-exposure, these events were not influenced by AAV2-IL-1 receptor antagonist. Hippocampal ∆FosB induction and corresponding social withdrawal in stress-sensitized mice after stress re-exposure were prevented by the AAV2-IL-1 receptor antagonist. Collectively, IL-1 signaling in glutamatergic neurons of the hippocampus was essential in neuronal-sensitization after social defeat and the recall of social withdrawal.

1. Significance statement

Anxiety is a common psychiatric disorder linked to both stress and inflammation. Traumatic or chronic psychological stress may promote stress-sensitization, in which individuals have increased vulnerability and reactivity to subsequent stressors. The mechanisms underlying stress-sensitization, however, are unknown. Here, we aimed to determine the role of IL-1 receptor signaling in glutamatergic (Vglut2+) neurons in stress-sensitization. We provide novel evidence that neuronal IL-1R1 was critical in the development of stress-sensitization in mice and the recurrence of cognitive impairment and social withdrawal. In addition, IL-1 signaling in glutamatergic neurons of the hippocampus was essential in neuronal-sensitization after social defeat and the recall of social withdrawal.

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2. Introduction

Psychosocial stress is associated with increased anxiety and depression (Faravelli and Pallanti, 1989; Kendler et al., 1998, 2003). Chronic stress is linked with stress-sensitization, which is represented by an enhanced vulnerability and reactivity to subsequent stressors (McLaughlin et al., 2010; Stroud et al., 2011). Thus, there is significant interest in understanding the role of inflammation and the immune system in stress-associated deficits in mood and behavior. For instance, inflammatory cytokines, including interleukin (IL-1) and IL-6, influence the central nervous system (CNS) and contribute to stress and anxiety in humans and rodents (Glaser and Kiecolt-Glaser, 2005; Danzter et al., 2008; Cohen et al., 2012; Haroon et al., 2012; Powell et al., 2013; Miller et al., 2014; Menard et al., 2017). Therefore, it is critical to determine the cell specificity by which cytokines signal in the brain and influence behavior.

Social defeat in mice is a stressor that promotes the convergence of neuronal, central inflammatory and peripheral immune pathways causing prolonged anxiety, social avoidance, and “stress-sensitization” (Wohleb et al., 2014c; McKim et al., 2018b). IL-1β is a key inflammatory cytokine that signals through the IL-1 receptor 1 (IL-1R1) and this signaling is regulated by IL-1 receptor antagonist (IL-1RA). With social defeat, inflammatory monocytes produce interleukin-1 beta (IL-1β) that signals through IL-1R1 on endothelia to augment anxiety-like behavior (Wohleb et al., 2014b; McKim et al., 2018b). IL-1β also signals through glutamatergic neurons in the hippocampus and induces social withdrawal and working memory deficits after social defeat (DiSabato et al., 2021). Thus, it is relevant to understand how cytokines influence neurons directly.

One consequence of this social defeat is stress-sensitization (Wohleb et al., 2014c). This stress-sensitization persists weeks after social defeat. It is important to highlight that anxiety-like behavior and cognition return to control levels by 2–3 weeks after social defeat (Wohleb et al., 2014c; McKim et al., 2016a, 2016b). Nonetheless, several indices of persistant stress persist at 30d including prolonged social avoidance of an aggressive intruder (Wohleb et al., 2014c), altered transcriptional profiles of microglia (Weber et al., 2019) and an increased reservoir of monocytes in the spleen (McKim et al., 2018a). As a result, exposure to one cycle of acute defeat at day 30 results in exaggerated neuro-inflammation and the recurrence of anxiety-like behavior. This acute defeat 30d after the initial stress sensitization was termed a “sub-threshold stressor” because it did not induce significant immune, neuronal, or behavioral changes in naive mice (Wohleb et al., 2014c; Weber et al., 2019). Based on previous studies, we propose that there is sensitization at three levels: neurons, myeloid cells in the spleen (Wohleb et al., 2014c; McKim et al., 2018b), and microglia in the brain (Weber et al., 2019). While we have previously published on sensitization of splenic myeloid cells and microglia, the mechanisms that cause neuronal sensitization are unclear. Stress-sensitized mice had increased neuronal phospho-cAMP-response element binding protein (pCREB) induction in the prelimbic cortex and dentate gyrus after exposure to acute defeat (Weber et al., 2019). In addition, pCREB induction preceded microglia reactivity (Iba-1 morphological restructing) and was paralleled by the recurrence of anxiety-like behavior (Weber et al., 2019). These data are interpreted to indicate that neuronal sensitization has a critical role in microglial reactivation and the recall of anxiety. The anxiety-like behavior at both the 14h time point immediately after stress (McKim et al., 2018b) and at the 30d post-stress-sensitized time point were associated with the accumulation of inflammatory IL-1-producing monocytes to the brain-endothelial interface, including within the hippocampus. Moreover, there is a high level of IL-1 receptor 1 (IL-1R1), the primary target for IL-1β signaling, particularly within the Vglut2+ glutamatergic neurons of the hippocampal granule cell layer (Liu et al., 2019). We have previously demonstrated that stress-induced microglial reactivity and peripheral monocyte inflammatory signaling target neuronal IL-1R1 and elicit anxiety-like behavior at 14h (DiSabato et al., 2021). Thus IL-1 signaling in neurons, especially in hippocampal (Vglut2+) neurons, may have an important role in the sensitization and re-activation of neurons during stress re-exposure.

Based on these data, the objective of this study was to determine if neuronal (Vglut2+) IL-1R1 in the hippocampus was essential in the establishment and recall of stress-sensitization after social defeat. We hypothesized that increased IL-1 signaling with social defeat sensitizes neurons and increases their reactivity to subsequent challenges. Here, we report that blockade of neuronal IL-1R1 signaling prevented key neuronal aspects in the establishment of stress sensitization. Furthermore, IL-1R1-mediated signaling in hippocampal neurons was critical in the recall of social withdrawal in stress-sensitized mice.

3. Materials and methods

**Mice:** Male C57BL/6 mice (wild-type or transgenic) were housed in cohorts of two per cage. Male CD-1 mice were individually housed and screened for aggression toward other mice. Wild-type C57BL/6 mice (6- to 8-week old) and CD-1 mice (12-month-old, retired breeders) were purchased from Charles River Laboratories (Wilmington, MA). Our previous work with IL-1R1 reporter mice indicate a high level of IL-1R1 expression in the hippocampus of adult mice, which can be targeted for deletion during development by Vglut2-Cre (DiSabato et al., 2021). The glutamatergic neuron-specific IL-1R1 knockout (Vglut2-IL-1R1−/−) transgenic mice were generated previously by crossing the Il1r1loxP/loxP mouse line (Robson et al., 2016) with the Vglut2-Cre (Slc17a8tm2(review) /+) mouse line until Cre+ Il1r1loxP/loxP homozygous mice were obtained and a colony was established (DiSabato et al., 2021). Here, Vglut2-IL-1R1−/− mice and Cre-negative Il1r1loxP/loxP littermates (IL-1R1+/−) were used in Figs. 1–4. The genetic backgrounds of these lines were confirmed by genotyping (Transnetsx, Inc). While Cre+ lines were confirmed, these mice were either hemizygous or homozygous for Cre. All mice were housed in polypropylene cages, with food and water ad libitum, in rooms maintained at 21°C under a 12h light/dark cycle. All behavior and biological measures were determined 14h after the last cycle of social defeat, at hour 2 of the light cycle. This time point was selected because sympathetic nervous system and hypothalamic-pituitary-adrenal axis activation returns to baseline by 14h after the last cycle of stress (Wohleb et al., 2011). All procedures were in accordance with the NIH Guidelines and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

**Social Defeat:** Mice were subjected to a modified version of repeated social defeat as previously described (Avitsur et al., 2002, 2003; DiSabato et al., 2021). In brief, experimental mice were placed individually into the cages of CD-1 aggressor mice for up to 1h (between 16:00 to 18:00) per night for six consecutive nights. During each cycle, submissive behaviors (e.g., upright posture, fleeing, crouching) were observed to ensure that the experimental mice showed signs of defeat. Mice were moved to a new aggressor’s cage if no attack occurred within the first 5 min or if the experimental mouse defeated the aggressor. At the end of the 1h period, the mice were removed and placed back into their home cage, left undisturbed until the following day when the paradigm was repeated. To avoid habituation, different aggressors were used on consecutive nights. The health of the experimental mice was monitored throughout the experiments. Mice that were significantly wounded or moribund were removed from the study. Less than 5% of mice met the early removal criteria, consistent with previous studies (DiSabato et al., 2021). Control mice were left undisturbed in their home cages.
Fig. 1. Social stress-induced \( \Delta \text{FosB} \) induction in the hippocampus was dependent on neuronal IL-1R1.

A) Male \( \text{Vglut2-IL-1R1}^{-/-} \) mice and IL-1R1 \( ^{+/+} \) (Cre \(^{-}\) littersmates) were subjected to 6 cycles of social defeat (Stress) or left undisturbed as controls (Control). Fourteen hours after the last cycle of social defeat, samples were collected for analyses. B) Representative labeling of Iba-1 \(^{+}\) microglia in the hippocampus. The dentate gyrus is outlined. C) Percent area of Iba-1 \(^{+}\) microglia labeling in the hippocampus (\( n = 4 \)). D) Total number of Iba-1 \(^{+}\) cells in the hippocampus (\( n = 4 \)). E) Representative labeling \( \Delta \text{FosB}^{+} \) cells in the dentate gyrus. F) Mean fluorescence intensity (MFI) of \( \Delta \text{FosB} \) labeling in the dentate gyrus (\( n = 4 \)). Scale bars = 100 \( \mu \)m. Bars represent mean \( \pm \) SEM. Means with (*) are different from control mice (\( P < 0.05 \)).
Stress-Sensitization: For stress-sensitization studies, mice were subjected to 6 cycles of social defeat as described (days 1–6) above while naïve mice were left undisturbed for the social stress period. Next, all mice were subjected to a single acute defeat (one cycle of social defeat) at day 30, similar to previous studies (Weber et al., 2019). Behavioral (locomotor activity and social interaction), cognitive (Y-maze), and biochemical analyses were completed 14h after the single cycle of acute defeat.

Social Interaction: Baseline locomotor activity and social interaction with a juvenile were determined 14h after the final cycle of social defeat as previously described (McKim et al., 2016b; DiSabato et al., 2021). In brief, mice were placed in the corner of the open field apparatus (40 × 40 × 25 cm Plexiglas box) and baseline activity was recorded for 5 min using an automated digital beam break system. Next, a 3–5 week old juvenile male C57BL/6 mouse was placed into an 18 × 8 × 8 cm metal wire enclosure, placed on one side of the open field arena. The duration of social investigation of the caged mouse by the experimental subject was determined over 5 min using Fusion software (Omnitech Electronics). Region of interaction was defined as within 2 cm of the caged mouse. After each test, both the wire enclosure and the open field box were cleaned with H2O and 70% ethanol to reduce odor cues. Movement and social interaction data were analyzed by an investigator blinded to treatment groups.

Working Memory: Spontaneous alternations in the Y-Maze were determined 14h after the final cycle of social defeat as previously described (DiSabato et al., 2021). In brief, mice were placed into the center of the Y-maze (7 × 40 × 12 cm, San Diego Instruments, Inc) and the number of entries into each arm were determined. A spontaneous alternation was defined as entering all three arms before revisiting a previously entered arm. The spontaneous alternations by each mouse were represented as a percentage of total 3-entry sets. After each test, the Y-Maze was cleaned with H2O and 70% ethanol to reduce odor cues. Movement and arm entries were analyzed by an investigator blinded to treatment groups.

Iba-1, ΔFosB and phospho-CREB Immunofluorescence: Iba-1, ΔFosB and phospho-CREB expression were labeled as previously described (DiSabato et al., 2021). Following behavioral assessments, mice were transcardially perfused with ice-cold PBS (pH 7.4) followed by 4% formaldehyde. Brain samples were post-fixed for 24 h, cryoprotected in 30% sucrose for 24 h, frozen using dry ice-cooled isopentane, sectioned (30 μm), and stored in cryoprotectant. Brain regions were identified using the Allen Mouse Brain Atlas (Allen Institute). Sections were washed in PBS with 1% bovine serum albumin (BSA), blocked with 5% normal donkey serum (1% BSA, 0.1% TritonX in PBS), and incubated with primary antibodies: rabbit anti-mouse ΔFosB (1:2000; Abcam; cat# ab184938), rabbit anti-mouse Iba-1 (1:1000; Wako Chemicals; cat# 019–19741), rabbit anti-mouse phospho-CREB (pCREB, 1:1000; Cell Signaling Technologies; cat# 9198S). Sections were incubated overnight at 4°C. Next, sections were washed in PBS and incubated with an appropriate fluorochrome-conjugated secondary antibody (Donkey anti-rabbit; AlexaFluor 488 or 594; Thermo Fisher Scientific). Sections were mounted on charged slides, cover-slipped with Fluoromount (Southern Biotech), and stored at −20°C. Slides were visualized and imaged using an EVOS M7000 imaging system (Thermo.
Fisher Scientific). For pCREB and GFP, cells with positive labeling were counted in each section. Iba-1 labeling was analyzed using digital image analysis. In brief, a threshold for positive staining was determined for each image that included all cell bodies and processes but excluded background staining. Data were processed by densitometric scanning of the threshold targets using ImageJ software. For ΔFosB, mean fluorescence intensity (MFI) was calculated within a region of interest defined around the dentate gyrus using ImageJ software. Values from 4 to 6 images per mouse were averaged, and these values were used to calculate group averages and variance for each experimental group.

Fig. 3. Neuronal IL-1R1-dependent pCREB and ΔFosB activation after acute defeat in stress-sensitized mice.
A) Male Vglut2-IL-1R1−/− mice and IL-1R1+/− (Cre− littermates) were subjected to 6 cycles of social defeat for stress-sensitization (SS) or left undisturbed as naïve controls (Naïve). Twenty-four days later, all mice (Naïve and Stress-Sensitized, SS) were exposed to acute defeat. Fourteen hours after acute defeat, samples were collected for analysis. B) Representative labeling of pCREB + cells and C) the number of pCREB + cells in the dentate gyrus after acute defeat (30d) (n = 4). D) Representative labeling of ΔFosB + cells and E) mean fluorescence intensity (MFI) of ΔFosB in the dentate gyrus after acute defeat (30d) (n = 4). F) Representative bivariate dot plots of CD11b+ and Ly6C+ labeling of monocytes in circulation. G) Number of CD11b+/Ly6C+ monocytes in the blood (n = 4–5). H) Representative bivariate dot plots of CD11b+ and CD45+ in the brain. I) Number of CD11b+/CD45+ monocyte/macrophages in the brain (n = 4–5). Scale bars = 100 μm. Bars represent mean ± SEM. Means with (*) are different from control mice (P < 0.05).
Investigators were blinded to experimental groups prior to all microscopy and throughout image analysis.

**Gene Expression Analysis:** Mice were euthanized following behavioral assessments and transcardially perfused with ice-cold PBS (pH 7.4). A 2 mm coronal brain section containing the cortex, hippocampus, striatum, and hypothalamus was taken using a brain matrix. Tissue was immediately snap-frozen in liquid nitrogen, stored at \(-80^\circ\text{C}\), and total RNA was extracted using the Tri-Reagent protocol (Sigma-Aldrich). Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to produce cDNA. Quantitative real-time (q)-PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems). In brief, experimental cDNA was amplified using qPCR such that target (IL-1\(\beta\), CCL2, ICAM-1) and reference cDNA (GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (FAM) and 3' non-fluorescent quencher (NFQ). Probes used include \(\text{Il1b}\) (Assay ID Mm00434228_m1), \(\text{Ccl2}\) (Mm00441242_m1), \(\text{Icam1}\) (Mm00516023_m1), and \(\text{Gadph}\) (Mm99999915_g1). When Taq DNA polymerase synthesizes a new strand and reaches the TaqMan probe, the FAM is cleaved from the NFQ and increases the fluorescent intensity.

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**Fig. 4. Neuronal IL-1R1-dependent social withdrawal and cognitive impairment after acute defeat in stress-sensitized mice.**

A) Male Vglut2-IL-1R1\(^{-/-}\) mice and IL-1R1\(^{+/-}\) (Cre\(^-\) littermates) were subjected to 6 cycles of social defeat for stress-sensitization (SS) or left undisturbed as naïve controls (Naïve). Twenty-four days later, all mice (Naïve and Stress-Sensitized, SS) were exposed to acute defeat. Fourteen hours after acute defeat, samples were collected for analysis. Social interaction with a novel juvenile C57BL/6 mouse was determined by B) total distance traveled, C) interaction time, and D) time spent in the corners of the arena (n = 7–8). Working memory was assessed in the Y-Maze by E) total arm entries and F) percentage of spontaneous alternations (n = 7–8). G) Spleen weight was determined after acute defeat (30d). Coronal (2 mm) brain sections were collected and mRNA levels of H) \(\text{Il1b}\), I) \(\text{Ccl2}\), and J) \(\text{Icam1}\) were determined (n = 4–5). Bars represent mean ± SEM. Means with (*) are different from control mice (P < 0.05).
proportional to the amount of amplicon synthesized. Fluorescence was determined using a QuantStudio 3 or 5 Real-Time PCR System (Applied Biosystems). Data were analyzed using the comparative threshold cycle (ΔΔCT) method and results are expressed as fold change from a control group.

**Flow Cytometry:** Following behavioral assessment, mice were euthanized via CO₂ asphyxiation. Blood was collected with EDTA-lined syringes by cardiac puncture and red blood cells were lysed. Mice were transcardially perfused with ice-cold PBS (pH 7.4) and brain samples were collected following extraction of the coronal brain section for PCR analysis. Brains were homogenized and labeling of cell surface antigens was performed as previously described (Wohleb et al., 2013). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience; cat# 553142). Cells were washed and then incubated with the appropriate antibodies for CD45 (BD Biosciences; cat# 550994), CD11b (eBioscience; cat# 17011283), Ly6G (BD Biosciences; cat# 560602), and Ly6C (eBioscience; cat# 45593282) for 1h at 4 °C. Cells were washed and then re-suspended in FACS buffer for analysis. Cell numbers were estimated using counting beads (ThermoFisher). Antigen expression was determined using a Beckton-Dickinson FACSCalibur cytometer with Cyteq nine-color upgrade. Data were analyzed using FlowJo software (FlowJo, LLC) and positive labeling for each antibody was determined based on isotype-controlled labels. Samples were first gated to reduce noise in the FSC<sup>low</sup>/SSC<sup>low</sup> signal range. Blood samples were gated for Ly6G-negative events before CD11b<sup>+</sup>/Ly6C<sup>+</sup> labeling was assessed. Brain samples were gated for Ly6G-negative events before appropriate antibodies for CD45<sup>+</sup>/CD45<sup>−</sup> labeling was assessed. Investigators were blinded to experimental groups during sample collection, flow cytometry, and data analysis.

**Intra-hippocampal AAV2-IL1RA Injections:** As previously described (D'Sabato et al., 2021), stereotactic injections were performed under isoflurane inhalation anesthesia using an automated stereotactic instrument (David Kopf Instruments). Mice were bilaterally injected with 1.0 × 10⁹ PFC/μL AAV2-IL1ra (AAV2.CMV.H.I1RN.ires.eGFP, Vector Biolabs, Lot, 190121#18) (AAV-IL1RA) into the hippocampus (coordinates AP -1.82, DV 2.26, ML ±0.75), and compared with AAV2-GFP-injected (AAV2.CMV.H.eGFP.WPRE.bGH, AddGene) mice (AAV-GFP). After injection, mice were afforded 3 weeks recovery before experimentation.

**Experimental design and Statistical analysis:** All experimentation and data analysis were conducted under blinded conditions. Number of mice per experiment including number of sections/images analyzed are included in the methods and figure legends for each experiment above. Mice were randomly assigned to either control or stress groups for all experiments. To determine significant main effects and interactions between main factors in multiple-group comparisons, data were analyzed using the General Linear Model procedures of SPSS statistical software (IBM). Two-way ANOVA were used to determine the main effects and interactions between treatments (P < 0.05). Tukey’s HSD (honest significant difference) was used for post-hoc analysis of the ANOVA data when main effects and/or interactions were significant. Tukey’s HSD analysis determined which specific group means were different from each other (P < 0.05). Denotations of this post-hoc significance are represented in the Figures. All data are presented as mean ± standard error of the mean (SEM).

4. Results

**Social stress-induced ΔFosB induction in the hippocampus was dependent on neuronal IL-1R1.** Repeated social defeat promotes the convergence of neuronal, central inflammatory and peripheral immune pathways causing prolonged anxiety, social avoidance, and “stress-sensitization” (Wohleb et al., 2014c; McKim et al., 2018b). IL-1β is increased by social stress and in the brain it signals through the IL-1 receptor 1 (IL-1R1) on endothelia (Wohleb et al., 2014b; McKim et al., 2018b) and on glutamatergic neurons (Liu et al., 2019; DiSabato et al., 2021). In this study, our goal was to further investigate the role of IL-1 receptor signaling in glummateric neurons with stress, stress-sensitization, and stress-reactivity (i.e., recall). The glutamate transporter VGLUT2 is expressed in relevant subcortical areas including the thalamus, amygdala, and hippocampus (Fremereau et al., 2001; Herzog et al., 2001). Thus, to target IL-1R1 signaling in hippocampal glutamatergic neurons, a Vglut2-Cre transgenic mouse line was used (Liu et al., 2019; DiSabato et al., 2021).

In the first experiment (Fig. 1A), IL-1R1<sup>+/−</sup> and Vglut2-IL1R1<sup>−/−</sup> mice were subjected to repeated social defeat (i.e., 6 cycles of defeat) and Iba-1 (Fig 1B) and ΔFosB (Fig 1E) labeling were determined in the hippocampus 14h later. Percent area of Iba-1 labeling was increased in the hippocampus by stress (F(1,12) = 59.4, P < 0.001; Fig 1C) and this increase was independent of IL-1R1 expression on Vglut2<sup>+</sup> neurons. Next, overall Iba-1<sup>+</sup> cell numbers were quantified and showed an IL-1R1-dependent increase after stress (F(1,12) = 9.7, P < 0.02; Fig 1D).

Neuronal activation (ΔFosB, MFI) was also increased by social defeat in the dentate gyrus of IL-1R1<sup>+/−</sup> mice (F(1,14) = 17.6, P < 0.002, Fig 1F). This ΔFosB increase was dependent on IL-1R1 expression in Vglut2<sup>+</sup> neurons (interaction, F(1,14) = 12.9, P < 0.004). Post hoc analysis showed that the Stress/IL-1R1<sup>+/−</sup> group had the highest MFI of ΔFosB labeling in the dentate gyrus compared to all groups, including the Stress/Vglut2-IL1R1<sup>−/−</sup> group (P < 0.001). Consistent with previous work (DiSabato et al., 2021), neuronal activation in the dentate gyrus after social defeat was dependent on IL-1R1 expression in Vglut2<sup>+</sup> neurons, but morphological restructuring of microglia was not.

In terms of a peripheral immune response, social defeat increased Ly6C<sup>hi</sup> mononcytes in circulation (F(1,16) = 7.0, P < 0.02, Fig 2A&B) and CD11b<sup>+</sup>/CD45<sup>−</sup> monocyte/macrophages in the brain (F(1,16) = 11.0, P < 0.005, Fig 2C&D). Knockout of IL-1R1 on Vglut2<sup>+</sup> neurons had no effect on this stress-induced monocyte release into circulation or recruitment to the brain. Next, mRNA levels of inflammatory mediators associated with microglia activation and monocyte recruitment were determined in a coronal brain section through the hippocampus. Social defeat increased mRNA levels of Il1b (F(1,16) = 14.1, P < 0.002; Fig. 2E), Ccl2 (F(1,16) = 7.6, P < 0.02; Fig 2F), and Icam1 (F(1,16) = 11.9, P < 0.004; Fig 2G) in the brain. These increases in Il1b, Ccl2, and Icam1 with social defeat were independent of IL-1R1 expression on Vglut2<sup>+</sup> neurons. Collectively, stress-associated activation of glummateric neurons in the dentate gyrus by IL-1 signaling was downstream of microglia activation, monocyte release, and monocyte recruitment to the brain.

**Neuronal IL-1R1-dependent pCREB and ΔFosB activation after acute defeat in stress-sensitized mice.** Repeated social defeat induces stress-sensitization that persists weeks after the initial stress exposure (Wohleb et al., 2014c; McKim et al., 2016b). Notably, anxiety-like behavior and cognition return to unstressed levels by 2–3 weeks after social defeat (Wohleb et al., 2014c; McKim et al., 2016a, 2016b). Nonetheless, several indexes of sensitization remain at 30d including altered microglial transcriptional profiles (Weber et al., 2019) and an increased reservoir of inflammatory monocytes in the spleen (McKim et al., 2018a). The functional consequence of this stress-sensitization is amplified inflammatory responses to an acute and subthreshold stressor at 30d with exaggerated neuroinflammation and the recurrence of anxiety (Wohleb et al., 2014c; McKim et al., 2016b; Weber et al., 2019). One of the unique indicators of stress-sensitization was the induction of pCREB<sup>+</sup> in the prelimbic cortex and dentate gyrus. This pCREB induction was only detected when stress-sensitized mice were exposed to acute defeat at 30d (Weber et al., 2019). Therefore, we sought to determine the role for neuronal (Vglut2<sup>+</sup>) IL-1R1 signaling in sensitization and reactivity to an acute stressor at 30d.

Here, IL-1R1<sup>+/−</sup> and Vglut2-IL1R1<sup>−/−</sup> mice were naïve or stress-sensitized (SS) by social defeat. All mice were exposed to a single cycle of acute defeat at 30d, which was 24 days after the completion of the initial social stress paradigm, and samples from the brain and blood were collected 14h later (Fig 3A). The hippocampus was sectioned for...
analysis of pCREB and ΔFosB activation. There was a main effect of stress-sensitization (SS) on the number of pCREB+ cells in the dentate gyrus at 30d (F(1,12) = 12.4, P < 0.01) and this effect was influenced by IL-1R1 expression in Vglut2+ neurons (interaction; F(1,12) = 9.8, P < 0.02, Fig. 3B&C). Post-hoc analysis shows that pCREB+ cells in the hippocampus were increased in stress-sensitized IL-1R1/-/- mice exposed to acute defeat compared to all other groups (P < 0.05, Fig. 3C). Stress-sensitized Vglut2-IL-1R1/-/- mice were not different than naïve controls. ΔFosB activation in the hippocampus paralleled the results of the pCREB activation. There was a main effect of SS on mean fluorescence intensity (MFI) of ΔFosB labeling in the dentate gyrus at 30d (F(1,12) = 12.7, P < 0.007, Fig. 3D&E) and this effect was dependent on IL-1R1 expression in Vglut2+ neurons (interaction; F(1,12) = 5.5, P < 0.04, Fig. 3E). Post-hoc analysis confirmed that stress-sensitized IL-1R1/-/- mice exposed to acute defeat had the highest MFI for ΔFosB labeling compared to all other groups (P < 0.03, Fig. 3E). Again, stress-sensitized Vglut2-IL-1R1/-/- mice were not different than naïve controls. Taken together, both neuronal reactivity (pCREB) and activation (ΔFosB) in the dentate gyrus after acute defeat in stress-sensitized mice (30d) were dependent on IL-1R1 in Vglut2+ neurons.

Next, the percentage of monocytes in circulation and in the brain were determined in this experimental design. As expected, there was a main effect of SS on the number of Ly6C(hi) monocytes in circulation (F(1,13) = 6.1; P < 0.03, Fig. 3F&G) and the percentage of CD11b+/CD45(hi) monocyte/macrophages in the brain (F(1,13) = 29.4, P < 0.0001, Fig. 3H&I). These increases with stress-sensitization were independent from the knockout of IL-1R1 on Vglut2+ neurons. For instance, all stress-sensitized groups had increased Ly6C(hi) circulating monocytes and brain monocytes upon acute defeat at 30d, regardless of IL-1R1 genotype. It is important to highlight that acute defeat had no effect on neuronal reactivity, activation, or these immune parameters in naïve mice. These data are consistent with our previous studies showing this acute defeat is a sub-threshold stressor (Wohleb et al., 2014; Weber et al., 2019). Collectively, IL-1R1 signaling in glutamatergic neurons has a key role in sensitization with social defeat and subsequent neuronal reactivity to acute defeat at 30d.

Neuronal IL-1R1-dependent social withdrawal and cognitive impairment with acute defeat in stress-sensitized mice. Next, hippocampal-dependent behaviors influenced by social stress (DiSabato et al., 2021) were assessed using the stress-sensitization and acute defeat experimental design (Fig. 4A). Again, IL-1R1/-/+ and Vglut2-IL-1R1/-/- mice were assigned as naïve controls or stress-sensitized by 6 cycles of social defeat. All mice were exposed to acute defeat at 30d and social interaction and working memory were assessed 14h later.

For social interaction, we first assessed locomotion during testing. Fig. 4B shows that total distance traveled during social interaction testing was similar across all four treatment groups. Interaction time with the novel juvenile, however, was dependent on stress-sensitization (F(1,27) = 10.2, P < 0.008, Fig. 4C) and this response was influenced by IL-1R1 expression in Vglut2+ neurons (interaction; F(1,27) = 8.6, P < 0.007, Fig. 4C). Post hoc analysis confirmed that SS/IL-1R1/-/+ mice exposed to acute defeat had the lowest social interaction compared to all groups (P < 0.004). Stress-sensitized Vglut2-IL-1R1/-/- mice exposed to acute defeat were not different than naïve controls in social interaction. Time spent in the corner paralleled the results of the social interaction, with a main effect of stress sensitization (F(1,27) = 15.1, P < 0.006, Fig. 4D) that was dependent on IL-1R1 expression in Vglut2+ neurons (interaction; F(1,27) = 7.6, P < 0.04). Post hoc analysis confirmed that SS/IL-1R1/-/+ mice spent the most time spent in the corner compared to all other groups (P < 0.04).

For cognitive assessment, we first assessed total activity during the Y-Maze testing. Total arm entries during Y-maze testing were similar across all four treatment groups (Fig. 4E). Percentage of alternations, however, were dependent on stress-sensitization (F(1,27) = 11.7, P < 0.002, Fig. 4F) and this effect was influenced by IL-1R1 expression in Vglut2+ neurons (interaction; F(1,27) = 14.5; P < 0.007, Fig. 4F). Post hoc analysis confirmed that SS/IL-1R1/-/+ mice exposed to acute defeat had the lowest percentage of spontaneous alternations compared to all other groups (P < 0.02). Stress-sensitized Vglut2-IL-1R1/-/- mice exposed to acute defeat were not different than naïve controls in spontaneous alternations. In addition, spleen weight was unaffected by stress-sensitization at 30d (Fig. 4G).

Next, mRNA levels of mediators associated with microglia activation and monocyte recruitment were determined in a coronal brain section through the hippocampus. There was a main effect of stress sensitization on mRNA levels of Il1b (F(1,13) = 6.9, P < 0.03; Fig. 4H), Ccl2 (F(1,13) = 16.2, P < 0.002; Fig. 4I), and Icam1 (F(1,13) = 9.6, P < 0.009; Fig. 4J). These increases in mRNA levels with stress-sensitization were independent of IL-1R1 expression on Vglut2+ neurons (Fig. 4H–J). Collectively, IL-1R1 signaling in Vglut2+ glutamatergic neurons caused recurrence of cognitive impairment and social withdrawal after stress-sensitization and acute defeat re-exposure at 30d.

IL-1RA in the hippocampus blocked the recall of stress-sensitized social withdrawal after acute defeat. Although the Vglut2-IL-1R1/-/- genotype reduced the reactivity of stress-sensitized mice to acute defeat, the IL-1R1 gene was already knocked out during the initial process of stress-sensitization. Therefore, we next sought to determine if intervention with IL-1RA in the hippocampus after stress-sensitization prevented neuronal and behavioral recall to acute defeat at 30d. To address this idea, mice were stress-sensitized with social defeat and then received either AAV2-GFP or AAV2-IL1RA injections into the hippocampus 48h later (Fig. 5A). In this AAV2 design, IL-1 receptor antagonist was augmented selectively in hippocampal neurons after stress-sensitization, but prior to acute defeat at 30d. Fig. 5B shows that the AAV2 construct successfully induced GFP expression in the dentate gyrus at 30d. This validates the localized AAV2-mediated expression within the hippocampus after bilateral administration.

The hippocampus was sectioned for analysis of pCREB and ΔFosB activation. As before, there was a main effect of stress sensitization on the number of pCREB+ cells in the dentate gyrus (F(1,18) = 46.3, P < 0.001, Fig. 5C&D). This activation of pCREB+ with stress sensitization, however, was independent of AAV2-IL1RA expression in the hippocampus. There was a tendency for main effect on neuronal ΔFosB activation in the dentate gyrus (F(1,19) = 7.9, P = 0.10, Fig. 5E&F). This activation of ΔFosB with stress sensitization was influenced by AAV2-IL1RA (interaction; F(1,19) = 67.7, P < 0.0001, Fig. 5F). Post hoc analysis confirmed that SS/AAV2-GFP mice exposed to acute defeat had the highest MFI of ΔFosB labeling in the dentate gyrus compared to all other groups (P < 0.002). Collectively, the AAV2-IL1RA intervention was insufficient to reverse pCREB-related sensitization but was effective in preventing the recapitulation of the ΔFosB activation with acute defeat.

All mice were exposed to acute defeat at 30d, and social interaction and spontaneous alternation were assessed 14h later. As before, Fig. 5G shows that total distance traveled during social interaction testing was similar across treatment groups. There was a main effect of stress sensitization (SS) on interaction time with the novel juvenile (F(1,20) = 7.7, P < 0.01, Fig. 5H) and this was influenced by IL-1RA expression in the hippocampus (interaction; F(1,20) = 8.3, P < 0.01). Post hoc analysis confirmed that stress-sensitized mice exposed to acute defeat spent more time in the corner than naïve mice (F(1,20) = 10.1, P < 0.005, Fig. 5I). SS/IL-1RA mice exposed to acute defeat were not different than naïve controls.

For cognitive assessment, we first assessed locomotion during the Y-maze testing. Total arm entries during Y-maze testing were similar across all four treatment groups (Fig. 5J). The percentage of spontaneous alternations in the Y-maze were decreased by stress-sensitization (F(1,20) = 10.6; P < 0.004, Fig. 5K). This decrease in spontaneous alternations in stress-sensitized mice, however, was independent of IL-1RA expression in the hippocampus. In summary, selective intervention with IL-1RA in the hippocampus prevented the stress-sensitized recall of social withdrawal after acute defeat, but did not affect
Fig. 5. IL-1RA in the hippocampus blocked the recall of stress-sensitized social withdrawal after acute defeat.

A) Male wild-type C57BL/6 mice were subjected to 6 cycles of social defeat or left undisturbed as controls. Two days after stress-sensitization, AAV2-IL-1 receptor antagonist (IL1RA) or AAV2-GFP (GFP) was bilaterally injected into the hippocampus. Twenty-four days later, both Stress-Sensitized (SS) and Naive mice were exposed to acute defeat. Fourteen hours after acute defeat, behavior was determined and samples were collected for analysis.

B) Confirmation of GFP expression in the hippocampus (30d) after AAV2 injection. C) Representative labeling and D) quantification of pCREB$^+$ cells in dentate gyrus after acute defeat (30d) (n = 5–6). E) Representative labeling and F) mean fluorescent intensity (MFI) of ΔFosB$^+$ cells in dentate gyrus after acute defeat (30d) (n = 5–6). Social interaction with a novel juvenile C57BL/6 mouse was determined by G) total distance traveled, H) interaction time, and I) time spent in the corners of the arena (n = 6). Working memory was assessed in the Y-maze by J) total arm entries and K) percentage of spontaneous alternations (n = 6). Scale bars = 100 μm. Bars represent mean ± SEM. Means with (*) are different from control mice (P < 0.05).
deficits in working memory. The divergence in these behaviors was mirrored by differential signaling pathways in neurons with pCREB and $\Delta$FosB activation.

5. Discussion

We have reported that social defeat in mice promotes stress sensitization that affects several cellular compartments including splenic monocytes, brain microglia, and neurons (Wohleb et al., 2014a; McKim et al., 2016b, 2018a; Weber et al., 2019). As a result of stress sensitization, exposure to acute defeat at 30 days results in the augmentation of neuroinflammatory pathways and the recurrence of anxiety-like behavior. Recently we also reported that neuronal (Vglut2+) IL-1R1 signaling in the hippocampus was associated with cognitive impairment and social withdrawal (DiSabato et al., 2021). Thus, the main goal of this study was to determine the extent to which neuronal IL-1 receptor signaling was involved in the establishment and recall of stress-sensitization after social defeat. We began by confirming that increased neuronal activity ($\Delta$FosB) in the hippocampus after social defeat was dependent on neuronal (Vglut2+) IL-1R1 mediated signaling. The inflammatory and immune components to repeated social defeat, however, were independent of this neuronal IL-1R1 signaling. In the context of stress sensitization, acute defeat at 30d induced neuronal activation ($\Delta$FosB and phospho-CREB) in the hippocampus of stress-sensitized mice, and this required functional IL-1R1 signaling in neurons. Moreover, acute defeat-induced social withdrawal and working memory impairment in stress-sensitized mice required functional neuronal IL-1R1 signaling. As expected, monocyte release and recruitment to the brain in stress sensitized mice after stress re-exposure occurred independent of neuronal IL-1R1 signaling. Next, an AAV2-IL-1 receptor antagonist construct in the hippocampus was used in stress-sensitized mice 2 days after the 6th cycle of social defeat. This intervention selectively prevented hippocampal $\Delta$FosB induction and social withdrawal in stress-sensitized mice after stress re-exposure at 30d.

One key finding of this study was that neuronal reactivity and IL-1R1-driven activation (pCREB/$\Delta$FosB) in stress-sensitized mice exposed to acute defeat (30d) were dependent on neuronal IL-1R1. These data reinforce previous work with stress sensitization and elimination of microglia. For instance, pCREB reactivity was evident only when stress-sensitized behaviors were recalled by acute defeat at 30d (Weber et al., 2019). Our previous work indicates that Vglut2-Cre-IL-1R1 knockout abrogated the increase of $\Delta$FosB in the dentate gyrus 14h after social defeat (DiSabato et al., 2021). This was recapitulated in the current study. Moreover, we have extended this finding to our model of stress-sensitization. For instance, Vglut2-IL-1R1$^{1/-}$ mice did not have increased $\Delta$FosB in the dentate gyrus after acute defeat at 30d. This correlated with reversal of stress-induced social withdrawal and working memory deficits in the Vglut2-IL-1R1$^{1/-}$ mice. As noted above, these neuron-mediated events are temporally downstream of the increased IL-1$^+$ monocytes in the brain and microglia activation. Therefore, without functional IL-1 receptor on glutamatergic neurons, the IL-1$^+$ released by inflammatory monocytes, microglia, or other cells after acute defeat in stress-sensitized mice cannot activate IL-1R1 in neurons. Without this IL-1R1 pathway in neurons, the social withdrawal and cognitive deficits with stress re-exposure (acute defeat) at 30d were prevented.

An important point for discussion is that our data indicate that neuronal IL-1R1 signaling in the hippocampus is an endpoint in the response to social defeat. For example, neuronal (Vglut2+) IL-1R1 knockout did not prevent the social defeat-induced release of monocytes in circulation or the recruitment of monocytes to the brain. In addition, social defeat-induced IL-1$^+$, CCL2, and ICAM-1 mRNA were not influenced by this neuronal IL-1R1 knockout. While sympathetic outflow from the CNS to the periphery after stress is an initiator of the myeloid response to social defeat (Wohleb et al., 2011; Ramirez et al., 2015), neuronal IL-1R1 signaling is downstream of the subsequent microglia activation, monocyte recruitment, and cytokine expression. Indeed, all of these inflammatory signals and deficits in cognition and sociability were still evident after social stress in the Vglut2-IL-1R1$^{1/-}$ mice. These data are interpreted to indicate that IL-1$^+$ acts on neuronal IL-1R1 in the dentate gyrus to recapitulate the social and cognitive deficits at 30d. Previous findings implicate hippocampal gene expression changes (Sathyarayanas et al., 2017), noradrenergic sensitization (Le Dorze et al., 2019), and epigenetic alterations (Floiri-Servou et al., 2018; Chaby et al., 2020) in the establishment of stress-sensitization. These alterations are critical to the understanding of stress-sensitized behaviors, but do not account for increased immune-to-brain signaling that is a critical aspect of social defeat. Here, our data point to a unique role for IL-1R1 signaling in extended neuronal and behavioral sensitization after social defeat.

Another relevant finding was that social withdrawal and cognitive deficits were recalled at 30d after acute defeat and these behaviors were dependent on neuronal IL-1R1 signaling in the hippocampus. As discussed above, the IL-1R1 on neurons is an end terminal of the inflammatory response to social defeat. It is relevant to note that social defeat-induced IL-1 in our previous work, a unique splenic monocyte population was responsible (McKim et al., 2016b, 2018a). In fact, stem cells from the bone marrow colonized the spleen and led to increased extra-medullary hematopoiesis in the spleen that persisted 24d later. Splenic monocytes were released into circulation after exposure to acute stress at 30d (McKim et al., 2016b) and trafficked to the brain-vascular interface. Taken together, it was the acute defeat-mediated reactivation of neurons in the hippocampus by IL-1$^+$, produced by CNS microglia, splenic monocytes, or other cells, which mediated the social withdrawal and the deficit in working memory in the Y-maze.

A novel aspect of this study was the use of viral (AAV2)-mediated intervention with IL-1 receptor antagonist (IL-1RA) that was adminis-
tered in the dentate gyrus of the hippocampus 2 days after stress sensitization and prior to acute defeat at 30d. This provided insight into both brain region and time dependency for IL-1$^+$ signaling. For instance, this dentate gyrus-specific intervention blocked social withdrawal from a juvenile mouse in stress-sensitized mice exposed to acute defeat. Thus, the recurrence of social withdrawal after acute defeat was blocked by IL-1RA overexpression. These results are interpreted to indicate that renewed IL-1$^+$ signaling in dentate gyrus neurons was required to recall stress-sensitized social withdrawal. These data therefore indicate that pharmaceutical intervention is possible after stress-sensitization to reduce the recapitulation of stress reactivity at later endpoints. Tying into these behaviors, increased $\Delta$FosB neuronal activity in stress-sensitized mice after acute defeat was prevented by IL-1RA overexpression in the dentate gyrus. In contrast, neither decreased Y-maze spontaneous alternations nor neuronal pCREB+ labeling in stress-sensitized mice after acute defeat were affected by this IL-1RA intervention. This implies that neurons in the hippocampus were altered in their activity or structure due to neuronal IL-1R1 activation during the initial exposure to social defeat. These results pair $\Delta$FosB labeling with social behavioral deficits, and pCREB labeling with cognitive deficits in the Y-maze. Thus, different pathways of activity in the dentate gyrus are responsible for $\Delta$FosB and social withdrawal compared to pCREB and working memory deficits.

Another relevant point for discussion is the potential source of IL-1 (alpha or beta) that would activate the IL-1 receptor on neurons after social stress. IL-1 is produced by several CNS cell types including astrocytes (Jones et al., 2018), neurons (Watt and Hobbs, 2000), oligodendrocytes (Blasi et al., 1999; Bocuzzi et al., 2021), microglia, and monocytes (Hsi and Remick, 1995; Liu and Quan, 2018; Kaneko et al., 2019). Based on our previous studies, the major source of social defeat-induced IL-1$^+$ is from myeloid populations, resident microglia and peripheral monocytes. For instance, PLX5622-mediated elimination of microglia blocked monocyte recruitment and IL-1 signaling in the brain (McKim et al., 2018b; Weber et al., 2019). Moreover, knocking out
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