Published in final edited form as:

Brain Behav Immun. 2016 January ; 51: 154–168. doi:10.1016/j.bbi.2015.08.011.

GABAergic modulation with classical benzodiazepines prevent stress-induced neuro-immune dysregulation and behavioral alterations

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Abstract

Objective—Psychosocial stress is associated with altered immunity, anxiety, and depression. Repeated social defeat (RSD), a model of social stress, triggers egress of inflammatory myeloid progenitor cells (MPCs; CD11b⁺/Ly6C^{hi}) that traffic to the brain, promoting anxiety-like behavior. In parallel, RSD enhances neuroinflammatory signaling and long-lasting social avoidant behavior. Lorazepam and clonazepam are routinely prescribed anxiolytics that act by enhancing GABAergic activity in the brain. Besides binding to the central benzodiazepine binding site (CBBS) in the central nervous system (CNS), lorazepam binds to the translocator protein (TSPO) with high affinity causing immunomodulation. Clonazepam targets the CBBS and has low affinity for the TSPO. Here the aims were to determine if lorazepam and clonazepam would: 1) prevent stress-induced peripheral and central inflammatory responses, and 2) block anxiety and social avoidance behavior in mice subjected to RSD.

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Methods—C57/BL6 mice were divided into experimental groups, and treated with either lorazepam (0.10mg/kg), clonazepam (0.25 mg/kg) or vehicle (0.9%NaCl). Behavioral data and tissues were collected the morning after the last cycle of RSD.

Results—Lorazepam and clonazepam were effective in attenuating mRNA expression of CRH in the hypothalamus and corticosterone in plasma in mice subjected to RSD. Both drugs blocked stress-induced levels of IL-6 in plasma. Lorazepam and clonazepam had different effects on stress-induced enhancement of myelopoiesis and inhibited trafficking of monocytes and granulocytes in circulation. Furthermore, lorazepam, but not clonazepam, inhibited splenomegaly and the production of pro-inflammatory cytokines in the spleen following RSD. Additionally, lorazepam and clonazepam, blocked stress-induced accumulation of macrophages (CD11b⁺/CD45^{high}) in the CNS. In a similar manner, both lorazepam and clonazepam prevented neuroinflammatory signaling and reversed anxiety-like and depressive-like behavior in mice exposed to RSD.

Conclusion—These data support the notion that lorazepam and clonazepam, aside from exerting anxiolytic and antidepressant effects, may have therapeutic potential as neuroimmunomodulators during psychosocial stress. The reversal of RSD-induced behavioral outcomes may be due to the enhancement of GABAergic neurotransmission, or some other off-target effect. The peripheral actions of lorazepam, but not clonazepam, seem to be mediated by TSPO activation.

Keywords

psychosocial stress; social defeat; benzodiazepines; anxiety-like behavior; social avoidance; microglia

1. Introduction

Psychosocial stress promotes brain-to-immune and immune-to-brain communication that can impact neurobiology and behavior. For example, exposure to stress is capable of causing peripheral immune dysregulation and also enhanced neuroinflammatory signaling by repeated activation of neuroendocrine and autonomic pathways. Activation of these pathways may contribute to the development of diseases and mental health disturbances. Two of the most prevalent behavioral outcomes induced by psychosocial stressors are anxiety and depression. Both are extremely debilitating and have a great impact on the quality of life. Depression has been reported in patients suffering from anxiety and anxiety has been reported in depressed patients. Both disorders share common symptoms and are associated with increased release of peripheral cytokines (Maes et al., 2011; Hodes et al., 2014).

Repeated social defeat (RSD), a model of psychosocial stress in mice, provides a valuable and predictable probe to study the mechanisms leading to stress-related alterations in inflammation in both the periphery and central nervous system (CNS), and associated anxiety (Kinsey et al., 2007; Wohleb et al; 2011) and depressive-like behaviors (Wohleb et al., 2013). RSD evokes a “fight or flight” response that causes neuronal and microglia activation within brain regions associated with fear, anxiety and threat appraisal, including the prefrontal cortex, hypothalamus, amygdala, and the CA3 and dentate gyrus of the hippocampus (Wohleb et al., 2015). RSD activates the hypothalamic-pituitary-adrenal

(HPA) axis and the sympathetic nervous system (SNS), highlighted by increases in systemic glucocorticoids (GC)s that trigger release of catecholamines and pro-inflammatory cytokines (Avitsur et al., 2001; Hanke et al., 2012). RSD promotes increased levels of corticosterone in serum and IL-6 in plasma (Hanke et al., 2012). In addition, published studies indicate, RSD enhances myelopoiesis and promotes the development, priming, and egress of glucocorticoid (GC) resistant CD11b⁺/Ly6C^{high} myeloid progenitor cell (MPC) population from the bone marrow (BM) to spleen, lung and circulation (Engler et al., 2004; Curry et al., 2010; Powell et al., 2013). Moreover, RSD-induced neuronal and microglia activation triggers the production of pro-inflammatory molecules that promote the development of a reactive endothelium (Wohleb et al., 2015). Vascular brain endothelial cells increase cell adhesion molecule expression, which facilitates the adherence and extravasation of peripherally derived monocytes that differentiate into perivascular and parenchymal macrophages (Wohleb et al., 2015). The increased accumulation of macrophages in the CNS elicited by RSD, enhanced neuroinflammatory signaling. Neuroinflammatory mediators such as IL1- β , TNF- α , and IL-6, are related in the neurobiological changes that reinforce fear/anxiety and threat circuitry (Wohleb et al., 2015), promoting the development and maintenance of anxiety-like behavior (Reader et al., 2015)

Interestingly, recent findings from our laboratory showed that RSD-induced anxiety-like behavior persisted for at least 8 days after social defeat but was resolved by 24 days, when markers of immune alterations associated with RSD, such as splenomegaly, plasma IL-6, and the number of circulating CD11b⁺ cells returned to control levels.

It should be noted, nevertheless, that social avoidance behavior developed after 1 cycle of social defeat (Wohleb et al., 2014), and long-lasting social avoidance to an aggressor was present 24 days after stress cessation. Previous findings from our laboratory (Wohleb et al., 2014, Ramirez et al., 2015) suggest social avoidance is associated with sensitized microglia. For example, at 24 days after stress termination, microglia from RSD mice have a primed phenotype, with an increased production of pro-inflammatory cytokines in response to mitogen stimulation (Ramirez et al., 2015). These findings suggest that social avoidance behavior is related to neuronal activation and microglia stimulation.

In order to relieve anxiety and depression accompanying stress, physicians resort to anxiolytic drugs, such as benzodiazepines (BDZs). The action of BDZs depends on the activation of binding sites, such as the central benzodiazepine binding site (CBBS) and the translocator protein (TSPO). The CBBS is present in several regions of the CNS and is a component of the GABAA receptor (Papadopoulos et al., 2006). Binding to this site, enhances GABAergic neurotransmission (Ferrarese et al., 1993), which plays an inhibitory effect in the CNS by counteracting stress-induced hyperactivation of the HPA axis. BDZs also stimulate neuroendocrine effects that seem to be mediated at the hypothalamic and/or suprahypothalamic level by suppressing the production of corticotropin-releasing hormone (CRH) (Arvat et al., 2002). On the other hand, the TSPO is structurally and functionally different from the GABA-A receptor. TSPO is mostly, but not only, located in peripheral tissues, (e.g., heart, kidney, liver, lung, and adrenal glands (Papadopoulos et al., 2006). Specifically, the TSPO is present in the mitochondria coupled to an anion channel, in platelets, lymphocytes, mononuclear cells, endothelium, vascular smooth muscle, mast cells,

and also in microglia and neurons. Several *in vitro* studies have shown that BDZs that bind to the TSPO can affect cellular and immune functions by suppressing cytokine secretion (Taupin et al., 1991; Kim et al., 2006; Joo et al., 2009; Yousefi et al., 2013), modifying cell proliferation, affecting immune cell migration (Ruff et al., 1985) and cellular phagocytic activity (Jin et al., 2013). Immune cells express GABA receptor transcripts and it has been reported GABA treatment decreases the production of pro-inflammatory cytokines in peripheral macrophages (Bhat et al., 2009).

Taken together, these clues prompted us to explore the use of lorazepam and clonazepam in RSD, to evaluate the effect of these two drugs on the neuroimmune system during psychosocial stress. Lorazepam and clonazepam are BDZs that act as positive allosteric modulators of GABA action with similar pharmacological mechanisms and share anxiolytic properties (Verleye et al., 2008; Saari et al., 2011). These two drugs have been used extensively to distinguish between the CBBS and the TSPO (Casellas, 2002). Lorazepam acts directly on the GABAA-R complex, and has high affinity for the TSPO. On the other hand, clonazepam is a selective agonist of the GABAA-R (CBBS) but does not bind with high affinity to the TSPO (Anholt et al., 1986). These two different positive allosteric modulators of the GABAA-R produce enhancement of GABAergic activity (Möhler, 2006; Uusi-Oukari and Korpi, 2010) by locking the GABA receptor complex into a conformational change that is able to bind GABA with high affinity (Nutt and Malizia, 2001; Saari et al., 2011). Since both drugs affect functions of the endocrine system, it would be logical to expect that these two drugs might modulate the neuroendocrine regulation of inflammation during psychosocial stress by decreasing stress-induced neuroinflammatory signaling, thus, modifying stress-associated behavioral changes. Therefore, the aims in the present study were to determine if lorazepam and clonazepam would 1) prevent stress-induced peripheral and central inflammatory responses, and 2) block anxiety and social avoidance behavior in mice subjected to RSD.

2. Materials and Methods

2.1 Animals

Male C57BL/6 (6–8 weeks old) and CD-1 (12 months, retired breeders) mice were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts) and allowed to acclimate to their surroundings for 7–10 days prior to initiation of experiments. C57BL/6 mice were housed in cohorts of three and CD-1 mice were singly housed in 11.5 × 7.5 × 6 inch polypropylene cages. Mice were maintained at 21°C under a 12:12 h light: dark cycle with *ad libitum* access to water and rodent chow in the animal facility at The Ohio State University. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

2.2 RSD

RSD was performed as described previously (Avitsur et al., 2001; Wohleb et al., 2011). In brief, an intruder male CD-1 mouse was introduced into home cages of male C57BL/6 mice (three per cage) for 2 hours (hrs) on 6 consecutive nights. Behavior was observed to make

certain that the intruder was aggressive. If the CD-1 mouse did not initiate an attack within 5-10 minutes or was attacked by resident mice, a new CD-1 mouse was introduced. At the end of the 2 hrs the CD-1 mouse was removed and the resident mice were left undisturbed until the next day when the same paradigm was repeated. During RSD, resident mice display submissive behaviors such as upright posture, fleeing, and crouching (Avitsur et al., 2001; Stark et al., 2001; Hanke et al., 2012). Home cage control (HCC) cohorts were left undisturbed in a separate room.

2.3 Pharmacological treatments and administration procedures

Figure 1a shows experimental schematic. C57BL/6 mice were randomly selected for inclusion into experimental treatment groups using two different drugs, lorazepam and clonazepam. For lorazepam experiments, adult male C57BL/6 mice were allocated into different experimental groups. The groups were: RSD treated with vehicle (VRSD), RSD treated with lorazepam (LRSD), HCC treated with vehicle (VCON) and HCC treated with lorazepam (LCON). Lorazepam (100 µg/kg) was diluted in distilled water, which is a good diluent for lorazepam, and an appropriate vehicle control solution (0.9% NaCl) was administered. The dose of lorazepam (100µg/kg/day), was chosen based on prior studies (Miller et al., 1987; de Angelis et al., 1995). A pilot study to determine dose-response in the open field test was also performed at this concentration in our laboratory. In this pilot study, only HCC were injected for eight days with lorazepam (100 µg/kg) or vehicle (n=6 per group) and locomotor activity was assessed at day eight. No differences were detected in the open field test in total distance traveled, total time spent in the periphery, and total time spent in the center. Spleens were weighed and no differences were detected between the groups (data not shown). These results demonstrated that lorazepam, at this concentration, did not affect locomotor activity in these mice or spleen weight. In experiments using vehicle administration, preliminary studies indicated that the response of vehicle-injected and non-treated mice, HCC, were indistinguishable (data not shown).

Lorazepam, or vehicle, was administered by intraperitoneal (i.p.) injection in a volume of 0.01ml/g body weight (i.e. for a mouse that weights 30 grams the volume injected was 0.3ml). Mice were pre-treated with lorazepam or vehicle, two days before starting RSD at 1900 EST. From day 1 to day 6 of RSD, LRSD and VRSD groups each received their respective treatment immediately after the RSD cycle. LCON and VCON groups were injected at this time as well. It was expected that mice would become sedated with lorazepam ~2 hours post injection, but they could can still ambulate and remain responsive.

For clonazepam experiments, the groups were: RSD treated with vehicle (VRSD), RSD treated with clonazepam (CRSD), HCC treated with vehicle (VCON) and HCC treated with clonazepam (CCON). Clonazepam was diluted in saline (0.9% NaCL) and an appropriate vehicle control solution was administered. The dose of clonazepam (50µg/kg) was chosen based on literature in a model of restraint stress (Verleye, et al., 2008). A pilot study was conducted with HCC in order to obtain the same dose-response behavioral and spleen weight data obtained with lorazepam (data not shown). Clonazepam or vehicle was administered i.p. in a volume of 0.01 ml/g body weight (i.e. for a mouse that weighed 30 grams the volume injected was 0.3ml). Mice were pre-treated with clonazepam or vehicle,

two days before starting RSD at 1900 EST. From day 1 to day 6 of RSD, CRSD and VRSD groups received their respective treatment immediately after the RSD cycle. VCON and CCON groups were injected at this time too.

2.4 Plasma corticosterone determination

Blood was collected from the submandibular vein immediately following the last cycle of RSD (i.e. at 1900 EST). Blood samples were immediately placed on ice and plasma was collected after centrifuging samples at 1800 x g for 15 minutes at 4°C. Plasma was stored at -80°C until assayed in duplicate using a Corticosterone ELISA kit from Enzo Life Sciences (Farmingdale, New York) following manufacturer's instructions (n=6-7 per group).

2.5 Anxiety-like behavior testing and social avoidance test

Behavior was determined the morning following the last cycle of RSD as previously described (Wohleb et al., 2011). Each behavioral test was assessed in different cohorts of mice. Anxiety-like behavior was measured in the open field test, consisting of a 40 × 40 × 25 cm square plexiglass box with a solid floor and a 6 × 6 grid drawn on the floor separating the open field into 36 identical squares. This test takes advantage of mice's natural tendencies to explore the environment while avoiding open spaces. For this experiment, the dependent variables were: total time spent in the center of the open field, latency to entering the center and the number of entries into the center of the open field in a 5 min period. The test apparatus was cleaned with water between experimental subjects. Behavior was taped, digitized and coded using the Observer program (Noldus Information Technologies, Netherlands) (n=7-11 per group for lorazepam experiment; n=6 per group for clonazepam experiment).

The social avoidance test was performed as previously described (Wohleb et al., 2014). Briefly, the social avoidance test consists of two trials. In the first 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 second trial, the experimental mouse was placed in the arena with an unfamiliar CD-1 mouse in the wire mesh cage and activity was recorded for the same amount of time. Time in the interaction zone and time spent in the corners was video-recorded and analyzed using Noldus EthoVision Software (Leesburg, Virginia). Fourteen hours after the sixth cycle of RSD, mice were killed by CO₂ asphyxiation after the completion of either anxiety-like behavior testing or the social avoidance test and tissues were collected for processing (n=9-11 for lorazepam experiment; n=8-9 per group for clonazepam experiment).

2.6 Plasma IL-6 determination

Blood was collected by cardiac puncture, centrifuged, and plasma was collected and stored at -80°C until assayed. IL-6 levels were determined using the BD OptEIA Mouse IL-6 ELISA as previously described (Wohleb et al., 2011) according to the manufacturer's instructions (BD Biosciences, San Jose, California) (n=6 per group).

2.7 Hypothalamus and slice of hippocampus/hypothalamus RNA isolation and real-time PCR

For RNA isolation, brains were removed and dissected for the different brain regions: hypothalamus and a 1 mm coronal brain section (slice) through the hippocampus/hypothalamus and immediately flash frozen in liquid nitrogen. RNA was isolated from homogenized brain regions using tri-reagent/isopropanol precipitation, RNA was reverse transcribed to cDNA using an RT-RETROscript kit (Ambion®, Life Technologies, Grand Island, New York) and RNA concentration was determined by spectrophotometry (Eppendorf, Hauppauge, New York) as described previously (Wohleb et al., 2013). Quantitative PCR was performed using the Applied Biosystems Assay-on-Demand Gene Expression protocol as previously described (Wohleb et al., 2011). In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (e.g., IL-1 β , IL-6, TNF- α for brain slice and CRH for hypothalamus) 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 (nonfluorescent quencher). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems®, Life Technologies, Grand Island, New York). Data were analyzed using a comparative threshold cycle method and results are expressed as a fold difference from GAPDH (n=5-6 per group for hypothalamus; n=6 per group for brain slice).

2.8 Microglia isolation and flow cytometry

Microglia was isolated from whole-brain homogenates as previously described (Henry et al., 2008; 2009; Wohleb et al., 2011). In brief, brains were homogenized in Hanks' balanced salt solution (HBSS), with a pH of 7.4, by mashing the brain through a 70 μ m nylon mesh cell strainer. The homogenates were then centrifuged at 500 X g for 6 min at 10°C. Supernatants were decanted and the obtained pellets were resuspended in 70% isotonic Percoll (GE Healthcare, Pittsburgh, Pennsylvania) at room temperature. A Percoll density gradient was layered in this manner: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged at 2000 X g for 20 minutes at 10°C and microglia was taken by aspirating the interphase between the 50% and 70% Percoll layers (Nair et al., 2007; Wohleb et al., 2011). The retrieved cells were washed and resuspended in sterile HBSS and centrifuged at 600 X g for 6 minutes at 10°C. The supernatant was decanted and viable cells were counted using 0.1% trypan blue staining in an automated cell counter (Luna-FL™ dual fluorescence cell counting, Logos Biosystems, Annandale, Virginia). Each brain yielded approximately 6.5×10^5 cells. Studies (Henry et al., 2009; Wynne et al., 2010) demonstrate that viable cells isolated from brain homogenates through this protocol yields >90% microglia. For flow cytometry, staining of microglia surface antigens was executed as previously described (Henry et al., 2009 ; Wohleb et al., 2011). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, California). The cells were then incubated with anti-CD11b-APC, anti-CD45-FITC, and anti-MHC-II-PE antibodies (eBioscience, San Diego, California). Expression of these surface receptors was evaluated using a Becton-Dickinson FACSCalibur™ four-color cytometer. Ten thousand events were recorded and microglia were identified by the expression of CD11b⁺ and CD45^{low} markers and macrophages by the expression of CD11b⁺ and CD45^{high} (Nair and Bonneau, 2006). For

each antibody, gating was determined based on appropriate isotype-stained controls. Flow data were analyzed using FlowJo software (Tree Star, San Carlos, California).

2.9 Microglia RNA isolation and real-time PCR

RNA from Percoll-isolated microglia was isolated using the RNeasy plus mini-kit (Qiagen®). RNA concentration was determined by a spectrophotometer (Eppendorf, Hamburg, Germany). RNA was reverse transcribed to get cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) and quantitative PCR was done using the Applied Biosystems® by Life Technologies Assay-on-Demand Gene Expression protocol as previously described (Godbout et al., 2005; Wohleb et al., 2011). Briefly, amplification of cDNA was performed by real-time PCR. A target cDNA (IL-1 β , IL-6, TNF- α , TLR4, CCL2, and CX3CR1) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase) were amplified at the same time using an oligonucleotide probe with fluorescent reporter dye (FAM). An ABI PRISM® 7300-sequence detection system (Applied Biosystems® by Life Technologies, Grand Island, New York) was used to assess fluorescence. Data were analyzed by the comparative threshold cycle and the results are given as the fold difference detected (n=8-11 per group for lorazepam experiments; n=6 per group for clonazepam experiments).

2.10 Total splenocytes RNA isolation and real-time PCR

Spleens were collected, weighted, and flash frozen in liquid nitrogen. Samples were stored at -80°C until assayed. Samples were homogenized using a tissue tearor and then centrifuged at 4°C and 12,000g for 10 minutes. Supernatant was collected and each sample received 200ul of chloroform, followed by vortexing and centrifugation at 12,000 x g for 15 minutes. The upper aqueous phase was decanted into a fresh tube, to which isopropanol was added to precipitate the RNA. This solution was centrifuged at 12,000 x g for 10 minutes, supernatant was removed, and the resulting pellet was washed with 1ml 75% EtOH to remove residual protein. The pellet was resuspended in 20ul nuclease free water. RNA concentration was measured by spectrophotometry (Implen, Westlake Village, CA) and RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using a Taqman Gene Expression Assay to observe for potential changes in expression of pro-inflammatory cytokines (i.e. IL-1 β , IL-6, TNF- α). Expression was analyzed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA) using the 2-Ct method with normalization glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (n= 8-11 per group for lorazepam experiment; n=6 per group for clonazepam experiment).

2.11 Flow cytometry of bone marrow, blood, and spleen

Blood was collected after cardiac puncture with an EDTA filled syringe and bone marrow was removed, placed in ice-cold HBSS and processed. Bone marrow was flushed from bone into a 70 μm strainer with 1xPBS into a 50 ml conical, until it reached a volume of 7.5 ml. Samples were centrifuged at 1800rpm for 8 minutes. Supernatant was poured off and the pellet of cells were brought up in 1ml FACS buffer and vortexed. Fifty μl of each sample was added into a flow tube. Fc receptors of bone marrow cells and blood leukocytes were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, California) as previously

described (Wohleb et al., 2014). Cells were washed and then incubated with the appropriate antibodies (CD45, CD11b, CD34; eBioscience, San Diego, California), and Ly6C (BD Biosciences, San Jose, California) for 1 h at 4°C. Cells were washed and re-suspended in FACS buffer for analysis. Nonspecific binding was assessed by using isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCalibur four-color cytometer (BD Biosciences, San Jose, California).

For flow cytometric assessment of splenocytes, spleens were collected, weighted, placed in ice-cold HBSS and disrupted mechanically to obtain single cell suspensions as previously described (Hanke et al., 2012). Red blood cells were lysed by adding 2 ml of room temperature lysis buffer (0.16 M NH₄Cl, 10 mM KHCO₃, and 0.13 mM EDTA) for 2 min, followed by one wash with HBSS/10% heat inactivated fetal bovine serum (FBS). Each cell pellet was resuspended in HBSS, filtered and washed a final time in HBSS. Cells were counted and samples were resuspended (2.5×10^6 cells/ml) in supplemented RPMI medium (10% heat-inactivated FBS, 0.075% sodium bicarbonate, 10 mM HEPES buffer, 100 U/ml penicillin G, 100 mg/ml streptomycin sulfate, 1.5 mM L-glutamine, and 0.0035% 2-mercaptoethanol). Single cell suspensions derived from spleen tissue (1×10^6 cells per sample) were incubated with 1 µg each of fluorescently labeled monoclonal antibodies (or the appropriate isotype controls). Antibody labeling was performed at 4 °C for 45 min. The cells were then washed twice in PBS containing 1% FBS and 0.09% NaN₃. All antibodies were obtained from BD PharMingen (San Jose, California), APC-labeled anti-CD11b, PerCP-labeled anti-Gr-1/Ly-6C. Leukocytes were gated based on forward versus side scatter and a total of 10,000–100,000 events were analyzed on a FACSCalibur flow cytometer using Cell Quest and Cell Quest Pro analysis software (Becton-Dickenson; San Jose, California).

3. Statistical Analysis

To ensure a normal distribution, gathered data were subjected to Shapiro-Wilk tests using SPSS Statistics version 21 (IBM, Armonk, New York). Data are expressed as means $\pm$ SEM. To determine significant main effects and interactions between variables being evaluated, data were subjected to one-way (trial, stress, lorazepam, clonazepam), two-way (trial $\times$ stress); (trial $\times$ lorazepam); (trial $\times$ clonazepam); (stress $\times$ lorazepam); (stress $\times$ clonazepam) or three-way (trial $\times$ stress $\times$ lorazepam); (trial $\times$ stress $\times$ clonazepam) analysis of variance (ANOVAs). When appropriate, differences between treatment group means were analyzed by an *F*-protected *t* test using the least-significant difference method. In all cases, the level of significance was set at $p < .05$.

4. Results

4.1 Lorazepam and clonazepam attenuated RSD-induced mRNA expression of CRH in the hypothalamus and corticosterone in plasma

It has been suggested BDZs have an anti-stress effect caused primarily by the inhibition of CRH release, followed by a decrease in corticosterone in response to stress (File, 1991). Therefore, the objective of this experiment was to assess mRNA expression of CRH in the hypothalamus of mice exposed to RSD and the effect of the BDZs lorazepam and

clonazepam on this expression. Furthermore, other objectives were to determine the effect of BDZs on stress-induced increased levels of plasma corticosterone and norepinephrine. The day after the last cycle of RSD, brains were collected and the hypothalamus was dissected, immediately flash frozen, and processed for RNA isolation and RT-qPCR. Figure 1B and 1C show RSD mice displayed higher expression of CRH in this brain region and this response was attenuated with lorazepam treatment. In the same manner, clonazepam diminished expression of CRH in the hypothalamus of RSD mice.

Previous studies demonstrated that mice had significantly elevated levels of serum corticosterone immediately after the last cycle of RSD compared to HCC mice (Avitsur et al., 2001; Hanke et al., 2012). Figure 1D and 1E show that immediately after the last cycle of RSD, increased levels of corticosterone were found in plasma of mice subjected to RSD. The increase plasma corticosterone was attenuated with both lorazepam and clonazepam treatment.

RSD increased levels of norepinephrine in plasma, immediately after six cycles of social defeat, and returned to control levels by three hours after termination of the stressor (Hanke et al., 2012). Thus, we collected blood via submandibular vein immediately after the last cycle of RSD from the different experimental groups to evaluate if BDZs lorazepam and clonazepam mitigated levels of norepinephrine in plasma. As shown in Figures 1F and 1G, there were amplified levels of norepinephrine in plasma of socially defeated mice. Lorazepam decreased norepinephrine levels in stressed mice comparable to HCC ($p>0.05$). On the other hand, clonazepam treatment attenuated norepinephrine levels in stressed mice, but they were still high compared to HCC ($p<0.05$). These data demonstrated both lorazepam and clonazepam were effective in attenuating stress-induced enhanced mRNA expression of CRH in the hypothalamus and increased corticosterone levels in plasma. Regarding norepinephrine in plasma, lorazepam reduced levels of this hormone in stressed mice comparable to HCC, however stress-induced increased levels of norepinephrine remained high with clonazepam treatment compared to HCC, levels were just attenuated with treatment.

4.2 Lorazepam and clonazepam prevented stress-induced increase of IL-6 in plasma

We had previously shown that RSD consistently promoted increased plasma levels of IL-6 (Stark et al., 2001; 2002). Thus, the IL-6 response was used as a biomarker to gauge the robustness of the response to RSD (Hanke et al., 2012). Blood was collected the day after the last cycle of RSD and plasma IL-6 levels were determined. As expected, social defeat increased IL-6 levels in plasma (Figure 2A and 2B), and this increase was blocked by lorazepam and clonazepam treatment, respectively. Together, these data indicate that anxiolytics such as lorazepam and clonazepam effectively decreased high levels of circulating IL-6 in plasma of C57BL/6 mice subjected to social defeat.

4.3 Effect of lorazepam and clonazepam on stress-induced alteration of bone marrow hematopoiesis

RSD enhances myelopoiesis and promotes the development, priming, and egress of a MPC population from the BM (Engler et al., 2004). Hence, the aim of this experiment was to

determine the effects of BDZs, lorazepam and clonazepam, on stress-induced alterations in hematopoiesis in the BM (Figures 3A to 3D). Figure 3C shows, RSD mice treated with vehicle, had a decreased percentage of lymphocyte progenitors in the BM and this effect was attenuated with lorazepam treatment in RSD mice, nonetheless, lymphocyte progenitors in RSD mice treated with lorazepam was still low compared to HCC ($p<0.05$). Lorazepam blocked stress-induced decrease in erythrocyte progenitors in the BM. Moreover, lorazepam blocked stress-induced increase percentage of granulocyte and attenuated stress-induced increase of monocyte progenitors in the BM. Monocyte progenitors in mice subjected to RSD and treated with lorazepam, were still high compared to HCC ($p<0.05$).

Clonazepam did not have an effect on the reduction of lymphocyte progenitors in the BM as a consequence of RSD. Stress-induced decrease of erythrocyte progenitors in the BM was attenuated with clonazepam, but they were still low compared to HCC ($p<0.05$). Additionally, stress-induced increase of granulocytes and monocytes was not affected by clonazepam treatment in RSD mice. There were still an increased percentage of these cells in the BM compared to HCC ($p<0.05$). In sum, the BDZs lorazepam and clonazepam have different effects on stress-driven hematopoiesis.

4.4 Both lorazepam and clonazepam inhibit stress-induced increased percentage of monocytes and granulocytes in circulation

As reported previously (Hanke 2012; Powell et al., 2013; Wohleb et al., 2013) and shown in Figure 4A, mice exposed to RSD had increased numbers of monocytes and granulocytes in circulation. Figure 4C shows lorazepam decreased stress-induced increase in monocytes and granulocytes released from bone marrow into circulation (Figure 4D). In the same manner, in the clonazepam experiment, mice subjected to RSD and treated with vehicle, had an increased pool of monocytes and granulocytes in circulation. Clonazepam prevented the stress-driven increase in monocytes in circulation (Figure 4E), such that this percent was equal to HCC ($p>0.05$). Also, clonazepam prevented stress-induced increase in granulocytes (Figure 4F) in circulation. These data suggest that lorazepam and clonazepam prevented the release of monocytes and granulocytes from the bone marrow into circulation, in mice subjected to social defeat.

4.5 Lorazepam, but not clonazepam, inhibited stress-induced splenomegaly

It was reported previously that RSD caused an increase in the number and percentage of monocytes and granulocytes in the spleen, which was associated with the development of splenomegaly (Avitsur et al., 2003). In line with this idea, total splenocytes were analyzed using flow cytometry to determine if lorazepam and clonazepam would affect the pool of these cells in the spleens of mice subjected to RSD (Figures 5A and 5B). Flow cytometric analysis indicated that stress-induced increase in monocytes (Figure 5C) was completely blocked by lorazepam treatment and this drug also reversed the stress driven increase in splenic granulocytes (Figure 5D). A greater percentage of monocytes (Figure 5E) and granulocytes (Figure 5F) were still observed in the spleen of RSD animals treated with vehicle and clonazepam.

As mentioned previously, RSD has been shown to increase spleen size and weight (i.e., splenomegaly) that is associated with an increased number of monocytes/macrophages (Avitsur et al., 2003). Therefore, we next aimed to determine if treatment with lorazepam and clonazepam would attenuate RSD-induced splenomegaly. Lorazepam treatment significantly inhibited social defeat induced splenomegaly (Figure 5G). Interestingly, clonazepam attenuated splenomegaly, nonetheless, post-hoc analysis indicate spleen weight in RSD mice treated with this drug was still high compared to HCC ($p < 0.05$) as shown in Figure 5H.

We also sought to determine if lorazepam and clonazepam would block RSD increased pro-inflammatory cytokine production by total splenocytes, specifically, IL-1 β , IL-6, and TNF- α (Table 1A and Table 1B). Lorazepam blocked mRNA expression of IL-1 β , IL-6, and TNF- α in total splenocytes. On the contrary, clonazepam did not have an effect on stress-induced increase of mRNA expression of IL-1 β . However, clonazepam did not decrease mRNA expression of IL-6 and TNF- α in total splenocytes of mice subjected to RSD. Post-hoc analyses revealed these latter cytokines in the RSD group treated with clonazepam were not significantly different from HCC and RSD mice treated with vehicle ($p > 0.05$). In sum, RSD promoted a pro-inflammatory phenotype in the spleen, increased the pool of granulocytes and monocytes in the spleen, and splenomegaly, and these three effects were completely suppressed by lorazepam but only partially suppressed with clonazepam treatment.

4.6 Lorazepam and clonazepam, prevented stress-induced trafficking of bone marrow-derived CD11b⁺ myeloid cells to the brain

RSD has been shown to alter the phenotype of myeloid-derived CD11b⁺ cells in the periphery (Bailey et al., 2009; Powell et al., 2009; Curry et al., 2010) and increase their propensity to traffic from the bone marrow to the brain (Wohleb et al., 2011). Therefore, we next sought to determine the effect of lorazepam and clonazepam on the trafficking of circulating MPCs to the CNS in RSD mice. Figures 6A and 6B show representative bivariate dot plots of CD11b and CD45 staining for macrophages (CD11b⁺/CD45^{high}) (Nair et al., 2007) for the various experimental groups. The percentage of macrophages after RSD was increased, however, this was ablated by both lorazepam and clonazepam treatment as seen in Figures 6C and 6D. These data suggest that BDZs, lorazepam and clonazepam were effective in preventing RSD-induced accumulation of macrophages in the brain.

4.7 Lorazepam and clonazepam ameliorated neuroinflammatory signaling

To determine if the RSD-induced neuroinflammatory profile was attenuated by lorazepam and clonazepam treatment, gene expression of pro-inflammatory cytokines and activation markers was examined in microglia (Tables 2A and 2B). For lorazepam experiments, RSD mice treated with vehicle had increased relative gene expression of IL1- β , IL-6, TNF- α , and activation markers TLR-4, CCL2, and CX3CR1 and this effect was prevented with lorazepam (stress $\times$ lorazepam interaction; $p < 0.05$ for all molecules evaluated). Similarly, clonazepam experiments showed a blockade of stress-induced increase of mRNA expression of IL1- β , IL-6, TNF- α , TLR-4, CCL2, and CX3CR1 in microglia. Post-hoc analyses indicated there was no difference in mRNA expression of these pro-inflammatory molecules

evaluated between RSD mice treated with clonazepam and HCC ($p < 0.05$ for all molecules assessed).

To further characterize neuroinflammatory signaling, mRNA expression of IL-1 β , IL-6, and TNF- α was determined in a 1mm coronal brain slice of the hippocampus/hypothalamus (Tables 3A and 3B). These pro-inflammatory cytokines were increased in RSD mice treated with vehicle and blocked with lorazepam treatment. Clonazepam was also effective in blocking mRNA expression of IL-1 β , IL-6, and TNF- α .

These data suggest that lorazepam and clonazepam may reduce RSD-induced neuroinflammatory signaling by decreasing gene expression of pro-inflammatory molecules in the CNS.

4.8 Lorazepam and clonazepam reversed stress-induced anxiety-like behavior

Consistent with previous studies, RSD promoted anxiety-like behavior in the open field test (Wohleb et al., 2011). The day after the last cycle of RSD, individual mice were placed in the corner of an open field box and allowed to freely explore the apparatus. Figures 7A and 7D show mice subjected to RSD had a decreased number of center entries compared to HCC mice, and this was reversed by both lorazepam and clonazepam treatment. Figures 7B and 7E show that mice subjected to RSD and treated with vehicle took more time to first enter the center of the open field compared to HCC treated with vehicle and this was reversed with lorazepam and clonazepam treatment. Likewise, Figures 7C and 7F show that both drugs were effective in rescuing stress-induced decreased time spent in the center of the open field in mice subjected to RSD. Neither RSD nor benzodiazepine treatment of RSD mice, affected the time spent moving in the open field (main effect of stress; $p > 0.05$; stress $\times$ BDZ interaction; $p > 0.05$; for both drugs) (data not shown), indicating that differences were not due to changes in locomotion or activity level.

4.9 Lorazepam and clonazepam reversed social avoidance behavior

Along with promoting anxiety-like behavior, RSD triggers depressive-like behavior that can be measured as social avoidance (Wohleb et al., 2013). Therefore, in this experiment the aim was to determine if stress-induced social avoidance behavior could be reversed in mice exposed to RSD with anxiolytics such as lorazepam and clonazepam. The day after the 6th cycle of RSD, social avoidance was determined using a two-trial interaction paradigm with an empty social target trial (Trial 1) followed by a social target trial (Trial 2). Figures 8A and 8B show that mice subjected to RSD spent less time in the interaction zone when an intruder was introduced in both lorazepam and clonazepam experiments. Lorazepam was effective in reversing stress-induced social avoidance behavior in mice subjected to RSD. In the same manner, clonazepam blocked social avoidance behavior in mice subjected to RSD. Together, these data indicate that BDZ, lorazepam and clonazepam effectively reversed stress-induced depressive-like behavior in the test of social avoidance.

5. Discussion

BDZs are the most widely used drugs in anxiety disorders and treatment of stress symptoms, and their biochemical effects have been used as a tool to elucidate mechanisms and

behavioral outcomes in response to stress (Ferrarase et al., 1992). Therefore, the aim of the present study was to evaluate the effects of BDZs on the neuroimmune system and behavior during psychosocial stress. These data show that lorazepam and clonazepam potentially ameliorated neuroimmune responses in mice subjected to RSD, and blocked stress-related anxiety and depressive-like behaviors.

It has been suggested that clinical actions of BDZs are mediated by their inhibitory influence on the HPA axis, mediated by CRH suppression, and reduction of ACTH, and corticosterone release (Arvat et al., 2002). We found lorazepam and clonazepam attenuated stress-induced mRNA expression of CRH in the hypothalamus, without affecting basal levels in HCC. Prenatal administration of diazepam in rats attenuated the reduction in hypothalamic norepinephrine induced by restraint stress, and at the same time, inhibited corticosterone response to stress (Keim and Sigg, 1977) and attenuated stress-induced changes in plasma corticosterone in a dose-related manner (Simmons et al., 1984). In agreement with this study, we found both BDZs to attenuate stress-induced levels of corticosterone in plasma of mice subjected to RSD. Also, lorazepam attenuated stress-induced increased levels of norepinephrine in plasma and clonazepam attenuated this response in mice exposed to RSD. In humans, the administration of alprazolam, a benzodiazepine that targets both the CBBS and TSPO like lorazepam, decreased stress-induced norepinephrine levels (Santagostino et al., 1996).

Studies in humans (Kiecolt-Glaser and Glaser, 2002) and animals (Hanke et al., 2012) confirm that psychosocial stress increases peripheral cytokine production, and patients with major depressive disorder and post-traumatic stress-disorder have higher levels of cytokine IL-6 (Cole et al., 2010; Baune et al., 2012). There is evidence that psychosocial stress triggers activation of IL-6 neurons in the hypothalamo-neurohypophyseal system, and this activation is paralleled by an increase in plasma IL-6 (Jankord et al., 2010). Studies indicate magnocellular neurons produce IL-6, and then it is transported to the neurohypophysis, where it is released into the peripheral circulation (Ghorbel et al., 2003; Jankord et al., 2007). In the present study, we found higher levels of IL-6 in plasma of animals subjected to RSD and treated with vehicle, nonetheless this increase was blunted in mice subjected to RSD and treated with either lorazepam or clonazepam. This decrease of IL-6 in plasma was accompanied by dampened neuroinflammatory signaling in the latter experimental groups. This indicates that central neural pathways may trigger secretion of IL-6 into the circulation in response to psychosocial stress. In agreement with another study using repeated social defeat stress (Hodes et al., 2014), this decrease in plasma IL-6 was paralleled by a reversal in social avoidance behavior in mice subjected to chronic social stress, which confirms the idea that IL-6 levels contribute to stress-induced behavioral alterations (Zorilla et al., 2001).

Microglia are also implicated in producing pro-inflammatory mediators and have a causal role in behavioral responses to stress (Wohleb et al., 2011; 2014). It is thought that activation of microglia triggers release of IL-1 β within the hypothalamus (Goshen and Yirmiya, 2009). There is strong evidence that noradrenaline and IL-1 β in the brain play pivotal roles in the regulation of stress-induced IL-6 in plasma (Ishikawa et al., 2001). Consistent with this idea, we previously showed that β -adrenergic receptor antagonism with propranolol (a non-selective β -adrenergic receptor antagonist) prevented, IL-6 in serum, anxiety-like behavior

and microglia neuroinflammatory signaling in mice subjected to RSD. Similarly, we prevented anxiety-like behavior and microglia neuroinflammatory signaling in mice subjected to RSD and treated with lorazepam and clonazepam. TSPO has been detected in microglia (Park et al., 1996) and macrophages (Zavala et al., 1984), thus in addition to the anxiolytic activity of BDZs, these drugs have been reported to be anti-neuroinflammatory agents (Ha et al., 2010) agent. Via interaction with the TSPO, BDZs, have been shown to inhibit microglial activation and to reduce inflammation *in vitro* (Wilms, 2003).

RSD is associated with increased myelopoiesis in the BM (Engler et al., 2004). It is hypothesized that enhanced myelopoiesis observed in the BM is due, at least in part, to an increase in the production of GM-CSF and norepinephrine (Engler et al., 2005; Hanke et al., 2012). We previously reported that neutralizing GM-CSF with an antibody, or pre-treatment with propranolol before each stress cycle blocked the RSD-induced increase in monocytic and granulocytic MPCs (Powell et al., 2013). In the same manner, lorazepam inhibited stress-induced increase in monocytes and granulocytes in the BM, while clonazepam had no effect on granulocytic or monocytic progenitors. Since the BM is a variable and dynamic microenvironment we cannot conclude exactly how these drugs affected myelopoiesis. However, it is plausible that lorazepam may be acting on myelopoiesis enhancement provoked by stress, via the action at the TSPO. Clear labeling of the BM using whole-body autoradiographic activity, showed high density of this receptor in this organ (Anholt et al., 1985). Another possible explanation is that lorazepam may also be inhibiting the activation of the SNS in mice subjected to RSD. In the present study, lorazepam prevented stress-induced increase in norepinephrine, right after the last cycle of RSD. It has been suggested BDZs may influence the HPA axis by modulating noradrenergic function (Arvat et al., 2002).

As previously reported by our group and others (Powell et al., 2013; Heidt et al., 2014) repeated stress resulted in a significant accumulation of monocytes and granulocytes in circulation. It is hypothesized that enhanced production and release of monocytes and granulocytes in the BM and subsequently their substantial accumulation in circulation was dependent upon SNS activity. In accordance with this idea, we showed that propranolol treatment in mice subjected to RSD prevented enhanced myelopoiesis and transient increase of these cells in the bloodstream (Hanke et al., 2012; Powell et al., 2013). Likewise, both BDZs, lorazepam and clonazepam inhibited stress-induced increase in monocytes and granulocytes in circulation. BDZs have been shown to be potent stimulators of human monocytes *in vitro*, and it is thought this effect is mediated through the TSPO (Ruff et al., 1985). The mechanism leading to this reduction in circulating monocytes and granulocytes in mice subjected to RSD and treated with BDZs needs to be further elucidated.

A hallmark characteristic of the RSD response is splenomegaly correlating with an increased accumulation of CD11b⁺ monocytes and granulocytes (Avitsur et al., 2002). Our hypotheses is that repeated activation of the neuroendocrine systems causes the mobilization of monocytes and granulocytes from the BM to the spleen (Reader et al., 2015). In the present study, we found that lorazepam attenuated the stress-induced increase percentage of monocytes and granulocytes in the spleen, and at the same time prevented splenomegaly. It has been reported that diazepam, a benzodiazepine that targets both CBBS and TSPO like

lorazepam, prevents changes in the subpopulation composition of the spleen, by inhibiting social defeat induced increased percentage proliferation index of splenocytes, and decreasing the percentage of S and G2-M cells (Kudryavtseva et al., 2011) in this organ. In this study, clonazepam had no effect on stress-induced increase percentage of monocytes and granulocytes, while only attenuating splenomegaly. This suggests that the increase of monocytes and granulocytes from the latter experimental group might be cells produced in the spleen *per se*, since myelopoietic activity in the adult spleen can be stimulated by various challenges (Ohno et al., 1993).

Previous studies reported that murine splenocytes exhibit abundant TSPO, particularly the macrophage and T-cell populations (Zavala et al., 1984). We found lorazepam, but not clonazepam, inhibited stress-induced increased mRNA expression of pro-inflammatory cytokines in stressed mice. Diazepam, has been found to be a potent inhibitor of phosphodiesterase type 4 (PDE4) (Cherry et al., 2001). Inhibitors of PDE4 increase intracellular levels of cyclic AMP, which can reduce the activation of a wide range of pro-inflammatory responses, including the production of pro-inflammatory cytokines (Spina, 2008). Furthermore, midazolam, a benzodiazepine that targets both the CBBS and TSPO like lorazepam, was found to block the p38MAPK pathway in a lipopolysaccharide-stimulated monocyte-macrophage lineage, while inhibiting NF- κ B activation *via* suppression of I κ B- α degradation and thus, NF- κ B translocation (Kim et al., 2006).

We found that lorazepam and clonazepam prevented stress-induced accumulation of macrophages in the CNS. Equivalently, we found both drugs were effective in decreasing stress-induced elevated mRNA expression of pro-inflammatory cytokines and activation markers in isolated microglia. Furthermore, we found dampened neuroinflammatory signaling in brain slices dissected from the hippocampus/hypothalamus of socially defeated mice treated with both BDZ, both of these regions are stress-responsive areas of the brain (Wohleb et al., 2014).

The interpretation of stress involves neuronal activation within brain areas associated with fear and threat appraisal, and these signals are relayed to the immune system via the HPA axis and SNS neuroendocrine pathways (Wohleb et al., 2014). It is thought neuronal activation precedes microglial activation, since cytokine expression was seen after 3 cycles of RSD (Wohleb et al., 2015). Moreover, we had demonstrated, pre-treatment with propranolol blocked stress-induced neuronal activation as well as microglia morphology (Wohleb et al., 2011). Other studies indicate ablation of noradrenergic locus coeruleus projections diminished stress-induced IL-1 β production in the hippocampus (Johnson et al., 2005). In concert, BDZs have been reported to inhibit cell firing and noradrenergic activation in the locus coeruleus, which provides to CRH neurons central noradrenergic inputs, while CBBS have been found on presynaptic axons and nerve terminals of noradrenergic neurons localized in the cerebral cortex and hippocampus (Arvat et al., 2002). Since GABA is the main inhibitory neurotransmitter in the CNS (Trincavelli et al., 2012), it is possible that by enhancing GABAergic neurotransmission with BDZ, stress-induced pathways were alleviated, downregulating the activity of the HPA axis and SNS, thus the neuroimmunomodulatory effects we found in the present study.

The neuroendocrine effects of BDZs have been reported previously in the literature (Arvat et al., 2002), however, we provide evidence that BDZs are potent neuroimmunomodulators during psychosocial stress. We confirm BDZs can decrease stress-induced IL-6 plasma levels in response to 6 cycles of RSD, and block stress-associated behavior alterations such as prolonged anxiety- and depressive-like behaviors. In recent years, it has been postulated that GABAA-R modulators may serve as novel antidepressants (Möhler, 2012). We provide evidence that BDZs improved stress-induced deficits in social avoidance associated with depression-like behavior. Additionally, the anti-inflammatory actions produced by targeting the TSPO during psychosocial stress in the periphery and CNS, offers new clinical therapeutic opportunities. By exploiting this knowledge, new tools for diagnosis and therapeutic interventions can be developed in order to treat stress-related disorders.

Acknowledgments

This research was supported by NIH/NIMH R01 MH097243-02 and R01 MH093473-03.

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Highlights

- Classical benzodiazepines attenuated stress-induced mRNA expression of CRH in the hypothalamus and corticosterone in plasma
- Lorazepam and clonazepam blocked stress-induced levels of IL-6 in plasma
- Lorazepam and clonazepam had different effects on stress-related enhancement of myelopoiesis and inhibited trafficking of monocytes and granulocytes in circulation
- Lorazepam, but not clonazepam, inhibited splenomegaly and the production of pro-inflammatory cytokines in the spleen following social defeat
- Lorazepam and clonazepam, blocked stress-induced accumulation of macrophages (CD11b⁺/CD45^{high}) in the CNS, neuroinflammatory signaling, and reversed anxiety-like and depressive-like behavior

Figure 1.

(A) Experimental schematic is shown. C57BL/6 mice were randomly allocated in different experimental groups and subjected to 6 cycles of RSD or left undisturbed and treated with daily i.p. injections of lorazepam, clonazepam, or vehicle starting two days before stress exposure. The next day after 6 cycles of RSD the hypothalamus was dissected in each experimental mice and processed for RT-q PCR to assess mRNA expression of CRH in this brain region. RSD animals treated with vehicle had increased mRNA expression of CRH in the hypothalamus (main effect of stress in lorazepam experiment; $F_{(1,22)} = 17.15$, $p < 0.01$; main effect of stress in clonazepam experiment; $F_{(1,23)} = 50.12$, $p < 0.01$), and this was attenuated with (B) lorazepam (stress $\times$ lorazepam interaction; $F_{(1,22)} = 4.09$, $p = 0.05$) and (C) clonazepam (stress $\times$ clonazepam interaction; $F_{(1,23)} = 2.88$, $p = 0.10$) treatment. Additionally, animals subjected to RSD and treated with vehicle had high levels of corticosterone in plasma (main effect of stress in lorazepam experiment; $F_{(1,24)} = 13.02$, $p < 0.01$; main effect of stress in clonazepam experiment; $F_{(1,23)} = 16.59$, $p < 0.05$), immediately after the last cycle of RSD. Animals subjected to RSD and treated with either (D) lorazepam (stress $\times$ lorazepam interaction; $F_{(1,24)} = 5.45$, $p < 0.05$) or (E) clonazepam (stress $\times$ clonazepam interaction; $F_{(1,23)} = 4.59$, $p < 0.05$) had increased levels of corticosterone than HCC treated with vehicle, lorazepam or clonazepam, but lower than RSD animals treated with vehicle. (F) Lorazepam decreased levels of norepinephrine in plasma (main effect of stress; $F_{(1,23)} = 73.59$, $p < 0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)} = 35.57$, $p < 0.01$), immediately following the last cycle of RSD, while (G) clonazepam (main effect of stress; $F_{(1,22)} = 41.42$, $p < 0.01$; stress $\times$ clonazepam interaction; $F_{(1,22)} = 5.61$, $p < 0.05$) attenuated levels of norepinephrine in plasma at this time point. Bars represent the mean $\pm$ SEM. Means with different letters (a,b,c) are significantly different from each other ($p < 0.05$). Control=Home cage controls; RSD=Repeated Social Defeat

Figure 2.

Levels of IL-6 were assessed in plasma of mice in the different experimental groups the next day after the last cycle of RSD. Mice subjected to RSD and treated with vehicle had increased levels of IL-6 in plasma. Levels of IL-6 in plasma of mice subjected to RSD and treated with benzodiazepines (**A**) lorazepam (main effect of stress; $F_{(1,23)} = 8.54$, $p < 0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)} = 9.01$, $p < 0.01$) and (**B**) clonazepam (main effect of stress; $F_{(1,23)} = 47.61$, $p < 0.01$; stress $\times$ clonazepam interaction; $F_{(1,23)} = 43.05$, $p < 0.01$) had lower levels of this cytokine, comparable to HCC. Bars represent the mean $\pm$ SEM. Means with different letters (a,b) are significantly different from each other ($p < 0.05$).

Control=Home cage controls; RSD=Repeated Social Defeat

Figure 3.

RSD mice treated with vehicle had increased percentage of (M) monocytic and (G) granulocytic lineage-committed progenitors and decreased percentage of (L) lymphocytic and (E) erythrocytic lineage-committed progenitors in the bone marrow (BM). **A** and **B** are representative bivariate dot plots of CD31/Ly-6c staining. Average numbers of M (CD31+/Ly-6chigh), G (CD31+/Ly-6c+), L (CD31+/Ly-6c-), E (CD31-/Ly-6c-) lineage-committed-progenitor-cells are illustrated. (**A** and **C**) Lorazepam (main effect of stress; $F_{(1,22)} = 25.50$, $p < 0.01$; stress $\times$ lorazepam interaction; $F_{(1,22)} = 16.52$, $p < 0.01$) reversed stress-induced decrease of E progenitors in the bone marrow and attenuated stress-induced decrease of L progenitors (main effect of stress; $F_{(1,22)} = 41.00$, $p < 0.01$; main effect of lorazepam; $F_{(1,22)} = 9.00$, $p < 0.01$) in the bone marrow. Stress (main effect of stress; $F_{(1,22)} = 40.02$, $p < 0.01$) increased G progenitors in the bone marrow was reduced with lorazepam (stress $\times$ lorazepam interaction; $F_{(1,22)} = 14.96$, $p < 0.01$) treatment. M progenitors in both stress groups were higher than HCC, nonetheless this stress-induced increase was attenuated with lorazepam (main effect of stress; $F_{(1,22)} = 47.89$, $p < 0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)} = 16.67$, $p < 0.01$). (**B** and **D**) Clonazepam (main effect of stress; $F_{(1,20)} = 3.57$, $p < 0.05$; stress $\times$ clonazepam interaction; $F_{(1,20)} = 8.02$, $p = 0.01$) attenuated E progenitors in the BM. However, this drug had no effect on the stress driven decrease in L progenitors (main effect of stress; $F_{(1,20)} = 5.88$, $p < 0.01$; stress $\times$ clonazepam interaction; $F_{(1,20)} = 0.05$, $p > 0.05$) or stress-induced increase in G and M progenitors (main effect of stress; $F_{(1,20)} = 4.97$, $p < 0.05$; main effect of stress; $F_{(1,20)} = 3.17$, $p = 0.05$; respectively). Bars represent the mean $\pm$ SEM. Means with different letters (a,b,c) are significantly different from each other ($p < 0.05$). HCC=Home cage controls; RSD=Repeated Social Defeat; VCON=Vehicle Controls;

LCON=Lorazepam Controls; VRSD=Vehicle Repeated Social Defeat; LCON=Lorazepam Controls; CCON=Clonazepam Controls; CRSD=Clonazepam RSD.

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Figure 4.

Following behavioral testing, mice were euthanized and blood was collected by cardiac puncture **A** and **B** are representative flow bivariate dot plots of Ly-6C/CD11b labeling in the blood showing that RSD mice treated with vehicle had elevated percentage of blood monocytes (M) (CD11b⁺/Ly6C^{high}) and blood granulocytes (G) (CD11b⁺/Ly6C^{intermediate}) in circulation (main effect of stress in lorazepam experiment; $F_{(1,22)} = 12.30$, $p < 0.01$; main effect of stress in clonazepam experiment; $F_{(1,22)} = 4.26$, $p = 0.05$). (**C and D**) Lorazepam decreased the percentage of M (stress $\times$ lorazepam interaction; $F_{(1,22)} = 4.60$, $p < 0.05$) and G (stress $\times$ lorazepam interaction; $F_{(1,22)} = 14.96$, $p < 0.01$) in circulation. (**E and F**) Likely, clonazepam ameliorated stress-induced increase in circulating M (main effect of stress; $F_{(1,20)} = 4.46$, $p = 0.05$; main effect clonazepam; $F_{(1,20)} = 12.09$, $p = 0.01$) and G (main effect of stress; $F_{(1,20)} = 12.09$, $p < 0.01$; stress $\times$ clonazepam interaction; $F_{(1,20)} = 12.09$, $p < 0.01$) in mice subjected to RSD. Bars represent the mean $\pm$ SEM. Means with different letters (a,b) are significantly different from each other ($p < 0.05$). Control=Home cage controls; RSD=Repeated Social Defeat

Figure 5.

Spleens were collected the next day after the last cycle of RSD from mice in the different experimental groups and flow cytometry was used to evaluate the percentage of myeloid progenitor cells (Ly-6c/CD11b) in this organ. **A** and **B** show bivariate dot plots of Ly-6C/CD11b labeling in the spleen demonstrating that RSD mice treated with vehicle had elevated percentage of monocytes (M) (CD11b⁺/Ly6C^{high}) and (G) granulocytes (CD11b⁺/Ly6C^{intermediate}) in the spleen. (**C and D**) Lorazepam blocked stress-induced increase of M (main effect of stress; $F_{(1,22)}=10.32$, $p<0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)}=4.65$, $p<0.05$) and G (main effect of stress; $F_{(1,22)}=8.06$, $p=0.01$; stress $\times$ lorazepam interaction, $F_{(1,22)}=4.18$, $p=0.05$) in the spleen in mice subjected to RSD. Mice subjected to RSD and treated with (**E and F**) clonazepam still displayed a high percentage of M (main effect of stress; $F_{(1,20)}=4.97$, $p<0.05$; stress $\times$ clonazepam interaction; $F_{(1,20)}=1.48$, $p>0.05$) and G (main effect of stress; $F_{(1,20)}=4.34$, $p=0.05$; stress $\times$ clonazepam interaction; $F_{(1,20)}=2.79$, $p>0.05$; respectively) when compared to HCC. Moreover, a hallmark of RSD is splenomegaly, or increased spleen weight. As expected, mice subjected to RSD and treated with vehicle had increased spleen weight compared to HCC. (**G**) Lorazepam treatment in RSD mice inhibited splenomegaly (main effect of stress; $F_{(1,46)}=44.98$, $p<0.01$; stress $\times$ lorazepam interaction, $F_{(1,46)}=31.77$, $p<0.01$). On the contrary, (**H**) mice subjected to RSD and treated with clonazepam still displayed a higher weight of the spleen when compared to HCC (main effect of stress; $F_{(1,47)}=34.72$, $p<0.01$; stress $\times$ clonazepam interaction; $F_{(1,47)}=5.98$, $p<0.05$), nonetheless RSD mice treated with vehicle had the highest spleen weight of the experimental groups ($p<0.05$). Bars represent the mean $\pm$ SEM. Means with different letters (a,b,c) are significantly different from each other ($p<0.05$). Control=Home cage control; RSD=Repeated Social Defeat

Figure 6.

Brains were collected the day after the last cycle of RSD, CD11b⁺ cells were enriched by Percoll gradient separation, and CD45, CD11b expression levels were determined through flow cytometry. **(A and B)** Representative bivariate dot plots of the different experimental groups with CD11b/CD45 staining, showing that **(C)** lorazepam block stress-induced increased percentage of macrophages in the brain (main effect of stress; $F_{(1,22)} = 5.72$, $p < 0.05$; stress $\times$ lorazepam interaction; $F_{(1,22)} = 4.52$, $p < 0.05$). **(D)** Likewise, clonazepam reversed stress-induced increased percentage of macrophages in the brain (main effect of stress; $F_{(1,20)} = 3.13$, $p = 0.05$; $p < 0.05$; stress $\times$ clonazepam interaction; $F_{(1,20)} = 6.19$, $p < 0.05$). RSD mice treated with vehicle had increased percentage of (CD11b⁺/CD45^{high}) macrophages in the CNS. Bars represent the mean $\pm$ SEM. Means with different letters (a,b) are significantly different from each other ($p < 0.05$). Control=Home cage controls; RSD=Repeated Social Defeat

Figure 7.

Anxiety-like behavior was evaluated the next day after the last cycle of RSD in the open field test. Mice subjected to RSD and treated with vehicle, had significantly less number of center entries than HCC and RSD mice treated with (A) lorazepam (main effect of stress; $F_{(1,41)}=15.10$, $p<0.01$; stress $\times$ lorazepam interaction; $F_{(1,41)}=7.84$, $p<0.01$) and (D) clonazepam (main effect of stress; $F_{(1,23)}=9.91$, $p<0.01$; stress $\times$ clonazepam interaction; $F_{(1,23)}=10.61$, $p<0.01$). Mice treated with vehicle and subjected to RSD took more time to enter the center of the open field and this was reversed with (B) lorazepam (main effect of stress; $F_{(1,41)}=4.05$, $p=0.05$; stress $\times$ lorazepam interaction, $F_{(1,41)}=5.73$, $p<0.05$) and (E) clonazepam (main effect of stress; $F_{(1,23)}=9.91$, $p<0.01$; stress $\times$ clonazepam interaction; $F_{(1,23)}=10.61$, $p<0.01$). A decrease of total time spent in the open field was assessed in RSD mice treated with vehicle and this was prevented with (C) lorazepam (main effect of stress; $F_{(1,41)}=38.00$, $p<0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)}=46.53$, $p<0.01$) and (F) clonazepam (main effect of stress; $F_{(1,23)}=7.72$, $p<0.05$; stress $\times$ clonazepam interaction; $F_{(1,23)}=7.27$, $p<0.05$) treatment in mice subjected to RSD. Bars represent the mean $\pm$ SEM. Means with asterisk (*) are significantly different from each other ($p<0.05$). Control=HCC; RSD=Repeated Social Defeat; #= number; (s)=seconds

Figure 8.

The morning after the last cycle of RSD, depressive-like behavior was evaluated using the social avoidance test. Social withdrawal was exacerbated when an aggressor was introduced in the social target trial (Trial 2) in RSD mice treated with vehicle, with a decreased time spent in the interaction zone (main effect stress in lorazepam experiment; $F_{(1,41)}=9.27$, $p<0.01$; main effect stress in clonazepam experiment; $F_{(1,34)}=5.01$, $p<0.05$), and this was reversed with (A) lorazepam (main effect trial; $F_{(1,38)}=14.12$, $p<0.01$; $F_{(1,38)}=14.12$; trial $\times$ stress interaction; $F_{(1,38)}=13.82$, $p<0.01$; trial $\times$ lorazepam; $F_{(1,38)}=16.81$, $p<0.01$; trial $\times$ stress $\times$ lorazepam interaction; $F_{(1,38)}=9.49$, $p<0.01$) and (B) clonazepam treatment (main effect trial; $F_{(1,31)}=15.50$, $p<0.01$; trial $\times$ stress interaction; $F_{(1,31)}=10.02$, $p<0.01$; trial $\times$ clonazepam; $F_{(1,31)}=8.17$, $p<0.01$; trial $\times$ stress $\times$ clonazepam interaction; $F_{(1,38)}=9.32$, $p<0.01$). Bars represent the mean $\pm$ SEM. Means with asterisk (*) are significantly different from each other ($p<0.05$). Trial 1: time without intruder; Trial 2= time with intruder; RSD=Repeated Social Defeat; (s)=seconds

Table 1

Total splenocytes of RSD animals treated with vehicle had increased mRNA expression of pro-inflammatory cytokines. **(A)** Lorazepam was effective in decreasing significantly mRNA expression of IL-1 β (main effect of stress; $F_{(1,40)} = 6.15$, $p < 0.05$; stress $\times$ lorazepam interaction; $F_{(1,40)} = 5.67$, $p < 0.05$), IL-6 (main effect of stress; $F_{(1,40)} = 12.94$, $p < 0.01$; stress $\times$ lorazepam interaction; $F_{(1,40)} = 3.84$, $p = 0.05$), and TNF- α (main effect of stress; $F_{(1,40)} = 4.5$, $p < 0.05$; main effect of lorazepam; $F_{(1,40)} = 14.68$, $p < 0.01$) in mice subjected to RSD, while **(B)** clonazepam treated mice subjected to RSD had increased mRNA expression of IL-1 β when compared to HCC (main effect of stress; $F_{(1,23)} = 14.23$; stress $\times$ clonazepam interaction; $F_{(1,23)} = 0.82$, $p > 0.05$). Relative gene expression of IL-6 and TNF- α in RSD mice treated with clonazepam was not significantly different to HCC and RSD mice treated with vehicle (main effect of stress; $F_{(1,23)} = 9.30$, $p < 0.01$; stress $\times$ clonazepam interaction; $F_{(1,23)} = 0.22$, $p = 0.07$; main effect of stress; $F_{(1,23)} = 7.99$, $p = 0.01$; stress $\times$ clonazepam interaction, $F_{(1,23)} = 0.17$, $p = 0.08$; respectively).

A. Total Splenocytes mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Lorazepam	
	HCC	RSD	HCC	RSD
<i>IL-1β</i>	1.06 $\pm$ 0.34 ^a	10.50 $\pm$ 3.53 ^b	0.72 $\pm$ 0.61 ^a	0.92 $\pm$ 1.32 ^a
<i>IL-6</i>	1.01 $\pm$ 0.10 ^a	1.62 $\pm$ 0.12 ^b	0.60 $\pm$ 0.09 ^c	0.78 $\pm$ 0.13 ^{a,c}
<i>TNF-α</i>	0.99 $\pm$ 0.34 ^a	2.06 $\pm$ 0.47 ^b	0.09 $\pm$ 0.07 ^c	0.43 $\pm$ 0.18 ^{a,c}

B. Total Splenocytes mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Clonazepam	
	HCC	RSD	HCC	RSD
<i>IL-1β</i>	0.99 $\pm$ 0.44 ^a	3.92 $\pm$ 0.91 ^b	1.53 $\pm$ 0.36 ^a	3.32 $\pm$ 0.65 ^b
<i>IL-6</i>	1.01 $\pm$ 0.31 ^a	3.62 $\pm$ 0.74 ^b	1.01 $\pm$ 0.34 ^a	2.10 $\pm$ 0.85 ^{a,b}
<i>TNF-α</i>	1.02 $\pm$ 0.34 ^a	3.95 $\pm$ 1.26 ^b	0.97 $\pm$ 0.26 ^a	1.97 $\pm$ 0.41 ^{a,b}

Means with different letters (a,b,c) are significantly different from each other ($p < 0.05$). HCC= Home cage controls; RSD= Repeated Social Defeat.

Table 2

Following behavioral testing, microglia were collected by Percoll gradient separation. Increased neuroinflammatory signaling was observed in RSD mice treated with vehicle and ameliorated in RSD mice treated with lorazepam and clonazepam. RSD markedly increased relative gene expression of pro-inflammatory cytokines and activation markers. **(A)** Lorazepam significantly blocked mRNA expression of IL-1 β (main effect stress; $F_{(1,37)}=4.17$, $p<0.05$; main effect of lorazepam; $F_{(1,37)}=4.30$, $p<0.05$), IL-6 (main effect of stress; $F_{(1,23)}=8.32$, $p<0.01$; main effect of clonazepam; $F_{(1,23)}=5.49$, $p<0.05$), TNF- α (main effect stress; $F_{(1,37)}=8.02$, $p<0.01$; main effect lorazepam; $F_{(1,37)}=6.17$, $p<0.01$), TLR-4 (main effect stress; $F_{(1,36)}=4.36$, $p<0.05$; main effect lorazepam; $F_{(1,36)}=11.20$, $p<0.01$), CCL2 (main effect stress; $F_{(1,23)}=11.24$, $p<0.01$; main effect lorazepam; $F_{(1,23)}=9.56$, $p<0.01$) and CX3CR1 (main effect stress; $F_{(1,23)}=6.77$, $p<0.01$; $F_{(1,23)}=4.70$, $p<0.05$) in microglia of mice subjected to RSD. In the same manner **(B)** clonazepam dampened mRNA expression of IL-1 β (main effect of stress; $F_{(1,23)}=6.98$, $p=0.01$; main effect of clonazepam; $F_{(1,23)}=9.95$, $p<0.01$), IL-6 (main effect of stress; $F_{(1,23)}=8.32$, $p<0.01$; main effect of clonazepam; $F_{(1,23)}=5.49$, $p<0.05$), TNF- α (main effect of stress; $F_{(1,23)}=6.60$, $p=0.01$; main effect of clonazepam; $F_{(1,23)}=6.45$, $p<0.05$), TLR-4 (main effect of stress; $F_{(1,23)}=12.23$, $p<0.01$; main effect of clonazepam; $F_{(1,23)}=7.22$, $p=0.01$), CCL2 (main effect of stress; $F_{(1,23)}=4.53$, $p=0.05$; main effect of clonazepam; $F_{(1,23)}=4.54$, $p<0.05$), and CX3CR1 (main effect of stress; $F_{(1,23)}=5.58$, $p<0.05$; main effect of clonazepam; $F_{(1,23)}=5.14$, $p<0.05$) in microglia.

A. Microglia mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Lorazepam	
	HCC	RSD	HCC	RSD
IL-1 β	1.02 $\pm$ 0.27 ^a	2.46 $\pm$ 0.52 ^b	0.69 $\pm$ 0.49 ^a	1.15 $\pm$ 0.20 ^a
IL-6	0.99 $\pm$ 0.20 ^a	3.03 $\pm$ 0.65 ^b	1.27 $\pm$ 0.28 ^a	1.42 $\pm$ 0.25 ^a
TNF- α	1.22 $\pm$ 0.21 ^a	1.75 $\pm$ 0.20 ^b	0.65 $\pm$ 0.25 ^a	0.94 $\pm$ 0.21 ^a
TLR-4	1.07 $\pm$ 0.67 ^a	3.66 $\pm$ 0.91 ^b	0.37 $\pm$ 0.20 ^a	0.38 $\pm$ 0.19 ^a
CCL2	1.01 $\pm$ 0.32 ^a	4.36 $\pm$ 1.04 ^b	0.68 $\pm$ 0.31 ^a	1.15 $\pm$ 0.17 ^a
CX3CR1	1.19 $\pm$ 0.27 ^a	1.99 $\pm$ 0.41 ^b	0.79 $\pm$ 0.25 ^a	1.11 $\pm$ 0.07 ^a

B. Microglia mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Clonazepam	
	HCC	RSD	HCC	RSD
IL-1 β	1.01 $\pm$ 0.83 ^a	4.02 $\pm$ 0.94 ^b	0.18 $\pm$ 0.04 ^a	0.52 $\pm$ 0.17 ^a
IL-6	1.02 $\pm$ 0.79 ^a	5.11 $\pm$ 1.13 ^b	0.34 $\pm$ 0.15 ^a	0.72 $\pm$ 0.33 ^a
TNF- α	1.02 $\pm$ 0.56 ^a	5.10 $\pm$ 1.08 ^b	0.20 $\pm$ 0.09 ^a	0.38 $\pm$ 0.05 ^a
TLR-4	1.07 $\pm$ 0.37 ^a	5.44 $\pm$ 1.53 ^b	1.06 $\pm$ 0.59 ^a	1.59 $\pm$ 0.66 ^a
CCL2	1.28 $\pm$ 0.26 ^a	2.85 $\pm$ 0.90 ^b	0.77 $\pm$ 0.29 ^a	2.48 $\pm$ 0.82 ^a
CX3CR1	1.10 $\pm$ 0.34 ^a	5.11 $\pm$ 1.65 ^b	1.15 $\pm$ 0.67 ^a	1.18 $\pm$ 0.21 ^a

Means with different letters (a,b) are significantly different from each other ($p<0.05$). HCC= Home cage controls; RSD= Repeated Social Defeat.

Table 3

The next day after the last cycle of RSD, brains were collected and a 1mm coronal section of the hippocampus/hypothalamus was dissected to assess mRNA expression of pro-inflammatory cytokines. **(A)** Lorazepam prevented RSD-induced increased mRNA expression of IL-1 β , IL-6, and TNF- α (main effect of stress; $F_{(1,23)}=8.76$, $p<0.01$; stress $\times$ lorazepam interaction; $F_{(1,23)} = 4.74$, $p<0.05$; main effect of stress; $F_{(1,23)}=5.77$, $p<0.05$; stress $\times$ lorazepam interaction; $F_{(1,23)}=4.52$, $p<0.05$; main effect of stress; $F_{(1,23)} = 5.38$, $p<0.05$; stress $\times$ lorazepam interaction; $F_{(1,23)} = 4.58$, $p<0.05$; respectively). In the same manner, **(B)** clonazepam ameliorated neuroinflammatory signaling by decreasing mRNA expression of IL-1 β , IL-6, and TNF- α (main effect of stress; $F_{(1,23)}=13.75$, $p<0.01$; stress $\times$ clonazepam interaction; $F_{(1,23)}=4.99$, $p<0.05$; main effect of stress; $F_{(1,23)} = 4.99$, $p<0.05$; stress $\times$ clonazepam interaction; $F_{(1,23)}=8.45$, $p<0.01$; main effect of stress; $F_{(1,23)}=4.93$, $p<0.05$; stress $\times$ clonazepam interaction; $F_{(1,23)}=4.73$, $p<0.05$; respectively).

A. Brain slice hypothalamus/hippocampus mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Lorazepam	
	HCC	RSD	HCC	RSD
<i>IL-1β</i>	0.99 $\pm$ 0.32 ^a	2.90 $\pm$ 0.63 ^b	0.17 $\pm$ 0.08 ^c	0.46 $\pm$ 0.19 ^{a,c}
<i>IL-6</i>	1.01 $\pm$ 0.11 ^a	3.07 $\pm$ 0.84 ^b	0.66 $\pm$ 0.24 ^a	0.53 $\pm$ 0.24 ^a
<i>TNF-α</i>	0.99 $\pm$ 0.35 ^a	6.04 $\pm$ 2.21 ^b	0.39 $\pm$ 0.18 ^a	0.60 $\pm$ 0.27 ^a

B. Brain slice hypothalamus/hippocampus mean relative gene expression

Gene	(6 Days After RSD)			
	Vehicle		Clonazepam	
	HCC	RSD	HCC	RSD
<i>IL-1β</i>	1.02 $\pm$ 0.06 ^a	2.42 $\pm$ 0.42 ^b	0.96 $\pm$ 0.09 ^a	1.31 $\pm$ 0.21 ^a
<i>IL-6</i>	1.03 $\pm$ 0.13 ^a	1.86 $\pm$ 0.28 ^b	1.12 $\pm$ 0.09 ^a	1.01 $\pm$ 0.09 ^a
<i>TNF-α</i>	1.01 $\pm$ 0.15 ^a	3.05 $\pm$ 0.91 ^b	1.03 $\pm$ 0.09 ^a	1.05 $\pm$ 0.11 ^a

Means with different letters (a,b) are significantly different from each other ($p<0.05$). HCC= Home cage controls; RSD= Repeated Social Defeat.