The Role of the Spleen in Re-Establishment of Macrophage Trafficking to the Brain and Anxiety in Stress-Sensitized Mice

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in the undergraduate colleges of The Ohio State University

By

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Abstract: Psychosocial stress is associated with immune dysfunction and anxiety. Previous studies demonstrated that stress-induced bone marrow-derived monocytes trafficked to the brain and contributed to neuroinflammation and prolonged anxiety following repeated social defeat (RSD). Moreover, 24 days post RSD, mice remained stress-sensitized (SS) to acute stress. For example, acute stress in SS mice caused monocyte trafficking from the spleen to the brain that promoted increased neuroinflammation and the re-establishment of prolonged anxiety. Therefore, we hypothesize that a unique monocyte population in the spleens of SS mice was released into circulation by acute stress leading to neuroinflammation and anxiety. Thus, we sought to ascertain the effects of splenectomy on brain-macrophage trafficking and anxiety in SS mice exposed to acute stress 24 days following initial exposure to RSD. Preliminary studies indicated that removal of the spleen two weeks before RSD did not affect stress-induced neuroinflammation or anxiety. However, splenectomy prevented the re-establishment of brain-macrophage trafficking, neuroinflammation and anxiety in SS mice following acute stress. Furthermore, because the spleen is innervated by the sympathetic nervous system (SNS), we sought to determine the degree to which ablation of the SNS would prevent the release of monocytes from the spleen. To prevent SNS-mediated release of splenic myeloid cells, guanethidine (50mg/kg) was injected prior to acute stress 24 days after initial exposure to RSD. Guanethidine treatment reduced the release of monocytes in circulation, reduced brain-macrophage trafficking, diminished brain cytokine expression and anxiety in SS mice. Taken together, these data demonstrate that the spleen is a key reservoir of inflammatory monocytes that play a role in the reactivation of anxiety. This observation may provide a potential target for intervention in prolonged anxiety and perhaps posttraumatic stress disorder.

Introduction:

Psychological stress in humans contributes to the development of mental health disturbances, including anxiety and depressive disorders (Wohleb et al., 2015). Moreover, psychological stress is associated with activated neuroimmune pathways. For example, the sympathetic nervous system (SNS), known as the fight or flight response, is activated in response to stress, leading to the increase of norepinephrine in circulation. Moreover, norepinephrine targets immune organs such as the bone marrow and spleen (Felten et al.,1985). Furthermore, SNS activation acts upon the bone marrow leading to an increase in monocyte production, particularly pro-inflammatory Ly6C^{hi} monocytes (Powell et al., 2013) (Fig.1A). Additionally, many of the pro-inflammatory effects of stress are attributed to the increase of Ly6C^{hi} monocytes in circulation (Powell et al., 2013). Moreover, there is evidence that psychiatric illness in humans is correlated to immune activation and monocytic release (Beumer et al., 2012). Recent studies in a murine model of psychosocial stress indicate that repeated social defeat (RSD) increases monocyte production in the bone marrow that leads to an increase in circulating Ly6C^{hi} monocytes. Additionally, these pro-inflammatory monocytes traffic to the brain and induce microglial activation and pro-inflammatory cytokine production (IL-1β, TNF-α, and IL-6) which leads to the development of neuroinflammation and behavioral deficits. This is important because stress-induced disorders including anxiety and depression are associated with neuroinflammation (Wohleb et al. 2012). However, 24 days after RSD, RSD-induced parameters return to baseline. This temporally corresponds with a reduction in neuroinflammation and anxiety. Furthermore, recent evidence shows that RSD causes long-term sensitization in which mice with previous exposure to RSD have exaggerated immunological and behavioral responses following subsequent exposure to an acute stressor (Wohleb et al. 2014). In this study, RSD-exposed mice were termed “stress-sensitized” (SS) because exposure to a single cycle of social defeat 24 days later re-established monocyte trafficking and anxiety-like behavior in SS mice but not in control

(Naïve) mice. Additionally, splenectomy in SS mice prevented the re-establishment of monocyte trafficking and anxiety-like behavior 24 days after RSD. This data indicates that monocyte trafficking from the spleen to the brain caused the re-establishment of anxiety in stress-sensitized mice (Wohleb et al., 2014). Moreover, the role of the spleen serving as a reservoir of inflammatory monocytes has recently been reported in several studies (Swirski et al. 2009; Seifert et al. 2012). Thus, we hypothesize that the spleen acts as reservoir for pro-inflammatory monocytes during the 24 day period after RSD and that sympathetic activation of the spleen induces the redistribution of monocytes, brain-macrophage trafficking and anxiety-like behavior.

Materials and Methods:

Animals: Male C57BL/6 (6-8weeks) mice and male CD-1 (12months) mice were purchased and allowed to acclimate to their surroundings for one week prior to initiation of experimental procedures. C57BL/6 mice were housed in cohorts of three under a 12 hour light-dark cycle with access to rodent chow and water. All procedures were performed in accordance with NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat (RSD): Repeated social defeat consisted of introducing a male intruder CD-1 mouse into a cage of established male cohorts of C57BL/6 mice for 2 hours between 17:00-19:00. Intruders made intermittent attacks on the resident mice and the resident mice showed submissive behavior. After 2 hours, the intruder was removed and the resident mice were left undisturbed until the following evening. Different intruder mice were used each night. Naïve mice were housed in similar cohorts in a separate room. To ascertain the effects of stress sensitization following RSD, Naïve and SS mice (mice with pre-exposure to 6 cycles RSD) were exposed to an acute social defeat, consisting of 1 cycle of RSD, 24 days later. Tissue collection followed 14 hours later.

Splenectomy: Two weeks prior to experimentation, mice received a sham or splenectomy surgery as previously described (Wohleb et al., 2014). In brief, mice were anesthetized with isoflurane and small incisions were made for spleen removal. Splenic nerves and vasculature were cauterized. The incisions were closed with sutures and surgical staples. All surgeries were performed in sterile conditions and tools were sterilized with a hot bead sterilizer (Fine Science Tools). Mice received subcutaneous injection of buprenorphine (0.05 mg/ml) immediately after surgery and 24 hours later to relieve pain. To control for the effect of general surgical procedure, sham surgeries were performed, which consisted of making a small incision without spleen removal or contact with splenic tissue. All surgical procedures followed IACUC guidelines.

Chemical Sympathectomy: Twenty-four hours prior to acute social defeat, mice received subcutaneous injections of guanethidine 50mg/kg (Santa Cruz Biotechnology, Dallas, TX) or vehicle (sterile water). Injection dosage and procedure was based on a previous report (Donello et al., 2011).

Anxiety-like Behavior: The morning after acute social defeat, mice were subjected to an open-field behavioral test. For open field activity, mice were placed individually into the Plexiglas test apparatus (40x40x25cm) and activity was recorded for 5 min by an automated system (AccuScan Instruments Columbus, OH). Anxious behavior in mice is characterized by increased time to enter the center of the apparatus and decreased time spent in the center.

Isolation of Brain CD11b$^+$ cells: Whole brains were homogenized and passed through a 70 μm cell strainer. Homogenates were centrifuged at 600 x g for 6 min. Supernatants were removed and cell pellets were layered in a Percoll density gradient (50%, 35%, and 0%) to isolate brain CD11b$^+$ cells (microglia/macrophages). The gradient was centrifuged for 20 min at 2000 x g and cells were

collected from the interface between the 70% and 50% Percoll layers. These cells were referred to as enriched brain $CD11b^+$ cells based on previous studies (Wohleb et al., 2014). Once isolated, the cells were labeled with the following antibodies: $CD11b^+$, CD45, and CD14. CD45 and CD 14 are markers for macrophages. In particular, resident microglia are $CD45^{lo}$, therefore CD45 differentiates peripherally derived macrophages ($CD45^{hi}$) from the resident microglia. The labeled cells were analyzed by flow cytometry in a FACS caliber machine.

Blood Analysis: Blood was labeled with the following antibodies: CD115, Ly6C, and $CD11b^+$. CD115 is a marker that is only expressed on monocytes and pro-inflammatory monocytes are characterized as $Ly6C^{hi}$. Labeled blood cells were analyzed by flow cytometry in a FACS caliber machine.

RNA isolation and real-time PCR: RNA was collected from enriched brain $CD11b^+$ cells using USB PrepEase RNA spin kit. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed using the Applied Biosystems (Foster, CA) Assay-on-Demand Gene Expression protocol to amplify the genes of interest (IL-1β, TNF-α, IL-6, CD14, CCL2).

Ex vivo culture with LPS: As previously reported, spleen cells were treated with 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0127:B8, Sigma-Aldrich, St. Louis, Missouri) (Hanke et al., 2012). Cells were incubated for 18 hours to measure supernatant cytokine levels. Supernatant IL-6 was determined by ELISA (BDBiosciences; San Diego, CA).

Statistical analysis: To determine significant main effects, data were analyzed using two-way ANOVA using the General Linear Model procedures of SAS (Cary, NC). ANOVA results are presented in the Results section. When there was a main effect of experimental treatment or a treatment interaction

effect, differences between means were evaluated by an *F*-protected t-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± SEM.

Results:

Exposure to acute stress 24 days post-RSD led to the re-establishment of monocyte trafficking from the spleen to the brain in SS mice.

We have previously reported that removal of the spleen after RSD prevented both monocyte trafficking to the brain and the re-establishment of anxiety-like behavior in stress-sensitized (SS) mice (Wohleb et al., 2014). For this study, mice were stress-sensitized by 6 cycles of RSD (SS) or left undisturbed (Naïve). Twenty-four days later, mice were exposed to acute social defeat or left alone as naïve controls (Fig. 2A). Acute social defeat increased circulating $Ly6C^{hi}$ monocytes in the blood (Fig. 2B, $p<0.05$) and $CD45^{hi}$ brain-macrophages (Fig. 2C, $p<0.05$) in SS mice. Additionally, exposure to acute social defeat increased mRNA expression of pro-inflammatory cytokines such as IL-1β, TNF-α, and CD14 in the brain of SS mice but not in naïve mice (Fig. 2D, $p<0.05$). Coinciding with an increase in neuroinflammation, acute social defeat increased anxiety-like behavior in SS mice but not in naïve mice. For example, SS mice exposed to acute stress took longer to enter the center (Fig. 2E, $p<0.05$) and spent less time in the center of the open field (Fig. 2F, $p<0.05$). Therefore, this data support our previous findings that RSD causes brain-macrophage trafficking and the development of anxiety-like behavior. Furthermore, these RSD-induced parameters return to baseline 24 days later and can be re-established following exposure to acute social defeat.

Additionally, stress-sensitization increased the number of $Ly6C^{hi}$ monocytes in the spleen at baseline compared to naïve controls (Fig 2G, p=0.1). Furthermore, after acute social defeat, there was a significant reduction in the number of $Ly6C^{hi}$ monocytes in the spleen of SS mice (Fig. 2G,

p<0.05). These novel data demonstrate that release of Ly6C^{hi} monocytes from the spleen is critical for increasing pro-inflammatory monocytes in circulation after exposure to acute stress. Thus, these data support the hypothesis that the spleen maintains inflammatory monocytes following stress-sensitization.

Splenectomy prior to RSD attenuated the re-establishment of brain-macrophage trafficking and anxiety-like behavior in SS mice.

Our previous work shows that removal of the spleen after stress-sensitization prevents monocyte trafficking to the brain following exposure to acute stress (Wohleb et al., 2014). Therefore, to determine if the spleen serves as a reservoir for releasable inflammatory monocytes, splenectomy was performed prior to stress sensitization (Fig. 3A). Sham SS mice exposed to acute stress had increased Ly6C^{hi} monocytes in circulation (Fig. 3B&C, *p*<0.05), increased CD45^{hi} brain-macrophages (Fig. 3D&E, *p*<0.05), and increased IL-1β, TNF-α, and CD14 mRNA expression in the brain (Fig. 3F, p<0.1). Furthermore, these RSD-induced parameters were attenuated by splenectomy (Fig.3B-F). Additionally, SS mice exposed to acute social defeat exhibited anxiety-like behavior in the open field with increased time to enter the center (Fig.3G, p=0.1) and reduced time spent in the center (Fig.3H, *p*<0.05). These behavioral deficits were attenuated by splenectomy (Fig. 3G&H). These data are interpreted to indicate that the spleen is a unique reservoir of releasable inflammatory monocytes following stress-sensitization.

Splenectomy prior to RSD did not affect circulating monocytes, brain-macrophage trafficking or the development of anxiety-like behavior.

Our data indicate that monocyte release and anxiety-like behavior following acute stress in SS mice was dependent on the spleen (Fig.3). Therefore, we sought to address whether the spleen was

necessary for the initial monocyte trafficking after exposure to RSD. To address this, mice were splenectomized prior to RSD, and behavioral and biological measures were determined 14 hours later (Fig. 4A). As seen previously, RSD led to an accumulation of primed myeloid cells in the spleen (Engler et al. 2004), coinciding with an increased spleen weight in sham mice (Fig. 4B, $p<0.05$). Moreover, RSD increased the percentage of $Ly6C^{hi}$ monocytes in circulation (Fig. 4C&D, $p<0.05$), the percentage of $CD45^{hi}$ macrophages in the brain (Fig. 4E&F, $p<0.05$), as well as mRNA expression of IL-1β, IL-6, TNF-α, CD14, and CCL2 ($p<0.05$, data not shown) in the brain independent of splenectomy. Additionally, RSD mice exhibited anxiety-like behavior with increased time to enter the center (Fig. 4G, $p<0.05$) and decreased time spent in the center of the open field (Fig. 4H, $p<0.05$) independent of splenectomy. Taken together, these data show that the spleen was not required for the initial increase in circulating monocytes, brain-macrophage trafficking or anxiety-like behavior 14 hours after RSD.

Stress-sensitization established a population of pro-inflammatory monocytes in the spleen that had an exaggerated response to *ex vivo* LPS challenge.

Data presented here demonstrate that the spleen is necessary for maintaining a pool of releasable monocytes following stress-sensitization by RSD. To identify the phenotype of these monocytes residing in the spleen during the 24 day period, splenocytes were collected from SS mice and reactivity to *ex vivo* LPS challenge 24 days after RSD was determined (Fig. 5A). Splenocytes from SS mice produced more IL-6 following LPS stimulation compared to cells from naïve mice (Fig. 5B, $p<0.05$). This exaggerated splenocyte response to LPS stimulation demonstrates that the spleen maintains primed monocytes following stress-sensitization.

Sympathetic activation of the spleen released primed monocytes into circulation, thereby re-establishing brain-macrophage trafficking and anxiety-like behavior.

Data shown here shows that the spleen has an important role in maintaining a primed and releasable monocyte population in SS mice. Additionally, the spleen is heavily innervated by the SNS (Nance and Sanders, 2007). Therefore, the effect of guanethidine, a peripheral sympathetic inhibitor, on monocyte release was quantified (Fig. 6A). Acute stress in SS mice increased the percentage of $Ly6C^{hi}$ monocytes in circulation (Fig. 6B&C, $p<0.05$) and the percentage of $CD45^{hi}$ brain-macrophages (Fig. 6D&E, $p<0.05$). Along with an increase in macrophage trafficking, mRNA levels of pro-inflammatory cytokines, IL-1β and TNF-α, were increased after acute social defeat (Fig. 6F, $p<0.05$). Guanethidine pre-treatment attenuated these parameters in SS mice (Fig. 6B-F). Moreover, prevention of primed monocyte release with guanethidine coincided with attenuation of anxiety-like behavior in SS mice. For example, acute stress in vehicle-treated SS mice increased time to enter the center (Fig. 6G, $p<0.05$) and reduced time spent in the center of the open field (Fig. 6H, $p<0.05$). These behavioral deficits were prevented by guanethidine treatment (Fig. 6G&H, $p<0.05$). Therefore, sympathetic activation initiates the release of primed monocytes from the spleen, eliciting brain-macrophage trafficking and thus the development of anxiety-like behavior.

Discussion:

Results shown here demonstrate that the spleen plays a critical role in maintaining and redistributing a population of primed monocytes weeks after stress-sensitization by RSD. First, we show that acute stress 24 days after RSD re-established anxiety-like behavior by promoting monocyte trafficking to the brain only in mice previously sensitized by RSD. Furthermore, we show that splenectomy prior to stress-sensitization prevented monocyte re-distribution and the re-establishment of anxiety-like behavior in stress-sensitized mice. Additionally, splenectomy prior to RSD did not affect monocyte priming, trafficking, or development of anxiety-like behavior 14 hours after RSD. Therefore, the spleen was necessary for maintaining a releasable pool of monocytes after stress-sensitization, but was not necessary for the initial trafficking of primed monocytes or

development of anxiety 14 hours after RSD. These results confirm previous studies showing that bone marrow-derived monocytes lead to the initial onset of neuroinflammation and anxiety-like behavior (Engler et al.,2004; Wohleb et al., 2013).

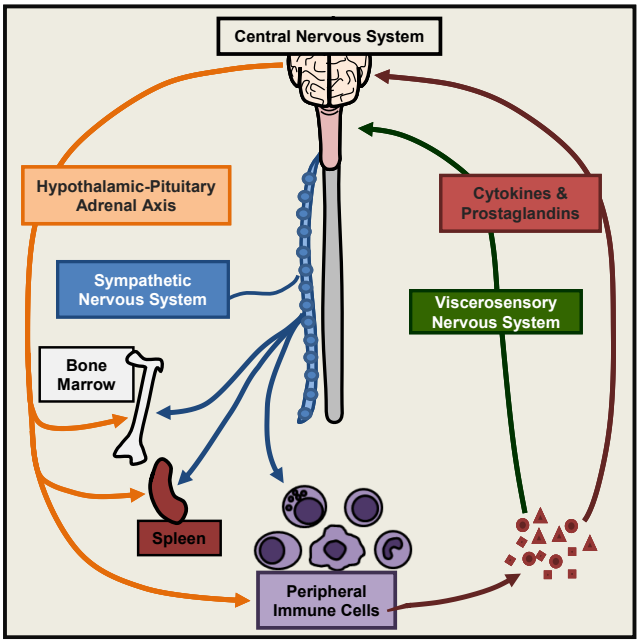
The next experiment showed that monocytes residing in the spleen during the 24 day period in which RSD-induced parameters returned to baseline, retained a pro-inflammatory phenotype in stress-sensitized mice, as seen with an exaggerated response to *ex vivo* LPS challenge. This suggests that RSD primed monocytes that initially trafficked to the spleen. Additionally, after exposure to acute social defeat, there was a decrease in the number of pro-inflammatory monocytes residing in the spleen. Therefore, this population of monocytes that initially trafficked to the spleen comprised the releasable pool of monocytes that contributed to the re-establishment of anxiety following acute stress 24 days later.

Additional experimentation addressed the signaling mechanism needed to initiate the release of monocytes from the spleen. The spleen is heavily innervated by the SNS (Nance and Sanders, 2007). Therefore, an SNS inhibitor, guanethidine, was used to block SNS innervation of the spleen. Guanethidine blocks SNS innervation by competitively inhibiting the release of norepinephrine from its vesicles, thus preventing its effect on its target tissue. These studies showed that pretreatment with guanethidine prior to acute social defeat prevented monocyte redistribution and trafficking, which coincided with an attenuation of anxiety in stress-sensitized mice. Therefore, sympathetic activation triggers the release of primed monocytes from the spleen, which can in turn traffic to the brain and re-establish anxiety-like behavior.

Overall, these studies demonstrate that RSD leads to neuroimmune sensitization. Additionally, RSD leads to the increase of pro-inflammatory monocytes that seed the spleen. Furthermore, the spleen maintains this unique population of pro-inflammatory monocytes, which can be redistributed upon activation of the SNS leading to immunological and behavioral deficits. Thus, increased susceptibility to immune and behavioral complications observed in stressed individuals may be related to the priming and redistribution of peripheral myeloid cells (Anisman, 2009; Dantzer and

Kelley, 2007). These findings may lend insight into the pathophysiology mediating persistent anxiety disorders, such as posttraumatic stress disorder, and provide an innovative target to prevent recurrent anxiety symptoms.

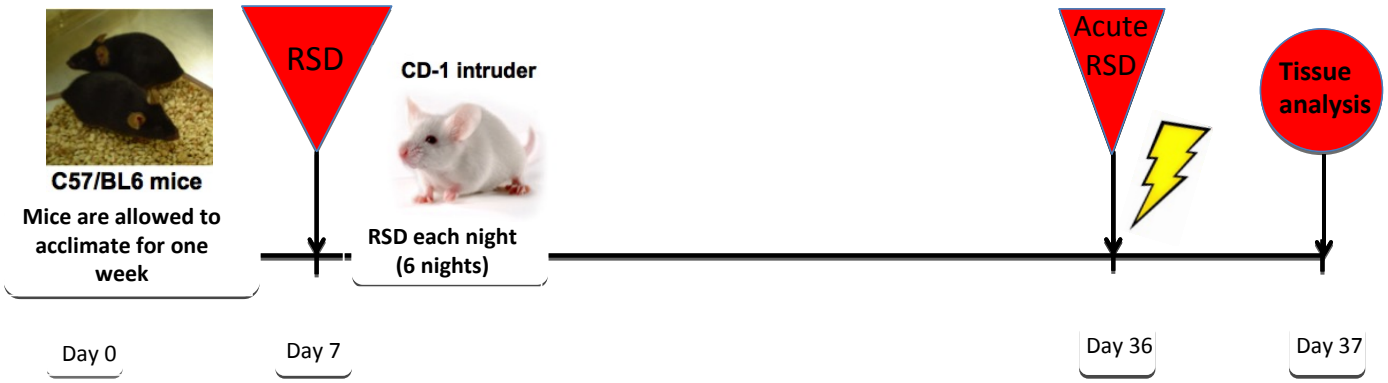
Figure 1



(Wohleb and Godbout, 2013)

Figure 2

A.



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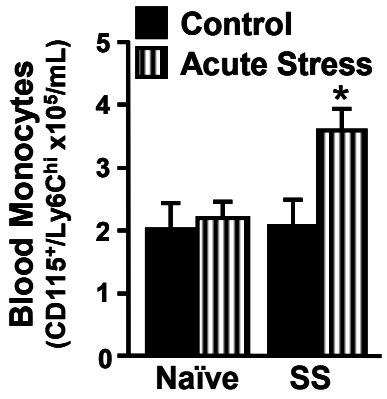
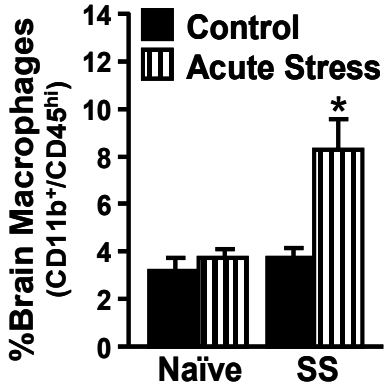


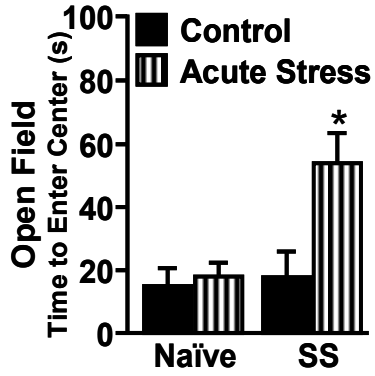
C.



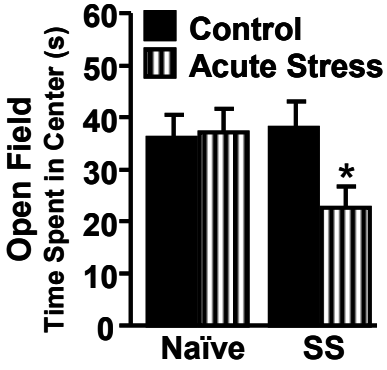
D

mRNA Expression in Brain (Fold Δ)				
	Naïve		Stress-Sensitized	
Gene	Control	Acute Stress	Control	Acute Stress
IL-1β	1.05±0.09	1.33±0.12	0.98±0.11	2.09±0.31 *
TNF-α	1.02±0.06	1.09±0.11	1.10±0.09	1.93±0.31 *
IL-6	1.01±0.04	1.08±0.06	1.13±0.09	0.97±0.10
CD14	1.03±0.04	0.97±0.04	0.99±0.03	1.85±0.37 *
CCL2	1.01±0.04	1.13±0.07	1.01±0.09	1.35±0.10

E.


Control
Acute Stress
Open Field
Time to Enter Center (s)
100
80
60
40
20
0
*
Naïve
SS

F.


Control
Acute Stress
Open Field
Time Spent in Center (s)
60
50
40
30
20
10
0
*
Naïve
SS

G.

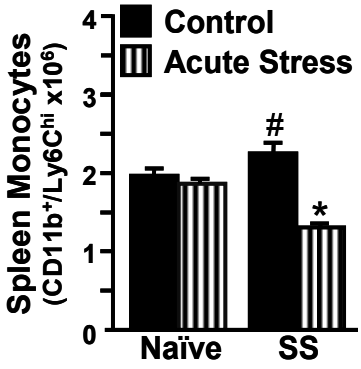
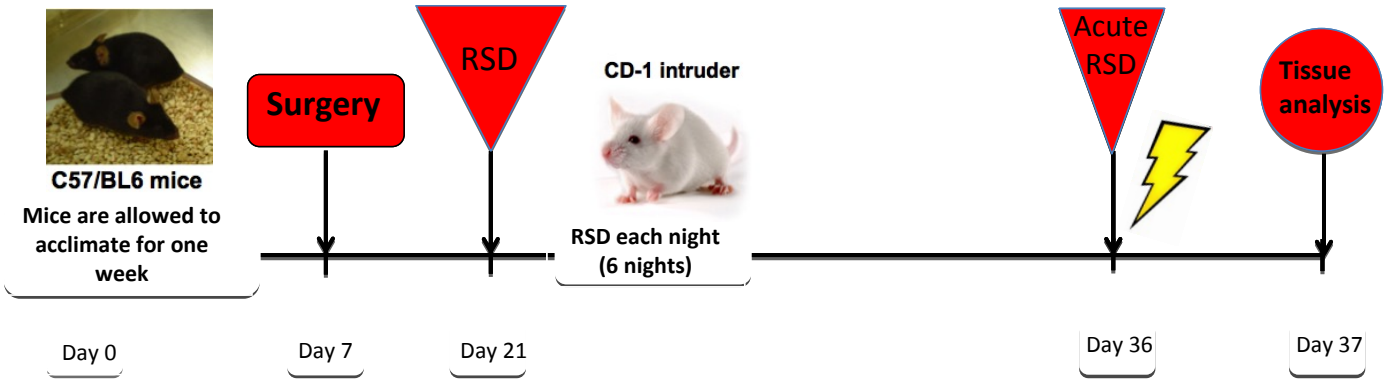
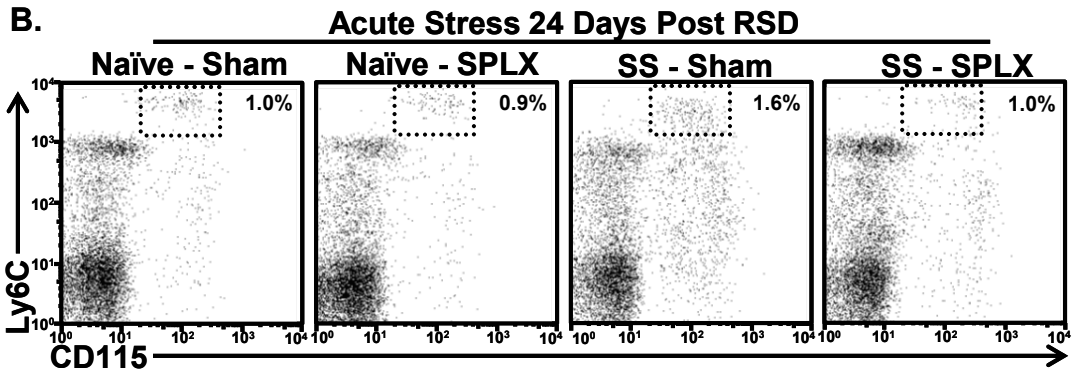
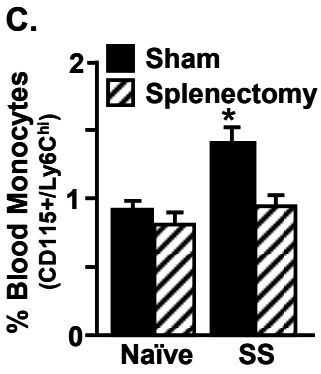
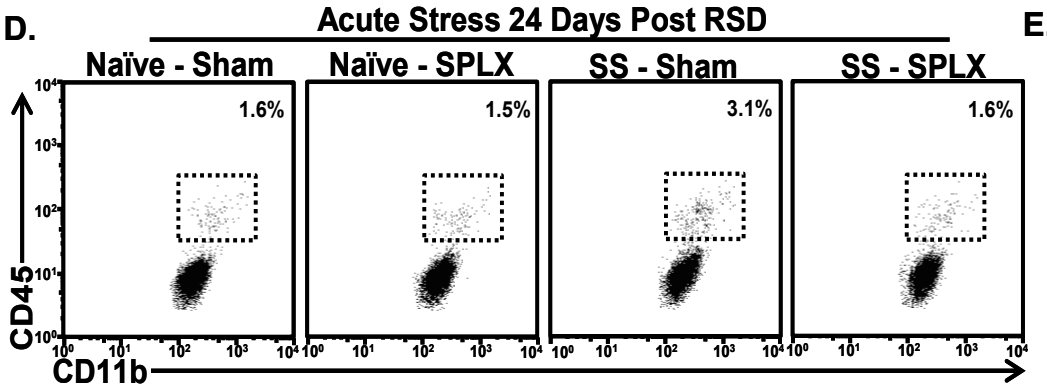

Control
Acute Stress
Spleen Monocytes
(CD11b+/Ly6Chi x10^6)
4
3
2
1
0
#
*
Naïve
SS

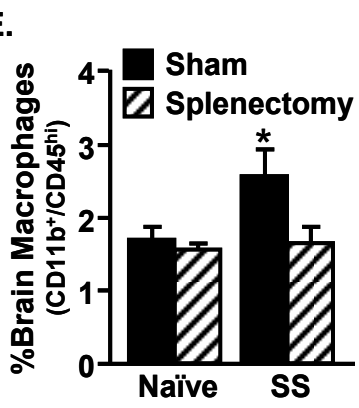
Figure 3


A.
C57/BL6 mice
Mice are allowed to acclimate for one week
Surgery
RSD
CD-1 intruder
RSD each night (6 nights)
Acute RSD
Tissue analysis
Day 0
Day 7
Day 21
Day 36
Day 37


B.
Acute Stress 24 Days Post RSD
Naïve - Sham
Naïve - SPLX
SS - Sham
SS - SPLX
1.0%
0.9%
1.6%
1.0%
Ly6C
CD115


C.
Sham
Splenectomy
% Blood Monocytes (CD115+/Ly6Chi)
Naïve
SS

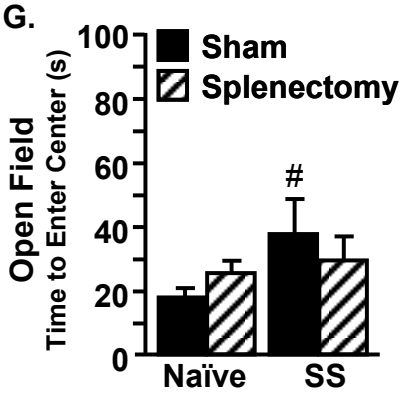

D.
Acute Stress 24 Days Post RSD
Naïve - Sham
Naïve - SPLX
SS - Sham
SS - SPLX
1.6%
1.5%
3.1%
1.6%
CD45
CD11b


E.
Sham
Splenectomy
%Brain Macrophages (CD11b+/CD45hi)
Naïve
SS

F.

mRNA Expression in Brain (Fold Δ)				
	Naïve		Stress-Sensitized	
Gene	Sham	SPLX	Sham	SPLX
IL-1β	1.09±0.19	1.08±0.10	2.18±0.43 *	0.98±0.10
TNF-α	1.03±0.14	0.97±0.10	1.59±0.16 #	1.10±0.31
IL-6	1.03±0.11	1.03±0.14	1.02±0.10	1.05±0.04
CD14	1.02±0.09	0.93±0.08	1.40±0.08 *	1.13±0.12
CCL2	1.03±0.09	0.97±0.15	1.17±0.14	1.08±0.09

G.



H.

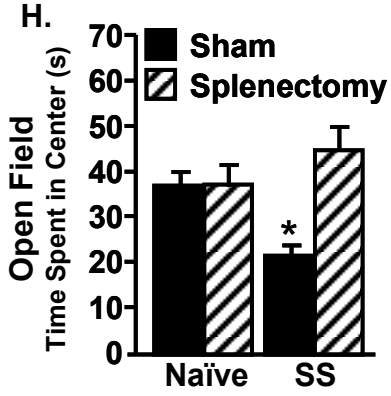
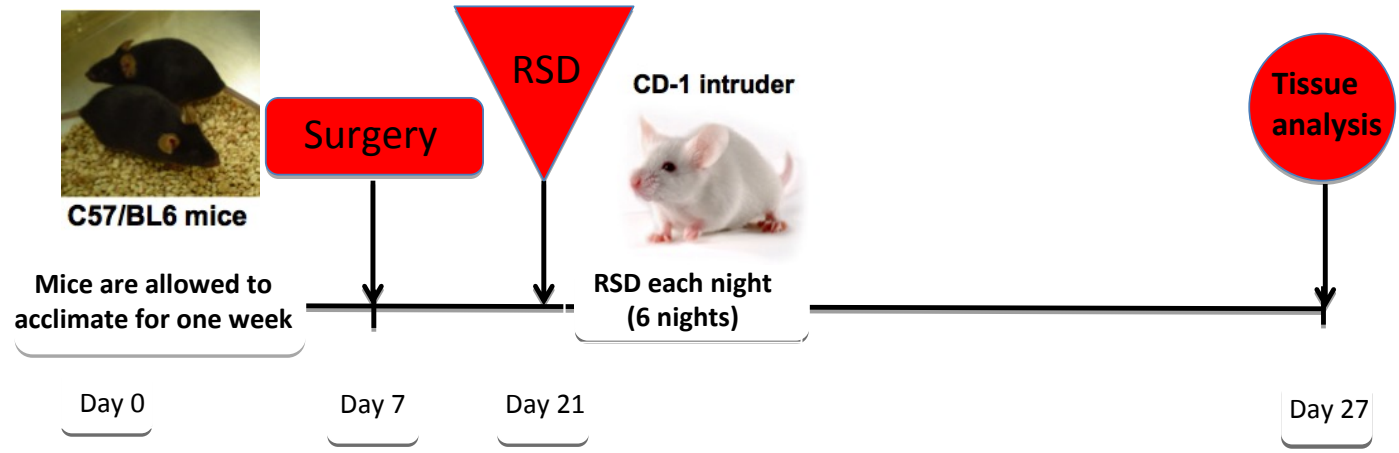


Figure 4

A.



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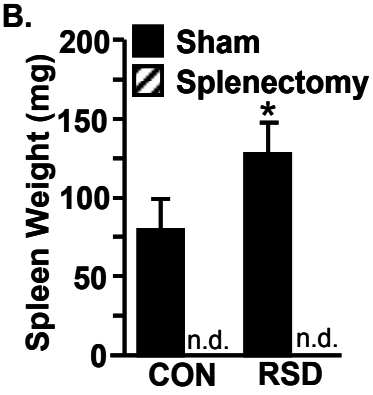
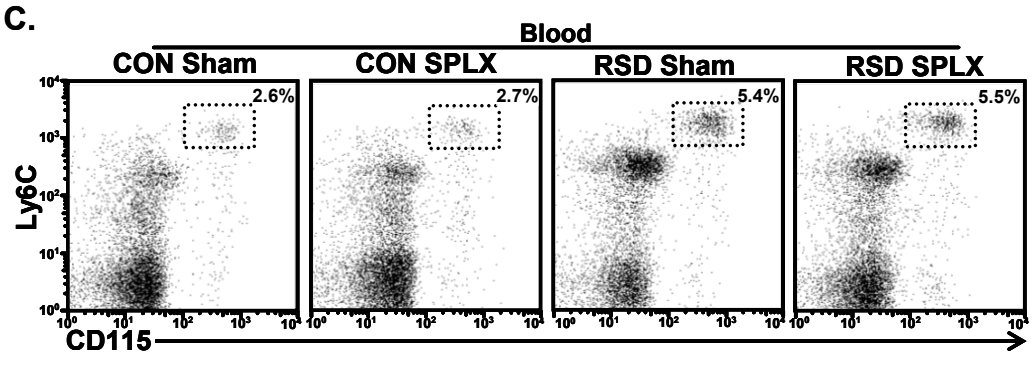
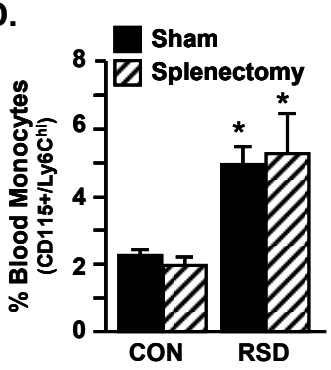


C.



D.



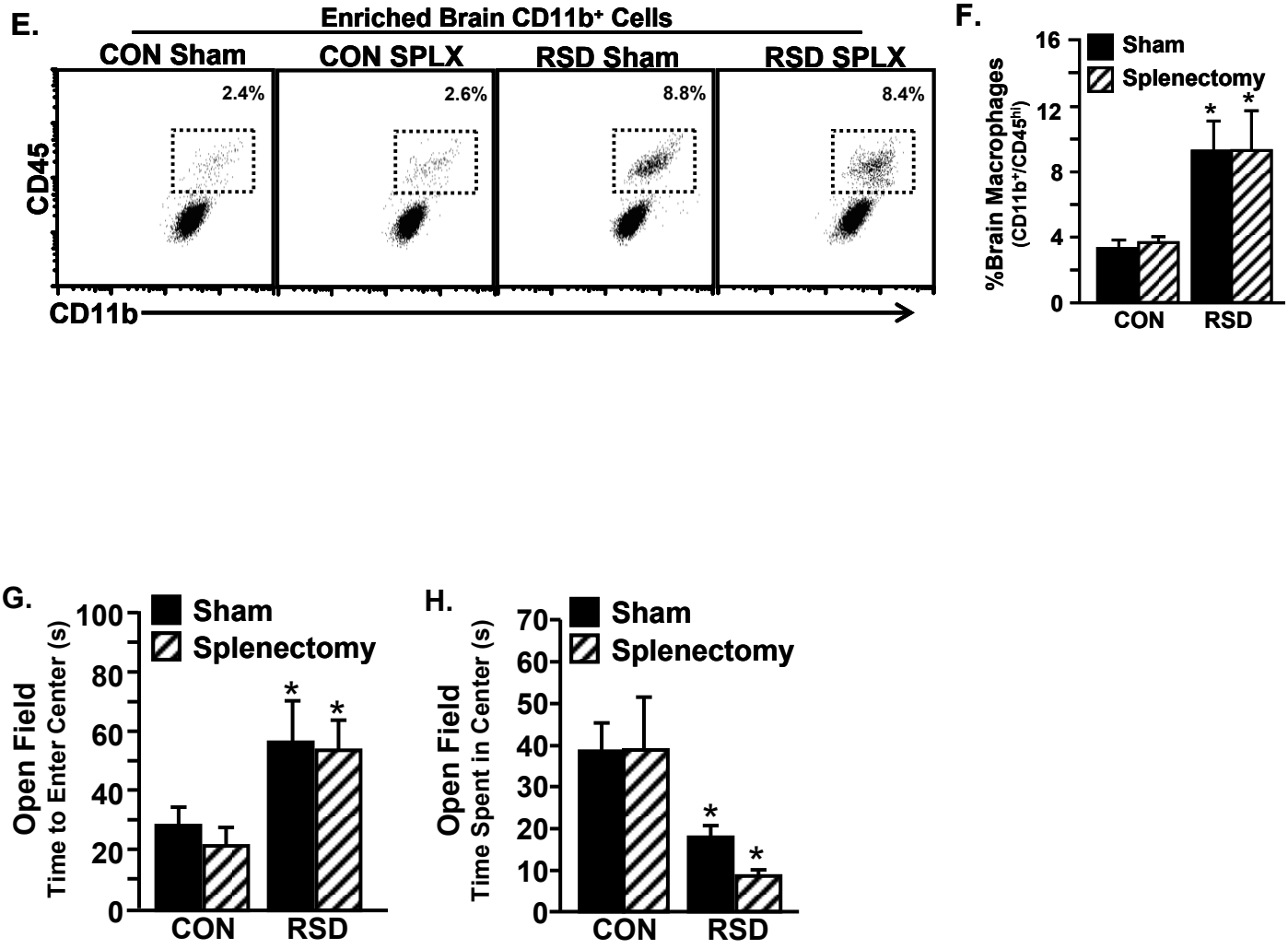
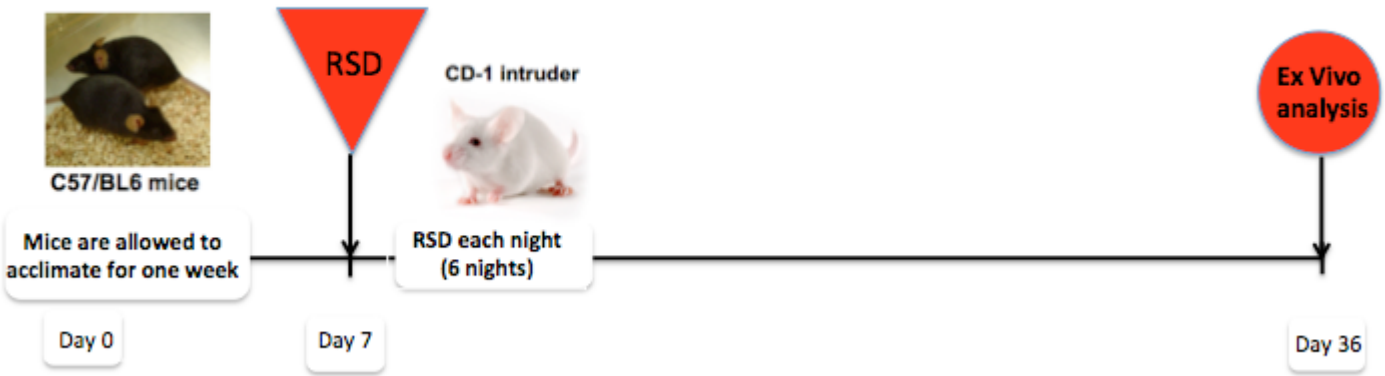

E.
Enriched Brain CD11b+ Cells
CON Sham
CON SPLX
RSD Sham
RSD SPLX
2.4%
2.6%
8.8%
8.4%
CD45
CD11b
F.
Sham
Splenectomy
%Brain Macrophages
(CD11b+/CD45hi)
16
12
8
4
0
CON
RSD
G.
Sham
Splenectomy
Open Field
Time to Enter Center (s)
100
80
60
40
20
0
CON
RSD
H.
Sham
Splenectomy
Open Field
Time Spent in Center (s)
70
60
50
40
30
20
10
0
CON
RSD

Figure 5

A.


C57/BL6 mice
RSD
CD-1 intruder
Ex Vivo analysis
Mice are allowed to acclimate for one week
RSD each night (6 nights)
Day 0
Day 7
Day 36

B.

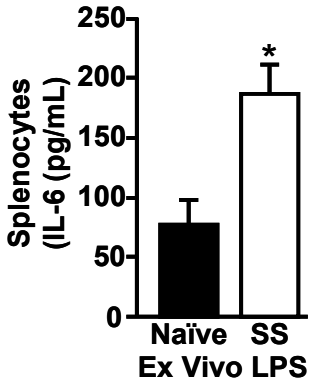
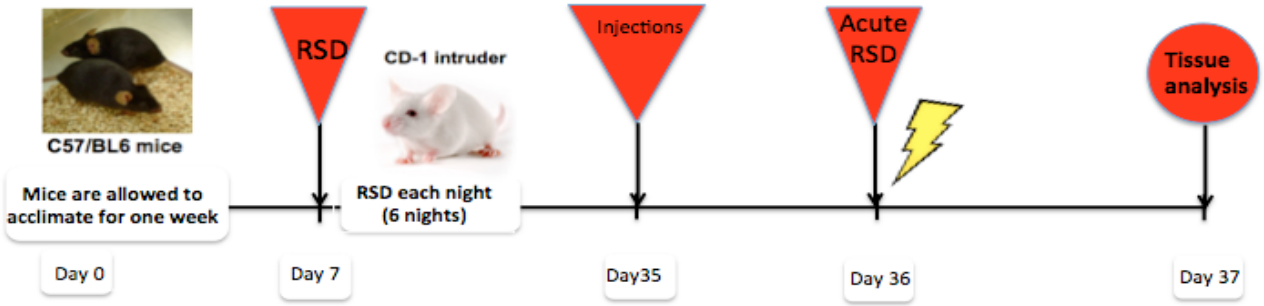
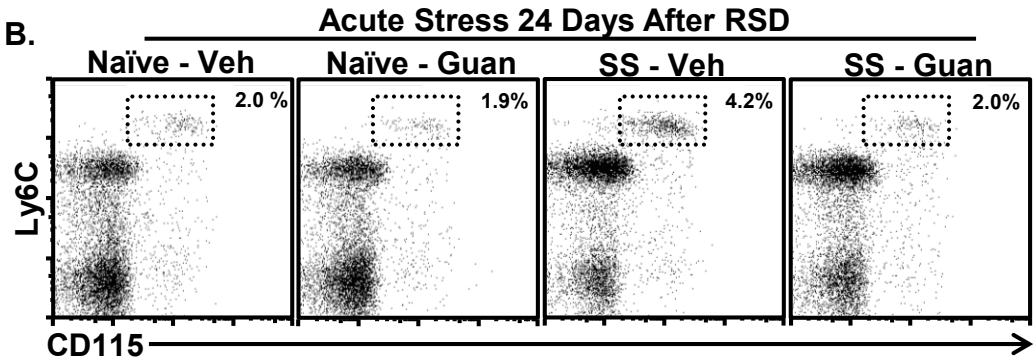
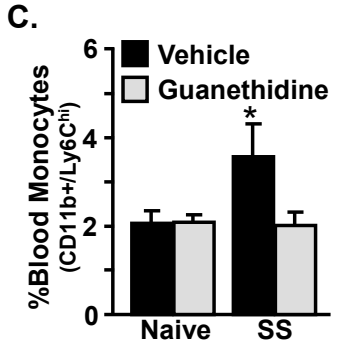

Splenocytes (IL-6 (pg/mL)
250
200
150
100
50
0
*
Naïve
SS
Ex Vivo LPS

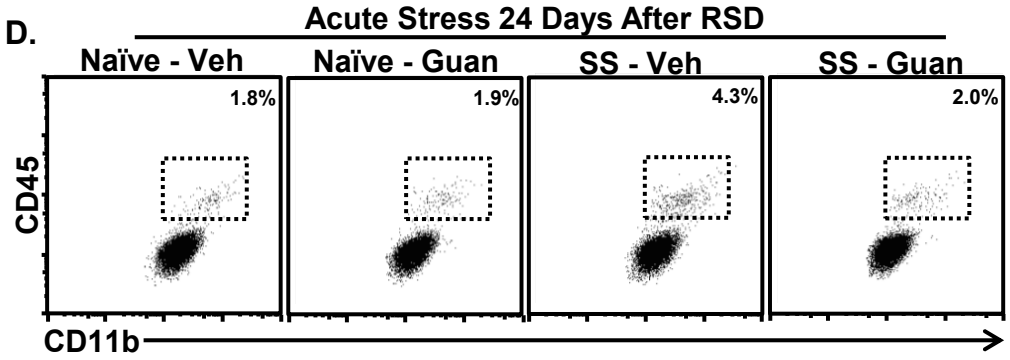
Figure 6

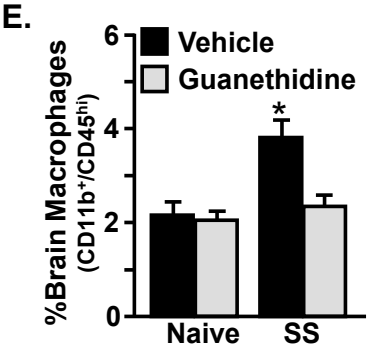
A.


C57/BL6 mice
Mice are allowed to acclimate for one week
Day 0
RSD
CD-1 intruder
RSD each night (6 nights)
Day 7
Injections
Day35
Acute RSD
Day 36
Tissue analysis
Day 37


B.
Acute Stress 24 Days After RSD
Naïve - Veh
Naïve - Guan
SS - Veh
SS - Guan
2.0 %
1.9%
4.2%
2.0%
Ly6C
CD115


C.
Vehicle
Guanethidine
%Blood Monocytes
(CD11b+/Ly6Chi)
Naive
SS

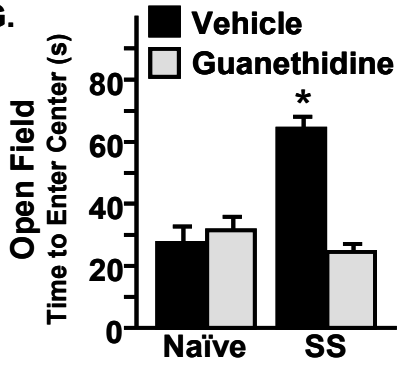

D.
Acute Stress 24 Days After RSD
Naïve - Veh
Naïve - Guan
SS - Veh
SS - Guan
1.8%
1.9%
4.3%
2.0%
CD45
CD11b


E.
Vehicle
Guanethidine
%Brain Macrophages
(CD11b+/CD45hi)
Naive
SS

F.

mRNA Expression in Brain (Fold Δ)				
	Naïve		Stress-Sensitized	
Gene	Vehicle	Guan	Vehicle	Guan
IL-1β	1.10±0.19	0.86±0.08	1.51±0.07 *	1.11±0.08
TNF-α	1.03±0.09	1.05±0.09	1.53±0.23 #	0.97±0.06
IL-6	1.01±0.04	0.84±0.08	0.87±0.09	0.89±0.07
CD14	1.02±0.04	1.19±0.24	0.95±0.12	0.86±0.10
CCL2	1.00±0.02	1.22±0.10	0.90±.0.08	0.95±0.25

G.



H.

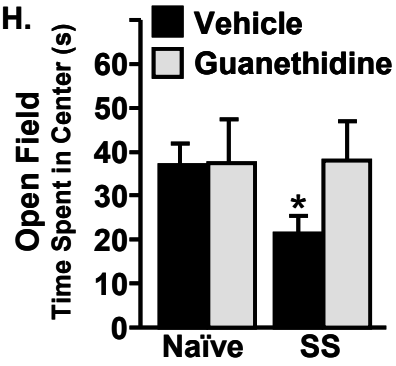


Figure Legends

Fig. 1 Overview of the neuroimmune pathways that respond to stress. Stress initiates bidirectional communication between the central nervous system and immune system. This communication influences myeloid cell function. Re-distribution of primed myeloid cells establishes immunological and behavioral deficits (Wohleb et al., 2015).

Fig. 2 Acute stress in stress-sensitized (SS) mice re-established monocyte trafficking and anxiety-like behavior. A) Male C57BL/6 mice were stress-sensitized (SS) by 6 cycles of RSD or left undisturbed as controls (Naïve). Mice were subjected to acute social defeat 24 days later and behavioral and biological analyses were completed 14 hours later. **B)** Acute social defeat increased the percentage of $Ly6C^{hi}$ monocytes in circulation in SS mice. **C)** Acute social defeat increased the percentage of brain macrophages in SS mice. **D)** Acute social defeat increased several inflammatory mediators: IL-1β CCL2, TNF- α, and CD14 in SS mice. Stress-Sensitized mice exposed to acute social defeat exhibited anxiety-like behavior in the open field with **E)** increased time to enter the center and **F)** reduced time spent in the center. **G)** Acute social defeat reduced the number of monocytes in the spleen. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON (p<0.05) according to *F*-protected *post hoc* analysis.

Fig.3 Splenectomy prior to stress-sensitization prevented the re-establishment of monocyte trafficking and anxiety-like behavior following exposure to acute stress. A) Male C57BL/6 mice were subjected to sham or splenectomy (SPLX) surgery and were allowed to recover for 14 days. Mice were then stress-sensitized (SS) by 6 cycles of RSD or left undisturbed as controls (Naïve). Twenty-four days later, mice were subjected to acute social defeat, behavioral and biological analyses were completed 14 hours later**. B)** Representative flow Bi-variate dot plots of CD115 and

Ly6C labeling of blood cells**. C)** The percentage of Ly6C^{hi} monocytes in circulation was increased in SS mice exposed to acute social defeat and this effect was blocked by splenectomy. **D)** Representative flow Bi-variate dot plots of CD11b and CD45 labeling on enriched brain macrophages (MΦ) and microglia (MGL). **E)** The percentage of brain macrophages was increased by acute stress in SS mice and this effect was blocked by splenectomy. **F)** Acute social defeat increased mRNA expression of IL-1 β, TNF- α and CD14 in SS mice and this was attenuated by splenectomy. Stress-sensitized Sham mice exhibited anxiety-like behavior in the open field with **G)** increased time to enter the center and **H)** reduced time spent in the center. These behavioral deficits were attenuated by splenectomy. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON (p<0.05) and means with (#) tended to be different from CON (p<0.1), according to *F*-protected *post hoc* analysis.

Fig. 4 Splenectomy prior to RSD did not influence myelopoiesis, monocyte redistribution, or the establishment of anxiety-like behavior. A) Male C57BL/6 mice were subjected to sham or splenectomy (SPLX) surgery and were allowed to recover for 14 days. Mice were then exposed to 6 cycled of repeated social defeat (RSD) or left undisturbed as controls (CON), and 14 hours after the final cycle, behavioral and biological analyses were completed. **B)** RSD increased spleen weight in sham mice. **C)** Representative flow Bi-variate dot plots of CD115 and Ly6C labeling of blood cells. **D)** RSD increased the percentage of Ly6C^{hi} monocytes in circulation independent of splenectomy. **E)** Representative flow Bi-variate dot plots of CD11b and CD45 labeling on enriched brain macrophages (MΦ) and microglia (MGL). **F)** RSD increased the percentage of brain macrophages independent of splenectomy. **G)** RSD mice exhibited anxiety-like behavior in the open field with increased time to enter the center and **H)** decreased time spent in the center. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON (p<0.05) according to *F*-protected *post hoc* analysis.

Fig. 5 Stress-sensitization primed splenocytes as demonstrated by an exaggerated response to *ex vivo* LPS challenge. A) Male C57BL/6 mice were stress-sensitized (SS) by 6 cycles of RSD or left undisturbed as controls (Naïve). Twenty-four days later, biological analyses were completed. **B)** Splenocytes from SS mice had increased IL-6 secretion following LPS challenge compared to splenocytes from naïve mice. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from Naive (p<0.05) according to *F*-protected *post hoc* analysis.

Fig. 6 Guanethidine attenuated monocyte trafficking and the re-establishment of anxiety in stress-sensitized mice. A) Male C57BL/6 mice were stress-sensitized (SS) by 6 cycles of RSD or left undisturbed as controls (Naïve). Twenty-three days later, mice were pretreated with guanethidine (Guan) or Vehicle (Veh), prior to acute social defeat. Fourteen hours after acute social defeat, behavioral and biological analyses were completed. **B)** Representative flow Bi-variate dot plots of CD115 and Ly6C labeling of blood cells. **C)** Acute stress increased the percentage of $Ly6C^{hi}$ monocytes in the blood of SS-Veh mice but not naïve mice. **D)** Representative flow Bi-variate dot plots of CD11b and CD45 labeling of enriched brain macrophages (MΦ) and microglia (MGL). **E)** The percentage of brain macrophages was increased by acute stress and this effect was blocked by guanethidine. **F)** Acute social defeat increased mRNA expression of IL-1 β and TNF- α in SS-Veh mice but not SS-Guan mice. SS-Veh mice exhibited anxiety-like behavior in the open field with **G)** increased time to enter the center and **H)** reduced time spent in the center. This affect was attenuated by guanethidine pretreatment. Bars represent the mean ± SEM. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON (p<0.05) and means with (#) tended to be different from CON (p<0.1), according to *F*-protected *post hoc* analysis.

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