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# Peripheral and Central Effects of Repeated Social Defeat Stress: Monocyte Trafficking, Microglial Activation, and Anxiety

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## Abstract

The development and exacerbation of depression and anxiety are associated with exposure to repeated psychosocial stress. Stress is known to affect the bidirectional communication between the nervous and immune systems leading to elevated levels of stress mediators including glucocorticoids (GCs) and catecholamines and increased trafficking of proinflammatory immune cells. Animal models, like the repeated social defeat (RSD) paradigm, were developed to explore this connection between stress and affective disorders. RSD induces activation of the sympathetic nervous system (SNS) and hypothalamic-pituitary (HPA) axis activation, increases bone marrow production and egress of primed, GC-insensitive monocytes, and stimulates the trafficking of these cells to tissues including the spleen, lung, and brain. Recently, the observation that these monocytes have the ability to traffic to the brain perivascular spaces and parenchyma have provided mechanisms by which these peripheral cells may contribute to the prolonged anxiety-like behavior associated with RSD. The data that have been amassed from the RSD paradigm and others recapitulate many of the behavioral and immunological phenotypes associated with human anxiety disorders and may serve to elucidate potential avenues of treatment for these disorders. Here, we will discuss novel and key data that will present an overview of the neuroendocrine, immunological and behavioral responses to social stressors.

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repeated social defeat; GC-insensitive monocytes; macrophages; cell trafficking; sensitization; inflammation; microglia; anxiety-like behavior

## 1. Introduction

Although the biological mechanisms are not fully understood, the individual response to prolonged or severe stressors contributes to the development and exacerbation of depression and anxiety (Maes *et al.*, 1998; Kalynchuk *et al.*, 2004; Raison *et al.*, 2006; Norman *et al.*, 2010; Capuron & Miller, 2011; Gilman *et al.*, 2013). One potential contributor to the etiology of stress-related mental health disorders involves the bidirectional communication between the immune system and the central nervous system (CNS) (Miller *et a.*, 2009). In humans, the experience of chronic stress is associated with proinflammatory leukocytic phenotypes that are unresponsive to the anti-inflammatory actions of glucocorticoids (GCs) (Cohen *et al.*, 2012) and a transcriptional profile that is consistent with the expansion and priming of myeloid-derived cells (Miller *et al.*, 2008; Powell *et al.*, 2013). The mechanistic association between inflammation and depression is particularly well-established (Raison *et al.*, 2006; Dantzer *et al.*, 2008; Miller *et al.*, 2009; Norman *et al.*, 2010; Capuron & Miller, 2011), while the case continues to build for the mechanistic association between inflammation and anxiety (Maes *et al.*, 1998; Pitsavos *et al.*, 2006; O'Donovan *et al.*, 2010; Pace & Heim, 2012).

The murine repeated social defeat (RSD) paradigm recapitulates many key immunological and behavioral features associated with psychosocial stress in humans. In the bone marrow, RSD increases production, egress, and trafficking of proinflammatory immune cells that are insensitive to GCs (Avitsur *et al.*, 2002; Engler *et al.*, 2004; Engler *et al.*, 2005; Kinsey *et al.*, 2007; Hanke *et al.*, 2012; Wohleb *et al.*, 2012) (Figure 1a) and traffic to the spleen (Engler *et al.*, 2004), lung (Curry *et al.*, 2010), and brain (Wohleb *et al.*, 2013) (Figure 1b). Furthermore, data from the RSD paradigm support a conserved inflammatory transcriptional response in leukocytes that is comparable to that of chronically-stressed human populations (Powell *et al.*, 2013).

RSD not only precipitates a proinflammatory environment within the periphery, but also activates brain regions associated with fear, anxiety, and threat appraisal that appear to contribute to behavioral changes. In many of these stress-responsive brain regions, neuronal activation co-occurs with neuroinflammatory events like microglial activation and recruitment of primed monocytes from the periphery (Wohleb *et al.*, 2013). Studies suggest a connection between monocyte trafficking to the brain and RSD-induced anxiety-like behavior (Wohleb *et al.*, 2013; Wohleb *et al.*, 2014a). Moreover, RSD induces long-term stress-sensitization that is related to monocyte trafficking from the spleen to the brain (Wohleb *et al.*, 2014a). In this review, we will introduce the concept of social defeat stress and provide an overview of studies that have used the RSD paradigm to elucidate the peripheral and central effects of social stress. Finally, we will discuss monocyte trafficking to the CNS and its relationship to anxiety-like behavior.

### 1.1 Stress and Inflammation

Inflammation is an adaptive biological response to tissue injury and other immune challenges and is a key indicator of mental and physical disease. Inflammation is characterized, in part, by an increase in primed and activated immune cells and the subsequent release of proinflammatory immune products (Black & Garbutt, 2002). In particular, stress majorly impacts the expression of cytokine and chemokine genes that are instrumental in mobilizing immune cells to aid in the resolution of an insult and restoration of tissue function (Black & Garbutt, 2002). In response to stress, a milieu of hormones, peptides, and neurotransmitters are released by the nervous and endocrine systems that pleiotropically function to modulate the immune system. Specifically, the response to stress activates the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) subsequently releasing GCs and catecholamines, respectively. Depending on the nature, intensity, and duration of the stressor and the type of immune challenge, stress effects on the immune system differ. On one hand, chronic, unrelenting stress (e.g., prolonged restraint stress) is able to induce immunosuppression and cause a skew towards an anti-inflammatory immune cell phenotype that increases susceptibility to disease (Cohen *et al.*, 2012). On the other hand, bouts of repeated defeat stress (e.g., RSD) can induce immunoenhancement and cause a skew towards a proinflammatory phenotype by inducing a state of GC-insensitivity to occur in innate immune cells; this insensitivity prevents the GC-induced suppression of inflammation through immune cell apoptosis and inhibition of nuclear factor κ B (NFκB) pathways (Chrousos *et al.*, 1996; Barnes & Adcock, 2009). This enhanced inflammation can be described as a double-edged sword. In the short-term, inflammation beneficially eradicates the immune challenge, but, over time, inflammation can worsen tissue damage and negatively impact disease outcome. Associated with the experience of social stress, it is this prolonged inflammatory state that has the potential to contribute to the etiology of depression and anxiety (Meduri & Yates, 2004; Barnes & Adcock, 2009).

### 1.2 Social Stressors

Numerous studies indicate that social status modulates the immunological and physiological responses to stress. Some of the first studies in this field examined subordinate non-human primates, who, in response to social reorganization (i.e., a shift in social hierarchy) had reduced body weights, higher levels of circulating GCs (i.e., cortisol), and increased susceptibility to upper respiratory infections (Cohen *et al.*, 1997; Sapolsky, 2005). In humans, longitudinal studies of social defeat involving bullying or loss of social status in the workplace have demonstrated increased incidence of depression, anxiety, loss of self-esteem, increased incidence of illness, and other aberrant physiological and behavioral symptoms (Marmot & Feeney, 1997; Griffin *et al.*, 2002; Stansfeld *et al.*, 2003; Bilgel *et al.*, 2006). The commonality and grave impact of social stress on humans led to the development of several animal models.

In the laboratory, murine social stress is modeled using resident-intruder paradigms whereby the response to social stress involves protecting the resources of the cage from intruders (Tamashiro *et al.*, 2005). Competition for access to resources causes the formation of social hierarchies within the cage consisting of a dominant alpha, co-dominants, and subordinates

(Ginsburg and Allee, 1975). These hierarchies are disrupted when an intruder vies to enhance social status by challenging and defeating the dominant alpha male (Ginsburg and Allee, 1975). Social status changes among cage mates often involve episodes of physical defeat occurring through the expression of aggression and acceptance of subordination. This disruption is not only psychologically stressful for the mice involved, but also entails a physical component whereby there is a risk of injury. Defeated subordinate animals receive bite wounds and experience pain that is not life-threatening, but contributes to significant changes in the immune system, cellular activation in the CNS, and expression of prolonged anxiety-like behavior (Bailey *et al.*, 2004).

### 1.3 Mouse Model of Social Stress: Repeated Social Defeat Paradigm (RSD)

RSD is an ethologically-relevant resident-intruder paradigm involving intermale aggression and loss of social status by the disruption of an established social hierarchy (Avitsur *et al.*, 2001; Stark *et al.*, 2001). Around the beginning of the active cycle (17:00), an aggressive mouse is introduced into a cage of resident mice that have an established social hierarchy. Aggressor mice are older, larger, and, due to retired breeder status, have had previous social experience with being a sole dominant in a cage.

Within minutes of introduction, the aggressive intruder engages the residents in brief, yet consistent, attacks for the length of the 2 h cycle. During these attacks, the residents display classic behavioral signs of fear and submissiveness that are dependent on social status (Blanchard & Blanchard, 1977; Avitsur et al., 2002; Avitsur et al., 2007).During these attacks, the residents display classic behavioral signs of fear and submissiveness that are dependent on social status (Blanchard & Blanchard, 1977; Avitsur *et al.*, 2002; Avitsur *et al.*, 2007). For instance, the threatened resident mouse, especially if it is a dominant alpha or co-dominant, may choose to fight with the intruder in an effort to reestablish dominance over the territory and resources or, alternatively, the resident mouse may attempt to flee (Avitsur *et al.*, 2007). By the end of 6 cycles of RSD, the ultimate behavior displayed by the defeated residents is subordination, which involves huddling in a corner and, upon approach, rearing on hind legs to bare their ventral body surface to the aggressor (Blanchard & Blanchard, 1977; Avitsur *et al.*, 2007). In all, this unique paradigm of social stress has elucidated many mechanisms regarding the effects of stress on the peripheral immune system and inflammatory processes.

## 2. Peripheral Effects of Repeated Social Defeat Stress

RSD causes gross anatomical changes to peripheral organs that are indicative of substantial immune and endocrine alterations (e.g., thymic involution, adrenal hypertrophy, and splenomegaly) (Engler *et al.*, 2005). Data generated over the last decade has yielded insight into the tissue-specific production, egress, and trafficking of bone marrow-derived, GC-insensitive monocytes/macrophages in mice that have undergone RSD treatment. These cells have a unique phenotype in that they are not only GC-insensitive, but also possess a primed and activated phenotype, i.e., they have elevated levels of Toll-like receptors (TLRs), adhesion molecules, and co-stimulatory receptors on their surface (Avitsur *et al.*, 2003; Engler *et al.*, 2004; Bailey *et al.*, 2007; Powell *et al.*, 2009). When stimulated, these cells

produce high levels of proinflammatory cytokines and chemokines, thus promoting an immunoenhancive environment within the periphery.

Additionally, RSD induces the increase of plasma and tissue catecholamines (norepinephrine and epinephrine) and plasma GCs (corticoisterone) (Avitsur *et al.*, 2001; Hanke *et al.*, 2012). RSD-induced levels of GCs increase as the cycle number increases indicating that habituation to RSD as a stressor does not occur (Avitsur *et al.*, 2001; Stark *et al.*, 2001; Bailey *et al.*, 2004; Engler *et al.*, 2005). Furthermore, there is no break in the circadian rhythm of the RSD-induced catecholamine or GC responses. The morning after the last RSD cycle, catecholamine and GC levels return to baseline (Avitsur *et al.*, 2002; Hanke *et al.*, 2012). Additionally, *in vivo* dexamethasone suppression tests indicate that RSD mice maintain a normal GC negative feedback loop as dexamethasone equivalently suppresses plasma GC levels in RSD and control mice (Avitsur *et al.*, 2002). Overall, the peripheral immunological and endocrine effects of RSD are tissue-specific and many significant changes are seen in the spleen, bone marrow, and circulation.

## 2.1 Spleen

Six cycles of RSD results in splenomegaly, a hallmark characteristic of the RSD response, which causes a near doubling of the spleen mass (Avitsur *et al.*, 2002). Cycle-dependent increases in spleen mass correspond with increasing accumulation of $CD11b^+$ monocytes and granulocytes (Avitsur *et al.*, 2002). CD11b is a cell adhesion molecule expressed on both granulocytes and monoctyes and is considered to be an indicator of activation. *In vitro*, monocytes from RSD spleens have enhanced cell viability in the presence of lipopolysaccharides (LPS) and varying concentrations of corticosterone. GC-insensitive monocytes also have enhanced IL-6 responses in the presence of LPS and GCs that is initiated somewhere between 3 and 6 cycles of RSD and persists for at least 10 days past the termination of the stressor (Avitsur *et al.*, 2002). While GCs normally reduce cell viability and suppress the production of proinflammatory cytokines in response to stress, RSD-treated splenocytes are unresponsive to GCs. This phenomenon of GC-insensitivity is also tissue-specific. For example, monocytes from the spleens of RSD mice are GC-insensitive, while peritoneal monocytes from RSD mice are not (Avitsur *et al.*, 2002). Data indicate that $CD11b^+$ monocytes derived from the bone marrow as a consequence of the stressor are responsible for this phenomenon of GC-insensitivity. When $CD19^+$ B-cells and $CD11b^+$ macrophages were selectively depleted from splenocyte cultures, it was the depletion of $CD11b^+$ cells, but not $CD19^+$ cells, which abolished the RSD-induced GC-insensitivity and cytokine responses (Stark *et al.*, 2001). It is important to note that not all RSD mice develop this state of GC-insensitivity in the spleen; it is most robust in mice that exhibited more subordinate behaviors during the stress treatment (Avitsur *et al.*, 2007).

GC-insensitivity presents in diseases and disorders that include asthma, infection, depression, and anxiety. It is possible that cytokines or neuroendocrine mediators induce GC-insensitivity by altering the number or function of GC receptors (Kino *et al.*, 2003). Quan *et al.*, (2003) examined the molecular mechanisms of GC-insensitivity in RSD splenocytes and found that the GC receptor in RSD splenocytes ($CD11b^+$ cells) did not have the ability to suppress NFκB activity due to a failure of the GC receptor to translocate to the

nucleus (Quan *et al.*, 2003). This change of GC receptor function could not be detected at the pre-translational level suggesting this change is not permanent (Quan *et al.*, 2003). A previous study demonstrated that GC sensitivity was recovered 30 days after the termination of RSD (Avitsur *et al.*, 2002). Overall, RSD-induced GC-insensitivity in the presence of LPS is, at least in part, due to the inability of the GC receptor to translocate to the nucleus and interact with NFκB.

The question was raised about the origin of the increased number of GC-insensitive $CD11b^+$ leukocytes in the RSD spleen. One hypothesisis that the repeated activation of neuroendocrine systems caused the mobilization of neutrophils and monocytes from the bone marrow to the spleen (Figure 1b). The murine spleen has low myelopoietic activity and most of the granulocytes and monocytes that circulate come from the bone marrow (Ohno *et al.*, 1993). It is well known that stress and severe infection causes a rapid release of neutrophils and monocytes into circulation referred to as neutrophilia and monocytosis, respectively subsequently leading to the increased production of these cells in the bone marrow (Stefanski & Engler, 1998; Stefanski, 2000; Powell *et al.*, 2013). Therefore, attention turned from RSD effects on the spleen toward examination RSD effects on the bone marrow.

### 2.2 Bone Marrow

The bone marrow is the site from which all circulating blood cells are generated. These cells originate from a common hematopoietic stem cell that commits to and differentiates along specific cell lineages (e.g., erythrocytic, granulocytic, monocytic, or lymphocytic) (Dorshkind, 1990). Stromal cells and macrophages produce colony stimulating factors like granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) that induce the proliferation and maturation of immature bone marrow cells (Lieschke & Burgess, 1992). The stromal cells are organized into hematopoietic microenvironments that are composed of nutrient arteries, capillaries, and emissary veins (Dorshkind, 1990). Influx and efflux of cells and mediators to and from the bone marrow affects the differentiation and release of immune cells (Dorshkind, 1990). In the case of the stress response, activation of the HPA axis and the SNS and the subsequent release of GCs and catecholamines alter the function, timing of the release, and migration of these bone marrow cells (Engler *et al.*, 2004; Engler *et al.*, 2005).

Repeated exposure to RSD is associated with increased cell mobilization and myelopoiesis in the bone marrow and an accumulation of neutrophils and monocytes in circulation and in the spleen (Engler *et al.*, 2004). Furthermore, RSD treatment is associated with the depletion of B-cells and erythrocytes in the bone marrow and blood (Engler *et al.*, 2004). This decrease in cellularity could be due to the continued GC- sensitivity of these cells and resultant GC-induced apoptosis. Interestingly, bone marrow cells from control mice are GC-insensitive, but, after 6 cycles of RSD, these cells disappear from the bone marrow and the remaining cells are GC-sensitive (Engler *et al.*, 2005). This is the opposite for the spleen whose cells, at the start of RSD, begin GC- sensitive and become insensitive after RSD. Data suggest that the unique RSD-induced microenvironment of the bone marrow produces an increase in primed, activated and GC insensitive immune cells. By the $6^{th}$ cycle of RSD,

continued trafficking of immune cells from the bone marrow causes not only a depletion in mature cells, but also the primed, activated, GC insensitive cells as they have been mobilized to organs such as the spleen and brain.

The enhanced myelopoiesis observed in the bone marrow of RSD mice is likely due to combination of increased production of GM-CSF leading to myeloid-derived cell survival in the periphery (Figure 1b). These colony stimulating factors likely act as chemotactic signals for circulating myeloid-derived cells to leave circulation and enter the spleen. GM-CSF, in particular, is a potent chemoattractant, and, by up-regulating CD11b expression, increases the adhesion of blood neutrophils and monocytes to the vascular endothelium (Socinski *et al.*, 1988; Kirby *et al.*, 2007). As the number of RSD cycles increases, GM-CSF expression, but not M-CSF, is increased in the bone marrow (Engler *et al.*, 2005) (Figure 1b). In the spleen, only after the 2$^{nd}$ cycle does GM-CSF and M-CSF increase, and by the 6$^{th}$ cycle the expression returns to baseline (Figure 1b).

Finally, there is a substantial increase in the proportion of immature myeloid-derived cells (defined as CD11b$^{+}$/Gr-1$^{low}$) following the 4$^{th}$ cycle in RSD groups that corresponds to the increase in GM-CSF expression (Engler *et al.*, 2005). Neutralizing GM-CSF with an antibody or pre-treatment with propranolol (a non-selective β-adrenergic receptor (βAR) antagonist) before each stress cycle inhibited RSD-induced increases in monocytic and granulocytic bone marrow progenitor cells (Powell *et al.*, 2013). Overall, in the bone marrow, RSD induces GC-insensitivity in immune cells and increases the production of myeloid progenitor cells and GM-CSF, and decreases mature CD11b$^{+}$ cells. These data point to the bone marrow as the most likely origin of the peripheral and central accumulation of proinflammatory GC-insensitive CD11b$^{+}$ cells.

### 2.3 Circulation

RSD stimulates proinflammatory cytokine release into circulation. These cytokines enhance inflammation and mobilize immune cells to enter circulation and tissues. In circulation, several cytokines are increased, e.g., IL-6, TNF-α, KC, MIP-2, and MCP-1 CCL2. The production of many of these cytokines is βAR-mediated in that pretreatment with the βAR antagonist propranolol abrogates RSD-induced increases in IL-6, TNF-α, and CCL2 (Wohleb *et al.*, 2011; Hanke *et al.*, 2012). Additionally, RSD induces an increase in adrenocorticotropic hormone (ACTH), with a peak after the 2$^{nd}$ cycle (Engler et al., 2005) and cycle-dependent decreases after this peak. Conversely, levels of corticosterone begin to elevate at the 2$^{nd}$ cycles and peak at the 6$^{th}$cycle. This inverse relationship of ACTH and GC suggests that the negative GC feedback mechanism on the pituitary gland is intact indicating that RSD does not result in a central state of GC-insensitivity (Engler *et al.*, 2005).

### 2.4 IL-1R1 and βAR Signaling Mediate Peripheral RSD Responses

Through use of antagonists and knockout (KO) mice, data indicate that the immunological effects of RSD are mediated by interleukin-1 receptor type 1 (IL-1R1) and βARs. IL-1R1 is a receptor that binds to both IL-1α and IL-1β and is an important mediator involved in the inflammatory response. Comparable to wild-type mice, IL-1R1 knockout (IL-1R1$^{KO}$) mice develop a stress phenotype that includes adrenal hypertrophy, thymic involution, and

elevated serum corticosterone in response to RSD (Engler *et al.*, 2008). However, unlike wild-type RSD mice, $CD11b^+$ cells do not accumulate in spleen, blood, or bone marrow of $IL\text{-}1R1^{KO}$ RSD mice and splenocytes from these mice fail to develop GC-insensitivity (Engler *et al.*, 2008). After RSD, there is an increase in IL-1 with IL-1β gene expression increased in the spleen and liver and IL-1α increased in the liver (Engler *et al.*, 2008). Further, IL-1β, but not IL-1α, protein is elevated in the plasma (Engler *et al.*, 2008). The lack of splenic accumulation of immune cells in $IL\text{-}1R1^{KO}$ mice could be one reason for the lack of GC-insensitivity in $IL\text{-}1R1^{KO}$ mice. Stress-associated release of immune cells from the bone marrow and signaling of IL-1 are likely important components contributing to the induction of GC-insensitivity in RSD immune cells.

In the context of stress, βAR activation appears to primarily mediate the proinflammatory actions of the high surge of stress-induced catecholamines and is able to increase the production of cytokines in immune cells (e.g., Johnson et al., 2013; Kim et al., 2013; Tan et al., 2007; Izeboud et al., 1999). When mice are pretreated with propranolol (βAR antagonist) 1 h before each cycle of RSD, the RSD-induced splenomegaly and plasma increases of IL-6, TNF-α, and CCL2 are prevented (Wohleb *et al.*, 2011; Hanke *et al.*, 2012). Pretreatment with propranolol did not alter GC levels indicating that blockade of βAR signaling does not affect HPA axis activation (Wohleb *et al.*, 2011). Flow cytometric analyses revealed that propranolol prevented the accumulation of $CD11b^+$ cells in RSD spleens and prevented the increases in splenic monocyte expression of TLR2 and 4 and CD86 following RSD (Hanke *et al.*, 2012). TLR2 and 4 are membrane-bound receptors that induce inflammatory responses through the activation of NF-κB. TLR2 recognizes a great variety of bacterial, fungal, viral, and endogenous molecules. TLR4 recognizes components of the Gram-negative cell wall such as LPS and lipid A. CD86 is a cell surface activation marker on monocytes that works with CD80 to prime T cells and provide the costumulatory signals necessary for T cell activation and survival. Additionally, supernatants from 18 h LPS-stimulated *ex vivo* cultures of splenocytes from propranolol-treated RSD mice contained less IL-6 than vehicle-treated RSD mice (Hanke *et al.*, 2012). Propranolol pretreatment *in vivo,* eliminated the development of GC-insensitivity of $CD11b^+$ monocytes *ex vivo* when compared to splenocytes from vehicle-treated RSD mice (Hanke *et al.*, 2012) suggesting that priming of GC-insensitive monocytes by RSD is a consequence of SNS activation. Taken together, RSD induces circulating and tissue-specific increases in catecholamines, GM-CSF, and major changes in the immune system that include increases in primed and activated cells and proinflammatory cytokines in the spleen, bone marrow, and circulation (Figure 1b).

## 3. RSD-Induced Behavioral and CNS Effects

While many of the previous data using the RSD paradigm had an intense focus on the stress-induced modulation of peripheral immune compartments (Avitsur *et al.*, 2001; Bailey *et al.*, 2004; Engler *et al.*, 2004; Engler *et al.*, 2008; Hanke *et al.*, 2012), recent focus has shifted toward the effects of social defeat on behavior and the brain. These studies demonstrate powerful connections between neuroinflammatory signaling, peripheral myelopoietic activity, and anxiety-like behavior.

One response to RSD is the development of anxiety-like behavior, which is evident in tests like open field, novel object, and light/dark assays (Kinsey *et al.*, 2007; Bailey *et al.*, 2009; Wohleb *et al.*, 2011; Hanke *et al.*, 2012). It is important to note that behavioral observations in this paradigm are measured the morning after the final cycle of social defeat (around 09:00h which is ~14h post RSD) when the neuroendocrine mediators (e.g., corticosterone, epinephrine, norepinephrine, etc. ) of the arousal systems have returned to baseline (Kinsey et al., 2007; Wohleb et al., 2013; Hanke et al., 2012). Anxiety-like behavior is most robustly observed following the 6 cycles of RSD, but also occurs in a cycle-dependent manner persisting to at least 8 days past the termination of the stressor (Wohleb *et al.*, 2013; Kinsey *et al.*, 2007). The development of RSD-induced anxiety strongly corresponds to immunological events in the periphery. For instance, the RSD-induced establishment and resolution of GC-insensitivity (Avitsur *et al.*, 2002) and increased myelopoiesis (Engler *et al.*, 2004; Wohleb *et al.*, 2013) follow similar kinetics to the development of anxiety-like behavior (Wohleb *et al.*, 2013). These strong temporal relationships between RSD-induced peripheral immunomodulation and anxiety support the hypothesis that prolonged behavioral changes following RSD are dependent on both peripheral and neuroinflammatory signaling.

### 3.1 Neuronal and Microglial Activation Co-Occur in Stress-Responsive Brain Regions

Initial studies regarding RSD-induced anxiety-like behavior examined the pattern of neuronal activation in response to RSD. Neuronal c-Fos expression, as determined by immunohistochemistry, was used as an indicator of regional neuronal activation (Wohleb *et al.*, 2011). This study revealed distinct patterns of increased neuronal c-Fos expression within specific regions of the brain. Following RSD, c-Fos$^{+}$ cells were increased in the prefrontal cortex (PFC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), and medial amygdala (Me AMYG) (Wohleb *et al.*, 2011), which are brain regions associated with fear, anxiety, and threat appraisal (LeDoux, 2000). Interestingly, increased c-Fos expression was observed following a single cycle of RSD indicating that substantial activation of fear, anxiety, and threat appraisal pathways preceded the major immunological events associated with additional cycles of RSD (i.e., GC-insensitivity, splenomegaly, thymic involution, increases in proinflammatory cytokines occur between 3 and 6 cycles) (Wohleb *et al.*, 2011). Furthermore, 6 cycles of RSD enhanced microglial activation as seen through Iba1 immunohistochemistry (Wohleb *et al.*, 2011). Pretreatment with propranolol prevented both RSD-induced neuronal and microglial activation as assessed by c-Fos and Iba1 labeling (Wohleb *et al.*, 2011). Overall, data indicate that brain region-specific neuronal activation is dependent on β-AR signaling and this activation appears to precede central and peripheral immune events associated with RSD. Thus, these data suggestthat neuronal activation is an upstream event that contributed to RSD-induced immunomodulation (Figure 1a).

Region-specific microglial and neuronal activation associated with chronic stress is observed in multiple models of repeated stress. Resembling work by Hinwood *et al.*, (2012), neuronal activity in RSD mice was regionally associated with local alterations in microglial morphology including increased cell size, and thickening and shortening of cell processes, i.e., deramification (Wohleb *et al.*, 2011) (Figure 1a). These microglia appear to be of an activated phenotype that are more inflammatory and phagocytic (Walker *et al.*, 2014).

Consistent with this observation, enriched microglia isolated from the brains of RSD mice have increased proinflammatory cytokine gene expression and increased protein expression of inflammatory markers (Wohleb *et al.*, 2011) (Figure 1a). These findings also strongly resemble work by Tynan et al., (2010) that revealed region-specific microglial activation following chronic restraint stress in rats.

There is a spatial coincidence of the activation of neurons and microglia that suggests a relation between these cell types. And the temporal kinectics of activation suggests a causative role of stress-induced neuronal activity in the local activation of microglia. This interpretation is supported by the fact that neuronal activation kinetically precedes microglial activation and cytokine production (Wohleb *et al.*, 2011; Wohleb *et al.*, 2013) (Figure 1a). Although it is possible that these two events may act independently of each other, it is well recognized that neuronal activity can regulate local microglial activation through specific chemokine signaling pathways (Kettenmann et al., 2013). One canonical pathway involves neuronal fractalkine ($CX_3CL1$) and microglial fractalkine receptor ($CX_3CR1$) (Biber *et al.*, 2007).

Under homeostatic conditions within the CNS, fractalkine is highly and primarily expressed by neurons (Bazan *et al.*, 1997; Kim *et al.*, 2011), while the fractalkine receptor is highly expressed by microglia (Jung *et al.*, 2000). Fractalkine receptor stimulation of brain microglia is anti-inflammatory (Cardona *et al.*, 2006; Corona *et al.*, 2010) and promotes microglial quiescence under homeostatic conditions (Wolf *et al.*, 2013). Consistent with this observation, microglial activation following RSD is associated with reduced expression of brain fractalkine (Wohleb *et al.*, 2013) and microglial fractalkine receptor (Wohleb *et al.*, 2014b). These findings demonstrate that reduced neuronal fractalkine expression in response to RSD contributes to increased microglial activation (Figure 1a). Neuronal fractalkine expression, or lack thereof, is involved in microglial chemotaxis and calcium regulation and plays a fundamental role within neuronal and microglial communication (Harrison et al., 1998). Our data suggest that neuronal activity may cause regional reductions in fractalkine expression that result in region-specific microglial activation (Figure 1a). This hypothesized relationship remains to be fully tested.

### 3.2 RSD Induces Distinct Patterns of Monocyte/Macrophage Trafficking to the Brain

Using flow cytometric assessment of $CD11b^+/CD45^{hi}$ monocyte/macrophages, RSD was found to induce monocyte trafficking to the brain (Wohleb *et al.*, 2011). Despite vascular perfusion, the increase in this $CD11b^+/CD45^{hi}$ population persisted suggesting that these $CD11b^+/CD45^{hi}$ monocyte/macrophages were perivascular or vascular-adherent monocyte/macrophages (Wohleb *et al.*, 2012). Functional studies from other labs identified this $CD11b^+/CD45^{hi}$ population as brain macrophages (Ford *et al.*, 1995; Sedgwick *et al.*, 1991). Brain macrophages can be defined as peripherally-derived monocytes that either accumulate in the perivascular space ($CD11b^+/CD45^{hi}$) or cross the blood brain barrier into the brain parenchyma ($CD11b^+/CD45^{lo}$). If these cells enter the brain parenchyma, CD45 expression is reduced and these cells morphologically and phenotypically become indistinguishable from resident microglia (Mildner *et al.*, 2007). Based on data from flow cytometric scatter

properties and Ly6C expression, the CD11b$^{+}$/CD45$^{hi}$ brain macrophages were likely derived from circulating Ly6C$^{hi}$ monocytes (Wohleb *et al.*, 2011).

These findings were further corroborated using lysozyme M-green fluorescent protein (LysM-GFP) knock-in mice (Wohleb *et al.*, 2013). These mice express GFP under the LsyM promoter that drive myeloid-derived cells to strongly express GFP (e.g., monocytes and granulocytes) (Rieger *et al.*, 2009). In this study, RSD induced an increase in CD11b$^{+}$/CD45$^{hi}$/LysM-GFP$^{+}$ cells as detected by flow cytometry. Immmunofluorescent histology demonstrated increased rod/circular GFP$^{+}$ cells that co-localized with the vasculature throughout the brain (Wohleb *et al.*, 2013). It should be noted that as with CD45 expression, LysM expression is also reduced in monocytes once they extravasate into the brain parenchyma (Wohleb *et al.*, 2013). Because GFP is driven by the LysM promoter, extravasated monocytes lose GFP expression (i.e., become GFP$^{-}$) and are indistinguishable from resident microglia. Taken together, RSD robustly increased monocyte recruitment to the brain, but the specific sub-anatomical localization of these cells was not yet verified.

Monocyte trafficking to the brain is usually observed in association with substantial CNS-related tissue pathology and inflammation, e.g., multiple sclerosis, traumatic injury, stroke, Alzheimer's disease, or infection (Hafler *et al.*, 2005; Gate *et al.*, 2010; Donnelly *et al.*, 2011; Hawthorne & Popovich, 2011; McGavern & Kang, 2011; de Vries *et al.*, 2012). Therefore, the possibility that RSD could induce monocyte trafficking to the brain was intriguing because it would occur in the absence of CNS tissue pathology (Wohleb *et al.*, 2013). To better assess this possibility of monocyte trafficking, green fluorescent protein positive (GFP$^{+}$) bone marrow chimeric mice were created. Wild-type bone marrow was reconstituted with bone marrow-derived donor cells that ubiquitously expressed GFP (Wohleb *et al.*, 2013). In this study, the resident microglia were identified as negative for GFP (GFP$^{-}$), while bone marrow-derived cells were GFP$^{+}$. It is important to note that when Evan's blue dye was intravenously injected, vascular permeability of the blood brain barrier was not observed in these chimeric mice demonstrating that the drug-induced bone marrow ablation did not compromise the blood brain barrier (Wohleb *et al.*, 2013). Additionally, the control chimeric mice in these studies displayed little or no GFP$^{+}$ cells within the CNS parenchyma, which is consistent with previous studies demonstrating that microglia are not bone marrow-derived under homeostatic conditions (Kierdorf *et al.*, 2013). However, when mice underwent RSD, GFP$^{+}$ bone marrow-derived monocytes extravasated into the brain parenchyma in a region- and cycle-dependent manner (Wohleb *et al.*, 2013). Visually, these GFP$^{+}$ parenchymal macrophages demonstrated a ramified, microglia-like morphology with elevated expression of Iba1 (Wohleb *et al.*, 2013). This substantial influx of parenchymal ramified GFP$^{+}$ macrophages was only observed following 6 cycles of RSD (Wohleb *et al.*, 2013). Similar to the pattern of c-Fos and Iba1 expression (Wohleb *et al.*, 2011), increased ramified GFP$^{+}$ cells were specifically observed in brain regions associated with fear, anxiety, and threat appraisal including the PFC, PVN, LS, BNST, and AMYG, but were not observed in other brain regions like the motor cortex, striatum, somatosensory cortex, or cerebellum (Wohleb *et al.*, 2013). This regional specificity of RSD-induced trafficking reinforced the patterns of neuronal and microglial activation that were observed in other studies (Wohleb *et al.*, 2011; Wohleb *et al.*, 2013). Overall, in the absence of tissue

pathology or global permeability of the blood brain barrier, RSD caused selectively permissible and region-specific extravasation of monocytes into brain regions associated with fear, anxiety, and threat appraisal.

Other murine models of chronic stress have reported monocyte trafficking to the brain. In particular, the Fujimiya lab demonstrated that brain macrophage infiltration occurs under other stressors (Ataka *et al.*, 2013; Brevet *et al.*, 2010; Sawada *et al.*, 2014). For example, following five days of foot shock stress, bone marrow-derived monocytes trafficked to the ventral hippocampus and demonstrated a ramified, microglia-like morphological phenotype (Brevet *et al.*, 2010). Interestingly, the stress associated with simply observing other mice undergoing foot shock was sufficient to recruit monocytes to the brain parenchyma (Ataka *et al.*, 2013). Additionally, both CCR2 and βAR signaling contributed to the trafficking of bone marrow-derived monocytes to the brain (Ataka *et al.*, 2013; Sawada *et al.*, 2014). In a more recent chronic neuropathic pain study, partial sciatic nerve ligation induced anxiety-like behavior and the infiltration of bone marrow-derived monocytes into the AMYG (Sawada *et al.*, 2014). Oral administration of a CCR2 antagonist or microinjections of IL-1 receptor antagonist to the AMYG prevented monocyte trafficking (Sawada *et al.*, 2014).

Stress tends to cause distinct patterns of monocyte recruitment to the brain: global and region-specific. Globally, RSD increased diffuse perivascular monocyte recruitment that was observed through flow cytometry and immunohistochemistry in both wild-type and LysM-GFP mice. On the other hand, in a region-specific manner, monocytes extravasated into the brain parenchyma following RSD in $GFP^{+}$ bone marrow chimeric mice. It is important to note the distinction between these trafficking patterns, as they are likely caused by distinct mechanisms that lead to differential effects on brain and behavior. For instance, global increases in perivascular monocytes are likely more peripherally regulated (e.g., circulating cytokines) while region-specific monocyte infiltration are likely more causally connected to regional neuronal and microglial activation. In fact, global increases in perivascular brain macrophages are proportionally linked to increases in circulating $Ly6C^{hi}$ inflammatory monocytes, and the trafficking pattern of these cells occurs throughout the brain even in areas that are unrelated to the expression of fear, anxiety, and threat appraisal (Wohleb *et al.*, 2013). Taken together, research from multiple labs demonstrates that repeated psychological stress causes distinct patterns of monocyte trafficking to the brain.

### 3.3 Stress-induced Brain Macrophage Trafficking Promotes Anxiety-like Behavior

One key finding is that the establishment of anxiety-like behavior following RSD is promoted by myeloid-derived cell trafficking to the brain (Wohleb *et al.*, 2013). All circulating monocytes express $CX_3CR1$ (Yona et al., 2012), but not all monocytes express CCR2. Additionally, brain macrophage trafficking and anxiety-like behavior following RSD was absent in both $CCR2^{KO}$ (CCL2 receptor KO) and $CX_3CR1^{KO}$ (fractalkine receptor KO) mice (Wohleb *et al.*, 2013). More specifically, $CCR2^{KO}$ and $CX_3CR1^{KO}$ mice lacked both global perivascular-monocyte trafficking and region-specific extravascular trafficking following RSD (Wohleb *et al.*, 2013). However, these KO mice did demonstrate peripheral immunomodulatory effects of RSD comparable to wild-type mice including increased circulating $Ly6C^{\mathbf{hi}}$ monocytes. Therefore, it was concluded that monocyte trafficking to the

brain was likely responsible for the promotion of anxiety-like behavior following RSD. This is consistent with a previous finding regarding βAR- and IL-1R1-dependent trafficking and anxiety-like behavior. Specifically, propranolol (βAR antagonist) or using IL-1R1$^{KO}$ prevented both RSD-induced anxiety-like behavior and brain monocyte trafficking (Wohleb *et al.*, 2011; Wohleb *et al.*, 2013). Together, these data support the idea that RSD-induced anxiety-like behavior is dependent upon monocyte trafficking to the brain and is mediated by SNS and IL-1 signaling (Figure 1a,c).

The hypothesis that monocyte trafficking to the brain is a factor that precipitates the behavioral responses to stress is relatively novel, and thus other reports of similar phenomena are relatively sparse. Despite this, there are a few examples of brain macrophage trafficking affecting behavior in the literature. For instance, in an inflammatory liver disease model, the Swain laboratory demonstrated an important role for brain macrophage trafficking in the development of fatigue and depressive-like behavior (D'Mello *et al.*, 2009). Additionally, in a neuropathic pain model, region-specific trafficking of monocytes to the AMYG was CCR2/CCL2-dependent and this trafficking was found to promote pain-induced anxiety-like behavior (Sawada *et al.*, 2014). Thus, we contend that monocyte trafficking to the brain is a unique pathway in which the immune system communicates with the brain and has important implications in the context of stress and inflammation-related mental health complications.

The specific molecular mechanisms connecting monocyte trafficking to stress-induced changes in behavior are not fully elucidated, but it is likely that brain macrophages contribute to RSD-induced anxiety through the propagation of cytokine signaling from the periphery to the brain. For example, even though they do not physically cross the blood brain barrier, it is well established that both peripheral and central cytokine signaling potently influences behavior through receptors located on either side of endothelial cells (Dantzer *et al.*, 2008; Capuron & Miller, 2011). Because the brain is highly sensitive to increases in proinflammatory cytokines, microglia tend to be highly regulated by anti-inflammatory ligands and receptors (e.g., CD200/CD200R (Lyons et al., 2007), CX3CR1/CX3CL1 (Cardona et al., 2006), or TGFβ/TGFβR (Butovsky et al., 2014)). Although similar in morphology and phenotype to microglia, peripherally-derived brain macrophages tend to be more inflammatory and less subject to immune regulation (Perry & Teeling, 2013).Along these same lines, RSD primes and activates peripherally-derived monocytes inducing increased cytokine expression and resistance to the immunosuppressive effects of GCs (Engler *et al.*, 2005; Wohleb *et al.*, 2011; Powell *et al.*, 2013; Wohleb *et al.*, 2014a). Moreover, Sawada *et al.*, (2014) demonstrated that pain-induced anxiety-like behavior associated with monocyte trafficking to the AMYG was prevented by local microinjection of IL-1R1 antagonist. Likewise, in a model of inflammatory liver disease, Kerfoot *et al.*, (2005) demonstrated that recruitment of TNF-α-expressing monocytes to the brain promoted depressive-like behavior and fatigue. These reports further suggest that monocyte trafficking to the brain promotes changes in behavior through central cytokine signaling. Nonetheless, the contribution of perivascular trafficking relative to parenchymal extravasation in the promotion of anxiety is yet unclear.

### 3.4 Neuron-Microglia-Endothelia Dynamics In The Context Of Psychological Stress: Evidence for An Immunological Neurovascular Unit

The region-specific trafficking patterns observed using bone marrow chimeric mice (Brevet *et al.*, 2010; Ataka *et al.*, 2013; Wohleb *et al.*, 2013) provide insight into the dynamic relationships among neurons, microglia, cerebral vasculature (i.e., endothelial cells) and the recruitment of monocytes to the brain in the context of psychological stress. For instance, increases in neuronal and microglial activation are region-specific and co-occur in fear, anxiety, and threat appraisal brain regions (Wohleb *et al.*, 2011). Furthermore, parenchymal infiltration of peripherally-derived monocytes is also region-specific and co-occurs within many of these same areas (Wohleb *et al.*, 2013). Accumulation of perivascular brain macrophages tends to be global and is not coupled to region-specific neuronal and microglial activation. Moreover, data suggest that RSD-induced microglial activation is likely an upstream event of monocyte trafficking to the brain and this is dependent on receptors such as CCR2 and $CX_3CR1$ in that increased microglia cytokine expression was still observed in $CCR2^{KO}$ and $CX_3CR1^{KO}$ mice that lacked both perivascular and parenchymal trafficking (Wohleb *et al.*, 2013). In all, data suggest that the infiltration of peripherally-derived monocytes is regionally coupled to neuronal and microglial activation giving cause to speculate that these events may be causally linked.

Microglial activation that is spatially coupled to local neuronal activation results in increased cytokines and chemokines, e.g., IL-1β, IL-6, TNF, and CCL2 (Wohleb *et al.*, 2011; Wohleb *et al.*, 2013; Wohleb *et al.*, 2014b) (Figure 1c). In other contexts, these same microglia-derived cytokines and chemokines are capable of increasing cell adhesion molecule (CAM) expression on the luminal surface of vascular endothelial cells and increasing the respective receptors on vascular monocytes (Kim *et al.*, 1996; Wang *et al.*, 2007; Shi *et al.*, 2011). CAMs on endothelial cells and their associated receptors on leukocytes (e.g., CD11b) are required for extravasation across the blood brain barrier into the parenchyma. Therefore, brain region-specific trafficking following RSD would require increased expression of these CAMs in the same regions. Indeed, RSD caused an exposure- and brain region-dependent increases in both gene and protein expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (Sawicki, in press, 2014). Furthermore, vascular ICAM/VCAM expression was increased in stress-responsive brain regions where neuronal and microglial activation co-occurred with macrophage trafficking (Sawicki, in press, 2014). Therefore, region-specific trafficking is likely related to region-specific activation of brain vascular endothelium and increases in CAMs through local stimulation by microglia-derived cytokines and/or chemokines that facilitate monocyte adhesion and extravasation (Figure 1c).

This idea that neuronal activity locally influences brain vasculature is reminiscent of the concept of a neurovascular unit (Hawkins & Davis, 2005). The concept of the neurovascular unit was initially used as a framework to explain the coupling of neuronal activity to alterations in local cerebral blood flow. However, this concept has expanded to explain vascular-neuronal interactions in the context of inflammatory CNS pathology, e.g., Alzheimer's disease and stroke (Iadecola & Gorelick, 2004). Arguments presented regarding RSD suggest a top-down association among neuronal activity, microglial activation, and

local vascular-facilitation of leukocyte diapadesis in the context of psychological stress. For instance, reports from both Wohleb and Hanke *et al.*, (2011) and Hinwood *et al.*, (2012) indicate a region-specific coupling between neuronal and microglial activation in the context of two different stress paradigms. Related to this, reports from our lab and the Fujimiya lab demonstrated that psychological stressors caused monocytes to extravasate into stress-responsive brain regions (Brevet *et al.*, 2010; Ataka *et al.*, 2013; Wohleb *et al.*, 2013; Sawada *et al.*, 2014). Futhermore, following RSD, substantial overlap occurs between monocyte extravasation and microglial activation within stress responsive brain regions (e.g., AMYG, PFC, and PVN) (Wohleb *et al.*, 2011; Wohleb *et al.*, 2013). Thus,the spatial co-occurrence of region-specific neuronal activity with microglial activation and monocyte extravasation and the kinetic timing of these events during RSD treatment provide evidence that there may exist a top-down causative link among these events (Figure 1c). More interventional studies must be performed before causation can be definitively determined.

### 3.5 Endothelial IL-1R1 Expression Contributes to RSD-Induced Anxiety

Mounting evidence implicates an important role for IL-1R1 signaling in the propagation of inflammatory signaling into, and within, the brain following RSD. Initial studies demonstrated that IL-1R1$^{KO}$ mice do not exhibit RSD-induced anxiety-like behavior or microglial activation despite having increased c-Fos$^{+}$ neurons (Wohleb *et al.*, 2011). These data indicate that although fear, anxiety, and threat appraisal-associated brain regions were activated, manifestation of RSD-induced anxiety-like behavior and the activation of microglia were dependent on intact IL-1R1 signaling (Wohleb *et al.*, 2011). The interpretation of these data is complicated by the fact that IL-1R1 contributes to inflammatory signaling in both the periphery and the CNS. For example, IL-1R1$^{KO}$ mice do not develop tissue-specific GC-insensitivity of myeloid-derived cells nor do they exhibit RSD-induced brain monocyte trafficking (Wohleb *et al.*, 2014b) suggesting that abrogation of anxiety-like behavior in IL-1R1$^{KO}$ mice was due to the lack of priming and trafficking of peripheral monocytes. However, subsequent experiments in an endothelial-specific knock down model (eIL-1R1kd) indicated that when IL-1R1 expression on vascular cells of the blood brain barrier were knocked down RSD continued to induce monocyte trafficking, but not anxiety-like behavior, suggesting that IL-1R1 plays a different role specifically within the CNS.

eIL-1R1kd mice exhibited intact peripheral immune responses to RSD in that there were increased circulating monocytes, primed splenic macrophages, and increased monocyte trafficking to the brain (Wohleb *et al.*, 2014b). Despite the monocyte trafficking and the immunoenhanced environment within the periphery, RSD-treated eIL-1R1kd mice did not exhibit increased microglial cytokine expression nor did they exhibit anxiety-like behavior. These data suggest that endothelial IL-1R1 plays a role in microglial cytokine expression and the precipitation of anxiety-like behavior.

### 3.6 Temporal Kinetics and Clinical Relevance Of RSD-Induced Anxiety-Like Behavior And Monocyte Trafficking

Thus far, findings from the RSD murine model implicate microglial activation and monocyte trafficking to the brain in the establishment of anxiety-like behavior (Wohleb *et*

*al.*, 2011; Wohleb *et al.*, 2013). This work was further extended to reveal strong temporal associations in the resolution and recurrence of anxiety-like behavior and brain monocyte infiltration (Wohleb *et al.*, 2013; Wohleb *et al.*, 2014a; Wohleb *et al.*, 2014b). Initial work demonstrated a cycle dependent increase in both anxiety-like behavior and brain monocytes (Wohleb *et al.*, 2011). Further work revealed that both anxiety-like behavior and recruited monocytes remained in the parenchyma for at least 8 days following the last cycle of RSD and that both of these parameters were resolved by 24 days (Wohleb *et al.*, 2014a) further reinforcing that the establishment and resolution of both anxiety-like behavior and brain monocyte trafficking were temporally linked. Despite the resolution of these parameters by 24 days, RSD-exposed mice remained sensitized to the behavior and immunomodulary effects of the stressor. At 24 days post RSD, sub-threshold stress exposure (i.e., one cycle of RSD) re-established monocyte trafficking and anxiety-like behavior in the stress-sensitized mice (Wohleb *et al.*, 2014a). One cycle of RSD was considered sub-threshold because it did not affect the behavioral or immunological parameters in control mice that were not exposed to the stressor (i.e., naïve mice). Therefore, comparable to the establishment and resolution, the recurrence of anxiety-like behaivor and monocyte trafficking was also temporally coupled.

One interesting finding was that myeloid cell redistribution in stress-sensitized mice after sub-threshold RSD exposure was not associated with alterations in myeloid cell production, egress, or trafficking from the bone marrow, but, instead, the redistribution of these cells was dependent upon a splenic myeloid cell reservoir (Wohleb *et al.*, 2014a). These data supported the hypothesis that myeloid-derived cell trafficking in the stress-sensitized animal originated not from the bone marrow, but from an alternative source. The idea that the spleen serves as an alternative source of monocyte trafficking is seen in several studies involving inflammatory challenges including myocardial infarction (Swirski *et al.*, 2009) and stroke (Seifert *et al.*, 2012).

The hypothesis that spleen-to-brain monocyte trafficking contributed to the reestablishment of anxiety-like behavior was tested. Control and RSD-sensitized mice were splenectomized prior to exposure to sub-threshold stress (Wohleb *et al.*, 2014a). Splenectomy prevented monocyte trafficking to the brain as well as the re-establishment of anxiety-like behavior in stress-sensitized mice (Wohleb *et al.*, 2014a). These data indicated that trafficking of a splenic monocyte population was necessary for promoting the recurrence of anxiety in sensitized mice. Additionally, sub-threshold stress augmented microglial cytokine expression in stress-sensitized mice, which was prevented by splenectomy. Thus, it was concluded that re-establishment of anxiety-like behavior and augmented microglial cytokine expression were dependent upon monocyte trafficking from the spleen to the brain (Wohleb *et al.*, 2014a).

These findings have exciting implications for mental health conditions involving recurring anxiety, yet several important points remain unclear. First, the mechanism by which RSD induces sensitization of splenic myeloid cell trafficking is unknown. However, previously published research sheds insight into this phenomenon. Although the literature regarding myeloid-derived cell trafficking from the spleen is relatively sparse, in the context of cardiovascular disease and stroke, substantial gains have been made (Leuschner *et al.*, 2012;

Seifert *et al.*, 2012). In a myocardial infarct model, monocytes recruited to damaged tissues were replenished by extramedullary monocytopoiesis that occurred in the spleen (Leuschner *et al., 2012*). The Pennypacker laboratory has elucidated regulatory mechanisms in spleen-to-brain myeloid trafficking within the context of cerebral artery occlusion. In their model, substantial splenic myeloid cell egress is observed following stroke (Seifert *et al.*, 2012). This increased egress was associated with more severe pathology (Ajmo et al., 2008) that was dependent upon peripheral noradrenergic signaling (Ajmo et al., 2009). Egress of splenic myeloid-derived cells was not dependent upon direct SNS innervation of the spleen, but rather circulating norepinephrine signaling through adrenergic receptors within the spleen.. This is a relevant finding because even after just one cycle, RSD induces an increase in norepinephrine in both circulation and the spleen (Hanke et al., 2012). When this catecholaminergic signaling is blocked by propranolol pretreatment, myeloid-derived cell production and trafficking is abrogated Thus, it is clear from our data and others that norepinephrine appears to play a critical role in spleen-to-brain myeloid-derived cell trafficking.

One last point of discussion is the clinical relevance of RSD as a model of stress-sensitization and recurring anxiety. Although RSD-sensitization is not considered a model of post-traumatic stress disorder (PTSD), studying stress-sensitization within the RSD paradigm may have relevance. For example, both RSD and PTSD involve a sensitizing event that is often both physical and psychological in nature (Bailey *et al.*, 2004; American Psychiatric Association, 2013; Freeman *et al.*, 2013). Analogous to RSD, the sensitizing event, or trauma, predisposes the individual to recapitulate the behavioral and physiological responses following exposure to either generalized cues or subsequent stressful events (American Psychiatric Association, 2013; Wohleb *et al.*, 2014a). Furthermore, both PTSD and RSD involve chronic maintenance of psychosocial deficits (American Psychiatric Association, 2013). Despite these commonalities, PTSD remains a complex and distinctly human disorder that likely cannot be fully modeled by RSD-sensitization in mice. However, it is increasingly appreciated that neuroimmune signaling contributes to the development and maintenance of PTSD and other chronic anxiety disorders. As reviewed by Pace and Heim (2012), strong clinical associations exist between recurring anxiety and inflammatory signaling. With the RSD paradigm recapitulating many of the behavioral and immunological phenomena associated with PTSD, mechanisms associated with RSD-sensitization (e.g., spleen-to-brain myeloid cell trafficking) may have clinical relevance to recurring stress and anxiety in PTSD and other human anxiety disorders.

## 4. Conclusion

Following prolonged and repeated stimulation of relevant pathways, psychological stress leads to negative physical and mental health outcomes. In summary, Figure 1 defines the proposed mechanisms by which RSD alters immune functioning and behavior ultimately leading to brain region-specific trafficking of monocytes that are associated with the development of anxiety-like behavior. First, in the CNS, Figure 1a depicts the scenario whereby RSD may induce neuronal activation through the release of norepinephrine and resultant βAR signaling in brain regions associated with fear, anxiety, and threat appraisal. This βAR signaling is hypothesized to result in reduced neuronal fractalkine ($CX_3CL1$)

production (a chemokine involved in communicating with microglia and regulating microglial activation) leading to microglial activation as shown by increases in Iba1. These activated microglia would now have the potential to increase production of proinflammatory cytokines and chemokines like IL-1β and CCL2. RSD then stimulates the peripheral SNS activation resulting in increases of epinephrine and norepinephrine in the bone marrow, spleen, and circulation. We propose that GM-CSF expression and βAR signaling promotes the production, release, and trafficking of these primed, GC-insensitive, immune cells from the bone marrow into circulation and various tissues including the spleen and brain (Figure 1b).

In the CNS, cells that comprise the neurovascular unit, which includes neurons, microglia, and endothelial cells, facilitate the bi-directional communication between the brain and the periphery. Figure 1c depicts the relationship among the members of the neurovascular unit in aiding the region-specific extravasation of monocytes following RSD. Microglia communicate with endothelial cells lining the blood brain barrier and this communication increases brain region-specific expression of CAMs that work to recruit monocytes. Proinflammatory cytokines and chemokines, specifically IL-1β and CCL2, interact with endothelial cells inducing selective permissibility of peripheral monocytes to the brain. In all, the complex communication among the members of the neurovascular unit directs psychosocial stress-induced monocyte trafficking and anxiety-like behavior. It is important that future studies place an emphasis on exploring the components of the neurovascular unit in order to further elucidate the roles of the numerous cellular and molecular components and pathways involved.

Recent findings from long-term studies suggest that RSD promotes the accumulation and longevity of primed splenic myeloid cells such that a exposure to a single cycle of RSD in stress-sensitized mice is sufficient to re-establish anxiety-like behavior and myeloid-derived cell redistribution. This finding is of great importance in that numerous mental health complications are linked to or exacerbated by stress even years after the initial stressor. Further research is needed to determine how long sensitization persists and to identify the mechanism(s) inducing sensitization and the reestablishment of monocyte trafficking and anxiety-like behavior. Furthermore, determining whether microglial priming contributes to stress sensitization in the periphery could also be beneficial. Clinically, these issues could be tested by assessing the association of stressful life events and proinflammatory markers in PTSD patients as well as those with other anxiety-related disorders. Overall, data from the RSD paradigm have contributed insight into mechanisms by which stress impacts behavior. These insights may be useful in generating new therapeutic approaches for those suffering from mental health complications and neurological diseases and disorders.

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## Abbreviations

| | |
|---|---|
| **ACTH** | adrenocorticotropic hormone |
| **αAR** | alpha adrenergic receptor |
| **AMYG** | amygdala |
| **βAR** | beta adrenergic receptor |
| **BNST** | bed nucleus of the stria terminalis |
| **CAM** | cell adhesion molecule |
| **CCL2** | chemokine (C-C motif) ligand 2 |
| **CCR2** | chemokine (C-C motif) receptor 2 |
| **CD11b** | cluster of differentiation molecule 11b |
| **CD19** | cluster of differentiation 19 |
| **CD45** | cluster of differentiation 45 |
| **CD86** | cluster of differentiation 86 |
| **CNS** | Central Nervous System |
| **$CX_3CL1$** | Chemokine (C-$X_3$-C motif) ligand 1 |
| **$CX_3CR1$** | chemokine (C-$X_3$-C motif) receptor 1 |
| **eIL-1R1kd** | endothelial-specific knock down |
| **GC** | glucocorticoid |
| **GM-CSF** | granulocyte macrophage-colony stimulating factor |
| **HPA** | hypothalamic-pituitary-adrenal |
| **Iba1** | ionized calcium-binding adapter molecule 1 |
| **ICAM-1** | intracellular adhesion molecule-1 |
| **IL-1** | Interleukin 1 |
| **IL-1R1** | interleukin-1 receptor type 1 |
| **IL-1α** | Interleukin-1 alpha |
| **IL-1β** | Interleukin-1 beta |
| **IL-6** | Interleukin-6 |
| **KC** | keratinocyte chemoattractant |
| **KO** | knockout |
| **LPS** | lipopolysaccharide |
| **LS** | lateral sulcus |
| **LsyM** | lysozyme M |

| | |
|---|---|
| **Ly6C** | lymphocyte antigen 6 C |
| **GFP** | green fluorescent protein |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **M-CSF** | macrophage-colony stimulating factor |
| **MeAMYG** | medial amygdala |
| **MIP-2** | macrophage inflammatory protein-2 |
| **NFκB** | nuclear factor κ B |
| **NGF** | nerve growth factor |
| **PFC** | prefrontal cortex |
| **PTSD** | post-traumatic stress disorder |
| **PVN** | paraventricular nucleus |
| **RSD** | repeated social defeat |
| **SNS** | sympathetic nervous system |
| **TLR2** | toll like receptor 2 |
| **TLR4** | toll like receptor 4 |
| **TLRs** | Toll-like receptors |
| **TNF-α** | tumor necrosis factor alpha |
| **VCAM-1** | vascular cell adhesion molecule-1 |

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## Highlights

- Social stress induces bidirectional responses between the brain and the immune system.
- Activation of the sympathetic nervous system alters hematopoiesis towards myelopoiesis.
- Primed myeloid cells (PMC) are proinflammatory and traffic to the brain in response to social stress.
- Trafficking of PMC to the brain is associated with prolonged anxiety-like behavior.

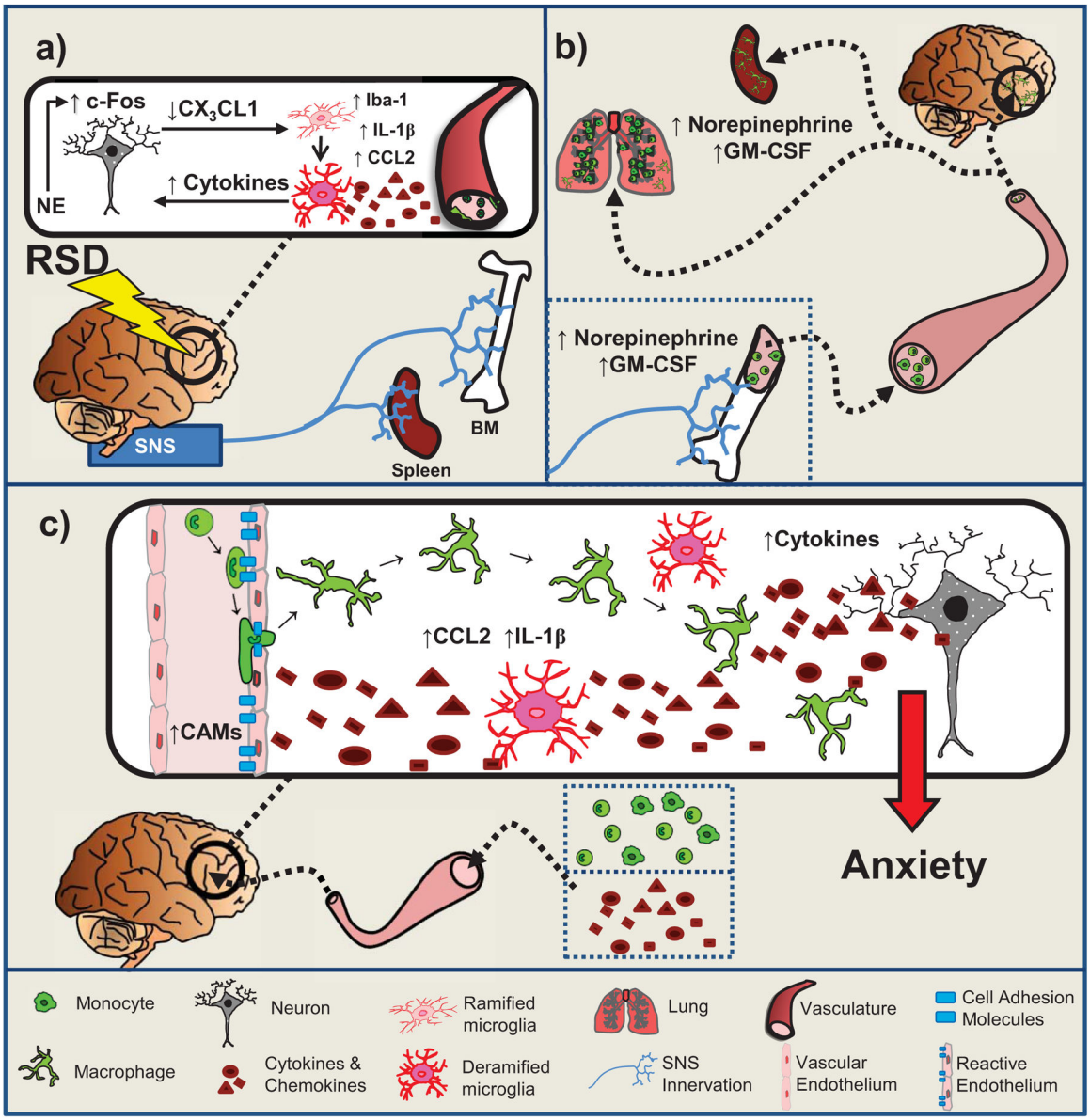


**Figure 1. Repeated Social Defeat and Anxiety**

**a.** Neuronal and microglia activation co-occur in brain regions associated with fear/anxiety and threat appraisal. SNS activation results in increased NE in BM, spleen, & circulation.

**b.** β-AR signaling promotes release of GC insensitive myeloid-derived cells that traffic to spleen, lungs, and brain and contributes to increased tissue-specific and circulating inflammatory cytokines.

**c.** Reactive endothelium facilitate monocyte trafficking to the perivascular and extra-vascular spaces. Brain monocyte/macrophage trafficking reinforces fear/anxiety and threat circuitry, promoting the development and maintenance of prolonged anxiety-like behavior.