Roles for the Ventromedial Prefrontal Cortex in Resistance to Acute Social Stress

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Dedication

For the one who first inspired my curiosity and determination to understand how health-related changes of the brain lead to inexplicable and surprising behaviors.

For that same person who taught me all one really needs to know about stress resilience.

For he who bought my first microscope and then pricked his finger to introduce me to a whole new world filled with unanswered questions.

For the one who read to me and later insisted I learn to read using "ValueTale" storybooks about dedication, perseverance, patience, honesty, respect, optimism, learning, self-belief, self-respect, creativity, imagination, leadership, humility, and so many more concepts that I've used daily to pursue this degree, but moreover, to pursue a life well-lived.

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Abstract

The ventromedial aspect of the prefrontal cortex (vmPFC) is an evolutionarily conserved region of the frontal lobe with primary roles in social processing and emotional regulation. During particularly stressful situations, neurons of the vmPFC are recruited and, through dynamic circuit-based activity patterns, help the organism integrate pertinent sensory cues and act in a context-specific manner. In the dissertation that follows, I discuss investigations aimed at determining how social experience prior to acute trauma modifies vmPFC activity and shifts behavioral responses in a manner suggesting resilience or susceptibility to stress. Using a Syrian hamster model of social status and stress responsivity, we have previously shown that socially defeated hamsters with dominant and subordinate social statuses display reduced or increased social avoidance, respectively, when compared with no-status controls. Further, vmPFC neural activity is enhanced dominant hamsters yet suppressed subordinates. Additionally, pharmacological inhibition of vmPFC neurons renders subordinate-like avoidance following stress, suggesting that vmPFC projections are necessary for resistance to acute traumatic stress. In Chapter 2, I demonstrate that dominant hamsters recruit a greater number vmPFC neurons that inhibit the dorsal raphe nucleus (DRN), a stress effector region implicated in social stress-induced avoidance. Moreover, subordinate hamsters failed to recruit this pathway during stress. In Chapter 3, I review follow-up experiments aimed at discerning whether the failure of vmPFC recruitment in subordinates could be due to immune-induced disruptions of vmPFC structural integrity. Results indicate that acute social defeat stress leads to cellular and synaptic degradation within the vmPFC and that these changes corresponded with increases in markers of proinflammatory activity of vmPFC immune cells, particularly in subordinate hamsters. Moreover, degradation was reduced by the antibiotic, minocycline. Taken together, the results of Chapter 3 implicate neuroimmune responses in stress-induced tissue degeneration in a social status-dependent manner. These projects collectively inform our understanding of how prior social conflict differentially influences responses to stress via changes in neural and immune mechanisms within the vmPFC. Accordingly, the results from this dissertation may inform future studies focused on the cellular mechanisms of stress resilience as well as provide potential targets for pharmacotherapeutic treatments promoting resilience to acute traumatic stress.

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Introduction

General Introduction

While stress is a contributing factor for an array of psychopathologies, not everyone responds to stress similarly (Southwick & Charney, 2018). Elucidating the neural mechanisms of stress resilience could therefore provide promising intervention strategies for vulnerable populations. Recently, several neurobiological studies have indicated that neuronal activity in the ventromedial aspect of the prefrontal cortex (vmPFC) is a critical mediator of individual differences in stress responding. In this dissertation, I present data using an animal model of acute social stress (*i.e*. acute social defeat) wherein I and colleagues investigated cellular mechanisms within the vmPFC that promote or impede the display of behavior that is consistent with stress resilience in humans.

According to human neural imaging studies, the vmPFC can become active in the presence of a threatening stimulus (Mobbs et al., 2007; Sinha et al., 2016; Urry et al., 2006). However, as perception of the threat's imminence increases, blood oxygenation, a commonly used proxy for neural firing, decreases within the vmPFC while increasing in autonomic centers in the brain stem that contribute to behavioral and physiological stress responses (Mobbs et al., 2007). Among these brain stem regions is the dorsal raphe nucleus (DRN), which is strongly implicated in individual differences in stress responses (Cooper et al., 2015, 2017; Maier & Watkins, 2010; Warden et al., 2012; Wood et al., 2015). This differential pattern of activity, often called 'inverse coupling', suggests a direct neural connection between regions and, in stressful conditions, has been similarly shown to exist between the vmPFC and other limbic regions as well (*e.g.* amygdala; Sinha et al., 2016; Urry et al., 2006). Collectively, these reports suggest that the vmPFC is an integral component of multiple stress-responsive circuits and, moreover, that its activity, via

neural projections to downstream regions such as the DRN, is critical for resilience to the effects of acute traumatic stress (Baratta et al., 2009; Dulka et al., 2018a; Sinha et al., 2016).

Using a well-characterized assay of the behavioral consequences of acute social stress in Syrian hamsters (*Mesocricetus auratus*) known as conditioned defeat (CD), our laboratory has shown that maintaining a dominant social status for 14 days promotes a reduced CD response relative to subordinate counterparts and dominance status controls (DSC; Morrison et al., 2011, 2014). Resistance to acute social stress therein depends on vmPFC neuronal activity, as pharmacological inhibition of the vmPFC with muscimol returns the CD response to the levels of subordinates (Morrison et al., 2013). These vmPFC neurons appear to be important regulators of stress-induced neural responses that control negative affective states given that dominants more readily recruit vmPFC efferent projections to the amygdala (Dulka et al., 2018a). Dominant animals also show reduced neural activation of DRN serotonergic cells during stress (Cooper et al., 2015, 2017). This finding is consistent with other models of stress resilience wherein vmPFC-driven inhibition of the DRN is recruited during stress (Baratta et al., 2009; Warden et al., 2012). Nevertheless, it has yet to be shown whether dominant hamsters recruit a direct vmPFC-DRN circuit during social defeat stress. Interestingly, subordinated hamsters are most vulnerable to the effects of social defeat, showing greater CD in a manner that corresponds with reduced activity in the vmPFC (Morrison et al., 2011, 2012, 2013, 2014) as well as increased DRN activity (Cooper et al., 2015, 2017) and a prolonged activation of the hypothalamic pituitary adrenal (HPA) axis (Dulka et al., 2018b).

While the mechanisms impeding vmPFC recruitment in subordinate hamsters during acute social defeat stress are unknown, we have recently shown that socially stressed

subordinates display elevated risk of oxidative stress in the brain, including the vmPFC (Dulka et al., 2017). Innate immune-induced inflammation is cyclically linked with oxidative stress and, together, the two are associated with destruction of cellular tissue and have been strongly implicated in promoting vulnerability to prolonged stress (Menard et al., 2017; Pfau & Russo, 2015; Salim, 2014). It is therefore plausible that acute social defeat may drive inflammatory processes in the vmPFC that contribute to or result from oxidative stress. Accordingly, innate immune hyperactivity may correspond with cellular and synaptic damage that disrupts input from regions upstream of the vmPFC and thus impairs vmPFC output during and/or after stress exposure.

The key aims of this dissertation are to elucidate the role of the vmPFC in conferring stress resilience and vulnerability to acute social defeat stress in Syrian hamsters. Specifically, I discuss experiments focused on direct vmPFC-DRN projections that I hypothesized would be recruited to a higher degree during stress in dominant hamsters (Chapter 2). I also discuss experiments seeking to determine whether dominant and subordinate hamsters display differential acute stress-induced cellular and synaptic degradation of vmPFC tissue in a manner dependent on neuroimmune function (Chapter 3). These findings importantly advance the field of behavioral neuroscience by strengthening our understanding of the neural correlates of stress responding, which may contribute to the advancement of therapeutic and preventative treatment regimens to promote resistance to traumatic stress.

Understanding Resilience and Vulnerability to Stress

Exposure to traumatic stress is a risk factor for an array of psychiatric conditions, though not everyone shares the same degree of stress susceptibility. For instance, while 9 in 10 individuals will experience a traumatic stressor in their lifetime, only 1 in 10 will develop posttraumatic stress disorder (PTSD; Southwick & Charney, 2018). This has prompted numerous efforts to elucidate mechanisms mediating individual differences of stress responsivity, thus permitting the development of novel therapeutic approaches to promote resilience to stress-induced pathologies on a global scale. Accordingly, several environmental factors and neurobiological mechanisms have been characterized regarding those who are more or less vulnerable to the effects of stress, especially within animal models. Importantly, the consensus from such pursuits holds that stress resiliency is characterized not by an absence of stress-derived consequences (*i.e*., alterations of physiology or behavior), but a reduction of long-term effects due to a general ability to 'bounce back' (Franklin et al., 2012; Southwick & Charney, 2018). In that regard, stress vulnerability or, as is sometimes used synonymously, susceptibility, is characterized by an exaggerated expression of inappropriate responses that can lead to persistent states of physiological and/or psychological stress (Franklin et al., 2012; Southwick & Charney, 2018).

During stressful experiences, recruitment of neural activity in the vmPFC appears key in driving resilience to the consequences of stress (Sinha et al., 2016). Our data show that resistance to the effects of acute social defeat stress in Syrian hamsters coincides with achieving a dominant social status (Morrison et al., 2012, 2014) and depends on vmPFC activity (Morrison et al., 2013), which is elevated in stress resistant, dominant hamsters (Cooper et al., 2015, 2017; Dulka et al.,

2018a). While the endogenous mechanisms mediating the differences between those more or less vulnerable to the effects of acute social stress are largely unknown, research into the brain's innate immune response to prolonged social stress suggests that inflammation-dependent disruptions of neuroanatomical integrity may contribute to stress vulnerability. This implies that discrete forms of social subordination have the ability to drive mechanisms of the innate immune system within the brain, particularly in key regulatory regions such as the vmPFC. To date, no groups, to my knowledge, have studied whether acute social defeat stress (*i.e.* < 1 day of stress) drives inflammatory activity in the vmPFC in a disruptive manner. Moreover, it is unknown whether status-dependent changes in neuroinflammation mediate the reductions of vmPFC activity found in populations vulnerable to acute stress. Inasmuch, the projects described hereafter were designed to interrogate whether increased vmPFC neuronal activity during acute social stress recruits specific downstream neural circuits implicated in stress resilience in dominant hamsters. Further, I sought to investigate whether stress-vulnerable, subordinate hamsters fail to recruit these circuits due to inflammation-based disruptions of vmPFC anatomical integrity.

The vmPFC and stress resilience

The anatomy, hodology, and function of the vmPFC

The prefrontal cortex (PFC) is an evolutionarily conserved set of (sub)regions of the vertebrate brain that has been argued to increase in relative size with both phylogenetic complexity and ontogenetic development and functions at a basic level to organize, represent,

and execute a diverse range of goal-directed behaviors (Fuster, 2001, 2008, 2015). Such behaviors are subserved by a perception-action cycle, within which the PFC is thought to regulate an organism's interactions between its internal state(s) and environment (Fuster, 2001, 2008). This is possible due to substantial neuronal input from thalamic and various cortical, subcortical, and brain stem regions which, alongside rich reciprocal efferent projections as well as dense interconnectivity within the PFC itself, permit the integration of sensory, memory, cognitive, and internal state information (*i.e.* emotional and motivational) (Nauta, 1972; Wood & Grafman, 2003). In mammals, the PFC can most simply be divided into three subregions: the orbital, medial, and lateral (Fuster, 2001), though these are often further subdivided and assigned taxonomically specific nomenclature which are, at times, inconsistent and fraught with controversy (Petrides et al., 2012; Uylings et al., 2003). While each of these broad PFC regions differentially receive and project information in a fairly topographically organized fashion, the orbital and medial regions of the PFC are distinguished by their predominating connections with other cortical and subcortical regions implicated in memory, reward, emotion, and social behavior (Fuster, 2015; Öngür & Price, 2000). This is especially true of the more evolutionarily conserved, vmPFC, which, in a largely reciprocal manner, connects heavily with the amygdala, DRN, hippocampus, nucleus accumbens (NAcc), ventral tegmental area (VTA), and locus coeruleus, among others (Gabbott et al., 2005; Hoover et al., 2007; Vertes, 2004). These connections position the vmPFC as a central processor and regulatory hub for mood and emotion in a range of contexts, affording 'top-down' control of various affective circuits (Challis & Berton, 2015; Etkin et al., 2011; Johnstone et al., 2007; Ochsner & Gross, 2005; Quirk & Beer, 2006).

In rodents, the vmPFC is often subdivided into the prelimbic (PL) and infralimbic (IL) cortices, which are believed to be homologs to Broadmann's areas 25 and 32 in the primate brain, respectively (Myers-Schulz & Koenigs, 2012). As in humans and monkeys, the rodent PL and IL contain, at times, overlapping yet often discrete efferent and afferent projections to and from various limbic regions (Hoover et al., 2007; Vertes, 2004). Long thought dissociable by their distinctive roles in mediating responses to aversive or appetitive stimuli, recent studies indicate that the IL and PL have more integrative and dynamic functions, possibly due to consequential interconnections. For instance, from their differential projections to neuronal subnuclei of the amygdala, the PL has repeatedly been shown to drive the development and expression of fear-related memories while the IL promotes the extinction of fear memories (Sierra-Mercado et al., 2011), a process argued to be integral for resilience to traumatic stress (Southwick & Charney, 2018). However, recent work from Pankaj Sah's lab shows that the PL and IL may not have such specific roles. Instead, Sah and colleagues found that the PL actually drives the IL-dependent extinction of fear memories via PL-IL glutamatergic projections, thus demonstrating that internal processing within the vmPFC can influence how the PL and IL regulate select behaviors (Marek et al., 2018a,b). While this certainly calls into question earlier findings regarding the mechanisms by which PL activity promotes stress vulnerability, it highlights the context-specificity of PL neuronal projections and underscores a critical need to further characterize the dynamic processing within the vmPFC in other stress-related contexts.

Neural activity of the vmPFC is critical for the expression of stress resilience. Human neuroimaging studies indicate that the vmPFC, at times, activates in the presence of a laboratory stressor (Mobbs et al., 2007; Sinha et al., 2016; Urry et al., 2006). Interestingly, this activation is

time-dependent and differs across individuals, with blood oxygenation gradually increasing alongside the duration of stress exposure only in those also demonstrating resilient stress-coping strategies, both outside of and within the laboratory setting (Sinha et al., 2016). For instance, greater vmPFC recruitment during an acutely stressful experience corresponds with fewer self-reports of stress-related discomfort as well as less HPA reactivity (Sinha et al., 2016). The notion that the vmPFC is necessary for adaptive functioning under duress is further supported by case studies of war- and injury-related lesions of the vmPFC wherein such patients often have difficulty properly regulating emotion and emotion-related behavior in laboratory tasks as well as their daily lives (Damasio, 2006).

In as much as can be modeled in animals, these findings are consistent with discoveries in our own laboratory. After male Syrian hamsters form dominance hierarchies in weight- and age-matched dyads in an experimentally controlled fashion, dominant animals display greater vmPFC neural activity during a social defeat stressor, as indicated by elevated expression of the immediate early gene, cFos, in both the PL and IL (Dulka et al., 2018a; Morrison et al., 2012, 2014). This vmPFC recruitment during acute stress corresponds with a reduced CD response 24 hours after an acute social defeat experience, which indicates a resistance to the adverse effects of the stressor (Morrison et al., 2012, 2014). Furthermore, we have found that dominant and subordinate hamsters display different rates of extinction of HPA reactivity following acute social defeat, wherein cortisol of subordinates returns to baseline more slowly than in dominants (Dulka et al., 2018b). Interestingly, vmPFC recruitment is necessary for the expression of resistance to CD, as pharmacological inactivation of the vmPFC via an injection of the GABA receptor agonist, muscimol, eliminates the expression of stress resistance in dominant hamsters

(Morrison et al., 2013). Thus, physiological disruption of vmPFC neural activity impedes the vmPFC-dependent circuitry necessary for stress resilience.

vmPFC Neural Activity Recruits Top-Down Regulatory Circuits

The discovery that the vmPFC plays a role in amygdala-dependent fear learning has prompted numerous studies into the mechanisms of vmPFC top-down behavioral regulation. Inasmuch, several neural imaging studies in humans indicate an inverse relationship in activity between the vmPFC the amygdala (Etkin & Wager, 2007; Johnstone et al., 2007; Rauch et al., 2006; Shin et al., 2004; Sinha et al., 2016; Urry et al., 2006). Moreover, disruptions in such inverse coupling between the vmPFC and amygdala are frequently reported in stress-related psychopathologies such as PTSD (Etkin & Wager, 2007; Rauch et al., 2006; Shin et al., 2004) and major depressive disorder (MDD; Heinz et al., 2005; Johnstone et al., 2007). In fact, it has been suggested that the efficacy of deep brain stimulation as a treatment for PTSD and treatment resistant depression lies in artificially driving neural activation of the vmPFC in a manner that corresponds with reduced amygdala activity (Marin et al., 2014; Reznikov et al., 2018). This implies that the vmPFC sends net-inhibitory neural projections to the amygdala, which may be disrupted in stress vulnerable populations.

As in humans and other primates, the rodent vmPFC sends dense glutamatergic projections throughout the forebrain, especially to limbic regions (Hoover et al., 2007; Vertes, 2004). The projections to the amygdala are well characterized, wherein vmPFC neurons synapse onto GABAergic interneurons to produce a net inhibition of the amygdala during fearful or stressful conditions (Pare et al., 2004; Urlich-Lai & Herman, 2009). Accordingly, disruptions in this

pathway have been a long-recognized factor in permitting the characteristic amygdalar hyperactivity seen in vulnerable populations during stress (Quirk & Beer, 2006; Russo et al., 2012; Sotres-Bayon & Quirk, 2010). In that regard, we too have recently shown that a vmPFC-basolateral amygdala pathway is more greatly recruited in stress-resistant, dominant male hamsters during an acute social defeat (Dulka et al., 2018a). On the other hand, their stress-vulnerable, subordinate counterparts appear to have no stress-induced recruitment of this pathway, which is consistent with reports from other animal models and humans (discussed above). Indeed, chemogenetic activation of this vmPFC-basolateral amygdala pathway during a stressful experience greatly enhances resistance to CD in subordinates and controls without dominance status. (Dulka, 2018). Thus, top down regulation from the vmPFC, particularly of the amygdala, confers resistance to the effects of stress.

Resilience and vulnerability are multifaceted constructs whose scenario-specific interpretations rely on an array of contextual factors. Inasmuch, the varied nature of the stressor itself can recruit different neural substrates in a stimulus-specific manner. For instance, years of research combined with the ease of study have distilled the fundamental processes of cued fear conditioning and extinction to dynamic actions within amygdala and PFC circuitry, though other effector regions can influence learning efficacy. However, daily experiences for most organisms are rarely so distilled and often require more complex interplay between various neural processes in order to navigate a context-appropriate behavioral response. For example, recall that greater stress-induced vmPFC recruitment occurs in a time-dependent manner though only in those also scoring highly on measures of stress resilience (Sinha et al., 2016). The researchers noticed a similar yet inverse time-dependent pattern of inactivation in the amygdala. Given that

as vmPFC time-dependently increased in activation, that the amygdala also time-dependently decreased activation supports the notion that the vmPFC inhibits the amygdala via the vmPFC-amygdala pathway. Moreover, the vmPFC-amygdala circuit is important in driving resilience to stress. That said, other regions held similar coupling patterns of inactivation. For instance, as vmPFC activity increased, activity time-dependently decreased in the brain stem as well, including the DRN (Sinha et al., 2016). A similarly graded activity pattern has been shown in vmPFC and DRN activity in a rat model of learned controllability (Amat et al., 2005). This is important given that acute stress drives DRN activity (Kollack-Walker et al., 1997), which leads to several behavioral and neuroendocrine consequences of stress (Maier et al., 1995; Graeff et al., 1996). Thus, in ethologically valid laboratory stressors that more robustly mimic those encountered in everyday life, the vmPFC may recruit multiple top-down pathways to confer resilience to stress; this includes an inhibitory vmPFC-DRN circuit.

Multiple forms of behavioral resilience to stress have been characterized to date. Importantly, while several experiences produce stress resistance via similar and/or overlapping neural mechanisms, other experiences engage separate neural circuits. For example, physical exercise and the learning of safety signals promotes resilience to acute stress, but neither appear to require vmPFC activity, as indicated by pharmacological inactivation studies (Christianson & Greenwood, 2014). In the case of safety signals, the insular cortex is instead critical for blunting the physiological and behavioral consequences of an acute tail shock (Christianson et al., 2008). Prolonged physical exercise can reduce many of the physiological and behavioral sequelae of various forms of stress, yet these effects are mediated by an exercise-dependent reduction of net DRN activity and are similarly unaffected by pharmacological inactivation of the vmPFC

(Greenwood et al., 2013). Therein, exercise drives neuroplastic changes of DRN serotonergic neurons which permit greater self-regulation via autoinhibition through serotonin 1a (5-HT1a) receptors (Greenwood et al., 2013; Christianson & Greenwood, 2014). Other forms of stress resistance similarly rely on reduced DRN activity, yet utilize glutamatergic vmPFC projections onto GABAergic DRN interneurons as a critical mediator. For instance, optogenetic stimulation of the vmPFC-DRN circuit during acute forced swim stress reduces both the latency and cumulative duration of immobility or otherwise passive swimming behaviors (Warden et al., 2012). That is, artificial activation of vmPFC terminals within the DRN mimicked the effects of various stress-relieving pharmacotherapeutics that have been demonstrated for decades (Porsolt et al., 1977; Wieland & Lucki, 1990; Can et al., 2012). Interestingly, this optogenetic-induced increase in proactive, escape-oriented behavior during the forced swim test was not seen if the vmPFC was artificially activated in a more general manner (Warden et al., 2012). That is, general activation of the vmPFC did not confer resilience to acute forced swim whereas specific recruitment of the net-inhibitory vmPFC-DRN pathway did.

That vmPFC-DRN recruitment is necessary for resistance to swim stress is mirrored in another prominent model of stress resilience, learned controllability (Maier et al., 1993; Amat et al., 2005; Maier, 2015; Maier & Seligman, 2016). Across a career exceeding 50 years, Steve Maier and colleagues have shown that when rats learn to exert control over the duration of a stressor (*i.e.* turning a wheel that ceases an acute tail shock), glutamatergic projections are preferentially recruited from the PL cortex of the vmPFC (Baratta et al., 2009). Importantly, these vmPFC projections then synapse onto GABAergic interneurons within the DRN, therefore leading to feedforward inhibition of DRN activity (Jankowski & Sesack, 2004; Baratta et al., 2009; Challis &

Berton, 2015). The vmPFC-induced activation of GABAergic microcircuits therefore leads to a reduction of numerous DRN-dependent consequences of the stress, such as helplessness (*i.e*. failure to attempt escape), loss of dominance status, anhedonia, potentiated fear conditioning, and attenuated fear extinction, among others (Maier et al., 1995; Graeff et al., 1996; Amat et al., 1998). Interestingly, while it is possible that the behavioral consequences of learned controllability could be due to a similar recruitment of multiple downstream targets of the vmPFC, the vmPFC-DRN circuit appears critical, as direct pharmacological inhibition of the DRN removes these negative consequences (Maier et al., 1993, 1995; Maier & Watkins, 2010). Moreover, Maier argues that the reduced fear conditioning and enhanced fear extinction that comes from learned controllability is achieved not by recruiting the vmPFC-amygdala pathway directly (though this is acknowledged as a possibility), but by indirect inhibition of amygdala by way of a vmPFC-DRN-amygdala circuit during times of stress (Maier & Watkins, 2010; Maier, 2015; Maier & Seligman, 2016). Taken together, several forms of behavioral resilience are mediated by a vmPFC-DRN circuit that drives DRN inhibition.

Recall that during an acute social defeat, stress-resistant, dominant hamsters recruit greater activity of vmPFC neurons (Morrison et al., 2011, 2012, 2014), some of which (roughly 10%) are vmPFC-amygdala projecting (Dulka et al., 2018a). On the other hand, stress-vulnerable, subordinate counterparts recruit little, if any, of these cells. It is interesting to note that within the projection region of most vmPFC-amygdala terminals (basolateral and central subnuclei of the amygdala), we have not found clear status-dependent differences of neural activation (*i.e.* cFos expression), despite having looked frequently (Cooper et al., 2015; Morrison et al., 2012, 2014). That is, if vmPFC-amygdala projections were solely responsible for conferring the

dominance-dependent resistance to the effects of acute social defeat stress (*i.e*. reduced CD, reduced HPA reactivity), this should be reflected in clearly differentiable levels of amygdala activity (*i.e.* dominants should have fewer stress-activated amygdala neurons than subordinates). However, we have repeatedly shown social status-dependent differences in DRN activity following multiple laboratory stressors, which mimic vmPFC activation patterns and human neural imaging data regarding patterns of stress resilience. Following a social defeat stressor (Cooper et al., 2015) or physical restraint (Cooper et al., 2017), dominant hamsters display reduced neural activation of DRN neurons when compared to subordinate counterparts as indicated by cFos expression. Thus, experiments of this dissertation were designed, in part, to determine if resistance to CD in dominant hamsters is specifically linked to a vmPFC-DRN pathway, thus extending our knowledge of the cortical circuits that support resistance to social stress (see Chapter 2).

The vmPFC and stress vulnerability

Neural activity of the vmPFC is critical for the expression of resilience in both pre-clinical models and humans. In that regard, stress vulnerability is often promoted by inactivation of the vmPFC, whether by artificial, experimental manipulations or through natural, endogenous processes. In our own hands, we have shown a gradual development of stress vulnerability in subordinate animals, which suggests that chronic subordination (Cooper et al., 2015; Morrison et al., 2014) might drive mechanisms which impede vmPFC function. The sources for vmPFC impedance can be categorized into three main possibilities. First, effectors upstream of the vmPFC may inhibit recruitment of otherwise normally functioning vmPFC neurons. For instance,

catecholaminergic inputs have the potential to inhibit vmPFC activity. For example, VTA terminals releasing dopamine within the vmPFC may activate inhibitory D2 receptors acting as autoreceptors on VTA terminals, inhibitory D2 postsynaptic receptors on pyramidal neurons, or excitatory D1 receptors on GABAergic terminals within the vmPFC, thereby suppressing their action in a context-specific manner (Vander Weele et al., 2018). Second, various intracellular mechanisms within vmPFC neurons might be up- or down-regulated in a manner that alters neuronal responsiveness during stress. For instance, experiential factors prior to stressor exposure could induce changes in protein expression patterns via epigenetic, transcriptomic, or post-translational modifications, among others. Third, recruitment of vmPFC neurons could be impeded by extracellularly derived disruptions of the structural integrity of vmPFC tissue, which might limit top-down control of limbic and hindbrain structures by impinging on synaptic transmission. For instance, typically neurosupportive glial cells can, in some extreme conditions, endanger natural function through hyperproduction of pro-inflammatory cytokines and, indirectly, increased oxidative stress. Traditionally, this latter possibility would be discarded as impertinent to acute stress conditions, in large part due to a traditional view that only chronically administered stressors drive excessive neuroinflammatory processes and, instead, acute stressors suppress immune activity. That said, recent discoveries regarding the reactivity of the innate immune system, including the brain's resident immune cells, microglia, call this view into question. Thusly, experiments of this dissertation were designed, in part, to investigate this third potential source of disrupted vmPFC functionality following acute stress. More specifically, I sought to determine whether elevated activity of the innate immune system in subordinate

animals corresponds with neural, glial, and/or synaptic degradation within the vmPFC and promotes vulnerability to acute stress (Chapter 3).

The vmPFC as a vulnerable target of neuroinflammation-induced impairment in acute stress

Mounting evidence suggests that immune system activity in the brain, *i.e.* neuroinflammation, may play a critical role in stress vulnerability (Wood & Bhatnagar, 2015; Wood et al., 2015; Menard et al., 2017). In that regard, many stress-related psychopathologies are characterized by systemic inflammation, for example MDD and PTSD (Friedrich, 2014) and some anti-inflammatory treatments have recently been shown to reduce negative emotional states (Hinwood et al., 2013; Hurley et al., 2014). Furthermore, an artificially induced inflammatory response in healthy subjects leads to psychopathology-like psychological affect (*e.g.,* depression, malaise, and social withdrawal; Dantzer, 2001; Haroon et al., 2012). Moreover, these symptoms can be reduced with administration of commonly prescribed antidepressants (Avitsur et al., 2017; Liu et al., 2011; Maciel et al., 2013), thus providing further support that inflammation and emotional dysfunction are cyclically linked. Taken together, these findings imply that immune responses to psychological stress may underlie subsequent emotional and behavioral impairments and hyperactive innate immune function in the brain can be a key component in the development of stress vulnerability (Wood et al., 2015).

In animal models of stress, especially in cases of prolonged stress exposure, the morphology of vmPFC cells is particularly sensitive to stress-induced perturbations. It has long been known that prolonged restraint stress leads to shortened neuronal dendrites within the vmPFC, especially of more superficial cortical layers (*i.e.* layers II/III; Cook & Wellman, 2004;

Radley et al., 2004). Similarly, I have shown that pharmacological buffering of prolonged physical restraint coincides with the protection of synaptic densities in the vmPFC of C57 mice, as indicated by increased immunolabeling of the presynaptic marker, synaptophysin (Grizzell et al., 2014) and postsynaptic marker, PSD-95 (Patel et al., 2014). Although less frequently reported, studies have also shown that acute stressors, such as acute tail shock or forced swim, reduce dendritic length of vmPFC neurons (Izquierdo et al., 2006; Nava et al., 2017). These findings support the notion that the integrity of grey matter in the vmPFC is sensitive to acute stress and that stress-induced changes occur more rapidly than in other stress-sensitive regions, including hippocampus (Arnsten, 2009). Therein, duration and stressor intensity appear to be factors in such disruptions of structural integrity (Izquierdo et al., 2006; Arnsten, 2009).

Models using more ethologically relevant stressors, such as predator exposure and socially derived stress, have also shown robust changes in vmPFC neuronal morphology. In a chronic social defeat paradigm, those mice more susceptible to the effects of social stress had shorter vmPFC dendrites than those considered stress resilient (Shinohara et al., 2018). Moreover, the authors suggested that these effects were brought on by stress-induced inflammation. In another study, vmPFC synaptophysin and PSD-95 levels were reduced when acute predator exposure stress was coupled with prolonged social disruption stress (Ogundele et al., 2017). Inflammatory activity was again implicated as stress-exposed rats displayed elevation in CD11b expression, a cell-surface marker of proinflammatory macrophagic cells including microglia and peripherally derived monocytes (Fischer & Reichmann, 2001; Duan et al., 2016).

Many additional studies suggest neurodegenerative consequences of interactions between social stress and the innate immune system, as many others have posited that

neuroinflammation can drive detrimental changes through a wide array of cellular mechanisms (Hodes et al., 2014; Wood et al., 2015; Menard et al., 2017; Nie et al., 2018). For instance, social stressors increase the risk of oxidative stress in the vmPFC (Zlatkovic et al., 2014; Herbet et al., 2017; Solanki et al., 2017). Furthermore, oxidative stress may influence the positioning of social rank within the dominance hierarchy (Hollis et al., 2015) and socially subordinate hamsters who have been subjected to acute social defeat stress display increased risk of oxidative stress in the vmPFC as well as nucleus accumbens (Dulka et al., 2017).

Oxidative stress is caused by excessive levels of unstable radicals (*i.e.* any chemical species containing unpaired electrons) also called reactive oxygen species (ROS), which can rapidly and fairly indiscriminately alter proximal molecular structures through chained reduction-oxidation (redox) reactions (Betteridge, 2000). While ROS production is a regular consequence of numerous cellular processes, such as aerobic and anaerobic metabolism, potentially disruptive electron transference to nearby molecules is often buffered by anti-oxidative defense mechanisms. These so-called antioxidants constitute any chemical species that reduces the rate of oxidation of a substrate by terminating chained redox reactions (Gutteridge, 1995). In normal conditions, antioxidant levels exceed those of naturally produced ROS to suppress redox reactions and maintain physiological equilibrium. However, various perturbations can result when ROS rise to levels beyond the capabilities of antioxidant defenses. Unchecked ROS production leads to near-instantaneous chain reactions that can weaken or break chemical bonds and thus alter confirmation and function of many biomolecules, including DNA, protein, and lipid-rich cellular membranes (Alberts et al., 2007). In extreme cases, imbalanced ROS production results in catastrophic cellular damage or death (Gutteridge, 1995). As referenced above, ROS

are most often produced during energy consumption within mitochondria, but elevations of ROS are also associated with degradative processes such as catabolism, apoptosis, synaptic pruning, and pathogen or debris clearance (Yost & Fridovich, 1974; Halliwell & Gutteridge, 1985; Gotz et al., 1994; Carmody & Cotter, 2001), though whether these processes cause or result from ROS is incompletely understood. Indeed, due to the instability of ROS and the rapidity of redox reactions, it is difficult to assess ROS generation or activity directly, especially in behaving animals. On the other hand, given the highly destructive nature of ROS, indirect measurements of oxidative stress are more readily available, such as assays of proinflammatory activity or cellular and synaptic degeneration and debris deposition.

Importantly, whether tissue damage results from or drives ROS elevations (or both), the degenerative fallout coincides with proinflammatory activity of myeloid-derived cells of the innate immune system. Within the brain, this most heavily implicates microglia, though peripherally derived monocytes may also infiltrate the blood brain barrier in extreme or prolonged inflammatory conditions. Indeed, ROS activity throughout the brain can promote but also result from microglial activity (Bordt & Poster, 2014), which can be inferred from morphometric analyses of microglial cells or from assessments of production/release of proinflammatory signaling molecules called cytokines.

Microglial proinflammatory activity in the vmPFC has been implicated in numerous models of psychological stress vulnerability. For example, researchers have recently shown that microglia were more active following prolonged restraint stress and, moreover, were responsible for much of the behavioral and neural consequences of restraint (*e.g.* stress-induced disruptions of working memory and reduced vmPFC recruitment; Hinwood et al., 2012, 2013). When treated

with minocycline, an antibiotic that inhibits proinflammatory activity of microglia and monocytes, rats subjected to prolonged restraint stress displayed fewer indices of stress-induced disruption. This suggests that microglial/monocyte proinflammatory activition during stress directly contributes to stress-induced impairments. More recent findings using genetic disruption of microglia in chronically defeated mice support the hypothesis that innate immune activity in the brain contribute to disruptions of structural integrity (*i.e.* dendritic retraction) and that behavioral responses to stress (*i.e.* social avoidance) depend on recruitment of proinflammatory microglial activity (Nie et al., 2018). Indeed, increased activity of microglia in various subregions of the human PFC has been linked to mental illness and suicide (Schnieder et al., 2014; Steiner et al., 2008). Thus, adverse life experiences, such as social subordination and/or traumatic stress, may drive inflammatory processes and ROS production in the brain, which together can disrupt vmPFC anatomical integrity and reduce the capacity for stress resilience.

Microglial expression, structure, and function in normal and stressed states

Although initially studied in the contexts of neural development and infectious disease, appreciation for microglial function has exponentially grown in recent years to include investigations of innumerable brain-related dysfunctions, especially in models of neurodegeneration and various psychopathologies. As the resident immune cells of the central nervous system, microglia have been argued to maintain a quiescent immune-related functional status in healthy conditions, in essence quietly surveilling surrounding tissue for threat of intruding pathogens. With the use of pattern recognition receptors (PRRs), microglia readily detect invading microbes due to their expression of pathogen associated molecular patterns

(PAMPs; MacPherson & Austyn, 2012). Recent studies confirm early theories (Matzinger, 1994) that even in sterile conditions, select endogenous molecules (*e.g.* $HMGB_1$ and ATP) serve as signals of tissue damage and bind to PRRs when released extracellularly (or leaked during lysis), even in sterile conditions (Lu et al., 2014; Feldman et al., 2015; Venereau et al., 2015). These aptly named damage- (also called danger-) associated molecular patterns (DAMPs) are like PAMPs in many respects in that they also drive microligal recruitment, surveillance, and activity. Once PRRs bind PAMPs or DAMPs, microglia secrete various inflammation-related signaling molecules called cytokines that can trigger a vast array of processes within the brain in a stimulus-, region-, and cell type-dependent manner. As referenced above, nitrous oxide and, at times, various ROS are also produced alongside proinflammatory cytokines and each of these has the capacity to recruit additional microglia to the affected area to target the signaled insult for destruction and clearance through phagocytosis and ROS- or enzyme-induced denaturation. Together, these are also destructive of surrounding molecules when unchecked by anti-inflammatory or antioxidative defense, thus triggering additional release of DAMPs. Cyclically, DAMP binding itself can further drive neurodegeneration (Thundyil & Lim, 2015), even if localized to synaptic components (*i.e.* dendrites and terminals; Fujita et al., 2016). Taken together, these well-defined principles form the basis for an array of theories implicating inflammatory mechanisms in the etiology of a wide variety of diseased states.

In most cases, microglial recruitment facilitates efficient clearance of any pathogen or endogenous threat as well as any cellular debris left in their wake, which is then balanced by a return to immunological quiescence via self-regulatory, anti-inflammatory cytokine release mechanisms. However, in the continued presence of PAMPs, DAMPs, ROS, and/or

proinflammatory cytokines, microglia may progress through quantifiable, dynamic stages that coincide with separable structural and functional consequences (see Figure 1.1 of Appendix A; Hinwood et al., 2012, 2013; Nie et al., 2018). While the ionized calcium binding adaptor protein-1 (Iba1) is expressed in all four of these states, it is substantially upregulated once the microglia progresses to and beyond the second stage, often referred to as a "hyper-ramified" state due to increased branching of microglial extensions. In contrast, microglia at the first stage have very little Iba1 expression and, as referenced above, have sometimes been referred to as "quiescent" due to the arguably fallacious presumption that no immune system functions occur beyond basic surveillance (Sierra et al., 2014). However, upon PRR binding and subsequent progression to the second state or further, Iba1 is upregulated, proinflammatory cytokines are released, and the microglial branches proliferate to greatly expand their surveillance capabilities. If the threat persists (*i.e.* continued PRR binding), microglia then progress toward a third "reactive" state, which consists of shorter cellular branches, a slightly enlarged and often-spheroid soma, continued hyperexpression of Iba1, proinflammatory cytokine release, ROS production, and increased likelihood of phagocytic activity through engulfment of extracellular contents via now-thickening processes as the membrane retracts toward the soma. In extreme conditions (*i.e.* traumatic brain injury or stroke), microglia then progress toward a final state aptly termed "phagocytic", which is marked by increasing expression of protein CD-68 alongside Iba1. In this fourth state, the cellular processes fully retract yielding a greatly enlarged soma that can more efficiently phagocytize larger quantities of potential threats or cellular debris. Interestingly in hippocampal culture, microglia displaying spheroid morphology can be found engulfing neuronal somas just before "spontaneous" death, with fully amoeboid arriving minutes later for cell debris

clearance (Peterson & Daily, 2004). In that regard, changes in microglial morphology are largely indicative of the presence of proinflammatory activity (see Figure 1.1 in Appendix A; Alberts et al., 2007; Tremblay et al., 2011; MacPherson & Austyn, 2012; Hinwood et al., 2012, 2013; Perry & Teeling, 2013; Nie et al., 2018).

While anti-inflammatory cytokines mitigate the effects of microglial-implicated destruction of tissue in healthy states, in diseased or aversive situations, microglia may remain in a proinflammatory state even after the initial insult has been neutralized. Through continued proinflammatory cytokine and ROS production (and thus, PRR binding), microglia thus continue promoting oxidative stress and further microglial recruitment, including in earlier "hyper-ramified" inflammatory states (Bisht et al., 2016). This inflammatory response proves problematic for nearby healthy brain tissue. For instance, whilst targeting cellular debris or damaged cells, phagocytic microglia can also engulf and endanger components of healthy neural and glial cells (*e.g.* astrocytes), which can lead to fewer synapses, reduced dendritic length, and even cellular death (Tremblay & Majewska, 2011; Tremblay et al., 2011). Furthermore, the possibility of an arrested proinflammatory state has been suggested wherein microglia cannot leave the hyper-ramified stage, both structurally and functionally (Hinwood et al., 2013; Streit et al., 1999). While such arrested states are uncommonly reported, they are argued to occur in aging (Streit et al., 1999) and continued psychological stress exposure (Hinwood et al., 2013), among others. Notably, both of these conditions are widely associated with persistent inflammatory activity and damaged neural tissue, particularly in crucial brain regions implicated in stress resilience such as the vmPFC. Thus, highly salient psychological stressors may contribute to microglial activity within the vmPFC, which results in disruptions to anatomical integrity via non-

selective phagocytosis and ROS production. Together, these increase the probability of stress-induced cellular and synaptic degradation which can impede vmPFC regulatory firing within limbic circuits.

Reduced neural inflammation in stress resilience

Various animal models have been introduced in recent decades providing valuable insight into the mechanisms promoting stress resilience. Some of these models elucidate the complex relationship between stress and inflammation in a manner suggesting that resilience is associated with reduced immune system activity following stress. For instance, animals responding to repeated stress in a proactive manner display fewer cytokines both in the periphery and brain (Wood & Bhatnagar, 2015; Wood et al., 2015). Moreover, experimentally halting the inflammatory profile of chronically defeated mice yields a behavioral display indistinct from those of resilient mice (*e.g.* social avoidance; Hodes et al., 2014; Nie et al., 2018). As mentioned above, pharmacological (Hinwood et al., 2012) or genetic inactivation (Nie et al., 2018) of microglia promotes fewer behavioral patterns of stress-induced impairments such as poor memory performance and social avoidance, respectively. Thus, reduced inflammatory activity following prolonged stress yields a resilient-like behavioral phenotype.

With this said, the vast majority of these studies investigate prolonged stress conditions. In fact, very little is known regarding the relationship between acutely traumatic stressors and neuroinflammatory processes. This is problematic given that despite the fact that both PTSD and MDD share similar increases in inflammatory profiles, they have very different etiologies and often require separable approaches to investigate relevant mechanisms via acute or chronic

stress exposure, respectively. Clearly, the immune system is disrupted in conditions stemming from acutely traumatic stressors, yet few researchers have reported on the relationships between acute stress and neuroinflammation. One possibility for this omission is the limited strength of available models of acute traumatic stress, especially when seeking to compare resilient and susceptible populations. On the other hand, some dismiss the utility of acute models, including them only as a control for chronic models or arguing their inclusion as altogether irrelevant for a discussion of inflammation and stress-linked psychopathology. Inasmuch, a model of individual differences to acutely traumatic stress would provide a novel opportunity to elucidate the role of neuroinflammation in stress susceptibility.

Some research groups have tried to bridge the gap in knowledge regarding acute vs. chronic stress-induced immune activity without fully dismissing acute stress-induced changes in neuroinflammation. In the "learned helplessness" model (introduced above), a subset of animals are trained to turn a lever to eliminate or "escape" an acute tail shock (Maier & Seligman, 2016). At the same time, another subset of yoked animals receive identical amounts of shock but are not given the opportunity to act on their environment to cease the stressful experience. Thus, these animals are assigned to the "inescapable" condition. Numerous studies using this model have shown that animals exposed to escapable stress respond to various subsequent stressors with reduced behavioral and physiological consequences when compared to inescapable counterparts. With regard to inflammation though, researchers were unable to detect differences between these two groups in Iba-1 expression of hippocampal microglia, suggesting that microglia play no role in the stress resistance shown by animals exposed to escapable tailshock (Frank et al., 2007). However, acute tail shock did *prime* subsequent microglial

activation following an immune system challenge 24 hours after the stress equally in both groups [*i.e.* systemic exposure to the endotoxin, lipopolysaccharide (LPS)]. On the other hand, others have shown that a high intensity, acute water immersion stress does indeed drive microglial activation in the brain stem (Sugama et al., 2009). However, this model did not afford an opportunity to determine if stress resilient phenotypes display reductions of microglial activity. Moreover, none of these studies investigated microglial activity in the vmPFC, and therefore do not provide any insight into whether traumatic stress may drive neuroinflammatory processes that disrupt vmPFC anatomical integrity in a manner that confers stress vulnerability.

Conclusion to Introduction

Use of an acute social defeat model in Syrian hamsters permits interrogation of individual differences in the response of the vmPFC to acute traumatic stress. Inasmuch, the experiments that are described in the upcoming chapters will address whether the vmPFC-DRN circuit is recruited in stress resilience and whether stress and neuroinflammation interact to increase degredation of vmPFC, particularly in stress vulnerable populations, in a manner that might impede this and other critical downstream vmPFC projections.

Overview and Aims of Conducted Experiments

In the experiments that follow, I sought to test the following *overarching hypothesis:* Increased vmPFC neuronal activity during acute stress in dominant male hamsters recruits a vmPFC-DRN circuit, which has been implicated in promoting acute stress resilience in multiple laboratory stress paradigms. Further, subordinate male hamsters fail to recruit this circuit, show

inflammation-dependent degradation of vmPFC tissue, and display increased anxiety-like behavior in social contexts following social defeat stress.

Specific Aim 1 (Chapter 2)

The goal of this aim was to determine if dominant male hamsters recruit a direct vmPFC-DRN circuit during acute social defeat stress. By stereotaxically injecting a retrogradely transported neural tracer [cholera toxin B, (CTB)] directly into DRN, I identified immunolabeled CTB-positive vmPFC neurons in dominant, subordinate, dominance status control (DSC), and no-stress control hamsters. I then compared colocalization of CTB and the defeat-induced expression of the immediate early gene cFos within vmPFC-containing tissue. I predicted that dominant hamsters would show greater defeat-induced neural activity within a vmPFC-DRN pathway compared to subordinates and controls and, further, that subordinate hamsters would show less activity within this pathway than DSCs (see Chapter 2).

Specific Aim 2 (Chapter 3a)

The goal of this aim was to determine if acute social defeat stress alters microglial/monocyte-specific protein expression and morphology in a manner suggesting that acute stress-induced proinflammatory activities occur within the vmPFC. This aim was addressed in three of four experiments presented in Chapter 3.

Experiment 1 Acutely defeated male hamsters and no-stress controls were euthanized 28-hours after stress (or stress control) procedures. The goal of this experiment was to determine if acute social defeat stress altered the structure and increased the immunolabeling within vmPFC

of the proteins *ionized calcium binding adaptor protein-1* (Iba1) and *cluster of differentiation 68* (CD68), indicators of microglial proinflammatory cytokine release and phagocytic activity, respectively. This would therefore demonstrate that acute social defeat induces functional and structural changes to macrophagic cells within vmPFC tissue consistent with proinflammatory activity. Because recent reports suggest acute foot-shock stress does not drive transcription of these proteins directly, but does sensitize their future expression following a subsequent immune challenge (Frank et al., 2007), all hamsters in Experiment 1 were also systemically injected 24-hours after stress (4-hours prior to euthanasia) with either saline or progressive doses of LPS, a component of Gram negative bacteria known to elicit system-wide inflammatory processes. I predicted that social defeat would interact with LPS to dose-dependently increase microglial/monocytic activity as indicated by elevated Iba1 and CD68 expression and morphological changes reflective of progression to phagocytic states.

Experiment 2 Acutely defeated hamsters were left undisturbed for 7 days before immunohistochemical quantification of microglial/monocytic expression of Iba1 and CD68 in the vmPFC. The goal of this experiment was to determine whether any acute stress-induced changes in macrophagic cells persisted for 1 week. I predicted that some, but not all, changes in microglial/monocytic expression patterns would persist for at least 1 week following stress.

Experiment 3 Hamsters were treated for 5 days with water or the microglial/monocyte inhibitor, *minocycline*, and then exposed to social defeat stress and tested for the CD response. Upon euthanasia (48-hours after stress), immunohistochemical labeling of Iba1, CD68, synaptophysin, and aminocupric binding in vmPFC-containing tissue were quantified. The goal of this experiment was to determine whether minocycline-sensitive mechanisms (*i.e.* phagocytosis

and proinflammation) contributed to decreased synaptic densities (*i.e.* reduced synaptophysin levels) and increased deposition of cellular debris (*i.e.* increased aminocupric binding) within the vmPFC in a manner that corresponds with changes in social anxiety behaviors. I predicted that minocycline treatment would reduce the expression of social avoidance as assayed in the CD response as well as increase the time spent investigating a novel conspecific in a social interaction test (SIT). Furthermore, I predicted that these behavioral changes would correspond with reduced vmPFC Iba1 and CD68 expression alongside fewer changes in cellular morphology that are collectively reflective of proinflammatory and phagocytic activities. Finally, I predicted that any markers of cellular or synaptic degeneration brought on by social defeat stress would be protected with minocycline treatment.

Specific Aim 3 (Chapter 3b)

The goal of this aim (one experiment) was to determine if subordinate male hamsters show elevated microglial/monocytic proinflammatory activity and markers of structural degradation of the vmPFC before or after acute social defeat stress. Dominant and subordinate male hamsters received social defeat stress or not (4 groups total) and the immunohistochemical analyses of anatomical integrity and inflammatory activity in the vmPFC were quantified using parameters identical to those described in Experiment 3 of Specific Aim 2 (above). Briefly, I sought to assess the following immunolabeled markers: Iba1 to determine microglia/monocyte activity levels and morphology; CD68 to determine active phagocytosis at euthanasia; aminocupric staining to determine degree of neurodegeneration; and synaptophysin to determine synaptic density. I also conducted CD and SIT testing in a counterbalanced order to

determine whether any markers correlated with social avoidance in each tests. I predicted that subordinate hamsters, whether socially defeated or not, would display increased social avoidance in both CD and SIT tests alongside increased Iba1, CD68, and aminocupric staining and reduced synaptophysin staining within the vmPFC when compared with stressed and unstressed dominants (Chapter 3). Importantly, water-drinking animals from Experiment 3 of Aim 2 were run concurrently with those from Aim 3 and, accordingly, were used as DSC and no-stress controls for all comparisons in Aim 3.

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Appendix A

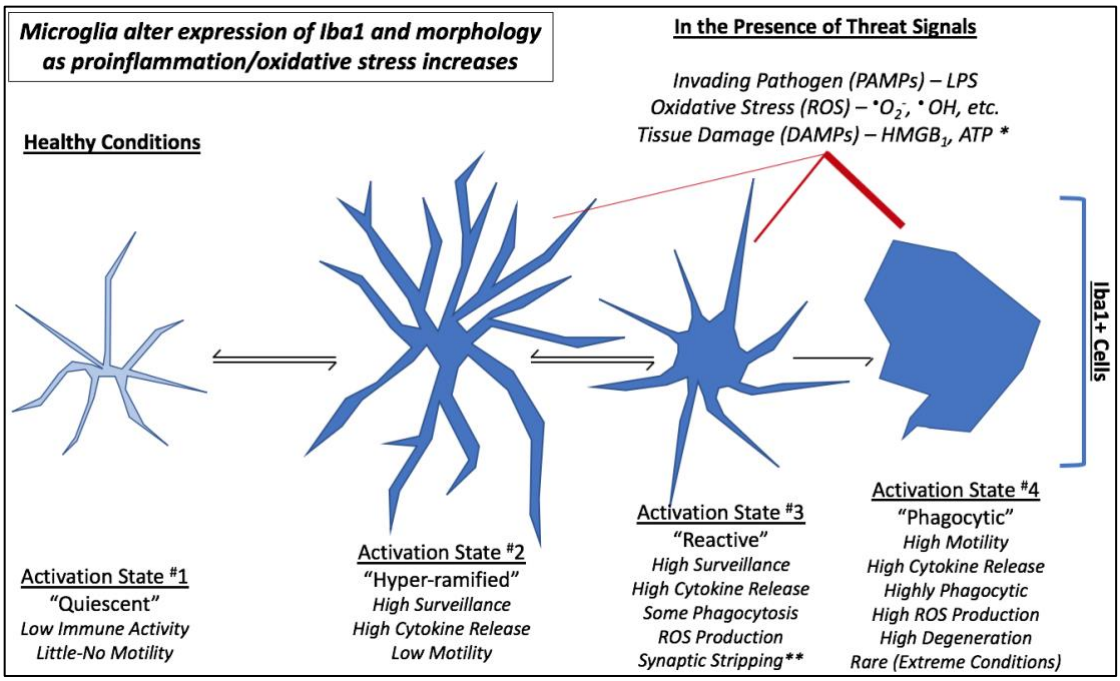

Figure 1.1. Microglial Activation States Correspond with Inflammatory Activity and Potential for Tissue Damage. Under progressively threatening conditions, microglia alter expression of *ionized calcium binding adaptor molecule 1* (Iba1) alongside changes in cell morphology across four activation states that are indicative of overlapping yet distinct proinflammatory profiles. Under healthy conditions (left, Activation State #1), microglia express low levels of Iba1, are relatively immobile within brain tissue, and engage in few, if any, proinflammatory activities such as cytokine release, reactive oxygen species (ROS) production, or phagocytosis. However, microglia progressively change their proinflammatory activity (Activation States #2-4) in the presence of molecular signaling that indicates potential threat. Invasive pathogens express molecular patterns (pathogen associated molecular patterns, PAMPs) which bind to specialized receptors which recognize these patterns (pattern recognition receptors, PRRs). Hyperproduction of ROS leads to damage of surrounding tissue and the subsequent release of endogenous molecules which are similarly recognized by PRRs. Together, these damage-associated molecular patterns (DAMPs) and PAMPs trigger the release of proinflammatory signals, cytokines, from Iba1-expressing cells which recruit additional Iba1 cells to the affected area. In the 2nd activation state, Iba1 expression increases as branching increases to facilitate greater surveillance of the environment. If threat signals persist or increase, Iba1-expressing cells progress to the 3rd activation state, where processes begin to retract inward to facilitate ROS production and phagocytosis. In extreme conditions, such as illness, stroke, and traumatic brain injury, Iba1 expressing cells can adopt a fully amoeboid appearance as branching fully retracts. In this confirmation, macrophagic activity is at its highest, alongside continued ROS and cytokine production. It is believed that microglia can freely alter their activation states in this linear fashion, moving back and forth among proinflammatory profiles given the concentration of threat signals in the extracellular milieu (with the exception of those entering the 4th activation state; black arrows). Red lines = relative concentration of threat signals.

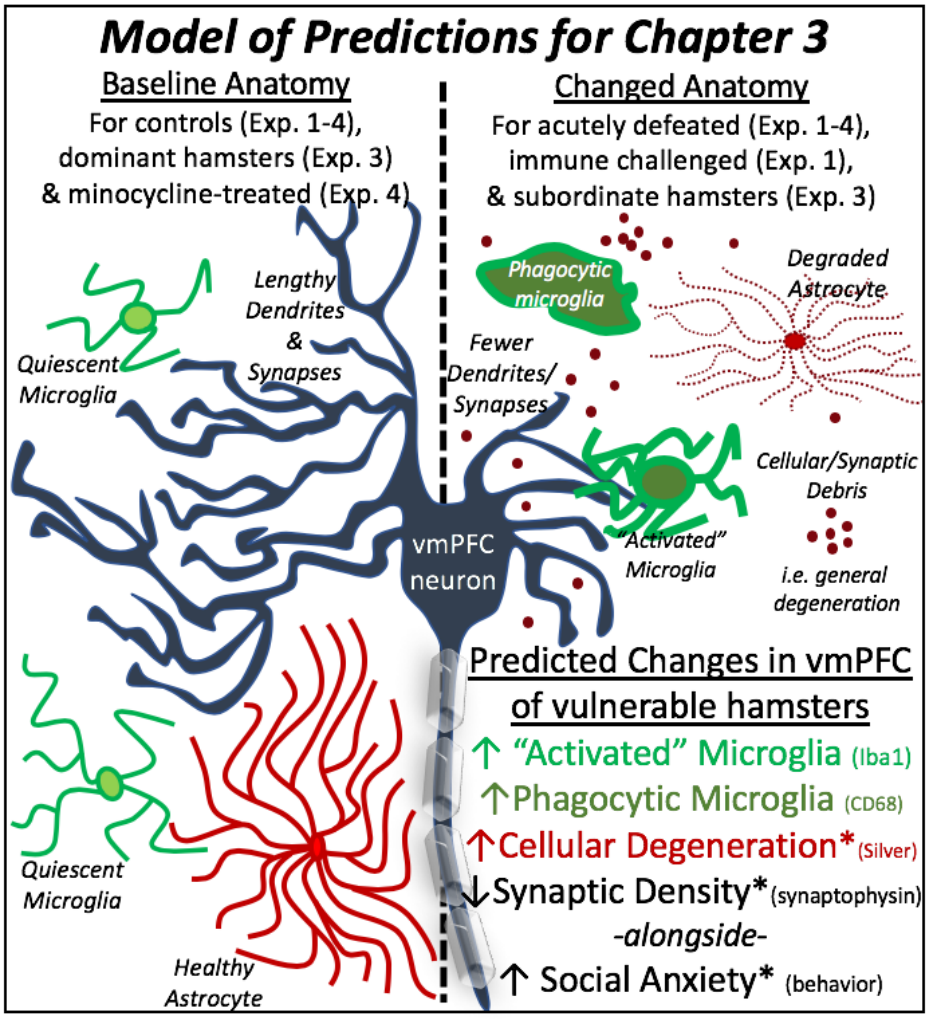

Figure 1.2. Model of Predicted Changes Across All Experiments from Chapter 3. Under basal conditions (left), neuronal dendrites are lengthy and numerous leading to high synaptic densities (indicated by high synaptophysin immunolabeling). Iba1-expressing cells remain in a low proinflammatory profile (see Fig. 1.1) and signals of cellular and synaptic debris remain low (indicated by low aminocupric, or "silver" immunolabeling). I have predicted that animals in the following conditions will reflect these anatomical signatures: all no-stress controls (Experiments 1-4) with the exception of no-stress subordinates (Exp. 3) and minocycline-treated animals regardless of stress (Exp. 4). I have also predicted that acute social defeat stress (Exp. 1-4) or subordination (Exp. 3) will result in increased signatures of degraded structural integrity. This includes decreased immunolabeling of synaptophysin and increased immunolabeling of Iba1, CD68, and silver deposition, which indicate lower synaptic densities and higher proinflammatory activity, phagocytosis, and cellular debris deposition, respectively. I also predicted that morphometric analyses of Iba1+ cells would suggest increased activation states only for those in the "changed anatomy" category (not depicted). Finally, I predicted that those in the "changed anatomy" category would display elevations of social anxiety. * = behavior and silver and synaptophysin stains were only conducted in Exp. 3 and 4.

Chapter 2:

Activity of a vmPFC-DRN Neural Circuit Corresponds with Resistance to Acute Social Defeat Stress

This chapter is adapted for dissertation formatting from the following publication submission:

Grizzell, J. A., Clarity, T. T., Graham, N. B., Dulka, B. N., & Cooper, M. A. (2019). Activity of a vmPFC-DRN neural circuit corresponds with resistance to acute social defeat stress. *Frontiers of Neural Circuitry.* (submitted November, 2019)

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My primary contributions to this paper include: (i) designing experiments, (ii) piloting experiments, (iii) conducting experiments, (iv) analyzing and interpreting data, (v) researching background information, (vi) writing the manuscript.

Abstract

The ventromedial prefrontal cortex (vmPFC) plays a critical role in stress resilience through top-down inhibition of key stress-activated limbic and hindbrain structures, including the dorsal raphe nucleus (DRN). In a model of experience-dependent stress resistance developed in our laboratory, socially dominant Syrian hamsters display fewer signs of distress following an acute social defeat stressor when compared to subordinate or control counterparts. Further, dominants activate vmPFC neurons to a greater degree during stress than subordinates and, moreover, become stress-vulnerable following pharmacological inhibition of the vmPFC. In addition, dominants display fewer stress-activated DRN neurons than subordinates, suggesting they engage an inhibitory vmPFC-DRN pathway during social defeat stress while subordinates do not. To test this hypothesis, we stereotaxically injected the retrograde tracer, Cholera Toxin B

(CTB), into the DRN of dominant, subordinate, and control hamsters and used a dual-labeling immunohistochemical approach to identify vmPFC neurons co-labeled with CTB and the defeat-induced expression of the immediate early gene, cFos. Results indicate that dominant hamsters display more cFos and cFos + CTB labeled cells in both the infralimbic (IL) and prelimbic (PL) subregions of the vmPFC compared to other animals. These findings suggest that the reduced behavioral consequences of acute social defeat in dominant hamsters correspond with recruitment of a vmPFC-DRN pathway during stress. Overall, this study provides evidence for the experience-dependent engagement of a monosynaptic vmPFC-DRN circuit, which has clinical implications for improving therapeutic interventions across various stress-related psychopathologies such as post-traumatic stress disorder and major depressive disorder.

Introduction

While exposure to traumatic psychological stress is a key risk factor for the development of various psychopathologies such as post-traumatic stress disorder (PTSD) and major depressive disorder (MDD), resilience to stress appears to be the rule rather than the exception. For example, of the 9 in 10 individuals estimated to encounter a traumatic stressor during their lifetime, only about 1 in 10 develop PTSD (Southwick & Charney, 2012). Accumulating evidence confirms that stress resilience can be learned, thereby implicating experience-dependent changes within the brain, which might be therapeutically recruited to promote resistance to stress-related impairments. This realization has encouraged numerous research efforts focused on elucidating the relationship between brain regions that underlie individual differences in

stress processing, for example the ventromedial aspect of the prefrontal cortex (vmPFC) and the dorsal raphe nuclei (DRN).

During acutely stressful experiences, vmPFC neurons are more greatly active in stress-resilient humans (Sinha et al., 2016) and non-human animals (Baratta et al., 2009; Morrison et al., 2014). In contrast, reduced activity of the vmPFC during stress corresponds with greater stress vulnerability. Similarly, several acutely administered laboratory procedures argued to model traumatic stress experiences drive neural activation of serotonin cells in the DRN, which positively correlates with stress susceptibility (Amat et al., 2005; Paul et al., 2011; Cooper et al., 2017). Taken together, these findings indicate that an inverse relationship between vmPFC and DRN activity coincides with resistance to the adverse effects of acute traumatic stress.

The DRN receives substantial glutamatergic projections from the vmPFC, which preferentially synapse onto inhibitory GABAergic microcircuits (Challis et al., 2014). In that regard, recruitment of this pathway during adversity is thought to promote resistance by suppressing stress-induced serotonergic activity, particularly during acutely traumatic experiences. For instance, in a model of learned controllability, rats who learn to exert control over the duration of an electric shock more readily recruit vmPFC-DRN projections when compared with those not having learned control (Baratta et al., 2009, 2019). Importantly, learning control in this model coincides with reduced consequences of uncontrollable stress, such as learned helplessness, downward mobility of social dominance, decreased aggression, anhedonia, neophobia, and various impairments of fear learning, among others (Maier & Seligman, 2016). Furthermore, learning control produces resilience toward future stressors that occur in the absence of control (Amat et al., 2008; Baratta et al., 2019). In another model,

optogenetic stimulation of the vmPFC-DRN circuit during an acute forced swim stressor promotes proactive coping responses (Warden et al., 2012), which have been argued to be a marker of stress resilience (Wood et al., 2010). In our laboratory, we have shown that Syrian hamsters who maintain social dominance for two weeks exhibit reduced behavioral (Morrison et al, 2014), neuroendocrine (Dulka et al., 2018b), and neurochemical (Dulka et al., 2017) consequences of social defeat stress compared to subordinates or controls. Importantly, these status-dependent changes in behavior require recruitment of vmPFC neurons (Morrison et al., 2013) and correspond with reduced activity of the DRN during acute administration of both social and nonsocial stressors (Cooper et al., 2017). That said, it is unknown whether the relationship between enhanced vmPFC activity and reduced DRN activity in dominant hamsters is merely coincidental, indirect, or instead due to top-down control via a monosynaptic vmPFC-DRN pathway.

In this study, we tested the hypothesis that recruitment of a monosynaptic vmPFC-DRN pathway corresponds with stress resilience. More specifically, we predicted that stress-resistant dominant Syrian hamsters recruit DRN-projecting neurons in the vmPFC to a greater degree than subordinate or control counterparts during acute social defeat stress. To address this question, we used a retrograde tracing approach to identify vmPFC neurons that project to the DRN and co-labelled the immediate early gene, cFos, to index neural activity following acute social defeat stress.

Materials and Methods

Subjects

Male Syrian hamsters, *Mesocricetus auratus*, bred in-house from progenitors obtained from Charles River Laboratories, were group housed until adulthood (9 weeks old, 120-180g). All animals were housed in a humidity and temperature controlled colony room (21 ± 2°C) on a 14:10h light:dark cycle in polycarbonate cages (12cm x 27cm x 16cm) with corncob bedding, cotton nesting materials, and wire mesh tops with food and water available ad libitum. All behavioral procedures were performed during the first 3h of the dark phase. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Formation and Maintenance of Dominance Relationships

Upon adulthood, subjects were individually housed for 7 days for territory establishment and handled several times to habituate them to human interaction. Hamsters (n=20) were then weight-matched into dyads, randomly assigned "resident" or "intruder" status, and paired in daily encounters to establish (day 1) and maintain (days 2-16) a dominance relationship (see Fig. 2.1a in Appendix B). To ensure that a dominance relationship was formed on day 1, the first dyadic encounter persisted for 10 min and each encounter thereafter was 5 min daily. Hamsters were classified in real-time as dominant or subordinate by trained experimenters based on the direction of aggressive and submissive/defensive behaviors (Fig 2.1b). To verify stability of

dominance relationships throughout this period, the presence of 4 types of aggressive behavior and 4 types of submissive behavior were recorded daily (i.e. yes = 1 or no = 0) during the early, middle, and/or late phase of each encounter (i.e. max score of 3 per behavior or 12 per aggressive/submissive category). Scores were summated daily into behavioral indices and organized by dominance status as depicted in Fig. 2.1c. During this period, control hamsters (n=16) were individually housed for 8 days without exposure to daily dyadic encounters.

Stereotaxic Surgery

On day 9, daily dyadic encounters were paused and all animals, anesthetized with isoflurane, received stereotaxic microinjections of the retrograde tracer cholera toxin B (CTB; List Biological Laboratories) directly into the DRN. Injections were administered with a 20° entry angle through the left hemisphere to avoid puncturing the fourth ventricle. Injections were targeted 4.6 μm posterior and 2.0 μm lateral to bregma and 5.4 μm below dura. CTB solution (25 nL; 1% concentration) was administered during a 5-min period and diffused for 15 min to reduce backflow. Animals were permitted 48 h of surgical recovery before returning to daily dyadic encounters on day 11.

Acute Social Defeat Stress

On day 17, dominant (n = 10) and subordinate (n = 10) as well as control hamsters without exposure to dyadic encounters (n = 8, termed “dominance status controls”), were subjected to acute social defeat stress as described in greater detail elsewhere (e.g. Dulka et al., 2018a,b). Briefly, a subject was placed consecutively into the home cages of three novel conspecifics who

were larger, older, sexually-experienced, and pre-screened for aggression (termed "resident aggressors"). Upon submission to the resident aggressor, each encounter persisted for 5-min at 5-min inter-trial intervals. Finally, additional control hamsters (n = 8, termed "handled controls") experienced no social defeat procedures but were similarly exposed to empty resident aggressors' cages. Amount of aggression initiated by resident aggressors was indexed as described for dominance relationships. Any animals wounded by the social defeat procedure were removed from analysis a priori (n = 1 dominant).

Immunohistochemistry and Analyses

Immunohistochemical labelling and analyses (detailed in Supplementary Materials) were conducted as previously described (Dulka et al., 2018a). Briefly, DRN-projecting vmPFC neurons were detected using CTB immunoreactivity (IR; goat anti-CTB, List Biological Laboratories, 1:40,000) which was amplified using an avidin-biotin complex (ABC Kit, Vector Laboratories) and visualized with the chromogen 3,3'-diaminobenzadine (DAB; Sigma-Aldrich). Stress-activated neurons were similarly labelled using cFos IR (rabbit anti-cFos, 1:5000, Santa Cruz: sc-52), though with a nickel-enhanced DAB reaction. Cells expressing cFos, CTB, or cFos + CTB IR in the infralimbic (IL) and prelimbic (PL) vmPFC subregions were counted separately using 2 image boxes per region per hemisphere (i.e., 8 boxes/slice) at 20x magnification (see Fig. 2.2a). cFos + CTB dual-labelling was confirmed on a cell-by-cell basis at 40x with progressive focus throughout the tissue to control for overlapping, single labelled cells. Although cFos-IR was apparent throughout the vmPFC, CTB-IR cells were localized within deep cortical layers (V/VI), which is consistent with anatomical studies of DRN-projecting cells within vmPFC (Vertes, 2004).

Therefore, all boxes were centered on layers V/VI. Only viable tissue sections were quantified, with each subject averaging 5 slices (40 image boxes) along a rostro-caudal axis.

Statistical Analyses

Parametric data were analyzed using a paired Student's t-test or analysis of variance (ANOVA) followed by Tukey's post-hoc tests, where appropriate. Nonparametric data were analyzed using Kruskal-Wallis tests followed by Dunn's post-hoc tests. For all immunohistochemical analyses, no differences were found along rostro-caudal axes or across hemispheres, so data were pooled accordingly. Statistical significance was set at $p < 0.05$ and all data are reported as mean ± SEM.

Results

Behavioral Data

All dyads formed dominance relationships on day 1, which remained stable throughout the dyadic encounters, including after surgical recovery (Fig. 2.1c). During social defeat encounters on day 17, there were no differences between groups in duration of aggression received ($F_{2,24} = 0.3236$, $p = 0.7266$) or total attacks received ($F_{2,24} = 0.3273$, $p = 0.7240$). However, a Kruskal-Wallis test revealed differences among groups in latency to submit [$\chi^2(2) = 8.910$, $p = 0.0116$, Fig. 2.1d] and duration of fighting back [$\chi^2(2) = 14.58$, $p = 0.0007$, Fig. 2.1e] during the first defeat encounter. Post-hoc analyses revealed that dominant animals took longer to submit and fought back more than subordinates, yet differed from dominance status controls only in duration of fighting back. Overall, 8 of 9 dominants, 2 of 8 dominance status controls, and 0 of

10 subordinates fought back against the resident aggressor in the first defeat encounter. All animals submitted immediately upon the onset of the second and third defeat encounters.

Immunohistochemistry

Localization of CTB Injections into DRN

While most injections filled the DRN (n = 5-7/group), some spread minimally outside of the DRN (n = 0-2/group). Of these, the majority diffused into the ventral periaqueductal grey (vPAG), particularly in the left hemisphere along the needle tract (n = 1-4/group). Min/max spreads are depicted in Fig. 2.2b.

Quantification of vmPFC Cells

To determine whether dominant animals recruit the vmPFC-DRN pathway to a greater degree during acute social defeat compared to subordinate and control animals, CTB, cFos, and CTB + cFos IR were quantified in the PL and IL cortices (Fig. 2.3). Dominants, subordinates, dominance status controls, and handled controls did not significantly differ in the number of CTB-IR cells in layers V/VI of the PL ($F_{3,31}$ = 1.301, p = 0.2910) or IL ($F_{3,31}$ = 0.365, p = 0.7785). To determine if the PL and IL differed in the number of CTB-IR cells, all animals were pooled and a paired Student's t test was conducted, which revealed no significant difference between these vmPFC subregions (t = 1.364, df = 34, p=0.18).

With regard to stress-induced neural activation, there were significant differences between groups in total cFos expression in layers V/VI of the PL ($F_{3,31}$ = 16.66, p < 0.0001; Fig.

2.4a) as well as IL ($F_{3,31}$ = 12.08, p < 0.0001; Fig. 2.4b). Post-hoc analyses reveal that dominants and dominance status controls differed significantly from subordinates and handled controls, but not from each other in either subregion.

Finally, there were significant differences between groups in the colocalization of CTB and cFos in the PL ($F_{3,31}$ = 26.22, p < 0.0001; Fig. 2.4c) and IL ($F_{3,31}$ = 21.34, p < 0.0001; Fig. 2.4d). Post-hoc analyses revealed that dominant animals differed significantly from subordinate, dominance status controls, and handled controls in both subregions.

Off-Site Injections into Ventral Periaqueductal Gray and Fourth Ventricle

We performed multiple analyses to account for diffusion into the lvPAG and/or fourth ventricle. First, all animals with any CTB-IR in lvPAG were removed from analyses and statistics were recalculated. Importantly, CTB + cFos IR results changed minimally in PL ($F_{3,23}$ = 27.52, p < 0.0001) and IL ($F_{3,23}$ = 19.04, p < 0.0001) and dominants remained significantly different from others. Second, we performed a subset of surgeries (n=8/region; 4 dominant, 4 subordinate) wherein the injection locations were centralized to the lvPAG (-5.275μm below dura) or fourth ventricle (1.6μm lateral from bregma; -4.4μm below dura). Among these animals, no double-labelled cells were detected in the vmPFC.

Discussion

In this study, we demonstrate that hamsters that have maintained social dominance for 14 days selectively activate a vmPFC-DRN circuit during an acute social defeat experience to a greater degree than subordinate or control counterparts. Interestingly, this effect of social

dominance occurs despite displaying total cFos-IR in deep layers (V/VI) of the vmPFC at levels similar to dominance status controls, from which daily dominance interactions were withheld. These findings suggest that the experience of maintaining social dominance drives neuroplastic processes that result in greater recruitment of a vmPFC-DRN pathway during traumatic stress.

Given the robustness of findings that dominant hamsters display resistance to acutely administered stressors (Morrison et al., 2013, 2014; Cooper et al., 2017; Dulka et al., 2017, 2018a,b), it is plausible that such resistance is made possible, at least in part, by selectively activating a vmPFC-DRN pathway during stress. Indeed, greater activity of DRN-projecting vmPFC cells has been associated with indices of stress resilience following other common laboratory stressors such as tail shock (Baratta et al., 2009) and forced swim stress (Warden et al., 2012). Accordingly, the vmPFC-DRN pathway has recently been dubbed "the hope circuit" (Maier & Seligman, 2016).

This report extends our understanding of the neurobiology of stress resilience in several ways. First, while studies have shown that artificial excitation of the vmPFC can facilitate resilience-like behaviors (Amat et al., 2008; Covington et al., 2010), Deisseroth and colleagues showed that non-selectively driving vmPFC activity via optogenetic stimulation failed to alter swimming behavior during an acute forced swim stress (Warden et al., 2012). However, when vmPFC terminals within the DRN were targeted and thus a monosynaptic vmPFC-DRN projection was selectively recruited, there was a dramatic photic stimulation-dependent increase in escape-oriented swimming behavior. Importantly, escape-oriented swimming indicates a proactive coping strategy and proactive responses are thought to promote stress resilience (e.g. Wood et al., 2010). Given that dominant Syrian hamsters appear to be more resilient than their

subordinate and control counterparts (Morrison et al., 2014), our results are consistent with these reports on two fronts. First, despite showing similar c-Fos expression in the vmPFC when compared to dominance status controls, dominant hamsters exhibited a 3-fold increase in selective activation of a vmPFC-DRN pathway. That is, similar to Warden et al.'s report, simply expressing greater neural activity within the vmPFC does not itself produce a stress resistant phenotype. Instead, it is critical to selectively recruit target-specific projections such as those terminating in DRN. Second, dominant hamsters also displayed greater latencies to submit during social defeat and were more likely to fight back against the resident aggressor than their stressed counterparts. This is important given that long latencies to submit during defeat in rats also indicates proactive coping strategies and corresponds with reduced neuroendocrine and behavioral correlates of psychopathology (Wood et al., 2010).

Second, our results show that 14 days of daily subordination appears to drive processes that render deep cortical layers of the vmPFC unresponsive to acute social defeat stress. Following social defeat, subordinate hamsters expressed substantially fewer cFos+ neurons in the vmPFC when compared to dominants or dominance status controls, and they were indistinguishable from those not receiving stress (i.e. handled controls). This suggests that social subordination for two weeks may render the deep layers of the vmPFC damaged (e.g. oxidative stress, dendritic remodeling), silenced (e.g. active inhibition), or simply not recruited (e.g. loss of excitatory input). On the other hand, in both resilient humans (Sinha et al., 2016) and rats resistant to learned helplessness (Maier & Seligman, 2016), the vmPFC is not immediately activated by stressor onset, but requires a gradual, time-dependent recruitment as the stressor frequency and/or duration continues. It is possible that vmPFC neurons in subordinate animals

would eventually activate with a more prolonged stress exposure (i.e. that their vmPFC-DRN recruitment is more greatly delayed than dominants), though this has yet to be shown. However, repeated optogenetic stimulation of the vmPFC-DRN pathway during sensory contact with an aggressor immediately after chronic stress appears to instead confer vulnerability (Challis et al., 2014), suggesting that vmPFC-DRN-driven resilience could be time- and context-dependent.

Third, these results suggest the vmPFC can confer resistance to the effects of social stress via multiple downstream targets. Using this approach, we recently reported that dominant hamsters similarly show increased defeat-induced activation of a vmPFC-to-basolateral amygdala pathway (Dulka et al., 2018b). Inasmuch, behavioral resistance to social defeat stress in hamsters requires neuronal activity of the vmPFC (Morrison et al., 2013) and appears to rely on the vmPFC's ability to gate stress-induced activation of basolateral amygdala, DRN, and perhaps other downstream targets. Indeed, neural imaging studies in humans suggest that resilience to stress corresponds with functional connectivity between the vmPFC and multiple stress-sensitive brain regions (Sinha et al., 2016). Similarly, the vmPFC projects to numerous cortical and subcortical regions throughout the rodent brain (Vertes, 2004), many of which are involved in processing stressful events. Importantly, we found that only around 10% of cFos+ neurons co-expressed CTB, which suggests that only a small proportion of activated vmPFC neurons send projections to the DRN. While our injections did not fill the entire DRN in every animal and terminals may not take up CTB with 100% efficiency, these findings are consistent with the possibility that dominants recruit additional ensembles of vmPFC projections during social defeat stress.

Fourth, this study demonstrates a striking similarity in vmPFC and vmPFC-DRN activity across models of experience-dependent stress resilience. For example, Maier and colleagues have shown that male rats learning to exert control over a stressor's duration by turning a wheel to abate and thus escape a tail shock stress (ES) display reduced stress-related consequences when compared to those exposed to the same amount of shock yet in an uncontrollable manner (inescapable stress or "IS"; Maier & Seligman, 2016). During stress, neural activity of ES and IS rats differ in patterns quite similar to those discussed above in dominant and subordinate hamsters. This includes cFos IR in the vmPFC and DRN, requirement of vmPFC for stress resistance, and stress-induced recruitment and plasticity of the vmPFC-DRN pathway (Amat et al., 2005, 2008; Baratta et al., 2009, 2019). Moreover, ES rats are protected against the social deficits seen in IS rats such as reduced aggression and downward mobility in dominance status as well as increased avoidance of non-threatening, juvenile conspecifics (Maier & Seligman, 2016). Dominant hamsters are similarly protected against these deficits, which are reliably observed in subordinate hamsters (Morrison et al., 2013, 2014). ES rats are also resilient to a subsequent, uncontrollable stressor (e.g. uncontrollable shock or acute social defeat), a phenomenon termed, immunization (Amat et al., 2008; Baratta et al., 2019). Maier's group argues that the vmPFC "detects" whether an organism has control over a stressor through reciprocal projections with the dorsal medial striatum. The vmPFC then "acts" accordingly by engaging a separate ensemble of vmPFC neurons that project to and inhibit the DRN. By repeating such experiences, the connections necessary for re-engaging these DRN-projecting vmPFC neurons are strengthened, permitting its recruitment even in uncontrollable scenarios and thereby promoting immunization and thus the resilience associated with "expected" control

(Maier & Seligman, 2016). Similarly, Syrian hamsters may experience a sense of control when they acquire and maintain social dominance, which thereby strengthens a vmPFC-DRN pathway through repeated stimulation during daily dyadic encounters. Then, upon uncontrollable social defeat stress (or forced restraint, see Cooper et al., 2017), this vmPFC-DRN ensemble is recruited once more, thereby suppressing DRN activity and reducing the behavioral consequences of social defeat.

Fifth, the similarities between Deisseroth's, Maier's, and our findings that suggest a vmPFC-DRN pathway drives resilient-like behaviors are even more intriguing given that, collectively, they contrast with reports from Olivier Berton's laboratory. Briefly, Berton's findings using a model of chronic social defeat stress suggest that repeated engagement of a vmPFC-DRN circuit instead promotes social avoidance and thus, a phenotype marked by susceptibility to chronic stress (Challis et al., 2013, 2014). Generally speaking, this model comprises c57-derived mice which are exposed daily (for 10 days) to a novel resident aggressor mouse from another strain (CD1) for a brief period of physical aggression (usually 5-10 minutes) followed immediately by separation with a perforated barrier to allow only sensory contact for the remainder of a 24 hour period. In a series of studies, Berton and colleagues found that, following physical aggression, the first 20 minutes of sensory contact was both necessary and sufficient for the expression of defeat-induced social avoidance in the susceptible phenotype and, moreover, that the expression of this phenotype corresponded with increased cFos and ΔFosB in GABAergic cells within the lateral wings of the DRN (Challis et al., 2013). Given that vmPFC neurons frequently terminate in this area, Challis et al. (2014) optogenetically targeted vmPFC-DRN cells during 20 minutes of sensory contact for 10 days following social defeat. Importantly, photostimulation of

vmPFC terminals within DRN significantly suppressed social approach behaviors in both defeated and non-defeated controls. On the other hand, photoinhibition of vmPFC terminals significantly increased social approach in defeated mice, though no changes were seen in controls. Together, these findings suggest that artificial stimulation of DRN-projecting vmPFC neurons in mice can suppress social approach if occurring during a sensory exposure period. While these findings are admittedly difficult to reconcile with the contrasting reports discussed above, there are several key methodological differences worth mention. First, the time period of vmPFC-DRN activity may be critical and activation of this pathway during vs. following a stressor might lead to dissociable outcomes. Second, the consequences of vmPFC-DRN activation may fundamentally differ in acute vs. chronic stress. Third, repeated optogenetic stimulation of this pathway may lead to several compensatory responses and/or stimulation of collaterals that are not characteristic of acute activation. Fourth, optogenetic stimulation might engage the vmPFC-DRN pathway more broadly and, furthermore, do so at supraphysiological levels (Marton & Sohal, 2016), thereby driving patterns of behavior not apparent upon more restricted activation. Finally, species differences in vmPFC organization may also be a critical factor. For example, although the rat DRN receives far more input from the PL than IL (Vertes, 2004), we were unable to detect a significant difference in the number of CTB+ cells between these regions of Syrian hamsters.

Our study is not without limitations. First, all findings presented and reviewed above were conducted in male hamsters only. This is a critically important caveat given that females are at a greater risk than males for developing stress-related psychopathologies (e.g. Southwick & Charney, 2012). Moreover, a recent study showed that female ES rats fail to display the resilience conferred by learning to control shock exposure (Baratta et al., 2019). While it is possible that

stress and/or training procedures were optimized for males and thusly do not adequately reflect the effects of stress controllability in female rats, this has meaningful implications for how our results are interpreted. Given that female hamsters, unlike most other laboratory rodents, will aggressively defend a territory, current work in our laboratory is focused on characterizing potential stress resistance conferred by social dominance in female Syrian hamsters and, accordingly, shedding additional light on this crucial shortcoming of trends within neuroscience research (Shansky, 2019). Second, we present no behavioral data in this report to confirm a causal role of the vmPFC-DRN circuit in stress resilience, rendering all data strictly correlational. Nonetheless, our lab has repeatedly shown that dominant hamsters display reduced behavioral and physiological consequences of acute social defeat, as reviewed above. Finally, it is possible that our results are not reflective of the total percentage of DRN-projecting vmPFC neurons activated by stress for two key reasons. First, cFos was the only immediate early gene probed in this study, rendering the possibility that other CTB+ neurons were active yet undetectable given our approach. Second, hamsters are rarely targeted for the optimization of molecular tools and our dual-labeling approach required that we reduce primary antibody concentrations to avoid non-specific binding. Inasmuch, it is possible that some cFos IR fell below our detection limits, rendering a potential reduction in total cell counts. With this said, our focus on relative differences between groups supports the overall conclusion of this report: achieving and maintaining social dominance in Syrian hamsters permits increased activity of a vmPFC-DRN pathway during acute social defeat stress and further implicates this pathway in resilience to acute traumatic stress.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

J.A.G. and M.A.C. designed the experiments. J.A.G. lead and T.T.C., N.B.G., and B.N.D. assisted in conducting experiments. J.A.G. analysed the experiments and wrote the manuscript. All authors edited and/or reviewed the manuscript for publication.

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Appendix B

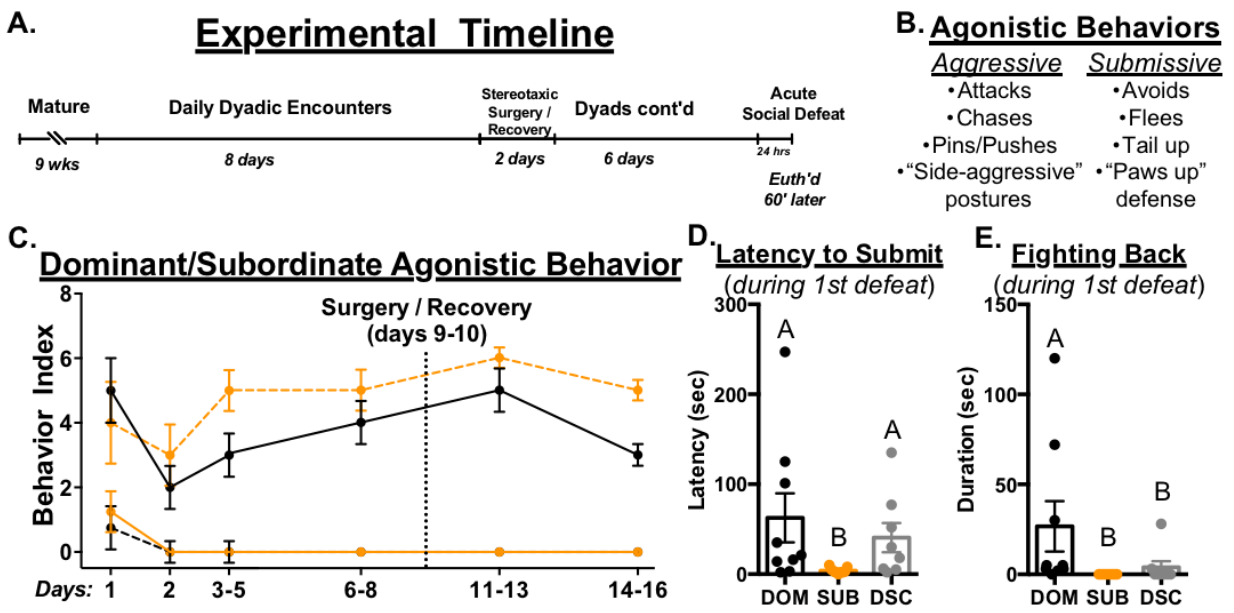

Figure 2.1 The Experimental Timeline, Dominance Status Formation, and Behavior During Acute Social Defeat. Upon maturation, male Syrian hamsters were assigned to a dyad with a weight- and age-matched conspecific and paired 5-mins daily for a total of 14 days. Dyadic encounters were paused on days 9-10 to permit stereotaxic injection of retrograde tracer, CTB, directly into the DRN. After surgical recovery, daily encounters resume on days 11-16. On day 17, animals receive an acute social defeat followed 60-mins later by euthanasia to time-lock cFos expression within stress-activated neurons. During dominance and defeat encounters, aggressive and submissive behaviors were scored per the ethogram summarized in (B). The occurrence of each behavior was recorded daily to index dominance status (C). Black and orange lines indicate summated behaviors of dominants and subordinates, respectively. Solid and dashed lines indicate aggressive and submissive behaviors, respectively. Importantly, once dominance relationships were formed on day 1, hierarchies remained stable throughout the remaining encounters. In response to the first of three social defeat encounters with a resident aggressor on day 17, the latency to submit (D) and duration of fighting back (E) were quantified. Dominants [DOM] took longer to submit than subordinates [SUB] and spend more time fighting back than SUB and dominance status controls [DSC].

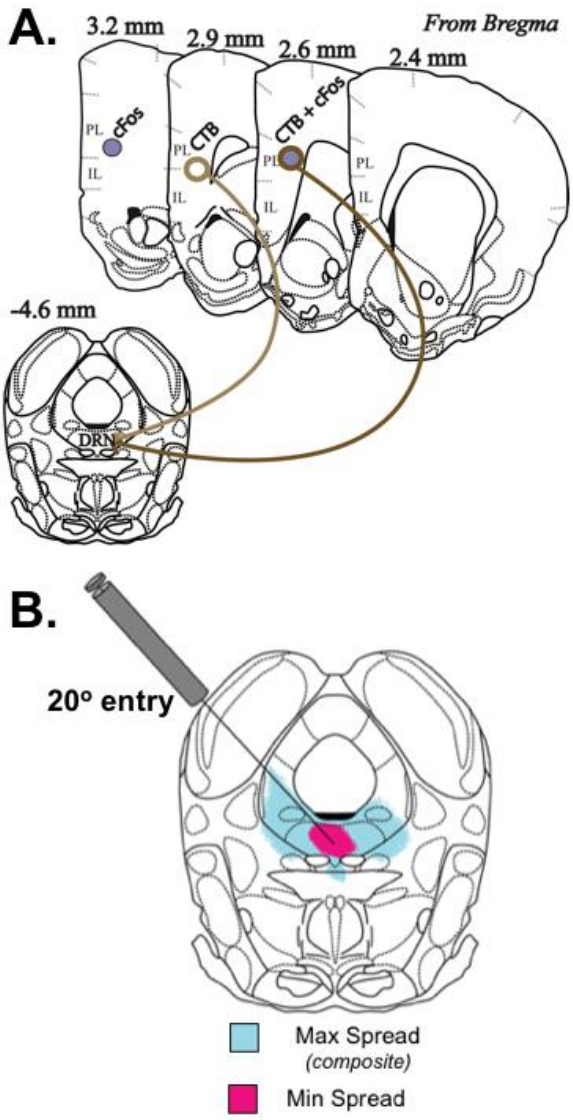

Figure 2.2. Pictographs Depicting the Dual Label Approach. The graphic in (A) depicts the anatomical location of CTB injection into the DRN (bottom) as well as quantification regions within the vmPFC (top). cFos immunoreactivity was detectable by the presence of purple, solid, nuclear staining and CTB immunoreactivity was detectable by the presence of brown, opened, circular staining of neuronal membranes. Dual-labeled cells, characterized by brown membranous staining around purple nuclei, indicate a DRN-projecting neuron with somas localized in the prelimbic [PL] or infralimbic [IL] subregions of vmPFC. Cells containing any of these three labels were counted within two, stacked image "boxes" (at 20x magnification) that were centered on deep cortical layers (V/VI). This permitted a quantification area of 440 μm x 660 μm per subregion per hemisphere per 40 μm-thick slice. The minimum, in pink, and maximum, in blue, spreads of CTB in the DRN are depicted in (B).

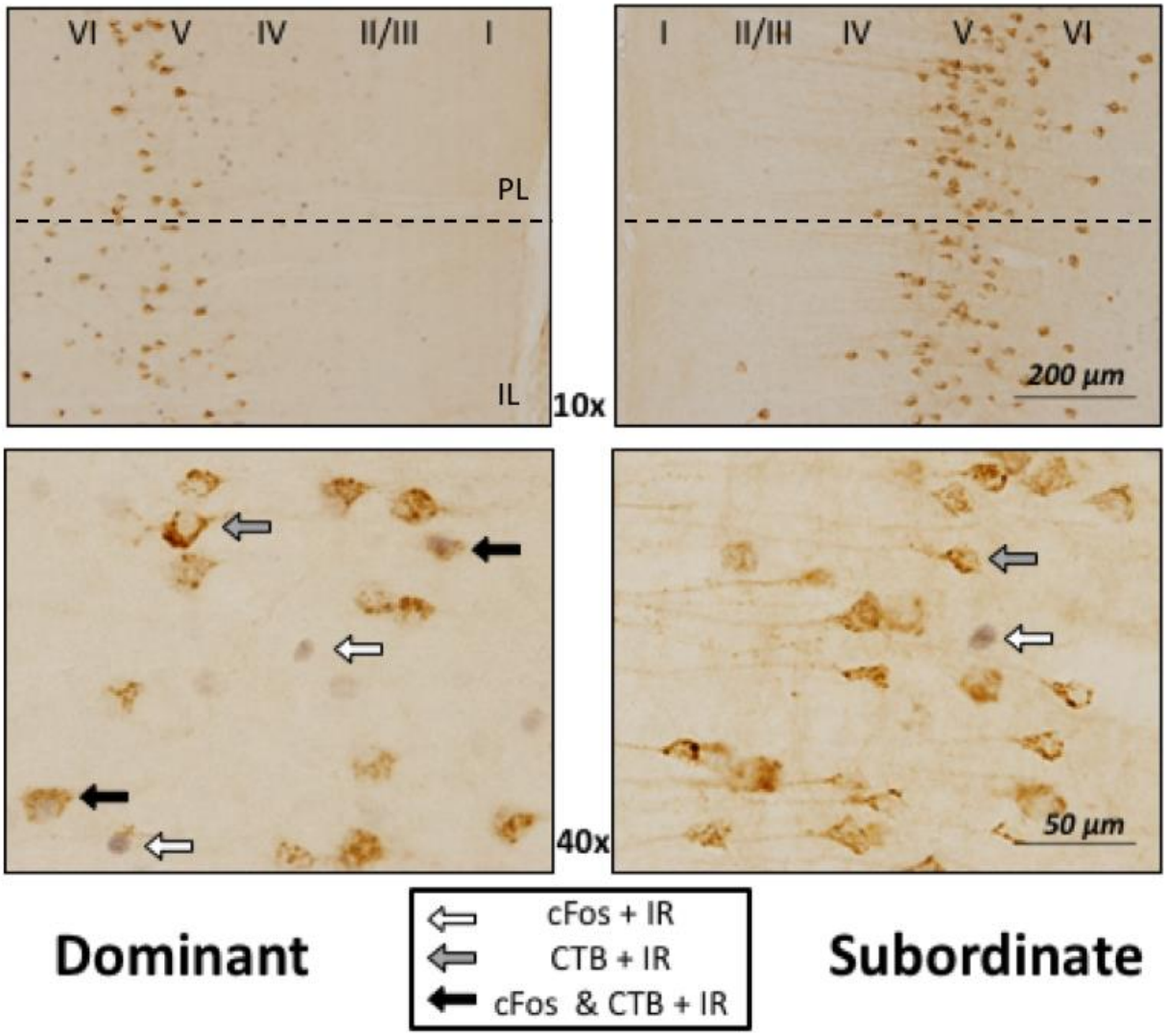

Figure 2.3. Representative Histology. Representative histology of dominant (left) and subordinate (right) hamsters depict relative differences of cFos-only (white arrows), CTB-only (grey arrows), and cFos + CTB cells (black arrows). Expression of CTB was localized primarily in deep cortical regions (V/VI) as depicted in top panels at 10x magnification. Confirmation of dual-labeling required progressive focus at 40x magnification as depicted in bottom panels.

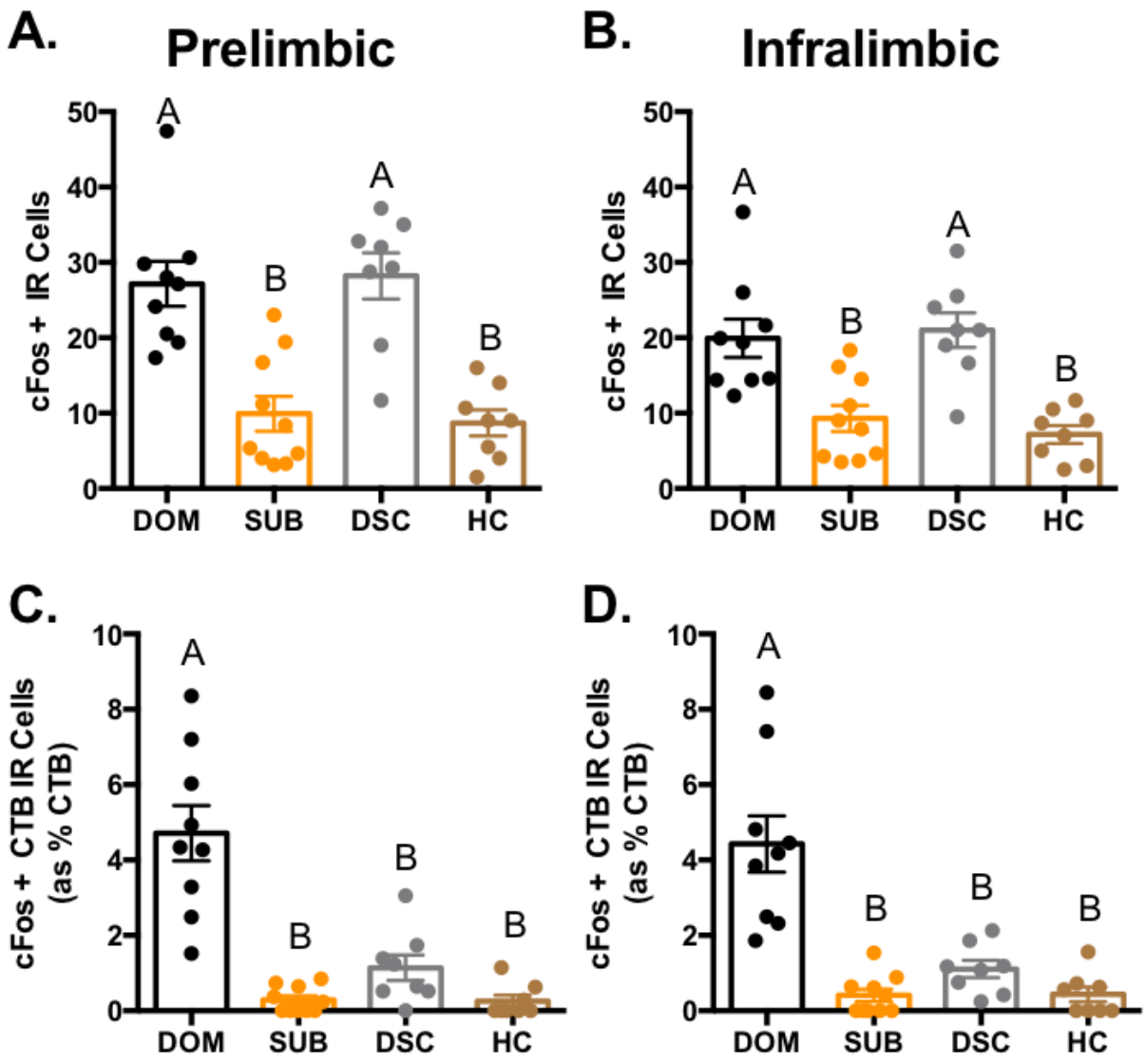

Figure 2.4. Dominant Hamsters Recruit More vmPFC and vmPFC-DRN Neuronal Activity when Compared with Subordinate and Control Hamsters. Total number of cFos+ cells within deep cortical layers (V/VI) are shown per slice in prelimbic (A) and infralimbic cortices (B). In both subregions, dominant hamsters [DOM] differed significantly from subordinate [SUB] and handled controls [HC] but not dominance status controls [DSC]. The total number of dual-labeled cells (as a percentage of total CTB+ cells) are shown per slice in prelimbic (C) and infralimbic cortices (D). Bars with separate letters indicate significant differences ($p < 0.05$).

Chapter 3:

An Investigation of Microglial Response to Acute Social Defeat Stress in Syrian Hamsters

Abstract

Given that stress-related psychopathologies are estimated to negatively impact at least 10% of the population, studies within basic neuroscience are necessary to better understand the neural correlates of stress-induced impairment. It has been well-described that prolonged psychological stress can contribute to enhanced activity of the brain's innate immune system that, at times, leads to tissue damage and, accordingly, disruption of neural functioning. However, whether acute traumatic psychological stress may trigger similar neuroimmune mechanisms is poorly understood. Within the ventromedial prefrontal cortex (vmPFC), the recruitment of neuronal activity during an acute social defeat stressor is associated with resistance to the negative effects of stress exposure, which can be quantified in rodent models using social avoidance assays. In that regard, animals with low recruitment of vmPFC neurons have marked increases of social avoidance, which mirrors studies of stress vulnerability in humans. We report on four studies in Syrian hamsters that focus on whether neuroimmune factors, such as the recruitment of microglia, contribute to disruptions of vmPFC structural integrity following acute social defeat stress. We found that, following social defeat stress, vmPFC microglia displayed increased markers of proinflammatory activity, such as an upregulation of *ionized calcium binding adapter molecule 1* (Iba1) and alterations of microglial cellular morphology. Importantly, these changes are consistent with an increased probability of proinflammatory cytokines release, reactive oxidative species production, and phagocytosis, all of which are highly destructive to surrounding tissue if unchecked. Further, social defeat stress increased markers of tissue degradation, including reduced expression of synaptophysin, a vesicular adhesion molecule that serves as a marker for functional synapses. Interestingly, we

also found that increased markers of proinflammatory microglial activity and tissue degradation were more pronounced in animals that also displayed enhanced social avoidance, though the stress-induced synaptic degradation occurred equally in all treatment groups. On the other hand, Iba1 expression was correlated with tissue degeneration state, suggesting that proinflammatory activity Iba1-expressing is associated with tissue degeneration. Finally, to determine causality within this relationship, an additional cohort of hamsters were treated with the antibiotic *minocycline* in drinking water, which halts proinflammatory activity of Iba1-expressing cells. Minocycline treatment protected vmPFC tissue from cellular and synaptic degradation and rendered more quiescent microglial morphological states. However, minocycline treatment did not reduce the defeat-induced expression of social avoidance yet did increase active vigilance behaviors in a subset of hamsters, which may reflect a shift toward a more proactive risk assessment strategy following stress. Taken together, acute social defeat stress induces cellular and synaptic degradation in the vmPFC alongside changes in microglial morphology that are consistent with proinflammatory activity. Further, perturbations of vmPFC anatomical integrity coincide with enhanced social avoidance following acute social defeat and vmPFC protection with minocycline treatment may confer more proactive coping strategies following acute trauma.

Introduction

While known for decades that psychological stress and the immune system share a complex and dynamic relationship, the biological mechanisms mediating their interactions are poorly understood. Many stress-related psychopathologies are characterized by systemic inflammation, for example major depression (MDD) and post-traumatic stress disorder (PTSD; Friedrich, 2014). Interestingly, an artificially induced inflammatory response in healthy subjects leads to negative affect such as depression, malaise, and social withdrawal (Dantzer, 2001; Haroon et al., 2012). Moreover, these symptoms can be reduced in rodents with administration of commonly prescribed antidepressants (Liu et al., 2011; Maciel et al., 2013; Avitsur et al., 2017). These findings suggest that immune responses to psychological stress underlie subsequent emotional and behavioral impairments. Inasmuch, stress vulnerability appears to be linked with hyperactive innate immune function, especially within the brain (Wood et al., 2015).

Recent discoveries show that microglia, the brain's resident immune cells, drive processes through which stress leads to elevated neuroinflammatory states and behavioral dysfunction (Hinwood et al., 2012, 2013; Nie et al., 2018). Indeed, innate immune activity in the brain leads to rapid proliferation and mobilization of microglia, as indicated by increased expression of the *ionized calcium binding adaptor molecule 1* (Iba1) and alterations of microglial morphology (see Fig. 1.1, Appendix A). These changes correspond with an array of disruptive consequences associated with inflammation, for instance, cytokine release, phagocytosis, and the production of reactive oxygen species (ROS), which can induce oxidative stress. Cytokines are signaling molecules which primarily serve to drive (proinflammatory) or inhibit (anti-inflammatory) the immune response by recruiting additional microglia in a feed-forward manner. However,

cytokines can also modulate brain activity through their own protein receptors on other cell types (*e.g.* neurons and astrocytes) to trigger various intra- and extracellular signaling cascades (Colton et al., 2000; Wohleb et al., 2013; Calcia et al., 2016; Hong et al., 2016). In extreme conditions, proinflammatory microglial activity can weaken the blood brain barrier (Yenari et al., 2006) to allow infiltration of peripherally derived macrophages (Persidsky et al., 1999), which also express Iba1 given their shared cellular lineage (Ohsawa et al., 2000). Microglia may also progress toward phagocytotic activity states themselves, which are often marked by a more spheroid soma or fully amoeboid appearances resulting from branch withdrawal (see Fig. 1.1; Kettenmann, 2007; Hinwood et al., 2012, 2013). Together, macrophagic activity coupled with ROS and subsequent oxidative stress, can disrupt the structure and function of regions that are particularly sensitive to stress- and inflammation-induced neurodegeneration, such as the ventromedial aspect of the prefrontal cortex (vmPFC; Arnsten, 2015). Indeed, in animal models of prolonged stress, the Iba1+ macrophagic cells can be found within vmPFC activity alongside, at times, reduced dendritic length, low neuronal activity, and increased depression- and/or anxiety-like behavior (Calcia et al., 2016; Hinwood et al., 2012, 2013; Nie et al., 2018). However, oral administration of the antibiotic *minocycline,* when treated concurrently with prolonged stress exposure, reduces Iba1+ cellular activity while returning neuronal activation within the vmPFC and attenuates stress-induced memory and anxiety-like impairments (Hinwood et al., 2012, 2013; Garrido-Mesa et al., 2013; Wang et al., 2018).

Disruption of vmPFC structural integrity can potentiate stress-related cognitive, emotional, and social impairments. Ample evidence demonstrates that the vmPFC plays a critical role in emotion regulation and stress reduction in both acute and chronic stress conditions. For

example, functional magnetic resonance imaging (fMRI) in human participants suggests that the vmPFC becomes active during acutely administered laboratory stressors in a manner that corresponds with stress resilience whereas stress vulnerability corresponds with reduced vmPFC recruitment (Sinha et al., 2016). Within animal models, chronic stress procedures such as prolonged restraint lead to deleterious alterations of various limbic structures, including reductions in neuronal dendritic length and branching (Radley et al., 2005; Goldwater et al., 2009; Nie et al., 2018), synaptic densities (Grizzell et al., 2014a; Patel et al., 2015), and neuroprotective growth factors implicated in maintaining the efficacy of the blood brain barrier (Grizzell et al., 2014b) within the vmPFC and hippocampus. Although studied to a much lesser degree, evidence suggests that acutely administered laboratory stressors may similarly result in reduction and remodeling of neuronal processes in vmPFC in a manner that corresponds with a loss of stress resilience (Izquierdo et al., 2006; Nava et al., 2017).

Research in our laboratory indicates that acute social defeat stress in Syrian hamsters activates vmPFC neurons, though to a smaller degree in those who have developed and maintained a subordinate social status prior to social defeat stress (Morrison et al., 2012). Importantly, subordinate hamsters also display exaggerated social avoidance to non-aggressive conspecifics following acute defeat when compared with dominant and dominance status control (DSC) counterparts. Moreover, vmPFC activity during social defeat appears necessary for stress resistance given that pharmacological inhibition of vmPFC neurons eliminates the reduced social avoidance characteristic of dominants (Morrison et al., 2013). Stress resistance in dominant hamsters also corresponds with selective activation of vmPFC neurons projecting to the amygdala (Dulka et al., 2018) and dorsal raphe nucleus (Grizzell, Chapter 2 of this dissertation),

both of which are regions highly active during traumatic stress and necessary for the display of defeat-induced social avoidance in a "conditioned defeat" (CD) test (Jasnow & Huhman, 2001; Cooper et al., 2008). On the other hand, stress vulnerability in subordinate animals corresponds with little to no recruitment of BLA-projecting or DRN-projecting vmPFC neurons. Given that an elevated CD response and a loss of stress-induced vmPFC activity gradually appear as hamsters maintain a subordinate relationship for two weeks with a familiar partner (Morrison et al., 2014), and that subordinate hamsters display reductions of key antioxidants which are necessary defenses against oxidative stress (Dulka et al., 2017), it follows that daily subordination may facilitate persistent proinflammatory activity and oxidative stress in the vmPFC which could, in turn, lead to stress vulnerability via disruptions of vmPFC structural integrity.

Very little is known regarding the active role of microglia/macrophages in disrupting vmPFC activity in acutely stressful conditions, though acute stress does prime microglia for an enhanced response to a subsequent immune challenge (*i.e.* lipopolysaccharide/"LPS" exposure; Frank et al., 2006). This provides support for this chapter's overarching hypothesis: acute stress drives mechanisms that enhance the proinflammatory profiles of vmPFC microglia/macrophages (**Studies 1-4**) in a manner promoting cellular degradation and reduced synaptic densities (**Studies 3-4**). These were addressed across four studies, with the last two running in concert. The primary goal for **Study 1** was to determine whether acute social defeat stress, either alone or when interacting with an acute immune challenge (*i.e.* systemic LPS administration), drives changes in Iba1 expression and morphology on Iba1+ cells that are consistent with states of proinflammation. We predicted that socially defeated hamsters would display increased Iba1 expression alongside increased diameters of soma size and soma-to-total cell size ratios in Iba1+

cells. Moreover, we predicted that these changes would be exaggerated in defeat + LPS groups. The goal for **Study 2** was to determine if the changes in Iba1 expression and morphology observed in Study 1 persisted for 7 days. We predicted that Iba1 expression and morphology may display some return to baseline, but that we would still see some persistent morphology changes consistent with proinflammatory profiles. The goal for **Study 3** was to determine whether subordinate hamsters display increased signals of proinflammation within Iba1+ cells alongside increased signals of cellular or synaptic degradation. We predicted that, whether experiencing social defeat or not, subordinate hamsters would display increased (relative to dominants and controls) Iba1 expression and morphological changes in Iba1+ cells which would be consistent with those predicted for study 1. Secondly, we predicted that subordinate hamsters, whether defeated or not, would display increased silver deposition following an amino cupric stain and decreased synaptophysin staining, which signify cellular debris and synaptic densities, respectively. The goal for **Study 4** was to determine whether the observed changes in Iba1+ cells, cellular debris deposition, and synaptic densities observed in Study 3 were sensitive to oral administration of minocycline. We predicted that minocycline administration would protect against acute stress-induced changes in Iba1 expression, Iba1+ cell morphology, silver deposition in the amino cupric stain, and synaptophysin labeling by rendering them no different than non-stressed controls. Finally we predicted that minocycline treatment would protect against acute social defeat-induced social avoidance as assayed in the conditioned defeat (CD) and social interaction (SIT) tests.

Methods

Subjects

Male Syrian hamsters (*Mesocricetus auratus*) were bred in-house and allowed to mature to adulthood (9 weeks old, 120-180g) while housed in groups of 3-4 in polycarbonate cages (12 cm x 27 cm x 16 cm) with cotton nesting materials, corncob bedding, and wire mesh lids with food and water available *ad libitum*. Upon adulthood, all subjects were individually housed for 7 days prior to any experimentation for territory establishment and, during this time, were frequently handled to habituate them to the inherent stress of human interactions. All housing occurred in an all-male vivarium room that was set on a 14:10-hour light:dark cycle with temperature and humidity controlled. All behavioral procedures were conducted within the first 3 hours of the dark phase. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

A total of 165 animals were used for all experiments in this report, not including those necessary for preliminary data, positive controls, or training of undergraduate assistants. A total of four studies were conducted as described below and depicted in Fig. 3.1 of Appendix C. Each study was split into multiple cohorts of animals to ensure feasibility of experimentation and replicability of findings. Each cohort ranged in size from 10-20 animals total. Importantly, **Studies 3 and 4** were conducted in parallel, which reduced the number of control animals required.

Throughout all studies, the social defeat procedure (described below) was held standard and was in all cases administered at least 24 hours following 7 days of territorial establishment (*i.e*. 7 days of individual housing in **Studies 1, 2, and 4**). In **Study 3**, social defeat procedures occurred 24 hours following cessation of dyadic, dominance encounters. In **Studies 3 and 4**, assays for stress-induced avoidance behavior were administered 24 hours following social defeat stress in a counterbalanced manner and all animals were euthanized 24 hours after avoidance assays for extraction of vmPFC tissue. This 48-hour, post-defeat interval for euthanasia was chosen to grant optimal immunohistochemical labeling of cellular degeneration via the amino cupric "silver stain", given that degenerated dendritic and axonal processes are not immediately detectable upon insult and are typically cleared as early as three days later (*personal communication*: Switzer, 2018; Switzer, 2000).

<u>Study 1: Acute Social Defeat-Induced Priming of vmPFC Microglia/Macrophages to Subsequent Immune Challenge with Lipopolysaccharide (LPS)</u>

The goal of this study was to determine whether acute social defeat alone or when followed 24-hours later by LPS injection drives changes in Iba1 expression and Iba1+ cell morphology. We predicted that social defeat would elevate Iba1 expression in vmPFC tissue and that Iba1+ cells would alter cell morphology in a manner consistent with increased proinflammatory profiles (see Fig. 1.1). Furthermore, we predicted that acute social defeat stress would prime an enhanced expression of Iba1 and greater changes in Iba1+ cell morphology.

To complete this study, animals (n = 58) were divided into stress and no stress conditions. Twenty-four hours after acute social defeat stress and control procedures, each group was

subdivided into one of four treatment levels to receive intraperitoneal injections of either 0, 20, 100, or 500 μg/kg of LPS (Sigma Aldrich) dissolved in sterile saline. These log-based concentrations were chosen due to limited information in the literature regarding optimal dosages for LPS in behavioral assays in Syrian hamsters. Inasmuch, our goal was to determine the lowest necessary dose to detect stress X LPS interactions in neuroimmune function. Accordingly, animals were left minimally disturbed following LPS administration and monitored regularly for 4 hours before euthanasia. During this time, visual observations of sickness-behavior displays (*e.g.* lethargy, kyphosis, piloerection, immobility, pallor, etc.) were noted. Importantly, only 3 animals, all of which received the 500 μg/kg dose and were assigned to the no-defeat condition, displayed any detectable sickness behavior. These animals showed lethargy, reduced mobility, and reduced arousal, which appeared approximately 2.5 hours after injection and continuing until euthanasia. No behavioral changes were observed in remaining animals across all dosages. Following euthanasia, the intensity of Iba1 labeling as well as the morphology of Iba1+ microglia/macrophages were assayed in the vmPFC (as described below).

Study 2: Prolonged Effects of Acute Social Defeat on Patterns of Iba1 Expression

The goal of this study was to determine whether acute social defeat stress induced changes in Iba1+ cells consistent with proinflammatory activity that persisted for 7 days. We predicted that acutely defeated hamsters would display increased Iba1 expression and proinflammatory morphological states.

To complete this study, animals (n = 19) were divided into either acute social defeat stress or no stress conditions and left undisturbed in their home cages for 7 days until euthanasia.

Following euthanasia, the intensity of Iba1 labeling as well as the morphology of Iba1+ cells were assayed in the vmPFC.

Study 3: Dominance Status-Dependent Differences in Acute Social Defeat Stress-Induced Alterations of vmPFC Integrity

The goal of this study was to determine whether, when compared with dominant and DSC hamsters, subordinate hamsters display differences in Iba1+ cell expression and morphology, synaptic densities, and cellular debris indicative of degeneration. A second goal of this study was to determine if, following acute social defeat stress, dominant hamsters display altered expression among the aforementioned parameters. We predicted that subordinate hamsters, whether defeated or not, would display increased Iba1+ expression, Iba1+ cell morphology consistent with proinflammatory states, reduced synaptophysin labeling, and increased amino cupric "silver stain" binding. Secondly, we predicted that dominant hamsters that had been subjected to acute social defeat stress would display fewer of these signals when compared with defeated subordinates or DSCs.

Animals (n = 62) in this study were divided into six groups in a 2 x 3 (stress x dominance status) multifactorial design with the groups labeled as follows: (1) *Dominance Status Control* (i.e. "DSC") *+ No Defeat*; (2) *DSC + Social Defeat*; (3) *Dominant + No Defeat*; (4) *Dominant + Social Defeat*; (5) *Subordinate + No Defeat*; and (6) *Subordinate + Social Defeat*.

Following territory establishment, all hamsters assigned to the *Dominant* and *Subordinate* groups were weight-matched into dyads, randomly assigned "resident" or "intruder" status, and paired in daily encounters for two weeks to establish (day 1) and maintain (days 2-14) a

dominance relationship. To ensure that a dominance relationship was formed on day 1, the first dyadic encounter persisted for 10 minutes and each encounter thereafter was 5 minutes daily. Hamsters were classified in real-time as dominant or subordinate by trained experimenters based on the persistent direction of aggressive and submissive/defensive behaviors (as described in Chapter 2 of this dissertation). To ensure stability of dominance relationships throughout this period, the presence of 4 types of aggressive and submissive behavior were recorded daily (*i.e*. yes = 1 or no = 0) during the early, middle, and/or late phases of the encounter (*i.e*. max score of 3 per behavior or 12 per agonistic category). Subsequently, dyadic encounter scores were summated daily into behavioral indices and organized by dominance status. Then, 24 hours after the final dyadic encounter (*i.e*. Day 15), subjects were exposed to the acute social defeat procedure. To control for dominance status, animals assigned to the *DSC* groups were exposed to the acute social defeat procedure 24 hours after territory establishment (*i.e.* Day 1).

Twenty-four hours after social defeat or no stress control exposure, all animals were exposed to the conditioned defeat (CD) and social interaction test (SIT) assays for acute stress-induced social avoidance (described below). To control for order effects, CD and SIT procedures were counterbalanced across all animals with a 30-60 minute inter-assay interval. All behavioral testing occurred under low light conditions and animals were returned to their home cages in a staging area located in a separate room that was kept quiet and dark for minimal stimulation. Then, 24 hours after social avoidance assays, all animals were euthanized via transcardial perfusion and vmPFC tissue extracted. Following euthanasia, tissue was processed for several immunolabeling procedures, which granted quantification of: (1) microglial/macrophage "activation" via Iba1 immunoreactivity; (2) microglial/macrophage "activation state" via

morphometric analysis of Iba1+ cells; (3) cellular degeneration via amino cupric "silver stain"; and (4) synaptic density via synaptophysin immunoreactivity.

Study 4: Effects of *Minocycline* on Acute Stress-Induced Changes in Social Avoidance and vmPFC Integrity

The goal of this study was to determine whether minocycline-sensitive mechanisms, such as proinflammatory activity of Iba1+ cells, are causally linked with cellular debris deposition, synaptic density reduction, and social avoidance following acute social defeat stress. We predicted that socially defeated animals treated with minocycline would demonstrate reduced Iba1+ expression and cellular morphology indicative of reduced inflammatory states. We also predicted that minocycline treatment would correspond with reduced amino cupric binding and increased expression of synaptophysin. Finally, we predicted that minocycline treated animals would display less social avoidance following acute social defeat stress, but would show no changes in socially avoidant behavior in the no-stress group.

To complete this study, animals (n = 21) were assigned to either the acute social defeat stress or no stress control conditions and orally administered the antibiotic *minocycline* (Sigma Aldrich) in sterilized drinking water (4 mg/ml), which was provided *ad libitum* in drip-resistant bottles, which were protected from light exposure. This amount was chosen based on the average water consumed by 9-10 week-old hamsters in our laboratory (5 ± 2 mL/day), which would yield a target dosage of approximately 120-140 mg/kg/day. Importantly, little is known regarding minocycline dosage in Syrian hamsters, particularly in behavioral studies. Nonetheless, these doses are consistent with others in hamsters (*e.g.* Tully et al., 2011). Moreover, preliminary

data indicated that lower dosages did not reduce Iba1 expression or alter stress-induced microglial morphology after 3 days of administration. Throughout minocycline administration, all animals were monitored for alterations of weight, in-cage behavior (*e.g*. locomotion and sickness displays), or distress associated with antibiotic administration (*e.g.* excessive itching, diarrhea). Importantly, no such signals were detected and the average daily consumption of minocycline was 4 ± 1 mL/day to yield an approximate dose of 106 ± 15 mg/kg/day.

Freshly made minocycline solution was adjusted to pH = 7.4 and provided daily for 5 total days beginning on the 5th day of territory establishment. On the third day of administration (7 days after territory establishment), hamsters were exposed to the acute social defeat procedure and, 24 hours later, the CD and SIT assays alongside (and identical to) subjects in **Study 3**. Inasmuch, the DSC groups in **Study 3** served as water-drinking controls for minocycline drinkers and all behavioral and immunohistochemical data are expressed as a percentage of *DSC + No Stress* controls. It is noteworthy that minocycline was continually administered until 5 minutes prior to euthanasia, at which point animals were permitted access to regular drinking water. At this time, all animals assigned to minocycline drinking groups quickly consumed drinking water and were allowed to drink until satiated (30+ seconds each), which indicated a natural aversion to the minocycline solution.

Social Defeat Procedures

For all studies, acute social defeat stress was administered using a resident:intruder paradigm wherein subject hamsters were sequentially placed into the home cages of three, larger, sexually experienced hamsters who were prescreened for aggression, termed *resident*

aggressors. Each defeat episode was allowed to persist for 5 minutes after the resident aggressor attacked and the subject displayed submissive behavior (*i.e*. 15 minutes of social defeat exposure total). Between each social defeat encounter, subjects were returned to their home cages for 5-minute inter-trial intervals. To control for social defeat stress, subsets of hamsters in each study were similarly exposed to the empty home cages of three resident aggressors for three 5-minute exposures, which accounted for exposure to a novel environmental and olfactory cues. All defeat procedures were monitored by trained experimenters who quantified the aggression received by each subject in real-time similar to that described in Chapter 2. Briefly, the frequency of attacks and other aggressive behaviors (*e.g.* chase, push) was recorded. In each of these cases, an average was calculated to ascertain whether there were differences in aggression received by treatment condition. Whether the subject fought back against the resident aggressor was also recorded. Following social defeat encounters, whether an animal was wounded with signs of tissue damage was recorded as well. In such cases, experimenters assigned a categorical score based on the estimated diameter of the wound, which included lesions ranging from a non-penetrative abrasion to a laceration that penetrated the hypodermis. In the event that an animal displayed multiple wounds, diameters were summated when assigning a wound score (0 = no signals of injury; 1 = > 1 mm cumulative diameter; 2 = 1-2 mm; 3 = 2-5 mm; 4 = > 5 mm). Animals with a wound score of 4 were removed from the study *a priori*. Any animals with wound scores of 3 or lower were treated with antiseptic solution and monitored. To control for the effects of wounding, all animals showing any lesions were removed from analysis and statistical tests were recalculated. Spearman correlations were used to assess the relationship between wound scores and each dependent variable.

Social Avoidance Assays

Conditioned Defeat

The CD assay is based on observations that, following a social defeat experience, Syrian hamsters will forego species-typical territorial defense when a non-threatening intruder is placed in their home cage (Potegal et al., 1993; Huhman et al., 2003). Briefly, subject hamsters were tested in a 5-minute encounter in their home cage wherein a younger, non-aggressive intruder was introduced. All encounters were monitored in real-time by trained experimenters and recorded digitally for subsequent behavioral analysis of the resident subject (Noldus Observer). A full ethogram is provided in Appendix D. Briefly, mutually exclusive durations of social, nonsocial, submissive/defensive, and aggressive behaviors were calculated, except where noted. Inter-rater reliability was established for aggressive and submissive/defensive behavior by reaching >90% agreement on total durations.

Social Interaction Testing

For SIT assays, subjects were placed into a neutral cage in two, sequential 5-minute trials (denoted as "Trial 1" and "Trial 2"). During Trial 1, an empty perforated box (7 x 14 x 7 cm) was placed against the wall of the cage to habituate the animal to the novelty of the arena and measure baseline locomotion. At the completion of Trial 1, the subject was momentarily removed, a second perforated box containing a novel conspecific replaced the empty box, and the subject was reintroduced to the novel arena for Trial 2. In both trials, the testing arena was divided into three zones: the *far zone* (1/2 of arena not containing the target box), the *interaction zone* (all space within 3 cm of target box), and the *near zone* (remaining portion within the 1/2 of

the arena containing target box while excluding the *interaction zone*). Testing was digitally recorded from overhead and trained observers quantified the duration of time the subject spent in each zone as well as specific types of submissive, social, and non-social behavior (*i.e.* avoid, flee, stretch attend, flank mark, and self-grooming, see Appendix D). Importantly, subjects were able to climb onto the perforated box while exploring the cage walls and ceiling. To control for such non-social exploration within the *interaction zone*, the animal was only scored as occupying the *interaction zone* if actively attending to the perforated box (*i.e.* snout oriented toward and within 3 cm of target box). In that regard, when animals occupied the *interaction zone* while visually attending elsewhere, they were scored as occupying the *near zone*. Except where noted, data are presented as cumulative durations in each zone per trial or as a ratio of zone durations (*i.e.* Trial 2:Trial 1), which accounts for baseline investigation and neophobia.

Euthanasia, Tissue Processing, and Immunohistochemistry

All animals in this report were euthanized via transcardial perfusion with approximately 100 mL of 0.1M phosphate buffer (PB) followed by approximately 100mL of 4% paraformaldehyde in 0.1M PB (PFA) for tissue fixation. In **Studies 1 and 2**, brains were immediately removed from the skull following perfusion and placed in PFA at a 1:10 (v:v) ratio on an orbital shaker at room temperature for 24 hours. Then, brains were treated for 48 hours in 30% sucrose in 0.1M PB and prepared for tissue sectioning. In **Studies 3 and 4**, brains remained in the decapitated skulls following perfusion and were similarly placed in agitated PFA at room temperature but for 48 hours at a 1:100 ratio. This step is necessary to avoid contaminated amino-cupric staining known as the Cammermeyer effect, wherein silver may be absorbed by

neurons that are not fully fixed by PFA and were physically damaged during extraction, rather than through naturally occurring or experimental treatments (Cammermeyer, 1978; de Olmos et al., 1994). Tissue was then carefully removed from the skull and returned to fresh PFA at a 1:10 ratio for 24 hours. Afterward, tissue was treated with 20% glycerol and 2% dimethysulfoxide to prevent freeze-artifacts during sectioning.

For sectioning of tissue in **Studies 1 and 2**, brains were placed in cold (6-8^{o}C) 0.1M PB and sectioned at 40μm on a vibrating microtome. Coronal tissue slices containing vmPFC were immediately placed in cryoprotectant solution and stored at -20^{o} C until immunohistochemical staining. For sectioning of tissue in **Studies 3 and 4**, cryoprotected brains were embedded in a gelatin matrix using MultiBrain® Technology (Neuroscience Associates Inc). Blocks were then rapidly frozen after curing by immersion in 2-methylbutane and chilled with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. Blocks were then sectioned coronally at 40μm and placed in an antigen preserve solution (49.5% phosphate buffered saline pH 7.0, 49.5% ethylene glycol, 1% polyvinyl pyrrolidone) and stored until immunohistochemical staining.

All immunohistochemical procedures followed a generalized protocol (except where noted) that was conducted at room temperature on an orbital shaker for constant agitation. Briefly, free floating slices (**Studies 1 and 2**) or MultiBrain® sections (**Studies 3 and 4**) were washed in 0.1M Tris-buffered saline (TBS), incubated for 30 minutes in 0.9% hydrogen peroxide solution in TBS with 0.02% Triton X-100 (TBS-Tx), and washed again before incubating overnight in TBS-Tx containing stain-specific primary antibody (summarized below). The following day, tissue was rinsed in TBS and then incubated for 1 hour in secondary solution directed against

each primary's host (1:1000, Vector Labs) which was dissolved in TBS-Tx. Tissue was rinsed again and incubated for 2 hours in an avidin-biotin-horseradish peroxidase complex (ABC Vectastain®, Vector Labs) per kit instructions. Tissue was rinsed again and allowed to react for 10 minutes with the chromogen, diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich) with nickel enhancement (Iba1 stains only) and hydrogen peroxide. Tissue was then rinsed and mounted on microscope slides (slides used for **Studies 3 and 4** were gelatin coated) before air dried overnight, dehydrated in alcohols, cleared in xylene, and coverslipped using toluene as a mountant.

Tissues from **All Studies** were immunohistochemically stained using a monoclonal primary antibody raised in rabbit and directed against Iba1 (Abcam, 1:10,000) with a nickel-enhanced DAB reaction for microglial/macrophage detection (every 6th slice for **Studies 1 and 2**; every 8th slice for **Studies 3 and 4**). Importantly, Iba1 is expressed in both brain-resident microglia and peripherally derived monocytes that can act as macrophages (Oshawa et al., 2000). While it is likely that most, if not all, Iba1+ cells identified across Studies 1-4 were microglial (*i.e.* brain resident immune cells), we were unable to definitively differentiate cellular origin using the procedures described in this report.

A subset of sections from **Study 1** were stained using polyclonal primary antibody raised in rabbit and directed against CD68 (Rockland, 1:1,000 – 1:100,000), however almost no signal was detected at any concentration despite confirmation of staining in positive controls. Inasmuch, a review of CD68 procedures, as well as a review of positive controls for all applicable histology, can be found in Appendix E but will not be discussed further.

Additional tissue sections from **Studies 3 and 4** were stained using a monoclonal primary antibody raised in mouse and directed at synaptophysin (Sigma Aldrich, 1:50,000) to quantify

synaptic densities of the vmPFC. Finally, additional sections from **Studies 3 and 4** were stained using the de Olmos amino cupric "silver stain" technique (de Olmos et al., 1994) according to propriety protocols (Neuroscience Associates Inc.) and counterstained with Neutral Red to quantify cellular degeneration in the vmPFC.

Image Analyses

For **All Studies**, bright-field images of the vmPFC were captured using an Olympus BX51 microscope. To permit quantifications for each stain of the PL and IL cortices separately, 8 images per stain per subregion per animal were collected using Olympus DP Controller along a rostro-caudal axis at a 20x (Iba1 and synaptophysin) or 10x (silver stain) magnification. Thusly, each image was 439μm x 330μm (width x height) for Iba1 and synaptophysin and 878μm x 660μm for silver stain analyses. This yielded approximately 5,300 images for analysis across all applicable immunohistochemical stains. Importantly, no differences were detectable across rostro-caudal, hemispheric, or dorsal-ventral axes (except where noted) and images were pooled for analyses accordingly. To expedite quantification of histology, the 20x magnification was selected to facilitate multiple analyses per whole image (*i.e*. optical densities and morphometry of Iba1+ microglia/macrophages) within the PL and IL subregions only; lesser magnifications produced images that included tissue lateral and dorsal of PL (*e.g.* corpus callosum and anterior cingulate) and lateral and ventral to IL (*i.e.* accumbens and dorsal tenia tecta). Moreover, by centering the viewfinder within the IL or PL, a 20x magnification produced more accurate analyses of microglial/macrophage dendrites for morphometry as well as an inclusion of cortical layers II-VI. Inasmuch, this magnification was also selected for quantification of synaptophysin

immunoreactivity, however the viewfinder was positioned more medially to reliably include cortical layers I-V, as *a priori* predictions based on qualitative appraisals from silver stain analyses yielded greater degeneration along the midline, which contain greater concentration of neuronal dendrites and axon terminals. Therefore, the 10x magnification was selected for analyses of silver stain images to expedite qualitative analyses of larger tissue areas in a selective manner based on anatomical localization permitted by counterstaining.

Quantifications of optical densities (OD) of Iba1 and synaptophysin immunoreactivity were conducted using Fiji software (Fiji is just Image J, National Institutes of Health) according to common standardized protocols. Briefly, following web-directed calibration procedures, images were converted to 8-bit and the thresholds for detection were adjusted on an image-by-image basis using Fiji's auto-thresholding feature. All OD data are presented in arbitrary units (AU) produced by Fiji, except where noted.

Morphometric analyses of microglia/macrophages were conducted by selecting four, optimally focused, non-overlapping Iba1+ cells that were fully captured in the image and resided closest to the outermost corners of each of the four quadrants of an image. This approach was selected for several reasons. First, it allowed an unbiased selection of cells in the dorsal and ventral aspects of the image that were most closely associated with cortical layers II/III and V/VI of the PL and IL cortices. Second, it permitted an unbiased selection of cells that could be more readily replicated by multiple observers to ensure high inter-rater reliability. Third, it permitted an unbiased selection of cells that were optimally focused for morphometry measurement procedures, which were derived from approaches used elsewhere (see Cogut et al., 2018) and are discussed below. Importantly, while cortical layers II/III and V/VI were targeted *a priori* for

cell selection in morphometric analyses, the aforementioned selection criteria coupled with higher magnification occasionally forced the selection of cells more centralized within each quadrant.

Morphometric analyses of microglial/macrophage were conducted to permit inferences of activation state by manually tracing the maximal diameters of the cell soma and the cell branches. This approach permits a calculation of both maximal soma diameter and a ratio of cell soma to total cell size. This is important given that, in low states of inflammation within cortical tissue, microglial cells occupy a surveillant state marked by small somatic diameters and lengthy, though minimally branched, dendritic processes (see Fig. 1.1; Hinwood et al., 2012, 2013). Furthermore, there is low occurrence of peripherally derived monocytes occupying macrophagic states. Together, quantification of somatic diameters as well as ratios of somatic diameters to total cell diameters of Iba1+ immune cells within cortical tissue at low inflammatory states is expected to yield a smaller value when compared to higher proinflammatory/phagocytic states. This change in diameter is due to the fact that increased threat detection and detection/release of proinflammatory cytokines leads to a progressive expansion of somatic diameter coupled with gradual retraction of dendrite-like branches. In that regard, microglia occupying higher proinflammatory states should yield larger soma diameters as well as ratios that more closely approach a value of "1" (*i.e.* an "amoeboid" appearance and the highest potential for phagocytosis and tissue destruction). Furthermore, infiltrating macrophages occur at much higher levels in high proinflammatory states, at which point a detected macrophage would similarly reveal a ratio approximating a value of "1". Accordingly, we traced each selected cell

using the manual, linear tracing function in MCID Core image analysis software (InterFocus Imaging).

For analyses of stress-induced degeneration of grey matter in the vmPFC, silver stained tissue slices were analyzed using qualitative appraisals of the degree of degeneration on a 0-4 scale (0 = no signals of degeneration, 4 = maximal degeneration; see Appendix C for exemplars). This approach was necessary due to an array of inevitable artifacts resulting from silver stain procedures that would confound OD measurements (see Switzer, 2000) and analysis occurred only after training by histologists with expertise in the silver stain approach at NeuroScience Associates (Knoxville, TN). Because of the qualitative nature of this approach, degeneration states were determined for the vmPFC as a whole. For an additional analysis of stress-induced degeneration, we counted the number of degenerated cells in the IL and PL that were labeled with the silver stain, which included cells characteristic of neurons (*e.g.* apical dendrites extending toward the midline) and non-neurons (*e.g.* "starburst" pattern; See Fig. 3.5 and Appendix E).

To ensure reliability of measurements from all histological analyses across multiple experimenters, thresholds for inter-rater reliabilities during training were set at no less than 90% agreement. For all analyses, experimenters were blind to treatment conditions.

Statistical Analysis

The majority of data were analyzed using Student's T-test and multivariate factorial analysis of variance (ANOVA) followed by Tukey's post hoc analyses, where appropriate. One-way ANOVAs were calculated to determine whether the aggression received during social defeat

episodes differed by group assignment. Wounding and fighting back during social defeat were analyzed using a Chi-squared test and lesion size categories were analyzed using the non-parametric Kruskal-Wallis test. Bartlett's test was used to assay equality of variance, where noted. If homogeneity of variance was not confirmed, data were analyzed using the Kruskal-Wallis or Mann-Whitney U tests, where appropriate. Because **Studies 3 and 4** were run in concert with treatment levels mixed within each of 5 cohorts, data for experimental groups in **Study 4** were calculated as a percentage of *DSC + No Stress* controls, whose raw data were included in the analyses for **Study 3.** Non-parametric, Spearman correlation coefficients were calculated for each reported dependent variable against wound size categories as well as wound frequency, and these analyses confirmed that wounding patterns were not associated with stress-induced changes in vmPFC tissue and social avoidance behavior. Pearson correlation coefficients were calculated for Iba1+ expression against histology conducted in **Study 3**, where noted. Scatter-plotted data are presented to include means and standard deviations. Alpha levels for all analyses were set at $p = 0.05$.

Results

Social Defeats (All Studies)

For **All Studies** in this report, the amounts of aggression received during acute social defeat did not significantly differ between each treatment level or by study assignment. One-way ANOVAs on total attacks ($F_{5, 60} = 1.44$; $p = 0.23$) indicate that defeat-, drug-, or status-dependent differences were not associated with systematic variation in intensity of aggression received

during social defeat. Within **Studies 1, 2, and 4,** there were no significant differences in the proportion of animals fighting back against the aggressor, however a Chi-Square test revealed significant differences in the number of animals fighting back in **Study 3** ($X^2 = 5.630$; $p = 0.018$; $df = 1$). Specifically, 4 of 10 dominant hamsters, 0 of 11 subordinate hamsters, and 0 of 9 DSCs fought back against their respective aggressors. Fighting back only occurred during the first of three defeat episodes. There were no significant differences in wound size across treatment conditions of all studies (Kruskal Wallis test, $X^2 = 10.04$; $p = 0.26$; $df = 8$). Of 95 animals engaging in agonistic encounters across the 4 studies of this report, 37% (n = 35) showed at least one lesion. In a majority of cases (74%; n = 25), this amounted to one or two visibly small abrasions with no detected epidermal lacerations. However, 4 animals were removed from their respective studies due to lacerations that exceeded 5mm in diameter when all wounds were summated and 5 animals received small lacerations that averaged 2.25 ± 2.23 mm in cumulative diameter; however, in no cases did lacerations penetrate the hypodermis. For all dependent variables elsewhere in this report, Spearman correlation coefficients were calculated against wound category. The only scenario where a studied outcome significantly correlated with wounding was "infralimbic cellular degeneration/starbursts" ($r = -.3361$; $p = 0.039$). Importantly, a total of 24 correlations coefficients were calculated against wounding across all studies, yielding an expectation that approximately 1.2 contrasts would produce a type I statistical error. In that regard, interpretation of this association should be made with caution.

Acute Social Defeat-Induced Alterations of Microglia/Macrophage: Interactions with Subsequent LPS Challenge

In **Study 1,** we sought to determine whether acute social defeat stress, either alone or when preceding a systemic injection of the endotoxin, lipopolysaccharide (LPS), led to altered Iba1 expression patterns in microglia/macrophagic cells within the vmPFC. Using a 2x4, multifactorial ANOVA (Stress x LPS dosage) to determine changes in Iba1 OD, we found that there was a significant main effect of stress in the PL ($F_{1, 50}$ = 11.41, p = 0.001), which suggests that acute social defeat stress drives an increase in Iba1 expression within the PL (Fig. 3.2b). Of note, post hoc tests revealed no significant effect of defeat in saline-treated animals (p = 0.19), yet at 20 mg/kg, there was a significant increase in Iba1 expression brought on by acute social defeat stress (p < 0.05). Furthermore, likely due to heterogeneity of variance between defeated and non-defeated groups, there were no significant differences in Iba1 expression brought on by defeat at either to 100 mg/kg or 500 mg/kg doses, although variance in Iba1 OD was greater in defeated animals. Within the IL (Fig 3.2c), we found similar significant main effects of stress ($F_{1, 50}$ = 4.879; p = 0.032) and dosage ($F_{3, 50}$ = 3.658; p = 0.019) and post hoc analyses revealed a significant difference brought on by social defeat at the 20 mg/kg dose only (p < 0.05).

Together, these data suggest that stress drives neuroinflammatory mechanisms that facilitate an increased expression of Iba1 within immune cells of the vmPFC following LPS administration. This change was most uniformly detectable at the 20 mg/kg dose of LPS. As LPS dosage increased, defeat-induced changes in Iba1 expression became more variable in both the PL and IL. Importantly, as the proinflammatory activity increases, the morphology of Iba1+ cells changes and the geometric confirmations that result are indicative of proinflammatory function

(Folkman & Moscana, 1978; McWhorter et al., 2013; Gordon & Pludermann, 2017). Accordingly, measurements of the soma size (i.e. maximal diameter) as a ratio of total cell size permits an inference of morphological changes where increased somatic diameters as well as increased ratios of soma to total cell size suggest higher proinflammatory activity. Inclusion of this alternative measurement provides more powerful insights of Iba1+ cellular activity given that OD measurements alone may vary as a function of total number of pixels. This is particularly relevant given that extensive branching may increase the number of quantified pixels without accounting for morphological states of assayed cells.

To first determine if stress-induced changes in cell morphology occurred across all dosage levels, three cells per image were randomly chosen in a subset of subjects (n = 5) from each of the 8 treatment levels and two trained experimenters who were blinded to subjects' group assignments rated these cells on their activation state (see Fig. 1.1; Hinwood et al. 2012, 2013). Qualitative appraisals suggested that the vmPFC Iba1+ cells of subsets of subjects at higher dosages did appear to occupy higher proinflammatory activation states as indicated by morphometry (data not shown). Accordingly, we used an objective, morphometric approach that included the lowest-administered dosage necessary to induce detectable changes in both Iba1 expression and qualitative appraisals of morphometry (20 mg/kg) and compared them to LPS controls (0 mg/kg). A multifactorial 2x2 (Stress x LPS) ANOVA on the diameters of somas of Iba1+ cells in the PL revealed a significant main effect of LPS ($F_{1, 29} = 5.639$; $p = 0.024$; Fig. 3.2D). Post hoc analyses indicate that this effect was likely carried by larger soma diameters in the group that was not stressed yet received 20 mg/kg LPS treatment ($p < 0.05$ when compared with saline-treated groups). There were also marginal increases in somatic diameter brought on by stress in

the groups receiving LPS ($p = 0.12$). Secondly, an analysis of the ratio of cell soma diameters to total cell diameters in the PL revealed no significant differences between groups (Fig. 3.2F).

Morphometric analyses in IL revealed similar patterns as those observed in PL. We found a significant interaction in somatic diameters across stress and LPS conditions ($F_{1, 29} = 4.447$; $p = 0.044$; Fig. 3.2E). Post hoc analyses reveal that this interaction is due to larger diameters in non-stressed subjects receiving 20 mg/kg LPS, which is consistent with patterns occurring in the PL ($p < 0.05$ when compared with saline-treated groups). There was also a marginal increase in somatic diameters brought on by stress in those animals receiving LPS treatment ($p = 0.10$). A subsequent analyses on the ratio of somatic to total cell diameters also revealed a significant interaction ($F_{1, 29} = 4.517$; $p = 0.042$; Fig. 3G). Post hoc analyses indicate that this interaction is likely carried by small ratios in the no-stress, no-LPS controls which clustered tightly around the mean ($p < 0.05$ when compared with no stress 20 μg/kg and social defeat saline groups). On the other hand, all other groups displayed a wider spread of ratio scores.

It should be noted that for each of the statistical analyses described above, the Bartlett's test was also conducted to assay equality of variance across all groups within a given contrast and, where appropriate, the data were recalculated using the Kruskal-Wallis test. Importantly, the contrasts of Iba1 OD in PL and IL violated the assumption of homogeneity of variance. However, subsequent reanalysis with the Kruskal-Wallis test produced similar findings (data not shown). The Bartlett's test also frequently revealed marginally significant results for morphometric analyses (*i.e.* soma diameter in PL: $p = 0.06$; ratios in PL: 0.05; ratios in IL: 0.13). Together, the results of these assays support the qualitative observation across all treatment levels that social defeat stress, LPS administration, and their interaction generally induces greater

variability of immune-cell structures and Iba1 expression when compared to controls, which may be due to individual differences in the innate immune system's response within the brain.

Prolonged Activation of Microglia/Macrophages Following Acute Social Defeat Stress

The results of **Study 1** suggested that acute social defeat stress, in the absence of LPS, drives mechanisms within Iba1+ cells that facilitate proinflammatory activity, at least within subsets of socially defeated animals. To determine if these changes persisted in time, hamsters in **Study 2** were subjected to an acute social defeat stress or non-stress control procedures and allowed to remain undisturbed in their home cages for 1 week following stress. A Student's T-test revealed that acute social defeat induces a marginal increase in Iba1 OD within the PL ($t = 1.48$; $df = 16$; $p = 0.16$; Fig. 3.3A) but not IL ($t = 0.12$; $df = 17$; $p = 0.90$; Fig. 3.3B). With regard to morphometry, acute social defeat stress elicited no detectable changes in somatic diameters in either subregion (Fig. 3.3C,E). However, social defeat lead to significant increases in the ratios in diameters of somas to total cell size within PL ($t = 2.75$; $df = 16$; $p = 0.01$; Fig. 3.3D) and IL ($t = 2.61$; $df = 16$; $p = 0.02$; Fig. 3.3F). Testing for homogeneity of variance within these ratios using Bartlett's test revealed a significant difference in the PL ($p = 0.02$) and a marginal difference in the IL ($p = 0.06$), suggesting that social defeat increased the variance in ratios between Iba1+ somas and total cell diameters. Subsequent non-parametric tests revealed marginally significant differences in the PL (Mann-Whitney U; $p = 0.082$) an IL ($p = 0.126$).

Acute Social Defeat-Induced Alterations of Microglia/Macrophages and vmPFC Integrity: Interactions with Dominance Status

The results from **Studies 1 and 2** collectively suggest that acute social defeat induced alterations within Iba1+ cells, which may induce proinflammatory and neurodegradative states, at least within subsets of acutely defeated hamsters. Moreover, these states may persist for as long as one week. Importantly, it is possible that the increased variability of scores within groups receiving stress in both studies may be due to individualized responses to acute social defeat stress, perhaps due to dissociable social experiences these subjects received prior to experimentation through the formation and maintenance of social dominance statuses.

Accordingly, **Study 3** was designed to investigate whether social dominance may account for the differences in Iba1 expression and morphometry of microglia/macrophages within the PL and IL. Further, we sought to determine whether stress- and dominance status-induced changes in Iba1+ cells corresponded with alterations in cellular degeneration and synaptic densities. Finally, to test whether stress- and status-dependent neurohistochemical changes were associated with changes in social behavior, we measured social avoidance in counterbalanced CD and SIT assays that were administered 24-hours prior to euthanasia.

Proinflammatory Activity within vmPFC: Iba1+ OD and Morphometry

Using a multifactorial, 2x3 (Stress x Social Status) ANOVA of Iba1 OD in dominant, subordinate, and DSC animals, we found a main effect of stress in the PL ($F_{1, 55} = 10.31$; $p = 0.002$; Fig. 3.4A). Post hoc analyses indicate that social defeat stress induced an increase in Iba1 OD in dominant ($p < 0.05$) and subordinate animals ($p < 0.05$), as socially defeated dominants and

socially defeated subordinates were statistically different from all other groups. Furthermore, there were no differences detected between DSCs who were or were not defeated. Moreover, dominant and subordinate animals, whether defeated or not, did not differ from one another. When analyzing Iba1 OD within the IL, we found an interaction of stress and social status conditions ($F_{2, 56} = 4.421$; $p = 0.017$; Fig. 3.4B). Post hoc analyses of Iba1 OD in the IL yielded patterns similar to those found in PL ($p < 0.05$ for all applicable posthoc tests).

Morphometric analyses of Iba1+ cells in the PL yielded no significant differences in somatic diameter or ratios of somatic and total cell diameters (Fig. 3.4 C,E). However, this was not true of stress- and status-dependent changes in cellular morphology within the IL. With regard to somatic diameters, we found main effects of stress ($F_{1, 56} = 11.36$; $p = 0.001$) and status ($F_{2, 56} = 18.93$; $p < 0.0001$; Fig. 3.4D). Post hoc analyses revealed several important findings. First, there were clear effects of social status on baseline somatic diameters. Specifically, non-defeated subordinate animals displayed longer soma diameters than non-defeated dominants ($p < 0.05$). Also, acute social defeat stress induced an increase in Iba1+ soma size in DSCs when compared to non-defeated DSCs ($p < 0.05$). On the other hand, there were no other detected differences brought on by stress when comparing the effects of acute defeat within either dominant or subordinate social statuses. That is, acute social defeat did not induce a shift in somatic diameters within dominant or subordinate animals.

With regard to the ratio of somatic and total cell diameters in the IL, we found an interaction between stress and dominance status ($F_{2, 56} = 4.601$; $p = 0.014$; Fig. 3.4F). Post hoc analyses revealed the following. First, in the absence of stress, there were differences in baseline ratios when subordinate animals were compared to dominants ($p < 0.05$) and DSCs ($p < 0.05$)

wherein subordinate hamsters had larger ratios, suggestive of a higher proinflammatory activation state. Second, this pattern continued following acute social defeat stress, as socially defeated subordinates again had higher ratios than socially defeated dominant ($p < 0.05$) and DSC counterparts ($p < 0.05$). Third, social defeat stress induced larger ratios within the subordinate social status only ($p < 0.05$). Finally, dominant and DSC hamsters did not significantly differ from one another in either the stress or no stress conditions.

Taken together, these results suggest the following regarding vmPFC Iba1+ cells of animals in **Study 3**. First, the IL is more vulnerable than the PL to dominance status- or defeat-induced insults. Second, social subordination induces changes in Iba1 OD and Iba1+ cellular morphology, which are reflective of higher proinflammatory states. Third, acute social defeat stress induces changes in markers of proinflammatory activity of Iba1+ cells, especially in animals with a prior subordinate status. Finally, despite exposure to an acute social defeat stressor, dominant animals appear to be protected from alterations of Iba1+ cells such as enlargement of soma size.

Cellular Degeneration

Using an amino cupric "silver" stain, we next sought to determine whether there were increased markers of cellular degeneration within the vmPFC. First, we counted markers characteristic of fully degenerated (i.e. "dead") neurons (see Appendix E). However, across all subjects (including in **Study 4**), we were able to detect a total of only 5 degenerated cells that appeared to be neuronal. These degenerated cells were distributed across treatment levels and, accordingly, no further analyses were conducted.

Given that the silver stain procedure positively labels cellular debris, which could result from degenerated dendrites or terminals of surviving neuronal cells, we also sought to qualitatively characterize the degree of total grey matter degeneration in the vmPFC. Using this procedure, we were not able to detect differences in qualitative appraisals of degeneration state across PL and IL subregions and data were pooled, accordingly. Secondly, across all tissues containing signals of silver-impregnated cellular debris, we noticed greater staining concentrated near the midline, which roughly occupied cortical layers I-III. Results from a 2x3 (Stress x Status) ANOVA indicated that there was a main effect of stress ($F_{1, 56} = 16.85$; $p < 0.0001$; Fig. 3.6.A) on the total degeneration states within the vmPFC. Post hoc analyses revealed a several key findings. First, acute social defeat led to an increase in degeneration in DSC ($p < 0.05$) and subordinate ($p < 0.05$), but not dominant, hamsters. Also, while there was no difference between treatment groups in non-stressed animals, defeated dominants displayed significantly less degeneration markers than did defeated subordinates ($p < 0.05$) or defeated DSCs ($p < 0.05$), both of which did not differ from one another.

During our qualitative analyses, we identified a "starburst" pattern of cellular debris present in tissues from each treatment level. While the origin of these "starbursts" cannot be verified, their structural remains were reminiscent of astrocytes. To determine whether there was a status- or stress-dependent difference in these signatures, we counted the number of starbursts occurring within the PL and IL in each treatment condition. Therein, we found a significant interaction between stress and status groups in the PL ($F_{2, 56} = 3.513$; $p = 0.037$; Fig. 3.6B). Post hoc analyses revealed that acute social defeat induced an increase in starburst debris in DSCs ($p < 0.05$) that were greater even than dominants ($p < 0.05$) and subordinates ($p < 0.05$)

having received social defeat. On the other hand, there were only marginally significant differences ($p < 0.10$) between dominant or subordinate animals that either did ($p = 0.08$) or did not ($p = 0.10$) receive social defeat. The trend in both cases was toward increased starburst patterning in subordinate animals. A similar trend toward increased starburst patterns was found within subordinate animals such that defeat marginally increased degenerated cell counts ($p = 0.10$). Analysis of starburst signatures occurring within the IL revealed similar results. There was a significant interaction ($F_{2, 55} = 3.375$; $p = 0.045$; Fig. 3.6C) and post hoc analyses revealed nearly identical outcomes ($p < 0.05$ for all applicable comparisons) with one exception: no-defeat dominants and no-defeat subordinates did not differ in starburst patterning ($p = 0.86$).

Taken together, these data suggest four key findings. First, regardless of defeat or dominance status, there were very few signs of whole-neuron death. Second, while there were treatment-level differences in markers of degeneration in the vmPFC, including in grey matter and in starburst debris, these signatures were present in all animals, even those not having received acute social defeat stress. Third, across all tissues with elevated markers of tissue degeneration, there were greater concentrations of silver-impregnated cellular debris along the synapse-rich midline. Finally, acute social defeat stress promotes an increase in grey matter and cellular (putative astrocyte) degeneration, though this effect is greater in DCS animals that lack previous experience in agonistic encounters. In that regard, any potential protection conferred by agonistic encounters appears to be highest in dominant hamsters.

Synapse Degeneration

Given the qualitative observations that acute stress induced an increase in grey matter degeneration within vmPFC, particularly along the midline portions where synaptic densities are greatest, we sought to determine whether there were stress- and/or status-dependent differences in synaptophysin OD. A 2x3 (Stress x Status) ANOVA of the PL revealed significant main effects of stress ($F_{1, 56} = 33.40$; $p < 0.0001$) and status ($F_{2, 56} = 3.896$; $p = 0.026$; Fig. 3.6D). Post hoc analyses revealed that social defeat stress induced reductions of synaptophysin across each dominance status group ($p < 0.05$ for each dominance status group). However, there were no detected differences between social status groups in either the defeat or non-defeat conditions. Within the IL subregion, ANOVA revealed a significant main effect of stress ($F_{1, 56} = 38.55$; $p < 0.0001$; Fig. 3.6E). Post hoc analyses of synaptophysin densities in IL revealed identical outcomes to those reported for PL ($p < 0.05$ for all applicable comparisons). Together, these data suggest that acute social defeat, regardless of social status, induced a decrease in synaptic densities within the PL and IL.

Given the observation that Iba1+ expression may be linked with changes in silver deposition and synaptic densities, we conducted Pearson's correlations for each of the aforementioned expression patterns against Iba1+ optical density in the more stress-sensitive IL of the vmPFC. As expected, there were no correlations between Iba1 OD and synaptophysin levels ($p = 0.372$) and no relationship detected among Iba1 OD and starbursts ($p = 0.428$). However, there was a detected relationship between Iba1 OD and degeneration state ($p = 0.011$), which suggests that the collective amount of silver-bound cellular debris may influence proinflammatory activity of Iba1+ cells.

Social Avoidance Behavior

Neurohistological analyses of animals in **Study 3** suggest that dominance status and stress can independently and collectively influence Iba1+ cells as well as cellular and synaptic degradation within the vmPFC. To test whether the observed cellular changes corresponded with alterations of social avoidance behavior, we analyzed CD and SIT behavior, which occurred 24 hours after acute social defeat and 24 hours before euthanasia. For the analyses of CD that follow, the durations of time subjects displayed submissive, defensive, and risk-assessing vigilance behaviors were combined into one category (also called the "conditioned defeat response"). For the analyses of SIT that follow, the durations of time spent in the far side of the arena were significantly correlated with those of the interaction zone and, accordingly, only the far side durations are presented.

For durations of submissive/defensive/risk assessment behavior (*i.e.* CD response) in the CD test, a 2x3 (Stress x Status) ANOVA showed a significant interaction between stress and status ($F_{2, 56} = 5.296$; $p = 0.008$; Fig. 3.7A). Post hoc analyses reveal that acute defeat induced a significant increase in CD response in DSCs ($p < 0.05$) and subordinate ($p < 0.05$), but not dominant hamsters. Socially defeated dominant hamsters also displayed significantly shorter durations in CD response than did socially defeated DSCs ($p < 0.05$) and subordinates ($p < 0.05$). Finally, socially defeated subordinates showed the greatest CD response, as they differed significantly from all other groups ($p < 0.05$ for all applicable comparisons), including socially defeated DSCs ($p < 0.05$). Importantly, these findings are consistent with previous reports from our laboratory that acute social defeat stress induces increased durations of submissive/defensive/risk assessment behaviors in a CD test and, moreover, that dominant hamsters displayed the smallest

increases in submissive/defensive behavior while subordinates display the largest (*e.g.* Morrison et al., 2012, 2014).

For durations of time spent in the far side of the neutral cage in the SIT, results of a 2x3 (Stress x Status) ANOVA revealed an interaction of stress and status ($F_{2, 52} = 5.022$; $p = 0.010$; Fig. 3.7B). Post hoc analyses indicate that, while there were no status-dependent differences in comparisons of far-side durations in animals not having received acute social defeat stress, there was increased defeat-induced avoidance in dominant ($p < 0.05$) and subordinate ($p < 0.05$) hamsters when compared to DSCs. On the other hand, there were no differences between socially defeated dominant and subordinate hamsters. Social defeat stress did increase the time spent in the far side by DSCs ($p < 0.05$).

Because this is the first report to conduct CD and SIT within the same Syrian hamsters following acute social defeat, we also conducted a linear regression analysis of behavior between these two tests among all animals having received social defeat. Results demonstrate that there is no significant correlation between the far side duration in the SIT during Trial 2 and the CD response ($R^2 = 0.11$; $p = 0.128$; Fig. 3.7C). Further, there were no detected effects of test order in the counter-balanced approach. This is also the first report to differentiate active avoidance from the CD response. Results indicate that while there was a marginal effect of stress ($F_{1, 37} = 4.010$; $p = 0.053$) there were no significant differences in active avoidance by status ($F_{1, 37} = 0.054$; $p = 0.82$).

Acute Social Defeat-Induced Alterations of Microglia/Macrophages and vmPFC Integrity: Interactions with Minocycline

The results from **Studies 1-3** suggest that acute social defeat induces changes in Iba1+ cells of the vmPFC that are consistent with increased proinflammatory activity and, moreover, that these changes likely correspond to individual differences derived from the development and maintenance of a social dominance status. Accompanying these defeat- and status-induced changes in Iba1+ cells were increased signals of cellular and synaptic degeneration in the vmPFC, which may be due to immune-induced disruptions of vmPFC anatomical integrity. To address this latter possibility, the subjects in **Study 4** were given the antibiotic, *minocycline*, in their drinking water for 48 hours prior to defeat and continuing daily until just prior to euthanasia. Because all animals from Studies 3 and 4 were run in parallel, data from Study 4 are presented as a percentage of no-stress DSCs.

To determine whether minocycline reduced the activity of Iba1+ cells either alone or in the presence of acute social defeat stress, 2x2 (Stress x Drug) ANOVAs were conducted in the PL and IL on Iba1 OD and cellular morphology in tissue collected 48 hours following defeat or control procedures. Results of Iba1 OD analyses indicate that there were significant main effects of drug in both the PL ($F_{1,38} = 4.245$; $p = 0.046$; Fig. 3.8A) and IL ($F_{1, 38} = 4.121$; $p = 0.049$; Fig. 3.8B). In both cases, post hoc analyses reveal significant increases in Iba1 expression brought on by acute social defeat within minocycline-treated animals ($p < 0.05$ for all applicable comparisons).

With regard to microglial morphology in the PL, there was a significant main effect of somatic diameter ($F_{1, 38} = 10.54$; $p = 0.002$; Fig. 3.8C). Post hoc analyses indicate that minocycline treatment reduced Iba1+ soma size in the PL generally ($p < 0.05$ for both comparisons between

defeated and no defeat groups) and, moreover, that there was a significant difference in soma size between socially defeated water and minocycline drinkers ($p < 0.05$). In contrast, an analysis of somatic by total cell size diameter within the PL revealed no significant differences between water- and minocycline-drinking animals, regardless of social defeat stress (Fig. 3.8E).

Analyses of microglial morphology in the IL revealed more dramatic changes. With regard to diameter of Iba1+ somas, there were main effects of stress ($F_{1, 38} = 10.98$; $p = 0.002$) and drug ($F_{1, 38} = 19.11$; $p < 0.0001$; Fig. 3.8D). Post hoc analyses indicate that while there were no differences in somatic diameter in non-defeated animals, there were differences between those that received defeat ($p < 0.05$), wherein minocycline protected against defeat-induced increases in soma size. These patterns were similar to the ratio of cell soma to total cell size, with main effects of stress ($F_{1, 38} = 9.211$; $p = 0.004$) and drug treatment ($F_{1, 38} = 15.89$; $p = 0.0003$; Fig. 3.8F) and identical outcomes of post hoc analyses ($p < 0.05$ for all applicable analyses).

Next, we analyzed markers of cellular degeneration following minocycline treatment in defeated and non-defeated subjects. ANOVA of qualitative appraisals of degeneration states of vmPFC tissue in Study 4 revealed main effects of stress ($F_{1, 38} = 11.11$; $p = 0.002$) and drug treatment ($F_{1, 38} = 8.668$; $p = 0.006$; Fig. 3.9A). Post hoc analyses indicate that minocycline treatment generally reduced markers of degenerated grey matter within vmPFC ($p < 0.05$ for applicable comparisons). Moreover, there is a significant difference between water- and minocycline-drinking, socially defeated subjects ($p < 0.05$). Within the PL, there was also a significant interaction of stress and drug treatment on the presence of starburst patterns of cellular degeneration ($F_{1, 38} = 13.12$; $p = 0.0009$; Fig. 3.9B). Post hoc analyses reveal that minocycline reduces the number of degenerated cells that resemble the starburst pattern ($p <$

0.05). Furthermore, while stress induces an increase in the number of degenerated cells per tissue slice, minocycline protects against this change ($p < 0.05$). These patterns are mimicked in the IL, where there was a significant interaction of stress and drug treatment in starburst patterns ($F_{1, 38} = 9.126$; $p = 0.005$; Fig. 3.9C). Post hoc analyses similarly reveal that minocycline protects against the defeat-induced increase in starburst staining ($p < 0.05$ for all applicable comparisons).

To determine whether minocycline's protection of cellular and grey matter degradation extended to a protection of synaptic densities, we conducted analyses on synaptophysin within the PL and IL cortices. The results of ANOVA revealed a significant interaction of stress and drug in the PL ($F_{1, 38} = 8.861$; $p = 0.005$; Fig. 3.9D) and post hoc tests indicate that synaptophysin expression patterns were consistent with degradation analyses above ($p < 0.05$ for all applicable analyses). Minocycline appears to protect against reductions of synaptic densities in both the stress and no-stress groups. These patterns were fully replicated within IL (interaction effect: $F_{1, 38} = 5.355$; $p = 0.026$; and $p < 0.05$ for all applicable post hoc contrasts; Fig. 3.9E).

Finally, we tested whether the cellular protection conferred by minocycline treatment corresponded with reduced social avoidance. Intriguingly, while there was a dramatic increase in submissive/defensive behaviors in the CD test in animals having received acute social defeat (main effect of stress: $F_{1, 38} = 30.55$; $p < 0.0001$; Fig. 3.10B), minocycline did not protect against this increase. This having been said, there were notable minocycline-induced differences in the amount of active vigilance, a sub-category of risk-assessment behavior that may convey proactive coping strategies in the face of adversity. When analyzed separately, there was a significant interaction of stress and drug on the duration of time animals spent engaging in active vigilance ($F_{1, 38} = 6.852$; $p = 0.013$; Fig. 3.10C). Post hoc analyses indicated that socially defeated animals

having received minocycline displayed significantly more active vigilance than any other group, including water-drinking, socially defeated counterparts ($p < 0.05$).

Within the SIT test, minocycline similarly failed to prevent defeat-induced social avoidance. There was a significant interaction of stress and drug treatment on time spent in the far side of the arena ($F_{1, 38} = 4.312$; $p = 0.045$; Fig. 3.10A) and post hoc analyses confirmed that minocycline-drinking, socially defeated animals avoided a caged intruder at levels no different than water-drinking counterparts ($p < 0.05$).

Discussion

Ample evidence supports the notion that chronic psychological stress can induce neuroinflammatory processes throughout the brain that can be particularly consequential in regions vulnerable to insult such as the vmPFC (Hinwood et al., 2012, 2013; Hodes et al., 2014; Arnsten, 2015; Menard et al., 2017; Nie et al., 2018). Furthermore, it has been well described that chronic psychological stress can drive degradative processes within the vmPFC that include structural retraction and remodeling of neurons alongside a reduction of synaptic densities (Radley et al., 2004; Goldwater et al., 2009; Grizzell et al., 2014; Patel et al., 2015). Across the four studies discussed in this report, we support the claim that acutely traumatic psychological stress in Syrian hamsters may do the same, particularly via recruitment of minocycline-sensitive activity in vmPFC cells, which strongly implicates neuroinflammatory processes of the brain such as the activation of microglia and, potentially, recruitment of peripherally derived monocytic macrophages.

To summarize, acute social defeat stress drove an increase in Iba1 expression alongside morphometric shifts toward proinflammatory states that, at least on some measures, may persist for at least a week. Furthermore, stress-induced increases in proinflammatory activity were potentiated in a dose-dependent manner by a subsequent immune challenge (*i.e.* LPS) administered 24 hours following stress. Intriguingly however, these changes occur to variable degrees that also appear to be dose-dependent, suggesting that individual differences in Iba1+ cellular responses to social defeat are more readily identifiable as LPS dosage increases. Given that hamsters rapidly and stably form dominance relationships in a resident-intruder procedure, our laboratory has previously identified phenotypic responses to stress that are associated with resilience and vulnerability based on social dominance status. Inasmuch, we have reported on mechanisms promoting resilience in dominant hamsters, such as increased activity of vmPFC neurons (Morrison et al., 2012-2014) in a circuit-specific manner (Dulka et al., 2018a; Grizzell, Chapter 2), and increased androgen receptor density in the medial amygdala (Clinard et al., 2016). On the other hand, the mechanisms promoting stress vulnerability in subordinate hamsters are poorly understood. However, we have reported that subordinate hamsters show increased risk of oxidative stress within vmPFC (Dulka et al., 2017), which suggests that immune dysfunction or hyperactivation of immune cells may correspond with mechanisms promoting stress vulnerability in animals with low social status. Interestingly, a recent study from another group demonstrates that the constituency of the microbial community in the gut of Syrian hamsters is predictive of whether the animal will become dominant or subordinate (Partrick et al., 2018). Furthermore, the shifts seen in microbiota following agonistic encounters are

indicative of an enhanced risk of inflammatory activity (Maes, 2008; Chaissang et al., 2015; Partrick et al., 2018).

With this in mind, we hypothesized that dominance status confers varied Iba1 expression patterns (*i.e.* OD and morphology) following acute social defeat stress, which might be similar to the patterns seen in socially defeated groups also receiving LPS injections in Study 1 (where dominance status was not assessed when animals were group-housed prior to the experiment). Such status-dependent changes in microglia and peripheral monocytes following acute social defeat might therefore correspond with enhanced proinflammatory activity as well as greater disruptions of anatomical integrity in the vmPFC. Inasmuch, vulnerability of immune responses or the oxidative stress that follows might therefore explain why vmPFC neurons were not as readily recruited during traumatic stress exposure in the subordinate hamsters across an array of studies from our laboratory (Morrison et al., 2012, 2014; Dulka et al., 2018; Grizzell, 2019: *Chapter 2*).

Following the establishment and two-week maintenance of social dominance, we found that acute defeat led to an increase of Iba1 expression in both dominant and subordinate animals, but that dominant hamsters were protected against the morphological changes occurring in Iba1+ cells. These effects were especially pronounced in the IL of socially defeated subordinate hamsters as well as socially defeated DSCs. Consistent with our hypothesis, we also found that markers of proinflammatory activity and a subordinate social status corresponded with increased markers of tissue degeneration in the vmPFC, yet these changes were again uniquely absent in dominant hamsters. With this said, we did not detect a status-dependent, defeat-induced reduction of synaptic densities, as social defeat stress instead reduced vmPFC

synaptophysin levels uniformly. Taken together, our data suggest that although stress-induced degeneration of vmPFC tissue is greatest in subordinates and DSCs, acute social defeat drives mechanisms that reduce synaptic densities regardless of previous dominance status. Inasmuch, we cannot be certain whether synaptic degeneration in the vmPFC is mediated by proinflammatory activity in a status-dependent manner. Accordingly, we propose a few alternative explanations for this discrepancy. First, defeat may induce status-dependent differences in synaptic degradation, yet synaptophysin OD measurements are not sensitive or selective enough to detect such changes. Second, defeat-induced reductions of synaptic densities are not consequential for dominance status-dependent behavioral and/or the neural responses to acute social defeat. In that regard, synaptic reductions may not, themselves, be a marker of stress-induced impairment, but of stress-induced remodeling necessary for plasticity associated with vmPFC-dependent learning following stress. Third, Iba1+ cell activity in dominant hamsters may be more efficient within the vmPFC, clearing synaptic densities and/or cellular debris more selectively. In that regard, subordinate and DSC Iba1+ cells may clear or strip synapses at the same rate, yet in a non-selective manner. Fourth, it is possible that acute social defeat-induced synaptic degradation and tissue degeneration occur uniformly, yet the Iba1+ cells of dominant hamsters are more efficient at debris clearance. Alternatively, Iba1+ cells of dominant hamsters may not be more efficient than those of subordinates or DSCs, yet the environmental milieu within the dominant vmPFC may be more protective against the ROS and subsequently destructive oxidative stress inevitably produced by phagocytosis and debris clearance. This possibility is supported by the fact that metabolomics analyses of dominant vmPFC and nucleus accumbens, when compared to those of subordinates and no-stress controls, revealed higher

levels of molecules that promote anti-oxidation and thus reduced oxidative stress, such as metathione and fumarate (Dulka et al., 2017).

Because microglia activity can correlate with each of the stress- and status-dependent changes described above, we lastly tested whether systemic treatment with minocycline, a tetracycline known to halt Iba1+ proinflammatory activity (Kim & Suh, 2009), could reverse many of the putative deficits of vmPFC anatomical integrity shown in Studies 1-3. Although minocycline led to an upregulation of Iba1 OD, it also normalized Iba1+ cellular morphology to non-stress levels and significantly reduced markers of cellular and synaptic degradation. While the OD data may appear disparate at first glance, it is possible that this effect is due by a stress-induced increase in microglial activation, which was not fully protected by minocycline treatment. That said, early phases of microglial recruitment are marked by an extension of microglial dendritic length and increased branching, yet unchanged soma sizes (Hinwood et al., 2012, 2013). Moreover, across all levels of proinflammatory activity, this early-phase activation state is also marked by the lowest levels of cytokine release, ROS production, microglial phagocytosis, and peripheral monocyte recruitment. For that matter, OD measurements might poorly reflect activation states if IHC procedures are optimized to show maximum Iba1 labeling in ramified cells. Taken together, these neurohistology data demonstrate that stress-induced perturbations of vmPFC morphology can be protected by minocycline administration. This strongly implicates proinflammatory microglial activity in the acute stress-induced reduction of vmPFC cellular and synaptic degradation. Additionally, these data do not rule out peripherally derived monocytes in such degradative processes, and more studies would be needed to confirm the source and precise function of each of these immune cell types.

We were unable to demonstrate that minocycline treatment and its associated protection of vmPFC tissue translated to a reduction of defeat-induced social avoidance in the conditioned defeat (CD) and social interaction (SIT) tests. However, two interesting observations emerged. First, there was a wide distribution of social avoidance scores following acute social defeat in both the SIT and CD tests for water-drinking subjects and within the CD test for minocycline drinkers. This is consistent with the observations from Study 1 where quantification of Iba1+ cellular responses to stress yielded more widely spread distributions than in non-stressed groups, suggesting that there are individual differences in stress-induced social avoidance much like there were individual differences in neuroimmune responses to stress + LPS treatments. Importantly, we did not identify or experimentally facilitate dominance status relationships in animals assayed in Study 4. Second, we observed a minocycline-induced shift in the behavior patterns of a subset of socially defeated hamsters in the CD test. Briefly, the CD test is based on the premise that naïve Syrian hamsters will reliably defend their territory upon intrusion by a conspecific. However, following defeat, hamsters will display submissive and defensive postures in response to an invading animal, even if that animal is younger and displays no aggressive behavior (Potegal et al., 1993; Huhman et al., 2003). Accordingly, the CD response is generally reported as a summation of the time spent submitting to the intruder as well as time spent engaged in defensive behavior, including various forms of risk assessment. Upon careful inspection in the current study, we noticed that a subset of minocycline drinkers were bolder, yet highly cautious, in their risk-assessment strategies when investigating the intruder. More specifically, minocycline-drinking hamsters spent greater durations of time cautiously yet repeatedly approaching and investigating the intruding conspecific, often circling around to

repeat their observations. Importantly, these animals rarely approached to within 10 cm and their slow approaches were marked by stretched yet mobile bodily postures that were punctuated by rapid avoidance in response to changes in the intruder's behavior. Interestingly, similar "vigilance" behaviors were recently reported in female California mice following social defeat, where animals avoided close proximity yet frequently attended to a caged intruder, even when not situated in the most distal zones of a novel interaction arena (Duque-Wilkins et al., 2018; Trainor, 2019 - personal communication). Our observations in minocycline-treated hamsters are in stark contrast to water-drinking animals following defeat, which often remained in their nests and avoided eye contact. This was evidenced by a "flagging" of head orientation or auditory attendance marked by limited motor movement with no clear visual fixation point. Water-drinking animals also appeared to take greater effort to avoid physical proximity, often remaining within their nests until the intruder approached, at which point they would quickly move to the opposite end of the cage and wait until the nest was clear before returning. Given these observations, we characterized the former behavior pattern as "active vigilance" given that it could clearly be differentiated from within-nest monitoring and distal head flagging, which we termed "passive vigilance". This is in keeping with nomenclature used to describe behavioral coping strategies in response to stress, which have been well described and are usually dichotomized into "active" (also called proactive) or "passive" (also called reactive). Inasmuch, active patterns of coping behavior often include engagement of the subject with the potential threat, where passive coping patterns include limited engagement and high avoidance, particularly from the most distal locations possible. Furthermore, active coping strategies often correspond with reductions of stress-induced consequences and are therefore highly correlated

with resilience-like behaviors (Koolhaas et al., 1999; Wood et al., 2010, 2015). Accordingly, we found that while defeated minocycline animals as a group spent the same amount of time in submissive and defensive patterns of behavior, about 1/3 of animals showed substantially greater active vigilance. With this said, more research would be needed to determine the neural correlates of active vigilance behavior. Importantly, when active and passive vigilance behaviors were quantified in Study 3, few socially defeated hamsters displayed high levels of active vigilance, regardless of dominance status.

It is worth mention that minocycline has been shown to reverse stress- and inflammation-induced behavioral responses such as sickness behavior (Henry et al., 2008), memory impairments (Hinwood et al., 2012, 2013; Reus et al., 2014; McKim et al., 2016), anxiety-like behavior (Neigh et al., 2009; Siopi et al., 2012; McKim et al., 2018; Wang et al., 2018), and depressive-like behavior (Henry et al., 2008; Miyaoka et al., 2012; Rosenblat & McIntyre, 2018). However, the finding that minocycline does not rescue stress-induced social avoidance is not without precedent. For instance, McKim et al. (2016) found that while minocycline did rescue repeated social disruption-induced deficits of spatial memory, there were no changes in stress-induced social avoidance. This finding was replicated when, despite rescuing anxiety-like behavior in an open field, stress-induced social avoidance remained unaltered by minocycline treatment (McKim et al., 2018). This suggests that social avoidance, unlike other assays of stress-induced behavior, may be a poor marker of stress x inflammation-induced disruptions of vmPFC-dependent behavior.

Our results from **Study 1** are consistent with reports of microglial priming in response to brief stress exposure (Quan et al., 2001; Johnson et al., 2002, 2003; Frank et al., 2007; Niraula et

al., 2017), though our results are the first to demonstrate that priming may be possible with a short duration social stressor (*i.e.* 15 minutes total). While the mechanisms of microglial priming are still being elucidated, it appears that they are based on glucocorticoid exposure when a stressor serves as the priming event (as opposed to repeated LPS administration, see Niraula et al., 2017 for review). For example, in rats receiving acute tail shock 24 hours before LPS exposure, treatment with either an adrenalectomy or the glucocorticoid receptor antagonist, RU486, abolished stress-induced priming effects in microglia (Frank et al., 2012). This finding may appear paradoxical, given that glucocorticoids are regarded as anti-inflammatory (Barnes, 1998). However, research using a repeated social disruption task, wherein an aggressive animal is placed into the cage of three, smaller group-housed residents who are reliably subordinated by the intruding aggressor, indicates that immune factors of subordinated animals display a resistance to the anti-inflammatory effects of glucocorticoids (Avitsur et al., 2001; Stark et al., 2001). This is argued to be an adaptive mechanism in the face of continued adversity, such as the case in social subordination, wherein immune cells, through the development of resistance, are able to serve in their capacity for wound healing and tissue cleanup despite repeated glucocorticoid release in response to continued stress and potential injury (Avitsur et al., 2001).

Interestingly, it appears that social stress-induced glucocorticoid resistance requires a secondary signal to serve as a ligand at the Toll-like receptor 4 (TLR4), for instance LPS (Avitsur et al., 2003). In that regard, more recent studies in microglial priming show that acute tail shock-induced priming depends on the release of the danger-associated molecular pattern, high-mobility group box 1 (HMGB-1) by way of the nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3 (NLRP3) inflammasome in rat hippocampus (Weber et al.,

2015). Importantly, HMGB-1 acts as an endogenous ligand at TLR4, present on microglia and peripheral macrophages, during acutely induced inflammation (Park et al., 2004). Moreover, TLR4 (alongside TLR2) within vmPFC microglia were found to not only mediate the social avoidance response in chronically stressed mice, but were also responsible for stress-induced reduction of neuronal dendrites (Nie et al., 2018). By removing TLR2/4 activity during repeated social defeat, Nie et al. (2018) showed that Iba1 expression was lowered, apical dendrites of the vmPFC were spared, and social avoidance behavior was attenuated. These data are consistent with reports from Hinwood et al., (2012, 2013), who showed that repeated restraint stress induced an upregulation of Iba1 in microglia which corresponded with reduced expression of the immediate early gene, ΔFosB in vmPFC tissue, which serves as a marker of cumulative neuronal activation. Moreover, minocycline treatment reduced the expression of Iba1, increased vmPFC ΔFosB, and reduced the behavioral consequences of prolonged stress (Hinwood et al., 2012), suggesting that stress-induced microglial activity is responsible for vmPFC-related neuronal and behavioral consequences of stress. Altogether, it appears that stress may drive signaling cascades resulting in TLR4 activation on microglia that can lead to neuronal degradation and social avoidance. However, whether these are the precise mechanisms occurring following acute social defeat stress in hamsters, particularly subordinate hamsters, remains to be determined.

In light of our findings that acute social defeat increased cellular and synaptic degradation, which was reversed by minocycline treatment, it is noteworthy that we found no direct evidence that microglia (or peripherally derived monocytes) adopted a phagocytic state. In addition to the findings summarized above, Nie et al. (2018) also reported that repeated social defeat in mice induced the upregulation of CD68, a protein that is expressed upon phagocytotic

activity of microglia. However, when TLR2/4 were knocked out, CD68 expression was reduced and apical dendrites of vmPFC neurons were spared (Nie et al., 2018). In pilot studies for this report, we were unable to detect CD68 in the vmPFCs of stressed, LPS injected hamsters, despite the presence of immunolabeling in a positive control study (see Appendix E). Furthermore, within all quantified microglia in Studies 1-4, we were also unable to detect a soma:cell ratio of "1", which would indicate a fully amoeboid appearance and thus the highest degree of phagocytosis. While it is possible that the occurrence of phagocytotic activity may have peaked and then subsided prior to euthanasia, it is of greater likelihood that microglia did not, themselves, adopt phagocytosis, at least not in the amoeboid state that is commonly referenced in studies of neurodegeneration. However, it is possible that microglial-dependent phagocytosis, debris clearance, and synaptic pruning/stripping can occur without microglial cells fully progressing to an amoeboid shape. Indeed, repeated social stress can weaken the blood brain barrier to permit trafficking of highly mobile, peripherally derived macrophages (Reader et al., 2015), though it is noteworthy that this has been contested (Lehmann et al., 2016). Alternatively, microglia in a ramified state have been shown to phagocytize neuronal processes in the dentate gyrus of the healthy adult hippocampus following pruning and apoptosis (Tremblay et al., 2011). Indeed, electron microscopy reveals that microglia reside within close proximity to synapses (Tremblay et al., 2011; Bisht et al., 2016) and it has been well documented that cells in such a scenario are heavily involved in pruning during the development and maturation of the central nervous system (Schafer et al., 2013). That having been said, while it is generally accepted that microglia contribute to cellular stripping and remodeling in even healthy brains, the mechanisms are not fully understood (Perry et al., 2010; Tremblay et al., 2011). For instance, in a model of Rett

Syndrome, MECP2-deficient microglia induce synaptic damage through excessive glutamate release (Maezawa & Jin, 2010). Furthermore, recent discoveries implicate a newly characterized, "dark" microglia, which are differentiable by their electron dense cytoplasm through transmission electron microscopy and are particularly active in pathological states, including stress (Bisht et al., 2016). Inasmuch, dark microglia approach and encircle synapses more readily than other non-amoeboid microglia, are highly phagocytic, produce ROSs in high quantities, and display Iba1 only faintly relative to other, less active microglia (Bisht et al., 2016). Taken together, while we were unable to definitively show microglial-specific phagocytosis via CD68 expression or morphometry, our results from Study 4 suggest that microglia contribute to stress-induced cellular and synaptic degeneration in Syrian hamsters given that these effects are halted by minocycline.

In conclusion, we provide data supporting the hypothesis that acute social stress can activate the innate immune system within the vmPFC, which has important roles in top-down control of aversive and appetitive motivational pathways. Moreover, this immune activity can disrupt the vmPFC anatomical integrity, which could impede neuronal activity during or following a high intensity stressor. We also show that dominant animals appears to be protected against innate immune-mediated insults to vmPFC structure following stress, providing additional mechanistic insight into the neural mechanisms controlling status-dependent changes in vulnerability to traumatic stress. Given the high burden that stress-induced impairments place on the global society, it is critical that more research be conducted to facilitate greater understanding of the biological underpinnings of resilience and vulnerability so that more

effective therapeutic and preventative treatment regimens can be developed for exposure to traumatic stress.

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Appendix C

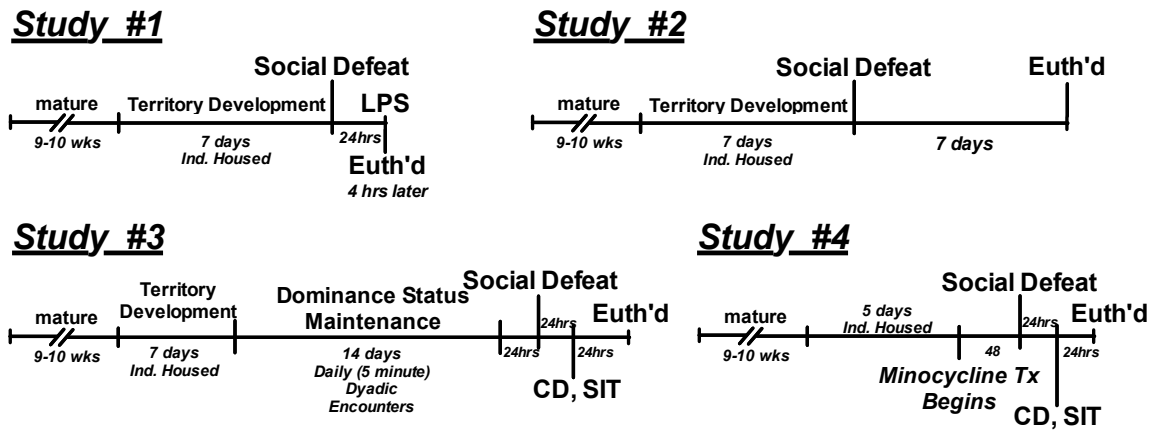

Figure 3.1. Experimental Timelines for Studies 1-4. For all studies, male Syrian hamsters bred in-house were allowed to mature and were individually housed for 7 days to permit territory establishment. In Study 1, 24 hours after acute social defeat stress, animals were administered 0, 20, 100, or 500 μg/kg of lipopolysaccharide (LPS) via intraperitoneal injection and euthanized (Euth'd) 4 hours later for vmPFC extraction. In Study 2, following social defeat, animals were left undisturbed in their home cages for 7 days and Euth'd for vmPFC extraction. In Study 3, following territorial development, animals were weight matched into dyads and permitted 14 days of daily agonistic encounters to establish and maintain dominance relationships. The following day, animals were subjected to social defeat. Control animals, termed dominance status controls (DSC), were subjected to social defeat following territorial development. Twenty-four hours after social defeat, all animals were administered the conditioned defeat (CD) and social interaction (SIT) tests in a counter-balanced manner to assay social avoidance. Twenty-four hours later, all animals were Euth'd for vmPFC extraction. In Study 4, animals were provided 4 mg/ml *minocycline* via drinking water during the final two days of territory development and treatment continued until Euth'd (5 days total). On the third day of *minocycline* treatment, animals were subjected to social defeat and, 24 hours later, CD and SIT tests as in Study 3. Animals were Euth'd 24 hours following completion of social avoidance assays.

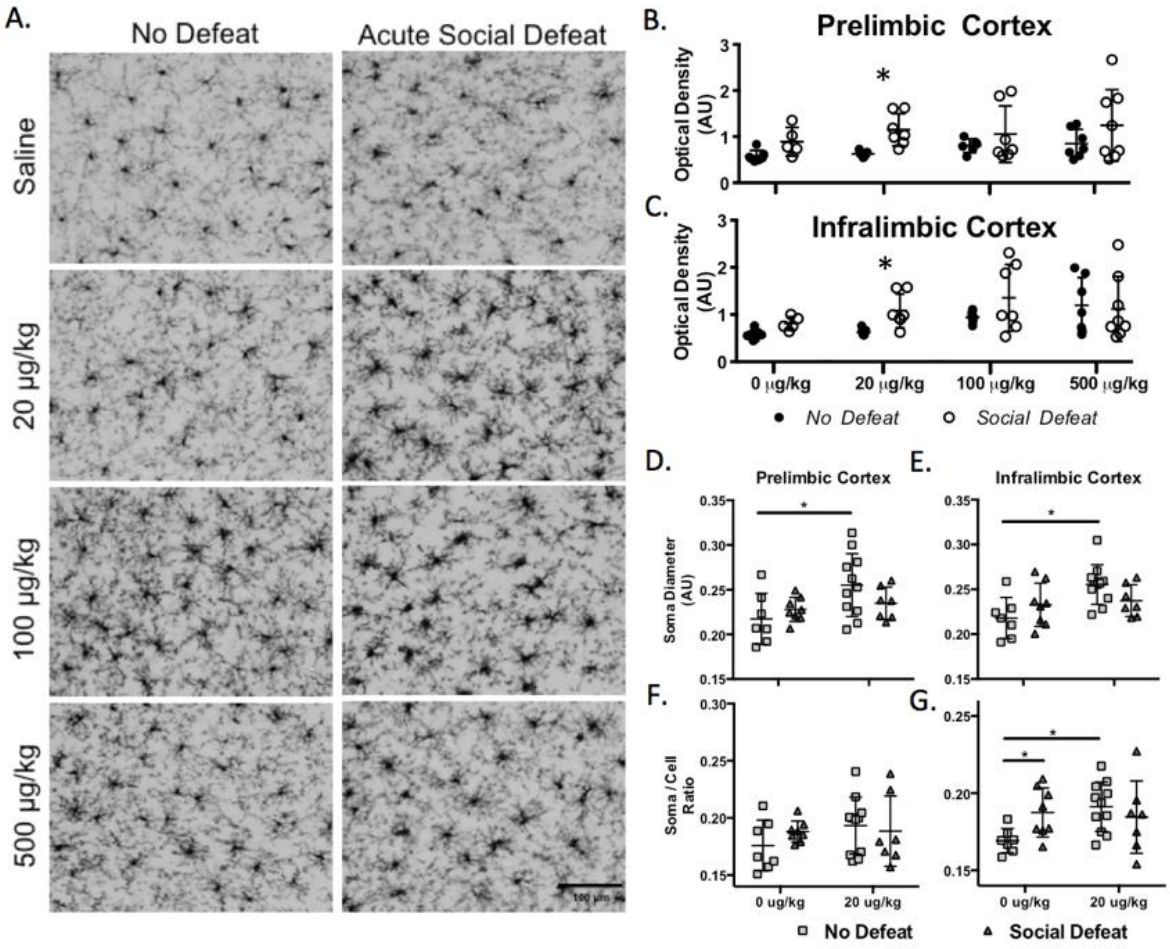

Figure 3.2. Stress Alters Iba1 Expression Patterns in the vmPFC. The results of Study 1 indicate that stress increases Iba1 expression in both the prelimbic and infralimbic cortices, especially when combined with subsequent injections of LPS, as can be qualitatively observed in representative images (A) and are quantitatively presented (B-C). Further analyses of saline- and 20 µg/kg LPS-injected hamsters indicate that Iba1+ cellular morphology shifts toward shapes consistent with proinflammatory activity following stress and/or LPS injections. Specifically, LPS induces an increase in Iba1+ soma diameter in the prelimbic (D) and infralimbic (E) cortex. Stress and LPS interact to increase the ratio of cell soma to total cell size in the infralimbic (G). AU: arbitrary units. * indicates treatment groups that significantly differ ($p < 0.05$). Data presented as mean and standard deviations. N = 7-11/group.

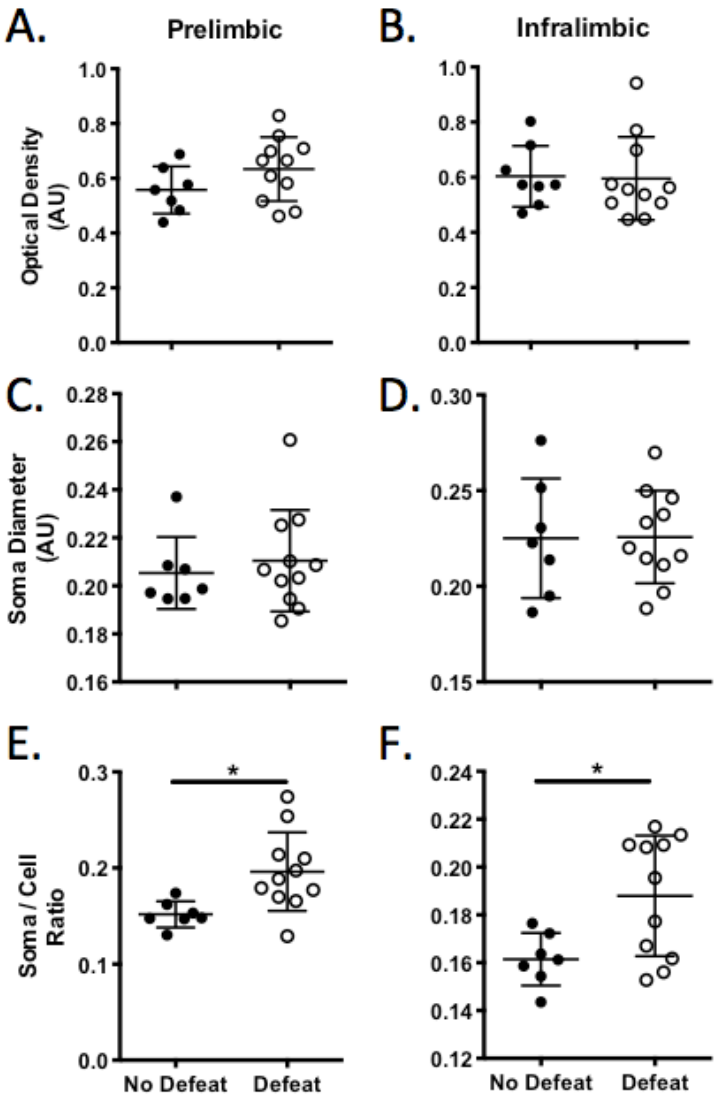

Figure 3.3. Persistent Stress-Induced Activity of Iba1 Cells in vmPFC. The results from Study 2 indicate that acute social defeat does not induce an upregulation in Iba1 expression (A, B) or soma diameter (C, D) in subregions of the vmPFC that persists 7 days following stress exposure. In contrast, there is an increase in the ratio of cell soma to total cell size in the prelimbic (E) and infralimbic (F) cortex 7 days post-stress, suggesting that acute social defeat stress induces morphological shifts in Iba1+ cells in the vmPFC that are consistent with a prolonged inflammatory response. * indicates $p < 0.05$. Data presented as means and standard deviations. N = 7-11/group.

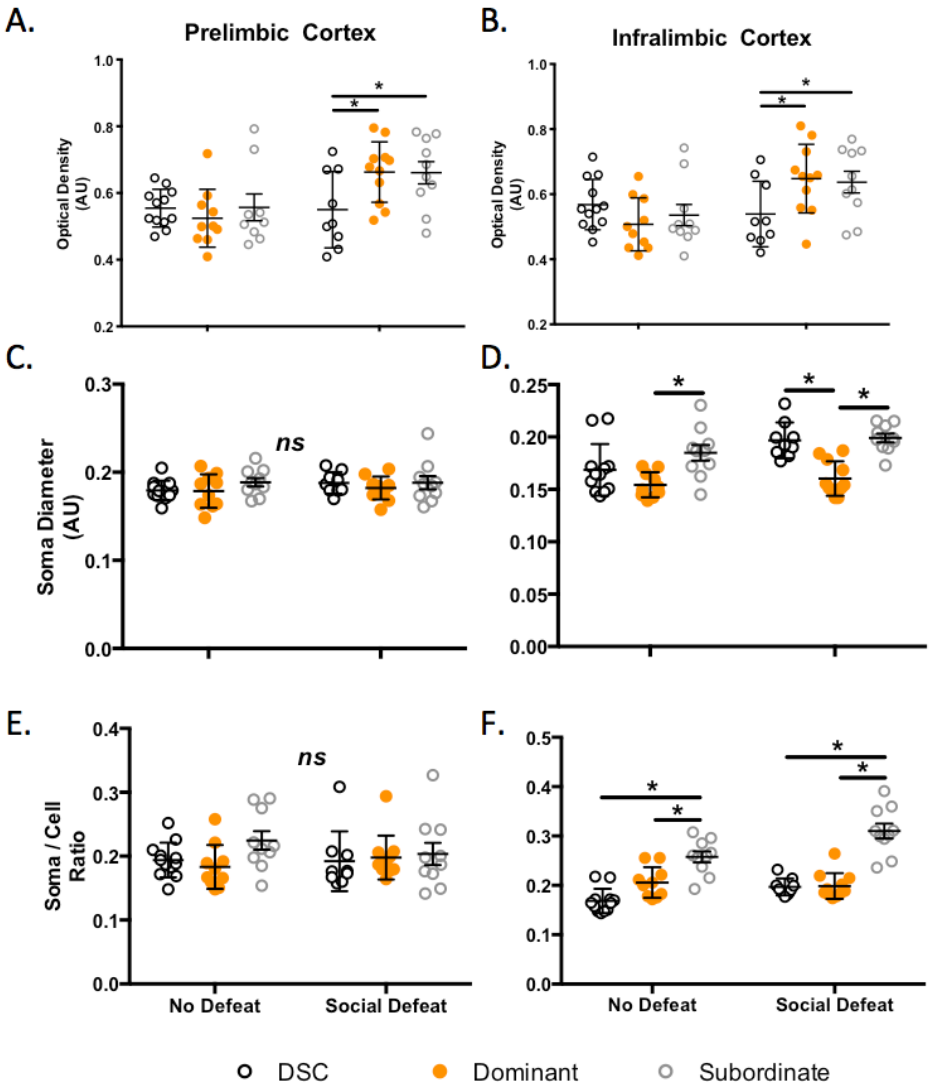

Figure 3.4. Stress-Induced Activity of Iba1+ Cells is Social Status Dependent. Results from Study 3 indicate that Iba1+ cells generally display dominance status- and defeat-dependent expression patterns in the vmPFC. With regard to optical density, social defeat stress interacted with dominance status to induce an upregulation of Iba1 in dominant and subordinate hamsters but not dominant status controls (DSC) in the prelimbic (A) and infralimbic (B) cortex. With regard to Iba1+ cellular morphology, there were no shifts in soma diameter in the prelimbic (C) but there were clear increases in soma diameter in the infralimbic (D). Post hoc tests demonstrate that subordinate hamsters display an increased soma diameter both without and with social defeat stress when compared to dominants. Also, socially defeated dominants displayed smaller Iba1+ somas when compared with socially defeated DSCs. The ratios of soma diameter to total cell diameter were not different in the prelimbic cortex (E). However, ratios were significantly greater in the infralimbic (F) in subordinate hamsters, where both defeated and non-defeated subordinates had greater ratios than corresponding DSCs and dominants. AU: Arbitrary Units. * indicates $p < 0.05$. ns = Not Significant. Data presented as means and standard deviations. N=7-11 per group.

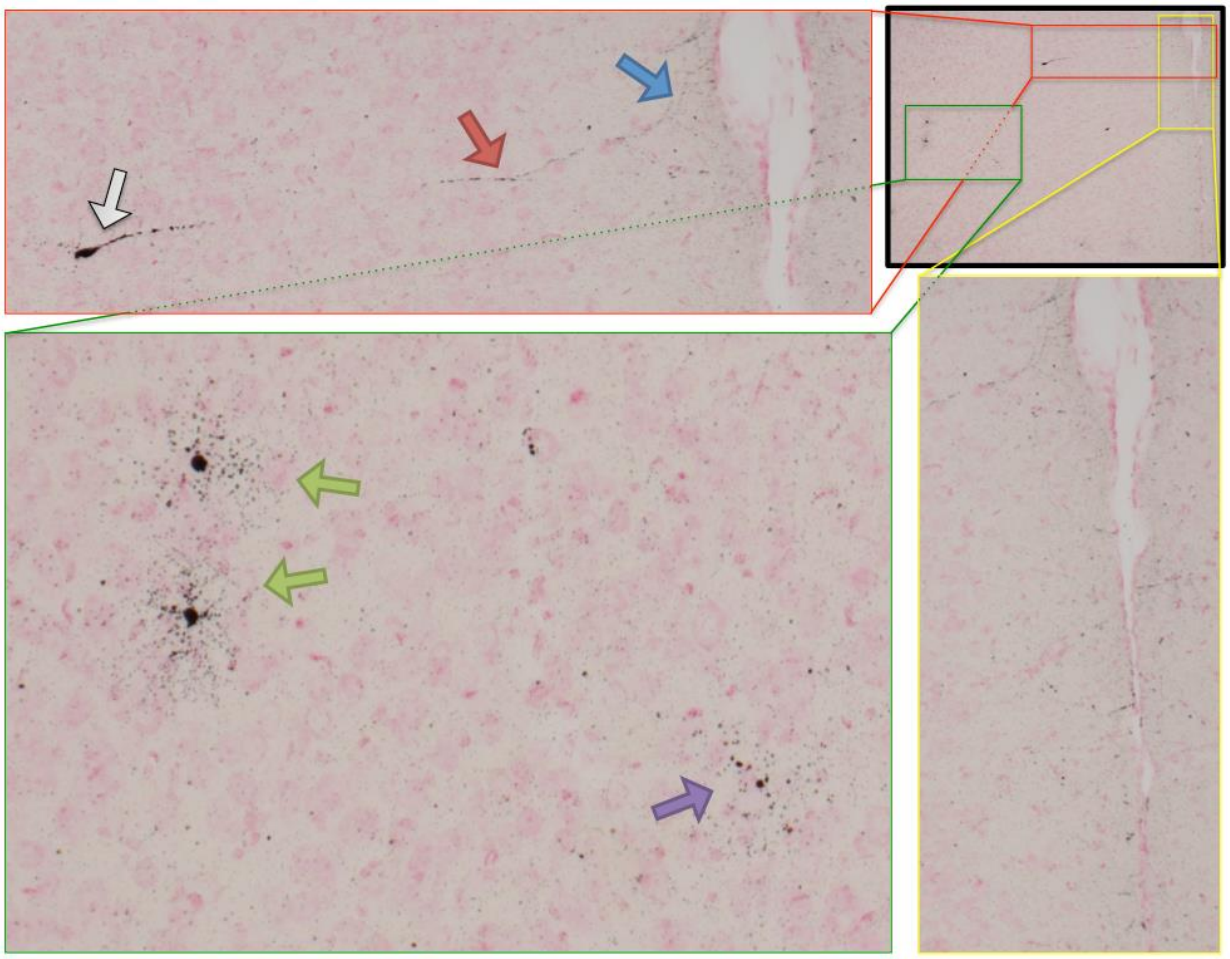

Figure 3.5. Cellular and Synaptic Debris in an Amino Cupric "Silver" Stain Exemplar. At 10x magnification, the original image (black border, top right panel) was taken of the prelimbic cortex. The surrounding images were enlarged to exemplify a degenerated neuron (red border, top left), degenerated glial cells (putative astrocytes; green border; bottom left panel), and general cellular debris (putative neuronal dendrites and terminals; yellow border; bottom right panel). The white arrow indicates a neuronal soma and apical dendrite, which is again visible as it progresses toward the midline (red arrow) and branches at the midline (blue arrow). Putative astrocytes are marked with green arrows. The purple arrow indicates debris that may also belong to the cell population identified with green arrows however, due to the absence of a clear soma, debris such as this was not included in degenerated cell counts. General markers of cellular debris were most commonly present along the midline as depicted in the image at the bottom right (yellow border), but can be visualized throughout the tissue. Qualitative appraisals of degeneration state were made on a 0 – 4 scale with 4 representing maximal degeneration. This exemplar received a qualitative degeneration score of 1. To determine anatomical location, slices were counterstained with Neutral Red.

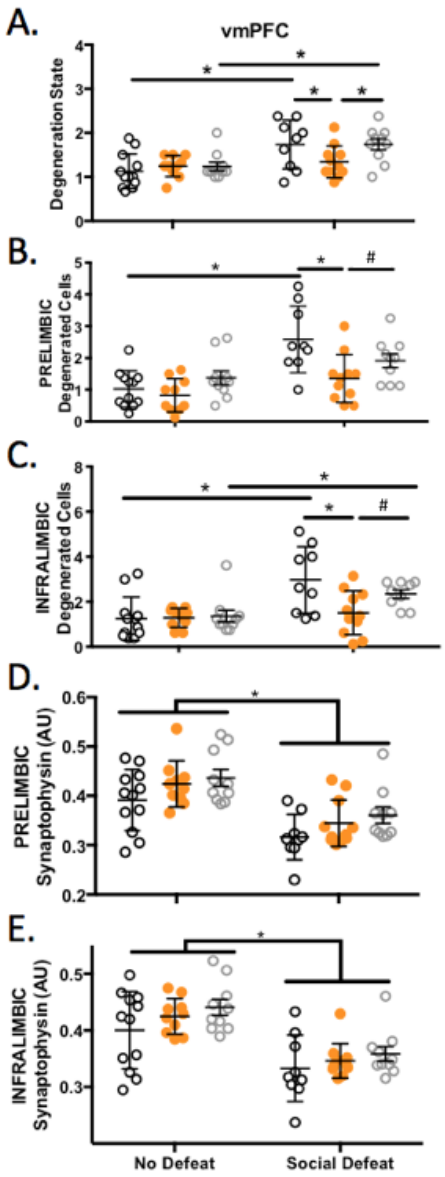

Figure 3.6. Cellular and Synaptic Degradation but not Synaptic Density in vmPFC is Social Status Dependent. Results from Study 3 indicate that social defeat stress and dominance status influence tissue degeneration in the vmPFC. Analyses of qualitative appraisals of degeneration state within the vmPFC (A) indicate that social defeat leads to significant increases in silver impregnation of cellular debris in DSCs (black circles) and subordinates (grey circles) but not dominants (orange circles). Moreover, following defeat, there was greater degeneration in DSCs and subordinates than in dominants. Social defeat also significantly increased the number of degenerated cells (putative astrocytes) in the prelimbic (B) and infralimbic (C) of DSCs and subordinates, but not dominants. Across both subregions, defeated dominants also displayed fewer degenerated cells than defeated DSCs and subordinates. Finally, social defeat stress led to a significant decrease in total synaptophysin expression in the prelimbic (D) and infralimbic (E) cortices. AU: Arbitrary Units; * indicates $p < 0.05$. # indicates $p < 0.10$. N = 7-11 per group.

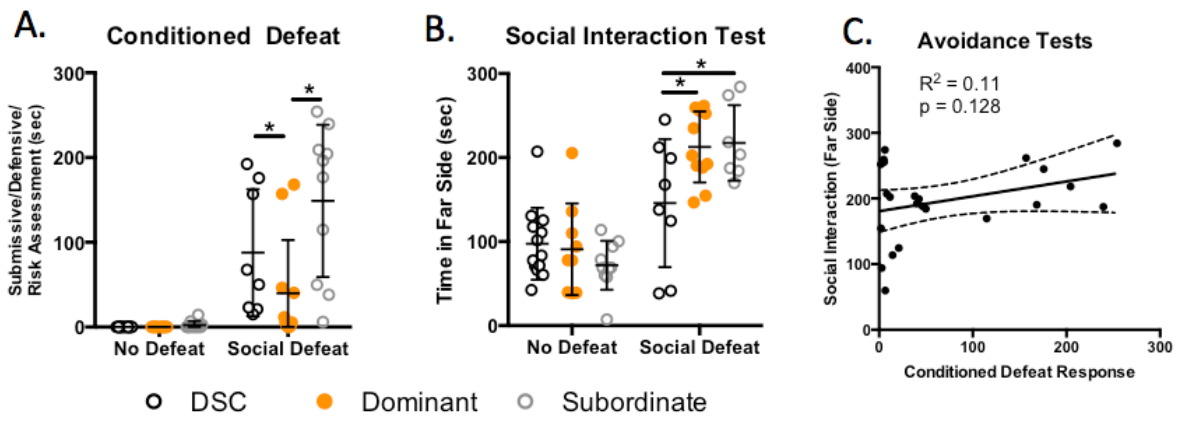

Figure 3.7. Defeat Stress-Induced Social Avoidance is Poorly Correlated Across Two Common Behavioral Assays. Results from Study 3 confirm previous reports from our lab that acute social defeat stress induces social avoidance of a novel, non-aggressive conspecific. Using a conditioned defeat test within the subject's home cage (A), we found that socially defeated dominants (orange circles) display a significantly lower duration of submissive, defensive, and risk assessment behaviors when compared to dominance status controls (DSC; opened black circles) and subordinates (opened grey circles). In the social interaction test (B), both dominant and subordinate hamsters spent more time in the far side of a neutral arena than DSCs. Importantly, these two tests assay differing constructs of social avoidance following social defeat, as there was no detected relationship in a linear regression analysis (C). * indicates $p < 0.05$. Data in A and B are expressed as mean with standard deviation.

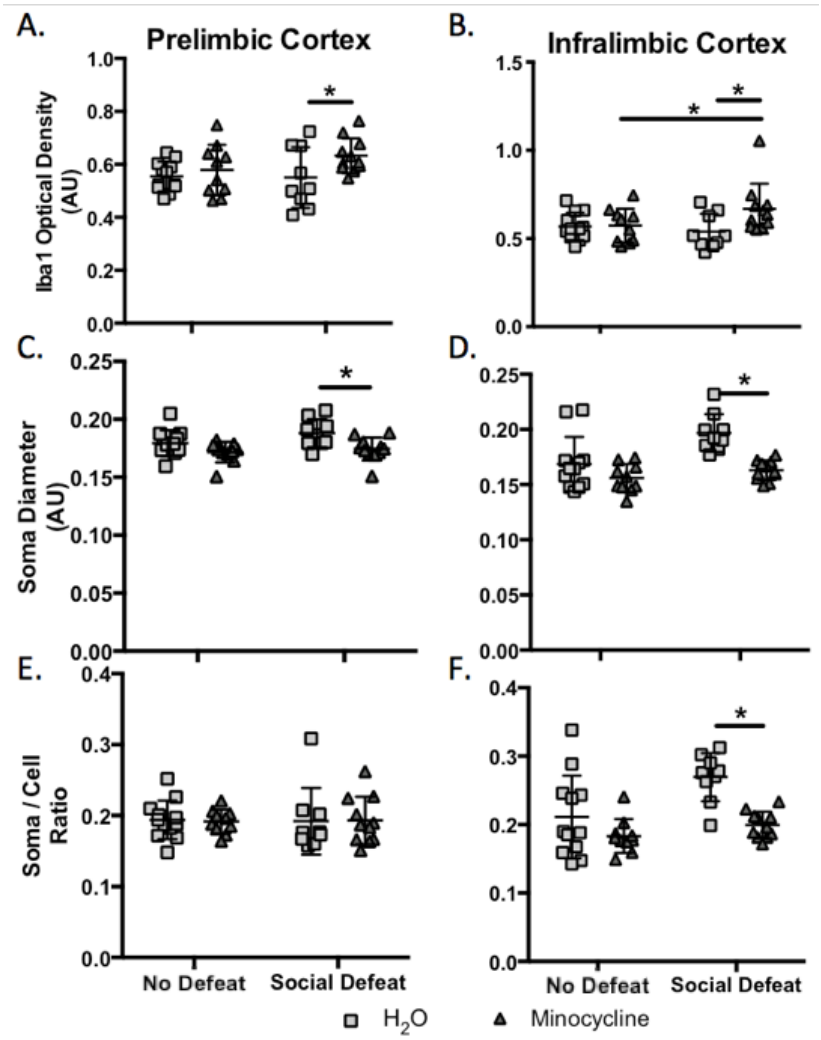

Figure 3.8. Effects of Minocycline on Iba1 Expression Patterns in vmPFC. Results from Study 4 indicate that while treatment with the Iba1-suppressing antibiotic, *minocycline* (triangles) did not reduce optical densities when compared to water-drinking counterparts (squares) in the vmPFC (A, B), it did reduce expression patterns of Iba1+ cells toward states that are consistent with reduced proinflammatory activity (C-F). In the prelimbic cortex, minocycline failed to reduce Iba1 optical density (A). Moreover, there was a significant increase in Iba1 optical density in minocycline-treated, socially defeated animals. This pattern extended to the infralimbic cortex as well (B). However, minocycline significantly reduced soma diameters in the prelimbic (C) and infralimbic (D) cortices of socially defeated hamsters. While there were no effects of minocycline on soma to total cell size ratios in the prelimbic cortex (E), there was a significant reduction in the ratios brought on by minocycline in socially defeated hamsters (F). AU: Arbitrary Units. * indicates $p < 0.05$. N = 8-12 per group.

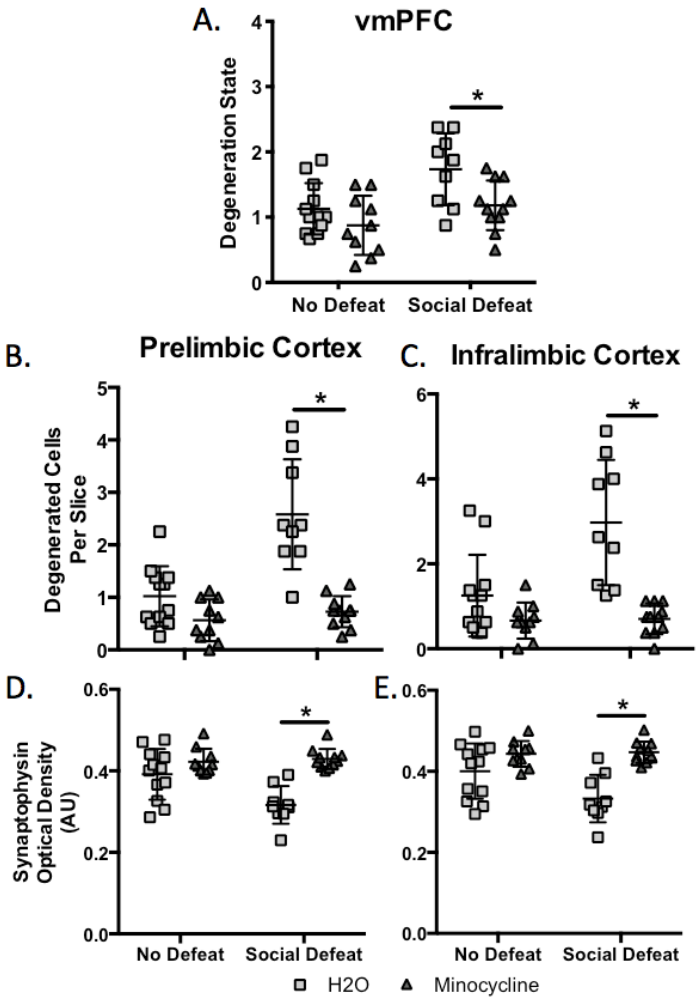

Figure 3.9. Minocycline Protects Against Acute Social Defeat Stress-Induced Cellular and Synaptic Degradation. Results from Study 4 indicate that minocycline protected the anatomical integrity of socially defeated vmPFCs. Qualitative appraisals of vmPFC tissue following defeat (A) indicated significantly less silver-impregnated cellular debris in minocycline treated (triangles) hamsters when compared to water-drinking controls (squares). There were also significantly fewer degenerated cells (putative astrocytes) in both the prelimbic (B) and infralimbic (C) cortices of minocycline, defeated hamsters. Finally, we found that minocycline treatment led to significantly greater synaptophysin optical densities in the prelimbic (D) and infralimbic (E) cortices. AU: Arbitrary Units. * indicates $p < 0.05$. Data are presented as means and standard deviations. N = 8-12 per group.

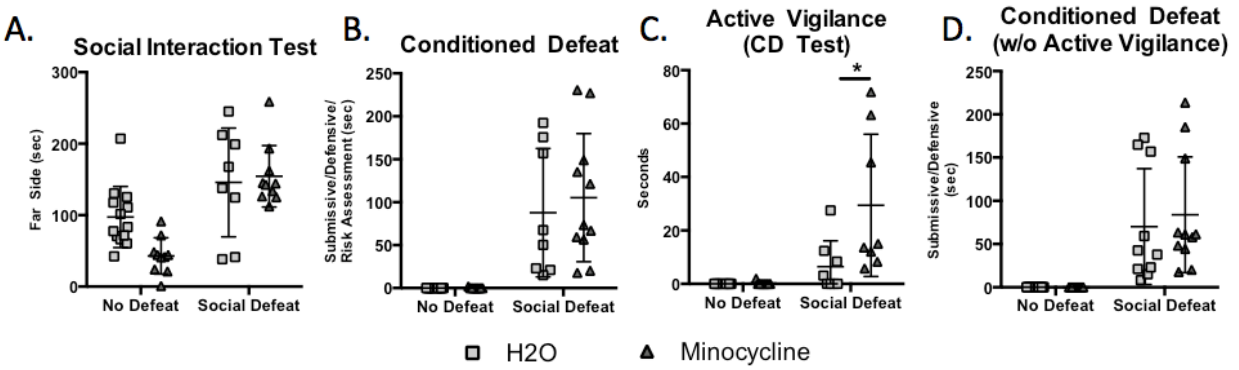

Figure 3.10. Minocycline Does Not Reduce Avoidance, but Does Bias Active Vigilance Following Acute Social Defeat Stress. Results from behavioral analyses of social avoidance indicate that, generally, minocycline did not protect hamsters from the avoidance-conferring effects of social defeat. In the social interaction test (A), defeated hamsters, regardless of minocycline treatment, displayed an increase in time spent in the far side of a neutral arena. There was also no difference in the conditioned defeat (CD) test (B). However, a subset of socially defeated minocycline animals did display significantly more active vigilance behavior (C), a subcategory of risk assessment behavior that may indicate a more proactive coping strategy following defeat. However, when active vigilant behaviors were removed from consideration, minocycline-treated hamsters still did not differ from water-drinking controls in the remaining submissive, defensive, and risk assessment behaviors (D). * = $p < 0.05$. Data expressed as means and standard deviations. N = 8-12 per group.

Appendix D

Ethogram for Social Behavior in Syrian Hamsters

This ethogram can used for *Conditioned Defeat* and *Social Investigation Test* (*SIT, also called Social Avoidance Test*) assays. The cumulative durations of the mutually exclusive behavioral categories (Nonsocial, Affiliative, Aggressive, Submissive, and Defensive) are calculated based on the presence of the behaviors described below. In all cases, the presence of a behavior listed below each category must occur in order to consider a shift between behavioral categories. For example, if an animal is locomoting, it is considered to be Nonsocial until a behavior from another category begins, such as fleeing from the opponent in the arena. In other words, the behavioral category would switch to Submissive **not** when locomotion *ends* but when fleeing *begins*.

Behavior	Description
Nonsocial	
Locomotion	walking about cage that may include exploration of cage and climbing of cage walls; does not include walking (or running) directly toward intruder/caged-conspecific if immediately culminating in social investigation (*i.e.* "approach") or attack (*i.e.* "chase")
Self-grooming	manipulation of one's own body, hair, or feces with paws or mouth
Nesting	picking up, pouching, carrying, piling, or arranging next materials

Feeding	picking up, pouching, carrying, piling, or arranging chow
Sleeping	characteristic crouched position with eyes closed

Affiliative

Social Approach	movement toward a conspecific (may be slow); subject must move to within 5 cm of conspecific to qualify; quick approach should be considered a "chase" (see below) and, if appropriate, would NOT constitute an affiliative behavior
Investigate	sniffing/manipulating conspecific's head, body, and/or anogenital region; when occurring in the SIT (*i.e.* social interaction test, also called social avoidance test), this occurs when sniffing, orienting, or otherwise focusing on the perforated box containing conspecific; this should only be scored if it occurs within 5 cm of intruder (in CD) or perforated box containing conspecific (in SIT)
Nose-to-nose	nose of two individuals in close proximity; investigation of face

Flank mark	animal arches side and rubs flank region* against inanimate object such as the walls of the cage or perforated box (with or without caged opponent) * - NOTE: when scoring frequencies, award one flank mark for each occurrence that the flank physically touches the inanimate object; flank marks should NOT be scored in bouts
Mounting	animal places forepaws on the hindquarters of conspecific; includes attempt to bring pelvic region into contact with conspecific's anogenital region

Aggressive *(CD only)*

Offensive upright/ side posture	animal is oriented toward conspecific and is either: [1] standing on rear paws when upright, [2] on three paws when on one side, or [3] on its back yet underneath the opponent; also called "side-aggressive posture" or "SAP"; offensive postures are characterized by moving the snout closer to the opponent, especially the opponent's flank or abdomen (as if preparing to bite the opponent) * - **NOTE**: this posture closely resembles the "defensive upright / side posture" described below and identification of this behavior

	can be informed by contextual information including: [1] behavior immediately preceding or immediately following the postural display in question, [2] the behavior of the opponent (*i.e.* while simultaneously aggression is possible, simultaneous submission and thus simultaneous defensive upright/side postures is unlikely), [3] orientation of the head, or [4] degree of extension of paws (*i.e.* defensive postures are marked by large if not full extension of forepaws)
Chase	rapidly approaching conspecific WITHOUT continuous contact; opponent MUST be fleeing (see below) in order for subject's movement to qualify as a chase; slower "following" behavior does not qualify, even if preceded and/or followed by other aggression
Attack	a bite or bite attempt* that often occurs from upright or side aggressive postures (above) but also while animal is on all four paws; the opponent is often held with the front paws; bites are most often directed toward the flank and abdomen, though bites to the face are common when opponents are fighting back; bite attempts are characterized by the rapid movement of the snout into contact with the opponent

	* - **NOTE**: few failed attempts should be scored as an attack; poor attempts that should NOT be scored are marked by: [1] a clear miss, [2] poor aim, [3] low speed, and/or [4] lunges toward opponent
Sparring	subject attempts to attack while the other defends with paws
Rolling fight	both animals attempt to bite while holding on to each other and rolling on the ground; only scored if both animals are aggressive
Fighting back	any aggressive behavior by subject that occurs simultaneously with that of opponent

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Submissive

Flee / flight	explosive movement away from the other; often displayed after receiving an attack or sudden movement by opponent; must move at least 6-8 inches in one motion (or ½ cage length); in the SIT (also called social avoidance test), extreme caution should be taken when considering whether the subjects' movement qualifies as a flee if it jumped from atop the perforated box (and thus 'away' from the opponent)

Full submissive posture	animal lies unmoving on back with limbs raised (may continue after opponent has moved away)
Tail-up	tail is raised (often while the back is arched) while moving away from opponent or while in a frozen position

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Defensive and Risk Assessment

Defensive upright/ side posture	animal is oriented toward opponent and is standing on rear paws when upright, three paws when on its side, or on its back (underneath the opponent); defensive postures are characterized by pushing the opponent away and largely (if not fully) extended forepaws
Paw up defense	animal lifts and holds forepaw(s) in midair, oriented toward opponent; subject may also turn away from approaching opponent with paws outstretched toward opponent
Avoid	animal moves away from conspecific (less than ½ a cage length); at times, subjects engaging in avoids appear to be *displaced* by the opponent, giving physical space to the subject; in the social

	interaction test (SIT, sometimes called social avoidance test), subjects can be scored as having displayed an avoid behavior even though the caged intruder cannot physically displace the subject
Head flag	subject alternates head orientation toward and away from the opponent; subject turns its head and/or shoulders toward and away to monitor the conspecific while also avoiding eye contact or facing intruder directly; head flags can only serve as behaviors initiating “defensive/risk assessment” if occurring for either: [1] over 3 seconds or [2] for more than 3 consecutive flags
Startle	animal jumps or flinches rapidly during the approach or sudden movement of the opponent
Stretch-attend	slow approach with body stretched and lowered that does NOT result in the subject’s nose coming within 5cm of opponent*; posture is often rigid; eyes must be fixed on opponent; subject is often (but not always) sniffing/investigating opponent during the stretch-attend; rear feet are often planted in or very near place of origin (most often in the nest); posture should be held for at least 1 second; stretch attends are a form of vigilance behavior***

* - **NOTE**: Stretch attends are nearly always followed by an avoid or a shift to a non-social behavior** – *HOWEVER* – on occasion, stretch attends culminate in an approach and/or social investigation of the opponent. In the scenario where affiliative behavior follows, a stretch attend should ONLY be scored if occurring for over 3 seconds BEFORE the approach began. ANY stretch attend-like behaviors lasting for *less* than 3 seconds before affiliative behavior should instead be scored as "approach" from the Affiliative Behavior category

** - **NOTE**: Most stretch attends end when the subject disengages in the posture described above in order to groom or locomote away from the opponent (or toward opponent, as described above) – *HOWEVER* – on occasion, the opponent approaches the subject thus forcing a disengagement in stretch attend-like behavior. If the opponent approaches the subject and the subject has not held the characteristic stretch attend posture for more than 3 seconds, a stretch attend should be scored ONLY if the subject does NOT engage in affiliative behavior upon the opponent's approach

*** - **NOTE**: Stretch attends should be considered a subcategory of "active vigilance" if: [1] the stretch attend culminates in an

approach toward the opponent (after holding the stretch attend posture for 3 seconds or more, as above), [2] the stretch attend culminates in subject-initiated "social" investigation or other affiliative behavior (after 3 seconds or more, as above) or [3] the stretch attend occurred with hind-paws fixed anywhere outside of the nest****. Any stretch attends occurring with hind-paws in the nest avoid should be considered "passive vigilance"

**** - **NOTE**: If the opponent occupies the nest, a defensive/submissive/avoidant subject will often relocate. In this scenario, out-of-nest stretch attends should only qualify as "active vigilance" if they do NOT occur with hind-paws fixed in a position of maximal possible distance (*i.e.* from the farthest point from the nest within the arena) AND meet all other requirements as above

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Subcategories of Defensive and Risk Assessment Behaviors

Cautious approach — A slow, locomotive approach toward the opponent without coming within 5cm and occurring for at least 3 seconds; cautious approaches taking longer than 3 seconds should be scored

	accordingly, but should be toggled to "approach" from the Affiliative Behavior category once the subject comes within 5cm of the opponent; cautious approaches are often indirect (*i.e.* non-linear); cautious approaches may also occur repetitively, for instance marked by "circling" behavior that consists of approach-like behavior (not within 5cm of opponent) followed immediately by retreating locomotion (*i.e.* "avoids" or "flees") yet then immediately followed by another cautious approach in a seamless manner and while sustaining attention on the opponent
Cautious monitoring	behavior (often preceded by some other submissive or defensive behavior) that is marked or punctuated by slow movements or freezing which includes either: [1] a fixed visual gaze toward the opponent, [2] head flagging, [3] a frozen posture that cannot be reasonably explained by stimuli emanating from outside of the arena, or [4] a combination of these; cautious monitoring should only be scored if the subject's behavior persist for at least 3 seconds
Passive vigilance	passive vigilance is a subcategory of behaviors including: [1] cautious monitoring, [2] head flags, and [3] stretch attends occurring within the nest (or distal-most position if intruder occupies nest); passive vigilance should NOT be scored if any

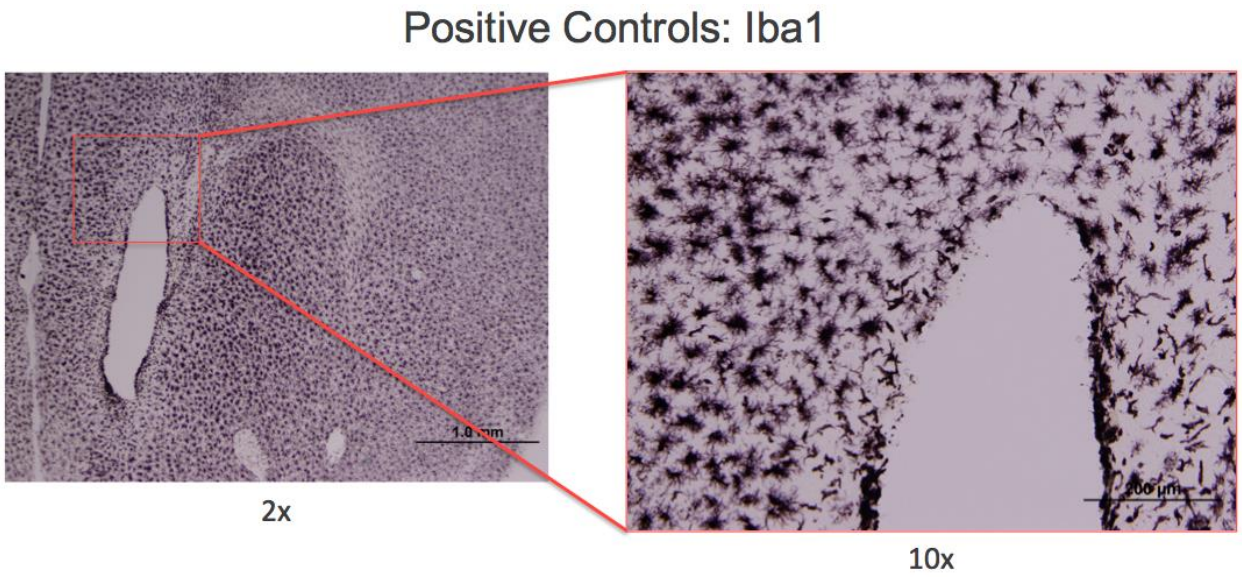
	movements result in approach toward a conspecific UNLESS those behaviors first occurred in a stationary or retreating manner for over 3 consecutive seconds – In other words, an animal must be passively vigilant for over 3 seconds in order to be scored as such if the passive vigilance is followed by an affiliative approach and/or engagement of other affiliative behaviors (*i.e.* if the opponent approaches the subject)
Active vigilance	active vigilance is a subcategory of behaviors that occur either outside of nest or in an approaching manner and, therein, may include: [1] cautious approach, [2] cautious monitoring, [3] head flags, and [4] stretch attends; active vigilance is also marked by aforementioned behaviors that, within 3 seconds of onset, do NOT culminate in subject-initiated affiliative investigation (*i.e.* subjects approaching within 5cm of intruder/conspecific cautiously but in under 3 seconds should be scored as "approach" from the Affiliative Behavior category)

Appendix E

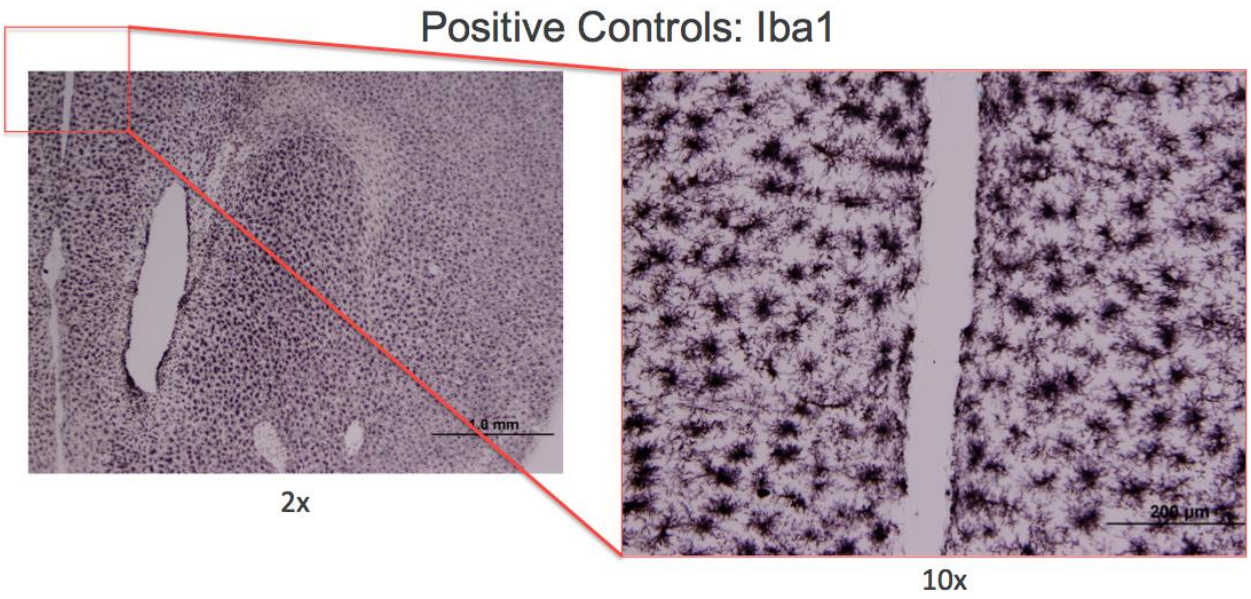
Positive Controls

The following positive controls were conducted for all experiments conducted in Chapter 3. Two stereotaxically mounted retired resident aggressors anesthetized with isoflurane gas (3.0%) were bilaterally lesioned in the vmPFC with a Hamilton syringe directed at the infralimbic cortex. Animals were then sutured, treated with ketoprofen and returned to their home cages for 48 hours, and euthanized. Tissue was processed identically to that of Studies 3 and 4.

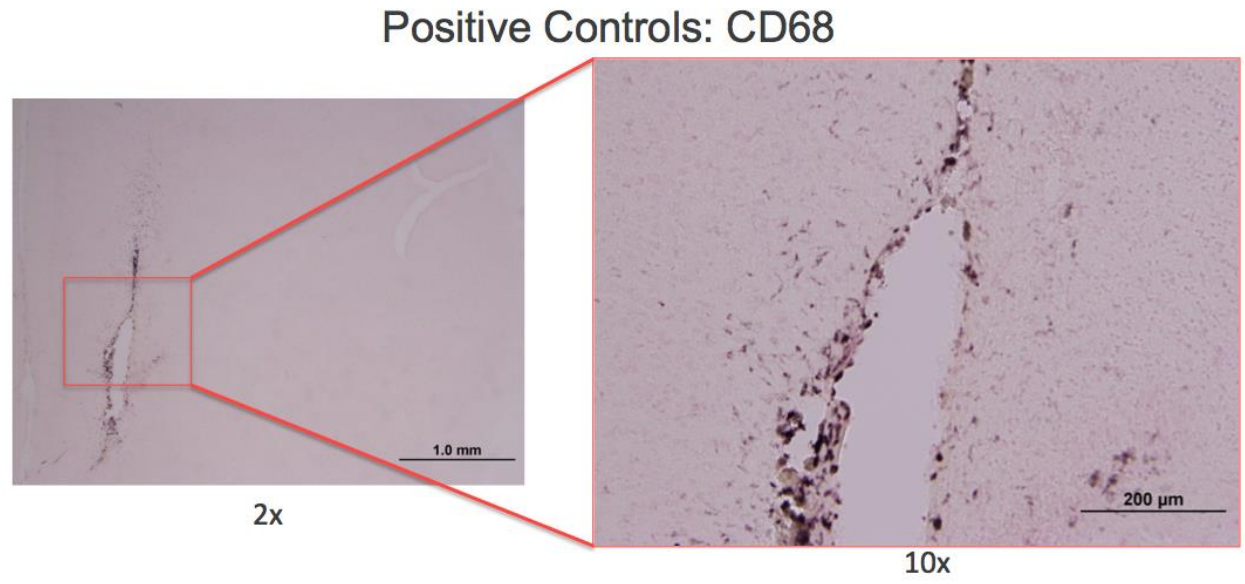
Iba1 Staining:



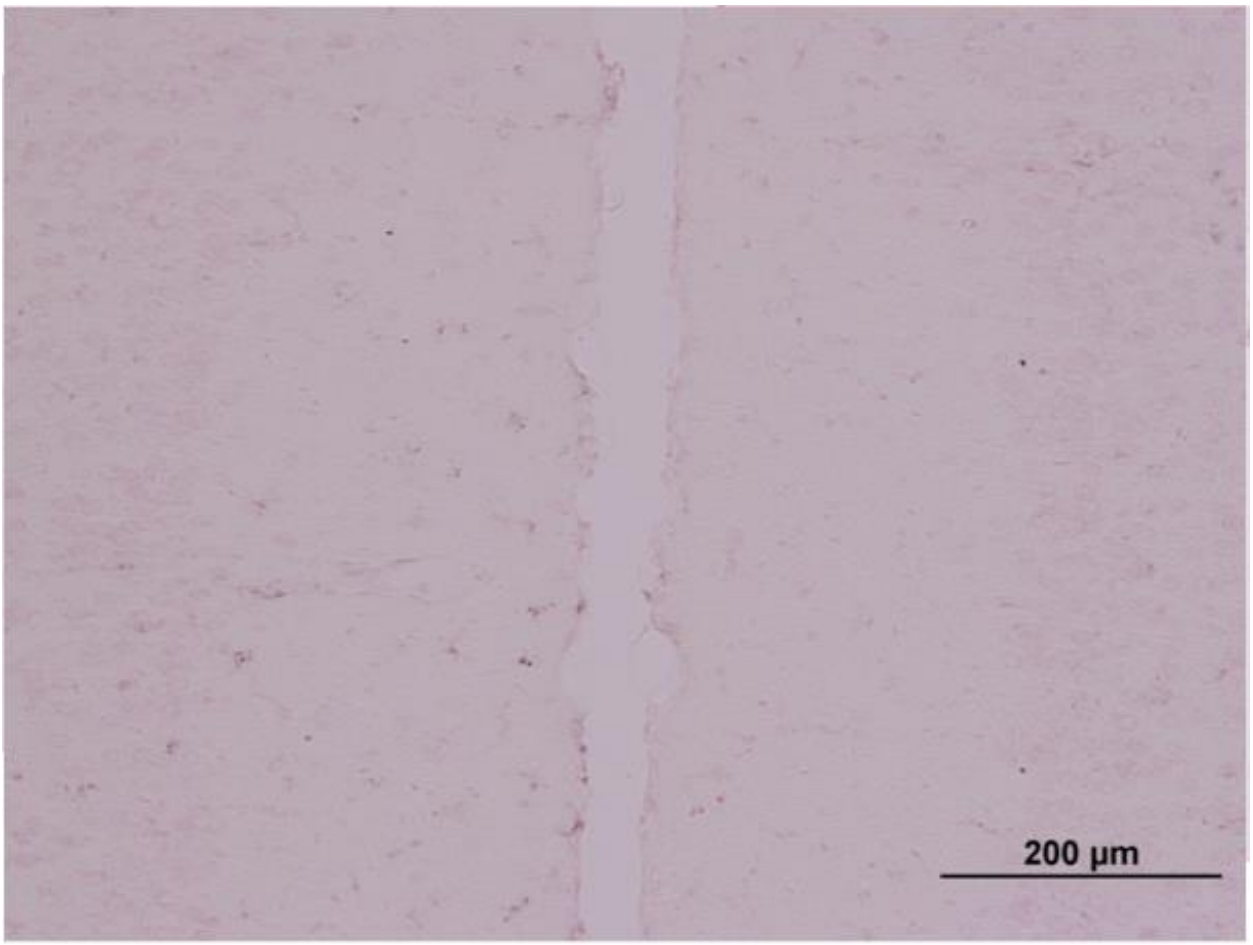
Iba1+ cells around the lesion site within the vmPFC. The panel in the right contains microglia/macrophages at various degrees of activation, including fully phagocytic microglia lining the walls of the cavity that resulted from the tissue lesion.



Iba1+ cells in the prelimbic cortex of the lesioned animal depicted above demonstrate how microglia/macrophages throughout the tissue display high levels of Iba1 expression and morphological shifts, which are consistent with high degrees of proinflammatory activity and phagocytosis.

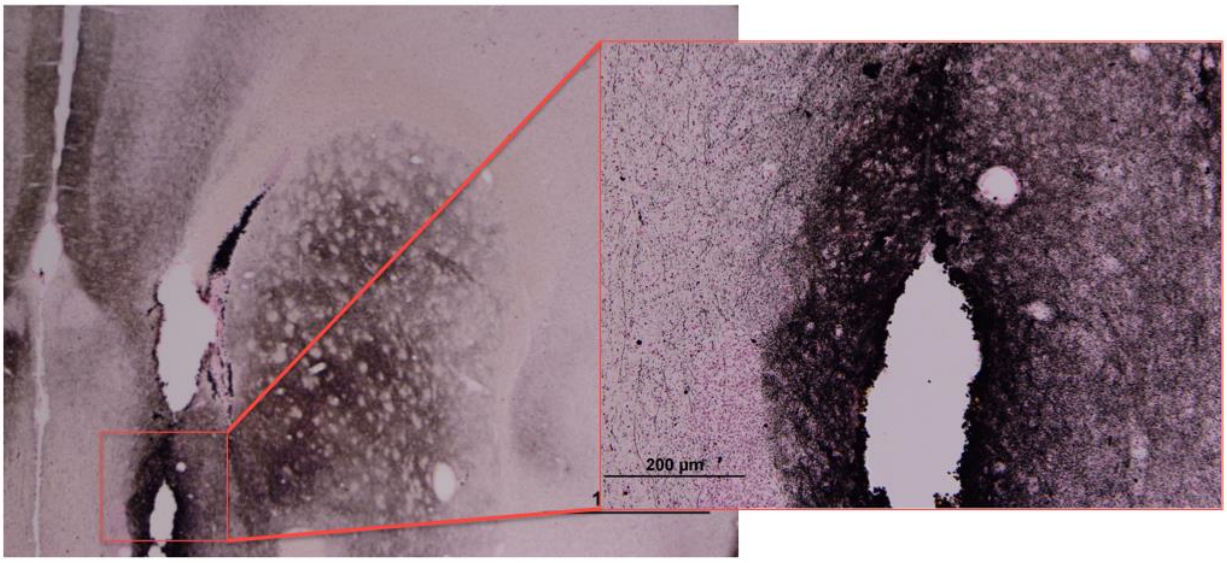


The CD68 protein is upregulated in microglia/macrophages that have entered phagocytosis. Importantly, this is an adjacent tissue slice rostral to that depicted above for Iba1. It is noteworthy how few cells display CD68+ labeling near the lesion site within the vmPFC.



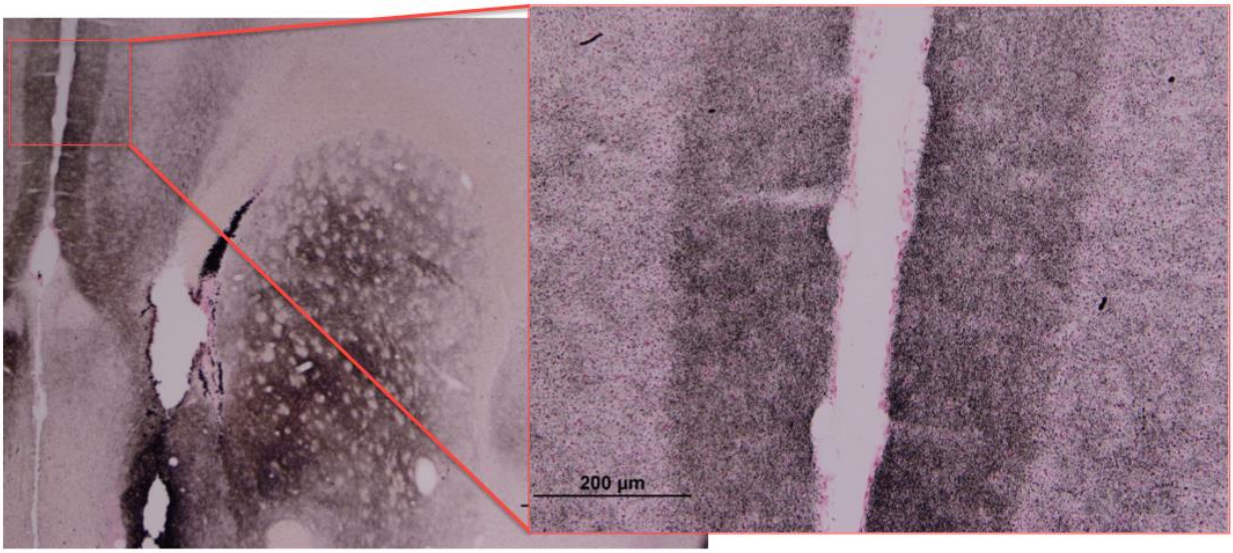
CD68 staining of the prelimbic cortex within the same tissue slice as that presented above. It is noteworthy that very little CD68+ labeling appears within this region, despite the high degree of Iba1+ cell activity and tissue degradation (below).

Positive Controls – Silver Stain



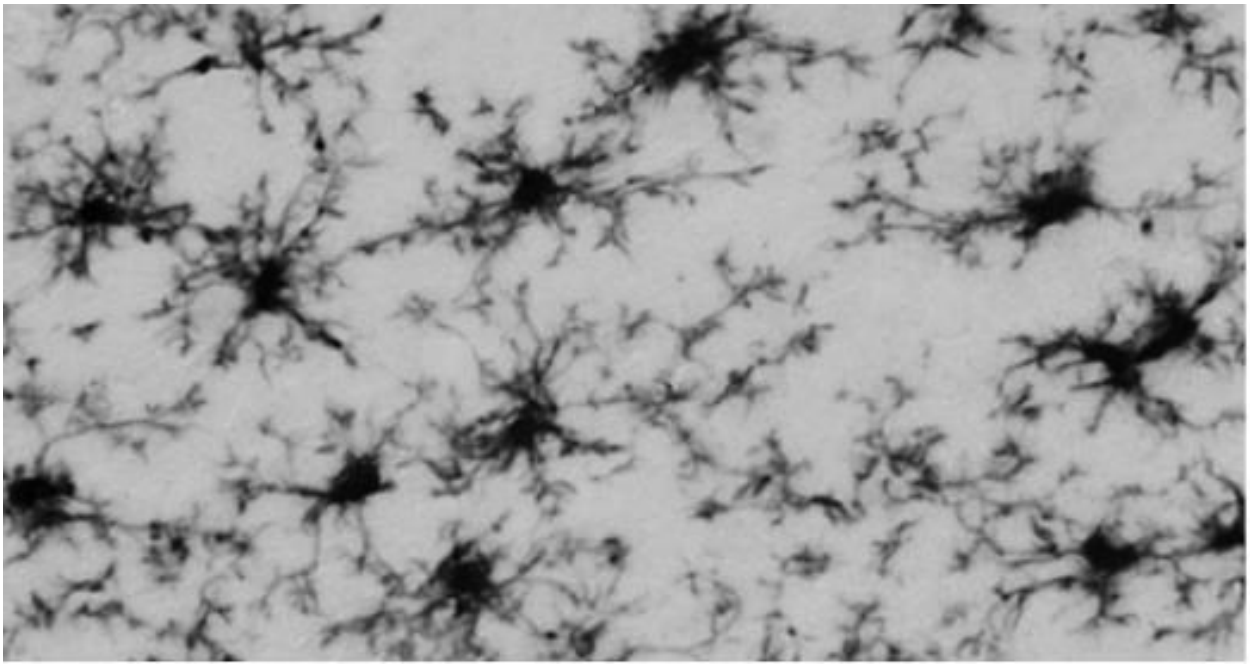
Amino cupric “Silver” staining is derived from Golgi staining procedures wherein silver atoms are taken up by degrading cellular debris (de Olmos et al., 1994; Switzer, 2000). These images were taken in tissue immediately caudal to the Iba1 positive control depicted above. The black coloration indicates a high degree of silver impregnation of debris surrounding the lesion site. Also detectable is silver-impregnated axons of the caudate nucleus to the right of the lesion site and below the corpus callosum.

Positive Controls – Silver Stain

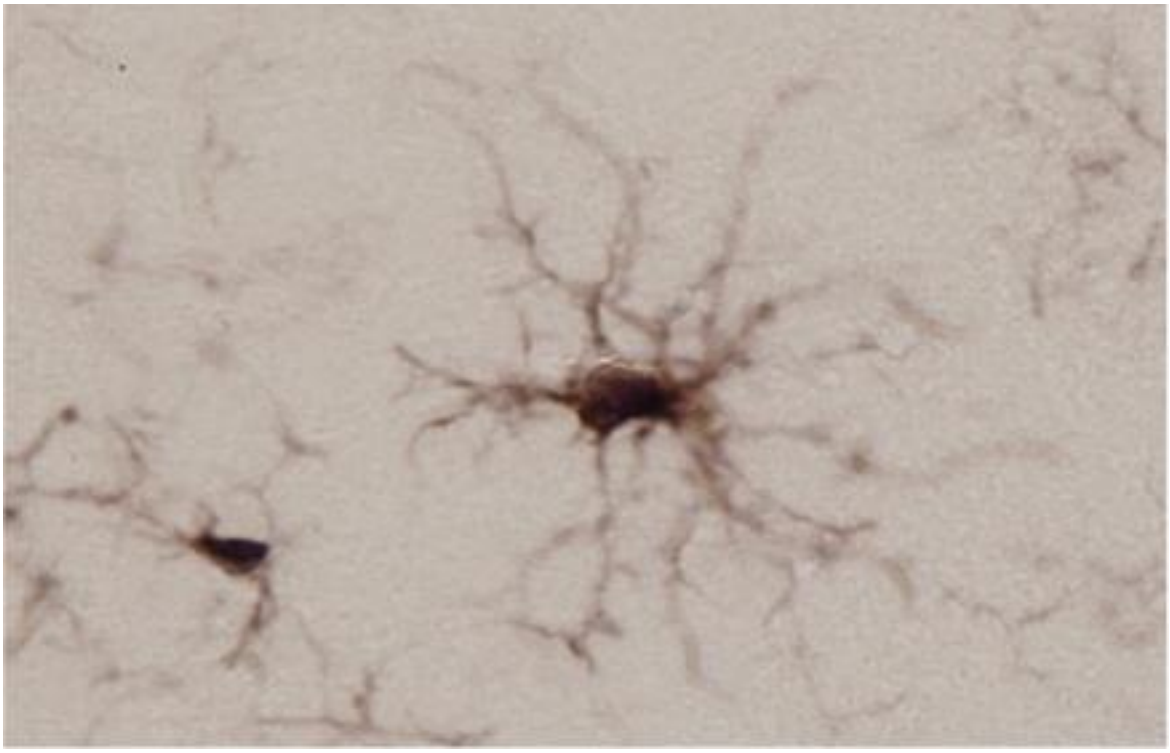


Within the same tissue slice as that depicted above, these images demonstrate the large degree of silver-impregnated debris in the prelimbic and infralimbic cortices. Noteworthy are: (1) the high degree of degenerated tissue, especially along the midline in cortical layers I-III; and (2), the occasional artifacts which render optical density measurements easily confounded. Accordingly, qualitative appraisals of degeneration state are necessary. This image was used as a reference point for the highest possible score, “4”.

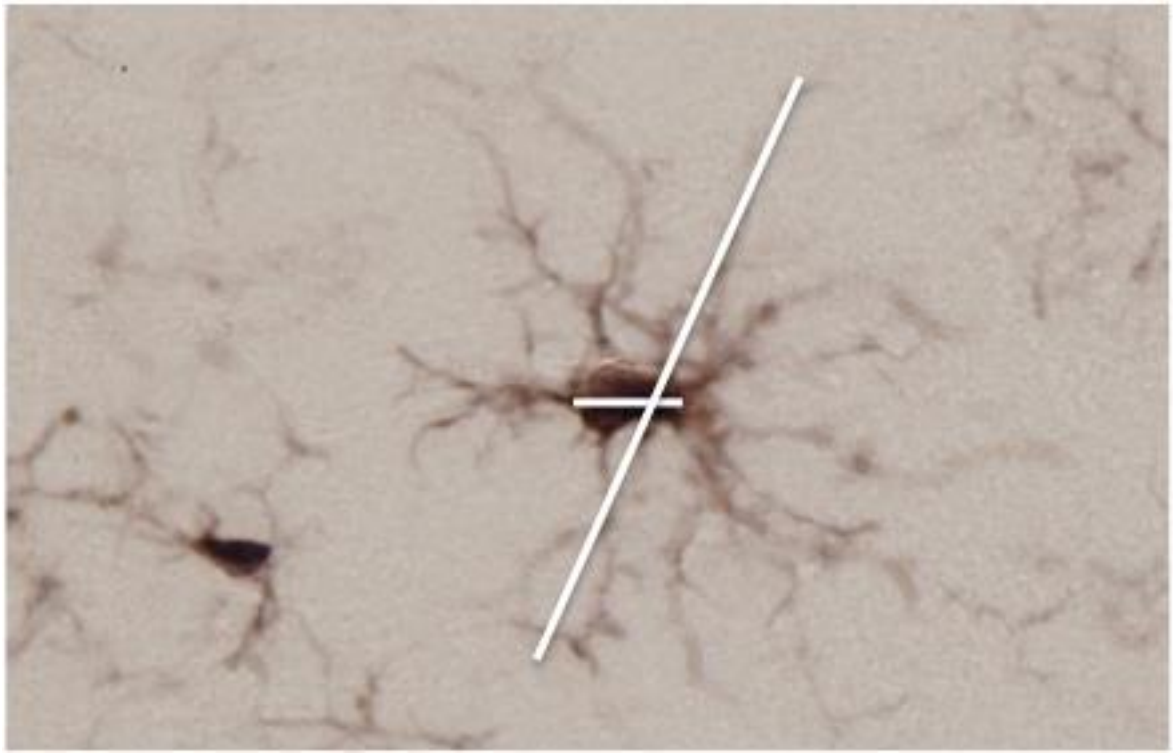
Examples of Detected Microglia at Various States of Activation and Morphology and Depiction of Quantification Approach



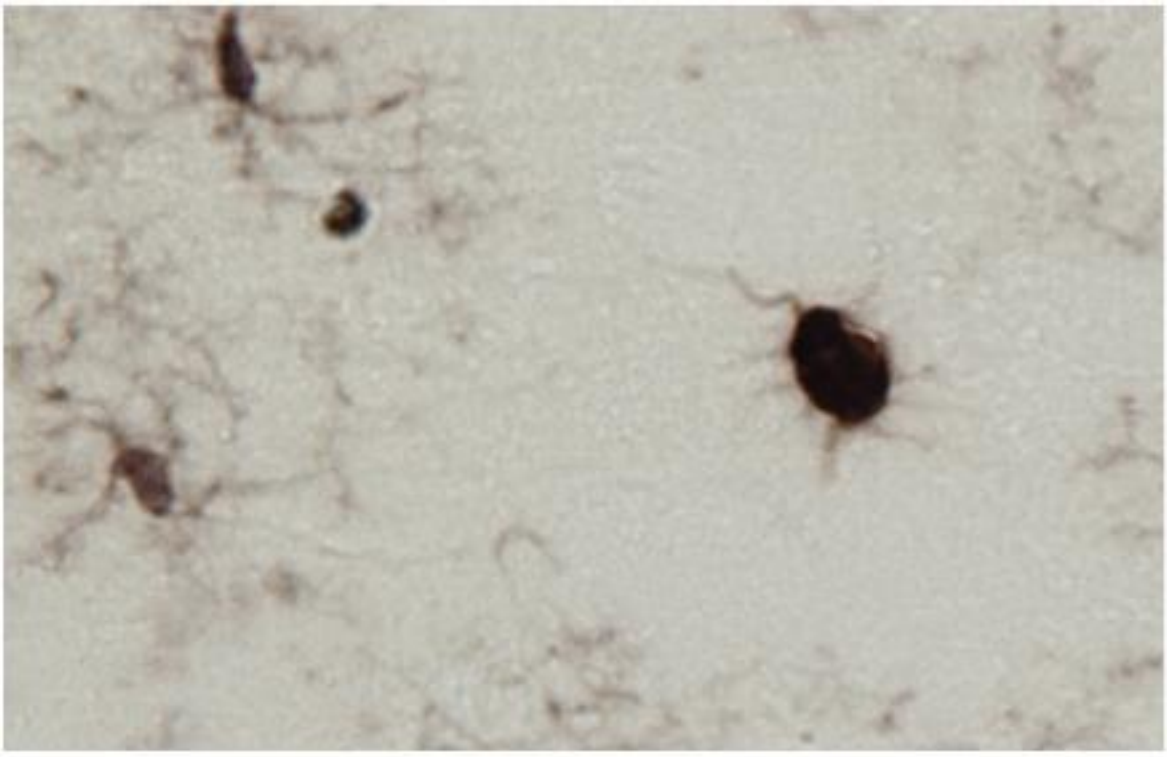
This image depicts an enlarged image from an animal in Study 1 that was socially defeated and treated with 500 μg/kg twenty-four hours later.



This image depicts microglia at two planes of visualization. Importantly, only the Iba1+ cell in the center of the image would be chosen for quantification. The image below indicates the method of quantification of cellular morphology, wherein the white lines demonstrate how the maximal diameters of soma and total cell would be traced.



The final image (below) indicates the most progressed example of an Iba1+ cell, which approximated but did not fully reflect an amoeboid state.



Chapter 4:

Conclusions

Situating the Results of this Dissertation within the Literature on Resistance to Acute Social Defeat Stress

A key aim of this dissertation was to further elucidate neural mechanisms of acute stress resilience in male Syrian hamsters, an alternative animal model of stress-induced impairment that provides a comparative approach for investigating the neural correlates of affective processing and psychopathologies in humans. My lab predecessors have shown that, relative to controls, the acquisition and maintenance of social dominance confers a reduced expression of submissive and defensive behaviors when exposed to a non-aggressive, intruding conspecific 24 hours after an acute social defeat stressor (Morrison et al., 2012). This behavioral phenomenon, referred to as "conditioned defeat" (CD), is readily and persistently expressed among male hamsters (Potegal et al., 1993; Huhman et al., 2003) and the attenuated CD response in dominant hamsters serves as a behavioral model to better inform the study of stress resilience in humans (Cooper et al., 2015). We have also shown that reduced CD in dominant hamsters is dependent on the duration of dominance status maintenance (Morrison et al., 2014) as well as defeat-induced neural activity within the vmPFC (Morrison et al., 2013). To further determine the role of status-dependent recruitment of vmPFC projection neurons in mitigating CD, we have more recently shown that dominance-dependent resilience to stress is likely conferred by selective activation of a vmPFC-basolateral amygdala (BLA) circuit, as assayed by increased expression of the immediate early gene, cFos (Dulka et al., 2018). Moreover, artificial activation of the vmPFC-BLA pathway through chemogenetic means is sufficient to reduce the CD response in subordinate and DSC hamsters, which display an enhanced CD response relative to dominant counterparts (Dulka et al., 2019, under review). It is noteworthy that reduced CD responses are also dependent

on gonadal hormones within the medial nucleus of the amygdala (MeA). Given that MeA neurons are similarly recruited to a greater degree in dominant than subordinate and control hamsters during defeat (Morrison et al., 2014), we also recently reported that pharmacological blockade of androgen receptors (AR) in the MeA during defeat prevents the acquisition of CD resistance in dominants (Grizzell et al., 2019). One possible explanation for these findings is that dominant hamsters, which upregulate AR during the development of social dominance (Clinard et al., 2016; Grizzell et al., 2019), may activate AR+ neurons in the MeA with a surge in plasma testosterone at the onset of a social defeat encounter. Although this possibility has yet to be demonstrated, it provides an explanation for stress-induced activation of AR+ MeA neurons during the defeat encounter. Inasmuch, the downstream targets of stress-activated AR+ MeA neurons may demonstrate alternative neural circuits permitting stress resilience in dominants. Although the MeA was not a focus of this dissertation, these findings support the conclusion that multiple neural circuits and neurochemical mechanisms contribute to resistance to acute traumatic stress exposure.

vmPFC-DRN Circuit Recruitment Confers Resilience to Acute Social Defeat Stress: Integrating Key Findings, Limitations, and Future Directions

To test the hypothesis that multiple brain mechanisms contribute to stress resistance, the experiments described in Chapters 2 and 3 of this dissertation were conducted to explore some alternative mechanisms that may collectively influence stress resistance in male hamsters. In Chapter 2, I presented findings that an acute social defeat episode in hamsters recruits vmPFC projections to the dorsal raphe nucleus (DRN), which is a stress effector region responsible for

distributing serotonin throughout the brain during various affective states. As per our hypothesis, we also found that dominant hamsters activate this pathway to a much greater degree than subordinate or dominant status control (DSC) counterparts. Furthermore, I described how we replicated earlier findings that subordinate animals fail to recruit neural activity within the vmPFC during social defeat stress.

In addition to these key results, the findings presented in Chapter 2 further inform the collective literature on animal models of stress resilience in multiple ways. First, it is noteworthy that tract-tracing studies in rats indicate a preferential targeting of DRN from the prelimbic (PL) subdivision, which is ventral to the anterior cingulate cortex and dorsal to the infralimbic (IL) subregion of the vmPFC (Vertes, 2004). However, we found no differences in DRN-targeted neuronal projections across these subregions. In that regard, it is possible that the vmPFC in Syrian hamsters is not organized identically to rats and mice. Indeed, reports of vmPFC-mediated resilience to learned helplessness implicate greater recruitment of the PL-DRN pathway in rats resistant to the effects of acute tail shock (Baratta et al., 2009, 2019). This observation is also important given frequent reports of differential function between the PL and IL in an array of behavioral paradigms. For instance, the PL has been shown to promote fear memory formation while the IL promotes fear memory extinction in rats (Sotres-Bayon et al., 2004; Sotres-Bayon & Quirk, 2010). The interpretation that vmPFC subregion organization differs between hamsters and other laboratory rodents is supported by our earlier report that PL-BLA activity is also more readily recruited in dominant hamsters than in subordinates, despite showing a reduced conditioned defeat response following a social defeat encounter (Dulka et al., 2018). This having been said, there were greater total vmPFC projections to the BLA that originated from the IL

cortex, and stress-resistant, dominant hamsters recruited this pathway more readily than subordinates or controls, which demonstrates some consistency with the functional neuroanatomy of rats and mice given that the IL-BLA pathway is associated with enhanced fear extinction and thus stress resilience. Indeed, the species differences in vmPFC anatomical organization do not appear to alter layer-specific cortical efferents because both the DRN-projecting and BLA-projecting neurons in the vmPFC, regardless of subregion, originate from cortical layers V/VI and II/III, respectively, which is consistent with anatomical studies in rats (Vertes, 2004) and mice (Challis et al., 2014).

Another observation regarding the results from Chapter 2 informs the collective understanding of the role of the DRN in response to acute social stress. Previous reports demonstrate changes in DRN neural activity in response to acute social defeat stress. We and others have shown that acute defeat induces DRN neural activity, as evidenced by the immediate early gene cFos, in losers and, to a lesser extent, winners (Kollack-Walker et al., 1997; Cooper et al., 2009). Furthermore, social defeat stress-induced neural activity in the DRN also appears causally linked with vulnerability, as pharmacological blockade and activation of self-inhibiting, serotonin-1a autoreceptors (5-HT1aR) in DRN both enhances (*i.e.* disinhibits DRN) or reduces (*i.e.* inhibits DRN) the CD response, respectively (Cooper et al., 2008). Importantly, 5-HT1aR binding by serotonin self-regulates not only DRN neural activation, but also serotonin release throughout the brain by inhibiting serotoninergic release via pre-synaptic autoinhibition. Interestingly, 5-HT1aR mRNA is downregulated in losers of defeat encounters but not in winners (Cooper et al., 2009). Our results further support a conclusion that stress resilience can be conferred via cellular mechanisms that regulate DRN activity in dominant animals, including vmPFC fibers that

innervate GABAergic microcircuits within the DRN. Indeed, we have also recently found that dominant hamsters express less cFos in the DRN compared to subordinates following acute social defeat or acute restraint, even though all animals were exposed to identical stress durations and intensities (Cooper et al., 2017).

Informing the literature at large, the results from Chapter 2 are also important in that they importantly extend observations from the learned controllability model to the social realm. By learning to exert control over the duration of a tail shock stressor, Steve Maier and colleagues have demonstrated that a vmPFC-DRN pathway is recruited to a greater degree in male rats than in those without such control (Baratta et al., 2009, 2019). A critique of this model has been that the tail shock stressor is not ethologically relevant, as rats in the wild rarely survive restraint coupled with electrocution. Inasmuch, the demonstration that learning control drives neural plasticity that confers stress resilience had yet to be shown within more naturalistic laboratory paradigms, at least to my knowledge. That said, the results presented in Chapter 2 suggest that not only might recruitment of a vmPFC-DRN circuit occur naturally, but that it extends to social settings. This is interesting given that more recent reports have shown that female rats fail to demonstrate vmPFC-DRN circuit recruitment in manner that confers resilience following learned controllability training (Baratta et al., 2018, 2019). Our results set a precedent that suggests a necessity to investigate the role of this circuit in female hamsters following stress, thereby providing further support for the role of vmPFC-DRN projections in stress resistant rodents generally and, accordingly, doing so would provide greater understanding of sex differences in behavioral and neuronal responses to stress.

Our findings from Chapter 2 also combine with those of Dulka et al., 2018 to demonstrate that acute stress resistance in more natural settings recruits multiple pathways responsible for conferring resilience. Both the learned controllability and fear conditioning paradigms, which respectively recruit vmPFC-DRN and vmPFC-amygdala circuits, generally utilize unnatural stressors to elicit robust circuit-specific consequences of acute stress. Social defeat stress integrates these circuits more generally, and Chapter 2 of this dissertation substantiates the claim that multiple circuits likely confer resilience in more natural scenarios.

Intriguingly, the results of Chapter 2 are inconsistent with those obtained by Berton and colleagues, which were briefly presented in the discussion of Chapter 2 but will be elaborated upon here. Utilizing an array of techniques, Berton's group convincingly demonstrate that artificial stimulation of DRN-projecting vmPFC neurons in mice suppresses social approach if occurring during a sensory-exposure period following chronic social defeat stress (Challis et al., 2013, 2014; Challis & Berton, 2015). These findings are at odds with previous reports on the role of the vmPFC-DRN circuit in stressful scenarios. As referenced above, Maier's group demonstrated that vmPFC-DRN recruitment occurred alongside resilience in rats learning control over a stressor. Similarly, Warden et al. (2012) showed that photic stimulation of vmPFC terminals in the DRN drive increased mobility during a forced swim task, which has been repeatedly argued as a proactive response to the inherent stress of being placed in an inescapable tank of water. Prior to our report, a potential explanation for the disparity between Berton's findings and others was based on the social nature of Berton's group's stress procedure. However, with the results of Chapter 2, it is now clear that this argument requires revision. Reconciliation between Berton's and our own findings, although difficult, is made easier given

the consistency of findings in our, Maier's, and Warden et al., (2012)'s reports. While a full review of methodological differences and their implications across these studies is probably necessary to fully appreciate a nuanced reconciliation of these reports, to do so would be too lengthy for this discussion. However, several key elements are worth noting.

First, the time period of vmPFC-DRN activity probed relative to stress exposure differs considerably in these models. Whereas all others, including ours, investigated neural activity *during* the physical exposure to their respective stressors, Berton and colleagues stimulated vmPFC-DRN activity immediately *following* the physical stress period for 10 days. The consequences of this disparity are difficult to surmise, as no other studies (to my knowledge) have interrogated neural responses after physical social defeat encounters. However, key in Berton's design is the notion that the sensory contact period is both necessary and sufficient to confer social avoidance behaviors following chronic defeat (Challis et al., 2013; Challis & Berton, 2015). This is predicated on the observation over numerous publications within C57Bl6 mice (or transgenic lines within this strain) that 10 minutes of a physical defeat followed by 23 hours and 50 minutes of sensory exposure to the aggressor induces social avoidance if occurring daily (with novel aggressors each day) for 10 days. Challis et al. (2013) demonstrated that they could obtain similar rates of social avoidance if C57 mice were exposed to the aggressor in the same cage but with a perforated, plastic divider for only 20 minutes of this sensory exposure period (rather than 23:50) following each of the 10-minute physical defeat encounters for 10 days. From this finding, Berton's artificial vmPFC-DRN stimulations occurred only during this 20-minute sensory exposure period, presumably due to the risk of physical entanglement of aggressor mice and optrode wires protruding from the subjects' head caps. Using this approach, vmPFC-DRN activity was enhanced

only during the period when the subject was protected from continued physical harm, yet in the enduring presence of the aggressor. When considering our findings from Chapter 2, it is therefore possible that vmPFC-DRN activation *during* a physical stressor confers resilience, while vmPFC-DRN activation in a rumination period *after* a physical stressor may critically differentiate the consequences of this circuit's activation.

The second noteworthy disparity in methodology is that in our and Maier's models, only a small subset of DRN-projecting vmPFC neurons were endogenously recruited during stress (Baratta et al., 2009). It is possible that distinct neuronal ensembles within the collective vmPFC-DRN fibers promote separable behavioral consequences. In that regard, unadulterated, endogenous recruitment of vmPFC neurons during stress is likely input specific, with selective firing onto vmPFC dendrites given context-specific recruitment of circuits upstream of DRN-projecting vmPFC synapses. In that regard, artificial circuit activation approaches likely target larger populations of cells which are selectively targeted by location (i.e. viral injection coordinates), viral injection spreads, transfection efficacy, and expression promoter (*CaMKIIa* in Berton's studies and Warden et al., 2012). It is therefore plausible that optogenetic studies may hyper-engage a pathway based on the physical presence of projecting neurons rather than their physiological relevance. Taken further, photic stimulation of virus-expressing vmPFC-DRN neurons may even induce activation beyond physiologically relevant levels, thereby additionally stimulating competing or compensatory mechanisms such as other regions (*e.g*. axonal collaterals), DRN subregions (*e.g*. non-GABAergic cells outside of lateral wings), downstream targets (*e.g*. via DRN projections to central amygdala or bed nucleus of stria terminalis), or additional ensembles not otherwise recruited in the tested scenarios where artificial stimulation

does not occur. Still, this does not explain the disparity between Berton's and Warden et al.'s (2012) studies, which might be better addressed by those listed in the paragraphs below. With this second critique having been levied, an important limitation of our and Baratta et al. (2009)'s report must be considered. In both cases, the reported levels of circuit activation were made possible by immunolabeling retrograde tract tracers alongside the expression of the immediate early gene, cFos. As is typically the case with any interrogative approaches within neuroscience research, there are several limitations to this experimental design. First, identification of stress-recruited vmPFC-DRN neurons depends on immunolabeling of both epitopes and, accordingly, leaves several potential sources of unintended variation. Indeed, we were forced to reduce the primary antibody concentrations in our dual-labeling approach due to poor specificity within Syrian hamster tissue and subsequent non-specific binding. This potentially lowered the threshold of detection to such a degree that we could incorrectly assume low levels of total vmPFC-DRN circuit recruitment. Second, while cFos identification does correlate highly with neuronal activation, given its roles in neuron-activated gene transcription, it is an indirect measure of circuit activation and may only reflect a subset of neurons activated based on differential presence of immediate early gene subtypes (*i.e.* arc, ΔFosB, etc.). Third, cFos activation is a poor indicator of degree or time of activation. While we and Baratta et al. (2009) did time lock our euthanasia to typical cFos decay rates (*i.e.* 90 minutes from stressor onset), there is no way to determine from this approach alone how often or exactly when these neurons fired. In that regard, those with lower cFos immunolabeling could have recruited vmPFC-DRN neurons at lower, inconsistent, or differening temporal levels. Even with this said, it is important to acknowledge the relative differences of these patterns of immunolabeling given the

standardized approaches, which controlled time-to-euthanasia, antibody concentrations, and more. Accordingly, it is safe to infer that those lacking controllability in Baratta et al. (2009) and of a subordinate social status in Chapter 2 engaged the vmPFC-DRN pathway to different and lesser degrees during stress exposure, and that the differential engagement corresponds with enhanced social avoidance alongside other markers of stress vulnerability.

The third and fourth considerations refer to the timeline of vmPFC-DRN recruitment. Berton's studies utilized 10 days of vmPFC-DRN pathway stimulation, and it is unknown whether one day of stimulation would produce similar social avoidance. Indeed, Warden et al. (2012) artificially stimulated the vmPFC-DRN circuit for a brief period on just one day, and Maier's and our studies incorporated endogenous vmPFC-DRN activation resulting from an acute presentation of stress on one day only. This said, it is likely (in fact, hypothesized) that dominant hamsters repeatedly activated the vmPFC-DRN circuit during dominance maintenance procedures occurring for 14 days prior to the single acute social defeat, though whether this occurred identically to the artificial activation conducted in Berton's studies is unknown. Future studies are needed to address this potential and would ideally be aimed at not only determining how long the vmPFC-DRN circuit is recruited by dominant hamsters in our model (*i.e*. days or time following agonistic encounter), but also whether it is necessary to dissect which neurons within the vmPFC-DRN circuit are activated during dyadic encounters and/or stress, for instance interrogating which terminal fields or cell populations within the DRN are targeted by winning encounters in dominant hamsters. One potential approach to address this would be the use of robust activity marking system (RAM), which permits time specific identification of neuronal recruitment and, moreover, whether neurons activated during different epochs coexpress the

RAM signal alongside an immediate early gene, such as cFos (Sorenson et al., 2016). In such a scenario, dual labeling of RAM and cFos would confirm: (1) whether dominant hamsters activate vmPFC-DRN neurons during dyadic encounters; and (2) whether those same neurons are reactivated during the social defeat experience. With this said, even if vmPFC-DRN neurons are activated over the 14 days of dyadic encounters in our dominant hamsters, it is highly likely that maintaining dominance status during this time engages collective neuronal activity differently than acute social defeat experiences. In Berton's studies, the 10 days of defeats differed from 14 days of dominance maintenance from our studies in that each were marked by repeated *receipt* vs. repeated *delivery* of physical and psychological attacks (*i.e*. body postures by aggressor), respectively. While there are undoubtedly increases in HPA reactivity during both of these procedures, future studies are needed to confirm whether the physiological stress responses differ between models. However, results obtained from Chapter 3 of this dissertation confirm a stark difference in the social avoidance that results from each paradigm. That is, following 10 days of chronic social defeat, Berton's studies and others demonstrate reliably that a large subset of mice avoid a novel conspecific in a social interaction test. However, in dominant hamsters that received 14 days of repeated dyadic encounters but not an acute social defeat, there was no social avoidance in a similar test (SIT, see Fig. 3.7 in Appendix C). In that regard, if the vmPFC-DRN circuit was repeatedly recruited by dominance maintenance procedures (as hypothesized), it did not yield social anxiety as demonstrated following 10 days (20 minutes daily) of repeated, artificial vmPFC-DRN artificial (Challis et al., 2014; Challis & Berton, 2015). Logically, if there are differing degrees of HPA reactivity between chronic defeat from Berton's model and the dominance maintenance period from our model ***and*** vmPFC-DRN recruitment occurred similarly

in each of these, then it follows that higher degrees of HPA reactivity may interact with vmPFC-DRN recruitment to shift affective states as they pertain to social avoidance. Indeed, DRN activity is dependent on corticotropin releasing hormone (CRH), and CRH has been shown to be a potent modulator of DRN neural activity and subsequent serotonergic release (Lowry et al., 2000; Hammack et al., 2002, 2003; Cooper & Huhman, 2007). Therefore, future studies directed at dissecting long-term modulation of stress and vmPFC-DRN activation in socially derived stress could more greatly inform this disparity.

Fourth, assuming that HPA reactivity during dominance status maintenance is low in our model given that there was only one day of social "defeat", the disparity between Berton's and our, Maier's, and Warden et al.'s (2012) studies could most parsimoniously be considered as a comparison of the effects of acute vs. chronic stress. While many similarities exist between the physiological responses to acute and chronic stress, key differences lie in the type and degree of plasticity-based changes occurring with such prolonged exposure. Given the potential for over-engagement using artificial means as discussed above, it is difficult to determine whether daily optogenetic recruitment also drives confounding compensatory changes or simply exaggerates meaningful phenomena, such as mechanisms underlying social avoidance. On the other hand, it is possible that only transient engagement of the vmPFC-DRN circuit confers resiliency by suppressing an acute-stress induced rise in serotonin, whereas prolonged engagement of this DRN-inhibiting pathway instead drives serotonergic hypofunction, which is consistent with clinical reports of MDD and PTSD and is evidenced by better-than-chance efficacy rates of SSRI treatment regimens. Further studies are needed to address the potential for artificially induced

serotonergic hypofunction and would benefit by including an acute stress condition as an additional means of control.

The fifth observation regards inherent differences between investigations across species. Warden et al.'s and Maier's studies were conducted with outbred rats where Berton's group used inbred mice. While important to acknowledge that outbred laboratory strains do not perfectly model the genetic diversity present in the wild, inbred lines run the risk of artificiality and/or masking natural phenomena. Further, as discussed earlier, our study confirms species differences given that the rat DRN receives sparse projections from the PL (Vertes, 2004) and, in Syrian hamsters, we were unable to detect any differences between IL and PL in CTB+ cells. This strongly supports the supposition that rats and hamsters differ in regional organization of vmPFC cells. Collectively, species differences highlight a need for more comparative analyses to increase translational potential.

The final observation refers to the nature of the very test used to assay the degree of affect following defeat procedures. In Warden et al.'s study, social avoidance was not assayed. In Maier's model, social avoidance was assayed in early reports but not when directly observing vmPFC-DRN recruitment, which is similarly true of our report in Chapter 2. In both of these latter cases, however, the inference of vmPFC-DRN involvement is drawn from social investigation assays using non-aggressive, juvenile conspecifics that are permitted to freely roam the testing arena. On the other hand, Berton's studies introduce a caged mouse from another strain (CD1), which more reliably elicits avoidance behavior than through the exposure to an age-matched or juvenile conspecific. Even further, ours and Maier's social investigation tests permit unbridled access and thus a more rich social interaction, whereas the "social interaction test" (SIT) used by

Berton's studies permit only olfactory and auditory cues, with limited visual stimulation and no physical interaction. Indeed, we were unable to detect a correlation between these two approaches within dominant hamsters when they were assayed in the experiments presented in Chapter 3 (see Fig. 3.7, Appendix C). In that regard, it may require more direct physical interactions, such as those permitted in the social investigation approaches used by our and Maier's models, to identify more subtle differences in social approach/avoidance as they pertain to the expression of stress resilience.

Taken together, there are multiple possibilities that may account for such a disparity between our and Berton's reports on vmPFC-DRN recruitment in chronic stress scenarios. Furthermore, this disparity may inform future studies of considerations when using artificial stimulation, particularly if not accounting for collateral and/or off-target activation, as well as the approach used to assay social avoidance following stress. Given that the social nature of the stressor is no longer a sufficient explanation for these disparities, future studies are warranted to further dissect the role of vmPFC-DRN activity, particularly in prolonged scenarios where plastic mechanisms within the DRN may produce a shift in the brain's response to social stress.

Although the data from Chapter 2 are highly informative, more studies are needed to confirm the conclusions drawn therefrom. The presented results are largely correlational and would be further substantiated by manipulations of the vmPFC-DRN circuit prior to, during, or following acute social defeat. Indeed, it is unknown what permits a greater recruitment of vmPFC-DRN projections in dominants or whether that recruitment is causally linked to CD resistance. It is plausible that plastic mechanisms during dominant status maintenance (*i.e.* prior to defeat) facilitate a more rapid recruitment during the subsequent social defeat stressor, and

therefore are necessary for the observed vmPFC-DRN recruitment in Chapter 2. This could be delineated using artificial neural activation procedures, though the conclusions ought to be taken with caution given the concerns raised above. That said, chemogenetic activation (or inhibition) of the vmPFC-DRN circuit during dominance status maintenance, but not during or following the defeat, would confirm whether this circuit's activity during stress: (1) is sufficient (or necessary) to produce resistance to social defeat stress as evidenced by reduced social avoidance; (2) is sufficient (or necessary) to drive plastic changes within the DRN prior to defeat (*e.g.* changes in 5-HT1aR expression); and (3) can rescue the neural signature or behavioral response of subordinates to defeat (in circuit activation studies) or produce subordinate-like patterns in dominants (in circuit inhibition studies). On the other hand, vmPFC-DRN projecting neurons may not be affected during dominance status maintenance, but may be recruited through alternative means during the defeat itself. For that matter, vmPFC-DRN recruitment during acute social defeat stress may be correlational yet not functionally necessary for CD resistance in dominant hamsters. For example, dominance status maintenance could drive plasticity within the DRN during dominance status maintenance that permits greater autoinhibition via 5-HT1aRs during stress and its recruitment during stress is inconsequential. In this case, the reduced CD response could result from plasticity mechanisms within the DRN that are independent of vmPFC involvement during the stressor itself. In that regard, similar chemogenetic or optogenetic circuit-manipulation studies to those described above could be conducted with the manipulations occurring instead during the defeat episodes. Such an approach would establish whether vmPFC-DRN recruitment during the defeat is necessary or sufficient for CD resistance.

Future studies aimed at establishing a causal role of vmPFC-DRN recruitment are important given that the observations from Chapter 2 are linked to behavioral outcomes based only on previous literature. Inasmuch, circuit manipulation and artificial activation/inactivation would permit direct observation of behavioral changes in response to acute social defeat stress. This having been said, such an approach would need to be conducted and interpreted carefully due to two key issues raised above in reference to Berton's reports in chronically defeated, optogenetically stimulated mice. First, studies increasingly report collateralization of axons throughout neural projections of the brain. In that regard, it is difficult to know for sure, without also accounting for collateral projections, whether an artificially activated circuit is solely responsible for observed behavior. Second, artificial activation likely recruits neurons in an anatomical but not functional manner. That is, studies increasingly report that minimally invasive observations of neuronal activation *in vivo* recruit ensembles rather than the entire population of neurons within a projecting circuit (Buzsaki, 2004; Baratta et al., 2009, 2019; Cruz et al., 2013; Josselyn et al., 2015; Sakurai et al., 2016; Carillo-Reid et al., 2017). Indeed, optogenetic activation of somas within the vmPFC *did not* yet the far more selective targeting of vmPFC terminals within the DRN *did* alter swimming behavior in Walden et al.'s aforementioned study (Walden et al., 2012). Given the likelihood of differential targets or region- or input-mediated titration of neural activity, it is possible that artificial activation would also create a conflicting or unnatural neural/behavioral state.

Given these challenges, it is noteworthy that technological advancements within biomedicine have provided some alternative methods to address some of these problems. For correlational approaches, the RAM system described earlier allows for viral expression of a

fluorescent marker tied to an immediate early gene promoter (Sorenson et al., 2016; Dolzani et al., 2018). Inasmuch, event-activated cells can be selectively visualized based on the permitted window of expression between doxycycline administrations, which suppress the expression of the virus. Because the expression of some immediate early genes peak around 90 minutes (*e.g*. cFos), this approach would essentially allow visualization of neural activity days after the targeted event (*i.e*. social defeat). Moreover, the added time between social defeat and RAM visualization would permit behavioral validation of the dual-labeled, tract-tracing approach taken in Chapter 2. For manipulation approaches, selective ensemble-based photic stimulation/inhibition is now theoretically possible given newly developed technology which: (1) permits real-time mapping of stress-activated neurons in a 3-dimensional space using two-photon, *in vivo* calcium imaging and, therefore, (2) permits rapid two-photon activation or inhibition of opsin-expressing individual neurons or neuron ensembles (Yang et al., 2018). Inasmuch, only the visualized, event-responsive ensembles could be artificially stimulated, permitting a selective dissection of vmPFC-DRN projections. This said, a limitation to this approach, aside from its limited availability and potential need for troubleshooting (especially within hamsters), is that photic-stimulation of select stress-activated ensembles may be restricted to dominant hamsters, given that subordinate hamsters express little or no such activity.

As referenced above, Chapter 2 and Dulka et al., 2018 together identify two key neural pathways recruited by dominants in response to stress. However, only a small percentage of cFos+ cells coexpressed CTB in each of these studies, indicating that there are other circuits recruited by acute social defeat stress in these animals. In that regard, future studies could identify additional downstream targets of the vmPFC, such as the bed nucleus of stria terminalis.

Alternatively, it is unknown which regions upstream of the vmPFC drive the status-dependent neural activation seen during social defeat stress. For that matter, within deep layers of the vmPFC, there were no overall differences in total stress-induced cFos+ cells between dominants and DSCs, despite the fact that dominants preferentially recruited DRN-projecting neurons during defeat. In that regard, the mechanisms permitting selective recruitment of this pathway remain unknown. Inasmuch, cell-type specificity studies would be substantially informative. It is possible that cFos-expressing GABAergic interneurons modulate the output of vmPFC in DSCs and, to some extent, subordinates. On the other hand, dominants may display a reduced ratio of GABAergic to glutamatergic activation by comparison. Furthermore, it is possible that differential levels of neuromodulation account for status- and/or stress-induced changes of vmPFC activity. For instance, ventral tegmental inputs deliver dopamine to vmPFC and could activate D1 or D2 receptors differentially across groups. In that regard, I would predict that dominant hamsters display fewer inhibitory D2 receptors than subordinate counterparts. While this could be confirmed histologically, it would not inform whether differential levels of dopamine are released in the vmPFC. Inasmuch, *in vivo* fiber photometry studies of GCaMP-expressing terminals via cre-dependent viral infection of VTA-vmPFC afferents would permit observation of firing rates of dopaminergic cells in the vmPFC. Similarly, a fast-scan cyclic voltammetry approach could confirm differences in dopamine levels released from inputs within vmPFC tissue, though this approach has noted limitations and is considered by many to be antiquated. On the other hand, recent developments in bioengineering have produced synthetic receptors which fluoresce upon ligand binding and can be directed at specific neurotransmitters, such as glutamate, GABA, dopamine, and more (Sun et al., 2018). These genetically encoded, g-protein coupled receptor-activation-

based (GRAB) sensors remain limited in that they cannot determine levels of neurotransmitter release per se due to the presence of transporters, reuptake, or synaptic degradation, but permit a more precise identification of synaptic concentrations of targeted ligands within sub-second timescales. Inashmuch, a "GRAB-DA" sensor would permit real-time interrogations of dopamine binding potential in selectively targeted neuronal projections, and accordingly, whether ventral tegmental dopamine (or any other upstream target/ligand) plays a role in status-dependent recruitment of vmPFC-DRN neurons.

Taken together, we have shown the vmPFC-DRN circuit recruitment corresponds with reduced avoidance following acute social defeat stress in a dominance status-dependent manner. These findings will advance the knowledge base both with regard to the use of Syrian hamsters in biomedical research and also the field as a whole as we seek to elucidate the neural mechanisms underlying resilience in humans. This having been said, there is more work to be done and the findings presented in Chapter 2 of this dissertation contribute to the literature by providing further directions for such pursuits.

vmPFC Neuroinflammation in Syrian Hamsters and the Effects of Dominance Status and Acute Social Defeat: Integrating Key Findings, Limitations, and Future Directions

The finding presented in Chapter 2 that stress in subordinates engaged the fewest vmPFC neurons, including those projecting to the DRN, led me to search for mechanisms which may impede this recruitment. The status-dependent disparity in vmPFC cFos expression suggests that 14 days of subordination drives changes within the brain that prevent vmPFC neural activation in the face of stress. While there are innumerable plausible mechanistic explanations for such an

impedance, I questioned whether, for male Syrian hamsters, the process of maintaining a subordinate position in a hierarchy might serve as a low-level chronic stress experience that drives tissue degradation and neuroinflammatory activity. Indeed, while the relationship between social status and stress is highly dynamic and context-dependent, subordination does correspond with increased risk of stress-induced impairment for many species, including humans (Marmot et al., 1984; Blanchard et al., 1993; Sapolsky, 2004, 2005, 2017). If such were the case for Syrian hamsters, prolonged psychological stress could, even at low levels, plausibly lead to elevations of stress-induced innate immune activity. This process might therefore drive risk of increased reactive oxygen species (ROS) production, damage-associated molecular pattern (DAMP) molecule release, subsequent microglial recruitment, associated phagocytosis, and in more extreme conditions, blood brain barrier breakdown and peripheral monocyte infiltration. Collectively, these changes could also drive an array of processes which could degrade vmPFC cells and thus impede neural recruitment during stress in those maintaining a subordinate social status. Given that this hypothesis is consistent with the observation that dominant hamsters displayed elevated levels of metabolites with antioxidant properties in the vmPFC and elsewhere (Dulka et al., 2017), I accordingly began to address this possibility by conducting pilot studies to quantify plasma IL-6 levels via enzyme-linked immunosorbent assay (ELISA). IL-6 is a principle proinflammatory cytokine with demonstrable elevations in an array of psychopathologies (Howren et al., 2009). However, due to poor availability of molecular tools targeted at hamsters as well as the inability to acquire sufficiently large volumes of blood in survival procedures, I was unable to complete these assessments. That said, I describe a series of studies in Chapter 3 that were conducted to systematically address this possibility from an alternative angle. Instead of

investigating changes within the periphery, we targeted changes occurring within neuroimmune cells of the vmPFC, specifically the defeat-induced changes in expression patterns of Iba1 as well as the morphometry of Iba1+ cells, which include microglia and, potentially, peripherally derived monocytes.

To do this, I had to first determine whether acute social defeat stress (i.e. < 30 minutes of defeat exposure) induced a proinflammatory state, as this was unknown prior to beginning this dissertation. Indeed, if a highly salient, acute defeat could not induce immune activity recruitment, lower salience dyadic encounters would unlikely do so as well. While authors of previous studies demonstrating robust neuroinflammation may characterize their defeat procedures as "acute", they often persisted for at least 4-5 days, at times for hours each day. On the other hand, an acute (60 minute) tail-shock stress procedure primed a proinflammatory responses to a subsequent immune challenge, but even when observed *in vitro*, the isolated microglia did not express an increase in Iba1 expression to tail shock only (Frank et al., 2007). In Syrian hamsters, there is no evidence that acute stress induces changes of the neuroimmune system function, though it has been shown that defeat (acute and chronic) induced a suppressing effect on humoral immunity in the periphery. Specifically, social defeat led to a reduced IgG response to injections of keyhole limpet hemocyanin (KLH; Jasnow et al., 2001). Importantly, IgG responses to KLH mark the efficiency of the *acquired* immune system and, accordingly, are only minimally reflective of *innate* immune responses, which is a primary subject of Chapter 3. With this said, a recently published report on the Syrian hamster microbiome provides support for the hypothesis that social defeat may drive alterations of immune activity. Specifically, social defeat altered gut microbiota in both winners and losers of the social defeat encounter, and this

occurred in both acute and chronic conditions (Partrick et al., 2018). Interestingly, because gut microbes can be repetitively and non-invasively assayed from fecal boli, Partrick and colleagues were also able to demonstrate that certain levels of bacteria were predictive of whether animals would win or lose an aggressive encounter. That is, the likelihood of an animal's future dominance status may be linked to the bacterial environment within the microbiome. For instance, by comparison with hamsters that would become subordinate, (future) dominant hamsters displayed fewer bacteria of the phylum *Firmicutes*, which has also been shown to be higher in patients with major depressive disorder when compared with healthy controls (Jiang et al., 2015; Partrick et al., 2018). Further, the observed shifts in these and other gut microbiota indicated an enhanced risk of proinflammatory activity (Maes, 2008; Chaissang et al., 2015) and Partrick and colleagues suggested that an investigation of inflammatory activity using the acute defeat model in Syrian hamsters was warranted.

Therefore, the results from Chapter 3 substantiate the claim that acute social defeat stress alters innate immune processes, at least in Syrian hamsters. While there were only mild increases in the expression of Iba1 in the vmPFC of acutely stressed animals, Iba1 expression was gradually potentiated in subsets of hamsters following progressive doses of the endotoxin, lipopolysaccharide (LPS). Inasmuch, there was a clear main effect of social defeat stress across all groups indicating that defeat episodes drive inflammatory mechanisms in the vmPFC which generally increase Iba1, a protein linked with proinflammatory activity of microglia and peripheral macrophagic monocytes.

The widely distributed responses of Iba1+ cells in defeated hamsters injected with LPS described in Study 1 alongside the effects of social dominance in Study 2 of Chapter 3 also provide

insight for future studies into status-dependent differences in neuroimmune reactivity in Syrian hamsters. While our report may be the first to demonstrate that such a short duration social stressor (*i.e*. 15 minutes total) can perturb inflammatory activity, it is not the first within rodents to also characterize individual differences in neuroinflammatory activity as it pertains to stress resilience and vulnerability. Rats who display short latencies to submit to an aggressor respond to stress with high neuroimmune activity, which corresponds with passive coping behaviors (Wood et al., 2010, 2015). On the other hand, longer latencies to submit to an aggressor are characterized by active coping, which corresponds with increased markers of stress resilience and fewer signals of inflammatory actions in the brain. Our findings are in agreement with Wood and colleagues in that subordinate hamsters also submit rapidly to a potentially threatening conspecific and display greater neuroinflammatory activity in response to stress. On the other hand, dominants regularly resist an aggressor's early attempts to subdue them, which is similar to Wood et al.'s "long latency" rats. Moreover, dominant hamsters displayed resilient-like reductions of social avoidance and fewer markers and consequences of immune activity, despite receiving similar amounts of aggression as subordinate and DSC counterparts. The short/long-latency-to-submit model, as well as other models of resilience, has been integral to the understanding of stress and neuroimmune interactions but importantly require longer, multi-day social defeat procedures and can only be used to assay males.

Our discovery that Syrian hamsters may serve is an alternative model of individual differences in stress-induced inflammation is therefore valuable for multiple reasons. First, exposure to a brief social defeat stressor permits more pointed investigations of immediate inflammatory responses, though it is possible that subordination instigates a greater degree of

microglial priming or low-level inflammatory activity in hamsters. Second, use of Syrian hamsters as a model provides a comparative perspective that is lacking among biomedical research. Third, investigations using Syrian hamsters provide an ability to interrogate sex differences within the same model species while also controlling for variations of stress delivery. Indeed, female hamsters also form easily identifiable dominance hierarchies and will readily and reliably defend their territories against an intruder (Huhman et al., 2003; Faruzzi et al., 2005; Solomon et al., 2007). Importantly, there are stark differences in rates of stress-induced dysfunction across the sexes and, given the inextricably interwoven relationship between stress and innate immune activity, it should be of no surprise that demonstrable sex differences in microglial and neuroinflammatory responses to stress abound (Rohleder et al., 2001; Bollinger et al., 2016; Bordeleau et al., 2019). Indeed, gonadal steroids modulate immune activity dynamically, at times performing proinflammatory or anti-inflammatory actions depending on the biochemical environment (Cutolo et al., 2004). The use of ethologically valid models of stress that permit the inclusion of both males and females are therefore of high importance, especially given recent critiques against the face validity of a wide array of traditional laboratory techniques in living rodents (Shansky, 2019; Reardon, 2019).

The findings described in Chapter 3 further inform a study of social status-mediated changes in innate immune activity. Previously, we found dominance status-dependent changes in proinflammatory activity and/or the tissue-protective responses to oxidative stress in multiple regions, including the vmPFC (Dulka et al., 2017), which includes prior shifts in inflammation brought on by two weeks of subordination alone (*i.e.* non-stressed subordinates). While there was an increase in Iba1 expression in both the IL and PL following social defeat in both dominants

and subordinates, there were more pronounced increases in soma diameters as well as soma size to total cell size ratios in subordinate animals both with and without preceding acute social defeat stress. The increase in Iba1 expression in both dominants and subordinates (though not in DSCs) suggests that 14 days of agonistic encounters likely sensitizes the brain's immune system to a subsequent defeat episode, much like the observations from the first study of Chapter 3 that suggests the possibility of social stress-induced priming of Iba1+ cells. Dominance status likely impacts the degree of this effect though, as morphometric analyses suggest that these changes are more consequential for subordinate hamsters. That is, subordinates' Iba1+ cell shapes shifted in a manner reflective of greater proinflammation, which includes elevated proinflammatory cytokine release, increased ROS production, and a greater likelihood of phagocytosis. Moreover, non-defeated subordinates also displayed morphological changes of Iba1+ cells consistent with progressed states of neuroinflammation, suggesting that the process of daily subordination, itself, may drive inflammatory processes within the vmPFC.

The observation that Iba1+ cells were more active following stress, especially in subordinates, is strengthened in that subordinates, as well as DSCs, had increased markers of tissue degradation following acute social defeat stress. Analyses of amino cupric "silver stain" labeling indicated significantly greater silver impregnation of debris within all cortical layers of vmPFC, especially along the synapse rich midline. Given the numerous reports of chronic stress-induced retraction of dendrites within vmPFC neurons, this is unsurprising, especially if two weeks of subordination induces a low level, chronic stress-like experience. To confirm that the qualitative, albeit blinded, analyses of degeneration states reflected consequential changes in tissue integrity, we also stained for the protein synaptophysin, a component of the snare complex

that is present in functional neuron terminals. To our surprise, while acute social defeat stress did reliably and dramatically reduce synaptophysin expression in the vmPFC, it did so regardless of dominance status. This is an important observation for several reasons. First, it demonstrates that acute social defeat in Syrian hamsters induces a reduction of vmPFC synapses. Second, it may suggest that the immune responses of dominant hamsters are more efficient, clearing the cellular debris resulting from acute stress-induced degeneration and synaptic stripping more rapidly. Third, it may suggest the possibility that the observed status-dependent differences in tissue degradation, immune activity, and stress-induced avoidance are unrelated to synaptic densities. For that matter, the additional debris observed in subordinates and DSCs could have originated from sources other than active synapses, such as degenerated glia, deconstructed extracellular matrices and/or perineuronal nets, or even recently dismantled proteins via proteolytic mechanisms. While the identity of the debris' source cannot be verified, we found that subordinate and DSC animals showed an increase in the number of "starburst" patterns, which appeared to be glial in nature. Fourth, synaptophysin immunolabeling may instead be a poor proxy for status-dependent changes of vmPFC structure and alternative approaches may provide greater insights. For examples, commonly used neuroanatomical methods include measurements of dendritic length and branching via Golgi staining procedures as well as dendritic spine segment measurements at high magnification, including morphological changes of spine number, shape, diameter, and distance between spines. It is also noteworthy that positive labeling of the synaptophysin protein documents the presence of vesicle-containing terminals but not necessarily the efficiency of synaptic communication itself. In that regard, further studies could be conducted to confirm whether acute stress-induced reductions of

synaptophysin are functionally consequential. For instance, using *ex vivo* whole-cell, patch clamp analyses or *in vivo* electrophysiological recordings of neuronal fields, one could quantify spontaneous excitatory- or inhibitory-post-synaptic potentials within vmPFC neurons to demonstrate if there are stress- or status-induced differences of firing among afferent neuron terminals in response to selected stimuli. This could also yield greater identification of stress-induced changes in GABAergic tone, which could be increased in subordinates, thus providing a net inhibition of efferent fibers and therefore reduced cFos expression as discussed in Chapter 2. This approach, if coupled with laser-stimulated opsin-expressing terminals, could also more selectively dissect status- and/or stress-induced disruptions of communication by identifying affected cell types and origins. For example, catecholaminergic inputs to vmPFC have been implicated in differences in stress responding (Arnsten, 2015; Wang et al., 2016; Vander Weele et al., 2019). It is therefore plausible that the stress-induced reductions of synaptic densities described in Chapter 3 could influence, perhaps in a status-dependent manner, the efficacy of dopaminergic or noradrenergic inputs to vmPFC cells, thereby accounting for observed differences in vmPFC neuron recruitment in stressed hamsters described previously.

As an additional consideration, the presence of cellular debris does not guarantee a causal role of Iba1+ cells in immune-generated tissue destruction. Instead, the increased inflammation patterns could reflect a response to the presence of tissue degeneration, rather than a cause. Indeed, cellular debris must be cleared by various mechanisms of the brain, especially microglia, in order to ensure optimal function. This having been said, the final study described in Chapter 3 weakens this consideration while supporting the previously posed hypothesis that immune activities contributed to stress-induced destruction of vmPFC tissue. Minocycline administration,

which blocks inflammatory activity of Iba1+ cells, produced a neuroprotective effect in stressed hamsters by reducing signals of degeneration as indicated by the silver stain, as well as preserving synaptophysin expression in stressed animals to levels no different than non-stressed counterparts. Intriguingly however, minocycline did not noticeably reduce Iba1 expression itself in vmPFC, regardless of stress, though it did lead to changes in the morphology of Iba1+ cells that were less reflective of proinflammatory activities. This observation was surprising, as we had hypothesized a substantial reduction in Iba1 optical densities alongside the observed reductions of proinflammatory morphology. This disparity could be explained in a couple of ways. First, it is possible that the chosen dosage of minocycline was not high enough to completely abate microglial recruitment in stressful conditions, though reductions in microglia activity were enough to block proinflammatory morphological changes. Second, it is possible that our detection methods for Iba1+ optical densities require optimization. For instance, the proinflammatory-based morphological changes described in Chapter 3 may have resulted in a reduced number of quantifiable pixels due to Iba1+ cellular branch retraction alongside an undetected increase of Iba1 expression within single pixels, as likely occurs in more extreme conditions of inflammation, such as traumatic brain injury. This claim is substantiated by the observation of substantially greater Iba1 expression coupled with a proinflammatory morphology in positive controls, which are presented in Appendix E.

Although ancillary, another contribution to the literature with regard to Syrian hamsters is in further describing cautious and avoidant social behavior following acute social defeat. In Chapter 3 and Appendix D, I describe the observed differences in risk-assessment referred to as “active” and “passive vigilance”. Interestingly, when scoring behavior in animals from Study 3, I

did not find significantly elevated active vigilance in DSC, dominant, or subordinate hamsters. This pattern of behavior only emerged in a subset of minocycline-treated hamsters in Study 4. While the mechanisms underlying this change in social vigilance are unknown, it is noteworthy that minocycline-drinking animals rapidly consumed regular drinking water when given the opportunity five minutes prior to euthanasia. Inasmuch, active vigilance could be a pattern of socially avoidant behavior that emerges following combinatorial social and non-social stress (*i.e.* water deprivation). Presently, it is difficult to know for sure whether or to what degree minocycline at 4 mg/ml is aversive to hamsters, but it is notable that there were no detected reductions in weight in minocycline drinkers at euthanasia. Taken together, the results from the minocycline studies suggest that although halting Iba1+ cellular function does not reduce social avoidance following acute social defeat stress, it may confer proactive coping strategies, at least within subsets of the population. Finally, these results confirm that Iba1+ cellular activity within the vmPFC plays a role in cellular and synaptic degeneration following acute social defeat, which supports the hypothesis that neuroinflammation is consequential for structural integrity of the vmPFC following traumatic stress.

Conclusions after Integrating Chapters 2 and 3

When collectively integrating the findings of this dissertation (see Figure 4.1 of Appendix F) and the existing literature, the conclusions of this dissertation are as follows. Acute social defeat stress inherently drives activation of vmPFC neurons, which include those that project to the DRN and other stress effector regions such as the BLA. Importantly, prior social experiences (*e.g.* acquisition and maintenance of social status) modify vmPFC neuronal recruitment in a

manner that corresponds with shifts in social avoidance. In that regard, increased vmPFC recruitment, including to the DRN and BLA, promotes reduced social avoidance and a return of species typical behavior such as territorial defense. On the other hand, acute social defeat also has the ability to drive inflammatory-based changes within the vmPFC which may perturb neuronal function and structural integrity. Moreover, this degradative physiological response to acute stress is also modified by prior social experience (*e.g.* acquisition and maintenance of social status) and in a consistent manner. In that regard, maintaining a subordinate social status prior to an acutely traumatic experience increases the likelihood of social avoidance and territory submission alongside vmPFC cellular and synaptic degradation, proinflammatory activity, and risk of oxidative stress which correspond with a failure to recruit vmPFC neurons, including vmPFC-DRN and vmPFC-BLA projections. On the other hand, maintaining a dominant social status prior to an acutely traumatic experience enables a resilience to these changes that corresponds with more efficient function of immune and antioxidant activities. Moreover, the prior social experience of maintaining social dominance permits an increase in vmPFC recruitment during stress, which is a necessary step in reducing social avoidance following acute stress likely via the vmPFC's ability to suppress BLA and DRN activity during the traumatic experience.

These findings are collectively important given their high translational value to humans. Accordingly, the results described in this dissertation can be applied to the advancement of therapeutic approaches, which include pharmaceutical regimens of anti-inflammatories and anti-oxidants as well as various vmPFC-engaging behavioral treatments. Inasmuch, treatments that drive vmPFC recruitment and/or protect vmPFC anatomical integrity may facilitate greater suppression of brain regions whose activity have been implicated in negative affective responses

to stress. Accordingly, such prefrontal suppression of negative affect may link with cognitive perceptions of control and enhanced self-efficacy in the face of adversity, which together might reduce the rates of various stress-related psychopathologies.

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Appendix F

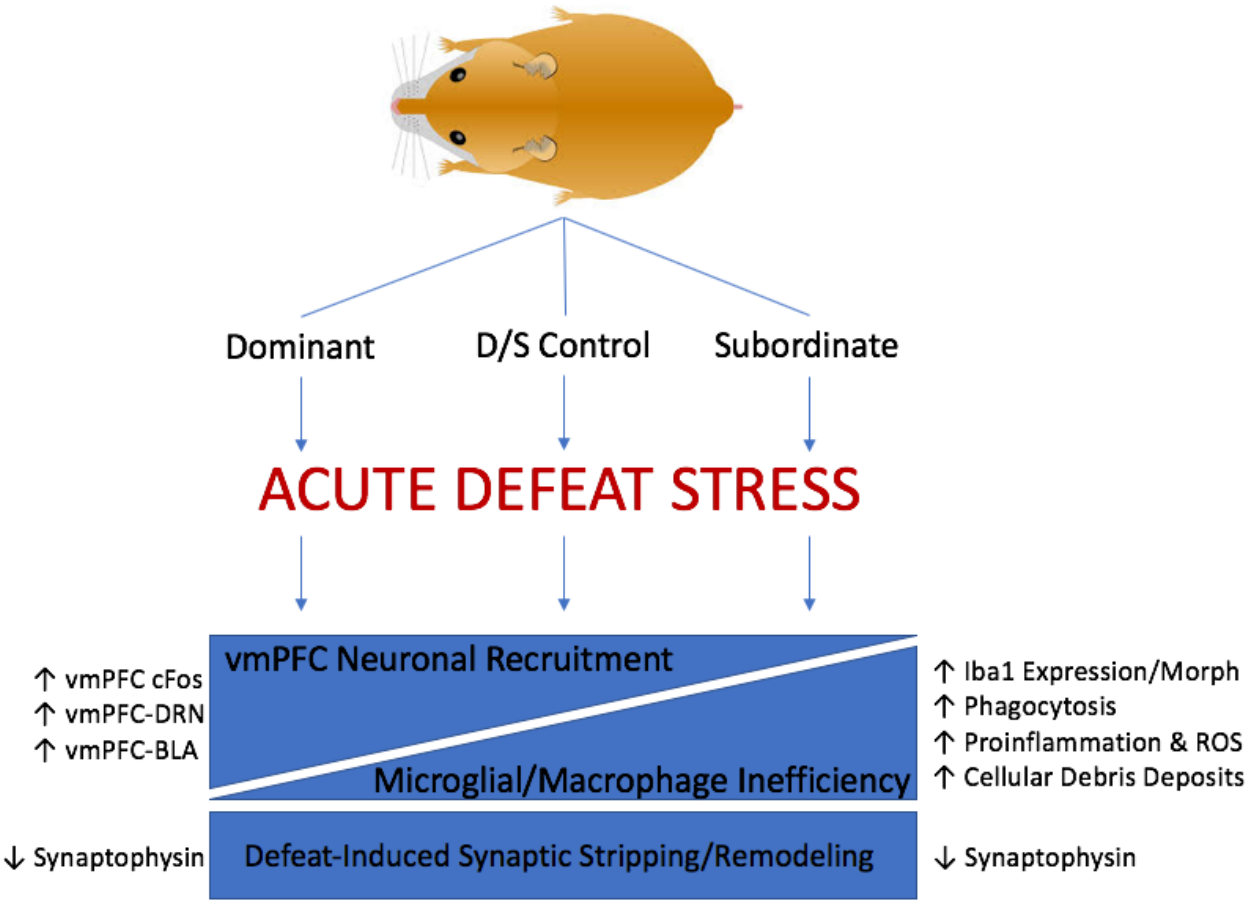

Figure 4.1 Integrated Model of Findings and Implications/Interpretations. Collectively, the results of this dissertation suggest that the acquisition and maintenance of a dominant social status permits increased vmPFC recruitment (*i.e*. increased expression of the immediate early gene, cFos) including in neurons that project to downstream stress effector regions such as the dorsal raphe (DRN) and basolateral amygdala (BLA). On the other hand, acquisition of a subordinate social status greatly reduces the likelihood of these neurons being recruited during stress. Having acquired and maintained a subordinate social status increases the likelihood of proinflammatory activity and oxidative stress, which leads to tissue degradation and cellular debris deposition. Observations that these all occur despite seeing no difference in synaptic densities suggest that defeat-induced synaptic stripping and/or remodeling occurs irrespective of social status. Together, this suggests that stress-induced reductions in synaptophysin are not responsible for the exaggerated defeat-induced social avoidance reliably seen in subordinate hamsters and may instead reflect more efficient inflammatory function in dominant hamsters coupled with an enhancement of anti-oxidant defenses.

Vita

Johnathon Alexander Grizzell, or as he is more commonly referred, “Alex”, was born in 1982 in Atlanta, GA to parents Cynthia N. Grizzell and John C. Grizzell. He is the first of two children, with 5.5 years separating him from his younger brother, Joshua M.. Alex attended elementary school at Midway Elementary in Alpharetta, GA before relocating to Woodstock, GA to attend Woodstock Middle and later, Woodstock High schools. Upon graduation in 2001, Alex attended Harding University in Searcy, AR. Frequently working multiple jobs to support his education, Alex eventually graduated with a BA in Psychology and minor in Sociology in May, 2008. He was then accepted into a PhD program of Psychology and Neuroscience at Baylor University in Waco, TX in the laboratory of Dr. Bradley Keele. At this time, he also served as a Graduate Teaching Assistant and, later, Teacher of Record for the upper-level course, Learning and Behavior, in Baylor’s Psychology Department under the tutelage of Dr. Hugh Riley.

Alex specialized his research at Baylor on the characterization of the hyperpolarization current of pyramidal neurons of the basolateral amygdala using whole cell, patch clamp electrophysiology techniques. His doctoral thesis would focus on how serotonin influenced this mechanism of amygdala activity in stressful conditions and, moreover, whether these two factors combined to impact the vmPFC via an amygdala-to-vmPFC neural circuit. However, this project would not be realized as, in 2010, Alex’s father died tragically due to complications following a brain surgery that was conducted in 2008 to remove the epileptogenic loci within his right amygdala and hippocampus. As a result, Alex accepted a MA in Neuroscience from Baylor University in May, 2011 and took some time away from his graduate studies to recover and evaluate his career trajectory. It was at this time that he joined the laboratory of Dr. Valentina

Echeverria at Bay Pines Veterans Affairs Hospital in Bay Pines, FL. There he led the effort to characterize the pharmacotherapeutic value of the nicotine metabolite, cotinine, on mouse models of PTSD, depression, and Alzheimer's disease. Due to funding complications, Alex remained in this position for only 8 months but accrued enough data to publish six peer-reviewed papers, four of which he served as first author. To ensure steady income while writing up these manuscripts with his beloved advisor, "Val", Alex then joined the laboratory of Dr. Jamie Fernandez in the department of Psychiatry and Neurosciences at nearby University of South Florida in Tampa, FL. There, he led research on the characterization of estrogen receptor fluctuations as well as the influence of nicotine on a non-invasive animal model of postpartum depression that he and Dr. Fernandez developed under the co-advisement of Drs. Lynn Wecker and Rex Philpot. Altogether, Alex garnered three years of experience on an array of behavioral and molecular techniques that complimented those gained at Baylor and the VA hospital. In this time at USF, he also served as second author for two peer-reviewed publications.

In the summer of 2015, Alex joined the laboratory of Dr. Matthew Cooper in the Neuroscience and Behavior tract of the Experimental PhD program in The University of Tennessee, Knoxville's Department of Psychology. Alex was welcomed to the program as an awardee of the prestigious J. Wallace and Katie Dean fellowship, which acknowledges and supports incoming students who have excelled in work prior to matriculation and show promise for outstanding graduate work in excellent and demanding programs at UT. While in Knoxville, Alex led several projects and developed multiple collaborations on topics ranging from the influence of alcohol and cotinine on acute stress resilience in laboratory mice to the impact of anthropogenic noise on foraging and agonistic behaviors of non-migratory, aviary *Paridae* flocks

during winter months in the forests of East Tennessee. Alex also acquired financial support for his research on neuroinflammatory mechanisms of stress resilience (*i.e.* Chapter 3 of this dissertation) across several internal and external funding sources which, collectively, eclipsed the $100,000 valuation mark. His research also extended to the classroom, where he served as coauthor to two peer-reviewed papers investigating *open education resources* and *non-disposable assignments* in university settings. He also coauthored a paper proposing a model of peer mentoring among graduate students. His passion for education and mentoring is reflected in his devotion to the classroom as well. In his second semester at UT, Alex began teaching a 400-level Special Topics of Neuroscience course in the Biochemistry and Cellular and Molecular Biology department, to be followed by 300-level courses on Motivation and Behavioral Neuroscience in the department of Psychology. In 2018, he was named the Graduate Student Teacher of the Year by UT's Department of Psychology and completed the Scholar's level training in the Center for the Integration of Research, Teaching, and Learning (CIRTL) Network. Alex also served on the UT's Department of Psychology task force to redesign the general education requirements in 2016 and helped design multiple courses within UT's psychology major, including two upper-level neuroscience courses.

Alex was also heavily involved in service while at UT. In addition to volunteering yearly in elementary classrooms to provide an introduction to neuroscience research for local youths, Alex served as the Director of the 2018 Brain Awareness Campaign at UT, which received the *Innovative Program of the Year Award* in 2019 by The University of Tennessee's Division of Student Life. While serving in this capacity, he united over 100 undergraduate and graduate student volunteers across 10 student organizations to develop a month-long celebration of brain

and mental health research for students of UT as well as community members and their children. Furthermore, Alex's fundraising efforts for this month-long program exceeded $30,000 across several donations from within and outside of the UT network, including service grants from The Pat Summitt Foundation and local biotech company, NeuroScience Associates.

Alex completed his graduate studies at UT in four years and entertained multiple job offers upon completion of his PhD. He accepted a postdoctoral fellowship in The Center for Neuroscience and The Department of Psychology and Neuroscience at The University of Colorado, Boulder in the laboratory of Dr. Michael Saddoris to study the intersection of stress resilience, controllability, and cocaine-based disruptions in reward learning. His career intentions upon publication of this document were to eventually run his own laboratory in a university setting much like that he experienced while enrolled at The University of Tennessee, Knoxville.