



NEUROINFLAMMATION AND BEHAVIOUR

EDITED BY: Luba Sominsky, Adam K. Walker and Deborah M. Hodgson

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NEUROINFLAMMATION AND BEHAVIOUR

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The brain and immune system are involved in an intricate network of bidirectional communication. This relationship is vital for optimal physiological and psychological development and functioning but can also result in unwanted outcomes. In particular, this interaction plays an important role in cognition, mood and behaviour. Neuroinflammation is known to contribute to neurological and affective disorders including impaired learning and memory, depressive, anxiety and schizoaffective symptoms, as well as pain. The development of these conditions often occurs on the backdrop of pre-existing physical illnesses which give rise to increased activation of the immune system, such as cancer, obesity, infection and autoimmune disorders. Similarly, psychological states can alter regulation of the immune system. This has been most extensively studied in the context of stress and immune function.

Understanding the underlying mechanisms that lead to the onset of inflammation-induced neuropathology and stress-induced immune suppression will contribute to the development of novel and effective treatment strategies for both the disease and its neurological side effects. In this research topic we explored the relationship between the immune system and the brain throughout life. We include both original research and review papers from animal, clinical and molecular perspectives.

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Editorial: Neuroinflammation and behavior

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Keywords: neuroinflammation, neuroimmune, neuroendocrine, proinflammatory, mood disorders, microglia, perinatal programming, aging

Neuroimmune regulation plays a major role in many facets of human health. In the last 30 years our understanding of this intricate relationship between the brain and immune system has progressed immensely. Pioneering work in the late 1980s and early 1990s began to establish an understanding of the bi-directional communication between these previously considered distinct systems, and its implications in the regulation of mood and cognition (Dantzer and Kelley, 1989; Kelley and Dantzer, 1990; Bluthé et al., 1991). Since then, significant advances in the field of neuroimmunology have been made, and we now know considerably more about the mechanisms responsible for neuroimmune regulation of health and behavior. In this Frontiers research topic *Neuroinflammation and Behavior*, 10 groups of researchers contributed their expertise to discuss the recent knowledge in the field of neuroimmunology, focusing on the neuroinflammatory mechanisms in affective disorders, early life programming of neuroimmune function, as well as neuroimmune interactions in the aging brain and its associated pathologies.

Anisman and colleagues begin this topic with a review on the role of proinflammatory cytokines in depressive disorders, and the ability of social experiences to cause, exacerbate or mitigate cytokine imbalance and mood disorders. The authors provide evidence for a particular contribution of individual factors, such as sex, age, genetic, and other differences, to the effectiveness of social support in alleviating the symptoms of depression and neuroinflammatory signaling (Audet et al., 2014). Hutchinson and colleagues expand on this discussion by exploring the specific contribution of the innate immune pattern recognition receptor Toll-like receptor 4 (TLR4) in the pathophysiology of depression. The authors suggest that TLR4-mediated mechanisms could underpin the neural, immune, and neuroendocrine alterations seen in patients with major depressive disorder (Liu et al., 2014). Wohleb et al. propose a novel model of neuroimmune interactions that underlie psychosocial stress-induced immune and behavioral changes. In response to stress, simultaneous activation of peripheral monocytes and microglia induces peripheral and central inflammation. Neuroendocrine and sympathetic stress responses then promote monocyte trafficking to the brain and exacerbate microglial activation. This in turn contributes to the behavioral phenotype, such as persistent anxiety-like behavior (Wohleb et al., 2014). Baune and colleagues discuss the role of inflammasomes in neuroinflammation. Inflammasomes, by promoting the processing and activation of proinflammatory cytokines, play an instrumental role in the pathophysiology of neuroinflammation in the aging brain, as well as in the etiologies of several neurological diseases. Inflammasome-driven inflammation is also involved in the development of metabolic disorders that are known to be associated with mental illness (Singhal et al., 2014). This review highlights the therapeutic potential of targeting inflammasome-related pathways to combat neuroinflammatory diseases.

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Several articles highlight the importance of early life events in the development of the neuroimmune circuitries. Campbell et al. investigate the role of the early microbial environment in the development of stress and nociceptive circuitries. The authors demonstrate that neonatal exposure to an immune challenge suppresses behavioral responses to nociceptive stimuli in adulthood. This is associated with increased activation of orexin neurons, implicated in stress and pain processing, in hypothalamic subregions (Campbell et al., 2015). These findings may have important implications for targeting the orexin system for treatment of chronic pain conditions, as well as highlighting the critical role of the early life environment in determining later health outcomes. The latter is further demonstrated by Spencer and colleagues. These authors examine the long-term effects of early nutritional environment on neuroinflammatory profile. Neonatal overfeeding induces basal microgliosis and increased neuronal activation in response to an immune challenge in the paraventricular hypothalamus. Interestingly, this early life intervention reduces central responses to the inflammatory effects of adult short-term high-fat diet. Whether these findings represent a suboptimal ability of neonatally overfed animals to counter the adversity of high-fat diet or whether this is an adaptive response, remains to be established (Cai et al., 2014). Jasoni et al. elaborate on the impact of perinatal immune-brain interaction by looking at maternal obesity-induced inflammation and predisposition of offspring to neurobehavioral and metabolic diseases. This review points to the common link between metabolic imbalance and neuroimmune activity and to the ability of the maternal immune milieu to alter fetal brain development and function long-term (Jasoni et al., 2014). The neuroinflammatory effects of obesity and other metabolic disorders persist throughout life, inducing cognitive dysfunction and other neuropathologies. Jenkins and colleagues review preclinical and clinical evidence concerning obesity and cognitive decline, focusing on the potential inflammatory mechanisms

underpinning this neuropathology in obesity. The authors conclude that exercise may be the most efficient strategy to alleviate obesity-related cognitive impairment (Nguyen et al., 2014).

Fuggle et al. review the evidence for the impact of neuroimmune interactions in rheumatoid arthritis (RA) on the pathophysiology of this autoimmune disease. RA patients suffer from greater susceptibility to infections, further exacerbating joint pain and swelling. Current biologic therapies target the suppression of major inflammatory pathways involved in joint inflammation, improving disease control and quality of life. This, however, may result in even a greater susceptibility to infections, stimulating disease flares (Fuggle et al., 2014). This article highlights the importance of further research into therapeutic interventions for RA, sufficient to maintain disease remission, while preventing the degree of immunosuppression that may increase the risk of infections and trigger RA flares.

Finally, Walker and colleagues review the evidence that neuroinflammation plays a role in the neurobehavioral toxicities of chemotherapy. They focus on the common symptoms, such as cognitive decline, neuropathy and fatigue. While neuroinflammation has been thought to be the major mediator of these symptoms, the authors propose additional mechanisms may be involved, such as damage associated molecular patterns (DAMPs) and mitochondrial dysfunction (Vichaya et al., 2015). This article highlights the exciting potential for novel interventions to alleviate chemotherapy-induced neurotoxicities, significantly improving quality of life of many cancer patients and survivors.

In summary, the articles presented in this research topic provide a valuable insight into the importance of neuroimmune regulation in health and disease, encompassing a wide scope of conditions and presenting evidence for novel and intriguing mechanisms that underlie the reciprocal nature of brain-immune interactions.

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Cytokine variations and mood disorders: influence of social stressors and social support

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Stressful events have been implicated in the evolution of mood disorders. In addition to brain neurotransmitters and growth factors, the view has been offered that these disorders might be provoked by the activation of the inflammatory immune system as well as by *de novo* changes of inflammatory cytokines within the brain. The present review describes the impact of social stressors in animals and in humans on behavioral changes reminiscent of depressive states as well as on cytokine functioning. Social stressors increase pro-inflammatory cytokines in circulation as well as in brain regions that have been associated with depression, varying with the animal's social status and/or behavioral methods used to contend with social challenges. Likewise, in humans, social stressors that favor the development of depression are accompanied by elevated circulating cytokine levels and conversely, conditions that limit the cytokine elevations correlated with symptom attenuation or reversal. The implications of these findings are discussed in relation to the potentially powerful effects of social support, social identity, and connectedness in maintaining well-being and in diminishing symptoms of depression.

Keywords: aggression, depression, IL-6, pro-inflammatory cytokines, social status, social stressors, social support, trauma

Without dismissing the importance of other stressful experiences in promoting mood disturbances, particular attention had been devoted to the influence of social stressors in the emergence and continuation of depressive disorders. Indeed, an array of social stressors have been found to act in this capacity, including separation/divorce, unhealthy and unsupportive relationships, loss of a friendship, social rejection, and social alienation (McQuaid et al., 2014a; Slavich and Irwin, 2014). This focus has, in part, stemmed from the frequent findings concerning the importance of social identity and connectedness to psychological well-being (Cruwys et al., 2013, 2014), as well as studies in rodents showing that social challenges have profound and long-lasting effects on neurobiological processes that have been implicated in depressive disorders and adaptation (Chaudhury et al., 2013; Azzinnari et al., 2014; Francis et al., 2014).

In addition to monoamine [e.g., serotonin (5-HT), norepinephrine (NE)], neuroendocrine [e.g., corticotropin-releasing hormone (CRH)] and growth factor [e.g., brain-derived neurotrophic factor (BDNF)] variations, it has been suggested that depressive symptoms may stem from activation of the inflammatory immune system, especially that of pro-inflammatory cytokines (e.g., signaling molecules normally released by immune cells in the presence of infectious microorganisms). In this regard, social stressors in rodents, especially those that involve trauma in the form of aggressive social interactions, promoted several neurobiological changes that have been implicated in depression. These have included elevated circulating corticosterone levels (Audet and Anisman, 2010; Audet et al., 2011), increased

expression of CRH and its receptors (McQuaid et al., 2013), variations of monoamine turnover and levels (Audet and Anisman, 2010), and changes of neurotrophins such as BDNF (Berton et al., 2006; Fanous et al., 2010) in brain areas that subserve stressor appraisal processes as well as depression [prefrontal cortex (PFC), hippocampus, amygdala, paraventricular nucleus (PVN) of the hypothalamus, and nucleus accumbens]. In addition, social stressors also affected pro-inflammatory cytokines both peripherally and within stress-sensitive brain regions (Bartolomucci et al., 2003; Audet et al., 2010, 2011), supporting the view that activation of these inflammatory markers may contribute to the pathogenesis of stress-related disorders.

The effects elicited by social challenges may depend on the animal's social status or defensive strategies adopted during aggressive interactions. For instance, active coping in the face of aggressive encounters (e.g., active defense, hyperactivity, dominance, aggression) limited or even prevented neuroendocrine and neurochemical effects ordinarily elicited by the social stressor, whereas strategies that involve less resistance (e.g., passive defense, immobility, submissiveness) were related to more profound stressor-induced variations (Bartolomucci, 2007; Audet and Anisman, 2010; Audet et al., 2010; Gómez-Lázaro et al., 2011). These outcomes may have been related to pre-existing neurobiological features associated with genetic dispositions or previous stressor experiences, which might have promoted vulnerability to adverse outcomes in response to social conflicts. The present review delineates the pro-inflammatory impacts of social stressors, with a focus on how these effects in vulnerable

individuals may influence the emergence of later mood disturbances and mental health problems, and it is suggested that the positive effects of social support and social connectedness on mood states and well-being may be related to attenuation of pro-inflammatory functioning. **Figure 1** describes some of the factors that may moderate the effects of social stressors on cytokine functioning which then promote depressive states.

IMPLICATIONS OF THE INFLAMMATORY IMMUNE SYSTEM IN DEPRESSIVE ILLNESSES

It has been about two decades since the suggestion was offered that over-activation of the inflammatory immune system might contribute to the pathogenesis of stress-related disorders, especially that of depressive illnesses (Maes, 1995). The inflammatory hypothesis of depression postulated that elevated circulating levels of pro-inflammatory cytokines might promote the evolution and maintenance of depressive symptoms (Maes, 1995, 2008). Meta-analyses have, in fact, indicated that in the absence of infectious pathogens, peripheral concentrations of pro-inflammatory cytokines, especially that of interleukin (IL)-6 and tumor necrosis factor (TNF)- α , were higher in non-medicated individuals with depression than in non-depressed individuals (Dowlati et al., 2010; Liu et al., 2012). Variations of IL-1 β in relation to depressive illnesses were less consistently established (Dowlati et al., 2010; Liu et al., 2012), likely because of the difficulty in detecting the very low levels of this cytokine in human circulation. In addition to elevated pro-inflammatory levels, low serum levels of the anti-inflammatory cytokine IL-10 were negatively correlated to depressive symptoms in drug-free depressed individuals (Dhabhar et al., 2009), suggesting that a shift toward a pro-inflammatory state, comprising variations of either or both pro- and anti-inflammatory functioning, may underlie depressive symptoms.

Additional support for the inflammatory hypothesis of depression has come from reports showing that the prevalence of depressive symptoms was relatively elevated in non-medicated patients with chronic inflammatory diseases (e.g., chronic hepatitis C, multiple sclerosis) or acute inflammatory conditions (e.g., surgery, stroke) (Musselman et al., 2001; Cremeans-Smith et al., 2009). As well, immunotherapy with the pro-inflammatory cytokine interferon (IFN)- α for chronic hepatitis C and some types of cancers promoted depressive features (Capuron and Miller, 2004). The latter findings not only support the view that inflammatory factors could play a provocative role in depression, but it appeared that the depressive effects of IFN- α could be attenuated by antidepressant treatments (Raison et al., 2006).

SOCIAL STRESSORS AND STERILE INFLAMMATORY RESPONSES

The possible role for pro-inflammatory cytokines in depression has been reinforced by the observation that stressful experiences enhanced inflammatory activity in the absence of infectious pathogens. In this regard, when experienced acutely, social stressor challenges increased circulating concentrations of pro-inflammatory cytokines, especially that of IL-6 and IL-1 β (Steptoe et al., 2007). Inflammatory activation has also been reported in individuals chronically exposed to stressful life events, including

long-term care of a spouse with dementia (Kiecolt-Glaser et al., 2003), bereavement (Schultze-Florey et al., 2012), prolonged isolation (Yi et al., 2014), effort-reward imbalance at work (Bellingrath et al., 2013), and low socioeconomic status (Gimeno et al., 2007; Loucks et al., 2010). As these stressors may be associated with depression, these data are in keeping with the view that chronic low-grade inflammation resulting from prolonged stressor exposure may be fundamental in the onset of stress-related depressive symptoms.

Among the different stressors that can be experienced, one of the most significant for the emergence of neuropsychiatric symptoms are those involving a violent or traumatic component (e.g., in the form of neglect, maltreatment/abuse, aggression). Based on a meta-analysis, it was concluded that trauma exposure during either childhood or adulthood was positively associated with levels of IL-1 β , IL-6, and TNF- α , (but not of IL-2, IL-4, IL-8, or IL-10) and these associations were especially pronounced in individuals who had developed neuropsychiatric symptoms, irrespective of their nature (Tursich et al., 2014). Associations between early-life adversities and higher levels of inflammatory factors have been reported among adults (Danese et al., 2007; Hartwell et al., 2013) but also among children and adolescents (Slopen et al., 2013). Thus, the view was taken that increased inflammatory activity associated with trauma exposure could be initiated shortly after trauma and persist over an extended period of time (or be re-induced, and even exacerbated, upon re-experiencing stressors), possibly progressively fostering sensitivity to later health or mood disturbances. In fact, in addition to having higher baseline cytokine levels, individuals at risk for the development of neuropsychiatric symptoms (owing to previous traumatic experiences) appeared to be more sensitive to the cytokine effects normally induced by a socially stressful experience or to the low-grade inflammation associated with chronic stressors. Higher cytokine levels in individuals with a history of childhood adversities were more pronounced in those currently experiencing high levels of distress, either acutely or on a chronic basis (Carpenter et al., 2010; Kiecolt-Glaser et al., 2011). Thus, the heightened vulnerability to psychiatric conditions in individuals who had been confronted with traumatic events might be related, at least in part, to a fragility or hyper-responsiveness of inflammatory processes resulting from earlier negative experiences (Anisman et al., 2008).

PERIPHERAL vs. CENTRAL CYTOKINE PROCESSES IN DEPRESSION

The significance of peripheral inflammatory activation in relation to depression is uncertain, especially as mood improvements elicited by medication are not consistently accompanied by a normalization of cytokine levels (Eller et al., 2009; Hannestad et al., 2011). Whereas circulating cytokines may directly or indirectly contribute to the evolution of depressive symptoms, their increased presence in circulation may also be a reflection of the distress experienced by depressed individuals (particularly as depression would itself act as a stressor). Ultimately, however, depression is probably more closely aligned with cytokine variations that occur in the brain or with effects secondary to such changes, including variations of brain neurotransmitters,

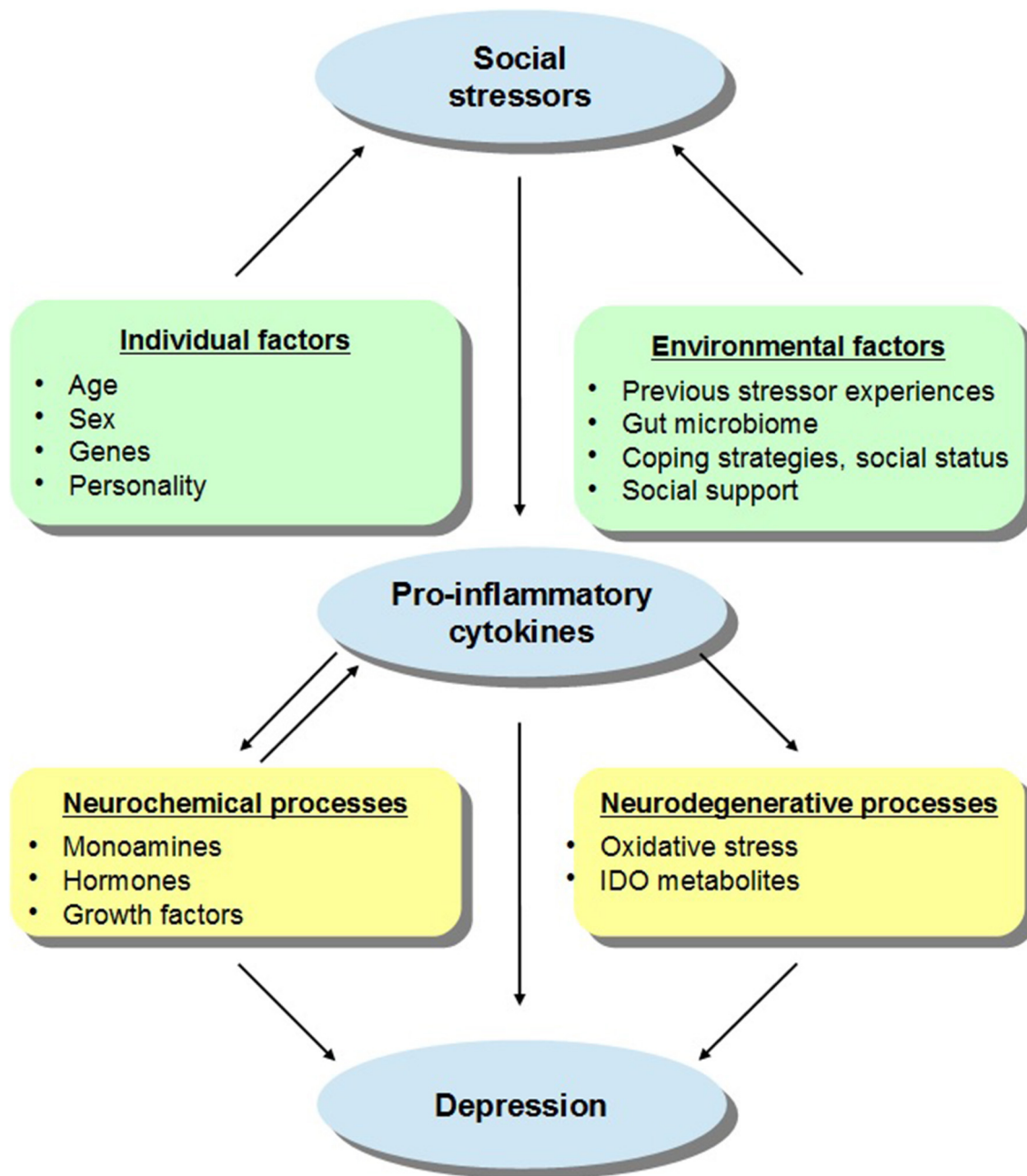


FIGURE 1 | A schematic representation of the relations between social stressors, pro-inflammatory cytokines, and depressive states, with a particular focus on the individual and environmental factors that may moderate these effects. It is suggested that in addition to sex and age, the capacity of social stressors to promote inflammatory variations that might lead to depression may be influenced by the presence of genetic and personality factors. For instance, individuals carrying specific gene combinations or polymorphisms (e.g., variants of IL-6, IL-1 β , TNF- α) may be more vulnerable to the depressive effects of inflammatory activation provided that they also encounter social stressors. In addition, it is proposed that environmental factors may also impact on stress-related cytokine responses and thus on depressive symptoms. For example, previous stressor experiences in the form of prenatal or early-life adversity or immunological challenges as well as gut bacterial disturbances may

influence inflammatory processes and sensitize immune responses to subsequent stressors, thus favoring the emergence of depressive symptoms. However, in the context of adequate coping strategies, higher social status, or in the presence of effective social support, the cytokine effects of stressors may be limited thus buffering against mood disturbances. The activation of pro-inflammatory processes may directly or indirectly influence depressive states. Elevations of cytokines may influence monoamine (e.g., 5-HT, NE), hormone (e.g., CRH), and growth factor (e.g., BDNF) activity which might favor the evolution of depression (and basal hormonal and neurochemical functioning may impact cytokine processes). Alternatively, cytokine variations may stimulate the enzyme indoleamine 2,3-dioxygenase (IDO) and promote the release of neurotoxic metabolites, including kynurenic acid, quinolinic acid, or 3-hydroxykynurenine, and cause oxidative stress, culminating in depression.

hormones, or growth factors (see Audet and Anisman, 2013). Several reports also point to the possibility that processes in other organs that share reciprocal connections with the brain, including the microbiota present in the gastrointestinal tract, may also play an important role in this regard (Cryan and Dinan, 2012; Haroon et al., 2012).

A small portion of the pro-inflammatory cytokines released into circulation may access the brain at circumventricular sites (Vitkovic et al., 2000) or through saturable transport systems (Banks, 2006). Thus, cytokine elevations that occur peripherally among stressed and/or depressed individuals might be reflected in the central nervous system, especially as several of the cytokines altered in circulation were also dysregulated in the brain of individuals with depression or with conditions that had been related to and/or are comorbid with the disorder. It is particularly significant, however, that pro-inflammatory cytokines may also be produced endogenously by brain microglia in response to inflammatory or stressor stimuli (Quan et al., 1998; Dantzer et al., 2008; Sukoff Rizzo et al., 2012; Schwartz et al., 2013). The few studies that examined brain cytokine variations in relation to depression revealed that several pro- and anti-inflammatory cytokines (e.g., IL-1 α , IL-2, IL-3, IL-5, IL-8, IL-9, IL-10, IL-12A, IL-13, IL-15, IL-18, IFN- γ , and TNF) were up-regulated in post-mortem frontal cortex of depressed patients who had died by natural causes or through suicide (Dean et al., 2010; Shelton et al., 2011). In contrast, in the PVN of the hypothalamus, changes in TNF- α or IL-1 β mRNA expression were not observed in depressed patients relative to healthy controls (Wang et al., 2008). A potential role for brain cytokine elevations in depression has also been confirmed in studies using rodent models of the disorder. Social conflicts in male mice increased plasma IL-6 and IL-1 β levels and altered mRNA expression of the same cytokines in the PFC and hippocampus (Bartolomucci et al., 2003; Audet et al., 2010, 2011). Moreover, genetic deletion of the IL-1 receptor type 1 on endothelial cells limited brain elevations of IL-1 β , TNF- α , and IL-6 mRNA expression elicited by repeated social defeat, indicating that IL-1 signaling likely plays a role in social stress-induced activation of pro-inflammatory cytokines (Wohleb et al., 2014). This is not to say that social stressors are uniquely capable of provoking such changes, as strong physical stressors known to elicit depressive-like behaviors, such as those involving shock and immobilization, increased IL-1 β protein and mRNA in serum and in the hypothalamus (O'Connor et al., 2003; Deak et al., 2005) as well as IL-6 in the frontal cortex (Sukoff Rizzo et al., 2012). Likewise, a chronic unpredictable mild stressor regimen that promoted depressive-like features also increased TNF- α levels in the PFC (Liu et al., 2013).

NEW PLAYERS IN THE CYTOKINE HYPOTHESIS OF DEPRESSION

Beyond variations of the traditional pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α , depression has been associated with elevations of circulating IL-18, which is involved in cell-mediated immunity (Merendino et al., 2002; Prossin et al., 2011), and macrophage migration inhibitory factor (MIF), which is considered to be a pro-inflammatory cytokine that has neurogenic actions (Musil et al., 2011; Cattaneo et al., 2013). Consistent

with these peripheral changes, IL-18 expression in the neocortex increased in subordinate rats after an agonistic encounter (Kroes et al., 2006). As well, genetic deletion of MIF blocked the increased cell proliferation normally elicited by the antidepressant fluoxetine (Conboy et al., 2011), and limited the antidepressant effects and the increased hippocampal BDNF expression ordinarily elicited by exercise (Moon et al., 2012). In addition to pro-inflammatory cytokines, several growth factors including BDNF as well as basic fibroblast growth factor (FGF-2), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and erythropoietin (EPO) have been implicated in depressive disorders (see Audet and Anisman, 2013 for a detailed description). These growth factors are also influenced by stressors (Cirulli and Alleva, 2009) and as indicated previously (Audet and Anisman, 2013), they may interact with cytokines in affecting behavioral phenotypes.

SOCIAL STRESSORS, CYTOKINES, AND DEPRESSION: DOES SEX MATTER?

The clinical observation that vulnerability to mood disturbances is more pronounced in women than in men has raised the possibility that males and females might also differ with respect to biological variations that could be linked to depressive symptoms. However, it was reported that females might be more resilient to the cytokine variations associated to depressive states or to stressor exposure. For instance, IL-6 elevations elicited by an acute stressor occurred earlier in men than in women (Edwards et al., 2006). Moreover, elevated levels of IL-6 were apparent in depressed men, but not in depressed women with a late onset of symptoms (Vogelzangs et al., 2012) or suffering from social isolation (Häfner et al., 2011). Animal studies also showed that increased depressive-like behaviors were observed in female but not male IL-10 knockout mice (Mesquita et al., 2008). As well, sensitized IL-1 β expression in the PFC and hippocampus apparent after a second stressor exposure in stressed males was not apparent in females (Hudson et al., 2014). Consistent with a role for sex hormones in such outcomes, in females foot-shocks increased IL-1 expression in the PVN during the diestrus, proestrus, and estrus stages, whereas no elevations were observed during the metestrus stage, suggesting that endogenous progesterone may play a role in the PVN IL-1 response to stressors (Arakawa et al., 2014). This complex picture implies that additional players may contribute to the mood disturbances apparent in females and the question that has to be asked is whether sex hormones (e.g., estrogen, progesterone) play a more provocative role in inflammatory processes than what was initially thought.

CYTOKINE VARIATIONS, AGGRESSIVENESS, AND IMPULSIVITY

It is interesting that the inflammatory variations observed among victims of traumatic events are also apparent in individuals perpetrating aggressive or violent acts, and it was suggested that activity of specific cytokines might modulate aggressive and impulsive behaviors. For instance, being physically aggressive during childhood and adolescence predicted reduced baseline levels of pro- (IL-6, IL-1 α) and anti- (IL-4, IL-10) inflammatory cytokines in early adulthood (Provençal et al., 2013). Likewise,

in mice, peripheral administration of IL-1 β suppressed aggressive actions during agonistic encounters (Cirulli et al., 1998). Moreover, among mice that did not express IL-6 the frequency of aggressive postures was elevated and affiliative behaviors were low, whereas overexpression of IL-6 was accompanied by elevated affiliative behaviors (Alleva et al., 1998). Curiously, however, higher plasma IL-6 concentrations in individuals with intermittent explosive disorder were directly correlated with their history of actual aggressive behaviors (Coccaro et al., 2014). Likewise, gene expression of TNF-related cytokines was related to aggression scores in adolescents with bipolar disorder (Barzman et al., 2014). Additional support for a role for inflammatory markers in impulsive or violent behaviors have come from reports showing enhanced levels of pro-inflammatory cytokines among individuals showing deliberate self-harm (Westling et al., 2011), high rage scores (Pesce et al., 2013), or pronounced hostile behaviors (Marsland et al., 2008). Why reduced vs. enhanced cytokine levels were apparent among individuals with aggressive and/or impulsive features in these studies is not entirely clear. Higher levels of childhood maltreatment have been reported in adults with intermittent explosive disorder compared with psychiatric and healthy controls (Fanning et al., 2014). Considering that early-life trauma has been linked to a subsequent increase of inflammatory activity, it might be suggested that the roots and trajectory of aggression (being physically abused vs. being physically aggressive as a child) might have played a role in the different inflammatory patterns observed in these studies.

Consistent with the suggestion that violent and/or impulsive behaviors may be linked to neuropsychiatric conditions, increased IL-1 β , IL-6, and TNF- α in post-mortem PFC were observed in teenaged individuals that died by suicide, irrespective of whether they had been diagnosed with major depression (Pandey et al., 2012). Increased protein levels of Toll-like receptors 3 and 4 (associated with increased production of pro-inflammatory cytokines) were also found in post-mortem PFC of depressed adults that had died by suicide compared to that evident in depressed individuals that had died from a cause other than suicide (Pandey et al., 2014). The fact that increased inflammatory activation had been observed in post-mortem brains of suicide individuals is of particular importance, as it raises the possibility that central cytokine variations are linked to suicide rather than depression *per se*. Although suicidal thoughts or ideations may be a symptom of major depression, actual suicide attempts are not necessarily reflective of depressive illnesses, but might be indicative of additional factors, such as impulsivity and extreme or violent behaviors. In this regard, elevated IL-6 levels found in the cerebrospinal fluid of suicidal individuals were more pronounced in those that made efforts of violent suicide (Lindqvist et al., 2009).

SOCIAL TRAUMA: SOCIAL STATUS AND DEFENSE STRATEGIES MATTER

It has long been known that social hierarchy, depending on whether the organism is at the top or bottom of the hierarchy, can be particularly stressful and can have marked effects on health and well-being (Sapolsky, 2005). Indeed, the stability of the hierarchy and the availability of resources can have

considerable sway on the adaptive neurobiological processes that occur in dominant and submissive/subordinate animals, as can affiliative behaviors of a group, gender, and individual difference factors (personality). Considering that individuals with aggressive profiles may also be vulnerable to neuropsychiatric disorders, the investigation of the potential victim/aggressor dichotomy in relation to inflammatory variations prompted further investigation. Preclinical studies indicated that although aggressive social interactions promoted comparable corticosterone elevations in submissive and dominant mice, some of the cytokine variations elicited were influenced by social status (Audet et al., 2010). Specifically, circulating IL-6 elevations after social conflicts were more pronounced in submissive mice (Audet et al., 2010). As well, higher spleen levels of TNF- α and IL-6 were apparent among mice using passive behaviors (e.g., immobility, decreased reactivity, and low aggression) during aggressive encounters compared to those using active defense strategies (e.g., aggression and territorial control) or to non-stressed controls (Gómez-Lázaro et al., 2011). In contrast, in dominant/winner mice, higher levels of the anti-inflammatory cytokine IL-10 (Stewart et al., 2014) and IL-6 up-regulations were apparent in the PFC (Audet et al., 2010). Together, these findings suggest that active coping strategies during aggressive interactions may be more effective in moderating the cytokine impacts of stressors involving social conflicts than strategies that involve less resistance (Gómez-Lázaro et al., 2011). How these cytokine variations come to be translated into specific behavioral changes is uncertain, nor is it known how chronicity of social stressors will affect cytokine-related disturbances, although it seems that chronic social stressors favor the development of varied pathological conditions (Sapolsky, 2005).

Similar distinctions based on the behavioral strategies used during aggressive encounters in rodents have been reported with respect to other neurochemical markers. In this regard, the increased NE utilization within the PFC and hippocampus observed after social conflicts was more pronounced in submissive than in dominant mice (Audet and Anisman, 2010). As well, latency to escape from an aggressor during a social defeat episode was negatively correlated with BDNF mRNA expression in the hippocampus, suggesting that an active response strategy during stressor exposure might be associated with higher hippocampal BDNF (Arendt et al., 2012). Likewise, depressive-like behaviors and hippocampal BDNF protein reductions were only apparent in those defeated mice that displayed a passive profile during agonistic interactions (Gómez-Lázaro et al., 2011).

THE CYTOKINE HYPOTHESIS OF DEPRESSION FACING NEW CHALLENGES

There are several unresolved issues pertaining to the relationships between social stressors, pro-inflammatory activation, and depression. Beyond individual differences related to sex, social status, or defense strategies described earlier, whether inflammatory variations and depressive features will emerge as a result of stressor exposure may not always occur and the multiple processes by which this comes about remain to be fully established. For instance, it is uncertain whether a single cytokine is of particular relevance in regard to the provocation of depression, or whether symptoms (or clusters of symptoms) evolve owing to

multiple cytokine actions or to interactions between cytokines and other factors. Most studies that examined the relations between circulating cytokines and depression have considered the illness from a broad perspective rather than one that focused on specific symptoms or on depression subtypes. However, it seems that HPA axis and sympathetic nervous system activity (Gold and Chrousos, 2002), as well as that of circulating IL-1 β and IL-6 (Anisman et al., 1999; Dunjic-Kostic et al., 2013), may vary greatly in individuals with typical vs. atypical features of major depression. In line with this view, a positive correlation between severity of depressive symptoms and IL-6 concentrations was found in melancholic patients, whereas a negative correlation was apparent in depressed patients with atypical features (Karlović et al., 2012). Whether links exist between other cytokines and specific characteristics of the illness remains to be determined, but specific attention needs to be paid to whether inflammatory activation is linked to specific depressive symptoms and not to others.

The fact that traditional antidepressant medication may limit depressive symptoms without affecting pro-inflammatory cytokine activity (Anisman et al., 1999; Eller et al., 2009; Hannestad et al., 2011) and that anti-inflammatory treatments are not systematically effective in limiting depressive symptoms (Köhler et al., 2014), also begs the question as to what the specific contribution of circulating pro-inflammatory cytokines might be in mediating depressive symptoms. Are these cytokines merely peripheral markers of depression or are they involved in the provocation of illness? Are these cytokines reflective of an ongoing biological dysfunction that would inform treatment resistance or vulnerability to relapse? If depression (or most likely specific depressive subtypes) is driven by inflammation, then traditional antidepressants would be expected to improve mood through cytokine-dependent mechanisms, and anti-inflammatory treatments would be effective in attenuating symptoms. However, this would only occur in those patients in whom depressive symptoms were precipitated by inflammatory factors. In this regard, there has been growing acknowledgment of the limited success in defining the genetic and biological complements that are associated with depression, and the suggestion was made that individualized treatment for the illness, based on biologically relevant markers, may be instrumental in guiding treatment selection and enhancing outcomes. Indeed, the identification of specific cytokine markers that might be aligned with particular symptoms or subtypes of depression could be especially useful in the prediction of treatment efficacy.

NEW AVENUES FOR THE INFLAMMATORY HYPOTHESIS OF DEPRESSION

As already intimated, the processes by which cytokine elevations are elicited by social stressors, and how these come to affect brain functioning, are still not known. In addition to cytokine variations that occur in the brain, neurobiological alterations elicited by stressors at various peripheral sites that interact with brain processes may be relevant to stressor-related disorders, including depression. In this regard, increasing interest has focused on the inflammatory variations that might occur along the gut-brain axis (Cryan and Dinan, 2012; Haroon et al., 2012). It has been suggested that microorganisms that inhabit the gastrointestinal

tract, referred to as the gut microbiota, might play an important role in inflammatory responses elicited by stressors. The gut is inhabited by 10^{13} – 10^{14} commensal bacteria that interact with each other and modulate other systems, including the immune functioning. Alterations in the composition and diversity of the gut microbiota by environmental insults, including stressors, have been correlated with plasma cytokine elevations (Bailey et al., 2011). Moreover, reduction of the gut microbiota by antibiotics prevented the stressor-induced cytokine elevations, indicating that bacteria that live in the gut were necessary for an inflammatory stress response to develop (Bailey et al., 2011). Given that microbiota may have immunoregulatory effects through actions on immune cells, it was suggested that probiotic bacteria (i.e., “good” bacteria that when ingested confer a benefit for the host), by their interactions with commensal gut bacteria, may also influence inflammatory responses elicited by stressors. In support of this view, plasma elevations of pro-inflammatory cytokines provoked by a psychological stressor were prevented in rats treated with the probiotic *Bifidobacterium infantis* (Desbonnet et al., 2010).

Accumulating data have also indicated that the gut microbiota may interact with the central nervous system and that alterations of microbiota composition could modulate brain functions and behaviors (Cryan and Dinan, 2012; Dinan and Cryan, 2012). For example, reduced anxiety-like behaviors as well as altered expression of BDNF and of a variety of NMDA and 5-HT receptors were reported in germ-free mice (i.e., mice that had been maintained in a sterile environment and never been exposed to bacterial microbe) and in mice treated with antibiotics that disrupted gut bacteria (Sudo et al., 2004; Bercik et al., 2011a; Neufeld et al., 2011). Moreover, administration of particular probiotic strains attenuated anxiety and depressive behaviors in rodents (Bravo et al., 2011) and in healthy humans (Messaoudi et al., 2011). As well, ingestion of probiotics in rodents affected central expression of GABA receptor subunits (Bravo et al., 2011), BDNF (Bercik et al., 2011b), and two markers of microglial activation, CD68 and CD11b (Distrutti et al., 2014), although the mechanisms by which this occurred have not been identified.

INFLAMMATORY GENE VARIANTS AS RISK FACTORS FOR DEPRESSION AND AS PREDICTORS OF TREATMENT RESPONSE

It has been suggested that inflammatory cytokine activity might serve as a biomarker to predict how individuals cope with stressors, whether they develop depression, and/or whether they respond to different treatment strategies (Anisman et al., 2008; Yoshimura et al., 2009; Cattaneo et al., 2013). Beyond protein or gene expression variations, allelic variants of genes [e.g., in the form of gene polymorphisms, including single-nucleotide polymorphisms (SNPs)] that promote higher transcription of pro-inflammatory cytokines appear to be related to inflammatory variations normally elicited by stressors and in some cases to predict the emergence of depressive symptoms (Bufalino et al., 2013). For instance, cytokine elevations in bereaved individuals were apparent in homozygous carriers of the mutant G allele of the IL-6-174C polymorphism (associated with high IL-6 transcription), but not in those carrying the low transcription

C allele (Schultze-Florey et al., 2012). Considering that a positive association between depression and mortality risk has been found in homozygous carriers of the high transcription G allele of the IL-6-174C polymorphism (Cole et al., 2010), it was suggested that the low transcription C allele may protect against physical and mental health problems associated with psychological distress (Schultze-Florey et al., 2012). In support of these findings, depressive symptoms elicited by IFN- α immunotherapy in patients with chronic hepatitis C were reduced in those carrying the C allele of the IL-6-174C polymorphism (Bull et al., 2009). In addition to IL-6, variants of the genes encoding for IL-1 β and TNF- α have also been associated with elevated risk for depression (Bufalino et al., 2013). Parenthetically, paralleling the effects of stressors, the depressive effects of immunotherapy were less pronounced among individuals carrying the two long (L/L) alleles of the serotonin transporter gene-linked polymorphic region (5-HTTLPR) relative to individuals carrying the short (S) allele, pointing to the possibility that IFN- α immunotherapy operates like stressors in predicting the development of depression (Bull et al., 2009). Thus, in addition to cytokine sensitivity elicited by external factors (e.g., traumatic events), polymorphic variations on inflammatory genes might also contribute to the vulnerability to social stressors and to the development of depressive illnesses. Whether environmental and genetic influences interact in this regard remains to be investigated, but given the influence of stressors on cytokine functioning, such interactions would be expected.

Just as increased levels of IL-6 (Lanquillon et al., 2000; Yoshimura et al., 2009) as well as up-regulated expression of IL-1 β and TNF- α (Cattaneo et al., 2013) predicted a lack of response to different classes of antidepressants, polymorphisms of the IL-1 β , IL-6, and IL-11 genes, which were associated with increased cytokine production, were accompanied by a diminished response to antidepressant medication (Uher et al., 2010; Bufalino et al., 2013). Based on these findings, it had been suggested that depressed individuals who repeatedly fail to respond to traditional antidepressants may exhibit a distinct pro-inflammatory profile. This possibility was confirmed in animal studies showing that mice engineered to overexpress IL-6 in the frontal cortex and hippocampus as well as mice that had received intracerebroventricular injections of IL-6 showed a blunted response to the antidepressant effects of fluoxetine (Sukoff Rizzo et al., 2012). In essence, elevated levels of particular cytokines may predict the development of depression, and although IL-6 and TNF- α levels may decline with appropriate treatment (Lanquillon et al., 2000; Yoshimura et al., 2009), if the cytokine levels are too high prior to treatment commencing, possibly reflecting a disturbance of regulatory/inflammatory processes, the effects of antidepressant treatments will be muted. It is equally possible that the elevated cytokine levels that sometimes persist despite positive mood changes elicited by antidepressant treatment may reflect a harbinger for illness recurrence (see Anisman et al., 2008).

CYTOKINE VARIATIONS AND POSITIVE ENVIRONMENTS—THE GOOD AND THE BAD

If negative events promote cytokine disturbances that undermine well-being, is it the case that positively interpreted events and

experiences act against cytokine disturbances and the emergence of depression? Studies in animals have indicated that positive interventions in animal models of depression are associated with reductions of stress-induced cytokine elevations. For instance, increased TNF- α and IL-6 mitogen-stimulated splenocyte production induced by separation from a mom or littermates was attenuated in young piglets that were paired with an age-matched conspecific (Tuchscherer et al., 2014). As well, in mice, long-term exposure to an enriched environment reduced IL-1 β and TNF- α elevations in the hippocampus induced by influenza infection (Jurgens and Johnson, 2012). That said, it has also been shown that environmental enrichment in male mice enhanced the cytokine effects normally elicited by social defeat, probably owing to the aggressive behaviors promoted by enrichment in male mice (McQuaid et al., 2013).

The data derived from human studies have similarly revealed that positive experiences may act against cytokine variations, which could influence mood states. For instance, psychosocial measures of coping and self-esteem were inversely correlated to IL-6 levels (Sjögren et al., 2006) and improvements in coping strategies and resilience after self-administered hypnosis were associated with IL-6 reductions (Schoen and Nowack, 2013). A meta-analysis also demonstrated that mind-body therapies (e.g., Tai Chi, meditation, yoga) tended to reduce IL-6 levels in both healthy and clinical populations (Morgan et al., 2014). Importantly, reductions of depressive symptoms after daily yogic meditation intervention (Black et al., 2013), a mindfulness treatment program (Carlson et al., 2007; Rosenkranz et al., 2013), and cognitive-behavioral therapy (Gazal et al., 2013) were all related to pro-inflammatory cytokine reductions.

Social factors, and particularly having social support, have long been known to diminish some of the adverse effects of stressors. By example, the availability of social support was accompanied by reduced levels of cortisol both in a natural setting and within a laboratory context (Heinrichs et al., 2003). Further, in a stress test the right PFC activation and diminished amygdala activity that were ordinarily elicited could be attenuated by having social support available (Taylor et al., 2008), and having received social support over several days blunted the cortisol response ordinarily elicited by a social stressor and enhanced neuronal activity within the anterior cingulate cortex (Eisenberger et al., 2007). There have similarly been reports showing that social support could influence the plasma cytokine response otherwise elicited by stressors, and could thus influence mood state (Slavich and Irwin, 2014). In fact, it has broadly been reported that among cancer survivors who had social support prior to treatment, later well-being was enhanced. In fact, in the individuals with lower pretreatment social support, the levels of IL-6 increased with illness progression, which predicted the elevation of depressive symptoms (Hughes et al., 2014).

A similar link between social support, cytokines, and general well-being has also been observed among medically healthy individuals. Specifically, low social status was accompanied by elevated IL-6 in response to a stressor (Derry et al., 2013), and social strain emanating from family and friends increased circulating IL-6, whereas having social support modestly protected against

this outcome (Yang et al., 2014). Moreover, the rise of IL-6 associated with anger was not evident among individuals who perceived themselves as having high social support (Puterman et al., 2013).

The data available concerning the influence of social stressors and social support on cytokine levels is still limited. Nonetheless, it is certain that social stressors, especially those of an interpersonal nature, such as social rejection and social adversity, can be particularly aversive and it has been suggested that the depressive actions of such experiences may involve activation of pro-inflammatory processes (Slavich and Irwin, 2014). However, it might not always be the case that social support will be prophylactic or remedial in attenuating the effects of stressful experiences, especially if efforts of support are interpreted as being insufficient or actually a reflection of an unsupportive interaction (McQuaid et al., 2014b).

CONCLUDING COMMENTS REGARDING CYTOKINE INVOLVEMENT IN DEPRESSION AND ITS COMORBIDITIES

The specificity of cytokine disturbances to depression has frequently been questioned. In this regard, it was demonstrated that despite the overlap that exists with respect to the symptoms of IFN- α -induced and idiopathic depression, there are differences between the two, leading to the suggestion that cytokines preferentially affect neural circuits associated with psychomotor activity, but have less of an effect on the processes that govern cognitive distortions concerning self-appraisal (Capuron et al., 2009). Furthermore, increased inflammatory activity has been reported in a variety of stress-related disorders, including bipolar disorder (Modabbernia et al., 2013), schizophrenia (Altamura et al., 1999), and post-traumatic stress disorder (Lindqvist et al., 2014), and has been associated with a number of chronic diseases including cancers, heart diseases, metabolic syndrome, diabetes and obesity, auto-immune illnesses, disorders of the digestive system (i.e., inflammatory bowel disease), and neurodegenerative disorders (Anisman et al., 2008). In essence, it is possible that altered cytokine functioning might create a general milieu that favors the development of pathology, but the specific disturbance that is expressed depends on the presence of still other factors being affected. This said, as most of these conditions are often comorbid with depression, it has been suggested that increased inflammation might be a common denominator underlying depressive symptoms across many neuropsychiatric and physical/medical conditions. Indeed, the link between depression and the development of illnesses, such as heart disease, is sufficiently strong to have prompted the suggestion that the presence of depression ought to be viewed as a marker for later physical illnesses (Hayley and Anisman, 2013). Just as stressful events, particularly those that involve social challenges, promote cytokine variations and several pathological conditions, social support has been effective in attenuating these outcomes. Considerable evidence indicates that the effectiveness of support depends on whom the support comes from, and the individual's social identity and social connectedness may be involved in the resolution of depression (Cruwys et al., 2013, 2014). It remains to be determined whether the positive effects of social identity and connectedness in attenuating depression operate through inflammatory processes.

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Toll-like receptor 4: innate immune regulator of neuroimmune and neuroendocrine interactions in stress and major depressive disorder

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Major depressive disorder (MDD) poses one of the highest disease burdens worldwide. Yet, current treatments targeting serotonergic and noradrenaline reuptake systems are insufficient to provide long-term relief from depressive symptoms in most patients, indicating the need for new treatment targets. Having the ability to influence behavior similar to depressive symptoms, as well as communicate with neuronal and neuroendocrine systems, the innate immune system is a strong candidate for MDD treatments. Given the complex nature of immune signaling, the main question becomes: What is the role of the innate immune system in MDD? The current review presents evidence that toll-like receptor 4 (TLR4), via driving both peripheral and central immune responses, can interact with serotonergic neurotransmission and cause neuroendocrine disturbances, thus integrating with widely observed hallmarks of MDD. Additionally, through describing the multi-directional communication between immune, neural and endocrine systems in stress, TLR4—related mechanisms can mediate stress-induced adaptations, which are necessary for the development of MDD. Therefore, apart from exogenous pathogenic mechanisms, TLR4 is involved in immune changes as a result of endogenous stress signals, playing an integral part in the pathophysiology, and could be a potential target for pharmacological treatments to improve current interventions for MDD.

Keywords: toll-like receptor 4, TLR4, HPA, neuroendocrine, neuroimmunology, stress, depression, MDD

INTRODUCTION

Major depressive disorder (MDD) represents a combination of disturbances to mood, cognition, sleep and appetite, which causes impairment to individual functioning lasting a minimum of 2 weeks (American Psychiatric Association, 2013). MDD consistently ranks within the top 4 highest “years lived with disability” (Vos et al., 2012), and accounts for 7.4% of total disability-adjusted life years worldwide (Whiteford et al., 2013). In addition, depressive disorders have an estimated 93.5% comorbidity with other diseases, most commonly with chronic pain, anxiety, bipolar disorder, post-traumatic stress disorder, diabetes and neurological disorders (Gadernann et al., 2012). Major depression thus bears one of the highest disease burdens, with matching economic and societal costs. Yet, current pharmacological treatments using serotonin and noradrenaline reuptake inhibitors (SSRI and SNRI, respectively) are inefficient, requiring to treat 7 patients in order to gain one positive outcome (Arroll et al., 2009). This suggests that alterations to the serotonergic pathway are only partly responsible for MDD, and other mechanisms must be involved. Thus, it is sensible to study other systems, in order to improve current treatments for MDD.

Ever since early observations of increased immune markers in psychiatric patients, the immune system has become increasingly associated with various psychosomatic illnesses (Solomon et al.,

1969). In the case of MDD, patients exhibit increased circulating peripheral cytokines, which are immune signaling molecules that can be pro or anti-inflammatory (Anisman and Hayley, 2012; Lichtblau et al., 2013). Additionally, a decrease in depressive symptoms is coupled with a normalization of immune signaling levels (Gazal et al., 2013), suggesting that there may be immune involvement in MDD.

NEUROINFLAMMATORY EVENTS: NEUROINFLAMMATION AND NEUROKINE SIGNALING IN MDD

Although immune signaling in the brain is comprised of signals from resident glial cells, peripheral to central immune communications and migration of peripheral cells into central compartments, it is important to note the phenotypic differences between different neuroinflammatory diseases. The term “Neuroinflammation” is commonly used to encompass increased immune activation in the CNS. However, the central nervous system (CNS) can take on different inflammatory states, and there is a distinction between the magnitude of immune responses in the CNS by varied causes. For example, neurodegenerative disorders including Alzheimer's and Parkinson's disease are characterized by widespread immune signaling in the CNS, oxidative stress, and increased immune trafficking into the brain, resulting in systemic inflammation accompanied with progressive damage

(Heneka et al., 2014). On the other hand, acute and sub-maximal immune challenges such as that triggered by opioid exposure can also cause increased central immune signaling, but the resulting inflammatory response is of a much lower magnitude (Stevens et al., 2013). This sub-inflammatory state is attributed to the direct actions of opioids on CNS expressed toll-like receptor 4 (TLR4), since opioids readily cross the BBB, rather than the indirect peripheral to central immune response following bacterial infections (Hutchinson and Watkins, 2014; Jacobsen et al., 2014).

Classically, inflammation involves swelling, heat, and pain, coupled with a coordinated infiltration of various immune cells into the affected area. In many neurological conditions, this large-scale damage is not seen unless in terminal stages, or in cases of major BBB compromise. Thus, the use of “neuroinflammation” to refer to central immune activity can become confusing, and a clear distinction between high magnitude and submaximal immune states is required.

Neurokinins refer to neurally active cytokines (Nathanson, 2012), which can be upregulated as a result of peripheral infections or innate immune activation without the infectious agent crossing the BBB (McCusker and Kelley, 2013). An important differentiation between increased neurokinin signaling and neuroinflammation is the reversibility of the effects. Central cytokine increase resulting from low-grade infections, exercise and stress result in reversible neuronal changes (McCusker and Kelley, 2013). These neuronal changes include the upregulation of AMPA and NMDA receptors, as well as decreased expression of GABA receptors on neurons by cytokines IL-1 β and TNF- α , causing reversible increased excitation (see Viviani et al., 2014 for a review). We thus propose the use of the terms “increased neurokinin signaling” or “increased central immune signaling” to apply to these sub-inflammatory states, and only when there is large-scale damage as a result of immune cell derived neurotoxicity and inflammation should the term “neuroinflammation” be applied in order to reduce confusion within the literature.

In regards to MDD, current evidence indicates a milder immune signaling phenotype more akin to increased neurokinin signaling, rather than neuroinflammation. Compared with the continued accumulated loss of function observed in neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease, MDD has periods of active disease characterized by depressive episodes followed by remission. During remission, peripheral blood expression of TNF superfamily 12–13 mRNA is lower than in active disease (Otsuki et al., 2010). This indicates that the immune signaling is influenced by the state of the disorder, but critically, the degree of immune signaling required for the presentation of a disease symptom may be significantly higher than that needed for MDD to be observed, as illustrated by the development of depressive-like behavior prior to the development of significant demyelination in experimental autoimmune encephalitis (Acharjee et al., 2013). Furthermore, structural changes in hippocampal volume measured using MRI appear reversible in patients with MDD (Frodl et al., 2004). Antidepressants (Horikawa et al., 2010; Alboni et al., 2013; Obuchowicz et al., 2014) and cognitive-behavioral therapy (Gazal et al., 2013) are also able to reduce cytokine expression in depressive disorders. Taken together, there is strong evidence for the

reversibility of inflammatory markers that closely relates to the state of depressive symptoms. Along with the bi-directional neuroimmune connection, this reversibility and liability of the condition suggests that targeting the central immune system could be a promising treatment option for MDD.

THE ROLE OF TOLL-LIKE RECEPTOR 4 IN MDD

Recently, TLR4 has come to the forefront of research linking neuroimmune signaling and MDD, through driving immune to brain communication. TLR4 is an innate immune pattern recognition receptor, which is part of the Interleukin-1 Receptor/Toll-Like Receptor Superfamily containing a toll-like/IL-1 Receptor (TIR) domain and Leucine-rich repeat motif in the extracellular domain. TLR4 recognizes endogenous danger associated molecular patterns (DAMPs) including heat shock proteins (HSP) and high mobility group box 1 (HMGB1), exogenous pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), as well as microbiome/microbe associated molecular patterns (MAMPs) (Akira and Takeda, 2004). Unlike other toll-like receptors, activation of TLR4 triggers pro-inflammatory transcription via 2 adaptor proteins, Myeloid differentiation primary response 88 (MyD88) and TIR domain-containing adapter inducing IFN- β (TRIF), which induces transcription factors NF- κ B, AP-1, and IRF3 (Xu et al., 2000; Watters et al., 2007). Activation of these transcription factors causes the production of pro-inflammatory cytokines including IL-1 β , TNF- α , IL-6, and CXCL10, along with an upregulation of proteins including cyclooxygenase 2 (COX-2), resulting in pro-inflammatory signaling (Akira and Takeda, 2004).

In the CNS, TLR4 is predominantly expressed on microglia, and is also to a lesser extent expressed on neurons (Zhao et al., 2014). Through modulation of neuroimmune activity, TLR4 is implicated in various neuropathological conditions that afflict the CNS, including neuropathic pain (Lewis et al., 2012) and neurodegenerative diseases (Heneka et al., 2014). Increasingly, these disorders are recognized as existing on a spectrum from altered neuroimmune signaling (e.g., neuropathic pain) through to gross neuroinflammation (e.g., Neurodegenerative diseases).

MDD, being increasingly classified as a neuroimmune disorder, may also have TLR4 involvement (Gárate et al., 2011; Hines et al., 2013). Recent evidence has found that peripheral blood mononuclear cells (PBMC) of patients with MDD express higher levels of TLR4 (Kéri et al., 2014). Importantly, the authors showed that this heightened expression was reduced following treatment, and paralleled improvement in depressive symptoms. This responsiveness toward depressive states indicates that TLR4 activity could directly be involved in the pathophysiology of MDD.

Given the involvement of the immune system in numerous neurological disorders, it is now accepted that the CNS is not an immune privileged organ that exists in isolation of the immune system. The CNS can no longer be thought of as resistant to immune signals, or protected from immune damage (Ransohoff and Brown, 2012). Rather than separate entities, immune, neuronal and endocrine systems appear to be in constant bidirectional communication and a dysregulation of these systems can result in pathological states within the CNS (see Kelley and McCusker, 2014 for review). The current review thus posits

that TLR4, being central to the immune to brain, and immune to neuroendocrine communication, underlies the neuroimmune signaling events observed in the pathophysiology of stress-related disorders including MDD, across systemic and cellular levels.

TLR4 ACTIVITY CAN INFLUENCE BEHAVIOR AND MDD

Using LPS as an agonist, peripheral TLR4 activation is sufficient to cause changes in motivational state and can trigger sickness behavior (Hines et al., 2013). Sickness behavior is characterized by increased anhedonia, lethargy, loss of locomotion and anorexia following immune challenges (Dantzer and Kelley, 2007). Once thought to stem from altered energy balance, it is clear that these immune signaling initiated behavioral adaptations are driven by specific cytokine-dependent signaling cascades (Dantzer, 2004). Thus, an immune stimulus can drive complex higher order behavioral adaptations, providing direct evidence for the link between the immune system and symptoms of depression.

Although the idea of sickness behavior may appear surprising since pathogens themselves are imperceptible by our classic sensory organs, it makes sense that the body needs to know when it encounters pathogens in order to make behavioral adjustments. Through reduction in locomotion, sickness behavior can promote recovery from the immune challenges. Sickness behavior can therefore be considered as adaptive since it promotes homeostasis within the conditions of the stressor (Dantzer and Kelley, 2007).

The effect of peripheral immune activation on behavior is thought to be mediated by three main mechanisms: (1) peripheral release of pro-inflammatory cytokines can cross the blood brain barrier (BBB) via “leaky” subventricular organs, thus directly increasing neurokinine signaling, (2) These activated immune cells can also cross the BBB directly to cause neuroimmune signaling, and (3) peripheral cytokines can also stimulate afferent pathways such as the vagus nerve, causing behavioral changes via neural mechanisms (see McCusker and Kelley, 2013 for review). Besides these mechanisms, emerging evidence have demonstrated that monocytes can adhere and roll along the cerebral vasculature, and can cause increased central immune signaling without crossing the BBB, and this is associated with increases in inducible Nitric Oxide Synthase (D’Mello et al., 2013). The authors further showed that inhibiting this mechanism could improve sickness behavior as a result of peripheral inflammation, thus suggesting another mode of peripheral to central communication with behavioral implications.

Importantly, the trigger for behavioral changes need not originate from pathogenic mechanisms, since cytokine administration is sufficient to elicit sickness behavior similar to bacterial stimulation (Tazi et al., 1988; Dantzer, 2004). This means that other potential sources of immune activation, including endogenous danger signals, or neurogenic activation could trigger this response.

Strong parallels can be drawn between sickness behavior and depressive behavior, namely reduced locomotion, anhedonia, and dysregulated sleep and food intake (McCusker and Kelley, 2013). Since MDD can be chronic and recurring, the question arises whether patients with MDD are just displaying chronic dysregulation in inflammation or immune signaling? Indeed, patients

suffering from MDD display heightened circulating cytokine levels, indicative of increased immune signaling (Anisman and Hayley, 2012; Lichtblau et al., 2013). Furthermore, there is some evidence showing that celecoxib, an NSAID that inhibits COX-2, can improve depression scores as well as increase remission rates in patients receiving anti-depressants (Faridhosseini et al., 2014; Na et al., 2014). In rodent models, NSAID administration can improve depressive-like behavior, measured using the forced swim test and tail suspension tests (Maciel et al., 2013; Guan et al., 2014). Notably however, these studies indicate that the anti-depressive effect of NSAID requires a higher dose in order to achieve comparable results to SSRIs, and are more effective in immune stressor induced depressive-like behavior. Additionally, there is some debate about the efficacy of these treatments since NSAIDs can also reduce efficacy of SSRI treatment when given in conjunction, in both animals and patients (Warner-Schmidt et al., 2011). MDD thus appears to have an immune component, but the exact mechanisms behind this interaction require more investigation.

CELL MEDIATED MECHANISMS OF CENTRAL IMMUNE SIGNALING EVENTS ASSOCIATED WITH MDD

It has been confirmed that both peripheral and central administration of LPS can induce sickness behavior, showing that both peripheral and central immune signaling are involved in perpetuating this behavioral change (Huang et al., 2008; Hines et al., 2013). This TLR4 mediated Innate immune signaling in the CNS is mainly undertaken by 2 cell populations, the resident glial cells, and infiltrating peripheral immune cells.

Glial cells, consisting of oligodendrocytes, astrocytes and microglia, vastly outnumber neurons by an estimated 10–50-fold (Temburni and Jacob, 2001; Banati, 2003). These cells were previously regarded as inert support cells that do not directly influence neurotransmission. However, there is increasing evidence that glial cells are integral to both physiological functions and pathophysiological states in the CNS.

Astrocytes

Astrocytes are the most abundant cell type in the CNS, providing structural and trophic support to neurons. They have star-shaped morphology, with processes that can be in contact with up to 100,000 neurons (Halassa et al., 2007). Although astrocytes are not capable of producing action potentials, neurotransmitter binding to receptors present on astrocytes can induce Ca²⁺ waves (Sharma and Vijayaraghavan, 2001; Schipke et al., 2011). In addition to forming close appositions with synapses, astrocytes can influence the chemical environment through secretion of various compounds such as ATP, adenosine and acetylcholine, a process known as gliotransmission. Astrocytes can therefore influence neurotransmission on a synaptic level and could play a role in aggregating neural responses. Collectively, astrocyte involvement with synaptic activity is termed the tripartite synapse (Araque et al., 2014).

Across the CNS, astrocytes display different phenotypes and can adopt different states (Sun and Jakobs, 2012). Activated astrocytes display less branching morphological characteristics, and this is associated with glial fibrillary acid protein (GFAP)

upregulation (Pekny and Nilsson, 2005). Activated in injury, astrocytes can be both protective by releasing factors facilitating recovery (Gimsa et al., 2013), and disruptive to neuronal functioning via inhibiting axon growth through formation of glial scars after extended activation (Smith-Thomas et al., 1994; Yuan and He, 2013). Moreover, radial glia located at the sub-ventricular zones also express GFAP, but act as precursor cells capable of differentiating into neurons and astrocytes, which can migrate to several areas including the cortex, displaying ability for adult neurogenesis and cell renewal (Sundholm-Peters et al., 2005). The role of astrocytes is therefore varied and important to normal physiology, but astrocytes can also participate in both neuroimmune signaling and neuroinflammatory disease progression.

In MDD, astrocytes may be involved in the progression of the disease through participating in the reuptake of serotonin. In the presence of TNF- α , serotonin transporters expressed on astrocytes increase reuptake of serotonin in a dose-dependent manner, and this effect is attenuated by administration of SSRIs (Malynn et al., 2013). Interestingly, SSRI are also able to influence astrocytic Ca²⁺ waves in a similar way to serotonin administration (Schipke et al., 2011). Although unclear whether astrocytes are an integral target for anti-depressant treatment, it appears that astrocytes can play a role in serotonin neurotransmission, and therefore be involved in the pathophysiology of MDD.

Microglia

Microglia are the resident immunocompetent cells in the CNS, and play an active role in neuroinflammatory actions by release of cytokines, chemokines phagocytosis and removal of debris, directly modulating neuroimmune activity. Microglia are thus the main cell type investigated in neuroimmune signaling events and neuroinflammatory/neurodegenerative conditions. Being implicated in synaptic pruning during CNS development, microglia not only perpetuate damage, but also are integral for normal functioning (Schwartz et al., 2013). Microglia too have different activation states, commonly referred to as M1 and M2, the pro and anti-inflammatory phenotypes, respectively (Olah et al., 2011). When activated to an M1 phenotype, microglia display a more amoeboid morphology, and secrete cytokines and chemokines to signal for other immune cells. Microglia can also function as antigen presenting cells through MHC-II expression, therefore possessing the ability to trigger the adaptive immune responses within the CNS (Harms et al., 2013). Expressing pattern recognition receptors such as TLR4, microglia are responsive to DAMPs, MAMPs, and PAMPs. Moreover, TLR4 activation can shift microglia toward a M1 phenotype, inducing pro-inflammatory responses in the CNS (Ajmone-Cat et al., 2013).

Besides functioning as an antigen-presenting cell, microglia can influence neuronal functions through expression of glutamate transporters (Persson et al., 2005), but to a lesser extent as compared to astrocytes (Beschorner et al., 2007). In addition, microglia in the “resting” state actively survey the surrounding area for chemical changes, and can rapidly respond to stimuli (Nimmerjahn et al., 2005). Thus, even during an immunologically dormant state, microglia are able to influence

neurotransmission and can rapidly respond to danger signals. Aiding this responsiveness are the toll-like receptors, which can recognize multiple stimuli and modulate the activation states of microglia.

Microglia, through TLR4-dependent signaling (Hines et al., 2013), and production of cytokines (Henry et al., 2009; Dobos et al., 2012), are regarded as mediators of central immune signaling in animal models of sickness behavior. In addition, changes in microglial reactivity states are also associated with the induction of stress-induced depressive like behavior (Pan et al., 2014). In a recent study, minocycline, an antibiotic that has also shown the ability to suppress central immune signaling by acting on microglia, can prevent the development of depressive-like behavior, tested using sucrose preference and social exploration (Kreisel et al., 2014). This reduction of depressive-like behavior was found in conjunction with changes to microglia morphology and proliferation. On the other hand, the study illustrated that simply inhibiting microglia chronically would not be a viable treatment option, since microglial activation states change from an initial proliferative state to later decline from acute to chronic models, and central immune suppression exacerbates depressive-like behavior in a chronic model. Instead, the authors proposed that depression is related to either an over or under activation of microglia, and treatments should strive toward a balance in activation states. Taken together, microglia appear important in central immune signaling and immune to brain communication in MDD, but this relationship is not uni-directional, and appears to be time-dependent.

Peripheral immune cells and their actions on the CNS

Although the CNS is protected from many peripheral factors by the BBB, peripheral immune cells have been found to infiltrate and drive inflammation within the CNS (Schweingruber et al., 2014; Vogel et al., 2014). Perivascular macrophages and circulating monocytes can cross the BBB into the CNS through the expression of adhesion molecules such as ICAM and VCAM on the endothelium of blood vessels surrounding the CNS (Wong et al., 2007). Additionally, peripheral leukocytes can also roll and adhere to the cerebral vasculature, and cause increased central immune signaling without entering the CNS, thus communicating across the BBB (D’Mello et al., 2013). Glial cells themselves can also release chemokines such as CCL2, which can trigger peripheral immune cell extravasation into CNS tissue (Williams et al., 2013; Shieh et al., 2014). Thus, the central immune system can actively signal for peripheral immune cells to cross the BBB.

Contrary to other models of neuroinflammatory/neurodegenerative disorders Wohleb et al. (2014) have recently shown that peripheral bone marrow-derived cell infiltration is not just evident in models of BBB compromise, but can also occur in sub-inflammatory conditions as well. Interestingly, increased infiltration of peripheral monocytes can influence anxiety-like behavior, once again illustrating the ability for immune signaling to influence higher-order CNS function (Wohleb et al., 2014). This result, however, still needs more investigation, as it is widely held that immune-cell infiltration is a sign of neuroinflammation, and is not evident in sub-maximal levels of central immune signaling.

There is little direct evidence showing peripheral immune cell migration in MDD due to the lack of a representative animal model of MDD. Nevertheless, social defeat and psychological stress can trigger increased trafficking of peripheral cell infiltration (Brevet et al., 2010; Wohleb et al., 2013). Given that MDD is considered a stress disorder (for more information, see Section “what does stress have to do with it?”), this indicates that peripheral cell infiltration could be involved in MDD. However, isolating the exact cause of immune signaling in MDD is challenging, and the extent of peripherally driven immune responses in MDD patients is thus still unknown.

EFFECT OF INCREASED IMMUNE (TLR4) ACTIVITY ON CNS NEUROTRANSMITTER ACTIVITY

So far, the best-characterized neuropathophysiology of MDD within the CNS is the dysregulation in serotonin neurotransmission, but the exact cause of this alteration is still unclear. There is growing evidence that glia are able to influence neurotransmission, and through these mechanisms, glia can contribute to the neuronal adaptations in MDD (Burke et al., 2014; Kreisel et al., 2014). Through close appositions with synapses in tripartite and tetrapartite arrangements, glia have access to the chemical environment of the synapse. Functionally, astrocytes can contribute to glutamate homeostasis by clearing excess glutamate from synapses. During situations of increased neuroimmune signaling, this process is impaired due to the down regulation of astrocyte glutamate transporters (EAAT1 and EAAT2), causing glutamate neurotoxicity and subsequent neuronal death (Tilleux and Hermans, 2008; Persson et al., 2009; Fang et al., 2012).

In relation to MDD, glia express serotonin transporters (Malynn et al., 2013), and can also directly inhibit serotonin production during neuroinflammation through the production of indoleamine-2,3-dioxygenase (IDO). IDO further interferes with the synthesis of serotonin by catalyzing tryptophan, forming quinolinic acid and 3-hydroxy-kynurenine, which can further result in neurotoxicity (O'Connor et al., 2009). Through this pathway, the increase in IDO reduces serotonin signaling as seen in MDD by impairing serotonin production, and can also cause direct damage to serotonergic neurons. Indeed, patients with MDD display and increased circulating kynurenine to tryptophan ratio, suggesting increased IDO activity (Quak et al., 2014). Moreover, this effect is thought to mediate depressive like behavior as a result of immune activation, as pharmacological inhibition of IDO is able to attenuate the increase in central immune signaling and depressive-like behavior in response to LPS administration in rodent models (Corona et al., 2010; Dobos et al., 2012). The ability of glia to influence serotonergic neurotransmission illustrates that rather than replace earlier notions of what causes depressive symptoms—that is, an impairment in serotonin metabolism—neuroimmune mechanisms instead contribute to and supplement neural mechanisms of disease.

EFFECT OF MDD TREATMENTS ON IMMUNE SIGNALING

The immune to brain communication is not uni-directional, since neuronal functions can also influence the activity of the immune system. This is especially evident in current treatments

of MDD using SSRIs or SNRIs that work via alterations to serotonin and noradrenaline, respectively. Anti-depressants have shown to reduce LPS induced peripheral IL-6 and TNF- α production (Manikowska et al., 2014). SSRI administration can also attenuate CRH, TNF- α , and IL-1 β mRNA expression in the hypothalamus after chronic treatments (Alboni et al., 2013). Glia are responsive to anti-depressant treatments, since SSRIs can decrease gliotransmission (Dhimi et al., 2013), and can partially attenuate microglial secretion of TNF- α in response to LPS. Pharmacologically blocking the reuptake of serotonin can also reduce microglial reactivity and inhibit LPS-induced changes in microglia morphology (Horikawa et al., 2010; Obuchowicz et al., 2014). In addition, there is also evidence that antidepressants are protective against microglial- (Zhang et al., 2012) and MPTP-induced neurotoxicity (Chung et al., 2011). Thus, alterations to serotonergic neurotransmission can also influence glial and central immune activity, and this may contribute to the anti-depressive effects of SSRI. Together with evidence of immune modulation of neurotransmission, this illustrates the bi-directional communication between the neural and immune systems in both normal and pathophysiology.

WHAT DOES STRESS HAVE TO DO WITH IT?

Stress refers to a challenge to the body's homeostatic state, and can be classified broadly as psychological, physiological and immunological in origin. According to the diathesis-stress model of depression, stress is essential to the development of MDD, as stress is required in order to unmask the underlying individual predisposition to the disorder (Monroe and Simons, 1991). Biological and environmental factors thus interact to produce the physiological and psychological depression phenotype. Stress, regardless of type, activates the hypothalamus pituitary and adrenal (HPA) axis, which forms the neuroendocrine stress response. The HPA axis is therefore the most investigated link between stress and MDD. Furthermore, TLR4 activation is considered an immunological stress, and recent research demonstrates that it is deeply intertwined with the neuroendocrine stress response. Given the innate immune component of MDD as outlined earlier, we propose that the role of TLR4 in MDD is mediated at least in part by its interaction with the HPA axis.

THE CLASSICAL HPA AXIS

When activated, the HPA axis works to restore homeostasis following different stressors (Chrousos, 2009). Activation of the HPA axis begins with neurons in the paraventricular nucleus of the hypothalamus (PVN), which secrete corticotropin releasing hormone (CRH). CRH then stimulates the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the blood circulation. Upon binding to melanocortin 2 receptor (MC2R) expressed on the zona fasciculata layer of the adrenal cortex, ACTH stimulates glucocorticoid (GC) production *de novo*. GC binds to cytoplasmic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at a higher affinity (Sapolsky et al., 2000). Due to this higher affinity, the actions of baseline GC are thus associated with MR binding, whereas GR actions are attributed to upregulated GC by stressors.

GR binding causes receptor translocation into the nucleus through the aid of chaperone proteins such as heat shock protein 70 and 90 (HSP70, HSP90, respectively), where it can either dimerise and bind to glucocorticoid response elements (GRE) on DNA to either increase transcription of glucocorticoid responsive genes, or interact with other proteins such as transcription factors NF- κ B and AP-1 (see Silverman and Sternberg, 2012 for review). GC actions are therefore extremely complex. Furthermore, most cells express GR, and thus GCs can influence the functions of multiple systems through diverse endocrine actions. GCs are also able to cross the BBB to influence CNS function including feeding back upon its own secretion by signaling via GR in the hippocampus to increase GABAergic tone on the PVN.

HPA ACTIVITY ALTERATIONS IN MDD

The HPA response is markedly changed during depression. Specifically, patients with MDD exhibit heightened cortisol levels in the morning, and possess a flatter diurnal slope throughout the day (Dinan, 1994; Jarcho et al., 2013). This indicates that a dysregulation in HPA activity is involved in the pathophysiology of depression, and further raises important questions as to what causes this HPA dysregulation, as well as how this fits in with the immune adaptations outlined earlier.

The hypersecretion of cortisol could mean either a problem with the feed-forward secretory pathway, or the negative feedback loop within the HPA axis. The former is unlikely true, as adrenal responsiveness to ACTH is normal in patients with depression (Rubin et al., 2006), showing that adrenal function itself is not altered in depressive disorders. On the other hand, patients exhibit lower responsiveness to the dexamethasone suppression test (DST), which tests GR mediated negative feedback onto HPA activity (Holsboer-Trachsler et al., 1991). Dexamethasone was also found to be less effective in suppressing immune activity in patients with depressive disorders (Maes et al., 1994). Taken together, GR function is modified in MDD, and patients develop what is termed GC resistance. Given that the actions of GR influence multiple systems in the body, GC resistance could serve as a link between MDD and immune dysregulation (Quan et al., 2003; Silverman and Sternberg, 2012).

HPA hyperactivity may be sufficient to cause depressive moods, since patients with Cushing's syndrome, characterized by hypersecretion of cortisol, often exhibit depressive symptoms (Starkman et al., 1981). In rodent models, administration of corticosterone can also elicit depressive-like behavior, and this effect is blocked by spironolactone, an MR antagonist (Wu et al., 2013). Since MR is associated with baseline GC actions, the increased secretion of cortisol in MDD patients may be in part mediating the disorder via MR activation. HPA dysregulation indeed presents itself to be integral to depressive symptoms. However, blocking GC signaling is not feasible due to its widespread effects on cardiovascular, reproductive, metabolic and immune function. Thus, recent attention has been turned toward specific upstream modulators of HPA activity in stress and MDD. Out of these investigations, the immune component appears to be the most likely target due to the overlaps between immune activation, the monoamine system, and the HPA axis.

ACUTE TLR4 ACTIVATION TRIGGERS THE HPA AXIS

HPA activity is thought to mediate the immune involvement in MDD. Using LPS, previous research has been able to show that TLR4 signaling is able to stimulate the HPA axis (Mohn et al., 2011). Further studies showed that TLR4 activation is sufficient to cause GC release from adrenal cells (Vakharia and Hinson, 2004; Kanczkowski et al., 2013). TLR4 activation can also cause CRH gene upregulation in paraventricular neurons in the hypothalamus (Loum-Ribot et al., 2006), as well as increased CRH in serum (Goebel et al., 2011). Pituitary cells stimulated by LPS also produce ACTH, but there is contrasting data on whether this effect is CRH dependent (Elenkov et al., 1992; Mehet et al., 2012).

Despite clear evidence that innate immune activation via TLR4 can strongly stimulate the HPA axis, the exact mechanism is still unclear, as it is not known if TLR4 can directly modulate neuronal activity, or cause steroidogenesis via intracellular signaling. Part of the problem is, as illustrated earlier, where in the system LPS has the most effect, since it appears that TLR4 is present and can trigger increased secretion of CRH, ACTH, and GC. The other part of the problem lies in the many levels of immune activation that can also influence neuroendocrine activity. The following section reviews how TLR4 signaling can trigger the HPA axis and its implications for MDD.

Cytokines and HPA activity

Cytokines are secreted by immune competent cells as a result of innate immune stimulation, including TLR4 activation by various ligands. As previously stated, cytokine administration is sufficient to cause sickness behavior. Cytokines can also upregulate HPA axis signaling on multiple levels through two main ways: (1) through reducing negative feedback on HPA signaling, and (2) by directly stimulating HPA activation. IL-1 β , IL-6, and TNF- α are able to reduce the efficacy of GR, thus disinhibiting GR induced negative feedback on HPA activity (Pace et al., 2007; Bogaert et al., 2010). This effect could be due to protein-protein interactions with GR, which enables pro-inflammatory cytokines to influence GR translocation, ligand binding affinity and GR binding to GRE within the nucleus (for review, see Pace and Miller, 2009).

Secondly, cytokines can amplify the feed-forward signaling within the HPA axis. IL-6 has been shown to potentiate CRH activation of ACTH (Mehet et al., 2012), while IL-1 β and stress have an additive effect on CRH secretion from the PVN (Chover-Gonzalez et al., 1993). Interestingly, peripheral and central IL-1 β is upregulated in response to acute stress within 1 h, and 3 h after stress onset, respectively, and could therefore endogenously prime the HPA response. Moreover, this study also found that the administration of an IL-1 β receptor antagonist diminished ACTH responses to restraint stress (Gądek-Michalska et al., 2011), showing that cytokines may partially mediate stress induced HPA activation. Given that an increase in peripheral IL-1 β and circulating cortisol is observed in patients with MDD (Maes et al., 1991), this permissive effect on ACTH secretion could be in part driving the dysregulation between the immune and HPA secretion in MDD.

TLR4-related COX-2 production as part of steroidogenesis pathway

Besides cytokine interactions, COX-2 has also been shown to mediate TLR4 involvement in modulation of HPA activity.

COX-2, an enzyme upregulated by TLR4 activation, catalyzes arachidonic acid into prostaglandin E2, which is part of the steroidogenesis pathway. COX-2 is therefore involved in the synthesis of GC at the level of the adrenal, and is shown to mediate LPS induced steroidogenesis within adrenal cells (Vakharia and Hinson, 2004). Furthermore, pharmacologically blocking COX-2 systemically is able to inhibit restraint stress induced GC both *in vivo* (Mouihate et al., 2010; Ma et al., 2013), and *in vitro* (Martinez Calejman et al., 2011). The effects of COX-2 extend beyond the adrenal gland, as expression of COX-2 in the PVN is important for sympathetic activation in response to restraint stress (Yamaguchi et al., 2010). In addition, COX-2 inhibition can influence higher order functions, buffering the effects of immobilization stress by reduced anxiety behavior and improve locomotor functioning and learning (Kumari et al., 2007).

Long-term effects of TLR4 activity on HPA axis function

Not only can TLR4 activation result in short-term stimulation of the HPA axis, but TLR4 can also influence HPA activity long after the stressor is resolved. For example, a single LPS challenge during early-life is sufficient to hyper-sensitize the CRH and ACTH response to both subsequent LPS and restraint stress when tested in adulthood, without baseline HPA differences when compared to vehicle controls in a rodent model (Mouihate et al., 2010). Early-life TLR4 activation also results in an increase in anxiety behavior during adulthood (Sominsky et al., 2013), thus fundamentally changing the stress response system. This suggests that TLR4 activity during developmentally sensitive periods may shape the HPA system, priming the system toward hyper-reactivity, and may even be changing individual predisposition toward stress-related disorders.

A good way of investigating the developmental consequence of TLR4 is through the use of genetic knockout models. TLR4 knockout mice are observed to have different HPA phenotypes when compared to match wild-type C57Bl/6 counterparts, showing increased adrenal gland volume and correspondingly higher baseline circulating glucocorticoid levels (Zacharowski et al., 2006). The direction of this change is not universally found however, as preliminary findings from our laboratory in Balb/c background TLR4 knockout mice have yielded opposite results. We have found that TLR4 genetic knockout mice have smaller adrenal glands as well as lower circulating GC (unpublished). Additionally, circulating ACTH was also elevated in mice lacking TLR4 when compared to matched controls.

Despite the differences in adrenal size and baseline ACTH levels, we found no difference between wild type and TLR4 knockout mice, in terms of adrenal responsiveness to ACTH administration in the same study (unpublished). Our findings support observations that systemic rather than adrenal MyD88 expression is important in regulating HPA activity (Kanczkowski et al., 2013). Taken together, these results suggest that although the TLR4 pathway can influence HPA activity, the innate immune system has little direct impact on adrenal function, and the observed HPA differences are likely to be driven by mechanisms within the CNS instead.

STRESS AND SYSTEMIC IMMUNITY

It is becoming clear that mood disorders such as depression have both immune and neuroendocrine components. Through interactions with the neuroendocrine system and central immune signaling, TLR4 is central to the physiological responses to immune stressors as well as baseline HPA activity. Yet at the same time, GCs classically suppress the immune system including the TLR4 pathway. When GCs bind to GR, immune suppression can occur in two main ways. Firstly, GR can directly interact with transcription factors NF- κ B and AP-1 through protein-protein interactions, and in the process interfere with the pro-inflammatory transcription (Ratman et al., 2013). Following nuclear translocation, GR can also dimerise and bind to GRE on DNA, upregulating transcription of anti-inflammatory or repressing inflammatory genes such as IL-6 receptor gene (Muzikar et al., 2009). Both mechanisms appear important in driving anti-inflammatory actions of GCs as DNA binding appears important for the resolution of high-dose LPS induced inflammatory and behavioral response (Silverman et al., 2013), while GR can interfere with NF- κ B induced transcription of proinflammatory genes (Novac et al., 2006). GR not only functions on the intracellular level, but can also downregulate macrophage expression of TLR4 mRNA in a dose and time dependent manner (Du et al., 2012). Given the varied roles of glucocorticoids in immune suppression, how does stress, which strongly triggers the HPA axis, cause increased immune signaling seen in MDD?

The answer likely lies in stress-induced adaptations, as recent evidence in rodent models has shown that stress itself can be both pro- and anti-inflammatory. Chronic footshock stress can induce bone-marrow derived monocytes infiltration into the hippocampus, thus increasing immune activity (Brevet et al., 2010). Acutely, footshock stress can also result in concurrent neuroendocrine and immune activation, characterized by increased hypothalamic IL-1 β and TNF- α , adrenal IL-6, and COX-2, in addition to circulating ACTH and GC increase (Hueston and Deak, 2014). The authors also showed that injection of ACTH and CRH induced adrenal IL-6 and COX-2 mRNA expression, indicating that HPA activation can be pro-inflammatory. The increase in immune signaling seen in MDD may therefore be driven by stress itself.

The elevation of neurokinine signaling appears stressor specific. Illustrating this, a meta-analysis showed that stress-induced IL-1 β in the hypothalamus is most reproducible in footshock and immobilization stress models (Deak et al., 2005). On the other hand, social defeat stress increases prefrontal cortex IL-1 β , IL-6, and TNF- α expression (Audet et al., 2011), in addition to increased monocyte infiltration (Wohleb et al., 2014). These variations in regional cytokine levels thus indicate a complex relationship between stressor type and the innate immune system within the CNS.

To reconcile the biphasic actions of HPA activation, in a recent review, Frank et al. (2013) argued that the timing of immune challenges and measurements is important in determining the direction of glucocorticoid actions. The authors proposed that glucocorticoids are anti-inflammatory during the stressor, but sensitizes the immune response after the stressor has ended, during what the “recovery phase” following the resolution of the stressor (Frank et al., 2013). Thus, timing of the

“second-hit” as well as measurements of immune functioning following both stressors is therefore imperative to measuring GC actions on immune function. At this point, it is still unclear what mechanisms drive this biphasic effect, and how long this pro-inflammatory state persists following stress. In the following section, we review potential mechanisms, as well as present the case for TLR4 involvement in mediating the pro-inflammatory actions of the HPA system.

MECHANISMS OF GLUCOCORTICOID INDUCED PRO-INFLAMMATORY RESPONSES

DIRECT MECHANISMS THROUGH HPA ACTIVATION

The HPA axis can directly influence immune signaling in two main ways, by reducing the inhibitory effects of glucocorticoid actions, or by directly stimulating the immune system. As mentioned in the previous section, there appears to be some form of GR adaptation, thus disrupting the actions of GR on the immune system and negative feedback onto the HPA system. This effect termed glucocorticoid resistance. Glucocorticoid resistance is predominantly thought to be due to either a reduced GR expression, or a selective reduction in GR α and a corresponding upregulation of GR β , the inactive splice variant of the receptor that is unable to bind glucocorticoids (Silverman and Sternberg, 2012). Increased expression of pro-inflammatory cytokines correspond to elevated expression of GR β , which could drive the disinhibition to immune response (Carvalho et al., 2014). Glucocorticoid resistance not only reduces glucocorticoid mediated immune suppression, but can in itself increase NF- κ B responses in PBMC when exposed to glucocorticoids, thus reshaping the response to a previously anti-inflammatory stimulus (Dawson et al., 2012).

Contrary to classical actions, HPA activation can also directly trigger the immune response. CRH, which is secreted by PVN cells, may also directly stimulate the innate immune system. When exposed to CRH, mast cells have been shown to undergo degranulation, releasing cytokines into the extracellular space (Theoharides et al., 1995; Alysandratos et al., 2012). This directly implicates CRH in the increased central immune or neurokinine signaling in stress-related disorders (Aguirre et al., 2013). On the other hand, CRH can also induce microglial apoptosis in the nanomolar range (Ock et al., 2006). It is therefore still unclear how the pro and anti-inflammatory effects are balanced in stress induced CRH release.

Pharmacological inhibition of GR reduces VCAM and ICAM expression in the microvasculature (Gregory et al., 2009), indicating the GR specific actions on immune cell migration. Indeed, low doses can be pro-inflammatory by stimulating phagocytosis and chemotaxis of macrophages via a GR mediated mechanism (Zhong et al., 2013). It has been previously established that GCs not only suppress the immune system, but at low doses directly induces production of macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine (Calandra et al., 1995). MIF can function as a chemokine, and stimulate CCL2 production when administered to the microvasculature, thus promoting migration of inflammatory cells to the site of damage (Gregory et al., 2009). Physiologically, MIF is involved in wound healing through promoting migration of endothelial progenitor cells to wounds (Grieb et al., 2012). Conversely, MIF is also implicated

in the development of neuropathic pain (Alexander et al., 2012; Lerch et al., 2014). The exact reason for immune activation following the HPA response is still debated, but one of the more accepted reasons for this effect is that by increasing immune signaling, MIF constrains the HPA response in order to counteract glucocorticoid induced immune cell apoptosis. Through these mechanisms, it is thus possible for heightened HPA activity and immune activation to co-exist in patients with MDD.

TLR4 MEDIATED MECHANISMS OF STRESS-INDUCED PRO-INFLAMMATORY RESPONSE

TLR4 mediates immune priming effects of stress

Given the often-contradictory results on HPA function, the complexity of glucocorticoid signaling is becoming more appreciated. In order to explain the complex actions of glucocorticoids Sapolsky et al. (2000) proposed 4 categories of glucocorticoid action - permissive, inhibitory, excitatory and priming, encapsulating the different receptors activated, timing of response relative to stressors and type of tissue activated. Illustrating this, glucocorticoids are excitatory in terms of heart function but inhibit vascular function, cause the release of glucose aiding energy expenditure, yet also trigger stockpiling of fat in adipose tissue. At the same time, basal glucocorticoid expression can be permissive toward sympathetic activation of the adrenal medulla, thus influencing stress responses even before the HPA activation even occurs. Thus, glucocorticoids have different dose and time response relationships across different tissues, and their actions are dependent on basal or activated HPA states.

Recent studies are beginning to classify the priming or sensitizing effect of stress and HPA activity on immune function. Chronic variable and acute social disruptive stress can sensitize HPA and immune response to subsequent LPS challenge, differentially inducing larger neurokinine and peripheral cytokine responses (Gibb et al., 2013). Repeated social defeat stress can also prime immune signaling in peripheral monocytes and dendritic cells in response to LPS, coupled with glucocorticoid resistance during the first 48 h after stressor, measured by immune cell expression following GC administration *in vitro* (Powell et al., 2009). Importantly, this immune priming is also seen in microglial populations 24 h after glucocorticoid treatment (Frank et al., 2011). Moreover, GC administration *in vivo* has been confirmed to emulate stress induced immune sensitization (Frank et al., 2010; Dey et al., 2014). This sensitizing effect was further blocked by a GR antagonist, indicating that GR signaling is essential (Frank et al., 2012). TLR2 and TLR4 activity could also be integral to glucocorticoid-induced immune priming in microglia, as administration of their respective antagonists prior to tailshock stress can prevent increased sensitivity in hippocampal tissues collected 24 h after the end of the stressor (Weber et al., 2013). Taken together, there appears to be crosstalk between the GR and TLR4 pathways, and both receptors appear to be important in driving immune cell sensitization and increased central immune signaling following stress.

The immune-priming effect of stress is proposed to mediate stress-induced side effects such as allodynia (Loram et al., 2011), and drug abuse (Frank et al., 2011), and could potentially be involved in MDD as well. PBMCs isolated from patients admitted

for severe depressive episodes are more responsive to interferon- γ (IFN- γ) stimulation (Schlaak et al., 2012). Along with increased TLR4 mRNA and expression on PBMCs of patients with MDD (Kéri et al., 2014), the increased immune signaling in MDD patients could be indicative of a primed immune system, rather than chronic inflammation.

DAMPs released during stress cause TLR4 activation

DAMPs, or alarmins, are released endogenously from stressed, dead and dying cells as a signal for danger. They include HMGB1, various HSP, ATP, and Uric acid. During normal physiological activation, DAMPs have a non-inflammatory function within the cell. Conversely during situations of tissue damage, when released into the extracellular space, DAMPs alert the immune system to the damage in order to promote repair and direct traffic toward the damaged tissue, thus triggering the inflammatory response. This inflammatory response is in part driven by TLR4, since DAMPs including HMGB1 and various HSPs can activate the TLR4 pathway (Hutchinson et al., 2009; Laird et al., 2014). Furthermore, ATP can trigger innate immune signaling by activating a protein complex known as the inflammasome, which induces the maturation and release of cytokines IL-1 β and IL-18 via a caspase-1 dependent mechanism (Chen et al., 2013). This effect is known to augment the inflammatory response to LPS, therefore amplifying TLR4 signaling (Ghonime et al., 2014).

DAMPs including HMGB1, uric acid and HSP72 are also released following tail-shock stress (Faraco et al., 2007; Maslanik et al., 2013), and thus are not limited to situations of tissue damage or cell death. DAMPs could therefore mediate the effect of stress on triggering or sensitizing the immune response, and this increased immune signaling may have wider implications for MDD. It is notable, however, that the mechanisms regulating the secretion of DAMPs in response to stress is not well characterized.

High mobility group box 1. HMGB1 functions as a chaperone protein within the cell by binding to proteins and transporting them between the cytoplasm and nucleus. During damage however, HMGB1 can be released into the extracellular space via an inflammasome mediated mechanism (Lu et al., 2012). Neural tissue is capable of releasing HMGB1 in response to glutamatergic excitotoxicity and glial activation as a result of LPS administration *in vitro* (Faraco et al., 2007). HMGB1 can also activate central immune signaling, as it can trigger TLR4 similar to LPS, via the MD2 and CD14 complex, and requires adaptor protein MyD88 to trigger downstream inflammatory actions (Kim et al., 2013). Moreover, psychological stress itself can induce an increase in HMGB1. For example, thymocytes are responsive to 15 min restraint stress, and release HMGB1 via GR signaling (Billing et al., 2012). There is further evidence showing that HMGB1 and GR can form complexes within the chromatin, increasing the residence time of GR when bound to DNA (Agresti et al., 2005). The functional consequence of this interaction, however, is still unclear. Given that HMGB1 is responsive to stress, interacts with GR, and is able to increase peripheral and central immune signaling, stress-induced immune sensitization through neurokinine signaling could therefore be partially mediated by HMGB1.

HMGB1 can also cause an upregulation of Matrix metalloproteinase 9 (MMP9), an enzyme that results in the breakdown of the extracellular matrix (Qiu et al., 2010). Interestingly, increased MMP9 in circulation is associated with mood disorders such as depression and bipolar disorder (Domenici et al., 2010; Rybakowski et al., 2013). Together with evidence that MMP9 can also be upregulated as a consequence of microglial activity (Lively and Schlichter, 2013), MMP9 could be a possible result of stress induced HMGB1 upregulation and immune signaling within the CNS in MDD.

Heat shock proteins. HSPs were first discovered in the drosophila model to be produced in response to hyperthermia, and vary in protein weights ranging up to 110 kDa. HSP regulate the folding and unfolding of other proteins, and are released in response to cellular damage. Signaling danger, extracellular HSP can activate the innate immune system (Colaco et al., 2013). Within the HSP family, HSP70 and HSP90, the 70 and 90 kDa variants, are of most relevance to glucocorticoid and TLR4 signaling. HSP70 and HSP90 can bind TLR4, resulting in release of pro-inflammatory cytokines (Gong et al., 2009; Colaco et al., 2013). Furthermore, HSP90 also serves as a chaperone protein for TLR4, triggering endocytosis in response to ligand binding (Triantafilou and Triantafilou, 2004). Through these mechanisms, HSP90 plays an integral role in TLR4 signaling and in TLR4 related neuropathic pain (Hutchinson et al., 2009). Stress can also induce HSP expression, notably decreasing the ratio between GR and HSP70 and HSP90 expression in the hypothalamus (Simic et al., 2012).

Interestingly, HSP90 is a well-characterized chaperone protein for GR nuclear translocation and permits GC binding to GR (Rickertson et al., 2007). Although important for binding and translocation, Increased HSP90 expression can also impair GR function (Matysiak et al., 2008). This effect increases in chronic stress as compared to acute models of stress, and thus is proposed as one facet of glucocorticoid resistance. Stress induced changes in HSP can therefore either directly activate TLR4, change the trafficking TLR4 and GR, as well alter GR binding capacity. However, to what extent each of these mechanisms is involved, and the magnitude of the change has yet to be investigated in models of depressive-like behavior. Nevertheless, given the involvement in TLR4 and GR signaling, HSP could be an avenue for further research in linking stress and increased immune signaling in MDD.

TLR4 activation as a result of gut translocation of microbes

One way in which TLR4 ligands are upregulated by stress is through intestinal translocation. Recently, the role of gut microbiota in potentiating differences in mood and behavior is gaining traction within the literature (Hsiao et al., 2013). It has been hypothesized that stress can cause a disruption in intestinal tight junctions, which would increase translocation of microbiota into the system, thus inducing inflammatory responses. Indeed, GR appears to be involved in gut HSP70 upregulation and intestinal permeability in response to restraint stress (Ait-Belgnaoui et al., 2012, 2014).

There is some evidence showing that intestinal decontamination using orally administered antibiotics is able to block the tight

junction disruption, as well as inflammatory and HPA responses to psychological stress (Gárate et al., 2011). Furthermore, antibiotic treatment is shown to mirror the ability of TLR4 antagonist in blocking stress induced depressive-like behavior (Gárate et al., 2011). This hypothesis is thus showing promise for developing new medication targeting the gut-brain axis in regulating behavior. On the other hand, the exact mechanisms of this gut-brain communication in respect to stress and MDD are unclear, since both ascending pathways and peripheral immune signaling could potentially be involved. In addition, the extent of intestinal translocation in the acute stress response requires more study, since it is not known if this effect is stressor specific.

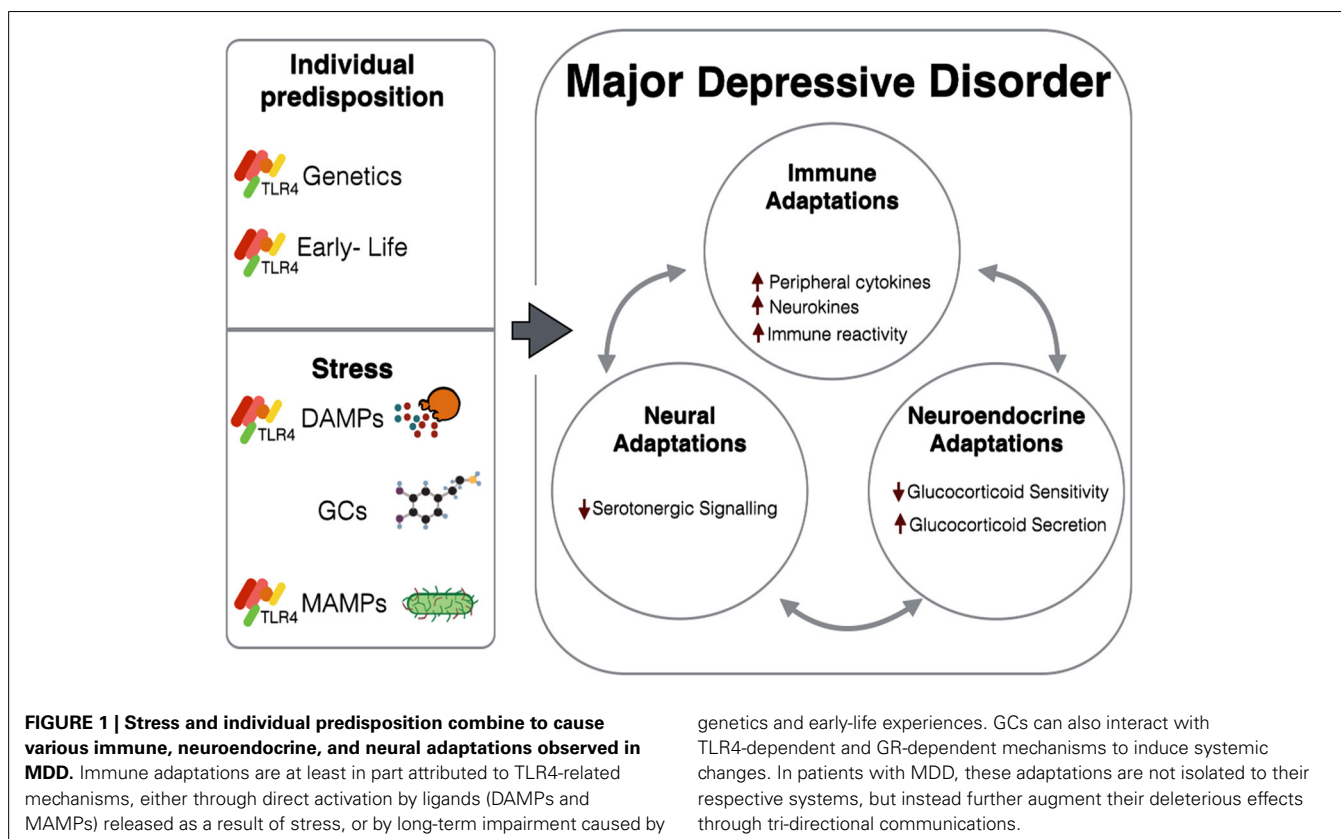
CONCLUSIONS

It is evident that the immune, neural and neuroendocrine systems are in constant multi-directional communication, and in the case of stress and MDD, patients exhibit a dysregulation of all three systems. Thus, the difficulty in finding treatment targets lies in untangling the multi-layered relationships. In the current review, we presented evidence centered on immune modulation of CNS and stress-induced adaptations observed in models of MDD. Stress is regarded as a necessary factor for the development of MDD through adaptations to the neuroendocrine and immune responses.

Through use of knockout models as well as pharmacological agonists and antagonists, TLR4 activation has been shown to elicit depression-like symptoms in animal models both behaviorally and physiologically. Additionally, TLR4 could potentially

mediate stress-induced immune signaling both in the periphery and within the CNS, as well as underlie stress-induced immune activity, through interactions with DAMPs, MAMPs, and GC signaling (Figure 1). TLR4, an innate immune receptor, could therefore be important in investigating the immune involvement in the pathophysiology of MDD.

On the other hand, this direct relationship between TLR4 and depression is still not fully understood, although timing and location of TLR4 activation appears to be important. The mode in which TLR4 influences MDD is not established, even though cytokines appear to be essential to the development of sickness behavior, other mechanisms such as direct interaction between TLR4 signaling pathway and other receptors (for example, GR) or intracellular signaling molecules could also play a role in the development of the disorder. Furthermore, due to the many compounds that can trigger TLR4 activation, an obvious question would be to identify which of those are most relevant to MDD. Critically, there is a need to move away from the use of LPS in the investigation of MDD, since it constrains the generalizability of conclusions to infectious factors. Although the immune system appears to be involved in the pathophysiology of MDD, it is unlikely that bacterial infections are the main factor, especially given that cytokines themselves can induce behavioral change in the absence of sickness. Instead, individual differences in immune activity could originate from alterations to immune signaling during critical periods during development, genetic disposition, or epigenetic changes that contribute to predispositions (Bilbo and Schwarz, 2009). Moreover, due to the



sub-inflammatory and state driven nature of heightened immune signaling in MDD, endogenous mechanisms including DAMPs, neuroendocrine, neurogenic signals, or an increase in gut translocation of microbiomes are more likely involved. Thus, in the search for a more efficacious treatment of MDD, the impact on neural, neuroendocrine and immune systems must be considered within representative models of the disorder.

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Monocyte trafficking to the brain with stress and inflammation: a novel axis of immune-to-brain communication that influences mood and behavior

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HIGHLIGHTS

- Psychological stress activates neuroendocrine pathways that alter immune responses.
- Stress-induced alterations in microglia phenotype and monocyte priming leads to aberrant peripheral and central inflammation.
- Elevated pro-inflammatory cytokine levels caused by microglia activation and recruitment of monocytes to the brain contribute to development and persistent anxiety-like behavior.
- Mechanisms that mediate interactions between microglia, endothelial cells, and macrophages and how these contribute to changes in behavior are discussed.
- Sensitization of microglia and re-distribution of primed monocytes are implicated in re-establishment of anxiety-like behavior.

Psychological stress causes physiological, immunological, and behavioral alterations in humans and rodents that can be maladaptive and negatively affect quality of life. Several lines of evidence indicate that psychological stress disrupts key functional interactions between the immune system and brain that ultimately affects mood and behavior. For example, activation of microglia, the resident innate immune cells of the brain, has been implicated as a key regulator of mood and behavior in the context of prolonged exposure to psychological stress. Emerging evidence implicates a novel neuroimmune circuit involving microglia activation and sympathetic outflow to the peripheral immune system that further reinforces stress-related behaviors by facilitating the recruitment of inflammatory monocytes to the brain. Evidence from various rodent models, including repeated social defeat (RSD), revealed that trafficking of monocytes to the brain promoted the establishment of anxiety-like behaviors following prolonged stress exposure. In addition, new evidence implicates monocyte trafficking from the spleen to the brain as key regulator of recurring anxiety following exposure to prolonged stress. The purpose of this review is to discuss mechanisms that cause stress-induced monocyte re-distribution in the brain and how dynamic interactions between microglia, endothelial cells, and brain macrophages lead to maladaptive behavioral responses.

Keywords: stress, neuroimmune, microglia, monocytes, macrophages, anxiety, depression, post-traumatic stress disorder

INTRODUCTION

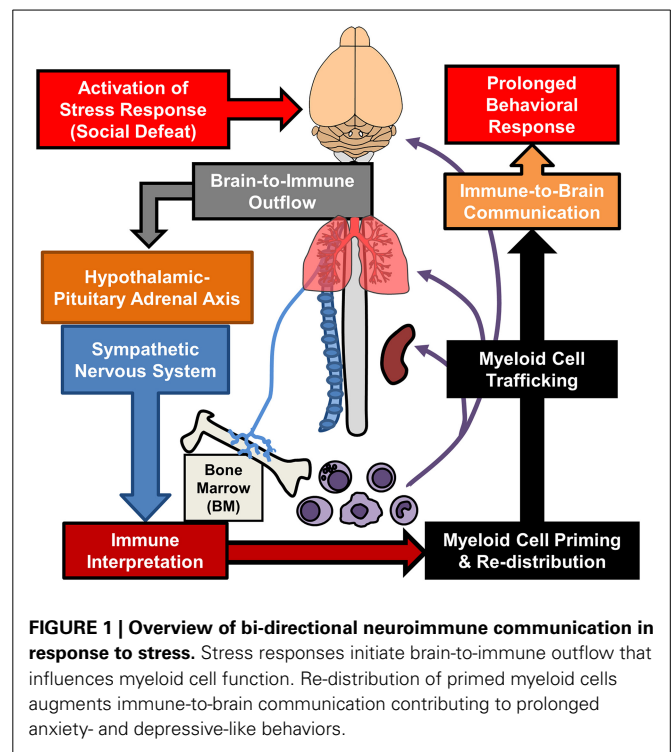
Psychological stress in humans promotes development of mental health disturbances, including anxiety and depressive disorders (Kendler et al., 1999; McLaughlin et al., 2010; Gilman et al., 2013). Nonetheless the biological mechanisms connecting stress to mental health complications are not well-understood. Recent evidence indicates that inflammation and altered immune signaling significantly contribute to the etiology of many psychiatric

symptoms and disorders (Evans et al., 2005), particularly in the context of chronic stress (Miller et al., 2009), depression (Raison et al., 2006), and anxiety (Pace and Heim, 2012). Indeed, chronic psychological stress in humans causes a “transcriptional fingerprint” on peripheral monocytes that is characterized by increased pro-inflammatory-related gene expression, particularly relating to the NF- κ B transcriptional control pathway (Miller et al., 2008, 2014; Cole et al., 2011, 2012; Powell et al.,

2013). This “transcriptional fingerprint” of stress on monocytes is linked to exaggerated pro-inflammatory responses following *ex vivo* immune stimulation and reduced anti-inflammatory responses initiated by glucocorticoid (GC)-mediated transcription (Miller et al., 2002; Rohleder et al., 2009; Cohen et al., 2012; Rohleder, 2012). Thus, chronic psychological stress substantially enhances the pro-inflammatory profile of peripheral monocytes.

These associations between stress and inflammation are relevant because immune activity potently regulates mood and behavior (Dantzer et al., 2008). For example, patients treated with inflammatory cytokines experience severe mood disturbances and have increased prevalence of depression (Udina et al., 2014). Moreover, peripheral innate immune stimulation increased self-reported anxiety symptoms (Reichenberg et al., 2001). These links between inflammation and behavior are well-established, particularly in the context of depression (Raison et al., 2006) and recurrent anxiety disorders (Pace and Heim, 2012). Thus, these studies show that the associations between stress and inflammation are critical for understanding the underlying neurobiology of stress-related mood disorders. Many of these stress-related immune phenomena are recapitulated in rodent models of stress including restraint stress, chronic variable stress, inescapable foot shock, and repeated social defeat (RSD). The objective of this review is to discuss novel data regarding brain-to-immune and immune-to-brain communication that function to regulate mood and behavior.

Additional sections of this review will focus on recent findings from the RSD model that have elucidated new components of neuroimmune facilitation of stress-induced behavioral adaptations. One important concept is that RSD and other stressors are physiological in nature and are interpreted in the brain within discrete stress-responsive neurocircuitry. Activation of this neurocircuitry subsequently leads to activation of the sympathetic nervous system (SNS) and the hypothalamic pituitary adrenal axis (HPA). Activation of the SNS and HPA with stress allows the CNS to communicate with the immune system and to profoundly influence its functions (Sternberg, 2006). We will discuss mechanisms regarding how the immune system responds to prolonged stress and how immune modulation then feeds back to the brain to modulate mood and behavior. For example, as illustrated in **Figure 1**, RSD exposure causes SNS-dependent monocyte trafficking from the bone marrow (BM) to the brain. This leads to dynamic interactions between BM-derived monocytes, endothelial cells, and resident microglia. Together, these signals converge to augment neuroinflammatory signaling and promote prolonged anxiety. A final topic reviewed here is the recent evidence that a similar neuroimmune circuit contributes to recurring anxiety following RSD-induced stress-sensitization. These recent studies show that this neuroimmune circuit was re-activated following exposure to a secondary acute stress, and this resulted in the re-establishment of anxiety-like behavior that was dependent on monocyte re-distribution and microglia sensitization. Overall these novel findings indicate that psychological stress initiates a cascade of neuroimmune responses involving brain-to-immune and immune-to-brain signaling that converge to influence mood and behavior.



INTERPRETATION OF STRESSFUL STIMULI ENGAGES CORTICO-LIMBIC BRAIN REGIONS AND ACTIVATES NEUROENDOCRINE RESPONSES

In both rodents and humans, physiological stress is interpreted by the brain within fear and threat appraisal circuitry that results in both neurobiological and behavioral responses. It is important to understand the cellular and molecular mechanisms mediating these behaviors because psychological stress in humans is associated with the development of anxiety, social withdrawal, and major depression (Ressler and Mayberg, 2007; Price and Drevets, 2010). Early studies examining the neurocircuitry of stress-responses in rodents showed that key stress-responsive areas of the brain are activated, including the pre-frontal cortex (PFC), hypothalamus (HYPO), amygdala (AMYG), and the CA3 and dentate gyrus of the hippocampus (HPC) (Kollack-Walker et al., 1997; Martinez et al., 2002). This is pertinent because there is evidence that activation of this neurocircuitry can manifest as anxiety or depression in humans (Sheehan et al., 2004). Thus, understanding fear and threat appraisal circuitry can help elucidate the neurobiology underlying the development of mood disturbances related to chronic stress.

Stress-responsive patterns of brain region activity are recapitulated in animal models of stress, such as RSD and restraint. This notion is supported by studies using c-Fos immunolabeling. c-Fos is an immediate early gene used as a functional marker of neuronal activation (Kovacs, 1998). For instance, social stress in rodents increased the number of cFos-expressing neurons in the PFC, HYPO, AMYG, and HPC (Kollack-Walker et al., 1997; Wohleb et al., 2011). In addition, several limbic brain regions implicated in regulating mood, including the bed nucleus of the stria terminalis (BNST), lateral septum (LS), and

nucleus accumbens (NAc) are activated in response to social stressors (Martinez et al., 1998). While these forebrain and mid-brain networks are interacting, visceral sensory information is simultaneously transmitted to cortico-limbic structures via brain-stem nuclei that are innervated by several ascending pathways. For example, the vagus nerve communicates peripheral sensory events into these circuits via innervation of the nucleus of the solitary tract, which sends secondary projections to brain-stem nuclei including the locus coeruleus (LC). This is relevant because stress-induced autonomic activation can initiate arousal pathways originating in the LC. Projections from the LC release norepinephrine into distant regions, including the PFC, AMYG, and HPC, that cause enhanced attention and vigilance (Morilak et al., 2005; Radley et al., 2008; Samuels and Szabadi, 2008). Collectively these inter-connected brain regions constitute the key brain regions activated during stress exposure. Despite the fact that these brain regions appear to be detached, feedback mechanisms linking all of these structures together coordinate adaptive physiological and behavioral responses. Taken together, as illustrated in the top half of **Figure 2**, interpretation of stress involves neuronal activation within brain regions associated with fear and threat appraisal.

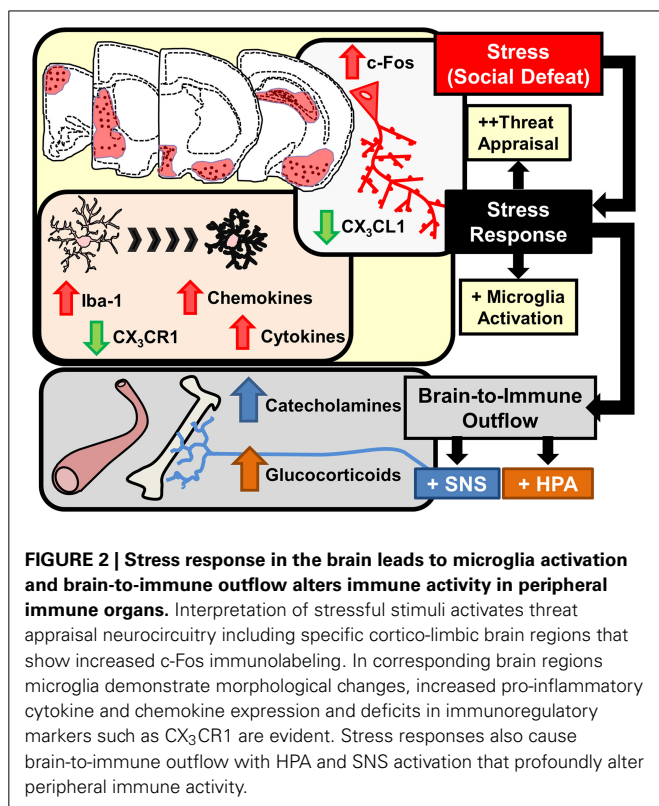
Several studies indicate that anxiety- and depressive-like behaviors are caused by stress-associated neurobiological alterations in brain regions, including the PFC, AMYG, and HPC (Ressler and Mayberg, 2007; Price and Drevets, 2010). For instance, stress exposure in rodents caused both retraction of neuronal dendrites and (Radley et al., 2004) and decreased dendritic spine density in the PFC (Magarinos et al., 1996). In addition, chronic stress impaired hippocampal neurogenesis (Gould et al.,

1997, 1998). The hypothalamic-pituitary-adrenal (HPA) axis was implicated in these studies because exogenous glucocorticoid (GC) administration or blockade of endogenous stress-induced GCs resulted, respectively, in enhancement or reversal of these neurobiological effects. There are dichotomous findings in other regions such as the amygdala that show functional activation of the amygdala is enhanced in mood disorders (Sapolsky, 2003). Indeed, stress promoted neuronal hypertrophy and increased dendritic complexity in the amygdala of rodents (Vyas et al., 2002; Mitra et al., 2005). Neurobiological changes in the amygdala were also facilitated by hormonal release of GCs and central noradrenergic pathways (Roozendaal et al., 2009). Based on these data initial propositions suggested that stress hormones and neurotransmitters caused intrinsic neuronal adaptations leading to mood disorders (Krishnan and Nestler, 2008; Christoffel et al., 2011a,b).

Interpretation of psychological stress in the brain causes activation of neuroendocrine pathways that signal into the periphery, including the hypothalamic-pituitary-adrenal (HPA) axis and the SNS. HPA activation leads to release of glucocorticoids (GC) in circulation and activation of the SNS leads to increased release of catecholamines in circulation (epinephrine, Epi) and in tissues (norepinephrine, NE). Collectively, HPA and SNS activation with acute stress synergize to influence physiology with increased breakdown of glucose, increased heart rate and muscle tone (Sternberg, 2006). This response is termed the “fight or flight” response and provides the organism with increased energy availability and heightened awareness to respond to aversive challenges. Another major element of HPA and SNS activation is that these signals are relayed to the immune system (Irwin and Cole, 2011; Wohleb and Godbout, 2013). Thus, an integral component of the stress response is to relay information from the brain to peripheral organs and immune system via HPA and SNS neuroendocrine pathways (**Figure 2**).

STRESS-INDUCED NEURONAL RESPONSES ARE ASSOCIATED WITH MICROGLIA ACTIVATION AND INCREASED NEUROINFLAMMATORY SIGNALING

Integration of stress-induced signaling is facilitated in the brain by microglia, the resident immune cells of the brain, through propagation of neuroinflammatory signaling that modulates neuronal and endocrine responses to stress. Microglia are integral cellular components of the brain and partake in many homeostatic processes, including removal of apoptotic neurons, pruning of synapses, phagocytosis of excess proteins, and regulation of neurotransmitter levels (Tremblay et al., 2011). Microglia also have a role in immune surveillance, and when they become activated, they provide similar immune function as peripheral macrophages (Pocock and Kettenmann, 2007; Ransohoff and Perry, 2009; Kettenmann et al., 2011, 2013). This includes the production of inflammatory mediators, such as prostaglandins, cytokines, and chemokines. Based on their functional role in the brain, microglia are implicated in neuroinflammatory and behavioral responses to stress. For instance, numerous studies have revealed microglia activation and neuroinflammatory signaling occurs following prolonged stress exposure (Johnson et al., 2005; Frank et al., 2007a, 2012; Tynan et al., 2010; Wohleb et al., 2011, 2012, 2013; Bian et al., 2012; Hinwood et al., 2012, 2013; Kopp



et al., 2013). Moreover, recent studies indicate that microglia activation and neuroinflammatory signaling have a causal role in behavioral responses to chronic stress (Blandino et al., 2006; Hinwood et al., 2012; Wohleb et al., 2013; Kreisel et al., 2014). In this context alterations in microglia physiology likely contribute to maladaptive neurobiological responses that underlie stress-induced mental health disorders. In this way, microglia may contribute to the maladaptive neurobiological interpretation of stress within the brain.

One common finding is that stress exposure altered microglia morphology in similar fear and threat appraisal areas of the brain that are activated by stress. For example, prolonged stress exposure caused neuronal activation and altered microglia morphology in overlapping stress-responsive regions, including the PFC, HYPO, AMYG, and the CA3 and dentate gyrus (DG) of the HPC (Tynan et al., 2010; Wohleb et al., 2011; Hinwood et al., 2013). In these studies, stress-induced morphological changes in microglia were consistent with an activated profile. In support of this idea, altered microglia morphology following RSD, foot shock, and chronic unpredictable stress were all associated with increased pro-inflammatory cytokine mRNA expression and exaggerated pro-inflammatory responses to immunological challenges (Frank et al., 2007a; Wohleb et al., 2011, 2012). In the context of psychological stress, the term “activated microglia” refers to changes in morphology (e.g., increased soma size) that corresponds with increased mRNA expression of inflammatory mediators including cytokines and chemokines. Thus, as highlighted in **Figure 2**, prolonged stress exposure caused microglia activation that regionally correlated with neuronal activation in stress-responsive areas of the brain.

The regional co-occurrence of neuronal and microglial activation suggests a causative link between these two events. In support of this, evidence from RSD revealed that increased region-specific neuronal activation was evident after a single cycle of social defeat. This neuronal activation preceded elevated cytokine expression that was observed only after at least 3 cycles. These findings are relevant because they indicated that neuronal activation is an upstream event of microglia activation. Moreover, pre-treatment with propranolol, a β -adrenergic receptor antagonist, blocked stress-induced neuronal activation as well as alterations in microglia morphology (Wohleb et al., 2011). Thus, β -adrenergic receptor-dependent neuronal activity was implicated in stress-induced microglia activation. Moreover, other reports revealed that central administration of β -adrenergic receptor agonists alone elicited pro-inflammatory cytokine production, and ablation of noradrenergic locus-coeruleus (LC) projections reduced stress-induced IL-1 β production in the HPC (Johnson et al., 2005). Furthermore, in a recent study, *in vitro* application of norepinephrine caused microglia morphological changes, including retraction of processes that was consistent with an activated phenotype (Gyoneva and Traynelis, 2013). These results indicate that central noradrenergic responses have a substantial contribution to stress-induced microglia activation and neuroinflammatory signaling.

The notion that neuronal activity regulates microglia activation is supported by the fact that microglia actively monitor

neurons through CD200/CD200R interactions, chemokine signaling (i.e., CX₃CL1), growth factors (i.e., M-CSF), ATP release, and neurotransmitter levels (Kettenmann et al., 2011; Kierdorf and Prinz, 2013; Dissing-Olesen et al., 2014). In the RSD model, region-specific microglia activation corresponded with reduced anti-inflammatory regulation through neuronal-derived fractalkine ligand (CX₃CL1) and reduced fractalkine receptor (CX₃CR1) on microglia (**Figure 2**). During physiological conditions CX₃CL1 expression is highly enriched within neurons and its homeostatic expression promotes a less-inflammatory profile of microglia that ubiquitously express CX₃CR1 (Cardona et al., 2006; Wynne et al., 2010). However, RSD caused significant reductions in CX₃CL1 expression (Wohleb et al., 2013) that were compounded by decreased CX₃CR1 expression in enriched microglia (Wohleb et al., 2014b). Moreover, the region specificity of microglia activation is also explained by the notion that neuronal CX₃CL1 expression is reduced in an activity dependent manner (Harrison et al., 1998). Thus, region-specific neuronal activation following stress exposure corresponded with reduced CX₃CL1 and CX₃CR1 expression that may contribute to activation of microglia within stress-responsive areas of the brain.

In numerous models of stress, microglia are implicated as the source of neuroinflammatory signals. In these studies, minocycline, an antibiotic that limits microglia responses, prevented stress-induced pro-inflammatory cytokine expression in the brain. For example, minocycline prevented increased IL-1 β expression in the brain following foot shock (Blandino et al., 2009), reduced swim stress-induced microglia NF- κ B signaling (Bradesi et al., 2009), and attenuated restraint stress-induced microglial activation (Hinwood et al., 2012). A compelling observation with chronic restraint stress is that minocycline treatment also attenuated persistent neuronal activation, as indicated by reduced FosB neuronal labeling (Hinwood et al., 2012). This observation suggests that microglia activation may enhance or reinforce chronic stress-induced neuronal activity. Consistent with this, inhibition of microglia activation by minocycline or other anti-inflammatory interventions corresponded with attenuation of cognitive deficits, depressive-like behavior, and anxiety-like behavior following restraint stress (Hinwood et al., 2012) and chronic variable stress (Kreisel et al., 2014). Moreover, there is substantial evidence that microglia activation and brain cytokine signaling following stress-exposure augment neuroendocrine outflow that may further reinforce stress-related behaviors (Goshen et al., 2008). It is thought that microglia activation potentiates HPA responses via release of IL-1 β within the hypothalamus (Goshen and Yirmiya, 2009). As illustrated in **Figure 2**, stress-induced microglia activation occurs within discrete stress-responsive brain regions, reinforces stress-responsive neuronal circuits, and augments neuroendocrine activation. This is important because these events influence immune and behavioral responses to stress.

NEUROENDOCRINE PATHWAYS SIGNAL TO THE IMMUNE SYSTEM AND INCREASE THE RELEASE OF INFLAMMATORY MYELOID CELLS FROM THE BONE MARROW

As previously discussed, activation of the HPA axis and SNS relays stress interpretation from the brain to the immune system

(bottom half of **Figure 2**). The best example of this communication is the hardwiring of the SNS into primary and secondary lymphoid tissues, including bone marrow (BM), lymph nodes, and spleen (Felten et al., 1985). In this context, stress-induced SNS activation causes direct release of catecholamines into these immune organs. This is pertinent because peripheral immune cells express receptors for NE, and stimulation of these receptors causes functional responses that influence their development, inflammatory phenotype, and migrational capacity (Bierhaus et al., 2003; Nance and Sanders, 2007; Grisanti et al., 2010). In the context of prolonged or repeated activation of the SNS, such as with chronic stress, increased NE in the BM promotes the production and release of myeloid cells, including monocytes and granulocytes (Dhabhar et al., 2012; Hanke et al., 2012). The increased cycling of myeloid cells in the BM with stress shifts the phenotype of peripheral monocytes to be less mature and more “inflammatory” (Engler et al., 2004, 2005; Hanke et al., 2012; Heidt et al., 2014). These monocytes are termed “inflammatory” because they are able to traffic throughout the body and have enhanced capacity to release pro-inflammatory cytokines upon entering tissue and becoming effector cells. This is relevant because stress-induced trafficking of inflammatory monocytes contributes to the exacerbation of both mental and physical health conditions (Dutta et al., 2012; Hanke et al., 2012; Liezmann et al., 2012; Seifert et al., 2012; Wohleb et al., 2013, 2014a; Heidt et al., 2014).

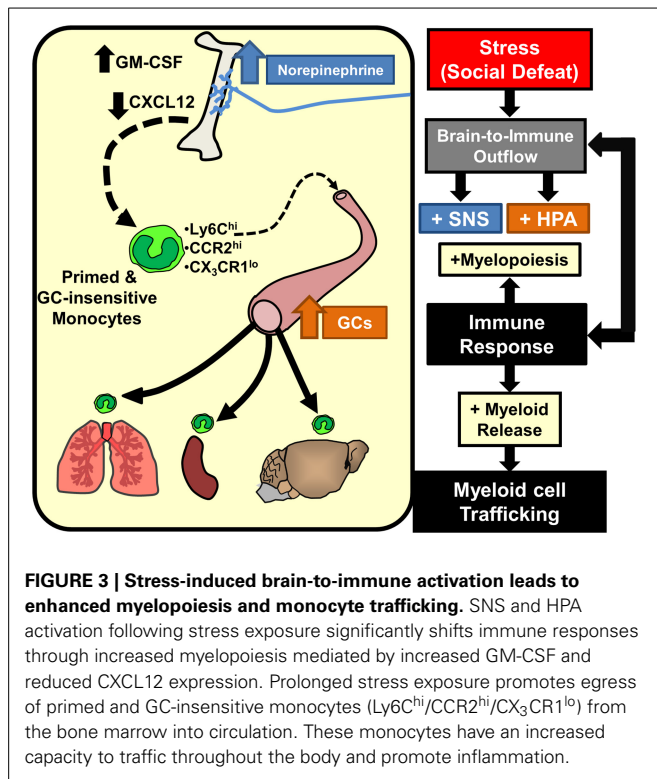
Numerous studies in mice and humans revealed that the SNS directly innervates the BM. The most salient evidence of this is the presence of tyrosine hydroxylase-expressing (TH⁺) axons observed throughout the BM (Afan et al., 1997; Nance and Sanders, 2007). Moreover studies revealed that this innervation regulates immune function under homeostatic conditions and during various challenges, such as inflammation and stress (Felten et al., 1985; Nance and Sanders, 2007). It is well-documented that various types of psychological stress enhance SNS signaling in the BM via dichotomous mechanisms depending on the duration of the stress. For example, acute stress enhances the turnover and release of NE within the BM (Tang et al., 1999; Hanke et al., 2012), while chronic stress causes structural alterations, characterized by increased innervation of TH⁺ axons that corresponds with enhanced sympathetic signaling (Heidt et al., 2014). These effects are also observed in other lymphoid tissues in which psychological stress caused re-organization and increased innervation by SNS inputs (Sloan et al., 2008). Thus, the SNS acts as an integral relay of stress-signals from the brain to the immune system.

Related to the effects of stress on SNS activity, stress duration also has dichotomous effects on BM functions. For example, acute and chronic stress-exposures have differential effects on the release, proliferation, and inflammatory capacity of progenitors in the BM that are related to the temporal dynamics of SNS signaling. With acute stress, SNS activation is associated with increased egress of leukocytes, especially myeloid cells (e.g., monocytes and granulocytes), from the BM that transiently accumulate in circulation (Engler et al., 2004; Dhabhar et al., 2012). Similar to acute stress, chronic stress maintains increased leukocyte release from the BM. However, the dichotomy of stress-duration is evident in

the selective enhancement of myeloid but not lymphocyte proliferation in the BM. For example, chronic stress caused sustained monocyte and granulocyte egress from the BM that resulted in substantial accumulation of these cells in circulation (Powell et al., 2013; Heidt et al., 2014). These myeloid-enhancing effects of chronic stress are amplified by proliferation and expansion of myeloid progenitor cells in the BM that occurs concomitantly with reductions in lymphocytes and erythrocytes (Engler et al., 2004; Powell et al., 2013; Heidt et al., 2014). Moreover, this selective enhancement of myelopoiesis was dependent upon SNS activation. For example, β -adrenergic receptor blockade with propranolol or selective β_3 -receptor antagonism prevented stress-induced enhancement of myelopoiesis in both RSD and chronic variable stress models (Wohleb et al., 2011; Hanke et al., 2012; Heidt et al., 2014). Chronic variable stress, that is also often referred to as “chronic mild stress” and “chronic unpredictable stress,” is similar to RSD in that it repeatedly promotes increased neuroendocrine activation with reoccurring stress (Yalcin et al., 2005). Thus, enhanced production and release of monocytes and granulocytes in the BM was dependent upon SNS activity and resulted in substantial accumulation of these cells in circulation.

Studies in the RSD and chronic variable stress (CVS) models revealed key downstream signals provided by chemokines and growth factors that mediate SNS-dependent enhancement of myelopoiesis. With the CVS model, reduced CXCL12 signaling contributed to increased egress of myeloid cells from the BM (Heidt et al., 2014). This is consistent with the known function of CXCL12 to promote the retention of leukocytes (Katayama et al., 2006). Moreover, CXCL12 expression in BM-stromal cells was reduced by stress and rescued by β_3 -adrenergic blockade (Heidt et al., 2014). Thus, SNS-dependent reduction in stromal CXCL12 expression is a key regulator in the expansion of BM progenitors during chronic stress. Related to this, studies in the RSD model revealed that increased granulocyte-monocyte colony stimulating factor (GM-CSF) signaling mediates selective enhancement of myeloid expansion within the BM (Powell et al., 2013). It is relevant to note that myeloid cells share a common progenitor called a granulocyte-macrophage colony forming unit (GM-CFU) that also shares the common stimulatory growth factor, GM-CSF. Thus, increased expansion of granulocytes and monocytes in the BM following stress requires proliferation of GM-CFUs that is enhanced by GM-CSF signaling (Hamilton and Achuthan, 2013). In support of this notion, RSD increased BM GM-CSF expression in an exposure-dependent manner that temporally correlated with enhancement of myelopoiesis following 3 and 6 cycles of social defeat (Engler et al., 2005). Moreover, treatment with anti-GM-CSF antibody prevented enhanced myelopoiesis following RSD (Powell et al., 2013). Collectively, as illustrated in **Figure 3**, SNS stimulation of the BM results in reduced CXCL12 and increased GM-CSF that promotes the enhancement of myelopoiesis with prolonged stress exposure.

Activation of the SNS with prolonged stress not only regulates the production and release of myeloid cells but also enhances their pro-inflammatory profile. For example, numerous studies in humans reveal that chronic stress caused substantial enhancement of monocytic inflammatory potential. This is evidenced by exaggerated responses to *ex vivo* innate immune challenge



(Miller et al., 2002; Rohleder et al., 2009; Cohen et al., 2012; Rohleder, 2012; Powell et al., 2013). The notion of stress-induced inflammation is best encapsulated in works published by Steve Cole, Gregory Miller, and colleagues, where they describe it as a “conserved transcriptional response to adversity” (Cole et al., 2011; Powell et al., 2013; Miller et al., 2014). These reports demonstrate that the “transcriptional fingerprint” associated with chronic stress is mainly characterized by up-regulation of pro-inflammatory transcription control pathways, particularly the NF- κ B pathway (Miller et al., 2008, 2014; Cole et al., 2011, 2012). Moreover, chronic stress is associated with down-regulation of transcriptional activity mediated by the glucocorticoid (GC) receptor, resulting in functional glucocorticoid insensitivity and loss of anti-inflammatory feedback associated with GC signaling (Miller et al., 2002; Rohleder et al., 2009; Cohen et al., 2012; Rohleder, 2012). These SNS-mediated pro-inflammatory effects of stress are recapitulated in rodent models. For example, myeloid cells isolated from RSD-exposed mice exhibited a similar pro-inflammatory “transcriptional fingerprint” as humans exposed to chronic stress (Powell et al., 2013). Also similar to humans, RSD enhanced pro-inflammatory cytokine production following innate immune challenge (Avitsur et al., 2001, 2002, 2003; Stark et al., 2001, 2002; Quan et al., 2003; Bailey et al., 2004; Engler et al., 2005, 2008; Hanke et al., 2012). In these studies, increased cytokine production in response to immune challenge was associated with the development of GC-insensitivity in peripheral myeloid cells, in which they were resistant to apoptosis following treatment with high levels of GCs in *ex vivo* cultures. These data indicate that pro-inflammatory effects of chronic stress in humans are recapitulated with RSD.

A relatively unappreciated notion is that many of the pro-inflammatory effects of chronic stress are simply a function of enhanced myelopoiesis. For example, in both humans and rodents, enhanced myelopoiesis during prolonged stress results in selective accumulation of immature monocytes in the periphery (Engler et al., 2004; Wohleb et al., 2011; Heidt et al., 2014) that was directly linked to increased cycling and release of monocytes in the BM (Engler et al., 2004; Hanke et al., 2012; Heidt et al., 2014). The immature monocytes released during stress represent an inflammatory subset that are identified as Ly6C^{hi} in mice and as CD14⁺/CD16[−] in humans (Geissmann et al., 2003; Heidt et al., 2014). They are considered immature because they are functional precursors of the matured and immunoregulatory Ly6C^{lo} or CD14[−] subset (Murray and Wynn, 2011; Yona et al., 2013). Moreover, these cells are termed “pro-inflammatory” because they readily traffic to inflamed tissue and have robust capacity to secrete pro-inflammatory cytokines once they enter tissue and become effector cells (Serbina and Pamer, 2006). The immature nature of these monocytes is pertinent as it may also account for the development of GC-insensitivity following prolonged stress. For example, immature BM monocytes are functionally GC-insensitive (Fitting et al., 2004; Engler et al., 2005). Thus, accumulation of these innately GC-insensitive immature monocytes corresponds with the development of GC-insensitivity during chronic stress. In support of these points, blockade of stress-induced myelopoiesis by β -adrenergic antagonism prevented accumulation of Ly6C^{hi} monocytes (Hanke et al., 2012; Powell et al., 2013; Heidt et al., 2014), and this was associated with prevention of GC-insensitivity (Hanke et al., 2012) and reversal of pro-inflammatory transcriptional profiles following RSD (Powell et al., 2013). However, priming of splenic macrophages during RSD involves additional priming events associated with TLR ligation (Bailey et al., 2006, 2007, 2011). Taken together, SNS-dependent enhancement of myelopoiesis underlies peripheral inflammation following chronic stress via the accumulation of innately pro-inflammatory and GC-insensitive immature monocytes.

Another key characteristic of the pro-inflammatory monocyte phenotype associated with prolonged stress is their enhanced capacity to traffic and promote pro-inflammatory signaling throughout the body. For example, several studies using RSD demonstrated increased trafficking of Ly6C^{hi} monocytes to peripheral tissues that was associated with exaggerated cytokine responses (Wohleb et al., 2011, 2012; Hanke et al., 2012) and the exacerbation of inflammatory conditions (Bailey et al., 2009a,b; Curry et al., 2010; Dong-Newsom et al., 2010; Mays et al., 2010; Tarr et al., 2012). This is similar to work in the CVS paradigm, where prolonged stress increased trafficking of Ly6C^{hi} inflammatory monocytes that promoted inflammation and exacerbated pathology of vascular plaques of ApoE^{−/−} mice (Heidt et al., 2014). Thus, prolonged stress exposure substantially increased circulating Ly6C^{hi} monocytes that trafficked to tissue, promoted inflammation, and exacerbated pathology. It is important to note that peripheral inflammation and exacerbated pathology were reversible by the blockade of SNS-enhancement of myelopoiesis. For example, β_3 -adrenergic receptor antagonism attenuated monocyte trafficking and prevented the exacerbation

of vascular plaques in ApoE^{-/-} mice exposed to CVS (Heidt et al., 2014). Similarly, propranolol prevented RSD-induced monocyte accumulation in BM, circulation, spleen, and brain that corresponded with reduced pro-inflammatory cytokine production in these tissues (Wohleb et al., 2011; Hanke et al., 2012). As summarized in **Figure 3**, increased trafficking of inflammatory monocytes following prolonged stress-exposure is due to SNS-mediated enhancement of myelopoiesis that results in the release of immature monocytes from the BM. These points are relevant, because it was recently demonstrated that stress causes these inflammatory monocytes to traffic and accumulate in the brain.

INFLAMMATORY MONOCYTES RELEASED FROM THE BONE MARROW TRAFFIC TO THE BRAIN

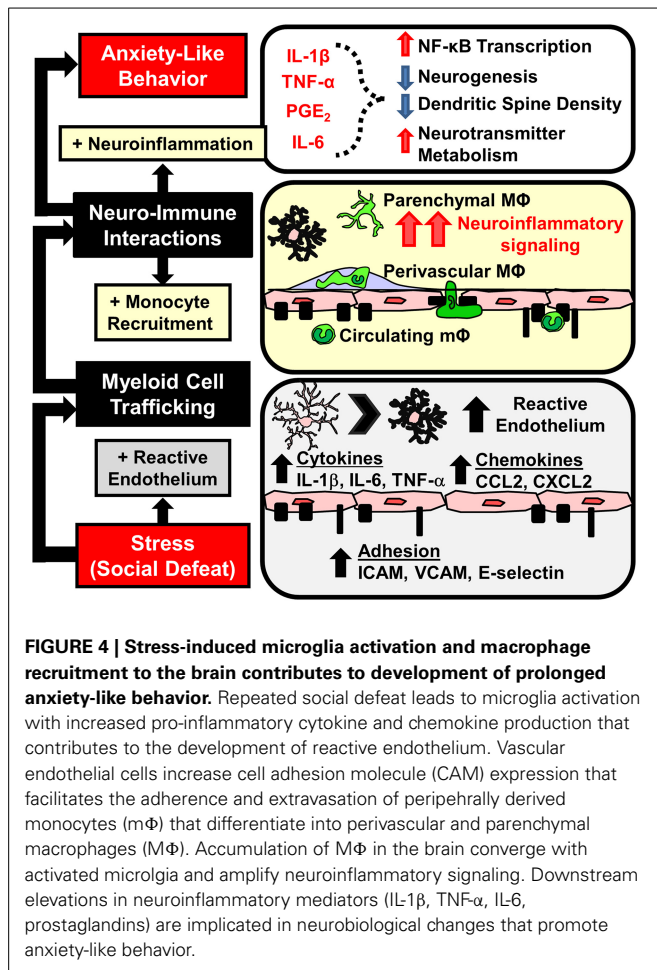
It is well-described in models of trauma, neurological disease, or infection that inflammatory BM-derived monocytes traffic to and are recruited into inflamed tissue, including the brain and spinal cord (Donnelly and Popovich, 2008; Kigerl et al., 2009; McGavern and Kang, 2011). Recent evidence also indicates that there is significant trafficking and recruitment of peripherally derived monocytes to the brain with psychological stress (Brevet et al., 2010; Wohleb et al., 2011; Ataka et al., 2013; Wohleb et al., 2013, 2014a; Sawada et al., 2014). In these studies, monocytes traffic to the brain and differentiate into brain macrophages that promote inflammatory signaling. These reports are somewhat surprising because they indicate that monocyte trafficking to the brain occurs in the absence of overt tissue pathology or injury. Nonetheless trafficking of monocytes with stress is important because their accumulation in the brain can influence neuroinflammatory signaling and behavior (Terrando et al., 2011; Wohleb et al., 2011, 2013; Beumer et al., 2012; D'Mello et al., 2013; Degos et al., 2013; Sawada et al., 2014). In addition, increased presence of vascular-associated macrophages in the brain was recently implicated in depression. In this study, depressed individuals who committed suicide had increased perivascular Iba-1 immunolabeling compared to non-depressed controls (Torres-Platas et al., 2014). Thus, the trafficking of BM-derived monocytes to the brain with psychological stress represents a cellular pathway in which the immune system communicates back to the brain to regulate behavior.

BM-derived macrophages comprise an integral component of the innate immune system in the brain. These immunosurveillance brain macrophages reside within the perivascular space, meninges, and choroid plexus, and represent a functionally distinct population in the brain separate from the resident microglia. These peripherally-derived macrophages (CD11b⁺/CD45^{hi}/Iba-1^{-/lo}) are steadily renewed every 3–4 weeks (Hickey and Kimura, 1988; Bechmann et al., 2001). Although brain macrophages comprise a small portion of resident immune cells, their proximity to vascular and parenchymal cells in the brain makes them an important transducer of peripheral immune signals to the brain (Serrats et al., 2010). Moreover, brain macrophages are significantly more efficient at antigen presentation and elicit more robust pro-inflammatory responses relative to microglia (Hickey and Kimura, 1988; Walker, 1999; Galea et al., 2007). The relative rarity of these brain macrophages

is important because recent studies show that inflammatory conditions profoundly increased the presence of macrophages associated with the brain. Thus, it is plausible that accumulation of primed, GC-insensitive macrophages in the brain significantly influences neuroinflammatory signaling in response to stress.

In support of this idea, recent studies show that RSD increased the presence of peripherally derived macrophages in the brain. For example, RSD caused a two to three fold increase in the number of CD11b⁺/CD45^{hi} cells in the brain (Wohleb et al., 2011, 2013, 2014a), which were not resident microglia (Sedgwick et al., 1991; Ford et al., 1995). Additionally, these cells were Ly6C^{hi} and had scatter properties consistent with trafficking monocyte/macrophages (Wohleb et al., 2012). Based on these profiles, these cells were referred to as brain macrophages. The RSD-induced increases in brain macrophages persisted after perfusion of the vasculature (Wohleb et al., 2012) and occurred despite no disruption in the blood-brain barrier (BBB) (Wohleb et al., 2013). In addition, studies using transgenic LysM-GFP⁺ mice confirmed that CD11b⁺/CD45^{hi} brain macrophages were derived from peripheral myeloid cells. Notably, LysM is expressed on monocytes and macrophages but not on resident microglia (Faust et al., 2000; Kim et al., 2009). Further histological analysis revealed that RSD increased rod/circular LysM-GFP⁺ macrophages within the perivascular regions of the brain (Wohleb et al., 2013). While these LysM-GFP⁺ macrophages were apparent around the vasculature, LysM expression appeared to be down-regulated as the cells reached the parenchyma of the brain. Thus, parenchymal infiltration could not be assessed using these mice. Taken together, initial studies using flow cytometric markers and LysM-GFP expression revealed that RSD increased macrophages in the brain.

BM-chimera mice were used to further enable characterization of BM-derived macrophages in the brain. In these studies, host BM was ablated using low doses of busulfan and was then reconstituted with donor BM from mice that expressed green fluorescent protein (GFP) under a ubiquitous promoter. Thus, in these BM chimera mice, BM-derived immune cells would express GFP, while host-derived microglia would lack GFP expression. It is important to note that the low dose busulfan regimen used in these studies was not associated with trafficking of cells into the brain of control mice (Kierdorf et al., 2013; Wohleb et al., 2013). These studies confirmed the increased presence of BM-derived myeloid cells (GFP⁺/CD11b⁺) in the brain after RSD. The GFP⁺/CD11b⁺ cells in the brain after RSD were both CD45^{hi} and CD45^{lo} consistent, respectively, with perivascular and parenchymal localization (Mildner et al., 2007). Indeed, immunohistochemical studies detected GFP⁺ macrophages in both parenchymal and perivascular compartments of the brain. GFP⁺ macrophages persisted in the brain for at least 8 days after the cessation of RSD and were no longer present 24 days later (Wohleb et al., 2013, 2014a). Thus, studies using WT, LysM-GFP⁺, and BM chimera mice all detected macrophage accumulation in the brain following RSD. Overall, these data were interpreted to indicate that RSD increased the trafficking of BM-derived monocytes to the brain that differentiated into perivascular and parenchymal macrophages (**Figure 4**).



An important aspect of these studies with RSD was the selective infiltration of BM-derived monocytes into the brain parenchyma of stress-responsive regions (Wohleb et al., 2013). Notably, GFP⁺ macrophages accumulated in the same threat appraisal areas that had increased c-Fos and Iba-1 activation after RSD (Wohleb et al., 2011). Increased ramified GFP⁺ cells were specifically observed in brain regions associated with fear, anxiety, and threat appraisal, including the PFC, HYPO, AMYG, and CA3 and DG of the HPC, but were not observed in other brain regions like the motor cortex, striatum, somatosensory cortex, or cerebellum (Wohleb et al., 2011, 2013). Moreover, monocytes that infiltrated the parenchyma differentiated into ramified and Iba-1⁺ macrophages. In addition, infiltrating cells reduced expression of typical macrophage markers (e.g., CCR2, Ly6C, CD45, and LysM). Similar to prior reports, infiltrating macrophages had a microglia-like appearance, but had a less ramified morphology compared to resident microglia (Varvel et al., 2012; Elmore et al., 2014). Another point of interest was that similar to microglia activation (Wohleb et al., 2011), increases in parenchymal GFP⁺ macrophages were not observed until after 6 cycles of RSD (Wohleb et al., 2013). Thus, there was an exposure-dependent increase in brain macrophages after RSD. Overall, in the absence of BBB permeability, RSD caused region-specific infiltration of

monocytes into brain regions associated with fear, anxiety, and threat appraisal.

The trafficking and recruitment of these monocytes with stress were also dependent on key chemokine receptors. For example, Wohleb et al. (2013) showed that CCR2^{KO} and CX3CR1^{KO} mice did not exhibit increased brain macrophages following RSD. Notably, RSD caused the release of myeloid cells into circulation independent of CCR2 or CX3CR1 expression, but neither CCR2^{KO} nor CX3CR1^{KO} mice had increased brain macrophages after RSD. These results indicate that stress-associated monocyte release alone was insufficient to gain access to the brain. In support of these findings, wild-type BM-chimera mice reconstituted with CCR2^{KO} or CX3CR1^{KO} progenitor cells also showed reduced brain macrophage trafficking following RSD. In addition, RSD increased CCL2 expression in the brain (Wohleb et al., 2013). These data indicate that monocytes were actively recruited to the brain after RSD using dynamic chemokine receptor interactions. The necessity for both CCR2 and CX3CR1 is supported in other models. For instance, infiltrating monocytes expressed CCR2 and CX3CR1 in the brain of experimental autoimmune encephalomyelitis and herpes-infected mice (Saederup et al., 2010; Boivin et al., 2012). Taken together, recruitment of monocytes to the brain with stress was prevented by deletion of key chemokine receptors, CCR2 and CX3CR1.

The notion that psychological stress increased the presence of BM-derived macrophages in the brain is supported by other studies. For example, BM-derived macrophages were detected in the ventral HPC in mice after five days of footshock stress (Brevet et al., 2010). These studies used a GFP⁺ BM-chimera generated by radiation in which the head was shielded. Consistent with studies using RSD, GFP⁺ cells showed a de-ramified, microglia-like morphological phenotype (Brevet et al., 2010). A similar set of studies in mice showed that simply observing cage mates undergoing foot shock was sufficient to cause influx of peripherally derived macrophages into brain parenchyma (Ataka et al., 2013). In these studies, both CCR2 and β-adrenergic receptor signaling contributed to the trafficking of BM-derived monocytes to the brain (Brevet et al., 2010; Ataka et al., 2013). In a more recent chronic neuropathic pain study, partial sciatic nerve ligation induced infiltration of BM-derived monocytes into the AMYG (Sawada et al., 2014), and this was blocked by oral administration of either a CCR2 antagonist or microinjections of IL-1 receptor antagonist. Thus, there is evidence for stress-induced brain-macrophage trafficking with several stress paradigms.

A relevant point to highlight is that infiltrating BM-derived monocytes/macrophages may not be identifiable with conventional lineage-specific markers (i.e., CD45, Ly6C, CD163, CCR2, LysM). This is because micro-environmental cues from the brain parenchyma cause macrophages to resemble microglia in both morphology and marker expression (Mildner et al., 2007). Numerous studies have reported that stress increased Iba-1⁺ cell numbers in stress-responsive brain regions (Frank et al., 2007b; Tynan et al., 2010; Bian et al., 2012; Kreisel et al., 2014). These data are often interpreted as microglia proliferation, but the increase in Iba-1⁺ cells may also be caused by influx of BM-derived macrophages in the brain. Thus, it is important to distinguish the role of resident microglia and

infiltrating monocytes when studying stress and neuroinflammatory signaling. Collectively, several lines of evidence reveal that stress causes the recruitment and accumulation of BM-derived macrophages in the brain that involve chemokine signaling.

NEUROVASCULAR DYNAMICS FACILITATE STRESS-INDUCED MONOCYTE TRAFFICKING

Previous studies demonstrate that exposure to psychological stress elicited monocyte trafficking to the brain (Brevet et al., 2010; Wohleb et al., 2011, 2013, 2014a,b; Ataka et al., 2013; Sawada et al., 2014). It was discussed how neuroendocrine outflow from the brain to the immune system promotes the release of myeloid cells from the BM into circulation (Hanke et al., 2012; Heidt et al., 2014) (**Figure 3**). However, the release of inflammatory monocytes from the BM is insufficient to cause their active recruitment to the brain. For example, recruitment of monocytes to the brain following stress required chemokine signaling involving CX₃CR1 and CCR2 (Ataka et al., 2013; Wohleb et al., 2013; Sawada et al., 2014). Moreover, accumulation of macrophages in the brain occurs with chronic peripheral inflammation that is also regulated by similar chemokine receptor and adhesion molecule dynamics (Kerfoot et al., 2006; D'Mello et al., 2009, 2013; Terrando et al., 2011; Degos et al., 2013). Thus, in the absence of frank neuropathology, the recruitment of monocytes into perivascular and parenchymal regions of the brain likely involves dynamic interactions between cells of the neurovascular unit, including endothelial cells, microglia, and neurons (**Figure 4**). The notion of a neurovascular unit is used to describe micro-dynamic interactions between the vasculature and local parenchymal cells, such as microglia and neurons (Stanimirovic and Friedman, 2012). This notion is appreciated in models of neurological diseases with inflammatory components, where neurovascular cells facilitate macrophage trafficking that can regulate disease progression (Prinz and Priller, 2010, 2014; Stanimirovic and Friedman, 2012). Here it is important to note that RSD promotes microglia activation and neuroinflammatory signaling that is independent of monocyte-trafficking. For example, deletion of CCR2 and CX₃CR1 prevented stress-induced monocyte trafficking to the brain, but it did not prevent increased cytokine mRNA expression following RSD (Wohleb et al., 2013). It is also important to note that macrophage influx was observed within the same stress responsive regions where RSD caused increased neuronal c-Fos expression and altered microglia morphology (Wohleb et al., 2011, 2013). Related to this, recent evidence indicates that expression of key immune cell adhesion molecules (CAMs) occurs in specific brain regions after stress. For instance, ICAM-1 and VCAM-1, which are integral to monocyte-vasculature adhesion, were both increased on endothelial cells in the PFC and PVN following RSD (Sawicki et al., 2014). Similar to the patterns of macrophage trafficking (Wohleb et al., 2013), increased ICAM-1 and VCAM-1 regionally correlated with previous reports of neuronal and microglia activation within specific stress-responsive brain regions. These findings are consistent with the notion that neuronal-microglial-endothelial cross talk results in regions specific facilitation of macrophage trafficking via neurovascular CAM and chemokine

expression (**Figure 4**). These dynamics are important because the presence of these monocytes/macrophages is implicated in pathogenesis of mental health disorders (Beumer et al., 2012; Torres-Platas et al., 2014). Thus, a key question is: to what degree does influx of monocytes influence neurocircuitry and behavior?

MONOCYTE TRAFFICKING TO THE BRAIN INFLUENCES BEHAVIOR

Several clinical and pre-clinical studies indicate that stress promotes the onset of anxiety- and depressive-like behaviors (Kendler et al., 1999; McLaughlin et al., 2010; Gilman et al., 2013). In the RSD model, anxiety-like behavior in the open field and light-dark preference tests developed after RSD (3–6 cycles) and persisted for at least 8 days (Wohleb et al., 2013, 2014a). It is important to note that behavioral responses to RSD are determined hours, days, and weeks after the cessation of the stressor. Thus, the measured behaviors are uncoupled from the transient induction of neuronal and endocrine arousal responses (Kinsey et al., 2007; Hanke et al., 2012). As previously outlined, studies with RSD indicate that inflammatory monocytes are released into circulation and they traffic to the brain. In this section, we will discuss the evidence that myeloid trafficking to the brain is necessary for the development of prolonged anxiety-like behavior following RSD (**Figure 4**). This section will review additional reports from other models that monocyte-trafficking is an important regulator of behavior.

With RSD, there are temporal relationships between the development, resolution, and recurrence of stress-induced anxiety and neuroimmune signaling. For example, anxiety, pro-inflammatory cytokine production in the brain, and brain macrophage accumulation were all observed in an exposure dependent manner after RSD. All parameters were moderately increased after 3 cycles and peaked after 6 cycles of social defeat (Wohleb et al., 2013). Similar to this, the resolution of anxiety and brain monocyte trafficking was also correlated. For instance, both anxiety-like behavior and increased brain macrophages persisted for at least 8 days after RSD and both of these parameters were resolved by 24 days later (Wohleb et al., 2014a). It should be noted, however, that social avoidance to an aggressor mouse developed after 1 cycle of social defeat and was maintained 24 days later (Wohleb et al., 2014a). Based on the timing and the lack of macrophages in the brain at these early and late time points, social avoidance occurs independent of monocyte trafficking to the brain. Overall, the development, maintenance, and resolution of anxiety, neuroinflammatory signaling, and brain monocyte trafficking in RSD were temporally correlated.

A key aspect to determining the cause and effect relationship between anxiety-like behavior and brain-monocyte trafficking was experimental interventions that prevented monocytes from trafficking to the brain. For example, initial studies used interventions that prevented release of monocytes from the BM (Engler et al., 2008; Wohleb et al., 2011), and this corresponded with blockade of both anxiety-like behavior and monocyte trafficking to the brain. For example, pretreatment with propranolol prevented the release and trafficking of monocytes from the BM to the brain (Wohleb et al., 2011), and this was associated with

the absence of the stress-induced anxiety-like behavior in treated mice. Moreover, IL-1 receptor type-1 (IL-1R1)-knockout mice do not exhibit stress-induced brain-monocyte trafficking, and this was also associated with the absence of anxiety-like behavior (Wohleb et al., 2011, 2014b). These initial data were circumstantial in that they reveal strong correspondence between trafficking of monocytes to the brain and the development of anxiety-like behavior. In addition, subsequent studies revealed compelling evidence that direct interference of monocyte trafficking to the brain prevented anxiety-like behavior in RSD exposed mice.

To specifically interfere with monocyte trafficking without affecting stress-interpretation or monocyte priming, Wohleb et al. (2013) used transgenic mice deficient in key monocyte chemokine receptors. This study showed that both CCR2 and CX₃CR1 expression were required for RSD-induced trafficking of monocytes to the brain. For example, both CCR2^{KO} mice and CX₃CR1^{KO} mice had stress-induced changes in the circulating Ly6C^{hi} monocytes but neither had increased macrophages in the brain (Wohleb et al., 2013). It is important to note that chemokine deficiency was associated with blockade of stress-induced accumulation of both CD45^{hi} perivascular macrophages and GFP⁺ parenchymal macrophages, as studied in both naïve and BM-chimeric mice (Wohleb et al., 2013). In these examples, when macrophages were not able to reach the brain, mice did not exhibit anxiety-like behavior 14 h after RSD. Notably, prevention of brain-monocyte trafficking in knock-out mice was not associated with attenuation of increased IL-1 β mRNA expression. These results suggest that intrinsic neuroinflammatory signaling is not sufficient to promote extended anxiety-like responses. Thus, the initial interpretation of these data is that brain-monocyte trafficking synergistically promotes the development of prolonged anxiety-like behavior following RSD.

A more generalized interpretation of these data is that trafficking of inflammatory monocytes to the brain is an independent axis of immune-to-brain signaling that regulates mood and behavior. In fact, there is additional evidence from other models to support this interpretation. For example, in a model of neuropathic pain, trafficking of macrophages to the amygdala promoted the development of anxiety-like behavior (Sawada et al., 2014). In this study, blockade of macrophage influx in the amygdala by CCR2 and IL-1R1 inhibition prevented pain-induced anxiety-like behavior. Additionally, the notion that monocyte trafficking to the brain influences behaviors has been reported in several models of peripheral inflammation. For example, experimental liver inflammation and experimental colitis both promote monocyte trafficking to the brain that is dependent upon CCL2/CCR2 signaling, tumor necrosis factor receptor expression, and P-selectin (Kerfoot et al., 2006; D'Mello et al., 2009, 2013). In these studies, blockade of brain-monocyte trafficking prevented inflammation-induced sickness behaviors. Moreover, brain monocyte trafficking promoted the development of cognitive decline following post-operative recovery from peripheral surgery (Terrando et al., 2011; Degos et al., 2013). These studies showed that general surgery caused accumulation of ramified CCR2⁺ macrophages in the hippocampus. Macrophage accumulation was prevented by depletion of peripheral phagocytes by injection of clodronate-loaded liposomes (Degos et al., 2013)

and by inhibition of peripheral macrophage NF κ B signaling (Terrando et al., 2011). Prevention of macrophage accumulation corresponded with prevention of postoperative cognitive decline (Terrando et al., 2011; Degos et al., 2013). Taken together, there is mounting evidence that trafficking of inflammatory monocytes to the brain with stress and peripheral inflammation promotes negative behavioral outcomes, such as anxiety, sickness, and cognitive decline. As illustrated in **Figure 1**, these data support the notion that monocyte trafficking to the brain is a key axis in immune-to-brain signaling that influences mood and behavior.

PROPAGATION AND CONVERGENCE OF NEUROIMMUNE SIGNALING IS MEDIATED BY ENDOTHELIAL IL-1R1 EXPRESSION

Related to previously discussed points, interactions at the neurovascular interface are critical for propagation of neuroinflammatory signaling related to RSD-induced monocyte trafficking to the brain. Classical neuroimmune communication studies showed that transduction of pro-inflammatory cytokines across the BBB and through circumventricular organs leads to microglia activation. Following activation by cytokines, microglia then produce secondary signals that can directly influence neuronal pathways (Quan and Banks, 2007). This is relevant in the context of RSD because peripherally-derived monocytes/macrophages potentiate neuroinflammatory signaling in proximity to the neurovascular interface. Indeed recent findings reveal that peripheral IL-1 β is an important modulator of RSD-induced neuroinflammation and anxiety. For instance, development of primed myeloid cells, macrophage trafficking in the brain, and altered microglia morphology are prevented in IL-1R1^{KO} mice after RSD (Engler et al., 2008; Wohleb et al., 2011, 2014b). The lack of stress-associated neuroinflammation coincided with decreased anxiety-like behavior in IL-1R1^{KO} mice after RSD. Because microglia do not respond robustly to direct IL-1 stimulation (An et al., 2011), it is likely that another cellular intermediate such as vascular endothelial cells are necessary to propagate peripheral inflammatory signals. Thus, propagation of myeloid-derived signals into the brain requires complex signaling events at the neurovascular interface.

Recent evidence using novel transgenic tools with RSD revealed that endothelial IL-1R1 expression plays a critical role in the propagation and convergence of neuroimmune signaling. In these studies mice with selective knockdown of IL-1R1 on endothelial cells (eIL-1R1KD) (Li et al., 2012) had reduced neuroinflammatory gene expression and decreased anxiety-like behavior after RSD (Wohleb et al., 2014b). Contrary to findings with the ubiquitous IL-1R1^{KO} mice, eIL-1R1KD mice developed primed monocytes in circulation that successfully trafficked to the brain following RSD. These data indicate that peripheral myeloid cells recruited to the brain with RSD communicate with the endothelial cells using IL-1. Moreover, unlike IL-1R1^{KO} mice, eIL-1R1KD mice exhibited microglia activation following RSD (Wohleb et al., 2014b). Thus, IL-1 signaling was not necessary for stress-induced alterations in microglia morphology. Despite this, eIL-1R1KD reduced cytokine mRNA expression in microglia isolated from RSD-exposed mice. These data were interpreted to indicate that stress-induced neuroinflammatory

signaling originated from both microglia and macrophages and was propagated by endothelial cells. Therefore, convergent signals from microglia, neurovascular endothelial cells, and monocytes are critical in immune-to-brain signaling that promotes anxiety-like behavior following RSD (**Figure 4**).

REPEATED STRESS EXPOSURE LEADS TO NEUROIMMUNE SENSITIZATION AND SUSCEPTIBILITY TO RECURRENT ANXIETY-LIKE BEHAVIOR

An important clinical component of stress research is the duration that stress-related behavioral adaptations persist. Findings from animal models revealed that microglia activation and trafficking of monocytes to the brain contribute to behavioral adaptations to stress (Wohleb et al., 2011, 2013; Hinwood et al., 2012; Kreisel et al., 2014; Sawada et al., 2014), but the role of neuroimmune signaling in long-term and recurrent stress-related behavioral disorders had not been addressed. For example, various chronic stressors cause neuronal atrophy and deficits in HPA activity that are implicated in long-lasting anxiety- or depressive-like behavioral changes (Vyas et al., 2003; Schmidt et al., 2007; Mizoguchi et al., 2008; Philbert et al., 2011), but the contribution of neuroimmune signaling was not considered. In fact, many of these neuronal and neuroendocrine alterations can be caused by pro-inflammatory cytokines and microglia activation. For example, pro-inflammatory transcriptional activity associated with microglia activation reduced neurogenesis in the hippocampus (Koo and Duman, 2008, 2009) and was implicated in reduced synaptic protein expression in the PFC (Kang et al., 2012). Thus, persistent neuroimmune sensitization may promote long-term behavioral and neurobiological adaptations to stress.

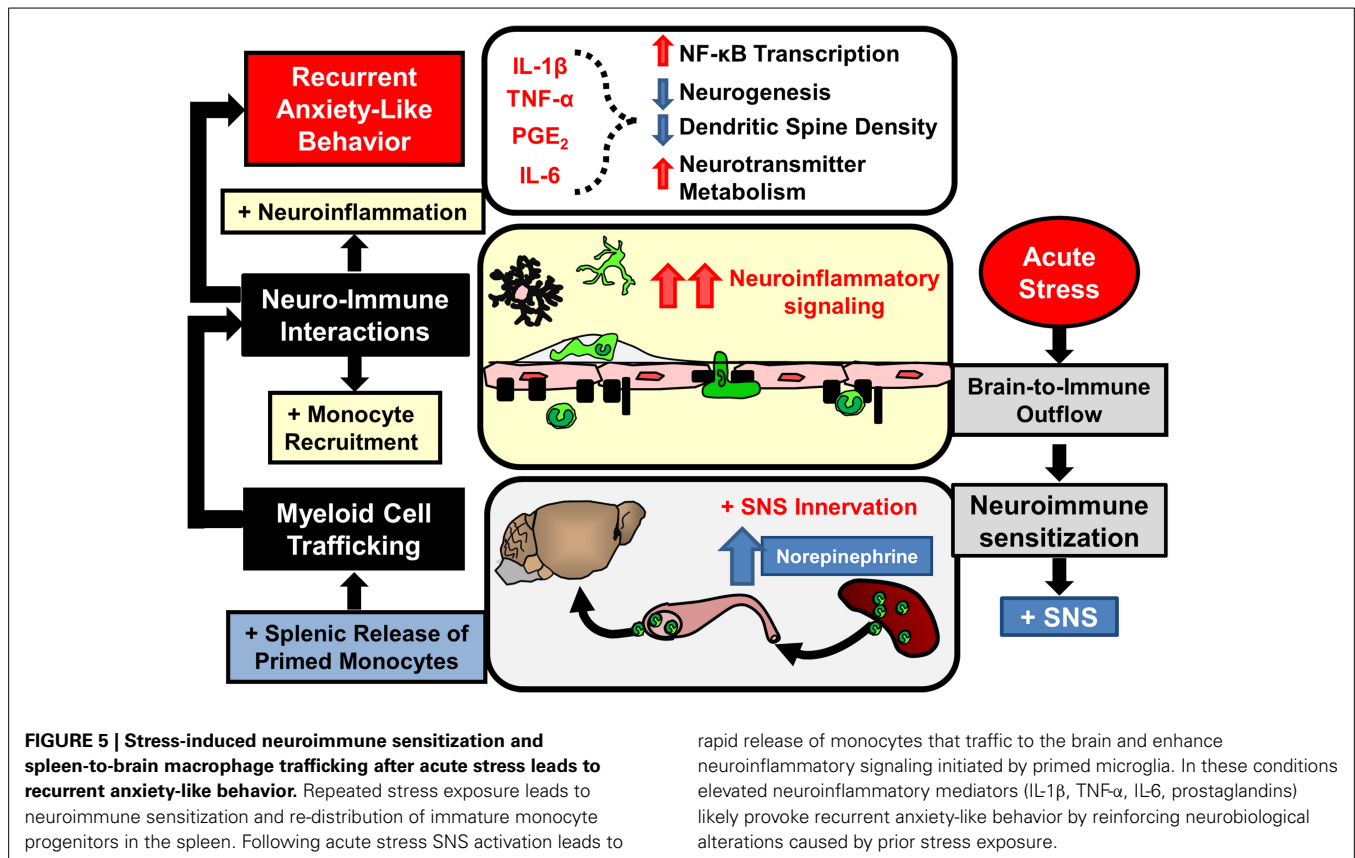
Similar to previously discussed reports, work in the RSD model revealed long-lasting changes in behavior, but in this case, brain monocytes and neuroimmune signaling had a significant role in the maintenance and re-occurrence of stress-induced anxiety-like behavior. For instance, mice exposed to RSD showed elevated pro-inflammatory cytokine expression in enriched microglia and increased macrophage populations in the brain that corresponded with prolonged anxiety-like behavior 8 days after stress cessation (Wohleb et al., 2014a). In addition, macrophages in the brain and microglia pro-inflammatory cytokine expression were no longer detected 24 days after stress, and this coincided with resolution of anxiety-like behavior at this 24 day time point. Despite the resolution of these parameters by 24 days, certain parameters were still persistently altered. This indicates that RSD caused priming or sensitization of neuroimmune responses. For example, enriched microglia showed increased expression of IL-6, CD14, and CX₃CR1 that was associated with altered morphology 24 days after RSD. Moreover, examination of peripheral immune organs showed that RSD caused significant re-distribution of monocyte progenitors in the spleen that persisted for at least 24 days (Wohleb et al., 2014a).

Recent findings indicate that RSD caused prolonged neuroimmune alterations that persist after stress contribute to exaggerated neuroinflammatory responses following acute stress at later time points. Indeed, RSD-exposed mice remained sensitized to subsequent stress exposure 24 days after RSD (Wohleb et al., 2014a). For example, at this 24 day time point, re-exposure to a single

cycle of social defeat caused the re-establishment of anxiety-like behavior and brain-monocyte trafficking. It should be noted that the single cycle of social defeat had no detectable effect on naïve mice, but caused the re-establishment of monocyte trafficking and anxiety in RSD-sensitized mice. Thus, one cycle of social defeat was considered to be a sub-threshold stressor. Moreover, because previous exposure to RSD caused mice to have an exaggerated response to a sub-threshold stress, RSD-exposed mice were termed to be “stress-sensitized” (Wohleb et al., 2014a). Collectively, these data showed strong kinetic relationships between the maintenance and recurrence of anxiety and brain monocyte trafficking. Furthermore, these kinetic relationships provide strong evidence that brain monocyte-trafficking re-established anxiety-like behavior in stress-sensitized mice.

Notably, increased monocyte re-distribution following sub-threshold stress in sensitized mice was not associated with altered monocyte production or egress from the BM. In contrast, myeloid cell progenitors were increased in the spleen following RSD and sub-threshold stress caused release of monocytes from this splenic monocyte reservoir (Wohleb et al., 2014a). Other studies in models of myocardial infarction (Swirski et al., 2009), atherosclerosis (Dutta et al., 2012), and stroke (Seifert et al., 2012) revealed that the spleen acts as an important source of monocytes during inflammatory conditions. The role of spleen-to-brain monocyte trafficking in stress-sensitized mice was substantiated by the fact that splenectomy prevented brain monocyte trafficking and prevented re-establishment of anxiety-like behavior following sub-threshold stress (Wohleb et al., 2014a). As depicted in **Figure 5**, these results indicated that prior exposure to RSD caused primed monocytes to persist in the spleen for 24 days that subsequently trafficked to the brain and promoted the reoccurrence of anxiety following exposure to sub-threshold stress. In addition, pro-inflammatory cytokine levels in isolated microglia were augmented after sub-threshold stress in stress-sensitized mice and this response was attenuated in splenectomized mice. Thus, as illustrated in **Figure 5**, this study revealed that spleen-to-brain monocyte trafficking played a prominent role in the re-establishment of anxiety-like behavior and neuroinflammatory signaling in stress-sensitized mice (Wohleb et al., 2014a).

The mechanisms driving neuroimmune sensitization in the spleen following RSD have not yet been determined. Nevertheless, studies involving spleen-monocyte trafficking in other contexts provide some insight into these phenomena. For example, there are a couple of groups that have reported on the idea of spleen-monocyte trafficking in the context of cardiovascular disease and stroke (Swirski et al., 2009; Leuschner et al., 2012; Seifert et al., 2012). In particular, recent work revealed that spleen-monocyte trafficking was implicated in the acceleration of atherosclerosis in ApoE^{-/-} mice following myocardial infarction (Dutta et al., 2012). Myocardial infarction transiently increased the release of myeloid progenitors from the BM that seeded the spleen and contributed to extramedullary production of inflammatory monocytes that persistently trafficked to vascular lesions and promoted the progression of vascular lesions. This is a relevant finding because RSD also caused the release of immature and progenitor-like monocytes that seeded the spleen (Hanke et al., 2012; Wohleb et al., 2014a). In fact, there is evidence



that RSD caused increased extramedullary monocytopoiesis in the spleen that persists in stress-sensitized mice. For example, increased CD11b⁺/Ly6C⁺/CD34⁺ monocytes were observed in the spleen following RSD that persisted in stress-sensitized mice for up to 24 days (Wohleb et al., 2014a). Thus, chronic induction of splenic monocytopoiesis may contribute to enhancement of spleen-monocyte trafficking in stress-sensitized mice.

There are several reports relating to the regulation of splenic monocyte egress that may be relevant to stress-sensitization. For example, following myocardial infarction, angiotensin II signaling contributes to the release of inflammatory Ly6C^{hi}/CCR2⁺ monocytes that rapidly traffic to the lesion site and significantly contribute to pathology (Swirski et al., 2009). In these studies, deletion or inhibition of angiotensin II type 1a receptor prevented spleen monocyte trafficking and attenuated myocardial infarct volume. Moreover, angiotensin II administration independently caused monocyte egress from the spleen. This is relevant to stress-sensitization, because both acute and chronic stress increase angiotensin II in circulation (Jezova et al., 1998; Saavedra et al., 2005; Saavedra and Benicky, 2007). Similar to these reports, the role of spleen-to-brain monocyte trafficking in the exacerbation of stroke has also been reported on Seifert et al. (2012). In this report, cerebral artery occlusion caused monocyte trafficking from the spleen to the brain that augmented infarct size (Ajmo et al., 2008). For example, splenectomy prior to cerebral artery occlusion decreased infarct volume by about 80%. In these studies, egress of splenocytes is dependent upon peripheral

noradrenergic signaling (Ajmo et al., 2009). This is particularly relevant because one cycle of RSD (i.e., sub-threshold stress) increased norepinephrine in both circulation and in the spleen (Hanke et al., 2012). Thus, these reports implicate noradrenergic and angiotensin II signaling in the release and trafficking of splenic monocytes in stress-sensitized mice.

Based on these reports, norepinephrine and angiotensin II are key regulators of spleen monocyte trafficking, yet it is unclear why sub-threshold stress only caused splenic monocyte trafficking in stress-sensitized mice and not naïve mice. One possibility is that stress-sensitization alters the sensitivity of spleen monocytes to egress-related signals (e.g., angiotensin II or norepinephrine). In fact, there is evidence for this. Stress-sensitized mice exhibited increased monocyte progenitors in the spleen (Wohleb et al., 2014a), and in the context of cardiovascular disease, splenic monocyte progenitors have a higher propensity for egress and trafficking than the pool of monocytes available under homeostatic conditions (Dutta et al., 2012). An alternative possibility is that exaggerated monocyte trafficking in stress-sensitized mice occurs as a function of the magnitude of sympathetic response to the acute stress. Despite these possibilities, the underlying mechanisms of splenic sensitization following RSD are not fully understood.

One last point of discussion is the clinical relevance of RSD as a model of stress-sensitization and recurring anxiety. Although RSD-sensitization is not considered a model of post-traumatic stress disorder (PTSD), it can be argued that studying

stress-sensitization within the RSD paradigm may have some basic relevance to this distinctly human condition. For instance, both RSD and PTSD involve a sensitizing event that is often both physical and psychological in nature (Bailey et al., 2004; American Psychiatric Association, 2013; Freeman et al., 2013). Similar to RSD, the sensitizing event or trauma predisposes the individual to recapitulate the behavioral and physiological responses following exposure to either generalized cues or subsequent stressful events (American Psychiatric Association, 2013; Wohleb et al., 2014a). Furthermore, both PTSD and RSD involve chronic maintenance of psychosocial deficits (American Psychiatric Association, 2013; Wohleb et al., 2014a). Despite these commonalities, PTSD remains a complex and distinctly human disorder that is unlikely to be fully modeled by RSD-sensitization in mice. However, mechanisms contributing to stress-sensitization following 6 cycles of RSD may be relevant to the biological events associated with PTSD in humans. In fact, it is increasingly evident that neuroimmune signaling contributes to the development and maintenance of PTSD and other chronic anxiety disorders. As reviewed by Pace and Heim (2012), there are strong clinical associations between recurring anxiety and inflammatory signaling, and these same associations are recapitulated in the RSD model. Taken together, evidence in the RSD-sensitization model, indicates that spleen-to-brain monocytes trafficking may be relevant to disorders involving recurrent anxiety.

SUMMARY

Exposure to chronic psychological stress promotes brain-to-immune and immune-to-brain communication that directly influences neurobiology and behavior (Figure 1). This review highlighted studies that show psychological stress promotes simultaneous activation of microglia and peripheral monocytes that directly influenced behavioral responses to stress. These studies revealed that noradrenergic signaling in the brain contributed to microglia activation within threat appraisal regions (Figure 2). Next, sympathetic outflow to the periphery enhanced the production and release of inflammatory monocytes from the bone marrow that trafficked throughout the body (Figure 3). Furthermore, we reviewed studies that show both stress and peripheral inflammation promote the accumulation of monocytes and macrophages in the brain. These studies revealed that brain monocyte trafficking involves dynamic interactions between cells of the neurovascular unit. These cells produce cytokines, chemokines, and cell adhesion molecules that facilitate accumulation of macrophages in the brain (Figure 4). Signals from BM-derived macrophages and activated microglia converge within the brain to promote neuroinflammatory signaling leading to the development of prolonged anxiety-like behavior (Figure 4). Moreover, this review covered recent evidence that spleen-to-brain monocyte trafficking contributes to stress-sensitization and recurring anxiety (Figure 5). Taken together, these studies reveal a novel axis of immune-to-brain communication involving monocytes trafficking to the brain that influences mood and behavior.

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Inflammasomes in neuroinflammation and changes in brain function: a focused review

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Recent literature has pointed to the existence of inflammasome-mediated inflammatory pathways in central nervous system (CNS) disorders and associated changes in behavior. Neuroinflammation, which is an innate immune response in the CNS against harmful and irritable stimuli such as pathogens and metabolic toxic waste, as well as to chronic mild stress, is mediated by protein complexes known as inflammasomes. Inflammasomes activate pro-inflammatory caspases 1 and 5, which then cleave the precursor forms of pro-inflammatory cytokines IL-1 β , IL-18, and IL-33 into their active forms. These pro-inflammatory cytokines have been shown to promote a variety of innate immune processes associated with infection, inflammation, and autoimmunity, and thereby play an instrumental role in the instigation of neuroinflammation during old age and subsequent occurrence of neurodegenerative diseases, cognitive impairment, and dementia. In particular, NLRP inflammasomes may also have a role in the etiologies of depression, Alzheimer's disease (AD) and in metabolic disorders, such as Type II diabetes, obesity and cardiovascular diseases that have been shown to be co-morbid with psychiatric illnesses. It has been reported that while these inflammasomes may be activated through TNF- α dependent pathways, other cytokines, like IFN- γ , may assist in inhibiting their activation and thus delay disease progression. Furthermore, some other cytokines, including IL-6, may not have a direct role in inflammasome-mediated diseases. An array of recent research suggests that NLRP inflammasomes targeted therapies could be used for alleviating neuroinflammation and for treatment of associated psychiatric illnesses, although this still remains a challenge and necessitates further extensive research. This review examines the complex inflammatory signaling pathways involved in the activation of NLRP inflammasomes and the role they play in promoting neuroinflammation and subsequent behavioral changes.

Keywords: inflammasomes, NLRP, neuroinflammation, cytokines, IL-1, aging, depression, Alzheimer's disease

INTRODUCTION

The discovery of inflammasomes by Martinon et al. (2002) has prompted considerable interest in the role that inflammasomes play in the mechanism of inflammation and associated disease patterns. Of late, an emerging body of literature points to the existence of inflammasome-mediated inflammatory pathways in central nervous system (CNS) disorders.

Neuroinflammation is a known factor in the pathogenesis of neurodegenerative diseases (Frank-Cannon et al., 2009), and psychiatric illnesses such as depression (Walker et al., 2014), Alzheimer's disease (AD) (Pimplikar, 2014), Parkinson's disease (PD) (Hirsch et al., 2012), Huntington's disease (Möller, 2010), and multiple sclerosis (Frohman et al., 2006). It has also been implicated in sickness behavior (Biesmans et al., 2013), diminished cognition (Ownby, 2010), and memory (Hein and O'Banion, 2009), as well as in age-related increased sensitization of the immune system to extrinsic and intrinsic stimuli (Godbout et al., 2005; Sparkman and Johnson, 2008). Pattern recognition receptors (PRRs) play an integral role in the innate

immune response through recognition of pathogen specific proteins (PAMPs) and damage associated proteins (DAMPs). They are primarily expressed by glial cells, macrophages and oligodendrocytes within the brain and can be membrane bound (toll-like receptors) or within the cytoplasm [Nod-like receptors (NLRs)]. Activation of these NLRs leads to the assembly and activation of cytosolic protein complexes known as inflammasomes which then enable the activation of pro-inflammatory caspases, particularly caspase-1. This then leads to the activation of pro-inflammatory cytokines interleukin (IL)-1 β , IL-18, and IL-33 (Arend et al., 2008; Chakraborty et al., 2010), which promote a number of innate immune processes associated with infection, inflammation and autoimmunity (Davis et al., 2011), thereby responsible for neuroinflammation and associated brain diseases (Tha et al., 2000; Cacquevel et al., 2004; Felderhoff-Mueser et al., 2005; Godbout and Johnson, 2009; Mawhinney et al., 2011; Zhang et al., 2014).

It has been known for some time that immunosenescence, in addition to neurodegenerative changes with age, predisposes

the brain to higher risk of acquiring neuroinflammatory disorders. Considerable findings during the last decade have suggested an instrumental role of inflammasomes in the pathophysiology of neuroinflammation during neuronal ageing, and its associated neurodegenerative diseases such as dementia, leading to loss of memory and cognitive impairment (Simi et al., 2007; Chakraborty et al., 2010; Mawhinney et al., 2011; Liu and Chan, 2014). In particular, NLRP (NLR family, containing pyrin domain) inflammasomes have been shown to have a role in the etiologies of several neurological diseases such as depression (Zhang et al., 2014), AD (Tan et al., 2013), PD (Cedillos, 2013), and multiple sclerosis (Gris et al., 2010; Fischer et al., 2012). Systemically, NLRP inflammasome-driven inflammatory responses also play a role in the development of Type II diabetes (Grant and Dixit, 2013; Lee et al., 2013), obesity (Stienstra et al., 2011), and cardiovascular diseases (Garg, 2011), as well as cancer (Zitvogel et al., 2012). Given that metabolic disorders can predispose to the development of psychiatric disorders, it is possible that inflammasome-driven inflammatory pathways may be a potential mechanism driving this co-morbidity.

A number of studies have investigated the innate immune pathways associated with the activation of NLRP inflammasomes and the subsequent production of IL-1 β , IL-18, and IL-33 from their precursors. The aim of this review is to examine these complex inflammatory signaling pathways associated with NLRP inflammasomes activation, and leading to neuroinflammation and behavioral changes that have commonly been observed during various psychiatric disorders and brain aging.

MATERIALS AND METHODS

PRISMA CRITERIA

The guidelines prescribed by PRISMA (Preferred reporting items for systematic reviews and meta-analyses) (Liberati et al., 2009; Moher et al., 2009) were followed while constructing this review. The checklist items from PRISMA as relevant to this review, for example those related to search and writing approaches, were included and the items not relevant, for example those related to meta-analyses, were excluded.

SEARCH AND SELECTION PROCESS

An electronic database search of PubMed and Google Scholar with several key terms in various permutations was performed. These included but were not limited to: inflammasomes, neuroinflammation, NLRP, NALP (NACHT, LRR, and PYD domains containing proteins), cytokines, IL-1, IL-18, IL-33, TNF, cellular, humoral, immune, aging, depression, AD, PD, Huntington's disease, multiple sclerosis, cognition, behavior, metabolic disorders, diabetes, obesity, cardiovascular disease, cancer, pathogen associated molecular patterns, damage associated molecular patterns, toll like receptors, and glial cells. At each stage of the search, titles and abstracts were scrutinized and the most appropriate organized into separate folders using End Note X6.0.1 software. In addition, articles relevant to our discussion were retrieved from the reference list of other online articles on each subtopic. This in total yielded 1563 papers. After placing all inclusion and exclusion criteria into our search (depicted in **Figure 1**), 164 articles

closely related to the aims set forth for this review were selected and hence utilized.

INCLUSION AND EXCLUSION CRITERIA

The emphasis of this review has been on inflammatory pathways associated with inflammasome activity in the brain, and as such articles investigating inflammasomes, in particular NLRP inflammasomes and their mechanism of actions in CNS disorders were selected for detailed analysis. In addition, articles addressing the effects of IL-1 family of cytokines in the brain were read thoroughly to understand and analyze the various mechanisms of actions of these cytokines, especially in the brain and their association with inflammasomes. Other immune factors related to inflammasomes and the role of inflammasomes in systemic diseases was also investigated while writing this review. All articles included in this review have been published between 1989 and 2014. Articles without the full text available and with anecdotal evidence were excluded from the review.

STRUCTURE OF NLRP INFLAMMASOMES

The NLR family, pyrin domain containing inflammasomes (NLRP) are the most studied and best characterized protein complex during inflammation (Stutz et al., 2009). NLRs are intracellular PRRs and function in association with Toll-Like Receptors to sense the presence of PAMPs which are found in a variety of microorganisms that enter cell through phagocytosis (infectious stimuli), and DAMPs such as nuclear and cytosolic protein characteristics of tissue injury/stress (non-infectious stimuli). In turn, this activates the innate and acquired immune response (Inohara et al., 2005; Kanneganti et al., 2007; Franchi et al., 2009). A key part of this process is the assembly of inflammasome complexes that generally have three main components: a cytosolic PRR (either from the NLR family or the pyrin and HIN domain containing family-PYHIN), caspase-1 and an adaptor protein ASC (apoptosis-associated speck like protein). The NLR family contains a leucine rich repeat domain (LRR), a central NACHT domain and a variable amino-terminal domain, which in the NLRP subfamily is an N-terminal pyrin domain (PYD). Activation of NLRPs leads to the recruitment of ASC which contains a caspase activation and recruitment domain (CARD). ASC then interacts with the CARD of pro-caspase-1. There are exceptions to this sequence, with for example NLRP1, directly interacting with pro-caspase 1, without necessarily needing ASC. Nonetheless the interaction with pro-caspase-1 leads to its conversion to caspase 1, which then converts pro forms of IL-1 β , IL-18, and IL-33 into their active forms, initiating an inflammatory response (Martinon et al., 2002; Petrilli et al., 2005). The NLRP3 is the largest and most studied inflammasome of all known at this stage (Stutz et al., 2009).

See **Figure 2** for the schematic representation of the structures of different NLRP inflammasomes as described above.

THE ROLE OF IL-1 FAMILY OF CYTOKINES IN INFLAMMATION, PATHOLOGICAL STATES, AND HOMEOSTATIC RESPONSE IN THE BRAIN

Pro-inflammatory cytokines function to attract leucocytes and enhance their proliferation at the site of inflammation. They

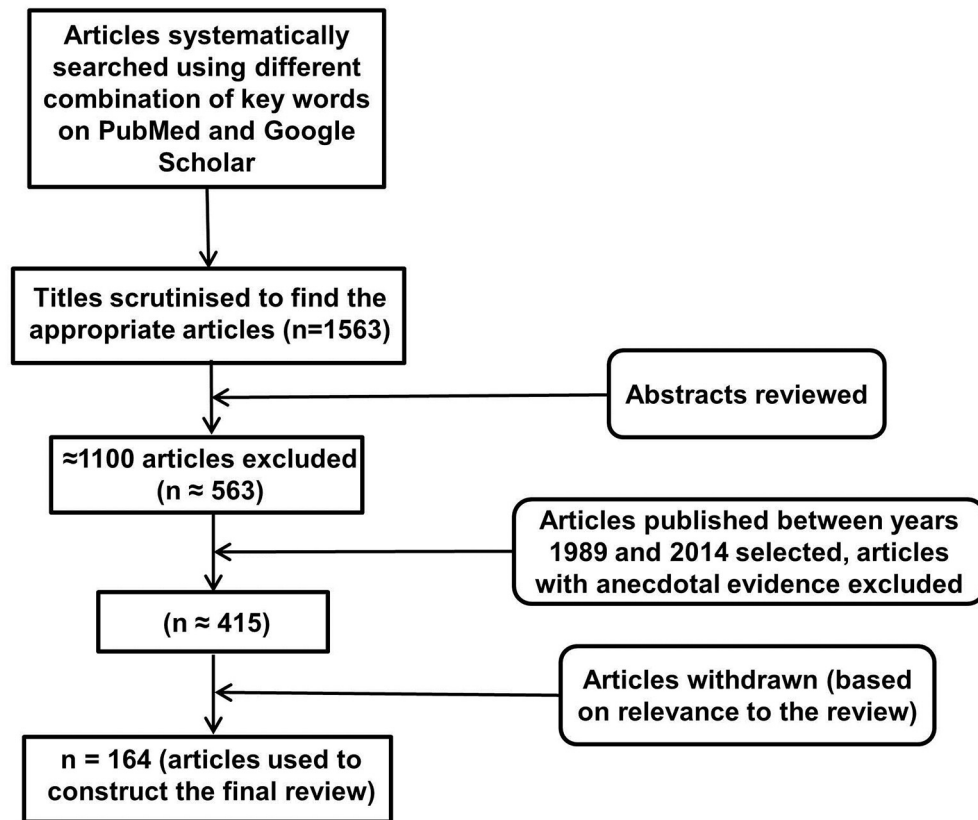


FIGURE 1 | Study inclusion flowchart. It depicts the methodology for search and collection of relevant articles for this review, following PRISMA guidelines (Liberati et al., 2009; Moher et al., 2009).

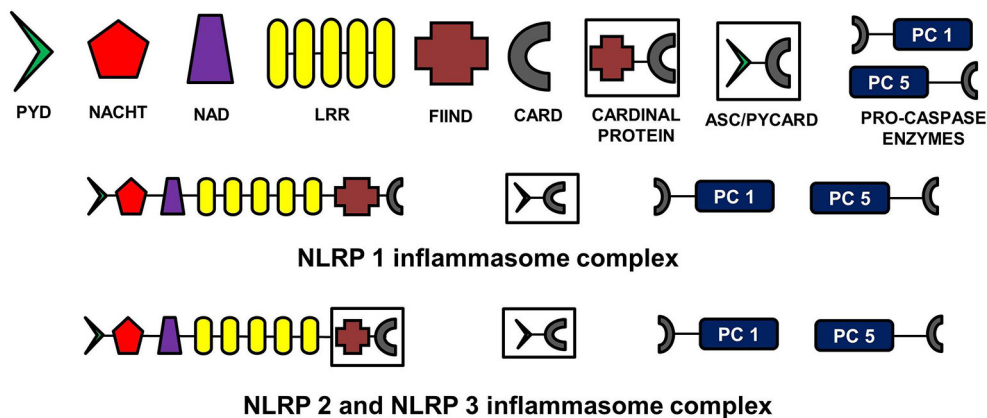


FIGURE 2 | Structure of NLRP inflammasomes. NLRP inflammasomes are intracellular protein complexes consisting of NLRP (NACHT, LRR, and PYD domains containing proteins 1, 2, or 3), the adapter protein ASC/Pycard, enzyme pro-caspases 1 and 5, and cardinal proteins. NLRP1 has Pyrin (PYD) domain on the amino (N)-terminal. This PYD domain is bonded to a NACHT domain followed by a NACHT-associated domain (NAD), several leucine-rich repeats (LRR), FIIND domain and the caspase recruitment domain (CARD) at the carboxy (C)-terminal. The molecular

structures of NLRP2 and NLRP3 are similar to NLRP1, except that instead of being directly linked to FIIND domain, LRRs are linked to a cardinal protein which consists of FIIND domain on N-terminal and CARD domain on the C-terminal. NLRPs with the adapter protein ASC/Pycard and pro-caspase enzymes form the inflammasome complex responsible for converting pro-IL-1 cytokines into their active forms within the cytoplasm of glial cells primarily. ASC, apoptosis-associated speck-like protein containing a CARD.

stimulate cytotoxicity, release of proteolytic enzymes, synthesis of prostaglandins, and synthesis and secretion of secondary cytokines. This in turn promotes inflammation and increases thermoregulatory set point, generally associated with symptoms such as fever, tissue destruction, shock, and even death (Cannon, 2000). The IL-1 family of cytokines comprises 11 secreted factors, including IL-1 α , IL-1 β , IL-18, and IL-33, which are known for playing a role in host defense and immune system regulation in inflammatory diseases (Barksby et al., 2007; Arend et al., 2008; Dinarello, 2009; Sims and Smith, 2010). These cytokines have been shown to be involved in a variety of immune reactions as well as in the initiation, regulation, and maintenance of inflammation (Dinarello, 2000). In particular, cytokine mediated processes have been shown to result in long term neuropsychiatric disorders and were found to be related to major depression, dementia, and AD (Licastro et al., 2000; Cacquevel et al., 2004; McAfoose and Baune, 2009). The presence of IL-1 β has been demonstrated in cerebrospinal fluid and plasma of patients with AD (Licastro et al., 2000; Tarkowski et al., 2003). Similarly, the roles of IL-18 and IL-33 in neuroinflammation and neurodegenerative diseases have also been well established (Felderhoff-Mueser et al., 2005; Arend et al., 2008; Liew et al., 2010).

However, it is important to note that although pro-inflammatory cytokines (IL-1 and TNF family of cytokines) have been shown to result in neuroinflammation and neurodegenerative diseases when expression is high, at constitutive levels they are required for normal physiological functioning, particularly in the molecular and cellular mechanisms responsible for learning, memory and cognition (McAfoose and Baune, 2009). They influence and maintain homeostasis in monoamine metabolism, neuronal genesis and survival, Hypothalamic-Pituitary-Adrenal (HPA) axis sensitivity to cortisol and certain cellular neuroimmune functions (Eyre and Baune, 2012). However, levels of both pro-inflammatory and anti-inflammatory cytokines have been shown to be elevated in many brain disorders, including AD, PD, and age related dementia, indicating their role in cognitive and memory deficits with age. When pro-inflammatory cytokines are overexpressed, anti-inflammatory cytokines potentially function to suppress the gene expression for pro-inflammatory cytokine production and control the pro-inflammatory response. For instance, gene knockout mice for anti-inflammatory cytokines, such as IL-1ra, IL-10, and TGF- β 1 showed enhanced inflammatory reactions (Dinarello, 2000). However, no study describes the effects of anti-inflammatory cytokines on activated inflammasomes. Activated microglia and astrocytes are the main source of cytokines in the brain (Rothwell et al., 1996; Hanisch, 2002).

Figure 3 shows the cytokine cascade in brain following stimulation with an infectious agent, metabolic waste or foreign material. As this includes increase in the levels of IL-1 β cytokine which can only be activated from its precursor in the presence of enzyme caspase 1, it indicates an active involvement of inflammasome action during this cytokine cascade.

LINK BETWEEN AGING OF BRAIN, IL-1 CYTOKINES, AND BRAIN DISORDERS

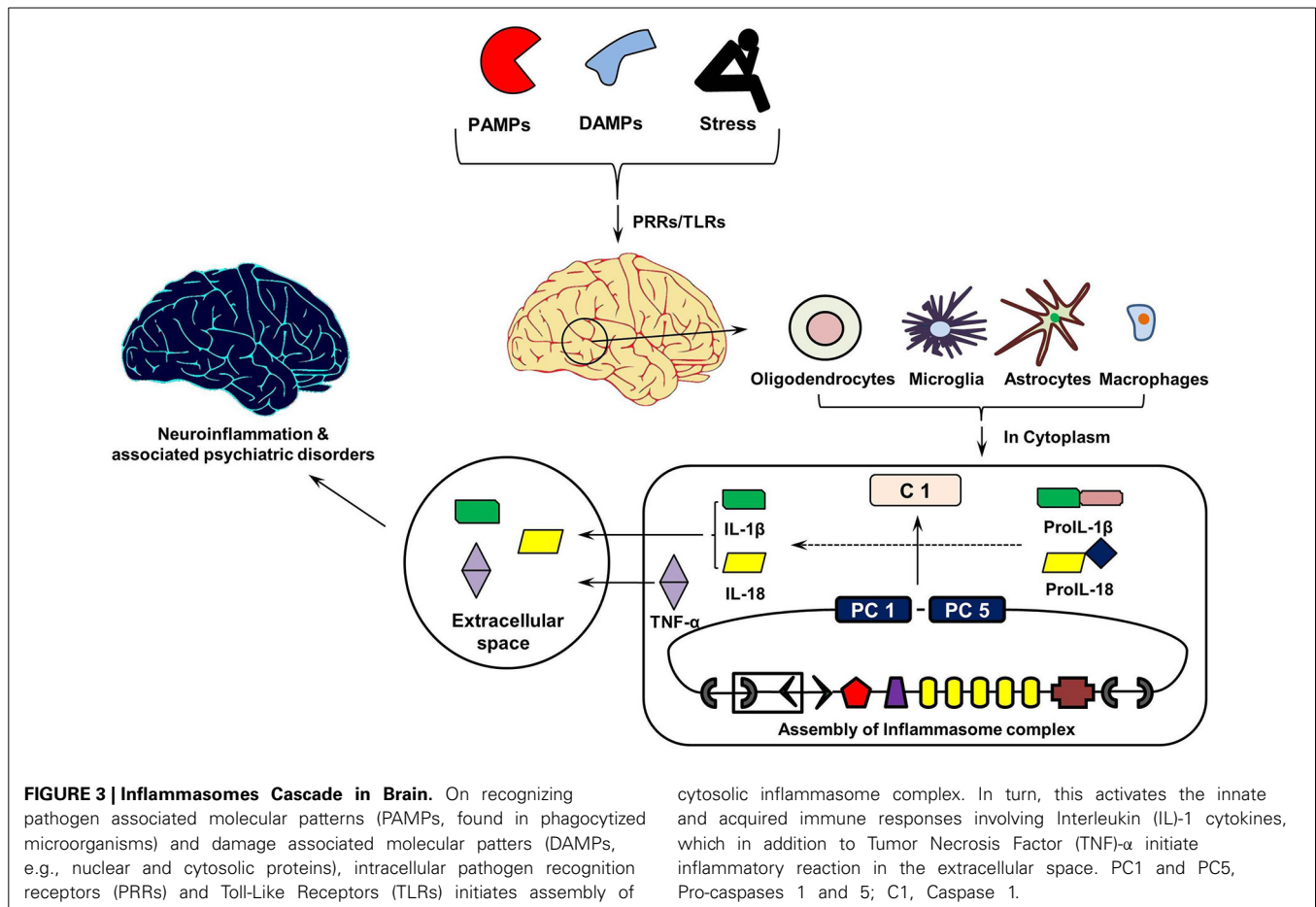
Aging of the brain has been shown to be associated with many cognitive and memory deficit disorders and is believed to be

regulated by extrinsic (e.g., environmental) and intrinsic (e.g., genotype) factors (van der Staay, 2002). Several brain disorders, such as AD and PD, are the products of chronic neuroinflammation and resultant neurodegeneration (Heneka et al., 2010; Hirsch et al., 2012), the symptoms which are also common to the aging brain (McGeer and McGeer, 2004). Dementia, decline in cognitive abilities and impairment of spatial memory are often seen during aging and are associated with neuroinflammatory changes within the brain accumulated over a period of time. Indeed, a significant association has been found between age related depression and level of pro-inflammatory cytokines in the brain (Godbout et al., 2008). Moreover, the risk of infections increases with age, mainly due to immunosenescence (Aw et al., 2007) and a rise in circulating autoantibodies and lymphoproliferative disorders, hence contributing toward greater morbidity and mortality in old age (Shinkai et al., 1998; Senchina and Kohut, 2007).

Structural and functional changes in the brain are coordinated by a range of intracellular signaling molecules. Consistent findings suggest that an increase in the expression of pro-inflammatory cytokines by astrocytes and microglia within the brain results in neuroinflammation followed by neurodegeneration, eventually resulting in cognitive and memory deficit and exacerbated sickness and depressive-like behavior (Mrak and Griffin, 2005; Huang et al., 2008). Microglia are primed with aging and upon secondary stimulation, these microglia release excessive quantities of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 (Dilger and Johnson, 2008). The increase in the number of microglia, astrocytes and percentage of GFAP in the brain with age has been reported in rodents, which is subsequently found to be related to cognitive and memory impairment (Sugaya et al., 1996; Rozovsky et al., 1998), and to neurodegenerative diseases such as AD (Mrak and Griffin, 2005). The effects of aging on microglial functions in the brain has been reviewed in detail by Conde and Streit (2006). During aging, glial cells, particularly microglia show increased activation and expression of pro-inflammatory cytokines, such as IL-1, however, they also become increasingly dysfunctional and lose neuro-protective properties which predispose the brain to the neurodegenerative disorders in line with other genetic and acquired environmental risks (Mrak and Griffin, 2005; Streit, 2005). Similarly, astrocytes are the immune effector cells, which express cytokines (IL-1, IL-6, IL-10, interferons α and β , TNF α and β) and chemokines, and mediate inflammation and immune reactivity in the brain. The under-expression or over-expression of astrocytes can also lead to neurodegenerative diseases (Dong and Benveniste, 2001).

INFLAMMASOME MEDIATED INFLAMMATORY PATHWAYS IN THE AGING BRAIN

It was not known until recently whether inflammasomes play any role in causing or aggravating neuroinflammation during neuronal aging. Considerable recent findings have suggested an instrumental role of inflammasomes in the pathophysiology of neuroinflammation during neuronal ageing, and associated neurodegenerative diseases, cognitive impairment and dementia (Simi et al., 2007; Chakraborty et al., 2010; Mawhinney et al., 2011; Liu and Chan, 2014). There is upregulation in the expression of several genes that signal inflammasome assembly



and activation of caspase 1 (e.g., thioredoxin-interacting protein, P2X7, and pannexins), as well as signaling of TLRs (e.g., CD14, TLR2, TLR4, TLR7, TOLLIP, MYD88) in different regions of the brain such as hippocampus, post-central gyrus, and superior frontal gyrus during aging (Cribbs et al., 2012). TLRs are evolutionary conserved microbe specific structural motifs (e.g., PAMPs) and endogenous molecule (e.g., DAMPs) recognition transmembrane or endosomal membrane proteins, primarily expressed in various sentinel cells such as dendritic cells, macrophages, and plasmotoid dendritic cells that form the first line of defense (Kumar et al., 2009). The interaction between TLRs and LRRs on cytosolic NLRs trigger the assembly and activation of inflammasomes culminating in caspase 1 catalyzing pro-IL-1 cytokines into their active forms (van de Veerdonk and Netea, 2011). The increased activity of both TLRs and NLRs in the aging brain can therefore act as a deterrent to the negative regulation of their expression and a sustained expression could result in chronic neuroinflammation and associated neurodegenerative diseases. Moreover, production of reactive oxygen species (ROS) from dysfunctional mitochondria and increased NF- κ B signaling with aging could also potentiate the priming of NLRP3 inflammasomes in the brain resulting in an inflammatory response (Salminen et al., 2012). Formation of mutant α -synuclein and A β fibrils, seen during PD and AD respectively, further pose greater danger in old age as they act to enhance the

activity of inflammasomes in the brain (Salminen et al., 2009; Tschopp and Schroder, 2010; Cedillo, 2013).

Enhanced inflammasome activity could manifest in the form of cognitive decline in an aging population, as shown in 18 month old male Fisher 344 rats, however spatial learning improved when rats were treated with an anti-inflammatory drug probenecid (Mawhinney et al., 2011) which reduced NLRP1 inflammasome activation. However the mechanism for this is not yet completely understood.

ASSOCIATION BETWEEN NEUROINFLAMMATION AND MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) is characterized by a distinct change of mood accompanied by sadness, irritability, loss of interest in all activities and events, as well as psychophysiological changes (Belmaker and Agam, 2008). A number of studies, both experimental and meta-analytic, have revealed increased expression of pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, in the brain of MDD patients leading to neuroinflammation (Maes et al., 1997; Howren et al., 2009; Dowlati et al., 2010; Hannestad et al., 2011). Role of chronic mild stress in neuroinflammation and subsequent occurrence of depression has also been established (Farooq et al., 2012), although it is not clear if inflammasomes have any role in causing neuroinflammation in response to chronic mild stress.

INFLAMMASOME MEDIATED INFLAMMATORY PATHWAYS ASSOCIATED WITH MAJOR DEPRESSIVE DISORDER

The increase in IL-1 β levels and neuroinflammation in the brain of MDD patients potentially suggests a role for inflammasomes in MDD. Researchers have indeed recently shown the involvement of the NLRP3 inflammasome in lipopolysaccharide (LPS)-induced mouse depressive-like behavior (Zhang et al., 2014). Similar findings were seen in human participants when activated NLRP3 inflammasomes were detected in blood mononuclear cells from depressive patients (Alcocer-Gómez et al., 2014). To investigate the etiological role of inflammasomes in depression, a panel of researchers conducted a clinical trial in major depression and schizophrenic patients. They concluded from the study that inflammasome-related inflammation is an ongoing process in psychiatric patients during diseased states (Hohmann et al., 2014). Moreover, the recent finding that mice lacking caspase-1 are resistant to LPS-induced depressive-like behavior further supports the role of inflammasomes in depression (Moon et al., 2009). Some authors recently reviewed the role of inflammasomes in MDD and its comorbidity with systemic illnesses, and proposed a new inflammasome hypothesis of depression and related comorbid systemic illnesses (Iwata et al., 2013). The review highlights the central mediator role that inflammasomes play in the contribution of psychological and physical stressors to the development of depression and its association with systemic illnesses. The activation of inflammasomes, particularly NLRP3, therefore could be indirectly related to the pathophysiology of depression and its comorbidity with other systemic diseases through an inflammatory response in the brain.

ASSOCIATION BETWEEN NEUROINFLAMMATION AND ALZHEIMER'S DISEASE

AD is characterized by a debilitating chronic and progressive neurodegeneration leading to major clinical hallmarks of loss of memory, cognitive deficit, dementia, and behavioral impairment. Although, the prevalence of AD is higher in people over 60 and it increases proportionally with every 10 years of age (Younger/Early Onset Alzheimer's and Dementia: Alzheimer's Association, 2014), early onset of AD has also been reported in people in their 40s and 50s (Kim et al., 2014). Several factors such as genetic predisposition (Bertram and Tanzi, 2009; Kamboh et al., 2012), reduced synthesis of excitatory neurotransmitter acetyl choline (Babic, 1999), extracellular deposition of amyloid beta (A β) in the brain (Palop and Mucke, 2010), abnormalities in tau protein forming neurofibrillary tangles leading to disintegration of microtubules (Ballatore et al., 2007), and oxidative stress and inflammatory cascades mediated by primed glia cells (Agostinho et al., 2010) have been proposed to cause AD. These different hypotheses have been established after years of independent research; however, recent efforts toward finding the common link between the causal factors for AD have pointed toward the inflammatory cascade linking them in the brain. Indeed, damaged neurons, highly insoluble A β deposits and neurofibrillary tangles could provide stimuli for neuroinflammation (Wenk, 2003). Similarly, neurotransmitter acetylcholine has been shown to be involved in inhibiting the release of pro-inflammatory cytokines from microglia and monocytes (Tabet, 2006), an anti-inflammatory mechanism that could

be disturbed during acetylcholine deficiency. This suggests that all above etiologies for AD, when accompanied by chronic neuroinflammation, lead to progressive neurodegeneration and behavioral impairment with age, characteristics of symptoms of AD. In the absence of neuroinflammation, these etiologies may not provide sufficient pathology to cause AD. This is supported by the finding that significant amyloid deposition could be present in the brain of healthy elderly individuals without cognitive impairment (Aizenstein et al., 2008). Likewise, while higher quantities of tau proteins have been reported in the brain of AD patients than unaffected individuals (Avila et al., 2004), some authors have challenged the tau protein hypothesis and proposed that tau phosphorylation is a compensatory mechanism to protect neurons against oxidative stress (Lee et al., 2005). Nonetheless, this suggests overall that a single factor alone may not be sufficient to cause AD, and irrespective of the causative factor, neuroinflammation essentially provides a central pathway to the onset of AD which is mediated by various pro-inflammatory cytokines and chemokines, including IL-1 family of cytokines that are activated by inflammasomes.

Indeed, IL-1 β and IL-18 over-expression has been shown to initiate inflammatory process in the brain of AD patients (Rubio-Perez and Morillas-Ruiz, 2012; Liu and Chan, 2014). This over-expression has been detected in microglia, astrocytes as well as neurons, and found to be co-localized with both A β plaques and tau. Interestingly, it has also been suggested that chronic inflammation could be the cause for increase in A β and tau phosphorylation in the brain (Meraz-Ríos et al., 2013). In support of this, studies on transgenic mice with LPS-induced neuroinflammation have shown enhanced intracellular deposition of A β (Sheng et al., 2003; Lee et al., 2008) and tau phosphorylation (Kitazawa et al., 2005) in the brain of mice. Overall, this suggests a chain of continuous adverse events in the brain of AD patients, mediated by IL-1 family of pro-inflammatory cytokines.

INFLAMMASOME MEDIATED INFLAMMATORY PATHWAYS ASSOCIATED WITH ALZHEIMER'S DISEASE

Recently, the role of inflammasomes, particularly NLRP3, in oxidative stress-induced neuroinflammation and impaired amyloid metabolism seen in AD brains has been evaluated and recognized (Halle et al., 2008; Marchesi, 2011; Tan et al., 2013). Neuronal injury caused by insoluble A β oligomers and fibrils releases DAMPs which are sensed by PRRs (NLR domain) on NALP inflammasomes initiating a chain of events leading to the maturation of proIL-1 β and proIL-18 cytokines and release of their active forms as the final event (Halle et al., 2008; Salminen et al., 2009). Moreover, A β can interact with neuronal membranes to create ion channels that allow potassium ion (K $^{+}$) efflux mediated by ATPase enzyme, activating inflammasomes and in turn secretion of the active IL-1 family of cytokines (Salminen et al., 2009; Tschopp and Schroder, 2010). Reduction in intracellular K $^{+}$ to 90 mM though has been found to be a requirement for the activation of NLRP3 inflammasomes (higher intracellular concentration of K $^{+}$ inhibits activation of inflammasomes) (Petrilli et al., 2007). Some authors however, found impaired activity of Na $^{+}$ /K $^{+}$ ATPase in AD patients (Hattori et al., 1998) that is required for the active efflux of K $^{+}$ across the cell membranes,

which therefore raises the question whether efflux of K^+ is essential for the activation of inflammasomes. ATPase is required to catalyze ATP into ADP and a phosphate ion with the release of energy that activates the purinergic P2X₇ receptor. This receptor in turn decreases intracellular K^+ levels (Perregaux and Gabel, 1994; Solle et al., 2001). Purinergic signaling has also been shown to control the cerebral vascular tone and this has been implicated in learning and memory, locomotor and feeding behavior and sleep (Burnstock, 2013). Nonetheless, diminished activity of Na^+/K^+ ATPase reduces the gradient of ions across the cell membranes causing an excitotoxic cellular response resulting in neuronal death (Hattori et al., 1998). This causes a release of DAMPs from dead neurons that potentially act as the activators of NLRP3 inflammasome dependent innate immune response (Rubartelli, 2014). It has also been shown that disease-associated extracellular amyloid and unique protein aggregates caused by inappropriate oligomerization or misfolding are sensed by NLRP3 inflammasomes (Masters and O'Neill, 2011), likely as DAMPs within the resident macrophages after engulfment in the brain. Research has also suggested that the brain in AD is under increased oxidative stress and A β peptides generates free radicals that together further enhance neuron degeneration and death (Markesbery, 1997). Mitochondrial ROS released during tissue injury/death could enhance oxidative damage and signal inflammasome activation up-regulating pro-inflammatory cytokine levels in brain (Martinon, 2010; Tschopp and Schroder, 2010; Naik and Dixit, 2011), potentially resulting in neuroinflammation.

Significant pathology and behavioral deficits characteristics of chronic neuroinflammation do not manifest until advanced age. This has been attributed to the capacity of the brain to compensate for the presence of chronic neuroinflammation by regulating the glutamatergic system (Brothers et al., 2013). This suggests that neuroinflammation in itself does not cause AD; however it acts as an initiator, enhancer and sustainer of AD disease during old age which is reinforced by various other etiologies. Since IL-1 cytokines are key contributors of chronic neuroinflammation and associated neurodegenerative diseases, including AD, inflammasomes provide a possible answer for the mechanism of IL-1 action and the ways in which IL-1 activity is regulated during chronic neuroinflammation in old age (Allan et al., 2005).

IMMUNE FACTORS ASSOCIATED WITH THE ACTIVITY OF INFLAMMASOMES

It has been well established that both TNF- α and IL-1 β stimulate each other's secretion and exhibit overlapping and synergistic effects (Akira et al., 1990; Ikejima et al., 1990; Knofler et al., 1997). For instance, while TNF- α enhances migration of leucocytes in inflamed tissue and promotes apoptosis, IL-1 β acts as a potent pyrogen and decreases the threshold of pain by inducing the transcription of cyclooxygenase 2 enzyme, thereby enhancing production of prostaglandins E₂, which is responsible for pain and fever. This raises a possibility that inflammasomes, which catalyze IL-1 β precursors, may stimulate TNF- α secretion through an indirect pathway. Contrary to this, it has recently been reported that inflammasomes may also be activated independently of PRRs, through TNF- α dependent pathways. TNF- α has been shown to trigger the activation of caspase 1 and in turn

secretion of IL-1 β (Alvarez and Munoz-Fernandez, 2013), suggesting a possible bidirectional cause-effect relationship between inflammasomes and TNF- α . Although the precise mechanism for this has not yet been elucidated, this study indicated that TNF- α may potentially substitute for a TLR mediated stimulus required for inflammasome activation. Moreover, recent findings also suggest that TNF- α induces production of IL-33 in keratinocytes (Taniguchi et al., 2013) and regulates expression of IL-18 in dendritic precursor-like cell line KG-1 and cardiomyocytes (Chandrasekar et al., 2003; Koutoulaki et al., 2010), further supporting the hypothesis that TNF- α has a role in inflammasome activation, although this direct relation between TNF- α , and IL-18 and IL-33 is yet to be established in the brain. Furthermore, other cytokines such as Type I interferon (IFN) gamma have been shown to inhibit caspase-1 cleavage and reduce IL-1 β secretion in rodents (Guarda et al., 2011). Conversely, although IFN-gamma does not cause inflammation by activating inflammasomes directly, it has been shown to augment TNF activity (Dinarello, 2000). Although IL6 is a reliable inflammatory marker it may not always be directly involved in inflammasome mediated inflammation as seen in an IL-6 knock in mouse model (McGeough et al., 2012). Overall, this suggests a complex and intricate immune pathway mediated by various cytokines that may be involved in the activation of inflammasomes in the brain, and resultant neuroinflammation and changes in brain function; however this requires validation through extensive research.

INFLAMMASOME-INDEPENDENT NEUROINFLAMMATION-MEDIATED BRAIN PATHOLOGIES

TNF- α is another pro-inflammatory cytokine, in addition to IL-1 cytokines, that has been primarily implicated in neuroinflammation. Elevated levels of TNF- α in particular have been shown to cause a reduction in hippocampal volumes through the neurodegenerative TNFR1 pathway (Baune et al., 2012) and can lead to the development of depressive-like behavior (Eyre et al., 2013). Glial cells, microglia, and astrocytes, are the primary immune effector cells and express various cytokines in the CNS (Rothwell et al., 1996; Hanisch, 2002). Though glial cells are neuroprotective, their over expression or sustained stimulation can result in enhanced production of cytokines (e.g., IL-1 β and TNF- α) (Sawada et al., 1989; Dong and Benveniste, 2001; Hanisch, 2002) resulting in severe neuroinflammation, neurodegeneration and subsequent cognitive dysfunction and psychiatric diseases, such as AD.

While levels of both pro-inflammatory and anti-inflammatory cytokines in the peripheral circulation and CNS have been reported to rise during several brain disorders such as depression, schizophrenia and AD (Schwarz et al., 2001), other humoral immune factors, such as mitogen-activated protein kinases (MAPK), C reactive protein (CRP), the complement system and chemokines have also been reported to modify brain anatomy and functions. MAPKs are specific protein kinases (serine-threonine specific) that elicit pro-inflammatory and immunomodulatory functions (Lee et al., 1994; Dong et al., 2002). Similarly, CRP is an acute phase reactant protein which enhances inflammation and tissue damage by promoting phagocytosis by opsonization (Du Clos, 2000) and activating the complement system (Padilla

and Perez, 2003). High levels of CRP in the brain have been linked to neuroinflammation and associated cognitive impairment and dementia (Kuo et al., 2005), and AD (McGeer et al., 2000). Researchers have observed upregulation of the complement system in human brain during AD and other neurodegenerative diseases (McGeer and McGeer, 1995; Yasojima et al., 1999). The complement system consists of distinct plasma proteins that act as opsonins and initiate a series of inflammatory responses (Janeway et al., 2001). Chemokines promote neuroinflammation by attracting leucocytes to the point of inflammation (Proost et al., 1996; Mélik-Parsadaniantz and Rostène, 2008). Neuroinflammation in turn has been implicated for the impairment of brain function (Campbell, 2004; Ownby, 2010; Tansey and Goldberg, 2010).

Further to the role of inflammasomes and humoral immune factors, several cellular immune factors such as granulocytes, monocytes/macrophages, NK cells, and T lymphocytes have also been shown to have a role in the pathophysiology of neuroinflammation (Petersen and Pedersen, 2005, 2006). The role of NK cells in various brain disorders such as depression, AD and PD has been reviewed and validated by some researchers (Poli et al., 2013). Likewise, the exchange of B cells across the BBB has been reported in patients with multiple sclerosis and associated with the development of autoimmunity in the CNS (von Büdingen et al., 2012).

DISCUSSION

NEUROINFLAMMATION AND CYTOKINES

Neuroinflammation is an innate mechanism to ward off any stimuli that may be harmful to the host and has been shown to be mediated by various immune factors, particularly cytokines (Cacquevel et al., 2004) and chemokines (Ubogu et al., 2006). In particular, pro-inflammatory cytokines, such as TNF- α and the IL-1 family of cytokines, are credited for initiating the inflammatory reactions in the brain in response to an adverse stimulus, for continuation of neuroinflammation by attracting leucocytes at the site of inflammation and activating other pro-inflammatory factors, as well as for the anti-inflammatory pathway by enhancing IL-6 production that in turn stimulates production and expression of anti-inflammatory cytokines and immune factors (Cannon, 2000). However, what is more important here is the mechanism for the activation of these pro-inflammatory cytokines in response to an adverse stimulus in the first instance. While the concept of an increase in concentration of pro-inflammatory cytokines within the brain during aging and infection is now established, less is known about the mechanisms of cell signaling that result in the pro-inflammatory cytokine gradients within the brain.

NLRP INFLAMMASOMES IN NEUROINFLAMMATION

While several theories have been postulated to explain this mechanism, recent findings suggest the role of inflammasomes to be important in particular for the IL-1 family of cytokines (Martinon and Tschoop, 2006; Stutz et al., 2009; Schroder and Tschoop, 2010). In response to PAMPs and DAMPs, trans-membranous TLRs that are present in semantic cells such as macrophages and dendritic cells, interact with NLRs on inflammasomes to

recognize the stimulus, initiating an inflammasome cascade leading to the release of caspase 1 enzyme in the cytoplasm. Caspase 1 cleaves the pro-forms of the IL-1 family of cytokines to form their active forms (Inohara et al., 2005; Kanneganti et al., 2007; Franchi et al., 2009) that may result in neuroinflammation as a result of increased pro-inflammatory cytokine gradients. Although the acute neuroinflammatory response includes activation of resident tissue macrophages in the CNS and subsequent release of various cytokines and chemokines, this may also cause oxidative and nitrosative stress, which is a first line preventative mechanism against pathogenic extrinsic and intrinsic proteins and is less likely to cause long term damage to neurons (Frank-Cannon et al., 2009). However, it could still result in neurodegenerative changes, as well as in short-term cognitive impairment and exacerbated sickness behavior, as seen in rodent trials after LPS-induced acute neuroinflammation characterized by heightened pro-inflammatory cytokine response (Morimoto et al., 2002; Huang et al., 2008). Nevertheless, a bigger danger is posed by chronic neuroinflammation that is generally seen during old age (Sparkman and Johnson, 2008) and responsible for some brain pathologies such as depression (Wager-Smith and Markou, 2011), AD (Hauss-Wegrzyniak et al., 1998), PD (Tansey and Goldberg, 2010), and multiple sclerosis (Frischer et al., 2009).

For a chronic neuroinflammation to be sustained, the stimuli need to be continuous, potent and self-replicating. This could be explained from the findings that AD patients in old age suffer from a persistent degenerative condition that involves consistent increases in the various proposed etiologies, be it A β oligomerization or Tau phosphorylation, in the presence of neuroinflammation (Meraz-Ríos et al., 2013). A similar scenario could be plausible in the case of PD where formation of α -synuclein fibril aggregate increases in the presence of neuroinflammation. Accelerated formation of the mutant α -synuclein fibrils has been linked with the onset of PD (Conway et al., 1998, 2000). Although it has been shown that aggregated α -synuclein in microglia-like cells potentially activate the assembly of NLRP3 inflammasomes by inducing vesicle rupture in THP-1 cells that are sensed as danger signals (Cedillos, 2013), the opposite scenario still need to be studied (Cedillos, 2013). Consistent findings have also established the link between various etiologies of depression and neuroinflammation (Maes et al., 1997; Howren et al., 2009; Dowlati et al., 2010; Hannestad et al., 2011).

A SHORT NOTE ON THE ROLE OF INFLAMMASOME MEDIATED NEUROINFLAMMATORY PATHWAYS IN THE COMORBIDITY OF SYSTEMIC ILLNESSES AND PSYCHIATRIC DISORDERS

High incidences of chronic inflammatory diseases such as cancer (Il'yasova et al., 2005), diabetes (De Rekeneire et al., 2006), osteoarthritis (Stannus et al., 2013), and cardiovascular disease (Volpato et al., 2001) have been demonstrated by prospective and correlative studies in aged cohorts. Investigation at the molecular level suggests increased levels of systemic pro-inflammatory cytokine IL-1 β , in addition to TNF- α and IL-6, and acute phase proteins (e.g., CRP). Moreover, significant findings have confirmed an association between age related depression and level of pro-inflammatory cytokines in the brain (Godbout et al.,

2008). This suggests a mechanism whereby pro-inflammatory cytokines migrate from systemic circulation to the brain and vice versa, especially during old age. Indeed, the pathways for the transport of pro-inflammatory cytokines to brain from systemic circulation have been described in a review by Capuron and Miller (2011) (See Figure 4). Rodent studies have shown increased production and expression of IL-1 β in the brain after LPS-induced systemic inflammation (Cunningham et al., 2005) and changes in mood and behavior similar to depression after systemic administration of pro-inflammatory cytokines (Pollak and Yirmiya, 2002). This transport of pro-inflammatory cytokines into the brain and increase in their expression could be the reason for the comorbidity of systemic illnesses with psychiatric disorders in old age. Comorbid conditions, such as Type II diabetes (Grant and Dixit, 2013; Lee et al., 2013), obesity (Stienstra et al., 2011), cardiovascular diseases (Connat, 2011), and cancer (Fallowfield et al., 2001) with psychiatric illnesses therefore supports the hypothesis that inflammasomes play a large role in

immunosenescence associated with aging and formation of psychiatric and systemic illnesses with age, the top-most reasons for deaths worldwide as mentioned by World Health Organization (2014). However, future research into the role of inflammasomes in these pathways during aging could possibly explain the link between age-related psychiatric and systemic illnesses.

The above mentioned link between systemic inflammatory conditions and CNS neurological disorders via activation of inflammasomes provides a molecular platform on which to develop therapies to prevent the initiation of those pro-inflammatory chronic cascades which are detrimental to the CNS. However, although hypothesized a number of times, it is yet to be seen if these therapies can be used to treat diseases such as cancer, diabetes, CVD, and auto-inflammatory disorders (Wilson and Cassel, 2010) that are major killers worldwide and comorbid with brain disorders. Inflammasomes, a molecular platform, could therefore be regarded as an advent to the innovation of therapies in the near future.

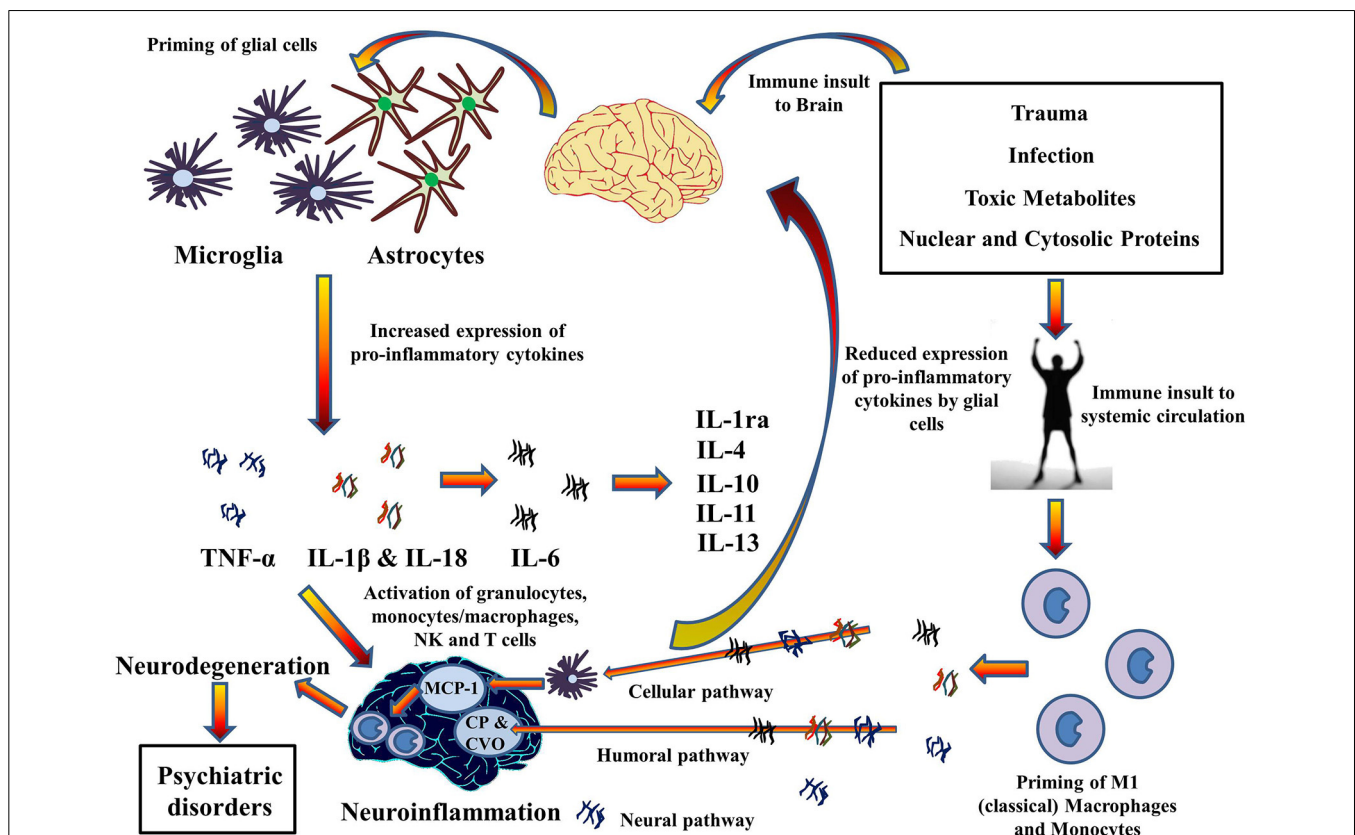


FIGURE 4 | Cytokines hypothesis of neuroinflammation: Implications in comorbidity of systemic illnesses with psychiatric disorders.

Pro-inflammatory cytokines can migrate between systemic circulation and brain in both directions which could explain the comorbidity of systemic illnesses with psychiatric disorders. There are three pathways for the transport of pro-inflammatory cytokines from systemic circulation to brain as described by Capuron and Miller (2011): Cellular, Humoral, and Neural. Moreover, PAMPs and DAMPs from trauma, infection, and metabolic waste can prime glial cells to express pro-inflammatory cytokines TNF- α , IL1 β , and

IL-6. When expressed, these cytokines activates granulocytes, monocytes/macrophages, Natural Killer, and T cells and together contribute to the pathophysiology of neuroinflammation. Chronic neuroinflammation could result in neurodegeneration and associated psychiatric disorders. These pro-inflammatory cytokines also stimulate production and expression of anti-inflammatory cytokine by glial cells that function as negative feedback to reduce the expression of pro-inflammatory cytokines, subsiding the neuroinflammation. MCP-1, Monocyte chemoattractant protein-1; CP, Choroid plexus; CVO, Circumventricular organ.

CONCLUDING REMARKS

Taken together, it is clear that the discovery of the role of inflammasomes in neuroinflammation has opened an array of research opportunities to investigate the inflammasome targeted therapies for age-related and pathological changes in the brain. It is also clear from the above discussion that the inflammasome activation pathway is complex and may involve the role of other immune factors such as cytokines, as well as mutant protein aggregates such as A β and α -synuclein fibrils. However, further research into these mechanisms and inflammasome-targeted therapies is advisable for constructing and assessing the complete profile of inflammasome-driven inflammatory pathways in brain.

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Recruitment of hypothalamic orexin neurons after formalin injections in adult male rats exposed to a neonatal immune challenge

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Exposure to early life physiological stressors, such as infection, is thought to contribute to the onset of psychopathology in adulthood. In animal models, injections of the bacterial immune challenge, lipopolysaccharide (LPS), during the neonatal period has been shown to alter both neuroendocrine function and behavioral pain responses in adulthood. Interestingly, recent evidence suggests a role for the lateral hypothalamic peptide orexin in stress and nociceptive processing. However, whether neonatal LPS exposure affects the reactivity of the orexin system to formalin-induced inflammatory pain in later life remains to be determined. Male Wistar rats ($n = 13$) were exposed to either LPS or saline (0.05 mg/kg, i.p.) on postnatal days (PND) 3 and 5. On PND 80–97, all rats were exposed to a subcutaneous hindpaw injection of 2.25% formalin. Following behavioral testing, animals were perfused and brains processed for Fos-protein and orexin immunohistochemistry. Rats treated with LPS during the neonatal period exhibited decreased licking behaviors during the interphase of the formalin test, the period typically associated with the active inhibition of pain, and increased grooming responses to formalin in adulthood. Interestingly, these behavioral changes were accompanied by an increase in the percentage of Fos-positive orexin cells in the dorsomedial and perifornical hypothalamus in LPS-exposed animals. Similar increases in Fos-protein were also observed in stress and pain sensitive brain regions that receive orexinergic inputs. These findings highlight a potential role for orexin in the behavioral responses to pain and provide further evidence that early life stress can prime the circuitry responsible for these responses in adulthood.

Keywords: orexin, hypocretin, pain, nociception, formalin, lipopolysaccharide, early life stress

INTRODUCTION

Chronic pain is a debilitating condition for which effective treatments and the underlying neurobiological mechanisms responsible are yet to be fully identified (Derish et al., 2002). Pain characteristically evokes emotional responses in individual sufferers and is often comorbid with negative affective states including anxiety and depression and sleep disturbances (Neugebauer et al., 2004; Katon et al., 2007). Interestingly, the normal maturation of the nociceptive system is dependent on the uninterrupted development of sensory inputs in early life (Fitzgerald, 2005). Exposure to adverse events during this sensitive period of development, when the nociceptive circuitry is undergoing fine-tuning, can precipitate maladaptive pain processing in later life. For example, in humans, early life physical stressors have been associated with an increased risk for developing chronic pain conditions in adulthood (Davis et al., 2005). Similarly, recent preclinical research conducted by our group has indicated that early life exposure to the immune challenge lipopolysaccharide (LPS) resulted in

hyperalgesia in response to formalin, a model of inflammatory pain, in young rats (Zouikr et al., 2014b). Despite recent efforts, the neural circuitry modulating the increased response to pain following early life stress remains to be fully determined.

Importantly, the hypothalamus controls neuroendocrine stress responses and nociceptive processing (Hsieh et al., 1996; Matthews, 2002). Direct afferent and efferent relays connect hypothalamic nuclei to brain areas involved in the active modulation of pain and nociception such as the dorsal horn of the spinal cord and the periaqueductal gray (PAG; Holland and Goadsby, 2007; Todd, 2010). Additionally, subregions such as the perifornical and lateral hypothalamic areas are key brain structures coordinating behavioral and autonomic stress responses and receive significant corticolimbic input (Millan, 2002). Recently, a subpopulation of hypothalamic neurons which produce the neuropeptides orexins (hypocretins), have been identified as possible substrates in the modulation of pain and stress through projections to the PAG, brainstem and paraventricular thalamus

(PVT; Peyron et al., 1998; Marcus et al., 2001). For example, injections of orexin-A into the rostral ventromedial medulla produced antinociceptive-like behavior in response to formalin in male rats (Azhdari-Zarmehri et al., 2014). Interestingly, maternal separation was found to alter orexin system reactivity to psychological stress in adulthood (James et al., 2014). Despite these results, only a few studies have assessed orexin system recruitment and also studied behavioral responses following noxious stimuli. Watanabe et al. (2005) demonstrated that prepro-orexin knockout mice exhibited increased nociceptive behaviors in response to peripheral inflammation and reduced stress-induced analgesia following footshock stress in adulthood compared to wild type mice. Further, Heidari-Oranjaghi et al. (2012) found that intracerebroventricular (i.c.v.) injections of the OX₁ orexin receptor antagonist SB-334867, resulted in increased pain responses to formalin, but only following both restraint and swim stress. Recently, we demonstrated that rats exposed to neonatal LPS displayed enhanced formalin-induced flinching but not licking responses in adolescence at postnatal day (PND) 22 (Zouikr et al., 2014b). These behavioral changes were accompanied by attenuated Fos-protein cell counts in the rostral dorsal PAG as well as the rostral and caudal axes of the ventrolateral PAG. But, whether exposure to early life immune challenge alters orexin system function in response to a noxious stimulus in adulthood (PND 80–97) has not been determined. This information will improve our understanding as to how neonatal physical and emotional insults can rewire the brain pathways involved in pain and stress processing.

Therefore, the aim of the present study was to assess the effect of neonatal LPS exposure on the reactivity of the orexin system, as assessed by Fos-protein expression, to formalin challenge in adulthood (PND 80–97). Given the relationship between stress, nociception and the orexin system, it was hypothesized that rats exposed to LPS in early life would demonstrate increased pain and grooming responses to formalin in adulthood. We predicted that these behavioral responses would be accompanied by evidence of increased orexin cell activity, and that this recruitment pattern would also be reflected in Fos-responses in downstream projection targets of this system.

METHODS AND MATERIALS

ETHICS STATEMENT

All experiments performed were approved by the University of Newcastle Animal Care and Ethics Committee, and carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific practice.

ANIMALS

Four experimentally naïve female Wistar rats were obtained from the University of Newcastle Animal house and bred with two experimentally naïve males in the University of Newcastle vivarium. On PND 3 and 5 a random subset of animals from each litter were administered LPS as a neonatal immune challenge (detailed below). A total of 13 male offspring were included in this study, 6 LPS-exposed animals and 7 saline animals. On PND 21, animals were weaned with 2 animals/cage (41.5 × 28 × 22 cm cages; Mascot Wire Works, Sydney). Food (Rat and Mouse Pellets, Glen

Forest, Western Australia) and water were available *ad libitum* and rats were maintained on a 12 h light (0600–1800): 12 h dark cycle. Temperature was maintained at 20 ± 2°C and humidity was kept at 34 ± 2%.

NEONATAL LPS CHALLENGE

The neonatal LPS procedure was performed as per previously published procedures (Walker et al., 2009). Between 0900 and 1000 h on PND 3 and 5 (birth as PND 1), pups in the LPS treatment condition were briefly removed from their home cages and administered 0.05 mg/kg LPS (intraperitoneally, i.p., LPS from *Salmonella enterica*, serotype *enteritidis*, Sigma-Aldrich, USA, dissolved in 20 µl sterile pyrogen-free saline). Saline controls were given an equal volume of sterile saline (Livingstone International, Australia). The timing of injection and dose were selected as it had been previously shown to produce a sustained immune response with no mortality (Walker et al., 2006).

FORMALIN TEST

This test has been previously described in our laboratory (Zouikr et al., 2014c) and is a well-validated model of behavioral responses to nociceptive stimuli (early phase or phase 1, first 5 min), inhibition of nociceptive responding (interphase, 5–15 min) and inflammation (late phase or phase 2, 15–60 min; Wheeler-Aceto and Cowan, 1991; Tjølsen et al., 1992; Henry et al., 1999; Fischer et al., 2014). Between PND 80 and 97 animals were removed from their home cage and a subcutaneous injection of formalin (2.25%) was administered into the plantar surface of the right hindpaw of all rats (50 µl formaldehyde 36.5–38%; Biolab Ltd, Victoria, Australia; sodium chloride injection BP 0.9% Pfizer, Australia). This volume and concentration of solution has been previously shown by our group to produce the biphasic response of the formalin test (Zouikr et al., 2013). A saline control injection into the hindpaw was not included, as this has been found to produce no pain-induced behaviors including licking and flinching (Guy and Abbott, 1992; Butkevich and Vershinina, 2001). The behavioral response to formalin was examined in transparent plexiglas boxes (30 × 30 × 30 cm) for 1 h. A researcher blind to experimental conditions scored behavioral responses using JWatcher ethograph software (version 0.9, Macquarie University, Sydney, Australia). Pain behaviors were measured by the number of flinches of the injected paw and the time spent licking the injected paw. Exploratory behavior was measured as the time spent rearing during the formalin test and grooming behavior during the formalin test was analyzed as the time spent grooming the forepaws.

IMMUNOHISTOCHEMISTRY

Ninety minutes following formalin injections, rats were deeply anaesthetized with an overdose of sodium pentobarbitone (200 mg/kg; i.p.; Virbac, Australia) and transcardially perfused with 200 mL of 0.1 M phosphate buffered saline followed by 500 mL of 4% paraformaldehyde (pH 9.5). Brains were then removed and postfixed in 4% paraformaldehyde (24 h at 4°C) and then stored in 15% sucrose until sectioning. Serial coronal sections of the rostral forebrain (40-µm) and caudal mid-brain (50-µm) were cut using a freezing microtome (Leica

Microsystems, SM2000R). A 1-in-4 series of sections from the hypothalamus (bregma -2.28 to -3.24), the PVT (bregma -2.28 to -3.24), the paraventricular nucleus of the hypothalamus (PVN; bregma -1.46 to -1.78) and the amygdala (bregma -2.28 to -3.08), and a 1-in-5 series of sections from the PAG (bregma -6.69 to -8.19) were processed for immunohistochemical detection of Fos-protein (72 h, 1:10000, rabbit polyclonal, sc-52, Santa Cruz Biotechnology, CA, USA) as described previously in detail (Dayas et al., 2008; James et al., 2014). Following primary antibody application, sections were incubated in a secondary antibody (2 h, 1:300, donkey anti rabbit, 711-065-152, Jackson IR, PA, USA). Hypothalamic sections were dual-labeled for orexin-A, also likely detecting pre-pro orexin (48 h, 1:15000, orexin-A antibody, goat polyclonal, sc-8070, Santa Cruz Biotechnology). The selectivity of this antibody has been illustrated in a recent study by Blanco-Centurion et al. (2013). Please see Supplementary Material S1 outlining the details of the specificity of the orexin-A antibody. Following orexin primary antibody application, sections were subsequently incubated in a secondary antibody (2 h, 1:400, donkey anti goat, 705-065-147, Jackson IR, PA, USA). An equal number of animals from each treatment group were included in each individual immunohistochemical run.

Bilateral counts of single-labeled Fos-positive cells were made in the PVT, PVN, basolateral amygdala (BLA), medial nucleus of the amygdala (MeA), the central nucleus of the amygdala including both lateral and medial subdivisions (CeL, CeM respectively), the dorsal PAG (including both the dorsomedial and dorsolateral columns), and the lateral and ventrolateral PAG using Metamorph Imaging System Software (Version 7.5; Molecular Devices Analytical Technologies) at 10x magnification (Olympus CX40). Quantification of Fos-positive cells was determined by creating a region of interest for each brain structure and a thresholding procedure was used to quantify Fos expression. Counts of Fos-positive orexin cells were made in the dorsomedial hypothalamus (DMH), the perifornical area (PFA), and the lateral hypothalamus (LH) by one observer, blind to treatment, using a 20x microscopic objective (Olympus CX40). The DMH was defined as the area between the third ventricle and the medial side of the PFA, the PFA was defined as the area surrounding the fornix and the LH was defined as the area from the lateral side of the PFA to the optic tract (Laorden et al., 2012; James et al., 2014). All brain coordinates were adapted from the Paxinos and Watson atlas (Paxinos and Watson, 2007).

DATA ANALYSIS

Initial analysis of covariance (ANCOVA) analyses revealed no significant effect of litter size on both behavioral and brain comparisons. Behavioral data was analyzed across neonatal treatment group using one-way between subjects ANOVAs for each phase of the formalin test. Using area under the curve calculations for the formalin test, phase 1 was considered the first 5 min, the interphase was the sum of 6–15 min, and phase 2 was the sum of responses from 16–60 min. Fos-protein immunohistochemical data was analyzed using two-way between subjects ANOVAs comparing neonatal treatment and brain region where appropriate, alternatively one-way ANOVAs were used. *Post-hoc* comparisons were assessed using least significant differences tests. Pearson's

correlations were used to examine the relationship between the percentage of Fos-positive orexin cells in the subregions of the hypothalamus and behavioral responses of animals in phase 1 and the interphase of the formalin test. All statistical analyses were conducted using IBM SPSS V21 with an alpha value of 0.05. All figures are represented as means with standard errors.

RESULTS

EFFECT OF NEONATAL LPS EXPOSURE ON FORMALIN-INDUCED NOCICEPTIVE BEHAVIOR

One-way between subjects ANOVAs revealed no significant effect of neonatal treatment on flinching behavior in any phase of the formalin test (p 's > 0.05 ; **Figures 1A,B**). An analysis of licking responses revealed a significant effect of neonatal treatment during the interphase with LPS animals displaying reduced time spent licking compared to saline controls [$F_{(1, 12)} = 3.795$, $p = 0.042$; **Figures 1C,D**].

EFFECT OF LPS ON FORMALIN-INDUCED EXPLORATORY AND GROOMING BEHAVIORS

One-way between subjects ANOVAs revealed a significant effect of neonatal treatment on the total time spent grooming in the interphase of the formalin test with LPS-treated animals spending more time grooming compared to saline animals [$F_{(1, 12)} = 6.96$, $p = 0.014$; **Figures 2A,B**]. LPS-treated animals also displayed significantly increased time grooming from phase 1 to the interphase of the formalin test compared to saline animals [$F_{(1, 12)} = 5.538$, $p = 0.022$; **Figure 2B**]. ANOVA also revealed no significant effects of neonatal treatment on time spent grooming during phase 1 or phase 2, nor for time spent rearing in any phase of the formalin test (p 's > 0.05 ; **Figures 2C,D**).

EFFECT OF NEONATAL IMMUNE CHALLENGE ON OREXIN CELL REACTIVITY IN RESPONSE TO FORMALIN IN ADULTHOOD

There were no differences found in the total number of orexin-positive cells between LPS animals and saline controls in any subregion of the hypothalamus (p 's > 0.05 , **Table 1**). However, a two-way between subjects ANOVA revealed a significant main effect of neonatal treatment on the percentage of Fos-positive orexin cells with LPS-exposed rats exhibiting a greater percentage of Fos-positive orexin cells compared to saline-treated animals [$F_{(1, 246)} = 11.863$, $p = 0.001$, **Figure 3**]. Additionally, there was a significant interaction between neonatal treatment and hypothalamic subregion on the percentage of Fos-positive orexin cells [$F_{(2, 246)} = 3.387$, $p = 0.035$]. *Post-hoc* comparisons revealed that LPS animals displayed significantly greater percentages of Fos-positive orexin cells in the DMH and PFA compared to saline controls (p 's < 0.05) however, no differences were found in the LH ($p > 0.05$, **Figure 3**).

EFFECT OF LPS ON FOS-PROTEIN EXPRESSION IN THE PVT, PVN, AMYGDALA AND PAG

A one-way between subjects ANOVA revealed that LPS-treated animals exhibited significantly greater numbers of Fos-positive cells in the PVT and PVN compared to saline controls [$F_{(1, 82)} = 59.055$, $p < 0.001$; $F_{(1, 31)} = 9.370$, $p = 0.005$; **Figures 4A,B**].

A two-way between subjects ANOVA revealed a significant interaction of neonatal treatment and amygdala subregion on the

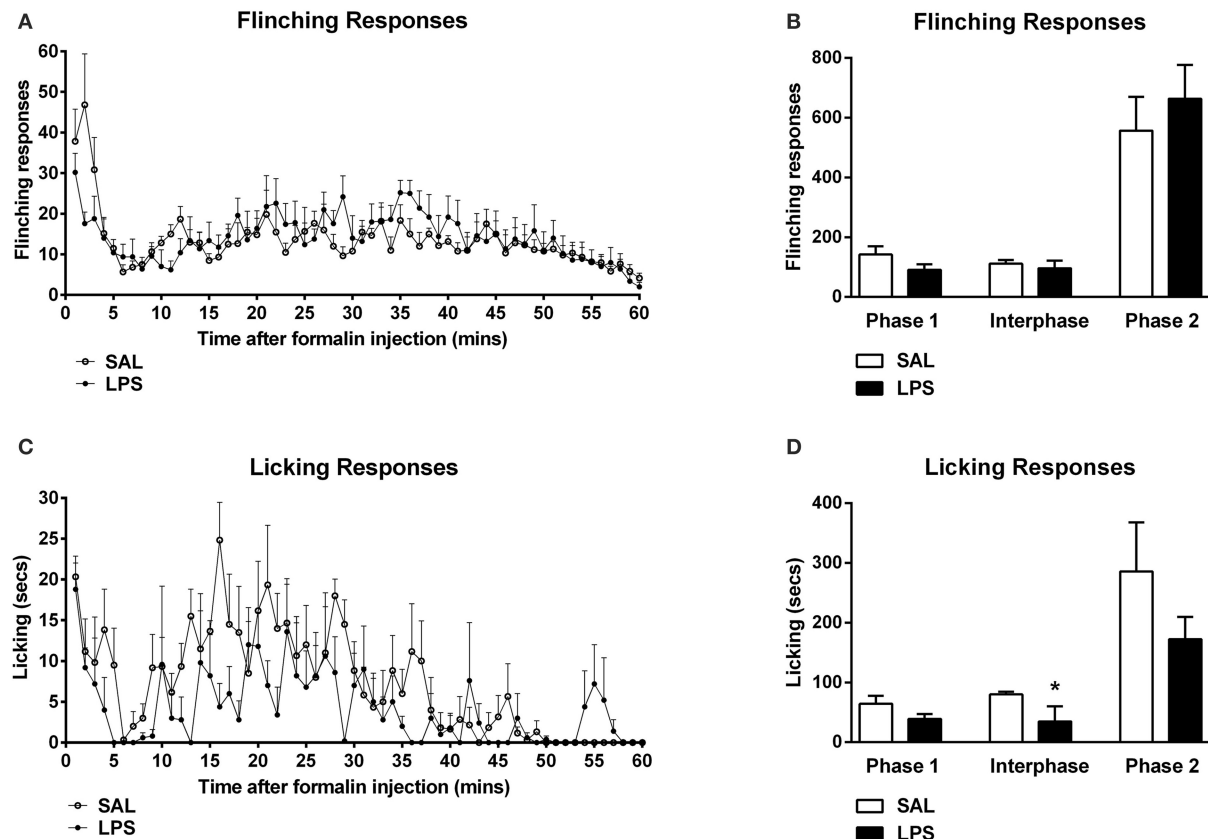


FIGURE 1 | Neonatal LPS exposure enhanced pain suppression of licking behaviors during the interphase in response to formalin in adulthood.

Time course of flinching and licking responses for LPS and saline rats exposed to formalin in adulthood (A,C). No differences were observed in flinching behavior across any phase of the formalin test between LPS-treated

rats and saline controls (B). LPS-exposed rats exhibited a potentiated inhibitory pain response to formalin in licking behaviors during the interphase, with no effect of neonatal treatment on phase 1 or phase 2 of the formalin test (D). Data are presented as mean + standard error. * $p < 0.05$. SAL: $n = 7$; LPS: $n = 6$.

number of Fos-positive cells [$F_{(3, 280)} = 2.896$, $p = 0.036$]. Least significant differences comparisons revealed that LPS-treated animals displayed a significantly greater number of Fos-positive cells in the MeA compared to saline animals ($p = 0.001$; Figure 4C).

A two-way between subjects ANOVA revealed a main effect of neonatal treatment on the number of Fos-positive cells within the PAG [$F_{(1, 204)} = 38.440$, $p < 0.001$]. *Post-hoc* comparisons revealed that LPS-treated animals displayed significantly greater numbers of Fos-positive cells in the dorsal ($p = 0.022$), lateral ($p < 0.001$) and ventrolateral PAG ($p < 0.001$) compared to saline animals (Figure 4D).

CORRELATIONS BETWEEN OREXIN CELL ACTIVITY AND BEHAVIORAL RESPONSES TO FORMALIN IN ADULTHOOD

Correlation analyses revealed a negative correlation between the total time spent licking in phase 1 of the formalin test and the percentage of Fos-positive orexin cells in the DMH ($r = -0.806$, $p = 0.005$) and the PFA ($r = -0.691$, $p = 0.027$; Figures 5A,B). In the interphase of the formalin test, total time spent grooming was also positively correlated with the percentage of Fos-positive orexin cells in the DMH ($r = 0.634$, $p = 0.049$) and PFA ($r = 0.673$, $p = 0.033$, Figures 5C,D).

DISCUSSION

In the current study we show that animals exposed to LPS in early life exhibit altered behavioral responses to a formalin challenge in adulthood. LPS-treated animals displayed increased orexin cell activity, as assessed by Fos-like immunoreactivity in the DMH and PFA but not LH. Additionally, increases in numbers of Fos-positive neurons were observed in stress and pain sensitive brain regions that express orexin receptors including the PVT, PVN, MeA, and PAG.

INCREASED OREXIN CELL REACTIVITY TO FORMALIN FOLLOWING AN EARLY LIFE IMMUNE CHALLENGE

The primary aim of this study was to examine the response of orexin neurons to an acute formalin injection in adulthood following early life LPS exposure. Using Fos-protein immunohistochemistry, we observed an increase in the recruitment of DMH and PFA orexin neurons in LPS-exposed rats compared to controls. Interestingly, no change in orexin cell reactivity to formalin was observed in the LH. This differential recruitment pattern is interesting given the recent suggestions of a dichotomy of function between medial and lateral orexin cell populations (Estabrooke et al., 2001; Harris and Aston-Jones, 2006). For

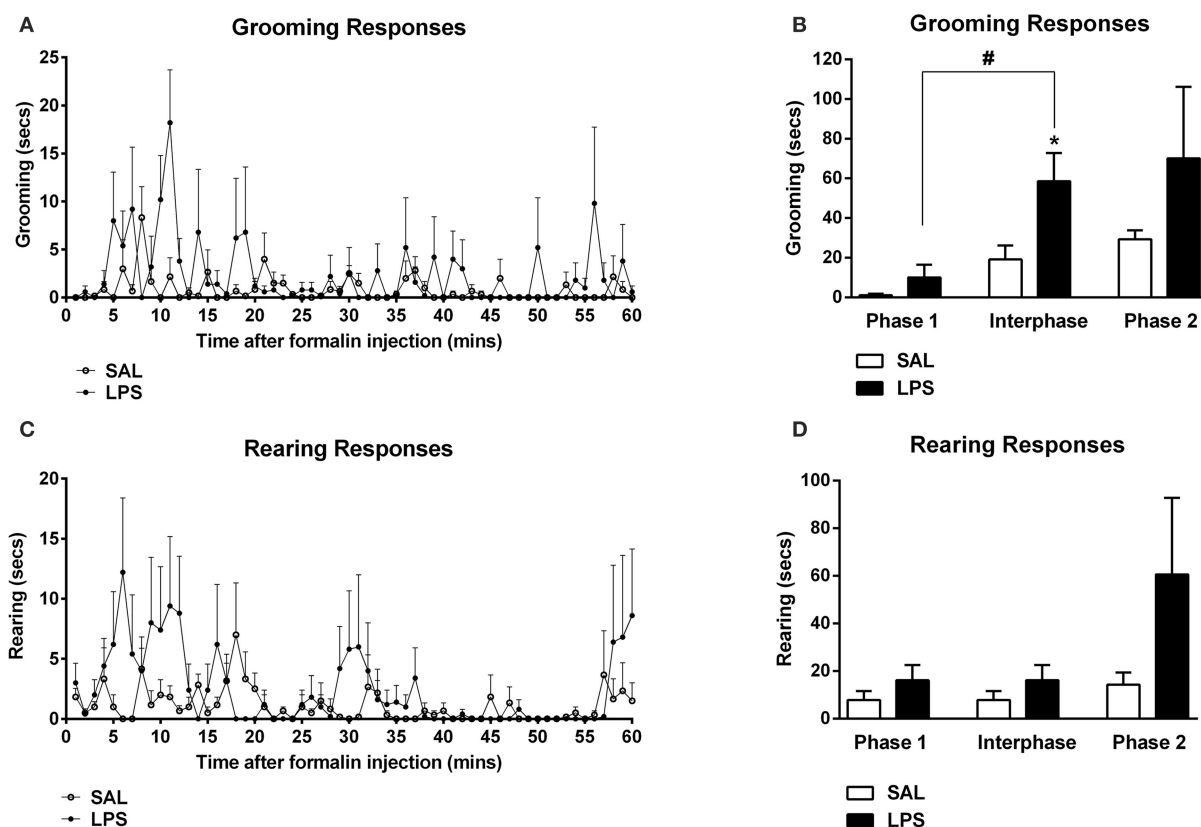


FIGURE 2 | An early life immune challenge increased self-grooming responses to formalin in adulthood. Time course of grooming and rearing responses for LPS and saline animals (A,C). LPS-treated rats displayed increased grooming behaviors in response to formalin when compared to

saline animals (B). There was no effect of neonatal treatment on rearing behavior across all phases of the formalin test (D). Data are presented as mean + standard error. * $p < 0.05$, # $p < 0.05$ phase 1 vs. interphase. SAL: $n = 7$; LPS: $n = 6$.

Table 1 | Orexin cell expression in each subregion of the hypothalamus.

Treatment	Region	Orexin number	Fos-Orexin number
Saline ($n = 6$)	DMH	30.77 ± 3.65	11.97 ± 1.55
	PFA	111.33 ± 10.56	19.99 ± 2.71
	LH	35.18 ± 2.33	3.35 ± 0.41
LPS ($n = 6$)	DMH	30.89 ± 2.85	16.89 ± 2.34
	PFA	101.59 ± 7.02	29.62 ± 3.77
	LH	39.60 ± 3.13	4.72 ± 0.63

Data presented as average number of orexin cells or Fos-positive orexin cells \pm standard error mean in the dorsomedial hypothalamus (DMH), perifornical area (PFA), and lateral hypothalamus (LH).

example, orexin neurons in the DMH and PFA have been linked with arousal and the modulation of the stress response whereas those located in the LH have been linked with reward (Harris et al., 2005). In support, rats administered sodium-lactate to induce panic anxiety exhibited increased activation of DMH-PFA but not LH orexin neurons (Johnson et al., 2010). Thus, arousal and stress responsive orexin cells may be preferentially sensitized by early life immune stress.

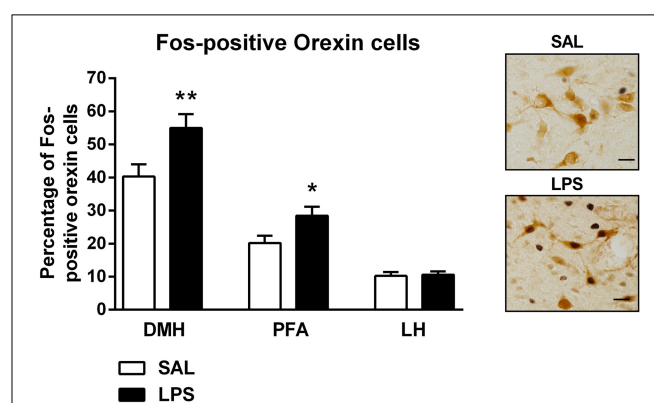


FIGURE 3 | LPS resulted in an increase in the percentage of Fos-positive orexin cells in both the dorsomedial (DMH) and perifornical (PFA) subregions of the hypothalamus. The percentage of Fos-positive orexin cells in the DMH and PFA was significantly higher in LPS-exposed animals compared to saline controls. No differences were observed in orexin cell activity in the lateral hypothalamus (LH) across neonatal treatment groups. Photomicrographs of coronal sections of the hypothalamus immunolabeled for Fos-protein and orexin. Data are presented as mean + standard error. * $p < 0.05$, ** $p < 0.01$, scale bar 20 μ m. SAL: $n = 6$; LPS: $n = 6$.

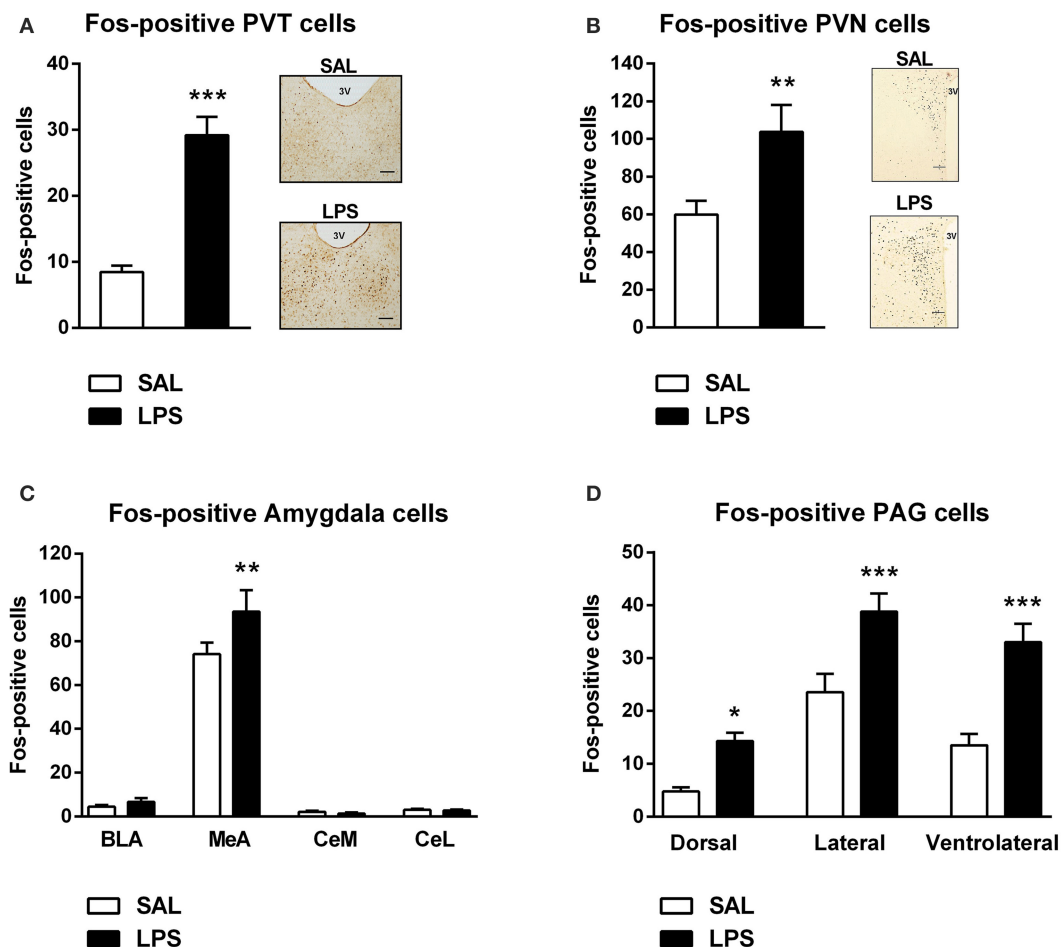


FIGURE 4 | Neonatal LPS treatment resulted in an increase in the number of Fos-positive cells in the paraventricular thalamus (PVT), paraventricular nucleus (PVN), the medial nucleus of the amygdala (MeA) and the periaqueductal gray (PAG). Early life LPS treatment resulted in an increase in Fos activity in both the PVT and PVN (A,B). Photomicrographs of coronal sections of the PVT and PVN immunolabeled

for Fos-protein (A,B). LPS-treated animals also exhibited an increased number of Fos-positive cells in the MeA (C). There was no effect of neonatal treatment on any other subregion of the amygdala. LPS-exposed rats also had increased Fos-protein expression in the dorsal, lateral and ventrolateral PAG (D). Data are presented as mean + standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, scale bar 100 μm , 3V = third ventricle. SAL: $n = 6$; LPS: $n = 6$.

It is unclear from the present study which pain sensitive afferent pathways may have been involved in the recruitment of orexin neurons. Brain sites that receive direct afferent inputs from lamina I projection neurons in the superficial dorsal horn include the ventrolateral medulla, nucleus of the solitary tract, lateral parabrachial nucleus, PAG and the thalamus (Millan, 2002; Gauriau and Bernard, 2004; Todd, 2010). The parabrachial nucleus is interesting in this regard possessing efferent projections to the amygdala and the LH (Bernard et al., 1993; Bester et al., 1997; Gauriau and Bernard, 2002). These inputs may directly recruit orexin neurons. Additionally, the spinal cord sends direct projections to the hypothalamus. For example, Burstein et al. (1987) demonstrated direct projections from three regions of the spinal gray matter, including the lateral reticulated area, the area surrounding the central canal and the marginal zone, to the hypothalamus (Burstein et al., 1987; Giesler, 1995). However, the direct input from these spinal cord regions to orexin neurons is yet to be examined. Interestingly, the stress responsive

central nucleus of the amygdala (CeA) and MeA also project to the LH (Peyron et al., 1998; Yoshida et al., 2006) and may provide “top-down” afferent input to the LH. Clearly, further work will be required to determine whether the activation of orexin neurons occurs through ascending nociceptive pathways or descending inputs from stress responsive centers such as the amygdala. It is important to acknowledge that because previous work has shown that hindpaw saline injections cause no pain-induced behaviors including licking and flinching (Guy and Abbott, 1992; Butkevich and Vershinina, 2001), we did not include a Fos control group for the formalin challenge. While it is difficult to determine the direction of change from baseline in our Fos-induced orexin cell reactivity, prior studies have shown that rats subjected to hindpaw saline injections showed no Fos labeling in the lumbar spinal cord (Yi and Barr, 1995). Additionally, Barr (2011) reported that 14 day old rat pups given saline into their hindpaw did not exhibit Fos labeling in the PVN or dorsal/lateral PAG.

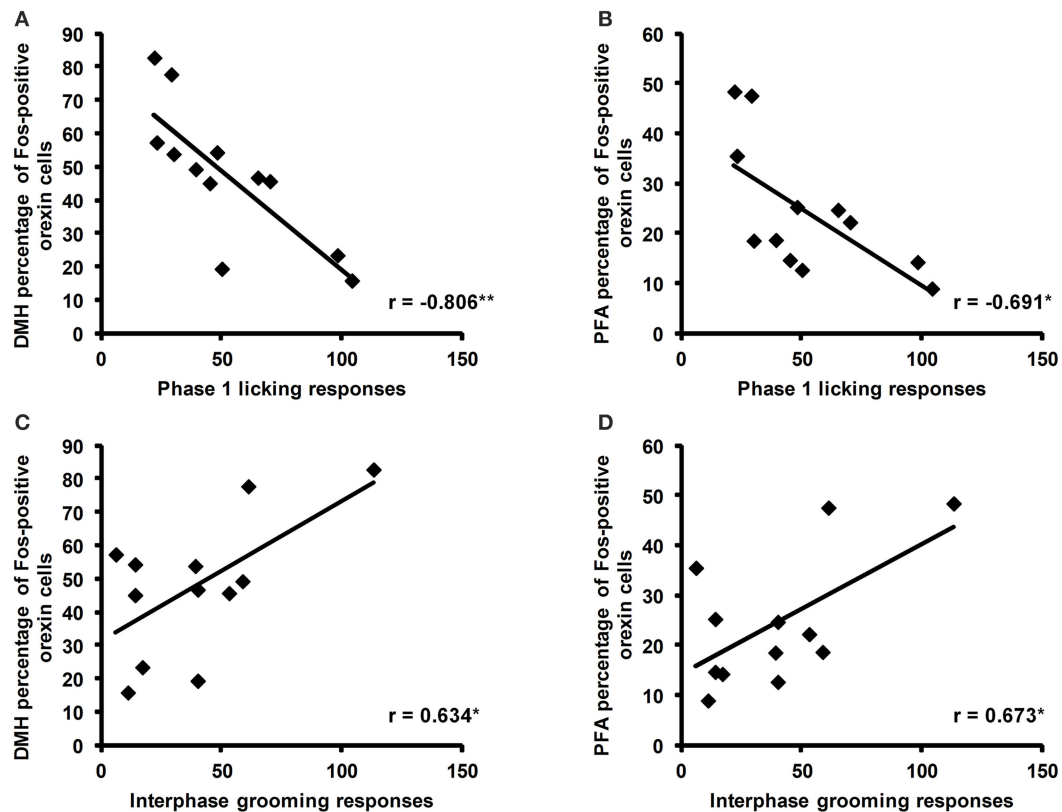


FIGURE 5 | Correlations of the percentage of Fos-positive orexin cells with licking and grooming behaviors during the formalin test. Significant negative correlations were found between the time spent licking (secs) during phase 1 of the formalin test and the percentage of Fos-positive cells in the

dorsomedial hypothalamus (DMH) and perifornical area (PFA; **A,B**). Significant positive correlations were found between the time spent grooming (secs) during the interphase of the formalin test and the percentage of Fos-positive orexin cells in the DMH and PFA (**C,D**). $^{*}p < 0.05$, $^{**}p < 0.01$. SAL: $n = 6$, LPS: $n = 6$.

ENHANCED FORMALIN-INDUCED INHIBITORY PAIN RESPONSE IN LPS-TREATED RATS

The formalin test is a well-established animal model of persistent pain (Tjølsen et al., 1992). Three distinct behavioral responses are commonly associated with the formalin test. The early phase, or phase 1, involves the direct chemical stimulation of nociceptors, the interphase denotes the active inhibition of pain and the late phase, or phase 2, represents the inflammatory pain response (Dubuisson and Dennis, 1977; Tjølsen et al., 1992; Franklin and Abbott, 1993; Henry et al., 1999; Fischer et al., 2014). Importantly, of these three phases, the interphase has received the least attention. Here, we demonstrated decreased licking behaviors in the interphase after formalin challenge in LPS animals. This is perhaps not surprising given that increased grooming may override or mask changes in licking behavior. Although a slight trend was observed in phase 2 we found no significant changes in flinching behavior in response to formalin between treatment groups in male rats. This is in contrast with our recently published work whereby adult rats exposed to a neonatal LPS challenge displayed a significantly increased flinching response during the late phase of the formalin test (Zouikr et al., 2014a). This discrepancy could be attributed to methodological differences, presently, we analyzed the interphase whereas Zouikr et al. (2014a) focused on phase 2 of the formalin test. Further, differences in behavioral

scoring methods (manual vs. software) may have contributed to this discrepancy. Lastly, enhanced grooming in the current cohort may have masked changes in flinching. The putative enhancement of pain suppression observed in the interphase of LPS-treated rats is interesting given the increase in orexin cell reactivity in LPS-exposed rats. Furthermore, overall licking behaviors of LPS-treated rats negatively correlated with the percentage of Fos-positive orexin cells in the DMH and PFA. These findings are consistent with data implicating the orexin system in descending inhibitory pain pathway control (Bingham et al., 2001).

In the current study we also observed an increase in Fos-protein in all subregions of the PAG in neonatally LPS-treated rats compared to controls. It is important to note that the PAG is anatomically organized into longitudinal columns including the dorsal PAG, the lateral PAG and the ventrolateral PAG and each column plays distinct roles in the response to both stress and pain (Bandler and Shipley, 1994). The PAG has a well-characterized role in analgesia and stress coping and is the recipient of orexin-ergic innervation (Peyron et al., 1998; Keay and Bandler, 2002; Gebhart, 2004; Chapman et al., 2008). It is possible that the increase in orexin cell activity and the enhanced inhibitory pain response to formalin contribute to the active inhibition of pain through projections to the PAG. In support, Azhdari-Zarmehri et al. (2011) found that microinjections of orexin-A into the

PAG enhanced inhibitory pain responses in the interphase in response to formalin in adulthood. Indeed, orexin-A has been shown to reduce inhibitory postsynaptic currents in ventrolateral PAG neurons that directly project to the rostral ventromedulla (Ho et al., 2011). Of the PAG columns, the lateral PAG tends to receive stronger orexin inputs, which is interesting given its role in active coping strategies in the response to pain (Bandler and Shipley, 1994; Yoshida et al., 2006). Accordingly, the pattern of Fos activity we observed in the PAG may reflect affective coping strategies in response to formalin-evoked stress. For example, Keay and Bandler (2001) demonstrated that increased Fos activity in both the ventrolateral and lateral PAG is linked with altered emotional coping responses to persistent pain. These results may help explain the affective behavioral changes observed in response to formalin as described in more detail below.

EARLY LIFE LPS EVOKED AN AFFECTIVE-LIKE BEHAVIORAL RESPONSE TO FORMALIN

We also examined the affective-like responses to formalin in adulthood by assessing both grooming and rearing. Self-grooming is thought to reflect a coping mechanism to produce de-arousal (Spruijt et al., 1992; Kalueff and Tuohimaa, 2005; Lariviere et al., 2011). In our study, LPS-exposed animals spent significantly more time grooming in response to formalin in adulthood compared to saline controls. No differences were observed in rearing behavior. In keeping with our findings, Aloisi et al. (1998) demonstrated that exposure to acute restraint stress in adulthood increased self-grooming during the interphase of the formalin test. Interestingly, our study found that an increase in orexinergic activity was correlated with an increase in grooming behavior following formalin injection. These data are in line with previous research implicating dysregulated orexin system function in affective behavioral responses to stress or adverse experiences in adulthood (Johnson et al., 2010; Li et al., 2010; James et al., 2014; Yeoh et al., 2014). Further, Low and Fitzgerald (2012) have demonstrated an increase in the number of Fos-positive orexin cells in animals exposed to neonatal pain followed by later life pain. Low and Fitzgerald (2012) also found this orexinergic activity to be negatively correlated with rearing behavior. Together these results suggest that the orexin system may be susceptible to early life immune or emotional challenges, which promote neuroadaptations that manifest as dysregulated behavioral and neural responses to painful stimuli in later life.

FOS-PROTEIN EXPRESSION IN STRESS RESPONSIVE BRAIN REGIONS

We identified an increased pattern of Fos-protein in brain regions that are known to receive strong orexinergic input and are involved in the neuroendocrine and behavioral response to stress or pain modulation (Peyron et al., 1998; Marcus et al., 2001; Vanegas and Schaible, 2004). The brain regions we examined were the PVT, PVN, amygdala and PAG. LPS-exposed animals displayed increased Fos-protein expression in the PVT and PVN. The PVN and PVT both play an important role in the neuroendocrine and autonomic responses to stress. PVN corticotrophin-releasing factor cells constitute the apex of the hypothalamic-pituitary-adrenal (HPA) axis and the PVT is involved in regulating the HPA axis response to chronic stressors

(Bhatnagar and Dallman, 1998; Dayas et al., 2004; Kirouac et al., 2005). Increased Fos immunoreactivity in the PVN and PVT is therefore consistent with other studies demonstrating activation of these stress response systems to a variety of physical stressors including painful stimuli such as cold and formalin-induced pain (Pacák and Palkovits, 2001).

Surprisingly, we found no changes in the numbers of Fos-positive CeA nuclei between treatment groups. This result contrasts previous research demonstrating increased CeA activity in response to physical stressors including persistent pain and acute pain or stress (Dayas et al., 2001; Neugebauer et al., 2004). Notably, orexin-immunoreactive fibers and orexin receptors are also observed in other subregions of the amygdala including the MeA (Peyron et al., 1998; Marcus et al., 2001). The MeA is typically sensitive to psychological stressors and has recently been identified as a central site mediating repetitive self-grooming behaviors, which has linked it to a range of neuropsychiatric disorders (Dayas et al., 1999, 2001; LeDoux, 2000; Hong et al., 2014). Interestingly, we observed a significant increase in Fos-positive nuclei in the MeA of LPS-exposed animals, an effect associated with elevated self-grooming responses to formalin. It is possible that ascending spinoparabrachial projections may recruit an orexin → MeA pathway resulting in affective-like behavioral responses to formalin. However, the MeA also provides input to DMH/PFA and may be recruited by ascending pain sensitive-pathways. Further work will be required to understand the hierarchical sequence for how these brain regions are recruited by formalin.

Taken together, the behavioral data presented here confirmed that animals exposed to an early life immune challenge exhibited an enhanced inhibition of pain during the interphase. These changes were associated with increased grooming behavior, which was strongly correlated with numbers of Fos-positive orexin neurons in the DMH/PFA. Our results are interesting given evidence that patients suffering from chronic pain disorders tend to suffer more from the affective disturbances of pain than frank pain itself (Crombez et al., 1999). Further, processes modulated by the orexin system eg. sleep, feeding, and motivation, are often disturbed in people suffering chronic pain states (Dersh et al., 2002). Thus, pharmacological or non-pharmacological interventions that restore normal orexin system function may prove beneficial in the treatment of chronic pain states.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fnins.2015.00065/abstract>

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Neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term high fat diet

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Neonatal obesity predisposes individuals to obesity throughout life. In rats, neonatal overfeeding also leads to early accelerated weight gain that persists into adulthood. The phenotype is associated with dysfunction in a number of systems including paraventricular nucleus of the hypothalamus (PVN) responses to psychological and immune stressors. However, in many cases weight gain in neonatally overfed rats stabilizes in early adulthood so the animal does not become more obese as it ages. Here we examined if neonatal overfeeding by suckling rats in small litters predisposes them to exacerbated metabolic and central inflammatory disturbances if they are also given a high fat diet in later life. In adulthood we gave the rats normal chow, 3 days, or 3 weeks high fat diet (45% kcal from fat) and measured peripheral indices of metabolic disturbance. We also investigated hypothalamic microglial changes, as an index of central inflammation, as well as PVN responses to lipopolysaccharide (LPS). Surprisingly, neonatal overfeeding did not predispose rats to the metabolic effects of a high fat diet. Weight changes and glucose metabolism were unaffected by the early life experience. However, short term (3 day) high fat diet was associated with more microglia in the hypothalamus and a markedly exacerbated PVN response to LPS in control rats; effects not seen in the neonatally overfed. Our findings indicate neonatally overfed animals are not more susceptible to the adverse metabolic effects of a short-term high fat diet but may be less able to respond to the central effects.

Keywords: inflammation, microglia, neonatal, obesity, paraventricular nucleus of the hypothalamus (PVN)

INTRODUCTION

The developmental origins of health and disease hypothesis suggests the early life period is one of significant vulnerability to programming of physiology by environmental influences (Forsdahl, 1977; Barker and Osmond, 1986; Wadhwa et al., 2009; Spencer, 2012). In particular, early life nutrition is important in programming the development of central and peripheral mechanisms regulating feeding and metabolism, and subsequent susceptibility to overweight or obesity (Spencer, 2012, 2013a,b). As such, perinatal overfeeding has major short- and long-term physiological consequences [e.g., reviewed in (Spencer, 2012, 2013a; Habbout et al., 2013)].

We, and others, have reported neonatal overfeeding in a rodent model leads to accelerated weight gain in early life that persists long-term and is linked with immune and hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Plagemann et al., 1992; Boullu-Ciocca et al., 2005; Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012). These findings parallel those of human studies where childhood obesity significantly increases the risk an individual will become an obese adult (Whitaker et al., 1997; Stettler et al., 2005; Biro and Wien, 2010). Obese children are also more likely to suffer from immune and HPA axis disturbances as they grow up (Reeves et al., 2008; Lee, 2009; Brune and Hochberg, 2013).

Although there are clear effects of early life nutrition on later susceptibility to overweight/obesity and its pathophysiological sequelae, it is also clear not all overweight children become obese adults (Potter and Ulijaszek, 2013). Similarly, several studies of neonatal overfeeding in rodents have shown that long-term exacerbated weight gain is mild and the animals do not always exhibit hyperphagia or indices of diabetes. For instance, while some studies have demonstrated being suckled in small litters leads to increased food intake in adulthood (Oscari and McGarr, 1978; Rodrigues et al., 2007, 2009), this tends to normalize when corrected for overall body weight (Mozes et al., 2004; Xiao et al., 2007; Stefanidis and Spencer, 2012). Studies also differ in their reporting of whether neonatal overfeeding influences glucose utilization (Plagemann et al., 1999; Xiao et al., 2007). Although some neonatally overfed cohorts show insensitivity to a glucose load in a glucose tolerance test (GTT), differences in glucose uptake into adipocytes, and differences in insulin signaling (Plagemann et al., 1999; Boullu-Ciocca et al., 2005; Rodrigues et al., 2007), indicating a pre-diabetic phenotype, we have seen only mild changes in metabolic parameters (Stefanidis and Spencer, 2012). In this regard neonatal overfeeding appears to result in a moderate predisposition to excessive weight gain, with some indications of diabetic symptoms and significant, but mild, metabolic impairment.

From a pathophysiological perspective, a single adverse event or period is unlikely to be the only factor influencing long-term physiology, however. A sustained high fat diet consumed in adult rodents and humans can lead to excessive weight gain, adiposity, and indices of diabetes such as glucose intolerance and insulin resistance (Rosini et al., 2012). In this study we therefore hypothesized that the mild metabolic phenotype induced by neonatal overfeeding would predispose an animal to more substantial metabolic disturbances later in life if it is also exposed to the “second hit” challenge of a short or medium term high fat diet.

Neonatal overfeeding by suckling rat pups in small litters induces notable but moderate changes in weight gain, feeding, and metabolism throughout life that may be exacerbated by later exposure to high fat diet. However, neonatal overfeeding also causes significant and substantial peripheral and central inflammation, including a pro-inflammatory profile in systemic tissue and the hypothalamus, as well as exacerbated pro-inflammatory response to a neuroimmune challenge with bacterial mimetic lipopolysaccharide (LPS) (Tapia-Gonzalez et al., 2011; Clarke et al., 2012; Ye et al., 2012; Ziko et al., 2014). For this reason we also hypothesized the systemic and central inflammatory profile would be further exacerbated by high fat diet in adulthood in the neonatally overfed rats.

In this study we manipulated litter sizes so that Wistar rats were suckled in litters of four (small litter; SL) or 12 (control litter; CL). The former have greater access to their dam's milk, consume milk that is higher in fat, and show accelerated growth and weight gain that is maintained into adulthood (Fiorotto et al., 1991; Mozes et al., 2004). The pups were weaned onto *ad libitum* normal rat chow, but in adulthood were given either 3 days (3D) or 3 weeks (3W) high fat diet (45% kcal as fat). At the end of this period we assessed changes in weight and indices of diabetes, as well as central and peripheral markers of inflammation. We also examined liver cytokine expression and central neuronal activation in response to i.p. LPS.

MATERIALS AND METHODS

ANIMALS

We obtained timed-pregnant Wistar rats from the Animal Resources Centre, WA, Australia. After arrival at the RMIT University Animal Facility, we housed the dams at 22°C on a 12 h light/dark cycle (7 a.m. to 7 p.m.) with free access to pelleted rat chow and water. We conducted all experiments in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All procedures were approved by the RMIT University Animal Ethics Committee.

LITTER SIZE MANIPULATION

On postnatal day (P) 0, the day of birth, we removed all pups from their dams and randomly fostered them to new dams in litters of 12 (CL; controls) or 4 (SL; neonatally overfed) as we have previously described (Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014). Birth litters included in this study had a range of 8–17 pups, a mean of 13.9 ± 0.36 , and mode of 14. No

dam received any of her own pups and each new litter was made up of 50% males and 50% females. Excess pups were culled. We have previously shown this litter size manipulation results in SL pups having accelerated growth and weight gain so that they are significantly heavier by around P7 and maintain greater weights into adulthood (Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014).

EFFECTS OF NEONATAL OVERFEEDING ON SUSCEPTIBILITY TO HIGH FAT DIET

To test long-term susceptibility to the effects of high fat diet after neonatal overfeeding, we weaned the rats into same-sex litter-mate pairs on normal rat chow and kept them until P56. At this time they were allocated to the 3D or 3W high fat diet or chow groups. 3D high fat diet (23.5% fat; 45% kcal from fat; Specialty Feeds, WA, Au) was commenced at P74 and 3W high fat diet (as above) was commenced at P56. On P76, i.e., 2 days or 20 days after the onset of the high fat diet, or equivalent in chow fed (4.8% fat) controls, we gave the rats an i.p. glucose tolerance test (GTT). Rats were fasted for 3–4 h prior to testing to standardize basal glucose levels. We then quickly took each rat from its cage and nicked the end off the tail with a sharp razor blade to extract ~20 µL of baseline blood sample into a heparinized capillary tube for measurement of plasma triglycerides. These and liver triglycerides were later determined using calorimetric enzymatic GPO-PAP assays (Roche Diagnostics, IN, USA). Blood samples were kept on ice until the end of the experiment, when they were centrifuged and the plasma aliquots stored at –20°C until assayed. We also measured basal glucose levels at this time using an Accu-Chek Performa blood glucose meter (Roche Diagnostics; Castle Hill, NSW, Au). We then injected each rat with 1.5 g/kg glucose and measured glucose levels at 15, 30, 45, 60, and 90 min after injection.

Two days later, i.e., after 4 or 22 days high fat diet (or chow), the pairs of rats were then randomly allocated into the saline or LPS group. We gave each rat an i.p. injection of LPS (*E. coli*, serotype 026:B6; L-3755; Sigma, St Louis, MO, USA; 100 µg/kg), or pyrogen-free saline. At 120 min after injection, we deeply anesthetized the rats with Lethobarb (~150 mg/kg pentobarbitone sodium, i.p.). We hemisected each rat below the diaphragm and used it for fresh tissue collection and for cardiac perfusion to obtain fixed brains. Thus, we removed livers and male epididymal or female perirenal fat pads. Tissues were weighed and snap-frozen in liquid nitrogen. For the brains, we perfused the rats transcardially with phosphate buffered saline (PBS; 4°C, pH 7.4) followed by 4% paraformaldehyde in PBS (4°C, pH 7.4). We then removed the brains and post-fixed them for 4 h in the same fixative before placing them in cryoprotectant with 20% sucrose in PBS (4°C). We cut forebrains into 30 µm coronal sections using a cryostat. All experiments were initiated between 0900 and 1200 h to limit potential effects of circadian rhythms on any parameters measured.

INFLAMMATORY GENE EXPRESSION

To assess changes in peripheral markers of inflammation, we measured mRNA expression levels of the free fatty acid and

LPS receptor, toll-like receptor 4 (TLR4), downstream transcription factor, nuclear factor κ B (NF κ B), as well as representative pro- and anti-inflammatory cytokines, interleukin (IL)-10, tumor necrosis factor (TNF) α , IL-1 β , and IL-6 in the liver and adipose tissues. We isolated RNA from our snap-frozen liver and fat samples using QIAzol and an RNeasy purification kit (QIAGEN, Valencia, CA, USA). The extracted RNA (1 μ g) was transcribed to complementary DNA with an iScript cDNA synthesis kit; (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. We then performed rt-PCR with Taqman Gene Expression Assays (Applied Biosystems, Mulgrave, Vic, Au). We measured fold differences in target mRNA expression with the δ -cycle threshold method by comparison with the house-keeping gene, 18S (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Data are expressed as mRNA relative fold change as described previously (Mouihate et al., 2010; Clarke et al., 2012; Spencer et al., 2012).

LIVER CYTOKINE EXPRESSION

To further assess changes in peripheral markers of inflammation, we examined concentrations of a number of pro- and anti-inflammatory cytokines in the liver using a Bio-Plex assay allowing multiple analytes to be assessed in one sample. Liver samples were lysed using Bio-Plex cell lysis kit (Bio-Rad) according to the manufacturer's instructions. The total protein concentration of the lysates was determined using the bicinchoninic acid (BCA) assay (PierceTM BCA Protein Assay Kit, Thermo Scientific). Samples were then diluted in Bio-Plex Sample Diluent (containing 0.5% BSA) and assayed in a final concentration of 500 μ g/mL using a magnetic beads-based Bio-Plex Pro rat TH1/TH2 12-Plex (Bio-Rad) assay. The assays were performed using the Bio-Plex MAGPIXTM instrument and the data were analyzed using Bio-Plex Manager Software 6.1 (Bio-Rad). Female IL-13, granulocyte macrophage colony-stimulating factor, and interferon gamma were not detectable and these were low and not significantly different between groups in the males, so are not reported here.

BRAIN MICROGLIA AND NEURONAL RESPONSES TO IMMUNE CHALLENGE

To assess the influence of early life overfeeding and adult high fat diet on central inflammation, we immunolabelled sections through the hypothalamus for ionized calcium-binding adapter molecule-1 (Iba-1; a marker for microglia/macrophages), seen as amber staining or Fos (a marker of neuronal activation), seen as a black nuclear deposit, as previously described (Spencer et al., 2004a,b; Mouihate et al., 2010; Ziko et al., 2014). Briefly, we incubated separate one-in-five series of forebrain sections from each animal in primary antibody overnight at 4°C (Iba-1, 1:1000; rabbit; Wako Chemicals USA Inc., Richmond, VA, USA or Fos, 1:10,000; rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by secondary antibody (1.5 h; 1:200, Iba-1, 1:500, Fos; biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite kit; Vector). The sections were then incubated in diaminobenzidine (DAB) with (black; Fos) or without (amber; Iba1) nickel and colbalt, to

visualize the HRP activity. The reactions were terminated once an optimal contrast between specific cellular and non-specific background labeling was reached. Randomly selected brains from each of the treatment groups were processed at the same time in batches. Sections were then air-dried, dehydrated in a series of alcohols, cleared in histolene, and coverslipped.

CELL COUNTS

An experimenter blinded to treatment condition assessed the sections for differences in numbers of cells with Iba-1 labeling and in density of Iba-1 labeling using photomicrograph images imported into image analysis software Image J (National Institutes of Health, Bethesda, MD, USA), as we have previously described (Beynon and Walker, 2012; Radler et al., 2014; Ziko et al., 2014). Briefly, we took all photomicrograph images from an Olympus upright microscope (Olympus BX41; Olympus, Melbourne, Vic, Au) with a 20 times objective lens using an Olympus DP72 digital camera (Olympus) and LabSens image capture software v1.6 (Olympus) software. Images were taken at 4140 \times 3096 pixel density. They were then imported into and processed using Image J. We auto-subtracted background and converted each image to 16 bit for analysis, then cropped each image to take a representative 1602 \times 1602 pixel sample from each region of interest within each section. We then assessed numbers of Iba-1-positive cells and density of staining using the thresholding method, as described (Beynon and Walker, 2012; Radler et al., 2014; Ziko et al., 2014), in the paraventricular nucleus of the hypothalamus (PVN; \sim 1.80 and 1.95 mm caudal to bregma) and in the arcuate nucleus (ARC; \sim 2.04 to $-$ 3.09 mm relative to bregma). Brain regions were identified according to the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson, 2009). For each region, we sampled the left and right sides across two sections of the PVN and five sections of the ARC, 150 μ m apart. We saw no differences between left and right hemispheres or rostrocaudal level for any of the regions, so we then took the sum of the images as our sampled result.

An experimenter, blinded to the group treatments, also carried out counts of cells positive for Fos-immunoreactivity in the PVN over two sections (\sim 1.80 and 1.95 mm caudal to bregma), in the dorsal (d) and ventral (v) bed nucleus of the stria terminalis (BNST) over four sections (\sim 0.24 to $-$ 0.36 mm relative to bregma), in the medial preoptic area (MPOA) and vascular organ of the lamina terminalis (OVLt) over two sections (\sim 0.36 and 0.51 mm rostral to bregma) and in the ventromedial (VM) POA over two sections (at and 0.15 mm caudal to bregma).

DATA ANALYSIS

We compared pre-weaning body weights between CL and SL rats using an analysis of variance (ANOVA) with repeated measures, with litter size as the between factor and age as the repeated measure. When a significant interaction was found between litter size and age we performed Student's unpaired *t*-tests for each time point. We compared adult parameters using multi-factorial ANOVAs with litter size, sex, adult diet, and LPS treatment as between factors where appropriate, with Tukey *post-hoc* comparisons where significant main effects or interactions were found. We also included time (min) as a repeated measure in analysis of

plasma glucose concentrations. Data are presented as the mean + standard error of the mean (SEM). Statistical significance was assumed when $P \leq 0.05$. Statistical details are reported in the figure legends.

RESULTS

WEIGHT GAIN WITH NEONATAL OVERFEEDING

As we, and others, have previously reported (Spencer and Tilbrook, 2009; Clarke et al., 2012; Ziko et al., 2014), being suckled in SL leads to accelerated weight gain and this is maintained into adulthood compared with rats from CL. Thus, being raised in SL led to pups being significantly heavier by as early as P7 and this was maintained throughout the suckling period (Figure 1A) and into adulthood (Figure 1B).

WEIGHT GAIN, FOOD INTAKE, AND CALORIC EFFICIENCY WITH HIGH FAT DIET IN ADULTHOOD

Neonatal overfeeding did not cause significant differences in the weight gained with the 3D high fat diet in males or females (Figures 2A,E). There were significant effects of sex and diet, with females gaining less weight over the period than males, and those on high fat diet gaining less weight than those on standard rat chow, but there were no differences between relevant groups with *post-hoc* comparisons. After 3W of high fat diet, all female groups had gained less weight than all male groups. There was also an effect of litter size, with SL gaining more weight than CL but no differences between relevant groups with *post-hoc* comparisons (Figures 2I,M).

Consistent with their size, females ate less than males in both the 3D and 3W analyses. There was also a significant effect of diet on food intake after 3W, with high fat diet-fed rats eating fewer grams of food than standard chow-fed rats, in total and for each of the 3 weeks (Figures 2B,F,J,N).

Calculations of total energy consumption revealed the high fat diet groups consumed more energy than the chow groups at 3D and 3W, and males ate more than females. However, there was no influence of neonatal overfeeding on total energy consumption (Figures 2C,G,K,O).

Caloric efficiency is a measure of the ability to convert calories into body weight. Thus, a reduced caloric efficiency reflects the need to consume more calories to maintain body weight. 3D high fat diet significantly reduced caloric efficiency in SL but not

CL male and female rats (Figures 2D,H). The 3W high fat diet significantly reduced caloric efficiency in SL but not CL females (Figures 2L,P).

FAT MASS AND TRIGLYCERIDE CONTENT WITH HIGH FAT DIET IN ADULTHOOD

Surprisingly, there were also no differences in total or percentage fat between any of the CL and SL groups (Figures 3A,B,E,F). We did not make a sex comparison in this analysis since the fat pads were different. There was a significant effect of litter size on plasma triglyceride concentrations, with generally increased triglyceride levels in rats from SL. There was also an effect of sex, with females of each group having lower triglyceride levels than their male counterparts (Figures 3C,G). We also detected significant effects of litter size and diet on liver triglyceride concentrations, with SL and the high fat diets increasing these levels (Figures 3D,H).

GLUCOSE UTILIZATION WITH HIGH FAT DIET IN ADULTHOOD

In accordance with the minimal effects of the high fat diet seen on overt measures of weight gain and adiposity, we also saw no significant differences in fasting glucose levels, or tolerance to glucose among the groups in males or females (Figure 4).

PERIPHERAL INFLAMMATION WITH HIGH FAT DIET IN ADULTHOOD; GENE EXPRESSION

We have previously reported neonatal overfeeding influences peripheral and central immune profiles (Clarke et al., 2012; Ziko et al., 2014). We therefore tested if neonatal overfeeding exacerbates the peripheral and central response of inflammatory markers to high fat diet. In the liver there was an increase in TLR4 mRNA after 3D high fat diet in both CL and SL males compared with their chow-fed counterparts. Interestingly, this increase in TLR4 did not persist, but had returned toward baseline values after 3W (Figure 5A). There were no significant differences between the female groups with *post-hoc* tests and no sex differences, but CL females did show a tendency to have elevated TLR4 after 3D high fat diet compared with chow-fed females (Figure 5B).

In liver there was a significant effect of sex on NF κ B, IL-10, and IL-1 β mRNA, with females expressing more of these three genes than males, but there were no significant differences with *post-hoc* tests except in that there was more IL-1 β in females after 3W high fat diet than in males. There were no differences between the groups in liver TNF α mRNA and IL-6 was undetectable in this tissue (Figure 5).

We analyzed male epididymal and female perirenal fat separately as the fat was taken from different regions. There was a significant effect of litter size on fat NF κ B in the males, with SL having more NF κ B than CL, but there were no significant differences between the individual groups with *post-hoc* tests (Figure 5G). There was also a significant effect of diet on male IL-10 and IL-1 β with the high fat diets reducing expression of these cytokines, but again there were no *post-hoc* differences and no further significant differences in male or female fat TLR4, NF κ B, TNF α , or IL-6 mRNA (Figure 5).

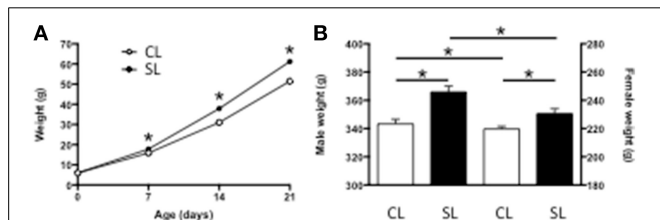


FIGURE 1 | Effects of neonatal overfeeding on body weight. (A) Pre-weaning (age, litter size interaction [$F_{(3, 78)} = 28.83$, $P < 0.001$] and (B) Adult (P56; significant effect of litter size [$F_{(1, 84)} = 25.96$, $P < 0.001$] and sex [$F_{(1, 84)} = 1559.46$, $P < 0.001$] body weights of rats raised in control (CL) and small (SL) litters. Data are mean + SEM. * $P < 0.05$.

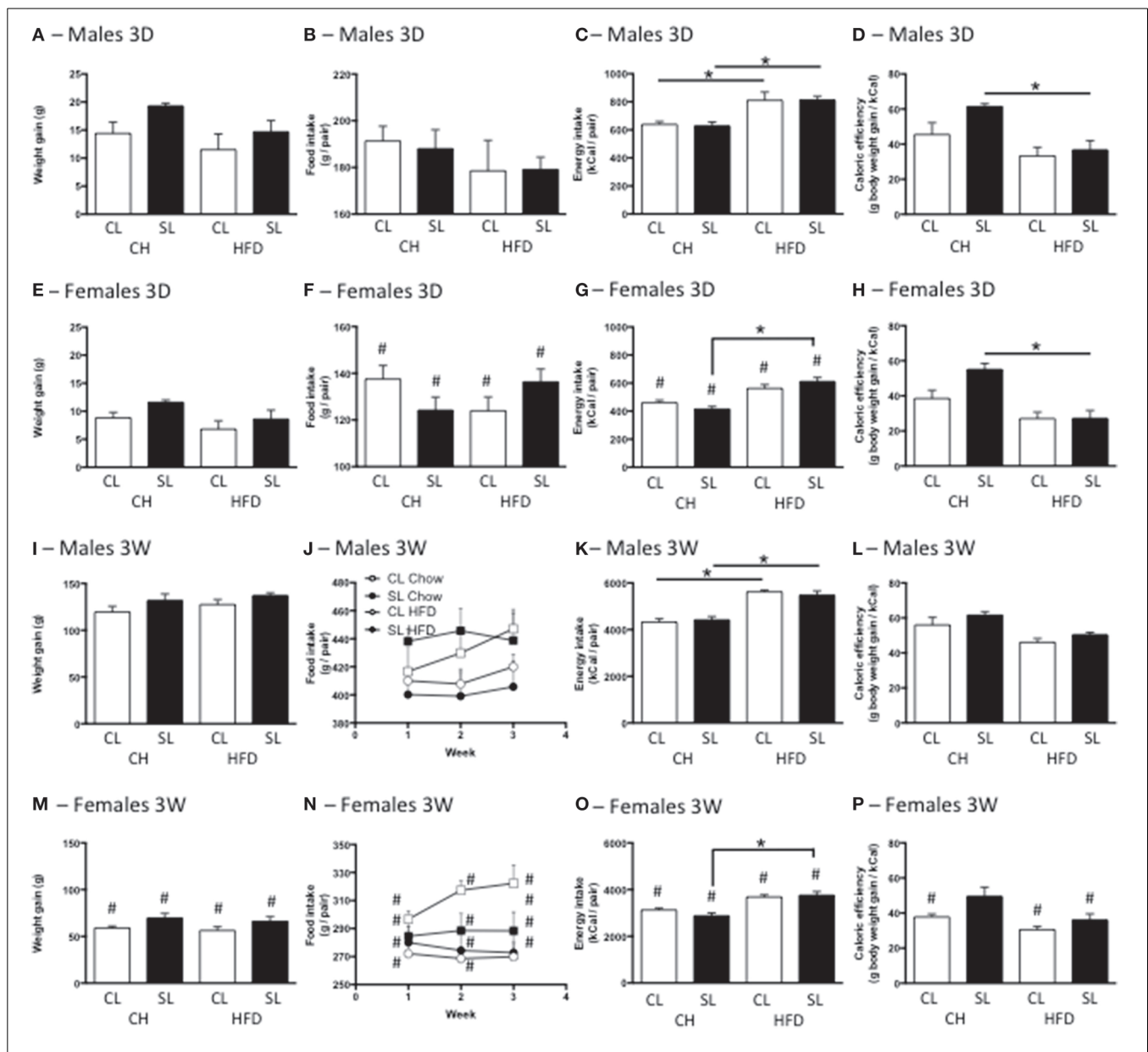


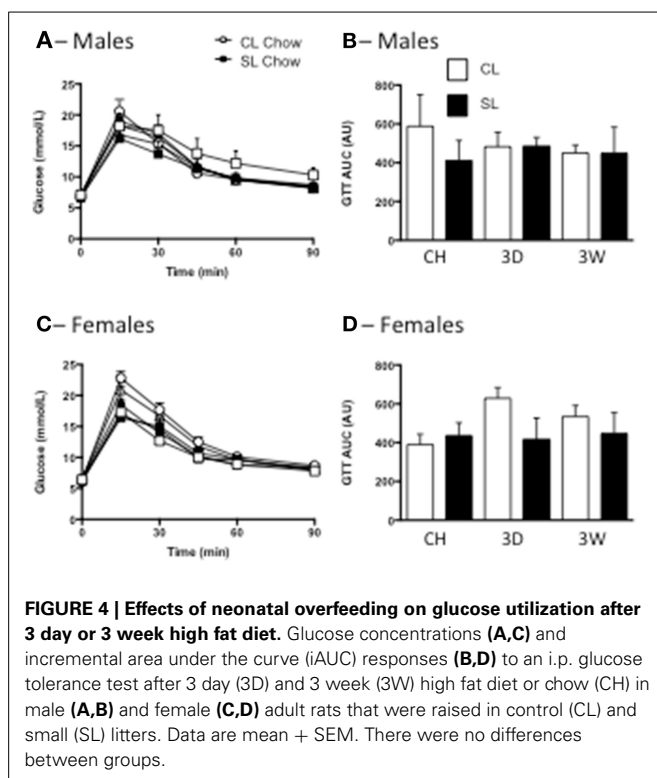
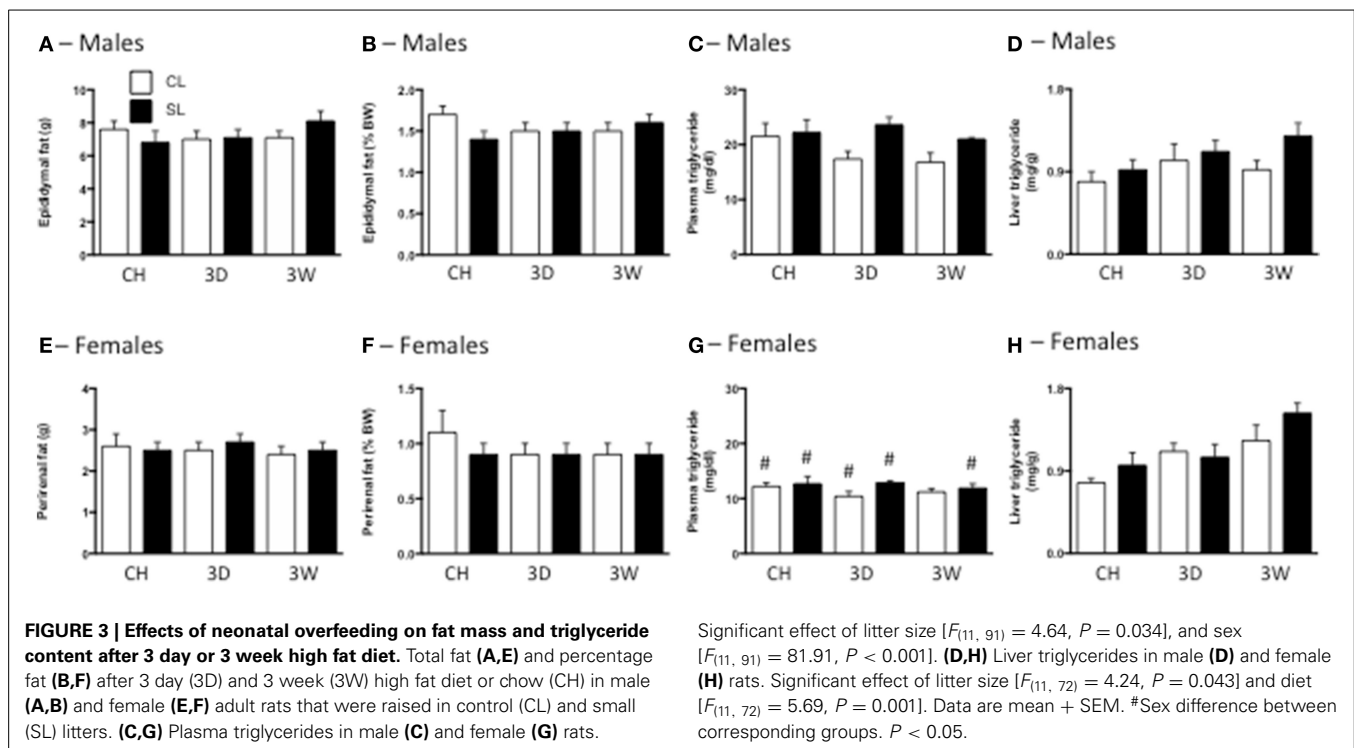
FIGURE 2 | Effects of neonatal overfeeding on weight gain and food intake after 3 day or 3 week high fat diet. (A,E,I,M) Weight gain with 3 day (3D; **A,E**) and 3 week (3W; **I,M**) high fat diet (HFD) or chow (CH) in male (**A,I**) and female (**E,M**) adult rats that were raised in control (CL) and small (SL) litters. 3D HFD: significant effect of sex [$F_{(1, 48)} = 26.77$, $P < 0.001$] and diet [$F_{(1, 48)} = 7.16$, $P = 0.01$]. 3W HFD: significant effect of sex [$F_{(1, 50)} = 374.35$, $P < 0.001$] and litter size [$F_{(1, 50)} = 9.78$, $P = 0.003$]. (**B,F,J,N**) Food intake with 3D (**B,F**) and 3W (**J,N**) HFD in male (**B,J**) and female (**F,N**) rats. 3D HFD: significant effect of sex [$F_{(7, 48)} = 110.74$, $P < 0.001$]. 3W HFD: significant effect of sex [$F_{(7, 46)} = 285.23$, $P < 0.001$] and diet [$F_{(7, 46)} = 12.03$, $P = 0.001$]. (**C,G,K,O**) Energy intake with 3D (**C,G**) and 3W (**K,O**) HFD in male (**C,K**)

and female (**G,O**) rats. 3D HFD: significant effect of sex [$F_{(7, 45)} = 88.54$, $P < 0.001$], and diet [$F_{(7, 45)} = 53.58$, $P < 0.001$]. 3W HFD: significant effect of sex [$F_{(7, 46)} = 266.19$, $P < 0.001$], and diet [$F_{(7, 46)} = 93.34$, $P < 0.001$], significant sex \times diet interaction [$F_{(7, 46)} = 5.54$, $P = 0.023$]. (**D,H,L,P**) Caloric efficiency with 3D (**D,H**) and 3W (**L,P**) HFD in male (**D,L**) and female (**H,P**) rats. 3D HFD: significant effect of litter size [$F_{(7, 45)} = 6.83$, $P = 0.012$], sex [$F_{(7, 45)} = 4.53$, $P = 0.039$], and diet [$F_{(7, 45)} = 30.73$, $P < 0.001$], significant litter size \times diet interaction [$F_{(7, 45)} = 4.45$, $P = 0.041$]. 3W HFD: significant effect of litter size [$F_{(7, 46)} = 10.75$, $P = 0.002$], sex [$F_{(7, 46)} = 51.85$, $P < 0.001$], and diet [$F_{(7, 46)} = 25.39$, $P < 0.001$]. Data are mean \pm SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$.

PERIPHERAL INFLAMMATION WITH HIGH FAT DIET AND LPS IN ADULTHOOD; LIVER PROTEIN

Analysis of liver concentrations of a suite of pro- and anti-inflammatory cytokines revealed no notable effects of diet at

3D or 3W in any of the groups, and no notable effects of the litter size except where IL-2 was suppressed in SL relative to CL. LPS significantly increased liver IL-1 α , IL-1 β , IL-6, and TNF α across the groups, but there were no significant differences



with the *post-hoc* tests except in IL-1 α CL after 3D high fat diet. We also found significant sex differences, with less of all the cytokines measured in females than in males except IL-1 α (Table 1).

MICROGLIAL CHANGES WITH HIGH FAT DIET IN ADULTHOOD

In agreement with our previous findings (Ziko et al., 2014), neonatal overfeeding significantly increased PVN microglial numbers so that under chow-fed conditions, male SL rats had more microglia than CL in this region (Figures 6A,I). In males, the 3D high fat diet caused a substantial increase in microglial numbers and density in CL rats but, interestingly, caused a reduction in microglial numbers in SL rats compared with the chow diet (Figures 6A,I). After 3W high fat diet, microglial numbers remained elevated in CL compared with under chow conditions, but there was no effect of the 3W diet on SL rats (Figures 6A,I). Similar trends were seen in microglial density. In this case, the 3D high fat diet increased microglial density in CL but not SL and the 3W high fat diet had little effect (Figure 6B). In females the responses were more ambiguous, with neonatal overfeeding and adult diet having no significant effects (Figures 6E,F).

In the ARC there were significant effects of litter size, diet, and sex on microglial numbers, with females having fewer microglia and neonatal overfeeding reducing microglial numbers overall, but there were no relevant differences with *post-hoc* comparisons (Figures 6C,G). There was also a significant effect of sex on microglial density in this region with *post-hoc* tests revealing female CL but not SL rats had reduced microglial density compared with males after 3W high fat diet (Figures 6D,H).

NEURONAL ACTIVATION WITH HIGH FAT DIET IN ADULTHOOD

As previously demonstrated (Clarke et al., 2012), neonatal overfeeding exacerbates the PVN response to LPS in male rats, with SL males having approximately twice as many mp and mg PVN neurons activated after LPS as CL (Figures 7A,C). In male CL rats, the

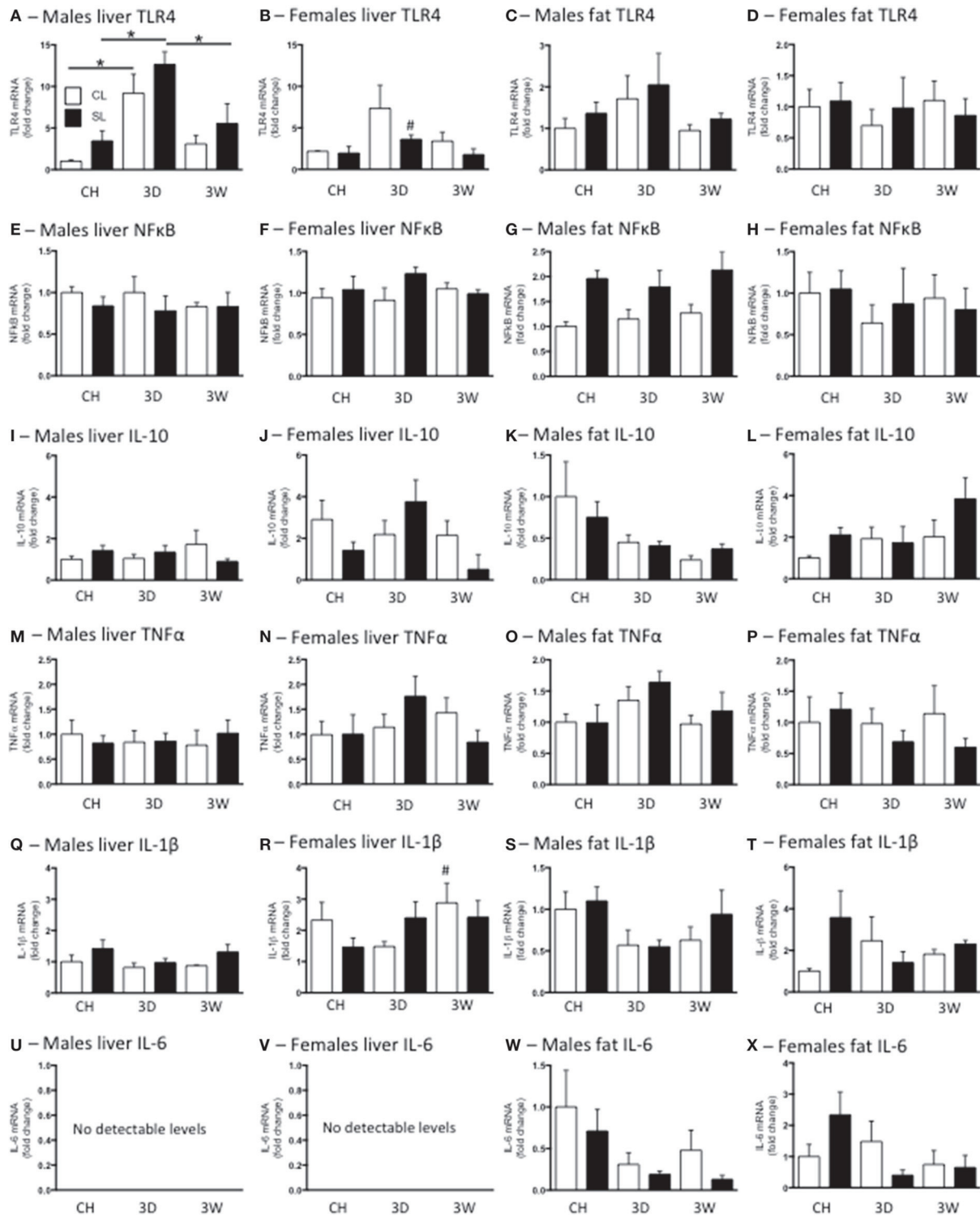


FIGURE 5 | Effects of neonatal overfeeding on peripheral inflammatory gene expression after 3 day or 3 week high fat diet. Liver and fat TLR4 (A–D), NFκB (E–H), interleukin (IL)-10 (I–L), TNFα (M–P), IL-1β (Q–T) and IL-6 (U–X) after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male and female adult rats that were raised in control (CL) and small (SL) litters. Liver TLR4: significant effect of diet [$F_{(11, 60)} = 18.71$, $P < 0.001$] and sex [$F_{(11, 60)} = 8.25$, $P = 0.006$], significant litter size × sex interaction [$F_{(11, 60)} = 7.47$,

$P = 0.008$], significant diet × sex interaction [$F_{(11, 60)} = 3.39$, $P = 0.04$]. Liver NFκB: significant effect of sex [$F_{(11, 56)} = 4.12$, $P = 0.047$]. Male fat NFκB: significant effect of litter size [$F_{(5, 33)} = 14.80$, $P = 0.001$]. Liver IL-10 significant effect of sex [$F_{(11, 57)} = 11.25$, $P = 0.001$]. Male fat IL-10 significant effect of diet [$F_{(5, 30)} = 4.81$, $P = 0.015$]. Liver IL-1β significant effect of sex [$F_{(11, 59)} = 26.42$, $P < 0.001$]. Male fat IL-1β significant effect of diet [$F_{(5, 30)} = 3.29$, $P = 0.051$]. Data are mean + SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$.

Table 1 | Liver cytokine (pg/mL) responses to lipopolysaccharide (LPS) after 3 days (3D) and 3 weeks (3W) in rats that were raised in control (CL) or small (SL) litters.

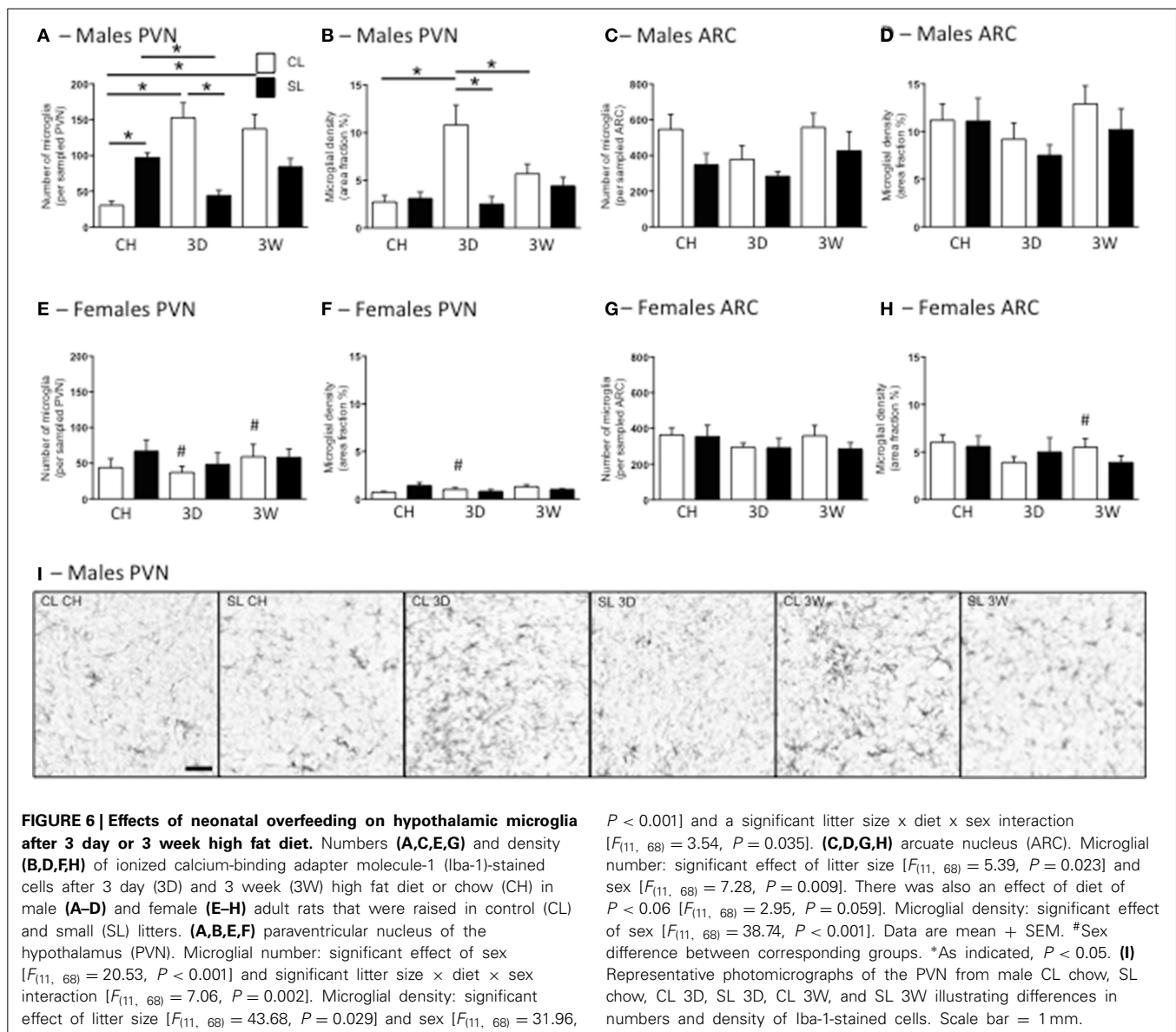
	CL CH Sal	SL CH Sal	CL 3D Sal	SL 3D Sal	CL 3W Sal	CL 3W Sal	CL CH LPS	SL CH LPS	CL 3D LPS	SL 3D LPS	CL 3W LPS	CL 3W LPS	Main effects
MALES													
IL-1 α	58.1 (10)	40.9 (6)	50.9 (5)	44.1 (5)	70.3 (11)	41.5 (6)	247.5 (88)	188.4 (79)	206.9 (60)*	190.7 (75)	186.5 (68)	116.1 (31)	LPS
IL-1 β	807.7 (120.2)	543.5 (66)	682.6 (65)	614.9 (71)	896.1 (132)	626.0 (32)	1533.3 (260)	1453.8 (358)	1559.5 (282)	1487.7 (463)	1643.0 (455)	1114.6 (242)	LPS, SEX
IL-2	174.2 (23)	129.3 (17)	155.4 (14)	132.9 (10)	177.3 (13)	142.2 (6)	164.2 (6)	150.4 (22)	148.8 (13)	151.3 (34)	166.7 (21)	116.8 (15)	LITTER, SEX
IL-4	1018.5 (190)	843.0 (309)	884.5 (297)	682.0 (182)	1040.4 (336)	853.6 (195)	856.6 (232)	1031.4 (341)	721.0 (185)	1163.8 (516)	1081.3 (415)	738.1 (212)	SEX
IL-5	1205.4 (167)	966.9 (185)	1056.8 (223)	905.0 (142)	1163.2 (253)	1034.3 (163)	1007.9 (175)	1151.6 (266)	879.10 (100)	1160.0 (311)	1099.5 (247)	964.1 (181)	SEX
IL-6	84.9 (12)	57.4 (12)	72.7 (10)	56.2 (7)	88.3 (10)	68.8 (11)	106.0 (14)	102.5 (29)	94.2 (18)	109.1 (36)	103.7 (26)	71.4 (10)	LPS, SEX
IL-10	4885.6 (654)	3760.2 (896)	4331.9 (794)	3655.3 (472)	5228.0 (1058)	4326.8 (660)	4764.5 (699)	4952.8 (1105)	4140.0 (567)	5372.0 (1667)*	5021.6 (1108)	3824.8 (680)	SEX
IL-12	341.2 (59)	260.9 (64)	277.0 (84)	224.4 (55)	314.5 (94)	257.9 (61)	263.5 (61)	311.1 (84)	233.2 (49)	316.1 (112)	289.9 (95)	249.0 (61)	SEX
TNF- α	205.7 (31)	157.0 (36)	173.9 (22)	158.8 (19)	221.3 (20)	168.6 (23)	287.2 (47)	232.4 (58)	243.8 (45)	257.9 (67)	284.7 (77)	194.2 (20)	LPS, SEX
FEMALES													
IL-1 α	40.7 (3)	35.6 (5)	44.1 (5)	37.7 (4)	34.5 (2)	68.0 (14)	155.3 (41)	179.0 (28)	292.0 (71)	136.2 (35)	151.8 (26)	167.4 (14)	
IL-1 β	424.1 (54)	324.3 (56)	459.6 (45)	390.7 (35)	377.0 (30)	524.9 (76)	975.4 (172)	1328.1 (227)	1403.6 (241)	930.8 (157)	964.0 (166)	1084.8 (113)	
IL-2	122.1 (7)	93.5 (10)	126.1 (12)	111.6 (11)	107.2 (8)	111.6 (8)	102.9 (14)	100.4 (11)	105.3 (9)	93.5 (13)	106.3 (7)	119.1 (14)	
IL-4	406.5 (56)	313.9 (88)	586.7 (133)	414.1 (116)	369.1 (59)	465.9 (72)	366.1 (96)	287.8 (49)	271.4 (43)	405.7 (83)	350.1 (48)	498.3 (129)	
IL-5	440.0 (36)	399.3 (37)	526.6 (50)	417.1 (37)	423.0 (25)	563.7 (69)	405.4 (58)	412.3 (47)	354.7 (35)	427.3 (59)	397.2 (13)	500.8 (61)	
IL-6	56.0 (7)	41.7 (6)	55.0 (6)	46.3 (5)	42.5 (4)	51.7 (7)	62.8 (8)	68.9 (10)	81.7 (16)	55.1 (9)	55.7 (5)	74.3 (10)	
IL-10	2206.2 (116)	1798.6 (162)	2223.9 (150)	1995.1 (226)	2021.2 (165)	2009.6 (85)	1849.1 (226)	1865.4 (143)	2002.2 (176)	1891.4 (162)	2043.1 (84)	2212.5 (128)	
IL-12	69.8 (7)	50.7 (9)	80.9 (11)	65.5 (8)	61.1 (6)	88.0 (14)	60.7 (11)	67.1 (13)	52.8 (7)	65.8 (11)	60.6 (5)	82.1 (13)	
TNF- α	162.2 (27)	124.4 (19)	129.2 (15)	129.0 (21)	105.1 (13)	133.0 (25)	159.4 (23)	182.2 (32)	203.0 (52)	142.0 (25)	145.9 (13)	182.8 (32)	

Interleukin (IL)-1 α : significant effect of LPS [$F_{(23, 118)} = 70.78$, $P < 0.001$]. IL-1 β : significant effect of LPS [$F_{(23, 117)} = 77.74$, $P < 0.001$]; significant effect of sex [$F_{(23, 117)} = 14.28$, $P < 0.001$]. IL-2: significant effect of litter size [$F_{(23, 119)} = 8.47$, $P = 0.004$]; significant effect of sex [$F_{(23, 119)} = 52.93$, $P < 0.001$]. IL-4: significant effect of sex [$F_{(23, 120)} = 34.36$, $P < 0.001$]. IL-5: significant effect of sex [$F_{(23, 120)} = 101.25$, $P < 0.001$]. IL-6: significant effect of LPS [$F_{(23, 120)} = 15.14$, $P < 0.001$]; significant effect of sex [$F_{(23, 120)} = 22.68$, $P < 0.001$]. IL-10: significant effect of sex [$F_{(23, 120)} = 93.59$, $P < 0.001$]. IL-12: significant effect of sex [$F_{(23, 120)} = 95.10$, $P < 0.001$]. Tumor necrosis factor (TNF) α : significant effect of LPS [$F_{(23, 120)} = 14.08$, $P < 0.001$]; significant effect of sex [$F_{(23, 120)} = 20.84$, $P < 0.001$]. Data are mean (SEM). *Versus saline group. #Versus female group. $P < 0.05$.

3D high fat diet led to a markedly increased PVN response to LPS compared with that seen in chow fed CL rats. This response was not seen in the SL group after 3D high fat diet, where there was a tendency for the PVN response to be reduced compared with chow SL. With 3W high fat diet, neuronal activation after LPS was

again similar to that seen in chow-fed rats in both CL and SL, but the difference between these two groups was no longer present.

Interestingly, the females had a different profile of Fos expression in response to LPS (**Figures 7B,D**). Although there were no significant differences between the relevant groups with *post-hoc*



tests, the trend was for chow-fed SL rats to have a smaller Fos response than CL in both the mp and mg PVN. There was also a trend for the 3W high fat diet to attenuate the response with no effect of the 3D diet. The response to LPS was also significantly higher in males than in females in the CL 3D diet group but, despite an attenuated female response overall compared with males, there were no other significant sex differences with *post-hoc* tests. In the dpPVN there were significant main effects of litter size [$F_{(23, 140)} = 14.86, P < 0.001$], LPS [$F_{(23, 140)} = 13.57, P < 0.001$], and sex [$F_{(23, 140)} = 49.32, P < 0.001$] but no relevant differences with *post-hoc* comparisons (data not shown).

We also examined neuronal activation in several other brain regions involved in fever regulation and the response to LPS. Although the pattern was not as clear as for the PVN, similar responses were also seen in the vBNST and VMPOA in males, with LPS leading to increased Fos in these regions compared with

saline after 3D high fat diet in CL but not SL rats (Figure 7). Specifically, there was an increase in vBNST Fos in LPS-treated 3D CL males compared with saline-treated 3D CL males, but no other relevant differences. In the dBNST there was an LPS, sex interaction [$F_{(23, 126)} = 4.54, P = 0.035$], a litter size, sex interaction [$F_{(23, 126)} = 4.45, P = 0.037$], and a litter size, diet interaction [$F_{(23, 126)} = 3.76, P = 0.026$], but there were no differences with *post-hoc* tests (data not shown). In the VMPOA there was again a significant increase in Fos in LPS-treated 3D CL males compared with saline-treated 3D CL males, but no other relevant differences. In the MPOA there were effects of LPS [$F_{(23, 130)} = 4.48, P = 0.036$] and litter size [$F_{(23, 130)} = 7.95, P = 0.006$], but no differences with *post-hoc* tests (data not shown). In the OVLT there were no relevant differences with *post-hoc* tests except that in females there were more Fos-positive cells with LPS after 3W high fat diet in CL rats than in SL.

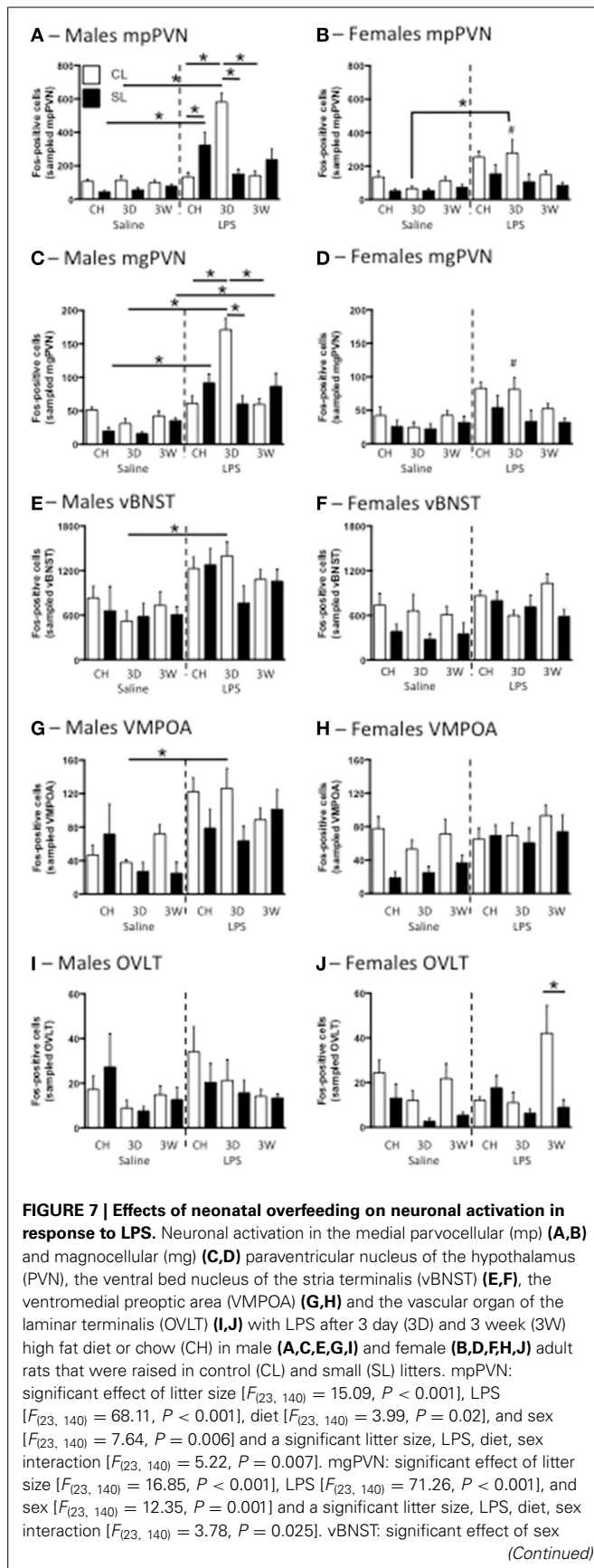


FIGURE 7 | Continued

[$F_{(23, 131)} = 15.76, P < 0.001$], LPS [$F_{(23, 131)} = 31.73, P = 0.051$], and litter size [$F_{(23, 131)} = 8.05, P = 0.005$] and a significant litter size, LPS \times diet \times sex interaction [$F_{(23, 131)} = 3.05, P = 0.051$]. VMPOA: significant effect of LPS [$F_{(23, 130)} = 31.81, P < 0.001$] and litter size [$F_{(23, 130)} = 11.70, P = 0.001$] as well as a significant litter size \times LPS \times diet \times sex interaction [$F_{(23, 130)} = 3.91, P = 0.023$]. OVLT: significant effect of diet [$F_{(23, 133)} = 5.34, P = 0.006$] and litter size [$F_{(23, 133)} = 7.64, P = 0.007$]. There was also a diet \times sex interaction of $P < 0.06$ [$F_{(23, 133)} = 2.90, P = 0.058$]. Data are mean \pm SEM. *Sex difference between corresponding groups. *As indicated, $P < 0.05$.

DISCUSSION

The perinatal nutritional environment is important in long-term metabolic programming and, as such, rats that are overfed as neonates by being suckled in small litters show early accelerated weight gain that is maintained into young-adulthood (Spencer and Tilbrook, 2009; Clarke et al., 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014). This model was established as early as the 1960s (McCance, 1962) and these findings have been consistently replicated by several groups (e.g., Plagemann et al., 1992, 2010; Xiao et al., 2007; Chen et al., 2008). Our data now show that despite this weight gain, neonatally overfed rats are only marginally more susceptible to the metabolic/obesogenic effects of a short or medium-term high fat diet. Neonatally overfed rats had an overall increase in plasma and liver triglyceride content on a high fat diet compared with CL rats. They also had a lower caloric efficiency after the high fat diets compared with their chow counterparts, indicating they gained less weight for the same calorie intake, whereas CL rats did not. However, contrary to our hypothesis that SL rats would be more susceptible to the metabolic effects of a high fat diet, they did not eat more, or gain more visceral fat mass, and they did not display early glucose intolerance that might suggest a pre-diabetic profile. They also had no differences in inflammatory gene expression in the liver or fat or in liver cytokine concentrations. We should note that different fat pads can respond differently to dietary influences and a comprehensive analysis of the different depots may still reveal differences between the groups. However, we did not find differences in any other metabolic or inflammatory parameters to suggest this is a strong possibility.

Interestingly, these neonatally overfed rats did show differences in susceptibility to the central pro-inflammatory effects of short-term (3 days) high fat feeding. Thus, 3 days high fat diet led to significant microgliosis in the PVN in male CL rats, but this was not seen in SL. This increase in microglial numbers in the PVN was still evident at the 3 week mark. Similarly, the PVN response to LPS was markedly enhanced in CL rats by 3 days of high fat diet, but not in SL, although this response was resolved to control levels at 3 weeks. These differences were not seen in the females.

In a recent study, Thaler and colleagues have suggested the early (3 day) inflammatory response to a high fat diet is actually an adaptive one and that it is only with longer-term (e.g., 3 weeks) high fat feeding that a maladaptive pro-inflammatory response ensues (Thaler et al., 2012). Thus, at 3 days on a high fat diet, rats and mice in the Thaler study showed hypothalamic microgliosis and an increase in hypothalamic pro-inflammatory

gene expression. This profile disappeared by 7 days but returned after 21 days of high fat diet (Thaler et al., 2012). In light of this work, our findings would suggest that the absence of microgliosis or an exacerbated response to LPS after 3 days of high fat diet in SL is maladaptive; reflective of an inability to effectively respond to the high fat diet. However, if this interpretation is correct, one would expect to see differences between the groups at 3 weeks, which we did not see in this study.

In this investigation, we deliberately selected relatively short periods of high fat feeding. Our hypothesis suggested neonatally overfed rats may be more susceptible to a high fat diet and it was therefore essential to give a metabolic challenge mild enough to avoid a ceiling effect. As with the present study, other groups have seen 3 weeks of high fat diet is not usually sufficient to induce overt body weight and fat mass differences. For example, Maric and colleagues have shown 32% calorie by fat diets for 8 weeks do not cause a difference, compared with chow fed, in fat pad weight in Wistar rats and only cause a significant increase in total weight gain if the diet is butter based (and not if it is coconut oil-based) (Maric et al., 2014). Significant metabolic and inflammatory effects of both a 3 day and 3 week high fat diet have been reported (Thaler et al., 2012), suggesting the dietary challenge in this study would be sufficient to induce inflammation and allow us to detect any differences between the neonatally overfed and control rats. However, it is possible that while an initial adaptive response to the high fat diet was evident at 3 days, 3 weeks was insufficient to reveal susceptibility to the metabolic effects of the challenge. If this is the case, we would expect the neonatally overfed rats to respond differently after a longer period of high fat diet. It is interesting that our 3 day high fat diet actually caused an overall reduction in the normal weight increase. This is likely to be related to the novelty of the new diet, since other experimental factors would also have influenced the chow groups. What this means for the inflammatory outcome is unclear, especially since the elevated energy intake at 3 days in the high fat diet-fed groups implies they were consuming the high fat chow as expected and any reductions in weight gain may therefore be due to non-nutrient factors. It is possible an adaptive anti-inflammatory response to acute high fat diet (Thaler et al., 2012) is aided in rodents by food-novelty-related elevations in glucocorticoids, but this possibility remains to be tested.

One of the more interesting findings to come out of the present study is our evidence of central pro-inflammatory changes in the absence of a significant change in the metabolic or peripheral pro-inflammatory profiles. Apart from an increase in liver TLR4 mRNA in both CL and SL groups at 3 days high fat diet, there were no significant changes in peripheral indicators of obesity or inflammation in the tissues we examined. These data support recently published evidence (Thaler et al., 2012; Maric et al., 2014). Although it has long been recognized obesity is associated with peripheral inflammation, including elevated pro-inflammatory cytokines in circulation (Hotamisligil et al., 1995; Hotamisligil, 2006), more recent evidence, and our own from this study, is suggesting central inflammation and neuronal injury with high fat diet actually precedes peripheral inflammation. The systemic inflammatory response to diet or weight gain is derived from excess macrophage infiltration to the adipose tissue and

subsequent excess production of pro-inflammatory cytokines. It is likely this is a relatively chronic process and possible it is driven, to a degree, by central inflammation (Weisberg et al., 2003; Xu et al., 2003). Thaler and colleagues have shown markers of inflammation in the hypothalamus are elevated as early as 24 h after the onset of a high fat diet. Within a week this is reflected in neuronal injury. Indices of peripheral inflammation, however, are not evident until weeks to months of the diet (Thaler et al., 2012). Similarly, Maric and colleagues have shown diet high in saturated fat leads to central inflammation in the absence of peripheral even as late as 8 weeks after onset (Maric et al., 2014). Our current findings tend to support these suggestions that central inflammation, at least in terms of microgliosis and susceptibility to an immune challenge, occurs early after the commencement of a high fat diet, and precedes the development of metabolic dysregulation or an obese profile. In this regard, it will be interesting to examine how high fat diet influences acute pro-inflammatory circulating signals such as leptin in these neonatally overfed populations, since adipokines such as leptin are important in influencing central inflammation (Gao et al., 2014).

Our findings also suggest that a short period of high fat diet feeding may actually leave the individual seriously vulnerable to bacterial infection at this time. A hypersensitive HPA axis after 3 days high fat diet may be an adaptive attempt to curtail inflammation through glucocorticoid production (Thaler et al., 2012). However, our CL rats given 3 days high fat diet responded to LPS with a six-fold increase in neuronal activation in the PVN. Although we did not see differences from the chow-fed groups in LPS/fever-regulatory brain regions (VMPOA, OVLT, BNST) and we did not measure fever and sickness behavior directly, a response of this magnitude in the PVN is likely to reflect a more severe illness with LPS (Tarr et al., 2012). Several studies have shown microglia behave differently depending upon their background or basal state. For instance, early life immune challenge can leave microglia “primed” to more readily respond to a similar challenge later on (Bland et al., 2010; Williamson et al., 2011). We have recently shown neonatal overfeeding has a similar effect, with neonatally overfed rats having an exaggerated microglial, febrile, cytokine, and HPA axis response to LPS (Clarke et al., 2012; Ziko et al., 2014). The present work suggests that instead of exacerbating this response, the 3 day and 3 week high fat diets dampen it, at least in terms of PVN neuronal activation after LPS, uncovering the possibility of an interaction between the “primed” microglial state and subsequent diet.

Another notable finding of the present study is that the sexes responded quite differently to the high fat diet. While CL males were affected by 3 days high fat diet in a number of parameters, females were not. We deliberately did not control for cycle stage in our females as this imposes an additional stressor on the animals. However, we believe cycle stage is unlikely to account for these sex differences since the variability in the data was similar for females as for males. Although few investigators have examined both males and females in the same study, our findings do concur with reported literature. For instance, male mice develop insulin resistance after a short period of diet high in saturated or unsaturated fat. Female mice retain their insulin sensitivity with the same diet (Senthil Kumar et al., 2014). Likewise, female rats

are relatively protected against the metabolic effects of a high fructose or sucrose diet, whereas males develop insulin resistance and hypertension under the same conditions (Galipeau et al., 2002). Our data thus illustrate female rats are likely to be more resilient to the effects of short-term high fat diet than males. These data also highlight the importance of including both sexes as study subjects, or at least exercising care when extrapolating data from one sex to another.

In summary, rats made overweight by early life overfeeding are unlikely to be substantially more vulnerable to a short-term adult-onset high fat diet than control rats in terms of developing further obesity or a diabetogenic profile. On the other hand, neonatally overfed rats were less responsive to the central pro-inflammatory effects of a 3 day high fat diet than controls. Whether this represents a maladaptive inability to combat the central effects of the high fat diet or, rather, a resilience to the challenge, remains to be determined in future work.

AUTHOR CONTRIBUTIONS

Guohui Cai, Juan Molero and Sarah J. Spencer conceived of and designed this study. Guohui Cai, Ilvana Ziko, Stanley M. H. Chan, Xiao-Yi Zeng, Songpei Li, Juan Molero, and Sarah J. Spencer ran the animal studies and collected samples. Guohui Cai, Tara Dinan, Joanne M. Barwood, Simone N. De Luca, Alita Soch, and Sarah J. Spencer analyzed samples and interpreted the data. Sarah J. Spencer wrote the manuscript. All authors revised the manuscript critically. All authors give final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Do all roads lead to Rome? The role of neuro-immune interactions before birth in the programming of offspring obesity

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The functions of the nervous system can be powerfully modulated by the immune system. Although traditionally considered to be quite separate, neuro-immune interactions are increasingly recognized as critical for both normal and pathological nervous system function in the adult. However, a growing body of information supports a critical role for neuro-immune interactions before birth, particularly in the prenatal programming of later-life neurobehavioral disease risk. This review will focus on maternal obesity, as it represents an environment of pathological immune system function during pregnancy that elevates offspring neurobehavioral disease risk. We will first delineate the normal role of the immune system during pregnancy, including the role of the placenta as both a barrier and relay of inflammatory information between the maternal and fetal environments. This will be followed by the current exciting findings of how immuno-modulatory molecules may elevate offspring risk of neurobehavioral disease by altering brain development and, consequently, later life function. Finally, by drawing parallels with pregnancy complications other than obesity, we will suggest that aberrant immune activation, irrespective of its origin, may lead to neuro-immune interactions that otherwise would not exist in the developing brain. These interactions could conceivably derail normal brain development and/or later life function, and thereby elevate risk for obesity and other neurobehavioral disorders later in the offspring's life.

Keywords: inflammation, cytokines, *in utero*, brain development, DOHaD, pregnancy, epigenetics

Introduction

The period of life before birth is the most important time in our lives. During this time, developmental mechanisms put into place the cellular foundations on which our functioning, thinking, and feeling bodies will exist, in sickness and in health, across our lifespan. During the period of embryonic and fetal development, the mother's health appears to be absolutely critical to the later life health of her offspring.

This concept was first appreciated when epidemiological studies examined the long-term health outcomes of individuals who were *in utero* during the Dutch hunger winter (1944–1945). The offspring of these undernourished mothers had low birth weight and impaired glucose tolerance later in life compared to individuals born in flanking years (Ravelli et al., 1998). Interestingly, the timing of exposure to famine within the gestational period is also critically important.

Thus, people exposed to famine in early gestation in the Dutch hunger winter were at greater risk of developing coronary heart disease and obesity in later life, whereas those exposed to famine in mid and late gestation were not similarly affected (Ravelli et al., 1976; Roseboom et al., 2000). Moreover, impaired glucose tolerance in offspring appeared more likely to result from undernutrition in late gestation (Ravelli et al., 1998). These observations inspired the concept that an individual's risk of disease across their lifespan could be shaped by events that occurred much earlier in their lives; indeed during their time in the womb (Barker, 2004). With the recent massive increase in obesity across the western world, much focus has now shifted to understanding the long-term health outcomes of individuals whose mothers were obese during pregnancy. Curiously, the epidemiological data paint a similar picture to undernutrition. Individuals whose mothers were obese during pregnancy show a significantly higher risk for later life obesity, and "the metabolic syndrome" (Law et al., 1992; Gale et al., 2007; Armitage et al., 2008; Crozier et al., 2010; Tamashiro and Moran, 2010; Alfaradhi and Ozanne, 2011; Ornoy, 2011). However, they also show increased risk for a constellation of behavioral and mental health problems including autism, attention deficit/hyperactivity disorder, developmental delay, anxiety, and depression (Herva et al., 2008; Rodriguez, 2010; Van Lieshout and Boyle, 2011a,b; Colman et al., 2012; Halmoy et al., 2012; Krakowiak et al., 2012; Moore et al., 2012; Wojcik et al., 2013).

Although nutrition during gestation and nutrition after birth are key contributors to neurobehavioral disease risk across the lifespan, the mechanisms that link these factors, either alone or together, to health risks and outcomes remain poorly understood. Animal models, typically rodent, sheep, or non-human primate (NHP), have been invaluable in shedding light on the cellular and molecular details occurring behind the scenes. In these models, research has traditionally focused on defining the deregulated function of offspring peripheral organs involved in metabolism, such as the pancreas, liver, adipose depots, and skeletal muscle whose function is impaired in obesity and the metabolic syndrome. However, since body weight and metabolic function are regulated by the brain (Stanley and Leibowitz, 1984; Fan et al., 1997; Cowley et al., 1999; Horvath et al., 1999; Cone et al., 2001; Elmquist et al., 2005), and since maternal nutrition during pregnancy programs an assortment of offspring neurological abnormalities, studies examining changes in the offspring brain have become increasingly prevalent.

In pregnancies complicated by maternal obesity, changes in the regions of the fetal brain that will regulate body weight later in life, including the arcuate nucleus of the hypothalamus (ARC) and the paraventricular nucleus of the hypothalamus (PVN), and brain areas associated with reward and food seeking have been reported. In the body weight regulating areas, the adult offspring of obese mothers have been reported to show: (i) altered expression of the appetite-regulating neuropeptides, including agouti-related peptide (AgRP, reduced) and pro-opiomelanocortin (POMC, reduced), in the ARC; (ii) altered expression of receptors for appetite-regulating neuropeptides, including neuropeptide Y (NPY) receptor Y1 and melanocortin receptor MC4R (both increased), in the PVN; (iii) altered

neurogenesis leading to increased numbers of body weight-regulating neurons expressing orexin, melanin concentrating hormone (MCH), galanin, enkephalin, and dynorphin (Chang et al., 2008; Chen et al., 2009); (iv) ARC neuron leptin resistance, as judged by reduced pSTAT3 in response to exogenous leptin administration; (v) and developmental alterations in the neural circuitry that regulates body weight (Bouret et al., 2008; Kirk et al., 2009; Sanders et al., 2014). In the reward pathways, there are reports of altered gene expression in the opioid system (Grissom et al., 2014), changes in the mesolimbic dopamine pathways, including increased dopamine synthesis in the nucleus accumbens (NAc) and ventral tegmental area (VA), and altered dopamine responsiveness in the NAc accompanied by reduced dopamine receptor D2 expression in the VTA (Naef et al., 2008, 2011). In the offspring hippocampus, abnormalities have been observed in neural circuit formation, neurogenesis and cell death both in the late gestation fetus and in the early post-natal period (Niculescu and Lupu, 2009; Tozuka et al., 2009, 2010), increased oxidative stress, and reduced brain derived neurotrophic factor (BDNF) mRNA and protein (Tozuka et al., 2009, 2010). Such offspring also exhibited behavioral changes that are consistent with the associations between maternal obesity and some aspects of offspring mental illness observed in humans. For example, the offspring of obese non-human mothers show defects in spatial learning in the Barnes maze test (Tozuka et al., 2010), elevated anxiety (Sasaki et al., 2014), and altered reward seeking behaviors (Naef et al., 2008, 2011).

Although not comprehensive, the above summary demonstrates that substantial progress has been, and is being, made in defining the spectrum of changes that eventuate after birth when an individual has undergone gestation in an obese mother. What remains to be discovered is what happens during gestation, what molecular factors are important, and how they act on the developing organism to affect the changes that increase disease risk much later in life. Although many factors could be at work, the potential importance of the immune system stands out for two main reasons. Firstly, it has a critical and highly regulated role at the fetal-maternal interface during normal pregnancy. Secondly, pregnancies complicated by obesity, be they human or non-human, are accompanied by elevated cytokine levels in both the maternal (Catalano et al., 2009; Madan et al., 2009; Roberts et al., 2011; Kepczynska et al., 2013) and fetal circulation (Heerwagen et al., 2013; Kim et al., 2014) across gestation (summarized in **Table 1**). Moreover, if one considers additional complications of pregnancy that also elevate offspring risk for metabolic disease and behavioral abnormalities, one feature that they all have in common is abnormal immune system activation and elevated cytokine levels during gestation.

The Placenta and Immune System During Pregnancy

There are multiple changes occurring in a mother's body during pregnancy, including increased adiposity and low-grade inflammation (Mor et al., 2011; Zhang et al., 2011). Such dynamic

TABLE 1 | Summary of inflammatory response in maternal obesity across different species.

Species	Source	Elevated cytokines	Developmental stage
Human	Maternal circulation	Leptin (Stewart et al., 2007; Challier et al., 2008; Catalano et al., 2009; Madan et al., 2009), CRP (Stewart et al., 2007; Challier et al., 2008; Catalano et al., 2009; Madan et al., 2009), IL6 (Challier et al., 2008; Catalano et al., 2009; Roberts et al., 2011), MCP-1 (Madan et al., 2009)	Second trimester (Madan et al., 2009), first to third trimester (Stewart et al., 2007), Pre-labor (Challier et al., 2008; Catalano et al., 2009), Term (Roberts et al., 2011)
	Placental sample (isolated placental macrophages)	IL6 (Challier et al., 2008), IL1 (Challier et al., 2008; Roberts et al., 2011), IL8 (Roberts et al., 2011), TNF (Challier et al., 2008), MCP1 (Challier et al., 2008)	Term (Challier et al., 2008; Roberts et al., 2011)
	Fetus (umbilical cord blood)	Leptin (Catalano et al., 2009), IL6 (Catalano et al., 2009)	Term (Catalano et al., 2009)
Non-human primate	Maternal circulation	Leptin (Frias et al., 2011)	Day 130 (of 170–180 d gestation)(Frias et al., 2011)
	Placental sample	MCP1 (Frias et al., 2011), IL1B (Frias et al., 2011)	Day 130 (of 170–180 d gestation)(Frias et al., 2011)
Sheep	Placental sample	TNF (Zhu et al., 2010), IL6 (Zhu et al., 2010), IL8 (Zhu et al., 2010), IL18 (Zhu et al., 2010)	Day 75 (of 135 d gestation)(Zhu et al., 2010)
Mouse	Maternal circulation	IL1B (Heerwagen et al., 2013; Kim et al., 2014), IL6 (Heerwagen et al., 2013; Ingvorsen et al., 2014; Kim et al., 2014), IL10 (Kim et al., 2014), IFNG (Heerwagen et al., 2013; Kim et al., 2014), TNF (Heerwagen et al., 2013; Ingvorsen et al., 2014; Kim et al., 2014), CXCL1 (Heerwagen et al., 2013), MIP2a (Heerwagen et al., 2013), CCL25 (Heerwagen et al., 2013), GM-CSF (Heerwagen et al., 2013), IL2 (Heerwagen et al., 2013), IL3 (Heerwagen et al., 2013), IL9 (Heerwagen et al., 2013)	GD15.5 (Kim et al., 2014), GD17.5 (Kim et al., 2014), GD18.5 (Heerwagen et al., 2013; Ingvorsen et al., 2014)
	Placental sample	IL6 (Kim et al., 2014), IL1b (Heerwagen et al., 2013; Kim et al., 2014), TNF (Kim et al., 2014), IL10 (Kim et al., 2014), iNOS (Heerwagen et al., 2013)	GD17.5 (Kim et al., 2014), GD18.5 (Heerwagen et al., 2013)
	Fetal circulation	IL6 (Kim et al., 2014), IL17A (Kim et al., 2014), IFNG (Kim et al., 2014)	GD17.5 (Kim et al., 2014)

pregnancy-specific changes are believed, at least in part, to be responsible for the maternal adaptation to the presence of the developing fetus. Indeed, the fetus, despite being immunologically foreign, is not rejected by the maternal immune system. In mammals, this protection is partly provided by the placenta, a transient organ that forms at the interface between the mother and fetus. The placenta is composed of both maternal and fetal cells and develops upon implantation in humans and early gestation in rodents (Watson and Cross, 2005). Despite the variability in placentation across species, placental function is strikingly similar among most placental mammals (Enders and Blankenship, 1999; Malassine et al., 2003).

Normal Function of the Placenta

One of the most critical functions of the placenta is to act as an immunological barrier, which protects the fetus from the maternal immune system (Kanellopoulos-Langevin et al., 2003; Mor

et al., 2011). Although the full spectrum of functions that the placenta performs in its role as an immunological barrier are not well-understood, current understanding indicates that placental and decidual immune cells, such as macrophages, have an immunosuppressive function, and thus dampen the maternal immune response toward the immunologically distinct fetus (Chang et al., 1993; Lin et al., 1993; Heikkinen et al., 2003; Gustafsson et al., 2008; Houser et al., 2011; Arck and Hecher, 2013). The placenta can also respond to cytokines (Jones et al., 2009; Hsiao and Patterson, 2011); and it has been argued that this serves as a mechanism for relaying information from the maternal environment to the fetus. The ability of maternal cytokines to cross the placental barrier and directly affect the fetus is still debatable. Previous studies have shown that some cytokines, when injected into pregnant rodent dams, are able to cross the placenta and enter the fetal circulation. Some cytokines have also been shown to cross the human placenta *ex vivo* (Zaretsky et al.,

2004; Dahlgren et al., 2006). This is contrasted, however, by other studies in humans and rodents, which have demonstrated a lack of cytokine transport from maternal to fetal circulation (Carbo et al., 1998; Aaltonen et al., 2005). Reconciling these datasets will require improved understanding of developmental timing in order to interweave better our knowledge of the development of cytokine-specific transport mechanisms in the placenta, changing cytokine production across gestation (Steinborn et al., 1995, 1998; Mark et al., 2013), and how both cytokine-specific and non-specific placental permeability change across gestation (Atkinson et al., 1991; Kent et al., 1994; Dahlgren et al., 2006). Nevertheless, what is clear is that the normal placenta, and its resident immune cells, performs a critical balancing act in order to be both the barrier and communicator between the mother and fetus.

Altered Placental Function in Maternal Obesity May Mean Fetal Exposure to Cytokines and Other Immune System Modulators

Human and animal studies have shown that maternal obesity alters placental morphology and vascularization (Hayes et al., 2012; Hayward et al., 2013; Kim et al., 2014). In rodent models of maternal obesity, decreased layer thickness, reduced trophoblast proliferation, reduced vascular supply and resultant hypoxia in the labyrinth were described (Hayes et al., 2012; Kim et al., 2014). In humans, maternal obesity caused reduced chorionic plate artery function, which may reduce blood supply to the fetus (Hayward et al., 2013). In addition, maternal obesity led to placental inflammation and altered cytokine production in human, rodent, and ovine models (Challier et al., 2008; Zhu et al., 2010; Roberts et al., 2011; Kim et al., 2014), including elevation in IL-6, IL-1 β , and TNF production (all models, see **Table 1** for complete list and associated animal model), as well as an increase in infiltrating monocytes and activated macrophages (Challier et al., 2008; Roberts et al., 2011; Kim et al., 2014). Also, since cytokines can alter placental nutrient transport, and maternal obesity can disrupt the placental vascular system (Jones et al., 2009; Hayes et al., 2012; Hayward et al., 2013), it is likely that fetuses developing in obese mothers would be exposed to factors from maternal and placental sources that would not be present in a normal pregnancy.

Evidence for Altered Brain Development and Offspring Obesity Consequent to Pathological Cytokine Exposure: A Common Link?

The idea that inappropriate exposure of the fetal brain to cytokines can interfere with neural development is an interesting idea, but has yet to gain traction in the field of developmental programming by maternal obesity, with currently only one publication suggesting that inappropriate exposure to inflammatory cytokines specifically may derail brain development (Sanders et al., 2014). Thus, we may be well-served to look to other models of developmental programming for clues to both common and diverse mechanisms leading to offspring neurobehavioral disorders. If activation of the immune system were to be a shared

causative agent in the offspring programming of metabolic and mental health disorders, then any pregnancy conditions that lead to these health outcomes should also exhibit immune system activation. Below we briefly review several instances where the inflammatory environment is changed during gestation, and where adverse neurobehavioral consequences to the offspring are observed (summarized in **Table 2**).

Maternal Immune Activation

Since the late 1980s it has been acknowledged that maternal infection may play a major role in the development of offspring mental illness. Many studies have reported an association between influenza infection (as well as rubella and respiratory tract infections) during pregnancy and an increased risk for schizophrenia and autism spectrum disorders (ASD) in the offspring (Mednick et al., 1988; McGrath et al., 1994; Izumoto et al., 1999; Brown et al., 2001, 2004; Brown, 2006, 2012). However, none of these epidemiological studies in humans have been able to prove causation or suggest a mechanism beyond pointing to the link between maternal immune system activation and adverse offspring outcomes.

Because very different pathogens seem to cause the same offspring phenotype, and since it is unlikely they are able to directly infect the fetus (Williams and Mackenzie, 1977; Irving et al., 2000; Shi et al., 2005), the maternal immune response has been implicated. Maternal immune activation (MIA) rodent models have been particularly helpful to examine in more detail the *in vivo* effects of the activated maternal immune system on the developing fetal brain. These models predominantly administer either bacterial lipopolysaccharide (LPS) or double-stranded viral RNA mimic polyinosinic:polycytidylic acid (poly I:C) to pregnant rats or mice. This induces an immune response, and increases circulating levels of multiple cytokines without the confounding effects of the introduction of a viral or bacterial pathogen itself. Offspring of these MIA rodents have been reported to show a “schizophrenic-like” phenotype, including deficits in prepulse inhibition (PPI), latent inhibition (LI), anxiety, locomotion and social interaction (Smith et al., 2007; Meyer et al., 2008), as well as behaviors reminiscent of ASD (Hsiao et al., 2012; Malkova et al., 2012).

Efforts to tease apart the mechanism have found that the inflammatory cytokine IL-6 appears to play a significant role in MIA's effects on offspring behavioral programming. Inducing MIA while simultaneously blocking IL-6 actions, via IL-6 receptor deletion or IL-6 function blocking antibody, was able to prevent the ability of MIA to induce a schizophrenic-like phenotype in offspring (Smith et al., 2007). Additionally, injection of IL-6, but not the other MIA-induced cytokines IL-1 α , tumor necrosis factor α (TNF α), or interferon γ (IFN γ), during pregnancy was enough to cause PPI deficits, a hallmark of schizophrenia, in offspring (Smith et al., 2007).

There is a curious link between MIA and metabolic disease, raising the possibility of some shared mechanistic underpinnings. Firstly, humans with schizophrenia appear to be more likely to develop metabolic disease (Ryan et al., 2003; Thakore, 2004; Meyer and Stahl, 2009). In addition, there is some evidence from MIA rodents that metabolic symptoms may also

TABLE 2 | Summary of different pregnancy states and their metabolic or behavioral sequelae in the offspring.

Maternal state	Offspring phenotype	Animal models	Human data
Maternal infection	Neurobehavioral disorders	Maternal immune activated (MIA) rodents produce offspring with schizophrenia and ASD – like phenotypes (Smith et al., 2007; Meyer et al., 2008; Hsiao et al., 2012; Malkova et al., 2012) Offspring of IL-6 injected rats display a subset of the MIA phenotype (Smith et al., 2007)	Maternal influenza, rubella, and respiratory tract infections during pregnancy increases offspring risk for Schizophrenia and ASDs (Williams and Mackenzie, 1977; Irving et al., 2000; Smith et al., 2007; Meyer et al., 2008; Hsiao et al., 2012; Malkova et al., 2012)
	Metabolic disease	Offspring of MIA and IL-6 injected rodents display metabolic symptoms (Dahlgren et al., 2006; Pacheco-López et al., 2011) IL6 affects fetal hypothalamic circuitry development (Sanders et al., 2014)	Schizophrenic patients more likely to develop metabolic disease (Ryan et al., 2003; Thakore, 2004)
Maternal autoimmune disease	Neurobehavioral disorders	N/A	Maternal autoimmune diseases associated with increased risk of offspring neurodevelopmental disorders such as learning disabilities and ASD (McAllister et al., 1997; Ross et al., 2003; Neri et al., 2004)
	Metabolic disease	N/A	Maternal systemic lupus erythematosus associated with low birthweight – a risk factor for offspring obesity (Baer et al., 2011)
Maternal smoking	Neurobehavioral disorders	Prenatal cigarette exposure mouse model results in offspring displaying hyperactive behaviors with disrupted memory (Balsevich et al., 2014)	Maternal smoking a risk factor for offspring aggressive behavior, inattention and conduct disorder, and attention deficit disorder (Fergusson et al., 1993, 1998; Orlebeke et al., 1999; Linnet et al., 2003)
	Metabolic disease	Mice exposed to cigarette smoke during pregnancy have offspring with increased body weight and plasma leptin (Chen et al., 2011)	Maternal smoking predisposes offspring to obesity in adolescence (Power and Jefferis, 2002; Al Mamun et al., 2006)
Air pollution exposure	Neurobehavioral disorders	Offspring of diesel exhaust exposed mice display decreased locomotion, changes to dopamine levels, and neurodevelopmental changes (Hougaard et al., 2010; Suzuki et al., 2010; Jackson et al., 2011)	Children exposed <i>in utero</i> to pollutants from coal fired power plant had motor, language and social developmental delays (Tang et al., 2008)
	Metabolic disease	Offspring of diesel exhaust exposed mice display increased bodyweight, insulin resistance particularly on high fat diet (Bolton et al., 2012)	N/A

result from prenatal immune challenge (Pacheco-López et al., 2011). Also, injection of IL-6 into pregnant rats can produce offspring with increased adipose tissue and body weight (Dahlgren et al., 2006). Finally, IL-6 appears to affect fetal hypothalamic neural circuit development in maternal obesity (Sanders et al., 2014), and has been reported to have many neurotrophic roles (Spooren et al., 2011). Given these links, it seems plausible that inflammation, perhaps IL-6 in particular, irrespective of how it becomes elevated, impinges on fetal brain development. The big remaining questions relate to whether cytokines have their effects directly or indirectly on the brain, and/or whether they cooperate with other cytokines or abnormally elevated factors to stimulate downstream events that impinge on brain development.

Maternal Autoimmune Disease

Curiously, the recent increase in obesity prevalence across the world has been mirrored by a similar rise in autoimmune disease prevalence. The literature suggests that obesity is a major contributing risk factor for the development of autoimmune disease in adults. Maternal autoimmune disease represents a spectrum of maternal disorders in which the fetus is exposed to a proinflammatory environment, and where neurobehavioral abnormalities in the offspring have been identified. For example, children whose mothers had systemic lupus erythematosus (SLE), had normal health and intelligence scores, but a higher rate of learning disabilities such as dyslexia (McAllister et al., 1997; Ross et al., 2003; Neri et al., 2004). Additionally, primary antiphospholipid syndrome, is associated with increased risk of ASD and learning disabilities

in the offspring (Nacinovich et al., 2008; Abisror et al., 2013). Although there is not yet a retrospective study examining body weight and metabolic parameters in the offspring of mothers with SLE, maternal SLE is associated with babies who are small for gestational age (Baer et al., 2011), itself a risk factor for developing obesity later in life (Barker, 2006; Gluckman et al., 2008).

Maternal Smoking

Maternal cigarette smoking, in addition to its direct effects on maternal and fetal health, predisposes offspring to increased risk of several later life adverse neurobehavioral outcomes, including aggressive behavior (Orlebeke et al., 1999), inattention and conduct disorder (Fergusson et al., 1993, 1998), and attention deficit disorder (Linnet et al., 2003). Animal models of prenatal cigarette smoke exposure have reported behavioral changes in adult offspring which parallel these human disorders (Balsevich et al., 2014). Further to this, maternal smoking is also associated with increased risk of obesity and the metabolic syndrome in the offspring, which show increased plasma leptin and impaired glucose tolerance in both humans and animal models (Power and Jefferis, 2002; Al Mamun et al., 2006; Chen et al., 2011). Although it can be difficult to tease apart the various effects of smoking during pregnancy, and exposure during early postnatal life, observations currently suggest a critical prenatal window during the first trimester of human pregnancy as the time when smoking is most likely to confer adverse health outcomes on the offspring (Toschke et al., 2003; Oken et al., 2005; Al Mamun et al., 2006).

Here, as with other complications of pregnancy that lead to developmental programming of neurobehavioral and metabolic disorders in the offspring, there appears to be chronic inflammation with elevated cytokines and other markers of immune system activation during gestation (Bermudez et al., 2002; Yanbaeva et al., 2007; Gosker et al., 2009).

Air Pollution

Human studies have found that children exposed *in utero* to pollutants from a nearby coal fired power plant had motor, language and social developmental delays (Tang et al., 2008). Animal models have shown that the air pollution present in modern day cities could cause a proinflammatory state during pregnancy, and increase the risk of metabolic and psychological disorders in the offspring. Pregnant mice exposed to common nanoparticle pollutants or diesel exhaust, showed elevated proinflammatory cytokines including IL-1 β and IL-6, some of which were detected in the fetal brain (Hougaard et al., 2010; Bolton et al., 2012; Jackson et al., 2012). The offspring of mice exposed to diesel exhaust displayed decreased locomotion, changes in the levels of the neurotransmitter dopamine, and neurobehavioral changes, including a tendency to avoid the central zone in an open field test and enhanced prepulse inhibition (Hougaard et al., 2010; Suzuki et al., 2010; Jackson et al., 2011). In addition, these offspring showed increased body weight and insulin resistance, classic features of the metabolic syndrome, particularly when placed on a high-fat diet (Bolton et al., 2012).

Taking these events or exposures together with their effects on cytokine abundance and offspring outcomes, it is tempting to speculate that abnormal exposure of the fetus to

immuno-modulatory molecules is the critical element linking adverse pregnancy experiences with developmental programming of obesity and neurobehavioral risks for the offspring.

How Might Immuno-Modulatory Molecules Elevate Offspring Risk of Neurobehavioral Disease?

Many of the changes to offspring phenotype that result from prenatal cytokine exposure are likely to be underpinned by changes in brain function, including cellular physiology, neural connectivity, and gene expression. The consequences are that the offspring's neural control of body weight and behavior are perturbed and/or are less robust to environmental challenge. How prenatal exposure affects later-life function is an area of much interest, but currently very few mechanisms have been revealed to explain this curious relationship. One possibility is that prenatal brain development is altered by abnormal exposure to cytokines, leading to structural changes in the brain that might underpin suboptimal function. Another is that abnormal cytokine exposure during fetal life alters the epigenetic regulation of genes whose expression and regulation later in life is critical to appropriate body weight regulation and behavior. These options and their potential consequences to the later life ability of the brain to regulate body weight are summarized in **Figure 1**. Similar mechanisms might be invoked to account for other abnormal offspring behaviors, such as socialization and learning, consequent to maternal obesity; and in these cases brain areas outside hypothalamic feeding centers (e.g., those that regulate the behaviors in question) are likely to be affected.

Access of Cytokines to the Fetal Brain

During normal development, the embryonic and fetal brain are believed to have limited exposure to cytokines because either their circulating levels are low (early pregnancy), or because entry into the brain is regulated (mid-late pregnancy). The blood-brain barrier (BBB) is believed to become functional between GD15 and GD17 in the mouse (Ben-Zvi et al., 2014). This time is theorized to relate approximately to late second/early third trimester in the human, but the actual time of BBB formation in humans is not well-characterized. Thus, prior to BBB formation, if there were elevated cytokines in the fetal circulation they might simply diffuse into the developing brain. Later in gestation, early life and into adulthood, when the BBB is intact, the main route into the brain for blood-borne molecules is via specific transporters that move molecules, including leptin, insulin, and growth factors, from the blood into the brain (Pohl et al., 2013; Banks, 2015; Koch et al., 2014). Some molecules may also enter the brain via fenestrated capillaries in circumventricular organs or the choroid plexus. How molecules that pass through the choroid plexus endothelium and into the ventricles then pass into the brain parenchyma are not well-characterized, but transporters as well as transcellular signaling have been implicated (Balland et al., 2014). The time in development at which the genes encoding these transporters begin to be expressed is currently unknown, but abnormally elevated cytokines might gain access to the fetal

brain through these normal transport mechanisms. Perturbation of either BBB formation or transporter function/gene expression in the brains of offspring developing in obese mothers are interesting further possible mechanisms that could account for abnormal exposure of the fetal brain to elevated cytokines. Another possible source of cytokines in the developing brain are microglia. These brain-resident immune cells of the monocyte lineage, are generated in the yolk sac around GD10, and can be found in the fetal brain several days later (Saijo and Glass, 2011). These seem likely candidates for producing cytokines within the brain proper, but they would need to become activated before they could do so. In support of this, recent evidence from the non-human primate suggests that microglia become abnormally activated in the prenatal hypothalamus of fetuses developing in high-fat diet-fed mothers (Grayson et al., 2010). These same fetuses also showed elevated cytokine gene expression in the hypothalamus, indicating that resident cells, such as activated microglia, provided an endogenous source of cytokines (Grayson et al., 2010).

Direct Effects of Cytokines on the Developing Brain

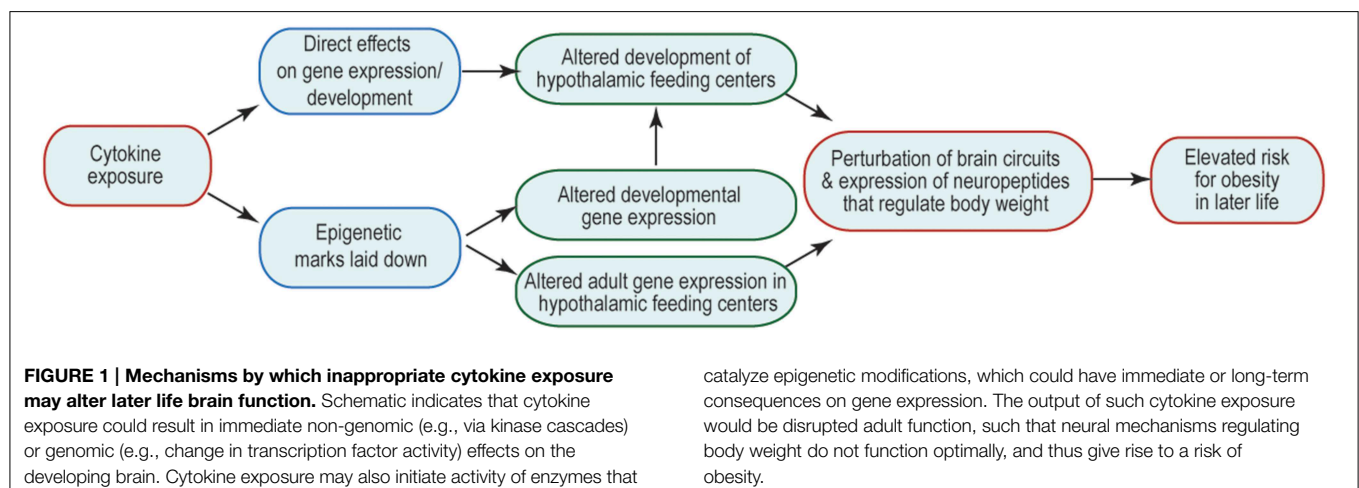
Although there is no apparent role for immune system-derived cytokines in normal brain development, the activation of cytokine signal transduction pathways, including JAK-STAT and MEK/ERK, are well-known to modulate brain development, including neurogenesis, cell type specification, migration, and axon growth (Schwartz et al., 2006; Markham et al., 2007; Qin and Zhang, 2012; Lee et al., 2013; Urayama et al., 2013). Thus, should cytokines gain access to the fetal brain inappropriately, they have the potential for substantial interference with normal neural development (Figure 1). Should such a mechanism be occurring, it is predicted that individuals who were exposed to cytokines *in utero*, would show structural changes in their brains at birth. In both humans and animal models, there are numerous reports of offspring brain changes that are evident beyond the neonatal period and in adulthood. However, few studies report structural or other alterations in the brain that are evident at birth, and which are related either to pregnancy complications or later-life outcomes. On the day of birth in a mouse model of

gestational obesity, the neural network regulating body weight is malformed in the offspring's brain, with fewer neural projections from the ARC reaching their targets (Sanders et al., 2014). Further exploration of the underlying mechanism revealed a role for IL-6, which altered axon growth and underlying developmental gene expression (Sanders et al., 2014). Also evident on the day of birth in a rat model of maternal obesity, were: (i) increases in the expression of orexigenic peptides, orexin and melanin concentrating hormone, in the lateral hypothalamic area; and (ii) increased galanin, enkephalin, and dynorphin expression in the PVN (Chang et al., 2008). Further exploration of the underlying mechanism revealed altered neurogenesis, leading to an increase in the generation of these cell types in the brains of fetuses developing in obese mothers (Chang et al., 2008). Also in a rat model of maternal obesity, CD11b and TLR4, both markers of activated microglia, were elevated on the day of birth (Bilbo and Tsang, 2010). This further suggests the possibility of an inflammatory state during the prenatal period. Better defining brain abnormalities as having their origins during prenatal or postnatal development (or some elements of each) will be critical to understanding underlying mechanisms, and ongoing research is beginning to provide this key information.

Effects of Cytokines on Epigenetic Regulators

Another possibility is that genes whose expression is critical for later life neural function are epigenetically modified at the time of cytokine exposure. Such genes may not necessarily be expressed during gestation, but the epigenetic marks laid down before birth would be there to affect gene expression whenever such genes were needed for normal neural function later in life; should such changes alter normal neural function, disease risk would be elevated. There is no need for the two mechanisms to be mutually exclusive (or exclusive of other possibilities), so it is likely that multiple mechanisms are at play when a fetus develops in an environment complicated by inappropriate cytokine exposure (Figure 1).

In many cases where life experience is associated with phenotypic change, alterations in DNA methylation, histone modifications, regulatory RNAs or some combination thereof have



been observed in multiple offspring tissues. What we currently do not understand in relation to maternal obesity is: which environmental factors are important modulators of the molecules that execute epigenetic changes; how particular genes become targeted for epigenetic change by certain factors; and how a constellation of epigenetic modifications occurring across the genome affect gene function at discrete loci and at particular times during development and adult life.

Should cytokines be causative agents in developmental programming via an epigenetic mechanism, then they should be able to modulate the activity of the proteins that enact epigenetic change, but how they might do so is less clear. It is known from cancer studies that inflammation-induced DNA methylation and histone changes are associated with polycomb-group target genes, which are significant here because their regulation relies on chromatin remodeling (Hahn et al., 2008). Also in cancer cell lines, IL-6 is able to affect both the expression and nuclear translocation of DNA methyltransferase 1 (DNMT1), consequently changing its activity within the cell (Hodge et al., 2001, 2007; Foran et al., 2010), and potentially targeting methylation to specific gene promoter regions (Li et al., 2012). As many epigenetic modifying enzymes are regulated by phosphorylation cascades (Jeltsch and Jurkowska, 2014), and since cytokines are known to stimulate multiple kinase cascades, including ERK, Jnk, PI3 kinase/Akt (Hirano et al., 1997; Mak and Yeh, 2002; Pestka et al., 2004a,b), the mechanistic links seem apparent, and available for further testing. In addition, transcription factors play a key role in directing epigenetic changes to particular genomic regions (Ding et al., 2008; Hashimoto et al., 2010; Feldmann et al., 2013). Cytokines also notoriously signal through the JAK/STAT pathway, which leads to DNA binding and transcriptional modulation by the activated STAT proteins (Murray, 2007). STAT proteins already have been described for their ability to recruit DNA binding and transcriptional regulatory molecules to specific DNA sequences, so this represents another signaling pathway through which cytokine exposure could lead to epigenetic modifications across the genome (Vahedi et al., 2012; Hedrich et al., 2014; Li et al., 2014).

Can Anti-Inflammation Treatment Protect the Offspring Brain?

If abnormally elevated inflammatory mediators play a causative role in the acquisition of offspring phenotype, then ameliorating them should protect normal offspring physiology. This line of reasoning has been used to test the efficacy of a number of pharmacological and physiological strategies that reduce inflammation. The literature here is growing rapidly, and this section is not intended to be exhaustive, but rather to give a glimpse into where the field is headed, and to bolster the hypothesis that increased exposure of the developing brain to inflammatory mediators is a key player in the developmental programming of offspring neurobehavioral disorders, including deregulated body weight control.

Transgenic elevation of the anti-inflammatory n-3 polyunsaturated fatty acids (PUFA) relative to the pro-inflammatory

n-6-PUFAs in a mouse model of maternal obesity, reduced the metabolic phenotype of offspring, including normalizing liver triglycerides and insulin sensitivity, and reducing body weight (Heerwagen et al., 2013). Unfortunately, inflammatory markers were not examined in the brain. As the transgene would presumably have been active during development, then it is likely that an anti-inflammatory milieu during gestation played a key role in determining the offspring phenotype, however, this was not examined directly. Several other agents with broad effects including anti-inflammatory, such as resveratrol (Roberts et al., 2014) and taurine (Li et al., 2013), have also been trialed in non-human primate or rat models of maternal obesity, respectively. Both reported mixed effects and confounding details that limit their practical utility, and neither examined inflammation in the brain.

Manipulation of diet and exercise also have been used to target inflammation as a means to combat offspring consequences of maternal obesity. Reversing maternal diet from high fat to control during the lactation period and after weaning in a mouse model of maternal obesity, was able to reverse social deficits and brain inflammation in offspring (Kang et al., 2014). This suggests that the neural circuits regulating social behavior rely on a constant exposure, particularly in the neonatal period, to inflammatory mediators in order to form and/or function improperly later in life. At least some of the neural circuits governing social behaviors show a great deal of plasticity in the early postnatal period, suggesting that once inflammation is reduced, these brain areas have the ability to form/reform normally. Other brain areas, governing other behaviors, will likely have different schedules of development and plasticity. Identifying these opportune times for plasticity will be critical to applying anti-inflammatory interventions to reduce offspring metabolic disease.

Exercise is another intervention that has been used to reverse the effects of maternal obesity on offspring health. Although exercise has been used to combat a variety of health disorders, the mechanisms underlying its effects are not well-understood. In a rat model of maternal obesity, several studies have observed that exercise, in either the early postnatal period or adulthood, was able to improve the offspring metabolic dysfunction caused by maternal obesity (Bahari et al., 2013; Rajia et al., 2013). The effects of early life voluntary exercise have not examined inflammatory marker expression in the brain (Rajia et al., 2013). Exercise in the adult offspring of obese dams who received either control or high fat diet after weaning showed a trend toward reduced IL-6 mRNA levels in the ARC and IL-1 β in the hippocampus following exercise compared to animals with similar diet history, but no exercise (Bahari et al., 2013).

Conclusions

Although the causative factors that link pregnancy complications or exposures to offspring health and disease risks later in life have yet to be fully cataloged, cytokines, classically known for their roles in immune system modulation, are surfacing as key players. Doubtless there are many more factors remaining to be identified. In addition, the mechanisms by which such factors impinge on the developing fetal brain and other organs remain very poorly explored at present. Identifying these mechanisms

and linking them causally with later life disease susceptibility will be a monumental challenge. Such understanding, however, is critical to developing therapeutic approaches to protect the developing brain in the face of adverse pregnancy complications. Of important further consideration, is the role of factors, including those from the immune system, in the postnatal developmental period, where the brain's extensive plasticity could be harnessed. The body of literature in this area also is growing at a rapid pace, and the interplay among prenatal and postnatal factors, immune activation, and brain development and function later in life are well on their way to being more clearly defined.

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- The next great challenges will be to translate this mechanistic understanding into preventative and directed interventions aimed at reducing the burden of disease risk that stems from pregnancy complications and exposures that are not currently easily preventable.
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Obesity and cognitive decline: role of inflammation and vascular changes

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The incidence of obesity in middle age is increasing markedly, and in parallel the prevalence of metabolic disorders including cardiovascular disease and type II diabetes is also rising. Numerous studies have demonstrated that both obesity and metabolic disorders are associated with poorer cognitive performance, cognitive decline, and dementia. In this review we discuss the effects of obesity on cognitive performance, including both clinical and preclinical observations, and discuss some of the potential mechanisms involved, namely inflammation and vascular and metabolic alterations.

Keywords: memory, hippocampus, cytokines, dementia, Alzheimer's disease, neuroinflammation

The incidence of obesity, classified by a body mass index (BMI, body mass divided by the square of one's height) $>30 \text{ kg/m}^2$, is rising steadily throughout the world's population. Attributed to unhealthy diets (that is over-consumption of food and beverages with a high content of fats, sugars, and salt) and physical inactivity, figures from the Organization for Economic Co-operation and Development 2014 Obesity report (OECD, 2014) suggest that worldwide 18% of the adult population are obese, with more than one in three adults in Mexico, New Zealand and the United States, and more than one in four in Australia, Canada, Chile, and Hungary included in this category.

Obesity can have damaging effects on many organ systems. Many of the comorbid conditions are related to metabolic syndrome, characterized by a large waist measurement, high triglyceride levels, glucose intolerance, and hypertension and thus risk factors for the development of non-insulin-dependent (type II) diabetes mellitus, systemic hypertension, coronary artery diseases, and heart failure. Moreover, the incidence of respiratory diseases such as obstructive sleep apnoea, gastrointestinal, and musculoskeletal disorders, thromboembolism, stroke and cancer are increased with obesity (Grundy, 2004; Haslam and James, 2005).

In addition, associations between obesity and impaired cognitive function, as well as risk of dementias such as Alzheimer's disease, have more recently been recognized. When we consider the growing population of overweight and obese people worldwide, along with an increasingly aging population, understanding the pathophysiology of obesity on the central nervous system and in particular those subregions important in learning, memory and executive functioning is essential. In this review we will focus on clinical evidence that obesity is associated with cognitive dysfunction and an increased risk of dementia, and

complement this with preclinical data from animal models of excess weight gain and cognitive impairment. We will then discuss brain pathological changes that have been observed in these populations, focusing largely on brain regions important in learning and cognition, namely the hippocampus and frontal cortex, before ending with an assessment of the current understanding of dietary-induced systemic and central inflammation within these regions.

OBESITY AND COGNITIVE DYSFUNCTION

MILD COGNITIVE IMPAIRMENT

A growing body of research indicates that obesity in mid-life is a predictor of mild cognitive impairment at old age. Cognitive aging is a normal process where in older adulthood there is a structural and functional change that results in a deterioration of cognitive ability (Glisky, 2007). However, even when controlling for cognitive aging, studies show a negative correlation between BMI and global cognitive performance (Elias et al., 2005; Jeong et al., 2005; Hassing et al., 2010). A cross-sectional longitudinal study of over 2000 middle aged workers supported the linear association between BMI and cognitive function determined by the word-list learning test, which evaluates verbal learning and memory, and Digit-symbol Substitution test (DSST), which assesses attention, response speed, and visuo-motor coordination. Obese people recalled fewer words from the list in the word-list learning test and took longer to complete DSST relative to normal weight individuals (Cournot et al., 2006). In another study combining ages from 20 to 82, overweight and obese people exhibited poorer executive function test performance than normal weight adults with no evidence of a BMI x age interaction (Gunstad et al., 2007). Across studies, the different cognitive domains analyzed make

it difficult to draw absolute comparisons, but impairment of specific cognitive domains such as executive function and short-term memory have been consistently identified in obese individuals when compared to normal weight counterparts (Cournot et al., 2006; Mond et al., 2007; Lokken et al., 2009; Sabia et al., 2009).

DEMENTIA AND ALZHEIMER'S DISEASE

Obesity is associated with not only an increased risk of development of mild cognitive impairment, but additionally, late-life dementia and Alzheimer's disease (Solfrizzi et al., 2004; Whitmer et al., 2005; Gustafson et al., 2012; Besser et al., 2014). The relative risk of the development of dementia and Alzheimer's disease for obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) and overweight ($\text{BMI} = 25\text{--}29.9 \text{ kg/m}^2$) individuals in midlife compared to normal weight individuals was 2.04 and 1.64, respectively (Anstey et al., 2011). Epidemiological studies have shown that obesity in middle age increases the risk of developing dementia and Alzheimer's disease, irrespective of associated medical conditions such as diabetes or vascular disease (Solfrizzi et al., 2004; Whitmer et al., 2005; Panza et al., 2010; Gustafson et al., 2012; Besser et al., 2014). For example Whitmer and colleagues reported that being overweight at age 40–45 increased ones risk of developing dementia by 35%, while being obese increased this risk to 74% when compared to normal weight individuals (Whitmer et al., 2005). The link of elderly obesity with dementia and Alzheimer's disease is complicated. Several studies have found an age dependent relationship with Alzheimer's disease and late-life obesity (Elias et al., 2003; Gustafson et al., 2003, 2009), while others have shown no or even negative correlations (Buchman et al., 2005; Stewart et al., 2005; Luchsinger et al., 2007; Fitzpatrick et al., 2009). A possible explanation of the confounding results is that weight loss is strongly associated with Alzheimer's disease and occurs before any presentation of cognitive impairment (Buchman et al., 2005; Stewart et al., 2005).

ANIMAL MODELS OF WEIGHT GAIN

Animal models, which allow for more accurate control of diet and other confounding factors than studies in humans, have also found that there is a detrimental effect of diet-induced obesity on cognition. Indeed, in high fat feeding models of obesity, impairments of working memory (Jurdak et al., 2008), learning (Molteni et al., 2002; Murray et al., 2009), and memory performance (Granhölm et al., 2008; Kanoski and Davidson, 2010; Kosari et al., 2012) have been observed. A rodent study showed that consumption of a high fat diet (45%) for 3 months caused obesity, insulin resistance, and poor performance in the operant based delayed matching to position task examining short-term information retention and executive function (McNeilly et al., 2011). Furthermore, acquisition rates in learning have been observed to be impaired in rats fed a high fat diet (25%) for 3 months, as evaluated by radial arm water maze, where fat-fed rats took longer and made more errors trying to locate a hidden platform compared to control (Alzoubi et al., 2013). Interestingly, a further study has shown that rats fed a high fat diet (60%) for 3 months have impaired spatial memory that is independent of weight gain and blood pressure change (Kosari et al., 2012).

ASSOCIATIONS WITH NEUROPSYCHIATRIC ILLNESS

There is widespread prevalence of psychiatric symptomatology in individuals diagnosed with mild cognitive impairment and Alzheimer's disease (Lyketsos et al., 2002; Enache et al., 2011), and extensive comorbidity of psychiatric illness with obesity (Luppino et al., 2010; Megna et al., 2011). Medical issues and mobility restrictions associated with being overweight or obese can negatively impact on an individual's psychological well-being, and can lead to depression (Wardle and Cooke, 2005). In turn, mental health issues can lead to unhealthy lifestyle choices, such as diminished physical activity, increased appetite and poor food choices, smoking, and excessive alcohol intake (De Wit et al., 2010; Hoare et al., 2014). The use of psychiatric medicines, such as antipsychotics and antidepressants, to manage mental health issues in obese individuals may be problematic as there is a clear association between psychiatric medicines and significant weight gain (Reynolds and Kirk, 2010; Serretti and Mandelli, 2010; Hasnain et al., 2012). While many patients with a psychiatric illness are highly susceptible to cardiovascular disease, diabetes, and metabolic syndrome (De Hert et al., 2009; Pan et al., 2012), there is growing understanding of a role for hypothalamic-pituitary-adrenal axis dysfunction and basal systemic low-grade inflammation in the relationship between psychiatry and obesity. While this is beyond the scope of this review, recent researchers discuss the complex relationship between obesity and psychiatric illness (Hryhorczuk et al., 2013; Jaremka et al., 2013; Castanon et al., 2014; Miller and Spencer, 2014).

OBESITY AND BRAIN PATHOPHYSIOLOGY

The negative systemic effects of obesity on cardiovascular and metabolic physiology are well-recognized, and it is now clear that the brain is also negatively affected by obesity. Alterations in brain pathology of overweight/obese individuals who are otherwise healthy are supported by preclinical studies, demonstrating the possible underlying mechanisms by which obesity impairs higher cerebral function and exacerbates aging-related dementia remain wide and varied.

BRAIN ATROPHY

Increased adiposity has been correlated with reduced volume in a number of brain regions. In a longitudinal study in a group of female patients born between 1908 and 1922, women with atrophy of the temporal lobe were found to have a higher BMI, with risk of temporal atrophy increased 13–16% per 1 kg/m^2 BMI rise (Gustafson et al., 2004). More recent brain scanning techniques demonstrated that a group of obese individuals (BMI average 39) had significantly lower gray matter density in the post-central gyrus, frontal lobe, putamen, and middle frontal gyrus compared to a group of controls with a BMI of 22 (Pannacciulli et al., 2006). A further analysis in over 1400 Japanese healthy individuals revealed a significant negative correlation in men, though not in women, between BMI and brain gray matter ratio with temporal, occipital, and frontal lobes and the anterior lobe of the cerebellum showing reduced volume with increased BMI (Taki et al., 2008).

The hippocampal formation, a structure essential for learning and memory, is particularly susceptible to aging (Jack et al., 2000; Raji et al., 2009). It is also well-recognized that reduced

hippocampal volumes predict cognitive decline and dementia in the general population (Elias et al., 2000; Amieva et al., 2005; Den Heijer et al., 2010). As we described previously, a majority of studies have found that obesity in mid-life is associated with an increased risk of developing dementia in later life, and consistent with this there is evidence from the Framingham Offspring Cohort Study of increased rates of hippocampal brain atrophy and executive function decline with mid-life obesity (DeBette et al., 2011). However, this effect of obesity on hippocampal functioning is also found earlier: Adolescents with metabolic syndrome showed significantly smaller hippocampal volumes along with impaired attention and mental flexibility compared to non-obese children of similar ages (Yau et al., 2012).

Pre-clinical experimental rodent studies have also provided insight into the potential mechanisms underpinning obesity-related cognitive impairment. The affected cognitive domains involved in learning, memory, and executive function are mainly subserved by the hippocampus and prefrontal cortex. Long-term potentiation (LTP) is considered to be the major cellular mechanism that contributes to learning and memory where there is a synaptic change that leads to the formation of a stronger synapse (Bliss and Collingridge, 1993). In rodent models, high fat levels impair hippocampal LTP in the dentate gyrus (Karimi et al., 2013) and CA1 regions (Stranahan et al., 2008). Moreover a diet high in triglycerides was shown to diminish hippocampal long-term synaptic potential maintenance (Farr et al., 2008) suggesting a possible mechanism by which triglycerides mediate cognitive dysfunction associated with obesity.

At a cellular level, hippocampal changes are observed when diet is manipulated. Consumption of a high fat diet produces a reduction in molecules involved with neurogenesis, synaptic function and neuronal growth. A decrease in hippocampal neurogenesis in the dentate gyrus was observed after 4 weeks of feeding of a 42% fat diet (Lindqvist et al., 2006), while reduced levels of hippocampal markers of cellular proliferation (Kim et al., 2009) and hippocampal brain-derived neurotrophic factor (Molteni et al., 2002; Wu et al., 2003) have also been reported. Additionally consumption of dietary fats induces hippocampal (Rivera et al., 2013) and hypothalamic (Moraes et al., 2009) neuronal apoptosis and a reduction in hippocampal weight (Calvo-Ochoa et al., 2014) showing that high fat consumption impairs both new neuronal production and cell survival. It should be noted that not all diet manipulations have a negative effect on hippocampal function: Mice fed a diet rich in polyphenols and polyunsaturated fatty acids were observed to have more newly generated cells in the dentate gyrus (Valente et al., 2009).

Meanwhile in the prefrontal cortex reduced levels of dopamine (Geiger et al., 2008; Hansen et al., 2013) and acetylcholine (Morganstern et al., 2012) and increased markers of oxidative stress (Souza et al., 2007) have been observed in both obese-prone rat models and after high fat feeding, suggesting a dysfunction in this region which may contribute to associated observed behavioral deficits.

CEREBROVASCULAR

Vascular dementia is caused by cerebrovascular disease. Increasing evidence suggests that the vascular effects of obesity

have a key role in the development of vascular cognitive impairment in aged people (Gorelick et al., 2011) by promotion of atherosclerosis in large cerebral arteries and alterations at the level of the cerebral microcirculation (Zlokovic, 2011). Indeed in a recent rodent study, mice fed a high fat diet displayed disruptions in cerebral vascular function including neurovascular coupling and functioning of arteries (Li et al., 2013; Lynch et al., 2013). Moreover, aging exacerbated obesity-induced decline in microvascular density in the hippocampus and cerebral cortex which was positively correlated with hippocampal-related cognitive function. Aging also exacerbated the obesity-induced oxidative stress and impaired cerebral blood flow indicating the possible effects of both aging and obesity and brain vascular integrity (Tucsek et al., 2014b).

ALZHEIMER'S DISEASE RELATED PATHOLOGY

Amyloid plaques and neurofibrillary tangles containing tau protein are the pathological markers of Alzheimer's disease (Serrano-Pozo et al., 2011), accompanied by microglia activation and astrogliosis (Beach et al., 1989; Itagaki et al., 1989). Pathological progression is somewhat consistent with plaques, tangles, neuronal, and synaptic loss observed in medial temporal cortical regions such as entorhinal and perirhinal cortex, followed by hippocampus and cerebral cortex (National Institute on Aging, 1997). The mechanisms by which obesity influences risk of Alzheimer's disease remain to be fully understood. Higher levels of Amyloid-beta ($A\beta$, the main component of amyloid plaques) precursor protein (APP) and tau expression have been reported in hippocampal sections from morbidly obese patients without cognitive impairment, compared to a cohort of non-obese controls (Mrak, 2009). Indeed increased levels of plasma amyloid proteins have been found in a number of studies of obese individuals (Lee et al., 2009; Jahangiri et al., 2013) suggesting a possible mechanism linking midlife obesity with the later development of Alzheimer's disease.

A number of experimental studies have examined markers of Alzheimer's disease-related pathology in rodents receiving diets high in fat. Mice receiving a high fat diet displayed increased expression of amyloid precursor protein and APP processing enzyme (Thirumangalakudi et al., 2008; Puig et al., 2012) along with tau phosphorylation (Koga et al., 2014). Moreover in rats fed a high fat diet followed by streptozotocin injection to induce a model of type 2 diabetes, hippocampal APP-cleaving enzyme and $A\beta$ were found to be present, and raised compared to controls, in the hippocampus (Zhang et al., 2009).

Similarly, diet-induced obesity has been shown to increase amyloid and tau pathology in transgenic mouse models of Alzheimer's disease. In the double-mutant presenilin (PS)-APP model just 7 weeks of diet modification resulted in both hypercholesterolemia and significantly increased levels of $A\beta$ peptides in the brain that were strongly correlated with the levels of both plasma and brain total cholesterol (Refolo et al., 2000). Meanwhile, much longer dietary interventions, for example 10 months of a high fat (35%) formula to the triple transgenic (3xTg-AD) mice increased $A\beta$ 40 and 42 concentrations and tau, suggesting that high-fat consumption promotes Alzheimer's disease-like neuropathology (Julien et al., 2010).

BLOOD BRAIN BARRIER

A functioning blood-brain barrier (BBB) has an important role in maintaining a precisely regulated microenvironment for reliable neuronal signaling by allowing entry into the central nervous system of essential nutrients and protecting the brain from blood-borne toxins (Ballabh et al., 2004). The chemical consequences of a high fat diet (including elevated fatty acids and sugars) may also influence the brain by disrupting the integrity of the BBB.

BBB dysfunction is associated with both Alzheimer's disease and vascular dementia (Skoog et al., 1998), and can be related to clinical vascular factors (Blennow et al., 1990). In a longitudinal study being overweight or obese in mid-life was correlated with lower BBB integrity almost a quarter of a century later (Gustafson et al., 2007). Further evidence is available from animal studies: Rats fed a Western diet for 3 months had a decrease in expression of tight junction proteins in the choroid plexus and BBB (Kanoski et al., 2010). Moreover, Western diet consumption in rats produces, as a consequence of this BBB dysfunction, increased permeability to a peripheral fluorescent tracer in the hippocampus (Kanoski et al., 2010; Davidson et al., 2012). Reduced BBB integrity and increased microgliosis in the hippocampus was also observed in rats fed a high-saturated-fat and cholesterol diet for 6 months (Freeman and Granholm, 2012) demonstrating that the hippocampus appears to be particularly vulnerable to diet-induced BBB disruption.

Mechanisms linking obesity to BBB dysfunction, and subsequent neuronal impairment, memory loss and dementia are not yet fully established. As stated previously obesity has been associated with increased levels of circulating plasma amyloid proteins (Lee et al., 2009; Jahangiri et al., 2013) and there is some suggestion that peripheral A β can impair BBB integrity by pathologically affecting the cerebrovasculature (Su et al., 1999). Further support for the relationship between obesity and degeneration of the BBB suggests that high circulating levels of fat impair active transport of consummatory regulatory hormones such as leptin and ghrelin through the BBB (Banks et al., 2004, 2008), perhaps inhibiting their positive roles in synaptic plasticity via actions in the hippocampus (Shanley et al., 2001; Diano et al., 2006). It should also be considered that obesity leads to increased circulatory inflammatory markers which in turn gain access to the hypothalamus by increasing BBB permeability and/or via areas that lack an effective BBB.

SYSTEMIC INFLAMMATION

In obesity there is an accumulation of white adipose tissue which is the key site facilitating systemic inflammation (Odegaard and Chawla, 2013). Particularly, both hypertrophied adipocytes and adipose tissue-resident immune cells (primarily lymphocytes and macrophages) contribute to increased circulating levels of pro-inflammatory cytokines where there is an increase of tumor necrosis factor (TNF)- α , important feeding-related peptides such as leptin and resistin, plasminogen activator inhibitor 1, C-reactive protein and interleukins (IL)-1 β and IL-6 (Visser et al., 1999; Yudkin et al., 1999; Ouchi et al., 2011) in obese individuals. Those with a higher waist circumference and waist-hip ratio also showed higher C-reactive protein and IL-6 concentrations, with IL-6 positively associated with total body fat (Hermsdorff et al., 2011)

suggesting that these measures may be more highly correlated to inflammatory markers than increases in BMI (Hermsdorff et al., 2011; Thewissen et al., 2011).

Another mechanism by which chronic low grade inflammation occurs is through T-cells. A cross-sectional study of obese women found that T-cell derived cytokines (IL-23 and IL-17) were increased independent of increased abdominal fat and insulin resistance (Sumarac-Dumanovic et al., 2009). This has also been corroborated in a diet-induced obese mouse study (Winer et al., 2009). Obesity has also been shown to induce the accumulation and activation of macrophages in adipose tissue in both mice and humans (Weisberg et al., 2003; Xu et al., 2003; Drake et al., 2011).

Systemic inflammation can contribute to cognitive decline and dementia. The first functional link between obesity and inflammation was found in obese mice where adipose tissue was observed to secrete TNF- α (Hotamisligil et al., 1993). Further pre-clinical data have demonstrated that after a lipopolysaccharide (LPS) challenge in diet-induced obese rats, an exacerbated and prolonged fever was observed as well as an increase in plasma TNF- α , IL-6, and IL-1 α levels compared to lean controls (Pohl et al., 2009). Cytokines, such as IL-1 β and IL-6 have been shown to disrupt neural circuits involved in cognition and memory (Jankowsky and Patterson, 1999; Gemma and Bickford, 2007). A recent meta-analysis identified that increased plasma levels of C-reactive protein and IL-6 is associated with an increase of dementia (Koyama et al., 2013). Elevated plasma IL-6 and IL-12 levels were also associated with impaired processing speed and executive function assessed via Stroop interference and digit symbol testing in a group of elderly participants between the ages of 70 and 90 (Trollor et al., 2012). Furthermore, an imaging study conducted by Harrison and colleagues showed that after inducing systemic inflammation by injection of *Salmonella typhi* vaccine an acute decline in spatial memory (but not medial temporal lobe independent procedural memory) was observed in humans (Harrison et al., 2014), suggesting that the medial temporal lobe is acutely sensitive to systemic inflammation.

CENTRAL INFLAMMATION

Peripheral cytokines can act on the brain to induce local production of cytokines (Dantzer et al., 2008). As such, central inflammation is observed after high fat feeding and in genetic models of obesity, particularly in the hypothalamus (for review see Miller and Spencer, 2014). When we consider areas important in cognition, in db/db mice, a model of metabolic syndrome where obesity arises as a result of leptin receptor insensitivity (Hummel et al., 1966), IL-1 β , TNF- α , and IL-6 mRNA expression levels in the hippocampus are increased when compared to wild type controls (Dinel et al., 2011). Moreover, in mice fed a 60% high fat diet for 20 weeks, raised TNF- α expression has been observed in the hippocampus (Jeon et al., 2012). Juvenile high fat diet intake did not influence basal expression of pro-inflammatory cytokines in the brain, but potentiated the enhancement of TNF- α expression specifically in the hippocampus after a peripheral immune challenge with LPS (Boitard et al., 2014). Chronic high fat diet consumption has also been shown to exacerbate LPS-induced cytokine mRNA expression of TNF- α and interferon- γ

in the hippocampus as well as IL-6 and suppressor of cytokine signaling-3 in the hypothalamus (Andre et al., 2014). At this stage the prefrontal cortex is yet to be investigated.

MICROGLIA AND ASTROCYTES

Microglia, the primary mediators of the central nervous system's immune defense system release pro-inflammatory cytokines, chemokines, nitric oxide, and superoxide species (Loane and Byrnes, 2010). While the relationship between obesity-induced microglia expression within hypothalamic regions in animal models is well-documented (Miller and Spencer, 2014), new data indicate that brain regions involved in cognition and memory also show exacerbated microglial expression. In the db/db mouse, increased levels of microglial activation markers are observed throughout the hippocampus (Erion et al., 2014). Moreover in aged (24 months) mice, hippocampal microglial activation was shown to be exacerbated by 5 months treatment with a high fat diet (Tucsek et al., 2014a). In addition, treatment of cultured primary microglia with sera derived from these aged obese mice resulted in significantly more pronounced microglia activation and oxidative stress (Tucsek et al., 2014a).

Astrocytes are the most abundant glial cell within the central nervous system and respond to all forms of insults through a process referred to as reactive astrogliosis (Sofroniew and Vinters, 2010). Within the hypothalamus, astrocytes produce cytokines that drive inflammatory responses (Garcia-Caceres et al., 2013) although new data suggest central inflammation can extend beyond the hypothalamus in obesity regimes to affect areas directly related to cognition. Astrocytes from the CA3 region of hippocampus showed longer and less abundant projections in high fat diet mice (Cano et al., 2014). In obese Zucker rats a similar pathology is observed with a reported significant increase in the number of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes throughout all subfields of the hippocampus as well as frontal and parietal cortices (Tomassoni et al., 2013).

CONCLUSION

It is abundantly evident that there is a deleterious effect of obesity/high fat feeding on cognitive performance. In human clinical studies, obesity has been shown to increase the risk of the development of mild cognitive impairment, in the form of short-term memory and executive function deficits, as well as dementia and Alzheimer's disease. Genetic and diet-induced models of obesity further support this link with obese animals displaying deficits in working memory, learning, and memory performance. The exact mechanisms or mediators that underlie the connections between obesity and the risk of cognitive impairment are still unknown but potential avenues of further research include brain atrophy, disruption in cerebrovascular function, development of Alzheimer's disease related pathology, BBB breakdown, and systemic and central inflammation.

Only a limited number of therapeutic options are currently available to treat dementia. These pharmaceutical agents have shown some potential to improve cognition but are effective for only some of the population, may be useful for only a limited time, and do not change the underlying disease process (Craig

and Birks, 2005; Birks, 2006). Moreover, it is evident that obesity not only negatively impacts brain function and structure in adulthood and dementia, but clearly causes changes in the developing brain during childhood and adolescence (Liang et al., 2014), with researchers still coming to understand the long-term negative consequences of a life-time of being overweight on the brain. As such interventions that focus on education and life-style related factors to improve cognitive health (Lucke and Partridge, 2013) appear to be the most promising option. Increasing physical activity is certainly beneficial in many instances of cognitive decline as well as other neurological disturbances (Loprinzi et al., 2013) and may be the best treatment preference until the development of therapeutic options to treat cognitive deficits and/or prevent cognitive decline in obesity are available.

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New insights into the impact of neuro-inflammation in rheumatoid arthritis

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Rheumatoid arthritis (RA) is considered to be, in many respects, an archetypal autoimmune disease that causes activation of pro-inflammatory pathways resulting in joint and systemic inflammation. RA remains a major clinical problem with the development of several new therapies targeted at cytokine inhibition in recent years. In RA, biologic therapies targeted at inhibition of tumor necrosis factor alpha (TNF α) have been shown to reduce joint inflammation, limit erosive change, reduce disability and improve quality of life. The cytokine TNF α has a central role in systemic RA inflammation and has also been shown to have pro-inflammatory effects in the brain. Emerging data suggests there is an important bidirectional communication between the brain and immune system in inflammatory conditions like RA. Recent work has shown how TNF inhibitor therapy in people with RA is protective for Alzheimer's disease. Functional MRI studies to measure brain activation in people with RA to stimulus by finger joint compression, have also shown that those who responded to TNF inhibition showed a significantly greater activation volume in thalamic, limbic, and associative areas of the brain than non-responders. Infections are the main risk of therapies with biologic drugs and infections have been shown to be related to disease flares in RA. Recent basic science data has also emerged suggesting that bacterial components including lipopolysaccharide induce pain by directly activating sensory neurons that modulate inflammation, a previously unsuspected role for the nervous system in host-pathogen interactions. In this review, we discuss the current evidence for neuro-inflammation as an important factor that impacts on disease persistence and pain in RA.

Keywords: neuroinflammation, rheumatoid arthritis, tumor necrosis factor alpha, neuroimaging, infection, lipopolysaccharide

INTRODUCTION

Rheumatic diseases include some of the most common chronic disorders worldwide. Of these, rheumatoid arthritis (RA) is considered to be, in many respects, an archetypal autoimmune disease (Feldmann and Maini, 2010). RA causes activation of pro-inflammatory pathways resulting in joint and systemic inflammation and remains a major clinical problem (McInnes and Schett, 2011). Treatments with immune-modulatory drugs, including biologic therapies, have revolutionized its management. New

therapies target relevant cytokines such as tumor necrosis factor alpha (TNF α) and immune cells such as B cells. In RA, biologics have been shown to reduce joint inflammation, limit erosive change, reduce disability and improve quality of life (Scott et al., 2010). Biologics are mainly co-administered with disease-modifying drugs such as methotrexate when the latter are found to achieve insufficient disease control on their own (Firestein, 2003). Recent work using functional neuroimaging has suggested that TNF inhibitors may also reduce central nervous system activity related to inflammation-induced pain in people with RA using brain neuroimaging (Rech et al., 2013). Classically, the term "neuro-inflammation" has implicated the activation of microglia and astroglia which in turn activate the expression of pro-inflammatory cytokines and chemokines in a variety of conditions including Alzheimer's disease (AD) (O'Callaghan et al., 2008). However, the trigger(s) for activation of a potential neuro-inflammatory process and furthermore its measurement in clinical disease is not always straightforward to decipher. Several groups have utilized animal models to discover that although certain features of inflammation are typical for the pro-inflammatory response systemically e.g.,

Abbreviations: ACPA, Anti-Citrullinated Protein Antibody; AD, Alzheimer's Disease; BBB, blood-brain barrier; Cho, Choline; Cr, Creatine; COX, Cyclo-Oxygenase; CS, Corticosteroids; CSSERP, Chemo-Somatosensory Event-Related Potentials; DAMPs, damage associated molecular patterns; DMARD, Disease-Modifying Anti-Rheumatic Drug; DRG, Dorsal Root Ganglia; DTI, Diffusion Tensor Imaging; ESR, Erythrocyte Sedimentation Rate; fMRI, Functional Magnetic Resonance Imaging; ICAM, intercellular adhesion molecule; IFN- β , Interferon- β ; IL, Interleukin; ISG, Interferon-Stimulating Genes; LPS, lipopolysaccharide; MRI, Magnetic Resonance Imaging; MRS, Magnetic Resonance Spectroscopy; NAA, N-Acetyl Aspartate; PADIV, Peptidyl Arginine Deaminase type IV; RA, Rheumatoid Arthritis; RNA, ribonucleic acid; SE, Shared Epitope; SNP, Single Nucleotide Polymorphism; TACE, Tumor necrosis factor alpha converting enzyme; Th, T helper cell; TLR, Toll-like receptor; TNF α , tumor necrosis factor alpha; WM, White Matter.

interleukin-6 (IL-6), interferon- β (IFN β), these are not observed in the brain, but activation of TNF α does appear to maintain pro-inflammatory activity in the brain as it does systemically (Skelly et al., 2013; Thomson et al., 2014). Some of the basic science observations can be extended to RA, where Chou et al. (2010) observed that people with RA receiving TNF inhibitor treatment (infliximab, etanercept, adalimumab) showed a reduction in the risk of developing AD compared to controls (Chou et al., 2010). The risk of AD was not affected by exposure to other Disease-Modifying Anti-Rheumatic Drugs (DMARDs) used in RA. Recent work by Detrait et al. (2014) has also shown that peripheral administration of TNF inhibitors in mice counteracts the amyloid-induced increase of hippocampal TNF α levels and memory deficits in mice. In this review, we explore the theme of neuro-inflammation in RA. Although as yet, the cause-and-effect relationship between infection, autoimmunity, neural correlates and RA disease activity are not understood mechanistically, we review the current status of the field.

CURRENT CONCEPTS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

RA is an immune-mediated pro-inflammatory disease, often resulting in chronic disability, early mortality, systemic complications and high socioeconomic burden on society as a whole (McInnes and Schett, 2011). There has been a vast improvement in our understanding of this condition over the last few decades, resulting in improved treatments which are now initiated early in order to maximize and maintain disease remission (van Vollenhoven, 2010).

Risks for developing RA involve a complex interplay between genotype, environment and lifestyle factors such as smoking (Mahdi et al., 2009). Twin studies have shown a concordance rate of 15–30% among monozygotic twins and 5% among dizygotic twins (MacGregor et al., 2000). Genome-wide association analyses have uncovered immune regulatory factors that may underlie the disease; including PTPN22 among the Single Nucleotide Polymorphisms (SNPs) identified (Wellcome Trust Case Control Consortium, 2007). An association with HLA-DRB1 has been established for RA patients who are positive for rheumatoid factor or anti-citrullinated peptide antibodies (ACPA) (Gregersen et al., 1987). In keeping with the role of HLA-DRB1 in antigen presentation, a number of studies over the last 2 decades have shown that auto-reactive immune responses are mediated by T-cell repertoire selection, antigen presentation or changes in peptide affinity (Panayi, 2006). The shared epitope (SE), carried by the vast majority of RA patients, is a 5-aa sequence motif in the third allelic hypervariable region of the HLA-DR β chain. Proposed explanations for the link between RA and the SE include molecular mimicry of the SE by microbial proteins, increased T cell senescence induced by SE-containing HLA molecules and a potential pro-inflammatory signaling function that is unrelated to the role of the SE in antigen recognition (Weyand and Goronzy, 1990; De Almeida et al., 2010).

Gene-environment interactions are also important in RA development. Smoking and other environmental risks to the lung

such as silica exposure, increase the risk of RA in people with susceptibility HLA-DR4 alleles (Symmons et al., 1997; Klareskog et al., 2008). Smoking and HLA-DRB1 alleles synergistically increase one's risk of developing the anti-citrullinated protein antibodies (ACPA) that are present in the majority of patients with RA (Klareskog et al., 2008). It has therefore been proposed that environmental stress in the lung or other mucosal surfaces may promote post-translational modifications through activation of peptidyl arginine deiminase, type IV (PADIV), resulting in citrullination of mucosal proteins. Loss of tolerance to the neoepitopes generated by citrullination can be detected clinically in people with RA by the ACPA response (Vincent et al., 1999).

It has long been recognized that infectious agents such as *cytomegalovirus*, *E. coli*, *Epstein Barr virus*, *parvovirus*, and *proteus* species may play a role in RA. Recently, the oral pathogen *Porphyromonas gingivalis* has been implicated in the pathogenesis of RA (Mikuls et al., 2014). Products of infectious agents e.g., heat shock proteins and enzymes responsible for citrullination have been shown in several models to induce immune reactivity. For example, several citrullinated self-proteins can be identified in ACPA assays, including alpha enolase, keratin, fibrinogen, fibronectin, collagen, and vimentin (van der Woude et al., 2010). Although unifying mechanisms for the link between infection and RA autoimmunity are not entirely established, the theory of molecular mimicry has been proposed (van Heemst et al., 2014). The formation of immune complexes during infection may trigger the induction of rheumatoid factor, which is a high affinity autoantibody against the Fc portion of immunoglobulin, often used in the diagnosis of RA (De Rycke et al., 2004). A link has been described between RA and periodontal disease: *Porphyromonas gingivalis* produces PADIV which can promote citrullination of mammalian proteins (Wegner et al., 2010). Recently, the gastrointestinal microbiome has also been implicated in the development of autoimmunity (Scher et al., 2012).

Emerging data suggests there is an important impact of bidirectional communication between the brain and immune system that has a significant impact on RA symptoms. The onset of RA is associated with adverse life events and infectious triggers, where links between the hypothalamic-pituitary-adrenal axis and autoimmunity have been shown (Sokka et al., 2009; Capellino et al., 2010). The impact of external factors such as infections and their impact on disease activity in RA including joint pain/swelling will be discussed further in the following sections. One feature of established RA includes the presence of rheumatoid factor, which is an IgM complex of antibodies to IgG found circulating in RA (Mellor, 1959). Cerebrospinal fluid rheumatoid factor has been demonstrated in case reports of central nervous system manifestations of RA. (Markenson et al., 1979; Inan et al., 2011) Recent studies suggest that circulating immune complexes can elicit a neuro-inflammatory response in the brain. Teeling et al. (2012) showed that in the presence of antigen, antibodies can lead to local immune complex-mediated inflammatory reaction in the brain parenchyma and directly induce local tissue damage through the recruitment and activation of microglia through Fc γ receptors (Teeling et al., 2012).

THE ROLE OF CYCLO-OXYGENASE AND PROSTANOIDS IN NEURO-INFLAMMATION

Several of the brain effects induced by cytokines such as TNF α are thought to be mediated by regulating the expression of cyclo-oxygenase (COX) enzymes (mainly COX-2) and generation of prostanoids. Under usual physiological conditions, these inflammatory mediators are absent in the brain or found in low levels. After brain injury or insult, there can be induction of pro-inflammatory mediators in astrocytes, microglia, neurons and endothelial cells which results in the development of neuro-inflammatory conditions. Recently, the COX-2 inhibitor celecoxib has been shown to reduce functional connectivity measured by functional MRI in an animal model of osteoarthritis (Upadhyay et al., 2013). In their study, Upadhyay et al. (2013) showed a reduction in blood oxygenation in recognized brain pain centers including the thalamus, hippocampus, periaqueductal gray matter and nucleus accumbens in arthritic rodents treated with celecoxib. Human studies in patients with chronic knee osteoarthritis have also shown that COX inhibitors e.g., valdecoxib, reduce spontaneous brain pain activation signals measured by functional brain MRI (Parks et al., 2011).

One of the earliest studies in human subjects by Ho et al. (2001) demonstrated that neuronal COX-2 expression was increased in post-mortem samples of people with Alzheimer's disease and correlated with clinical progression of dementia (Ho et al., 2001). In Alzheimer's disease, the classical neuropathological changes include deposition of neurofibrillary tangles and β amyloid (A β). Alzheimer's dementia is typified by activation of microglia and astrocytes in response to A β deposition, leading to a release of a variety of factors including the cytokines TNF α and IL-1 β , free radicals such as nitric oxide, superoxide and cyclo-oxygenase pathway derived prostanoids (Combs et al., 2001; Hull et al., 2006; Medeiros et al., 2007). Evidence for the activation of the COX pathway has come from neuro-inflammatory diseases that include Alzheimer's disease, Parkinson's disease and multiple sclerosis. For example, in a mouse model of Parkinson's disease, Hunter et al. (2007) showed that celecoxib, a COX-2 inhibitor, inhibited neuronal effects in rats injected with LPS. They found that a number of effects were reduced in rats treated with celecoxib, including inflammation and nigral dopaminergic neuronal loss. It is therefore possible that non-steroidal anti-inflammatory drugs (NSAIDs) such as celecoxib could have an inhibitory effect on neuro-inflammation and could have an impact on delaying neurodegenerative effects.

CAN INFECTION INFLUENCE RHEUMATOID ARTHRITIS PAIN?

The central nervous system is involved in immune regulation and homeostasis, with neuro-immunological interactions regulating disease development in animal models of arthritis (Chiu et al., 2013). Peripheral inflammation triggered by synovial inflammation in RA stimulates the increased release of cytokines (Taylor, 2014). Cytokine inhibitors administered to patients with a high disease load have also been shown to suppress RA arthritic disease activity (Feldmann and Maini, 2010). More recently, microarray analysis of brain extracts from mice injected peripherally with the gram-negative bacterial stimulus of lipopolysaccharide

(LPS) has indicated an increase in cytokine expression (Thomson et al., 2014). The cytokine up regulation observed was found to be elicited by a Toll-Like Receptor (TLR)-mediated interferon response in mice injected peripherally with LPS (Thomson et al., 2014).

Data has emerged from animal models to suggest that microbial components including LPS can activate sensory neurons directly (Chiu et al., 2013). In their elegant studies, Chiu et al. (2013) applied heat-killed *S. aureus* to dorsal root ganglia (DRG) sensory neurons and demonstrated an induction of calcium flux and induced action potential firing. *S. aureus* was found to directly activate nociceptors in the Nav1.8-lineage (Chiu et al., 2013). Other experiments showed that neuronal responses by the Nav 1.8 nociceptor could be activated through heat-killed *streptococcus*, *Listeria monocytogenes*, *Mycoplasma fermentans*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Variations in the pattern of nociceptor responsiveness between bacteria could indicate that strain-specific ligands act through different mechanisms.

THE IMPORTANCE OF TNF AS A PRO-INFLAMMATORY CYTOKINE IN RHEUMATOID ARTHRITIS

Biologic therapies have been used to treat severe RA since 1997 and have been manufactured to target specific elements of inflammation pathways. They include anakinra, an IL-1 antagonist; abatacept, which down-regulates T cell activation; rituximab, a chimeric anti-CD20 antibody which reduces B cell activation and infliximab, adalimumab and etanercept which inhibit TNF activity (Malaviya and Ostor, 2012). The European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) provide guidance on suitable indications for commencing biologic therapy for RA recommending treatment for disease which is active despite optimization of conventional DMARDs or for patients with high disease activity and risk factors for poor outcome (ACPA and rheumatoid factor positivity and erosive disease on radiography) (Smolen et al., 2014).

As mentioned above, TNF α is a key cytokine in the pathogenesis of synovial inflammation in RA, however, it has also been shown to play a vital role in fighting infections in animal models (Parks et al., 2011). TNF α is a type II transmembrane protein, cleaved by TNF- α converting enzyme (TACE) to a soluble form. It acts as a ligand for two receptors, TNFR1 and TNFR2, to enable transduction of anti-apoptotic, pro-inflammatory signals. TNF α -mediated functions include phagosome maturation, autophagy inhibition and apoptosis following activation of caspase 8 by TNFR1 (Harris and Keane, 2010). By inhibiting these actions, anti-TNF therapy causes suppression of the immune system (Tracey et al., 2008) and improves outcomes for suppressing inflammation and function clinically. However, TNF inhibitor therapy has also been shown to induce neurological events (Kaltsonoudis et al., 2014).

TYPES OF INFECTIONS

The risk of developing infections is heightened in RA. In the following sections we describe the more common infections observed in people with RA. Since TNF inhibitor therapy can increase the incidence of infections in RA, it is possible that

increased infections observed in RA during TNF inhibitor therapy could be a cause of RA flares, which in turn could have an impact on RA disease activity.

BACTERIAL INFECTIONS

Bacterial infections contribute to three quarters of infections in patients taking corticosteroids (CS), DMARDs or biological agents for RA and spondyloarthritis with respiratory tract infections being the most common (Germano et al., 2014). A recent systematic review of over 23,000 patients using anti-TNF therapy in the treatment of RA, juvenile idiopathic arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis and Crohn's disease, has further characterized infections associated with biologics. It showed that infections leading to a cessation in treatment included pneumonia (6.6%), bacterial arthritis (2.8%), gastrointestinal abscess (2.1%), and cellulitis (1.1%). In this study the highest rates of serious infection events were seen in RA and, within this subset of patients, cellulitis and pneumonia were the most common types of infection (Burmester et al., 2013).

MYCOBACTERIAL INFECTIONS

TNF α is a key mediator in the host immune defense against mycobacterium tubercule bacillus (TB) infections leading to activation of macrophages, cell recruitment, granuloma formation and maintenance (Bean et al., 1999). In murine models, the neutralization of TNF α increases susceptibility to primary TB and injection of soluble TNF α receptors can cause activation of TB in infected mice (Senaldi et al., 1996). TNF inhibitor therapy may also result in active tuberculosis in humans carrying a latent infection (Keane et al., 2001). A 3 year study by Tubach (Tubach et al., 2009) found 69 cases of tuberculosis (40 in RA) in patients treated with anti-TNF α . Tuberculosis has been found to occur more frequently in patients taking anti-TNF α therapy than in the general population with a standardized incidence rate varying between 12 and 35 (Ramiro et al., 2014). There is data to suggest that the risk of tuberculosis is not constant for all anti-TNF α medications, with soluble TNF α receptors resulting in a lower risk of infection than monoclonal antibodies (Tubach et al., 2009).

VIRAL AND OPPORTUNISTIC INFECTIONS

Some studies separate opportunistic infections from a broader definition of infections, serious infections, or serious infection events. In a study of 23, 458 patients on anti-TNF α therapy 20 patients (14 patients with RA) developed opportunistic infections (excluding TB or oral candidiasis) including oesophageal candidiasis, aspergillosis, candida sepsis, coccidiomycosis, cytomegalovirus, herpes zoster, and nocardiasis (Burmester et al., 2013).

Analysis of a French registry for opportunistic infections in patients taking biologic therapy for any condition found that 43 patients had opportunistic infections; 29 on infliximab, 10 on adalimumab and 4 on etanercept (Salmon-Ceron et al., 2011). Twenty-six of these were experienced by patients with RA and, of the total 43, 10 patients required intensive care and four died. When interpreting these findings it should be noted that patient information included in registry data is unlikely to be subject to

the close scrutiny and follow-up afforded as those from clinical trials. Thus, systematic reviews of randomized controlled trials and open-label trials may provide more robust data.

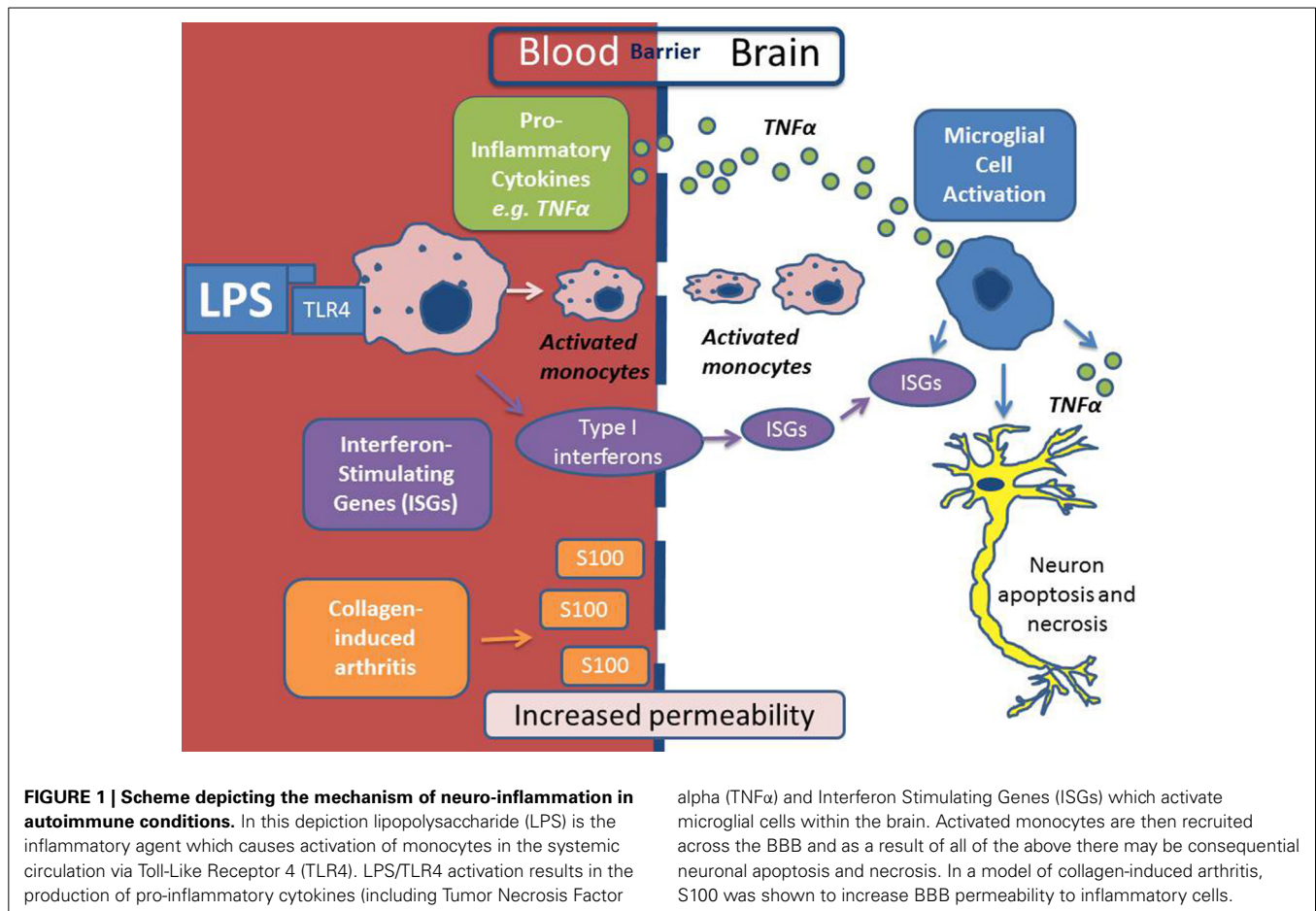
The risk of herpes zoster infection is higher in those with autoimmune conditions than in the general population (Wolfe et al., 2006) and rates of the disease are significantly increased for those receiving anti-TNF therapy (and monoclonal antibodies in particular), with an increased rate with greater age and higher disease activity at baseline (Strangfeld et al., 2009). However, a more recent systematic review including three studies investigating skin infections in patients taking biologic therapies calculated an adjusted hazard ratio range of 1.0–1.7 for herpes zoster infection, reflecting no significant risk of the infection when taking biological DMARDs (Ramiro et al., 2014).

RISK OF INFECTION

Randomized controlled trials are mixed in their demonstration of an association between biologic therapy and infectious adverse events (Lipsky et al., 2000; Keystone et al., 2004; Wallis et al., 2004). It has been demonstrated that of the patients with RA who acquire serious infections whilst taking biologics, 32% permanently discontinue therapy (Burmester et al., 2013) leading to significant compromise in therapeutic options for the treatment of the patient's arthritis.

The higher rate of infectious events may result from an increased risk of infection inherent to RA (Doran et al., 2002a) (though this appears to have reduced over the last 50 years (Ni Mhuirheartaigh et al., 2013), which is thought to be secondary to immune disturbance associated with disease pathogenesis and the use of immune-modulators to control the condition (Doran et al., 2002a,b). An inherent risk of infection makes establishing a causal link between biologic DMARDs and infections more difficult. For this reason systematic reviews and meta-analyses of clinical trials and registry data are useful tools to investigate the rare adverse events, by pooling data for large cohorts of patients.

Recent work has suggested that the blood brain barrier (BBB) may be compromised in RA with macrophages being recruited from the systemic circulation via intercellular adhesion molecule (ICAM) expression on the endothelium (Jacobs and Tavitian, 2012). Increased cerebrovascular permeability has been demonstrated in mice challenged with the collagen-induced arthritis (CIA) model (Nishioku et al., 2011). The same group have also proposed that the S100A4 protein mediates disruption of the BBB by increasing vascular permeability (Nishioku et al., 2011). Further evidence that the BBB is not as immunologically a privileged site as previously thought is supported by the recent observations in mice with systemic inflammation triggered by a peripheral LPS challenge show activation of interferon-stimulated genes (ISGs) and TNF α in the brain (Thomson et al., 2014), as illustrated in **Figure 1**. Chronic inflammatory diseases, including RA, are observationally associated with neuropsychiatric features such as depression, anxiety, pain and fatigue. It was thought that these features were simply secondary to the peripheral manifestations of the disease. However, the above findings suggest the BBB may be more porous in RA (Nishioku et al., 2011) and that intracerebral TNF α activity has been demonstrated and (Thomson et al., 2014), thus, opens the possibility of a primary, cerebral



etiology for neuropsychiatric symptoms as proposed by Rech et al. (2013).

The influence of infections in triggering and then maintaining a pro-inflammatory response in RA is not fully understood. Laboratory-based studies have focused on the well-characterized pro-inflammatory response induced by agents such as LPS, which is clearly specific to gram negative bacteria. However, the risk of infection for patients on biologics for RA can also be influenced by patient characteristics including age, disease activity, leukopenia, with co-morbidities including dementia, chronic lung disease, alcoholism and diabetes mellitus, baseline steroid use and female sex. An early systematic review of risk of serious infections in 5014 patients with RA taking anti-TNF therapy (infliximab and adalimumab with etanercept excluded due to differences in structure) in placebo-controlled trials found that serious infections requiring anti-microbial therapy or hospitalization occurred in 126 patients in the treatment arm and 26 patients on placebo (Bongartz et al., 2006). This demonstrated an increased risk of infection with an odds ratio of 2.0, which changed to 2.3 when stratified for dose (Bongartz et al., 2006). Similar findings have been found in other studies with a 2.7–2.8-fold increase in serious infections associated with infliximab and etanercept treatment (Listing et al., 2005) and an incidence rate of 1.79 with subcutaneous abatacept (Alten et al., 2014).

The most recent systematic review at the time of writing was an analysis of data to inform an update of EULAR recommendations for biologic therapies. This review concluded that most articles referring to serious infection demonstrated a significantly increased risk of infection in those treated with anti-TNF therapy when compared to conventional DMARD treatment (adjusted hazard ratio 1.0–1.7) (Ramiro et al., 2014).

A meta-analysis and simple pooling of data from 18 studies (8808 subjects) was used by Leombruno and colleagues to investigate the safety of TNF inhibitor therapy in RA (Leombruno et al., 2009). Etanercept was included in the analysis as the investigators considered that similarities out-weighed the differences between the agents. In this study there was no significant difference in the rate of serious infections between patients on anti-TNF treatment and those not. The mean duration of therapy was less than 1 year, therefore the authors recommended caution in drawing conclusions with regard to long-term safety of biologics. However, a later systematic review investigating the safety of further biologic treatment after initial anti-TNF failure found that serious infection rates were not significantly different to placebo (although only four trials met the eligibility criteria for the review) (Schoels et al., 2012), lending weight to the findings of Leombruno et al. (2009). A further meta-analysis of 12 randomized controlled trials investigating the risk of serious infections during rituximab, abatacept, and anakinra treatment for RA found no increased risk

of infections for abatacept, rituximab or low-dose anakinra but an increased risk for high-dose anakinra (OR 3.4) (Salliot et al., 2009).

The risk of infection for patients on biologic therapy is affected by the other medications used to treat the condition. A study by Germano and colleagues demonstrated that, compared to the incidence ratio of infection in patients taking conventional DMARDS and corticosteroids, those taking anti-TNF and DMARDS had a two-fold increase in incidence ratio, and those taking anti-TNF and corticosteroids had a three-fold increase in incidence ratio (Germano et al., 2014). This dropped to a 2.5-fold increase with anti-TNF, corticosteroid and DMARD suggesting that corticosteroids increase the risk of infection when used with anti-TNF. Infection risk (though not serious infection risk) whilst taking adalimumab has been shown to increase with increasing dose of concurrent methotrexate therapy in biologic and DMARD naïve patients (Burmester et al., 2014). The risk of infections appears to vary depending on the biologic agent, with studies showing that opportunistic infections were less common when using etanercept compared to the monoclonal agents infliximab (OR 17.6) and adalimumab (OR 10.0) (Salmon-Ceron et al., 2011).

TIMING OF INFECTIONS IN THE CONTEXT OF ANTI-TNF OR IMMUNOSUPPRESSANT TREATMENT

Animal models have shown the peak of reported effects in the brain to occur within a few hours following the acute phase response (Serrats and Sawchenko, 2009). Peripheral LPS injection in mice has been shown to elicit Interferon Stimulating Genes (ISG) induction between 6 and 48 h after exposure (Thomson et al., 2014). However, Thomson and colleagues found that the ISG up regulation was not wholly TNF dependent, as shown by the lack of up regulation of all ISGs with TNF α alone (Thomson et al., 2014). The same group showed that IL-1 β and TNF α were both independently up regulated in the brain following multiple LPS challenges (Thomson et al., 2014). Nadeau and Rivest (1999) have also suggested that LPS can have rapid effects on the hypothalamic-pituitary-adrenal axis was determined by measuring the transcriptional activity of corticotropin-releasing factor and plasma corticosterone levels. It is therefore conceivable that clinical RA disease activity, which can flare after acute infection, may also be linked to neuro-inflammation during episodes of clinical infection.

Askling and Dixon (2008) found that infection risk was highest in the first few months of anti-TNF treatment, and then declined in frequency. This effect was echoed in another study demonstrating that the risk of serious infection was greater at 12 weeks duration of TNF inhibitor therapy (odds ratio 2.08) than at 104 weeks duration (odds ratio 0.97). This finding may be due to a shift in the drug safety profile of TNF inhibitor agents, with persistent blockade of the TNF pathway leading to up-regulation of an alternative immune pathway and thus a compensation of immunity (Rosenblum and Amital, 2011). However, the decline in infection risk with time may be due to confounding factors. As the active RA is controlled by the anti-TNF agent, the dose of immunosuppressant CS is reduced, leading to a possible recovery of host immunity. It is also hypothesized that some patients

may be at an inherently higher risk of contracting infections. If they are included within the biologic treatment arm of a randomized controlled trial they may develop an infection soon after starting therapy, the drug will then be stopped and they will leave the cohort leaving a healthier cohort of patients. The latter is known as the “healthy user effect” (Rosenblum and Amital, 2011).

In terms of opportunistic infections (excluding TB), the median time from commencement of treatment to time of first opportunistic infection is 16.2 months (6.0–26.0) (Salmon-Ceron et al., 2011), much longer than the 3 months of highest risk, suggesting that immunosuppression secondary to biologics is, in fact, delayed in onset. It should be noted that studies since that performed by Dixon and colleagues (Askling and Dixon, 2008) have shown that the risk of first serious infection was stable throughout treatment.

Numerous murine and human models have demonstrated how LPS-induced gram-negative infections induce inflammation accompanied by somatic or visceral pain (Teeling et al., 2012; Chiu et al., 2013; Thomson et al., 2014). Such features have been attributed to sensitisation of nociceptors by inflammatory mediators released by immune cells. Sensitisation of nociceptors by inflammatory mediators released by peripheral immune cells occurs through activation of the TLR4 signaling pathway by LPS a toxic product of bacterial lysis. Meseguer et al. (2014) recently proposed that LPS induces activation of TRPA1 in a murine model, a transient receptor potential cation channel that transduces environmental stimuli to nociceptor activity. The group also found that acute vascular reactions, including neurogenic inflammation as indicated by CGRP release (measured by enzyme immunoassay), were mediated through TRPA1 and were independent of TLR4 activation. Such observations suggest that not only are infectious agents such as LPS inducing the pain response, but also produce a neurovascular response which could explain the missing link between peripheral infection and central brain neuro-inflammation.

NEURO-IMMUNE INTERACTION IN RHEUMATOID ARTHRITIS

Neuroinflammation is conducted, in the CNS, by microglial cells and astrocytes through the production of pro-inflammatory mediators and cytokines. This inflammation results in cell death and neuronal loss. RA is characterized by chronic inflammation which leads to a constant stimulation of nociceptor and excitation of afferent neurons to the brain. As such it has neurological similarities to a chronic pain state and central sensitization is thought to occur in as a result (Woolf, 1983). Evidence for enhanced hyperalgesia has been demonstrated in RA patients when compared to controls using a capsaicin and pin-prick method (Morris et al., 1997).

Lee and colleagues have suggested central sensitization as a mechanism for explaining chronic pain in rheumatic diseases such as RA (Lee et al., 2011). Central sensitization and changes in nociceptive processing in response to repeated stimulation by chemo-somatosensory event-related potentials (CSSERP) have been demonstrated encephalographically in RA patients (Wendler et al., 2001). Although increases in α_1 and β_1

range are thought to be secondary to a chronic pain state it may be that these changes are in fact a result of direct neuroinflammation secondary to the arthritis itself. Schweinhardt et al. found that people with painful RA demonstrate higher activation of brain regions associated with emotional cognition of pain including the medial pre-frontal cortex (Schweinhardt et al., 2008).

Further evidence of neuro-immune interplay has also emerged suggesting that use of biologic therapies such as TNF inhibitors can have central brain effects in people with RA well before a reduction of inflammation within affected joints occurs. In a recent study functional MRI (fMRI) was used to assess the neurological effects of TNF inhibitor therapy in 10 patients with active RA (Rech et al., 2013). The fMRI signal is sensitive to concentration of deoxyhemoglobin (which is paramagnetic), hence can detect areas of higher neuronal activity where there is greater blood flow due to increased brain metabolism associated with a task or stimulus (Ogawa et al., 1990). Rech et al. (2013) analyzed the fMRI response to compression of finger joints in RA patients at baseline and 3, 7, and 28 days after subcutaneous treatment with the TNF inhibitor adalimumab. Clinical disease activity was assessed at the same time points by applying the validated DAS28 (Disease Activity Score), which is based on the assessment of the number of swollen and tender joints, the ESR and the patient's global rating of disease activity. Inflammatory tissue was also measured anatomically by MRI of the dominantly affected hand at baseline and 28 days. At baseline there was a greater volume of functional activation in response to finger joint compression of the patients that responded to TNF treatment than those that did not respond. The main regions activated by joint compression at baseline were the somatosensory cortex, insular cortex and dorsolateral prefrontal cortex, thus suggesting a greater perception of pain. However, the brain activation was then significantly reduced as early as 3 days after treatment and further decreased after 7 days, whereas the disease activity score was only significantly reduced by day 28. Data from this study support the concept that chronic inflammation in RA leads to central sensitization, with the possibility of modulating pain perception with appropriate treatment.

A neuro-immune interaction is not isolated to RA or TNF α . Neuropsychiatric symptoms have shown improvement with cytokine blockade, for example IL-1 inhibition in Sjögren's and diabetes mellitus reduced fatigue (Norheim et al., 2012). Within "pure" neuroinflammatory disorders such as Alzheimer's there is increasing murine data to show an improvement in memory and reduced hippocampal TNF α levels as a result of anti-TNF α receptor fusion protein administration (Detrait et al., 2014). This has led to a move toward considering anti-TNF α as a therapy for Alzheimer's dementia (Cheng et al., 2014). Auranofin, a gold-containing medication and established treatment for RA, dampens inflammation through manipulation of the anti- and pro-inflammatory interleukin balance. Interestingly a recent *in vitro* study has demonstrated a reduced production of cytotoxic mediators by microglia in response to gold therapy (Medeiros et al., 2007).

Diamond and Tracey recently proposed the concept of the immunological homunculus of the brain (Diamond and Tracey, 2011) where the brain acts as a sensory organ, allowing real-time

transmission of information such as infections, tissue damage and inflammation to the central nervous system. Indeed, they draw attention to the concept of an "inflammatory reflex," in which the neurotransmitter acetyl choline interacts with cytokine production via $\alpha 7$ nicotinic acetylcholine receptors on immune cells.

MAGNETIC RESONANCE IMAGING IN RHEUMATOID ARTHRITIS

Magnetic resonance imaging (MRI) provides a variety of techniques for assessing neurological involvement of disease processes by detecting changes in brain morphometry [T1 weighted imaging (T1w)] and tissue microstructure [T2 weighted imaging (T2w), diffusion weighted imaging (DWI), diffusion tensor imaging (DTI), and magnetisation transfer imaging (MTI)], brain metabolism by magnetic resonance spectroscopy (MRS) and actual brain functionality by fMRI as described above. However, there are far fewer reports of neuroimaging abnormalities for RA compared to other related autoimmune diseases. In Sjögren's syndrome significant loss of brain tissue and presence of white matter lesions are observed (Akasbi et al., 2012; Lauvsnes et al., 2014). Lupus can lead to microstructural changes in white matter (WM), white matter lesions and metabolic abnormalities (Axford et al., 2001; Zivadinov et al., 2013) with associated neuropsychological sequelae. A summary of the imaging techniques utilized so far in demonstrating inflammatory changes in autoimmune-mediated conditions is summarized in **Table 1**.

T2 weighted imaging allows detection of lesions associated with inflammatory and degenerative changes in the brain and in terms of number and size of WM hyperintensities, lesion load is higher with age in the normal population and associated with microvascular disease (King et al., 2014). A quantitative T2w MRI study showed no significant difference in WM lesion load between RA patients and controls (Bekkelund et al., 1995) although WM lesions in RA patients have been associated with higher levels of the protein S100B, a potential plasma marker of BBB disruption and neurodegenerative effects (Hamed et al., 2012). Hamed and colleagues performed cognitive testing and found that RA patients had greater depression than controls, but this did not correlate with cognitive scores, whereas cognition did correlate with S100B levels (Hamed et al., 2012). In a rodent model of arthritis the presence of the inflammation stimulated protein S100A4 was strongly associated with BBB disruption (Nishioku et al., 2011). Thus a cerebrovascular component is likely in RA, but not one specifically detectable by conventional MRI in the general RA population.

T1 weighted imaging provides high spatial resolution of the brain with excellent contrast between gray and white matter structures to allow assessment of the shape and size of different anatomical regions. A recent study with T1w MRI demonstrated no global difference of intracranial volume between RA patients and controls, but observed significant increases in the volume of basal ganglia structures (Wartolowska et al., 2012). The caudate nucleus in particular showed the most significant increase, suggesting these changes are related to pain processing rather than the disease itself. However, there are reports of both increased and decreased gray matter structure volumes in patients with chronic

Table 1 | A table displaying the imaging modalities used to investigate neuroinflammation.

Imaging modality	Method of action	Relevance to rheumatoid arthritis
T2-Weighted MRI	Inflammatory and degenerative changes are demonstrated in the form of white matter hyperintensities	White matter lesions are associated with higher levels of S100B which is a potential surrogate marker for blood brain barrier disruption (Hamed et al., 2012)
T1-Weighted MRI	Provides high spatial resolution imaging of the brain allowing volumetric assessment of cerebral anatomy	Has demonstrated increased volume of basal ganglia in RA patients compared to controls (Steens et al., 2005)
Diffusion weighted imaging	Quantitative parameters relate to neuronal density, structural integrity and water content	Potential for assessing inflammatory and neurodegenerative changes (Steens et al., 2005; Zivadinov et al., 2013)
Magnetisation transfer imaging	Quantitative parameters relate to magnetisation exchange between macromolecules (e.g., myelin, proteins) and bulk tissue water	Potential for assessing inflammatory and neurodegenerative changes (Steens et al., 2005)
Magnetic resonance spectroscopy	Relative levels of specific metabolites, including N-acetyl aspartate, creatine, choline, myo-Inositol and some neurotransmitters	Increased choline/creatine ratio is proportional to rise in ESR and disease activity Increased choline levels seen in neuroinflammation in systemic lupus (Axford et al., 2001; Emmer et al., 2009)

pain indicating there may be a complex mix of neurodegenerative or adaptive changes dependent on disease, duration and consequent lifestyle changes, as well as treatment (Wartolowska et al., 2012). Currently it is known that only in patients with longstanding (i.e., >15 years) RA is there a reduction in brain volume that may relate to neurodegenerative changes caused by RA (Bekkelund et al., 1995).

Treatment for RA commonly includes CS to reduce inflammation and the effect of low-dose CS treatment on the brain has been investigated with quantitative MRI (Steens et al., 2005). Parameters derived from DWI and MTI relate to neuronal density and myelin-tissue water magnetisation exchange respectively, hence are sensitive to inflammatory and neurodegenerative changes. Whole brain histograms of the apparent diffusion coefficient (ADC) and magnetization transfer ratio (MTR) showed no significant difference between controls and RA patients with or without CS treatment. Although the results of Steens et al. (2005) indicate no global differences of MRI parameters, a more focused study that separates gray and white matter structures is warranted as performed for lupus and with the increased sensitivity of modern 3T MRI systems (Zivadinov et al., 2013). In addition, DTI has much greater sensitivity than DWI to detect microstructural changes in WM, and can provide detailed anatomical maps that describe regional differences of neurodegenerative change (Barrick et al., 2010).

There is some evidence of metabolic changes in the brain due to RA. In the Steens study (Steens et al., 2005) 1H MRS of a single white matter region adjacent to the left ventricle almost reached a significant difference in metabolite levels between RA patients and controls. A subsequent retrospective analysis showed that a high choline to creatine (Cho/Cr) metabolite ratio was associated with high ESR levels and correlated with ESR and disease activity after correction for disease activity and duration (Emmer et al., 2009). There was no difference in N-acetyl aspartate to creatine (NAA/Cr) ratio or correlation with ESR, suggesting there was no neuronal damage, which is consistent with the lack of major

neurological symptoms in RA, unlike for lupus. Elevated choline is found in lupus and other neuroinflammatory diseases (Axford et al., 2001). Choline has a function in cell membrane synthesis and its elevation in RA may relate to microglial activation or monocyte infiltration (Emmer et al., 2009). Brain metabolic changes have been associated with pain processing regions in a number of studies and elevation of the combined neurotransmitters glutamate and glutamine observed in fibromyalgia patients (Fayed et al., 2010) and also with induced pain (Gussek et al., 2010). The significance of these results for development of novel treatment or monitoring treatment response is yet to be established.

To date there are far fewer neuroimaging studies in RA patients compared to other diseases with cerebrovascular, neuroinflammatory and neurodegenerative components (e.g., stroke, lupus and dementias). This reflects the lower incidence of neurological symptoms and the more subtle effects of cerebrovascular involvement that have been so far detected.

CONCLUSION

The concept of neuroinflammation as a significant component of disease pathophysiology in RA has only recently been recognized. Animal models have recently demonstrated that infectious agents including LPS can directly activate the immune system stimulate pain responses. Coupled with the strong link between RA and incidence of infection, it may be time to reconsider the balance between maintaining disease remission using disease-modifying immune-modulatory drugs and preventing disease flares induced by infection. However, the link between infection and immune-modulatory therapies remains controversial and comparison of studies is made difficult by differing biologic therapies used, different diseases treated and different, concurrent medications regimes. Further, high-powered studies are required to establish the period of highest risk and susceptibility to infections and the overall risk of infections for patients taking biological treatments for RA.

Future work is needed to understand how the RA disease process impacts on pain activation in RA, whether sensitisation is a general phenomenon in RA or found in specific subsets of patients and what specific infectious triggers contribute to an autoimmune pro-inflammatory network that leads to chronic pain and inflammation.

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Mechanisms of chemotherapy-induced behavioral toxicities

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While chemotherapeutic agents have yielded relative success in the treatment of cancer, patients are often plagued with unwanted and even debilitating side-effects from the treatment which can lead to dose reduction or even cessation of treatment. Common side effects (symptoms) of chemotherapy include (i) cognitive deficiencies such as problems with attention, memory and executive functioning; (ii) fatigue and motivational deficit; and (iii) neuropathy. These symptoms often develop during treatment but can remain even after cessation of chemotherapy, severely impacting long-term quality of life. Little is known about the underlying mechanisms responsible for the development of these behavioral toxicities, however, neuroinflammation is widely considered to be one of the major mechanisms responsible for chemotherapy-induced symptoms. Here, we critically assess what is known in regards to the role of neuroinflammation in chemotherapy-induced symptoms. We also argue that, based on the available evidence, neuroinflammation is unlikely the only mechanism involved in the pathogenesis of chemotherapy-induced behavioral toxicities. We evaluate two other putative candidate mechanisms. To this end we discuss the mediating role of damage-associated molecular patterns (DAMPs) activated in response to chemotherapy-induced cellular damage. We also review the literature with respect to possible alternative mechanisms such as a chemotherapy-induced change in the bioenergetic status of the tissue involving changes in mitochondrial function in relation to chemotherapy-induced behavioral toxicities. Understanding the mechanisms that underlie the emergence of fatigue, neuropathy, and cognitive difficulties is vital to better treatment and long-term survival of cancer patients.

Keywords: chemotherapy, inflammation, fatigue, neuropathy, cognition, DAMP, cellular metabolism, mitochondria

Introduction

When someone describes his/her battle with cancer, the discussion inevitably intertwines their experience of the disease with their experience of the treatment. This is because the toxicities of cancer treatment are commonly debilitating and can drastically reduce quality of life. Indeed, often these side effects persist for weeks, months, or years after patients are cancer-free. Furthermore, symptoms can be so severe that physicians may be forced to deviate from the optimal treatment strategy for a patient, which can directly influence survival.

It has also been found that high symptom expression is related to increase risk of mortality. For example, Innominato et al. (2013) found fatigue to be a negative predictor of survival of metastatic cancer which highlights the importance of studying symptoms to both improve quality of life of cancer patients and potentially impact survival.

While there are many anti-cancer drugs used with widely varying mechanisms of action, there appear to be a common set of symptoms induced by many of these agents which include fatigue, cognitive dysfunction, and peripheral neuropathy (Cleeland et al., 2003). No FDA-approved treatment is currently available for treatment or prevention of these symptoms. In addition, the underlying mechanisms of chemotherapy-induced symptoms are poorly understood. The current dogma of the mechanisms responsible for the symptoms of chemotherapy largely revolves around neuroinflammation (Cleeland et al., 2003; Miller et al., 2008; Dantzer et al., 2012). This has primarily been driven by preclinical and clinical studies in non-cancer contexts demonstrating that propagation of peripheral inflammatory signals to the brain results in acute behavioral symptoms of sickness which can transition into chronic conditions. For instance, it is clear that there is a temporal dissociation between the symptoms of sickness and the development of persistent cognitive, neuro-pathic or mood, and behavioral changes after the illness has dissipated (Capuron et al., 2002). During the acute phase response to a disease and/or inflammatory response, reduced mood, increased pain and fatigue are adaptive processes to aid in the recovery from illness. However, when these symptoms remain after the disease has cleared then they have transitioned into a chronic and pathological condition (Walker et al., 2014). Such findings made neuroinflammation an attractive mechanistic target to explain the behavioral toxicities in response to cancer and chemotherapy given that many of the side-effects of chemotherapy remain long after treatment has ceased.

On the basis of the data on inflammation-induced behavioral phenomena, a great deal of research into the symptoms of cancer and chemotherapy has focused on peripheral and central cytokine signaling as a possible common inducer of these toxicities as well. However, cancer and its treatment appear to exist as a particularly unique circumstance. Cancer-related neuroinflammation may be a consequence of peripheral inflammatory signaling due to the effect of therapy on the tumor or other peripheral tissues or may be a direct consequence of chemotherapy agents localizing to cells of the central nervous system (CNS) (Giurgio et al., 1997; Cavaletti et al., 2001). Now after over a decade of research on the role of neuroinflammation in chemotherapy-induced symptoms, it is imperative to re-evaluate the available evidence for the role of neuroinflammation in chemotherapy-induced symptoms. Doing so will provide a clear account of what we have learned and an understanding of where we are heading. In this review we will discuss the role of neuroinflammation in chemotherapy-induced fatigue, cognitive dysfunction, and peripheral neuropathy and pain, as well as highlight potential novel mechanistic candidates for future investigation. We recognize that the relationship between chemotherapy-induced symptoms and cancer-related symptoms is complex. Based on the current literature and minimal data

for pre-diagnosis and treatment naïve patients the two cannot be fully disentangled. However, much of what is known is derived from studies carried out in non-tumor bearing rodents treated with chemotherapy. Although these symptoms are apparent in patients with both CNS and non-CNS cancers, CNS cancers hamper the study of the specific effects of chemotherapy because of the possible confounding effects of the tumor. To avoid such confusion, we will focus our discussion on the relationship between neuroinflammation and symptoms in non-CNS cancer patients. Similarly, additional symptoms such as cachexia/anorexia induced both by the cancer and chemotherapy are thought to be regulated by central cytokine signaling (reviewed in Illman et al., 2005) and probably potentiate neuroinflammation and chemotherapy-induced fatigue, cognition and neuropathy. This interplay alone could serve as a topic for review. We have decided therefore, to limit the scope of this review specifically to what is known about chemotherapy-induced fatigue, cognitive dysfunction and neuropathy/pain.

Neuroinflammation in Chemotherapy-induced Behavioral Toxicities

Fatigue

Fatigue is one of the most common symptoms experienced by cancer patients (Cleeland, 2007). In some studies up to 60% of patients receiving chemotherapy have been found to exhibit symptoms of fatigue (Bock et al., 2014). While the experience of fatigue often declines shortly after treatment, for many survivors their fatigue persists long after treatment cessation. Indeed, it is estimated that between 19 and 38% of cancer survivors still suffer from fatigue after treatment has stopped (Cella et al., 2001; Prue et al., 2006; Berger et al., 2010). Fatigue significantly impairs one's quality of life by exerting its effects at the physical, psychological, and social levels (Curt, 2000). While the term fatigue has become common parlance, many of us take for granted the complexity of discrete neurological and biobehavioral components that comprise it. At a basic level fatigue can be divided into peripheral fatigue and central fatigue (Davis, 1995; Chaudhuri and Behan, 2000). Peripheral fatigue refers to physical exhaustion and is often described in terms of muscle fatigue and lack of physical energy. Central fatigue refers to the set of discrete central processes that drive the cognitions associated with fatigue, which include a lack of motivation to engage in a given behavior. When studies also assess the motivational components of fatigue, the incidence of fatigue in cancer patients and survivors rises to 50% or higher (Curt et al., 2000; Sadler et al., 2002; Van Belle et al., 2005). Understanding the discrete units of central fatigue is complicated and only recently, the topic has entered the forefront of scientific pursuit. A consideration of fatigue cannot avoid mentioning the high degree of convergence between fatigue and depression. Fatigue is indeed part of the diagnostic criteria for depression, and approximately 73% of patients with depression report a lack of energy and fatigue (Lecrubier, 2006). These rates are even higher in cancer patients experiencing depression, with somatic depression-related symptoms being reported as more prominent

than affective symptoms (Wedding et al., 2007). A meta-analysis by Brown and Kroenke (2009) revealed an overall correlation of 0.56 between fatigue and depression in patients with cancer. This indicates that while fatigue and depression are related, they still do have independent components. This is further evidenced by studies indicating that the progression for fatigue and depression are different over the course of treatment in patients (Visser and Smets, 1998; Brown and Kroenke, 2009). At the methodological level, the overwhelming majority of studies that have investigated the link between cancer and its treatment with fatigue rely on patient self-report of fatigue on an unidimensional scale, therefore, omitting any consideration of the various components of fatigue. Below we will describe what has been discovered in regards to fatigue in preclinical and clinical models for cancer and chemotherapy in relation to neuroinflammation.

Neuroinflammation has been overwhelmingly proposed as the mechanism to account for cancer-related fatigue (Dantzer et al., 2014). This has partly been driven by evidence for a role of neuroinflammation in fatigue in patients from non-cancer contexts such as rheumatoid arthritis and multiple sclerosis. However, understanding the mechanisms underlying fatigue in cancer patients receiving chemotherapy may require a completely different mechanism of induction and/or maintenance than inflammation-induced fatigue. For many studies, particularly those at the clinical level, dissociation between chemotherapy-induced fatigue vs. that induced by the disease or by additional treatment strategies is difficult. In contrast, few preclinical studies investigate the synergistic effect of the disease and chemotherapy on fatigue, but choose to most often look at each in isolation. One murine study, however, did examine fatigue-related behaviors in mice with non-inflammatory Lewis Lung Carcinoma cell tumors that received the chemotherapeutic agent Etoposide (Wood et al., 2006). Etoposide significantly reduced voluntary wheel running activity used as an index of fatigue despite its intrinsic complexity (Novak et al., 2012) with a concomitant increase in serum IL-6 but causation cannot be inferred.

Human studies allow us to infer exacerbation of symptoms by chemotherapy on existing fatigue in cancer patients. For example, a recent study showed that children with acute lymphoblastic leukemia had reduced muscle strength, bone density, and fitness at diagnosis prior to treatment (Ness et al., 2014). However, the severity of these symptoms did not appear as great as those that were observed in such patients following treatment with chemotherapy, which is suggestive of a significant role of chemotherapy in the development of these symptoms. It should be noted that children receiving chemotherapy for acute lymphoblastic leukemia also receive high doses of the synthetic glucocorticoid dexamethasone which is likely to also contribute to symptoms of fatigue.

Additionally, most clinical studies that included investigation of chemotherapy-related fatigue relied upon the measurement of peripheral markers of inflammation as a proxy for central inflammatory processes. For example, Wang et al. (2012) found that fatigue as measured by the fatigue item of the MD Anderson Symptom Inventory (MDASI) was positively associated with serum interleukin (IL)-6 and soluble tumor necrosis factor-receptor 1 (sTNF-R1) concentrations for colorectal and

oesophageal cancer patients treated with combined chemotherapy and radiotherapy. Importantly, symptoms peaked at the end of treatment suggestive of the cumulative effects of treatment toxicity. More specific to chemotherapy alone, Pertl et al. (2013) investigated fatigue and depression symptoms in patients with breast cancer. The acute phase protein C-reactive protein (CRP) at baseline predicted changes in fatigue as measured by Functional Assessment of Cancer Therapy—Fatigue Scale in patients receiving chemotherapy and was independent of depression. It should be noted that other inflammatory markers including the cytokines interferon (IFN)- γ , IL-6 and TNF- α were assessed and no such relationship with fatigue emerged. However, circulating levels of cytokines are often very low and close to undetectable in many cases making it hard to draw firm conclusions. Nevertheless, the relationship with CRP may indicate the importance of the baseline level of inflammatory activity to predict fatigue severity in response to chemotherapy. A recent murine model was used to investigate the development of fatigue, as measured by decreased voluntary wheel running in response to systemic injection of cyclophosphamide, doxorubicin, and fluorouracil (Weymann et al., 2014). Reduced wheel running and elevated pro-inflammatory cytokine expression in the brain were observed which were attenuated with a central injection of orexin—a neuropeptide responsible for arousal and wakefulness. While the data provide compelling evidence of a role for neuroinflammation in chemotherapy-induced fatigue, it is important to note that these effects are not observed across all studies and that not all chemotherapeutic agents induce inflammation.

Research into fatigue prevalence in survivors has also been conducted, and gives us some insight into the transition from acute symptoms of treatment to long-term fatigue. Researchers recently, followed breast cancer patients from just prior to adjuvant chemotherapy through to 1 year post-treatment (Moore et al., 2014), and noted a tendency for patients to report high levels of fatigue at baseline which worsened during chemotherapy and had not fully resolved by 1 year post-treatment. Such a finding is not uncommon and many studies cite evidence of inflammation as a contributing factor. Alfano et al. (2012) found that breast cancer survivors had a 1.8 fold greater chance of suffering from fatigue if they exhibited high serum CRP levels. Moreover, higher CRP levels showed a significant positive correlation with higher scores for behavioral, sensory, and total fatigue on the Piper Fatigue Scale. Fatigue in breast cancer survivors has also been shown to correlate positively with peripheral CRP levels and leukocyte counts but not with IL1-receptor antagonist (RA), IL-6, and soluble TNF-Receptor1 (sTNF-R1), which again diminishes the role of a cytokine-specific mechanism. Collado-Hidalgo et al. (2006) compared the *ex vivo* monocyte response to lipopolysaccharide (LPS) between breast cancer survivors with chronic fatigue and those without. The *ex vivo* response of peripheral monocytes to LPS was significantly greater for survivors with fatigue compared to their control counterparts.

The question remains, however, what causes the transition from acute symptoms to chronic fatigue after chemotherapy—and where might inflammation fit into this transition? Smith et al. (2014) attempted to answer this question. They hypothesized that inflammation may persist into survivorship and

cause chronification of fatigue via changes to the epigenome. They looked at DNA methylation patterns of peripheral blood mononuclear cells in response to chemotherapy in breast cancer patients. They were able to observe an association between plasma sTNFR1 and fatigue but no epigenetic mechanism could be supported by the data. Reinertsen et al. (2011) investigated single nucleotide polymorphisms (SNPs) for IL-1 β and IL-6R but found no relationship with fatigue. Hence, while there is compelling evidence to implicate neuroinflammation with fatigue emergence during and after a variety of chemotherapy agents, it has not been possible so far to demonstrate causation. Identifying cause-and-effect relationships between chemotherapy and behavioral toxicities is further complicated by the widely varying mechanisms of action of different chemotherapeutic agents. For instance, inflammation is a likely candidate for etoposide-induced fatigue as it activates p38 MAPK pathway (Wood et al., 2006), while bortezomib inhibits NF- κ B (Ma et al., 2003; Mitsiades et al., 2006), and therefore, would not be expected to induce an inflammatory response. Despite the variations in the degree to which different chemotherapeutic agents induce inflammation, fatigue appears to remain a constant and common outcome of chemotherapy. The reason for this may lie in the possibility that treatment-related fatigue is not primarily or solely caused by inflammatory mediators, but is induced by treatment-induced intracellular metabolic changes in the target tissue such as direct mitochondrial damage (discussed below).

Cognitive Dysfunction

Chemotherapy-induced cognitive impairment (CTCI), also referred to as “chemobrain” or “chemofog,” is experienced by 15–80% of cancer patients and survivors (Cleeland et al., 2003). The variance in incidence rates of CTCI can be attributed to different treatment modalities as well as methodological variations across studies such as use of different definitions, objective vs. subjective tests of CTCI, and times of assessment of CTCI (Hutchinson et al., 2012; O’farrell et al., 2013). The most robust effects of chemotherapy are reported for executive function, memory, and processing speed (Cleeland et al., 2003; Jones et al., 2013; Seretny et al., 2014)—all of which involve frontal regions of the brain. Brain imaging studies indeed show subtle reductions in white and gray matter volume and density and frontal hypo—as well as hyperactivity during memory-related cognitive tasks in chemotherapy treated breast cancer survivors (Wieseler-Frank et al., 2005; Hutchinson et al., 2012; O’farrell et al., 2013). While these changes in brain volume and activity improve over time after cessation of treatment, subtle changes are still apparent years into survivorship (Jounai et al., 2012). Several mechanisms underlying cognitive impairment have been proposed including direct neurotoxic injury, decreased neurogenesis, hormonal pathways, and neuroinflammation (Seigers et al., 2013). Neuroinflammation as a possible explanatory mechanism for cognitive dysfunction has been studied both in clinical and animal studies.

Several clinical studies have now been published that focus on the relation between peripheral inflammatory markers, as a proxy for neuroinflammation, and cognitive performance (see Seretny et al., 2014 for a recent review). Overall, results from

these studies tentatively point to a role for inflammation in CTCI (Seretny et al., 2014). Ganz et al. (2013) reported an association between soluble TNF receptor type II (sTNF-RII), a marker for TNF- α activity, and subjective memory complaints in breast cancer survivors. Higher levels of sTNF-RII were associated with greater memory complaints approximately 3 months post treatment and a decrease in sTNF-RII over the 12 months post treatment was related with improvements in self-reported memory. Of note, the observed relation between sTNF-RII and subjective complaints disappeared when controlling for fatigue, suggesting an intertwining of self-reported fatigue and cognitive symptoms. Reporting on a subset of the same breast cancer survivor sample, Pomykala et al. (2013) showed a positive association between several cytokine markers (among which sTNF-RII) and subjective memory complaints as well as cerebral metabolism both at 3 and 12 months post treatment. Janelins et al. (2012), reporting on a different cohort, found an association between increases in the chemokine MCP-1 during two cycles of doxorubicin-based chemotherapy and less subjective cognitive problems at the end of the two cycles in breast cancer patients. Although not significant, increases in the cytokines IL-6 and IL-8 were associated with more subjective cognitive problems, suggesting that the relation between CTCI and inflammation is intricate and might not readily be captured with the assessment of single inflammatory markers. In the same study, no association was found between any of the inflammatory markers and subjective cognitive difficulties in breast cancer patients treated with a methotrexate-based chemotherapy cocktail, indicating that the relation between inflammation and CTCI might be chemotherapy-agent-specific. Kesler et al. (2013) reported an interaction between IL-6 and TNF- α on performance on a verbal learning test in chemotherapy-treated breast cancer survivors. IL-6 and TNF- α were also related to lower left hippocampal volume, suggesting that inflammation possibly reduced cognitive function through effects on the hippocampus. On the other hand, Gan et al. (2011) did not observe any relationship between objectively assessed cognitive function and inflammatory biomarkers in head and neck cancer survivors. However, considering the small sample size of this study ($n = 10$), this null finding needs to be interpreted with caution.

The above described peripheral markers of inflammation are considered a proxy for neuroinflammation and indeed seem to be associated with brain metabolism and volume, implicating that the peripheral markers are representative of a central mechanism. However, the use of more direct measures of neuroinflammation, such as inflammatory markers in cerebrospinal fluid or assessment of microglia activation with positron emission tomography (Dickens et al., 2014) would significantly increase our understanding of the role of neuroinflammation in CTCI. Of course, such measures are not always feasible due to their invasiveness for the patient and high costs. Clinical studies also do not allow for an easy disentanglement of the effects of the tumor and its treatment on subsequent cognitive difficulties. There is evidence of disease-driven cognitive dysfunction, such that subtle cognitive impairments accompanied by subtle differences in brain volume and activity are already apparent before the start of chemotherapy (Cleeland et al., 2003;

O'farrell et al., 2013). Furthermore, an association between inflammatory markers and cognitive impairment has also been observed prior to chemotherapy (Bernard et al., 2012). These disease-driven impairments and their possible association with inflammation can be addressed with longitudinal study designs that incorporate assessments prior to the onset of chemotherapy. Such studies have already been undertaken with regard to CTCI showing the feasibility of these designs but, unfortunately, measures of inflammation have not yet been included.

Animal studies do allow for the study of the effects of chemotherapy alone (i.e., without tumor interference) and also for more direct measures of neuroinflammation through the assessment of cytokines concentrations in the brain and microglia activation. Another advantage of the use of animal models is the relatively easy assessment of both the acute and long-term neuroinflammatory response to chemotherapy. In most rodent studies published up to now, measures of inflammation served as a secondary outcome and more direct, mechanistic investigations between neuroinflammation and chemotherapy-induced cognitive dysfunction are required. Nevertheless, results from rodent studies do suggest that neuroinflammation might be related to cognitive dysfunction in specific chemotherapy models (Lecrubier, 2006).

Seigers et al. (2010) reported an increase in the number of active microglia in the hippocampus 1 and 3 weeks after methotrexate treatment. However, they did not find an effect of methotrexate on cytokine levels in the hippocampus or on microglial activation as assessed by PET ([¹¹C]PK11195). Furthermore, methotrexate appeared to reduce peripheral levels of cytokines (Topp et al., 2000). The latter finding is not surprising considering the anti-inflammatory properties of methotrexate (Cutolo et al., 2001) and indicates that the observed increase in the number of active microglia may represent activation of anti-inflammatory M2 microglia (Cherry et al., 2014). Briones and Woods (2014) showed that treatment with a combination of cyclophosphamide, methotrexate, and fluorouracil led to an increase in IL1- β and TNF- α in the corpus callosum of rats and a decrease in the anti-inflammatory cytokine IL-10 approximately 4 weeks after chemotherapy. These changes in cytokine levels were accompanied by reduced performance on a working memory task. Administration of a COX-2 inhibitor normalized the cytokine concentrations and attenuated the deficit seen in cognitive performance, strengthening the assumption of a direct relationship between the observed neuroinflammation and cognitive impairment. Findings from this animal study stand in contrast to Janelsins' report on patients receiving the same combination of chemotherapeutic agents in whom no increase in peripheral markers of inflammation were observed (Janelsins et al., 2012), possibly indicating that the neuroinflammation found in animals cannot be translated to peripheral inflammation. Impaired performance in a working/spatial memory task was also observed in rats treated with either cyclophosphamide or doxorubicin 3 weeks prior to assessment of cognitive performance. Cyclophosphamide only led to inflammation in the hippocampus assessed as an increased number of activated microglial cells (Dina et al., 2001). Finally, microglial activation throughout the brain was observed in one out of ten mice treated with

fluorouracil (Schaefer, 2014) at 1 day post-treatment. Cognitive performance was not assessed in this study.

In sum, clinical studies indicate that peripheral inflammation might be related to cognitive impairments after chemotherapy, suggesting a role for neuroinflammation in CTCI. This notion is corroborated by findings from animal models showing that chemotherapy can lead to both neuroinflammation and impairments in cognitive function. Interestingly, these associations are observed immediately as well as some weeks after therapy. Both clinical and animal studies indicate that a neuroinflammatory mechanism underlying CTCI is probably restricted to specific chemotherapeutic agents, stressing the importance of studying CTCI in different patient populations and models of chemotherapy.

Neuropathy

Peripheral neuropathy characterized by pain, numbness, and temperature sensitivity is another common side effect of chemotherapy known as chemotherapy-induced peripheral neuropathy (CIPN) (Dougherty et al., 2004; Wolf et al., 2008). CIPN occurs in about 60% of cancer patients (Rowinsky et al., 1993a,b; Windebank and Grisold, 2008; Wolf et al., 2008; Cavaletti et al., 2011; Seretny et al., 2014) and can cause dose limitations or early cessation of treatment making it a challenge for effective cancer treatment (Cavaletti et al., 1992; Uhm and Yung, 1999; Polomano and Bennett, 2001; Mielke et al., 2006). As reported for fatigue and cognitive deficits, CIPN can persist after completion of treatment thereby contributing to the reduction in quality of life of cancer survivors.

Chemotherapy-treated individuals frequently report an acute pain phase in the days immediately following treatment (Gamelin et al., 2002; Grothey et al., 2011; Park et al., 2011). This acute phase usually subsides. However in some cases acute CIPN symptoms transition into a chronic pain phenotype (Seretny et al., 2014). Both the acute and chronic CIPN symptoms can be problematic for patients. Intense acute pain symptoms can lead to the necessity of decreasing the dose of the drug or number of treatment cycles. Persistent chronic pain states can also adversely affect quality of life both during and following completion of chemotherapy treatment (Vichaya et al., 2013). CIPN symptoms are most frequently reported in a "glove and stocking" distribution in which patients report neuropathy symptoms in their hands and feet (Kim et al., 2015). These neuropathy symptom profiles are reported across different classes of chemotherapeutic agents including taxanes, platinum, proteasome inhibitors, and vinca-alkaloids. Why many different chemotherapeutic agents result in similar neuropathy profiles is unclear. More importantly molecular/cellular cause(s) of CIPN remain unknown.

In this section we shall highlight research on inflammation as a potential cause of CIPN. Human studies discussed above measured chemotherapy-induced increases in peripheral pro-inflammatory cytokine levels corresponding with behavioral toxicities such as cognitive deficits, fatigue, and neuropathy. Animal studies have enabled investigators to further elucidate effects of inflammation on neuronal tissues such as peripheral sensory neurons as a potential cause of CIPN. Several investigators have measured increased pro-inflammatory cytokines,

such as IL-1 β , IL-6, and TNF- α , at the site of peripheral sensory neurons (either in the dorsal root ganglia or spinal cord) of chemotherapy treated rodents (White et al., 2005; Ledeboer et al., 2007; Xiao et al., 2011; Wang et al., 2012; Zhang et al., 2012, 2013; Pevida et al., 2013; Janes et al., 2014a). Studies in inflammatory pain have shown that endogenous or exogenous increases in pro-inflammatory cytokines can sensitize peripheral sensory neurons leading to spontaneous discharge and neuropathic pain in the absence of chemotherapy treatment (Topp et al., 2000; Dina et al., 2001; Wieseler-Frank et al., 2005; Schafers and Sorkin, 2008). Due to the negative effects that pro-inflammatory cytokines have on peripheral sensory neurons, cytokines were investigated in the context of CIPN. It quickly became clear that pro-inflammatory cytokines were actively contributing to chemotherapy-induced neuropathic symptoms as blockade via cytokine antagonists such as IL-1 receptor antagonist or anti-TNF- α attenuated chemotherapy-induced neuropathy (Ledeboer et al., 2007; Cata et al., 2008; Ale et al., 2014). Furthermore, these pro-inflammatory cytokine effects could be regulated through changing the pro-inflammatory vs. anti-inflammatory cytokine balance at neuronal tissue sites. Ledeboer et al. (2007) demonstrated that intrathecal administration of the anti-inflammatory cytokine IL-10 could attenuate paclitaxel-induced neuropathy. Another group also found that increasing anti-inflammatory cytokine levels, IL-10 and IL-4, in the spinal dorsal horn via an S1PR₁ antagonist could also prevent CIPN in rodents (Janes et al., 2014a). Others have shown that thalidomide, a biological agent shown to inhibit TNF- α , reduced chemotherapy and bone cancer induced neuropathy (Cata et al., 2008; Gu et al., 2010) in rodent models. Conversely, when thalidomide was used in the treatment of multiple myeloma in patients, thalidomide administration induced neuropathic symptoms (Mileshkin et al., 2006; Chowdhury et al., 2013). For the most part studies have demonstrated that increases in pro-inflammatory cytokines either in the dorsal root ganglia or spinal cord corresponds with symptoms of CIPN. Prevention of these pro-inflammatory cytokines can attenuate neuropathy symptoms. However, the therapeutic effect of inhibition of these cytokines in humans has yet to be attained.

These initial discoveries were highly supportive of the hypothesis that CIPN can be driven by an inflammatory mechanism and drove researchers to investigate which specific cell type(s) were responsible for chemotherapy-induced production of pro-inflammatory cytokines. Monocytes/macrophages, a component of the innate immune system, are major producers of peripheral pro-inflammatory cytokines during infection and at injury sites. Neuronal cells have also been shown to produce pro-inflammatory cytokines as well as chemokines. Zhang et al. (2013) found that chemotherapy induced the production of monocyte-chemoattractant-protein-1 (MCP-1, also known as CCL2) in murine DRGs, which corresponded with macrophage infiltration of the DRGs. It was also shown that blockade of MCP-1 prevented macrophage infiltration and symptoms of CIPN (Pevida et al., 2013; Zhang et al., 2013). Furthermore, treatment with minocycline, an FDA-approved antibiotic also known to inhibit macrophages as well as pro-inflammatory cytokine production, prevented CIPN across a range of chemotherapeutic agents in murine systems (Boyette-Davis and Dougherty, 2011;

Boyette-Davis et al., 2011; Drouin-Ouellet et al., 2011; Gwak et al., 2012). The pre-clinical positive results on the use of minocycline in CIPN prevention has led to current clinical trials investigating the efficacy of minocycline in the prevention of CIPN in patients. The success of macrophage/microglia blocking agents in prevention of CIPN was unexpected as chemotherapy administration has mainly been shown to induce astrocyte activation but not microglia activation in DRGs and spinal cord (Di Cesare Mannelli et al., 2013, 2014; Janes et al., 2014b; Robinson et al., 2014).

Chemotherapy administration has been shown to greatly reduce the density of intraepidermal nerve fibers (IENFs) crossing the basement membrane into the epidermis (Dougherty et al., 2004; Boyette-Davis and Dougherty, 2011; Boyette-Davis et al., 2011; Kosturakis et al., 2014; Mao-Ying et al., 2014). This reduction, but not total loss of IENFs is hypothesized to leave remaining neurons highly sensitized and a potential reason for neuropathic outcomes. It is unclear what leads to IENF retraction. Some researchers propose it to be the result of altered mitochondrial function and energy states in the sensory neurons (discussed below). Others have suggested that the nerve terminals are the most vulnerable part of sensory neurons and therefore, most easily damaged by chemotherapy administration (Milteneburg and Boogerd, 2014). Chemotherapy-induced increases in cytokine levels or macrophage infiltration at nerve terminals has yet to be investigated.

Alternative Mechanisms for Chemotherapy-induced Behavioral Toxicities

Above we have presented evidence in support of the role of chemotherapy-induced neuroinflammation in the symptoms of fatigue, cognitive dysfunction, and neuropathy. There is certainly evidence to indicate that neuroinflammation is involved in each of these symptoms. However, there is limited evidence to support a causal relation between neuroinflammation and these chemotherapy-induced symptoms, calling for the consideration of additional pathways.

Damage-associated Molecular Patterns

Damage-associated molecular patterns—also known as danger-associated molecular patterns, cell death-associated molecules, or DAMPs—are endogenous intracellular molecules released due to compromised membrane integrity during cellular death and injury (Kaczmarek et al., 2013). DAMPs can activate membrane receptors like the receptor for advanced glycation end product (RAGE) and pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), NOD-like receptors (NLRs), and purinergic receptors on target cells to initiate inflammatory responses (Chen and Nunez, 2010). Coincidentally, TLRs and NLRs also recognize pathogens and are a shared pathway for infectious and non-infectious inflammation (Pradere et al., 2014). Most often released as the result of decreased plasma membrane integrity of injured cells, DAMPs can be classified as proteins (Rubartelli and Lotze, 2007), nucleic acids (Bernard et al., 2012; Jounai

et al., 2012; Paludan and Bowie, 2013), purines (Schaefer, 2014), and other non-protein molecules such as reactive oxygen species (ROS). Interestingly, many of the DAMPs that are released during necrosis as well as their receptors are also overexpressed in tumor cells (Castellani et al., 2014). Here, we examine function of the high-mobility group box-1 (HMGB1) protein, DNA and RNA fragments, purines such as adenosine triphosphate (ATP) and adenosine, and ROS and provide possible links to tumorigenesis and chemotherapeutic agents.

High-mobility Group Box-1

HMGB1 is perhaps the best characterized DAMP. Synthesized as a nuclear protein, HMGB1 is normally bound to DNA acting as a transcription factor and is released during cellular damage or injury. It is released less during programmed cell death or apoptosis where the up-regulation of histone 2B inhibits the dissociation of HMGB1 from DNA (Lotze et al., 2007). Extracellular HMGB1 can promote angiogenesis, stem cell migration, as well as neutrophil recruitment and subsequent pro-inflammatory immune responses via the activation of TLR2, TLR4, and RAGE. Conversely, activated T-cells or natural killer cells (Lotze and Tracey, 2005) as well as many chemotherapeutic agents promote the release of HMGB1 from tumor cells and healthy tissues (Tang et al., 2010). Hence HMGB1 liberation may be promoted by chemotherapy-induced cell death. Additionally, HMGB1 can also activate numerous immune cells including macrophages and dendritic cells via TLR and RAGE to stimulate the release of cytokines such as TNF- α , interleukin (IL)-1 α , IL-1 β , and IL-6 (Lotze and Tracey, 2005). Therefore, HMGB1 likely contributes to the elevations in inflammatory markers observed in patients treated with chemotherapy.

HMGB1 has been linked to muscle function and strength and, therefore, could play a role in peripheral fatigue (Grundtman et al., 2010). Furthermore, several studies have indicated a role for HMGB1 release in the development of non-chemotherapy-induced neuropathies, such as nerve injury (Shibasaki et al., 2010; Feldman et al., 2012) and cognitive impairment following surgery or sepsis (Chavan et al., 2012; Li et al., 2013; Vacas et al., 2014). While HMGB1 has not yet directly been shown to mediate these symptoms in the context of chemotherapy, the known release of HMGB1 in response to many chemotherapeutic agents indicates that research down this avenue is warranted.

Reactive Oxygen Species

Primarily generated in the mitochondria, ROS are produced as a part of normal respiration and energy metabolism. In the physiological state, ROS are rapidly converted to hydrogen peroxide and ultimately to water and oxygen in the cytosolic space which is rich in oxidoreductases and non-protein thiols, such as thioredoxin and glutathione. The accumulation of ROS in the cytosol signals the activation of caspases, mainly caspase-1, via the NLRP3 inflammasome, and subsequently promotes inflammation. Additionally, ROS can also activate the executioner molecule of apoptosis, caspase-3, via the release of cytochrome c and caspase-9 leading to apoptosis (Circu and Aw, 2010).

The intracellular space promotes a reducing environment in healthy cells. During pathological states, the reducing capacity of the cytosol can drastically decrease and thus promote oxidation of many proteins, including HMGB1, and indirectly stimulate the production of secondary DAMP signaling (Lotze et al., 2007). Interestingly, approximately 40% of all FDA-approved anticancer drugs have been shown to induce ROS (Chen et al., 2007). Oxidative stress can produce behavioral toxicities, such as chronic fatigue syndrome (Logan and Wong, 2001; Kennedy et al., 2005), mild cognitive impairment (Fukui et al., 2002; Pratico et al., 2002), and diabetic neuropathy (Nagamatsu et al., 1995; Low et al., 1997; Vincent et al., 2004). Furthermore, there is evidence to suggest that chemotherapy-induced neuropathy (Areti et al., 2014) and cognitive impairment (Aluise et al., 2010) may also be mediated by oxidative stress.

Nucleic Acids

Classically associated with bacterial or viral infections, nucleic acids such as DNA and RNA can elicit an innate immune response via TLR activation (mainly TLR-3 for double-stranded RNA (Alexopoulou et al., 2001), TLR-7 and 8 for single-stranded RNA (Heil et al., 2004), and TLR-9 for unmethylated DNA (Hemmi et al., 2000)). Typically sequestered within the cell, host DNA and RNA are normally considered as unrecognizable by these membrane bound receptors. However, nucleic acids can be released from host cell due to damage or death and can signal as DAMPs. During normal apoptosis nucleotides liberated from membrane-bound organelles are rapidly degraded by nucleases such as DNase and RNase, but during damage or unprogrammed cell death, nucleic acids can also be released into the extracellular space as immune stimulators. Furthermore, resident macrophages and dendritic cells can engulf circulating nucleotides to form endosomes (Yasuda et al., 2005) and subsequently stimulate innate immune responses (see review by Ishii and Akira, 2005). Interestingly, mitochondrial DNA (mtDNA) and bacterial DNA are both rich in CpG motifs which is the primary ligand of TLR-9, suggesting that mitochondrial damage induced release of mtDNA can be a potent stimulator of the immune system via TLR-9 activation (Zhang et al., 2010). Platinum-based chemotherapeutic agents, such as cisplatin, target the purine bases of DNA to inhibit replication, transcription, and repair (Jamieson and Lippard, 1999). This may be devastating for the healthy cells of the peripheral and CNS needed to regulate cognition, pain sensation, and behavior. While most neurons are in a post-mitotic state, other cells in the CNS, such as glial cells, still proliferate and are thus susceptible to chemotherapy-induced shortening of telomeres. Therefore, it is conceivable that chemotherapy may accelerate cellular aging leading to senescence and apoptosis (Flanary and Streit, 2004). Furthermore, when cisplatin crosslinks DNA it promotes the cleavage to short nucleic acid fragments and the breakdown of the cell membrane (Barry et al., 1990). Short DNA fragments can leak into the circulation and can act as immunostimulatory agents (Zhang et al., 2010).

Interestingly, the DNA fragmentation that occurs following chemotherapy treatment is also observed in other states of cognitive impairment such as Alzheimer's disease (Lassmann et al., 1995; Stadelmann et al., 1998), aging-related early dementia

(Troncoso et al., 1996), and traumatic brain injury (Mattson, 2000). There is a parallel increase in microglia activation and subsequent pro-inflammatory responses (Gehrmann and Banati, 1995). Taken together, these studies indicate that chemotherapy-induced cognitive deficits may be due, in part, to directly increasing DNA damage of neuronal cells, or by promoting accelerated aging via the shortening of telomere.

Purines

Purine nucleosides, mainly adenosine and ATP, are physiologically sequestered in the intracellular space and are involved in a multitude of biological functions including energy balance (Leist et al., 1997) and synthesis of nucleic acids (Hartman and Buchanan, 1959). However, extracellular purines are also immunomodulatory and can act as danger signals (Inoue, 2002). Many chemotherapeutic agents elicit anti-tumor effects by stimulating ATP release from tumor cells (Martins et al., 2009), subsequently recruiting dendritic cells (Aymeric et al., 2010) and lymphocytes via P2X7 (an ATP purinergic receptor), and promote phagocytosis and autophagy (Michaud et al., 2011). Furthermore, ATP can also attract monocytes and microglia while simultaneously promoting the production of inflammatory cytokines including IL-1 β (Aymeric et al., 2010). Interestingly, increased extracellular ATP concentration has been associated with pain sensation (Tominaga et al., 2001) by the depolarization of sensory neurons (Cook et al., 1997) via the P2X receptors (Rassendren and Ullmann, 2014). Taken together these data indicate that increased extracellular ATP might play a role in CIPN.

Degradation of ATP yields adenosine. Extracellular adenosine concentration drastically increases in response to increased extracellular ATP (Dunwiddie et al., 1997). In many physiological states, adenosine serves as a counter-modulator of synaptic firing by hyperpolarizing neurons (Dulla and Masino, 2013) inhibiting neurotransmitter release (Boison, 2007, 2008) and thus decreasing cerebral activity (Dulla and Masino, 2013). Adenosine also functions as a regulator of sleep and wakefulness in a way that the extracellular concentration of adenosine increases during the waking hours (Huston et al., 1996; Porkka-Heiskanen et al., 1997). Taken together an increase in extracellular adenosine may be an important mediator of chemotherapy-induced fatigue associated with sleep disorders. Indeed, central inhibition of adenosine signaling, via caffeine administration, has been shown to decrease muscle fatigue as well as to increase motor activity (Davis et al., 2003). Furthermore, cognitive disorders such as Alzheimer's (Angulo et al., 2003) and Parkinson's (Schwarzschild et al., 2006) disease are associated with elevated circulating adenosine levels. However, inhibition of adenosine signaling has been associated with cognitive deficits in models of hypoxia (Chiu et al., 2012) and Alzheimer's disease (Dall'igna et al., 2007), as well as with depressive- (Sarges et al., 1990) and anxiety-like behaviors in rodents (Florio et al., 1998; Chiu and Freund, 2014; Chiu et al., 2014).

Finally, it is important to note that extracellular purine is ultimately degraded to uric acid (Becker, 1993). Accumulation and precipitation of uric acid can form monosodium urate crystals

to stimulate NOD-like receptors in immune cells and subsequently produce inflammatory cytokines including IL-1 β and IL-18 (Gasse et al., 2009). The most obvious example of uric acid-mediated inflammation is gout, where monosodium urate crystals induce arthritis that is characterized by localized pain and inflammation (Martinon et al., 2006; Schumacher et al., 2009). Interestingly, a high plasma uric acid level is also seen after chemotherapy (Liu et al., 2005) and can lead to a high uric acid buildup in both the tumor microenvironment (Hu et al., 2004) and circulation (Liu et al., 2005). Taken together it appears that elevated plasma uric acid after chemotherapy treatment can promote a pro-inflammatory response leading to inflammatory pain. Indeed, studies have shown that acute gout and associated arthritis and inflammatory pain can develop in patients receiving chemotherapeutics such as gemcitabine (Bottiglieri et al., 2013), paclitaxel (Alexandrescu et al., 2009), and capecitabine (Peixoto et al., 2014).

Cellular Metabolism

Chemotherapy is also capable of inducing symptoms by altering the brain's and peripheral nervous system's bioenergetic status. Mitochondria are at the center of cellular bioenergetics as they mediate the production and distribution of ATP. Typically energy production begins with the process of glycolysis within the cytoplasm of a cell. During glycolysis, glucose is broken down into pyruvate. The pyruvate molecules can then either enter the mitochondrial matrix or be converted to lactate. Within the mitochondria, pyruvate is oxidized into citric acid and enters the tricarboxylic acid (TCA) cycle and electron transport chain. Historically it has been thought that lactate formation only occurs in response to a lack of oxygen (i.e., anaerobic conditions) or when there is a disruption in oxidative metabolism. However, despite glucose being considered the primary fuel for normal brain activity (see review by Dienel, 2012), recent evidence suggests that brain lactate production may serve as a signaling molecule and an alternative source of fuel (Gibbs and Hertz, 2008; Suzuki et al., 2011; Tang et al., 2014). Furthermore, lactate produced by the tumor microenvironment serves an important fuel for tumor cell energy metabolism, which is at the basis of the well-known Warburg effect (Pavlidis et al., 2009). The interaction between tumor-associated lactate production and brain lactate is still unknown.

Association between Mitochondrial Dysfunction and Behavioral Changes

The brain is the most energetically demanding organ in the body. Therefore, agents that result in even minor changes in mitochondrial energy metabolism are capable of impacting brain function and producing behavioral changes. For example, there is significant evidence to suggest that mood and psychiatric disorders, such as bipolar disorder, autism, and schizophrenia, are associated with impaired brain energy metabolism (Prabakaran et al., 2004; Young, 2007; Quiroz et al., 2008; Rezin et al., 2009; Rossignol and Frye, 2012). Furthermore, mitochondrial dysfunction has been implicated in the pathophysiology of chronic fatigue syndrome (Myhill et al., 2009, 2013; Murrough et al., 2010) as

well as fatigue in patients with multiple sclerosis (Roelcke et al., 1997), and fatigue in rodents treated with an inflammatory agent (Sheng et al., 2011) or exposed to stressors (Tanaka and Watanabe, 2008). For example, higher ventricular lactate levels (an indirect indication of mitochondrial dysfunction) have been observed in patients with chronic fatigue syndrome compared to healthy volunteers (Murrough et al., 2010). Mitochondrial impairment or damage has also been implicated in cognitive impairment such as that associated with aging (Liu et al., 2002; Wang et al., 2006; Liu, 2008), traumatic brain injury (Sauerbeck et al., 2011), HIV-associated dementia (Valcour and Shiramizu, 2004), and Alzheimer's disease (Corona et al., 2010; Dragicevic et al., 2010). HIV/AIDS-related neuropathy (Dalakas et al., 2001) and diabetic peripheral neuropathy (Srinivasan et al., 2000; Chowdhury et al., 2013) have also been associated with mitochondrial damage. Further, there is evidence to suggest that protecting mitochondrial integrity is able to protect against ischemic brain damage as well as the resulting cognitive and motor impairment (Nijboer et al., 2011, 2013).

There is growing evidence that chemotherapy-associated behavioral toxicities are also associated with mitochondrial dysfunction. For example, cisplatin is capable of significantly inhibiting electron chain transport complexes I–IV resulting in a 70% reduction in ATP production (Kruidering et al., 1997). Furthermore, animal models of CIPN show mitochondrial dysfunction within the peripheral nerves and the dorsal root ganglion, axonal mitotoxicity (swollen, vacuolated mitochondria), and poorer antioxidant defense in response to a wide array of chemotherapy agents, including taxanes, vinca alkaloids, platinum agents, and bortezomib (Jin et al., 2008; Melli et al., 2008; Podratz et al., 2011; Xiao et al., 2011; Zheng et al., 2011, 2012). Given that peripheral nerves do not have the protection of the blood brain barrier, it is not unexpected that evidence for mitochondrial dysfunction was first noted here. However, brain mitochondrial function is also affected by chemotherapy. For example, a recent study in patients showed that chemotherapy can induce transient changes in glucose metabolism within the brain (Baudino et al., 2012). Peripheral cisplatin administration was shown to enhance mitochondrial lipid peroxidation levels and protein carbonyl content within the brain of rats (Waseem and Parvez, 2013). Moreover, in a mouse model it has been shown that doxorubicin administration results in an acute reduction in brain complex I function and an increase in pro-apoptotic proteins such as p53 and Bax in brain mitochondria (Tangpong et al., 2006). Finally, it has been shown that doxorubicin treatment increases the susceptibility of rat brain mitochondria to damage from excessive calcium and oxidative stress (Cardoso et al., 2008).

It is important to note that the mitochondrial effects of chemotherapy are often observed in the presence of a tumor. Tumor cells are metabolically demanding and, therefore, have altered metabolic profiles. Furthermore, they can induce metabolic changes that extend to the tumor microenvironment to provide for their metabolic needs (Pavlides et al., 2009; Bonucci et al., 2010). Therefore, it is important for future studies to explore how chemotherapy agents affect energy metabolism in the presence of a tumor.

Potential Mechanisms of Chemotherapy-induced Mitochondrial Dysfunction

While there is growing evidence that chemotherapy is capable of altering mitochondrial function, the mechanism by which this occurs is still unclear. The effect could be an indirect result of increased inflammation and/or oxidative stress or a direct effect of chemotherapy on mitochondria. These potential mechanisms are briefly discussed below.

Mitochondria and inflammation

There is both *in vitro* and *in vivo* evidence that mitochondria are sensitive to inflammation. This has been most directly shown by treating cells or mice with the cytokine stimulant, LPS. In both cases significant evidence of mitochondrial metabolic changes were observed (Xie et al., 2004; Hunter et al., 2007). Moreover, decreased brain oxidative phosphorylation has also been observed in a mouse model of sepsis (D'Avila et al., 2008). These models induce high levels of inflammation, severe mitochondrial dysfunction, and cellular death (Welty-Wolf et al., 1996; Crouser et al., 2002; Hunter et al., 2007). While this situation is particularly relevant to the symptoms associated with the neurodegeneration observed in Parkinson's disease, the inflammation induced by chemotherapy treatment would likely be markedly milder. Therefore, further investigation is needed to determine if a similar phenomenon is observed in the brain.

Mitochondria and oxidative stress

Oxidative stress is an inherent aspect of mitochondrial function. At baseline levels, approximately 1–5% of oxygen used by the cells is converted to ROS (Chance et al., 1979). However, when there is insult to the mitochondria these levels dramatically increase. As mentioned previously, a high proportion of chemotherapeutic agents result in production of ROS. This imbalance in ROS production can lead to cellular damage and mitochondrial damage in particular (reviewed by Adam-Vizi and Chinopoulos, 2006; Areti et al., 2014). Mitochondrial complex I and II of the electron transport chain and mitochondrial DNA (Wallace, 2005) are particularly vulnerable. In addition to expressing genes encoded by the nuclear genome, mitochondria have their own functional genome (mtDNA). The mtDNA has a higher mutation rate than nuclear DNA and a more limited repair capacity than nuclear DNA (Tuppen et al., 2010). This mechanism likely contributes to chemotherapy-induced mitochondrial dysfunction. However, blocking ROS has been shown to be insufficient to prevent cisplatin-induced mitochondrial dysfunction within the kidney (Kruidering et al., 1997) suggesting that chemotherapy may be capable of inducing mitochondrial damage via multiple pathways.

Mitochondrial p53

In response to cellular stress there is a rapid accumulation of p53 to the mitochondrial membrane which increases mitochondrial membrane potential, cytochrome c release, and caspase-3 activation (Marchenko et al., 2000). The phosphorylation of p53 by c-Jun N-terminal kinase (JNK) protects p53 from ubiquitination and degradation, thereby enhancing its activity (Fuchs et al., 1998). Using a model of ischemic brain damage,

it has been demonstrated that interfering with the mitochondrial JNK/p53 pathway, by inhibiting p53 accumulation [such as with the small molecule inhibitor pifithrin- μ (PFT- μ); (Nijboer et al., 2011)] or by inhibiting the activity of JNK (with the use of TAT-JBD, D-JNKi, and Sab_{kin1}; Nijboer et al., 2010, 2013), is neuroprotective and can attenuate damage-associated behavioral deficits. Given that the activity of p53 is a critically involved in chemotherapy-induced tumor cell apoptosis for a wide variety of agents (Pritchard et al., 1997; Hwang et al., 2001; Tan et al., 2002; Bragado et al., 2007), it follows that it is a candidate therapeutic target for the neurotoxic effects of these agents. Further, we have preliminary evidence that that PFT- μ can also inhibit chemotherapy-induced neuropathy (Krukowski et al., 2014, under review).

Mitochondrial DNA adducts

Another possible mechanism by which chemotherapy may disrupt mitochondrial function is through the formation of DNA adducts. For example, platinum-based antineoplastic agents act by crosslinking DNA and, consequently, interfering with cellular division and repair, which causes mitochondria to release apoptotic proteins. This effect does not require the formation of adducts between nuclear DNA and cisplatin, but can occur as a direct effect of cisplatin on mtDNA (Yang et al., 2006). Not only can cisplatin-mtDNA adducts form in cancer cells, but these adducts have been noted to develop in other cells throughout

the body including the brain (Johnsson et al., 1995; Giurgiovich et al., 1996, 1997). Furthermore, cisplatin has also been noted to accumulate in high levels within the dorsal root ganglion (McDonald et al., 2005). This data along with the p53 data would suggest that chemotherapy can induce mitochondrial damage and, consequently behavioral toxicities, via non-inflammation based mechanisms.

Conclusion

In this review we have evaluated the available evidence for the role of neuroinflammation in chemotherapy-induced behavioral toxicities. Despite neuroinflammation being the clear “mechanism of choice” for many researchers, close examination of the literature forces one to be open to the possibility that other mechanisms also play a critical role, either in conjunction with neuroinflammation or independently. As we point out, clinical studies are rarely designed to allow delineation between inflammatory markers that arise from the cancer vs. those that emerge and dissipate with the start and finish of chemotherapy regimens. This makes it difficult to understand what chemotherapy is precisely doing to the body and brain outside of their effects on tumor progression. On the other hand many preclinical models in the field fail to focus on the causal role of neuroinflammation in many of the symptoms of chemotherapy which leaves us with having to interpret the meaning of associations between central and peripheral markers

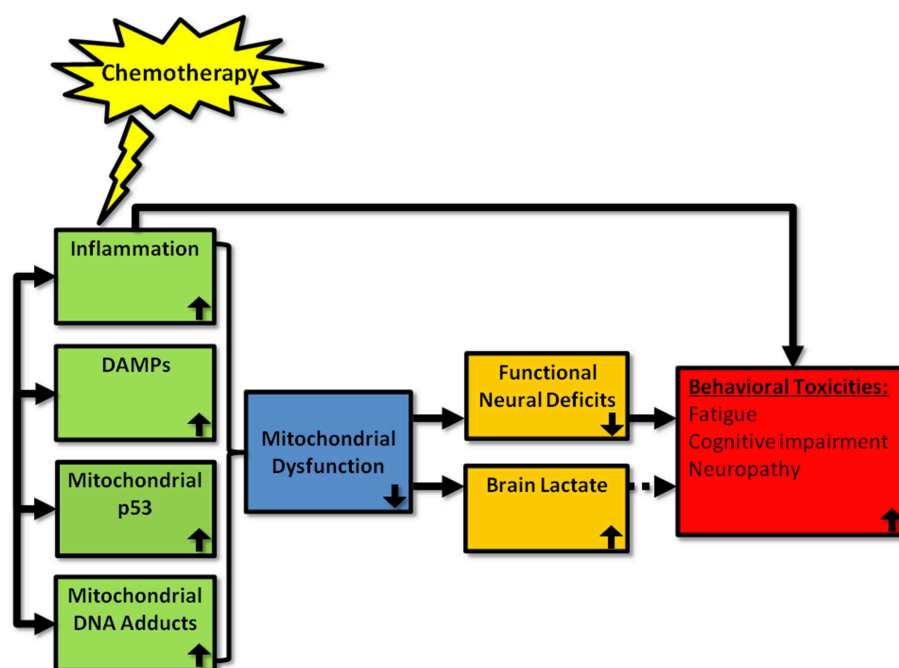


FIGURE 1 | Proposed mechanisms by which chemotherapy can induce behavioral toxicities. Chemotherapy has been shown to induce peripheral inflammation, DAMP, mitochondrial p53, and mitochondrial adducts. We propose that chemotherapy also induces these processes within the brain, which leads to mitochondrial dysfunction. This, in turn, leads to neural deficits and increased brain lactate. Depending upon the

localization of these neuronal deficits in the brain, behavioral toxicities—such as fatigue, cognitive impairment, and neuropathy—are likely to emerge. Whether lactate production is a byproduct or inducer of symptoms is as yet unclear. Further, it is possible that chemotherapy-induced inflammation may also act to induce behavioral toxicities via non-mitochondrial related pathways. Up and down arrows represent the direction of the effect

of inflammation with chemotherapy-induced behaviors. Nevertheless, remarkable progress has been made in the field which places us in an opportune position to assess what we have learned and where we should aim toward.

It is clear that the evidence for neuroinflammation contributing to some symptoms and for particular agents is more convincing than for others. Much more work has been conducted in the field of chemotherapy-induced neuropathy and there is a strong foundation of support for peripheral inflammation as a mediator of pain sensation. More still needs to be done on the central components of pain assessment and experience and inflammation, and many other mechanisms have also been put forward in lieu of neuroinflammation. Much less work has been conducted in the fields of fatigue and cognitive dysfunction following chemotherapy but there remains evidence in favor of the neuroinflammation hypothesis. Unfortunately many studies looking at inflammation and chemotherapy-induced fatigue and cognitive decline report mixed findings and even negative results suggesting that alternative mechanisms need to be considered while also investigating the role of neuroinflammation with greater rigor.

Promising alternative mechanisms for chemotherapy-induced behavioral toxicities are DAMPs and the bioenergetics status of cells of the CNS (Figure 1). These avenues of investigation

are growing rapidly and need to be integrated into the field more widely. In regards to DAMPs, most work has been conducted in relation to HMGB1 but a range of other DAMPs are known to be activated in response to chemotherapies, and the activation of specific DAMPs may be chemotherapy agent-specific. The prospect that DAMPs may be a major player in chemotherapy-induced behavioral symptoms is particularly convincing given that they often cause downstream production of pro-inflammatory cytokines which may suggest that the focus of many of us in the field on neuroinflammation *per se* has been a matter of “putting the cart before the horse.” The same may also be said for the field of bioenergetics and symptoms of chemotherapy given the relationship between mitochondrial dysfunction and inflammation. However, the evidence that is emerging also indicates that alterations in mitochondrial energy metabolism and production of metabolites such as lactate are likely to contribute to cancer-related symptoms in an independent fashion also. Clearly, the literature is currently somewhat scarce for DAMPs and mitochondrial dysfunction in the field of chemotherapy-induced behavioral toxicities but they represent exciting new avenues of research that should complement our understanding of the mechanisms at the origin of cancer-related symptoms.

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