Microglia: neuroimmune-sensors of stress

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Abstract

Exposure to stressors disrupts homeostasis and results in the release of stress hormones including glucocorticoids, epinepherine and norepinepherine. Interestingly, stress also has profound affects on microglia, which are tissue-resident macrophages in the brain parenchyma. Microglia express a diverse array of receptors, which also allows them to respond to stress hormones derived from peripheral as well as central sources. Here, we review studies of how exposure to acute and chronic stressors alters the immunophenotype and function of microglia. Further, we examine a causal for stress hormones in these effects of stress on microglia. We propose that microglia serve as immunosensors of the stress response, which puts them in the unique position to sense and respond rapidly to alterations in homeostasis and integrate the neural response to threats.

Key words: stress; microglia; neuroinflammation; innate immunity.

1. Introduction

Exposure to stressors, whether physical and/or psychological in nature, perturbs the homeostasis of several biological systems including the endocrine, autonomic, immune and central nervous systems (CNS). These perturbations manifest in the mobilization of key molecules, which allow organisms to respond to threats, otherwise known as the fight/flight response. Interestingly, the molecular effectors of the fight/flight response directly impinge upon microglia, which are tissue-resident macrophages occupying the brain parenchyma. Indeed, microglia express the necessary repertoire of receptors to integrate and respond to stress-induced endocrine (e.g., glucocorticoids), immune (e.g., interleukin-1) and neural (e.g., norepinepherine) products of the stress response. This interaction with microglia during stress exposure can result in profound microglial cellular alterations characterized by modifications in immunophenotype, number, morphology and/or function. In turn, microglia secrete a number of immunological factors, which modulate the motivational state and cognitive function of the organism. Here, we will explore the notion that microglia serve as immunosensors of the stress response, thereby providing a critical immunological input to neural circuits to help shape an organism's behavioral response to threats.

2. Microglia

Microglia are a type of mononuclear phagocyte or macrophage that occupy the brain parenchyma [1]. With a highly ramified cell morphology and small cell soma, microglia are derived from erythromyeloid progenitors in the yolk sac [2, 3] and are maintained in the brain through a process of self-renewal [4]. Remarkably, Huang and

colleagues recently demonstrated that after acute depletion of microglia, repopulation of brain microglia was derived from a small fraction (<1%) of the surviving cells [5].

Microglia were once considered a resting and passive inhabitant of the CNS. As Crotti and Ransohoff note "it has been stated innumerable times that the primary role of microglia is to protect the CNS from pathogenic insults". Further, they contend that there is little evidence in support of this assertion [6]. This antiquated view presumes that microglia passively wait for pathogenic intruders to enter the brain parenchyma and upon encounter with a pathogen, to then launch an immune assault to neutralize the pathogen. Rather, microglia are now viewed as a dynamic and active participant in the CNS, which maintain brain homeostasis and surveil the specialized niche of the brain parenchyma for disruptions in homeostasis [7]. Nimmerjahn and colleagues demonstrated that microglia actively extend and retract their fine processes while moving through the CNS microenvironment as if sensing the milieu for disturbances in homeostasis. Recent evidence from Madry and colleagues demonstrates that the microglial potassium channel THIK-1 plays a critical role in this surveillance function of microglia [8].

Beyond their critical role in neurodevelopment (for review see [9]), microglia perform critical functions in the mature healthy brain including the removal of cellular debris and apoptotic or necrotic cells through their phagocytic capacity [6]. Moreover, microglia lend trophic support to neurons, modulate neuronal activity, and modify synaptic connections and plasticity [10]. Tremblay and colleagues note that "microglia display functional features of synaptic partners" [11]. Indeed, microglia express an array of ion channels, neurohormone receptors and neurotransmitter receptors typically found

in neurons [12], which permit microglia to function as "synaptic partners" and directly respond to alterations in key mediators of the stress response including glucocorticoids (GCs) and catecholamines (i.e., norepinepherine and epinepherine). As immunocompetent cells, microglia also are capable of producing an array of proinflammatory and antiinflammatory factors, some of which play a critical role in synaptic plasticity [13] as well as memory processes [14] under physiological conditions.

As the primary innate immune residents of the parenchyma, microglia are sequestered behind the blood brain barrier (BBB), which normally excludes exposure to microorganisms as well as peripheral leukocytes except during periods of CNS infection or trauma. Indeed, the BBB effectively sequesters the brain parenchyma from blood products and it is thus considered an immune privileged site because it excludes entry of leukocytes of the adaptive (e.g. T and B cells) or innate (e.g. monocytes and dendritic cells) immune system under non-pathological conditions [15]. However, it is important to note that peripheral leukocytes do gain entry and occupy other CNS compartments such as the meninges, choroid plexus and ventricular space, which are not encapsulated by the BBB. Rather, these CNS compartments contain a blood cerebrospinal fluid barrier, which is more permissive to the extravasation of peripheral leukocytes [15].

As noted, surveillant or homeostatic microglia display a highly ramified morphology with a small cell soma. Homeostatic microglia exhibit a unique transcriptional signature (*P2ry12*, *Tmem119*, *Siglech*, *Gpr34*, *Socs3*, *Hexb*, *Olfml3*, and *Fcrls*), which differentiates microglia from other myeloid cell types as well as microglial cell lines [16]. Disruptions in CNS homeostasis, such as occurs during aging and

neurodegenerative disease, results in the suppression of this homeostatic transcriptional signature or immunophenotype, concomitant with induction of an immunophenotype that characterizes degeneration- or disease-associated microglia (DAM)[17]. Microglia also undergo a variety of morphological changes that are typically classified under the umbrella term "activated". In general, activated microglia are characterized by shorter and thicker processes and a larger cell soma. At the extreme end of microglia activation, microglia exhibit a de-ramified or amoeboid morphology [1]. Alternatively, microglia can enter a hyper-ramified state under certain pathological conditions [18]. The notion of microglia activation as a binary state (activated vs non-activated) has been superseded by evidence suggesting that microglia lie along a spectrum of activation states depending upon the CNS microenvironment, regulatory milieu, stage of development and environmental factors [19]. These states are characterized by varying blends of immunophenotypes and cytokine profiles. Of note, a primed activation state may be induced in microglia under several neuroinflammatory conditions [20] including aging [21] and stress [22], which we expand upon in section 3.3.1.

The activation state of microglia in the healthy brain can be modulated by either peripheral or central inflammatory insults. Importantly, inflammatory insults can be characterized as either sterile (e.g. closed head injury) or non-sterile (e.g. *E. coli* infection). Typically, sterile inflammatory responses occur in response to tissue damage in the absence of infectious processes, which results in the release of damage-associated molecular patterns (DAMPs) or alarmins. These include effector molecules such as heat shock proteins, S100 proteins and high-mobility group box-1 (HMGB1).

DAMPs elicit proinflammatory responses in innate immune cells including microglia via a diverse set of germ-line encoded receptors [23]. Of note, exposure to stressors (see section 4.5), which are largely sterile, also have been found to induce DAMPs in the brain and modulate the proinflammatory properties of microglia [24]. Interestingly, microglia also respond to distal immune insults such as peripheral infection, trauma or sterile injury even though microglia are not directly exposed to the initiating stimulus. For example, intraperitoneal injection of LPS, which is a pathogen-associated molecular pattern (PAMP) derived from gram-negative bacteria [25], is a potent stimulus of proinflammatory immune responses in peripheral tissues such as liver [26]. LPS binds to LPS binding protein (LBP), which is then bound by cluster of differentiation (CD)14. The LBP-CD14 complex delivers LPS to the pattern recognition receptor (PRR) toll-like receptor 4 (TLR4) in complex with MD-2. This complex is expressed on innate immune cells including microglia to elicit a proinflammatory response [27]. This immune response is characterized by the production of proinflammatory cytokines (e.g. IL-1β) as well as chemokines from peripheral innate immune cells such as tissue macrophages (e.g. liver Kupffer cells). When administered peripherally, LPS is prevented from entering the brain parenchyma due to the unique structural properties of the BBB [28] and thus microglia fail to directly "see" the initiating stimulus. However, despite this restriction of the BBB, LPS elicits a proinflammatory response in microglia. This indirect effect of LPS on microglia is achieved through well-characterized neural and humoral immune-to-brain signaling pathways [29], which allow the immune system to communicate peripheral perturbations in homeostasis or threats (e.g. infection or injury) to the CNS. Blood-borne LPS is capable of binding TLR4 receptors expressed on

endothelial cells of the BBB, which then communicate proinflammatory signals to the brain parenchyma. In addition, blood-borne cytokines can bind their cognate receptors expressed on endothelial cells of the BBB, they can be actively transported across the BBB or enter the brain by volume diffusion at the circumventricular organs [28]. Alternatively, in cases of visceral infections, PAMPs such as LPS can induce neuroinflammatory responses via neural routes of communication such as vagal afferents [29]. In turn, these immunological signals are transduced in the brain into an array of neural, endocrine and behavioral responses, which facilitate the organism's response to environmental threats in threat appraisal regions of the brain including the hippocampus, amygdala and prefrontal cortex. As the predominant innate immune cell in the CNS, it can be argued that microglia play a pivotal role in shaping this response to peripheral immune signals. However, immunocompetency in the CNS is not restricted to microglia as other mononuclear phagocytes (e.g. perivascular, meningeal and choroid plexus macrophages)[30] as well as astrocytes [31] exhibit this property. Thus, these cell types likely play a role in stress effects described henceforth, but will not be the focus of the present review.

It is worth noting that the immune system has been conceptualized as a sixth-sense [32], and as a component of the mononuclear phagocyte system, microglia might be considered a critical node of this sensory network in the brain. In many instances, the central cytokine response mirrors the peripheral cytokine response [33]. Of particular relevance to the present topic, effector molecules of the proinflammatory response in the brain, such as the cytokine IL-1β, elicit a constellation of behavioral and physiological changes known as the sickness response [34]. This response is

characterized by decreased motor activity, social withdrawal, reduced food and water intake, increased slow-wave sleep, increased core body temperature and altered cognition. In addition, the sickness response involves increased pain sensitivity and induction of anxiety- and depressive-like behaviors. Notably, some aspects of the sickness response are recapitulated in animals exposed to stressors [35], which also induce alterations in microglia immunophenotype, morphology and function.

3. Stress modulation of microglia: density, morphology, immunophenotype and function

Indeed, the effect of stress exposure on the sickness response is remarkably similar to the induction of the sickness response by a proinflammatory immune challenge such as LPS. As noted, proinflammatory cytokines in the CNS play a causal role in the sickness response to immune challenge. Further, several aspects of the sickness response resemble a number of endophenotypes that characterize neuropsychiatric disorders such as major depression, PTSD and anxiety disorders [34]. Of relevance here, stress is considered a key predisposing factor in the etiology of neuropsychiatric disorders [36]. Given the parallel phenomena induced by stress and immune challenge, the neuroinflammatory effects of stress became a subject of considerable research. Subsequently, interest in the effects of stress on microglia stemmed, in large part, from a number of studies, which demonstrated that exposure to stressors induce proinflammatory signaling molecules in the CNS. These studies suggest that proinflammatory molecules play a mediating role in the behavioral response to stress [35]. These studies constitute an extensive literature, which are beyond the scope of the present discussion. Indeed, pharmacological blockade or

genetic manipulations of proinflammatory signaling, in particular IL-1β signaling, can attenuate or block the stress-induced sickness response (see review by [35]). IL-1β is considered a key mediator of the stress response in the CNS given its pleiotropic properties [37] and function as a gatekeeper of neuroinflammation [38].

It is worth noting here that it is often said stressors and peripheral inflammatory events such as LPS induce “neuroinflammation” in the brain parenchyma. However, what is actually measured are brain cytokines, chemokines, or DAMPs. Elevations in these proteins is typically equated with neuroinflammation, but it is worth bearing in mind that while proinflammatory cytokines are pivotal for the induction of neuroinflammation, they are not sufficient for neuroinflammation, which would be indicated by the infiltration of circulating leukocytes. Rather, much of the evidence gathered to date implicates proinflammatory cytokines as well as DAMPs in the behavioral effects of stress without the induction of overt neuroinflammation in the brain parenchyma. Of note, emerging evidence is beginning to suggest that stressors can induce the recruitment of peripheral leukocytes (i.e., inflammatory monocytes) to CNS structures outside the brain parenchyma [39], findings we elaborate upon in section 4.4.

3.1. Microglia density

The preponderance of stress studies has examined the expression of a small set of myeloid-associated proteins in the CNS including ionized calcium binding adaptor protein-1 (Iba-1), CD11b, CD45, fractalkine receptor (CX3CR1) and major histocompatibility class II (MHCII). Expression of these proteins has been used to quantify the number or density of microglia in specific brain regions. Of note, these proteins are expressed on microglia as well as other CNS macrophages. Originally, Nair

and Bonneau found that exposure to repeated-restraint stress resulted in a transient increase in the proportion of microglia (CD45 low/CD11b+) in the mononuclear cell fraction isolated from total mouse brain [40]. Flow cytometry was utilized here to quantify the number of microglia in a heterogeneous population of cells isolated by density gradient centrifugation, which is problematic as it obscures regional brain variations in microglia density [41] and likely does not capture all microglia from a tissue of interest. Microglia are characterized by low CD45 staining compared to other macrophages and thus, the expression level of this antigen can be used to delineate microglia from other brain macrophages. A flow cytometric approach is useful for assessing antigen expression or immunophenotype on a per cell basis, but is of limited utility for inferring cell density *in situ*. Subsequent studies of stress effects on microglia cell density have largely relied upon immunohistochemistry or immunofluorescent approaches *in situ*. Indeed, we found that an acute stressor (tailshock) increased the number of MHCII+ microglia immediately after stress exposure in the CA3 and dentate sub-region of the rat hippocampus [42]. MHCII co-localized with Iba-1 in cells with a highly ramified morphology, which is a characteristic of microglia. MHCII failed to co-localize with glial fibrillary acidic protein (GFAP; an astrocyte antigen) staining suggesting that the tailshock-induced increase in MHCII+ cells was unique to microglia. In addition, hippocampal microglia isolated 24h post-tailshock exhibited an upregulation of MHCII gene expression. In home cage control animals, MHCII staining was largely undetectable, which is consistent with a large body of evidence suggesting that microglia express very low cell surface MHCII levels except under conditions such as aging [43]. Sugama et al. found that an acute heterotypic stressor (i.e, cold-water

immersion in combination with restraint) increased the number of CD11b as well as MHCII immunopositive cells in hypothalamus, hippocampus, and thalamus [44, 45]. Notably, stress failed to increase proinflammatory cytokine expression (IL-1β) in CD11b+ cells as measured by double immunofluorescent staining in hypothalamus [45]. Several studies using a variety of chronic unpredictable stress (CUS) models demonstrated that stress exposure increased the number of cells immunopositive for the microglia/macrophage-associated proteins Iba-1 [46, 47], MHCII [48-50] and CD11b [51] in a number of different brain regions. Similar effects on Iba-1 staining were observed using repeated restraint stress [52-54]. However, several studies have found that CUS fails to affect the number of Iba-1 immunopositive cells in brain [52, 55]. Wohleb and colleagues found that repeated social defeat (RSD) increased the proportional area of Iba-1 staining within hippocampus, prefrontal cortex and amygdala [56-58], however Iba-1+ microglia cell density was not assessed. Using a fractalkine receptor (CX3CR1)-green fluorescent protein (GFP) transgenic mouse, Kreisel et al. demonstrated that short-term exposure (2 days) to unpredictable stress increased the number of CX3CR1-GFP+ microglia in the dentate gyrus [59]. Unpredictable stress also increased the number of bromodeoxyuridine-labeled microglia in the dentate gyrus suggesting that unpredictable stress increased the proliferation of microglia. At the same time, unpredictable stress increased the number of apoptotic microglia as measured by caspase-3 immunofluorescence. Alternatively, CUS (5 wk of exposure) decreased the number of CX3CR1-GFP+ microglia in the dentate gyrus. Treatment with macrophage-colony stimulating factor (M-CSF) or granulocyte-macrophage (GM)-CSF, which signal through the CSF1 receptor on microglia to induce proliferation, blocked the

effect of CUS on microglia number. Of note, M-CSF and GM-CSF were effective at blocking the behavioral effects of CUS including reductions in sucrose preference and social exploration, which are considered measures of anhedonia and anxiety, respectively. These findings suggest that acute stress exposure induces a transient proliferation of microglia, while protracted exposure to stress results in apoptosis of microglia and reductions in microglia cell number. This microglial cytopenia appears to play a critical role in the behavioral effects of stress, but the mechanism of action is unclear. Similarly, Lehmann and colleagues found that acute social defeat (SD; 5 min/day for 3 days) increased the number of CX3CR1-GFP+ microglia throughout the brain, while chronic SD (5 min/day for 14 days) failed to alter microglia cell number [60]. Acute SD also increased the co-localization of the S phase proliferation marker (proliferating cell nuclear antigen) with microglia, suggesting that acute stress increased microglia proliferation throughout the brain. It is important to note that CX3CR1-GFP+ transgenic mice lack a copy of CX3CR1. When bound to its cognate ligand CX3CL1, CX3CR1:CX3CL1 signaling serves as a checkpoint mechanism on microglial immune activity [61]. Thus, lack of CX3CR1 in this transgenic model confounds these studies.

Of note, a particular concern with SD models is the likelihood of non-sterile injuries stemming from direct agonistic encounters between the resident/dominant mouse and the sub-ordinate/intruder mouse. Non-sterile injuries in the form of bites could lead to infection and/or damaged tissue, which could then drive peripheral proinflammatory immune reactions and thus potentially impact microglia and neuroinflammatory process via immune-to-brain pathways discussed previously. Clearly, neuroimmune effects brought about by infection would be confounded with the

neuroimmune effects of defeat-induced psychological stress. Notably, this risk of infection was mitigated by trimming the mandibular incisors of the resident mouse [60]. It is worth noting that alternate models of SD such as the RSD model permit non-sterile injuries such as bites to the subordinate animal, which are considered a necessary element of the model to generate primed immune cells [62]. Thus, this element increases risk of infection and the ensuing immune response that would follow. This is an important consideration when attempting to explain heterogeneous findings between stress models that employ social defeat.

Taken together, these findings suggest that exposure to acute and chronic stressors increases the number of cells immunopositive for microglial/macrophage-associated proteins such as Iba-1. However, it is unclear whether this actually reflects an increase in microglial cell number or a microgliosis in the brain. One possibility is that stress simply upregulates protein expression, thereby increasing the detection of microglia *in situ*. In large part, the studies reviewed here did not quantify myeloid protein expression on a per cell basis, but much of the immunohistochemical images clearly show that protein expression such as Iba-1 appears upregulated on a per cell basis. Alternatively, evidence suggests that RSD induces the recruitment of peripheral macrophages/monocytes to the brain parenchyma [58], which also stain positive for Iba-1 and thus may mediate reports of stress-induced increases in microglial density. However, recent work suggests that RSD recruits peripheral monocytes to vascular endothelial cells in the brain and not into the parenchyma per se [39], which we discuss in section 4.1. The work of Lehmann et al. and Kreisel et al. provide compelling evidence that acute or short-term stress induces microglia proliferation [59, 60]. As a

whole, the findings reviewed here largely fail to clarify what stress-induced upregulation of Iba-1 immunostaining in the brain represents biologically.

3.2. Microglia morphology

A number of studies reviewed in section 3.1 presented immunohistochemical findings that show a clear alteration in microglia morphology even though morphological changes were not formally quantified. For example, high-resolution images of CD11b staining demonstrate that acute stress (1-2 hr of restraint plus cold water immersion) induces what has been termed a "bushy" phenotype characterized by less ramification, shorter and thicker processes and a larger cell soma [45]. Similar effects on microglia phenotype were observed with MHCII staining using the same stressor [44]. Likewise, high-resolution images show that RSD induces a "bushy" or de-ramified microglia phenotype in multiple brain regions [56-58].

Several studies have taken a more formal approach to quantifying stress-induced morphological changes. Hinwood et al. undertook one of the first detailed studies of microglia morphological changes in the medial prefrontal cortex of animals exposed to repeated restraint (6h/day for 21 days). They found that stress exposure resulted in increased branching or a hyper-ramification of microglia. This effect was most prominent in large microglia cells and was blocked by treatment with minocycline [63], which is considered a microglial activation inhibitor. However, it is important to consider that while minocycline exerts anti-inflammatory effects on microglia, it also exerts effects on other cells of the innate immune system (e.g. monocytes and neutrophils) and adaptive immune system (e.g. T cells) through a number of mechanisms [64]. Thus, the

experimental use of minocycline to examine a causal role for microglia is problematic given minocycline's non-specific cellular effects.

Kreisel et al. found that 2 days of unpredictable stress increased the size of microglial soma, while 2 and 4 days of unpredictable stress decreased microglial process length, which is indicative of a de-ramified morphology. However, exposure to CUS decreased both cell soma size and process length [59]. Milior et al also found that CUS (each stressor applied for 24h over a 2 wk period) induced a decrease in microglia ramification, but cell body size was unaltered [55]. Franklin and colleagues recently demonstrated that 4 weeks of CUS increased microglia cell soma size, branch length, branch number and branch volume in the dorsal hippocampus [65], suggesting that stress-induces a hyper-ramified phenotype. However, the study by Lehmann and colleagues found that both acute and chronic SD failed to alter several microglia morphological parameters including cell area, soma area, cell spread, eccentricities, cell perimeter length and roundness [60]. Importantly, they used an automated method [66], which examined 6716 microglia across three stress-responsive brain regions. In addition, *Cx3cr1*$^{wt/gfp}$ mice were utilized in which GFP is endogenously expressed at high levels in microglia. This method of detection permits complete visualization of the cell and obviates the limitations of immunohistochemical approaches such as antibody specificity and non-specific binding. In addition, Lehmann et al. included a positive control by exposing *Cx3cr1*$^{wt/gfp}$ mice to the immunogen LPS and found that LPS did induce increases in cell soma and roundness, and decreased cell perimeter length. It is important to consider that the lack of an effect of SD on microglia morphology might be due to the milder form of stress (5 min/day for 3 and 14 days) utilized by Lehmann et al.

compared to other forms of SD (e.g., 2h of RSD/day for 6 days) as used by Wohleb and colleagues.

With regard to stress effects on microglia morphology, a clear pattern of effects is not evident from this limited set of studies. It seems that the heterogeneity of these findings reflects or parallels the heterogeneity in the stress models used to study stress effects on microglia morphology. A troubling consideration and one that bears emphasizing, especially given concerns regarding questions of "rigor" or replication of findings in neuroscience [67], is that each of the findings discussed heretofore as well as the following might simply be unique to the narrow combination of experimental parameters that comprise each stress model and might not generalize beyond those parameters. In addition, strain differences in the stress response are a critical variable that might also contribute to this heterogeneity. These points are worth bearing in mind when attempting to reconcile divergent findings in this stress field of study.

3.3. Microglia immunophenotype and function *ex vivo*

Flow cytometry is a powerful analytical tool for characterizing and quantifying stress-induced alterations in microglia antigen expression on a per cell basis. Originally, Sedgwick and colleagues utilized density gradient centrifugation followed by flow cytometry to characterize the immunophenotype of microglia from whole rat brain [68, 69]. Flow cytometric analyses revealed that microglia expressed lower levels of the CD45 (protein tyrosine phosphatase, receptor type, C) antigen compared to other CNS mononuclear phagocytes such as perivascular macrophages, which can be used to differentiate these cell types in a heterogeneous cell preparation. We built upon this method to purify highly enriched and viable microglia from discrete brain regions, which

are devoid of other CNS mononuclear phagocytes as well as other immunocompetent CNS elements such as astrocytes [70]. The power of this approach is that it allows one to interrogate how an *in vivo* condition, such as stress exposure, specifically modulates functional characteristics of microglia *ex vivo.* For example, direct exposure of microglia to an immunogen such as LPS *ex vivo* and assessing the level of proinflammatory cytokine production is one approach. Another involves directly exposing microglia to fluorescently labeled apoptotic cells or microbeads and assessing the phagocytic capacity of microglia. However, such *ex vivo* approaches are not without limitations. That is, the procedures used to isolate cells result in the loss of microglial processes, presumably through cell retraction. In addition, the removal of microglia from their native microenvironment results in the loss of checkpoint mechanisms (e.g. CD200: CD200R1 signaling)[61], which might induce a primed activation state in microglia. Despite these limitations, isolated microglia appear to retain immunophenotypic characteristics found *in situ* [70].

3.3.1. Microglia priming

We utilized this *ex vivo* approach to test whether exposure to a severe acute stressor (tailshock) sensitizes or primes the microglial proinflammatory response to an immune challenge (i.e. LPS) *ex vivo* [42]. Studies had found that exposure to prior tailshock potentiates the neuroinflammatory response in hippocampus to a subsequent immune challenge (LPS), which was administered *in vivo* (intraperitoneal injection)[71, 72]. While these studies demonstrated that prior stress exposure potentiated the neuroinflammatory response to a subsequent immune challenge, the design precluded determination of the biological substrate(s) that was primed by stress. In other words,

neuroinflammatory priming is not equivalent to microglial priming. For example, stress might prime a peripheral substrate such as a liver macrophage (Kupffer cell) resulting in a potentiated liver cytokine response to LPS. In turn, this cytokine response would result in enhanced immune-to-brain signaling and potentiation of the microglia proinflammatory response. To test whether an antecedent condition primes the immune response of a biological substrate, it is necessary to isolate and purify the substrate and directly expose it to an immunogen. A potentiated immune response to the immunogen would then be indicative of a primed activation state. To address this design issue of priming, we isolated hippocampal microglia 24h after offset of the stressor and directly exposed microglia to several concentrations of LPS. Prior stress exposure potentiated the microglial pro-inflammatory response to LPS *ex vivo,* suggesting that stress induces a primed activation state in hippocampal microglia. Of note, this finding does not exclude the possibility that other biological substrates are primed by prior stress exposure or that this phenomenon varies across brain regions. Subsequently, we demonstrated that this phenomenon of stress-induced microglial priming is mediated by glucocorticoids [73], the pattern recognition receptors TLR2 and TLR4 [74], the alarmin HMGB1 [75] and the microglial inhibitory receptor CD200R1 [76]. We discuss and integrate these findings in more detail in section 4. In addition, stress-induced microglial priming is time of day dependent [77].

A similar phenomenon was observed using a chronic homotypic stressor [57]. Mice were subjected to six days of RSD (2h/day) and microglia were then isolated from whole brain and directly exposed to LPS. Prior exposure to RSD potentiated the microglial proinflammatory cytokine response to LPS including IL-6, TNFα and MCP-1.

In addition, prior RSD, independent of LPS, increased microglia mRNA expression of IL-1β and upregulated microglial cell surface expression of CD14, TLR4 and CD86. As noted in section 2, CD14 and TLR4 are required for LPS signaling. Thus, this impact of RSD on CD14 and TLR4 might mediate the priming effects of RSD. These findings, as a group, suggest that stress-induced microglial priming occurs in stress models of differing chronicity (acute vs chronic) as well as type of stressor (tailshock vs SD). In a subsequent study, LPS was injected intraperitoneally 14h after cessation of RSD and proinflammatory endpoints were assessed in isolated microglia 4 and 24h after LPS treatment. As with priming assessed *ex vivo* [57], prior RSD potentiated the microglia response (IL-1β, TNFα, iNOS and CD14 mRNA) to LPS *in vivo* [56]. In addition, prior RSD potentiated LPS-induced Iba-1 immunostaining in the hippocampus, suggesting that upregulated expression of Iba-1 might represent a shift in the activation state of microglia towards a primed immunophenotype. More recent work by this group has demonstrated that microglia must be present during the stress exposure in order to develop a primed phenotype. Microglia that were eliminated prior to RSD with a CSF1R antagonist (PLX5622) did not show evidence of priming when they were allowed to repopulate following the stressor [78].

Importantly, prior stress exposure also potentiates the sickness response (e.g., increased core body temperature, reduced activity and increased anxiety-like behavior) to an immune challenge [56, 72], which parallels the stress-induced priming of neuroinflammatory and microglial proinflammatory responses. An important consideration is how these priming effects of stress might play a role in stress as a predisposing factor to psychiatric disorders [22]

3.3.2. Microglia phagocytic function

As noted in section 2, the phagocytic function of microglia plays a pivotal role in removing cellular debris, apoptotic and necrotic cells, and synaptic pruning. Following injury, microglia first take on a classical activation state, which is typified by the production of proinflammatory cytokines and reactive oxygen species. Microglia subsequently transition towards a state of alternative activation, in which anti-inflammatory factors are upregulated, and microglia participate in wound repair and debris clearance [79]. Indeed, proinflammatory microglia phagocytose less efficiently [80], whereas microglia treated with antiinflammatory factors (e.g., IL-4) increase phagocytic capacity [81]. Several studies have investigated whether exposure to stress modulates phagocytic function. Milior et al. exposed mice to a chronic heterotypic stressor for 2 weeks and assessed microglia phagocytosis in the CA1 area of the hippocampus using electron microscopy [55]. They found that exposure to stress increased the number of phagocytic inclusions per Iba-1+ microglial process, which were defined as vacuoles or endosomes containing neuronal elements such as axon terminals and dendritic spines. Similarly, Wohleb and colleagues utilized a Thy-1-GFP transgenic mouse to examine whether CUS (14 days) modulates microglial phagocytosis of neuronal elements in the medial prefrontal cortex [50]. When excited, GFP fluoresces exclusively in neurons, which provides a sensitive measure of Iba-1+ microglia with GFP+ inclusions. Exposure to CUS increased the percentage and number of microglia with GFP+ inclusions as well as the number of phagocytic cups per microglia. Further, the lysosomal antigen CD68 co-localized with GFP+ inclusions. These findings suggest that CUS increases microglial phagocytosis of neuronal

elements and thus might play a role in stress-induced neuronal remodeling. Interestingly, phagocytosis of neuronal elements was mediated by resident microglia and not by peripheral monocytes/macrophages. The authors further demonstrated that CUS increased CSF1 levels in medial prefrontal cortex, which was necessary for the stress-induced increase in microglial phagocytic function. Using an *ex vivo* phagocytosis assay, Lehmann and colleagues isolated whole brain microglia from mice exposed to acute (3 days) and chronic (14 days) SD [60]. Microglia were then incubated with fluorescently labeled ultraviolet-irradiated neural cells, which are apoptotic due to irradiation and thus elicit a phagocytic response in microglia. While acute SD failed to alter microglia uptake of apoptotic neural cells, chronic SD strongly upregulated uptake suggesting that chronic SD increased microglial phagocytic function. In addition, chronic, but not acute SD increased the proportion of microglia that were immunopositive for CD68, which is a lysosomal antigen thought to play a role in phagocytosis. Of note, in the studies discussed above, there was no evidence of a proinflammatory shift in microglia following stress. For example, Wohleb et al found microglia upregulated anti-inflammatory factors (Cx3cr1 and TGFbr1) and downregulated proinflammatory cytokines (IL-1β and TNF-α). In contrast, we found that the proinflammatory shift in mice exposed to an acute stressor (2h of tailshock) was associated with suppressed microglial phagocytic function [82]. 24h after stress exposure, hippocampal microglia were isolated and incubated with fluorescently labeled latex beads. Stress decreased uptake of latex beads in both male and female rats, suggesting that acute stress exposure decreases the phagocytic function of microglia.

These contrasting findings are likely due to multiple factors including the type of stressor and timing of the phagocytosis assay following stress.

These findings suggest that the phagocytic function of microglia is upregulated in three different models of chronic stress, suggesting that chronic stressors might induce neuronal remodeling via enhanced microglia phagocytic function. An alternate explanation is that chronic stress might induce cell death or apoptosis of CNS elements, which would then elicit an enhanced phagocytic response in microglia designed to remove dead and dying cells and thus mitigate the release of proinflammatory factors (e.g. DAMPs) from dying cells. Kreisel et al. demonstrated (see section 3.1) that unpredictable stress increased the number of apoptotic microglia and that CUS resulted in loss of microglia [59]. A recent study by Lehmann et al. demonstrates that chronic social defeat in susceptible mice induces microbleeds in the parenchyma of the brain suggesting that chronic stress might induce damage to the CNS vasculature [83]. As noted in section 4.2, a number of studies have found that both acute and chronic stressors induce DAMPs in the CNS, which might be indicative of tissue injury. Further, stress hormones such as GCs have been found to induce a dystrophic phenotype in neurons as well as to induce neuronal damage and cell death [84]. Clearly, additional studies are required in this domain to clarify the role of microglia phagocytic function in the effects of stress on the brain given the correlative nature of these studies. A key question is whether inhibition of microglia phagocytic function during chronic stress exposure would alter the neuroinflammatory and behavioral effects of stress.

4. Mechanisms of stress effects on microglia

Exposure to acute and chronic stressors drives activation of the HPA axis and sympathetic nervous system (SNS), resulting in the systemic release of stress hormones including GCs and epinepherine from the adrenal glands, and norepinepherine from the SNS as well as stress-responsive brain regions. A number of studies have implicated GCs in stress-induced neuroinflammatory priming [85], while catecholamines have been implicated in the neuroinflammatory effects of stress exposure as well as the mobilization and recruitment of peripheral monocytes to the CNS [62]. In light of these findings, a number of studies have investigated the effects of stress hormones on microglia.

4.1. GCs and catecholamines

GCs are critical mediators of stress-induced microglia priming. GCs are potently upregulated following stress and implicated in mediating a number of the negative effects of stress. A causal role for GCs has been demonstrated by blocking GC signaling through to 2 key approaches. One approach involves utilizing a GC receptor antagonist, such as mifepristone (RU486), to block GC signaling. An alternate approach is to adrenalectomize (ADX) subjects, which effectively prevents the stress-induced increase in peripheral as well as brain GC levels.

We conducted a study in which subjects were either treated with mifepristone or underwent ADX [73]. It is important to note that basal levels of GCs were maintained in the ADX subjects—thus, only the stress-induced increases from baseline were prevented. Subjects were then exposed to tailshock, which we have found to prime the microglia proinflammatory response *ex vivo* [42]. 24 hours after stress exposure, hippocampal microglia were isolated and treated with the immunogen LPS. Both

mifepristone treatment and ADX blocked the priming effect of stress on microglia, suggesting that stress-induced GCs play a causal role in this priming phenomenon. We conducted additional studies demonstrating that both acute and chronic administration of exogenous GCs primes the proinflammatory response of hippocampal microglia *ex vivo* [82, 86, 87], suggesting that GCs are sufficient to recapitulate the effects of stress on microglial priming. Similar priming effects of GCs have been found *in vivo* [82, 86, 88]. It is important to consider that GCs have been utilized extensively, since their discovery in the 1950s, as powerful anti-inflammatory and immunosuppressive drugs [89]. However, GCs also have been found to exert a number of so called permissive effects, which includes priming of neuroinflammatory processes [90].

Catecholamines have also been implicated in mediating the effects of stress on microglia priming. Systemically treating mice with a β-adrenergic receptor antagonist (propranolol) blocked the effects of RSD on microglia IL-1β mRNA, cell surface expression of CD14 and upregulation of Iba-1 immunostaining in the amygdala, prefrontal cortex and hippocampus [57]. Propranolol also blocked the RSD-induced anxiety-like behavior. Similar effects occurred using an IL-1 receptor knockout mouse suggesting that IL-1 signaling mediates the effects of RSD on microglia and behavior [57], which were downstream of RSD effects on catecholamine signaling. These findings are consistent with *in vivo* data demonstrating that catecholamines mediate the neuroinflammatory effects of tailshock [91]. Further, administration of a β-adrenergic agonist (isoproterenol) recapitulates the neuroinflammatory effects of stress [91]. Isoproterenol administration *in vivo* also primes the proinflammatory cytokine response

of hippocampal microglia to LPS *ex vivo* [92] suggesting that stimulation of β-adrenergic receptor signaling *in vivo* is sufficient to prime hippocampal microglia.

Stress-induced catecholamines also play a critical role in leukocyte mobilization and redistribution of leukocytes from primary immune compartments such as spleen and bone marrow [93]. Sheridan, Godbout and colleagues have conducted a number of compelling studies demonstrating that stress (RSD)-induced catecholamines increase the number of inflammatory macrophages ($Ly6C^{high}$) in the CNS [57] and that these macrophages are derived from bone marrow-derived monocytes [94]. Recent evidence from this group demonstrates that stress induces microglia to recruit proinflammatory monocytes from the bone marrow, likely via release of the chemokine CCL2 [39]. These inflammatory monocytes, which express high levels of CCR2, are then recruited to brain vascular endothelial cells where they release IL-1β, which bind the type I IL-1 receptor (IL-1R1) expressed by endothelial cells. IL-1R1 then transduces the IL-1β signal into the brain parenchyma, which is thought to mediate the effects of stress on anxiety-like behavior.

These studies provide converging evidence that the hormonal response to stress plays a causal role in stress effects on microglial immunophenotype and function, which produces inflammatory microenvironments within threat appraisal regions of the brain including the hippocampus, amygdala and prefrontal cortex. However, it is important to note that the pharmacological interventions used to manipulate GC and catecholamine signaling *in vivo* have a number of off-target effects, which are limitations of these studies. Much of this work has focused on examining the role of proinflammatory cytokines in stress-induced behavioral outcomes, however a number of

studies now implicate alarmins or danger-associated molecular patterns (DAMPs) in the neuroinflammatory effects of stress.

4.2. DAMPs

As noted in section 2, DAMPs are host proteins that are typically sequestered within cellular compartments under non-pathological conditions. However, in response to injury or tissue trauma, DAMPs are released from stressed or dying cells, which then signal through receptors on innate immune cells to elicit proinflammatory immune reactions. Touched upon in section 3.3.2, GCs can induce a dystrophic or stressed phenotype in neurons as well as cell death [84]. Thus, DAMPs might play a role in the neuroinflammatory effects of stress. Indeed, a number of studies demonstrate that stress exposure increases expression of DAMPs (e.g. HMGB1 and ATP) in the brain [65, 75, 76, 95-98].

To examine whether stress-induced increases in the DAMP HMGB1 plays a causal role in microglia priming, we injected an HMGB1 antagonist (box A) into the cisterna magna, which blocks HMGB1 signaling either through TLR4 and/or RAGE [75]. Rats were exposed to tailshock, and 24hr after offset of the stressor, hippocampal microglia were isolated and treated with LPS. Blocking HMGB1 signaling prevented the stress-induced potentiation of the microglial proinflammatory response to LPS suggesting that HMGB1 plays a causal role in stress effects on microglial priming. Of note, exposure to tailshock also induced the release of HMGB1 from hippocampal microglia isolated immediately after stress exposure, suggesting that microglia are a source of stress-induced HMGB1.

In light of these findings, we examined the mechanism whereby stress increased expression of HMGB1 in the hippocampus. We had found that GCs down-regulate the expression of CD200R1 on hippocampal microglia [99]. Briefly, CD200R1 is expressed exclusively on microglia in the brain parenchyma, and binds CD200 expressed by neurons and endothelial cells [100, 101]. CD200 is thought to constitutively inhibit myeloid cell function via engagement of CD200R1 [102]. Indeed, disruption of CD200:CD200R1 signaling potentiates the pro-inflammatory response of microglia to immune stimuli [103, 104]. The CD200:CD200R1 signaling dyad is considered a checkpoint mechanism that maintains microglia in a surveillant or homeostatic phenotype [105]. Given that GCs down-regulated expression of CD200R1 on microglia, we explored the possibility that stress might down-regulate microglial CD200R1 expression and mediate the increase in HMGB1 and microglial priming. Indeed, exposure to tailshock down-regulated CD200R1 mRNA and protein expression in hippocampus as well as in hippocampal microglia [76, 97]. Of note, we recently demonstrated that treatment with a psychobiotic (*Mycobacterium vaccae*) blocked the effects of tailshock on CD200R1 in hippocampal microglia [97]. To examine a causal role for CD200:CD200R1 signaling in stress-induced HMGB1 and microglial priming, we injected a CD200R1 agonist (mCD200-Fc) intra-cisterna magna just prior to stress exposure. mCD200-Fc blocked stress-induced increases in hippocampal HMGB1 as well as the priming of microglia, suggesting that tailshock dis-inhibits microglia via reductions in CD200R1, thereby resulting in the release of HMGB1 and priming of microglia. Further, exogenous administration of the pro-inflammatory form of HMGB1 (disulfide HMGB1) is sufficient to induce a primed microglia phenotype [106].

Consistent with the above, Franklin and colleagues recently reported that 28 d of CUS in mice increased expression of RAGE protein and mRNA in hippocampal microglia. As noted, RAGE is a receptor for HMGB1. Notably, the effect of CUS on RAGE protein was also observed 28 and 42 d following CUS exposure [65]. As noted in section 3.1.1, Wohleb and colleagues found that RSD upregulated the expression of TLR4 on microglia, which also serves as a receptor for HMGB1 [57].

Thus, stress-induced DAMPs might play a mediating role in the effects of stress on microglia. With regard to HMGB1, it is important to consider that all nucleated cells contain HMGB1 in the nucleus and the cellular source of stress-induced HMGB1 is largely unknown. Additionally, HMGB1 is capable of binding proinflammatory cytokines such IL-1 and thereby amplifies IL-1 signaling [107]. This facet of HMGB1 function raises the intriguing possibility that HMGB1 might act in concert with IL-1 to mediate the behavioral effects of stress.

5. Sex differences, stress, and microglia

There are well-established sex differences in the stress response and the innate immune system that implicate sex as an important variable to consider in stress induced microglia priming. Sex differences in the stress response exist throughout the mammalian lifespan and relate to both organizational and activational effects of gonadal hormones [108]. In adulthood, female humans and rodents exhibit more robust and prolonged behavioral and physiological responses to stress. For example, the GC response to a variety of stressors is enhanced in both female rodents and humans [82, 109]. Sex differences in the GC response are largely attributed to differential expression of sex steroids [109]. As discussed in section 4, GCs are a proximal signal through

which acute and chronic stress primes neuroinflammatory responses [82, 86]. Thus, sex differences in GC responses may manifest in distinct neuroimmune profiles following stress.

Given the substantial sex differences in both stress responses and microglia form and function, it follows that there could be sex differences in stress-induced priming of microglia. Indeed, sex-specific neuroimmune changes occur in several rodent stress models [50, 82, 110]. For example, recent work from our group demonstrated that while male and female rats exhibit comparable behavioral responses to an immune challenge following stress, the cellular mediators of neuroinflammatory priming may differ [82]. Male and female rats that underwent tailshock stress exhibited potentiated anxiety- and depressive-like behaviors to an LPS challenge compared to non-stressed controls. Consistent with these exaggerated behavioral deficits, the induction of proinflammatory cytokines was enhanced in the hippocampus of both male and female rats that received stress prior to LPS treatment. Inflammatory priming was associated with a downregulation of key anti-inflammatory neuron-microglia signaling dyads: CD200R1 and CX3CR1 were suppressed in the hippocampus of male and female rats exposed to tailshock. However, while microglia isolated from male rats that underwent prior tailshock exhibited a primed phenotype when challenged *ex vivo* with LPS, microglia isolated from stressed female rats paradoxically exhibited a downregulation in LPS elicited cytokine responses. Despite the blunted proinflammatory cytokine response in female microglia, microglia isolated from male and female rats showed similar decreases in phagocytic activity following tailshock. Thus, these results suggest that female microglia were not shifting toward a more anti-inflammatory phenotype;

rather, microglia from stressed female rats were simply less pro-inflammatory. Interestingly, female, but not male rats that underwent prior tailshock exhibited LPS-elicited increases in peripheral cytokines. Taken together, these results suggest that stress exaggerates neuroinflammatory and behavioral responses in male and female rats via distinct mechanisms [82]. Future studies could further explore mechanisms mediating stress-elicited neuroinflammatory processes in female rats.

Sex differences in stress-elicited neuroinflammatory processes also occur in other rodent stress models, including CUS stress protocols and restraint stress [50, 111]. For example, exposure to 3 days of CUS elicited a proinflammatory cytokine response (increased IL-1β mRNA expression) in the prefrontal cortex and hippocampus of male, but not female mice [112]. In contrast, following acute or chronic restraint stress, female but not male rats exhibit a reduced number of microglia with a primed morphology in the prefrontal cortex [111]. Furthermore, Wohleb et al showed that mice that underwent 2 weeks of CUS had altered gene expression of factors that mediate neuron-microglia interactions in the prefrontal cortex. These neuroimmune changes and phagocytic activity were more robust in microglia from the prefrontal cortex of male as compared to female mice [50].

Overall, there are many complexities to consider regarding sex and the neuroimmune system. Sex differences in microglia exist during development, in the adult, between species, and even between brain areas in a single animal [113]. Further, stress-elicited neuroimmune priming likely occurs via sex-specific mechanisms: stress-elicited priming is caused by microglia in male, but not female rats (82). Future studies could establish the mechanism driving stress-induced priming in female rats, how stress

affects neuroinflammatory responses and priming throughout development, and how stress or immune challenge relates to neuroinflammation in humans.

6. Conclusions

The findings reviewed here suggest that microglia serve as immunosensors of peripheral as well as central perturbations of homeostasis (**Fig. 1**). As a result of stress exposure, organisms undergo profound alterations in metabolic and endocrine function to mobilize resources to cope with threats. As stress hormones rise, microglia appear to take on a hyper-vigilant state with up-regulation of antigens, receptors, cytokines and chemokines. However, it is important to consider that the effect of stress on microglia is likely not limited to stress hormones, but might involve an array of immune molecules such as reactive oxygen species and alarmins. With threats may come harm and injury, thus hyper-vigilant microglia may be in a state of preparedness to respond rapidly to harm. It is interesting that microglia exhibit the unique combination of properties of cell motility, the capability to sculpt synapses (phagocytosis), provide trophic support to cells and generate cytokines such as IL-1β that subjugate the brain's neurocircuitry, thereby altering the motivational state of the organism. These properties allow microglia to interface and impact all other cell types within the CNS as well as to respond to signals derived from outside the CNS. In addition, microglia release signals, which recruit leukocytes to the CNS. Indeed, microglia display an amazing repertoire of functions that put them in the unique position to sense and respond rapidly to alterations in homeostasis and integrate the neural response to threat.

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Figure Caption

Figure 1. Microglia as immunosensors of the stress response. Acute and chronic stressors induce a spectrum of alterations in microglia function and immunophenotype. These alterations are mediated, in part, via stress hormones/transmitters including GCs and NE. We propose that, given the diverse functional capacity of microglia, these innate immune cells serve as immunosensors of perturbations in homeostasis, which allows them to respond to a spectrum of peripherally and centrally derived signals. In doing so, microglia play a pivotal role in shaping the neural and thus the behavioral response to stress in threat appraisal regions in the brain.

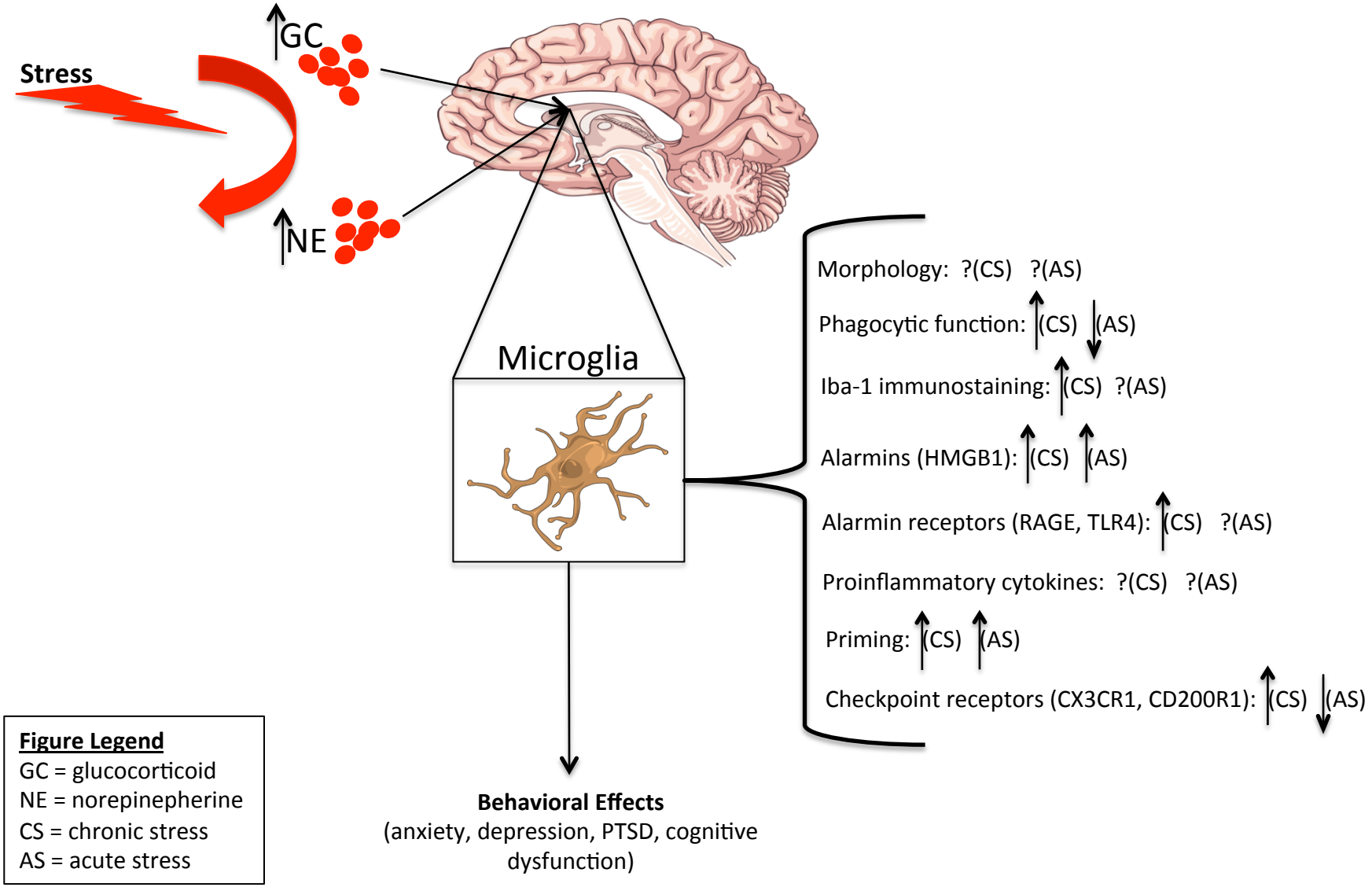

Figure Legend
GC = glucocorticoid
NE = norepinepherine
CS = chronic stress
AS = acute stress