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Stereological Analysis of Microglia in Aged Male and Female Fischer 344 Rats in Socially Relevant Brain Regions

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Abstract—Aging is associated with a substantial decline in the expression of social behavior, as well as increased neuroinflammation. Since immune activation and subsequent increased expression of cytokines can suppress social behavior in young rodents, we examined age and sex differences in microglia within brain regions critical to social behavior regulation (PVN, BNST, and MEA) as well as in the hippocampus. Adult (3-month) and aged (18-month) male and female F344 ($N = 26$, $n = 5$–8/group) rats were perfused and Iba-1 immunopositive microglia were assessed using unbiased stereology and optical density. For stereology, microglia were classified based on the following criteria: (i) thin ramified processes, (ii) thick long processes, (iii) stout processes, or (iv) round/ameboid shape. Among the structures examined, the highest density of microglia was evident in the BNST and MEA. Aged rats of both sexes displayed increased total number of microglia number exclusively in the MEA. Sex differences also emerged, whereby aged females (but not males) displayed greater total number of microglia in the BNST relative to young adult counterparts. When morphological features of microglia were assessed, aged rats exhibited increased soma size in the BNST, MEA, and CA3. Together, these findings provide a comprehensive characterization of microglia number and morphology under ambient conditions in CNS sites critical for the normal expression of social processes. To the extent that microglia morphology is predictive of reactivity and subsequent cytokine release, these data suggest that the expression of social behavior in late aging may be adversely influenced by heightened inflammation.

Key words: aging, senescence, microglia, morphology, social behavior, rat, limbic system, Fischer 344.

INTRODUCTION

Neuroinflammation is a significant consequence of normal aging (for review, see Barrientos et al., 2015a,b; Eggen et al., 2013; Jurgens and Johnson, 2012; Norden and Godbout, 2013), and inflammation or illness is associated with a reduction in social behavior as part of a larger repertoire of 'sickness behavior'. In fact, aged mice exhibit prolonged expression of sickness-related reductions in social interaction (Godbout et al., 2005; Huang et al., 2008), supporting a role for age-related neuroinflammation in the regulation of social behavior. Furthermore, aging is accompanied by a decline in the expression of social behavior, even in the absence of overt illness (Salchner et al., 2004; Hunt et al., 2011; Perkins et al., 2016). The consequences of decreased social behavior may be substantial, since positive social interactions are beneficial for overall health (Carter, 1998; DeVries et al., 2007; Grippo et al., 2007). Thus, identification of mechanisms by which social behavior is reduced in late aging remains critical.

Microglia represent a unique population of immune cells, as they are found exclusively within the central nervous system (Lawson et al., 1990; Kettenmann et al., 2011). Derived from myeloid progenitor cells that infiltrate the brain predominantly before the formation of the blood brain barrier (Ginhoux et al., 2010), microglia are the resident immune cells of the brain, performing numerous functions that include surveillance of the brain microenvironment. Microglia also respond to injury or illness by releasing pro-inflammatory cytokines (such as IL-1β and TNFα) and chemokines (Dantzer, 2004). However, recent advances in the understanding of microglia function have shown that in addition to their role as immune cells, microglia are integral in the formation and pruning of synapses across the lifespan (Tremblay, 2011; Paolicelli et al., 2011; Kettenmann et al., 2013; Salter and Beggs, 2014), help regulate neurogenesis (Sierra et al., 2010), and even contain receptors for neurotransmitters and neuropeptides that allow them to respond to neuronal activity (Pocock and Kettenmann, 2007; Kettenmann et al., 2011). Thus, microglia help maintain homeostasis in the CNS and respond to pertur-

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bations in homeostasis (e.g., pathogens) by releasing a cascade of inflammatory factors.

Importantly, natural aging is associated with tell-tale signs of neuroinflammation that can manifest in multiple ways (for review, see Barrientos et al., 2015a,b; Eggen et al., 2013; Jurgens and Johnson, 2012; Norden and Godbout, 2013). For example, microglia isolated from the aged brain have enhanced expression of MHCII (Griffin et al., 2006; Henry et al., 2009), CD86 (Griffin et al., 2006), CD68 (Wong et al., 2005), CD11b (Stichel and Luebbert, 2007; Hart et al., 2012), IL-1β (Griffin et al., 2006; Sierra et al., 2007; Stichel and Luebbert, 2007), and IL-6 (Sierra et al., 2007). Other studies have demonstrated that the aged brain is 'primed' in response to an immune challenge (reviewed in Norden and Godbout, 2013; Barrientos et al., 2015a,b). In this case, 'primed' indicates that there are no basal differences in neuroimmune markers, but rather that a secondary challenge is required for age-related alterations in neuroimmune function to become evident. For example, administration of LPS produces a robust increase in central IL-1β and IL-6 mRNA expression that is exacerbated in aged mice (Godbout et al., 2005; Huang et al., 2008). In addition, hippocampal microglia isolated from aged rats exhibit a sensitized cytokine response to peripheral immune challenge with LPS (Frank et al., 2010b) or *Escherichia coli* (Frank et al., 2010a). However, the mechanisms that drive age-related neuroinflammation are not well understood. One possibility is that aging is associated with increased number or reactivity of microglia, particularly in response to injury or immune challenge. Indeed, microglia isolated from the hippocampus (Huang et al., 2008; Abraham and Johnson, 2009), cortex (Henry et al., 2009), and cerebellum (Huang et al., 2008) of aged mice exhibit a prolonged pro-inflammatory response to endotoxin administration. Aging is also accompanied by a significant decline in the expression of fractalkine (CX_3CL1), a negative regulator of microglia activation (Fenn et al., 2013). Interestingly, CX_3CR1 knockout mice display altered patterns of social behavior, providing evidence for a functional role of microglia in social behavior regulation (Zhan et al., 2014). In addition, aged rats have high levels of corticosterone and this is related to greater glucocorticoid receptor activation within the hippocampus, both of which contribute to the sensitization of microglia (Barrientos et al., 2015b). Taken together, late aging is clearly associated with significant alterations in the inflammatory environment that may contribute to altered social behavior.

Increased neuroinflammation in late aging may be related to changes in the number and/or morphology of microglia in senescence. For example, using *in vivo* 2-photon microscopy, Hefendehl et al. (2014) found that cortical microglia in the aged murine brain are abnormal, exhibiting increased soma size and decreased process length (Hefendehl et al., 2014). These characteristics are thought to result in a decrease in the ability of microglia to surveil the brain parenchyma and respond to perturbations of homeostasis. Using unbiased stereology, Mouton et al. (2002) found that microglia number in the dentate gyrus and CA1 increased with age in female C57 mice; females also exhibited greater numbers of microglia regardless of age (Mouton et al., 2002). Other studies have found no late aging- or sex-differences in microglia number in the hippocampus (Kohman et al., 2013; Khan et al., 2015). Despite a rich literature examining microglia number and morphology in late aging, no studies have examined microglia specifically in CNS structures that play an established role in social behavior regulation.

The neural circuitry governing social behavior is well established in younger animals and is referred to as the social behavior neural network (SBNN) or social salience network (SSN) (Freeman and Young, 2016; Johnson et al., 2017; Johnson and Young, 2017). Oxytocin (OT), a neuropeptide heavily involved in social behavior, is produced in the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON). OT receptor is expressed in the prefrontal cortex, nucleus accumbens, bed nucleus of the stria terminalis (BNST), hippocampus (HPC), and medial amygdala (MEA) in the rodent brain (Knobloch and Grinevich, 2014; Freeman and Young, 2016). The PVN receives substantial brainstem inputs and as such is a key site of integration of ascending signals and the neuroendocrine responses to stress (Pacak and Palkovits, 2001; Myers et al., 2017). The BNST is involved in social recognition (Dumais et al., 2016b) and exhibits sex-specific patterns of immediate early gene (c-Fos) activation following a social experience (Perkins et al., 2017). The amygdala has been implicated in social information processing in humans (Adolphs, 2003) and the MEA specifically exhibits induction of c-Fos in response to social interaction in adult rats that is blunted in aged rats (Salchner et al., 2004). Thus, these three regions (PVN, BNST, and MEA) were chosen to assess potential age and sex differences in microglia morphology that may relate to social behavior. The HPC was assessed since age-related changes in neuroimmune function have been extensively characterized in this structure, including examination of microglia morphology (Mouton et al., 2002; Khan et al., 2015) and age-related microglia priming (Frank et al., 2010b; Huang et al., 2008; Wynne et al., 2010).

Given the strong ability for illness and inflammation-related events to suppress social behavior, we reasoned that late aging-associated reductions in social behavior might be a result of increased number and/or altered activational states of resident microglia within these socially relevant nuclei. Thus, to examine age-related changes in microglia, we used immunohistochemistry and unbiased stereology to estimate total microglia number, average area (μm^2), and average volume (μm^3) in three socially relevant brain structures: BNST, PVN, and MEA in male and female F344 rats of different ages. Microglia were also assessed within the HPC as a referent site to help contextualize measures of morphology within CNS sites more critically involved in social behavior regulation, and because the extant literature includes numerous assessments of microglia in this structure.

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