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Behavioral and Physiological Consequences of Microembolic Stroke

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Behavioral and Physiological Consequences of Microembolic Stroke

By

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B.A., Hamilton College, 2006

Advisor: Gretchen N. Neigh, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Neuroscience
2014

Abstract

Behavioral and Physiological Consequences of Microembolic Stroke
By Christina L. Nemeth

An estimated 15% of the population over age 65 suffers from some form of depression and the risk of depression increases with age. The already multifarious nature of depressive disorders makes it especially difficult to diagnose and treat in the elderly due to pre-existing health conditions, related medications, and concurrent anhedonia associated with aging in many individuals. Silent cerebral infarction, or microembolic stroke, stems from arteriosclerotic risk factors and occurs frequently within the general population. Microembolic stroke correlates highly with the manifestation of depression and cognitive decline. To explore this relationship, we used a microsphere embolism model to induce behavioral disruption in adult rats in order to study the mechanisms of acute ischemic damage. We found that while the degree or location of microvascular injury did not correlate with altered behavior, ME procedures did lead to the increased expression of several inflammatory markers at a time-point that correlates with behavior. Inhibition of inflammatory activity via the use of specific and generalized anti-inflammatory therapeutics reverses behavioral deficits; however, timing of pharmacological intervention, with respect to injury, is important. These findings shed light on a common, but under-appreciated, model of depression and suggest that depression and cognitive dysfunction of a vascular origin may be ameliorated by careful control of cardiovascular risk factors and by anti-inflammatory therapeutics.

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Acknowledgments

The chapters that follow describe years of hard work done through collaboration, meetings, and teamwork. First and foremost, Dr. Neigh has provided me with the utmost professional and personal support to persevere when life argues otherwise. Her best interests were always mine and her own dedication and commitment to her career and family will serve as a model for my own. Special thanks to my committee, Dr. Kerry Ressler, Dr. Malú Tansey, Dr. Lary Walker, and Dr. Mark Wilson, as their guidance and enthusiasm over many years sometimes fueled my work. Within the lab, Emily Hardy, Sean Kelly, and Constance Harrell were dedicated lab partners, reliable hands, solid sounding boards, comic relief, and most importantly, good friends to work with on a daily basis. Dr. CJ Barnum, Dr. David Gutman, Dr. Ebrahim Haroon, and Dr. Andrew Miller have also provided project guidance and contributed to my overall knowledge and understanding of both the science and the scientific process. Many thanks to my classmates for the nurturing environment that is Emory Neuroscience; I am blessed to have been able to find life-long friends during this journey and for their friendship I am grateful. Thanks to my family, especially Steve, whose jokes will never cease to improve my day, your own hard work and quest for knowledge has been an inspiration to me. Joe, your support, optimism, confidence, and hilarity have been sources of my strength through this first of many adventures. Lastly, to my mom and dad, the respective inspiration and motivation to my life: thank you for the means to get here, I only wish I didn't have to take the journey without you.

Of course, a special recognition of the rats who help to advance our understanding of health and disease every day.

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List of Figures and Tables

CHAPTER ONE

HEARTSICK: PSYCHIATRIC IMPLICATIONS OF CEREBROMICROVASCULAR DISEASE

Adapted from: *International Journal of Geriatric Psychiatry*
Nemeth CL, Haroon E, Neigh GN
2014 Jun;29(6):577-85; DOI: 10.1002/gps.4046

Cerebral Microvascular Disease: An Epidemic

Every 25 seconds one American has a coronary event, every 40 seconds one American has a stroke, and every minute one American dies from a cardiovascular event (Roger et al., Circulation 2012). These statistics, while profound and startling, are only the tip of the iceberg when considering the number of people afflicted with diseases of the cardiovascular system. When focused on cardiovascular disease specific only to the central nervous system (cerebromicrovascular disease; CMVD), reports indicate that as much as 87% of the population over age 65 is affected (Wong et al. 2002). Physical changes associated with CMVD include thickening of arterial walls, microvascular lesions, and microembolic strokes (Chen et al., 2010; Farkas & Luiten, 2001; Vermeer et al., 2007). CMVD is linked to a greater risk of ischemic stroke (Chen et al. 2010), an increased risk of depression (Kales et al., 2005; Santos et al., 2009),

and an increased incidence of mild-cognitive impairment (Grau-Olivares et al. 2009), dementia (Knopman 2007), and Alzheimer's disease (Farkas et al. 2001; Purandare et al. 2012).

A Succinct Review of Blood Vessels in the Body

The peripheral vasculature is a dynamic organ system designed to deliver oxygen and nutrients to the body while maintaining a homeostatic state. The dynamic nature of the peripheral vascular system is best illustrated by its vast design: blood vessels range in size from the largest vessels (2-3 cm) to the smallest capillary (5-10 μm), a diameter so small that blood cells must flow single file (Iaizzo 2005); when stretched end to end these vessels cover over 100,000 kilometers (Anitei 2007). Considering the body's density of blood vessels, it is no surprise that the integrity of blood vessels critically important to overall health. Atherosclerosis (or the build-up of plaques in blood vessels) is the leading cause of death in the Western hemisphere. Non-modifiable risk factors of atherosclerosis include sex, age, and genetics, while modifiable risk factors include hypertension, obesity, and smoking, to name a few. The risk factors that facilitate hypertension and the formation of plaques in peripheral vascular diseases and myocardial infarction are synonymous with risk factors that contribute to cerebral vessel disease both at the macro- and micro-vessel scale (Figure 1.1). While great strides have been made to educate the population on the importance of "heart health" and the effects of poor cardiovascular lifestyles on the body, the effects of poor cardiovascular lifestyles are equally as dangerous to the brain.

Cerebral (Macro) Vasculature

Stroke is a leading cause of death and the greatest cause of disability in the United States (Chen et al. 2010; Li et al. 2011) and occurs by the same processes that lead to blockages within

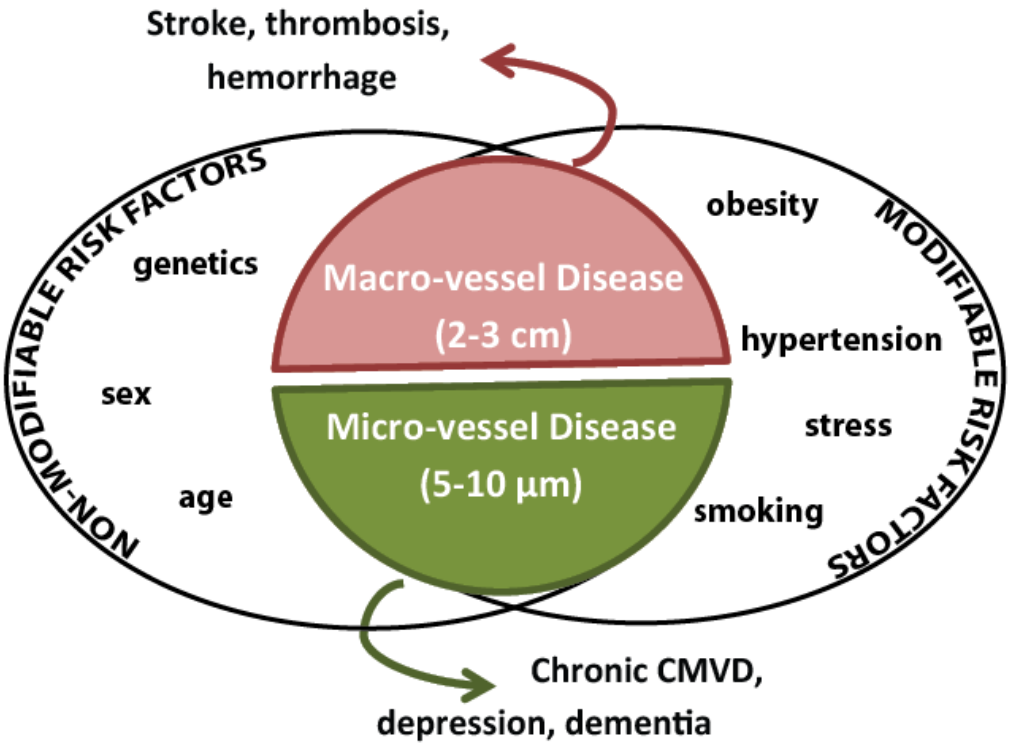

Figure Error! No text of specified style in document.**1.1 Cerebrovascular disease risk factors**

Modifiable and non-modifiable risk factors contribute to the manifestation of vessel disease at the macro and micro scale. Chronic microvessel disease in the brain can lead to vascular depression and dementia, while macrovessel disease can lead to more pronounced stroke, thrombosis, or hemorrhage.

vessels of the body. In short, a vessel carrying blood within the brain becomes blocked, or a clot ruptures, preventing the flow of vital oxygen and nutrients to brain tissue. Additional tissue injury can occur following reperfusion of the blocked artery due to a shift of the original clot or collateralization, or via the production of reactive oxygen species and the activation of damaging inflammatory pathways that promote cell and tissue death (Wang et al. 2007). Stroke damage in the brain is particularly detrimental as the amassment of necrotic tissue diminishes functionality in the corresponding brain region. Blockage of cerebral small vessels also occurs, and though the damage incurred from each clot is less catastrophic, the accumulation of damage over time can be as devastating as a large scale occlusion. To illustrate these consequences, Figure 1.2 shows neurological deficits that result from region-specific large vessel and small vessel (discussed in the next section) ischemia.

Though the same atherosclerotic processes can occur in every large vessel of the body, the pathologies manifest quite differently, and the repercussions are wide-spread. For example, peripheral vascular events first manifest as pain such as in peripheral artery disease or preceding a heart attack, while macrovascular events in the brain are quite pronounced and functionally devastating. In contrast, microvascular diseases are associated with much more subtle effects, manifesting as behavioral and cognitive changes. These behavioral changes, often considered to be a consequence of old age, have a biological basis and can be hindered by early awareness and prevention.

Cerebral Microvasculature

Microvascular disease most often stems from arteriolosclerotic mechanisms arising from common vascular risk factors such as age, hypertension, glucose-intolerance, atrial fibrillation, and cardiovascular disease (Lee et al. 2000; Kales et al. 2005). Microvascular disease first

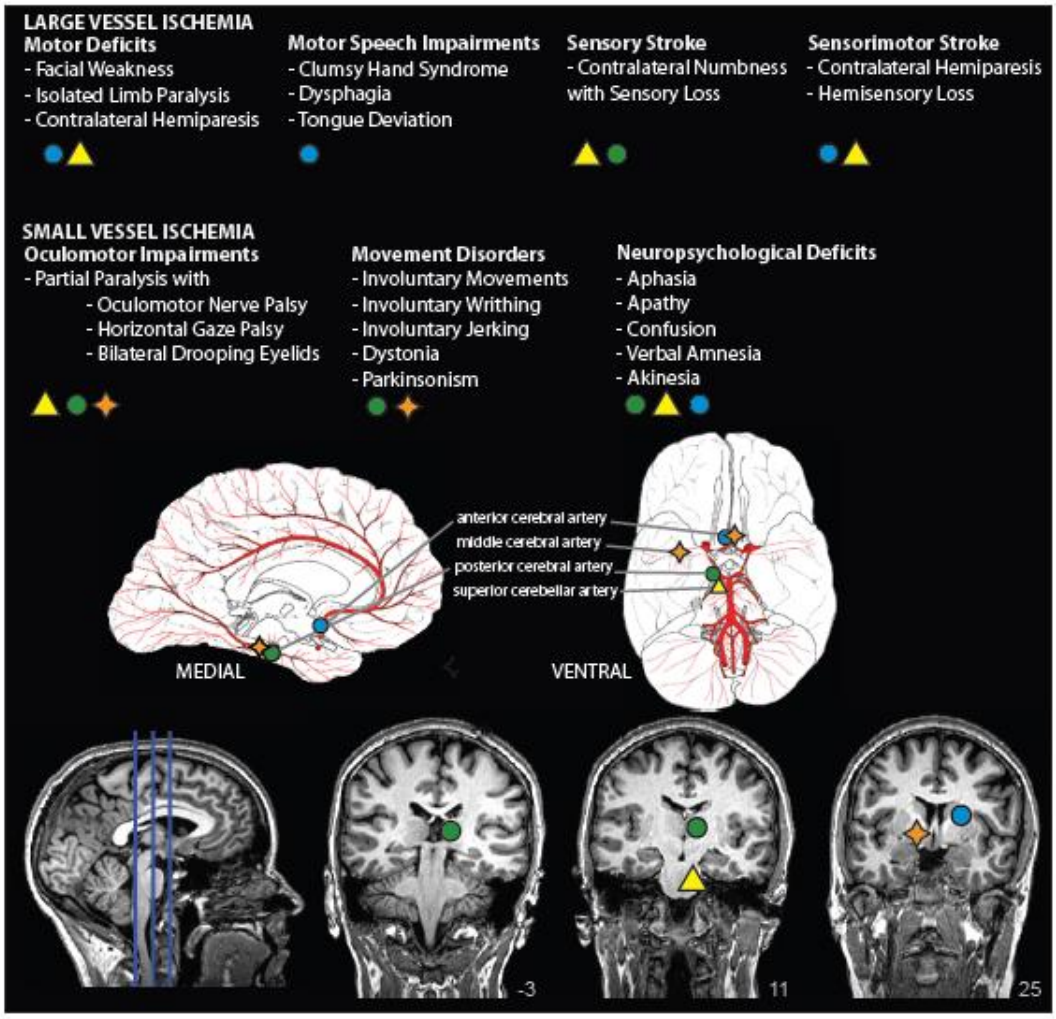

Figure 1.2 Functional correlates of vascular injury

Shaded symbols under each clinical deficit (top) correspond to location of possible vessel occlusion (center) and region of neurologic damage in coronal brain sections (bottom). Images standardized to Talairach atlas.

affects the small penetrating arteries that largely lack distal collateralization (Ringelstein et al. 2005) leaving them more susceptible to injury. Though both the human and rodent brains have been noted for their remarkable tolerance to embolization in larger vessels because of collateralization, occlusion is not limited to larger vessels but also affects arterioles, venules, and capillaries. The arterioles most commonly implicated in CMVD feed much of the deep subcortical matter, and thus lesions are most frequently observed in the gray matter, basal ganglia, brain stem, and areas of the brain that are the most highly vascularized (Feil et al. 1999; del Zoppo et al. 2003). Similarly, ischemia of these penetrating capillaries most often leads to disruption of white matter tracts (Feil et al. 1999).

Due to the nature of microvascular diseases, clinically assessing and diagnosing the associated pathologies has proved challenging. Microvascular lesions in the brain are often incidentally detected following a larger ischemic event or during post-mortem brain analysis. In the clinic, microvascular lesions are best detected via magnetic resonance imaging (MRI) rather than computed tomography (CT) due to the greater sensitivity of MRI to such small events (Vermeer et al. 2007). Not surprisingly, variations in imaging availability and techniques among hospitals make categorization and diagnosis of microvascular events difficult and often unreliable. Adding to the difficulty, individual microvascular lesions confer no overt phenotype, hence these "silent" lesions progress via subtle injury until the accumulation of lesions manifests as a more serious event or behavioral disruption (del Zoppo et al. 2010). As a result, defining the CMVD patient population has been one of the most challenging aspects of approaching the disease in the clinic, complicating treatment strategies (Sneed et al. 2008).

Vascular-Induced Behavioral Disruption: Evolution of the Vascular Depression Hypothesis

Over 100 years ago, Gaupp first described "arteriosclerotic depressive states" and the

increased prevalence and severity of depression in the aged (Gaupp 2000). Since then, the relationship between depression and small vessel pathology has been revisited and redefined, with the conception of the "vascular depression hypothesis" which posits that the formation of vascular lesions precipitates changes in mood and cognitive functioning (Fujikawa et al. 1993; Alexopoulos et al. 1997; Alexopoulos 2006). About a decade later, the theory developed further to suggest that the presence of minute lesions in the brain is associated with the acceleration of late life depression and cognitive decline, as well as the progression and severity of Alzheimer's disease (3; 7; 8; 27; 37). Within this theory, the manifestation of vascular induced depression exists along a continuum (Figure 1.3) with vascular risk factors (highlighted in red) precipitating peripheral and central symptoms of disease (green) and resulting in a myriad of disease states (blue). Because work in humans precludes the determination of causality within these relationships, clinical assessment of CMVD and associated behavioral disruptions are still largely based on correlation. Despite this, there are well-established links between vascular risk factors and geriatric disease states in the clinic, and the use of appropriate animal models can accelerate both the mechanistic understanding and treatment approach.

Vascular-Induced Behavioral Disruption: Mechanisms

Microembolic lesions in the aged brain cause chronic hypoperfusion in white matter areas that are largely infiltrated by the small vessels (Román 2002). Over time this accumulation leads to changes in cerebral blood flow, and affects metabolism, neuronal regulation, and cerebrovasculature (Margolis et al. 1985). Though some hypothesize that specific disruption to the caudate nucleus, basal ganglia, and thalamus manifest as the apathy and mood changes cited in vascular depression (Román 2002), the mediating factors to these lesions remain a mystery.

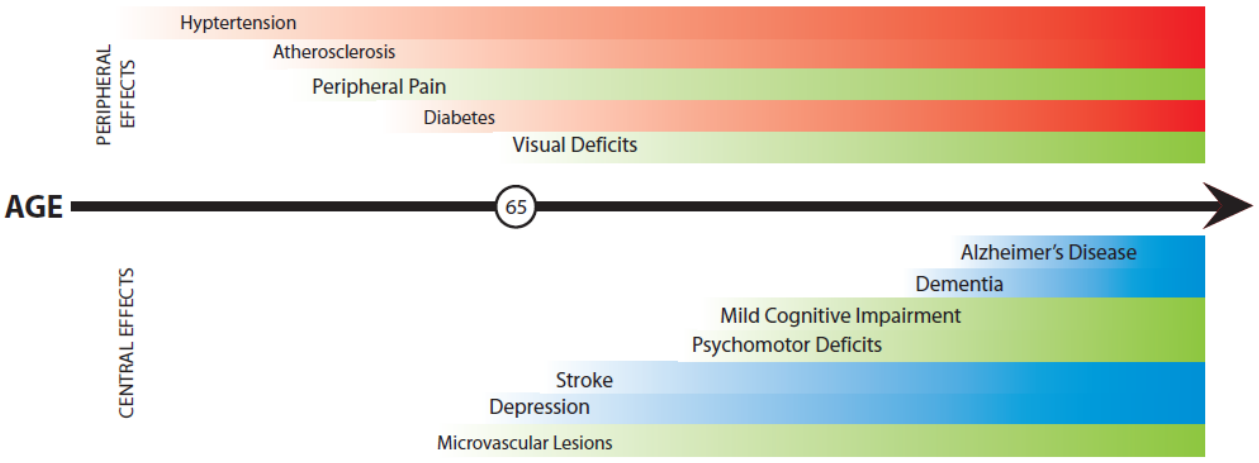

Figure 1.3 Timeline of vascular disruption

The emergence of vascular disruption is slow and progressive and begins later in life. The presence of risk factors (highlighted in red) facilitates peripheral and central symptoms (green) of vascular disease. Over time, the formation of microvascular lesions and other deficits further perpetuate the likelihood of ischemic events and the manifestation of diseases (blue) such as depression and Alzheimer's disease.

Many systems are affected by the slow progression of vascular pathologies, and accordingly, several mechanisms may be in play: structural factors, influence of the HPA axis, and neurochemical activation.

Structural:

As early as the 1980s, associations have been made between lesion location following focal stroke and the severity of post-stroke depression. Robinson and colleagues (1984) proposed that depressive-symptoms correlate with the distance of the lesion to the frontal pole during the acute post-stroke period. Similarly, other groups have found that lesion laterality plays a large role in the manifestation of depression (Robinson & Coyle, 1980; Tupler et al., 2002). Many of the theories regarding lesion laterality and location have been debated (Shimoda et al. 1999; Carson et al. 2000); however, the correlation between brain vasculature, late life depression, and dementia remains strong (Baldwin 2002).

Hypothalamic Pituitary Adrenal Axis Interactions:

The hypothalamic-pituitary-adrenal (HPA) axis, a tonically active system that mediates the body's response to stressors, is hyperactive following an ischemic event. Levels of corticotropin-releasing factor and corticosterone are elevated in the brain and periphery following stroke-like events (Milot et al. 2011) and cardiac arrest (Neigh, Karelina, Zhang, et al. 2009). These changes are likely related to damage to specific brain regions such as the hippocampus, which leads to disruption of the negative feedback loop of the HPA axis and ultimately behavior (Milot et al. 2011; O'Connor et al. 2000; Olsson et al. 1992). Over time, this chronic dysfunction can contribute to reproductive and immune issues (Maccari et al. 2007), as well as increased susceptibility and incidence of depressive and anxiety disorders (Holsboer 2001).

Neurochemical Activation:

Parallel to the activation of the HPA axis, ischemia is characterized by the interruption of neurotransmission, an effect observed in both humans (Fang et al. 2009) and rodents (Hayashi et al. 1998). It is hypothesized that during certain forms of ischemia, ascending biogenic amine-containing axons become disrupted, thus decreasing the overall production of neurotransmitters implicated in mood, such as norepinephrine and serotonin (Fang et al. 2009). Additionally, increased enzyme activity leads to the production of indoleamine 2,3-dioxygenase (IDO) which decreases the overall availability of tryptophan, again leading to deficits in serotonin (Spalletta et al. 2006). Regardless, the exact roles and degrees of influence of HPA activity and neurotransmission in microvascular-induced behavioral disruption are still unclear.

Excitotoxicity accounts for a significant portion of necrosis following ischemia and may contribute to disrupted mood. Hypoxic conditions trigger dysfunction of ATP production, cell depolarization, and a subsequent influx of calcium ions leading to glutamate release and AMPA and NMDA receptor activation. The perpetuation of this cycle by continued glutamate release and activation of these receptors begets excitotoxicity, cell membrane and mitochondrial dysfunction, and apoptosis. Thus, attempts to abate excitotoxicity following ischemia is a target of research, and has been shown preclinically to reduce experimental lesion area; however, these successes have not translated well to the clinic (Chang et al. 2012) because many models of focal ischemic insult do not accurately mimic human stroke (Macrae 2011). Alternative pharmacological attempts have focused on the role of enhanced astrocyte mitochondrial metabolism to control lesion size. Enhanced calcium signaling (via IP_3-mediated pathways, including through stimulation of G-protein coupled purinergic receptors) promotes post-ischemic cell survival by increasing cellular resistance to oxidative stress (Wu et al. 2007; Zheng et al.

2010). Agonists targeting purinergic receptors have shown neuroprotective promise by enhancing mitochondrial metabolism and reducing cellular edema, cell death, and infarct size (Zheng et al. 2010; Wu et al. 2007). Adenosine agonists have also been explored as novel agents to reduce inflammation and injury following experimental ischemia (Choi et al. 2011). Such therapies, however, have been associated with severe side effects, and their value in the clinic remains under consideration.

Vascular-Induced Behavioral Disruption: An Inflammatory Disease?

A theory that has gained popularity in the last decade suggests that neuroinflammation contributes to the manifestation of depression following brain ischemia (Spalletta et al. 2006; Fang et al. 2009; Hakim 2011). Neuroinflammation, specifically through the activity of pro-inflammatory cytokines, is triggered following brain injury and participates in cell death, tissue damage, and the perpetuation of the inflammatory response (Lakhan et al. 2009; Valente et al. 2009). While neuroinflammation is intricately linked to both the HPA axis and neurotransmission, inflammation, alone, is sufficient to alter mood. Recent reports have shown that clinically depressed patients have increased elevated levels of inflammatory cytokines, including monocyte chemoattractant protein 1 (MCP1), a marker implicated in stroke and other vascular events (Miller 2010). Furthermore, cytokine therapy induces sickness and depressive-like behavior in rodents (Song et al. 2011), as well as in humans (Raison et al. 2006). Moreover, the administration of anti-inflammatory drugs (including a specific tumor necrosis factor alpha [TNF] inhibitor, infliximab (Raison et al. 2012)) may relieve symptoms of depression while anti-depressants have the same attenuating effect on inflammatory activity (Raison et al. 2006). These strategies will be discussed in Chapters 7 and 8.

In experimental stroke, ischemia leads to increased production of inflammatory cytokines

(Maddahi et al. 2010), a process involved in the manifestation of behavioral disturbances (Craft et al. 2006). Similar acute and prolonged inflammatory responses occur in humans (Spalletta et al. 2006; Kriz et al. 2009), and once initiated contribute to changes in nitric oxide bioavailability, neuronal and vascular injury, and damage to the blood-brain barrier (Hakim 2011). In cases of CMVD, and dissimilar to stroke, the damage is perpetual, and thus so are body and brain responses, leading to long-term activation of these pleiotropic processes and the accumulation of significant damage over time. The aged brain, in particular, provides a suitable host for ischemic events as normal aging is accompanied by chronic low-grade inflammation and an altered cellular response to ischemic events (Popa-Wagner et al., 2007).

Recent manipulations using rodent vessel preparations have demonstrated the reactivity of brain vasculature to inflammatory agents. In such a vessel preparation, exogenous administration of recombinant TNF increased release of iNOS, NF-κB, and induced cell death, and endothelial dysfunction (Csiszar et al. 2007). In aged animals, inactivation of TNF improved vascular responses, reduced endothelial oxidative stress, and decreased DNA fragmentation, illustrating the potency of TNF in age-related changes to vasculature (Csiszar et al. 2007). Imaging has also been explored as a method to better understand atherosclerosis in waking animals. MRI in combination with PET scanning is currently being used to identify and track the distribution of macrophages within vessels (Jarrett et al. 2010). Macrophages, capable of releasing cytokines and other factors that promote atherosclerotic plaque rupture, are densely packed in highly unstable areas of vasculature. The ability to visualize and track inflammatory activity within vessels *in vivo* allows researchers, and even clinicians, to identify at-risk patients and predict areas vulnerable to vessel rupture. Moreover, as part of clinical research paradigms, vascular inflammation models are employed as a method to examine vascular reactivity in pre-

existing systemic inflammation. Patients with small vessel vasculitis had increased levels of inflammatory cytokines as well as greater levels of arterial stiffness as compared to age-matched healthy control subjects (Booth et al. 2004). Arterial stiffness, recognized as a risk factor for CMVD, appears directly correlated to levels of systemic inflammation and is reduced by anti-inflammatory treatments (Booth et al. 2004). Taken together, both rodent and clinical models support the direct involvement of inflammatory mediators in the reactivity and tone of central and peripheral vasculature. Taken one step further, the independent involvement of inflammation with mood systems in combination with its effects on vessel health provide a strong case for the need to approach age-related vascular disease with an eye on inflammation.

Vascular-Induced Behavioral Disruption: In the Lab

From the most basic perspective, translational research relies on the ability to predict preclinical and clinical therapies. Rodent models are a physiologically and ethologically relevant method to better examine the relationship between vascular inflammation and functional outcome. A range of species are used as animal models to cover a range of pathologies in an attempt to better understand the biological basis of disease. From these models research scientists can determine the genes, endophenotypes, and pathophysiologies of diseases that are then interpreted to form diagnoses and interventions.

It was recognized more than 10 years ago that current animal models of ischemia recapitulate fewer than a quarter of human stroke events (Small et al. 2000). Still, rodent models of ischemia have shed light on the ischemic cascade and recovery from stroke. Since that time, modifications of ischemic models have allowed for the refinement and exact study of various mechanisms of stroke and brain repair, simulating the variation observed in the clinic. For example, to better understand the effects of vascular burden on behavior, microsphere-induced

embolic models accurately mimic the physical presentation of lesions observed in human CMVD. Compared to permanent focal occlusion or global models of stroke, microsphere induction models provide a more accurate representation of the clinical situation (Mayzel-Oreg et al. 2004; Small et al. 2000), though they are used less commonly. Though microembolism models are considered variable in the sense that the location of the clots are difficult to control, several groups have lauded the model because the dose and size of injected microspheres can be easily regulated to provide reproducible and clinically-relevant results (Zhu et al. 2012; Mayzel-Oreg et al. 2004; Hossmann 1998). Microsphere-induced embolic models have been used extensively to study several parameters from cerebral blood flow and brain metabolism, to activity and concentration of specific neurochemicals.

The experiments described within this document utilize a microsphere embolism model to generate acute ischemic infarcts in the rodent brain. Further, these experiments were designed to simulate small cerebral infarcts which occur regularly in the human brain as a result of arteriosclerotic processes. The location of damage and subtle symptom presentation in the rodent model resembles the histological and behavioral manifestation of silent infarction observed in the clinic. For example, and as described fully in the next chapter, the generation of diffuse lesions in the rat brain results in the manifestation of depressive- and anxiety-like behaviors (Nemeth et al. 2012). Interestingly, and similar to what is observed in cases of vascular depression and vascular dementia, depression precedes cognitive deficits in these rodents (Barnes et al. 2012; Nemeth et al. 2012). Expansions upon these initial findings, including pharmacological interventions, will be described in full throughout the document.

Vascular-Induced Behavioral Disruption: In the Clinic

Embolization or occlusion at different levels of vasculature has varying histological

consequences and pathological outcomes (Zhu et al. 2012; Ringelstein et al. 2005). For example, the penetrating arterioles most commonly implicated in CMVD feed much of the deep subcortical matter, and thus lesions are most frequently observed in the gray matter, basal ganglia, brain stem, and areas of the brain that are the most highly vascularized (Feil et al. 1999; del Zoppo et al. 2003). Similarly, ischemia of capillaries most often leads to disruption of white matter tracts (Feil et al. 1999). Due to the nature of microvascular diseases, however, clinically assessing and diagnosing the associated pathologies has proven challenging. Microvascular lesions in the brain are often incidentally detected following a larger ischemic event or during post-mortem analysis of the brain. Adding to the difficulty of identification and diagnosis, individual microvascular lesions confer no overt phenotype, hence these "silent" lesions progress via subtle injury until the accumulation of lesions manifests as a more serious event or behavioral disruption (del Zoppo et al. 2010). In this vein, and given that a strikingly high proportion of individuals with microvascular lesions present with depression (Fujikawa et al. 1993), depression should be recognized as a potential behavioral manifestation of underlying CMVD and the patient should be referred for cardiovascular assessment before the condition progresses further.

Other potential methods to confirm the presence of small vessel disease, such as imaging techniques, are complex; hallmark white matter intensities are not unique to depression and are in fact observed in bipolar disorder and schizophrenia (Kempton et al. 2011). Furthermore, the time and cost of such procedures far outweigh the benefit in diagnoses in the absence of stroke and other neurological disorders. Even without visual confirmation, the vascular nature of late-life depression is accepted as heuristically valid based on thoughtful analyses (Alexopoulos et al., 1999; Blazer & Hybels, 2005).

No neuropathological studies have directly tested the causality of CMVD and depression in humans, though evidence of atherosclerosis has been found in cases of late life depression (Thomas et al. 2001). The presentation of depression late in life is unique from clinical depression diagnosed earlier in life in that it is characteristically non-responsive to anti-depressant treatments (Baldwin 2002), shows no pattern of inheritance (Kales et al. 2005; Blazer et al. 2005), and is accompanied by increased motor impairments (Kales et al. 2005; Chen et al. 2010). Due to the prevalence of CMVD in the elderly population, and the prevalence of depression alongside other vascular diseases, most cases of late life depression are considered vascular in etiology. Further, because of the growing relationship between vascular-based depression and dementia, and the identification of depression as a prodrome of dementia, depression is treated in an attempt to stave off cognitive impairment. Indeed, control of additional vascular risk factors becomes important to slow the progression of symptoms and the development of cognitive impairment (Sun et al. 2008). Despite the frequency of vascular-induced depression diagnoses in the clinic, treatments are not targeted towards the disease pathology (due to aforementioned difficulties in diagnosis) but rather parallel treatment strategies of classic depression.

The presence of white matter lesions in the brains of patients with late life depression suggests not only a vascular origin for behavioral disturbance, but also that the progression of this subtype of depression may be preventable and even treatable (Thomas et al. 2001). Lesions associated with CMVD are considered predictors of both treatment-resistance, poor outcome, and death (Thomas et al. 2001; Santos, Gold, et al. 2009). First, appropriate control of risk factors forestalls disease development. Second, with the determination of disease mechanism comes the opportunity for target-specific therapeutics. The timely approach to CMVD is

important as the progression of CMVD or the development of comorbid disease states, such as Alzheimer's disease, parallels greater dysfunction and is associated with more rapid functional decline (Dempsey et al., 2010; Grau-Olivares & Arboix, 2009; Purandare et al., 2012).

Vascular-Induced Behavioral Disruption: Call for Translation

The transition from science in a laboratory to treatment in a clinic is an ambitious task that requires a critical re-evaluation of the research model. The scientific process must expand to focus beyond the hypothesis, experiment, and analysis of data, to include a method in which application is a required and indispensable step. Expansion of the classic translational research model to include steps beyond T1 (transfer of new laboratory methods into new clinical methods of diagnosis, therapy, and prevention) and T2 (transfer of clinical trial results to new daily clinical practice), are dubbed T3 and T4. In 2007, Westfall and colleagues proposed T3 to include changes to daily clinical practice encompassing these new research beliefs (Westfall et al. 2007). Furthermore, within this stage of research, scientists learn new ways to employ laboratory findings in real-world settings. Finally, T4 was proposed to challenge the daily lives of people outside of the clinic: to impose community-wide alterations of both health habits and public health perception (Woolf 2008), and to use outcome studies to determine the effect of treatment implementation.

Vascular depression is no exception, for efficient translation to the clinic will require an emphasis on the use of an appropriate model; one that incorporates accurate preclinical models, appropriate analyses and interpretation, and efficient adaptation to a clinical setting. Accurate preclinical models are necessary because relevant clinical data are lacking, and because appropriate behavioral endpoints cannot be achieved due to variability in clinical samples. Furthermore, clinical data from suspected cases of vascular-induced late-life depression are

correlative and often retrospective, precluding the ability to create testable hypotheses. With this in mind, accurate preclinical models should place greater emphasis on the clinical trajectory of vascular disruption, focusing on motor impairment, behavioral and cognitive disruption, and premature death.

Steps have been taken in the clinic to address the relationship between inflammatory activity and depressive states, though little progress has been made in terms of treatment. Various preclinical studies have highlighted the anti-depressant properties of non-steroidal anti-inflammatory drugs (NSAIDS) and cyclooxygenase-2 (COX-2) inhibitors (Hayley, 2011; Müller et al., 2011). NSAIDS and COX-2 inhibitors are typically used in conjunction with classic anti-depressants, though the exact therapeutic mechanisms of anti-inflammatories remain unclear (Hayley 2011). As of 2009, fewer than 10% of drugs designed to target inflammatory activity in rodent models continued to clinical trials (Lakhan et al. 2009) and even when a tested compound makes it to clinical trials, the short therapeutic window of ischemia introduces a confounding factor. In light of CMVD, this becomes a more serious issue because microembolic strokes occur without perception.

Summary

Despite the growing evidence of CMVD and its implications for depression, dementia, Alzheimer's disease, and mortality, few outside the field acknowledge silent infarction as a major contributor to cerebral decline. The often underappreciated role of cerebrovascular health in the normal functioning of the nervous system, mental health and cognition, is highlighted by the dearth of scientific articles regarding cerebrovascular health and behavior in journals outside the subspecialty of cardiovascular biology. Furthermore, the lack of appreciation for the role of cerebrovascular health in brain function and behavior may cause psychiatrists to miss important

behavioral harbingers of underlying cardiovascular disease. The chapters within this dissertation describe experiments conducted using a rodent model of diffuse microvascular damage and subsequent behavioral disruption to, 1) determine causality, 2) mechanism, and 3) alleviate behavioral consequences via the administration of target-specific approaches. The model described within the following chapters sheds light on the relationship between vascular disruption and behavioral changes. Furthermore, these findings suggest that the appropriate recognition of vascular risk factors and target-specific therapeutics can alleviate patient discomfort, reduce symptom severity and progression, and lessen the prevalence of this subset of depression.

CHAPTER TWO

MICROEMBOLISM INFARCTS LEAD TO DELAYED CHANGES IN AFFECTIVE-LIKE BEHAVIORS FOLLOWED BY SPATIAL MEMORY IMPAIRMENT

Adapted from: *Behavioral Brain Research*
Nemeth CL, Shurte MS, McTigue DM, Nemeroff CB, Neigh GN
Volume 234, Issue 2, 1 October 2012, Pages 259–266

Cerebral microvascular disease is common among the adult population and the incidence is increasing rapidly as the population ages (van Norden et al. 2011; Prins et al. 2005). Microvascular disease consists of minute vascular events including arterial wall thickening, microvascular lesions, and microembolic strokes (Grau-Olivares et al. 2009) which collectively lead to localized brain areas deprived of oxygen and nutrients. Due to the lack of overt pathophysiology, the minute vascular events precipitated by microvascular disease often remain undetected until a larger ischemic event occurs. Indeed, the presence of microvascular disease confers a greater than two-fold increase in the risk of a larger ischemic event (Vermeer et al. 2007).

In addition to the risk of overt infarction, changes in vasculature are now known to contribute to late-life depression (Alexopoulos et al. 1997; Baldwin 2002; Kales et al. 2005; Sheline et al. 2010) and the pathophysiology of Alzheimer's disease (Knopman 2007). An

estimated 94% of patients presenting with late-onset depression in the elderly harbor small lesions characteristic of microvascular disease (Fujikawa et al. 1993). Furthermore, microvascular disease has been associated with subtle deficits in motor and cognitive function, and microvascular lesions are correlated with the severity and rapidity of cognitive decline in Alzheimer's disease (Purandare et al. 2012; Vermeer et al. 2007). In an early report on microvascular lesions and depression in the clinic, Fujikawa et al. (1993) hypothesized that the behavioral changes accompanying microvascular events exist on a continuum, such that changes in affective behavior induced by microvascular events beget cognitive deficits and dementia. To date, this hypothesis has not been tested empirically. Despite this clinical evidence, the difficulty of temporally tracking microvascular events combined with the often delayed diagnosis of depression has made the relationship between behavioral changes and microvascular disease difficult to fully establish.

Rodent models of ischemia have provided insight into the neurobiological mechanisms which underlie changes in affective-like behaviors following middle cerebral artery occlusion and cardiac arrest and cardiopulmonary resuscitation (Neigh et al. 2004; Neigh et al. 2009; DeVries et al. 2001); however, the effects of minor, diffuse cerebral damage, resembling the magnitude of microvascular disease, on affective-like behavior have not been assessed. Previous research has shown that the injection of microbeads into the carotid artery of rats produces microvascular damage that recapitulates the cognitive deficits of microvascular disease (Craft et al. 2005; Miyake et al. 1993; Norio Takagi et al. 1997), but these studies did not examine the potential temporal relationship between deficits in affective-like behavior and cognitive deficits. Given that disturbances in affective-like behavior have been proposed to result from microvascular disease, the current study tests the hypothesis that diffuse microembolic (ME)

infarcts are sufficient to induce depressive and anxiety-like behaviors in a rodent model. To address this hypothesis we used the same ME infarct model which has successfully been used to demonstrate the role of ME in cognitive decline. Furthermore, we hypothesized that cognitive impairment would manifest subsequent to depressive-like behaviors and therefore spatial memory was assessed at short and long-term time-points after microembolism. Finally, the use of a rodent ME model facilitated an assessment of potential mediators of ME-induced changes in behavior.

MATERIALS AND METHODS

Animals and Surgery

Adult male Wistar rats (3 months of age, Charles River) were pair housed until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity-controlled vivarium. Throughout the duration of the study, animal caretakers provided *ad libitum* food and water. We performed all experiments in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Following a one week acclimation period, the rats were randomly assigned to sham or ME surgical groups. Rats received ME surgery as previously described (Craft et al. 2005). Briefly, animals received an anesthetizing dose of isoflurane, an incision was made and the common carotid artery was isolated and ligated with suture followed by ligation of the external carotid artery at the bifurcation with the internal carotid artery. The surgeon injected microspheres (New England Nuclear Inc., Boston, MA; 50 μm in diameter; suspended in 10% Dextran and 0.01% Tween in isotonic saline; PerkinElmer Instruments; Shelton, CT; ≈2500 spheres in 50 μL) with a 30 G needle inserted into the left internal carotid artery. Direct pressure

applied to the injection site facilitated the cessation of bleeding from the injection site, then ligatures were released and blood flow returned. Sham rats underwent an identical procedure without the infusion of microbeads. Animals recovered in their home cage prior to assessment of behavior.

Behavioral Testing

Three separate cohorts of rats underwent behavioral testing. For affective-like behaviors: one cohort was tested beginning 4 days following ME [Short Recovery (SR)] and a second cohort was assessed beginning 14 days following ME procedures [Long Recovery (LR)]. We assessed performance in a spatial memory task in the third cohort, and their training began 14 days after microembolism. Affective-like behavioral testing included the open field paradigm and the sucrose consumption test: sham (SR: n = 9; LR: n = 19) and ME (SR: n = 10; LR: n = 13). The SR group underwent open field testing on day 4 post-op and sucrose preference testing on post-operative day 6. The LR group underwent open field testing on day 14 post-op and sucrose preference testing on post-operative day 16. In addition, a subset of LR rats were assessed in the social interaction test to expand our characterization of depressive-like behaviors on post-operative day 17 (sham: n = 6; LR: n = 7). The social interaction task was not assessed in the SR groups because further expanding the testing protocol would have begun to exceed the short recovery time window.

The open field paradigm provided a validated assessment of anxiety-like behavior (Prut et al. 2003). We conducted the open field test within the first three hours of the animals' dark phase, videotaped the behavior and later scored the behavior with Cleversys, Inc. behavioral tracking software (Reston, VA). The open field apparatus consisted of an enclosed box (1 m x 1 m x 1 m). We interpreted time spent in the center (60 cm x 60 cm) versus the periphery of the

box as an index of exploratory anxiety (Prut et al. 2003). We used total distance traveled as an indicator of locomotor activity. The open field test lasted 10 minutes and started with placement of the rat in the center of the apparatus.

We used the sucrose consumption test as an index of depressive-like behavior such that decreased sucrose consumption reflects an anhedonic state (Willner et al. 1987). Following a one day habituation, we assessed sucrose consumption by recording total grams of each solution consumed (1% sucrose solution versus tap water) over a 24 hour period in the home cage.

The social interaction test was used as an additional metric of affective-like behavior in the LR group (File et al. 1978), 17 days after ME. The experimental rat was placed in the center of an enclosure (75m x 75m) containing an ovariectomized female as the stimulus animal. Interactions were videotaped and hand scored by an investigator blind to condition. Latency to first interaction (3 second limit of detection) and total time interacting were tabulated. Each session lasted 10 minutes.

Rats from the third cohort underwent spatial memory assessment in the Barnes Maze (sham n = 4; ME n = 5). We used a naïve cohort because activity in the Barnes Maze depends on the rats' innate dislike of open spaces, and previous testing in the open field could confound Barnes Maze results (Hölscher 1999). Two weeks following surgery rats began testing in the Barnes Maze. On days 1-4 of Barnes Maze testing, rats began in the start box with a 30 second delay and then explored freely. After the rat located the escape box, the entry was covered for 90 seconds. If the rats failed to find the escape box, they were placed in the box after 3 minutes where they remained for 90 seconds with the entry/exit covered. The second trial followed the same sequence, immediately following the first trial on the first day and with a five minute delay on days two-four. The fifth day of Barnes Maze exposure began with the same acquisition style

trial followed 5 minutes later by an evaluation trial in which the escape box was replaced with a fake box (similar to 19 other boxes). Evaluation of time spent in each area of the maze lasted 3 minutes. Data from the fifth day of Barnes Maze testing corresponded to 19 days after ME or sham surgery. In order to determine the long-term retention of the location of the escape box, rats were retested with an additional probe trial 14 days later, approximately 33 days after ME or sham surgery.

Volume Assessment and Analysis

Ischemic lesions undergo delayed expansion and although markers of neurodegeneration may be evident early after ME (Moxon-Emre et al. 2010; Lakhan et al. 2009), lesions are not fully developed for weeks following ME induction. Therefore, lesion volume was assessed only in the LR survival group, 17 days after surgery. Rats were rapidly decapitated and the brains removed. Brains were frozen on dry ice and stored at -80^{o}C until sectioning. Brains were sectioned by cryostat at a thickness of 50 µM and slices mounted on clear Superfrost Plus Microscope Slides (Fisher Brand Scientific, Pittsburgh, PA). The sections were stained with cresyl violet and cover-slipped with Permount (Fisher Brand Scientific, Pittsburgh, PA) prior to stereological assessment of lesion volume.

An unbiased stereological approach estimated mean lesion volumes of both the left and right hemispheres. Initial histological assessment revealed that ME-induced damage occurred in only subcortical structures of the brain; therefore we sampled the middle third of each brain with a high section sampling frequency (ssf = 1/6). A priori narrowing of the region of interest facilitated a more stringent assessment of the damage-susceptible areas; however, to ensure no major volumetric differences existed, we sampled half of the brains along their entire axis (n=8 per condition; selected randomly; ssf = 1/15). An ssf of 1/6 for experimental and sham brains

resulted in 16-19 sections for each hemisphere for total volume assessment. Surface area calculations employed the Cavalieri principle with point counting under a low power objective (2X) and thickness estimates used a high power objective (60X). Each sampling session began with a random start and all results were calculated within Stereologer 2000 software (Systems Planning and Analysis, Alexandria, VA). We maintained a coefficient of error at half or less than biological variability, approximately 10% (Gundersen et al. 1999). Mean hemispheric volumes were calculated for each group.

In order to calculate lesion volume, we subtracted ventricular volume of the control hemisphere from lesion plus ventricular volume of the experimental hemisphere. All asymmetrical cystic spaces with a diameter greater than 300 μm were considered lesions in the analysis. Sham hemispheres were subtracted from each other at random.

Immunohistochemical Staining

In addition to the behavioral cohorts, we generated an additional cohort for further histological analysis: amoeboid microglial cells (OX-42), activated macrophages (CD68), astrocyte activity (glial fibrillary acidic protein; GFAP), and neuronal death (Fluoro-Jade C) in sham (n = 3) and ME (n = 4) tissue for both the SR and LR groups. All animals were transcardially perfused with 4% paraformaldehyde, brains removed and stored at 4^{o}C until sectioning at 30 μM.

OX-42: Sections were double-labeled for myelin and axons using eriochrome cyanine (EC; Sigma-Aldrich, St. Louis, MO) and an anti-neurofilament (RT97; Developmental Studies Hybridoma Bank) antibody as previously described (Tripathi et al. 2008). Briefly, sections were mounted on Superfrost Plus Microscope Slides, pre-incubated in blocking solutions (PBS solution containing 4% bovine serum albumin, 0.1% Triton X-100 [Sigma-Aldrich]) followed by

treatment with 1:2000 mouse anti-neurofilament overnight at 4 ^{o}C. The next day, sections were rinsed and treated with secondary antibody for 1 hour at room temperature. Sections were rinsed and treated with 6% hydrogen peroxide in methanol, followed by exposure to the Elite avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) and visualized via a DAB solution (Vector Laboratories). Slides were rinsed, treated with acetone for 5 minutes, rinsed again and immersed in EC solution for 30 minutes. Sections were differentiated in 5% iron alum and completely differentiated in borax ferricyanide. Slides were dehydrated, cleared and coverslipped.

To label microglia and macrophages, sections were rinsed in PBS and placed in the same blocking serum as above. Sections were rinsed then treated with anti-CD11b primary antibody (1:1000; OX-42 clone, Serotec, Raleigh, NC) overnight at 4 ^{o}C. Sections were then rinsed and treated with secondary antibody for 1 hour at room temperature. Sections were rinsed, treated with hydrogen peroxide in methanol, followed by Elite ABC and DAB as above. Sections were rinsed, dehydrated and coverslipped with Permount.

CD68 and GFAP: Sections were incubated free-floating in blocking buffer (PBS solution containing 10% normal goat serum [Vector Laboratories], 0.4% Triton X-100) for 2 hours at room temperature in preparation for GFAP and CD68 staining. Blocking was followed by incubation in primary antibody for GFAP (mouse anti-GFAP, 1:500; BD Biosciences, Sparks, MD) or CD68 (mouse anti-CD68, 1:500; AbD Serotec) overnight at 4^{o}C. Sections were washed and incubated in secondary (biotinylated goat anti-mouse, 1:250; Vector Laboratories) for 2 hours at room temperature. Sections were washed again and exposed to ABC according to manufacturer's instructions. Following a wash, sections were incubated in a DAB solution for approximately 5 minutes. Sections were washed, mounted on Superfrost Plus Microscope Slides

and coverslipped.

Fluoro-Jade C: Sections was slide mounted for Fluoro-Jade C staining. Once dry, slides were incubated in an ethanol solution (1% sodium hydroxide in 80% ethanol) and rinsed in 70% ethanol followed by distilled water. Slides were incubated in a 0.06% potassium permanganate solution for 15 minutes, rinsed in distilled water for 2 minutes and immersed in a 0.0004% fluoro-jade C (Millipore, Billerica, MA) solution for 20 minutes. Sections were thoroughly rinsed, allowed to dry, cleared with xylene and coverslipped with Permount.

Histological Analysis

OX-42: The area of microglial/macrophage immunoreactivity was quantified in the ipsilateral and contralateral sides of brains from sham and ME rats. For this, microscopic images of OX-42 immunolabeled sections were digitized using image analysis software (MCID Elite, Imaging Research Inc., Canada). The ipsilateral and contralateral hemisphere of each animal was outlined separately and the area of OX-42 immunopositivity was divided by the sample area to provide the proportional area of tissue immunoreactive for OX-42.

CD68, GFAP and Fluoro-Jade C stains were visualized with a Nikon Eclipse 90i microscope (Melville, NY) fitted with Micro Bright Field Stereo Investigator 9.14.1 (Williston, VT). The Optical Fractionator probe was used to estimate the total number of GFAP+ cells and CD68+ macrophages within the CA1 and CA3 subfields of the hippocampus in both hemispheres with regions centered by the pyramidal cell layer. We chose to focus further assessments on the CA1 and CA3, subfields of the hippocampus that are especially susceptible to cell death following ischemia and regions where functional and/or morphological changes are observed in patients with depression (Ballmaier et al. 2008). Approximately 100 frames across six sections from each time-point were assessed for GFAP and CD68 reactivity in the CA1 and

CA3 regions of the hippocampus. For all, the sampling frame was set to 300 μm^2 and counting grid was set to 100 μm^2. Cells counts were estimated by the Optical Fractionator probe and expressed as the estimated number of cells per region. The investigator was blind to treatment group for all assessments.

To assess Fluoro-Jade C staining, a 20X image was taken of the CA1 and CA3 subfields in each hemisphere across 6 sections from each time-point with regions centered by the pyramidal cell layer. Each image was converted to an 8-bit file in Image J 1.44 (NIH) and the threshold was appropriately adjusted to reflect the staining in the original sample image. Particle values (size and circularity) were set to best capture Fluoro-Jade C tagged cells. The total number of counted cells were averaged by group and reported as the number of cells per mm^2 of tissue.

Data Analysis

All data were assessed for normality and equal variance prior to statistical analysis. Surgical weights were assessed using a two-way repeated measures ANOVA with condition (sham vs ME) and post-surgical day (SR vs LR) as factors. We assessed behavioral data from the sucrose preference test and the open field with a one-way ANOVA to compare sham, SR and LR ME groups. Behavior in the social interaction test was analyzed with a t-test. Acquisition in the Barnes Maze was assessed with a two-way repeated measures ANOVA across days. For assessment of probe trials, one-tailed t-tests compared sham and ME. Volumetric data from the LR cohort were examined by a two-tailed unpaired t-test to time-matched sham rats. Analysis of CD68, GFAP and Fluoro-Jade C cell counts employed a two-way repeated measures ANOVA with side (ipsilateral, contralateral) as the within-subjects factor and condition (sham, SR ME, LR ME) as the between-subjects factor. When appropriate, Bonferroni *post-hoc* tests were used

to further delineate group differences. Results were considered significant at $P < 0.05$. All results were calculated using GraphPad Prism 5 and are reported as mean ± standard error of the mean (SEM).

RESULTS

Microemboli Do Not Cause Gross Physical Impairment

Mean body mass for both ME groups decreased following the procedures; however, weights stabilized and terminal weights did not differ from sham controls in the SR ($p > 0.05$; sham: 457.9 ± 12.9 g; ME: 441.6 ± 11.1 g) or LR groups ($p > 0.05$; SHAM: 481.1 ± 13.0 g; ME: 464.9 ± 11.9 g). Assessment of locomotor activity in the open field confirmed that ME did not alter activity for either the SR or LR groups as compared to sham operated rats (4 or 14 days following surgery, respectively) (Figure 2.1A; $p > 0.05$). To rule out behavioral effects due to laterality, a subset of rats (sham n = 13; ME n=15) received microsphere surgery to the right internal carotid artery and we assessed their behavior after a two week recovery, consistent with the LR group. Behavior of right hemisphere ME animals did not differ from left ME animals on any metric and was therefore not included (data for right ME not shown; $p > 0.05$). Data from sham animals with right-sided arterial ligations did not differ from those with left-sided ligations and therefore these sham values were collapsed for all behavioral metrics.

Microemboli Induce a Delayed Increase in Anxiety-like Behaviors

Exploratory anxiety was assessed via time in the center of a novel open field 4 (SR group) or 14 (LR group) days following ME procedures. As stated above, ME did not alter overall activity in the open field at either time-point (Figure 2.1A; $p > 0.05$). Likewise, rearing, dips, and stretch attends did not differ among groups (data not shown; $p > 0.05$), suggesting overall locomotor ability was intact in both SR and LR groups. Central tendency did not differ

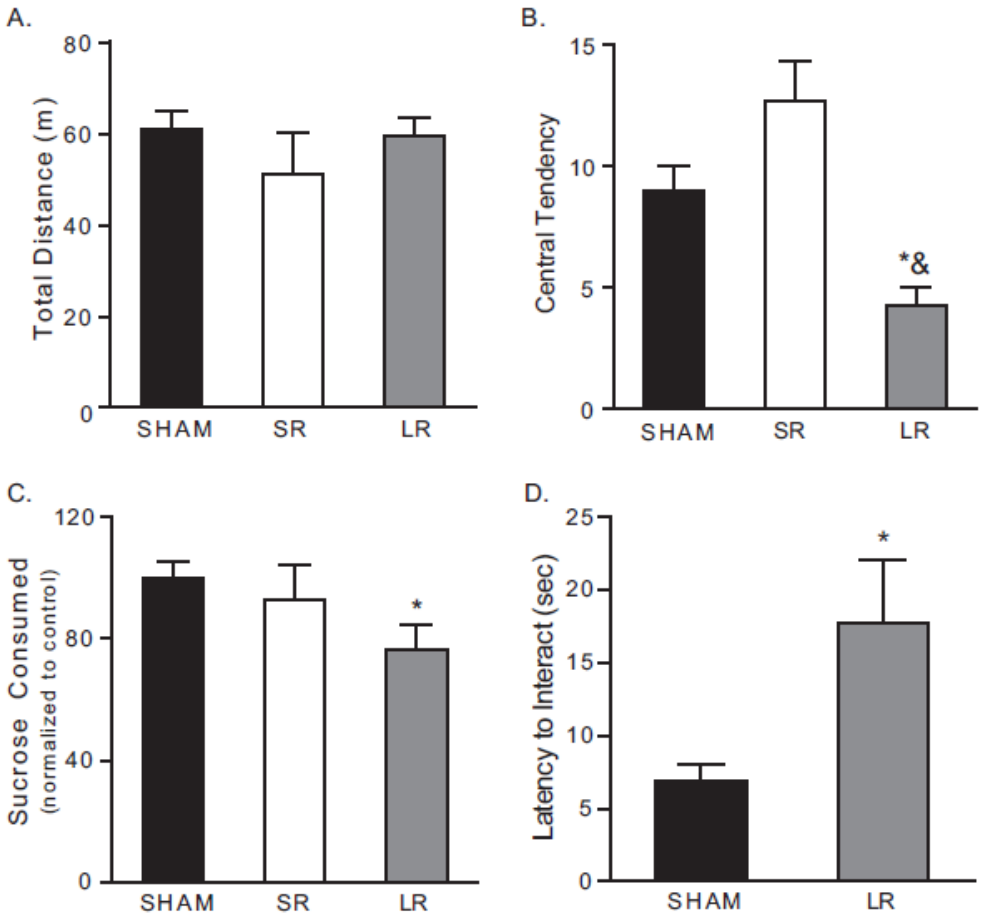

Figure 2.1 ME infarcts alter affective behavior after long-term recovery

Affective-like behavior was tested in sham and microembolism (ME) animals at short (SR; 4-6 days post-operative) and long-term (LR; 14-17 days post-operative) time-points. Anhedonia was assessed with a two bottle sucrose consumption test in the home cage while anxiety-like behavior was assessed in a ten minute open field test. Social interaction was assessed with an ovariectomized female. A*)* There was no effect of ME on distance traveled in the open field for either the SR or LR groups as compared to the sham group suggesting overall mobility was unaffected by ME infarcts ($p > 0.05$). *B)* Within two weeks of ME, there was no effect on central tendency in the open field, a measure of anxiety-like behavior, relative to sham (SR vs. sham, $p > 0.05$); however, rats in the LR group demonstrated impaired central tendency as compared to sham-operated rats indicating an increase in anxiety-like behavior at the later time-point (* indicates $p < 0.05$). C) Sucrose consumption did not differ between sham-operated rats and rats in the SR ME group ($p > 0.05$). Conversely, rats with ME in the LR group consumed less sucrose than sham operated rats, indicating a delayed manifestation of anhedonia (* indicates $p < 0.05$ from sham). D) Social interaction with an ovariectomized female demonstrated that rats with ME in the LR group had an increased latency to initiate social contact (* indicates $p < 0.05$). Data presented as mean ± SEM.

between SR and LR sham -operated animals and therefore values were collapsed. Central tendency did not differ between rats in the SR group and those in the sham -operated group (Figure 2.1B; $p > 0.05$). In contrast, rats in the LR group showed a reduction in the percent of time spent in the center of the open field compared to the sham -operated group (Figure 2.1B; $F(2, 45) = 10.03$, $p < 0.05$).

Microemboli Induce a Delayed Increase in Depressive-like Behaviors

Sucrose consumption was measured over a 24 hour period for sham and ME rats in both the SR and LR groups. A one-way ANOVA revealed no differences between sham, SR and LR ME rats (Figure 2.1C; $p > 0.05$); however, an *a priori* comparison demonstrated that LR ME-operated rats consumed less sucrose than sham -operated rats ($t(28) = 2.20$, $p < 0.05$). Therefore, sucrose consumption was reduced 16 days (LR group), but not 6 days (SR group) following ME.

Social interaction was only assessed in the LR group due to difficulty accommodating repeated behavioral testing in the SR timeline. LR ME rats had an increased latency to initiate social contact with an ovariectomized female 17 days following microembolism as compared to sham -operated rats at the same time-point (Figure 2.1D; $t(11) = 2.24$, $p < 0.05$). There was no difference in total time spent in contact with the ovariectomized female (data not shown; $p > 0.05$). As stated above, locomotor activity in the open field was not compromised in the LR group, and therefore, motor impairments do not account for the delayed initiation of social interaction. Although we cannot determine if social interaction deficits had a delayed manifestation, we can conclude that this behavior deficit was evident at the LR time-point over two weeks following ME.

Microemboli Induce a Delayed Deficit in Spatial Memory

ME did not alter acquisition of the location of the escape box in Barnes Maze testing conducted 14 days following surgery (Figure 2.2A; $p > 0.05$); a time-point consistent with the affective-like behavioral testing conducted in the LR group. In addition, ME did not alter performance in a probe trial immediately following training (Figure 2.2B; $p > 0.05$). Reversal training, 21 days following surgery, demonstrated no difference in ability to learn the new escape box location for ME rats compared to sham rats (Figure 2.2C; $p > 0.05$). However, long-term retention testing two-weeks after reversal, and over one month following ME surgery, revealed a deficit in spatial memory in ME rats compared to sham rats with an increased latency to locate the escape box (Figure 2.2D; $t(6) = 2.406$, $p < 0.05$), increased latency to enter the escape box ($t(6) = 2.107$, $p < 0.05$), and a trend towards increased nose poke errors ($t(6) = 1.847$; $p = 0.05$).

Microembolism Damage Is Diffuse and Variable

A thorough stereological assessment of approximately 1,000 sections from 32 brains revealed only a small subset of ME brains to exhibit visible lesions (see Table 1; representative image Figure 3A, B). Lesion volume totaled 2.404 mm^3 compared to 0.153 mm^3 in sham tissue, which is likely accounted for by asymmetrical ventricular volumes (Figure 2.3C; $p > 0.05$). Quantification of damage revealed no correlation between behavior and lesion volume; for example, lesion volume did not predict central tendency (ME: $r = 0.003$; $p > 0.05$) or consumption in the sucrose preference test (ME: $r = 0.071$; $p > 0.05$).

Microembolism Damage Does Not Trigger Cellular Response or Neuronal Damage

Staining for eriochrome cyanine and neurofilament revealed tissue and myelin loss in the internal and external capsules of ME brains as compared to sham animals (not quantified). OX-42 was used as a marker of amoeboid microglial activity in whole brain slices prior to a more

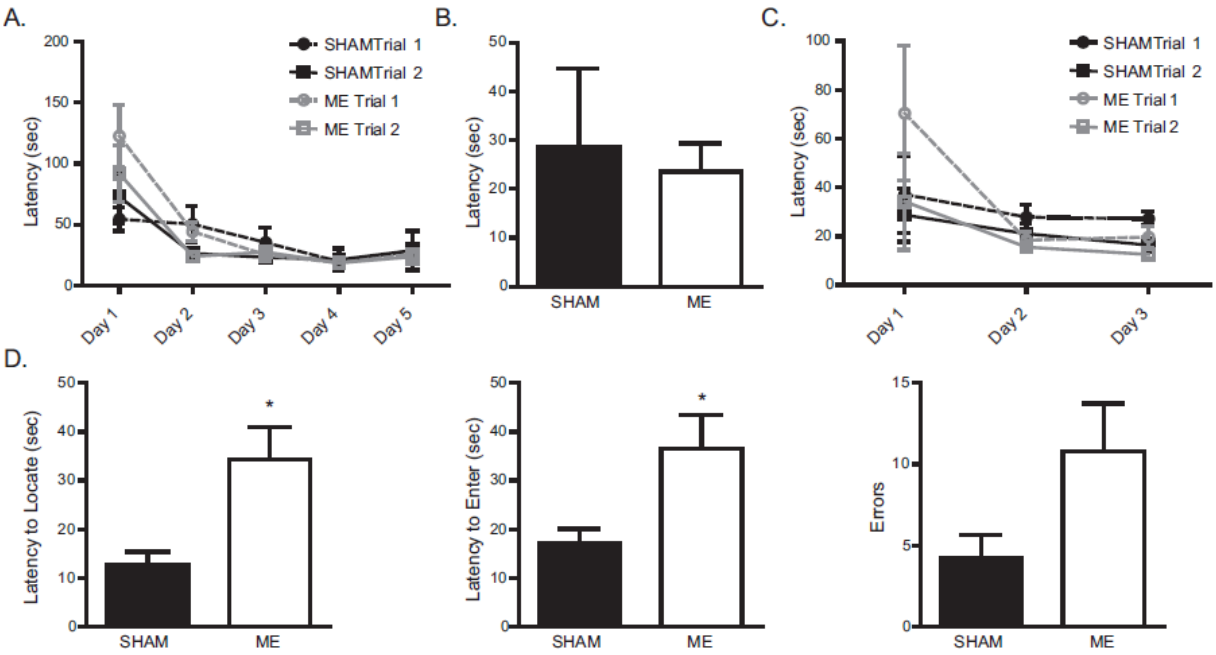

Figure 2.2 ME infarcts alter Barnes Maze performance

Spatial memory was tested in the Barnes Maze over an 8 day acquisition, evaluation and reversal training schedule. Two weeks later, long-term retention of spatial memory was evaluated by an additional evaluation probe trial. *A*) Acquisition training revealed no differences in the ability of sham and microembolism (ME) animals to learn the location the escape box ($p > 0.05$). *B)* Latency to approach the escape box during the evaluation trial of Day 5 revealed no differences in the ability of sham and ME animals to locate the escape box ($p > 0.05$). *C*) During reversal training, the location of the escape box was moved to a new area of the maze. Assessment of reversal training revealed no differences in the latencies of sham or ME animals to re-learn the task with the escape box in a new location ($p > 0.05$). *D)* Spatial memory was disrupted in ME animals as compared to sham animals two weeks following initial Barnes Maze training, over one month after the ME procedure. ME animals had a greater latency to locate and enter (* indicates $p < 0.05$) the box as compared to sham animals (left and center panels, respectively). Additionally, ME animals showed a trend towards an increased number of errors locating the escape box as compared to sham rats ($p = 0.05$; right panel). Data presented as mean ± SEM.

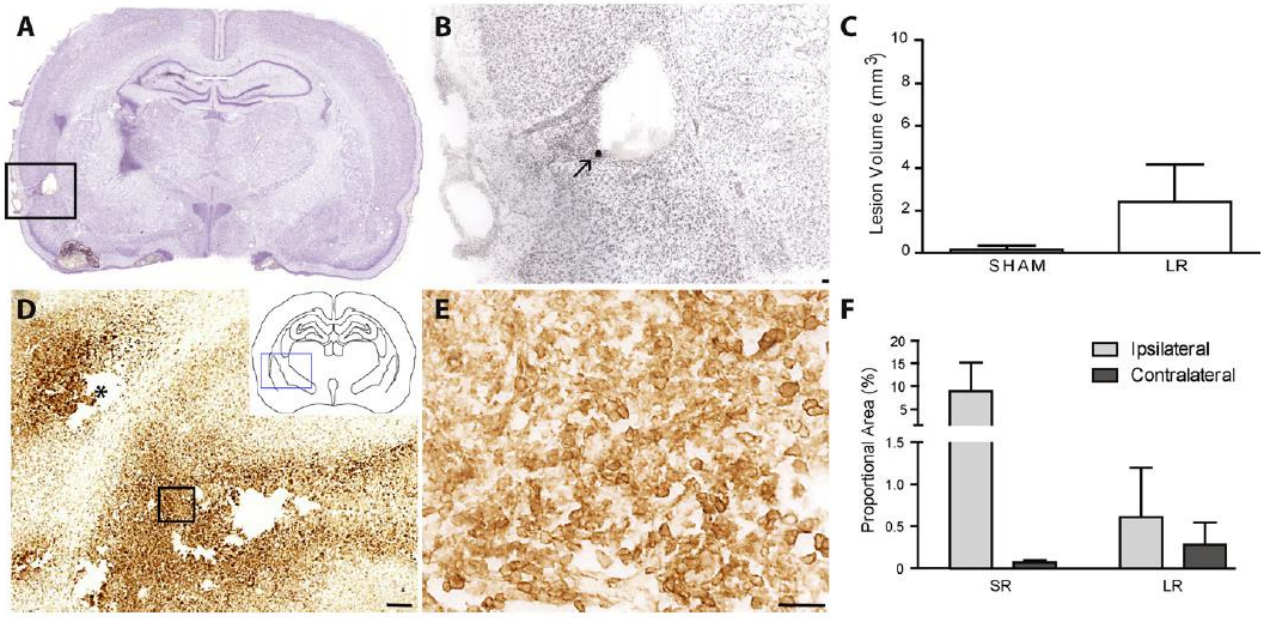

Figure 2.3 ME infarcts result in minor physical lesions without long-term cellular activation

Sections were double-labeled to visualize myelin and axons using eriochrome cyanine (EC) and an anti-neurofilament antibody (RT97). Adjacent sections were stained with OX-42 for the visualization of amoeboid microglial cells. A) Whole brain image showing ME cortical lesion in a LR animal. Cell aggregation in basal ganglia and hippocampal regions of lesioned hemisphere suggest additional ME-induced damage without the creation of cystic spaces. B) High power view of boxed region in A. Arrow points to microsphere lodged in tissue. *C)* A stereological volume assessment of ME animals at 14 days revealed a small subset of animals to exhibit lesion damage. ME resulted in mean lesion volume of 2.404 ± 1.778 mm^3. *D*) View of lesioned side of brain immunolabeled for microglia/macrophages (Ox42). Region shown corresponds to area in square on insert. Note the robust areas of OX-42 immunoreactivity revealing marked microglial and macrophage activation. *E*) High power view of boxed region in D. Most of the profiles show an activated and phagocytic phenotype. *F)* OX-42 staining of both brain hemispheres shows as Data presented as mean ± SEM. Scale bar in B, E, 50 µm; D, 400 µm.

focused assessment of the CA1 and CA3 subregions of the hippocampus. Quantification of OX-42 stain showed robust areas of OX-42 immunoreactivity in SR ME treated tissue (Figure 2.3D, E) as compared to LR ME rats, including clusters of macrophages in cortical regions. Despite this, a repeated measures two-way ANOVA revealed no difference between groups (Figure 2.3F; $F(1,3) = 1.025$, $p > 0.05$).

Quantification of CD68, a more specific surface marker of activated macrophages, revealed a modest but non-significant increase in staining in the SR group. This increase was specific to the ME treated hemisphere and observed in CA1 and, to a lesser degree, CA3 subregions of the hippocampus when compared to the contralateral hemisphere and both hemispheres of sham rats (Figure 2.4A-B; $p > 0.05$). CD68 staining in the LR group revealed fewer tagged macrophages overall as compared to SR group and no overall difference between sham and ME animals (data not shown; $p > 0.05$).

GFAP-expressing astrocytes were observed in both the CA1 and CA3 subfields of the hippocampus; however, the estimated number of GFAP+ cells remained unchanged between sham and ME infarcted rats in the SR group (Figure 2.4C-D, respectively; $p > 0.05$). Similarly, the LR group and sham operated rats did not differ (data not shown; $p > 0.05$).

Fluoro-Jade C staining of degenerating neurons was uniformly low throughout the CA1 and CA3 subregions of the hippocampus; however, we observed a non-significant increase in the CA1 of the treated hemisphere of ME animals (Figure 2.4E; $p > 0.05$). No difference in Fluoro-Jade C staining was detected in CA3 (Figure 2.4F; $p > 0.05$).

DISCUSSION

Collectively, these data demonstrate that diffuse ischemic damage is sufficient to induce changes in behavior. Depressive and anxiety-like behaviors manifest by 14 days following ME;

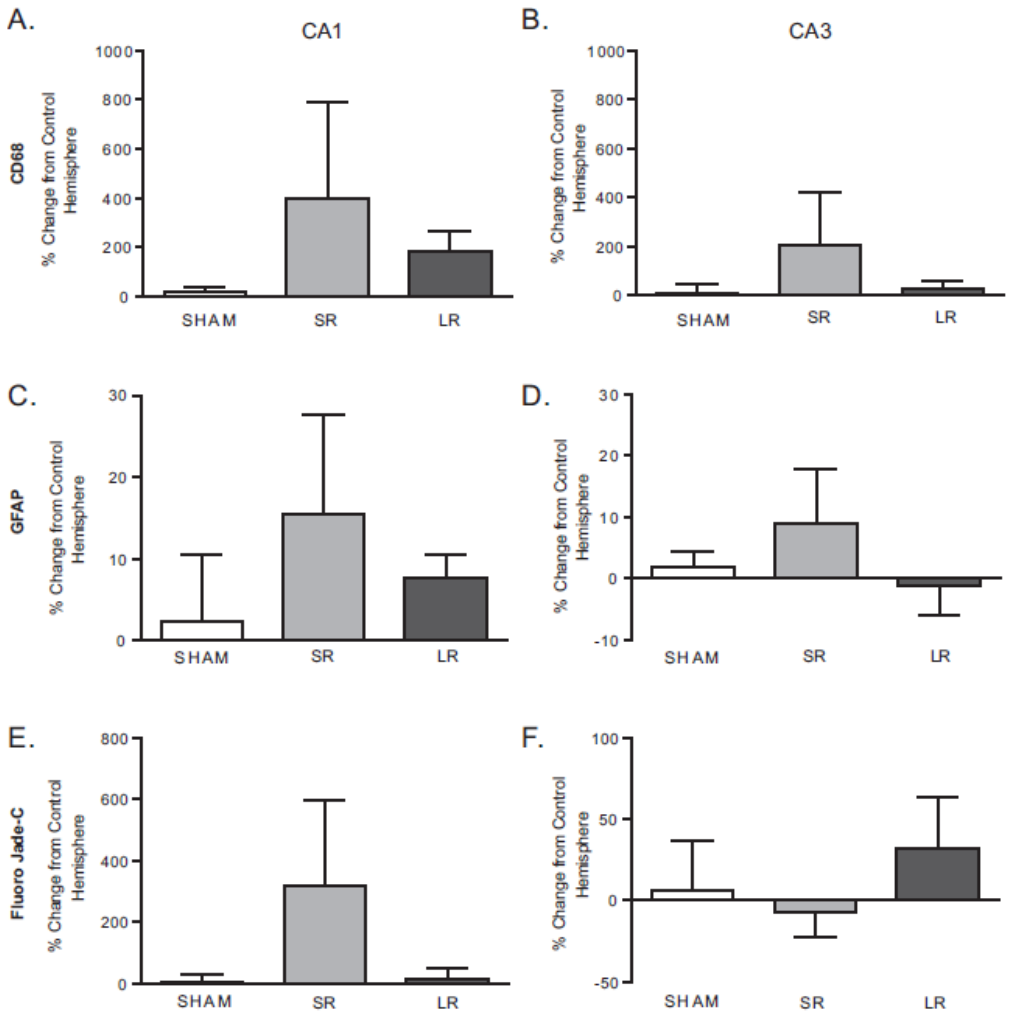

Figure 2.4 ME infarcts do not produce long-term alterations to cellular activity

Cell counts of CD68-positive macrophages, GFAP-positive astrocytes and Fluoro-Jade C-positive degenerating neurons were compared in the CA1 and CA3 subfields of each hemisphere across 7 animals. All data represented as percent change from control hemisphere. *A)* Though not significant, macrophages were increased in the CA1 subfield of the treated hemisphere of 4 Day microembolism (ME) animals as compared sham -treated animals ($p > 0.05$). *B)* Similar increases were observed in the CA3 subfield of the hippocampus, though to a lesser degree ($p > 0.05$). *C, D)* Analysis of GFAP staining revealed similar patterns of increased positive staining in 4 Day ME animals as compared to 14 Day and sham rats, in the CA1 or CA3 subfields of the hippocampus ($p > 0.05$). *E*) No differences were observed in Fluoro-Jade C stained neurons in the CA1 subfield of the treated hemisphere of 4 or 14 Day ME animals as compared to sham -treated animals ($p > 0.05$). *E*) No differences were observed in Fluoro-Jade C staining in the CA3 region of either hemisphere for sham or ME treated animals ($p > 0.05$). Data presented as mean ± SEM.

however, deficits in spatial memory are further delayed and are not detected until over a month following injury. Multiple histological assessments indicated that the observed behavioral changes were not directly explained by lesion volume, frank lesions in any one area of the brain, overt neurodegeneration, or rampant neuroinflammation. Given the established role of the hippocampus in depressive-like behaviors, additional histological analyses were aimed at a more refined assessment of the hippocampus which also indicated that neither overt neuropathology nor profound neuroinflammation were candidates for the mechanisms underlying behavioral changes. Importantly, the available data set does indicate that the nature of the neuronal changes following injury is prolonged and dynamic. Furthermore, the absence of overt neuropathology suggests that the mechanisms that underlie the observed behavioral consequences of diffuse ischemic damage may be rooted in alterations in the connectivity and/or function or surviving neurons either proximal or distal to the site of damage.

Previous studies established the presence of decreased sucrose consumption following stroke in rodent models (Craft & DeVries, 2006; Wang et al., 2009). Similarly, post-stroke depression patients report anhedonia symptoms in the absence of other cardinal symptoms of depression, such as disrupted sleep and loss of appetite (Miller et al., 2006). There is evidence that disruption of reward and motivational pathways, such as the mesolimbic dopamine system, underlie the decreased sucrose consumption following the ME procedure (Hajnal et al., 2004). To this end, the presence of anhedonic-like behaviors in the LR group, but not SR group, could be indicative of delayed secondary damage to the mesolimbic dopamine pathway triggered by cerebral ischemia. Future studies will be necessary to address the potential for ME to disrupt neurotransmission in limbic brain regions. Given that the ME model produces delayed anhedonic and anxiety-like behavior in the absence of gross motor impairment, this model may

provide useful for the assessment of neuronal mechanisms of behavioral changes associated with microvascular compromise such as late-life depression.

We also observed a delayed memory impairment subsequent to the advent of changes in affective behavior. Acquisition in the Barnes Maze was unaltered by ME suggesting that the impairments may be specific to recall. However, it is also possible that the cognitive deficits evolve over time; Barnes Maze training was conducted two weeks following the ME procedure and the final probe trial was almost five weeks after surgery. In order to fully assess the long-term impact of ME on the separate processes of learning and memory, additional studies would be necessary that initiate training at a time-point consistent with memory deficits documented here as well as training prior to ME. We can, however, conclude that an early learning deficit is not responsible for the impairment detected because acquisition was normal. Our data are consistent with clinical reports documenting memory impairments following microemboli. In patients with atrial fibrillation, which frequently is associated with cerebral microembolism, memory is impaired in the absence of evidence of stroke (Knecht et al. 2008). Furthermore, a community-based study on aging and dementia demonstrated that minute cortical and subcortical brain infarcts are associated with deficits in memory and cognitive performance (Blum et al. 2012), and that silent brain infarcts increase the risk of dementia (Vermeer et al. 2003). The ME-induced progression from altered affective-like behavior to memory impairments may prove useful given that a cerebrovascular link has previously been proposed between late-onset depression and memory impairments (Hakim 2011). Future studies will further characterize the progression of memory alterations following ME and focus on altered neuronal function as a potential mechanism given that the data reported here indicate that neither frank lesions nor overt activity of primary immune cells account for the documented behavioral changes.

Although we documented behavioral changes in multiple cohorts over three different types of behavioral tests, gross histological changes in terms of neuroinflammation or lesions were either transient and preceded behavioral changes or were absent entirely. Variations of microsphere models have been used reliably to examine changes in cerebral blood flow (Miyake et al. 1993), metabolism (Miyake et al. 1993), neurotransmitter activation (Taguchi et al. 1993), and attentional performance (Craft et al. 2005). Consistent with these studies, we also found that the generation of ME infarcts results in functional changes in the rodent brain without producing large lesions or altering the expression of macrophage/microglia and astrocytes. In fact, dose response studies of experimental microembolization show that the size (25, 45 and 90 μm) and volume (150, 500, and 1000 microspheres) of injected microspheres produce little variation in lesion volume or the degree of cellular death, which indicate that the mechanism of disruption is not a function of lesion load (Zhu et al. 2012). Furthermore, although ME models produce variations in lesions, others have shown that neither lesion size nor the number of damaged neurons predict behavioral outcome in rodent models of stroke or cardiac arrest, suggesting that the functionality of surviving neurons may underlie alterations in behavior following the event (Zhu et al. 2012; Neigh, Kofler, et al. 2004; DeVries et al. 2001). A separate study examining neuronal and glial depolarization after the injection of microspheres observed lesions in fewer than half of their experimental animals but perturbations of signal transduction in more than half, again suggesting that overt lesions are not necessary to lead to functional changes (Nozari et al. 2010). Various mechanisms exist that may account for behavioral and/or functional changes following ischemic damage, such as changes in the hypothalamic-pituitary-adrenal axis (Olsson et al. 1992; Neigh, Karelina, Zhang, et al. 2009), glucose availability (Kwan et al. 1999), structural connectivity (Whyte et al., 2004), and neuroinflammation (Whyte et al. 2004; Caso et

al. 2008).

Together, these data demonstrate for the first time that diffuse microembolic infarcts are sufficient to induce increases in anxiety-like and depressive-like behaviors, providing causal evidence in support of the proposed relationship between microvascular disease and late-life depression (Alexopoulos et al. 1997; Baldwin 2002; Kales et al. 2005; Sheline et al. 2010). Furthermore, ME-induced changes in affective-like behavior are associated with the manifestation of spatial memory impairments, providing support for the hypothesized relationship between microvascular disease, affective disturbances, and progression to cognitive decline (Vermeer et al. 2007; Purandare et al. 2012). Finally, using the current metrics, behavioral changes were not explained by frank lesions or neurodegeneration in the hippocampus, leading to the alternative hypothesis that behavioral deficits may be the result of more subtle alterations in other brain regions, or the functioning of surviving neurons. Future analyses will target the mechanisms that underlie the function of surviving neurons and activity in other brain regions that may account for the observed changes in behavior, potentially leading to novel treatment strategies.

CHAPTER THREE

MICROEMBOLISM INFARCTS INDUCE A FUNCTIONAL AND HISTOLOGICAL RESPONSE THAT IS SEXUALLY DIMORPHIC

Adapted from: *Journal of Neuroinflammation*
Nemeth CL, Reddy R, Bekhbat M, Bailey J, Neigh GN
Forthcoming

For most of life, women experience reduced incidence of coronary heart disease, preserved vascular endothelial function, better cerebral blood flow following injury, as well as decreased levels inflammatory markers, increased anti-apoptotic factors, and even reduced β-amyloid production in the presence of Alzheimer's disease (Hemmings et al. 2004; White et al. 2010; Wagner et al. 2004). Then, starting at age 50, women outnumber men in both prevalence and mortality rates of cardiovascular-related diseases (Vitale et al. 2007; Steiner 2011). In both sexes, aging positively correlates with the incidence and prevalence of cardiovascular-related disease, as age remains one of the greatest risk factors for the manifestation of the disease (Pekmezovi et al. 2011; Baños et al. 2011); however, in later life, cardiovascular disease emerges as the leading cause of death among women (Steiner 2011; Baños et al. 2011).

Menopause marks the cessation of ovarian function, effectually halting estrogen

modulation of numerous central and peripheral processes. The cyclical secretory patterns of estrogen and progesterone during menstrual years affect function at the cellular level: promoting cell survival, synaptic transmission, and exerting anti-oxidant effects (Garcia-Segura et al. 2001). System wide, estrogen is known to alter neurotransmitter and stress-response sensitivity, which collectively "protects" women from the detrimental effects of disease and injury. The somewhat rapid depletion of sex steroids following menopause in women is accompanied by the increased risk of illness and a worsened prognosis after illness. Pre-menopausal women experience reduced incidence of coronary heart disease, preserved vascular endothelial function, better cerebral blood flow following injury, as well as decreased levels inflammatory markers, increased anti-apoptotic factors, and even reduced β-amyloid production in the presence of Alzheimer's disease (Hemmings et al. 2004; White et al. 2010; Wagner et al. 2004). In cases of traumatic brain injury, women experience reduced secondary edema (likely due to anti-inflammatory effects; (White et al. 2010)), and better functional outcome compared to men, a finding consistent with rodent models of injury (Xiong et al. 2007). In ischemic animal models, histological damage is reduced in females compared with males and it has been suggested that the mechanisms mediating ischemic cell death pathways may be sexually dimorphic (Luine 2002; Hurn 2003; Semenas et al. 2010).

As discussed throughout this dissertation, microvascular lesions in the brain affect roughly one third of the population over age 65 and a striking 94% of patients presenting with their first depressive episode after age 65 (Fujikawa et al. 1993). Preclinical studies and clinical observations suggest that the accumulation of microvascular lesions in the brain is associated with depressive-behaviors, cognitive decline, and the acceleration of Alzheimer's disease (Nemeth et al. 2012; Alexopoulos et al. 1997; Kales et al. 2005; Purandare et al. 2012).

However, while men are at a greater risk of developing cardiovascular conditions, women suffer a worse prognosis and have higher rates of mortality (Steiner, 2011; Steiner et al., 2003).

Though estrogen's role in neuroprotection has been well evidenced, the mechanisms are less clear. While a singular pathway is likely not responsible for the myriad of estrogen's benefits, this study sought to further explore the role of sex in histological and functional outcome following an acute cerebrovascular event. Previously, we have shown that the formation of microembolic infarcts is sufficient to induce behavioral disruption after long-term recovery in male rats (Nemeth et al. 2012). In the current set of experiments, we used the same microsphere embolism (ME) model to measure the behavioral and cellular response in both male and female rats to determine whether ME-induced cerebral modifications were consistent between male and female rats. Specifically, we examine the expression of IBA1-positive activated microglia and sphingosine 1-phosphate receptor 1 ($S1P_1$) in the brains of both male and female rats to determine if the response to ME is sex-dependent and to determine if and to what extent sphingosine phosphate pathways mediate female protection. Specifically, we hypothesize that following ME, adult female rats will not manifest behavioral or histological change to the same extent as male rats.

MATERIALS AND METHODS

Adult male and female Wistar rats (3 months of age, Charles River) were pair-housed by sex until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity- controlled vivarium with *ad libitum* food and water. We performed all experiments in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Following a one week acclimation period, rats were randomly assigned to sham (male n = 10; female n = 10) or microembolism (ME; male n = 10; female n = 11) surgical groups. Rats received ME surgery as described in Chapter 2. As before, animals recovered in their home cage prior to assessment of behavior.

Elevated Plus Maze Testing

Behavioral consisted of a five minute elevated plus maze (EPM) exposure on Day 14 during the animals' dark cycle. Behavior was videotaped and blindly scored using Cleversys, Inc. behavioral tracking software (Reston, VA). Time spent in the open arms and closed arms, total activity, and stretch attend postures (risk assessment behavior defined as stationary hind paws while the head and forepaws are extended) were measured.

Histological Assessment

Twenty-four hours following EPM behavior, animals were transcardially perfused with 4% paraformaldehyde, brains were removed and stored at 4^{o}C until sectioning at 40 µM.

Alternating sections (section sampling frequency = 12) were taken for $S1P_1$ and IBA1 immunohistochemistry. All $S1P_1$ and IBA1 sections were incubated free-floating in blocking buffer (PBS solution containing 10% normal goat serum [Vector Laboratories], 0.4% Triton X-100 [Sigma Aldrich]) for 2 hours at room temperature followed by incubation in primary antibody (rabbit anti-IBA1, 1:500; Wako, Richmond, VA; or rabbit anti-EDG1, 1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Sections were washed and then incubated in secondary (biotinylated goat anti-rabbit, 1:250; Vector Laboratories, Burlingame, CA). All sections were washed and exposed to Vectastain ABC Elite (Vector Laboratories) according to manufacturer's instructions. After a DAB incubation, sections were washed, mounted, and coverslipped.

Histological Analysis

$S1P_1$ and IBA1 stains were visualized with a Nikon Eclipse 90i microscope (Melville, NY) fitted with Micro Bright Field Stereo Investigator 9.14.1 (Williston, VT) as previously described (Chapter 2; Nemeth et al. 2012). For measurements of $S1P_1$, two whole brain sections from four animals within each surgical condition (male and female, sham and ME) were imaged at 2X, outlined and measurements of optical density were completed using Image J Software (National Institutes of Health, version 1.47) . IBA1 cell counts were conducted in the hippocampus, amygdala, and caudate nucleus. An unbiased stereological approach estimated cell counts of IBA1 using the Optical Fractionator probe. For all, the sampling frame was set to 300 μm^2 and counting grid was set to 100 μm^2, which resulted in the assessment of approximately 100 frames in each of six sections per animal. From here, the number of cells within each counting frame was counted and expressed as the estimated number of cells.

Cell morphology in the hippocampus was assessed from the same sections in which 25-30 cells were chosen at random, converted to 8-bit, cleaned with a Gaussian filter, binarized, and analyzed for the number of branches, the number of junctions, and average branch length using the ImageJ AnalyzeSkeleton plugin. For all, the investigator was blind to treatment groups.

Data Analysis

All data were assessed for normality and equal variance prior to statistical analysis. EPM behavior and histological endpoints were analyzed using a two-way ANOVA with sex (male, female) and surgery (sham, ME) as factors. When necessary, Bonferroni *post-hoc* tests delineated additional group differences. Results were considered significant at $p < 0.05$, calculated using GraphPad Prism 5, and are reported as mean ± standard error of the mean (SEM).

RESULTS

ME procedures increased anxiety-like behavior in male, but not female rats

The elevated plus maze (EPM) was used as a measure of anxiety-like behavior which consisted of a five minute exposure on Day 14 during the animals' dark cycle. While general locomotor behavior did not differ between surgical groups ($p > 0.05$; Figure 3.1A), females were more active in the EPM compared to male rats ($F_{1,36} = 14.92$, $p < 0.05$). Furthermore, analysis of time in the open arms of the EPM revealed a significant interaction of sex and surgery, such that ME procedures affected male, but not female, behavior at two weeks ($F_{1,43} = 7.140$, $p < 0.05$; Figure 3.1B). Bonferroni posttests revealed a difference between male sham and male ME time spent in the open arms of the maze (mean difference: 49.03 s, $p < 0.05$). Females that underwent the ME procedure did not demonstrate any evidence of increased anxiety-like behavior as defined by differences in time spent in the open arms of the maze, or in stretch attend postures. Conversely, males had an increased display of stretch attend postures compared to sham-operated male and female rats (interaction: $F_{1,42} = 7.004$, $p < 0.05$; posttest: mean difference: 12.80, $p < 0.01$; Figure 3.1C).

ME procedures increased expression of IBA1 in a sex- and region-dependent manner

A two-way ANOVA confirms that in the hippocampus, both male and female rats showed increased estimated counts of IBA1+ cells following ME surgery (main effect of surgery: $F_{1,14} = 8.018$, $p < 0.05$; Figure 3.2A). Conversely, a main effect of sex was present in the amygdala ($F_{1,11} = 7.68$, $p < 0.05$; Figure 3.2B) indicating an overall decreased microglial detection in both sham and ME female rats. Though no differences were detected via a two-way ANOVA in the caudate, separate examination of male and female rats using *a priori* Student's t-test reveals an increase in female IBA1 cells following ME and suggests that the cellular

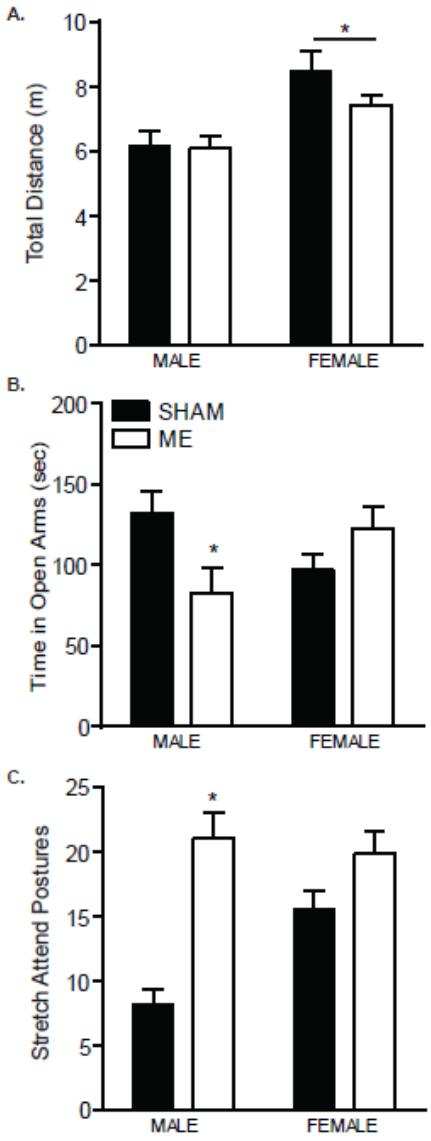

Figure 3.1 ME induces anxiety-like behavior in male, but not female, rats

Male and female SHAM and microembolism (ME) rats were run in a five-minute elevated plus maze. (**A**) Total distance traveled of male and female sham and ME rats indicates a significant effect of sex, such that female rats traveled more overall as compared to male rats ($p < 0.05$). (**B**) A significant interaction between sex and surgery was detected in the elevated plus maze, specifically, compared to male SHAM, male ME animals spent less time in the open arms of the elevated plus maze, indicative of an anxiety-like state. No difference was detected between female SHAM and ME animals ($p < 0.05$). (**C**) Similarly, the number of stretch attend postures were significantly higher in male ME rats compared to both SHAM and female animals ($p < 0.05$). For all, error bars represent standard error of the mean (S.E.M.) and * indicates $p < 0.05$.

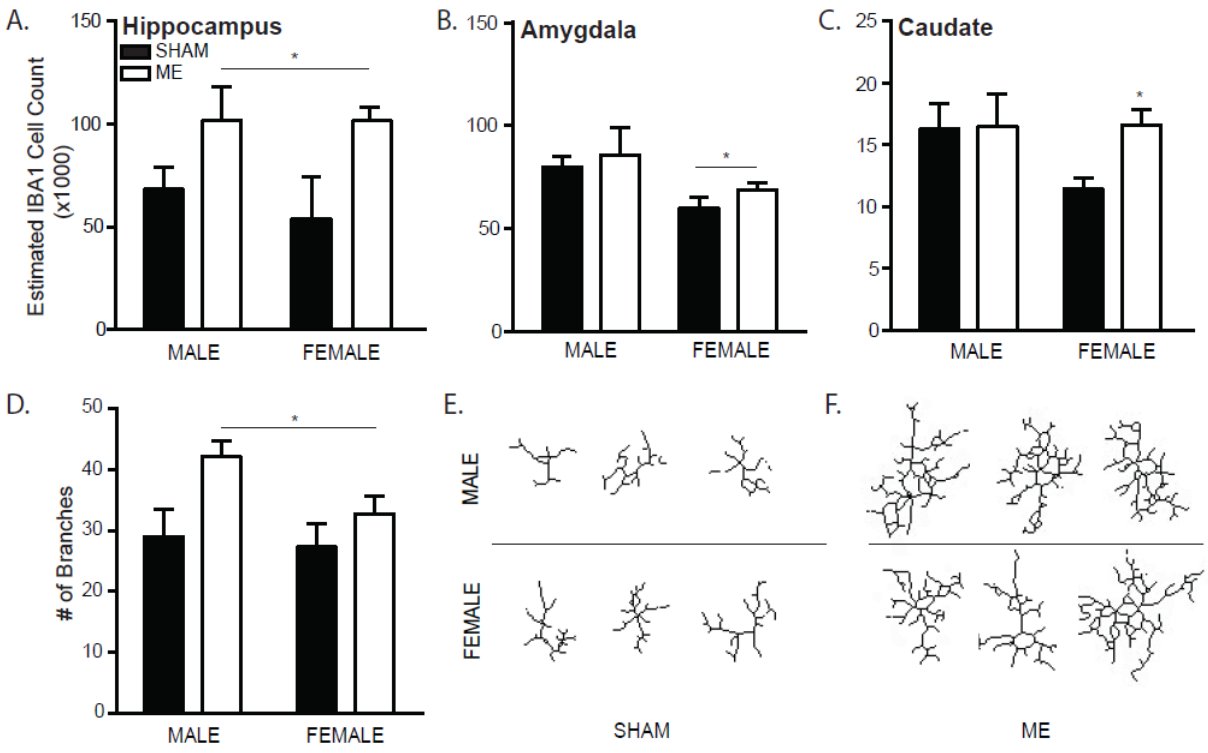

Figure 3.2 IBA1^{+} cell counts are region and sex dependent and exhibit a "primed" morphology within the hippocampus

In-depth microglia/macrophage activation was determined by cellular counts of ionized calcium-binding adapter molecule 1 (IBA1)-stained cells in the brains of male and female SHAM and ME rats. (A) IBA1 cell counts were estimated in the hippocampus of male and female SHAM and ME animals and compared with a two-way ANOVA. Analysis revealed a main effect of surgery such that surgery increased the number of activated microglial cells independent of sex. (B) In contrast, staining in the amygdala revealed an effect of sex, with females having fewer overall IBA1+ cells as compared to males ($p < 0.05$). (C) A priori t-test of the estimated cell counts in the caudate show an increased number of stained cells ($p < 0.05$); though higher at baseline, males showed no effect of ME. (D) Morphological assessment of IBA1+ cells in the hippocampus illustrates increased branching and a hyper-ramified state in both male and female ME rats compared to sex-matched sham animals ($p < 0.05$).

response in the caudate may be more sensitive in females compared to males ($t(6) = 3.40$, $p < 0.05$; Figure 3.2C).

ME procedures lead to a "primed" activational state in hippocampal IBA1$^+$ cells

Analysis of cellular morphology within the hippocampus of sham and ME rats reveals a significant "priming" effect of ME surgery. Specifically, a two-way ANOVA of the morphology of IBA1+ cells in the hippocampus shows no effect of sex; however, ME surgery significantly increased both the number of branches ($F_{1,12} = 7.73$, $p < 0.05$; Figure 3.2D) as well as the number of junctions ($F_{1,12} = 7.57$, $p < 0.05$) without affecting the average branch length ($p > 0.05$). Representative cells are depicted for sham and ME rats in Figures 3.2E and 3.2F, respectively.

ME procedures increases expression of S1P$_1$ in male, but not female, rats

One-way ANOVA analysis reveal no differences when considering whole brain $S1P_1$ expression in male and female rats; however, separate examination of male and female rats using *a priori* Student's t-test reveal differences in male, but not female, ME animals. Specifically, optical density measurements demonstrate a significant increase of $S1P_1$-positive staining in the ME males (0.229 ± 0.014) compared to sham males (0.181 ± 0.017; $t(9) = 2.13$, $p < 0.05$, Figure 3.3). No difference in staining intensity was detected between female sham (0.222 ± 0.026) and ME (0.222 ± 0.015) animals ($t(9) = 0.02$, $p > 0.05$).

DISCUSSION

Collectively, these data demonstrate that males are more susceptible than females to the heightened anxiety-like behaviors following ME procedures. Furthermore, following ME,

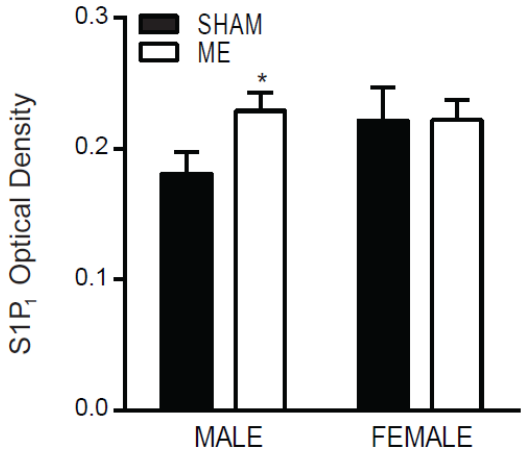

Figure 3.3 $S1P_1$ was increased in males, but not females, following ME procedures

As an estrogen-regulated bioactive sphingolipid involved in regulating vascular tone, expression levels of sphingosine 1-phosphate receptor 1 ($S1P_1$) were measured in the brains of male and female sham and ME rats. While no differences in expression levels of $S1P_1$ were detected by two-way ANOVA, *a priori* t-tests revealed greater expression in male ME compared to male sham, and no differences between female SHAM and ME animals as determined by optical density measurements.

microglial activation is brain region specific and is not coupled to the emergence of anxiety-like behaviors in female rats. In regard to the males, the data extend previous documentation of the behavioral effects of ME to include an additional metric of anxiety-like behavior, the elevated plus maze. Previously we have shown that male rats manifest anhedonia, alterations in social behavior, and increased anxiety-like behavior in the open field following ME (Nemeth et al. 2012). In addition, our previous work documented the effects of ME on lesion volume and the general presence of microglia, but the current work refines our understanding of the histological effect of ME by documenting an increase in reactive microglia, an altered morphology of these cells, and an increase in $S1P_1$. A causal relationship between microglial activation and anxiety-like behavior has been demonstrated following global ischemia (Neigh, Karelina, Glasper, et al. 2009) and these data suggest that a similar relationship may exist following ME in male rats.

The absence of altered behavior following ME infarction in females, despite clear evidence of microglial activation, may illustrate a resilience that stems from the neuroprotective effects of endogenous estrogen. Clinical and rodent literature support the role of estrogen as a neuroprotectant following stroke or other brain trauma (Li et al. 2011; Wagner et al. 2004) and studies of other rodent models of ischemia note reduced infarct size in female rodents compared to both males and ovariectomized female counterparts (Alkayed et al. 1998) (thoroughly reviewed in 2), though no studies have examined the long-term effects of ME lesions in male and female rodents. We have previously reported that frank lesions are rare following the ME procedure in male rats (Nemeth et al. 2012), and in the current set of experiments, we demonstrate that microglial activation is region-dependent, and that microglia within the hippocampus adopt a hyper-ramified state. The enhanced ramification, illustrated by increased branching and junctions, observed in these cells is likely a heightened response to ME which

stems from systemic inflammation and serves to protect the damaged area from additional injury (Hinwood et al. 2013; Moisse et al. 2008; Perry et al. 2014). In addition, these specific cellular adaptations do not associate with behavior, suggesting that the mechanism for the behavioral difference in the current study is not at the level of neuronal damage. The possibility remains that female sex steroids may be modifying the functional consequences of ME-induced microglial activation as has been reported for chronic stress (Pyter et al. 2013). Interactions among estrogen and microglia are diverse and plentiful [15], and estrogen has been shown to simultaneously stimulate microglia and neural repair (Zhang et al. n.d.). Alternatively, sex differences in neuronal or signaling reorganization and plasticity unrelated to microglial/macrophage activation may mediate these differences in behavior (Smith et al. 2014).

The sex difference in response to ischemic conditions in the current study may be attributed to the endogenous circulating hormones and therefore increased baseline level of $S1P_1$ in female SHAM and ME rats. Sphingolipids mediate cell death and survival in ischemia (Pfeilschifter et al. 2011), and S1P is the most potent of the intracellular signaling sphingolipids (Pfeilschifter et al. 2011). $S1P_1$, the main receptor of S1P, is associated with favorable vascular function in premenopausal women via enhanced cerebral blood flow, and reduced leukocyte and platelet blockade of microvasculature (Semenas et al. 2010). In male rats, middle cerebral artery occlusion (MCAO) results in deficits in $S1P_1$ in the core of the infarct and administration of the $S1P_1$ agonist, FTY720, reduces damage (Hasegawa et al. 2010); however, peri-infarct areas maintain expression of $S1P_1$. The divergent result observed in the current study may be due to either the relatively small nature of ME damage in comparison to MCAO or to the assessment of the tissue at 2 weeks rather than 24 hours after injury. Although previous reports have largely focused on the protective role of S1P1 in females (Hemmings et al. 2004; Hofmann et al. 2009),

these data demonstrate that S1P1 may also be an important neuroprotectant in males exposed to ischemia.

Sex-dependent expression of anxiety-like behaviors and differential activation of microglial cells in response to ischemic conditions evident in the current set of experiments may be attributed to the presence of endogenous circulating hormones. In female sham and ME rats, this may translate to an increased baseline level of $S1P_1$. Sphingolipids mediate cell death and survival in ischemia (Pfeilschifter et al. 2011) and S1P is the most potent of the intracellular signaling sphingolipids (Pfeilschifter et al. 2011). $S1P_1$, the main receptor of S1P, is associated with favorable vascular function in premenopausal women via enhanced cerebral blood flow, and reduced leukocyte and platelet blockade of microvasculature (Semenas et al. 2010). In male rats, middle cerebral artery occlusion (MCAO) results in deficits in $S1P_1$ in the core of the infarct, and administration of the $S1P_1$ agonist, FTY720, reduces damage (Hasegawa et al. 2010); however, peri-infarct areas maintain expression of $S1P_1$. The divergent result observed in the current study may be due to either the relatively small nature of ME damage in comparison to MCAO or to the assessment of the tissue at 2 weeks rather than 24 hours after injury. Although previous reports have largely focused on the protective role of S1P1 in females (Hemmings et al. 2004; Hofmann et al. 2009), these data demonstrate that S1P1 may also be an important neuroprotectant in males exposed ischemia.

Collectively, these data demonstrate that both male and female rats exhibit evidence of neuroinflammation with increased numbers of "primed" microglial cells, but that females do not exhibit corresponding anxiety-like behaviors. A relationship between anxiety-like behavior and neuroinflammation has been previously demonstrated in male rats in models of global ischemia (Neigh, Karelina, Glasper, et al. 2009) and chronic stress (Wohleb et al. 2014; Wohleb et al.

2013). The current data set suggests that this relationship cannot be extrapolated to the female brain. Future studies investigating the mechanisms by which females exhibit normal behavior in the presence of microglial activation will provide important insight for treatment strategies.

CHAPTER FOUR

MICROEMBOLISM INFARCTS INDUCE ANHEDONIA BUT NO DETECTABLE CHANGES IN WHITE MATTER INTEGRITY IN AGED RATS

Adapted from: *PLoS One*
Nemeth CL, Gutman DA, Majeed W, Keilholz SD, Neigh GN
Volume 9, Issue 5, 8 May 2014: e96624.

The incidence of microvascular disease is growing among the aged population (Chen et al., 2010). As discussed previously, small vessel pathology, including within the context of microvascular pathology, and microvascular events are known contributors to depressive behaviors late in life (Kales et al. 2005; Rubin et al. 2010; Alexopoulos et al. 1997; Santos, Kovari, et al. 2009; Xekardaki et al. 2012; Sheline et al. 2010; Lyness 2002), and have been implicated in the progression of cognitive impairment and Alzheimer's disease (Grau-Olivares et al. 2009; Purandare et al. 2012). Despite these links and the high correlation between microvascular disruption and behavioral deficits, the mechanisms behind this relationship remain a topic of debate (Santos, Gold, et al. 2009). Due to the slow progression of these symptoms, the phenotypically silent nature of these lesions, and the difficulty of directly addressing this relationship in the clinic, establishing a cause and effect relationship has been difficult. Finally,

because ME lesions are typically detected following a larger ischemic event or after death, preventative measures and treatment strategies are greatly hindered.

As illustrated in Chapter 2, rodent microsphere embolism (ME) models have been used to successfully recapitulate the cognitive deficits of microvascular pathology (Craft et al., 2005; Taguchi et al., 1993) and the clinical features of stroke (Mayzel-Oreg et al., 2004; Small and Buchan, 2000) including alterations to cognitive, depressive- and anxiety-like behaviors (Nemeth et al. 2012). In these studies, however, the relationship between microsphere lesions and behavioral disruption was not paralleled by cellular death, macrophage activation, or astrocyte activity measured by conventional histology. Therefore, the current study employed diffusion tensor imaging (DTI) as a method of assessing microstructural differences in tissue to complement previous histological findings in this model. In our study, we adopted high-throughput *ex vivo* imaging, a novel method to more efficiently image multiple perfused brains simultaneously (Gutman et al. 2012; Dyrby et al. 2011). Assessment of DTI indices in microsphere-lesioned brains may provide a more translatable correlate compared to slice histology.

DTI is a powerful tool that allows for the assessment of white matter tract directionality and tissue integrity. Because anisotropy and diffusivity of water in brain tissue depend upon tissue microstructure, fractional anisotropy (FA) and mean diffusivity (MD) values can be used as indices of healthy tracts and tissues. The sensitivity of DTI allows for the detection of subtle changes in cell, axon, and myelin morphology and has become a useful tool in predicting functional outcome following stroke and other brain injuries (Jiang et al. 2010; Pitkonen et al. 2012). Despite growing popularity of DTI for neuroimaging, little is known about the relationship between DTI-derived measures and affective behavior in the context of

microvascular pathology. Further, to the best of our knowledge, this has not been examined in an aged model.

We have previously established that the induction of microsphere lesions results in behavioral disruption in adult animals that does not correlate with cell death, or macrophage/astrocyte activity as detected by conventional histology. Thus we sought to more closely explore the interactions of microvascular structure and normal aging. To accomplish this, we elected to compare the effects of ME infarction in adult and aged rats. Through the examination of behavior in aged rodents, we tested the hypothesis that subtle ME-induced lesions result in structural changes to white matter integrity and gray matter tissue, as measured by DTI, that correspond to the manifestation of behavioral deficits.

MATERIALS AND METHODS

Animals

Adult (3 months of age) and aged (16 months of age, Charles River) male Wistar rats were pair housed until surgery. Rats were maintained on a reverse 14:10 light:dark cycle in a temperature- and humidity-controlled AAALAC-approved facility. To promote healthy aging prior to the start of the study, aged rats were provided cage enrichments and maintained at a healthy weight by limited food ration. At the time of ME or sham surgery (described later), and throughout the duration of the study, cage enrichments were removed and food and water were available *ad libitum* regardless of age. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University, the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and *The Code of Ethics of the World Medical Association for experiments involving laboratory animals*.

Surgery

Following a minimum one week acclimation period, rats were randomly assigned to sham (adult n = 7; aged n = 7) or ME (adult n = 9; aged n = 9) surgical groups. ME surgery was conducted as previously described (Nemeth et al. 2012). Animals were anesthetized with isoflurane and a neck incision was made to locate the common, internal, and external carotid arteries. The common carotid artery was isolated and ligated with suture and the external carotid artery was ligated at the bifurcation with the internal carotid artery. Microspheres (New England Nuclear Inc., Boston, MA; 50 μm in diameter; suspended in 10% Dextran and 0.01% Tween in isotonic saline; PerkinElmer Instruments; Shelton, CT; ≈2500 spheres in 50 μL) were injected using a 30 G needle to the left internal carotid artery. Pressure was applied to the injection site until bleeding stopped, at which point ligations were released and blood flow was allowed to return to normal. Sham animals underwent an identical procedure without the infusion of microbeads. The wound was closed with surgical staples and antiseptic was applied. Surgical staples were removed on post-surgical day 5. Animals recovered in their home cage prior to assessment of depressive- and anxiety-like behaviors (aged animals) and imaging (adult and aged animals).

Behavioral Testing

We have previously established that ME infarcts in adult animals induce depressive- and anxiety like behaviors when tested at 14, but not four, days after the procedure (Nemeth et al. 2012). In the current experiment, only aged animals were tested in the open field paradigm and the sucrose consumption test to determine the effect of ME infarction on susceptibility of the animals to behavior deficits. Behaviors were assessed as previously described (Nemeth et al. 2012), with one test conducted per day.

In order to assess anxiety-like behavior, the open field test (Prut et al. 2003) was conducted during the animals' dark phase, and was videotaped and hand-scored by an observer blind to surgery groups. Time spent in the center (60 cm x 60 cm of 1 m^3 open box) versus the periphery of the box, or central tendency, was interpreted as exploratory anxiety (Prut et al. 2003) and locomotor activity was assessed by counting the total number of squares crossed. The open field test lasted 10 minutes and started with placement of the rat in the center of the apparatus.

The sucrose consumption test measures animals' preference for a sucrose solution over tap water and serves as an indicator of reward state, such that decreased sucrose consumption reflects an anhedonic state (Willner et al. 1987). Sucrose consumption was assessed over 48 hours in the animals' home cages. During this time, rats had equal access to a 0.8% sucrose solution and tap water. A 24-hour habituation period was followed by a 24-hour evaluation period. Bottle presentation was reversed after the first 24 hours to prevent side bias (Nemeth et al. 2012).

Tissue Preparation

In order to reduce the time required to scan the brains, we adopted a high-throughput imaging paradigm used by Gutman *et al.* (2012). Following behavioral testing (post-ME day 18), all animals were transcardially perfused with 4% paraformaldehyde. Brains were then removed and stored at 4 $^{\circ}$C until they were embedded in an agarose matrix for imaging. Three to four rat brains were embedded into each of seven tubes for imaging. The embedding mixture was composed of a matrix of 1% agarose (Sigma, St. Louis, MO) doped with an insoluble mixture of 1 mM gadolinium oxide (Fisher Scientific, Pittsburgh, PA); the gadolinium serves to suppress the signal from the agarose, providing better separation of the brains from the background.

Imaging

All imaging experiments were performed on a Bruker 9.4T horizontal scanner using a 72 mm volume coil (Bruker, Billerica, MA). Sham, adult, and aged rat brains were randomly assigned to imaging tubes. For each tube of brains, T2-weighted images were first acquired at 161 micron isotropic resolution (echo time [TE] = 25 ms, matrix 256 x 512, 12 averages, ~16 hours scan time). DTI images were acquired using a spin-echo based sequence with 200 micron isotropic resolution (TE = 26.9 ms, TR = 27.5 s, matrix size of 256 x 128, ~ 55-60 axial slices, 64 gradient directions with b = 2000 s/mm^2, 3 images with b = 0, scan time = ~61 hours).

Segmentation and Registration

Prior to further processing, the images from each scan that contained one or more individual rat brains were manually segmented into individual files. For both the T2-weighted and DTI-scans, masks were generated for each individual rat brain using BET, (FSL, www.fmrib.ox.ac.uk/fsl) followed by manual editing to generate individual brain masks for each animal. A study-specific high-resolution rat template was generated by nonlinearly registering each T2-weighted brain to a single reference image, and then creating a composite image (FNIRT,www.fmrib.ox.ac.uk/fsl). A transformation matrix for each rat DTI dataset to this rat-standard space was then generated using FLIRT (FSL, www.fmrib.ox.ac.uk/fsl) using a 12-DOF affine warp. Brain region and white matter tract volumes of interest (VOIs) areas were subsequently defined on each individual reference image (9 total VOIs including: the genu of the corpus callosum, and the ipsilateral and contralateral regions of the amygdala, external capsule, CA1 and CA3 subfields of the hippocampus; Figure 4.1). The mean intensity from the FA map was then extracted from each VOI and averaged as described below.

Data Analysis

All data were assessed for normality and equal variance prior to statistical analysis. Animal weights were assessed using a three-way repeated-measures ANOVA with age (adult vs aged), surgery (sham vs ME), and days post-surgery (1 vs 7 vs 30) as factors. For each age group, further group differences were delineated using a two-way repeated-measures ANOVA with surgery (sham vs ME) and day (1 vs 7 vs 30) as factors. Sucrose consumption was assessed using a two-way repeated-measures ANOVA with surgery (sham vs ME) and day (Day 1 vs Day 2) as factors. Open field behavior was averaged by group (sham vs ME) and compared using a Student's t-test.

For each VOI, FA and MD values were computed and averaged by group. For both adult and aged animals, values measured for each region and tract were analyzed by a two-way repeated-measures ANOVA with surgery (sham vs ME) and hemisphere (ipsilateral vs contralateral) as factors. FA and MD values of the genu of the corpus callosum were compared to sham treated animals using a Student's t-test. Differences in lesion frequency were determined using a two-sample t-test of proportions. For all comparisons, results were considered significant when $P < 0.05$. All results are reported as means ± the standard error of mean (S.E.M.) and all analyses were performed with GraphPad Prism 5.

RESULTS

Microsphere injection induces depressive-like behaviors in aged rats with no effect on overall health

Sucrose consumption was measured as an index of anhedonic-like behaviors in aged animals over a two day period in the home cage. A two-way repeated measures ANOVA demonstrated a main effect of surgery, such that aged ME rats consumed less sucrose over a 48

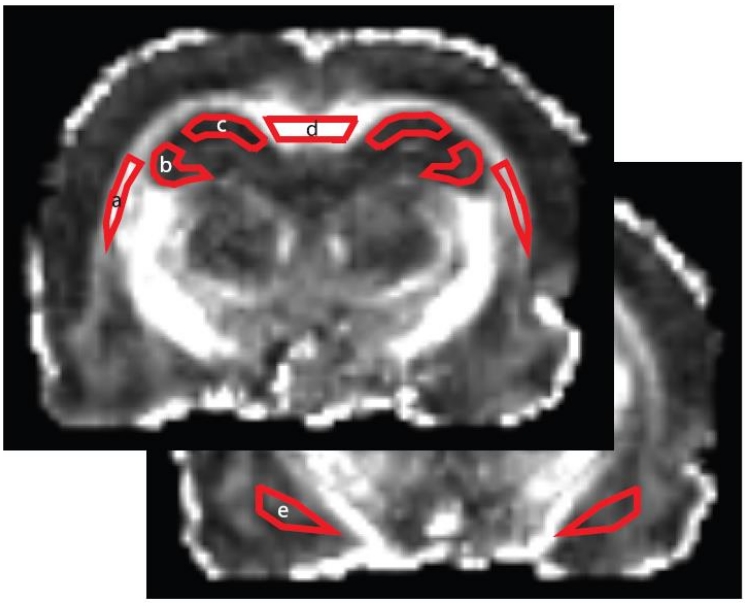

Figure 4.1 Representative image of volume of interest regions

Volume of interest (VOI) regions were individually defined on an image stack to create a 3D image on which analysis was conducted. The following regions were measured for fractional anisotropy (FA) and mean diffusivity (MD): external capsule (a), CA3 subfield of the hippocampus (b), CA1 subfield of the hippocampus (c), genu of the corpus callosum (d), and the amygdala (e). For all regions, except the corpus callosum, the corresponding contralateral regions were also measured.

hour period compared to aged sham rats (Figure 4.2A; $F_{1,14} = 4.966$, $p < 0.05$). This difference in consumption is not due to differences in body weight between the groups, for weight in each age group did not differ as a function of surgery (post-behavior weights: adult sham 508.6 ± 13.29; adult ME 538.56 ± 12.77; aged sham 667.43 ± 27.78; aged ME 681.90 ± 17.81). Although aged animals weighed significantly more than adult animals ($p < 0.05$), a three-way repeated measures ANOVA revealed no interaction between age, surgery, or post-surgery day ($F_{1,28} = 1.32$, $p > 0.05$). Furthermore, a two-way repeated measures ANOVA of adult ($F_{1,28} = 1.13$, $p > 0.05$) or aged ($F_{1,30} = 0.12$, $p > 0.05$) animals revealed no effect of surgery.

Additionally, analysis of behavior in the open field showed no differences in the amount of time spent in the center of the arena between aged sham and ME animals (Figure 4.2B; $p > 0.05$). Similarly, no differences in total mobility in the open field were found between surgery groups, suggesting no physical impairment (Figure 4.2C; $p > 0.05$).

Microsphere injection does not cause long-term alterations in tract or tissue diffusivity

FA and MD serve as indices of pathology in diffusion tensor scanned brains. Figure 4.3 shows representative high resolution images of sham, adult ME, and aged ME rat brains. At more than two weeks following surgery, aged rats exhibited no differences in FA in either the CA1 or CA3 subfields of the hippocampus or in the amygdala. Analysis of white matter tracts revealed similar findings; no differences were detected in the strength of directionality (FA) in the external capsule as compared to the contralateral hemisphere or in the corpus callosum as compared to adult or aged sham animals (Figure 4.4 shows representative FA map; adult and aged group means presented in Table 1; $p > 0.05$).

As expected, analysis of MD paralleled FA data. Gray matter diffusivity was unchanged across hemispheres in the CA1, CA3, and amygdala, with no effect of age. MD values for the

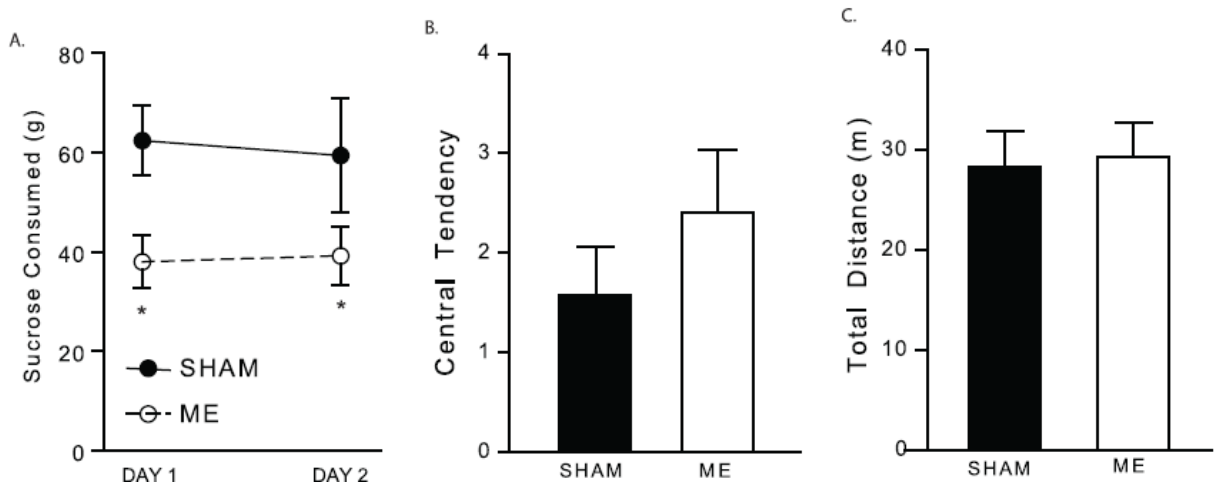

Figure 4.2 ME infarcts reduce preference for sucrose in aged animals

Aged sham and ME animals were evaluated in the sucrose consumption test and open field for depressive- and anxiety-like behaviors, respectively. (A) Aged ME animals in the sucrose consumption test consumed less sucrose over a two day period compared to aged sham animals (* indicates $P < 0.05$). (B,C) In the open field, no differences existed in the time spent in the center of the arena, or in the total distance traveled between aged sham and ME animals. Error bars indicate standard error of mean (S.E.M.).

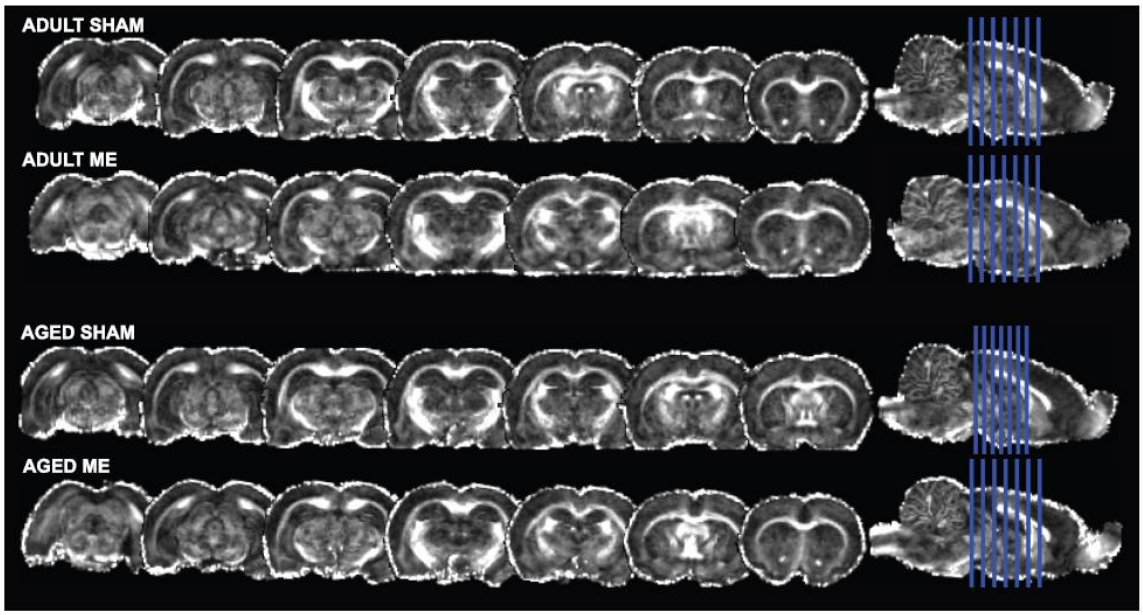

Figure 4.3 Representative multislice display of sham, adult ME, and aged ME brains

Aged and adult sham and ME perfused brains were secured in agarose and simultaneously scanned by DTI using a Bruker 9.4T horizontal scanner. Figure shows representative multislice display of sham, adult ME, and aged ME brains.

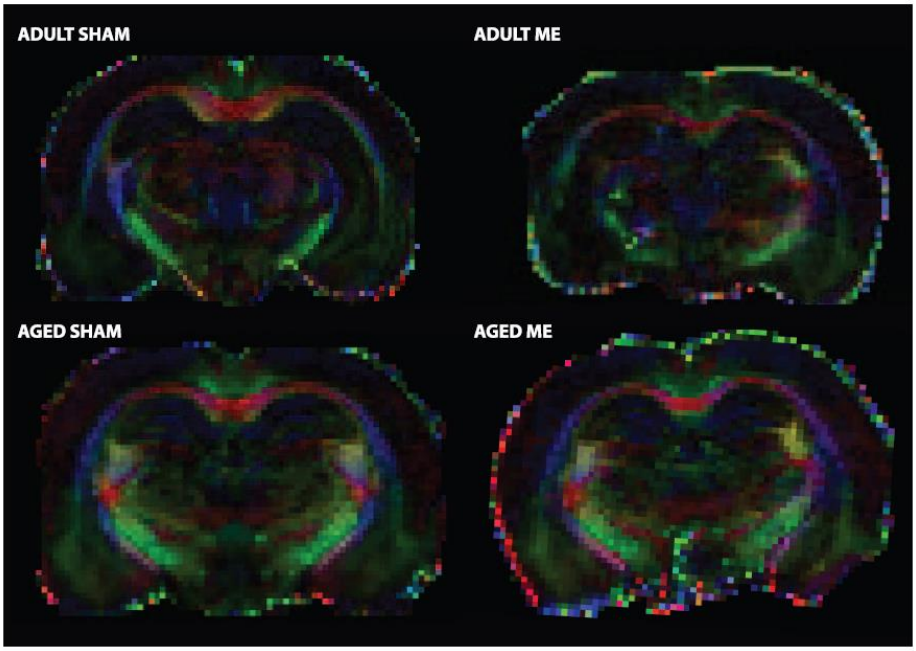

Figure 4.4 Representative fractional anisotropy color map of sham and ME brains

Representative color maps of fractional anisotropy (FA) values for sham - (top) and microsphere- (ME; bottom) treated adult animals. Colors represent tract strength and directionality; red, green, and blue, indicate transverse, rostrocaudal, and anterior-posterior, respectively.

external capsule and corpus callosum were unchanged by surgery, regardless of age (Table 4.1; $p > 0.05$).

Visual observations of tissue cavitation were noted in a subset of ME animals comprising 3 adult and 2 aged rats (Figure 4.5). Table 4.2 outlines lesion location and frequency within adult and aged animals. A two-sample t-test between proportions revealed no differences in the lesion frequency between adult and aged rats in any brain region observed ($p > 0.05$). Analysis of animals with tissue cavitation demonstrated reductions in FA in the external capsule of adult ME animals compared to age-matched sham operated controls (main effect of surgery: $F_{1,8} = 5.651$, $p < 0.05$). No changes in FA or MD were observed in any region of aged ME animals as compared to aged-matched sham operated controls (data not shown, $p > 0.05$). Furthermore, comparisons of the cavitated subset of aged animals to either non-damaged aged ME animals or sham rats was not predictive of behavior (data not shown, $p > 0.05$).

DISCUSSION

Our results demonstrate that the induction of diffuse microsphere lesions is not sufficient to produce lasting alterations to metrics of DTI in adult or aged animals two weeks following the procedure. However, consistent with previous findings in adult rodents (Nemeth et al. 2012), we found that aged animals exhibit increased depressive-like behavior as a result of ME damage. These data indicate that ME lesions do not affect the pathology of cerebral tracts or tissue and that damage does not predict behavioral disruption.

High-throughput e*x vivo* DTI analysis of ME-treated aged rodent brains revealed no changes in tract strength or gray matter tissue integrity (as measured using FA and MD) in any brain region measured. Selection of VOIs for the current experiment was based on previous

Table 4.1 Fractional anisotropy and mean diffusivity values by region

	ADULT				AGED			
FA	SHAM	±SEM	ME	±SEM	SHAM	±SEM	ME	±SEM
Ipsi- CA1	0.1198	0.001	0.1029	0.010	0.1429	0.018	0.1176	0.070
Contra- CA1	0.1014	0.004	0.1168	0.005	0.1500	0.016	0.1186	0.007
Ipsi- CA3	0.1231	0.023	0.1816	0.063	0.1226	0.005	0.1214	0.006
Contra- CA3	0.1150	0.017	0.1297	0.005	0.1235	0.005	0.1234	0.006
Ipsi- Amygdala	0.1559	0.001	0.1389	0.016	0.1331	0.012	0.1458	0.093
Contra- Amygdala	0.1268	0.007	0.1375	0.011	0.1322	0.010	0.1485	0.088
Ipsi- Ext. Capsule	0.4011	0.019	0.3603	0.015	0.3929	0.019	0.3868	0.015
Contra- Ext. Capsule	0.3756	0.037	0.3035	0.034	0.3872	0.017	0.3920	0.017
Corpus Callosum	0.5255	0.044	0.4951	0.032	0.4966	0.025	0.5308	0.024

	ADULT				AGED			
MD	SHAM	±SEM	ME	±SEM	SHAM	±SEM	ME	±SEM
Ipsi- CA1	0.7150	0.014	0.6205	0.075	0.5912	0.038	0.6356	0.068
Contra- CA1	0.7535	0.001	0.6810	0.031	0.5456	0.054	0.6433	0.076
Ipsi- CA3	0.5947	0.081	0.4950	0.012	0.5560	0.040	0.5678	0.050
Contra- CA3	0.5857	0.088	0.7290	0.122	0.5428	0.033	0.5452	0.048
Ipsi- Amygdala	0.6930	0.014	0.6478	0.032	0.5623	0.061	0.5707	0.063
Contra- Amygdala	0.6850	0.003	0.7135	0.010	0.5585	0.064	0.6097	0.079
Ipsi- Ext. Capsule	0.4253	0.056	0.4480	0.018	0.4177	0.018	0.4128	0.049
Contra- Ext. Capsule	0.4653	0.080	0.4280	0.019	0.4353	0.015	0.3992	0.039
Corpus Callosum	0.4753	0.041	0.4812	0.120	0.4052	0.029	0.4814	0.073

Table shows values of fractional anisotropy (FA; top) and mean diffusivity (MD, $x10^{-3}$ (mm^2/s); bottom) of sham and microembolism (ME) animals for adult (left column) and aged (right column) animals. For each age group, no significant differences in FA or MD were detected across the nine brain regions analyzed.

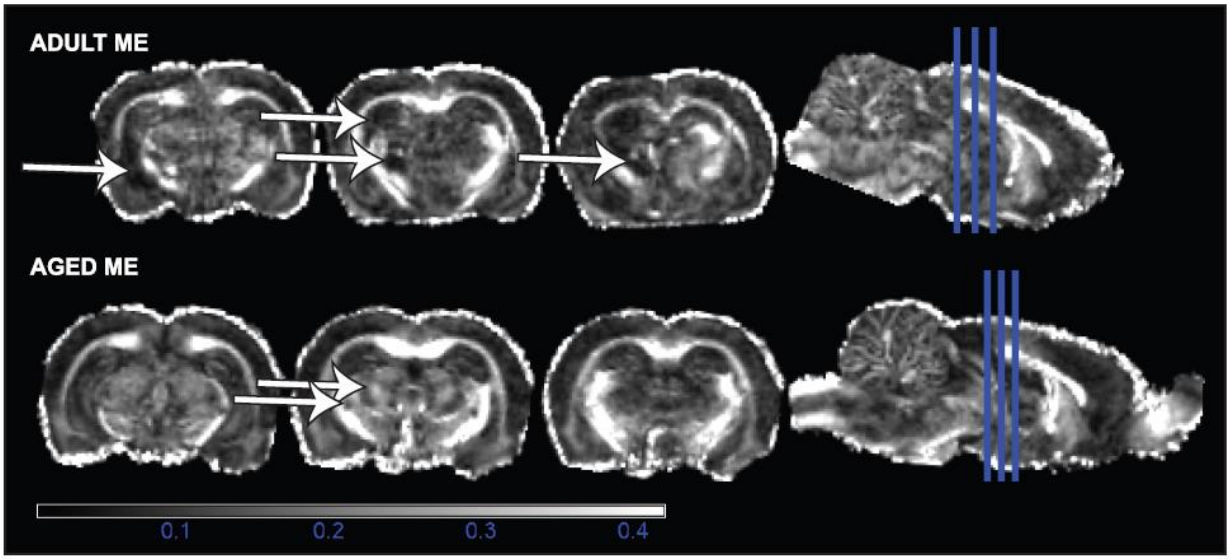

Figure 4.5 Representative sections of adult ME cavitated brain

Representative coronal brain sections from an adult ME animal show cavitation in areas of the ipsilateral amygdala, hippocampus and thalamus. Scale bar represents values of FA.

Table 4.2 Summary of lesion location and infarct rate

	% Adult	% Aged	% Total
Lesions			
Amygdala	14.3	33.3	23.1
Caudate	29.6	50.0	38.5
Cortex	29.6	50.0	38.5
Hippocampus	14.3	33.3	23.1

Lesion location and frequency are listed for adult ME and aged ME animals as visible by T2-weighted scans.

observations of region-specific tissue susceptibility related to ME infarction (Nemeth et al. 2012), stroke (Shereen et al. 2011), and cerebral hyperintensities in the presence of small vessel disease (O'Sullivan et al. 2001). Following acute strokes in humans and experimental stroke in rodents, it is reported that in these regions of susceptibility (white matter tracts, subcortical regions), ischemic lesions, and cellular swelling peak by seven days and normalize by day 14 (Sotak 2002; Pitkonen et al. 2012). Similarly, indices of DTI, such as FA and MD, change parallel to the evolution of damage following ischemic injury. In the current study, we can speculate that cellular swelling and edema have subsided prior to imaging, which may have led to normalization of DTI measures. Furthermore, we do not suspect that paraformaldehyde fixation or tissue preparation related to *ex vivo* imaging resulted in tissue compromise or confounded results, as previous studies have reported microstructural stability and high quality data sets following fixation and postmortem imaging (Gutman et al. 2012; Dyrby et al. 2011).

Given the susceptibility of the aged human brain to ME lesions, we would expect aged rats in the current study to manifest a greater degree of damage (Chen et al., 2010; Hecht et al., 2012; Rothwell et al., 2005). In contrast, increased susceptibility of aged rodents to ischemia has been poorly replicated in various rodent models as differences in infarct size between young and aged rodents are rarely observed (Andersen et al., 1999). Aged rats had a higher proportion of cavitation compared with adult rats; however, this difference failed to reach significance and did not correlate to any other measured metric. Further, in one examination of adult and aged rats following microsphere injection, adult rats showed neurological deficits shortly after the procedure, while aged animals' deficits were gradual and far less severe (Shapira et al., 2002). Though the behavioral manifestation of stroke-related impairment in rodents appears to contrast clinical data, the neurochemical factors influencing poor stroke recovery among the elderly

human population are paralleled in rodents and are characterized by increased rates of cell death and scar formation following trauma, in addition to slower response rates of neurotrophic and neuroprotective mechanisms (Popa-Wagner et al. 2007). Despite discrepancies in the rates of actions between the adult and aged brains, in long-term assessments (greater than 1 week) of recovery, responses to ischemic events appear to conclude at the same time (Popa-Wagner et al. 2007) and our findings likely reflect the stabilization of damaged areas (Fukuchi et al., 1999).

Diffusivity in the adult rodent brain was consistent with aged findings, such that group differences in FA and MD did not differ from age-matched sham operated controls. Similarly in this cohort, we believe that the normalization of values reflects recovery of acute edema and cellular responses to ME procedures. In the adult population, we and others have observed variable instances of vasogenic edema that develops into cavitation (Nemeth et al. 2012; Zhu et al. 2012). In fact, the ratio of cavitated to undamaged brains in our experiment is consistent with previous reports of microsphere injection models (Fukuchi et al. 1999; Zhu et al. 2012). In this cohort of cavitated animals, adult, but not aged animals, exhibited reductions in FA in the external capsule only (see Table 4.1), supporting previous work that suggests the external capsule to be more sensitive to ischemic damage (Shereen et al. 2011), therefore quantifiable damage to the external capsule is evident in more severe instances of ME infarction. Reductions of FA in the external capsule indicate cell membrane and myelin disruption, and demonstrate our ability to detect changes in DTI metrics following microsphere damage in adult and aged rats.

Aged rodents in the current experiment exhibited increased depressive-like behaviors more than two weeks following ME procedures. We elected to use aged animals in this assessment because aged rodent models of microvascular pathology are clinically more relevant than models involving young adult animals (Popa-Wagner et al. 2007), and this would allow us

to extend our previous findings that ME alters affective-like behaviors in adult male rats (Nemeth et al. 2012). Though these data support the Vascular Depression Hypothesis (Alexopoulos et al. 1997), a full characterization of behavioral deficits was not possible in aged animals during the current experiment as a lack of movement in the aged cohort confounded interpretation of these and other behavioral results that rely on motor activity. In all, adult and aged animals manifest similar behavioral disruptions following ME procedures in the absence of altered metrics of DTI, suggesting that adult animals may be a sufficient population in which to study microvascular events without the confound of reduced motor activity.

Using DTI, we have determined that ME infarcts are not sufficient to alter FA and MD in a manner that is associated with the manifestation of behavioral disruption. As stated, in the subset of animals with observed cavitation, we were able to detect minimal structural damage with DTI demonstrating that our ME procedure induces long lasting structural damage visible on the macro scale in a subset of the animals. Despite the presence of cavitation in a proportion of animals, behavioral assessment of these cavitated animals compared to sham or non-damaged ME animals did not differ, suggesting that behavioral changes were not mediated by overt damage as assessed by DTI, findings consistent with previous histological comparisons (Nemeth et al. 2012; Neigh, Glasper, et al. 2004). Given that previous work by our group (Nemeth et al. 2012), and others (Craft et al., 2005; Miyake et al., 2002; Takagi et al., 1997), has demonstrated that traditional histological analyses of microsphere-induced damage are inconclusive and may not be sensitive enough to detect microsphere-induced damage, we limited the current hypothesis to include only metrics of DTI. Specifically, we hypothesized that the gestalt assessments of brain morphology that are possible with DTI would be able to detect microsphere-induced structural changes in the brain. Based on our findings, we do not suspect that our methods were

not thorough enough to detect changes of FA or MD using DTI. In fact, our data may reflect tissue stabilization two weeks following injury. Though independently, values of FA can reflect white matter changes, FA is determined from a ratio of eigenvalues and should be used in conjunction with additional parameters (mean, radial, and axial diffusivity; Pitkonen et al. 2012) to provide a more thorough understanding of tissue structure following damage. MD is affected by myelin disruption, tissue cavitation, edema, and cell death, all factors that evolve temporally, and complement information provided by FA in diffusion based studies (Betz et al., 2012; Pitkonen et al., 2012). Furthermore, while other DTI indices are available, the use and interpretation of radial and axial diffusivity is somewhat controversial and not well understood (Shereen et al., 2011; Xekardaki et al., 2011) and were deemed unnecessary for the current analyses. MD and FA remain the most frequently reported indices of tissue and tract integrity, and FA appears most predictive of stroke outcome (Jiang et al. 2010), thus making it appropriate for our analyses.

The present experiments tested the hypotheses that 1) adult and aged animals experience ME-induced structural damage as measured by DTI; and 2) that these structural damages correlate to the manifestation of behavioral disruption in aged rats. While aged animals showed depressive-like behaviors following ME, as compared to sham operated animals, this was not paralleled by altered DTI metrics in the ME aged group. In adult brains and aged brains, no group level differences were observed in FA or MD following ME procedures, suggesting ME infarction to be too acute to generate permanent structural alterations to tract or tissue integrity, as detectable by DTI. In conclusion, our results indicate that the subtle presentation of ME lesions does not alter metrics of DTI in the rodent brain, and thus factors that influence FA and MD, as measured by DTI, do not contribute to the manifestation of behavioral deficits following

ME lesions. These findings are of importance because they suggest that the functioning of remaining cells and increased activity of other systems underlie behavioral perturbations following ME formation. In light of this, the following chapters discuss both the role of inflammatory cytokines and activation of the HPA axis in mediating and perpetuating disrupted behaviors at long-term time-points following ME damage. Furthermore, the activity of these systems as a target for functional recovery following ME will be explored in the following chapters.

CHAPTER FIVE

MICROEMBOLISM INFARCTS AND BEHAVIOR: INFLUENCE OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Microvascular ischemia is linked to cardiovascular disease pathology, alterations in mood, cognition, and risk of disease (Thomas et al. 2001; Taylor et al. 2013; Alexopoulos 2006; Kales et al. 2005; Purandare et al. 2012). Activation of the body's stress response, the hypothalamic-pituitary-adrenal (HPA) axis, occurs immediately following ischemia (de la Tremblaye et al. 2014; Radak et al. 2013; Johansson et al. 1997) and is similarly linked to changes in mood, and risk for disease, including stroke (de la Tremblaye et al. 2014; Loubinoux et al. 2012). The HPA axis response to several stressors, including ischemia, is well characterized, however, given the role of the HPA axis in both ischemia and behavioral change, little is known about HPA activity following microvascular ischemia.

Hypercortisolism, or the prolonged release and exposure to glucocorticoids, is observed

following stroke (Johansson et al. 1997). Additionally, increases in inflammatory cytokines are observed shortly after stroke, and together with heightened HPA axis activity, may precipitate change in behavior and susceptibility to future injury or comorbid disease (Feng et al. 2014; Craft et al. 2009). In the absence of stroke, but seemingly parallel, major depressive disorder patients are noted for hypercortisolism and over-activity of the HPA axis (Loubinoux et al. 2012) in addition to elevated levels of circulating inflammatory cytokines (Slavich et al. 2014; Leonard et al. 2009; Feng et al. 2014), again suggesting a role of both systems in the pathogenesis of depressive behaviors. While activity of the HPA axis alone is correlated with stroke severity and infarct size, and indeed prolonged stress exposure increases the risk of a stroke event, the physiological mechanisms by which stroke, on any scale, leads to behavioral changes likely involves the influence of both the HPA axis and the immune system.

The current set of experiments uses the same rat microsphere embolism (ME) model of microvascular ischemia to first determine if ME alters corticosterone (CORT) or glucocorticoid co-chaperone levels at baseline, two weeks following the surgical procedure. Second, we determined if ME alters the HPA axis response following a subsequent acute stressor. Finally, due to intricate interactions of the HPA axis with inflammatory cytokines and the parallel activity of the two systems, we sought to determine if chronic stress subsequent to ME alters the long-term expression of inflammatory cytokines within a stress-sensitive brain region.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 months of age, Charles River) were pair-housed until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity- controlled vivarium with *ad libitum* food and water. We performed all experiments in accordance with the Institutional Animal Care and Use Committee of Emory

University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Following at least a one week acclimation period, rats were randomly assigned to sham or ME surgical groups. Rats underwent surgery as previously described (Nemeth et al. 2012; Craft et al. 2005). Briefly, under isoflurane anesthesia, rats were secured in the supine position and an incision was made to locate the common carotid artery (CCA). After isolation and ligation of the CCA, the external carotid artery (ECA) was ligated at the bifurcation with the internal carotid artery (ICA). Microspheres (New England Nuclear Inc., Boston, MA; 50 μm in diameter) were injected into the left ICA using a 30 G needle. Following removal, pressure was applied to the injection site, ligatures were released, surgical staples sealed the incision, and animals recovered in their home cage. Sham animals underwent an identical procedure without the infusion of microbeads.

Acute and Chronic Stress

Acute stress consisted of a 20 minute restraint using plastic restrainers designed to minimize movement (Braintree Scientific, Braintree, MA). Chronic stress consisted of 14 consecutive days of social defeat stress in which sham and ME rats were placed in the home cage of a male aggressor rat and allowed five minutes of interaction before 25 minutes of separation by a wire mesh divider. Chronic social defeat stress is an ethologically relevant model that has consistently been shown to produce depressive-like behaviors in rodents (Rygula et al. 2005) and altered metrics of GR (Buwalda et al. 2001). Aggressor rats were cycled to prevent acclimation. Non-stressed (control) sham or ME rats remained in the home cage.

Corticosterone Assessment

Two weeks after ME injection (baseline) or an acute stress exposure, rats were rapidly decapitated and trunk blood was collected. Plasma corticosterone was assessed using the

ImmunoChem ^{125}I Corticosterone RIA kit with a sensitivity of 1 ng/mg (MP Biomedicals, Orangeburg, NY).

Gene Expression

Two weeks after ME injection or chronic stress, rats were decapitated and brains were frozen and dissected under RNAse-free conditions with regions bilaterally isolated for gene expression analysis. Due to damage susceptibility in stroke and its role in HPA axis regulation of behavior, the hippocampus was the focus of gene expression analyses. RNA was extracted using the Qiagen RNeasy Mini Kit (Valencia, CA) and reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Resulting cDNA was quantified and normalized using the PicoGreen method (Invitrogen, Grand Island, NY). All samples were prepared in triplicate using 10 pg of sample and carried out on the same Applied Biosystems HT7900 Fast Real-Time PCR system (Carlsbad, CA). For HPA axis modulators, genes of interest were limited to glucocorticoid receptor (*Gr:* Rn00561369_m1), *Fkbp5* (Rn01768371_m1), and *Ppia* (Rn00690933_m1). For inflammatory endpoints, genes included monocyte chemotactic protein-1 (*Mcp1*: Rn00580555_m1*)*, secreted phosphoprotein 1 (*Spp1*; Rn01449972_m1), and the chemokine receptor, *Xcr1* (Rn03037149_s1).

Data Analysis

Serum corticosterone values were averaged by group and analyzed via Student's t-test. Body mass data were averaged by group and a two-way ANOVA of surgery (sham and ME) and stress (control and stress) was used to detect differences on Day 28. Results of rt-pcr experiments (GR and co-chaperone, inflammatory endpoints) were averaged by group and analyzed via the $2^{-\Delta\Delta CT}$ method. Specifically, fold change was calculated from cycle threshold, standardized to a housekeeping gene (*Tfrc*; Rn01474701_m1), and normalized to sham samples.

Baseline GR and co-chaperone results were compared via Student's t-test, while 28 Day data and chronic stress effects were measured using a two-way ANOVA with surgery and stress as factors. Inflammatory endpoints were similarly analyzed using a two-way ANOVA. For all sets, data are presented as mean ± standard error of the mean (S.E.M.) and data are considered significant when $p \leq 0.05$.

RESULTS

ME rats exhibit a heightened CORT response to acute stress

Baseline plasma corticosterone was not different in ME rats compared to sham rats two weeks following surgery (Figure 5.1A; $p > 0.05$). In contrast, plasma corticosterone measured immediately following a 20-minute restraint stressor was elevated in ME rats compared to sham levels (Figure 5.1B; $t(43) = 2.96$; $p < 0.05$).

Chronic social defeat stress alters weight gain

To monitor overall rat health and demonstrate efficacy of social defeat stressor, rat weights were measured at surgery, upon staple removal, and at the conclusion of the experiments. A two-way ANOVA of surgery and stress on Day 28 of the experiment reveals a significant effect of stress illustrating the strength of the social stressor (Figure 5.2; $F_{1,30} = 20.29$, $p < 0.05$). Measurements on Day 28 show no differences in mass between the ME surgical group and the sham surgical group, suggesting no adverse health effect of the ME procedure, and good overall rat health ($p > 0.05$).

ME does not alter HPA axis activity at baseline or following chronic stress

Hippocampal gene expression levels of *Gr* and its co-chaperones *Fkbp5* and *Ppia* were measured in both ME and sham rats at baseline and again at two weeks following surgical

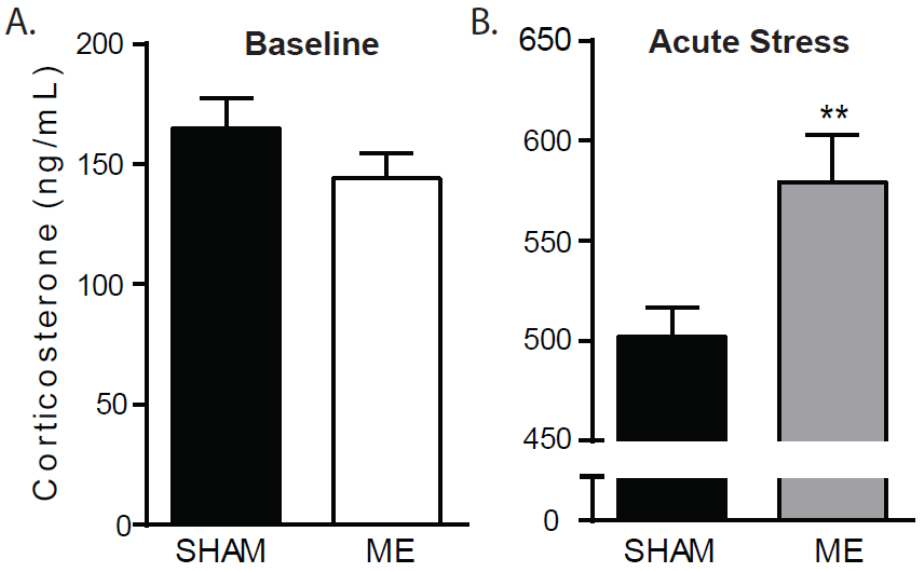

Figure 5.1 ME rats show heightened corticosterone response to an acute stressor

Corticosterone concentrations measured at baseline do not differ from one another (*A*); however, immediately following an acute stressor, ME rats show a heightened response to a subsequent acute stress (*B*; $p < 0.05$). A sensitivity to an acute stress may suggest that HPA functioning is primed thus showing a hyper-responsiveness to stressful stimuli.

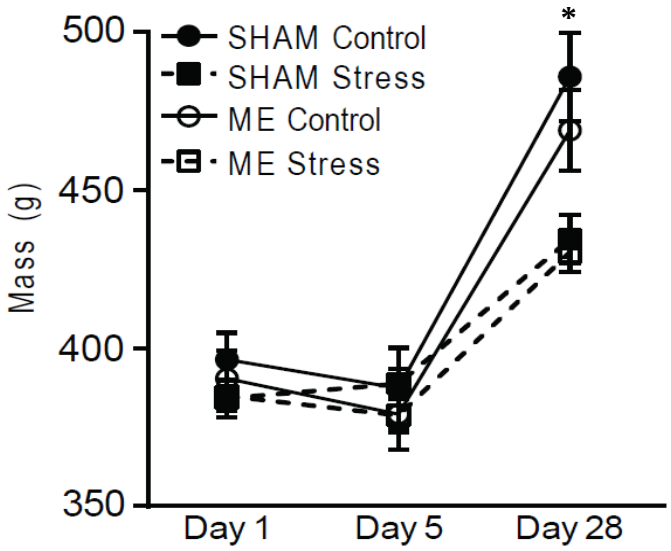

Figure 5.2 Two weeks of social defeat stress reduce weight gain in adult male rats independent of injury

Body mass was monitored throughout the experiment and summarized graphically depicting weights prior to surgery (Day 1), upon staple removal (Day 5), and prior to rapid decapitation (Day 28). Regardless of surgery, socially defeated rats (dashed lines) show reduced weight gain by Day 28 as compared to sham and ME non-stressed (control) rats ($F_{1,30} = 20.29$, $p < 0.05$).

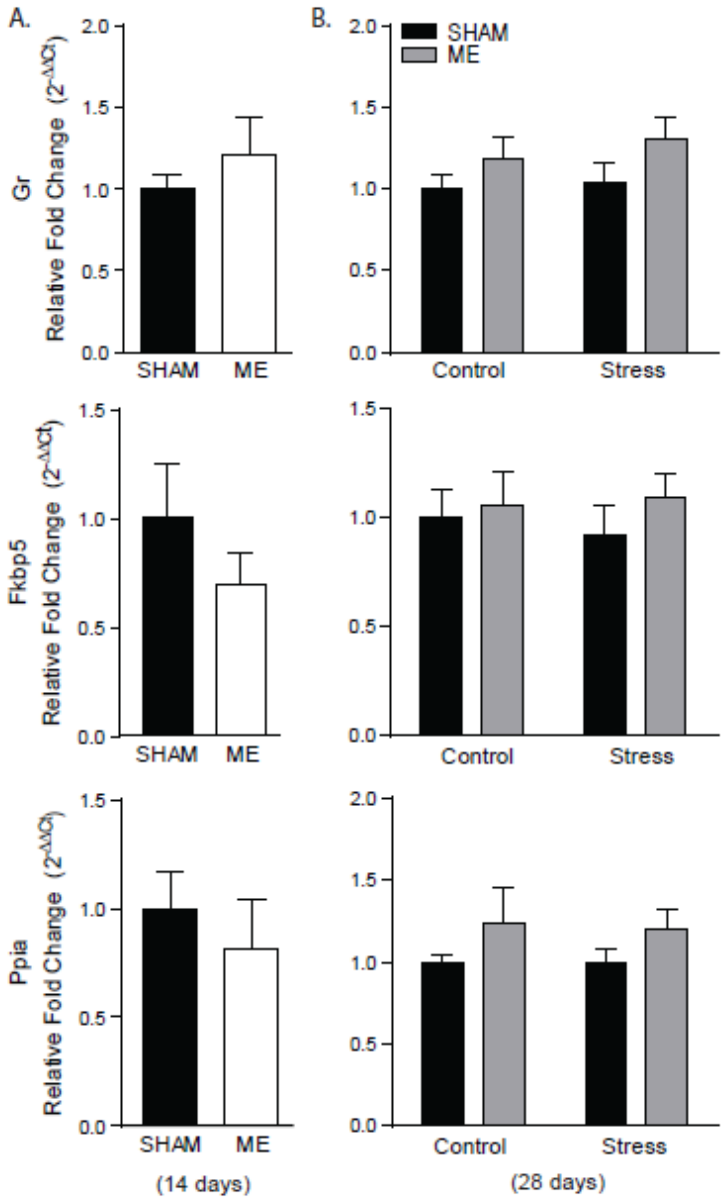

Figure 5.3 ME does not alter hippocampal gene expression of glucocorticoid receptor (Gr) or Gr co-chaperones at baseline or following social defeat stress.

At baseline (two weeks following ME-injury) hippocampal gene expression of the glucocorticoid receptor (*Gr*) and co-chaperones *Fkbp5* and *Ppia* was measured. No differences were detected between sham levels and ME-treated rats, suggesting normal functioning of the receptor and translocation to the nucleus two weeks after ME injury (*A; p* > 0.05). Similarly, following two weeks of social defeat stress, no differences were detected between non-stressed and stressed sham and ME rats, indicating that a history of microvascular ischemia does not alter the Gr functional response to a subsequent stressor (*B; p* > 0.05).

procedures. Gene expression levels of ME rats were not significantly different from those of sham rats (Figure 5.3A; $p > 0.05$). Similarly, following two weeks of chronic social defeat stress, and four weeks following sham or ME procedures, gene expression of *Gr*, *Fkbp5* and *Ppia* were again assessed. For each marker measured, the same pattern emerged in which surgery lead to an increased gene expression of *Gr*, *Fkbp5*, and *Ppia*; however, only in measures of *Gr* did values approach significance (Figure 5.3B; main effect of surgery: $F_{1,28} = 3.14$, $p = 0.088$).

ME does not alter the inflammatory response to chronic stress

In the same cohort of chronically stressed rats, inflammatory markers were measured a total of four weeks after surgery and two weeks following exposure to chronic social defeat stress. A two-way ANOVA of *Mcp1* hippocampal gene expression reveals a main effect of surgery, illustrating an increase of the cytokine as a result of ME, while showing no effect of chronic stress on inflammatory expression levels (Figure 5.4A; $F_{1,31} = 7.93$, $p < 0.05$). Similar expression patterns were observed for *Spp1*, although this comparison failed to reach significance (Figure 5.4B; $p > 0.05$). Levels of chemokine receptor, *Xcr1*, were increased as a result of ME and reduced by chronic stress, though again, this comparison failed to reach statistical significance (Figure 5.4C; $p > 0.05$).

DISCUSSION

The presence of microvascular ischemia alters subsequent responses to an acute stressor. In contrast, microvascular ischemia did not alter modulators of HPA axis activity, specifically glucocorticoid receptor functioning as indicated by gene expression of the *Gr* and co-chaperones *Fkbp5* and *Ppia* in the hippocampus at baseline or following a subsequent chronic stressor.

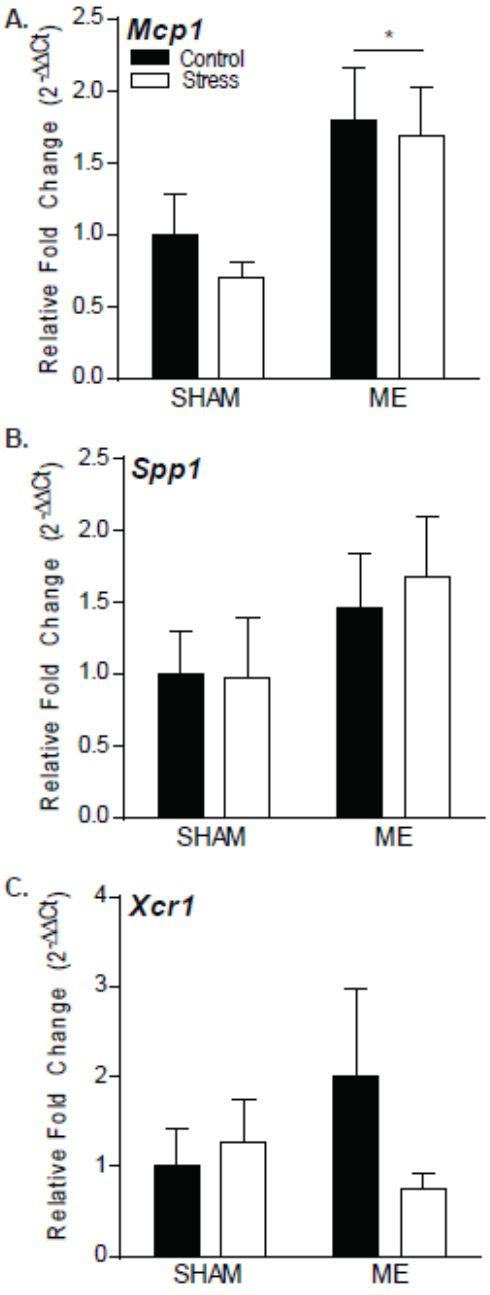

Figure 5.4 Chronic social defeat stress after ME injury does not alter hippocampal expression of inflammatory cytokines.

At four weeks post-ME injury, no differences in expression of *Il-1β*, *Spp1*, and *Xcr1* were detected. Expression levels of chemokine *Mcp1* were increased in both ME stress and non-stressed rats, suggesting a prolonged activation of inflammation that is not influenced by chronic stress (main effect of surgery, $F_{1,31} = 7.93$, $p < 0.05$) .

Additionally, ME damage, in combination with two weeks of chronic social defeat stress had no effect on prolonging ME-induced inflammatory activation.

The current findings are in agreement with previous reports demonstrating altered CORT responses following global ischemia (de la Tremblaye et al. 2014). In such cases, transient elevations in CORT levels precede a return to baseline; however, for up to one month following injury, CORT responses to subsequent stressors are enhanced (de la Tremblaye et al. 2014). These findings are replicated in the current set of experiments though overall effects of ME on HPA axis function may be dampened due to both the acute nature of ME injury and the young age of the rats used in the current experiment (Merrett et al. 2010).

Independently, stress exposure and ischemia both up-regulate components of the HPA axis and increase inflammatory activity (Zunszain et al. 2011). Such stress-induced changes, particularly in the hippocampus, are well documented (Koo et al. 2010) and stress induced increases in inflammatory cytokines have been detected in response to chronic mild unpredictable stress (Grippo et al. 2005; Koo et al. 2010), social stress (Slavich et al. 2014), and foot-shock (Blandino et al. 2009). When functioning normally, inflammatory cytokines can stimulate glucocorticoid secretion and conversely, glucocorticoids temper the inflammatory response (Turnbull et al. 1999). In the current study, however, levels of inflammation are not attenuated by chronic stress in the presence of ME which may suggest no HPA axis involvement at this late time-point; however, more work will be necessary to determine the exact nature and duration of HPA axis activity and its influence over inflammation.

In our experiment, chronic social defeat stress was used to evoke heightened HPA axis activity in order to determine if more severe stressors were necessary to elicit and detect subtle dysfunctions or sensitivities in HPA axis function. Social defeat stress was an effective stressor

as evidenced by suppressed weight gain in stress exposed animals; however, no stress specific changes were detected in Gr levels or function, as indicated by co-chaperone expression levels. Previous work has shown that the effects of stress compounded with acute ischemia exacerbate the physiological effects of ischemia alone (Craft et al. 2009; Caso et al. 2008); however, though two weeks of chronic social defeat stress was an effective stressor, in our hands, it was not sufficient to elicit an exacerbated inflammatory response immediately after stress exposure. Specifically, hippocampal gene expression levels of *Mcp1* and *Spp1* were maintained two weeks following stress, and four weeks following surgical procedures. *Mcp1* and *Spp1* are of noted importance in both ischemia and behavior and will be discussed further in Chapters 6, 7, and 8 as they relate to ME. *Mcp1* is commonly increased following ischemia, correlates to neurologic dysfunction (Strecker et al. 2011) and is increased in depression (Raison et al. 2006). *Spp1,* or osteopontin, involved in cell adhesion, chemoattraction, and modulation of immune cells (Zollo et al. 2012), is frequently used as a marker of inflammation and has been noted to be neuroprotective following ischemia in rodents (Chen et al. 2011).

An additional mechanism by which endocrine/immune interactions occur is via gene activation. Glucocorticoids bound to the Gr activate the receptor to migrate to the nucleus. At physiological baseline, release of Gr from co-chaperones commences translocation. Among many other genes transcribed by glucocorticoids are those targeted towards anti-inflammatories (*e.g.* secretory leukoprotease inhibitor) and towards inhibiting inflammatory pathways (*e.g.* mitogen-activated protein kinase phosphatase-1, which inhibits MAP kinase). Furthermore, cytokines can reduce Gr nuclear translocation, therefore reducing Gr function and the functional response (Barnes 2010). In our set of experiments, expression levels of *Gr*, as well as its co-chaperones, were not significantly different from sham values, suggesting unaltered gene

transcription. Moreover, at four week post-injury, levels of inflammatory cytokines in non-stressed animals versus chronic stressed animals were not significantly different, and only *Xcr1* shows evidence for glucocorticoid reduction of inflammatory activity, though this relationship failed to reach significance (see Figure 5.4C). Despite these findings, others report stress to lead to changes in behavioral anhedonia, increased CORT, as well as increases in TNF and IL1β (Grippo et al. 2005), illustrating parallel activity of the two systems.

Measuring HPA output and cytokine levels at times distant from injury are not unfounded. Following injury, stroke patients are at an increased risk of infection, atherosclerosis (McColl et al. 2009), and depression for up to two years (Whyte et al. 2004) and increases in cytokine expression and circulating glucocorticoids have been detected in patients for months after ischemia. In experimental stroke, antagonization of regulatory T lymphocytes, thymus-derived T cells responsible for modulating the immune response, reduced infarct size and the infiltration of damaging TNF and interferon-y (IFN- y) (Liesz et al. 2009). Furthermore, infarct size reduction has also been accomplished via administration of minocycline (to inhibit microglial activity), and by blocking IL-1 or TNF. Similarly, following stroke in a rat four-vessel occlusion model, increases in Gr, CRF, and CRF1 were detected in the brain, with increased CORT secretion persisting for seven days following injury. Additionally, acute stress led to a greater stress response in stroke animals 27 days following stroke, demonstrating long-term HPA axis dysregulation (de la Tremblaye et al. 2014). Taking these long-term effects into consideration, this relationship was nicely addressed in a work by Craft and DeVries (2006) in which BL/6 mice were administered 60 minutes of unilateral middle cerebral artery occlusion (MCAO) or sham surgery and four days later administered a Gr antagonist, Gr synthesis inhibitor, or an inhibitor of the IL-1 receptor. Interestingly, only inhibition of cytokine activity

reversed the anhedonic effect of the MCAO surgery and no treatment-altered infarct size. These findings suggest that inflammation influences the behavioral response to focal ischemia more so than Gr. Furthermore, these findings support the dissociation between behavior and infarct volume following stroke injury. Still, the mechanisms behind the brain and behavior interaction are not clear, and may still include an indirect influence of endocrine signaling.

The experiments described within this chapter begin to address the complexity of endocrine and immune responses to microvascular ischemia. As mentioned earlier, the immune-endocrine interaction in response to focal stroke has been widely studied in rodents, and these findings have both answered questions and sparked hypotheses. Much of our understanding of immune-endocrine crosstalk is independent of ischemia, as we know that catecholamines and glucocorticoids regulate the expression of cytokines, and that immune stimuli can regulate adrenal function (Deak 2008; Blandino et al. 2006). Further, peripheral administration of both IL-1 and TNF resulted in increased circulation of corticotropin releasing factor (CRF) as well as release of CRF from specific nuclei of the hypothalamus (Turnbull et al. 1999). To demonstrate the reverse, psychological stress-induced activation of Toll-like receptor 4, a receptor active in the innate immune system's detection of pathogens, resulted in increased production of NF-κB and the subsequent transcription of IL-1β and TNF in the brain (Caso et al. 2008). Thus, more work is necessary to determine how ischemia affects this complex set of relationships.

As discussed, mechanisms of immune-endocrine interactions are numerous, and the results are strongly influenced by species, strain, and the timing and nature of injury. With these variables and the intricate nature of immune-endocrine interactions, it is difficult to determine a causative or exacerbating role for inflammation in HPA axis activity following ME injury. It is our goal that the experiments described within this chapter lay the foundation for the further

study of HPA axis and inflammatory interactions in the context of microvascular injury. The use of specific inhibitors of inflammation (*e.g.* minocycline) or CRF (*e.g.* dexamethasone) and altered timing of stress exposure will be fruitful in continuing to disentangle the roles of these systems in microvascular ischemia and functional outcome. Based on these findings, however, and the prolonged activation of inflammatory cytokines following ME, further studies address the role of inflammation in mediating ME-induced behavioral disruption.

CHAPTER SIX

MICROEMBOLISM INFARCTS INDUCE LONG-TERM NEUROINFLAMMATION

Immediately following an ischemic event, a series of biochemical events are set into motion which include activation of inflammatory pathways (Lakhan et al. 2009; Kriz et al. 2009; Xia et al. 2010). The response launched by the immune system is characterized by the activation of various cell types including microglia, astrocytes, neutrophils, and peripherally infiltrating B and T cells. These cells act in a pleiotropic fashion, releasing inflammatory cytokines, chemokines, as well as neurotrophic and cytotoxic factors that migrate to affected regions of the brain.

Inflammatory cytokines are well known for their self-propagating activity (Turnbull et al. 1999) and pleiotropic effects: triggering apoptotic pathways and promoting the health and

survival of neurons (Frank-Cannon et al. 2009). Following brain injury, nuclear factor kappa B (NF-κB), a transcription factor critical for regulating numerous inflammatory pathways, is increased and capable of inducing the transcription of several other inflammatory markers (Lakhan et al. 2009). Upregulated cytokines, such as interleukin-1 beta (IL1β), tumor necrosis factor-alpha (TNF), and interleukin 6 (IL6), are correlated to ischemic infarct size and rate of survival, such that inhibition of cytokine activity reduces infarct size and increases the rate of survival (Craft et al. 2006; Yamasaki et al. 1996; Spalletta et al. 2006).

More recently, dysregulated inflammatory activity has been linked to changes in mood (Raison et al. 2006; Song et al. 2011) - a relationship that is frequently observed in autoimmune and neurodegenerative diseases (Zunszain et al., 2011) and the elderly (Taylor et al. 2013). Because inflammatory cytokines are known to interact with a wide range of processes involved in changes of mood, including HPA activity, neurotransmitter metabolism, and neuroendocrine function, to name a few (Miller et al., 2009; Song & Wang, 2011; Zunszain et al., 2011), there are several mechanisms by which inflammatory cytokines may influence behavior. Animal studies have demonstrated that the direct application of IL1β to the brain or periphery induces anxiety-like behaviors, memory impairment, and even sickness behaviors that may last beyond the duration of cytokine treatment (reviewed in Song & Wang, 2011). Similarly, in humans, sickness behaviors are present in those with elevated circulating levels of inflammatory markers, and those administered cytokines (lipopolysaccharide or interferon-α, for example) exhibit an increase in anxiety and depressed mood, the symptoms of which are often severe enough to satisfy DSM-V criteria (Miller et al. 2009).

The salience of peripheral inflammation and the crosstalk between peripheral and central markers of inflammation are of growing recognition in terms of controlling injury after an

ischemic event and in treatment of these injuries (Xia et al. 2010; Besedovsky et al. 2011). Inflammatory crosstalk is a long-lasting event following brain ischemia; however, few study the peripheral response to brain injury despite its potential therapeutic benefit (Xia et al. 2010; Sekeljic et al. 2012).

In the current set of experiments, we use the same rodent microsphere (ME) injection model that has been used previously to demonstrate the sufficiency of diffuse microembolic lesions to produce delayed behavioral disruptions (Nemeth et al. 2012) to test the hypothesis that diffuse microembolic lesions are sufficient to produce long-term elevations of innate immune system activity. Importantly, this set of experiments will also reveal if ME lesions are sufficient to lead to activation of peripheral inflammatory processes and increased disruption of blood-brain barrier permeability. These findings may shed light on specific inflammatory markers that, 1) play a role in chronic inflammation following ischemic injury; and 2) may serve as potential therapeutic targets.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 months of age, Charles River) were pair-housed until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity-controlled vivarium. *Ad libitum* food and water were available throughout the duration of the study. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Surgical Procedure

Following a one week acclimation period, the rats were randomly assigned to sham or

ME surgical groups. Rats were anesthetized with isoflurane and secured in a supine position. An incision was made and the common carotid artery was isolated and ligated with suture followed by ligation of the external carotid artery at the bifurcation with the internal carotid artery. The surgeon injected microspheres (New England Nuclear Inc., Boston, MA; 50 µm in diameter; suspended in 10% Dextran and 0.01% Tween in isotonic saline; PerkinElmer Instruments; Shelton, CT; ≈2500 spheres in 50 µL) with a 30 G needle inserted into the left internal carotid artery. Following a complete injection, direct pressure applied to the injection site facilitated the cessation of bleeding from the injection site, then ligatures were released and blood flow returned. Sham animals underwent an identical procedure without the infusion of microbeads.

Gene Expression

Brain tissue was collected 14 days following ME procedures, frozen and then dissected under RNAse-free conditions. Regions were bilaterally isolated for analysis. Due to its involvement in mood disorders (Campbell et al. 2004; Stockmeier et al. 2004) and its sensitivity to ischemia (Böttiger et al. 1999), the hippocampus was the focus of gene expression analyses. Genes were standardized to lactate dehydrogenase A and normalized to sham animals in accordance with $2^{-\Delta\,\Delta CT}$ calculations. The array was processed according to manufacturer's instructions and carried out on an Applied Biosystems HT7900 Fast Real-Time PCR system (Carlsbad, CA).

For all other time-points (1 day, 2 days, 3 days, 7 days, 28 days, and 42 days), genes of interest were limited to tumor necrosis factor α (*Tnf*; Rn01525859_g1), monocyte chemotactic protein-1 (*Mcp1*: Rn00580555_m1*)*, interleukin 1β (*Il-1β*; Rn00580432_m1), secreted phosphoprotein 1 (*Spp1*; Rn01449972_m1), nuclear factor kappa-light-chain-enhancer of

activated B cells inhibitor-alpha (Nfκbia; Rn01473657_g1), and complement receptor 3 (*Cd11b*; Rn00709342_m1), because these markers are among the most active as detected by the SABiosciences gene array plate and relevant to mood disruption (Zunszain et al. 2011; Raison et al. 2006; Song et al. 2011). For confirmation purposes, RNA from the Day 14 samples was run against each individual primer. Additionally, to determine the degree to which inflammation generalizes to the contralateral hemisphere, *Tnf* and *Mcp1* were assessed in the contralateral hippocampus of Day 14 samples. Gene expression of peripherally infiltrating T and B cells was measured in ipsilateral Day 14 samples via hippocampal detection of *Cd3* (using forward primer CAGAACTGTGTGGAGCTGGA and reverse TGCTCGTTCTTCAACAGGAC) and *Cd19* (using forward primer CAGTGTGGCTCTGGCTGTT and reverse CCTAGCAGGGTCGGTCATT), respectively. For all, brains were dissected as above, homogenized, and RNA was extracted using the Qiagen RNeasy Mini Kit (Valencia, CA) according to manufacturer's instructions. RNA samples were reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Resulting cDNA was then quantified and normalized using the PicoGreen method (Invitrogen, Grand Island, NY). All samples were prepared in triplicate using 1 µg of sample and carried out on an Applied Biosystems HT7900 Fast Real-Time PCR system.

Blood-Brain Barrier Permeability

Perfused sham and ME tissues were rinsed in 0.3% H_2O_2 for 10 minutes followed by a one hour blocking step (10% normal goat serum, 0.2% triton x-100 in PBS), and an overnight incubation in a primary antibody solution (1:1000 biotinylated anti-rat IgG; Vector Labs). Visualization was completed with a one hour incubation in ABC solution, according to the manufacturer's instructions, and an approximate five minute incubation in a DAB solution

(Sigma) until the desired intensity was reached. Sections were mounted on Superfrost Plus Microscope Slides, imaged on a Nikon Eclipse 90i microscope (Melville, NY), and analyzed using Image J 1.44 (NIH).

Data Analysis

Array data were analyzed by $\Delta\Delta C_t$ based fold-change calculations using the SABiosciences PCR Array web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and results were considered significant when relative mRNA expression was two-fold different from that of sham animals. Standard PCR data were averaged by group and analyzed via the $2^{-\Delta\Delta CT}$ method. Specifically, fold change was calculated, standardized to a housekeeping gene (*Tfrc*; Rn01474701_m1), and normalized to sham (gene expression time-line) or vehicle treated (pharmacological inhibition). For the gene expression time-line, data were analyzed using a one way ANOVA. When appropriate, specific differences were detected using Bonferroni's multiple comparison tests, with each time-point compared to the sham value. A two-way Student's t-test was used to detect differences in hippocampal gene expression in contralateral (to ME injection) tissue. For all, differences were considered significant when $\alpha < 0.05$.

RESULTS

ME infarction increases gene expression of inflammatory cytokines for up to 14 days post-injury

Figure 6.1 shows the results of a gene array demonstrating an increase in 43 of 84 measured inflammatory cytokines and receptors on Day 14 following ME procedures (summarized in Table 6.1). Identified candidate inflammatory cytokines and receptors likely involved in the manifestation of behavioral deficits as determined by the gene array were isolated

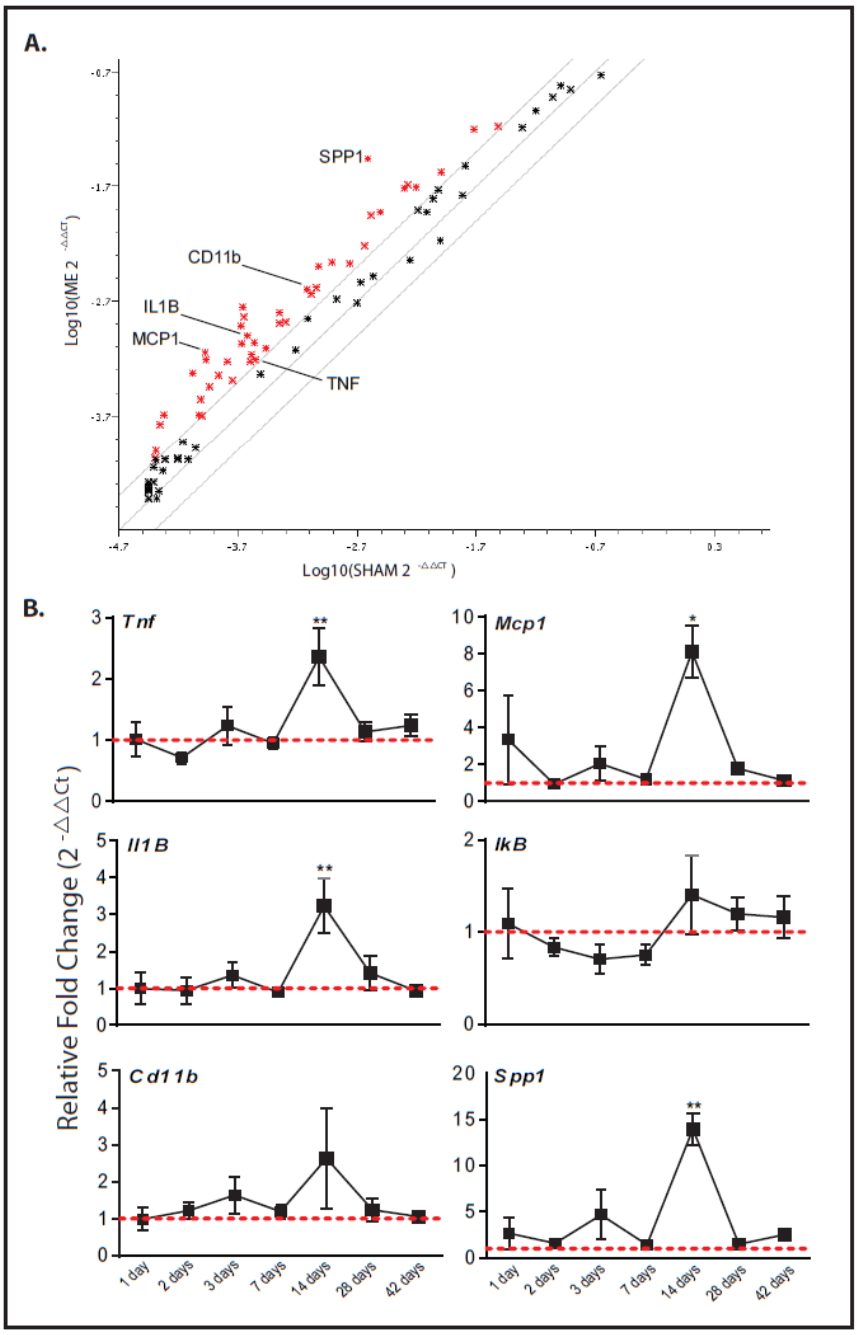

Figure 6.1 ME infarcts increase hippocampal gene expression of cytokines and receptors

Genes related to inflammatory cytokines and receptors are elevated in microembolism (ME) relative to sham. (A) Scatter plot of data at two weeks post-lesion reveals 43 of 84 genes related to inflammatory cytokines and receptors are elevated beyond a two-fold change (dots located above midlines). Graph represents the log10 of normalized gene expression levels. Increases in gene expression for *Tnf*, *Mcp1*, *Il1β*, and *Spp1* were detectable 14 days following ME infarction and were examined over an extended timeline (B). Graphs depict significant increases in gene expression 14 days following ME infarction for *Tnf*, *Mcp1*, *Il1β*, and *Spp1*. Dotted line represents sham levels. Error bars indicate standard error of the mean (S.E.M.). * indicates $p < 0.05$.

Table 6.1 Table of hippocampal genes upregulated following ME.

	Fold Δ	95% CI
Cxcr5	2.68	(0.00001, 6.25)
C3	3.20	(0.00001, 9.62)
Casp1	3.82	(0.00001, 8.99)
Ccl11	3.88	(0.00001, 8.18)
Ccl12	5.69	(0.00001, 15.97)
MCP1	8.11	(0.00001, 24.17)
Ccl24	2.62	(0.00001, 5.45)
Ccl3	6.56	(0.00001, 21.52)
Ccl4	5.81	(0.00001, 17.98)
Ccl5	3.36	(0.01, 6.71)
Ccl6	4.33	(0.00001, 10.28)
Ccl7	4.23	(0.00001, 11.44)
Ccl9	5.83	(0.00001, 18.79)
Ccr1	2.87	(0.58, 5.16)
Ccr2	3.17	(0.00001, 7.34)
Ccr3	3.68	(0.00001, 8.97)
Ccr5	3.86	(0.00001, 10.98)
Ccr6	2.85	(0.00001, 6.73)
Ccr7	2.00	(0.00001, 4.03)
Cx3cr1	3.28	(0.05, 6.51)
Cxcl1	2.31	(0.00001, 5.02)

	Fold Δ	95% CI
Cxcl10	4.24	(0.00001, 10.80)
Cxcl11	6.72	(0.00001, 19.38)
Cxcl2	2.40	(0.05, 4.75)
Cxcl9	2.21	(0.00001, 5.35)
Il10ra	3.60	(0.00001, 7.48)
Il11	2.27	(0.80, 3.74)
Il13ra1	2.87	(0.00001, 7.98)
Il15	2.46	(0.00001, 6.18)
Il1a	2.80	(0.00001, 6.34)
Il1b	3.23	(0.00001, 7.01)
Il1r2	4.01	(0.10, 7.92)
Il2rb	3.37	(0.00001, 6.91)
Cxcr2	2.20	(0.00001, 4.47)
CD11b	3.51	(0.00001, 7.63)
Itgb2	3.84	(0.00001, 9.08)
SPP1	13.96	(0.00001, 47.31)
Tgfb1	2.64	(0.00001, 5.40)
TNF	2.36	(0.00001, 5.15)
TNFrsf1b	2.66	(0.00001, 5.61)
Tollip	2.18	(0.00001, 4.98)
Xcr1	4.29	(0.00001, 9.99)

Table shows inflammatory cytokines and receptors upregulated beyond a two-fold change two weeks after microsphere embolism (ME) procedures.

and measured over an expanded time-course that included 1, 2, 3, 7, 14, 28, and 42 days. A one-way ANOVA of *Tnf* values revealed a significant difference of expression over time (Figure 6.1B *;* $F_{7, 49} = 4.205$, $p < 0.01$). Furthermore, *Tnf* at Day 14 was significantly elevated above sham values, and was also higher than *Tnf* expression at the 1, 2, 7, 28, and 42 day time-points. Similarly, a one-way ANOVA of *Mcp1* data revealed a significant effect of time on gene expression (Figure 6.1B*;* $F_{7, 49} = 6.681$, $p < 0.01$) and levels of *Mcp1* were increased 14 days after ME relative to both sham levels of gene expression and expression levels at other time-points post-ME. Analysis of ME rats compared to sham revealed a difference of *Il-1β* ($F_{7, 49} = 4.205$, $p < 0.01$; Figure 6.1B). Specifically, sham values differed from ME rats at the 14 day time-point, and the 14 day time-point differed significantly from each other time-point. Similarly, differences were also detected in expression levels of *Spp1* (Figure 6.1B) such that Day 14 levels differed significantly from sham values. In contrast, microembolism procedures had no effect on hippocampal *IκB* or *Cd11b* expression levels, as values did not differ in comparison to sham or any other time-point, $p > 0.05$ (Figures 6.1B.

Analysis of the contralateral hippocampus of ME-injected rats on Day 14 revealed an increase in gene expression of *Mcp1* as compared to sham rats (Figure 6.2; $t(10) = 2.51$, $p = 0.031$). In contrast, levels of *Tnf* in the contralateral hemisphere of ME rats did not different from sham levels ($p > 0.05$).

ME infarction increases blood brain barrier permeability and CNS expression of B cells

Optical density analysis of a representative sham and ME brain show increased staining of IgG throughout brain tissue, (Figure 6.3; $t(14) = 4.405$, $p < 0.001$). Further, infiltrating T and

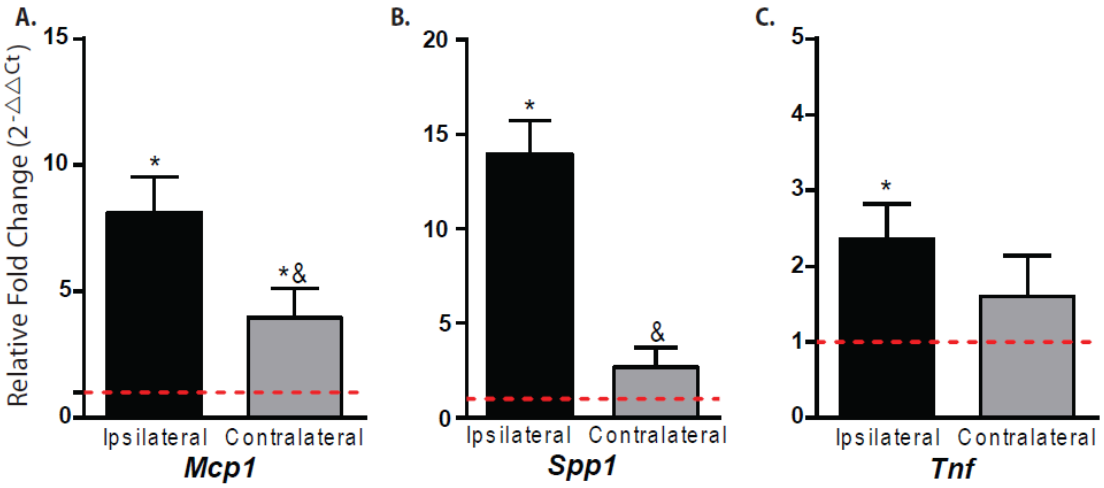

Figure 6.2 Elevations of inflammatory cytokines not restricted to site of injury

Gene expression of *Mcp1* (A), *Spp1* (B), but not *Tnf* (C), in the contralateral hippocampus was increased following microsphere embolism (ME). Fold change calculations were standardized to sham rats and significance was determined as a difference from sham. Dotted line indicates sham levels. * indicates difference from sham; & indicates difference from ipsilateral levels. For all, error bars indicate standard error of the mean (S.E.M.) and significance is defined as $p < 0.05$.

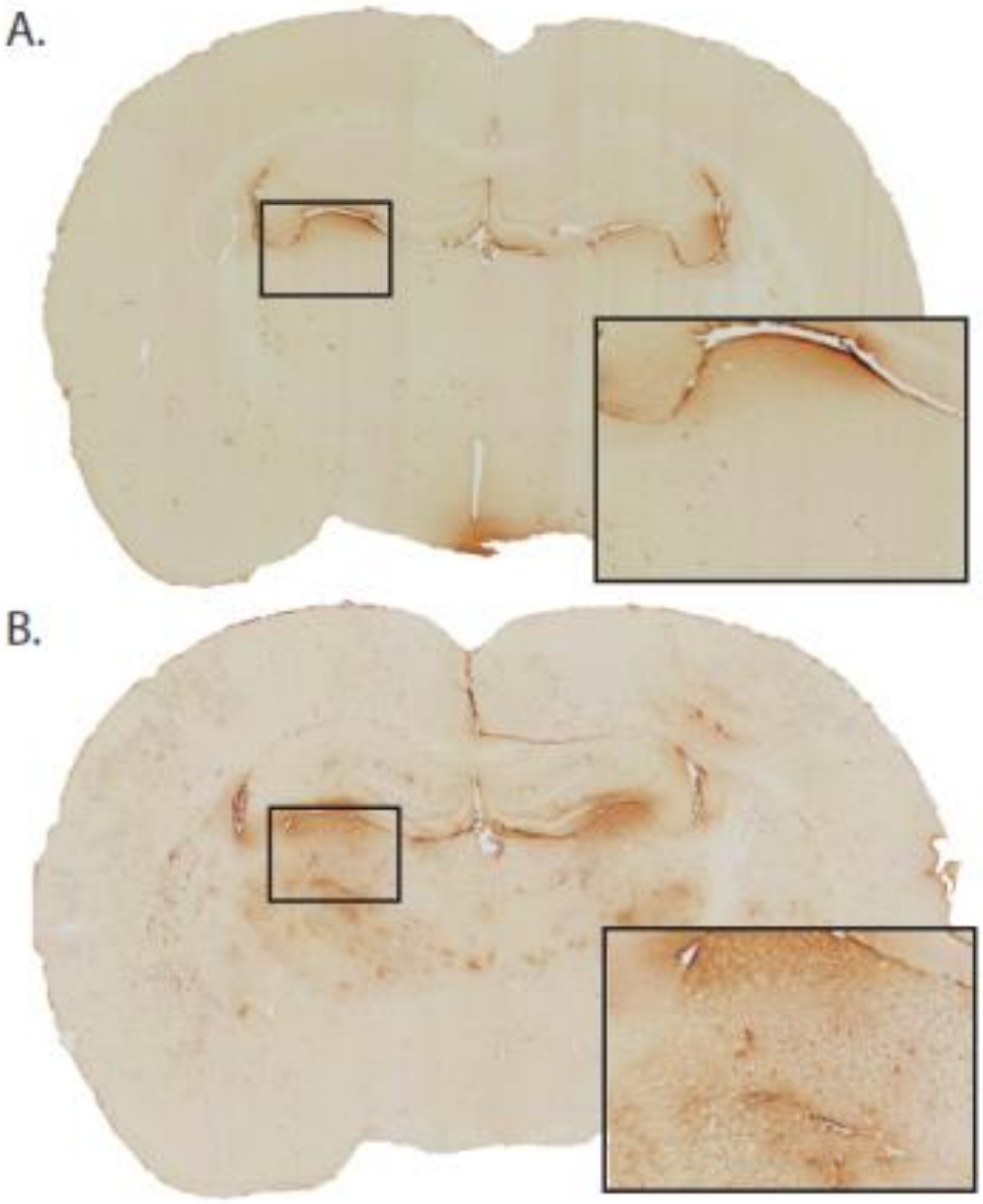

Figure 6.3 ME infarcts increase permeability of the blood-brain barrier

Representative brain sections of sham (A) and ME (B) operated brains were stained for rat IgG to demonstrate leakage of the blood-brain barrier. Optical density measurements reveal an increase in IgG stain in ME tissue compared to sham controls when compared with a Student's t-test.

B cells were measured in the hippocampus of both hemispheres two weeks following ME infarction. While no differences in gene expression levels of CD3+ T cells were detected, ME animals showed an almost 3-fold increase in ipsilateral hippocampal gene expression of *Cd19*+ B cells compared to the contralateral hemisphere and sham -operated controls (Figure 6.4; $F_{2,17} = 5.294$, $p = 0.018$).

DISCUSSION

The induction of ME infarction generates increases in an array of inflammatory cytokines at a time-point distant from injury demonstrating a mechanism by which behavioral disruption may occur. A more detailed examination of gene expression levels following microsphere embolism over time reveals a peak in inflammatory activity at 14 days, a time that correlates with increases in depressive-like and anxiety-like behaviors previously described in the same model (Nemeth et al. 2012). Additionally, inflammatory markers are increased in the hemisphere contralateral to the injury site, suggesting a globalization of physiological modifications in response to the unilateral injury that included an almost 3-fold increase in *Cd19*+ B cells, paralleled by increased permeability of the blood-brain barrier as demonstrated by IgG+ staining in ME tissue.

An unbiased gene array led to the identification of several upregulated gene targets that may participate in the manifestation of behavioral disruption following ME. Of particular interest is the delayed expression of peak levels of inflammatory markers (see Figure 5.1), time-point that is temporally consistent with previously reported behavioral disruption (see Figures 2.1 and 2.2; Nemeth et al. 2012). The sharp increase in inflammatory cytokine activity 14 days following the injection of microbeads has not previously been reported, although secondary activation of inflammatory cascades are not uncommon following ischemia

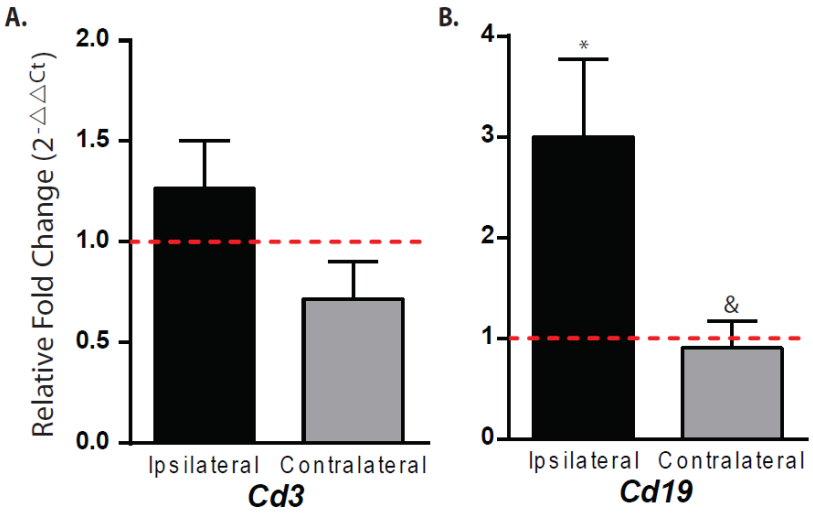

Figure 6.4 ME infarcts increase hippocampal expression of T and B cells

Gene expression of T and B cell markers, *Cd3* and *Cd19*, respectively, were measured in the ipsilateral and contralateral damaged hemispheres of the hippocampus two weeks following ME. While no changes in *Cd3* were detected (A), CD19+ B cells were upregulated in the ipsilateral-to-damage hemisphere when compared to both sham and contralateral levels (B). Dotted line represents sham levels of gene expression. For all, error bars indicate standard error of the mean (S.E.M.) and * indicates $p < 0.05$ difference from sham; & indicates difference from ipsilateral levels.

(Rodriguez-Grande et al. 2012; Mirabelli-Badenier et al. 2011) or other brain injury (Mannix et al. 2012). The peak of these inflammatory cytokines at 14 days is accompanied by the increased transcription of, at least, *Mcp1* in the hippocampus of the hemisphere opposite to direct insult, suggesting a more widespread generalization of inflammation that is not specific to the injury site. Increased and prolonged inflammatory activity in the brain contributes to secondary damage and may contribute to long-term behavioral and functional deficits (Loane et al. 2010).

Assessment of peripheral inflammatory markers in the hippocampus revealed an increase of CD19+ B cells, illustrating global inflammatory activity not limited to the innate response. B cell recruitment to the brain occurs rapidly following a cerebral event, and though B cells are capable of differentiation locally within the CNS, increases in blood-brain barrier permeability and increased chemotaxis from cytokines such as *Mcp1*, as described here, facilitate migration of peripheral cells to the brain (Meinl et al. 2006; Man et al. 2012). Few studies examine peripheral inflammation following cerebral ischemia; however, infiltrating immune mediators contribute to ischemic damage and perpetuate central inflammation via local cytokine production, and therefore may serve as potential therapeutic targets (Xia et al. 2010; Meinl et al. 2006).

The time window of increased inflammation following ME is interesting due to its temporal overlap with increased depressive- and anxiety-like behaviors. As referenced in Chapter 2, ME-treated rats show disrupted affective like behavior when evaluated 14, but not 4 days, following ME procedures (Nemeth et al. 2012). Similarly, as discussed within this chapter, inflammation is not significantly increased at short-term time-points. Like focal stroke, microvascular ischemia is characterized by primary injury, and by a delayed sequence of secondary injury. These delayed effects evolve beginning minutes after injury and can progress for months after initial injury (Karki et al. 2010; Moxon-Emre et al. 2010; Kumar et al. 2012;

McColl et al. 2009). Mechanisms of secondary injury include disrupted ionic homeostasis, mitochondrial dysfunction, apoptosis and necroptosis, the release of neurotransmitters, and the activation of the inflammatory and immune responses (thoroughly reviewed in Kumar et al. 2012). Disrupted behavior observed in ME rats may therefore be a response initiated by the delayed activation and upregulation of cytokine activity. Along these lines, several studies have supported the differential role of inflammatory cytokines during the primary verses secondary phases of injury and the idea that the accumulation of these processes contribute to ischemic damage and poor prognosis. (Kumar et al. 2012; Ziebell et al. 2010) Finally, and as discussed in later sections, the timing of secondary injury may be an important consideration in therapeutic strategies, as "windows" of treatment are a concern following traditional stroke injury.

Importantly, these findings illustrate that acute and diffuse ME lesions are sufficient to induce long-term inflammatory activity that is not restricted to the site of injury. Increases of pro-inflammatory cytokines are well evidenced in other animal models of ischemia (Maddahi et al. 2011) as well as in acute ischemia in humans (Supanc et al. 2011) and in cases of post-stroke depression (Spalletta et al. 2006), major depression (Raison et al. 2006; Miller et al. 2009), and anti-depressant resistance (Raison et al. 2012). The relationship between activation of inflammatory cytokines and mood disturbances, independent of the presence of brain trauma, supports the use of anti-inflammatories to alleviate depressive symptoms and will be discussed in the next chapter (Loftis et al., 2010; Miller et al., 2009).

In conclusion, the data presented demonstrate increased and prolonged inflammatory activity following the induction of ME via microsphere-mediated damage. Based on the temporal congruence of the peak in cytokine expression with previously reported increases in depressive-like and anxiety-like behaviors (Nemeth et al. 2012), we hypothesized that there may

be a causal relationship between the elevated cytokine expression and behavioral disruption. The current findings are significant because they demonstrate that inflammation is both global and long lasting in a rodent model of microsphere embolism. We can speculate that in humans, acute microembolism, though appearing to be phenotypically silent, can result in chronic activation of inflammatory markers that progresses towards damaging cellular and functional consequences. These damaging processes may then potentially sensitize the inflammatory response to subsequent embolic events. Therefore, the identification and targeted inhibition of such markers or related mechanisms may lead to the development of novel therapeutics to address this subset of behavioral disorders.

CHAPTER SEVEN

INHIBITION OF SOLUBLE TNF ATTENUATES MICROEMBOLISM-INDUCED NEUROINFLAMMATION AND IMPROVES BEHAVIORAL OUTCOME

As discussed throughout this document, vascular damage initiates a sequence of events leading to stroke-like brain injury (Maddahi et al. 2010; del Zoppo et al. 2010). Even at the microvascular level, the activation of inflammatory processes following ischemic damage results in subtle brain injury that accumulates over time. The contribution of these inflammatory processes to the progression of cerebromicrovascular disease and the manifestation of mood and cognitive effects is recognized both in the laboratory (Nemeth et al. 2012) and in the clinic (Fang et al. 2009; Hoshi et al. 2005). However, though several studies have examined inflammatory messengers as biomarkers of depression and the effectiveness of anti-inflammatories in combination with anti-depressant treatment regimens (reviewed in Hayley, 2011), critical gaps in knowledge preclude the advancement of anti-inflammatory treatments.

Studies conducted in the microsphere embolism (ME) model, and described within

Chapter 6, demonstrate that in rodents, microvascular ischemia induces long-term alterations to the inflammatory profile in both the brain and the periphery as well as subtle changes to behavior. In humans, the inflammatory consequences of microvascular ischemia are less well defined; however, aging is associated with an increase in peripherally circulating cytokines, and pro-inflammatory states in this population are associated with declining cognition, executive function, and memory (Taylor et al. 2013). Thus, the identification of specific inflammatory biomarkers as potent participants in the progression of injury following ischemia and chronic inflammatory processes has become the goal of many research endeavors.

Increases in plasma TNF are detected in patients with major depressive disorders (Raison et al. 2012; Dannehl et al. 2014) and TNF is rapidly upregulated in the brain following injury (Maddahi et al. 2011; Spalletta et al. 2006). Like many inflammatory cytokines, TNF is pleotropic and serves to promote necroptosis, apoptosis, as well as angiogenesis and survival (Maddahi et al. 2011; Maeda et al. 2014). TNF activation initiates a cascade of sustained and prolonged cytokine activity, contributing to both delayed and chronic expression of inflammatory players (Yarilina et al. 2008). Contributing to both cell death and the further activation of inflammatory participants, TNF-induced 'programmed cell death', or necroptosis, indirectly fuels the maintenance of the inflammatory response (Davidovich et al. 2014; Maeda et al. 2014). During necroptosis and upon TNF binding, receptor-interacting protein kinase (RIP1 or RIP3) activation signals the release of intact mitochondria which serves as a 'danger signal' triggering the release of pro-inflammatory cytokines from neighboring immune cells (Maeda et al. 2014). This relatively new revelation demonstrates that programmed cell death, unlike apoptosis, can contribute to the pathogenesis and worsening of various disease states, including ischemia (Fayaz et al. 2014).

TNF signals via two receptors, TNF-Receptor 1 (R1) and TNF-R2. TNF-R1 is found in all cell types and is the primary signaling receptor for TNF, mediating both membrane-bound (tmTNF) and soluble forms of TNF (solTNF; Maddahi et al. 2011; McCoy et al. 2008). In contrast, TNF-R2 is the main signaling receptor type for membrane-bound forms of TNF, and recent studies have confirmed that the signaling cascades of the two receptor types are quite diverse. TNF-R1, containing an intracellular death domain (DD), promotes cell apoptosis, while TNF-R2 (lacking a DD) is associated with survival, neurogenesis, and proliferation (McCoy et al. 2008; Aggarwal 2003; Chadwick et al. 2009). Given the pleiotropic nature of inflammatory cytokines and the disparate signaling pathways of TNF-R1 versus TNF-R2, selective inhibition of pathways which promote cell death and toxicity may be beneficial to recovery after injury (Maeda et al. 2014).

As a proof of concept experiment, the current study tests the efficacy of a specific inhibitor of solTNF, XPro1595® (Steed et al. 2003; Brambilla et al. 2011; Barnum et al. 2014), to reduce ME-induced increases in hippocampal inflammation and to attenuate ME-induced changes of behavior. Specifically, XPro1595 was administered at two different time-points: immediately, to test the efficacy of inhibition when injury occurs concurrently; and delayed, to test the efficacy of inhibition when treatment occurs *after* ischemic and inflammatory cascades have been set into motion. Together, these experiments determine the extent to which solTNF and its downstream pathways regulate post-ischemic inflammation and functional outcome.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 months of age, Charles River) were pair-housed until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity-controlled vivarium. *Ad libitum* food and water were available

throughout the duration of the study. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Surgical Procedures

Animals were isoflurane anesthetized and secured in a supine position. Body temperature was maintained at 37 ± 0.5 °C throughout surgery via the use a homeothermic blanket system (Harvard Apparatus, Holliston, MA). An incision was made to the neck, and the common carotid artery was isolated and ligated with suture followed by ligation of the external carotid artery at the bifurcation with the internal carotid artery. Microspheres (New England Nuclear Inc., Boston, MA; 50 µm in diameter; suspended in 10% Dextran and 0.01% Tween in isotonic saline; PerkinElmer Instruments; Shelton, CT; ≈2500 spheres in 50 µL) were injected using a 30 G needle inserted into the left internal carotid artery. Following a complete injection, direct pressure applied to the injection site facilitated the cessation of bleeding from the injection site, then ligatures were released and blood flow returned. Sham rats underwent an identical procedure without the infusion of microbeads.

XPro1595® Dosing

Rats were randomly assigned to surgical and drug groups (ME/Vehicle; ME/XPro1595 Immediate; ME/XPro1595 Delayed; n=5 per surgical group). Because microvascular lesions occur silently and are incidentally detected in humans, we elected to test XPro1595 efficacy at two different time-points. Under this strategy we would be able to determine if such a pharmacological intervention could be effective *after* disease progression is initiated. Therefore, XPro1595 (10 mg/kg; *s.c.*) or saline was administered every third day beginning immediately after surgery (Me/XPro1595 Immediate) or on Day 7 (ME/XPro1595 Delayed; saline injections

prior to Day 7). XPro1595 is an engineered protein containing specific mutations of human solTNF that disrupt its binding to TNF receptors (Steed et al. 2003). Therefore, XPro1595 is detectable via standard protein detection systems and levels of XPro1595 in the cerebrospinal fluid (CSF) were quantified using a MesoScale Discovery (Rockville, MD) Human TNF kit (catalog no. K151BHC-2) just prior to euthanasia, and two days after the last XPro1595 injection.

Behavior

On Day 13 following surgery, rats were tested in the open field paradigm as a metric of locomotor and anxiety-like behavior. During their light cycle, animals were placed in the center of a 90 cm x 90 cm arena and allowed to explore for 10 minutes. Behavior was videotaped and analyzed using Cleversys Inc TopScan software (Reston, VA) by an individual blinded to treatment groups.

Gene Expression

Rats were rapidly decapitated and brain tissue was collected on Day 15 following sham or ME procedures. Tissue was dissected frozen under RNAse free conditions. Similar to assessments in Chapter 6, the ipsilateral hippocampus was used for all gene expression analyses. Gene expression analysis included quantification of monocyte chemoattractant protein 1 (*Mcp1*), cluster of differentiation molecule 11B (*Cd11b*) and tumor necrosis factor alpha (*Tnf*). Gene expression analyses were completed via SYBR® Green real time PCR quantification techniques. Genes were standardized to cyclophilin A and normalized to sham animals in accordance with $2^{-\Delta\Delta CT}$ calculations. All samples were prepared in triplicate using 1 µg of sample and carried out on an Applied Biosystems HT7900 Fast Real-Time PCR system (Carlsbad, CA).

Data Analysis

XPro1595 protein level in CSF, open field behavior, and gene expression data were averaged by group and separately compared via a one-way ANOVA with drug as the factor (vehicle, XPro1595 Immediate, XPro1595 Delayed). For all, findings were considered significant when $p \leq 0.05$; all analyses were calculated with Graph Pad Prism6.

RESULTS

XPro1595 administration did not alter metrics of overall health

Body mass was altered by neither ME procedures nor XPro1595 on Day 14 (Sham/veh 354.00 ± 9.36 g; ME/veh 366.17 ± 6.51 g; ME/Xpro1595 Immediate 376.83 ± 11.47 g; ME/Xpro1595 Delayed 366.80 ± 10.69 g). Further, overall distance traveled was not significantly different between groups administered vehicle or XPro1595 immediately or seven days following surgery (delayed), suggesting no effects of surgery or drug on overall locomotor activity ($F_{3,18} = 1.48$; $p = 0.25$).

XPro1595® blocked inflammatory gene expression in the hippocampus and rescued open field activity

To determine whether selective inhibition of solTNF could alter the inflammatory and functional outcome responses to ME infarction, XPro1595 was administered subcutaneously every third day as previously described (Brambilla et al. 2011; Barnum et al. 2014) with treatment starting either immediately at the time of injury or one week following ME-induction. XPro1595 delivered peripherally was detected in the CSF of Immediate and Delayed- dosed animals, with no detection in vehicle-treated rats, though high levels of variability within samples precluded a statistically significant difference (Figure 7.1; $F_{3,18} = 1.245$, $p = 0.25$, *ns*).

Assessment of hippocampal gene expression levels of *Tnf* following a 14 day recovery

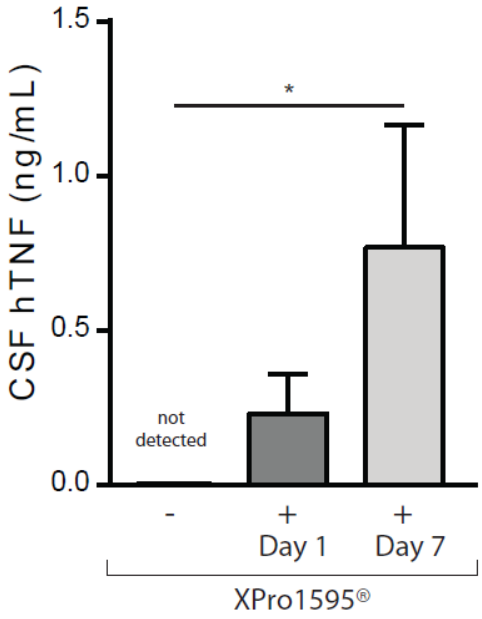

Figure 7.1 Protein expression of XPro1595 in cerebrospinal fluid of vehicle and drug treated animals

Protein levels of human TNF were measured in the cerebrospinal fluid (CSF) prior to tissue collection at two weeks using the MesoScale Discovery Human TNF-alpha kit. XPro1595 was detectable in the CSF at levels appropriate for target engagement demonstrating that XPro1595 was present within the CNS two days after the last dose. Results indicate similar levels of XPro1595 in the CSF of animals regardless of when dosing begins in relation to ME infarction, or duration of administration. For all, error bars indicate standard error of the mean (S.E.M.).

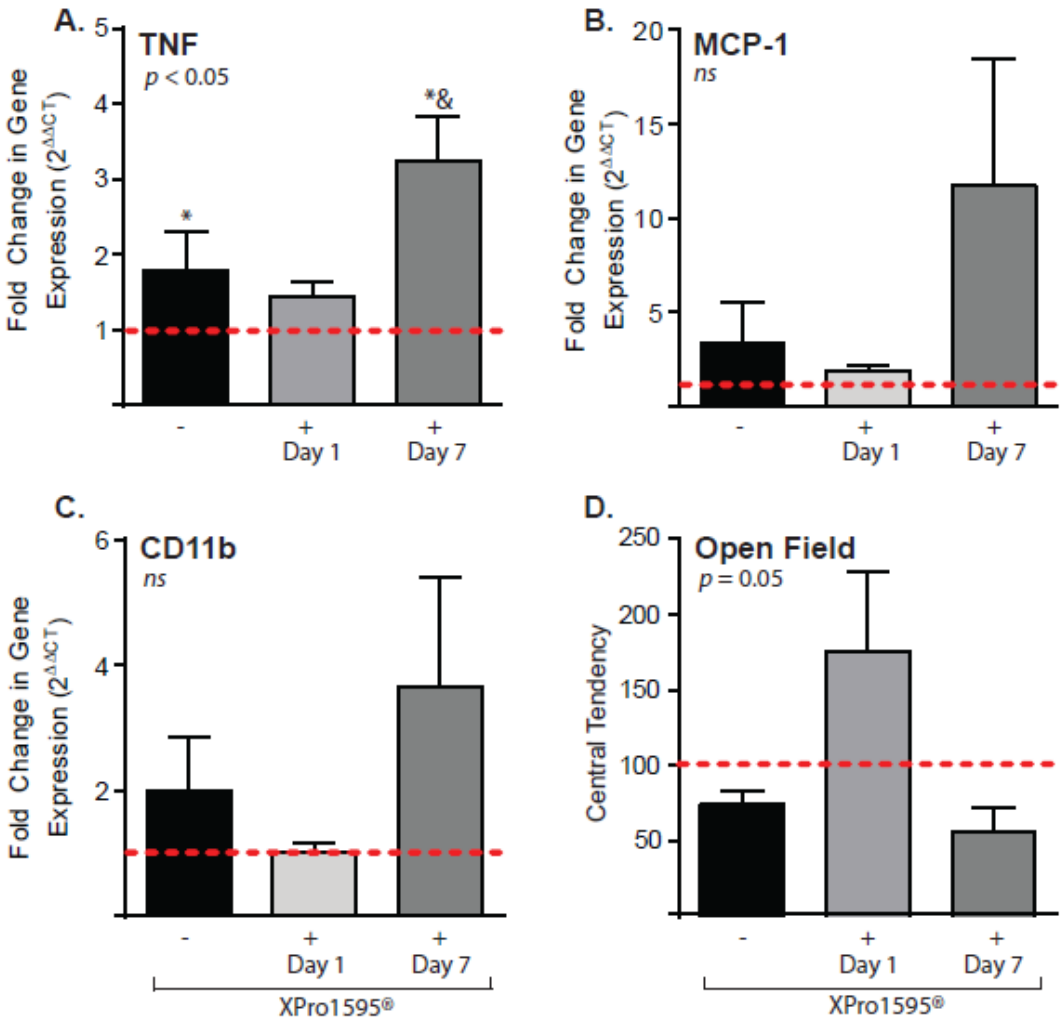

Figure 7.2 Administration of XPro1595 altered inflammatory and functional response to injury

Peripheral administration of XPro1595, an inhibitor of soluble *Tnf*, altered gene expression of in the hippocampus and modified behavior. XPro1595 attenuated *Tnf* mRNA in the hippocampus two weeks following the induction of microsphere infarcts (A; $F_{2,15} = 4.81$, $p < 0.05$). Bonferroni *post-hoc* tests confirm a differential effect of immediate versus delayed administration of XPro1595 (* indicates difference from sham; & indicates differences from XPro1595 Immediate, Day1; $p < 0.05$). Though patterns were consistent, no changes were detected in *Mcp1* and *Cd11b* in the hippocampus following XPro1595 administration (B,C). Similarly, a main effect of drug revealed differences in the ratio of distance traveled in the center versus the periphery of the open field (D; $F_{(2,16)} = 3.54$, $p = 0.05$). Dotted line represents sham levels. Data presented as percent of sham values. For all, error bars indicate standard error of the mean (S.E.M.) and * indicates $p < 0.05$.

revealed a significant effect of drug across ME groups ($F_{2,15} = 4.81$, $p < 0.05$). Bonferroni's multiple comparisons tests further show that expression levels of *Tnf* were dependent upon the dosing schedule of XPro1595, such that levels differ depending on whether XPro1595 was administered immediately (Day 1) or delayed until well surgery (Day 7; Figure 7.2A; $p < 0.05$).

Expression levels of *Mcp1*, and *Cd11b* in the hippocampus followed similar numeric patterns with immediate administration of XPro1595 most effectively inhibiting ME-induced hippocampal inflammation; however, these data failed to reach significance (Figure 7.2B,C; *Mcp1*: $F_{2,15} = 1.93$, $p = 0.18$, *ns*; *Cd11b*: $F_{2,15} = 1.63$, $p = 0.23$, *ns*). Activity in the open field was measured following XPro1595 dosing in ME-induced animals. Consistent with gene expression, administration of XPro1595 altered the ratio of distance traveled in the center versus the periphery of the open field (Figure 7.2D; $F_{2,16} = 3.54$, $p = 0.05$). Differences in velocity did not differ as a result of XPro1595 administration or timing of administration (data not shown; $p > 0.05$).

DISCUSSION

These findings demonstrate the proof of concept that a specific inhibitor of solTNF is sufficient to alter both hippocampal inflammation as well as open field behavior following ME in a time-dependent manner. In cases of microvascular ischemia, inflammatory cascades are set in motion covertly and therefore ideal treatment strategies begin after injury. Thus, we used a specific inhibitor of solTNF, XPro1595, to block solTNF through TNF-R1 and sought to explore if attenuation of this activity could occur at an early or delayed stage following ME.

Inflammation following brain injury is multi-faceted and includes processes that promote both cell survival and cell death pathways. The complex and often conflicting roles of individual cytokines are dependent upon several factors. For example, actions of inflammatory cytokines

vary based on location of action (core versus penumbra of damaged region) and timing (immediately after injury versus periods of recovery). Additionally, the pleiotropic functions of TNF are reported to be differentially mediated such that solTNF promotes inflammation within the brain while transmembrane TNF is neuroprotective (Maddahi et al. 2011; Yarilina et al. 2008; Chen et al. 2002; Taoufik et al. 2011). As mentioned, solTNF signals primarily through TNF-R1 and membrane-bound forms of TNF signal via TNF-R2 (TNFR2; McCoy et al. 2008; Maddahi et al. 2011) and therefore, complete inhibition of cytokine activity (thus preventing TNF signaling through both receptor types) can result in increased infarct size and poorer rates of survival (van den Tweel et al. 2006; Browne et al. 2006; Nijboer et al. 2008). As evidenced within the current set of experiments and others using XPro1595, timing of such inflammatory blockade is of importance (Nijboer et al. 2008; Barnum et al. 2014).

Peripherally administered XPro1595 resulted in alterations to both hippocampal gene expression of TNF and behavior in the open field, though insufficient power in our experimental groups precludes the detection of significant differences between specific experimental groups. Still, while inflammation in rats dosed immediately resembles vehicle-treated groups, delayed administration exacerbates inflammation, suggesting no effect of the drug. Our data support the tight regulation of TNF receptor function and suggest that timing of drug administration plays a critical role in its efficacy as an intervention. Furthermore, solTNF appears to be a necessary early component of the prolonged inflammatory response following ME; however, the ability of solTNF-specific pharmacological inhibition to attenuate inflammation is time-dependent. Because inhibition of solTNF with XPro1595 at a later time-point does not appear to alter levels of cytokine expression, despite XPro1595 being detectable within the brain to the same degree as seen following immediate administration (see Figure 7.1), chronic or increased cytokine activity

may not be dependent on solTNF alone. These findings are consistent with another study showing efficacy of XPro1595 when administration was begun three, but not 14 days, following experimental injury (Barnum et al. 2014). Other mechanisms of inflammatory activation may fuel cytokine increases. Mcp1, for example, has been shown to stimulate TNF expression following ischemia and LPS exposure (Rankine et al. 2006), further supporting the idea that in our experiment, inhibition of TNF may not be sufficient to attenuate inflammatory cascades once they have been set in motion. Alternatively, chronic activation of TNF may lead to activation and expression of other inflammatory genes that drive further inflammation. In this scenario, specific inhibition of TNF after these other cascades have been set in motion may do little to reduce overall inflammation.

In summary, timing is an important factor in the dosing strategy, as XPro1595 administered seven days after injury, and after the acute effects of injury had been initiated, was not effective in altering either behavior or hippocampal cytokine levels. Together, results from XPro1595 experiments support the idea of a 'therapeutic window' in which disease-modification can occur. Sustained cytokine signaling has been implicated in a wide variety of human diseases (Chen et al. 2002; Frank-Cannon et al. 2009; Tansey et al. 2010), and in the case of ME, initiation of these processes is often unnoticed, blurring the identification of the 'therapeutic window' and complicating therapeutic options. Experiments described in Chapter 8 highlight the use of commonly prescribed medications as a prophylactic option towards reducing inflammation and subsequent behavioral disruption.

CHAPTER EIGHT

MICROEMBOLISM INFARCTS INDUCE FUNCTIONAL DEFICITS THAT ARE ATTENUATED BY ANTI-INFLAMMATORY, BUT NOT ANTI-DEPRESSANT, TREATMENT

Despite being the most commonly prescribed treatment for late-life depression, anti-depressant response among the elderly population is poor (Baldwin et al. 2004; Kales et al. 2005; Taylor et al. 2013). The estimated percentage of elderly depressed patients with confirmed silent infarction who achieve sustained remission is 5%, compared with an estimated remission rate of 36% in patients without silent infarction (Yamashita et al. 2010). Compared to responders, non-responders exhibit higher degrees of depression, cognitive impairment, and impaired executive function (Baldwin et al. 2004). The poor response rate to anti-depressant therapies within the population suggests that neurochemical imbalances do not underlie this subset of depressive behaviors; however, few alternate mechanisms have been explored.

As discussed in previous chapters, processes related to inflammatory activation have

gained popularity in the field of depression as well as in the phenomenon of treatment resistance within depression. Recent studies in humans demonstrate that increased concentrations of inflammatory cytokines in blood plasma predict non-response to anti-depressant treatments (Raison et al. 2012), further supporting the idea of alternate mechanisms behind late-life depression. In Chapter 6, we illustrated that the induction of microvascular damage in rats leads to depressive-like behaviors and concurrently increased central and peripheral inflammation, and that that inhibition of inflammation *after* initiation of inflammatory pathways neither attenuates inflammation within the brain nor alters behavior (Chapter 7). Therefore, the experiments described in this chapter used the same model of microvascular damage to compare the effectiveness of prophylactically administered anti-inflammatories and anti-depressants in alleviating both the functional disruption and cerebral inflammation.

Patients with apparent cardiovascular risk factors are frequently maintained on low dose non-steroidal anti-inflammatory drugs (NSAIDS) to reduce the risk of ischemic events, and similarly, patients experiencing depressive episodes (of a vascular origin or not) are prescribed anti-depressant medications. In both of these scenarios, it is possible for low-grade and chronic inflammation to underlie disease progression. Thus, the current set of experiments mimic the chronic dosing of a commonly used NSAID and selective serotonin reuptake inhibitor (SSRI), meloxicam and fluoxetine, respectively, to test the effect of prophylactically administered therapeutics on cerebral inflammation and behavior. The experiments described herein shed light on the use of anti-depressants in vascular induced depression as well as the ability of anti-inflammatories to alleviate long-term inflammation and functional disruption.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 months of age, Charles River) were pair-housed until surgery. An AAALAC-approved facility maintained the rats on a reverse 14:10 light:dark cycle in a temperature- and humidity-controlled vivarium. *Ad libitum* food and water were available throughout the duration of the study. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Prophylactic Meloxicam and Fluoxetine Administration

In a separate cohort of rats and following a one week facility acclimation period, rats were habituated to handling with five-minute daily handlings for three days prior to meloxicam or vehicle administration. Rats were randomly assigned to vehicle (sham n = 12; ME n = 13) or meloxicam (sham n = 12; ME n = 13; 1 mg/kg) groups, and intraperitoneal dosing began four days prior to surgery and continued daily throughout the duration of the study. Surgery was performed as described above.

Fluoxetine was tested in a separate cohort of animals. Animals were habituated as before and were randomly assigned to vehicle (sham n = 12; ME n = 14) or fluoxetine (sham n = 11; ME n = 15; 10 mg/kg) groups. Intraperitoneal dosing began four days prior to surgery and continued daily throughout the duration of the study.

Surgical Procedures

Animals were isoflurane anesthetized and secured in a supine position. Body temperature was maintained at 37 ± 0.5 °C throughout surgery via the use a homeothermic blanket system (Harvard Apparatus, Holliston, MA). An incision was made to the neck, and the common carotid artery was isolated and ligated with suture followed by ligation of the external carotid artery at the bifurcation with the internal carotid artery. Microspheres (New England

Nuclear Inc., Boston, MA; 50 μm in diameter; suspended in 10% Dextran and 0.01% Tween in isotonic saline; PerkinElmer Instruments; Shelton, CT; ≈2500 spheres in 50 μL) were injected using a 30 G needle inserted into the left internal carotid artery. Following a complete injection, direct pressure applied to the injection site facilitated the cessation of bleeding from the injection site, then ligatures were released and blood flow returned. Sham rats underwent an identical procedure without the infusion of microbeads.

Behavior

On Day 13 following surgical procedures, rats were tested in the open field paradigm to measure anxiety-like behavior. During their light cycle, animals were placed in the center of a 90 cm x 90 cm arena and allowed to explore for 10 minutes. This behavior was repeated on Day 14 during the animals' dark cycle. Light-cycle behavior was used as a metric of locomotor activity while dark-cycle behavior was used to assess differences in anxiety-like behavior. All activity was videotaped and analyzed using Cleversys Inc TopScan software (Reston, VA) by an individual blinded to treatment groups.

Also beginning on Day 13, and after open field testing, rats were granted access to identical bottles of tap water and a 0.9% sucrose solution. Bottles were available for 30 consecutive hours, with placement of bottles switched after 15 hours to prevent side bias. Following the 30 hour liquid exposure, rats were deprived of liquid for an additional 16 hours and then re-presented with the bottles for a one-hour probe trial. Bottle weights were recorded over each time period to determine consumption.

Gene Expression

Brain tissue was collected 15 days following surgical procedures and dissected frozen under RNAse free conditions. Similar to previous experiments, the ipsilateral hippocampus was

used for all gene expression analyses. All other tissue was preserved for future analyses. Gene expression analyses of monocyte chemoattractant protein 1 (*Mcp1*), nuclear factor kappa-light-chain-enhancer of activated B cells (*IkB*), cluster of differentiation molecule 11B (*Cd11b*) and tumor necrosis factor alpha (*Tnf*) were completed via SYBR® Green real time PCR quantification techniques. Genes were standardized to cyclophilin A and normalized to sham animals in accordance with $2^{-\Delta\Delta CT}$ calculations. All samples were prepared in triplicate using 1 µg of sample and carried out on an Applied Biosystems HT7900 Fast Real-Time PCR system (Carlsbad, CA).

Data Analysis

Rat body mass was averaged by group and compared using a three-way ANOVA with surgery (sham, ME), drug (vehicle, drug), and day (Day 1, Day 15) as factors. Distance traveled in the open field was analyzed via a three-way ANOVA with surgery (sham, ME), drug (vehicle, drug), and cycle (light cycle, dark cycle) as factors. Other behavior, such as time spent in the center of the open field, was analyzed using a one-way ANOVA with drug (vehicle, meloxicam, fluoxetine) as the factor. Sucrose consumption data were averaged by group and analyzed via one-way ANOVA. Sucrose consumption data presented are initial 30 hour consumption totals. For analysis of RT-PCR data, cycle threshold values were averaged by group and analyzed via the $2^{-\Delta\Delta CT}$ method. Specifically, fold change was calculated, standardized to a housekeeping gene and normalized to sham. Meloxicam and fluoxetine data were analyzed separately by two-way ANOVA with surgery (sham, ME) and drug (vehicle, drug) as factors. For all, differences were considered significant when $\alpha \leq 0.05$. When appropriate, specific differences were further delineated using Tukey's multiple comparison test.

RESULTS

Surgical procedures or drug treatment did not alter measures of overall health

Terminal weights did not differ between sham and ME animals regardless of treatment (vehicle, meloxicam, or fluoxetine) as detected by a three-way ANOVA ($p > 0.05$). Vehicle-treated sham rats weighed 417.7 ± 8.7 g at the end of the study and did not statistically differ from vehicle-treated ME rats (414.0 ± 6.3 g), meloxicam-treated sham rats (396.5 ± 5.0 g), meloxicam-treated ME rats (399.0 ± 5.3 g), fluoxetine-treated sham animals (405.7 ± 12.0 g), or fluoxetine-treated ME rats (389.8 ± 8.9 g). Distance traveled in the open field was measured as an additional indicator of overall health and a one-way ANOVA of total distance traveled between sham, vehicle-treated, meloxicam-treated, and fluoxetine-treated ME rats revealed no difference between groups ($F_{3,45} = 0.88$; $p = 0.46$; data not shown).

Meloxicam, but not Fluoxetine, blocked inflammation and improved functional outcome

Daily administration of meloxicam or fluoxetine starting four days prior to ME surgeries resulted in a significant alteration of ME-induced increases in hippocampal gene expression of *Spp1* as compared to sham-treated (Figure 8.1A; $F_{3,66} = 3.564$; $p < 0.05$). *Post-hoc* analyses of *Spp1* data showed levels of *Spp1* in fluoxetine-treated animal to be significantly elevated. Meloxicam-treated rats showed levels of *Spp1* similar to sham controls. While numeric patterns of *Mcp1* gene expression were similar with meloxicam-treated levels of *Mcp1* resembling sham rat levels, these findings failed to reach significance (Figure 8.1B; $F_{3,48}=0.35$, $p > 0.05$).

Behavior in the open field was significantly altered by prophylactic treatment (Figure 8.2A; $F_{3,29} = 5.03$, $p < 0.01$). As expected, an increase of anxiety-like behavior was evident as

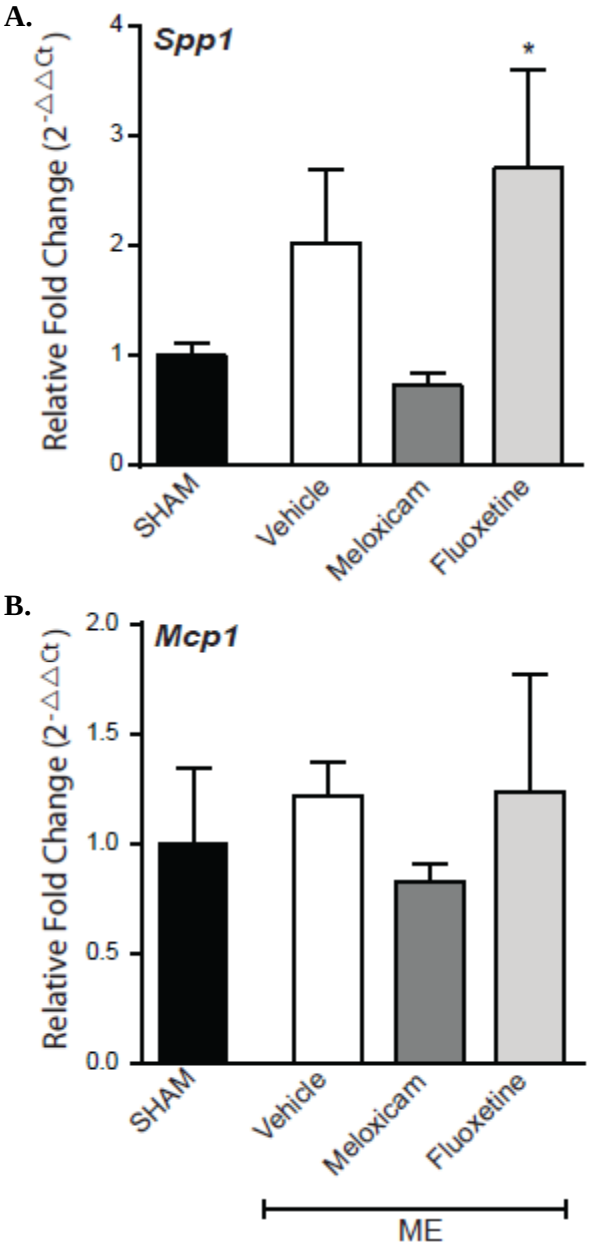

Figure 8.1 Administration of meloxicam, but not fluoxetine, alters inflammatory response to injury

Hippocampal gene expression levels of *Spp1* and *Mcp1* were measured 15 days after sham or ME injury. Animals were prophylactically dosed with vehicle, meloxicam, or fluoxetine beginning four days prior to surgery. A one-way ANOVA revealed a significant effect of drug on *Spp1* levels. *Post-hoc* analyses of *Spp1* show fluoxetine to be significantly increased compared to sham levels, with meloxicam-treated levels resembling sham-treated levels. Though numeric patterns are similar for measures of *Mcp1*, these values failed to reach significance. Error bars indicate standard error of the mean (S.E.M.) and * indicates $p < 0.05$.

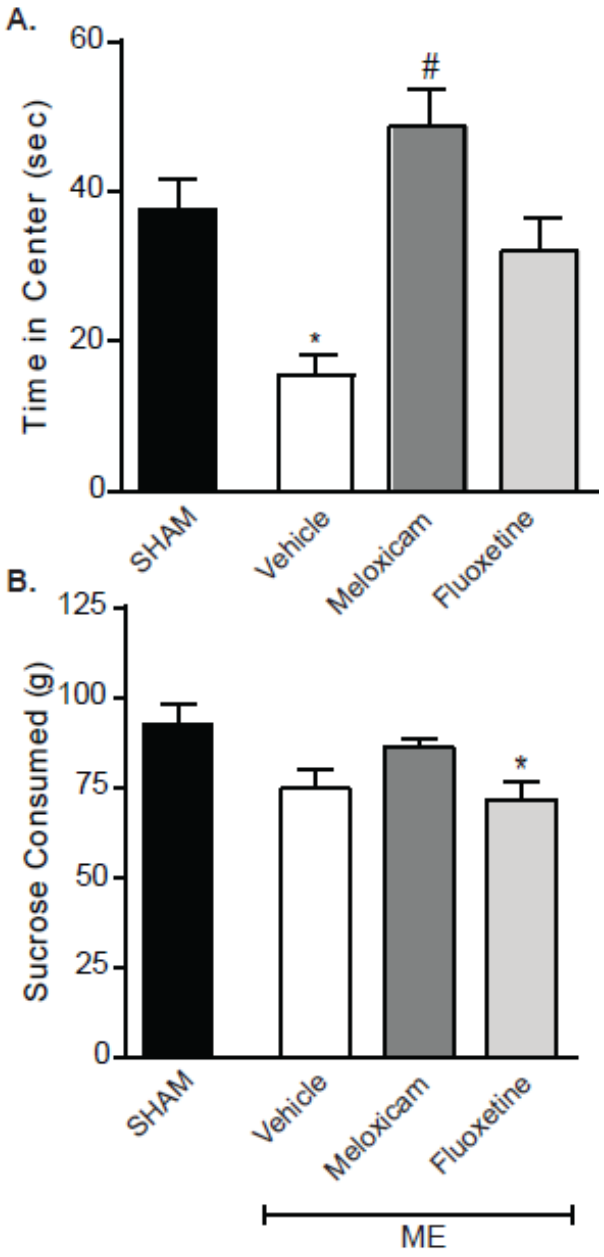

Figure 8.2 Administration of meloxicam, but not fluoxetine, attenuates effects of injury

Beginning 13 days following ME surgery, rats were tested in an open field arena and with a sucrose consumption test for anxiety- and depressive-like behaviors, respectively. (A) Vehicle-treated ME rats spent less time in the center of an open field arena, indicative of heightened anxiety. Meloxicam treatment increased time in the center compared to vehicle treated rats. No differences were detected between fluoxetine- and vehicle-treated rats, suggesting fluoxetine to have a moderate attenuating effect on open field behavior. (B) Consumption of sucrose is used as a metric of anhedonia with decreased consumption indicative of a loss of pleasure. While differences were not detected between vehicle- or meloxicam-treated animals, fluoxetine-treated rats consumed significantly less sucrose when compared to sham rats, suggesting that fluoxetine-treatment results in a depressive-like phenotype. * indicates $p < 0.05$ from sham; # indicates $p < 0.05$ from vehicle-treated ME rats.

post-hoc analyses revealed a significant difference between vehicle-treated sham and vehicle-treated ME rats. Similarly, a reduction of anxiety-like tendencies was evidenced by a significant difference between vehicle-treated and meloxicam-treated ME rats. In contrast, time spent in the center of the open field in fluoxetine-treated rats resembled that of vehicle-treated sham rats. Finally, sucrose consumption was used as an index of anhedonia, and analysis by one-way ANOVA revealed a significant effect of prophylactic drug treatment ($F_{3,47} = 3.90$, $p < 0.05$). *Post-hoc* analysis reveals fluoxetine-treated ME rats to consume significantly less of a sucrose solution compared to vehicle-treated sham rats, indicative of an increased anhedonic state (Figure 8.2B).

DISCUSSION

Collectively, these data demonstrate that both specific (XPro1595; Chapter 7) and generalized anti-inflammatory treatments reduce neuroinflammation and improve metrics of anxiety and anhedonia in a rodent model of microvascular damage. Furthermore, the use of fluoxetine, a selective serotonin reuptake inhibitor, was less effective than meloxicam at attenuating increases in inflammation and alterations to depressive-like behavior. These findings support the use of the ME model in mimicking treatment-resistance. More importantly, these findings suggest that anti-inflammatory treatments show promise in reducing the physiological effects of microvascular infarction.

Because the efficacy of XPro1595 depended on immediate administration- an unrealistic therapeutic option in the clinical sense- we tested the ability of a low-dose non-steroidal anti-inflammatory drug (NSAIDs; meloxicam) and a common SSRI (fluoxetine) to complete the same objectives. In elderly patients, chronic treatment with NSAIDs or SSRIs is common; therefore, we tested the effects of ME infarction on a system already primed by these

therapeutics. Collectively, our data indicate that meloxicam is more effective than fluoxetine in the reduction of central inflammation and behavioral deficits, which suggests that the mechanisms responsible for physiologic dysfunction may be, at least in part, inflammatory in nature. Meloxicam and fluoxetine were both marginally effective in reversing the effects of ME in the open field (as time in the center did not differ between fluoxetine- and vehicle-treated rats); however, anhedonia as measured by the sucrose consumption test was not altered by SSRI administration. Following ME, depressive- and anxiety-like behaviors are dissociated in terms of their behavioral responsiveness to drugs. Differential responses are not uncommon, and in the current experiments, the moderate effects of fluoxetine on open field behavior may stem from serotonergic control of generalized anxiety (Graeff et al. 1996; Ping et al. 2012), while conversely, deficits in dopaminergic signaling related to motivation and reward may preclude ameliorating effects of fluoxetine administration on sucrose consumption (Hajnal et al. 2001; Hajnal et al. 2004). In addition to differential neurochemical signaling patterns, inflammatory activation likely plays a large role in behavioral disruption following ME. One murine model of stress-induced depression found that susceptible mice, or mice with anhedonic symptoms beyond a predetermined threshold, expressed higher levels of inflammatory cytokines and IDO within the brain compared with resilient mice, or stress-exposed mice with reduced anhedonic behavior (Couch et al. 2013). These mice, regardless of stress sensitivity, all expressed increased expression levels of 5-HT_{2A} and COX-1 in addition to having increased numbers of IBA^{+} microglial cells (Couch et al. 2013). These findings illustrate impaired neurotransmission within all mice experiencing stress-induced depressive-like behaviors, but disrupted neuroinflammation within the most heavily affected subset, which suggests that anti-inflammatory treatments may be successful in ameliorating behavioral deficits within these

animals.

In summary, our findings suggest that the use of non-specific, anti-inflammatory therapeutics to alter the response to ME infarction is a more effective strategy than classic SSRI treatments. Indeed, several factors influence the development and progression of CMVD and thus the response of CMVD to therapeutics. Evidence supports the anti-inflammatory potential of anti-depressant treatments (Tynan et al. 2012; Müller et al. 2011), as anti-depressants also carry anti-platelet activity, and have proven effective at reducing symptoms of depression in patients with coronary artery disease (Nemeroff et al. 2012). However, drug responses may be different in an environment in which the full spectrum of disease-related processes is present, such as in cerebromicrovascular disease. Despite the complexity, these findings highlight the potential utility of anti-inflammatory drugs for the reduction of chronic inflammation and the attenuation of vascular-induced behavioral disruptions that may be perpetuated by inflammatory activity.

CHAPTER NINE

GENERAL DISCUSSION

The goal of the experiments described within this dissertation is to contribute to the understanding of the mechanisms that underlie behavioral change subsequent to diffuse and acute cerebral ischemia. While a complicated relationship, the experiments described within answer questions pertinent to this overall goal. Multiple theories have developed and evolved over a span of hundreds of years to explain this relationship. In the 1980s, however, Robert Robinson and colleagues laid groundwork for studies of post-stroke depression and the idea that lesion location and size correlated to the degree and nature of behavioral change. Theories developed further to specify prefrontal-subcortical circuits that may be involved in depressive pathology. In parallel, 'arteriosclerotic depressive states', described since the early 20th Century, began to be recognized as a clinical condition (Gaupp 2000).

The correlation between vascular damage and changes of mood is quite striking, as discussed throughout this dissertation. Arteriosclerotic and atherosclerotic mechanisms, themselves, are quite complex and include several different processes, all of which contribute to the manifestation of various behavioral or functional consequences. For example,

arteriosclerotic mechanisms include increased inflammatory activity, alterations to vascular integrity, endothelial cell damage and dysfunction, and damage to tissue. Together, behavioral changes are likely the culminated manifestation of several different processes.

The experiments included within this document address the relationship between ischemia and behavior from several different angles. In light of these experiments, it is important to note that the variables of arteriosclerotic and atherosclerotic processes are not a factor; tissue and vascular damage, cellular and inflammatory activity, and behavioral effects are derived from consequences of microsphere embolism alone. Therefore, while cardiovascular risk factors may contribute to the generation of such embolic damage in humans, the preceding studies address the downstream effects of microvascular damage. Figure 9.1 illustrates the complex interaction of processes that have been shown to contribute to behavioral dysfunction in the literature and the processes that are experimentally addressed within this document.

Determining Causality

In the first set of experiments (discussed in Chapter 2) it was determined that lesion size or location did not correlate to the presence or degree of damage incurred by microsphere emboli (ME). Assessment of lesion size included unbiased assessment using the Cavalieri estimation technique and included volume estimation in sham rats as well as in rats that have been treated with microspheres to the left or right hemispheres. Results of this experiment found no correlation between frequency of lesions or lesion volume and any metric of anxiety-like or depressive-like behaviors. Later analysis using a more sensitive technique, diffusion tensor imaging (DTI; discussed in Chapter 4), revealed similar findings that the diffusivity of water to flow through white matter tracts or gray matter tissue was not affected by microsphere lesions

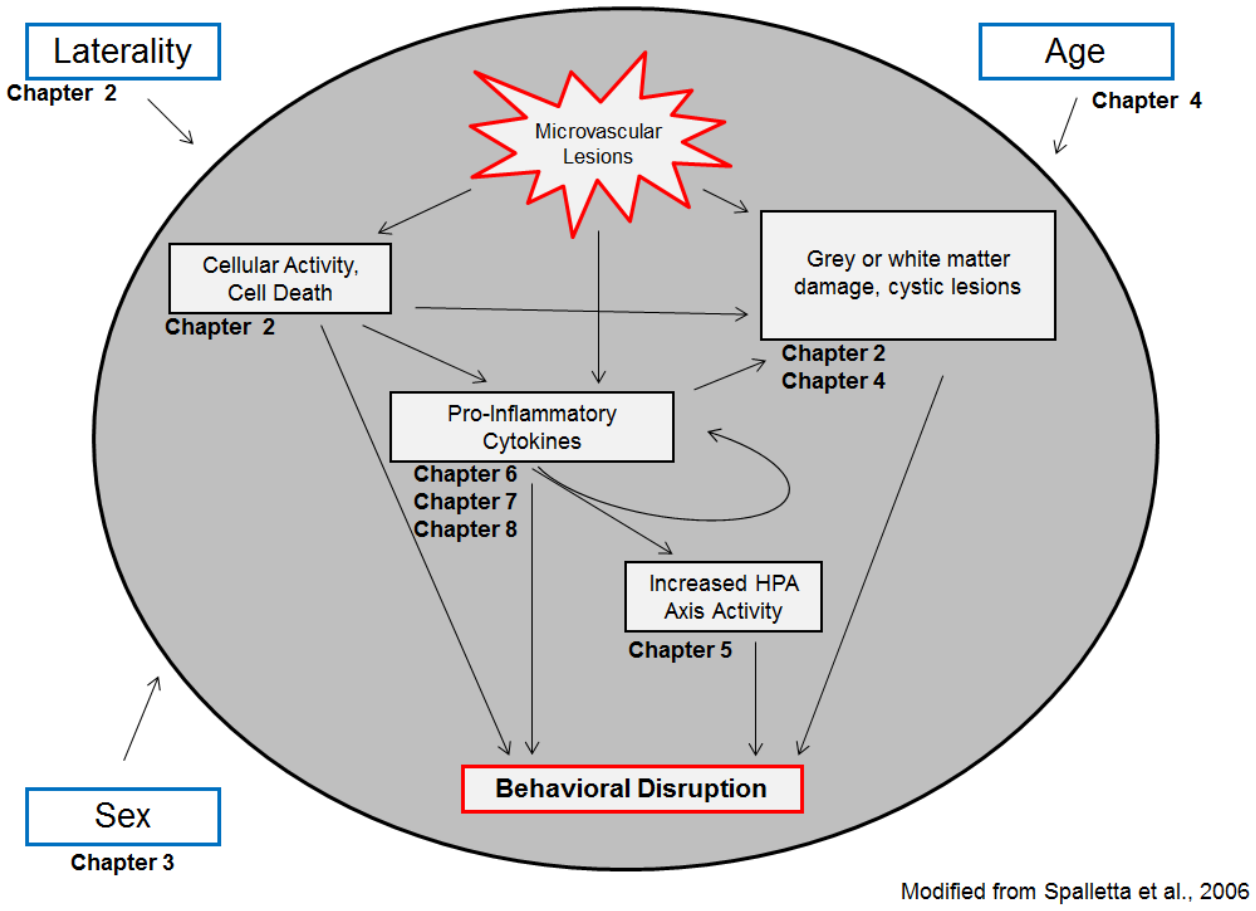

Figure 9.1 Schematic of potential mechanisms of microvascular-induced behavioral disruption

and did not correlate to behavior. These findings are in agreement with recent reports which suggest that lesion location does not influence depression after injury (Carson et al. 2000). There is, however, contention surrounding this field as several variables including stroke boundary assessment, time since stroke, and different sensitivities of imaging techniques may explain inter-study discrepancies in the literature (Shimoda et al. 1999; Alexander et al. 2010).

Similarly, lesion laterality failed to predict behavioral response to ME. Similar to reports surrounding lesion size, this topic is often debated. In studies of lesion laterality, two specific pathways are of importance: the limbic-thalamic-cortical circuit, and the limbic-cortical-striatal-pallidal-thalamic circuit. Within these pathways, mood changes are most commonly associated with damage which disconnects the cortex from subcortical/temporal regions (Tupler et al. 2002; Soares et al. 1997; Blazer et al. 2005). In our studies, no change of tissue integrity in ME compared to sham rats was detected when using DTI; however, subtle changes in tractography cannot be fully evaluated by measures of brain region diffusivity, and more sophisticated tractography techniques may reveal finer alterations to these pathways. Further, work will be necessary to determine if changes in cell signaling (for example, interruptions in amine signaling) in frontal-subcortical projections underlie some degree of behavioral disruption in the model.

Searching for a Mechanism

Given the failure of ME infarcts to correlate with behavioral outcome, we sought next to examine on a cellular level, the effects of ME. Chapter 2 discusses data of macrophage and astrocyte activity as well as the degree of cell loss as determined by Fluoro-Jade C staining. Astrocytes are activated following ischemic injury, and through the secretion of inflammatory cytokines, these cells contribute to inflammation following injury (Strecker et al. 2011; Xia et al.

2010). Astrocyte activation occurs within 30 minutes of injury (Strecker et al. 2011), in contrast to the three to seven day timeline of activation for blood-derived macrophages (Chiba et al. 2013). Studies report, however, the long-term presence of pro-inflammatory cytokines, suggesting an ongoing inflammatory process (Karki et al. 2010). In our study, we observed increases of TNF, MCP1, IL1β, and SPP1 when measured four days following injury, though levels returned to baseline within fourteen days. Findings reported in Chapter 3 show an increased number of microglia, the brain's resident macrophages, as indicated by Iba1 positive staining fourteen days following injury and a primed morphology of these cells. While astrocytes, macrophage, and microglia are all sources of inflammatory cytokine production, together, the data suggest that only microglia may be contributing to inflammatory levels at this time-point.

The focus of studies thereafter shifted to understanding how other system processes adapted to ME injury. Activation of the HPA axis after cerebral injury occurs immediately and long-term increases in glucocorticoid signaling can lead to neuronal damage, hypersensitivity of the HPA axis response, and behavioral impairments (Radak et al. 2013; Johansson et al. 1997; Neigh et al. 2009; de la Tremblaye et al. 2014). Work in Chapter 5 demonstrates that the presence of ME injury contributes to an exaggerated response of CORT to an acute stressor (Figure 5.1) despite unchanged levels of baseline CORT, or GR and co-chaperones FKBP5 and PPIA. Though subtle, these findings are in agreement with previous reports of altered HPA axis functioning that persist in the short-, but not long-term (de la Tremblaye et al. 2014). Furthermore, evaluation of inflammatory cytokines in conjunction with HPA axis activation led to the finding that inflammatory cytokines are indeed increased two weeks following ME and that the addition of subsequent chronic stressors after this time-point (two weeks of social defeat

stress) does not lead to differential expression of GR, FKBP5, or PPIA. Additionally, two weeks of chronic social stress does not lead to an exacerbated inflammatory response in the presence of ME. Rather, at four weeks following ME, and two weeks of chronic stress, inflammation, for the most part, remains steady. As inflammation remains a major component of the brain-behavior relationship, especially in the context of brain injury, we sought to further explore how inflammatory cytokines change as a function of microvascular injury in rats and determine how these signaling molecules may contribute to impairments in behavior.

Due to the involvement of inflammatory cytokine expression in behavior, mood disorders, and response to injury, examining the inflammatory response to ME is a critical piece to understanding mechanisms of ME- induced behavioral change. At two weeks, 43 of 84 measured inflammatory genes are upregulated beyond a two-fold change, confirming the sufficiency of microvascular lesions to induce long-term changes in brain physiology (see Figure 6.1). Expanding this timeline to determine the dynamics of the inflammatory response reveals a varied and low-level inflammatory response among several measured inflammatory markers, but more profoundly, a peak at 14 days which correlates with detected deficits in behavior. Brain injury, including stroke, is noted to be multiphasic with acute and more long-term responses which influence functional outcome (Strecker et al. 2011). These findings strongly suggest a role for inflammation in mediating behavioral change following ME; however, questions arise as to the source of inflammation, the state of inflammation in the periphery, and if inflammation can be tempered to reduce the behavioral burden.

To begin to understand the influence of ME infarcts on peripheral infiltration, we first stained sections of sham and ME tissue with rat antibody. Under normal conditions, rat IgG does not enter the brain tissue due to a lack of transport; however, with increased permeability of the

blood-brain barrier due to chronic inflammation from injury, illness, or stress, these plasma proteins more readily cross through vessel walls and become detectable in tissue. IgG staining of ME tissue reveals bilateral indicators of blood-brain barrier leakage suggesting the effects of ME to extend beyond the specific sites of injury. Furthermore, as discussed in Chapter 6, gene expression analyses for *Cd3* and *Cd19* confirm the presence of these transcripts in brain tissue. CD3 and CD19 are markers for T cells and B cells, respectively, found in peripheral blood mononuclear cell populations. Together these findings indicate inflammatory activation beyond the site and time of injury- suggesting a long-term and profound inflammatory response that may influence behaviors. Other studies report behavioral changes triggered by immune challenges and in one such case, lipopolysaccharide (LPS) administration triggers sickness behavior and increased central and peripheral inflammation (Bay-Richter et al. 2011). Though the effects of LPS are short-lived, after 24 hours behavioral deficits and central inflammation persisted demonstrating a longer lasting effect and greater influence of central inflammation. Similarly, in a separate study, blockage of NF-κB reverses stress-induced inhibition of neurogenesis and stress-induced decreases of sucrose consumption, again depicting a clear relationship between inflammatory activation and behavior (Koo et al. 2010). Peripheral inflammation is evident; however, the nature of these cells, the timing of activation, and their direct contribution to behavioral despair is still unknown. Future work including cell isolation and sorting (*i.e.* flow cytometry) will be necessary to identify specific populations of peripheral and central inflammatory markers. Furthermore, immediate and long-term analysis will be necessary to determine how these cell populations change as ME damage progresses.

Putting the Mechanism to the Test: Reversal

The use of anti-inflammatories to combat chronic increase in cytokine circulation and

changes in behavior has been achieved in the pre-clinical setting (Casolini et al. 2002; Koo et al. 2008; Craft et al. 2006). Several groups have used specific or generalized anti-inflammatories to alter functional output (thoroughly reviewed in Hayley 2011). These studies have made evident the pleiotropic nature of inflammatory cytokine involvement in disease states and sickness behaviors. As discussed in Chapters 6 and 7, the overexpression of inflammatory cytokines contributes to cardiovascular disease risk and progression, anemia (Ferrucci et al. 2010), cancer (Kundu et al. 2008), neurodegenerative disorders (Frank-Cannon et al. 2009), and changes of behavior (Raison et al. 2006) and most importantly, that the complete inhibition of inflammatory processes does not equate to a healthy outcome.

Given the need to maintain this careful balance of cytokine activity, the first tested anti-inflammatory drug (XPro1595) inhibits soluble TNF and allows for the full signaling potential of transmembrane TNF. Furthermore, given the silent presentation of cerebromicrovascular disease, a treatment administered at the time of injury is implausible, thus we sought to test the drug's ability to be effective at a time-point distant from injury. However, as discussed in Chapter 7, XPro1595 failed to reduce measured inflammatory cytokines after the ischemic processes had been set in motion.

Next, to mimic SSRI and NSAID treatments prescribed to patients with cardiovascular risk factors, or patients undergoing cardiac procedures, we dosed sham and ME rats prophylactically with fluoxetine (a classic SSRI) or meloxicam (a generalized anti-inflammatory) to test the ability of each of these drugs to lower inflammation and alleviate the behavioral phenotype. We elected to compare these two drugs for two reasons. First, patients with suspected vascular-induced depression or dementia are prescribed SSRIs to combat depression and forthcoming cognitive decline despite a high rate of treatment-resistance within this

population. Secondly, ME formation is frequently detected following coronary artery bypass grafting (CABG) procedures (Gasparovic et al. 2013; Kim et al. 2011; Hassell et al. 2013; Goto et al. 2014), and similarly to vascular depression, is linked to increases in circulating inflammatory cytokines (Dimaria-Ghalili et al. 2013; de Amorim et al. 2014; Goto et al. 2014), and post-procedure deficits in cognition (Knopman 2007; Purandare et al. 2012; Gasparovic et al. 2013) which are exacerbated by depressive symptoms (Tully et al. 2012). Patients in this cohort are administered NSAIDs to reduce ischemic complications, improve graft patency, and prevent platelet aggregation via inhibition of COX-1; however, again, treatments are only 25% effective (Mannacio et al. 2012; Floyd et al. 2014).

Many of the long-term detriments of CABG occur following surgery when platelet levels drop and then rebound to pre-surgical levels resulting in extensive inflammatory activation (Floyd et al. 2014). This chronic inflammation contributes to aspirin resistance (Larsen et al. 2013), changes of behavior, and increased mortality (Slavich et al. 2014) following surgery. In such cases and in suspected cases of vascular depression, SSRIs and anti-inflammatory treatments are among the most frequently prescribed medications despite evidence that their therapeutic benefits are mixed (Chocron et al. 2013; Mannacio et al. 2012; Larsen et al. 2013). SSRIs following CABG (Chugh et al. 2013; Abdel-Salam et al. 2004), improve quality of life in patients but do not affect overall bleeding or mortality (Chocron et al. 2013) and in fact also inhibit platelet activity (Serebruany et al. 2003). Unlike COX-1 inhibitors (*e.g.* NSAIDs), COX-2 inhibitors (*e.g.* meloxicam) do not have the same aggregatory effect on platelets as traditional NSAID drugs since COX-2 has a reduced effect on thromboxane A as compared to COX-1 inhibitors (Floyd et al. 2014). Thus, COX-2, through inhibition of TNF and NF-κB, may act more on the inflammatory mediators that contribute to behavior without influencing overall

mortality (Ackerman et al. 2005). Findings from Chapters 7 and 8 support this idea and suggest that inhibition of cytokine activity, such as with a COX-2 inhibitor, may lower wide-spread inflammatory activity, its downstream effects, and attenuate behavioral deficits.

Together, it is the goal of these experiments to have provided a foundation for the understanding and appreciation of vascular events in the participation of endocrine, inflammatory, and behavioral alterations. Though the relationship is complex, the highly influential role of inflammatory mediators is apparent and future work exploring the nature of the inflammatory response is warranted. From a "big picture" perspective (Figure 9.2), each minute microvascular event launches a physiological response, and depending on the frequency of microvascular events, the accumulation of endocrine, inflammatory, and neurochemical responses contribute to a threshold of injury. Under such a model, attenuating these responses and addressing the source of damage may help to limit the overall burden and reduce the likelihood of macrovascular events, behavioral changes, or death. Lastly, we hope that these experiments have shaped the framework for future studies to address questions and hypotheses that have arisen from the work and are discussed below.

Important Considerations: Interactions of Age and Sex

Advanced age and male sex are cardiovascular risk factors and predict the incidence of cerebral ischemia; however, the interaction of age and sex within the context of ischemia is much more complex. Although young males are at a higher risk of stroke throughout much of life, women in peripartum (approximately ages 19 to 30) or peri-menopause (ages 45 to 54) experience surges of higher risk (Ritzel et al. 2013; Quillinan et al. 2014; Haast et al. 2012). Thereafter, and after the age of 50, both men and women experience increased risk (as compared to those under 50 years of age) and by aged 85, women surpass men in incidence of ischemia

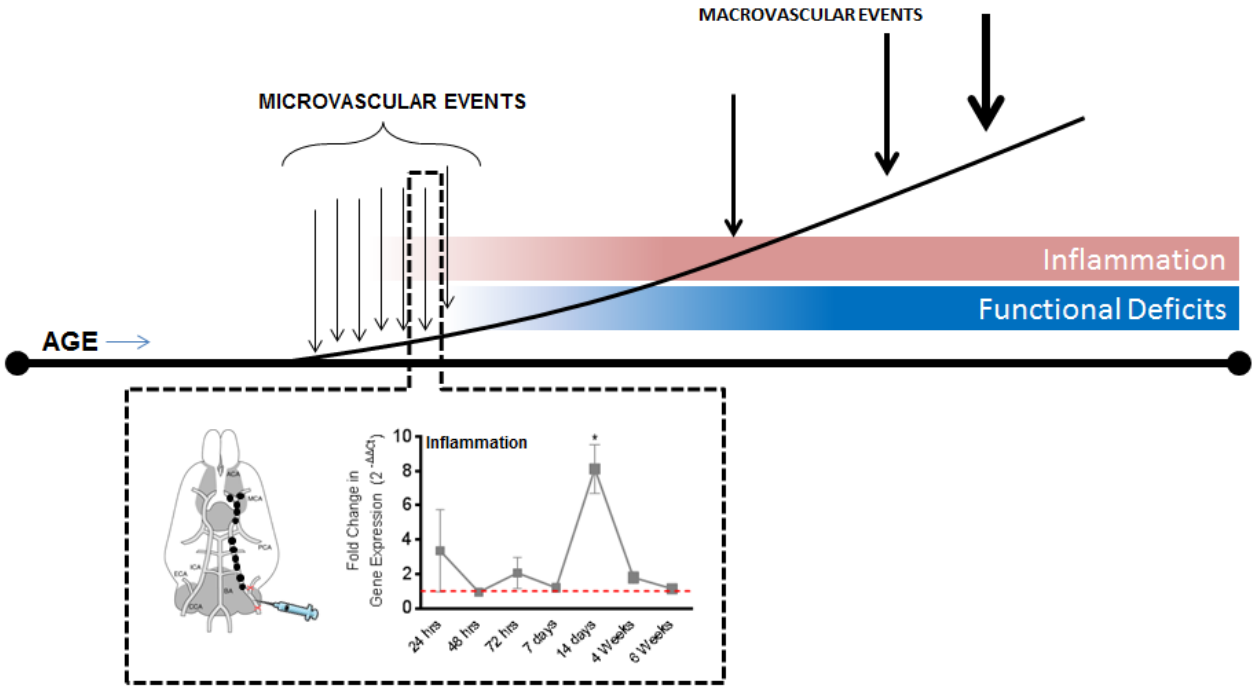

Figure 9.2 Hypothesized timeline of microvascular damage

Cardiovascular risk factors lead to the formation of microvascular events within the periphery and brain. Each event initiates a cascade of stroke-like responses, including activation of the inflammatory system (as mimicked by the ME model and depicted within the box). Slowly, accumulation of these events and responses contributes to increased inflammation over time which precipitates macrovascular events and facilitates deficits in function. Ideal treatment strategies would be most effective and practical if inhibition could occur after such deleterious processes had been set in motion. Anti-inflammatories remain a plausible and promising therapeutic option.

(Ritzel et al. 2013). Despite the irrefutable relationship between age and sex, experiments described herein examined each risk factor separately, warranting explanation.

The majority of studies described within this document assessed the effects of ME in adult (3 months of age) rats. Although the ME model is intended to mimic a disease primarily affecting the elderly, several factors influence the decision to use adult, and not aged, animals. Aged rats are prone to spontaneously occurring tumor growth (Pollard 1973) confounding assessment of behavior and microembolic effects. Furthermore, aged rats accumulate weight and develop symptoms and signs of metabolic syndrome (Ghezzi et al. 2012). Thus, in addition to poor health, the increased adiposity alters performance in locomotor-dependent behavior tasks and makes interpretation of depressive- and anxiety-like behaviors becomes increasingly difficult (Nemeth, Gutman, et al. 2014). In addition to the high cost and historically strict restrictions on obtaining aged and un-manipulated rats, determining basic behavioral effects of ME injury was most time-, cost-, and energy-efficient in adult animals. Therefore, to study the basic neurobiological and behavioral changes associated with the injection of microspheres, adult rats were ideal to determine a baseline.

Considering how advanced age many influence the progression of ME is an interesting topic and worthy of discussion. Though work described within this document shows that aged (18 months of age) rats manifest depressive-like behaviors in the sucrose consumption test, they do not appear more susceptible to tissue cavitation or ME-inducted structural changes (Nemeth, Gutman, et al. 2014). Similarities of structural effects following ischemia in aged and young rats have been previously reported (Popa-Wagner et al. 2007) and further work has shown that although aged rats are capable of mounting a cellular response to brain injury, the response is temporally delayed, profoundly affecting recovery (Popa-Wagner et al. 2007). With this said,

aging in itself is associated with increases in pro-inflammatory cytokine production (Casolini et al. 2002; Ritzel et al. 2013), and increased inflammation in aged rats may have resulted in a heightened inflammatory profile following ME than was observed in adult animals (Chapter 6).

Anti-inflammatory agents were used to prevent both increases in hippocampal inflammation and behavioral changes induced by ME procedures in adult rats. Based on the findings presented herein, we concluded that prophylactic and early-dose therapeutic administration was most effective because these drugs coincided with a 'window' of plasticity reflecting therapeutic efficacy. Given that aged animals have increased production of inflammatory cytokines (Casolini et al. 2002), it would be interesting to test the efficacy of agents such as XPro1595 or meloxicam in aged animals. Work by Casolini et al. (2002) touches on this idea and shows that in a rodent model of natural aging, 22 month-old rats manifest altered cognitive and affective behavior that can be reversed by a COX-2 inhibitor (celecoxib) if treatment is begun early, by 12 months of age. In contrast, if rats were treated beginning at 18 months of age, no difference in behavior or cerebral inflammation was detected between treated- and non-treated rats at 22 months. These findings are important because they suggest that consequences of inflammation, natural (i.e. aging) or induced (i.e. ME), are more difficult to reverse once a certain threshold of inflammation has been reached, or once the cellular responses of the aged brain can no longer respond effectively. We can therefore speculate that anti-inflammatory treatment would be less effective in aged rats following ME; however, these same experiments conducted in adult rats allow us to measure fluctuations of inflammatory cytokines implicating inflammation in the mechanism of ME-induced behavioral and physiological changes. Clinically speaking, for treatments to be most effective, patients at risk for silent infarction or behavioral impairments as a result of CMVD will need to be identified and treated

early.

In addition to age, the response to ischemia or other forms of brain injury is highly dependent upon the biological sex of the subject. Though females and males differ vastly in the response to injury (Steiner 2011; Liu et al. 2012; Bushnell et al. 2014), and the study of sex differences if of utmost interest (Clayton et al. 2014), our studies of ME focus mainly on effects in the male brain. To first establish a foundation on which to build additional experiments, male rats were employed to characterize the behavioral and basic physiological responses to ME injury. Female comparisons were later established to add clinical relevance to the model and to determine the extent to which female protection existed following ME injury.

Compared to adult female rats, adult males show a worsened behavioral response to ME, despite no differences in the cellular response to injury (Chapter 3; Nemeth, Reddy, et al. 2014). While the neuroprotective effects of estrogen during reproductive years have been well established (Geary et al. 2000; Morissette et al. 2007; Ritzel et al. 2013) our study focus on the characterization of behavioral differences, the cellular response to ME injury, and the potential role of S1P1, an estrogen-regulated signaling phospholipid, as a mechanism of female protection. While it is clear from our studies that females experience a level of protection against ME-triggered behavioral disruption, independent of microglial activation, our results fail to explain how females are protected. Previous studies speak towards the potential relationship between sex and inflammatory contribution and have identified estrogen as a potent anti-inflammatory, reducing activity of Nf-κB, IκB, NOS, and TNF (Ritzel et al. 2013). Furthermore, downstream signaling pathways related to estrogen receptor activation including changes in COX mediated prostaglandin activity (actions which promote constriction or dilatation of blood vessels) may account for sex-specific responses to injury (Haast et al. 2012). In any case, and further

supporting a protective role of estrogen, adult males treated with estrogen experienced reduced infarct size and improved behavioral response following ischemic injury (Ritzel et al. 2013). We can speculate that within the ME model, ovariectomized (OVX) adult females would fare worse, manifesting behavioral responses similar to age-matched male rats. Furthermore, hormone replacement in adult OVX-females would produce a phenotype similar to that observed in ovary in-tact adult females.

It appears that in many disease states and aging models, major fluctuations is gonadal hormone concentrations (such as during peripartum or menopause) account for female susceptibility and vulnerability to ischemia or disease (Quillinan et al. 2014; Ritzel et al. 2013). Similarly, in males, drastic changes in androgen concentrations are linked to susceptibility to ischemia (Quillinan et al. 2014), suggesting that the maintenance of gonadal hormone concentrations within a specific range favors cerebral health and functioning. These data further supports the idea that hormone replacement therapies may be most effective, in preclinical models and in humans, when begun before or shortly after hormonal fluctuations occur (Ritzel et al. 2013).

Future work examining the activational and organizational role of sex differences in the manifestation of and response to ME may be warranted. These experiments would include the comparison of in-tact and OVX females, in addition to estrogen replacement groups (activational effects) to determine how estrogen affects ME injury. Furthermore, use of genetically modified "four core genotype" mice would allow for the assessment of how chromosomal (organizational) effects determine the response to ischemic injury. In these mice, gonadal males expressing either the X- and Y-chromosome or two X-chromosomes are compared to gonadal females expressing either XX or XY chromosomes. These comparisons allow for the purely genetic effects to be

identified, but also simultaneously allows for the examination of hormonal effects (gonads intact) as well as the interaction of genetic and hormonal effects (Du et al. 2014; Arnold et al. 2009). Within the context of ischemia, these experiments would illustrate how genes regulate the response to brain injury, independent of hormonal influence. For example, genes located on the Y-chromosome are associated with higher blood pressure and hypertension compared with females, suggesting males are at a higher risk of ischemic events than their female counterparts even when hormonal influence is excluded (Haast et al. 2012).

Sex and age influences are major contributors to the response of the brain to injury and the interaction of these two factors, in particular, help determine outcome. The use of adult rats in the experiments described above helped to determine a cellular mechanism of response following ME, while the use of males allowed for a full characterization of behavior and histological activity prior to the comparison to female rats. While the study of sex differences is pertinent to any disease state, a solid foundation of information must first exist. From this point, within the context of ME, age- and sex-specific experiments can be designed to study the influence of these factors on microvascular damage formation and recovery.

Future Directions

Though several questions have been answered during the course of these experiments, several new inquiries and challenges have emerged. Most importantly, and in light of the promising effects of anti-inflammatory treatments following microvascular damage, we must build upon our knowledge of inflammation within microvascular ischemia. An important aspect to refining the efficacy and utility of anti-inflammatory drugs is to better define the inflammatory reaction, including source of inflammation, timeline of activation, and abundant inflammatory

mediators. As previously mentioned, flow cytometry would allow for the powerful analysis of specific cell populations which would allow for the identification of peripherally infiltrating cells (neutrophils, lymphocytes). Furthermore, though immunohistochemical techniques have shed some light on these data, macrophage and microglial activity can be measured and confirmed through the detection of CD45 and CD11b (of which varying concentrations signify specific activation states). Such analyses over multiple time-points would be informative as to the nature of the immune response which will provide additional direction on how best to target anti-inflammatory therapeutics.

An even more powerful technique, liquid chromatography-mass spectrometry, permits the highly sensitive analysis of the metabolomic response to ME. Analysis with LC-MS can be region-specific and can provide a highly detailed analysis of ME-induced proteomics. Previous analyses of inflammatory proteins have proven unsuccessful due to the subtle changes in protein concentration and a moderate threshold for detection. LC-MS offers a lower limit of detection and the identification and profiling of hundreds, if not thousands, of proteins may reveal new players and complex relationships key to the understanding of the pathogenesis of microvascular ischemia.

The preceding chapters describe several experiments; however, none touch upon the neurochemical changes that may result from ME. Ischemia elicits the release of monoaminergic neurotransmitters within minutes of injury (Toner et al. 1996; Nellgård et al. 1999; Toner et al. 2001). Additionally, the release of glutamate during anoxic or ischemic conditions triggers cell death linked to excitotoxicity. Reversal or blockade of these mechanisms results in decreased tissue damage and cell death, though these effects are highly dependent upon timing of administration and mechanism of antagonism (Nellgård et al. 1999; Toner et al. 1996). In our

experiments, increased cell death was observed four, but not fourteen, days after ME, which suggests that apoptosis had largely ceased and cell clearance mechanisms were efficient (Elliott et al. 2010). Despite the apparent recovery of cell-death processes, long-term altered neurotransmitter release (Sánchez-Mendoza et al. 2013) may still occur with downstream consequences to the duration and degree of the inflammatory response (Levite 2008). Previous work with a rat microsphere embolism model conducted in the late 1990s has established microsphere-induced changes to norepinephrine uptake (Hayashi et al. 1998), cholinergic neurons and fibers (Takagi et al. 1997; Taguchi et al. 1993; Craft et al. 2005) and learning/memory and attention (Takagi et al. 1997; Craft et al. 2005). To build upon these findings, microdialysis would be a useful technique to further explore the extracellular medium of the waking rat at varying time-points following ME injury. The understanding of neurochemical release and activity following ischemia may prove useful to rendering a new method with which to slow microvascular injury directly or indirectly via the influence of other biological processes.

Relevant to neurotransmitter release, and as discussed in Chapter 5, activity of the HPA axis following ischemia is important to injury response and functional outcome (Turnbull et al. 1999). Though some experiments have been conducted which focus on the HPA axis following microvascular injury, future work could focus on the effects of *pre*-exposure to stress on outcome from ME damage. Several works have cited stress as a precursor or risk factor for ischemia (Merrett et al. 2010), and pre-exposure to stress has been shown to alter the post-injury response (Neigh, Karelina, Glasper, et al. 2009), thereby highlighting vulnerable mechanisms previously masked. Furthermore, assessing the immediate and waking CORT response and recovery in the presence of ME through the use of repeated blood collections via a jugular

catheter may shed light on HPA axis function and further highlight the dynamics of observed subtle HPA axis functioning changes discussed in Chapter 5.

Finally, and in addition to highly innovative and highly sensitive techniques, a more thorough assessment of behavior is warranted. As depression is considered a prodrome of cognitive dysfunction in patients with suspected vascular depression, full characterization of cognitive deficits in the ME model will promote validity of the model. Comprehensive analysis of affective behavior has defined ME-induced increases in depressive- and anxiety-like disorders. Cognitive functioning following ME was evaluated briefly employing the Barnes Maze as discussed in Chapter 2. A more thorough examination of the dynamics of cognitive effects to include working memory (delayed alternations; radial arm maze; spontaneous exploration; and delayed non-matching to sample task) and recognition memory (novel object recognition) will complement errors previously detected in the Barnes Maze to provide a more complete picture of cognitive disruption (Dudchenko 2004).

Microvascular ischemia is a silent injury with profound effects on several bodily systems and behavior. The microsphere induced embolic model described herein demonstrates utility in the study of ME and its long-term effects on affective behavior, cognition, neuroimmune activation, and stress response. These studies address and confirm the complex relationship and interplay among inflammation, stress, behavior, and ischemia, and have demonstrated that, under certain conditions, this relationship can be modified with beneficial results in terms of behavior. With this, we have made progress towards a better understanding of microvascular ischemia, the mechanisms behind the far-reaching effects, and offer methods by which symptom severity and progression can be alleviated.

References

Abdel-Salam, O., Baiuomy, A., Arbid, M. 2004. Studies on the anti-inflammatory effect of fluoxetine in the rat. *Pharmacol Res* 49(2): p.119–131.

Ackerman, W.E., Zhang, X.L., Rovin, B.H., Kniss, D. a. 2005. Modulation of cytokine-induced cyclooxygenase 2 expression by PPARG ligands through NFkappaB signal disruption in human WISH and amnion cells. *Biol Reproduction* 73(3): p.527–35.

Aggarwal, B.B. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3(9): p.745–56.

Alexander, L.D., Black, S.E., Gao, F., Szilagyi, G., Danells, C.J., McIlroy, W.E. 2010. Correlating lesion size and location to deficits after ischemic stroke: the influence of accounting for altered peri-necrotic tissue and incidental silent infarcts. *Behav Brain Funct* 6: p.6.

Alexopoulos, G. 2006. The vascular depression hypothesis: 10 years later. *Biol Psych* 60(12): p.1304–5.

Alexopoulos, G.S., Bruce, M.L., Silbersweig, D., Kalayam, B., Stern, E. 1999. Vascular depression: a new view of late-onset depression. *Dialogues Clin Neurosci* 1(2).

Alexopoulos, G.S., Meyers, B., Young, R., Campbell, S., Silbersweig, D., Charlson, M. 1997. "Vascular depression" hypothesis. *Arch Gen Psychiat* 54: p.915–22.

Alkayed, N.J., Harukuni, I., Kimes, a. S., London, E.D., Traystman, R.J., Hurn, P.D., Grady, P. a. 1998. Gender-Linked Brain Injury in Experimental Stroke Editorial Comment. *Stroke* 29(1): p.159–166.

De Amorim, C.G., Malbouisson, L.M.S., da Silva, F.C., Fiorelli, A.I., Murakami, C.K.F., Carmona, M.J.C. 2014. Leukocyte depletion during CPB: effects on inflammation and lung function. *Inflammation* 37(1): p.196–204.

Andersen, M.B., Zimmer, J., Sams-Dodd, F. 1999. Specific behavioral effects related to age and cerebral ischemia in rats. *Pharmacol Biochem Behav* 62(4): p.673–82.

Anitei, S. 2007. 7 Things about heart and blood vessels. Available at: http://news.softpedia.com/news/7-Things-About-Heart-and-Blood-Vessels-73405.shtml [Accessed September 11, 2012].

Arnold, A.P., Chen, X. 2009. What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? *Front Neuroendocrinol* 30(1): p.1–9.

Baldwin, R., Jeffries, S., Jackson, A., Sutcliffe, C., Thacker, N., Scott, M., Burns, A. 2004. Treatment response in late-onset depression: relationship to neuropsychological, neuroradiological and vascular risk factors. *Psychol Med* 34(1): p.125–36.

Baldwin, R.C. 2002. Vascular basis of late-onset depressive disorder. *Br J Psychiatry* 180(2): p.157–160.

Ballmaier, M., Narr, K.L., Toga, A.W., Elderkin-Thompson, V., Thompson, P.M., Hamilton, L., Haroon, E., Pham, D., Heinz, A., Kumar, A. 2008. Hippocampal morphology and distinguishing late-onset from early-onset elderly depression. *Am J Psychiat* 165(2): p.229–37.

Baños, G., Guarner, V., Pérez-Torres, I. 2011. Sex steroid hormones, cardiovascular diseases and the metabolic syndrome. *Cardiovasc Hematol Agents Med Chem* 9(3): p.137–46.

Barnes, D.E., Yaffe, K., Byers, A.L., McCormick, M., Schaefer, C., Whitmer, R.A. 2012. Midlife vs Late-Life Depressive Symptoms and Risk of Dementia. *Arch Gen Psychiatry* 69(5): p.493–8.

Barnes, P.J. 2010. Mechanisms and resistance in glucocorticoid control of inflammation. *J Steroid Biochem Molecular Biol* 120(2-3): p.76–85.

Barnum, C.J., Chen, X., Chung, J., Chang, J., Williams, M., Grigoryan, N., Tesi, R.J., Tansey, M.G. 2014. Peripheral administration of the selective inhibitor of soluble Tumor Necrosis Factor (TNF) XPro®1595 attenuates nigral cell loss and glial activation in 6-OHDA hemiparkinsonian rats. *J Parkinson's Dis* 4(3): p.349–60.

Bay-Richter, C., Janelidze, S., Hallberg, L., Brundin, L. 2011. Changes in behaviour and cytokine expression upon a peripheral immune challenge. *Behav Brain Res* 222(1): p.193–9.

Besedovsky, H.O., del Rey, A. 2011. Central and peripheral cytokines mediate immune-brain connectivity. *Neurochem Res* 36(1): p.1–6.

Betz, J.F., Zhuo, J., Roy, A., Shanmuganathan, K., Gullapalli, R.P. 2012. Prognostic value of diffusion tensor imaging parameters in severe traumatic brain injury. *J Neurotrauma* 1305: p.1292–1305.

Blandino, P., Barnum, C.J., Solomon, L.G., Larish, Y., Lankow, B.S., Deak, T. 2009. Gene expression changes in the hypothalamus provide evidence for regionally-selective changes in IL-1 and microglial markers after acute stress. *Brain Behav Immun* 23(7): p.958–68.

Blandino, P.J., Barnum, C.J., Deak, T. 2006. The involvement of norepinephrine and microglia in hypothalamic and splenic IL-1B responses to stress. *J Neuroimmunol* 173: p.87–95.

Blazer, D.G., Hybels, C.F. 2005. Origins of depression in later life. *Psychol Med* 35(9): p.1241–52.

Blum, S., Luchsinger, J.A., Manly, J.J., Schupf, N., Stern, Y., Brown, T.R., DeCarli, C., Small, S. a, Mayeux, R., Brickman, a M. 2012. Memory after silent stroke: hippocampus and infarcts both matter. *Neurology* 78(1): p.38–46.

Booth, A., Jayne, D.R.W., Kharbanda, R.K., McEniery, C.M., Mackenzie, I.S., Brown, J., Wilkinson, I.B. 2004. Infliximab improves endothelial dysfunction in systemic vasculitis: a model of vascular inflammation. *Circulation* 109(14): p.1718–23.

Böttiger, B.W., Teschendorf, P., Krumnikl, J.J., Vogel, P., Galmbacher, R., Schmitz, B., Motsch, J., Martin, E., Gass, P. 1999. Global cerebral ischemia due to cardiocirculatory arrest in mice causes neuronal degeneration and early induction of transcription factor genes in the hippocampus. *Mol Brain Res* 65(2): p.135–42.

Brambilla, R., Ashbaugh, J.J., Magliozzi, R., Dellarole, A., Karmally, S., Szymkowski, D.E., Bethea, J.R. 2011. Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination. *Brain* 134(Pt 9): p.2736–54.

Browne, K.D., Iwata, A., Putt, M.E., Smith, D.H. 2006. Chronic ibuprofen administration worsens cognitive outcome following traumatic brain injury in rats. 201: p.301–307.

Bushnell, C., Reeves, M., Zhao, X., Pan, W., Prvu-Bettger, J., Zimmer, L., Olson, D., Peterson, E. 2014. Sex differences in quality of life after ischemic stroke. *Neurol* Epub ahead.

Buwalda, B., Felszeghy, K., Horváth, K.M., Nyakas, C., de Boer, S.F., Bohus, B., Koolhaas, J.M. 2001. Temporal and spatial dynamics of corticosteroid receptor down-regulation in rat brain following social defeat. *Physiol Behav* 72(3): p.349–54.

Campbell, S., Macqueen, G. 2004. The role of the hippocampus in the pathophysiology of major depression. *J Psych Neurosci* 29(6): p.417–26.

Carson, A., MacHale, S., Allen, K., Lawrie, S., Dennis, M., House, A., Sharpe, M. 2000. Depression after stroke and lesion location: a systematic review. *Lancet* 356(9224): p.122–6.

Caso, J.R., Pradillo, J.M., Hurtado, O., Leza, J.C., Moro, M.A., Lizasoain, I. 2008. Toll-like receptor 4 is involved in subacute stress-induced neuroinflammation and in the worsening of experimental stroke. *Stroke* 39(4): p.1314–20.

Casolini, P., Catalani, A., Zuena, A.R., Angelucci, L. 2002. Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat. *J Neurosci Res* 68(3): p.337–43.

Chadwick, W., Magnus, T., Martin, B., Keselman, A., Mark, P., Maudsley, S. 2009. Targeting TNF-α receptors for neurotherapeutics. *October* 31(10): p.504–511.

Chang, P.K.-Y., Verbich, D., McKinney, R.A. 2012. AMPA receptors as drug targets in neurological disease--advantages, caveats, and future outlook. *Eur J Neurosci* 35(12): p.1908–16.

Chen, G., Goeddel, D. V. 2002. TNF-R1 Signaling : A Beautiful Pathway The Fas Signaling Pathway : More Than a Paradigm. *Science* 296(May): p.1634–1635.

Chen, R.-L., Balami, J.S., Esiri, M.M., Chen, L.-K., Buchan, A.M. 2010. Ischemic stroke in the elderly: an overview of evidence. *Nature reviews. Neurology* 6(5): p.256–65.

Chen, W., Ma, Q., Suzuki, H., Hartman, R., Tang, J., Zhang, J.H. 2011. Osteopontin reduced hypoxia-ischemia neonatal brain injury by suppression of apoptosis in a rat pup model. *Stroke* 42(3): p.764–9.

Chiba, T., Umegaki, K. 2013. Pivotal roles of monocytes/macrophages in stroke. *Mediators Inflamm* 2013: p.759103.

Chocron, S., Vandel, P., Durst, C., Laluc, F., Kaili, D., Chocron, M., Etievent, J.-P. 2013. Antidepressant therapy in patients undergoing coronary artery bypass grafting: the MOTIV-CABG trial. *Ann Thorac Surg* 95(5): p.1609–18.

Choi, I.-Y., Lee, J.-C., Ju, C., Hwang, S., Cho, G.-S., Lee, H.W., Choi, W.J., Jeong, L.S., Kim, W.-K. 2011. A3 adenosine receptor agonist reduces brain ischemic injury and inhibits inflammatory cell migration in rats. *Am J Pathol* 179(4): p.2042–52.

Chugh, P.K., Kalra, B.S., Kaushik, N., Tekur, U. 2013. Evaluation of anti-inflammatory activity, effect on blood pressure & gastric tolerability of antidepressants. *Indian J Med Res* 138(July): p.99–103.

Clayton, J.A., Collins, F.S. 2014. NIH to balance sex in cell and animal studies. *Nature* 509: p.282–3.

Couch, Y., Anthony, D.C., Dolgov, O., Revischin, A., Festoff, B., Santos, A.I., Steinbusch, H.W., Strekalova, T. 2013. Microglial activation, increased TNF and SERT expression in the prefrontal cortex define stress-altered behaviour in mice susceptible to anhedonia. *Brain Behav Immun* 29: p.136–46.

Craft, T.K.S., DeVries, A.C. 2006. Role of IL-1 in poststroke depressive-like behavior in mice. *Biol Psych* 60(8): p.812–8.

Craft, T.K.S., DeVries, A.C. 2009. Vulnerability to stroke: implications of perinatal programming of the hypothalamic-pituitary-adrenal axis. *Front Behav Neurosci* 3: p.54.

Craft, T.K.S., Mahoney, J.H., Devries, A.C., Sarter, M. 2005. Microsphere embolism-induced cortical cholinergic deafferentation and impairments in attentional performance. *Eur J Neurosci* 21(11): p.3117–32.

Csiszar, A., Labinskyy, N., Smith, K., Rivera, A., Orosz, Z., Ungvari, Z. 2007. Vasculoprotective effects of anti-tumor necrosis factor-alpha treatment in aging. *Am J Pathol* 170(1): p.388–98.

Dannehl, K., Rief, W., Schwarz, M., Hennings, A., Riemer, S., Selberdinger, V., Stapf, T., Euteneuer, F. 2014. The predictive value of somatic and cognitive depressive symptoms for cytokine changes in patients with major depression. *Neuropsych Dis Treat* 10: p.1191–1197.

Davidovich, P., Kearney, C.J., Martin, S.J. 2014. Inflammatory outcomes of apoptosis, necrosis and necroptosis. *Biol Chem* 395(10): p.1163–1171.

Deak, T. 2008. Immune cells and cytokine circuits: toward a working model for understanding direct immune-to-adrenal communication pathways. *Endocrinology* 149(4): p.1433–5.

Dempsey, R.J., Vemuganti, R., Varghese, T., Hermann, B.P. 2010. A review of carotid atherosclerosis and vascular cognitive decline: a new understanding of the keys to symptomology. *Neurosurgery* 67(2): p.484–93; discussion 493–4.

DeVries, A., Nelson, R.J., Traystman, R.J., Hurn, P.D. 2001. Cognitive and behavioral assessment in experimental stroke research: will it prove useful? *Neurosci BioBehav Rev* 25(4): p.325–42.

Dimaria-Ghalili, R.A., Sullivan-Marx, E.M., Compher, C. 2013. Inflammation, Functional Status, and Weight Loss During Recovery From Cardiac Surgery in Older Adults: A Pilot Study. *Biol Res Nursing*.

Du, S., Itoh, N., Askarinam, S., Hill, H., Arnold, A.P., Voskuhl, R.R. 2014. XY sex chromosome complement, compared with XX, in the CNS confers greater neurodegeneration during experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 111(7): p.2806–11.

Dudchenko, P.A. 2004. An overview of the tasks used to test working memory in rodents. *Neurosci Biobehav Rev* 28(7): p.699–709.

Dyrby, T.B., Baaré, W.F.C., Alexander, D.C., Jelsing, J., Garde, E., Søgaard, L. V. 2011. An ex vivo imaging pipeline for producing high-quality and high-resolution diffusion-weighted imaging datasets. *Human Brain Mapping* 32(4): p.544–63.

Elliott, M.R., Ravichandran, K.S. 2010. Clearance of apoptotic cells: implications in health and disease. *J Cell Bio* 189(7): p.1059–70.

Fang, J., Cheng, Q. 2009. Etiological mechanisms of post-stroke depression: a review. *Neurol Res* 31(9): p.904–9.

Farkas, E., Luiten, P.G.. 2001. Cerebral microvascular pathology in aging and Alzheimer's disease.

Fayaz, S., Kumar Suvanish, V., Rajanikant, G. 2014. Necroptosis: who knew there were so many interesting ways to die? *CNS Neurol Disord Drug Targets* 13(1): p.42–51.

Feil, D., Kumar, A. 1999. The neuropsychiatry of subcortical ischemic brain disease. *Curr Psych Rep* 1(1): p.69–77.

Feng, C., Fang, M., Liu, X.-Y. 2014. The neurobiological pathogenesis of poststroke depression. *ScientificWorldJournal* 2014: p.521349.

Ferrucci, L. et al. 2010. Proinflammatory state, hepcidin, and anemia in older persons. *Blood* 115(18): p.3810–6.

File, S.E., Hyde, J.R. 1978. Can social interaction be used to measure anxiety? *Br J Pharmac* 62(1): p.19–24.

Floyd, C.N., Ferro, A. 2014. Mechanisms of aspirin resistance. *Pharmacol Therapeut* 141(1): p.69–78.

Frank-Cannon, T.C., Alto, L.T., McAlpine, F.E., Tansey, M.G. 2009. Does neuroinflammation fan the flame in neurodegenerative diseases? *Mol Neurodegen* 4: p.47.

Fujikawa, T., Yamawaki, S., Touhouda, Y. 1993. Incidence of silent cerebral infarction in patients with major depression. *Stroke* 24(11): p.1631–4.

Fukuchi, K., Kusuoka, H., Watanabe, Y., Nishimura, T. 1999. Correlation of sequential MR images of microsphere-induced cerebral ischemia with histologic changes in rats. *Invest Radiol* 24(11): p.698.

Garcia-Segura, L.M., Azcoitia, I., DonCarlos, L.L. 2001. Neuroprotection by estradiol.

Gasparovic, H., Borojevic, M., Malojcic, B., Gasparovic, K., Biocina, B. 2013. Single aortic clamping in coronary artery bypass surgery reduces cerebral embolism and improves neurocognitive outcomes. *Vasc Med* 18(5): p.275–81.

Gaupp, R. 2000. Depressive states in old age (Classic Text No. 42). *History of Psychiatry* 11(42): p.213–5.

Geary, G.G., Krause, D.N., Duckles, S.P. 2000. Estrogen reduces mouse cerebral artery tone through endothelial NOS- and cyclooxygenase-dependent mechanisms. *Am J Physiol Heart Circ Physiol* 279(2): p.H511–9.

Ghezzi, A.C., Cambri, L.T., Botezelli, J.D., Ribeiro, C., Dalia, R.A., de Mello, M.A.R. 2012. Metabolic syndrome markers in wistar rats of different ages. *Diabetol Metab Syndr* 4(1): p.16.

Goto, T., Maekawa, K. 2014. Cerebral dysfunction after coronary artery bypass surgery. *J Anesth* 28(2): p.242–8.

Graeff, F.G., Guimarães, F.S., De Andrade, T.G., Deakin, J.F. 1996. Role of 5-HT in stress, anxiety, and depression. *Pharmacol Biochem Behav* 54(1): p.129–41.

Grau-Olivares, M., Arboix, A. 2009. Mild cognitive impairment in stroke patients with ischemic cerebral small-vessel disease: a forerunner of vascular dementia? *Exp Rev Neurotherap* 9(8): p.1201–17.

Grippo, A.J., Francis, J., Beltz, T.G., Felder, R.B., Johnson, A.K. 2005. Neuroendocrine and cytokine profile of chronic mild stress-induced anhedonia. *Physiol Behav* 84(5): p.697–706.

Gundersen, H.J., Jensen, E.B., Kiêu, K., Nielsen J. 1999. The efficiency of systematic sampling in stereology--reconsidered. *Journal of microscopy* 193(Pt 3): p.199–211.

Gutman, D. a, Keifer, O.P., Magnuson, M.E., Choi, D.C., Majeed, W., Keilholz, S., Ressler, K.J. 2012. A DTI tractography analysis of infralimbic and prelimbic connectivity in the mouse using high-throughput MRI. *NeuroImage* 63(2): p.800–11.

Haast, R.A.M., Gustafson, D.R., Kiliaan, A.J. 2012. Sex differences in stroke. *J Cereb Blood Flow Metab* 32(12): p.2100–7.

Hajnal, A., Norgren, R. 2001. Accumbens dopamine mechanisms in sucrose intake. *Brain Res* 904(1): p.76–84.

Hajnal, A., Smith, G.P., Norgren, R. 2004. Oral sucrose stimulation increases accumbens dopamine in the rat. *Am J Physiol* 286(1): p.R31–7.

Hakim, A.M. 2011. Depression, strokes and dementia: new biological insights into an unfortunate pathway. *Cardiovasc Psychiatr Neurol* 2011(Mci): p.649629.

Hasegawa, Y., Suzuki, H., Sozen, T., Rolland, W., Zhang, J.H. 2010. Activation of sphingosine 1-phosphate receptor-1 by FTY720 is neuroprotective after ischemic stroke in rats. *Stroke* 41(2): p.368–74.

Hassell, M.E.C., Nijveldt, R., Roos, Y.B.W., Majoie, C.B.L., Hamon, M., Piek, J.J., Delewi, R. 2013. Silent cerebral infarcts associated with cardiac disease and procedures. *Nat Rev Cardiol* 10(12): p.696–706.

Hayashi, H., Hirota, S., Takeo, S. 1998. Microsphere embolism-induced changes in noradrenaline uptake of the cerebral cortex in rats. *Brain Res* 808(2): p.190–6.

Hayley, S. 2011. Toward an anti-inflammatory strategy for depression. *Front Behav Neurosci* 5(April): p.19.

Hecht, N., He, J., Kremenetskaia, I., Nieminen, M., Vajkoczy, P., Woitzik, J. 2012. Cerebral Hemodynamic Reserve and Vascular Remodeling in C57/BL6 Mice Are Influenced by Age. *Stroke*.

Hemmings, D.G., Xu, Y., Davidge, S.T. 2004. Sphingosine 1-phosphate-induced vasoconstriction is elevated in mesenteric resistance arteries from aged female rats. *Br J Pharmacol* 143(2): p.276–84.

Hinwood, M., Tynan, R.J., Charnley, J.L., Beynon, S.B., Day, T. a, Walker, F.R. 2013. Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. *Cerebral Cortex* 23(8): p.1784–97.

Hofmann, U., Burkard, N., Vogt, C., Thoma, A., Frantz, S., Ertl, G., Ritter, O., Bonz, A. 2009. Protective effects of sphingosine-1-phosphate receptor agonist treatment after myocardial ischaemia-reperfusion. *Cardiovasc Res* 83(2): p.285–93.

Holsboer, F. 2001. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *Journal of affective disorders* 62(1-2): p.77–91.

Hölscher, C. 1999. Stress impairs performance in spatial water maze learning tasks. *Behav Brain Res* 100(1-2): p.225–35.

Hoshi, T., Kitagawa, K., Yamagami, H., Furukado, S., Hougaku, H., Hori, M. 2005. Relations of serum high-sensitivity C-reactive protein and interleukin-6 levels with silent brain infarction. *Stroke* 36(4): p.768–72.

Hossmann, K.A. 1998. Experimental models for the investigation of brain ischemia. *Cardiovasc Res* 39(1): p.106–20.

Hurn, P.D. 2003. Estrogen and Stroke: A Balanced Analysis. *Stroke* 34(2): p.338–341.

Iaizzo, P. 2005. General features of the cardiovascular system. In P. Iaizzo (ed) The Handbook of Cardiac Anatomy, Physiology and Devices, Totowa, NJ: Humana Press

Jarrett, B.R., Correa, C., Ma, K.L., Louie, A.Y. 2010. In vivo mapping of vascular inflammation using multimodal imaging. *PloS One* 5(10): p.e13254.

Jiang, Q., Zhang, Z.G., Chopp, M. 2010. MRI of stroke recovery. *Stroke* 41(2): p.410–4.

Johansson, A., Olsson, T., Carlberg, B., Karlsson, K., Fagerlund, M. 1997. Hypercortisolism after stroke--partly cytokine-mediated? *J Neurol Sci* 147(1): p.43–7.

Kales, H.C., Maixner, D.F., Mellow, A.M. 2005. Cerebrovascular disease and late-life depression. *Am J Geriatr Psychiatry* 13(2): p.88–98.

Karki, K., Knight, R.A., Shen, L.H., Kapke, A., Lu, M., Li, Y., Chopp, M. 2010. Chronic brain tissue remodeling after stroke in rat: a 1-year multiparametric magnetic resonance imaging study. *Brain Res* 1360: p.168–76.

Kempton, M., Xalvador, Z., Munafo, M., Geddes, J., Simmons, A., Frangou, S., Williams, S. 2011. Structural neuroimaging studies in major depressive disorder. *Arch Gen Psychiatry* 68(7): p.675–690.

Kim, I.-C. et al. 2011. Incidence and predictors of silent embolic cerebral infarction following diagnostic coronary angiography. *Int J Cardiol* 148(2): p.179–182.

Knecht, S. et al. 2008. Atrial fibrillation in stroke-free patients is associated with memory impairment and hippocampal atrophy. *European heart journal* 29(17): p.2125–32.

Knopman, D.S. 2007. Cerebrovascular disease and dementia. *Br J Radiol* 80 Spec No: p.S121–7.

Koo, J.W., Duman, R.S. 2008. IL-1B is an essential mediator of the antineurogenic and anhedonic effects of stress. *Sciences-New York*.

Koo, J.W., Russo, S.J., Ferguson, D., Nestler, E.J., Duman, R.S. 2010. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *P Natl Acad Sci USA* 107(6): p.2669–74.

Kriz, J., Lalancette-Hébert, M. 2009. Inflammation, plasticity and real-time imaging after cerebral ischemia. *Acta Neuropathologica* 117(5): p.497–509.

Kumar, A., Loane, D.J. 2012. Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. *Brain Behav Immun* 26(8): p.1191–201.

Kundu, J.K., Surh, Y.-J. 2008. Inflammation: gearing the journey to cancer. *Mutat Res* 659(1-2): p.15–30.

Kwan, L.T., Reed, B.R., Eberling, J.L., Schuff, N., Tanabe, J., Norman, D., Weiner, M.W., Jagust, W.J. 1999. Effects of subcortical cerebral infarction on cortical glucose metabolism and cognitive function. *Archives of neurology* 56(7): p.809–14.

De la Tremblaye, P.B., Raymond, J., Milot, M.R., Merali, Z., Plamondon, H. 2014. Evidence of lasting dysregulation of neuroendocrine and HPA axis function following global cerebral ischemia in male rats and the effect of Antalarmin on plasma corticosterone level. *Horm Behav* 65(3): p.273–84.

Lakhan, S.E., Kirchgessner, A., Hofer, M. 2009. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med* 7: p.97.

Larsen, S.B., Grove, E.L., Kristensen, S.D., Hvas, a-M. 2013. Reduced antiplatelet effect of aspirin is associated with low-grade inflammation in patients with coronary artery disease. *Thrombosis and haemostasis* 109(5): p.920–9.

Lee, S.C., Park, S.J., Ki, H.K., Gwon, H.C., Chung, C.S., Byun, H.S., Shin, K.J., Shin, M.H., Lee, W.R. 2000. Prevalence and risk factors of silent cerebral infarction in apparently normal adults. *Hypertension* 36(1): p.73–7.

Leonard, B.E., Myint, A. 2009. The psychoneuroimmunology of depression. *Pharmacology* (January): p.165–175.

Levite, M. 2008. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr Opin Pharmacol* 8(4): p.460–71.

Li, J., Siegel, M., Yuan, M., Zeng, Z., Finnucan, L., Persky, R., Hurn, P.D., McCullough, L.D. 2011. Estrogen enhances neurogenesis and behavioral recovery after stroke. *J Cereb Blood Flow Metab* 31(2): p.413–25.

Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., Giese, T., Veltkamp, R. 2009. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 15(2): p.192–9.

Liu, F., McCullough, L.D. 2012. Interactions between age, sex, and hormones in experimental ischemic stroke. *Neurochemistry international* 61(8): p.1255–65.

Loane, D.J., Byrnes, K.R. 2010. Role of microglia in neurotrauma. *Neurotherapeutics* 7(4): p.366–77.

Loftis, J.M., Huckans, M., Morasco, B.J. 2010. Neuroimmune mechanisms of cytokine-induced depression: current theories and novel treatment strategies. *Neurobiol Dis* 37(3): p.519–33.

Loubinoux, I., Kronenberg, G., Endres, M., Schumann-Bard, P., Freret, T., Filipkowski, R.K., Kaczmarek, L., Popa-Wagner, A. 2012. Post-stroke depression: mechanisms, translation and therapy. *J Cell Mol Med* 16(9): p.1961–9.

Luine, V. 2002. Sex differences in chronic stress effects on memory in rats. *Stress* 5(3): p.205–16.

Lyness, J.M. 2002. The cerebrovascular model of depression in late life. *CNS spectrums* 7(10): p.712–5.

Maccari, S., Morley-Fletcher, S. 2007. Effects of prenatal restraint stress on the hypothalamus-pituitary-adrenal axis and related behavioural and neurobiological alterations. *Psychoneuroendocrinology* 32 Suppl 1: p.S10–5.

Macrae, I.M. 2011. Preclinical stroke research--advantages and disadvantages of the most common rodent models of focal ischaemia. *Br J Pharmacol* 164(4): p.1062–78.

Maddahi, A., Edvinsson, L. 2010. Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway. *J Neuroinflamm* 7: p.14.

Maddahi, A., Kruse, L.S., Chen, Q.-W., Edvinsson, L. 2011. The role of tumor necrosis factor-α and TNF-α receptors in cerebral arteries following cerebral ischemia in rat. *J Neuroinflamm* 8(1): p.107.

Maeda, A., Fadeel, B. 2014. Mitochondria released by cells undergoing TNF-α-induced necroptosis act as danger signals. *Cell Death Dis* 5(7): p.e1312.

Man, S., Tucky, B., Cotleur, A., Drazba, J., Takeshita, Y., Ransohoff, R.M. 2012. CKCL12-Induced monocyte-endothelial interactions promote lymphocyte transmigration across an in vitro blood-brain-barrier. *Sci Transl Med* 4(119): p.1–18.

Mannacio, V.A., Di Tommaso, L., Antignan, A., De Amicis, V., Vosa, C. 2012. Aspirin plus clopidogrel for optimal platelet inhibition following off-pump coronary artery bypass surgery: results from the CRYSSA (prevention of Coronary arteRY bypaSS occlusion After off-pump procedures) randomised study. *Heart* 98(23): p.1710–5.

Mannix, R.C., Whalen, M.J. 2012. Traumatic brain injury , microglia , and beta amyloid. *International Journal of Alzheimer's Disease* 2012: p.1–5.

Manwani, B., McCullough, L.D. 2011. Sexual dimorphism in ischemic stroke: lessons from the laboratory. *Women's Health* 7(3): p.319–39.

Margolis, R.L., Robinson, R.G. 1985. Right and left cortical lesions asymmetrically alter cerebrovascular permeability in the rat. *Brain research* 359(1-2): p.81–7.

Mayzel-Oreg, O., Omae, T., Kazemi, M., Li, F., Fisher, M., Cohen, Y., Sotak, C.H. 2004. Microsphere-induced embolic stroke: an MRI study. *Magn Reson Med* 51(6): p.1232–8.

McColl, B.W., Allan, S.M., Rothwell, N.J. 2009. Systemic infection, inflammation and acute ischemic stroke. *Neuroscience* 158(3): p.1049–61.

McCoy, M.K., Tansey, M.G. 2008. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J Neuroinflamm* 5: p.45.

Meinl, E., Krumbholz, M., Hohlfeld, R. 2006. B lineage cells in the inflammatory central nervous system environment: migration, maintenance, local antibody production, and therapeutic modulation. *Ann Neurol* 59(6): p.880–92.

Merrett, D.L., Kirkland, S.W., Metz, G. a. 2010. Synergistic effects of age and stress in a rodent model of stroke. *Behav Brain Res* 214(1): p.55–9.

Miller, A.H. 2010. Depression and immunity: a role for T cells? *Brain, Behavior, and Immunity* 24(1): p.1–8.

Miller, A.H., Maletic, V., Raison, C.L. 2009. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol Psych* 65(9): p.732–41.

Miller, J.M., Vorel, S.R., Tranguch, A.J., Kenny, E.T., Mazzoni, P., van Gorp, W.G., Kleber, H.D. 2006. Anhedonia after a selective bilateral lesion of the globus pallidus. *The American journal of psychiatry* 163(5): p.786–8.

Milot, M.R., Plamondon, H. 2011. Changes in HPA reactivity and noradrenergic functions regulate spatial memory impairments at delayed time intervals following cerebral ischemia. *Hormones and Behavior* 59(4): p.594–604.

Mirabelli-Badenier, M., Braunersreuther, V., Viviani, G.L., Dallegri, F., Quercioli, A., Veneselli, E., Mach, F., Montecucco, F. 2011. CC and CXC chemokines are pivotal mediators of cerebral injury in ischaemic stroke. *Thrombosis and Haemostasis* 105(3): p.409–20.

Miyake, K., Takeo, S., Kaijihara, H. 1993. Sustained decrease in brain regional blood flow after microsphere embolism in rats. *Stroke* 24(3): p.415–420.

Miyake, K., Yamamoto, W., Tadokoro, M., Takagi, N., Sasakawa, K., Nitta, A., Furukawa, S., Takeo, S. 2002. Alterations in hippocampal GAP-43, BDNF, and L1 following sustained cerebral ischemia. *Brain Research* 935(1-2): p.24–31.

Moisse, K., Welch, I., Hill, T., Volkening, K., Strong, M.J. 2008. Transient middle cerebral artery occlusion induces microglial priming in the lumbar spinal cord: a novel model of neuroinflammation. *J Neuroinflamm* 5: p.29.

Morissette, M., Jourdain, S., Al Sweidi, S., Menniti, F.S., Ramirez, A.D., Di Paolo, T. 2007. Role of estrogen receptors in neuroprotection by estradiol against MPTP toxicity. *Neuropharmacology* 52(7): p.1509–20.

Moxon-Emre, I., Schlichter, L.C. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemia. *J Neuropath Exp Neurol* 69(1): p.1–15.

Müller, N., Myint, A.-M., Schwarz, M.J. 2011. Inflammatory biomarkers and depression. *Neurotoxicity research* 19(2): p.308–18.

Neigh, G.N., Glasper, E.R., Kofler, J., Traystman, R.J., Mervis, R.F., Bachstetter, A., DeVries, A.C. 2004. Cardiac arrest with cardiopulmonary resuscitation reduces dendritic spine density in CA1 pyramidal cells and selectively alters acquisition of spatial memory. *Eur J Neurosci* 20(7): p.1865–72.

Neigh, G.N., Karelina, K., Glasper, E.R., Bowers, S.L.K., Zhang, N., Popovich, P.G., DeVries, A.C. 2009. Anxiety after cardiac arrest/cardiopulmonary resuscitation: exacerbated by stress and prevented by minocycline. *Stroke* 40(11): p.3601–7.

Neigh, G.N., Karelina, K., Zhang, N., Glasper, E.R., Owens, M.J., Plotsky, P.M., Nemeroff, C.B., DeVries, A.C. 2009. Cardiac arrest and cardiopulmonary resuscitation dysregulates the hypothalamic-pituitary-adrenal axis. *J Cereb Blood Flow Metab* 29(10): p.1673–82.

Neigh, G.N., Kofler, J., Meyers, J.L., Bergdall, V., La, K.M.D., Traystman, R.J., Devries, A.C. 2004. Cardiac arrest/cardiopulmonary resuscitation increases anxiety-like behavior and decreases social interaction. *J Cereb Blood Flow Metab* 24(4): p.372–82.

Nellgård, B.M., Miura, Y., Mackensen, G.B., Pearlstein, R.D., Warner, D.S. 1999. Effect of intracerebral norepinephrine depletion on outcome from severe forebrain ischemia in the rat. *Brain Res* 847(2): p.262–9.

Nemeroff, C.B., Goldschmidt-Clermont, P.J. 2012. Heartache and heartbreak--the link between depression and cardiovascular disease. *Nature reviews. Cardiology* 9(9): p.526–39.

Nemeth, C.L., Gutman, D.A., Majeed, W., Keilholz, S.D., Neigh, G.N. 2014. Microembolism induces anhedonia but no detectable changes in white matter integrity in aged rats. *PloS ONE* 9(5): p.e96624.

Nemeth, C.L., Reddy, R., Bekhbat, M., Bailey, J., Neigh, G.N. 2014. Microglial activation occurs in the absence of anxiety-like behavior following microembolic stroke in female but not male rats. *J Neuroinflamm*.

Nemeth, C.L., Shurte, M.S., McTigue, D.M., Nemeroff, C.B., Neigh, G.N. 2012. Microembolism infarcts lead to delayed changes in affective-like behaviors followed by spatial memory impairment. *Behav Brain Res* 234(2): p.259–266.

Nijboer, C.H., Heijnen, C.J., Groenendaal, F., May, M.J., van Bel, F., Kavelaars, A. 2008. A dual role of the NF-kappaB pathway in neonatal hypoxic-ischemic brain damage. *Stroke* 39(9): p.2578–86.

Van Norden, A.G. et al. 2011. Causes and consequences of cerebral small vessel disease. The RUN DMC study: a prospective cohort study. Study rationale and protocol. *BMC neurology* 11(1): p.29.

Nozari, A. et al. 2010. Microemboli may link spreading depression, migraine aura, and patent foramen ovale. *Ann Neurol* 67(2): p.221–9.

O'Connor, T.M., O'Halloran, D.J., Shanahan, F. 2000. The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia. *QJM* 93(6): p.323–33.

O'Sullivan, M., Jarosz, J.M., Martin, R.J., Deasy, N., Powell, J.F., Markus, H.S. 2001. MRI hyperintensities of the temporal lobe and external capsule in patients with CADASIL. *Neurology* 56: p.628–634.

Olsson, T., Marklund, N., Gustafson, Y., Nasman, B. 1992. Abnormalities at different levels of the hypothalamic-pituitary- adrenocortical axis early after stroke. *Stroke* 23: p.1573–1576.

Pekmezovi, T., Zidverc-trajkovi, J., Jovanovi, Z., Tomi, G. 2011. What are the differences between younger and older patients with symptomatic small vessel disease ? 113: p.762–767.

Perry, V.H., Holmes, C. 2014. Microglial priming in neurodegenerative disease. *Nat Rev Neurol* 10(4): p.217–24.

Pfeilschifter, W., Czech-Zechmeister, B., Sujak, M., Mirceska, A., Koch, A., Rami, A., Steinmetz, H., Foerch, C., Huwiler, A., Pfeilschifter, J. 2011. Activation of sphingosine kinase 2 is an endogenous protective mechanism in cerebral ischemia. *Biochem Biophys Res Comm* 413(2): p.212–7.

Ping, F., Shang, J., Zhou, J., Zhang, H., Zhang, L. 2012. 5-HT(1A) receptor and apoptosis contribute to interferon-α-induced "depressive-like" behavior in mice. *Neurosci Lett* 514(2): p.173–8.

Pitkonen, M., Abo-Ramadan, U., Marinkovic, I., Pedrono, E., Hasan, K.M., Strbian, D., Durukan, A., Tatlisumak, T. 2012. Long-term evolution of diffusion tensor indices after temporary experimental ischemic stroke in rats. *Brain Research* 1445: p.103–10.

Pollard, M. 1973. Spontaneous prostate adenocarcinomas in aged germfree Wistar rats. *J Natl Cancer Inst* 51(4): p.1235–41.

Popa-Wagner, A., Carmichael, S.T., Kokaia, Z., Kessler, C., Walker, L.C. 2007. The response of the aged brain to stroke: too much, too soon? *Current neurovascular research* 4(3): p.216–27.

Prins, N.D., van Dijk, E.J., den Heijer, T., Vermeer, S.E., Jolles, J., Koudstaal, P.J., Hofman, A., Breteler, M.M.B. 2005. Cerebral small-vessel disease and decline in information processing speed, executive function and memory. *Brain* 128(Pt 9): p.2034–41.

Prut, L., Belzung, C. 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European Journal of Pharmacology* 463(1-3): p.3–33.

Purandare, N., Burns, A., Morris, J., Perry, E.P., Wren, J., Mccollum, C. 2012. Association of cerebral emboli with accelerated cognitive deterioration in Alzheimer's disease and vascular dementia. *Am J Psychiatry* 169(3): p.300–8.

Pyter, L.M., Kelly, S.D., Harrell, C.S., Neigh, G.N. 2013. Sex differences in the effects of adolescent stress on adult brain inflammatory markers in rats. *Brain, Behavior, and Immunity* 30(January): p.88–94.

Quillinan, N., Deng, G., Grewal, H., Herson, P.S. 2014. Androgens and stroke: good, bad or indifferent? *Exp Neurol* 259: p.10–5.

Radak, D., Resanovic, I., Isenovic, E.R. 2013. Changes in Hypothalamus-Pituitary-Adrenal Axis Following Transient Ischemic Attack. *Angiology* 00(0): p.1–10.

Raison, C.L., Capuron, L., Miller, A.H. 2006. Cytokines sing the blues: inflammation and the pathogenesis of depression. *Trends Immunol* 27(1): p.24–31.

Raison, C.L., Rutherford, R.E., Woolwine, B.J., Shuo, C., Schettler, P., Drake, D.F., Haroon, E., Miller, A.H. 2012. A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: The role of baseline inflammatory biomarkers. *JAMA Psychiatry* 70(1): p.31–41.

Rankine, E.L., Hughes, P.M., Botham, M.S., Perry, V.H., Felton, L.M. 2006. Brain cytokine synthesis induced by an intraparenchymal injection of LPS is reduced in MCP-1-deficient mice prior to leucocyte recruitment. *The European journal of neuroscience* 24(1): p.77–86.

Ringelstein, E.B., Nabavi, D.G. 2005. Cerebral small vessel diseases: cerebral microangiopathies. *Current Opinion in Neurology* 18(2): p.179–88.

Ritzel, R.M., Capozzi, L.A., McCullough, L.D. 2013. Sex, stroke, and inflammation: the potential for estrogen-mediated immunoprotection in stroke. *Horm Behav* 63(2): p.238–53.

Robinson, R.G., Coyle, J.T. 1980. (Accepted September 13th, 1979). *Behavioral Science* 188: p.63–78.

Robinson, R.G., Starr, L.B., Lipsey, J.R., Rao, K., Price, T.R. 1984. A two-year longitudinal study of post-stroke mood disorders: dynamic changes in associated variables over the first six months of follow-up. *Stroke* 15(3): p.510–517.

Rodriguez-Grande, B., Blackabey, V., Gittens, B., Pinteaux, E., Denes, A. 2012. Loss of substance P and inflammation precede delayed neurodegeneration in the substantia nigra after cerebral ischemia. *Brain, Behavior, and Immunity* 29: p.51–61.

Román, G.C. 2002. Vascular dementia revisited: diagnosis, pathogenesis, treatment, and prevention. *Med Clin N Am* 86(3): p.477–99.

Rothwell, P.M. et al. 2005. Population-based study of event-rate, incidence, case fatality, and mortality for all acute vascular events in all arterial territories (Oxford Vascular Study). *Lancet* 366(9499): p.1773–83.

Rubin, R.R., Gaussoin, S. a, Peyrot, M., DiLillo, V., Miller, K., Wadden, T. a, West, D.S., Wing, R.R., Knowler, W.C. 2010. Cardiovascular disease risk factors, depression symptoms and antidepressant medicine use in the Look AHEAD (Action for Health in Diabetes) clinical trial of weight loss in diabetes. *Diabetologia* 53(8): p.1581–9.

Rygula, R., Abumaria, N., Flügge, G., Fuchs, E., Rüther, E., Havemann-Reinecke, U. 2005. Anhedonia and motivational deficits in rats: impact of chronic social stress. *Behav Brain Res* 162(1): p.127–34.

Sánchez-Mendoza, E., Bellver-Landete, V., Merino, J.J., González, M.P., Martínez-Murillo, R., Oset-Gasque, M.J. 2013. Review: could neurotransmitters influence neurogenesis and neurorepair after stroke? *Neuropathol Appl Neurobiol* 39(7): p.722–35.

Santos, M., Gold, G., Kövari, E., Herrmann, F.R., Bozikas, V.P., Bouras, C., Giannakopoulos, P. 2009. Differential impact of lacunes and microvascular lesions on poststroke depression. *Stroke* 40(11): p.3557–62.

Santos, M., Kovari, E., Hof, P.R., Gold, G., Bouras, C., Giannakopoulos, P. 2009. The impact of vascular burden on late-life depression. *Brain Research* 62(1): p.19–32.

Sekeljic, V., Bataveljic, D., Stamenkovic, S., Ułamek, M., Jabłoński, M., Radenovic, L., Pluta, R., Andjus, P.R. 2012. Cellular markers of neuroinflammation and neurogenesis after ischemic brain injury in the long-term survival rat model. *Brain Structure & Function* 217(2): p.411–20.

Semenas, E., Nozari, A., Sharma, H.S., Basu, S., Rubertsson, S., Wiklund, L. 2010. Sex differences in cerebral injury after severe haemorrhage and ventricular fibrillation in pigs. *Acta Anaesthesiologica Scandinavica* 54(3): p.343–53.

Serebruany, V.L. et al. 2003. Platelet/endothelial biomarkers in depressed patients treated with the selective serotonin reuptake inhibitor sertraline after acute coronary events: the Sertraline AntiDepressant Heart Attack Randomized Trial (SADHART) Platelet Substudy. *Circulation* 108(8): p.939–44.

Shapira, S., Sapir, M., Wengier, A., Grauer, E., Kadar, T. 2002. Aging has a complex effect on a rat model of ischemic stroke. *Brain Research* 925(2): p.148–58.

Sheline, Y.I. et al. 2010. Support for the vascular depression hypothesis in late-life depression. *Arch Gen Psychiatry* 67(3): p.277–285.

Shereen, A., Nemkul, N., Yang, D., Adhami, F., Dunn, R.S., Hazen, M.L., Nakafuku, M., Ning, G., Lindquist, D.M., Kuan, C.-Y. 2011. Ex vivo diffusion tensor imaging and neuropathological correlation in a murine model of hypoxia-ischemia-induced thrombotic stroke. *J Cereb Blood Flow Metab* 31(4): p.1155–69.

Shimoda, K., Robinson, R.G. 1999. The relationship between poststroke depression and lesion location in long-term follow-up. *Biol Psych* 45(2): p.187–92.

Slavich, G.M., Irwin, M.R. 2014. From stress to inflammation and major depressive disorder: a social signal transduction theory of depression. *Psychol Bull* 140(3): p.774–815.

Small, D.L., Buchan, A.M. 2000. Animal models. *British Medical Bulletin* 56(2): p.307–17.

Smith, A.L., Alexander, M., Rosenkrantz, T.S., Sadek, M.L., Fitch, R.H. 2014. Sex differences in behavioral outcome following neonatal hypoxia ischemia: insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury. *Exp Neurol* 254: p.54–67.

Sneed, J.R., Rindskopf, D., Steffens, D.C., Krishnan, K.R.R., Roose, S.P. 2008. The vascular depression subtype: evidence of internal validity. *Biol Psych* 64(6): p.491–7.

Soares, J.C., Mann, J.J. 1997. The anatomy of mood disorders--review of structural neuroimaging studies. *Biol Psych* 41(1): p.86–106.

Song, C., Wang, H. 2011. Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. *Prog Neuropsychopharmacol Biol Psych* 35(3): p.760–8.

Sotak, C.H. 2002. The role of diffusion tensor imaging in the evaluation of ischemic brain injury - a review. *NMR Biomed* 15(7-8): p.561–9.

Spalletta, G., Bossù, P., Ciaramella, A., Bria, P., Caltagirone, C., Robinson, R.G. 2006. The etiology of poststroke depression: a review of the literature and a new hypothesis involving inflammatory cytokines. *Molecular Psychiatry* 11(11): p.984–91.

Steed, P.M. et al. 2003. Inactivation of TNF signaling by rationally designed dominant-negative TNF variants. *Science (New York, N.Y.)* 301(5641): p.1895–8.

Steiner, M. 2011. Serotonin, depression, and cardiovascular disease: sex-specific issues. *Acta Physiol* 203(1): p.253–8.

Steiner, M., Dunn, E., Born, L. 2003. Hormones and mood: from menarche to menopause and beyond. *J Affect Dis* 74(1): p.67–83.

Stockmeier, C.A., Mahajan, G.J., Konick, L.C., Overholser, J.C., Jurjus, G.J., Meltzer, H.Y., Uylings, H.B.M., Friedman, L., Rajkowska, G. 2004. Cellular changes in the postmortem hippocampus in major depression. *Biol Psych* 56(9): p.640–50.

Strecker, J.-K., Minnerup, J., Gess, B., Ringelstein, E.B., Schäbitz, W.-R., Schilling, M. 2011. Monocyte chemoattractant protein-1-deficiency impairs the expression of IL-6, IL-1β and G-CSF after transient focal ischemia in mice. *PLoS ONE* 6(10): p.e25863.

Sun, X., D.C., S., Au, R., Folstein, M., Summergrad, P., Yee, J., Rosenberg, I., Mwamburi, M., Qiu, W.Q. 2008. Amyloid-Associated Depression: A prodromal depression of Alzheimer Disease? *Arch Gen Psychiatry* 65(5): p.542–550.

Supanc, J., Biloglav, Z., Kes, V., Demarin, V. 2011. Role of cell adhesion molecules in acute ischemic stroke. *Ann Saudi Med* 31(4): p.365–70.

Taguchi, T., Miyake, K., Tanonaka, K., Okada, M., Takagi, N., Fujimor, K., Takeo, S., Fujimori, K. 1993. Sustained changes in acetylcholine and amino acid contents of brain regions following microsphere embolism in rats. *Japan J. Pharmacol.1* 62: p.269–78.

Takagi, N., Miyake, K., Taguchi, T., Sugita, N., Takagi, K., Tamada, H., Takeo, S. 1997. Changes in cholinergic neurons and failure in learning function after microsphere embolism-induced cerebral ischemia. *Brain Res Bull* 43(1): p.87–92.

Takagi, N., Miyake, K., Taguchi, T., Tamada, H., Takagi, K., Sugita, N., Takeo, S. 1997. Failure in learning task and loss of cortical cholinergic fibers in microsphere-embolized rats. *Exp Brain Res* 114: p.279–87.

Tansey, M.G., Goldberg, M.S. 2010. Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. *Neurobiol Dis* 37(3): p.510–8.

Taoufik, E., Tseveleki, V., Chu, S.Y., Tselios, T., Karin, M., Lassmann, H., Szymkowski, D.E., Probert, L. 2011. Transmembrane tumour necrosis factor is neuroprotective and regulates experimental autoimmune encephalomyelitis via neuronal nuclear factor-kappaB. *Brain* 134(Pt 9): p.2722–35.

Taylor, W.D., Aizenstein, H.J., Alexopoulos, G.S. 2013. The vascular depression hypothesis: mechanisms linking vascular disease with depression. *Molecular Psychiatry* (October 2012): p.1–12.

Thomas, A.J., Ferrier, I.N., Kalaria, R.N., Perry, R.H., Brown, A., O'Brien, J.T. 2001. A neuropathological study of vascular factors in late-life depression. *J Neurol Neurosurg Psychiatry* 70(1): p.83–7.

Toner, C.C., Connelly, K., Whelpton, R., Bains, S., Michael-Titus, A., Mclaughlin, D.P., Stamford, J. 2001. Effects of sevoflurane on dopamine, glutamate and aspartate release in an in vitro model of cerebral ischaemia. *Br J Anaesth* 86(4): p.550–554.

Toner, C.C., Stamford, J. a. 1996. "Real time" measurement of dopamine release in an in vitro model of neostriatal ischaemia. *J Neurosci Methods* 67(2): p.133–40.

Tripathi, R.B., McTigue, D.M. 2008. Chronically increased ciliary neurotrophic factor and fibroblast growth factor-2 expression after spinal contusion in rats. *J Comp Neurol* 510(2): p.129–44.

Tully, P.J., Baker, R. a. 2012. Depression, anxiety, and cardiac morbidity outcomes after coronary artery bypass surgery: a contemporary and practical review. *J Geriat Cardiol* 9(2): p.197–208.

Tupler, L.A., Krishnan, K.R.R., McDonald, W.M., Dombeck, C.B., D'Souza, S., Steffens, D.C. 2002. Anatomic location and laterality of MRI signal hyperintensities in late-life depression. *J Psychosomat Res* 53(2): p.665–76.

Turnbull, A., Rivier, C. 1999. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol Rev* 79(1): p.1–71.

Van den Tweel, E.R.W., Kavelaars, A., Lombardi, M.S., Groenendaal, F., May, M., Heijnen, C.J., van Bel, F. 2006. Selective inhibition of nuclear factor-kappaB activation after hypoxia/ischemia in neonatal rats is not neuroprotective. *Ped Res* 59(2): p.232–6.

Tynan, R.J., Weidenhofer, J., Hinwood, M., Cairns, M.J., Day, T.A., Walker, F.R. 2012. A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. *Brain Behav Immun* 26(3): p.469–79.

Valente, S.A., Fallon, W.F., Alexander, T.S., Tomas, E.R., Evancho-Chapman, M.M., Schmidt, S.P., Gorski, R., Pizov, O., DeFine, L., Clark, A.J. 2009. Immunologic function in the elderly after injury--the neutrophil and innate immunity. *J Trauma* 67(5): p.968–74.

Vermeer, S.E., Longstreth, W.T., Koudstaal, P.J. 2007. Silent brain infarcts: a systematic review. *Lancet Neurol* 6(7): p.611–9.

Vermeer, S.E., Prins, N.D., den Heijer, T., Hofman, A., Koudstaal, P.J., Breteler, M.M.B. 2003. Silent brain infarcts and the risk of dementia and cognitive decline. *N Engl J Med* 348(13): p.1215–22.

Vitale, C., Miceli, M., Rosano, G.M.C. 2007. Gender-specific characteristics of atherosclerosis in menopausal women: risk factors, clinical course and strategies for prevention. *Climacteric* 10 Suppl 2(Suppl 2): p.16–20.

Wagner, A.K., Willard, L.A., Kline, A.E., Wenger, M.K., Bolinger, B.D., Ren, D., Zafonte, R.D., Dixon, C.E. 2004. Evaluation of estrous cycle stage and gender on behavioral outcome after experimental traumatic brain injury. *Brain Res* 998(1): p.113–121.

Wang, Q., Tang, X.N., Yenari, M.A. 2007. The inflammatory response in stroke. *J Neuroimmunol* 184(1-2): p.53–68.

Wang, S.H., Zhang, Z., Guo, Y.J., Zhou, H., Teng, G.J., Chen, B.A. 2009. Anhedonia and activity deficits in rats: impact of post-stroke depression. *J Psychopharmacol* 23(3): p.295–304.

Westfall, J.M., Mold, J., Fagnan, L. 2007. Practice-based research--“Blue Highways” on the NIH Roadmap. *JAMA* 297(4): p.403–406.

White, R.E., Gerrity, R., Barman, S.A., Han, G. 2010. Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. *Steroids* 75(11): p.788–93.

Whyte, E.M., Mulsant, B.H., Vanderbilt, J., Dodge, H.H., Ganguli, M. 2004. Depression after stroke: a prospective epidemiological study. *J Am Geriatr Soc* 52(5): p.774–8.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., Muscat, R. 1987. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology* 93(3): p.358–364.

Wohleb, E.S., Patterson, J.M., Sharma, V., Quan, N., Godbout, J.P., Sheridan, J.F. 2014. Knockdown of interleukin-1 receptor type-1 on endothelial cells attenuated stress-induced neuroinflammation and prevented anxiety-like behavior. *J Neurosci* 34(7): p.2583–91.

Wohleb, E.S., Powell, N.D., Godbout, J.P., Sheridan, J.F. 2013. Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. *J Neurosci* 33(34): p.13820–33.

Wong, T.Y., Klein, R., Sharrett, A.R., Couper, D.J., Klein, B.E.K., Liao, D.-P., Hubbard, L.D., Mosley, T.H. 2002. Cerebral white matter lesions, retinopathy, and incident clinical stroke. *JAMA* 288(1): p.67–74.

Woolf, S.H. 2008. The meaning of translational research and why it matters. *JAMA* 299(2): p.211–3.

Wu, J., Holstein, J.D., Upadhyay, G., Lin, D.-T., Conway, S., Muller, E., Lechleiter, J.D. 2007. Purinergic receptor-stimulated IP3-mediated Ca2+ release enhances neuroprotection by increasing astrocyte mitochondrial metabolism during aging. *J Neurosci* 27(24): p.6510–20.

Xekardaki, A., Giannakopoulos, P., Haller, S. 2011. White matter changes in bipolar disorder, alzheimer disease, and mild cognitive impairment: New insights from DTI. *Journal of Aging Research* 2011: p.1–10.

Xekardaki, A., Santos, M., Hof, P., Kövari, E., Bouras, C., Giannakopoulos, P. 2012. Neuropathological substrates and structural changes in late-life depression: the impact of vascular burden. *Acta neuropathologica* 124(4): p.453–64.

Xia, W., Han, J., Huang, G., Ying, W. 2010. Inflammation in ischaemic brain injury: current advances and future perspectives. *Clin Exp Pharmacol Physiol* 37(2): p.253–8.

Xiong, Y., Mahmood, A., Lu, D., Qu, C., Goussev, A., Schallert, T., Chopp, M. 2007. Role of gender in outcome after traumatic brain injury and therapeutic effect of erythropoietin in mice. *Brain Res* 1185: p.301–12.

Yamasaki, Y., Itoyama, Y., Kogure, K. 1996. Involvement of cytokine production in pathogenesis of transient cerebral ischemia damage. *Keio J Med* 45(3): p.225–9.

Yamashita, H., Fujikawa, T., Takami, H., Yanai, I., Okamoto, Y., Morinobu, S., Yamawaki, S. 2010. Long-term prognosis of patients with major depression and silent cerebral infarction. *Neuropsychobiol* 62(3): p.177–81.

Yarilina, A., Park-Min, K.-H., Antoniv, T., Hu, X., Ivashkiv, L.B. 2008. TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes. *Nat Immunol* 9(4): p.378–87.

Zhang, L., Nair, A., Krady, K., Corpe, C., Bonneau, R., Simpson, I., Vannucci, S. Estrogen stimulates microglia and brain recovery from hypoxia-ischemia in normoglycemic but not diabetic female mice. *J Clin Invest* 113(1): p.85–95.

Zheng, W., Watts, L.T., Holstein, D.M., Prajapati, S.I., Keller, C., Grass, E.H., Walter, C.A., Lechleiter, J.D. 2010. Purinergic receptor stimulation reduces cytotoxic edema and brain infarcts in mouse induced by photothrombosis by energizing glial mitochondria. *PloS ONE* 5(12): p.e14401.

Zhu, L., Hoffmann, A., Wintermark, M., Pan, X., Tu, R., Rapp, J.H. 2012. Do microemboli reach the brain penetrating arteries? *J Surg Res* 176(2): p.679–83.

Ziebell, J.M., Morganti-Kossmann, M.C. 2010. Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. *Neurotherapeutics* 7(January): p.22–30.

Zollo, M., Di, V., Daniela, D., Daniela, S., Liguori, L., Marino, N., Vastolo, V., Navas, L. 2012. Targeting monocyte chemotactic protein-1 synthesis with bindarit induces tumor regression in prostate and breast cancer animal models. *Clin Exp Metastasis* 29: p.585–601.

Del Zoppo, G.J., Gorelick, P.B. 2010. Innate inflammation as the common pathway of risk factors leading to TIAs and stroke. *Ann NY Acad Sci* 1207: p.8–10.

Del Zoppo, G.J., Mabuchi, T. 2003. Cerebral microvessel responses to focal ischemia. *J Cereb Blood Flow Metab* 23(8): p.879–94.

Zunszain, P.A., Anacker, C., Cattaneo, A., Carvalho, L.A., Pariante, C.M. 2011. Glucocorticoids, cytokines and brain abnormalities in depression. *Prog Neuro-Psychoph* 35(3): p.722–9.