SEX DIFFERENCES IN MICROGLIA MORPHOLOGY IN RESPONSE TO ISCHEMIC STROKE

By

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Abstract

In this study we assessed microglia morphology in male, pre-menopausal female, and post-menopausal female mice (Male n = 5-6; Pre M n = 6; PM n = 8) following 8 hours of reperfusion, as a measurable response to injury. We hypothesized that because the size of infarct is decreased in pre-menopause female versus post-menopause female and male mice, that changes in microglia morphology post stroke will vary according to sex group. Using skeletal and fractal analysis, we found that ramified morphology is decreased in proximity to injury (endpoints/cell region main effect: $F_{(3,68)} = 20.71$, $p < 0.0001$; process length/cell region main effect: $F_{(3,68)} = 11.63$, $p < 0.0001$); however there are no differences among sex groups for the endpoints/cell variable ($F_{(2,68)} = 0.6$, $p < 0.55$). In addition, fractal dimension decreased in proximity to the ischemic region with significant differences according to sex group (two-way ANOVA: region: $F_{(3,57)} = 36.80$, $p < 0.0001$; sex group: $F_{(2,19)} = 7.5$, $p < 0.01$). The focus of this study is on the basic discovery of microglia morphological response that can be used to develop treatments that have minimal variability among sex groups.

Chapter 1

Introduction

Strokes are the leading cause of disability in the United States and are a significant health concern that affects over 33 million people worldwide (CDC, 2018). Given that females account for more than half of all stroke deaths and have a greater lifetime risk for stroke, identifying the pathophysiology behind the sex-specific difference in immune cells may help identify interventions to improve outcomes. This thesis researches sex differences in microglia morphology as a measurable response to ischemic stroke and reperfusion. The initial chapters will discuss stroke etiology, the pathophysiology of ischemic stroke to include the brain cell types involved in post-stroke injury and repair with a focus on the role of microglia in the surveil of the parenchyma with ischemia, infarction, and dysfunction. The remaining chapters are devoted to the methodologies and results formed in this research, concluded by the clinical significance these findings play in understanding the role of sex in pathology and disease outcomes.

Types of Stroke

A stroke is the occlusion or eruption of a blood vessel in the brain that through a cascade of events leads to neuronal tissue ischemia and necrosis. As of 2016, strokes became the fifth leading cause of death in the United States (Mozzafarian et al., 2016). As a leading cause of death and occurring nearly every 40 seconds, strokes are a costly incidence, accounting for $34 billion each year in health care services, medications, and treatments (Mozzafarian et al., 2016). By understanding the pathophysiology of immune cells such as microglia and the role played in mitigating ischemic injury, personalized evidenced-based nursing interventions can improve healing and reduce the overall death, cost, incidence, and recurrence rates.

Hemorrhagic strokes. A hemorrhagic stroke occurs from the rupture of a weakened blood vessel that causes primary damage through ischemia of the parenchymal and secondary damage from increased intracranial pressure, cytotoxicity, oxidative stress and excitotoxicity (Shi, 2017). The main causes of a hemorrhagic stroke are cerebral aneurysms and arteriovenous malformations. A cerebral aneurysm develops at the bifurcation of arteries and is created from constant pressure changes that weaken the walls resulting in a ballooning of the vessel. An arteriovenous malformation (AVM) is the weakening of the area that connects arteries and veins together. As the wall weakens over time, microbleeds can occur and with a rapid change in pressure can lead to a hemorrhage. The primary result of bleeding produces a physical disruption of the cerebral architecture, while secondary cascades result in the deterioration of patients with a hemorrhage (Shi, 2017). As a result, inflammation, oxidative stress, and blood-brain barrier dysfunction lead to neuronal tissue injury.

Transient ischemic attack. A transient ischemic attack, or TIA, is a temporary blockage in blood flow to the brain, sometimes referred to a “mini-stroke”, despite being capable of causing true stroke-like ischemia. A TIA can result from a mired of complications and are categorized by embolic, lacunar, and large artery/low flow TIA. An embolic TIA is a single but prolonged episode of focal ischemia caused by a clot that has broken off from its original location and has traveled to an artery in the brain. A lacunar or small penetrating vessel TIA is caused by the stenosis of an intracerebral penetrating vessel that stems from the middle cerebral artery, basilar artery, vertebral artery, or Circle of Willis, while a large artery/low flow TIA is an atherosclerotic abrasion at the internal carotid (Hadjiev &Mineva, 2007). Though these attacks are classified by a symptom duration of less than an hour and do not show evidence of acute infarction, focal neurological damage can occur. Infarction does not occur because, during local

brain hypoperfusion, ion gradients are not irreversibly damaged, therefore decreasing the formation of the ischemic penumbra (Hadjiev &Mineva, 2007). It is from this that the benefits of neuroprotection and reperfusion are more obvious in TIA compared to more damaging forms of stroke.

Ischemic stroke. An ischemic stroke is a neuronal death caused by oxygen deprivation that ultimately leads to tissue infarction and focal brain injury. With ischemic strokes accounting for 87% of all strokes, it has become a major cause of mortality and disability across the globe (Mozzafarian et al., 2016). Though declining since 2010, the mortality rate of stroke continues to be a significant problem despite attention to modifiable risk factors such as smoking and hypertension because, among many reasons, of a lack of understanding and treatment based on sex differences (Morrison & Filosa, 2016). It is known that stroke is less prevalent in women until menopause when the incidence and injury surpass the incidence rate for men. In addition, the mortality rate for post-menopausal women with ischemic stroke is greatly increased and approximates 60% of all stroke deaths (Samai & Martin-Schild, 2015)

In general, cerebral ischemia in the brain can occur from acute events such as cardiac arrest, drowning, strangulation, choking, traumatic brain injuries (TBI), and carbon monoxide poisoning, or more chronic causes such as large artery atherosclerosis, cardioembolism, and small artery occlusions (Alexander, 2013). Depending on the subclassification of ischemic stroke, these events produce ischemia via thrombi, emboli, and/or hypercoagulability.

Thrombotic ischemic stroke. A thrombotic ischemic stroke occurs from arterial stenosis or the occlusion of vessels within the brain. Luminal changes caused by plaque accumulation within the main large arteries in the cerebral circulation (i.e. carotid artery, middle cerebral artery, anterior cerebral artery, posterior cerebral artery, vertebral artery, or basilar artery) or

small arterial occlusions of cerebral vessels are considered thrombotic (Alexander, 2013). Similar to coronary artery disease and peripheral vascular disease, large artery atherosclerosis of cerebral vessels results from the progressive accumulation of plaque within the vessel wall reducing the interior diameter of the lumen. The risk factors mentioned above in general cause increased vascular resistance. In addition to resistance, systemic and local factors such as increased platelet reactivity and tissue factors contribute to the formation of thrombi in vulnerable plaques which can become lodged in the lumen and impede flow (Alexander, 2013).

Embolic ischemic stroke. An embolic stroke occurs when a blood clot originating elsewhere in the body (artery-to-artery and cardiac embolism) dislodges and then enters the cerebral circulation. When the clot size exceeds the vessel diameter, an occlusion will occur and result in ischemia to the area supplied by the occluded artery. An artery-to-artery embolism is more likely to form from the develop from large artery atherosclerotic plaque, decreased forward flow, and the presence of luminal abnormalities (Deb, Sharma, & Hassan, 2010). When a portion of the plaque, clot, or thrombus becomes dislodged and travel to into the cerebral circulation to initiate an embolic stroke. A cardio-embolism is responsible for approximately 20% of all ischemic strokes and form from cardiac abnormalities such as atrial fibrillation, mechanical prosthetic valves, and valvular heart disease are associated with embolus formation that can lead to cerebral embolism and ischemic stroke (Furie et al., 2011). When an embolus originates in the heart, it can travel through the cardiac cycle and then be ejected from the left ventricle. The embolus then enters the cerebral circulation via the carotid arteries that originate in the aortic arch or into the vertebral arteries via the subclavian arteries.

Hypercoagulability/Thrombophilia. In ischemic strokes, thrombophilia, or the tendency to clot may result if there are hematologic clotting abnormalities that predispose an individual to

premature clotting. Primary thrombophilic states are thought to be responsible for 1–4% of all ischemic strokes (Furie et al., 2011). Prothrombin gene mutations found in patients with cryptogenic stroke and Patent Foramen Ovale (PFO), for example, may be associated with venous thromboembolism. This can result in ischemic stroke when a thrombus from the venous circulation shunts to the left side of the heart, becoming an arterial thrombus that travels to the brain.

Brain Cells Involved in Ischemic Stroke

Among immune-to-brain interactions, it is now recognized that there is a dynamic interaction between the central nervous system and the immune system that extends from pathology to homeostasis. With the immune system acting as a sensor of environment, different responses in tissue can be seen through microglial activation (Jakel & Dimou, 2017). However, before understanding the complex relationship between the two during an ischemic event, it is important to discuss the function of resident cells within the brain.

Neurons. Neurons are the information messengers of the central nervous systems. Using electrical impulses, signals can be transmitted from one area of the brain to the surrounding structures and components of the nervous system. The three types of neurons area sensory, motor, and interneurons. Sensory neurons, as inferred by the name, carry sensory information from the sensory organs to the brain. Motor neurons control muscle activity, while interneurons relay information between peripheral and central neurons. The basic part of a neuron is the cell body, axon, and dendrites. Within the cell body, the nucleus controls the cellular activity and contains the genetic material. The axon transmits a message, while the dendrites receive incoming chemical messages. In terms of stroke, the neurons suffer hypoxia from decreased perfusion secondary to diminished glucose and ATP viability (Jakel & Dimou, 2017).

Glia. The function of a glial cell is to provide support for the neuron. Upon discovery, these cells were named after the belief that they acted as the glue – derived from the Greek word glue -- for neurons, essentially holding them together (Jakel & Dimou, 2017). Recent findings have shown that there are vital roles that glial cells play in maintaining the neuronal environment (Jakel & Dimou, 2017), significantly extending their function in the healthy and injured brain beyond that of "glue" and support. Three main types of glial cells are astrocytes, oligodendrocytes, and microglia.

Astrocytes. Functionally, astrocytes maintain, mediate, and restore neuronal function during pathologic and physiologic conditions (Morrison & Filosa, 2016). An astrocyte is a highly branched glial cell that as a population, occupies a large amount of space within the brain and spinal cord. Through the development of branching, astrocyte processes form a scaffold within the CNS that provides structural support for neurons and other glial matter. During a traumatic event to the brain tissue, astrocytes will also proliferate and migrate to an area of injury and form a hypertrophic tissue from their processes that form a scar around the injured tissue, essentially barricading it from healthy tissue. Astrocytes also maintain homeostasis of the interstitial fluid through the regulation of ion gradients and synaptic maintenance. Lastly, these cells play a vital role in neuronal functioning under hypoperfused conditions. By producing lactate in an environment depleted of oxygen and glucose, neurons can maintain a level of function that enables the production of ATP used by the neuron for energy (Deb, Sharma, & Hassan, 2010). This in turn can prevent necrosis of brain tissue.

Oligodendrocytes. During development, oligodendrocytes develop from the subventricular zone of the lateral ventricles for the cerebellum (Jakel & Dimou, 2017). In the spinal cord, oligodendrocytes originate from the ventral regions of the neural tube and in the

optic nerve, they migrate into the nerve from the third ventricle (Jakel & Dimou, 2017). It is the oligodendrocyte precursor cells which migrate to their destination where they then differentiate into the more mature oligodendrocytes. The proliferation of the oligodendrocyte progenitor cells is controlled by the number of growth factors released from neurons and astrocytes such as platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) (Jakel & Dimou, 2017). Oligodendrocytes support the axon of the neuron through the production of a lipid substance called myelin. Myelin is wrapped around the axon of a neuron and serves as a layer of insulation that allows for the acceleration of electrical impulses up to 200 m/second, thus increasing neuronal functioning (Jakel & Dimou, 2017).

Microglia. Microglia, the local immune cells in the brain, functionally survey neuronal tissue through constant monitoring of the neuronal environment. During early embryogenesis, a significant proportion of these cells migrate from the yolk sac and populate in the neuroepithelium (Ma, Wang, & Yang, 2017). The primitive function of microglia in early development is the phagocytosis of apoptotic neuronal cells, a manifestation of the creation of circuitry in the developing CNS (Ma, Wang, & Yang, 2017). However, the primary function of microglia in the adult brain is to monitor neurons and the interstitial environment. In the healthy adult brain, microglia morphology is termed as ramified, meaning that they have small somas and highly branched or arborized processes. This ramified morphology facilitates their continual interaction with neurons and glia within their territory. Through alterations in morphology known as ramification, microglia can continually interact with neurons and glia within their territory with process activity. This morphologic structure distinguishes microglia from other systemic immune cells that infiltrate the brain during cerebral events. Microglia are distributed through the parenchyma of the brain and can vary in density depending on regional need and

density of other cell-types (De Biase et al., 2017). This distribution is optimal for immediate response during ischemic events in which deramification occurs upon activation of microglia causing a proinflammatory cascade and a decreasing number of projections creating an irregular appearance (Morrison & Filosa, 2016). This change in morphologic structure then becomes a measurable means of microglia activation in response to an acute physical or chemical injury.

Microglia as a first responder to ischemic stroke. Microglia are the first line of defense and after ischemic brain injury. These cells migrate toward the lesion site and exacerbate tissue injury by producing inflammatory cytokines and cytotoxic substances; however, microglia also contribute to tissue restoration and remodeling by phagocyting debris. Following an ischemic event, microglia activation is the first step in the creation of an inflammatory response in the brain, followed by the infiltration of immune cells such as neutrophils and macrophages (Ma, Wang, & Yang, 2017). Within minutes, activated microglia produce nitric oxide, proinflammatory cytokines, anti-inflammatory cytokines, growth factors, and plasminogen (Ma, Wang, & Yang, 2017). For example, tumor necrosis factor -alpha is a proinflammatory cytokine upregulated after an ischemic stroke that enhances the tolerance of immune cells to withstand oxidative stress and ischemic injury but causes an increase in swelling, infarction volume, and neurological deficits post-stroke (Perry & Teeling, 2013). This is also seen in proximal and distal activation of microglia to the site of infarction. Activated microglia in the infarct zone have a negative correlation with tissue preservation, but in the remote area, activated microglia have a positive correlation with tissue preservation (Morrison & Filosa, 2016).

Summary and Study Objectives

In summary, strokes can be differentiated into multiple classifications each with specific causes that lead to neuronal death and loss of brain function. Glial cells also contribute to brain

function and are exquisitely linked to neuronal action during health and disease and are therefore impacted by interactions with hypo-perfused neurons post-stroke. The objective of this study is to investigate changes in microglia morphology after ischemic stroke and 8 hours of reperfusion in female (pre-menopause and post-menopause) and male mice. We hypothesize that because the size of infarct is decreased in pre-menopause female versus post-menopause female and male mice, that changes in microglia morphology post stroke will vary according to sex group. In the next chapter, we discuss, in greater detail, the events that occur after ischemic stroke that results in brain injury and the influence of biological sex on post-stroke brain injury.

Chapter 2

Pathophysiology of Ischemic Stroke

As described above, ischemic stroke is a term that depicts conditions in which blood flow to the cerebral tissue is impeded resulting in tissue damage and infarction. Ischemic stroke can manifest in an embolic stroke, venous thrombosis, thrombotic stroke, or systemic hypoperfusion. Beyond cause, the vasculature supply is compromised, and neurons cannot recover from low energy reserves and a critical dependence on anaerobic metabolism. These factors put tissue at high risk for ischemia under such conditions. This damage occurs on a continuum where necrotic tissue correlates to blood flow below <10-25% of normal volume and is proximal to the vessel blockage, while the tissue distal to the site of stroke, the penumbra, is an area of hypo-perfused but viable tissue at risk for necrosis if ischemia is not resolved (Deb, Sharma, & Hassan, 2010). The penumbra is the target of medical and nursing therapeutics to increase blood flow to the brain or limit neuroinflammation. During an ischemic event, the inner core is critically hypo-perfused at less than 10 ml/100 g/min and is at risk of dying, while the penumbra is perfused at approximately 60 ml/100 g/min and is less likely to die (Deb, Sharma, & Hassan, 2010).

Neurons in the penumbra are mostly dysfunctional but may recover if perfused in time. This forms the basis of current treatments which favor early pharmacologic intervention for reperfusion of tissue.

Brain damage as a result of ischemia occurs through the ischemia cascade which causes local depletion of oxygen and glucose causing decreased production of energy compounds such as ATP (Deb, Sharma, & Hassan, 2010). With a loss in energy stores, cellular functioning cannot occur which culminates in injury and death. Depending on the cell's location of ischemia in conjunction with the severity and duration of infarction, the damage can be minuet causing minimal side effects or drastic which can lead to functional brain loss. Since neurons require constant oxygen and glucose, it is susceptible to changes under hypoxic conditions in the case of strokes. The changes involved in tissue injury are mitochondrial failure associated with depleted energy stores, loss of membrane ion function, excitatory neurotransmitters, production of reactive oxygen species, and apoptosis (Patel, 2008).

In hypoxic conditions, neuronal tissue function can only be sustained for a temporary amount of time. When storages of energy are depleted from mitochondrial reserves, the cell can be triggered to undergo apoptosis. Ischemia also causes a loss of ATP and potassium, which are important for energy exchange across the membrane. Though energy depletion rarely results in immediate cellular death, temporary occlusions or partial occlusions for prolonged periods of time can result in irreversible brain damage from deterioration of the ion gradients by the by-products of anaerobic metabolism (Patel, 2018). Under such conditions, potassium is a loss in exchange of sodium, chloride, and calcium ions, which in addition to an influx of water, results in cytotoxic edema, or rapid swelling of neurons and glia. Ischemia also directly results in dysfunction of the vasculature within the brain with a breakdown of the blood-brain barrier

occurring within 4-6 hours after infarction (Deb, Sharma, & Hassan, 2010). Following the barrier's breakdown, proteins and water flood into the extracellular space, leading to vasogenic edema. This produces greater levels of brain swelling and mass effect that peak at 3-5 days and resolve over the next several weeks with resorption of water and proteins.

During the ischemic cascade, cells release excitatory neurotransmitters such as glutamate that are toxic at high concentrations. Glutamate is responsible for sending signals between nerve cells, and under optimal conditions, this plays a role in learning and memory; however, during hypoxic conditions and ischemic areas, this activates N-methyl-d-aspartate (NMDA), -amino-3-hydroxy5-methyl-4-propionate (AMPA) which triggers Na+ and Ca2+ influx (Deb, Sharma, & Hassan, 2010). As a result, the neuronal membrane utilizes depleting ATP storages to maintain calcium balance and has unorganized activation of enzymes within the cell. These enzymes and their metabolic products, such as oxygen free radicals, damage cell membranes, genetic material, and structural proteins in the neurons, ultimately leads to cell death (Deb, Sharma, & Hassan, 2010). Though it is known that glutamate is released through these mechanisms, the pathway of release during ischemic injury is ambiguous. The activation of excitatory receptors causes neuronal depolarization which further causes the release of glutamate and the influx of calcium which activates degradative enzymes and leads to the destruction of cellular membranes and other structures (Deb, Sharma, & Hassan, 2010). This ultimately results in glutamate toxicity and the consumption of scarce resources.

During hypoxic conditions, the byproducts of cellular respiration produce reactive oxygen species that damage cellular and extracellular elements, specifically vascular endothelium. Through redox signaling, it also initiates apoptotic pathways that induce cellular death. In contrast to necrotic causes of cellular death in the ischemic core, apoptosis occurs in the

peripheral neurons in response to ischemic damage (Deb, Sharma, & Hassan, 2010). This ischemic damage triggers Bcl-2 and p53 gene expression, followed by the release of proapoptotic molecules such as cytochrome c and apoptosis-inducing factor from the mitochondria (Patel, 2018).

Within hours to days after a stroke, specific genes are activated, leading to the formation of cytokines and other factors that, in turn, cause further inflammation. Ultimately and if unresolved, the ischemic penumbra is consumed by these factors which result in the death of astrocytes, oligodendroglia and microglial cells. The infarcted tissue eventually undergoes necrosis and is removed by macrophages, with the development of functional tissue loss in the weeks to months following the infarction.

Pathophysiological Underpinnings of Sex Differences in Ischemic Stroke

Although lifetime risk of stroke is higher for men than for women, women tend to have more severe strokes, more stroke deaths, and increased post-stroke dysfunction compared to men (Mozzafarian et al., 2016). From ages 19–30, and again from ages 45 to 54, women have an increased stroke risk (Mozzafarian et al., 2016), and one plausible explanation for the increased risk of stroke during these time periods is alterations in estrogen status.

Role of sex hormones. It is well known that sex-specific hormones play a key role in the neuroprotection and neuromodulation of stroke response. Estrogen, progesterone and androgens all aid in the stroke response by altering cellular responses via activation and inhibition.

The role of estrogen. Estrogens are a steroid hormone that regulate female reproductive function and act upon the cardiovascular, immune, and central nervous system through binding to estrogen receptors. Apart from being highly expressed in reproductive and neuroendocrine regions like the hypothalamus, estrogen receptors are widely distributed in the brain. Sex

hormones modulate not only neuronal function; they exert their actions on different cellular targets, modulating an important number of physiological processes. Estrogen impact on stroke have been modeled in ovariectomized female mice that allow researchers to remove endogenous estrogen and resupply estrogens to evaluate therapeutic potential (Gibson, 2006). Overall, estrogens reduced lesion volume in a dose-dependent manner when administered up to a week before or up to four hours after transient or permanent cerebral ischemia (Gibson, 2006). The three forms of estrogen synthesized by the ovaries are estrone (E1), estradiol (E2), and estriol (E3) (Zarate, Stevnsner, & Gredilla, 2017). 17-beta-estradiol (17β-E2), which is involved in ischemic injury mediation following stroke, demonstrates the neuroprotective capabilities of estrogens through promoting transcription of genes involved in neuronal survival, expression of proteins, and the inhibition of neurodestructive factors (Petrone, Simpkins, & Barr, 2014). Following focal brain injuries, 17β-E2 administration in mice has shown an increased expression of proteins involved in cell survival including phosphoinositide 3-kinase, cyclic-AMP response element binding protein, superoxide dismutase, and protein phosphatase 2A (Petrone, Simpkins, & Barr, 2014). In addition, 17β-E2 inhibited the expression of pro-apoptotic proteins and cytochrome-c release (Petrone, Simpkins, & Barr, 2014).

Because the immune response following stroke dictates functional recovery and the extent of brain damage, 17β-E2 may be dually protective in stroke by also mediating the immune response. Following ischemia, there is a rapid local production of estrogen, indicating that the hormone may be involved in an immediate physiological response to limit tissue damage (Koellhoffer & McCullough, 2014). This early production of estrogen occurs simultaneously with the upregulation of innate immune responses. 17β-E2 has been shown to inhibit production of neutrophil chemoattractants in the ischemic region, preventing an excessive neutrophil

response that would increase inflammation (Koellhoffer & McCullough, 2014). This hormone also limits the adhesion of neutrophils to endothelial cells on the vasculature which prevents excess infiltration from circulating neutrophils (Koellhoffer & McCullough, 2014). 17β-E2 can also resolve an inflammatory response by assisting in the clearance of neutrophils from brain Koellhoffer & McCullough, 2014) and inducing anti-inflammatory cytokines to limit infarction following ischemic stroke (Petrone, Simpkins, & Barr, 2014).

The role of progesterone. Progesterone is another sex hormone that is characterized by reproduction regulation. Progesterone receptors are expressed widely throughout the brain and have been described to be present along glial cells (Zarate, Stevnsner, & Gredilla, 2017). Among others, progesterone reduces proinflammatory cytokines, modulates neurotransmission, suppresses microglial activation, and aids in myelin repair, which suggests that this sex steroid has positive effects on outcomes post-stroke (Spychala, Honarpisheh, & McCullough, 2017). These neuroprotective effects involve different mechanisms of action, among them, progesterone activates the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase/ serine/threonine kinase (PI3K/Akt) pathways which aid in cellular survival after injury (Zarate, Stevnsner, & Gredilla, 2017). However, when delivered in conjunction with estrogen, studies have found that progesterone often acts as an antagonist to estrogen within the CNS (Zarate, Stevnsner, & Gredilla, 2017). Though the precise mechanism is poorly understood, it is believed that the regulation of ER expression is responsible for this antagonistic effect (Zarate, Stevnsner, & Gredilla, 2017).

The role of androgens. Androgens are sex steroid hormones that generally control male sex traits and development, but also influence female sexual behavior. Androgens are capable of both detrimental and beneficial effects which suggest that maintaining androgen levels within the

adult ‘normal’ physiological range is likely the optimal condition to minimize risk for stroke. Several mechanisms have been identified that demonstrate the neurodestructive abilities of androgens; however, exogenous testosterone or steroids use can increase glutamate-induced calcium influx, thereby increasing excitotoxic injury in oligodendrocyte and glial cells (Quillinan, Deng, Grewal, & Herson, 2014). Moreover, in neuronal cultures using GABA as an excitatory neurochemical, testosterone has been shown to increase GABA induced calcium influx and injury (Quillinan, Deng, Grewal, & Herson, 2014). This hormone has also been observed to induce pro-apoptotic genes and increase injury through increased inflammation and altered cell-signaling (Quillinan, Deng, Grewal, & Herson, 2014). However, several studies have demonstrated that androgens can be neuroprotective under conditions of glucose deprivation in both neurons and astrocytes through increased antioxidant catalase activity, increased expression of salt-induced kinase 1, and CREB activation (Quillinan, Deng, Grewal, & Herson, 2014).

Role of menopause. While sex hormones as a unit are of importance as a risk factor for stroke, it is evident that menopause is attributed to the difference in incidence as evidenced by premenopausal women having a much lower incidence compared to young males yet doubling that of men at the end of menopause. Menopause is defined as the absence of menstrual periods for 12 consecutive months. The menopausal transition, stage of menopause-associated with a cessation in reproductive fertility caused by a decrease in hormone production, is associated with significant hormonal changes – most importantly, diminished production of estradiol by 60% (Lisabeth & Bushnell, 2011). As a consequence, estrogen levels decline steeply after menopause, whereas testosterone level remains more or less unchanged, leading to a state of relative androgen excess (Lisabeth & Bushnell, 2011). The drop in estrogen places tissue at a high risk since the anti-inflammatory properties are no longer at bay. Furthermore, the androgen

excess is positively correlated with insulin resistance and type-2 diabetes in the elderly population which increases the risk for stroke.

Neuroinflammation after Ischemic Stroke

Defining neuroinflammation. Neuroinflammation is the inflammatory response within the nervous system. Generally, an inflammatory response occurs as a reaction to foreign invasion or injury to the tissue that involves the release of proinflammatory mediators such as chemokines and cytokines. When uncontrolled, this inflammatory process can increase tissue damage and impair restoration of injury. Neuroinflammation is regulated by the production chemokines, cytokines, and reactive oxygen species (Cherry, Olschowka, & O'Banion, 2014). The extent to which neuroinflammation is initiated is dependent on scene, duration, and course of insult. This inflammatory process can then both serve supportive and destructive functions as part of its mediation of injury. The neuroprotective role of neuroinflammation is to increase glial cell activation and surveillance. For example, during low transient inflammation, the immune cell signals to the brain by increasing the expression of interleukin (IL)-1 cytokine, this then increases the surveillance role of glial cells in the brain if infected (Liu et al, 2017). The transient inflammation of traumatic CNS injury, following the expression of IL-4, has been shown to promote injury recovery and axonal regrowth (Liu et al, 2017). However, the neurodegenerative aspects of neuroinflammation increase the production of cytokines (IL-1 and tumor necrosis factor), reactive oxygen species (ROS), and other inflammatory mediators including inducible nitric oxide synthase (Liu et al, 2017). Following the acute phase of CNS trauma, the IL-1 and IL-6 give rise to collateral damage through driving a low-level and chronic inflammatory response that to cognitive impairments and reduced neuronal plasticity (Liu et al, 2017). Acute inflammation is a defensive response that creates a foundation for tissue repair. Chronic

inflammation, however, results from consistent stimuli. To identify the principles of general neuroinflammation, acute and chronic neuroinflammation will be defined in the context of ischemic stroke.

***Acute vs chronic neuroinflammation*.** Historically, the term "reactive gliosis" was used to describe the response of glial cells to CNS tissue injury. Reactive gliosis referred to the nonspecific change in glial cells such as proliferation, hypertrophy, or morphology (Streit, Mrak, & Griffin, 2004). However, it is now known that glial cells respond through activation into an aggressive role rather than the previous notion of passive response. In this activated form, glial cells release tissue factors that cause leukocytes infiltration or an increase in rapid activation of other glial cells (Streit, Mrak, & Griffin, 2004). This type of inflammation is seen with injury rather than a disease which is associated with chronic neuroinflammation. Chronic neuroinflammation produces excessive microglial activation in addition to infiltrating leukocytes that ultimately translate into a cycle of neurodegeneration (Streit, Mrak, & Griffin, 2004). In ischemic stroke, it is considered that the major contributors to ischemic neuronal injury are excitotoxicity, oxidative stress, and inflammation (Liu et al, 2017). During the process of reperfusion after ischemia, superoxide and nitric oxide produced from damaged cells and depletion of glutathione, an antioxidant against ROS occur. This results in acute inflammation from dying cells and tissue debris, especially because in ischemic stroke, a blockage prevents nutrients from perfusing cells while also inhibiting the removal of waste products.

Microglia's role in neuroinflammatory response to ischemic stroke. It is known that during ischemic stroke, neuroinflammation occurs as a result to ischemia-induced cell debris and increased ROS which activates microglia and enhances the production of these proinflammatory cytokines. During ischemic stroke, microglia transform their morphology from its ramified

surveillance state to a motile amoeboid state once reperfusion of the brain occurs. These activated cells then act as phagocytes and engulf endothelial cells which allow for the entrance of blood serum components (Cherry, Olschowka, & O'Banion, 2014). Several studies have demonstrated that this early activation of microglia in the neuroinflammatory response increases the infarcted area causing cells to undergo apoptosis. Contrary, microglial activation can promote active microglia migration to the penumbra in delayed stages of neuroinflammation and assist in cell survival (Streit, Mrak, & Griffin, 2004). During activation, microglia morphology is changed either to M1, the typically activated phenotype, or to M2, an alternatively activated phenotype, after stroke (Cherry, Olschowka, & O'Banion, 2014). M1 microglia activated by LPS and the pro-inflammatory cytokine interferon-gamma (IFN-γ) shows harmful effects after stroke (Colton, 2009). In contrast, M2 phenotype microglia contribute to stroke recovery through anti-inflammatory cytokines such as IL-4 (Colton, 2009). In ischemic stroke, the M2 phenotype is more likely to be induced by ischemic neurons to promote tissue restoration.

M1 microglial activation. The classical activation of microglia in neuroinflammation is the M1 state. This term was originally formulated after the association of IFN-γ produced by Th1 cells and the importance of it in polarizing microglia (Colton, 2009). It is now known that microglia partially control their own activation through autocrine and paracrine means. In many cases, this response is neuroprotective, however, chronic activation of these cells can lead to tissue damage. In addition, the ratio of cytokines has been used to identify inflammatory macrophages and in microglia, IL-12 and IL-10 production are used as a marker for distinguishing inflammatory cells Colton, 2009). Another potential distinction and an important component of M1 microglia is their ability to produce reactive oxygen species and reactive nitrogen species which correlate with an increase in apoptosis (Colton, 2009).

M2 microglial activation. Inflammation generated by stroke and ischemic reperfusion injury is generally considered harmful to the issue as the M1 phenotype is favored; however, M2 phenotype activation has been shown to downregulate inflammation and initiate cellular repair (Cherry, Olschowka, & O'Banion, 2014). This has been demonstrated in mice that lack IL-4 or IL-10 which results in an increase in the infarct zone (Cherry, Olschowka, & O'Banion, 2014). This response also promotes debris clearance, extracellular matrix deposition, and angiogenesis (Cherry, Olschowka, & O'Banion, 2014). Overall, the damage that results from an ischemic insult is dependent on the transition from the M1 to M2 phenotype to efficiently restore tissue function. However, in cases where the transition is not made and there is a constant activation of the M1 phenotype, the continued production of proinflammatory cytokines results in further tissue damage and an increase in the infarct zone (Cherry, Olschowka, & O'Banion, 2014).

Sex Differences in Microglia Function in Response to Stroke

The sex-differences in microglia abundance and phenotype are prominent in the brain, however, it is in the response to stimuli that marks sex-differences in outcomes rather than the number of cells. In a study conducted by Villa et al. (2018), microglia cells maintain sex-specific expression independent to circulating hormones as evidenced by transplantation of microglia into the opposite sex. Through defeminizing the brain using estrogens, the researchers found that the expression of certain genes was altered, suggesting that the sex of microglia is determined at birth (Villa et al., 2018). As discussed in chapter 1, perinatal microglia cells migrate to the brain in early fetal development and undergo precise temporal phases that characterize microglia development and sexual differentiated differences. (Matcovitch-Natan et al., 2016). This sexual dimorphism persists in the adult brain through varying amounts and morphology of microglia in different anatomical regions (Rahimian, Cordeau, & Kriz, 2018). It is likely that the variances in

early development lead to divergence in microglia inflammatory responses. Indeed, sex differences in microglial activation patterns following brain injuries have been reported by different groups. Bodhankar et al. (2015) found that after middle cerebral artery occlusion (MCAO), microglia from female mice had a milder inflammatory phenotype compared to males. In addition, other experimental stroke models have demonstrated the increase in microglial density at the lesion border in male mice (Caplan et al., 2017). Other models showed that separated male and female microglia perform differently when exposed to inflammatory triggers such as lipopolysaccharides (LPS). Male microglia showed a higher proinflammatory response to LPS compared to the female cells, yet the exposure to E2 resulted in an anti-inflammatory effect in male neonatal microglia and a proinflammatory effect in female neonatal microglia (Loram et al., 2012). Collectively, these observations suggest sex differences in the microglial activation patterns following injury and stress. It is possible that the observed sex differences in the microglial activation patterns after stroke may trigger distinct downstream signaling cascades and thus have a significant impact on the damage associated with ischemic injury.

While the meaning of this sexual differentiation is unclear, it has been shown that this biological event has functional repercussions on the damage caused by MCAO. As mentioned previously, males are associated with increased stroke incidence and poorer outcomes are attributed to the findings that female microglia are more apt to reduce ischemic damage compared to their male counterpart. By analyzing the downstream signaling pathways for responses to ischemic damage, the male and female stroke incidence and outcomes can be explained.

One example of the sexual dimorphic responses is caspase-mediated cell death. Female microglia primarily follow caspases mediate cell death whereas the caspase-independent Poly

(ADP-ribose) polymerase-1 (PARP-1) cell death and nitric oxide pathway predominate in males (Rahimian, Cordeau, & Kriz, 2018). The loss of either PARP1 or nitric oxide synthase reduces infarct volume only in males (Rahimian, Cordeau, & Kriz, 2018). In addition to cell death mechanisms, it has been reported that stroke-induced inflammatory and/or anti-inflammatory signaling is sexually dimorphic. Examples include peroxisome proliferator-activated receptor alpha (PPAR-α), and peroxisome proliferator-activated receptor gamma (PPAR-γ), high mobility group box 1, IL-4 receptor and IGF-1 signaling pathways (Rahimian, Cordeau, & Kriz, 2018). PPAR- α and PPAR- γ activation primarily affects microglia polarization and inhibition of inflammatory signaling cascades through decreasing nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells in neurons in experimental stroke (Dotson et al., 2016). Treatment with a PPAR- α agonist prior to stroke is only neuroprotective in males, probably because of the lower PPAR- α expression in the female brain (Dotson et al., 2016).

In addition to cell-death-associated pathways, the sex differences have been also reported in the levels of many cytokines and chemokines. Recent evidence suggests that neuroprotective cytokines such as IL-4 may stimulate sex-dependent signaling after stroke. Xiong et al. (2015) demonstrated that IL-4 is crucial for female neuroprotection after ischemic stroke through the stimulation of galectin-3 expression and release. Galectin-3 over-expression is a feature of M2 activation that binds to and cross-links to CD98 on macrophages (Xiong et al., 2015). In addition, IL-4 can initiate the activation of PPAR- γ via IL-4 receptor-dependent signaling pathways to provide neuroprotection against inflammation (Rahimian, Cordeau, & Kriz, 2018). In a study conducted by Lalancette-Herbert et al. (2012), galectin-3 played an instrumental role in microglial activation and proliferation in response to ischemic stroke and in male mice, was attributed to an increase in apoptotic neurons and an increase in the infarcted

tissue. Downstream of galectin-3 is insulin-like growth factor-1 (IGF-1). IGF-1 is a key molecule in galectin3-induced microglia proliferation and M2 activation that provides neuroprotection through decreasing the size of infarction and provoking neurogenesis (Sohrabji & Williams, 2013). When galectin-3 is removed, IGF-1 levels are markedly decreased which resulted in decreased M2 microglial proliferation and an increase in neuronal damage (Lalancette-Hebert et al., 2012). This evidence demonstrates a few of many sex-dependent responses that microglia undergo in response to ischemic stroke.

Summary

In summary, the sex-differences in ischemic stroke outcomes are highly connected to the sexual dimorphic environment and morphology of microglia which result in dynamically different responses to neuronal damage. The roles of sex hormones create the foundation for responses through acting as neuroprotective or inflammatory mediators. These then take part in the difference in microglial activation pathways and dimorphic functions. By understanding the specific pathways that male and female microglia undergo, the more specific stroke therapies can be developed to preserve tissue, decrease mortality and reduce disability.

Chapter 3

Methods to Study Ischemic Stroke

Developing effective clinical treatments for ischemic strokes is dependent on understanding the pathophysiology of injury. Developing this basis requires the use of animal models that mimic human insults so that reliable results can be replicated and restudied for further therapeutic modalities. As recommended by the Stroke Treatment Academic Industry Roundtable (STAIR), prehospital testing research and treatment management should demonstrate

evidence of safety in stroke models (Albers et al., 2011). The methods used in this project to study ischemic stroke were middle cerebral artery occlusion (MCAO) model.

Middle cerebral artery occlusion model. The brain is perfused via the internal carotid and vertebral arteries. The MCAO model is formulated around occluding the middle cerebral artery which is a distal branch of the internal carotid artery. As the supplier of most of the outer brain tissue including the anterior and posterior internal capsules and the basal ganglia, an occlusion of this vessel is not only a commonly found cause of human stroke but replicates clinical cerebral ischemia (Sutherland, et al., 2017). The middle cerebral artery can be accessed internally or externally and can result in temporary or permanent occlusion based on the study protocol. The intraluminal filament MCAO stroke model conducted in rats was developed by Koizumi in 1986 (Sutherland, et al., 2017). This model uses a temporary insertion of a filament through the internal common carotid artery to the ostea of the MCA to occlude the vessel resulting in ischemia to the tissue (Sutherland, et al., 2017). For this experiment, the ischemic period occurred for a continuous 60-minute period and produced at least a 70% reduction in blood flow and reperfusion of at least 70% return of blood flow when compared to the animal's baseline (Morrison & Filosa, 2013). The advantages of this method are eliminating the need for a craniotomy, production of a focal occlusion, and standardizing the area of infarct (Shahjouei et al., 2016). This procedure can also be reproduced multiple times and result in almost identical areas of ischemia which improves standardized outcomes in research.

Animals used in ischemic stroke models. There are a variety of animal stroke models that have been developed with the goal of identifying the underlying mechanisms behinds cerebral ischemia and to develop new therapies for strokes (Fluri, Chuhmann, & Kleinschnitz, 2015). Most stroke experiments are carried out in small animals such as rabbits, mice, and rats.

The use of small animals as subjects present clear advantages compared to studying acute strokes in humans in the clinical setting. Ischemic strokes in humans are vastly diverse in anatomic location, manifestations, and causes, whereas experimental strokes are highly reproducible and well-controlled which limit extraneous variables. In addition, the use of animal models allows for controlled reperfusion and vasculature which cannot be studied in in-vitro models (Fluri, Chuhmann, & Kleinschnitz, 2015). The most common animal subject of ischemic stroke is the rodent. Rodents are good subjects as the cerebral vasculature is similar to humans, the small body size allows for easy monitoring of hemodynamic parameters, and there is a relative homogeneity among strains which can improve the reproducibility of results (Fluri, Chuhmann, & Kleinschnitz, 2015).

Methods to model female post-menopause. Female C57Bl6/J mice were purchased at 5 weeks old in order to induce ovarian failure as a model of the human post-menopause period. We employed the post-menopause protocol as previously published (Haas, Christian, & Hoyer, 2007; Van Kempen et al., 2014). As a brief summary, 5-week-old female mice were injected for 21 days with 4-vinylcyclohexene diepoxide (VCD, 160 mg/kg/i.p./day). Follicle depletion and ovarian cessation was assessed ~65 days after first injection via vaginal lavage, and ovarian failure was confirmed by observing 15 days of persistent diestrus prior to tissue collection. At this point, 10 weeks after VCD injections and at 16 weeks of age, mice underwent a surgery to induce ischemic stroke as described in the section above.

Animal usage. All animal handling and experiments were performed according to methods approved by and in compliance with the University of Arizona Institutional Animal Care and Use Committee and according to the National Institutes of Health guide for the care

and use of laboratory animals (protocol approval number 14-539). All animals were housed in rooms with a 12-hr light/dark schedule (7am-7pm).

Methods to Study Microglia

Immunohistochemistry. Immunohistochemistry (IHC) is a microscope-based technique aimed at visualizing cellular components in a tissue sample. This technique uses target-protein binding within tissue to identify cellular components. For this data analysis, coronal sections of brain tissue underwent an immunohistochemistry assay to distinguish microglia from other resident brain cells. The classical IHC assay involves detection of epitopes expressed by a single protein-target within a tissue sample using an antibody capable of binding to those epitopes with high specificity, or selectivity to a specific binding site. After the epitope-antibody binding complex, a secondary antibody capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a reporter molecule and after the antibody-antibody binding event, a chemical substrate is added which reacts with the reporter molecule to produce a colored precipitate at the site of the whole epitope-antibody complex (Morrison & Filosa, 2013). This fluorescence created by the antibody-antibody binding complex can be detected using a fluorescent microscope to create an image of the selected cell type.

For this study, brain tissue was then collected from all animals after ischemic stroke and 8 hours of reperfusion. All animals were sedated with 5% isofluorane delivered via a 20% oxygen/80% air mixture and systemically perfused with ice-cold phosphate buffered saline via cardiac puncture, after which brain tissue was immediately removed. Brain tissue was fixed for 24h in a 4% paraformaldehyde solution followed by 72h in a 30% sucrose solution, after which the tissue was dried and stored at -80°C until sectioning into coronal sections and use for immunohistochemistry techniques.

All IHC was completed on tissue sectioned into 50µm coronal sections (Leica cryostat) and stored at -20°C in a cryoprotectant solution (50% 50mM PBS, 30% ethylene glycol, 20% glycerol) until IHC experiments. Our IHC protocol, similar to as previously published (Morrison & Filosa, 2013, 2016), results in staining of microglia with anti-IBA1. Free floating brain sections were first blocked in 10% horse serum solution (0.01M PBS, 0.05% Triton, and 0.04% NaN_3) for 1h followed by a 72h incubation with primary antibodies as appropriate: rabbit anti-iba1 at 1:1000 (Wako, 019-19741). A 4-h incubation of 1:250 secondary primaries (Jackson ImmunoResearch Laboratories) followed: donkey anti-rabbit Alexa 488 (711-546-152. All tissue was incubated in solutions common to all groups to avoid group/batch differences. All reactions were carried forward at room temperature; washes between incubations were done with 0.01M PBS for 15min. Slices were then mounted onto slides using Vectashield (Vector Laboratories, H-1000).

Photomicrograph Acquisition and Analysis. Microglia morphology was assessed in brain regions in proximity to the ischemic injury after ischemic stroke and 8 hours of reperfusion. Imaging was accomplished using a Zeiss 880 NLO [30-µm Z-stack at 1-µm intervals, 40X/1.3Na C PL-Apo oil objective, and a 212.6 µm x 212.6µm imaged area (1200 pixels x 1200 pixels)]. Photomicrographs were stacked and split using ImageJ plugins (National Institute of Health, https://imagej.nih.gov/ij/) in order to obtain maximum intensity projections of all channels.

Skeleton Analysis. A method used to quantify microglia morphology from photomicrographs was skeleton analysis. The goal of Skeleton Analysis is first model microglia from IBA-1 positive photomicrographs to simple “stick images”. Our first step, then, is to convert photomicrographs into skeletonized images as shown in Figure 1. In this process,

maximum intensity projections are created to visualize microglia processes. Next, a series of ImageJ software plugins are used to de-speckle the image (to eliminate single-pixel background fluorescence), convert to binary, and then skeletonized images. The AnalyzeSkeleton plugin is then applied to skeletonize the image and gather data to identify the number of endpoints per frame and process length. The information gathered from skeleton analysis aids in the identification of branching complexity and process lengths as a measure of response to injury severity. This process has been published in greater detail by (Young & Morrison, 2018).

Fractal Analysis. To help quantify the microscopic details of microglia shapes, and as a second method to quantify morphology, fractal analysis can be used and is summarized in Figure 2. Fractal analysis assesses the scaling of the subject and produces a statistical form of complexity called fractal dimension. This number measures scale-invariant detail or complexity of the image. For a pattern to have fractal scale-invariant detail means that the pattern repeats itself infinitely as the resolution increases (Karperien, Ahammer, & Jelinek, 2013). Box-counting, a subtype of fractal analysis, is used to quantify both gross and minute nuances of morphology that are vital in understanding microscopic details of microglia (Karperien, Ahammer, & Jelinek, 2013). Box-counting is a sensitive indicator of microglia structural features such as ramification, branching patterns, and membrane details. This proven method has become a standard for the characterization of microglial morphology. In a dataset, box-counting fractal analysis lays increasingly smaller grids over an image and counts the number of boxes containing pixels (Karperien, Ahammer, & Jelinek, 2013). Through calculating the box-counting dimension, the value produced represents the average rate of change in detail with a concurrent change in magnification of the sample image.

For this analysis, FracLac for ImageJ was applied to three randomly chosen single cells per photomicrograph used in each region. Randomly chosen microglia were first made binary through the process described for skeleton analysis via cell isolation. Binary images are then converted to outlines using ImageJ and analyzed using FracLac for Image J. A more thorough description of this process is provided by (Young & Morrison, 2018). Additional morphology parameters were assessed using FracLac for ImageJ: lacunarity, density, span ratio, and circularity (Figure 2). A full description of these calculations can be found: https://imagej.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm. Noting the difference between these two methods, skeleton analysis is carried out on the entire photomicrograph and is considered "high-throughput", fractal analysis is carried out on individual cells and is correspondingly labor intensive.

Statistical Analysis

All data are presented as mean ± standard error of mean (SEM). Sex and region differences were determined using a two-way ANOVA test with Bonferroni post-hoc testing. All reported p values have been adjusted for multiple comparisons. GraphPad Prism 6 was used for statistical analyses.

Summary

To summarize, the identification and replication of ischemic stroke in animal models are vital to understanding the cellular response to injury and developing clinical treatments specific to these pathways. The MCAO model allows for experimental designs to become standardize and mimic human pathophysiology. Following the induction of stroke and reperfusion, these microglia are studied through the identification of IHC and are further quantified with skeletal

and fractal analysis. This analysis helps to assess the differences between areas of ischemia to monitor microglia response to injury as it pertains to injury proximity and sex.

Chapter 4

Study Results

We quantified microglia morphology after 8-hour ischemia to test if there is a focal and sex differences in variance in microglial morphological responses to injury. Microglia are dynamic cells and changed microglia morphology may correspond to functional responses. We initially quantified the ramified morphology after 8-hour occlusion in male, pre-menopause (Pre M), and post-menopause (PM) mice in the necrotic core, proximal, distal, and contralateral regions using the skeleton analysis method summarized by Figure 1. Figure 3 summarizes findings on the ramified morphology among male, Pre M, and PM female mice. Cell ramification was operationalized using two variables: endpoints per cell and process length per cell. Endpoints/cell and process length/cell data are summarized in Figure 3A and 3B. We show that ramified morphology is decreased when in proximity to injury as determined by two-way ANOVA analyses (endpoints/cell region main effect: $F_{(3,68)} = 20.71$, $p < 0.0001$; process length/cell region main effect: $F_{(3,68)} = 11.63$, $p < 0.0001$). These data indicate that microglial branching complexity is greater in areas that are not affected by ischemic injury and indicative that morphology and proximity to injury are interdependent after ischemic stroke and 8 hours of reperfusion. However there were no differences among sex groups for the endpoints/cell variable (two-way ANOVA sex main effect: $F_{(2,68)} = 0.6$, $p < 0.55$) whereas significant sex group differences for the process length/cell variable (two-way ANOVA sex main effect: $F_{(2,66)} = 4.65$, $p < 0.05$. In particular, process length/cell was increased in the Pre M group at the proximal to injury region when compared to the matching region in the male sex group (Bonferroni post-hoc

comparison $p < 0.05$) In summary, microglia are de-ramified in relation to the focal injury with little influence of sex group to modulate these findings.

Next, using fractal analysis, we examined the complexity and shape of microglia to measure the degree of variance between region and sex group. As previously described and illustrated in Figure 2, FracLac for ImageJ was used to outline microglia and quantify fractal dimension, lacunarity, density, span ratio, and circularity; these data are summarized by Figure 4. In Figure 4A we show that, similar to ramification measures, fractal dimension decreases in proximity to the ischemic region with significant differences according to sex group (two-way ANOVA: region: $F_{(3,57)} = 36.80$, $p < 0.0001$; sex group: $F_{(2,19)} = 7.5$, $p < 0.01$). Specific sex group differences were noted in the PM and Pre M group versus male when comparing either the proximal to injury or adjacent to injury regions. While cell lacunarity (Figure 4B, shape heterogeneity) was largely unchanged according to region ($F_{(3,57)} = 1.68$, $p > 0.05$) sex group differences were present ($F_{(2,19)} = 13.80$, $p < 0.001$). Cell density (Figure 4C, cell size) was decreased in proximity to the ischemic stroke ($F_{(3,57)} = 21.63$, $p < 0.0001$) but without sex group differences. Analysis illustrate that no differences were noted for span ratio for either effect, region or sex group (two-way ANOVA: region $F_{(3,57)} = 2.9$, $p < 0.05$; sex group $F_{(2,19)} = 0.3$, $p > 0.05$). Lastly, microglia circularity was different according to region (two-way ANOVA; region $F_{(3,57)} = 3.39$, $p < 0.05$) but without sex differences ($F_{(2,19)} = 2.93$, $p > 0.05$). In all cases, no significant interaction effect was present between region and sex; specific Bonferroni post-hoc comparisons are noted in Figure 4.

Chapter 5

Discussion

As a leading cause of mortality and disability in the United States, ischemic stroke has significant clinical impact. As a first responder to neuronal injury, microglia response is a variable factor to study. Microglia, as an inflammatory mediator and phagocyte in response to ischemic stroke, can improve outcomes or cause trauma. This study tested the hypothesis that ischemia would elicit a morphological response to ischemic stroke injury hours after reperfusion and that this morphological response would be different among sex groups: male, premenopausal and post-menopausal females. It is imperative to understand the sex-specific variability of microglia responses post-injury because the existence of such differences would impact future stroke therapies. We assessed microglia morphology 8 hours after reperfusion at four locations in the brain in respect to the area of injury among three sex groups (male, pre-menopausal females, and post-menopausal females). Through skeleton analysis, a method that uses hyper- and de-ramification of microglial cells as an indicator of activation (Morrison & Filosa, 2013), we illustrate that after 8 hours of reperfusion, microglia expressed an increase in de-ramification in proximity to injury. However, there were no significant differences among sex groups after 8 hours of reperfusion.

Microglia cells have an immediate response to injury (Davalos et al., 2005; Morrison & Filosa, 2013; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Microglia tend to retract their branches after ischemia, leading to a reduction in the total length and the total number of microglial processes (Zhang, 2019). In this study, we have confirmed previous work showing that microglia morphologic responses are quantifiable and decreased hours after ischemic and from reperfusion (Morrison & Filosa, 2013). However, there were no differences according to sex group. This finding was different than previous investigations that occurred at an earlier time-point—immediately after ischemic stroke (Morrison & Filosa, 2016). We had expected a

difference between sex groups as there is a clear distinction in clinical outcomes among male and female stroke victims. We postulated that sex differences in microglia morphologic responses to ischemic injury at this early time-point post stroke may exist, indicative of a functional response, and therefore may be a source of variability contributing to sex differences in stroke injury. In our ongoing work, and data not presented here, preliminary results from 24-hour reperfusion time-point is similar to our findings shown here—a similarity in morphologic responses to injury among sex groups. As this is one of the few studies measuring sex differences in microglial morphology in response to ischemic stroke, further investigation is warranted to confirm the findings of this study. Moreover, coupling the study of morphology and function, while difficult, would be informative.

In this study, we present data investigating sex differences in microglia morphology as a measurable response to ischemic stroke and reperfusion. These findings are vital contributions towards understanding sex as a biological variable in science and medicine. A limitation of this study included small sample sizes which impact the ability to identify significant relationships among the sex groups. It is possible that a larger and more equal sample size could produce a sex difference that is clinically significant in terms of morphological response. The skeletal analysis protocol poses a potential bias. To avoid bias, all skeleton analysis is carried out by one individual blinded to experimental group, when possible. The skeleton and fractal analysis were carried out on a two-dimensional projection of microscopic images and as such could lead to a substantial loss of end points and ramification that may be present in three-dimensional cell shapes (Heidl et al., 2018). The fractal analysis was time consuming and is less efficient for a large number of cellular analysis and this limitation could affect a reproduction of this study. In addition, this cross-sectional descriptive study with data collection analysis a single-time point of

the very dynamic process of phagocytosis which could be a misrepresentation of microglial response at a different point in time. Further research should be conducted to compile sex group data at 24 hours of reperfusion.

Clinical Significance

In this study, we present a quantitative analysis of the sex differences in microglia morphology as a measure of microglia activation after ischemic injury. These findings are vital contributions towards understanding sex as a biological variable in science and medicine. As we continue to explore the reason behind dichotomous stroke outcomes between the sexes, we move closer towards the development of sex-specific stroke therapies (Morrison & Filosa, 2016). The closer to understanding and defining the morphological response cells have during ischemic injury, the greater are ability to understand their functional response to pathologic conditions becomes. The focus of this study is on the basic discovery of microglia morphological response that can be used to develop treatments that have minimal side effects and variability among sex groups. In this study it was also found that there is hyper- and de-ramification present in respect to the site of injury. This is significant as microglia-mediated response can be both neuroprotective and neurotoxic depending on the proximity to the area of injury and the presence of ramification vs de-ramification—a morphology that may be related to their function. A better understanding of microglial actions during stroke pathology will broaden our understanding of post-stroke neuroinflammation—a targetable stroke therapy

In this study, we focused on target discovery and validation for novel therapeutics to better balance microglia mediated post stroke neuroinflammation. To do that, it is imperative to develop extensive knowledge on microglia action during stroke physiology in both males and females. Although it is valuable to have both male and female sex group, we must distinguish

and acknowledge the role that hormones play in cellular response (Eldahshan, Fagan, & Ergul, 2017). Sex as a biological variable should be a factor in biomedical research as experimental results obtained from unisex studies may be inadvertently extrapolated. Though there has been an increase in sexual equality in clinical trials and studies, there is a lack of analysis to determine sex differences in outcomes (Lee, 2018). When experimental conditions are reported clearly, both positive and negative data correlations between sex groups are valuable. In relation to this study, the finding of no sex differences is significant as alterations in microglial morphology could be a potential target for therapies as on a cellular level, the cells remain unchanged. However, the dichotomy in sex-specific stroke outcomes still poses a question as it remains unknown how biological sex plays a role in the response ischemic stroke.

Resources

Alexander, S. A. (Eds.). (2013*). Evidence-Based Nursing Care for Stroke and Neurovascular Conditions*. Oxford, UK: John Wiley & Sons, Inc.

Arganda-Carreras, I., Fernandez-Gonzalez, R., Munoz-Barrutia, A., & Ortiz-De-Solorzano, C. (2010). 3D reconstruction of histological sections: Application to mammary gland tissue. Microsc Res Tech, 73(11), 1019-1029. doi:10.1002/jemt.20829

Bodhankar, S., Lapato, A., Chen, Y., Vandenbark, A. A., Saugstad, J. A., & Offner, H. (2015). Role for microglia in sex differences after ischemic stroke: Importance of m2. *Metabolic Brain Disease, 30*(6), 1515-1529. doi:10.1007/s11011-015-9714-9

Caplan, H.W., Cox C.S., & Bedi., S.S. (2017). Do microglia play a role in sex differences in TBI? *Journal of Neuroscience, 95*(1) doi:10.1002/jnr.23854

Centers for Disease Control and Prevention. (2019). Stroke. Retrieved from https://www.cdc.gov/stroke/index/html/.

Cherry, J. D., Olschowka, J. A., & O'Banion, M. K. (2014). Neuroinflammation and m2 microglia: The good, the bad, and the inflamed. *Journal of Neuroinflammation, 11*(98). doi: 10.1186/1742-2094-11-98

Colton, C. A. (2009). Heterogeneity of microglial activation in the innate immune response in the brain. *Journal of Neuroimmune Pharmacology*, *4*(4), 339-418.

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., . . . Gan, W. B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. In Nat Neurosci (Vol. 8, pp. 752-758). United States.

Deb, P., Sharma, S., & Hassan, K. M. (2010). Pathophysiologic mechanisms of acute ischemic

stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Journal of the International Society of Pathophysiology*, 17(3), 197-218. doi: 10.1016/j.pathophys.2009.12.001

Dotson, A.L., Wang, J., Chen, Y. et al. (2016). Sex differences and the role of PPAR alpha in experimental stroke. *Metabolic Brain Disease, 31*(3), 539-547. doi:10.1007/s11011-015-9766-x

Eldahshan, W., Fagan, S., & Ergul, A. (2017). Inflammation within the neurovascular unit: Focus on microglia for stroke injury and recovery. Pharmological Research, 147. doi: 10.1016/j.phrs.2019.104349

Fluri, F., Schuhmann, M. K., & Kleinschnitz, C. (2015). Animal models of ischemic stroke and their application in clinical research. *Drug design, development and therapy*, *9*, 3445–3454. doi:10.2147/DDDT.S56071

Furie et al. (2011). Guidelines for the prevention of stroke in patients with stroke or transient ischemic attack: A guideline for healthcare professionals from the american heart association/american stroke association. *Stroke* 42(1). doi: 10.1161/STR.0b013e3181f7d043

Hadjiev, D. I., & Mineva, P. P. (2007). A reappraisal of the definition and pathophysiology of the transient ischemic attack. *Medical Science Monitor*, 13(3). Retrieved from https://www.medscimonit.com/

Haas, J. R., Christian, P. J., & Hoyer, P. B. (2007). Effects of impending ovarian failure induced by 4-vinylcyclohexene diepoxide on fertility in C57BL/6 female mice. *Comp Med, 57*(5), 443-449.

Heindl, S., Gesierich, B., Benakis, C., Llovera, G., Duering, M., & Liesz, A. (2018). Automated

morphological analysis of microglia after stroke. *Frontier Cellular Neuroscience,* 12(106). doi: 10.3389/fncel.2018.00106

Jakel, S., & Dimou, L. (2017). Glial cells and their function in the adult brain: A journey through the history of their ablation. *Frontier Cellular Neuroscience,* 11(4). doi: 10.3389/fncel.2017.00024

Karperien, A. (1999-2013). FracLac for ImageJ. http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm .

Karperien, A., Ahammer, H., & Jelinek, H. F. (2013). Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in cellular neuroscience, 7,* 3. doi:10.3389/fncel.2013.00003

Koellhoffer, E. C., & McCullough, L. D. (2014). The effects of estrogen in ischemic stroke. *Translational Stroke Research, 4*(4), 390-401. doi:10.1007/s12975-012-0230-5

Lalancette-Henert, M., Swarup, V., Beaulieul, J. M., Bohacek, I., Abedelhamid, E., Weng, Y. C., Sato, S., Kriz, J. (2012). Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. *Journal of Neuroscience, 32*(30), 10383-10395. doi: 10.1523/JNEUROSCI.1498-12.2012

Lee SK. Sex as an important biological variable in biomedical research. *BMB Rep*. 2018;51(4):167–173. doi:10.5483/bmbrep.2018.51.4.034

Lei, B., Mace, B., Dawson, H. N., Warner, D. S., Laskowitz, D. T., & James, M. L. (2014). Anti-inflammatory effects of progesterone in lipopolysaccharide-stimulated bv-2 microglia. *PLOS One, 9*(7). doi:10.1371/journal.pone.0103969

Lisabeth, L., & Bushnell, C. (2012). Stroke risk in women: The role of menopause and hormone therapy. *The Lancet: Neurology, 11*(1), 82-91. doi:10.1016/S1474-4422(11)70269-1

Liu et al, (2017). Role of neuroinflammation in ischemic stroke. *Journal of Neuroimmunology and Neuroinflammation, 4*. doi:1 0.20517/2347-8659.2017.09

Loram, et al. (2012). Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats. Psychoneuroendocrinology, 37(10), 1688-1699. doi:10.1016/j.psyneuen.2012.02.018

Matcovitch-Natan O., Winter D.R., Giladi A., Vargas Aguilar S., Spinrad A., Sarrazin S., Ben-Yehuda H., David E., Zelada González F., & Perrin, P. (2016). Microglia development follows a stepwise program to regulate brain homeostasis. *Science, 353*(6301). doi: 10.1126/science.aad8670

Morrison, H. W., & Filosa, J. A. (2013). A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. *Journal of neuroinflammation, 10*, 4. doi:10.1186/1742-2094-10-4

Morrison, H. W., & Filosa, J. A. (2016). Sex differences in astrocyte and microglia responses immediately following middle cerebral artery occlusion in adult mice. *Neuroscience*. doi:10.1016/j.neuroscience.2016.09.047

Mozzafarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al., on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2016 update: a report from the American Heart Association. *Circulation* 2016;*133*(4):e38–360

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. In Science (Vol. 308, pp. 1314-1318). United States.

Patel, P. M. (2008). Cerebral ischemia. In K. A. & A. W. Gelb (Eds), *Essential of*

neuroanesthesia and neurointensive care (pp 36-42). doi: 10.1016/B978-141604653-0.10006-8

Petrone, A. B., Simkins, J. W., & Barr, T. L. (2014). 17β-Estradiol and inflammation: Implications for ischemic stroke. *Aging and Disease, 5*(5), 340-345. doi: 10.14336/AD.2014.0500340

Quillinan, N., Deng, G., Grewal, H., & Herson, P. S., (2015). Androgens and stroke: Good, bad, or indifferent? *Experimental Neurology, 259,* 10-15. doi:10.1016/j.expneurol.2014.02.004

Rahimian, R., Cordeau, P, & Kriz, J. (2018). Brain response to injuries: When microglia go sexist. *Journal of Neuroscience*. doi:10.1016/j.neuroscience.2018.02.048

Samai, A. A., & Martin-Schild, S. (2015). Sex differences in predictors of ischemic stroke: Current perspectives. *Vascular Health Risk Management,* 11, 427-436. doi: 10.2147/VHRM.S65886

Shahjouei, S., Cai, P. Y., Ansari, S., Sharififar, S., Azari, H., Ganji, S., & Zand, R. (2016). Middle Cerebral Artery Occlusion Model of Stroke in Rodents: A Step-by-Step Approach. *Journal of vascular and interventional neurology*, *8*(5), 1-8.

Shi Z. (2017) Pathophysiology of Hemorrhagic Stroke. In: Lapchak P., Yang GY. (eds) Translational Research in Stroke. Translational Medicine Research. Springer, Singapore. doi:10.1007/978-981-10-5804-2_5

Sohrabji F, Williams M (2013) Stroke neuroprotection: estrogen and insulin-like growth factor-1 interactions and the role of microglia. *Journal of Neuroendocrinology, 25*:1173–1181. doi:10.1111/jne.12059

Sohrabji, F., Okoreeh, A., & Panta, A. (2018). Sex hormones and stroke: Beyond estrogens.

Hormones and Behavior. doi:10.1016/j.yhbeh.2018.10.010

Spychala, M. S., Honarpisheh, P., & McCullough, L. D. (2017). Sex differences in neuroinflammation and neuroprotection after ischemic stroke. *Journal of Neuroscience Research, 95*(1-2), 462-471. doi:10.1002/jnr.23962

Streit, W. J., Mrak, R. E., & Griffin, W. S. T. (2004). Microglia and neuroinflammation: A pathological perspective. *Journal of Neuroinflammation, 1*(14). doi: 10.1186/1742-2094-1-14

Sutherland, B. A., Fordsmann, J. C., Martin, C., Neuhaus, A. A., Witgen, B. M., Piilgaard, H., … Buchan, A. M. (2016). Multi-modal assessment of neurovascular coupling during cerebral ischaemia and reperfusion using remote middle cerebral artery occlusion. *Journal of cerebral blood flow and metabolism: Official journal of the International Society of Cerebral Blood Flow and Metabolism*, *37*(7), 2494–2508. doi:10.1177/0271678X16669512

Van Kempen, T. A., Gorecka, J., Gonzalez, A. D., Soeda, F., Milner, T. A., & Waters, E. M. (2014). Characterization of neural estrogen signaling and neurotrophic changes in the Accelerated Ovarian Failure mouse model of menopause. Endocrinology, en20141190. doi:10.1210/en.2014-1190

Villa, A., Gelosa, P., Castiglioni, L., Cimino, M., Rizzi, N., Pepe, G., Lolli, F., Marcello, E., Sironi, L., Vegeto, E., … Maggi, A. (2018). Sex-specific features of microglia from adult mice. *Cell reports*, *23*(12), 3501-3511.

Xiong, X., Xu, L, Wei, L., White, R. E., Ouyang, Y., & Giffard, R. G. (2015). IL-4 is required for sex differences in vulnerability to focal ischemia in mice. *Stroke*, 46. doi: 10.1161/strokeaha.115.008897

Young, K., & Morrison, H. (2018). Quantifying Microglia Morphology from Photomicrographs of Immunohistochemistry Prepared Tissue Using ImageJ. *J Vis Exp, in press*

Zarate, S., Stevnsner, T., & Gredilla, R. (2017). Role of estrogen and other sex hormones in brain aging, neuroprotection, and DNA repair. *Frontiers in Aging Neuroscience, 9*. doi:10.3389/fnagi.2017.00430

Zhao, X., Wang, H., Sun, G., Zhang, J., Edwards, N. J., & Aronowski, J. (2015). Neuronal Interleukin-4 as a Modulator of Microglial Pathways and Ischemic Brain Damage. *The Journal of Neuroscience*, *35*(32), 11281-91.

Figure 1

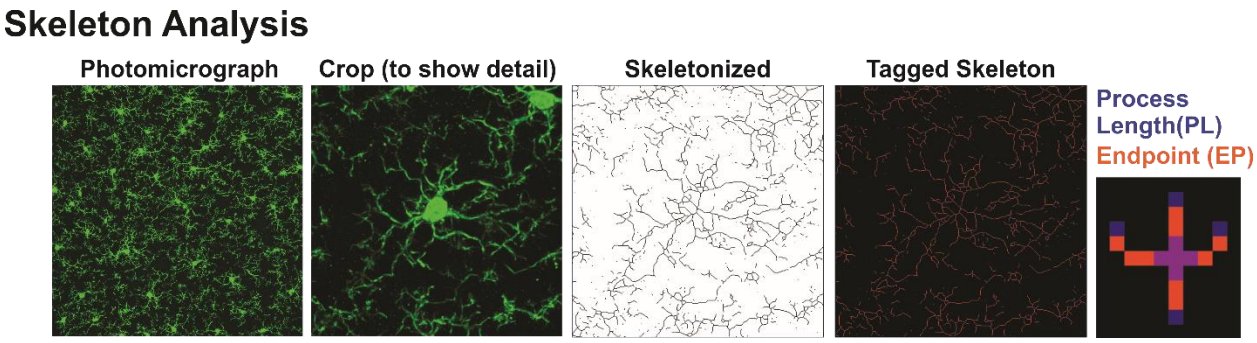

Figure 1. Typical photomicrograph of microglia immunohistochemistry (IHC; in *green*) and the skeleton analysis method to quantify microglia morphology in histological sections. Briefly, entire images were subjected to a series of ImageJ plugins to create a skeletonized model of all microglia in the frame (Young & Morrison, 2018). The cropped single cell shows the detail to visualize the analysis technique. Once skeletonized, the Analyze-Skeleton plugin tags skeleton elements, in the entire photomicrograph, as endpoints (*blue*), processes (*orange*), or junctions (*purple*)(Arganda-Carreras, Fernandez-Gonzalez, Munoz-Barrutia, & Ortiz-De-Solorzano, 2010). The number of endpoints/cell and process length/cell are summarized from data output to quantify microglia ramification.

Figure 2

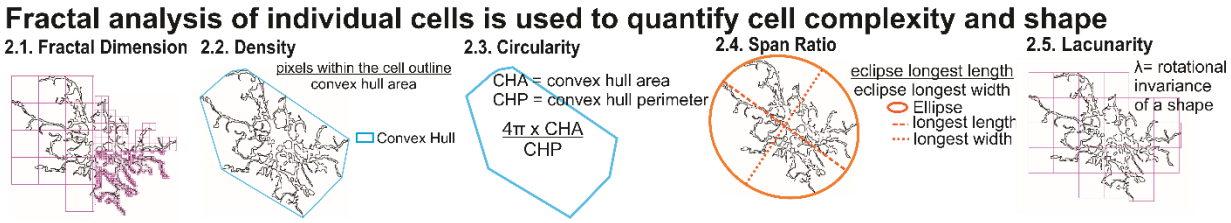

Figure 2. FracLac for ImageJ(Karperien, 1999-2013) was used to collect morphometrics such as fractal dimension, density, circularity, span ratio, and lacunarity. Single microglia are isolated from the entire photomicrograph and outlined using ImageJ plugins. Fractal dimension (**3.1**) is a measure of shape complexity and is calculated by FracLac for ImageJ algorithms using the number of boxes containing outlined pixels as the box size decreases. Larger fractal dimension values are indicative of increased shape complexity. Density (**3.2**), an indicator of cell size or solidity, is calculated by dividing the number of pixels in a cell shape by the area the cell occupies (convex hull area, blue). Circularity is an indicator of cell roundness (**3.3**), whereas span ratio is an indicator of shape elongation (**3.4**). Lacunarity (**3.5**) measures shape heterogeneity and the variability in how a cell shape occupies space. A high lacunarity value indicates that the different parts of a cell's shape are not symmetrical.

Figure 3

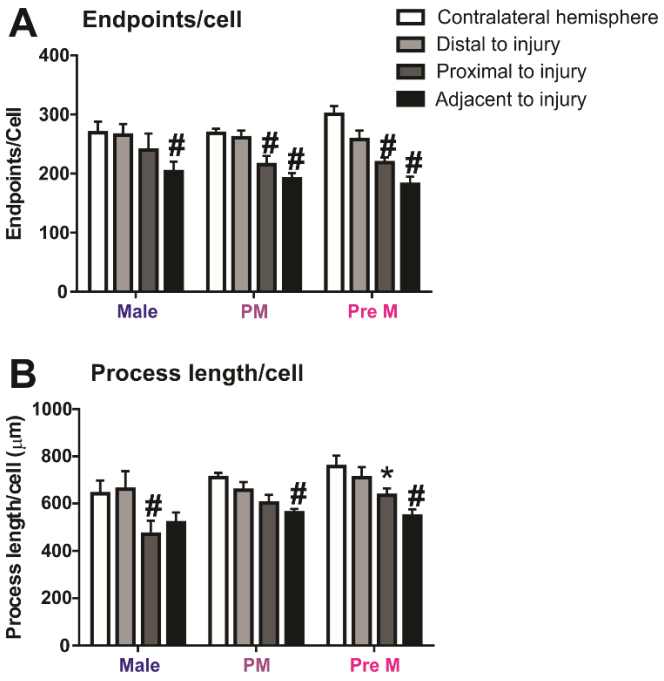

Figure 3. Microglia ramified morphology is decreased in proximity to brain injury after ischemic stroke in male, pre-menopause (Pre M) and post-menopause (PM) female mice. **A)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia endpoints/cell in brain regions following ischemic stroke and 8hr of recovery. The number of microglia endpoints/cell is different than the contralateral hemisphere ($F_{(3,68)} = 20.71$, $p < 0.0001$) with Bonferroni post-hoc comparisons reported in the figure (# $p < 0.05$). There are no differences in the number of endpoints/cell ($F_{(2,68)} = 0.6$, $p < 0.55$) among sex groups. **B)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia process length/cell in brain regions following ischemic stroke and 8hr of recovery. The microglia process length/cell is different than the contralateral hemisphere ($F_{(3,68)} = 11.63$, $p < 0.0001$) with Bonferroni post-hoc comparisons reported in the figure (# $p < 0.05$). In one region (proximal to injury), microglia process length/cell was different among sex groups ($F_{(2,66)} = 4.65$, $p < 0.05$) with Bonferroni

post-hoc comparisons reported in the figure (* $p < 0.05$). Sample size: Male n = 5-6; PM n = 8; Pre M n = 6

Figure 4

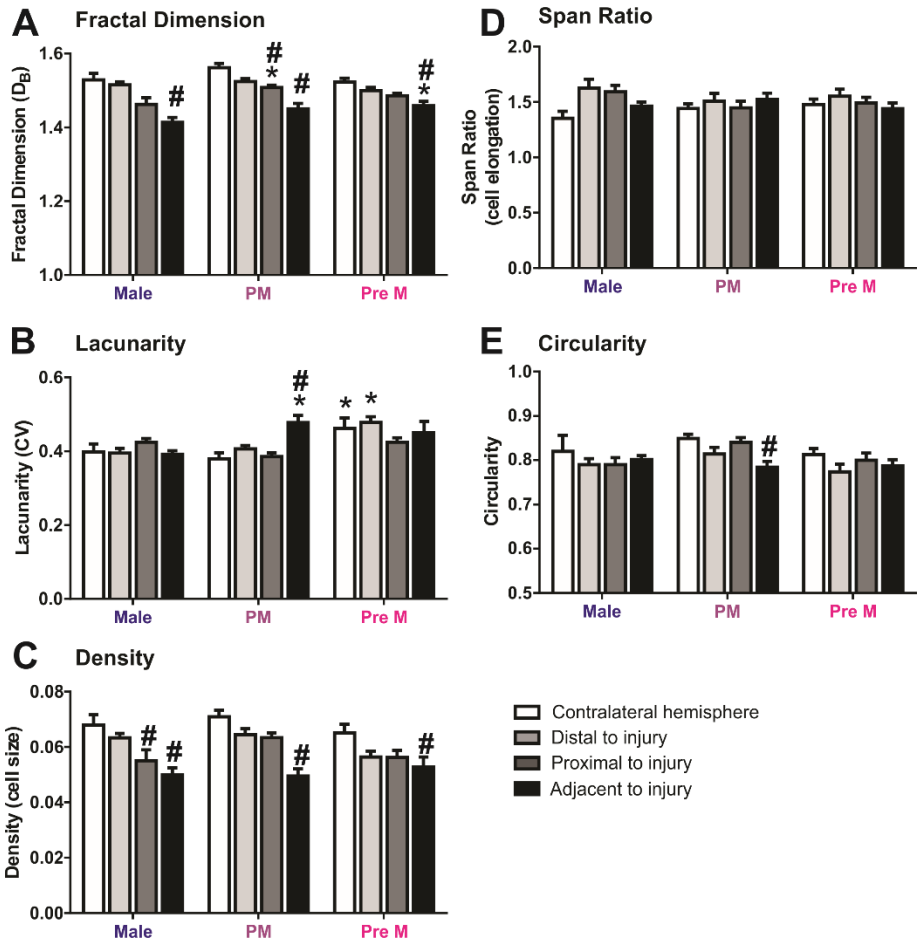

Figure 4. Microglia shapes are changed proximity to brain injury after ischemic stroke in male, pre-menopause (Pre M) and post-menopause (PM) female mice. **A)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia fractal dimension in brain regions following ischemic stroke and 8hr of recovery. Microglia fractal dimension is decreased versus the contralateral hemisphere ($F_{(3,57)} = 36.80$, $p < 0.0001$) with Bonferroni post-hoc comparisons

reported in the figure (# $p < 0.05$). Fractal dimension is also different according to sex group ($F_{(2,19)} = 7.5$, $p < 0.01$) with Bonferroni post-hoc comparisons reported in the figure (* $p < 0.05$). **B)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia lacunarity in brain regions following ischemic stroke and 8hr of recovery. Microglia Lacunarity is unchanged versus the contralateral hemisphere ($F_{(3,57)} = 1.68$, $p > 0.05$; # $p < 0.05$ vs contralateral) but with differences among sex groups ($F_{(2,19)} = 13.80$, $p < 0.001$) and significant interaction ($F_{(6,57)} = 3.62$, $p > 0.01$) with Bonferroni post-hoc comparisons reported in the figure (* $p < 0.05$). **C)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia density following ischemic stroke and 8hr of recovery. Microglia density is different than the contralateral hemisphere ($F_{(3,57)} = 21.63$, $p < 0.0001$) with Bonferroni post-hoc comparisons reported in the figure (# $p < 0.05$) but without differences among sex groups ($F_{(2,19)} = 2.39$, $p > 0.05$) or interaction ($F_{(3,57)} = 3.62$, $p > 0.05$). **D)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia span ratio following ischemic stroke and 8hr of recovery. Microglia span ratio is different than the contralateral hemisphere ($F_{(3,57)} = 2.9$, $p < 0.05$) but without significant Bonferroni post-hoc comparisons, differences among sex groups ($F_{(2,19)} = 0.3$, $p > 0.05$) or interaction ($F_{(3,57)} = 1.37$, $p > 0.05$). **E)** Summary data (mean and SEM) and statistical analysis (two-way ANOVA) of microglia circularity following ischemic stroke and 8hr of recovery. Microglia circularity is different than the contralateral hemisphere ($F_{(3,57)} = 3.39$, $p < 0.05$) with Bonferroni post-hoc comparisons reported in the figure (# $p < 0.05$) but without differences among sex groups ($F_{(2,19)} = 2.93$, $p > 0.05$) or interaction ($F_{(3,57)} = 0.92$, $p > 0.05$). Sample size: Male n = 7; PM n = 8; Pre M n = 7