MECHANISMS OF SOCIAL NEUROPROTECTION AFTER CEREBRAL ISCHEMIA

DISSERTATION

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ABSTRACT

Social isolation has long-term physiological and psychological consequences. The benefits associated with social support are well described in cerebrovascular disease patients; however, the mechanisms by which social interactions influence disease outcome are unknown. The present body of work examined the effects of social interaction on stroke outcome in mice. The goals of this dissertation are to describe the phenomenon and consequences of social interaction-mediated neuroprotection in a mouse model of cerebral ischemia, as well as to describe a neuroendocrine basis by which social interactions may mediate stroke-induced neuroinflammation and functional outcome.

Social housing conditions influence measures of stroke outcome in a mouse model of transient focal cerebral ischemia. Male mice housed with an ovariectomized female have attenuated ischemic injury, improved post-stroke survival rate and enhanced functional recovery. Importantly, this neuroprotective effect requires the physical contact component of social interaction, as removal of this component using a barrier (while preserving all other sensory stimuli associated with social interactions), eliminates both the neuroprotection and locomotor recovery in socially housed mice. Additionally, the reduction in ischemic damage in socially housed mice is accompanied by an anti-inflammatory response, characterized by altered central and systemic

markers of inflammation. Specifically, interleukin-6, a cytokine that is both modified by social interactions and predicts stroke outcome, is differentially regulated in socially housed and isolated mice, suggesting that social housing may alter the trajectory of ischemia outcome in part by attenuation of inflammation.

Finally, the role of oxytocin, a neuropeptide released during social contact, was assessed as a potential mediator of social neuroprotection. Administration of oxytocin to socially isolated animals reproduces the neuroprotection conferred by social housing, and blockade of oxytocin action via administration of an oxytocin receptor antagonist blocks these effects in socially housed animals. Importantly, oxytocin receptors on microglia, critical immune effectors in ischemia, appear to modulate microglia activation in response to an inflammatory stimulus. These findings support the hypothesis that oxytocin is neuroprotective against physiological and behavioral consequences of cerebral ischemia, and provide insight into the mechanism by which social influences impact stroke outcome.

This dissertation is dedicated to my mother

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Very shortly after I began my rotation in Courtney DeVries' lab I knew that I wanted to stay. At our first meeting just as I started my rotation, I brought up some ideas about studying the role of oxytocin as a mechanism of what I now call "social neuroprotection" in cerebral ischemia. I hadn't realized then that I was outlining my dissertation work before I had even officially joined the lab. Over the years, Courtney has pushed me to take every opportunity to apply for awards/grants, publish, and present data, and I owe the sum of my success as a scientist to her belief in me. I have tried to make the most of every opportunity I have been given in this lab and graduate program, and plan to take the lessons I have learned here with me throughout my career.

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CHAPTER 1

INTRODUCTION

Social interactions shape humans from early development through senescence and have a strong impact on many aspects of physiology and behavior. Indeed, social interaction is essential for proper cognitive, affective and behavioral development (Dawson and Dawson, 2006). Among adults, the social environment remains an important determinant of health and well being; ample evidence suggests that positive social support improves and accelerates patient recovery from cancer, cerebrovascular and cardiovascular disease (CVD), atherosclerosis, and other chronic diseases with an inflammatory component (Barry et al., 2006; Cohen et al., 2007b; Seeman, 2000; Strating et al., 2006). This has led to a substantial interest in the capacity to which the social environment impacts physiological systems, particularly during health challenges. The benefits of a positive social environment are particularly salient in chronic disease states, in which social support is often perceived as being equally or more important than instrumental and informational support (i.e. Arora et al., 2007). In contrast, social isolation and loneliness can have profoundly detrimental effects on mental and physical health (Boden-Albala et al., 2005).

While this observation is not novel in the medical community (Berkman and Syme, 1979; Kaplan et al., 1977; Wolf, 1969), it has only relatively recently begun to gain momentum in both clinical and animal research. The addition of evaluating patients' social, along with cognitive and physical states, while not yet considered common practice, is gaining acceptance in hospitals and clinics worldwide (S.I.G.N., 2002; Stone and Whincup, 1994). However, despite growing evidence implicating the social environment as a modifying factor in disease outcomes, little is known regarding the mechanisms through which psychosocial factors influence disease pathogenesis. Importantly, converging evidence from experimental research suggests that socially isolated and socially housed animals mount a quantitatively different pathophysiological response to disease and physical trauma.

The focus of this dissertation is specifically on the psychobiological determinants of ischemia outcome. This focus is embedded in the overarching theme of social influences on disease states. This dissertation also describes the extent to which animal models are an invaluable tool for bridging the gap in knowledge about environment-biology interactions. The term **social neuroprotection** is introduced here to broadly describe the phenomenon by which social interactions are observed to attenuate neuronal injury and consequently improve behavioral outcomes and survival in a cerebral ischemia model. **Chapter 3** describes a series of experiments that expand on observations of social neuroprotection in a mouse model of cerebral ischemia. These experiments were designed to characterize the extent to which social interaction influences both the physiological and behavioral components of ischemia outcome, as

well as to identify the specific component of social interaction that confers neuroprotection. The experiments in **chapter 4** identify the specific role of the inflammatory component in ischemic injury. Social interaction-mediated attenuation of ischemic injury is accompanied by a suppressed inflammatory response (both centrally and peripherally), representing a potential proximate mediator of social neuroprotection. Finally, **chapter 5** describes a series of experiments aimed at identifying a potential neuroendocrine mechanism by which psychosocial experiences influence cerebral ischemia pathophysiology and outcome. The purpose of the work described in this document is to both describe the phenomenon of social neuroprotection in ischemia, and to convey the potential cost with neglecting to identify environmental factors (i.e. stress, social support) as risk factors for disease.

1.1 Social modulation of disease

Social support and disease: evidence from clinical research

Chronic diseases such as cardiovascular and cerebrovascular disease, diabetes, cancer, and autoimmune disorders accounted for 70% of all deaths in the United States in 2005 (Kung et al., 2008). Among those individuals living with chronic disease, many experience long-term disability as well as concomitant psychological distress such as anxiety/depression. A substantial research effort has elucidated a number of risk factors (e.g. smoking, alcohol consumption, high blood pressure, cholesterol, etc) that

are common across most chronic disease states, as well as identified causal relationships and mechanisms by which these factors influence disease onset and outcome. Interestingly, even after statistically accounting for these risk factors, there still exists substantial inter-individual variability in susceptibility to disease and recovery. Recent research has identified an additional class of risk factors: psychological stress (including social isolation or perceived lack of social support), that is predictive of disease outcome independently of other "traditional" risk factors (Cacioppo and Hawkley, 2003; Orth-Gomer et al., 1998).

The term "psychological stress" broadly encompasses emotional and environmental distress to which nearly all individuals are susceptible. More specifically, the focus of this thesis is on the state of social isolation. It should be noted that social isolation can be both "real", measured quantitatively as individuals reporting few or no acquaintances or friends and/or avoidance of social support or communication; or "perceived", described colloquially as loneliness (Hawkley et al., 2006; Peardon et al., 2010). The term "social support" can refer to various supportive behaviors or concepts, including physical and emotional comfort, as well as instrumental, and informational support (Peardon et al., 2010; Thoits, 1986). A notable caveat in the literature on the role of psychosocial experiences in disease outcomes is that there is no clear baseline from which to interpret this complex relationship. Both the clinical and animal studies are divided into two schools of thought that are by no means mutually exclusive: 1) social support (in humans) and affiliative social interactions (in humans and animals)

provide protection, and 2) social isolation (real or perceived) and other forms of social stress exacerbates disease onset/progression. In other words, it is unclear whether social interaction *improves,* or social isolation *exacerbates* disease states. Given the social structures of the species being reviewed in this document, a social environment is the most likely baseline. After all, most species' fitness depends in some way on maintenance of complex social structures. Nonetheless, because social experience is not truly quantifiable (particularly the emotional and cognitive components), a true baseline is difficult to define. Until a true baseline can be determined, it must be assumed that social interactions have the capacity to both support and exacerbate health outcomes. As such, the existing literature alternates focus between the benefits of social support and the consequences of social isolation.

A prevailing hypothesis of the mechanism by which the psychosocial environment affects physiology is that social support increases health promoting behaviors. Indeed, social support increases behaviors that serve to enhance health, including better medical compliance, physical exercise, proper nutrition and low-to-moderate tobacco and alcohol consumption (Cohen and Lemay, 2007). While it is not surprising that social and peer support increases the likelihood of engaging in health behaviors (whether because of the pressure to conform to social norms or a potential increase of tangible resources), statistically controlling for these behavioral changes indicates that the benefits of social support remain substantial (Caspi et al., 2006; Orth-Gomer et al., 1998; Uchino et al., 1996). Importantly, a number of clinical studies have

elucidated that socially integrated individuals often present with quantitatively different physiological measures that may reduce susceptibility to disease, as compared to individuals who report experiencing social isolation (Hawkley et al., 2006; McDade et al., 2006). Indeed, perceived social isolation is linked to the development of coronary calcification (Kop et al., 2005), atherosclerosis (Wang et al., 2005), and chronic low-grade inflammation (Ford et al., 2006). The major implication of these findings is the need to identify an endogenous mechanism by which positive social behaviors are identified and interpreted by an organism as a pro-health message.

To date, it has been difficult to definitively identify a causal relationship between psychosocial factors and disease among the clinical population, however, a number of successful longitudinal studies (Eaker et al., 1992; Hawkley et al., 2006; McDade et al., 2006), have identified social isolation as a predictor of physiological measures that are known risk-factors for disease, as well as onset of CVD, even 20 years following initial assessment (Caspi et al., 2006; Eaker et al., 1992). Specifically, psychosocial variables such as self-reported loneliness have a substantial impact on 20-year incidence of CVD. Risk of myocardial infarction or coronary death was increased by more than 3-fold in "lonely" women (Eaker et al., 1992). Moreover, childhood social isolation predicted a greater number of CVD and stroke risk factors, including high body mass index, high blood pressure and cholesterol and low oxygen consumption in adulthood (Caspi et al., 2006). Importantly, this relationship remained significant even after authors statistically accounted for socioeconomic status and health compromising behaviors. These data

demonstrate that the relationship between social isolation and disease is not strictly correlational; however, a complicating factor in the study of the relationship of social behaviors and disease is that social isolation may manifest as a sickness behavior, leading medical professionals and researchers to rule out social isolation as a potential causal factor requiring therapeutic intervention. Thus, the crucial implication of these longitudinal studies is that even early experiences of social isolation predict the development of risk factors well into adulthood.

Two important questions emerge in light of these studies. (1) What are the shared pathophysiological features in disease states that are influenced by social experiences, and (2) in what way are they modifiable by the psychosocial environment? One common mechanism of disease that underlies the multiple pathophysiologies described in this review is inflammation. The immune system actively regulates and sustains a state of homeostasis under normal physiological conditions. However, following trauma, the organism enters a pro-inflammatory and pro-thrombotic state (Medzhitov, 2008). This state of systemic inflammation involves activation of macrophages and T cells, release of soluble factors such as cytokines and chemokines, leukocyte extravasation and upregulation of adhesion molecules among a multitude of other immune effectors (Bramlett and Dietrich, 2004; Medzhitov, 2008; Wang et al., 2007). Whereas acutely this is a coordinated adaptive response intended to restore homeostasis and remove pathogens and dead/dying cells, there are serious consequences to tipping the balance toward a chronic pro-inflammatory state. Indeed,

chronic inflammation increasingly ranks as an important novel risk factor for stroke and CVD, as well as many cancers, Alzheimer's disease and major depression disorder (Arenillas et al., 2003; Heneka and O'Banion, 2007; Lavie et al., 2009; Miller et al., 2009; Schetter et al., 2010). For example, patients presenting with systemic inflammation, such as systemic lupus erythematosus and rheumatoid arthritis, have a 4-10 fold increased risk of developing CVD (reviewed in Van Leuven et al., 2008).

Notably, although the influence of social experiences on pathophysiology is evident in a wide range of disease states, this relationship is most commonly reported in stroke and CVD patients. It is likely that this is due to the strong inflammatory component of both disease states. In both coronary and cerebral ischemia, inflammation is relevant both as an indicator of an underlying cause (i.e. atherosclerosis Nissen et al., 2005) and as an important mediator (Lavie et al., 2009), rather than just a biomarker of ischemia. As such, chronic inflammation has become a main focus for monitoring and prevention of stroke and CVD. In particular, systemic levels of acute phase proteins such as C-reactive protein (CRP) and proinflammatory cytokines (primarily interleukin 6; IL-6) predict the risk and prognosis of stroke and CVD (Arenillas et al., 2003; Rost et al., 2001). CRP is a nonspecific but sensitive marker of systemic inflammation and reliably predicts the onset of atherosclerosis (Arici and Walls, 2001) and consequent ischemic events (both coronary and cerebral) (Arenillas et al., 2003; Arici and Walls, 2001; Shah et al., 2009). Therapeutic interventions that reduce CRP expression are associated with a reduction of cerebrovascular and cardiovascular events

(Krupinski et al., 2008), and direct CRP antagonists are being developed for clinical trials for stroke and CVD patients (Pepys et al., 2006). In addition, elevated plasma IL-6 (the cytokine that induces CRP production in hepatocytes) is among the early measurable markers of systemic inflammation after stroke (Emsley et al., 2003). Both peak plasma IL-6 and CRP concentrations correlate with infarct (dead tissue as a consequence of reduced blood supply) and are strongly associated with survival (Smith et al., 2004).

Given that inflammation is a key underlying mechanism of disease in stroke and CVD, it is likely that factors that affect stroke/CVD (including psychosocial experiences) may do so by influencing an inflammatory response. Indeed, a particularly robust finding in the clinical literature is an inverse relationship between social support and circulating markers of inflammation such as C-reactive protein (CRP) and IL-6. Emerging evidence indicates that serum CRP and IL-6 concentrations are elevated among socially isolated individuals (Ford et al., 2006; Loucks et al., 2006; McDade et al., 2006). Further, stroke morbidity and mortality rates (which correlate significantly with circulating CRP and IL-6 concentrations) are significantly greater in socially isolated individuals (Ikeda et al., 2008). Taken together, the known causal relationship between elevated circulating CRP and IL-6 concentrations with CVD and cerebrovascular disease (Arenillas et al., 2003; Kuo et al., 2005; Ladenvall et al., 2006; Rost et al., 2001) establishes a potential link between lack of social support and increased risk of CVD and stroke (Boden-Albala et al., 2005).

The relationship between social isolation and stress represents another widely hypothesized link between social interactions and pathophysiology. Indeed, socially isolated individuals may suffer increased levels of stress; which consequently negatively impacts measures of inflammation. Elevated circulating concentrations of glucocorticoids (physiological markers of stress) exacerbate CRP as well as pro-inflammatory cytokine concentrations (Zhou et al., 1993). Moreover, increased cortisol and epinephrine are linked to hypertension in isolated individuals, which may partially account for increased presence of atherosclerosis and endothelial dysfunction in these individuals (Hawkley et al., 2006). Conversely, the benefits of social support are often attributed to "social buffering" – or the idea that positive social experiences serve as a buffer against psychological stress, essentially dampening the hypothalamic-pituitary-adrenal (HPA) axis and reducing the glucocorticoid-mediated stress response (reviewed in DeVries et al., 2003). While the data on the detrimental effects of stress (particularly chronic stress) on stroke outcome are well known, the impact of stress on disease outcome in the context of social interactions (i.e. social isolation stress) is not well understood.

Overall, the mechanisms by which psychosocial factors influence the pathophysiological response to disease remain unknown because the necessary research cannot be ethically conducted on the clinical population. On the other hand, environmental factors such as social housing are easily modifiable in rodents, and as such can be used to establish causation and the mechanisms underlying exacerbation of

disease. A common argument against relying on the use of animal models in the context of socio-biological interactions is rooted in the definition of and terminology used to describe social support in the clinical population. As noted above, the bulk of the clinical literature on social influences on health relies largely on self-reported measures of "perceived" social isolation. In fact, it has been suggested that perceived isolation is as strong or stronger a predictor of poor health outcomes as "real" isolation (Uchino et al., 1996). The notion of perceived social isolation, colloquially referred to as "loneliness", is a concept that implies emotional distress which is believed by many to be uniquely experienced by primates and higher-order non-primate species. Although the notion of emotional intelligence and even empathy has been reported in rodents (Tuttle, 2008), the difficulty with qualifying the emotional distress of social isolation (or the emotional benefits of social interactions) remains a limitation of experimental research. Nonetheless, animal models are increasingly able to account for measures of distress associated with social isolation, and the consistent and robust detrimental effects of social isolation on health in animal models are in parallel with clinical findings. Above all else, these findings (described in detail below) indicate that the negative health impacts of social isolation are reproducible in multiple disease and trauma models. Moreover, in keeping with the clinical findings, the benefits of social interaction are measured as a quantitatively different physiological response to disease.

Social stress in animals

As the impact of social housing conditions (i.e. single vs. pair/group housing) on rodent welfare gains attention, it is becoming increasingly evident that for some species, social isolation is a profound psychological stressor. Chronic individual housing of rodents induces symptoms of "isolation syndrome" including depressive-like behavior (Grippo et al., 2007a; Martin and Brown, 2010), stress and anxiety-like behaviors (Ferrari et al., 1998; Weiss et al., 2004), as well as aggression (Olsson and Westlund, 2007; but see, Arndt et al., 2009). In addition, the physiological consequences of social isolation include autonomic dysregulation (Grippo et al., 2007b), altered metabolism, heart rate, and core body temperature (Bartolomucci et al., 2009; Rettich et al., 2006; Spani et al., 2003), and even suppression of adult neurogenesis (Stranahan et al., 2006). Many of the negative consequences of social isolation are rescued simply by manipulating housing conditions. For example, numerous studies have indicated that environmental enrichment rescues depressive-like and anxiety-like phenotypes typically observed after social isolation (reviewed in Fox et al., 2006; Laviola et al., 2008) and improves cognitive and functional outcome after stroke (Dahlqvist et al., 2004; Nygren and Wieloch, 2005). Enriched cages typically house multiple rodents and consist of a running wheel, and various objects such as plastic tubes, platforms, etc. Because environmental enrichment involves enhancing both physical activity and social contact, it is important to tease apart the influence of these stimuli. Importantly, social housing (Ferrari et al., 1998; Grippo et al., 2007b; Stranahan et al., 2006), even in the absence of other forms of

enrichment, is sufficient for rescuing the detrimental effects of social isolation. Indeed, affiliative social interactions typically ameliorate stress responses and enhance physiological defenses against disease and trauma, indicating the powerful influence of the social environment on physiology and behavior.

Social interactions and disease: evidence from animal models

A growing body of experimental literature has provided evidence that the behavioral and physiological consequences of social isolation include increased susceptibility to disease, prolonged wound healing, and a disruption of functional recovery. Existing animal models of disease, in particular rodent models of ischemia, neuropathic pain, and wound healing – disease states well known to be influenced by social factors in the clinical population – are all sensitive to social manipulations. There is a high degree of agreement among the clinical and emerging rodent data on the positive impacts of social interaction on the pathogenesis of these disease states (DeVries et al., 2007), and these data are now being extended to study the mechanisms by which social factors influence disease pathophysiology.

In a series of studies employing well-characterized rodent models of focal (stroke) and global (cardiac arrest) cerebral ischemia (Craft et al., 2005; Karelina et al., 2009b; Weil et al., 2008a), social housing condition (social isolation vs. multiple or pair housing) has been shown to significantly impact ischemia outcome. Socially housed

(paired) animals are less likely to develop atherosclerosis (Bernberg et al., 2008; McCabe et al., 2002) and have smaller ischemic infarcts compared to isolated animals (Craft et al., 2005), and while the mechanisms of social neuroprotection in these models are still under investigation, it is evident that many of the factors known to exacerbate ischemic outcome are influenced by social factors. Socially housed mice have lower circulating concentrations of CRP (Craft et al., 2005) and plasma triglycerides (Bernberg et al., 2008), and decreased vascular oxidative stress (Nation et al., 2008). Social housing also attenuates inflammatory response to trauma, measured as suppressed reactive gliosis (Weil et al., 2008a) and cytokine release (IL1 beta Norman et al., 2010; TNFalpha Özlem eri et al., 2009). In addition to attenuation of factors that exacerbate ischemic outcome, social interaction has also been shown to up-regulate protective factors including brain-derived neurotrophic factor (Scaccianoce et al., 2006) and IL-6 (a cytokine shown to be neuroprotective in cerebral ischemia; Karelina et al., 2009b). Taken together, the mechanisms of social neuroprotection appear to involve not only a suppression of inflammation and oxidative stress, but also an up-regulation of neuroprotective factors.

Functional recovery following an ischemic event is equally, if not more, important than infarct volume as a measure of ischemic outcome. Cerebral ischemia typically causes sensorimotor deficits characterized by problems with general motor coordination, balance, and postural and sensory reflexes (Bouët et al., 2007; Gerlai et al., 2000). Interestingly, whereas social interaction prior to an ischemic event is sufficient for reducing infarct volume, the positive impact of social interaction on

sensorimotor recovery appears to require continued social interaction immediately following the ischemic event (Johansson and Ohlsson, 1996; Risedal et al., 2002; Silasi et al., 2008). Further, as will be discussed in chapter 3, the degree of sensorimotor recovery appears to be dependent on physical contact during social interaction. For example, socially housed mice that are separated by a barrier that prevents physical contact do not exhibit the same degree of functional recovery as mice that are socially housed without a barrier (Karelina et al., 2009a). The extent and timing of the social interaction may be a critical determinant of social neuroprotection in ischemia. These data provide strong support for the assertion that the psychosocial environment is an independent predictor of disease outcome.

A substantial research effort has been focused on the role of elevated circulating glucocorticoids and altered inflammatory responses as mediating variables in the relationship between social interaction and disease. Although basal glucocorticoid concentrations are typically similar between socially housed and isolated rodents (Arndt et al., 2009; Martin and Brown, 2010; Scaccianoce et al., 2006), stress reactivity is attenuated in socially housed animals (Detillion et al., 2004; Weiss et al., 2004; Williams et al., 2009; but see Sanchez et al., 1998). Although acute stress responses may be adaptive in many circumstances, chronic stress is well-documented as being detrimental to health. In fact, chronic stress (such as chronic social isolation) leads to altered immune function (DeVries et al., 2007; Padgett and Glaser, 2003), hypertension, myopathy (Lucini et al., 2005; Radley and Morrison, 2005), and a range of psychological

disorders (Pace et al., 2007; Southwick et al., 2005). Further, social stress, such as hierarchy disruption and intruder aggression results in glucocorticoid insensitivity and tips the balance toward a pro-inflammatory state (Avitsur et al., 2009), leaving the organism more susceptible to disease. However, despite the increasing attention being paid to the role of stress in disease, it continues to be among the biggest challenges facing experimental and clinical research, in part because stress influences psychological (affective), behavioral, and biological processes in ways that are most likely to converge to influence the onset and progression of disease (Cohen et al., 2007a). Thus the detrimental effect of stress on disease outcome is multifactorial and highly integrated with other risk factors. One approach to reducing the deleterious effects of stress is to identify the psychosocial factors that increase an individual's susceptibility to both psychological and physical stressors.

The buffering effect of affiliative social interaction against stress represents one potential mechanism of social neuroprotection. For example, acute restraint elevates circulating cortisol concentrations and prolongs wound healing in Siberian hamsters (*P. sungorus*), however, pair-housing eliminates the stress-induced activation of the HPA axis and ameliorates the effect of stress on wound healing (Detillion et al., 2004). In fact, restraint stress has no impact on wound healing latency among pair housed hamsters. Moreover, the effect of restraint stress on wound healing in socially isolated hamsters was likely mediated by endogenous cortisol secretion, because adrenalectomized hamsters healed more rapidly (Detillion et al., 2004). In addition,

social disruption (an experimental model in which established social hierarchies are disrupted via introduction of an aggressive intruder animal) substantially elevates circulating glucocorticoid concentrations and increases susceptibility to viral infection (Sheridan et al., 2000) and a variety of inflammatory diseases including pulmonary inflammation (Curry et al., 2010), asthma (Sheridan et al., 2006) and influenza (Powell et al., 2008). Taken together, the consequences of over-stimulation of the HPA axis and hypersecretion of glucocorticoids may be ameliorated by stable and affiliative social interaction

1.2 Ischemia Pathophysiology

Stroke is the third leading cause of death in the United States and is a leading cause of long-term disability, including paralysis, as well as speech and affective disorders (Camarata et al., 1994). To date the only treatment for stroke approved by the FDA is thrombolytic therapy through the use of tissue plasminogen activator (tPA). This treatment is aimed at rescuing viable tissue within a short therapeutic window and has been met with some clinical success (Ribo et al., 2005). However, while tPA treatment can reduce some of the damage caused by stroke, its use is limited because 1) it must be administered within 3 hours of symptom onset, 2) fewer than 15% of stroke patients are eligible for tPA treatment, and 3) the use of tPA can cause serious complications such as intracranial hemorrhage (Bambauer et al., 2006; Cocho et al., 2005; Dawson and Dawson, 2006). Thus, the paucity in available treatments as well as

the dramatic effect of stroke on quality of life necessitates further research into the mechanisms underlying the progression of stroke-related damage.

The risk factors for stroke are well known and include: smoking, high cholesterol, high blood pressure, diabetes mellitus, heart disease, physical inactivity, infection, and social isolation (AHA, 2008; Elkind, 2007; Greenwood et al., 1996). However, despite the growing clinical and experimental literature on factors influencing stroke outcome, the underlying mechanisms by which many of these factors influence the physiological and behavioral measures following a stroke are not yet established. The growing depth of knowledge of the modulatory role of social factors in ischemia merits further research into the mechanisms by which they alter pathophysiological responses to ischemia.

Neuroinflammation in ischemic injury

Transient focal cerebral ischemia (stroke) produces cell death; however, regional differences in the extent of neuronal injury provide a small therapeutic window during which some recovery is possible. Focal cerebral ischemia results in irreversible necrotic cell death within the core (center) of the infarct, as a result of a severe reduction of blood flow. Severe damage mediated by oxygen and glucose deprivation in the core leads to energy failure and a loss of membrane potential. Immediately surrounding the core of the infarct is a border of tissue (termed the penumbra) situated between the

irreversibly dead and healthy brain tissue (Stoll et al., 1998). Unlike the core, tissue within the penumbra has received some blood flow during an ischemic event, typically via collateral blood flow. While functionally inactive, tissue within the penumbra remains viable for several hours, thus stroke treatments are typically designed to rescue penumbral tissue by means of slowing or reducing the converging cascade of pathophysiological events that contribute to the extent of cell death. Although several endogenous mechanisms contribute to ischemic injury, including oxidative damage and excitotoxicity (Camacho and Massieu, 2006), there is increasing interest in the role of inflammatory processes and how they contribute to the progression of neuronal damage (Crack and Taylor, 2005; De Simoni et al., 2002; Huang et al., 2006; Mergenthaler et al., 2004; Stoll et al., 1998). Under hypoxic conditions (i.e. inadequate supply of oxygen due to reduced blood flow), neurons, astrocytes, microglia, endothelial cells and oligodendrocytes produce proteolytic enzymes (i.e. matrix metalloproteinases) that degrade the extracellular matrix and compromise the integrity of the blood-brain barrier (BBB; Rosenberg et al., 1998). BBB disruption permits the infiltration of inflammatory cells including monocytes/macrophages, neutrophils, T-cells, B-cells and natural killer cells to the site of injury. Lymphocyte migration is further facilitated by the induction of chemokines and adhesion molecules (Feuerstein, 2001). The net effect is an invasion of the CNS by inflammatory cells which trigger further induction of immune mediators and contribute to the development of CNS injury.

The transcription of many inflammatory genes, including cell adhesion molecules, interferons and cytokines is dependent on activation of the transcription factor nuclear factor κB (NF-κB) (Hayden and Ghosh, 2008). NF-κB signaling can occur in most cell types, including neurons, astrocytes and microglia (Mattson and Meffert, 2006). Under normal conditions, NF-κB proteins are bound to an inhibitory protein (IκB) which keeps the transcription factor inactive in the cytosol. However, once stimulated (i.e. in cerebral ischemia), IκB is phosphorylated, polyubiquitinated and degraded. This permits NF-κB proteins to translocate to the nucleus where it stimulates the transcription of inflammatory genes (Schwaninger et al., 2006).

The neuroinflammatory response to ischemic injury is triggered by activated microglia and astrocytes, which are an important early local source of pro-inflammatory cytokines (Huang et al., 2006; Wang et al., 2007). Among the most studied cytokines associated with cerebral ischemia are interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNFα), and interleukin-6 (IL-6). These cytokines are produced and secreted by activated glia soon after ischemic injury and are thus able to contribute significantly to the extent of neuronal damage following MCAO (Huang et al., 2006; Wang et al., 2007). Indeed, treatment with IL-1β (Stroemer and Rothwell, 1998) or TNFα (Arvin et al., 1996) exacerbates infarct volume in mice; moreover, infarct size is attenuated following administration of the IL-1 receptor antagonist (Stroemer and Rothwell, 1997) and in mice deficient in TNF receptors (Bruce et al., 1996). Further, blockade of either IL-1β (Boutin et al., 2001) or TNFα (Nawashiro et al., 1997) signaling is associated with

decreased infarct size. There are conflicting data on the functional role of IL-6 up-regulation following ischemic stroke (Ali et al., 2000; Clark et al., 2000; Loddick et al., 1998). Studies demonstrate that central expression of this cytokine plays a critical neuroprotective role during an ischemic event (Ali et al., 2000; Loddick et al., 1998). For example, intracerebroventricular administration of IL-6 reduces infarct size, possibly through a mechanism involving suppressed excitotoxicity (Ali et al., 2000). Likewise, blockade of IL-6 signaling results in increased apoptotic cell death and infarct size, as well as poor neurological outcome (Yamashita et al., 2006). Conversely, as described above, increased circulating concentrations of IL-6 initiate acute phase protein induction and are generally considered to be associated with a poor prognosis of stroke outcome (Dziedzic, 2008).

Oxidative stress in Ischemic Injury

The damaging effects of oxidative stress are well recognized in a variety of neurological disorders including traumatic brain injury, cerebral ischemia and neurodegenerative diseases (Maier and Chan, 2002; Saito et al., 2005). In stroke, while the return of oxygenated blood is vital for the potential recovery of reversible tissue damage in the penumbra, it can also be deleterious due to increased reactive oxygen species (ROS) production (Crack and Taylor, 2005). Indeed, excessive production of ROS during the rapid return of blood following occlusion (reperfusion) results in damage to lipids, protein and nucleic acids and contributes to the progression of cell death (Loh et

al., 2006; Saito et al., 2005). The CNS is particularly vulnerable to oxidative damage for several reasons: 1) neurons are metabolically expensive and require high consumption of glucose and oxygen, 2) the high content of polyunsaturated fatty acids are targets for oxidative damage, and 3) the CNS is relatively ill equipped with antioxidant defenses against ROS (Crack and Taylor, 2005).

An imbalance between the antioxidant defense and ROS production results in a state of oxidative stress. The generation of free radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxinitrite, contributes to lipid peroxidation, excitotoxicity and inflammation (Warner et al., 2004). The best defense against free radicals is provided by antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Crack and Taylor, 2005; Maier and Chan, 2002). Briefly, SOD catalyzes O_2^- into oxygen and H_2O_2 (Maier and Chan, 2002), whereas catalase and GPx further convert H_2O_2 to water and oxygen (reviewed in Crack and Taylor, 2005). Thus, these enzymes play an essential protective role in mediating the degree of oxidative stress following ischemia/reperfusion injury. For example, transgenic mice that over-express Cu/Zn-SOD (an SOD isoenzyme that is primarily localized to the cytosol) have a reduction in cell death and smaller infarcts following MCAO (Kinouchi et al., 1991). Accordingly, both MnSOD (an SOD isoenzyme localized to the mitochondria) and Cu/Zn-SOD knockout mice have significantly increased infarct volume and edema following cerebral ischemia (Kondo et al., 1997; Murakami et al., 1998). GPx also plays an integral role in neuroprotection against cell death induced by

oxidative stress. GPx knockout mice have increased infarct volume and cell death, through a mechanism that involves increased NF-κB activation (Crack et al., 2006). Conversely, over-expression of GPx inhibits the pro-apoptotic pathway, increases neuronal survival within the infarct, and inhibits glial activation following MCAO (Hoehn et al., 2003; Ishibashi et al., 2002). Likewise, catalase over-expression prior to (but not following) MCAO is neuroprotective against oxidative damage (Gu et al., 2004).

Under non-pathological conditions, antioxidant levels are sufficient for scavenging free radicals, however a number of factors can tip the balance toward elevated free radical production. In addition to physical trauma and disease, psychosocial stress can also lead to oxidative damage. It has been demonstrated that social isolation induces oxidative damage and decreases antioxidant content in the CNS and periphery (Huong et al., 2005; Nishio et al., 2007). Specifically, chronic social isolation results in increased oxidative DNA damage in peripheral blood cells (Nishio et al., 2007), as well as oxidative damage in the brain via enhanced production of nitric oxide, increased lipid peroxidation, and decreased GPx content (Huong et al., 2005).

Importantly, while a number of treatments aimed at each of these processes have been successful in animal models, nearly all have failed in late phase clinical trials (De Keyser et al., 1999; Gladstone et al., 2002; Ikonomidou and Turski, 2002). The failure of these clinical trials has been attributed to the complicated interaction of pathophysiological events in ischemia, improper dosing or administration, dangerous side effects, minimal efficacy, and a small therapeutic time window (De Keyser et al., 1999). In contrast, the

relative success of similar treatments in animal studies speaks to lack of translational value in using treatments that directly target a specific aspect of ischemia pathophysiology in a controlled laboratory environment, as well as the caveat of using infarct size as a primary measure of ischemic outcome. One step toward closing this gap is to use the naturally occurring variability in stroke outcome to identify underlying factors that lead to neuroprotection in some individuals, but not others. The benefits of social support, while commonly reported, remain little understood; however, as will be discussed in detail in chapters 3-5, the differential pathophysiological response to ischemia in socially housed and isolated animals (or socially integrated or isolated patients in the clinical literature) merits further mechanistic research.

1.3 Oxytocin

A rapidly growing collection of studies on oxytocin (OT), a neuropeptide that is released during social behaviors, shed light on one such potential mechanism. OT is a nine amino acid peptide that is structurally similar to arginine vasopressin (the structures differ in two of nine amino acid residues Gainer and Wray, 1992). OT is produced in high concentrations in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, which in turn project to the posterior pituitary, where OT is released to its central and peripheral targets (Gainer and Wray, 1992).

The biological activity of OT is mediated by an oxytocin receptor (OTR) and to a lesser extent, three vasopressin receptors. The OTR, a G protein-coupled receptor, is abundantly present in several regions, both in the brain and periphery. OTR expression in the brain is nearly ubiquitous, however, several structures are particularly important for the onset and maintenance of the behavioral effects of OT. Specifically, OT receptors are abundant in the hippocampus, lateral septum, olfactory bulbs and amygdala (Gimpl and Fahrenholz, 2001), and OT binding at these sites facilitates the onset and maintenance of social behaviors (reviewed in Winslow and Insel, 2002). The peripheral actions of OT, including cardiovascular regulation and uterine contractions during labor, are mediated in part by OTRs on the heart, myometrium and uterus; OTRs are also present on the kidney, adrenal medulla, thymus, pancreas, and fat cells (Gimpl and Fahrenholz, 2001).

Oxytocin regulation of social behaviors

Numerous studies have implicated a role for OT in mediating social behaviors. Indeed, central release of OT regulates a wide range of social behaviors, including pair-bonding, mother-infant bonding, social recognition, and aggression (Carter, 2003; Hammock and Young, 2006; Wang et al., 1996). However, the effects of OT are to some degree species-specific and depend on the social structure of the species. There are distinct differences in OTR distribution and binding patterns between some socially monogamous and polygynous vole species (Insel and Shapiro, 1992; Young, 1999). This

difference in receptor distribution speaks to the role of receptor-mediated OT action on social behaviors such as pair bonding which is observed in monogamous, but not polygynous voles (Young and Wang, 2004). Central infusion of OT facilitates the onset of social behaviors (Pedersen and Prange Jr, 1979; Witt et al., 1990; Witt et al., 1992), while an OT receptor antagonist (OTA) nearly completely eliminates them (Young et al., 2001). OT also suppresses the HPA axis in several species (reviewed in DeVries et al., 2007), which further facilitates aspects of social behavior such as social recognition and formation of pair-bonds (reviewed in DeVries et al., 2003). Important converging evidence from OT knockout (OTKO) studies further implicates a role for this neuropeptide in maintaining social behaviors. Specifically, OTKO mice fail to develop social memory (Crawley et al., 2007; Ferguson et al., 2001; Ferguson et al., 2000; Winslow and Insel, 2002). When assessed for social approach behaviors during repeated pairings with a conspecific, OTKOs exhibit decreased olfactory investigation and approach behaviors toward the stimulus mouse (Ferguson et al., 2000). Similar results have been achieved with OTA administration to wildtype mice, and social amnesia in OTKOs is reversed with central administration of exogenous OT (Ferguson et al., 2001).

The role of OT as a mediator of social behaviors is also evident in clinical studies. Exogenous OT administration has been shown to increase pro-social behaviors in humans, including the ability to interpret emotions of others (Domes et al., 2007), interpersonal communication, social approach behavior (Shamay-Tsoory et al., 2009)

and trust (Kosfeld et al., 2005). In contrast, abnormalities in social processing and behaviors have led to a recent surge in identifying a potential modulatory role for OT in autism, social phobia and borderline personality disorder (reviewed in Bartz and Hollander, 2006). Anxiolytic properties of OT are also evident in clinical studies. Indeed, a particularly robust finding in the clinical literature is the relationship between endogenous OT (which is elevated during lactation) and stress hyporesponsiveness. Lactating and nursing women have attenuated stress responses (Heinrichs et al., 2001) and lower blood pressure (Jonas et al., 2008) compared to non-lactating controls. Moreover, the stress buffering effects can be mimicked with exogenous OT administration (Heinrichs et al., 2003). Taken together, the role of OT in meditating social behaviors makes it an attractive potential mediator of social influences on pathophysiology.

Oxytocin as a mediator of environment-biology interaction

Recent research developments suggest that there may be clear physiological benefits to engaging in social behavior: protection from wounds, illness, and the long-term effects of repeated stress. Indeed, such benefits extend beyond pair bonding species to those that are polygynous but highly social. For example, pair-housing facilitates wound healing in hamsters, an effect that requires central OT signaling (Detillion et al., 2004). Indeed, socially isolated hamsters treated with an OT agonist displayed similar wounds and healing time as pair-housed hamsters. However,

treatment of pair-housed hamsters with an OT receptor antagonist (OTA) increased wound size and delayed healing (Detillion et al., 2004). In addition to wound healing, OT is involved in pain modulation, and has been shown to have potent anti-nociceptive effects (Martínez-Lorenzana et al., 2008). Indeed, reduced allodynia (pain in response to a stimulus that does not typically evoke pain) in pair-housed mice is also mediated by central OT signaling (Norman et al., 2010). The critical finding in both of these studies is that social facilitation of pain and healing is blocked by OTA, indicating a receptor-dependent mechanism for this environment-physiology interaction.

Following a stroke, the neuroinflammatory response serves the important function of clearing debris, producing neurotrophins and growth hormones, and promoting angiogenesis. However, it is apparent that a dysregulated, overactive immune response also significantly contributes to neuronal damage. Therefore, a goal of stroke treatment should be aimed at identifying manipulations that reduce the inflammatory response and maintain it at therapeutic levels. Recent research suggests that OT has both anti-inflammatory and antioxidant properties. OT administration alleviates tissue damage in a variety of animal models of injury including renal (Tugtepe et al., 2007) and hepatic (Dusunceli et al., 2008) ischemia/reperfusion injury, as well as sepsis-induced multiple organ damage (Iseri et al., 2005b), skin injury (Iseri et al., 2008) and colitis (Iseri et al., 2005a). The protective actions of OT in these models are primarily anti-inflammatory, resulting in decreased levels of TNFα and IL-6 (Dusunceli et al., 2008; Tugtepe et al., 2007) as well as decreased neutrophil infiltration to the site of

injury (Dusunceli et al., 2008; Iseri et al., 2005a; Iseri et al., 2005b; Tugtepe et al., 2007). Additionally, increased OT is correlated with an up-regulation of nerve growth factor (Luppi et al., 1993) as well as insulin-like growth factor-1 (Petersson et al., 1998), which are neuroprotective in ischemic injury (Lee et al., 1998; Liu et al., 2004).

In addition, several peptide hormones, including OT, have recently been shown to have antioxidant properties (Moosmann and Behl, 2002). Subcutaneous OT treatment increases peripheral antioxidant content and alleviates oxidative stress in a number of disease and injury models (Iseri et al., 2008; Iseri et al., 2005a; Iseri et al., 2005b; Tugtepe et al., 2007). Indeed, OT scavenges peroxinitrite, prevents oxidation of low-density lipoprotein and inhibits lipid peroxidation (Moosmann and Behl, 2002). Taken together, OT represents a neuroendocrine mediator ideally suited to coordinate environmental inputs (i.e. upregulation of OT during social or stressful interactions) with physiology (i.e. inflammation). To date, there are no known studies on the role of OT as a mediator of social neuroprotection in a stroke model.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Animals

Adult male C57/BL6 mice (Charles River, Wilmington, MA) were maintained on a 14:10 light/dark cycle in a temperature and humidity-controlled vivarium. All animals were allowed *ad libitum* access to food and water. Experimental animals were either housed singly (isolated) or paired (socially housed) with an ovariectomized female. All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by the institutional animal care and use committee.

Stroke Surgery

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). The mice were anesthetized with 1.5% isofluorane in oxygen-enriched air provided though a face mask. Body temperature was maintained at 37 ± 0.5°C through the use of a homeothermic blanket system. Briefly, unilateral right MCAO was achieved by insertion of a 6-0 nylon monofilament into the internal carotid artery

to a point 6mm beyond the internal carotid-pterygopalatine artery bifurcation. Once the occluder was secured, the wound was sutured and the animal was allowed to awaken from anesthesia. After 60 minutes of occlusion, the animal was re-anesthetized and reperfusion was initiated by removal of the occluder. For SHAM surgery, the internal carotid artery was exposed, but not disturbed, all other aspects of the surgery remained the same. Sixty minutes following MCAO surgery, a neurological score was assigned to each animal as previously described (Hattori et al., 2000). This model of cerebral ischemia allows long-term survival lasting months following acute stroke (i.e. Bouët et al., 2007; Yano et al., 2005).

Ovariectomy Surgery

In all experiments described in chapters 3-5, mice assigned to the social housing condition were housed with an ovariectomized female. The rationale for choosing ovariectomized females as the social stimulus was three-fold. First, when adult males are forced to cohabitate under laborartory conditions, they exhibit aggression, territoriality, and intolerance against same-sex conspecifics (Van Loo et al., 2003). Second, ovariectomy eliminates the confound of estrous state influencing male behavior (Ingersoll and Weinhold, 1987). Finally, males were paired rather than group-housed in order to eliminate the consequences of complex social structures such as hierarchy formation, particularly because hierarchy status is known to influence behavior (Ferrari et al., 1998) as well as susceptibility to disease (Sapolsky, 2004). The surgical protocol for ovariectomy is as follows: stimulus females were anesthetized with 1.5% isofluorane

in oxygen-enriched air provided through a face mask. The females were placed on their ventrum, and a 2 cm^2 patch of fur shaved, cleaned with iodine, and one mid-line dorsal incision was made through the skin. The surgical opening was shifted to the one side and an incision made through the muscle layer. The ovarian fat pad and fallopian tube were extended through the incision, clamped, and cauterized. The ovary was removed, and the site was sutured with sterile 00 monofilament suture. The second ovary was removed using the same procedure. The skin incision was then closed using sterile suture clips. Mice were allowed 10 days of recovery prior to pairing with an experimental male.

Intracerebroventricular Cannulation

The mice were anesthetized with 1%-1.5% isofluorane in oxygen-enriched air and were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An incision was made along the midline to locate bregma. A guide cannula, aimed at the left lateral ventricle, was implanted 1 week before experimental stroke surgery. The cannula (2.75mm below the pedestal, Plastics One, Roanoke, VA) was positioned at +0.02mm posterior and +0.95mm lateral to bregma, and secured with surgical glue. Once the glue was dry, a dummy cannula was inserted into the guide cannula, and the mice were replaced into their home cages for recovery.

Determination of Post-stroke Edema

Brain tissue was collected at 48 hours of reperfusion immediately following transcardial perfusion with 20mL of 0.9% saline. The brain tissue was divided into ipsilateral (right) and contralateral (left) hemisphere and an initial wet weight was obtained (W_R and W_L respectively). The tissue was then dehydrated in an oven maintained at 70˚C, and dry weights of right and left hemispheres (D_R and D_L respectively) were obtained at 24-hour intervals until two consecutive weights yielded the same mass (Tanaka et al., 1997). An index of edema was calculated using the formula $E = [(W_R/D_R – W_L/D_L)/(W_L/D_L)] X 100$.

Determination of Stroke Volume

Immediately following cervical dislocation and decapitation, fresh brains were removed, sectioned into five 2-mm-thick coronal sections and incubated for 15-minutes with 2,3,5-triphenyltetrazolium (TTC) at 37°C, which stains live mitochondria. Slices were post-fixed with 10% buffered formalin for 3-5 days before image analysis, at which point the slices were photographed and infarct area throughout the cerebrum was analyzed using Inquiry software (Loats Associates, Inc. Westminster, MD). Infarct size was determined as a percentage of the contralateral hemisphere after correcting for edema, using the following formula: [1-(total ipsilateral hemisphere- infarct)]/total contralateral hemisphere] X100.

Histochemistry

The TTC-stained sections were stored in 10% formalin for an additional 10 days. The sections were then embedded in paraffin blocks and further sectioned on a microtome at a thickness of 5µm and mounted on slides. The product of the TTC stain, red formazan, was dissolved during the embedding process allowing the tissue to be used for additional stains (Seidler, 1980). Serial sections were used for additional stains.

Astrocytes

Slides were deparafinized, rinsed in distilled water, quenched in H_2O_2, and then blocked with goat serum. Slides were then incubated for 24 hours at room temperature with antibodies to GFAP (1:500, Dako, Carpinteria, CA) in phosphate buffered saline containing 0.3% Triton-X and goat serum. Slides were then rinsed and incubated with anti-rabbit secondary antibody (1:500, Vector Labs, Burlingame, CA) for 2 hours. Sections were then treated with Elite ABC reagent and then visualized with DAB containing nickel (Vector Labs, Burlingame, CA). Slides were photographed and the glial scar area was outlined using ImageJ (NIH) according to the morphology of the GFAP-positive cells which clearly delineated the glial scar.

Microglia

The procedure for microglial analysis was similar, except tissue was blocked with bovine albumin serum and incubated for 4 hours in biotynilated isolectin B4, a lectin from Griffonia (Bandeiraea) simplicifolia (1:75, Vector Labs, Burlingame, CA), rinsed,

then treated with Elite ABC and visualized with DAB containing nickel. Photographs of the striatum and overlying cortex were taken with a Nikon E800 microscope at a magnification of 20x. Images were digitized and proportional stained areas were assessed using ImageJ (NIH). Briefly, fixed size rectangular boxes were superimposed over the images and the proportion of stained area within the defined region was recorded.

Serum Corticosterone Radioimmunoassay

Trunk blood samples were collected immediately following rapid cervical dislocation and decapitation. The samples were centrifuged at 6,000 rpm for 30 minutes at 4°C; sera were collected and stored at -80°C until assayed. Corticosterone (CORT) concentrations were determined by using an I^{125} corticosterone kit (MP Biomedical, Solon, OH). The standard curve was run in triplicate and samples were run in duplicate. All samples within an experiment were run in a single assay.

IL-6 ELISA

Following MCAO, bilateral samples from the cortex and striatum as well as blood serum were collected for analysis of central and peripheral IL-6 protein expression. For protein extraction, brain tissue was homogenized in RIPA buffer with protease inhibitors (Pierce, Rockford, IL). Brain tissue lysates and serum samples were diluted 1:5 and assayed using a sandwich ELISA kit (BD Biosciences, San Jose, CA) according to manufacturer's protocol.

Real-Time PCR

Bilateral samples were dissected from the cortex and striatum, and total RNA was extracted using a homogenizer (Ultra-Turrax T8, IKA Works, Wilmington, NC) and an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's protocol. Extracted RNA was suspended in 30µL of RNase-free water and RNA concentration was determined by a spectrophotometer (NanoDrop ND-1000, Wilmington, DE). A TaqMan 18S rRNA primer and probe set (labeled with VIC dye: Applied Biosystems, Foster City, CA) were used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Sequencing Detection System by using Taqman Universal PCR master mix. The universal two-step RT-PCR cycling conditions used were: 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min.

Oxidative Stress Assays

Whole hemispheres were homogenized in cold 20mM Tris-buffered saline and centrifuged at 8500 x g at 4°C for 10 minutes. Supernatants were collected, aliquoted, and stored at -80°C until use for determination of antioxidant enzyme activity. GPx activity was measured using a commercial kit (Calbiochem, San Diego, CA) according to manufacturer's protocol. All samples were run in duplicate in a single assay. One unit of GPx is defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH per minute. Data are presented as unit per mg protein, as measured by the Bio-Rad protein assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA).

Oxidative stress was measured as a ratio of reduced (GSH) to oxidized (GSSG) glutathione, using a commercial kit (Oxford Biomedical Research, Oxford, MI). Glutathione peroxide reduces hydrogen peroxide and lipid hydroperoxides to water and oxygen, during which time reduced glutathione (GSH) becomes oxidized glutathione (GSSG). Exposure to oxidative stress decreases the GSH/GSSG ratio due to increasing accumulation of GSSG, thus the GSH/GSSG ratio is a common and useful indicator of oxidative stress (Warner et al., 2004). Samples were prepared according to assay instructions with slight modifications. Briefly, 200µL of tissue homogenates were diluted with 200µL of cold assay buffer and 5% metaphosphoric acid, centrifuged and new supernatants collected. The GSSG sample was further added to 15µL of 2-vinylpyridine prior to the assay. All samples were run in duplicate in a single assay.

Behavioral Assays

Animals underwent paw preference and open field behavioral testing. Both tests were conducted at 24 hours prior to MCAO for baseline analysis, and again after 36-72 hours of reperfusion. Behavioral testing took place between 10:00 -13:00 hours, during the animals' light cycle, and scored by an individual who was not aware of group assignment. The apparatuses were thoroughly cleaned between animals using a 70% alcohol solution.

Cylinder Test

Each animal was placed individually inside a clear plastic cylinder (8cm internal diameter, 12cm height) for 5 minutes and videotaped simultaneously from four angles.

Contralateral paw use (paw preference) was recorded as whether the left (contralateral) or right (ipsilateral) paw was first to contact the cylinder during rearing. Only the initial paw placement for each rear was recorded. If both paws were placed on the cylinder in such rapid succession that slow motion analysis could not determine which paw was placed first, then the placement was scored as being "simultaneous". An index of contralateral paw preference was determined using the following formula: [left/(left + right + simultaneous)]X100.

Open Field

Exploratory behavior was assessed in an open field apparatus using Flex Field photobeam activity (San Diego Instruments, San Diego, California). The apparatus was enclosed in a sound attenuating chamber equipped with a ventilating fan which provided masking noise. A clear Plexiglas insert (40 X 40 X 37.5 cm) was fitted inside a metal frame consisting of 16 equally spaced infrared photocell detectors. Interruptions in the infrared light sources by the experimental animal were recorded in the associated computer program. Animals were individually placed inside the apparatus for 60-minute sessions and data were analyzed to determine general locomotor activity, and relative amount of activity occurring in the periphery versus the center of the apparatus.

CHAPTER 3

SOCIAL CONTACT INFLUENCES HISTOLOGICAL AND BEHAVIORAL OUTCOMES FOLLOWING CEREBRAL ISCHEMIA

Traditional risk factors for stroke include elevated cholesterol and blood pressure, smoking, and prior transient ischemic attack or stroke occurrence (Rosamond et al., 2007); however, more recently, environmental influences such as stress and psychosocial factors are being recognized for predicting stroke outcome, as well as, or better than traditional risk factors (Harmsen et al., 2006; Surtees et al., 2007). Further, social isolation exacerbates ischemic damage in both global and focal rodent models of ischemia (Craft et al., 2005; Karelina et al., 2009b; Weil et al., 2008a; Woodlee and Schallert, 2006). The mechanism by which social interaction influences ischemic outcomes is multifaceted and likely involves altered neuroendocrine (DeVries et al., 2007), inflammatory (Karelina et al., 2009b; Mulcahy et al., 2003; Weil et al., 2008a), and synaptic plasticity (Silasi et al., 2008) factors. Importantly, social housing conditions (isolation vs. paired or grouped housing) have additional wide ranging influences on the behavior and physiology of rodents, including changes in anxiety and aggression (Olsson and Westlund, 2007; Pietropaolo et al., 2008), altered metabolism (Bartolomucci et al., 2009), heart rate, and core body temperature (Rettich et al., 2006; Spani et al., 2003).

These factors, in turn, can have a dramatic influence on stroke outcome. Indeed, cerebral ischemia-induced behavioral and affective outcomes correlate with measures of ischemic injury and functional deficits (DeVries et al., 2001b). Further, heart function and body temperature are critical determinants of the severity of neuronal damage and functional recovery following cerebral ischemia (Barber et al., 2004; Butcher et al., 1993; Corbett and Thornhill, 2000; Kollmar et al., 2007; Phanithi et al., 2000). Thus, in order to elucidate the mechanisms through which social housing impacts cerebral ischemia outcome, it is important to determine the contribution of the specific behavioral and physiological components associated with social housing (or isolation) in rodents.

Social interaction is not only an alteration of the social environment but also introduces a number of complex auditory, olfactory, visual, and physical stimuli. Identifying the individual contribution (as well as interaction) of these components to health outcomes will provide further insights into the mechanisms by which social interactions influence disease pathophysiology and recovery. For example, social housing differences in ischemia outcome may be explained by findings that social interactions influence body temperature (T_b) by passive transfer of body heat. The effects of T_b on cerebral ischemia outcome are well known (Caso et al., 2007; Corbett and Thornhill, 2000; Hawthorne, 2008; Phanithi et al., 2000), but to our knowledge no studies have assessed social housing effects on spontaneous changes in T_b following cerebral ischemia. Social interaction also may influence locomotor activity. Indeed, social isolation-induced hyperactivity and reduced habituation (Pietropaolo et al., 2008) is well described and is often associated with an increase in stress/anxiety-like behavior (Weiss et al., 2004),

which is a factor well known to exacerbate stroke outcome (Caso et al., 2007; DeVries et al., 2001a).

We have previously reported reduced neuronal damage in paired, relative to socially isolated, animals (see Chapter 5; Craft et al., 2005; Karelina et al., 2009b). The social housing differences in ischemic damage and functional outcome are accompanied by altered inflammatory and neuroendocrine measures, and have been attributed to the beneficial effects of psychosocial interaction. The focus of the current study is on the role of one aspect of social interaction, namely physical contact, and its effect on core body temperature regulation, locomotor activity, and infarct size in a model of cerebral ischemia. Through the use of standard and partition housing, we assessed the extent to which physical contact prior to and following cerebral ischemia is necessary for establishing and maintaining social housing differences in histological and behavioral measures of stroke outcome.

Materials and Methods

Animals

Adult male C57/BL6 mice (60-80 days old; 23-28g; Charles River, Wilmington, Mass) were housed either individually (socially isolated) or with an ovariectomized female (paired) in standard mouse cages (31.8 x 17.1 x 14 cm) or grid partitioned cages. The cages with grid partitions were divided into two separate compartments (24.9 x 23.5 x 15.2 cm and 19.8 x 23.5 x 15.2 cm), and experimental animals housed in

partitioned cages were always housed in the larger compartment (housing assignment and schedule described in Figure 3.1). The study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by the institutional animal care and use committee.

Surgery

All experimental mice were implanted intraperitoneally with a radiotelemetric transmitter (PDT-4000; Minimitter, Bend, OR) under 1.5% Isofluorane anesthesia and allowed one week to recover prior to initiation of data collection. The home cages were placed on receivers and connected to a personal computer which provided temperature and locomotor activity output collected in 30 minute intervals (VitalView ® software). Emitted temperature frequencies were converted to temperatures based on preprogrammed calibration curves from each transmitter. Radiotelemetry recordings of body temperature (T_b) and locomotor activity were collected beginning 24 hours before and continued through 72 hours following MCAO or SHAM surgery.

Experiment 1

Mice were randomly assigned to one of four experimental groups: Isolated-MCAO, n = 5; Paired-MCAO, n = 5; Isolated-SHAM, n = 5; Paired-SHAM, n=5. Mice were housed in standard mouse cages either individually (isolated) or with an ovariectomized female (paired) starting 2 weeks prior to MCAO or SHAM surgery and throughout reperfusion. Collection of core T_b and locomotor activity began 24 hours prior to

surgery and continued throughout 72 hours of reperfusion. Tissue was collected for infarct volume assessment at 72 hours of reperfusion.

Experiment 2

Experiment 2 was designed to control for the element of physical contact during pair housing, this experiment included the use of standard rat cages fitted with a grid partition in the middle that allowed the experimental mouse to see, hear and smell its partner, but not engage in physical contact. In order to assess the role of peri-ischemic physical contact on T_b, locomotor activity and infarct volume, mice were assigned to partition housing either throughout the experiment or starting two days prior to surgery (see Figure 3.1 for schematic and group assignment details). All mice in Experiment 2 underwent MCAO surgery, and were randomly assigned to one of three experimental groups: partition housed throughout the experiment (Partition-Housed, n = 6); paired in standard cages, then transferred to partition housing 48 hours prior to MCAO (Paired → Partition, n = 6); or socially isolated in standard cages, then transferred to partition housing 48 hours prior to MCAO (Isolated → Partition, n = 5) as a control group for any effects as a consequence of transfer to partitioned cages.

In order to control for the possibility that transfer into a new cage selectively introduced a stressor to the mice in Experiment 2, all mice in Experiment 1 were transferred into new standard cages 48 hours prior to MCAO or SHAM surgery.

Statistical Analysis

Results for body weight and neurological score were analyzed via a one-way ANOVA (factor was group). Infarct volume was analyzed via an independent samples t-test for Experiment 1 and a one-way ANOVA for Experiment 2 (factor was housing condition). Locomotor activity and T_b data were collapsed into 6 hour bins as well as across the light/dark phase of the light cycle and assessed using a 2-way ANOVA for Experiment 1 (factors were surgery and housing) and a one-way ANOVA for Experiment 2 (factor was housing condition). Rhythmicity measures were derived by a Fast Fourier transform on the 72 hours of post surgical activity and T_b data. Spectral power was then integrated over bands encompassing 0-20 hours, 20-28 hours and 28 – 72 hours to determine potential differences in the periodicity of activity and T_b. Between-groups differences in periodicity of activity and T_b were then compared using a 2-way ANOVA for Experiment 1 (factors were surgery and housing) and a one-way ANOVA for Experiment 2 (factor was housing condition). All significant ANOVA results (P<0.05) were followed by a Tukey HSD post hoc test.

Results

Infarct volume and surgical parameters

Housing condition significantly impacted infarct volume. In Experiment 1, pair-housing significantly decreased infarct volume relative to social isolation ($t_9 = 2.033$, $P < 0.05$; Figure 3.2A). In Experiment 2, there was also a significant effect of housing condition on infarct volume ($F_{2,17} = 3.718$, $p < 0.05$, Figure 3.2B). A Tukey post-hoc analysis confirmed that Paired → Partition mice significantly decreased infarct volume

relative to Isolated → Partition mice ($p < 0.05$). However, Partition-Housed mice had infarct volumes that did not differ significantly from the other groups in Experiment 2. Statistical analysis revealed that observed power for infarct analysis was 0.59 in Experiment 1, and 0.63 in Experiment 2.

In Experiment 1, there were no group differences in body mass on the day of surgery ($F_{3,19} = 1.023$, $P > 0.05$) or body temperature during surgery ($F_{3,19} = 1.355$, $P > 0.05$). Among MCAO mice in Experiment 1, there were no housing differences in neurological score ($F_{1,9} = 0.000$, $P > 0.05$). In Experiment 2, there were no group differences in body mass on the day of surgery ($F_{2,16} = 0.075$, $P > 0.05$), body temperature during surgery ($F_{2,16} = 0.726$, $P > 0.05$), or neurological score ($F_{2,16} = 1.571$, $P > 0.05$).

Among MCAO animals, the average survival rate to reperfusion day 3 was 67%, there were no group differences in survival ($P > 0.05$).

Locomotor Activity

In Experiment 1, there were no group differences in locomotor activity during the 24 hours prior to surgery. Following surgery, there were significant group differences in locomotor activity during the dark (active) phase of day 1 ($F_{3,19} = 5.488$, $P < 0.01$) and day 2 ($F_{3,19} = 10.395$, $P < 0.01$) of reperfusion (Figure 3.3A). A Tukey post-hoc analysis revealed that following MCAO, socially isolated mice significantly reduced locomotor activity relative to SHAM mice ($P < 0.05$), however, pair-housed mice that

underwent MCAO maintained locomotor activity levels comparable to SHAMs ($P > 0.05$). There was no effect of housing on post-surgical locomotor activity among SHAM mice ($P > 0.05$). Spectral analysis did not indicate a main effect of housing or surgery on the 0-20 hour, 24 hour, or 28-72 hour rhythms of activity (all $P > 0.05$); however, there was a significant interaction of housing X surgery during the 0-20 hour ($F_{1,19} = 4.505$, $P = 0.05$) and the 24 hour($F_{1,19} = 8.113$, $P < 0.05$) rhythms of activity. Activity rhythmicity was disrupted among socially isolated MCAO mice relative to all other groups, while, pair-housed MCAO mice maintained rhythms of activity that did not differ from SHAM groups. Statistical analysis of observed power for locomotor activity during each 24-hour period ranged from 0.51 to 0.99.

In Experiment 2, there was a significant effect of housing condition on locomotor activity in the 24 hours prior to MCAO ($F_{2,16} = 6.151$, $P < 0.05$). Partition-Housed mice had reduced locomotor activity during the dark (active) phase prior to MCAO, compared to other groups (Figure 3.3B). Following MCAO surgery, all mice reduced locomotor activity relative to baseline, however, by day 3 of reperfusion, Paired → Partition mice significantly recovered (increased) some locomotor activity during the dark phase relative to Isolated → Partition ($F_{2,16} = 3.572$, $p = 0.05$). Spectral analysis indicated that 0-20, 24, and 28-72 hour rhythms of locomotor activity were disrupted following MCAO in all mice in Experiment 2, however, there were no group differences ($P > 0.05$). Statistical analysis of observed power for locomotor activity during each 24-hour period ranged from 0.56 to 0.81.

Body Temperature

In Experiment 1, there were no group differences in T_b during the 24 hours prior to surgery. Following surgery, T_b did not vary by housing conditions; however, a significant reduction in T_b in MCAO relative to SHAM mice was evident at each time point throughout the reperfusion period (all $P < 0.01$; Figure 3.4A). Spectral analysis revealed a significant effect of surgery on the 0-20 hour ($F_{1,19} = 4.706$, $P < 0.05$), 24 hour ($F_{1,19} = 18.544$, $P < 0.0001$), and 28-72 hour ($F_{1,19} = 45.181$, $P < 0.0001$) rhythms of T_b. All mice that underwent MCAO surgery exhibited significant post-surgical hypothermia and a disruption T_b rhythmicity relative to SHAMs. There were no housing differences in T_b rhythm. Statistical analysis of observed power for T_b during each 24-hour period ranged from 0.52 to 1.00.

In Experiment 2, there were no group differences in Tb during the 24 hours prior to surgery. Overall, post-surgical T_b did not vary by group in Experiment 2. Spectral analysis indicated that the 0-20, 24, and 28-72 hour rhythms of T_b were significantly disrupted in all mice in Experiment 2; however, there were no group differences ($P > 0.05$).

Discussion

Affiliative social interaction improves cerebral ischemia outcome, however, the contributions of the individual components of social interaction (social and sensory) are unknown. Data from the current study confirm previous reports of a reduction of infarct volume among paired versus isolated mice (Figure 3.2A). Importantly, infarct

volume was also reduced in Paired → Partition compared to Isolated → Partition mice (Figure 3.2B). Because the Paired → Partition group was housed in pairs in standard housing until 48 hours prior to MCAO then separated by a barrier, these data suggest that 12 days of physical contact prior to an ischemic event was sufficient for a neuroprotective effect in the absence of peri-ischemic or post-ischemic contact. On the other hand, Partition-Housed mice, which had never physically interacted, had infarct sizes that were intermediate between the Isolated → Partition and Paired → Partition groups (Figure 3.2B), which lends further support to the hypothesis that physical contact prior to an ischemic event is important for maintaining the neuroprotective effect of social interaction.

Social interaction also influenced locomotor activity among standard-housed MCAO groups. Following MCAO, isolated mice had significantly reduced locomotor activity, as well as a significant disruption of locomotor rhythms (Figure 3.3A). This is consistent with previous findings of ischemia-induced disruptions of daily rhythms of melatonin (Meng et al., 2008) secretion as well as both T_b and activity rhythms (Noppens et al., 2004). The hypothalamus, which contains the brain's master biological clock regulator, the suprachiasmatic nucleus (SCN), was not directly damaged by MCAO in this study, as measured histologically; however, MCAO-induced glutamate excitotoxicity and oxidative stress may have indirectly influenced SCN function, thus contributing to the abolished activity rhythm among socially isolated mice. Additionally, the disruption of locomotor rhythms among socially isolated MCAO mice appears to be driven by a near complete inhibition of locomotor activity during the first 48 hours post-

MCAO, particularly during the dark cycle (active phase). Thus, the possibility remains that the disruption in locomotor activity is a function of reduced motor function, rather than a specific disruption of the daily rhythm. Reduced motor function is often observed following MCAO (Yonemori et al., 1998), as well as traumatic brain injury (Fujimoto et al., 2004), and is associated with the extent of the injury. It remains to be determined whether the reduction in post-ischemic locomotor activity observed in the current study is a function of increased neuronal damage, or whether it is a contributing factor to the developing ischemic injury. Indeed, early recovery of motor function often predicts reduced injury level (Lee et al., 2009; Will et al., 2004) and reduced functional deficits (Zhao et al., 2005); thus, the possibility remains that enhanced locomotor activity in the paired mice conferred neuroprotective effects, possibly by promoting synaptogenesis (Zhao et al., 2005) and neuronal plasticity (Kleim et al., 2003). In the current study, socially isolated mice begin to regain locomotor activity rhythmicity by 72 hours post-MCAO, however it is unknown whether, or when, this group would return to SHAM levels of locomotor activity and rhythm.

Interestingly, locomotor activity levels and rhythmicity in paired MCAO mice were indistinguishable from SHAMs. The recovery of locomotor activity in paired MCAO mice suggests that they may have been influenced by cues from their healthy cagemates, resulting in synchronous activity rhythms in the experimental mice. Indeed, social stimuli are capable of altering daily rhythms of behavior, including locomotor activity (Mistlberger and Skene, 2004). The reduction in infarct volume in paired mice likely also contributed to improved functional recovery in this group, however it is

unlikely that the reduction in damage was alone sufficient for recovery, because while Paired → Partition mice also had smaller infarcts than the Isolated → Partition mice, these two groups did not differ on measures of locomotor activity during the first 48 hours following MCAO (Figure 3.3B). However, by 72 hours, Paired → Partition mice began to show a recovery of locomotor activity, indicating that a delayed entrainment to non-physical sensory cues may also influence locomotor recovery. Overall, the dissociation between infarct volume and the extent of locomotor deficits suggests that while the social condition prior to ischemia is relevant to infarct development, the magnitude of the infarct does not directly predict the development of locomotor deficits. Rather, the social condition immediately following ischemia has a direct effect on the recovery of locomotor activity.

It should be noted that in a recent study (see Chapter 5; Karelina et al., 2009b), we reported no housing differences in post-MCAO locomotor activity. There are several possible explanations for the discrepancy in conclusions drawn about locomotor activity in this recent study and the current study, 1) the animals in the previous study were assessed for exploratory behavior and locomotor activity in an open field apparatus for 60 min rather than continuously in their home cage, 2) for feasibility of analysis, paired animals in the previous study were separated from their cagemates during locomotor activity testing, which was not necessary in the current study because we used telemetry, and 3) the behavioral assessment in the previous study took place during the light phase of the circadian cycle, while in the current study we noted the greatest differences in locomotor activity during the dark phase of the circadian cycle (the

animals' active period). The current study provides a more relevant assessment of behavioral activity following MCAO in the homecage, with high temporal resolution. Activity assessment beyond 72 hours would be necessary to determine if and when locomotor recovery occurs in socially isolated mice.

Finally, all animals that underwent MCAO developed spontaneous hypothermia as well as a disruption of T_b rhythmicity; however, T_b measures were not influenced by social housing condition (Figure 3.4A). Further, physical contact did not influence T_b pre- or post-MCAO, as there were no differences in T_b among groups housed in partition cages (Figure 3.4B). Indeed, T_b measures were similar for MCAO animals in both experiments. These data confirm a previous report of spontaneous hypothermia in mice as a consequence of cerebral ischemia (Noppens et al., 2004). Importantly, T_b measures were unrelated to both infarct volume and locomotor activity following MCAO. Taken together, these data allow us to rule out changes in core T_b as a factor influencing cerebral ischemia outcome under various housing conditions. However, it remains unknown whether social housing influences brain temperature, which is typically lower than T_b (DeBow and Colbourne, 2003). We have previously reported increased brain interleukin-6 (IL-6) mRNA and protein in paired, relative to isolated MCAO mice (Karelina et al., 2009b). Elevated central IL-6 is neuroprotective in stroke (Ali et al., 2000; Loddick et al., 1998), and may influence stroke outcome in part via its pyrogenic properties (Herrmann et al., 2003). Thus, further studies are necessary to determine whether the social housing-induced increase in brain IL-6 reflects changes in post-MCAO brain temperatures.

The mechanism of neuroprotection is unclear in this study. In addition to a reduction in ischemic injury, psychosocial factors have been reported to influence the neuroinflammatory response to cerebral ischemia. For example, social isolation-induced exacerbation of infarct size and cell death following cerebral ischemia (see Chapter 5; Craft et al., 2005; Karelina et al., 2009b; Weil et al., 2008a) is accompanied by a pro-inflammatory profile as measured by both an increase in reactive gliosis and pro-inflammatory cytokine gene expression and a decrease in anti-inflammatory cytokine gene expression at the site of the ischemic injury. Further, social stress exacerbates infarct size (Sugo et al., 2002) in part by suppression of endogenous neuroprotective mechanisms (DeVries et al., 2001a), while environmental enrichment may influence infarct development by mechanisms involving enhanced synaptic plasticity (Silasi et al., 2008) as well as attenuation of astrogliosis (Buchhold et al., 2007). Taken together, these studies suggest that an altered neuroinflammatory response is a critical component of psychosocial influences on the development of ischemic injury, and subsequently on functional outcomes. An important future direction will be to establish a mechanistic link between social interactions and neuroinflammation in ischemia.

In summary, physical contact during social interactions influences stroke outcome. Paired animals had smaller infarct volumes, even if they were physically separated starting 48 hours prior to MCAO. Further, social housing conditions had a profound influence on post-stroke functional recovery, and only paired animals that were in unobstructed physical contact exhibited a recovery of locomotor activity following MCAO. Taken together, these data are an important step toward identifying

the roles of the individual components of social interaction on cerebral ischemia outcome. In particular, the dramatic influence of peri- and post-ischemic physical contact on functional recovery merits further clinical research to determine the influence of physical contact, as a component of social support immediately following ischemic stroke, on patient recovery.

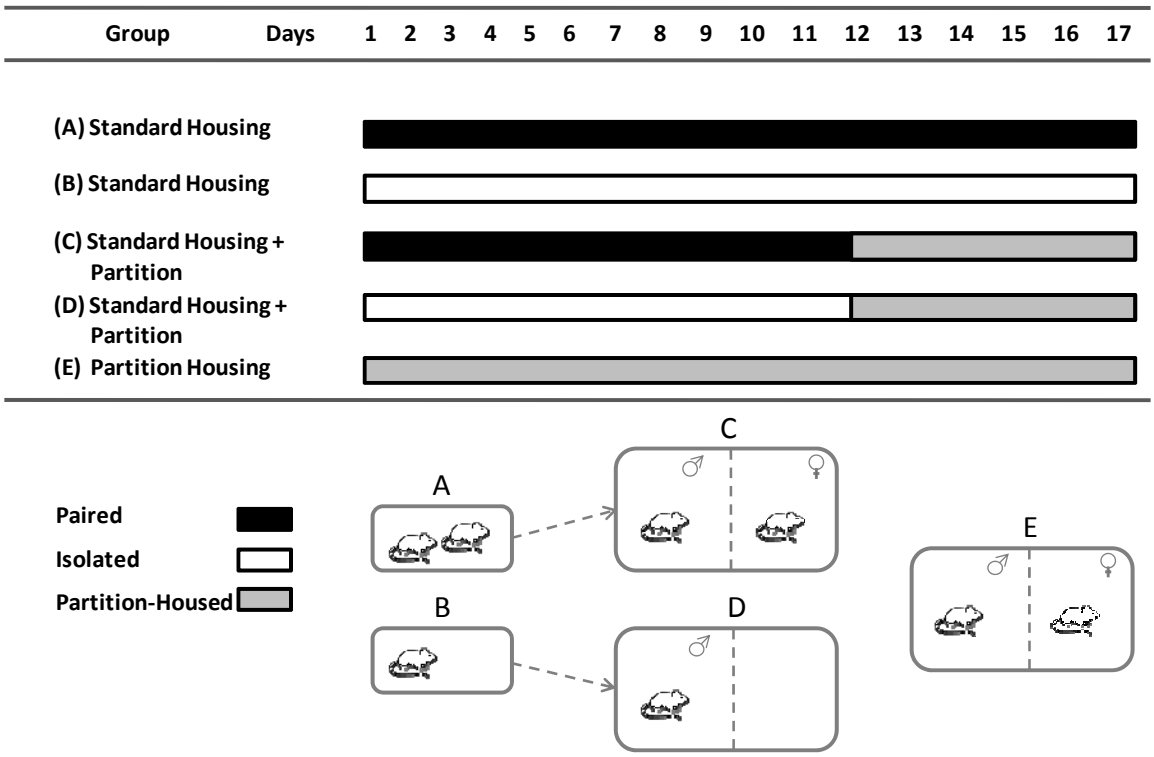


Figure 3.1: Housing condition assignment and schedule.

In groups A and B, mice were maintained in standard mouse cages and were paired with an ovariectomized female (A) or socially isolated (B) for two weeks prior to surgery and throughout the reperfusion period. Mice in groups C and D were maintained in identical housing conditions as A and B respectively, but were transferred to partition housing on day 12 and throughout reperfusion, at which point paired mice (C; Paired → Partition) were separated by the partition and socially isolated mice (D; Isolated → Partition) were housed singly on one side of the barrier to serve as a control for the new cage dimensions. Mice in group E consisted of paired mice partition housed for the duration of the experiment (Partition-Housed). MCAO or SHAM surgery took place on day 14 and body temperature/locomotor activity telemetric recordings were collected starting day 13 (baseline) and continuing until tissue collection on day 17.

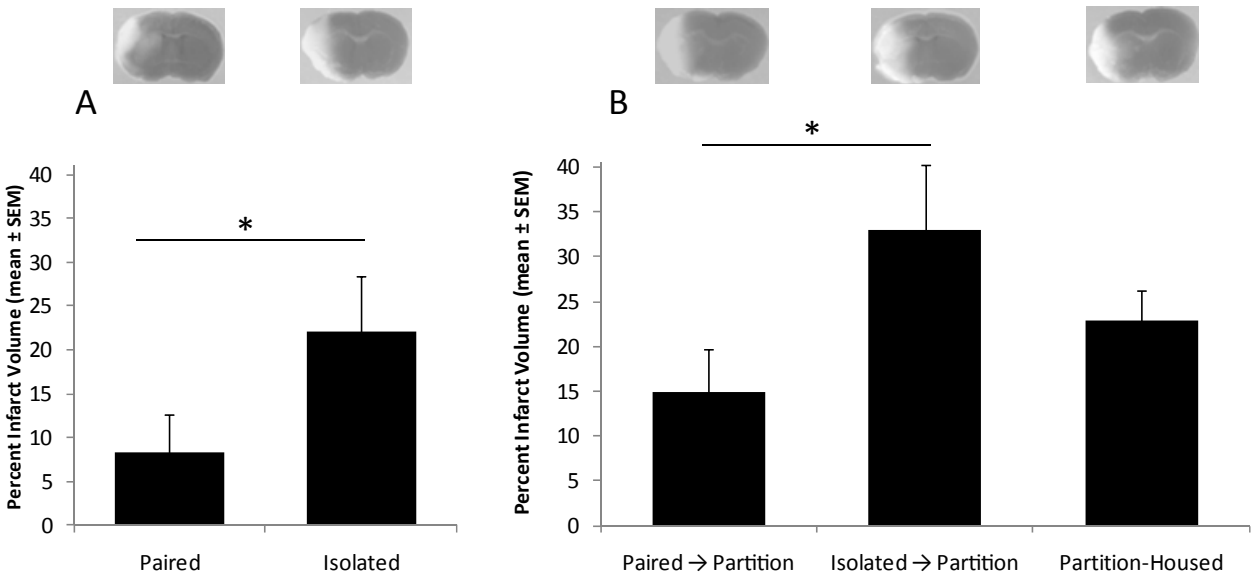


Figure 3.2: Social housing and physical contact influences on infarct size

A) In Experiment 1, pair housing reduced infarct volumes compared to social isolation. B) In Experiment 2, a reduction in infarct volume in paired mice was still evident in Paired → Partition relative to Isolated → Partition mice. Partition-Housed mice had infarcts that did not differ from the other groups. Representative photomicrographs are provided above each group; dashed outlines indicate infarct lesion borders. An asterisk indicates a statistically significant difference at $P < 0.05$.

Figure 3.3: Homecage locomotor activity beginning 24 hours prior to MCAO and continuing throughout 72 hours of reperfusion.

In Experiment 1, baseline locomotor activity did not differ by housing condition, but was significantly reduced following MCAO in socially isolated mice. Paired mice that underwent MCAO maintained locomotor activity levels comparable to SHAMs. An asterisk (*) indicates significantly different from all other groups. B) In Experiment 2, baseline locomotor activity was significantly lower in Partition-Housed mice, however there were no significant group differences in locomotor activity following MCAO until day 3 of reperfusion. An asterisk (*) indicates significantly different from all other groups, a pound sign (#) indicates significantly different from mice that were paired then separated by a partition 48 hours prior to MCAO. Group differences are considered statistically different at $P < 0.05$. Data are collapsed across time and plotted in 6 hour bins. Dashed lines represent time of surgery.

Figure 3.3

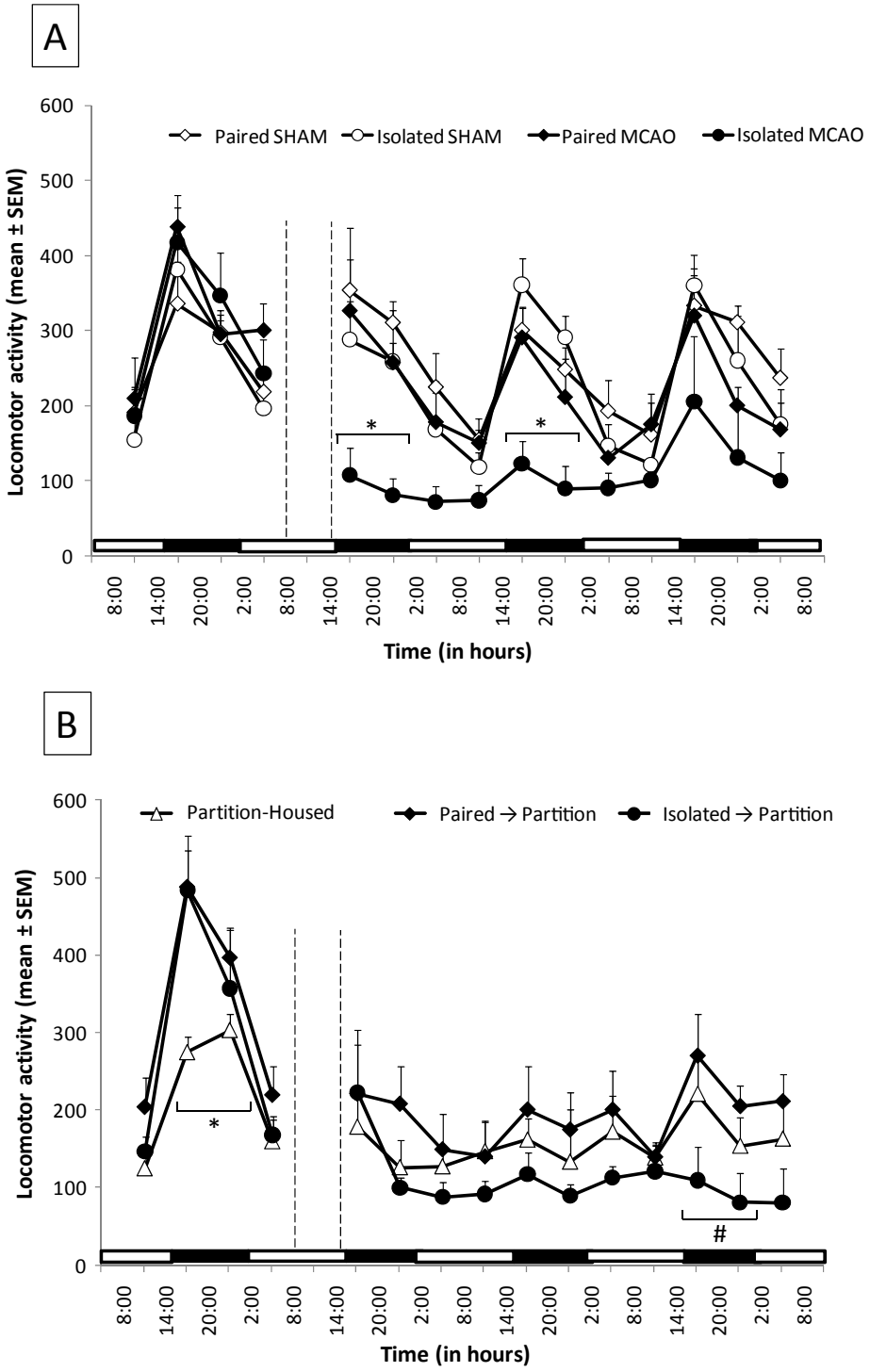
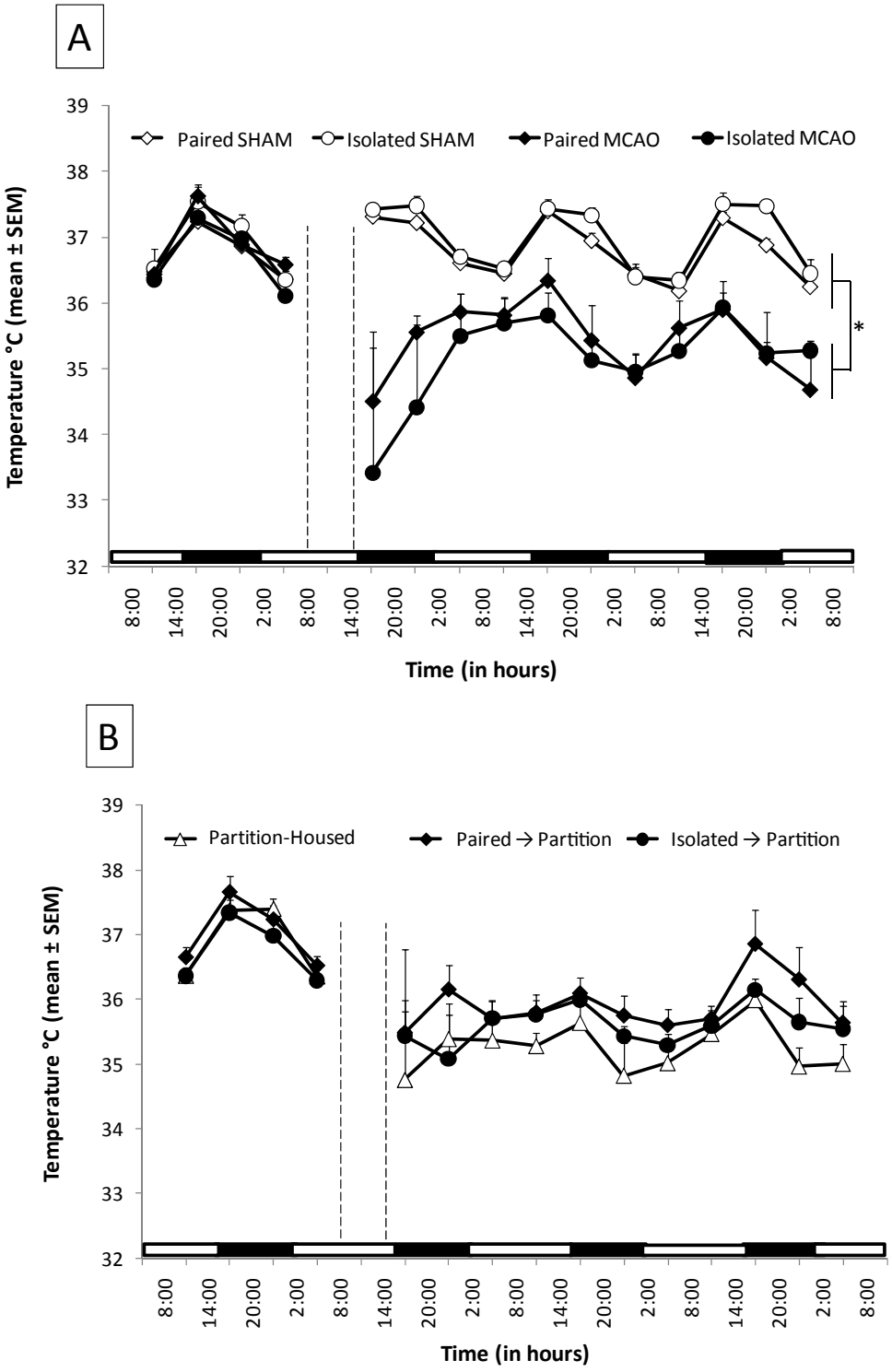

A
Paired SHAM
Isolated SHAM
Paired MCAO
Isolated MCAO
Locomotor activity (mean ± SEM)
600
500
400
300
200
100
0
*
*
8:00
14:00
20:00
2:00
Time (in hours)
B
Partition-Housed
Paired → Partition
Isolated → Partition
Locomotor activity (mean ± SEM)
*
#
Time (in hours)

Figure 3.4: Core body temperature beginning 24 hours prior to MCAO and continuing throughout 72 hours of reperfusion.

A) In Experiment 1, baseline body temperature did not differ by housing condition, but was reduced following MCAO compared to SHAMs. A significant reduction in body temperature following MCAO was evident at every time point throughout 3 days of reperfusion ($P < 0.01$). B) In Experiment 2, body temperature did not differ by housing condition, but was reduced following MCAO ($P > 0.05$). Data are collapsed across time and plotted in 6 hour bins. Dashed lines represent time of surgery.

Figure 3.4


A
Paired SHAM
Isolated SHAM
Paired MCAO
Isolated MCAO
Temperature °C (mean ± SEM)
Time (in hours)
*
B
Partition-Housed
Paired → Partition
Isolated → Partition
Temperature °C (mean ± SEM)
Time (in hours)

CHAPTER 4

SOCIAL ISOLATION ALTERS NEUROINFLAMMATORY RESPONSE TO STROKE

Social interaction is an important modulator of both mental and physical health. Social relationships perceived as being supportive are associated with improved health, whereas perceived social isolation and stressful social interactions can be detrimental to health. Within the clinical literature, low perceived social support and social isolation predict the onset of depression, as well as increased morbidity and mortality from cardiovascular and cerebrovascular disease (Barry et al., 2006; Boden-Albala et al., 2005; Ikeda et al., 2008; Lett et al., 2007). Despite growing evidence implicating limited or negative social interactions as risk factors for cerebrovascular disease, little is known regarding the mechanisms through which psychosocial factors influence stroke pathogenesis. The health benefits of social interaction in humans are typically attributed to improved health behaviors such as decreased smoking, decreased alcohol consumption, better nutrition, or better medical compliance, which in turn improve cerebrovascular health (Cohen and Lemay, 2007). However, both social isolation and perceived lack of social support are predictive of disease outcome independent of

health behaviors (Cacioppo and Hawkley, 2003; Seeman, 2000). Furthermore, the negative effects of social isolation on stroke and cardiac arrest outcome reproduced in mice, and the data suggest that socially isolated and socially housed mice mount a quantitatively different pathophysiological response to ischemic damage (Craft et al., 2005; Weil et al., 2008a).

Inflammatory processes have a fundamental role in the pathophysiology of ischemic injury. Indeed, chronic and acute infection, as well as low-grade systemic inflammation (i.e. elevated serum C-reactive protein; CRP) are predictive of future strokes, as well as death from stroke and cardiac arrest (Everett et al., 2006; Kuo et al., 2005; Ladenvall et al., 2006; Muir et al., 2007; Spencer et al., 2007). CRP is an acute phase protein which increases substantially in response to pro-inflammatory cytokine release, and as such is used clinically as an index of chronic low-grade inflammation (Dziedzic, 2008). Importantly, emerging evidence indicates a relationship between the social environment and systemic inflammation (Cole et al., 2007; McDade et al., 2006), and in otherwise healthy humans, low social integration is associated with increased CRP concentrations (Ford et al., 2006; Loucks et al., 2006). Further, socially isolated mice exhibit increased intra-ischemic serum CRP concentrations relative to socially housed animals following experimental stroke (Craft et al., 2005). Although a direct causative role for CRP on the extent of ischemic injury has not been established, both the clinical (Cole et al., 2007; Everett et al., 2006; Ford et al., 2006; McDade et al., 2006)

and animal (Craft et al., 2005) data provide evidence of a strong correlation between social factors and the inflammatory response typically associated with ischemic injury.

The goal of the current study was to examine the influence of social housing on stroke outcome. Specifically, post-stroke cytokine expression, edema formation and infarct development were compared in socially housed and isolated mice.

Materials and Methods

Animals

Adult male C57/BL6 mice (23-30g; Charles River, Wilmington, Mass) were maintained on a 14:10 light/dark cycle in a temperature and humidity-controlled vivarium. All animals were allowed *ad libitum* access to food and water. Experimental animals were housed either individually (socially isolated) or with an ovariectomized female (socially housed) for a period of 2 weeks prior to surgery and throughout the reperfusion period. The study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by the institutional animal care and use committee.

Experimental Procedures

The influence of social housing on measures of stroke outcome was assessed in separate cohorts of mice at 5 different reperfusion periods. In experiment 1, mice were

assessed for post-stroke behavior, blood corticosterone concentration (CORT), and infarct size at 24 hours (pair-MCAO, n = 10; single-MCAO, n = 10), 72 hours (pair-MCAO, n = 13; single-MCAO, n = 11; pair-SHAM, n=6; single-SHAM, n = 6) or 7 days of reperfusion (pair-MCAO, n = 8; pair-SHAM, n = 10; single-MCAO, n = 4 (6 died prior to sampling); single-SHAM, n = 10). Edema was determined at 48 h, the earliest time point at which secondary damage is observed following MCAO (pair-MCAO, n = 6; single-MCAO, n = 6).

In experiment 2, gene expression of inflammatory markers was measured in the cortex and striatum following stroke. Tissue was collected from separate cohorts of animals at 12 and 24 h of reperfusion (pair-MCAO, n = 6 per time point; single-MCAO, n = 6 per time point). Serum corticosterone levels were also measured in all mice in experiment 2.

Experiment 3 was designed to test the role of central and peripheral levels of IL-6 in mediating the effects of social interaction on stroke outcome. In experiment 3a, blood and tissue were collected at 24 hours of reperfusion (pair-MCAO, n = 6; single-MCAO, n = 6) for protein assay. In experiment 3b, mice were treated intracerebroventricular (ICV) with IL-6 neutralizing antibody (10ng in 2 µL vehicle; R&D Systems, Madison, WI) or vehicle (aCSF) 1 hour prior to MCAO (see Chapter 2, page 31 for cannulation methods). This dose has been used successfully to neutralize IL-6 signaling in mice (Meagher et al., 2007). According to the manufacturer, this dose is within range of the 50% neutralization dose determined in the presence of 0.25 ng/ml

rmIL-6 (R&D Systems anti-mouse IL-6 Ab, AF-406-NA). The solutions were administered over 30sec using a 5µL Hamilton syringe. Correct cannula placement was confirmed through cresyl violet staining. Blood and brain tissue were harvested at 24 hours of reperfusion and assessed for infarct volume and circulating IL-6 protein concentration (pair-MCAO-IL6 antibody, n = 7; pair-MCAO-aCSF, n = 6; single-MCAO-IL6 antibody, n = 7; single-MCAO-aCSF, n = 7). The ELISA assay for IL-6 requires a large amount of blood, among socially housed mice 2 samples from the IL-6 antibody group and 2 samples from socially isolated mice were not sufficiently large to allow the assay.

Real-time PCR

RT-PCR was conducted at 12 and 24 hours of reperfusion following MCAO. Bilateral samples were dissected from the cortex and striatum and RNA extraction and PCR were conducted as described in Chapter 2 (page 35). The following inventoried primers and probes (Applied Biosystems, Foster City, CA) were used: glial fibrillary acidic protein (GFAP), MAC-1, interleukins IL-6 and IL-1β, tumor necrosis factor alpha (TNFα), cyclooxygenase 2 (COX-2), and transforming growth factor beta (TGF-β).

Data Analysis

Results for surgical parameters, survival, infarct volume, edema, corticosterone concentrations, and serum IL-6 protein concentrations were analyzed via a 2-way ANOVA (factors were surgery and housing), a one-tailed *t* test where appropriate

(edema), or using non-parametric statistics (Mann-Whitney U). Gene expression and brain protein expression data were analyzed via 3-way ANOVA (factors were hemisphere, reperfusion period and housing). Further, PCR data were also expressed as a ratio of ipsilateral to contralateral hemisphere (R/L) gene expression and were analyzed via 2-way ANOVA (factors were reperfusion period and housing). Significant ANOVA results were followed by a Tukey HSD post hoc test. Behavior was analyzed by independent 2-way ANOVAs (factors were surgical assignment and housing assignment) at baseline and 72 hrs post-surgery because novelty of the testing environment may have differentially influenced behavior at these two time points; for the purpose of this study, across group comparisons at the post-surgical time point are more informative than within group comparisons between baseline and the post-surgical time point. When the data did not meet assumptions of normality (ex. serum IL-6), a log10 transformation was conducted prior to analysis. Data were considered significant at $P \leq 0.05$, and effect sizes (r for non-parametric and eta squared, η^2, for parametric data) are reported for all relevant data.

Results

Social Interaction Influences Post-Stroke Survival and Ischemic Damage

Housing condition was a strong determinant of post-stroke survival rate. Only 40% of socially isolated mice survived 7 days, compared to 100% of socially housed MCAO mice ($U = 20.00$, $P < 0.05$, $r = 0.63$), which limits interpreting the day 7 infarct and

behavior data as being truly representative of the two experimental groups. However, it is interesting to note that the four surviving mice in the socially isolated group were similar in infarct size and behavior to the socially housed group on post-stroke day 7 (P>0.05; although we caution that this comparison suffers from the statistical limitation inherent in having a small sample size in one of the experimental groups).

To address the issue of differential long-term survival, all remaining measurements were made 24-72 hr after initiation of reperfusion, when survival rates were not statistically different between groups (90% socially isolated, 100% socially housed on day 3; U = 45.00, P > 0.05, r = 0.22). Social isolation exacerbated infarct volume at 24 and 72 hours (24 hours, t_{20} = 1.738, P < 0.05; η^2 = 0.12; 72 hours, t_{20} = 2.568, P < 0.05, η^2 = 0.11 Figure 4.1A). Social isolation also significantly exacerbated cerebral edema 48 hours following MCAO; socially isolated animals experienced a 2-fold increase in edema relative to socially housed animals (t_9 = 1.801, P = 0.05, η^2 = 0.16; Figure 4.1B).

Within the open field, there were no effects of social housing on locomotor activity or exploratory behavior measured 24 hours prior to surgery (all P > 0.05). A two-factor ANOVA (factors were surgery and housing condition) revealed an effect of surgery on rearing behavior 72 hours post-MCAO or SHAM surgery ($F_{1,26}$ = 28.61, P < 0.05). Following MCAO, mice reared significantly less than SHAM, however, there were no effects of housing condition on total locomotor activity or exploratory behavior (all P > 0.05, Figure 4.2). Additionally, there were no social housing differences in open field

central tendency (a measure of anxiety-like behavior) at the pre-surgical or post-surgical time points ($P > 0.05$). Further, during rearing in a cylinder, there was no significant effect of housing condition on contralateral paw use pre- or post-surgery (all $P > 0.05$).

There were no significant housing effects on body mass ($F_{1,52} = 2.189$, $P > 0.05$, $\eta^2 = 0.04$) body temperature during surgery ($F_{1,52} = 0.038$, $P > 0.05$, $\eta^2 = 0.0004$), or neuroscore ($F_{1,52} = 2.901$, $P > 0.05$, $\eta^2 = 0.05$) across or within the experiments.

Social Interaction Alters the Neuroinflammatory Response to Stroke

Post-stroke gene expression of macrophage antigen complex-1 (MAC-1), a pattern recognition complement receptor protein expressed on macrophage-lineage cells, ($F_{1,96} = 5.699$, $P < 0.05$, $\eta^2 = 0.05$) and glial fibrillary acidic protein (GFAP), an intermediate filament protein that is up-regulated in astrocytes following injury, ($F_{1,92} = 5.519$, $P < 0.05$, $\eta^2 = 0.05$) were significantly elevated in the ipsilateral (ischemic) relative to the contralateral (non-ischemic) hemisphere across both time points following MCAO (Figure 4.3). At the 12 hour time point, there was a main effect of housing within the striatum on MAC-1 ($F_{1,10} = 8.709$, $P < 0.05$, $\eta^2 = 0.46$) and GFAP ($F_{1,15} = 6.63$, $P < 0.05$, $\eta^2 = 0.31$) gene expression and a post-hoc analysis revealed that both glial markers were significantly elevated in socially isolated animals relative to socially housed animals ($P < 0.05$; Figure 4.4). Cortical gene expression of MAC-1 ($F_{1,15} = 0.297$, P

> 0.05, $\eta^2 = 0.02$) and GFAP ($F_{1,15} = 2.67$, $P > 0.05$, $\eta^2 = 0.16$) did not vary significantly by housing conditions ($P > 0.05$).

Overall, relative gene expression of pro-inflammatory cytokines interleukin-1 beta (IL-1β; $F_{1,146} = 11.429$, $P < 0.05$, $\eta^2 = .07$), tumor necrosis factor alpha (TNF-α; $F_{1,136} = 30.876$, $P < 0.05$, $\eta^2 = 0.17$), and interleukin-6 (IL-6; $F_{1,127} = 15.180$, $P < 0.05$, $\eta^2 = 0.10$) as well as transforming growth factor beta (TGF-β; $F_{1,128} = 7.886$, $P < 0.05$, $\eta^2 = 0.22$) and cyclooxygenase-2 (COX-2; $F_{1,147} = 7.773$, $P < 0.05$, $\eta^2 = 0.05$) were significantly up-regulated in the ipsilateral (ischemic) hemisphere relative to the contralateral (non-ischemic) hemisphere across both time points (Figure4.5). Post hoc analyses revealed that there were no effects of housing on IL-1β, TNF-α, TGF-β, or COX-2 expression (all $P > 0.05$). However, IL-6 gene expression was significantly lower in socially isolated mice than socially housed mice at 12 hours (striatum, $F_{1,10} = 5.689$, $P < 0.05$, $\eta^2 = 0.36$). Further, brain IL-6 protein expression was significantly lower (cortex: $F_{1,10} = 8.711$, $P < 0.05$, $\eta^2 = 0.49$), while serum IL-6 concentrations were significantly higher, in socially isolated relative to socially housed mice ($F_{1,15} = 9.297$, $P < 0.05$, $\eta^2 = 0.39$; Figure 4.6).

IL-6 Antibody Infusion Eliminates the Influence of Social Interaction on Ischemic Outcome

Treatment with an IL-6 neutralizing antibody significantly increased infarct volume. A two-factor ANOVA revealed main effects of treatment ($F_{1,24} = 16.081$, $P <$

0.05), housing ($F_{1,24}$ = 5.057, P < 0.05), and a treatment by housing interaction ($F_{1,24}$ = 7.315, P < 0.05) on infarct volume (η^2 = 0.32). Among vehicle (artificial cerebrospinal fluid) treated mice, a Tukey post-hoc analysis revealed that infarct volume was significantly larger in socially isolated than socially housed mice (P < 0.05), but the infarct size was equivalent between animals in both housing conditions that received IL-6 antibody treatment.

Further, across both treatment conditions, a two-factor ANOVA revealed a main effect of housing on serum concentration of IL-6 protein ($F_{1,21}$ = 7.984, P < 0.05; η^2 = 0.28). A Tukey post-hoc revealed that socially isolated mice had significantly higher concentrations of circulating IL-6 protein compared to socially housed mice in the vehicle treated group (P < 0.05). However, central administration of the IL-6 neutralizing antibody eliminated the difference in circulating IL-6 between socially housed and isolated mice. Thus, central IL-6 immunoneutralization in turn eliminated social influences on both post-ischemic infarct volume and peripheral IL-6 concentration (Figure 4.7).

Post-stroke Serum Corticosterone Concentrations

A two-factor ANOVA revealed a main effect of reperfusion time on corticosterone concentration (CORT; $F_{1,47}$ = 10.975, P < 0.05; η^2 = 0.18). Corticosterone concentrations were highest at 12 hours and decreased significantly by 24 hours.

Among mice that underwent behavioral testing at the 72 hour reperfusion time point, CORT concentrations were elevated in socially isolated, relative to socially housed mice ($P < 0.05$; Figure 4.8).

Discussion.

Social environment influences immune function and disease outcome (Loucks et al., 2006; McDade et al., 2006). However, the mechanisms underlying the interaction of psychosocial factors and pathophysiology in ischemic injury require clarification. Data from the current study indicate that social housing condition is a strong determinant of the pathophysiology and long-term survival following experimental stroke. The survival rate to 7 days following experimental stroke was 100% for socially housed mice, compared to only 40% of socially isolated mice. The biased distribution in survival may reflect increased damage in socially isolated animals that consequently did not survive to day 7. Indeed, infarct and edema analyses at earlier time points indicate significantly greater ischemic damage in socially isolated mice than socially housed mice (Figure 4.1). These data confirm and extend previous reports that social isolation potentiates the pathophysiological response to ischemia (Craft et al., 2005; Weil et al., 2008a), and suggest that social isolation contributes to early differences in the trajectory of ischemic injury development.

A separate cohort of animals was used to determine whether the increase in infarct size among socially isolated mice was associated with a difference in the neuroinflammatory response to MCAO. The inflammatory response is triggered by activated microglia and astrocytes (i.e. reactive gliosis), as well as an up-regulation of pro-inflammatory cytokine release in response to neuronal damage (Aschner, 1998; Huang et al., 2006; Wang et al., 2007). As expected, there was increased gene expression of MAC-1 and GFAP in the ipsilateral relative to the contralateral hemisphere following MCAO (Figure 4.3). Importantly, within the ipsilateral hemisphere, gene expression of both MAC-1 and GFAP was increased in socially isolated mice relative to socially housed mice (Figure 4.4). These data complement a recent report on social isolation-induced potentiation of neuroinflammatory responses in a model of global cerebral ischemia (Weil et al., 2008a). The functional role of glia in ischemic injury is multifactorial, studies report both neuroprotective and damaging effects of glial products following an ischemic event (Lai and Todd, 2006; Neumann et al., 2006; Neumann et al., 2008; Trendelenburg and Dirnagl, 2005; Watanabe et al., 2000). Although the current study does not indicate a causal relationship between the up-regulated glial markers and infarct volume, there is evidence that inhibition of microglial activation (via administration of minocycline) reduces stroke damage (Nagel et al., 2008). Thus, taken together with increased infarct volume in socially isolated animals, it is possible that the secondary processes triggered by increased glial activation, exacerbate neuronal damage.

We further conducted mRNA gene expression profiles on several genes that are central to the inflammatory responses in cerebral ischemia. Key among these genes are the cytokines IL-1β, TNFα, IL-6, TGF-β and the COX-2 enzyme. These inflammatory mediators are produced and secreted by activated glia within hours of ischemic injury and thus contribute significantly to the extent of neuronal damage following MCAO (Huang et al., 2006; Wang et al., 2007). Our data indicate that gene expression of IL-1β, TNF-α and COX-2 is significantly up-regulated in the ipsilateral relative to the contralateral hemisphere (Figure 4.5), but, contrary to our initial hypothesis, these inflammatory markers do not appear to be influenced by social housing conditions. In contrast, IL-6 signaling is significantly altered by housing conditions; gene expression of striatal IL-6 is decreased in socially isolated, relative to socially housed mice (Figure 4.6A). These data were confirmed through protein analysis, which also indicated a decrease in central IL-6 protein expression in socially isolated mice (Figure 4.6B).

Despite conflicting data on the functional role of IL-6 (Ali et al., 2000; Clark et al., 2000; Loddick et al., 1998; Yamashita et al., 2006), studies demonstrate that central expression of this cytokine plays a critical neuroprotective role during an ischemic event (Ali et al., 2000; Loddick et al., 1998). Intracerebroventricular administration of IL-6 reduces infarct size, possibly through a mechanism involving suppressed excitotoxicity (Ali et al., 2000; Loddick et al., 1998). Likewise, blockade of IL-6 signaling results in increased apoptotic cell death and infarct size, as well as poor neurological outcome (Yamashita et al., 2006). To address a role for central IL-6 as a mediator of the social

housing effects on stroke outcome, mice were treated with an IL-6 neutralizing antibody or vehicle (aCSF) prior to MCAO. Treatment with the IL-6 antibody increased infarct volume in the socially housed group, and eliminated the effect of social housing condition on infarct size (Figure 4.7A). In contrast to reported effects of IL-6 on infarct volume (Loddick et al., 1998), antibody treatment in our study did not affect infarct volume of socially isolated mice. One possible explanation for this is that post-stroke central gene expression and protein concentrations of IL-6 in isolated mice were similar (or even lower) within the ischemic, compared to the non-ischemic hemisphere in our study; however, IL-6 was significantly elevated in the ischemic hemisphere of socially housed mice. Thus, the use of neutralizing antibody may reveal a 'floor effect' whereby IL-6 levels in the socially isolated mice cannot be further reduced. On the other hand, preventing the increase in IL-6 signaling via the neutralizing antibody potentiated infarct development in socially housed mice.

In addition to measuring central IL-6 protein levels, we assessed circulating concentrations of IL-6. Our data indicate that while central IL-6 is down-regulated (Figure 4.6B), peripheral levels of IL-6 protein are up-regulated (Figure 4.6C) in isolated relative to socially housed mice. This is consistent with the clinical literature on serum IL-6 concentration and stroke outcome. Within the clinical literature, elevated peripheral IL-6 is a reliable predictor of stroke occurrence, severity, and mortality (De Simoni et al., 2002; Smith et al., 2004). The relationship between peripheral IL-6 and stroke outcome is indicative of an increased pro-inflammatory state, largely due to IL-6

mediated signaling of acute phase protein induction (i.e. CRP) following stroke (Dziedzic, 2008; Rost et al., 2001). Thus, contrary to its central actions, peripheral IL-6 is pro-inflammatory and is therefore a target of ongoing clinical trials for stroke patients (Shenhar-Tsarfaty et al., 2008). Data from the current study indicate that social housing condition influences both the neuroinflammatory and systemic inflammatory response to stroke. Importantly, both the central and peripheral IL-6 protein expression assays were performed in the same cohort of animals. Taken together, an up-regulation of peripheral IL-6, along with low central IL-6 expression, is consistent with an altered inflammatory state that contributes to poorer ischemic outcome in the socially isolated mice. Further, the increase in serum IL-6 among socially isolated mice is consistent with a previous report of increased intra-ischemic serum CRP concentrations in isolated relative to socially housed mice (Craft et al., 2005). Additionally, ICV treatment with the IL-6 antibody eliminated this group difference in serum IL-6 concentrations (Figure 4.7B). An increase in serum IL-6 likely reflects an increase in the systemic inflammatory response to the substantial increase in infarct volume that occurred following treatment with the IL-6 antibody. In the current study, serum IL-6 concentrations are related to infarct size and do not appear to be independently modulated by social interaction in the post-ischemic period.

Another physiological system known to contribute to the extent of ischemic injury is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis functions in part to coordinate the body's physiological response to stressors by regulating glucocorticoid

release (Sorrells and Sapolsky, 2007). CORT plays an important modulatory role in ischemic cell death (Caso et al., 2007; DeVries et al., 2001a; Sugo et al., 2002). Following restraint stress, elevated post-ischemic serum CORT concentrations influence infarct size and functional outcome (DeVries et al., 2001a); in humans, post-stroke cortisol concentration predicts mortality (Marklund et al., 2004). Because social isolation is a stressor among several species, including *mus*, (Bartolomucci, 2007; DeVries et al., 2007), and is often associated with altered HPA axis responsivity (Serra et al., 2005), circulating CORT was measured in the current study at 12hr-72hr following experimental stroke. CORT concentrations were similar between socially housed and socially isolated mice at early time points, despite a housing difference in infarct size in the 24hr cohort (Figure 4.8). Although the data from the current study do not support a role for CORT underlying housing effects on infarct size, it remains possible that there may have been group differences in CORT concentration at earlier time points, or that the stress of social isolation may be influencing IL-6 and infarct through a corticosterone-independent mechanism. Elevated CORT in socially isolated animals assessed 72hr post-stroke likely reflects a stress response to the behavioral tasks performed at this time point. Indeed, because behavioral effects are difficult to identify at earlier time points (mice dramatically reduce locomotor activity during the first 48hrs post-MCAO, see Figure 3.3 in Chapter 3), behavioral analysis could only be conducted at 72hrs of reperfusion. Social isolation is often insufficient for increasing circulating CORT concentrations until a stressor has occurred, thus elevated CORT concentrations in isolated, relative to socially housed animals, likely reflect an enhanced stress response

to the behavioral task. Although it is unclear from these data whether CORT influenced the trajectory of the ischemic injury or functional recovery, it is apparent that social isolation negatively impacts recovery from the ischemic injury. Additional research is necessary to identify the upstream mechanisms underlying the effects of social isolation on ischemic outcomes.

In spite of significant differences in infarct size and edema, it was not apparent through the behavioral testing conducted at 72 hrs that a reduction in ischemic damage was associated with a reduction in behavioral deficits (Figure 4.2). One possible explanation is that among socially housed mice, there remained sufficient damage to surviving neurons, which contributed to functional deficits in these mice. We have previously reported functional outcome deficits in socially isolated mice following stroke (Craft et al., 2005). However, the behavioral assessments in the previous study were conducted at a later time point, suggesting that over time, socially housed mice may be better able to recover from functional deficits than socially-isolated mice. Measures of *perceived* social isolation or social support are as powerful, and in some cases more powerful, predictors of outcome than measures of *actual* social isolation or support in clinical studies examining health and well-being (Cacioppo et al., 2002; Hawthorne, 2008; Uchino et al., 1996). It is not possible to differentiate between actual and perceived social isolation in mice, nor is there a measure in mice that would be comparable to social support in humans; however, the current study provides evidence that the presence or absence of a cohabitating conspecific is sufficient to alter stroke

pathogenesis and outcome. Furthermore, this study identifies differential expression of IL-6 as one factor contributing to the difference in infarct size between socially housed and isolated mice. Both social isolation and elevated serum IL-6 concentrations are associated with poor outcome in human stroke patients (Boden-Albala et al., 2005; De Simoni et al., 2002; Smith et al., 2004), but whether there is a causal link between these two factors in humans, as there appears to be in mice, will need to be empirically tested. Additional studies comparing the effects of social interaction on IL-6 expression in human stroke patients would also be informative.

In summary, socially isolated mice were less likely to survive a stroke and had increased infarct volumes and edema compared to socially housed mice. The increase in ischemic damage among socially isolated mice was accompanied by an altered neuroinflammatory response that was consistent with a neurocompromising influence of social isolation. Post-stroke IL-6 signaling was down-regulated in the CNS and up-regulated in the periphery among socially isolated mice. Further, treatment with the IL-6 neutralizing antibody eliminated the effect of social housing on infarct size. Although numerous reports exist on neuroinflammatory measures in ischemia, they rarely describe housing conditions of the experimental mice, making it difficult to interpret those data independent of social/environmental influences. The current study is the first to investigate the modulation of neuroinflammatory responses by social housing following experimental stroke. Taken together, these data support a causal role for IL-6 underlying the increase in ischemic injury associated with social isolation and provide

evidence that social modulation of immune function can significantly influence stroke outcome.

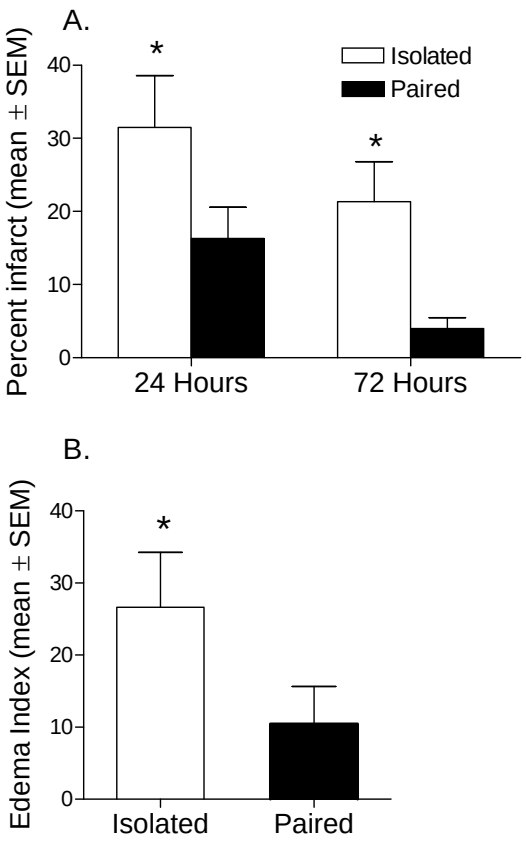


Figure 4.1: Social interaction influence on infarct size and brain edema

(A) Percent infarct relative to the contralateral hemisphere is significantly increased in socially isolated mice after 24 hours and 72 hours of reperfusion. (B) Index of edema is also significantly increased in socially isolated mice at 48 hours of reperfusion. *Significantly different from socially housed mice, $P < 0.05$.

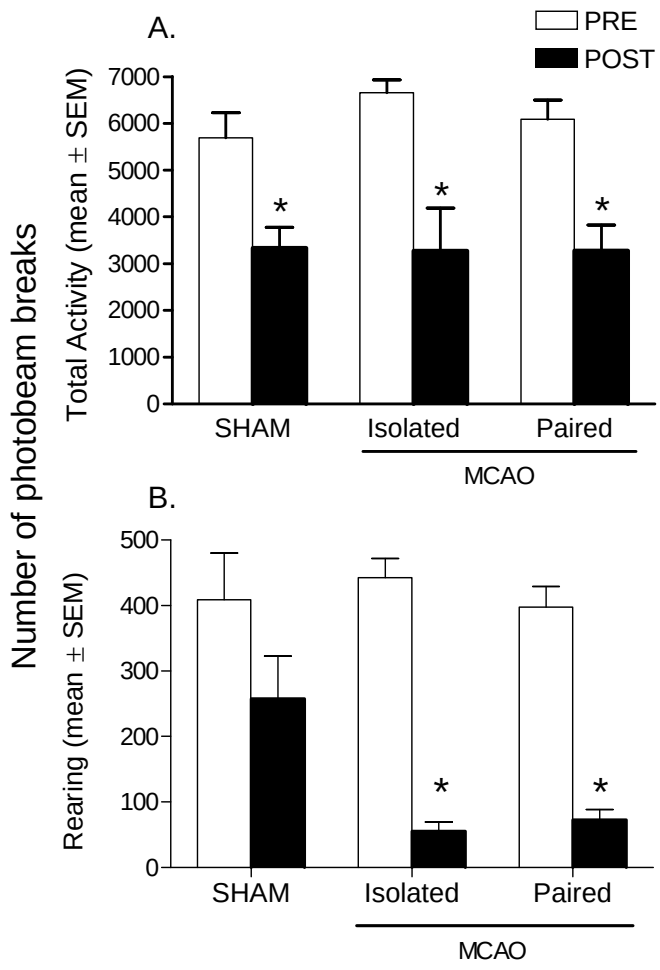


Figure 4.2: Baseline and post-stroke locomotor and exploratory behavior in the open field.

(A) Total locomotor activity and (B) frequency of rearing measured one day prior to MCAO or SHAM (PRE) and at 72 hours (POST), significantly decreases after surgery in both MCAO and SHAM mice, but does not vary by housing condition. *Significantly different from baseline behavior (PRE), $P < 0.01$.

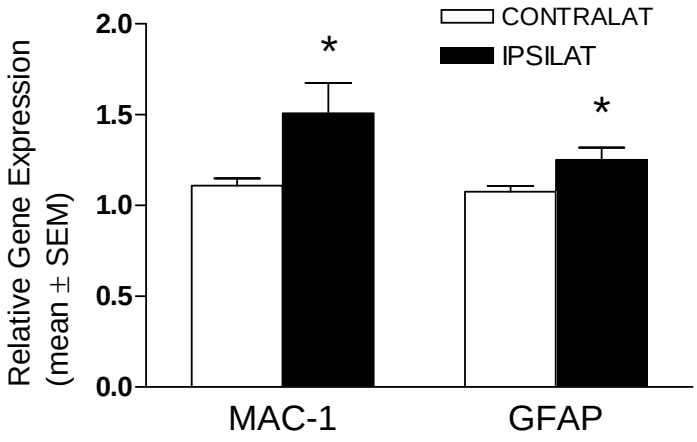


Figure 4.3: Relative gene expression of MAC-1 and GFAP in ipsilateral and contralateral hemispheres after MCAO.

Relative gene expression of MAC-1 and GFAP in striatum is up-regulated in the ipsilateral ischemic (IPSILAT) relative to the contralateral non-ischemic (CONTRALAT) hemisphere. Data are collapsed across the 12- and 24-hour reperfusion timepoints. *Significantly different from contralateral hemisphere, $P < 0.05$.

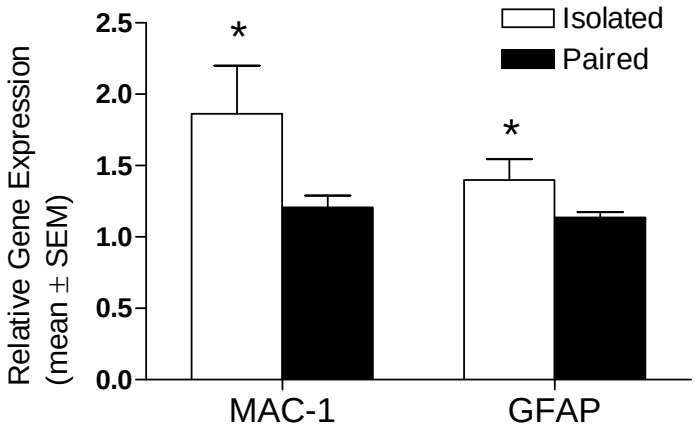


Figure 4.4: Social housing influences on post-stroke gene expression of MAC-1 and GFAP in striatum.

Within the ipsilateral hemisphere, both MAC-1 and GFAP are significantly up-regulated in socially isolated relative to socially housed mice. *Significantly different from socially housed mice, $P < 0.05$.

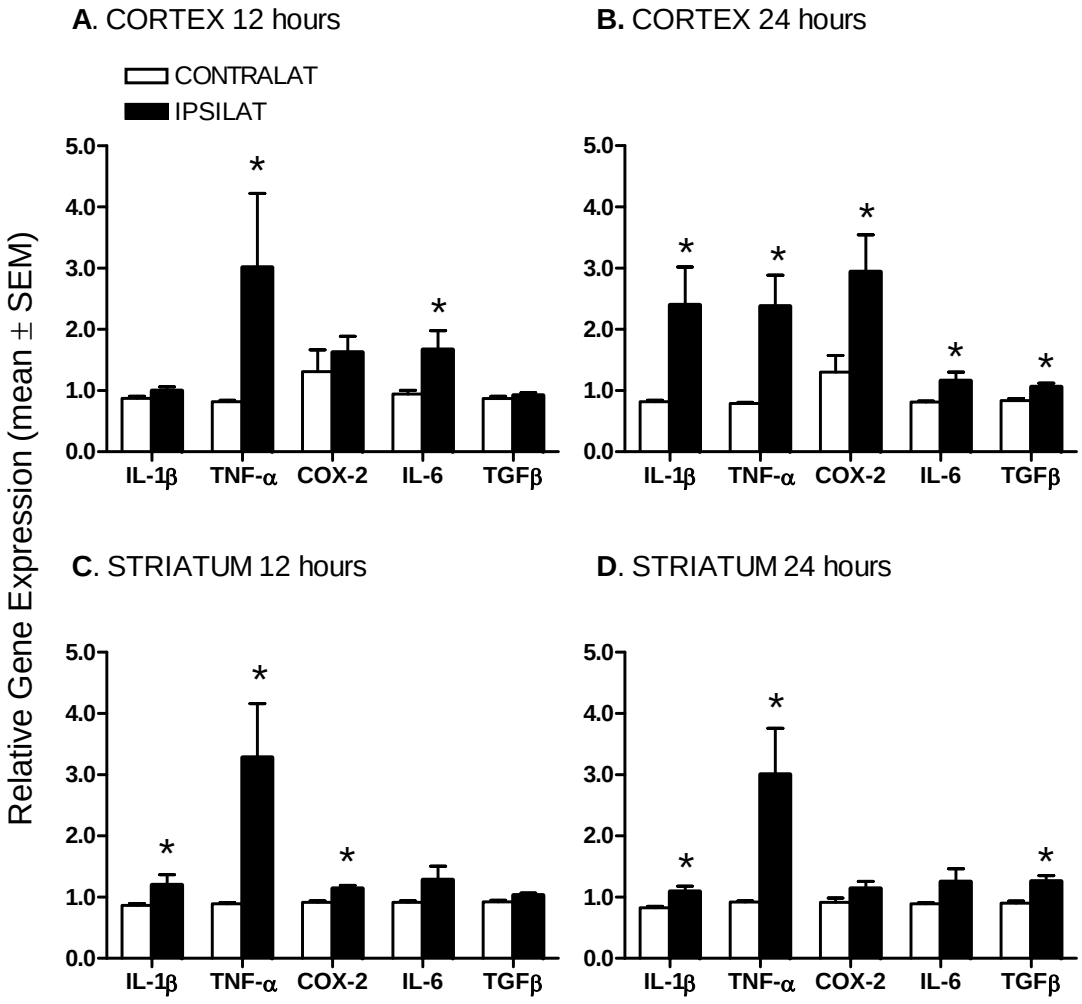


Figure 4.5: Relative gene expression of inflammatory markers following MCAO measured via RT-PCR.

Relative mRNA gene expression of IL-1β, TNF-α, COX-2, IL-6, and TGF-β are significantly up-regulated in the ipsilateral, relative to the contralateral hemisphere. *Significantly different from contralateral hemisphere, $P < 0.05$.

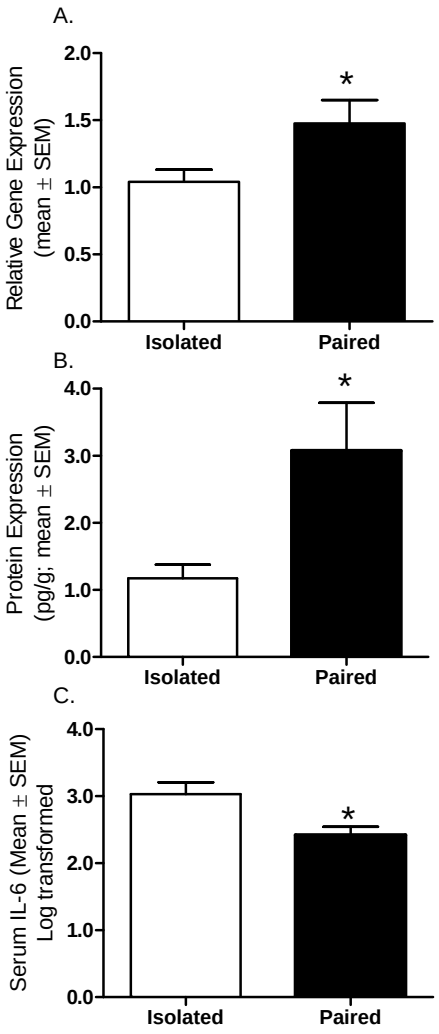


Figure 4.6: Relative gene expression and protein concentration of post-stroke IL-6.

In the CNS, (A) striatal IL-6 mRNA gene expression measured via RT-PCR and (B) cortical protein concentration measured via ELISA are significantly up-regulated in the ischemic hemisphere of socially housed, relative to isolated mice. (C) Serum IL-6 measured via ELISA is down-regulated in socially housed mice. Gene and protein expression data in the CNS are presented as a ratio of ischemic to non-ischemic hemisphere concentrations. *Significantly different from socially isolated mice, $P < 0.05$.

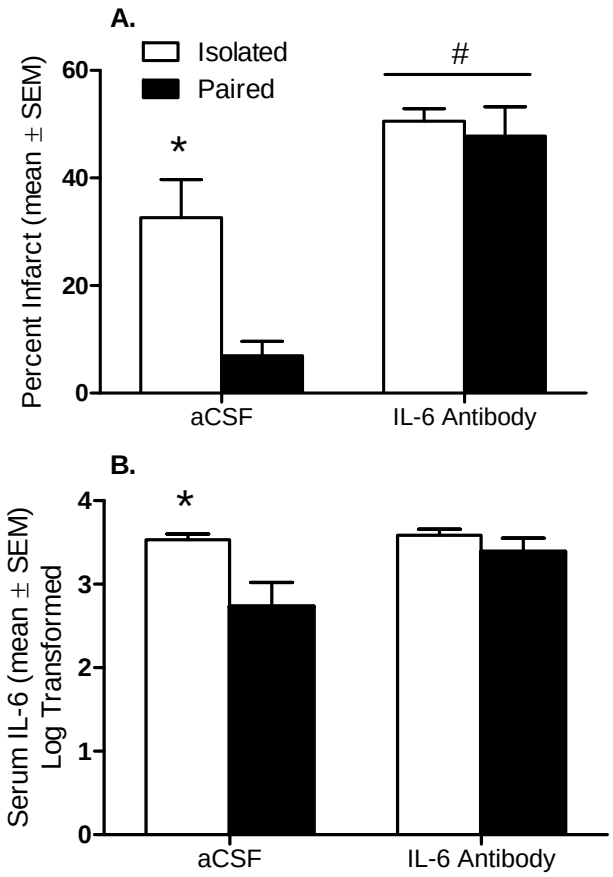


Figure 4.7: Infarct volume and serum IL-6 protein concentrations following ICV treatment with IL-6 neutralizing antibody.

(A) Treatment with 10 ng of IL-6 neutralizing antibody significantly increases infarct volume of socially housed mice and eliminates the effect of social interaction achieved with vehicle treatment. (B) Serum IL-6 is down-regulated in vehicle-treated socially housed mice relative to isolated mice, but treatment with the IL-6 neutralizing antibody eliminates the difference in serum IL-6 concentrations. * Significantly different from socially housed mice, # Significantly different from aCSF treated mice, $P < 0.05$.

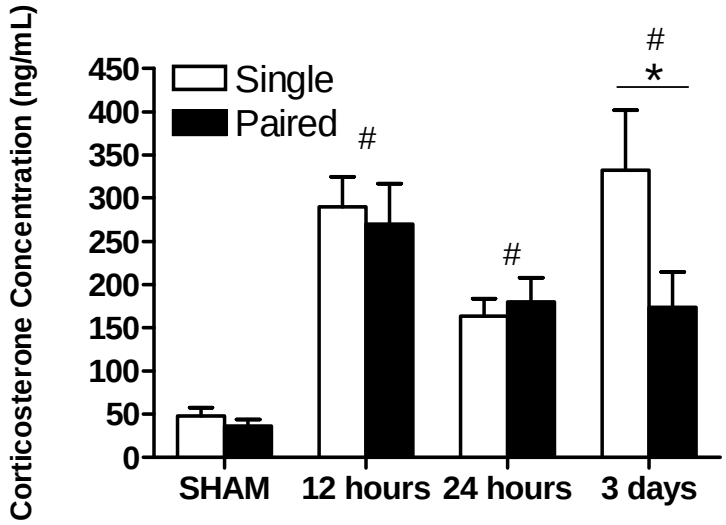


Figure 4.8: Post-stroke serum corticosterone concentration

Serum corticosterone concentrations are elevated relative to SHAMs at each time-point assessed post-stroke. In the cohort assessed at 72 hours, post-stroke serum corticosterone was elevated in isolated, relative to socially housed mice. * Significantly different from SHAMs, # Significantly different from aCSF treated mice, $P < 0.05$.

CHAPTER 5

OXYTOCIN MEDIATES SOCIAL NEUROPROTECTION AFTER CEREBRAL ISCHEMIA

Social factors have a fundamental influence on disease outcome (McDade et al., 2006). For instance, the diverse negative health effects of social isolation and lack of social support extend to rheumatoid arthritis (Strating et al., 2006), renal disease (Cohen et al., 2007b), and cancer (Spiegel and Sephton, 2001). The influence of social interactions on disease outcome is particularly evident in the context of vascular disease, including cerebrovascular and cardiovascular disorders (Ikeda et al., 2008); an effect that has been replicated in animal models of global (Weil et al., 2008a) and focal cerebral ischemia (Craft et al., 2005; Karelina et al., 2009b), as well as atherosclerosis (McCabe et al., 2002), a common risk factor for vascular disease. As such, the social environment is becoming recognized as a key contributor to the substantial individual variability in patient susceptibility to stroke outcome, thus, the need to identify the mechanism by which psychosocial experiences influence disease physiology is increasingly important.

One likely mediator of psychosocial influences on disease outcomes is oxytocin (OT), a nonapeptide produced in the paraventricular and supraoptic nuclei of the hypothalamus. OT is both induced by and facilitates social behaviors (Uvnas-Moberg, 1997); exogenous administration of OT potentiates social behaviors (Carter, 2003; Neumann, 2008) and central blockade of OT signaling disrupts social memory(Ferguson et al., 2000), parental behaviors (Takayanagi et al., 2005), and pair-bonding (Cho et al., 1999). Additionally, both exogenous and endogenous OT promote a buffering effect against stress and anxiety via suppression of the HPA-axis response to stress (DeVries et al., 2003). The role of OT as a neuroendocrine mediator in response to the psychosocial environment is evident throughout species of highly variable social structures, thus additional roles for OT beyond regulation of social behaviors are now being identified.

Indeed, in addition to social buffering, data indicate that OT may also have anti-inflammatory and anti-oxidant properties. Exogenous OT administration alleviates tissue damage in a variety of animal models of injury including renal (Tugtepe et al., 2007), hepatic (Dusunceli et al., 2008) and cardiac (Houshmand et al., 2009; Ondrejcakova et al., 2009) ischemia/reperfusion injury, as well as sepsis-induced multiple organ damage (Iseri et al., 2005b) and colitis (Iseri et al., 2005a). Co-administration of an oxytocin receptor antagonist (OTA), blocks the cardioprotective effects of OT on cardiac infarct development in rats (Houshmand et al., 2009). The protective actions of OT in these models may be associated with decreased levels of peripherally circulating tumor necrosis factor-α and interleukin-6 (IL-6) (Dusunceli et al., 2008; Tugtepe et al., 2007) as well as decreased neutrophil infiltration to the site of

injury (Dusunceli et al., 2008; Iseri et al., 2005a; Iseri et al., 2005b; Tugtepe et al., 2007). Additionally, increased OT is correlated with an up-regulation of nerve growth factor (Luppi et al., 1993), as well as insulin-like growth factor-1 (Petersson et al., 1998), which are neuroprotective in ischemic injury in peripheral organ systems (Lee et al., 1998; Liu et al., 2004).

Taken together, the role of endogenous OT as a mediator of social behaviors and the evidence supporting a protective role of exogenous OT in the pathophysiological responses in several disease models makes it a compelling candidate as the mediator of social influences on disease outcome. Indeed, exogenous OT administration protects against several of the physiological and behavioral consequences of social isolation, including autonomic dysfunction and anhedonia (Grippo et al., 2009), and slower wound healing (Detillion et al., 2004), as well as stress-induced HPA axis activation and anxiety-like behavior (Windle et al., 1997). Further, social housing attenuates the proinflammatory response to disease (Karelina et al., 2009b; Nation et al., 2008; Weil et al., 2008a). However, to our knowledge, no studies have assessed the role of OT in mediating social influences on any of the major causes of human morbidity and mortality.

We have previously reported reduced ischemic damage in socially housed, compared to socially isolated mice (Craft et al., 2005; Karelina et al., 2009b; Weil et al., 2008a). Given the growing research on the importance of OT in regulating physiology in the context of both the psychosocial environment and disease state, the present study was designed to investigate the effects of exogenously administered OT in mediating

social interaction influences on stroke outcome. Specifically, we examined the effects of OT treatment and social housing conditions on histological, behavioral, neuroendocrine, inflammatory and antioxidant measures following cerebral ischemia.

Materials and Methods

Animals

Adult male C57/BL6 mice (23-30g; Charles River, Wilmington, Mass) were maintained on a 14:10 light/dark cycle in a temperature and humidity-controlled vivarium. All animals were allowed *ad libitum* access to food and water. Experimental animals were housed either individually (socially isolated) or with an ovariectomized female (pair housed) for a period of 1 week prior to surgery and throughout the reperfusion period. The study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by the institutional animal care and use committee.

Experimental Procedures

The role of OT in mediating social housing influences on measures of stroke outcome was assessed in separate cohorts of mice at two different reperfusion periods. In Experiment 1, mice were assessed for post-stroke behavior, blood corticosterone concentration, and infarct size after 72 hours of reperfusion. In Experiment 1a, socially housed animals were treated with the vehicle, artificial cerebrospinal fluid, (aCSF), 50ng OTA, 500ng of OTA delivered daily (dose reflects amount delivered across a 24-hour

period for the duration of the experiment) [aCSF-MCAO, n = 8; 50ng OTA-MCAO, n = 8; 500ng OTA-MCAO, n = 9; aCSF-SHAM, n = 4; 50ng OTA-SHAM, n = 5; 500ng OTA-SHAM, n = 5]. In Experiment 1b, socially isolated animals were treated with aCSF, 2ng OT, 20ng of OT, or a cocktail of 20ng OT with 50ng OTA delivered daily, [aCSF-MCAO, n = 8; 2ng OT-MCAO, n = 8; 20ng OT-MCAO, n = 11; 50ng OTA-MCAO, n = 6; 20ng OT+50ng OTA, n = 6; aCSF-SHAM, n = 4; 2ng OT-SHAM, n = 5; 20ng-SHAM, n = 4]. The doses for both manipulations were chosen based on previous research demonstrating their efficacy (Detillion et al., 2004; Liu and Wang, 2003).

Experiment 2 was designed to assess the role of social housing and oxytocin in post-ischemic neuroinflammation and systemic inflammation. Gene expression of inflammatory markers was measured in the cortex and striatum, and protein expression of IL-6 was measured in serum. All animals in Experiment 2 underwent MCAO surgery and tissue was collected after 24 hours of reperfusion, the most effective dose of OT (20ng) and OTA (50ng) was used based on the outcome of Experiment 1, resulting in four groups [Isolated-aCSF, n = 7; Isolated-OT, Isolated-OTA, n = 8; n = 8; Social-aCSF, n = 6; Social-OT, n = 6; Social-OTA, n = 7].

Experiment 3 assessed the role of social housing and oxytocin in post-ischemic antioxidant defenses. Glutathione peroxidase (GPx) activity was measured in a separate cohort of animals. Brain tissue was collected in a separate cohort of animals after 24 hours of reperfusion, drug dose and regimen was the same as described in Experiment 2 Isolated-aCSF, n = 7; Isolated-OT, n = 6; Social-aCSF, n = 6; Social+OTA, n = 7].

Experiment 4 was designed to confirm and quantify the presence of oxytocin receptors (OTR) on neurons, astrocytes and microglia. Brain tissue was collected from a separate cohort of animals that did not undergo cerebral ischemia [Isolated, n = 7, Social, n = 7]. An additional cohort was used to assess LPS-challenge induced MHC class II induction following OT incubation.

Intracerebroventricular Cannulation and Drug Administration

Mice were implanted with an Alzet minipump (Model 1002, Durect, Cupertino, CA) connected via tubing to an ICV cannula (2.75mm projection, Plastics One, Roanoke, VA) implanted into left lateral ventricle. The cannulation procedure was performed as described in Chapter 2 (page 31). The pumps delivered aCSF, OT (Bachem Biosciences Inc, King of Prussia, PA), or a selective oxytocin antagonist [(OTA); desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT, generously donated by Dr. Maurice Manning, The University of Toledo] at a rate of 0.25µL/hour. Drug infusion was initiated 1 week prior to MCAO or SHAM surgery, and continued until tissue collection after 24 or 72 hours of reperfusion. Correct cannula placement was confirmed through cresyl violet staining.

Histochemistry

Immediately following cervical dislocation and decapitation, fresh brains were removed and processed for TTC, microglia and astrocytes histochemistry as described in chapter 2 (page 33).

Real-time PCR

RT-PCR was conducted at 24 hours of reperfusion following MCAO. Bilateral samples were dissected from the cortex and striatum and RNA extraction and PCR were conducted as described in Chapter 2 (page 35). The following inventoried primers and probes (Applied Biosystems, Foster City, CA) were used: IL-6, CD11b (a pattern recognition complement receptor protein expressed on macrophage-lineage cells) and glial fibrillary acidic protein (GFAP; an intermediate filament protein that is up-regulated in astrocytes following injury).

Cell isolation, microglial isolation and flow cytometry

Brain tissue was obtained at indicated experimental time points immediately following euthanasia. Single-cell suspensions were obtained by passage through cell strainers. At least 10^6 cells/sample were resuspended in staining wash buffer. Cell surface Fc receptors were blocked by incubation with anti-CD16/32 antibody (eBioscience, San Diego, CA) and then washed. All antibody incubations were performed on ice in the absence of light. The cells were incubated with antibodies to CD11b (eBioscience, San Diego, CA, 1:200), NeuN (a neuronal marker, Millipore, Billerica, MA, 1:100), GFAP (Santa Cruz, Santa Cruz, CA, 1:66) and oxytocin receptor (OTR: rabbit anti-OTR, Abcam, Cambridge, MA, 1:100) for 1 hour. The cells were further incubated with AlexaFluor 647 conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA, 1:500) secondary antibody.

Microglial cultures were prepared from brain tissue isolated from socially isolated mice immediately following euthanasia. Single cell suspensions were resuspended in 70% Pecoll. A density gradient was set up as follows: 70%, 50% and 0% Percoll. The gradient was centrifuged for 45 minutes at 1200g. The middle interface between 70% and 50% consisting of enriched microglia was removed, washed. Microglia were incubated for 2 hours in the presence or absence of OT and OTA, followed by a 22 hour LPS challenge (1 μg/mL, serotype 0127:B8, Sigma Aldrich, St. Louis, MO). The following conditions were compared; 1) control: no stimulation or treatment, 2) LPS treatment only, 3) LPS + 0.1 μM OT and 4) LPS + 1μM OT. Reactive microglia were measured by MHC class II expression (1:16,000 eBioscience, San Diego, CA). Cell surface Fc receptors were blocked by incubation with anti-CD16/32 antibody and then washed. All antibody incubations were performed on ice in the absence of light. Flow cytometry data was acquired using a BD LSRII instrument (Davis Heart and Lung Flow Core Facility at OSU) and analyzed using FlowJo software (TreeStar, OR). For any given marker, all of the analysis gates were identical in size and position for all groups.

Data Analysis

Results for infarct size were analyzed as a two-way ANOVA (housing X drug). At no point did SHAM operated animals differ significantly across housing condition or drug treatment ($P>0.05$), so surgical parameters, behavioral data and corticosterone

concentrations were analyzed via a seven-level one-way ANOVA (group). PCR data were expressed as a ratio of ipsilateral to contralateral hemisphere gene expression and were analyzed via a nonparametric Mann-Whitney U test. Significant ANOVA results were followed by a Tukey HSD post hoc test. Data were considered significant at $P \leq 0.05$.

Results

Social housing condition and OT influence infarct size

Socially housed or isolated mice underwent middle cerebral artery occlusion (MCAO) or SHAM surgery, and were assessed for neuronal damage (infarct size relative to the contralateral hemisphere) after 72 hours of reperfusion. As shown in Figure 5.1, housing condition significantly influenced infarct size. Among artificial cerebrospinal fluid (aCSF) vehicle treated groups, social housing decreased infarct size relative to social isolation ($F_{1,14} = 7.598$, $P = 0.016$). To determine whether the neuroprotective effect of social housing is mediated by endogenous OT, socially housed mice were treated with OTA (50ng or 500ng per day). Treatment of socially housed mice with either dose of OTA increased infarct size ($F_{1,20} = 13.914$, $P = 0.001$) relative to aCSF. A dose-dependent effect of OTA treatment was not observed, as both doses comparably increased infarct size. Importantly, the infarct sizes in OTA treated mice were equivalent to the socially isolated, vehicle treated group, thus the neuroprotection afforded by social housing was essentially eliminated by oxytocin receptor (OTR) blockade, indicating a potential mechanism of neuroprotection mediated by OT signaling. To further address the potential for a direct neuroprotective role of OT,

socially isolated mice were treated with 2ng or 20ng OT per day in order to assess the ability of exogenous OT to mimic the effects of social housing. OT treatment dose-dependently reduced infarct size among socially isolated mice ($F_{4,30}$ = 3.417, *P* = 0.020). A Tukey post-hoc analysis revealed that the high dose (20ng/day) but not the low dose treatment (2ng/day) reduced infarct size relative to aCSF among socially isolated mice (*P* = 0.045). Moreover, co-infusion of 50ng OTA with the effective OT dose reversed the neuroprotection conferred by OT treatment (post hoc, *P* = 0.999 relative to aCSF), indicating a receptor mediated effect of OT treatment. Importantly, OTA infusion does not exacerbate ischemic injury, as treatment of isolated mice with 50ng of OTA alone did not significantly alter infarct volume relative to aCSF (post hoc, *P* = 0.996).

There were no group differences in body mass (*P* > 0.05) or neurological score among MCAO mice (*P* > 0.05) in these experiments. During surgery, body temperature was elevated in mice undergoing MCAO relative to SHAM surgery ($F_{7,119}$ =32.754, *P* = 0.001), however, there were no differences in body temperature among MCAO groups (*P* > 0.05).

Post-MCAO serum corticosterone concentrations

The physiological benefits of social interaction are often attributed to "social buffering", whereby social interactions attenuate the physiological (i.e. circulating glucocorticoids) and behavioral stress responses (DeVries et al., 2003). Because stroke is itself a potent stressor, and social isolation exacerbates stress-induced glucocorticoid release (DeVries et al., 2003), circulating corticosterone concentrations were assessed in

all drug and housing conditions. Circulating corticosterone concentrations measured after 72 hours of reperfusion ($F_{1,78}$ = 46.147, *P* = 0.0001) were elevated in MCAO relative to SHAM groups. A Tukey post-hoc analysis revealed that only one MCAO group, socially housed aCSF-treated mice, did not differ from SHAM mice (*P* > 0.05). Further, among socially housed groups, treatment with 50ng OTA further increased circulating corticosterone relative to the aCSF group (*P* = 0.013) (Table 1). OT treatment of isolated mice did not reduce circulating corticosterone relative to aCSF, indicating that the neuroprotective effects of the high dose of OT may be independent of circulating glucocorticoids.

Post-MCAO sensorimotor recovery

Focal cerebral ischemia elicits profound sensorimotor deficits (Hattori et al., 2000), thus functional recovery was assessed in the same cohort of mice. The cylinder test is a measure of forepaw use deficits in the affected limb (contralateral to ischemic hemisphere). While groups did not differ in contralateral paw use during baseline testing (24 hrs prior to surgery), post-surgical testing revealed a main effect of surgery whereby contralateral paw use was reduced in mice that underwent MCAO relative to SHAM surgery ($F_{1,62}$ = 21.217, *P* = 0.0001). A notable exception is that both doses of OT treatment partially reduced this deficit in socially isolated mice (2ng OT, *P* = 0.08; 20ng OT, *P* = 0.144 relative to SHAM). Among socially housed mice, contralateral paw use did not differ by drug treatment (all *P* > 0.05) (Table 2).

There were no differences in open field activity among groups during baseline testing (activity in center of open field, total activity, and rearing behavior: all $P > 0.05$). During post-surgical testing, there was a dramatic reduction of rearing behavior in the open field in mice that underwent MCAO surgery ($F_{1,73} = 188.525$, $P = 0.000$), however, total activity and activity in the center of the open field were not significantly affected by surgery (all $P > 0.05$). There were no significant effects of drug treatment or dose on open field activity in either socially housed or socially isolated mice (all $P > 0.05$). OT has been shown to influence locomotor activity (Uvnäs-Moberg et al., 1994); however, in the present study OT did not affect activity in the open field (Table 2), indicating that the doses used may have been insufficient for directly influencing these measures of motor activity.

Social housing and OT influence interleukin-6 expression

Focal cerebral ischemia triggers a marked neuroinflammatory response, particularly in the cortical and striatal regions of the ischemic hemisphere. Central interleukin-6 (IL-6) has been shown to play a neuroprotective role in ischemia (Loddick et al., 1998) and we recently reported a role for IL-6 expression as a proximate mediator of the neuroprotection conferred by social housing (Karelina et al., 2009b). The OT receptor gene contains a response element for nuclear factor IL-6 (Schmid et al., 2001) and OT has been shown to influence IL-6 secretion (Szeto et al., 2008). Using real-time PCR analysis, we observed differential expression of the mRNA encoding IL-6 in the striatum following MCAO. Among aCSF-treated groups, social housing increased

striatum IL-6 mRNA expression (U = 6.0, P = 0.032) relative to social isolation. However, IL-6 mRNA expression was reduced in socially housed mice treated with OTA (U = 3.0, P = 0.05). Additionally, OT was administered to socially housed mice to determine whether this would result in an additive effect, however, while OT treatment resulted in increased striatum expression of IL-6 mRNA relative to OTA treatment (U = 1.0, P = 0.014), we did not observe an additive effect of social housing and OT treatment. OT has a bi-phasic therapeutic effect when administered in a model of cardiac ischemia (Houshmand et al., 2009), thus the potential for an "ideal" additive benefit of social interaction as well as OT treatment warrants further careful titering of OT dosing. On the other hand, treatment of socially isolated mice with OT increased IL-6 mRNA expression relative to aCSF (U = 7.0, P = 0.05). Further, OTA administration to socially isolated mice did not alter IL-6 mRNA expression relative to aCSF treatment, indicating 1) that endogenous OT signaling is low in isolated mice and not further antagonized with the 50ng dose of OTA, and 2) central administration of this dose of OTA does not independently influence the neuroinflammatory response to cerebral ischemia. A correlation between brain IL-6 mRNA and protein expression measured after 24 hours of reperfusion was recently confirmed (Karelina et al., 2009b). There were no group differences in cortical IL-6 mRNA expression (all $P > 0.05$; Figure 5.2).

In contrast to central IL-6 actions in ischemia, elevated peripheral IL-6 concentrations are predictive of increased likelihood of stroke occurrence, severity and mortality (Smith et al., 2004). Serum concentrations of IL-6 protein, measured after 24 hours of reperfusion, varied significantly among the groups ($F_{3,33}$ = 3.191, P = 0.038). A

Tukey post-hoc analysis revealed that there were no differences by housing condition among aCSF-treated groups; however, among socially isolated groups, OT treatment reduced circulating IL-6 relative to aCSF treatment ($P = 0.023$). OTA treatment did not influence circulating IL-6 in socially housed mice ($P > 0.05$) (Table 1).

Post-MCAO antioxidant capacity and oxidative stress

The generation of free radicals during cerebral ischemia is well known to contribute to inflammation and cell death and experimental conditions that inhibit free radical production or increase antioxidant defenses attenuate ischemic injury (Warner et al., 2004). Several studies have established that OT has antioxidant properties; OT scavenges peroxinitrite, inhibits lipid peroxidation, and attenuates NADPH-dependent superoxide activity (Iseri et al., 2005a; Moosmann and Behl, 2002; Szeto et al., 2008). To that end, the effect of OT and OTA treatment on oxidative stress was assessed in socially housed and isolated mice. Brain antioxidant levels (glutathione peroxidase; GPx) were increased in socially housed ($F_{1,13} = 7.816$, $P = 0.016$) and OT-treated mice ($F_{1,12} = 9.949$, $P = 0.009$) relative to aCSF-treated socially isolated mice (Figure 5.3A). Oxidative stress was measured as a ratio of reduced (GSH) to oxidized (GSSG) glutathione, thus a smaller ratio is indicative of greater oxidative stress. Oxidative stress was attenuated by social housing ($F_{1,11} = 4.660$, $P = 0.05$) as well as OT treatment ($F_{1,11} = 5.066$, $P = 0.048$) relative to aCSF treated isolated mice. Among socially housed mice, OTA treatment significantly increased oxidative stress ($F_{1,15} = 4.722$, $P = 0.045$: Figure 5.3B).

Social housing and OT influence glial expression

Accumulating evidence suggests that microglia and astrocytes play a central role in the progression of neuronal damage following an ischemic event (Wang et al., 2007). A strong induction of CD11b and GFAP mRNA was evident in the ischemic hemisphere after MCAO. CD11b mRNA gene expression differed across groups; both social housing (U = 2.0, P = 0.017) and OT treatment (U = 5.0, P = 0.028) reduced striatal CD11b mRNA expression relative to social isolation. CD11b mRNA expression was also increased in socially housed mice treated with OTA (U = 1.0, P = 0.011). Finally, GFAP mRNA gene expression also differed across groups. Again both social housing (U = 6.0, P = 0.05) and OT treatment (U= 6.0, P = 0.05) reduced striatal GFAP mRNA expression relative to social isolation, however, there was no effect of OTA treatment on GFAP mRNA expression (P > 0.05). There were no group differences in cortical GFAP mRNA expression (all P > 0.05) (Figure 5.4A,B).

Protein expression of microglia and astrocytes were assessed histologically in the ischemic hemisphere. As expected, MCAO induced microglial activation (as measured by isolectin B4 expression) in the ischemic hemisphere; however, among socially isolated mice, the extent of cortical microglial activation was suppressed by OT treatment ($F_{1,18}$ = 4.811, P = 0.043). There was no drug effect on microglial activation among socially housed mice (P > 0.05) and there were no significant effects of housing or drug treatment in the striatum (P > 0.05). MCAO also induced astroyctosis (that is, GFAP-positive staining) and produced glial scarring. The glial scar area was reduced by

OT treatment of socially isolated mice ($F_{1,16}$ = 5.930, P = 0.026). There was no drug effect on glial scar area among socially housed mice (P > 0.05). (Figure 5.4C-F).

Evidence for oxytocin receptor expression on glia and neurons

The ability of OT (as well as OTA) to modulate the inflammatory response to cerebral ischemia indicates the presence of OTR on cell populations that mediate neuroinflammation. OTR expression on neuronal and glial cell populations was assessed in socially housed and isolated mice using flow cytometry. As previously reported, both neuronal (NeuN-positive) and astroglial (GFAP-positive) cells express OTR (Wang and Hatton, 2006). Interestingly, the percent of NeuN-positive cells that express OTR was greater in socially housed, relative to isolated mice (t_9 = 2.200, P = 0.05), a relationship that merits further research. Additionally, approximately 16 % of microglia (CD11b-positive cells) express OTR. The objective of this experiment was to identify OTR expression on cell populations in normal, healthy tissue, thus this cohort of mice did not undergo cerebral ischemia. Circulating macrophages are unlikely to have migrated to the brain parenchyma in the absence of trauma (Stoll et al., 1998), thus our data indicate the presence of OTR on resident microglia in the CNS (Figure 5.5).

OT modulates microglial reactivity

In order to determine whether microglial OTR play a regulatory role during microglial activation, enriched microglia were incubated with or without OT (0.1 µM or 1 µM), then challenged with 1µg/mL of lipopolysaccharide (LPS). LPS is a bacterial

endotoxin, and was chosen because of its rapid induction of microglial activation (Triantafilou and Triantafilou, 2005). Once activated, microglia act as antigen-presenting cells and up-regulate major histocompatibility complex class II (MHC class II) expression, thus MHC class II expression was used as a measure of microglial reactivity. Primary microglial cultures stimulated with LPS increased expression of MHC class II relative to the non-stimulated control (t_{12} = 9.715,*P* = 0.0001). OT dose-dependently attenuated LPS-stimulated MHC class II expression relative to LPS alone (t_{12} = 2.578, *P* = 0.024). Taken together, these data indicate a direct role for OT *in vitro* for antagonizing microglial reactivity (Figure 5.6).

Discussion

The influence of social interaction on disease outcome suggests that an endogenous signaling pathway links the psychosocial environment to disease pathophysiology. Data from the current study provide evidence for a role of OT as a mediator of social interaction-induced neuroprotection against the detrimental physiological and behavioral consequences of ischemia. The present findings indicate that relative to social isolation, either social interaction or chronic central administration of OT leads to a reduction in ischemic damage (Figure 5.1), as well as measures of neuroinflammation (Figures 5.2 and 5.4) and oxidative stress (Figure 5.3) after cerebral ischemia. A mechanism for OT in this model was further supported by the finding that chronic OTR antagonism during social housing blocks the protection conferred by social

interaction. Together, these data lend support to the hypothesis that the buffering of stroke outcome via social interaction may be mediated by OT.

Inflammatory components of stroke have long been a target of treatment. Cerebral ischemia promotes glial cell activation, leukocyte infiltration and the consequent release of soluble and toxic factors such as cytokines, chemokines and nitric oxide within hours of an ischemic event. Although this inflammatory response is linked with the repair process and debris removal, it is also a major contributor to cell death, and experimental interventions that block peri-ischemic neuroinflammation reduce ischemic injury and improve outcome (Stoll et al., 1998). Nonetheless, therapeutic interventions that target these inflammatory components in stroke patients have largely failed in late phase clinical trials (Feuerstein and Chavez, 2009). In contrast, the protection conferred by social support has been consistently reported in cerebrovascular and cardiovascular disease (Ikeda et al., 2008) and there is evidence that social interactions attenuate inflammation in both clinical (McDade et al., 2006) and animal studies (Karelina et al., 2009b; Weil et al., 2008a). These studies predict the existence of one or several potent endogenous neuroprotective factors that are engaged by social interaction and render discovery of these pathways of paramount importance.

Data in the current study indicate an increase in brain IL-6 mRNA expression in socially housed and OT-treated mice. Further, serum concentrations of IL-6 in the same cohort of mice were reduced in OT-treated mice (Table 5.1). This is consistent with an anti-inflammatory IL-6 profile, as central IL-6 signaling is neuroprotective in ischemia

(Loddick et al., 1998); however, contrary to its central actions, peripheral IL-6 promotes acute phase protein induction and is thus indicative of a pro-inflammatory state (Smith et al., 2004). While these data may reflect a reduction in neuronal injury in the socially housed or OT-treated mice, we have recently reported that a single treatment with an IL-6 neutralizing antibody prior to an ischemic event is sufficient for both increasing infarct size and eliminating the social buffering effect (Karelina et al., 2009b). Studies also support a direct reciprocal relationship between OT and cytokine signaling both *in vivo* and *in vitro* (Dusunceli et al., 2008; Iseri et al., 2005b; Schmid et al., 2001; Szeto et al., 2008; Tugtepe et al., 2007). The OTR gene contains response elements for inflammatory mediators including nuclear factor-IL6, as well as nuclear factor-κB, acute phase response factor, and binding sites for activator protein-1 (Murasawa et al., 1995; Schmid et al., 2001). Up-regulation of proinflammatory cytokines such as IL-6 or interleukin-1β modulates both OT and OTR gene transcription (Rauk and Friebe-Hoffmann, 2000; Schmid et al., 2001). Additionally, OT attenuates LPS-stimulated IL-6 secretion from cultured macrophages and endothelial cells, likely via a mechanism involving suppressed NADPH oxidase activity (Szeto et al., 2008).

Oxidative stress, marked by production of free radicals, antioxidant depletion and lipid peroxidation occurs rapidly after the onset of ischemia and is further a detrimental and unintended consequence of the rapid return of blood flow to the affected brain area during reperfusion (Warner et al., 2004). Reactive oxygen and nitrogen species are potent inducers of inflammation and cell death, and therapeutic treatments aimed at reducing oxidative stress have successfully attenuated ischemic

injury in animal models (Warner et al., 2004). In the current study, both social housing and exogenous OT treatment increased antioxidant activity (GPx) and attenuated oxidative stress (Figure 5.3), which corresponds to reduced ischemic damage and neuroinflammation in similarly treated cohorts. It remains to be determined whether the reduction in oxidative stress in the current study is a function of reduced neuronal damage in OT-treated and socially housed mice, or whether it is a contributing factor to the developing ischemic injury. However, OT has been shown to act as a free radical scavenger and reduces lipid peroxidation (Moosmann and Behl, 2002; Szeto et al., 2008). Further, the anti-oxidant capacity of OT has also been reported in models of renal and hepatic ischemia/reperfusion injury (Dusunceli et al., 2008; Tugtepe et al., 2007). Importantly, the reduction of oxidative damage in these studies was accompanied by an attenuation of pro-inflammatory cytokines (Dusunceli et al., 2008; Tugtepe et al., 2007). Thus it is likely that the neuroprotective and anti-inflammatory effects of OT in cerebral ischemia are in part mediated by its antioxidant properties.

Further support for a potent anti-inflammatory component of OT-mediated neuroprotection comes from the discovery that OT attenuates gliosis and glial reactivity to both ischemia and endotoxin challenge. In the current study, social housing and OT treatment attenuated CD11b and GFAP expression; while, OTA treatment increased CD11b expression similar to levels observed in socially isolated mice (Figure 5.4). Microglia and astrocytes play a critical role in the development of ischemic injury. Glial activation occurs rapidly following an ischemic event, and generates reactive changes including production of reactive oxygen species and release of cytotoxic factors (Huang

et al., 2006; Wang et al., 2007). CD11b-positive microglia contribute substantially to the progression and extent of ischemic injury, as treatment that inhibits microgliosis reduces infarct size and attenuates pro-inflammatory cytokine production (Yrjanheikki et al., 1999). Moreover, the identification of OTR expression on CD11b-positive cells in the brain (Figure 5.5) provides a mechanistic link that may further explain attenuation of neuroinflammation by OT. We report evidence of OT-mediated attenuation of LPS-induced MHC class II expression in cultured microglia. OT reduces LPS-stimulated IL-6 and superoxide production in cultured macrophages and endothelial cells (Szeto et al., 2008). Given the role of microglia in the production of pro-inflammatory cytokines and reactive oxygen species following cerebral ischemia, these data indicate that the anti-oxidant and anti-inflammatory effects of OT may be driven by OTR signaling directly on resident microglia and invading macrophages.

Perhaps more relevant to patient recovery, in addition to attenuating neuronal injury and pathophysiology, OT treatment improves functional outcome following cerebral ischemia (Table 5.2). Sensorimotor deficits are a leading cause of reduced quality of life and affective disorders following stroke, and rehabilitation services that enhance functional recovery greatly reduce psychological distress in stroke patients (Robinson-Smith et al., 2000). It is unlikely that the improved functional recovery in this study is solely a product of reduced damage, as social housing did not improve functional recovery in spite of attenuated neuronal damage. Thus, although the protective effects of OT on stroke outcome generally recapitulate those of social housing, OT appears to have an independent effect on functional outcome. Further

research will be necessary to identify the extent and mechanisms by which OT affects sensorimotor deficits following stroke.

The results from this study lend support to the growing body of evidence that social interaction and support positively influence stroke outcome. On nearly all measures assessed in the current study, the neuroprotection conferred by social housing can be mimicked in socially isolated mice via chronic central infusion of OT. These data extend recent research on the neuroinflammatory and antioxidant properties of OT and emphasize that OT is uniquely suited to integrate psychosocial stimuli with pathophysiological responses to central and peripheral tissue injury, or trauma. Taken together, these data support a causal role for OT as a mediating factor of social modulation of stroke outcome.

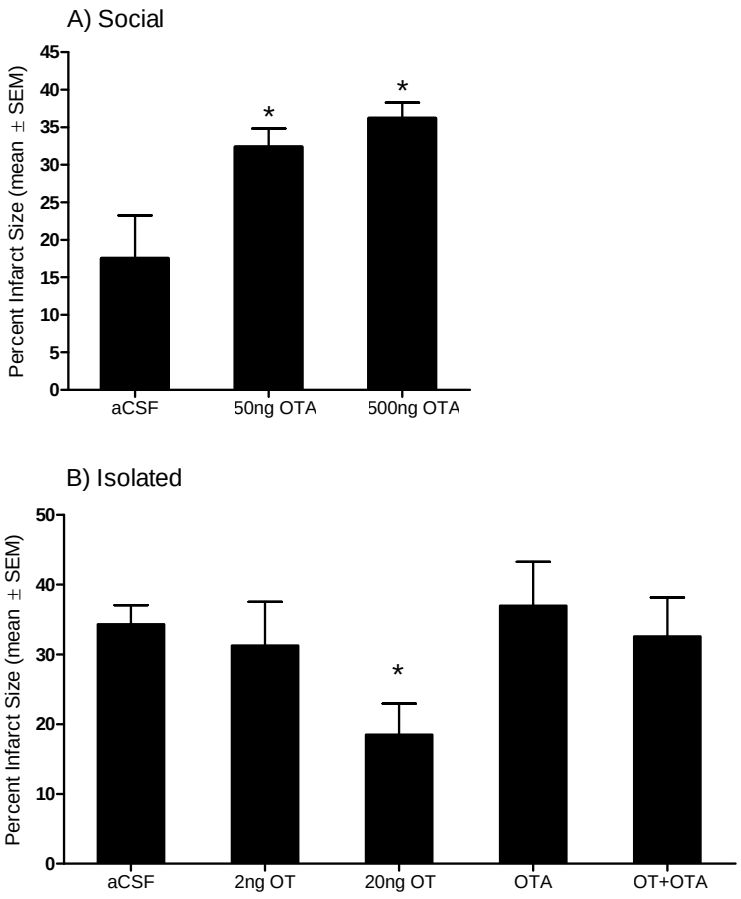


Figure 5.1: Social housing condition and oxytocin influence infarct size and functional recovery.

(A) Social housing reduces infarct size (aCSF: social n = 8, isolated n =8); however, daily treatment of socially housed mice with OTA (50ng n = 8 and 500ng n = 9) eliminates the neuroprotective effect of social housing on infarct size. (B) Daily treatment of socially isolated mice with 20ng (n = 11) OT (but not 2ng, n = 8) reduces infarct size. OTA infusion alone (n = 6) or with OT (n = 6) does not affect infarct size. An asterisk (*) indicates a statistically significant difference from the socially housed aCSF-treated mice ($P < 0.05$). A pound sign (#) indicates a statistically significant difference from socially isolated aCSF-treated mice ($P < 0.05$).

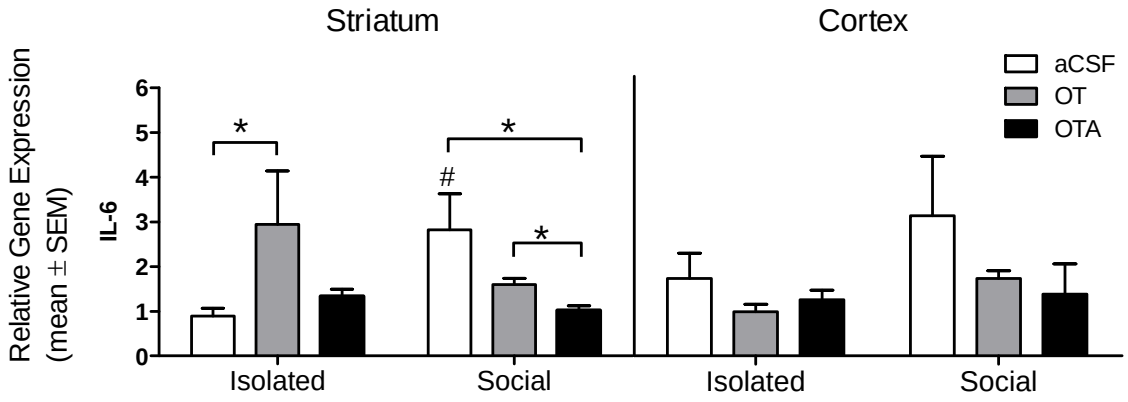


Figure 5.2: Relative gene expression of interleukin-6 following MCAO.

Striatal mRNA gene expression of IL-6 is elevated in socially housed (aCSF n = 6) and OT-treated mice (n = 7) relative to social isolation (aCSF n = 7). OT treatment (n = 6) increased IL-6 expression relative to OTA (n = 7) in socially housed mice, while OTA treatment (n = 8) did not influence IL-6 expression in isolated mice. An asterisk (*) indicates a statistically significant difference between indicated groups ($P < 0.05$). Data are expressed as a ratio of ischemic to non-ischemic hemisphere. A pound sign (#) indicates a statistically significant difference from socially isolated aCSF-treated mice ($P < 0.05$).

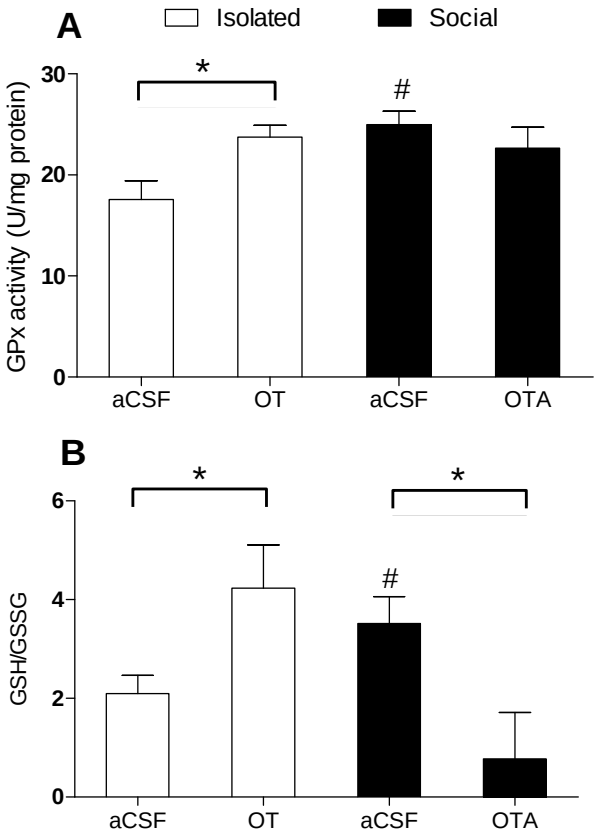


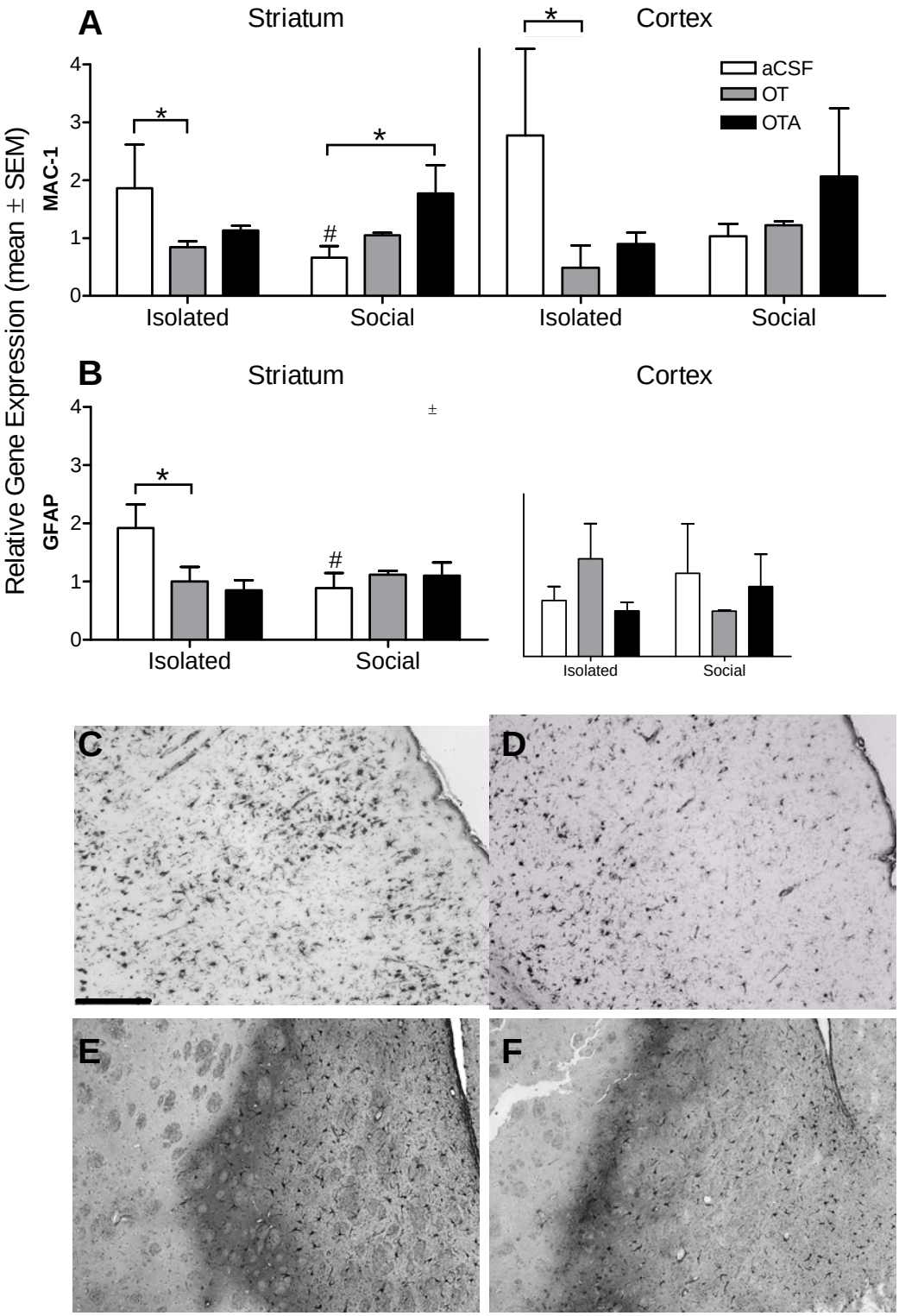
Figure 5.3: Antioxidant enzyme activity and oxidative stress levels.

(A) GPx activity is increased in OT-treated (n = 6) and socially housed (n = 6) mice relative to social isolation (n = 7). (B) Likewise, relative to social isolation, oxidative stress is reduced following OT treatment or social housing, but is significantly elevated in socially housed mice following OTA treatment (n = 7). An asterisk (*) indicates a statistically significant difference between indicated groups ($P < 0.05$). A pound sign (#) indicates a statistically significant difference from socially isolated aCSF-treated mice ($P < 0.05$).

Figure 5.4: Gene and protein expression of glial markers following MCAO.

(A-B) Striatal and cortical mRNA gene expression of MAC-1 and GFAP is significantly altered by social housing and OT treatment. An asterisk (*) indicates a statistically significant difference between indicated groups ($P < 0.05$). Data are expressed as a ratio of ischemic to non-ischemic hemisphere. A pound sign (#) indicates a statistically significant difference from socially isolated aCSF-treated mice ($P < 0.05$). (C-F) Representative images of cortical microglial activation in socially isolated (C) aCSF and (D) OT-treated mice, and GFAP-positive glial scars of (E) aCSF and (F) OT-treated mice. Scale bar = 200 μ

Figure 5.4


A
Striatum
Cortex
aCSF
OT
OTA
Relative Gene Expression (mean ± SEM)
MAC-1
Isolated
Social
B
GFAP
C
D
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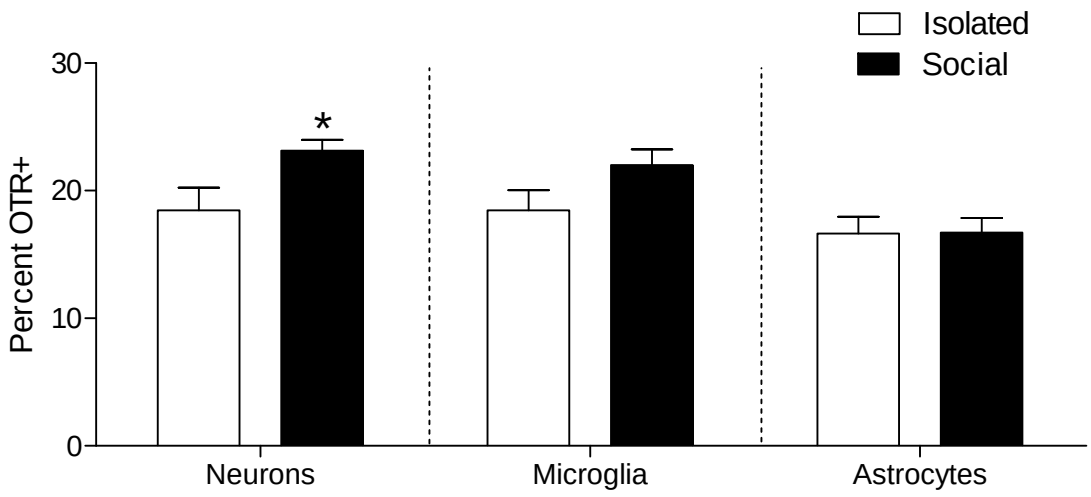

Figure 5.5: Neuronal and glial oxytocin receptor expression in socially housed and isolated mice.

Percent OTR expression in socially housed and isolated mice is shown on neuronal (NeuN-positive), astroglial (GFAP-positive) and microglial (CD11b-positive) cell populations, n = 5-6 per group. An asterisk (*) indicates a statistically significant social condition difference, ($P > 0.05$).

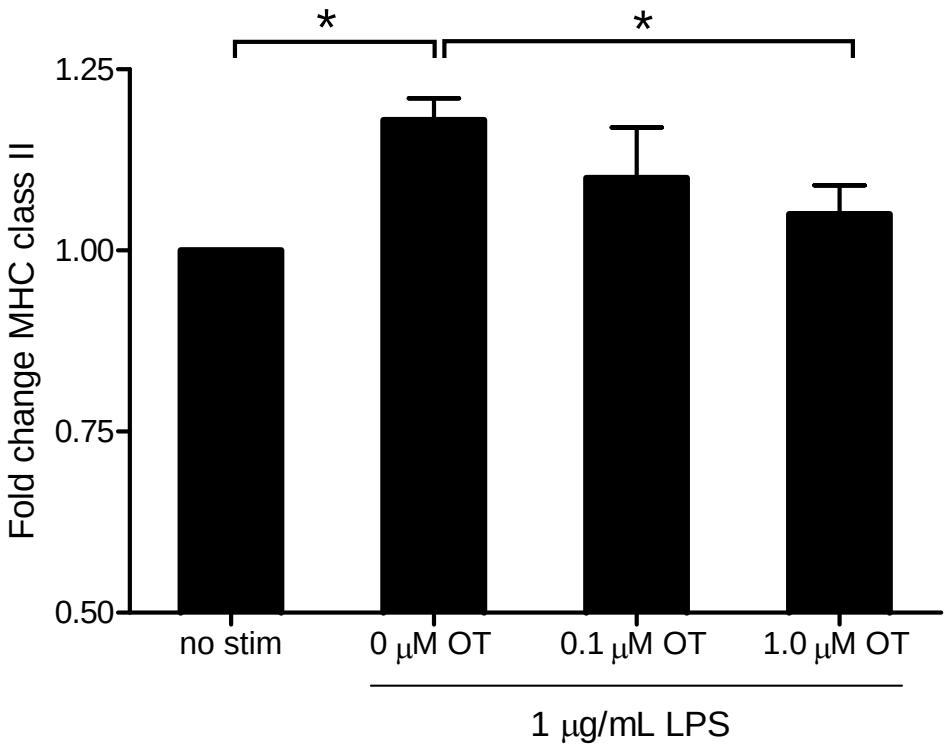

Figure 5.6: Oxytocin attenuates LPS-stimulated microglial reactivity.

Microglial MHC class II expression is up-regulated following a 24 hour LPS challenge (1μg/mL). Pre-incubation with the high dose (1 μM) but not the low dose (0.1 μM) OT attenuates LPS-stimulated MHC class II expression. An asterisk (*) indicates a statistically significant difference between indicated groups, ($P > 0.05$).

Table 5.1. Circulating corticosterone and interleukin-6 concentrations.

Group	Corticosterone (ng/mL)	Interleukin-6 (pg/mL)
SHAM	62.67 (7.44)	----------
Isolated + aCSF	295.97 (49.98)*	454.70 (127.07)
Isolated + 2ng OT	470.94 (99.49)*	----------
Isolated + 20ng OT	344.60 (78.81)*	179.35 (46.27)†
Social + aCSF	193.89 (90.00)	327.44 (61.96)
Social + 0.05µg OTA	582.83 (66.94)*#	283.70 (55.23)
Social + 0.5µg OTA	395.64 (84.96)*	----------

Shown are circulating corticosterone and interleukin-6 concentrations, mean (SEM).

* = significantly different from SHAM ($P < 0.05$)

= significantly different from Social + aCSF ($P < 0.05$)

† = significantly different from Isolated + aCSF ($P < 0.05$)

Table 5.2: Baseline and post-stroke behavioral measures

Shown are open field activity and paw preference data as measured 24 hours before surgery (PRE) and 72 hours after surgery (POST). All open field data (Total activity, Rearing activity, and activity in the Center of the open field chamber) are measured in number of photobeam breaks. Paw preference data are presented as percent contralateral paw use. All data are presented as mean (SEM). Behavioral indices were not affected by housing or treatment conditions in SHAM operated mice and are collapsed across all SHAM groups.

* = significantly different from SHAM ($P < 0.05$)

Table 5.2 Baseline and post-stroke behavioral measures

Group		Total		Rearing		Center		Paw Preference
SHAM								
	PRE	5430.80 (230.44)	PRE	409.85 (20.51)	PRE	774.95 (31.65)	PRE	47.78 (3.30)
	POST	5173.79 (263.09)	POST	376.89 (19.44)	POST	705.89 (48.90)	POST	44.06 (6.16)
SOC A.								
aCSF	PRE	4862.29 (286.01)	PRE	404.00 (42.50)	PRE	727.57 (65.96)	PRE	38.59 (5.61)
	POST	3983.71 (845.76)	POST	166.20 (46.45)*	POST	428.83 (52.62)	POST	10.00 (6.67)*
50ng OTA	PRE	6155.00 (395.45)	PRE	443.13 (49.53)	PRE	777.25 (63.24)	PRE	35.81 (5.82)
	POST	6811.71 (1687.28)	POST	29.13 (8.23)*	POST	1405.66 (317.62)	POST	4.21 (2.78)*
500ng OTA	PRE	5387.33 (309.32)	PRE	398.67 (44.80)	PRE	771.67 (57.69)	PRE	45.71 (4.27)
	POST	3925.80 (958.87)	POST	76.00 (11.84)*	POST	1140.00 (386.58)	POST	8.33 (6.18)*
ISOLATED								
aCSF	PRE	5390.75 (290.56)	PRE	422.00 (44.43)	PRE	783.88 (76.77)	PRE	42.35 (2.29)
	POST	3545.83 (527.42)	POST	87.38 (25.51)*	POST	579.71 (102.83)	POST	16.51 (7.42)*
2ng OT	PRE	5562.00 (392.36)	PRE	416.71 (38.35)	PRE	779.14 (85.75)	PRE	39.70 (2.33)
	POST	2739.60 (483.09)	POST	124.50 (45.64)*	POST	297.40 (49.48)	POST	22.46 (9.77)
20ng OT	PRE	5749.50 (281.71)	PRE	403.08 (43.93)	PRE	839.00 (51.30)	PRE	35.42 (4.42)
	POST	5057.38 (807.42)	POST	91.92 (23.93)*	POST	944.46 (214.74)	POST	27.20 (10.04)
50ng OTA	PRE	6450.50 (707.99)	PRE	522.25 (69.01)	PRE	940.50 (64.74)	PRE	39.00 (6.31)
	POST	3181.25 (986.58)	POST	11.00 (9.02)*	POST	581.00 (288.77)	POST	14.00 (7.81)*
CT+OTA	PRE	6205.17 (307.61)	PRE	421.83 (63.56)	PRE	910.17 (95.45)	PRE	51.33 (4.19)
	POST	4530.67 (2005.15)	POST	36.67 (16.03)*	POST	1060.33 (492.28)	POST	12.50 (12.50)*

CHAPTER 6

GENERAL DISCUSSION

Positive social interactions promote psychological and physiological health. While the mechanisms underlying social influences on disease outcome remain unknown, growing interest in the phenomenon of social buffering and social neuroprotection has drawn the attention of clinical and experimental research. The goals of this dissertation were to assess the phenomenon and mechanisms of social neuroprotection in a mouse model of focal cerebral ischemia.

This phenomenon of social neuroprotection has been previously described (Craft et al., 2005; Norman et al., 2010; Weil et al., 2008a); however, the specific sensory requirements for social neuroprotection had not been addressed. The studies described in **chapter 3** were designed to identify the extent to which the type and timing of social interaction are important determinants of social neuroprotection. Animals were maintained either in standard housing conditions (standard mouse cages) or larger rat cages fitted with a partition which prevented the physical contact component of social interaction. While social housing under standard housing conditions attenuated the ischemic injury and improved functional recovery, partition housing (for the entire

duration of the study) only modestly reduced infarct size and did not improve functional outcome relative to social isolation. Importantly, standard social housing for 12 days, followed by partition housing beginning 2 days prior to ischemia also reduced infarct size, but this group did not exhibit the same extent of functional recovery as was observed in the paired group in standard housing (Figure 3.2). These data speak to the sensory requirements of social interaction, particularly during the critical period immediately surrounding the ischemic event. Although all other sensory stimuli (i.e. visual, auditory and chemosensory) were preserved with the use of partition housing, completely depriving the mice from physical contact around the time of the ischemic event was sufficient to block functional recovery following stroke. Thus, the type of social interaction is a critical determinant of stroke outcome. One hypothesis regarding the social neuroprotection effect in this model is that close contact during social housing may influence locomotor activity or body temperature, both factors that impact stroke outcome. The data in **chapter 3** indicate that neither factor is affected by social housing alone (see baseline data in Figures 3.3 and 3.4). Additionally, while core body temperature was reduced in all mice that underwent cerebral ischemia, housing condition did not influence body temperature following stroke. Taken together, these data demonstrate the profound influence of social interaction on ischemic injury and functional recovery, as well as the role of the social contact component of social interaction in cerebral ischemia outcome.

The type and timing of social interaction are apparently critical determinants of both ischemic injury and functional recovery. Under standard social housing conditions, does social interaction impact the pathophysiological response to ischemic injury? Cerebral ischemia induces a cascade of pathophysiological events that converge to contribute to cell death. Among these events, the neuroinflammatory response is among the earliest, most pervasive and potentially detrimental consequences of ischemia. There are several components to the inflammatory response. The early, local response is mediated largely by resident microglia and astrocytes which, once activated, become a major source of pro-inflammatory cytokine and chemokine production (Stoll et al., 1998). Within 48 hours, this is followed by progressively increasing macrophage infiltration and activation within the injured tissue (Schilling et al., 2003). There is considerable evidence that both the local inflammatory response and infiltration of inflammatory cells contribute to infarct development and are detrimental for the ischemic brain (Lai and Todd, 2006; Streit, 2000; Yrjanheikki et al., 1999). In addition, within 48 hours, damage to the blood-brain barrier contributes to the formation of edema, or increased brain water content. Inflamed edematous nerve tissue can precipitate *s*econdary pathophysiological processes such as increased intracranial pressure that is a significant cause of neurological deficits and death. Further, these secondary inflammation-provoked injuries are particularly challenging to treat once there has been extensive blood-brain barrier breakdown (Raslan and Bhardwaj, 2007). Data in **chapter 4,** demonstrate that in addition to reducing infarct size, social housing attenuates the inflammatory response, as measured by reduced mRNA gene expression of microglial

and astrocytic markers, as well as modulation of central and circulating IL-6 content. In addition, social housing attenuated the development of cerebral edema following stroke. To the extent that uncontrolled inflammation represents a maladaptive response to stroke, identifying a mechanism for naturally occurring variation of inflammation in stroke may yield important advances in the search for effective therapies for ischemic disease. The overall suppression of inflammatory responses has proven to be a largely ineffective strategy in clinical cerebral ischemia populations (De Keyser et al., 1999; Gladstone et al., 2002; Ikonomidou and Turski, 2002). The failure of anti-inflammatory agents in clinical trials likely has several causes but key among them is that the immune and inflammatory responses after cerebral ischemia are not universally deleterious (Stoll et al., 1998; Weil et al., 2008b). A strategy aimed at maximizing the pro-survival aspects of immune responses (e.g. debris clearance and neurotrophic support) while minimizing maladaptive damaging factors (e.g. extracellular matrix breakdown and the expression of inflammatory cytokines) is likely to meet much greater success than simply blocking inflammatory mediators. Exploiting natural variation such as has been described in this dissertation may well yield the roadmap for designing these types of interventions.

Reduced inflammation, particularly altered IL-6 content, represents a proximate mediator of social neuroprotection. These initial findings left open a major question: what is the proximate mechanism that transduces social cues into a physiological signal? The indispensability of somatosensory contact for social neuroprotection as described in chapter 3 suggested the possibility that the neurohormone oxytocin (OT) was involved.

There is growing evidence that OT may be released during physical contact (Uvnas-Moberg, 1997). Indeed, OT is an ideal candidate mediator of social neuroprotection based on its up-regulation during social interactions and its known anti-inflammatory and anti-oxidant properties. As hypothesized, OT was shown to be a mediator of social neuroprotection in **chapter 5**. Treatment of socially isolated mice with OT beginning one week prior to ischemia and continuing throughout reperfusion recapitulated social neuroprotection, and OTA treatment antagonized the benefits of social housing. OT treatment was shown to reduce infarct size and improve functional recovery, as well as attenuate inflammation and oxidative stress following cerebral ischemia. These data complement findings of an anti-inflammatory and anti-oxidant effect of OT on disease states, including peripheral ischemic disease (Detillion et al., 2004; Iseri et al., 2008; Iseri et al., 2005a; Iseri et al., 2005b; Ondrejcakova et al., 2009; Özlem eri et al., 2009; Petersson et al., 1998). To our knowledge the study described in **chapter 5** is the first to establish a neuroprotective mechanism of OT action during cerebral ischemia. In addition, LPS-stimulated microglia were shown to down-regulate MHC class II activation in the presence of OT. These *in vitro* data implicate a direct anti-inflammatory action of OT on microglia. Future studies will be necessary to tease apart the role of OT on immune effector cells during cerebral ischemia.

Taken together, the data presented in this dissertation contribute to the growing interest in the phenomenon of social buffering against disease. As described in **chapter 1**, a common explanation for the social buffering effect is that individuals who experience

substantial social support may be more likely to engage in "health behaviors" (i.e. behaviors that serve to enhance health). The data presented here support the hypothesis that the benefits of social interaction extend beyond encouragement of a healthy lifestyle and indeed account for neuroendocrine mediated differences in disease pathology. Overall, the dramatic difference in ischemia outcome between socially housed and isolated mice speaks to the key modulatory effect of social interactions on disease outcome. Although assessment of social relationships is becoming a more common practice in hospitals and stroke clinics worldwide (S.I.G.N., 2002; Stone and Whincup, 1994), the full impact of this variable is not yet understood. Importantly, as data on the mechanisms of social buffering and social neuroprotection are emerging, the potential costs of neglecting environmental and social influences on disease outcome become increasingly evident. Encouraging social interaction to patients represents a feasible approach to treatment that confers profound protection with no known side effects and merits serious consideration.

A great majority of clinical trials have failed to replicate successful experimental studies (De Keyser et al., 1999; Gladstone et al., 2002; Ikonomidou and Turski, 2002). By studying the naturally occurring variability in susceptibility to stroke damage, we have identified a potential underlying factor that leads to neuroprotection. The benefits of social support appear to be mediated at least in part by OT. Substantial research remains to be conducted in order to understand the cellular/molecular mechanism of OT action during ischemic injury; however these data are an important first step toward

identifying a potential therapeutic role for OT in stroke. Synthetic OT (pitocin®) is an FDA approved agent commonly used for labor induction (Ramsey et al., 2000). Systemically injected OT does not cross the blood-brain barrier in appreciable quantiti; however, intranasal administration of OT has been tested safely for the treatment of autism spectrum disorder and schizophrenia (Bakharev et al., 1986; Hollander et al., 2003) with minimal side effects. Taken together, the data presented in this dissertation provide preliminary evidence that OT treatment might prove beneficial to stroke victims.

As previously described, social isolation potentiates response to future stressors. Basal circulating corticosterone concentrations do not generally differ by housing condition (i.e. Figure 4.8 and Table 5.1); however, stress-induced corticosterone release is exacerbated after social isolation (Weiss et al., 2004). The corticosterone data in this dissertation do not necessarily conflict with the hypothesis that social isolation stress exacerbates stroke outcome. Future studies should utilize more sensitive stress assays (i.e. corticotrophin releasing hormone and its receptors, adrenocorticotropin hormone, sympathetic nervous system output) to identify the role of social isolation stress on exacerbating stroke outcome.

Finally, another question regards the structural and functional similarities between OT and the closely related neuropeptide vasopressin. A single receptor has been identified for OT (OTR), however OT also binds the vasopressin receptor V1a and to a lesser extent V1b and V2 (Audigier and Barberis, 1985). Moreover, vasopressin exhibits

a mere 10-fold selectivity for the V1a receptor over OTR (Chen et al., 1999). This leads to the question of the role of vasopressin and its receptors in social neuroprotection. Not surprisingly, there is also evidence for a role of vasopressin in social behaviors (Donaldson and Young, 2008). More importantly vasopressin, so called because of its role as a vasoconstrictor, is implicated as an exacerbating factor in heart disease (Blair et al., 2008). Additionally, vasopressin regulates brain and serum osmolarity, and contributes to the development of edema in cerebral ischemia (Chang et al., 2006). Thus, while experimental data indicate a therapeutic potential for OT in ischemic disease (Dusunceli et al., 2008; Tugtepe et al., 2007; chapter 5), both experimental and clinical data suggest a deleterious effect of vasopressin in vascular disease. Given that both endogenous and exogenous neuropeptides OT and vasopressin bind both receptors types (Audigier and Barberis, 1985; Chen et al., 1999), before clinical therapies using OT can be developed it will be necessary to elucidate the potentially contradictory role of OT and vasopressin during an ischemic event.

By elucidating the basic mechanisms that predispose individuals to increased damage following cerebral ischemia, we can take further steps toward developing therapeutic techniques for stroke patients. The studies included in this dissertation were aimed at identifying a neuroendocrine basis by which social interaction influences stroke outcome. Taken together, these data will increase our understanding of the basic pathophysiological mechanisms underlying the influence of psychosocial interactions on stroke outcome. The MCAO mouse stroke model is sensitive to manipulations of

environment and physiological processes, making it possible to determine a causal relationship between the social environment and stroke outcome. Ultimately, understanding these processes will increase our understanding of the individual differences that contribute to the extent of ischemic injury and will fill a gap in knowledge about the relationship between psychosocial state and the neurological and functional outcome following a stroke.

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