

PSYCHOBIOLOGICAL FACTORS ALTER HEALTH OUTCOME

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of
Philosophy in the Psychology Program of The Ohio State University

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The Ohio State University
2006

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ABSTRACT

Both exposure to positive social interactions and stress can influence health outcome, via alterations in the hypothalamic-pituitary-adrenal (HPA) axis and changes in immune functioning. The first series of experiments examined the roles of social interactions and stress on wound healing and HPA axis activity in *Peromyscus* species exhibiting various social structures. Social facilitation decreased wound size in male monogamous versus male polygamous *Peromyscus*; this relationship was mediated by physical contact. Further investigation of social facilitation and stress effects on wound healing elucidated the interactions between pair housing and wound healing by examining various housing dyads. Positive social interactions facilitate wound healing in monogamous and polygynous *Peromyscus* regardless of the sex of the experimental or stimulus mouse. However, the effects of pair housing on wound healing are limited; male and female polygynous mice benefit from pair housing, but only under non-stressed conditions. Thus, differences in social systems can influence the extent to which social interactions may be beneficial to wound healing.

The second series of experiments examined the impact of endogenous glucocorticoids on physiological parameters following experimental stroke. Stress exacerbates post-stroke neuronal damage, via increases in circulating corticosteroid

concentrations and greater cerebral edema, compared to mice not experiencing stress or those allowed a temporal window between the conclusion of stress and the onset of stroke.

The third series of experiments examined the effects of experimental stroke on peripheral immune function. An augmentation in the peripheral immune response is observed following stroke; however, stroke failed to alter the rate of cutaneous wound healing. This augmentation in peripheral immune function was mitigated when neuronal damage was prevented via hypothermia. Pair housing also mitigated the effects of stroke on cell-mediated immune function and reduced wound size, regardless of the surgical condition. Restraint stress increases neuronal damage, which can lead to a decrease in humoral immune function when coupled with stress following stroke; however, stress, in the absence of neuronal damage, increases humoral immune function, thereby further adding to the literature elucidating stress effects on immune function. In conclusion, the work described in this dissertation adds to the body of literature that elucidates the relationship between social interactions, stress, and health.

Dedicated to my father, mother, and sister.

ACKNOWLEDGMENTS

“No matter what accomplishment you make, somebody helps you.”

Althea Gibson

There are so many people that I wish to thank, however, I must first give praise to the one who truly made this possible. God has blessed me tremendously while pursuing my doctorate. Without my faith, I know that I would have thrown my hands up more than a few times. I am forever grateful and will praise your name always. When praises go up, blessings truly do come down!

I must thank my immediate family. Many, many thanks to Clyde Glasper, Iantha Spalding, and Yolanda Reid. I could not have had a more supportive family. Daddy, I love you. I can see your work ethic in myself and am so thankful for these strong genes. Mom, thank you for being my cheer leader when I thought things were getting tough. You saw me through some rough ones, but I made it thanks to your motivation and support. Lon, thanks for being the best “big sis” a girl could have. You definitely inspired me to pursue a higher education and to stick it out no matter what. I am thankful for the relationship that we have developed over the years. Collectively, I thank you all for standing behind me as I pursued and finished my education. Every encouraging

word, visit, and prayer was truly appreciated throughout my journey. I hope I have made all of you proud.

True friendship is a rarity these days and I have been blessed to make and keep two dear friends throughout the years. Melanie, we have known each other for so long and have both been tried and put through the ringer while pursuing our goals. Keep going, you are almost there. To Ruth, it seems like we have known each other our whole lives. You are definitely my sister. Can you believe we made it? We did it girl!! God is so good!

I've seen a few people come and go while at Ohio State, but there have been some constants in my life for which I am thankful. Tara, you have been an encourager, a confidant, and a friend. It seems like we have hit every milestone together and I couldn't have gone through this with a better person. You are truly my friend. Tammy and Michelle, my two sister girls at OSU: Thank you for being my outlet away from the lab. We have really had some great years, from laughing until it hurts to just sitting in silence because there was nothing more to say. From coffee at Easton, to watching Oprah when we should have been working...all of those moments I will cherish forever. I will never forget those times, and will be in touch always. Good luck at your internships!

There are many people in the Nelson/DeVries lab that helped train me and make this dissertation possible. Gretchen, I thank you for your wisdom. Your guidance was most helpful and I appreciated all that you taught me. Leah, the newest mommy, thank you for teaching me many of my molecular skills. Good luck in your new position in life! I can't wait to see more pictures of your new bundle of joy. Ning, I know my dissertation would not be what it is without your surgical assistance. You are amazing

and were so willing to help me with my studies. Thank you from the bottom of my heart. Jessa, the remaining DeVries Lab member. These few years have been a joy. You have made me crack up laughing almost on a daily basis. From your overload on sugar and caffeine to your refusal to try new foods, I have enjoyed every laugh. Keep pursuing your interests and never stop asking questions. Stephanie, you have been so helpful to me since my first day in the lab. But not only that, you have been a great friend, and for that I am very thankful.

Many people have assisted me with animal care. Many thanks to Janet Crossland of the *Peromyscus* Genetic Stock Center at the University of South Carolina. Also, I have to thank Trica Uhor of The Ohio State University. To my undergraduate assistants, Ryan Blair and Amy Hasely, thank you both for your assistance with the various studies in this dissertation. You made it possible to be in two places at one time. Much success in your later years.

Finally, I must thank two people who have made my success here at Ohio State possible. Dr. Nelson, how do I say thank you for all that you have done for me. It has really been great having you as an “unofficial” co-advisor. Thanks for always popping in with your witty banter just at the right time or sending hilarious emails to start off the work day. Hopefully, I will continue to get those great emails (science related or not!) once I leave. Last, but certainly not least, I wish to thank my advisor, A. Courtney DeVries, for her encouragement, intellectual stimulation, and enormous enthusiasm. You have not only been a wonderful influence as a scientist, but you have also been a positive female role model. Thanks for seeing the potential in me from the beginning and pushing me to do my best. I can only hope to be as successful as you are!

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2. Neigh, GN, Glasper ER, Kofler, J, Traystman, RJ, Mervis, R, Bachstatter, A, & DeVries, AC. (2004). Cardiac Arrest/Cardiopulmonary resuscitation reduces dendritic spine density in CA1 pyramidal cells and selectively alters acquisition of spatial memory. *European Journal of Neuroscience*, 20(7): 1865-1872.
3. Detillion, CE, Craft, TKS, Glasper, ER, Prendergast, BJ, & DeVries, AC. (2004). Social facilitation of wound healing. *Psychoneuroendocrinology*, 29(8): 1004-1011.
4. Glasper, ER & DeVries, AC. (2005). Social structure influences effects of pair housing on wound healing. *Brain, Behavior, and Immunity*, 19(1): 61-68.
5. Craft, TKS, Glasper, ER, McCullough, L, Zhang, N, Sugo, N, Otsuka, T, Hurn, PD, & DeVries, AC (2005). Social interaction improves stroke outcome. *Stroke*, 36(9): 2006-11.
6. Neigh, GN, Glasper, ER, Bilbo, SD, Traystman, R J, & DeVries, AC. (2005). Cardiac Arrest/Cardiopulmonary resuscitation augments cell-mediated immune function

and transiently suppresses humoral immune function. *Journal of Cerebral Blood Flow & Metabolism*, 25: 1424-1432.

7. Love, GA, McNamara, I, Morgan, M, Banks, M, Wightman, N, Glasper, ER, DeVries, AC, Torrey, N, Kinsley, C, & Lambert, KG. (2005). Maternal experience produces long lasting behavioral and neuroanatomical modifications in rat, *Behavioral Neuroscience*, 119(4): 1084-1096.

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9. Craft, TKS, Zhang, N, Glasper, ER, Hurn, PD, & DeVries, AC. (2006). Neonatal factors influence stroke outcome in adult mice, *Psychoneuroendocrinology*, 31: 601-613.

FIELDS OF STUDY

Major Field: Psychology

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CHAPTER 1

INTRODUCTION

Social support in humans and affiliative behaviors in other animals can have positive effects on health (House et al., 1990; Uchino et al., 1996). In 1948, the World Health Organization defined health as the presence of well-being—physical, mental, and social—not the absence of disease (Ray, 2004). Social relationships could potentially promote health in several ways, but emphasis was placed on the role of social relationships in moderating or acting as a buffer against the potentially deleterious health effects of psychosocial stress (Cassel, 1976; Cobb, 1976). For example, individuals with high levels of social support have lower blood pressure compared to individuals with lower levels of social support (Uchino et al., 1999). High levels of social support are also associated with stronger natural killer cell responses (i.e., ability to kill susceptible tumor cells; Uchino et al., 1999). Additionally, social support improves outcome or recovery following a wide variety of human illnesses and medical conditions, including systemic lupus erythematosus (Bae et al., 2001), cardiovascular disease (Grace et al., 2002), back pain (Penttinen et al., 2002), cancer (Spiegel and Sephton, 2001), and stroke (Glass et al., 1993). Lower levels of social support are also associated with attenuated immune function (reduced cellular immunity and more days of upper respiratory infections;

Kiecolt-Glaser et al., 1991), and impaired mental and physical health compared to individuals who reported higher levels of social support (Cacioppo et al., 2000).

Despite a large literature demonstrating that social interactions modulate the hypothalamic-pituitary-adrenal (HPA) axis and improve health outcome, very little is known concerning the physiological mechanisms underlying this phenomenon. The goal of this dissertation is to describe how both positive and negative social interactions alter HPA axis activity and peripheral immune function in mouse species that exhibit various social structures. Also, this thesis will examine the effects of both positive and negative social interactions on post-stroke peripheral immune function.

What is Stress?

Activation of the HPA axis is the most thoroughly characterized neuroendocrine response to stress. Adrenocorticotropin hormone (ACTH), corticotropin releasing hormone (CRH), and glucocorticoids, such as cortisol (or corticosterone in rodents), are the three primary hormones of the HPA axis. Collectively, these three hormones are often referred to as “stress” hormones because they increase following stress and return to basal concentrations after the termination of the stressor. Nonetheless, these hormones are critical even under non-stressful condition, as they are essential in maintaining homeostasis and energetic balance (McEwen and Wingfield, 2003; Sapolsky et al., 2000).

The term “stress” was originally used in the field of engineering to describe a force that exerts physical strain on a structure. The profound physiological consequences of stress in the biological sense were first shown empirically in Hans Selye’s seminal

paper (Selye, 1936). He reported that exposure to stressors caused adrenal gland enlargement, atrophy of thymus and lymph nodes, increased cardiovascular tone, immune-system suppression, and ulcerations.

Although the term stress has become part of the vernacular, no consensus has been reached for a precise definition of biological stress in the literature (McEwen, 2000). Often, elevated concentrations of HPA axis hormones are used as indices of stress and the presence of stressors. Stressors can be either physical or psychological. The important parameter is the organism perceives and reacts to the stressor (Kim and Diamond, 2002). In general, a stressor is defined as any stimulus that increases HPA axis activity. However, this definition varies from researcher to researcher; a cardiovascular researcher may define a stressor as something that increases heart rate and blood pressure (Burleson et al., 2002). With this in mind, the definition of stress used throughout this thesis will be any threat, real or implied, to homeostasis that results in an activated HPA axis (McEwen and Wingfield, 2003).

Following a short exposure to a stressor, there are measurable increases in CRH released from the hypothalamus, which in turn causes the release of ACTH from the anterior pituitary. ACTH then stimulates the release of glucocorticoids from the adrenal cortex. Proper regulation of the HPA axis via negative feedback at the levels of the hypothalamus and anterior pituitary minimizes the effects of long-term exposure of glucocorticoids, which can have deleterious effects (Sapolsky et al., 2000).

Acute versus Chronic Stressors

Although stress is generally thought to be harmful and damaging to an organism, many recent studies have illuminated both the beneficial and detrimental effects of a

stressor on an organism. In general, the stress response is potentially adaptive in the short-term, but maladaptive in the long-term (Nelson, 2005). Acute stressors produce a stress response that aims to maintain homeostasis, or maintains a range of physiological parameters essential for survival when the organism is challenged by an external and internal stimulus (Bartolomucci et al., 2005). This is adaptive in many ways; however, when these acute responses are overused or inefficiently managed, due to chronicity, damage may occur (McEwen, 1998). Yet, there is no definitive point at which a stressor switches from being acute to chronic. Likewise, the transition from acute stress to chronic stress is usually dependent on the type of stressor used (Armario et al., 1990).

Every system of the body responds to acute and chronic challenges by trying to maintain balance (McEwen, 2004). For example, acute stress promotes immune function by enhancing the movement of cells to places in the body where they are needed to defend against the possibility of an invading pathogen (Dhabhar, 2000; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1996; Dhabhar et al., 1994). Chronic stress, however, suppresses immune function via use of hormonal mediators (Dhabhar, 2000; Dhabhar and McEwen, 1997; Millan et al., 1996). Similar effects are observed in the cardiovascular system, where blood pressure rises and falls throughout the day to meet daily challenges, such as physical and emotional demands; however, repetitive elevations of blood pressure may generate atherosclerotic plaques that cause damage to coronary walls (Manuck et al., 1988). Metabolism responds to stressors in a similar fashion. Glucocorticoids are so named because of their ability to promote the conversion of protein and lipids into usable carbohydrates, which can benefit the body in the short run. Inactivity and lack of energy expenditure, however, can create a situation where chronic

elevations of glucocorticoids can promote increased food intake (McEwen, 2004) and the deposition of body fat (Brindley and Rolland, 1989). Lastly, the nervous system is able to influence all other physiological systems, thereby regulating the response to daily stressors. Acute exposure to glucocorticoids improves memory; however, if exposure is prolonged or repeated over many weeks, neuronal atrophy results (decreased dendritic branching and reduced numbers of neurons in the dentate gyrus of the hippocampus) resulting in impaired memory (McEwen, 2004). Therefore, duration determines the beneficial or detrimental effects of the stressor on an organism.

Stress and the Social Environment

Social interactions can be a source of conflict, stress, and tension. Social stress is a recurring factor in the lives of almost all vertebrate species, and because of this, social factors are a key stimulus for the evolution of stress mechanisms (Sapolsky, 1992). Several recent studies have shown that chronically induced activation of the HPA axis leads to dysfunction of physiological systems. For example, aggressive social interactions produce higher and more prolonged corticosterone concentrations following defeat compared with traditional psychological and physical stressors (Bailey et al., 2004; Engler et al., 2005; Haller et al., 1999; Koolhaas et al., 1997; Sgoifo et al., 1999). Studies have examined the relationship between social status and HPA axis activity. Much of the studies involving animals and social stress have focused on the influence of rank; specifically, animals of different ranks experience different patterns of stress (Sapolsky, 2005). It was originally postulated that animals that were higher in ranking experienced the most stress, whereas those lower in ranking experienced the least stress (Brady et al.,

1958). However, after examining a wide range of species it has become clear that rank means different things in various species. It is now understood that ranks that experience the most physical and psychological stressors tend to exhibit the most stress-related disorders (Sapolsky, 2005).

In some species, rank is inherited, thereby lifelong, and limits the amount of social unrest that would bring about stress and stress-related pathologies to those who are dominant. It is the subordinate that experiences the greatest stress (Virgin and Sapolsky, 1997). Yet, under periods of hierarchical instability, the dominant individual is usually at the center of the tension and typically experiences the greatest amount of stress (Sapolsky, 2005). In this case, however, once stability returns, it is the subordinate that again experiences the greatest physiological indices of stress (Sapolsky, 1993). It appears that the mechanism behind increased physiological indices of stress (increased cortisol concentrations) observed in subordinate males is due to a dysregulation of the HPA axis negative feedback system at the level of the central nervous system (Sapolsky, 1989; 1983), as a result of a change in set point in the neuroendocrine regulation of the adrenocortical axis (Abbott et al., 2003).

In contrast, among species in which rank shifts, frequently dominant animals behave aggressively toward lower ranking individuals, even in the absence of a challenge to the hierarchy. Examples include dwarf mongooses, African wild dogs, and ring-tailed lemurs, in which the dominant individuals routinely exhibit greater indices of stress from subordinant individuals, reflecting the physiological demands of frequent fighting (Cavigelli, 1999). In other species where dominance is maintained by non-aggressive

means (i.e., eye contact; common marmosets and cotton top tamarins), the subordinate rank is associated with the greatest physiological indices of stress (Abbott et al., 1998; Bercovitch and Clarke, 1995; Cavigelli, 1999; Sapolsky, 1990).

Mouse models of chronic psychosocial stress have proven to be very useful experimental models of social stress, exploiting the natural behavior of male mice (i.e., acquiring and defending a territory; Bartolomucci et al., 2001). Chronic social defeat results in prolonged activation of the HPA axis, which results in many physiological and behavioral alterations. Motor activity is the behavior most frequently studied in animal models of chronic stress. Subordinate mice show decreased activity, while dominant mice slightly increase their home cage motor activity (Bartolomucci et al., 2003a; Kramer et al., 1999). This paradigm results in chronically elevated concentrations of glucocorticoids and a dysregulation of HPA axis regulatory feedback (McEwen, 2000; Sapolsky, 1992). Mice experiencing social defeat also experience reductions in aggressive-like behaviors as well as decreases in food and water intake (Albonetti and Farabollini, 1994; Potegal et al., 1993). Immunologically, when mice are exposed to chronic social defeat, a reduction in *in vitro* lymphocyte proliferation, T helper cell numbers, and natural killer cell (NKC) activity, as well as cytokine and antibody production, is observed (Fleshner et al., 1989; Gryazeva et al., 2001; Raab et al., 1986; Stefanski, 2001; 1998; Stefanski et al., 1996). Reductions in immune function have also been observed in hamsters that have experienced chronic social defeat; specifically, reductions in anti-keyhole limpet hemocyanin (KLH) immunoglobulin G (IgG) antibody production (Jasnow et al., 2001). The interaction between chronic stress and immune function is further elaborated below. Defeated animals display changes in endocrine

function as well. Increased HPA axis activity usually accompanies chronic social defeat, as evidenced by increased release of ACTH, β -endorphin, cortisol, and corticosterone concentrations (Blanchard et al., 1995; Huhman et al., 1990; Huhman et al., 1991; Huhman et al., 1992; Jasnow et al., 2001; Kramer et al., 1999; Spencer et al., 1996).

Stress and Immune Function

It is commonly believed that stressful experiences suppress the ability of the immune system to respond to challenges and increases susceptibility to infectious disease. Most of the original studies investigating the effects of stress on immune functioning focused exclusively on the immunosuppressive effects. Recent studies, however, have provided evidence that the immune system responds differently to various stressors. To better understand the divergence in the data, stressors have been qualified as either acute or chronic. These two distinctions are based on the duration and intensity of the stressor. Acute stress usually lasts for a period of minutes to hours and has enhancing effects on the immune system, while chronic stress usually lasts for days to months and has suppressing effects on the immune system (Dhabhar, 2000).

Given the differences in the types of stressors, specifically the duration and magnitude, it is not surprising that there are paradoxical observations regarding the effects of stress on immune function. Two paradoxes are presented when reviewing the literature examining the effects of stress on immune function (Dhabhar, 2000). First, it is perplexing that an organism would evolve to suppress immune function during stressful times, when this is the time that the organism needs an active immune response to survive and fight off infection. Stress is thought to suppress immunity and increase susceptibility

to infections and cancer (Cohen and Herbert, 1996; Cohen et al., 1997; Cohen et al., 1991). Secondly, stress has also been reported to exacerbate inflammatory disease (Chida et al., 2005; Sieve et al., 2004); however, glucocorticoids, or stress hormones, are used clinically to treat these diseases. With these paradoxes in mind, the following will review studies examining varying conditions of stress and how they may enhance or suppress immunity.

Acute Stress Effects on Immune Function

Although many Psychoneuroimmunology studies have focused on the immunosuppressive effects of stress, many studies have reported increases in immune function following acute stress. Acute stress-induced enhancement of immune function may be adaptive, preparing the organism for potential challenges to the system (Dhabhar and McEwen, 1997; 1999; Dhabhar and McEwen, 1996). For example, adrenal steroids are the major mediators of leukocyte redistribution (Dhabhar et al., 1996). During a stress response, several hormones and neurotransmitters allow an organism to mobilize its resources to cope with a challenge. One of the effects at the level of the immune system is that cells of the immune system move from their storage areas (e.g., blood, spleen) to areas where they are needed (e.g., bone marrow, lymph nodes, skin) and can exert their function. Because the skin is one of the targets to which leukocytes travel during stress, it was hypothesized that stress may increase immune surveillance in the skin and improve immune function following challenge.

Using an *in vivo* test of cell-mediated immunity, Dhabhar and colleagues used a rodent model of the delayed-type hypersensitivity (DTH) response. Rodents were sensitized with an antigen on the dorsum. This is termed the sensitization phase of the

DTH response, where the organism develops an immunologic memory (through the generation of T cells) for the antigen (Dhabhar, 2000). Following sensitization, the ability for the organism to mount a response against the antigen is examined by administering a smaller amount of the antigen on the dorsal portion of the pinna (i.e., ear). This phase of the response is known as the challenge phase, which involves recruitment of memory T cells developed during the previous sensitization. The DTH response was measured as an increase in pinna thickness. Acute stress administered immediately prior to challenge and at the time of sensitization resulted in an increase in the DTH response (Blecha et al., 1982; Dhabhar and McEwen, 1999; Dhabhar and McEwen, 1996; Dhabhar and Viswanathan, 2005; Viswanathan et al., 2005). This finding is in support of the adaptiveness of the acute stress response—the skin is one of the primary routes of access to the body for a pathogen—promoting immunoprotection in case of wounding, infection, or vaccination. Immune responses during acute stress tasks are thought to be mediated by the HPA axis (McEwen, 2000). Exposure of rodents to acute stress has been shown to increase mitogen-induced T-cell proliferation (Bauer et al., 2001) and both T-cell dependent (Millan et al., 1996; Silberman et al., 2003) and independent antibody production (Millan et al., 1996).

Likewise, there is much evidence that experimental acute stressors activate the immune system in humans. Previous studies have reported that the number of natural killer (NK) cells and NK cell activity increase during acute stress tasks, such as mental arithmetic (Burleson et al., 1998; Delahanty et al., 1996; Edwards et al., 2005; Isowa et al., 2004; Pike et al., 1997; Wang et al., 1998; Willemsen et al., 2002), cold pressor tests

(Delahanty et al., 1996; Isowa et al., 2004; Willemsen et al., 2002), public speech (Burleson et al., 1998; Marsland et al., 2001; Mills et al., 1996; Wang et al., 1998), and the Stroop test (Bachen et al., 1992).

Chronic Stress Effects on Immune Function

As mentioned previously, most studies have examined the immunosuppressive effects of stress. An excellent study performed on first-year medical students revealed that the stress of exams weakened the students' immune systems and led to more infections and illness (Kiecolt-Glaser et al., 1984) compared to times of vacation. Other human studies investigating the effects of chronic stress on immune function revealed interesting results as well. The chronic stress of caring for a loved one with dementia resulted in immunosuppression; IgG antibody titers to the pneumococcal vaccine fell over a 6-month period in the caregivers compared to no change in IgG antibody titer among control and former caregiver subjects (Glaser et al., 2000). Among parents of cancer patients, dexamethasone's (a synthetic glucocorticoid antagonist) capacity to suppress interleukin (IL) -6 production was significantly reduced compared with parents of healthy children (Miller et al., 2002). This study, along with others, suggests a novel mechanism through which chronic psychological stress could influence the onset and/or progression of conditions that involve excessive inflammation.

In contrast to acute stress enhancing the skin DTH response to antigen challenge, chronic stress decreases the skin DTH response to antigen challenge in mice (Dhabhar and McEwen, 1997). Chronic immobilization stress has been found to have a major impact on immune function, causing changes in the normal distribution of immune cells (Dhabhar and McEwen, 1997; Dhabhar et al., 1995; Dominguez-Gerpe and Rey-Mendez,

2001) and also decreasing proliferation of immune cells (Wang et al., 2002). Chronic social stress has also been linked to increased susceptibility to infection in non-human primates; specifically, unstable social environments influence the probability of developing infection after viral exposure (Cohen et al., 1997). The chronic stress of an illness, such as Theiler's virus, an animal model of multiple sclerosis, has revealed more compelling evidence that chronic stressors suppress the immune system. Four weeks of restraint stress (Sieve et al., 2004) or repeated social disruption stress (Johnson et al., in press, 2006) prior to the onset of the demyelinating condition developed a worse course of the disease compared to mice not experiencing restraint. Noise exposure, a highly relevant environmental and clinical stressor, has also been linked to immunosuppression. Three weeks of chronic, intermittent, unpredictable noise resulted in suppressed splenic NK cells compared to control rats (Van Raaij et al., 1996).

Chronic stress delays wound healing in studies using rodents (Mercado et al., 2002a; Padgett et al., 1998a) through a mechanism that involves reduced inflammation. The delay in healing is significantly correlated with serum corticosterone concentrations (Padgett et al., 1998a). This suggests that disruption of neuroendocrine homeostasis modulates wound healing. The influence of chronic stress on wound healing has also been studied in humans. Psychological stress causes the dysregulation of cytokine production. Women caring for a relative with Alzheimer's disease had wounds that healed more slowly (required 24% longer) than age-matched controls (Kiecolt-Glaser et al., 1991). Furthermore, women who reported higher levels of life-stress exhibited lower production of pro-inflammatory cytokines at blister wound sites than women who reported low levels of life-stress (Glaser et al., 1999).

Given the numerous studies examining the effects of chronic stress on immune function, it is reasonable that many have investigated mechanisms controlling stress-induced decreases in immune function. Consequently, adrenal steroids have been reported as mediating the bidirectional effects of stress on immune function. Chronic stress reduces wound cellularity and delays wound closure (Broadbent et al., 2003) through a mechanism that involves stress-induced increases in circulating corticosteroid concentrations. In mice, chronic restraint stress delayed wound closure by 3.1 days as compared to controls. An increase in serum corticosterone concentrations is correlated with delayed wound healing. Chronic stress resulted in the activation of the HPA axis and sustained elevations in serum corticosterone concentrations (Detillion et al., 2004; Padgett et al., 1998a). Indeed, studies have shown that treatment with dexamethasone, a high-affinity synthetic glucocorticoid, also delays wound healing (Gordon et al., 1994; Hubner et al., 1996). Mice treated with dexamethasone, for one week prior and either three or five days after wounding, exhibited reduced expression of pro-inflammatory cytokines, which are necessary for repair of damaged tissue (Hubner et al., 1996). On the contrary, removal of glucocorticoids has the opposite effect. Siberian hamsters (*Phodopus sungorus*) undergoing adrenalectomy 48 h prior to wounding displayed decreased wound size as compared to SHAM-adrenalectomized hamsters, indicating that wound healing is inhibited due to stress-induced secretions of cortisol (Detillion et al., 2004). Likewise, treatment with a glucocorticoid receptor antagonist (RU40555) prior to the onset and throughout the course of the stress paradigm (Padgett et al., 1998a), ameliorates the effects of stress on wound healing.

Even though the biphasic effects of stress on immune function have been examined in numerous studies, the model does not sufficiently explain findings linking chronic stress with disease processes associated with inadequate immunity (infectious and neoplastic disease) and disease outcomes associated with excessive immunity (allergic and autoimmune disease; reviewed in (Segerstrom and Miller, 2004). Some researchers have chosen to resolve this paradox by focusing on how chronic stress might shift the balance of the immune response. A well known interpretation is that chronic stress elicits both simultaneous enhancement and suppression of the immune response by altering patterns of cytokine secretion (Marshall et al., 1998). Th1 cytokines, which activate cellular immunity to provide defense against many kinds of infections and some kinds of neoplastic disease are suppressed by chronic stress. This suppression has permissive effects on production of Th2 cytokines, which activate humoral immunity and exacerbate allergy and many kinds of autoimmune diseases. A shift in Th1/Th2 balance can occur in response to stress. Glucocorticoid hormones differentially modulate the expression of cytokines, resulting in a polarized shift of T-cell responses to the Th2 subset (Elenkov et al., 1996; Glaser et al., 2001; Viveros-Paredes et al., 2006). The polarization of T-helper cell responses can be driven *in vivo* by the stress-induced elevation of endogenous glucocorticoid hormones (Dobbs et al., 1996). A Th1-to-Th2 shift changes the balance of the immune response without necessarily changing the overall level of activation or function within the system. Because a diminished Th1 mediated cellular immune response could increase vulnerability to infectious and neoplastic disease, and an enhanced Th2 mediated humoral immune response could

increase vulnerability to autoimmune and allergic diseases, this cytokine shift model also is able to reconcile patterns of stress-related immune changes with patterns of stress-related disease outcomes (Marshall et al., 1998).

Social Facilitation of the Stress Response and Immune Function

It has long been noted that there is a connection between social relationships and health. In 1948, the World Health Organization defined health as the presence of well-being—physical, mental, and social—not the absence of disease (Ray, 2004). Today, a number of studies have examined the effects of social support and health outcome; however, social support is not easily defined or measured. Generally, social support characterizes the environment or those who surround individuals in their network (Helgeson, 2003). It has also been defined as “the experience or information that one is loved or cared for, valued, and esteemed, and [the ability] to count on others should the need arise” (Cobb, 1976). People who have strong perceived social support systems tend to have a lower incidence of physical and mental disorders than counterparts who are socially isolated or less socially integrated (Uchino et al., 1996). Other studies have found evidence of relationships between perceived loneliness and survival (Herlitz et al., 1998; Seeman, 2000) and loneliness and cancer (Fox et al., 1994).

Small social networks, few close relationships, and low perceived adequacy of social support are linked to depressive symptoms (Hashimoto et al., 1999; Heinrichs et al., 2003; Kawachi and Berkman, 2001; Mohr and Genain, 2004). Those with large social networks are also more likely to live longer compared to those with small social networks (Lyyra and Heikkinen, 2006; Mookadam and Arthur, 2004). Specifically, when all

factors were controlled in an early study exploring relationship size and an individual's social network and that individual's health and chance of dying, the probability of an individual's death over nine years was related to the size of his or her social system (Berkman and Syme, 1979). Similarly, the level of perceived social support was predictive of improved functioning and quality of life in stroke survivors and those with less social support declined in functional ability and quality of life (Boden-Albala et al., 2005; Glass et al., 1993; Gottlieb et al., 2001; Tsouna-Hadjis et al., 2000). Health behaviors have a large effect on morbidity and mortality; however, studies examining the relationship between social isolation and health behaviors have not found health behaviors to account for the differences in health outcome between the socially connected and the socially isolated (Cacioppo et al., 2002; Seeman, 2000).

Experimental studies also have demonstrated the detrimental effects of isolation on an organism's health outcome. Social isolation has been shown to exacerbate autoimmune disease and reduce survival rate (Chida et al., 2005; Kerr et al., 1997), as well as impair immune functioning; specifically, studies from our lab have demonstrated reduced rates of wound healing in socially isolated animals compared to pair housed animals (Detillion et al., 2004; Glasper and DeVries, in review; 2005; Martin et al., 2006) and larger infarcts following stroke (Craft et al., 2005). Separation studies, where individuals are removed from social housing conditions and placed in social isolation also results in alterations in HPA axis activity, as well as other detrimental outcomes, such as stress-induced immunosuppression (Castro and Matt, 1997; Glasper and DeVries, 2005; Martin et al., 2006; Mendoza and Mason, 1986; Shanks et al., 1994; van Reenen et al., 2000; Westenbroek et al., 2005; Wu et al., 2000).

Social Buffering of Stress

Cohen and Wills newly emphasized the idea that social environments could have strong implications on health outcome and general quality of life (Cohen and Wills, 1985). There were two different hypotheses formulated to explain social influences on health: 'main effects' versus 'stress-buffering'. The main effects hypothesis described that the more social support an individual has, the better the quality of life, regardless of the level of stress experienced by the individual. This posited a linear relationship between social support and quality of life. The stress-buffering hypothesis; however, described that the relationship between social support and quality of life depends on the individuals level of stress. Specifically, if there is no stress or very little stress, then social support is unrelated to quality of life. However, under situations of high stress, social support would serve as a buffer against the adverse effects of the stressor (Cohen and Wills, 1985). Given this explanation, one could reason that an individual facing high stress with support is almost as well off as an individual not experiencing the stressor. This hypothesis was tested in Chapter 2, where I investigated the effects of stress and social facilitation in three species of mice, two demonstrating monogamous social structure and one demonstrating polygynous social structure.

To the contrary, not all social interactions have positive effects on health. Depending on the circumstances, social interactions can provide a buffer against the deleterious effects of stress or can become a source of stress. This was clearly evidenced in a study examining college students with or without their romantic partners, comparing how people who had a secure versus an insecure attachment style responded to stress (Carpenter and Kirkpatrick, 1996). Insecure respondents increased in blood pressure and

heart rate when their partner was present. Thus, the insecure persons did not benefit by having their partner present. Whether or not an individual benefits from social interactions depends on many factors, including the type and nature of social interaction and the individual's status. Therefore, not all social interactions have positive effects on health outcome.

Indeed, social stress can increase the incidence or severity of many diseases and health conditions. Chronic social stress in mice can lead to the reactivation of latent herpes simplex virus (Padgett et al., 1998b) and an increased susceptibility to bacterial endotoxic shock (Quan et al., 2001). Non-human primate models demonstrate increases in simian immunodeficiency virus (SIV; Capitanio et al., 1998) and upper respiratory infections following chronic social stress (Cohen et al., 1997). Social stress also affects non-infectious conditions such as increase neuronal death (DeVries et al., 2001a; Fuchs et al., 2001; Kozorovitskiy and Gould, 2004; Sugo et al., 2002) or impaired functional recovery following stroke in mice (Sugo et al., 2002). Humans also are susceptible to the negative effects of stress on health. Chronic life stress has been shown to delay recovery following an acute psychological stressor (Pike et al., 1997). Likewise, chronic stress impairs the immune system's response to anti-inflammatory signals (Miller et al., 2002).

Mechanisms Underlying Social Buffering of the HPA Axis

The physiological mechanism through which social support improves health in humans is not known; however, hypotheses concerning the psychophysiologic basis have been well investigated (Bovard, 1985). Oxytocin (OT) is a behaviorally active peptide

hormone that is probably best known for the role it plays in lactation, suckling, mating, maternal behavior, and pair-bonding (Carter et al., 1995; Febo et al., 2005; Ferguson et al., 2002; Insel and Young, 2001). However, OT is also associated with suppressed HPA axis activity (DeVries, 2002; DeVries et al., 2003). Most studies have examined lactating females and the ability of lactation to dampen HPA axis activity to physical or psychological stressors. For example, nipple stimulation in lactating and non-lactating women is associated with an increase in OT concentrations, whereas decreasing ACTH and cortisol concentrations (Amico et al., 1994; Chiodera et al., 1991). Lactation failed to alter the endocrine profile of women on the Trier Social Stress Test (TSST); however, speech preparation and delivery during the TSST caused an equivalent increase in anxiety and plasma concentrations of ACTH and cortisol in both lactating and non-lactating women (Altemus et al., 2001). On the contrary, a similar study investigating women who breastfed their infants 30 min prior to the TSST exhibited a significant increase in epinephrine, norepinephrine, and cortisol concentrations in response to a stressor; however, their cortisol concentrations were attenuated compared to women who did not breastfeed and those that just held their infants without breastfeeding (Heinrichs et al., 2001).

Recently, the effect of positive social contact was reported to alter the release of OT has been investigated. Not surprisingly, high partner support was related to elevated OT in both men and women, with OT having greater potential for cardioprotective effects in women (Grewen et al., 2005). Taken together, these clinical studies suggest that lactation dampens HPA axis reactivity to stress during positive social interactions. With these findings, lactation and social support provide a basis to study the physiological

relationship between OT and glucocorticoids. A single intranasal OT administration, combined with social support, decreases cortisol concentrations in response to psychological stress in humans (Heinrichs et al., 2003). Furthermore, social interaction facilitates wound healing through a mechanism that involves dampened HPA axis responses to stress (Detillion et al., 2004) by way of OT. OT is also able to provide a buffer against chronic stress in the absence of social support. Chronic intranasal administration of OT prior to acute social isolation attenuates the ACTH response to stress in monkeys; however, no effect of OT was observed on the cortisol response to stress (Parker et al., 2005).

Pharmacological studies in men suggest that OT suppresses HPA axis activity at the levels of the pituitary and adrenal glands (Chiodera et al., 1991; Legros et al., 1988), which may explain why peripheral injections of OT are successful in suppressing the HPA axis, presumably by decreasing the release of glucocorticoids, such as cortisol or corticosterone. The rapid actions of glucocorticoids on magnocellular neuroendocrine cells of the hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN) demonstrate how glucocorticoids have fast inhibitory effects on neurohypophyseal OT and vasopressin (VP) secretion, and how this secretion is mediated by steroid actions similar to those seen in PVN parvocellular neuroendocrine cells (Di et al., 2005). Glucocorticoid-induced suppression of glutamate synaptic input occurs in both oxytocinergic and vasopressinergic magnocellular neurons, which suggests a generalized rapid inhibitory feedback role of glucocorticoids in the regulation of magnocellular peptide hormone secretion.

Focus of Theses Experiments

Given the influences that positive and negative social interactions have on immune function and how social support can provide a buffer against the negative effects of stress on immune function, the first half of my dissertation examines how varying social structures and stress can alter the healing rate following wounding in numerous species of *Peromyscus*, which display different social systems. The latter half of my dissertation focuses on physiological outcomes following stroke. Specifically, I characterized central inflammation and peripheral immune function following stroke in mice. Following this characterization, I aimed to mitigate the negative effects of stroke on immune function using positive social interactions. I also aimed to exacerbate the negative effects of stroke on peripheral immune function using restraint stress prior to stroke.

Monogamy

Monogamy is commonly defined as a prolonged social relationship between one adult female and one adult male for the purpose of reproduction (Moller, 2003). Monogamy is particularly common in birds (90% of species; Lack, 1968), but occurs infrequently in invertebrates, fish, amphibians, reptiles, and mammals (<3%; Carter et al., 1995; Kleiman, 1977). There are three general categories of monogamy (Reichard, 2003). Sexual, or reproductive monogamy, that is defined as an exclusive relationship between a female and a male based on sexual monogamy. Genetic monogamy, defined as male-female relationships when DNA analyses can confirm that a male-female pair reproduces exclusively with each other. The form of monogamy that is of primary

concern in this dissertation is social monogamy, which refers to a male and female social living arrangement, but does not necessarily imply a sexually exclusive relationship.

Social monogamy does not evolve from a common, single origin, but arises independently through various evolutionary pressures and along different pathways in numerous lineages (Reichard, 2003). Social monogamy in birds and mammals represents just one possible outcome of the compromise between the reproductive interests and strategies of the sexes under specific conditions. The three components that influence the occurrence of social monogamy are: the amount of paternal care necessary for successful rearing of young, the mode of resource access, and mate choice (Chapman and Partridge, 1996; Davies, 1992; 1991).

Social monogamy is likely to become the reproductive strategy of choice when females within a species cannot rear young successfully without the direct help of a male (i.e., carrying, feeding, warming, defending). The importance of biparental care in the evolution of social monogamy has attracted a great deal of attention because most socially monogamous species exhibit this characteristic. Indeed, the need for biparental care also has been suggested as one of the important factors explaining the prevalence of social monogamy in humans; most human cultures practice polygyny (Barash and Lipton, 2001).

For the purpose of this thesis, monogamy in rodents is characterized by an adult male and female pair sharing a nest and home range, preferential copulating with the mate, males participating in parental care, and vigorous defending of the nest against intruders (Dewsbury, 1987). Alternative forms of rodent social organization include polygamy, defined as cohabitation with multiple mates, and promiscuity, characterized

by an absence of long-term social relationships. As mentioned before, a number of factors influence monogamy: need for biparental care, mode of resource access, and mate choice. However, neuroendocrine mechanisms influence the behavioral components of monogamy. Microtine rodents have been thoroughly studied to understand the substrates underlying monogamy. Species within the genus *Microtus* display diverse forms of social organization (Young et al., 1998). The prairie vole, *Microtus ochrogaster*, is highly social and forms long lasting pair bonds (Carter and Getz, 1993), while the montane vole, *M. montanus*, is relatively asocial and breeds promiscuously (Jannett, 1980). There are many modulators of social preference in prairie voles. The effects of stress and corticosterone on partner preference in prairie voles are sexually dimorphic (DeVries et al., 1996). Socially naïve females form preferences with a novel male when corticosterone concentrations are low. In contrast, male prairie voles form partner preferences after exposure to a stressor, when circulating corticosteroids are higher (DeVries et al., 1995). Central CRF has also been demonstrated to modulate pair bonding in the monagamous prairie vole (DeVries et al., 2002; Lim et al., 2005). Prairie voles usually establish pair bonds as a result of mating. Because vagino-cervical stimulation in mammals results in central release of OT, it was hypothesized that prairie vole mating stimulated OT release and would facilitate attachment. Other hormones (VP) and a neurotransmitter (dopamine) also are involved in partner preference in prairie voles. VP, but not OT, directly facilitates partner preference in male prairie voles (Winslow et al., 1993), while OT facilitates partner preference in female prairie voles (Williams et al., 1994), potentially by reducing circulating corticosteroids. Dopamine

seems to play a role in the reward and reinforcement of social bonding in both male and female prairie voles. In males, the interaction between dopamine and corticosteroids may contribute to stress-induced pair bonding (Young et al., 2001).

Peromyscus

Peromyscus are a very useful group in which to study social behaviors, because the different populations of *Peromyscus* display various social behaviors (Wolff, 1989). Above all social behaviors, social monogamy is by far the most uncommon in the *Peromyscus* species, with only four of nine *Peromyscus* displaying monogamous behaviors. The most thoroughly described monogamous species within the genus *Peromyscus* is *Peromyscus californicus*. This species is both socially and reproductively monogamous (Ribble, 2003). Male care is critical for offspring survival and is the salient feature of monogamy in this species. In nature, mated pairs of *P. californicus* remain together as long as both members remain alive (Eisenberg, 1963; Ribble, 1991; Ribble, 1992). Most re-mate only after their first mate dies (Ribble, 1991).

As is the case with *P. californicus*, biparental care is an important characteristic of this socially monogamous species. In an experimental study performed under natural conditions, the need for male *P. californicus* in offspring survival was confirmed (Gubernick and Teferi, 2000). Significantly fewer offspring survived weaning if the father was removed compared to when the father was allowed to remain with the dam and pups through weaning. Warming of the pups by the male is essential to survival in *P. californicus* (Ribble, 2003). *P. eremicus* exhibit several features of monogamy, including high tolerance of pups and increased social contact with mates (Ribble, 2003). Furthermore, in the field, male-female *P. eremicus* pairs do form under some

circumstances, but the associations are not necessarily enduring (Eisenberg, 1963). These data suggest that *P. eremicus* exhibit a social system that resembles facultative monogamy. *P. leucopus* are one of the only two *Peromyscus* species characterized as polygynous (Ribble, 2003). This species is known for its roving spacing of territories, where one male overlaps with many female territories; there is no evidence for male-female nest sharing or paternal care in this species. If females can successfully wean progeny without paternal care, then mating with several females can increase the reproductive success of the male in this species (Davies, 1992). Given the differences in social structure in these three species, studying the effects of social structure on health outcome proves beneficial to the greater understanding of the relationship between social support and health.

Wound Healing

The involvement of the immune system in cutaneous wound healing protects the wound site from infection and also prepares it for the repair, or healing, process. The healing of cutaneous wounds normally progresses through three stages: [1] the inflammatory stage; involving platelet aggregation, blood coagulation, and migration of inflammatory cells to the wound, [2] the proliferative phase; which involves migration and proliferation of keratinocytes, fibroblasts, and endothelial cells and leads to re-epithelialization and granulation tissue formation; and [3] a long remodeling phase (Hubner et al., 1996).

Immune function plays a critical role in this three-step process. Pro-inflammatory cytokines, such as IL-1 and tumor necrosis factor (TNF) are essential factors during the inflammatory stage of the healing process. These cytokines help protect the wound

against infections and prepare the tissue for phagocytic cell recruitment and activation (Lowry, 1993). IL-1 can regulate the production, release, and activation of metalloproteinases that are important in the remodeling of the wound. IL-1 also stimulates the production of other cytokines important for wound healing, such as IL-2, IL-6, and IL-8 (Lowry, 1993). Stress has been demonstrated repeatedly to negatively influence wound healing, an integrative measure of immune functioning. It is the inflammatory stage of wound healing that is most likely altered by stress and glucocorticoids (Padgett et al., 1998a).

Stress-induced activation of the HPA axis has been well-characterized (Jacobson and Sapolsky, 1991) and repeatedly shown to negatively impact wound healing. For example, women caring for a relative with Alzheimers disease had wounds that healed more slowly than age-matched controls (Kiecolt-Glaser et al., 1991). Furthermore, women who reported higher levels of life-stress exhibited a lower production of pro-inflammatory cytokines at blister wound sites than women who reported low levels of life-stress (Glaser et al., 1999). Likewise, wound healing was negatively correlated with perceived stress, while positively correlated with dispositional optimism (Ebrecht et al., 2004). Rodent studies have also demonstrated stress-induced delays in wound closure; specifically, mice undergoing restraint stress had impaired wound closure by >46% compared to non-restrained counterparts (Horan et al., 2005). It should be noted that delays in wound closure have been directly linked to increased scarring and infection (Robson, 1997; Rojas et al., 2002).

Mechanistic studies in rodents suggest that chronic stress reduces wound cellularity and delays wound closure through a mechanism that involves stress-induced

increases in circulating corticosteroid concentrations (Padgett et al., 1998a). Indeed, treatment with dexamethasone, a high-affinity synthetic glucocorticoid, delays wound healing (Gordon et al., 1994; Hubner et al., 1996), whereas adrenalectomy (Detillion et al., 2004), or treatment with a glucocorticoid receptor antagonist prior to the onset of stress (Padgett et al., 1998a), reduces the effects of stress on wound healing.

Stroke

The transient middle cerebral artery occlusion (MCAO) model of ischemic stroke, originally developed in rats (Tamura et al., 1981), is a widely used experimental model of stroke. The pathophysiological consequences of MCAO have been thoroughly studied. In the ischemic brain, there are typically two areas of damage—the core of the infarct and the surrounding area, termed the penumbra (Astrup et al., 1981). Different mechanisms of cell death, necrosis, and apoptosis, occur in each of these regions. In the core, the massive reduction in blood flow leads to tissue loss, which is rapid, as a consequence of cell death from hypoxia and a reduction in energy substrates. The result is immediate cell death within minutes. As ischemia progresses and reperfusion of the penumbral tissue commences, various processes ensue including inflammation, excitotoxicity, nitric oxide production, free radical damage, and apoptosis (reviewed in Dirnagl et al., 1999; Liang et al., 2004; Mergenthaler et al., 2004). These mechanisms are collectively known as the ischemic cascade.

Stroke-Induced Activation of the HPA axis

With an increased incidence and a mortality rate of 30% (Dirnagl et al., 1999), stroke can be considered one of the primary causes of death or long-term disability and suffering. Increased incidence of morbidity and mortality seen in stroke patients could be

due to the higher cortisol response, as measured by plasma cortisol concentrations and catecholamines (Feibel et al., 1977). Activation of the HPA axis is one of the first measurable physiological responses to cerebral ischemia, which leads to sustained increases in glucocorticoid concentrations (Fassbender et al., 1994; Olsson, 1990; Olsson et al., 1992). Increased activity of the HPA axis, as well as the sympathoadrenal system, is commonly seen in various forms of acute stress, including stroke (Feibel et al., 1977). Elevated cortisol concentrations after stroke are significantly correlated with increased morbidity and mortality in humans (Davalos et al., 1994; Fassbender et al., 1994; Feibel et al., 1977) and increased infarct size (measurable damage to cerebral hemispheres) in mice (DeVries et al., 2001b). Soon after stroke, abnormalities at various levels of the HPA axis have been documented in several studies, demonstrating an increased cortisol production rate, both in urine (Olsson, 1990) and in plasma (Fassbender et al., 1994; Mulley et al., 1989; Murros et al., 1993; O'Neill et al., 1991). Repeated stressors, such as cardiovascular complications, infection, and emotional reactions, are frequent in stroke victims (Blake and Ridker, 2002; Emsley and Tyrrell, 2002; Kattapong et al., 1996; Truelsen et al., 2003; Wolf et al., 2003). These repeated stressors may prolong the hypercortisolism seen in stroke patients following the initial neurological insult.

To better understand physiological mechanisms underlying neuronal death, researchers have investigated factors that mimic clinical features of stroke. Focal brain ischemia elevates circulating glucocorticoid concentrations in a variety of species, including humans (Sapolsky and Pulsinelli, 1985). Glucocorticoid receptors are present in the brain and high glucocorticoid concentrations, both endogenous and exogenous, have been suggested to be detrimental to neuronal cells, especially in the hippocampus (Auer et al.,

1989; Sapolsky et al., 1986). Furthermore, the hippocampus is very sensitive to ischemic damage. Chronic social stress prior to cerebral ischemia increases infarct size in mice (DeVries et al., 2001a; Madrigal et al., 2003; Sugo et al., 2002). Immediate administration of glucocorticoids following transient ischemia exacerbates ischemic neuronal damage, whereas both immediate adrenalectomy or adrenalectomy 24 h after ischemia are associated with partial protection from hippocampal damage (Morse and Davis, 1990; Sapolsky and Pulsinelli, 1985), as well as administration of RU38486, which selectively blocks glucocorticoid receptors (Antonawich et al., 1999). However, if an inhibitor of glucocorticoid production is administered prior to ischemic insult, the ischemia-induced rise in corticosterone is prevented (Smith-Swintosky et al., 1996). Thus, hypercortisolism might exacerbate the ischemic damage to hippocampal neurons following stroke. Furthermore, hypercortisolism may induce various negative effects on body functions (e.g., immune function), possibly rendering stroke patients more susceptible to a number of complications.

Stroke and Inflammation

There are several lines of evidence suggesting that inflammation plays a key role in ischemic stroke (reviewed in Emsley and Tyrrell, 2002; Zhang and Stanimirovic, 2002). First and foremost, there are consistent reports of increased infection among patients experiencing ischemic stroke (reviewed in Emsley and Tyrrell, 2002; Grau et al., 2001; Grau et al., 1995; Kawamoto et al., 2003; Mandrioli et al., 2004; Prass et al., 2003). The mechanism linking chronic infections and ischemic stroke is a continuously low-grade inflammatory process, activated by mononuclear leukocytes (Danesh et al., 1997). Also, white cell counts are elevated soon after ischemic stroke (Grau et al., 1994;

Marquardt et al., 2005) and leukocyte infiltration has been detected within ischemic lesions (Chuaqui and Tapia, 1993; Liao et al., 2001; Pozzilli et al., 1985; Reichmann et al., 2002). Although the percentage of all strokes caused by immune-mediated diseases is small, the risk of further strokes in immuno-compromised individuals may increase (Futrell, 1995). Specifically, recent studies demonstrate that influenza vaccinations may prevent stroke (Grau et al., 2005; Meyers, 2003; Smeeth et al., 2004).

Cytokines are important chemical molecular signals between cells in the inflammatory response to ischemic stroke (Iadecola and Alexander, 2001). Interleukins are among the most important humoral mediators of inflammation and their production is consistently altered following cerebral ischemia. IL-6, a pro-inflammatory cytokine, has consistently been shown to increase in concentration following cerebral ischemia (Dziedzic et al., 2004; Offner et al., 2006; Perini et al., 2001; Tarkowski et al., 1995c; Vila et al., 2000). TNF- α , another known pro-inflammatory cytokine, is also upregulated following cerebral ischemia (Gong et al., 1998; Hill et al., 1999; Hosomi et al., 2005; Offner et al., 2006; Vila et al., 2000; Wang et al., 2004), while neutralizing TNF- α reduces infarct size (Hosomi et al., 2005). IL-1 is one of the most powerful pro-inflammatory cytokines known to be upregulated following cerebral ischemia (Hill et al., 1999; Perini et al., 2001); increased expression of IL-1 increases infarct size (Allan et al., 2005) and induces depressive-like behaviors (Craft and DeVries, in press).

Stroke and Peripheral Immune Function

The nervous system is able to communicate with the immune system in various ways, from sympathetic nerve damage to cytokine trafficking. Many researchers have investigated whether damaging the nervous system directly influences immunologic

status. There is evidence suggesting that the immune system is altered following stroke. Inflammation plays an important role in the pathophysiology of ischemic stroke (Barone and Feuerstein, 1999). As mentioned previously, white blood cell counts are elevated soon after stroke, as well as leukocyte infiltration (Chuaqui and Tapia, 1993; Pozzilli et al., 1985; Schroeter et al., 1994). Others have demonstrated how stroke up-regulates the systemic T-cell response (Tarkowski et al., 1991) and lateralizes T-cell dependent cutaneous inflammation (Tarkowski et al., 1996). The effects of central neuronal damage correlate with changes to cutaneous sympathetic nerve traffic (Tarkowski et al., 1995b). These results indicate that early after ischemic stroke, the immune system is compromised and can have severe effects on clinical outcome. In consideration of the long-term increase in post-stroke mortality (Bordin et al., 2003; Mitosek-Szewczyk et al., 2002; Sramek et al., 2003), it is important to determine how post-stroke neurological changes may also influence peripheral immune responses. Previous research has demonstrated that lesions of the anterior hypothalamus in rats significantly decrease CD4/CD8 ratio of peripheral blood lymphocytes and spleen cells (Mori et al., 1993). Specifically, the anterior hypothalamus has some influence on the control of cellular immunological functions at the peripheral level. Lesions in the limbic system (Brooks et al., 1982; Devi and Namasivayam, 1991), substantia nigra (Neveu et al., 1992), and cerebral cortex (Neveu, 1992; Neveu et al., 1989; Renoux et al., 1987; Renoux et al., 1983) alter immune function.

More relevant to this thesis are findings that brain lesions caused by stroke lead to lateralization of T-cell dependent (i.e., DTH) cutaneous inflammation (Tarkowski et al., 1991; Tarkowski et al., 1995b). As well, brain structures in the frontal lobe are involved

in the regulation of DTH responses both in the early and chronic phases of stroke (Tarkowski et al., 1998). Many studies have investigated how neurological changes influence peripheral immune responses. These changes in the immune system can then cascade into other diseases and infections that accompany stroke. Stroke survivors have a 15-fold increase in further vascular events compared to the general population (Redfern et al., 2002). Also, acute and chronic infections have been associated with ischemic stroke, as previously described. These concomitant infections may lead to the development of atherosclerotic plaques or in triggering complications due to ischemia (Lavalley et al., 2002). Risk for influenza increases post-stroke and influenza vaccinations may protect against brain infarction by preventing viral infections or bacterial infections that accompany influenza (Poole et al., 2000). The literature concerning stroke and its effects on the organism is numerous; however, what remains to be shown is how environmental interventions might reduce the effects of stroke leading to a decrease in the concomitant diseases that follow.

Summary

Hence, studying the effects of social interaction on health outcome in closely related species displaying various social structures and in a rodent model of stroke may help to understanding the relationship between social interactions and health outcome. The second chapter of this dissertation aimed to characterize wound healing in two monogamous and one polygamous species of *Peromyscus*. I hypothesized that wound healing would be facilitated in those species that benefit from pair housing, whereas the polygamous species, that would only be found in a pair bond in the wild during mating

season, would not benefit from experimental social facilitation. Also, social contact should be necessary for the benefits of social interaction on wound healing in the monogamous species. The third chapter of this dissertation will further examine the effects of positive social interactions on wound healing in a monogamous and polygynous species. However, unlike Chapter 2, Chapter 3 will investigate various housing dyads by changing the sex of the experimental and stimulus mouse. This variation in the experimental design aims to elucidate the interactions between same sex pairs and wound healing. Given the vast literature on intersex competition and its effects on various biological outcomes, as mentioned previously, Chapter 3 will add to the understanding of both positive and negative social interactions and wound healing.

Chapter 4 will then turn to examine the effects of endogenous glucocorticoids on physiological parameters following stroke. Acute stress should exaggerate neuronal damage, inflammation, and corticosterone concentrations following stroke. Chapter 5 will characterize post-stroke cellular and humoral immune function. Neuronal damage should alter immune function following stroke; however, by protecting the brain from neuronal damage, immune function should be similar in mice not experiencing stroke-induced neuronal damage. Chapter 6 will assess immune function following stroke in mice provided positive social interactions via pair housing and comparing those to socially isolated mice. Pair housing should mitigate the effects of stroke on peripheral immune function evidenced in Chapter 5. Chapter 7 will then examine the effects of restraint stress on peripheral immune function. Restraint stress should further exacerbate

the effects of stroke on peripheral immune function. Taken together, these chapters should add to the current understanding of the relationship between social interactions and health outcome.

CHAPTER 2

SOCIAL STRUCTURE INFLUENCES THE EFFECTS OF PAIR HOUSING ON WOUND HEALING

Social support in humans and affiliative behaviors in other animals can have positive effects on health (House et al., 1990; Uchino et al., 1996). Social support improves outcome or recovery following many types of human illnesses and medical conditions (Bae et al., 2001; Grace et al., 2002; Penttinen et al., 2002; Spiegel and Sephton, 2001). For example, low levels of social support are associated with attenuated immune function (reduced cellular immunity and more days of upper respiratory infections; Kiecolt-Glaser et al., 1991) and impaired mental and physical health compared to individuals who report high levels of social support (reviewed in Cacioppo et al., 2000).

Although these findings provide evidence for a clear relationship between perceived social support and immune function, the physiological mechanism through which social support improves health in humans is not known. It is believed that a putative mediating factor between social support and wound healing is glucocorticoids; however, many of the studies have been case studies (Jones, 2003) and have not manipulated levels of social support, due to the unethical nature of the task, and measured

immune functioning. However, in female Siberian hamsters, *Phodopus sungorus* social interaction facilitates wound healing through a mechanism that involves dampened hypothalamic-pituitary-adrenal (HPA) axis responses to stress (Detillion et al., 2004). Stress-induced activation of the HPA axis has been well-characterized (Jacobson and Sapolsky, 1991) and repeatedly shown to impact wound healing negatively. For example, women caring for a relative with Alzheimers disease had wounds that healed more slowly than age-matched controls (Kiecolt-Glaser et al., 1991). Furthermore, women who reported higher levels of life-stress exhibited a lower production of pro-inflammatory cytokines at blister wound sites than women who reported low levels of life-stress (Glaser et al., 1999). Likewise, wound healing was negatively correlated with perceived stress, while positively correlated with dispositional optimism (Ebrecht et al., 2004). Rodent studies have also demonstrated stress-induced delays in wound closure; specifically, mice undergoing restraint stress had impaired wound closure by >46% compared to non-restrained counterparts (Horan et al., 2005). It should be noted that delays in wound closure have been directly linked to increased scarring and infection (Robson, 1997; Rojas et al., 2002).

Mechanistic studies in rodents suggest that chronic stress reduces wound cellularity and delays wound closure through a mechanism that involves stress-induced increases in circulating corticosteroid concentrations (Padgett et al., 1998a). Indeed, treatment with dexamethasone, a high-affinity synthetic glucocorticoid, delays wound healing (Gordon et al., 1994; Hubner et al., 1996), whereas adrenalectomy (Detillion et al., 2004), or treatment with a glucocorticoid receptor antagonist prior to the onset of stress (Padgett et al., 1998a), reduces the effects of stress on wound healing.

In the current series of experiments, the effects of social housing on wound healing and stress-induced alterations in HPA axis function were examined in two mouse species that have been characterized in nature as exhibiting behaviors consistent with a monogamous social system (*Peromyscus californicus* and *P. eremicus*) and one mouse species exhibiting behaviors consistent with a polygynous social system (*P. leucopus*). *P. californicus* are monogamous (obligate) in the field and exhibit a strong tendency to form life-long bonds with their mates (Eisenberg, 1963; Ribble, 1991; Ribble, 1992). *P. eremicus* exhibit several features of monogamy, including high tolerance of pups and increased social contact with mates (Ribble, 2003). Furthermore, in the field, male-female pairs do form under some circumstances, but the associations are not necessarily enduring (Eisenberg, 1963). These data suggest that *P. eremicus* exhibit a social system that resembles facultative monogamy. *P. leucopus* are one of the two *Peromyscus* species characterized as polygynous (Ribble, 2003). I predicted that mice from the monogamous *Peromyscus* species (*P. californicus* and *P. eremicus*), but not the polygynous species (*P. leucopus*), would exhibit improved wound healing and HPA axis regulation upon pairing with a female partner. Furthermore, I predicted that physical contact would be a necessary component in the social facilitation of wound healing and that the benefits of social housing would not continue beyond pair dissolution.

Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The protocol was approved by the local Institutional Animal Care and Use Committee. *P. californicus*, *P. eremicus*, and *P. leucopus* were purchased from The *Peromyscus* Genetic Stock Center at the University of South Carolina (Columbia, South Carolina, USA). Male mice (>60 days of age) were either individually housed or pair housed with an ovariectomized female (>60 days of age) upon delivery to our laboratory. The mice habituated to the vivarium for two weeks before commencement of the study. All mice were maintained on a 14L:10D light-dark cycle and allowed *ad libitum* access to food and water, except during periods of restraint, as described below.

Experimental Procedures

In Experiment 1, the effects of stress and social pairing on wound healing and corticosterone concentrations were determined using four experimental groups: Socially Isolated-No Stress (SI-NS), Paired-No Stress (P-NS), Socially Isolated-Stress (SI-S), and Paired-Stress (P-S), for all species. The stressed mice were exposed to 2 h of restraint for 10 days following wounding, as described below. In Experiment 2, the importance of physical contact in wound healing was examined by housing male and female *P. californicus* and *P. eremicus* pairs in cages separated via a double mesh screen barrier: Socially Isolated (SI) and Barrier-Pair (B-P). In Experiment 3, the effects of separating

established *P. californicus* and *P. eremicus* pairs on wound healing and corticosterone concentrations were determined using three experimental groups: Socially Isolated (SI), Paired (P), and Separated (SEP).

Wounding Procedure

Male mice were anesthetized with isoflurane in O₂-enriched air. A 3x3 cm patch of fur was shaved on the dorsal surface and cleansed with ethanol. Dorsal, mid-scapular, cutaneous wounds were produced using a 3.5 mm dermal punch biopsy tool (Miltex Instruments, New York, USA).

Analysis of Wounds

Each day, beginning with the day of wounding, the wound site was photographed using a digital camera. Each photograph included a standard-sized circle (3.5 mm ID) placed on the skin near the wound. The wound size for each mouse was determined using Canvas 8.0 (Deneba Systems, Florida, USA) and expressed as the ratio of the wound area to the area of the standard circle in the photograph (in pixels).

Restraint

In each experiment, restraint was initiated 24 h post-wounding. Restraint consisted of placing the mice into small, ventilated, Plexiglas tubes (3 cm ID, 10 cm in length) for 2 h per day for 10 consecutive days. The restraint tubes allowed for minimal, confined movement. Restraint was administered during the light phase of the daily cycle. Control mice remained undisturbed in their home cages.

Determination of Corticosterone Concentrations

Blood samples (50 µl) were collected on the final day of the experiment and centrifuged at 4°C for 25 min at 2,500 g. Plasma was collected and stored at -70°C.

Corticosterone concentrations were determined in duplicate samples using an ^{125}I radioimmunoassay kit (ICN Pharmaceuticals, Costa Mesa, California, USA). One outlier in Experiment 3 and two outliers in Experiment 4 were removed from analyses because they were greater than two standard deviations from the mean.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze wound size (repeated measures) and corticosterone concentrations. Post-hoc comparisons were conducted using Scheffe test.

Results

Experiment 1

There was a significant effect of housing ($F(1, 76) = 36.75, p < 0.05$), species ($F(2, 76) = 9.60, p < 0.05$), and an interaction between species and housing ($F(2, 76) = 4.79, p < 0.05$) on wound size (Figure 2.1-2.3). On the whole, pair-housed mice had smaller wound sizes than cohorts that were socially isolated. Within species analysis revealed that *P. californicus* pair-housed mice had significantly smaller wounds than socially isolated mice on days 1-7 (main housing effect: $F(1,30) = 33.56, p < 0.05$; Figure 2.1). Pair-housed *P. eremicus* also had significantly smaller wounds than socially isolated mice on days 1-11 (main housing effect: $F(1,31) = 16.14, p < 0.05$; Figure 2.2). Pair-housing had no significant effect on wound size in *P. leucopus* ($F(1,16) = 1.82, p > 0.05$; Figure 2.3).

Overall, *P. leucopus* mice had significantly smaller wounds than *P. californicus* mice ($F(2,76) = 9.60, p < 0.05$). Post-hoc analysis of wound size on individual days

indicated that *P. californicus* wounds were significantly larger ($p < 0.05$) than *P. eremicus* wounds on days 3-5, 9, and 11 and significantly larger than *P. leucopus* wounds on days 0 and 3-7. As well, *P. eremicus* wound size was significantly larger than *P. leucopus* on day 7, and days 9-11. To increase clarity, wound size data from socially isolated (Figure 2.4) and paired (Figure 2.5) mice are presented separately.

Overall, no stress effect was observed ($F(1, 76) = 3.14, p > 0.05$). Within species analysis revealed no significant differences in wound size between restrained and non-restrained *P. californicus* ($F(1,30) = 0.58; p > 0.05$) and *P. eremicus* ($F(1,31) = 0.10, p > 0.05$) mice. However, *P. leucopus* mice that were restrained daily had significantly smaller wound sizes on days 3 and 7 than non-restrained mice ($F(1, 16) = 7.32, p < 0.05$; Figure 2.3).

A significant effect of housing ($F(1, 63) = 11.77, p < 0.05$) and species ($F(2, 63) = 11.69, p < 0.05$) also was observed in basal corticosterone concentrations. Overall, pair-housed mice had lower corticosterone concentrations as compared to their socially isolated counterparts (Figure 2.6). Furthermore, corticosterone concentrations were significantly lower in *P. californicus* and *P. leucopus* mice than *P. eremicus* mice. Within species analysis revealed significant differences between pair-housed and socially isolated *P. californicus* ($F(1, 20) = 33.65, p < 0.05$) and *P. eremicus* mice ($F(1, 31) = 6.833, p < 0.05$). There was no effect of housing on corticosterone concentration in *P. leucopus* ($F(1, 14) = .001, p > 0.05$). There were no differences in basal corticosterone concentrations between restrained and non-restrained mice from any species when measured 24 h following removal from restraint ($F(1, 63) = 0.00, p > 0.05$).

Experiment 2

Because social pairing had no effect on wound size in *P. leucopus* in Experiment 1, this species was not included in subsequent experiments. To determine if physical contact was necessary for the facilitatory effect of social housing on wound healing, *P. eremicus* and *P. californicus* mice were pair-housed, but prevented from interacting physically via a double screen barrier and compared to socially isolated mice. There was no effect of housing condition on wound healing ($F(1, 22) = 0.61, p > 0.05$). Wound size was similar in pair-housed mice separated via barriers and socially isolated mice. Again, overall wound size was smaller in *P. eremicus* mice than *P. californicus* mice ($F(1, 22) = 6.38, p < 0.05$; Figure 2.7).

There also was no significant effect of housing on corticosterone concentrations in *P. californicus* or *P. eremicus* mice ($F(1, 21) = 2.14, p > 0.05$; Figure 2.8). However, within species analysis revealed that corticosterone concentrations in *P. californicus* mice were significantly lower in the barrier-pair group compared to the socially-isolated group ($t(12) = 2.68, p < 0.05$). *P. eremicus* corticosterone concentrations were similar in both socially-isolated and barrier-pair mice ($t(10) = 0.81, p > 0.05$). There was an overall effect of species on corticosterone concentration; *P. californicus* corticosterone concentrations were significantly lower than those of *P. eremicus* ($F(1, 21) = 5.33, p < 0.05$).

Experiment 3

Experiment 1 indicated that pair housing facilitates wound healing in both *P. eremicus* and *P. californicus* mice. To determine if the positive effects of social housing continued after pair dissolution, male mice were housed with an ovariectomized female

for two weeks. The pair was then permanently separated two days prior to wounding. The three experimental groups included socially isolated mice (SI), pair-housed (P), and separated (SEP). There was a significant effect of housing ($F(2, 40) = 13.89, p < 0.05$) and species ($F(1, 40) = 4.49, p < 0.05$) on wound size (Figure 2.9). Within group analysis revealed that *P. californicus* pair-housed mice had smaller wounds than separated mice on days 2-6 and 10, and smaller wounds than socially isolated mice on days 2-6 and 10. *P. eremicus* pair-housed mice had smaller wounds than socially isolated mice on days 2, 4, 6-9, and 11. There were no significant differences in wound size between socially isolated and separated mice at any time point.

There was a significant effect of both species ($F(1, 39) = 8.64, p < 0.05$) and housing condition ($F(2, 39) = 4.83, p < 0.05$) on corticosterone concentration. Post hoc analysis revealed that pair-housed mice had lower corticosterone concentrations than both separated and socially isolated mice ($p < 0.05$). As described in Experiments 1 and 2, corticosterone concentrations were significantly lower in *P. californicus* than *P. eremicus* mice ($p < 0.05$; Figure 2.10).

Discussion

Experimental males from two monogamous mouse species (*P. californicus* and *P. eremicus*) exhibited smaller wounds and lower corticosterone concentrations when housed with an ovariectomized female versus housed alone (Figure 2.1, 2.2, and 2.6). A decline in basal corticosteroid concentrations following social pairing has been observed in several other pair bonding species, including prairie voles (DeVries et al., 1995; DeVries et al., 1997), dwarf hamsters, (Reburn and Wynne-Edwards, 1999) and black

tufted-ear marmosets (Smith and French, 1997a). The mechanism through which social interaction suppresses corticosteroid concentrations likely involves oxytocin-induced suppression of the HPA axis (Detillion et al., 2004; DeVries et al., 2003). Oxytocin is released in response to physical contact and has been shown to facilitate social bonding in monogamous prairie voles (Cho et al., 1999; Insel and Hulihan, 1995; Williams et al., 1994). Indeed, oxytocin-induced suppression of the HPA axis mediates social facilitation of wound healing in sibling pairs of Siberian hamsters (Detillion et al., 2004). Thus, it is likely that in the current study, the pair-housed *P. californicus* and *P. eremicus* mice healed more quickly compared to their socially isolated cohorts because corticosteroid concentrations were lower in the pair-housed than socially isolated mice, possibly due to increases in oxytocin released during physical contact.

Physical contact is an important component of social facilitation of wound healing in monogamous mice. When *P. californicus* and *P. eremicus* pairs were housed in the same cage, but prevented from physically interacting by a double screen barrier, wound size was similar to socially isolated mice (Figure 2.7). Housing prairie vole pairs in a similar manner also prevents the normal formation of a social bond (Shapiro et al., 1986). In *P. eremicus* mice, corticosteroid concentrations were similar between socially isolated and barrier-pair groups. In contrast, within *P. californicus*, barrier-pair mice had significantly lower corticosterone concentrations than socially isolated mice. However, comparison of corticosterone concentrations in Experiments 1 and 2 indicates that corticosteroid concentrations in the barrier-pair group are significantly lower than socially isolated mice (socially isolated values were similar between experiments), but corticosterone values in barrier-pair mice were still significantly higher than in pair

housed mice. Thus, for *P. californicus*, being able to see, hear, and smell a female conspecific reduces corticosteroid concentrations significantly compared to socially isolated mice, but corticosterone concentrations are further reduced when physical interaction is allowed. Despite the decrease in corticosterone concentrations in *P. californicus* barrier-pair mice relative to the socially isolated group, there were no significant differences in wound healing between the two groups.

The beneficial effects of social interaction for wound healing do not persist beyond pair dissolution in either *P. californicus* or *P. eremicus*. Overall, the separated mice had corticosteroid concentrations that were similar to socially isolated mice, and both of these groups exhibited corticosteroid concentrations that were significantly higher than the pair-housed mice (Figure 2.10). Disruption of social pairs often leads to elevated glucocorticoid concentrations (Castro and Matt, 1997; Mendoza and Mason, 1986; Ramage-Healey et al., 2003), which can negatively impact wound healing (Detillion et al., 2004; Gordon et al., 1994; Hubner et al., 1996; Padgett et al., 1998a) and other measures of well-being (Castro and Matt, 1997; Crawley, 1984).

In contrast to *P. californicus* and *P. eremicus* mice, housing conditions had no effect on wound size or corticosterone concentrations in the polygynous *P. leucopus* mice. Thus, it does not appear that social pairing alters HPA axis activity or facilitates wound healing in this typically solitary species. Evidence from studies in monogamous and polygynous voles (*Microtus*) and mice (*P. californicus* and *P. maniculatus*) suggest that developmental differences in oxytocin and vasopressin receptor expression in the brain may contribute to species differences in propensity to form social bonds (Bales et al., 2004; Bales and Carter, 2003; Insel et al., 1991; Insel and Shapiro, 1992; Lim et al.,

2005; Young et al., 2001). Thus, if oxytocin mediates the effects of social interaction on HPA axis activity and subsequent wound healing (Detillion et al., 2004), then it is possible that the brains of polygynous species, such as *P. leucopus*, are not organized to respond to oxytocin release during social interaction in a manner similar to monogamous or highly social species. Indeed, for some species, pair or group housing may actually induce social stress and have deleterious effects on health measures (Gattermann et al., 2002; Hannes and Franck, 1983; Sachser et al., 1998). Of course, the direction in which social interaction affects corticosteroid concentrations and immune function may also be impacted by several factors not examined in the current study, including the sex of the experimental and stimulus animals (Klein and Nelson, 1997; 1999), social status (Bartolomucci et al., 2003b; Devoino et al., 2003; Spencer et al., 1996), group size (Grewal et al., 1997; Karp et al., 1997), and level of aggression among group members (reviewed in (Coe, 1993; de Groot et al., 2002).

Exposure to daily restraint stress did not alter basal corticosteroid concentrations in any of the three *Peromyscus* species. In contrast to previous studies in male inbred mouse strains (Mercado et al., 2002a; Padgett et al., 1998a; Rojas et al., 2002; Sheridan et al., 2004) and female Siberian hamsters (Detillion et al., 2004), stress did not delay wound closure in *P. californicus* or *P. eremicus* mice (Figure 2.1 and 2.2). The absence of a stress-induced delay in wound healing has been reported in male Siberian hamsters (*P. sungorus*) housed under conditions similar to those used in the current study (Kinsey et al., 2003). However, in *P. leucopus* mice, exposure to restraint stress facilitated wound healing relative to mice not exposed to stress (Figure 2.3). Discrepancies in the effects of stress on wound healing may reflect sex differences, species differences, and procedural

differences among studies. For example, in the studies using inbred mouse strains, the restraint procedure was initiated several days prior to wounding and each restraint session lasted 12-15 h during the dark cycle (Mercado et al., 2002a; Padgett et al., 1998a; Rojas et al., 2002) compared to restraint for 2 h during the light phase, beginning one day post-wounding in the current study. Thus, it is possible that the short duration of restraint and absence of pre-wounding restraint sessions led to immuno-enhancement during the initial stage of wound healing. These results are consistent with immuno-enhancement observed in response to acute stress (reviewed in (Dhabhar, 2003), rather than the immuno-suppression previously reported in wound healing studies (Mercado et al., 2002a; Padgett et al., 1998a; Rojas et al., 2002). Interestingly, short-term exercise, an acute stressor, has been linked to enhancement of wound healing rate in older adults compared to those not experiencing the exercise (Emery et al., 2005).

A species effect on wound healing and corticosterone concentration was observed across experiments. On the whole, *P. eremicus* and *P. leucopus* wounds were smaller than *P. californicus* wounds on days 2-5, 9, and 11 for *P. eremicus* and on day 0 and days 3-7 for *P. leucopus* (Figure 2.4). Corticosterone concentration was significantly higher in *P. eremicus* than the other two species. Whether species differences in immune responses to wounding or other properties of the skin account for the observed differences in actual wound size remains to be determined. It also is not appropriate to make across-species comparisons as to the role of absolute concentrations of corticosterone on wound healing. In the present study, *P. eremicus* had the higher corticosteroid concentrations, but the smaller overall wound size compared to *P. californicus*. However, alterations in factors such as corticosterone binding globulin may

compensate for the higher baseline corticosterone concentrations observed in *P. eremicus* (Taymans et al., 1997) thereby making it difficult to draw conclusions about the effects of absolute concentrations of corticosterone on wound healing across species.

Taken together, the data from the current study suggest that social contact facilitates wound healing via a mechanism that involves decreased HPA axis activity in two monogamous mouse species. However, despite facilitation of wound healing in pair-housed mice, the benefits of social contact observed in the monogamous species were not apparent following pair dissolution or when the pairs were prevented from interacting physically. In contrast to the monogamous species, the polygynous mouse did not exhibit lower corticosteroid concentrations or smaller wounds when pair-housed versus socially isolated. Thus, the beneficial effects of social housing are not equivalent for all species, and may be limited to those that are monogamous or highly social.

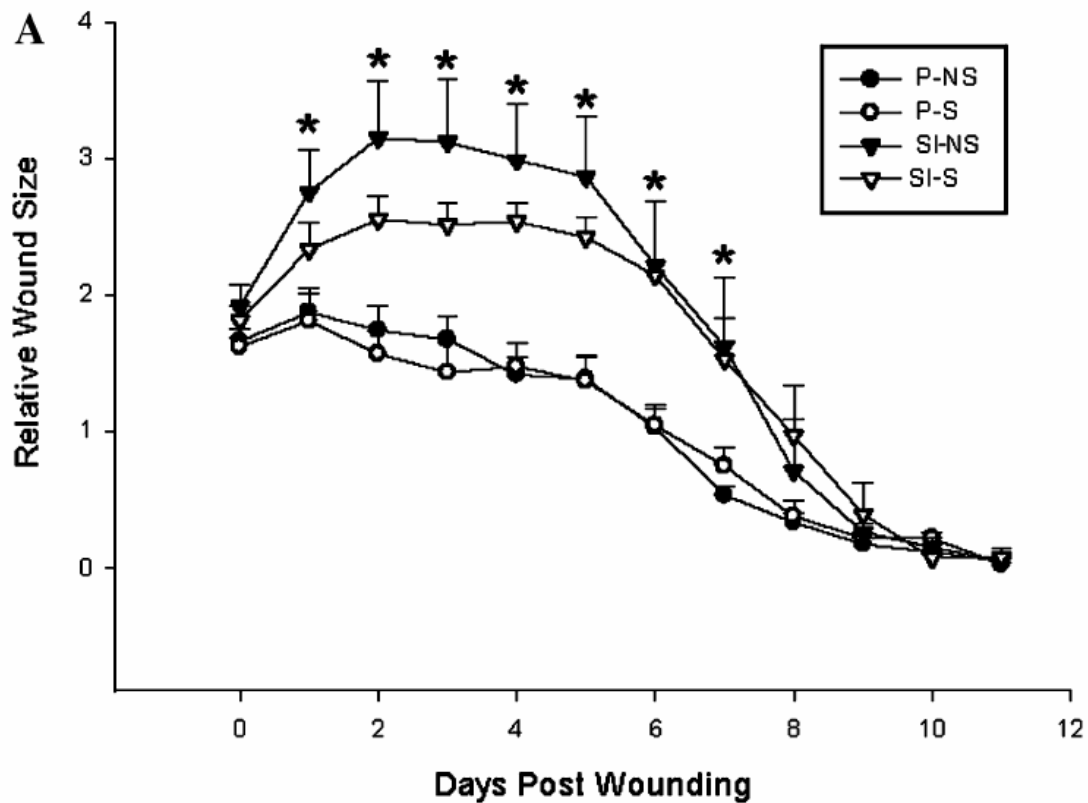


Figure 2.1: Effect of housing condition and stress on relative wound size of pair housed *P. californicus*.

Male mice were pair housed (P) with an ovariectomized female or socially isolated (SI) for two weeks prior to wounding. Stress (S) consisted of 2 h of restraint beginning 24 h after wounding. The non-stressed mice (NS) remained undisturbed in their home cages. There was a significant effect of housing ($p < 0.05$) and species ($p < 0.05$) on wound size. An asterisk (*) indicates statistical significance at $p < 0.05$ between the pair housed - no stress and socially isolated – no stress groups. Pair-housed *P. californicus* mice had significantly smaller wounds on days 1-7 as compared to socially isolated mice. (Mean $\pm$ SEM)

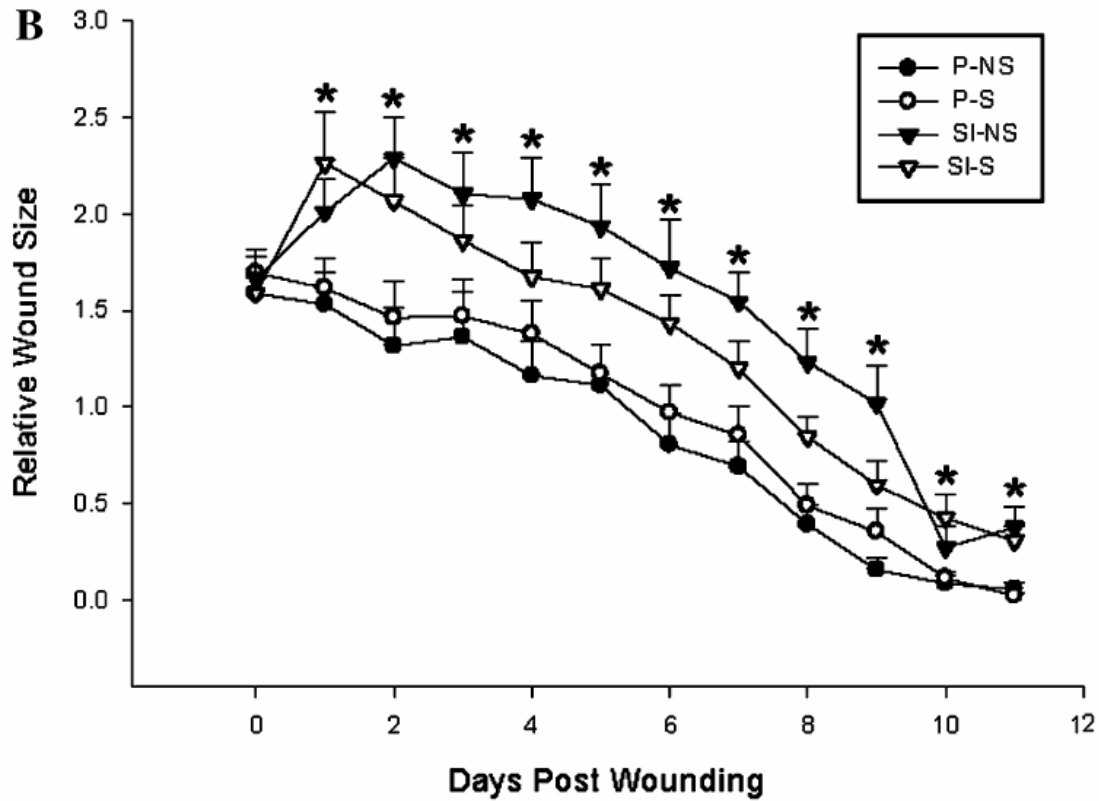


Figure 2.2: Effect of housing condition and stress on relative wound size of pair housed *P. eremicus*.

Male mice were pair housed (P) with an ovariectomized female or socially isolated (SI) for two weeks prior to wounding. Stress (S) consisted of 2 h of restraint beginning 24 h after wounding. The non-stressed mice (NS) remained undisturbed in their home cages. There was a significant effect of housing ($p < 0.05$) and species ($p < 0.05$) on wound size. An asterisk (*) indicates statistical significance at $p < 0.05$ between the pair housed - no stress and socially isolated - no stress groups. Pair-housed *P. eremicus* had smaller wounds compared to socially isolated mice on days 1-11. (Mean $\pm$ SEM)

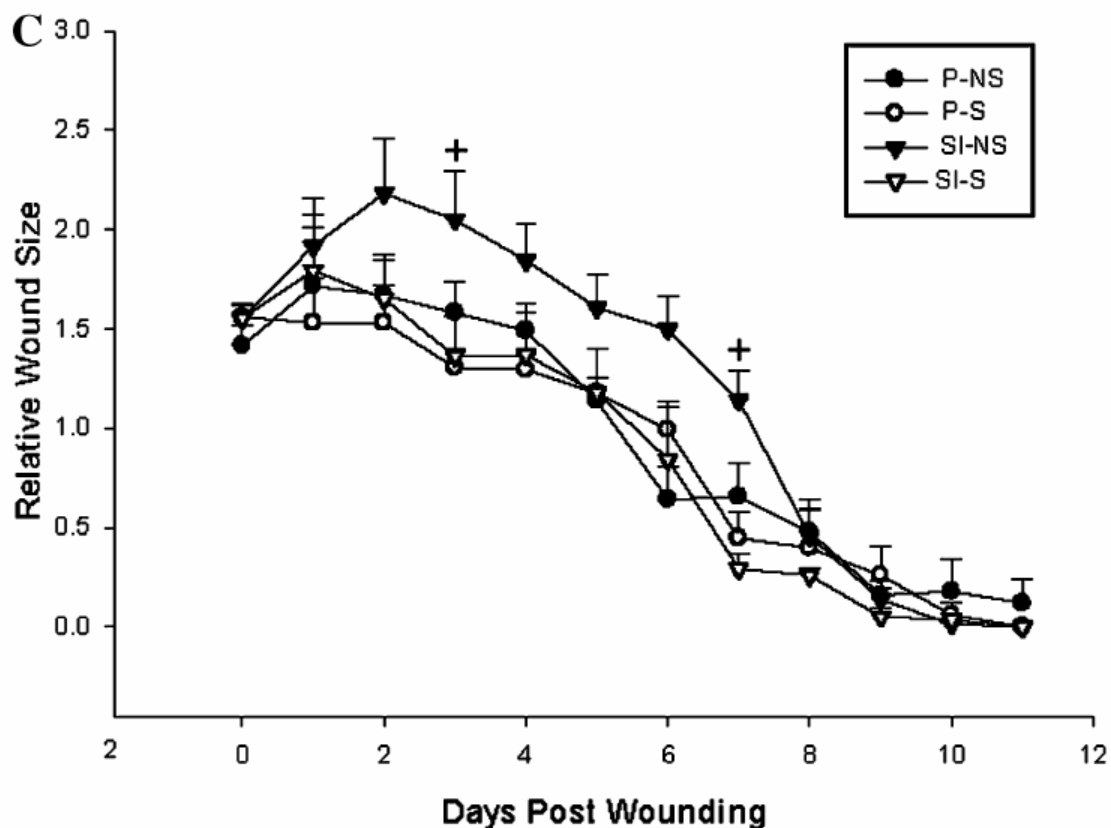


Figure 2.3: Effect of housing condition and stress on relative wound size of pair housed *P. leucopus*.

Male mice were pair housed (P) with an ovariectomized female or socially isolated (SI) for two weeks prior to wounding. Stress (S) consisted of 2 h of restraint beginning 24 h after wounding. The non-stressed mice (NS) remained undisturbed in their home cages. An (+) indicates statistical significance at $p < 0.05$ between stress and no stress groups. No housing effect was observed in *P. leucopus*; however, *P. leucopus* that were restrained (S) had smaller wounds compared to no stress mice. (Mean $\pm$ SEM)

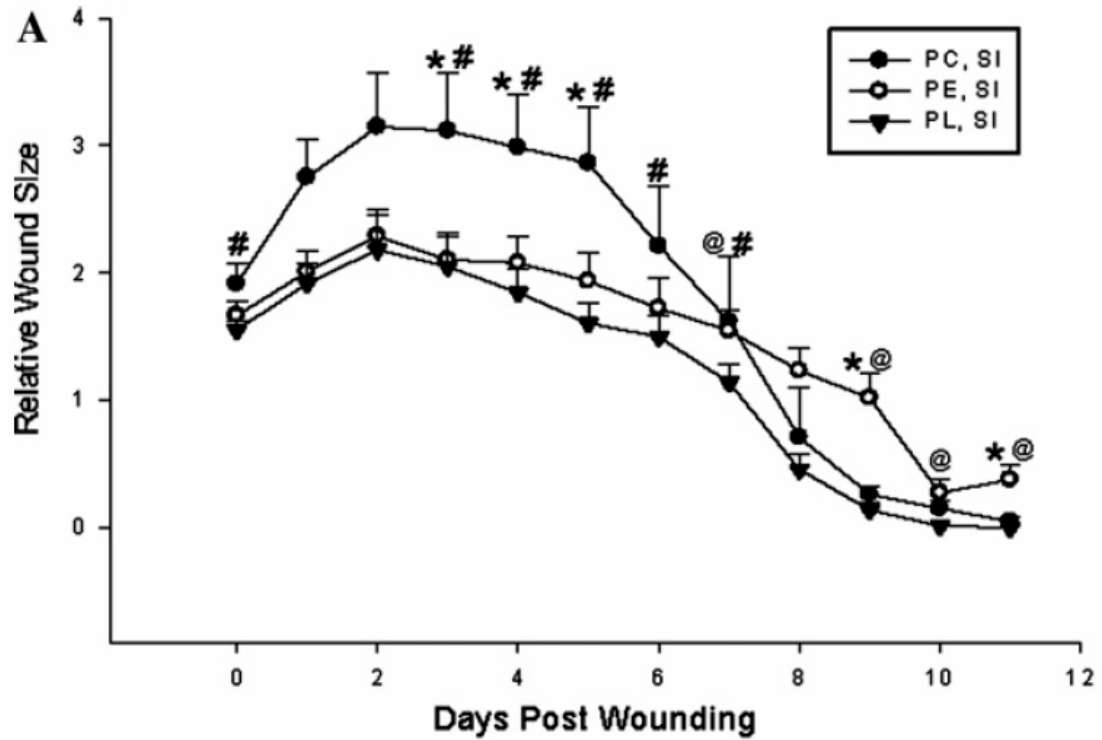


Figure 2.4: Relative wound size of socially isolated mice of three *Peromyscus* species.

Relative wound size on each day post wounding of socially isolated mice (Mean $\pm$ SEM). An asterisk (*) indicates statistical significance at $p < 0.05$ between *P. californicus* and *P. eremicus*. A number sign (#) indicates statistical significance at $p < 0.05$ between *P. californicus* and *P. leucopus* wound size. An ampere sign (@) indicates statistical significance at $p < 0.05$ between *P. eremicus* and *P. leucopus*.

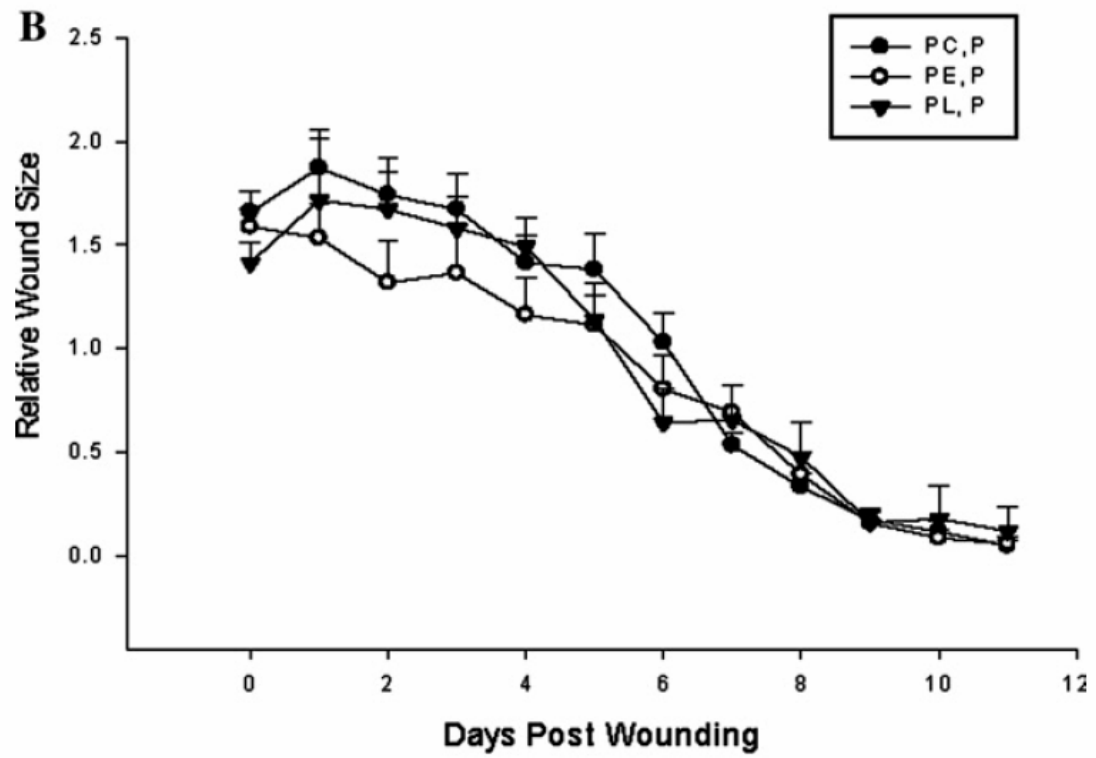


Figure 2.5: Relative wound size of pair housed mice of three *Peromyscus* species.

Relative wound size on each day post wounding of pair housed mice.

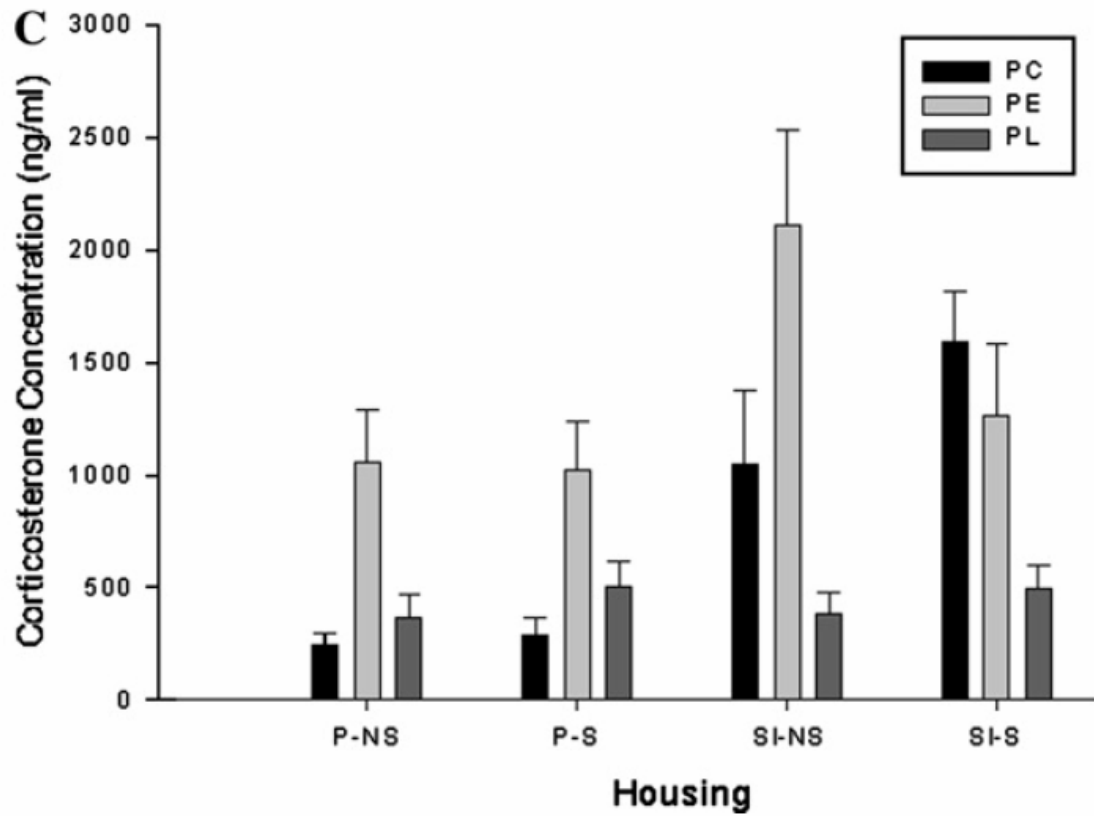


Figure 2.6: Corticosterone concentrations of socially isolated and pair housed mice of three *Peromyscus* species.

Corticosterone concentrations (ng/ml) were taken 11 days post-wounding. A significant effect of housing ($p < 0.05$) and species ($p < 0.05$) was observed.

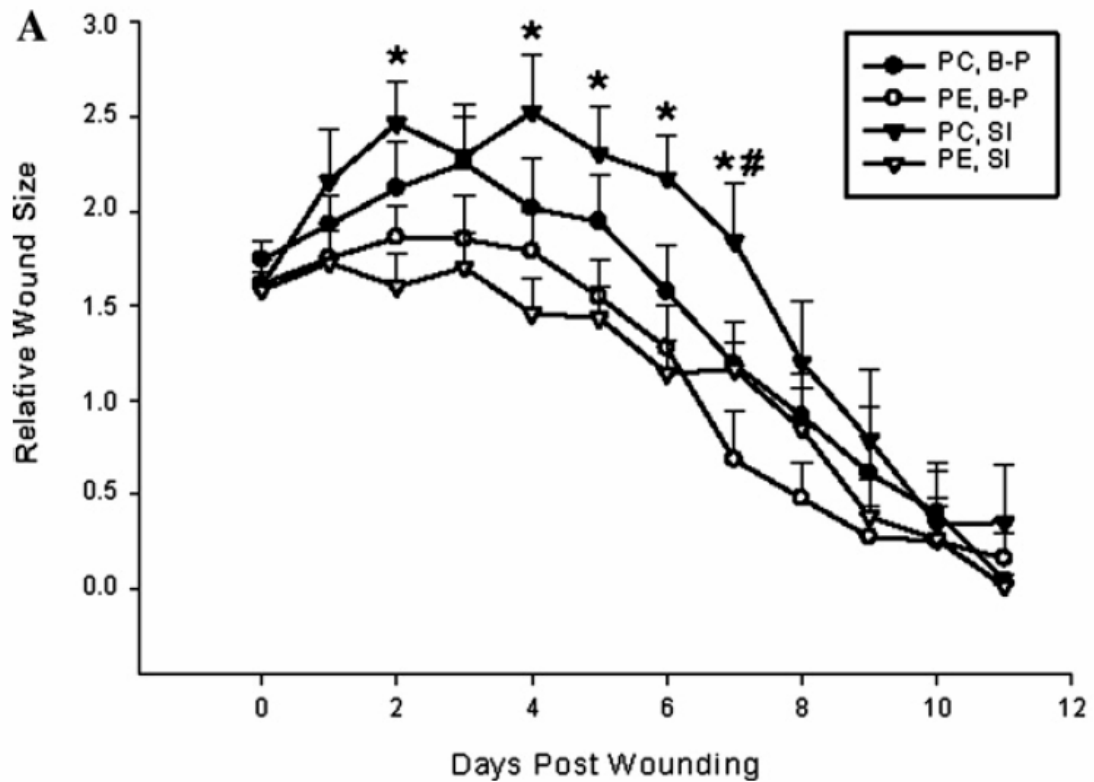


Figure 2.7: Relative wound size following barrier-pair housing or social isolation.

Male mice were either housed alone (SI) for 2 weeks prior to wounding or housed in a cage with an ovariectomized female for 2 weeks, separated by a double mesh barrier screen. *P. eremicus* mice had smaller wounds than *P. californicus* ($p < 0.05$), regardless of housing condition. An asterisk (*) indicates statistical significance at $p < 0.05$ between *P. californicus* and *P. eremicus*. A number sign (#) indicates statistical significance at $p < 0.05$ between socially isolated and barrier-pair wound size. (Mean $\pm$ SEM)

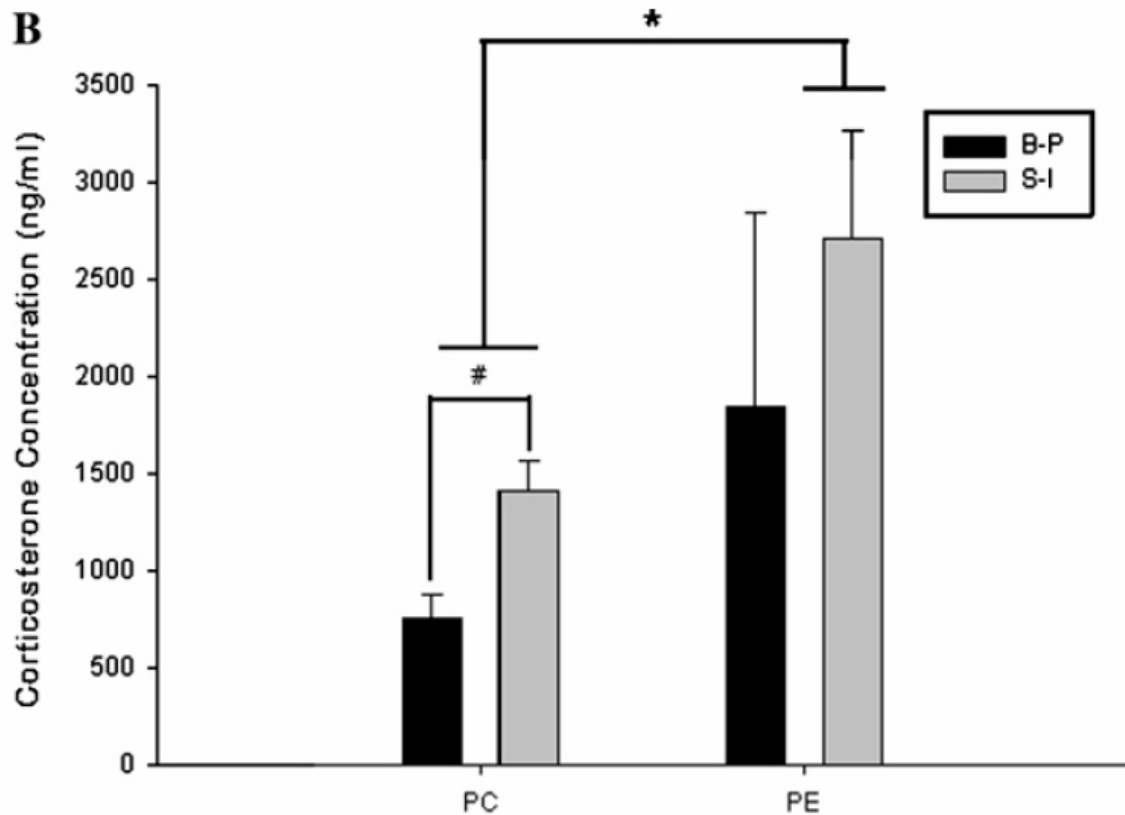


Figure 2.8: Corticosterone concentrations following barrier-pair housing or social isolation.

P. californicus barrier-pair mice have significantly lower corticosterone concentrations than *P. californicus* socially isolated mice. However, *P. californicus* corticosterone concentrations were lower than *P. eremicus* in both housing conditions ($p < 0.05$). An asterisk (*) indicates statistical significance at $p < 0.05$ between *P. californicus* and *P. eremicus*. A number sign (#) indicate statistical significance at $p < 0.05$ between barrier-pair and socially isolated *P. californicus* mice. (Mean $\pm$ SEM)

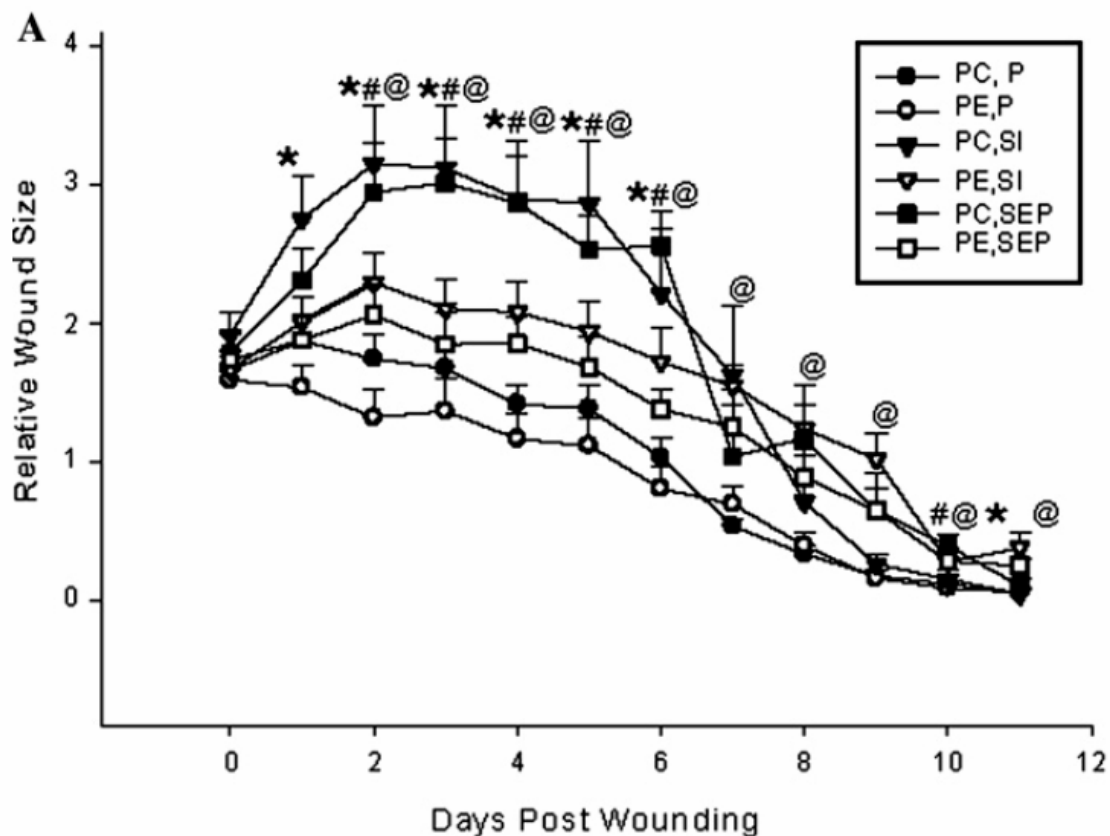


Figure 2.9: Relative wound size following pair housing, social isolation, and separation housing.

Male mice were pair-housed with an ovariectomized female, socially isolated (SI), or paired for two weeks then separated 48 h prior to wounding. There was a significant effect of housing ($p < 0.05$) and species ($p < 0.05$) on wound healing. An asterisk (*) indicates statistical significance at $p < 0.05$ between *P. californicus* and *P. eremicus*. A number sign (#) indicates statistical significance at $p < 0.05$ between socially isolated and pair-housed wound size in *P. californicus* mice. An ampere sign (@) indicates statistical significance at $p < 0.05$ between separated and pair-housed *P. californicus* mice. Post hoc analysis revealed that pair-housed mice healed faster than both separated and socially isolated mice. (Mean $\pm$ SEM)

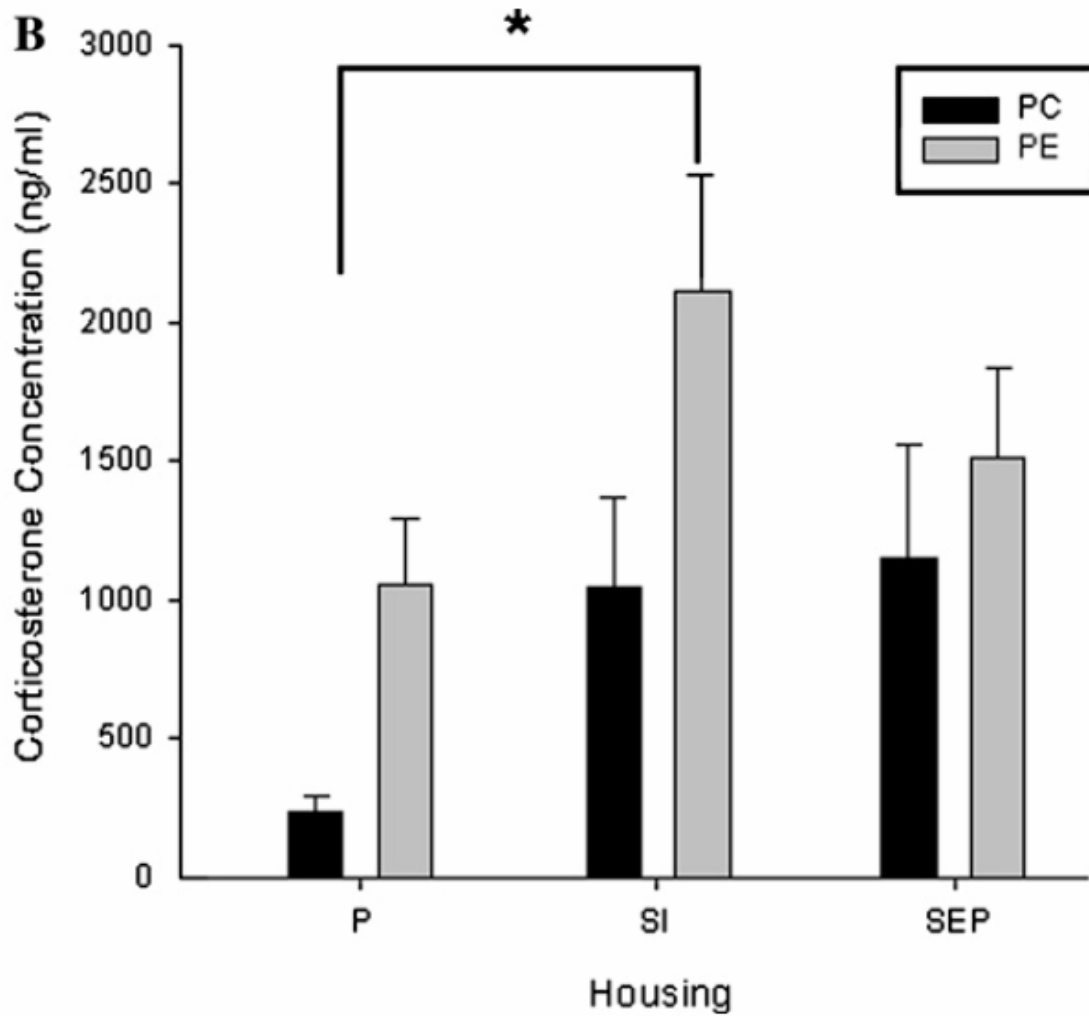


Figure 2.4: Corticosterone concentrations following pair housing, social isolation, and separation housing.

Corticosterone Concentrations (mean $\pm$ SEM) 11 Days Post-Wounding. An asterisk (*) indicates statistical significance at $p < 0.05$ between socially isolated and pair-housed corticosterone concentration.

CHAPTER 3

POSITIVE SOCIAL INTERACTIONS ALTER WOUND HEALING AND HPA AXIS ACTIVITY

Social factors impact health and well-being in a variety of species, including humans (DeVries et al., 2003; Kiecolt-Glaser et al., 2002; Seeman, 2000). Positive social relationships moderate or act as a buffer against the deleterious effects of psychosocial stress, and improve recovery from numerous human illnesses and medical conditions (Cassel, 1976; Cobb, 1976). Although the mechanism through which social support improves health outcome is not known in humans, recent studies demonstrate positive associations between social support and health, specifically by influencing corticosteroid concentrations. Laboratory studies demonstrate that social support suppresses cortisol responses to psychological stressors in humans (Heinrichs et al., 2003; Kirschbaum et al., 1995; Thorsteinsson and James, 1999) via modulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system. Reduced HPA axis activity among socially bonded animals also has been demonstrated in several rodent species (Detillion et al., 2004; DeVries et al., 1997; Giralt and Armario, 1989; Glasper and DeVries, 2005; Gonzalez et al., 1981; Hoffman-Goetz et al., 1992; Sachser and Kaiser, 1997; Smith and French, 1997b).

To the contrary, negative social interactions can compromise health (reviewed in DeVries et al., 2003). Stress-induced activation of the HPA axis has been well-characterized (Jacobson and Sapolsky, 1991) and repeatedly shown to negatively impact wound healing. For example, wounds heal more slowly among women caring for a relative with Alzheimers disease than age-matched controls (Kiecolt-Glaser et al., 1991). Furthermore, women who report higher levels of life-stress exhibit a lower production of pro-inflammatory cytokines at blister sites than women who report lower levels of life-stress (Glaser et al., 1999). Rodent studies also have demonstrated stress-induced delays in wound closure (Horan et al., 2005; Padgett et al., 1998a).

Chronic stress reduces wound cellularity and delays wound closure through a mechanism that involves stress-induced increases in circulating corticosteroid concentrations (Horan et al., 2005; Padgett et al., 1998a). Indeed, treatment with dexamethasone, a high-affinity synthetic glucocorticoid, delays wound healing (Gordon et al., 1994; Hubner et al., 1996), while adrenalectomy (Detillion et al., 2004), or treatment with a glucocorticoid receptor antagonist prior to the onset of stress (Padgett et al., 1998a), reduces the effects of stress on wound healing. Delays in wound closure have been directly linked to increased scarring and infection (Robson, 1997; Rojas et al., 2002) and could be particularly detrimental to immuno-compromised patients.

In the present study, the effects of pair versus social housing, sex, and exposure to stress on wound healing and corticosterone response were determined in *P. californicus* and *P. leucopus* mice. *P. californicus* are monogamous (obligate) in nature and exhibit a strong tendency to form life-long bonds with their mates (Eisenberg, 1963; Ribble, 1991; Ribble, 1992). *P. leucopus* are one of two *Peromyscus* species characterized as

polygynous (Ribble, 2003). Mixed-sex, pair housing in monogamous, but not polygynous, species increases healing rate and lowers corticosteroid concentrations (Glasper and DeVries, 2005). I predicted in the current study that mice from the monogamous *Peromyscus* species (*P. californicus*), but not the polygynous species (*P. leucopus*), would exhibit improved wound healing and decreased HPA axis activity upon pairing with a partner, regardless of the sex of the experimental or stimulus mouse. I also predicted that pair housing would ameliorate the effects of restraint stress on corticosterone concentrations and wound healing in *P. californicus*, but not *P. leucopus* mice.

Materials and Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The protocol was approved by the local Institutional Animal Care and Use Committee. *P. californicus* and *P. leucopus* were purchased from The *Peromyscus* Genetic Stock Center at the University of South Carolina (Columbia, South Carolina, USA). Male mice (>60 days of age) were either socially isolated (n=15, *P. californicus*; n=15, *P. leucopus*) or pair housed with an ovariectomized (OVX) female (n=16, *P. californicus*; n=16, *P. leucopus*; >60 days of age) or with a castrated (CAST) male (n=16, *P. californicus*; n=16, *P. leucopus*; >60 days of age). Female mice (>60 days of age) were either socially isolated (n=17, *P. californicus*; n=16, *P. leucopus*) or pair housed with an OVX female (n=16, *P. californicus*; n=16, P.L; >60 days of age) or a CAST male (n=15, *P. californicus*; n=16,

P. leucopus; >60 days of age) for two weeks after habituation (one week following arrival). All mice were maintained on a 14L:10D light-dark cycle and allowed *ad libitum* access to food and water, except during periods of restraint, as described below.

Experimental Procedures

The effects of social housing, sex of experimental mouse, sex of partner, and stress on wound healing and corticosterone concentrations were determined using six experimental groups. Among paired groups, sex of the experimental mouse precedes the slash, while sex of the stimulus mouse follows the slash: male, female, male/male, male/female, female/female, and female/male for both species. All stimulus mice were gonadectomized prior to pair housing. Half of the mice in each group were exposed to 3 h of daily restraint (stress), beginning three days prior to wounding and continuing for 5 days post wounding, as described below. Control mice were left in their home cages until wounding, and then were photographed daily, as described below.

Wounding Procedure

Mice were anesthetized with isoflurane in O₂-enriched air. A 3x3 cm patch of fur was shaved on the dorsal surface and cleansed with ethanol. Dorsal, mid-scapular, cutaneous wounds were produced using a 3.5 mm dermal punch biopsy tool (Miltex Instruments, New York, USA).

Analysis of Wounds

Each day, beginning with the day of wounding, the wound site was photographed using a digital camera. Each photograph included a standard-sized circle (3.5 mm ID)

placed on the skin near the wound. The wound size for each mouse was determined using Canvas 8.0 (Deneba Systems, Florida, USA) and expressed as the ratio of wound area to the area of the standard circle in the photograph (in pixels).

Stressor

Restraint was initiated 72 h prior to wounding and consisted of placing the mice into small, ventilated, polycarbonate tubes (3 cm ID, 10 cm in length) for three hours per day for eight consecutive days (Padgett et al., 1998a). The restraint tubes allowed minimal, confined movement. Restraint was administered during the light phase of the daily cycle. Control mice remained undisturbed in their home cages.

Determination of Corticosterone Concentrations

Blood samples (50 µl) were collected following two weeks of pair or individual housing (baseline), immediately following the first restraint session (post-restraint), and on the final day of the experiment (terminal), via the retro-orbital sinus under anesthesia (less than two minutes) with isoflurane in O₂-enriched air. All samples were collected within a four h window during the light phase to control for circadian variation. The samples were centrifuged at 4°C for 25 min at 2,500 g. Plasma was collected and stored at -70°C. Corticosterone concentrations were determined in duplicate samples using ¹²⁵I radioimmunoassay kits (ICN Pharmaceuticals, Costa Mesa, California, USA). Inter-assay variance was less than five percent.

Statistical Analysis

Analysis of variance (ANOVA) analyses were used to analyze wound size (repeated measures) and corticosterone concentrations. When appropriate ($p < 0.05$ on main effect), repeated measures post-hoc comparisons were conducted using Scheffe test.

Group differences were considered statistically significant at $p < 0.05$. Six corticosterone values from male *P. californicus* (1 unstressed M, 1, unstressed M/F, 1 unstressed M/M, 2 stressed M/F, 1 stressed M/M), five corticosterone values from female *P. californicus* (1 unstressed F, 1 unstressed F/M, 2 stressed F, 1 stressed F/F), three corticosterone values from male *P. leucopus* (2 stressed M, 1 stressed M/M), and five corticosterone values from female *P. leucopus* (1 unstressed F/F, 2 stressed F, 2 stressed F/F) were treated as outliers and removed from analysis because they were >3 standard deviations from the mean.

Results

Effects of species and sex on wound healing and corticosterone concentrations

Wound healing. Analysis of wound size of male and female, socially isolated *P. californicus* and *P. leucopus* revealed a significant main effect of species ($F(1, 28) = 17.609$, $p < 0.05$), day ($F(8, 224) = 54.295$, $p < 0.05$), and an interaction between species and day ($F(8, 224) = 4.695$, $p < 0.05$). Overall, wound size among *P. californicus* was significantly larger than wounds of *P. leucopus*, beginning on day1 post-biopsy. No significant main effect of sex was detected ($p > 0.05$; Figure 3.1).

Corticosterone Concentrations. There were no main effects of species ($p > 0.05$) or sex ($p > 0.05$) on corticosterone concentration among male and female, socially isolated *P. californicus* and *P. leucopus* mice.

Effects of housing on wound healing and corticosterone concentrations

Wound healing among male *P. californicus*. There were significant main effects of housing ($F(2, 21) = 29.673$, $p < 0.05$), day post-wounding ($F(8, 168) = 35.578$, $p < 0.05$),

and an interaction between housing and day ($F(16, 168) = 6.228, p < 0.05$). Overall, pair housed male *P. californicus* had significantly smaller wounds across days compared to socially isolated males, regardless of the sex of the stimulus mouse (Figure 3.2).

Corticosterone concentrations among male *P. californicus*. Corticosterone concentrations of male *P. californicus*, assessed at baseline and at the termination of the experiment, were not significantly different across the three housing conditions ($p > 0.05$).

Wound healing among female *P. californicus*. Wound size among female *P. californicus* mice varied significantly as a factor of housing ($F(2, 21) = 13.31, p < 0.05$) and day post-wounding ($F(8, 168) = 37.135, p < 0.05$). There also was a significant interaction between housing and day ($F(16, 168) = 3.834, p < 0.05$). Post-hoc analysis revealed that pair housed female *P. californicus* had significantly smaller wounds across days compared to socially isolated females, regardless of the sex of the stimulus mouse in the pair (Figure 3.3).

Corticosterone concentrations among female *P. californicus*. Corticosterone concentrations of female *P. californicus*, assessed at baseline and at the termination of the experiment, revealed no significant difference among the three housing conditions ($p > 0.05$).

Wound healing among male *P. leucopus*. There was a significant main effect of housing ($F(2, 21) = 6.697, p < 0.05$), a main effect of day ($F(8, 168) = 56.440, p < 0.05$), and an interaction between housing and day ($F(16, 168) = 3.639, p < 0.05$) on wound size. Male *P. leucopus* housed with females had significantly smaller wound sizes compared to socially isolated males ($p < 0.05$); however, males housed with males did not differ in

wound size compared to socially isolated males ($p>0.05$). Male *P. leucopus* housed with females had significantly smaller wounds than males housed with males ($p<0.05$; Figure 3.4).

Corticosterone concentrations among male *P. leucopus*. CORT concentrations from male *P. leucopus*, assessed at baseline and at the termination of the experiment, revealed no significant difference among the three housing conditions ($p>0.05$).

Wound healing among female *P. leucopus*. Among female *P. leucopus* mice, there was a significant main effect of housing ($F(2, 21) = 4.661$, $p<0.05$), a main effect of day ($F(8, 168) = 65.441$, $p<0.05$), and an interaction between housing and day ($F(16, 168) = 1.956$, $p<0.05$). Socially paired female *P. leucopus*, housed with either a female or male stimulus mouse, had significantly smaller wound sizes compared to socially isolated females ($p<0.05$; Figure 3.5).

Corticosterone concentrations among female *P. leucopus*. A significant main effect of housing was observed for corticosterone concentrations of female *P. leucopus* at baseline ($F(2, 18) = 4.15$, $p<0.05$); corticosterone was significantly elevated in socially isolate females relative to those pair housed with other females. There was no significant effect of housing on terminal corticosterone concentrations ($p>0.05$).

Effects of stress on wound healing and corticosterone concentrations

Stress and Wound Healing. Exposure to stress did not alter the pattern of social influences on wound healing observed among restrained *P. californicus* mice. Both male and female experimental mice exposed to restraint stress had smaller wounds if they were pair housed versus individually housed ($F(2,20)=11.19$, $p<0.05$ and $F(2,21)=42.35$, $p<0.05$, respectively; Figure 3.6 and 3.7). In contrast, male *P. leucopus* mice exposed to

stress no longer benefited from social housing; socially isolated and pair housed males had wounds of similar sizes ($F(2,20)=0.07$; $p>0.05$; Figure 3.8). Following stress exposure, socially isolated female *P. leucopus* had wound sizes that were not significantly different from either pair housed group, although wound sizes were significantly larger among females paired with a male versus females paired with female stimulus mice ($F(2,21)=3.93$, $p<0.05$; Figure 3.9).

Stress and Corticosterone Concentrations. At baseline, corticosterone concentrations were similar among the experimental groups for male and female *P. californicus* and *P. leucopus* ($p>0.05$ for each ANOVA). As expected, corticosterone concentrations were elevated relative to baseline in all groups upon removal from restraint ($p<0.05$). Housing condition did not influence corticosterone concentrations immediately following restraint among male *P. californicus* or either sex of *P. leucopus* ($p>0.05$ for each ANOVA). However, there was a significant interaction between housing conditions and exposure to stress among female *P. californicus*; corticosterone concentrations were significantly higher in the socially isolated group than the group paired with a male stimulus mouse, while the female/female group had corticosterone concentrations that were intermediate, but not significantly different from either group ($F(2,21)=4.25$, $p<0.05$). Also, at the termination of the study, corticosterone concentrations were not higher in previously stressed, socially isolated male *P. californicus* mice; however, corticosterone concentrations were higher in female *P. californicus* mice compared to their pair housed cohorts [$F(2,17)=1.454$, $p>0.05$] and

($F(2,22)=11.61$, $p<0.05$), respectively; Figure 3.10 and 3.11]. In contrast, among male and female stressed *P. leucopus* mice, housing did not have a significant effect on terminal corticosterone concentration ($p>0.05$, Figure 3.12 and 3.13).

Discussion

The positive effects of social support and affiliative behaviors on health have been demonstrated in both humans and rodents (House et al., 1990; Uchino et al., 1996). Although the mechanisms underlying the effects of social support on health have not been elucidated in humans, rodent studies suggest that social interaction facilitates wound healing via dampened HPA axis activity (Detillion et al., 2004; Glasper and DeVries, 2005). The current study suggests that the benefits of pair housing for wound healing are not limited to male *P. californicus* mice housed with ovariectomized females (Glasper and DeVries, 2005). Both male and female *P. californicus* had smaller wounds if pair housed rather than socially isolated (Figure 3.2 and 3.3). Furthermore, pair housing with same-sex and opposite-sex mice was equally effective at reducing wound size among *P. californicus*.

Although there was a species difference in wound size beginning on day 1 post-wounding (Figure 3.1; *P. californicus* experienced greater post-biopsy inflammation and had larger wounds than *P. leucopus*), there was no sex difference in wound size within either species (Figure 3.1). In contrast to our previous study (Glasper and DeVries, 2005), unstressed males of the polygynous species, *P. leucopus*, demonstrated reductions in wound size, relative to socially isolated males, if they were housed with an ovariectomized female (Figure 3.4). Pair housing with a male stimulus mouse had no

impact on wound healing among male *P. leucopus*. Similar to female *P. californicus*, female *P. leucopus* had smaller wounds if pair housed with either a male or female stimulus mouse, relative to being socially isolated (Figure 3.5). These data are consistent with another study in which housing male *P. leucopus* with either an intact male littermate or unrelated female decreased the inflammatory response associated with delayed-type hypersensitivity, compared to socially isolated males (Pyter et al., 2005). Importantly, however, in the current study the benefits of social housing on wound healing are eliminated among *P. leucopus* when the experimental mice are exposed to repeated stress prior to and following wounding (Figure 3.8 and 3.9).

Despite clear evidence of social facilitation of wound healing among *P. californicus* under both stress and non-stress conditions, basal corticosteroid concentrations were not significantly different between socially isolated and pair housed *P. californicus* at baseline or termination of the current study, unless the mice had been previously exposed to stress (Figure 3.7). At the termination of the study, socially isolated female *P. californicus* that had been previously exposed to stress had significantly higher corticosterone concentrations than pair-housed cohorts (Figure 3.7). Furthermore, corticosterone concentrations upon removal from restraint also were significantly higher in socially isolated female *P. californicus* compared to cohorts housed with a male. Baseline corticosterone concentrations among *P. californicus* are approximately 50% as high in the current study as we documented in the previous study (Glasper and DeVries, 2005). The only protocol difference between the two studies involves housing the mice in the current study in microisolator cages versus freestanding cages in the previous study. The microisolator cages may have filtered out odor cues

from mice in nearby cages, and thereby lead to reduced HPA axis activity in *P. californicus*, as reported in inbred mice (Neigh et al., 2005a). A decline in basal and stress-induced corticosterone concentrations has been observed in a number of species, including two monogamous mouse species (Glasper and DeVries, 2005), prairie voles (DeVries et al., 1995; DeVries et al., 1997), dwarf hamsters (Detillion et al., 2004; Reburn and Wynne-Edwards, 1999), and black tufted ear marmosets (Smith and French, 1997a). No group differences were observed in male *P. leucopus* baseline, stress-induced, or terminal corticosterone concentrations. However, non-stressed socially isolated female *P. leucopus* had significantly higher corticosterone concentrations than cohorts paired with males. Again, exposure to stress eliminated social influences on HPA axis activity among *P. leucopus*.

Stress has been repeatedly shown to negatively impact wound healing and pro-inflammatory cytokine production at wound sites in humans (Glaser et al., 1999; Kiecolt-Glaser et al., 1991). Mechanistic studies in inbred mouse strains suggest that chronic stress delays wound closure by increasing circulating corticosteroid concentrations (Horan et al., 2005; Padgett et al., 1998a) and also by reducing pro-inflammatory cytokine expression, which in turn promotes cell growth and healing (Horan et al., 2005; Mercado et al., 2002b). However, in the current study, daily restraint did not influence wound healing in *P. californicus* mice (Figures 3.6 and 3.7). These results are similar to our previous study which also did not demonstrate a delay in wound healing following daily restraint stress (Glasper and DeVries, 2005). However, daily restraint stress did eliminate the benefits of pair housing on wound healing in both male and female *P. leucopus* mice (Figures 3.8 and 3.9); when stress was imposed, no significant differences

were observed between socially isolated mice and those housed with stimulus mice. Both species of *Peromyscus* responded to 3 h of restraint stress with significant increases in corticosterone concentrations. Discrepancies in the effects of stress on wound healing may reflect species differences and duration of the stressor. For example, in the studies using inbred mouse strains, the restraint procedures lasted 12-15 h during the active portion of the daily cycle (Mercado et al., 2002b; Padgett et al., 1998a; Rojas et al., 2002) compared to 3 h of restraint daily during the non-active portion of the light-dark cycle in the current study. Thus, it is possible that a longer duration stressor, delivered during the dark phase, could impact wound healing in *P. californicus*.

Sex differences in immune function have been consistently observed in humans. In general, women produce more vigorous cellular and humoral immune reactions compared to men, and this difference is partially due to the suppressive effects of testosterone and the enhancing effects of estrogen on immune function (Bouman et al., 2005). Women also are more resistant to bacterial infections, and suffer a higher incidence of autoimmune diseases compared to men (Beeson, 1994; Bouman et al., 2005; Hochberg and Spector, 1990). Rodent studies further demonstrate the sexual dimorphism in immunologic disease susceptibility (reviewed in Shames, 2002; Tanriverdi et al., 2003). In addition to mechanistic explanations of sex differences in immune function, adaptive functional explanations suggest that sex differences in immune function should be more pronounced among polygynous species than monogamous species, in part due to the generally higher testosterone concentrations of polygynous males (Zuk, 1990). However, in non-domesticated rodent species raised under standard laboratory conditions, sex differences in immune function have not been consistently observed

(Klein et al., 1997; Klein and Nelson, 1997). Social interactions appear to facilitate the expression of cell-mediated immune function differences in polygynous vole species compared to monogamous vole species (Klein et al., 1997). The current study, using wound healing as an integrated measure of immune functioning, provides additional evidence that sex differences in immune function are absent in some laboratory-raised, outbred rodent species. Males and females of both species of *Peromyscus* demonstrated healing rates that were similar (Figure 3.1). Thus, the present study failed to demonstrate sex differences in rate of wound healing in a polygynous species of mouse following social interactions (Figure 3.8 and 3.9; Klein and Nelson, 1997).

Taken together, the data from the current study suggest that positive social interaction facilitates wound healing in a monogamous mouse species regardless of the sex of the experimental or stimulus mouse. Both male and female *P. californicus* exhibit reductions in wound size following pair housing. However, the effects on wound healing are limited; male and female polygynous mice did benefit from pair housing, but only under non-stressed conditions. Thus, differences in social system can influence the extent to which social interactions may be beneficial to wound healing.

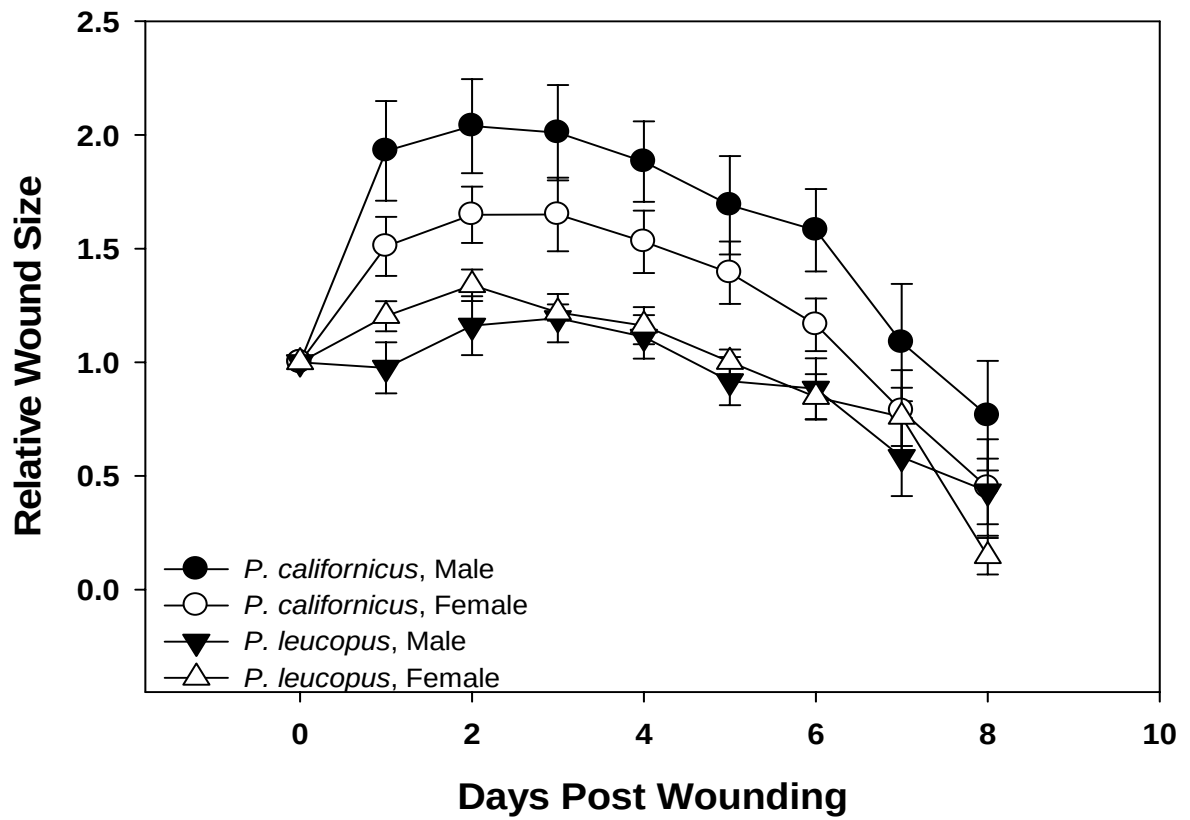


Figure 3.1: Effects of species and sex on wound healing.

Wound size among *P. californicus* was significantly larger than wounds of *P. leucopus* ($p < 0.05$), beginning on day1 post-biopsy. (Mean $\pm$ SEM)

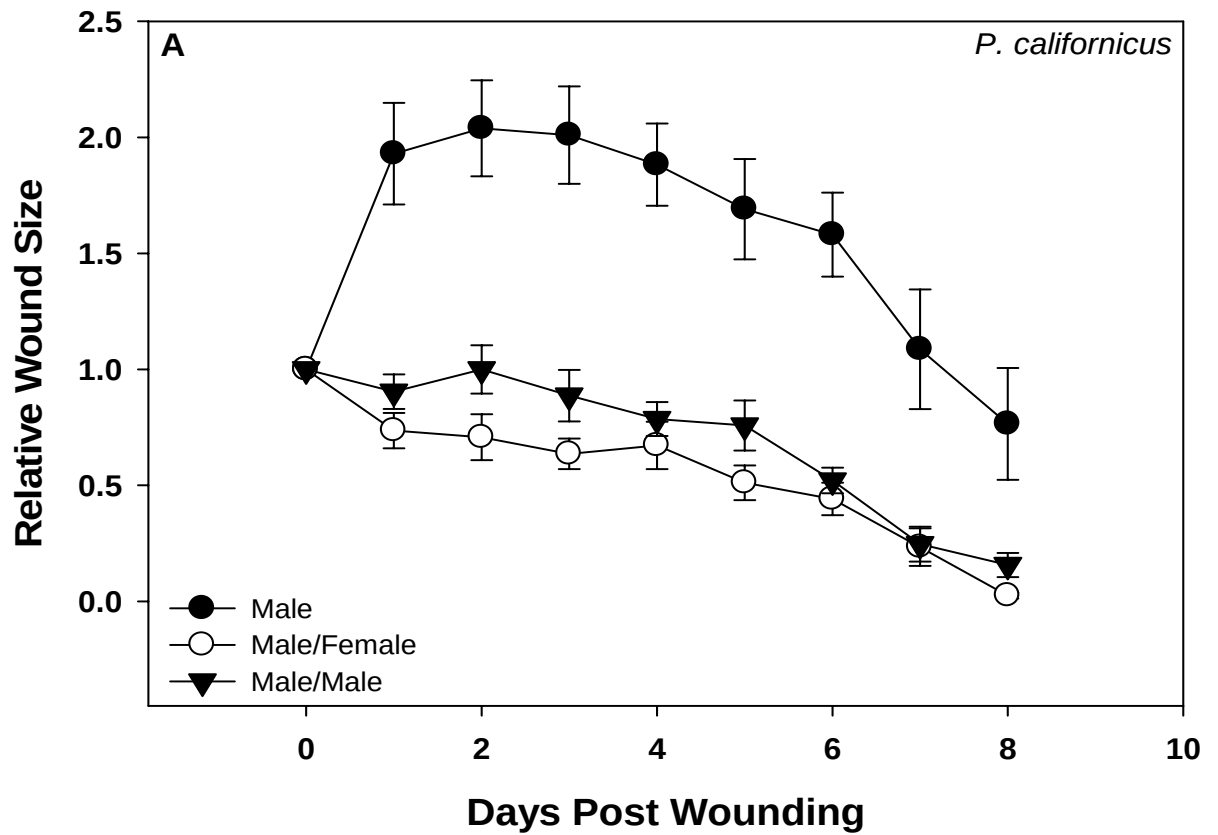


Figure 3.2: Effect of pair housing on wound size of male non-stressed *P. californicus*.

Pair housing decreased wound size across days in male pair housed *P. californicus*, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

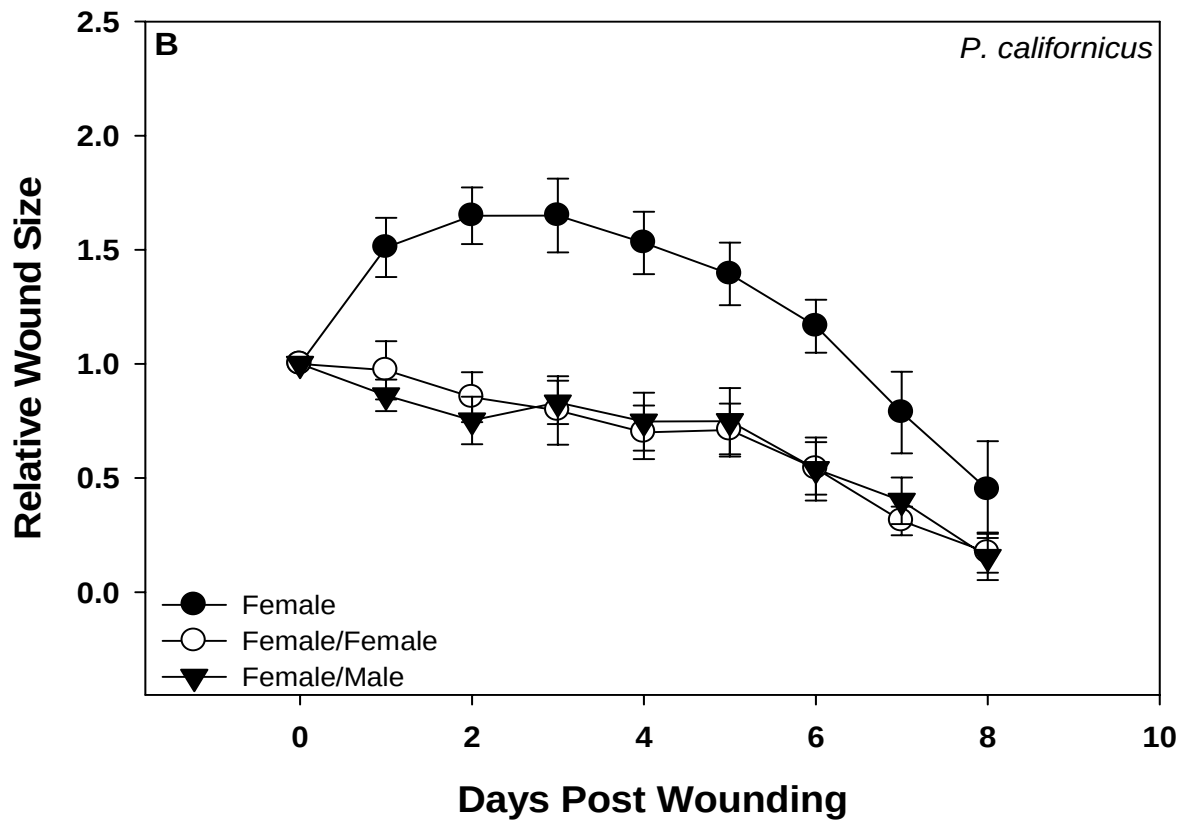


Figure 3.3: Effects of pair housing on wound size of female non-stressed *P. californicus*.

Pair housing decreased wound size across days in female pair housed *P. californicus*, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

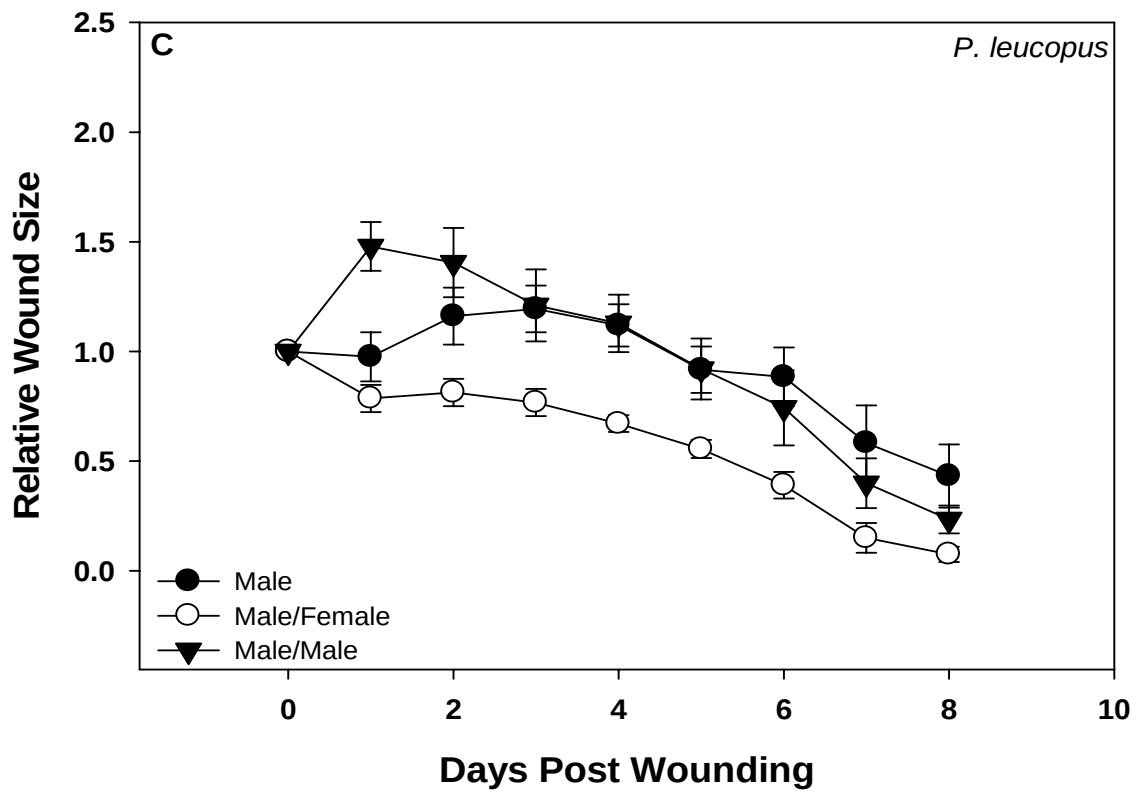


Figure 3.4: Effects of pair housing on wound size of male non-stressed *P. leucopus*.

Pair housing male *P. leucopus* with females significantly reduced wound size compared to socially isolated males; however, males pair housed with males did not differ in wound size compared to socially isolated males. Male *P. leucopus* housed with females had significantly smaller wounds than males housed with males. (Mean $\pm$ SEM)

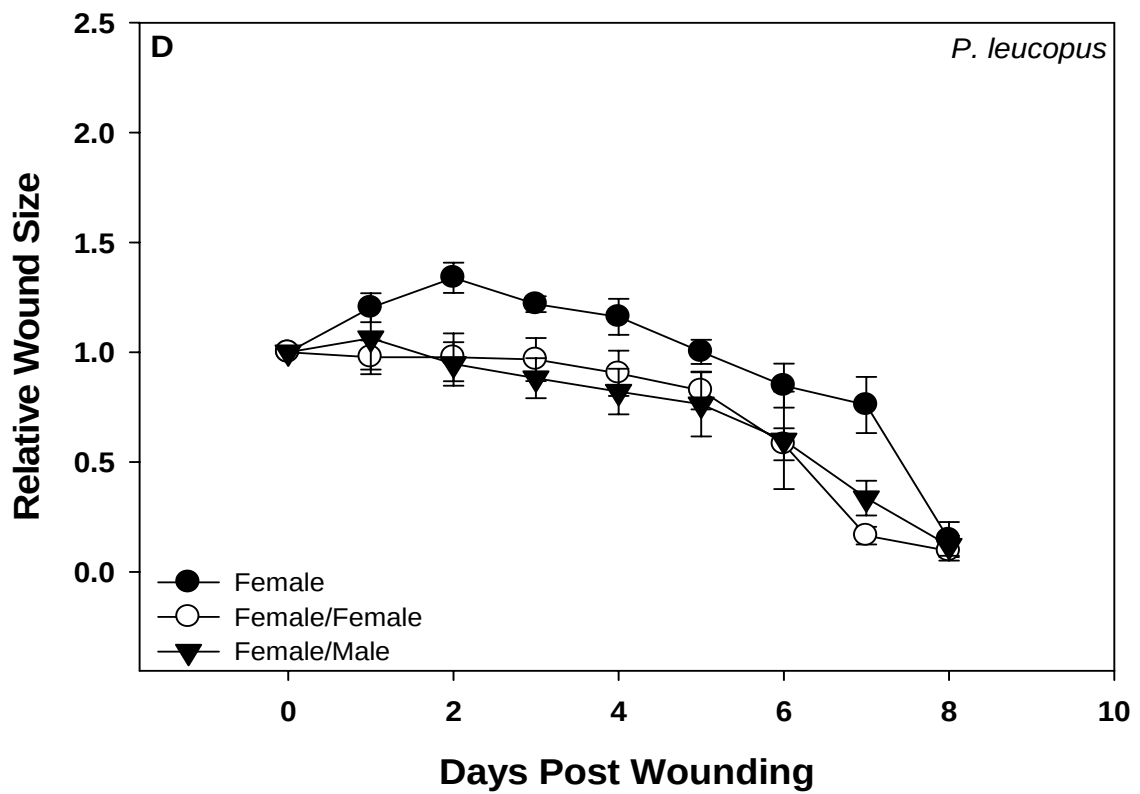


Figure 3.5: Effects of pair housing on wound size of female non-stressed *P. leucopus*.

Pair housing decreased wound size across days in female pair housed *P. leucopus*, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

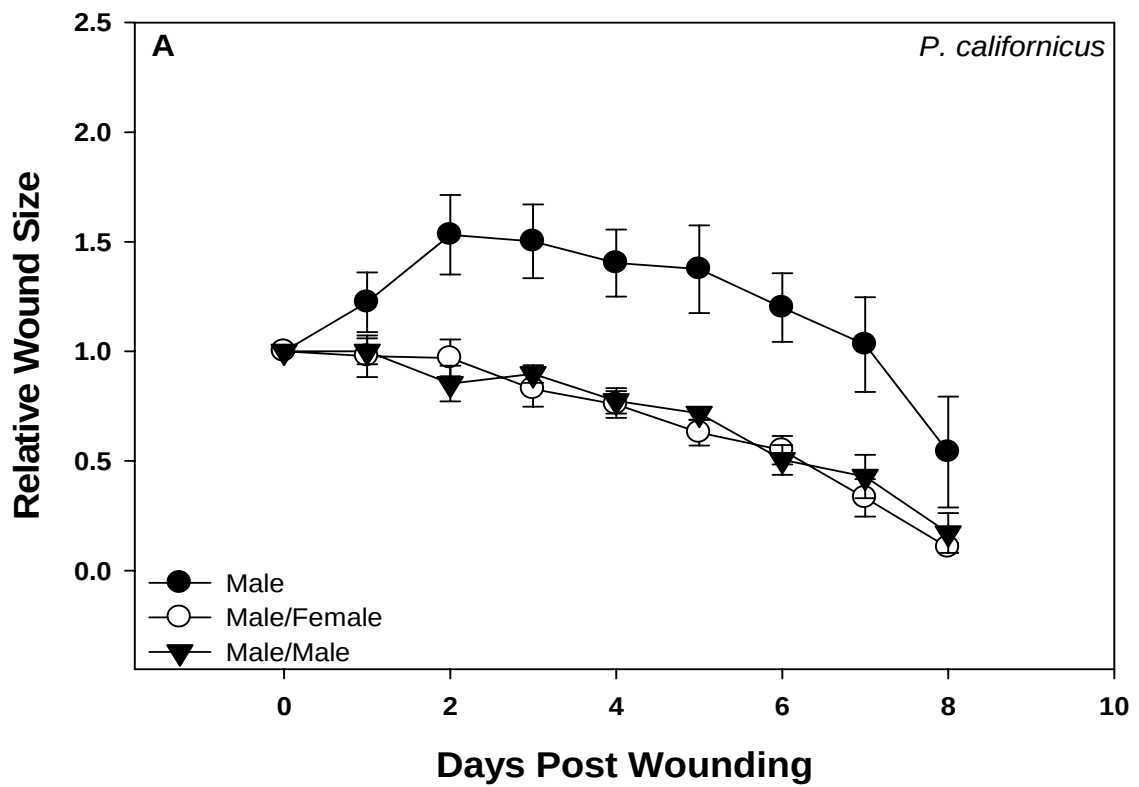


Figure 3.6: Effect of pair housing and restraint stress on wound size of male stressed *P. californicus*.

Pair housing decreased wound size across days in male pair housed *P. californicus* that received restraint stress, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

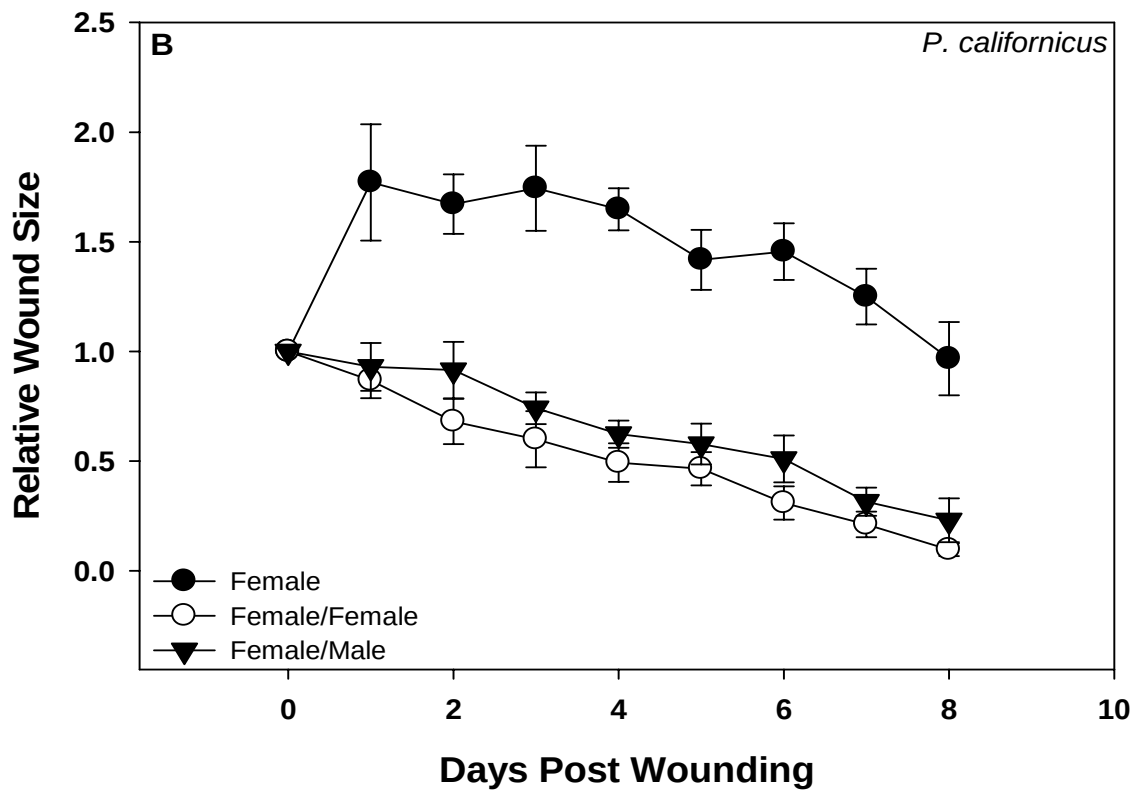


Figure 3.7: Effect of pair housing and restraint stress on wound size of female stressed *P. californicus*.

Pair housing decreased wound size across days in female pair housed *P. californicus* that received restraint stress, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

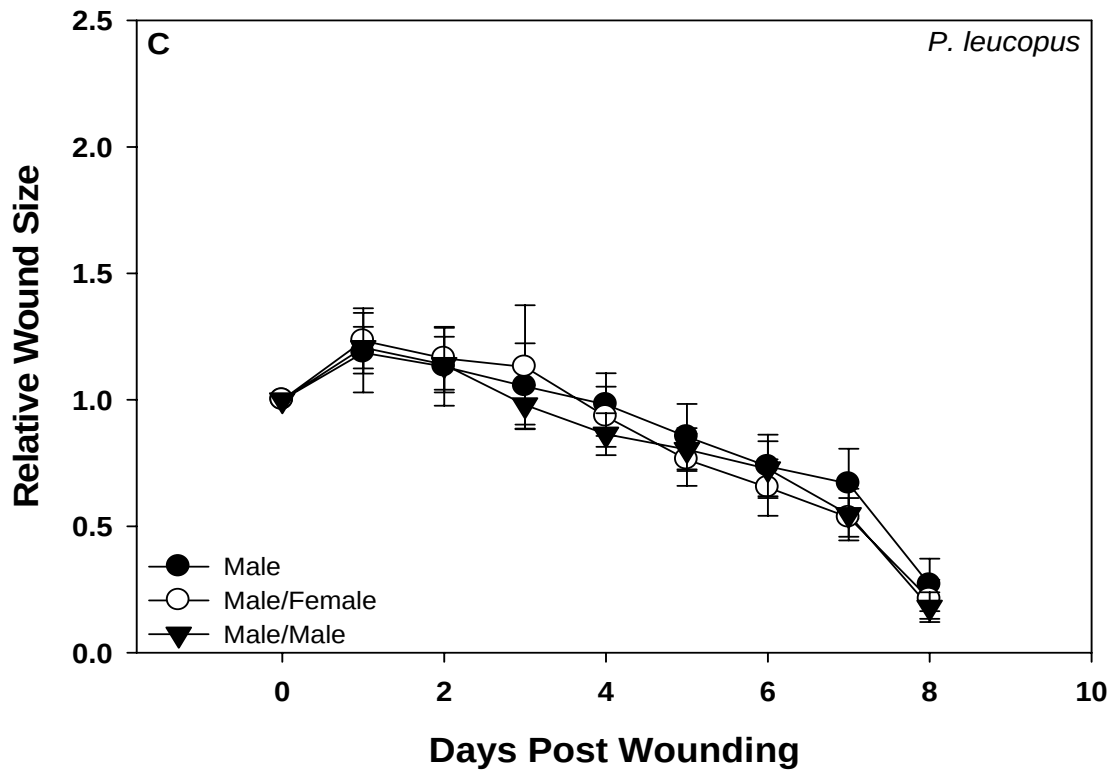


Figure 3.8: Effect of pair housing and restraint stress on wound size of male stressed *P. leucopus*.

Under conditions of stress, pair housing no longer facilitated wound size among male *P. leucopus* mice; socially isolated and pair housed males had wounds of similar sizes.
(Mean $\pm$ SEM)

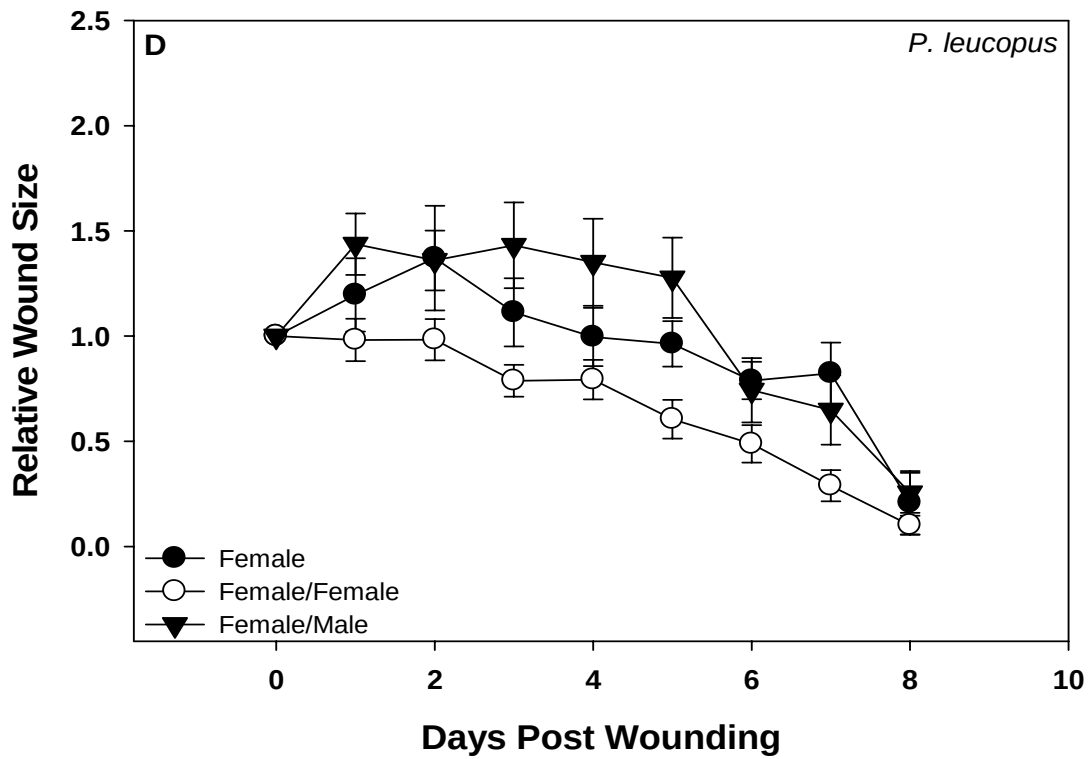


Figure 3.9: Effect of pair housing and restraint stress on wound size of female stressed *P. leucopus*.

Under conditions of stress, pair housing no longer facilitated wound size among female *P. leucopus* mice; wound sizes were significantly larger among females paired with a male versus female stimulus mice. (Mean $\pm$ SEM)

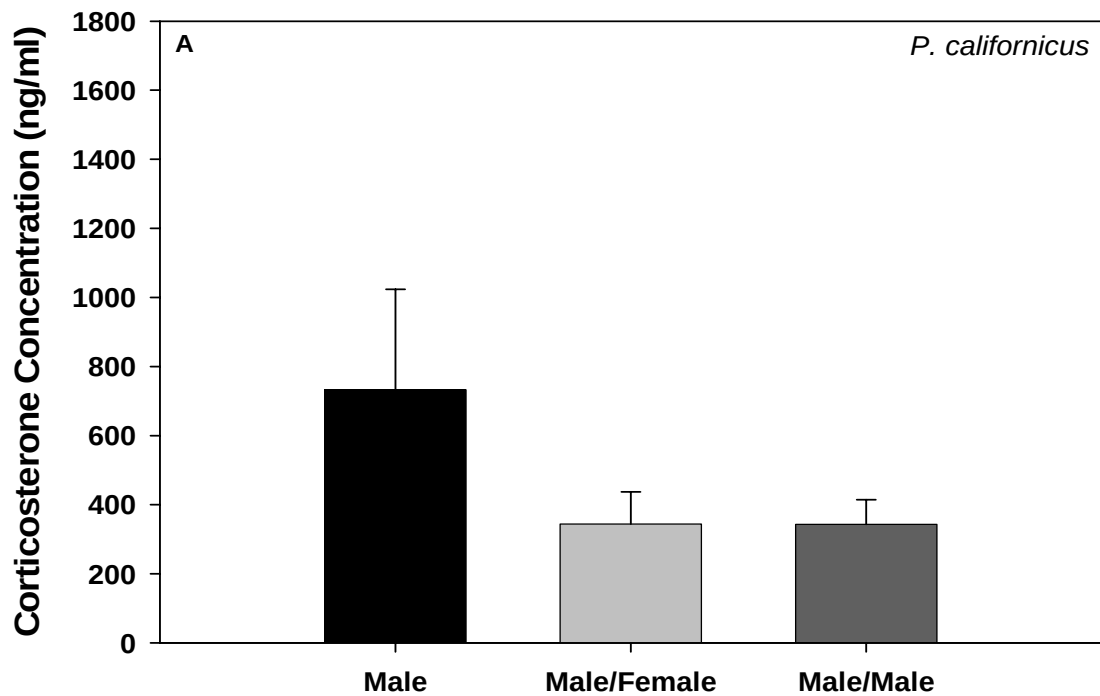


Figure 3.10: Effects of pair housing on terminal corticosterone concentrations of stressed male *P. californicus*.

Pair housing did not significantly reduce terminal corticosterone concentrations among stressed, pair housed male *P. californicus*, regardless of the sex of the stimulus mouse. (Mean $\pm$ SEM)

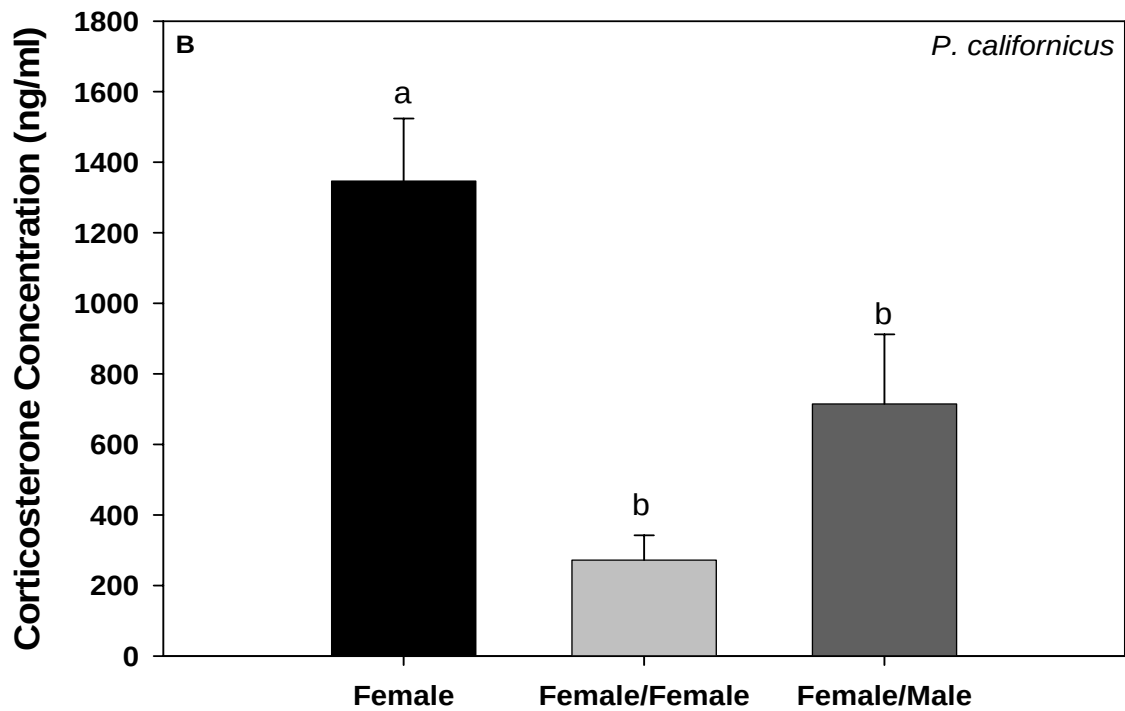


Figure 3.11: Effects of pair housing on terminal corticosterone concentrations of stressed female *P. californicus*.

Pair housing stressed, female *P. californicus* significantly reduced terminal corticosterone concentrations, irrespective of the sex of the stimulus mouse (b). (Mean $\pm$ SEM)

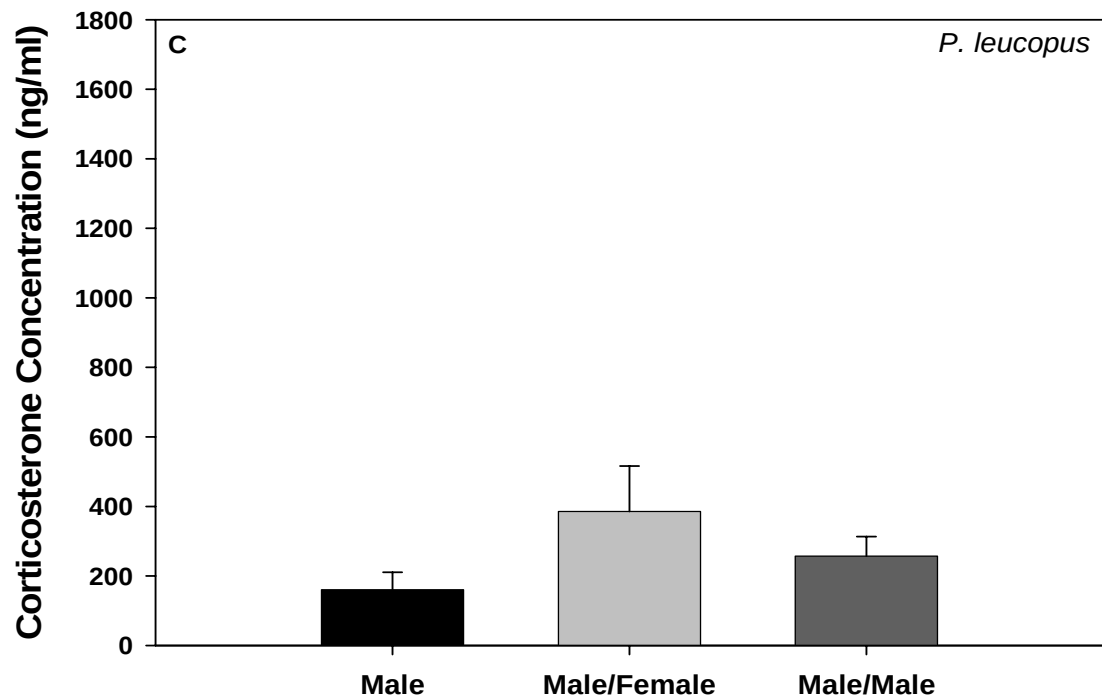


Figure 3.12: Effects of pair housing on terminal corticosterone concentrations of stressed male *P. leucopus*.

Pair housing did not alter terminal corticosterone concentrations among stressed, male *P. leucopus* mice. (Mean $\pm$ SEM)

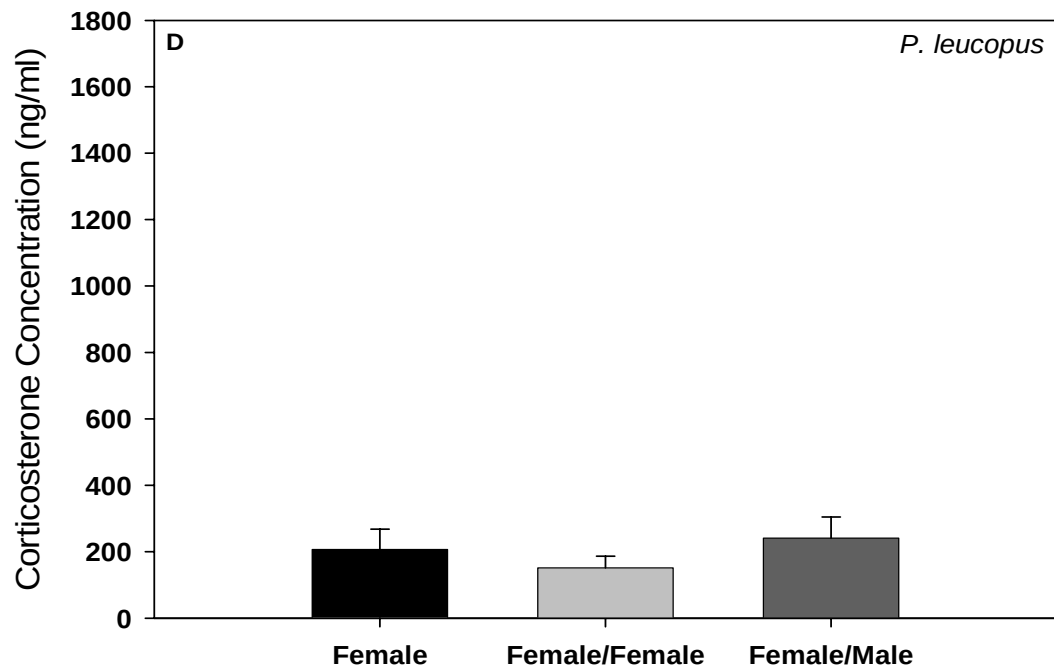


Figure 3.13: Effects of pair housing on terminal corticosterone concentrations of stressed female *P. leucopus*.

Pair housing did not alter terminal corticosterone concentrations among stressed male *P. leucopus* mice. (Mean $\pm$ SEM)

CHAPTER 4

RESTRAINT STRESS ALTERS NEURONAL SURVIVAL AND INFLAMMATION FOLLOWING FOCAL ISCHEMIA

Stress, and its relationship to disease, has been well documented in both basic science and clinical research and the general public often mentions stress as an important risk factor for stroke. Although many studies and case reports have provided support for a relationship between stress and stroke (Kattapong et al., 1996; Kothari et al., 1997; Pancioli et al., 1998; Sug Yoon et al., 2001; Yoon and Byles, 2002), there are others, however, that have failed to find a relationship between emotional factors, such as stress, and stroke (Agewall et al., 1998; Macko et al., 1996). These contrasting results likely reflect differences in the types of stressors and method for determination of stroke. Clinical and pre-clinical studies have provided evidence suggesting a link between peri-ischemic glucocorticoid concentrations and stroke outcome. Glucocorticoids, cortisol in humans and corticosterone in most rodents, increase during stressful events (Fassbender et al., 1994; Morgan et al., 2004; Olsson, 1990; Olsson et al., 1992). Increased morbidity and mortality in humans have been associated with elevated cortisol concentrations following stroke (Feibel et al., 1977; Marklund et al., 2004).

Rodent studies have demonstrated increased neuronal death following stress and exogenous administration of corticosterone (DeVries et al., 2001a; Madrigal et al., 2003; Sugo et al., 2002), while surgical or pharmacological suppression of corticosterone is neuroprotective (Antonawich et al., 1999; Karishma and Herbert, 2002; Sapolsky et al., 1986; Sapolsky and Pulsinelli, 1985). Additionally, a causal link between corticosteroids and stroke outcome is suggested by rodent studies in which manipulating blood corticosteroid concentrations during or after an ischemic event alters infarct size (Koide et al., 1986; Morse and Davis, 1990; Sapolsky and Pulsinelli, 1985; Smith-Swintosky et al., 1996).

The purpose of the present study was to evaluate the influence of acute restraint stress on infarct size, corticosterone concentration, and edema index following 60 min of experimental stroke. I hypothesized that endogenous glucocorticoids, produced via restraint, would exacerbate post-stroke outcome by increasing corticosteroid concentrations and promoting cerebral edema.

Materials and Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the local institutional animal care and use committee. Adult male C57BL/6 mice (Charles River, Wilmington, MA, USA) were housed individually in polycarbonate cages (28 x 17 x 12 cm) from the onset of the study in rooms maintained on a 14:10-h light:dark cycle at $20^{\circ} \pm 4^{\circ}$ C and relative humidity of $50 \pm 5\%$. Tap water and food (TekLab 8620) were

available *ad libitum*. Mice were randomly assigned to one of three experimental groups: (1) no stressor, (2) one hour of stress immediately followed by stroke (immediate stressor), and (3) one hour of stress, return to home cage and stroke commencing two hours later (stressor + 2 h delay).

Restraint

Mice were individually placed in well-ventilated, polystyrene tubes (50 ml). The tubes allowed for confined movements and postural adjustments. The “no stressor” mice remained in their home cages until the stroke procedure commenced.

Laser Doppler Flowmetry

Relative blood flow and blood gases were assessed during ischemia and reperfusion in a separate cohort of mice (no stressor, n=5; immediate stressor, n=6; stressor + 2 h delay, n=5). Femoral arterial blood pressure and cortical laser-Doppler (DRT4, Moor Instruments, LTD, Devon, England) were determined during occlusion and the first 30 min of reperfusion. A shallow indentation was made in the parietal skull (2 mm posterior, 3 mm lateral to Bregma) for the placement of the LDF probe. A thin oil interface and the probe were applied with a hood to block ambient light. The LDF signal was recorded continuously and averaged over 15 min intervals for comparison among groups. Arterial blood samples (100 μ L sample volume) were analyzed for pH, PO₂, PCO₂, and standard base excess at baseline and at the end of ischemia.

Experimental Stroke

Transient focal cerebral ischemia was induced in adult male mice (no stressor, n=8; immediate stressor, n=7; stressor + 2 h delay, n=6; 23-25 g) by right middle cerebral artery occlusion (MCAO), as described (Hattori et al., 2000). Briefly, the mice were

anesthetized with 1-1.5% halothane in O₂ enriched air delivered through a facemask. Unilateral MCAO was achieved by using the intraluminal filament insertion technique. MCAO was achieved via inserting a 6-0 nylon monofilament into the right internal carotid artery to a point 6 mm distal to the internal carotid artery-pterygopalatine artery bifurcation. Once the filament was secured, the wound was sutured and the mouse was allowed to emerge from anesthesia in its home cage. After 60 min of ischemia, the mice were re-anesthetized, and reperfusion was initiated through withdrawal of the filament. Mice were given a 0.5 ml s.c. injection of lactated Ringer's solution at the conclusion of the surgical procedure.

Determination of Blood Corticosterone Concentrations

Blood samples were collected at 60 min (no stressor, n=9; immediate stressor, n=10; stressor + 2 h delay, n=10), 4 h (no stressor, n=9; immediate stressor, n=10; stressor + 2 h delay, n=10), and 12 h (no stressor, n=14; immediate stressor, n=14; stressor + 2 h delay, n=15) following insertion of the occluder in separate cohorts of mice. Samples were centrifuged at 6°C for 30 min at 3000 rpm. The serum was collected and then stored at -80°C. Corticosterone concentrations were determined using an I¹²⁵ radioimmunoassay kit (ICN Pharmaceuticals, Inc., CA). All samples were quantified in the same assay.

Determination of Stroke Volume

Brains were removed after three days of reperfusion and sectioned into five 2-mm thick coronal sections. Sections were incubated for 15 min in 2, 3, 5-triphenyltetrazolium maintained at 37°C. Following staining, sections were fixed in 10% formalin and

photographed. Images were analyzed and infarct size was expressed as percent of contralateral hemisphere after correcting for edema (Inquiry; Loats Inc, Rockville, MD, USA).

Edema

A dry/wet weight method was used to measure brain water content in mice that underwent MCAO under no stressor conditions (n=5), immediate stressor conditions (n=4), or stressor + 2 h delay conditions (n=4). The brains were placed on pre-weighed slides, reweighed, and dried in an oven at 60° C for 2 to 3 days until the weight of dry tissue was constant. The water content was calculated as the difference between wet and dry weight of the sample and then converted to a percentage of its wet weight.

Statistical Analysis

All data are reported as mean values $\pm$ standard error of the mean (SEM). Multiple comparisons between groups were made by one-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference among treatment groups ($p < 0.05$), Tukey's PLSD were used to compare among groups. When repeated measures ANOVA indicated a significant difference among treatment groups ($p < 0.05$), Scheffe's tests were used to compare among the groups.

Results

Laser Doppler Flowmetry

During MCAO, relative blood flow decreased to $<10\%$ of pre-ischemia baseline in the cortex of all three treatment groups. After withdrawal of the filament, blood flow was restored to $>90\%$ among each experimental group. There were no differences among

no stressor, immediate stressor, and stressor + 2 h delay in LDF analyzed at any measurement time point of MCAO ($p > 0.05$) in either mean arterial blood pressure (MABP), pH, PCO₂, or PO₂ (data not shown).

Infarct

A significant effect of treatment on infarct size was observed among mice receiving 60 min of MCAO ($F(2, 18) = 5.941$, $p < 0.05$; Figure 4.1). Post-hoc analysis revealed a significant difference between no stressor and immediate stressor mice ($p < 0.05$); specifically, no stressor mice had significantly smaller infarct sizes compared to immediate stressor mice. The stressor + 2 h delay mice were significantly different from immediate stressor mice ($p < 0.05$), with the immediate stressor mice having larger infarct sizes compared to the stressor + 2 h delay mice. No differences were observed between no stressor mice and stressor + 2 h delay mice ($p > 0.05$).

Corticosterone Concentration

Intra-ischemic corticosterone concentrations, sampled 60 min following insertion of the occluder, revealed no significant differences among the three treatment groups ($F(2,26) = 0.542$, $p > 0.05$; Figure 4.2). Corticosterone concentrations, sampled 4 h following insertion of the occluder, revealed no significant differences among the three treatment groups ($F(2,26) = 1.162$, $p > 0.05$; Figure 4.3). Corticosterone concentrations collected 12 h post-MCAO revealed no significant differences among the three treatment groups ($F(2,40) = 2.344$, $p > 0.05$; Figure 4.4); however, an independent t-test between the no stressor and immediate stressor groups revealed significantly higher circulating corticosterone concentrations among the immediate stressor mice at 12 h of reperfusion ($t(26) = 2.203$, $p = 0.04$).

Edema

A significant main effect of treatment was observed in total water content 24 h post MCAO ($F(2, 10) = 7.74, p < 0.05$; Figure 4.5). A significant difference in total water content was observed between no stressor mice and immediate stressor mice ($p < 0.05$). Similarly mice undergoing 60 min of restraint followed by MCAO differed significantly in total water content compared to mice that received a 2 h delay post stress ($p < 0.05$). However, no difference in water content was observed between no stressor mice and those that received a 2 h delay after stress prior to the induction of MCAO ($p > 0.05$). Thus, the pattern of edema is similar to the pattern of infarct size among the three groups; the greater the edema at 24 h, the greater the infarct size at 72 h.

Discussion

Glucocorticoids have been shown previously to increase neuronal destruction; specifically, animal studies have demonstrated increased neuronal death following stress and exogenous administration of corticosterone (DeVries et al., 2001a; Madrigal et al., 2003; Sugo et al., 2002). We now show that a stress-induced increase in infarct size is associated with a significant increase in cerebral edema; specifically, mice experiencing stress immediately prior to MCAO resulted in a larger infarct size and greater cerebral edema compared to mice undergoing MCAO without stress (Figures 4.1 and 4.5).

Equally important, neuronal protection from stress was observed in mice that experienced the same stressor but were allowed a delay before the onset of MCAO, as these infarct sizes and cerebral edema indices were comparable in size to mice not experiencing stress prior to ischemia (Figures 4.1 and 4.5). While intraischemic corticosterone

concentrations were comparable among the three groups at 60 min and 4 h following reperfusion (Figures 4.2 and 3), samples taken 12 h following MCAO revealed higher corticosterone concentrations in mice experiencing stress immediately prior to MCAO compared to mice not experiencing the stressor (Figure 4.4). In comparison to infarct size and cerebral edema, 12 h corticosterone concentrations in mice allowed a delay between stress and MCAO were similar to mice not experiencing the stressor (Figure 4.4).

Stress has been repeatedly shown to increase neuronal damage following both focal and global ischemia via corticosterone mediated mechanisms (DeVries et al., 2001a; Madrigal et al., 2003; Smith-Swintosky et al., 1996; Sugo et al., 2002). My observation that prior exposure to stress increases post-stroke infarct size extends previous studies demonstrating how glucocorticoids can potentiate neuronal damage. Glucocorticoids increase neuronal damage following stroke, while attenuating stress induced neuronal damage, via metyrapone, reduces neuronal damage (Smith-Swintosky et al., 1996). Glucocorticoids not only increase neuronal damage, but also increase cerebral edema, which leads to secondary neuronal damage (Stoll et al., 2002), caused by increased inflammatory cells and pro-inflammatory cytokine expression (Barone et al., 1997; Boutin et al., 2001; Dawson et al., 1996; Dinkel et al., 2003; Yang et al., 1998).

While attenuating stroke-induced neuronal damage via reductions in glucocorticoids via administration of a synthetic drug has been previously shown (Smith-Swintosky et al., 1996), the present study demonstrates that reduced circulating corticosteroids following stroke can prevent increased neuronal damage following when a temporal window is placed between the conclusion of the stressor and the induction of

stroke. A delay in the onset of stroke following restraint is the first experiment, to my knowledge, to demonstrate restraint stress as a method of preconditioning.

Preconditioning is operationally defined as a developed tolerance to a stressor that would otherwise be lethal or highly injurious (Ferriero, 2005). Given the decrease in neuronal damage, cerebral edema, and circulating corticosterone concentrations following stroke among the mice undergoing the preconditioning stress, I can conclude that circulating stress-induced corticosterone concentrations are important factors in the mediation of stroke-induced neuronal damage and cerebral edema.

Taken together, these data suggest that prior exposure to acute stress exacerbates histological stroke outcome, while decreased corticosteroid exposure following stress provides protection from ischemia-induced neuronal death and cerebral edema. This outcome is likely linked to glucocorticoid receptor-dependent corticosteroid mechanisms. Therefore, interventions that are aimed at decreasing the inflammatory reaction of the post-ischemic brain are an attractive therapeutic strategy in stroke, with a potentially wide therapeutic and temporal window.

Future Directions

Cerebral ischemia induces expression of various cytokines. Several studies have demonstrated a significant up-regulation of various cytokines, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) - α after cerebral ischemia (Berti et al., 2002; Dziedzic et al., 2004; Gong et al., 1998; Hill et al., 1999; Hosomi et al., 2005; Liu et al., 1993; Offner et al., 2006; Perini et al., 2001; Tarkowski et al., 1995c; Vila et al., 2000; Wang et al., 2004; Zaremba and Losy, 2001). Also, many experimental and clinical studies have demonstrated a correlation between infarct size and cytokine expression

(Clark et al., 1999; Fassbender et al., 1994; Tarkowski et al., 1995c; Wang et al., 2004; Zaremba et al., 2001), with increased expression of pro-inflammatory cytokines correlating with increased neuronal death. However, TNF- α has been demonstrated to protect cultured neurons against excitotoxic death induced by the glutamate receptor agonist *N*-methyl-D-aspartic acid (NMDA; (Bruce et al., 1996; Carlson et al., 1998; Carlson et al., 1999). Further, following an animal model of stroke, mice deficient for TNF- α receptors have enhanced sensitivity to ischemic brain damage following arterial occlusion (Bruce et al., 1996).

It has been proposed that stress exacerbates neuronal destruction via increases in inflammatory cytokines in the brain (MacPherson et al., 2005). Acute stressors increase cytokine production (Deak et al., 2005; Hayley et al., 2005; Johnson et al., 2005; Minami et al., 1991; Miyahara et al., 2000; Nguyen et al., 1998; Nguyen et al., 2000; O'Connor et al., 2003); however, not all acute stressors result in increased cytokine expression. Restraint stress (30 min) decreased expression of IL-6 in the hypothalamus and midbrain, while a longer duration (4 h) of restraint stress increased IL-6 mRNA in the midbrain (Shizuya et al., 1998). Likewise, immobilization stress and exogenous glucocorticoid injections did not induce IL-1 β expression in the brain (Quan et al., 2000). Studies have also demonstrated increases in brain pro-inflammatory cytokine expression following chronic stressors (Mormede et al., 2002; Quan et al., 2001). It is difficult to assess the effects of acute and chronic stress on brain cytokine expression. The contrasting results likely reflect, among other factors, differences in the types and duration of stressors. Given the divergence in whether pro-inflammatory cytokines have detrimental or

protective consequences to neurons, future studies will evaluate the influence of acute restraint stress on the time course of pro-inflammatory cytokine expression following 60 min of stroke.

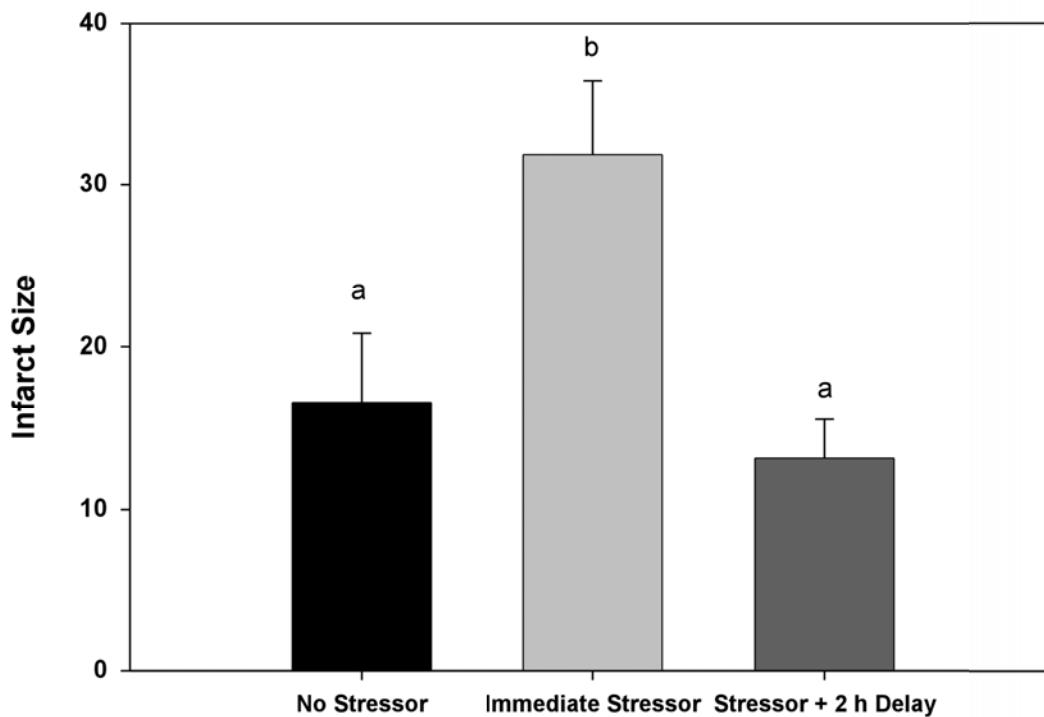


Figure 4.1: The effects of stress on infarct size.

Male mice underwent 60 min of middle cerebral artery occlusion (MCAO) either under conditions of no stressor, immediate stressor, or stressor + 2 h delay. Mice were survived for three days, when brain collections were done to assess post-stroke neuronal damage via TTC staining. A significant effect of treatment on infarct size was observed among mice receiving 60 min of MCAO ($F(2, 18) = 5.941, p < 0.05$). Post-hoc analysis revealed a significant difference between no stressor and immediate stressor mice ($p < 0.05$); specifically, no stressor mice had significantly smaller infarct sizes compared to immediate stressor mice (a). The stressor + 2 h delay mice were significantly different from immediate stressor mice ($p < 0.05$), with the immediate stressor mice having larger infarct sizes compared to the stressor + 2 h delay mice (b). No differences were observed between no stressor mice and stressor + 2 h delay mice ($p > 0.05$).

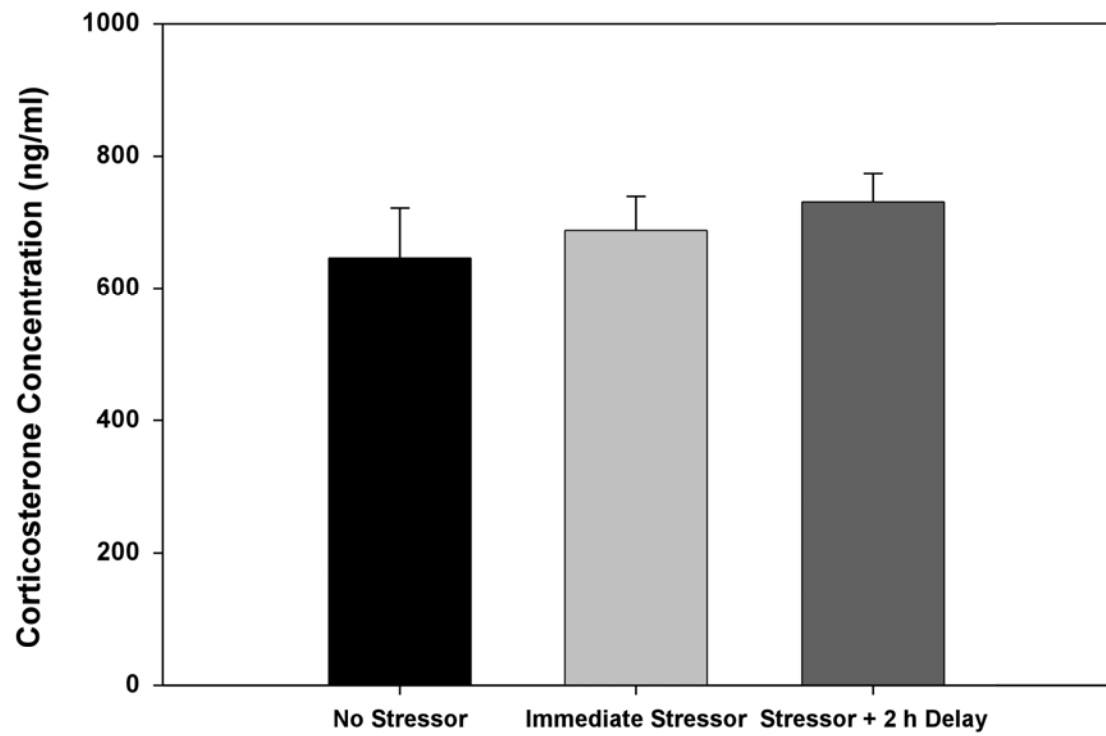


Figure 4.2: Intra-ischemic corticosterone concentrations among no stressor, immediate stressor, and stressor + 2 h delay mice.

Intra-ischemic corticosterone concentrations, sampled 60 min following insertion of occluder, revealed no significant differences among the three treatment groups.

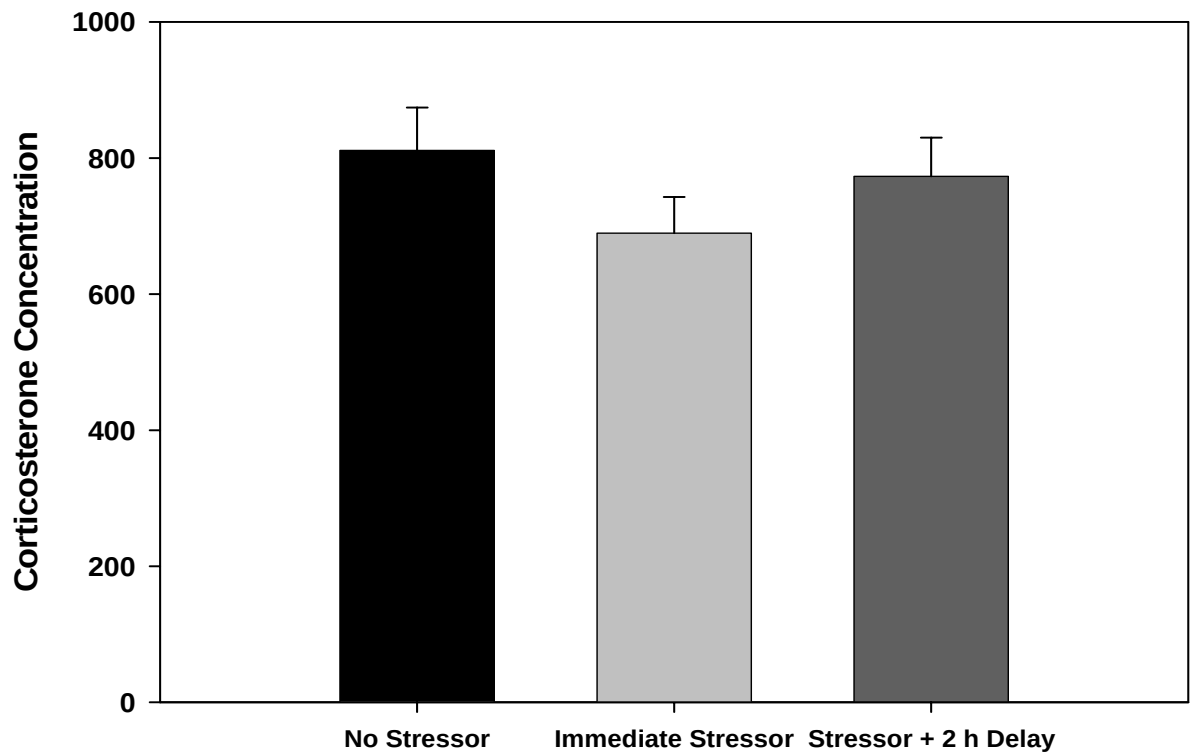


Figure 4.3: Corticosterone concentrations among no stressor, immediate stressor, and stressor + 2 h delay mice following 4 h of reperfusion.

Corticosterone concentrations, sampled 4 h following insertion of occluder, revealed no significant differences between treatment groups.

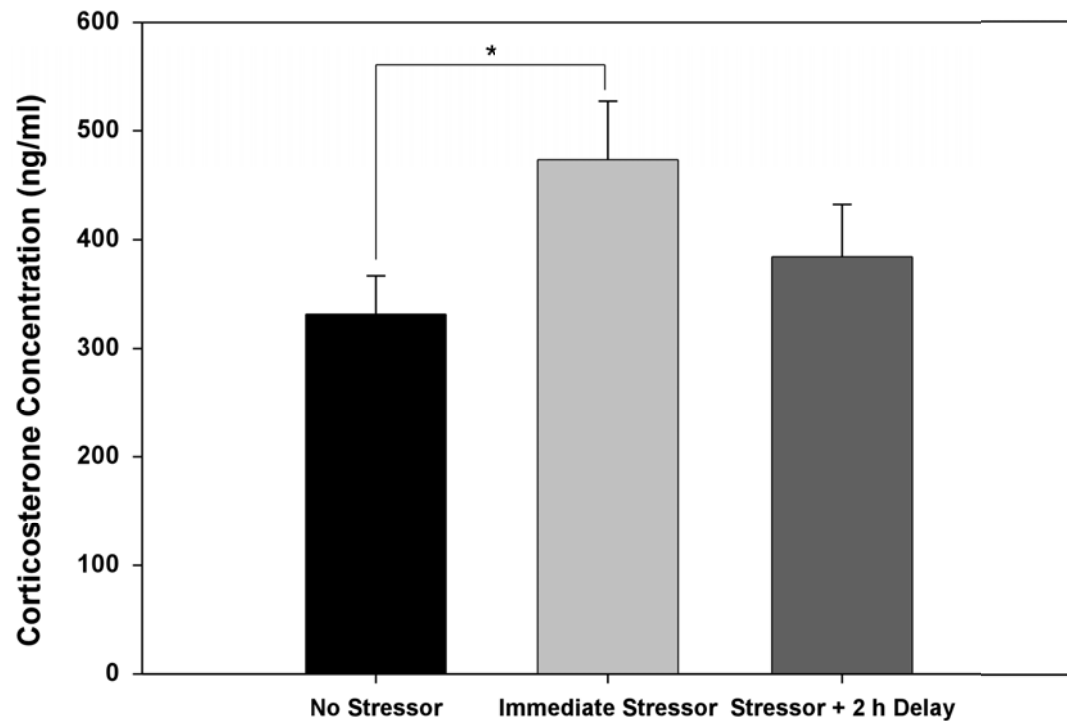


Figure 4.4: Corticosterone concentrations among no stressor, immediate stressor, and stressor + 2 h delay mice following 12 h of reperfusion.

Corticosterone concentrations, sampled 12 h following insertion of the occluder, revealed no significant differences among the three treatment groups. However, independent t-tests between the no stressor and immediate stressor groups revealed significantly higher circulating corticosterone concentrations following 12 h of reperfusion. An asterisk (*) indicates statistical significance ($p < 0.05$).

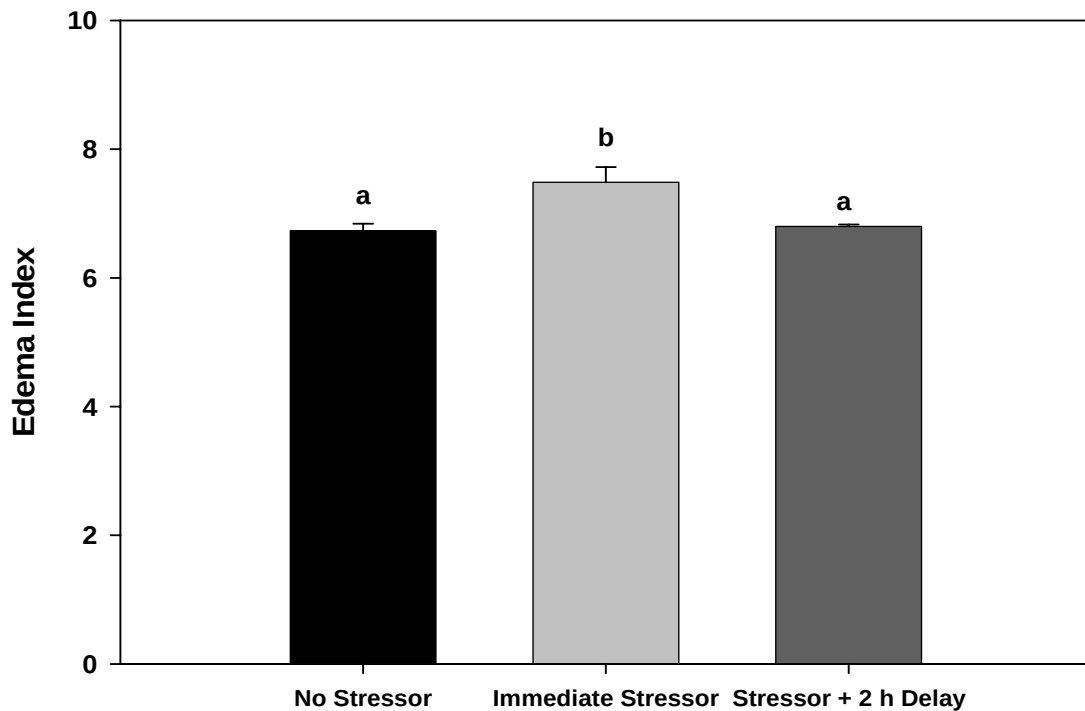


Figure 4.5: The effects of stress on edema index.

Male mice underwent 60 min of middle cerebral artery occlusion (MCAO) either under conditions of no stressor, immediate stressor, or stressor + 2 h delay. Mice were survived for 24 h, when brain collections were done to assess post-stroke water content. Only mice undergoing restraint stress immediately prior to MCAO demonstrated larger infarct sizes. Post-hoc analysis revealed a significant difference between no stressor and immediate stressor mice ($p < 0.05$), specifically, no stressor mice had significantly smaller infarct sizes compared to immediate stressor mice (a). The stressor + 2 h delay mice were significantly different from immediate stressor mice ($p < 0.05$), with the immediate stressor mice having larger infarct sizes compared to the stressor + 2 h delay mice (b). No differences were observed between no stressor mice and stressor + 2 h delay mice ($p > 0.05$).

CHAPTER 5

FOCAL ISCHEMIA AUGMENTS PERIPHERAL IMMUNE FUNCTION

The nervous system communicates with the immune system in various ways, from sympathetic nerve damage to cytokine trafficking (Ader et al., 1995; Blalock, 1994; Sternberg, 2001). Damaging the nervous system directly influences immunologic status. For example, there is evidence suggesting that the immune system is altered following stroke, via up-regulation of pro-inflammatory cytokines (Offner et al., 2006). Inflammation plays an important role in the pathophysiology of ischemic stroke (Barone and Feuerstein, 1999). White blood cell counts are elevated soon after stroke, as well as leukocyte infiltration (Chuaqui and Tapia, 1993; Pozzilli et al., 1985; Schroeter et al., 1994). Stroke up-regulates the systemic T-cell response (Tarkowski et al., 1991) and lateralizes T-cell dependent cutaneous inflammation (Tarkowski et al., 1996). The effects of central neuronal damage correlate with changes to cutaneous sympathetic nerve traffic (Tarkowski et al., 1995b), thereby suggesting that early after ischemic stroke, the immune system is altered and can have severe effects on clinical outcome.

Cerebral ischemia influences peripheral inflammatory responses. Brain lesions caused by stroke lead to lateralization of T-cell dependent cutaneous inflammation (Tarkowski et al., 1991; Tarkowski et al., 1995b) following antigenic challenge. Also,

brain structures in the frontal lobe are involved in the regulation of delayed-type hypersensitivity (DTH) responses both in the early and chronic phases of stroke (Tarkowski et al., 1998). Neurological changes influencing peripheral immune responses have been previously investigated. Cardiac arrest and cardiopulmonary resuscitation, which primarily damages the hippocampus, results in augmented T-cell dependent immune responses (Neigh et al., 2005b). These changes in the immune system can cascade into other diseases and infections that accompany stroke. Stroke survivors have a 15-fold increase in further vascular events compared to the general population (Redfern et al., 2002). The focus of the interaction between the immune system and the brain has been acute and chronic infection prior to ischemic stroke. These concomitant infections may lead to the development of atherosclerotic plaques or trigger complications due to ischemia (Lavalley et al., 2002). Risk for influenza increases post-stroke and influenza vaccinations may protect against brain infarction by preventing viral infections or bacterial infections that accompany influenza (Poole et al., 2000).

Given the increasing clinical evidence demonstrating altered peripheral immune responses following ischemia, a better understanding of the mechanisms behind these alterations will be addressed in this chapter. By understanding the underlying processes behind post-stroke peripheral immune function, interventions that could potentially mitigate these responses can be addressed (seen in Chapters 6 and 7). Therefore, I assessed the alterations in DTH response to antigenic challenge, wound healing rate, and primary immune response to a novel antigen following MCAO. I also examined how preventing MCAO-induced neuronal damage alters peripheral immune function.

Materials and Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the local institutional animal care and use committee. Adult male C57BL/6 mice (Charles River, Wilmington, MA, USA) were housed individually in polycarbonate cages (28 x 17 x 12 cm) from the onset of the study in rooms maintained on a 14:10-h light:dark cycle at $20^{\circ} \pm 4^{\circ} \text{C}$ and relative humidity of $50 \pm 5\%$. Tap water and food (TekLab 8620) were available *ad libitum*. Mice were randomly assigned to one of four experimental groups: (1) normothermic MCAO (n=31; head maintained at 37°C), (2) hypothermic MCAO (n=29; head lowered to 27°C), and (3) normothermic SHAM (n=33; head maintained at 37°C), or hypothermic SHAM (n=20; head lowered to 27°C).

Experimental Stroke

Transient focal cerebral ischemia was induced in adult male mice (23-25 g) by middle cerebral artery occlusion (MCAO), as described (Hattori et al., 2000). Briefly, the mice were anesthetized with 1-1.5% halothane in O_2 enriched air delivered through a facemask. Unilateral MCAO was achieved by using the intraluminal filament insertion technique, which involved inserting a 6-0 nylon monofilament into the right internal carotid artery to a point 6 mm distal to the internal carotid artery-pterygopalatine artery bifurcation. Once the filament was secured, the wound was sutured and the mouse was allowed to emerge from anesthesia in its home cage. After 60 min of ischemia, the mice were re-anesthetized, and reperfusion was initiated through withdrawal of the filament. Mice were given a 0.5 ml s.c. injection of lactated Ringer's solution at the conclusion of

the surgical procedure. A subset of MCAO mice were maintained at a body temperature of 27°C during surgery to serve as controls for other aspects of the MCAO surgery, including any immunological consequences of the introduction of the filament.

Laser Doppler Flowmetry

Femoral arterial blood pressure and cortical laser-Doppler (DRT4, Moor Instruments, LTD, Devon, England) were determined during occlusion and the first 30 min of reperfusion. A shallow indentation was made in the parietal skull (2 mm posterior, 3 mm lateral to Bregma) for the placement of the LDF probe. A thin oil interface and the probe were applied with a hood to block ambient light. The LDF signal was recorded continuously and averaged over 15 min intervals for comparison among groups.

Determination of Stroke Volume

Brains were removed after three days of reperfusion and sectioned into five 2-mm thick coronal sections. Sections were incubated for 15 min in 2, 3, 5-triphenyltetrazolium maintained at 37°C. Following staining, sections were fixed in 10% formalin and photographed. Images were analyzed and infarct size was expressed as percent of contralateral hemisphere after correcting for edema (Inquiry; Loats Inc, Rockville, MD, USA).

Assessment of Peripheral Immune Function

KLH. To assess primary humoral immunity, mice were injected subcutaneously, on post-MCAO day 7 with keyhole limpet hemocyanin (KLH), to which they were previously naïve (150 µg suspended in 0.1 ml sterile 0.9% saline). KLH evokes an acute immune response, but does not replicate or cause long-term fever or inflammation.

Immunoglobulin (Ig) G specific for KLH was measured on Day 4, Day 7, Day 14, Day 21, and Day 28 post-immunization for primary humoral immunity using a specific enzyme-linked immunosorbent assay (ELISA). Diluted serum samples (1:100) were added to antigen-coated plates that were pre-coated with 0.5% milk in PBS to reduce nonspecific binding. After incubation, secondary antibody (AP-conjugated anti-mouse IgG, 1:1000) was added, and after further incubation, the enzyme substrate p-nitrophenyl phosphate was added. The optical density (OD) of each well was determined using a BioRad plate reader (405 nm). Mean OD for each sample was expressed as a percent plate positive control for statistical analyses.

DTH. Delayed-type-hypersensitivity (DTH), a test of cell mediated immune function, was assessed in a separate cohort of mice following MCAO one week post recovery. Mice were sensitized to the antigen, 2-4-dinitro-1-fluorobenzene (DNFB, Sigma), placed on their dorsum for the assessment of an antigen-specific, cell-mediated, DTH reaction in the skin on two consecutive days two weeks prior to MCAO. To do so, mice were anesthetized with isoflurane vapor, and an area of approximately 2 x 3 cm was shaved on the dorsum. Twenty-five μ l of DNFB [0.5% (wt/vol) in 4:1, acetone:olive oil vehicle] was applied to the shaved skin. The thickness of both pinnae were measured before sensitization using a constant-loading dial micrometer (Mitutoyo, Japan) for later comparison during DTH induction. On day 7, or 1 week after MCAO and 3 weeks after sensitization, pinnae thickness was again measured and mice received a challenge of 20 μ l of DNFB [0.2% (wt/vol) in 4:1, acetone:olive oil vehicle] to the skin of the dorsal surface of the right pinna, and the left pinna was treated with the vehicle. Mice were again anesthetized with isoflurane for the procedure. Following challenge, mice were

returned to their cages and pinnae thickness was measured every 24 h for the next 8 days. All measurements were made on the same relative region of the pinnae. DTH responses were determined as percent increases of pinnae thickness over baseline for each mouse and compared among groups using repeated measures.

Wounding. On the seventh day following MCAO, mice were anesthetized with isoflurane in O₂-enriched air. A 3 x 3 cm patch of fur was shaved on the dorsal surface and cleansed with ethanol. Dorsal, mid-scapular cutaneous wounds were produced using a 3.5 mm dermal punch biopsy tool (Miltex Instruments, NY, USA). Each day, beginning with the day of wounding, the mice were anesthetized with isoflurane in O₂-enriched air and the wound site was photographed using a digital camera. Each photograph included a standard-sized circle (3.5 mm ID) placed on the skin near the wound. The wound size for each mouse was determined using Canvas 8.0 (Deneba Systems, FL, USA) and expressed as the ratio of wound area to the area of the standard circle in the photograph (in pixels).

Statistical Analysis

Infarct size, pinnae thicknesses, wound size, and anti-KLH IgG concentrations were compared using ANOVAs. Post hoc analysis (Scheffe test) was used to further distinguish among groups, and all differences were considered statistically significant if $p < 0.05$. When statistically appropriate ($p > 0.05$; Keppel, 1991), normothermic SHAM and hypothermic SHAM groups were collapsed into one group for further analyses and this group was designated as 'SHAM.'

Results

Infarct Size

Analysis of infarct size post-stroke revealed a main effect of temperature ($F(1, 8) = 24.092$, $p < 0.05$; Figure 5.1). Hypothermia (head temperature 27°C) significantly reduced infarct size compared to normothermic MCAO mice. SHAM surgery, under normothermic and hypothermic conditions, did not produce infarcts (data not shown).

Anti-KLH IgG

Analysis of post-MCAO induced KLH antibody response revealed a main effect of surgery ($F(2, 22) = 4.554$, $p < 0.05$) and a main effect of time ($F(4, 88) = 23.299$, $p < 0.05$). Post-hoc analysis revealed significant differences in anti-KLH IgG antibody production between normothermic MCAO and SHAM surgery mice (Figure 5.2). Neuronal damage induced by MCAO increases antibody production across days compared to mice not experiencing neuronal damage. Post-hoc analysis also revealed significant differences between normothermic and hypothermic MCAO mice. Preventing MCAO-induced neuronal damage, via hypothermia, blunted the increase in antibody production. Challenge with KLH did not alter survival rate following MCAO (data not shown).

Delayed-Type Hypersensitivity (DTH) Response

Analysis of the DTH response to challenge did not reveal a main effect of surgery ($F(2, 48) = 2.382$, $p > 0.05$); however, a main effect of time ($F(6, 288) = 11.289$, $p < 0.05$) and an interaction between surgery and time ($F(12, 288) = 2.661$, $p < 0.05$) were observed. Independent t-test analysis of pinnae edema on day six revealed a significant increase in pinna measurements in normothermic MCAO mice compared to SHAM mice

(Figure 5.3). Preventing neuronal damage, via hypothermia, reduced pinna edema in the hypothermic MCAO mice compared to normothermic MCAO mice. No difference in edema across days was observed between hypothermic MCAO and SHAM surgery mice. Sensitization and challenge with DNFB did not alter survival rate (data not shown).

Wound Healing

Analysis of the healing response to cutaneous wounds post-MCAO revealed no main effects of surgery on wound healing among the three experimental groups ($F(2, 40) = 1.678, p > 0.05$; Figure 5.4). Mice experiencing MCAO surgery did not exhibit delays in wound healing compared to SHAM groups. Likewise, preventing MCAO-induced neuronal damage did not alter healing rate among the hypothermic MCAO mice.

Discussion

Th1 cytokines, which activate cellular immunity to provide defense against many kinds of infections and some kinds of neoplastic disease, are suppressed by chronic stress (Elenkov and Chrousos, 2002). This suppression has permissive effects on production of Th2 cytokines, which activate humoral immunity and exacerbate allergy and many kinds of autoimmune disease. A shift in Th1/Th2 balance can occur in response to stress. Glucocorticoid hormones differentially modulate the expression of cytokines, resulting in a polarized shift of T-cell responses to the Th2 subset (Elenkov et al., 1996; Glaser et al., 2001). Based on the present data and other results, it can be concluded that stroke up-regulates the Th2 response to a novel antigen. Traumatic brain injury increases Th2 derived factors, such as IL-10 and IL-6 (Amick et al., 2001). Increased Th2-derived cytokines increase NGF synthesis, which may contribute to increased neuronal support

and reduced neurotoxicity associated with Th2 responses in the central nervous system (Brodie, 1996). In the present study, cerebral ischemia, which results in neuronal damage, via excitotoxicity, nitric oxide production, free radical damage, and apoptosis (reviewed in Dirnagl et al., 1999; Liang et al., 2004; Mergenthaler et al., 2004) resulted in an increase in anti-KLH IgG antibody production (Figure 5.2). Antibody production, via the humoral arm (Th2 response) of the immune system can be neuroprotective following different experimental models of brain injury (Arumugam et al., 2005). Since cell death following stroke may take several days, the goal is to salvage the penumbral tissue from apoptotic death. Therefore, the increased antibody production observed in the normothermic MCAO mice depicts the up-regulation of T-cells probably via Th2-type regulatory cells providing anti-inflammatory responses to the damaged tissue. This increase in antibody production was absent from the hypothermic MCAO mice because hypothermia prevented neuronal death, thereby mitigating the effect of MCAO on antibody production (Figure 5.2).

Cerebral ischemia, via damage to the nervous system, influences the immune system via sympathetic nerve traffic, hormones, neuropeptides, and cytokines (Mergenthaler et al., 2004). Major strokes up regulate systemic T-cell responses (Tarkowski et al., 1995a; Tarkowski et al., 1991), while minor strokes down regulate systemic T-cell responses to antigenic challenge (Tarkowski et al., 1996). While the present data failed to demonstrate a significant increase in the DTH response following stroke, normothermic MCAO increased the DTH response compared to SHAM MCAO mice ($p=0.1$; Figure 5.3). More so, preventing MCAO-induced neuronal damage, via hypothermia, failed to increase the DTH response compared to normothermic MCAO

mice. Cardiac arrest/cardiopulmonary resuscitation (CA/CPR), which targets hippocampal damage, increases the DTH response, while a hypothermic head prevents CA/CPR induced neuronal damage and enhances the DTH response (Neigh et al., 2005b).

Various models of neuronal damage have demonstrated the neuroprotective effects of hypothermia (Neigh et al., 2004; Polderman et al., 2005; Wright, 2005), with mechanistic studies reporting reductions in metabolic and enzymatic activity, glutamate release and re-uptake, inflammation, reactive oxidant production, and the expression of a host of other genes (reviewed in Erecinska et al., 2003). Recently, we have demonstrated that global ischemia, a rodent model of CA/CPR, increases the DTH response to challenge; however, preventing global ischemia-induced neuronal damage, via hypothermia, reduces the DTH response following CA/CPR (Neigh et al., 2005b). The present study demonstrates reduced neuronal damage via hypothermia; specifically, mice undergoing hypothermic MCAO demonstrate smaller infarct sizes compared to normothermic MCAO (Figure 5.1). More so, the data presented in this chapter demonstrate that hypothermia mitigates alterations in peripheral immune function following cerebral ischemia (Figures 5.2 and 5.3).

Stroke is a potent activator of the hypothalamic-pituitary-adrenal axis (HPA) and chronic stress elicits both simultaneous enhancement and suppression of the immune response by altering patterns of cytokine secretion (Marshall et al., 1998). Activation of the HPA axis is one of the first measurable physiological responses to cerebral ischemia, which leads to sustained increases in glucocorticoid concentrations (Fassbender et al., 1994; Olsson, 1990; Olsson et al., 1992). Although glucocorticoids have been negatively

associated with wound healing (Detillion et al., 2004; Gordon et al., 1994; Horan et al., 2005; Hubner et al., 1996; Padgett et al., 1998a), no differences in rate of healing was observed among the three treatment groups (Figure 5.4). These data suggest that central nervous system damage does not alter cutaneous wound healing following MCAO.

In conclusion, this study demonstrates an augmentation in the peripheral immune response to cerebral ischemia. The augmentation in peripheral immune function was mitigated when neuronal damage was prevented via hypothermia. These data further highlight the changes in immune function following neuronal damage and the therapeutic potential of hypothermia during an ischemic event.

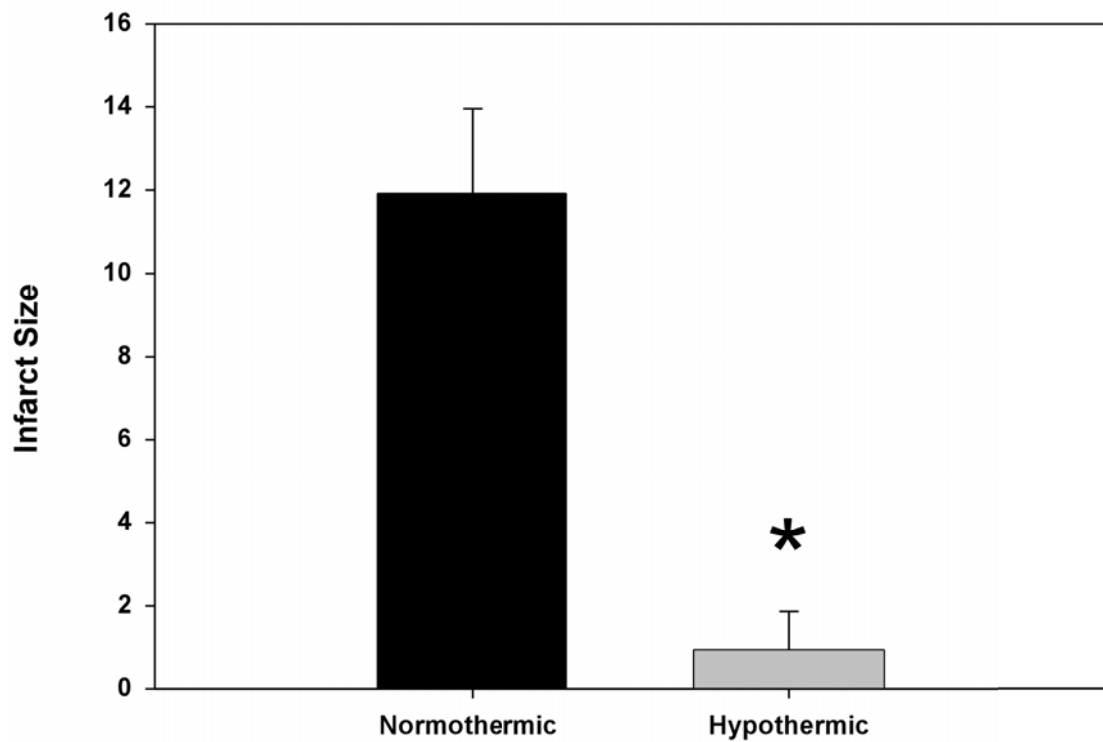


Figure 5.1: Hypothermia reduces neuronal damage following MCAO.

Mice underwent MCAO under normothermic (37° head temperature) or hypothermic (27° head temperature) conditions and survived for three days. Infarct size was significantly smaller among the hypothermic mice compared to the normothermic mice. An asterisk (*) indicates statistical significance ($p < 0.05$).

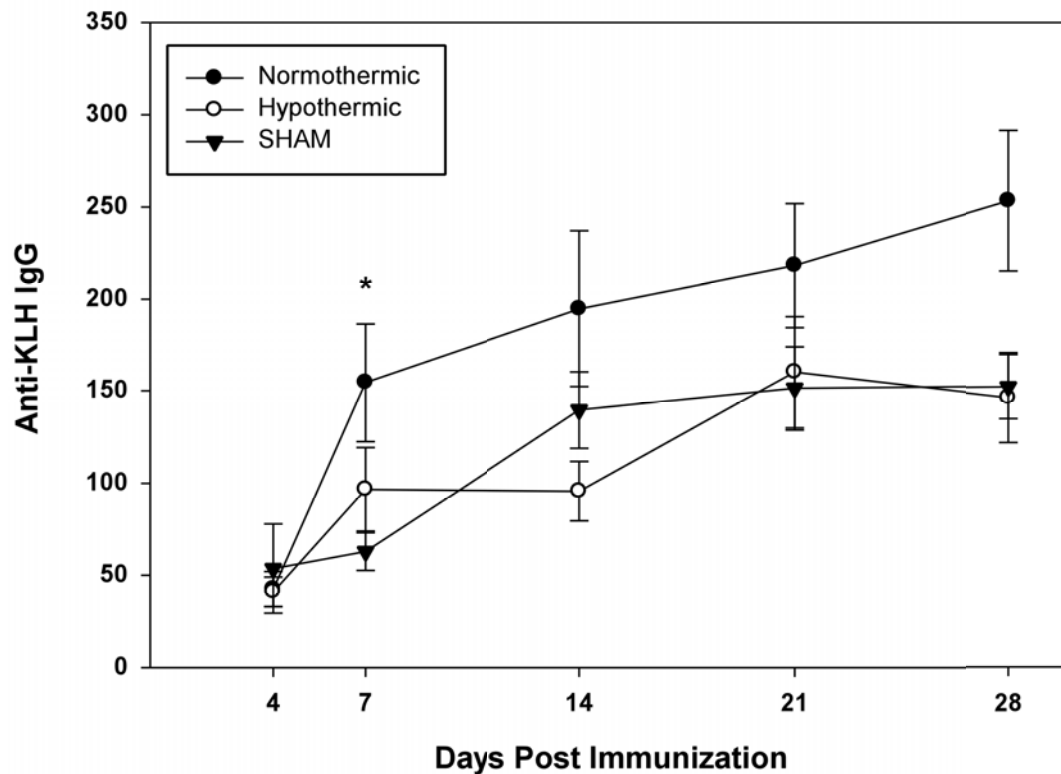


Figure 5.2: Anti-KLH IgG antibody response among normothermic, hypothermic, and SHAM MCAO mice.

Normothermic MCAO mice displayed significantly increased anti-KLH IgG antibody response compared to SHAM MCAO mice; however, preventing MCAO-induced neuronal damage, via hypothermia, resulted in blunted anti-KLH antibody production in hypothermic MCAO mice compared to normothermic MCAO mice. No difference in antibody production was observed between hypothermic MCAO mice and SHAM MCAO mice. An asterisk (*) indicates significant differences ($p < 0.05$) between normothermic MCAO and SHAM surgery.

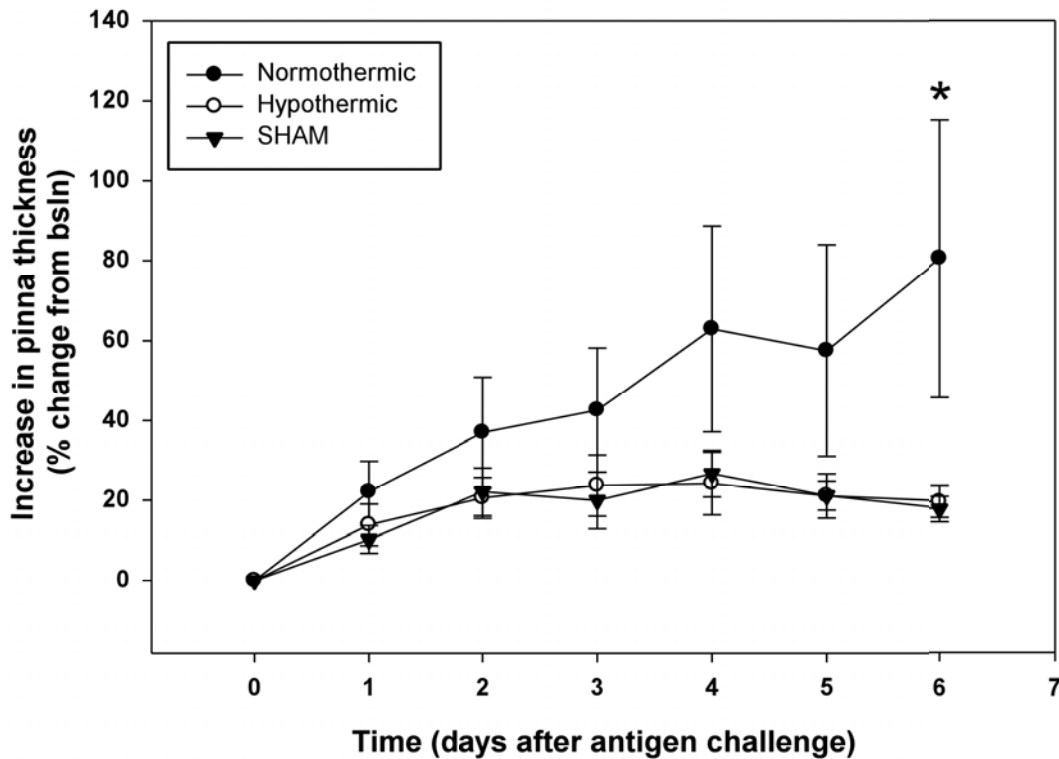


Figure 5.3: Delayed-type hypersensitivity response among normothermic, hypothermic, and SHAM MCAO mice.

Preventing MCAO-induced neuronal damage, via hypothermia, reduced the increase in edema in the hypothermic MCAO mice, compared to the normothermic MCAO mice. No difference in edema across days was observed between hypothermic MCAO and SHAM surgery mice. An asterisk (*) indicates significant differences ($p < 0.05$) between normothermic MCAO and SHAM surgery.

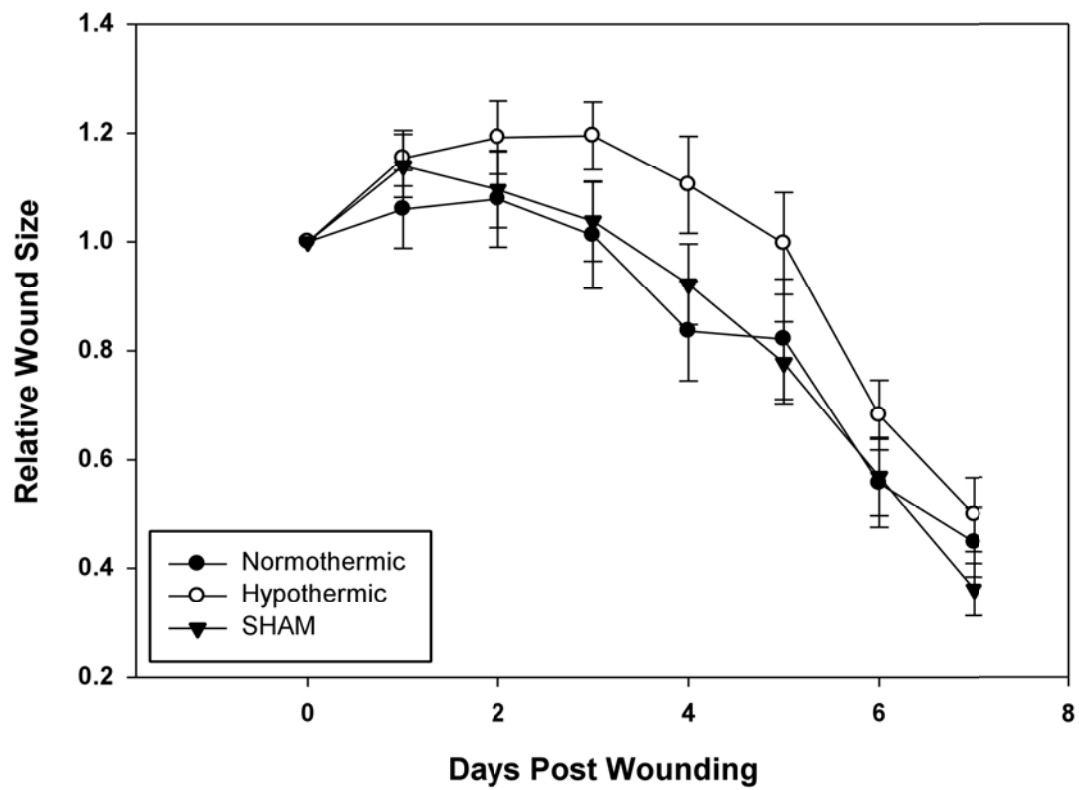


Figure 5.4: Wound size across days among normothermic, hypothermic, and SHAM MCAO mice.

Mice were given cutaneous wounds 7 days post MCAO or SHAM surgery. Wound size across days did not differ between MCAO or SHAM surgeries.

CHAPTER 6

POSITIVE SOCIAL INTERACTIONS MITIGATE THE EFFECTS OF FOCAL ISCHEMIA ON PERIPHERAL IMMUNE FUNCTION

Social support in humans and affiliative behaviors in other animals has positive effects on health outcome (House et al., 1990; Uchino et al., 1996). Isolation from positive social interactions predicts morbidity and mortality from numerous health conditions, including stroke and cerebrovascular diseases (Eng et al., 2002; Hawkey et al., 2003; Hawkey and Cacioppo, 2003; Kawachi et al., 1996). High levels of social support are also predictive of functional recovery following stroke (Colantonio et al., 1993; Glass et al., 1993; Koukouli et al., 2002).

Social influences on stroke have been thoroughly studied, most often under the context of functional outcome and cerebral damage following environmental enrichment. Environmental enrichment consists of several components, including increased social interaction, complex housing, and physical activity (i.e., running). These factors improve functional outcome following stroke (Grabowski et al., 1995; Held et al., 1985; Johansson, 1996; Risedal et al., 2002). Enriched environments following stroke improve functional recovery without decreasing infarct size (Grabowski et al., 1995; Johansson, 1996; Ohlsson and Johansson, 1995), through a mechanism that most likely involves

structural changes in the contralateral cortex (Johansson and Belichenko, 2002), altered gene expression (Dahlqvist et al., 2003; Dahlqvist et al., 1999), or neurogenesis (Komitova et al., 2005a; Komitova et al., 2005b; Matsumori et al., 2006).

Recently, we were able to demonstrate decreased cerebral damage and increased functional outcome in mice experiencing social facilitation prior to and following experimental stroke, compared to socially isolated counterparts (Craft et al., 2005). The literature concerning stroke and its effects on the organism are numerous; however, what has failed to be examined is how environmental interventions might reduce the secondary effects of stroke on health. Given the functional improvements following stroke in animals experiencing social facilitation, and the numerous alterations in immune function following stroke (see Chapter 5), the present study examined the effects of social isolation versus pair housing on stroke-induced alterations in immune function.

Materials and Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the local institutional animal care and use committee. Adult male C57BL/6 mice (Charles River, Wilmington, MA, USA) were housed individually in polycarbonate cages (28 x 17 x 12 cm) from the onset of the study in rooms maintained on a 14:10-h light:dark cycle at $20^{\circ} \pm 4^{\circ}$ C and relative humidity of $50 \pm 5\%$. Tap water and food (TekLab 8620) were available *ad libitum*. Mice were randomly assigned to one of eight experimental groups: (1) single housed normothermic MCAO (n=32; head maintained at 37°C), (2) pair housed

normothermic MCAO (n=16; head maintained at 37°C), (3) single housed hypothermic MCAO (n=37; head lowered to 27°C), (4) pair housed hypothermic MCAO (n=12; head lowered to 27°C), (5) single housed normothermic SHAM (n=33; head maintained at 37°C), pair housed normothermic SHAM (n=11; head maintained at 37°C), single housed hypothermic SHAM (n=20; head lowered to 27°C), or pair housed hypothermic SHAM (n=12; head lowered to 27°C). Pair housed mice were housed with ovariectomized females for two weeks prior to the commencement of the studies.

Laser Doppler Flowmetry

Femoral arterial blood pressure and cortical laser-Doppler (DRT4, Moor Instruments, LTD, Devon, England) were determined during occlusion and the first 30 min of reperfusion. A shallow indentation was made in the parietal skull (2 mm posterior, 3 mm lateral to Bregma) for the placement of the LDF probe. A thin oil interface and the probe were applied with a hood to block ambient light. The LDF signal was recorded continuously and averaged over 15 min intervals for comparison among groups.

Experimental Stroke

Transient focal cerebral ischemia was induced in adult male mice (23-25 g) by middle cerebral artery occlusion (MCAO), as described (Hattori et al., 2000). Briefly, the mice were anesthetized with 1-1.5% halothane in O₂ enriched air delivered through a facemask. Unilateral MCAO was achieved by using the intraluminal filament insertion technique, which involved inserting a 6-0 nylon monofilament into the right internal carotid artery to a point 6 mm distal to the internal carotid artery-pterygopalatine artery bifurcation. Once the filament was secured, the wound was sutured and the mouse was

allowed to emerge from anesthesia in its home cage. After 60 min of ischemia, the mice were re-anesthetized, and reperfusion was initiated through withdrawal of the filament. Mice were given a 0.5 ml s.c. injection of lactated Ringer's solution at the conclusion of the surgical procedure. A subset of MCAO mice were maintained at a body temperature of 27°C during surgery (prevents neuronal death, as described in Chapter 5, pg 111) to serve as controls for other aspects of the MCAO surgery, including any immunological consequences of introduction of the filament.

Assessment of Peripheral Immune Function

DTH. Delayed-type-hypersensitivity (DTH) was assessed in a separate cohort of mice following MCAO one week post recovery to test cell mediated immune function. To induce sensitization, mice were exposed to 2-4-dinitro-1-flourobenzene (DNFB, Sigma), on two consecutive days beginning two weeks prior to MCAO. Mice were anesthetized with isoflurane vapor, and an area of approximately 2 x 3 cm was shaved on the dorsum. Twenty-five µl of DNFB [0.5% (wt/vol) in 4:1, acetone:olive oil vehicle] was applied to the shaved skin. The thickness of both pinnae were measured before sensitization using a constant-loading dial micrometer (Mitutoyo, Japan) for later comparison during DTH induction. On reperfusion day 7, 1 week after MCAO, and 3 weeks after sensitization, pinnae thickness was again measured and mice were challenged on the skin of the dorsal surface of the right pinna with 20 µl of DNFB [0.2% (wt/vol) in 4:1, acetone:olive oil vehicle]. The left pinna was treated with the vehicle. Following challenge, mice were returned to their cages and pinnae thickness was measured every 24 h for the next 8 days. DTH responses were determined as percent increases of pinnae thickness relative to baseline for each mouse.

Wounding. On the seventh day following MCAO, mice were anesthetized with isoflurane in O₂-enriched air. A 3 x 3 cm patch of fur was shaved on the dorsal surface and cleansed with ethanol. Dorsal, mid-scapular cutaneous wounds were produced using a 3.5 mm dermal punch biopsy tool (Miltex Instruments, New York, USA). Each day, beginning with the day of wounding, the mice were anesthetized with isoflurane in O₂-enriched air and the wound site was photographed using a digital camera. Each photograph included a standard-sized circle (3.5 mm ID) placed on the skin near the wound. The wound size for each mouse was determined using Canvas 8.0 (Deneba Systems, Florida, USA) and expressed as the ratio of wound area to the area of the standard circle in the photograph (in pixels).

Statistical Analysis

Pinnae thicknesses and wound sizes were compared using repeated measures ANOVAs. Post hoc analysis (Scheffe test) was used to further distinguish among groups, and all differences were considered statistically significant if $p < 0.05$. When statistically appropriate ($p > 0.05$; Keppel, 1991), normothermic SHAM and hypothermic SHAM groups were collapsed into one group for further analyses and this group was designated as 'SHAM.'

Results

Delayed-type hypersensitivity response

Analysis of the DTH response to challenge did not reveal a main effect of surgery ($F(2, 75) = 1.442, p > 0.05$) or a main effect of housing ($F(1, 75) = 3.133, p > 0.05$); however, a main effect of time ($F(6, 450) = 12.057, p < 0.05$) was observed. Independent

t-test analysis of pinna edema on day six revealed a significant increase in pinna measurements in normothermic MCAO mice compared to SHAM mice (Figure 6.1). Preventing neuronal damage, via hypothermia, reduced pinna edema in the hypothermic MCAO mice compared to normothermic MCAO mice. No difference in pinna edema across days was observed between hypothermic MCAO and SHAM surgery mice. Sensitization and challenge with DNFB did not alter survival rate (data not shown). Pair housing did not significantly reduce pinna thickness across days ($p=0.08$); however, independent t-test of pinna edema on day two revealed significantly smaller pinna thickness among pair housed mice compared to single housed mice. No difference between hypothermic single housed MCAO and normothermic pair housed MCAO mice was observed ($p>0.05$). Likewise, no difference was observed between hypothermic pair housed MCAO and normothermic pair housed MCAO mice ($p>0.05$).

Wound Healing. Analysis of the healing response to cutaneous wounds post-MCAO revealed no main effect of surgery on wound healing among the experimental groups ($F(2, 66) = 0.230$, $p>0.05$; Figure 6.2). However, a significant main effect of housing ($F(1, 66) = 48.240$, $p<0.05$), time ($F(7, 462) = 106.026$, $p<0.05$), and an interaction between time and housing ($F(7, 462) = 8.357$, $p<0.05$) were observed. Mice experiencing MCAO surgery did not exhibit delays in wound healing compared to SHAM groups. Likewise, preventing MCAO-induced neuronal damage did not alter healing rate among the hypothermic MCAO mice. However, pair housing, under both normothermic and hypothermic conditions reduced wound size across days. Pair housing, however, did not further reduce wound size among hypothermic MCAO mice compared to pair housed normothermic mice ($p>0.05$).

Discussion

Enriched environments increase functional outcome following stroke (Grabowski et al., 1995; Held et al., 1985; Johansson, 1996; Risedal et al., 2002) and social facilitation prior to and after stroke decreases infarct size and exacerbates functional deficits (Craft et al., 2005). Decreased neuronal death leads to alterations in immune function following stroke (see Chapter 5), thereby decreasing neuronal death, via social facilitation, should improve immune function compared to socially isolated counterparts. Pair housing decreased wound size across days in male mice that were housed with a female prior to and following MCAO and reduced stroke-induced increases in pinna swelling (edema; Figures 6.1 and 6.2).

Social facilitation, via pair housing, decreased the DTH response in pair housed normothermic mice compared to socially isolated normothermic mice (Figure 6.1). Pair housing decreases edema associated with the DTH response (Pyter et al., 2005) regardless of the sex of the stimulus mouse. *In vivo* cell mediated immune responses [e.g., DTH, contact sensitivity (CS)] are mediated by sensitized T-cells recruited into tissues (e.g., skin) to bind antigen peptides to molecules on the surface of antigen-presenting cells (Askenase, 2001). This recognition causes T-cell activation, which produces cytokines that help orchestrate local inflammation (as demonstrated in normothermic socially isolated mice, see Chapter 5). Alterations in immune function following stroke are related to changes in T-cell responses. Major strokes up regulate systemic T-cell responses (Tarkowski et al., 1995a; Tarkowski et al., 1991), while minor strokes down regulate systemic T cell responses to antigenic challenge (Tarkowski et al., 1996). Although pair housing did not significantly ($p=0.08$) reduce edema associated

with antigenic challenge following stroke, there were no differences in DTH response between hypothermic single housed mice and pair housed normothermic mice ($p>0.05$; Figure 6.1). As mentioned in Chapter 5, hypothermia prevents stroke-induced neuronal damage (i.e., reduces infarct size). Likewise, individually housed mice undergoing hypothermic MCAO displayed reduced DTH responses following stroke relative to individually housed mice undergoing normothermic MCAO. Pair housing prior to and following MCAO reduces neuronal death and improves functional outcome (Craft et al., 2005), and presently, this study demonstrates decreases in DTH responses following MCAO, that likely results from reduced neuronal death following stroke in mice undergoing social facilitation, via pair housing.

Pair housing also decreased wound size following MCAO, regardless of surgical treatment (Figure 6.2). Social facilitation alters wound size and rate of healing following cutaneous wounding. Female Siberian hamsters (*Phodopus sungorus*) housed with female siblings demonstrate increases in healing rate compared to socially isolated females (Detillion et al., 2004). Social facilitation of wound healing, via physical contact, is also observed in two monogamous species of *Peromyscus* (Glasper and DeVries, 2005). Monogamous *Peromyscus* that are separated from their social partners demonstrate wound healing rates similar to socially isolated counterparts (Glasper and DeVries, 2005; Martin et al., 2006). Perceived social isolation is also highly correlated with wound-healing time in young adults (Cacioppo and Hawkley, 2003). As demonstrated in single housed mice, MCAO did not alter wound size among pair housed mice, suggesting that central nervous system damage does not alter cutaneous wound healing following MCAO.

Pair housing decreased wound size, in the absence of neuronal damage, as evidenced in the SHAM mice (Figure 6.2). Social facilitation increases immune function via reductions in circulating corticosteroid concentrations and increases in oxytocin (OT). Pair housed female Siberian hamsters exhibited reductions in corticosteroid concentrations compared to socially isolated counterparts (Detillion et al., 2004). In contrast, treating socially housed hamsters with an OT antagonist delayed wound healing compared to non-treated pair housed hamsters (Detillion et al., 2004). Likewise, pair housed *P. californicus* reduced corticosteroid concentrations, compared to socially isolated mice, after two weeks of physical contact (Glasper and DeVries, 2005). These data suggest that reductions in HPA axis activity mitigate the effects of social isolation on immune function. Recent data from our lab indicate that the effects of pair housing on infarct size is unlikely mediated by reductions in HPA axis activity; specifically, pair housing male mice for two weeks did not significantly reduce circulating corticosteroid concentrations following stroke (Craft et al., 2005). In contrast, social housing did significantly decrease c-reactive protein (CRP) concentrations compared to socially isolated mice. Lower levels of social support are associated with increased CRP concentrations in healthy humans (Schnorpfeil et al., 2003). Conversely, after an inflammatory stimulus, CRP concentrations are dramatically increased (Lindsberg and Grau, 2003).

In conclusion, this study further demonstrates an augmentation in the peripheral immune response to cerebral ischemia. The augmentation in peripheral immune function was mitigated when neuronal damage was prevented via hypothermia and pair housing; however, the additive effect of hypothermia and pair housing did not further mitigate the

alteration in post-stroke immune function. These data further highlight the changes in immune function following neuronal damage and the therapeutic potential of increased social facilitation prior to and following an ischemic event.

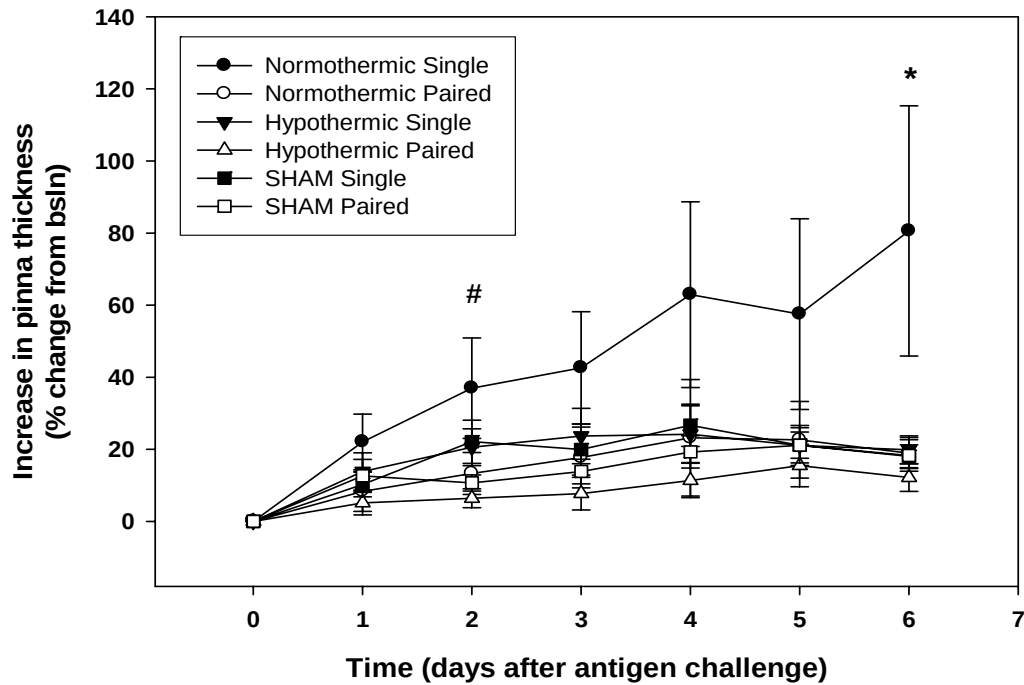


Figure 6.1: Delayed-type hypersensitivity response among normothermic, hypothermic, and SHAM MCAO mice under both single and pair housed conditions.

Preventing MCAO-induced neuronal damage, via hypothermia, reduced the increase in edema in the hypothermic MCAO mice, compared to the normothermic MCAO mice. No difference in housing condition was observed across days. No difference in edema across days was observed between hypothermic MCAO and SHAM surgery mice. A number sign (#) indicates significant differences ($p < 0.05$) between normothermic MCAO and SHAM surgery. An asterisk (*) indicates significant differences between single housed and pair housed mice.

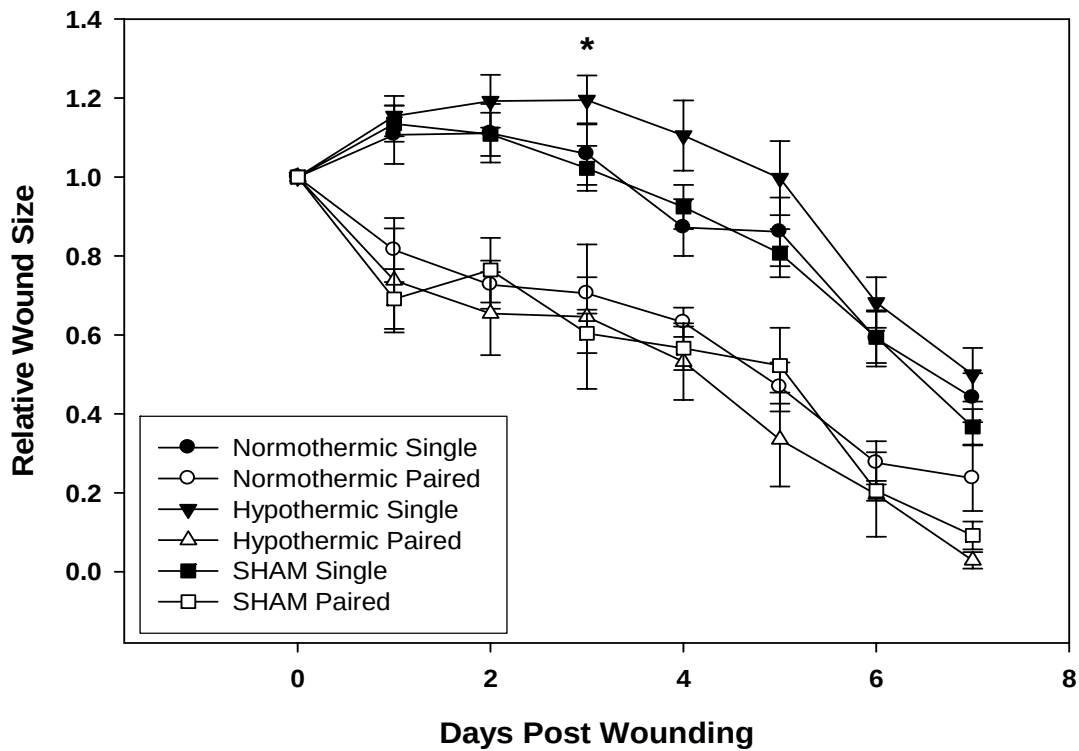


Figure 6.2: Wound size across days among normothermic, hypothermic, and SHAM MCAO mice under both single and pair housed conditions.

Mice were either single housed or pair housed prior to the administration of subcutaneous wounds 7 days post MCAO or SHAM surgery. Wound size across days did not differ between MCAO or SHAM surgeries; however, pair housed mice expressed smaller wound sizes across days compared to single housed mice. An asterisk (*) indicates a significant difference between single housed hypothermic mice and single housed SHAM mice.

CHAPTER 7

GLUCOCORTICOIDS ALTER THE EFFECTS OF FOCAL ISCHEMIA ON PERIPHERAL IMMUNE FUNCTION

Stress suppresses immunity and increases susceptibility to infections and cancer (Cohen and Herbert, 1996; Cohen et al., 1997; Cohen et al., 1991). Stress also exacerbates inflammatory disease (Chida et al., 2005; Sieve et al., 2004); however, glucocorticoids, the stress hormones, are used clinically to treat these diseases. Given this paradox, studies have investigated the effects of both chronic and acute stress on immune function; however, this chapter will address chronic stress effects on health, as stroke results in a chronic elevation of corticosteroids, observed within hours of occlusion (Slowik et al., 2002).

Many studies have examined the immunosuppressive effects of stress on health. Stress of exams weakened students' immune systems and lead to more infections and illness (Kiecolt-Glaser et al., 1984) compared to times of vacation. The chronic stress of caring for a loved one with dementia resulted in immunosuppression; immunoglobulin G (IgG) antibody titers to the pneumococcal vaccine fell over a 6 month period in the caregivers compared to no change in IgG antibody titer among control and former caregiver subjects (Glaser et al., 2000). Among parents of cancer patients,

dexamethasone's (a synthetic glucocorticoid antagonist) capacity to suppress interleukin (IL) -6 production was significantly reduced compared with parents of healthy children (Miller et al., 2002). This study, along with others, suggests a novel mechanism though with chronic psychological stress could influence the onset and/or progression of conditions that involve excessive inflammation.

In contrast to acute stress enhancing the skin DTH response to antigen challenge, chronic stress decreases the skin DTH response to antigen challenge in mice (Dhabhar and McEwen, 1997). Chronic immobilization stress has a major impact on immune function, causing changes in the normal distribution of immune cells (Dhabhar and McEwen, 1997; Dhabhar et al., 1995; Dominguez-Gerpe and Rey-Mendez, 2001) and also decreasing proliferation of immune cells (Wang et al., 2002). Chronic social stress also has been linked to increased susceptibility to infection in non-human primates; specifically, unstable social environments influence the probability of developing infection after viral exposure (Cohen et al., 1997). The chronic stress of an illness, such as Theiler's virus, an animal model of multiple sclerosis, has revealed more compelling evidence that chronic stressors suppress the immune system. Four weeks of restraint stress prior to the onset of the demyelinating condition developed a worse course of the disease compared to those mice not experiencing restraint (Sieve et al., 2004). Noise exposure, a highly relevant environmental and clinical stressor, has also been linked to immunosuppression. Three weeks of chronic intermittent unpredictable noise resulted in suppressed splenic NK cells compared to control rats (Van Raaij et al., 1996).

In the present study, the effects of restraint stress on peripheral immune function were assessed in both MCAO and SHAM surgery mice. Restraint stress experienced immediately prior to MCAO should decrease the stroke-induced DTH response and stroke-induced anti-KLH IgG antibody production.

Materials and Methods

Animals

This study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the local institutional animal care and use committee. Adult male C57BL/6 mice (Charles River, Wilmington, MA, USA) were housed individually in polycarbonate cages (28 x 17 x 12 cm) from the onset of the study in rooms maintained on a 14:10-h light:dark cycle at $20^{\circ} \pm 4^{\circ}$ C and relative humidity of $50 \pm 5\%$. Tap water and food (TekLab 8620) were available *ad libitum*. Mice were randomly assigned to one of four experimental groups: (1) No Stress-SHAM (n=10), (2) Stress-SHAM (n=11), (3) No Stress-MCAO (n=12), and (4) Stress-MCAO (n=18).

Restraint

Mice were individually placed in well ventilated, polystyrene tubes (50 ml) for 1 h prior to MCAO or SHAM surgery. The tubes allowed for confined movements and postural adjustments. The “no stressor” mice remained in their home cages until the stroke procedure commenced.

Laser Doppler Flowmetry

Femoral arterial blood pressure and cortical laser-Doppler (DRT4, Moor Instruments, LTD, Devon, England) were determined during occlusion and the first 30 min of reperfusion. A shallow indentation was made in the parietal skull (2 mm posterior, 3 mm lateral to Bregma) for the placement of the LDF probe. A thin oil interface and the probe were applied with a hood to block ambient light. The LDF signal was recorded continuously and averaged over 15 min intervals for comparison among groups.

Experimental Stroke

Transient focal cerebral ischemia was induced in adult male mice by right middle cerebral artery occlusion (MCAO), as described (Hattori et al., 2000). Briefly, the mice were anesthetized with 1-1.5% halothane in O₂ enriched air delivered through a facemask. Unilateral MCAO was achieved by using the intraluminal filament insertion technique. MCAO was achieved via inserting a 6-0 nylon monofilament into the right internal carotid artery to a point 6 mm distal to the internal carotid artery-pterygopalatine artery bifurcation. Once the filament was secured, the wound was sutured and the mouse was allowed to emerge from anesthesia in its home cage. After 60 min of ischemia, the mice were re-anesthetized, and reperfusion was initiated through withdrawal of the filament. Mice were given a 0.5 ml s.c. injection of lactated Ringer's solution at the conclusion of the surgical procedure.

Determination of Stroke Volume

Brains were removed after three days of reperfusion, in a separate cohort of mice (No Stress, n=5; Stress, n=6), and sectioned into five 2-mm thick coronal sections. Sections were incubated for 15 min in 2, 3, 5-triphenyltetrazolium maintained at 37°C. Following staining, sections were fixed in 10% formalin and photographed. Images were analyzed and infarct size was expressed as percent of contralateral hemisphere after correcting for edema (Inquiry; Loats Inc, Rockville, MD, USA).

Assessment of Peripheral Immune Function

KLH. To assess primary humoral immunity, mice was injected subcutaneously, on post-MCAO day 7 with keyhole limpet hemocyanin (KLH), to which they were previously naïve (150 µg suspended in 0.1 ml sterile 0.9% saline). KLH evokes an acute immune response, but does not replicate or cause long-term fever or inflammation. IgG specific for KLH was measured on Day 4, Day 7, Day 14, Day 21, and Day 28 post-immunization for primary humoral immunity using a specific enzyme-linked immunosorbent assay (ELISA). Diluted serum samples (1:100) were added to antigen-coated plates that were pre-coated with 0.5% milk in PBS to reduce nonspecific binding. After incubation, secondary antibody (AP-conjugated anti-mouse IgG, 1:1000) was added, and after further incubation, the enzyme substrate p-nitrophenyl phosphate was added. The optical density (OD) of each well was determined using a BioRad plate reader (405 nm). Mean OD for each sample was expressed as a percent plate positive control for statistical analyses.

DTH.

Delayed-type-hypersensitivity (DTH), used to test cell mediated immune function, was assessed in a separate cohort of mice following MCAO one week post recovery. Mice were sensitized to the antigen, 2-4-dinitro-1-flourobenzene (DNFB, Sigma), placed on their dorsum for the assessment of an antigen-specific, cell-mediated, DTH reaction in the skin on two consecutive days two weeks prior to MCAO. To do so, mice were anesthetized with isoflurane vapor, and an area of approximately 2 x 3 cm was shaved on the dorsum. Twenty-five μ l of DNFB [0.5% (wt/vol) in 4:1, acetone:olive oil vehicle] was applied to the shaved skin. The thickness of both pinnae were measured before sensitization using a constant-loading dial micrometer (Mitutoyo, Japan) for later comparison during DTH induction. On day 7, or 1 week after MCAO and 3 weeks after sensitization, pinnae thickness was again measured and mice received a challenge of 20 μ l of DNFB [0.2% (wt/vol) in 4:1, acetone:olive oil vehicle] to the skin of the dorsal surface of the right pinna, and the left pinna was treated with the vehicle. Mice were again anesthetized with isoflurane for the procedure. Following challenge, mice were returned to their cages and pinnae thickness was measured every 24 h for the next 8 days. All measurements were made on the same relative region of the pinnae. DTH responses were determined as percent increases of pinnae thickness over baseline for each mouse and compared among groups using repeated measures.

Statistical Analysis

Infarct size was compared using one way ANOVA. Pinnae thicknesses were compared using repeated measures ANOVAs. Post hoc analysis (Scheffe test) was used to further distinguish among groups, and all differences were considered statistically significant if $p < 0.05$.

Results

Infarct Size

A non-significant effect of stress on infarct size was observed among mice receiving 60 min of MCAO ($F(1, 9) = 3.668$, $p = 0.08$; Figure 7.1). However non-significant, infarct size was larger among mice experiencing 1 h of restraint stress immediately prior to MCAO compared to non-stressed counterparts.

KLH

Analysis of the anti-KLH IgG antibody response did not reveal a main effect of surgery ($F(1, 20) = 0.503$, $p > 0.05$) or stress ($F(1, 20) = 0.802$, $p > 0.05$); however, a main effect of day of sampling was observed ($F(4, 80) = 111.572$, $p < 0.05$). An interaction between surgery and stress ($F(1, 20) = 7.153$, $p < 0.05$) and an interaction between day of sampling, surgery, and stress was observed ($F(4, 80) = 2.930$, $p < 0.05$). Analysis of mice receiving SHAM surgery revealed no overall significant difference ($p = 0.09$) between No Stress and Stress mice; however, independent t-tests revealed significantly higher anti-KLH IgG antibody production among Stress mice on days 4 ($t(8) = -3.347$, $p = 0.01$) and 21 ($t(8) = -2.487$, $p = 0.04$) post-immunization (Figure 7.2). As observed in SHAM mice, mice receiving MCAO revealed no overall significant differences between No Stress and

Stress mice; however, independent t-tests revealed significantly higher anti-KLH IgG antibody concentrations among No Stress mice on day 14 ($t(12) = 2.24$, $p=0.05$; Figure 7.3).

DTH

Analysis of the DTH response to challenge did not reveal a main effect of surgery ($F(1, 23) = 0.474$, $p>0.05$) or a main effect of stress ($F(1, 23) = 2.014E-4$, $p>0.05$; Figure 7.4); however a main effect of day of ear measurement was observed ($F(7, 161) = 12.14$, $p<0.05$). No difference in pinna thickness was observed between MCAO or SHAM treated mice. Pinna thickness was also similar between No Stress and Stress mice, irrespective of their surgery treatment.

Survival

A main effect of surgery was observed in survival ($F(1, 64) = 26.398$, $p<0.05$; Figure 7.5) of mice sensitized with DNFB. Nine of 26 No Stress-MCAO mice survived after the MCAO procedure (35%), while only seven of 31 Stress-MCAO mice survived after the MCAO procedure (23%). No significant difference in survival was observed between No-Stress and Stress mice ($p>0.05$).

Discussion

Rodent studies have demonstrated increased neuronal death following stress and exogenous administration of corticosterone (DeVries et al., 2001a; Madrigal et al., 2003; Sugo et al., 2002), while surgical or pharmacological suppression of corticosterone is neuroprotective (Antonawich et al., 1999; Karishma and Herbert, 2002; Sapolsky et al., 1986; Sapolsky and Pulsinelli, 1985). Additionally, a causal link between corticosteroids

and stroke outcome is suggested by rodent studies in which manipulating blood corticosteroid concentrations during or after an ischemic event alters infarct size (Koide et al., 1986; Morse and Davis, 1990; Sapolsky and Pulsinelli, 1985; Smith-Swintosky et al., 1996). Although the infarct data in this experiment did not reach statistical significance ($p=0.08$; Figure 7.1), I have previously shown that 1 h of restraint immediately prior to MCAO results in larger infarct sizes compared to mice not experiencing restraint (see Chapter 4).

Restraint stress produced increases in anti-KLH IgG antibody production among SHAM mice compared to non-stressed SHAM mice (Figure 7.2). Stress-induced enhancements of humoral immunity have been demonstrated previously in the absence of neuronal damage. Exposure to acute stressors enhances humoral immunity to KLH, specifically serum anti-KLH IgG levels are significantly higher compared to non-stressed controls (Persoons et al., 1995; Wood et al., 1993). Cold stress (Blecha and Kelley, 1981; Sabiston et al., 1978), restraint stress (Berkenbosch et al., 1991), foot shock (Persoons et al., 1995; Wood et al., 1993), weaning stress (Blecha and Kelley, 1981), and alarm stress (Cocke et al., 1993) enhance humoral immune responses.

While restraint stress increases humoral immune responses in the absence of neuronal cell death, under conditions of neuronal damage and stress, humoral immune response is decreased compared to non-stressed mice undergoing experimental stroke (Figure 7.3). One hour of restraint stress is typically referred to as an acute stressor (Dhabhar, 2003) and is known to enhance immune function, as previously mentioned (see Chapter 1, page 4). However, stroke is considered a physiological stressor, with increases in corticosteroids as one of the first measurable responses to stroke (Slowik et

al., 2002). Therefore, the chronic stress of a stroke may have led to the decrease in humoral immune response among mice experiencing stroke coupled with 1 h of restraint stress (Figure 7.3. Chronic stress has been shown to suppress various immune parameters, examples of which include antibody production (Bhatnagar et al., 1996; Houry-Haddad et al., 2003; Jasnow et al., 2001; Neigh et al., 2005c; Silberman et al., 2003; Zalcman and Anisman, 1993).

Contrary to the stroke-induced DTH enhancements demonstrated in Chapters 5 and 6, this experiment failed to produce increases in pinna edema following MCAO and antigenic challenge (Figure 7.4). Interestingly, a high mortality rate was observed among this subset of mice. A 75-80% survival rate is typically observed in our laboratory following MCAO surgery; however, only 35% of the No Stress MCAO mice and only 23% of the Stress mice survived to receive antigenic challenge (Figure 7.5). Those surviving to challenge failed to show the stroke-induced increase in edema following MCAO and antigenic challenge seen in Chapter 5 and Chapter 6. Therefore, it may be that the immune response observed in this subset of mice is atypical and not reflective of stroke-induced augmentation of the DTH response, as well as the chronic stress-induced suppression of the DTH response to antigenic challenge. Chronic stress has been shown to suppress the DTH response (Basso et al., 1993; Basso et al., 1999; Dhabhar and McEwen, 1997; 1999; El-Lethey et al., 2003; Puebla-Perez et al., 2003).

Taken together, these data suggest that 1 h of restraint stress increases neuronal damage, which can lead to a decrease in humoral immune function when coupled with stroke; however, this same stressor, in the absence of neuronal damage, increases humoral immune function. Given these findings and the present literature, stress can

alter an organism's immune function; however, the effects of stress on immune function are context dependent and may depend on whether the organism is experiencing alterations in homeostasis other than the stressor.

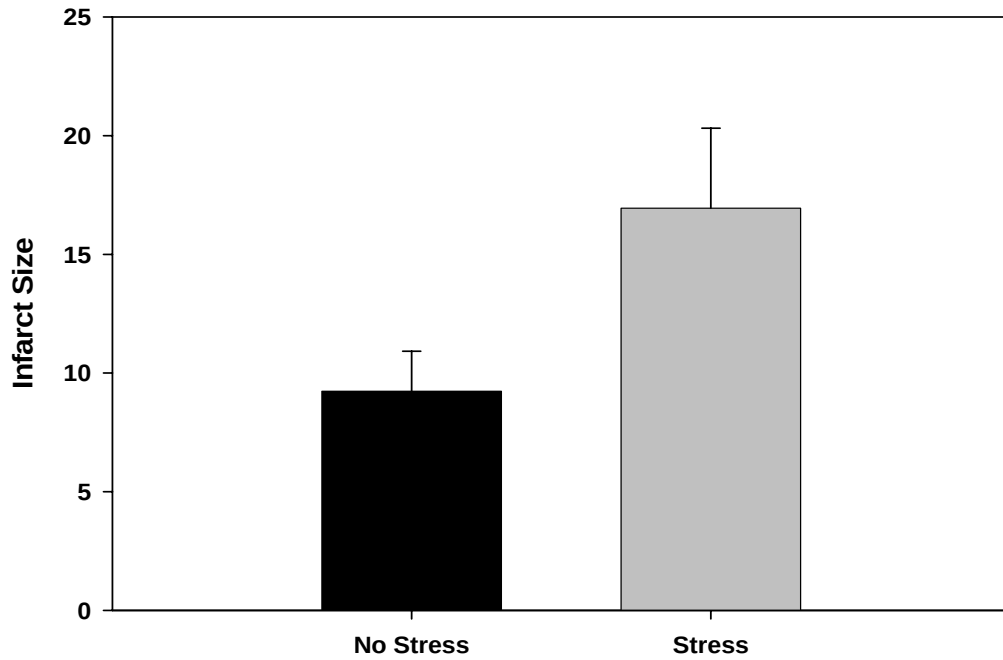


Figure 7.1: Effects of immediate stress on infarct size.

Infarct size was larger among mice experiencing 1 h of restraint stress immediately prior to MCAO compared to non-stressed counterparts ($p=0.08$). (Mean $\pm$ SEM)

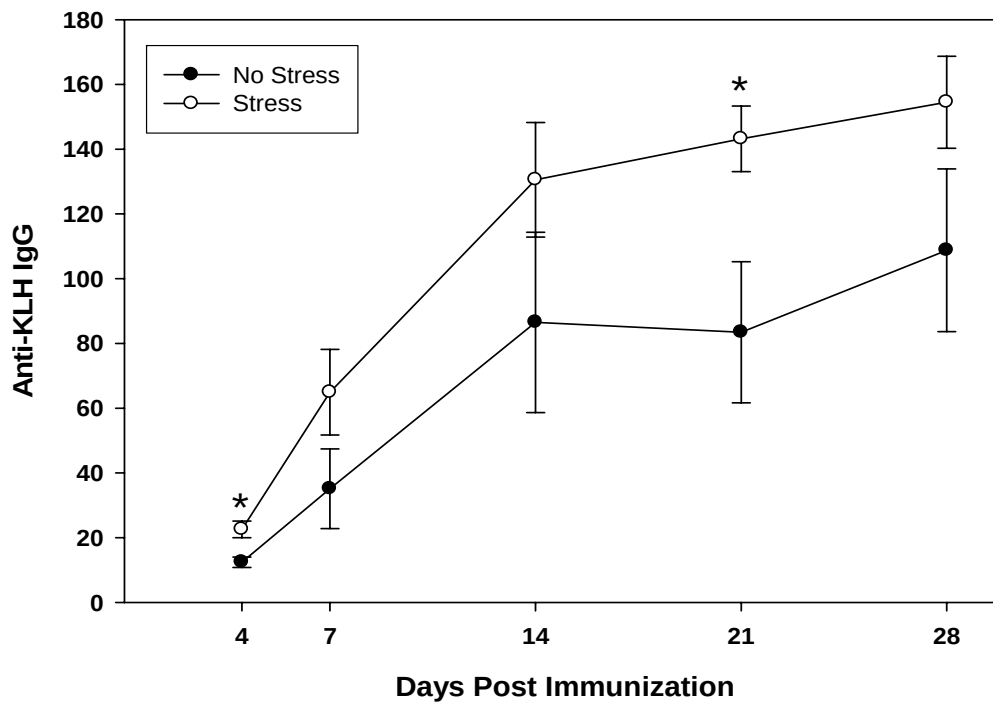


Figure 7.2: Anti-KLH IgG antibody response to SHAM surgery.

Anti-KLH IgG antibody production to SHAM surgery produced no significant differences across days; however, on days 4 and 21, antibody production was significantly higher in SHAM mice experiencing 1 h of restraint stress immediately prior to surgery compared to mice not experiencing stress. An asterisk (*) indicates statistical significance ($p < 0.05$). (Mean $\pm$ SEM)

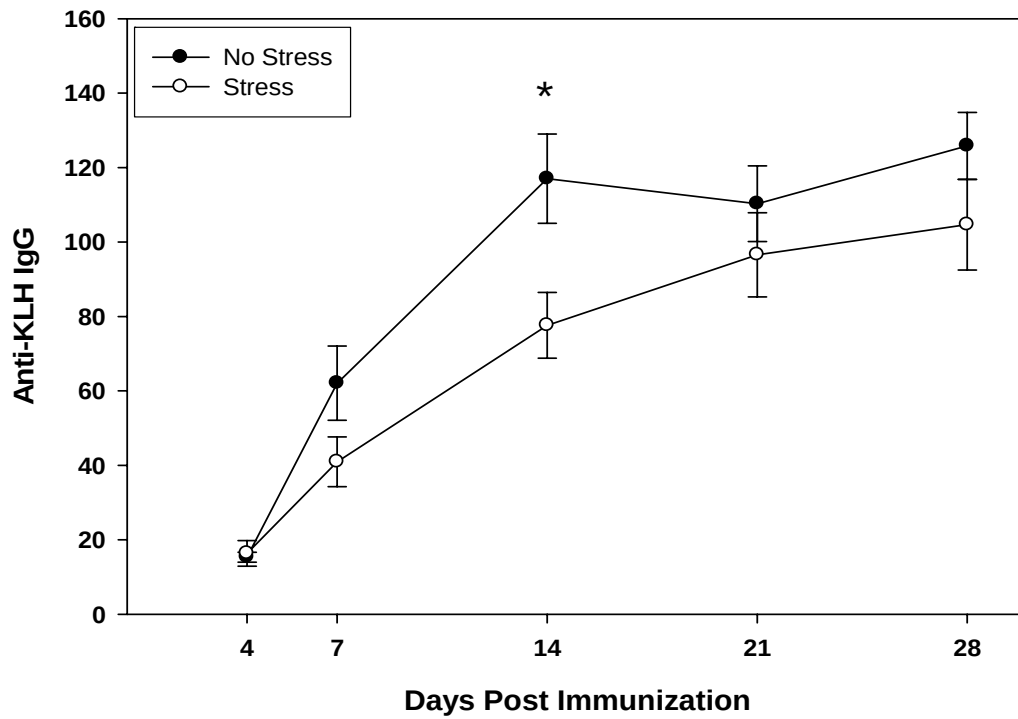


Figure 7.3: Anti-KLH IgG antibody response to MCAO surgery.

Anti-KLH IgG antibody production to MCAO surgery produced no significant differences across days; however, on day 14, antibody production was significantly higher in MCAO mice not experiencing 1 h of restraint stress immediately prior to surgery compared to mice that did experience stress. An asterisk (*) indicates statistical significance ($p < 0.05$). (Mean $\pm$ SEM)

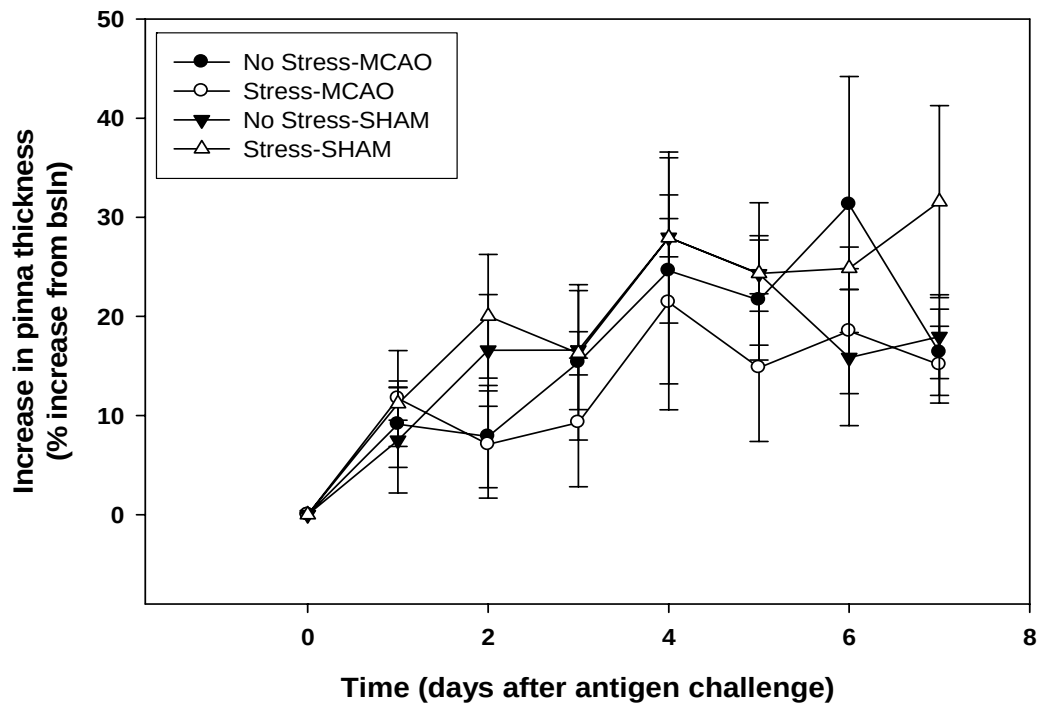


Figure 7.4: Delayed-type hypersensitivity response to MCAO and stress.

Stress did not alter the delayed-type hypersensitivity response to MCAO. No difference was observed between MCAO and SHAM surgery pinna thicknesses. (Mean $\pm$ SEM)

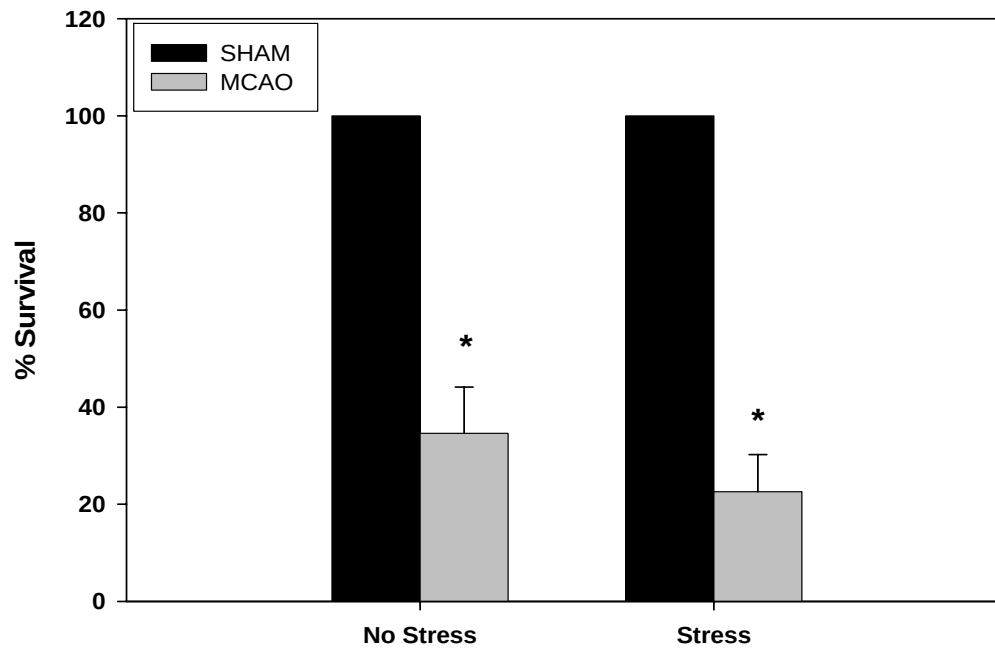


Figure 7.5: Percent survival of MCAO and SHAM mice sensitized with 2-4-dinitro-1-flourobenzene (DNFB).

One-hundred percent survival was observed among mice sensitized with DNB and recieving SHAM surgery; however, survival following sensitization and MCAO surgery was greatly decreased, irrespective of stress. An asterisk (*) indicates statistical significance ($p < 0.05$). (Mean $\pm$ SEM)

SUMMARY AND CONCLUSIONS

Social support in humans and affiliative behaviors in other animals can have positive effects on health (House et al., 1990; Uchino et al., 1996), whereas stressful experiences suppress the ability of the immune system to respond to challenges and increases susceptibility to infectious disease. The first part of the dissertation work examined the role of social facilitation on wound healing in *Peromyscus* species depicting varying social structures. The second section of this dissertation details the effects of stress on neuronal damage, corticosterone concentrations, and cerebral edema following middle cerebral artery occlusion (MCAO). The final studies described in this dissertation detailed the effects of stroke on peripheral immune function and how alterations in the environment (social facilitation and stress) alter post-stroke peripheral immune function.

I began by testing the hypothesis that social housing would mitigate the effects of wound healing and stress-induced alterations in HPA axis function in two mouse species that are characterized in nature as exhibiting behaviors consistent with a monogamous social system (*Peromyscus californicus* and *P. eremicus*) compared to one mouse species exhibiting behaviors consistent with a polygynous social system (*P. leucopus*). In order to test this hypothesis, I began by pair housing male mice of each species with ovariectomized females and compared their wound sizes across days to

socially isolated male mice. Pair housing decreased wound size and lowered circulating corticosteroid concentrations in the two monogamous species, however, not in the polygynous species (Figure 2.1, 2.2, and 2.6). Thus, it is likely that in this study, the pair-housed *P. californicus* and *P. eremicus* mice healed more quickly than the socially isolated cohorts *P. leucopus* because corticosteroid concentrations were lower in the pair-housed than socially isolated animals, possibly due to increases in oxytocin released during physical contact. I then tested the hypotheses that social contact was necessary for the immune enhancing effects of pair housing on wound healing. By examining the effect of pair dissolution and physically separating pairs, I was able to demonstrate that the beneficial effects of social interaction for wound healing and corticosterone concentrations do not persist beyond pair dissolution in either *P. californicus* or *P. eremicus* (Figures 2.10), and that physical contact is necessary to observe the effects of pair housing on wound healing and corticosterone concentrations (Figures 2.7 and 2.8). These observations suggest that the beneficial effects of social housing are not equivalent for all species, and may be limited to those that are monogamous or highly social.

The next chapter of my thesis focused on expanding the observations in Chapter 2. This chapter of my dissertation examined the effects of positive social interactions on wound healing in a monogamous and polygynous species. However, unlike Chapter 2, Chapter 3 investigated various housing dyads by changing the sex of the experimental and stimulus mice. This variation in the experimental design elucidated the interactions between same sex pairs and wound healing. Given the vast literature on intersex competition and its effects on various biological outcomes, as mentioned previously, this chapter adds to the understanding of both positive and negative social interactions and

wound healing. The data from Chapter 3 suggest that positive social interaction facilitates wound healing in a monogamous mouse species regardless of the sex of the experimental or stimulus mouse. Both male and female *P. californicus* exhibit reductions in wound size following pair housing (Figure 3.2 and 3.3). However, the effects on wound healing are limited; male and female polygynous mice benefit from pair housing, but only under non-stressed conditions. Thus, differences in social systems can influence the extent to which social interactions may be beneficial to wound healing.

In the second portion of this thesis, I examined the impact of endogenous glucocorticoids on physiological parameters following stroke. Chapter 4 details the progression of post-stroke corticosterone concentrations following stroke and the effects of prior stress on corticosterone concentrations. No differences in intra-ischemic and 4 h reperfusion corticosterone concentrations were observed among the three treatment groups (Figures 4.2 and 4.3); however, when stress was administered immediately prior to stroke, 12 h corticosterone concentrations were significantly higher compared to mice not experiencing stress (Figure 4.4). The same pattern was observed in infarct size (Figure 4.1) and cerebral edema (Figure 4.5), thereby suggesting that stress exacerbates post-stroke outcome.

The third and final portion of my thesis examines the effects of stroke on peripheral immune function. Chapter 5 characterized post-stroke cell-mediated and humoral immune function. This study demonstrates an augmentation in the peripheral immune response to cerebral ischemia (Figures 6.1 and 6.2); however cerebral ischemia failed to alter cutaneous wound healing rate (Figure 6.3). The augmentation in peripheral immune function was mitigated when neuronal damage was prevented via hypothermia

(Figure 6.1 and 6.2). These data further highlight the changes in immune function following neuronal damage and the therapeutic potential of hypothermia during an ischemic event. Chapter 6 examined immune function following stroke in mice provided positive social interactions via pair housing and comparing those to socially isolated mice. Social facilitation has positive effects on health and has been shown to decrease neuronal damage and increase functional outcome following stroke. Pair housing mitigated the effects of stroke on cell mediated immune function evidenced in Chapter 5; however as demonstrated in Chapter 5, stroke did not alter cutaneous wound healing. Pair housing did, however, reduce wound size, regardless of the surgical condition (Figure 6.2). Finally, Chapter 7 examined the effects of restraint stress on peripheral immune function. Stress has a biphasic effect on immune function and can enhance or dampen various immune parameters (Dhabhar, 2003). Restraint stress increases neuronal damage (Figure 7.1; see Chapter 4 also), which can lead to a decrease in humoral immune function when coupled with stress following stroke (Figure 7.3); however, this same stressor, in the absence of neuronal damage, increases humoral immune function (Figure 7.2), thereby further adding to the literature elucidating stress effects on immune function.

Collectively, the experiments in this dissertation suggest that 1) physical contact is necessary for the social facilitation of wound healing; 2) social facilitation of wound healing occurs in both male and female monogamous mice, regardless of the sex of the partner and whether the experimental mouse is exposed to stress. In contrast, stress eliminates the effects of pair housing on wound size of polygynous mice; 3) restraint stress exacerbates histological and physiological outcome following stroke; 4) stroke

augments cell-mediated and humoral immune function; 5) social facilitation can mitigate the stroke-induced alterations in cell-mediated immune function; and 6) restraint stress interacts with stroke, such that the effects of stress on humoral immune function are altered under the presence of neuronal damage. Taken together, these data suggest that exposure to stress or positive social interactions drastically affect health outcomes.

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