SEX DIFFERENCES IN THE INDUCED EXPRESSION OF HSP70 AND HSP27 IN THE BRAIN AND HEART OF RATS

by

Danielle A. Rioux

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
February 2013

© Copyright by Danielle A. Rioux, 2013

DALHOUSIE UNIVERSITY

DEPARTMENT OF MEDICAL NEUROSCIENCE

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "SEX DIFFERENCES IN THE INDUCED EXPRESSION OF HSP70 AND HSP27 IN THE BRAIN AND HEART OF RATS" by Danielle A. Rioux in partial fulfilment of the requirements for the degree of Master of Science.

Dated: February 11, 2013

Co-Supervisors: _____

_____

Readers: _____

_____

_____

DALHOUSIE UNIVERSITY

DATE: February 11, 2013

AUTHOR: Danielle A. Rioux

TITLE: SEX DIFFERENCES IN THE INDUCED EXPRESSION OF HSP70 AND HSP27 IN THE BRAIN AND HEART OF RATS

DEPARTMENT OR SCHOOL: Department of Medical Neuroscience

DEGREE: M.Sc. **CONVOCATION**: May **YEAR**: 2013

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions. I understand that my thesis will be electronically available to the public.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

_____

Signature of Author

I dedicate this thesis to my parents and my sisters whom provided me with, and continue to provide me with, enthusiasm to pursue my dreams.
"Ti amo; Je t'aime"

"Education is the most powerful weapon which you can use to change the world."
-Nelson Mandela

Table of Contents

List of Tables

List of Figures

Abstract

There are sex differences in degenerative disease prevalence in humans. Most models of degenerative disease use male animals. Examining female and male responses to stress may give insight into disease prevalence. Heat shock proteins are chaperones linked to damaged proteins in degenerative diseases and may be expressed differentially in females and males. My goal was to characterize the induced expression of Hsp70 and Hsp27 in the brain and the heart of female and male rats. Rats were heat shocked, brains and hearts were removed 24 hours after, and western analyses were done to quantify the expression of these proteins. Immunofluorescence was used to localize Hsp70 and Hsp27 in the hippocampus. Overall, male rats have significantly greater induced expression of both Hsp70 and Hsp27 in the brain. In the hippocampus, Hsp70 was localized in blood vessels and microglia, and Hsp27 was localized in astrocytes, following heat shock. Therefore, the difference in expression of heat shock proteins seen in the brain and the heart of male and female rats following heat shock suggests that sex hormones may have an impact on degenerative disease prevalence.

List of Abbreviations Used

Some abbreviations used in this thesis are in the *Stedman's Medical Dictionary 28th Edition* (published in 2005) and are not repeated here.

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
CA1, CA2, CA3	Cornu Ammonis 1, 2, and 3 regions of the hippocampus
CVD	Cardiovascular diseases
DNAJ	HSP40 kDa family of heat shock proteins
E2	17-beta estradiol
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
GFAP	Glial fibrillary acidic protein
HSF1	Heat Shock Transcription Factor 1
HSP	Heat shock proteins (not specific to one family)
Hsp27	HSPB1, the 27 kDa heat shock protein
Hsp70	HSPA1A, the highly inducible 70 kDa heat shock protein
HSP70	HSPA, the 70 kDa family of heat shock proteins
Hsc70	HSPA8, the constitutively expressed 70 kDa heat shock protein
HSP15-30	HSPB, the family of small heat shock proteins
HSP90	HSPC, the 90 kDa family of heat shock proteins
HSP110	HSPH, the 100 kDa family of heat shock proteins
NeuN	Neuronal nuclei protein
Iba1	Ionized calcium binding adaptor molecule 1
PD	Parkinson's disease
SEM	Standard error of the mean

Acknowledgements

I feel so lucky to have had the opportunity to work with Dr. Currie and I greatly appreciate how he always left his door open to me. I thoroughly enjoyed talking to him and learning from him. He provided me with invaluable lessons on data, lab techniques, science, research, and life. I will always appreciate the endless patience he had for me, and the help he provided me with, while reviewing my thesis. His love for teaching and research is inspiring and provided me with continual motivation for my research and future career plans. I would like to sincerely thank my supervisor, Dr. Currie, for all his optimism, support and guidance throughout my masters program and for making every bit of it an incredibly positive experience.

I was also very fortunate to work with Dr. Esser, who also always left his door open to me and provided me with guidance throughout my masters program and on my future career goals for medicine. He was a fountain of knowledge, especially on topics related to research, science and medicine. Dr. Esser had endless patience and advice and made every bit of my masters a positive experience. I would like to sincerely thank my co-supervisor, Dr. Esser, for inspiring me to work towards becoming the best student I can be-whether that be in research or in my plans for medicine.

Only after I met Kay did I realized the importance of beginning every project with a positive outlook, persevering through challenges, and remaining optimistic regardless of the outcome. Kay provided me with tireless help with my lab techniques, ideas while writing my thesis, and a life lesson on every topic that came my way. I am forever grateful that I met you during my masters program and I hope you know that you will never get rid of me! I would like to sincerely thank you for everything you've done for me, and your boundless confidence in me to pursue my future goal of medicine.

I would also like to thank my supervisory committee that included: Dr. Tara Perrot, Dr. Kazue Semba, Dr. Gary Allen, and Dr. William Baldridge. I will always appreciate our meetings together, your endless support and suggestions for my project and, the patience and the time you spent on reading and reviewing my final thesis. I would like to sincerely thank you all for your support and encouragement.

For all of those whom I befriended during my masters, I would like to take this opportunity to thank you for your continued support, encouragement, laughs, assistance, and sharing of ideas of science and future endeavors. The optimism you all provided me with was priceless and helped me to overcome any challenge I was faced with during my masters program. Many sincere thanks to: The Department of Medical Neuroscience, and The Department of Pharmacology, and, especially, the members of the (old) 15E lab.

Chikako and Quinn: I couldn't have asked for better lab mates and feel so fortunate to have worked beside you during my masters program. You are both very inspiring individuals and I learned so much from both of you. I wish you the best on all your future endeavors and I hope to always keep in touch.

Most importantly, I would like to thank my parents and my sisters. To my parents: you have provided me with endless support and unwavering love throughout my masters program. I will be forever grateful for your positive outlook, reassurance, and encouragement to do whatever brings me happiness. To my sisters: You are both amazing role models and have always pushed me to become the best I can be. Both of you have footsteps that are hard to follow behind, which continues to challenge me. I sincerely thank all of you.

Introduction

As a result of the increased life expectancy in developed countries, chronic diseases, like neurodegenerative diseases, are becoming more common (Hebert *et al.,* 2003). In the aging population there are several degenerative diseases that have a strong prevalence for either males or females. In the brain these disease include amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD). Similarly, in the heart, cardiovascular diseases have a strong bias for male or female, depending on age. However, most research into these diseases tends to be done in male animal models of disease utilizing males. One common feature of all these diseases of aging is the accumulation of abnormal protein aggregates and the cellular response to proteotoxicity with altered expression levels of various heat shock proteins. In this thesis, I will characterize the levels of two heat shock proteins in the brain and heart of normal and heat shocked adult female and male rats. This characterization may provide insights into female and male differences in disease prevalence.

Disease prevalence in the brain

Amyotrophic lateral sclerosis is a neurodegenerative disease of upper and lower motoneurons. In North America and European countries, the incidence of ALS is 1.5 times greater in men, compared to women, between the ages of 15-85. ALS incidences increase with age, with peak incidence in men being between the ages of 70-74 and peak incidence in women being between the ages of 65-74. Between the ages of 25-55 however, men are two times more likely than women to be diagnosed with ALS. In contrast, between the ages of 55-75, men are only 1.5 times more likely than post-

menopausal women to be diagnosed with ALS (Huisman *et al*., 2011a). This suggests that estrogen in women may be a factor in the prevalence of ALS in females and males.

Alzheimer's disease is a degenerative condition and the most common form of dementia in people over the age of 60. AD is most often diagnosed between the ages of 65-69 years of age, and especially in women, where prevalence is approximately twice that in men (Moschetti *et al*., 2012). This female prevalence appears to be linked to the levels of estrogen in women, as this disease occurs approximately 10-20 years past the age at which most women begin menopause (Kawas *et al.*, 1997; Gao *et al.*, 1998; Hampel *et al*., 2011). This suggests the decline in estrogen in post-menopausal women may make women vulnerable to AD.

Parkinson's disease is a degenerative disease of the central nervous system (CNS) resulting from death of dopaminergic neurons in the substantia nigra. PD is 1.5 to 2 times more common in men, compared to women, and the average age for diagnosis is usually between the ages of 50-60 years. Given that menopause affects women between the ages of 45-65 and the majority of cases of PD are diagnosed between the ages of 50-60, it appears that the age of menopausal women coincides with the age of greatest PD diagnoses (Van Den Eeden *et al.,* 2003), which highlights estrogen levels as a possible factor contributing to PD onset (Kompoliti, 2003).

Disease prevalence in the heart

In Canada and the United States, cardiovascular diseases (CVD) are the most common cause of death. The most common types of CVD are those affecting the vessels of the heart leading to blockages and total or partial ischemia. There are sex differences

in prevalence of cardiovascular diseases (Safford *et al.*, 2012) in that men are more likely to have a CVD than women. Estrogen levels appear to decrease the risk of cardiovascular disease in premenopausal women. However, the incidence of CVD in men and women equalize as the estrogen levels decrease in post-menopausal women (Masood *et al*., 2010). Men and women also differ in the severity of injury following a heart attack. Although pre-menopausal women are less likely to have a heart attack than men (Smiley & Khalil, 2009), men suffer less damage following a heart attack, compared to pre- and post-menopausal women (Canto *et al*., 2012). Estrogen likely plays a role in CVDs.

Sex bias in animal models of disease

According to research funding agencies, like the Canadian Institutes of Health Research, Heart and Stroke Foundation of Canada and the Dalhousie Medical Research Foundation, medical research is funded on the premise that the results, often achieved through the use of animal models, will one day be translatable to human conditions. Diseases affecting the cardiovascular and nervous systems affect males and females disproportionately. Despite the US National Institutes of Health Revitalization Act in 1993 mandating that women and minorities be included in research studies (Wald &Wu, 2010; Zucker & Beery, 2011), males remain the predominate sex used in animal studies of these diseases. Studies that use animal models for predicting the effectiveness of treatment strategies have remained controversial because of recurrent failure of results from animals to translate to clinical trials. Recent examples of these studies include treatments for stroke and for myocardial infarction (van der Worp *et al.*, 2010). Treatments for both conditions have been studied with animals in the laboratory, but have

failed in large clinical trials (van der Worp *et al.*, 2010). The lack of research done in female animal models may be a major factor contributing to the failures seen in clinical trials related to therapeutics.

Examination of over 2,000 animal studies revealed that 80% of published scientific articles on human diseases had a strong male bias, most pronounced being in neuroscience and pharmacology (Beery & Zucker, 2011). Surprisingly, 75% of the studies in three highly cited immunology journals did not provide the sex of the animal models used (Beery & Zucker, 2011). The paucity of research including female animals is often stated because of concerns with variations seen in data due to the fluctuations of estrogen levels during the estrous cycle. Female rats have a 4-day ovarian cycle that must be taken into consideration in experiments where hormones might influence results (Wald & Wu, 2010). In order to take into consideration the varying levels of estrogen during the estrous cycle, compared to males, four times as many female animals, corresponding to the four stages of the estrous cycle, may be required in a study. Taking estrogen levels into consideration is important in order to develop results that can be translatable to human populations for purposes of studying disease manifestation and therapeutics to treat and manage diseases.

Potential new therapeutics may go undiscovered by exclusion of female subjects in research. Certain estrogen replacement therapies may help to prevent heart diseases, like atherosclerosis, and neurological diseases, like ALS, PD, and AD (Brinton, 2008; Gold & Voskuhl, 2009; Fait & Vrablik, 2012). Furthermore, studying the use of therapeutics on male animal models of disease may not only eliminate the potential for further progress in treatment of diseases in women, but may also have potentially harmful

consequences when given to women. Sex differences in plasma volume, body weight, and gastric emptying time factor into pharmacokinetic variability, including the dimensions of drug bioavailability, volume of distribution, metabolism, and elimination (reviewed by Franconi *et al.*, 2007). Failing to include both sexes in research design and analyses has led to treating both men and women with drugs that were studied only in men. As a result, women have experienced various drug efficacy and side-effect profiles, compared to men, leading to the withdrawal of prescription drugs (Fish, 2008; Holdcroft, 2007). Due to adverse effects in women, in 2005, eight out of ten prescription drugs were withdrawn from the United States market (Simon, 2005). Sex differences in susceptibility to diseases, manifestation of diseases and therapeutic strategies indicates studying these factors in both sexes is important in order to fully understand the disease.

Studying the stress response in female and male animals in diseases should begin at a fundamental level. One common feature of neurodegenerative diseases is the accumulation of abnormally folded protein within cells. These intracellular protein aggregates seem to be proteotoxic and the proteotoxicity induces expression of various heat shock proteins (HSP). Heat shock proteins are in all cells of the body and protect the cells against physical and chemical stressors, including proteotoxicity. HSP act as molecular chaperones (Benarroch, 2011) and are involved in a variety of degenerative diseases, such as ALS, AD, PD and CVD buffering the effects of the various stressors, like reactive oxygen species **(**Renkawek *et al*., 1994; Xu & Wick, 1996; Maatkamp *et al*., 2004; Muchowski & Wacker, 2005; Huang *et al.,* 2006; Sharp *et al.,* 2007; reviewed by Xu *et al.,* 2012). Studying the characterization of these proteins will enable researchers and clinicians to better target the factors that are contributing to pathology associated

with degenerative diseases and the therapeutics to treat or better manage these diseases in both females and males.

Expression of heat shock proteins

Heat shock proteins were discovered in 1962, and are a set of highly conserved chaperone proteins found in eukaryotes and prokaryotes (Ritossa, 1962; Ritossa, 1996). Some HSP are constitutively expressed in cells to promote proper folding and assembly of polypeptides and are instrumental for normal signaling and protein trafficking. Other HSP are rapidly induced in response to cellular stress, like hyperthermia and ischemic injury, and function to prevent the formation of protein aggregates (Benarroch, 2011).

Mammalian HSP have been classified into five families according to their molecular mass (in kDa): HSP110 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP40 (DNAJ) and small HSP (HSPB, 15–30 kDa) including Hsp27 (Lindquist and Craig, 1988; Kampinga *et al*., 2008). Each family of HSP is composed of members that have constitutive and induced expression. For example HSP70 family includes two isoforms, Hsc70 (HSPA8) and Hsp70 (HSPA1A). Hsc70 is highly expressed under normal conditions, whereas, Hsp70 is expressed at high levels following stress (Currie & Tanguay, 1991; Tanguay *et al.,* 1993). Similarly, Hsp27 (HSPB1) is expressed normally in some cells and tissues and is highly inducible in other cells and tissues (Plumier *et al*., 1997a; 1997b; Krueger-Naug *et al*., 2000).

Synthesis of HSP is regulated by heat shock transcription factors. Several Hsp interact with the heat shock transcription factor 1 (HSF1) to maintain it in an inactive state in the cytoplasm. Following stress, with accumulation of damaged proteins,

regulatory Hsp such as Hsc70 or Hsp90 dissociate from HSF1, and bind to damaged protein. Freed HSF1 trimerizes, becomes phosphorylated and translocates to the nucleus where it binds to the heat shock element on DNA and initiates the transcription of new heat shock proteins (Shi *et al*., 1998). As Hsp are synthesized and accumulate, they renature damaged protein through their chaperone function and also to bind to monomers of HSF1 and inactivate its transcriptional activity (reviewed by Noble *et al.,* 2008).

Hsp70 and Hsp27 have several functions. Hsp70 is the highly inducible member of the 70 kDa family of HSP and functions at very low levels in most cells and tissues under normal conditions. Hsp70 is involved in the folding of proteins, and under stress Hsp70 prevents the aggregation of unfolded proteins and can even refold aggregated proteins (Hartl & Hartl, 2002). Many nonlethal but noxious stimuli induce a high expression of Hsp70 and other Hsp (Currie & Tanguay, 1991). Subsequent to the induction of Hsp70, cells and tissues have a remarkable resistance to further metabolic injury (Currie *et al.,* 1988; Currie *et al*., 1993). In addition, Hsp70 proteins ensure the coordinated regulation of protein translocation, import and folding, and limit cellular damage following stress by their ability to prevent protein aggregation and to restore the function of denatured proteins (Parsell & Lindquist, 1993).

Finally, Hsp70 also acts as a signaling molecule suppressing cell death pathways. Following potentially lethal stress, Hsp70 regulates the release of cytochrome c from mitochondria interfering with programmed cell death pathways. The interaction with cytochrome c also regulates the activation of procaspase 9 suppressing cell death. Both these actions appear to depend on the chaperone function of Hsp70 (Mosser *et al*., 2000).

In comparison, Hsp27 acts as a: 1) molecular chaperone (Diaz-Latoud *et al*., 2005) by inhibiting actin polymerization (Weiske, 2001), 2) an antioxidant by reducing the effect of tumor necrosis factor-α-mediated cell death, by phosphorylation (Mehlen *et al.*, 1995), and 3) suppressor of signaling events, leading to apoptosis, by blocking cytochrome c release therefore suppressing caspase-3 (Pandey *et al*., 2000). Importantly, the functional diversity of Hsp27 appears to be regulated by its phosphorylation state (Rogella *et al.*, 1996).

Under normal conditions in the cytosole, Hsp27 is in an oligomeric form and following a stress, the monomeric Hsp27 becomes phosphorylated by several mechanisms, including mitogen-activated-protein-kinases, which causes Hsp27 to either stay in a monomeric phosphorylated form or for it to dimerize. Monomeric phosphorylated Hsp27 can then bind to actin and prevent protein damage and apoptosis. In contrast, dimeric phosphorylated Hsp27 targets denatured proteins to the proteasome (Ferns *et al.,* 2006). Normal levels of Hsc70 and Hsp27 in the rat may provide neurons with a line of defense against protein misfolding and aggregation resulting from neurodegenerative diseases that impair normal neuronal functioning (Chen and Brown, 2007).

Aggregation, accumulation and deposition of abnormally folded proteins are mechanistic unifying features of degenerative disorders, like AD (Kopito & Ron, 2000) and CVD (Wang & Robbins, 2006). HSP70 and HSP27 families of HSP are implicated in degenerative diseases, because of their localization in the aggregates and by their constitutive and induced expression in organs like the brain and the heart (Wilhelmus *et al*., 2007; Xu *et al*., 2011). In order to understand the sex differences in disease

prevalence and in how these proteins function in the brain and the heart, we first need to recognize the constitutive and induced expression patterns in both male and female animals.

Sex differences in expression of heat shock proteins in the brain

There are sex differences in neurological disease prevalence (Huisman *et al.*, 2011b; Van Den Eeden *et al.,* 2003) and HSP are strongly associated with various degenerative diseases of the brain. For these reasons it seems important to examine the expression of HSP in both male and female animal models, as noted above. As noted above, most research on expression of HSP has been done in male animals.

Curiously, in a study that used various regions of the brain in both female and male rats to examine the constitutive levels of several Hsp, the results were reported for the females and males as a combined group, except in one region: the hypothalamus (Bodega *et al.,* 2002). Hsp70 levels were greater in the hypothalamus of 3-week old male rats, compared to same age female rats. By 8 months of age, female rats had greater levels if Hsp70 in the hypothalamus, compare to male rats.

In the brain of adult male rats, Hsp70 is present at very low levels (Krueger *et al.,* 1999). Following hyperthermic treatment, Hsp70 levels in male rats significantly increased in the hippocampus, specifically in glial cells, at 3, 6, 12 and 24 hours following hyperthermic treatment, compared to controls that were not heat shocked. Similarly, using female rats, Hsp70 was significantly increased within 4 hours following heat shock, and was detected in glial cells and vascular cells in the cerebral cortex and hippocampus (Krueger *et al.,* 1999; Bechtold *et al.,* 2000; Pavlik *et al.,* 2003).

Most studies examining the Hsp27 levels in the brain are done in male animals. Hsp27 is undetectable in the cerebral cortex of unstressed male rats, and is present normally in the brain stem and spinal cord (Plumier *et al.,* 1997b). After heat shock, by 2 hours, Hsp27 is expressed at high levels in many cells (mostly glial) in the male rat brain including the hippocampus (Krueger-Naug *et al.,* 2000; Bechtold & Brown, 2003). Hsp27 levels peaked at 24 hours following hyperthermia and returned to constitutive or basal levels only after more than 6 days (Krueger-Naug *et al.,* 2000). Thus, most research on the induced expression of Hsp70 and Hsp27 has been done in male rats and there is little or no information on the expression levels of Hsp70 and Hsp27 in the female rat brain.

Sex differences in expression of heat shock proteins in the heart

Cardiovascular diseases are also associated with protein damage and abnormally folded proteins. HSP are recruited to damaged proteins, and through their chaperone function they attempt to restore cellular and protein homeostasis (Lindquist & Craig, 1988). While there is less information available on expression of HSP in the female brain following heat shock treatment, some limited information is available in the female heart.

Three to four-month old male and female rats have differences in expression levels of Hsp70 and Hsp27. Baseline levels of Hsp70 are significantly greater in female rats, compared to male rats. In contrast, male rats had significantly greater levels of Hsp27, compared to female rats at baseline (Voss *et al.,* 2003). Nine weeks following ovariectomy, the cardiac levels of Hsp70 in female rats drop significantly, compared to Hsp70 levels before ovariectomy. The cardiac Hsp70 levels in ovariectomized female rats

were similar to cardiac levels in male rats. Ovariectomized female rats receiving E2 supplements had significantly greater cardiac Hsp70 levels, compared to female rats nine weeks post-ovariectomy (Voss *et al.,* 2003). Taking these results into consideration, estrogen appears to have an effect on cardiac levels of Hsp70 in three to four-month old Sprague Dawley rats (Voss *et al.,* 2003). Age of rats may also have an effect on cardiac Hsp70 levels because there are no sex differences in levels of Hsp70 in two-month old rats (Shinohara *et al.,* 2004; Paroo *et al.,* 1999; Bae & Zhang, 2005).

Following a stressor, like hyperthermia, there also appears to be sex differences in the expression of cardiac Hsp70 and Hsp27. Twenty-four hours following hyperthermic treatment, two-month old male rat hearts had significantly greater expression of Hsp70, compared to female hearts, or control male hearts (Shinohara *et al.,* 2004). Levels of Hsp70 in heat shock female hearts are similar to female control hearts (Shinohara *et al*., 2004).

Similarly, exercise training increases core temperature and cellular stress leading to the induction of heat shock proteins in both male and female rats. Control male and female two month-old rats have similar levels of cardiac Hsp70. Following an acute bout of exercise (treadmill running) male rats have significantly greater cardiac Hsp70 levels, compared to female rats (Paroo *et al.,* 1999). Following two weeks of daily E2 injections, male rats have significantly less cardiac Hsp70, compared to the control male rats. However, post-exercise female rats have similar levels of cardiac Hsp70, compared to post-exercise and E2-treated female and male rats (Paroo *et al.,* 1999). Following an acute bout of exercise, female cardiac Hsp70 levels are unaffected, whereas male cardiac Hsp70 levels significantly increased compared to control animals. However, E2

supplementation in male rats appears to suppress cardiac Hsp70 levels following acute exercise, whereas female cardiac Hsp70 levels are unaffected (Paroo *et al.,* 1999). Estrogen appears to be one factor contributing to the sex differences in cardiac Hsp70 levels following a stressor, like exercise.

In six-month old control rats, cardiac Hsp70 levels were similar in both male and female rats. Following 14 weeks of exercise, both male and female cardiac Hsp70 levels were increased significantly and more so in males (Thorp *et al.,* 2007). After ischemia and reperfusion injury, hearts from control (no exercise training) female rats recovered their ventricular function more strongly than male control hearts. After ischemia and reperfusion injury, hearts from exercise-trained male rats had significantly greater ventricular function, compared to control male rats, but hearts from exercise-trained female rats had similar ventricular function as control female rats. Therefore, acute exercise training is beneficial for male cardiac function following a stress like ischemia and reperfusion (Thorp *et al.,* 2007).

Expression of heat shock proteins in the diseased brain

Amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease are all neurodegenerative diseases that have an accumulation of intracellular and extracellular protein aggregates, protein misfolding, and neuronal loss in the central nervous system (Muchowski & Wacker, 2005). In ALS, motor neurons of the spinal cord and motor cortex are selectively lost (Martin, 1999). In PD dopaminergic neurons in the substantia nigra are lost (Martin, 1999). In AD neuronal loss is prominent in the entorhinal cortex and hippocampus (Martin, 1999; Bruijn *et al.,* 2004; Schapira & Olanow, 2004).

Neurons are post mitotic and are therefore unable to dilute the toxic effects of misfolded and damaged proteins through cell division. Thus neurons are especially vulnerable to aggregated and misfolded proteins and often die by apoptosis (Muchowski & Wacker, 2005). HSP function as chaperones to prevent apoptosis, protein misfolding, and protein aggregation. HSP are potent suppressors of neurodegenerative diseases (Renkawek *et al*., 1994; Muchowski & Wacker, 2005). Transgenic upregulation of Hsp27 and Hsp70 in animal models of neurodegenerative diseases seem to be protective by buffering the toxicity of aggregated or damaged proteins (Patel *et al.,* 2005).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a neurodegenerative disorder characterized by upper and lower motoneuron death. The most common cause of ALS is due to increased reactive oxygen species causing protein damage leading to cellular death. Superoxide dismutase-1 is an abundant cytoplasmic enzyme that neutralizes reactive oxygen species. Mutant superoxide dismutase-1 is related to 20% of familial forms of ALS and more than 90 genetic mutations of the superoxide dismutase gene are known that lead to motoneuron degeneration (reviewed by Cleveland & Rothstein, 2001). Aggregates of mutant superoxide dismutase-1 results in motoneuronal damage, such as calcium channel disregulation and activation of caspase-3 and potentially leading to cells death (reviewed by Cleveland & Rothstein, 2001).

Chaperone proteins, like Hsp70 and Hsp27 are key factors associated with preventing protein aggregations and blocking the mitochondrial apoptotic pathway, thus suppressing cellular death. When motoneurons express mutant forms of superoxide

dismutase-1, aggregates of superoxide dismutase are formed with Hsp70 promoting survival (Shinder *et al.,* 2001). A double transgenic mouse model overexpressing Hsp27 and the mutant superoxide dismutase showed delayed decline in motor strength and significantly greater improvement in the number of functional motor units and increased survival of spinal motor neurons, compared to the transgenic mouse that overexpressed only the mutant superoxide dismutase gene (Sharp *et al.,* 2007; Maatkamp *et al*., 2004).

Similarly, in several mutant superoxide dismutase mouse models of ALS, elevated levels of Hsp70 and Hsc70 were found in insoluble aggregates with the mutant proteins (Liu *et al*., 2005).

Alzheimer's disease

Heat shock proteins are also thought to have a role in AD. The pathology associated with AD is the presence of extracellular plaques and intraneuronal neurofibrillary tangles, which lead to the loss of synapses and neurons (reviewed by Nelson *et al.,* 2012). Neurofibrillary plaques are composed of amyloid beta peptides (reviewed by Nelson *et al.,* 2012), and accumulation of these peptides precedes the appearance of neurofibrillary tangles and senile plaques observed in regions affected early in AD, such as the hippocampus and entorhinal cortex (Gouras *et al.,* 2000). Specific amyloid beta proteins, like amyloid beta-42 are primary factors contributing to neuronal apoptosis, through activation of the p53-Bax pathway (Zhang *et al.,* 2002) seen in patients with AD. Apoptosis associated with activation of the p53-Bax pathway is related to the oxidative damage caused by the amyloid beta peptides. Products released from amyloid beta peptides create hydroxyl radicals and nitro-oxidative stress

(Butterfield *et al.,* 2002). Along with oxidative damage, protein aggregation of mis-folded proteins occurs in the brains of patients with AD. Aggregates of the amyloid beta protein form extra-cellularly in senile plaques, whereas the hyperphosphorylated tau protein accumulates intracellularly and forms neurofibrillary tangles (reviewed by Wilhelmus *et al.,* 2007).

Heat shock proteins are involved in protecting neurons against aggregations of mis-folded proteins and the oxidative stressors from reactive oxygen species created in the AD brain. Accumulation of misfolded protein leads to induction of the heat shock response where Hsp70 facilitates protein refolding in the endoplasmic reticulum. Alternately misfolded protein is degraded in the proteasomes or lysomes facilitating cellular survival. Mis-folded proteins in the brain cause the astrocytes and microglial cells surrounding the senile plaques and neurofibrillary tangles to increase the expression of Hsp27, which indirectly aid in the refolding of proteins (Shinohara *et al.,* 1993; Shimura *et al.,* 2004). Hsp70 has an ATP binding domain, which allows direct interaction with damaged proteins and to help to re-fold them into their proper conformation (reviewed by Wilhelmus *et al.,* 2007).

Although an increase in reactive oxygen species and aggregated proteins accumulate with age leading to increased intracellular levels of heat shock proteins, the levels of heat shock proteins remain inadequate in preventing AD. Increased levels of damaged and mis-folded proteins may be the result of an overall decrease in protein chaperone activity in aged cells (Csermely, 2001).

Parkinson's disease

PD is characterized by loss of midbrain dopaminergic neurons in the nigrostriatal dopaminergic pathway and the presence of alpha synuclein in cytoplasmic aggregates, termed Lewy bodies and Lewy neurites (Schapira, 1997; Aridon *et al.,* 2011). There are several factors that contribute to the pathology associated with PD. One factor is the aggregation of alpha synuclein that makes up the Lewy bodies. Alpha synuclein also has a role in neurotransmitter and vesicle release and turnover (Aridon *et al.,* 2011). Disrupted synaptic vesicle function leads to build up of catecholamines, like dopamine, in the cytoplasm of neurons. This is because mutations in alpha-synuclein in dopaminergic neurons may result in a reduced number of vesicles available for the storage of dopamine and the accumulation of this neurotransmitter in the cytoplasm (reviewed by Lotharius and Brundin, 2002; Aridon *et al*., 2011). Along with the accumulation of dopamine in the cytoplasm, there is also an accumulation of dopamine metabolites, such as superoxide ions, dopamine-quinone species, and hydroxyl radicals, which are cytotoxic and lead to oxidative damage if not converted to their non-cytotoxic forms. In PD, there is an abnormal increase in these reactive oxygen species (reviewed by Lotharius & Brundin, 2002; Bellucci *et al.,* 2012). Therefore, the mutations in alpha-synuclein may lead to dopaminergic neuron degeneration seen in PD (reviewed by Lotharius & Brundin, 2002; Bellucci *et al.,* 2012). In addition to mutations in alpha-synuclein, other mutations in PD-related genes, like PARK1, PARK2, and PARK3 may lead to oxidative stress through mitochondrial dysfunction and accumulation of misfolded proteins leading to neurodegeneration (Lotherius & Brundin, 2002). Like in ALS and AD, HSP prevent

oxidative damage and neurodegeneration seen in PD (Renkawek *et al*., 1994; Maatkamp *et al*., 2004; Muchowski & Wacker, 2005; Huang *et al.,* 2006; Sharp *et al.,* 2007**)**.

Heat shock proteins, act as suppressors of alpha synuclein aggregation by inhibiting alph synuclein fibril formation, therefore, preventing aggregation of proteins, vesicle malfunctioning and buildup of cytoplasmic dopamine (Huang *et al.,* 2006). Preventing metabolite buildup in neurons leads to decreased reactive oxygen species and cell death. In animal models of PD, Hsp70 interacts with the apoptosis protease activating factor-1 and blocks the assembly of a functional apoptosome, therefore preventing dopaminergic cell death. Apoptosis-inducing factor is blocked by Hsp70, which leads to prevention of chromatin condensation and apoptosis. Additionally, Hsp27 blocks cytochrome c, preventing cell death (Kalmar & Greensmith, 2009).

The common features of neurodegenerative diseases of the aging brain are accumulation of damaged or misfolded protein, and recruitment of various HSP to these aggregates of damaged protein, along with age related decline in the expression of HSP (Csermely, 2001). Current treatments for neurodegenerative diseases are targeted at managing the symptoms, rather than the factors that may cause the disease, such as accumulation of mis-folded proteins and decreased expression of chaperone proteins. A preventative strategy to combat the accumulation of mis-folded proteins might be to increase the levels of heat shock proteins in areas vulnerable to degeneration.

Expression of heat shock proteins in the diseased heart

Cardiovascular disease is a chronic inflammatory condition that usually begins at an early age but does not become pathological until later in life, once hyperlipidemia

leads to the aggregation of lipid proteins within blood vessels (Kilic & Mandal, 2012). Foam cells then form and propagate the inflammatory process (Kilic & Mandal, 2012). Pathology associated with CVD is inflammatory with generation of reactive oxygen species, and subsequent protein and cellular damage (reviewed by Xu *et al*., 2012). Other stressors, like physical exertion, drug toxicity, or hypertensive agents, lead to a rise in blood pressure that causes the arterial wall to stretch. Elevation in blood pressure causes protein damage in smooth muscle cells. Protein damage directly or indirectly activates the heat shock transcription factor-1 leading to increased expression of various HSP including Hsp70 (Xu & Wick, 1996). Elevated levels of Hsp70 appear to be beneficial during acute injury such as ischemia, but may be detrimental during chronic injury such as chronic hypertension (Xu & Wick, 1996). In diseased states, Hsp70 has protective effects but the stress may reach a level where the HSP can no longer provide protection from diseases (Zhu *et al.,* 2003).

Circulating levels of Hsp70 may also be associated with severity of coronary artery disease. For example, while a majority of patients with myocardial ischemia had detectable levels of serum HSP70, patients without coronary artery disease or ischemia had significantly higher Hsp70 serum levels (Zhu *et al.,* 2003). On the other hand, Hsp70 increases in response to stress, as seen in chronic heart failure. The concentration of circulating Hsp70 was significantly higher in patients with severe chronic heart failure, compared to patients with mild chronic heart failure (Gombos *et al*., 2008).

Hearts also respond to transient stressors such as hyperthermia. Mouse, rat and rabbit hearts all respond to heat shock treatment with increased levels of Hsp70 that peaks at about 24 hours and declines slowly still being detectable 2 weeks later

(Karmazyn *et al.,* 1990; Currie *et al.,* 1993; Plumier *et al.,* 1995). Most interesting is the acquisition of myocardial protection from ischemic injury that is associated with high levels of Hsp70 (Plumier *et al.,* 1995). After heat shock treatment, high levels of Hsp70 are found in blood vessels within the heart and this localization may protect the entire myocardium from reperfusion injury and the reintroduction of molecular oxygen and free radical injury (Leger *et al.,* 2000). The arterial wall is an integrated functional component of the cardiovascular system that must continually adapt to stress. Vascular cells express high levels of HSP, both constitutively and induced, which allows the vessel walls to maintain homeostasis (Xu & Wick, 1996; reviewed by Xu *et al.,* 2012).

Hsp27 is at high levels normally in skeletal and cardiac muscle cells (Voss *et al*., 2003; Leger *et al.,* 2000). Hsp27 is significantly greater in vascular cells of healthy individuals, compared to atherosclerotic plaques (Martin-Ventura *et al.,* 2004; Park *et al.,* 2006; Robinson *et al.,* 2010).

Hsp27 is also highly regulated in the heart after heat shock treatment (Currie & Tanguay, 1991). Hsp27 exists in the cytoplasm as interchangeable monomers, dimers, and oligomers, and each form has distinct functions. Hsp27 monomers regulate actin filament fragmentation by binding to the end of actin fibers and capping them (Guay *et al.,* 1997; Mounier & Arrigo, 2002). Large multimeric organization of Hsp27 has chaperone function in cells (Mounier & Arrigo, 2002).

Heat shock proteins protect cells against damage through maintenance of proper functioning of proteins. The maintenance of proper protein functioning is done by increasing the expression of Hsp70 and the expression and phosphorylation of Hsp27. Increased expression of HSP appears to help protect the cardiovascular system, as higher

levels of phosphorylated Hsp27 is correlated with healthy individuals (Martin-Ventura *et al.,* 2004; Park *et al.,* 2006; Robinson *et al.,* 2010).

Additionally, increased Hsp70 levels correlate with those suffering from cardiovascular diseases, but then levels appear to peak at a critical point that may differ depending on the individual and the disease. With age, the levels of HSP decrease and damaged proteins accumulate (Colotti *et al.,* 2005). Maintenance of HSP levels may help to prevent progression of cardiovascular diseases or limit damage to the cardiovascular system.

Sex specific gonadal hormones

Sex specific disease prevalence may be related to gonadal hormones. The most obvious hormonal differences between females and males are the circulating levels of estrogen and testosterone, respectively. Additionally, in females levels of progesterone may also influence disease prevalence. Age-related hormonal changes increase the risk of neurodegenerative and cardiovascular diseases (Kalin & Zumoff, 1990; Margo & Winn, 2006; Barron & Pike, 2012). Throughout the lifetime, the brain and heart are hormonally responsive organs and are therefore affected by the fluctuating levels of sex hormones, especially with age (Kalin & Zumoff, 1990; Margo & Winn, 2006; Barron & Pike, 2012). In this thesis, I will focus my discussion on the regulatory role of estrogen on heat shock proteins.

Estrogen in the brain and heart

There are three types of biologically active estrogens, estrone, estriole and 17-beta-estradiol (E2). E2 is produced by the ovaries and is the most concentrated in plasma during reproductive years and is the most potent in estrogenic effects. Plasma E2 concentrations fluctuate throughout the estrous cycle in females (Wojtys *et al*., 2002). For example E2 plasma level concentrations range from 499 to 573 pg/mL, in diestrus; 145 to 2178 pg/mL, in proestrus; 161 pg/mL, in estrus; and 401 pg/mL, in metaestrus (Shaikh, 1971).

Premenopausal women between 20 and 50 years of age have E2 plasma concentrations of approximately 81 pg/mL, whereas postmenopausal women between 51 and 65 years of age have E2 plasma concentrations of approximately 35 pg/mL. On average males have E2 plasma concentrations of approximately 18 pg/mL that is consistent between of 20 and 65 years of age (Massafra *et al.,* 2002).

Besides its physiological role in the female reproductive system, E2 modulates the cardiovascular and nervous systems by activation of the estrogen receptors that can alter gene transcription in the nucleus or acutely activate kinase signaling in the cytosol. There are two estrogen receptors, estrogen receptor-alpha (ER-α) with a molecular mass of 67 kDa and estrogen receptor-beta (ER-β) with a molecular mass of 59 kDa. Both estrogen receptors are highly homologous and expressed in both the nervous system and the cardiovascular system (reviewed by Kumar *et al.,* 2011).

In the hippocampus, ER are found in interneurons throughout the dentate gyrus and striatum radiatum with the number of positive cells and intensity of immunoreactivity is equivalent in female and male rats (Weiland *et al.,* 1997). However, there may be sex

differences in the localization of ER-α in the hippocampus. Researchers Romeo *et al.* (2005) show that ER-α may be more abundant in dendritic spines of CA1 region of the female hippocampus compared to the male hippocampus. In the female rat hippocampus, ER-α immunoreactivity is predominantly in the nuclei of interneurons, in the CA1 region and in the hilus of the dentate gyrus (Milner *et al.,* 2001). Some ER-α immunoreactivity is localized in the perikarya, in dendritic spines, axons and axon terminals of pyramidal and granular cells within the CA1 region. Some extranuclear ER-α immunoreactivity is localized in astrocytes throughout the hippocampal formation (Milner *et al.,* 2001).

In the male rat hippocampus, ER-α immunoreactivity is predominantly in the nuclei of pyramidal cells of the CA1 and CA3 regions (Sakuma *et al.,* 2009).

In comparison, ER-β immunoreactivity is localized to similar hippocampal regions in both female and male rats, including the dentate gyrus, CA1, and CA3 regions, and most is non-nuclear. Furthermore, ER-β immunoreactivity is localized in the cytoplasm, dendritic spines, dendrites, axons and axon terminals of pyramidal and granule cells (Azcoitia *et al.,* 1999; Milner *et al*., 2005; Sakuma *et al.,* 2009).

In the human heart, ER-α and ER- β are expressed in the vascular endothelial and smooth muscle cells and cardiomyocytes in both men and women (Grohé *et al.,* 1997). Similarly, in the mouse heart, there are no sex differences in the expression or distribution of ER-α or ER-β (Lizotte *et al.,* 2009). ER-α is expressed in the cytosolic, sarcolemmal and nuclear fractions from ventricular tissue. The sarcolemmal fraction contains the greatest abundance of ER-α (Lizotte *et al.,* 2009). ER- β is expressed in the cytosolic and nuclear fractions, and is barely detectable in the sarcolemmal fraction from ventricular tissue. The nuclear fraction contains significantly greater abundance of ER-β,

compared to the sarcolemma fraction (Lizotte *et al.,* 2009). Estrogen and estrogen precursor stimulation caused a greater activation of ER-α in female cardiomyocytes compared to male cardiomyocytes (Grohé *et al.,* 1997).

E2 binding to estrogen receptors can lead to either acute or long-term cellular changes, termed non-genomic or genomic signaling, respectively. Non-genomic signaling leads to rapid cellular changes activating membrane-bound receptors. E2 binding causes activation of phosphatidyl inositol-3-OH kinase/AKT pathway leading to increased nitric oxide synthase and inhibition of apoptosis through phosphorylation and degradation of pro-apoptotic proteins (reviewed by Stice & Knowlton, 2008).

Upon activation of the genomic pathway, the E2 and estrogen receptor complex translocates to the nucleus and binds to the estrogen response element and increases the transcription of genes that lead to upregulation of estrogen responsive genes (reviewed by Stice & Knowlton, 2008).

In addition to estrogen, Hsp90 also binds to estrogen receptors (Milne & Noble, 2008). The estrogen receptors are normally bound to Hsp90. When E2 binds to the receptor, Hsp90 is displaced and free to bind to the HSF1, leading to decreased trimerization of HSF1 and decreased transcription of HSP (Milne & Noble, 2008). Estrogen and the estrogen receptor translocate to the nucleus and bind to the estrogen response element, increasing transcription of estrogen regulated genes (Milne & Noble, 2008). However, if there is stress and damaged proteins present, Hsp90 chaperones the damaged proteins leaving HSF1 free to trimerize, translocate to the nucleus and increase the transcription of HSP (Milne & Noble, 2008). This is the proposed mechanism for estrogen regulation of HSP expression.

The effects of estrogen on the expression of heat shock proteins in the brain

Estrogen supplementation in males increases the levels of HSP in the hippocampus. Six hours after the administration of E2 in male rats, there is significantly more Hsp27 and Hsp70 immunoreactive cells in cerebral blood vessels, compared with the brains of control rats (Lu *et al.,* 2002). Additionally, immunoreactive Hsp27 levels in astrocytes and in hippocampal neurons are increased in the brains of E2 treated animals (Lu *et al.,* 2002). Also, in the brains of animals given E2, the heat shock transcription factor-1 levels are significantly greater, compared to animals that did not receive E2 supplementation (Lu *et al.,* 2002).

Similarly, in the cortex, HSF1 is at a lower level after heat shock treatment in ovariectomized female rats treated with estrogen rather than with testosterone, and perhaps Hsp70 levels were also lower in the cortex of estrogen-treated rats, compared to testosterone-treated rats (Papasozomenos & Papasozomenos, 2008). Hsp70 is linked with gonadal hormones and estrogen levels may influence the expression of heat shock protein in the brain.

The effects of estrogen on the expression of heat shock proteins in the heart

Normally, female rats have significantly more Hsp70 and less Hsp27 in the heart, compared to males (Voss *et al.,* 2003). Supplemented estrogen in males increases cardiac Hsp27 and Hsp70 mRNA and protein levels (Szalay *et al.,* 2006). E2 administration in males increased expression of Hsp32, Hsp60 and Hsp70 mRNA, compared to the sham-treated rats (Szalay *et al.,* 2006). E2 administration appears to increase Hsp70 mRNA levels by directly increasing the mRNA levels of the heat shock transcription factor-1

(Papacontantinou *et al.,* 2003). Although, other factors may influence heat shock protein expression in the heart, male animals given estrogen supplementation have increased levels of HSP. In females, estrogen supplementation does not significantly influence the levels of HSP (Voss *et al.,* 2003; Paroo *et al.,* 1999).

Following a stressor, like exhaustive exercise, E2 leads to decreased levels of Hsp70 in male but not female rats. E2 treated male rats subjected to a single bout of intense exercise (treadmill running) had 2-4 times less cardiac Hsp70, compared to vehicle treated male rats. E2 supplementation in females rats had no effect on Hsp70 levels in the hearts and levels of Hsp70 are comparable to those of exercised male rats (without E2 treatment) (Paroo *et al.,* 1999). Estrogen supplementation leads to decreased Hsp70 levels following exercise in the male heart (Paroo *et al.,* 1999).

Furthermore, 24 hours following heat-shock treatment, male rat hearts had significantly greater levels of Hsp70 and better myocardial contractile recovery after ischemia, compared to female hearts. After heat shock, hearts from ovariectomized females had levels of Hsp70 that were comparable to hearts of male rats. After heat shock, hearts from E2 treated ovariectomized females had levels of Hsp70 that were significantly less than hearts from males or intact females that were not treated with E2 (Shinohara *et al.,* 2004).

Summary, hypotheses and objective

It seems clear that in the heart there are basal and induced differences between females and males in the levels of Hsp70 and Hsp27. In the brain these male and female differences in levels of Hsp70 and Hsp27 have not been explored. However, it seems

reasonable to expect sex differences in levels of HSP because of the differences in prevalence of diseases of proteopathies in the brain and the heart. Due to differences in the prevalence of diseases associated with abnormal protein folding, my hypothesis is that there are sex differences in, either or both, the baseline and induced levels of Hsp70 and Hsp27 in the brain and the hearts of female and male rats.

Thus the objective of this work is to characterize the levels of Hsp70 and Hsp27 in the brain and heart of female and male control rats and in female and male rats 24 hours after heat shock treatment, and to identify cell types expressing Hsp70 and Hsp27 in the hippocampus of female and male rats.

Materials and Methods

Animal groups

Male and female Sprague-Dawley rats (Charles River, Quebec, Canada; 225-250 g) were cared for in accordance with the *Guide for Care and Use of Experimental Animals* of the Canadian Council on Animal Care. The experimental procedures used were approved by the Dalhousie University Committee on Laboratory Animals. There were four groups of rats: control females, heat shock females, control males, and heat shock males. All the rats were raised in the same colony room with 12 hour light/dark cycle (7 am/7 pm) and were provided with food and water *ad libitum.* A total of 32 rats were processed for immunofluorescence and 32 were processed for western blot analyses.

Vaginal Swabbing

All female rats were vaginally swabbed to determine their phase in the estrous cycle before control or heat shock treatment and again 24 hours later, immediately before the animals were killed. This procedure consisted of a person holding the rat while another person took a 1 mL syringe containing 0.2 mL of saline to flush the vagina. The 0.2 mL of saline was withdrawn from the vagina and was then placed on a slide for analysis. This procedure was performed in order to determine the stage of the estrous cycle for each female rat.

The vaginal smears were stained with a Diff-Quik staining kit (Dade Behring, B4132-1A). The kit is comprised of three solutions, in which the smears were dipped for 2 minutes each. They were dipped in the following order: Fixative (methanol), Solution 1

(Xanthene dye), and then Solution 3 (Thiazine Dye, Azure A and Methylene blue). The vaginal smears were left to dry overnight, and cells were then examined under a light microscope in order to categorize the stage of estrous.

Heat shock procedure

All animals were housed in the Carleton Animal Care Facility for at least one week following arrival, before receiving heat shock. All animals were handled and weighed each day to minimize stress on the day of heat shock.

The animals were brought to the laboratory early on the day of heat shock and allowed to settle. The animals were weighed and given an intra-peritoneal injection of sodium pentobarbital (CEVA, McGill, 35 mg/kg). Once the animals were anesthetized, based on pedal reflex, a lubricated thermometer was inserted rectally. The control rats were constantly monitored but were not warmed. The heat shock rats were placed on an insulated heating pad (48 °C), covered, and constantly monitored. Once the core body temperature reached 42 °C, this temperature was maintained for 15 minutes, by adjusting the heating pad. After 15 minutes at 42 °C, the animals were removed from the pad and given a subcutaneous injection of 3 mL of saline and then subsequent injections of 1 or 2 mL of saline throughout the day until the animals began drinking on their own. All control and heat shock rats were handled gently and encouraged to drink and eat. Once the rats became ambulatory and drank and ate on their own, they were placed in a clean cage. The control and heat shock rats were then returned to their homeroom and monitoring continued into the evening.

Tissue preparation

At 24 hours following control or heat shock treatment, 64 animals were deeply anesthetized with an intra-peritoneal injection of sodium pentobarbital (100 mg/kg). Animals were perfused transcardially with saline (0.9% sodium chloride) for approximately one minute to remove blood. The brains and hearts were removed from the rats. The whole hippocampus was removed from the brain and the hearts were cut and separated into the ventricles and atria. Tissues were stored at -70 °C for western analysis.

Protein fractionation

Hippocampal samples were homogenized with plastic wands chilled at 4 °C attached to a motorized pestle (Sigma) and the heart tissue was homogenized with a Bio-Gen homogenizer (Pro Scientific) both in a 0.32 M sucrose solution made in 0.1 M sodium phosphate buffer (pH = 7.4) with one protease inhibitor tablet (Roche). The protein concentration was determined by the Bio-Rad Bradford Assay and read in an ELx800 UV spectrophotometer (Bio-Tek Instruments, Inc.; Winooski, VT) at 595 nm. Samples were then aliquoted and stored at -70 °C.

Protein samples (n = 6, heat shock female and heat shock male; n = 5, control female and control male) containing 10 µg of hippocampal protein and 5 µg of heart protein were separated by electrophoresis on a Bio-Rad mini-Protean apparatus. Protein samples were solubilized in sodium dodecyl sulfate sample buffer (5% ß-mercaptoethanol + Laemmli Sample Buffer, Bio-Rad), heated for 10 minutes at 95 °C, and then loaded onto a 4% polyacrylamide stacking gel (made with 1.25 mL of upper Tris (0.5 M Tris-HCl, pH = 6.8, and 0.4% sodium dodecyl sulfate), 500 µL of 30%

acrylamide and 0.8% bis-acrylamide, 80 µL of ammonium persulfate, 5 µL of N,N,N',N'-tetramethylethylenediamine (TEMED), and 3.17 mL of distilled water). A Precision Plus Protein Kaleidoscope molecular weight marker (Bio-Rad) was also loaded beside the protein samples. The samples were migrated through the stacking gel at 75 V for approximately 20 minutes until stacked and then the samples were fractionated by electrophoresis in a 10% polyacrylamide separating gel (made with 2.5 mL of lower Tris (1.5 M Tris-HCl, pH = 8.8, and 0.4% sodium dodecyl sulfate), 3.32 mL of 30% acrylamide and 0.8% bis-acrylamide, 150 µL of ammonium persulfate, 5 µL of TEMED, and 4.03 mL of distilled water) at 125 V for approximately 1.5 hours.

Following electrophoretic separation, fractionated proteins were electroblotted from the polyacrylamide separating gel onto Immobolin-P polyvinylidene fluoride membranes (Millipore, Missisauga, ON). Membranes were wetted with methanol, and then with 1 x Tris Buffered Saline (0.02 M Tris-base and 0.14 M NaCl) with 0.1% Tween-20 (TBS-T, Sigma Aldrich). Proteins were electroblotted to the membrane at 100 V for approximately 2 hours. Membranes were then air dried for approximately 48 hours, so that protein can dry on the membrane.

Western analysis

After air drying for 48 hours, membranes were wetted with methanol, rinsed with TBS-T, and then put in 1 x TBS-T made with 5% low fat skim milk (blotto) for 1 hour to block non specific labeling. Each antibody was applied individually to the membrane. Before applying a new antibody, the membranes were washed overnight in TBS-T. Preparation of antibodies was as follows: primary mouse antibody specific for the

inducible Hsp70 (Enzo Scientific, ADI-SPA-810) was diluted at 1:2000 in blotto. Primary rabbit antibody specific for Hsp27 (Enzo Scientific, ADI-SPA-801) was diluted 1:10 000 in blotto.

Antibodies specific for ß-Actin and ß-Tubulin were used to control for the amount of protein loaded in each lane. The primary rabbit antibody specific for ß-Actin (Sigma Aldrich, A2066) was diluted at 1:25000 in blotto, and was incubated on the membranes containing heart proteins. The primary mouse antibody specific for ß-Tubulin (Sigma Aldrich, T4026) was diluted at 1:250 000 in blotto and was incubated on the membranes containing hippocampus proteins. ß-Actin and ß-Tubulin were levels were consistent and uninfluenced by stress, which was confirmed by replicat western blots (data not shown).

Incubation of the primary antibody on the membranes was overnight (approximately 15 hours) in the refrigerator (4 °C) with gentle agitation on an orbital shaker. In order to remove excess primary antibody, membranes were first dipped a few times in TBS-T and then washed 3 x 20 minutes with TBS-T on an orbital shaker at room temperature. For detection of Hsp27 and Hsp70, membranes were then incubated with secondary peroxidase conjugated chicken anti-rabbit IgG (Santa Cruz, sc-2963) and peroxidase conjugated chicken anti-mouse IgG (Santa Cruz, sc-2962), respectively. The incubations were done at a 1:10 000 dilution (in blotto) shaking at room temperature for one hour.

To remove excess secondary antibody, membranes were first dipped a few times in 1 x TBS-T and then washed 3 x 20 minutes in 1 x TBS-T at room temperature on an orbital shaker. Peroxidase-labeled proteins were detected using the enhanced

chemiluminescence plus (ECL+) Western Blot Detection Kit (Amersham, Arlington Height, IL) and visualized on a Typhoon (Amersham).

Densitometric measurement

Densitometric measurements for western analysis were performed using Image-J processing software analysis in Java. Hippocampal Hsp70 and Hsp27 density measurements were normalized to the density measurement of ß-tubulin in the same lane. Cardiac Hsp70 and Hsp27 density measurements were normalized to the density measurement of ß-actin in the same lane.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical anaylses were performed using GraphPad Prism 5.0 for Mac (GraphPad Software Inc.). One-way Analysis of Variance, and T-tests were used to determine significance, and Tukey's post-hoc comparison was used to determine the significant differences. $P < 0.05$ was used to determine significance.

Immunofluorescent microscopy

At 24 hours following control or heat shock treatment, 32 animals were deeply anesthetized with an intra-peritoneal injection of sodium pentobarbital (100 mg/kg). Animals were perfused transcardially with 0.9% saline for approximately one minute to remove blood. They were then perfused with 4% paraformaldehyde made in 0.1 M sodium phosphate buffer (pH = 7.4, 4 °C) for approximately two minutes. Tissues were

then harvested for immunofluorescence microscopy. Brains were then post-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.4) for approximately 48 hours at 4 °C, followed by immersion in 30% sucrose made up in 0.1 M phosphate buffer (pH = 7.4). Serial coronal 30 µm sections of the brain were cut on a freezing microtome (Leica) then stored in a refrigerator (4 °C) in Millonig's buffer (0.1M sodium phosphate with 0.03% sodium azide, pH = 7.4) until being processed for immunofluorescence microscopy.

Hsp70 immunofluorescence

The free-floating 30µm coronal brain sections were washed 3 X 10 minutes in PBS-T, and sections were incubated in 1% hydrogen peroxide made in PBS-T for 20 minutes. Sections were washed 3 x 10 minutes in PBS-T before incubating for one hour in 2% donkey serum in PBS-T (Millipore). Hippocampal sections immunoreacted with antibodies against Hsp70, were also immunoreacted with antibodies against either glial fibrillary acidic protein (GFAP), neuronal nuclei (NeuN), ionized calcium binding adaptor molecule 1 (Iba1), or Factor VIII. The sections were incubated for one hour at room temperature and then put at 4 °C on an orbital shaker for 3 days (see Table 1 for antibody information).

After 3 days of incubating in the primary antibodies, sections were washed 3 x 10 minutes in PBS. Sections were then incubated on an orbital shaker at room temperature for two hours in fluorescent secondary antibodies diluted in 2% donkey serum (see Table 1 for secondary antibody information).

Immunoreactivity specificity controls

For the primary antibodies against Hsp70, GFAP, Iba1, or Factor VIII, two specificity controls (no primary added and no secondary added) were done with 2 or 3 sections of hippocampus. For the primary NeuN polyclonal rabbit antibody conjugated to Alexa Fluor 488 the no primary specificity control sections were incubated with donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 and with donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555. After the two-hour incubation, sections were washed in PBS for two hours (12 X 10 minute washes).

In dim-light, sections were mounted on *Superfrost* brand slides and coverslipped with fluorescent mounting media (Sigma-Aldrich).

Hsp27 immunofluorescence

Free-floating 30 μm coronal brain sections were washed 3 X 10 minutes in PBS-T, and then incubated in 1% hydrogen peroxide made in PBS-T for 20 minutes. Sections were washed 3 x 10 minutes in PBS-T before incubating for one hour in 2% donkey serum (Millipore). Hippocampal sections immunoreacted with antibodies against Hsp27, were also immunoreacted with antibodies against either GFAP, NeuN, Iba1 or Hsp70. The sections were incubated for one hour at room temperature and then put at 4 °C on a shaker for 3 days (see Table 2 for antibody information).

After 3 days of incubating in the primary antibodies, sections were washed 3 x 10 minutes in PBS. Sections were then incubated on a shaker at room temperature for two hours in fluorescent secondary antibodies diluted in 2% donkey serum (see Table 2 for secondary antibody information).

Immunoreactivity specificity controls

For the primary antibodies against Hsp27, NeuN, Iba1, or Factor VIII, two specificity controls (no primary added and no secondary added) were done with 2 or 3 sections of hippocampus. For the primary GFAP monoclonal mouse antibody conjugated to Cy3 the no primary specificity control sections were incubated with donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 and with donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555. After the two-hour incubation, sections were washed in PBS for two hours (12 X 10 minute washes).

In dim-light, sections were mounted on *Superfrost* brand slides and coverslipped with fluorescent mounting media (Sigma-Aldrich).

Table 1. Hsp70, GFAP, NeuN, Iba1, and Factor VIII primary and secondary antibodies for Immunofluorescence microscopy

Primary antibodies	Secondary antibodies
Hsp70 monoclonal mouse antibody at 1:500 (Enzo Scientific, ADI-SPA-810)	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21202)
	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570)
GFAP polyclonal rabbit antibody at 1:500 (Dako, Z0334)	donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21206)
NeuN polyclonal rabbit antibody conjugated to Alexa Fluor 488 at 1:500 (Millipore, ABN78A4)	*
Iba1 polyclonal rabbit antibody at 1:500 (Wako, 019-19741)	donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21206)
Factor VIII polyclonal rabbit at 1:500 (Oncogene Research Products, PC313)	donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570)

* The NeuN polyclonal rabbit antibody conjugated to Alexa Fluor 488 no primary control sections were incubated with donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21206) and with donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570).

Table 2. Hsp27, GFAP, NeuN, Iba1, and Hsp70 primary and secondary antibodies for Immunofluorescence microscopy

Primary antibodies	Secondary antibodies
Hsp27 polyclonal rabbit antibody at 1:500 (Enzo Scientific, ADI-SPA-801)	donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21206)
GFAP monoclonal mouse antibody conjugated to Cy3 at 1:500 (Sigma, C92050)	*
NeuN monoclonal mouse antibody at 1:500 (Millipore, MAB377)	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570)
	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21202)
Iba1 monoclonal mouse antibody at 1:500 (Abcam, ab15690)	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570)
Hsp70 monoclonal mouse antibody at 1:500 (Enzo Scientific, ADI-SPA-810)	donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 at 1:500 (Invitrogen, A31570)

* The GFAP monoclonal mouse antibody conjugated to Cy3 no primary control sections were incubated with donkey anti-mouse IgG antibody conjugated to Cy3 at 1:500 (Jackson Immuno Research, 715-165-151) and with donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A21206).

Results

Whole hippocampus western analysis

Hsp70 and Hsp27 levels were detected by western analysis in the hippocampus of control and 24 hours heat shock female and male rats. β-tubulin levels were detected and served as a loading control and for normalization of the levels of Hsp70 and Hsp27.

By western analysis, little or no Hsp70 was detected in the hippocampus of control female and control male rats (Figure 3.1 A). Hsp27 was easily detectable and at similar levels in the hippocampus of control female and male rats (Figure 3.1 A). Statistical analysis, using an unpaired two-tailed t-test, revealed no differences in levels of Hsp70 (Figure 3.1 B) or Hsp27 (Figure 3.1 C) in the hippocampus between control female ($n = 5$) and male ($n = 5$) rats ($p > 0.05$).

Hsp70 and Hsp27 levels in the hippocampus were low in control female rats and appeared to be increased in female rats 24 hours following heat shock treatment (Figure 3.2 A). Statistical analysis, using an unpaired two-tailed t-test, revealed significantly greater levels of Hsp70 ($p < 0.05$; Figure 3.2 B) and Hsp27 ($p < 0.05$; Figure 3.2 C) in the hippocampus of heat shock female ($n = 6$) rats, compared to control female ($n = 5$) rats.

Hsp70 and Hsp27 levels in the hippocampus were low in control male rats and appeared to be increased in male rats 24 hours following heat shock treatment (Figure 3.3 A). Statistical analysis, using an unpaired two-tailed t-test, revealed significantly greater levels of Hsp70 ($p < 0.01$; Figure 3.3 B) and Hsp27 ($p < 0.01$; Figure 3.3 C) in the hippocampus of heat shock male ($n = 6$) rats, compared to control male ($n = 5$) rats.

Hsp70 and Hsp27 levels in the hippocampus were easily detectable 24 hours following heat shock treatment in both female and male rats (Figure 3.4 A). Heat shock male rats appeared to have greater levels of Hsp70 and Hsp27 than heat shock female rats. Statistical analysis, using an unpaired two-tailed t-test, revealed significantly greater levels of Hsp70 ($p < 0.05$; Figure 3.4 B) and Hsp27 ($p < 0.05$; Figure 3.4 C) in the hippocampus of heat shock male ($n = 6$) rats, compared to heat shock female ($n = 6$) rats.

Immunofluorescent microscopy

Immunoreactivity specificity controls

Hsp70 immunoreactivity was detectable in the hippocampus in a few cells in the control male rat (Figure 3.5 A), and in many cells in the heat shock male rat (Figure 3.5 B) with the primary Hsp70 monoclonal mouse antibody and the secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 or the donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488. No Hsp70 immunoreactive cells were detected in the hippocampus with omission of the primary Hsp70 monoclonal mouse antibody (Figure 3.5 C) or omission of the secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 (data not shown) or the secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Figure 3.5 D).

Hsp27 immunoreactivity was detected in the hippocampus in a few cells in the control male rat (Figure 3.5 E), and in many cells in the heat shock male rat (Figure 3.5 F) with the primary Hsp27 polyclonal rabbit antibody. No Hsp27 immunoreactive cells were detected with omission of the primary Hsp27 polyclonal rabbit antibody (Figure 3.5

G) or omission of the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Figure 3.5 H).

GFAP immunoreactivity was detected in many astrocytes in the hippocampus of the control rat with primary GFAP polyclonal rabbit antibody and the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Figure 3.6 A). No immunoreactivity was detected with omission of the primary GFAP polyclonal rabbit antibody (Figure 3.6 B) or with omission of the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Figure 3.6 C). GFAP immunoreactivity was also detected in many astrocytes in the hippocampus of the control rat with primary GFAP monoclonal mouse antibody conjugated to Cy3. No immunoreactivity was detected with omission of the primary GFAP monoclonal mouse antibody conjugated to Cy3 or with incubation with inappropriate secondary donkey anti-mouse IgG antibody conjugated to Cy3, or with secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (data not shown).

NeuN immunoreactivity was detected in many neurons in the hippocampus of the control male rat with the primary NeuN monoclonal mouse antibody and the donkey anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Figure 3.6 D). No immunoreactive neurons were detected with omission of the primary NeuN monoclonal mouse antibody (Figure 3.6 E) and with the omission of the donkey anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Figure 3.6 F). NeuN immunoreactivity was also detected in many neurons in the hippocampus of the control male rat with primary NeuN polyclonal rabbit antibody conjugated to Alexa Fluor 488. No immunoreactivity was detected with omission of the primary NeuN polyclonal rabbit

antibody conjugated to Alexa Fluor 488 or with incubation with inappropriate secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488, or with secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 (data not shown).

Iba1 immunoreactivity was detected in many microglia in hippocampus of a control male rat with the primary Iba1 polyclonal rabbit antibody and the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Figure 3.6 G). No immunoreactive microglia were detected with omission of the primary Iba1 polyclonal rabbit antibody (Figure 3.6 H) and with the omission of the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Figure 3.6 I). Iba1 immunoreactivity was also detected in many microglia in hippocampus of a control male rat with the primary Iba1 monoclonal mouse antibody and the secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555. No Iba1 immunoreactivity was detected with omission of the primary Iba1 monoclonal mouse antibody or the secondary donkey anti-mouse IgG antibody conjugated to Alexa Fluor 555 (data not shown).

Factor VIII immunoreactivity was detectable in and around small blood vessels in the hippocampus of the control male rat with primary Factor VIII polyclonal rabbit antibody and the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 555 (Figure 3.6 J). No immunoreactivity was detected with omission of the primary Factor VIII polyclonal rabbit antibody (Figure 3.6 K) and with omission of the secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor 555 (Figure 3.6 L).

Immunofluorescent double labeling for Hsp70 and GFAP, NeuN, Iba1, and Factor VIII.

Hsp70 immunoreactivity was undetectable in the hippocampus of control female rats (Figure 3.7 A1) and was detectable in many cells and small blood vessels 24 hours following heat shock in the hippocampus of female rats (Figure 3.7 B1). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus of control male rats (Figure 3.7 C1) and in many cells and small blood vessels 24 hours following heat shock in the hippocampus of male rats (Figure 3.7 D1). GFAP immunoreactivity was detectable in many cells in the hippocampus in control female (Figure 3.7 A2), heat shock female (Figure 3.7 B2), control male (Figure 3.7 C2), and heat shock male (Figure 3.7 D2) rats. Merged micrographs (Figure 3.7 A3, B3, C3, D3) of Hsp70 and GFAP immunoreactivity show no colocalization of Hsp70 and GFAP.

Immunofluorescent double labeling for Hsp70 and NeuN (Figure 3.8) revealed similar Hsp70 immunoreactivity as before. Hsp70 was undetectable in the hippocampus of control female rats (Figure 3.8 A1) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (Figure 3.8 B1). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus of control male rats (Figure 3.8 C1) and in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (Figure 3.8 D1). NeuN immunoreactivity was detectable in many cells in the hippocampus of control female (Figure 3.8 A2), heat shock female (Figure 3.8 B2), control male (Figure 3.8 C2), and heat shock male (Figure 3.8 D2) rats. Merged micrographs (Figure 3.8 A3, B3, C3, D3) of Hsp70 and NeuN immunoreactivity show no colocalization of Hsp70 and NeuN.

Immunofluorescent double labeling for Hsp70 and Iba1 (Figure 3.9) revealed similar Hsp70 immunoreactivity as before. Hsp70 immunoreactivity was undetectable in the hippocampus of control female rats (Figure 3.9 A1) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (Figure 3.9 B1). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus in control male rats (Figure 3.9 C1) and in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (Figure 3.9 D1). Iba1 immunoreactivity was detectable in many cells in the hippocampus of control female (Figure 3.9 A2), heat shock female (Figure 3.9 B2), control male (Figure 3.9 C2), and heat shock male (Figure 3.9 D2) rats. Merged micrographs (Figure 3.9 A3, B3, C3, D3) of Hsp70 and Iba1 immunoreactivity show some colocalization of Hsp70 and Iba1. After heat shock in the female, Hsp70 immunoreactivity (Figure 3.9 B3, arrows) was detected mostly in Iba1 negative cells and in some small blood vessels. After heat shock in the male, Hsp70 immunoreactivity (Figure 3.9 D3, arrows) was detected in some Iba1 immunoreactive cells and some small blood vessels.

Immunofluorescent double labeling for Hsp70 and Factor VIII (Figure 3.10) revealed similar Hsp70 immunoreactivity as before. Hsp70 immunoreactivity was undetectable in the hippocampus of control female rats (Figure 3.10 A1) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (Figure 3.10 B1). Hsp70 immunoreactivity was not detectable in the hippocampus of control male rats (Figure 3.10 C1) and was detected in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (Figure 3.10 D1). Factor VIII immunoreactivity was detectable in many small blood

vessels in the hippocampus of control female (Figure 3.10 A2), heat shock female (Figure 3.10 B2), control male (Figure 3.10 C2), and heat shock male (Figure 3.10 D2) rats. Merged micrographs (Figure 3.10 A3, B3, C3, D3) of Hsp70 and Factor VIII immunoreactivity show colocalization of Hsp70 and Factor VIII, particularly in small blood vessels after heat shock (Figure 3.10 B3, D3). Hsp70 immunoreactive cells that were negative for Factor VIII immunoreactivity are also evident (Figure 3.10 B3, D3, arrows).

Immunofluorescent double labeling for Hsp27 and GFAP, NeuN, Iba1, and Hsp70

Hsp27 immunoreactivity was undetectable in the hippocampus of a control female rat (a few fluorescent red blood cells are evident) (Figure 3.11 A1) and was detectable in small blood vessels and many astrocytes in the hippocampus 24 hours following heat shock in a female rat (Figure 3.11 B1). Hsp27 immunoreactivity was detectable at a low level in some astrocytes in the hippocampus of this particular control male rat (Figure 3.11 C1) and in some small blood vessels and many astrocytes 24 hours following heat shock in the hippocampus of a male rat (Figure 3.11 D1). GFAP immunoreactivity was detectable in many astrocytes in the hippocampus of control female (Figure 3.11 A2), heat shock female (Figure 3.11 B2), control male (Figure 3.11 C2), and heat shock male (Figure 3.11 D2) rats. Merged micrographs (Figure 3.11A3, B3, C3, D3) of Hsp27 and GFAP immunoreactivity show some colocalization of Hsp27 and GFAP (arrows), particularly after heat shock.

Immunofluorescent double labeling for Hsp27 and NeuN (Figure 3.12) revealed the more consistent pattern of Hsp27 immunoreactivity, particularly for the hippocampus

of the control male. Hsp27 immunoreactivity was undetectable in the hippocampus of control female rats (Figure 3.12 A1) and was detectable in small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in female rats (Figure 3.12 B1). Hsp27 immunoreactivity was undetectable in the hippocampus of control male rats (a few fluorescent red blood cells are evident) (Figure 3.12 C1) and was detectable in some small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in male rats (Figure 3.12 D1). NeuN immunoreactivity was detectable in many neurons in the hippocampus of control female (Figure 3.12 A2), heat shock female (Figure 3.12 B2), control male (Figure 3.12 C2), and heat shock male (Figure 3.12 D2) rats. Merged micrographs (Figure 3.12 A3, B3, C3, D3) of Hsp27 and NeuN immunoreactivity show no colocalization of Hsp27 and NeuN.

Immunofluorescent double labeling for Hsp27 and Iba1 (Figure 3.13) revealed a similar pattern of Hsp27 immunoreactivity as before. Hsp27 immunoreactivity was undetectable in the hippocampus of control female rats (a few fluorescent red blood cells are evident) (Figure 3.13 A1) and was detectable in small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in female rats (Figure 3.13 B1). Hsp27 immunoreactivity was undetectable in the hippocampus of control male rats (Figure 3.13 C1) and was detected in some small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in male rats (Figure 3.13 D1). Iba1 immunoreactivity was detectable in many cells in the hippocampus of control female (Figure 3.13 A2), heat shock female (Figure 3.13 B2), control male (Figure 3.13 C2), and heat shock male (Figure 3.13 D2) rats. Merged micrographs (Figure 3.13 A3, B3, C3, D3) of Hsp27 and Iba1 immunoreactivity show no colocalization of Hsp27 and Iba1.

Immunofluorescent double labeling for Hsp27 and Hsp70 (Figure 3.14) revealed similar patterns of Hsp27 immunoreactivity and Hsp70 immunoreactivity as seen before. Hsp27 immunoreactivity was detectable in a few small blood vessels in the hippocampus of control female rats (Figure 3.14 A1) and was detectable in small blood vessels and many astrocytes in the hippocampus 24 hours following heat shock in female rats (Figure 3.14 B1). Hsp27 immunoreactivity was detectable in a few small blood vessels in the hippocampus of control male rats (Figure 3.14 C1) and was detected in some small blood vessels and many cells in the hippocampus 24 hours following heat shock in male rats (Figure 3.14 D1). Hsp70 immunoreactivity (red) was undetectable in the hippocampus of control female rats (Figure 3.14 A2) and was detectable in many cells and small blood vessels in the hippocampus 24 hours following heat shock in female rats (Figure 3.14 B2). Hsp70 immunoreactivity was undetectable in the hippocampus of control male rats (Figure 3.14 C2) and was detectable in small blood vessels and in many cells in the hippocampus 24 hours following heat shock in male rats (Figure 3.14 D2). Merged micrographs (Figure 3.14 A3, B3, C3, D3) of Hsp27 and Hsp70 immunoreactivity show some colocalization of Hsp27 and Hsp70 in small blood vessels and in many cells, particularly after heat shock (Figure 3.14 B3, D3; arrows).

Heart western analysis

Hsp70 and Hsp27 levels were detected by western analysis in the heart of control and 24 hours heat shock female and male rats. β-actin levels were detected and served as a loading control and for normalization of the levels of Hsp70 and Hsp27.

By western analysis, little or no Hsp70 was detected in the heart of control female and control male rats (Figure 3.15 A). Hsp27 was detectable in the heart at similar levels in both control female and control male rats (Figure 3.15 A). From left to right on the membrane, control female rats were in the following stages of the estrous cycle: metaestrus, metaestrus, diestrus, metaestrus, and diestrus. Statistical analysis revealed no significant differences between Hsp70 (Figure 3.15 B) and Hsp27 (Figure 3.15 C) levels in hearts of control female ($n = 5$) and male ($n = 5$) rats ($p > 0.05$).

Hsp70 and Hsp27 levels in the heart were low in control female rats and appeared to be increased in female rats 24 hours following heat shock treatment (Figure 3.16 A). Statistical analysis revealed significantly greater levels of Hsp70 ($p < 0.05$; Figure 3.16 B) and Hsp27 ($p < 0.001$; Figure 3.16 C) in the hearts of heat shock female ($n = 6$) rats, compared to control female ($n = 5$) rats.

Hsp70 and Hsp27 levels in the heart were low in control male rats and appeared to be increased in male rats 24 hours following heat shock treatment (Figure 3.17 A). Statistical analysis revealed significant differences in Hsp70 (*, $p < 0.05$; Figure 3.17 B) and Hsp27 (**, $p < 0.01$; Figure 3.17 C) levels in the hearts of control male ($n = 5$) and heat shocked male ($n = 6$) rats.

Hsp70 and Hsp27 levels in the hearts of female and male rats were easily detectable 24 hours following heat shock treatment (Figure 3.18 A). From left to right on the membrane (Figure 3.18 A) heat shock female rats are in the following stages of the estrous cycle: estrus, proestrus, estrus, estrus, metaestrus, and proestrus**.** Statistical analysis revealed no difference in Hsp70 (Figure 3.18 B) levels and a significant

difference in Hsp27 (Figure 3.18 C) levels in the hearts of female (n = 6) and male (n = 6) rats (*, $p < 0.05$) 24 hours following heat shock treatment.

Figure 3.1: Western analysis of Hsp70 and Hsp27 levels in the hippocampus of control rats. Rats were perfused 24 hours following control treatment. Little or no Hsp70 was detected in both the hippocampus of control male and the control female rats (**A**). Hsp27 was detectable at similar levels in the hippocampus of both control male and female rats (**A**). From left to right on the membrane, control female rats were in the following stages of the estrous cycle: metaestrus, metaestrus, diestrus, metaestrus, and diestrus. Statistical analysis revealed no significant differences in Hsp70 (**B**) and Hsp27 (**C**) levels in the hippocampus of control male ($n = 5$) and female ($n = 5$) rats ($p > 0.05$). Data are presented as mean ± SEM.

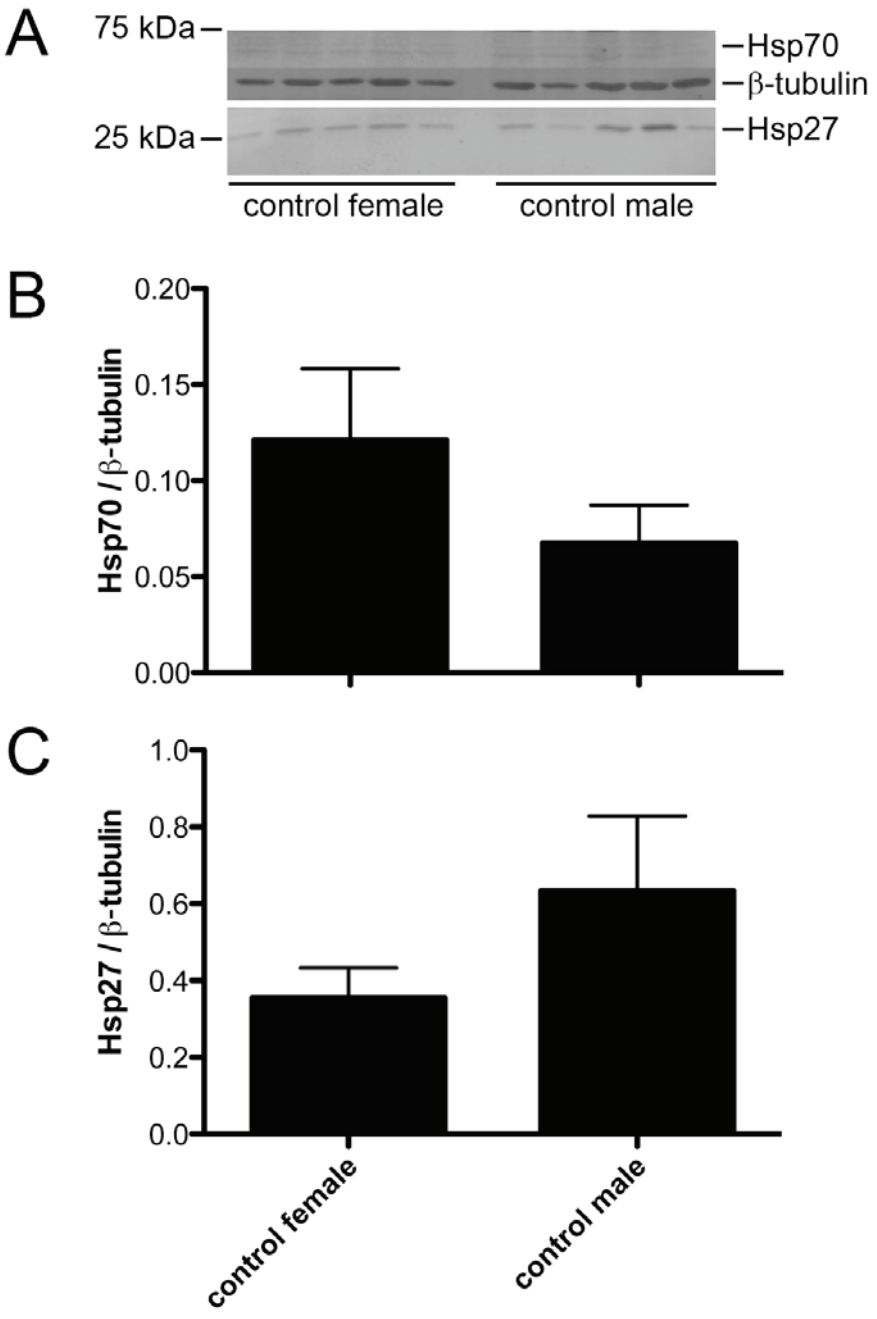

A
75 kDa
25 kDa
Hsp70
β-tubulin
Hsp27
control female
control male
B
Hsp70 / β-tubulin
0.20
0.15
0.10
0.05
0.00
C
Hsp27 / β-tubulin
1.0
0.8
0.6
0.4
0.2
0.0
control female
control male

Figure 3.2: Western analysis of Hsp70 and Hsp27 levels in the hippocampus of control and heat shocked female rats. Rats were perfused 24 hours following control treatment and 24 hours following heat shock treatment. Hsp70 and Hsp27 levels appeared to be increased 24 hours following heat shock treatment in the hippocampus (**A**). From left to right on the membrane (**A**), female sham treated rats are in the following stages of the estrous cycle: metaestrus, metaestrus, diestrus, metaestrus, and diestrus, and female heat shocked rats are in: estrus, proestrus, estrus, estrus, metaestrus, and proestrus**.** Statistical analysis revealed significant differences in Hsp70 (**B**) and Hsp27 (**C**) levels in the hippocampus of control female (n = 5) and 24 hours after heat shock female (n = 6) rats (*, $p < 0.05$). Data are presented as mean ± SEM.

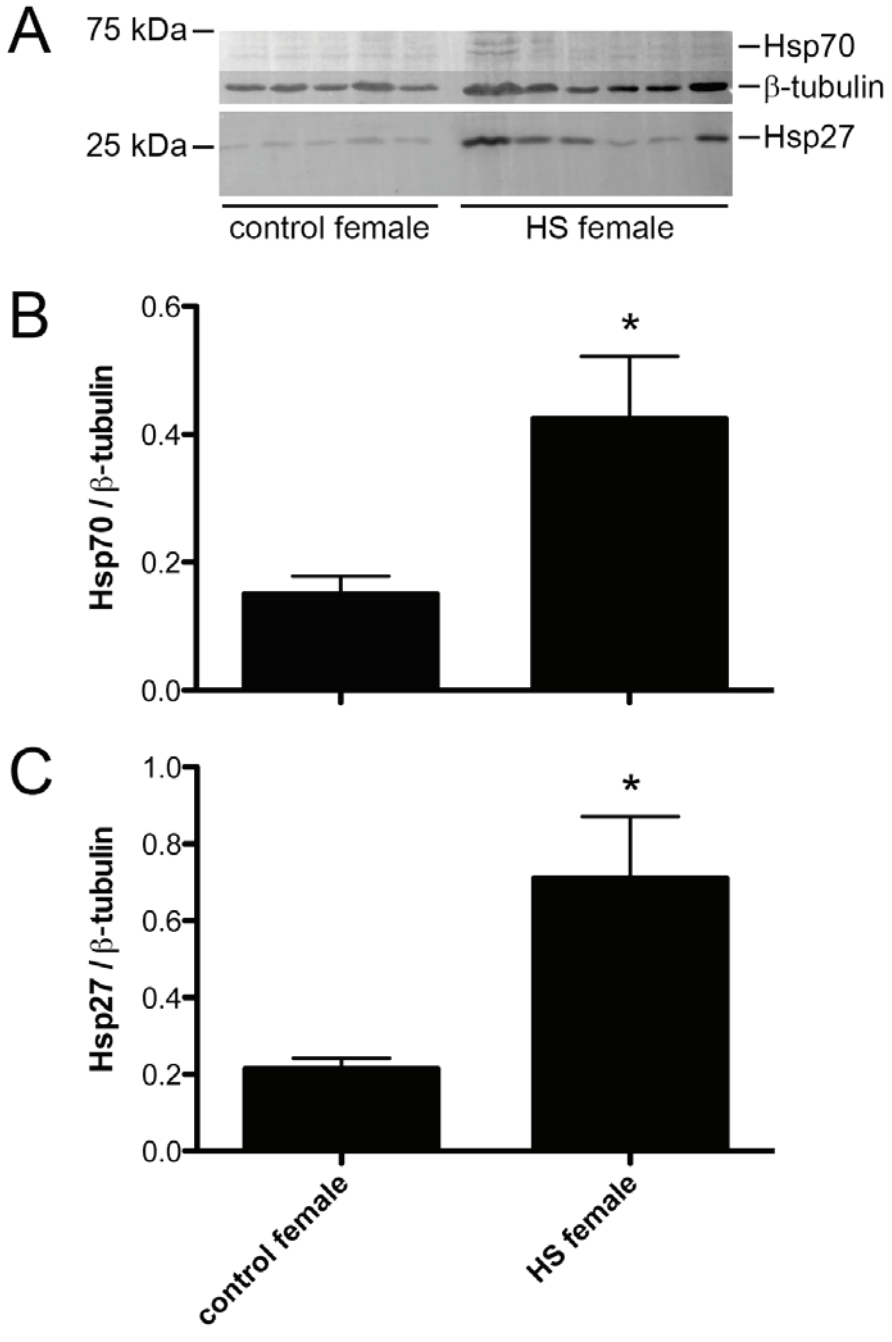

A
75 kDa
Hsp70
β-tubulin
25 kDa
Hsp27
control female
HS female
B
Hsp70 / β-tubulin
0.6
0.4
0.2
0.0
*
C
Hsp27 / β-tubulin
1.0
0.8
0.6
0.4
0.2
0.0
*
control female
HS female

Figure 3.3: Western analysis of Hsp70 and Hsp27 levels in the hippocampus of control and heat shocked male rats. Rats were perfused 24 hours following control treatment and 24 hours following heat shock treatment. Hsp70 and Hsp27 levels appeared to be increased 24 hours following heat shock treatment in the hippocampus (**A**). Statistical analysis revealed significant differences in Hsp70 (**B**) and Hsp27 (**C**) levels in the hippocampus of control male (n = 5) and 24 hours after heat shock male (n = 6) rats (**, $p < 0.01$). Data are presented as mean ± SEM.

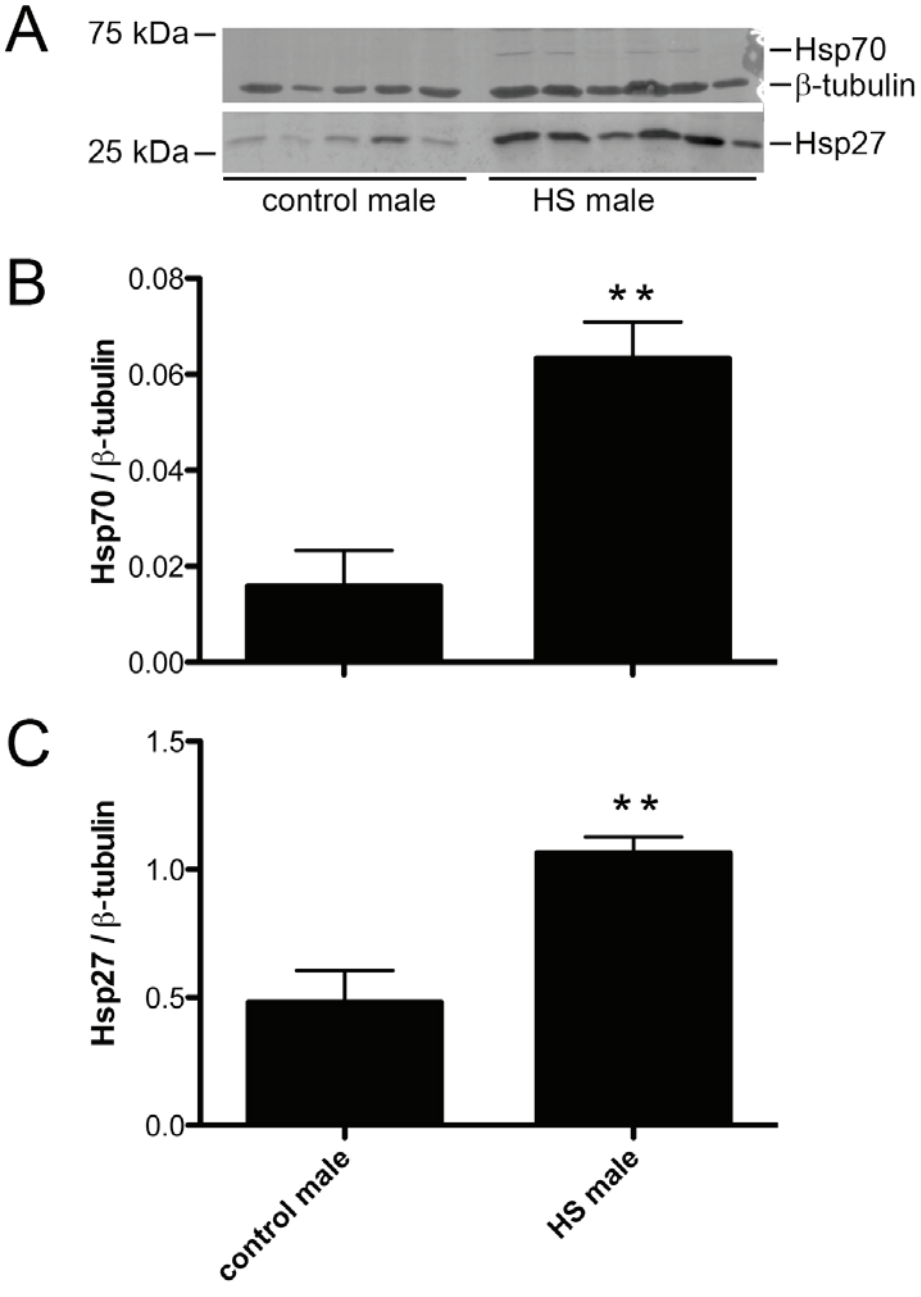

A
75 kDa
25 kDa
Hsp70
β-tubulin
Hsp27
control male
HS male
B
Hsp70 / β-tubulin
0.08
0.06
0.04
0.02
0.00
* *
C
Hsp27 / β-tubulin
1.5
1.0
0.5
0.0
* *
control male
HS male

Figure 3.4: Western analysis of Hsp70 and Hsp27 levels in the hippocampus of heat shock female and heat shock male rats. Rats were perfused 24 hours following heat shock treatment. Hsp70 and Hsp27 levels were easily detectable 24 hours following heat shock treatment in the hippocampus (**A**). From left to right on the membrane (**A**) female heat shock rats are in the following stages of the estrous cycle: estrus, proestrus, estrus, estrus, metaestrus, and proestrus**.** Statistical analysis revealed significant differences in Hsp70 (**B**) and Hsp27 (**C**) levels in the hippocampus of 24 hours after heat shock female ($n = 6$) and 24 hours after heat shock male ($n = 6$) rats (*, $p < 0.05$). Data are presented as mean ± SEM.

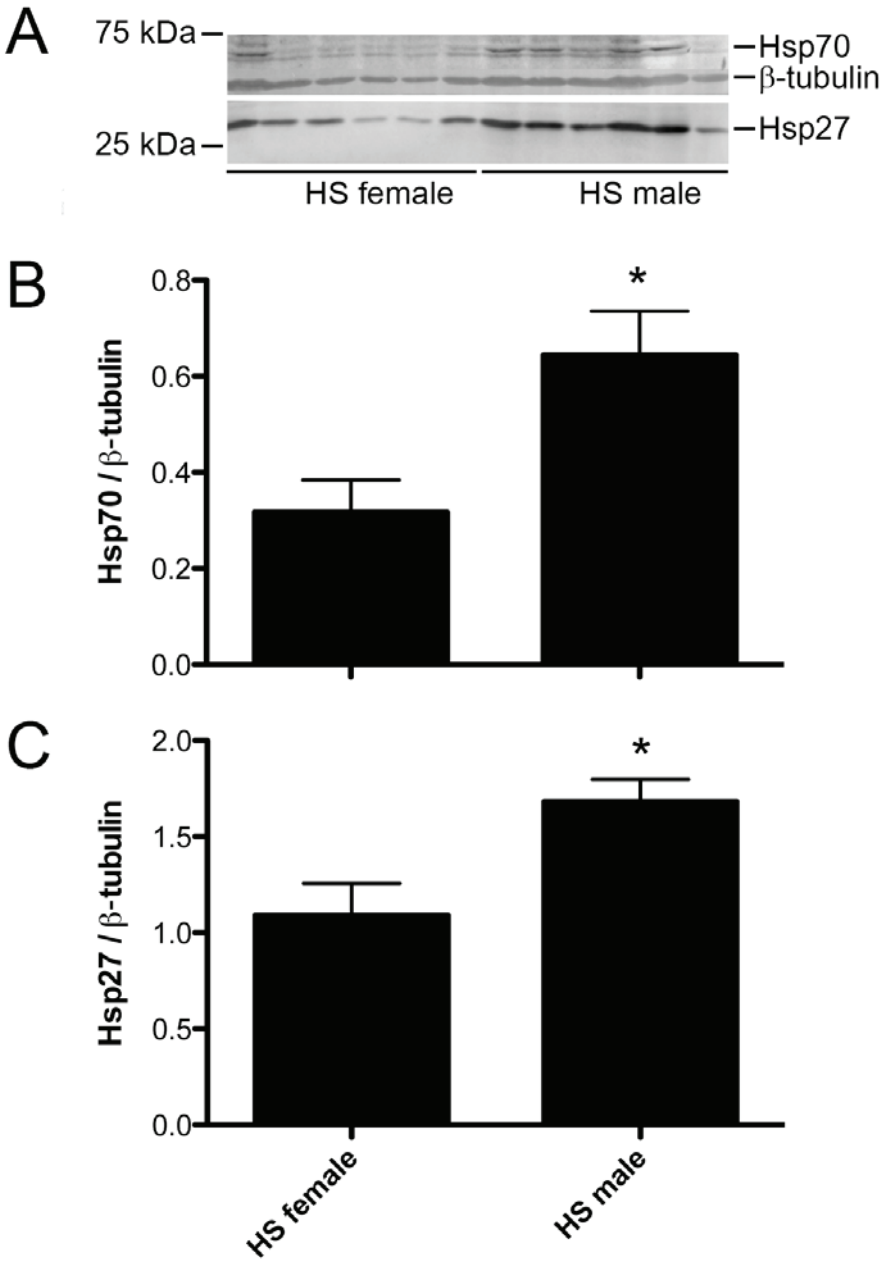

A
75 kDa
25 kDa
Hsp70
β-tubulin
Hsp27
HS female
HS male
B
Hsp70 / β-tubulin
0.8
0.6
0.4
0.2
0.0
*
C
Hsp27 / β-tubulin
2.0
1.5
1.0
0.5
0.0
*
HS female
HS male

Figure 3.5: Specificity of Hsp70 and Hsp27 immunoreactivity. Little or no Hsp70 immunoreactivity was detected in the hippocampus of a control male rat (**A**). Hsp70 immunoreactivity was abundant 24 hours after heat shock treatment (**B**). Immunoreactivity was not detected with omission of the primary Hsp70 monoclonal mouse antibody (**C**) or omission of the secondary Alexa Fluor 488 donkey anti-mouse antibody (**D**). A few cells were Hsp27 immunoreactive in the hippocampus of a control male rat (**E**). Hsp27 immunoreactivity was abundant 24 hours after heat shock treatment (**F**). Immunoreactivity was not detected with omission of the primary Hsp27 polyclonal rabbit antibody (**G**) or omission of the secondary Alexa Fluor 488 donkey anti-rabbit antibody (**H**) in hippocampus of a heat shock male rats. The scale bar represents 100 microns.

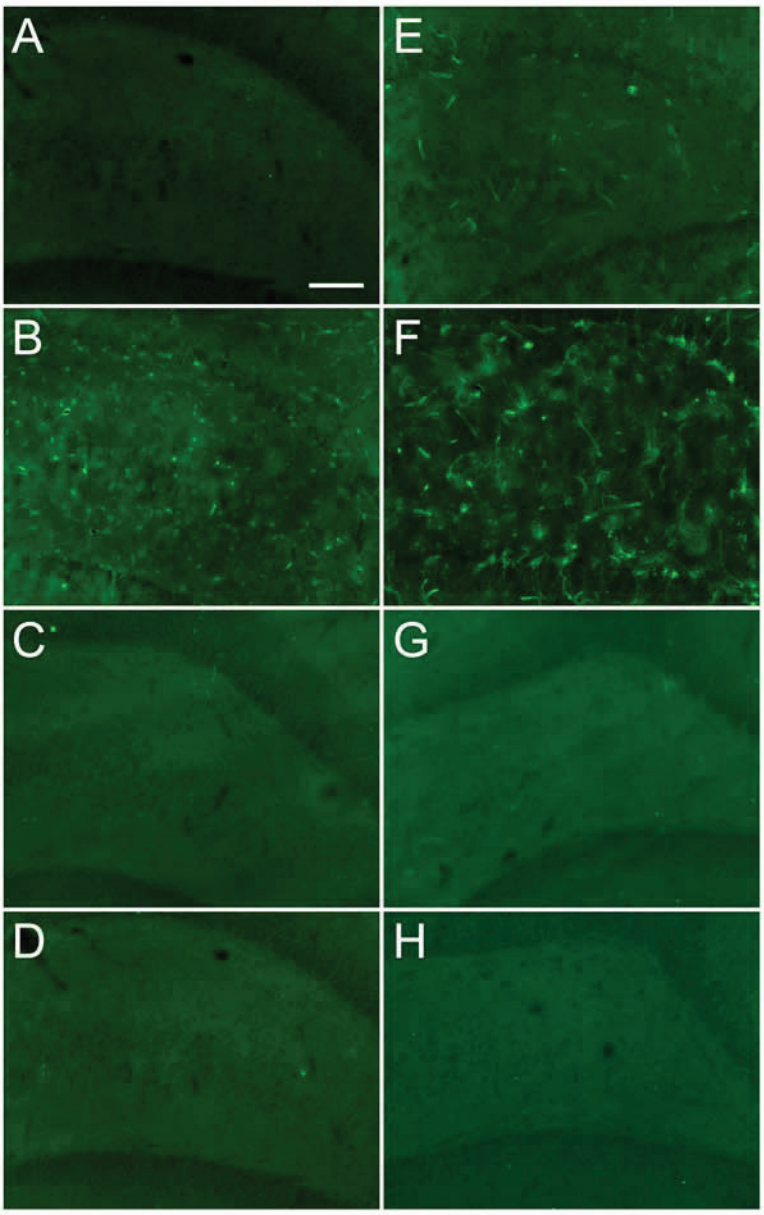

A
E
B
F
C
G
D
H

Figure 3.6: Specificity of GFAP, NeuN, Iba1, and Factor VIII immunoreactivity. Many cells were immune-reactive with primary GFAP polyclonal rabbit antibody (**A**). No immunoreactivity was detected with omission of the primary GFAP polyclonal rabbit antibody (**B**) and with the omission of the Alexa Fluor 488 donkey anti-rabbit antibody (**C**). Many cells were immunoreactive with primary NeuN monoclonal mouse antibody (**D**). No immunoreactivity was detected with omission of the primary antibody (**E**) and with the omission of the secondary Alexa Fluor 488 donkey anti-mouse antibody (**F**). Many cells were immunoreactive with primary Iba1 polyclonal rabbit antibody (**G**). No immunoreactivity was detected with omission of the primary polyclonal rabbit antibody (**H**) and with the omission of the Alexa Fluor 488 donkey anti-rabbit antibody (**I**). Many cells were immunoreactive with primary Factor VIII polyclonal rabbit antibody (**J**). No immunoreactivity was detected with omission of the primary Factor VIII polyclonal rabbit antibody (**K**) and with the omission of the Alexa Fluor 555 donkey anti-rabbit antibody (**L**). The scale bar represents 100 microns.

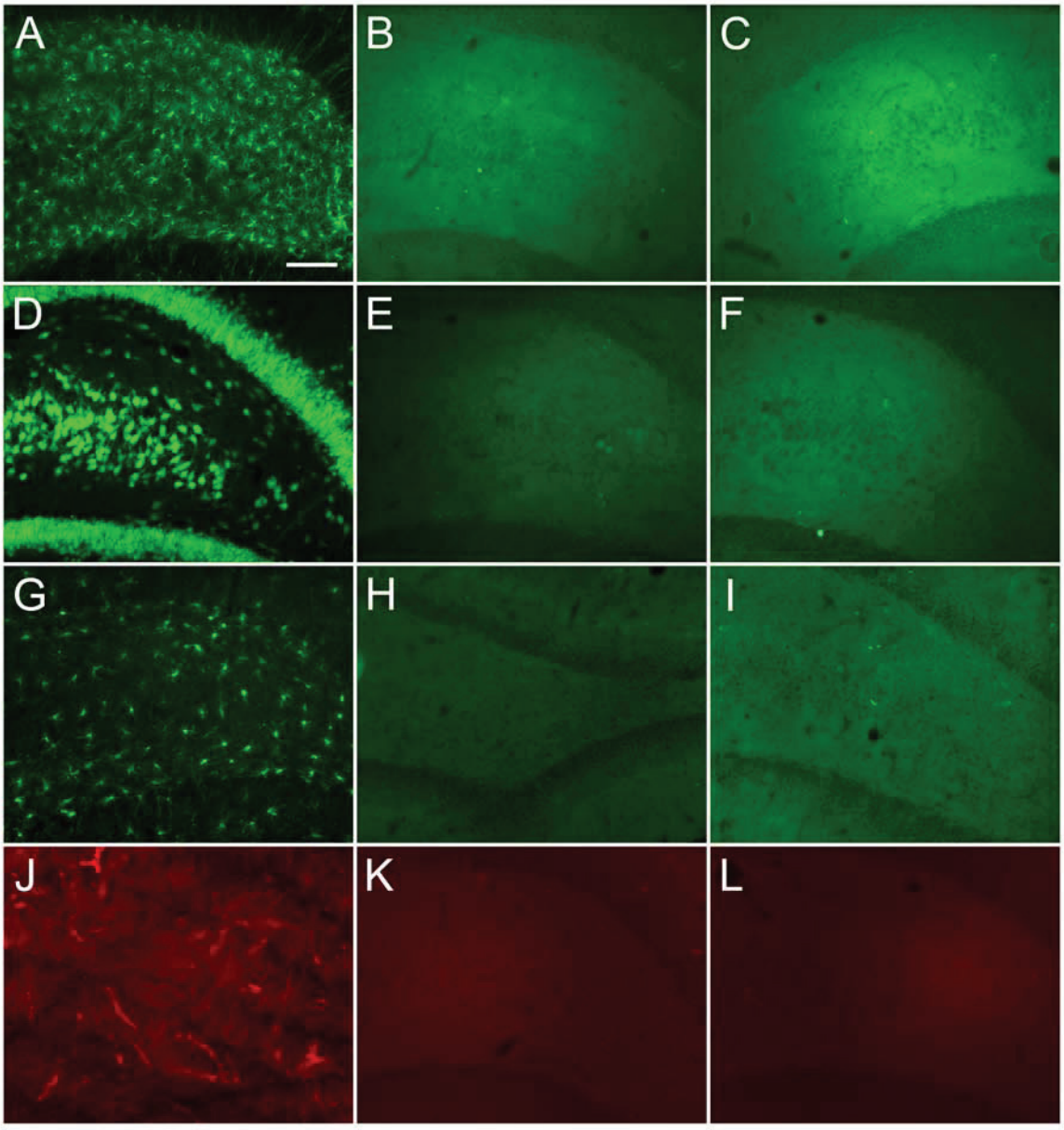

A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.7: Hsp70 and GFAP immunoreactivity in hippocampus of female and male rats. Hsp70 immunoreactivity (red) was undetectable in the hippocampus of control female rats (**A1**) and was detectable in many cells and small blood vessels 24 hours following heat shock in the hippocampus of female rats (**B1**). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus of control male rats (**C1**) and in many cells and small blood vessels 24 hours following heat shock in the hippocampus of male rats (**D1**). GFAP immunoreactivity (green) was detectable in many cells in the hippocampus in control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3, B3, C3, D3**) of Hsp70 and GFAP immunoreactivity show no colocalization of Hsp70 and GFAP. Arrows indicate Hsp70 immunoreactive cells. The scale bar represents 100 microns.

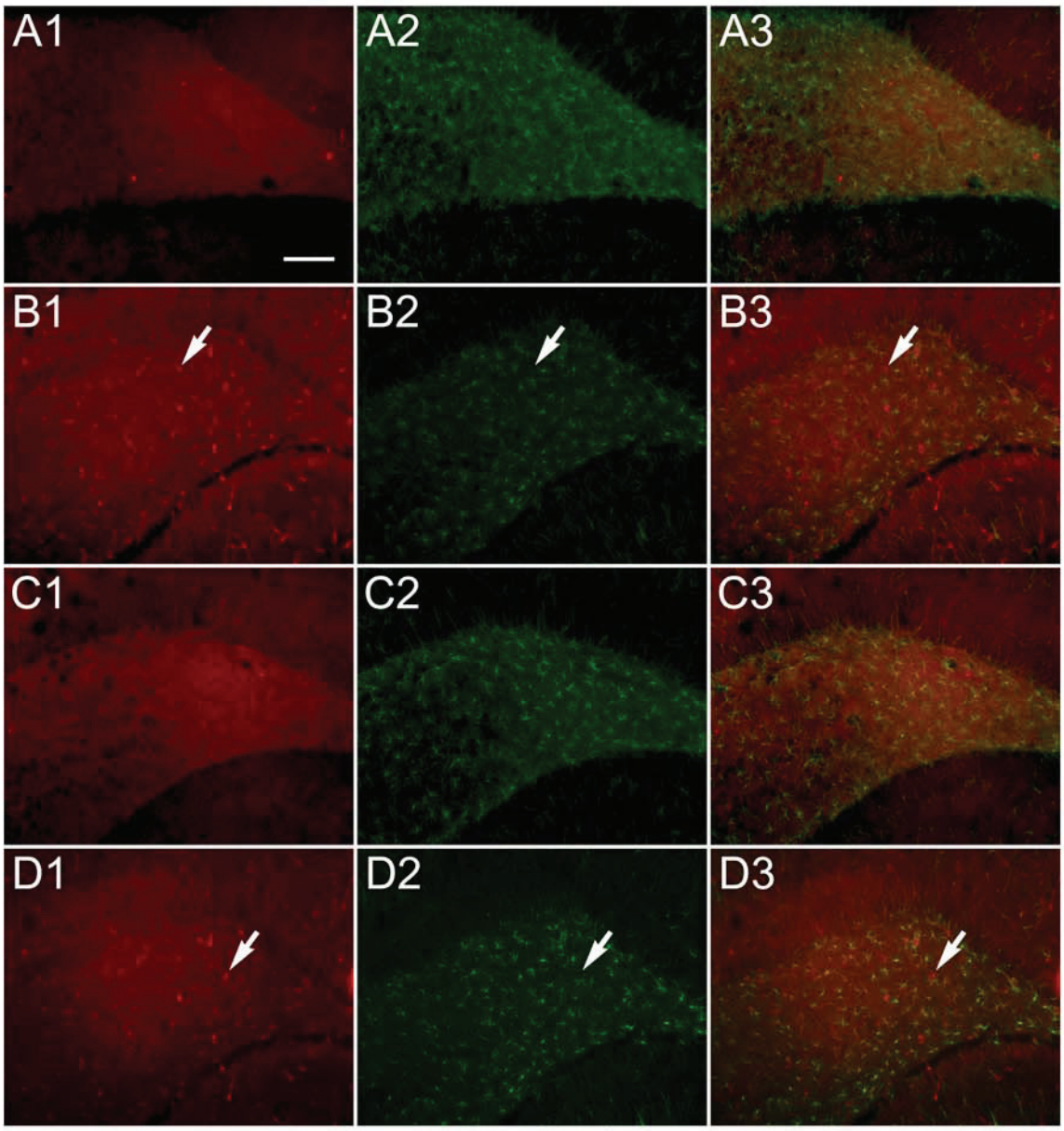

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.8: Hsp70 and NeuN immunoreactivity in the hippocampus of female and male rats. Hsp70 immunoreactivity (red) was undetectable in the hippocampus of control female rats (**A1**) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus of control male rats (**C1**) and in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (**D1**). NeuN immunoreactivity (green) was detectable in many cells in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3, B3, C3, D3**) of Hsp70 and NeuN immunoreactivity show no colocalization of Hsp70 and NeuN. Arrows indicate Hsp70 immunoreactive cells. The scale bar represents 100 microns.

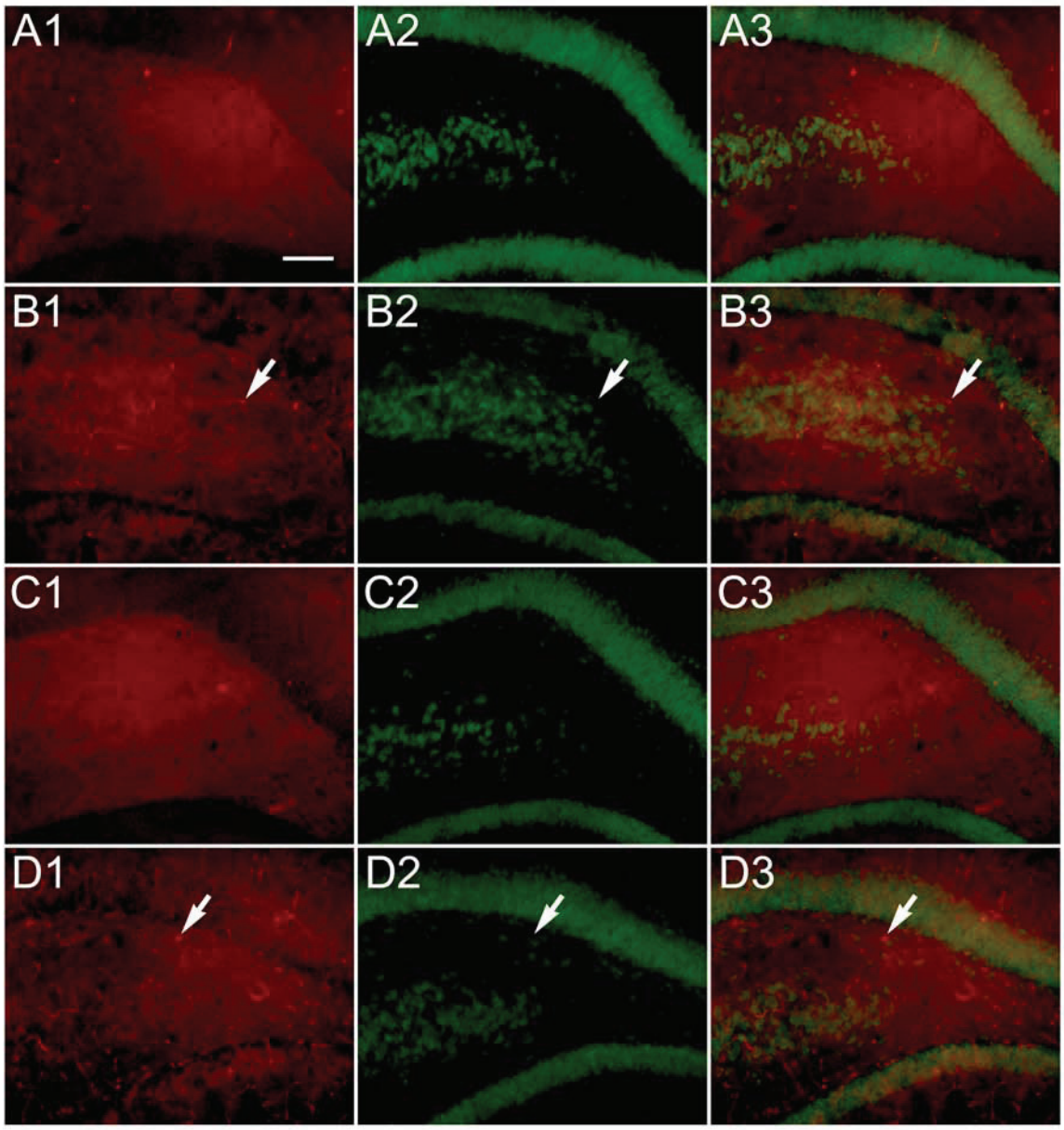

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.9: Hsp70 and Iba1 immunoreactivity in the hippocampus female and male rats. Hsp70 immunoreactivity (red) was undetectable in the hippocampus of control female rats (**A1**) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp70 immunoreactivity was detectable in a few cells of the hippocampus in control male rats (**C1**) and in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (**D1**). Iba1 immunoreactivity (green) was detectable in many cells in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3**, **B3**, **C3**, **D3**) of Hsp70 and Iba1 immunoreactivity show some colocalization of Hsp70 and Iba1. After heat shock in the female, Hsp70 immunoreactivity (**B3**, arrows) was detected mostly in Iba1 negative cells and in some small blood vessels. After heat shock in the male, Hsp70 immunoreactivity (**D3**, arrows) was detected in some Iba1 immunoreactive cells and some small blood vessels. The scale bar represents 100 microns.

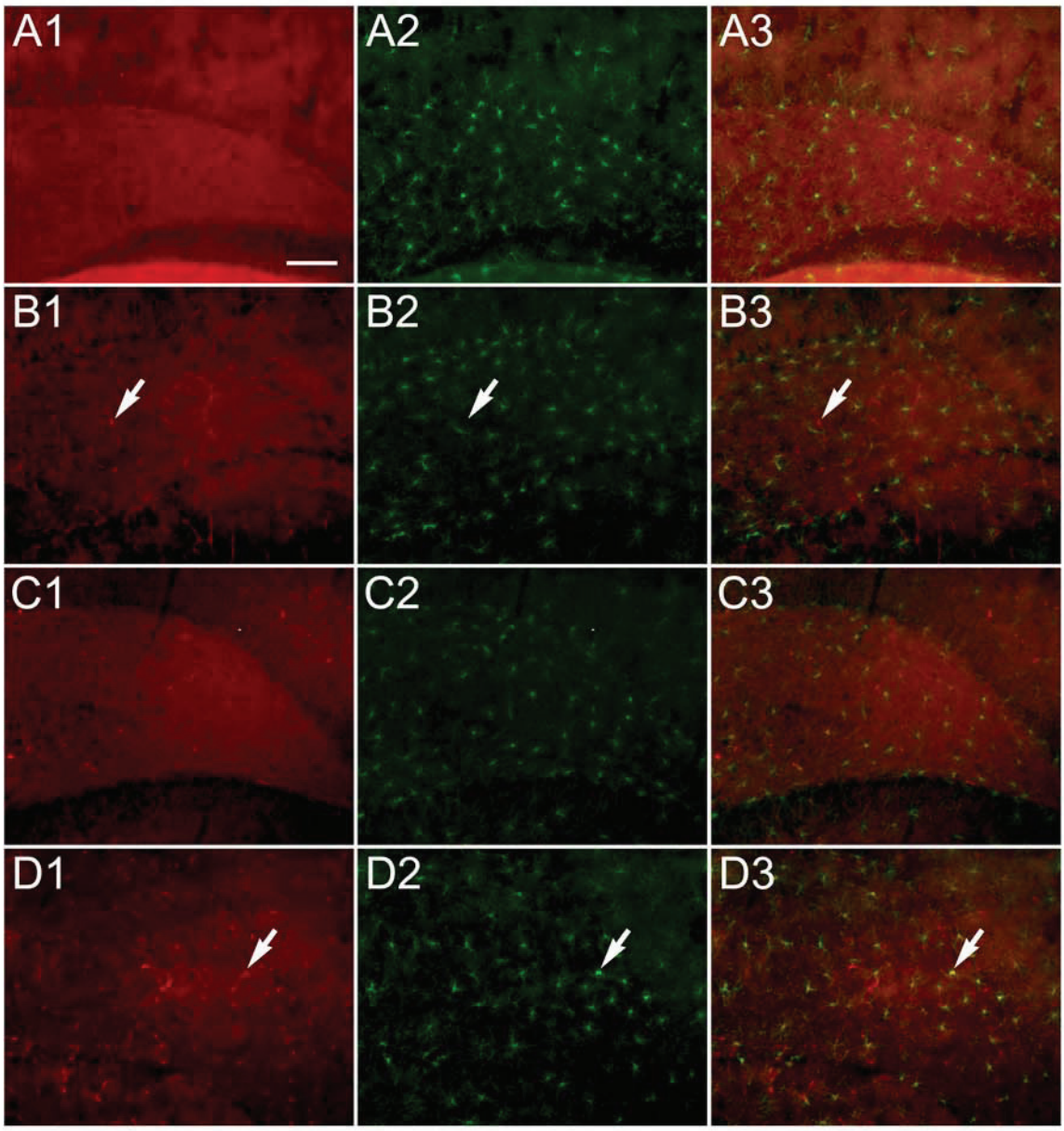

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.10: Hsp70 and Factor VIII immunoreactivity in the hippocampus of female and male rats. Hsp70 immunoreactivity (green) was undetectable in the hippocampus of control female rats (**A1**) and was detectable in many cells and small blood vessels of the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp70 immunoreactivity was not detectable in the hippocampus of control male rats (**C1**) and was detected in many cells and small blood vessels of the hippocampus 24 hours following heat shock in male rats (**D1**). Factor VIII immunoreactivity (red) was detectable in many small blood vessels in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3**, **B3**, **C3**, **D3**) of Hsp70 and Factor VIII immunoreactivity show colocalization of Hsp70 and Factor VIII, particularly in small blood vessels (**B3**, **D3**). Arrows indicate Hsp70 immunoreactive cells that were negative for Factor VIII immunoreactivity. The scale bar represents 100 microns.

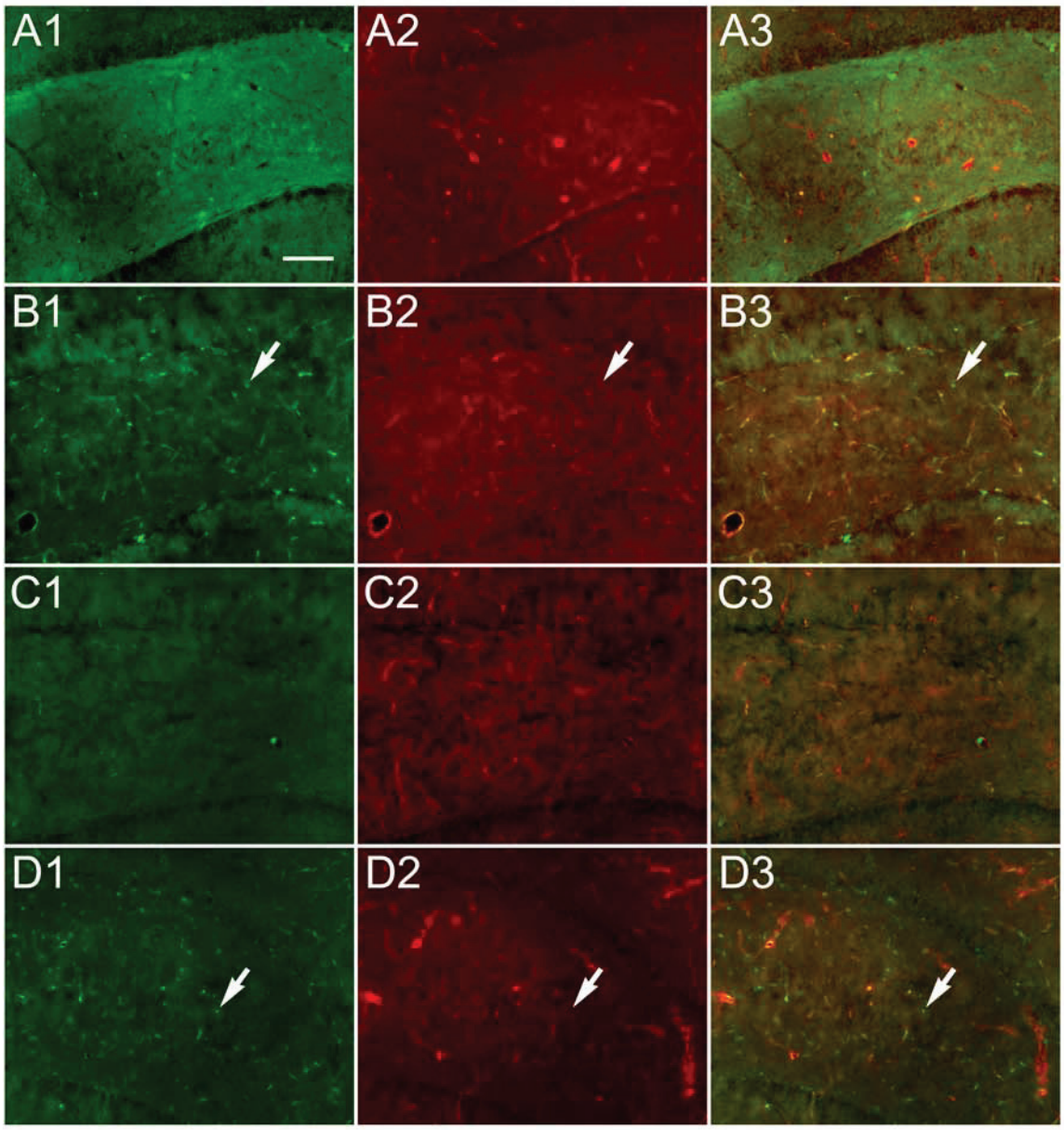

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3. 11: Hsp27 and GFAP immunoreactivity in the hippocampus of female and male rats. Hsp27 immunoreactivity (green) was undetectable in the hippocampus of control female rats (a few fluorescent red blood cells are evident) (**A1**) and was detectable in small blood vessels and many astrocytes in the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp27 immunoreactivity was detectable at a low level in some astrocytes in the hippocampus of control male rats (**C1**) and in some small blood vessels and many astrocytes 24 hours following heat shock in the hippocampus of male rats (**D1**). GFAP immunoreactivity (red) was detectable in many astrocytes in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3, B3, C3, D3**) of Hsp27 and GFAP immunoreactivity show some colocalization of Hsp27 and GFAP. Arrows indicate Hsp27 immunoreactive astrocytes that are also positive for GFAP immunoreactivity. The scale bar represents 100 microns.

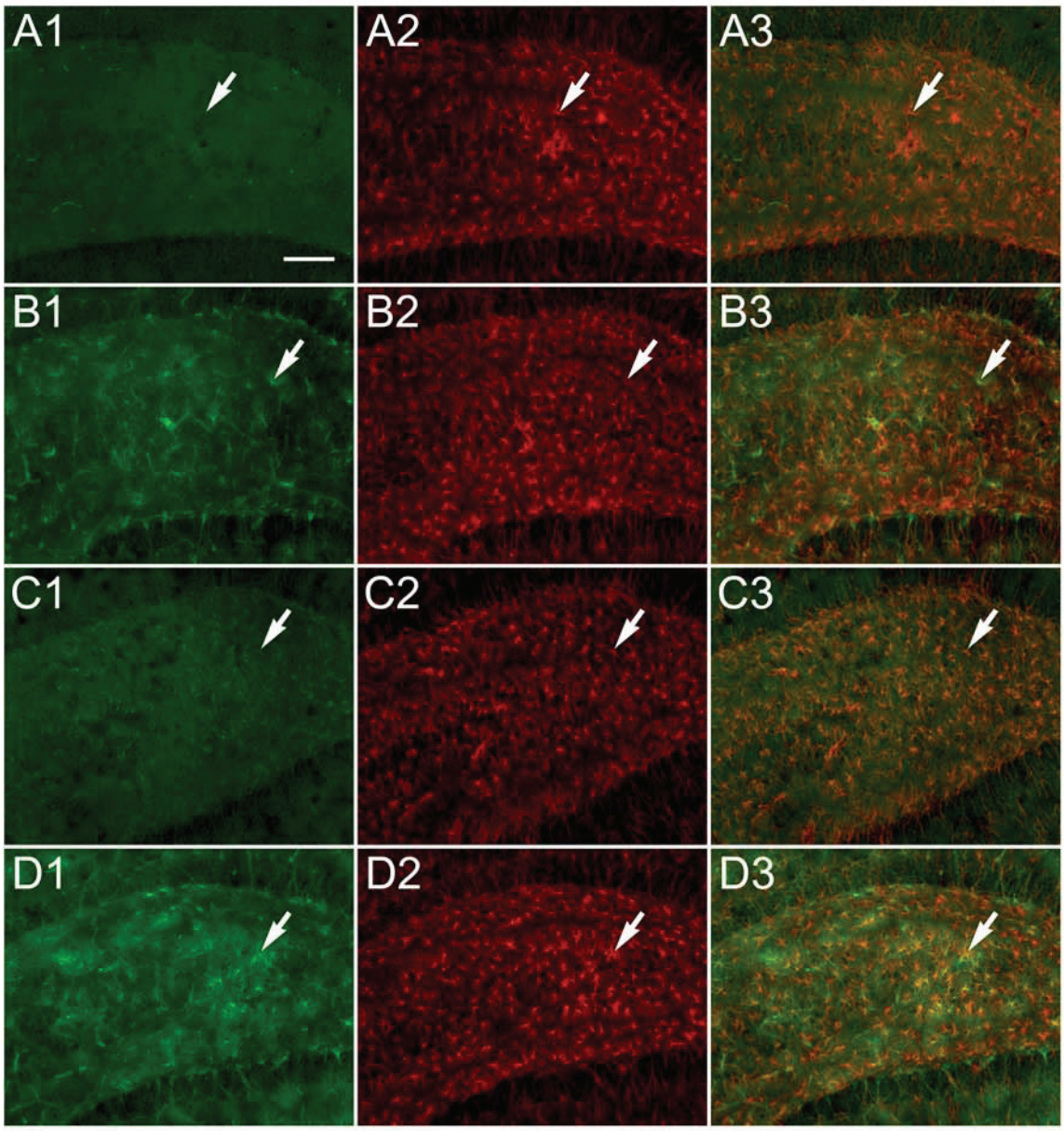

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.12: Hsp27 and NeuN immunoreactivity in the hippocampus of female and male rats. Hsp27 immunoreactivity (green) was undetectable in the hippocampus of control female rats (**A1**) and was detectable in small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp27 immunoreactivity was undetectable in the hippocampus of control male rats (a few fluorescent red blood cells are evident) (**C1**) and was detectable in some small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in male rats (**D1**). NeuN immunoreactivity (red) was detectable in many neurons in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3, B3, C3, D3**) of Hsp27 and NeuN immunoreactivity show no colocalization of Hsp27 and NeuN. Arrows indicate Hsp27 immunoreactive astrocytes. The scale bar represents 100 microns.

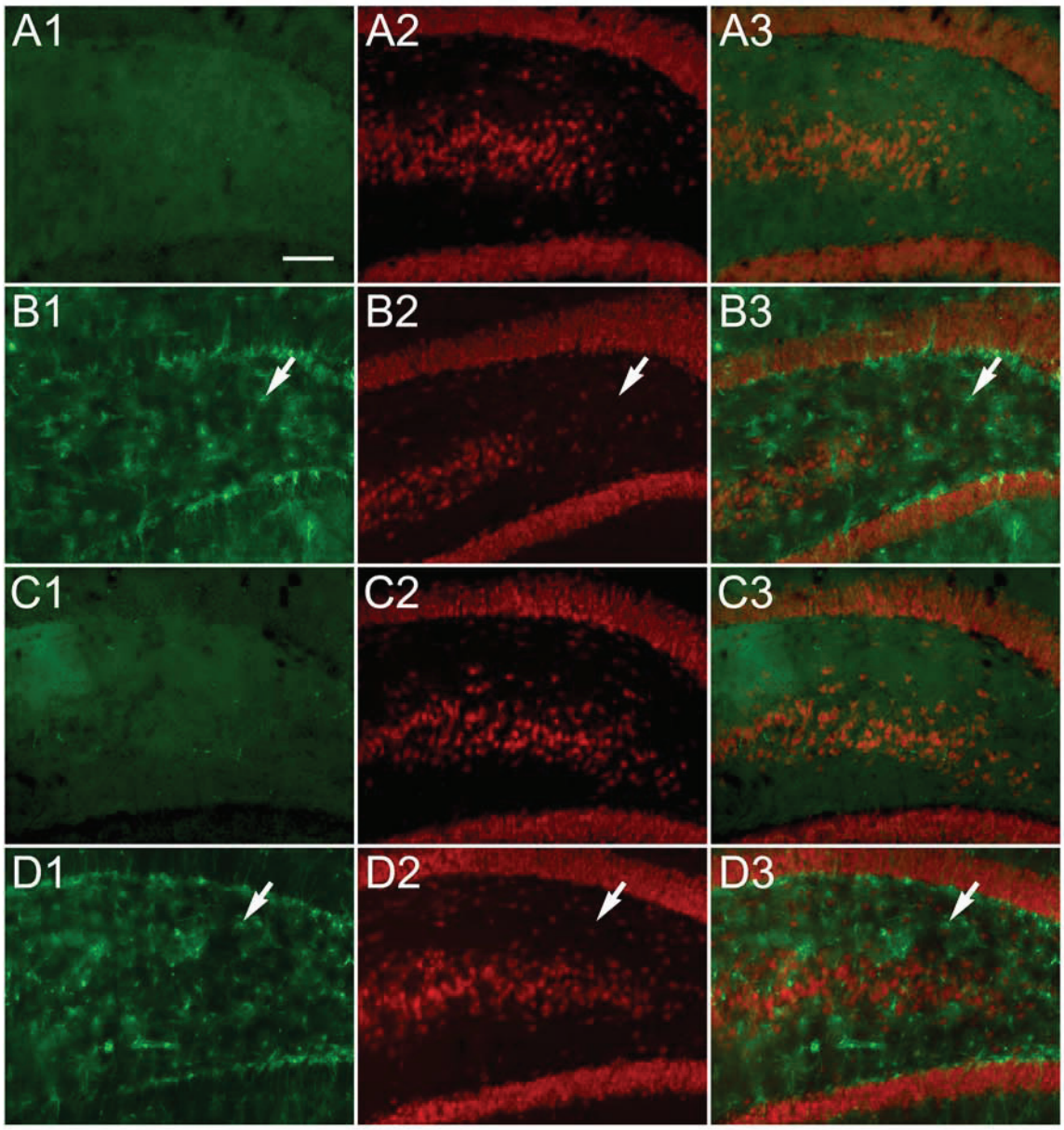

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.13: Hsp27 and Iba1 immunoreactivity in the hippocampus of female and male rats. Hsp27 immunoreactivity (green) was undetectable in the hippocampus of control female rats (a few fluorescent red blood cells are evident) (**A1**) and was detectable in small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp27 immunoreactivity was undetectable in the hippocampus of control male rats (**C1**) and was detected in some small blood vessels and many astrocytes of the hippocampus 24 hours following heat shock in male rats (**D1**). Iba1 immunoreactivity (red) was detectable in many cells in the hippocampus of control female (**A2**), heat shock female (**B2**), control male (**C2**), and heat shock male (**D2**) rats. Merged micrographs (**A3, B3, C3, D3**) of Hsp27 and Iba1 immunoreactivity show no colocalization of Hsp27 and Iba1. Arrows indicate Hsp27 immunoreactive astrocytes. The scale bar represents 100 microns.

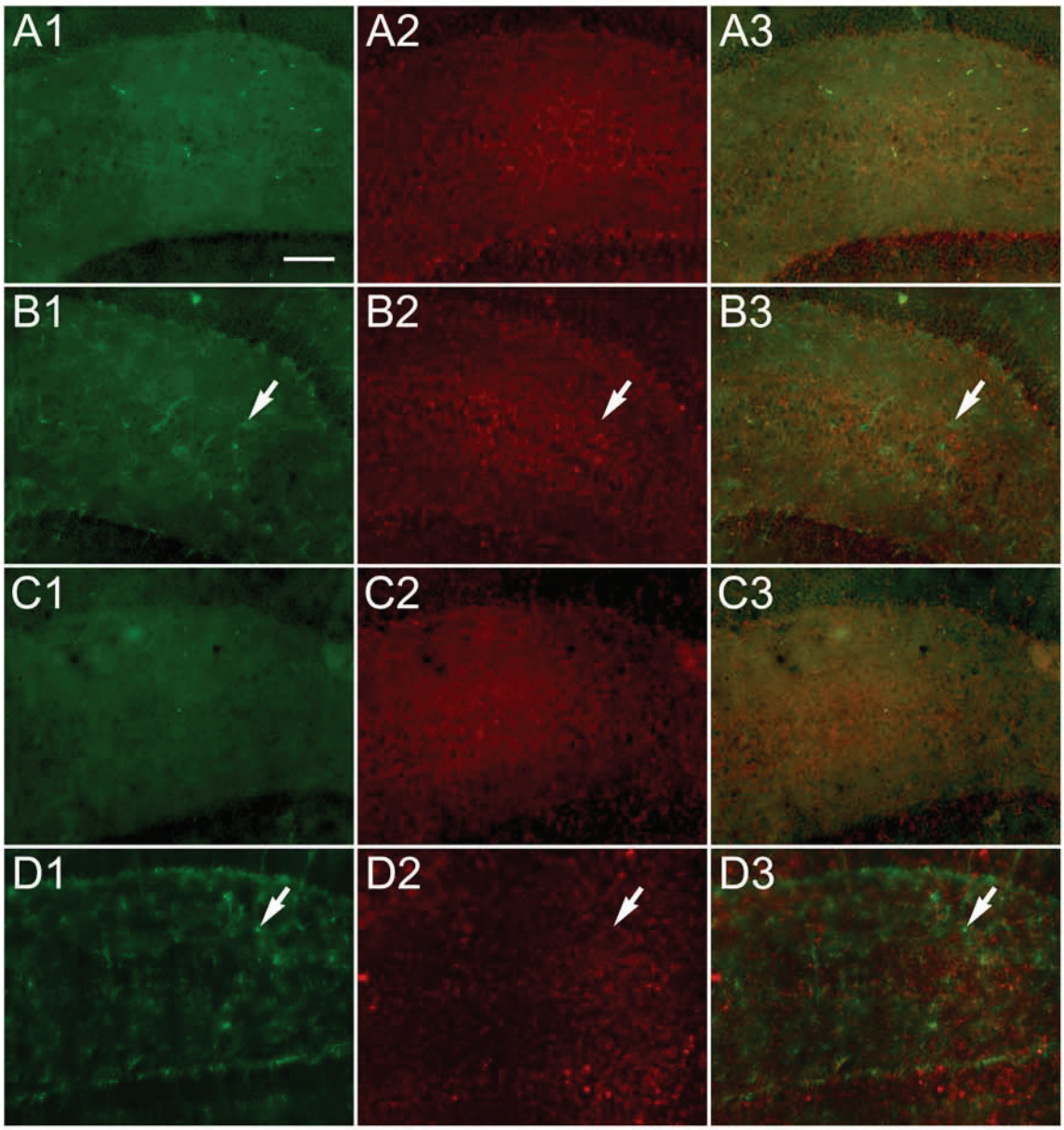

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.14: Hsp27 and Hsp70 immunoreactivity in the hippocampus of female and male rats. Hsp27 immunoreactivity (green) was detectable in a few small blood vessels in the hippocampus of control female rats (**A1**) and was detectable in small blood vessels and many astrocytes in the hippocampus 24 hours following heat shock in female rats (**B1**). Hsp27 immunoreactivity was detectable in a few small blood vessels in the hippocampus of control male rats (**C1**) and was detected in some small blood vessels and many cells in the hippocampus 24 hours following heat shock in male rats (**D1**). Hsp70 immunoreactivity (red) was undetectable in the hippocampus of control female rats (**A2**) and was detectable in many cells and small blood vessels in the hippocampus 24 hours following heat shock in female rats (**B2**). Hsp70 immunoreactivity was undetectable in the hippocampus of control male rats (**C2**) and was detectable in small blood vessels and in many cells in the hippocampus 24 hours following heat shock in male rats (**D2**). Merged micrographs (**A3**, **B3**, **C3**, **D3**) of Hsp27 and Hsp70 immunoreactivity show colocalization of Hsp27 and Hsp70 in small blood vessels and in many cells. Arrows indicate Hsp27 and Hsp70 immunoreactive cells. The scale bar represents 100 microns.

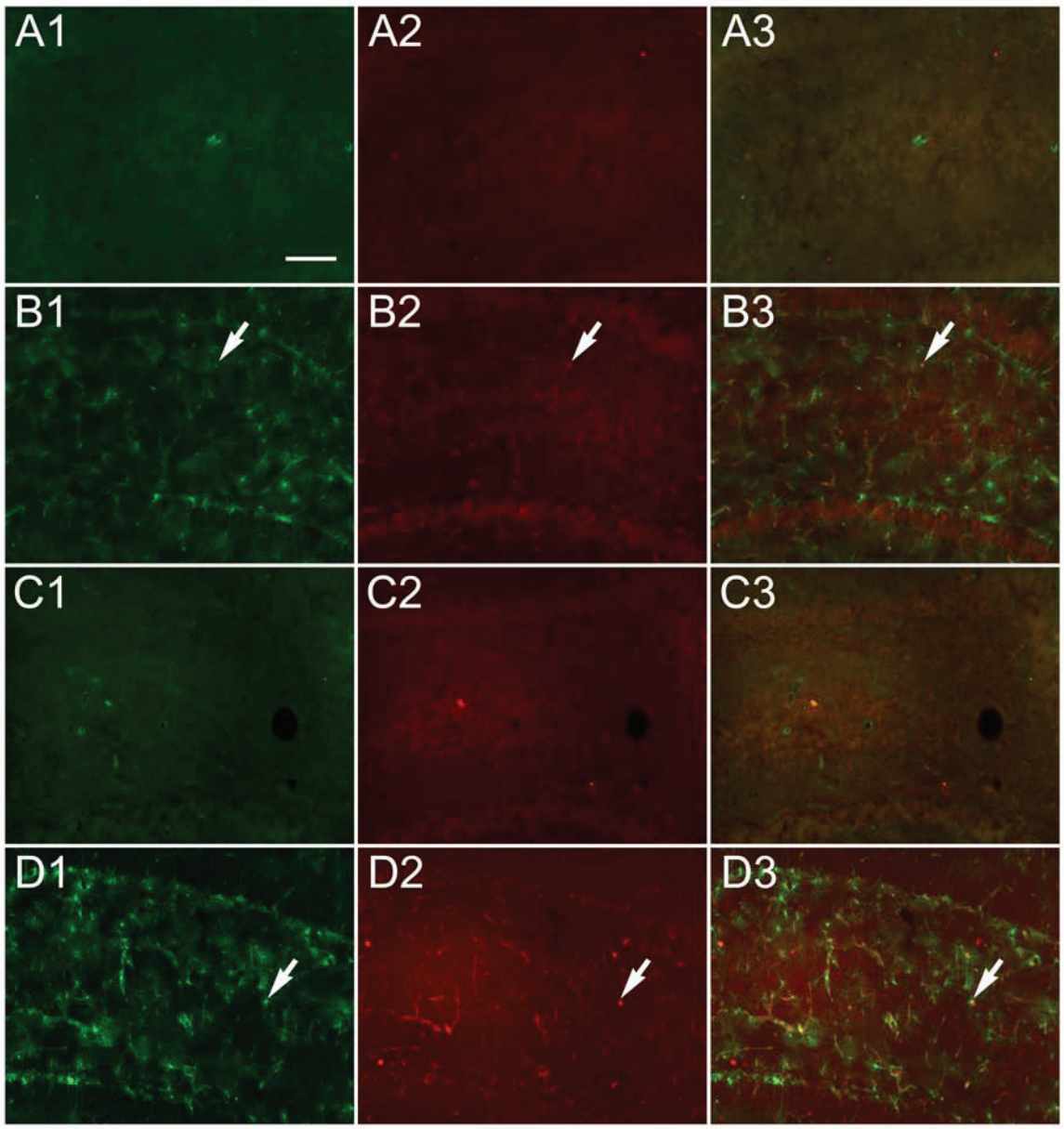

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 3.15: Western analysis of Hsp70 and Hsp27 levels in the hearts of control rats. Rats were perfused 24 hours following control treatment. No Hsp70 was detected in heart of both control female and control male rats (**A**). Hsp27 was detectable in the heart at similar levels in both control female and control male rats (**A**). From left to right on the membrane, control female rats were in the following stages of the estrous cycle: metaestrus, metaestrus, diestrus, metaestrus, and diestrus. Statistical analysis revealed no significant differences between Hsp70 (**B**) and Hsp27 (**C**) levels in hearts of control female ($n = 5$) and male ($n = 5$) rats ($p > 0.05$). Data is presented as mean ± SEM.

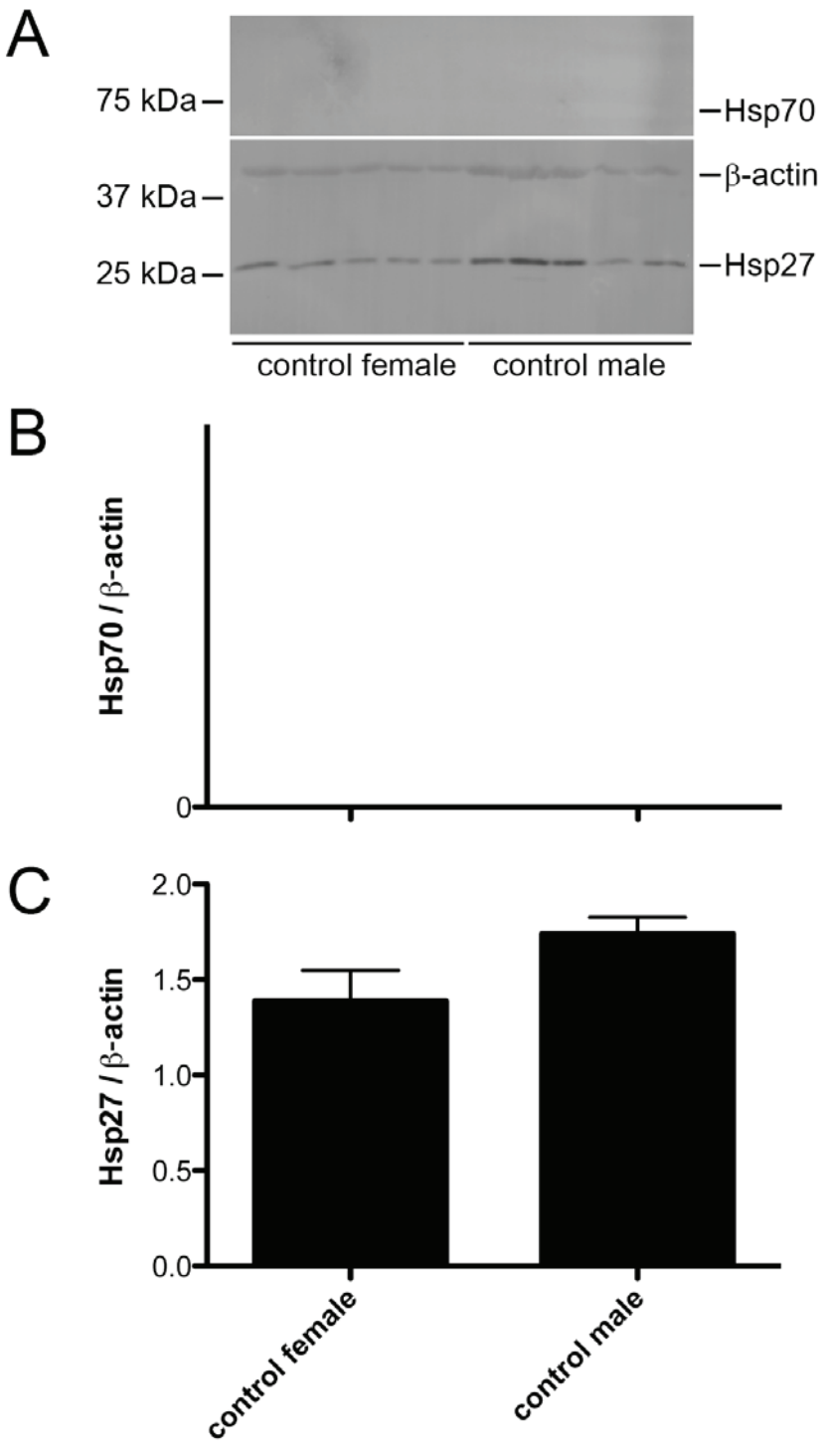

A
75 kDa
37 kDa
25 kDa
Hsp70
β-actin
Hsp27
control female
control male
B
Hsp70 / β-actin
0
C
Hsp27 / β-actin
2.0
1.5
1.0
0.5
0.0
control female
control male

Figure 3.16: Western analysis of Hsp70 and Hsp27 levels in the hearts of control and heat shock female rats. Rats were perfused 24 hours following control treatment and 24 hours following heat shock treatment. Hsp70 and Hsp27 levels appeared to be increased in the hearts 24 hours following heat shock treatment (**A**). From left to right on the membrane (**A**), control female rats are in the following stages of the estrous cycle: metaestrus, metaestrus, diestrus, metaestrus, and diestrus, and heat shock female rats are in: estrus, proestrus, estrus, estrus, metaestrus, and proestrus**.** Statistical analysis revealed significant differences in Hsp70 (*, $p < 0.05$) (**B**) and Hsp27 (***, $p < 0.001$) (**C**) levels in the hearts of control female ($n = 5$) and heat shock female ($n = 6$) rats. Data are presented as mean ± SEM.

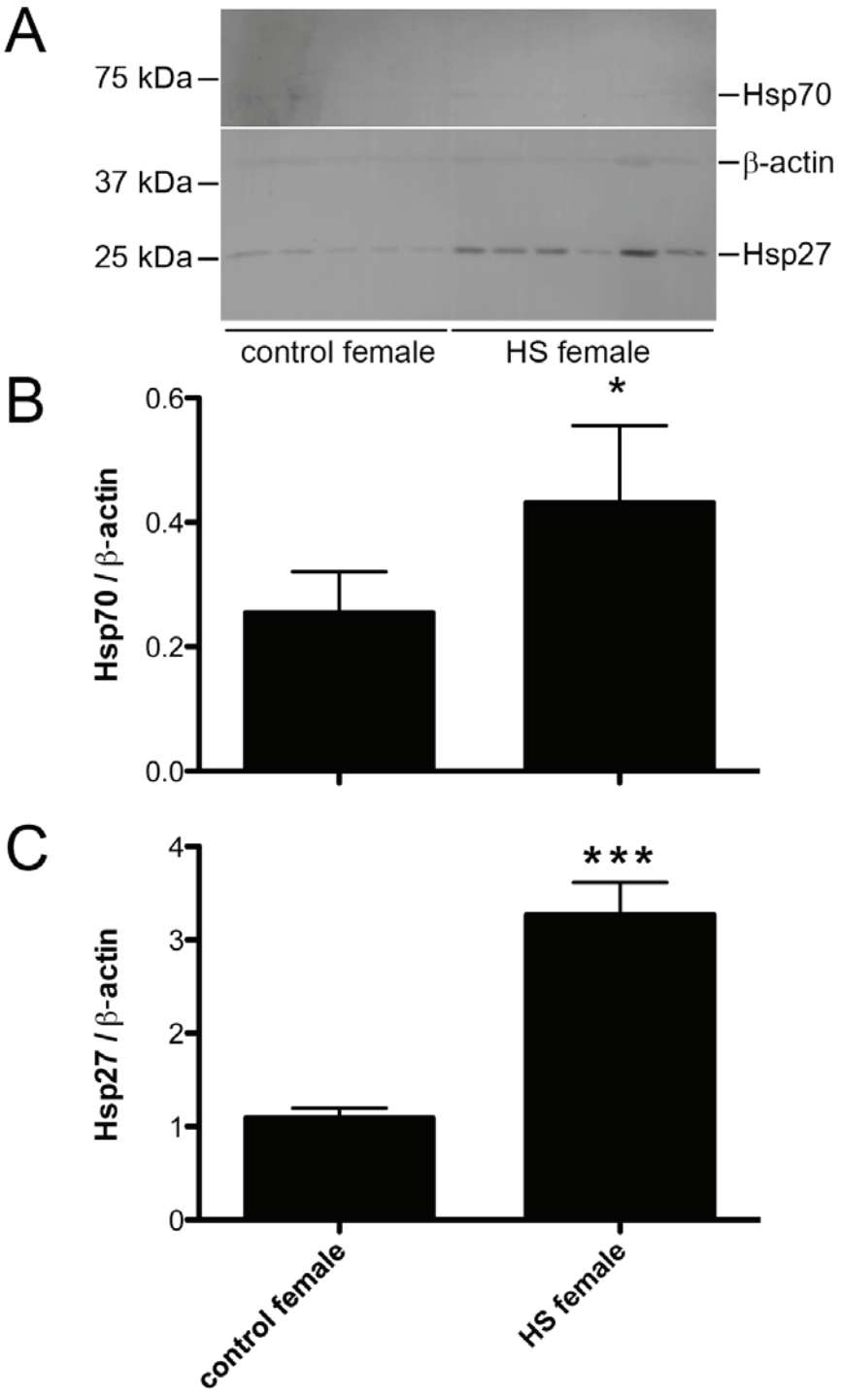

A
75 kDa
37 kDa
25 kDa
Hsp70
β-actin
Hsp27
control female
HS female
B
Hsp70 / β-actin
0.6
0.4
0.2
0.0
*
C
Hsp27 / β-actin
4
3
2
1
0
***
control female
HS female

Figure 3.17: Western analysis of Hsp70 and Hsp27 levels in the hearts of control and heat shock male rats. Rats were perfused 24 hours following control treatment and 24 hours following heat shock treatment. Hsp70 and Hsp27 levels appeared to be increased in the heart 24 hours following heat shock treatment (**A**). Statistical analysis revealed significant differences in Hsp70 (*, $p < 0.05$) (**B**) and Hsp27 (**, $p < 0.01$) (**C**) levels in the hearts of control male ($n = 5$) and heat shocked male ($n = 6$) rats. Data are presented as mean ± SEM.

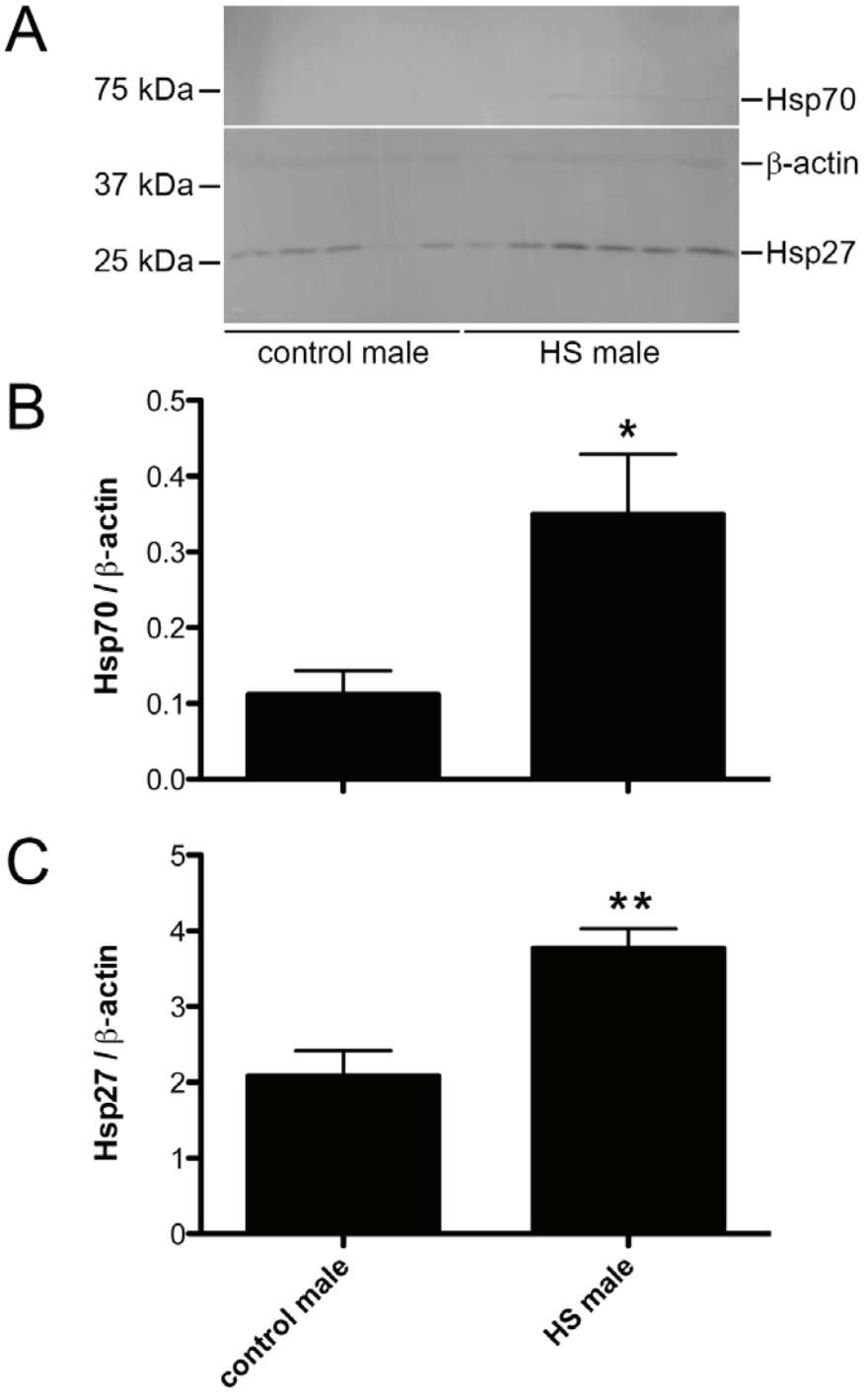
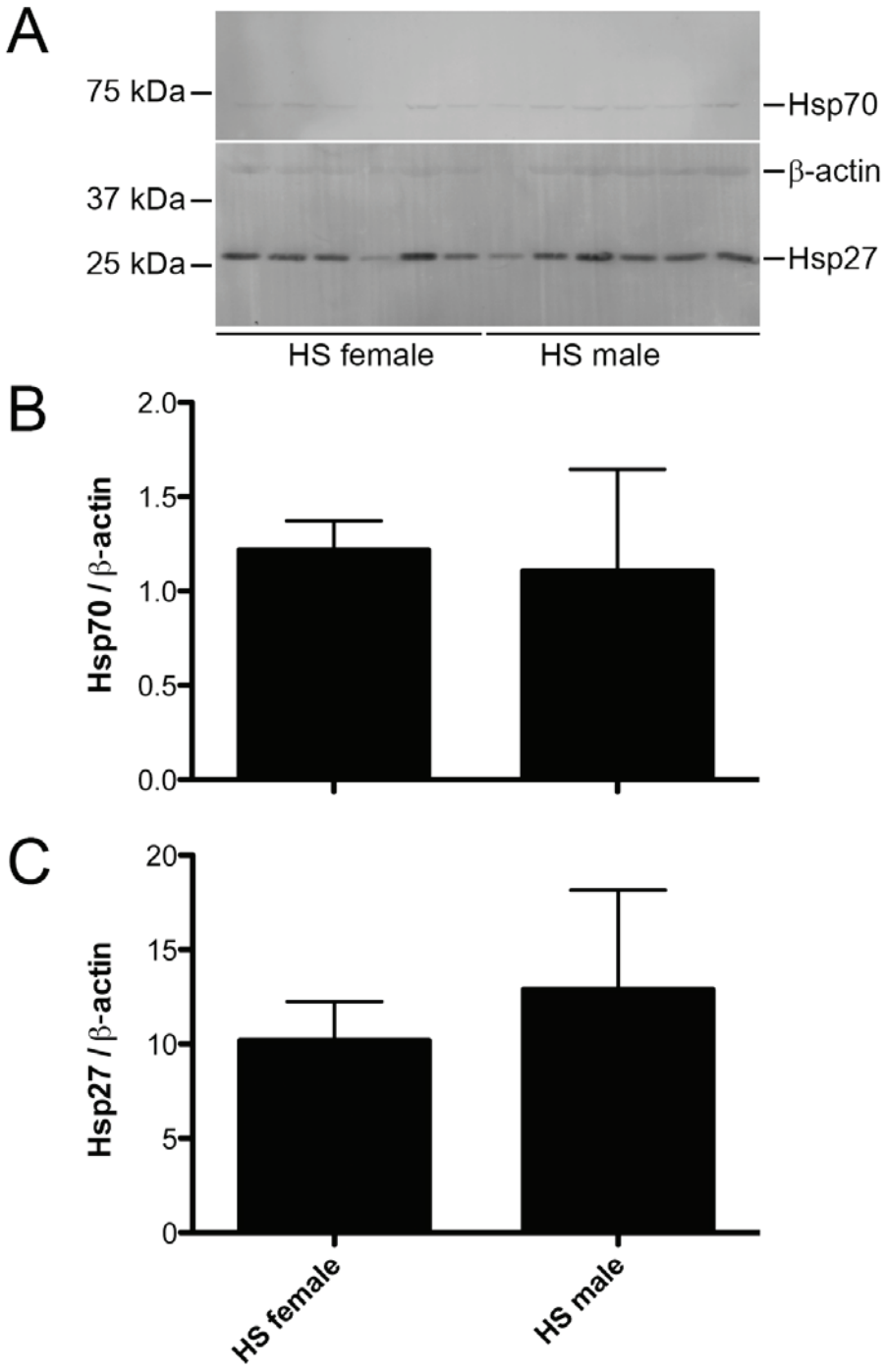

A
75 kDa
37 kDa
25 kDa
Hsp70
β-actin
Hsp27
control male
HS male
B
Hsp70 / β-actin
0.5
0.4
0.3
0.2
0.1
0.0
*
C
Hsp27 / β-actin
5
4
3
2
1
0
**
control male
HS male

Figure 3.18: Western analysis of Hsp70 and Hsp27 levels in the hearts of heat shock female and heat shock male rats. Rats were perfused 24 hours following heat shock treatment. Hsp70 and Hsp27 levels were easily detectable 24 hours following heat shock treatment in the hearts (**A**). From left to right on the membrane (**A**) heat shock female rats are in the following stages of the estrous cycle: estrus, proestrus, estrus, estrus, metaestrus, and proestrus**.** Statistical analysis revealed no difference in Hsp70 (**B**) or Hsp27 (**C**) levels in the hearts of female (n = 6) and male (n = 6) rats. Data are presented as mean ± SEM.


A
75 kDa
37 kDa
25 kDa
Hsp70
β-actin
Hsp27
HS female
HS male
B
Hsp70 / β-actin
2.0
1.5
1.0
0.5
0.0
C
Hsp27 / β-actin
20
15
10
5
0
HS female
HS male

Discussion

The major findings in this thesis are that: 1) In the hippocampus, Hsp70 and Hsp27 levels are significantly greater in male rats, compared to levels in female rats, 24 hours after heat shock treatment. 2) In the hippocampus, Hsp70 is colocalized in microglia and blood vessels, Hsp27 is colocalized in astrocytes, and Hsp70 and Hsp27 are closely associated in blood vessels and astrocytes, respectively, in female and male rats 24 hours after heat shock treatment. 3) In the heart, Hsp70 and Hsp27 levels are similar in female and male rats, at baseline and 24 hours after heat shock treatment. These results are important for understanding sex differences in neurodegenerative and cardiovascular disease prevalence.

Hsp70 and Hsp27 expression in the hippocampi of control rats

In the absence of stress, Hsp70 and Hsp27 are expressed at low levels in the brain. In this study, western analyses indicate that Hsp70 levels in the hippocampus are similar in control female male rats (Figure 3.1B). This finding is consistent with previous studies (Olazabal *et al.,* 1992a; Thorp *et al*., 2007) and supports the notion that there are no sex differences in the baseline levels of Hsp70 in the hippocampus of female and male rats. The low level of Hsp70 observed in the hippocampus of control animals is also similar to that seen in other studies done exclusively with male rats (Currie and White, 1983; David *et al.,* 1994; Armstrong *et al.,* 1996; Krueger *et al.,* 1999).

In the present study, Hsp27 levels in the hippocampus were not different between control female rats and control male rats (Figure 3.1 C). This finding appears to be the first comparison of constitutive Hsp27 levels in the hippocampus of female and male rats.

The low level of Hsp27 observed in the hippocampus of control animals is also similar to that seen in other studies done exclusively with male rats (Plumier *et al.,* 1996; 1997a; 1997b; Krueger-Naug *et al*., 2000). While Hsp27 is at low levels in the hippocampus, Hsp27 is abundant in the brain stem and spinal cord in control male rats (Plumier *et al.,* 1997b).

Hsp70 and Hsp27 expression in the hippocampi of heat shock rats

In the current study, hippocampal Hsp70 levels are greater in both the female (Figure 3.2 B) and male (Figure 3.3 B) rats compared control rats, 24 hours after heat shock treatment and hippocampal Hsp70 and Hsp27 levels are greater in male rats, compared to female rats, 24 hours after heat shock treatment (Figure 3.4 B). In fact, the highly inducible nature of Hsp70 in the brain or other tissues has been examined for many years (Schlesinger *et al.,* 1982; Currie & White, 1983; David *et al*., 1994; Armstrong *et al.,* 1996; Krueger *et al.,* 1999; Bechtold *et al.,* 2000). While these studies examined male rats, it is also clear that Hsp70 is inducible to high levels in the hippocampus of female rats (Pavlik *et al.,* 2003).

In my study, Hsp70 hippocampal induced levels were significantly greater in male rats, compared to female rats. (Figures 3.4 B). Similarly, following an acute stressor, such as tail shock, Hsp70 levels are significantly greater in the hippocampus in male rats, compared to female rats. Although I did not measure E2 plasma levels to compare to Hsp levels, Nickerson *et al*. (2006) found that the estrous cycle had no impact on induced levels of Hsp70 in the brain of female rats.

While Hsp70 is at low levels in the rat brain, the constitutively expressed Hsc70 is abundant in the brain (Manzerra *et al.,* 1997). Hsc70 has similar chaperone functions as Hsp70 and likely regulates HSF1. The lower levels of induced Hsp70 observed in the hippocampus of female rats may be related to higher levels of Hsc70 in the absence of stress (Paroo *et al.,* 1999). Hsp70 and Hsc70 may be involved in the interaction between estrogen and its receptor, since both Hsp70 and Hsc70 have been found to bind E2 (Gacad & Adams, 1998).

Hsp27 is expressed at high levels in the hippocampus after heat shock in both female (Figures 3.2 C) and male (Figures 3.3 C) rats and it is clear that Hsp27 expression is greater in males than in females after heat shock (Figures 3.4 C). My finding of the highly inducible nature of Hsp27 after heat shock is similar to other studies done with male rats (Krueger-Naug *et al*., 2000; Bechtold & Brown, 2003). My finding of greater expression of Hsp27 in the hippocampus of male rats after heat shock appears to be the first comparison of inducible Hsp27 levels in female and male rats.

Antibody specificity

Negative and positive Hsp70 immunofluorescence in the hippocampus of control male (Figure 3.5 A) and heat shock male (Figure 3.5 B) rats, respectively, suggests the immunoreactivity is specific for the inducible Hsp70. The negative immunofluorescence for the no primary antibody (Figure 3.5 C) and no secondary antibody (Figure 3.5 D) also suggest the specific nature of the immunoreaction for Hsp70.

Finding Hsp27 immunofluorescence in a few cells in the hippocampus of a control male rat (Figure 3.5 E) and many cells after heat shock in a male rat (Figure 3.5

F) suggests the immunoreactivity is specific for Hsp27. The negative immunofluorescence for the no primary antibody (Figure 3.5 G) and no secondary antibody (Figure 3.5 H) also suggest the specific nature of the immunoreaction for Hsp27.

GFAP immunofluorescence in the hippocampus of heat shock male rat (Figure 3.6 A) is specific for the glial acidic fibrillary protein in astrocytes, as suggested by the no primary antibody (Figure 3.6 B) and no secondary antibody (Figure 3.6 C) negative immunoreaction.

NeuN immunofluorescence in the hippocampus of heat shock male rat (Figure 3.6 D) is specific for the neuronal nuclei in neurons, as indicated by the no primary antibody (Figure 3.6 E) and no secondary antibody (Figure 3.6 F) negative immunoreaction.

Iba1 immunofluorescence in the hippocampus of heat shock male rat (Figure 3.6 G) is specific for the ionized calcium binding adapter molecular protein in microglia, as indicated by the no primary antibody (Figure 3.6 H) and no secondary antibody (Figure 3.6 I) negative immunoreaction.

Factor VIII immunofluorescence in the hippocampus of heat shock male rats (Figure 3.6 J) is specific for the factor VIII protein in and around blood vessels, as indicated by the no primary antibody (Figure 3.6 K) and no secondary antibody (Figure 3.6 L) negative immunoreaction.

Hsp70 and Hsp27 immunofluorescence in the hippocampus of rats

The low level of Hsp70 immunofluorescence in the hippocampus of control female and control male rats (Figures 3.7 - 3.11) is similar to that seen in previous studies

(Armstrong *et al.,* 1996; Pavlik *et al.,* 2003). By immunofluorescence, Hsp70 is not detected in control female rats (Pavlik *et al.,* 2003) and by immunohistochemistry Hsp70 is not detected in control male rats (Armstrong *et al.,* 1996). Hsp70 immunofluorescence 24 hours after heat shock treatment in female and male rat hippocampi is detectable and is colocalized with Iba1 (Figure 3.9 B and D) and with Factor VIII (Figure 3.10 B and D) immunofluorescence, which is indicative of immunoreactivity in microglia and blood vessels, respectively (Theilen & Kuschinsky, 1992; Maeda *et al*., 2008). Hsp70 was not however colocalized with either GFAP (Figure 3.7 B3 and D3) or NeuN (Figure 3.8 B3 and D3) immunofluorescence and is similar to previous findings (Lee *et al*., 2002).

My finding of Hsp70 localization in microglia and blood vessels is similar to previous studies showing Hsp70 colocalized in microglia, oligodendrocytes and blood vessels, using OX-42 antibody, GSII lectin antibody, and histological morphology, respectively (Pavlik *et al.,* 2003). Similarly, Hsp70 has also been localized in vimentin-positive glia (astrocytes) in the hippocampus of male rats after heat shock (Krueger *et al*., 1999).

In this study, Hsp27 immunofluorescence in the hippocampus of control female and control male rats was at low levels (Figures 3.11 - 3.14). This finding is similar to previous work done with only male animals (Plumier et al., 1996; 1997; Krueger-Naug *et al.,* 2000). Following heat shock, Hsp27 immunofluorescence is detectable and is colocalized with GFAP immunofluorescence (Figure 3.11 B3 and D3). Hsp27 immunofluorescence is not colocalized with NeuN or Iba1 immunofluorescence (Figure 3.12 or 3.13). Colocalization of Hsp27 with GFAP is indicative of immunoreactivity in astrocytes (Plumier *et al*., 1997a; Lee *et al*., 2002; Bechtold & Brown, 2003). Hsp27

appears not to be localized in neurons or microglia as indicated by the NeuN and Iba1 immunofluorescence, respectively (Lee *et al*., 2002; Maeda *et al*., 2008).

My finding of Hsp27 immunoreactivity in astrocytes in both female and males is similar to previous studies that were done in male animals only (Krueger-Naug *et al.,* 2000; Bechtold & Brown, 2003). My study appears to the first showing localization of Hsp27 in astrocytes in the female hippocampus.

Following heat shock, Hsp27 immunofluorescence is associated with some Hsp70 immunofluorescence in the hippocampus of female and male rats 24 hours after heat shock (Figure 3.14 B and D). The male rats have greater levels of both Hsp70 and Hsp27, compared to the female rats, 24 hours after heat shock treatment (Figure 3.14 B & D). As well, Hsp70 colocalized with microglia and in small blood vessels, Hsp27 is in astrocytes, and Hsp27-expressing astrocytes are in close proximity to Hsp70-expressing blood vessels, which was evident in the hippocampus of both females and males 24 hours after heat shock treatment (Figure 3.14). The resulting cellular damage within the blood vessels 24 hours following heat shock treatment likely induced the expression of Hsp70. Normally, astrocytes associate with endothelial cells in blood vessels in the brain to help protect the blood brain barrier, especially following stress (Bechtold & Brown, 2003). Heat shock treatment likely caused increased permeability in the endothelial cells and cellular damage. As a result, Hsp27-expressing astrocytes in association with the Hsp70-expressing endothelial cells in the blood vessel wall may be facilitating restoration of homeostasis following heat stress (Plumier *et al.,* 1996; Krueger-Naug *et al.,* 2000; Pavlik *et al.,* 2003).

Heat shock triggers thermal and osmotic stressors that induce protein instability and aggregation, leading to activation of the HSF1 in order to maintain protein homeostasis (Cotto & Morimoto, 1999). For example, heat shock triggers hypo-osmotic stress in mammalian cells leading to activation of HSF1 and iso-osmotic conditions restore activated HSF1 to normal levels (Caruccio *et al.,* 1997). Hsp27 levels are also induced following stress (heat shock, ischemia, etc.) and is a good marker of reactive gliosis (Plumier *et al.,* 1997a). Elevated expression of Hsp27 after heat shock facilitates protein repair and actin filament reorganization and stabilization (Lavoie *et al.,* 1995) and prevents apoptosis through inhibition of caspase activity (Garrido *et al.,* 1999).

Hsp70 and Hsp27 expression in the hearts of control rats

In control rats, cardiac Hsp70 levels were low. In this study, western analysis indicates that Hsp70 levels in the heart are similar in control female rats and control male rats (Figure 3.15 B) and this result is similar to that of a previous study comparing hearts of female and male rats (Thorp *et al*. 2007). In contrast, other research has shown sex differences in the expression of cardiac Hsp70 levels in control animals (Voss *et al.,* 2003). Western analysis revealed that Hsp70 expression in control female rat hearts is significantly greater, compared to the levels of Hsp70 in control male rat hearts (Voss *et al.,* 2003). While there may be sex differences in expression of Hsp70 in the hearts of control animals, it is clear that the cardiac level of Hsp70 in control male animals is low (Currie and White, 1983; Currie *et al.,* 1988; Karmazyn *et al.,* 1990; Plumier *et al.,* 1995; Leger *et al.,* 2000; Thorp *et al*. 2007).

In the present study, Hsp27 levels in hearts are detectable and are not significantly different between control female rats and control male rats (Figure 3.15 C). This finding appears to be in contrast to previous work showing that Hsp27 in control male rat hearts was significantly greater, compared to the levels of Hsp27 in control female rat hearts (Voss *et al.,* 2003) but in fact in my study, the trend was that control male hearts had more Hsp27 than the control female hearts. The level of Hsp27 observed in the hearts of control animals is also similar to that seen in other studies done exclusively with male rats (Leger *et al.,* 2000).

Hsp70 and Hsp27 expression in the hearts of heat shock rats

In the present study, Hsp70 is expressed at high levels 24 hours after heat shock treatment in both female (Figure 3.16 B) and male (Figure 3.17 B) hearts compared to control rats. The induced expression of Hsp70 in female and male hearts appears to be at approximately equal levels (Figures 3.18 B). This observation in my study is in contrast to male rats having significantly greater expression of Hsp70 in the heart, compared to hearts of female rats, after exercise training on a treadmill (Paroo *et al.,* 1999). Whether by exercise or heat shock, the highly inducible level of Hsp70 in the heart has been observed before (Schlesinger *et al.,* 1982; Currie & White, 1981; 1983; Currie *et al.,* 1988; Karmazyn *et al.,* 1990; Plumier *et al.,* 1995; Hoch *et al*., 1996; Shinohara *et al.,* 2004; Lam *et al.,* 2012). While these studies examined male rats, it is also clear that Hsp70 is inducible to high levels in the heart of female rats (Thorp *et al.,* 2007).

In my study, Hsp27 is expressed at high levels in the heart after heat shock in both female (Figures 3.16 C) and male (Figures 3.17 C) rats and Hsp27 expression is greater

in males than in females after heat shock (Figures 3.18 C). My finding of the inducible nature of Hsp27 in the heart after heat shock is similar to other studies done with male rats (Currie and Tanguay, 1991; Hoch *et al.,* 1996; Leger *et al.*, 2000). Similarly to male rats, female rats have induced cardiac Hsp27 levels compared to control rats, however the induction of Hsp27 levels may be less in female rats, compared to male rats. The lower level of induction of Hsp27 in female rats hearts may be related to other factors associated with the activation of HSF1, such as E2 plasma levels, levels of Hsp90 and cortisol levels. These factors are different in females and males (Massafra *et al.,* 2002; Li & Sanchez, 2005; Elakovic *et al.,* 2007) and any may modulate the heat shock response.

Effects of estrogen on expression of HSP

An obvious difference between females and males is the levels of circulating estrogen. Women have approximately 2 to 4 times greater plasma levels of E2, compared to men (Massafra *et al.*, 2002) and plasma levels of E2 in female rats is also greater compared to male rats (Pratchayasaku *et al.,* 2011). E2 functions by binding to ER-α or ER-β. Interestingly, the density and distribution of ER-α and ER-β are not different in female and male rat hearts (Grohé *et al.,* 1997; Lizotte *et al.,* 2009). In the hippocampus the overall density of ER-α and ER-β are not different between female and male rats. Similarly the distribution of ER-β in the hippocampus is not different between female and male rats. However, the distribution of ER-α in the female hippocampus is widespread, and is present in both nuclear and extranuclear sites in the pyramidal and granule cells and in their dendrites and axons (Milner *et al.,* 2001; 2005; Romeo *et al.,* 2005). In female rats, ER-α are also found in astrocytes throughout the hippocampus (Milner *et al*

2005). In male rats, the ER-α primarily located in pyramidal cells in the CA1 and CA2 regions of the hippocampus (Azcoitia *et al.,* 1999; Milner *et al*., 2001; 2005; Sakuma *et al.,* 2009).

The difference in ER-α distribution in the hippocampus may regulate the estrogen signaling pathways. Estrogen receptors in the nuclei act by genomic signaling and those bound to the plasma membrane, act by non-genomic signaling. The ER-α acts by both of these signaling pathways in the hippocampus, whereas the ER-β acts primarily by non-genomic signaling, as it is located in extranuclear sites in the hippocampus (Milner *et al.,* 2001; Milner *et al.,* 2005). The sex differences in plasma estrogen levels, signaling pathways and the hippocampal distribution of the ER-α, may contribute to some of the sex differences in expression of heat shock proteins in the hippocampus.

As mentioned above, in the female and male hearts there is no difference in ER-α and ER-β density or distribution (Grohé *et al.,* 1997; Lizotte *et al.,* 2009). In fact in the heart E2 may be regulating HSP independent of ER-α and ER-β. Tamoxifen, an estrogen receptor antagonist, does not alter Hsp70 levels in intact females, indicating that estrogen is regulating expression of HSP independently of nuclear ER-α and ER-β (Paroo *et al.,* 2002). While E2 levels may be the regulator of differential expression of HSP in the female and male hearts, but in my study, basal levels of Hsp70 are similarly low in control female and control male hearts. By western analysis, the low levels of Hsp70, particularly in the heart, make it a challenge to compare levels between females and males. However, analysis by enzyme-linked immunosorbent assay for Hsp70 in unstressed rats indicates approximately twice as much Hsp70 in the female heart compared to male heart (Voss *et al.,* 2003).

In this thesis, in unstressed rats, Hsp27 levels tend to be higher in male hippocampus and heart compared to female hippocampus and heart. Indeed, particularly in the heart, Hsp27 is significantly more abundant in unstressed males compared to unstressed female rats (Voss *et al.,* 2003). Removal of E2 by ovariectomy in unstressed female rats reduces the levels of Hsp70 in the heart to levels similar to male rats (Voss *et al.,* 2003). Replacement of E2 in ovariectomized females or supplementation of E2 in male rats elevated Hsp70 levels in the heart to the levels seen in unstressed intact females (Voss *et al.,* 2003). In unstressed conditions, it seems that estrogen may regulate levels of HSP differentially in females and males.

Estrogen may also regulate the expression of HSP following stressful conditions. In this thesis, the levels of Hsp70 and Hsp27 are greater in hippocampus of heat shock male rats compared to heat shocked female rats in the hippocampus. In the heart, the level of Hsp70 is slightly greater (not significantly) in the heat shock female rats, compared to the heat shock male rats, and the level of Hsp27 is slightly greater (not significantly) in the heat shocked male rats compared to heat shock female rats. Hsp70 levels are shown to be significantly greater in hearts of male rats compared to female rats after 2 weeks of treadmill exercise training (Paroo *et al.,* 1999). E2 supplementation in male rats suppressed the exercise induced increase of Hsp70 in the hearts to the level seen in hearts of female rats (Paroo *et al.,* 1999). Removal of E2 by ovariectomy significantly increased the level of Hsp70 in the hearts of exercised rats compared to intact female rats (Paroo *et al.,* 2002). A similar increase in Hsp70 in the heart is seen in ovariectomized rats compared to intact female rats after heat shock treatment (Shinohara

et al., 2004). Therefore, circulating estrogen levels may have an effect on the sex differences in expression of heat shock proteins in both the heart and the brain of rats.

As discussed in this thesis, estrogen seems to be regulating the expression of HSP differentially between males and females. Interestingly, ovariectomized rats that were treated with E2 had increased levels of Hsp90 in the hypothalamus, compared to ovariectomized rats that were not given E2 (Olazabal *et al.,* 1992b). This suggests that E2 in female rats may regulate higher Hsp90 levels in the cortex and hypothalamus, compared to that in male rats (Elakovic *et al.,* 2007). However in other tissues, including the heart, Hsp90 levels are higher in male rats (Voss *et al.,* 2003). Interaction of Hsp90 with both the estrogen receptor and the HSF1 may account for these differences. The estrogen receptor is maintained in the cytoplasm bound to Hsp90 (Milne & Noble, 2008). Estrogen freely penetrates cell membranes and binds with the estrogen receptor in the cytoplasm, releasing Hsp90. Freed Hsp90 can bind and inactivate any free HSF1 in the cytoplasm, thus suppressing the transcription of various heat shock genes regulated by HSF1. In normal conditions, Hsp90 and/or Hsc70 bind and maintain HSF1 in an inactive state in the cytoplasm (Zou *et al.,* 1998; Guo *et al.,* 2001; Anckar & Sistonen, 2011). Metabolic stressors such as heat shock, intense exercise, or ischemia leads to the accumulation of damaged or denatured proteins within cells. Various HSP including Hsp90 and Hsc70 are recruited to damaged proteins, freeing HSF1. HSF1 trimerizes, becomes phosphorylated, and translocates to the nucleus and binds to the heat shock elements in the promoter region of heat shock genes and increases their transcription (Morimoto, 1998; Anckar & Sistonen, 2011). Thus E2 interaction with its receptors and

Hsp90 may be regulating the differential expression of various HSP in females and males (Milne & Noble, 2008).

In addition to interacting with HSF1, and estrogen receptors, Hsp90 (and Hsc70) also interacts with glucocorticoid receptors (Pratt, 1993; Jones *et al.,* 2004). These interactions of Hsp90 may also explain the differential glucocorticoid levels in females and males after various stresses. Following a stressor, female rats have less activation of HSF1 and less expression of HSP, and higher cortisol levels compared to male rats (Jezova *et al.,* 1994; Beiko *et al.,* 2004; Li & Sanchez, 2005). High glucocorticoid levels binding to the glucocorticoid receptors free Hsp90 that binds and inactivates HSF1 (Li & Sanchez, 2005). These sex differences in response to stress may be indicative of differential activation of the hypothalamic pituitary adrenal axis in that following an acute stress, levels of cortisol secretion are greater and maintained at higher levels in females, compared to males (Galluci *et al.,* 1993; Kant *et al.,* 1983).

Estrogen may mediate protection through a mechanism that stabilizes cellular membranes and prevents protein denaturation, resulting in less oxidative stress and expression of heat shock proteins (Paroo *et al.,* 2002). E2 dampens oxidative stress by increasing antioxidants following injury (Essig & Nosek, 1997; Kher *et al.,* 2005). Oxidative stress leads to activation of the p38 mitogen activated protein kinase, nuclear factor kappa B and accumulation of inflammatory cytokines, like tumor necrosis factor, that may lead to activation of the apoptotic pathways (reviewed by Kher *et a*l., 2005). In a normal state, male rats have significantly greater phosphorylated (activated) p38 mitogen activated protein kinase, compared to females, indicating that normally females have lower levels of oxidative stress (Wang *et al.,* 1998). Lower levels of oxidative stress

in females may be a factor contributing to sex differences in degenerative disease prevalence, that disproportionately affect males, compared to females. As stated previously, this sex difference equalizes once women begin menopause, and E2 levels drop drastically. Low levels of E2 play a role in the development of degenerative diseases. In neurodegenerative and cardiovascular diseases, damaged proteins aggregate and lead to proteotoxicity and the release of reactive oxygen species (Muchowski & Wacker, 2005; reviewed by Xu *et al*., 2012).

Conclusions and future directions

HSP are differentially regulated in female and male rats. Overall, male rats have greater induced levels of Hsp70 and Hsp27 in the hippocampus and similar induced levels of Hsp70 and Hsp27 in the heart, compared to levels in female rats, 24 hours after heat shock treatment. In the hippocampus 24 hours after heat shock, Hsp70 is expressed in microglia and small blood vessels, and Hsp27 is expressed in astrocytes and astrocytic processes around small blood vessels. Sex differences in E2 regulation of the expression of HSP in the brain after stress may help to explain neurodegenerative disease prevalence. The age-related accumulation of damaged proteins in neurodegenerative and cardiovascular disease induces a sex-specific heat shock response that appears to be regulated at the level of E2 interacting with the estrogen receptor and Hsp90. This interaction may explain sex differences in disease prevalence.

In this thesis several questions have arisen that should be addressed. The levels of Hsp90, Hsc70, and HSF1 should be determined after heat shock in both females and males in order to understand the sex-specific induction of the HSP. In addition, estrogen

and HSP levels need to be examined in young and old animals to determine age-related changes in the interaction between E2 and HSP.

References

Anckar, J., Sistonen, L. (2011). Regulation of HSF1 function in the heat stress response: implications in aging and disease. Annual Reviews of Biochemistry *80*, 1089-1115.

Aridon, P., Geraci, F., Turturici, G., D'Amelio, M., Savettieri, G., Sconzo, G. (2011). Protective role of heat shock proteins in Parkinson's disease. Neurodegenerative Disease *8*, 155-168.

Armstrong, J.N., Plumier, J.C., Robertson, H.A., Currie, R.W. (1996). The inducible 70,000 molecular/weight heat shock protein is expressed in the degenerating dentate hilus and piriform cortex after systemic administration of kainic acid in the rat. Neuroscience *74*: 685-693.

Azcoitia, I., Sierra, A., Garcia-Segura, L.M. (1999). Localization of estrogen receptor beta-immunoreactivity in astrocytes of the adult rat brain. Glia *26*, 260-7.

Bae, S., Zhang, L. (2005). Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. Journal Pharmacology Experimental Therapy. *15*, 1125-1135.

Barron, A.M., Pike, C.J. (2012). Sex hormones, aging, and Alzheimer's disease. Frontal Biosciences *4*, 976-997.

Bechtold, D.A., Brown, I.R. (2003). Induction of Hsp27 and Hsp32 stress proteins and vimentin in glial cells of the rat hippocampus following hyperthermia. Neurochemistry Research *28*, 1163-1173.

Bechtold, D.A., Rush, S.J., Brown, I.R. (2000). Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain. Journal of Neurochemistry *74*, 641-646.

Beery, A.K., Zucker, I. (2011). Males still dominate animal studies. Nature *465*, 690.

Beiko, J., Lander, R., Hampson, E., Boon, F., Cain, D.P. (2004). Contribution of sex differences in the acute stress response to sex differences in water maze performance in the rat. Behaviour Brain Research *151*, 239-53.

Bellucci, A., Zaltieri, M., Navarria, L., Grigoletto, J., Missale, C., Spano, P. (2012). From α-synuclein to synaptic dysfunctions: new insights into the pathophysiology of Parkinson's disease. Brain Research *1476*, 183-202.

Bennaroch, E. (2011). Heat shock proteins: multiple neuroprotective functions and implications for neurologic disease. Neurology *76*, 660-667.

Bodega, G., Hernández, C., Suárez, I., Martin, M., Fernández, B. (2002). HSP70 constitutive expression in rat central nervous system from postnatal development to maturity. Journal of Histochemistry and Cytochemistry *50*, 1161-1168.

Brinton, R.D. (2008). Estrogen regulation of glucose metabolism and mitochondrial function: therapeutic implications for prevention of Alzheimer's disease. Advanced Drug Delivery Reviews *60,* 1504-1511.

Bruijn, L.I., Miller, T.M., Cleveland, D.W. (2004). Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annual Reviews of Neuroscience *27*, 723–749.

Butterfield, D.A., Griffin, S., Munch, G., Pasinetti, G.M. (2002). Amyloid beta-peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. Journal of Alzheimers Disease *4*, 193-201.

Canto, J.G., Rogers, W.J., Goldberg, R.J., Peterson, E.D., Wenger, N.K., Vaccarino, V., Kiefe, C.I., Frederick, P.D., Sopko, G., Zheng, Z.J. (2012). Association of age and sex with myocardial infarction symptom presentation and in-hospital mortality. Journal of American Medical Association *307*, 813-822.

Caruccio, L., Bae, S., Liu, A.Y., Chen, K.Y. (1997). The heat-shock transcription factor HSF1 is rapidly activated by either hyper- or hypo-osmotic stress in mammalian cells. Biochemistry Journal *327*, 341-347.

Chen, S., Brown, I.R. (2007). Neuronal expression of constitutive heat shock proteins: implications for neurodegenerative diseases. Cell Stress Chaperones *12*, 51-58.

Cleveland, D.W., Rothstein, J.D. (2001). From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nature Reviews Neuroscience *2*, 806-819.

Colotti, C., Cavallini, G., Vitale, R.L., Donati, A., Maltinti. M, Del. Ry. S., Bergamini, E., Giannessi, D. (2005). Effects of aging and anti-aging caloric restrictions on carbonyl and heat shock protein levels and expression. Biogerontology *6*, 397-406.

Cotto, J.J., Morimoto, R.I. (1999). Stress-induced activation of the heat-shock response: cell and molecular biology of heat-shock factors. Biochemistry Society Symposium *64*, 105-118.

Csermely, P. (2001). Chaperone overload is a possible contributor to 'civilization diseases'. Trends in Genetics *17*, 701-704.

Currie, R.W., Tanguay, R.M. (1991). Analysis of RNA for transcripts for catalase and SP71 in rat hearts after in vivo hyperthermia. Biochemistry and Cellular Biology *69*, 375-382.

Currie, R.W., Karmazyn, M., Kloc, M., Mailer, K. (1988). Heat-shock response is associated with enhanced postischemic ventricular recovery. Circulation Research *63*, 543-549.

Currie, R.W., Tanguay, R.M., Kingma, J.G. (1993). Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. Circulation *87*, 963-971.

Currie, R.W., White, F.P. (1981). Trauma-induced protein in rat tissues: a physiological role for a "heat shock" protein? Science *214*, 72-73.

Currie, R.W., White, F.P. (1983). Characterization of the synthesis and accumulation of a 71-kilodalton protein induced in rat tissues after hyperthermia. Canadian Journal of Biochemistry and Cell Biology *61,* 438-446.

David, J.C., Currie, R.W., Robertson, H.A. (1994). Expression and distribution of hsp71 and hsc73 messenger RNAs in rat brain following heat shock: effect of dizocilpine maleate. Neuroscience *62,* 945-954.

Diaz-Latoud, C., Buache, E., Javouhey, E., Arrigo, A.P. (2005). Substitution of the unique cysteine residue of murine Hsp25 interferes with the protective activity of this stress protein through inhibition of dimer formation. Antioxidant Redox Signaling *7*, 436-445.

Elaković, I., Brkljacić, J., Matić, G. (2007). Long-term imipramine treatment affects rat brain and pituitary corticosteroid receptors and heat shock proteins levels in a gender-specific manner. Journal of Neural Transmission *114*, 1069-1080.

Essig, D.A., Nosek, T.M. (1997). Muscle fatigue and induction of stress protein genes: a dual function of reactive oxygen species? Canadian Journal of Applied Physiology *22*, 409-428.

Fait, T., Vrablik, M. (2012). Coronary heart disease and hormone replacement therapy - from primary and secondary prevention to the window of opportunity. Neurology Endocrinology Letters *33*, 17-21.

Ferns, G., Shams, S., Shafi, S. (2006). Heat shock protein 27: its potential role in vascular disease. International Journal of Experimental Pathology *87*, 253-274.

Fish, E. (2008). The X-files in immunity: sex-based differences predispose immune responses. Nature Reviews: Immunology *8*, 737-744.

Franconi, F., Carru, C., Canu, S., Seghieri, G. (2007). Targeting gender difference in the introduction of new drugs for diabetes mellitus and metabolic disorders. Recent Patents on Endocrine, Metabolic and Immune Drug Discovery *1*, 152-156.

Gacad, M.A., Adams, J.S. (1998). Proteins in the heat shock-70 family specifically bind 25-hydroxyvitamin D3 and 17beta-estradiol. Journal of Clinical Endocrinology and Metabolism *83*, 1264-1267.

Gallucci, W.T., Baum, A., Laue, L., Rabin, D.S., Chrousos, G.P., Gold, P.W., Kling M.A. (1993). Sex differences in sensitivity of the hypothalamic-pituitary-adrenal axis. Health Psychology *12*, 420-425.

Gao, S., Hendrie, H.C., Hall, K.S., Hui, S. (1998). The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. Archives General Psychiatry *55*, 809-815.

Garrido, C., Bruey, J.M., Fromentin, A., Hammann, A., Arrigo, A.P., Solary, E. (1999). HSP27 inhibits cytochrome c-dependent activation of procaspase-9. The FASEB Journal *13*, 2061-2070.

Gold, S.M., Voskuhl, R.R. (2009). Estrogen treatment in multiple sclerosis. Journal of Neurological Science *28*, 99-103.

Gombos, T., Förhécz, Z., Pozsonyi, Z., Jánoskuti, L., Prohászka, Z. (2008). Interaction of serum 70-kDa heat shock protein levels and HspA1B (+1267) gene polymorphism with disease severity in patients with chronic heart failure. Cell Stress Chaperones *13*, 199-206.

Gouras, G.K., Xu, H., Gross, R.S., Greenfield, J.P., Hai, B., Wang, R., Greengard P. (2000). Testosterone reduces neuronal secretion of Alzheimer's beta-amyloid peptides. Proceedings of the National Academy of Science in the United States of America 97, 1202-1205.

Grohé, C., Kahlert, S., Löbbert, K., Stimpel, M., Karas, R.H., Vetter, H., Neyses, L. (1997). Cardiac myocytes and fibroblasts contain functional estrogen receptors. FEBS Letters *416*, 107-112.

Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J.N., Huot, J., Landry, J. (1997). Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. Journal of Cell Science *110*, 357-368.

Guo, Y., Guettouche, T., Fenna, M., Boellmann, F., Pratt, W.B., Toft, D.O., Smith, D.F.,Voellmy, R. (2001). Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. Journal of Biology and Chemistry *276*, 45791-45799.

Hampel, H., Prvulovic, D., Teipel, S., Jessen, F., Luckhaus, C., Frölich, L., Riepe, M.W., Dodel, R., Leyhe, T., Bertram, L., Hoffmann, W., Faltraco, F. (2011). The future of Alzheimer's disease: the next 10 years. Progressive Neurobiology *95*, 718-728.

Hartl, F.U., Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. Science *295*, 1852-1858.

Hebert, L.E., Scherr, P.A., Bienias, J.L., Bennett, D.A., Evans, D.A. (2003). Alzheimer disease in the US population prevalence estimates using the 2000 Census. Journal of the American Medical Association *60*, 1119-1122.

Hoch, B., Lutsch, G., Schlegel, W.P., Stahl, J., Wallukat, G., Bartel, S., Krause, E.G., Benndorf, R., Karczewski, P. (1996). HSP25 in isolated perfused rat hearts: localization and response to hyperthermia. Molecular Cellular Biochemistry *160-161*, 231-9.

Holdcroft, A. (2007). Gender bias in research: how does it affect evidence based medicine. Journal of the Royal Society of Medicine *100,* 2-3.

Huang, C., Cheng, H., Hao, S., Zhou, H., Zhang, X., Gao, J., Sun, Q.H., Hu, H., Wang, C.C. (2006). Heat shock protein 70 inhibits alpha-synuclein fibril formation via interactions with diverse intermediates. Journal of Molecular Biology *364*, 323-336.

Huisman, M.H., de Jong, S.W., van Doormaal, P.T., Weinreich, S.S., Schelhaas, H.J., van der Kooi, A.J., de Visser, M., Veldink, J.H., van den Berg, L.H. (2011a). Population based epidemiology of amyotrophic lateral sclerosis using capture-recapture methodology. Journal of Neurology Neurosurgery Psychiatry *82*, 1165-1170.

Huisman, M.H., de Jong, S.W., Verwijs, M.C., Schelhaas, H.J., van der Kooi, A.J., de Visser, M., Veldink, J.H., van den Berg, L.H. (2011b). Family history of neurodegenerative and vascular diseases in ALS: a population-based study. Neurology *4*, 1363-1369.

Jezová, D., Kvetnanský, R., Vigas, M. (1994). Sex differences in endocrine response to hyperthermia in sauna. Acta Physiologica Scandinavia *150*, 293-298.

Jones, T.J., Li, D., Wolf, I.M., Wadekar, S.A., Periyasamy, S., Sánchez, E.R. (2004).

Kalmar, B., Greensmith, L. (2009). Induction of heat shock proteins for protection against oxidative stress. Advanced Drug Delivery Reviews *61*, 310-318,

Kalin MF, Zumoff B. (1990). Sex hormones and coronary disease: a review of the clinical studies. Steroids *8*, 330-352.

Kampinga, H.H., Hageman, J., Vos, M.J., Kubota, H., Tanguay, R.M., Bruford, E.A., Cheetham, M.E., Chen, B., Hightower, L.E. (2009). Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones *14*, 105-111.

Kant, G.J., Lenox, R.H., Bunnell, B.N., Mougey, E.H., Pennington, L.L., Meyerhoff, J.L. (1983). Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology *4*, 421-428.

Kant, G.J., Lenox, R.H., Bunnell, B.N., Mougey, E.H., Pennington, L.L., Meyerhoff, J.L. (1983). Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology *8*, 421-428.

Karmazyn, M., Mailer, K., Currie, R.W. (1990). Acquisition and decay of heat-shock-enhanced postischemic ventricular recovery. American Journal of Physiology *259*, 424-431.

Kato, H. K, Kogure, K. T., Araki, Y., Itoyama. (1994). Astroglial and microglial reactions in the gerbil hippocampus with induced ischemic tolerance Brain Research *664*, 69–76.

Kawas, C., Resnick, S., Morrison, A., Brookmeyer, R., Corrada, M., Zonderman, A., Bacal, C., Lingle, D.D., Metter, E. (1997). A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. Neurology *4*, 1517-1521.

Kher, A., Wang, M., Tsai, B.M., Pitcher, J.M., Greenbaum, E.S., Nagy, R.D., Patel, K.M., Wairiuko, G.M., Markel, T.A., Meldrum DR. (2005). Sex differences in the myocardial inflammatory response to acute injury. Shock *23*, 1-10.

Kilic, A., Mandal, K. (2012). Heat shock proteins: pathogenic role in atherosclerosis and potential therapeutic implications. Autoimmune Disease *2012*, ID 502813, 1-9.

Kompoliti, K. (2003). Estrogen and Parkinson's disease. Front Biosciences *8*, 391-400.

Kopito, R.R., Ron, D. (2000). Conformational disease. Nature Cell Biology *2*, 207-209.

Krueger-Naug, A.M., Emsley. J.G., Myers, T.L., Currie, R.W., Clarke, D.B. (2003). Administration of brain-derived neurotrophic factor suppresses the expression of heat shock protein 27 in rat retinal ganglion cells following axotomy. Neuroscience *116*, 49-58.

Krueger-Naug, A.M., Hopkins, D.A., Armstrong, J.N., Plumier, J.C., Currie, R.W. (2000). Hyperthermic induction of the 27-kDa heat shock protein (Hsp27) in neuroglia and neurons of the rat central nervous system. Journal of Comparative Neurology *428*, 495-510.

Krueger, A.M., Armstrong, J.N., Plumier, J.C., Robertson , H.A., Currie, R.W. (1999). Cell specific expression of Hsp70 in neurons and glia of the rat hippocampus after hyperthermia and kainic acid-induced seizure activity. Molecular Brain Research *71*, 265-278.

Kumar, R., Zakharov, M.N., Khan, S.H., Miki, R., Jang, H., Toraldo, G., Singh, R., Bhasin, S., Jasuja, R. (2011). The dynamic structure of the estrogen receptor. Journal of Amino Acids *2011*, ID 812540, 1-7

Lam, K.K., Cheng, P.Y., Lee, Y.M., Liu, Y.P., Ding, C., Liu, W.H.,Yen, M.H. (2012). The role of heat shock protein 70 in the protective effect of YC-1 on heat stroke rats. European Journal of Pharmacology *699*, 67-73.

Lavoie, J.N., Lambert, H., Hickey, E., Weber, L.A., Landry, J. (1995). Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27 Molecular Cell Biology *15*, 505-516.

Lee, J.E., Yenari, M.A., Sun, G.H., Xu, L., Emond, M.R., Cheng, D., Steinberg, G.K., Giffard, R.G. (2001). Differential neuroprotection from human heat shock protein 70 overexpression in in vitro and in vivo models of ischemia and ischemia-like conditions. Experimental Neurology *170*, 129-139.

Lee, M.Y., Choi, Y.S., Choi, J.S., Min, D.S., Chun, M.H., Kim, O.N., Lee, S.B., Kim, S.Y. (2002). An immunohistochemical study of APG-2 protein in the rat hippocampus after transient forebrain ischemia. Brain Research *924*, 237-241.

Leger, J.P., Smith, F.M., Currie, R.W. (2000). Confocal microscopic localization of constitutive and heat shock-induced proteins HSP70 and HSP27 in the rat heart. Circulation *102*, 1703-1709.

Li, D., Sánchez, E.R. (2005). Glucocorticoid receptor and heat shock factor 1: novel mechanism of reciprocal regulation. Vitamines and Hormones *71*, 239-262.

Lindquist, S., Craig, E.A. (1988). The heat-shock proteins. Annual Reviews Genetics *22*, 631-677.

Liu, J., Shinobu, L.A., Ward, C.M., Young, D., Cleveland, D.W. (2005). Elevation of the Hsp70 chaperone does not effect toxicity in mouse models of familial amyotrophic lateral sclerosis. Journal of Neurochemistry *93*, 875-82.

Lizotte, E., Grandy, S.A., Tremblay, A., Allen, B.G., Fiset, C. (2009). Expression, distribution and regulation of sex steroid hormone receptors in mouse heart. Cell Physiology and Biochemistry *23*, 75-86.

Lotharius, J., Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nature Reviews Neuroscience *3*, 932-942.

Lu, A., Ran, R.Q., Clark, J., Reilly, M., Nee, A., Sharp, F.R. (2002). 17-beta-estradiol induces heat shock proteins in brain arteries and potentiates ischemic heat shock protein induction in glia and neurons. Journal of Cerebral Blood Flow and Metabolism *22*, 183-195.

Maatkamp A, Vlug A, Haasdijk E, Troost D, French PJ, Jaarsma D. (2004). Decrease of Hsp25 protein expression precedes degeneration of motoneurons in ALS-SOD1 mice. European Journal of Neuroscience *20*, 14-28.

Maeda, T., Nagata, K., Yoshida, Y. (2008). Exogenous l-DOPA induce no dopamine immuno-reactivity in striatal astroglias and microglias of adult rats with extensive nigro-striatal dopaminergic denervation. Neuroscience Letters *433*, 255-258.

Manzerra, P., Rush, S.J., Brown, I.R. (1997). Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. Journal of Cellular Physiology *170*, 130-137.

Margo, K, Winn, R. (2006) Testosterone treatments: why, when, and how? America Family Physician *73*, 1591-1598.

Martin JB. (1999). Molecular basis of the neurodegenerative disorders. New England Journal of Medicine *340*, 1970-1980.

Martin-Ventura, J.L., Duran, M.C., Blanco-Colio, L.M., Meilhac, O., Leclercq, A., Michel, J.B., Jensen, O.N., Hernandez-Merida, S., Tuñón, J., Vivanco, F., Egido, J. (2004). Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. Circulation *110*, 2216-2219.

Martin, L.J., Price, A.C., Kaiser, A., Shaikh, A.Y., Liu, Z. (2000). Mechanisms for neuronal degeneration in amyotrophic lateral sclerosis and in models of motor neuron death. International Journal of Molecular Medicine *5*, 3-13.

Masood, D.E., Roach, E.C., Beauregard, K.G., Khalil, R.A. (2010). Impact of sex hormone metabolism on the vascular effects of menopausal hormone therapy in cardiovascular disease. Current Drug Metabolism *11*, 693-714.

Massafra, C., Gioia, D., De Felice, C., Muscettola, M., Longini, M., Buonocore, G. (2002). Gender-related differences in erythrocyte glutathione peroxidase activity in healthy subjects. Clinical Endocrinology *57*, 663-667.

Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M.E., Arrigo, A.P. (1995). Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (hsp27) structural organization and phosphorylation in basal and tumour necrosis factor alpha-treated T47D human carcinoma cells. Biochemistry Journal *312*, 367-375.

Milne, K.J., Noble, E.G. (2008). Response of the myocardium to exercise: sex-specific regulation of Hsp70. Medicine and Science in Sports and Exercise *40*, 655-663.

Milner, T.A., Ayoola, K., Drake, C.T., Herrick, S.P., Tabori, N.E., McEwen, B.S., Warrier, S., Alves, S.E. (2005). Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. Journal of Comparative Neurology *491*, 81-95.

Milner, T.A., McEwen, B.S., Hayashi, S., Li, C.J., Reagan, L.P., Alves, S.E. (2001). Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. Journal of Comparative Neurology *429*, 355-371.

Morimoto, R.I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes and Development *12*, 3788-3796.

Moschetti, K., Cummings, P.L., Sorvillo, F., Kuo, T. (2012). Burden of Alzheimer's disease-related mortality in the United States, 1999-2008. Journal of American Geriatrics Society *60*, 1509-1514.

Mosser, D.D., Caron, A.W., Bourget, L., Meriin, A.B., Sherman, M.Y., Morimoto, R.I., Massie, B. (2000). The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Molecular Cell Biology *19*, 7146-7159.

Mounier, N., Arrigo, A.P. (2002). Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chaperones *7*, 167-176.

Muchowski, P.J., Wacker, J.L. (2005). Modulation of neurodegeneration by molecular chaperones. National Reviews Neuroscience *6*, 11-22.

Nelson, P.T., Head, E., Schmitt, F.A., Davis, P.R., Neltner, J.H., Jicha, G.A., Abner, E.L., Smith, C.D., Van Eldik, L.J., Kryscio, R.J., Scheff, S.W. (2012). Alzheimer's disease is not "brain aging": neuropathological, genetic, and epidemiological human studies. Acta Neuropathology *121*, 571-587.

Nickerson, M., Kennedy, S.L., Johnson, J.D., Fleshner, M. (2006). Sexual dimorphism of the intracellular heat shock protein 72 response. Journal of Applied Physiology *101*, 566-575.

Noble, E.G., Milne, K.J., Melling, C.W. (2008). Heat shock proteins and exercise: a primer. Applied Physiology Nutrition Metabolism *33*, 1050-1065.

Olazábal, U.E., Pfaff, D.W., Mobbs, C.V. (1992a). Sex differences in the regulation of heat shock protein 70 kDa and 90 kDa in the rat ventromedial hypothalamus by estrogen. Brain Research *596*, 311-314.

Olazábal, U.E., Pfaff, D.W., Mobbs, C.V. (1992b). Estrogenic regulation of heat shock protein 90 kDa in the rat ventromedial hypothalamus and uterus. Molecular Cell Endocrinology *84*, 175-183.

Pandey, P., Farber, R., Nakazawa, A., Kumar, S., Bharti, A., Nalin, C., Weichselbaum, R., Kufe, D., Kharbanda S. (2000). Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. Oncogene *19*, 1975-81.

Papaconstantinou, A.D., Goering, P.L., Umbreit, T.H., Brown, K.M. Regulation of uterine hsp90alpha, hsp72 and HSF-1 transcription in B6C3F1 mice by beta-estradiol and bisphenol A: involvement of the estrogen receptor and protein kinase C. Toxicology Letters *144*, 257-70.

Papasozomenos, S.C., Papasozomenos H. (2008). The activation of heat shock transcription factor 1 is differentially regulated in the brain of estrogen- and testosterone-treated heat-shocked rats. Journal of Alzheimers Disease *15*, 375-390.

Park, H.K., Park, E.C., Bae, S.W., Park, M.Y., Kim, S.W., Yoo, H.S., Tudev, M., Ko, Y.H., Choi, Y.H., Kim, S., Kim, D.I., Kim, Y.W., Lee, B.B., Yoon, J.B., Park, J.E. (2006). Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. Circulation *114*, 886-893.

Paroo, Z., Haist, J.V., Karmazyn, M., Noble, E.G. (2002). Exercise improves postischemic cardiac function in males but not females: consequences of a novel sex-specific heat shock protein 70 response. Circulation Research *90*, 911-917.

Paroo, Z., Tiidus, P.M., Noble, E.G. (1999). Estrogen attenuates HSP 72 expression in acutely exercised male rodents. European Journal of Applied Physiology Occupational Physiology *80*, 180-184.

Parsell, D.A., Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual Reviews Genetics *27*, 437-496

Patel, Y.J., Payne Smith, M.D., de Belleroche, J, Latchman D.S. (2005). Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells. Brain Research Molecular Brain Research *134*, 256-74.

Pavlik, A., Aneja, I.S., Lexa, J., Al-Zoabi, B.A. (2003). Identification of cerebral neurons and glial cell types inducing heat shock protein Hsp70 following heat stress in the rat. Brain Research *973*, 179-89.

Plumier, J.C., Armstrong, J.N., Landry, J., Babity, J.M., Robertson, H.A., Currie, R.W. (1996). Expression of the 27,000 mol. wt heat shock protein following kainic acid-induced status epilepticus in the rat. Neuroscience *75*: 849-856.

Plumier, J.C., David, J.-C., Robertson, H.A., Currie, R.W. (1997a). Cortical application of potassium chloride induces the low-molecular weight heat shock protein (Hsp27) in astrocytes. Journal of Cerebral Blood Flow and Metabolism *17*, 781-790.

Plumier, J.C., Hopkins, D.A., Robertson, H.A., Currie, R.W. (1997b). Constitutive expression of the 27-kDa heat shock protein (Hsp27) in sensory and motor neurons of the rat nervous system. Journal of Comparative Neurology *384*, 409-428.

Plumier, J.C., Ross, B.M., Currie, R.W., Angelidis, C.E., Kazlaris, H., Kollias, G., Pagoulatos, G.N. (1995). Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. Journal of Clinical Investigation *95*, 1854-60.

Pratchayasakul, W., Chattipakorn, N., Chattipakorn, S.C. (2011). Effects of estrogen in preventing neuronal insulin resistance in hippocampus of obese rats are different between genders. Life Sciences *89*, 702-707.

Pratt, W.B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. Journal of Biology and Chemistry *268*, 21455-21458.

Renkawek, K., Bosman, G.J., de Jong, W.W. (1994). Expression of small heat-shock protein hsp 27 in reactive gliosis in Alzheimer disease and other types of dementia. Acta Neuropathology *87*, 511-519.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in Drosphila. Experientia *18*, 571-573.

Ritossa, F. (1996). Discovery of heat shock proteins. Cell Stress Chaperones *1*, 97-98.

Robinson, A.A., Dunn, M.J., McCormack, A., dos Remedios, C., Rose, M.L. (2010). Protective effect of phosphorylated Hsp27 in coronary arteries through actin stabilization. Journal of Molecular Cellular Cardiology *49*, 370-379.

Rogalla, P., Rohen, C., Bonk, U., Bullerdiek, J. (1996). Telomeric repeat fragment lengths are not correlated to histological grading in 85 breast cancers. Cancer Letters *106*, 155-61.

Romeo, R.D., McCarthy, J.B., Wang, A., Milner, T.A., McEwen, B.S. (2005). Sex differences in hippocampal estradiol-induced N-methyl-D-aspartic acid binding and ultrastructural localization of estrogen receptor-alpha. Neuroendocrinology *81*, 391-399.

Safford, M.M., Brown, T.M., Muntner, P.M., Durant, R.W., Glasser, S., Halanych, J.H., Shikany, J.M., Prineas, R.J., Samdarshi, T., Bittner, V.A., Lewis, C.E., Gamboa, C., Cushman, M., Howard, V., Howard, G. (2012). Association of race and sex with risk of incident acute coronary heart disease events. Journal of American Medical Association *308*, 1768-1774.

Sakuma, S., Tokuhara, D., Hattori, H., Matsuoka, O., Yamano, T. (2009). Expression of estrogen receptor alpha and beta in reactive astrocytes at the male rat hippocampus after status epilepticus. Neuropathology *29*, 55-62.

Schapira, A.H. (1997). Pathogenesis of Parkinson's disease. Baillieres Clinical Neurology *6*, 15-36.

Schapira, A.H., Olanow, C.W. (2004). Neuroprotection in Parkinson disease: mysteries, myths, and misconceptions. Journal of the American Medical Association *291*, 358-364.

Schlesinger, M.J., Ashburner, M., Tissieres, A. (1982). Heat shock from bacteria to man. Cold Spring Harbor Laboratory, United States of America.

Sharp, P.S, Akbar, M.T., Bouri, S., Senda, A., Joshi, K., Chen, H.J., Latchman, D.S., Wells, D.J., de Belleroche, J. (2007). Protective effects of heat shock protein 27 in a model of ALS occur in the early stages of disease progression. Neurobiology Diseases *30*, 42-55.

Shaikh, A.A. (1971). Estrone and estradiol levels in the ovarian venous blood from rats during the estrous cycle and preagnancy. Biology of Reproduction *5*, 297-307.

Shi, Y., Mosser, D.D., Morimoto, R.I. (1998). Molecular chaperones as HSF1-specific transcriptional repressors. Genes Development *12*, 654-666.

Shimura, H., Miura-Shimura, Y., Kosik, K.S. (2004). Binding of tau to heat shock protein 27 leads to decreased concentration of hyperphosphorylated tau and enhanced cell survival. Journal of Biology and Chemistry *279*, 17957-17962.

Shinder, G.A., Lacourse, M., Minotti, S., Durham, H.D. (2001). Mutant Cu/Zn-Superoxide Dismutase Proteins Have Altered Solubility and Interact with Heat Shock/Stress Proteins in Models of Amyotrophic Lateral Sclerosis. Journal of Biology and Chemistry *276*, 12791-12796.

Shinohara, H., Inaguma, Y., Goto, S., Inagaki, T., Kato K. (1993). Alpha B crystallin and HSP28 are enhanced in the cerebral cortex of patients with Alzheimer's disease. Journal of Neurological Science *119*, 203-208.

Shinohara, T., Takahashi, N., Ooie, T., Ichinose, M., Hara, M., Yonemochi, H., Saikawa, T., Yoshimatsu, H. (2004). Estrogen inhibits hyperthermia-induced expression of heat-shock protein 72 and cardioprotection against ischemia/reperfusion injury in female rat heart. Journal of Molecular Cell Cardiology *37*, 1053-1061.

Simon, V. (2005). Wanted: Women in Clinical Trials. Science *308*, 1517.

Smiley, D.A., Khalil, R.A. (2009). Estrogenic compounds, estrogen receptors and vascular cell signaling in the aging blood vessels. Currrent Medical Chemistry *16,* 1863–1887.

Stice, J.P., Knowlton, A.A. (2008). Estrogen, NFkappaB, and the heat shock response. Molecular Medicine *14*, 517-527.

Szalay, L., Shimizu, T., Suzuki, T., Yu, H.P., Choudhry, M.A., Schwacha, M.G., Rue, L.W., Bland, K.I., Chaudry, I.H. (2006). Estradiol improves cardiac and hepatic function after trauma-hemorrhage: role of enhanced heat shock protein expression. American Journal of Physiology Regulatory Integrative Comprehensive Physiology *290*, 812-818.

Tanguay, R.M., Wu, Y., Khandjian, E.W. (1993). Tissue-specific expression of heat shock proteins of the mouse in the absence of stress. Developmental Genetics *14*, 112-118.

Theilen, H., Kuschinsky, W. (1992). Fluorescence labeling of the capillary network in rat brains. Journal of Cerebral Blood Flow and Metabolism *12*, 347-350.

Thorp, D.B., Haist, J.V., Leppard, J., Milne, K.J., Karmazyn, M., Noble, E.G. (2007). Exercise training improves myocardial tolerance to ischemia in male but not in female rats. American Journal Physiological Regulatory, Integrative and Comparative Physiology *293*, 363-371.

Van Den Eeden, S.K., Tanner, C.M., Bernstein, A.L., Fross, R.D., Leimpeter, A., Bloch. D.A., Nelson LM. (2003). Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. American Journal of Epidemiology *157*, 1015-1022.

van der Worp H.B., Howells, D.W., Sena, E.S., Porritt, M.J., Rewell, S., O'Collins, V., Macleod, M.R. (2010). Can Animal Models of Disease Reliably Inform Human Studies? PLoS Medicine *7*, e1000245.

Voss, M.R., Stallone, J.N., Li, M., Cornelussen, R.N., Knuefermann, P., Knowlton, A.A. (2003). Gender differences in the expression of heat shock proteins: the effect of estrogen. American Journal of Physiology Heart Circulation Physiology. *285*, 687-692.

Wald, C.,Wu, C. (2010). Biomedical research. Of mice and women: the bias in animal models. Science *327*, 1571–1572.

Wang, X., Robbins, R. (2006). Heart Failure and Protein Quality Control. Circulation Research *99*,1315-1328.

Wang, X., Martindale, J. L. Liu, Y. and Holbrook, N.J. (1998). The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochemistry Journal *333*, 291-300.

Weiland, N.G., Orikasa, C., Hayashi, S., McEwen, B.S. (1997). Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats. Journal of Comparative Neurology *388*, 603-12.

Weiske, J., Schöneberg, T., Schröder, W., Hatzfeld, M., Tauber, R., Huber, O. (2001). The fate of desmosomal proteins in apoptotic cells. Journal of Biological Chemistry *276*, 41175-41181.

Wilhelmus, M.M.M., de Waal, R.M.W., Marcel M. Verbeek, M.M. (2007). Heat Shock Proteins and Amateur Chaperones in amyloid-beta accumulation and clearance in Alzheimer's disease. Molecular Neurobiology *35*, 203–216.

Wojtys, E.M., Huston, L.J., Boynton, M.D., Spindler, K.P., Lindenfeld, T.N. (2002). The effect of the menstrual cycle on anterior cruciate ligament injuries in women as determined by hormone levels. American Journal of Sports Medicine *30*, 182-188.

Xu Q, Metzler B, Jahangiri M, Mandal K. (2012). Molecular chaperones and heat shock proteins in atherosclerosis. American Journal of Physiology Heart Circulation Physiology *302*, 506-514.

Xu, Q., Wick, G. (1996). The role of heat shock proteins in protection and pathophysiology of the arterial wall. Molecular Medicine Today *2*, 372-379.

Zhang, Y., McLaughlin, R., Goodyer, C., LeBlanc A. (2002). Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. Journal of Cell Biology *156*, 519-529.

Zhu, J., Quyyumi, A.A., Wu, H., Csako, G., Rott, D., Zalles-Ganley, A., Ogunmakinwa, J., Halcox, J., Epstein, S.E. (2003). Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. Arteriosclerosis Thrombosis Vascular Biology *23*, 1055-1059.

Zou, J., Guo, Y., Guettouche, T., Smith, D.F., Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell *94*, 471-480.