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Gender Differences In The Processing Of Acute And Repeated Stress

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DISSERTATION

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ABSTRACT

Chronic stress is implicated in the pathogenesis of a variety of diseases, including affective, immune and cardiovascular disorders, which are differentially experienced by men and women. Corticotropin-releasing factor (CRF), arginine vasopressin (AVP), and glucocorticoid receptor (GR) are the major regulators of the stress response and are widely expressed in the paraventricular nucleus of the hypothalamus (PVH), where adaptive responses to stress are generated. Adult male and female rats were subjected to single (acute) or 14 consecutive daily (repeated) 30 min restraint sessions, or maintained as unstressed controls. Rats were perfused after their final restraint, and their brain tissues sectioned and stained immunohistochemically for Fos and to co-localize Fos/CRF, Fos/AVP and Fos/GR in the PVH. Control rats expressed low levels of Fos, with females exhibiting significantly fewer Fos positive cells than males. Acute restraint increased the number of Fos-expressing cells in the PVH of both males and females, with a relatively greater increase seen in the males. In repeated restraint stress, Fos returned to levels not significantly different from control in both sexes, and habituated responses were seen following repeated stress. Fos/CRF, Fos/AVP and Fos/GR expression showed similar trends as Fos alone across all experimental groups. Female rats that were ovariectomized (OVX) with or with out estrogen replacement did not show similar trends in stress response for Fos/AVP and Fos/GR as those previously described. OVXed vehicle females showed high basal (control) levels for both Fos/AVP and Fos/GR, in comparison to OVXed control females that had either low or high estrogen replacement. Together, these data suggest that gender-specific responses to stress are evident at the level of neuronal activation, and may in part be due to effects of estrogen.

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CHAPTER I

Introduction:

”Stress” is a term used to describe a wide range of stimuli, both physiological and psychological, which can directly and indirectly contribute to disorders of the mind, and can have a major impact on the physical functioning of the human body [61]. As such, it is critically important to understand the central nervous system pathways and mechanisms that contribute to the initiation and termination of stress responses, in order to accurately identify targets for the treatment of stress-related illnesses. There are two types of stress: eustress, which is considered a “positive stress” and distress, which is considered a “negative stress”. Both raise the levels of adrenaline and corticosteroids in the body, which puts more physical demand on bodily organs. Individual stressors can be grouped into two broad categories, emotional (also called psychological, processive or neurogenic) and physiological (also known as homeostatic or systemic). These stressors are distinguished on the basis of the type of sensory input they provide to the brain, the extent to which they elicit affective responses, and the neural circuitry that mediates adaptive responses to them [48]. Adaptive responses include modifications to the neuronal pathways that convey relevant sensory information to appropriate effector neurons, and changes in hormonally based feedback mechanisms that serve to attenuate neuroendocrine components of the response [49].

Physiological stressors, such as hemorrhage or immune challenge, disrupt homeostasis and challenge the ability of an organism to maintain an internal equilibrium. The pathways that convey information regarding theses types of stressors to the brain are well characterized. Emotional stressors, in contrast, often have environmental cues as a significant component, which are perceived by the organism as potentially dangerous. Stressors of this type, including

restraint and footshock, are processed through sensory inputs that are poorly understood. Both types of challenges ultimately come together to activate common response systems but to differing degrees. First, stimulation of the adrenal medulla by the sympathetic nervous system results in the release of epinephrine which transiently increases heart and breathing rates, among other responses, with the goal of preparing the organism to escape or fight the threat. The hypothalamic-pituitary-adrenal (HPA) axis, in contrast, involves the secretion of corticotropin-releasing factor (CRF) from the paraventricular hypothalamic nucleus (PVH), which then stimulates adrenocorticotropic hormone (ACTH) release from the pituitary gland. ACTH, in turn, stimulates the release of glucocorticoids from the adrenal gland. This secondary response system promotes adaptation to and recovery from the stressor.

Acute stress is known to activate both CRF and vasopressin (AVP) hormones, whereas repeated stress preferentially activates AVP rather than CRF [38]. Acute stress increases CRF expression and secretion in the PVH [33], as well as the expression of the immediate-early gene, *c-fos* [11]. Fos is the protein product of the *c-fos* proto-oncogene and is commonly used as a marker to indicate neuronal activation, identifying cells and circuitries that become activated in response to various stimuli including stress [31]. However, repeated exposure to a stressor leads to habituation of the HPA axis as seen by progressively decreasing responses at all levels of the axis [13]. Glucocorticoid negative feedback in acute stress occurs through rapid inhibition of CRF release, while in chronic stress situations, it acts through down-regulation of CRF and AVP expression in PVH neurons [14].

Multiple studies have shown differences between male and female responses to stress, and gender is one of the most important predictors of health in humans. It has been found that sex influences many brain functions, including emotion, memory, vision, pain perception,

neurotransmitter levels, stress hormone action and disease states [7]. As a consequence of chronic stress, women suffer more often from autoimmune illnesses, while men are more at risk for coronary or infectious diseases [32]. Of the psychiatric disorders, women often develop anxiety, depression or panic disorders, while substance abuse and antisocial behavior are more common in men [32]. HPA hyperactivity is frequently found in patients suffering from major depression and also appears to be associated with susceptibility to infectious diseases, whereas hypoactivity has been associated with autoimmune diseases [28].

This project was designed to address the mechanisms of how the brain processes acute and repeated stress, by identifying the cell groups potentially involved in the adaptation process. The goal is to understand and identify the response mechanisms of the HPA axis during repeated stress, as repeated or chronic stress has been linked to the incidence and severity of numerous chronic disease states such as depression, cardiovascular disease and immune dysfunction. This project will also address differences between male and female rats in their mechanisms for coping with stressful conditions, with the goal of discovering strategies and targets for the prevention and treatment of gender specific illnesses.

Rationale:

The **goals of this research project** were to more clearly define the central nervous system pathways and mechanisms that are activated by stress and may contribute to stress-related illness, with special attention paid to how these pathways and mechanisms differ in males and females. Acute stress has been shown to induce a wide array of neurological and endocrine responses geared toward survival and the maintenance of homeostasis. Repeated or chronic stress, in contrast, leads to an adaptation of these responses, yet has been linked with the onset or

increased severity of a multitude of diseases including affective, immune and cardiovascular disorders. Furthermore, differential responses to stress have been seen in males and females, and sex-specific outcomes have been noted in terms of stress-related disease incidence, but the mechanisms through which these outcomes are mediated remain unclear. Our **working hypothesis** was that the activation of hypothalamic neurons that function in the initiation and termination of stress responses differs by stress condition and sex. The following experiments focused primarily on the paraventricular hypothalamic nucleus (PVH), where neuroendocrine responses to stress are initiated. The **specific hypotheses** tested were: 1) distinct subsets of neurons in the PVH are responsible for maintaining stress responses in the repeated compared to the acute condition, and can be identified by their anatomical location and neuropeptide phenotype; and 2) the mechanisms through which sex-specific responses to stress are mediated include neuroendocrine and feedback control systems involved in fluid and energy homeostasis, as these mechanisms are critical for the survival of females and their offspring.

One of the main components of this research was to study the differences between male and female rats that occur in the processing of stress, since sex is one of the most important predictors of health. Women suffer more often from autoimmune illnesses, while men are more at risk for coronary or infectious diseases [32]. Regarding psychiatric disorders, women more often develop anxiety, depression or panic disorders, while substance abuse and antisocial behaviors are more common in men [32]. Understanding the biological mechanisms responsible for these differences will provide information about the pathology of various disorders, and will aid the development of new preventative and therapeutic strategies [23].

The experiments outlined in this dissertation used acute and repeated physical restraint in rats as an animal model of emotional stress, and evaluated peptide- and mRNA-level changes in gene

expression in the PVH as a consequence of stress. The anatomy of the rat brain is similar to that of the human, allowing for correlations to be drawn between mechanisms that are implicated in our paradigm and may contribute to disease in humans. In addition, data from numerous experiments have previously been published using this stress model and provide a framework for these studies and the interpretation of our results.

For our purposes, the emotional stress model more closely approximates the human condition than would a physiological stressor, in that it involves higher brain circuitries and multiple neural pathways and has been implicated in disorders ranging from depression to obesity. Restraint also allows us to easily perform studies on the effects of acute vs. repeated stress exposure with minimal consequence to the experimental animal; this model does no obvious physical harm to the animal, making it ideal for a repeated stress study.

Our focus on the PVH is due to its unique ability to incorporate information regarding the homeostatic condition of an organism, and initiate responses that maintain or re-establish homeostasis when it is challenged. Adaptive responses have some specificity toward the stressor that causes them, but those responses may change if the severity or duration of the stress increases such as during chronic or repeated stress [30]. It is important to understand the organization, phenotype and properties of PVH neurons, as well as the inputs to and outputs from these cells, in order to determine how responses to different types or patterns of stress are generated. How these measures differ in females compared to males is another critical question.

CHAPTER II

THE ROLES OF FOS AND AVP OR GR IN THE STRESS RESPONSE

Introduction:

The hypothalamic-pituitary-adrenal (HPA) axis is a physiological control and regulatory mechanism that connects the central nervous system with peripheral hormone secretion and function. This stress-responsive neuroendocrine system helps mammalian organisms adapt to increased demands and maintain homeostasis after challenge, but is also vital for supporting normal physiological functioning. The PVH lies at the head of the HPA axis and, as mentioned before, is responsible for generating adaptive responses to stress.

The PVH is an aggregation of neurons in the hypothalamus that can be subdivided into magnocellular and parvocellular divisions. The magnocellular division contains large neurosecretory cells whose axons extend into the posterior pituitary gland and produce and secrete oxytocin and vasopressin (AVP). The parvocellular division, in contrast, contains five different subregions that are defined by their anatomical location within the nucleus: periventricular, anterior, medial, dorsal and lateral [59]. The medial parvocellular region of the PVH, in particular, contains neurosecretory cells that project to the median eminence, where their nerve terminals release peptides into blood vessels of the hypothalamic-pituitary portal circulation [59]. This specialized vascular system then carries the secreted neuropeptides to the anterior pituitary gland, where they regulate the release of various hormones into the systemic circulation. Included within this region are cells that produce and secrete corticotropin-releasing factor (CRF) and AVP, known regulators of the stress response [61]. Other regions of the parvocellular PVH house several populations of peptide-containing cells that project throughout and beyond the central nervous system to control neurochemical and behavioral responses,

including: a) oxytocin cells that project mainly to the brainstem and spinal cord for autonomic influence; b) AVP cells that project to areas of the hypothalamus and limbic system, as well as the brainstem and spinal cord to regulate blood pressure and osmolarity [59] ; and c) TRH neurons that project to the median eminence to stimulate the release of thyroid stimulating hormone (TSH), which regulates metabolism [3]. Together, these cell groups have the ability to affect all aspects of the stress response, making the PVH the most recognized and studied site in the brain for investigating stress responses and adaptations. In addition to quantifying Fos expression in the PVH of acutely and repeatedly stressed male and female rats, the studies were designed to determine if the phenotypes of Fos-expressing cells are different in acute and repeated restraint.

AVP is normally secreted by magnocellular cells of the PVH and functions in osmoregulation. During chronic or repeated stress, however, it can be released from parvocellular neurons of the PVH, at the median eminence, where it is an important releasing factor for adrenocorticotropin releasing hormone (ACTH) in the anterior pituitary gland. Indeed, at the pituitary level, the effects of CRF are enhanced by AVP, which is increasingly co-expressed and secreted from CRF neurons during prolonged stress [26]. AVP is believed to play an important role in sustaining pituitary responsiveness during chronic stress [38], and CRF and AVP are both essential for coordinating the behavioral and metabolic responses to stress [30].

Circulating GCs bind to glucocorticoid receptors (GRs), low affinity type II corticosteroid receptors that are expressed at high levels throughout the brain and pituitary [57]. A member of the nuclear hormone receptor family of ligand-activated transcription factors [68], GR is activated when corticosteroid concentrations are high, terminates stress responses through negative feedback, and mobilizes required energy resources [30]. Glucocorticoids are able to

exert their negative feedback actions through GR not only in the hypothalamus and pituitary gland, but also in suprahypothalamic regions that are able to influence HPA axis activity [10]. GR facilitates recovery from stress by interfering with transcription and repressing stress-induced responses such as CRF and AVP synthesis [30]. Circulating glucocorticoid concentrations are primarily mediated by the HPA axis [10], and their role in HPA axis inhibition serves to minimize the total tissue exposure to glucocorticoids, thus reducing the catabolic, lipogenic, anti-reproductive and immunosuppressive effects of these hormones [10]. Furthermore, GR promotes memory storage in preparation for future events [30]. Given the highly significant roles of AVP and GR in HPA axis regulation, we examined the PVH in male and female rats under control, acute stress and repeated stress conditions to determine if stress-sensitive neurons would also express these markers.

Aim 1. To identify and characterize PVH neurons that are differentially activated in response to acute compared to repeated stress.

Numerous studies have shown that PVH neurons are consistently activated in response to stressors of various types, and that these neurons tend to be CRF-expressing cells in the medial parvocellular region of the PVH. Responses to repeated stress exposure, however, are more variable and depend on stressor type, severity and duration. In repeatedly restrained animals, the level of Fos induction habituates, and the cells that remain immunoreactive for Fos are still largely CRF-positive but are localized to more ventral regions within the PVH [64]. This suggests that different subpopulations of CRF neurons may be involved in initiating responses to acute stress and maintaining HPA axis control in conditions of repeated or chronic stress. How these cells differ in terms of their recruitment or function, however, is unknown. This Aim was

designed to confirm earlier findings, and extend our investigation into the patterns of expression of other peptides and receptors and more specifically determine the phenotypes of stress-activated PVH cells. This work is novel, in that the markers used to identify the neural pathways involved in stress responses and adaptations have been shown to have stress-related roles, but their direct relationship to PVH control mechanisms is unknown.

Aim 2. To identify mechanisms that may contribute to sex-specific differences in stress processing by examining the activation of PVH neuronal types in stressed female compared to male rats.

Multiple studies have shown that females respond to stress differently than males, and that the consequences of stress exposure, particularly when the stress is prolonged or recurring, also differ by sex. While gonadal steroids likely contribute substantially to these effects, the detailed mechanisms through which stress modifies nervous system and other physiological functions, and the interplay between gonadal hormones and other neural factors controlling the HPA axis, however, remain largely unknown. This Aim was designed to identify and characterize the type and location of neurons in the PVH of female rats that are activated under conditions of acute compared to repeated restraint stress, and compare these findings against the results from Aim 1 in males. This work is novel, in that it is the first study to comprehensively evaluate numerous regulatory peptides in stressed females, with a view toward identifying sex-specific HPA axis control mechanisms that may contribute to the downstream incidence of stress-related disorders.

Methods:

Experimental animals.

Young adult (3-4 months of age) male and female Sprague Dawley rats (Harlan, Houston, TX) were used in the present experiments. All rats were individually housed in standard cages in a temperature-controlled animal facility maintained on a 12:12 hour light:dark cycle, with food and water provided ad libitum. Following shipment, the rats were allowed at least one week of acclimatization to the facility before experimentation was initiated. Rats were randomly assigned to Control, Acute restraint or Repeated restraint groups (n=5/group). Animal care and use were in accordance with the Guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

Restraint stress.

Emotional stress was applied in the form of acute or repeated restraint, which consisted of placing the rats in a plastic restraining device (Kent Scientific, Torrington, CT) for 30 min. Repeatedly stressed rats were restrained in their home cages for 30 min daily over 14 consecutive days. Acutely stressed rats were exposed to open restrainers for 30 min/d over the first 13 days, then restrained for 30 min on the 14th day only. Unstressed control rats were exposed to open restraining devices on each of 14 consecutive days, but were never restrained. All restraint and exposure occurred near the beginning of the light cycle, between the hours of 0900 and 1100.

Perfusion.

Rats were transcardially perfused 2 hours after the termination of restraint or exposure on the final day. Rats were weighed and deeply anesthetized by i.p. injection of 100 mg/kg sodium pentobarbital (Nembutal®; McKesson, Washington Courthouse, OH). Perfusion through the ascending aorta was done using ~100 mL of ice-cold 0.9% saline, followed by 400-500 mL of ice-cold 4% paraformaldehyde (JTBaker, Inc., Pittsburg, NJ) at pH 9.5 in 0.1 M borate buffer. Brain tissues were then collected and post-fixed for 5 hours at 4ºC, followed by cryoprotection overnight at 4ºC in 10% sucrose in KPBS.

Tissue Processing.

The following day, brains were removed from cryoprotectant, mounted on a tabletop freezing microtome (Model SM 2000R; Leica Microsystems, Bannockburn, IL), and serial frozen sections taken in the coronal plane from a block of brain tissue containing the hypothalamic paraventricular nucleus. Five 1:5 series at 30µm intervals were collected into antifreeze (30% ethylene glycol, 20% glycerol) and stored at -20ºC until used for immunohistochemical analyses.

Immunohistochemistry.

Brain tissue sections were immunohistochemically stained for peptides and receptors known to play key roles in the stress response. First, Fos immunoreactivity was measured using a nickel-intensified avidin-biotin-immunoperoxidase technique. Sections were washed in KPBS, then placed in 0.3% hydrogen peroxide to quench endogenous peroxidases and 1% sodium borohydride to reduce free aldehydes. After being washed thoroughly, the tissue was placed in primary antiserum (rabbit anti-Fos; Oncogene Science, Cambridge, MA) diluted 1:50,000 in

KPBS containing 0.3% Triton X-100 and 2% normal goat serum and incubated at 4ºC overnight with gentle agitation. On the following day, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution; Vector Laboratories, Burlingame, CA) for 1h. An avidin-biotin-complexing solution (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) was then applied for 1h, and a nickel-enhanced glucose oxidase method using diaminobenzidine (DAB) as a chromogen was utilized to visualize specific binding [17].

Subsequently, dual localization of Fos with other markers was accomplished using the same immunoperoxidase method as described above but with sequential staining for the second marker done without nickel enhancement. Cells expressing both markers were therefore visible as having black nuclei (Fos) and a brown cytoplasm (second marker). Due to the exothermic nature of the DAB reaction, the concentration of the Fos primary in these analyses was changed to a 1:10,000 dilution and the reaction carried out on ice in order to optimize our double staining. Fos was co-localized with arginine vasopressin (AVP) and the glucocorticoid receptor (GR), using primary antisera at 1:10,000 dilutions (Abcam, Inc., Cambridge, MA), to determine the number of stress-activated cells that also expressed these markers. In all cases, stained sections were mounted on gelatin-coated slides and allowed to dry overnight before being defatted through a graded ethanol series and xylene and coverslipped. Lastly, one series from each rat was stained with 0.25% thionin for Nissl material. These slides were used to evaluate the basic architecture of the brain regions to be examined, and served as a reference during analysis of staining for other markers.

Quantification of Staining.

Light microscopy (AxioScop; Carl Zeiss, Inc., Thornwood, NY) and simple cell counting methods were used to measure immunoreactivity in both single- and dual-staining experiments. Initial counts were made at 20x magnification for Fos-positive and doubly-labeled cells, with a higher magnification (40x) and different focal planes used for verification of double labeling. Cell counts were performed both manually and with the assistance of a digital microimaging system coupled to computer software (AxioCam with AxioVision software; Carl Zeiss, Inc., Thornwood, NY), and quantified in five sections through the PVH. Proper identification of the anatomical region of interest was assured through use of adjacent Nissl series as well as the The Rat Brain atlas of Paxinos and Watson (4th edition, 1998) [18]. Specifically, immunopositive cells were counted bilaterally; the number of cells expressing each marker per side was then summed, and group means, standard deviations and standard errors were calculated.

Statistical Analysis.

The mean number of immunopositive cells was compared and evaluated with a one-way analysis of variance (ANOVA) to determine overall effects, followed by Tukey-Kramer post hoc tests to determine individual differences (JMP program, version 7; SAS Institute, Inc., Cary, NC). Significance was determined at the $p<0.05$ level.

Results:

Body weight.

The body weights of all rats were recorded at the time of perfusion. While males were significantly larger than females in all treatment groups, as would be expected, no significant

differences were seen between treatment groups within either sex: Control males = 358 ± 8g, Acute males = 345 ± 7g, Repeated males = 332 ± 7g, Control females = 255 ± 8g, Acute females = 250 ± 7g, Repeated females = 241 ± 6g.

Immunohistochemical staining for neuroendocrine markers.

Single and dual immunostaining for Fos, AVP and GR yielded numerous cells that were subsequently visualized and quantified throughout the rostrocaudal extent of the PVH (Fig. 1). Localization of a nuclear Fos signal within neurons of the PVH was achieved in both sexes and all treatment groups. Similarly, cytoplasmic staining for AVP or GR was done in conjunction with the Fos labeling, allowing doubly-labeled cells to be seen and counted in tissue sections from all animals.

Fos expression in the PVH of stressed male and female rats.

The profile of neuronal activation in the PVH was similar in male and female rats exposed to acute or repeated restraint stress; significant sex differences were seen, however, in the acute stress paradigm (Fig. 2). The number of cells showing Fos immunoreactivity in the PVH was low in control males (159 ± 3), but increased significantly in response to acute restraint stress (657 ± 87). With repeated stress exposure, a habituation was seen in the number of activated cells (210 ± 47) such that Fos expression was restored to control levels in this group. Female rats displayed qualitatively similar responses to stress as the males, with acute stress increasing the number of Fos-positive cells in the PVH (329 ± 64) over that seen in controls (76 ± 13) and repeated restraint leading to a complete habituation of the Fos response (88 ± 11). It should be noted that the absolute level of Fos expression following acute restraint was significantly less in

females compared to males. No sex differences were found in the number of neurons expressing Fos in the control or repeated stress conditions. The overall effect of stress treatment on Fos expression was significant ($F=19.97$; $p<0.0001$).

Stress-induced activation of AVP-expressing neurons.

Stress-sensitive PVH neurons that also express AVP were identified by dual localization of Fos and AVP in individual cells, which were seen in all groups. The absolute number of Fos- and AVP-expressing neurons was increased by acute stress and decreased following repeated stress exposure in both males and females, resulting in an overall treatment effect of stress ($F=20.13$; $p<0.0001$), but no sex differences were seen within any of the stress treatment groups (Fig. 3A). Normalization of the data so that cells expressing both Fos and AVP are given as a percentage of the number of neurons staining positively for Fos alone, however, yielded some interesting trends (Fig. 3B). The overall effect of stress when the data are presented in this way was non-significant ($F=2.42$; $p=0.06$), but a strong trend toward a decrease in AVP neuronal activation after acute stress (21 ± 4) compared to control (34 ± 3) was seen in the males ($p=0.04$ by t-test). Likewise, a trend ($p=0.09$ by t-test) was observed between males and females during acute stress, with females tending to show a greater relative recruitment of AVP-expressing neurons.

Sex differences in the recruitment of GR-expressing neurons during stress.

The activation of neurons staining positively for GR was seen in all control and stressed rat groups, with an overall significant effect of stress on Fos and GR co-expression ($F=11.41$;

$p<0.0001$) (Fig. 4). Acute restraint increased the absolute number of Fos- and GR-labeled PVH cells in both males and females, and both sexes again had numbers of dually-stained neurons that were similar to control levels after repeated stress exposure (Fig. 4A). No sex differences were seen for any treatment group, but when the number of cells expressing both markers was normalized to the total number of Fos-expressing cells, significant sex-dependent responses then became apparent (Fig. 4B). Male rats showed equivalent numbers of Fos- and GR-expressing cells regardless of whether they were stressed or not, while females showed a trend (non-significant) toward increasing recruitment of GR-expressing neurons as their exposure to stress increased. In addition, females in the acute and repeated restraint groups displayed significantly greater numbers of neurons co-expressing Fos and GR than their male counterparts in the same treatment groups (43 ± 9 and 50 ± 8 compared to 15 ± 2 and 15 ± 3, respectively).

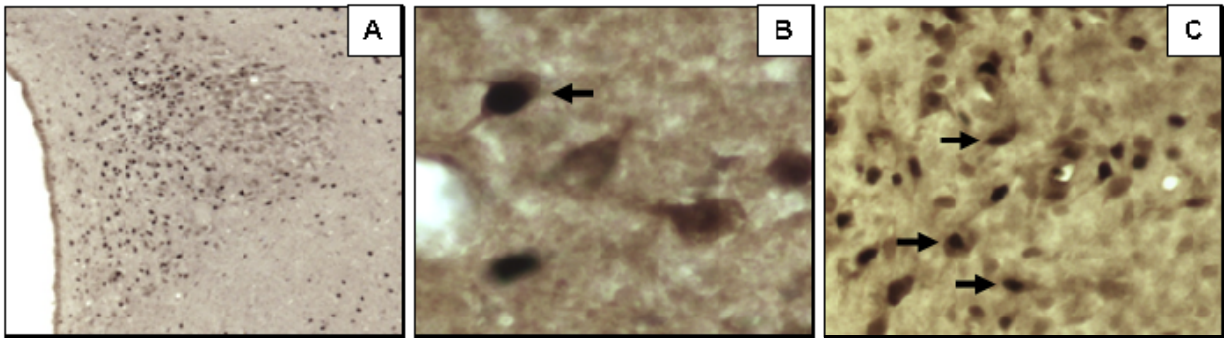

Figure 1. *Immunohistochemical staining for Fos, AVP and GR*. Neuronal markers were localized to PVH cells using single- or dual-label immunohistochemical staining, and an immunoperoxidase method with or without nickel enhancement. Numerous stress-sensitive neurons are seen in a representative photomicrograph (5x magnification) of the PVH from an acutely stressed male rat stained for Fos immunoreactivity in panel A. Co-localization of Fos with AVP was also demonstrated (panel B), in which dual staining for Fos (black nuclei) and AVP (brown cytoplasm) was performed. A single cell expressing both markers can be seen (black arrow) in this image, which was captured at 40x magnification. As shown in panel C, many cells were seen to co-express Fos and GR in the PVH, some of which are indicated by the black arrows in this image (20x magnification).

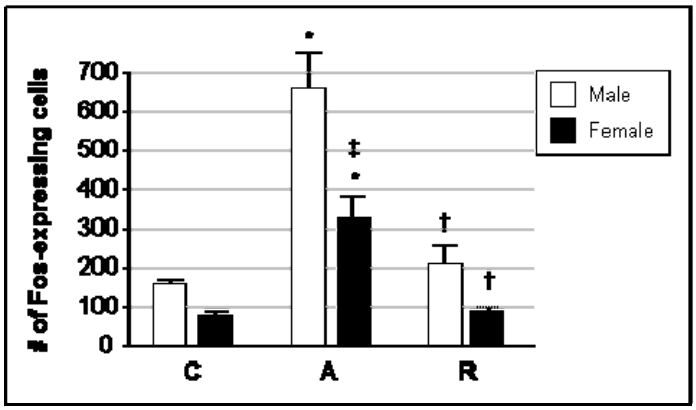

Figure 2. *Quantification of PVH cells for Fos.* The absolute numbers of cells expressing Fos were quantified in the PVH by simple cell counting. Counts were made at 20x magnification. Five sections were counted through the PVH for each animal (n = 5/group). C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Bars represent group averages + SE. Symbols indicate significant difference ($p>0.05$) from C (*) or A (†) or between genders (‡).

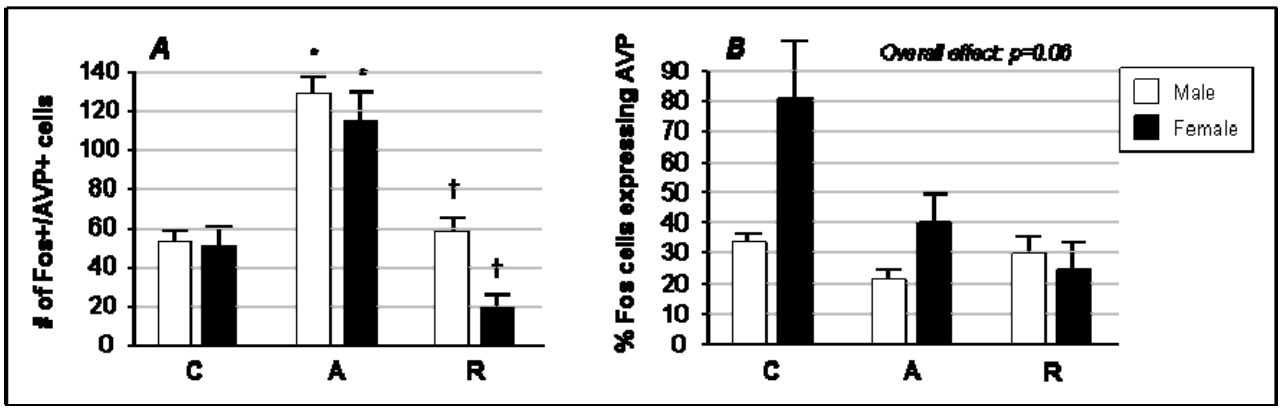

Figure 3A. *Quantification of PVH cells doubly-stained for Fos and AVP*. The absolute number of cells expressing both Fos and AVP were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). Bars represent group averages + SE. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from C (*) or A (†). No significant differences were seen between sexes.

Figure 3B. *Percentage of Fos+ cells that also express AVP*. The number of cells expressing both Fos and AVP is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats.

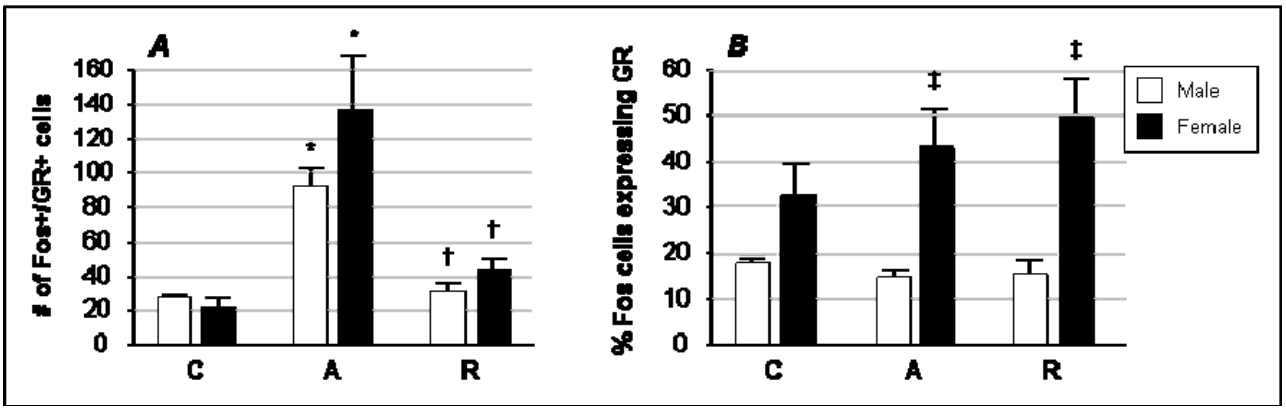

Figure 4A. *Quantification of PVH cells doubly-stained for Fos and GR.* The absolute number of cells expressing both Fos and GR were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from C (*) or A (†). No significant differences were seen between sexes.

Figure 4B. *Percentage of Fos+ cells that also express GR.* The number of cells expressing both Fos and AVP is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) between genders (‡).

Summary:

Table 1. Immunohistochemical data summary for stress related hormone expression in male/female rats for Fos, Fos/AVP and Fos/GR.

Male				Female		
Control	Acute	Repeated		Control	Acute	Repeated
X = ‡159 SE = 13	X = *‡657 SE = 87	X = †‡229 SE = 47	**Fos**	X = 76 SE = 13	X = * 329 SE = 64	X = † 88 SE = 11
X = 31 SE = 5	X = * 246 SE = 68	X = † 47 SE = 7	**Fos/AVP**	X = 27 SE = 5	X = * 246 SE = 78	X = * 64 SE = 19
X = 45 SE = 7	X = * 187 SE = 34	X = † 77 SE = 18	**Fos/GR**	X = 37 SE = 10	X = * 163 SE = 26	X = * 112 SE = 23

Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). Averages (X) and Standard Errors (SE) are given for C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from C (*), A (†) or between sex (‡) within the same experimental group.

Discussion:

Numerous studies have demonstrated that males and females differ in their responses to stress [3, 4, 19-21], and further evidence in support of these findings comes from the results of the present work. We have identified changes in neuronal activation and the recruitment of specific cell types within the PVH that may be differentially involved in the initiation and termination of stress responses through their ability to modulate HPA axis function. Hyperactivity of the HPA axis is frequently seen in patients suffering from major depression and has also been associated with increased susceptibility to infectious diseases, whereas HPA axis hypoactivity has been correlated with autoimmune diseases [19]. Therefore, understanding the biological mechanisms that underlie these differences and contribute to dysregulation of the HPA axis will provide information about the pathology and progression of stress-related disorders, and will aid the development of preventative and therapeutic strategies to alleviate these maladies [4]. In addition, sex has a significant influence over the normal functioning of the brain, with males and females showing differences in neurotransmitter levels, pain perception, emotion, memory, and vision, as well as in stress hormone action and the manifestation of various disease states [20]. The neurobiological correlates of stress identified in this study may affect the ability of males and females to cope with stressful situations, and may inform new approaches for the treatment of sex-specific illnesses that result from chronic stress exposure.

Neuronal activation in response to stress.

As shown previously [10], neurons expressing Fos as a consequence of stress were primarily localized within the medial parvocellular component of the PVH in both sexes. Acute restraint

led to an increased number of Fos-positive nuclei throughout this region, while the habituated response seen following repeated restraint exposure involved Fos-expressing cells that were more restricted to the ventral subregion of the medial parvocellular PVH. Therefore, while the entire PVH was quantified in our experiment, results on the numbers of cells doubly-labeled for Fos and AVP or Fos and GR, below, are driven by the pattern of Fos expression in this nucleus.

The response to acute stress was diminished in females, compared to males, in terms of the absolute number of PVH neurons expressing Fos as a consequence of a single exposure to restraint. This could suggest that females have a reduced sensitivity to stress, and recruit fewer neurons than males in response to the same stimulus at the time of initial exposure. Alternatively, given that the female adrenal gland has been shown to exhibit enhanced sensitivity to ACTH [21], perhaps a lesser level of PVH activation is sufficient to produce the appropriate neuroendocrine response needed to deal effectively with the stressor. A final possibility is that males and females may excite the same proportion of PVH neurons, but that the absolute number of activated cells is reduced in the females due to a smaller brain size or PVH nucleus volume. This possibility cannot be discounted at this time, as volumetric analysis of the PVH was not performed in this study. However, our data do demonstrate that the level of activation in acutely stressed females (433% of control) is similar to that in acutely stressed males (413% of control).

Body weight.

Body weights at the time of perfusion were not significantly different across treatment groups within either sex. There was a trend, however, toward decreasing body weights with increasing numbers of stress exposures. When compared by t-test, the p value was 0.055

between control males and males subjected to repeated restraint. Therefore, while these data do not demonstrate a significant effect of continued stress, they do suggest, as least, that the animals may have been subjectively experiencing stress throughout the study even though our findings show clearly habituated responses to repeated restraint in terms of neuronal activation.

The role of AVP in stress responses.

The number of stress-sensitive PVH neurons co-expressing AVP was increased in acutely stressed rats compared to unstressed controls, and the number of activated, AVP-expressing cells habituated with repeated compared to acute stress exposure. These effects are likely a function of changing levels of Fos expression in each stress condition, and do not reflect changes in AVP expression per se, as absolute counts of Fos-positive and Fos- and AVP-positive cells followed the same profile. No sex differences were observed in any of the treatment groups, but expressing the number of neurons doubly-labeled for Fos and AVP as a percentage of the total number of Fos-positive cells gave rise to the suggestion of possible sex differences in both control and acutely stressed groups. While the results were statistically non-significant, females tended to display greater numbers of activated AVP neurons under basal (unstressed) conditions and after acute restraint. Male rats, in contrast, appear to de-recruit AVP-expressing cells during acute exposure to restraint stress. These findings, although somewhat less than convincing, do not entirely preclude the possibility that females may require fewer PVH neurons to be activated in order to mount an effective response to an acute stressor, due to their increased utilization of AVP as a co-secretagogue for ACTH. This is particularly interesting in light of the fact that other researchers have suggested a greater role for AVP during situations of chronic, and not

acute, stress [22-26]. Our data allow for additional alternative interpretations, as well. First, the derecruitment of AVP neurons by females with increasing exposure to a homotypic stressor may indicate that other neuronal types or mechanisms play a more critical role in modulating the HPA axis and are becoming activated at the expense of this cell type. Second, as we have not quantified the number of AVP-expressing neurons that were Fos-negative, there is still room for the idea of an enhanced role for AVP during repeated stress. Finally, repeated exposures to corticosterone during stress applications could subsequently and progressively prevent the activation of AVP neurons, protecting the organism from excessive responses to that stressor. Future studies will be needed to further define the mechanisms at play in this system.

GR expression and implications for GC feedback.

Similar to the results seen in Fos- and AVP-expressing cells, the number of neurons co-expressing Fos and GR was elevated by acute restraint, habituated in response to repeated stress, and was not different between males and females in any treatment group. Once again, however, consideration of the number of cells expressing Fos and GR as a percentage of those expressing Fos alone yielded some interesting sex differences that were largely significant in this case. In all stress treatment groups, females had increased numbers of Fos- and GR-expressing PVH neurons compared to males, with the acutely and repeatedly stressed females showing statistically greater numbers of doubly-stained cells. Although not significant, the trend was for females to activate increasing numbers of GR-positive neurons as a function of increasing exposures to stress. This suggests that females may be more sensitive to GC negative feedback, which may serve to dampen their responses to stress or terminate those responses more quickly.

If, as stated above, females also show enhanced release of GCs from the adrenal gland after stimulation by ACTH, then they may also be able to respond to increases in circulating GCs at a more rapid pace than males.

It is possible that the increased number of GR-expressing cells that also stain positively for Fos in acutely restrained animals of either sex reflects a kind of compensatory mechanism. In this role, GR could allow for rapid termination of the stress response, which would be appropriate under the conditions of our paradigm in which the stress is emotional (i.e., not posing an immediate survival threat to the organism) and short-lived. Perhaps more interesting is the fact that females display a larger percentage of activated GR neurons than males, again suggesting that they may be more sensitive to negative feedback. Convincing evidence for sex differences in negative feedback sensitivity comes from Louvart et al. [27], in which females exposed to footshock showed enhanced corticosterone negative feedback to subsequent restraint, compared to males. In addition, Kudielka and Kirshbaum [3] discuss the ability of estrogen to regulate the HPA axis and its sensitivity to negative feedback. Lastly, Weiser and Handa [28] demonstrate that individuals with affective disorders have impaired glucocorticoid negative feedback, which is mediated at least in part by estradiol acting through the estrogen receptor α in the PVH.

Steroid hormone regulation in neuroendocrine responses to stress.

Basal sex differences in HPA axis function must also be considered in our findings on central nervous system activational responses to stress. It has been shown previously that circulating glucocorticoid levels are higher in females than males under resting conditions [29].

Furthermore, average daily corticosterone levels fluctuate according to estrus cycle stage in females, with females showing equivalent levels to males during estrus but higher values during proestrus, metestrus and diestrus [30]. It is therefore likely that our female subjects had higher concentrations of glucocorticoid hormones on board throughout the majority of our experimental paradigm, which could play a role in the central nervous system activational responses shown in our data. For example, heightened glucocorticoid negative feedback in females could contribute to the lower numbers of Fos-positive neurons seen after acute stress exposure, or the relatively higher numbers of activated AVP- or GR-expressing neurons in females at baseline.

It is also clear from the aforementioned references that estrogen has a significant impact on the HPA axis, and this has also been found to be true under conditions of stress. Estrogen has been shown to regulate both HPA axis function and the sensitivity of the axis to glucocorticoid negative feedback [3, 27]. In addition, Weiser and Handa [28] have demonstrated a mechanism through which estrogen can impair glucocorticoid negative feedback on the HPA axis by acting through the estrogen receptor-α in the PVH. Additional experiments are needed to more fully determine the basis for the sex differences seen in our results. However, we have identified critical differences in the stress-induced activation of neurons which may underlie sex-specific mechanisms involved in the neural processing of stress.

Future studies will need to include assessments of ACTH and/or corticosterone levels to more accurately outline the complex and interactive mechanisms responsible for our observed results. The existing literature shows larger responses to a multitude of stressors in females compared to males, but a majority of these studies show that the heightened responses in females originate from increased adrenal sensitivity to ACTH and higher adrenal hormone secretion. Less is known about sex differences in stress responses at the level of the central nervous system

and, specifically, the PVH level of the HPA axis. Our data show that females display fewer Fos-expressing cells than males after acute restraint (although the percent increase from control is similar), but habituate similarly to males after repeated restraint exposure. In addition, females have a higher level of activation of GR-expressing cells than males after both acute and repeated stress exposure, suggesting that they may be able to respond more rapidly to hormonal signals designed to terminate the response to stress. Whether or not PVH responses are actually terminated faster, or subsequent responses are dampened, cannot be determined by the present data. It is possible, however, that the longer-lasting circulatory hormone profiles previously seen in females could be due to changes in peripheral secretory or clearance mechanisms which are regulated separately from the activities of the central nervous system. Future work will be needed to appropriately address these questions.

CHAPTER III

FOS AND CRF OR MC4R IN THE PVH

Introduction:

As mentioned previously, CRF is widely known for its role in initiation of the stress response. Several studies have shown PVH activation due to stress, often measured by *Fos* and/or CRF mRNA or protein expression, as well as ACTH and corticosteroid levels in the blood [45, 18, 22, 29]. Fos expression in both acutely and repeatedly stressed animals is mostly seen in the neurons that express CRF, which gave rise to our suggestion that a distinct subset of CRF-expressing neurons in the ventral region of the medial parvocellular PVH is responsible for maintaining stress responses in the repeated condition. CRF is produced by parvocellular neurons in the PVH and released into the hypophyseal portal blood vessels to activate the HPA axis [26], producing a broad range of autonomic, electrophysiological and behavioral effects which are characteristic of neurotransmitter or neuromodulator roles in the brain [56]. Similar to Fos, CRF synthesis and secretion is increased in acutely stressed rats, and habituates with repeated stress exposure [11, 13].

Melanocortin-4-Receptor (MC4R) function in the hypothalamus modulates HPA axis activity as well as feeding, grooming and reproductive behaviors [9]. This receptor is expressed in many brain regions, including both the parvocellular and magnocellular regions of the PVH [9]. More importantly, MC4R mRNA is expressed in the same areas where both CRF and AVP are synthesized, and may regulate the release of these peptides [9]. Specifically, MC4R mRNA has been shown to overlap with CRF-expressing neurons in ventral regions of the medial parvocellular PVH, where Fos and CRF also have been co-localized following repeated restraint stress [35]. This suggests that PVH neurons that continue to be activated following repeated

stress may be linked to long-term physiological responses that are controlled by the melanocortin system. Therefore, the present study sought to further identify the different types of PVH neurons activated under acute and repeated stress conditions, by evaluating the stress-induced activation of CRF and MC4R neurons.

Methods:

Experimental animals.

Young adult (3-4 months of age) male and female Sprague Dawley rats (Harlan, Houston, TX) were used in the present experiments. All rats were individually housed in standard cages in a temperature-controlled animal facility maintained on a 12:12 hour light:dark cycle, with food and water provided ad libitum. Following shipment, the rats were allowed at least one week of acclimatization to the facility before experimentation was initiated. Rats were randomly assigned to Control, Acute restraint or Repeated restraint groups (n=5/group). Animal care and use were in accordance with the Guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

Restraint stress.

Emotional stress was applied in the form of acute or repeated restraint, which consisted of placing the rats in a plastic restraining device (Kent Scientific, Torrington, CT) for 30 min. Repeatedly stressed rats were restrained in their home cages for 30 min daily over 14 consecutive days. Acutely stressed rats were exposed to open restrainers for 30 min/d over the first 13 days, then restrained for 30 min on the 14th day only. Unstressed control rats were exposed to open restraining devices on each of 14 consecutive days, but were never restrained.

All restraint and exposure occurred near the beginning of the light cycle, between the hours of 0900 and 1100.

Perfusion.

Rats were transcardially perfused 2 hours after the termination of restraint or exposure on the final day. Rats were weighed and deeply anesthetized by i.p. injection of 100 mg/kg sodium pentobarbital (Nembutal®; McKesson, Washington Courthouse, OH). Perfusion through the ascending aorta was done using ~100 mL of ice-cold 0.9% saline, followed by 400-500 mL of ice-cold 4% paraformaldehyde (JTBaker, Inc., Pittsburg, NJ) at pH 9.5 in 0.1 M borate buffer. Brain tissues were then collected and post-fixed for 5 hours at 4ºC, followed by cryoprotection overnight at 4ºC in 10% sucrose in KPBS.

Tissue Processing.

The following day, brains were removed from cryoprotectant, mounted on a tabletop freezing microtome (Model SM 2000R; Leica Microsystems, Bannockburn, IL), and serial frozen sections taken in the coronal plane from a block of brain tissue containing the hypothalamic paraventricular nucleus. Five 1:5 series at 30µm intervals were collected into antifreeze (30% ethylene glycol, 20% glycerol) and stored at -20ºC until used for immunohistochemical analyses.

Immunohistochemistry.

Brain tissue sections were immunohistochemically stained for peptides and receptors known to play key roles in the stress response. Dual localization of Fos with other markers was

accomplished using the same immunoperoxidase method as described in chapter 1 but with sequential staining for the second marker done without nickel enhancement. Cells expressing both markers were therefore visible as having black nuclei (Fos) and a brown cytoplasm (second marker). Due to the exothermic nature of the DAB reaction, the concentration of the Fos primary in these analyses was changed to a 1:10,000 dilution and the reaction carried out on ice in order to optimize our double staining. Fos was co-localized with corticotropin-releasing factor (CRF) and the melanocortin-4-receptor (MC4R), using primary antisera at 1:10,000 dilutions (Abcam, Inc., Cambridge, MA), to determine the number of stress-activated cells that also expressed these markers. In all cases, stained sections were mounted on gelatin-coated slides and allowed to dry overnight before being defatted through a graded ethanol series and xylene and coverslipped. Lastly, one series from each rat was stained with 0.25% thionin for Nissl material. These slides were used to evaluate the basic architecture of the brain regions to be examined, and served as a reference during analysis of staining for other markers.

Quantification of Staining.

Light microscopy (AxioScop; Carl Zeiss, Inc., Thornwood, NY) and simple cell counting methods were used to measure immunoreactivity in both single- and dual-staining experiments. Initial counts were made at 20x magnification for Fos-positive and doubly-labeled cells, with a higher magnification (40x) and different focal planes used for verification of double labeling. Cell counts were performed both manually and with the assistance of a digital microimaging system coupled to computer software (AxioCam with AxioVision software; Carl Zeiss, Inc., Thornwood, NY), and quantified in five sections through the PVH. Proper identification of the anatomical region of interest was assured through use of adjacent Nissl series as well as the The

Rat Brain atlas of Paxinos and Watson (4th edition, 1998) [18]. Specifically, immunopositive cells were counted bilaterally; the number of cells expressing each marker per side was then summed, and group means, standard deviations and standard errors were calculated.

Statistical Analysis.

The mean number of immunopositive cells was compared and evaluated with a one-way analysis of variance (ANOVA) to determine overall effects, followed by Tukey-Kramer post hoc tests to determine individual differences (JMP program, version 7; SAS Institute, Inc., Cary, NC). Significance was determined at the $p<0.05$ level.

Results:

Stress-induced activation of CRF-expressing neurons.

Stress-sensitive PVH neurons that also express CRF were identified by dual localization of Fos and CRF in individual cells, which were seen in all groups. In figure 4A, the absolute number of Fos- and CRF-expressing neurons was increased by acute stress and significantly decreased following repeated stress exposure in both males and females ($p < 0.05$). However, no sex differences were seen in any of the stress treatment groups. When cells expressing both Fos and CRF are given as a percentage, females show a greater relative recruitment of CRF-expressing neurons; though this trend is non-significant (Fig. 4B).

Expression of MC4R-PVH neurons.

Fos positive neurons that are also positive for MC4R in the PVH, showed significant sex differences in all stress-treated groups ($p < 0.05$) (Fig. 5A). MC4R also showed to be unaltered due to the different stress paradigms, as the data shows to be non-significant among all three

groups ($p > 0.05$). When looking at the percent of Fos positive cells also expressing MC4R, although non-significat, females preferentially recruit more MC4R than males especially under basal or control conditions (Fig. 5B)

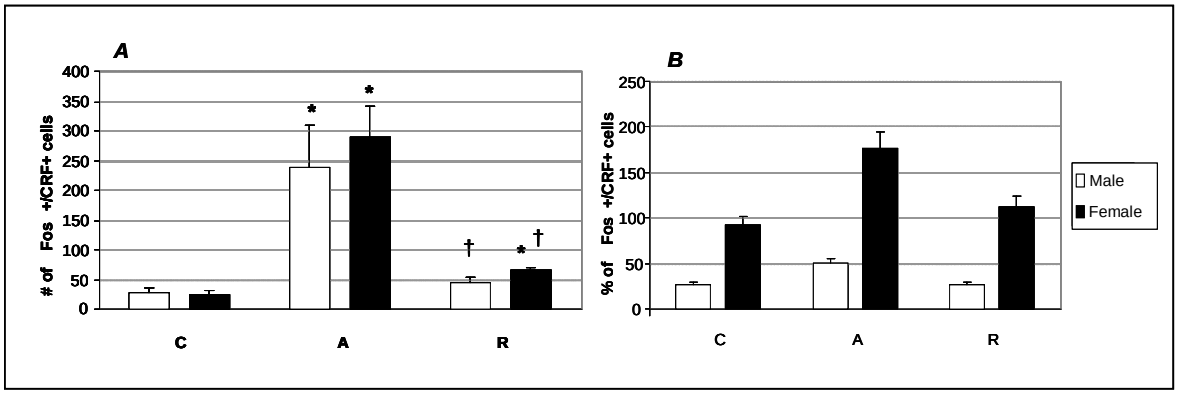

Figure 5A. *Quantification of PVH cells doubly-stained for Fos and CRF.* The absolute number of cells expressing both Fos and CRF were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from C (*) or A (†). No additional significant differences were seen between sexes.

Figure 5B. *Percentage of Fos+ cells that also express CRF.* The number of cells expressing both Fos and CRF is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats.

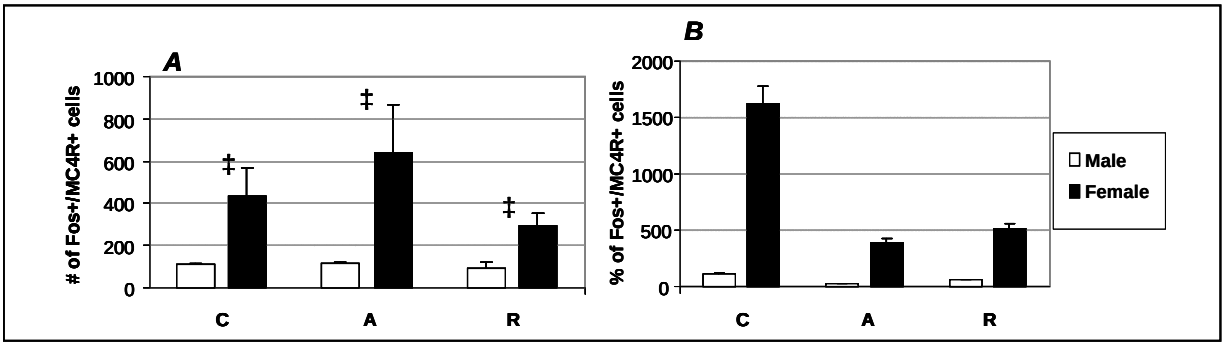

Figure 6A. *Quantification of PVH cells doubly-stained for Fos and MC4R.* The absolute number of cells expressing both Fos and MC4R were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant differences between sexes (‡). No significant differences were seen among experimental groups in each sex.

Figure 6B. *Percentage of Fos+ cells that also express MC4R.* The number of cells expressing both Fos and MC4R is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats.

Summary:

Table 2. Immunohistochemical data summary for stress related hormone expression in male/female rats for Fos/CRF and Fos/MC4R.

Male				Female		
Control	Acute	Repeated		Control	Acute	Repeated
X = 21 SE = 6	X = 204 SE = 56	X = † 34 SE = 9	**Fos/CRF**	X = 17 SE = 5	X = * 269 SE = 51	X = *† 57 SE = 15
X = ‡ 116 SE = 5	X = ‡ 128 SE = 6	X = ‡ 107 SE = 25	**Fos/MC4R**	X = 472 SE = 105	X = 559 SE = 154	X = 277 SE = 65

Counts were made at 20x magnification, and double staining was verified at 40x magnification. Five sections were counted through the PVH for each animal (n = 5/group). Averages (X) and Standard Errors (SE) given for C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from C (*), A (†) or between sex (‡) within the same experimental group.

Discussion:

The role of CRF in stress responses.

The number of stress-responsive neurons in the PVH co-expressing CRF was increased in acutely stressed rats compared to unstressed controls, and the number of activated, CRF-expressing cells habituated with repeated compared to acute stress exposure. No sex differences were observed in any of the treatment groups; however when looking at the percentage of doubly-labeled expressing both Fos and CRF, it gave rise to the suggestion of possible sex differences. While the results were statistically non-significant, females tended to display greater numbers of activated CRF neurons under all stress conditions. As mentioned before, do not entirely exclude the possibility that females may require fewer PVH neurons to be activated in order to mount an effective stress. It has been suggested that estradiol may directly enhance the CRF gene transcription in the hypothalamus by binding the estrogen responsive elements in the CRF gene [32]. Although non-significant, Estrogen's stimulating effects on the HPA axis may be, in part, responsible for females showing a higher percentage on CRF recruitment in both basal and stressed conditions compared to their male counterparts. Future studies will be needed to further define the mechanisms at play in this system.

The role of MC4R in stress responses.

The number of neurons co-expressing Fos and MC4R was highest and remained highest in females throughout all three stress-treated groups. Though the sex differences seen are significant, it doesn't seem to be as a result of the stress paradigm instilled for each group. Co-expression of Fos and MC4R remained low across all groups in males and fairly high in females. When we looked at the percentage of MC4R positive cells that were also positive for fos,

expression was highest in control females and then seemed to be de-recruited during acute and repeated stress. MC4R has been known to regulate feeding, which would be highest during non-stress (control) conditions, when the parasympathetic nervous system is in control. Not much is know yet about sex differences in MC4R as a function of stress; therefore further studies will have to be conducted to elucidate any role MC4R may play in the stress response.

CHAPTER IV

A ROLE FOR ESTROGEN IN THE STRESS RESPONSES

Introduction:

Given the sex differences identified in earlier experiments of this research project, the goal of this portion of the research project was to establish whether gonadal steroids, and estrogen, in particular, play a role in mediating sex differences in the response to acute or repeated emotional stress. While numerous effectors are known to contribute to the regulation of the HPA axis, previous studies in which we controlled for all variables other than gender still resulted in differential responses in male and female rats under both acute and repeated stress conditions. It remains unclear, however, whether the factors influencing HPA axis responsiveness involve or are independent of the activational effects of gonadal steroids [46]. Our working hypothesis was that the sex differences observed in PVH neuronal activation following stress exposure result from changes in HPA axis sensitivity, which is developmentally and functionally regulated by circulating gonadal steroids. The studies determined acute and repeated restraint stress responses in ovariectomized (OVX) female rats, and in OVX rats replaced with estradiol. We examined the levels of Fos induction as a marker of neuronal activation in these rats, and localized Fos expression within cells immunohistochemically staining for other proteins involved in HPA axis control and shown previously (Chapter 1) to differ between males and females in terms of their activation. Our specific hypotheses were that: 1) OVX would cause female rats to exhibit male-like patterns of Fos expression and co-localization with other neuronal markers in response to acute and repeated restraint stress; and 2) replacement with estradiol would re-establish the female patterns of stress responsiveness in OVX rats.

Data from the literature have stated that estrogen may play a role in enhancing HPA axis activity, by way of increasing CRF mRNA expression, leading to increased levels of circulating glucocorticoids, and conversely leading to a decrease in GR mRNA [51]. Hypercortisolemia can lead to a variety of disorders including depression, Cushing's disease, obesity and osteoporosis [30]. Several studies have shown sex differences in HPA axis activity via blood corticosterone levels in stressed male and female rats, with females exhibiting higher corticosterone secretions [51]. It is important to examine the role of estrogen on the generation of neural responses, in order to gain a better understanding on possible estrogenic mechanisms underlying stress-induced sex pathologies.

Sex-related differences in the stress response have been presented in the literature for more than 40 years, and variations in circulating gonadal steroids are often speculated to be the reason for observed disparities between males and females [46]. Sex differences in endocrine function are proposed to result from the two modes of action utilized by gonadal steroids, termed organizational and activational effects [42]. Gonadal steroids present around the time of birth are able to organize neuronal substrates, thus resulting in life-long alterations in endocrine function; activational effects, in contrast, are mediated by the influences of circulating levels of these hormones at any given time, and these effects can be reversed by castration or mimicked by steroid replacement [42].

Several studies have shown that circulating levels of estradiol increases the secretion of both ACTH and cortisol; while dihydrotestosterone, the non-aromatizable form of testosterone, decreases the secretion of both ACTH and cortisol in response to stress [46]. In rats, castrated males show an increase in both basal and stress-induced release of corticosterone in comparison to controls, providing support of an inhibitory role for testosterone [51]. Also, a decrease in

corticosterone levels were seen in ovariectomized rats, providing an indication of an excitatory role for estrogen [51]. Basal and stress activated levels of corticosterone release have also been shown to differ across the estrous cycle [37]. Basal corticosterone levels are higher during the proestrus phase of the estrous cycle, where estrogen levels tend to be the highest [51].

Estrogen is a neuroactive hormone that stabilizes neuronal functioning and supports neuronal viability [4]. Estradiol has been implicated in playing a major role in the brain: with altering neurotransmitter functioning, influencing differential limbic activity, influencing HPA axis functioning, as well as having an impact on neuronal plasticity [21]. In the estrous cycle, estradiol concentrations peak during the proestrus phase, which occurs every fourth to fifth day in rats [21]. Studies have confirmed that the amount of circulating estrogen in the female rat at any given time may have an effect on its endocrine responses to stress; consequently, CRF mRNA and corticosterone blood levels are highest during the proestrus phase of the estrous cycle [6]. Attributable to estrogen's vast role as a gonadal steroid and it's functioning in the sex differences exhibited in the stress response, it is vital to examine its influences in our research to gain a better understanding as to the existence of sex-specific pathologies seen in chronic stress exposures.

Aim 1. To examine the effect of OVX on the generation of neural responses to acute and repeated stress in females.

Differences seen in the activation of neurons of varying phenotypes in Chapter 1 might be explained by the presence of different gonadal steroid hormones in male and female rats. This Aim investigated the role of estrogen in controlling neural responses to acute and repeated stress in females, by performing OVX, with or without estradiol replacement, in these animals.

Aim 2. To clarify the influence on estrogen on the development and responsiveness of the PVH in adult females exposed to acute or repeated stress.

Ample evidence supports the idea that responses to stress that differ in females compared to males result from the influences of estrogen on the HPA axis and brain pathways and mechanisms that contribute to its activity. This Aim specifically tested the ability of estrogen to modify the activation levels of PVH neurons of different types, under conditions of acute and repeated stress.

Methods:

Experimental animals.

Young adult (3-4 months of age) male and female Sprague Dawley rats (Harlan, Houston, TX) were used in the present experiments. All rats were individually housed in standard cages in a temperature-controlled animal facility maintained on a 12:12 hour light:dark cycle, with food and water provided ad libitum. Following shipment, the rats were allowed at least one week of acclimatization to the facility before experimentation was initiated. Rats were randomly assigned to Control, Acute restraint or Repeated restraint groups (n=5/group). Animal care and use were in accordance with the Guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

Ovariectomies.

To investigate the role of estrogen in brain responses to stress, female rats were ovariectomized prior to being acutely or repeatedly stressed and evaluated for brain cell activation. All individuals underwent bilateral ovariectomy (OVX) between 9 and 11 weeks of

age. OVX was completed before 60 days of age, when normal cycling typically begins in females [41]. Rats were anesthetized with ~ 0.3 ml of Rompun mixture [ketamine/xylazine/acepromazine, 25:5:1 mg/kg, s.c.], with inhaled isoflurane gas anesthesia available as a supplement, using 1 cc syringes and 26G x 3/8" needles. Skin incisions were made bilaterally on the dorsal surface below the ribcage, and blunt dissection was used to isolate the ovaries in the abdominal cavity. The ovaries, located in fat pads just below the musculature, were grasped with forceps and exteriorized through the incision. Forceps were used to gently crush the fallopian tubes at the tip of the dorsal horn of the uterus, sutures were wrapped tightly underneath to cut off blood supply, and the ovaries were removed by cutting above to clamped area. Care was taken not to crush or otherwise disturb the ovary during excision. The remaining uterine and fat tissue was returned to the abdominal cavity, muscle tissue was sutured using 2-3 surgeon knots while being careful not to tear or pull tissue too tight, and skin incisions were closed using wound clips. These steps were then repeated on the contralateral side. Rats were allowed 6 days for recovery before being subjected to the restraint stress paradigm.

The methods employed for Aim 2 are identical to those described in Aim 1, above, except that OVX females were replaced with estradiol to determine whether the re-introduction of circulating estrogen levels leads to the recovery of normal female responses to acute and repeated restraint stress. Therefore, at the time of OVX, some of the OVX rats were implanted subcutaneoulsy with 17β-estradiol (1.5 mg/pellet, 120-day release; Innovative Research of America, Sarasota, FL, USA), to form a 17β-estradiol-treated OVX group (OVXE2). Rats in the sham OVX (SO) and OVX groups received control pellets subcutaneously (placebo for 17β-estradiol).

Restraint stress.

Emotional stress was applied in the form of acute or repeated restraint, which consisted of placing the rats in a plastic restraining device (Kent Scientific, Torrington, CT) for 30 min. Repeatedly stressed rats were restrained in their home cages for 30 min daily over 14 consecutive days. Acutely stressed rats were exposed to open restrainers for 30 min/d over the first 13 days, then restrained for 30 min on the 14th day only. Unstressed control rats were exposed to open restraining devices on each of 14 consecutive days, but were never restrained. All restraint and exposure occurred near the beginning of the light cycle, between the hours of 0900 and 1100.

Perfusion.

Rats were transcardially perfused 2 hours after the termination of restraint or exposure on the final day. Rats were weighed and deeply anesthetized by i.p. injection of 100 mg/kg sodium pentobarbital (Nembutal®; McKesson, Washington Courthouse, OH). Perfusion through the ascending aorta was done using ~100 mL of ice-cold 0.9% saline, followed by 400-500 mL of ice-cold 4% paraformaldehyde (JTBaker, Inc., Pittsburg, NJ) at pH 9.5 in 0.1 M borate buffer. Brain tissues were then collected and post-fixed for 5 hours at 4ºC, followed by cryoprotection overnight at 4ºC in 10% sucrose in KPBS. Blood samples were also collected for measurement of circulating estrogen levels at the time of perfusion, to confirm the success of the OVX procedure.

Tissue Processing.

The following day, brains were removed from cryoprotectant, mounted on a tabletop freezing microtome (Model SM 2000R; Leica Microsystems, Bannockburn, IL), and serial frozen sections taken in the coronal plane from a block of brain tissue containing the hypothalamic paraventricular nucleus. Five 1:5 series at 30μm intervals were collected into antifreeze (30% ethylene glycol, 20% glycerol) and stored at -20ºC until used for immunohistochemical analyses.

Immunohistochemistry.

Brain tissue sections were immunohistochemically stained for peptides and receptors known to play key roles in the stress response. First, Fos immunoreactivity was measured using a nickel-intensified avidin-biotin-immunoperoxidase technique. Sections were washed in KPBS, then placed in 0.3% hydrogen peroxide to quench endogenous peroxidases and 1% sodium borohydride to reduce free aldehydes. After being washed thoroughly, the tissue was placed in primary antiserum (rabbit anti-Fos; Oncogene Science, Cambridge, MA) diluted 1:50,000 in KPBS containing 0.3% Triton X-100 and 2% normal goat serum and incubated at 4ºC overnight with gentle agitation. On the following day, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution; Vector Laboratories, Burlingame, CA) for 1h. An avidin-biotin-complexing solution (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) was then applied for 1h, and a nickel-enhanced glucose oxidase method using diaminobenzidine (DAB) as a chromogen was utilized to visualize specific binding [17].

Subsequently, dual localization of Fos with other markers was accomplished using the same immunoperoxidase method as described above but with sequential staining for the second marker done without nickel enhancement. Cells expressing both markers were therefore visible as

having black nuclei (Fos) and a brown cytoplasm (second marker). Due to the exothermic nature of the DAB reaction, the concentration of the Fos primary in these analyses was changed to a 1:10,000 dilution and the reaction carried out on ice in order to optimize our double staining. Fos was co-localized with arginine vasopressin (AVP) and the glucocorticoid receptor (GR), using primary antisera at 1:10,000 dilutions (Abcam, Inc., Cambridge, MA), to determine the number of stress-activated cells that also expressed these markers. In all cases, stained sections were mounted on gelatin-coated slides and allowed to dry overnight before being defatted through a graded ethanol series and xylene and coverslipped. Lastly, one series from each rat was stained with 0.25% thionin for Nissl material. These slides were used to evaluate the basic architecture of the brain regions to be examined, and served as a reference during analysis of staining for other markers.

Quantification of Staining.

Light microscopy (AxioScop; Carl Zeiss, Inc., Thornwood, NY) and simple cell counting methods were used to measure immunoreactivity in both single- and dual-staining experiments. Initial counts were made at 20x magnification for Fos-positive and doubly-labeled cells, with a higher magnification (40x) and different focal planes used for verification of double labeling. Cell counts were performed both manually and with the assistance of a digital microimaging system coupled to computer software (AxioCam with AxioVision software; Carl Zeiss, Inc., Thornwood, NY), and quantified in five sections through the PVH. Proper identification of the anatomical region of interest was assured through use of adjacent Nissl series as well as the The Rat Brain atlas of Paxinos and Watson (4th edition, 1998) [18]. Specifically, immunopositive

cells were counted bilaterally; the number of cells expressing each marker per side was then summed, and group means, standard deviations and standard errors were calculated.

Statistical Analysis.

The mean number of immunopositive cells was compared and evaluated with a Student's t-test to determine overall effects, followed by Tukey-Kramer post hoc tests to determine individual differences (JMP program, version 7; SAS Institute, Inc., Cary, NC). Significance was determined at the $p<0.05$ level.

Results:

Fos expression in the PVH of stressed ovariectomized female rats with or with out E2 replacement.

The neuronal activation in the PVH was tested among ovariectomized females that were either replaced with low (OVX/LoE2) or high (OVX/HiE2) estrogen or received vehicle (OVX/Veh) pellets (Fig. 7) The number of cells showing Fos immunoreactivity in the PVH was low in control groups, but increased in acute restraint stress. With repeated stress exposure, the number of activated cells decreased in comparison to acutely stressed groups. Although this data is non-significant, these trends are similar to those seen in the Fos expression of both males and females from chapter 1. Significant differences were seen for both control and repeated OVX/Veh groups when compared with the acutely stressed OVX/Veh group.

Stress-induced activation of AVP-expressing neurons in ovariectomized females with or without estrogen replacement.

Stress-sensitive PVH neurons that also express AVP were identified by dual localization of Fos and AVP in individual cells, which were seen in all groups. In figure 8A, the absolute number of Fos- and AVP-expressing neurons yielded some interesting trends. In the OVX/Veh group, the number of Fos- and AVP-expressing neurons decreased as the number of stress exposure increased. OVX/LoE2 groups, showed an increase in Fos- and AVP- expressing neurons as the number of stress exposure also increased. In OVX/HiE2 groups, high expression of Fos/AVP neurons were seen in its control (non-stressed) group and lower levels of expression were seen in both acute and repeatedly stressed groups. Although this data is non-significant, these groups do not follow the stress trends seen in chapters 2 and 3 as well as those in figure 7. Significant differences were seen for both OVX/LoE2 and OVX/HiE2 control groups when compared to OVX/Veh control ($p < .05$). The OVX/Hi acute group was also significantly different from OVX/Veh actue group ($p < 0.05$).

Normalization of the data so that cells expressing both Fos and AVP are given as a percentage of the number of neurons staining positively for Fos alone, also yielded some interesting trends (Fig. 8B). All three control groups show a greater relative recruitment of AVP expressing neurons, while all acute and repeatedly stressed groups show a de-recruitment in comparison. These data are also different from the percentage of Fos cells also expressing AVP seen in intact females from chapter 2 (Fig. 3B).

Sex differences in the recruitment of GR-expressing neurons in ovariectomized female rats with or with out E2 replacement.

Figure 9A shows the activation of Fos neurons staining positively for GR. The OVX/Veh control group shows similar numbers of Fos/GR expression to that of the OVX/Veh acutely stressed group; while the OVX/Veh repeatedly stressed group had fewer numbers of Fos/GR expression. The OVX/LoE2 control group showed the least number of Fos/GR positive cells in the PVH out of all control groups, and when compared to OVX/LoE2 acute and repeatedly stressed groups. Although non-significant, OVX/HiE2 acute group had a greater increase in the number of Fos/GR cells in the PVH compared to its control group. The OVX/HiE2 repeatedly stressed group had fewer numbers of Fos/GR activated neurons compared to its respective acute group, and had similar numbers of expression to its respective OVX/HiE2 control group,

When the number of cells expressing both markers was normalized to the total number of Fos-expressing cells; the OVX/HiE2 control group showed to preferentially recruit more GR cells than any other group (Fig. 9B). Both OVX/HiE2 and OVX/Veh groups decreased in percentage of Fos cells expressing GR as stress exposure increased in their respective acute and repeatedly stressed groups. The OVX/LoE2 acute group showed de-recruitment of Fos/GR expression compared to the OVX/LoE2 control group; while the OVX/LoE2 repeatedly stressed group showed a similar amount of expression as its respective control group.

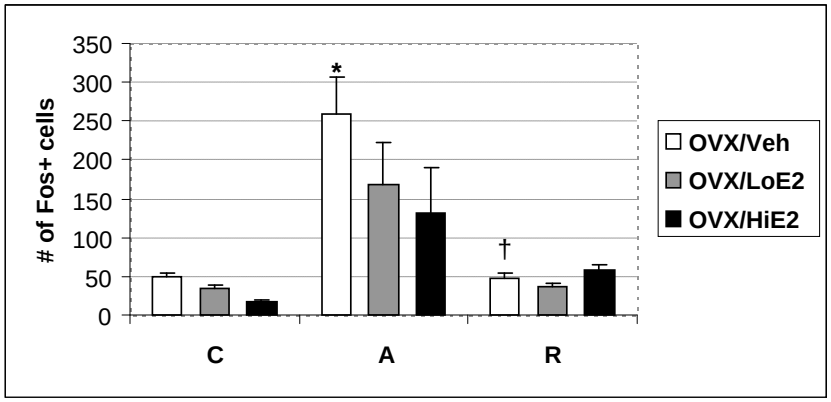

Figure 7. *Quantification of PVH cells for Fos in ovariectomized females.* Absolute number of cells expressing Fos was quantified in the PVH by simple cell counting. Counts were made at 20x magnification. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from OVX/Veh Control (*) and OVX/Veh (†).

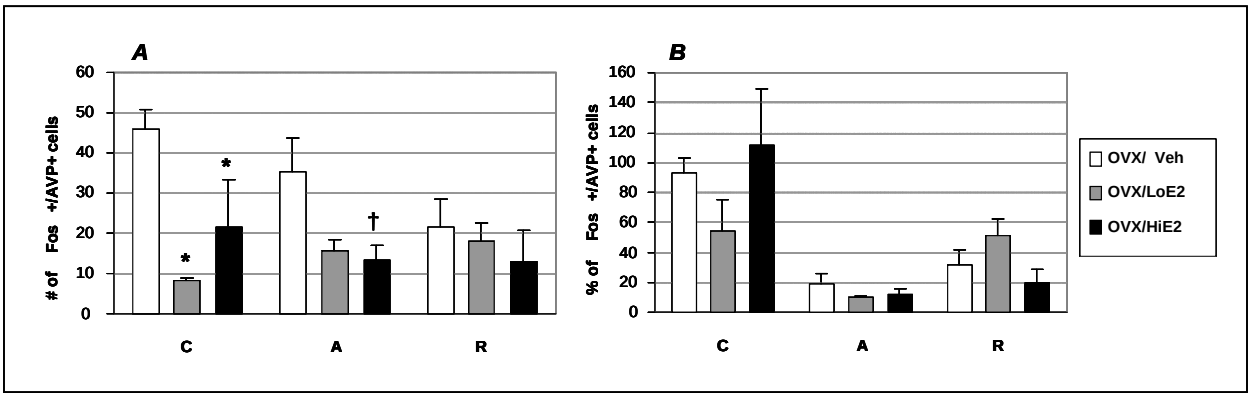

Figure 8A. *Quantification of PVH cells doubly-stained for Fos and AVP in ovariectomized females.* Absolute number of cells expressing both Fos and AVP were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats. Symbols indicate significant difference ($p>0.05$) from OVX/Veh C (*) or OVX/Veh A (†).

Figure 8B. *Percentage of Fos+ cells that also express AVP in ovariectomized females.* The number of cells expressing both Fos and AVP is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats.

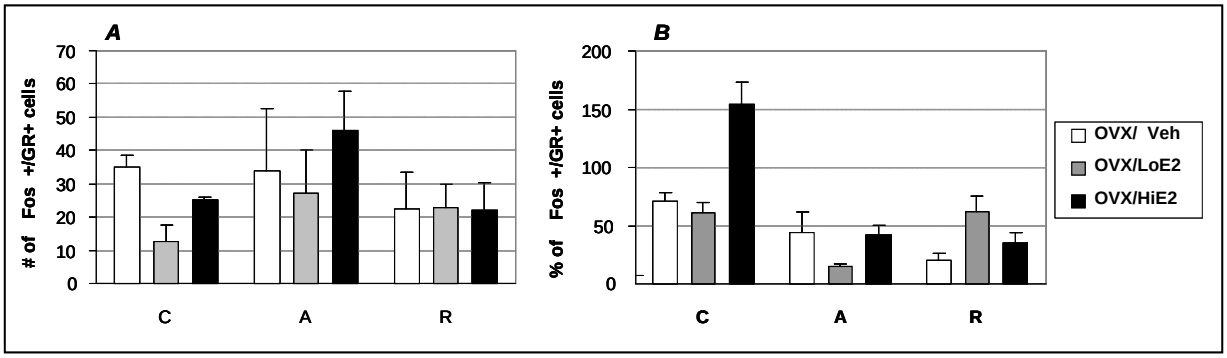

Figure 9A. *Quantification of PVH cells doubly-stained for Fos and GR in ovariectomized females.* Absolute number of cells expressing both Fos and GR were quantified in the PVH by simple cell counting. Counts were made at 20x magnification, and double staining was verified at 40x magnification. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats.

Figure 9B. *Percentage of Fos+ cells that also express GR in ovariectomized females.* The number of cells expressing both Fos and AVP is provided as a percentage of the total number of cells expressing Fos. C, control rats; A, acutely restrained rats; R, repeatedly restrained rats

Discussion:

A role for AVP in Ovxed females with or without E2 replacement.

The data for the number of Fos/AVP cells in the PVH in females that have been ovariectomized, didn't show the same stress-induced trends seen throughout chapters 2 and 3. Ovariectomized females that were implanted with a vehicle pellet had the highest number of Fos/AVP positive neurons in its control (non-stressed) group; while the females that were re-introduced to estrogen had lower levels of Fos/AVP positive neurons in their control groups. Both OVX/LoE2 and OVX/HiE2 groups were able to maintain lower numbers of activated neurons when exposed to acute restraint stress; while the OVX/Veh acute group had a higher stress response. Repeatedly stressed OVX/Veh females had lower levels of Fos/AVP expression compared to its respective acutely stressed group; thus showing similar levels of expression as the OVX/LoE2 and OVX/HiE2 acute groups.

When looking at the percentage of Fos+ cells that also expressed AVP, under conditions of both no E2 (OVX/Veh) and high E2 (OVX/HiE2) replacement, expression of AVP was highest in control (non-stressed) groups and seemed to be de-recruited when rats were subjected to restraint stress. In groups that were replaced with low E2 levels, both control and repeatedly stressed groups showed to recruit more AVP, whereas the acutely stressed groups showed to de-recruit of this hormone. It is possible that since basal (control) levels of AVP expression were already high, E2's stimulatory effects [51] had to be dampened by other mechanisms to compensate for possible hypersecretion of the HPA axis.

GR in Ovxed females with or without E2 replacement.

The number of Fos/GR positive cells in the PVH in ovariectomized females, was high in the OVX/Veh control group, and remained at similar levels in its respective acutely stressed group. Ovariectomized females that were replaced with low levels of estrogen had the lowest number of Fos/GR activated neurons in its control group. Fos/GR expression increased slightly in this same group when exposed to acute and repeated restraint stress. OVX/HiE2 also showed and increase in Fos/GR positive cells in acute restraint stress, compared to its respective control group; with fewer numbers exhibited in its respective restraint group.

The percent of Fos+ cells also expressing GR in ovxed females, showed similar trends to that seen above in the percent of Fos/ AVP data. Under conditions of both no E2 and high E2 replacement, expression of GR was highest in control groups and seemed to be de-recruited when rats were subjected to acute or repeated restraint stress. In ovxed/low E2 replacement groups, again it seems as though the acute group de-recruited GR, while control and repeatedly stressed groups had similar higher levels of GR expression. Assuming that the acute and repeatedly stressed groups also exhibited high levels of GR expression before exposed to restraint induced stress, it may possible that this provided a fast acting mechanism for GC's to turn off the stress response; thus no need to recruit more GR.

A role for Estrogen

As mentioned throughout the dissertation, estrogen is known to stimulate or enhance the stress response by targeting all levels; and here we show a possible new role for high estrogen levels in down-regulating stress hormones (AVP and GR), thus possibly dampening the stress response. It is also possible that there may be a saturation of circulating E2 levels in these

studies, since the OVX/HiE2 females showed similar results to the OVX/LoE2 females for all data points examined. We also show that OVXed females, both with and without estrogen replacement, have a higher number of neurons co-expressing Fos and GR under basal (non-stressed) conditions. This suggests the possibility of an enhanced capacity for rapid termination of the stress response and/or GC sensing in general. After the rats underwent the acute or repeated stress paradigms, decreasing activation of GR- and AVP- expressing neurons was seen compared to what was seen previously in stress-induced circumstances.

Conclusions:

We have observed significant differences in the activation of PVH neurons that co-express neuropeptides known to function in the regulation of the HPA axis. Expression profiles that differ by sex suggest that there are substantial variations in the mechanisms females use to generate responses to stress, as compared to males. Females appear to be less sensitive than males to acute stress, in that they have fewer Fos-positive cells in the PVH under this condition. However, they may, in fact, produce equal or even greater responses to acute stressors through the specific recruitment of AVP and, possibly, increased adrenal sensitivity to ACTH. The enhanced activation of GR-expressing neurons seen in the PVH of female rats may indicate a heightened sensitivity to negative feedback that is not seen in males. This could lead to constrained stress responses at the level of the PVH, decreased sensitivity to subsequent stressors, and/or more rapid termination once a stress response is initiated. Taken together, these data suggest that female rats may respond to acute stress in a manner that is equal to or even greater than that seen in males, but might also have an increased efficiency in their ability to process that stress. Our findings that females appear to respond to increasing stress exposure by

selectively activating more GR-expressing and fewer AVP-expressing neurons warrant further study, and may provide insight into the mechanisms through which men and women differentially manifest stress-related illnesses.

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CURRICULUM VITA

Jaidee Zavala was born in Reynosa, Tamaulipas Mexico and was raised in El Paso TX. She graduated from Montwood High School in 2001 and went on to pursue her bachelor's degree at New Mexico State University, in Las Cruces NM. At the end of her sophomore year, she transferred to Texas A&M University in College Station, TX. She graduated with a Bachelor of Science degree in Animal Science in 2004. During her time at Texas A&M, Jaidee went on a mission trip to Central America to gain hands on experience in the field of Veterinary Medicine. She also volunteered at animal shelters and veterinary clinics. In December of 2004, Jaidee returned to El Paso and began working for Adobe Animal Hospital until she joined the graduate program at the University of Texas at El Paso in the fall of 2005. In the summer of 2007, she entered the PhD program in Pathobiology at the University of Texas at El Paso.

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