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The Effects of Chronic Restraint Stress On Behavioral, Neuroendocrine, and Neurochemical Responses in Female Rats.

By

Rachel E. Bowman

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2002

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Abstract

THE EFFECTS OF CHRONIC RESTRAINT STRESS ON BEHAVIORAL, NEUROENDOCRINE, AND NEUROCHEMICAL RESPONSES IN FEMALE RATS.

By

Rachel E. Bowman

Adviser: Distinguished Professor Victoria N. Luine

While stress is a common to all animals, the stress response is sex dependent and limited work has shown sex differences in the male and female response to stress. Effects of chronic restraint stress, (6h/day, 21 and 28 days) in intact female rats were examined. Stressed rats had higher corticosterone levels during the stress period but levels returned to baseline at 15 days post-stress. Stress had no effect on estrous cycle lengths, but led to less weight gain. Following the stress period, subjects were tested for open field (OF) activity and radial arm maze (RAM) performance. Females stressed for 21 days showed enhanced spatial memory performance on the RAM, but females in proestrus, regardless of stress, had impaired acquisition. A longer period of restraint, 28 days, also led to less weight gain by stressed subjects and unaltered estrous cycle lengths, but was not associated with enhanced RAM performance, except stressed females in proestrus performed better than proestrus controls. Following sacrifice, brain areas contributing to learning and memory were assessed for neurotransmitter levels.

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Following both 21 and 28 days of stress, dopaminergic activity was increased in prefrontal cortex and regions of the hippocampus.

To assess ovarian hormone contributions to the female stress response, effects of 21-days of stress in ovariectomized (OVX) rats receiving either cholesterol or estradiol replacement were examined. OVX-stress subjects were not impaired and stressed subjects receiving estradiol showed the best RAM performance. Monoamine and amino acid levels changed in response to both stress (e.g., decreases dopamine metabolites in prefrontal cortex) and estradiol (e.g., increased CA3 levels of norepinephrine).

Finally, stress alone, without behavioral testing, did not alter neurotransmitter levels, but corticosterone was correlated with specific neurotransmitters in stressed and control subjects, suggesting that the experience of stress may alter neurochemical responses differentially following specific cognitive demands.

In summary, the current studies provide novel behavioral, neuroendocrine and neurochemical information about the stress response in female rats. Results show that estradiol may moderate stress effects on cognition, as compared to males, through both organizational and activational effects and that interactions at monoaminergic terminals may be important in mediating the effects.

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Dedication

You've been so kind and generous; I don't know how you keep on giving
For your kindness I'm in debt to you
And I never could have come this far without you
For everything you've done, you know I'm bound,
I'm bound to thank you for it.....

--Natalie Merchant

This thesis is dedicated to Sosimo - my husband and, perhaps more importantly, my best friend. Thank you for everything.

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This thesis would not have been possible without the help and friendship of many people. Most importantly, I wish to thank my thesis committee: Drs. Luine, Angulo, Frankfurt, Quinones, and Zrull. You have all been invaluable during this process. I am especially grateful to Dr. Victoria Luine for all her help and wisdom and for making it possible for me to come to Hunter. She has managed to provide the perfect balance of guidance and mentoring, while allowing me enough autonomy, which gave way to a wonderful working environment. Also, I thank Dr. Mark Zrull for initially encouraging me to enter into the science community. You have probably put up with more frantic phone calls than anyone should be expected to. Thank you. I am forever grateful to my entire committee for believing in my ability to juggle many tasks and roles and for providing me the encouragement to succeed in them all.

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And, many thanks to my wonderful daughter, Ava Jane, for making me laugh and keeping me on my toes. You have added an invaluable sense of perspective to my life.

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The Effects of Chronic Restraint Stress On Behavioral, Neuroendocrine, And Neurochemical Responses In Female Rats.

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General Introduction

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General Introduction

Stress

The experience of stress is common to all organisms. However, what constitutes a stressful event is variable for every animal. Nonetheless, it is clear that major life changes, personal threat, trauma, or physical illness constitute stressful events in most vertebrates. Physical and psychological stressors exert effects that range from the physiological to the cognitive level. While stressful events take many forms, their consequences can, in general, be classified as either acute (i.e., short-term and adaptive) or chronic (i.e., long-term and maladaptive).

When homeostasis is threatened by the experience of stress, the body undergoes a myriad of changes. One primary physiological change that occurs in response to stress is the release of glucocorticoids (GC) by the adrenal glands (Jacobson & Sapolsky, 1991), which is regulated by the hypothalamo-pituitary-adrenocortical (HPA) stress axis. The HPA axis is a complex neurocircuit controlled by a regulatory set of afferents, primarily those of the neurons in the paraventricular nucleus of the hypothalamus (PVN). In response to a stressful event, PVN neurons secrete corticotrophic releasing hormone (CRF) which triggers the pituitary gland to release adrenocorticoptrophic hormone (ACTH) that in turn signals adrenal glucocorticoid secretion (Herman & Cullinan, 1997). GC steroid receptors are located throughout the brain and work to provide negative feedback to terminate both neural and endocrine activation that results from stress.

Short-term glucocorticoid changes, in response to stress, are adaptive. However, prolonged exposure to stress and subsequent sustained, elevated GC levels has an adverse effect. The hippocampus has the highest density of GC target receptors and has been

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implicated in the regulation of HPA stress axis and behavioral response to stress (Diamond, *et al.*, 1996). Thus, the hippocampus provides a model for studying the neurobiological effects of increased levels of GC associated with stress.

Stress and the hippocampus

The circulating glucocorticoid in primates is cortisol and in rats is corticosterone (Bodnoff, *et al.*, 1995). In rats, both physical chronic stress (e.g., foot shock or cold water swim) and psychosocial stress (e.g., restraint or placement in a novel environment), affects hippocampal morphology (Bodnoff, *et al.*, 1995; Magarinos, *et al.*, 1997) and function. Stress and subsequent elevated corticosterone levels have been shown to block hippocampal long-term potentiation (LTP) (Foy, *et al.*, 1987) and primed burst potentiation (PB) (Diamond, *et al.*, 1990; Diamond, *et al.*, 1994). Both LTP, a long lasting increase in the excitability of postsynaptic neurons due to repeated electrical stimulation, and PB, a low-threshold form of LTP, are physiological processes involved in the regulation of learning and memory formation. Chronic stress leads to alterations in hippocampal morphology in CA3 pyramidal neurons following 21 days of daily restraint stress or 21 days of administration of exogeneous GC (Magarinos, *et al.*, 1997). The CA3 pyramidal neurons are a part of the major excitatory afferent pathway, utilizing glutamate as the predominate neurotransmitter, to relay information from the dentate gyrus to the CA1 level of the hippocampus (Magarinos, *et al.*, 1997). Structural changes in the hippocampus following stress include decreases in both apical dendritic branching as well as total dendritic length (Watanabe, *et al.*, 1992). The atrophy is mediated by GC levels and N-methyl-D-aspartate receptor-mediated excitatory input (Watanabe, *et al.*, 1992) and can be prevented by inhibiting GC secretion. In humans, the adverse effects of

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stress induced elevated GC levels are evident by hippocampal atrophy and overall loss of hippocampus volume in sufferers of posttraumatic stress disorder (Gurvits, *et al.*, 1996), prolonged depression (Sheline, *et al.*, 1996), and Cushing's syndrome (Starkman, *et al.*, 1992)

The morphological changes that are observed in the hippocampus in response to stress induced elevated corticosterone levels are associated with behavioral deficits in spatial learning and memory. Following 21 days of chronic restraint, male performance is impaired on the radial arm maze, a test of spatial memory (Luine, *et al.*, 1994). Stressed male rats make their first mistake sooner and have less correct responses in their first eight choices as compared to control males. The changes in spatial memory performance following chronic restraint stress are reversible and appear to be temporally constrained (Luine, *et al.*, 1994). For example, thirteen days of chronic stress improves radial arm maze performance and behavioral deficits are not observed until 21 days of stress (Luine, *et al.*, 1996). It is unclear what centrally located mechanisms are mediating the biphasic transition from beneficial to deleterious effects of stress exposure on behavior. Prolonged elevated corticosterone levels are also associated with impaired performance on a number of other tasks that require the use of spatial memory including the Y-maze (Conrad *et al.*, 1996), the Morris water maze (Bodnoff *et al.*, 1995) and the Barnes maze (Mclay, *et al.*, 1998). These mazes, like the radial arm maze, all test various aspects of mammalian spatial learning and memory performance.

Sex differences in response to stress

The stress response is regulated by the HPA axis and sex differences exist in resting levels of the HPA secretions. For example, female rats have higher basal levels of

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CORT (Chritchlow, *et al.*, 1963) and display greater diurnal changes in both adrenocorticotropin and CORT than males (Handa, *et al.*, 1994). A primary stress-induced physiological change is an increase in GC levels. In comparison to males, female rats have higher GC levels following stress (Chritchlow, *et al.*, 1963). These sex differences in CORT levels are responsive to ovarian hormones, as GC secretion fluctuates over the estrous cycle (Burgess & Handa, 1992; Carey *et al.*, 1995; Viau & Meaney, 1991).

Sex dependent morphological changes occur in the hippocampus in response to chronic stress. Following 21 days of chronic restraint stress, structural changes in the male rat hippocampus include decreases in both apical dendritic branching as well as total dendritic length (Watanabe, *et al.*, 1992). In contrast, female rats do not show atrophy of apical dendritic branches after chronic stress. However, significant decreases in the number of branch points within the basal dendritic area is observed in stressed female rats when compared to non-stressed controls (Galea, *et al.*, 1997). Stress induced neurochemical changes also appear to be sexually dimorphic. The dopamine (DA) system is activated in the prefrontal cortex (PFC) and amygdala of males, an effect not observed in females after chronic stress (Luine, *et al.*, 2001; Beck & Luine, 1998). Additionally, only males show changes in hippocampal GABA levels following stress, with increased levels seen in CA3, while stressed females had increased norepinephrine (NE) and serotonin (5HT) in CA1 (Luine, *et al.*, 2001).

Behavioral sex differences have been observed following both acute and chronic stress. For example, acute stress facilitates trace conditioning in male rats but is associated with impairments in females (Wood, *et al.*, 1998). Open field activity is

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influenced by 5 days of acute stress with decreases in locomotion behavior observed in females but not males (Haleem, *et al.*, 1988). Sex differences have been observed in non-spatial, non-reward tasks following 21 days of stress; males were impaired but females were not in the object recognition task (Beck & Luine, 1998). To the best of our knowledge, there is no other data regarding the influence of chronic stress on either non-spatial or spatial learning and memory performance in female rats. It also remains unclear how observed morphological changes in female brain structures may correlate with subsequent behavioral changes.

Stress and neurotransmitters

It appears that stress-dependent impairments in spatial memory may be mediated in part by the serotonergic system (Luine, *et al.*, 1993). Luine and colleagues found that male rats with the highest levels of plasma corticosterone had the most impairments performance on the radial arm maze and demonstrated enhanced hippocampal 5-HT and its metabolite, 5-hydroxyindole acetic acid, 5-HIAA activity in the dentate gyrus (DG). Decreases in both 5-HT and NE levels were observed in the frontal cortex of corticosterone treated animals (Luine, *et al.*, 1993). Additionally, 5-HT agonists (e.g., 8-OH-DPAT) have been shown to impair radial arm maze performance, further supporting that increases in serotonin activity may produce inhibitory effects on learning and memory performance (Winter & Petti, 1987). Finally, tianeptine, which enhances 5HT uptake and hence decreases its effects, blocks both stress induced morphological changes in the hippocampus and impaired spatial memory performance (Conrad, *et al.*, 1996; Luine, *et al.*, 1994).

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The effects of chronic stress on brain function are also associated with changes in dopaminergic (DA) systems (Camp & Robinson, 1988). Significant decreases in DA and its metabolite, DOPAC, correlate with impaired performance on the eight arm radial maze in aged rats (Luine, *et al.*, 1990). Glucocorticoids have a modulatory effect on both the synthesis and release of dopamine and serotonin through regulation of the levels of the enzymes that synthesize or deactivate these transmitters. At type I receptors, GC increase tryptophan uptake and tryptophan hydroxylase activity which stimulates serotonin synthesis in midbrain raphe (McEwen, *et al.*, 1986).

Corticosterone influences on stress induced behavioral deficits may also be mediated by excitatory amino acids, such as glutamate (Luine, *et al.*, 1994). For example, phenytion administration interferes with excitatory amino acid activity and leads to reversal of radial arm maze impairments (Luine, *et al.*, 1994). Phenytion also blocks the atrophy of CA3 dendrites associated with stress exposure (Watanabe, *et al.*, 1992). Glutamate is the neurotransmitter involved in relaying information from the mossy fibers of the dentate gyrus to CA3 neurons of the hippocampus. Glutamate has also been implicated in regulation of long term potentiation that is critical to hippocampus dependent memory (Pavlides, *et al.*, 1993). All of these stress dependent changes in neurotransmitters have been determined in male rats, and little information is available on effects of stress on neurotransmitter activity in females.

Stress, ovarian hormones, and memory

Hippocampal morphology and subsequent spatial memory performance is also influenced by gonadal hormones. Performance on the eight arm radial maze is sexually dimorphic, with male rats consistently outperforming females and in task acquisition

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(Luine & Rodriguez, 1994; Luine, *et al.*, 1998; Williams & Meck, 1990). The ovarian hormone estradiol has been implicated in enhanced spatial memory performance (for review, see Luine, 1997) and estrogen exerts both organizational, mediated primarily through genomic steroid effects, and activational influences on spatial performance. An example of organizational effects of estrogen on spatial performance is the ability of castration in neonatal male rats to produce female learning patterns in adults, and estradiol treatment of neonatal females to produce male-typical learning patterns in adulthood (Williams & Meck, 1991).

Administration of estradiol to OVX rats improves performance in the radial arm maze and in a delayed, win-shift paradigm of the eight arm radial maze and is indicative of estrogen's activational influence on the system. In this task, a varying time delay is interposed between the 4th and 5th choice and rats must then choose the four, previously unvisited arms. The enhancing effect of estradiol on spatial memory may be due to increased density of apical branches in CA1 hippocampal neurons (Luine, *et al.*, 1994). Furthermore, estradiol's influence on spatial learning and memory appears to be dependent on prolonged low-dose treatment, over approximately 3 weeks, in order to demonstrate behavioral changes. The requirement of prolonged exposure may underlie why no consistent significant differences in spatial learning and memory performance have been reported across the rat's 4-day estrous cycle (Stachman, *et al.*, 1997). Estradiol treatment has also been shown to improve human performance in delayed memory tasks (Philips & Sherwin, 1992) and estrogen replacement therapy in post-menopausal women leads to improved memory performance (Sherwin, 1997) and a 30% decrease in the

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incidence of Alzheimer's disease as compared to women not taking estrogen-replacement (Paganini-Hill & Henderson, 1994).

Specific research aims:

Studies in this and other laboratories have investigated the effects of stress on male spatial memory performance utilizing the radial arm maze (Luine, *et al.*, 1994; Luine *et al.*, 1996), the Barnes maze (Mclay, 1998), the Morris water maze (Bodnoff, 1995), and the Y-maze (Conrad, 1996; Mizoguchi, *et al.*, 2000). Based on the previously reported sex differences in behavioral, anatomical, neuroendocrine, and neurochemical responses to chronic stress (Atkinson, *et al.*, 1997; Beck & Luine, 1998; Galea, *et al.*, 1997; Luine, *et al.*, 1994; Luine, *et al.*, 1996; Luine, *et al.*, 2001), it seemed pertinent to examine the effect of chronic stress on female rats. The influence of stress was examined at the neuroendocrine, neurochemical, and behavioral levels in intact female rats and ovariectomized females with and without estrogen replacement. This series of experiments was designed to specifically address each of the following:

1. To examine the effects of chronic restraint stress on behavioral parameters in intact female rats. Stress, see Figure 1 for schematic of the HPA axis, was induced in female rats by restraining them for 6h/day for 21 (experiment 1) and 28 days (experiment 2) and assessing effects on overall locomotor activity using the open field and on spatial learning and memory using the eight arm radial maze.
2. Potential neuroendocrine changes in HPG and HPA axes induced by chronic stress in intact normal female rats were examined (i.e., estrous cyclicity, experiments 1 and 2), as well as possible influences of estrous cycle status on behavioral parameters

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following chronic stress. Furthermore, total (bound and free) and free corticosterone were measured at different time points during the 21-day stress period (experiment 1).

3. To assess possible interactions of the HPG and HPA axes on mediating stress effects, ovariectomized (stressed and non-stressed) female rats were compared to estrogen-replaced ovariectomized rats (stressed and non-stressed) (experiment 3). Stress effects on locomotor activity, spatial learning/memory, and neurotransmitter levels were examined.
4. To investigate stress effects on neurotransmitter levels as well as possible interactions between circulating hormone levels (corticosterone and progesterone) and monoamine and amino acid levels immediately following stress. Intact females were stressed for 21-days and immediately sacrificed (no behavioral testing) and possible correlations between neurotransmitter levels and hormone levels were investigated.
5. Following each experiment, neurochemical levels that may contribute to stress-dependent alterations in spatial learning and memory were measured in brain areas known to be involved in cognitive function, see Figures 1 and 2. These include dopamine (DA) and its metabolites, 3,4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-methoxy-4hydroxyphenylglycol (MHPG); and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA), as well as the amino acids glutamate and gamma-aminobutyric acid (GABA).

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Figure 1. A schematic representation of the brain areas comprising the memory circuit of interest, its relationship with the HPA axis, and the known sex differences in the stress response.

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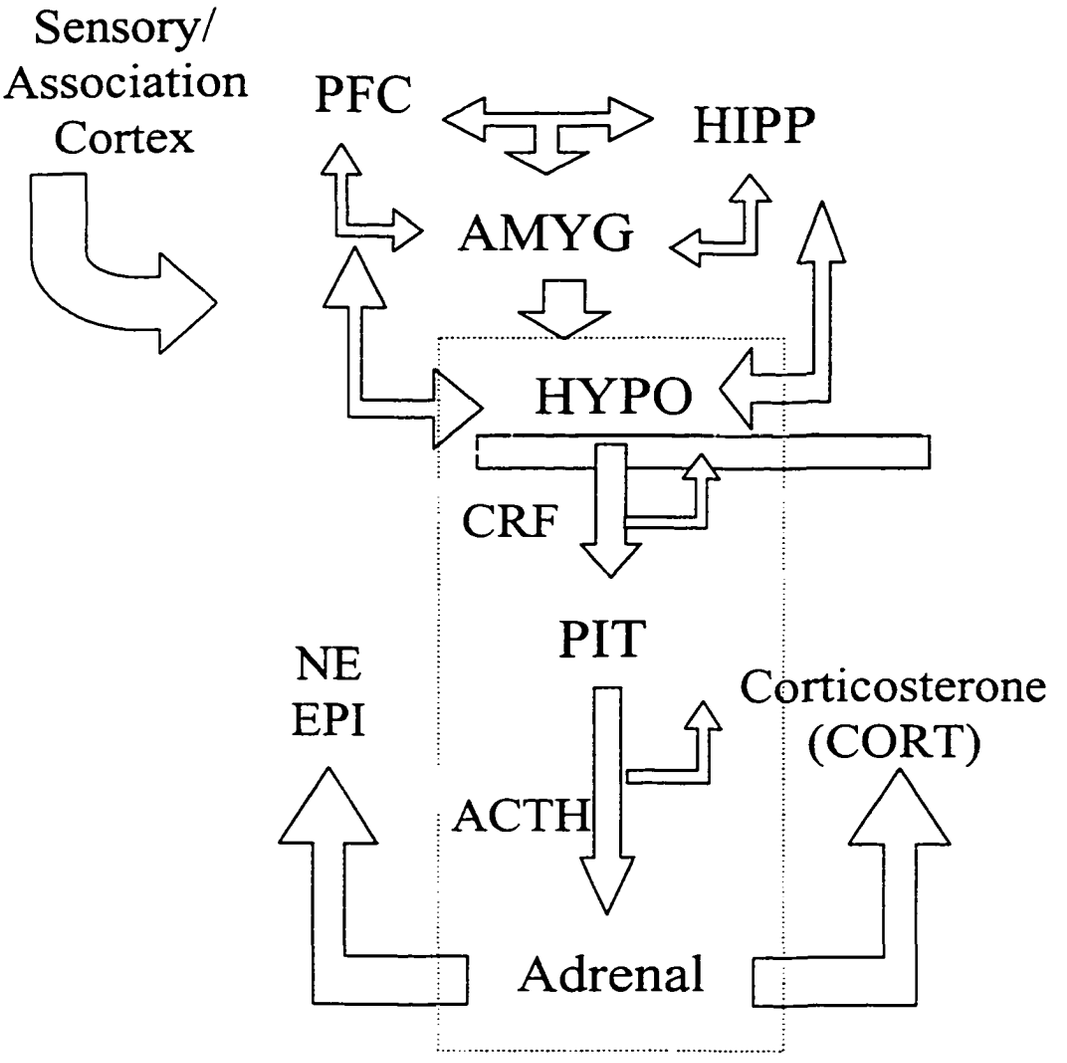

HPA function and sexually dimorphic responses:

- Basal CORT levels are higher in female rats than males
- Females display greater diurnal changes in both ACTH and CORT
- CORT levels are influenced by HPG changes, with highest CORT levels observed during proestrus

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Chapter 1 (experiment 1)- Effects of 21-days restraint stress on intact female rats: Assessment of behavioral, neuroendocrine, and neurochemical parameters.

Studies in this and other laboratories have investigated the effects of stress on male spatial memory performance utilizing the radial arm maze (Luine, *et al.*, 1994; Luine, *et al.*, 1996), the Morris water maze (Bodnoff, *et al.*, 1995), the Barnes maze (Mclay, *et al.*, 1998), and the Y-maze (Conrad, 1996; Mizoguchi, 2000). However, the effects of chronic stress on female radial arm maze performance have not been investigated. Based on the previously reported sex differences in behavioral, anatomical, neuroendocrine, and neurochemical responses to chronic stress (Atkinson & Waddell, 1997; Beck & Luine, 1998; Galea, *et al.*, 1997; Luine, *et al.*, 2001; Luine, *et al.*, 1996; Luine, *et al.*, 1994), it was hypothesized that females would be less sensitive to the effects of chronic restraint stress on spatial learning and memory than previously reported in male rats.

Experiment 1 assessed the effects of chronic restraint stress in intact, cycling female rats at the neuroendocrine, behavioral, and neurochemical levels. Specifically, the estrous cyclicity of all subjects was monitored for baseline levels and potential alterations during and following the stress paradigm were examined. Following the stress period, all animals were assessed for overall locomotor activity using the open field and for spatial memory using the eight-arm radial maze. Following behavioral assessment, all subjects were sacrificed and the brains processed for neurochemical analysis. Brain areas known to contribute to learning and memory process (e.g., the prefrontal cortex and hippocampus) were sampled and monoamines and their metabolites and amino acid levels were analyzed.

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Methods

Subjects

Forty female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), aged 55-60 days (upon arrival) served as subjects. All animals were maintained on a 14/10 hr light-dark cycle (lights on at 5:00 AM) and in accordance with the NIH Guide for Care and Use of Animals and allowed to acclimate to the housing environment for two weeks. During the acclimation period and the 21-day stress period, all subjects were double housed in plastic tubs. Animals were singly housed during behavioral studies. Throughout the acclimation period, stress period, and behavioral testing, all animals received daily vaginal lavages to determine their estrous cycle phase (2:00-3:00 PM). Briefly, the vagina is gently lavaged with physiological saline on a Q-tip and smeared on a slide. The slides are rinsed, stained with Coomassie Blue, rinsed, dried and examined under a microscope. The estrous cycle day was determined based upon the presence of leukocytes, nucleated epithelial or cornified epithelial cells. Rats that exhibited all phases of the estrous cycle, across a two-week period, were randomly assigned to either a stressed (N=15) or control (non-stressed, N=13) condition. Other intact females (N=12) were assigned to a quiet control group, which did not receive stress or behavioral testing and were used to obtain basal CORT levels. Animal body weights were obtained weekly.

During the 21-day stress period, stressed animals were placed in Plexiglas restrainer tubes manufactured by Harvard Apparatus for 6 hr (always beginning between 8:00 am and 9:00 am). The tube has air holes for ventilation and the animals have limited room for movement. Rats were placed in an isolation chamber with ventilation and maintained at room temperature. The chamber was located in a separate room, adjacent to

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the animal colony, which allowed for the separation of stressed and control animals during the stress period.

Immediately following the last stress day, all subjects were transferred to single housing and placed on food restriction. Because of the food-reward nature of the radial arm maze, food deprivation is necessary to reduce animals to 90% of their normal body weight. All subjects had free access to water and were weighed regularly. Subjects were given daily food rations (2-3 rat chow pellets) depending on their weight following behavioral testing. All subjects were sacrificed by rapid decapitation 14 days post-stress (cohort 1) and 15 days post-stress (cohort 2).

Corticosterone Radioimmunoassay

Blood samples were collected from the tail during the 1st hour of restraint stress on days 1, 7, 14, and 21 from the stress animals. Blood was sampled in the same fashion on day 1 from the quiet control animals, to provide basal CORT levels. Briefly, the tail was soaked in warm water and the tip quickly removed via a straight blade razor. The tail was palpated to collect the blood sample and then treated with antibiotic spray. To control for CORT levels across the estrous cycle (Atkinson, *et al.*, 1997; Raps, *et al.*, 1971; Viau & Meaney, 1991), stressed and quiet control females in proestrus (N=5, quiet controls, N=5, stress day 1 and N=6, stress days 7, 14, and 21) were selected and these samples were measured for analysis purposes. Total CORT levels were measured by Radioimmunoassay using the Coat-A-Count® assay kit available from Diagnostic Products Corporation, Los Angeles, CA (catalog number TKRC1). Free CORT was assessed using a modification of Edwards and colleagues (Edwards, *et al.*, 1999) technique. Briefly, Concanavalin-A conjugated to Sepharose-B ™ (Sigma, St. Louis,

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MO) was added to the serum sample to precipitate the corticosterone that is bound to corticosterone binding globulin protein. The samples were centrifuged and the supernatant, containing free CORT, was measured by RIA. Samples were analyzed in duplicates. In order to have sufficient volume to run the assay, serum samples were diluted to 300 ul (using the zero calibrator provided with the assay kits, as specified by the vendor) and calculations were made appropriately. All samples were run in one assay and the detection limit was 5.7 ng/ml, as defined by the 95% confidence limits of the zero standard. CORT levels are expressed as ng/ml.

Behavioral Measures

Open field

On day 1 post-stress, subjects were tested on the open field, which provides an overall measurement of locomotor activity. In random fashion, subjects were placed singly in the center of a 4 X 4 enclosed area with the floor marked out into 9" squares. All subjects remained on the open field for 6 min, during which time behaviors for the first 3 min and the second 3 min were tallied for each animal. These behaviors included sector visits (movement across squares), rears (raising up on haunches with forelimbs 3-4 cm off the floor), wall climbs, grooms, and defecations.

Radial arm maze

Spatial memory was assessed using the eight arm radial maze as described by Olton, Walker, and Gage (1978) and as previously used in this laboratory (Luine, *et al.*, 1994; Luine, *et al.*, 1996). One-quarter of a peanut is placed in a food receptacle located at the end of each arm of the maze, out of sight of the rat. A visit to the arm is scored if the subject traverses at least three-fourths of the arm, if the arm is completely entered and the

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food is not eaten, or if the arm is entered and the food is eaten. Errors are quantified as re-entries into previously visited arms during the same session. Choice accuracy is scored both by the number of correct choices in the first eight visits and the choice number at which the first error occurs.

Because of the food-reward nature of this spatial task, subjects are placed on food restriction. Rats are maintained at 90% of their normal body weight throughout training and testing sessions. Rats are trained to go the ends of the arms in order to retrieve the food reward over 10 shaping sessions (2 sessions/day for 5 days). After training, testing trials are given 2 trials/day, for a total of 4 trials. On these trails, subjects are required to complete the task within 5 minutes. Following completion of the regular trials, subjects receive two training sessions for delay trials. During training sessions, the delay is 15 minutes. During subsequent delay trials, each rat makes four choices in five minutes or less and is then removed from the maze to their home cage, remaining in the behavior room for a delay interval of 1-4 hours. After the delay, rats are then returned to the maze to complete the four remaining choices within another 5 minutes. As in regular trials, the number of correct choices in the first eight visits is scored. Also, the total number of errors made following the delay is calculated.

Generally, most young rats quickly acquire and perform this spatial task. One consideration is that occasionally, a rat will develop a non-spatial strategy such as sequentially entering each arm in order to complete the task. It has previously been noted that this non-memory strategy seems to develop when the rat is hungry and can be alleviated by increasing the animal's body weight or by feeding half a pellet before trials commence (Luine, personal observation). Nonetheless, some rats persist in this strategy

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and are removed from the study. This has not compromised results. In a recent study (Luine, *et al.*, 1998), 3 out of 68 total rats were removed.

Neurochemical Analyses

Rats were sacrificed by rapid decapitation during the delay period of the radial arm maze task and the brains rapidly frozen and stored at –70° C. A Reichert Histostat cryostat at –8° C was used to obtain serial sections, 300 μm thick, of the brain. Using a 500 μm diameter cannula, brain samples were punched from the frozen sections according to the atlas of Palkovits and Brownstein (1988). Between 3-12 punches were taken, dependent upon the area and neurotransmitter being sampled.

Monoamine and amino acid neurotransmitters levels were measured by dissolving the punches in 75 μl of acetic acid buffer, pH 6.5, and obtaining the released neurotransmitter through a process of freezing and thawing. Samples were then centrifuged at 12,000 rpm for 10 minutes and the supernatant removed for neurotransmitter analyses. Internal standards, homoserine for amino acids and α-methyl-dopamine for monoamines, were added. Sixty μl of the supernatant was used for injection into a high performance liquid chromatography (HPLC) system for monoamines and 15 μl was used for amino acid HPLC analysis. Protein was measured by redissolving the pellet in 100 of μl of 1.0 N NAOH.

High performance liquid chromatography (HPLC) with electrochemical analysis was used to quantify neurotransmitter levels. The supernatant (60 μl) was used in the detection of monoamines, including dopamine (DA) and its metabolites, 3,4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-methoxy-4hydroxyphenylglycol (MHPG); and serotonin (5-HT) and

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its metabolite 5-hydroxyindole acetic acid (5-HIAA). Monoamines were measured in a Waters Associates chromatographic system (Waters 2690) consisting of an Alliance module containing an automated refrigerated, injector, pump, C-18 reverse-phase column (Novapak 3 micron), and an ESA coulometric detector (+0.48-+0.50 V potential). The mobile phase, described elsewhere (Luine, *et al.*, 1990), contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile phase: 0.5% methanol).

Amino acids, including glutamate and gamma-aminobutyric acid (GABA) were measured following precolumn derivatization of the sample with o-phthalaldehyde and beta-mercaptoethanol. Samples are injected into the system using a Waters 717 automated, refrigerated injector and a 510 pump. A Waters Novapak C-18 reverse phase column is used for separation. An ESA model 5200 Coulochem II detector with a model 5011 analytical cell with electrodes set at +0.1 V to oxidize and remove derivatization contaminants and at +0.4 V to oxidize and detect derivatized amino acids.

Millenium software (Waters Assoc.) runs both chromatography systems. Concentrations of transmitters and metabolites are calculated by reference to standards and an internal standard using peak integration with the Millenium system. Monoamine levels were measured in prefrontal cortex (PFC), nucleus accumbens (nAc), vertical nucleus of diagonal bands in the basal forebrain (VDB), hippocampus (CA1, CA3, and dentate gyrus), and basolateral amygdala (BLA). Amino acid levels were measured in PFC, CA1, CA3, and DG areas of the hippocampus. Figure 2 shows the placement of micropunches sampled from the various brain regions.

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Figure 2. Brain areas sampled for monoamine and amino acid levels.

Panel A shows area of prefrontal cortex sampled (Interaural 12.2 mm and Bregma 3.2 mm). Panel B shows the area (Interaural 10.0 mm and Bregma1.0 mm) from which samples of VDB and nAc were sampled. Panel C (Interaural -1.8 mm and Bregma 4.0 mm) shows the brain area from which hippocampal regions CA1 and CA3 were sampled. Panel D (Interaural –2.0 and Bregma 3.8) shows the area from which hippocampal region DG as well as BLA were sampled.

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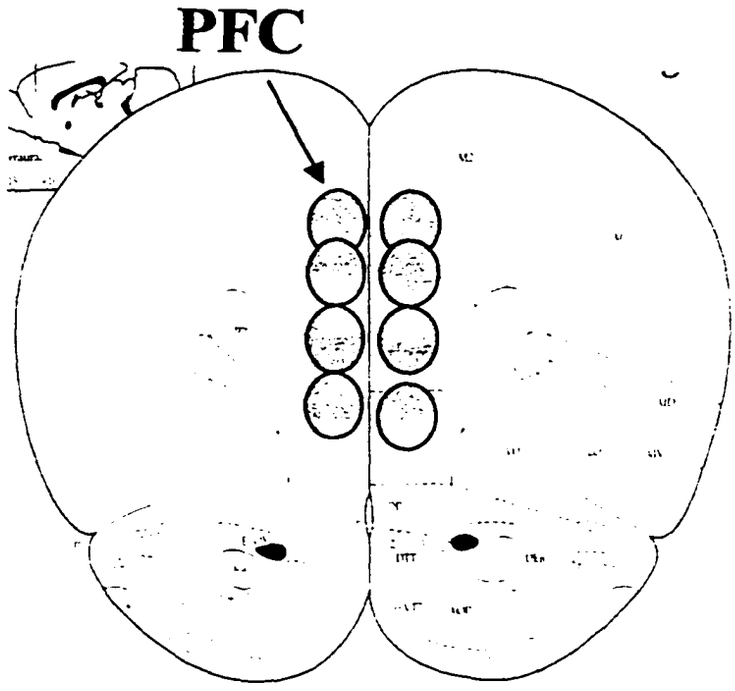
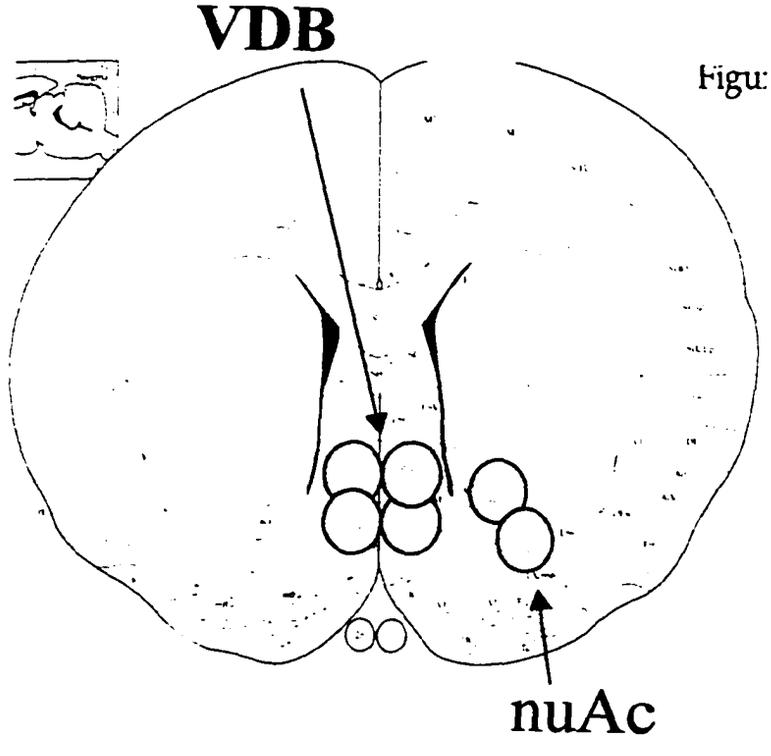
Panel A



Panel B



Figu

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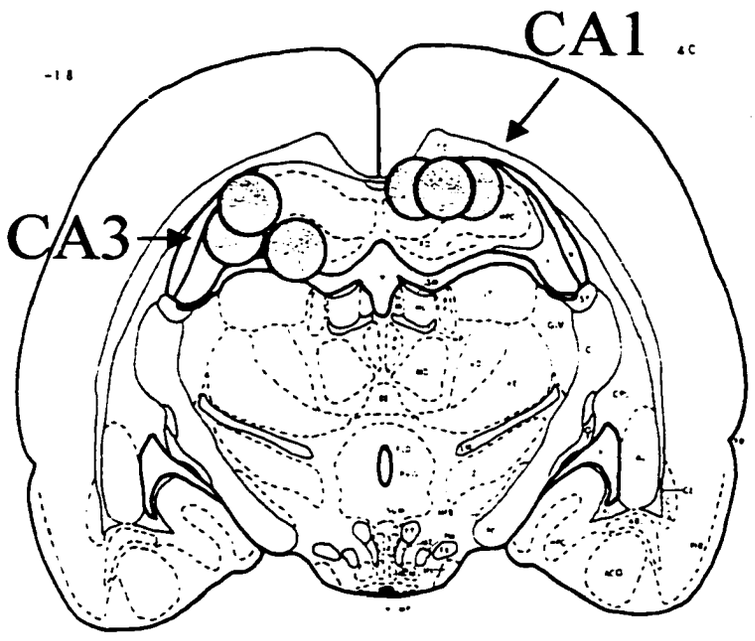
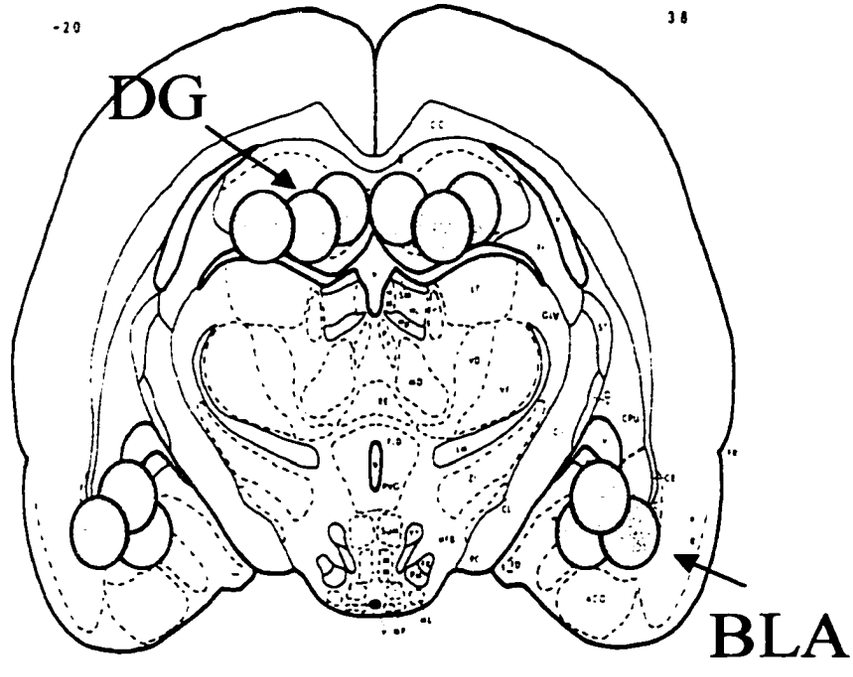
Panel C



Panel D



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Data Analysis

Measurements of animal weight gain, estrous cyclicity, and serum CORT level data were from a split-plot factorial design with treatment (between-subjects) and time (within-subjects or repeated) as design factors. A two-factor ANOVA was used to test for statistically significant differences in weight gain between stressed and non-stressed animals across the 21-day stress period. Mean estrous cycle lengths for the stressed and non-stressed animals during pre-stress, stress, and post-stress time periods were examined for statistically significant differences using ANOVA as well. Initially, an independent samples t-test was used to examine differences in serum CORT levels between stressed and quiet control animals on Day 1 of the stress paradigm. Subsequently, variance in CORT levels of the stressed animals across the 21 days was partitioned using a 2-factor (treatment X stress day) ANOVA, and Dunnett's post-hoc procedure was used to compare CORT values for stressed females across the stress period to values obtained for these animals on Day 1 for statistically significant differences. Finally, a dependent samples t-test was used to test for statistically significant differences in CORT levels obtained from the trunk blood of stressed and non-stressed females upon sacrifice after behavioral testing.

The behavioral data were also from a split-plot factorial design. Open field measures were analyzed using a 2-factor ANOVA with treatment (stressed, non-stressed) and time (first 3 min, second 3 min of the task) as between-subjects and within-subjects, or repeated, design factors, respectively. While radial arm maze data were from a 3-factor design, only the between-subjects factor (treatment) and one repeated factor (time) were experimenter-controlled. The second within-subjects design factor, estrous cycle, was

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subject-dependent and was not completely crossed with the time factor. Because time and estrous cycle formed an unbalanced, partial, and thus confounded interaction both relative to treatment and relative to the error terms necessary to use a 3-factor ANOVA accurately, stress treatment effects were analyzed initially across the regular and delay trials using a 2 X 4 (treatment X time [RAM testing day]) ANOVA without regard to estrous cycle of the animals. Subsequently, treatment and time effects were examined for each specific estrous cycle using 2 X 4 ANOVAs to provide a better description of treatment, time and treatment X time effects. While this method of analysis limited inferences that could be drawn from the data, it was necessary due to the confounding of time and estrous cycle variables.

Animal weight was measured in grams, and descriptive statistics for weight and behavioral data are expressed as Mean ± SEM. Monoamine and metabolite levels are expressed as pg/μg protein and amino acids are expressed as ng/μg protein. Regular RAM trials (2 per day) were averaged for each day. For purposes of determining statistical significance of effects, Type I error rate was set at 0.05 for individual global and families of post-hoc tests.

Results

Physiological measures

Weight

Animals' weights were monitored during the acclimation, stress and behavioral testing periods. There was no main effect of stress on weight gain. There were significant effects of time ($F_{3,115} = 13.05$, $p < 0.00000$) and the treatment X time interaction ($F_{3,115} =$

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8.07, $p < 0.00009$). Stressed animals weighed less than the controls from stress day-13 through the end of the stress paradigm, see Figure 3.

Estrous Cyclicity

Stressed females did not statistically differ from controls with respect to their average estrous cycle length. The average cycle length during the pre-stress period was 8.7 ± 0.45 and 7.9 ± 0.59 days (Mean $\pm$ SEM) for control and stressed animals, respectively. Cycle length during the stress period was 7.6 ± 0.46 days (controls) and 7.2 ± 0.34 days (stressed) and the post-stress period was 8.2 ± 0.39 days and 6.8 ± 0.47 days (control and stressed, respectively). A separate analysis was performed on the females that exhibited more typical estrous cycle lengths, 4-5 days. This subgroup of stressed females did not differ from controls with respect to their mean estrous cycle length. During the pre-stress period the mean cycle length was 5.5 ± 0.5 and 5.7 ± 0.37 days for control and stressed animals respectively. Average cycle length during the stress period was 5.5 ± 0.5 for controls and 5.6 ± 0.4 for stressed and during the post-stress period was 5.35 ± 0.35 and 5.34 ± 0.38 days (control and stressed, respectively).

Corticosterone Levels

Serum corticosterone (CORT) was measured in the stressed animals following one hour of restraint on days 1, 7, 14, and 21 of the stress period. A companion group of animals, neither stressed nor control, was sampled on Day 1 to provide baseline CORT measurements. Serum measurements of total CORT, as well as free CORT, were obtained. Significant differences between the stressed and quiet control animals in total CORT ($t = 2.43$, $p < 0.03$), as well as free CORT ($t = 3.13$, $p < 0.02$) were found. Table 1 shows the elevated serum CORT levels of the stressed animals as compared to those of

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the non-stressed companions. A significant effect of day of stress on levels of both total CORT ($F_{3,19}= 3.82$, $p< 0.02$) and free CORT ($F_{3,19}= 5.87$, $p<0.005$) was observed using ANOVA. Dunnett's Test post-hoc analysis, with Type I error controlled at 0.05 for the family of tests, identified significant differences in total CORT levels in stressed females on day 1 from levels on days 7, 14, and 21 (critical difference=184.28) and free CORT levels in stressed females on day 1 from those obtained on days 7, 14, and 21 (critical difference=144.54) (see Table 1). Animals were sacrificed by rapid decapitation 14-15 days after the stress period and their trunk blood was obtained. CORT levels returned to baseline 14 days after stress treatment; no significant difference was observed in stressed animals versus controls.

Behavioral Measures

Open Field

Open field measurements were quantified for the first three minutes and second three minutes of the task, Table 2. There was a significant difference between groups in outer sector crossings, $F_{1,27}=4.20$, $p=0.0504$, with stressed females making fewer overall visits (52.25 ± 2.99) than controls (61.38 ± 3.31). The total number of outer sector visits decreased over time for both groups ($F_{1,27}=19.19$, $p<0.0002$). No differences were observed between groups in total number of wall climbs; however, the number of wall climbs decreased across time for both groups ($F_{1,27}=20.87$, $p<0.0001$). No significant difference was observed for grooming, rearing, or inside sector crossing between the groups.

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Radial Arm Maze

Daily restraint stress for 21 days led to a small but significant difference in performance on the radial arm maze. As seen in Figure 4, stress enhanced performance as measured by the total number of visits required to complete the task. Stressed females completed the task in fewer visits than the controls, $F_{1,26}= 9.64$, $p<0.005$. Both groups took fewer visits to complete the task across time, $F_{3,160} = 4.52$, $p<0.005$, indicating that acquisition of the task was occurring. Additionally, stressed females made more correct choices in the first eight visits of the task as compared to the control animals, $F_{1,26}=5.19$, $p<0.03$, see Figure 5. There were no significant treatment effects for the visit in which the first error occurred across regular RAM trials.

In order to better describe and understand the main treatment and treatment X time effects, treatment effects were examined separately for females in proestrus, estrus, and diestrus. Additional behavioral differences among treatment groups were observed when estrous cycle day was held constant. While all stressed females required significantly fewer total visits to complete the task, the effect reached significance only for stressed subjects in diestrus (treatment X time, holding constant cycle), $F_{1,19}=5.09$, $p<0.04$. Additionally, animals in proestrus, both non-stressed and stressed, showed impaired RAM acquisition as evidenced by the visit in which the first mistake occurred (treatment X time, holding constant cycle), $F_{2,42}=3.20$, $p=0.0507$. Animals in estrus ($F_{2,30}=1.17$, $p>0.34$) and diestrus ($F_{3,47}=1.34$, $p>0.27$) did not show this acquisition effect, see Figure 6. Stress effects on other choice accuracy parameters during specific estrous cycle days were non-significant.

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No significant differences in performance between control and stress females, or during any estrous cycle stage, were observed during RAM trials with a 15 min, 1 h, 2 h, or 4 h delay between the fourth and fifth choice, see Table 3.

Neurochemical Analyses

Monoamines and metabolites

Monoamine levels differed among the control, stressed, and quiet control animals in select areas following the 21-day stressed period. Table 4 shows all the neurochemical values and Figure 7 shows a comparison between stress induced changes in females versus males. There was a main effect of stress treatment on prefrontal cortex dopaminergic activity, using a HVA/DA ratio ($F_{2,26}$=5.99, $p<0.008$). Post-hoc analysis revealed control group levels were lower than that observed in stressed and quiet control animals. Prefrontal cortex dopamine levels were higher in the control animals as compared to the naïve, but not stress animals; however, this trend failed to reached significance, $F_{2,29}$=2.81, $p<0.08$. Stress treatment also altered HVA levels in the CA3 region, $F_{2,29}$=3.72, $p<0.04$, with post-hoc analysis revealing elevated levels observed in the stress animals as compared to both control and naïve animals. There were no differences in monoamine levels among the treatment groups in the CA1, accumbens, or VDB areas.

Amino acids and metabolites

There was a main effect of stress treatment on glutamate levels in the CA3 region ($F_{2,28}$=4.23, $p<0.03$), with decreased levels observed in the stress animals as compared to controls, but not naïve animals. No changes in either glutamate or GABA were observed in the CA1, DG, or PFC regions, see Table 5.

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Figure 3. Twenty-one days of chronic restraint stress led to less weight gain in stressed females as compared to their counterpart controls.

Entries are the mean average body weight $\pm$ SEM (grams).

There was no main effect of stress on weight gain. There were significant effects of time ($F_{3,115} = 13.05$, $p < 0.00000$) and the treatment X time interaction ($F_{3,115} = 8.07$, $p < 0.00009$). Post-hoc revealed that stressed animals weighed less than the controls from stress day-13 through the end of the stress paradigm ($p<0.05$).

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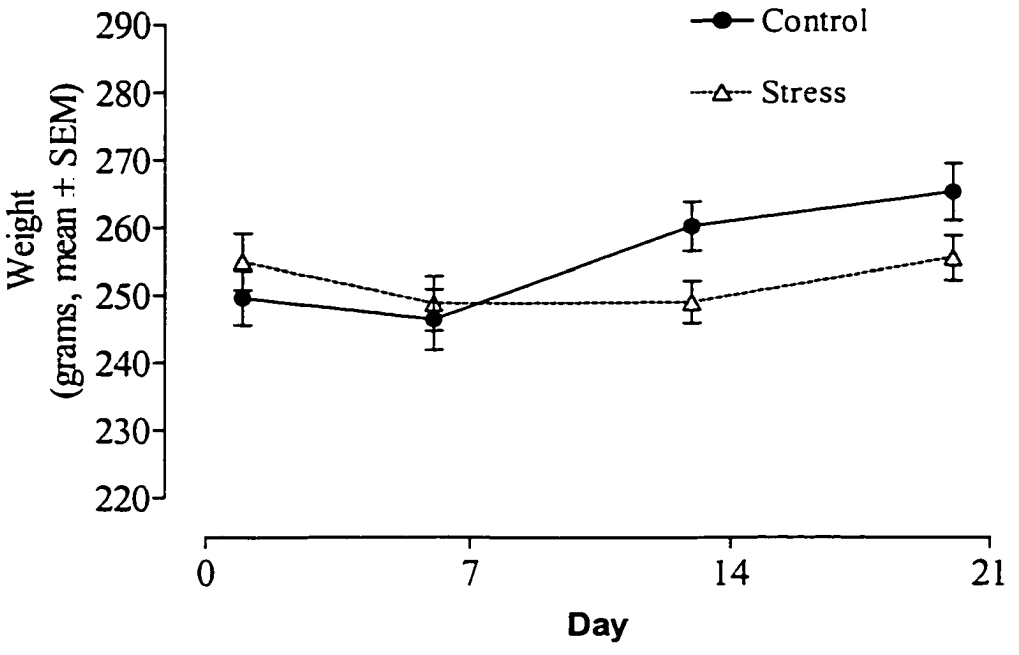

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Figure 4. Twenty-one days of chronic restraint stress enhances female radial arm maze performance as measured by the total number of visits required to complete the task. Entries are the mean $\pm$ SEM. A two-factor mixed ANOVA (treatment X time) indicated that all animals improved over time with regards to the total visits required to finish the task, $F_{3, 160}=4.52$, $p<0.005$; however, overall stressed females required fewer total visits (10.26 ± 0.24) to complete the task than controls (11.68 ± 0.36), $F_{1,26}=9.64$, $p<0.005$.

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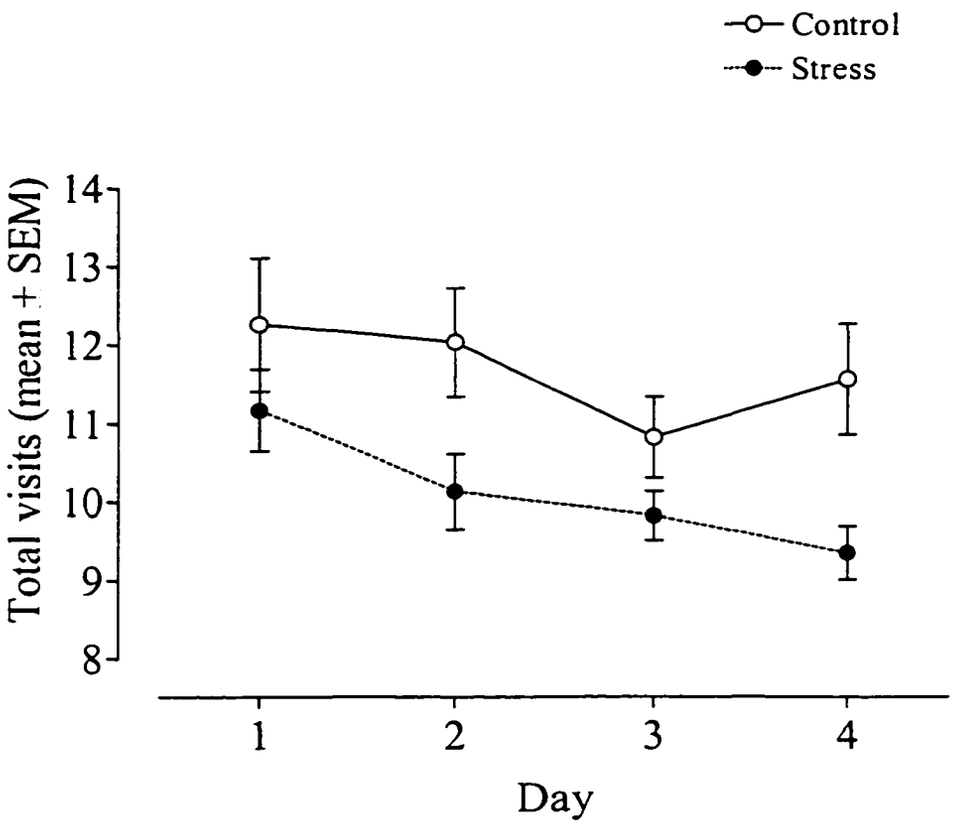

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Figure 5. Number of correct choices in first 8 visits is higher for stressed females than controls following 21 days of restraint stress.

Entries are the mean $\pm$ SEM. A two-factor mixed ANOVA (treatment X time) indicted that stressed females (6.91 $\pm$ 0.09) had more correct choices in the first eight visits of the task as compared to controls (6.59 $\pm$ 0.11), $F_{1, 26}=5.19$, $p<0.03$.

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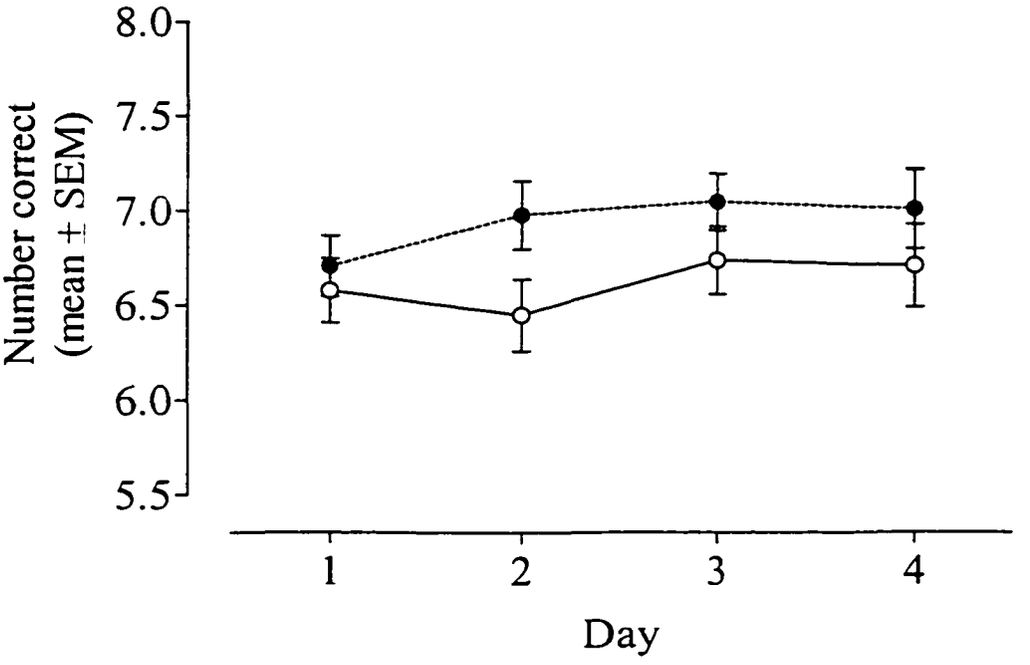

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Fig. 6. Effects of 21 days of stress on RAM performance in females at various days of the estrous cycle.

Entries are the mean $\pm$ SEM. Two-factor mixed ANOVAs (treatment X time, holding constant estrous cycle day) were used for analyses. Subjects in proestrus, regardless of treatment, improved over time on the visit in which the first mistake occurred ($F_{2,42}=3.20$, $p<0.0507$; o animals were in proestrus during behavioral testing on day 4). This improvement indicates that females in proestrus require longer acquisition of the task, while animals in estrus ($F_{2,30}=1.17$, $p>0.34$) and diestrus ($F_{3,47}=1.34$, $p>0.27$) did not show this acquisition effect.

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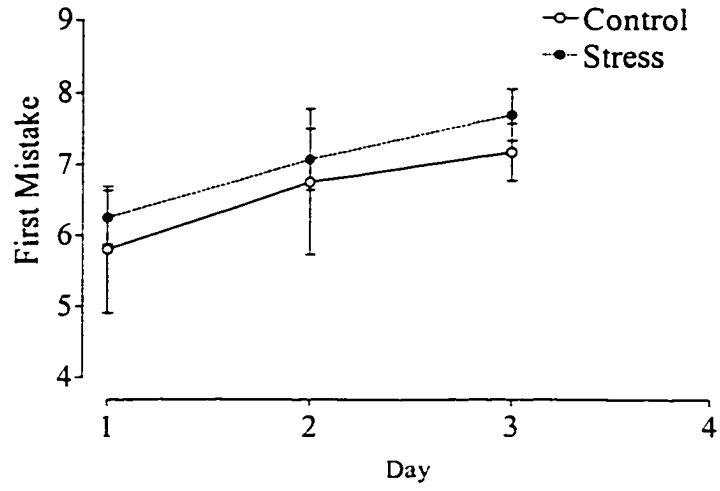
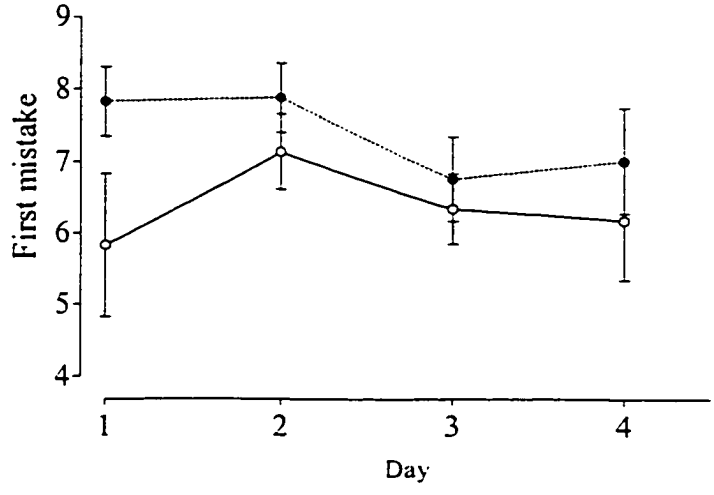
Proestrus



Estrus



Diestrus

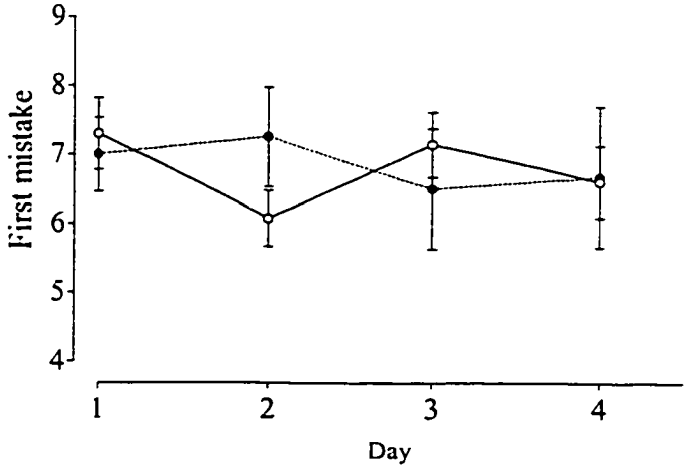


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Figure 7. A comparison of stressed induced neurochemical alterations in intact female rats following 21 days of chronic restraint stress to those previously observed in males.

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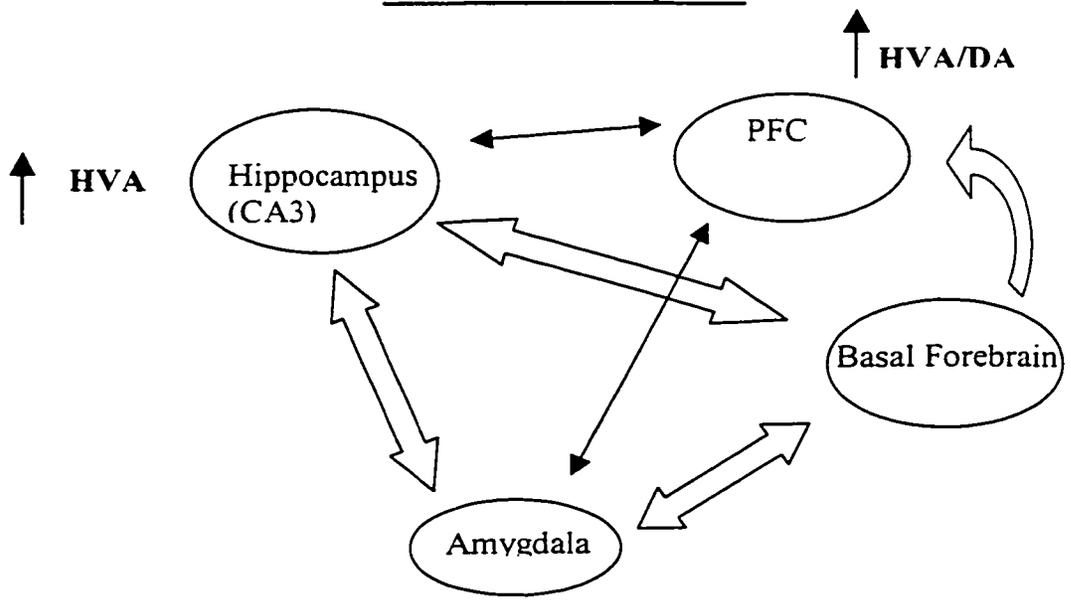
Female Stress Response:



Male Stress Response

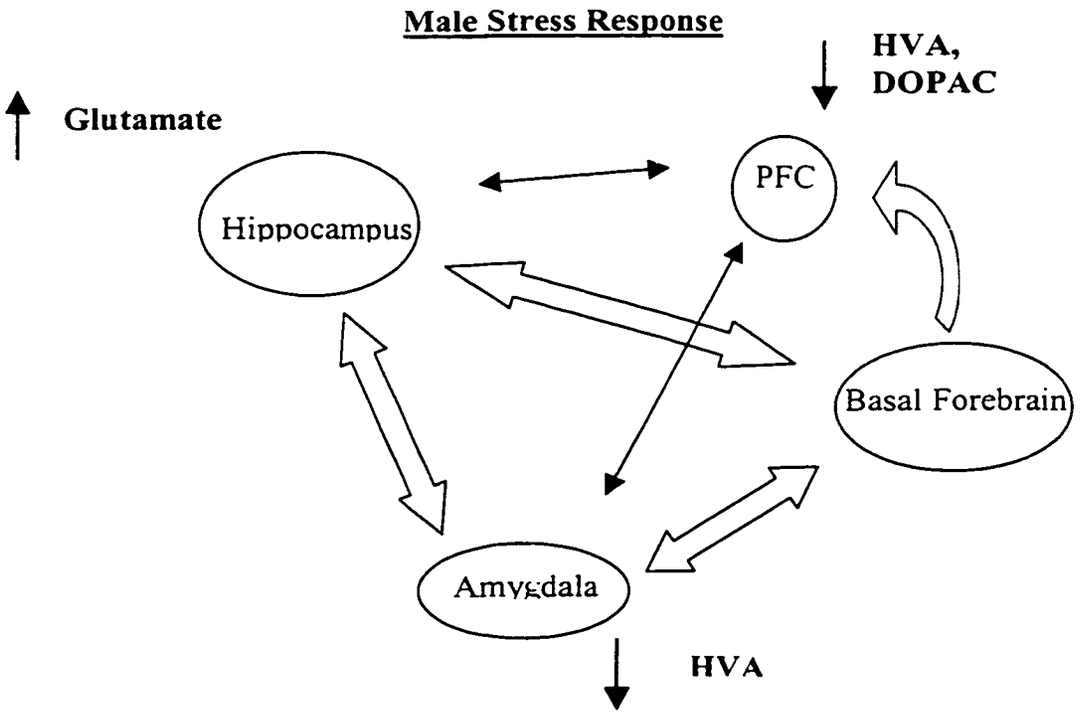


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Table 1. Comparison of total (bound and free) and free serum corticosterone (CORT) levels between stressed and quiet control female rats.

	Quiet Control Basal Levels	Day of chronic stress paradigm 1	7	14	21
Total CORT levels					
	232 ± 14	504 ± 112	310 ± 53	215 ± 36	291 ± 31
Free CORT levels					
	147 ± 14	404 ± 82	236 ± 38	151 ± 45	150 ± 26

Total and free serum corticosterone (CORT) levels are expressed as ng/ml. Entries are mean ± SEM. To account for differences in CORT levels across the estrous cycle, RIA analysis was performed on females in proestrus (N=5, quiet controls, N=5, stress day 1 and N=6, stress days 7, 14, and 21). A Two-sample t-test compared the means of stressed and quiet controls on Day 1 of the stress paradigm and indicated that stressed animals had higher levels of total and free CORT than controls. ANOVA assessed serum CORT levels in the stressed animals across the 21-days of chronic restraint indicating a main effect of stress day on total CORT ($F_{3,19}= 3.82$, $p< 0.02$) and free CORT ($F_{3,19}= 5.87$, $p<0.005$), Dunnett's post-hoc analysis indicated that both total CORT and free CORT measurements on day 1 were different from levels on days 7, 14, and 21.

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Table 2. Open field behaviors following 21 days of chronic restraint stress.

	Control		**Stress**	
Dependent variable	*Time 1*	*Time 2*	*Time 1*	*Time 2*
Outer sector crossing	**66.8 ± 3.4**	**56.0 ± 3.9**	**57.6 ± 3.7**	**46.9 ± 3.3**
Inner sector visits	5.2 ± 1.1	5.7 ± 1.5	5.8 ± 1.2	7.8 ± 1.4
Wallclimbs	**13.8 ± 1.3**	**10.0 ± 1.0**	**14.8 ± 1.1**	**10.2 ± 0.7**
Grooms	1.0 ± 0.4	2.3 ± 0.5	0.6 ± 0.2	1.1 ± 0.4
Rears (inner sectors)	0.7 ± 0.2	0.8 ± 0.3	0.3 ± 0.2	0.9 ± 0.4
Rears (outer sectors)	5.5 ± 0.8	5.4 ± 1.1	5.4 ± 1.0	4.9 ± 0.8

Entries are the mean ± SEM. Time 1 refers to the first three minutes on the field and Time 2 to the second three minutes on the field. There was a significant difference between groups in total outer sector crossings, $F_{1,27}=4.20$, $p=0.0504$, with stressed females making fewer overall outer sector visits (52.25 ± 2.99) than controls (61.38 ± 3.31). The total number of outer sector visits decreased over time for both groups ($F_{1,27}=19.19$, $p<0.0002$). No differences were observed between groups in total number of wall climbs; however, the number of wall climbs decreased across time for both groups ($F_{1,27}=20.87$, $p<0.0001$). No significant difference was observed for grooming, rearing, or inside sector crossing between the groups.

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Table 3. Twenty-one days of chronic restraint stress in intact females did not alter performance on RAM delay trials.

Delay	Group	Total Visits	First Mistake
15 min	Control	12.2 ± 0.6	5.6 ± 0.1
	Stressed	12.5 ± 0.9	5.8 ± 0.3
1 h	Control	12.3 ± 0.5	5.8 ± 0.2
	Stressed	10.9 ± 0.5	5.7 ± 0.3
2 h	Control	12.8 ± 1.4	6.2 ± 0.3
	Stressed	12.2 ± 0.7	5.6 ± 0.3
4 h	Control	12.3 ± 0.3	5.3 ± 0.2
	Stressed	10.7 ± 0.8	5.5 ± 0.2

There were no differences between stressed and control animals during RAM trials including either a 15 min, 1 h, 2 h, or 4 h delay between the fourth and fifth choices

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Table 4. Summary of monoamine and metabolite levels following 21 days of chronic restraint stress in intact females.

Area	Group	DA	HVA	HVA/DA	NE	5HIAA
PFC	Control	**1.1 ± .36**$^{+}$	.49 ± .16	**.52 ± .11***	3.2 ± .78	4.5 ± .88
	Stressed	**.84 ± .59**	.80 ± .17	**.90 ± .11**	4.3 ± .58	5.7 ± .63
	Naïve	**.39 ± .06**	.51 ± .09	**1.2 ± .13**	3.1 ± .48	4.5 ± .64
CA3	Control	.01 ± .003	**.003 ± .001***	.83 ± .32	3.4 ± .007	.02 ± .01
	Stressed	0.46 ± 0.17	**.05 ± .02**	.48 ± .11	4.7 ± .01	.02 ± .005
	Naïve	.017 ± .003	**.01 ± .002**	.37 ± .09	3.9 ± .004	.03 ± .005
CA1	Control	4.8 ± 1.3	1.3 ± 0.3	1.0 ± .45	10.5 ± 1.4	5.8 ± 1.1
	Stressed	9.2 ± 2.7	1.8 ± 0.4	0.7 ± .28	11.3 ± 2.1	7.7 ± 1.2
	Naïve	4.9 ± 2.7	1.0 ± 0.3	1.7 ± .82	6.5 ± 2.0	5.2 ± 1.9
nAc	Control	38.7 ± 9.3	3.3 ± 0.7	0.1 ± .02	3.6 ± 0.9	4.5 ± 0.8
	Stressed	43.5 ± 4.1	2.9 ± 0.3	0.1 ± .02	5.9 ± 1.1	3.0 ± 0.3
	Naïve	54.0 ± 7.7	3.1 ± 0.5	0.07 ± .04	4.7 ± 1.2	4.2 ± 0.6
VDB	Control	40.8 ± 8.2	5.0 ± 0.9	.13 ± .04	40.2 + 3.2	12.9 ± 1.9
	Stressed	50.6 ± 8.3	4.5 ± 0.8	.07 ± .01	35.8 ± 2.2	15.6 ± 1.8
	Naïve	52.4 ± 12.2	5.2 ± 1.1	.09 ± .01	40.6 ± 4.2	14.0 ± 2.2

Entries are the mean ± SEM. Values expressed are pg/μg protein. PFC dopaminergic activity, as measured by the HVA/DA ratio, was lowest in the control animals as compared to both the stressed and naïve groups, $F_{2,26}=5.99$, $p<0.008$. Prefrontal cortex dopamine levels were higher in the control animals as compared to the naïve animals, however this trend failed to reached significance, $F_{2,29}=2.81$, $p<0.08$. Stress treatment led to elevated HVA levels in the CA3 as compared to control but not naïve animals, $F_{2,29}=3.72$, $p<0.04$. An asterisk (*) represents a significant effect, $p<0.05$. A cross (+) represents a non-significant trend, $p>0.05$.

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Table 4 continued

Entries are the mean ± SEM. Values expressed are pg/μg protein. PFC dopaminergic activity, as measured by the HVA/DA ratio, was lowest in the control animals as compared to both the stressed and naïve groups, $F_{2,26}=5.99$, $p<0.008$. Prefrontal cortex dopamine levels were higher in the control animals as compared to the naïve animals, however this trend failed to reached significance, $F_{2,29}=2.81$, $p<0.08$. Stress treatment led to elevated HVA levels in the CA3 as compared to control but not naïve animals, $F_{2,29}=3.72$, $p<0.04$. An asterisk (*) represents a significant effect, $p<0.05$. A cross (+) represents a non-significant trend, $p>0.05$.

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Table 5. Glutamate and GABA levels following 21 days of chronic stress in intact females.

Amino Acid	**Area**	**Group**		
		Control	*Stress*	*Naïve*
GLU	CA1	57.9 ± 5.2	78.4 ± 9.9	50.3 ± 8.7
GABA		9.3 ± 1.6	17.4 ± 4.6	9.2 ± 2.4
GLU	CA3	**60.58 ± 5.7***	**38.9 ± 5.0**	**46.8 ± 2.8**
GABA		2.9 ± 0.8	2.6 ± 0.3	4.0 ± 0.9
GLU	DG	62.9 ± 13.7	48.9 ± 11.2	76.9 ± 17.5
GABA		48.0 ± 6.5	42.6 ± 4.2	66.6 ± 10.3
GLU	PFC	84.1 ± 16.3	61.3 ± 13.0	60.5 ± 11.0
GABA		13.3 ± 2.1	11.4 ± 2.1	13.6 ± 2.5

Entries are the mean ± SEM. Values are expressed as ng/ml protein. Control animals had higher glutamate levels in the CA3 region as compared to stressed, but not naïve, animals ($F_{2,28}=4.23$, $p<0.03$).

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Discussion

Physiological measures

Weight gain

All animals used in this experiment gained weight across time; however, stressed animals weighed less than their counterpart controls across 21 days of stress. This result is consistent with previous studies in males in which restraint stress causes a decrease in body weight in an indirect fashion (Akana, *et al.*, 1999). That is, restraint stress leads to increased levels of CORT, which in turn leads to an overall decrease in weight gain. This would be comparable to observations in which other stressors (e.g., cold stress) have been shown to interact with CORT on signals of energy balance (Akana, *et al.*, 1999).

Corticosterone levels

CORT exists in two forms in plasma, free or bound, with total CORT levels, as measured by RIA, being the sum of free and bound. Typically, studies have only investigated the effects of stress on total CORT levels, with little attention being paid to levels of circulating free CORT (Atkinson, *et al.*, 1997; Buckingham, *et al.*, 1978; Galea, *et al.*, 1997). Bound CORT is generally considered to be biologically inactive, while free CORT exerts the effects of this hormone (Fleshner, *et al.*, 1995). To determine the amount of biologically active CORT, we removed the bound fraction (see methods). We were specifically interested in assessing free CORT levels to determine if they could account for the lack of female sensitivity previously observed in response to chronic stress. Chronic restraint stress leads to elevated levels of both free and bound CORT (see Table 1). The levels of total CORT observed in the current studies are consistent with those previously reported (Atkinson, *et al.*, 1997; Buckingham, *et al.*, 1978; Raps, *et al.*, 1971; Viau & Meaney, 1991). The levels of total CORT in females are higher than that

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reported for males (Galea, *et al.*, 1997) and reflects the sexual dimorphic CORT release of the HPA axis following stress (Atkinson, *et al.*, 1997).

On stress day 1 total CORT levels were higher than those observed for stress days 7, 14, and 21, suggesting that the stressed females had habituated to the stress paradigm by day 7. Free CORT levels on stress day 1were also higher than those observed on day 7, 14 or 21. This suggest that other stress induced mechanisms may be involved in regulating the spatial memory enhancement as no significant difference in the amounts of biologically active (free) CORT exists by the end of the stress period (21 days). However, this does not exclude the possibility that the initial increase in free CORT levels triggers a cascade of events that results in the observed enhancement. Interestingly, the ratio of free: bound CORT is approximately 80% on stress day 1 and falls to 50% by day 21. It is possible that declining free: bound CORT levels are partly responsible for females' resistance to the effects of chronic restraint stress. Currently, little is known about the active levels of CORT in male rats following stress; if higher free: bound CORT levels exist in males, this could account for the detrimental effects of restraint stress observed in male rats. Future studies should investigate the profile of free: bound CORT ratios in males in response to stress.

Estrous cyclicity

Results from this study indicate that 21 days of chronic restraint stress leads to no significant differences in estrous cycle length in females with either normal length (4-5 days) or elongated cycles (up to 9 days), when compared to control females. While average cycle lengths were longer than that usually reported (4-5 days) for Sprague Dawley or other strains, they are not inconsistent with previously reported observations

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(Long & Evans, 1922). This increase in cycle length could be attributed to the subjects or methodology used. The females used in the current experiments were young, 10-12 weeks, and possibly had yet to establish mature cycling patterns. Alternatively, vaginal smearing could be responsible for this temporal increase in cyclicity.

Stress has been reported to cause alterations in reproductive function in humans, non-human primates, and rats. In humans, stressors such as psychological distress (Hjollund, *et al.*, 2000), environmental toxin exposure (Thursten *et al.*, 2000), and strenuous exercise (Beckvid Henriksson, *et al.*, 2000) leads to aberrations in menstrual cycles and/or amenorrhea. In rats, chronic exposure to physical stressors (forced swimming, foot shock, temperature extremes) leads to estrous cyclicity alterations (Axelson, 1987; Gonzalez, *et al.*, 1994; Rodriguez *et al.*, 1988). However, less severe stressors do not appear to disrupt estrous cycling in rats. Anderson *et al.* (1996) showed that a 14-day sustained stress paradigm in which rats received around-the-clock signaled intermittent foot shock and could either avoid/escape the shock or had no control over the shock did not experience estrous cycle alterations. Additionally, mild food deprivation (85% body weight) did not disrupt normal estrous cycle patterns in Sprague Dawley rats (Tropp & Markus, 2001). It appears that stressors of various severities have differential effects on the estrous cycle. Stressors that disrupt the rat estrous cycle are generally more severe and physical in nature. Restraint stress is believed to be a psychosocial stressor (Luine, 1997) and subsequently did not appear to disrupt estrous cyclicity of female rats in this study that exhibited either normal length or elongated cycles.

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Behavioral measures

Open field and RAM

The current data show that stressed subjects made fewer outer sector crossings and these results are consistent with previously reported data in which stressed subjects had decreased ambulation on the open field (Katz, *et al.,* 1981; Soblosky & Thurmond, 1986), (Table 2). The decreased ambulation of the stressed subjects in the current studies did not appear to compromise RAM performance, as there were no differences between the groups with regards to the time required to complete the spatial task.

The eight arm radial maze was used to evaluate the effect of chronic stress on female rat spatial memory. These experiments are, to the best of our knowledge, the first investigations of the effect of chronic stress on spatial memory in female rats. Our data indicates that 21 days of chronic restraint stress enhances spatial memory. That is, stressed females scored better than controls on both the total visits to complete the task, as well as, the number of correct choices in the first eight visits (see Figures 4 and 5). This result is different from data previously reported for male rats, which are impaired following 21 days of stress (Luine, *et al.*, 1994). Specifically, stressed males make their first mistake sooner and have less correct responses in their first eight choices as compared to controls following 21 days of chronic restraint stress (Luine, *et al.*, 1994). The dimorphic behavioral response to chronic stress is consistent with the sexual dimorphism observed in morphology (Galea, *et al.*, 1997) and chemistry (Beck & Luine, 1998; Luine, *et al.*, 2001) following restraint stress. As previously reported, in contrast to males, female rats do not show atrophy of apical dendritic branching following 21 days of restraint stress (Galea, *et al.*, 1997). Female rats also show a different pattern of

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neurochemical changes following stress than males, with neither frontal cortex dopamine or amygdala norepinephrine being affected (Beck & Luine, 1999). Both males and females show changes in hippocampal amino acid levels; but in males, changes are centered in CA3 and in females changes are centered in CA1 regions (Luine, *et al.*, 2001).

This sex difference on RAM performance following 21 days of chronic stress may be the result of influences exerted by gonadal hormones, specifically estrogen, on the stress response. We speculate that circulating estrogen results in female resistance to stress-induced impairments by either exerting a direct protective effect on the hippocampus or by modifying the HPA cascade in females (e.g., estrogen could be influencing CORT release or GC receptor density).

It has been well established that estrogens play a beneficial role in learning and memory in both humans and rats. Both estrogen treated gonadectomized males (Luine & Rodriguez, 1994) and estrogen-treated ovariectomized females (Luine, *et al.*, 1998) perform better during delay RAM trials than controls. Estrogen enhancements have also been reported on numerous other spatial memory tasks including two-way active avoidance (Sing, *et al.*, 1994) and T-maze acquisition (Fader, *et al.*, 1998). It is unclear at what level estrogen is exerting its moderating effect on the HPA stress response and whether the effect is activational or organizational. As stated, estrogen could be exerting a direct effect on behavior or could be affecting the CORT response at the neurochemical or molecular level (e.g., estrogen could be altering glucocorticoid receptor levels, Ferrini & De Nicola, 1991)

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Alternatively, estrogens could be exerting an effect on spatial learning and memory by increasing spine density in the CA1 hippocampal cells (Murphy & Segal, 1996). As previously mentioned, elevated CORT levels, in female rats, do not lead to apical hippocampal atrophy in CA3 neurons (as compared to males); however, there is resulting basal remodeling (Galea, *et al.*, 1997). Possibly, this remodeling in the female hippocampus following stress and the enhancing effects of estrogen on the CA1 pyramidal cells, are working together to lead to stress-dependent enhancements in females.

Interestingly, during delay trials, no effects of stress on female performance were observed. This result is consistent with previous results in male rats (Luine, *et al.*, 1996) and suggests that stress-dependent spatial memory enhancement is possibly not sufficient to overcome the high cognitive demand of delay trials. Alternatively, no effects of stress on female performance during delay trials may be because stress-dependent enhancements are temporally constrained. Luine, *et al.* has shown that male rats stressed for 21 days, but not tested on the RAM until 18 days post-stress, show no effects of stress on performance (Luine, *et al.*, 1994). Additionally, it has been shown that stress induced dendritic atrophy is reversed after termination of the stress paradigm (by 5-10 days, Conrad, *et al.*, 1999).

Female RAM performance, following stress, was also influenced by specific estrous cycle days. Following 21 days of stress, when treatment effects are examined at each stage of the estrous cycle, there is a trend for all stressed animals to perform better than controls; however, performance of stressed females is only significantly different than that of controls during diestrus. It is unclear why animals in diestrus, when hormone

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levels are low, are performing better. One possibility is that during diestrus, there are insufficient gonadal hormones to interact with the stress effect. Additionally, proestrus females, regardless of treatment, had impaired RAM acquisition following 21 days of stress. This proestrus associated impairment is similar to previously reported work in which spatial memory acquisition was impaired during proestrus when gonadal hormones are at their highest (Frye, 1995; Warren & Juraska, 1997).

Neurochemical assessment

Neurochemical changes were observed in brain regions believed to be involved in spatial learning and memory. Specifically, neurochemical changes were observed in the prefrontal cortex, an area involved in spatial working memory, and the CA3 region of the hippocampus, a brain region that is thought to be critical to optimal performance of the RAM. PFC dopaminergic activity, as measured by the HVA/DA ratio, was lowest in controls as compared to both the stressed and naïve animals. Elevated HVA levels in the CA3 were observed in stressed animals as compared to control and naïve animals. Stress treatment decreased glutamate levels in the CA3 region as compared to controls, but not naive.

The catecholamine system, specifically DA, has been implicated in learning and memory. It has been shown that DA has a beneficial impact on spatial working memory (Simon, 1981; Bubser & Schmidt, 1990); however, both excessive or insufficient levels of DA activity lead to impairments (Murphy, *et al.*, 1996; Zahrt, *et al.*, 1997). Control animals had the lowest level dopaminergic activity in the PFC (as measured by HVA/DA ratio) and suggest that this depletion may be contributing to the poorer performance of control animals on the RAM as compared to stressed subjects. Of interest is the

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observation that control animals had decreased dopaminergic activity as compared to both the stress and naïve animals. This seems to suggest that cognitive demand of behavioral testing lowers DA levels but that this process is attenuated by the accompaniment of stress. Additional changes in the DA system were observed in the CA3 region of the hippocampus, with stress treatment increasing HVA levels as compared to controls and naïve animals. Increased HVA levels suggest an increase in DA activity. The hippocampus is critical to rodent RAM performance and this result again suggests that changes in the DA system are contributing to stress-dependent enhancements in spatial memory. Interestingly, stressed rats had higher DA activity than controls in both PFC and CA3.

Additionally, it is important to note, that ovarian hormones are critical regulators of prefrontal cortex innervation and neurochemical profiles. Prefrontal cortex pyramidal neurons are innervated by both dopaminergic and serotonergic afferents (Williams & Goldman-Rakic, 1993, 1998; Jakob & Goldman-Rakic, 1998) and these afferents mediate PFC neuronal excitability. In the dorsolateral PFC in monkeys, OVX reduces, while subsequent estrogen and progesterone replacement restores, the density of axons immunoreactive for tyrosine hydroxylase (Kritzer & Kohama, 1999). Estrogen replacement to OVX animals attenuates the observed decreases in density of fibers immunoreateive for choline acetyltransferase, as well as the increases the density of axons immunoreactive for dopamine beta-hydroxylase (Kritzer & Kohama, 1999). Consistent with these observations, monoamine oxidase, an enzyme involved in the degradation of dopamine, is decreased in response to estradiol (McEwen, Biegon, Fischette, Luine, Parsons, and Rainbow, 1984).

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Similarly, ovarian hormones are critical regulators of the striatal DA systems, influencing both behavioral and biochemical aspects of non-reproductive behaviors. For example, DA metabolism, as measured by HVA levels, varies across the estrous cycle of the rat (Jori & Cecchetti, 1973) and hormone variations across the estrous cycle also modulate striatal D-1 DA receptors (Levesque & Di Paolo, 1990). Furthermore, the density of striatal DA uptake sites in OVX female rats is increased after an acute does of estradiol, but not of progesterone (Morisette, Biron, and Di Paolo, 1990). Because it is clear that ovarian hormones, particularly estrogen, influence the DA system, it is appealing to speculate that observed sex differences on spatial memory tasks may indeed result from fundamental sex differences in the DA system in areas critical to optimal performance of these tasks, most notably the prefrontal cortex.

Stress treatment decreased glutamate levels in the CA3 region as compared to control, but not naïve animals. The CA3 region plays a critical role in the transmission of information from the mossy fibers of the dentate gyrus to the CA1 region of the hippocampus. Glutamate is crucial for the induction of long-term potentiation (LTP), a likely synaptic mechanism underlying hippocampal memory. The decreased levels of glutamate observed in the stressed females could be indicative of increased glutamate activity. Thus, it seems tenable to suggest that the stress-dependent enhancements in RAM observed are due in part to changes in glutamate activity. Again, because control and naïve animals did not differ from one another, it appears that the neurochemical alterations are being induced by a synergistic effect of both cognitive demand and stress.

Changes in activation of both DA and glutamate by stress treatment in the CA3 region of females are notable because this is the site for CORT dependent decreases in

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dendritic length (Galea, *et al.,* 1997). Interestingly, stressed males show changes in apical dendritic length (Galea, *et al.*, 1997) and there are changes in the activity of CA3 GABA in males (Beck & Luine, 1999); however, females show changes in basal dendritic length and stress induced neurochemical alterations involve CA3 DA and glutamate. Thus, it seems tenable to suggest that sexually dimorphic neurochemical responses to stress may underlie the stress-induced sexually dimorphic morphological changes.

In summary, the current study provides novel information about the effects of chronic restraint stress on intact female rats. 21-days of restraint stress did not lead to alterations in estrous cyclicity. Additionally, unlike males, female rats are enhanced on RAM following 21-days of chronic restraint stress. Neurochemical changes in areas known to contribute to learning and memory were observed in response to stress and may underlay the observed stress-dependent enhancements. It is appealing to speculate that these differences are due, in part, to moderating effects of gonadal hormones (estrogen in particular) on the HPA response and/or hippocampal sensitivity to chronic stress.

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Chapter 2 (experiment 2) - Effects of 28-days chronic restraint stress on intact female rats: Assessment of behavioral, physiological, and neurochemical parameters

Results from experiment 1 showed that performance on a spatial memory task is enhanced in female rats following 21 days of chronic restraint stress. In contrast, previous work has shown that male rats are impaired following this stress paradigm (Luine, *et al.*, 1994); however, males that are stressed for 14 days show enhanced RAM performance (Luine, *et al.*, 1996). Taken together, these results suggest that females may require longer periods of stress than males to show impairment. Alternatively, females may not be impaired by chronic restraint stress. To test these possibilities, a second experiment was conducted in which the stress period was extended to 28 days.

Methods

Subjects

Twenty-four female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) aged 55-60 days old, upon arrival, served as subjects. Group assignment, daily vaginal lavages, periodic weighing, and behavioral assessment were as described in Experiment 1. No blood was collected from the animals. The stress period was extended to 28 days.

General Procedure

All post-stress procedures, including food restriction, open field assessment, RAM testing, and neurochemical assessment were identical to those described in Experiment 1.

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Data Analysis

Analysis of weight gain, estrous cyclicity, and behavioral data were identical to those described in experiment 1. Neurochemical differences between the stressed and non-stress groups were assessed using two-sample t-tests.

Results

Physiological Measures

Weight

There was no main effect of stress on weight gain. There were significant effects of time ($F_{4,75} = 36.94$, $p < 0.001$) and the treatment X time interaction ($F_{4,75} = 8.58$, $p < 0.001$). As seen in Figure 8, stressed females weighed less than controls from stress day 7 through stress day 21; however, by stress day 28 both stress and control animals weighed approximately the same.

Estrous Cycle

There were no effects of treatment on average cycle length. During the pre-stress period, the average cycle lengths were 8.5 ± 0.37 days and 7.6 ± 0.22 days for controls and stressed animals, respectively (Mean $\pm$ SEM). During stress, the lengths were 8.6 ± 0.30 days and 8.5 ± 0.28 days (controls and stressed, respectively) and during the post-stress period cycle lengths were 9.1 ± 0.38 days and 8.0 ± 0.43 days (controls and stressed, respectively). One stressed animal exhibited constant diestrus during the stress period and two controls exhibited constant estrous during the behavioral period. These three animals were not included in the estrous cycle means. A separate analysis was performed on the females that exhibited more typical estrous cycle lengths, 4-5 days. This subgroup of stressed females did not differ from controls with respect to their mean

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estrous cycle length. During the pre-stress period the mean cycle length was 4.6 ± 0.3 and 5.0 ± 0.1 days (Mean ± SEM) for control and stressed animals, respectively. Average cycle length during the stress period was 4.8 ± 0.4 for controls and 5.1 ± 0.1 for stressed and during the post-stress period was 5.5 ± 0.3 and 5.5 ± 0.3 days (control and stressed, respectively).

Behavioral Measures

Open Field Assessment

Following 28 days of chronic stress, there were no differences between the stressed and non-stressed subjects on any performance parameters on the open field task, Table 6. All subjects showed habituation during the second half of the task, making more visits to the inner sectors, $F_{1, 22}=5.42$, $p<0.03$ and fewer wall climbs, $F_{1, 22}=6.26$, $p< 0.02$.

Radial Arm Maze

There were no overall differences between stressed and control females on any RAM choice accuracy parameter following 28 days of stress, see Fig 9. All subjects improved over time on the number of correct choices in the first eight visits ($F_{1, 148}=3.04$, $p<0.03$), indicating an acquisition effect.

However, there were statistically significant differences among the treatment groups when evaluated at specific days during the animals' estrous cycles. Stressed animals in proestrus had better choice accuracy rates on the number of correct choices in first eight visits (6.77 ± 0.18) as compared to controls (6.28 ± 0.09), $F_{1,17}=4.74$,$p<0.04$, this effect was not significant for animals in estrus or diestrus. As in experiment 1, stress did not affect female RAM performance during Delay trials, see Table 7.

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Neurochemical analyses

Twenty-eight days of stress altered monoamine levels in several brain areas examined. Table 8 shows all the monoamine levels and Figure 10 illustrates the statistically significant changes. Prefrontal cortex levels of HVA were higher in stressed animals than in controls, $t=-2.17$, $p<0.04$. Both NE ($t=-2.24$, $p< 0.04$) and 5H1AA ($t=-2.82$, $p< 0.01$) levels were higher in stressed females than controls in the VDB. Additionally, NE levels ($t=-2.27$, $p<0.04$) and DA levels ($t=-3.68$, $p< 0.003$) were elevated in stressed animals in the DG region of the hippocampus. No changes were observed in the CA1, CA3, BLA, nAc regions.

Amino acid levels

Following 28 days of chronic restraint stress, elevated GABA levels in the DG were observed in stressed animals as compared to controls, $t=2.6$, $p<0.02$, see Table 9. No changes were observed in any other brain regions sampled.

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Figure 8.Weight gain across the 28-day stress period in control and stressed female rats. Entries are the mean $\pm$ SEM. While there was no main effect of stress on weight gain, there were significant effects of time ($F_{4,75} = 36.94$, $p < 0.001$) and the treatment X time interaction ($F_{4,75} = 8.58$, $p < 0.001$). Stressed females weighed less than controls from stress day 7 through stress day 21; however, by stress day 28 both stress and control animals weighed approximately the same.

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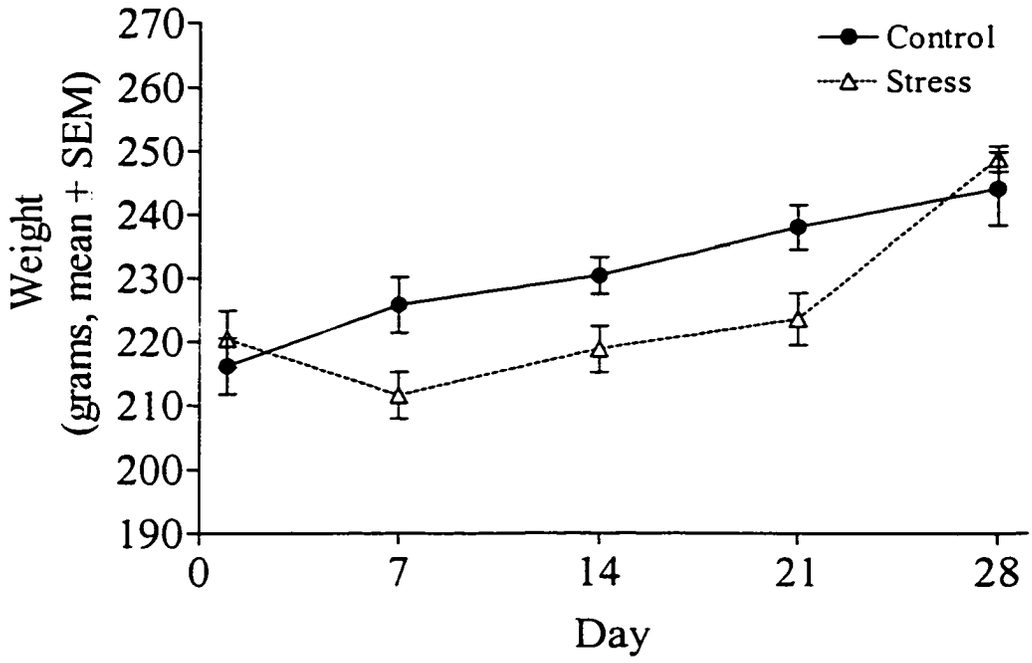

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Figure 9. Twenty-eight days of restraint stress did not alter female RAM performance. Entries are the mean $\pm$ SEM. There were no differences between the treatment groups on any RAM choice accuracy parameter including total visits to complete the task ($F_{1,27}=0.01$, $p>0.92$), number correct in first eight visits ($F_{1,27}=2.75$, $p>0.10$), and the choice on which the first mistake occurred ($F_{1,27}=0.15$, $p>0.70$). All subjects improved over time on the number of correct choices in the first eight visits ($F_{1, 148}=3.04$, $p<0.03$), indicating an acquisition effect.

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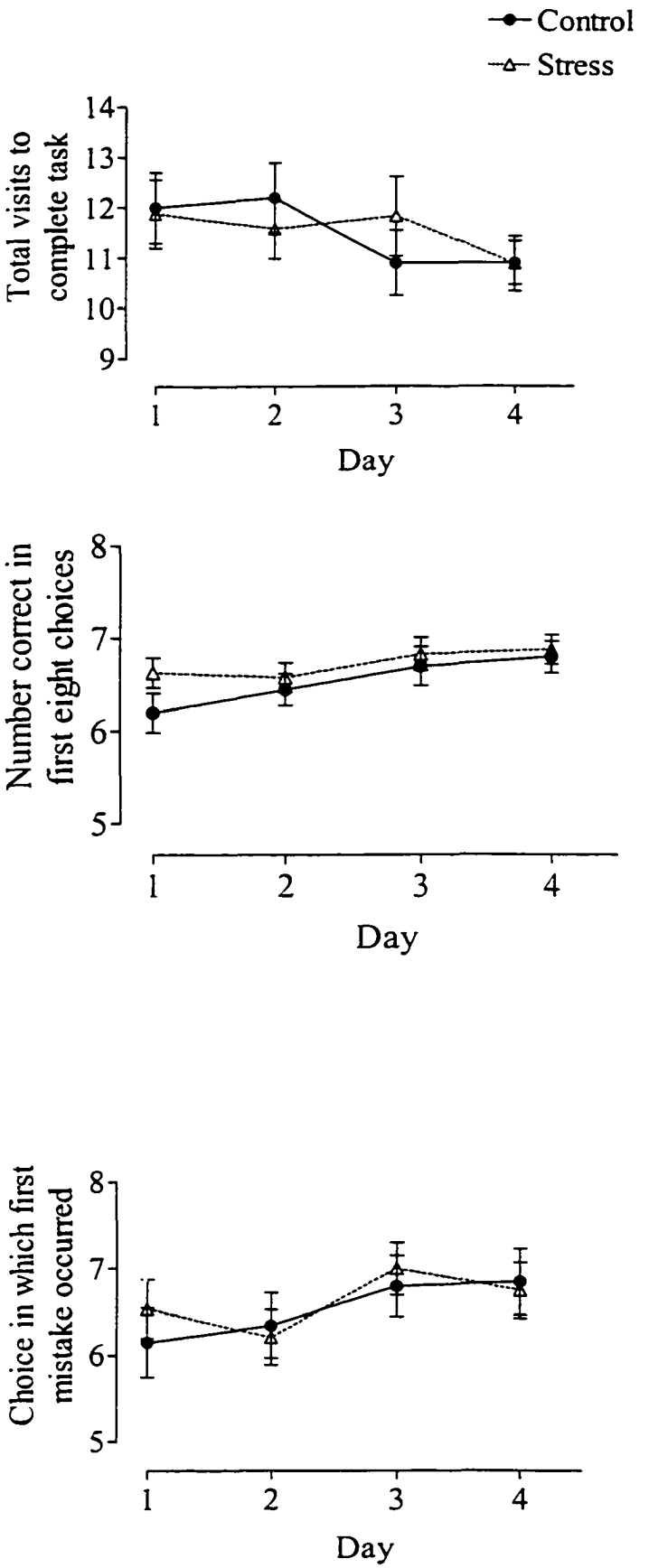

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Figure 10. Schematic illustration depicting stressed induced neurochemical changes in intact female rats following 28 days of chronic restraint stress.

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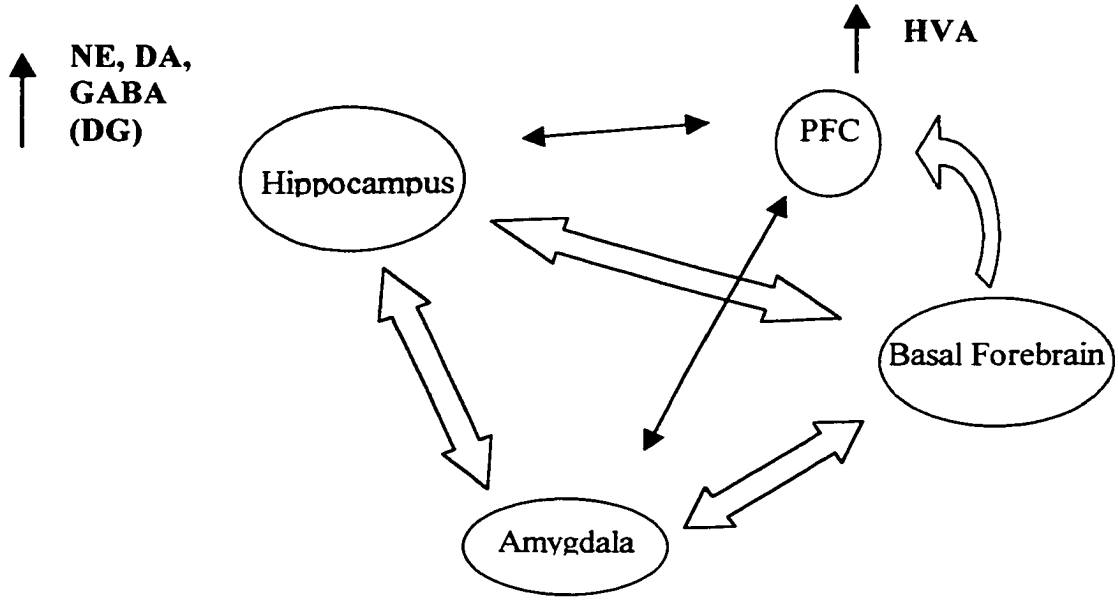

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Table 6. Open field behaviors following 28 days of chronic restraint stress.

	Control		Stress	
Dependent variable	*Time 1*	*Time 2*	*Time 1*	*Time 2*
Outer sector crossing	55.5 ± 3.6	58.3 ± 3.3	54.8 ± 5.4	53.8 ± 4.9
Inner sector visits	4.2 ± 1.1	7.3 ± 1.1	5.0 ± 1.2	6.2 ± 1.0
Wallclimbs	11.4 ± 1.0	9.7 ± 0.7	11.6 ± 1.3	9.8 ± 1.3
Grooms	0.8 ± 0.3	1.6 ± 0.4	1.3 ± 0.3	1.2 ± 0.3
Rears (inner sectors)	0.3 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0.3 ± 0.1
Rears (outer sectors)	1.9 ± 0.6	2.9 ± 0.9	2.4 ± 0.7	2.7 ± 0.9

Entries are the mean ± SEM. Time 1 refers to the first three minutes on the field and Time 2 to the second three minutes on the field. Following 28 days of chronic stress, there were no differences between the stressed and non-stressed subjects on any performance parameters on the open field task. All subjects showed habituation during the second half of the task, making more visits to the inner sectors, $F_{1, 22}=5.42$, $p<0.03$ and fewer wall climbs, $F_{1, 22}=6.26$, $p< 0.02$

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Table 7. Twenty-eight days of chronic restraint stress in intact females did not alter performance on RAM delay trials.

Delay	Group	Total Visits	First Mistake
15 min	Control	11.6 ± 1.1	6.3 ± 0.4
	Stressed	12.3 ± 0.5	5.9 ± 0.4
1 h	Control	13.2 ± 1.1	5.7 ± 0.3
	Stressed	11.3 ± 0.7	5.7 ± 0.2
2 h	Control	12.2 ± 0.4	5.8 ± 0.4
	Stressed	11.4 ± 0.7	5.8 ± 0.3
4 h	Control	12.1 ± 0.5	5.2 ± 0.3
	Stressed	11.8 ± 0.5	6.0 ± 0.3

There were no differences between stressed and control animals during RAM trials including either a 15 min, 1 h, 2 h, or 4 h delay between the fourth and fifth choices.

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Table 8. Summary of monoamine and metabolite levels following 28 days of chronic restraint stress in intact females.

Area	Group	DA	HVA	NE	MHPG	5HIAA
PFC	Control	0.8 ± 0.3	**0.23 ± 0.07**	1.7 ± 0.26	ND	2.7 ± 0.31
	Stressed	1.4 ± 0.3	**0.57 ± 0.13***	2.4 ± 0.36	ND	2.3 ± 0.34
CA1	Control	1.3 ± 0.56	30.8 ± 17.1	13.7 ± 7.1	0.4 ± 0.2	23.4 ± 11.8
	Stressed	2.5 ± 0.47	62.2 ± 61.4	3.3 ± 0.82	0.5 ± 0.2	4.3 ± 3.9
CA3	Control	4.4 ± 0.32	ND	3.5 ± 1.2	ND	ND
	Stressed	5.3 ± 1.1	0.1 ± 0.1	3.2 ± 1.2	ND	ND
DG	Control	**1.5 ± 0.9**	ND	**0.2 ± 0.13**	0.2 ± 0.13	ND
	Stressed	**9.4 ± 2.0***	ND	**2.4 ± 1.0***	4.1 ± 3.6	ND
BLA	Control	0.7 ± 0.2	0.77 ± 0.3	4.5 ± 1.0	0.11 ± 0.03	1.1 ± 0.3
	Stressed	1.3 ± 0.4	0.12 ± 0.05	4.7 ± 1.0	0.29 ± 0.2	1.0 + .22
nAc	Control	9.6 ± 1.9	3.34 ± 0.52	0.06 ± 0.06	1.6 ± 0.4	1.5 ± 0.39
	Stressed	7.5 ± 1.5	5.08 ± 1.2	1.0! ± 0.49	0.66 ± 0.3	2.3 ± 0.42
VDB	Control	7.7 ± 2.5	0.35 ± 0.11	**1.9 ± 0.65**	1.1 ± 0.73	**1.0 ± 0.21**
	Stressed	6.5 ± 2.4	0.36 ± 0.11	**3.9 ± 0.8***	1.0 ± 0.5	**2.0 ± 0.3***

Entries are the mean ± SEM. Values expressed are pg/μg protein. Stress treatment led to elevated HVA levels in the PFC, t=-2.17, p<0.04. NE levels were higher in the stressed animals in the VDB (t=-2.23, p<0.04) and DG regions (t=-2.27, p<0.04). Stress treatment also led to elevated 5HIAA levels in the VDB (t=-2.82, p<0.01) and elevated DA levels in the DG (t=-3.68, p<0.003). An asterisk (*) represents a significant main effect of stress treatment. ND represents values that were not detectable.

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Table 9. Glutamate and GABA levels following 28 days of chronic stress in intact females.

Amino Acid	**Area**	**Group**	
		Control	*Stress*
GLU	PFC	7.0 ± 1.6	5.2 ± 2.3
GABA		6.2 ± 1.1	9.3 ± 5.2
GLU	DG	21.6 ± 3.9	28.1 ± 6.8
GABA		**4.8 ± 1.4**	**13.9 ± 3.2***
GLU	nAc	11.5 ± 5.1	9.5 ± 2.7
GABA		7.9 ± 4.1	8.8 ± 2.6
GLU	Raphe	15.4 ± 2.7	14.8 ± 1.8
GABA		4.6 ± 0.8	4.9 ± 0.8

Entries are the mean ± SEM. Values are expressed as ng/μg protein. Stress treatment led to elevated GABA levels in the DG, $t=2.6$, $p<0.02$, as compared to controls. An asterisk (*) represents a significant main effect of stress ($p<0.05$).

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Discussion

Physiological measures

Weight gain and estrous cyclicity

Initial weight gain across the stress period was similar to that observed in experiment 1. That is, all animals gained weight across time, but stress animals weighed less than their counterpart controls for the first three weeks of the stress period. Interestingly, by 28 days of restraint, stressed subjects did not weigh less than controls. This result, coupled with habituating CORT levels in experiment 1, suggest that CORT's hypophagic action attenuates over time. Additionally, neither normal length nor elongated estrous cycles were altered by 28 days of stress. As previously discussed, it does not appear that the severity of restraint stress is sufficient to lead to alterations in estrous cycling.

Behavioral Measures

Open field and RAM

The current data show that stress-induced decreases in ambulation attenuate by stress day 28, as there were no differences between the groups on the open field assessment. This result is similar to previously reported results in which stress male rats stressed for 21 days did not show differences in ambulation on the open field (Beck & Luine, 1999). This observation raises the possibility that prolonged periods of stress are required to elicit male-like stress responses in females (i.e., 28 days of stress in females elicits similar behaviors to that observed in males following 21 days of stress).

21-days of chronic stress enhanced female RAM performance, while 28-days neither enhanced nor impaired performance. This pattern of results is different than those

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observed in male rats in which prolonged exposures to stress changes from enhanced spatial memory at 14 days of stress to maladaptive (RAM performance is impaired following 21 days of restraint) (Luine, *et al.*, 1996; Luine, *et al.*, 1994). Stress exerts a biphasic effect on the central and peripheral nervous systems, with limited amounts of stress being advantageous to an organism and prolonged stress being deleterious. For example, an inverted U relationship between the levels of CORT and hippocampal primed burst potentiation exists and both extremely high and low CORT levels block prime burst potentiation (PB) and intermediate levels enhance PB (Diamond, *et al.*, 1996). Additionally, a shifting time course from adaptive to maladaptive effects of stress on behavioral measures exists as evidenced by enhanced RAM performance following 13 days of stress and impaired performance following 21 days of stress in male rats. Based on the current results, it does not appear that stress exerts the same time course of action in female rats. It remains to be investigated if extremely prolonged periods (e.g., 35 days) of restraint stress leads to impairments in female rats.

Interestingly, 21 days of chronic restraint stress enhanced female spatial memory, while 28 days did not and it has been reported that following 21 days of restraint stress estrogen levels decrease in female rats (Galea, *et al.*, 1997). Thus, this decline in estrogen could be partly responsible for the lack of enhancing effects of chronic stress on female RAM performance.

There were no main treatment effects on spatial memory; however, RAM performance, following stress, was influenced by specific estrous cycle day. Unlike results reported for experiment 1, acquisition is not impaired during proestrus following 28 days of stress and rats in proestrus are actually showing benefits of stress on spatial

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memory, as compared to controls. Interestingly, estrogen levels are highest on proestrus and only these subjects show enhancing effects of stress on spatial memory. This, result supports the hypothesis that estrogens may be moderating the stress response observed in female rats. Following along these lines, it is possible that 28-days of chronic restraint stress is beginning to elicit a male-like response pattern (i.e., no stress enhancements); however, high estrogen levels are producing a 'buffering' effect against the stress action that allow the stress-dependent enhancements to be observed.

Neurochemical assessment

Neurochemical changes were observed in brain regions believed to be involved in spatial learning and memory. Specifically, neurochemical changes were observed in the prefrontal cortex (an area involved in spatial working memory), the DG region of the hippocampus (a brain region that is thought to be critical to optimal performance of the RAM), and the VDB (with projection sites to the hippocampus and cortex), an area critical to memory tasks. Stress increased HVA levels in the PFC. Stress elevated NE, DA, and GABA levels in the DG. Twenty-eight days of chronic restraint stress also elevated NE and 5HIAA levels in the VDB.

The catecholamine system, specifically DA, has been implicated in learning and memory. It has been shown that DA has a beneficial impact on spatial working memory (Simon, 1981; Bubser & Schmidt, 1990); however, excessive or insufficient levels of DA lead to impairments (Murphy, *et al.*, 1996; Zahrt, *et al.*, 1997). The current results indicate that 4 weeks of chronic restraint stress leads to elevated HVA levels in the PFC, indicating an increased activation of the dopaminergic system, and that this elevation is maintained 15 days post-stress. The current results indicate that this

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elevation is insufficient to alter female RAM performance, as there were no stress-induced impairments.

Interestingly, 21 days of chronic stress both elevated HVA levels and enhanced RAM performance; however, 28 days of chronic restraint stress elevated HVA levels but RAM performance was neither enhanced nor impaired. HVA levels following 28 days of stress are lower for both control and stressed animals than those observed following 21 days. It appears that the drop in HVA levels across time (i.e., from 21 to 28 days of stress) is insufficient to lead to behavioral enhancements. Following this hypothesis, 28 days of stress elevated DA levels in the DG. This increase in DA in the DG, an area critical to optimal performance of the RAM, could be approaching levels that are considered "excessive" and thus no stress-dependent enhancements were observed. Future studies are required to examine the stress response, and subsequent activation of the DA system, following longer periods of stress (e.g., 35 days of stress). Such experiments might provide information on determining how great a change in DA activity is required to impair cognitive function.

In the current study, changes were observed in the noradrenergic systems of the DG region of the hippocampus and the VDB, with stress-dependent increases in NE levels in both areas following 28 days of restraint stress. NE utilization and synthesis in the brain is increased in response to stress. An increase in an animal's vigilance (its ability to detect the occurrence of important stimuli) is thought to be a major behavioral effect of increased NE. Because DA is a precursor to NE, higher levels of NE may be indicative of previously high levels of DA. Stress induced increases in NE were observed in the DG and the VDB; however, these changes were not associated with changes in

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behavior. Again, it seems reasonable to suggest that the increased NE levels (and subsequently, previously elevated DA levels) are approaching the far right of the "inverted U" hypothesis of optimal catecholamine levels for spatial memory performance, thereby attenuating the stress-dependent enhancements observed following 21 days of stress.

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Chapter 3 (experiment 3) - Chronic stress effects on ovariectomized females with and without estrogen replacement therapy.

The results from experiments 1 and 2 provide novel data concerning the sexually dimorphic behavioral response to chronic stress. Several observations suggest that female gonadal hormones, most likely estradiol, may contribute to sexually dimorphic stress effects. First, GC levels vary depending on estrous cycle day (Burgess & Handa, 1992; Carey *et al.*, 1995; Viau & Meaney, 1991). Second, estrogen has been shown to have both neuroprotective (Dubal & Wise, 2001; Wise, *et al.*, 2000; Wise, *et al.*, 2001) and growth promoting effects (Toran-Allerand, *et al.*, 1999) that may underlie the sex differences in stress-dependent dendritic changes. Finally, estradiol alone enhances performance of several tasks requiring spatial memory, including the RAM (Luine & Rodriquez, 1994; Luine *et al.*, 1998; Sandstrom & Williams, 2001).

In the current study, we examined stress-induced changes in OVX females, with and without estradiol replacement, at the behavioral and neurochemical level. Following 21 days of restraint stress, subjects were assessed on the OF for locomotor activity and the eight-arm RAM was used to measure spatial memory performance. Following the behavioral testing period, subjects were sacrificed and brain areas implicated in memory function were assessed for monoamine, amino acid, and metabolite levels. Specifically, this experiment was designed to assess whether circulating estradiol contributes to the observed behavioral, morphological, and neurochemical resistance to stress exhibited by female rats. Because estradiol appears to contribute to the sexually dimorphic effects of stress, it was hypothesized that OVX rats would be more sensitive to the effects of

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chronic stress and that estradiol treatment during stress would moderate these effects. Specifically, because estrogen is known to moderate some aspects of HPA function, coupled with the previous observations that male rats, who have low circulating estrogen levels, are impaired on the RAM following stress (Luine *et al.*, 1994), it was hypothesized that stressed OVX females would be impaired on the RAM. Based on results from experiment 1, in which stress enhanced female RAM performance, it was hypothesized that estradiol treated OVX females receiving stress treatment would have enhanced RAM performance.

Methods

Subjects

Thirty-two female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) aged 55-60 days old (upon arrival) served as subjects. All animals were maintained on a 14/10-h light-dark cycle (lights on at 05:00 h) and in accordance with the NIH Guide for Care and Use of Animals. Animals were allowed to acclimate to the housing environment for two weeks. During the acclimation period and the 21-day stress period, all subjects were double housed in plastic tubs. All animals were weighed weekly during the acclimation and stress periods and daily during the behavioral period.

All 32 subjects were bilaterally ovariectomized (OVX) while under metophane anesthesia and immediately implanted with a 1 cm long Silastic capsule (0.058" i.d., and 0.077" o.d.). Sixteen rats received cholesterol filled Silastic implants and were randomly assigned to either a stressed (N=8) or non-stressed group (N=8). The additional 16 rats were implanted with 1 cm long Silastic capsules that contained a 10% estradiol-90% cholesterol mixture (Sigma Chemical Co, St. Loius) and were randomly assigned to

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either a stressed (N=8) or non-stressed group (N=8). All animals were allowed to recover for 10 days before the stress period began. Thus, there were 4 treatment groups: stressed + estradiol (ST-EST), stressed + cholesterol (ST-CHOL), non-stressed + estradiol (NS-EST) and non-stressed + cholesterol (NS-CHOL).

General Procedure

All animals were weighed weekly and behavioral assessment was as previously described. The stress period was 21 days. All stress and post-stress procedures, including food restriction, open field assessment, RAM testing, and neurochemical assessment were identical to those described in Experiment 1.

Data Analysis

Measurements of animal weight gain and behavioral data were from a spit-plot factorial design with stress and estradiol (between-subjects treatments) and time (within-subjects or repeated) as design factors. Statistical differences among the four groups in animal weight gain across the 21-day stress period were tested by a three-factor (stress X estradiol X time) repeated measure ANOVA. Three-factor (stress X estradiol X time) ANOVAs were also used to test for statistical differences among the groups on the OF task and the RAM. A two-factor ANOVA (stress X estradiol) was used to test for statistical differences among the groups in monoamine and amino acid levels.

Descriptive statistics for weight and behavioral data are expressed as mean $\pm$ standard error of the mean (SEM). Monoamine and metabolite levels are expressed as pg/μg protein. Type I error rate was set at 0.05 for determining statistical significance. Where appropriate, Fisher's LSD was used for post-hoc testing.

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Results

Weight

Animal weight gain was affected by stress ($F_{1,92}$=16.72, p<0.0001), estradiol ($F_{1,92}$=188.40, p<0.0001), and time ($F_{1,92}$=34.98, p< 0.0001). Weight gain across the daily restraint period was also influenced by the stress X time interaction ($F_{4,92}$=2.88, p<0.03) and the estradiol X time interaction ($F_{4,92}$=9.23, p<0.0001). As seen in Figure 11, stressed animals weighed less than the non-stress and estradiol treated females weighed less than their cholesterol controls across the stress period. At 21-days of stress, post-hoc analysis revealed that the ST-EST group gained the least weight across time (p<0.05).

Behavioral Measures

Open field

Open field measurements were quantified for the first three minutes and second three minutes of the task, Table 10. Stressed groups, regardless of estradiol treatment, made fewer total outer sector crossing than NS-EST and NS-CHOL ($F_{1,29}$=12.92, p<0.001) and all groups made fewer crossings during the second 3 minutes ($F_{1,27}$=29.95, p<0.0001). Inner sector visits were influenced by estradiol treatment ($F_{1,29}$=7.60, p<0.01), time ($F_{1,29}$=4.70, p<0.04) and the estradiol X time interaction ($F_{1,27}$=4.23, p<0.04), as evidenced by NS-EST and ST-EST groups making more inner visits during the second half as compared to NS-CHOL and ST-CHOL. Wall climbs were influenced by both time ($F_{1,27}$=23.80, p<0.0001) and the estradiol X time interaction ($F_{1,27}$=60.55, p<0.0001) as evidenced by all groups except NS-EST making fewer wallclimbs during

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the second 3 min on the field. There were no differences between the groups with respect to rears or defecations.

Radial arm maze

21-days of restraint stress led to a significant difference in RAM performance among the treatment groups as measured by the total number of visits required to complete the task. There was a main effect of estradiol treatment ($F_{1,255}=9.36$, $p<0.003$), with estradiol treated groups, with or without stress, performing better than cholesterol groups. There was no main effect of stress, however there was a significant stress X estradiol interaction ($F_{1,255}=8.85$, $p<0.002$) and post hoc testing showed that the ST-EST group required fewer visits to complete the task as compared to all other groups. Total visits to complete the task was also influenced by trial ($F_{7,255}=2.68$, $p<0.01$), stress X trial ($F_{7,255}=2.39$, $p<0.02$) and the stress X estradiol X trial interaction ($F_{7,255}=2.17$, $p<0.04$), see Fig. 12.

There was a similar stress X estradiol X trial interaction with regards to the number of correct choices in the first eight visits, but this trend failed to reach significance ($F_{7,255}=1.91$, $p<0.07$). There were no significant differences among the treatment groups for the first mistake choice accuracy parameter. Furthermore, no significant differences among the groups during delay trials were observed, see Table 11.

Neurochemical analyses

Monoamines and metabolites

Both stress and estradiol treatment altered monoamine and metabolite levels. Table 12 shows all monoamine and metabolite levels, and Fig. 13 shows only significant changes. There was a main effect of stress treatment on HVA levels in the prefrontal

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cortex with elevated levels observed in ST-EST and ST-CHOL groups as compared to NS-EST and NS-CHOL ($F_{1,19}$=19.5, p<0.0004). Estradiol treatment led to elevated BLA levels of MHPG ($F_{1,24}$=11.70, p<0.003), 5HIAA ($F_{1,24}$=7.72, p<0.01), and DA ($F_{1,24}$=13.36, p<0.001). Both stress and estradiol treatment lowered 5HIAA levels in the DG ($F_{1,21}$=20.57, p<00001 and $F_{1,21}$=4.84, p<0.04, respectively). MHPG levels in the DG were decreased by estradiol treatment ($F_{1,21}$=5.49, p<0.03), and HVA levels were affected by stress ($F_{1,21}$=6.23, p<0.02), with lower levels in ST-EST as compared to NS-EST. There was a main effect of estradiol on NE levels in CA3 ($F_{1,24}$=8.25, p<0.009) with lower levels observed for ST-CHOL as compared ST-EST and NS-EST groups. There were no significant changes in CA1 or nAc.

Amino acids

No changes were observed in glutamate or GABA levels in the CA1, PFC or nucleus accumbens following 21 days of chronic restraint stress, see Table 13.

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Fig. 11. Weight gain across the 21-day stress period in OVX rats.

Entries are the mean $\pm$ SEM. A three-factor ANOVA (stress X estradiol X time) was used to test for differences among the groups with respect to weight gain across the stress period. While all animals gained weight ($F_{1,92}=34.98$, $p< 0.0001$), stressed groups gained less weight than non-stressed animals ($F_{1,92}=16.72$, $p<0.0001$). Additionally, estradiol treated groups weighed less than their cholesterol controls ($F_{1,92}=188.40$, $p<0.0001$). Post-hoc analysis revealed that the ST-EST treated subjects weighed less than all other groups, $p<0.05$.

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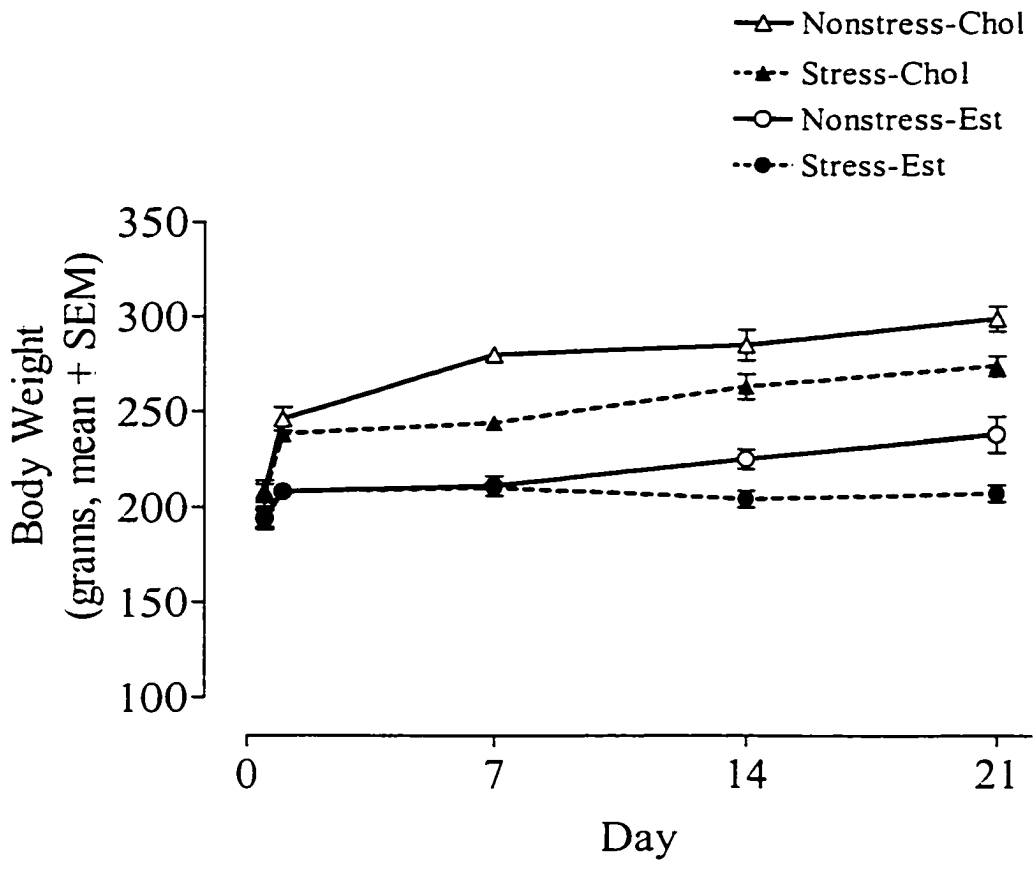

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Fig. 12. Effects of stress and estradiol treatment on radial arm maze performance. Entries are the mean $\pm$ SEM. Total visits to complete the task was influenced by estradiol treatment ($F_{1,255}=9.36$, $p<0.003$), stress X estradiol interaction ($F_{1,255}=8.85$, $p<0.002$), trial ($F_{7,255}=2.68$, $p<0.01$), stress X trial ($F_{7,255}=2.39$, $p<0.02$) and the stress X estradiol X trial interaction ($F_{7,255}=2.17$, $p<0.04$). Post hoc testing showed that the ST-EST group required fewer visits to complete the task as compared to all other groups ($p<0.05$). The main figure shows the differences between the groups across the 8 regular trials and the insert shows the averages for each group.

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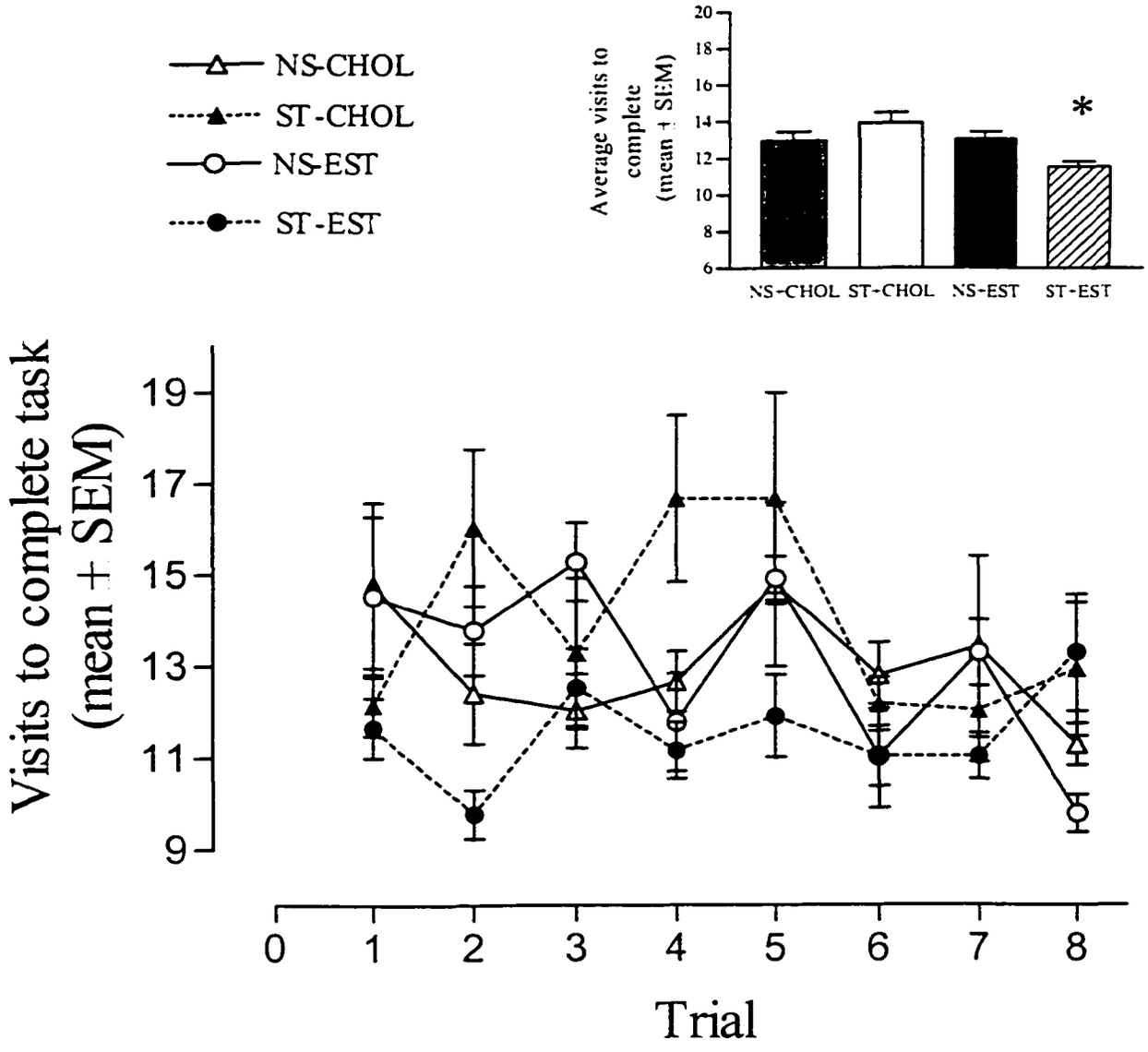

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Fig 13. Effect of stress and estradiol treatment on monoamine and metabolite levels in OVX rats.

All entries are the mean ± SEM. Stress treatment elevated HVA levels in the prefrontal cortex of the ST-EST and ST-CHOL groups as compared to NS-EST and NS-CHOL ($F_{1,19}=19.5$, $p<0.0004$), see panel A. There was a main effect of estradiol on NE levels in CA3 ($F_{1,25}=4.84$, $p<0.04$) with lower levels observed in the ST-CHOL group as compared to ST-EST and NS-EST groups, panel A. Estradiol treatment increased BLA levels of MHPG ($F_{1,24}=11.70$, $p<0.003$), 5HIAA ($F_{1,24}=7.72$, $p<0.01$), and DA ($F_{1,24}=13.36$, $p<0.001$), panel B. Both stress and estradiol treatment lowered 5HIAA levels in the DG ($F_{1,21}=20.57$, $p<00001$ and $F_{1,21}=4.84$, $p<0.04$, respectively), panel C. MHPG levels in the DG were decreased by estradiol treatment ($F_{1,21}=5.49$, $p<0.03$) and HVA levels were lowered by stress ($F_{1,21}=6.23$, $p<0.02$), with lower levels in ST-EST as compared to NS-EST. An asterisk (*) indicates a main effect of estradiol treatment and a cross (+) indicates a main effect of stress treatment. There were no significant changes in CA1 or nAc.

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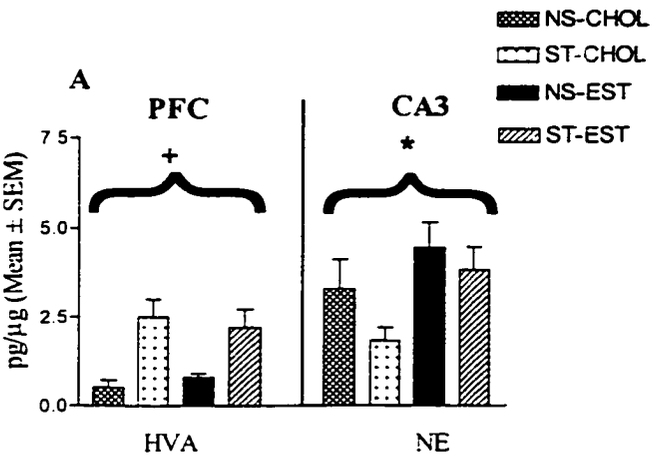

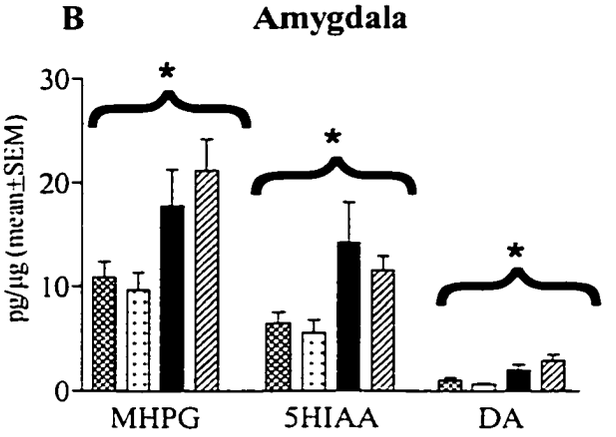

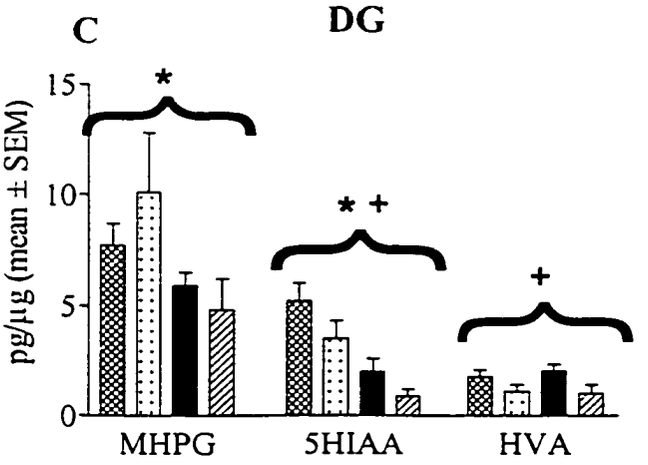

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Table 10. Summary of behavioral measures on the open field following 21 days of chronic stress in ovariectomized rats.

	Outer Crossings		*Inner Visits*		*Wallclimbs*	
Group	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
ST-CHOL	51.6 ± 6.1^{+}	31.5 ± 7.2	1.1 ± 0.7	0.9 ± 0.7	9.1 ± 1.6	3.5 ± 0.9
NS-CHOL	67.4 ± 4.7	53.5 ± 9.5	1.5 ± 0.8	1.9 ± 0.8	8.1 ± 1.5	4.1 ± 1.3
ST-EST	49.3 ± 6.3^{+}	35.6 ± 3.9	1.9 ± 1.0	5.6 ± 1.7*	6.9 ± 1.6	5.4 ± 1.3
NS-EST	68.4 ± 6.1	57.8 ± 3.3	2.9 ± 0.9	3.9 ± 0.9*	6.8 ± 0.7	6.9 ± 0.6

Entries are the mean ± SEM. Time 1 refers to the first 3 min on the field and Time 2 the second 3 min. A three-factor mixed ANOVA (stress X estradiol X time) with Fisher LSD post hoc testing was used to test for significant differences among the groups. There was a main effect of stress ($F_{1,29}$=12.92, $p<0.001$) on outer sector crossing with stressed groups (42.0 ± 3.8) making fewer total outer sector crossing than non-stress groups (61.8 ± 3.9). All groups showed habituation to the open field with a decrease in outer sector crossing across time ($F_{1,27}$=29.95, $p<0.001$). Inner sector visits were influenced by estradiol treatment ($F_{1,29}$=7.60, $p<0.01$), time ($F_{1,29}$=4.70, $p<0.001$), and the estradiol X time interaction ($F_{1,27}$=4.23, $p<0.04$) with post hoc testing revealing that estradiol treated groups (4.8 ± 0.5) made more inner visits during the second three min on the field than cholesterol groups (1.4 ± 0.6). Both time ($F_{1,27}$=23.80, $p<0.001$) and the estradiol X time interaction ($F_{1,27}$=60.55, $p<0.0001$) affected wallclimbs with all groups except NS-EST making fewer wallclimbs across time. An asterisk (*) represents a main effect of estradiol treatment and a cross (+) represents a main effect of stress.

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Table 11. Twenty-one days of chronic restraint stress did not alter performance on RAM delay trials in OVX females.

Delay	Group	Total Visits	First Mistake
15 min	NS-CHOL	15.1 ± 1.5	5.3 ± 0.4
	NS-EST	14.3 ± 1.6	5.0 ± 0.3
	ST-CHOL	14.5 ± 1.0	5.5 ± 0.5
	ST-EST	14.6 ± 0.9	5.4 ± 0.2
1 h	NS-CHOL	14.1 ± 1.5	5.4 ± 0.2
	NS-EST	13.0 ± 1.0	5.3 ± 0.4
	ST-CHOL	13.8 ± 0.9	4.9 ± 0.4
	ST-EST	13.3 ± 0.6	5.3 ± 0.5
2 h	NS-CHOL	13.0 ± 1.1	4.7 ± 0.6
	NS-EST	11.0 ± 0.6	6.1 ± 0.3
	ST-CHOL	15.1 ± 2.0	5.5 ± 0.4
	ST-EST	12.0 ± 0.7	5.6 ± 0.2
4 h	NS-CHOL	14.5 ± 1.2	5.3 ± 0.2
	NS-EST	16.1 ± 1.9	4.8 ± 0.3
	ST-CHOL	13.3 ± 0.7	4.6 ± 0.3
	ST-EST	12.6 ± 0.9	5.6 ± 0.5

There were no differences among the groups during RAM trials including either a 15 min, 1 h, 2 h, or 4 h delay between the fourth and fifth choices.

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Table 12. Monoamine and metabolite levels following 21 days of stress in OVX rats.

Area	Group	DA	HVA	NE	MHPG	5HIAA
PFC	ST+CHOL	0	**2.5 ± 0.5**$^{+}$	1.6 ± 0.5	0	6.1 ± 1.0
	NS+CHOL	0	**0.5 ± 0.2**	4.7 ± 2.3	0	6.4 ± 2.1
	ST+EST	0	**2.2 ± 0.5**	2.3 ± 0.7	0	8.5 ± 5.1
	NS+EST	0.2 ± 0.2	**0.8 ± 0.1**	1.9 ± 0.3	0	3.7 ± 0.7
BLA	ST+CHOL	**0.7 ± 0.1***	1.3 ± 0.5	2.6 ± 0.5	**9.7 ± 1.7***	**5.6 ± 1.2***
	NS+CHOL	**1.0 ± 0.3**	1.8 ± 0.4	4.7 ± 1.1	**10.9 ± 1.5**	**6.5 ± 1.1**
	ST+EST	**2.9 ± 0.6**	2.0 ± 0.7	4.8 ± 1.3	**21.2 ± 3.0**	**11.5 ± 1.4**
	NS+EST	**2.0 ± 0.5**	6.1 ± 2.3	8.1 + 2.0	**17.8 ± 3.5**	**14.2 ± 3.9**
CA3	ST+CHOL	1.5 ± 0.6	0.4 ± 0.2	**1.8 ± 0.4***	5.8 ± 3.1	2.3 ± 0.9
	NS+CHOL	2.4 ± 1.6	0.6 ± 0.3	**3.3 ± 0.8**	3.6 ± 1.2	2.3 ± 0.6
	ST+EST	1.6 ± 1.0	0.2 ± 0.1	**3.8 ± 0.7**	2.5 ± 0.7	2.7 ± 0.7
	NS+EST	1.8 ± 1.2	1.0 ± 0.8	**4.5 ± 0.7**	5.8 ± 2.3	1.3 ±0.2
DG	ST+CHOL	4.3 ± 1.0	**1.1 ± 0.3**$^{+}$	1.7 ± 0.4	**10.1 ± 2.7***	**3.5 ± 0.8***$^{+}$
	NS+CHOL	5.0 ± 1.6	**1.8 ± 0.3**	0.9 ± 0.3	**7.7 ± 1.0**	**5.2 ± 0.8**
	ST+EST	3.1 ± 1.1	**1.0 ± 0.4**	2.1 ± 0.7	**4.8 ± 1.4**	**0.9 ± 0.3**
	NS+EST	4.6 ± 1.1	**2.1 ± 0.3**	1.2 ± 0.3	**5.9 ± 0.6**	**2.0 ± 0.6**

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Table 12 continued

Entries are the mean ± SEM. Values expressed are pg/μg protein. Stress treatment elevated HVA levels in the prefrontal cortex of the ST-EST and ST-CHOL groups as compared to NS-EST and NS-CHOL ($F_{1,19}$=19.5, $p<0.0004$), see panel A. There was a main effect of estradiol on NE levels in CA3 ($F_{1,25}$=4.84, $p<0.04$) with lower levels observed in the ST-CHOL group as compared to ST-EST and NS-EST groups, panel A. Estradiol treatment increased BLA levels of MHPG ($F_{1,24}$=11.70, $p<0.003$), 5HIAA ($F_{1,24}$=7.72, $p<0.01$), and DA ($F_{1,24}$=13.36, $p<0.001$), panel B. Both stress and estradiol treatment lowered 5HIAA levels in the DG ($F_{1,21}$=20.57, $p<00001$ and $F_{1,21}$=4.84, $p<0.04$, respectively), panel C. MHPG levels in the DG were decreased by estradiol treatment ($F_{1,21}$=5.49, $p<0.03$) and HVA levels were lowered by stress ($F_{1,21}$=6.23, $p<0.02$), with lower levels in ST-EST as compared to NS-EST. An asterisk (*) indicates a main effect of estradiol treatment and a cross (+) indicates a main effect of stress treatment.

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Table 13. Glutamate and GABA levels following 21 days of chronic stress in OVX females.

Amino Acid	**Area**	**Group**			
		NS-CHOL	*ST-CHOL*	*NS-EST*	*ST-EST*
GLU	PFC	40.1 ± 16.0	34.0 ± 13.8	19.5 ± 5.9	30.3 ± 12.7
GABA		7.5 ± 3.2	7.3 ±2.2	4.6 ± 1.3	6.3 ± 1.1
GLU	CA1	37.6 ± 10.9	45.2 ± 11.2	33.3 ± 6.0	31.5 ± 7.0
GABA		4.7 ± 1.4	4.2 + 1.0	3.8 ± 0.7	4.7 ± 1.4
GLU	nAc	30.8 ± 2.9	23.1 ± 7.1	17.7 ± 3.4	22.9 ± 7.5
GABA		13.7 ± 5.2	6.1 ± 1.9	6.5 ± 1.3	10.6 ± 2.6

Entries are the mean ± SEM. Values are expressed as ng/μg protein. There were no significant differences between the stressed and control animals.

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Discussion

Body weight

All animals used in this study gained weight across time; however, the weight gain differed among the treatment groups. Stressed females gained less weight than non-stress females across the 21-days and this is consistent with previous studies in which both stress and CORT administration led to decreases in body weight (McLay, *et al.*, 1998; Beck & Luine, 1999; Akana, *et al.*, 1999; Bowman *et al.*, 2001). Additionally, all estradiol treated females weighed less than their cholesterol counterparts, which is characteristic of estrogen's hypophagic effect (Hamosh & Hamosh, 1975; Ramirez, 1981). Additionally, ST-EST group weighed the least, which provides further evidence of an interaction between the HPA and HPG axes.

Behavioral measures

Open field

The current data shows that stressed subjects, regardless of estradiol treatment, made fewer outer sector crossings and these results are consistent with previously reported data in which stressed subjects had decreased ambulation on the open field (Katz, *et al.*, 1981; Soblosky & Thurmond, 1986; Bowman *et al.*, 2001), (Table 10). The decreased ambulation of the stressed subjects in the current studies did not appear to compromise RAM performance, as there were no differences between the groups with regards to the time required to complete the spatial task. The observation that stressed subjects, regardless of estradiol treatment, made fewer outer sector crossings corresponds with the data from experiment one, in which stressed intact females had decreased ambulation as compared to non-stressed. Thus, it appears that intact and OVX respond

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the same, indicating that estrogen may be exerting an activational influence on this parameter.

Inner sector visits on the open field are considered a measure of overall anxiety, with increased visits to the inner sectors indicative of less anxiety. In the current study, estradiol treated groups made more inner sector visits during the second half of the task than cholesterol groups, (Table 10), suggesting the estradiol treatment decreased anxious behavior on the OF across time. This result is consistent with previously reported results that demonstrate estrogen's anxiolytic effects (Fernandez-Guasti & Picazo, 1990; Mora, *et al.,* 1996; Fernandez-Guasti & Picazo, 1999). Additionally, anxious behavior is thought to be, in part, mediated by the 5HT system (Iversen, 1984; Briley, *et al.,* 1990) and modifications in the 5HT system (e.g., increased binding in OVX rats following estradiol treatment) have been observed in response to estradiol treatment (Williams & Uphouse, 1989). In the current study, decreases were observed in 5HIAA in the DG in response to both stress and estradiol treatment. The decrease in anxious behavior of the estradiol treated groups, coupled with the estrogen-influenced changes in the serotonergic system, provides further evidence for estrogen's role in mediating anxious behavior.

Interestingly, habituation as evidenced by a decrease in wall climbs during the second half was also influenced by estradiol treatment, (Table 10); cholesterol treated groups made fewer wallclimbs than estradiol treated groups. It is unclear why estradiol treatment had an anxiolytic effect on one OF parameter (inner sector visits), but not on another (wallclimbs).

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Radial arm maze

Previous studies in this laboratory have shown that 21-days of stress impairs male rat performance on the RAM (Luine *et al.*, 1994) but enhances female performance (Bowman *et al.*, 2001) and we were interested in possible ovarian hormone mechanisms underlying this sexually dimorphic behavioral response to chronic restraint stress. It is widely recognized that estrogen enhances learning and memory process in both humans and animals (for review, Luine, 1997) and therefore we hypothesized that estrogen may mediate the enhancing effects of stress in female rats. The data from the current study show that both estradiol treatment alone and a stress by estradiol interaction enhance RAM performance following 21 days of daily restraint stress. Estradiol treated groups performed better than cholesterol groups on the RAM, and this result is similar to previously reported results. OVX rats receiving chronic estradiol replacement have enhanced performance on both spatial and non-spatial tasks including RAM, T-maze, and passive avoidance conditioning (Luine *et al.*, 1994; O'Neal, *et al.*, 1996; Daniel, *et al.*, 1997; Luine *et al.*, 1998).

Estrogen is known to exert both organizational and activational effects on the brain (McEwen, 1983; Toran-Allerand, 1984). In the current study we observed that RAM performance was enhanced in stress and estradiol treated subjects and that OVX rats remained insensitive to impairing effects of stress. This result is consistent with previous observations demonstrating that spatial learning and memory is influenced by early (Williams, *et al.*, 1990; Roof & Havens, 1992; Luine & Rodriquez, 1994) as well as late estrogen exposure (Luine & Rodriquez, 1994; Luine *et al.*, 1998). For example, Williams and colleagues (1990) showed that control male rats or females rats treated

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neonatally with estradiol benzoate had improved acquisition of a 12-arm radial maze. Further support for the organizational impact of estrogen on spatial memory comes from studies in which neonate females treated with testosterone (which is aromatized to estrogen in the brain) had enhanced performance on the Morris water maze (Roof & Havens, 1992). The HPA axis is also subject to activational influences of estrogen (e.g., CORT release varies across the estrous cycle) (Viau *et al.*, 1991; Burgess *et al.*, 1992; Carey *et al.*, 1995). Additionally, estrogen exerts an organizational (developmental programming) impact on HPA function (e.g., the gene expression of corticotrophin-releasing-hormone) (Patchev, *et al.*, 1995; McCormick, *et al.*, 1998; Patchev & Almeida, 1998). For example, Patchev and colleagues demonstrated that neonatal estrogen treatment masculinized regulatory functions of the HPA axis including CRH and hippocampal glucocorticoid receptor expression (1995). Thus, the decreased sensitivity of females, as compared to males, to chronic stress may be due to a combination of both organizational and activational effects of estrogen. Support for the organizational role of estrogen is provided by our observation that OVX rats are resistant to chronic stress. That is, following the chronic stress period the OVX rats were not impaired on the RAM, suggesting that neonatal exposure to estrogen has provided a protective effect. Additionally, the enhanced performance of the ST-EST group is indicative of the activational effects of estrogen.

Because estrogen is known to moderate some aspects of HPA function, coupled with previous observations that male rats, who have low circulating estrogen levels, are impaired on the RAM following stress (Luine *et al.*, 1994), we had originally hypothesized that OVX rats in the ST-CHOL group would be impaired on the RAM.

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Given our observation that OVX females were not impaired by chronic stress, we believe that future studies investigating the effect of chronic stress prenatally as well as gonadal hormone manipulations postnatally would prove informative in elucidating the role of estrogen in the stress response. For example, examining the effect of neonatal estrogen treatment on spatial memory and the stress response would provided insight on the necessity of early estrogen exposure on HPA function in adulthood.

Neurochemistry

Neurochemical changes were observed in brain regions believed to be involved in spatial learning and memory. In particular, neurochemical changes were observed in the CA3 and DG regions of the hippocampus, brain structures believed to be critical in the ability of rats to perform on the RAM. In CA3, an estradiol dependent increase in NE was observed. Decreases in the levels of MHPG and HVA in the DG were estradiol and stress dependent, respectively. Decreases in 5HIAA were observed in response to both stress and estradiol treatment in the DG. In regions outside the hippocampus, such as BLA and PFC (known to also contribute to learning and memory), contrasting changes in neurochemistry were observed. For example, estradiol treatment led to increases in MHPG, 5HIAA, and DA in the BLA, while a stress dependent increase in HVA was observed in the PFC.

The catecholamine system, specifically DA, has been implicated in learning and memory. It has been shown that DA has a beneficial impact on spatial working memory (Simon, 1981; Bubser & Schmidt, 1990); however, both excessive or insufficient levels of DA activity lead to impairments (Murphy, *et al.*, 1996; Zahrt, *et al.*, 1997). The current results indicate that 3 weeks of chronic restraint stress leads to elevated HVA levels in

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the PFC, indicating an increased activation of the dopaminergic system, and that this elevation is maintained 15 days post-stress. This stress dependant change is notable because gonadally intact females stressed for 21 days (experiment 1) and 28 days (experiment 2) also showed alterations the dopaminergic system. The current results indicate that this elevation of HVA levels does not impair OVX female RAM performance, as there were no stress-induced impairments; however, decreases in DA activity are associated with impaired performance in male (Luine, *et al.* 2001). It appears that decreased levels of DA impair male performance, while increased levels in females enhances performance. It is possible that DA in the PFC may contribute only to non-spatial memory. Chronic stress also enhances object recognition performance in intact females (Luine *et al.*, 2001), but it is unknown whether OVX alters the response. The increase in PFC DA activity in stressed OVX females shown here may be associated with impaired object recognition. Further studies are required to understand the relationship between PFC DA activity, stress, gonadal hormones, and memory function.

Furthermore, stress-dependent alterations in spatial memory appear to be mediated by the serotonergic system. Luine and colleagues (1993) found that RAM performance was impaired in rats with enhanced 5HT and 5HIAA activity in the DG. Additionally, 5HT agonists (e.g., 8-OH-DPAT) have been shown to impair RAM performance (Winter & Petti, 1987) while tianeptine, which enhances 5HT uptake, blocks impaired spatial memory performance (Conrad, *et al.*, 1996). Decreases in 5HT are associated with better RAM performance and the current data shows that both stress and estradiol treatment decreased 5HIAA levels in DG, with the lowest levels observed in the

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ST-EST group. Thus, it appears that the observed enhancements in RAM performance may be due in part to decreased serotonergic activity.

In the current study, changes were observed in the noradrenergic system of the CA3 region, with increased NE levels in response to estradiol treatment. Post-hoc testing revealed that the ST-CHOL group had significantly lower levels than the NS-EST group. Estrogen appears to be exerting a 'buffering' effect on NE, particularly in the presence of stress, as levels decrease in response to stress in OVX animals, but no such decrease is observed in ST-EST animals. Consistent with this observation, previous studies have shown that NE in the CA3 region is increased by stress in intact females (Luine *et al.*, 2001). Estradiol modulates noradrenergic receptor activity in the central nervous system, including the hippocampus, (Favit, *et al.*, 1991) and the noradrenergic system is involved in memory consolidation in other brain areas, such as the BLA (Ferry & McGaugh, 2000). Thus, it seems tenable to suggest that the observed estrogen dependent change of CA3 NE levels is influencing the observed behavioral changes.

Interestingly, the majority of the observed neurochemistry changes were estradiol dependent. The responsiveness of catecholamine synthesizing enzymes to estrogen, as well as stress is well documented (De Potter, *et al.*, 1976; Carr & Voogt, 1980; Kritzer & Kohama, 1999; Nankova, *et al.*, 1999; Sabban & Kvetnansky, 2001). Given our observation that estradiol leads to contrasting neurochemical changes in brain regions known to be involved in learning and memory, it is appealing to speculate that different transcriptional mechanisms might be involved. A number of estrogen receptors have been reported in the literature (Shughrue, *et al.*, 1997; Koenig, 2001) and their presence suggests the possibility that they may differentially affect signaling mechanisms and thus

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gene activity within target cells. In keeping with this notion, it is possible that levels of these estrogen receptor isoforms vary within the surveyed brain regions. Thus, changes in estradiol could lead to either an increase or decrease in the production/activity of catecholamine synthesizing enzymes, which may explain the observed neurochemical changes.

In light of the stress and estradiol interaction in influencing on RAM performance, it is surprising that neurochemical levels were not influenced by the stress X estradiol interaction. This result could be due to the temporally constrained aspect of stress effects. It has previously been shown that both male and female stress-dependent enhancements on RAM performance attenuate during delay trials, 10-12 days post-stress, or if RAM is assessed beginning 18 days post-stress (Luine *et al.*, 1996; Bowman *et al.*, 2001). Additionally, it has been shown that termination of a daily stress paradigm resulted in reversal of stress-induced dendritic atrophy (by 5-10 days post-stress) (Conrad, *et al.*, 1999). Alternatively, the failure to observe a stress X estradiol effect on neurochemistry may be due to a "ceiling effect". That is, either estradiol or stress treatment alone elicits a maximum neurochemical response and subsequently, there is no additive effect of the two combined on the system. Thus, it may be important that changes observed in CA3 levels of NE and PFC levels of DA were different in OVX females than intact females following stress. To better understand the role of estrogen and stress on neurochemical changes following a spatial learning task, future studies should consider sacrificing animals immediately following RAM regular trials and/or completing an estradiol dose-response curve.

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In summary, the current study provides novel behavioral and neurochemical information about the role of estradiol in mediating the stress response in OVX rats. Results suggest that estradiol, acting organizationally and activationally, alters the cognitive response to chronic stress in female rats.

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Chapter 4 (experiment 4)- Neurochemical and neuroendocrine assessments immediately following 21-days of chronic restraint stress

Results from experiments 1-3 show that intact female rats exhibit a markedly different behavioral response to chronic stress than males and that this sexually dimorphic response is due, at least in part, to both organizational and activational influences of estrogen. Because stress responses are known to attenuate over time, it was of interest to compare neurochemical values between intact control and stressed females immediately following 21 days of chronic restraint stress. Additionally, possible correlations between circulating CORT and monoamines and amino acid levels immediately following 21 days of chronic restraint stress were examined.

Methods

Subjects

Thirty-five female Sprague Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) aged 55-60 days old upon arrival served as subjects. Rats were allowed to acclimated to the housing environment for two weeks during which time they received daily vaginal lavages. Rats were randomly assigned to either a stressed (N=20) or non-stressed (control, N=15) condition; however, because data (i.e., hormone levels, neurochemical values) were not always available for each animal, a smaller number of subjects were often used in subsequent statistical analysis. Rats were stressed for 6 hours per day for 21 days. One hour after the stress regime began, on day 21, rats were sacrificed by rapid decapitation. Following decapitation, trunk blood was collected, centrifuged, and the

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plasma frozen, as previously described (experiment 1). The brains were also removed, frozen in dry ice, and stored at –80° C for subsequent neurochemical analysis, (see methods, experiment 1). Additionally, the control rats were sacrificed in the same manner (with the exception of no stress) to serve as a baseline comparison group.

Neurochemical analysis

The general procedures used for neurochemical analysis were identical to those described in experiment 1. Only brain regions that had previously shown neurochemical alterations in response to 21 days of stress in intact females were sampled and assessed (i.e., PFC, CA1, CA3, and DG).

Corticosterone radioimmuassay

Corticosterone radioimmunoassay were performed using trunk blood collected immediately upon sacrifice from both stress and control animals. The samples were centrifuged and the supernatant was removed and stored (-80° C) until assayed. Both total and free CORT were measured using standard RIA techniques as previously described (methods, experiment 1). Corticosterone values are expressed as ng/ml.

Data analysis

Differences in neurotransmitter levels and serum hormone levels between the stress and control animals were analyzed using two-sample t-tests. Correlational analyses were performed between circulating CORT levels and neurochemical values using Pearson Product Moment correlations.

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Results

Monoamine and metabolites

Using two-sample t-tests, no differences were observed between the stressed and non-stressed animals following 21 days of chronic stress in monoamine and metabolite levels in the PFC, CA1, CA3, or DG brain regions, see Table 14. While overall levels of neurochemicals were not altered by stress, circulating CORT did influence levels of neurochemicals in some brain areas as evidenced by correlational analysis between CORT and specific neurotransmitters. PFC HVA/DA ratio in the stressed rats significantly correlated with total CORT levels ($r= 0.7147$, $p<0.01$). Control levels of total CORT and 5HT were significantly correlated in the CA1 region ($r=-0.5828$, $p<0.04$). For control animals, CA3 levels of NE and total CORT were significantly correlated ($r=0.6034$, $p< 0.05$), as well as free CORT and DA activity, as assessed by a HVA/DA ratio ($r=-0.6357$). Total CORT levels in the DG region were significantly correlated with control levels of DA ($r=-0.6260$, $p<0.04$). Additionally, free CORT and 5HIAA were significantly correlated in the DG region of stressed animals ($r=-0.7693$, $p<0.01$). Significantly correlations observed in the control animals are shown in Figure 12 and significant correlations observed in the stressed animals are shown in Figure 14.

Amino acids

Using two-sample t-tests, no differences were observed between the stressed and non-stressed animals in glutamate or GABA levels in the PFC, CA1, CA3 or DG, see Table 15. Levels of total CORT and GABA were significantly correlated in the CA1 region of stressed animals ($r=-0.6918$, $p<0.01$) and the CA3 region of control animals ($r=0.6729$, $p<0.03$). Total CORT were significantly correlated with GLU levels in the DG

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region of control animals ($r=0.9288$, $p<0.01$). Figure 16 shows the significant correlations between total CORT levels and amino acids in hippocampal regions of control and stressed rats.

Hormone levels

Stress treatment did not lead to significant differences between the control and stress groups with regards to total CORT (206.1 ± 23.1 and 245.3 ± 28.9, control and stress, respectively) or free CORT (125.5 ± 18.2 and 162.7 ± 17.1, control and stress, respectively). While there were no main effects of either stress treatment or estrous cycle day on total CORT levels, there were apparent differences between CORT levels across the estrous cycle between the stress and control animals (stress X estrous cycle). While this interaction between stress treatment and estrous cycle failed to reached significance, $F_{2,29}=3.02$, $p=0.06$, it appears that CORT levels were highest during proestrus for control animals and estrus for stressed animals and control animals in estrus had the lowest levels, see Table 16.

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Figure 14. Correlations of total and free CORT levels and monoamine and metabolite levels in intact female control rats.

Entries are the mean ± SEM. CORT values are expressed as ng/ml and neurotransmitter levels are expressed as pg/μg protein. Control levels of total CORT and 5HT were significantly correlated in the CA1 region ($r=-0.5828$, $p<0.04$). CA3 levels of NE and total CORT were significantly correlated ($r=0.6034$, $p< 0.05$), as well as free CORT and DA activity, using a HVA/DA ratio ($r=-0.6357$). Total CORT levels in the DG region were significantly correlated with control levels of DA ($r=-0.6260$, $p<0.04$

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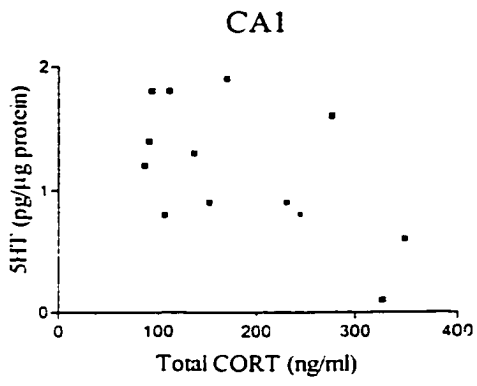

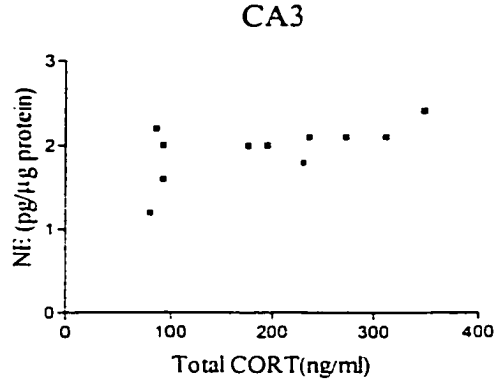

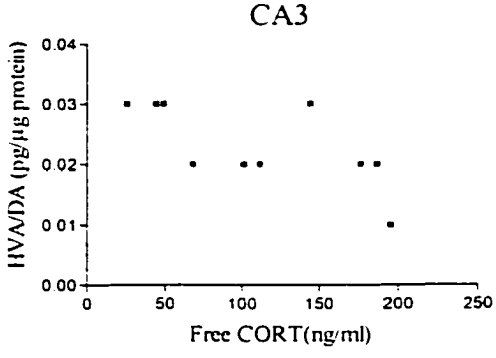

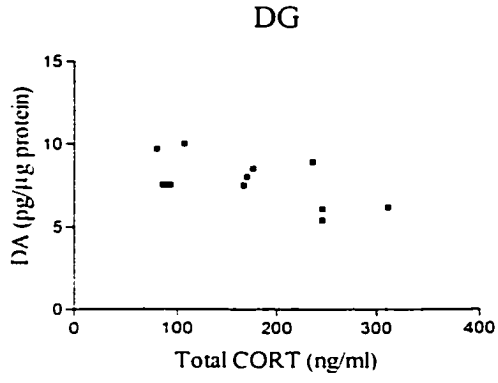

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Figure 15. Correlations between total and free CORT levels and monoamine and metabolite values in stressed rats immediately following 21 days of chronic restraint stress.

CORT values are expressed as ng/ml and neurotransmitter levels are expressed as pg/μg protein. DA activity, using a HVA/DA ratio, was significantly correlated with total CORT levels in the PFC ($r= 0.7147$, $p<0.01$). Additionally, free CORT and 5HIAA were significantly correlated in the DG region of stressed animals ($r=-0.7693$, $p<0.01$).

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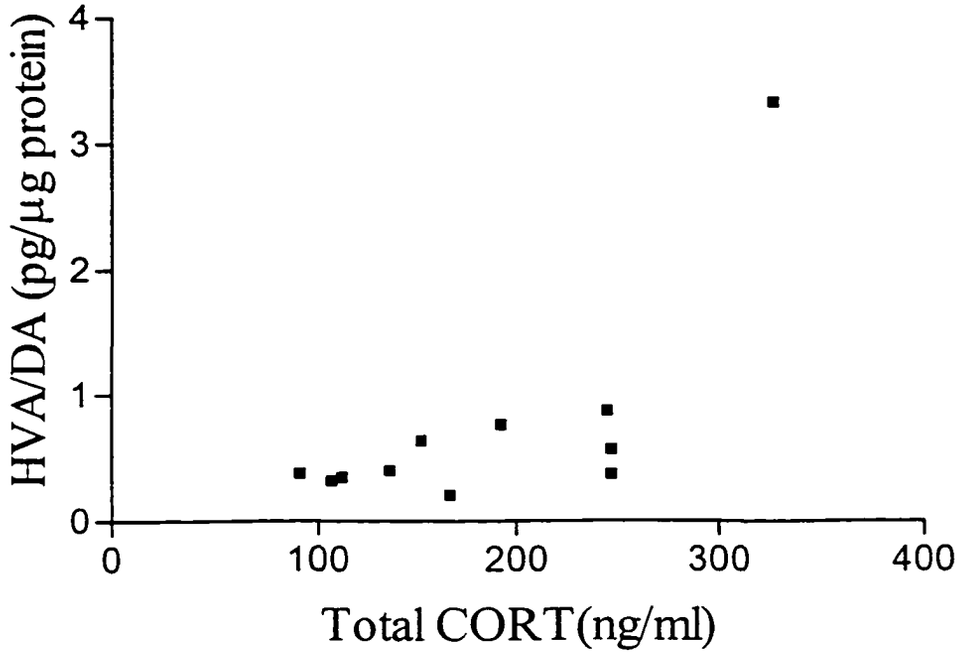
PFC



DG

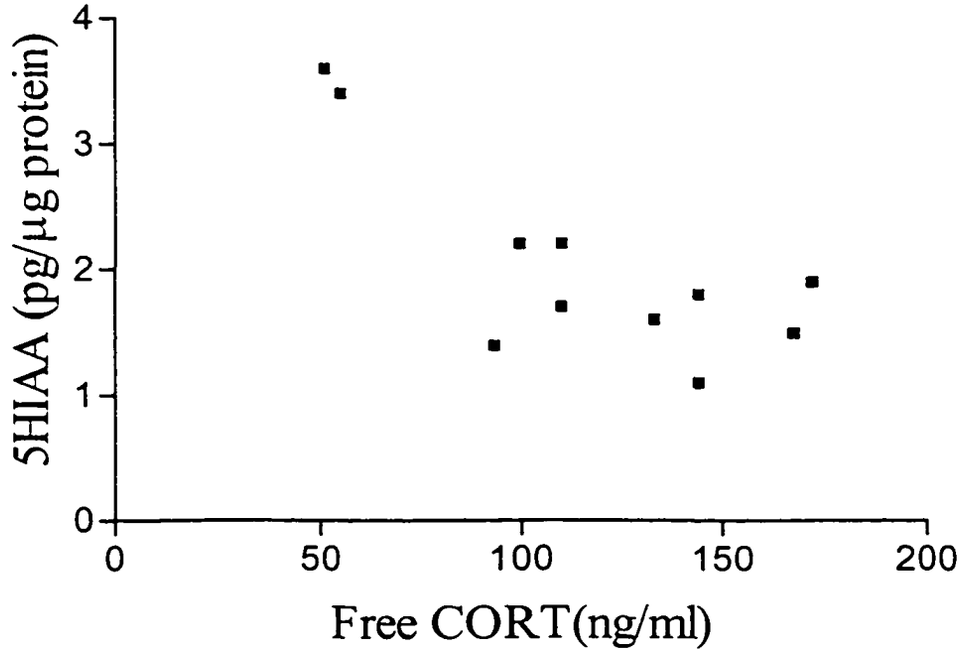


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Figure 16.

Correlations between total CORT and amino acid levels in hippocampal regions of control and stressed animals immediately following 21 days of stress.

Levels of total CORT and GABA were significantly correlated in the CA1 region of stressed animals ($r=-0.6918$, $p<0.01$) and the CA3 region of control animals ($r=0.6729$, $p<0.03$). Control animals had significantly correlated levels of total CORT and glutamate in the DG ($r=0.9288$, $p<0.01$).

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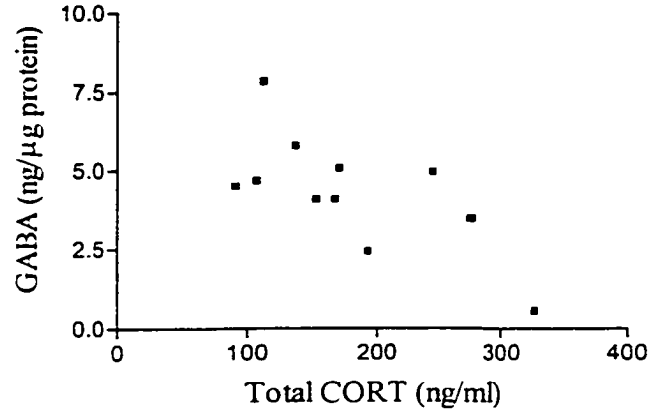
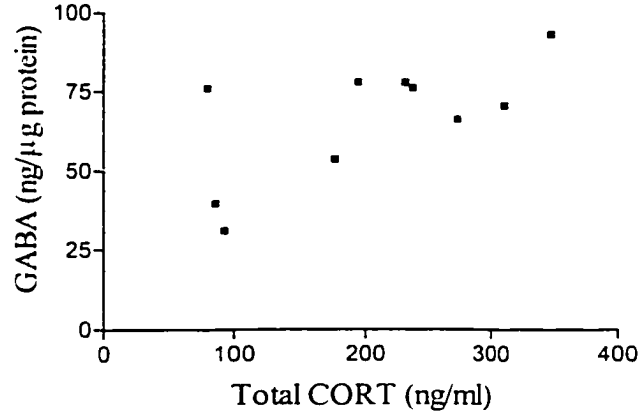
CA1 (stress)



CA3 (control)



DG (control)

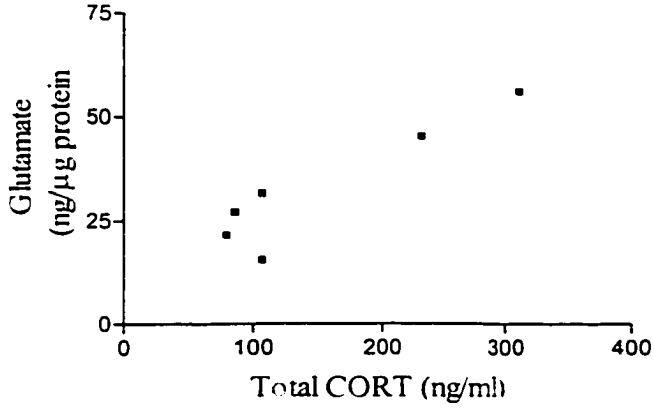


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Table 14. Monoamine and metabolite levels immediately following a 21-day chronic stress period.

Area	Group	DA	HVA	NE	MHPG	5HIAA
PFC	Control	0.87 ± 0.3	0.53 ± 0.2	6.5 ± 1.5	0.2 ± 0.05	1.5 ± 0.3
	Stressed	1.0 ± 0.2	0.42 ± 0.06	5.4 ± 0.7	0.24 ±0.06	1.3 ± 0.2
CA1	Control	0.4 ± 0.1	0.18 ± 0.03	4.6 ± 0.5	ND	1.2 ± 0.1
	Stressed	0.6 ± 0.1	0.25 ± 0.05	5.9 ± 0.7	ND	1.5 ± 0.2
CA3	Control	18.4 ± 1.0	0.43 ± 0.03	2.0 ± 0.1	ND	1.3 ± 0.2
	Stressed	17.5 ± 1.6	0.37 ± 0.05	1.8 ± 0.2	ND	1.2 ± 0.1
DG	Control	7.5 ± 0.5	0.5 ± 0.05	2.9 ± 0.5	0.07 ± 0.02	1.8 ± 0.1
	Stressed	6.1 ± 0.6	0.5 ± 0.04	3.1 ± 0.4	0.14 ± 0.03	2.0 ± 0.2

Entries are the mean ± SEM. Values expressed are pg/μg protein. Non-detectable values are indicated as ND. No differences were observed between the groups in monoamine or metabolite levels in the PFC, CA3, or DG brain regions.

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Table 15. Glutamate and GABA levels immediately following 21 days of stress.

Amino Acid	**Area**	**Group**	
		Control	*Stress*
GLU	PFC	25.09 ± 2.8	21.0 ± 2.2
GABA		2.2 ± 0.3	1.9 ± 0.2
GLU	CA1	52.1 ± 8.1	43.2 ± 6.0
GABA		5.3 ± 0.9	4.3 ± 0.5
GLU	CA3	23.6 ± 2.8	27.1 ± 4.2
GABA		66.2 ± 6.0	48.9 ± 7.5
GLU	DG	32.8 ± 6.2	30.5 ± 4.5
GABA		3.9 ± 0.5	4.2 ± 0.5

Entries are the mean ± SEM. Values are expressed as ng/μg protein. There were no differences between the stressed and control animals in neurotransmitter levels in any brain region sampled.

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Table 16. Total CORT levels across the estrous cycle in control and stressed animals.

Estrous Cycle Day	**Group**	
	Control	*Stress*
Proestrus	261.0 ± 24.0 (n=7)	190.0 ± 37.0 (n=6)
Estrus	125.0 ± 34.0 (n=4)	256.0 ± 51.0 (n=6)
Diestrus	167.0 ± 42.0 (n=4)	222.0 ± 38.0 (n=7)

Entries are the mean ± SEM. Values are expressed as ng/ml. There were no main effects of either stress treatment or estrous cycle day on total CORT levels. However, while the trend failed to reach significance ($p=0.06$), there were apparent differences in total CORT levels across the estrous cycle between the control and stressed animals, with highest CORT levels observed in proestrus controls and estrus stress animals and lowest levels observed in estrus controls.

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Discussion

Regions of the brain known to be involved in spatial learning and memory (i.e., PFC and the CA1, CA3, and DG regions of the hippocampus) were analyzed for neurochemical values in female rats immediately following 21 days of chronic restraint stress and in control rats. Surprisingly, there was no effect of stress on either monoamine and metabolite levels or amino acid levels in any brain area sampled. Additionally, there was no stress effect on CORT (either total or free) levels between the stressed and control animals immediately following the termination of the stress period. This result suggests that females have habituated to the stress paradigm, and, thus, no overall treatment effects are observed in either neurochemical or hormone levels. The observation that there were no differences between stress and control CORT levels are similar to those observed in experiment 1 in which stressed animals showed habituation of CORT levels by day 21 of the stress period. However, the current neurochemistry data is different from that previous observed in experiment 1, in which there were stress effects on neurochemical values in select brain areas. Thus, it is possible that previous neurochemical changes observed in experiments 1-3 are the result of the cognitive demand of behavioral testing more so than direct stress effects. Because there were significant effects of stress on neurochemical changes following behavioral testing (experiments 1-3), it appears that the experience of stress may alter subsequent neurochemical responses following cognitive demands.

While there was no significant main effect of either stress treatment or estrous cycle on total CORT levels, there were apparent differences in total CORT values across the estrous cycle between control and stressed animals, although this trend failed to reach

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significance (p=0.06). For control animals, CORT levels were highest on the day of proestrus and this is consistent with previously reported data (Burgess & Handa; 1992; Carey, *et al.*, 1995; Viau & Meaney, 1991) and had the lowest levels of CORT during estrus (when estrogen levels drop). Interestingly, stressed rats had the highest CORT levels during estrus and the lowest during proestrus. Previous studies have shown that stress-induced CORT elevations are more pronounced during proestrus (Carey, *et al.*, 1995; Viau & Meaney, 1991); however, these studies utilized acute stressors (e.g., 20 min of restraint, cold water swim). Thus, it appears that the typical CORT secretion across the estrus cycle is observed in control, but may be different in chronically stressed animals. It appears that the HPG (i.e., estrogen) is exerting a modifying effect on the HPA axis (i.e., CORT secretion) of stressed animals. While stress induces a overall rise in CORT levels, is appealing to speculate that in a chronically stressed female, the presence of estrogen works to buffer the negative effects of CORT.

In the DG region of control animals, both total and free CORT were correlated with increased levels of glutamate. It has previously been shown that ether stress-induced CORT release is accompanied with elevated glutamate levels (Abraham, *et al.*, 1998). More specifically, stress-induced glutamate release occurs in the hippocampus, as well as other brain areas (i.e., prefrontal cortex) and this accumulation is attenuated by adrenalectomy (Lowy, *et al.*, 1993; Moghaddam, *et al.*, 1994). It is intriguing that control animals, in the current study, are exhibiting this CORT – glutamate relationship, when the stress animals are not. One possibility is that the arousal of being sacrificed was a sufficient stressor to increase glutamate in the control, but not stress animals. Additionally, the stress animals may have 'learned' how to accommodate the rise in

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CORT during the 21-day stress period and therefore subsequent elevations in CORT alone are no longer sufficient to elicit increases in glutamate. In experiment 1, stressed animals had decreased levels of glutamate (indicative of increased glutamate activation) and had enhanced performance on the RAM. Because CORT was not correlated with increased levels of glutamate, it is appealing to speculate that activation of the HPA axis results in rapid use of glutamate and thus influences spatial performance (perhaps through LTP induction in the hippocampus).

Total CORT influenced hippocampal GABA levels in opposite ways in stressed and control animals. Total CORT was correlated with increased GABA levels in the CA3 region of control animals, but correlated with decreased GABA levels in the CA1 region of stressed animals. It has been shown using in situ hybridization that CORT treatment differentially affects mRNA levels for many hippocampal GABAA receptors (Orchinik, *et al.*, 1995) and that GABA(A) receptors are characterized by functional heterogeneity in the hippocampus (Orchinik, *et al.*, 2001). Thus, it seems possible that elevations in CORT between stress and control animals are having differential effects on GABA receptors in various regions of the hippocampus as well as subsequent neurotransmitter levels. Additionally, CORT levels in the stressed animals may be indicative of long-lasting HPA induced changes, while elevated CORT levels in the controls may be more indicative of a rapid response to the immediate stressor (sacrifice).

Serotonergic activity was differentially correlated with CORT in various brain regions in the stress and control animals. Total CORT was correlated with decreased CA1 levels of 5HT in the controls. Previous data (Luine, *et al.*, 1993) showed no association between CORT and neurotransmitter levels in the CA1 region of male rats. It is possible

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that the current data are unique to female rats or that these results attenuate over time, as Luine and colleagues analyzed monoamine levels 5 weeks following the CORT treatment. It would be of interest to examine the relationship between CORT and neurotransmitter levels immediately following the termination of the stress period in male rats. Both total and free CORT was correlated with serotonergic activity in the DG of stressed animals. Interestingly, the correlation between CORT levels and decreased 5HIAA levels is similar to results from the OVX experiment in which stress treatment decreased DG levels of 5HIAA. Thus, it appears that stress induced CORT changes are associated with neurochemical changes that underlie behavioral responses.

In summary, this experiment provides novel information about the relationships between CORT and neurochemical levels in stressed and control animals. Because CORT was correlated with different neurotransmitters in various brain regions for the control and stressed animals, it appears that CORT has differential effects in animals exposed to chronic stress than it does in controls. It is unclear what mechanism(s) are underlying these differential responses. Even though there were no overall differences in CORT levels between the control and stress animals, it is possible that sustained CORT across the 21-day period induces changes (e.g., changes in receptor levels or physiological characteristics of receptors) that result in differential relationships between the HPA axis and the neurochemical systems of stress and control animals.

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Chapter 5-Summary and Conclusion

In summary, the results from these experiments provide novel information concerning the effects of chronic restraint stress on intact and ovariectomized female rats, see Figure 17 for an overall summary. In general, females appear less sensitive to the effects of chronic restraint stress than males. While physiological measurements obtained during these experiments (i.e., decreased body weight and elevated CORT levels in the stressed animals) indicate that chronic restraint is a stessor for female, these stress-induced changes attenuate over time. CORT levels habituated by day 7 of the stress paradigm and no differences were observed in body weight between stressed and control subjects at 28 days of stress. Additionally, there were no alterations in estrous cyclicity during or following the stress period.

Experiment 1 and 2 investigated the effects of chronic restraint stress in intact females. Results show that 21 and 28 days of stress have differential effects on both locomotor activity and spatial learning and memory. Following 21 days, stressed females had decreased ambulation on the open field and enhanced radial arm maze performance. Following 28 days of chronic restraint stress, there were no differences between control and stressed animals on the open field and RAM performance was neither enhanced nor impaired.

Several current observations suggest a different temporal relationship between stress duration and behavioral changes between males and females. In general, longer periods of stress in females are required to achieve the same effect as in males. First, female performance on the open field is different following 21 and 28 days of stress. Twenty-one days of stress led to a decrease in overall ambulation , while 28 days of

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stress resulted in no differences in locomotor activity between the stressed and non-stress females. The activity of stressed females following 28 days of stress is similar to previously reported results in which stressed male rats did not show differences in ambulation on the open field following 21 days of stress (Beck & Luine, 1999). Second, the current female RAM data is different from that previously reported for male rats in stress effects on performance of memory tasks. It has previously been shown that 14 days of stress enhances male RAM performance (Luine, *et al.*, 1996), but that males are impaired following 21 days of chronic restraint stress (Luine, *et al.*, 1994). While 21 days of stress enhanced female RAM performance, 28 days neither enhanced nor impaired female RAM performance and this pattern of results is different than that observed in male rats in which stress effects change from adaptive to maladaptive over time (Luine, *et al.*, 1994; Luine, *et al.*, 1996). It is important to note, that these dimorphic behavioral responses to chronic stress are consistent with the sexual dimorphism observed in morphology (Galea, *et al.*, 1997) and chemistry (Beck & Luine, 1998; Luine, *et al.*, 2001) following restraint stress. Third, the current female neurochemical data is different than that previously reported for males following chronic restraint stress. Although it appears that, like the behavioral data, prolonged stress periods in females begin to elicit male-like stress-dependent neurochemical alterations. For example, 21-days of stress increased hippocampal CA3 GABA levels in male rats and was accompanied by spatial memory impairments, whereas 21-days of stress is not accompanied by GABA changes in females and there is spatial memory enhancement. However, when females are exposed to a prolonged period of stress (28 days), GABA levels increases in the hippocampal DG region and enhancement of a spatial task has attenuated. Based on the current results,

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stress does not exert the same time course of action in female rats. Longer periods of stress are required to produce stress-dependent impairments in females than in males. Thus, the time course of adaptive (14 days) to maladaptive (21 days) stress effects in males does not appear relevant to females. It remains to be determined if even more prolonged periods of stress (e.g., 35 days) would impair female performance on the RAM.

The mechanisms underlying the observed sexually dimorphic stress responses are not understood. However, it has been well established that estrogens play a beneficial role in learning and memory and several observations suggest that gonadal hormones, most likely estradiol, may contribute to the observed sexually dimorphic stress effects. First, GC levels vary depending on estrous cycle day (Burgess & Handa, 1992; Carey, *et al.*, 1995; Viau & Meaney, 1991) and thus, it seems possible that estrogen may also be affecting the CORT response at the neurochemical or molecular levels (e.g., estrogen could be altering glucocorticoid receptor levels (Ferrini & De Nicola, 1991)). For example, while both males and females have a similar distribution of glucocorticoid and mineralocorticoid receptors, male rats have higher levels of mineralocorticoid receptors in the principal cell fields of the hippocampus (MacLusky, *et al.*, 1996). Second, estrogen has been shown to have both neuroprotective (Dubal & Wise, 2001; Wise, *et al.*, 2000; Wise, *et al.*, 2001) and growth promoting effects (Toran-Allerand, *et al.*, 1999). Therefore, female levels of estrogen may have general protective effects against CORT, particularly in the CA3 region of the hippocampus (where stress-induced atrophy of apical dendrites occurs in males but not in females). Third, it has been shown that estrogen levels decrease following 21 days of restraint stress (Galea, *et al.*, 1997). This

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decline in estrogen is consistent with the current data in which the enhancing effects of chronic stress on female RAM performance are present following 21 days of stress, but diminish with extended periods of stress, during the time when stress levels of estrogen would apparently be lower than estrogen levels in non-stressed females.

Another observation that provides further support for the role of estrogens in the observed sexually dimorphic stress response is that estradiol alone enhances performance of several tasks requiring memory, including the RAM (Luine & Rodriquez, 1994; Luine *et al.*, 1998; Sandstrom & Williams; 2001; Williams, *et al.*, 1990). More specifically, the current results show that both estradiol treatment alone and estradiol treatment to stressed females enhances RAM performance as compared to OVX females of OVX stressed females following 21 days of stress. Enhanced performance in the estradiol treated groups is indicative of the activational effects of estrogen. Furthermore, the observation that OVX rats without estrogen replacement were not impaired on the RAM following the stress period provides further support for an organizational role of estrogen on the stress response. Taken together, these results suggest that estradiol is playing a role in protection from stress-induced corticosterone associated cognitive impairments.

Several neural systems are implicated in mediating stress effects on cognition, and these systems also appear to respond differentially to stress. Most notably, prefrontal cortex dopaminergic activity is critical for both spatial and non-spatial rodent memory, and there are distinct sex differences in dopamine activity following chronic stress. In general, an "inverted U" relationship exists between prefrontal cortex dopamine levels and memory function in which too little or too much dopamine interferes with memory (Murphy, *et al.*, 1996; Zhart, *et al.*, 1997). Previous studies have shown that stressed

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males who are impaired on the non-spatial object recognition task, have decreased dopamine metabolites (indicative of decreased dopamine activity) in prefrontal cortex (Luine, *et al.*, 2001). But, stressed females who are not impaired on spatial and non-spatial tasks show no changes in dopamine activity (Luine, *et al.*, 2001). In the current studies, stress led to increases in dopamine activity following 21 and 28 days in intact females, as well as increases in stressed OVX rats following 21 days. We have also demonstrated that in stressed animals, CORT levels are correlated with increased levels of PFC dopamine activity. Thus, in female rats, it appears that increases in DA activity helps with performance on memory tasks. These results suggest that both stress (adrenal) and gonadal (estradiol) hormones may be underlying sex dependent changes in memory function through actions on dopaminergic terminal in the PFC.

Another important observation is that stressed females show changes in both monoamine and amino acid activity in the DG region of the hippocampus, whereas stressed males do not. In the current studies, DG activity was influenced by both stress and estradiol treatment. The DG is the hippocampal region characterized by new neuron production during adulthood and this neurogenesis is influenced by circulating estrogen levels (Tanapat, *et al.*, 1999). Specifically, elevated estrogen levels associated with proestrus produces a transient increase in the DG neurogenesis. It is possible that this female specific neuron production is having a functional impact (e.g., axon extension to the CA3 region) on the observed sexually dimorphic morphological, behavioral, and neurochemical responses to stress.

In conclusion, the current studies provide novel information about the effects of chronic restraint stress on intact and OVX female rats. Female rats are enhanced on RAM

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following 21 days of chronic restraint stress, but these enhancements do not extend to 28 days of stress. Furthermore, neurochemical changes in areas known to contribute to learning and memory were present, and the changes varied following 21 and 28 days. Thus, hormone dependent effects on neurotransmitters may underlay the observed stress-dependent alterations in memory performance. The current studies also suggest that estradiol, acting organizationally and activationally, alters the cognitive response to chronic stress in female rats. Because some aspects of the sex differences in response to chronic stress appear to be hard-wired, it raises the interesting question as to whether these sex differences reflect an advantageous evolutionary development. That is, males may be better suited to short-term periods of stress (e.g., territorial defense, foraging) while females may be better adapted to sustained durations of stress (e.g., maintenance of nest and offspring). While a difficult hypothesis to address, future studies could focus on whether sex differences in the stress response are maintained across various animal species (e.g., in species where there is a higher paternal investment than maternal investment). In summary, the current data, when compared to previously reported data for male rats, suggest that sexually dimorphic responses to chronic stress may depend, in part, on the presence or absence of estradiol.

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Figure 17.

Summary of physiological, behavioral, and neurochemical responses to chronic stress in intact and OVX females.

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Dependent variable	Exp. 2 (21 days stress; intacts)	Exp. 1 (28 days stress; intacts)
Weight gain	stress ↓ weight gain	stress ↓ weight gain (habituates by day 28)
Estrous cyclicity	stress ↔ no changes	stress ↔ no changes
Open field	stress ↓ outer crossings	stress ↔ no changes
RAM	stress ↓ visits to complete stress ↑ correct choices proestrus: impaired acquisition	stress ↔ no changes proestrus: stress ↑ #correct
Neurochemistry	PFC: stress ↑ HVA/DA CA3: stress ↑ HVA CA3: stress ↓ glutamate	PFC: stress ↑ HVA DG: stress ↑ NE, DA, GABA VDB: stress ↑ NE, 5HIAA

Dependent variable	Exp. 3 (21 days stress; OVX)	Exp 4 (21 days stress; no behavior)
Weight	stress ↓ weight gain estradiol ↓ weight gain	
Open field	stress ↓ outer crossings estradiol ↑ inner visits	
RAM	estradiol ↓ visits to complete stress + estradiol ↓ RAM	
Neurochemistry	PFC: stress ↑ HVA BLA: estradiol ↑ MHPG, 5HIAA, DA DG: stress ↓ 5HIAA, HVA DG: estradiol ↓ MHPG estradiol ↓ 5HIAA CA3: estradiol ↑ NE	Correlations – Control CA1: 5HT - total CORT CA3: NE - total CORT CA3: HVA/DA – free DG: DA - total CA3: GABA - total DG: GLU – total DG: GLU – free Correlations – Stress PFC: HVA/DA – total DG: 5HIAA/5HT– total DG: 5HIAA – free CA1: GABA - total

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