Chronic Variable Stress: Effects of Gender, Chronic Fluoxetine Treatment and Early Life Maternal Care

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ABSTRACT

Exposure to prolonged stress is associated with an increased risk for mood disorders. However, only a small portion of those who experience such distress go on to develop these disorders, suggesting that individual and stress-related variables may increase the vulnerability to the damaging effects of chronic stress. Accordingly, the present series of experiments assess the influence of three factors on vulnerability to long-term exposure to chronic variable stress (CVS) in rats. In particular, we investigated the influence of gender, chronic fluoxetine treatment and early life maternal care on the behavioral and neuroendocrine response to CVS. The results from study 1 suggested that the direction of the behavioral and neuroendocrine response to CVS was in the opposite direction in male and female rats. Studies 2 and 3 revealed that fluoxetine treatment had a gender-dependent ability to reverse some of the effects of CVS, and this was mediated by increasing the rate of habituation to novel, acute stressors. Studies 4 and 5 demonstrated a gender-dependent effect of maternal care on behavior and the neuroendocrine response to an acute stress. In addition, maternal care influenced the impact of CVS exposure in a gender-dependent manner. The results suggested that maternal care affected coping processes in males and females that were expressed in different behavioral tests. Studies 5, 6 and 7 revealed that maternal care influenced the strength of associative fear conditioning to a context associated with shock administration, but did not influence conditioning to a discrete conditioned stimulus associated with shock. These results suggest that maternal care influences the strength of learned associations on a hippocampal-dependent task. Exposure to CVS eliminated the effect of maternal care on the strength of contextual fear conditioning. Thus, conditions in the post-weaning

environment, such as CVS exposure, may reverse the effects of maternal care on contextual fear conditioning. In conclusion, the results of this thesis suggest that there are gender, fluoxetine and early life maternal care influences on the behavioral and neuroendocrine response to CVS exposure. These factors appear to influence the response to CVS by modifying the capacity to adapt to novel stressors.

RESUME

L'exposition prolongée au stress chronique augmente les risques de développer des désordres de l'humeur. Cependant, seulement une faible proportion d'individus qui sont exposés au stress chronique développera de tels désordres, suggérant des différences individuelles au niveau de la vulnérabilité au stress. La présente série d'expériences évalue l'influence du genre (masculin ou féminin), traitement avec la Fluoxetine, ainsi que les soins maternels (SM) sur la vulnérabilité à l'exposition au stress chronique variable (SCV) chez le rat. Les résultats de l'étude 1 indiquent que les femelles sont plus sensibles aux effets du SCV que les males. Les études 2 et 3 révèlent que certain des effets du SCV sont renversés d'une façon dépendante du genre, et ce par une augmentation du taux d'habituation à un stress nouveau et aigue. Les études 3 et 4 démontrent que les SM influencent l'impact de l'exposition au SCV en affectant les processus de control des réponses au SCV d'une manière dépendante du genre. Les études 5, 6 et 7 révèlent que les SM influencent l'intensité du conditionnement à la peur (CP) dans un contexte associé avec l'administration d'un choc mais n'influence pas le conditionnement à un stimulus discret associé avec un choc, suggérant que les SM influencent la force des associations qui ont été apprises lors de l'exécution d'une tâche dépendante de l'hippocampe. En conclusion, les résultats suggèrent que le genre, la fluoxetine ainsi que les SM influencent les réponses comportementales et neuroendocriniennes suite au SCV en modifiant la capacité d'adaptation à des nouveaux stress.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine; serotonin
5-HTTLPR	5-HT transporter promotor
8-OH-DPAT	8-hydroxydipropylaminotetralin
11BHSD	11-β-hydroxysteroid dehydrogenase
ABN	arched-back nursing
ACTH	adrenocorticotropin hormone
ADX	adrenalectomy
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ASR	acoustic startle response
AUC	area under the curve
BNST	bed nucleus of the tractus solitarius
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding globulin
CBZ	central benzodiazepine receptor
CnAmy	central nucleus of the amygdala
CORT	corticosterone (rat), cortisol (human)
CRE	cAMP response element
CREB	CRE binding protein
CRH	corticotropin releasing hormone
CS	conditioned stimulus
CSF	cerebrospinal fluid
CVS	chronic variable stress
DA	dopamine
dB	decibel
DRN	dorsal raphe nucleus
DST	dexamethasone suppression test
EEG	electroencephalogram
EP	progesterone
EPM	elevated plus maze
FDA	food and drug administration
GABA	gamma-aminobutyric acid
GC	glucocorticoid
GR	glucocorticoid receptor
H	handled
HMS	handled, maternally separated
HPA	hypothalamic-pituitary-adrenal
HRT	hormone replacement therapy
ICV	intracerebroventricular
IP	inositol phosphate
LC	locus ceoruleus
LG	licking and grooming
MAO	monoamine oxidase
MAOi	monoamine oxidase inhibitor

MHPG	3-methoxy-4-hydroxy-phenylglycol
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MRN	median raphe nucleus
MS	maternally separated
NE	norepinephrine
NIH	National Institute of Health
NH	non-handled
NMDA	N-methyl-D-aspartate
OBX	olfactory bulbectomy
OCD	obsessive-compulsive disorder
PCA	para-chloramphetamine
PFC	prefrontal cortex
PKA	protein kinase A
PND	post-natal day
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TH	tyrosine hydroxylase
VP	vasopressin

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Study 1: The results of this study demonstrated that common behavioral tests used in stress research yield contrasting results in male and female rats. In addition, chronic variable stress induces gender dependent behavioral and neuroendocrine responses.

Study 2 and 3: This study demonstrated that fluoxetine had a gender-dependent ability to reverse some of the effects of CVS exposure. Fluoxetine appeared to reverse the effects of CVS by increasing the rate of habituation to a novel stressor.

Study 4 and 5: These studies showed that the influence of maternal care on the adult behavioral response to novelty is gender-dependent. The impact of CVS exposure on the behavioral and neuroendocrine response to a novel stress was also influenced by gender. Maternal care appeared to affect the behavioral response to a novel stress in part through increasing habituation, and coping mechanisms.

Study 6, 7 and 8: These studies demonstrated that early life maternal care modified the strength of a conditioned response to an unconditioned stimulus (US; footshock)-context association. However, maternal care did not appear to influence the strength of the association between the US and the conditioned stimulus (CS; auditory cue). These findings implicate the influence of early environmental effects on hippocampal functioning in the strength of the association between the US and the context. Exposure to CVS eliminated the effect of maternal care on the US-context association, suggesting

that some early environmental effects may be vulnerable to aversive events in the post-weaning environment.

CONTRIBUTION OF AUTHORS

Study 1: Emma Spreekmeester, Shakti Sharma, Michael Meaney, Joseph Rochford and Ettie Grauer.

Drs Joseph Rochford, Michael Meaney and Ettie Grauer supervised the research project. Shakti Sharma ran the corticosterone assays, Dr Ettie Grauer participated in the behavioral testing. The author of this thesis carried out the stress protocol, the majority of the behavioral testing, analyzed the data and wrote the manuscript.

Study 2 and 3: Emma Spreekmeester, Shakti Sharma, Michael Meaney, Joseph Rochford and Ettie Grauer.

Drs Joseph Rochford, Michael Meaney and Ettie Grauer supervised the research project. Shakti Sharma ran the corticosterone assays, Dr Ettie Grauer participated in the behavioral testing. The author of this thesis carried out the stress protocol, the majority of the behavioral testing, analyzed the data and wrote the manuscript.

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author of this thesis carried out the stress protocol, the majority of the behavioral testing, analyzed the data and wrote the manuscript.

Study 6, 7 and 8: Emma Spreekmeester, Joseph Rochford and Michael Meaney.

Drs Joseph Rochford and Michael Meaney supervised the research project. The author of this thesis carried out the stress protocol, all of the behavioral testing, analyzed the data and wrote the manuscript.

INTRODUCTION

1 GENERAL INTRODUCTION

Exposure to severe or prolonged stress has been shown to influence long-term health and well-being. For instance, stress has been associated with the development of anxiety and affective disorders as well as premature coronary artery disease (Smith and Ruiz, 2002) and premature osteoporosis (Michelson et al., 1996). However, while a large percentage of anxiety and affective disorder patients have been exposed to a severe stress or trauma, not all who experience such distress go on to develop a disease. This raises the issue of identifying traits that may render an individual more or less vulnerable to the impact of one or several of the risk factors for disease. While exposure to stress is a significant precipitating feature of such disorders, other factors, such as genetics and early environment, can also contribute to the outcome of an individual's health. The magnitude of the reaction and capacity for recovery from any given stressor is unique to each person and it has been hypothesized that these measures can be of predictive value for susceptibility to illness. As the factors that have been shown to affect stress reactivity, such as early environment and exposure to previous stressors, are the same as those used to predict susceptibility to affective illnesses, an examination of their contributing and additive elements could illuminate the traits that cause individual differences in the development of disease.

The variables that may be used to determine one's response to a stressor can be divided into two main categories; those that fall into a "pre-stressor" category, such as gender, early life experiences and genetics; or those that relate to the stressor itself, such

as degree of exposure to the stressor and subjective appraisal of perceived threat (Yule et al., 2000).

Major depression is among one of the leading causes of disease-related disability in the world (Murray and Lopez, 1996; Kessler, 2003). Estimates of the total population lifetime prevalence of major depression range between 6 and 17%. Major depression is a recurrent, lifetime illness, characterized by repeated remissions and exacerbations in over 75% of patients (Gold and Chrousos, 2002). Further, over 50% of patients that do recover will experience a second episode within 6 months unless they are given further antidepressant treatment (Gold and Chrousos, 2002). Typically, as a depressive illness progresses, it is characterized by more frequent episodes of depression, precipitated by less severe stressors, with more symptoms and an increased resistance to therapeutic intervention (Post, 1992). While there is a heritable component to depression (Gold and Chrousos, 2002) involving multiple genes (Kendler et al., 2001), epidemiological surveys have shown that the prevalence has increased significantly in the past few decades for both men and women. Since the population gene pool could not have changed this quickly, the change in environmental conditions is likely to account for this increase. A part of the increase in prevalence may be due to a change in diagnostic procedures that are more sensitive and better able to identify specific subtypes of depression. In addition, diagnostic procedures now recognize other disorders, such as anxiety and drug addiction that are comorbid with depression (Kessler, 2003). There is also evidence that depressed patients use less adaptive coping mechanisms in response to stress (Ravindran et al., 1995b).

There are two distinct clinical syndromes of depression. Melancholic depression describes a state of pathological hyperarousal (Gold and Chrousos, 2002). There is intense anxiety with feelings of worthlessness and recollections of past transgressions, failures and helplessness. Feelings of self-deficiency taint the outlook on future prospects and permeate thoughts and affect (Gold and Chrousos, 2002). Physiologically, melancholic patients display hypercortisolism, suppression of growth hormone and hormones regulating the reproductive axes, insomnia and loss of appetite. In addition, they exhibit a diurnal variation in the magnitude of their symptoms, with the most depressed mood occurring in the morning.

Atypical depression is characterized by feelings of emptiness and disconnectedness, with brief periods of emotional arousal in response to external events. Patients with atypical depression do not dwell on memories of past failures or negative emotional events, in fact, they have little connection to their own feelings. Physiologically, atypical depression is associated with lethargy, fatigue, excessive sleepiness, increased food intake and weight gain. Atypical depressed patients also display a diurnal variation in the magnitude of their symptoms however their symptoms worsen as the day progresses (Horwath et al., 1992).

It is noteworthy that only 25-30% of depressed patients exhibit pure melancholic symptoms, and only 15-30% present with pure atypical features (Gold and Chrousos, 2002). The majority of patients with major depression appear to have a mixture of symptoms. However, those with either melancholic or atypical features tend to show a more severe course of illness than those with mixed symptoms.

1.1 Physiology of the stress response

When danger presents, there is a series of physiological and behavioral responses that are relatively predictable and promote survival in a threatening situation. The physiological changes include increases in heart rate and blood pressure, redirection of blood flow to the brain and the stressed body part, as well as breakdown of tissue to mobilize fuel (Gold and Chrousos, 2002). In addition, there is a concurrent inhibition of neurovegetative functions, such as feeding, sleep, sexual behavior and endocrine programs for growth and reproduction, that would limit the likelihood of survival in a life threatening situation (Gold and Chrousos, 2002).

1.1.1 The Hypothalamic-Pituitary-Adrenal Axis (HPA)

Both physical and psychological stressors increase the activity of the hypothalamic-pituitary-adrenal (HPA) system, which mediates the ability to cope with stress (see Figure 1: adapted from (de Kloet et al., 1998a)). The neural, endocrine and behavioral adaptations induced by HPA activation help to restore homeostasis.

Stress causes the release of parvocellular CRH and vasopressin (VP) from the paraventricular hypothalamus (PVN) (Koob, 1999d). The released CRH stimulates pituitary adrenocorticotropin (ACTH) secretion, which in turn mobilizes cortisol (human) or corticosterone (rat) production and release from the adrenals (Koob, 1999c). It has been shown that cortisol facilitates adaptation to a stressor by increasing blood pressure and redirecting metabolism to free energy stores. Glucocorticoids also temporarily control the immune system by inhibiting the production of cytokines, and serve as a negative feedback signal to both the hypothalamus and pituitary, inhibiting further release of CRH and ACTH (O'Connor et al., 2000). While the HPA axis is activated almost

immediately upon the presentation of a stressor, it takes approximately 10-15 minutes for glucocorticoid levels to peak (de Kloet et al., 1998a). The length of time that they remain elevated depends upon the length and severity of the stressor as well as the coping ability of the individual.

Corticosterone levels change in response to circadian rhythmicity and to stress, however, under normal circumstances, the average 24 hr steroid concentrations are quite constant, and are known as a "set point" of homeostasis (Dallman et al., 1992). Access of free corticosterone to the target tissue depends on first, the regional availability of steroid-metabolizing enzymes, such as 11-β-hydroxysteroid dehydrogenase (11BHSD) type 1 and 2; and second, levels of corticosteroid-binding globulin (CBG), which binds to the steroid and prevent it from entering the target cell. There are two receptors for corticosterone in the brain, both of which are intracellularly located steroid receptors, with different affinities for the steroid (Truss and Beato, 1993). The mineralocorticoid receptor (MR) has a high affinity for corticosterone and also binds aldosterone (Reul and de Kloet, 1985). The glucocorticoid receptor (GR) has a lower affinity than MR. Therefore, low circulating levels of corticosterone will bind mainly to MR, while higher levels induced by stress or the diurnal peak will bind to both MR and GR. In the hippocampus, ion regulation and neurotransmitter synthesis and release depend on a finely tuned balance of MR- and GR-mediated actions.

Upon the termination of a stressor or successful coping, activity of the HPA axis is decreased via a negative-feedback action by corticosterone acting on GR receptors located in the hypothalamic parvocellular CRH neurons (de Kloet et al., 1998a). Occupation of GRs in the PVN will suppress the stress-induced synthesis

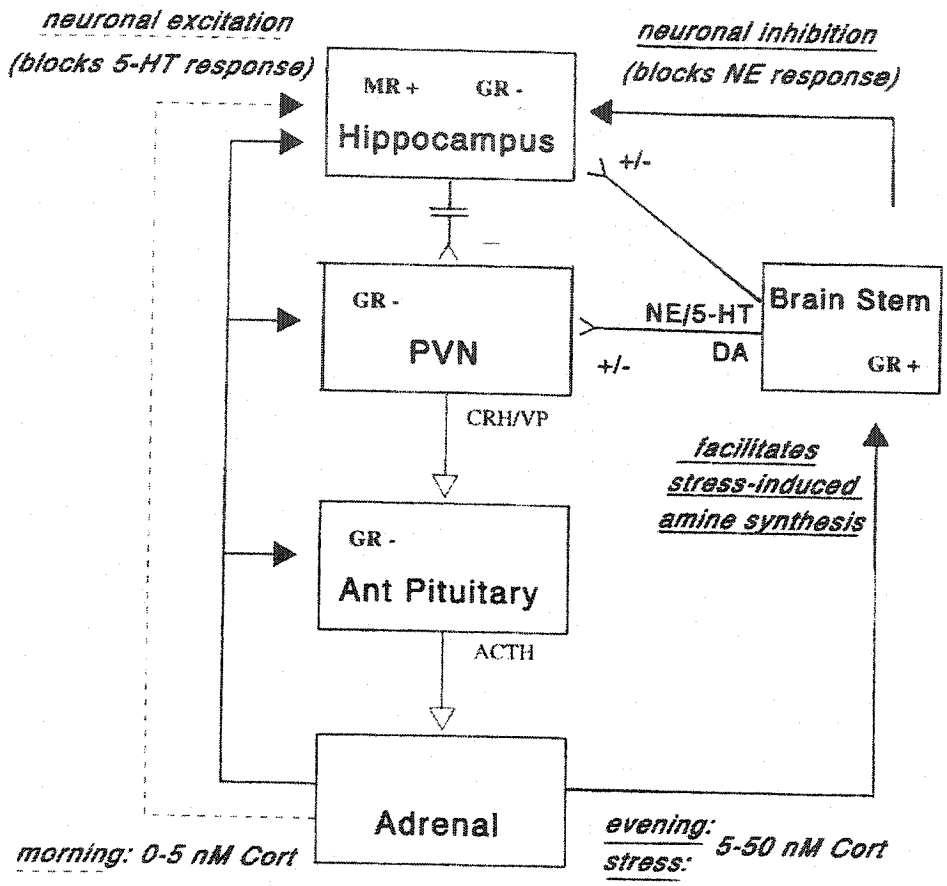

Figure 1. Schematic of MR- and GR-mediated effects in control of HPA axis. Stress induces aminergic input to the paraventricular nucleus (PVN) via stimulatory action of GRs. Primary feedback site: corticosteroids suppress stress-induced CRH and VP via GR in the PVN. MRs in the hippocampus exert an inhibitory tone on HPA. Occupation of GRs in during the circadian peak or after stress is thought to act as a switch that disinhibits the negative MR-mediated control over the HPA axis. The corticosterone concentrations represent the free fraction of the hormone.

of CRH, VP and other ACTH secretagogues (Vamvakopoulos and Chrousos, 1994). Occupation of GRs outside of the hypothalamus promotes the stress-induced activation of parvocellular CRH (de Kloet et al., 1998a).

Corticosteroids acting in the hippocampus also mediate HPA activity. Hippocampal GABA-ergic neurons synapse onto CRH neurons and inhibit their activity (Swanson, 1991). Under basal conditions, it is the hippocampal MRs maintain this inhibition over the CRH neurons (de Kloet et al., 1998a). Under conditions of stress, activation of GRs attenuates the GABA-ergic inhibition of CRH neurons.

1.1.2 The Corticotropin Releasing Factor – Norepinephrine (CRF-NE) System

There is evidence to indicate that in addition to it's role as a neurohormone within the HPA axis, CRH can also act as a neurotransmitter in conjunction with NE systems in mediating stress responsivity (Curtis et al., 1999; Lechner and Valentino, 1999; Wong et al., 2000). Activation of the CRH and noradrenaline systems can contribute to stress-induced pathologies on their own or in concert with the HPA axis (Coplan et al., 1996; Koob, 1999b; Wong et al., 2000; Heim et al., 2000b). The activity of the locus coeruleus (LC), the main noradrenergic nucleus of the brain, has been shown to increase in response to arousing stimuli and is positively correlated to the state of arousal (Abercrombie and Jacobs, 1987a; Abercrombie and Jacobs, 1987b; Foote et al., 1991; Nestler et al., 1999). It has also been shown that stress increases noradrenergic release and turnover in the terminal regions of LC neurons (Abercrombie et al., 1988).

Centrally administered CRH has been shown to induce arousal and behavioral profiles indicative of states of stress. For example, intracerebroventricular (ICV) infusion

of CRF facilitates fear responses (Britton et al., 1982; Imaki et al., 1987; Diamant et al., 1992; Lee and Davis, 1997a; Lee and Davis, 1997b). Conversely, ICV administration of CRF antagonists decrease stress and fear-related behaviors (Tazi et al., 1987; Kalin et al., 1988; Swerdlow et al., 1989; Korte et al., 1994; Koob, 1999a). Further, transgenic mice that overproduce CRF exhibit exaggerated fear-like responses to novelty as well as elevated levels of glucocorticoids and ACTH (Stenzel-Poore et al., 1994), while mice with a knockout of the CRF-1 receptor display a decreased behavioral and HPA response to stressful stimuli (Skutella et al., 1997; Smith et al., 1998).

The CRH neurons of the PVN contain GR receptors, are inhibited by glucocorticoids, and therefore play a primary role in negative feedback of HPA activity. Immunohistochemical studies employing retrograde tract tracing techniques have also identified CRH neurons in the bed nucleus of the tractus solitarius (BNST), the central nucleus of the amygdala (CnAmy), and Barrington's nucleus (Sakanaka et al., 1986; Valentino et al., 1993; Valentino et al., 1996; Van Bockstaele et al., 1998; Lechner and Valentino, 1999). However, unlike the neurons in the PVN, the CRH neurons from these other brain nuclei are activated by glucocorticoids (Makino et al., 1994). Of interest, CRH axons from the CnAmy, the BNST and Barrington's nucleus project to the peri-LC area where they synapse onto NE dendrites of the LC, and provide stimulation when activated (Van Bockstaele et al., 1996; Lechner and Valentino, 1999).

Infusion of CRF into the LC increases LC discharge rates and NE release in the terminal regions of LC neurons (Valentino and Foote, 1988; Smagin et al., 1995; Page and Abercrombie, 1997; Curtis et al., 1997; Curtis et al., 1999; Palamarchouk et al., 2000), and also increases cortical electroencephalogram (EEG) activity (Curtis et al.,

1997), whereas the administration of CRF antagonists directly into the LC has been shown to attenuate LC activation induced by physiological and psychological stressors (Berridge and Dunn, 1987; Curtis et al., 1993).

There is some evidence to indicate that CRF not only increases LC activity, but that LC fibers in turn, project to the PVN, BNST and CnAMY, where they synapse on CRF containing neurons (Liposits et al., 1986a; Phelix et al., 1994; Raber et al., 1995; Koob, 1999h). Both exogenous CRF administration and stress increase NE release and NE's major metabolite, 3-methoxy-4-hydroxy-phenylglycol (MHPG) to NE ratios in the PVN, BNST, CnAMY, prefrontal cortex (PFC), hypothalamus and the brainstem (Dunn and Berridge, 1987; Pacak et al., 1995a; Pacak et al., 1995b; Quirarte et al., 1998). Moreover, evidence that NE then stimulates the release of CRH provides a final link in a putative CRF-NE feed-forward circuit that is proposed to be activated by stress and is self perpetuating (Koob, 1999e). That is, stress is known to activate CRF-containing neurons, which project to the LC. The released CRF stimulates the LC, resulting in increased NE release at CRH cell bodies. The released NE then stimulates the production of CRF, resulting in a feed-forward system.

1.1.3 The Serotonergic System

There are several nuclei containing serotonin cell bodies that project to the periphery as well as to other areas in the brain. Serotonin neurons emanating from the pontine and medullary nuclei project to the spinal cord and are involved in the perception of pain, visceral regulation and motor control (Graeff, 1997). The dorsal raphe nucleus

(DRN) and the median raphe nucleus (MRN) project to the forebrain and are involved in cognitive, affective and neuroendocrine functions (Graeff, 1997).

The serotonergic cell bodies of the raphe nuclei also receive afferents from many regions including, the amygdala, hippocampus, striatum, cortex and periaqueductal grey, allowing these nuclei to receive constant information during times of stress (Jacobs and Azmitia, 1992; Chaouloff, 1993; Graeff, 1993).

The limbic forebrain areas are differentially innervated by the MRN and the DRN; the septum, frontal cortex and the dorsal hippocampus are innervated mainly by the MRN, whereas the amygdala and ventral hippocampus receive the majority of their 5-HT fibers from the DRN. The hypothalamus receives 5-HT input from both nuclei; the MRN projects to the lateral nucleus, and the DRN projects to the medial nucleus (Graeff, 1997). (There are many other 5-HT projections that will not be described here.)

The terminal fibers of the DRN are fine axons with small varicosities, are destroyed by halogenated amphetamines, such as para-chloramphetamine (PCA), and establish contact with post-synaptic 5-HT 2A/2C receptors. The fibers emanating from the MRN are thicker than those of the DRN, are beaded with large spherical varicosities, are resistant to PCA, and establish preferential contact with post-synaptic 5-HT1A receptors (Mamounas et al., 1991). Despite the morphological differences between the DRN and the MRN, they are interconnected and probably serve a mutual function (Mamounas et al., 1991). While several families of 5-HT receptors have been identified with many subtypes, the function of only a few of them have been recognized and implicated in psychiatric disorders.

5-HT1A receptors are located pre-synaptically on the cell body and dendrites of raphe neurons, and post-synaptically in the hippocampus, septum, neocortex, hypothalamus and in several nuclei of the amygdala (Graeff, 1997). The 5-HT1A receptor is negatively linked to adenylylcyclase, reduces cyclic adenosine monophosphate (cAMP) formation and therefore inhibits neuron firing when activated by 5-HT (Brandao et al., 1991; Graeff, 1997). Indeed, pre-synaptically, the 5-HT1A receptor decreases the activity of serotonin neurons, resulting in a reduced release of 5-HT at post-synaptic sites. Post-synaptically, activation of the 5-HT1A receptor will inhibit the firing rate of post-synaptic neurons.

The 5-HT1B receptor in mice and rats is the functional equivalent to the 5-HT1D receptor in humans. It is located mainly on 5-HT terminals, where its stimulation inhibits 5-HT release via a decrease in cAMP. While this receptor is abundant in rodents, it is scarce in humans. However, it may still play a role in the pathophysiology of mood disorders as chronic administration of the selective serotonin reuptake inhibitor (SSRI) class of antidepressants have been shown to desensitize the 5-HT1B/1D receptor, which is correlated with their therapeutic response (Blier et al., 1987; Chopin et al., 1994; Mongeau et al., 1997).

The 5-HT2A and 5-HT2C receptors are located on post-synaptic neurons and increase the second messenger inositol phosphate (IP) when stimulated by 5-HT, thereby increasing post-synaptic neuron firing rates (Brandao et al., 1991; Humphrey et al., 1993). Both receptors are located in the neocortex and hypothalamus, however, the 5-HT2A receptor is more abundant in amygdala, cingulated cortex, olfactory tubercle and

claustrum, whereas the 5-HT2C receptor predominates in the hippocampus, substantia nigra and globus pallidus (Graeff, 1997).

Serotonin is involved in many processes including anxiety, arousal, vigilance, aggression, mood, impulsivity as well as regulation of food intake. Evidence suggests that 5-HT may have an inhibitory or facilatory impact on stress responsivity that appears to be dependent on the type of stressor. A variety of acute stressors such as conditioned footshock, tail pinch and restraint stress have been shown to increase 5-HT turnover in the medial PFC, nucleus accumbens, amygdala, lateral hypothalamus and LC (Petty and Sherman, 1983; Adell et al., 1988; Pei et al., 1990; Inoue et al., 1994). However, following chronic electric shock, a learned helplessness behavior developed with a reduction in release of 5-HT in the frontal cortex is observed (Petty et al., 1992).

1.1.4 HPA and 5-HT Interactions

Numerous studies have demonstrated a reciprocal influence between the serotonergic system and the HPA axis. These interactions may be of relevance to the pathophysiology of mood disorders, such as depression, in which alterations in both the 5-HT system and the HPA axis have been evidenced.

Anatomical and functional evidence supports a stimulatory role of 5-HT on HPA activity (Weiner and Ganong, 1978; Tuomisto and Mannisto, 1985; Phelix et al., 1992; Dinan, 1996). For example, central 5-HT lesions and 5-HT synthesis inhibitors have been shown to decrease the amplitude of circadian ACTH and corticosterone (Weiner and Ganong, 1978; Tuomisto and Mannisto, 1985).

Serotonergic nerve fibers ascend from the dorsal and median raphe as well as the medial lemniscus and synapse onto CRF-containing fibers in the PVN (Steinbusch, 1981; Sawchenko et al., 1983; Liposits et al., 1986b; Chaouloff, 1993). Electrical stimulation of either the dorsal or median raphe has been demonstrated to increase 5-HT metabolism in the PVN (Petersen et al., 1989). Further, there are serotonergic neurons located entirely within the PVN (Beaudet and Descarries, 1979). Administration of 5-HT and 5-HT agonists increases hypothalamic CRF release (Calogero et al., 1989), and local infusion of 5-HT into hypothalamic tissues augments CRF release (Holmes et al., 1982). It appears as though the mechanism for CRF induced release by 5-HT in the PVN is via 5-HT2 receptors, as 5-HT and DOI, but not 8-OH-DPAT, elicit a bell-shaped dose response curve (Calogero et al., 1989). However, while some studies have shown an increase in CRF release by 5-HT1A receptor agonists in hypothalamic tissues, this effect seems to occur via 5-HT1A receptors located in the VMH as there are no 5-HT1A receptors located in the PVN (Chaouloff, 1993).

There is also evidence for a direct interaction of 5-HT with pituitary hormones, such as ACTH, due to the presence of serotonergic nerve terminals, and specific 5-HT uptake sites within the pituitary (Johns et al., 1982; Friedman et al., 1983; Mezey et al., 1984). Locally applied 5-HT stimulates ACTH release via 5-HT1A and 5-HT2 receptors (Spinedi and Negro-Vilar, 1983; De Souza, 1986).

Serotonin has also been shown to increase corticosterone secretion at the level of the adrenal gland (Bagdy et al., 1989). This effect appears to occur via the stimulation of local 5-HT1A and 5-HT2 receptors (Bagdy et al., 1989; Fuller, 1990).

The HPA axis has also been shown to regulate serotonin synthesis, metabolism and receptor activity. For example, adrenalectomy decreases both tryptophan hydroxylase activity and 5-HT synthesis, and this effect is reversed with corticosterone replacement (Azmitia, Jr. and McEwen, 1969; Azmitia, Jr. et al., 1970). However, the effect of corticosterone on 5-HT levels is dose dependent, and dual; while a low (1mg/kg) dose increased 5-HT levels in the hypothalamus, a high dose (10 mg/kg) had the opposite effect (Kovacs et al., 1977).

High doses of corticosterone have been shown to decrease 5-HT 1A and 5-HT 1B receptors in the dentate gyrus, an effect thought to be mediated through GR mechanisms (Mendelson and McEwen, 1992). Another study found that while low doses of corticosterone decrease post-synaptic effects of 5-HT1A stimulation in the hippocampus (most probably via stimulation of MR), higher doses more closely relevant to states of stress increase hippocampal 5-HT1A stimulation (through a GR mediated-mechanism) (Joels and de Kloet, 1992). However, the relationship between corticosterone and 5-HT1A receptors is dependent on the type of stressor, as well as individual characteristics such as gender. First, while repeated restraint stress has been shown to increase hippocampal 5-HT1A receptors, cold exposure caused a reduction in 5-HT1A receptor-dependent behavioral responses (Mendelson and McEwen, 1991; Zamfir et al., 1992). Second, gender-dependent effects of chronic restraint stress have been demonstrated on the regulation of 5-HT1A receptors (Kennett et al., 1986; Mendelson and McEwen, 1991).

1.1.5 Gender differences in stress physiology

The gender difference in the prevalence of depression has brought forth many psychological theories to explain this gender gap. However, studies on the onset, chronicity and recurrence of depressive episodes have shown that the gender gap is in fact limited to the time of life beginning at adolescence and ending at menopause (Kessler and Magee, 1993). These data suggest that in addition to and perhaps in concert with the psychological differences between the societal roles of men and women, ovarian steroids may also contribute to the greater vulnerability of women to chronic stress and depression. In fact, it has been shown that there are sex differences in HPA activity and a reciprocal relationship between gonadal steroids and corticosterone and CRH that could underlie the physiological differences in stress responsivity.

In rats, it has been shown that the corticosterone response to stress is greater in females than it is in males. Onset of release is faster, and the rate of rising of corticosterone is more pronounced, both of these events are necessary to elicit the glucocorticoid fast feedback in females (Jones et al., 1972). However, corticosteroid – binding globulin (CBG) which limits the availability of corticosterone to the target tissue, is higher in female rats due to it's upregulation by estrogen, suggesting that the absolute values of cortisol may not be sufficient to evaluate the stress response.

Both estrogen and progesterone can modulate HPA activity independent of their effects on CBG. The data indicate that estrogen increases the HPA axis response to stress. For example, rats treated with chronic estrogen display an elevated corticosterone response to stress, as well as a delay in the recovery from stress in comparison to ovariectomized females (Burgess and Handa, 1992). Viau and Meaney (1991)

investigated the effect of stress on cycling and ovariectomized rats maintained with estrogen, estrogen and progesterone (EP), or vehicle. They found that in cycling rats, the ACTH and corticosterone response to stress was greater during proestrus than estrus or diestrus, however, there were no differences in basal levels between any of the estrus stages. The ovariectomized plus estrogen treated rats displayed a greater ACTH response to stress than either the EP or the vehicle treated animals. Although the corticosterone response was similar in all of the groups, the estrogen and EP treated rats secreted more corticosterone after the termination of the stressor than the vehicle rats. Further, in humans, administration of an estrogen patch in men for 48 hours increases their response to a social stressor (Kirschbaum et al., 1996). One mechanism for this effect may reside on the CRH gene, which holds a partial estrogen response element that may allow an enhanced estrogen-induced CRH expression during stress in females (Vamvakopoulos and Chrousos, 1993). In contrast, there are data to suggest that estrogen may also have some protective effects against stress, as chronic estradiol treatment decreases the downregulation of hippocampal GR following chronic administration of RU 28362, a glucocorticoid agonist in rats (Burgess and Handa, 1992).

Progesterone and dexamethasone have been shown to bind to the same receptor in vitro (Rousseau et al., 1972; Svec, 1988), and progesterone acts as an antagonist of dexamethasone binding. Although progesterone binds at a different site on the GR than glucocorticoids, it can increase the rate of dissociation of glucocorticoids from GR, and thus diminish the effectiveness of glucocorticoid feedback on stress responsiveness (Rousseau et al., 1972; Keller-Wood et al., 1988; Svec, 1988). It has also been shown that female rats have a higher number of GRs in the hippocampus, and this is modulated

by progesterone (Turner and Weaver, 1985; Ahima et al., 1992). In addition to its effects on GR, progesterone has also been shown to bind to MR with an affinity similar to that of dexamethasone (Arriza et al., 1987). Finally, progesterone treatment increases MR binding in female rats (Carey et al., 1995).

1.1.6 Physiology of Major depressive Disorder

Repeated stress increases the co-expression of CRH with argenine vasopressin (AVP) from CRH neurons that terminate in the median eminence (Anisman and Merali, 1997). Argenine vasopressin and CRH work in concert to stimulate ACTH release, contributing to a "sensitized" HPA response by subsequent stressors (Tilders et al., 1993). This primed system may be in part responsible for the recurrence of depressive episodes and chronic depressive states.

The role of stress in the aetiology of depression has been investigated in numerous animal models. Both acute and chronic stress paradigms have been employed, demonstrating that the ensuing behavioral effects vary according to the length, intensity, and nature of the stress. Elevated CRH levels can in themselves induce physiological and behavioral indices that are used to model depressive symptoms in animals. For example, exogenous CRF administration increases anxiety and fear-related behaviors and decreases behaviors considered to have hedonic value such as sexual behavior and food consumption in a food-deprived animal (Nemeroff, 1988b; Liang et al., 1992; Lee and Davis, 1997a; Jezova et al., 1999; Koob, 1999f). This, together with data showing that stress induces NE release in the PVN, BNST and in the CnAmy, (Pacak et al., 1995a; Pacak et al., 1995c; Quirarte et al., 1998) suggests that the feed-forward structure of the

CRF-NE systems may be at least in part responsible for the progressive sensitization of the stress response (Koob, 1999g).

The pre-clinical data supporting a role in the sensitization of the CRH-NE systems mediating stress reactivity is backed by emerging clinical evidence of elevated CRH in patients with major depressive disorders (Nemeroff, 1988a; Bremner et al., 1997; Heim et al., 1997a; Heim and Nemeroff, 1999b; Heim et al., 2000b). It has been speculated that the hyperactive secretion of CRH in these patients may be due in part to high levels of previous stress exposure (Heim et al., 1997a; Heim and Nemeroff, 1999a; Horan et al., 2000b). This hypothesis stems from studies demonstrating that exposure to previous stress as well as adverse early life events increases the risk for the development of affective disorders (Heim and Nemeroff, 1999c; Horan et al., 2000a; Heim et al., 2000b).

1.2 Individual-Related Risk Factors for Depression

1.2.1 Genetics

It has been proposed that an individual's vulnerability to the damaging effects of stress may depend, in part, on their genetic makeup (Caspi et al., 2003). In fact, the risk of depression after a stressful event is elevated in those with a high genetic risk for depression compared to those with a low genetic risk (Kendler et al., 1995). Of interest, although the prevalence of depression in women is approximately double that of men, there is no gender difference in the heritability of depression. Thus, family history as a risk factor in depression has shown little specificity. That is, history of anxiety, alcoholism or other psychiatric disorders have been just as important as family history of mood disorders in predicting depression (Kessler, 2003). Both atypical and melancholic

depression are heritable and involve multiple genes (Kendler et al., 1993; Kendler et al., 2001).

Although a genetic heritability will increase the probability of developing depression over the lifespan, it is not sufficient in itself, but increases the vulnerability to a negative or stressful environment. Examples of gene by environment interactions can be seen in the literature on serotonergic functioning and stress responsivity. First, transgenic mice with a disrupted 5-HT transporter show greater behavioral and physiological responses to stress than those without the transporter alteration (Murphy et al., 2001). Second, rhesus monkeys that display variations in the length of the 5-HT transporter promotor (5-HTTLPR) region that are similar to those seen in humans, exhibit differences in serotonergic functioning as a consequence of both the promotor length and the level of environmental stress (Bennett et al., 2002). Monkeys with the short allele display a decrease in serotonergic functioning when they are reared in stressful conditions, but not in normal conditions. In humans, neuroimaging studies have shown that those with either one or two copies of the short 5-HTTLPR gene show greater activity in the amygdala in response to fearful stimuli compared to those with two long alleles (Hariri et al., 2002). Finally, a recent study by Caspi et al (2003), demonstrated that humans with a short 5-HTTLPR were more likely to develop an episode of major depression as a consequence of stressful life events than those with the long 5-HTTLPR. In the same cohort, childhood maltreatment predicted adult depression only among individuals carrying at least one short allele, but not among long allele homozygotes (Caspi et al., 2003).

Recently, studies using animals that are selected and bred for characteristics that are typically associated with mood disorders, have demonstrated an enhanced vulnerability to the effects of stress in comparison to the outbred controls (Beaulieu et al., 1994; Bornstein et al., 1999; Kabbaj et al., 2000; Overstreet et al., 2003). These studies highlight the importance of genetics in the capacity for adaptation and therefore the vulnerability to chronic stress.

1.2.2 Gender

The higher prevalence of mood disorders among women than men has been well documented and is a cross-cultural phenomenon (Kessler, 2003). However, the course of illness appears to be equivalent in men and women. That is, there no evidence to suggest a gender difference in the speed of episode recovery or the chronicity of their depressive episodes. The occurrence of major depression in women is approximately one and a half to three times that of men (Kessler et al., 1994a; Cyranowski et al., 2000; Kessler, 2003). Recent studies suggest that in contrast to some popular theories, the higher prevalence of mood disorders in women is not the result of a gender difference in self-reported psychological distress (Gove and Geerken, 1977; Kendler and Karkowski-Shuman, 1997).

Prepubescent girls are either equally or less likely to be depressed than boys. However, between the ages of 11 – 14, the gender gap in depression emerges and remains stable for the next 35 to 40 years (Cyranowski et al., 2000; Kessler, 2003). Examination of the factors that contribute to this change in the gender gap at adolescence may shed light on current research theories concerning major depression in adult women. Several theories have been put forward to explain the timing and mechanism of the increase in

depression in female adolescents. First, it has been hypothesized that changes in circulating gonadal hormones during puberty may exert either direct effects, or potentiate secondary effects, on the central nervous system that regulate mood (Susman et al., 1987; Angold et al., 1998). In conjunction with these hormonal changes, female morphological changes during puberty may have a negative effect on some girls, especially when the timing of the changes occurs before that of their peers (Ge et al., 1996; Cyranowski et al., 2000). However, recent studies indicate that the status of the female adolescent at puberty has a greater influence on the development of depression than their age or the timing of their puberty (Angold et al., 1998; Angold et al., 1999).

A second theory is that the gender gap in depression may appear as a result of the "turning on" of the endocrine system as females progress through puberty (Brooks-Gunn and Warren, 1989). However, while estrogen levels were found to account for 4% of the variance in negative affect, the interaction between negative life events and estrogen change accounted for 17% of the variance, indicating a more complicated relationship (Brooks-Gunn and Warren, 1989).

In addition, transitions in social roles, including changes in school environment, as well as changes in parental, peer and romantic/sexual relationships may play a large role in the onset of depression. While the importance of these psychosocial factors in the onset of depression is well documented in women, a recent review suggests that "correlated consequences", in which difficulties in adolescent transitions are experienced in combination with negative life events and a concurrent need for intensified affiliative relationships are more likely to lead to an episode of depression than any of these factors alone (Cyranowski et al., 2000). This theory takes into account the role of a strong

parental bond, which can ease the social transition, and act as a buffer when difficulties in new peer or romantic relationships fail (Armsden and Greenberg, 1987; Blain et al., 1993; Cooper et al., 1998). In addition, there is an increase in the positive correlation between stressful life events and depressogenic effects in girls, and a concurrent decrease in this relationship for boys that can at least partially be explained by the greater need for stronger affiliative relationships in girls (Feingold, 1994). Therefore, the "correlated consequences" theory explains why the majority of adolescent girls do pass through puberty without any occurrence of depression, and why adolescent boys appear to be less vulnerable to the negative effects of stressful life events.

Further evidence for a sex-role theory of depression in women stems from data that the gender difference is stronger for married women than the unmarried (Gove, 1972; Barnett et al., 1987). The main contention of this theory is that married women have higher levels of stress, with lower levels of fulfillment that come from performing more traditional sex-role responsibilities than single women. However, studies showing that the gender difference in the first onset of depression is the same among the married, never married and divorced women challenge this theory (Kessler, 2003). The stronger gender difference among married women than men instead appears to be a result of two other factors. First, depression has different effects on marital stability in women and men. Second, the key environment events that are depressogenic are more often related to family problems in women, whereas financial pressures are more depressogenic for men (McLeod et al., 1992).

It has been proposed that the gender difference in major depression is in part due to a prior existence of anxiety, which is more prevalent in women (Breslau et al., 1995;

Wilhelm et al., 1997). When previous anxiety is controlled for, the odds-ratio of gender predicting depression is significantly decreased. However, a more recent study that controlled for predictors that are more characteristic of men, such as alcohol/drug abuse, antisocial personality disorder and conduct disorder showed an increase in the odds-ratio of gender as a predictor (Kessler, 2000). These results are interesting in that they demonstrate that while a prior existence of a mental disorder does not explain the gender difference in depression, men and women do differ in the type of comorbidity that may lead to the onset of depression.

The human evidence highlights the complexity of the relationship between the stressors and gender on the vulnerability to depression. It is clear that numerous types of stressors impact men and women differently, and that these effects can be modified by individual physiology among many other details. However, the vast majority of animal studies presently use males to investigate the effects of stress on behavioral and physiological symptoms resembling depression in humans. While there is a general consensus on the types of stressors used in these experiments, there are few studies that examine the gender differences in response to these stressors. Given the predominance of women in depressed patients, and the significant contribution of stress to the development of that disorder, gender differences in response to common laboratory stressors would appear to be a relevant area of investigation.

1.2.3 Stress history

Some specificity has been found between the effects of stressful life experiences on the development of depression (Kessler, 2003). That is, exposure to stressors

involving loss tends to be associated with depression, while stressors involving danger are more likely to be associated with anxiety. In fact, it was found that exposure to a stressor that involved loss and was also dangerous results in a mixed anxiety-depression. However, another study that only looked at the development of major depression without taking anxiety into account, found that the number of adulthood traumatic events significantly predicted the maximum ACTH response to a subsequent stress, and the ACTH concentrations were positively correlated to severity of depression symptoms (Heim et al., 2002).

Of interest, a one-time stress in adult laboratory animals induced sensitization of the HPA axis response to a subsequent stress (van Dijken et al., 1993). Re-exposure to a stress after a period of time without any stress experience, causes an increase in the turnover of NE in the cortex, hippocampus, amygdala, hypothalamus and LC (Cassens et al., 1980; Nisenbaum et al., 1991; Tanaka et al., 2000). In the LC and hippocampus, the sensitized NE release is dose-dependently increased with subsequent stressors (Page and Abercrombie, 1997).

1.2.4 Early environment

The primary caregiver is one of the most important influences in early life experiences. In humans, exposure to abuse, trauma or neglect in childhood increases later vulnerability to affective and gastrointestinal disorders in comparison to adults from a nurturing early environment (Kendler et al., 1993; Leserman et al., 1996; Heim et al., 1997d)). There is little specificity to the experience of childhood abuse or trauma, that is,

many different types of adversity in childhood have an equally potent contribution to the development of depression in adulthood (Kessler, 2003).

For example, it has been shown that childhood abuse and negative parental behaviors impact greatly on the development of depression in adults (Bifulco et al., 2002a). Parental behaviors that were analyzed included: neglect, antipathy, role reversal, discipline, supervision, physical abuse, and sexual abuse. The data revealed a "dose response curve" for the effect of negative early environment on adult depression, suggesting that more severe childhood abuse is associated with the greatest incidence of chronic and recurrent adult depression. However, lifetime suicide behavior was related to any level of abuse, ranging from mild to severe. In addition, a study that examined the contribution of both exposure to childhood abuse as well as adult stressful experiences to the development of depression in adult women found that first, both childhood abuse and exposure to stressful events in adulthood were each positively correlated to the development of depression on their own (Heim et al., 2002). However, the interaction of childhood abuse and adulthood trauma was the best predictor of the stress response as measured by ACTH release. Thus, it seems as though women with an early childhood history of trauma were more vulnerable to the negative effects of adulthood trauma and displayed a sensitized HPA axis response to an acute stress. This study did not assess any of the variables in men; therefore, it is unknown whether the same interaction would hold true for males with the same experiences.

In addition, Krause et al (2003) examined the effect of emotional invalidation in childhood on adult psychological distress. Emotional invalidation by parents that consisted of minimization, distress in response to negative emotion and psychological

abuse, was associated with chronic emotional inhibition in adulthood. Emotional inhibition in adulthood is associated with thought suppression, ambivalence over emotional expression and avoidant stress responses. While childhood emotional inhibition may be functional in the short term in that it reduces parental distress, it leads to chronic psychological distress, including depression and symptoms of anxiety in adulthood (Krause et al., 2003).

Finally, the effect of the early environment has been shown to affect other mood disorders as well; children with previous records of learning, emotional or behavioral difficulties were more likely to develop post-traumatic stress disorder (PTSD) after exposure to a major trauma, than those without these hindrances (Yule et al., 2000). In addition, children with a strong system of social support appeared to be protected, while those with little or no social support were as likely to develop PTSD as those who came from a violent home. This example is important in that, first, it underlines the importance of the early environment as a possible defence against the impact of stressors on mental health, and second, it demonstrates that a wide range of conditions may impact negatively on one's health.

In animals, the relationship between early environmental experiences and health status in adulthood was first demonstrated in rats by Levine, Denenberg and Ader (Levine, 1957; Denenberg, 1964; Ader and Grota, 1969). Neonatal handling, separation of the pups from the dams for a brief time, was shown to reduce the expression of novelty-induced fearfulness and adrenal responsiveness to stressors, while maternal separation, a longer period of separation, had the opposite effect on the stress response (Levine et al., 1991; Pihoker et al., 1993; Plotsky and Meaney, 1993; Vazquez et al.,

1996; Ladd et al., 1996). Of interest, this change in stress responsivity is not limited to rats, but is rather a widespread phenomenon in response to the withdrawal of maternal care (Hennessy, 1997). A great deal of evidence indicates that distinct aspects of maternal care regulate the neuroendocrine and behavioral responsiveness to stress in offspring. When these maternal behaviors are missing or altered during critical periods of development, there are negative long-term consequences in the health of the offspring (Evoniuk et al., 1979; Schanberg et al., 1984; Jans and Woodside, 1990; Suchecki et al., 1993; Liu et al., 1997h).

In order to investigate the effects of maternal care on neuroendocrine and behavioral responsiveness to stress, Plotsky and Meaney (1993) designed a neonatal maternal separation protocol which mimicked the temporal patterns of separation observed in the wild. Rodent dams normally leave their pups for periods of approximately 15-30 min to forage for food, however, low ranking females may have to leave for longer periods of time due to the greater distance of their nests from the sources of food (Leon et al., 1978; Jans and Woodside, 1990). The typical "nursing bout" is initiated when the mother approaches her nest, licks and grooms the pups and then begins to nurse. Toward the end of the nursing bout, the dam licks and grooms her pups again before she leaves her nest. In the neonatal handling model, each litter was exposed to one of three rearing conditions from postnatal day 2 (PND2) to 14: a) handled rats, which were separated from their mothers for 15 min daily (HMS15), b) maternally separated rats, which were separated from their mothers for 180 min daily (HMS180), and c) non-handled, which were not handled for any purpose, including cage changing, from PND3-14.

A brief period of separation (HMS15) from the nest resulted in an increase in maternal care upon reunion with the offspring (Leon et al., 1978; Jans and Woodside, 1990; Huot et al., 2001). Dams retrieved their pups faster and initiated more nursing bouts, thereby increasing the total time spent licking and grooming, but not the total time feeding. In contrast, the longer period of separation (HMS180) resulted in reduced and disturbed maternal behavior (Huot et al., 1997).

The offspring of HMS 15 and HMS 180 dams were later compared for behavioral, central nervous system and neuroendocrine responses to acute stress exposure as adults. The HMS180 rats exhibited an impaired dexamethasone suppression of HPA axis activity in comparison to HMS15 animals (Ladd et al., 2000). This finding was supported by data showing that the HMS180 rats displayed a down-regulation of hippocampal GR accompanied by an increase in MR in comparison to HMS15 rats (Ladd et al., 1997). The changes in GR density were specific to the hippocampus, with no apparent changes in the septum, amygdala, hypothalamus or pituitary. The decrease in hippocampal GR density would presumably diminish the responsiveness of negative-feedback over the HPA axis, thereby increasing CRF and AVP synthesis and stress-induced release from the hypothalamus. In accord with this hypothesis, basal levels of CRF mRNA in the PVN, central nucleus of the amygdala and bed nucleus of the stria terminalis were elevated in the HMS180 animals as compared to HMS15 rats (Plotsky and Meaney, 1993). CRF peptide content was also found to be increased in the terminal fields of these neurons in the HMS180 rats.

HMS180 animals display exaggerated levels of anxiety and fearfulness when exposed to mild stressors, such as a novel environment, when tested in adulthood (Caldji

et al., 2000; Liu et al., 2000a). Corresponding to the behavioral measures, HMS180 rats also displayed an enhanced and prolonged corticosterone release, with no differences in the rate of metabolism of corticosterone or in the levels of corticosterone binding globulin (Rosenfeld et al., 1992). In addition, the stress caused an augmentation of corticotropin-releasing hormone (CRF) gene expression in the HMS180 rats (Caldji et al., 2000). In contrast, rat pups in the HMS15 groups displayed less fearfulness in a novel environment, and decreased corticosterone levels in response to a stressor (Caldji et al., 2000; Liu et al., 2000a).

Given the importance of norepinephrine (NE) in stress-induced release of CRF and the stimulation of the HPA axis, NE release was measured in the PVN during and following a 60-minute restraint stressor in HMS15 and HMS180 rats (Liu et al., 2000a). While stress induced an increase in NE release in all groups regardless of their rearing environment, the response in the HMS180 rats was significantly greater than the other animals. In addition, the pre-restraint levels of NE were also higher in the HMS180 rats. These results suggest that NE afferents to the hypothalamus are probably contributing the differences in HPA axis activation during stress. Furthermore, HMS180 rats displayed a decrease in alpha-2 receptor binding in the LC and NTS compared to HMS15 animals, highlighting another mechanism for the environmentally-induced differences in stress responsivity.

Anhedonia is a symptom of major depression and is characterized by the inability to feel pleasure. In rats, anhedonia is often assessed by measuring the quantity of sucrose or saccharine solution consumption when given a choice between one of the sweet solutions or regular water. Willner and colleagues (Willner et al., 1987; Muscat and Willner, 1992)

have shown that chronic mild stress can decrease the amount of sucrose solution ingested by rats when offered within the stress period, and that this effect is reversible by antidepressant treatment. When HMS180 and HMS15 rats were given a choice between sucrose solution and regular water, the HMS180 animals drank approximately 35% less sucrose compared to the HMS15 animals (Ladd et al., 2000).

The effects of maternal separation and handling on the stress response are thought to be mediated not by the experimenter-pup interactions, but instead via a change in the mother's behavior toward her pups. That is, the increase in tactile stimulation via the extra licking and grooming in the HMS15 pups is thought to mediate enduring changes in the neuroendocrine system that decrease the stress response in adulthood. Likewise, the disturbed behavior of the HMS180 dams is characterized by a decrease in licking and grooming and a subsequent increase in adult stress reactivity. In support of this hypothesis, cross-fostering of the HMS180 pups to HMS15 dams and vice versa was able to reverse the outcome of the rearing condition.

To investigate the effects of licking and grooming more directly, Meaney and associates (Liu et al., 1997g; Caldji et al., 1998) characterised pups from mothers who displayed naturally high or low levels of licking and grooming (LG) and arched back nursing (ABN). It was found that the behavioral and physiological measures of stress reactivity of the offspring of naturally high LG-ABN mothers differed remarkably from the offspring of naturally low LG-ABN mothers. For example, adult offspring of high LG-ABN mothers showed reduced fearfulness in response to a mild stress, dampened ACTH and corticosterone responses to an acute stress and enhanced glucocorticoid feedback sensitivity compared to offspring of low LG-ABN mothers (Liu et al., 1997f;

Caldji et al., 1998). Further, differences between the offspring of high and low LG-ABN mothers mirrored the differences between handled and non-handled animals respectively (Liu et al., 1997e; Caldji et al., 1998). The results of the maternal care studies support the hypothesis of maternally mediated effects of postnatal handling and suggest that distinct aspects of maternal behavior are critical in mediating the development of endocrine and neural systems mediating the stress response. In addition, the physiological and behavioral consequences of low maternal care in the adult offspring mirror those in depressed patients, suggesting that the maternal care model may be suitable to study individual differences in the susceptibility to the effects of chronic stress.

The behavioral and physiological effects of handling and maternal separation are compared to those found in the offspring of high and low LG dams in Table 1.

Measure	Handling	Maternal LG-ABN
ACTH response to acute stress	H < NH < MS	High < Low
CORT response to acute stress	H < NH < MS	High < Low
Hippocampal GC receptor mRNA expression	H > NH > MS	High > Low
PVNh CRF mRNA expression	H < NH < MS	High < Low
GC negative-feedback sensitivity	H > NH > MS	High > Low
Open-field exploration	H > NH =MS	High > Low
Novelty-suppression of feeding	H > NH > MS	High > Low
CBZ receptor		
Central nucleus of the Amygdala	H > NH = MS	High > Low
Lateral nucleus of the Amygdala	H > NH = MS	High > Low
Locus ceruleus	H > NH = MS	High > Low
Nucleus tractus solitarius	H > NH = MS	High > Low
Hippocampus	H = NH = MS	High = Low
Frontal cortex	H = NH = MS	High = Low
Medial prefrontal cortex	H = NH = MS	High = Low
CRF receptor		
Locus Ceruleus	H < NH = MS	High < Low
pPVN	H < NH = MS	?
Alpha2 adrenoceptor		
Locus ceruleus	H > NH = MS	High > Low
Nucleus tractus solitarius	H > NH = MS	High > Low
PVNh	H = NH = MS	High = Low

Table 1. Behavioral and physiological measures induced by variations in the early environment in the rat. Modified and used with permission from Caldji et al. (1998).

1.3 Stressor-Related Factors determining Neurochemical and Behavioral Responses to the Stressor

Exposure to acute stressors or environmental insults will induce neurochemical changes that are associated with adaptation and coping. However, certain characteristics of the stressor may cause detrimental changes in neurotransmitter functioning that impede coping, and lead to depressive symptoms (Weiss et al., 1976; Anisman et al., 1980; Anisman et al., 1984; Tsuda and Tanaka, 1985). Behavioral disturbances may result from changes in neurochemical functioning and the inability to cope with the stressor at hand.

A few of the stressor characteristics that are known to increase the demands on neurotransmitter systems are listed below:

Predictability:

One condition that may enhance or reduce neurochemical adaptation is the predictability of a stressor. It has been show that when a stressor is administered at unpredictable times, the adaptation process is slower than it is for a predictable schedule of stressors (Anisman and Zacharko, 1986). Likewise, a stressor regimen that involves different types of stressors will also slow adaptation in comparison to the repeated presentation of the same stress (Foa et al., 1992). The predictability of the stressor can be enhanced by specific cues or environmental contexts in which the stressor always occurs. For example, a stressor, such as a mild electric shock, can be paired with a conditioned stimulus (such as a tone or a light) to always predict the shock, or it can be unpaired to any obvious cue, and appear to occur randomly. In humans, it has been shown that subjects receiving a paired (conditioned) shock, display an enhanced startle to the

conditioned stimulus (CS) (tone/light) (Ameli et al., 2001). While subjects that received the unpaired, or random shocks did not display an enhanced startle to the CS itself, they displayed a heightened startle to the environment, and reported greater anxiety and arousal than the subjects receiving the conditioned, more predictable shock. Inescapable footshock induces a marked increase in NE release in the hypothalamus (Yokoo et al., 1990). Re-exposure to the shock environment, without presentation of a shock produces the same increase in hypothalamic NE release, suggesting that NE neurons are activated simply by exposure to a conditioned environment (Yokoo et al., 1990).

The stress of an unpredictable life-style can have long-term negative consequences for organisms experiencing the stress, as well as their offspring. Non-human primates were reared by mothers foraging under predictable or unpredictable conditions (Coplan et al., 1996). The infant monkeys that were raised by mothers foraging under unpredictable conditions displayed persistently elevated levels of cerebrospinal fluid (CSF) CRF in comparison to the monkeys raised by mothers in predictable rearing conditions. This study is especially interesting in that it demonstrates the interaction between the type of stressor and early environmental effects on the neurobiological mechanisms related to anxiety and mood disorders.

Controllability:

A well-used paradigm to study the effects of uncontrollable versus controllable stress is the shuttle escape task. Animals are first exposed to either an escapable or an inescapable (yoked) shock. Within this paradigm, two animals in adjacent chambers receive exactly the same amount of shock. However, the shock administered to one animal is completely dependent on the behavior of the other. That is, one has the ability

to escape the shock. If escape is successful, neither animal is shocked, if the animal fails to escape, both animals are shocked. Thus, while both animals receive equal shock exposure, only one has control over the stressor. Those animals previously exposed to the inescapable shock display an impaired escape performance in a subsequent test. That is, they display a passive behavior, failing to make an attempt to escape in the face of a new stressor. Therefore, although both sets of animals were exposed to the same stress, only those with no control over the stress developed the "depressive" behavior (Maier and Seligman, 1976)

Tanaka et al (Tanaka, 1999), investigated the effect of controllability on footshock-induced NE release. It was found that both controllable and uncontrollable footshock increased NE release, suggesting that it was the physical aspect of the stressor and not the psychological aspect that induced NE activity. However, when the stressors were continued for 5 consecutive days, increases in NE seen in the controllable group declined, whereas stress-induced NE release increased in the uncontrollable group increased. Further, if rats are allowed to display aggression, by biting a wooden stick, the formation of gastric ulcers as well as NE release in the amygdala were both attenuated (Tanaka et al., 1998).

Duration:

Weiss et al (1976) found that mice that were exposed to repeated inescapable shocks did not display the typical deficit in shuttle escape performance. Similarly, rats will bar press for electrical brain stimulation of the Nucleus Accumbens, and this is attenuated by previous exposure to inescapable shock (Zacharko et al., 1983). However, the decrease in responding is not seen when the rats are exposed to repeated inescapable

shocks as apposed to a single shock session. These two findings point towards a process of adaptation, rather than learned helplessness. It has been suggested that when exposure to a stressor is prolonged, an additional series of adaptive changes can occur to facilitate coping. For example, it is known that the stress-induced reduction in transmitter levels will cause the sensitivity of the β-NE receptor to be reduced and the synthesis of NE to increase (Kvetnansky et al., 1977; Roth et al., 1982; Irwin et al., 1986). This increase in synthesis can endure past the termination of the stress, causing amine levels to exceed those of control animals (Roth et al., 1982; Anisman and Zacharko, 1986; Irwin et al., 1986). In fact, both behavioral depression in the forced swim test and anorexia was reduced in conjunction with the development of the receptor subsensitivity (Platt and Stone, 1982; Stone and Platt, 1982).

These findings are paradoxical given the preponderance of evidence showing that chronic exposure to stress is damaging to one's health. There are several explanations for the above findings: first, as noted previously, factors such as intensity and predictability can modulate the adaptation to stress. Therefore it is possible that given a different set of shock variables, the rate of adaptation would not have been sufficient to deal with the stress. Second, these studies were conducted on standard laboratory animals. It is well known that the majority of individuals exposed to stress are able to cope successfully and do not develop a stress-related pathology. This suggests that the example above may be more suitable as model of successful adaptation to chronic stress.

However, exposure to prolonged stress does not always result in adaptation. In fact, it has been demonstrated that chronic stress can cause an enhanced reactivity to a subsequent stress. For example, while an acute stress, such as tail pressure, increases NE

efflux in the hippocampus as measured by microdialysis, rats that have previously been exposed to chronic cold (3 weeks) display an enhanced NE release in response to the same acute stressor than naïve rats (Nisenbaum et al., 1991). This suggests that prior exposure to chronic stress can sensitize the NE response to a subsequent acute stress. Finlay et al (1995) also found that acute tail pressure increased dopamine and norepinephrine in the medial frontal cortex of rats. Administration of the benzodiazepine, diazepam decreased basal levels of both dopamine and norepinephrine, but only attenuated the stress-induced increase in dopamine (DA) levels, without affecting stress-induced NE levels (Finlay et al., 1995). Therefore, the ratio of DA to NE in the medial PFC was altered in response to an acute stress after the administration of an anxiolytic. Of interest, in rats that had previously been exposed to chronic cold stress, diazepam no longer decreased the basal DA or NE levels, nor did it affect the stress-induced increase in DA or NE. Further, the chronically cold stressed rats, displayed an enhanced increase in NE in response to an acute stress compared to that seen in the naïve rats, supporting the previous finding of a sensitized NE release following chronic stress. The results of this experiment suggest that the stress-induced sensitization of NE may be mediated in part by a decrease in sensitivity to the inhibitory effects of GABA-ergic input to noradrenergic neurons. Therefore, while chronic stress increases the catecholaminergic response to a subsequent acute stress, it decreases the sensitivity to anxiolytic compounds (Finlay et al., 1995). These results may explain why antidepressants are able to decrease symptoms of anxiety as well as depression; however, anxiolytics are effective only for symptoms of anxiety.

Chronic cold exposure also increases the alpha-2 modulation of NE release and synthesis compared to control rats (Nisenbaum and Abercrombie, 1993). Chronic cold exposure increases locally applied k+ -induced release of NE from hippocampal nerve terminals (Nisenbaum and Abercrombie, 1993). However, these results were not replicated in the medial PFC (Finlay et al., 1997), and therefore it is possible that stress-induced changes in catecholaminergic functioning are mediated by different mechanisms in a region-dependent manner.

This effect of chronic cold exposure on stress-induced NE release is also dependent on the length of the stress protocol. Exposure to cold for one week was unable to evoke the enhanced NE release in the mPFC seen with 3 weeks of cold exposure (Finlay et al., 1997).

Intensity:

Additional studies using the shuttle escape task showed that the effects of uncontrollable stress could be further modified by varying the intensity and the duration of the inescapable shock (Glazer and Weiss, 1976; Anisman et al., 1978). When an inescapable shock of high intensity and short duration was used, the deficit in shuttle performance was short-lived. However, a long-term deficit in escape performance could be induced with an initial inescapable shock of moderate intensity and long duration. This example serves to illustrate that many parameters of a stressor can interact to bring about disparate degrees of adaptation and coping.

While the effects of chronic cold stress on NE release in the hippocampus and mPFC have been well documented (see *"duration"* above), changes in the stress protocol can attenuate the stress-induced enhancement of NE in these regions. For example,

exposure to footshock, either on a continuous or an intermittent schedule did not elicit an enhanced NE response to an acute tail shock (Jedema et al., 1999). Further, changing the chronic cold exposure from a continuous to an intermittent schedule attenuated the stress-induced enhancement of NE release, even though the total amount of cold exposure was the same (Jedema et al., 1999).

1.4 Chronic Stress

While a temporary change in metabolism and immune function by activation of the HPA axis is fundamental for the adaptation to a stressor and to the restoration of homeostasis, prolonged activation of the stress system has deleterious outcomes. A chronic surplus of cortisol can induce excessive fear, insulin resistance with visceral fat deposits as well as osteoporosis, inhibition of T helper-1 cellular immunity, and chronic suppression of mesolimbic dopaminergic reward system (Chrousos and Gold, 1992; Gold and Chrousos, 2002).

As noted previously, exposure to an acute uncontrollable stress leads to an increase in the turnover of biogenic amines (Anisman and Merali, 1997). With continued stress exposure, available stores of amines may not be sufficient to respond to a subsequent stress, which can result in a compensatory increase in the rate of amine synthesis (Anisman and Zacharko, 1982). In addition to the increase in amine synthesis, changes in receptor sensitivity also contribute to behavioral adaptation. For example, following a chronic stressor regimen, β-NE density is reduced in rat cortex, and the sensitivity of cAMP response to catecholamines is diminished (Anisman and Zacharko, 1990). The change in β-NE receptor is transient, as it is absent 24 hrs following the termination of the stress, however the cAMP response is still present 24 hrs later.

These changes in neuroendocrine and catecholamine activity associated with chronic stress may eventually give rise to depressive like symptoms, including alterations in sleep patterns, locomotor activity, lack of affect (anhedonia), and cognitive impairments (Anisman and Merali, 1997). In fact, it has been suggested that prolonged activation of the stress response is a principal feature of both unipolar and bipolar depression.

1.4.1 Effects of chronic stress on the HPA axis

Melancholic depression is associated with stressful life experiences and prolonged hypercortisolemia. Depressed patients also have elevated CSF CRH and an attenuated level of dexamethasone suppression, despite their elevated levels of cortisol (Nemeroff et al., 1984; Gold and Chrousos, 2002), indicating that the CRH system is not being regulated by the high levels of circulating cortisol.

To explain this effect, Makino et al (2002) investigated the effects of acute or chronic immobilization stress in sham vs. adrenalectomized, corticosterone replaced (ADX + CORT) rats. Following an acute stress, both CRH mRNA in the PVN and tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis mRNA in the LC were increased, however, the magnitude of the increase was greater in the sham animals, suggesting an intact negative feedback inhibition on these measures (Makino et al., 2002). While both mRNAs were also elevated after the repeated stress, the extent of the increase was similar in sham and ADX + CORT animals, indicating an attenuation of glucocorticoid feedback during chronic stress. Moreover, there was a reduction in GR mRNA in the hippocampus, the PVN and the LC following chronic stress only in the sham animals. These results suggest that the downregulation of GR mRNA by repeated stress (or high levels of glucocorticoids), is related to a diminished

capacity of cortisol to inhibit the hypothalamic secretaguogues that activate the pituitary during chronic stress. In support of these findings, many studies have found a downregulation of hippocampal GR by stress (Sapolsky et al., 1984; Jacobson and Sapolsky, 1991; Chao et al., 1993; Brooke et al., 1994) and an increase in CRH and AVP secretion in the PVN following lesions of the hippocampus (Sapolsky et al., 1989; Herman et al., 1989; Jacobson and Sapolsky, 1991). These data would explain the increased sensitization to stress that is seen in affective disorders.

There have been several findings however, that have called this model of HPA sensitization to chronic stress into question. First, not all chronic stressors lead to a downregulation of hippocampal or hypothalamic GR mRNA (Herman, 1993; Clark et al., 1994). It has been shown that starvation failed to decrease GR mRNA in these regions despite a significant increase in circulating corticosterone (Makino et al., 2001). This suggests that hippocampal GR is most probably regulated not only by circulating corticosterone, but also possibly by stress-mediated neurotransmitters. For example, it has been shown that catecholamines (through the β-NE receptor), N-methyl-D-aspartate (NMDA) or GABA-A receptors are able to regulate hippocampal GR and GR mRNA (Tritos et al., 1999). Therefore, the type and or severity of the stressor may specify whether there will be any long term consequences on HPA feedback via stress-induced changes in GR as well as the mechanism of GR regulation.

Second, there is now evidence indicating that hippocampal MR activation relays GABA-ergic inhibitory tone to the PVN (Joels and de Kloet, 1992; Herman and Cullinan, 1997; de Kloet et al., 1998b). This would suggest that glucocorticoids acting at hippocampal MR maintain an inhibitory tone on HPA activity. Glucocorticoids acting at

hippocampal GR suppress the inhibitory hippocampal relay, resulting in disinhibition of the HPA axis (Joels and de Kloet, 1992; de Kloet et al., 1998a). In support of this finding, it has been shown that while an intracerebroventricular (ICV) injection of a GR antagonist increased plasma ACTH and CORT levels, infusion directly into the hippocampus had an opposite effect, suggesting that hippocampal GR exerts a positive glucocorticoid feedback on the HPA axis (van Haarst et al., 1997). It is possible that the ICV glucocorticoid mediated inhibition of HPA activity is mediated instead by GR in the PVN. It has previously been demonstrated that administration of glucocorticoids into the PVN inhibits the biosynthesis of CRH (Kovacs et al., 1986; Sawchenko, 1988), it is therefore possible that the feedback inhibition through the PVN may override the positive feedback of GR in the hippocampus. If this were true, then the decrease in GR mRNA in the PVN rather than the hippocampus would be the critical factor mediating the attenuation of negative feedback of HPA activity after chronic stress.

Third, hippocampal MR not only plays a role on the basal inhibitory tone of HPA activity, but also has an inhibitory function during stress (Reul et al., 1997). It has also been shown that stressful events can increase hippocampal MR, and that this increase is correlated to the inhibition of HPA activity following stress (Gesing et al., 2001). Finally, chronic antidepressant treatment increases hippocampal MR (as well as GR) density, which is related to the inhibition of CRH in the PVN and the HPA axis activity (Brady et al., 1991; Seckl and Fink, 1992; Reul et al., 1993).

1.4.2 Effect of chronic stress on the 5-HT system

Chronic stress is associated with changes in serotonergic functioning that can either promote adaptation and coping, or conversely, underlie some of the behavioral

symptoms of mood disorders. The sensitivity of raphe 5-HT1A autoreceptors that control nerve firing activity has been shown to be decreased after chronic but not acute stress exposure (Laaris et al., 1997). This is supported by the delay in changes to 5-HT1A autoreceptor sensitivity after administration of corticosterone (Laaris et al., 1995).

A study examining the impact of escapable versus inescapable stress on 5-HT receptors found that first, 5-HT2A receptor density was decreased in the hypothalamus in rats subjected to inescapable stress, but not in those exposed to escapable stress (Wu et al., 1999). Second, 5HT2A receptor density was decreased in the hippocampus and amygdala in all rats exposed to stress, regardless of whether it was escapable or not. Third, serotonin transporter density was decreased in the mPFC in helpless rats compared to controls, but not in comparison to non-helpless rats. And finally, in this study, there were no changes in 5-HT1A receptors under any conditions. This last result is in contrast to data showing that chronic restraint stress does decrease 5-HT1A binding in the hippocampus (Mendelson and McEwen, 1991). To complicate the 5-HT-stress story further, it has been shown that social stress can increase 5-HT1A receptors in the hippocampus and dentate gyrus, and decrease 5-HT2 binding in the parietal cortex (McKittrick et al., 1995). In fact, chronic stress-induced changes in 5-HT1A receptor mRNA expression and binding are dependent on the severity and/or the predictability of the stressor (McKittrick et al., 1995; Lopez et al., 1998).

Serotonin mediates behaviors that are used in animal models of anxiety and depression. Learned helplessness type behaviors can be induced by the administration of serotonin antagonists (Sherman and Petty, 1980). Chronic electric shock that produced learned helplessness behavioral deficits were associated with reduced in-vivo release of

serotonin in the frontal cortex (Petty et al., 1992). Antidepressants that enhance 5-HT transmission, such as the selective serotonin reuptake inhibitors (SSRIs), can reverse learned helplessness (Martin et al., 1990). Further, animals that are given antidepressants before stress exposure do not exhibit the stress-induced decrease in 5-HT or the behavioral deficits (Petty et al., 1992).

1.5 Chronic Antidepressant Treatment

Given the paramount role of stress in the development of affective disorders, antidepressants may exert their clinical efficacy in part, by reducing the negative impact of stress. It has been shown that patients report reductions in the perceived intensity of stress, and increases in the use of more adaptive coping techniques upon recovering from depression (Veiel et al., 1992; Overholser, 1996; Ravindran et al., 1997).

The first antidepressants, known as the classical antidepressants were discovered over 40 years ago, quite by accident. Long after they were introduced, it was discovered that they worked by either inhibiting the enzyme monoamine oxidase (MAO) or by blocking the reuptake of monoamines (5-HT and NE) (Stahl, 1998). This discovery lead to the monoamine hypothesis of depression, which states that depression is due to a deficiency in one or more of three monoamines, being serotonin, norepinephrine and or dopamine. The theory stems from the knowledge that all antidepressants increase levels of these three monoamines. The classical antidepressants dominated the market from the 1950s, when they were introduced, until the 1980s, upon the introduction of serotonin selective reuptake inhibitors (SSRIs).

All of the antidepressants have a delayed therapeutic action, as well as a delay in development of tolerance to side effects, which coincide with the changes in the

sensitivity of function of various neurotransmitter receptors (Hyman and Nestler, 1996). Thus, the monoamine receptor hypothesis has evolved and postulates that changes in receptors result in an increased monoamine neurotransmission. Further, it is this increase in transmission that is thought to underlie the therapeutic effects of antidepressants (Hyman and Nestler, 1996; Stahl, 1998).

Each class of antidepressant has a different breadth of therapeutic potential as well as a variety of side effects that are dependent on their specific pharmacological properties. Given that the SSRIs are the most widely prescribed antidepressants today, this thesis will focus on the mechanism of action of this class of drugs only.

The immediate action of SSRIs upon administration, is to block the serotonin reuptake pump. This causes an increase in serotonin, mainly at the somatodendritic area of the neuron (Stahl, 1998), but not at the axon terminal. However, chronic administration of SSRIs will cause a desensitization of the somatodendritic 5-HT-1A autoreceptors. Since the role of the 5-HT1A autoreceptor is to inhibit neuron firing, the desensitization would lead to an increase in neuronal impulse flow, and more serotonin will be released from the axon terminal (Bel and Artigas, 1993; Blier and de Montigny, 1994). Following the increase in serotonin release at the axon terminals, postsynaptic serotonin receptors may also be desensitized (Charney et al., 1981; Stahl and Hauger, 1994). The delay in therapeutic actions and tolerance to the side effects of SSRIs may be due to the time it takes for these receptors to be downregulated (Blier et al., 1987; de Montigny et al., 1992).

There are many 5-HT pathways in the brain, and each is known to mediate different CNS functions (Cooper et al., 1991). Since SSRIs have a proven clinical efficacy not

only in depression, but also for panic disorder, obsessive-compulsive disorder (OCD), social phobia, post traumatic stress disorder, migraine, dysthymia, premenstrual dysphoric disorder as well as bulimia, it is possible that therapeutic actions are due to disinhibition of 5-HT transmission of different pathways (Stahl, 1998). For example, disinhibition of the pathway from the raphe nucleus to the prefrontal cortex may mediate the antidepressant effects of SSRIs (Mann et al., 1996; Stahl, 1998), while disinhibition of the pathway to the basal ganglia may improve symptoms of OCD (Baxter, Jr., 1992; el Mansari et al., 1995). This theory is supported by data showing that the initial and maintenance dosages as well as the severity of side effects of the SSRIs are different depending on the disorder being treated.

1.5.1 Effect of antidepressants on coping

The impact of aversive events is largely dependent on the individual's perceived ability to cope with stressful life events (Parker et al., 1986; Alloy and Clements, 1992). Many individual and stressor-related factors (listed above) contribute to the perceived coping capacity and style. It has been shown that depressed patients use more emotion-focused rather than problem-focused coping styles, leading to less adaptive responding to stress (McNaughton et al., 1992; Roy-Byrne et al., 1992).

Ravindran et al (1995) investigated the effects of antidepressants on adaptive behavior, depressive symptoms and coping strategies in depressed and dysthymic patients. Both major depression and dysthymia were associated with increased reports of minor stressors, feelings of loneliness, reduced uplifts and the use of inappropriate coping styles in comparison to non-depressed controls (Ravindran et al., 1995a). Eight weeks of

SSRI treatment resulted in a decrease in reports of minor stressors, although the number of reports still exceeded that of non-depressed controls, and no change in the number of uplifts. However, patients that recovered from depression changed their coping strategy from emotion to problem-focused styles. It is still unclear whether the change seen in coping strategy was due to a change in appraisal of daily stressors leading to an improvement in mood, or conversely, a decrease in depressive symptoms, leading to a change in coping strategy. Regardless, it is of interest that antidepressant medication may improve depressive symptomatology in part by changing the individual's ability to cope with stress.

1.5.2 Effect of antidepressants on HPA functioning

Elevated levels of CRF in the majority of depressed patients suggests that the hyperactivity of the HPA axis may contribute to the psychopathology of affective disorders (Nemeroff and Evans, 1984; Gold et al., 1988). Chronic antidepressant treatment and electroconvulsive therapy have been shown to decrease CRF concentrations and normalize both monoaminergic and HPA activity only in those patients that responded to treatment (Valentino, 1989; Valentino et al., 1990; Valentino and Curtis, 1991; Curtis and Valentino, 1991; Nemeroff et al., 1991; Artigas et al., 1996; Heuser et al., 1998). One mechanism for the normalization of the HPA system appears to be from the restoration of glucocorticoid-mediated inhibitory feedback as assessed by dexamethasone-CRH administration (Holsboer, 2000; Pariante and Miller, 2001; Zobel et al., 2001). Animal studies have shown that the number and functional capacity of GRs are increased following chronic antidepressant administration (Holsboer, 2000; Pariante

and Miller, 2001). In fact, transgenic mice that are deficient in CNS GR show an upregulation of GR and improved dexamethasone suppression test (DST) suppression as well as an amelioration of species typical behavioral deficits that are similar to those seen in human depressed patients (Holsboer, 2000).

The mechanism by which antidepressants increase GR expression is thought to occur via an increase in gene transcription of this receptor. It has been hypothesized that gene transcription for GR may be increased by two pathways that may be acting independently or concurrently. First, it has been shown in vitro that antidepressants are able to inhibit a membrane steroid transporter and thus increase intracellular concentrations of 3H-cortisol or dexamethasone in the presence of the drug (Pariante et al., 2001; Pariante et al., 2003). This effect has been shown for different classes of antidepressants, including tricyclic antidepressants (TCAs) and SSRIs (Pariante et al., 2001; Pariante et al., 2003). In fact, desipramine has been shown to cause translocation of GR to the nucleus even in the absence of hormone (Pariante et al., 1997). The second mechanism of facilitated GR function may be due in part to the activation of second messenger pathways that are involved in GR regulation such as, cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) (Chen and Rasenick, 1995; Thome et al., 2000). Transgenic mice with a cAMP response element (CRE)-LacZ reporter gene construct were administered acute or chronic TCAs, SSRIs or monoamine oxidase inhibitors (MAOIs) (Thome et al., 2000). Chronic but not acute antidepressant treatment significantly increased CRE-mediated gene transcription, as well as the phosphorylation of CRE binding protein (CREB). Further, activation of both cAMP and PKA have been shown to increase GR functioning (Rangarajan et al., 1992; Pariante and Miller, 2001).

Once GR functioning has been increased chronically and feedback inhibition is facilitated, GR upregulation by gene transcription may follow.

Support for this hypothesis stems from evidence that increased HPA axis negative feedback in laboratory animals and normalization of HPA axis activity in depressed patients is observed after 5-7 days of antidepressant treatment (Reul et al., 1993; Heuser et al., 1996; Deuschle et al., 1997). Therefore, the time-course of the therapeutic effects of antidepressants may correspond more closely to the upregulation of GR as well as the normalization of HPA activity.

1.5.3 Gender differences in antidepressant efficacy

A survey by Kinney et al (1981) reviewed the top pharmacology journals published in the previous year and found that only 12% of phase 1 and 21% of phase 2 clinical trials included women. Although the number of women included in studies increased in the following years, the U.S. Food and Drug Administration (FDA) found that only 50% of applications for new antidepressant compounds between 1988 and 1991 had actually analyzed the data by gender (Kinney et al., 1981). Consequently, the FDA announced new guidelines for applicants of new drugs, and congress mandated that the National Institutes of Health (NIH) follow these guidelines for the government-sponsored studies. While new information may be emerging regarding gender differences in the pharmacokinetics and pharmacodynamics of antidepressant treatments, the lack of basic animal studies using females has left a void in the knowledge of 1) biological processes underlying the disorder that may cause differences in treatment efficacy and 2) mechanisms and possible interactions of antidepressants with gender-specific gonadal steroids.

The majority of antidepressants are best absorbed under less acidic conditions (Bies et al., 2002). It has been hypothesized that the greater absorption of these drugs in women than men is due to less gastric acid, slower gastric emptying times and a longer colonic transit time in women (Bies et al., 2002). After absorption by the gut and the gastrointestinal tract, a drug enters systemic circulation and is delivered to the liver, where it is further metabolized or sent to other parts of the body (Yonkers and Brawman-Mintzer, 2002). There is evidence that gender differences exist in the levels of several hepatic enzymes that are involved in this metabolism, causing potential differences in blood levels of antidepressant drugs, and influencing both response to treatment as well as severity of side effects (Bies et al., 2002; Yonkers and Brawman-Mintzer, 2002). For example, it has been shown that women have higher plasma levels of tertiary amines, imipramine, amitriptyline and clomipramine (Moody et al., 1967; Preskorn and Mac, 1985). In addition, adipose tissue accounts for approximately 33% of body weight in women compared to 18% in men, which could prolong the half-life, without changing the plasma concentrations of a drug (Greenblatt et al., 1980). However, the clinical significance of the above findings has not been investigated.

A study that investigated plasma drug levels of the SSRI, sertraline, found that young men had plasma levels that were 27% lower than young women, older women or older men (Ronfeld et al., 1997). In another study using the same drug as well as a TCA, it was found that women were 10% more likely to respond to the SSRI than to the TCA, whereas men were 12% more likely to respond to the tricyclic antidepressants (TCA) (Kornstein et al., 2000). Of interest, the stronger efficacy in the SSRI was eliminated in the women who were postmenopausal. The hypothesis that the difference in SSRI

responsiveness between pre- and post-menopausal women may be due to the influence of gonadal hormones is supported by data showing that older women taking hormone replacement therapy (HRT) respond better to SSRIs than to post-menopausal women not taking HRT (Schneider et al., 1997).

The effects of hormonal status on SSRI efficacy may be due to changes in serotonin receptors and function induced by fluctuations in gonadal hormones. It has been shown that acute administration of estradiol increases 5-HT2 and decreases 5-HT1 receptor expression (Biegon and McEwen, 1982; Fink et al., 1996). In addition, estradiol increases the serotonin transporter activity by increasing serotonin reuptake (Tam et al., 1985; Klompenhouwer et al., 1990). However, progesterone is colocalized with serotonin receptors in the raphe, and it has been shown to reverse some of the effects of estrogen on serotonin accumulation in the hypothalamus and pituitary (Bethea, 1993; Bethea et al., 1996).

These results highlight the need to study changes in antidepressant metabolism and efficacy throughout the menstrual cycle. One such study demonstrated that trazodone and desipramine levels doubled at the mid-cycle (Kimmel et al., 1992). While the use of oral contraceptives on the efficacy of antidepressants has not been studied, it has been shown that low-dose estrogen contraceptives decrease the clearance of caffeine, suggesting an effect of hepatic enzymes on drug absorption (Abernethy and Todd, 1985). This evidence suggests that there may be a rationale for pulse dosing of antidepressants.

1.6 Objectives of the Present Study

Throughout the world, the majority of people are exposed to chronic stress or severe trauma during their lifetime and yet never develop a mood disorder. However, major depression is debilitating for the 15% of the population that develop this illness at some point in their lifetime. There are risk factors that can increase one's vulnerability to the damaging effects of stress and augment the probability of developing a mood disorder. It is well known that females are approximately twice as likely to develop a mood disorder as men, and yet the majority of basic research has failed to use female animals in stress studies. Therefore, the first objective of this thesis was to investigate any putative gender differences in the long-term consequences of chronic variable stress.

Antidepressant treatment is normally prescribed after the diagnosis of depression, following exposure to stress or trauma. However, most basic research has investigated the ability of antidepressant treatment to prevent the damaging effects of stress when administered either in advance of, or concurrently with the stress exposure. In addition, gender differences in antidepressant efficacy have largely been ignored. Therefore, the second objective of this thesis was to investigate the ability of chronic antidepressant treatment to reverse the damaging effects of CVS after the termination of the stress regimen in both male and female rats.

A negative early life environment is associated with an increased risk to develop a mood disorder in adulthood. In rats, it has been shown that a natural variation on the quality of maternal care influences the behavioral and physiological response to an acute stress in the adult offspring. Given the evidence that as adults, offspring of low LG/ABN mothers display less efficient behavioral responses to an acute stress in comparison to

offspring of high LG/ABN mothers, the offspring of low LG/ABN mothers may be thought of as a model of stress vulnerability due to adverse early-life events. Therefore, the third objective of this thesis was to test the effects of a chronic variable stress regimen on the offspring of high and low LG dams. In addition, we tested whether the effects of maternal care on stress responsivity would differ in males and females.

It has been hypothesized that a failure to return to a normal state of coping following exposure to a severe stress or trauma is in part related to abnormal "encoding" of the event. It has been shown that the quality of maternal care affects the response to stressful stimuli in adult rats. The fourth objective of this thesis was to investigate the influence of early maternal care on the learned association between stressful/fearful stimuli and their context.

CHAPTER 2 (STUDY 1)

2 The Consequences of Chronic Variable Stress on Behavioral and endocrine Measures in Male and Female Rats.

Emma Spreekmeester, Shakti Sharma, Joseph Rochford, Michael Meaney and Ettie Grauer

2.1 Study 1: The Consequences of Chronic Variable Stress on Behavioral Measures of Anxiety in Male and Female Rats.

2.1.1 Introduction

There is a higher prevalence of mood and anxiety disorders among women than men, and this is a cross-cultural phenomenon (Kessler, 2003). In spite such gender differences, the majority of the basic research on chronic stress has been conducted in males, with the assumption that any findings can be generalized to female subjects. Recently, there has been an emergence of a small number of studies that have looked at gender differences in the vulnerability to chronic stress. While a great deal of information still remains to be investigated in females, the studies that have taken this issue into account have uncovered gender differences in both behavioral and physiological reactivity to stress. For example, Beck and Luine (2002) found an interaction between housing conditions and gender after exposure to chronic stress. Males that were double housed performed poorly in a cognitive test of object recognition after chronic restraint stress, while the performance of doubly housed females was enhanced following stress. Single-housed rats displayed an increase in hippocampal CA1 serotonin levels independent of gender or stress, and, females had higher levels of serotonin and dopamine metabolites in the frontal cortex than males irrespective of stress or housing conditions (Beck and Luine, 2002). It has been shown that female rats have a different ratio of MR:GR in the hippocampus and hypothalamus compared to males, and that exposure to chronic (14 days) restraint stress differentially affected the pattern of these receptors in a gender-dependent manner (Karandrea et al., 2000). Chronic restraint stress has also been shown to impair object recognition and radial arm maze performance in males, while the performance of females was either enhanced or unaffected (Bowman

et al., 2003). Though the above studies do illustrate that chronic stress has gender dependent effects on behavior and physiology, the majority of these studies used a single stress (e.g., restraint) applied repeatedly, that may confer a degree of habituation. One study by Duncko et al (2001), investigated the effects of a chronic mild and variable stress paradigm in male and female rats and found that females had higher basal CRH mRNA in the PVN than males, but did not display the same stress-induced increase that was evident in males (Duncko et al., 2001b).

Another area that has not been investigated in females or males is the long-term consequences of chronic unpredictable stress, and the ability of the organism to return to normal baseline health measures following the termination of the stress protocol. The studies that have looked at gender differences in behavioral or neurochemical reactions to chronic stress have done so either during or immediately following the stress exposure. However, physiological and behavioral reactions to stress are not only normal, but adaptive in the short term, and it is hypothesized that mood disorders stem from an inability to return to homeostasis. It is possible that gender differences in the prevalence of mood disorders may be due in part to a difference in the capacity to adapt long term in males and females. Therefore, in this study we allowed a minimum of 3 days of recovery following the termination of the stress protocol, before the onset of behavioral testing, which continued for one month.

2.1.2 Experimental Design:

Animals:

Female and male Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were obtained from Charles River Canada Inc. (St Constant, Que). Throughout, rats were housed in same-sex groups of 2 per cage in standard (dimensions 60 x 30 x 25cm) polypropylene cages, on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when restriction was required by the stress protocol. All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Stress Regimens:

Female and male animals were randomly assigned to either control or variable stress, for a total of four groups: Female Control (n=8), Female Stress (n=7), Male Control (n=7) and Male Stress (n=6). All animals in the variable stress groups were subjected to a random 6 week schedule of variable unpredictable stressors that included: cold exposure (4^{0}C, 4 hrs), cage shaking (2 hrs), noise (80 Db, 3 hrs), 2 ten-minute footshock sessions (2 shocks per session of 0.4 mA, 1 second in duration at random time points), re-exposure to the shock environment (10 min), restraint (2 hrs), an overnight period of either: water deprivation, food deprivation, crowding with unfamiliar rats (n = 6 per cage), wet bedding, and continuous illumination (lights on). Rats in the unpredictable stress groups were exposed to one of the above stressors per day, at random time points. The stress regimen was ceased three days prior to the onset of behavior testing. Control animals remained undisturbed, except for routine cage maintenance for the entire six-

week period before behavioral testing. Both chronically stressed and non-stressed control animals were tested in the same order in the behavioral paradigms.

Behavioral Testing

Open Field (days 7 – 14 post chronic stress)

Animals were placed into the middle of a white 122 x 122 x 60 cm square arena in a well-lit room for 30 min per session for two consecutive days. The inside of the open field was divided into 25 (5 x 5) equal sized squares to measure locomotor activity. Behavior was monitored by a computerized visual tracking system (HVS, England), and later coded for total squares crossed, and ratio of inner to total squares crossed.

Acoustic Startle (days 16 – 21 post chronic Stress)

Startle testing was conducted in two identical stabilimeters (SR-LAB, San Diego Instruments, San Diego, CA). Each stabilimeter consisted of a Plexiglas tube 8 cm in diameter that rested on a 12 x 12 cm Plexiglas frame within a ventilated sound-proof chamber. A piezoelectric device mounted below the Plexiglas frame detected and transduced motion within the tube. The delivery of acoustic stimuli was controlled by a PC microcomputer and SR-LAB interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. Background noise (70 dB) and startle stimuli (120 dB) were delivered in each chamber through a Radio Shack Supertweeter (frequency response predominantly between 5 and 16 kHz) located 24 cm above the animal. Chambers were balanced across all experimental groups. Following a 5-min acclimation period in the stabilimeter with 70-dB background noise, animals were exposed to 100 - 120-dB, 40-ms noise bursts with a variable 15-150 sec inter-trial

interval. One hundred 1-ms readings were collected beginning at the stimulus onset. Startle amplitude was defined as the average of the hundred readings.

Corticosterone Stress Recovery (day 30 post chronic stress)

All rats were housed individually and transported to a blood sampling room one day prior to stressor testing to habituate the animals to the environment. Animals were restrained and a baseline blood sample was drawn within 2 minutes following removal from the homecage. Following this first blood sample, termed "pre", animals were restrained in Plexiglas restrainers for 20 minutes. A sample was taken from each rat during the restraint stress at 10 and 20 minutes. In addition, another 4 samples were taken at 20 minute intervals following release from the restrainer. Rats were returned to their homecage between each blood sample. Blood was collected into tubes coated with EDTA, centrifuged and the supernatant stored at -20 until assay.

Determination of plasma corticosterone:

Corticosterone was measured by the Radioimmunoassay of Krey et al. (1975) with a highly specific B antiserum (B3-163, Endocrine Sciences, Tarzana, CA), [3H-B] (101 Ci/mmol; New England Nuclear, Boston, MA) as tracer and 10 ml of plasma. The minimum level of detection of the assay was 10 pg/ml. The antiserum cross-reacts slightly with deoxycorticosterone (4%) but not with cortisol (<1%). Separation of bound from unbound hormone was achieved using dextran-coated charcoal. Samples were then decanted into mini-scintillation vials, filled with 4.5 ml of Liquiscint (National Diagnostics, Sommerville, NJ) and radioactivity determined in a Packard scintillation counter at 56% efficiency. The intra- and inter-assay coefficients of variation were 3.2% and 3.9% respectively.

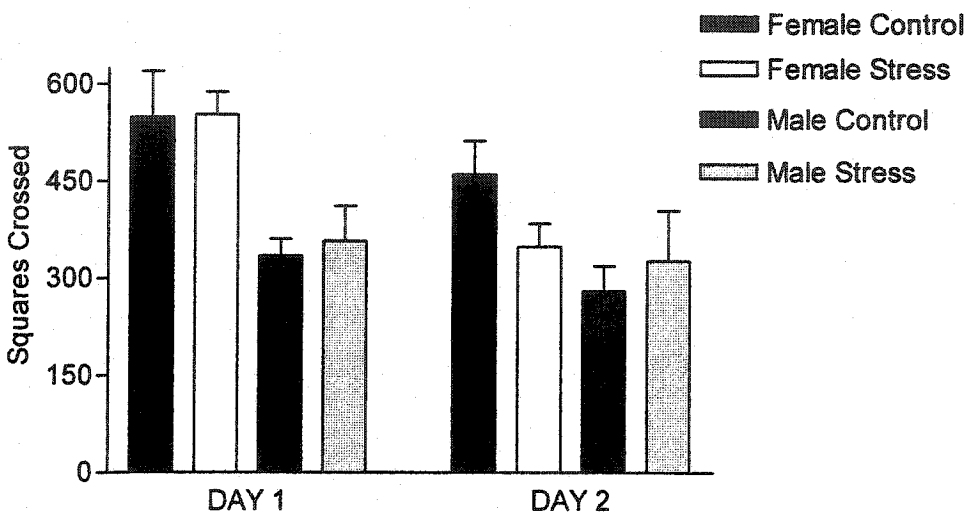

Figure 2. Total squares (mean + SEM) crossed in the open field for male and female rats with and without exposure to CVS. Female Control (n=8), Female Stress (n=7), Male Control (n=7), Male Stress (n=6).

2.1.3 Statistical Results

Open Field

Total Squares Crossed:

Data from 2 rats (Female control = 1, male control =1) were removed due to problems in tracking with the HVS system. A three-way analysis of variance with gender and stress as between subject factors and day as a repeated measures factor was performed (Figure 2). The results revealed that female rats crossed more squares in the open field than male rats ($F(1, 22) = 10.08$, $p = .0044$). There was also a main effect of day, such that the total number of squares crossed decreased from day 1 to day 2 ($F(1, 22) = 11.01$, $p = .0031$). Although the interaction of day x stress was not significant ($F(1, 22) = 3.36$, $p = .0804$), inspection of the graph suggests that the main effect of day is mainly due to the effect of CVS exposure on the number of squares crossed in females.

Ratio of Center to Total Squares Crossed:

There were no group differences in the ratio of center to total squares crossed in the open field over the two days of testing (data not shown).

Acoustic startle response

The results from the acoustic startle paradigm can be seen in Figure 3. A trend suggesting a threeway (gender x stress x intensity) interaction ($F(4, 108) = 2.25$, $p = .0684$) was observed, suggesting that chronic stress exposure decreased the startle response of the males and increased it in the females at the higher dBs ($F(1, 27) = 3.64$, $p = .0672$). Analysis with simple main effects revealed that non-stressed males displayed a

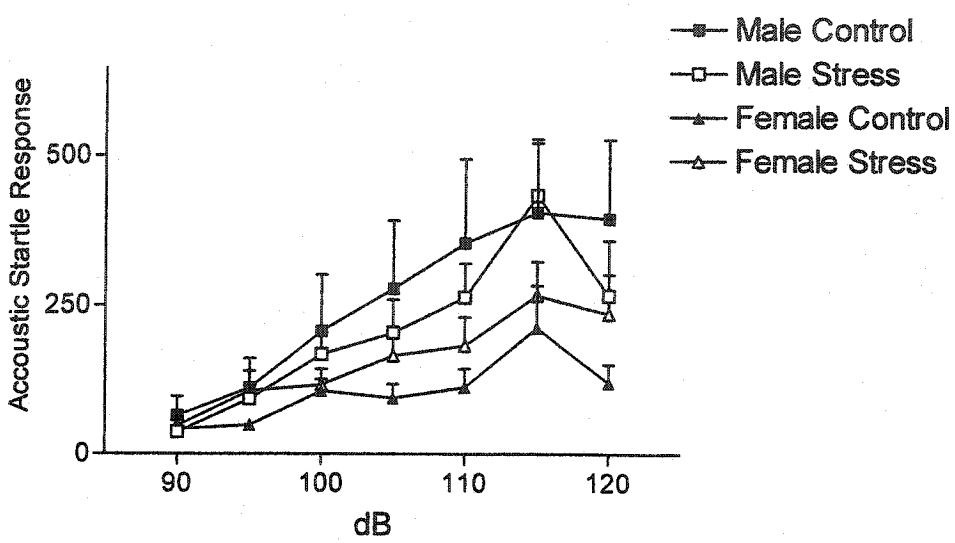

Figure 3. Mean (± SEM) acoustic startle response (ASR) in male and female rats with and without exposure to CVS. Female control (n=8), Female stress (n=7), male control (n=7) and male stress (n=6).

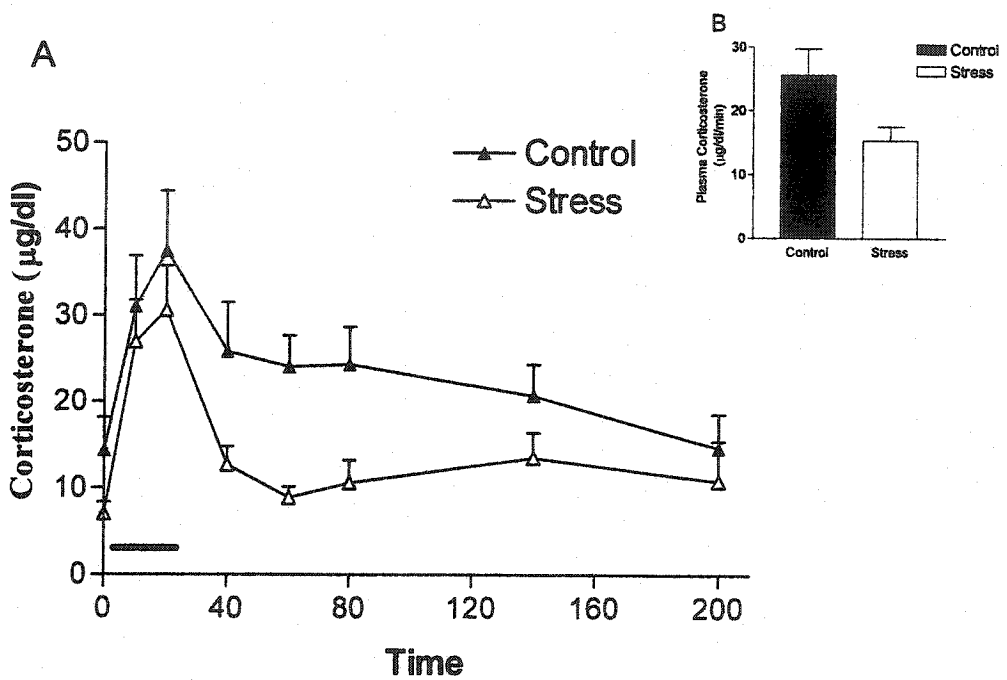

Figure 4. A) Female mean (+ SEM) plasma corticosterone levels in response to an acute (20 min) restraint stress (solid bar) in female control (n=6) and CVS exposed rats (n=6). B) Mean (+SEM) corticosterone values expressed as the area under the curve (AUC).

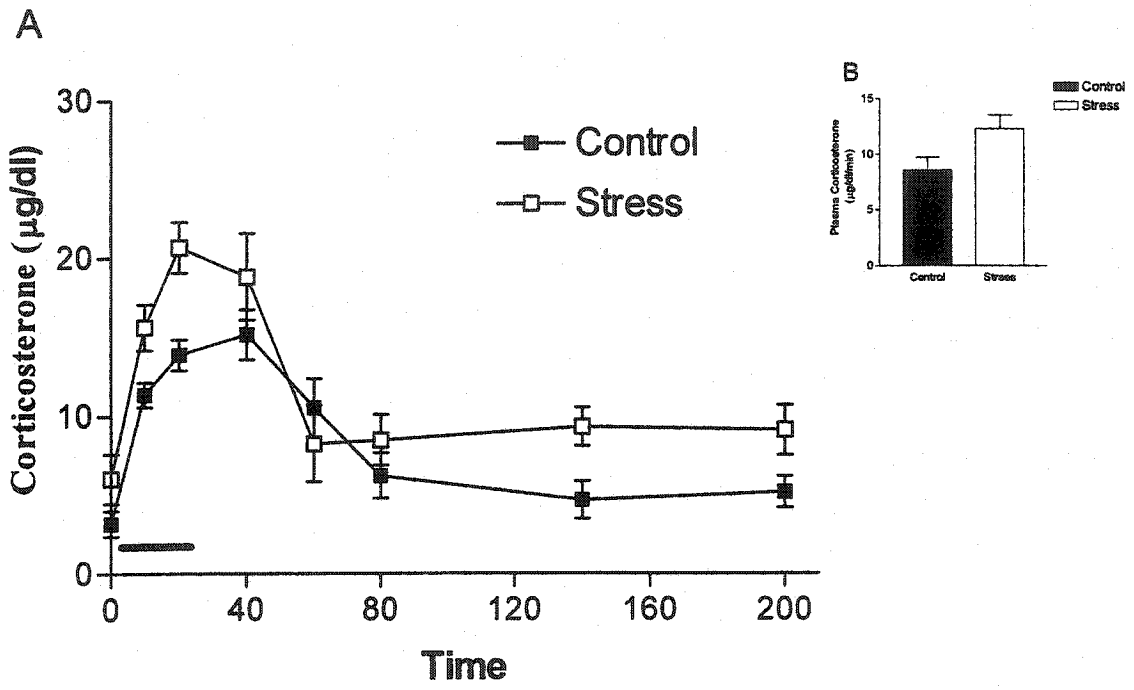

Figure 5. A) Male mean (± SEM) plasma corticosterone levels in response to an acute (20 min) restraint stress (solid bar) in male rats with (n=6) and without (n=7) exposure to chronic variable stress (CVS). B) Mean (+SEM) corticosterone values expressed as the area under the curve (AUC).

significantly higher acoustic startle than control females at the highest 3 dBs, ($F(1, 135) = 4.23$, $p = .0473$), ($F(1, 135) = 5.29$, $p = .0277$) and ($F(1, 135) = 3.81$, $p = .0593$).

Plasma Corticosterone

Female Corticosterone: Analysis of plasma corticosterone was done separately for males and females due to the large difference in basal total plasma corticosterone levels between the sexes. In females, prior exposure to chronic stress was associated with decreased levels of plasma corticosterone after the removal from the restraint stress in comparison to controls (Figure 4). Although the corticosterone levels of the CVS exposed rats appeared to be lower in comparison to the controls, the main effect of CVS exposure was not significant ($F(1, 70) = 4.27$, $p = .065$). However, as the corticosterone levels are expected to change over time, we employed the trapezoidal rule to determine the area under the curve (AUC) for each group over the entire testing period. The results of an independent samples t-test on the AUC revealed that CVS-exposed female rats displayed significantly lower levels of corticosterone over the testing session ($t(1, 10) = 4.81$, $p = .0531$).

Male Corticosterone: The two-way ANOVA revealed a significant interaction between CVS exposure and time of blood sample ($F(7, 91) = 2.21$, $p = .0407$)(Figure 5). Analysis of the interaction with a test of simple main effects revealed that CVS-exposed males displayed higher levels of plasma corticosterone at the two time points during the restraint stress ($F(1, 104) = 3.83$, $p = .0552$) and ($F(1, 104) = 9.84$, $p = .0027$). In addition, CVS-exposed males also had higher plasma corticosterone levels at the second to last recovery sample ($F(1, 104) = 4.52$, $p = .0379$). The results of the independent t-test performed on the AUC also revealed a significant effect of CVS-exposure on the

male rat (t (1, 13) = 4.78, p = .0476). Thus, CVS induced changes in plasma corticosterone that were dependent on gender.

2.1.4 Discussion

The aim of this study was to investigate any gender differences in behavioral testing that assesses reactivity to novelty following exposure to chronic variable stress.

The open field is commonly used as a test of general locomotor activity and exploration when looking at the total squares crossed as well as a measure of anxiety when assessing the ratio of center to total squares crossed. Both the total locomotor activity as well as the ratio of center:total squares have been shown to be affected by stress (D'Aquila et al., 2000; Heiderstadt et al., 2000; Bowman et al., 2002). We found that female rats were more active in the open field compared to non-stressed and stressed male rats. Stress had no effect on open field activity on the first exposure (day 1), but tended to decrease activity in the females on the second day of testing. The results of the CVS exposure on day 2 open field activity in the females, suggests that chronic stress may have affected habituation processes differently in male and female rats. This is interesting in that the ability to habituate or adapt to a novelty has been associated with the outcome of mental health in response to stress (Anisman and Merali, 1999; Olff, 1999). The gender difference in habituation to the open field may be due to the influence of gonadal hormones on HPA activity, or differences in coping mechanisms.

Exposure to CVS and gender did not influence the ratio of center to total squares crossed in the open field. This measure is typically used to assess anxiety. Therefore,

these results suggest that there was no gender difference in anxiety, and that CVS exposure did not influence anxiety in males or females in the open field.

The acoustic startle response (ASR) has been shown to be increased in patients with some mood disorders (Morgan, III et al., 1997; Yehuda et al., 1998; Grillon, 2002), and is therefore commonly used in animal stress research. Male rats displayed a higher ASR than female animals. In this study, there was a trend for CVS to decrease the ASR in male rats and increase it in females; however, neither of these effects was significant. Nevertheless, it is interesting that the trend for CVS in each gender was in the opposite direction, suggesting that this effect may be of interest for further investigation.

The corticosterone response to an acute stress is a common test employed to assess neuroendocrine functioning, and is typically found to be increased in rodents following a chronic stress paradigm (Blanchard et al., 1998; Bielajew et al., 2002; Veenema et al., 2003). Consistent with previous research, CVS exposure increased the plasma corticosterone levels in the male rats. Although direct comparisons were not made between the male and female corticosterone values, females tended to display higher plasma corticosterone in comparison to both groups of male rats, as is commonly seen in female rodent neuroendocrinology. Unexpectedly, CVS exposed female rats displayed a decrease in corticosterone compared to female controls. While this effect was surprising, there is previous evidence of stress-induced decreases in cortisol levels. In humans, while major depression is associated with increased cortisol levels, atypical depression as well as post traumatic stress disorder (PTSD) are associated with decreased cortisol secretion (Anisman et al., 1999; Gold and Chrousos, 1999; Yehuda, 2001). It is therefore possible that the certain features of the chronic stress paradigm used in this

experiment were able to elicit symptoms resembling either atypical depression or PTSD in the female rats and symptoms resembling a more typical major depression in the males. Gender differences in physiology, including the influence of gonadal hormones on HPA activity and monoamine functioning may be in part responsible for this gender difference in stress reactivity. This finding also illustrates that the stress response of females differs from that of males, and that the same stressor can have a different impact depending on gender.

CHAPTER 3 (STUDY 2 AND 3)

3 The Impact of Chronic Fluoxetine Treatment on the Long-Term Effects of Chronic Variable Stress in Male and Female Rats

Emma Spreekmeester, Shakti Sharma, Joseph Rochford, Michael Meaney and Ettie Grauer

3.1.1 Introduction

Exposure to stress precedes the onset of depression, and influences its severity and course (Frank et al., 1990; Kessler and Magee, 1994; Kessler et al., 1994b). An emerging theory is that depression results from the lack of adaptation to stress (Anisman and Zacharko, 1992; Duman et al., 1999c). The inability to make the appropriate adaptive response to stress may be attributed to the dysfunction of systems underlying neural plasticity (Anisman and Zacharko, 1992; Duman et al., 1999b). It has been hypothesized that antidepressants may work in part by increasing adaptation and coping, possibly via an increase in neuronal plasticity, thereby explaining the lag in time between antidepressant onset and therapeutic efficacy (Anisman and Zacharko, 1992; Ravindran et al., 1999; Duman et al., 1999a).

Antidepressants or other treatments for depression are normally prescribed when a patient presents with symptoms of depression that have lasted for a sufficient amount of time to be ruled out as an episode of "the blues". Most basic research protocols have administered antidepressants either prior to, or concurrently with the stress exposure. The prophylactic mechanism of antidepressants given prior to or in combination with stress exposure may differ from the mechanism required to reverse depressive symptomatology. Therefore an important query for this study was the ability of antidepressants to reverse any putative behavioral or neuroendocrine changes caused by chronic stress exposure after the termination of the stress protocol.

Second, given the evidence that Chronic variable stress does not have the same impact on males and females (see chapter 2), and that antidepressant treatment may have differential effects depending on gender (Yonkers et al., 1992), we assessed the effectiveness of antidepressant treatment to attenuate CVS induced deficits in both females (study 2) and males (study 3). We used fluoxetine hydrochloride since it is currently one of the most commonly prescribed antidepressant.

In addition, since the open field results of STUDY 1 suggested that stress may affect habituation processes differently in males and females, we employed a random effects regression model that provides an estimate of both the rate of habituation as well as the initial reactivity to the open field.

3.2 STUDY 2: The Impact of Chronic Fluoxetine Treatment on the Long-Term Consequences of Chronic Variable Stress in Female Rats

3.2.1 Experimental Design:

Female Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were obtained from Charles River Canada Inc. (St Constant, Que). Throughout, rats were housed in groups of 2 per cage in standard (dimensions 60 x 30 x 25cm) polypropylene cages, on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol. All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Stress Regimens

Animals were randomly assigned to four groups: Control (C-Veh) (n=10), Stress (S-Veh) (n=10), Control + Fluoxetine (C-Fluox) (n=10) and Stress + Fluoxetine (S-Fluox) (n=10). All animals in the unpredictable stress groups were subjected to the same random 6 week schedule of variable unpredictable stressors described in Study 1.

Behavioral Testing

Open Field (days 24 - 29 post chronic stress)

Open field testing was conducted in the same manner as described in Study 1.

Acoustic Startle (days 30 - 32 post chronic Stress)

Testing for the acoustic startle response was conducted in the same manner as described in Study 1.

Elevated Plus Maze (days 38 – 40 post chronic stress)

The elevated plus maze was constructed of wood covered with white enamel and had four arms 50 cm long and 10 cm wide at a height of 53 cm above the floor. The two enclosed arms had 40 cm high walls. Rats were placed in the center of the maze facing an open arm. Each session lasted 5 min and were recorded and later coded for open and closed arm locomotor activity.

Corticosterone Stress Recovery (day 47 post chronic stress)

Corticosterone was sampled in the same manner as described in Study 1.

Determination of plasma CORT

Plasma corticosterone assay is described in Study 1.

3.2.2 Drug Administration

Water consumption for each cage was measured for one week prior to the onset of antidepressant treatment. The quantity of fluoxetine added to the drinking water was based on average amount consumed in the previous week. Consumption of water was measured throughout the period of drug administration to ensure the proper dosage. Fluoxetine (10 mg/kg/day) was administered in the drinking water beginning the morning following the last day of stress exposure for a minimum of 21 days before the onset of behavioral testing. Water was weighed daily. Animals continued to receive fluoxetine in their drinking water throughout behavioral testing.

3.2.3 Results

Open Field

Total squares crossed

As shown in Figure 6, a threeway ANOVA (stress x drug x day) revealed a stress by day of testing interaction ($F(1, 36) = 7.60$, $p = .0091$). Control animals displayed an initial high rate of locomotor activity that decreased slightly on the second day of testing. In contrast CVS exposed rats started with a lower level of locomotor activity that increased on the second day. A test of simple main effects revealed that stress significantly affected the number of squares crossed on the second test day ($F(1, 72) = 8.89$, $p = .0045$), but not on day 1, when the open field held more novelty ($F(1, 72) = .69$, $p = .4102$). In addition, CVS exposure significantly affected the activity of the vehicle- ($F(1, 36) = 7.91$, $p = .0079$), but not the fluoxetine- ($F(1, 36) = .01$, $p = .9095$) treated animals, suggesting that fluoxetine was able to attenuate the effects of CVS. There was an overall main effect of stress, such that the CVS treated group crossed more total squares in the open field than the control animals ($F(1, 36) = 4.28$, $p = .0457$).

Time series

Time series data were extracted by taking totals for squares crossed at 5 min intervals. Curves for each group of animals were constructed and fit with a random effects exponential regression model (implemented using the NLINMIX macro within the SAS statistical system v6.12) as indicated below:

Behavior = A x exp (B x time)

The A-parameter (y-intercept) was operationally defined as reflecting the initial behavioral reactivity of the animal while the B-parameter (nonlinear slope estimate) was

defined as the rate of habituation. Various combinations of random and fixed coefficients were examined for these parameters and due to considerable variability between individual animals within a group, both A and B were included as random effects.

To assess goodness of fit, each regression model was also estimated using a least-squares approach and calculations of R^2 values indicated that all models accounted for at least %70 of the variance on the averaged data from each group of animals in each test. Linear and quadratic functions were also calculated using least squares for each data set but in each case yielded lower R^2 values than the respective exponential function.

Statistical comparisons of the regression parameters (A or B) between any two groups were made using a likelihood ratio test. Briefly, the minimum objective function (-2 log-likelihood) of fits in which the parameters of the curves were allowed to vary (full model) was compared with the objective functions of fits in which one of the parameters was constrained to be equal (reduced models). The difference between these objective functions has a χ^2 distribution with the difference in the number of parameters in the full and reduced models as the degrees of freedom. The accepted level of significance was a difference in objective functions associated with a P-value of $< .05$. In addition, all statistical comparisons were verified by evaluating the difference between the parameters against their pooled asymptotic standard error and assessed for significance employing a Bonferroni *t* correction to control for possible inflation of the alpha level.

Figure 7b and 7c display the reactivity (*A*-value) and habituation (*B*-value) estimates respectively, for the first day of open field testing. The stress-veh animals displayed a slower rate of habituation compared to the C-veh ($\chi^2(1) = 7.0$, $p < .01$), while there were no differences between any of the groups in the initial reactivity to the open

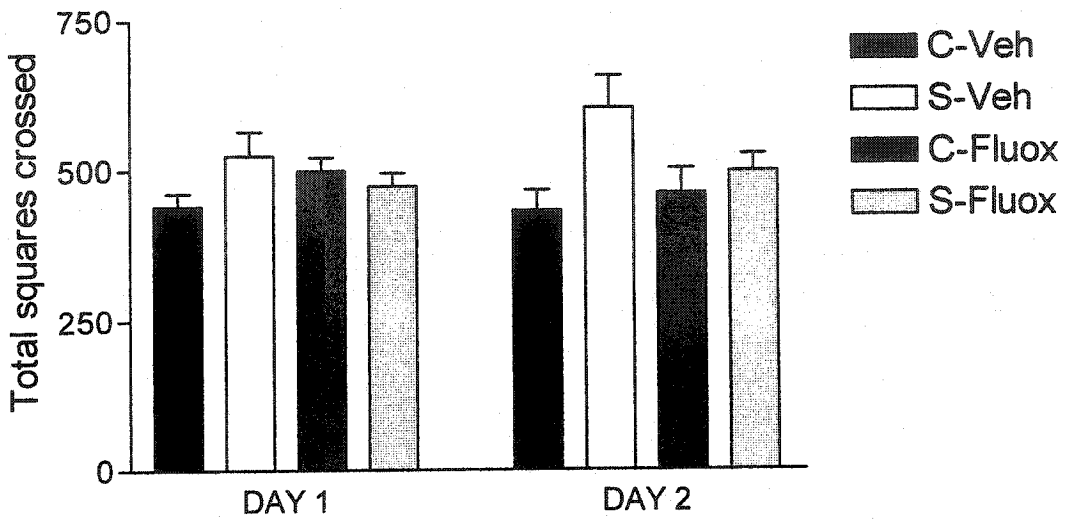

Figure 6. Mean (+SEM) total squares crossed in the open field in control -vehicle (n=10), stress-vehicle (n=10), control-fluoxetine (n=10), and stress-fluoxetine (n=10) female rats.

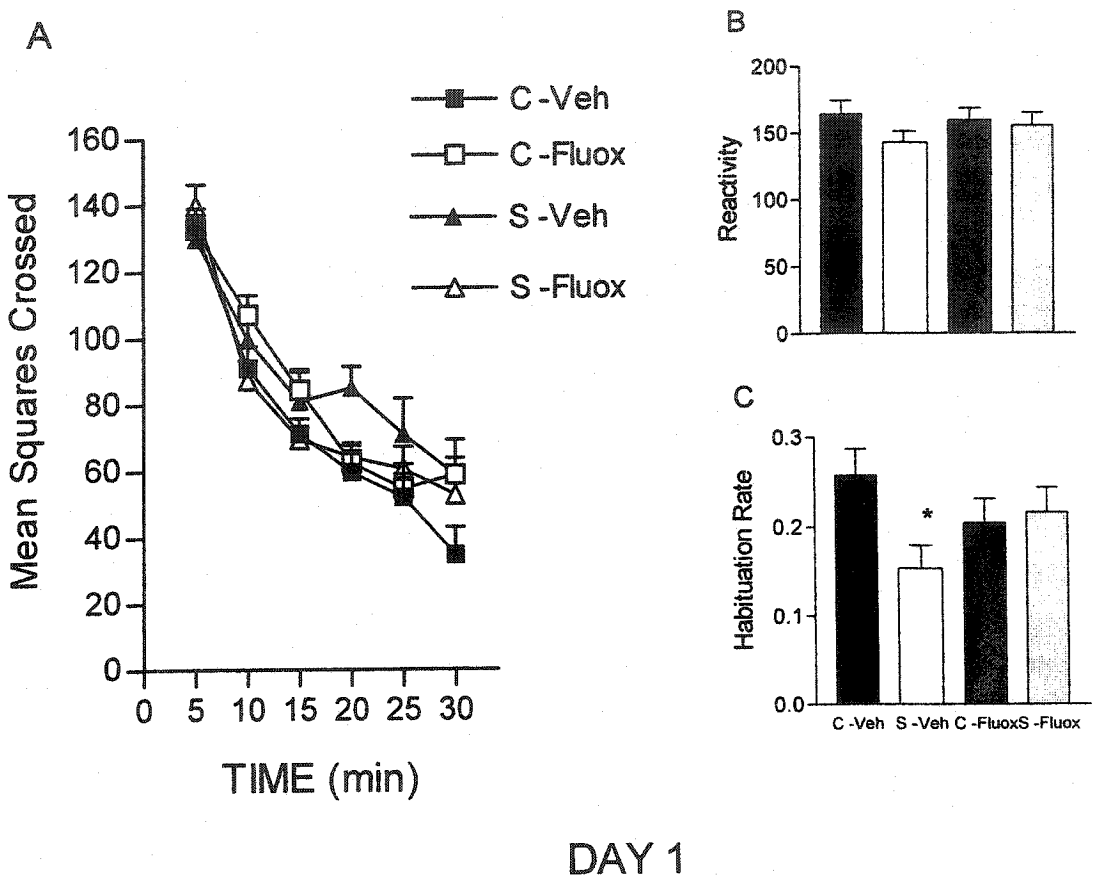

Figure 7. Day 1 A) Mean (+SEM) squares crossed in 5 min bins over 30 min in the open field. C-veh (n=10), C -Fluox (n=10), S-Veh (n=10), S -Fluox (n=10). B) Mean (+sem) initial reactivity score. C) Mean (+sem) rate of habituation. *Significant ($p < .05$) from C-Veh.

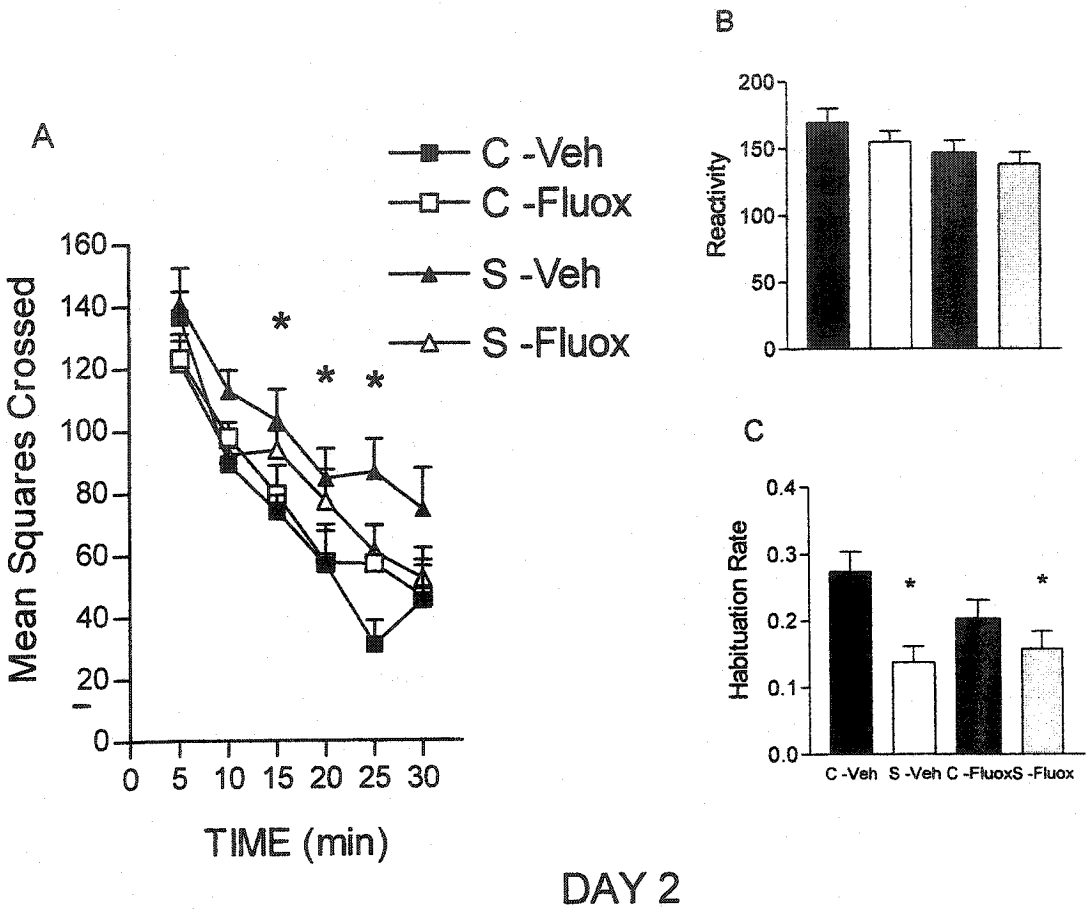

Figure 8. Day 2 A) Mean (+SEM) squares crossed in 5 min bins over 30 min in the open field. C-veh (n=10), C -Fluox (n=10), S-Veh (n=10), S -Fluox (n=10). B) Mean (+sem) initial reactivity score. C) Mean (+sem) rate of habituation. *Significant ($p < .05$) from C-Veh.

field on the first day. The reactivity and habituation estimates for the second day of testing can be seen in figure 2b and 2c respectively. Again, there were no differences in the initial reactivity even on the second open field test, and the habituation rate of the stress-veh group remained significantly slower than the C-veh group (χ^2 (1) = 12.2, p < .001). In addition, the stress-flu animals displayed a slow rate of habituation in comparison to the C-veh (χ^2 (1) = 8.5, p < .01) on the second day, suggesting an impairment in coping from the first to the second open field exposure in the fluoxetine treated stressed animals.

Acoustic startle response

The acoustic startle response in the CVS exposed animals was significantly increased in comparison to controls (F (1, 36) = 7.53, p = .0094) (Figure 9). Fluoxetine was without effect in the CVS exposed and control animals.

Elevated Plus Maze

The time spent on the open arms of the elevated plus maze is displayed in Figure 10. The ANOVA revealed a significant interaction between CVS exposure and fluoxetine treatment (F (1, 34) = 5.08, p = .0308). Further analysis with simple main effects revealed that CVS exposure decreased the amount of time spent on the open arms of the EPM (F (1, 34) = 6.45, p = .0158), but was not effective in fluoxetine treated rats (F (1, 34) = .42, p = .5218).

Plasma Corticosterone

A threeway ANOVA with stress x fluoxetine x blood sample was performed and revealed a main effect of fluoxetine treatment (F (1, 36) = 4.589, p = .039) (Figure 11).

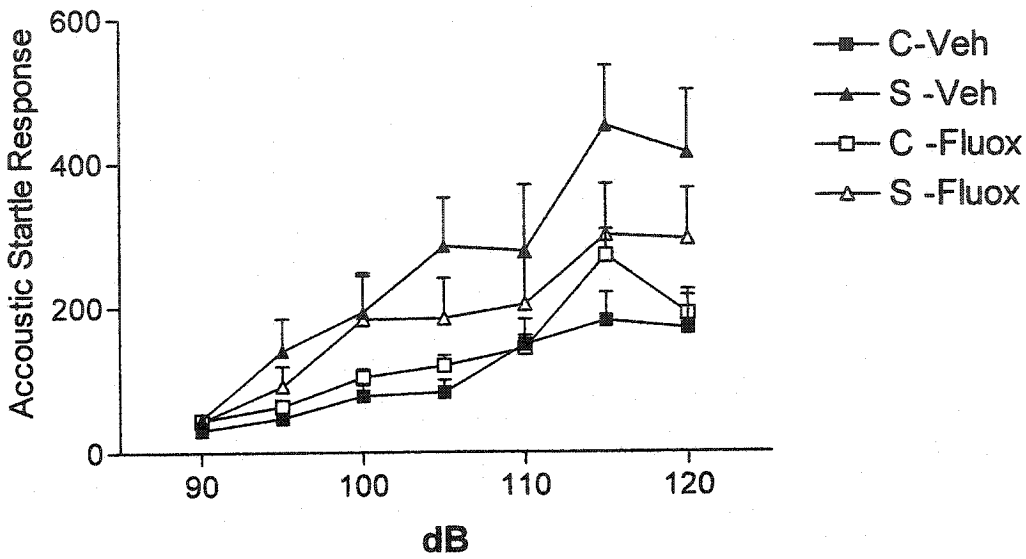

Figure 9. Mean (+SEM) acoustic startle response in control and CVS-exposed female rats treated with vehicle or chronic fluoxetine. Control vehicle (n=10), stress-vehicle (n=10), control-fluox (n=10), stress-fluox (n=10). *Significant effect of CVS.

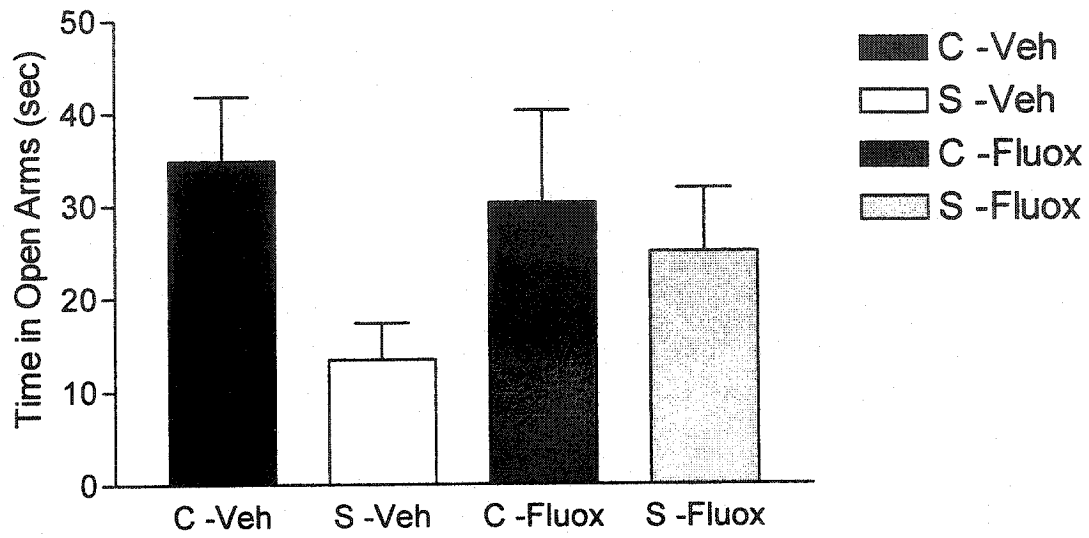

Figure 10. Mean (+SEM) time spent in the open arms of the elevated plus maze in control and CVS-exposed female rats treated with vehicle or chronic fluoxetine. Control vehicle (n=10), stress-vehicle (n=10), control-fluox (n=10), stress-fluox (n=10). * Significant interaction of CVS exposure and Fluoxetine treatment.

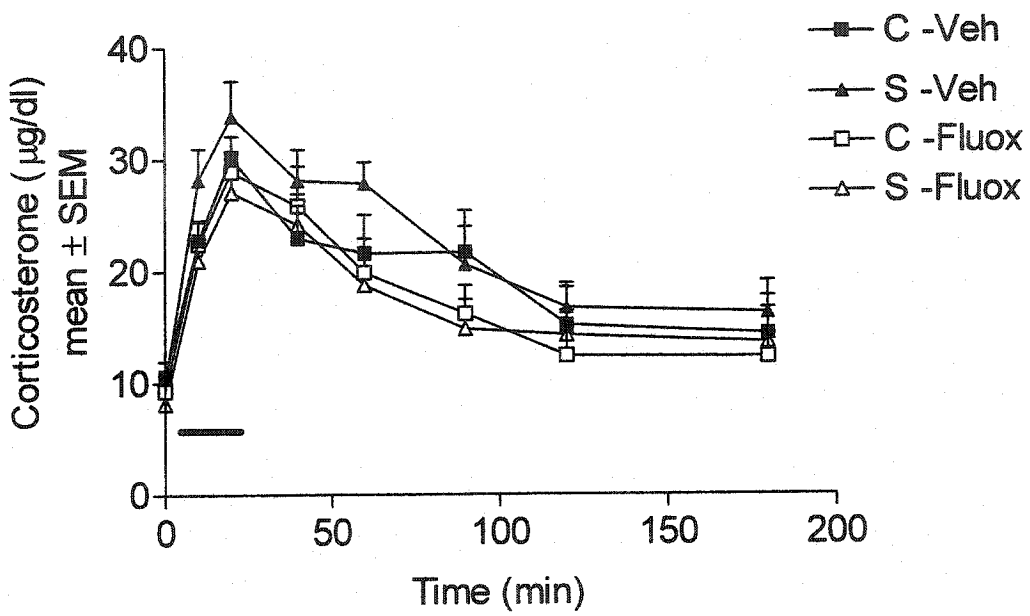

Figure 11. Mean (+SEM) plasma corticosterone response to a 20-min acute restraint stress (black bar) in vehicle- and fluoxetine-treated control and CVS-exposed (S) female rats. Control-vehicle (n=10), CVS-exposed-vehicle (S-Vehicle, n=10), control -fluoxetine (n=10), CVS-exposed fluoxetine (S-fluox, n=10). *Significant ($p < .05$) main effect of fluoxetine.

Fluoxetine decreased the plasma corticosterone response to an acute restraint stress in chronically stressed and control rats.

3.2.4 Discussion

Female rats exposed to CVS displayed an increase in open field activity in comparison to non-stressed controls, and this effect was reversed by chronic fluoxetine treatment. However, the response to novelty, such as a novel open field may be divided into two components; the initial reactivity and the rate of habituation to the stimulus. To assess the effects of CVS and fluoxetine treatment on each of these components in the open field, we used random effects regression modeling. This analysis revealed that neither CVS nor fluoxetine treatment affected the initial reactivity to the open field. In contrast, rats exposed to CVS displayed a slower rate of habituation than control rats across days, and this effect was reversed by chronic fluoxetine treatment on the first day of open field testing. The decrease in the rate of habituation seen in CVS-exposed rats suggests that the higher locomotor activity in these animals was not due to an increase in the initial reactivity to the environment, but to a lack of habituation within and across open field trials. In addition, fluoxetine administration decreased the total CVS-induced activity by increasing the rate of habituation. The effect of fluoxetine on the habituation to the open field has been demonstrated previously in another animal model of antidepressant efficacy, the olfactory bulbectomized (OBX) rat (Mar et al., 2002). However, in this experiment, fluoxetine failed to reverse the effect of CVS on habituation on the second day. The reason for this effect is unclear as previous studies using this

method of analysis have only conducted one open field trial, leaving the consequences of fluoxetine treatment on repeated testing unanswered.

Exposure to CVS in female rats enhanced the acoustic startle response (ASR) in comparison to non-stressed control animals. This finding is consistent with some reports showing an increase in the startle response in stressed females (Fullerton et al., 2001), however, it is in contrast to other studies (Stohr et al., 2000; Beck et al., 2002), suggesting that, at least in female rats, the startle response may not be a good indication of stress reactivity. While there was a trend for fluoxetine to reverse the effects of CVS on ASR, this was not significant. Fluoxetine has been shown to reduce the ASR (Mar et al., 2000), however, this effect was in male rats. These results suggest that an increase in ASR may not be a consistent effect of stress exposure, and that fluoxetine may not exert a reliable therapeutic effect on this measure.

Time spent on the open versus the closed arms of the elevated plus maze is generally thought to reflect levels of anxiety in rodents. The less anxious rat (or mouse) will generally spend more time in the open arms, while a more anxious rodent will largely remain in the closed, safer arms of the maze (Zurita et al., 2000; File et al., 2000). Exposure to CVS decreased the time spent on the open arms of the elevated plus maze, indicating that CVS had anxiogenic effects on this test in female rats. In addition, chronic fluoxetine was successful in reversing the effect of stress on open arm activity, suggesting that a part of the therapeutic efficacy following fluoxetine administration may be due to its effect on anxiety.

The plasma corticosterone response to an acute restraint stress was slightly elevated in CVS exposed females, but this effect was not significant. This effect was

surprising in that we did not replicate the previous finding of decreased corticosterone in CVS exposed females. Given the influence of genetics, early environment and many other factors on the individual stress response, it is possible that the lack of decrease in corticosterone in the CVS-exposed females in this experiment is due to another unknown variable. However, fluoxetine was effective in decreasing the total corticosterone levels in CVS-exposed and non-exposed rats.

3.3 STUDY 3: The Impact of Chronic Fluoxetine Treatment on the Long-Term effects of Chronic Variable Stress in Male Rats

3.3.1 Experimental design

Male Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were obtained from Charles River Canada Inc. (St Constant, Que). Throughout, rats were housed in groups of 2 per cage in standard (dimensions 60 x 30 x 25cm) polypropylene cages, on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol. All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the Douglas Hospital Animal Care Committee.

Stress Regimens

Animals were randomly assigned to four groups: Control (C –Veh) (n=10), Stress (S-Veh) (n=10), Control + Fluoxetine (C – Fluox) (n=10) and Stress + Fluoxetine (S – Fluox) (n=10). All animals in the CVS groups were subjected to the same stress protocol outline in Study 1 (chapter 2).

Behavioral Testing

Open Field (days 24 – 29 post chronic stress)

Open Field testing was conducted in the same manner as described in study 1.

Acoustic Startle (days 30 - 32 post chronic Stress)

Startle testing was identical to that described in Study 1.

Corticosterone Stress Recovery (day 60 post chronic stress)

Corticosterone sampling was performed as described in Study 1.

Determination of plasma CORT

Determination of plasma corticosterone was carried out as described in Study 1

3.3.2 Drug Administration

Fluoxetine was administered as described in Study 2.

3.3.3 Results

Open Field

Total Squares Crossed

The total squares crossed in the open field is displayed in Figure 12. The ANOVA revealed a main effect of antidepressant, indicating that fluoxetine decreased activity in comparison to veh-treated animals ($F(1, 35) = 7.39$, $p = .0102$). While the interaction between stress and fluoxetine treatment was not significant ($F(1, 35) = 3.61$, $p = .0657$), examination of the data revealed that the effect of antidepressant administration appeared to be largely due to fluoxetine's effect in CVS-exposed rats and not in the non-stressed controls. This is supported by a test of simple main effects that revealed a significant effect of fluoxetine in CVS-exposed animals ($F(1, 35) = 10.66$, $p = .0024$) and not in the non-stressed groups ($F(1, 35) = .33$, $p = .5668$).

Time series

Time series data were analyzed as described in Study 2.

Figure 13 b and 13 c display the reactivity (*A*-value) and habituation (*B*-value) estimates respectively, for the first day of open field testing. The S-veh ($\chi^2(1) = 8.6$, $p = .003$) group displayed a decreased initial reactivity compare to the C-fluox animals

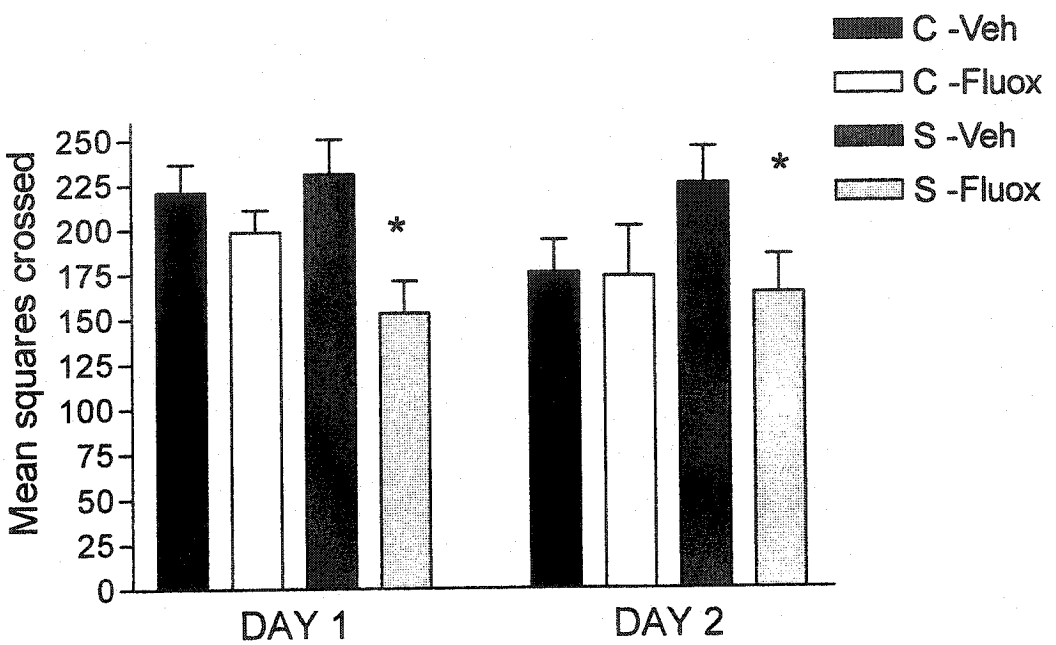

Figure 12. Mean (+SEM) squares crossed in the open field on day 1 and day 2 in vehicle- and fluoxetine-treated control and CVS-exposed male rats. Control-vehicle (n=10), CVS-exposed-vehicle (S-Veh, n=10), control-fluoxetine (n=10), CVS-exposed-fluoxetine (S-fluox, n=10). *Significant ($p < .05$) from S-Veh, main effect of fluoxetine.

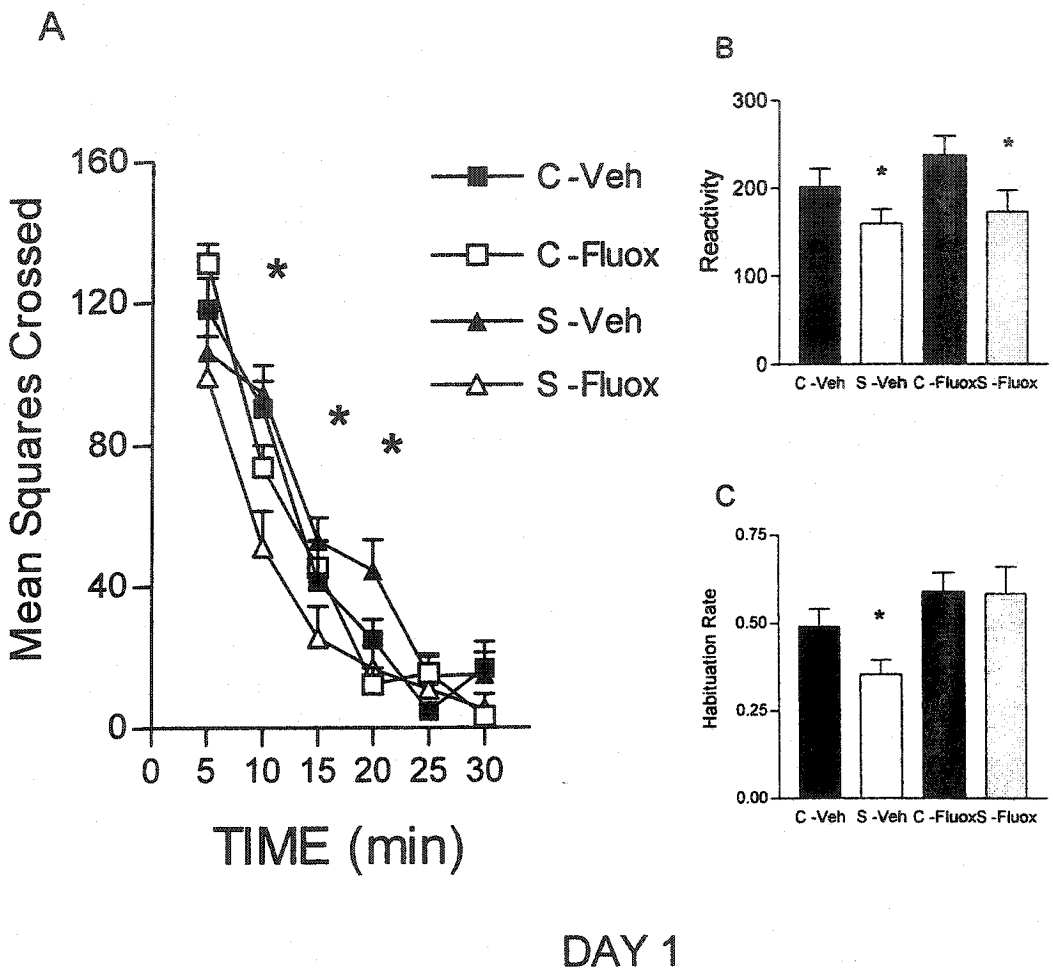

Figure 13. Day 1, A) Mean (+SEM) squares crossed in the open field by male rats divided into 5-min bins. B) Initial reactivity, *Significant ($p < .05$) relative to C-fluox. C) Rate of Habituatio, *Significant ($p < .05$) relative to all other groups. Control-vehicle (n=10), CVS-exposed-vehicle (S-Veh, n=10), control-fluoxetine (n=10), CVS-exposed -fluoxetine (S-fluox, n=10).

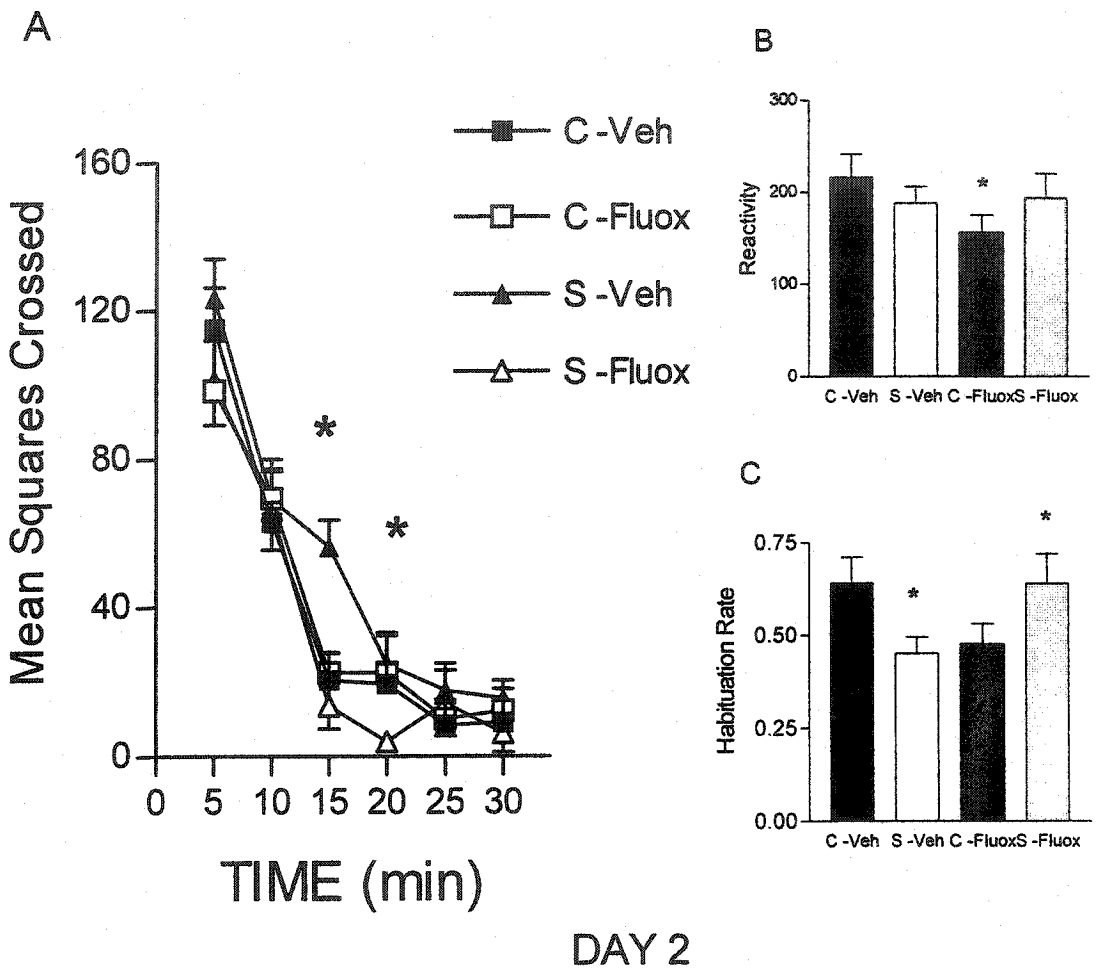

Figure 14. Day 2 , A) Mean (+SEM) squares crossed in the open field by male rats divided into 5-min bins. B) Initial reactivity, *Significant ($p < .05$) relative to C-Veh. C) Rate of Habituatio, *Significant ($p < .05$) relative to C-Veh and S-fluox. Control-vehicle (n=10), CVS-exposed-vehicle (S-Veh, n=10), control-fluoxetine (n=10), CVS-exposed -fluoxetine (S-fluox, n=10).

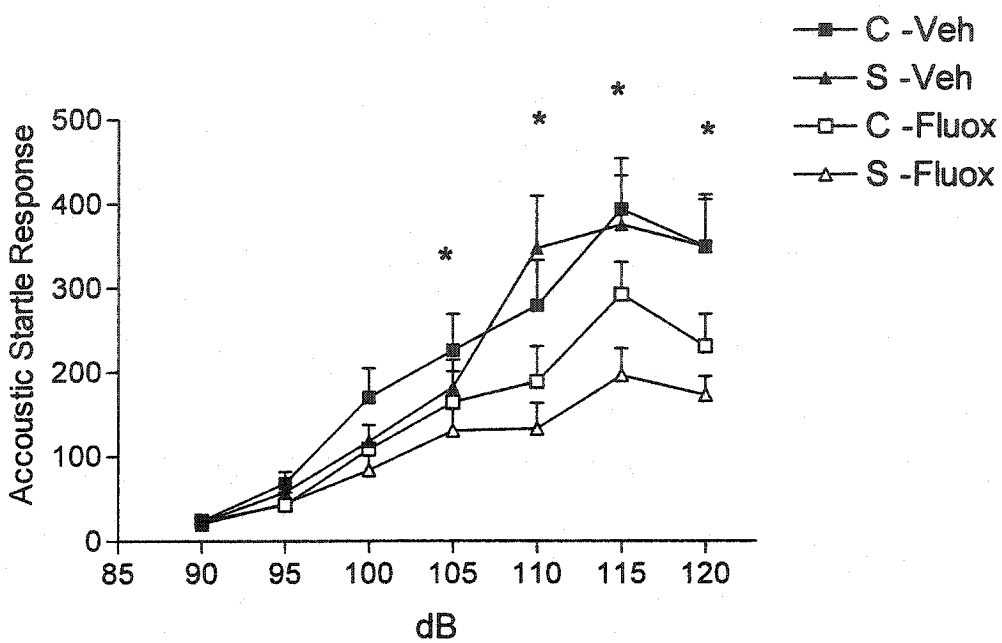

Figure 15. Mean (+SEM) startle amplitude in vehicle and fluoxetine treated male rats exposed not exposed to CVS. Control -Veh (n=10), CVS-exposed -Veh (S-Veh, n=10), Control-fluoxetine (n=10), CVS-exposed-fluoxetine (n=10). *Significant main effect of fluoxetine, and S-Veh vs S-Fluox.

only, and a slower rate of habituation compared to all other groups (C-veh (χ^2 (1) = 4.7, p = .03), C-Fluox (χ^2 (1) = 12.5, p = .0004), S-Fluox (χ^2 (1) = 9.1, p = .0025), suggesting that the decrease in the rate of habituation in the chronically stressed animals was reversed by fluoxetine treatment. On the second open field exposure (Figure 14), the C - fluox animals displayed a lower initial reactivity in comparison to C-veh animals (χ^2 (1) = 3.8, p = .05). The rate of habituation of the S-veh group remained significantly lower than the C-veh (χ^2 (1) = 5.9, p = .015) and the S-fluox groups (χ^2 (1) = 5.3, p = .02).

Acoustic Startle Response

The acoustic startle response was similar in both CVS-exposed and non-exposed male rats (F (1, 165) = 2.97, p = .0866). As can be seen in Figure 15, Chronic fluoxetine treatment decreased the acoustic startle response (F (1, 165) = 35.69, p <.0001). The interaction terms were non significant.

Plasma Corticosterone

There was no effect of CVS or chronic fluoxetine treatment on the plasma corticosterone levels in the male rats. (Figure 16).

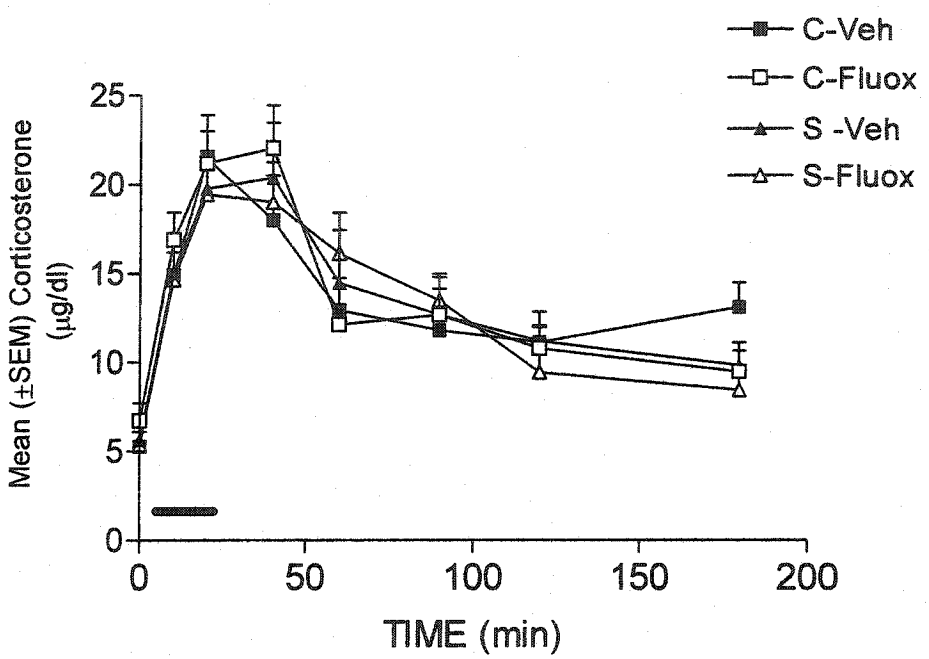

Figure16. Mean (+SEM) plasma corticosterone response to an acute restraint stress (black bar) in male rats. Control-Vehicle (n=10), Control-fluoxetine (n=10), CVS-exposed-vehicle (S-veh, n=10), CVS-exposed-fluoxetine (S-fluox, n=10).

3.3.4 Discussion

The results from this study confirmed the results obtained in male rats of study 1, as it revealed that CVS did not affect the total squares crossed in the open field in comparison to the control animals. As was also suggested by the results in Study 1, the present results showed that CVS exposure modified the total squares crossed in female rats. Despite the lack of effect of CVS exposure in the males, chronic fluoxetine treatment decreased activity in both CVS-exposed and non-exposed male rats. However, this effect appeared to be largely due to the influence of fluoxetine on CVS-exposed animals, and not in the non-stressed controls. Further analysis of the open field behavior with the random effects regression modeling revealed that CVS exposure decreased the initial reactivity and the rate of habituation on the first day of testing. The effect of CVS on the initial reactivity to the open field is in contrast to that found in CVS-exposed female rats, suggesting that the CVS-induced mechanism for this effect is gender dependent. In contrast, the CVS-induced decrease in the rate of habituation was apparent in both male and female rats. Chronic fluoxetine treatment reversed the effect of CVS on habituation in males and females, but was without effect on the initial reactivity in either gender. The effect of fluoxetine on open field habituation in both genders replicates a similar finding that fluoxetine specifically increases habituation in another animal model of antidepressant efficacy, the OBX rat (Mar et al., 2002). However, there may be a difference in the potency of fluoxetine on this behavior in the female rats compared to the male rats. While fluoxetine reversed the CVS-induced decrease in habituation across both days in the males, it was without effect on the second day in the females. It is possible that the perceived aversive properties of the open field changed

from day 1 to day 2 in the females, rendering the effects of fluoxetine ineffective. Conversely, the therapeutic effect of fluoxetine may not be capable of enduring through repeated exposures to novel or stressful environments in the female rats.

The results of CVS exposure and chronic fluoxetine administration on ASR in male rats were in contrast to those of female rats. First, CVS exposure did not affect the ASR in male rats. Gender differences in startle following stress have been reported previously, however, the direction of the difference has not been consistent (Stohr et al., 2000; Fullerton et al., 2001; Beck et al., 2002). In addition, fluoxetine decreased the startle response in both CVS-exposed and non-exposed male rats, despite the absence of a CVS-induced increase in ASR. These results are in also in contrast to the effects of fluoxetine on ASR in the female rats. In the females, stress increased the startle response, and while there was a trend for fluoxetine to attenuate this effect, it was not significant. These results underscore the gender difference in the vulnerability to chronic stress as well as in the response to specific antidepressant treatments.

Exposure to CVS had no effect on the plasma corticosterone response to a subsequent acute restraint stress in the male rats. This effect was surprising given the CVS-induced increase in corticosterone seen in the male rats in Study 1. The reason for this difference is unclear as the CVS paradigm was employed in the exact same manner in both studies. However, since half of the rats in this study were treated with fluoxetine before the commencement of the behavioral testing, all animals waited an extra month before the onset of behavioral testing. Therefore, it is possible that the extra lag of time allowed the activity of the HPA axis in the CVS-exposed rats to return to a normal level of functioning. In fact, in contrast to study 1, in the present study CVS did not induce

hypocortisolemia in the female rats, therefore the CVS-induced influence on corticosterone levels may be time-dependent. However, since we have not performed a time course study on the CVS effects on corticosterone, we cannot be sure that the inability of CVS to alter hormone levels in these two studies was not due to another variable. Fluoxetine also had no effect on the corticosterone response in the male rats. It appears that the impact of fluoxetine on this measure was gender dependent, as fluoxetine decreased plasma corticosterone in female rats.

In summary, the impact of CVS exposure and chronic fluoxetine were dependent on gender and on the test being employed. These results are consistent with data in humans showing a gender difference in the vulnerability to stress as well as in response to specific antidepressant treatments.

CHAPTER 4 (STUDY 4 AND 5)

4 The Impact of Early Maternal Care on the Vulnerability to the Effects of Chronic Variable Stress in Female and Male Rats.

E. Spreekmeester, J. Diorio, S. Sharma, J. Rochford, M. Meaney & E. Grauer

4.1 Study 4: Chronic Variable Stress in Female Rats: Modulation by Early Life Maternal Care

4.1.1 Introduction

While stress is a common experience to all organisms, the coping style adopted in response to any given stressor can vary across individuals. In humans, only a small percentage of those exposed to stress develop a psychopathology.

One of the risk factors that influences the vulnerability to severe or chronic stress and is associated with vulnerability for psychiatric disorders is the early life environment (Heim et al., 1997c; Bifulco et al., 2002a; Kessler, 2003). In particular, early childhood adversity dramatically increases the risk for developing either major depression or other anxiety disorders in adults (Young et al., 1997; Yule et al., 2000; Bremne and Vermetten, 2001; Wainwright and Surtees, 2002; Kendler et al., 2002). Of interest, parental antipathy and neglect are associated with equal risks for depression and PTSD as childhood sexual abuse (Yule et al., 2000; Harkness and Monroe, 2002). While a good system of social support and high self esteem can protect against the impact of an adverse early environment or chronic stress (Andrews and Brown, 1993; Brugha et al., 1997; Bifulco et al., 2002c), those with little or no social support are as likely to develop a mood disorder as those who come from a violent home (Yule et al., 2000).

The influence of the early environment on adult stress responsivity can also be demonstrated in rats. There is a natural variation that exists among dams in the quality of maternal care shown to their pups (Caldji et al., 1998; Francis et al., 1999a). That is, while all dams lick and groom their pups, there is a range in the quantity of licking and grooming behavior displayed by the mother. This range of behavior seen in the mothers

appears to influence the development of neuroendocrine systems mediating stress responsivity in her pups (Caldji et al., 1998; Caldji et al., 2000; Liu et al., 2000a). For example, the adult male offspring of dams that lick and groom frequently (HIGH LG), display a reduced startle response, higher ambulation in the open field and lower overall stress induced plasma corticosterone (CORT) response to an acute stress compared to the offspring of dams that lick and groom less frequently (LOW LG) (Caldji et al., 1998). However, the influence of maternal care in response to a chronic stress regimen has yet to be examined. This may provide a valuable model to study individual differences in vulnerability to the damaging effects of chronic stress.

In this study, we sought to examine the influence of the early life environment on the individual vulnerability to a regimen of chronic unpredictable stress in rats. Until now, little attention has been paid to any long term behavioral or neuroendocrine consequences after the termination of the stress exposure. However, there is evidence that some forms of psychopathology may develop not as an immediate response to stress exposure, but rather as a failure to adapt and return to homeostasis upon removal of the stress (Warshaw et al., 1993; McEwen, 2000a). Therefore, to assess the long term impact of chronic stress, we employed a six week regimen of chronic variable stress (CVS) on the offspring of high and low LG dams, followed by behavioral and plasma corticosterone testing. Testing was conducted between one and 12 weeks post stress exposure. Further, due to the predominance of mood and anxiety disorders in women, we used the female offspring of high and low LG dams in order to specifically assess the role of the early environment on CVS in females.

4.1.2 Experimental Design

Animals

Maternal behavior was observed in Long Evans dams during five 75-minute periods over the first 8 days postpartum. Mothers were scored for licking and grooming as well as nursing posture every three minutes. The licking and grooming scores were tallied for the entire 8 day period and the offspring of mothers that licked and groomed at least one standard deviation above or below the mean were taken as high and low LG offspring respectively. Rat pups were left with their mothers until day 21, at which point they were weaned and caged in pairs in standard (dimensions 60 x 30 x 25cm) polypropylene cages and left undisturbed until approximately 3 months of age when the stress regimen began. All animals were maintained on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol.

All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Stress Regimens

Animals were randomly assigned to four groups: Offspring of *high* LG mothers subjected to the variable stressor regimen (HVS) (n = 9), offspring of *high* LG mothers that received no stress (HNS) (n = 9), offspring of *low* LG mothers with variable stress (LVS) (n = 8) and offspring of *low* LG mothers with no stress (LNS) (n = 8). All animals

in the variable stress groups were subjected to the same random 6-week schedule of variable unpredictable stressors that is described in Study 1.

Behavioral Testing

Open Field (Days 14 - 27 post CVS)

Open field testing was conducted in the same manner as described in Study 1.

Acoustic Startle (days 25 - 26 post CVS)

The acoustic startle response was tested in the same manner as described in Study 1.

Voluntary Sucrose Consumption (Day 29 – 36 post CVS)

Animals were housed individually in the colony room and given access to both a 1% sucrose solution as well as water, for a period of 7 days. Both sucrose and water bottles were weighed and refilled daily in order to assess consumption.

Corticosterone Stress Recovery (day 44 post CVS)

Corticosterone samples were obtained in the same manner as described in Study 1.

Determination of plasma corticosterone

The protocol for the plasma corticosterone assay is described in Study 1.

4.1.3 Results

Open Field

Total Squares Crossed

Total squares crossed during 30 minutes of testing for days 1 and 2 was analyzed using a threeway ANOVA (maternal care x stress x day) (Figure 17). CVS exposure increased the number of squares crossed in both the offspring of high and low LG dams

(F (1, 30) = 6.53, p = .0159). There was a main effect of day indicating that the squares crossed decreased from day 1 to day 2 in all groups (F (1, 30) = 9.09, p = .0052).

Time series:

Time series analysis is described in Study 2.

Figure 18b and 18c display the reactivity (*A*-value) and habituation (*B*-value) estimates respectively, for the first day of open field testing. Compared to HNS (χ^2 (1) = 18.5, P < .01), LNS (χ^2 (1) = 13.9, P < .01) and HVS (χ^2 (1) = 17.4, P < .01) animals, LVS rats displayed a significantly lower initial reactivity. LVS animals also displayed a significantly slower rate of habituation than HNS (χ^2 (1) =14.3, P < .01), HVS (χ^2 (1) = 9.5, P < .01), and LNS (χ^2 (1) = 9.0, P < .01) rats.

The initial reactivity and the rate of habituation for the second open field exposure are displayed in Figure 19b and 19c respectively. While the initial reactivity of LVS animals was only lower than that of the HVS (χ^2 (1) = 5.7, p < .05) group on the second day of testing, the rate of habituation remained significantly decreased compared to HNS (χ^2 (1) = 14.8, p < .01), LNS (χ^2 (1) = 7.5, p < .01) and HVS (χ^2 (1) = 10.5, p < .01) animals.

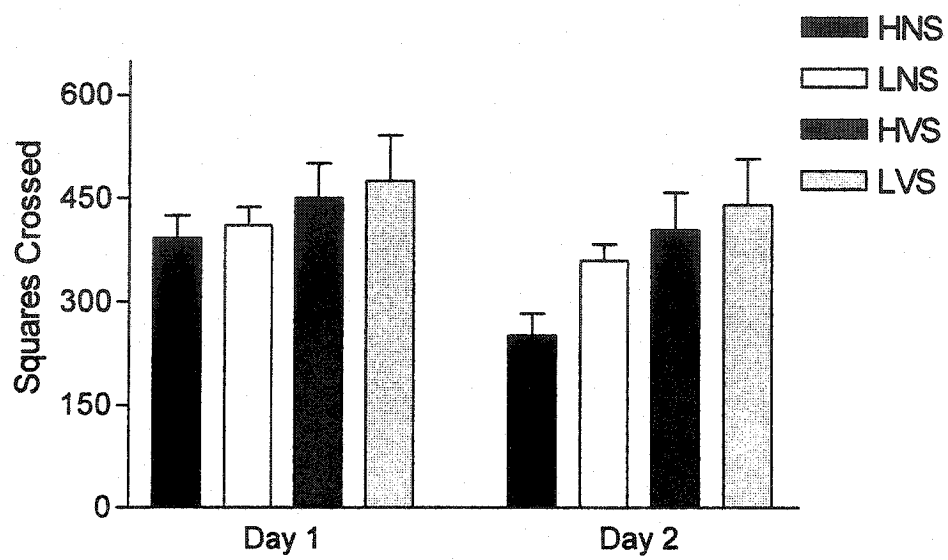

Figure 17. Mean (+SEM) total squares crossed in the open field on day one and two of testing, in control and CVS-exposed female offspring of high and low LG dams. High Control (HNS, n=9), Low Control (LNS, n=8), High-CVS (HVS, n=9), Low-CVS (LVS, n=8). *Main effect of CVS exposure.

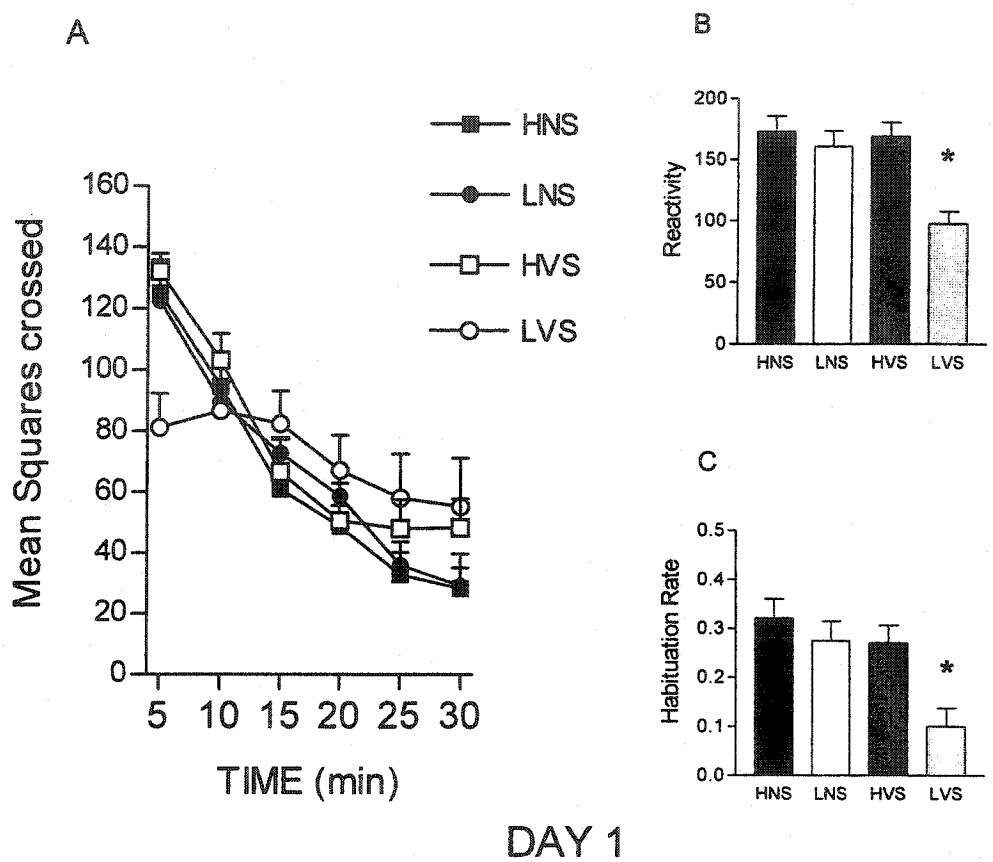

Figure 18. Day 1 Open Field, A) Mean (+SEM) squares crossed in 5-min bins, B) Initial reactivity, *LVS Significant ($p < .05$) relative to all other groups.C) Rate of habituation, *LVS significant relative to all other groups ($p < .05$). Female High control offspring (HNS, n=9), High CVS offspring (HVS, n=9), low control offspring (LNS, n=8), Low CVS offspring (n=8).

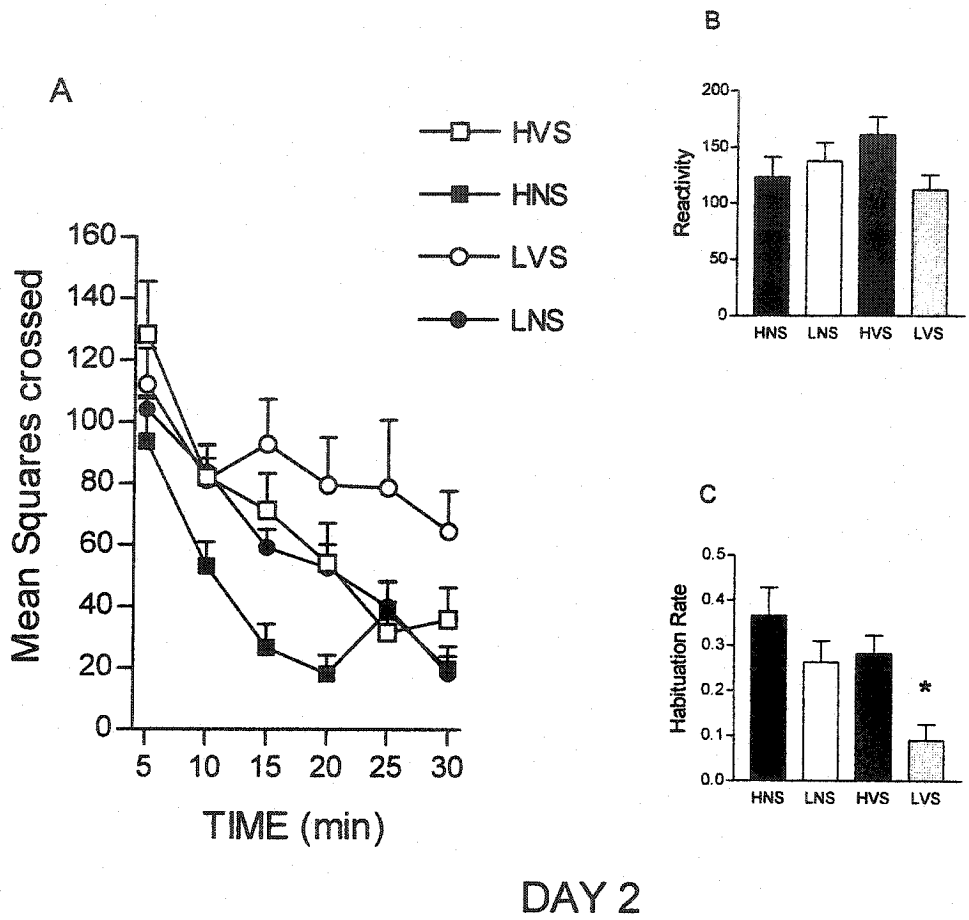

Figure 19. Day 2 Open Field, A) Mean (+SEM) squares crossed in 5-min bins, B) Initial reactivity, *LVS Significant ($p < .05$) relative to all other groups.C) Rate of habituation, *LVS significant relative to all other groups ($p < .05$). Female High control offspring (HNS, n=9), High CVS offspring (HVS, n=9), low control offspring (LNS, n=8), Low CVS offspring (n=8).

Acoustic Startle Response

Figure 20 presents the results of the acoustic startle response. The 3 way (maternal care x stress x intensity) repeated measures ANOVA revealed a significant three-way interaction ($F(4, 120) = 5.09$, $p=0.0008$). At higher decibels, the low offspring showed a higher startle response relative to the high offspring ($F(1, 30) = 10.43$, $p=0.0020$). CVS exposure decreased the startle response of low offspring ($F(1, 30) = 12.00$, $p= 0.0010$), but did not affect the offspring of high LG dams.

Voluntary sucrose consumption

To test for anhedonia, voluntary sucrose consumption was calculated as the ratio of sucrose:water consumed in 24 hours, over 7 days of testing and is displayed in Figure 21. A threeway repeated measures ANOVA (maternal care x stress x day) revealed that the offspring of high LG mothers consumed a greater ratio of sucrose:water than the offspring of low LG mothers ($F(1, 29) = 12.62$, $p=.0013$). The difference in sucrose consumption between the high and low offspring was significant at every day except for the first day of testing. Chronic variable stress did not change the preference for sucrose in either the high or low offspring. To ensure that any effect of sucrose consumption was not due to differences in weight, animals were weighed at the start of the sucrose test. There were no weight differences between any of the groups.

Plasma corticosterone

The response to a 20-minute restraint stress is shown in Figure 22. The ANOVA revealed a maternal care x time interaction ($F(7, 210) = 5.38$, $p<0.0001$) indicating that while both high and low offspring had a similar CORT response throughout the restraint stressor itself, the high offspring had elevated CORT during recovery at sample times 60,

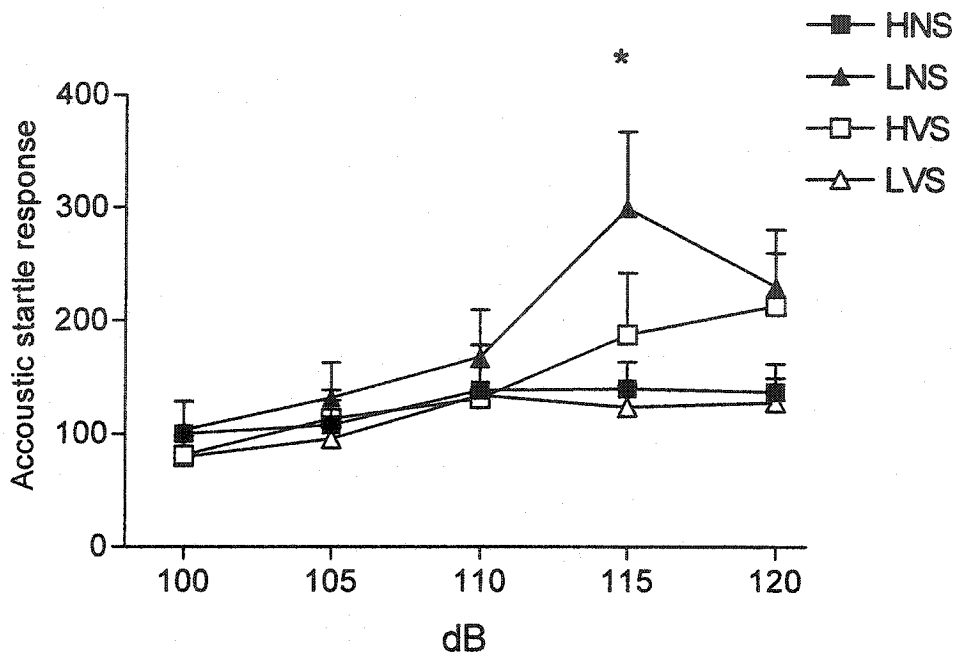

Figure 20. Mean (+SEM) acoustic startle response in control and CVS-exposed female offspring of high and low LG dams. High control (HNS, n=9), High-CVS (HVS, n=9), Low control (LNS, n=8), Low-CVS (LVS, n=8). *LNS significant ($p < .05$) relative to HNS and LVS.

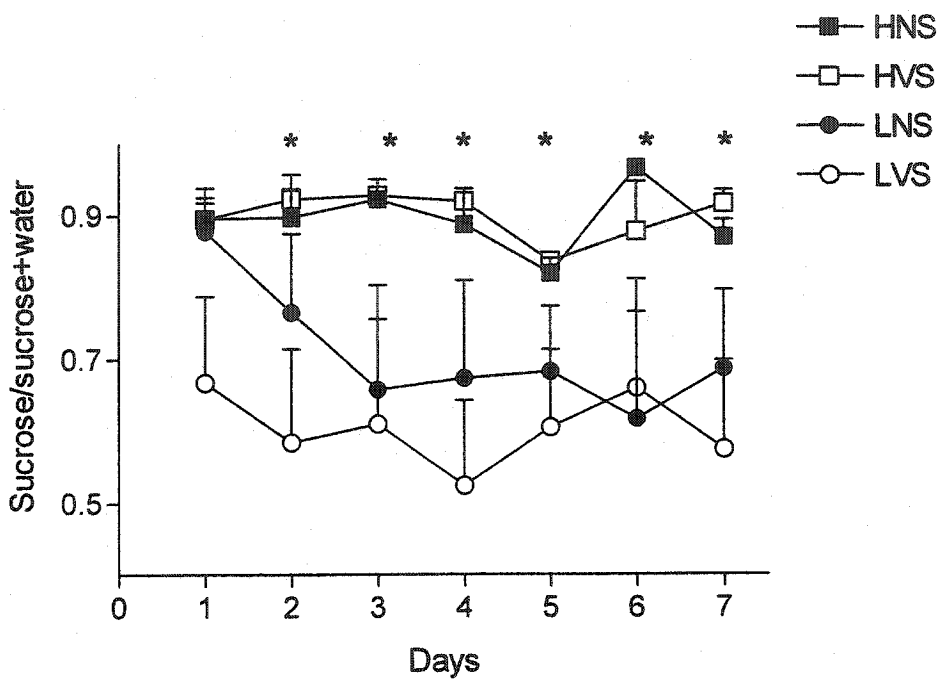

Figure 21. Mean (+SEM) sucrose preference in the control and CVS-exposed female offspring of high and low LG dams. High control (HNS, n=9), High-CVS (HVS, n=9), Low control (LNS, n=8), Low-CVS (LVS, n=8).

*Significant main effect of maternal care.

80 and 140 min. The chronic stress decreased the CORT response to restraint stress in both the high and low offspring ($F(1, 30) = 32.20$, $p<0.0001$). There was also a significant chronic stress x time interaction ($F(7,210) = 2.97$, $p=0.0055$) indicating that while chronic stress decreased the corticosterone response at both the baseline and stress time points, the CORT response during recovery was only partially reduced.

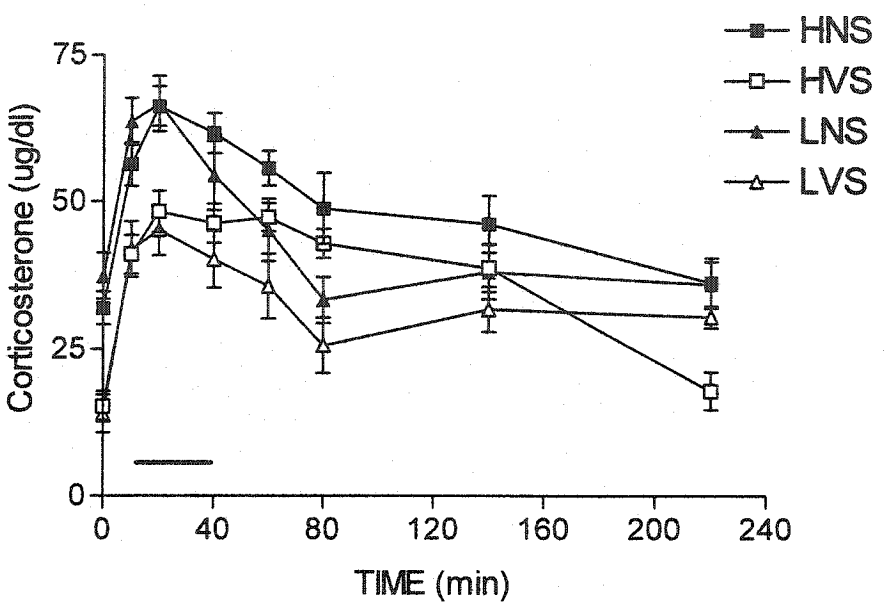

Figure 22. Mean (+SEM) plasma corticosterone response to an acute restraint stress (black bar) in control and CVS-exposed female offspring of high and low LG dams. High control (HNS, n=9), High -CVS (HVS, n=9), Low control (LNS, n=8), Low-CVS (LVS, n=8). *Significant maternal care by time interaction, high offspring have elevated cort relative to low offspring ($p < .05$). *Significant main effect of CVS ($p < .05$).

4.1.4 Discussion

This study explored the long-term effects of chronic unpredictable stress on behavioral and neuroendocrine responses in female rats. In particular, we examined the offspring of dams that displayed high or low levels of licking and grooming toward their pups in the first week of life, to assess the contribution of the early life environment on the vulnerability to damaging effects of CVS in adulthood. The findings of the present study support the hypothesis that the early environment exerts an influence on locomotor activity and habituation in the open field, acoustic startle reflex, voluntary sucrose consumption and the blood plasma corticosterone response to an acute restraint stress. In addition, the impact of CVS was dependent on early environment maternal behavior and the testing procedure.

Open Field

The analysis of open field behavior revealed that exposure to CVS increased locomotor activity in all animals, and that in general, activity decreased on the second day of testing indicating a degree of familiarity with the context. In addition, this paper applied a statistical model based on random effects regression, to investigate more closely the influence of maternal care and CVS exposure on the behavioral patterns in the open field. The model allowed us to analyze both the initial reactivity as well as the rate of habituation to the open field on both days of exposure. This analysis revealed that on the first day of testing, the LVS animals displayed a blunted initial reactivity, as well as a slower rate of habituation, in comparison to all other groups. On the second day, the

initial reactivity of LVS animals was comparable to those of the other groups, however, the rate of habituation remained significantly slower. Thus, the lower rate of habituation in this group is not simply the result of a blunted initial reactivity. Of interest, CVS did not affect the initial reactivity or the rate of habituation in the offspring of High LG dams on either day.

These results are consistent with recent reports of differential housing on open field behavior. Rats housed in isolation display enhanced activity to environmental novelty, whereas environmental enrichment accelerates the habituation to novelty (Larsson et al., 2002; Schrijver et al., 2002). Exposure to an acute stress further decreased the habituation rate in the impoverished animals on the first day, but only affected the activity of the enriched animals on the third day of testing. Taken together, the results from this study and from Larsson et al (2002) provide evidence that variations in the early environment (pre and post weaning) appear to affect systems regulating habituation in adulthood.

Both environmental enrichment and maternal care have been shown to induce structural and neurochemical changes in the hippocampus that result in enhanced learning and memory (Frankland et al., 1998; Van Praag et al., 2000; Liu et al., 2000b). Given the role of the hippocampus in the detection of novelty and habituation, since chronic stress has been shown to damage the hippocampus, it is possible that the low habituation rate seen in the LVS animals may be due to stress-induced damage of the hippocampus. The interaction between CVS exposure and maternal care in open field activity suggests that a high LG dam may impart protection against the impact of chronic stress on hippocampal integrity to her offspring. In fact, it has already been shown that the male offspring of

High LG dams have higher levels of hippocampal GR mRNA and NMDA receptors (Liu et al., 1997d; Liu et al., 2000b). It is therefore possible that the higher expression of these physiological markers in these animals would increase the capacity of hippocampal dependent behaviors, such as context recognition and habituation, and provide a buffer to the damaging effects of CVS on the hippocampus.

Acoustic Startle Reflex

In support of previous work in this lab, the results of this study showed that the offspring of low LG dams demonstrated a greater startle response than the offspring of high LG dams. Surprisingly, exposure to CVS decreased the ASR in the low offspring, and was without effect in the high-LG animals. One possible explanation for the effects of CVS on the startle response of the low LG offspring is the method of restraint in the startle apparatus. The restrainers used in the startle apparatus are identical to those used in the CVS protocol, suggesting that the animals may have at least partially habituated to the testing context. A decrease in the ARS of the high LG offspring after CVS may not be evident due to a floor effect (i.e., the already low ARS in that group).

Voluntary Sucrose Consumption

Anhedonia, as evidenced by a reduction in preference for a sweet solution, is commonly found under conditions of acute and chronic stress (Cheeta et al., 1994; D'Aquila et al., 1994; D'Aquila et al., 1997). In this study, the offspring of the low LG mothers exhibited lower sucrose preference than the offspring of high LG dams. The effect of maternal care on sucrose preference is consistent with previous reports that maternally separated monkeys display a diminished preference for sweetened water than

control monkeys (Paul et al., 2000). This is not likely due to neophobia since the non-stressed low LG offspring consumed the same amount of sucrose solution as the non-stressed high LG offspring on the first day of testing, supporting the notion that they only failed to demonstrate the typical increase in preference normally associated with a choice for the sweetened water. CVS decreased the preference for sucrose on the first day, but only in the offspring of low LG dams. Exposure to CVS had no effect on sucrose preference in the high offspring, indicating that the maternal care of this group may have afforded a degree of protection against the effects of CVS on this measure. In addition, the decreased preference for sucrose seen in the low LG offspring is not likely due to a lack of extinction of neophobia. If a lack of extinction of neophobia were the cause of this effect, then one would expect the consumption to remain constant throughout the testing period. However, the sucrose preference in the low LG offspring actually declined from day 1 to day 2.

Corticosterone Stress Recovery

The interaction between the early environment and time of blood plasma sampling revealed that the offspring of High LG dams had a slower corticosterone recovery from the restraint stress than the offspring of Low LG mothers. These results are surprising in that previous work from this laboratory has shown that in male rats, the offspring of High LG dams display both a lower corticosterone response as well as a faster recovery to restraint stress than Low offspring. However, these paradoxical effects of the early environment on corticosterone levels in male and female rats have also been found following maternal separation and handling (Shanks et al., 1995; Sutanto et al., 1996). While handled male rats have lower restraint induced corticosterone levels than

non-handled and maternally separated animals, the opposite is true of female rats (Shanks et al., 1995; Sutanto et al., 1996).

In addition, a finding of considerable interest was that CVS decreased basal and restraint induced plasma corticosterone levels in both HVS and LVS animals. This was surprising in that chronic stress is most often associated with an increase in corticosterone. There are however some instances where exposure to stress or trauma lead to a fast feedback of the HPA axis, resulting in lower corticosterone values (Yehuda et al., 1990; Blanchard et al., 1993; Albeck et al., 1997). It appears as though this is the case for post traumatic stress syndrome (PTSD), where PTSD patients have been shown to have lower baseline and stress induced cortisol relative to control values (Yehuda et al., 1990; Yehuda, 1997). This phenomenon has also been shown in rodents where a subgroup of chronically stressed subordinate rats displayed a reduced corticosterone response to an acute restraint stress (Blanchard et al., 1993; Albeck et al., 1997). The fact that all of the CVS exposed rats in our study displayed this downregulation, suggests first, that the effects of CVS supercede any protective effects of the early environment on corticosterone levels, and also brings forth the possibility that this chronic stress paradigm may provide a useful tool to study the mechanism for fast feedback of HPA activity in vulnerable populations.

The results from this study confirm the hypothesis that differences in the early neonatal environment influence processes affecting levels of hedonia and anxiety, and also modify the impact of CVS in adult female rats. The effect of maternal care on the influence of CVS is interesting in that it demonstrates a natural variation in individual susceptibility to the damaging effects of stress and trauma that is reflective of the clinical

literature (Brugha et al., 1997; Heim et al., 1997a; Bifulco et al., 2002b). Thus, the high and low offspring provide a valuable tool to elucidate environmentally regulated mechanisms that protect against the damaging effects of chronic stress on behavior.

It is noteworthy that all of the testing was conducted following the termination of the stress protocol, and that the effects were enduring three months after the stress was terminated. An immediate behavioral and physiological reaction to stress exposure is normal it is the lack of recovery that is required in order to establish the existence of pathology. However, the many animal studies conducted in the field of chronic stress assess its behavioral and physiological consequences either during or directly following the stress exposure (Rodriguez Echandia et al., 1988; Blanchard et al., 1998; McKittrick et al., 2000; Duncko et al., 2001a). We cannot assume that a normal stress reaction and a prolonged stress response with a lack of improvement are mediated by the same mechanism. Therefore it is important to assure that the stress course that is being evaluated models the state of a disorder in a clinical population and not a normal process that leads to eventual recovery.

The finding that female offspring of high and low LG dams differ from their male counterparts in respect to their plasma corticosterone response to an acute restraint stress, highlights a significant gender effect of maternal care on HPA functioning. The smaller behavioral stress response in the female offspring of High LG dams, as measured by open field activity and acoustic startle, appears to be incongruous with their plasma corticosterone response to the restraint stress. This raises an important void in the animal literature with regard to the effects of stress on females. Given the eminent role of early life experiences on the susceptibility to develop mood disorders, and the predominance of

females in the same population, it is possible that the early life environment may have a different impact on the development of the HPA axis, thereby producing a vulnerability to the effects of chronic stress in females. However, this gender difference in corticosterone may be misleading as we have not yet measured levels of CBG. In fact, there are known gender differences in plasma CBG levels, which may at least partially explain our results. Conversely, gender differences stemming from the early environment's effect on plasma corticosterone may represent a divergence between neuroendocrine functioning and behavioral measures of anxiety in female rats, and could have implications for the predominance of females in mood disorder patients. In addition, since no records were taken of estrous cycles, there is a need for further investigation into the possible impact of hormonal states on these behaviors.

4.2 Study 5: Chronic Variable Stress in Male Rats: Modulation by Early Life Maternal Care

4.2.1 Introduction

Recently, it has been shown that adaptation to chronic stress may be mediated in part by serotonergic activity. For example, individual differences in coping styles are associated with differences in the postsynaptic 5-HT 1A receptors (Korte et al., 1996), and selectively bred lines of rats that differ in hippocampal 5-HT1A receptors display markedly different behavioral levels of anxiety (Gonzalez et al., 1998). Of interest, preliminary data in our lab shows that the offspring of high LG dams have higher 5-HT transporter levels in the PFC and the amygdale than the offspring of low LG dams (unpublished data), suggesting that these two groups of animals may have underlying differences in serotonergic activity.

Accordingly, in this study, we sought to examine the influence of the early life environment on the individual vulnerability to a regimen of chronic unpredictable stress in rats. Until now, little attention has been paid to any long-term behavioral or neuroendocrine consequences after the termination of the stress exposure. However, there is evidence that some forms of psychopathology may develop not as an immediate response to stress exposure, but rather as a failure to adapt and return to homeostasis upon removal of the stress (Warshaw et al., 1993; McEwen, 2000b). Therefore, to assess the long term impact of chronic stress, we employed a six week regimen of chronic variable stress (CVS) on the offspring of high and low LG dams, followed by behavioral testing. Due to evidence from animal and clinical studies that postsynaptic 5-HT 1A receptors play an important role in the pathophysiology of mood disorders, we also investigated the effects of the maternal care and chronic variable stress on hippocampal

5-HT 1A receptor autoradiography. Testing was conducted between one and 12 weeks post stress exposure.

4.2.2 Experimental Design

Animals

Maternal behavior was observed in Long Evans dams during five 75-minute periods over the first 8 days postpartum. Mothers were scored for licking and grooming as well as nursing posture every three minutes. The licking and grooming scores were tallied for the entire 8 day period and the offspring of mothers that licked and groomed at least one standard deviation above or below the mean were taken as high and low LG offspring respectively. Rat pups were left with their mothers until day 21, at which point they were weaned and caged in pairs in standard (dimensions 60 x 30 x 25cm) polypropylene cages and left undisturbed until approximately 3 months of age when the stress regimen began. Only male offspring of high and low LG dams were used in this experiment. All animals were maintained on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol.

All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Stress Regimens

Animals were randomly assigned to four groups: Offspring of *high* LG mothers subjected to the variable stressor regimen (HVS) (n = 10), offspring of *high* LG mothers that received no stress (HNS) (n = 10), offspring of *low* LG mothers with variable stress

(LVS) (n = 10) and offspring of *low* LG mothers with no stress (LNS) (n = 10). All animals in the unpredictable stress groups were subjected to the same random 6 week schedule of variable unpredictable stressors described in Study 1.

Behavioral Testing

Open Field (days 3 -8 post chronic stress)

Open field testing was conducted in the same manner as described in Study 1.

Acoustic Startle (days 10 – 12 post chronic Stress)

The acoustic startle response was measured in the same manner as described in Study 1.

Voluntary Sucrose Consumption (days 16 – 22 post chronic stress)

Sucrose consumption was measured in the same manner as described in Study 4.

Elevated Plus Maze (days 24 – 26 post chronic stress)

Elevated plus maze testing was conducted in the same manner as described in Study 2.

Hippocampal 5-HT 1A autoradiography (day 38 post chronic stress)

The brains of 4 animals in each group were taken to perform receptor autoradiography. After decapitation, brains were rapidly removed, frozen on dry ice, and stored at -80 °C for later use. Coronal sections of 16 μm were cut on a cryostat microtome and thaw-mounted onto polylysine-coated glass slides. The slide-mounted sections were preincubated for 10 min at room temperature in 50 mM Tris-HCI buffer (pH 7.4) containing 4 mM $CaCl_2$ and 1nM $MgCl_2$. Then, sections were incubated for 1 hr at room temperature, in the same buffer with 100nM [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralyn ([^{3}H]8-OH-DPAT; specific activity-229 = 229 Ci/mmol, Perkin

Elmer) and 10 µl SB 269970 (Sigma). Non-specific binding was determined by incubation of adjacent sections in the presence of 10 µM WAY 100635 (Sigma). Subsequently, sections were washed two times for 5 min at 4 °C in preincubation buffer, dipped in water and air dried. The radiolabeled sections were then apposed to Kodak Biomax MS film for 10 weeks at room temperature. The films were developed in Kodak developer and Kodak Rapid Fixer. Autoradiograms were analyzed with a computer-assisted image analysis system (MCID) by an experimenter who was blind to the treatment of the rats. After shading and background correction, the optical density of [3H]8-OH-DPAT binding was determined and converted into femtomoles of radioligand bound per milligram of brain tissue according to curves that were determined with (Amersham) [3H]Microscale standards coexposed with the labelled sections. For each brain region, 4 sections were measured bilaterally and the average of the 8 measurements was used for the final statistical analysis.

4.2.3 Statistical Results:

Open Field:

Total Squares Crossed:

The ANOVA revealed a stress x day interaction ($F(1, 36) = 4.23$, $p = 0.047$) indicating that the total squares crossed by the non-stressed control animals decreased from day 1 to day 2 ($F(1, 36) = 14.58$, $p = .0005$), whereas, there was no change in the number of squares crossed in the CVS exposed animals ($F(1, 36) = .29$, $p = .5914$). There was no effect of maternal care on the number of squares crossed in the open field on either day of testing (Figure 23).

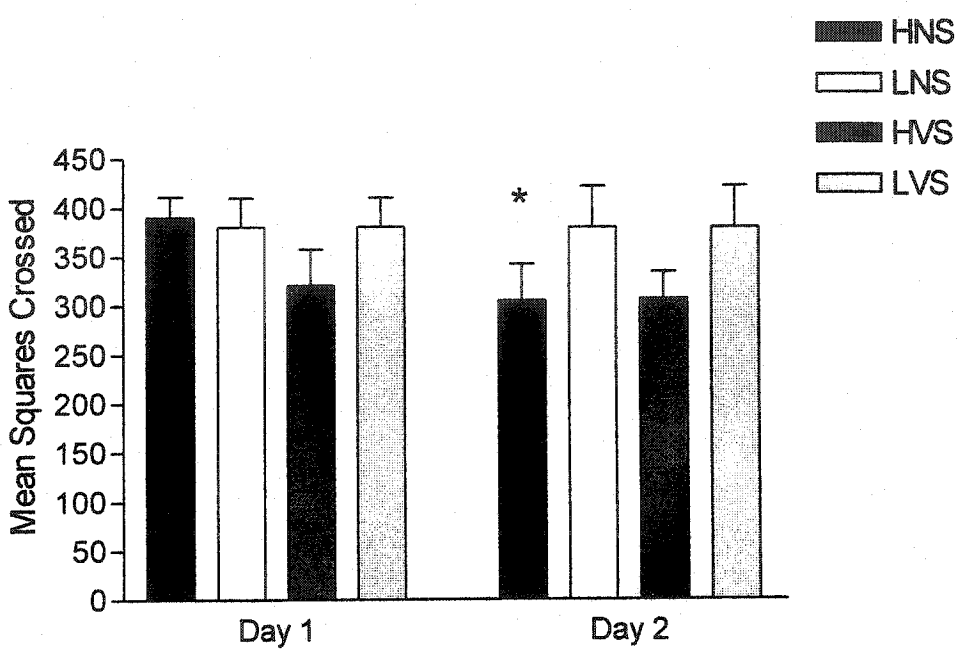

Figure 23. Mean (+SEM) total squares crossed in the open field in control and CVS-exposed male offspring of high and low LG dams. High control (HNS, n=10), High-CVS (HVS, n=10), Low control (LNS, n=10), Low-CVS (LVS, n=10). *Significant decrease in squares in from day 1 to day 2 in control animals.

Time series

Time series data were analyzed as described in Study 2..

Figure 24b and 24c display the reactivity (*A*) and habituation (*B*) estimates respectively, for the first day of open field testing. The initial reactivity of LVS (χ^2 (1) = 4.5, $p < .05$) as well as LNS (χ^2 (1) = 6.9, $P < .01$) rats was higher in comparison to HVS animals on the first open field exposure. There were no differences between any of the groups in the rate of habituation.

The initial reactivity and the rate of habituation for the second open field exposure are displayed in Figure 25b and 25c respectively. On the second day of testing, LVS rats displayed a higher initial reactivity to the open field in comparison to HNS animals (χ^2 (1) = 4.0, $P < .05$). There were no differences between the groups in the rates of habituation on the second day.

Inner/outer squares:

The ratio of inner to outer squares crossed is normally used as an index of fear or anxiety because a fearful rat will typically circle the outside perimeter of the open field while avoiding the center squares. Figure 26 illustrates the ratio of inner to outer squares crossed in the open field for the total 30 minutes over two days of testing. The ANOVA revealed a three-way interaction between rearing, stress and day of testing ($F (1, 36) = 9.62$, $p = 0.0037$). Closer analysis of the interaction revealed that while the offspring of high LG mothers displayed a greater ratio of inner to outer squares crossed than the offspring of low LG mothers ($F (1, 36) = 4.50$, $p = 0.04$), exposure to chronic stress

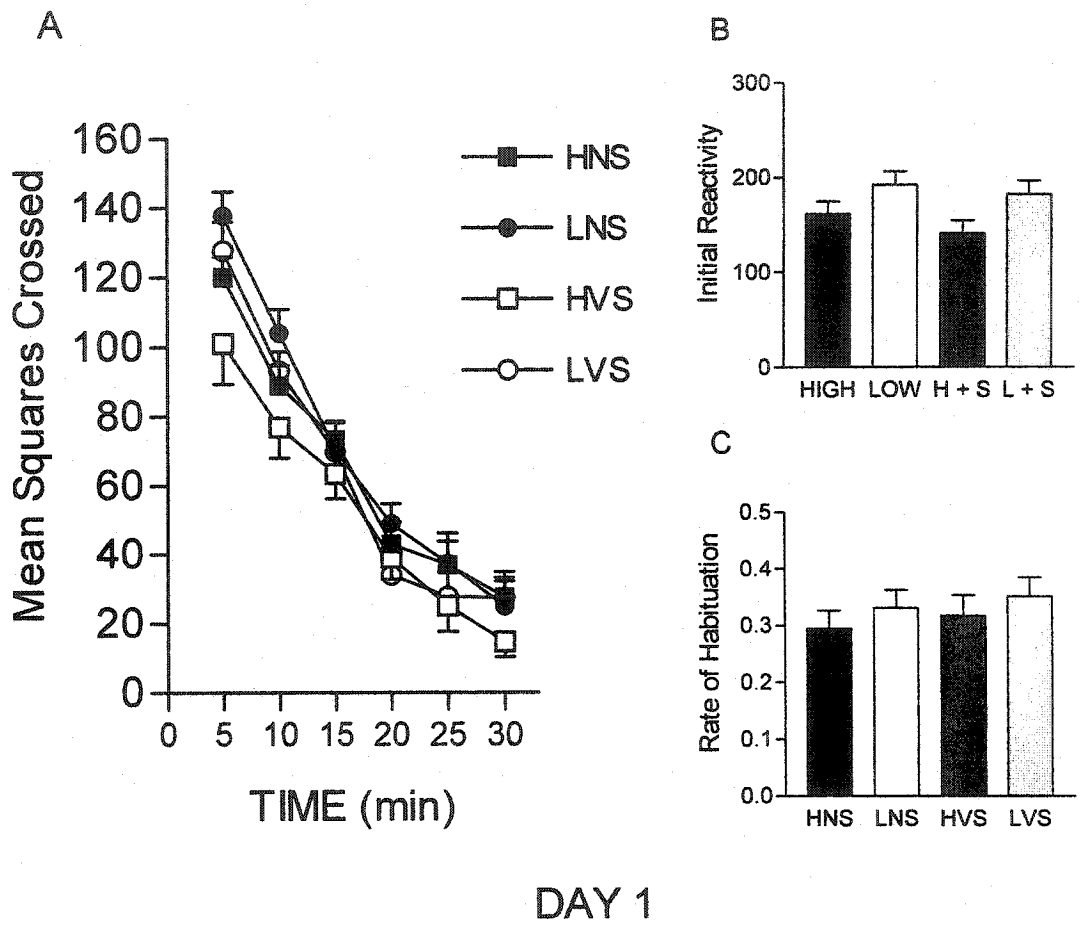

Figure 24.Day 1, Open field. A) Mean (+SEM) squares crossed in 5-min bins. B) Initial Reactivity, C) Rate of Habituation. High control (HNS, n=10), High-CVS (HVS, n=10), Low control (LNS, n=10), Low-CVS (LVS, n=10).

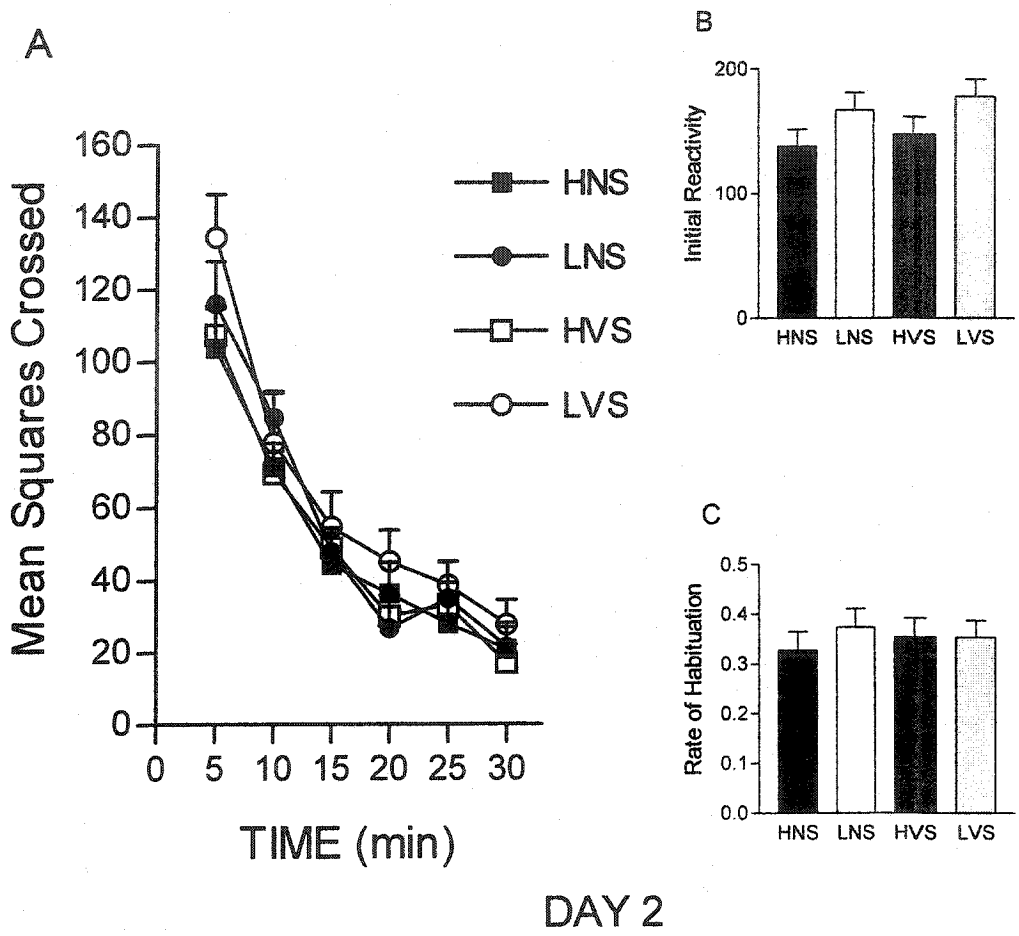

Figure 25. Day 2, Open field. A) Mean (+SEM) squares crossed in 5-min bins. B) Initial Reactivity, C) Rate of Habituation. High control (HNS, n=10), High-CVS (HVS, n=10), Low control (LNS, n=10), Low-CVS (LVS, n=10).

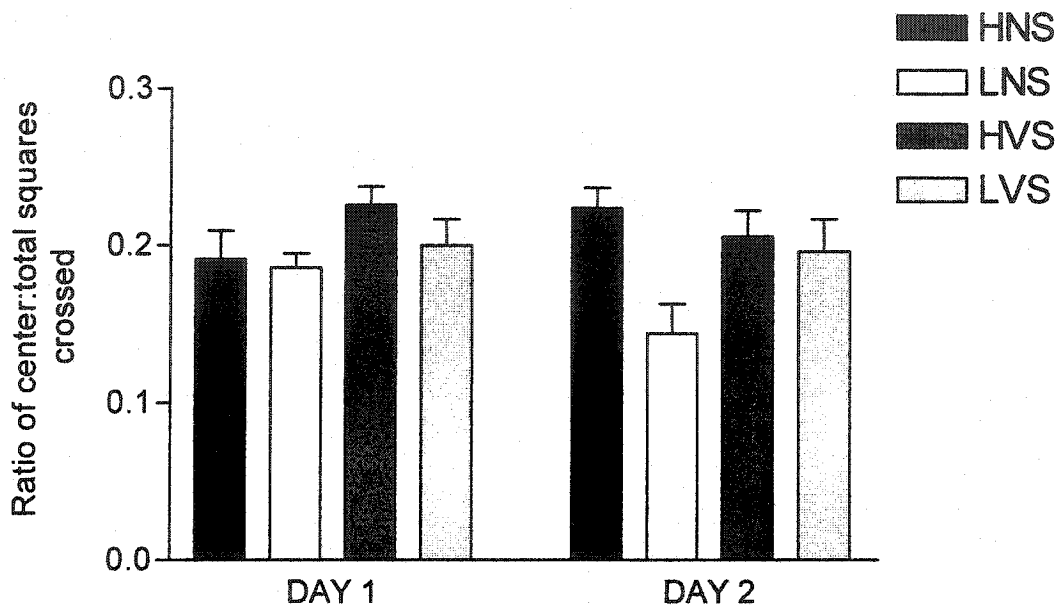

Figure 26. Mean (+SEM) ratio of innner to total squares crossed in the open field in control and CVS-exposed male offspring of high and low LG dams. High control (HNS, n=10), High-CVS (HVS, n=10), Low control (LNS, n=10), Low CVS (LVS, n=10). *Significant maternal care by CVS interaction, high control cross greater ratio of inner squares than low control, this effect is eliminated by CVS. *Significant maternal care by day interaction, the effect of maternal care only emerged on day 2.

eliminated the differences caused by the quality of the early environment ($F(1, 36) = 0.79$, $p = 0.37$). Further, the effect of maternal care only emerged on the second day of testing ($F(1, 36) = 7.85$, $p = 0.007$), indicating that familiarity with the environment may be necessary to differentiate the behavior of these two groups of animals.

Acoustic Startle Response:

The acoustic startle response of all of the groups is displayed in Figure 27. A threeway ANOVA (maternal care x stress x db) confirmed that there was no effect of maternal care or CVS on the startle response in this experiment.

Voluntary Sucrose Consumption:

Voluntary sucrose consumption was not affected by maternal care or CVS exposure as assessed by the threeway (maternal care x stress x day) ANOVA (data not shown).

Elevated Plus Maze:

The total activity in the open arms of the elevated plus maze is represented in Figure 28. A two-way ANOVA revealed that CVS exposure increased open arm activity ($F(1, 35) = 9.73$, $P = .0036$) in both the offspring of high and low LG dams. Although HNS rats tended to spend more time on the open arms than LNS rats, this effect was not significant.

5 – HT 1A receptor autoradiography:

Separate two-way (maternal care x stress) ANOVAs were performed for the dentate gyrus (DG), CA1, CA2, and the CA3 regions (Figure 29). In the DG, there was a higher optical density (look up how this is reported) in the offspring of low LG dams (F

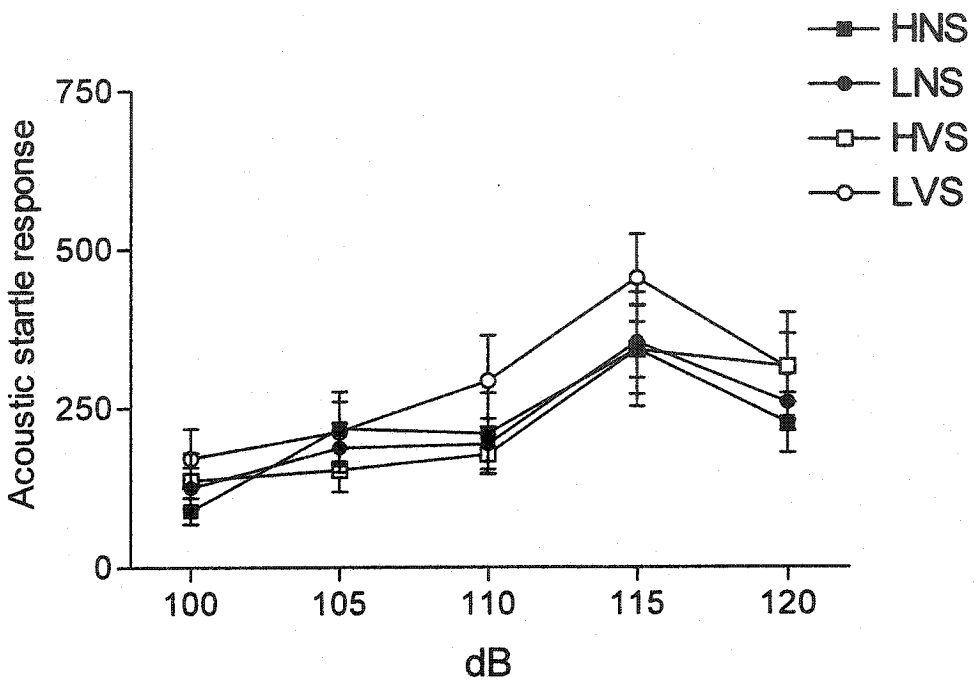

Figure 27. Mean (+SEM) acoustic startle response in control and CVS-exposed male offspring of high and low LG dams. High control (HNS, n=10), High CVS (HVS, n=10), low control (LNS, n=10), low-CVS (LVS, n=10).

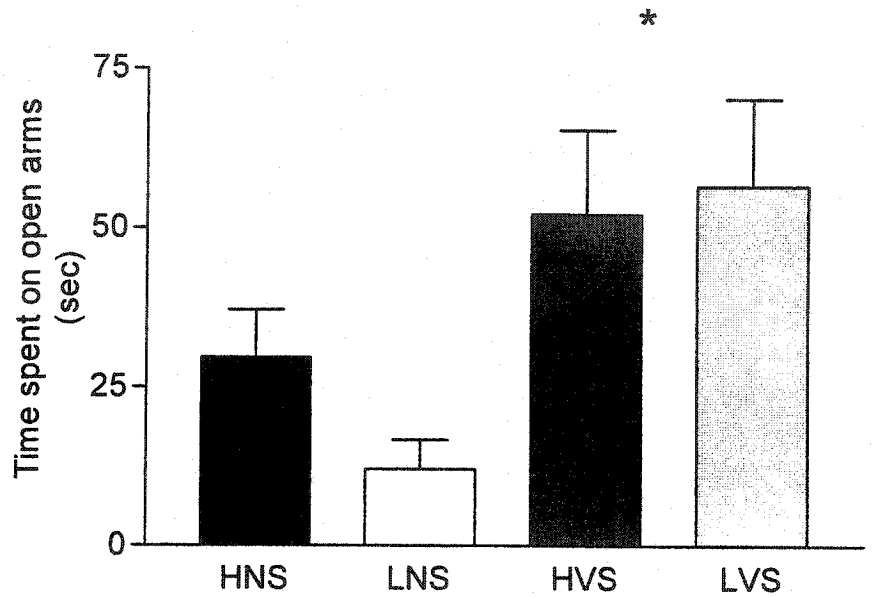

Figure 28. Mean (+sem) time spent in the open arms of the elevated plus maze in control and CVS-exposed male offspring of high and low LG dams. High control (HNS, n=10), High-CVS (HVS, n=10), low control (LNS, n=10), low-CVS (LVS, n=10). *Significant main effect of CVS.

(1, 25) = 8.80, p = .0066), and chronic stress increased binding in both high and low offspring (F (1, 25) = 28.48, p < .0001). In the CA1 region, 5HT 1A binding was increased in CVS exposed rats in comparison to non-stress controls (F (1, 20) = 7.45, P = .0129). There was no main effect of maternal care or CVS exposure on 5HT 1A binding in the CA2 region of the hippocampus, however, in CA3, binding was higher in the offspring of low LG dams (F (1, 20) = 10.38, P = .0034), and this was not affected by CVS. The level of 5-HT 1A binding in the dentate gyrus of the hippocampus was significantly correlated to the time spent in the open arms of the elevated plus maze (r = .58, p = .0296) (Figure 30).

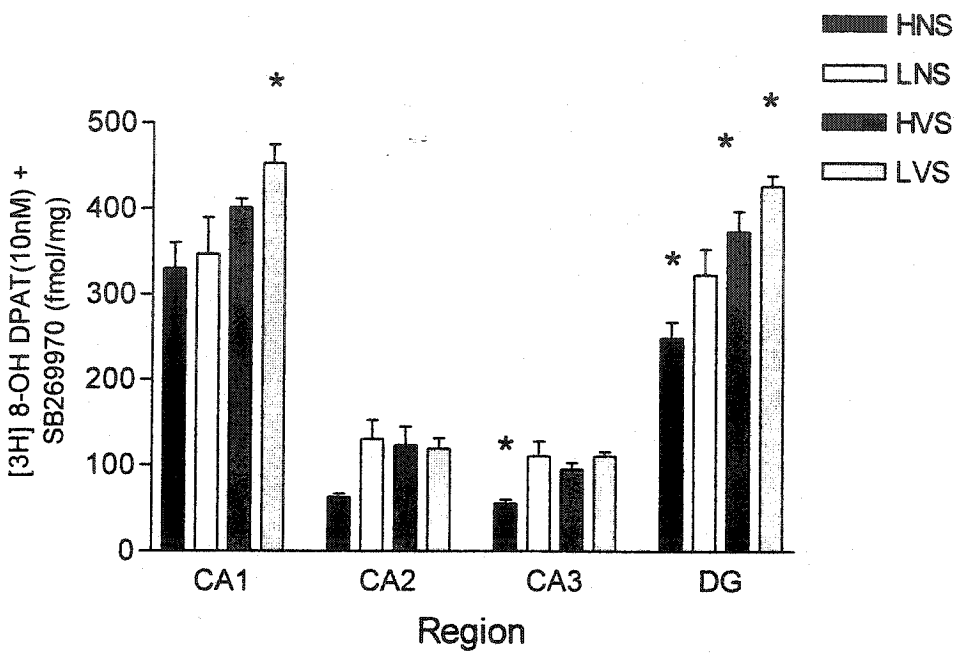

Figure 29. Mean (+SEM) 5-HT 1A receptor autoradiography in the hippocampus of control and CVS-exposed male offspring of high and low LG dams. High control (HNS, n=5), High-CVS (HVS, n=5), Low control (LNS, n=5), Low-CVS (LVS, n=5). Dentate gyrus (DG): *Significant effect of maternal care ($p < .05$), and significant effect of CVS ($p < .05$). CA1:*Significant effect of chronic stress. CA3: Significant effect of maternal care ($p < .05$).

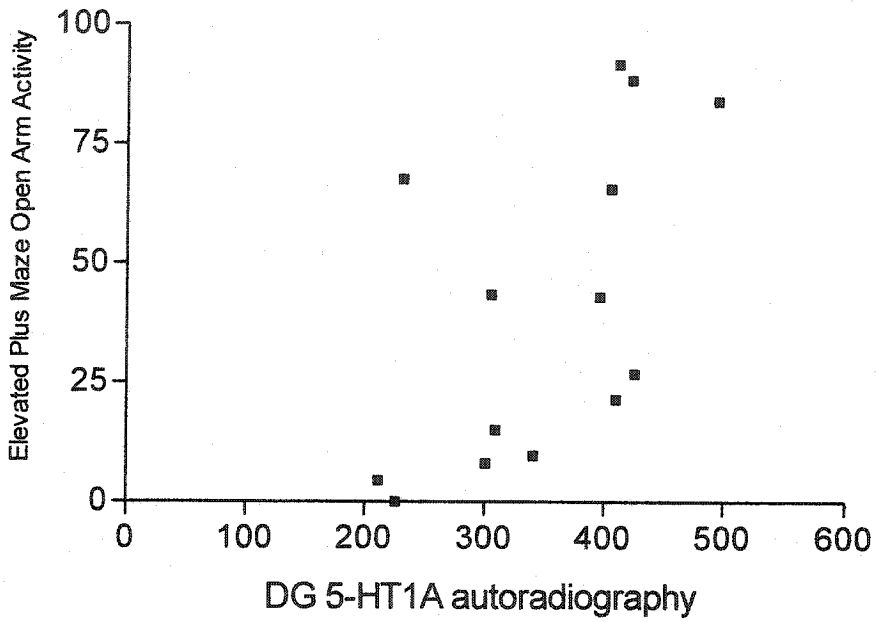

Figure 30. Correlation of dentate gyrus (DG) hippocampal 5-HT 1A autoradiography with time spent on the open arms of the elevated plus maze in control and CVS-exposed male offspring of high and low LG dams. ($r = .58$, $p = .0296$).

4.2.4 Discussion

In this study, we replicated the finding that the male offspring of high LG dams spent more time in the center of an open field than the offspring of low LG mothers, an indication that these animals are less anxious relative to the low offspring. The emergence of this behavioral difference on the second rather than the first day of testing was consistent with the results of previous work conducted in our laboratory suggesting that open field behavioral differences between high and low LG offspring are greatest in less stressful testing environments. That is, the greatest behavioral differences between the offspring of high and low LG dams have been found when the testing has been conducted in a black open field apparatus, in a dimly lit room (presumably less stressful). When the environment is brightly lit and presumably more stressful, as in this study, the differences in behavior are eliminated, at least on the first open field exposure. It is possible that some degree of familiarity with the arena on the second day of testing may have been necessary to draw out the differences seen previously in our lab when using a dark open field.

Neither maternal care nor exposure to CVS affected the rate of habituation in the open field. These results are in contrast to the effects of CVS on the female offspring of high and low LG dams and may reflect a gender difference in the maternal influence on habituation. However, these results are in contrast to those in chapter 2 and 3 where CVS exposed male rats did show a decrease in the rate of habituation.

However, in this study, maternal care appeared to affect the initial reactivity to the open field. Both CVS exposed and non-exposed offspring of low LG dams displayed a higher initial reactivity to the open field on the first day of testing. On the second open

field exposure, only the CVS exposed low offspring continued to show an enhanced initial reactivity in comparison to HNS rats. The effect of maternal care on the initial reactivity to the open field resembles that seen in the olfactory bulbectomized (OBX) rat model of antidepressant activity (Mar et al., 2000; Mar et al., 2002). The OBX rat also displays an elevated initial reactivity to a novel open field, which was not reversed by antidepressant treatment.

Neither maternal care nor CVS affected the startle reactivity to an acoustic (audible) stimulus. While the reason for this is unclear, it is possible that the rats were not adequately stimulated due to their previous experience with the restrainers due to the restraint stress during the stress regimen. Conversely, the acoustic startle test may not be sensitive to the effects of early environment or CVS exposure in male rats.

The elevated plus maze is commonly used to assess anxiety in rodents. While there was a trend for the offspring of high LG dams to spend more time on the open arms of the elevated plus maze (EPM), this effect was not significant. Given the previous characterization of the offspring of high LG dams to be less anxious, the results of the EPM were unexpected. However, the behavior in this test may have also been influenced by the brightness of the testing environment. As argued above, we have suggested that behavioral differences between high and low offspring are diminished in bright, presumably more stressful environments. In addition, it is possible that the duration of this test was too short for the animals to habituate to the environment and that differences in open arm activity may have increased (to significance) with continued testing.

The majority of studies using chronic stress have found an anxiogenic effect on EPM behavior. In contrast, we found that CVS exposure increased open arm activity in

all of our animals. While this effect was unexpected, there are previous reports of an anxiolytic effect of chronic stress on the EPM, as well as other behavioral measures of anxiety, consistent with our finding (D'Aquila et al., 1994; Rossler et al., 2000). While the reason for this effect in our experiment is uncertain, there is evidence to suggest that it may have been mediated by CVS induced changes in serotonergic activity. For example, it has been shown that behavior on the elevated plus maze can be modified by drugs acting at the 5-HT 1A receptor (Barf et al., 1996; Overstreet et al., 2003). While selective 5-HT 1A receptor agonists administered directly into the median raphe are anxiogenic, administration into the dorsal hippocampus increases the time spent on the open arms of the EPM, and this effect is reversed by the selective antagonist WAY-100135 (Guimaraes et al., 1993; File et al., 1996; File and Gonzalez, 1996; Menard and Treit, 1998; Overstreet et al., 2003). In this study, we found an upregulation of 5-HT1A receptor binding in the hippocampus of our stressed animals, and this effect was significantly correlated with the open arm activity on the EPM. Although we did not test this hypothesis directly, given the evidence at hand, it is possible that the anxiolytic effect of stress was mediated by a CVS induced upregulation of 5-HT 1A receptors in the dorsal hippocampus.

Other studies have also reported an increase in hippocampal 5-HT 1A receptor binding after stress exposure, which was instrumental in the behavioral adaptation to the stress exposure (Graeff et al., 1996). Animals that are restrained for 2 hrs display decreased open arm activity on the elevated plus maze 24 hrs after the restraint (Guimaraes et al., 1993). The effect of the restraint stress on open arm EPM activity is reversed by bilateral microinjection of 8-OH-DPAT, a selective 5-HT 1A agonist, into

the dorsal hippocampus. In addition, chronic stress has been shown to increase hippocampal 5-HT1A receptor binding, an effect that was further enhanced by antidepressant treatment (Papp et al., 1994). Further, hippocampal 5-HT 1A receptors have been linked to the coping style that an animal will display in the face of distress (Korte et al., 1996; Meerlo et al., 2001). Animals with lower 5-HT 1A receptor binding tend to display a passive coping style, while those with greater binding are more active. This theory fits with our finding that those rats with higher 8-OH DPAT binding in the hippocampus spent more time in the open arms of the elevated plus maze.

While the exact mechanism for this effect is still unknown, the majority of evidence to date suggests that glucocorticoids released during stress modulate 5-HT 1A receptor functioning in the hippocampus. For example, while low doses of corticosterone decrease post-synaptic effects of 5-HT1A stimulation in the hippocampus (most probably via stimulation of MR), higher doses more closely relevant to states of stress increase hippocampal 5-HT1A stimulation (through a GR mediated-mechanism) (Joels and de Kloet, 1992). However, the relationship between corticosterone and 5-HT1A receptors is dependent on the type of stressor, as well as individual characteristics such as gender. First, repeated restraint stress has been shown to increase hippocampal 5-HT1A receptors, yet, cold exposure caused a reduction in 5-HT1A receptor-dependent behavioral responses (Mendelson and McEwen, 1991; Zamfir et al., 1992). Given the chronic nature of the stress regimen in this study, one would expect the CVS exposed animals to have an increase the release of corticosterone, and therefore a decrease 5-HT 1A binding. One possible explanation for the increase in 8-OH DPAT binding seen in this experiment is that the variability in our stress regimen may have also acted as partial

environmental enrichment in these animals. Indeed, environmental enrichment has been shown to increase both GR and 5-HT 1A receptor mRNA in the dorsal hippocampus (Rasmuson et al., 1998; Dahlqvist et al., 1999). However, since this effect has been found previously after a variable stress regimen (Papp et al., 1994), there remains a potential for the upregulation of hippocampal 5-HT 1A receptors from chronic stress that may be dependent on the type of stress or another unknown factor. As mentioned previously, it has been hypothesized that adaptive coping to chronic stress is associated with an upregulation of hippocampal 5-HT 1A receptors (Graeff et al., 1996). Therefore, another potential explanation lies in the possibility that the change in hippocampal 5-HT 1A receptor status depends on the animal's capacity to adapt to the stress, which can be influenced not only by the type of stressor, but also by genetics, gender, the quality of the early life environment and previous history of stress.

In conclusion, the current experiment provides further evidence for an effect of maternal care on exploratory behavior that is eliminated by CVS exposure. The reason that this effect was not found in the EPM, a traditional test of anxiety in rodents is unclear, however it may have been due to the anxiogenic conditions of the testing environment. This experiment also showed an anxiolytic effect of CVS on the EPM that was correlated with 5-HT 1A receptor binding. The effect of CVS both in the open field and in the EPM was similar for the offspring of high and low LG dams, suggesting that maternal care did not affect the vulnerability to the damaging effects of CVS.

CHAPTER 5 (STUDY 6, 7 & 8)

5 Contextual and Cued Fear Conditioning: Mediation by Maternal Care and Chronic Variable Stress

Emma Spreekmeester, Ettie Grauer, Joseph Rochford and Michael Meaney

5.1 Introduction

Evidence from both clinical and basic studies has implicated the hippocampus in the pathophysiology of major depression. Several studies have found a reduced hippocampal volume in depressed subjects relative to healthy controls (Sheline et al., 1996; Sheline et al., 1999; Bremner et al., 2000; Sheline, 2000). Elevated glucocorticoid levels that mirror those seen in depression have been associated with hippocampal atrophy in rats and primates (Sapolsky et al., 1990; Sapolsky, 2000). MacQueen et al (2003) found that patients with major depression show dysfunction on hippocampal dependent memory recollection that predates any detectable hippocampal volume reductions. Patients presenting with either a first episode or a recurrent episode of depression scored lower than healthy controls on hippocampal memory function. However, only patients that had a history of previous depressive episodes displayed a reduced hippocampal volume. It has been hypothesized that this decrease in hippocampal function may result from prolonged exposure to elevated levels of glucocorticoids in depressed patients. In addition, detectable morphological changes may not be necessary to cause functional impairment (MacQueen et al., 2003).

Depressed patients have a biased autobiographical memory (Brittlebank et al., 1993). When asked to recall memories associated with positively valenced, negatively valenced or neutral words, depressed patients have difficulty identifying personal memories in response to the positively valenced cues. Given that corticosterone affects learning and memory (Lupien et al., 1997; Golier and Yehuda, 1998; Wolf et al., 2001;

Abercrombie et al., 2003), it is possible that emotional memories may be stored differently in depressed patients.

The relationship between learning and mood has also been shown in "healthy" subjects. Grillon (2002) has shown that individuals that fail to make an association between an unconditioned stimulus (mild electrical shock) and a conditioned stimulus display higher levels of anxiety and avoidance behavior. A failure to recognize a relevant cue or environmental that is associated with a stressor may enhance the perceived unpredictable nature of the stress, and therefore increase the individual stress response.

In rats, it has been shown that maternal care affects adult stress responsivity as measured by plasma corticosterone, as well as hippocampal morphology (Liu et al., 1997c; Liu et al., 2000b), suggesting that these animals may react differentially to a hippocampal-dependent behavioral task. Contextual fear conditioning is dependent on the hippocampus, as lesions to this area disrupt freezing to a context associated with an electric shock (Phillips and LeDoux, 1992). However, while learning the association between a cued (auditory) conditioned stimulus and an electric shock is spared by hippocampal lesions, it is disrupted by lesions to the amygdala (Phillips and LeDoux, 1992). Therefore differences in hippocampal or amygdaloid functioning can be assessed with the use of a contextual or cued conditioning paradigm. Given the differences in hippocampal and amygdala morphology (see Table 1 in Introduction) as well as the effects of chronic variable stress on the offspring of high and low LG dams, in this series of experiments we sought to assess the effects of maternal care on 1) contextual fear conditioning, 2) auditory fear conditioning and 3) contextual fear conditioning following exposure to chronic variable stress.

5.2 Study 6: The Impact of Early Maternal Care on Contextual Fear Conditioning in Adult Male Rats.

5.2.1 Experimental Design:

Animals

Male Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were the offspring of high and low LG/ABN mothers, originally obtained from Charles River Canada Inc. (St Constant, Que). Maternal behavior was observed in Long Evans dams during five 75-minute periods over the first 8 days postpartum. Mothers were scored for licking and grooming as well as nursing posture every three minutes. The licking and grooming scores were tallied for the entire 8 day period and the offspring of mothers that licked and groomed at least one standard deviation above or below the mean were taken as high and low LG offspring respectively. Rat pups were left with their mothers until day 21, at which point they were weaned and caged in pairs in standard (dimensions 60 x 30 x 25 cm) polypropylene cages and left undisturbed until approximately 3 months of age when the stress regimen began. All animals were maintained on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol.

All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the Douglas Hospital Animal Care Committee.

Apparatus

Fear conditioning occurred in two plastic conditioning chambers with a clear plastic top. Each conditioning chamber was placed inside a wooden sound attenuating box containing a 40 watt light bulb and a ventilation fan mounted inside. The front of each wooden box was left open to allow visualization of the rats by a video camera. The rod floors were wired to a shock generator and scrambler to deliver a 1-second, 0.8 mA shock. Beneath each conditioning chamber was disposable cardboard that was replaced after each rat. The rods of each chamber were rinsed with water after each rat. A speaker was mounted on the inside of the conditioning chambers, but was not used for this experiment. A video camera was placed approximately 2 feet away from each rat, and the shocks were delivered manually via an extended wire from outside of the testing room.

Fear Conditioning Procedure

Fear Conditioning

Rats were transported in groups of 2 to the testing room, and each rat was placed inside a conditioning chamber. Following 2 minutes of acclimatization to the chamber, rats were randomly exposed to 5, 1-sec shocks of 0.8 mA, in a 25 min period, with an average inter trial interval of 4 min.

Extinction

For each of 3 days following the conditioning day, rats were placed in the same chamber in which they had received conditioning for 12 min. Their behavior was recorded by a video camera and later coded for freezing behavior. Rats were determined to be freezing in the absence of any movement except for breathing.

5.2.2 Results

A two-way ANOVA with maternal behavior as a between-subjects factor and day of extinction as a repeated measure was used to assess freezing behavior (Figure 31). The ANOVA revealed a main effect of day, suggesting that freezing behavior declined with days of extinction ($F(2, 36) = 22.43$, $p < .0001$). While there was no main effect of maternal behavior, further analysis with simple effects tests revealed that the offspring of high LG dams displayed more freezing behavior than the offspring of low LG dams on the second day of extinction ($F(1, 54) = 4.93$, $p = .0335$).

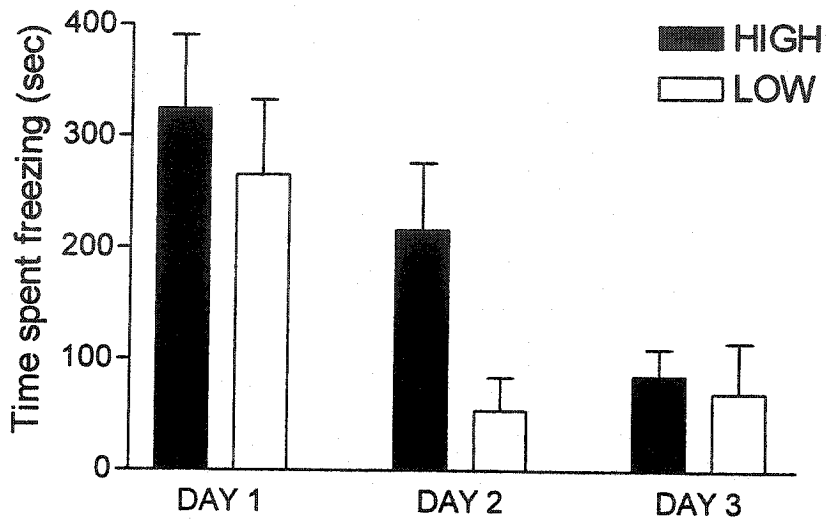

Figure 31. Mean (+SEM) time spent freezing in the context previously associated with footshock in the offspring of high and low LG dams.

5.3 Study 7: The Impact of Early Maternal Care on Auditory Fear Conditioning in Adult Male Rats.

5.3.1 Experimental Design:

Animals

Male Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were the offspring of high and low LG/ABN mothers, originally obtained from Charles River Canada Inc. (St Constant, Que). Maternal behavior was observed in Long Evans dams during five 75-minute periods over the first 8 days postpartum. Mothers were scored for licking and grooming as well as nursing posture every three minutes. The licking and grooming scores were tallied for the entire 8 day period and the offspring of mothers that licked and groomed at least one standard deviation above or below the mean were taken as high and low LG offspring respectively. Rat pups were left with their mothers until day 21, at which point they were weaned and caged in pairs in standard (dimensions 60 x 30 x 25cm) polypropylene cages and left undisturbed until approximately 3 months of age when the stress regimen began. All animals were maintained on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol.

All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the Douglas Hospital Animal Care Committee.

Apparatus

Fear conditioning apparatus was the same as that described in Study 6. However, in addition, a 20-second tone at 80 dB was used as the auditory stimulus, delivered through a speaker mounted on the inside of the conditioning chambers. A video camera was placed approximately 2 feet away from each rat, and the shocks were delivered via an extended wire from outside of the testing room.

Fear Conditioning Procedure

Pre-exposure

Rats were transported in groups of 2 to the testing room. Each rat was placed inside the conditioning chamber and allowed to explore for 20 minutes per session for three days prior to the conditioning day to remove any unconditioned fear to the context. All pre-exposures and testing occurred between 10:00 and 16:00 h.

Fear Conditioning

Rats were placed inside the same chamber to which they had been pre-exposed. Following 2 minutes of acclimatization to the chamber, rats were randomly exposed to 5, 1-sec shocks of 0.8 mA, which were preceded by a 20-sec tone of 80 dB in a 25 min period, with an average inter trial interval of 4 min.

Extinction

Three of the walls and the ceiling of each conditioning chamber were covered in black paper and the rod floors were covered with cardboard and bedding, in order to change the context. In addition, rats were placed in a different chamber to the one they had been conditioned in. Following 2 min of silence, rats were exposed to 10 min of the 80 dB tone in each of the 3 days following the conditioning day. Their behavior was

recorded by video camera and later coded for freezing behavior. Rats were determined to be freezing in the absence of any movement with the exception of breathing.

5.3.2 Results

Freezing during Tone

A two-way ANOVA was performed with maternal behavior as a between-subjects factor and day of extinction as a within-subjects factor. The ANOVA was performed on freezing behavior that only occurred in the presence of the conditioned stimulus (tone); these data are displayed in Figure 32. ANOVA revealed a significant main effect of maternal behavior ($F(1, 16) = 30.47$, $p < .0001$), suggesting that the offspring of high LG dams displayed more freezing behavior than the offspring of low LG dams to the tone. In addition, freezing behavior declined over extinction days ($F(2, 32) = 16.15$, $p < .0001$) in both groups of animals. The interaction term was not significant ($F(2, 32) = 0.68$, $p > .05$).

Freezing before the tone

A separate two-way (maternal behavior x days) ANOVA was performed to assess the extent of conditioning to the context during the two minutes that preceded the tone presentation (see Figure 33). . The ANOVA revealed a significant main effect of maternal behavior ($F(1, 16) = 30.42$, $p < .0001$), indicating that the offspring of high LG dams froze significantly more to the context than did the low LG offspring. Although the extent of freezing to the context appeared to increase over days, particularly in the high LG group, this was not confirmed statistically as both the main effect for day ($F(2, 32) = 2.57$, $p > .09$), and the maternal behavior x day interaction ($F(2, 32) = 2.24$, $p > .12$) were not significant.

The greater degree of freezing exhibited by the high LG group during both the pre-tone and the tone periods suggests that the animals in this group may have been freezing not to the tone, but to the context. To assess this possibility, we conducted an analysis of covariance (ANCOVA), in which freezing prior to the tone served as the covariate and freezing during the tone served as the dependent measure. This analysis revealed that when freeing during the tone was corrected for differences in pre-tone freezing, there were no differences between groups, as the main effect for maternal behavior ($F(1, 15) = 3.28$, $p > .09$) and the maternal behavior x days interaction ($F(2,31) = 0.64$, $p > .50$) were not significant. To provide a rough estimate of the relative strength of conditioning to the tone versus the context, we computed the percentage of time that animals spent freezing during the 2 minutes prior to tone presentation, and the percentage of time animals spent freezing during the 10 minute tone presentation. We then calculated "freezing ratios" of the form:

$$\frac{\text{percent freezing during the tone}}{\text{percent freezing during the tone + percent freezing prior to the tone.}}$$

With this measure a ratio of 0.5 reflects (proportionately) equal freezing to the tone and the context, a ratio above 0.5 reflects relatively more freezing to the tone, and a ratio below 0.5 reflects relatively more freezing to the context. The mean $\pm$ SEM freezing ratios for the low LG animals across the three days of extinction were, respectively: .938 $\pm$ .052, .782 $\pm$ .105 and .807 $\pm$ .106, whereas those for the high LG group were: .732 $\pm$ .048, .727 $\pm$ .066 and .469 $\pm$ .033. A maternal behavior x day ANOVA conducted on the freezing ratio measure yielded a significant main effect of maternal behavior ($F(1, 16) = 9.07$, $p < .001$), a main effect of day ($F(2, 32) = 4.86$, $p < .025$), but no significant interaction ($F(2, 32) = 2.47$ $p > .10$). The maternal behavior main effect suggests that the

relative strength of conditioning to the tone was lower in the high LG group in comparison to the low LG group. The day main effect indicates the relative reduction in freezing to the tone over days.

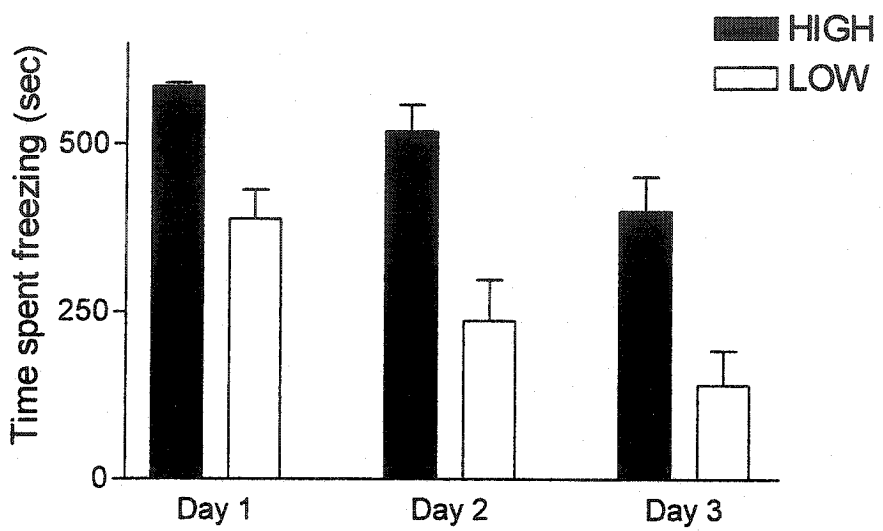

Figure 32. Mean (+SEM) time spent freezing (sec) during presentation of tone previously paired with footshock in male offspring of high and low LG dams. Significant effect of maternal care ($p < .0001$).

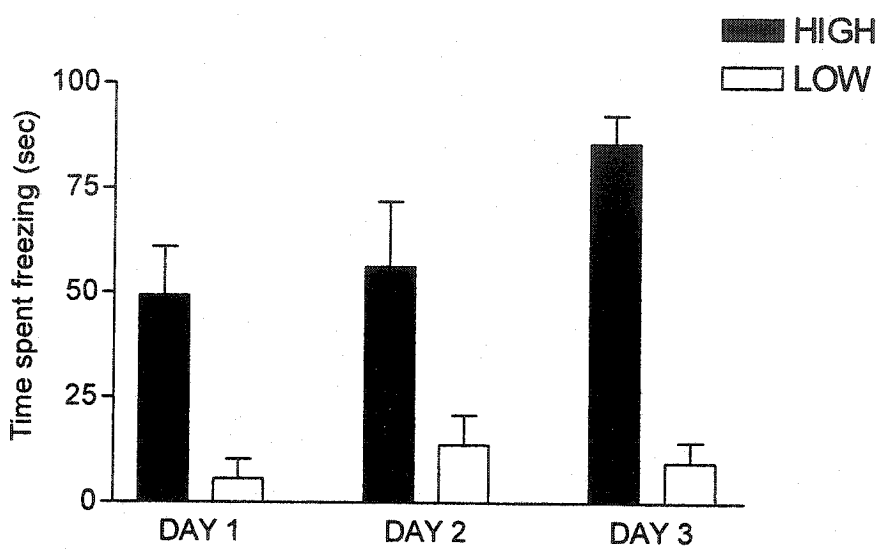

Figure 33. Mean (SEM) time spent freezing (sec) in the absence of the tone, in male offspring of high and low LG dams.

*Significant effect of maternal care ($p > .0001$).

5.4 Study 8: The Effect of Chronic Variable Stress on Contextual Fear Conditioning in the Male Offspring of High and Low LG Dams.

5.4.1 Experimental Design

Animals

Male Long-Evans rats approximately 3 months of age, and weighing between 250-300 g, were the offspring of high and low LG/ABN mothers, originally obtained from Charles River Canada Inc. (St Constant, Que). Maternal behavior was observed in Long Evans dams during five 75-minute periods over the first 8 days postpartum. Mothers were scored for licking and grooming as well as nursing posture every three minutes. The licking and grooming scores were tallied for the entire 8 day period and the offspring of mothers that licked and groomed at least one standard deviation above or below the mean were taken as high and low LG offspring respectively. Rat pups were left with their mothers until day 21, at which point they were weaned and caged in pairs in standard (dimensions 60 x 30 x 25cm) polypropylene cages and left undisturbed until approximately 3 months of age when the stress regimen began. All animals were maintained on a 12 hr light-dark cycle (lights on: 0800-2000 hr) in a temperature-controlled (19-22 ^{0}C) room, and were provided free access to chow and water, except when required by the stress protocol.

All experiments complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Stress Regimens

Animals were randomly assigned to four groups: Offspring of *high* LG mothers subjected to the variable stressor regimen (HVS) (n = 10), offspring of *high* LG mothers that received no stress (HNS) (n = 10), offspring of *low* LG mothers with variable stress (LVS) (n = 10) and offspring of *low* LG mothers with no stress (LNS) (n = 10). All animals in the variable stress groups were subjected to the same random 6 week schedule of variable unpredictable stressors that as described in Study 1.

Apparatus

Fear conditioning chamber was the same as that described in Study 6.

Fear Conditioning Procedure

Fear Conditioning

Rats were transported in groups of 2 to the testing room, and each rat was placed inside a conditioning chamber. Following 2 minutes of acclimatization to the chamber, rats were randomly exposed to 5, 1-sec shocks of 0.8 mA, in a 25 min period.

Extinction

For each of 3 days following the conditioning day, rats were placed in the same chamber in which they had received conditioning, and were allowed to explore for 12 min. Their behavior was recorded by a video camera and later coded for freezing behavior. Rats were determined to be freezing in the absence of any movement except for breathing.

5.4.2 Results

A three-way ANOVA was performed with maternal care and CVS exposure as between subject factors and day of extinction as a within-subject factor (Figure 34). The ANOVA revealed a main effect of maternal care, supporting previous findings that the offspring of high LG dams display more contextual freezing than the offspring of low LG dams ($F(1, 40) = 5.58$, $p = .0284$). In addition, there was a main effect of day ($F(2, 40) = 10.14$, $p = .0003$), as freezing behavior decreased over the three days of extinction. There was no effect of chronic variable stress on contextual conditioning, and the interaction terms were not significant.

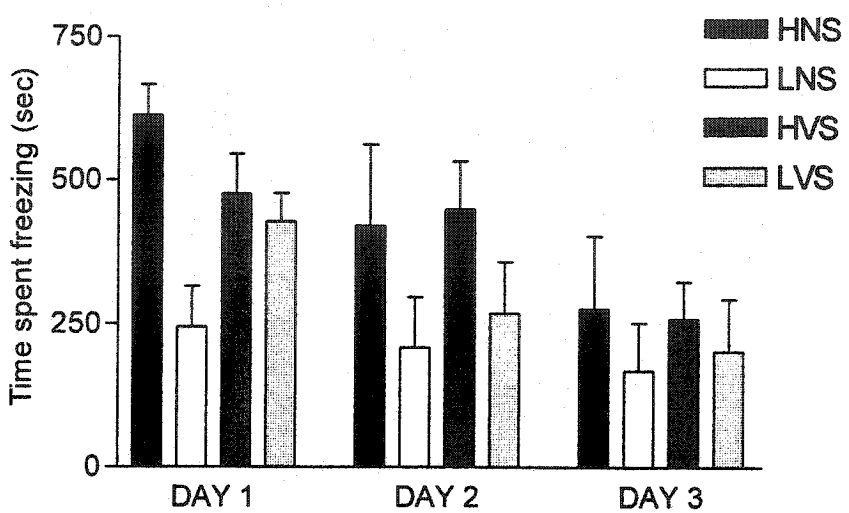

Figure 34. Mean (+SEM) time spent freezing to the context previously associated with footshock. *Significant main effect of maternal care.

5.4.3 Discussion

This series of studies examined the strength of contextual and auditory fear conditioning in the offspring of high and low LG dams. In addition, the effect of maternal care on contextual conditioning was assessed following the exposure to chronic variable stress. In the contextual conditioning studies, animals were re-exposed to the context in which they had previously received footshock 24, 48 and 72 hrs post-conditioning. The first study revealed that contextual fear conditioning, as assessed by the time spent freezing, was similar in both the offspring of both low and high LG dams 24 hrs post conditioning. However, group differences emerged 48 hrs post-conditioning, when the offspring of low LG dams showed a marked reduction in freezing that was not apparent in the high offspring. At 72 hrs post conditioning, the time spent freezing during re-exposure to the context was again similar in both groups. Differences in freezing behavior that were seen on the second context re-exposure may be due to a greater strength of the context-shock conditioned association. The offspring of high LG dams did display a slightly higher level of freezing on the first re-exposure however, the difference between groups was not significant. Another possible explanation may be that maternal care affects the rate of extinction for a learned aversive stimulus. It has been shown that the formation of US-context associations is mediated by a different mechanism than that required for extinction (Wilson et al., 1995).

Freezing in response to the auditory stimulus (CS) in a novel environment reflects the strength of the US-CS association. The offspring of high LG dams spent significantly more time freezing to the tone than the offspring of low LG dams. However, the animals

also displayed freezing behavior in the absence of the tone, suggesting that some remaining contextual cues may have elicited behavioral conditioning to the context. When freezing to the context (in the absence of the CS) was controlled for, the cued conditioning response was no longer significantly different between the groups, suggesting that the greater conditioned response in this study was also due to a stronger contextual conditioning. Contextual and cued conditioning are mediated in part by different brain regions. That is, lesions of the amygdala interfere with conditioning to a cue and to the context, whereas lesions of the hippocampus only attenuate conditioning to a context (Phillips and LeDoux, 1992). Maternal care has been shown to influence both hippocampal and amygdalar physiology (see Table 1, Introduction). Although the amygdala and the hippocampus are both involved in the formation of US-context association, it appears as though the contextual conditioning may be mediated by maternal care-mediated differences in hippocampal functioning. The similar response seen between the high and low offspring to the cued conditioning in study 7, suggests that the amygdala may not be as strongly implicated in the conditioning paradigms. One would expect that if maternal care-mediated differences in the amygdala were influencing these effects, then there should be a difference between the high and low offspring in the cued conditioning paradigm.

The significant difference in contextual conditioning that was seen between the offspring of high and low LG dams was no longer apparent after exposure to CVS. The effect of stress on conditioning may have been due to changes in hippocampal morphology following CVS. It is known that maternal care affects hippocampal dependent processes and that the offspring of high LG dams have higher levels of

hippocampal glucocorticoid mRNA as well as protein levels, NR2A and NR2B subunits of the NMDA receptor, synaptophysin and N-CAM all of which can contribute to learning. In addition, chronic stress has been shown to cause hippocampal damage (Magarinos et al., 1997; Ohl and Fuchs, 1999; McKittrick et al., 2000; Sandi et al., 2001; McEwen, 2001). Therefore, CVS-induced damage to the hippocampus may have eliminated the effect of maternal care on hippocampal physiology, and in turn, contextual conditioning.

CHAPTER 6

6 GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The present series of experiments were conducted to assess factors thought to influence stress sensitivity: gender, antidepressant treatment and early life environment. Given the gender differences in HPA activity, we hypothesized that CVS may affect female rats differently than male rats. To test this hypothesis, we exposed both male and female rats to 6 weeks of CVS and then examined their behavioral reactivity to novel conditions, as well as their neuroendocrine response to an acute stress. If CVS exposure sensitized the behavioral and neuroendocrine response to stress, then chronically stressed animals should display an exaggerated response to an acute stress. Second, we hypothesized that chronic antidepressant treatment may reverse the effects of CVS exposure differently in male and female rats. To test this, we exposed both male and female rats to 6 weeks of CVS, followed by 4 weeks of fluoxetine treatment. The reactivity and adaptation to novelty as well as the neuroendocrine response to an acute stress was then examined in all groups. Third, we hypothesized that CVS exposure would impact differently on the adult offspring of high and low LG dams. Therefore, we exposed both male and female adult offspring of high and low LG dams to 6 weeks of CVS. All groups were then assessed for their initial reactivity and their rate of habituation to novelty. The neuroendocrine response to an acute stress was also tested in the females, and the 5-HT1A receptor autoradiography was examined in the males. Fourth, we hypothesized that early life maternal care may affect conditioning to a context or cue associated with a mild electric shock. Therefore, we first exposed male high and low LG offspring to a mild electric footshock and then measured the amount of freezing to the context. We then exposed male high and low LG offspring to an auditory cue

associated with a mild electric footshock, and then measured the amount of freezing to the cue in a novel context. Finally, in order to assess the effect of CVS exposure on a hippocampal dependent task, we subjected the male adult offspring of high and low LG dams to six weeks of CVS, followed by a session of contextual fear conditioning.

6.1.1 Gender

The prevalence of depression is approximately doubled in women compared to men (Kessler, 2003). In addition, major depression is associated with the perception of increased experiences of negative life events or stressors, and the use of more emotion-focused rather than the adaptive problem-focused coping styles (McNaughton et al., 1992; Roy-Byrne et al., 1992; Ravindran et al., 1999). Given the gender differences in HPA activity and stress reactivity, it is possible that females are either more prone to identify new predicaments or difficulties as stressful, or use inappropriate coping skills that reduce the probability of successful adaptation. In fact, in Chapter 2, we showed that female rats react differently than males in a novel environment, showing more activity in an open field, and a smaller acoustic startle response. Female rats also have higher basal and stress induced plasma corticosterone levels than males, consistent with the gender difference in HPA activity. However, it has been shown that plasma CBG levels are higher in females (Jones et al., 1972), which were not measured in this study, and therefore the exact amount of corticosterone that was available to the target tissue in this study cannot be determined. Nevertheless, chronically high circulating levels of corticosterone can sensitize the HPA axis and impose damage on the hippocampus, causing a further decrease in negative feedback to HPA functioning (de Kloet et al., 1998b; McEwen and Lasley, 2003). Therefore, the increased activity in the open field,

and higher stress-induced corticosterone levels may indicate vulnerability in females to the impact of chronic stress.

At the initiation of this thesis, little research had been conducted in female animals within the field of chronic stress. Although data on chronic stress in females is now emerging, most of these studies have employed a single repeated stressor, instead of a variable stress regimen, which is known to have a different impact on the ability to adapt (Anisman and Zacharko, 1986; Coplan et al., 1996; Ameli et al., 2001). The CVS regimen used in this thesis produced gender-specific results that were dependent on the test. Although the results from experiment 1 do not support this conclusion, the results from the other experiments in this thesis suggest that CVS exposure decreased habituation in male and female rats. This suggests that CVS may have affected adaptation to a novel environment in a gender-independent manner. Motor habituation in the open field has been associated with hippocampal serotonergic activity (Bidzinski et al., 1998). A p-chlorophenylalanine pretreatment induced a decrease in 5-HT that was linked to an abolishment of motor habituation in the open field. Based on the present study, it is not possible to determine if the CVS-induced changes in open field exploration were due to gender differences in 5-HT activity, or another unknown mechanism.

The results of chapter 2 showed that males have a larger startle response (ASR) than females. Gender differences in ASR have been reported previously, however, the direction of these differences has not been consistent (Stohr et al., 2000; Fullerton et al., 2001; Beck et al., 2002). Exposure to CVS did not significantly affect the males or the females.

A finding of great interest in this study was the contrasting effects of CVS on the plasma corticosterone response to acute restraint stress in males and females. As expected, in the male rats prior exposure to CVS increased corticosterone levels in comparison to control males. However, CVS-exposed females displayed a blunted baseline and restraint stress-induced corticosterone response in comparison to control females. This effect was replicated (as seen in Chapter 4), in the female CVS exposed offspring of high and low LG dams. The CVS induced hypocorticoid seen in the females was surprising since exposure to stress had become synonymous with hypercortisolism. However, hypocortisolism was first described by Yehuda and colleagues (see (Yehuda, 1998) for review) who found a decrease in cortisol levels in patients with post-traumatic stress disorder (PTSD). Of interest, females are also more likely than men to develop PTSD following a traumatic event (Lipschitz et al., 2000; Fullerton et al., 2001). It is now known that patients with atypical depression may also show hypocortisolism (Gold and Chrousos, 1999). In addition, hypocortisolism has also been reported in healthy individuals living under conditions of chronic stress (Friedman et al., 1963; Bourne et al., 1967; Bourne et al., 1968; Mason et al., 1968; Caplan et al., 1979) suggesting that under activation of the HPA axis may be a more widespread phenomenon than previously thought. The few animal studies that have reported hypocortisolism following exposure to chronic stress applied a repeated stressor and allowed long time latencies between the stressor presentations. That is, animals were presented with the stressor once per week for several weeks, suggesting that a common attribute of those studies and the current CVS protocol is the animal's experience of stress over an extended period (Mason et al., 1968; Natelson et al., 1988). While the exact mechanisms that trigger hypocortisolism are

unknown, there are currently several theories that have been proposed such as 1) reduced biosynthesis or depletion at several levels of the HPA axis (CRF, ACTH, cortisol), 2) CRF hypersecretion and adaptive down-regulation of pituitary CRF receptors, 3) increased feedback sensitivity of the HPA axis and 4) morphological changes at different levels of the HPA axis (for review see: (Heim et al., 2000a)). However, we cannot deduce which of these may apply to explain the results of our studies. Still, it is of interest that the CVS-induced hypocortisolism was only found in the female rats, and not in males, suggesting a possible gender-induced vulnerability to CVS. Factors that may contribute to this vulnerability could include gonadal hormonal interactions with HPA or monoamine functioning, or gender differences in coping styles.

It has been shown that the estrus cycle can influence the activity of the HPA axis (Viau and Meaney, 1991). Because we did not track the estrus cycles in our female rats, it is possible that the gender differences in behavior and corticosterone levels were influenced by hormonal variations in the females. Therefore, the influence of both the female and male gonadal hormones on the behavioral and neuroendocrine response to CVS needs to be examined in greater detail in the future.

6.1.2 Chronic Fluoxetine Treatment

It is thought that antidepressant medications normalize mood, at least in part, through their actions on monoamine and neuroendocrine functioning (Barden et al., 1995; Stahl, 1998). Given that the symptoms of depression are thought to be induced in part by exposure to prolonged or severe stress, antidepressant medication might act by reversing stress-induced behavioral and neuroendocrine deficits. In Chapter 3, CVS exposure

decreased the rate of habituation to an open field in both male and female rats, and this effect was reversed by fluoxetine treatment. However, CVS also decreased the initial reactivity to the open field in the male rats only, and fluoxetine treatment was without effect on this measure. Thus, fluoxetine appears to normalize hyperactivity by promoting more rapid habituation. These findings partially replicate those from previous studies from our laboratory using another animal model of antidepressant activity, olfactory-bulbectomized (OBX) rats (Mar et al., 2000; Mar et al., 2002). Olfactory bulbectomy causes an increase in the initial reactivity to a well illuminated (presumably more stressful), but not a low luminance (presumably less stressful) open field, causing the total activity of these rats to increase only under stressful conditions. However, under closer examination of the data, the presumed hyperactivity of the OBX rats in the high luminance open field is in fact the result of a decrease in activity in sham-operated controls under stressful conditions. The decrease in activity seen in the sham-operated controls in the high luminance open field can be seen as an adaptive change in behavior dependent on the context. Therefore, the deficit in OBX behavior appears to result from an exaggerated initial sensitivity that does not permit the animal to inhibit behavior upon introduction to a stressful context. Of interest, however, whereas chronic fluoxetine treatment has no effect on the initial reactivity, it decreases the "hyperactivity" by increasing the rate of habituation to the open field. Therefore, while the OBX rats appear hyperactive at the onset of the open field session, they habituate faster, and decrease activity in comparison to sham-operated animals by the end of the testing period. Of interest, fluoxetine has no effect on OBX behavior under less stressful conditions. Thus, fluoxetine appears to promote the function of coping mechanisms that come into play

after the initial period of reactivity. We did not test our rats under less stressful conditions, and therefore we don't know if the increased activity seen in the CVS exposed animals was due to a decrease in non-stressed control rats under aversive conditions, or, conversely, represents a true CVS-induced hyperactivity.

In addition, there are other details of the open field data from this chapter that merit discussion. The OBX and CVS induced hyperactivity are caused by distinct mechanisms. That is, OBX rats display an elevated initial reactivity, while CVS exposed rats display either no difference in reactivity (in the females), or a reduced initial reactivity (in the males) only on the first open field test. The hyperactivity induced by CVS is instead caused by a decrease in the rate of habituation to the open field over the testing period. This suggests that while both models impair the adaptation to an aversive environment, the mechanisms for this impairment may be distinct. However, it is important to note that chronic fluoxetine treatment increases the adaptive response to the environment through the same mechanism (i.e., by increasing habituation), irrespective of the initial deficit. Another aspect of this data to consider is that in the CVS-exposed female rats, fluoxetine was unable to increase the rate of habituation on the second open field exposure. A possible cause for the slow rate of habituation in CVS-exposed females on the second day is that perhaps the influence of fluoxetine on habituation rates in the females is not strong enough to persist through repeated stressful open field exposures. However, it is also possible that for the female rats, the testing environment had already become familiar on the second day, at least to a certain degree, and therefore lost some of its perceived aversive qualities. This idea may be supported by the OBX studies that demonstrated a lack of antidepressant effects in less aversive open fields. Moreover, it is

possible that fluoxetine may not be as efficacious as other types of antidepressants, on this kind of coping mechanism in female rats. It has been shown that other types of antidepressants (e.g., amitriptyline, desipramine) with a stronger noradrenergic (NE) mechanism of action, also enhance habituation in the open field (Mar et al., 2000). However, it appears as though the antidepressants with greater 5-HT potency were especially proficient on this measure, suggesting that antidepressants with stronger 5-HT pharmacology are more efficacious in promoting "coping" at least in males. It is possible that other antidepressants with a stronger effect on other monoamines (e.g., NE) may be more effective in females. Gender differences in antidepressant efficacy have been found in humans, supporting this idea (Ronfeld et al., 1997; Kornstein et al., 2000). The effect of other antidepressants should be assessed in females to evaluate a possible gender difference in antidepressant induced coping mechanisms.

Activity in the elevated plus maze (EPM) is commonly used to assess anxiety in rodents. Time spent in the open arms is associated with less anxiety and conversely, time spent in the closed arms is linked to more anxiety. Female rats exposed to CVS spent less time in the open arms of the EPM than non-stressed controls, indicating that at least in females, CVS increases anxiety in this test. Chronic antidepressant administration increased the time spent in the open arms in the CVS-exposed rats only, suggesting that fluoxetine reversed the effect of CVS without affecting the behavior of non-stressed control animals. The effect of fluoxetine in the EPM was consistent with the well known anxiolytic effect of SSRIs seen in both animals and humans (Berton et al., 1999; Ballenger et al., 2000; Belzung et al., 2001; Huot et al., 2001). The effect of fluoxetine on open arm activity in the elevated plus maze and the rate of habituation in the open

field may be mediated by different mechanisms. Behavior in the elevated plus maze can be altered by both antidepressants and standard anxiolytics (e.g., benzodiazepines), whereas the rate of habituation in the open field is affected only by antidepressants, indicating that the mechanism controlling habituation is not the same as that controlling levels of anxiety (Mar et al., 2002). Therefore, the results of this study suggest that fluoxetine may exert therapeutic effects via different mechanisms that impact on anxiety as well as the ability to cope in a novel environment. The elevated plus maze was not performed in male rats, therefore it is unknown if CVS or chronic fluoxetine treatment would have gender-dependent effects in this test. It has been shown that maternal separation induces gender dependent effects on the elevated plus maze, however the effects of chronic variable stress and fluoxetine have not been compared across gender (Romeo et al., 2003)

Chronic fluoxetine treatment had differential effects on the results of acoustic startle depending on gender. In female rats, CVS increased the startle response, but fluoxetine treatment was without effect. In male rats, while there was no effect of CVS, chronic fluoxetine treatment decreased the startle response in both CVS-exposed and non-exposed rats. These results first support the findings of chapter 2, that the startle response is affected differently by both gender and CVS exposure. In addition, fluoxetine treatment does not appear to be effective in reversing CVS-induced deficits in female rats. The effects of CVS are in contrast to human studies that have shown an increased startle response in males but not females in response to stress (Beck et al., 2002). However, in accordance with our results, women with assault-related PTSD show an abnormal startle response (Morgan, III et al., 1997). The effect of fluoxetine on the

startle response in males is supported by a study showing that chronic fluoxetine treatment reduced the startle response in OBX rats (Mar et al., 2000). These results highlight the complexity of gender differences in response to stress as well as in response to fluoxetine treatment. In addition, as fluoxetine failed to reverse the CVS-induced increase in ASR seen in females, and decreased the "normal" ASR in males, this antidepressant may not be appropriate to reverse the startle deficit seen in mood disorders.

The results of the corticosterone response to an acute restraint stress in chapter 3 were in contrast to those found in chapter 2. Chronic variable stress did not affect the corticosterone levels following a subsequent acute stress in either male or female rats. The reason for this discrepancy is unclear, and may suggest that the effect of CVS on neuroendocrine functioning is not stable across groups or dependent on an additional unknown variable. As mentioned previously, corticosterone was sampled one month later in these studies due to the month of antidepressant treatment. It is therefore possible that any CVS-induced changes in plasma corticosterone may have normalized within this extended period before blood sampling was performed. While the plasma corticosterone levels were not changed by CVS in male or female rats in this experiment, CVS did alter the results of the other tests indicating that behavior deficits can be induced without concomitant or lasting changes in neuroendocrinology. In addition, fluoxetine treatment had a gender dependent effect on corticosterone levels. The corticosterone response to an acute restraint stress was decreased in fluoxetine treated CVS exposed and non-exposed female rats, while there was no effect of antidepressant treatment in any males.

Fluoxetine treatment had gender and test specific effects. Given the diversity of CVS induced results across gender and behavioral or neuroendocrine tests, it appears that the therapeutic effect of fluoxetine treatment may depend upon individual differences in the underlying pathophysiology. Further experiments would have to be conducted to directly investigate this theory. However, data demonstrating that different classes of antidepressants have a greater therapeutic outcome depending on the subtype of depression or on gender support the notion that the pathophysiology of the individual should be considered in prescribing medication (Kornstein, 1997; Schneider et al., 1997; Stahl, 1998).

6.1.3 Early environment

The quality of the early life environment has been shown to influence the susceptibility to mood disorders (Kendler et al., 1993; Leserman et al., 1996; Heim et al., 1997b). In rats, early maternal care impacts on the development of neural systems that mediate reactivity to stress (Caldji et al., 1998; Francis et al., 1999b; Caldji et al., 2000). In our study, we extended these findings by assessing the impact of maternal care on a chronic variable stress regimen in both male and female adult offspring. The results of the studies in chapter 4 demonstrated that the effects of maternal care are gender specific. It has been reported that the male offspring of high LG dams have lower corticosterone levels than the offspring of low LG dams following an acute restraint stress (Liu et al., 1997b). In contrast, the female offspring of high LG dams in our study showed a slightly elevated corticosterone response in comparison to the female offspring of low LG dams following a restraint stress, suggesting that the early environment may have disparate

effects on the development of neuroendocrine systems in males and females. In addition, female offspring of low LG dams showed a reduced preference for sucrose solution, indicating a lower hedonic state than the offspring of high LG dams. Maternal care had no effect on sucrose solution in the male rats, indicating that the early environment may render female rats more vulnerable to symptoms of anhedonia.

The impact of CVS on behavior and neuroendocrinology in the offspring of high and low LG dams was dependent on gender as well as maternal care. The rate of habituation to the open field was decreased only in the CVS-exposed female offspring of low LG dams. That is, female offspring of high LG dams and both male groups appeared to be protected against the impact of CVS on this measure. The results of CVS in the male offspring are surprising in that we did not replicate the effect of CVS on habituation in male rats seen in chapter 3. Although the rats were of the same strain, it is possible that other unknown variables may have affected the behavioral reactivity to animals ordered directly from a supplier or, conversely, of those that are born within the local animal facilities. For example, the early environment of the male rats used in the studies of chapter 3 is not known. However, the effect of CVS on the female offspring of low LG dams replicated the results of CVS induced changes in female rats displayed in chapter 3. Moreover, we extended those results by showing that the female offspring of high LG dams appear to be protected against the impact of CVS on habituation to a novel environment. Chronic variable stress did not affect the preference for sucrose in the high LG offspring, and only decreased the preference on the first day in the low LG offspring. The decrease in sucrose preference seen in thenon-stressed low LG offspring could not be due to neophobia since all groups consumed the same amount of sucrose on the first day.

In fact, sucrose consumption decreased over days in the non-stressed offspring of low LG dams, whereas, the non-stressed and the CVS-exposed offspring of high LG dams displayed a constant high level of preference for the sucrose over the 7 day period. Therefore, the offspring of low LG dams were initially as motivated to consume the sucrose, however, following the initial consumption, preference declined, presumably because these animals did not find the sucrose rewarding. In addition, the lack of an increase in sucrose preference in both the non-stressed low offspring as well as the CVS-exposed low offspring would suggest that the female offspring of low LG dams do not show the "adaptive" change in sucrose consumption that is normally observed (i.e., increased consumption over days). One drawback to this conclusion could be that the offspring of high LG dams also did not show increased consumption over days. However, note that consumption in these animals was near maximal across all days, including day 1. Thus, a ceiling effect may have prevented the observation of increased consumption over days.

Chronic variable stress decreased corticosterone levels in the female rats only, at time zero of restraint (before the application of the acute restraint stress), throughout the restraint period, and following release from the restrainers. There was no effect of the early environment, indicating that female rats may be more vulnerable to the effects of CVS on hypocorticosterone irrespective if the quality of maternal care. Of interest, the female CVS-exposed offspring of high did not display any behavioral deficits or anhedonia, suggesting that perhaps they were able to adapt to the chronic stress behaviorally, despite the CVS induced hypocortisolism. While changes in HPA activity are a principal component of mood disorders, there have been findings of

hypocortisolism in healthy people that are subjected to chronic stress (Friedman et al., 1963; Bourne et al., 1967; Bourne et al., 1968; Caplan et al., 1979).

As noted previously, the elevated plus maze is most often used to assess the levels of anxiety in rodents. Maternal care did not affect activity on the elevated plus maze in the male rats. Surprisingly, exposure to CVS increased the amount of time spent on the open arms in both the high and low offspring. Elevated plus maze activity has been shown to be mediated in part by 5-HT1A receptors in the hippocampus (Guimaraes et al., 1993; File et al., 1996; File and Gonzalez, 1996; Menard and Treit, 1998; Overstreet et al., 2003). In addition, the style of coping in the face of distress has also been linked to activity at the hippocampal 5-HT1A receptor (Korte et al., 1996; Meerlo et al., 2001). For example, Meerlo et al. (2001) found that rat pups exposed to corticosterone through their mother's milk had lower 5-HT 1A receptor binding in the hippocampus as adults, and displayed more passive coping styles in an aggression test and the shock probe defensive burying test. In addition, mice that are selectively bred for aggression display higher 5-HT1A mRNA levels in the dorsal hippocampus (Korte et al., 1996). In this study, we found that CVS increased hippocampal 5-HT1A receptor binding that was correlated with the time spent in the open arms of the elevated plus maze. It is possible that the offspring of both high and low LG dams exposed to CVS were able to develop coping mechanisms mediated in part by the 5-HT1A receptor in the hippocampus. This may also explain the inability of CVS to affect the rate of habituation in the open field, sucrose preference, or the acoustic startle response in these animals.

Habituation to a novel environment, such as the open field, is a form of non-associative learning that is dependent on the hippocampus (Izquierdo et al., 1999; Vianna

et al., 2000). The results of chapter 3 showed that exposure to CVS disrupted habituation to the open field in male and female rats. In addition, the results of chapter 4 extended these findings by demonstrating that, at least in female rats, the quality of the early environment could protect against the inhibitory effect of CVS on habituation. To test if the impact of maternal care would extend to associative forms of learning, we subjected the male offspring of high and low LG dams to both contextual and cued fear conditioning. Following the contextual conditioning, the offspring of high and low LG dams displayed a different rate of extinction across 3 days of re-exposure to the context. The time spent freezing in the context was similar in both groups on the first re-exposure. However, the offspring of low LG dams displayed a rapid decrease in the in freezing to the context, which is representative of extinction, on the second re-exposure. A similar decrease in freezing was not seen until the third context re-exposure in the offspring of high LG dams, suggesting that strength of the conditioned association may have been stronger in the high offspring. The results of the cued fear conditioning at first suggested that the offspring of high LG dams formed a greater association between the CS (auditory cue) and the US (footshock) than the offspring of the low LG dams. However, closer examination of the data revealed that despite the attempts to minimize the influence of contextual cues, contextual conditioning did take place, and influenced the results of the tone-footshock association. In fact, when the time spent freezing to the context in the absence of the cue was controlled for, the effect of maternal care was no longer significant. This suggests that maternal care influenced the strength of the conditioned response to the context, but perhaps not to the cue. In addition, the offspring of high LG dams may be especially adapt at recognizing subtle cues in the environment, as the

changes in the conditioning chamber were not sufficient to decrease the amount of time spent freezing to the context. Previous work from the Meaney laboratory has shown that the male offspring of high LG dams are in fact less fearful than the offspring of low LG dams (Caldji et al., 1998). At first, the results of the fear conditioning studies appeared to contradict the previous research findings. However, within our own experiment we also found that the offspring of high LG dams were less fearful than the offspring of low LG dams in the open field. For this reason, we believe that the results of the open field and the fear conditioning studies assessed two distinct behavioral measures. While the ratio of center to total squares in the open field assesses an instinctive level of fearfulness, the fear conditioning experiments require the acquisition of associative forms of learning. Therefore, we believe that the larger conditioned fear response in the offspring of the high LG dams represents a greater ability of these animals to form a learned association between a context and the stimulus. Although the mechanism for the effect of the early environment on fear conditioning is not known, hippocampal and amygdalar physiology has been shown to be mediated in part by maternal care (see Table 1 in introduction) (Liu et al., 1997a; Caldji et al., 2000; Liu et al., 2000b). Contextual conditioning is mediated by both the amygdala and the hippocampus, while only the amygdala is necessary for conditioning to an auditory cue (Phillips and LeDoux, 1992; Phillips and LeDoux, 1995). Since maternal care did not appear to influence conditioning to a cue, this would suggest that the greater contextual conditioning seen in the high offspring may be due to the effect of the early environment on hippocampal functioning.

Exposure to CVS eliminated the difference between the high and low offspring in contextual conditioning. It is possible that chronic stress caused morphological damage

to the hippocampus in the high offspring, thereby removing the maternal care-induced advantage in those offspring. Similarly, it has been shown that environmental enrichment reversed the effect of maternal care on hippocampal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor binding (Bredy et al., 2003). In addition, environmental enrichment reversed the effects of maternal care on two hippocampal dependent tasks, the Morris water maze and object recognition (Bredy et al., 2003). These results demonstrate that post-weaning environmental changes that impact on hippocampal morphology may also eliminate the protective effect of high levels of maternal care.

The effect of maternal care on fear conditioning was not assessed in the female offspring, and therefore, we cannot assume that associative forms of learning would also be affected in these rats. However, it is noteworthy that both associative and non-associative forms of learning are mediated by the hippocampus and amygdala (Vianna et al., 2000; Fischer et al., 2003), two brain structures shown to be affected by the early environment. The rate of habituation to the open field was not affected in the male offspring, raising the possibility that in male rats, maternal care may only influence the strength of associative forms of learning. It is of interest that chronic stress has been shown to cause damage to the hippocampus and decrease hippocampal functioning, suggesting a possible mechanism for the effect of CVS on learning (Magarinos et al., 1997; Ohl and Fuchs, 1999; McKittrick et al., 2000; Sandi et al., 2001; McEwen, 2001).

The results of this thesis suggest that the quality of the early environment affects reactivity to an acute stress as well as the impact of chronic stress in both males and females; however, these effects may be expressed differently. In females, maternal care

affected hedonia and was protective against the impact of chronic variable stress on the rate of habituation to a novel environment. In males, the offspring of high LG dams demonstrated a stronger association to conditioned fear and less anxiety, in a non-novel (habituated) open field, as indicated by the higher ratio of inner to total squares crossed. There appeared to be no protective effect of maternal care on the impact of CVS in the male offspring. It is possible that in these males, maternal care has a greater influence on the reactivity to an acute stress. It is also possible that the variable nature of the CVS protocol overruled the protective effect of maternal care, and that the offspring of high and low LG dams may show an enhanced adaptation to a repeated chronic stressor regimen.

6.1.4 Conclusion

In chapters 2, 3 and 4 we showed that the effects of CVS were gender and test specific. The CVS regimen caused a downregulation of plasma corticosterone in female rats, whereas it was either increased or unchanged in male rats. These results highlight the possibility that chronic stress can have dramatically different effects on HPA activity, depending on an individual's pre-existing physiology and coping abilities. It is unclear whether the CVS induces changes in HPA activity that are in part responsible for the behavioral deficits, or whether the changes in HPA activity and behavior both stem from a failure to cope with the chronic stress exposure. These questions may be answered in future experiments by using pharmacological manipulations of HPA functioning and subsequent behavioral testing. In addition, it is possible that gonadal hormones

contributed to the gender difference in corticosterone as a result of CVS, this issue should also be addressed in future experiments.

In chapters 3 and 4 we showed that CVS caused a decrease in the rate of habituation to a novel environment. This effect of CVS was consistent, except in the male offspring of high and low LG dams. It is possible that the effect of CVS on habituation is mediated in part through the damaging effects of stress on the hippocampus. In this respect, it is interesting that high levels of maternal care have been shown to affect hippocampal functioning, and that the female offspring of high LG dams were protected against the effect of CVS on habituation.

The effect of chronic fluoxetine treatment on CVS induced deficits was test and gender dependent, suggesting that fluoxetine has a differential efficacy on some behaviors in comparison with others. Fluoxetine did reverse the effects of CVS on habituation in the open field, which was consistent with previous studies that have used fluoxetine in other animal models of depression. This may be an important feature of antidepressant treatment as an increased ability to cope with and adapt to environmental challenges is associated with recovery from mood disorders.

The goal of this thesis was to assess the influence of gender, fluoxetine treatment and the early life environment on the impact of chronic variable stress. The results highlight the complexity of the individual stress response. The effects of CVS and fluoxetine treatment were gender and test dependent. In addition, the protective influence of the early life environment on the impact of CVS was expressed differently in male and female rats.

APPENDIX

McGill University Animal Use Protocol

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