CONTRIBUTIONS OF INDIVIDUAL DIFFERENCES IN STRESS REACTIVITIY TO POST-TRAUMATIC STRESS DISORDER VULNERABILITY AND RESILIENCE

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by

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ABSTRACT

CONTRIBUTIONS OF INDIVIDUAL DIFFERENCES IN STRESS REACTIVITY TO POST-TRAUMATIC STRESS DISORDER VULNERABILITY AND RESILIENCE

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Post-traumatic stress disorder (PTSD), an anxiety disorder precipitated by exposure to extreme emotional and/or physical stress, is characterized by persistent, intrusive memories of the precipitating trauma. Thus, the pathogenesis of PTSD has been conceptualized as involving a deficit in consolidation mechanisms underlying the extinction of fear memory. The mechanisms underlying this deficit have not been elucidated. In addition to intrusive memories, patients with PTSD display heightened sensitivity of the hypothalamic pituitary adrenocortical (HPA) axis to glucocorticoid negative feedback. As glucocorticoids are important modulators of memory consolidation, increased sensitivity to HPA negative feedback, by blunting glucocorticoid responses, may contribute to memory-related symptoms in PTSD patients. Emerging evidence in human patients suggests that the observed heightened negative feedback sensitivity in PTSD patients represents a marker of susceptibility to developing PTSD, rather than an effect of exposure to trauma, but this hypothesis has not been tested in animal studies. We examined individual differences in HPA responsiveness in rats displaying low (LR) and high (HR) locomotor responses to novelty. LR rats exhibited increased anxiety-like behaviors and less motility as compared to HR rats. HR rats displayed larger increases in corticosterone in response to restraint stress as compared to LR rats. LR and HR rats were subjected to contextual fear conditioning in order to examine consolidation, incubation, and extinction effects. LR rats exhibited increased freezing time and a reduction in the ability to extinguish fear memory as compared to HR rats. Additional animals were measured for acoustic startle prior to and following exposure the animal PTSD-model single-prolonged stress. LR rats subjected to a SPS exhibited a small increase in freezing indicative. HR rats expressed slightly lower levels of startle amplitude for most conditions, suggestive of habituation between trials. Overall, LR rats provide a working model to examine how individual differences in the HPA axis stress response play a role in the formation of PTSD-like behaviors.

DEDICATION

Jonathan Edward Hill, B.A.

I dedicate this work to my mom and dad:

Alice Virginia & Timothy Patrick

Your endless sacrifice and love provided the inspiration that all things are possible

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CHAPTER I

GENERAL INTRODUCTION

General Introduction

Posttraumatic stress disorder (PTSD) is a severe anxiety disorder that develops in response to extreme physical and/or emotional stress during which the individual experiences actual or perceived threat of safety to self or others; accompanied by an extreme sense of helplessness and fear (DSM-IV-TR, 2000). It is estimated that approximately 69% of Americans will experience some form of traumatic episode in their lifetime (Norris, 1992). The precipitating trauma can be varied, for example: natural disasters (Hussain *et al.*, 2011), rape (Rothbaum *et al.*, 1992), sudden loss of a loved one (Breslau *et al.*, 1999), severe automobile accidents, or combat (Javidi & Yadollahie, 2012). However, not all individuals who experience a traumatic episode develop PTSD. It is approximated that only 7.8% of all Americans are affected by PTSD (Kessler *et al.*, 1995).

Following exposure to the traumatic episode, individuals with PTSD can show a wide variety of symptoms. PTSD is characterized by a combination of hyperarousal and numbing symptoms and persistent, intrusive memories of the traumatic episode (DSM-IV-TR, 2000). Persistent and intrusive memories can manifest as generalized anxiety, nightmares, and flashbacks (Freud, 1962; Hartmann, 1984; Hanlon, 1987; Kavaler, 1987; Greenberg *et al.*, 1992; Stickgold, 2002). All are potential processes affected by memory dysfunction. Indeed, these characteristics have led to the hypothesis that the pathogenesis

of PTSD may involve a dysregulation in the consolidation mechanisms underlying the extinction of fear memory (Rauch *et al.* 2006).

Individuals with PTSD have been shown to exhibit decreased circadian plasma concentrations of the stress-hormone cortisol (Mason *et al.*, 1986; Yehuda *et al.*, 1995) as well as enhanced sensitivity to suppression of cortisol in response to treatment with dexamethasone (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997). In other words, individuals with PTSD display altered functioning of the hypothalamic-pituitary-adrenal (HPA) axis manifest through the expression of lower baseline cortisol levels and / or heightened sensitivity to glucocorticoid negative feedback. The question that arises is whether the observed differences in stress reactivity are a result of the precipitating trauma or of a genetic predisposition that may constitute a susceptibility factor. Previous studies demonstrate that dysregulation of the HPA axis and glucocorticoid imbalances can lead to pathology; such as hyperglycemia, adrenal suppression, depression, mania, or dysregulation of memory (McEwen & Stellar, 1993; Lansang & Hustak, 2011; Marques *et al*., 2009; McGaugh & Roozendaal, 2002). The question is whether the dysregulation of the HPA glucocorticoid system increases vulnerability to developing PTSD through the reduced availability of cortisol to interact with critical systems, or is a result of exposure to a traumatic insult.

Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenocortical (HPA) axis is the primary neuroendocrine component of the stress response. In response to stressful stimuli, sensory information from the periphery is integrated and activates cells in the paraventricular nucleus of the hypothalamus (PVN). Upon activation, parvocellular neurons in the PVN

release corticotrophin-releasing factor (CRF), and other neuropeptides, into the hypophyseal portal circulation system (Vale *et al.*, 1981; Rivier & Vale, 1983). Release of CRF causes subsequent release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH is released into the systemic circulation, whereupon it induces the production and secretion of glucocorticoids, namely cortisol (humans) or corticosterone (rodents), from the zona fasciculata of the adrenal cortex (Munck *et al.*, 1984) see Figure 1.1. Once released, glucocorticoids have a variety of actions throughout the body and brain. This includes activation of different cellular pathways regulating the redistribution of energy (Contarino *et al*., 2000; Richard *et al.*, 2002), organization and processing of memory (Croiset *et al*., 2000) and negative-feedback inhibition of the HPA axis to inhibit further corticosterone release (Widmaier & Dallman, 1984; de Kloet *et al.*, 2005). Previous studies have demonstrated that corticosterone concentrations in the brain are reflected in plasma concentrations collected from the periphery. More specifically, central corticosterone concentrations have been found to be a linear function of its plasma concentration (Weber, Eckert, & Müller, 2006). This is important because most studies examining corticosteroid levels sample plasma concentrations, underscoring how important it is to know whether plasma concentrations reflect, to some degree, central concentrations.

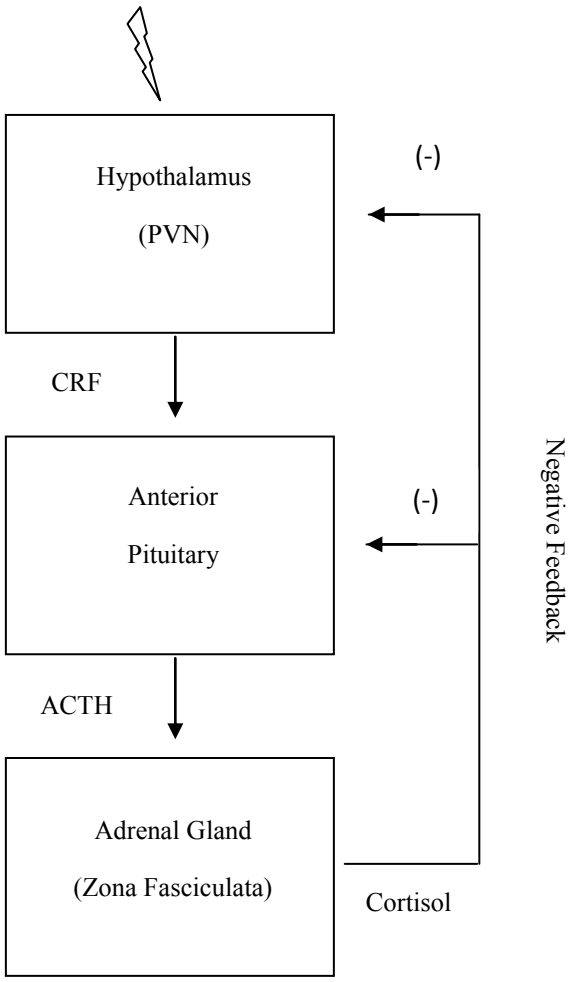

Figure 1.1 Diagram of the Hypothalamic-Pituitary-Adrenal (HPA) axis. In response to stress (lightning bolts) sensory information is integrated within the hypothalamus and subsequently activates parvocellular neurons in the paraventricular nuclei (PVN). This results in the secretion of corticotrophin-releasing factor (CRF), which enters the anterior pituitary and induces the release of adrenocorticotrophic hormone (ACTH). ACTH enters the systemic circulatory system and targets the adrenal cortex, where it induces the release of glucocorticoids from the zona fasciculata. At high enough concentrations, glucocorticoids will initiate negative feedback inhibition of further release by inhibiting the release of CRF from the PVN and ACTH from the pituitary, resulting in the deactivation of the stress response.

HPA Axis & PTSD

Individuals with PTSD have been shown to exhibit differences in the functioning of the HPA axis. The glucocorticoid cortisol, or corticosterone in rodents, is an important component of the stress response system, and as such has been a focus of research in understanding the underlying mechanisms of PTSD. Studies have demonstrated that individuals with PTSD have exhibited decreased concentrations of cortisol (Boscarino, 1996; Yehuda, 2002; Yehuda *et al.*, 1993, 1995), enhanced sensitivity to negative feedback inhibition of the HPA axis (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997) and expressed increased concentrations and responsiveness of the glucocorticoid receptor (Yehuda *et al*., 1995). Furthermore, individuals that expressed lower cortisol levels in the emergency room immediately following a traumatic experience, such as rape or motor vehicle accidents, had a greater incidence of developing PTSD over time as compared to individuals with higher levels of cortisol that underwent the same trauma and had similar trauma history (Resnick *et al*., 1995; McFarlane *et al*., 1997). This is interesting because it suggests a positive correlation between low cortisol levels following traumatic stress and the development of PTSD. Considered together, it is possible that by possessing decreased concentrations of cortisol, individuals with PTSD are unable to properly activate cortisol-dependent processes mediated through the glucocorticoid receptor during times of stress and therefore develop a variety of PTSD-like behaviors.

It is important to note however, that other studies have demonstrated that individuals with PTSD may exhibit a hyperactivity of the HPA response; sometimes resulting in higher levels of cortisol (Heim *et al*., 2000; Pittman & Orr, 1990; Rasmussen

et al., 2001), or no difference in cortisol levels from controls (Meewisse *et al.*, 2007; Young & Breslau, 2004). However, in the previous studies significant differences were observed in cortisol concentrations, but only within select subpopulations. For example, Young and Breslau (2004) reported that only when an individual with PTSD expressed a comorbidity with major depressive disorder did they observe a significant elevation of cortisol. This difference was not observed when analyzing PTSD patients without major depressive disorder.

Furthermore, a study by Bremner *et al.* (2003) found that although individuals with PTSD have demonstrated lower basal cortisol levels, when presented with a cognitive stress individuals with PTSD demonstrated no differences in cortisol concentrations in response to stress compared to individuals without PTSD. This study is important because it examines glucocorticoid levels following stress, not at a basal level. This study, while important, is subject to the same issues as the aforementioned studies when trying to understand the role glucocorticoids and the HPA axis play in PTSD.

One complication of trying to understand how differences in HPA response may contribute to PTSD is that there are no set parameters in how the question is addressed. As the above paragraphs introduce, there are many conflicting observations regarding cortisol levels in individuals with PTSD. This is a result of a combination of factors: different methodologies, demographics of participants, and pharmacological controls. Regardless of the differences in demographics and methodologies used, one critical question remains: what role do individual differences of the HPA axis play, if any, in the vulnerability or resilience to developing PTSD? Do lower basal cortisol levels, as observed in individuals with PTSD, contribute to the development of PTSD; and if so, is

it the result of an inability to activate critical glucocorticoid receptor-dependent signaling cascades?

Glucocorticoids

Glucocorticoids, such as corticosterone, are steroid hormones released from the adrenal glands in response to activation of the HPA axis. The effects of glucocorticoids are mediated mainly through specific intracellular receptors, the glucocorticoid and mineralocorticoid receptors, within the central nervous system (de Kloet *et al.*, 1998). Glucocorticoid receptors can be separated into two types. Type I, or mineralocorticoid, receptors (MR) are high affinity receptors that are typically occupied under basal conditions. Type II, or glucocorticoid, receptors (GR) are lower affinity receptors that are typically occupied only under stress conditions or pharmacological manipulation (de Kloet *et al.*, 1998). GR will be the focus for the remainder of this dissertation because of this receptor's involvement in stress and learning. The MR, while important, is thought to be saturated at basal conditions as a result of a 10-fold higher affinity for cortisol (Reul & de Kloet, 1985), and therefore is not as critical in playing a role in stress systems under fluctuating levels of glucocorticoids as compared to the GR.

Glucocorticoid receptors are ubiquitously expressed within the brain (Joëls, 2008) including areas known to be involved in cognition, such as the hippocampus, amygdala, and prefrontal cortex. The hippocampus is a limbic structure involved in declarative and spatial memory (Eichenbaum *et al.*, 1999; Squire, 1992). The amygdaloid nuclei play important roles in emotional memory (LeDoux, 2000; McGaugh & Roozendaal, 2002). The prefrontal cortex is important for short-term working memory and regulation of coping behaviors (Baddeley, 2001; Maier & Watkins, 2010). Therefore any reduction in

the activation of these receptors, possibly through reduced cortisol concentrations as seen in individuals with PTSD, could have serious impacts on a host of behavioral and physiological systems; especially memory.

Transactivation

Glucocorticoids diffuse across the cell membrane as a result of their cholesterol backbones. Inside the cellular cytoplasm, the GR exists as part of a larger protein complex that includes multiple chaperone proteins, including heat-shock and FK506-binding proteins (Schiene-Fischer & Yu, 2001). Once in the cytoplasm, glucocorticoids bind to the GR and cause a conformational change that results in the jettison of associated heat-shock proteins and FKBP51 from the receptor complex (Heitzer *et al.*, 2007). This allows for FKBP52 to bind to the protein complex and assist in translocation into the nuclear membrane through a dynein-cytoskeletal interaction (Silverstein *et al*., 1999; Davies *et al*., 2005; Wochnik *et al*, 2005). Inside the nucleus, glucocorticoids bind to specific DNA response elements in the promoter region of target genes and regulate gene expression. (Binder, 2009; Heitzer *et al.*, 2007).

Chaperone Proteins

FKBP51, encoded by the gene FKBP5, is a chaperone protein that is part of the glucocorticoid receptor complex and decreases its affinity for glucocorticoid binding (Wochnik *et al*, 2005). Upon binding to the associated GR complex, glucocorticoids cause a structural change that removes FKBP51 from the complex (Heitzer *et al.*, 2007), increasing affinity for glucocorticoid binding and allowing for FKBP2 binding, which

recruits dynein and induces translocation of the receptor complex across the nuclear membrane (Silverstein *et al*., 1999; Davies *et al*., 2005; Wochnik *et al*, 2005).

Current research suggests an additional role for FKBP51 in the modulation of the glucocorticoid response. In the absence of glucocorticoids binding to the receptor complex, FKBP51 is able to translocate the receptor complex into the nucleus (Zhang *et al.*, 2008). This effectively limits the capacity for glucocorticoid effects by reducing the number of GR complexes in the cytosol available for glucocorticoids to interact with and induce subsequent genomic responses.

Therefore, regulation of FKBP51 and FKBP52 expression is an important component in establishing an effective glucocorticoid response. Mineralocorticoids, such as progestins, have been demonstrated to have a weak effect on the promoter sequence of the FKBP5 gene (Hubler & Scammell, 2004). Glucocorticoids, however, have been shown to promote FKBP5 gene transcription (Hubler *et al.,* 2003) even though increased levels of FKBP5 reduce glucocorticoid sensitivity (Wochnik *et al*, 2005; Zhang *et al.*, 2008). It has been hypothesized that this creates a short intracellular negative feedback loop for glucocorticoid receptor activity (Vermeer *et al*., 2003), whereby expression of FKBP5 increases glucocorticoid resistance at the intracellular level.

In support of this hypothesis, Binder *et al.* (2008) observed that healthy individuals who expressed alleles for increased FKBP5 expression demonstrated lower dexamethasone suppression and therefore a high degree of glucocorticoid resistance (Binder *et al.*, 2008). Interestingly, in response to stressful stimuli, these individuals exhibited increased anxiety and decreased recovery of cortisol levels following exposure to a stressful stimuli (Ising *et al*., 2008). This suggests that alterations in expression of

FKBPs have: A) the capacity to increase or decrease glucocorticoid sensitivity and resistance, effectively modulating the genomic response (Wochnik *et al*, 2005; Zhang *et al.*, 2008), and B) are partially dependent upon differential activation of the HPA axis in response to stressful stimuli (Ising *et al*., 2008; Binder, 2009).

Low levels of FKBP5 mRNA expression have been positively correlated to the development of PTSD symptom (van Zuiden *et al*., 2012). Lower levels of FKBP5 could result in increased glucocorticoid sensitivity, supporting previous reports of individuals with PTSD exhibiting enhanced sensitivity to HPA negative feedback by cortisol (Yehuda, 1993; 1997). Furthermore, polymorphisms in the FKBP5 gene previously associated with psychopathologies have been found to be positively correlated with threat-related activation of the amygdala (White *et al*., 2012), an important center for the regulation of fear memory and response (McGaugh *et al.*, 1996; McGaugh, 2000).

Neuro-circuitry of Emotional Memory

As mentioned previously, glucocorticoids modulate neuronal function in multiple brain regions associated with cognition; the hippocampus, prefrontal cortex, and amygdala. The amygdala is perhaps the most important integration center for emotional learning and memory. The amygdala receives input from a number of brain nuclei, but the prefrontal cortex, ventral/medial regions (vmPFC), and hippocampus are two regions critical in amygdalar regulation of emotional memory. Insufficient output by the vmPFC is thought to underlie deficits in suppression of attention and response to trauma-related cues as well as deficits in extinction (Rauch *et al.*, 1998). Inadequate influence by the hippocampus may result in difficulties in the ability to identify safe contexts in addition to other memory deficits (Bremner *et al*., 1995). Combined, inadequate regulation of the

amygdala could result in hyper-responsivity and persistent intrusive memories, which could account for a majority of the observed symptoms of PTSD.

The amygdala can be divided into multiple sub-nuclei, each with specific functions and microcircuitry. The basal and lateral regions, known collectively as the basolateral (BLA) region, are important areas for mediating the effects of emotional arousal and fearful events on memory consolidation (McGaugh *et al.*, 1996; McGaugh, 2000). The mechanisms underlying glucocorticoid actions on consolidation are unknown, but research has suggested that the glucocorticoid cortisol (corticosterone in rodents) (van Stegeren *et al.*, 2007; McGaugh & Roozendaal, 2002) and the monoamine neurotransmitter norepinephrine (Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2008) are key components in regulating memory consolidation involving the amygdala and other regions.

Learning & Memory

Memory consolidation is a specific stage of memory processing. Memory can be separated into three primary stages; acquisition, consolidation, and retrieval (Abel & Lattal, 2001). In acquisition, the memory is initially learned and processed. Consolidation is the process by which the memory trace is stabilized and put into storage. The time between acquisition and consolidation is sensitive to modulation (Schafe *et al.*, 2001). Retrieval is the process by which the memory is evoked, or recalled. Current research has suggested that after evocation of a memory, it is temporarily labile, and subject to reconsolidation (Nader *et al*., 2000; Sara, 2000) This is potentially a mechanism whereby a previously stored memory can be edited. Another component of memory is extinction,

which is a process in which a context cue that was formerly associated with an adverse stimulus is no longer able to elicit a behavioral response.

Fear Conditioning

Fear conditioning is a Pavlovian model of learning in which a neutral stimulus is paired with a noxious stimulus, resulting in behavioral responses (fear) towards the formerly neutral stimulus (Johansen *et al*., 2011). For example, a rodent can be placed into a neutral context and have no particular behavioral response. The context can then be paired with a series of electrical footshocks; the footshocks inducing fear behaviors. This would create an association between the two, so that when subsequently exposed to the originally neutral context the rodent would respond fearfully to the context even without the presentation of the noxious stimuli, such as electric footshocks.

Stress Hormone Modulation of Memory Consolidation

Glucocorticoids and norepinephrine are potent regulators of memory consolidation (McGaugh & Roozendaal, 2002; Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2007,2008). Gold and van Buskirk (1975) presented the first evidence suggesting that an endogenous hormone could play a role in memory consolidation in a study that reported systemic administration of epinephrine enhanced retention of inhibitory avoidance behavior in a dose-dependent manner when administered after training. It has since been shown that during times of emotional arousal, and/or intense fear, norepinephrine is released within the BLA, inducing activation (McIntyre *et al.*, 2002; Pelletier *et al.*, 2005), further suggesting a role for norepinephrine in the mediation of consolidation of fear memory. Additional studies have shown that activation of β -

adrenoceptors in the amygdala enhances consolidation of fear memory when administered after training, while antagonism of β -adrenoceptors inhibits norepinephrine effects on memory consolidation (Liang *et al.*, 1986; Ferry & McGaugh, 1999; Liang *et al.,* 1990).

Glucocorticoids, which are released during emotionally arousing, or fearful events (Vermetten & Lanius, 2012), can also modulate the consolidation of fear memory. Administration of glucocorticoids shortly after fear training has an enhancing effect on memory consolidation similar to that observed in response to norepinephrine (Pugh *et al*., 1999; Sandi *et al.*, 1997; Cordero *et al.*, 1998; Roozendaal *et al*., 1999; Roozendaal, 2000). Additionally, administration of a GR antagonist, but not an MR antagonist, immediately following a training session for a spatial memory task impairs subsequent memory challenges (Oitzl & de Kloet, 1992; Roozendaal *et al*., 1996), indicating that any glucocorticoid effects on memory consolidation are due to activation of GR and not MR. Conversely, removal of the adrenal gland, effectively inhibiting glucocorticoid synthesis, reduces memory performance and retention (Roozendaal *et al.*, 1996). Furthermore, treatment with the corticosterone synthesis inhibitor metyrapone inhibits the enhancing effects observed with post-training administration of norepinephrine (Roozendaal *et al.*, 1996).

Interestingly, the enhancing effects of glucocorticoids on memory are dependent upon norepinephrine activation within the BLA (Roozendaal *et al.*, 2006). Administration of the β-adrenoceptor antagonist propranolol into the BLA blocks the corticosterone-induced enhancement of memory (Roozendaal *et al.*, 2006). Rats that received a systemic injection of corticosterone immediately following a 3-minute habituation trial to a novel

environment demonstrated enhanced retention 24 hours later, but not if they had prior habituation to the context, and thus lower novelty-induced emotional arousal (Okuda *et al*., 2004; Roozendaal *et al*., 2006). This suggests that in order for corticosterone to exhibit enhancing effects, a degree of heightened emotion is necessary. Furthermore, the enhancing effect of corticosterone was blocked by systemic or intra-BLA administration of propranolol, a β-adrenoceptor antagonist. Additionally, administration of yohimbine, an α_2-adrenoceptor, in the habituated animals resulted in enhanced memory (Roozendaal *et al*., 2006). Therefore, corticosterone by itself was not sufficient to enhance memory in the pre-habituated animals, but in combination with yohimbine-driven norepinephrine release memory was enhanced. This suggests that a component of the consolidation mechanism underlying fear memory extinction involves: A) the noradrenergic system, B) glucocorticoids, and C) the BLA. Imbalances, or dysregulation, of either of the two aforementioned neurochemical systems can have significant effects on the consolidation of extinction for fear memory.

Emotion & Consolidation

It is important to note that the enhancing effects of glucocorticoids are dependent upon a component of the experience being emotionally arousing. For example, rats that received post-training corticosterone injections immediately following an object recognition trial demonstrated enhanced 24-hr retention performance only if they were unhabituated to the context, thereby having an increased novelty-induced emotional arousal. Animals that were habituated to the context demonstrated no improved retention with post-training administration of corticosterone (Okuda *et al.*, 2004). Similarly, humans received a cortisol or placebo injection and were then shown a series of pictures

with varying degrees of emotional arousal. Participants were tested one week later and demonstrated enhanced memory for emotionally arousing images, but not neutral images, if they had previously received a cortisol injection, but not placebo (Buchanan & Lovallo, 2001). These, and additional studies, support the idea that in order for glucocorticoids to have a robust effect on memory enhancement, a component of the experience being consolidated has to be emotionally arousing (Cahill *et al.,* 2003; Abercrombie *et al.*, 2006).

Extinction

Extinction is a process in which a context cue that was formerly associated with an adverse stimulus is no longer able to elicit a behavioral response (Milad *et al*., 2006). Glucocorticoids have been demonstrated to play an important role in the maintenance and progression of extinction processes of fear memory. Specifically, systemic administration of the glucocorticoid receptor agonists dexamethasone and intra-amygdala infusion of RU28362 prior to extinction training resulted in a facilitation of extinction of conditioned fear in a dose-dependent manner (Yang *et al*., 2006). Furthermore, administration of the corticosteroid inhibitor metyrapone prior to extinction trials results in an inhibition of extinction to context-dependent fear responses in rats (Yang *et al*., 2006) and mice (Blundell *et al*., 2011). Importantly, administration of corticosterone immediately following extinction trials in mice that received a pre-trial injection of metyrapone demonstrated a rescue effect that allowed for extinction (Clay *et al.*, 2011). Together, these data suggest that glucocorticoids are important in the extinction of conditioned fear and indicate the amygdala as an important site of glucocorticoid action in extinction processes. Furthermore, the data suggest that decreased glucocorticoids during re-

activation of a fear memory may lead to a deficit in the extinction of fear memory. This is important because it suggests a possible mechanism which could explain why individuals with PTSD, who have decreased cortisol levels, have an inability to extinguish fear memory.

Organic Cation Transporter 3

Glucocorticoids and norepinephrine both act as potent modulators of memory consolidation (McGaugh & Roozendaal, 2002; Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2007,2008). Recent studies suggest that, in addition to exerting actions via the mineralocorticoid and glucocorticoid receptors (MR and GR), corticosteroids also act by inhibiting monoamine clearance mediated by uptake$_2$, a high-capacity, low-affinity transport system for norepinephrine, epinephrine, dopamine, histamine and serotonin (Baganz *et al*., 2008; Feng *et al*., 2009, Gasser, Lowry & Orchinik, 2006; Gasser *et al*., 2009). In contrast to uptake$_1$, which is mediated by a combination of the specific transporters for norepinephrine (NET), dopamine (DAT), and serotonin (SERT), uptake$_2$ is a higher-capacity but lower affinity transport system, and is acutely inhibited by corticosterone and other steroids (Iversen & Salt, 1970; Simmonds & Gillis, 1968). Inhibition of uptake$_2$ in cardiac or smooth muscle tissue by acute bath application of corticosteroids enhances the contractile effects of exogenously applied epinephrine, norepinephrine, serotonin and histamine (Horvath *et al.*, 2003; Eyre, Elmes & Strickland, 1979; Kalsner, 1975; Mikami *et al*., 1989; Purdy, Weber & Drayer, 1982; Purdy & Weber, 1983). Recent studies have identified a small group of transporters that mediate uptake$_2$-like transport and have demonstrated their expression in the brain (Gasser, Lowry & Orchinik, 2006; Gasser *et al*., 2009; Engel, Zhou & Wang, 2004; Amphoux *et al*.,

2006; Vialou *et al*., 2004). Thus, uptake$_2$ is a mechanism by which corticosteroids may act to enhance the actions of monoamines in the CNS as well as in peripheral targets.

Uptake$_2$ activity has been attributed to a group of broadly-specific organic cation transporters. These include the organic cation transporter (OCT) family: OCTs 1, 2 and 3, and the plasma membrane monoamine transporter (PMAT). Because OCT3 is the most sensitive of these transporters to inhibition by corticosterone, it has been described as the most important uptake$_2$ transporter (Grundemann *et al.*, 1998; Wu *et al*., 1998, Duan &Wang, 2010). However, all of the above transporters have uptake$_2$-like characteristics. All are broadly-specific organic cation transporters, capable of transporting, with varying efficiencies, norepinephrine, epinephrine, serotonin, dopamine and histamine, as well as the cationic neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) (Engel, Zhou & Wang, 2004; Grundemann *et al.*, 1998; Grundemann *et al.*, 1998; Grundemann *et al.*, 1999. All are sensitive to inhibition by corticosterone, though their sensitivities differ widely (Gasser, Lowry & Orchinik, 2006; Engel, Zhou & Wang, 2004; Wu *et al*., 1998; Duan *et al.*, 2010; Schomig, Lazar & Grundemann, 2006; Gorboulev *et al*., 2005), and all are inhibited by the pseudoisocyanine compound 1, 1'-diethyl-2, 2'-cyanine iodide (decynium-22) (Engel, Zhou & Wang, 2004; Hayer-Zillgen, Bruss & Bonisch, 2002).

All of the uptake$_2$ transporters are expressed, at varying levels and with distinct distributions, in rodent and human brain (Gasser, Lowry & Orchinik, 2009; Gasser *et al.*, 2009; Engel, Zhou & Wang, 2004; Amphoux *et al*., 2006; Vialou *et al.*, 2004), and recent studies suggest that they play important roles in monoamine clearance. Pharmacological inhibition of uptake$_2$ by direct application of decynium-22 decreases the rate of serotonin

clearance in mouse hippocampus (Baganz *et al*., 2008), and treatment of rats with the OCT3 inhibitor normetanephrine potentiates venlafaxine-induced increases in extracellular norepinephrine concentrations in rat prefrontal cortex (Rahman *et al.*, 2008).

Corticosterone inhibition of OCT3 has been suggested to play a role in the modulation of behavior. Specifically, corticosterone inhibition of OCT3 results in the potentiation of drug-seeking behavior (Graf *et al.* unpublished), providing a mechanism by which glucocorticoids can modulate behavior through inhibition of OCT3. For example, a model of how low cortisol levels may contribute to the development of PTSD-like behaviors is as follows. Under stress conditions, cortisol inhibits OCT3 and results in subsequent increases of norepinephrine that may be necessary for proper consolidation and extinction during times of emotional arousal, such as traumatic stress. Individuals with PTSD, who express lower levels of cortisol, may be unable to inhibit OCT3, thereby allowing for the continuation of norepinephrine clearance from the extra-synaptic space within the amygdala and other memory-important areas of the brain. This could result in insufficient levels of norepinephrine and prevent the modulatory effects of norepinephrine on memory, resulting in dysregulation of memory processes.

Negative Feedback of the HPA Axis

Glucocorticoid involvement in the HPA axis response is complicated. Not only do glucocorticoids have a variety of peripheral roles, but they also have very specific roles within the central nervous system. Glucocorticoids cross the blood brain barrier and target specific receptors within the brain (Widmaier & Dallman, 1984; de Kloet *et al.*, 2005). In order to limit further release, thereby providing negative feedback, glucocorticoids will bind to GRs in the hypothalamus and the pituitary. This causes the

subsequent inhibition of secretion for CRF (hypothalamus) and ACTH (pituitary), which leads to less stimulation of downstream products including glucocorticoid secretion from the zona fasciculata of the adrenal cortex.

The actions of glucocorticoids in HPA axis negative feedback can be broken down into two major time frames: fast and slow feedback (Keller-Wood & Dallman, 1984). The slow response is the most classical in nature, in that it refers to glucocorticoid actions at the level of the genome. Glucocorticoids induce decreases in mRNA encoding for proopiomelanocortin (POMC), the precursor for ACTH, thereby reducing levels of ACTH (Heitzer *et al.*, 2007; Keller-Wood & Dallman, 1984). The overall effect causes the reduction of downstream glucocorticoid secretion. The fast feedback response is much quicker as its name implies. Studies have shown that the fast feedback response can occur in as little as 15 minutes (Hinz & Hirschelmann, 2000). This is interesting because it suggests that the mechanism behind fast feedback inhibition of ACTH secretion is not genomic in nature.

Recent studies suggest that individuals with PTSD exhibit alterations in rapid negative feedback systems, not just in glucocorticoid concentrations. Yehuda *et al*. (2006) reported that individuals with PTSD demonstrated a greater decline in ACTH concentrations following an injection of cortisol, suggesting a greater sensitivity to cortisol effects at the pituitary. This is important because, accompanied with previous reports of individuals with PTSD exhibiting enhanced negative feedback through dexamethasone suppression, these data suggest that central, and peripheral, negative feedback inhibition may be altered in PTSD. The difference in fast negative feedback may be a result of differential expression of GR (Liberzon *et al*., 1999). The stress-

restress animal model of PTSD has found that animals that receive a traumatic stress episode followed by a non-stress interval, and not a chronic stress interval, exhibit enhanced negative feedback during testing a week later (Liberzon *et al.*, 1997). This study was repeated but with a focus on changes in glucocorticoid receptor mRNA expression in the hippocampus. Animals that demonstrated enhanced negative feedback also demonstrated an increase in GR mRNA and a decrease in MR mRNA (Liberzon *et al*., 1999). Furthermore, it has been observed that individuals with PTSD express a greater number of glucocorticoid receptors in peripheral lymphocytes (Yehuda *et al*., 1991), but show a decrease in these glucocorticoid receptors following administration of DEX that is not observed in control subjects (Yehuda *et al*., 1995), suggesting an enhancement of negative feedback systems at some level of the HPA axis .

Imbalances in these feedback mechanisms, or HPA negative feedback in general, may lead to pathological conditions (McEwen & Stellar, 1993; Lansang & Hustak, 2011; Marques *et al*., 2009), such as post-traumatic stress disorder, hyperglycemia, depression or mania. Increased sensitivity to HPA negative feedback by cortisol could account for the reduced basal cortisol levels observed in individuals with PTSD (Mason *et al.*, 1986; Yehuda *et al.*, 1995). Reduced cortisol levels could prevent activation of the glucocorticoid receptor or a lack of OCT3 inhibition, which could result in a number of behavioral and physiological deficits, such as activation of amygdala circuits which have been shown to be critical in the extinction of conditioned fear memory (Yang *et al.*, 2006). Therefore it is important to understand how an individual's innate cortisol response to stress may contribute to the development of pathological conditions. Increasingly, research has focused on whether or not an individual's specific stress

reactivity plays a role in his or her vulnerability or resilience to developing such a disorder (Levay *et al*., 2005; Kabbaj *et al*., 2000; Padilla *et al*., 2010; Duclot *et al*., 2011).

Individual Differences in HPA Reactivity

One way to examine individual differences in HPA response is to separate rats into groups based upon locomotor activity in response to a novel stress (Kabbaj *et al.*, 2000; Liebsch, 1997). In detail, rats are placed within a locomotor chamber for a set period of time, typically an hour, and allowed to explore freely. During the trial, locomotor activity is recorded as infrared beam breaks. At the end of exposure, rats can be separated into two distinct behavioral phenotypes based upon locomotor activity: low and high responders (Jama *et al.*, 2008).

It has been reported that rats with a higher novelty-induced locomotor response (HRs) exhibit heightened levels of corticosterone in response to stress, whereby rats with a lesser locomotor response to novelty (LRs) exhibit lower levels of corticosterone (Piazza *et al*. 1991). This is the first study to suggest that locomotor response to novelty is an indicator of individual differences in HPA reactivity.

Low responders and high responders have been observed to exhibit multiple behavioral differences in response to a novel, mild stress environment (Kabbaj *et al.*, 2006). As the names imply, LR animals locomote less within a novel environment as compared to HR animals (Piazza *et al.*, 1989; Pierre & Vezina, 1997;1998). Additional behaviors, such as sniffing and rearing have been observed to be different between LR and HR animals (Hooks *et al*., 1994). This is interesting because these behaviors, sniffing and rearing, can be considered measures of active surveillance or vigilance; which is a behavior altered in many people with PTSD (Paulus, Argo, and Egge, 2013). Low

responders typically rear less and spend less time in the center, indicative of higher anxiety.

Low responders have been shown to exhibit significant differences in both phenotype and genotype as compared to HR counterparts (Kabbaj, 2004; Blanchard *et al.*, 2009). Low responder rats exhibit lower levels of CRF mRNA in the paraventricular nucleus of the hypothalamus, an area important for activating the HPA response, as compared to high responder rats (Kabbaj *et al*., 2000). This could provide a mechanism underlying the differences in HPA reactivity. Specifically, lower CRF mRNA could result in less CRF available to activate downstream components of the HPA axis, thus resulting in lower corticosterone being secreted during stress. Additionally, HR animals but not LR, exhibited lower basal levels of CRF mRNA in the central amygdala, an area important for emotional modulation of learning and memory (Kabbaj *et al*., 2000). This could suggest an explanation as to why HR animals typically show lower anxiety and fear-like behaviors in general. It is possible that by having reduced CRF mRNA, HRs have lower CRF in the amygdala, and thus are unable to activate as robust a fear / anxiety response. In essence, they could be protected by lower CRF and require stronger emotional and fear inputs to demonstrate fear and anxiety-like behaviors.

HR rats have been shown to have an increase in novelty-seeking behaviors (Kerman *et al*., 2011; Kabbaj, 2006), a predilection that has suggested they be termed as more "thrill-seeking" in behavior. In response to administration of cocaine, HRs demonstrated higher increases of extracellular dopamine compared to LRs (Hooks *et al.*, 1991). This could be a result of the fact that HR rats have been shown to have fewer D2 binding sites in the nucleus accumbens and the striatum as compared to LR rats, both

areas important for motivated behaviors and addiction (Hooks *et al.*, 1994). HR rats also display an increased preference for sucrose (Duclot *et al*., 2011; Hollis *et al*., 2011), and acquire self-administration of cocaine at low doses faster than LR controls (Beckmann *et al*., 2011) as well as amphetamine (Piazza *et al*.1991).

Current Studies

Individuals with PTSD exhibit differences in HPA reactivity as compared to individuals without PTSD. Specifically, a number of individuals with PTSD exhibit decreased basal concentrations of the stress hormone cortisol (Boscarino, 1996; Yehuda, 2002; Yehuda *et al.*, 1993, 1995). It has also been demonstrated that individuals with PTSD may also display enhanced sensitivity of the HPA axis to negative feedback inhibition of cortisol (Yehuda, 1997). Patients with PTSD have demonstrated increased suppression of cortisol following administration of dexamethasone, indicating enhanced sensitivity to negative feedback (Stein *et al*., 1997; Yehuda *et al*., 1993). This is further supported by experiments demonstrating individuals with PTSD exhibiting a decrease in glucocorticoid receptors on peripheral lymphocytes in response to dexamethasone, but not in individuals without PTSD (Yehuda *et al*., 1995). Conversely, studies have found that when cortisol negative feedback of the HPA axis is inhibited by administration of the 11β-hydroxylase inhibitor, metyrapone, individuals with PTSD demonstrated increases in ACTH and 11-deoxycortisol, the precursor to cortisol (Yehuda *et al*., 1996), further supporting increased feedback inhibition by cortisol.

Decreased cortisol concentrations, possibly through enhancement of HPA sensitivity to glucocorticoid negative feedback, could result in an inability to occupy and activate the glucocorticoid receptor or inhibit the corticosteroid-sensitive monoamine

transporter, OCT3. This could result in dysregulation of a host of behavioral and physiological processes resulting in the expression of pathological symptoms. It has been shown that corticosteroids are important for the extinction of fear memory (Yang *et al*., 2006; Blundell *et al*., 2011; Clay *et al.*, 2011) and that without sufficient levels this process is disrupted and results in persistent fear behaviors similar to those expressed in PTSD. Furthermore, it has also been observed that norepinephrine is critical in the modulation of fear memory alongside of corticosterone (McIntyre *et al.*, 2002; Pelletier *et al.*, 2005; Roozendaal, 2006). A failure to inhibit the monoamine transporter, OCT3, could potentially result in a lack of available norepinephrine that would typically be available under stress conditions and allow for proper consolidation of memory. Therefore, a reduction in basal levels of cortisol, as seen in PTSD, could indicate an underlying deficiency in available cortisol that contributes to the expression of PTSD-like behaviors.

The current set of experiments utilize the LR/HR animal model to take advantage of a model in which individual HPA responses are readily identifiable, and most importantly, prior to exposure to any traumatic episode. These experiments seek to understand whether differences in stress reactivity are a contributing factor in developing PTSD-like behaviors. Additionally, these experiments aim to more fully understand the behavioral and physiological differences between LR and HR animals. Furthermore, the following experiments aim to understand how individual differences in stress reactivity play a role in the consolidation and extinction of fear memory, and the exhibition of PTSD-like behaviors.

CHAPTER II

BEHAVIORAL AND HYPOTHALAMIC-PITUITARY ADRENOCORTICAL AXIS PHENOTYPES IN LOW AND HIGH RESPONDING RATS

Introduction

Posttraumatic stress disorder (PTSD) is an anxiety disorder precipitated by exposure to extreme physical and/or emotional stress (DSM-IV-TR, 2000) that affects approximately 8% of all Americans (Kessler *et al.*, 1995). Due to a combination of symptoms including but not limited to: hyperarousal, generalized numbing, and persistent and intrusive memories, it has been hypothesized that the pathogenesis of PTSD may involve a deficit in consolidation mechanisms underlying the extinction of fear memory (Rauch *et al.* 2006). One important factor involved in memory consolidation is glucocorticoid concentration which is dependent upon activation of the hypothalamic-pituitary-adrenal axis stress response system.

The hypothalamic-pituitary-adrenal (HPA) axis is critical in the adaptation and response to stressful stimuli. Upon activation, corticotrophin-releasing factor (CRF) is released by cells in the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal portal circulation system (Vale *et al.*, 1981; Rivier & Vale, 1983). This stimulates the release of adrenocorticotrophic hormone (ACTH) into the systemic circulatory system, leading to the secretion of glucocorticoids from the zona fasciculata of the adrenal glands (Munck *et al.*, 1984). Once released into systemic circulation, glucocorticoids exert potent influences on multiple processes including the organization

and processing of memory (Croiset *et al*., 2000) and energy redistribution (Contarino *et al*., 2000; Richard *et al.*, 2002).

Alterations in the functioning of the HPA axis have been observed in individuals with PTSD. The glucocorticoid cortisol, or corticosterone in rodents, is an important component of the stress response system, and as such has been a focus of research in understanding the underlying mechanisms of PTSD. Individuals with PTSD have been observed to express varying levels of cortisol. Studies have demonstrated that individuals with PTSD have exhibited decreased concentrations of cortisol (Boscarino, 1996; Yehuda, 2002; Yehuda *et al.*, 1993, 1995), enhanced sensitivity to negative feedback inhibition of the HPA axis (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997) and expressed increased concentrations and responsiveness of the glucocorticoid receptor (Yehuda *et al*., 1995). Furthermore, individuals that expressed lower cortisol levels in the emergency room immediately following a traumatic experience, such as rape or motor vehicle accidents, had a greater incidence of developing PTSD over time as compared to individuals with higher levels of cortisol that underwent the same trauma and had similar trauma history (Resnick *et al*., 1995; McFarlane *et al*., 1997). This is interesting because it suggests a positive correlation between low cortisol levels following traumatic stress and the development of PTSD. Considered together, it is possible that enhanced sensitivity to negative feedback could result in decreased cortisol in response to stress. Therefore, by possessing decreased concentrations of cortisol, individuals with PTSD are unable to properly activate cortisol-dependent processes mediated through the glucocorticoid receptor during times of stress and therefore develop a variety of PTSD-like behaviors.

It is important to note however, that other studies have demonstrated that individuals with PTSD may exhibit a hyperactivity of the HPA response; sometimes resulting in higher levels of cortisol (Heim *et al*., 2000; Pittman & Orr, 1990; Rasmussen *et al*., 2001), or no difference in cortisol levels from controls (Meewisse *et al.*, 2007; Young & Breslau, 2004). However, in the previous studies significant differences were observed in cortisol concentrations, but only within select subpopulations. For example, Young and Breslau (2004) reported that only when an individual with PTSD expressed a comorbidity with major depressive disorder did they observe a significant elevation of cortisol. This difference was not observed when analyzing PTSD patients without major depressive disorder.

To examine whether individual differences in stress reactivity contribute to development of PTSD we used an animal model that examines individual differences in HPA reactivity based upon locomotor activity in response to novelty (Kabbaj *et al.*, 2000; Liebsch, 1997). Specifically, rats are allowed to explore a novel locomotor chamber for an hour. During the trial, locomotor activity is recorded by infrared beam breaks, which can then be used to separate participating rats into two distinct behavioral phenotypes based upon locomotor activity: low and high responders. Low responders (LR), those who score within the bottom 33rd percentile of locomotor activity, have been shown to exhibit significant differences in both phenotype and genotype as compared to high responder (HR) counterparts who score in the top 33rd percentile (Kabbaj, 2004; Blanchard *et al.*, 2009). Additionally, LRs have been observed to have less corticosterone in response to exposure to a novel environment as compared to HRs (Piazza *et al*., 1991).

This is the first study to suggest that locomotor response to novelty is an indicator of individual differences in HPA reactivity.

Low responders and high responders have also been observed to exhibit multiple behavioral differences in response to a novel, mild stress environment (Kabbaj *et al.*, 2006). As the names imply, LR animals locomote less within a novel environment as compared to HR animals (Piazza *et al.*, 1989; Pierre & Vezina, 1997;1998). Additional behaviors, such as sniffing and rearing have been observed to be different between LR and HR animals (Hooks *et al*., 1994). Low responders typically exhibit reduced rearing and less time spent in the center of the open field.

Individuals with PTSD express altered HPA activity characterized by lower cortisol levels (Yehuda, 2002), suggesting that individuals with PTSD express a blunted stress response which may underlie alterations in the consolidation and/ or extinction of fear memory. The current set of experiments utilizes the LR/HR animal model in which individual HPA responses are readily identifiable prior to exposure to traumatic stress. These experiments aim to more fully understand the behavioral and physiological differences between LR and HR animals. Specifically, these experiments seek to understand the differences, if any, between LR and HR animals in anxiety-like behaviors including open field and light/dark box behavior and to describe HPA axis functioning in the two groups by measuring ACTH and corticosterone concentrations following exposure to a restraint stress. We predict that LRs, as previously reported possessing lower levels of corticosterone and increased anxiety-like behaviors, will exhibit increased anxiety-like behaviors, enhanced sensitivity to glucocorticoid negative feedback, and lower peak corticosterone levels in response to stress as compared to HRs.

Materials and Methods

Animals

Adult male Spraque-Dawley rats (Harlan Laboratories, St. Louis, MO) were housed in cages of two under a 12:12 hour light:dark cycle (lights on 7:00 AM) in a temperature and humidity controlled facility. All procedures were approved by the Marquette University Institutional Animal Care and Use Committee and performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Animal housing facilities were accredited by AALAC.

Identification of Low and High Responders

All behavioral tests were conducted 2 hours into the light phase. Adult male Sprague-Dawley rats were placed into photocell cages that served as a novel locomotor chamber (AccuScan, Columbus, OH) and allowed to explore for 1 hour. Locomotor behavior (photocell beam breaks) was monitored by software provided by the manufacturer. Rats were separated into LR and HR groups based upon cumulative locomotor activity over the 1 hour test. Rats scoring in the bottom 33 percentile were classified as LR, and those in the top 33 percentile as HR.

Light/Dark Activity

Anxiety-like behaviors were evaluated using a modified light/dark box (Crawley and Goodwin, 1980) consisting of a 94.0 cm long×30.5 cm wide×15.2 cm high acrylic glass chamber divided into a 71.1 cm long×30.5 cm wide light compartment with a white floor and walls and a 22.9 cm long×30.5 cm wide dark compartment with a black floor,

walls, and a removable black lid. Movement between compartments was possible through a 10.2-cm passageway. There was no appreciable illumination in the dark box. Time spent in and entries and explorations into the light and dark compartments and latency to enter the light compartment were measured. An entry was defined as introduction of all four paws into a compartment. An exploration was defined as introduction of fewer than four paws into a compartment. The apparatus was cleaned with 70% ethanol solution before testing each rat.

In one study, rats were placed into the light compartment facing one of the side walls. In another study rats were placed into the dark compartment facing one of the side walls at the beginning of the session. The varying of starting points between experiments was to determine any inherent differences between LR and HR animals, specifically in locomotor activity versus anxiety. All variables were assessed in regards to exploration and entries into the light chamber.

Effects of cortisol pretreatment on restraint-induced corticosterone in LR and HR rats

To examine potential differences in the sensitivity of LR and HR rats to rapid glucocorticoid-induced negative feedback, the effects of pretreatment with cortisol on restraint stress-induced increases in ACTH and corticosterone (in separate experiments) were examined in LR and HR rats. LR and HR rats (22 LR, 37 HR) received an injection of cortisol (2 mg/kg, ip, Steraloids, Newport, RI) or vehicle and were subjected to a 30 minute full-body restraint. Restraint studies were conducted in a room distinct from that used for housing. Rats were placed into plexiglass tubes (6.5 cm i.d., 20.5 cm long) sealed at one end with tape to still allow airflow. This configuration prevented movement

in any direction but did not fully immobilize the rat. At the conclusion of the 30 minute restraint period the rat was released and placed back into the cage.

Blood samples were collected using a modified tail incision protocol (Fluttert *et al.,* 2000). A scalpel was used to make a 4-6 mm diagonal incision approximately 10 mm from the end of the tail. At designated time points (0,15, 30, 60, or 90 minutes for restraint stress) blood was collected directly into commercially available EDTA coated capillary tubes (T-MQK, Terumo, Elkton, MD). Samples were centrifuged for 10 min and plasma was collected and stored at -20 ºC for future use.

Corticosterone Radioimmuno Assay

Plasma samples were assayed for corticosterone using a commercially available ^{125}I radioimmunoassay (RIA) kit (Cat. No. 07120103, MP Biomedicals, LLC, Irvine, CA). Dialysates were diluted (1:200) with steroid diluent according to the standard RIA kit protocol. A standard curve was generated with standards provided by the kit with the addition of two additional lower concentration standards (12.5 ng/mL and 6.25 ng/mL). Standards and samples were run in triplicate. ^{125}I tracer and anti-serum were added to all standard and sample tubes (plastic, 12 × 75 mm). Tubes were then vortexed briefly and incubated at room temperature for 2 hours. Following incubation, a precipitant solution was added to all tubes before tubes were vortexed thoroughly. All assay tubes were then centrifuged for 15 minutes at 2500 r.p.m. (1000 x g). Once centrifuged, all tubes were decanted and allowed to drain for 10 minutes. The precipitate was counted in a gamma counter (Genysys GenII Series Gamma Counter, Laboratory Technologies, Inc., Maple Park, IL).

ACTH

Plasma samples were assayed as described previously (Nicholson *et al*., 1984). Standards and samples were assayed for ACTH concentration by way of a competitive radioimmunoassay courtesy of Dr. Robert Spencer, University of Colorado, Boulder, CO.

Statistical Analysis

The significance of differences in behaviors of locomotor activity and within the light/dark box were determined using individual unpaired *t* tests as appropriate. The significance of between-group (LR v HR) and within-group (Drug Treatment) differences in HPA reactivity between LR and HR rats were determined using a two-way analysis of variance (ANOVA), followed by Bonferroni *post-hoc* analysis when appropriate.

Results

Identification of Low and High Responders

Locomotor activity counts, as determined by total distance traveled, followed a unimodal distribution (see Figure 2.1). The median locomotor activity score for the group including all rats (n=399) was 6085. Rats scoring in the top 33 percentile with scores of 7063 and above were assigned to the high responder (HR) group (n=131, mean ± SE activity: ±91.27) and those scoring in the bottom 33 percentile with scores of 5153 and below were assigned to the low responder (LR) group (n=133, mean ±SE activity: ±64.26). The remaining rats with intermediate activity scores between 5160 and 7061

value were labeled as the middle group (MR) (n=135, mean ± SE activity: ±46.90). Total distance traveled in LR and HR animals can be observed in Figure 2.2A.

Center time, the amount of time spent in the center of the locomotor chamber is a measure of anxiety-like behavior (Katz, Roth, & Carroll, 1980). The longer the amount of time spent in the center is indicative of decreased anxiety-like behavior. As expected, LRs were found to spend significantly less time in the center as compared to HRs (Figure 2.2B: $t_{268} = 10.09$, $p < 0.0001$).

Rearing, a risk assessment behavior involving posturing on the hind legs and surveying the environment, can also be considered to be a measure of anxiety (Escorihuela *et al*., 1999). Specifically, lower rearing scores represent higher anxiety because the animal is not actively engaging in the environment. LRs were found to spend significantly less time engaged in rearing behavior compared to HRs (Figure 2.2C: $t_{268} = 10.21$, $p < 0.0001$).

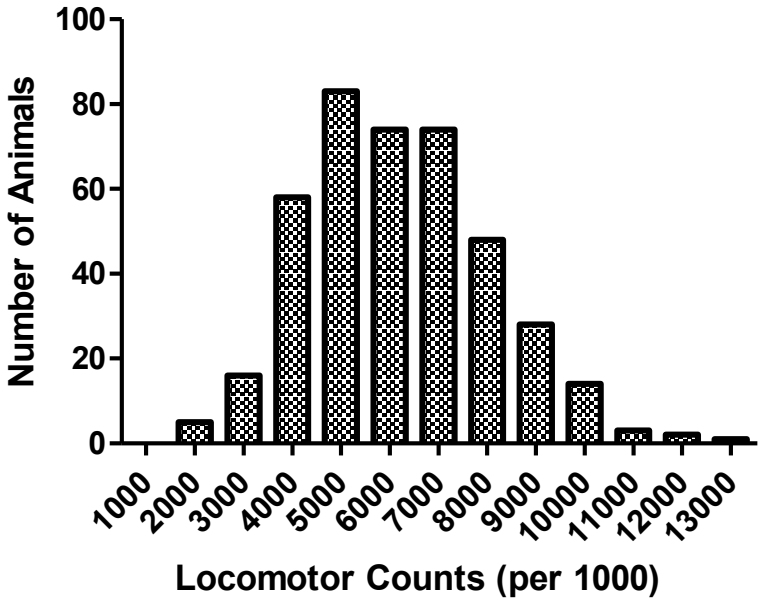

Figure 2.1 Distribution of locomotor activity between LR and HR rats in a novel environment. Rats were placed into the novel environment and separated into 1000 count bins (n=406).

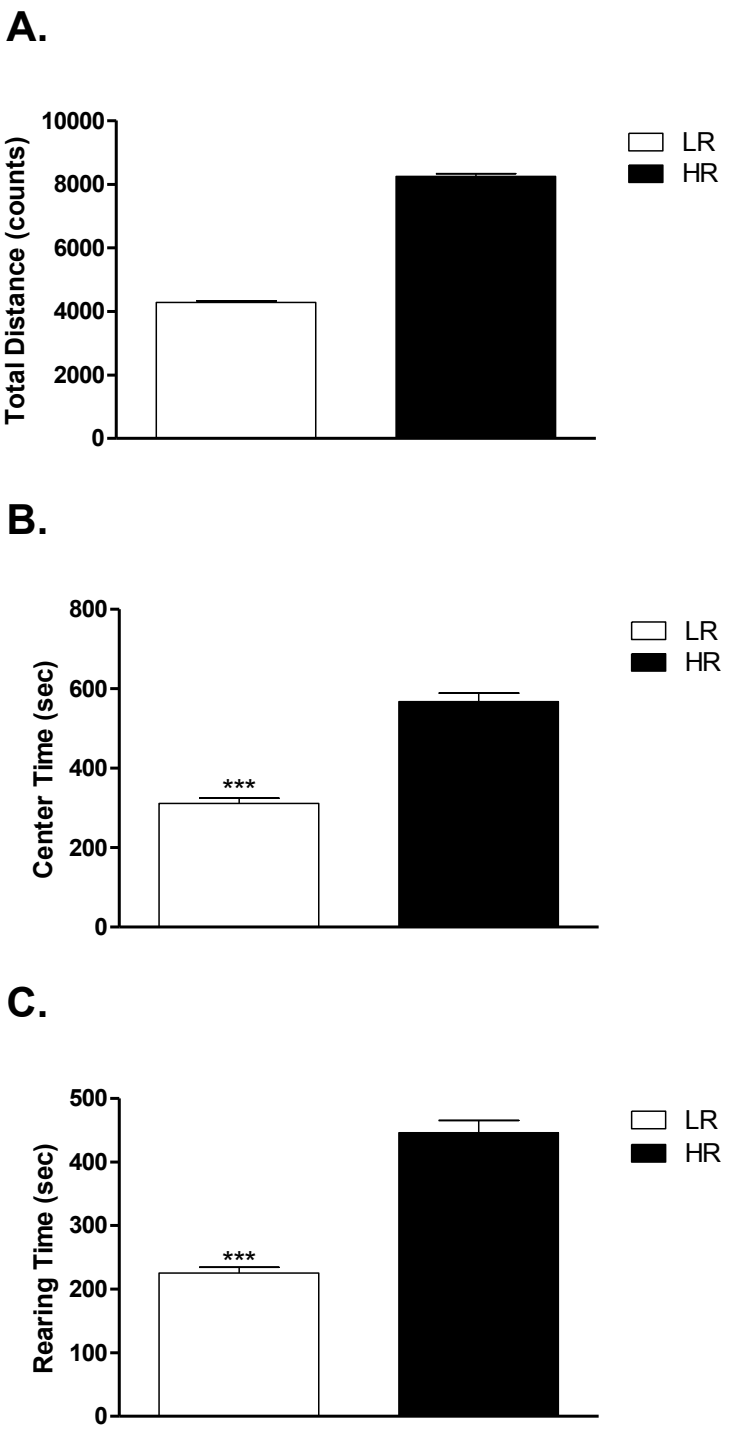

Figure 2.2. Behavioral assessment of LR and HR rats in a novel environment. Rats were placed into the novel environment and behaviors were recorded for total distance (A) (n=133:LR, 131:HR), center time (B) (n=135:LR, 135:HR), and rearing (C) (n=135:LR, 135:HR). All values are mean ± SEM. *** $p<.0001$.

Light-dark box

A total of 39 rats were tested in the light/dark box (11 LR and 28 HR) in which placement started in the dark chamber. The number of entries into the light chamber, number of explorations, latency to enter the light compartment, and total time in light compartment are shown (see Figure 2.3). Low responders explored the light chamber significantly less than HRs (Figure 2.3A: $t_{37} = 2.185$, $p < 0.05$), entered the light chamber significantly less compared to HRs (Figure 2.3B: $t_{37} = 2.064$, $p < 0.05$), and displayed a greater latency to enter the light chamber (Figure 2.3C: $t_{37} = 2.255$, $p < 0.05$).

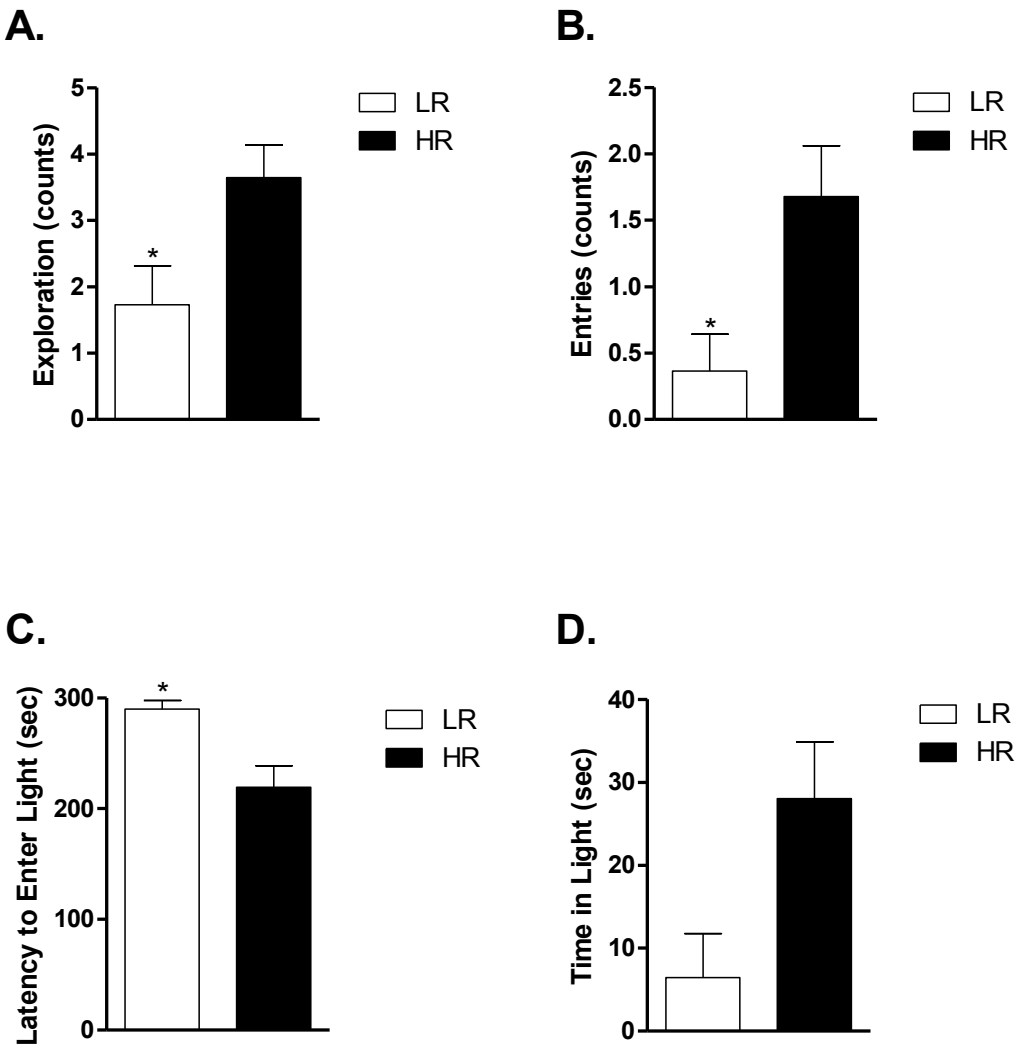

Figure 2.3. Behavior of LR and HR rats in the light/dark box test. Rats were placed in the dark chamber and allowed to explore for 5-minutes. Behavior was recorded for exploration (A), entries (B), latency to enter light compartment (C), and time in light chamber (D). All values are mean ± SEM (n=11:LR, 28:HR). * $p<.05$.

Dark-light box

A total of 65 rats were tested in a reversed placement light/dark box (27 LR and 38 HR). One HR animal was removed due to consistent significant outlier data points (meaning the value was less than or equal to the first quartile minus 3 times the interquartile range). Therefore, 27 LR and 37 HR rats were used for analysis. Rats initiated activity in the light compartment, with latency to enter the dark component as a measure of general activity in addition to number of entries into the light chamber subsequent to entry into the dark chamber. Low responders did not differ from high responders in latency to enter the dark (Figure 2.4A: $t_{63} = 0.2560$, n.s.). Low responders demonstrated significantly fewer entries into the light chamber once they reached the dark chamber (Figure 2.4B: $t_{63} = 2.879$, $p < 0.01$). Exploratory behavior and total time in light were also measured (see Supplementary Figure S1).

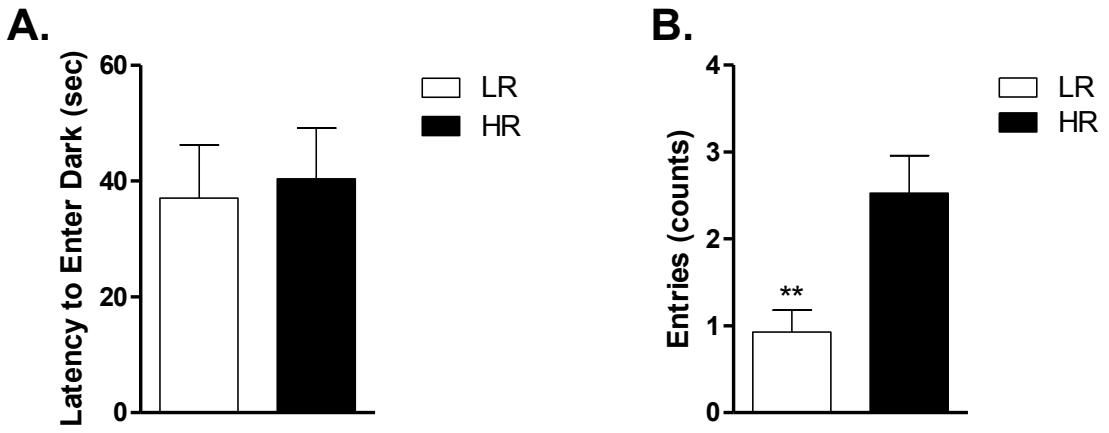

Figure 2.4. In a separate experiment from Figure 2, behavior of LR and HR rats in the light/dark box test. Rats were placed in the light chamber and allowed to explore for 5-minutes. Latency to enter the dark compartment was recorded (A). The number of re-entries into the light chamber was revealed to be significantly different between LRs and HRs (B). All values are mean ± SEM (n=27:LR, 37:HR). $^{**}p<0.01$.

HPA Reactivity

Corticosterone

Thirty seven rats were tested for corticosterone reactivity to stress (9 LR and 28 HR). The effects of restraint stress on plasma corticosterone concentrations were examined by measuring plasma corticosterone concentrations at multiple time points in LR and HR rats following injections of vehicle (see Figure 2.5A) or cortisol 2mg/kg/i.p. (see Figure 2.5B). Low responders displayed less corticosterone in response to restraint-stress as compared to high responders when subjected to pretreatment with vehicle. When subjected to a cortisol (2mg/kg/i.p.) pretreatment, there was no reduction in corticosterone values in LRs. There was however, a reduction in corticosterone levels in HRs. In order to test the hypothesis that LRs would exhibit lower peak corticosterone responses compared to HRs, we used a two-way ANOVA to examine the relationship between phenotype and drug treatment on peak corticosterone concentrations in LR and HR rats. Analysis revealed a significant drug treatment by phenotype interaction ($F_{1,33}$ = 4.163, p <0.05) and a main effect of phenotype ($F_{1,33}$ = 4.606, p <0.05). Bonferroni *post hoc* analysis revealed a significant effect of phenotype in the vehicle treated animals only (t_{27} = 3.667, p<0.01). Specifically, LRs had significantly lower peak corticosterone response than HRs. Additionally, Bonferroni *post hoc* analysis revealed a significant effect of drug treatment in HRs only (t_{26} = 2.335, p<0.05). Specifically, HRs had significantly lower peak corticosterone levels following a cortisol pretreatment (see Figure 2.5C)

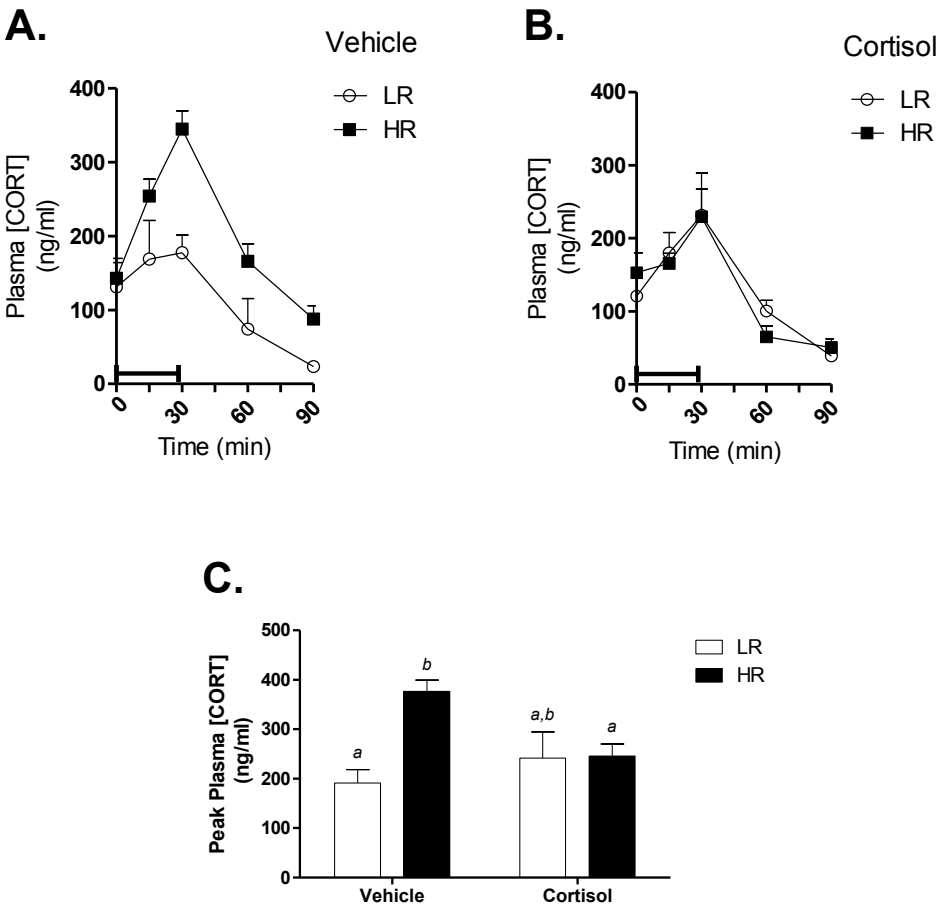

Figure 2.5. Effects of restraint stress on plasma corticosterone concentrations between LR and HR animals. Rats received ip injections ten minutes prior to placement in a plexiglass restraint tube. Blood samples were collected by tail nick at the indicated times. Plasma corticosterone concentrations over time between LR and HR animals following injections of saline (A) or cortisol (B) (2mg/kg, ip). Differences in peak plasma corticosterone concentrations following injections of saline or cortisol (2mg/kg, ip) in LR and HR rats (C). All values are mean ± SEM (n=LR:9, HR:28).

ACTH

Twenty four rats were tested for ACTH reactivity to stress (10 LR and 12 HR). The effects of restraint stress on plasma ACTH concentrations were examined by measuring plasma ACTH concentrations at multiple time points in LR and HR rats following injections of vehicle (see Figure 2.6A) or cortisol 2mg/kg/i.p. (see Figure 2.6B). Both LR and HR rats displayed an increase in plasma ACTH concentrations in response to restraint stress in addition to a reduction in ACTH following administration of a cortisol pretreatment. In order to test the hypothesis that LRs would exhibit lower peak ACTH responses compared to HRs, we used a two-way ANOVA to examine the relationship between phenotype and drug treatment on peak ACTH concentrations in LR and HR rats. Analysis revealed a main effect of drug treatment ($F_{1,18} = 21.21$, $p < 0.001$), but failed to reveal an effect of phenotype ($F_{1,18} = 1.639$, ns). Bonferroni *post hoc* analysis revealed a significant effect of cortisol in both LRs ($t_8 = 2.557$, $p < 0.05$) and HRs ($t_{10} = 4.030$, $p < 0.01$) (see Figure 2.6C).

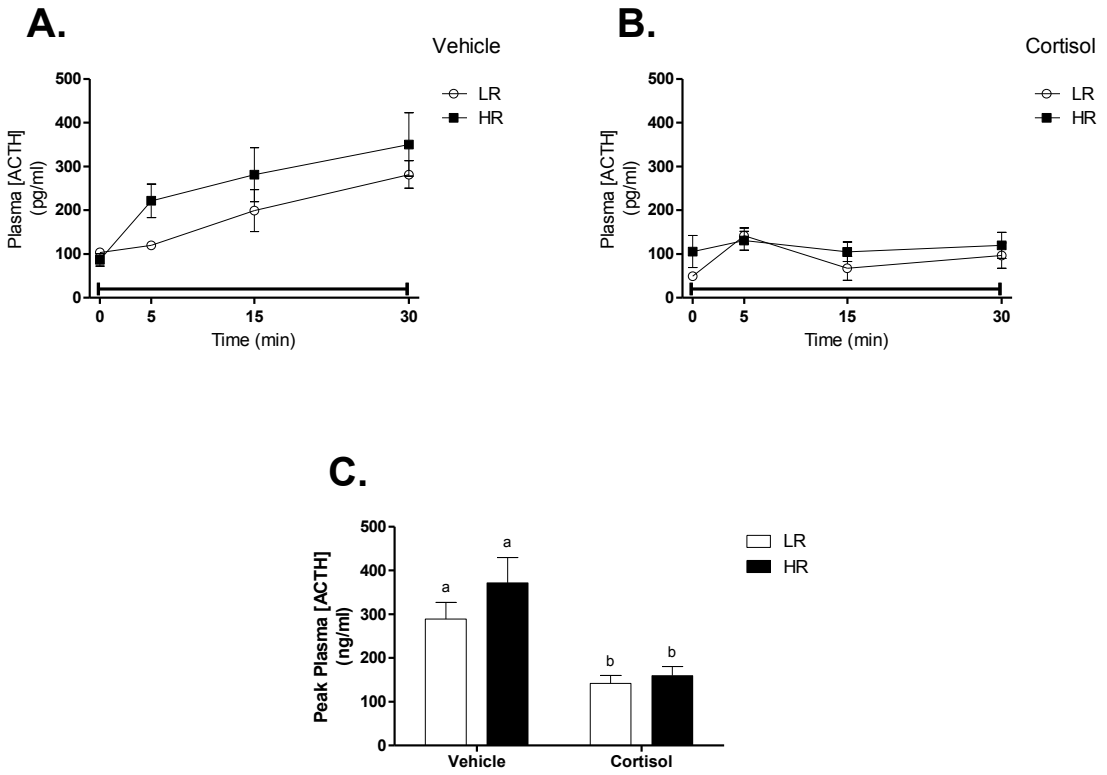

Figure 2.6 Effects of restraint stress on plasma ACTH concentrations between LR and HR animals. Rats received i.p. injections ten minutes prior to placement in a plexiglass restraint tube. Blood samples were collected by tail nick at the indicated times. Plasma ACTH concentrations over time between LR and HR rats following administration of vehicle (A) or cortisol (2mg/kg, ip) (B). Differences in peak plasma ACTH concentrations following injections of saline or cortisol (2mg/kg, ip) in LR and HR rats (C). All values are mean ± SEM (n=LR:10, HR:12).

Histogram Analysis of Locomotor Activity and Peak Stress-Induced Corticosteron

A histogram examining the relation between locomotor activity and peak corticosterone response, regardless of time, was revealed to be non-significant (r^2 = 0.055, $F_{1,49}$ = 2.0889, p<0.0955) (see Figure 2.7).

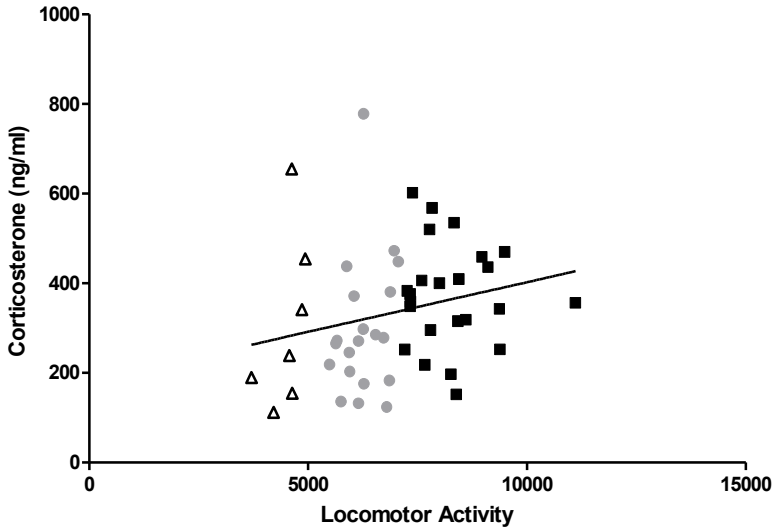

Figure 2.7 A histogram comparing locomotor activity with peak corticosterone response to restraint stress for a subset of animals (n=51). Δ designated as LRs, ○ designated as MRs, ■ designated as HRs.(r^2 = 0.055, $F_{1,49}$ = 2.0889, p<0.0955)

Conclusions

As expected, animals exposed to a novel environment, a mild stressor, separated into distinct behavioral phenotypes as determined by locomotor activity. Locomotor differences between all animals resulted in the emergence of LR (bottom 33%) and HR (top 33%) groups. As expected, LRs exhibited more anxiety-like behavior as demonstrated by spending less time in the center and reduced rearing. Behavioral activity in the light-dark chamber demonstrated a pronounced difference between LR and HR animals. Low responders displayed significantly fewer explorations and entries into the light chamber than did high responders, suggestive of a preference for the dark chamber, which would be in line with the dark chamber representing a safe and less anxiety-provoking environment (Crawley, 1980). It was also observed that LRs displayed a longer latency to enter the light chamber, preferring to spend more time in the dark chamber.

When the light/dark experiment was reversed, and animals began the session in the light chamber, there were no significant differences in latency to enter the dark between LR and HR animals. Thus, LRs moved to escape the light chamber and enter the "safety" of the dark with the same latency as HRs. This finding is important because an alternative theory for the emergence of distinct behavioral phenotypes in the locomotor test could be that LRs represent a more lethargic, or less mobile, group as compared to a more anxious phenotype. This is unlikely because when the placement was reversed LRs moved just as quickly as HRs to get out of the more stressful light environment, and continued to demonstrate elevated anxiety-like behaviors by re-entering the light chamber significantly less than HRs. Furthermore, one could posit that if locomotor differences

were a result of differences in motility then LRs would weigh more than HRs; but we found no weight differences between groups regardless of locomotor score (see Supplementary Figure S2). Finally, in rats selectively bred for divergent stress responses, similar observations in locomotor activity and anxiety-like behaviors were made supporting the idea that behavioral differences exhibited in light/dark box behavior are a result of exposure to novelty (Steimer & Driscoll, 2003).

When subjected to restraint stress, both LRs and HRs exhibited a rise in plasma corticosterone concentrations, which are indicative of corticosterone concentrations within the brain (Weber, Eckert, & Müller, 2006). As expected, based on previous reports indicating that locomotor response to novelty can indicate HPA reactivity (Piazza *et al*. 1991), LRs exhibited significantly less corticosterone in response to stress than did HRs. Additionally, LRs and HRs did not differ in corticosterone under basal non-stressed conditions, suggesting that LRs exhibit a blunted corticosterone response to stress. It is possible that this provides a mechanism by which fear learning and memory processes are altered in individuals with PTSD, but other forms of memory not dependent upon emotional arousal remain unaffected.

In order to investigate enhanced sensitivity to negative feedback, another hallmark of PTSD (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997), rats were pre-treated with cortisol. High responders expressed a reduction in corticosterone similar to what would be expected if negative feedback systems were being activated. Low responders failed to demonstrate a reduction in corticosterone concentrations, suggesting insensitivity to negative feedback. Therefore, to further elucidate the differences in negative feedback sensitivity between phenotypes we examined ACTH concentrations.

Low responders and high responders both demonstrated increases in plasma ACTH concentrations as a result of exposure to restraint stress. There were no differences between groups during basal non-stressed conditions. However, it is possible that significant difference could emerge as a result of an increased sample size, seeing that the current data suggest that in response to stress LRs secrete less ACTH than HRs. In response to pre-treatment with cortisol both LRs and HRs exhibited similar reductions in ACTH, suggesting that at the level of the pituitary the mechanisms responsible for negative feedback inhibition of glucocorticoid release are functional in both groups. Therefore, LRs do not exhibit enhanced sensitivity to negative feedback.

Together, these data suggest that there are no differences in LRs and HRs at the level of the anterior pituitary. Negative feedback systems seem to be functional and similar in both phenotypes. At the level of the adrenal gland, there is a difference in adrenal output between LRs and HRs. Low responders fail to display a reduction in corticosterone following a cortisol-pretreatment, suggesting a lack of adrenal sensitivity to ACTH or that outside physiological systems are modulating adrenal output independent of the HPA response. Specifically, the sympatho-adreno-medullary axis could be exerting influence on the adrenals that results in adrenal output, but is specific to LRs or inactive in HRs. During stress the autonomic nervous system can stimulate the adrenal medulla through the splanchnic nerve (Jasper & Engeland, 1994) and cause the release of cortisol in addition to norepinephrine. Studies have demonstrated that splanchnic nerve innervation can increase adrenal responsivity to ACTH (Ulrich-Lai, Arnhold, & Engeland, 2006). Therefore, as a result of this potential change in ACTH

responsivity LRs secrete corticosterone even under inhibition by cortisol-driven negative feedback.

These data, in combination with previous studies, show that there are group differences between LR and HR rats in HPA reactivity at the level of the hypothalamus, the pituitary, and the adrenals. While we may not be able to directly identify the mechanisms of these differences, or clearly state what the full ramifications of these differences are, we can show that there are distinct and fundamental differences between LR and HR rats in regards to the HPA axis. As such, the LR/HR model provides an invaluable tool to attempt to ascertain the effects of such differences on a variety of PTSD-like behaviors and within PTSD-like animal paradigms. Specifically, LRs provide a means by which we can examine the effects that lower corticosterone levels have on behaviors disrupted in individuals with PTSD, possibly as a result of lower cortisol levels.

Together, these data support the idea that LR animals may provide a model in which we can begin to tease apart the question of whether blunted HPA responses contribute to vulnerability to developing PTSD-like behaviors. Individuals with PTSD display enhanced negative feedback (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997) and decreased levels of cortisol (Mason *et al.*, 1986; Yehuda *et al.*, 1995), the human equivalent of corticosterone. Decreased corticosterone levels and increased anxiety-like behaviors, but not enhanced sensitivity to negative feedback, are characteristics observed in the LR animal phenotype, suggesting that the LR/HR model is a valid model to investigate stress differences, or more specifically; individual differences in the hypothalamic-pituitary-adrenal axis.

CHAPTER III

CONSOLIDATION AND PERSISTENCE OF FEAR MEMORY IN LOW AND HIGH RESONDING RATS

Introduction

Posttraumatic stress (PTSD) disorder is a debilitating anxiety disorder estimated to affect 7.8% of Americans (Kessler *et al.,* 1995). While considered to be mainly a wartime disorder, PTSD is increasingly being recognized in other types of trauma such as natural disasters, severe automobile accidents, diagnosis of a life threatening illness, sudden loss of a loved one, or rape (Hussain *et al.*, 2011; Javidi & Yadollahie, 2012; Breslau *et al.*, 1999; Rothbaum *et al.*, 1992). A study by Norris (1992) found that approximately 69% of Americans experience some form of traumatic stress within their lifetime. However, only about 1 in 13 people in the United States will develop PTSD. Therefore, understanding what contributes to some developing the disorder while the majority do not is of utmost importance.

PTSD develops in response to an extreme physical and/or emotional stress during which the individual experiences actual or perceived threat of death or injury to self or others. This is typically accompanied by a strong sense of horror and paralyzing helplessness (DSM-IV-TR, 2000). Following the traumatic episode, individuals suffering from PTSD can show a wide variety of symptoms, such as persistent, intrusive memories of the traumatic episode and a combination of hyperarousal and numbing symptoms. Early evidence showing individuals with PTSD as having decreased 24-hour mean cortisol concentrations (Yehuda *et al.*, 1995; Mason *et al.*, 1986) suggest that

dysregulation of the neuroendocrine stress response may play a role in the pathogenesis of PTSD. Additionally, research has shown that individuals with PTSD demonstrate enhanced sensitivity to suppression of cortisol in response to treatment with dexamethasone (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997). However, it remains unknown whether the dysregulation of the HPA axis observed in individuals with PTSD is a result of exposure to trauma or was present prior to trauma, potentially representing a marker of vulnerability to PTSD.

Glucocorticoids, such as cortisol, are hormones released from the adrenal glands in response to activation of the HPA axis. The effects of glucocorticoids within the brain are mediated mainly through specific intracellular receptors known as mineralocorticoid (MR) and glucocorticoid receptors (GR) (de Kloet *et al.*, 1998). Mineralocorticoid receptors possess a 10-fold affinity for glucocorticoids such as cortisol, and are typically thought to be occupied under basal non-stressed conditions (Reul & de Kloet, 1985). Therefore, much research has focused on the glucocorticoid receptor (GR) which requires a higher concentration of cortisol, typically observed during stress exposure, in order to be activated. Glucocorticoid receptors (GRs) are found throughout the brain, including areas known to be involved in cognition, such as the hippocampus, amygdala, and prefrontal cortex.

The amygdala, with particular emphasis on the basal and lateral (BLA) regions, is critical for mediating the effects of emotional arousal and fearful events on memory consolidation (McGaugh *et al.*, 1996; McGaugh, 2000). The exact consolidation mechanisms underlying extinction of fear memory remain widely unknown. Research has shown that mediators of the neuroendocrine stress response, such as norepinephrine

(Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2008) and cortisol (corticosterone in rodents) (van Stegeren *et al.*, 2007; McGaugh & Roozendaal, 2002) are key regulators of memory consolidation. Gold and van Buskirk (1975) reported that systemic injections of epinephrine enhanced retention of inhibitory avoidance in a dose-dependent manner when administered after training. This provided the first evidence suggesting that an endogenous hormone could play a role in memory consolidation. It has since been shown that during times of emotional arousal, and/or intense fear, norepinephrine is released within the BLA, inducing activation (McIntyre *et al.*, 2002; Pelletier *et al.*, 2005). Hence, further suggesting a role for norepinephrine in the mediation of consolidation of fear memory. Other research has shown that activation of β –adrenoceptors enhances memory consolidation when administered after training, while antagonism of β -adrenoceptors inhibits norepinephrine effects on memory consolidation. (Liang *et al.*, 1986; Ferry & McGaugh, 1999; Liang *et al.,* 1990).

In a variety of memory tests, administration of glucocorticoids after behavioral training sessions, but not prior to, has enhancing effects on memory consolidation similar to that observed in response to norepinephrine treatment (Pugh *et al*., 1999; Sandi *et al.*, 1997; Cordero *et al.*, 1998; Roozendaal *et al*., 1999; Roozendaal, 2000). Furthermore, the enhancing effects of glucocorticoids on memory have been shown to be dependent upon norepinephrine activation within the BLA (Roozendaal *et al.*, 2006). Treatment with metyrapone, a corticosterone synthesis inhibitor, inhibits the enhancing effects observed with post-training administration of norepinephrine, suggesting that a component of the consolidation mechanism underlying fear memory extinction may involve both the noradrenergic system and glucocorticoids. Imbalances or dysregulation of either system

can have significant effects on the consolidation of extinction for fear memory. This is important because one of the underlying deficits of PTSD is thought to be a deficit in consolidation mechanisms underlying the extinction of fear memory (Rauch *et al.* 2006).

Glucocorticoids play an important role in the maintenance and progression of extinction processes of fear memory. Extinction is a process in which a context cue that was formerly associated with an adverse stimulus is no longer able to elicit a behavioral response (Milad *et al*., 2006). Systemic administration of dexamethasone and intra-amygdala infusion of RU28362, glucocorticoid receptor agonists, prior to extinction training facilitated extinction of conditioned fear in a dose-dependent manner (Yang *et al*., 2006). Conversely, administration of metyrapone, a corticosteroid inhibitor, prior to extinction trials resulted in an inhibition of extinction to context-dependent fear responses in rats (Yang *et al*., 2006) and mice (Blundell *et al*., 2011). Extinction was rescued by administration of corticosterone immediately following extinction trials in mice that received a pre-trial injection of metyrapone (Clay *et al.*, 2011), reinforcing the idea that corticosterone is a critical modulator in learning and memory.

Individuals with PTSD may also exhibit an exacerbation of the fear response over time in the absence of extinction (Frueh *et al*., 2009; Smid *et al.*, 2011). This phenomenon can be considered an incubation of fear. Fear incubation is best described as an increase in fear and anxiety that occurs over time following exposure to fear conditioning (Pickens *et al*., 2010; Schreurs *et al*., 2011). Therefore, an individual with PTSD experiences the traumatic event and forms associative memory traces between the trauma and cues in the environment. Over time, the fear response is elevated even without re-exposure to the cue or the environment. This is counter to what occurs in non-

PTSD individuals, where a memory will typically extinguish barring re-exposure to the noxious cue. As a result, exposure therapy has been utilized for many with PTSD in order to provide a context similar to the precipitating trauma so that they might be able to extinguish the fear memory and to counter the effects of fear incubation (Myers *et al.*, 2007; Beckett, 2002; Quirk *et al*., 2008).

Overall, these data suggest that glucocorticoids are critical in the consolidation and extinction of conditioned fear and indicate the amygdala as an important component of these memory processes. Furthermore, these data suggest that decreased glucocorticoids during re-activation of a fear memory may lead to a deficit in the extinction of fear memory. This is important because it suggests a possible mechanism which could explain why individuals with PTSD, many of whom display decreased basal cortisol levels, have an inability to extinguish fear memory. Specifically, reduced cortisol levels could prevent proper activation of GR and inhibition of the high-capacity monoamine transporter organic cation transporter 3 (OCT3), resulting in a reduction in downstream signaling cascades resulting in dysregulation of consolidation and extinction of fear memory.

Organic cation transporter 3 (OCT3) is a corticosterone-sensitive low-affinity, high-capacity transport mechanism capable of clearing norepinephrine, epinephrine, dopamine, histamine, and serotonin from the extracellular space within the brain (Grundemann *et al.*, 1998; Wu *et al*., 1998, Duan &Wang, 2010; Baganz *et al.*, 2008; Feng *et al*., 2009; Gasser *et al.*, 2006; Gasser *et al.*, 2009). Of particular importance, is that OCT3 is stress-sensitive and under high concentrations of glucocorticoids monoamine transport is directly inhibited (Iversen & Salt, 1970; Simmonds & Gillis,

1958; Hill *et al.*, 2010). Corticosterone inhibition of OCT3 has been implicated in the expression of altered behavioral responses. Specifically, inhibition of OCT3 has been demonstrated to play a role in the potentiation of drug-seeking behaviors (Graf *et al.* unpublished). This is especially relevant because it provides a mechanism by which glucocorticoids can modulate learning and memory processes reflected by behavioral changes. It is possible that glucocorticoid inhibition of OCT3 modulates the norepinephrine response within the amygdala. When there is insufficient corticosterone available, OCT3 continues to clear norepinephrine from the extracellular space limiting its reported enhancing effects on memory, thus contributing to dysregulation of consolidation mechanisms underlying the extinction of fear memory.

To address this question we turned to animal models whereby we could examine individual differences in HPA responsiveness in the context of Pavlovian fear conditioning and subsequent differences in memory consolidation, extinction and incubation. Previous studies have focused on separating Spraque-Dawley rats into distinct phenotypic groups based upon locomotor activity during exposure to novelty (Kabbaj *et al.*, 2000) as an indicator of HPA reactivity. Animals scoring in the bottom 33% percentile were labeled as low responders (LR) and animals scoring in the top 33% percentile were labeled as high responders (HR). Any intermediate scoring animals were identified as median responders (MR). Kabbaj *et al.* (2000) also demonstrated that in response to restraint stress, rats designated as LRs had significantly lower peak corticosterone responses than HR rats. This suggests that at the level of HPA responsiveness, LR rats display a pre-existing blunted stress response, similar to what would be expected in an individual with enhanced sensitivity to negative feedback.

Proper regulation of the neuroendocrine stress response is critical in the regulation of glucocorticoids and norepinephrine, both of which are mediators in the consolidation mechanisms underlying extinction of fear memory. Therefore the aim of this study was to examine the effects of blunted HPA responsiveness on consolidation and persistence of contextual fear memory in LR and HR rats. We predicted that LR rats, which as a group display blunted corticosterone responses to stress, would display a prolonged and more pronounced fear response, a deficit in the extinction of the fear response, and lower corticosterone levels following extinction.

Materials and Methods

Animals

Adult male Spraque-Dawley rats (Harlan Laboratories, St. Louis, MO) were housed in cages of two under a 12:12 hour light:dark cycle (lights on 7:00 AM) in a temperature and humidity controlled facility. All procedures were approved by the Marquette University Institutional Animal Care and Use Committee and performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Animal housing facilities were accredited by AALAC.

Identification of Low and High Responders

Adult male Sprague-Dawley rats were placed into photocell cages that served as a novel locomotor chamber (AccuScan, Columbus, OH) and allowed to explore for 1 hour. Locomotor behavior (photocell beam breaks) was monitored by software provided by the manufacturer. Rats were separated into Low and High responders based upon cumulative

locomotor activity over the 1hour test. Rats scoring in the bottom 33% were classified as low (LR), and those in the top 33% as high (HR) responders.

Experimental Design

Experiment 1: Consolidation and extinction of contextual fear memory in LR and HR rats.

Two versions of this experiment were carried out. In Experiments 1A and 1B, rats (Experiment 1A: 14LR, 9HR; Experiment 1B: 8LR, 14HR) were subjected to a contextual fear conditioning session (D1) followed by a consolidation test (D3) and then a series of extinction sessions over the following week (D6-10). Rats in experiment 1B then received an extinction challenge on D32.

On Day 1, rats were trained in a Pavlovian contextual fear conditioning procedure. Rats were tail-marked within their respective colony room before transport to the laboratory. Rats were placed in the conditioning chambers and allowed to explore for 4 minutes before receiving a series of 3 electric foot shocks (1mA intensity, 2 second duration, 3 minutes inter-shock interval). Animals were removed from the chamber 30 seconds after the final shock and returned to their home cages.

On Day 3, rats were returned to the training chambers for a test of consolidation of contextual fear memory. After a 10-min period for observation, rats were returned immediately to the home cage.

For the remainder of the experiment, rats were exposed daily to the training context for 10 minutes, and behavior was recorded.

In Experiment 1B, rats were trained and put through extinction as in experiment 1A. Animals were returned to the conditioning chambers on D32 to examine the persistence of extinction memory. Rats were then immediately returned to the home cage.

Experiment 2: Incubation effects on consolidation of contextual fear memory in LR and HR rats.

Experiment 2 was designed to examine potential differences between LR and HR animals in incubation of fear responses, or more specifically if the lack of repeated extinction training had an effect on behavior. LR and HR rats (8 LR, 12HR) were subjected to a contextual fear conditioning session (D1) followed by re-exposure to the training chambers on D10 and D32.

On Day 1, rats were trained in a Pavlovian contextual fear conditioning procedure as in Experiment 1. After training, rats were returned to their home cages for 9 days, during which time they received standard care, including cage cleaning.

On Day 10, rats were returned to the training chambers for a 10 minute period for observation, during which behavior was recorded, and returned to their home cages. Rats remained in home cages for 22 days with routine care and cage cleaning.

On Day 32, rats were returned to the conditioning chambers for a 10 minute period for observation during which behavior was recorded. Rats were then immediately returned to the home cage.

Experiment 3: Plasma corticosterone concentrations following a series of extinction trials.

Thirty-seven rats were used for sampling following a fear conditioning paradigm (20 LR, 17 HR). Rats were subjected to contextual fear conditioning as described in

Experiment 1A. On Day 10, rats were placed within the context and allowed to explore for 10 minutes, and immediately following the 10 minute extinction trial rats were subjected to blood sampling.

Experiments 1A, 1B, 2, and 3 utilized different groups of animals.

Fear Apparatus

Animals were trained in squads of four, in four identical conditioning chambers (30 cm × 24 cm × 21 cm; Med Associates Inc., St. Albans, VT). The chambers were installed on a stainless-steel rack in a brightly lit room (8 100-W overhead incandescent bulbs). The ceiling and back wall of each chamber were constructed of opaque white plastic. The sides of each chamber were constructed of aluminum. The front wall/door was constructed of clear polycarbonate plastic. The floor of each chamber was constructed of a removable grid and waste pan. Before each squad of animals was conditioned, the chambers were cleaned with a 1% acetic acid solution (in tap water) and dried thoroughly with paper towels and a hair dryer. A thin film of the same solution was placed in the waste pan of each chamber as well. The grid floor was composed of 36 stainless steel rods (3 mm dia., spaced 8 mm apart center to center). The grid floor, when placed in the chamber, made contact with a printed circuit board through which shock was delivered. The presentation of all stimuli was programmed with a PC running MedAssociates software (Med Associates, Inc., St. Albans, VT). During training and testing, background white noise (60 dB) was provided by a standard HEPA air filter. Sound levels for the background white noise were calibrated and monitored with a Radio Shack dB meter (A scale). Shock intensity was confirmed for each grid of each chamber before the introduction of each squad of animals, with a storage oscilloscope (B&K

Precision Corporation, Yorba Linda, CA) and a 10 kΩ resistor. Context testing and extinction took place in the same chambers and under the same ambient conditions as did training but in the absence of any other stimuli.

Collection of Plasma

Blood samples were collected using a modified tail incision protocol (Fluttert *et al.,* 2000). A scalpel was used to make a 4-6 mm diagonal incision approximately 10 mm from the end of the tail. Immediately following a 10 minute extinction session on Day (10), blood (from a separate group of animals) was collected directly into commercially available EDTA coated capillary tubes (T-MQK, Terumo, Elkton, MD). Samples were centrifuged for 10 min and plasma was collected and stored at -20 ºC for future use.

Corticosterone Radioimmuno Assay

Plasma samples were assayed for corticosterone using a commercially available ^{125}I radioimmunoassay (RIA) kit (Cat. No. 07120103, MP Biomedicals, LLC, Irvine, CA). Dialysates were diluted (1:200) with steroid diluent according to the standard RIA kit protocol. A standard curve was generated with standards provided by the kit with the addition of two additional lower concentration standards (12.5 ng/mL and 6.25 ng/mL). Standards and samples were run in triplicate. ^{125}I tracer and anti-serum were added to all standard and sample tubes (plastic, 12 × 75 mm). Tubes were then vortexed briefly and incubated at room temperature for 2 hours. Following incubation, a precipitant solution was added to all tubes before tubes were vortexed thoroughly. All assay tubes were then centrifuged for 15 minutes at 2500 r.p.m. (1000 x g). Once centrifuged, all tubes were decanted and allowed to drain for 10 minutes. The precipitate was counted in a gamma

counter (Genysys GenII Series Gamma Counter, Laboratory Technologies, Inc., Maple Park, IL).

Behavioral analysis

During all contextual fear procedures, a single camera recorded behavior from all four chambers simultaneously. The video signal was sent to a DVD recorder for storage and records were digitized later to Quicktime files on a Macintosh G5. For freezing measures, video records were digitized at 1 Hz. A human observer counted the number of samples, per minute, in each resulting video file during which the animal made any movement. Freezing behavior simply was quantified as the percentage of samples during which no movement was detected. Freezing percentage scores for the context tests were averaged across the first 4 minutes (pre-shock) and extinction tests were averaged across the total 10 minute exposure.

Statistical Analysis

Between-group and within-group differences in consolidation and extinction behaviors between LR and HR rats were examined using a two-way repeated measures Phenotype x Day ANOVA followed, when appropriate, by Bonferroni *post hoc* analysis.

Results

Experiment 1A: Consolidation and extinction of contextual fear memory in LR and HR rats

In experiment 1A, twenty three rats were used for extinction testing (14 LR and 9 HR). A repeated measures two-way ANOVA (Phenotype x Day) examining differences between LRs and HRs in freezing behavior showed a significant main effect of day ($F_{6,126}$

=27.10; $P<0.0001$) (see Figure 3.1A). Bonferroni *post hoc* analysis revealed both LRs ($t_{12} = 9.684$, $p<0.001$) and HRs ($t_7 = 7.878$, $p<0.001$) displayed significant increases in freezing behavior from Day 1 to Day 3. LRs, but not HRs displayed a significant reduction in freezing behavior from Day 3 to Day 6 ($t_{12} = 2.934$, $p<0.01$). However, compared to Day 3, LRs (see Figure 3.1B) and HRs (see Figure 3.1C) showed significant reductions in freezing behavior on Day 7 (LR: $t_{12} = 3.171$, $p<0.01$; HR: $t_7 = 2.926$, $p<0.01$), Day 8 (LR: $t_{12} = 3.604$, $p<0.001$; HR: $t_7 = 3.916$, $p<0.001$), Day 9 (LR: $t_{12} = 3.266$, $p<0.01$; HR: $t_7 = 3.975$, $p<0.001$), and Day 10 (LR: $t_{12} = 4.258$, $p<0.001$; HR: $t_7 = 4.495$, $p<0.001$). Additionally, HRs demonstrated a significant reduction in freezing on the last day of extinction (Day 10) as compared to the first day of extinction (Day 6) ($t_7 = 2.537$, $p<0.05$). No significant differences in freezing where revealed between LRs and HRs on any day (see Figure 3.1D).

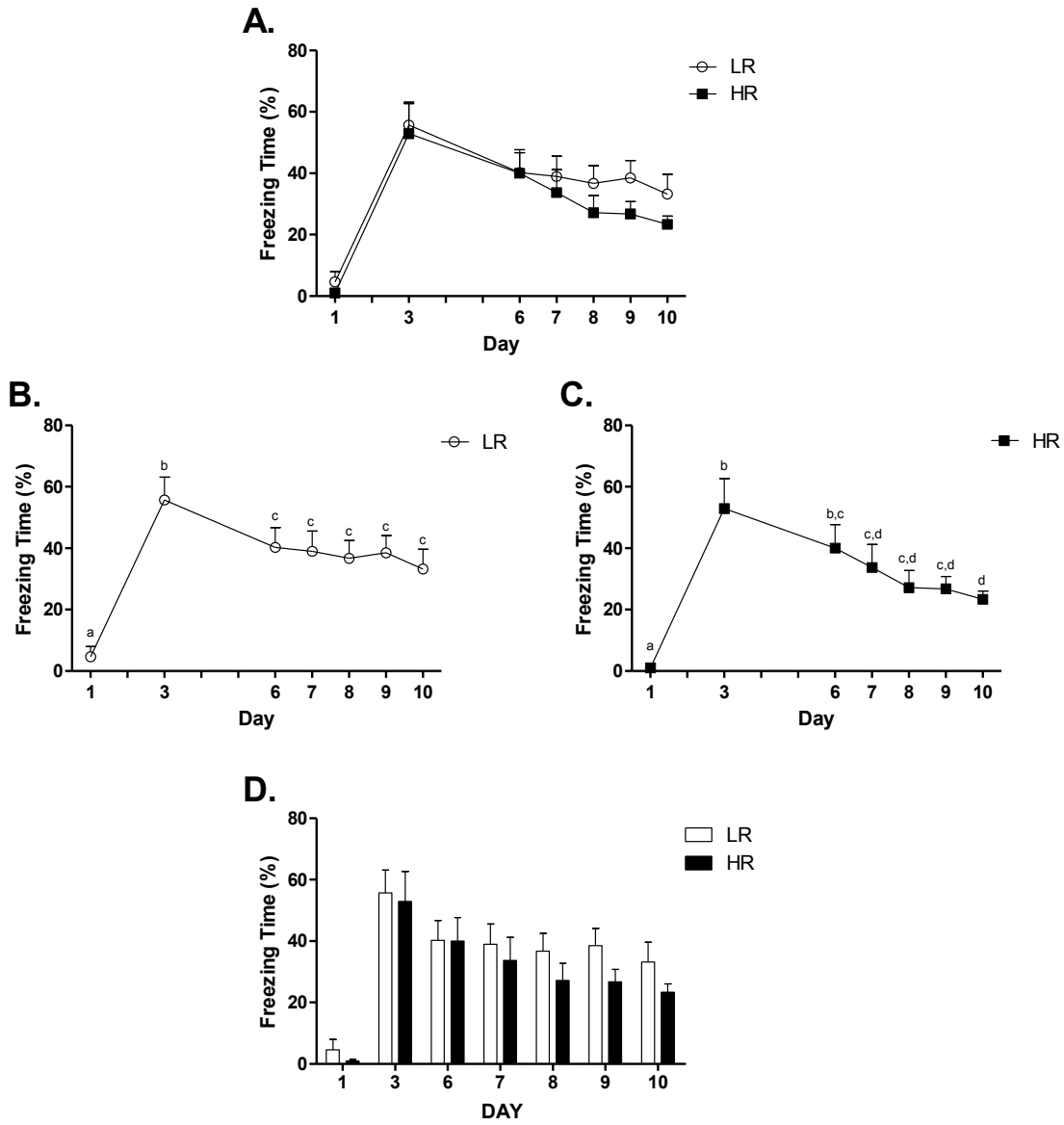

Figure 3.1. Experiment 1A. Consolidation and extinction of contextual fear memory in LR and HR rats. Rats received a training trial followed by a consolidation test and subsequent extinction trials to measure consolidation of fear memory through freezing behavior (A). Freezing behavior between LR (B) and HR rats over time (C). Repeated measures two-way ANOVA showed main effect of Day, but failed to show any significant interaction between Phenotype and Day. There were no significant differences between LRs and HRs at any point (D). All values are mean ± SEM (n=14LR, 9HR).

Experiment 1B: Consolidation and extinction of contextual fear memory in LR and HR rats

In the second experiment, twenty two rats were used for extinction testing (8 LR, 14 HR). A repeated measures two-way ANOVA (Phenotype x Day) examining differences between LRs and HRs in freezing behavior showed a significant main effect of day ($F_{7,140}$=46.70; p<0.0001) and a main effect of phenotype ($F_{1,140}$=4.859; p<0.05) but failed to reveal a significant interaction between phenotype and day ($F_{7,140}$=1.249; ns) (see Figure 3.2A).

Bonferroni *post hoc* analysis revealed both LRs (t_6 = 11.39, p<0.001) and HRs (t_{12} = 10.37, p<0.001) displayed significant increases in freezing behavior from Day 1 to Day 3. Low responders displayed significantly higher freezing on Day 3 compared to HRs (t_{20} = 3.030, p<0.05) see Figure 3.2D).

Compared to Day 3, LRs (see Figure 3.2B) and HRs (see Figure 3.2C) showed significant reductions in freezing behavior on all consecutive days: Day 6 (LR: t_6 = 5.967, p<0.001; HR: t_{12} = 6.030, p<0.001), Day 7 (LR: t_6 = 7.844, p<0.01; HR: t_{12} = 7.987, p<0.01), Day 8 (LR: t_6 = 8.790, p<0.001; HR: t_{12} = 9.330, p<0.001), Day 9 (LR: t_6 = 9.230, p<0.01; HR: t_{12} = 9.788, p<0.001), Day 10 (LR: t_6 = 9.776, p<0.001; HR: t_{12} = 10.03, p<0.001), and Day 32 (LR: t_6 = 8.197, p<0.001; HR: t_{12} = 9.170, p<0.001).

Compared to the first extinction trial (Day 6) LRs and HRs showed significant reductions in freezing behavior in subsequent extinction trials from Day 8 through Day 10: Day 8 (LR: t_6 = 2.823, p<0.05; HR: t_{12} = 3.300, p<0.01), Day 9 (LR: t_6 = 3.263, p<0.01; HR: t_{12} = 3.758, p<0.001), Day 10 (LR: t_6 = 3.810, p<0.001; HR: t_{12} = 4.004, p<0.001). When exposed to a delayed extinction trial on Day 32, only HRs continued to

display significantly less freezing compared to the first extinction trial (Day 6): (LR: t_6 = 2.231, ns; HR: t_{12} = 3.140, p<0.01).

When compared to acquisition on Day 1, LRs and HRs displayed increased freezing behavior on Day 6 (LR: t_6 = 5.425, p<0.001; HR: t_{12} = 4.338, p<0.001) and Day 7 (LR: t_6 = 3.548, p<0.001; HR: t_{12} = 2.382, p<0.05). On Day 8, only LRs displayed significantly higher freezing compared to Day 1 (LR: t_6 = 2.602, p<0.05; HR: t_{12} = 1.039, p<0.05). Neither group displayed significantly different freezing behaviors on Days 9 or 10 compared to Day 1. However, when exposed to a delayed extinction trial on Day 32, LRs only exhibited a significant increase in freezing behavior compared to Day 1 (LR: t_6 = 3.194, p<0.01; HR: t_{12} = 1.198, ns).

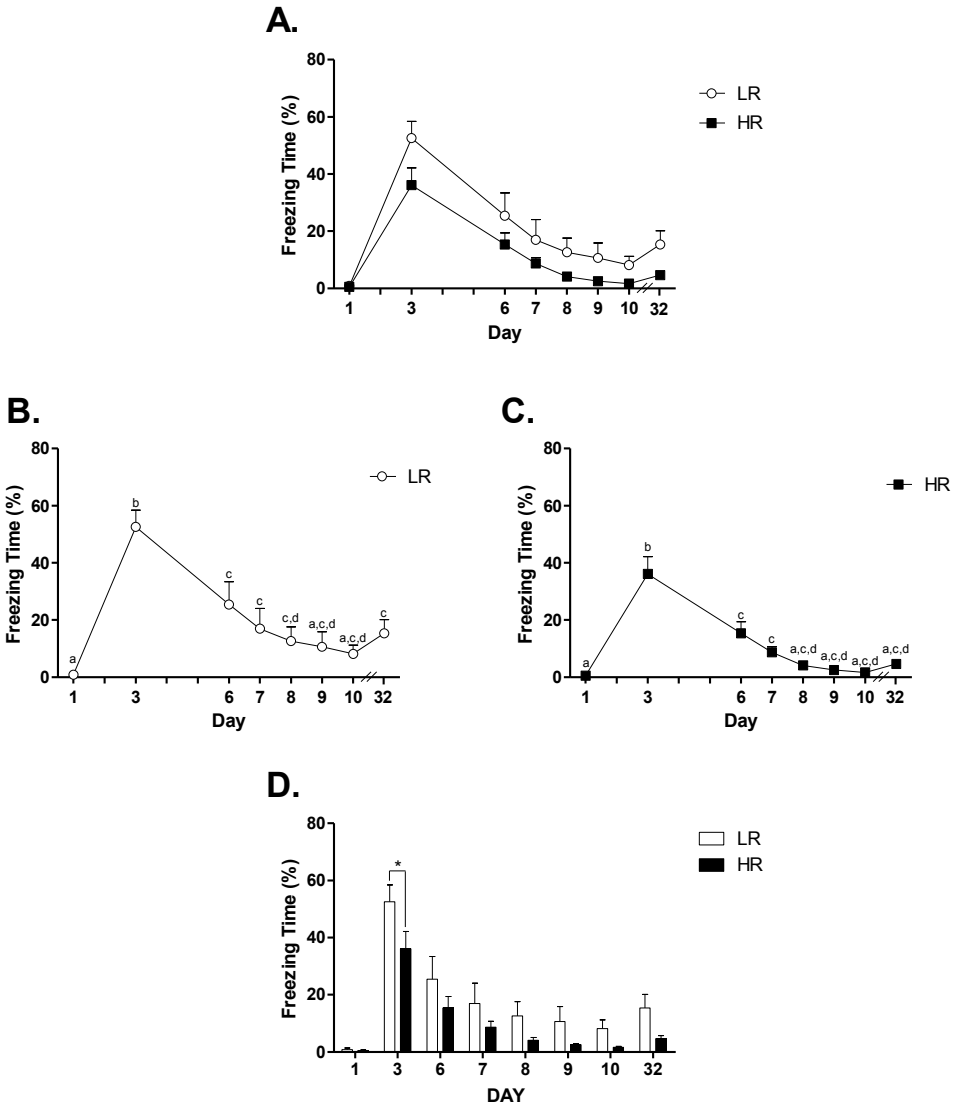

Figure 3.2. Experiment 1B. Consolidation and extinction of contextual fear memory in LR and HR rats. Rats received a training trial followed by a consolidation test and subsequent extinction trials to measure consolidation of fear memory through freezing behavior. Rats underwent delayed extinction trial to measure persistence of fear memory on Day 32 (A). Freezing behavior in LR (B) and HR (C) rats over time. Repeated measures two-way ANOVA revealed a main effect of Day and Phenotype, but failed to reveal any significant interaction between Phenotype and Day. Freezing behavior was significantly different between LRs and HRs on Day 3 (D). All values are mean ± SEM (n=8LR, 14HR).

Experiment 2: Incubation effects on consolidation of contextual fear memory in LR and HR rats.

Twenty rats were used for incubation testing (8 LR, 12 HR). A two-way repeated measures ANOVA (Phenotype x Day) showed a significant main effect of day ($F_{2,36}$ =63.70; P<0.0001) but failed to reveal a significant effect of phenotype ($F_{1,36}$ =2.667; ns) (see Figure 3.3A). *Post hoc* analysis using Bonferroni t-test revealed significant increases in freezing behavior between Day 1 and Day 10 in both LRs (t_6 = 7.967, p<0.001) and HRs (t_{10} = 6.803, p<0.001). The elevation in freezing behavior was maintained over time and found to be significant between Day 1 and Day 32 in both LRs (t_6 = 6.768, p<0.001) and HRs (t_{10} = 5.752, p<0.001) (see Figure 3.3B).

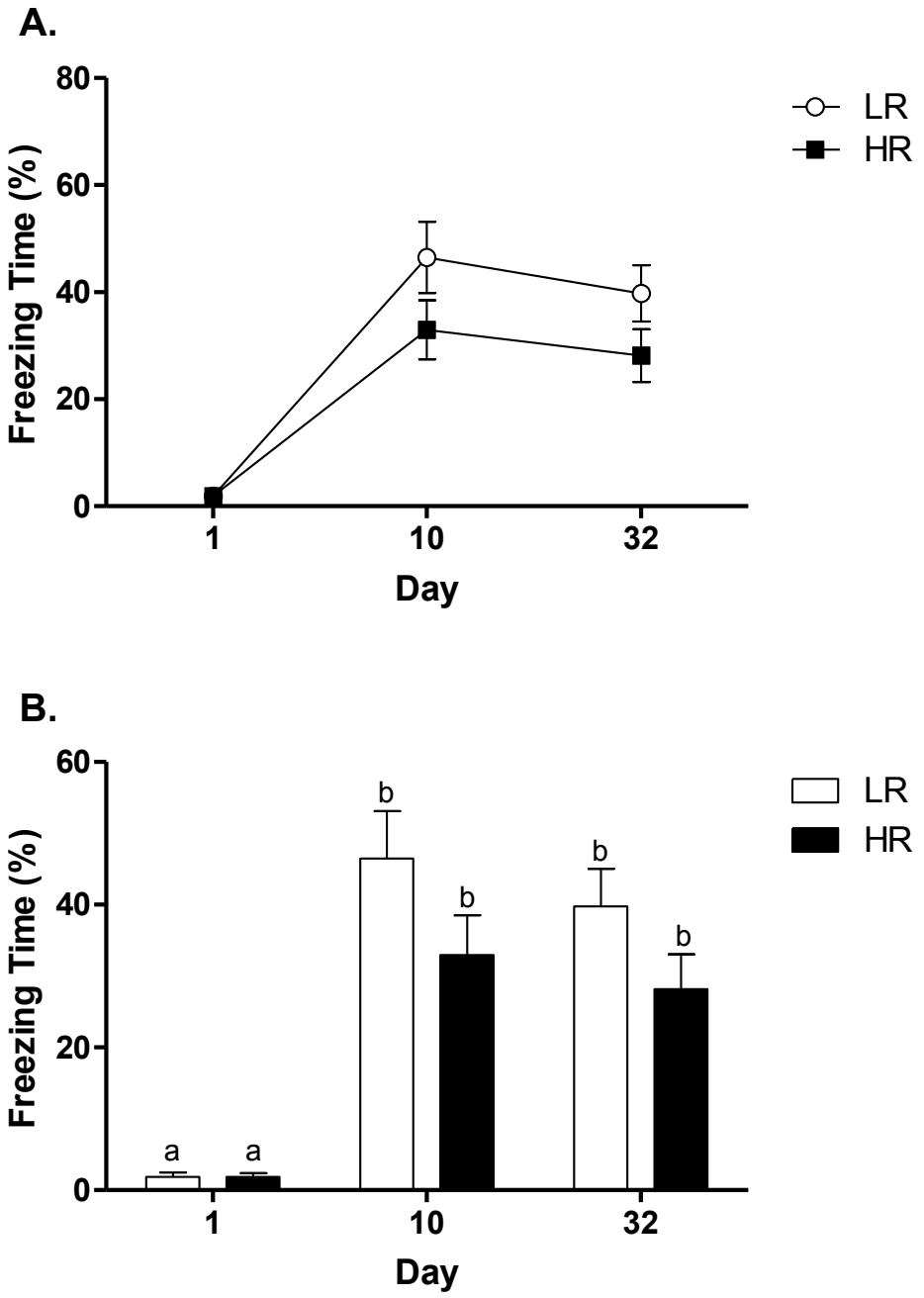

Figure 3.3. Experiment 2. Incubation effects on consolidation of contextual fear memory in LR and HR rats. Rats received a training trial followed by a consolidation test on D10 and an extinction trial on D32 to investigate any differences in behavioral response as a result of an incubation effect. Freezing behavior between LR and HR rats over time (A). Repeated measures two-way ANOVA revealed significant main effect of Day, with both LRs and HRs displaying significantly elevated freezing on Day 10 and 32 compared to Day 1 (B). All values are mean ± SEM (n=8LR, 12HR).

Experiment 3: Plasma corticosterone concentrations following a series of extinction trials.

Thirty-seven rats were used for sampling following a fear conditioning paradigm (20 LR, 17 HR). Analysis using individual unpaired *t* test comparing LR and HR corticosterone levels following extinction revealed LRs to display significantly lower corticosterone than HRs ($t_{35} = 2.886$, $p < 0.01$) see Figure 3.4.

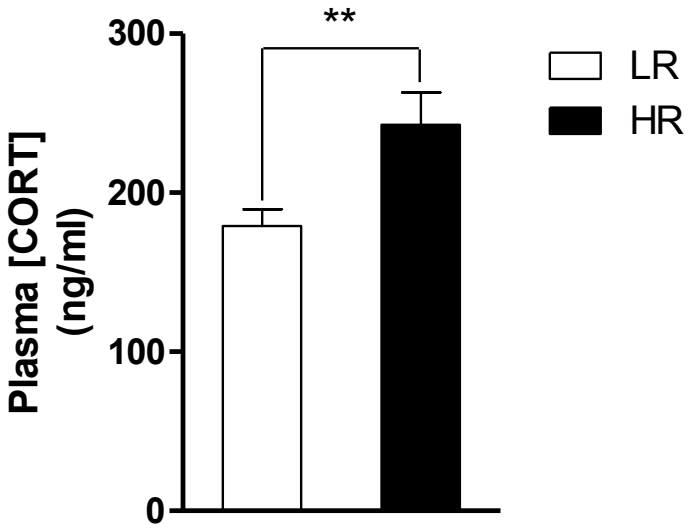

Figure 3.4. Plasma corticosterone concentrations in LRs and HRs following extinction. On the last day of extinction rats were placed within the training context for 10-minutes and allowed to explore freely. Immediately following extinction, rats were removed and subjected to blood sampling and plasma corticosterone concentrations were measured. All values are mean ± SEM (n=20LR, 17HR). ** $p<.001$.

Conclusions

In the current study, we examined the consolidation and the persistence of fear memory in LR and HR rats. Experiments 1A and 1B examined differences between LRs and HRS over the course of fear memory acquisition followed by a series of extinction trials. Experiment 2 examined maintenance and persistence of fear memory in LR and HRs rats not subjected to repeated extinction. In an experiment similar to experiment 1A, we examined the levels of plasma corticosterone immediately following exposure to a series of successive extinction trials.

In experiment 1A, both LRs and HRs showed significant increases in freezing behavior on the day of consolidation (Day 3) compared to Day 1, demonstrating that both phenotypes learned fear. Of note, LRs and HRs did not exhibit differences in freezing behavior on the day of acquisition. Over the course of extinction trials both LRs and HRs displayed reductions in freezing behavior compared to Day 3, suggesting that both phenotypes were capable of extinguishing the fear response. Low responders, but not high responders, failed to show significant reductions in freezing behavior on the last day of extinction compared to the first extinction trial. This is the first time we observed LRs to be potentially resistant to extinction, or maintaining a longer and more persistent fear response compared to HRs.

There were no significant differences in freezing behavior between LRs and HRs at any point in time. It was noted however, that a difference between LRs and HRs started to emerge following repeated extinction trials, but never reached statistical significance, suggesting a possible emergence of a differential extinction response that could be significant if we increased our sample size. However, the lack of a significant difference

in freezing behavior between LRs and HRs was not expected. Low responders have repeatedly been found to exhibit decreased exploratory and locomotor behaviors (Kabbaj, 2004; Blanchard *et al.*, 2009; Kerman *et al*., 2011; Kabbaj, 2006). It was expected that following acquisition there would be a distinct separation between LRs and HRs, with LRs exhibiting increased freezing.

Experiment 1B revealed similar results in that both LRs and HRs did not display any initial differences in freezing on Day 1 alone, but both groups did display significant increases in freezing behavior on Day 3 as compared to Day 1, suggesting that again both phenotypes learned fear. However, LRs displayed significantly higher freezing on Day 3 compared to HRs, suggesting that LRs consolidated fear memory better than HRs. Conversely, it is possible that HRs did not consolidate the fear memory as well, but based on the significant increase in freezing compared to acquisition, HRs were still able to demonstrate some degree of learning. Throughout extinction, both LRs and HRs demonstrated reductions in freezing behavior compared to consolidation testing, indicating that both groups were extinguishing. However, LRs took longer to display significant reductions in freezing as a result of extinction than did HRs. Again, LRs are maintaining a longer and more persistent fear response than HRs. Furthermore, when exposed to a delayed extinction trial on Day 32, only LRs displayed significant elevations in freezing compared to Day 1. Together, these data indicate that LRs extinguish slower and maintain a longer and more persistent fear response as compared to HRs, and display heightened fear responses when re=exposed to the context after a long delay.

Experiments 1A and 1B displayed similar, but conflicting data in regards to consolidation and extinction of fear memory in LRs and HRs. To resolve the conflicting

results between experiments, we examined any differences in experimental design between animals. Animals in experiment 1A were handled daily for nine days, once per day for 5 minutes per animal by laboratory personnel regardless of involvement in experimentation, meaning that some of the handlers were not the experimenters. Animals in experiment 1B were handled daily for 2 weeks, 2 times per day for approximately 2 minutes per animal per session by laboratory personnel involved in experimentation. Handling has been demonstrated to reduce glucocorticoid secretion (Vallée *et al*., 2008) and thereby reduce stress-like behaviors (Costa *et al.*, 2012). Therefore, a lack of handling could result in the observed behaviors, as compared to experiment 1B. Low responders in general would be expected to demonstrate higher levels of freezing compared to HRs (Kabbaj, 2004; Blanchard *et al.*, 2009; Kerman *et al*., 2011; Kabbaj, 2006). The difference in handling could contribute to the elevation of freezing behaviors in experiment 1A compared to experiment 1B. Thus, HRs demonstrating heightened freezing could be demonstrating elevated stress behaviors due to a lack of inurement, not in response to the context. The increased handling in experiment 1B provided increased habituation to physical manipulation, a necessary component of fear conditioning, thereby potentially reducing anxiety and stress as a result of experimenter contact with the animal.

Experiment 2 examined the persistence of fear memory in LRs and HRs not subjected to repeated context re-exposure after conditioning. Animals were exposed to the context only twice, on days 10 and 32. This could also be considered an examination of incubated fear, assuming both phenotypes acquire fear learning as displayed in the previous two experiments. Fear incubation is described as the increase in fear and anxiety

that occurs over time following exposure to fear conditioning (Pickens *et al*., 2010; Schreurs *et al*., 2011). Individuals with PTSD have been shown to exhibit incubation-like effects on fear behavior; mainly that fear responses are exacerbated and delayed in onset over time following exposure to the precipitating trauma (Frueh *et al*., 2009; Smid *et al.*, 2011).

As in the previous experiments, LRs and HRs displayed similar freezing on the day of acquisition, and both groups displayed significant increases in freezing behavior from Day 1 to Day 10, indicating that both groups successfully acquired fear learning. The elevation in freezing was maintained in both groups and was still significantly higher on Day 32, as compared to Day 1. However, at no time point did LRs and HRs differ significantly from each other. That being said, HRs consistently displayed lower mean freezing behavior than did LRs, suggesting that there may possibly be a difference in vulnerability to incubated fear if we increased our sample size. This would suggest that while HRs are less likely to express elevated fear in response to a lack of extinction trials, LRs are more likely to demonstrate exacerbated fear responses and therefore may be more prone to developing PTSD-like behaviors (Frueh *et al*., 2009; Smid *et al.*, 2011). As a result, LRs could benefit from exposure therapy which has been utilized for many with PTSD in order to provide a context similar to the precipitating trauma so that they might be able to extinguish the fear memory and to counter the effects of fear incubation (Myers *et al.*, 2007; Beckett, 2002; Quirk *et al*., 2008).

Immediately after the last extinction trial, corticosterone levels were significantly lower in LR than in HR rats. This is important, because as previously mentioned, lower cortisol levels following a traumatic stress have been positively correlated with

developing PTSD in the future (Resnick *et al*., 1995; McFarlane *et al*., 1997). It is possible that LRs are more vulnerable to developing PTSD-like behaviors, such as the observed prolonged fear response and resistance to extinction, as a result of decreased corticosterone.

Low responders display a blunted corticosterone response to stress (Piazza *et al*. 1991), which could result in the inability of low responders to properly activate critical GR-mediated cellular processes necessary for learning and memory. Specifically, corticosterone levels in LRs could be insufficient to enhance memory as observed in previous studies (McGaugh & Roozendaal, 2002). This could be due to an inability to inhibit OCT3, the corticosterone-sensitive high-capacity monoamine transporter (Grundemann *et al.*, 1998; Wu *et al*., 1998, Duan &Wang, 2010). This potentially would result in a lack of stress-induced elevations of norepinephrine, which have also been shown to be critical in the processing and maintenance of fear memory (Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2007,2008). Together, low corticosterone concentrations could affect a multitude of memory processes resulting in the exacerbation and persistence of fear memory following a traumatic event.

CHAPTER IV

MODULATION OF ACOUSTIC STARTLE BY EXPOSURE TO SINGLE-PROLONGED STRESS IN LOW AND HIGH RESPONDING RATS: VULNERABILITY OR RESISTANCE ASSESSMENT

Introduction

Posttraumatic stress disorder (PTSD) is a severe anxiety disorder precipitated by exposure to extreme physical and / or emotional stress (DSM-IV-TR, 2000). The traumatic event can be varied, such as natural disasters (Hussain *et al.*, 2011), rape (Rothbaum *et al.*, 1992), or combat (Javidi & Yadollahie, 2012). However, not all individuals who experience a traumatic episode develop PTSD. Approximately 69% of Americans experience a traumatic episode in their lifetime (Norris, 1992), but only 8% develop PTSD (Kessler *et al.*, 1995).

Following exposure to trauma, individuals with PTSD show a variety of symptoms including hyperarousal, numbing symptoms, and persistent, intrusive memories of the traumatic episode (DSM-IV-TR, 2000). One key physiological characteristic of PTSD is altered levels of the stress hormone cortisol. Individuals with PTSD may exhibit decreased circadian plasma concentrations of cortisol (Mason *et al.*, 1986; Yehuda *et al.*, 1995). Additionally, it has been observed that individuals with PTSD may also express increased sensitivity to cortisol negative feedback of the HPA axis (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997). These characteristics combined could result in decreased, or blunted, cortisol levels during a traumatic/ stressful event. Thus, decreased cortisol levels could increase vulnerability to developing PTSD by insufficiently activating critical signaling pathways during stress. It has been

shown that in emergency room trauma patients low levels of cortisol following the trauma are positively correlated with future development of PTSD (Resnick *et al*., 1995; McFarlane *et al*., 1997). The previous chapters have discussed the importance of memory consolidation and the persistent, intrusive memories associated with PTSD. The current chapter focused on vulnerability to developing PTSD-like behaviors utilizing acoustic startle as a measure. Exaggerated acoustic startle, a component of the hyperarousal behaviors observed in PTSD, could result in part from blunted corticosterone responses to stress.

Acoustic startle, or the startle response, is a behavioral response in reaction to an abrupt and intense stimulus that has been demonstrated by a wide variety of species (Bradley *et al*., 1999; Grillon and Baas, 2003). However, it has been demonstrated that individuals with PTSD can display exaggerated acoustic startle responses (Shalev *et al*., 1992; Orr *et al*., 1995; Garrick *et al*., 2001). Grillon *et al.* (1998) demonstrated that veterans with PTSD exhibited increased startle magnitudes as compared to veterans and civilians without PTSD in a trial during which they were under an experimental stressor, fear of electric shock. This exaggerated effect was not demonstrated in non-stress conditions, which suggests that the stress-response system may play a role in the mechanisms underlying acoustic startle. Importantly, the exaggeration of the startle response seems to be dependent upon the situation being stressful in nature, as no significant increases in startle were observed in control conditions.

Cortisol is believed to exert a modulatory influence over acoustic startle response. Studies examining the interaction between startle response and cortisol levels have demonstrated that healthy individuals exhibited a lower startle response in the mornings

and a higher startle response in the evenings (Miller and Gronfier, 2006). This is important because cortisol levels are higher in the morning and lower in the evening, suggesting a relationship between glucocorticoid concentrations and acoustic startle. Specifically, lower cortisol levels may be associated with higher startle responses, which is interesting considering individuals with PTSD have been observed to exhibit reduced levels of cortisol (Boscarino, 1996; Yehuda, 2002; Yehuda *et al.*, 1993, 1995).

A study measuring acoustic startle in humans found that the startle response was reduced in participants who ingested oral doses of hydrocortisone (cortisol). The reduction in startle was only observed at the 20mg dose, as compared to the 5mg dose (Buchanan *et al*., 2001), suggesting the reduction was due in part to saturation of the glucocorticoid receptor by cortisol. Additional studies demonstrated that peripheral injection of corticosterone in rats decreased startle magnitude (Sandi *et al*., 1996), supporting the idea that low cortisol or corticosterone levels results in heightened startle responses. Furthermore, administration of the cortisol-suppressing drug metyrapone results in an enhancement of startle in human participants (Roemer *et al*., 2009).

In order to examine the question as to whether low levels of cortisol contribute to vulnerability in developing PTSD, we turned to an animal model. Previous studies have demonstrated that rats which exhibit locomotor responses to novelty in the bottom 33rd percentile of a population (LR animals) display significantly lower peak plasma corticosterone responses to restraint stress (Kabbaj *et al.*, 2006). This suggests that at the level of HPA responsiveness, LR rats demonstrate a pre-existing blunted stress response, similar to what would be expected in an individual with enhanced sensitivity to negative feedback.

To examine the responses of LR and HR rats to a severe stress, we used the single-prolonged stress model of PTSD (SPS) (Liberzon *et al.*, 1997) to determine the effects of severe stress on acoustic startle behavior. In short, animals are subjected serially to a restraint stress, forced swim trial, and ether anesthesia. This test has been used as a means to observe a combination of behavioral abnormalities, such as enhanced anxiety, expressed in PTSD (Khan and Liberzon, 2004; Harada *et al*., 2007).

Rats that underwent SPS have demonstrated increased acoustic startle responses compared to rats that did not undergo SPS (Khan & Liberzon, 2004). Additionally, rats exposed to SPS had increased freezing in contextual fear conditioning (Imanaka *et al.*, 2006; Takahashi *et al*., 2006). Animals that underwent SPS have also been found to exhibit an upregulation of mRNA for the glucocorticoid receptor (GR) and a downregulation in mRNA for the mineralocorticoid receptor (MR) in the hippocampus 1 week after SPS (Liberzon *et al*., 1999). This is important because it is the first evidence to suggest that rats subjected to SPS demonstrate enhanced negative feedback. A study by Liberzon, Krstov, and Young (1997) demonstrated that animals exposed to SPS expressed enhanced sensitivity to negative feedback during a stress test 1 week later. Furthermore, these same animals that were exposed to SPS did not differ in ACTH or corticosterone.

One key physiological characteristic of PTSD is altered levels of the stress hormone cortisol. Individuals with PTSD can exhibit decreased circadian plasma concentrations of cortisol (Mason *et al.*, 1986; Yehuda *et al.*, 1995). Exaggerated acoustic startle could result from decreased levels of cortisol. Previous studies have demonstrated that reduced cortisol levels result in enhanced startle response in humans (Miller and

Gronfier, 2006) and that administration of cortisol reduces the startle response (Buchannan *et al.*, 2001; Sandi *et al*., 1996). Animal models have demonstrated that the SPS animal model of PTSD enhances startle response in animals following exposure (Khan and Liberzon, 2004; Harada *et al*., 2007). However, nobody has examined the effects of SPS on groups of rats with differential stress responses.

As a result of expressing reported blunted corticosterone levels similar to those observed in individuals with PTSD, LR animals make an appealing choice in trying to understand the role stress reactivity plays in PTSD-like symptoms. Therefore the aim of this study was to examine the effects of individual differences in HPA responsiveness on the susceptibility to developing PTSD-like behaviors. We predicted that LR animals would be more sensitive to SPS and express more PTSD-like behaviors as compared to HR animals. Specifically, we predicted SPS would cause greater increases in acoustic startle response in LRs as compared to HRs. Furthermore, we examined whether there were initial differences in acoustic startle response between phenotypes prior to exposure to severe stress in order to determine whether or not enhanced startle behavior is a pre-existing characteristic or a result of trauma.

Materials and Methods

Animals

Adult male Spraque-Dawley rats (Harlan Laboratories, St. Louis, MO) were housed in cages of two under, a 12:12 hour light:dark cycle (lights on 7:00 AM) in a temperature and humidity controlled facility. All procedures were approved by the Marquette University Institutional Animal Care and Use Committee and performed in

accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Animal housing facilities were accredited by AALAC.

Identification of Low and High Responders

Adult male Sprague-Dawley rats were placed into photocell cages that served as a novel locomotor chamber (AccuScan, Columbus, OH) and allowed to explore for 1 hour. Locomotor behavior (photocell beam breaks) was monitored by software provided by the manufacturer. Rats were separated into Low and High responders based upon cumulative locomotor activity over the 1 hour test. Rats scoring in the bottom 33% were classified as Low (LR), and those in the top 33% as High responders (HR).

Acoustic Startle

Rats were placed on a platform that rested on a force transducing plate to measure the amplitude of startle responses to loud noise. Following a 5 minute habituation period in a sound attenuating chamber (27.62x35.56x49.53cm) (Hamilton Kinder, CA, USA) that, rats were exposed to a series of 30 trials over 15 minutes (30 seconds interval) consisting of a single auditory stimulus (pulse stimulus; 51dB above the 57dB background noise, 50ms duration). In all experiments, rats were subjected to an acoustic startle trial to determine baseline response 2 weeks prior to single-prolonged stress. Following exposure to SPS, all rats were subjected to a second acoustic startle trial 2 weeks following exposure to severe stress.

Single-Prolonged Stress

Animals underwent single-prolonged stress, as previously described (Liberzon, *et al.*, 1997). Briefly, 2 hours into their light cycle, animals were exposed to a single session

of prolonged stress consisting of restraint for 2 hour in a clear plexiglass container followed immediately by a forced swim for 20 minutes in 24°C water. Animals were allowed to recuperate for 15 minutes and then were exposed to ether vapors until loss of consciousness. The animals were then returned to their home cages and left undisturbed for two weeks. Forced swim was conducted in a 59.7cm x 46.7cm x 41cm container. Four rats at a time were subjected to swim stress in the same container. Animals were closely watched and prevented from escaping.

Analysis of Acoustic Startle

Total startle amplitude over the course of all 30 trials was examined and represented as the mean and standard error for each animal subgroup. The first three trials were used to habituate and stabilize startle responding similar to previous reports (Risbrough *et al.*, 2009), and subsequently were excluded from analysis. Startle responses were averaged across trials 4-30 and represented as mean startle amplitude with mean standard error.

Data Analysis

The differences in acoustic startle response between LR and HR rats were determined using a two-way ANOVA (Phenotype x Stressor) followed, when appropriate, post-hoc testing using Bonferroni *t* test.

Results

Acoustic Startle

Thirty eight rats were used for startle response testing (14 LR, 24 HR) [LR : Control (7) / SPS (7) ; HR: Control (12) / SPS (12)]. There were no significant differences in acoustic startle response between startle trials in LRs (Figure 4.1A) or HRs (Figure 4.1B) that did not receive an intervening stressor.

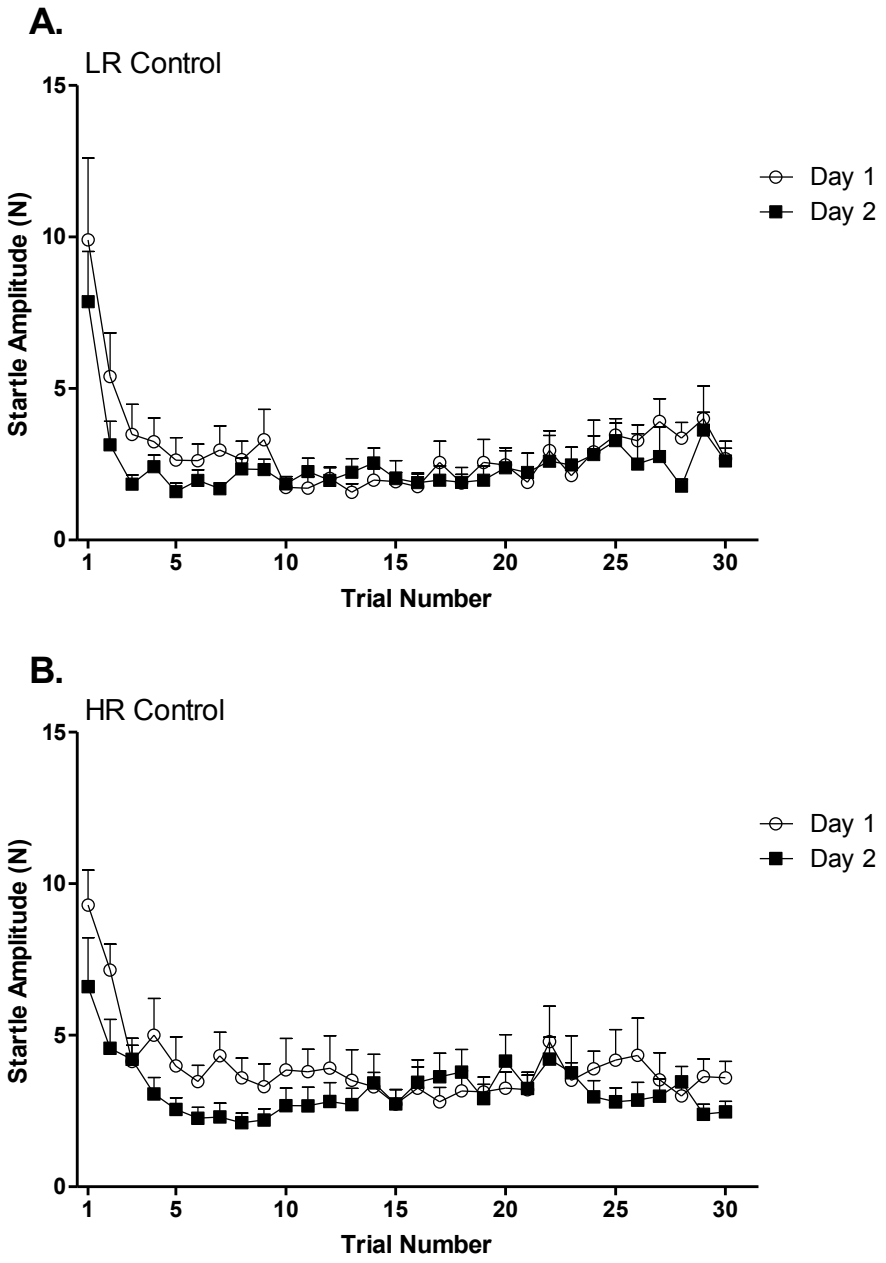

Figure 4.1. Effects of repeated startle trials on LRs and HRs. Animals were subjected to an acoustic startle session consisting of 30 individual trials. Startle amplitude for each day is reported for LR rats that underwent no stress (A) and HR rats that underwent no stress (B). Values are reported as mean and SEM. (LR = 7, HR = 12)

Effects of Startle Stimuli Following Exposure to SPS

A two-way ANOVA (Phenotype x Day) examining differences between LRs and HRs in startle response failed to reveal a significant effect of condition ($F_{1,34}$=0.3930; ns) or phenotype ($F_{1,34}$=1.927; ns). (see Figure 4.2). Data do suggest an increase in acoustic startle in LRs following SPS, which could become significant if sample size is increased.

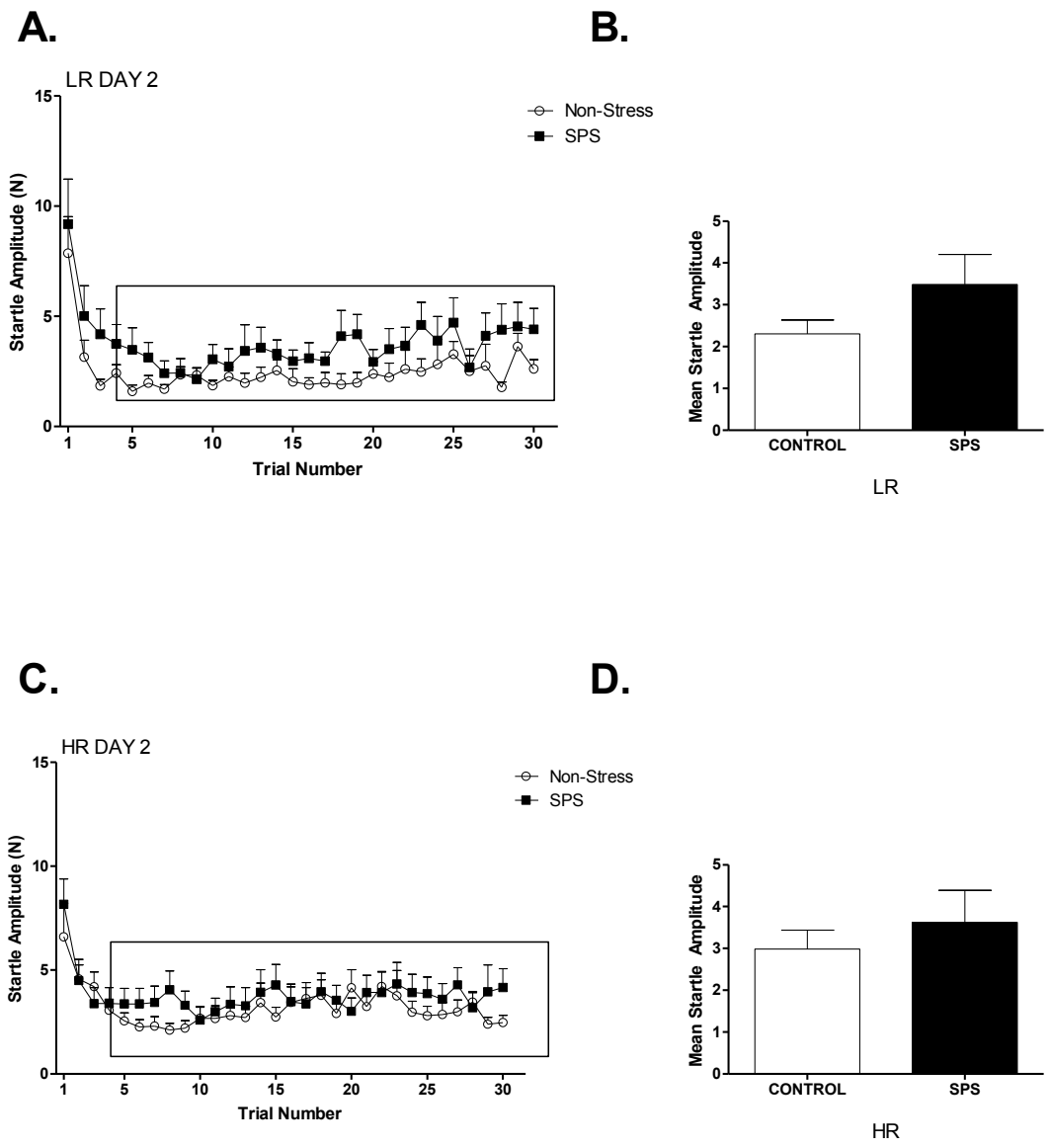

Figure 4.2 Effects of startle stimuli on LRs and HRs that received SPS. Animals were subjected to an acoustic startle session consisting of 30 individual trials. Startle amplitude for Day 2 (post-SPS) is reported for LR rats that underwent SPS (A) and HR rats that underwent SPS (C) compared to control animals. The rectangle indicates the trials examined (4-30) for statistical analysis. Mean differences in startle amplitude across all trials for LRs (B) and HRs (D) is represented. Values are reported as mean and SEM. (LR = 7, HR = 12)

Conclusion

Low responders and high responders subjected to repeated acoustic startle trials without any intervening stress did not reveal any significant differences in startle behavior over time or between phenotypes. This suggests that there are no inherent differences in startle behavior between phenotypes prior to exposure to trauma. Furthermore, it also suggests that repeated acoustic startle training has no significant effects on behavior. If anything, both LRs and HRs displayed a slight reduction in freezing behavior during the second startle trial compared to the first trial.

Animals that underwent SPS demonstrated no significant differences in the second acoustic trial (post-stress) than did animals in the control group during their second acoustic trial. When comparing LR and HR controls and LR and HR SPS animals against their respective second acoustic startle trials, LRs displayed a greater increase in startle amplitude following SPS than did HRs.

The lack of significant differences between stress conditions and / or phenotypes could be a result of a small sample size. Another consideration for why there was a lack of significant changes in startle behavior could be the result of a habiutation effect. As observed in control non-stressed animals, there seems to be a slight habituation effect where freezing is reduced in the second startle session. By exposing the animals to acoustic startle prior to trauma they may become habituated to the context and result in decreased startle responses upon secondary testing. Therefore, in order to observe any significant changes in behavior a given phenotype has to increase startle significantly to counter any habituation effects. This is one main reason why the question of pre-existing differences remains unstudied. There are not many working models.

Another potential reason for the lack of a response could be experimenter error in the execution of the acoustic startle. This could be due to insufficient restraint of the animal during testing. If the animal was not properly installed in the recording apparatus, the normal force of startle could be reduced, resulting in inaccurate and blunted responses. Additionally, if the chamber was not properly sealed, with specific attention to the insulating plug, startle responses could have been modulated by ambient noise or leakage of the startle noise. This is again, unlikely because each startle apparatus was calibrated for sound prior to testing.

Together, the data suggest that there are no inherent differences in LRs and HRs prior to trauma, and that repeated acoustic startle sessions alone have no significant effects on startle behavior outside of a slight habituation effect. While not significant, the data suggest a trend in which LRs displayed heightened elevations in freezing behavior following exposure to the traumatic SPS model as compared to non-stressed LRs. This increase was not observed in the HR phenotype.

CHAPTER V

NATURAL AND SYNTHETIC CORTICOSTEROIDS INHIBIT UPTAKE$_2$-MEDIATED TRANSPORT IN CNS NEURONS

Introduction

Natural and synthetic corticosteroids are powerful modulators of neuronal physiology and behavior. By actions at a variety of cellular targets, they initiate both rapid and delayed effects on central nervous system (CNS) function. Recent studies suggest that, in addition to exerting actions via the mineralocorticoid and glucocorticoid receptors (MR and GR), corticosteroids also act by inhibiting monoamine clearance mediated by uptake$_2$, a high-capacity, low-affinity transport system for norepinephrine, epinephrine, dopamine, histamine and serotonin (Baganz *et al*., 2008; Feng *et al*., 2009, Gasser, Lowry & Orchinik, 2006; Gasser *et al*., 2009). In contrast to uptake$_1$, which is mediated by a combination of the specific transporters for norepinephrine (NET), dopamine (DAT), and serotonin (SERT), uptake$_2$ is a higher-capacity but lower affinity transport system, and is acutely inhibited by corticosterone and other steroids (Iversen & Salt, 1970; Simmonds & Gillis, 1968). Inhibition of uptake$_2$ in cardiac or smooth muscle tissue by acute bath application of corticosteroids enhances the contractile effects of exogenously applied epinephrine, norepinephrine, serotonin and histamine (Horvath *et al.*, 2003; Eyre, Elmes & Strickland, 1979; Kalsner, 1975; Mikami *et al*., 1989; Purdy, Weber & Drayer, 1982; Purdy & Weber, 1983). Recent studies have identified a small group of transporters that mediate uptake$_2$-like transport and have demonstrated their expression in the brain (Gasser, Lowry & Orchinik, 2006; Gasser *et al*., 2009; Engel,

Zhou & Wang, 2004; Amphoux *et al*., 2006; Vialou *et al*., 2004). Thus, uptake$_2$ is a mechanism by which corticosteroids may act to enhance the actions of monoamines in the CNS as well as in peripheral targets.

Uptake$_2$ activity has been attributed to a group of broadly-specific organic cation transporters. These include the organic cation transporter (OCT) family: OCTs 1, 2 and 3, and the plasma membrane monoamine transporter (PMAT). Because OCT3 is the most sensitive of these transporters to inhibition by corticosterone, it has been described as the most important uptake$_2$ transporter (Grundemann *et al.*, 1998; Wu *et al*., 1998, Duan &Wang, 2010). However, all of the above transporters have uptake$_2$-like characteristics. All are broadly-specific organic cation transporters, capable of transporting, with varying efficiencies, norepinephrine, epinephrine, serotonin, dopamine and histamine, as well as the cationic neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) (Engel, Zhou & Wang, 2004; Grundemann *et al.*, 1998; Grundemann *et al.*, 1998; Grundemann *et al.*, 1999. All are sensitive to inhibition by corticosterone, though their sensitivities differ widely (Gasser, Lowry & Orchinik, 2006; Engel, Zhou & Wang, 2004; Wu *et al*., 1998; Duan *et al.*, 2010; Schomig, Lazar & Grundemann, 2006; Gorboulev *et al*., 2005), and all are inhibited by the pseudoisocyanine compound 1, 1'-diethyl-2, 2'-cyanine iodide (decynium-22) (Engel, Zhou & Wang, 2004; Hayer-Zillgen, Bruss & Bonisch, 2002).

All of the uptake$_2$ transporters are expressed, at varying levels and with distinct distributions, in rodent and human brain (Gasser, Lowry & Orchinik, 2009; Gasser *et al.*, 2009; Engel, Zhou & Wang, 2004; Amphoux *et al*., 2006; Vialou *et al.*, 2004), and recent studies suggest that they play important roles in monoamine clearance. Pharmacological

inhibition of uptake$_2$ by direct application of decynium-22 decreases the rate of serotonin clearance in mouse hippocampus (Baganz *et al*., 2008), and treatment of rats with the OCT3 inhibitor normetanephrine potentiates venlafaxine-induced increases in extracellular norepinephrine concentrations in rat prefrontal cortex (Rahman *et al.*, 2008). Extracellular concentrations of dopamine are elevated in the striatum of OCT3 knockout mice (Cui *et al*., 2009). These studies demonstrate that uptake$_2$ transporters play important roles in regulating extracellular concentrations of monoamines in the rodent brain.

Each of the identified uptake$_2$-like transporters is inhibited by corticosterone, though they differ in their sensitivity to this corticosteroid. OCT3 is the most sensitive (IC_{50} = 0.04 – 0.2 μM), followed by OCT2 (IC_{50} = 4 μM), OCT1 (IC_{50} = 150 μM) and PMAT (K_i = 450 μM) (Gasser, Lowry & Orchinik, 2006; Engel, Zhou & Wang, 2004; Wu *et al.*, 1998; Duan & Wang, 2010; Schomig, Lazar & Grundemann, 2006; Gorboulev *et al*., 2005). Studies in peripheral tissues suggest that uptake$_2$-mediated transport is also inhibited by a variety of natural and synthetic corticosteroids. In vascular smooth muscle, the mineralocorticoids aldosterone and 11-deoxycorticosterone enhance the contractile effects of epinephrine (Weber & Purdy, 1982; Weber, Purdy & Drayer, 1983), and OCT3-mediated norepinephrine clearance in bronchial smooth muscle is inhibited by the synthetic corticosteroids budesonide, methylprednisolone, and fluticasone (Horvath *et al*., 2007). These studies suggest that uptake$_2$ inhibition may play an important role in mediating the effects of both natural and synthetic corticosteroids on neuronal physiology and behavior.

Given the powerful behavioral effects attributed to a wide variety of both natural and synthetic corticosteroids, it is important to understand the potential contribution of uptake$_2$ inhibition to their actions in the CNS. However, the structure activity relationships among the natural and synthetic corticosteroids for inhibition of uptake$_2$ in neurons have not been determined. We recently demonstrated that rat cerebellar granule neurons (CGNs) in culture accumulate [^{3}H]-MPP$^+$ in a corticosterone-sensitive manner, and that they express the uptake$_2$ transporter OCT3, but not the uptake$_1$ transporter DAT (Shang *et al*., 2003). In the present studies, we fully characterized the expression of uptake$_1$ and uptake$_2$ transporters in CGNs, and examined the sensitivity of uptake$_2$-mediated accumulation of [^{3}H]-MPP$^+$ to various natural and synthetic corticosteroid hormones. This information is important for a full understanding of the mechanisms by which corticosteroids influence CNS function.

Materials and Methods

Materials

All steroids were purchased from Steraloids (Newport, RI). Timed pregnant female Sprague-Dawley rats were purchased from Harlan (Madison, WI). 1-[^{3}H]-Methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$) (specific activity of 86.4 Ci/mol) was purchased from Perkin Elmer (Waltham, MA). Primers for RT-PCR were synthesized by Invitrogen (Carlsbad, CA).

Culture of Cerebellar Granule Neurons

CGNs were prepared from 6-8-day-old rat pups of either sex as described previously (Hillard *et al*., 1997) except that cerebellae were incubated in 40 rather than

20 U/mL of papain. Cells were plated onto 6-well culture plates (2 ml, $1x10^6$ cells/mL) and were maintained in basal minimal Eagle's media (BME; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 25 mM KCl, 5 mM glutamine, and 0.01 mg/ml ampicillin. After 24 h, cytosine arabinoside (10 μM) was added to the cultures to inhibit glial proliferation. After 5 days in culture, 600 μl of the media was removed and replaced with BME supplemented as above, except that B-27 (Invitrogen) was substituted for fetal bovine serum.

[^{3}H]-MPP$^+$ Uptake Assay

Uptake experiments were carried out at room temperature on CGNs that had been in culture for 6-8 days. Cells were washed and pre-incubated in 1.8 mL transport buffer (25 mM Tris Base, pH 8.5, 280 mM mannitol, 5.4 mM KCL, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose). To test the effects of putative $uptake_2$ inhibitors on MPP^+ accumulation, CGNs were incubated in the presence of vehicle or increasing concentrations of inhibitors for 5 minutes before the addition of 20 nM [^{3}H]-MPP$^+$. Uptake was terminated after two minutes by aspiration of transport buffer, followed by two washes with 1mL ice-cold transport buffer. CGNs were lysed by scraping in 500 μl water. Radioactivity in the cell lysate was determined by liquid scintillation spectroscopy. Uptake experiments were repeated at least three times, except where noted. The effects of the following inhibitors were tested: 11-dehydrocorticosterone (10 nM – 5.0 μM); 11-deoxycorticosterone (10 nM- 1 μM); aldosterone (10 nM – 1 μM); betamethasone (10 nM – 1 μM); corticosterone (10 nM – 5 μM); cortisol (10 nM – 10 μM); cortisone (10 nM – 5 μM); decynium-22 (0.01 nM – 0.1 μM); dexamethasone (0.1 nM – 1 μM); prednisolone (10 nM – 5 μM); RU38486 (10 nM – 5 μM).

Reverse Transcriptase-PCR

After 7 days in culture, CGNs were washed twice with transport buffer and total RNA was isolated using 1 mL TRIzol Reagent (Invitrogen) per well according to the manufacturer's protocol. One microgram of the resulting total RNA was reverse transcribed in the presence and absence SuperScript^{TM}II Reverse Transcriptase (Invitrogen) using oligo$(dT)_{20}$ primers. Two microliters of the resulting cDNA were used as a template for PCR reactions using GoTaq Green Master Mix (Promega, Madison, WI) and gene specific primers (Invitrogen) at 1 μM. Sequences for gene-specific primers and the corresponding PCR cycling parameters are shown in Figure 5.1.

Figure 5.1. Primers and cycling conditions for amplification of MPP^+ transporters in CGNs. All reactions were initiated by a 2 min incubation at 94°C, were run for 32 cycles, and ended with 10 min at 72°C.

Gene	Primers (5'-3')	Product Size (bp)	Cycling Parameters
rOCT1a	Forward-GAT CTT TAT CCC GCA TGA GC Reverse-TTC TGG GAA TCC TCC AAG TG	477	45 s at 94°C, 30 s at 55°C, 45s at 72°C
rOCT1b	Forward-TGC AGA CAG GTT TGG CCG TAA Reverse-TCG AGG CCG CTA TTG GGT AGA	722	45s at 94°C, 30s at 55°C, 45s at 72°C
rOCT2	Forward -CGT TGG GTA GAA TGG GCA TC Reverse -GTG AGG TTG GTT TGT GTG GG	460	45 s at 94°C, 30 s at 57°C, 45 s at 72°C
rOCT3	Forward -TCT TCA CCC TCG GAA TCA TC Reverse -TGA TAC ACC ACG GCA CTT GT	351	45 s at 94°C, 30 s at 55°C, 45 s at 72°C
rDAT	Forward -TCC CTG ACAA GCT TCT CC Reverse -GCC AGG ACA ATG CCA AGA	305	1 min at 95°C, 45 s at 56°C, 45 s at 72°C
rNET	Forward -ACA TCG GGA AAG GTT GTC TG Reverse -GTG GCG ACA TCC TCA ATC TT	370	1 min at 95°C, 45 s at 60°C, 45 s at 72°C
rPMAT	Forward -ACC GCT ACC ATG CCA TCT AC Reverse -AAG GCC AGG AGG TAA CCT G	238	1 min at 95°C, 45 s at 58°C, 45 s at 72°C
rSERT	Forward -GTA CCA CCG AAA CGG GTG CA Reverse -TGG TGG ATC TGC AGC ACA TG	300	45 s at 95°C, 45 s at 60°C, 45 s at 72°C

Data Analysis

Results of uptake assays are expressed as means ± SEM from independent replicates. Uptake data were analyzed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Transport data were analyzed by fitting untransformed data to appropriate equations using iterative, least-squares curve-fitting techniques. IC_{50} values for inhibitors were determined by fitting the pooled data from independent experiments to the one-site competition equation using non-linear regression.

Results

Expression of transporter mRNA in CGNs

RT-PCR was used to determine the expression of mRNA for $uptake_1$ (DAT, NET and SERT) and $uptake_2$ (OCT1, OCT2, OCT3 and PMAT) transporters in CGNs. All primer pairs were tested for their ability to amplify the target genes from cDNAs obtained from tissues known to express each of the transporters. All primer pairs were able to amplify products of the expected size in cDNA from positive control tissue: midbrain for OCT3, OCT2, PMAT, DAT, NET and SERT; and kidney for OCT1(a and b) and OCT2; data not shown). As shown in Figure 5.2, mRNAs for OCT1, OCT3, and PMAT were clearly detected in CGNs, while mRNA for SERT was detected at very low levels, and mRNAs for OCT2, DAT, and NET were not detected. Because the results of the current OCT1 RT-PCR contradicted our previous results (Shang *et al*., 2003), we repeated the OCT1 PCR using either the primer pair that we used in the earlier publication (OCT1b), or the new primer pair (OCT1a). Using the OCT1a primer pair, we detected OCT1 mRNA in CGNs, as well as in forebrain, liver, kidney and lung. Using the OCT1b

primer pair, we failed to detect OCT1 mRNA in CGNs or in rat forebrain, but did detect OCT1 mRNA in kidney, liver and lung tissue (data not shown).

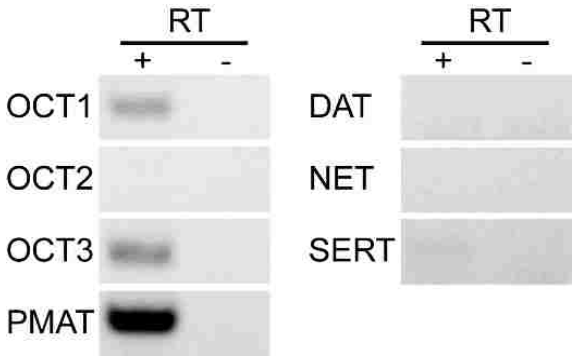

Figure 5.2. Expression of monoamine transporters in CGNs. RT-PCR was performed using total RNA extracted from CGNs and primers specific to OCTs 1, 2, and 3, plasma membrane monoamine transporter (PMAT), dopamine (DAT), norepinephrine (NET) or serotonin reuptake transporter (SERT). PCR was performed on cDNA samples generated in the presence (+) or absence (-) of reverse transcriptase. Only RT-PCR that included reverse transcriptase (+) produced distinct bands for any of the transporters. mRNAs for OCT1, OCT3 and PMAT were clearly detected, and a very small amount of mRNA for SERT was detected, in CGNs.

[^{3}H]-MPP$^+$ uptake assays

Previous studies demonstrated saturable accumulation of [^{3}H]-MPP$^+$ by cultured CGNs, and presented evidence that this accumulation was mediated by OCT3 (Shang et al, 2003). We tested the sensitivity of CGN accumulation of [^{3}H]-MPP$^+$ to the potent uptake$_2$ inhibitor decynium-22, and to a variety of natural and synthetic corticosteroids. Uptake of [^{3}H]-MPP$^+$ was inhibited by both decynium-22 and corticosterone in a dose dependent manner, with decynium-22 inhibiting a larger fraction of MPP$^+$ accumulation than did corticosterone (Figure 5.3, Figure 5.4). Accumulation of [^{3}H]-MPP$^+$ was also partially inhibited by other naturally occurring corticosteroids and their metabolites, including aldosterone, cortisol, 11-deoxycorticosterone (11-DOC), and cortisone (Figures 5.5 and 5.6, Figure 5.3). These steroids inhibited [^{3}H]-MPP$^+$ uptake with efficacies similar to that of corticosterone (Figure 5.4). 11-Dehydrocorticosterone had only a slight inhibitory effect on [^{3}H]-MPP$^+$ uptake (Figure 5.6, Figure 5.4). Synthetic corticosteroids inhibited [^{3}H]-MPP$^+$ uptake with varying efficacies. Betamethasone inhibited uptake with an efficacy similar to that of corticosterone, while dexamethasone and prednisolone were less effective (Figure 5.7, Figure 5.4). The glucocorticoid receptor antagonist RU38486 inhibited [^{3}H]-MPP$^+$ uptake, but was less effective than corticosterone (Figure 5.7, Figure 5.4). Decynium-22 inhibited a larger fraction of uptake than did any of the steroids examined (Figure 5.3, Figure 5.4).

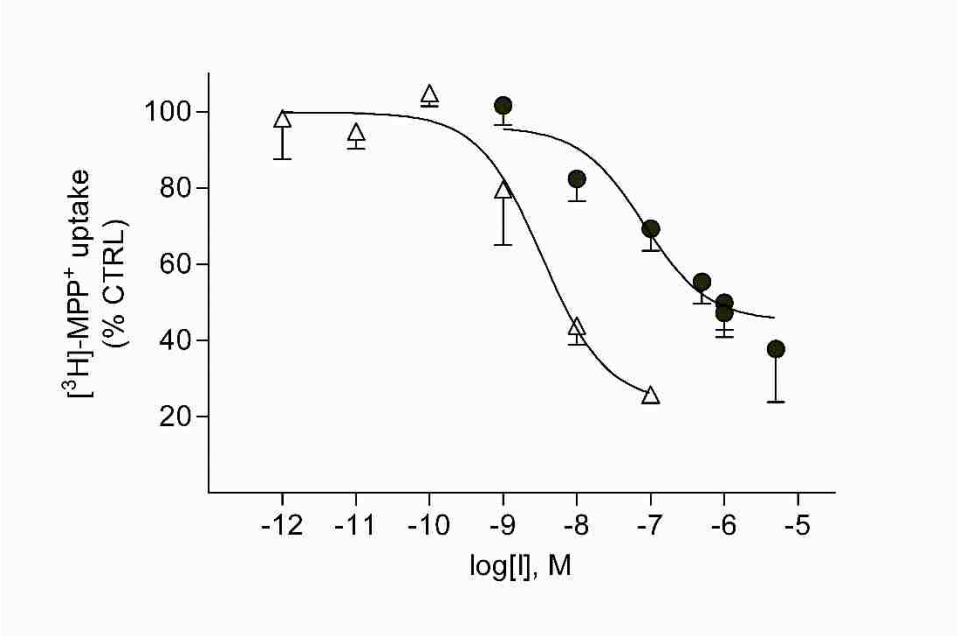

Figure 5.3. Effects of Decynium-22 and corticosterone on CGN accumulation of [^{3}H]-MPP$^+$. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of inhibitors for 5 min, after which 20 nM [^{3}H]-MPP$^+$ was added and uptake was measured after 2 min. Decynium-22 (Δ) and corticosterone (•) inhibited [^{3}H]-MPP$^+$ accumulation in a concentration-dependent manner. Error bars represent − SE from n = 3 (decynium-22) or 8 (corticosterone) replicate wells.

Figure 5.4. Potencies of inhibitors of [^{3}H]-MPP$^+$ accumulation by CGNs

Inhibitor	IC_{50} nM (95% CI)		Maximal Inhibition (%)
Decynium-22	3.4	(2.4 – 4.8)	76 ± 5
11-dehydrocorticosterone	23	(2.3 – 200)	13 ± 5
11-deoxycorticosterone	9.25	(4.25 – 20)	58 ± 7
Aldosterone	18	(7.5 – 43)	49 ± 7
Betamethasone	33	(15 – 72)	50 ± 8
Corticosterone	81	(42 – 200)	55 ± 5
Cortisol	1.1μM	(0.5 – 2.5)	47 ± 8
Cortisone	82	(28 – 240)	32 ± 6
Dexamethasone	0.1	(0.03 – 0.6)	25 ± 4
Prednisolone	6.4	(1.2 – 33)	32 ± 7
RU38486	44	(11.5 – 200)	25 ± 7

IC_{50} values were determined by fitting pooled data from independent experiments to the one-site competition equation using nonlinear regression.

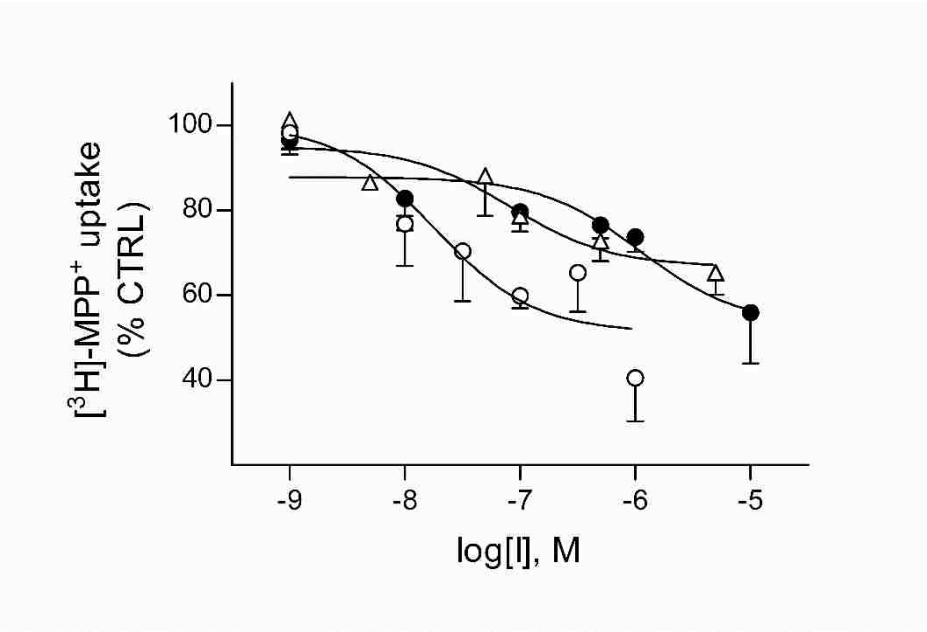

Figure 5.5. Effects of corticosteroids on CGN accumulation of [^{3}H]-MPP$^+$. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of inhibitors for 5 min, after which 20 nM [^{3}H]-MPP$^+$ was added and uptake was measured after 2 min. Aldosterone (o), cortisol (•) and cortisone (Δ) inhibited [^{3}H]-MPP$^+$ accumulation in a concentration-dependent manner. Error bars represent − SE from n = 3 (aldosterone, cortisone) or 4 (cortisol) replicate wells.

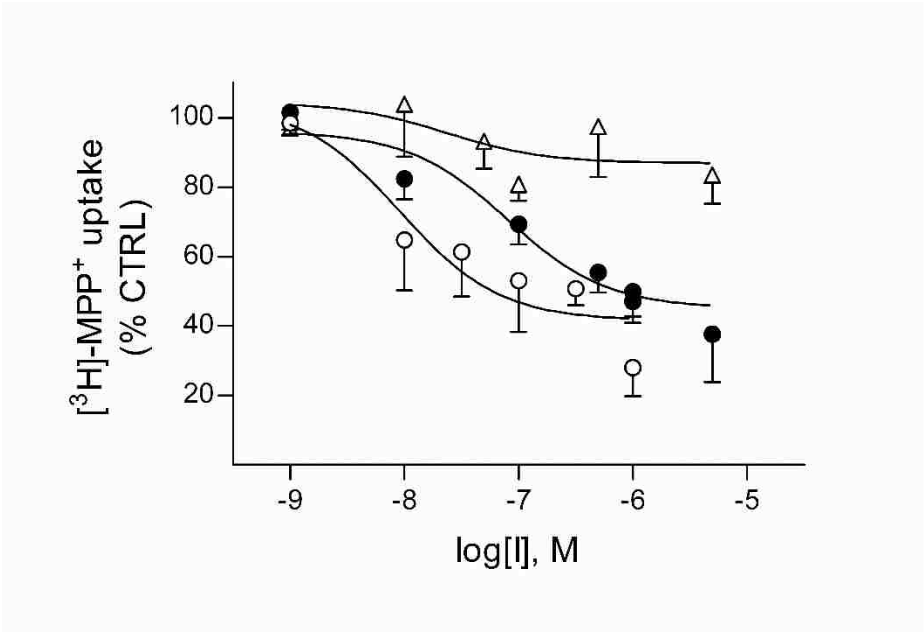

Figure 5.6. Effects of corticosterone and its metabolites on accumulation of [^{3}H]-MPP$^+$ by CGNs. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of steroids for 5 min, after which 20 nM [^{3}H]-MPP$^+$ was added and uptake was measured after 2 min. The data for corticosterone (•) are from Figure 2, presented here for comparison to the other steroids. Both 11-deoxycorticosterone (o) and 11-dehydrocorticosterone (Δ) inhibited [^{3}H]-MPP$^+$ accumulation in a concentration-dependent manner, though with different efficacies. Error bars represent − SE from n = 3 (11-dehydrocorticosterone, 11-deoxycorticosterone) or 8 (corticosterone) independent wells.

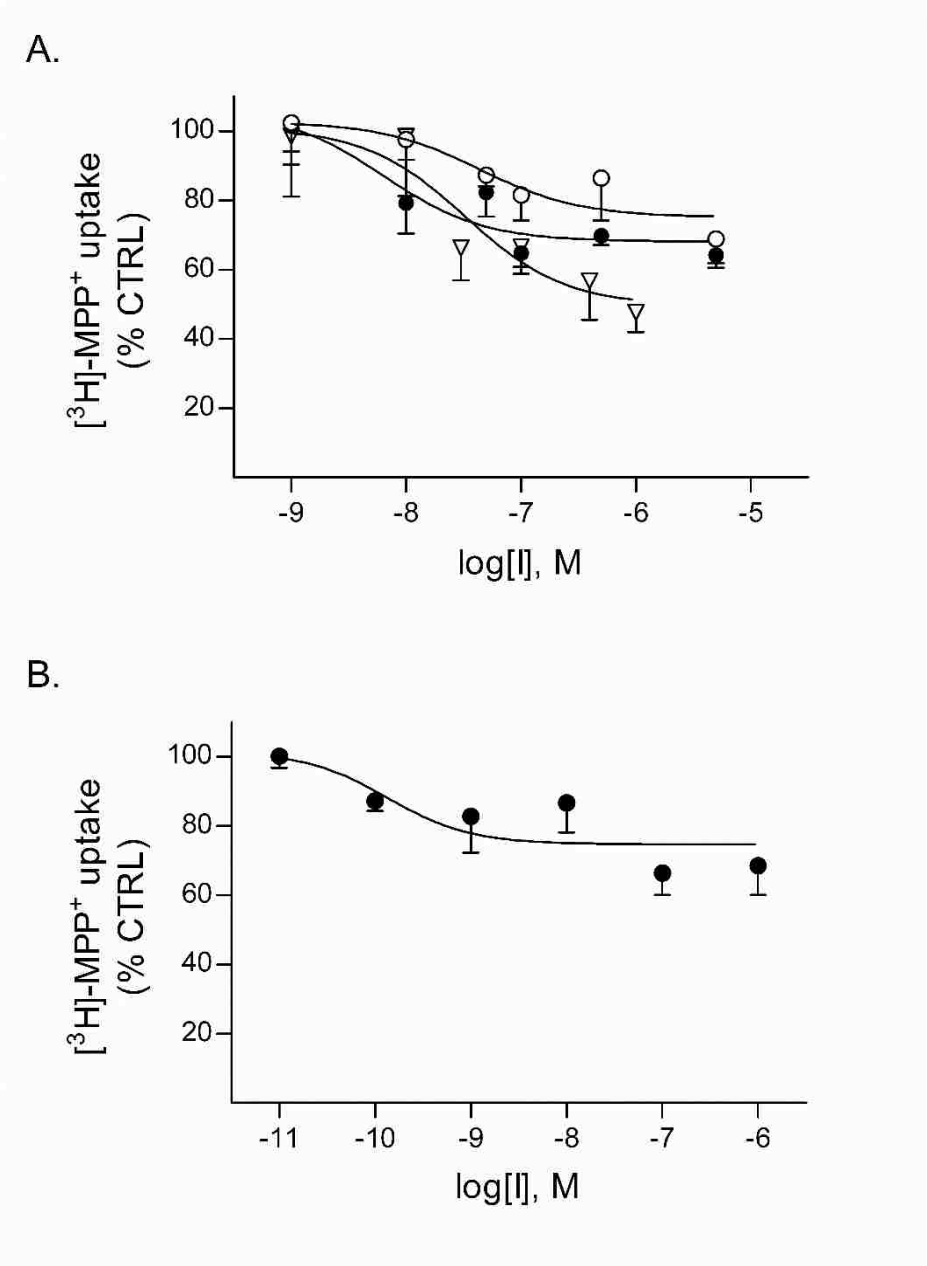

Figure 5.7. Effects of synthetic corticosteroids on accumulation of [^{3}H]-MPP$^+$ by CGNs. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of steroids for 5 min, after which 20 nM [^{3}H]-MPP$^+$ was added and uptake was measured after 2 min. **A.** Betamethasone (∇), prednisolone (•) and RU38486 (o) dose-dependently inhibited [^{3}H]-MPP$^+$ accumulation. **B.** Dexamethasone (•) dose-dependently inhibited [^{3}H]-MPP$^+$ accumulation. Error bars represent − SE from n = 3 (betamethasone, prednisolone, RU38486) wells or − SD from two (dexamethasone) wells.

Conclusion

This study is the most detailed characterization of the corticosteroid sensitivity of uptake$_2$-mediated transport, and the first in CNS cells. The data from expression and functional studies indicate that uptake$_2$ is the primary monoamine transport system in CGNs, and that, as in peripheral tissue, uptake$_2$ activity in the CNS is broadly sensitive to inhibition by both natural and synthetic corticosteroid hormones. These data are consistent with the hypothesis that natural and synthetic corticosteroids exert modulatory actions in the CNS via inhibition of uptake$_2$ -mediated clearance of monoamines, and that inhibition of uptake$_2$-mediated monoamine clearance may underlie some of the effects of these hormones on neuronal physiology and behavior.

The data from RT-PCR studies indicate that [^{3}H]-MPP$^+$ accumulation by CGNs is mediated primarily by three uptake$_2$ transporters: OCT1, OCT3 and PMAT. These data are consistent with previous studies demonstrating expression of OCT3, OCT1 and PMAT protein or mRNA expression in the cerebellar granule layer (Gasser *et al.*, 2009; Amphoux *et al.,* 2006; Dahlin *et al.*, 2007). However, our detection of OCT1 mRNA expression in CGNs is not consistent with our previous report that OCT1 mRNA is not expressed in these cells (Shang *et al.*, 2003). This discrepancy may be due to the use of different primer sets in the two studies. In the present study, we used a primer set (OCT1a) that targets the 3' end of the OCT1 cDNA, whereas in the previous study, we used a primer set (OCT1b) that targets a region in the middle of the OCT1 cDNA. Both primer sets detected OCT1 mRNA in cDNA from kidney, raising the possibility that kidney and brain OCT1 mRNAs represent alternatively spliced forms of the gene, as has been reported for human OCT1 (Hayer *et al.,* 1999). To address this discrepancy, we

performed parallel PCR using each of the OCT1 primer pairs to amplify product from CGN and kidney cDNAs. Using the OCT1b primers (from our earlier publication), we again detected OCT1 mRNA in kidney, but not in CGN cDNA. Using the OCT1a primers, we detected OCT1 mRNA in both kidney and CGN. These results suggest that the OCT1 mRNA we detected in CGNs represents an alternatively spliced form of the gene. Further studies are required to test this hypothesis.

The data from RT-PCR studies further demonstrate that CGNs do not express DAT and NET, and that they express only very low levels of SERT. These results are consistent with previous studies examining DAT and SERT mRNA expression in CGNs (Shang *et al.*, 2003; Zusso *et al*., 2008). Our data on the expression of these $uptake_1$ transporters seem to contradict previous studies demonstrating the expression of DAT, NET and SERT in the cerebellar granule layer. However, these studies measured radioligand binding in intact brain sections, or uptake of substrates by acutely-prepared synaptosomes (Freund *et al.*, 2003; Giompres & Delis, 2001; Gonzalez-Polo *et al*., 2001; Le *et al*., 1998; Strazielle *et al.*, 1999). Data obtained using these techniques likely reflect transporters expressed on monoaminergic terminals in the cerebellar granule layer (Ikai *et al*., 1992; Kerr & Bishop, 1991; Verney, Grzana & Farkas, 1982), rather than specifically in CGNs. Our data reflect transporter expression almost exclusively in CGNs.

Our functional studies are also consistent with a primary role of $uptake_2$ in CGN transport. Two well-characterized $uptake_2$ inhibitors, decynium-22 and corticosterone, each inhibited substantial fractions of MPP^+ uptake in the present studies. All of the transporters we detected in CGNs are inhibited by decynium-22, though their sensitivities

are not equivalent. OCT3 and PMAT are the most sensitive to decynium-22 inhibition (IC_{50} = 9-100 nM for OCT3, 100 nM for PMAT), while OCT1 is significantly less sensitive (IC_{50} = 0.5-1μM) (Engel, Zhou & Wang, 2004; Hayer-Zillgen, Bruss & Bonisch, 2002; Grundemann *et al*., 1997; Martel *et al.*, 1996; Russ *et al*., 1992). Some reports have suggested that, at very high concentrations, decynium-22 can inhibit NET and SERT (Russ *et al.*, 1993; Martel *et al*., 2003). In the present studies, decynium-22 inhibited approximately 80% of [^{3}H]-MPP^+ accumulation with the same potency (IC_{50} = 3.4 nM) as reported in our previous studies (Shang *et al*., 2003). As the concentrations of decynium-22 used in the current study (0.01 nM – 0.1 μM) did not reach the previously reported IC_{50} for OCT1, the data suggest that the decynium-22-sensitive fraction of MPP^+ accumulation reported here is mediated primarily by PMAT and OCT3, and that the remaining fraction of MPP^+ accumulation is mediated by OCT1, with a possible contribution of SERT.

While all of the $uptake_2$ transporters expressed in CGNs are inhibited by corticosterone, they differ greatly in their sensitivity. OCT3 is the most corticosterone-sensitive, followed by OCT1 and PMAT (Gasser, Lowry & Orchinik, 2006; Engel, Zhou & Wang, 2004; Grundemann *et al*., 1998; Wu *et al.*, 1998l Duan & Wang, 2010; Arndt *et al*., 2001). The concentrations of corticosterone used in the current study did not reach the previously-reported IC_{50}s for inhibition of OCT1 (150 μM) or PMAT (450 μM). Thus, the *corticosterone*-sensitive MPP^+ uptake (50-60% of total uptake) observed in the present study is likely mediated primarily by OCT3, with the corticosterone-insensitive fraction (40-50% of total uptake) mediated by PMAT and OCT1. Given the high levels of PMAT mRNA we detected in relation to OCT3, it is surprising that the corticosterone-

insensitive fraction of MPP^+ accumulation in CGNs is not larger. This may be due to differences in the efficiencies with which OCT3 and PMAT transport MPP^+. In support, recently published studies demonstrated that the V_{max} for OCT3-mediated MPP^+ uptake is two-fold higher than that of PMAT (Duan & Wang, 2010).

Previous studies demonstrated that corticosterone-induced inhibition of OCT3-mediated transport is a non-genomic, GR-independent phenomenon (Horvath *et al.,* 2003). The present studies are consistent with a non-genomic mechanism, in that inhibition of MPP^+ uptake by all tested corticosteroids was evident with only 5 minutes of steroid exposure, and that the GR antagonist RU38486 also inhibited MPP^+ accumulation. The IC_{50} for corticosterone inhibition of MPP^+ accumulation reported in the present study (81 nM) is consistent with previous studies (Gasser, Lowry & Orchinik, 2006; Shang *et al.*, 2003), and is within the physiological range reported for stress-induced corticosterone concentrations the rat brain (Droste *et al.*, 2008; Droste *et al.,* 2009).

While each of the individual $uptake_2$ transporters is inhibited by corticosterone, their sensitivities to other corticosteroids have not been thoroughly characterized. Early studies in peripheral tissues demonstrated that a variety of natural corticosteroids inhibit $uptake_2$ activity or enhance the actions of epinephrine (Purdy, Weber & Drayer, 1982; Purdy & Weber, 1983). However, the effects of corticosteroids other than corticosterone on individual $uptake_2$ transporters have been examined only for OCT3. Those studies demonstrated that OCT3 is inhibited by budesonide, methylprednisolone, and fluticasone (Horvath *et al.*, 2003; Horvath *et al.*, 2007). Our data indicate that $uptake_2$-mediated transport in the CNS is broadly sensitive to inhibition by natural and synthetic

corticosteroids. While it is likely that these corticosteroids exert their effects at least in part by inhibiting OCT3, further studies are required to determine the specific contributions of each of the expressed transporters to corticosteroid-sensitive uptake. The mineralocorticoids aldosterone and 11-DOC also inhibited MPP^+ accumulation by CGNs. This is consistent with previous studies, which demonstrated that these two mineralocorticoids could inhibit the clearance of epinephrine and norepinephrine, and potentiate their actions in vascular or cardiac tissue (Purdy , Weber & Drayer, 1982; Purdy & Weber, 1983; Bell & McLachlan, 1979; Martel, Azevedo & Osswald, 1993). This is the first study demonstrating mineralocorticoid-sensitive transport in CNS cells, and suggests that mineralocorticoids also exert actions in the CNS by inhibiting $uptake_2$-mediated monoamine clearance. Interestingly, OCT3 knockout mice display alterations in salt intake that are comparable to those exhibited by aldosterone-treated animals (Vialou *et al.,* 2004). As OCT3 protein and mRNA are expressed in brain regions involved in regulation of salt intake, including circumventricular organs and medial amygdala, these data raise the possibility that this particular $uptake_2$ transporter represents a target for mineralocorticoid action on ingestive behavior (Gasser *et al*., 2009; Vialou *et al.,* 2004).

Comparison of the effects of corticosterone, 11-DOC and 11-dehydrocorticosterone on MPP^+ accumulation indicates that slight differences in corticosteroid structure significantly alter potency and efficacy of steroid-mediated inhibition of $uptake_2$. These three steroids differ in structure only at C11 of the steroid backbone. 11-Deoxycorticosterone, which differs from corticosterone only by the lack of a hydroxyl group at C11, was a more potent and slightly more effective inhibitor of MPP^+

accumulation than was corticosterone, suggesting that the lack of the C11 hydroxyl group may increase the potency of 11-DOC at OCT3 and/or make it a more effective inhibitor of PMAT and/or OCT1. 11-Dehydrocorticosterone, which contains a keto- group at C11, was a much *less* effective inhibitor than either corticosterone or 11-DOC. Further studies are necessary to determine the effects of these steroids on individual uptake$_2$ transporters. The present studies are the first to demonstrate sensitivity of uptake$_2$ activity to dexamethasone, prednisolone, and betamethasone, and to demonstrate that uptake$_2$-mediated transport in the CNS is sensitive to synthetic corticosteroids. These results are consistent with studies demonstrating that OCT3-mediated monoamine transport in bronchial smooth muscle cells is inhibited by budesonide, fluticasone and methylprednisolone (Horvath *et al.*, 2003; Horvath *et al*., 2007). Again, further studies are required to determine the relative contributions of OCT3, OCT1 and PMAT to the effects of each of the tested synthetic steroids. However, these studies raise the interesting possibility that synthetic corticosteroids may acutely and potently enhance monoaminergic neurotransmission in the CNS, and that these actions may in part underlie previously described acute effects of synthetic corticosteroid treatment on behavior in humans (Greeves, 1984; Swinburn *et al*., 1988; Wolkowitz *et al*., 1997).

Both natural and synthetic corticosteroids have powerful effects on monoamine-regulated behaviors, (Greeves, 1984; Swinburn *et al*., 1988; Wolkowitz *et al*., 1997; de Quervain *et al*., 2000; Mizoguchi *et al.*, 2004; Roozendaal *et al*., 2006; Sandi, Venero & Guaza, 1996; Wada *et al.,* 2000). Accumulating evidence suggests that uptake$_2$ transporters may play important roles in regulating monoaminergic neurotransmission in specific brain regions (Baganz *et al*., 2008; Gasser, Lowry & Orchinik, 2006; Rahman *et*

al., 2008; Cui *et al*., 2009). The present studies demonstrate that uptake$_2$ represents an important target for corticosteroid action in the brain and that inhibition of uptake$_2$-mediated clearance of monoamines must be taken into consideration when interpreting the results of acute treatment with both natural and synthetic corticosteroid hormones.

CHAPTER VI

ORGANIC CATION TRANSPORTER 3 IS DENSELY EXPRESSED IN THE INTERCALATED CELL GROUPS OF THE AMYGDALA: ANATOMICAL EVIDENCE FOR A STRESS HORMONE-SENSITIVE DOPAMINE CLEARANCE SYSTEM

Introduction

The amygdala is a critical component of the neural circuitry mediating anxiety and fear (Davis *et al.*, 1994; Ledoux, 2000). Effective regulation of anxiety and fear-related behaviors requires strict control over the activity and excitability of neurons in the basolateral amygdala (BLA) and the central amygdala (CeA), so that anxiety and fear responses are suppressed under most conditions, but can be rapidly expressed when necessary. This regulation is accomplished in part by a complex inhibitory network surrounding the amygdala which, driven primarily by cortical projections, suppresses BLA and CeA activity and thus prevents inappropriate expression of fear and anxiety responses (Davis & Myers, 2002; Quirk & Gehlert, 2003; Sanders & Shekhar, 1995).

When these responses are appropriate, however, inhibitory control of amygdala output must be released. Recent studies have demonstrated that the dopaminergic system plays a critical role in releasing cortical inhibition of amygdala activity, and thus is a potent regulator of fear and anxiety responses (de la Mora *et al*., 2010; Fadok *et al*., 2009; Guarraci *et al*., 1999). Acting primarily through D1 receptors, dopamine increases the activity of BLA pyramidal neurons by attenuating inhibitory influence from the infralimbic prefrontal cortex (PFC) and enhancing sensory cortical inputs, resulting in overall enhancement of BLA-mediated behaviors (Greba *et al*., 2001; Lamont &

Kokkinidis, 1998; Rosenkranz & Grace, 2001). Thus, D1 receptors are thought to function as a switch, facilitating the transition of the BLA from a PFC-controlled, relatively inhibited state to a disinhibited, more excitable state (Rosenkranz & Grace, 2002). Recent studies suggest that the effects of dopamine on amydala activity result in large part from its actions on neurons in the intercalated cell groups of the amygdala (ITC) (Marowsky *et al*., 2005).

The ITCs consist of dense clusters of D1 receptor-expressing small-to-medium-sized GABAergic neurons surrounding the BLA, and are subdivided into the main (I_M), anterior (I_A) and lateral and medial paracapsular (I_{lp} and I_{mp} respectively) islands (Millhouse, 1986). ITC neurons receive prominent excitatory inputs from the PFC (Berretta *et al*., 2005; Quirk *et al*., 2003; Sesack *et al*., 1989; Vertes, 2004) and send projections to the central and basolateral amygdaloid nuclei, as well as to adjacent ITC cell groups (Royer *et al*., 1999; Royer *et al*., 2000). ITC neurons are activated during fear expression, extinction training and extinction retrieval (Busti *et al*., 2011), and in response to pharmacological activation of infralimbic PFC neurons (Berretta *et al*., 2005).

Recent studies have demonstrated that the intercalated cell groups are functionally heterogeneous, and suggest that they form a complex interconnected network that controls the sensitivity of the amygdala to afferent neuronal signals and regulates communication between distinct nuclei within the amygdala. These studies suggest that cells in I_{mp} and I_M regulate the flow of neuronal signals from the BLA to the central nucleus (Royer *et al.*, 1999; Manko *et al.*, 2011), while I_{lp} neurons regulate PFC-to-amygdala signals (Marowsky *et al*., 2005). Neurons in the I_A may modulate communication between right and left amygdalae (Marcellino *et al*., 2012). In addition,

the ITCs are functionally and anatomically interconnected, such that activation of one intercalated cell group may result in inhibition of another. Stimulation of I_M, I_{lp} and I_{mp} neurons by infralimbic PFC projections results in inhibition of BLA and CeA projection neurons (Marowsky *et al*., 2005; Amir *et al*., 2011), and lesions of I_{mp} neurons result in increases in anxiety and fear, and deficits in extinction of conditioned fear (Pape & Pare, 2010; Likhtik *et al*., 2008).

Intercalated cell groups receive dense dopaminergic projections from the VTA (Asan, 1998; Freedman & Cassell, 1994; Moore & Bloom, 1978), and express the highest concentration of dopamine D1 receptors in the mammalian amygdala (Fuxe *et al*., 2003). Activation of D1 receptors hyperpolarizes and decreases the firing rates of GABAergic neurons in both the I_{mp} and the I_M (Marowsky *et al*., 2005; Manko *et al*., 2011). In the I_{mp}, this effect is mediated by D1-receptor-induced activation of G protein-coupled inwardly rectifying potassium (GIRK) channels, decreasing the sensitivity of ITC neurons to PFC stimulation, and resulting in disinhibition of BLA and CeA activity (Marowsky *et al.*, 2005). Thus extracellular dopamine, by regulating ITC-mediated inhibitory tone, is a critical determinant of amygdala excitability and plasticity. Stimuli, including stress-related stimuli, which increase dopamine concentrations in these cell groups, would lead to altered sensitivity of BLA neurons to excitatory signals.

Neuroanatomical studies of the ITC indicate that a substantial portion (up to 50%) of dopaminergic neurotransmission within these areas occurs via volume transmission (Fuxe *et al*., 2003; Fuxe *et al.*, 2005; Marcellino *et al*., 2012). Thus, mechanisms, including presynaptic or postsynaptic transport, that regulate extracellular dopamine concentration are likely to be important determinants of amygdala function. Previous

studies have demonstrated that the pattern of dopamine transporter (DAT) immunoreactivity in the amygdala overlaps with that of tyrosine hydroxylase (TH), with high densities observed within the ITCs, and lower densities elsewhere (Revay *et al*., 1996). Recent descriptions of TH immunostaining have described a spatial "mismatch" between these dopaminergic terminals and D1 receptors in the ITC, with distances of 1 μm or more separating D1 receptors from TH-immunoreactive terminals, suggesting that other clearance mechanisms besides the presynaptic DAT may be involved in regulating dopaminergic neurotransmission in these areas (Marcellino *et al*., 2012; Fuxe *et al.*, 2003).

We have recently described the expression in the amygdala of an additional dopamine clearance mechanism, organic cation transporter 3 (OCT3), with particular enrichment in dense clusters of small cells surrounding the BLA (Gasser *et al*., 2009). In contrast to the DAT, OCT3 has higher capacity and lower affinity for dopamine, is sodium-independent, and has the capacity to transport norepinephrine, serotonin, and other monoamines (Duan & Wang, 2010; Grundemann *et al*., 1998; Grundemann *et al*., 1999). Interestingly, OCT3-mediated transport is directly and acutely inhibited by the stress hormone corticosterone (Grundemann *et al.*, 1998). Thus, OCT3 may represent a stress-sensitive dopamine clearance mechanism, and may act within the amygdala to control extracellular dopamine concentrations. Because of the profound functional implications of high-density expression of a previously uncharacterized dopamine transporter in this important brain region, and in order to understand the contribution of OCT3 to the regulation of dopaminergic neurotransmission, we sought in this study to more fully describe the distribution of OCT3-immunoreactivity in the ITC, to examine its

relationship to D1 receptors and dopaminergic terminals in the ITC, and to identify the phenotype(s) of OCT3-expressing cells.

Materials and methods

Animals

Male Sprague Dawley rats (Harlan Laboratories, Inc., St Louis, MO, USA), weighing 275-325 g, were housed individually in a temperature- and humidity-controlled, AAALAC-accredited vivarium under a 12h/12h light-dark cycle (lights on at 0700 h) with *ad libitum* access to food and water. Housing conditions and experimental protocols approved by the Marquette University Institutional Animal Care and Use Committee, and were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication number 80-23, revised 1996).

Perfusion and Histology

Rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and were transcardially perfused with ice-cold 0.05 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). Following perfusion, brains were removed and post-fixed in the 4% paraformaldehyde solution for 12 h at 4 °C, and were rinsed twice in 0.1 M PB for 12h. The brains were incubated in 30% sucrose in 0.1 M PB for approximately 72 h. Brains were then blocked into two pieces with a cut in the coronal plane at the caudal border of the mammillary bodies (approximately –5.30 mm bregma) using a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA). Brains were frozen rapidly in dry-ice-chilled liquid isopentane and stored at –80 °C until sectioning. Forebrain sections (25

μm) including the basolateral amygdala were cut across the coronal plane using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA), and stored as 6 alternate sets of sections in cryoprotectant (30% ethylene glycol (w/w)/20% glycerol (w/w) in 0.05 M PB, pH 7.4) at –20 °C until immunostaining.

Antibodies

For immunodetection of OCT3, an affinity-isolated antibody (rabbit anti-OCT3, cat # OCT31A, Alpha Diagnostics International, San Antonio, TX, USA) raised against an 18-amino acid sequence in the large intracellular loop of rat OCT3 (amino acids 313-330: HLSSNYSEITVTDEEVSN) was used. This amino acid sequence is 100% conserved in mouse and rat OCT3, and has no significant sequence homology with other OCTs or with any organic cation/carnitine transporters. The specificity of this antibody was confirmed previously in immunohistochemical and immunofluorescence applications (Gasser et al., 2006; Gasser et al., 2009; Lips et al., 2005; Vialou et al., 2004). For immunodetection of tyrosine hydroxylase (TH) a monoclonal antibody (mouse anti-TH, cat # MAB318, Millipore, Billerica, MA, USA), previously used to characterize TH expression in the rat amygdala (Muller et al., 2009), was used at a dilution of 1:1600. For Dopamine D1 receptor immunodetection, a monoclonal antibody (mouse anti-D1a receptor, cat # MAB5290, Millipore) was used at a dilution of 1:5000 (immunohistochemistry) or 1:250 (immunofluorescence). This antibody has been thoroughly characterized and shown to be specific for D1 receptors, and to display no cross-reactivity for dopamine D5 receptors (Luedtke et al., 1999). For immunodetection of the neuronal marker NeuN, a monoclonal antibody (mouse anti-NeuN, cat# MAB377, Millipore) was used at a dilution of 1:400.

Immunohistochemistry

Free-floating sections were incubated in 24-well tissue culture plates and gently shaken on an orbital shaker throughout the staining process. For single-label immunostaining, sections were first rinsed in 0.05 M PBS for 15 min, then treated with 1% hydrogen peroxide in 0.05 M PBS for 15 min, washed again for 15 min in PBS, pre-incubated in PBS containing 0.3% Triton X-100 (PBST), and then incubated overnight at room temperature with primary antibody (OCT3 (1:500) or D1r (1:5000)) in 0.1% PBST. Sections were rinsed twice for 15 min in 0.05 M PBS, after which they were incubated for 90 min with secondary antibody (biotinylated swine anti-rabbit IgG, cat. no. E0353, DAKO, Ely, UK) diluted 1:200 in 0.05 M PBS. After two more 15-min rinses in PBS, sections were incubated for 90 min with an avidin-biotin complex (Elite ABC reagent; Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS. Sections were then rinsed for 15 min in 0.05 M PBS, and incubated in a solution containing 0.001% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.0015% hydrogen peroxide in PBS for 20 min or until staining was clearly visible. Stained sections were rinsed for 15 min in 0.1 M PB, dipped briefly in water, and mounted onto SuperFrost slides (VWR, Arlington Heights, IL, USA). After drying at room temperature overnight, sections were dehydrated in ascending alcohol concentrations and slides were mounted with cover slips using Entellan mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

Separate sections were processed for double-label immunostaining for OCT3 and TH. Procedures for OCT3 immunostaining were conducted as above. After the DAB reaction, sections were rinsed twice in 0.05 M PBS, followed by an additional treatment with 1% hydrogen peroxide in 0.05 M PBS for 15 min, rinsing, and overnight incubation

in mouse anti-TH in 0.1% PBST. Sections were then washed twice in 0.05 M PBS, followed by a 90-min incubation with secondary antibody (biotinylated donkey anti-mouse IgG (Millipore, 1:200 in 0.05 M PBS). After rinsing, sections were incubated for 90 min with Elite ABC reagent (diluted as above), rinsed, and incubated for 10 min with a peroxidase substrate solution (Vector SG; Vector Laboratories) diluted as recommended by the vendor. Sections were transferred into 0.05 M PBS to stop the chromogen reaction, rinsed briefly in distilled water, mounted onto glass microscope slides, dehydrated and coverslipped as above.

Immunofluorescence

Separate coronal sections (25 μm) containing the BLA were used for combined detection of OCT3 and either dopamine D1 receptor (1:250), tyrosine hydroxylase, or NeuN. After rinsing in PBS, sections were incubated overnight with anti-OCT3 antibody (1:250) and anti-DA D1r antibody (1:250) in 0.1% PBST. Sections were rinsed the next day, and incubated 2 h with fluorophore-conjugated secondary antibodies (AlexaFluor594-conjugated donkey anti-rabbit and AlexaFluor488-conjugated donkey anti-mouse IgG antibodies (1:200; Invitrogen)). Sections were then rinsed briefly in PB, mounted onto SuperFrost microscope slides, dried briefly and coverslipped with Vectashield antifade mounting medium containing DAPI for visualization of nuclei (Vector Laboratories). All immunostaining studies were repeated a minimum of three times with similar results.

Imaging

Photomicrographs were acquired using a Nikon 80i microscope fitted with a Retiga 2000R digital camera (QImaging, Surrey, BC, Canada) linked to a computer running NIS Elements-D software (Nikon Instruments, Melville, NY, USA). Color bright field images were captured using a liquid crystal RGB color filter (QImaging RGB-HM-S-IR). Distances and cell diameters were estimated using a utility in the NIS Elements-D software, which measures the length of a line drawn across the diameter of each cell of interest. Care was taken to exclude proximal processes from the diameter estimates. Mean cell diameters were calculated by averaging the estimated diameters of at least 25 cells within the region of interest.

Results

OCT3 immunostaining in the intercalated cell groups: Comparison with DA D1r immunostaining.

Immunohistochemical localization revealed a consistent pattern of OCT3-like immunoreactivity in the BLA and intercalated cell groups in the brains of each rat (n = 4). OCT3-like immunoreactivity was seen as a brown reaction product concentrated in perikarya, with diffuse punctate brown reaction product visible at higher magnification (Fig. 6.1A,C). As previously reported, this staining pattern was not observed in tissue sections incubated in the absence of primary antibody (data not shown). OCT3-like immunoreactive (OCT3-ir) perikarya were observed at low density in the BLA and at high density in small clusters of cells on the dorsal, medial and lateral borders of the BLA, and in one large cluster ventromedial to the BLA (Fig. 6.1C). These clusters

corresponded to the areas in which dense D1 receptor immunoreactivity were observed (Fig. 6.1B,D), and thus to the locations of the main, paracapsular and anterior intercalated cell groups, respectively (Fuxe *et al*., 2003; Marcellino *et al*., 2012). In both the BLA and intercalated clusters, OCT3-ir perikarya were small, ranging from 6 – 12 μm in diameter. OCT3-ir perikarya in the BLA were slightly, but significantly, smaller than those in the intercalated clusters (ITC mean diameter (n=50 cells) = 9.85 ± 1.1 μm, range 7.1 – 12.39 μm; BLA mean diameter (n=30 cells) = 8.84 ± 1.29 μm, range 6.4-11.28 μm; unpaired *t*-test, $p < 0.001$).

The distribution of D1 receptor immunoreactivity in the BLA and ITC was similar to that of OCT3, with low densities in the BLA, and high densities in the ITC. D1 receptor immunoreactivity was observed as punctate brown reaction product, and occurred at high density in small clusters dorsal, medial and lateral to the BLA, and in one large cluster ventromedial to the BLA. D1 receptor immunoreactivity was also observed, though at much lower density, on perikarya throughout the BLA (Fig. 6.1B, D).

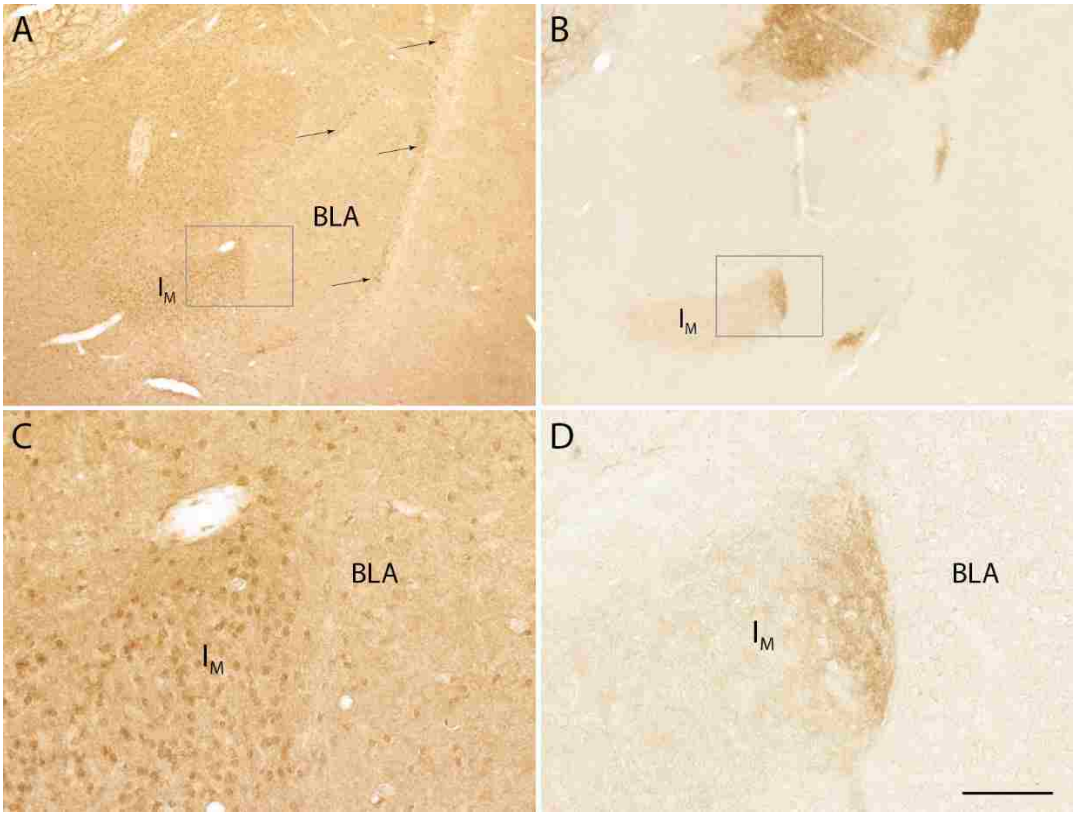

Figure 6.1. OCT3 and dopamine D1 receptor are expressed at high density in the main, anterior and paracapsular intercalated cell groups of the amygdala. Bright field photomicrographs of sections immunostained with antibodies directed against OCT3 (A, C) and dopamine D1 receptor (B, D). Boxes in A and B indicate regions shown at higher magnification in C and D, respectively. **A:** Low power photomicrograph showing the basolateral amygdaloid complex at approximately –2.04 mm bregma. Arrows indicate dense clusters of small-diameter OCT3-ir perikarya dorsal, lateral and medial to the BLA (medial is to the left). A larger dense cluster of OCT3-ir perikarya is visible ventromedial to the BLA (I_M). **B:** Low power photomicrograph of the basolateral amygdaloid complex at approximately –2.04 mm bregma showing dense D1-like immunoreactivity in clusters dorsal, lateral and ventromedial to the BLA (medial is to the left). **C:** Higher power photomicrograph showing OCT3-immunoreactive perikarya at low density in the BLA, and at high density in the adjacent main intercalated cell group (I_M). **D:** Dense D1 immunostaining in I_M ventromedial to the BLA. Scale bar = 500 µm (A, B); 100 µm (C, D). BLA – basolateral amygdala; I_M – main intercalated cell group; I_A – anterior intercalated cell group; I_P – paracapsular intercalated cell group.

OCT3 and D1 receptor dual immunofluorescence.

Dual-label immunofluorescence confirmed co-distribution of OCT3- and D1-like immunoreactivities in the intercalated cell groups. OCT3-ir perikarya and were most densely distributed in the intercalated cell groups, with lower density in the BLA (Fig. 6.2A). In contrast to the immunohistochemical staining, in which OCT3-ir punctae were not readily distinguished, immunofluorescence revealed a dense distribution of discrete OCT3-ir punctae in the I_M (Fig. 6.2B). Dense clusters of OCT3-ir perikarya and punctae were also observed in the anterior and paracapsular ITCs (data not shown). OCT3 immunostaining overlapped with dense D1 receptor-immunoreactive punctae. Examination of the spatial relationships of D1- and OCT3-immunoreactive punctae at high magnification revealed close apposition of the two in the I_M (Fig.6. 2 H, mean distance between D1- and OCT3-ir punctae (n=90) = 0.56 ± 0.25 μm, range 0.28 – 1.53 μm).

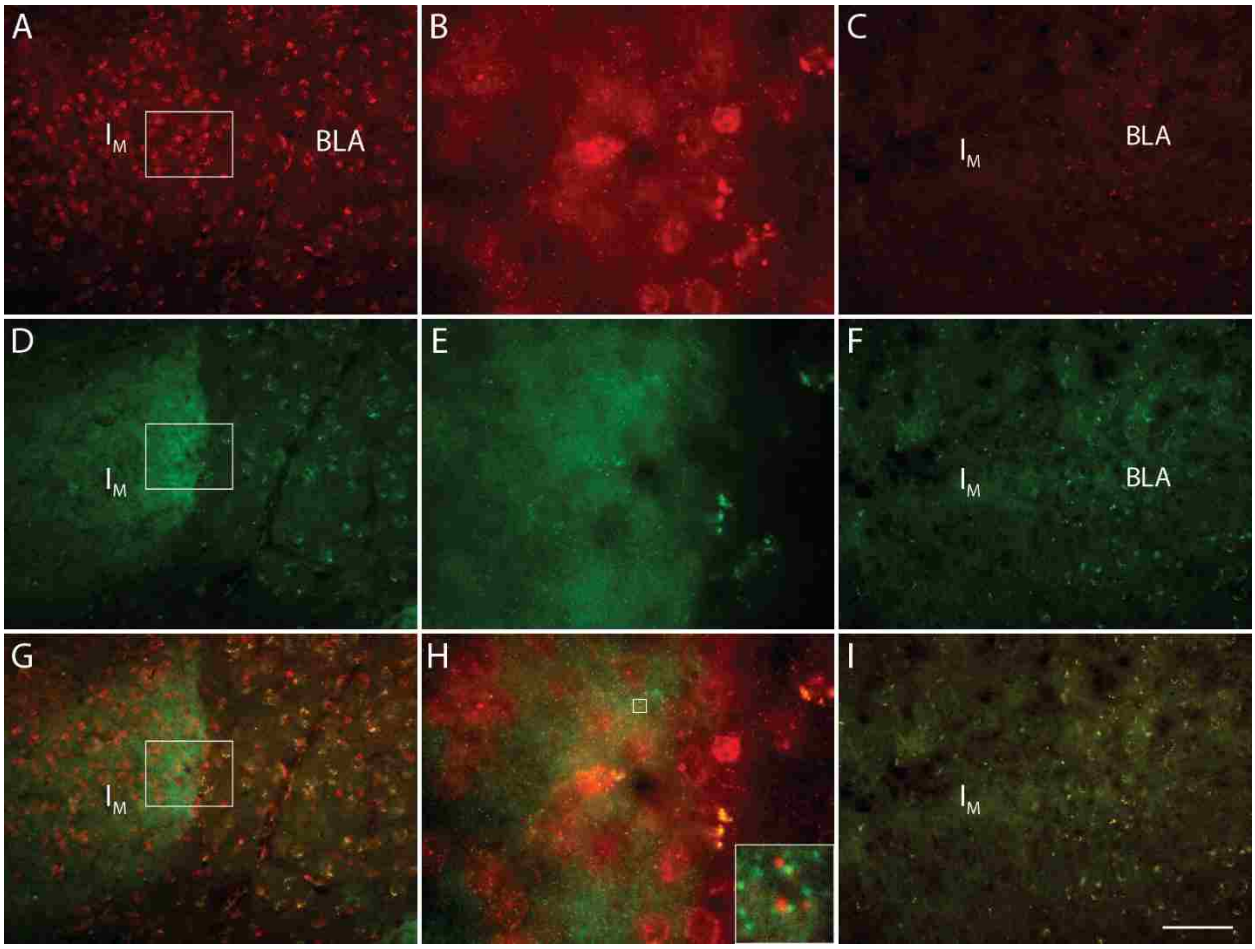

Figure 6.2. Distribution pattern of OCT3 (red) and dopamine D1 receptor (green) immunostaining. Fluorescence photomicrographs of dual-label immunofluorescence localization of D1- and OCT3-immunoreactivity in the main intercalated cell group. Panels depict sections incubated in the presence (A-B, D-E, G-H) or absence (C, F, I) of primary antibodies. Boxes in A, D and G indicate regions shown at higher magnification in adjacent panels. OCT3-ir perikarya (A, B) and puncta (B) in the main intercalated cell group and in the BLA were not observed in sections incubated in the absence of the primary antibody (C). D1-ir puncta (D, E) observed in the main intercalated cell group were not observed in sections incubated in the absence of the primary antibody (F, I). OCT3-ir perikarya were observed in close proximity to D1-ir puncta in the main intercalated cell group (G-H). Box in H indicates area shown at higher magnification in the inset. Non-specific (autofluorescent) signal was detected mainly in the BLA in sections incubated in the absence of either primary antibody (C, F) and was observed as yellow signal in (I). Scale bar = 100 µm (A, C, D, F, G, I); 20 µm (B, E, H); 4 µm (inset H). BLA – basolateral amygdala; I_M – main intercalated cell group.

OCT3 and tyrosine hydroxylase dual immunolabeling in the amygdala.

Tyrosine hydroxylase-immunoreactive fibers were observed at high density in the striatum and amygdalostriatal transition area, at low-to-moderate density in the BLA, and at high density in the main, anterior and paracapsular intercalated cell groups (Fig. 6.3, 6.4). In the main intercalated cell group, TH-ir fibers were most densely distributed in the region most proximal to the BLA, and decreased in density ventromedially (Fig. 6.3A). OCT3-ir perikarya and puncta were observed at high density in all intercalated cell groups, including I_{mp} (Fig. 6.3B), I_A (Fig. 6.3C), I_{lp} (Fig. 6.3D) and I_M (Fig.6. 3E). In all ITCs, OCT3-ir perikarya occurred in very close proximity to TH-ir fibers. Dual-label immunofluorescence revealed a similar pattern of OCT3- and TH-immunoreactivity in the amygdala (Fig.6. 4). At high magnification, OCT3-ir punctae were observed in close proximity to, but only rarely overlapping with, TH-ir fibers (Fig. 6.4C).

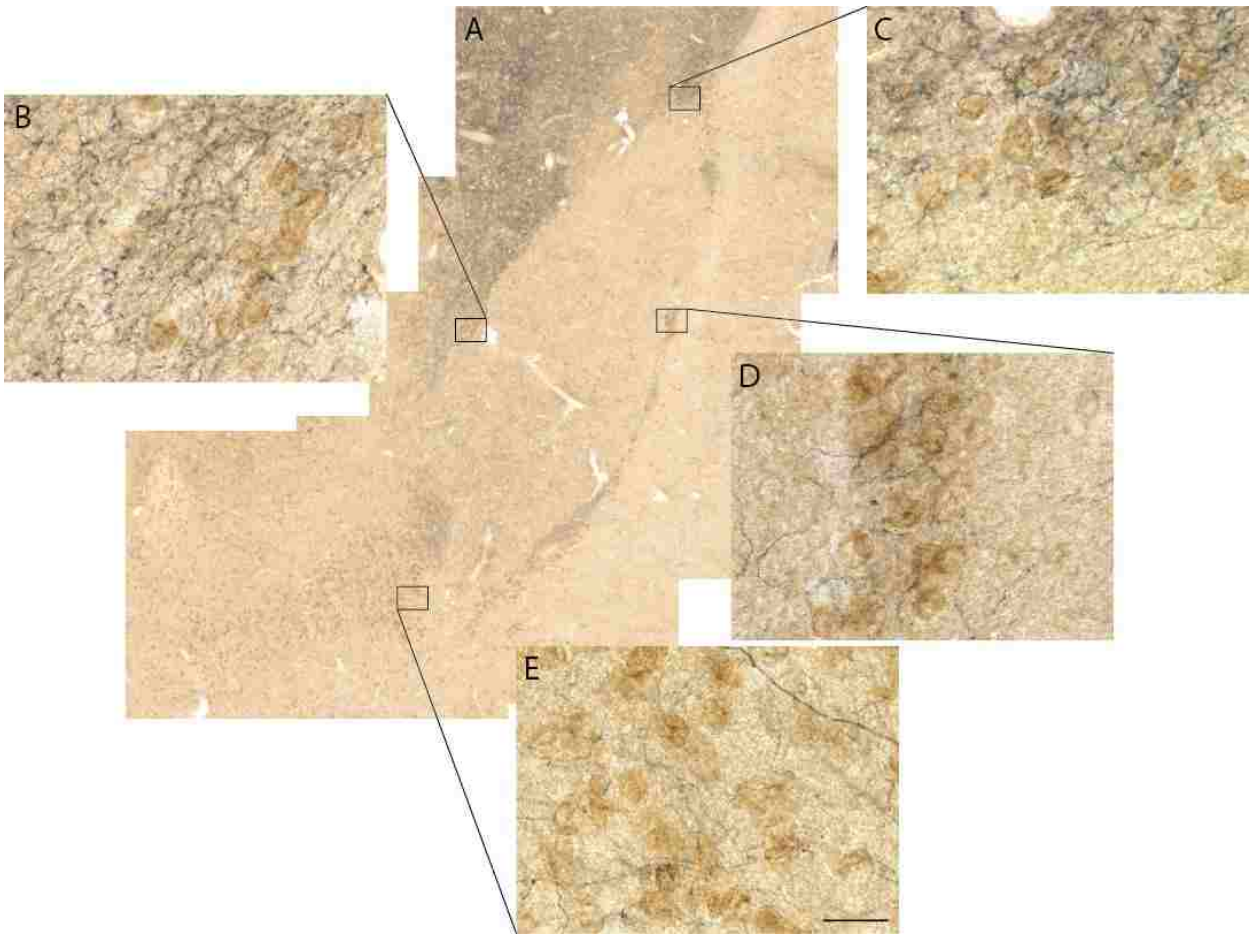

Figure 6.3. Dual-label immunohistochemistry for OCT3 and tyrosine hydroxylase in intercalated cell groups. Photomicrographs depicting sections immunostained for OCT3 (brown) and TH (black). **A:** Composite photomicrograph depicting dense clusters of OCT3-ir perikarya and D1-ir fibers in the main, anterior and paracapsular intercalated cell groups. Boxes in A indicate areas depicted at higher magnification in panels **B-E**. At high magnification, OCT3-immunoreactivity was observed both concentrated in perikarya and as diffuse brown reaction product adjacent to black TH-immunoreactive fibers in the medial paracapsular (B), anterior (C), lateral paracapsular (D) and main (E) intercalated cell groups. Scale bar = 250 µm (A); 20 µm (B-E).

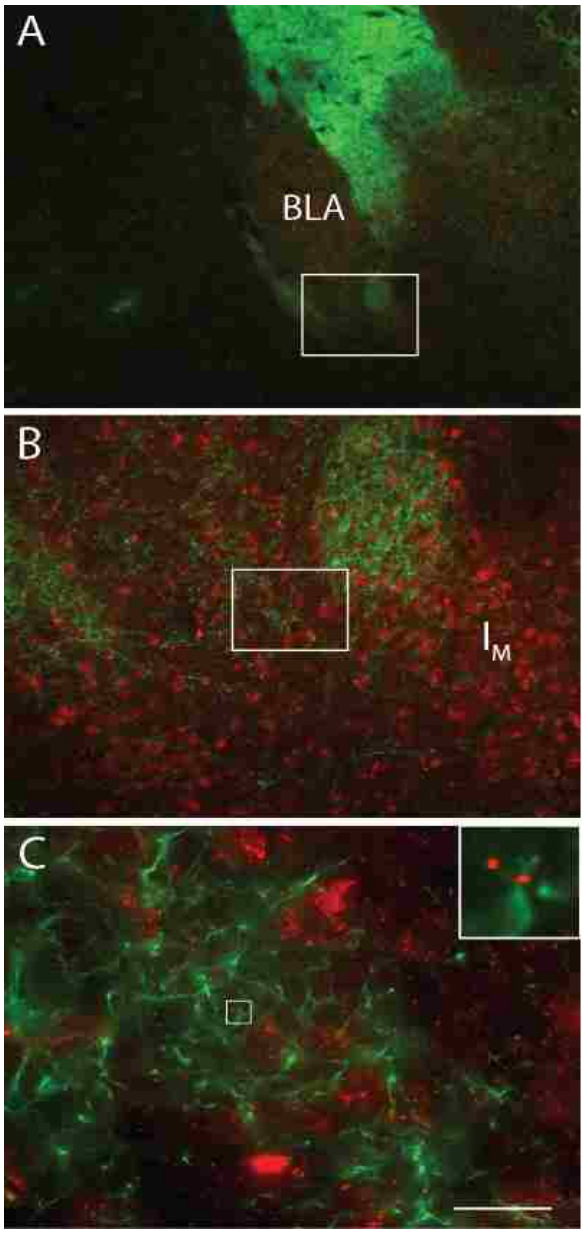

Figure 6.4. Fluorescence photomicrographs of sections depicting OCT3 (red) and TH (green) immunoreactivity in the BLA and intercalated cell groups. Boxes in **A** and **B** indicate areas depicted at higher magnification in B and C, respectively. **C:** OCT3-ir perikarya and puncta in close proximity to TH-ir fibers in the main intercalated cell group. Small box in **C** indicates area shown at higher magnification in the inset. Scale bar = 500 µm (A); 100 µm (B); 20 µm (C); 4 µm (inset C).

OCT3 and NeuN dual immunolabeling of the main intercalated cell group.

The intercalated cell groups were visible as dense clusters of NeuN-immunoreactive perikarya on the borders of the BLA. Most NeuN-immunoreactive perikarya within the intercalated cell groups also displayed OCT3 immunoreactivity (Fig. 6.5).

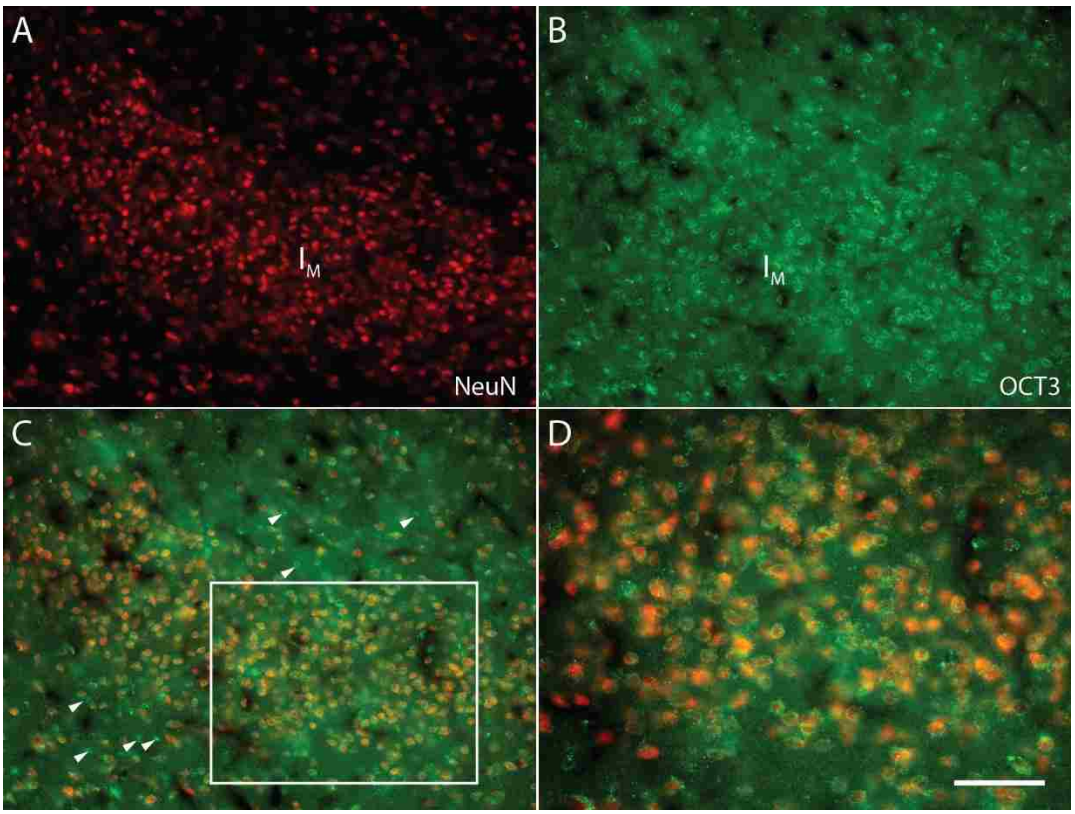

Figure 6.5. Neuronal phenotype of OCT3-ir cells in I_M. Fluorescence photomicrographs of sections dual-labeled with NeuN (red) and OCT3 (green). Box in (C) indicates region shown at higher magnification in (D). **A, B**: Dense clusters of NeuN-ir nuclei (A) and OCT3-ir perikarya (B) were observed in the main intercalated cell group. **C, D:** Most OCT3-ir perikarya in the I_M were also positive for NeuN-immunoreactivity. A few OCT3-ir, NeuN-immunonegative perikarya were observed outside the I_M (arrowheads in C). Scale bar = 100 μm (A-C); 50 μm (D). I_M – main intercalated cell group.

Conclusion

These studies provide anatomical evidence that OCT3, a high-capacity transporter for dopamine and other monoamines (Grundemann *et al*., 1998), may play a prominent role in controlling dopaminergic neurotransmission in the intercalated cell masses of the amygdala. The dense expression of OCT3, its close proximity to dopamine D1 receptors, and its relationship to catecholaminergic terminals, most of which are likely to be dopaminergic terminals (Marcellino *et al*., 2012), suggest that OCT3-mediated clearance may be a critical determinant of dopamine volume transmission and of the activation of D1 receptors in these areas. The expression of OCT3 in ITC neurons and not on catecholaminergic terminals, indicates that this transporter, in contrast to the DAT, does not function as a pre-synaptic reuptake mechanism, and suggests that OCT3 may mediate dopamine clearance on dopamine target cells in the this area. As OCT3-mediated transport is directly inhibited by corticosterone (Grundemann *et al*., 1998; Horvath *et al*., 2003), OCT3 may represent a mechanism by which acute stress enhances dopaminergic neurotransmission in the amygdala.

The distribution of OCT3 expression described in the present study is consistent with our previous studies (Gasser *et al*., 2009), and the extensive co-distribution with areas of dense D1 receptor expression confirms our previous assertion that the dense clusters of OCT3-expressing cells surrounding the BLA correspond to the D1 receptor-rich intercalated cell masses. The sizes of OCT3-expressing cell bodies in all ITC cell groups, and their co-expression of the neuronal marker NeuN, indicate that they represent the small- to medium-sized GABAergic neurons most abundant in the ITCs (Marowsky *et al*., 2005; Millhouse, 1986).

In addition to OCT3-expressing cell bodies, we observed a dense distribution of OCT3-ir punctae in the ITCs that were not localized on cell bodies. The fact that these punctae were not observed in tissue incubated in the absence of OCT3 antibody indicates that they, like the cell bodies, represent sites of OCT3 expression. While the OCT3-expressing cell bodies very likely represent neurons, the cellular identity of the diffuse OCT3-ir punctae is not as clear. Based on the restricted distribution of dense OCT3 labeling to the ITC groups, we hypothesize that OCT3-ir punctae in these areas represent transporter expressed on ITC GABAergic cell dendrites, which are largely confined within the ITCs (). This interpretation is also consistent with the absence of OCT3-ir punctae co-localized with TH+ catecholaminergic terminals in the ITCs. However, while these observations indicate that OCT3 is not expressed on dopaminergic terminals, they do not rule out the possibility that OCT3 is expressed presynaptically on glutamatergic or other afferent terminals. A clear determination of the subcellular localization of OCT3 will require additional studies.

Our description of amygdala D1 receptor expression is consistent with previous anatomical studies demonstrating dense D1 receptor mRNA and protein expression on ITC GABAergic neurons (Fuxe *et al*., 2003; Maltais *et al*., 2000; Marcellino *et al*., 2012; Pinto & Sesack, 2008), and low levels of D1 receptor expression on neurons within the BLA (Pickel *et al*., 2006; Pinto & Sesack, 2008), and with functional studies demonstrating D1 receptor-mediated actions of dopamine in both BLA and ITC neurons (Marowsky *et al*., 2005; Rosenkranz & Grace, 2002). The dense expression of OCT3 in the ITCs suggests that dopamine clearance in these areas is accomplished by the combined activity of the presynaptic dopamine transporter (DAT) and OCT3, and that

OCT3-mediated transport plays a particularly prominent role in controlling extracellular dopamine concentrations in the main, anterior and paracapsular ITCs. The close apposition of OCT3-ir puncta in the ITCs to D1 receptors suggests that the transporter, by controlling dopamine levels immediately surrounding receptors, may be a key determinant of D1 activation in ITC neurons.

Previous studies examining the spatial relationships between D1 receptor-immunoreactive puncta and tyrosine hydroxylase-immunoreactive fibers in the ITC have suggested that at least 50% of dopaminergic neurotransmission in these areas occurs via extrasynaptic volume transmission (Fuxe *et al*., 2003; Fuxe *et al*., 2005; Marcellino *et al*., 2012; Marowsky *et al*., 2005). These studies have demonstrated that, while the ITC receive the densest dopamine innervation in the amygdala, the density of TH-immunoreactive terminals within these cell groups is not uniform. Dopamine terminals are concentrated in the rostral ITC and, in the I_M, in the dorsolateral portion of the cell group, while D1 receptors are expressed at uniformly high levels throughout the I_M (Fuxe *et al*., 2003; Marcellino *et al*., 2012; Pinto & Sesack, 2008). We observed a similar polarized distribution of D1 receptors and TH-ir fibers in the I_M (Fig. 1, 3). Thus, at areas distant from dopaminergic terminals, DAT is expected to play a less prominent role in controlling dopamine concentrations surrounding many D1 receptors. In the present studies, we observed uniformly dense OCT3 distribution throughout the intercalated islands, suggesting that OCT3 plays a prominent role in controlling dopaminergic transmission in these areas. Indeed, OCT3-mediated clearance may be the primary determinant of dopamine volume transmission in the DAT-poor ventromedial and caudal portions of the I_M.

The dense expression of a high-capacity monoamine transporter in the ITCs has important implications for the dopaminergic regulation of amygdala function. OCT3-mediated monoamine clearance is acutely and directly inhibited by corticosterone via a non-genomic, glucocorticoid receptor-independent mechanism (Grundemann *et al*., 1998; Hill *et al*., 2011; Horvath *et al*., 2003; Gorboulev *et al*., 2005). We and others have demonstrated that OCT3-mediated transport is inhibited at concentrations of corticosterone within the range induced by acute exposure to stress-related stimuli (Gasser *et al*., 2006; Hill *et al*., 2011; Shang *et al.*, 2003). Thus, OCT3 may represent a stress-sensitive component of the monoamine clearance system in the ITC and other areas, with OCT3-mediated clearance restraining volume transmission under basal conditions, and corticosterone-induced inhibition of clearance removing that restraining influence.

The expression of OCT3 on ITC neurons, and its close proximity to D1 receptors suggests that OCT3 plays a central role in determining dopamine concentrations immediately surrounding these important dopamine targets. Thus, during non-stress (low corticosterone) conditions, ongoing OCT3 activity may limit the influence of dopamine (and other monoamines) on intercalated neurons, ensuring that only large dopamine-releasing stimuli would disinhibit BLA and CeA activity. During stress, corticosterone-induced inhibition of OCT3-mediated clearance would be expected to increase the peak concentration, duration and physical spread of released dopamine, facilitating dopamine-induced disinhibition of amygdala function. Such actions may be an important mechanism by which stress regulates the activity and plasticity of neurons in the BLA and CeA.

CHAPTER VII

GENERAL DISCUSSION

Discussion

Individuals with PTSD have been shown to exhibit alterations in the functioning of the HPA axis. Cortisol, a glucocorticoid, is an important component of the stress response system, and as such has been a focus of research in understanding the underlying mechanisms of PTSD. Studies have demonstrated that individuals with PTSD have exhibited decreased concentrations of cortisol (Boscarino, 1996; Yehuda, 2002; Yehuda *et al.*, 1993, 1995), enhanced sensitivity to negative feedback inhibition of the HPA axis (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997) and expressed increased concentrations and responsiveness of the glucocorticoid receptor (Yehuda *et al*., 1995). Furthermore, individuals that expressed lower cortisol levels in the emergency room immediately following a traumatic experience, such as rape or motor vehicle accidents, had a greater incidence of developing PTSD over time as compared to individuals with higher levels of cortisol that underwent the same trauma and had similar trauma history (Resnick *et al*., 1995; McFarlane *et al*., 1997). This is interesting because it suggests a positive correlation between low cortisol levels following traumatic stress and the development of PTSD. Combined with decreased basal cortisol levels, increased sensitivity to negative feedback, and low cortisol following stress exposure it is possible that individuals with PTSD have low cortisol during the traumatic stress as well. Considered together, it is possible that by possessing decreased concentrations of cortisol, individuals with PTSD are unable to properly activate cortisol-dependent processes

mediated through the glucocorticoid receptor during times of stress and therefore develop a variety of PTSD-like behaviors.

We utilized an animal model in which we could examine individual differences in the stress response system and investigate as to whether those difference contributed to the development of PTSD-like behaviors. Spraque-Dawley rats were placed within a novel environment and allowed to investigate for an hour. Locomotor activity was recorded as infrared beam breaks, and summed for each animal across the session. Rats with scores in the lowest 33% of locomotor activity were labeled low responders and those in the highest 33% were labeled high responders (Kabbaj *et al.*, 2000; Liebsch, 1997; Jama *et al*., 2008). Previous research has demonstrated that locomotor activity can reflect HPA response to novelty stress (Piazza *et al*. 1991). Specifically, LRs exhibit lower corticosterone responses to stress than HRs. This provided us a model to examine whether or not individual differences in the stress response system contributed to developing PTSD.

We examined the behavioral phenotype of LRs and HRs following locomotor sorting and revealed that LRs have an overall higher anxiety profile than do HRs. Specifically, we demonstrated that LRs spent significantly less time in the center zone, reared less, explored and entered the light chamber of the light-dark apparatus significantly less than did HRs, and had a greater latency to enter the light chamber. These are all measures of increased levels of anxiety (Katz, Roth, & Carroll, 1980; Escorihuela *et al*., 1999; Crawley and Goodwin, 1980), which were not observed in HRs.

The next thing was to investigate the physiological differences between LRs and HRs in response to stress. It was revealed that under basal non-stressed conditions, LRs

and HRs did not differ from each other in regards to plasma corticosterone or plasma ACTH concentrations. In response to restraint stress, both LRs and HRs demonstrated an increase in both plasma corticosterone and plasma ACTH. However, LRs displayed significantly less corticosterone in response to stress than did HRs, demonstrating a difference in the stress response between phenotypes. Plasma ACTH concentrations were also less in LRs than HRs, but not significantly different. Either way, LRs responded differently than HRs in response to restraint stress by exhibiting significantly lesser corticosterone. It is possible that this blunted corticosterone response to stress could contribute to the development of PTSD.

Our prediction of LRs exhibiting enhanced rapid negative feedback was not supported. Both LRs and HRs displayed a reduction in ACTH following administration of cortisol. These data indicate that at the level of the pituitary the mechanisms for negative feedback are functional, but not significantly different between phenotypes. Interestingly, there seems to be a difference between LRs and HRs at the level of adrenal output. Following administration of cortisol, HRs demonstrated a reduction in plasma corticosterone which is in line with a typical negative feedback response. Low responders, while exhibiting initially lower levels of corticosterone than high responders, displayed no change in corticosterone following administration of cortisol, suggesting a lack of adrenal sensitivity to ACTH or that outside physiological systems are modulating adrenal output independent of the HPA response. Specifically, the sympatho-adreno-medullary axis could be exerting influence on the adrenals that results in adrenal output, but is specific to LRs or inactive in HRs. During stress the autonomic nervous system can stimulate the adrenal medulla through the splanchnic nerve (Jasper & Engeland, 1994)

and cause the release of cortisol in addition to norepinephrine. Studies have demonstrated that splanchnic nerve innervation can increase adrenal responsivity to ACTH (Ulrich-Lai, Arnhold, & Engeland, 2006). Therefore, as a result of this potential change in ACTH responsivity LRs secrete corticosterone even under inhibition by cortisol-driven negative feedback. However, we detected no differences in adrenal weight between phenotypes (see Supplementary Figure 3). Additionally, chronic stress and / or prolonged affective disorders may result in dissociation between activation of the HPA axis and the adrenal cortex (Ehrhart-Bornstein *et al*., 1998; Pignatelli *et al*., 1998). Therefore, it is possible that ACTH may not be an accurate mechanism to determine negative feedback sensitivity.

Together, these data suggest that LR animals may provide a model in which we can begin to tease apart the question of whether blunted HPA responses contribute to vulnerability to developing PTSD-like behaviors. Individuals with PTSD display enhanced negative feedback (Yehuda *et al.*, 1993; Yehuda *et al.,* 2002; Stein *et al.*, 1997) and decreased levels of cortisol (Mason *et al.*, 1986; Yehuda *et al.*, 1995), the human equivalent of corticosterone. Decreased corticosterone levels and increased anxiety-like behaviors, but not enhanced sensitivity to negative feedback, are characteristics observed in the LR animal phenotype, suggesting that the LR/HR model is a valid model to investigate stress differences, or more specifically; individual differences in the hypothalamic-pituitary-adrenal axis and how they may contribute to the development of PTSD-like behaviors.

Contextual fear conditioning is a behavioral paradigm that we utilized in order to investigate whether a blunted stress response had any effect on the consolidation and

extinction of fear memory. We predicted that LRs would display enhanced fear, through freezing behavior, and maintain a fear response longer than HRs, similar to the lack of extinction of fear memory observed in individuals with PTSD. In all trials, LRs and HRs displayed significant increases in freezing following acquisition, suggesting that both phenotypes learned, and that any differences in freezing behavior over time were the result of extinction processes. Our data demonstrated that LRs did not extinguish fear memory as rapidly as HRs and were more susceptible to demonstrating enhanced fear responses following a delayed fear extinction trial. Furthermore, LRs displayed significantly less plasma corticosterone following extinction than did HRs. This is important because reports show that emergency room patients who displayed lower cortisol following a traumatic experience had a higher incidence of developing PTSD than counterparts who had higher levels of cortisol following similar traumas (Resnick *et al*., 1995; McFarlane *et al*., 1997). Therefore, low levels of corticosterone during a traumatic stress could contribute to the development of PTSD. Specifically, lower corticosterone levels during stress, or recall of a traumatic experience, could result in an inability to properly activate glucocorticoid receptor-driven processes and inhibit the monoamine transporter, OCT3.

Failure to activate the glucocorticoid receptor (GR) could result in a variety of memory deficits considering the GR is expressed in a number of brain regions critical for different types of memory such as the hippocampus, amygdala, and prefrontal cortex. The hippocampus is a limbic structure involved in declarative and spatial memory (Eichenbaum *et al.*, 1999; Squire, 1992). The amygdaloid nuclei play important roles in emotional memory (LeDoux, 2000; McGaugh & Roozendaal, 2002). The prefrontal

cortex is important for short-term working memory and regulation of coping behaviors (Baddeley, 2001; Maier & Watkins, 2010). Together, dysregulation of GR, which induces important learning processes such as gene transcription (Binder, 2009; Heitzer *et al.*, 2007), could disrupt multiple types of memory and how they are maintained and processed.

Specifically, corticosterone has been demonstrated to be a potent modulator of memory consolidation (McGaugh & Roozendaal, 2002; Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2007,2008). Administration of glucocorticoids shortly after fear training has an enhancing effect on memory consolidation similar to that observed in response to norepinephrine (Pugh *et al*., 1999; Sandi *et al.*, 1997; Cordero *et al.*, 1998; Roozendaal *et al*., 1999; Roozendaal, 2000). Additionally, administration of a GR antagonist, but not an MR antagonist, immediately following a training session for a spatial memory task impairs subsequent memory challenges (Oitzl & de Kloet, 1992; Roozendaal *et al*., 1996), indicating that any glucocorticoid effects on memory consolidation are due to activation of GR and not MR. Conversely, removal of the adrenal gland, effectively inhibiting glucocorticoid synthesis, reduces memory performance and retention (Roozendaal *et al.*, 1996) as does treatment with the corticosterone synthesis inhibitor metyrapone (Roozendaal *et al.*, 1996).

Glucocorticoids have also been demonstrated to have an effect on extinction processes. Systemic administration of the glucocorticoid receptor agonists dexamethasone and intra-amygdala infusion of RU28362 prior to extinction training resulted in a facilitation of extinction of conditioned fear in a dose-dependent manner (Yang *et al*., 2006). Furthermore, administration of the corticosteroid inhibitor

metyrapone prior to extinction trials results in an inhibition of extinction to context-dependent fear responses in rats (Yang *et al*., 2006) and mice (Blundell *et al*., 2011). Importantly, administration of corticosterone immediately following extinction trials in mice that received a pre-trial injection of metyrapone demonstrated a rescue effect that allowed for extinction (Clay *et al.*, 2011). Therefore, it is possible that blunted corticosterone responses to stress could contribute to the development of extinction deficits as a result of insufficient activation of critical GR-mediated memory processes.

Additionally, insufficient corticosterone could result in a lack of available norepinephrine, which has also been demonstrated to modulate memory (Lalumiere *et al.*, 2003, van Stegeren *et al.*, 2007,2008). The enhancing effects of glucocorticoids on memory are dependent upon norepinephrine activation within the BLA (Roozendaal *et al.*, 2006). Administration of the β-adrenoceptor antagonist propranolol into the BLA blocks the corticosterone-induced enhancement of memory (Roozendaal *et al.*, 2006). Rats that received a systemic injection of corticosterone immediately following a 3-minute habituation trial to a novel environment demonstrated enhanced retention 24 hours later, but not if they had prior habituation to the context, and thus lower novelty-induced emotional arousal (Okuda *et al*., 2004; Roozendaal *et al*., 2006). This suggests that in order for corticosterone to exhibit enhancing effects, a degree of heightened emotion is necessary. Furthermore, the enhancing effect of corticosterone was blocked by systemic or intra-BLA administration of propranolol, a β-adrenoceptor antagonist. Additionally, administration of yohimbine, an α_2-adrenoceptor, in the habituated animals resulted in enhanced memory (Roozendaal *et al*., 2006). Therefore, corticosterone by itself was not sufficient to enhance memory in the pre-habituated animals, but in

combination with yohimbine-driven norepinephrine release memory was enhanced. This suggests that a component of the consolidation mechanism underlying fear memory extinction involves: A) the noradrenergic system, B) glucocorticoids, and C) the BLA. Imbalances, or dysregulation, of either of the two aforementioned neurochemical systems can have significant effects on the consolidation of extinction for fear memory.

As described previously, OCT3 is a high-capacity monoamine transporter that is inhibited by corticosterone and expressed in the brain (Iversen & Salt, 1970; Simmonds & Gillis, 1968; Grundemann *et al.*, 1998; Wu *et al*., 1998, Duan &Wang, 2010; Gasser, Lowry & Orchinik, 2006; Gasser *et al*., 2009; Engel, Zhou & Wang, 2004; Amphoux *et al*., 2006; Vialou *et al*., 2004). Furthermore, OCT3 has been expressed within the basal and lateral regions of the amygdala, areas important in fear and emotional memory (LeDoux, 2000; McGaugh & Roozendaal, 2002).Under non-stressed conditions corticosterone is not available in significant concentrations to inhibit OCT3, thereby allowing continuous clearance of norepinephrine from the extracellular space. Under stress conditions, corticosterone concentrations would rise, thus inhibiting norepinephrine clearance and allowing for a rapid elevation of norepinephrine. This could result in the aforementioned enhancing effects of norepinephrine on memory. However, in individuals with lower corticosterone levels, OCT3 would not be sufficiently inhibited, and norepinephrine clearance would continue. This could result in a lack of norepinephrine-driven memory enhancement as well as an inability for norepinephrine-dependent glucocorticoid enhancement of memory. Together, low corticosterone levels could contribute to a vulnerability to development of PTSD by preventing proper activation of

GR-dependent memory processes and inhibition of OCT3-mediated clearance of norepinephrine from the extracellular space.

Behavioral differences following exposure to stress manipulations provided some insight into how individual differences in HPA reactivity may be associated with susceptibility to developing PTSD-like other behaviors. Single prolonged stress, an animal model for traumatic stress thought to induce PTSD-like responses, did not have as strong an effect on startle behavior as expected. Following exposure to SPS, LRs demonstrated an increase in mean startle amplitude while HRs did not. However, this effect was not significant, but suggestive of a difference between groups. A larger sample size might allow for the determination of significance. Low responders, but not high responders, demonstrated higher initial startle amplitude in trials 1-5 following exposure to SPS than did non-stressed controls. Therefore, it is possible that LRs do have significant elevations in startle behavior, but are quickly habituated to the startle chamber. It would be interesting to see if the elevated startle behavior was maintained in LRs that were not previously exposed to the startle chamber in a baseline pre-SPS test.

The mechanisms underlying the differences in corticosterone concentrations remain unknown. Additionally, the mechanisms underlying the differences in extinction of fear memory remain unexplored. However, future research could examine noradrenergic differences between LRs and HRs. Individuals with PTSD display elevated twenty-four-hour norepinephrine urine excretion (Kosten *et al*., 1987; Yehuda *et al*., 1992). Furthermore, treatment with yohimbine, an α_2-adrenergic receptor antagonist, has been shown to induce panic attacks in individuals with PTSD (Southwick *et al*., 1997). These studies are highly suggestive of there being differences between individuals with

and without PTSD in the noradrenergic system, therefore it would be interesting to see if the LR / HR model could account for these differences. Imbalances in norepinephrine could affect everything from initial activation of the fight or flight response to the processing and encoding of fear in the amygdala alongside of glucococorticoids. In fact, the glucocorticoid and norepinephrine interaction in fear and emotional learning provides an entirely separate field of research.

Furthermore, inhibition of OCT3 has been suggested to play a role in the potentiation of addictive behavior (Graf *et al.* unpublished). This is important because it provides a mechanism by which glucocorticoids can not only modulate learning and memory, but also associated behaviors. Of particular note is that glucocorticoid inhibition of OCT3 has been suggested to play a role in the potentiation of drug seeking behaviors, seeing that individuals with PTSD have a high incidence of comorbidity with drug addiction (Logrip *et al*., 2012). Therefore, dysregulation in stress-induced corticosterone inhibition of OCT3 could alter norepinephrine concentrations in such a way as to exacerbate certain types of behaviors.

Glucocorticoids also have been demonstrated to have rapid non-genomic effects regarding HPA negative feedback, which could also partially account for the differences in LR and HR behaviors. Studies demonstrate that glucocorticoids bind membrane receptors on PVN neurons expressing CRF, and activate intracellular signaling cascades that cause the synthesis of endocannabinoids (Herman *et al*., 2012). Endocannabinoids are then able to inhibit glutamate release, which would reduce the overall activity of PVN neurons (Di *et al.*, 2003). Inhibition of endocannabinoid receptors inhibits negative feedback inhibition of ACTH, and subsequent glucocorticoid release (Evanson *et al*.,

2010). Additionally, administration of a membrane-impermeant glucocorticoid conjugate is equally as effective as an unconjugated steroid in inhibiting glucocorticoid release (Evanson *et al*., 2010), suggesting that the mechanisms underlying fast feedback inhibition are at, or near, the level of the cellular membrane. However, the exact mechanism remains unknown and could be the result of a number of different mechanisms.

In summary, examination of stress reactivity in the context of this dissertation highlights a small component of potential mechanisms by which an individual is more or less susceptible to developing PTSD. It would be interesting to examine whether or not LRs and HRs differentially express norepinephrine, and how that would alter fear processing. Furthermore, it would be interesting to investigate the effects of corticosterone-induced inhibition of OCT3 on behavior and physiology. Lastly, understanding what role, if any, endocannabinoids may play in determining potential risk or maintenance of PTSD-like behaviors is critical.

The data indicate that LR rats exhibit similarities to individuals with PTSD. For example, LRs have increased baseline anxiety and fear-like behaviors. Emergency room patients who displayed lower cortisol concentrations following trauma were more likely to develop PTSD (Resnick *et al*., 1995; McFarlane *et al*., 1997), suggesting that low cortisol levels contributed to a vulnerability to PTSD. Combined with decreased basal cortisol levels and enhanced sensitivity to negative feedback, it is possible that individuals with PTSD have lower cortisol levels during traumatic stress in addition to before and after. This would be similar to what is observed in LRs, who possess a blunted corticosterone response to stress.

This blunted stress response may underlie the deficits in extinction of fear memory that LRs tend exhibit. It is possible that reduced corticosterone is preventing proper memory consolidation by insufficiently activating GR-mediated gene transcription in critical memory areas and failing to inhibit OCT3-mediated clearance of norepinephrine. Furthermore, when exposed to an animal model of PTSD, animals that demonstrated a pre-existing blunted stress response seemed to be slightly more susceptible to exhibiting exaggerated startle. Together, the LR/HR model is a useful tool to investigate how individual differences in the stress response contribute to vulnerability or resilience to the development of PTSD.

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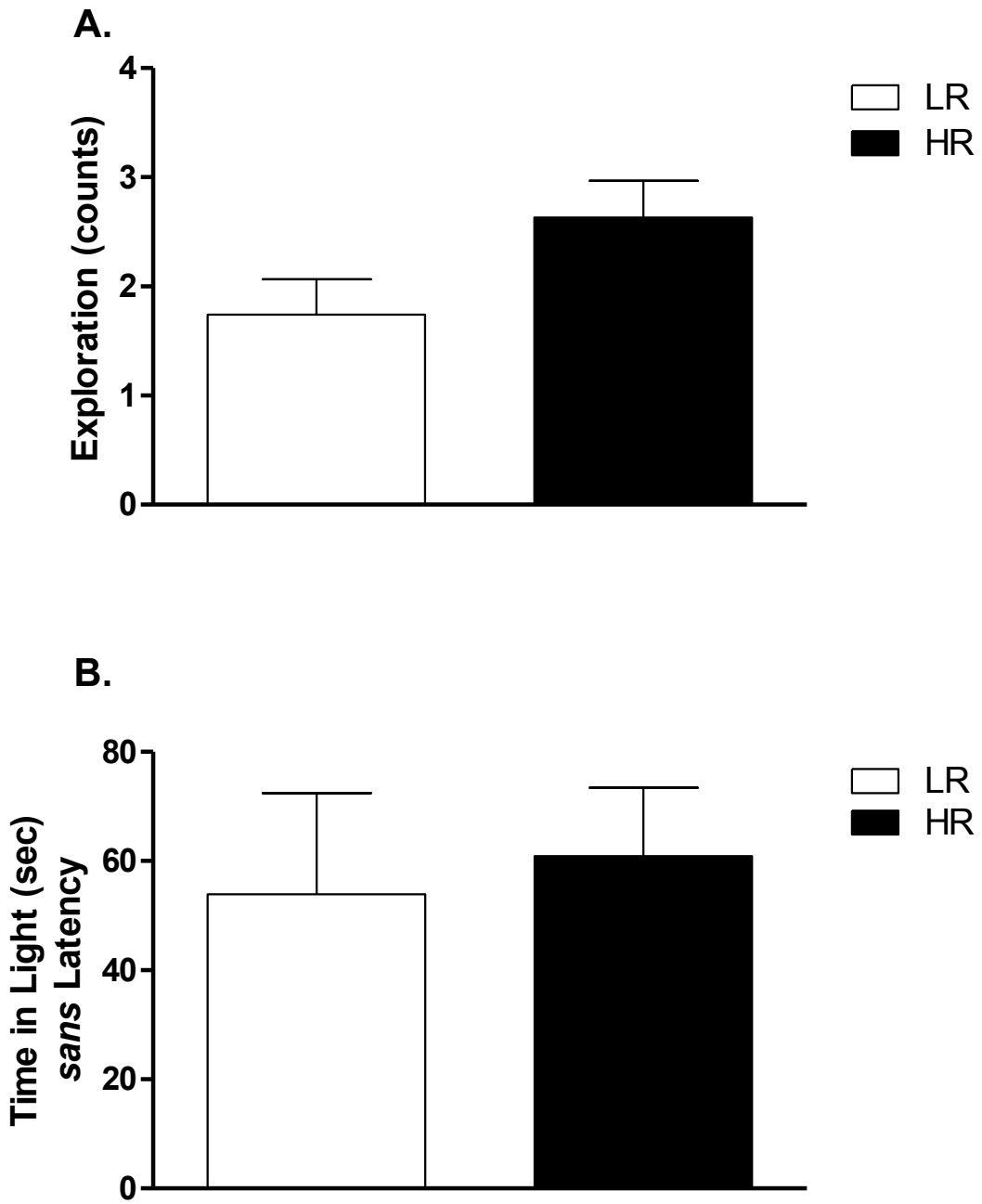
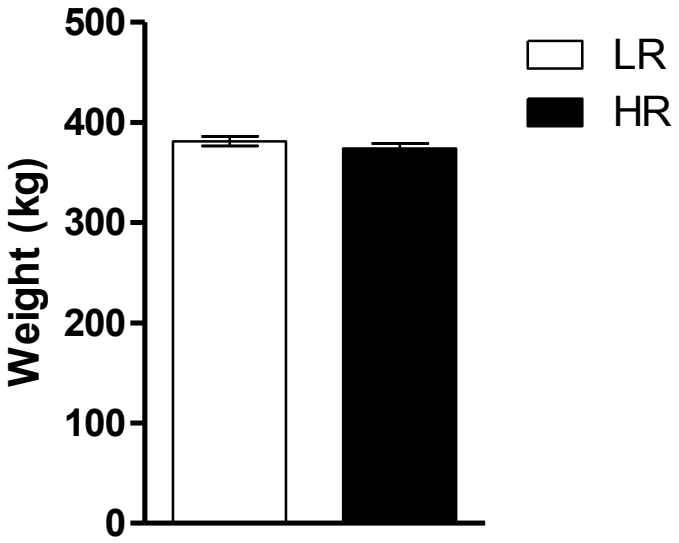
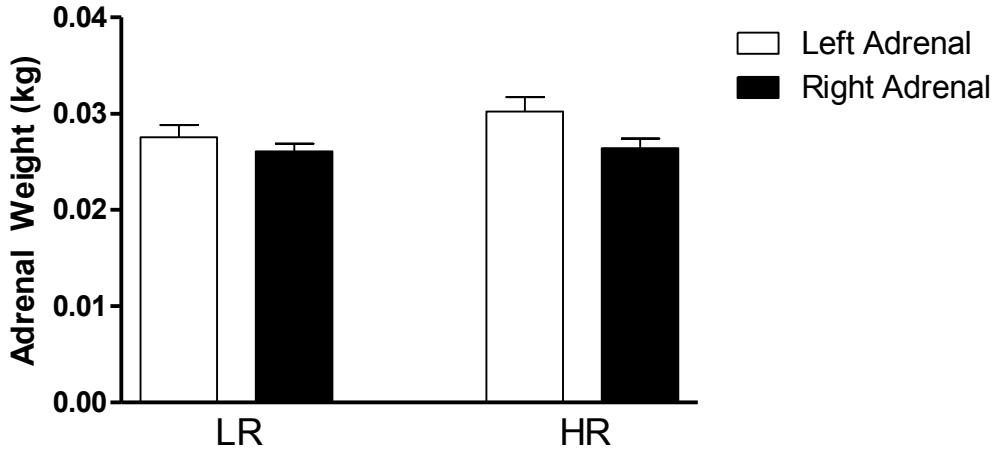


Supplemental Figure S1. Behavior of LR and HR rats in the light/dark box test. Rats were placed in the light chamber and allowed to explore for 5-minutes. Behavior was recorded for exploration (A) and time in the light chamber minus the latency to enter the dark compartment (B). All values are mean ± SEM (n=27:LR, 38:HR).



Supplemental Figure S2.Weight mean and standard error of both LR and HR rats at the time of locomotor sorting. All values are mean ± SEM (*n*= 11LR, 28HR).



Supplemental Figure S3.Weight mean and standard error of left and right adrenals of both LR and HR rats. All values are mean ± SEM (*n*= 11LR, 12HR).