Endocrine Adaptation to Repeated Psychological Stress in the Adult Male Rat Depends on Central Activation of Vasopressin V1a Receptors

by

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B.Sc., The University of Guelph, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies (Neuroscience)

The University of British Columbia (Vancouver)

July 2012

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Abstract

The aim of this thesis was to investigate the brain pathways regulating the decline or habituation of glucocorticoid responses to repeated exposure of psychological stress. In the first study, adult male rats were exposed to repeated bouts of restraint stress and tested for changes in endocrine responses and neuropeptide expression in the brain. Stress-induced elevations in glucocorticoids decreased as restraint was repeated, and this decline was associated with increased mRNA levels of the neuropeptide vasopressin in the medial amygdala and the bed nucleus of the stria terminalis. This suggested that central vasopressin signaling might be involved in coordinating glucocorticoid habituation. In a follow up experiment, adult male rats were again repeatedly restrained, however this time animals received continuous intracerebroventricular infusion of saline or an antagonist for the vasopressin V1a receptor. As expected, saline treated males showed stress-induced increases in vasopressin mRNA levels and habituation of glucocorticoid responses. Blocking activation of the vasopressin V1a receptor had no effect on basal or acute stress glucocorticoid levels. Antagonism did however attenuate habituation of glucocorticoid responses during subsequent restraint exposures. These findings indicated that habituation of endocrine stress responses depends on central vasopressin signaling. In the final set of experiments, saline and antagonist treated male rats were again repeatedly restrained, but this time brains were analyzed for changes in cellular activation using immunohistochemical detection of Fos protein. Antagonism had no effect on basal Fos expression, but blunted the habituation of repeated stress-induced Fos responses within the hypothalamus and select forebrain regions. Furthermore, a separate experiment revealed that repeated restraint induces region-specific changes in central V1a receptor binding levels which included decreases in the hippocampus, thalamus and central amygdala, but increases in the septum and bed nucleus of the stria terminalis. Overall, these studies show that habituation of glucocorticoid responses depends on central vasopressin V1a receptor signaling. The findings also suggest that stress-induced increases in V1a receptor binding within the bed nucleus and septum might be especially relevant in promoting habituation of glucocorticoid responses.
Preface

The research design, interpretation of results, and preparation of this dissertation was accomplished with the assistance and guidance of Dr. Victor Viau.

A version of chapter 2 has been published: Gray M, Bingham B, and Viau V (2010) A comparison of two repeated restraint stress paradigms on hypothalamic-pituitary-adrenal axis habituation, gonadal status and central neuropeptide expression in adult male rats. Journal of Neuroendocrinology 22:92-101. I performed all the animal work with assistance from Dr. Viau, and I completed all the data analysis with helpful guidance from Brenda Bingham. I also wrote the manuscript for this chapter that was subsequently revised by Dr. Viau.

Chapter 3 is submitted: Gray M, Innala L, Viau V (2012) Central vasopressin V1a receptor blockade impedes hypothalamic-pituitary-adrenal habituation to repeated restraint stress exposure in adult male rats. I performed all the surgeries, animal experiments and data analysis, with tremendous assistance from Leyla Innala during the testing phase. I wrote the manuscript for this chapter that was subsequently revised and edited by Dr. Victor Viau.

A version of chapter 4 is completed, but pending approval for submission: Gray M, Innala L, Myung C, Flores J, Viau V (2012) Central vasopressin V1a receptor blockade prevents glucocorticoid habituation during repeated restraint and selectively alters regional changes in cellular activity. I performed all the surgeries, experiments and data analysis with invaluable help from Leyla Innala and Clara Myung during the surgical days and animal testing. Jacques Flores assisted with the receptor binding analysis. I wrote the manuscript for this chapter that is currently under review by Dr. Victor Viau. This work was approved by the UBC animal care committee (Application Number A07-0235).
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropin hormone</td>
</tr>
<tr>
<td>ADH</td>
<td>anti-diuretic hormone</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>aBST</td>
<td>anterior bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>pBST</td>
<td>posterior bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>CA1</td>
<td>cornu ammonis 1, CA1 field of the hippocampus</td>
</tr>
<tr>
<td>CA3</td>
<td>cornu ammonis 3, CA3 field of the hippocampus</td>
</tr>
<tr>
<td>CB1</td>
<td>cannabinoid receptor 1</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRH-R1</td>
<td>corticotropin-releasing hormone receptor 1</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>dMPOA</td>
<td>dorsal, medial preoptic area</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>DVC</td>
<td>dorsal vagal complex</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotrophin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA axis</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HSD</td>
<td>honestly significant difference</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>MeA</td>
<td>medial nucleus of the amygdala</td>
</tr>
<tr>
<td>MPOA</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>MR</td>
<td>mineralcorticoid receptor</td>
</tr>
<tr>
<td>N.Acc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>N.Amb</td>
<td>nucleus ambiguus</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OVLT</td>
<td>organum vasculosum of the lamina terminalis</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>PE</td>
<td>periventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PETHal</td>
<td>thalamic periventricular nucleus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PNVdp</td>
<td>paraventricular nucleus of the hypothalamus, dorsal parvocellular</td>
</tr>
<tr>
<td>PVNmpd</td>
<td>paraventricular nucleus of the hypothalamus, medial parvocellular dorsal</td>
</tr>
<tr>
<td>PVNmpv</td>
<td>paraventricular nucleus of the hypothalamus, medial parvocellular ventral</td>
</tr>
<tr>
<td>PVNmam</td>
<td>paraventricular nucleus of the hypothalamus, posterior magnocellular</td>
</tr>
<tr>
<td>pvTHAL</td>
<td>posterior paraventricular nucleus of the thalamus</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus of the hypothalamus</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>v1SEPT</td>
<td>ventrolateral septum</td>
</tr>
<tr>
<td>V1aR</td>
<td>vasopressin 1a receptor</td>
</tr>
<tr>
<td>V1bR</td>
<td>vasopressin 1b receptor</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
</tr>
<tr>
<td>V2</td>
<td>vasopressin 2 receptor</td>
</tr>
</tbody>
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Acknowledgements

I would like to thank my advisor and committee for their much appreciated guidance and feedback over the years. I am also grateful to Dr. Elena Choleris and Dr. Mansel Griffiths for inspiring me to pursue this degree.

Many thanks to members of the Viau lab past and present for their assistance and support. Whether it was a conference, hiking trail or afternoon kayaking, it was always good company. Special thanks to Leyla who played a key role in the execution of these experiments, including some secret pilot studies that proved fruitful.

My training has benefited from many thoughtful discussions with colleagues Dr. Nicola Grissom and Dr. Seema Bhatnagar for which I am thankful. The anatomical training and mentorship I have received while acting as a teaching assistant under Dr. Claudia Krebs and Professor Betty Akesson has also been amazing. I could not have asked for two better role models.

The highlights of this degree are intertwined with competitive rowing at UBC. Thank you to Victor and Craig Pond for allowing me to do both. Racing and training with national level rowers will remain some of my best, and most fulfilling memories so far.

Special thanks to my close friends and fellow graduate students Jenny, Julien, Conan, Tiffany and Chris, who certainly understand the challenges that come with this job. We have celebrated everything from Nature papers and weddings, to Ironman races and the West Coast Trail. The many days we spent cycling or hiking up mountains were unforgettable, and your friendships have been invaluable. Lastly, thanks to my wonderful family who have always impressed me with their hardwork, dynamic personalities, and incredible personal and professional success. My partner Chris, parents Peggy and Bill, and siblings Courtney, Simon and Hilary also deserve awards for patience. This degree took longer than expected, and your humor, professional advice, and love helped every step of the way.
Chapter 1: Introduction

1.1 Introduction

By 2020 depression and anxiety disorders are expected to join heart disease as the most debilitating illnesses worldwide (Hirschfeld, 2001). Although the causes of depression and anxiety disorders remain elusive and effective treatments limited, remission of their clinical symptoms is associated with the dampening and normalization of plasma glucocorticoid hormone levels (Holsboer, 2003; Inder et al., 2001; Wolkowitz et al., 2009). This has placed pressure on scientists to widen our understanding of the systems controlling glucocorticoid secretion so that we can determine how dysregulation might contribute to the onset of mental disorders, or exacerbate their symptoms. Many mental illnesses that exhibit abnormal glucocorticoid levels also show abnormal central and/or peripheral levels of the signaling peptide vasopressin. This symptomology is found in depression (Gold et al., 1981; van Londen et al., 1997; Purba et al., 1996; Frank et al., 2000), suicide (Inder et al., 1997), schizophrenia (Goldman, 2009) and obsessive compulsive disorder (Altemus et al., 1994). However, our understanding of why or how these associated abnormalities arise is limited (Scott & Dinan, 2002). Therefore, the goal of this dissertation was to examine the basic mechanisms controlling and restricting glucocorticoid secretion, with a particular interest on the neurotransmitter vasopressin.

1.2 Endocrine stress response

1.2.1 Initiation

When ‘systemic’ or physical threats to homeostasis arise, brainstem and hypothalamic neural centers respond by initiating changes to promote survival and the maintenance of homeostasis (Li et al., 1996; Dayas et al., 2001). These challenges which are referred to as ‘stressors’ can include thermal, metabolic, osmotic, immune and hypoxic disturbances. The changes initiated by stressors are considered ‘stress responses’ and can include changes in behavioral, autonomic, and endocrine output (Mayer & Faneslow, 2003). Stress responses can be triggered in the absence of physical threats, if stressors are perceived or anticipated. This is referred to as ‘psychological’ stress, and is initiated by cognitive
centers including the hippocampus, amygdala and cortex, which process and integrate sensory input (de Kloet et al., 2011; Cullinan et al., 1995; Reyes et al., 2003). Human studies using functional magnetic resonance imaging (fMRI) have found that patients with mental disorders show activational differences in these regions when processing negative visual cues or negative words. These cues that included imagery of war and catastrophes elicit hyper-activation of the amygdala and hippocampus, but hypo-stimulation of the cortex relative to controls (Siegle et al., 2007; Root et al., 2008). These neural differences are associated with abnormal glucocorticoid elevations, which suggests that dysregulation of forebrain processing could be the basis for hyperglucocorticoid secretion in these diseases (Root et al., 2008). However, the mechanisms explaining how forebrain circuitry impinges on glucocorticoid regulating pathways is not fully understood (Herman et al., 2005).

1.2.2 Glucocorticoid secretion

Glucocorticoids mobilize glucose, which is an essential energy source for the brain and body (Strohle & Holsboer, 2003). Since physical and psychological threats place increased demands on cognitive, cardiovascular, and muscular functioning, rapid increases in glucocorticoids are a critical component of the integrated stress response. Glucocorticoid secretion is coordinated by the hypothalamic-pituitary-adrenal (HPA) axis, which receives descending input from three routes. Stimulation from stress-activated (1) autonomic brainstem regions and (2) forebrain cognitive centers, as well as (3) circadian inputs from hypothalamic nuclei. Unlike stress-provoked afferents, circadian signaling from the suprachiasmatic nucleus (SCN) provides a constant source of communication to the HPA axis. This allows glucocorticoid rhythms to synchronize with our light-cycle so basal levels are high during the day but decline prior to our sleep phase (Kalsbeek et al., 2003, 2011; Droste et al., 2009). SCN signaling is also dynamic, enabling our bodies to adjust to new time zones and seasonal changes in daylight periodicity (Herbert et al., 2006; Hoffman et al., 1993).

The net influence of circadian and stress-activated input leads to the stimulation of neuroendocrine cells in the medial parvocellular dorsal (mpd) division of the hypothalamic paraventricular nucleus (PVN) (Figure 1-1). Stimulation of these cells leads to the release of
corticotrophin-releasing hormone (CRH) from the hypophyseal portal system into the anterior pituitary. From here activation of CRH-R1 receptors stimulates the release of adrenocorticotropic hormone (ACTH) (Vale et al., 1981; Aguilera et al., 2004). In rats, 50% of the cells in the PVNmpd co-express the peptide vasopressin (Antoni, 1993). When vasopressin is released with CRH into the anterior pituitary, activation of V1b receptors (V1bR) potentiate the discharge of ACTH into circulation (Gilles et al., 1982). ACTH activation of adrenal melanocortin-2 receptors then stimulates the synthesis and release of glucocorticoids (Wikberg & Mutulis, 2008). Synthesis varies across species; cortisol is the main glucocorticoid in humans, whereas in rodents corticosterone predominates (Giannopoulos & Keichline, 1981). However, the time course of ACTH and glucocorticoid responses across humans and rodents is comparable (Checkley, 1996).

1.2.3 Glucocorticoid feedback

In the face of ‘stressors’ glucocorticoids promote efficient glucose usage by suppressing processes that are not immediately practical like reproduction, metabolism and immune function (Ziegler & Herman, 2002). This is rapidly achieved as the lipophilic properties of glucocorticoids allow them to easily penetrate tissues in the brain and periphery. Although beneficial in the short-term, sustained glucocorticoid elevations can be debilitating and lead to infertility, diabetes, autoimmune disorders, and cognitive deficits (Chrousos, 2009; Starkman et al., 2001). To avoid these effects, membrane bound and cytosolic glucocorticoid receptors (GR) and mineralcorticoid receptors (MR) are positioned throughout the HPA axis and its afferents to provide multiple sites for negative feedback (De Kloet et al., 2011). Both cortisol and corticosterone bind GRs and MRs with different affinities allowing a wide spectrum of concentrations to be detected. Under non-stress conditions, basal or low levels of glucocorticoids preferentially bind to high-affinity MRs. In this scenario MRs typically show 90% occupancy while GRs show 50% (Strohle & Holsboer, 2003). However, when stress-induced increases occur, hyperglucocorticoid levels promote full occupation of the lower-affinity GRs. This had led researchers to perceive GR signaling as an important
Figure 1-1. Central regulation of glucocorticoid hormones. Descending circadian and stress activated circuitry (psychological and physical) stimulates cells in the medial parvocellular dorsal zone (mpd) of the hypothalamic paraventricular nucleus (PVN). This leads to the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophysial portal system which extends from the median eminence. Once released into the anterior pituitary, CRH and AVP have a synergistic effect on ACTH secretion. ACTH stimulation of the adrenals then leads to the synthesis and release of glucocorticoid hormones into peripheral circulation. Independent of the pituitary, glucocorticoid secretion is also influenced by autonomic projections directing adrenal sensitivity to ACTH.
mechanism controlling stress-induced glucocorticoid secretion (de Kloet et al., 2008). It has also been postulated that this process could entail an important interplay of MR and GR signaling (Pace & Spencer, 2005).

GR expression is ubiquitous in the brain of mature adults, but most abundant in the hippocampus, septum, amygdala, cortex, and PVN (de Kloet et al., 2011). This has been confirmed across rodent and primate species with few differences noted, except that primates tend to show higher levels of GR mRNA in the neocortex (Pryce, 2008). Although GR is found in regions regulating the HPA axis, the cortex and hippocampus do not directly project to the PVN, and direct efferents from the septum and amygdala are limited. However, tract-tracing studies suggest all of these regions communicate indirectly with the HPA axis. The septum and amygdala project proximately to the PVN (Prewitt & Herman 1998; Csaki et al., 2000), whereas the hippocampus, cortex and additional septal and amygdalar fibers project to the bed nucleus. Hence the bed nucleus is generally regarded as a key relay to the HPA axis (Dong & Swanson, 2001; Vertes, 2004). Given that these forebrain sites can contribute to both the initiation and termination of stress responses, this pathway is referred to as the “limbic-HPA axis.” Impaired glucocorticoid negative feedback signaling is associated with psychiatric diseases like depression and panic disorder (Strohle & Holsboer, 2003; Raison & Miller, 2003). Therefore scientists have speculated that abnormal functioning of the limbic-HPA axis might contribute to glucocorticoid elevations observed in mental illnesses.

1.3 The Limbic-HPA axis

1.3.1 Neuroanatomy

Studies which use restraint to evoke activation of the HPA axis generally rely on the use of clear, inescapable Plexiglas ventilated tubes as stimuli. Placing animals in these confined spaces reliably activates increases in glucocorticoid secretion without posing a real physical threat, therefore restraint is generally considered a mostly psychological stressor. Studies using restraint as a psychological stress have shown that lesioning the cortex (Diorio et al., 1993; Radley et al., 2006), hippocampus (Sapolsky et al., 1984; Herman et al., 1992), septum (Dobrakovova, 1982), medial preoptic area (Viau & Meaney, 1996), or the posterior bed nucleus of the stria terminalis (BST) (Choi et al., 2007) increases stress-induced HPA
activation and glucocorticoid concentrations. Although the consequence of similar manipulations has not been studied during repeated stress conditions, these regions are thought to promote HPA axis inhibition (Figure 1-2). Conversely, lesioning the medial amygdala (Dayas et al., 1999; Dayas & Day, 2002), infralimbic cortex (Radley et al., 2006), or the anterior BST (Choi et al., 2007) decreases stress-induced glucocorticoid elevations. Based on these acute studies these regions are conversely considered to promote HPA axis stimulation. Many of these findings have been tested using local stimulation techniques in the hippocampus (Casady & Taylor, 1976; Dunn & Orr, 1984), BST (Dunn, 1987; Casada & Dafny, 1991), medial preoptic area, and the medial amygdala (Feldman, 1981). Overall, these studies have established a general model wherein the cortex (prelimbic, anterior cingulate) and hippocampus are thought to exert an inhibitory influence by recruiting the downstream GABAergic HPA relay, the posterior BST (Figure 1-2). The posterior BST is then thought to project to the PVN surround triggering HPA axis inhibition (Cullinan et al., 1993; Herman et al., 2005).

Opposite to the posterior BST, the anterior BST is regarded as providing a stimulatory influence on the HPA axis through direct connections to the PVNmpd (Dong & Swanson, 2006; Choi et al., 2007), and vicinity of the PVN (Dong & Swanson, 2004). The anterior BST is also regarded as a conduit of descending limbic influences, however its sources differ from the posterior BST and include the brainstem (Forray & Gysling, 2004), infralimbic cortex (Vertes, 2004; Takagishi & Chilba, 1991), the central and medial amygdala (Sun, 1991; Prewitt & Herman, 1998). The medial amygdala can modulate the PVN indirectly via afferents to the BST (Dong et al., 2001). The septum also modulates the PVN indirectly, but via projections to the anterior hypothalamus that follow a course that is similar to the BST-hypothalamic pathways (Risold & Swanson, 1997). As well, both the medial amygdala and septum can modulate the PVN via different projections which target a separate population of cells just ventral to the PVN (Prewitt & Herman, 1998; Csaki et al., 2000). It should therefore be noted these multi-synaptic avenues of communication have made it difficult to discern how the septum and amygdala exert their actions on the HPA axis. Although an inhibitory septal influence is supported by the work of this dissertation, the actual role of the septum on HPA axis output has yet to be convincingly shown across multiple studies (Herman et al., 2003). Exceptions to this general model also exist; one study
Figure 1-2. Diagram illustrating the inhibitory (A) and excitatory (B) circuits composing the limbic-HPA axis based on lesion and stimulation experiments. Regions include: pBST; posterior bed nucleus of the stria terminalis, PVN; hypothalamic paraventricular nucleus, aBST; anterior BST, MeA; medial amygdala. (A) shows that activation of stimulatory projections can act on inhibitory relays to the PVN to dampen HPA axis activation. (B) shows that stimulatory HPA axis influences generally involve a cascade of excitatory pathways targeting the PVN.
surprisingly found a discrete population of limited GABAergic cells in the anterior BST that represent an additional route for HPA axis inhibition on the PVN (Radley et al., 2009).

Exceptions aside, this general framework has assisted researchers in determining how forebrain nuclei differentially modulate HPA axis activity. This model also guided the experiments of this thesis as we examined the process of stress adaptation, and specifically habituation of HPA axis responses.

1.3.2 Habituation of limbic-HPA axis responses

Under normal conditions exposing adult male and female rats to a psychological stress like novel confinement, or ‘restraint’, provokes increases in PVNmpd cellular activation (Zavala et al., 2011) and robust increases in plasma ACTH and corticosterone (Lunga & Herbert., 2004; Vahl et al., 2005). These responses reliably decrease, or habituate as the stressor is subsequently reintroduced (Thompson & Spencer, 1966; Dallman, 2007). Stress responses to physiological threats do not habituate which suggests that the mechanisms coordinating habituation of stress responses are likely confined to the limbic-HPA axis (Grissom & Bhatnagar, 2009). The observation that habituation of stress responses often occurs without a change in basal HPA tone additionally suggests this process is independent of both circadian and brainstem HPA circuits (Ma et al., 1999; Bhatnagar et al., 2005; Grissom et al., 2007; Cole et al., 2000; Girotti et al., 2006). Based on these findings, the limbic-HPA axis has remained a focal point for subsequent human and rodent studies examining the process of habituation.

1.3.3 Habituation in humans and rodents

Habituation of HPA responses has been studied in both humans and rodents using a range of psychological tests. In humans, math tests, public speaking, the first week of school for five year olds, and mock parachute jumps during military training have been used to study changes in endocrine response and individual variability (Gutteling et al., 2005; Schommer et al., 2003; Deinzer et al., 1997; Kirschbaum et al., 1995). Habituation of infant cortisol responses during repeated blood sampling and inoculations have also been examined (Gunnar et al., 1989; Davis & Granger, 2009). In rodents this process has been studied using
ferret odor (Weinberg et al., 2009), cat exposure (Figueiredo et al., 2003), non-aversive banana and peppermint odor (Grisom & Bhatnagar, 2007), noise (Campeau et al., 2002; Armario et al., 1984) and restraint (Dallman, 2007). This field has also benefited from rodent paradigms using stressors that are more physical in nature such as repeated immobilization (Chen & Herbert, 1995; Ons et al., 2010; Stamp & Herbert, 1999), footshock (Li & Sawchenko, 1998) and water immersion (De Boer et al., 1990). Consistent across human and animal studies, this literature has demonstrated that endocrine stress responses habituate as psychological stressors are repeated in a predictable manner. However there are noteworthy caveats. In the rodent literature, the capacity to habituate during psychological stress appears to develop during adolescence (see for review Romeo, 2010). As well in the human literature, although age-specific effects have yet to be examined, exceptions have been observed. In a study by Gutteling et al, (2005) they found that five year olds starting the first week of school may require a duration exceeding seven days for habituation of stress responses to be detected. Interestingly, this suggests that the first week of school is an intense stressor for a five year old as habituation is generally quickly expressed during mild, but not severe stressors (Grisom & Bhatnagar, 2009). This study also raises the question as to whether humans also show age-dependent differences in their capacity to habituate to psychological stress.

1.3.4 Proposed mechanisms

Although habituation of HPA responses and negative feedback signaling appear to be complementary processes, inhibition of the HPA axis does not solely depend on glucocorticoid signaling. When glucocorticoids are abolished through adrenalectomy, male rats continue to show robust cellular activation in the PVNmpd and significant increases in ACTH levels during acute restraint stress (Melia et al., 1994; Jaferi & Bhatnagar, 2006). This suggests HPA activation is not compromised. Surprisingly, continued re-exposure to the same stimulus still produces normal declines in cellular activity (Melia et al., 1994) and ACTH responses (Jaferi & Bhatnagar, 2006). These studies illustrate that habituation does not rely on negative feedback, and indicate other central processes within the limbic-HPA axis must be involved.
Studies further investigating the neural basis for stress habituation have provided insight through the use of pharmacological and lesion techniques. Lesioning the anterior medial bed nucleus has no effect on normal glucocorticoid declines during repeated restraint (Fernandes et al., 2002), while discrete lesions of the medial amygdala merely delay the onset of habituation (Carter et al., 2004). These studies indicate that excitatory HPA afferents may have a minimal contribution to the expression of habituation. Manipulations focused on inhibitory HPA afferents however have been more promising. Lesioning the posterior paraventricular thalamus (pvTHAL) has no effect on acute stress response (Jaferi et al., 2003), but blocks habituation of endocrine responses during repeat restraint (Bhatnagar et al., 2002). Inactivation of the medial prefrontal cortex using muscimol microinfusion also blocks the expression of habituation (Weinberg et al., 2010). These studies have highlighted that the nuclei contributing to endocrine stress responses during acute and repeated exposures are different, and that the two processes may rely on different signaling pathways. This has been confirmed by antagonist studies demonstrating that inactivation of certain signaling pathways blocks habituation of HPA responses with no effect on acute endocrine response. This has been demonstrated with local injections of GR and MR antagonist into the posterior pvTHAL (Jaferi & Bhatnagar, 2006), peripheral administration of MR antagonist (Cole et al., 2000), peripheral and intra-amygdalar administration of CB₁ receptor antagonist (Hill et al., 2010), and local administration of β-adrenergic antagonist into the basolateral amygdala (Grissom & Bhatnagar, 2011). Together these studies have demonstrated that the process of habituation relies on multiple signaling pathways and the coordinated influence of different forebrain structures.

Further work by Grissom et al., (2007) has also emphasized that the process of habituation relies on constant environmental and sensory processing. Using half hour episodes of restraint, repeated daily for 8 days, they showed that subtle changes in environmental context on day 8 reversed habituated endocrine responses. This was demonstrated by pairing restraint with a banana or peppermint odor on days 1-7, then switching the background odor on day 8. This suggests that habituation of endocrine stress responses relies on constant cognitive and sensory processing. These data also suggest that a variety of limbic neurotransmitters implicated in cognitive and sensory functioning may be contributing factors. These include but are not limited to acetylcholine, dopamine, serotonin,
epinephrine, oxytocin, CRH and vasopressin (Zarrindast, 2006; Roozendaal & McGaugh, 2011). Vasopressin therefore became a key interest of this dissertation based on its ability to modulate forebrain processing. The overlap of hyperglucocorticoid and vasopressin abnormalities in mental disorders also encouraged our interests in this direction.

1.4 Vasopressin signaling

Vasopressin, also known as arginine vasopressin (AVP), is a neuropeptide signaling molecule composed of nine amino acids which is made in the brain (du Vigneaud, 1954). Vasopressin receptors are G protein-coupled and show variations in second messenger systems. V2 receptors initiate adenylate cyclase and cyclic-AMP signaling, while the V1a and V1b subtypes trigger phosphatidylinositol and calcium signaling pathways (Butlen et al., 1978; Michell et al., 1979). Vasopressin actions in the periphery are coordinated by three receptors (V1a, V1b and V2), whereas in the brain only V1a and V1b have been consistently documented (Ostrowski et al., 1994; Vaccari et al., 1998; Ostrowski et al., 2002). V1b receptor (V1bR) mRNA in the brain is found in discrete regions including the olfactory cortex and bulb, substantia nigra, motor nucleus of CNX, and in hypothalamic nuclei including the supraoptic, suprachiasmatic, and dorsomedial zone (Figure 1-3) (Vaccari et al., 1998). Although V1bR protein is prominent in the pituitary, V1bR protein levels are low in the brain, making it difficult to detect and study this receptor with autoradiography techniques (Hernando et al., 2001). On the other hand, V1a receptor (V1aR) mRNA and binding is found throughout the brain and corresponds with vasopressin immuno-reactivity (Figure 1-3) (Szot et al., 1994; Ostrowski et al., 1994, De Vries & Miller, 1998; De Vries & Panzica, 2006). Receptor binding levels have been mapped in various species including vole (Wang & Young, 1997), hamster (Delville et al., 1995; Caldwell & Albers, 2003), rat (Tribollet et al., 1990; Tribollet et al., 1999; Johnson et al., 1993), rhesus monkey (Young et al., 1999), and marmoset (Wang et al, 1997; Schorscher-Petcu et al., 2009). Although distribution and expression varies across species, V1aR consistently appears in similar forebrain nuclei including the septum, amygdala, bed nucleus, thalamus, and hypothalamus (Young et al., 1999). This suggests that neural processes mediated by V1aR could have
**Figure 1-3.** Diagrams illustrating distribution of V1a receptors (V1aR) (A), and V1b receptors (V1bR) based on mRNA and binding studies (Ostrowski et al., 1994; Philips et al., 1988; Vaccari et al., 1998). Abbreviated regions include: BST; bed nucleus of the stria terminalis; CeA; central amygdala, DMH; dorsomedial hypothalamus, LH; lateral hypothalamus, N.Acc; nucleus accumbens, PE; periventricular nucleus, SCN; suprachiasmatic nucleus, SON; supraoptic nucleus, subPVN; sub-paraventricular nucleus of the hypothalamus.
functional similarities across species, making this system ideal for studying basic mechanisms that might be clinically relevant to primates.

1.4.1 Vasopressin production and functions

Central vasopressin pathways can be categorized into five systems regulating (1) circadian, (2) autonomic, (3) endocrine, (4) limbic processing, and (5) peripheral vasopressin secretion. In order to modulate these various systems, vasopressin production and vasopressin fibers are found in different parts of the brain (Figure 1-4). Within the hypothalamus vasopressin is produced in magnocellular neurons of the supraoptic nucleus and the PVN that coordinate peripheral release from the posterior pituitary. Vasopressin in the periphery is often referred to as anti-diuretic hormone (ADH) due to its ability to stimulate renal V2 receptors, which coordinate water absorption. Conversely, vasopressin produced in parvocellular neurons of the PVN project to the hypophyseal portal system where upon its release into the anterior pituitary act on V1b receptors to potentiate ACTH release (Lolait et al., 1995; Rabadan-Diehl et al., 1995; Spiga et al., 2009a), or stimulate V1a receptors on pituitary gonadotropes (Orcel et al., 2002). Lastly in the hypothalamus, vasopressin production from the SCN coordinates circadian rhythms including basal HPA activation through the stimulation of V1a receptors in the PVN surround (Kalsbeek et al., 2002; Sage et al., 2001).

Outside the hypothalamus vasopressin is made by neurons in the posterior bed nucleus and medial amygdala with innervations extending throughout limbic and brainstem regions (Caffe et al., 1987; De Vries & Panzica, 2006). Vasopressin from these sources is thought to mediate arousal, thermal, nociperception, and forebrain cognitive processes (Ostrowski et al., 1994, Chen & Herbert, 1995; Arnauld et al., 1989). Given that all these factors are affected in stress-provoking circumstances, it is not surprising that neural vasopressin receptors are distributed throughout stress-responsive and HPA regulating circuitry.
Figure 1-4. Diagram illustrating hypothalamic (A) and limbic (B) sources of arginine-vasopressin (AVP) in bold, and their corresponding projections. Abbreviated regions include: MPOA; medial preoptic area, OVLT; organum vasculosum of the lamina terminalis, PE; hypothalamic periventricular nucleus, PEThal; thalamic periventricular nucleus, DMH; dorsomedial nucleus of the hypothalamus, subPVN; sub-paraventricular nucleus of the hypothalamus, ME; median eminence, DVC; dorsal vagal complex, N.Amb; nuclues ambiguus.

Adapted from Ring (2005), and the work of De Vries and Panzica (2006) and Kalsbeek et al., (2010).
1.4.2 Vasopressin regulation of the HPA axis

In acute stress conditions activation of V1b receptors in the pituitary increases glucocorticoid secretion. Upstream from the pituitary, however, vasopressin appears to exert an opposite and inhibitory effect, which is mediated by the V1a receptor (V1aR). Initial studies investigating the role of vasopressinergic fibers from the SCN revealed that SCN lesions increase basal HPA tone as well as HPA responses during acute novel cage exposure (Buijs et al., 1993). This finding suggested that the HPA axis is regulated by the SCN, and suggested an inhibitory influence. Additional studies that locally administered V1aR antagonist into the PVN surround (Kalsbeek et al., 2002) or the supraoptic nucleus have also documented an increase in basal glucocorticoid levels, and higher stress-induced ACTH and corticosterone responses to social and osmotic stress (Wotjak et al., 1996, 2002). These studies have confirmed that central V1aRs can inhibit basal HPA tone and stress-provoked activation. However, the contribution of V1aRs in modulating endocrine responses during chronic stress or stress adaptation has yet to be investigated.

V1aR distribution is notable in many hypothalamic nuclei including the arcuate, stigmoid, SCN, periventricular, and lateral hypothalamus (Ostrowski et al., 1994). Moderate V1aR transcripts have been observed in the parvocellular PVN (Ostrowski et al., 1994). However the presence of V1aR mRNA or binding levels has not been conclusively confirmed in the PVNmPd region, which regulates HPA axis endocrine output. Some reports have failed to detect V1aR in the PVN of rats (Phillips et al., 1988), rhesus monkey (Young et al., 1999), and marmoset (Schorscher-Petcu et al., 2009), while other studies using rats have not commented on the presence of V1aR binding in the PVN (Tribollet et al., 1990; Tribollet et al., 1999; Johnson et al., 1993). V1aRs are however heavily distributed in sensory and cognitive centers communicating directly and/or indirectly with the PVN. These include the hippocampus, septum, amygdala, thalamus, and bed nucleus (Ostrowski et al., 1994). Therefore, we hypothesized that limbic-HPA axis regulation during repeated psychological stress might depend, or be susceptible to vasopressin neuro-modulation.
1.4.3 Vasopressin changes during repeated restraint

Studies examining the consequences of repeated restraint, or the process of HPA habituation have generally given limited attention to extrahypothalamic vasopressin signaling. Most studies instead have confined their examination of CRH and vasopressin to the PVN. Some studies, but not all forms of repeated restraint have found stress-induced increases in vasopressin mRNA within the PVNmpd (Ma et al., 1997, 1999; Gomez et al., 2004; Viau & Sawchenko, 2002). The inability for this change to consistently appear in habituated rodents, and especially those exposed to mild forms of restraint, suggest this change is not necessary for the expression of habituation. However, it is likely that through downstream actions on pituitary V1b receptors, that this increase in transcript expression is adaptive by maintaining HPA axis responsiveness (Aguilera, 1994; Spiga et al., 2009a; Spiga et al., 2009b; Stewart et al., 2008a; Stewart et al., 2008b).

Studies exposing adult male rats to daily episodes of three hour (Gomez et al., 2004), or half hour (Bingham et al., 2005) repeated restraint have shown that habituation of endocrine stress responses is associated with increased limbic vasopressin expression. This was found in the form of increased levels of mRNA and vasopressin positive cell counts in the posterior BST and medial amygdala. This suggests stress adaptation during repeated restraint may involve limbic vasopressin circuits, however this has yet to be tested. Based on the prominence of V1aR within the limbic-HPA axis, and its established role in promoting HPA axis inhibition, the focus of this dissertation centered on the hypothesis that limbic vasopressin signaling, and activation of V1aRs might contribute to the habituation of HPA axis responses during psychological stress.

(see Figure 1-5 for summary of proposed model)

1.5 Summary and specific hypotheses

The goal of this thesis was to increase our knowledge of the basic mechanisms coordinating declines in glucocorticoid response during repeated stress exposures. Based on the distribution of vasopressin fibers and vasopressin receptors within stress regulating circuits, the initial starting point of this project focused on gauging vasopressin as a potential contributing factor. Following some promising preliminary findings, we next
pharmacologically blocked vasopressin signaling and successfully implicated this peptide in the process of stress habituation. Lastly, our final work has begun mapping the anatomical extent of our antagonist effects so that future work can further delineate and confirm the precise neural circuits mediating vasopressin dependent inhibition of stress activated glucocorticoid responses.

The main hypotheses presented in this dissertation include:

1.5.1 Chapter 2. Hypothesis 1

**Stress-induced vasopressin mRNA increases within limbic regions is a common feature of different repeated restraint paradigms**

This first experiment compared two forms of repeated restraint differing in frequency and duration, which are commonly used by various labs. The two paradigms used were five consecutive episodes of three hour daily restraint, and ten consecutive episodes of half hour daily restraint. Previous studies have reported increases in hypothalamic vasopressin mRNA following repeated restraint, however this observation has not been consistently reported. To the best of our knowledge even fewer studies have examined and reported vasopressin mRNA increases in extrahypothalamic sites (Gomez et al., 2004; Bingham et al., 2005). Therefore the aim of this study was to compare and contrast the neuropeptide changes caused by different types of repeated restraint in order to detect common underlying features. In addition to vasopressin, expression of CRH, and gonadotrophin releasing hormone (GnRH) was assessed in order to detect and/or rule out other neurotransmitters of possible interest.

1.5.2 Chapter 3. Hypothesis 2

**Central activation of vasopressin V1a receptors is necessary for glucocorticoid responses to decline during repeated restraint**

This study used continuous intracerebroventricular (icv) administration of vasopressin antagonist superimposed with repeated restraint exposure. Continuous pharmacological blockade was chosen to avoid the stress of daily drug injections, and 3h repeated restraint was selected because this paradigm revealed the most prominent changes in vasopressin and CRH expression following repeated restraint. In addition to assessing the effects of
antagonism on glucocorticoid responses, possible changes in neuropeptide expression were also surveyed. Upon finding a drug effect on stress-induced endocrine responses, an additional experiment examined if our antagonist manipulation might have also altered circadian HPA axis activity.

1.5.3 Chapter 4. Hypothesis 3

**Central activation of V1a receptors is necessary for normal Fos responses to decline during repeated restraint**

Using the early immediate gene Fos protein as a marker of cellular activation, this study tested if normal Fos increases induced by stress are altered by antagonism. Fos protein shows temporary stress-induced increases during restraint, which habituate in response as restraint exposures are repeated. By using this marker to examine patterns of cellular activity, the aim of this experiment was to determine which brain regions might be directly and/or indirectly affected by antagonism.

1.5.4 Chapter 4. Hypothesis 4

**Repeated restraint is associated with increases in V1a receptor expression**

This study used receptor autoradiography to measure receptor binding levels in animals that had been repeatedly restrained compared to non-stressed controls. By detecting possible changes in receptor expression it was our hope that we might be able to identify, and begin mapping possible regions and circuits coordinating vasopressin-mediated habituation. It was also our intention that this data might provide a context for, and complement our Fos study.
**Figure 1-5.** Diagram of the vasopressin pathways mediating regulation of the hypothalamic-pituitary-adrenal axis and overall secretion of ACTH and glucocorticoids. Circuits mediating circadian and stress-induced (psychological and physical) glucocorticoid output show a reliance on inhibitory V1a receptors acting on the HPA axis. Although poorly understood, circadian HPA axis tone is also thought to involve changes in adrenal sensitivity to ACTH which are also suspected to involve V1a receptor influences. In this former pathway V1a receptor activation is thought to act on brain-stem autonomic centers to dampen excitation of adrenal splanchic projections influencing adrenal sensitivity to ACTH. Conversely, pituitary V1b receptors have a stimulatory influence on ACTH release. Signaling pathways which have been shown to inhibit glucocorticoid release during psychological stress are acknowledged, including: MR/GR; mineralcorticoid and glucocorticoid negative feedback, β-AR; β-adrenergic receptor signaling, CB₁-R; cannabinoid receptor 1 activation.
Chapter 2: A comparison of two repeated restraint stress paradigms on hypothalamic-pituitary-adrenal axis habituation, gonadal status and central neuropeptide expression in adult male rats

2.1 Introduction

Daily functioning of the hypothalamic-pituitary-adrenal (HPA) axis controlling glucocorticoid secretion, cortisol in humans and corticosterone in the rat, is regulated by diurnal signals and influenced by threats to homeostasis (i.e. stress). Stress-induced elevations in circulating glucocorticoids are adaptive, at least acutely, as they coordinate the activity of a variety of systems essential for survival, including increasing glucose mobilization, cardiovascular, and cognitive functioning; while temporarily suppressing, in some cases, immune, reproductive, and digestive function. Just as critical, once the demands of homeostatic threat are met, is the efficient termination of increased HPA activity and glucocorticoid release to minimize the detrimental effects of sustained suppression of these systems (Dallman, 2003). Importantly, stress-induced elevations in adrenocorticotrophic hormone (ACTH) and corticosterone release decline over repeated exposures to the same (homo-typic) stimulus, including restraint, water immersion, and foot-shock (Barnum et al., 2007; Retana-Marquez et al., 2003). This process of HPA habituation is adaptive as it limits overall exposure to circulating glucocorticoids, but allows the system to remain fully responsive to new challenges (Bhatnager & Dallman, 1998).

Wide assortments of repeated restraint have been used to study the basis for HPA habituation in the rodent (Viau & Sawchenko, 2002; Carter et al., 2004; Zelena et al., 2004; Melia et al., 1994; Gomez et al., 2004), involving a spectrum of different combinations of duration (0.5 to 3h) and number (3 to 15 bouts) of restraint exposures. Based on the similarities by which the ACTH and corticosterone responses decline during these forms of

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repeated restraint, the process of habituation is generally inferred as equivalent between paradigms of repeated stress. This may not be entirely true, however, as longer lasting episodes of restraint demand relatively higher levels of corticosterone exposure. This has been duly noted as influencing the central nervous systems requirements for HPA habituation, including, for example, effects on stress-induced drive to the PVN motor neurons driving ACTH release, as well as on corticotrophin-releasing hormone (CRH) expression within the hypothalamic paraventricular nucleus (PVN) and extended amygdala.

Often ignored as well, is the fact that plasma testosterone concentrations also vary over the course of repeated stimulus exposure, either negatively or positively, depending on the type and duration of stressor imposed (Retana-Marquez et al., 2003). Despite the potency and regularity by which variations in testosterone inhibit the HPA axis, at least under acute stress conditions, there is a tendency to treat changes in testosterone release as a consequence of repeated stress, rather than a potential requirement for HPA habituation. Recent studies by Dallman's group, however, have made clear that dynamic age- and situation-dependent changes in testosterone secretion render the HPA axis flexible to different types of challenges, as well as facilitate the process of HPA habituation seen under repeated episodes of 3h restraint or cold exposure (Gomez et al., 2004; Gomez et al., 2002).

As emphasized in our previous study (Viau et al., 1999), corticosterone negative feedback regulation of restraint-induced ACTH release is attenuated in castrated male rats, and reinstated with testosterone replacement. Further, corticosterone regulation of basal levels of CRH and arginine vasopressin (AVP) expression in the PVN, as well as within the amygdala and bed nucleus of the stria terminalis (BST), appear to be secondary to changes in testosterone (Viau et al., 2001). As the decline in HPA activity during repeated stress is associated with alterations in CRH and AVP expression within the PVN and its afferent pathways, testosterone may very well play a role in the process of HPA axis habituation.

As a first step towards this possibility, the aim of the present study was to systematically compare how two commonly used paradigms of repeated restraint alter central and peripheral indices of adrenal and male gonadal function. The data illustrate that while the two forms of repeated restraint caused comparable declines in stress-induced ACTH and corticosterone release, as well as increases in forebrain GnRH, these were met by marked
differences in CRH and AVP, in addition to testosterone in circulation. Taken together, these findings underscore the importance of examining male gonadal status when pursuing possible mechanisms underlying individual, age-related, and stress-specific changes in HPA function.

2.2 Methods

2.2.1 Animals

Male Sprague-Dawley rats (Charles River, St. Constant, Canada) were used, weighing 200-230 g on arrival (46-49 days old). Animals were pair housed under controlled temperature (23 ± 2°C) and lighting conditions (12:12-hour light:dark cycle, lights on at 0600 hours), with food (Labdiet; Rat diet 5012) and water available ad libitum. All experimental protocols were approved by the University of British Columbia Animal Care Committee.

2.2.2 Repeated restraint

The experiments were initiated using two groups of male rats, either 53 or 58 days old on the first day of testing so that both groups of restrained animals would be of equal age on the last day of testing (63 days old). Rats were housed and restrained in separate colony rooms, adapted to handling and daily weighing at 0800h each day for 1 week prior to stress testing. One group of rats (n=6) was exposed to 10 daily episodes of 0.5h restraint stress (0830-0900h), and the other (n=6) to 5 daily episodes of 3h restraint stress (0830-1130h). On the first and last day of testing, after immediate removal from the home cage and placement into Plexiglas restrainers, blood samples were collected from a razor nick over the lateral tail vein (pre-stress, time 0). Additional samples were collected at 30 and 60 min after the onset of 0.5h restraint or collected at 30, 90, and 180 min after the onset of 3h restraint exposure. Samples of 300 ul were collected into ice-chilled, EDTA- and aprotinin-treated tubes, centrifuged at 4°C. Plasma was stored at -20°C in separate aliquots to avoid repeated freeze-thawing of individual samples. Rats were returned to their home cages at the end of testing, and left undisturbed until the next day. 24h after the last day of repeated restraint or after daily weighing and handling in control animals, all rats were anesthetized for perfusion under basal conditions at 0800h.
2.2.3 Tissue collection

In order to compare relative levels of neuropeptide expression across restraint stress and control groups, animals were anesthetized with chloral hydrate (700 mg/kg, intraperitoneal injection) and sequentially perfused via the ascending aorta with 0.9% saline and then 4% paraformaldehyde (pH 9.5) at 4°C, delivered over 5 and 20 min, respectively, at a flow rate of 20–25 ml/min. Brains were post-fixed 4 hours and cryo-protected overnight with 10% sucrose in 0.1M potassium phosphate buffered saline (KPBS) pH 7.3 prior to slicing (Leica microtome, model SM2000R). Five one-in-five series of coronal sections (30 µm) were collected and stored in antifreeze (30% ethylene glycol, 20% glycerol in 0.05 M sterile KPBS) at -20°C until processing.

2.2.4 Plasma hormone assays

Plasma levels of ACTH, corticosterone (CORT) and testosterone were measured in duplicate using RIA kits from MP Biomedicals, Inc. (Solon, OH), with [\(^{125}\)I] as the tracer. To render detection of hormones both above the minimal detection limit and within the linear part of the standard curve for each assay, the following volumes and dilutions were employed: 25µl testosterone, 5µl corticosterone diluted 1:100 (basal) and 1:400 (stress), 50µl ACTH (basal), and 25µl diluted 1:1 (stress). The intra- and interassay coefficients of variation for all assays typically ranged from 1-7 and 2-13%, respectively. The testosterone antibody (liquid phase) cross-reacts 100% with testosterone and slightly with 5-dihydrotestosterone (3.40%), 5-androstane-3β, 17β-diol (2.2%), and 11-oxotestosterone (2%), but does not cross-react with progesterone, estrogen, or glucocorticoids (all <0.01%). The standard curve ED\(_{50}\) for testosterone was 0.684 ng/ml, and the detection limit of this assay was 0.2 ng/ml. The corticosterone antibody cross-reacts 100% with corticosterone, slightly with deoxycorticosterone (0.34%), testosterone, and cortisol (0.10%), but does not cross-react with progestins or estrogens (<0.01%). The standard curve ED\(_{50}\) for corticosterone was 138.8 ng/ml, and the detection limit of the assay was 7.7 ng/ml. The ACTH antibody cross-reacts 100% with ACTH\(_{1-39}\) and ACTH\(_{1-24}\), but not with β-endorphin, β-MSH, or β-lipotropin (all <0.8%). The standard curve ED\(_{50}\) for ACTH was 89.11 pg/ml, with a detection limit of 5.7 pg/ml.
2.2.5 Hybridization histochemistry

A hybridization approach was used to determine the relative effects of the two repeated restraint paradigms on AVP, CRH, and gonadotropin releasing hormone (GnRH) mRNA using [\(^{33}\)P] UTP-labeled (Amersham Biosciences Inc., GE Healthcare, Arlington Heights, IL) antisense cRNA probes. The AVP probe was transcribed from a 230-bp cDNA fragment encoding the vasopressin-specific 3’ end of AVP, the CRH probe from a full-length (1.2-kb) cDNA encoding CRH mRNA, and the GnRH probe from a 360-bp fragment.

Techniques for riboprobe synthesis, hybridization, and the patterns of hybridization for these probes are described in greater detail elsewhere (Viau et al., 2001; Simmons et al., 1989). Based on the strength of autoradiographic signal on X-ray film (Amersham), the hybridized slides were then coated with Kodak NTB2 liquid autoradiographic emulsion and exposed at 4°C in the dark with desiccant. Exposure time to emulsion was optimized to ensure that mRNA levels detected were within the linear range of the assay and could be quantified by making relative comparisons in optical density (OD) levels; 6, 25, and 28 days for AVP mRNA in the PVN, BST, and medial amygdala, respectively; 3.5, 12, and 14 days for CRH mRNA in the PVN, central amygdala, and BST, respectively; and 8 days for GnRH mRNA through the forebrain, including within the medial septum, diagonal band of Broca, and rostral preoptic area. Using standard reference frames, average OD values were determined bilaterally on regularly spaced 150 µm intervals through each region of interest, and corrected by background subtraction. Hybridized tissue series between animals were aligned using white matter morphology illuminated under dark-field conditions and by cytoarchitectonic features provided by an adjacent series of Nissl-stained material. The highly dispersed nature by which neurons express GnRH mRNA allowed us to quantify the number of cells expressing this transcript. This was achieved by simply counting the number of nuclei that contained clusters of silver grains whose density was more than five times background.

Parceling of the rat brain followed the mapping of CRH and AVP mRNA in the PVN, BST, and amygdala, and GnRH mRNA in the forebrain as defined by the morphological features provided by thionin staining of an adjacent series of tissue. Terminology was based on the work of Swanson (2004), Swanson and Kuypers (1980), and Viau and Sawchenko (2002) to describe the PVN, Dong and Swanson (2006) and Viau et al., (2001) to describe
the anterior and posterior divisions of the BST, Swanson and colleagues (Canteras et al., 1995; Dong et al., 2001) to describe the central and medial amygdala, and of Spratt and Herbison (1997); Silverman, et al (1987); and Merchenthaler et al (1989) to describe forebrain GnRH. Light- and dark-level images were captured using a Retiga 1300 CCD digital camera (Q-imaging, Burnaby, BC), analyzed using Macintosh OS X-driven, Open Lab Image Improvision v. 3.0.9 (Quorum Technologies, Guelph, ON) and ImageJ v. 1.38 software (NIH, Bethesda, MD), exported to Adobe Photoshop (v. 10.0, San Jose, CA), where standard methods were used for final image assembly.

2.2.6 Statistics

Grouped data from the hybridization histochemical analyses were compared with a one-way ANOVA for restraint condition (basal-unstressed, 0.5h and 3h repeated restraint), followed by post hoc pairwise comparisons using Tukey’s HSD test. Hormone responses under each restraint condition were analyzed using a two-way within-subjects repeated measures ANOVA. To gauge the relative changes in testosterone and HPA output in response to 0.5h and 3h repeated restraint, integrated hormone levels were determined with the trapezoidal rule, and the data expressed over time of sampling (area under the curve/time) were analyzed using a two-way (between restraint condition, within subject) mixed design ANOVA. Data are expressed as mean ± SEM.

2.3 Results

2.3.1 Growth rate responses to 0.5h and 3h repeated restraint

Growth rates normalized as a function of daily body weight gain among control, 0.5h, and 3h stress groups and were 8.2 ± 0.6, 8.7 ± 0.4, and 5.4 ± 0.4 g/day, respectively [F (2, 15) = 14.2; P = 0.0003]. Post hoc analysis confirmed lower daily weight gains in rats exposed to 3h repeated restraint compared with control and 0.5h stress groups (P < 0.5).
2.3.2 Plasma testosterone responses to 0.5h and 3h repeated restraint

The time courses for the testosterone responses on the first and last day of the two repeated restraint paradigms are shown in Figure 2-1 (panels A and B, respectively). Within the 0.5h group, there was no significant effect of repeated restraint \([F (1, 30) = 1.2; P = 0.07]\), no significant effect of time \([F (2, 30) = 0.59; P = 0.4]\), but a significant interaction between repeated restraint and time \([F (2, 30) = 2.0; P = 0.03]\). This interaction was attributed to significantly higher plasma testosterone concentrations at 30 min on the last day compared to the first day of 0.5h restraint.

Within the 3h group, there was a significant effect of repeated restraint \([F (1, 42) = 6.5; P = 0.02]\), no significant effect of time \([F (3, 42) = 1.5; P = 0.22]\), but a significant interaction between repeated restraint and time \([F (3, 42) = 8.3; P = 0.0001]\). Part of this interaction was attributed to significantly lower plasma testosterone concentrations at 0 and 30 min on the last day compared to the first day of 3h restraint. In addition, whereas animals displayed a significant decline in testosterone at 180 min on the first day of 3h restraint, there was a trend \((P = 0.06)\) towards testosterone secretion at 90 min on the last day of 3h restraint.

Analyses of the integrated (area under the curve/time) testosterone response revealed a significant effect of group \([F (1, 10) = 18.2; P = 0.0017]\), no significant effect of repeated restraint \([F (1, 10) = 0.2; P > 0.5]\), and no significant interaction between group and repeated restraint \([F (1, 10) = 1.5; P = 0.24]\). As illustrated in Figure 2-1 (panel C), plasma testosterone concentrations remained significantly higher in the 0.5h compared to the 3h group regardless of acute or repeated restraint exposure.

2.3.3 Plasma ACTH responses to 0.5h and 3h repeated restraint

The time courses for the ACTH responses on the first and last day of the two repeated restraint paradigms are shown in Figure 2-1 (panels D and E, respectively). In the 0.5h group, there was a significant effect of repeated restraint \([F (1, 30) = 16.0; P = 0.0005]\), a significant effect of time \([F (2, 30) = 16.1; P < 0.0001]\), and a significant interaction between repeated restraint and time \([F (2, 30) = 9.0; P = 0.0011]\). This interaction was attributed to significantly lower plasma ACTH concentrations at 30 min on the last day compared to the first day of 0.5h restraint.
Within the 3h group, there was a significant effect of repeated restraint \([F (1, 42) = 53.8; P < 0.0001]\), a significant effect of time \([F (3, 42) = 22.1; P < 0.0001]\), and a significant interaction between repeated restraint and time \([F (3, 42) = 16.1; P < 0.0001]\). This interaction was attributed to significantly lower plasma ACTH concentrations at 30 and 90 min on the last day compared to the first day of 3h restraint.

Analyses of the integrated (area under the curve/time) ACTH response revealed no significant effect of group \([F (1, 10) = 0.66; P = 0.4]\), a significant effect of repeated restraint \([F (1, 10) = 0.2; P < 0.0001]\), and no significant interaction between group and repeated restraint \([F (1, 10) = 1.5; P > 0.5]\). As illustrated in Figure 2-1 (panel F), after 0.5h and 3h repeated restraint animals showed nearly equivalent declines in ACTH (66 ± 8.2 and 67 ± 5.3 %, respectively).

### 2.3.4 Plasma corticosterone responses to 0.5h and 3h repeated restraint

The time courses for the corticosterone responses on the first and last day of the two repeated restraint paradigms are shown in Figure 2-1 (panels G and H, respectively). In the 0.5h group, there was a significant effect of repeated restraint \([F (1, 30) = 5.1; P = 0.033]\), a significant effect of time \([F (2, 30) = 21.0; P < 0.0001]\), and a significant interaction between repeated restraint and time \([F (2, 30) = 4.0; P = 0.041]\). This interaction was attributed to significantly lower plasma corticosterone concentrations at 30 min on the last day compared to the first day of 0.5h restraint.

Within the 3h group, there was a significant effect of repeated restraint \([F (1, 42) = 34.1; P < 0.0001]\), a significant effect of time \([F (3, 42) = 64.4; P < 0.0001]\), and a significant interaction between repeated restraint and time \([F (3, 42) = 16.1; P = 0.0006]\). This interaction was attributed to significantly lower plasma corticosterone concentrations at 30 and 180 min on the last day compared to the first day of 3h restraint.

Analyses of the integrated (area under the curve/time) corticosterone response revealed a significant effect of group \([F (1, 10) = 7.9; P = 0.0187]\), a significant effect of repeated restraint \([F (1, 10) = 34.1; P = 0.0002]\), and no significant interaction between group and repeated restraint \([F (1, 10) = 0.7; P = 0.42]\). As illustrated in Figure 2-1 (panel I), after
0.5h and 3h repeated restraint animals showed similar declines in corticosterone (45 ± 9.5 and 35 ± 7.0 %, respectively).

To confirm that the animals assigned to each of the two stress groups were comparable in neuroendocrine capacity, grouped hormone data was assessed at 0 and 30 min of acute 0.5h and 3h restraint exposure. At 0 min, there was no significant effect of group on testosterone [F (1, 10) = 0.03; P > 0.5], ACTH [F (1, 10) = 0.05; P > 0.5], or corticosterone [F (1, 10) = 3.1; P = 0.11]. At 30 min, there was no significant effect of group on testosterone [F (1, 10) = 0.4; P > 0.5], ACTH [F (1, 10) = 1.4; P = 0.3], or corticosterone [F (1, 10) = 3.6; P = 0.7].

2.3.5 GnRH mRNA in the rostral preoptic area

As gonadotropin-releasing hormone (GnRH) cells drive the gonadal axis and respond to testosterone in circulation, GnRH mRNA levels in the region of the rostral preoptic area (POA) were assessed as a function of repeated restraint exposure. It has been suggested that the rostral POA may be partitioned into dorsal and ventral regions that give rise to overlapping, but primarily hypothalamic and hypophysiotropic (median eminence-directed) projections (Silverman et al., 1987; Merchenthaler et al., 1989). Because we were unable to distinguish the dorsal and ventral parts in routine Nissl preparations, for the purpose of quantitative analyses we arbitrarily divided the rostral POA in half, using the roof of the third ventricle as a horizontal midpoint. Between these proposed divisions, there was a significant effect of group on GnRH cell numbers in the ventral part [F (2, 15) = 5.9; P = 0.013], but not within the dorsal part [F (2, 15) = 0.1; P > 0.5] of the rostral POA. Post hoc analysis confirmed that the number of GnRH cells in the ventral part of the POA was significantly higher in animals exposed to 3h repeated restraint compared with controls (Figure 2-2), while there was a trend towards significance in the 0.5h group (P = 0.06).

2.3.6 CRH and AVP mRNA in the PVN and SON

Assessment of neuropeptide mRNA expression within the hypophysiotropic, medial parvocellular dorsal (mpd) part of the PVN, revealed no significant effect of group on CRH
[F (2, 15) = 2.7; P = 0.1], but a significant effect on AVP mRNA [F (2, 15) = 10.4; P = 0.001]. Post hoc analysis confirmed that AVP expression in the mpd was significantly higher in animals exposed to 3h repeated restraint compared with controls (Figure 2-3). There was no significant effect of group on AVP mRNA within the posterior magnocellular part of the PVN [F (2, 15) = 0.1; P > 0.5], nor within magnocellular neurons of the supraoptic nucleus [F (2, 15) = 2.5; P = 0.1].

2.3.7 CRH and AVP mRNA in the bed nucleus of the stria terminalis

Within the anterior division of the bed nucleus of the stria terminalis (BST), there was a significant effect of group on CRH mRNA within the dorsal and ventral regions of the anteromedial area [F (2, 15) = 23.5; P < 0.001], but not within the oval [F (2, 15) = 1.6; P = 0.2] and fusiform [F (2, 15) = 0.004; P > 0.5] nuclei. Post hoc analysis confirmed that CRH expression within the anteromedial area of the BST was significantly higher in animals exposed to 3h repeated restraint compared with controls (Figure 2-4, top). There was a significant effect of group on AVP mRNA within the posterior division of the BST [F (2, 15) = 5.8; P = 0.015]. Post hoc analysis confirmed that AVP expression in the posterior BST was significantly higher in animals exposed to 3h repeated restraint compared with controls (Figure 2-4, bottom). Although we did not distinguish changes in AVP expression between individual nuclei, qualitative analyses of the distribution and strength of hybridization signal for AVP and morphological features provided by adjacent thionin stained material suggested an influence of 3h restraint on AVP within the interfascicular and transverse nucleus.

2.3.8 CRH and AVP mRNA in central and medial amygdala

There was a significant effect of group on CRH mRNA in the central amygdala [F (2, 15) = 13.0; P < 0.001]. Post hoc analysis confirmed that CRH expression was significantly higher in animals exposed to 3h repeated restraint compared with controls (Figure 2-5). There was a significant effect of group on AVP mRNA in the medial amygdala [F (2, 15) = 5.8; P = 0.014]. Post hoc analysis confirmed that AVP expression was significantly higher in animals exposed to 0.5h repeated restraint compared with controls (Figure 2-5).
Figure 2-1. Mean + SEM plasma testosterone (TEST) (A, B), adrenocorticotropic hormone (ACTH) (D, E), and corticosterone (CORT) (G, H) concentrations on the first (Acute) and last day (Repeat) of 0.5 and 3h restraint. *P < 0.05 versus acute response; #P < 0.05 versus 0 min (n = 6 per group). Mean + SEM integrated (area under the curve/time) plasma hormone responses to show a main effect of repeated restraint on ACTH (F) and CORT (I), but not on testosterone (C), *P < 0.05 versus acute response.
Figure 2-2. Dark-field photomicrograph of a coronal section of the rostral preoptic area to show the distribution and relative strength of hybridization signal for gonadotrophin-releasing hormone (GnRH) (A). Scale bar = 250 μm. Mean + SEM cell count estimates of the total number of GnRH expressing neurons through proposed dorsal (B) and ventral (C) divisions of the rostral forebrain and preoptic area in unstressed control (CTL), 0.5 and 3h stress groups. *P < 0.05 versus control animals (n = 6 per group). 3v, third ventricle.
Figure 2-3. Hybridization histochemical localization of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the paraventricular nucleus of the hypothalamus (PVN). Dark-field photomicrographs of coronal sections through comparable levels of the PVN to show the distribution and relative strength of hybrization signal for CRH (A, B) and for AVP (D, E) in unstressed control (A, D) and 3h stress (B, E) groups. Scale bar = 250 μm (applies to all). Mean + SEM relative levels of CRH (C) and AVP (F) mRNA within the medial parvocellular dorsal part of the PVN (boxed region) in unstressed control (CTL), 0.5 and 3h stress groups. *P < 0.005 versus control animals (n = 6 per group). OD, optical density.
Figure 2-4. Hybridization histochemical localization of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the bed nuclei of the stria terminalis (BST). Dark-field photomicrographs of coronal sections show the distribution and relative strength of CRH hybridization within the anteromedial (A-C) area of the BST, and AVP in the posterior BST (E-G) of unstressed control (A, E), 0.5h (B, F) and 3h (C, G) stress groups. Based on Swanson (2004), structures are labelled for reference: ac, anterior commissure; am, dorsal and ventral regions of the anteromedial BST; if, interfascicular nucleus; tr, transverse nucleus of the posterior BST. Scale bar = 500 μm (A-C); 250 μm (E-G). Mean ± SEM relative levels of CRH mRNA in the anteromedial BST (D), and AVP mRNA through the posterior division (H) of the BST in unstressed control (CTL), 0.5 and 3h stress groups. *P < 0.05 versus control animals (n = 6 per group). OD, optical density.
Figure 2-5. Hybridization histochemical localization of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the central (CeA) and medial amygdala (MeA). Dark-field photomicrographs of coronal sections to show the distribution and relative strength for CRH hybridization signal in the CeA (A, B) and for AVP in the MeA (D, E) in unstressed control (A, D), 3h (B) and 0.5h (E) stress groups. Scale bar = 250 μm (applies to all). Mean ± SEM relative levels of CRH (C) and AVP (F) within the CeA and MeA, respectively, in unstressed control (CTL), 0.5 and 3h stress groups. *P < 0.05 versus control animals (n = 6 per group). OD, optical density.
2.4 Discussion

Based on the regularity by which the HPA axis shows lower responses to repeat homo-typic stress, the central requirements for HPA habituation are often assumed as equivalent or overlapping between identical stimuli that differ in duration and frequency of exposure. Independent studies have previously shown that repeated exposures to 0.5h and 3h repeated restraint cause significant declines in ACTH and corticosterone responses. However, no study to date has directly compared how the central nervous system responds to these two commonly used paradigms of repeated restraint. Differences in the duration of corticosterone exposure, higher during 3h repeated stress, could prove instrumental for determining the stress specific nature by which HPA habituation occurs. However, the potential effects of these two forms of repeated restraint on the male gonadal axis must also be considered, given the shared inhibitory characteristics by which testosterone and corticosterone operate on the HPA axis. Although our current findings are descriptive, they provide several new entry points for determining how testosterone and corticosterone may come to influence or interact on the stress-specific habituation of the HPA axis.

While stress has been shown to inhibit the male gonadal axis in general, our findings underscore how the magnitude of the testosterone response reflects the metabolic demands underlying each of the repeated restraint paradigms. Thus, decreasing or muting the anabolic effects of testosterone exposure during repeated 3h restraint may be necessary to permit the sustained catabolic actions of corticosterone, including extended gluconeogenesis, for example (Gomez & Dallman, 2001). This point is underscored by the observation that the 3h group showed lower daily body weight gains over the course of repeated restraint exposure compared to the 0.5h group.

Stress-induced elevations in corticosterone in the rat have been shown to decrease testosterone secretion as a consequence of inhibiting testosterone synthesis and release at the level of the testes, luteinizing hormone release from anterior pituitary gonadotropes, and/or GnRH synthesis and drive to the gonadal axis (Kalra et al., 1997; Rivier & Rivest, 1991; Hu et al., 2008; Chichinadze & Chichinadze, 2008). Thus, relative to the 3h group, testosterone secretion during 0.5h repeated restraint could reflect a diminution of the regulatory effects of corticosterone on the gonadal axis. As the 3h group showed a reliable increase in GnRH
expression, an underlying drive to the gonadal axis might also be common to both forms of repeated restraint. Consistent with this idea, adrenalectomized animals provided with fixed levels of corticosterone secrete successively higher levels of testosterone over the course of repeated 3h restraint exposure (Gomez et al., 2004). Furthermore, in contrast to male rats with testes, castrated male rats show a marked increased in plasma lutenizing hormone concentrations after 10 daily episodes of 0.5h restraint (Bingham et al., 2005). Thus, stimulation of the male gonadal axis could provide a mechanism for increasing or sustaining minimal levels of testosterone secretion in animals exposed to 0.5h and 3h repeated restraint, respectively.

The experimental rats were between 52-58 days old at the start and 63 days old at the end of repeated restraint exposure. Depending on the parameter chosen, adolescence in the rodent is considered to continue up to about 55 days, and that 63 days is also not quite full adulthood (Viau et al., 2005). Importantly, the inhibitory effect of testosterone on the stress-induced activation of the HPA axis emerges during pubertal development or adolescence (Gomez et al., 2004). Further, the time course, plasma concentrations, and responses of testosterone to restraint in the current study are remarkably similar to the profiles of testosterone observed in older, 77 day old rats (Romeo et al., 2004). Based on these findings, even though the animals used in the current study were not adults, we can assume that any possible influences of testosterone and/or corticosterone on stress HPA habituation were already in place. This is not to downplay the importance of age, as the activity of both the adrenal and gonadal systems change through the entire lifespan of the organism, and thus may continue to shape HPA function.

At the level of the hypophysiotropic zone of the PVN, the two forms of restraint were not distinguished by CRH, but by differences in AVP mRNA, elevated in animals exposed to 3h repeated restraint. This does not rule out a role for CRH, but suggests a greater requirement for AVP particular to this restraint condition. AVP itself is a weak ACTH secretagogue, but potentiates the stimulatory effects of CRH on ACTH release. Thus, our findings remain in keeping with the idea that an increase in AVP synthesis and release are required in animals exposed to 3h repeated restraint to sustain ACTH release in the face of relatively longer durations of elevated corticosterone exposure (Lightman, 2008; Aguilera et al., 2008).
On the basis of previous studies showing overlapping influences of adrenal and gonadal status on neuropeptide expression within limbic-related, PVN projecting nuclei, differences in corticosterone and testosterone secretion could have a bearing on the group differences in CRH and AVP within the BST and amygdala. In response to 3h repeated restraint, elevations in steady state levels of CRH mRNA occurred within defined interconnected sub-regions of the central extended amygdala, including the anteromedial nucleus of the BST. Adrenalectomy decreases CRH mRNA levels in the central amygdala (CeA), while injections of high levels of corticosterone increase CRH mRNA levels in the BST, as well as within the CeA (Makino et al., 1994). Testosterone appears to inhibit CRH expression in the CeA, at least in an intact adrenocortical system (Viau et al., 2001), and the same may hold true for CRH neurons in the anterior BST. Thus, the results suggest that the elevation in CRH mRNA within the CeA and BST unique to rats exposed to 3h repeated restraint, depends on corticosterone, but may also require a decrease in testosterone.

Basal expression of AVP mRNA in the medial amygdala (MeA) increased only after 0.5h repeated restraint, whereas AVP expression in the posterior BST increased only after 3h repeated restraint. AVP in these regions increase in response to exogenous testosterone and positively relate to variations in plasma testosterone concentrations under basal conditions (De Vries & Panzica, 2006). Adrenalectomy causes a testosterone dependent decrease in AVP expression in the MeA, but not in the BST (Viau et al., 2001), suggesting that AVP neurons in the MeA may be more sensitive to changes in testosterone than AVP neurons in the BST. Otherwise, testosterone and corticosterone may uniquely interact on AVP neurons in the MeA. Taken together with our current findings, the changes in AVP within the MeA may rely on testosterone only partially, requiring a concomitant decrease in corticosterone over the course of 0.5h repeated restraint. In line with this reasoning, AVP expression in the PVNmpd is also testosterone dependent and negatively correlates with adrenal status (Viau et al., 2001), whereas high exogenous levels of corticosterone prevent the normal increase in AVP mRNA during repeated restraint (Pinnock & Herbert, 2001). The basis for the changes in AVP expression in the posterior BST during 3h repeated restraint remains less clear, although our findings appear to suggest that this occurs independently of testosterone.
2.4.1 Perspectives and significance

A hallmark response of animals exposed to repeated homo-typic stimuli is to show facilitated HPA and behavioral responses to subsequent introduction of a novel (heterotypic) stressor (Dallman, 2003; Fernandes et al., 2002; Grissom et al., 2007). On the basis of connectivity, CRH and AVP expressing nuclei within the BST and amygdala issue projections to a vast array of forebrain and hindbrain regions implicated in subserving emotional, behavioral, autonomic, as well as adrenal and testicular neuroendocrine responses (reviewed in (Williamson et al., 2005; Herman et al., 2003; Koolhaas et al., 1998; Caldwell et al., 2008; Koob et al., 1993). Based on our current findings, therefore, we anticipate animals emerging from 0.5h and 3h repeated restraint to show marked departures in HPA and behavioral coping responses to subsequent novel stimuli (Bhatnagar et al., 2003). As variations in testosterone are important for the formation of individual differences in aggressiveness, active coping strategies, as well as HPA function (Linfoot et al., 2009), we anticipate that the 0.5h group will also show a greater reliance on testosterone for the process of repeated stress-induced facilitation to occur.

Stress-induced CRH expression within forebrain regions, including within the CeA and the BST, has been shown to occur in response to more chronic or variable types of repeated stress that require or induce sustained elevations in corticosterone release (reviewed in Jankord et al., 2008). Based on these studies, broad inferences have been made to the effect that CRH within these regions provides a tonic stimulatory drive to the PVN and HPA axis. Our current results challenge this notion, however, and appear to reflect a general stimulatory influence of stress-induced corticosterone to promote CRH expression within limbic-related regions. On this point, whether alterations in limbic CRH actually assist in the process of HPA habituation remains far from clear. Stronger lines of evidence continue to relate the inhibitory influence of the gonadal axis on HPA function in male rats, both under acute and repeated stress conditions (Gomez et al., 2004), to testosterone-dependent increases in extrahypothalamic AVP (reviewed in Williamson et al., 2005). Thus, elevations in AVP expression within the MeA and posterior BST, unique to animals exposed to 0.5h and 3h repeated restraint, respectively, might form a basis for the decline in HPA output in response to homo-typic challenges. Motivated by this hypothesis we are currently examining the
effects of central AVP receptor blockade on stress HPA habituation (Gray et al., 2009), as we predict animals during repeated restraint will show a shift towards AVP utilization.

In summary, we have shown that the expected declines in HPA output in two forms of repeated restraint were met by marked differences in CRH and AVP expression within the PVN and brain regions known to regulate ACTH release. These findings are key to broadening our perspective on stimulus-specific stress circuitry by illustrating that identical stimuli of varying duration can lead to diverging recruitment of neural pathways. We suspect that the effects of stress and differential requirements for testosterone and corticosterone to mediate HPA habituation are not limited to CRH and AVP, nor restricted to the regions described.

While we have compared the propensity of two commonly used paradigms of repeated restraint to evoke changes in neuroendocrine and neuropeptide responses, we have yet to consider the nature by which the differences observed actually depend on the frequency over and beyond the duration of restraint exposure. To illustrate this point, one could imagine a return to baseline levels for CRH in the amygdala in the 3h group if these rats were exposed to 10 days of restraint. In this context, based on the exquisite sensitivity of the adrenal and gonadal axes to different forms of restraint as realized here, the requirements for testosterone and corticosterone to regulate HPA adaptation may ultimately depend on both the duration and frequency of aversive stimulus exposure. Nonetheless, based on our current findings we now have a testable framework for exploring the relative contributions of testosterone and corticosterone both within and between the two stress paradigms of interest. The significance of such future studies and the present findings, are underscored by the fact that several types of psychiatric disorders are met by abnormal changes in androgens (Cherrier, 2009; Rubinow & Schmidt, 1996) as well as by glucocorticoids. Thus, insofar as HPA adaptation may rely on changes in testosterone secretion appropriate to the type or severity of the stressor, abnormal testosterone responses to stress may also be primary to the development of affective disorders.
Chapter 3: Central vasopressin V1a receptor blockade impedes hypothalamic-pituitary-adrenal habituation to repeated restraint stress exposure in adult male rats

3.1 Introduction

Glucocorticoids are important for survival as they regulate essential physiological responses during real (physical or predatory) and perceived (anticipated or psychological) threats to homeostasis. Glucocorticoids promote homeostasis by reallocating energy resources to support bodily functions of immediate priority such as increased glucose mobilization, cardiovascular tone and cognitive functioning, while temporarily suppressing immune, reproductive and metabolic systems. Although adaptive in the short term, the detrimental effects of sustained glucocorticoid elevations are readily observed in several affective and metabolic disorders that share common symptomology including immune, neurological and physiological disturbances (Chrousos, 2009). Thus, the effective termination or attenuation of hypothalamic-pituitary-adrenal (HPA) axis responses once a threat is removed is equally important.

Studies in humans and rats have shown that when exposure to the same psychological stimulus is repeated in a predictable manner, the magnitude of glucocorticoid responses readily decreases or habituates during successive bouts (Kirschbaum et al., 1995). This process is stimulus-specific, therefore habituation is thought to be adaptive by minimizing glucocorticoid exposure while maintaining HPA responses to new or unanticipated challenges. Several brain regions and multiple neuropeptide systems, including corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and oxytocin, have been implicated in mediating chronic stress-induced changes in behavioral, autonomic and endocrine responses (Herbert, 1993). However, relatively few studies have attempted to systematically link these neuropeptide systems to adaptive responses and normal HPA axis habituation.

AVP regulation of the HPA axis is coordinated by two G-protein coupled receptors, the V1a and V1b subtypes. V1b receptors are less prominent centrally, but are highly

2 This chapter has been submitted for peer-review.
expressed within the anterior pituitary (Vaccari et al., 1998). CRH producing neurons of the hypothalamic paraventricular nucleus (PVN) co-express AVP, which potentiates the stimulatory effect of CRH on ACTH secretion via V1b receptors in pituitary corticotropes (Aguilera et al., 2008). In contrast to V1b receptors, the V1a isoforms shows a wide spread distribution in the brain (Ostrowski et al., 1994). There is also evidence to suggest central V1a receptor activation can inhibit the HPA axis. Injections of a V1a receptor antagonist into the vicinity of the PVN and supraoptic nucleus increases basal glucocorticoid levels and acute social stress-induced glucocorticoid responses (Kalsbeek et al., 2002; Wotjak et al., 1996). Repeated restraint induces declines in HPA output which are associated with increased numbers of AVP positive cells and/or higher AVP mRNA expression within the medial amygdala (MeA) and posterior bed nucleus of the stria terminalis (BST) (Gomez et al., 2004; Gray et al., 2010). AVP circuits involving these extrahypothalamic nuclei target a variety of V1a receptor expressing cell groups, including brain regions and hypothalamic relays that are in a position to regulate HPA effector neurons of the PVN. Therefore, this raises the possibility that V1a receptors may modulate glucocorticoid responses during repeated restraint.

To test this hypothesis, we examined the effects of V1a receptor antagonism on central and peripheral indices of HPA axis activity during episodes of 3h restraint, repeated daily for 5 consecutive days. Based on the widespread distribution of vasopressin-containing projections and V1a receptors, an intracerebroventricular approach for antagonist delivery was chosen. Previous studies have shown that stress dependent differences in neuroendocrine responses are associated with changes in AVP release and/or expression within the PVN, suprachiasmatic and supraoptic hypothalamic nuclei (Kalsbeek et al., 1992; Wotjak et al., 2002). Thus, in addition to extrahypothalamic AVP, we assessed whether these nuclei may be recruited to show changes in AVP mRNA in response to repeated restraint and V1a receptor antagonism. As extrahypothalamic AVP is sensitive to variations in gonadal status, treatment and stress effects on testosterone in circulation were also explored.
3.2 Methods

3.2.1 Animals

Adult male Sprague Dawley rats (Charles River, Canada) were used, weighing 250-260 g on arrival (50 days old). Rats were pair housed under controlled temperature (23 ± 2°C) and lighting conditions (12:12 hour light:dark cycle, lights on at 0700 hours) with food and water available ad libitum. All protocols were approved by the University of British Columbia Animal Care Committee.

3.2.2 Surgery and treatment

Upon arrival rats had one week of acclimatization prior to surgery. Rats were anesthetized and implanted with Alzet osmotic (model 2004) minipumps (Cupertino, CA), designed to deliver vehicle (saline) or a V1aR antagonist d(CH2)5Tyr(Me)AVP (10 ug/day, Sigma V2255) into the right lateral ventricle using the following stereotaxic coordinates: anteroposterior, -0.40 mm from bregma; mediolateral, + 1.50 mm; dorsoventral, - 4.50 mm from skull. Sham controls followed the same surgical procedures, but without pump implantation. All animals received continuous infusion of vehicle or drug for 7 days prior to the first episode of restraint to provide appropriate surgical recovery, and to ensure adequate drug delivery prior to testing. Separate cohorts of stress-naïve animals including surgical shams, vehicle and drug treated animals were handled daily, but never restrained. Evans Blue dye (50 µl, 0.02%) was injected into indwelling cannula at the time of perfusion to assess dye penetration and cannula patency. Animals showing evidence of improper placement or blocked cannula were removed from analysis. Final endocrine analysis included sham (n=8), vehicle (n=11) and antagonist (n=14) group sizes. Subsets of animals (n=7 per treatment condition) were randomly selected for subsequent hybridization-histochemical analysis.

3.2.3 Blood sampling

Restraint was performed for 5 consecutive days for 3h each day (0900 to 1200 hours), using Plexiglass restrainers. Initial restraint exposure began when rats were 65 days old. Blood samples (300 µl) obtained from the tail vein were collected in ice-chilled tubes.
containing aprotinin and EDTA, then centrifuged at 10,000 rpm for 20 min at 4°C. Blood samples on the first and last (fifth) day of restraint were obtained from individual animals immediately following home cage removal (0 min), and at 30, 60 and 90 min from restraint onset. This sampling interval is optimal for minimizing blood-sampling load and sufficient for detecting stress-induced changes in HPA responses (Gray et al., 2010). To test for possible drug effects on circadian HPA tone, a separate set of unstressed vehicle and antagonist treated animals (n=6 per group) were sampled for 5 consecutive days, 2 hours after lights on (0900 hr, light phase) and 2 hours after lights off (2100 hr, dark phase).

3.2.4 Radioimmunoassays

Plasma hormone concentrations were measured using commercial RIA kits (MP Biomedicals, OH) with [125I] as tracer, as previously described (Gray et al., 2010). The standard curve ED50 for ACTH, corticosterone and testosterone was 83.65 pg/ml, 166.9 ng/ml, and 0.688 ng/ml, with detection limits of 7.9 pg/ml, 6.42 ng/ml, and 0.1 ng/ml, respectively. The intra- and inter-assay coefficients of variation for these assays ranged from 1-7 and 2-13%, respectively.

3.2.5 Hybridization histochemistry

To explore stress and drug interactions on neuropeptide expression, rats were anesthetized for perfusion (Gray et al., 2010) using a lethal dose of chloral hydrate (700 mg/kg), 24 hours after the last restraint exposure or after an identical number of post-surgical days in unstressed animals. Five 1-in-5 series of frozen 30 µm-thick coronal sections were collected and stored in antifreeze until processing. Adjacent series of tissue from each animal were used for in situ hybridization and morphological analysis. Tissue series between animals were aligned using white matter morphology illuminated under dark-field conditions and by cytoarchitectonic features provided by an adjacent series of Nissl-stained sections. In situ hybridization was performed using 33P-labeled antisense cRNA probes as previously described (Gray et al., 2010). The AVP probe was transcribed from a 230-bp cDNA fragment encoding the vasopressin-specific 3’ end of AVP, and the CRH probe from a full-length (1.2-kb) cDNA encoding mRNA. Based on the strength of autoradiographic signal on
X-ray film (Amersham), hybridized slides were coated with autoradiographic emulsion and exposed for: 5, 25, and 27 days for AVP mRNA in the hypothalamus, posterior BST and MeA, respectively; and 9 days for CRH mRNA in the PVN. Semi-quantitative densitometric analysis of relative levels of AVP and CRH mRNA were performed under dark-field illumination of emulsion-coated slides. Optical densities were determined bilaterally on regularly spaced 150 µm intervals through each region of interest, and corrected by background subtraction. Dark-field images were captured using a Retiga 1300 CCD digital camera (Q-imaging, BC), analyzed using Openlab Image Improvision v.3.0.9 (Quorum Technologies, ON) and ImageJ v.1.38 software (NIH, Bethesda, MD), then exported to Adobe Photoshop (v.10.0) for final figure assembly.

3.2.6 Statistics

Hybridization data was compared using two-way ANOVAs for stress (no stress, stress) and treatment (sham, vehicle, drug). Three-way ANOVAs (between treatment, within subject) were used to analyze hormone responses using restraint (acute, repeat) and time as repeated measures. To gauge treatment effects on habituation, total hormone responses (area under the curve) on the last day of restraint was calculated as a percentage of hormone responses on the first day of restraint, and compared using a one-way ANOVA for treatment. Three-way ANOVAs (between treatment, within subject) were used to analyze the diurnal rhythm of basal HPA activity using day of sampling and cycle phase (AM, PM) as repeated measures. When appropriate, post hoc comparisons were made using Newman-Keuls and Tukey-Kramer.

3.3 Results

3.3.1 Hormone responses to restraint

ACTH and corticosterone:

Significant three-way interactions were revealed between treatment, restraint and time for ACTH [F (6, 90) = 5.26; P = 0.0001] and corticosterone [F (6, 90) =6.15; P < 0.0001]. As expected, both ACTH and corticosterone showed a significant effect of restraint [F(1, 30) =
143.19, P < 0.0001], [F(1, 30) = 73.11, P < 0.0001], respectively, and a stress by time interaction [F(3, 90) = 87.98, P < 0.0001], [F(3, 90) = 18.36, P < 0.0001], respectively. ACTH and corticosterone responses did not vary as a function of treatment status during initial restraint exposure (Figure 3-1, A & B). Post hoc comparisons found stress-induced ACTH increases were significantly lower during repeated restraint compared to acute responses in all treatments (<0.0001), while corticosterone responses only habituated in sham and vehicle treated rats (<0.0001). Antagonism attenuated habituation of ACTH and corticosterone responses at 30 min (P < 0.001), (<0.001), at 60 min (P < 0.002), (<0.0001), and at 90 min (<0.0001), (P < 0.004), respectively (Figure 3-1, A & B).

**Testosterone:**

Three-way analysis revealed no significant effect of treatment [F (2, 30) = 0.52; P = 0.59], but significant interactions between treatment and restraint [F (2, 30) = 5.053; P = 0.013] and between restraint and time [F (3, 90) = 11.92; P < 0.0001] (Figure 3-1, C). These interactions were credited, in part, to a general decline in plasma testosterone concentrations between 0 and 90 min of exposure on the first day of restraint. In contrast, post hoc analysis confirmed significant increases in plasma testosterone at 60 (P < 0.018) and 90 min (P < 0.009) of exposure in sham and vehicle control groups on the last day of restraint, but not for antagonized animals (Figure 3-1, C).

**Hormone habituation:**

To determine the basis for repeated restraint and treatment interactions, total ACTH, corticosterone and testosterone responses (area under the curve) on the last day were calculated as a percentage of the hormonal responses on the first day of restraint (Figure 3-2, A & B). Significant effects of treatment were revealed for the percent change in ACTH [F (2, 30) = 18.47; P < 0.0001], corticosterone [F (2, 30) = 21.25; P < 0.0001] and testosterone [F (2, 30) = 3.61; P = 0.039] responses. Post hoc analysis confirmed greater declines in ACTH and corticosterone responses to repeated restraint exposure in vehicle and sham controls compared to antagonized animals (Figure 3-2, A & B). Total testosterone responses
remained comparable in vehicle and sham controls, whereas antagonized animals showed a relative decline in testosterone response between the first and last day of restraint (Figure 3-2, C).

3.3.2 Basal ACTH and corticosterone

To test for possible drug effects on circadian HPA tone, a separate set of unstressed vehicle and drug treated animals were sampled for 5 consecutive days during the light and dark phases of the cycle (Figure 3-3, A & B). For ACTH there was a significant effect of cycle phase \[F (1, 40) = 31.65; P = 0.002\], but no significant effect of treatment \[F (1, 10) = 0.61; P = 0.45\] and no significant interaction between treatment, day and cycle phase \[F (4, 40) = 0.15; P = 0.96\]. Corticosterone levels likewise revealed a significant effect of cycle phase \[F (1, 40) = 259.84; P < 0.0001\], no significant effect of treatment \[F (1, 10) = 0.53; P = 0.49\], and no significant interaction between treatment, day and cycle phase \[F (4, 40) = 0.45; P = 0.77\]. Post hoc analysis confirmed higher plasma levels of ACTH and corticosterone during the PM phase of the cycle, which were comparable between vehicle and V1a receptor antagonized animals (Figure 3-3, A & B).

3.3.3 CRH and AVP expression in the PVN

Densitometric analysis of relative levels of CRH mRNA in the medial dorsal parvocellular (mpd), anterior pituitary-regulating zone of the PVN (Figure 3-4, C) revealed no significant effects of treatment \[F (2, 41) = 0.003; P > 0.5\] and stress (no stress, stress) \[F (1, 41) = 0.95; P = 0.34\], and no significant interaction between treatment and stress \[F (2, 41) = 0.84; P = 0.44\]. Using adjacent sections for AVP mRNA detection in the PVNmpd region, there was no significant effect of treatment \[F (2, 41) = 0.063; P > 0.5\] and no significant interaction between treatment and stress \[F (2, 41) = 0.14; P > 0.5\]. However, there was a significant effect of stress \[F (1, 41) = 21.66; P < 0.001\], credited to significant increases in AVP mRNA across treatment groups (Figure 3-4, F). For AVP in the PVN posterior magnocellular division, there was no significant effect of stress \[F (1, 41) = 0.25; P > 0.5\] or treatment \[F (2, 41) = 0.18; P > 0.5\], and no significant interaction between treatment and stress \[F (2, 41) = 0.18; P > 0.5\].
3.3.4 AVP expression in the supraoptic and suprachiasmatic nuclei

AVP in the supraoptic nucleus showed no significant effect of stress [\( F (1, 41) = 0.06; P > 0.5 \)] or treatment [\( F (2, 41) = 0.61; P > 0.5 \)], and no significant interaction between treatment and stress [\( F (2, 41) = 0.21; P > 0.5 \)]. For the suprachiasmatic nucleus, there was no significant effect of stress [\( F (1, 41) = 0.08; P > 0.5 \)] or treatment [\( F (2, 41) = 0.67; P > 0.5 \)], and no significant interaction between treatment and stress [\( F (2, 41) = 0.004; P > 0.5 \)], consistent perhaps with the absence of treatment effects on basal HPA drive.

3.3.5 AVP expression within extrahypothalamic regions

For the posterior BST, there was no significant effect of treatment [\( F (2, 41) = 0.31; P > 0.5 \)] and no significant interaction between treatment and stress (no stress, stress) [\( F (2, 41) = 0.15; P > 0.5 \)]. However, there was a significant effect of stress [\( F (1, 41) = 63.18; P < 0.001 \)], credited to significant increases in AVP mRNA across treatments (Figure 5c). For the MeA, there was no significant interaction between treatment and stress [\( F (2, 41) = 1.80; P = 0.18 \)]. However, significant effects of stress [\( F (1, 41) = 99.02; P < 0.001 \)] and treatment [\( F (2, 41) = 8.98; P < 0.001 \)] were found. Post hoc analysis confirmed repeated restraint increased AVP mRNA in the BST and MeA across all treatments. Antagonism however enhanced this stimulatory effect of repeated restraint in the MeA (Figure 3-5, F).
Figure 3-1. Mean ± SEM ACTH (A), corticosterone (B), and testosterone (C) responses on the first (acute) and last day (repeat) of restraint exposure in surgical sham (n = 8), vehicle treated (n = 11) and V1aR antagonized (n = 14) animals. *P < 0.05 vs sham and vehicle groups on the last day of restraint. bP < 0.05 vs basal (time 0).
Figure 3-2. Total ACTH (A), corticosterone (B) and testosterone (C) responses on the last day as a percentage of the total hormone response on the first day of restraint in surgical sham (n =8), vehicle treated (n = 11) and V1aR antagonized (n =14) animals. *P < 0.05 vs sham and vehicle groups.
Figure 3-3. Mean ± SEM plasma ACTH (A) and corticosterone (B) concentrations averaged over 5 consecutive days of sampling in the AM and PM phases of the cycle in vehicle treated \((n = 6)\) and V1aR antagonized \((n = 6)\) animals. *\(P < 0.05\) vs AM.
Figure 3-4. Representative photomicrographs showing the distribution and relative strength of hybridization signal for CRH and AVP mRNA in the PVN under basal conditions in sham unstressed animals (A and D, respectively) and those exposed to repeated restraint (B and E, respectively). Repeated restraint results in a marked increase in AVP, but not CRH expression within the dorsal body of the medial parvocellular (mpd) part of the PVN (boxed region). Scale bar = 200 μm (applies to all). Mean ± SEM relative optical density (OD) levels of CRH (C) and AVP (F) mRNA in the PVNmpd as a function of treatment condition in surgical sham, vehicle treated and V1aR antagonized animals (n = 7 per group). *P < 0.05 vs unstressed counterparts.
**Figure 3-5.** Representative photomicrographs showing the distribution and relative strength of hybridization signal for AVP mRNA within the posterior BST and medial amygdala under basal conditions in sham unstressed animals (A and D, respectively) and those exposed to repeated restraint (B and E, respectively). Scale bar = 200 μm (applies to all). Mean + SEM relative optical density (OD) levels of AVP mRNA in the posterior BST (C), and medial amygdala (F), as a function of treatment condition in surgical sham, vehicle treated and V1aR antagonized animals (n =7 per group). The results show that repeated restraint increases relative levels of AVP mRNA within both extrahypothalamic regions, and that V1aR antagonism enhances this effect in the medial amygdala. *P < 0.05 vs unstressed counterparts; †P < 0.05 vs repeatedly restrained sham and vehicle treated animals.
3.4 Discussion

Our goal here was to test the hypothesis that HPA habituation of male rats requires the activation of central arginine-vasopressin (AVP) receptors. This was based on our previous findings that declines in glucocorticoid response during repeated restraint are associated with increased levels of limbic AVP mRNA in the bed nucleus (BST) and medial amygdala (MeA) (Gray et al., 2010). Central antagonism of hypothalamic V1a receptors (V1aRs) increases basal glucocorticoid levels (Kalsbeek et al., 2002), and increases glucocorticoid responses during social stress (Wotjak et al., 1996). However the influence of V1aRs on HPA activity during repeated stress conditions has yet to be tested. V1aRs are found in many HPA-regulating regions including; the hippocampus, septum, thalamus, hypothalamus and brainstem (Ostrowski et al., 1994). Given the potential that V1aRs could be acting in any one, if not all of these regions to influence glucocorticoid output, we employed intracerebroventricular antagonism.

Antagonism had no effect on basal levels of ACTH and corticosterone at the onset of restraint (0 min), or on the magnitude of HPA responses to initial stress exposure. On the last day of stress compared to the first restraint experience both surgical and vehicle control groups showed reduced ACTH and corticosterone responses. Antagonism completely blocked corticosterone responses from declining across restraint exposures, whereas habituation of ACTH responses was only attenuated. This might suggest that circulating ACTH was less sensitive to antagonism. Or conversely that repeated restraint, and/or antagonism altered autonomic pathways sub-serving adrenal sensitivity (Engeland & Arnhold, 2005; Kalsbeek et al., 2011). Otherwise, the ACTH habituation observed in the antagonist group should have been sufficient to promote habituation of corticosterone.

Although antagonism did not alter basal HPA measures, we nevertheless wondered if the effects of antagonism might be explained by changes in basal HPA tone not readily detected during the circadian trough of HPA activity. A study using a separate cohort of unstressed animals showed no effect of continuous antagonism on AM-PM phase differences for ACTH and corticosterone. Additionally, in unstressed animals there was no treatment effect on AVP expression within the hypothalamus and extrahypothalamic regions. These findings suggest that our antagonist manipulation did not have a significant effect in regions
regulating basal ACTH and corticosterone levels, including; the suprachiasmatic, medial preoptic, and dorsomedial nuclei of the hypothalamus, as well as the periventricular PVN, and paraventricular thalamus (Buijs et al., 1993; Kalsbeek et al., 2002). These data also indicate that the influence of V1aR antagonism on repeated restraint cannot be explained by effects exerted in advance of stress exposure.

Similar to HPA responses, plasma testosterone levels showed no treatment effect during acute restraint. Antagonism did however suppress plasma testosterone levels during the last restraint exposure at 60 and 90 min from stress onset. Since corticosterone inhibits GnRH and lutinizing hormone secretion (Rivest & Rivier, 1991), this may explain our observed treatment differences. Nonetheless, these findings suggest that control animals displaying HPA habituation were better equipped to maintain normal testosterone levels in the face of repeated stress.

All treatment groups showed comparable increases in AVP mRNA within the PVNmpd after repeated restraint, and no change in CRH expression. Complementary to the lack of antagonist effect on basal HPA tone, these data support the perspective that the influence of V1aRs on HPA habituation appears to be mediated by receptor populations upstream from the PVN. However, our findings do not rule out a role for the PVN, or hypothalamic sources of AVP in contributing to V1aR-mediated HPA habituation. AVP can gain access to central V1aRs by diffusion from several distant release points, including magnocellular neurons of the PVN, suprachiasmatic and supraoptic nuclei (Leng & Ludwig, 2008). While we found no evidence to suggest that magnocellular cell groups of the hypothalamus are recruited to express higher levels of AVP, these nuclei should not be discounted as representing important sources of potential influence during repeated stress exposure. As previously mentioned, the mpd is the anterior pituitary-regulating zone of the PVN, and AVP from this region potentiates ACTH secretion via V1b receptors in pituitary corticotropes (Aguilera et al., 2008). On this basis, we suspect the functional role of stress-induced AVP mRNA increases in the PVNmpd is to facilitate HPA responses via its neurohypophyseal projections, as opposed to promoting HPA habituation. The ability of AVP parvocells in the PVNmpd to release peptides through perikarya, axons and dendrites however, cannot exclude a potential role for mpd AVP to also contribute to HPA habituation.
As expected stress-induced elevations of AVP mRNA were observed in the posterior BST and MeA. Specific to the amygdala, antagonism significantly enhanced AVP induction in the MeA. AVP expression in the MeA and BST positively correlates with gonadal status (Szot & Dorsa, 1994; Viau et al, 2001), and does not appear to be directly influenced by changes in circulating corticosterone or dexamethasone (Urban et al., 1991). Given that there was no treatment effect on basal testosterone levels, we suspect that increased AVP mRNA expression within the MeA of antagonist treated animals could reflect increased activation within the MeA, and specifically that of second messenger signaling cascades which are known to increase AVP expression (i.e. adenylate cyclase, cAMP, PKA, AP2) (Iwasaki et al, 1997). The extent to which AVP containing neurons of the MeA and posterior BST are actually recruited to alter HPA activity during repeated restraint also remains unclear. Based on previous functional and connectivity studies, both of these regions may regulate PVN neuroendocrine neurons directly or indirectly through cortical, subcortical and limbic-related pathways to the PVN surround. V1aRs follow the distribution of these AVP-containing projections, most apparent within the septum, BST, central amygdala, hippocampus, and entorhinal cortex (Tribollet et al., 1988; Ostrowski et al., 1994). Lesion studies have also revealed that many of these targets containing V1aR populations have an inhibitory influence on the HPA axis, with particular attention to the hippocampus, septum and posterior BST (Herman et al., 2005; Dobrakovova et al., 1982; Choi et al., 2007). Therefore, anyone of these structures could represent viable targets mediating the endogenous effects of AVP on HPA habituation.

Corticosterone selectively increases V1aR mRNA and binding expression in the BST and septum, as found using adrenalectomy and glucocorticoid replacement (Watters et al., 1996). Given that AVP projections from the MeA innervate both these nuclei it appears corticosterone uniquely promotes AVP signaling within this circuit, and that this circuit might be particularly influential to the process of habituation. Therefore, glucocorticoid negative feedback and V1aR signaling, may represent complementary and converging processes directing HPA inhibition, which future investigations should consider.
3.4.1 Conclusions

The current results underscore a critical role for central AVP and V1aRs in the process of stress habituation. The distribution of V1aRs in the brain indicates that AVP can access a variety of response systems to coordinate metabolic, autonomic, behavioral, and neuroendocrine responses to homeostatic threat. All of these systems are debilitated to overlapping degrees in humans suffering from post-traumatic stress disorder, anxiety and depression. Furthermore, emerging evidence suggests that individual variations in central AVP release could be a predisposing factor underlying these disorders (Frank & Landgraf, 2008). Prevailing models of neuroendocrine habituation suggest a broad array of circuits and contributing factors (Hill et al., 2010; Grissom & Bhatnagar, 2011), and a variety of mechanisms by which normal declines in HPA output may be achieved (reviewed in Herbert, 1993; Grissom & Bhatnagar, 2009). These would include stimulus specific changes in inhibitory and stimulatory input to the HPA axis (Girotti et al., 2006), and alterations in glucocorticoid-mediated negative feedback efficacy (Cole et al., 2000). Changes in central AVP signaling during repeated stress could conceivably play an active role in linking several of these processes, and our findings provide several new starting points in pursuit of this possibility.
Chapter 4: Central vasopressin V1a receptor blockade prevents glucocorticoid habituation and selectively alters regional changes in cellular activity

4.1 Introduction

Public speaking and academic tests are robust stressors that can be used to study human endocrine changes in plasma glucocorticoids, as well as examine individual differences in stress coping (Gutteling et al., 2005; Schommer et al., 2003). Pharmacological and lesion studies using rodents have revealed that cognitive and limbic brain centers including the hippocampus, amygdala, cortex, and hypothalamus are responsible for coordinating both the initiation and termination of glucocorticoid responses during psychological stress. However, our understanding of the neural mechanisms mediating glucocorticoid inhibition is limited, making it difficult in clinical scenarios to treat individuals exhibiting abnormal hyperglucocorticoid levels (Wolkowitz et al., 2009).

Under normal conditions stress-induced glucocorticoid increases will decline, or habituate, in response to repeated exposures of the same psychological stimulus. Rodent studies have shown that this habituation of glucocorticoid responses during psychological stress is associated with increased vasopressin expression in the bed nucleus of the stria terminalis (BST) and medial amygdala (Gray et al., 2010; Gomez et al., 2004). The BST and amygdala heavily innervate stress modulating areas like the hippocampus, septum, brainstem and hypothalamus (De Vries & Panzica, 2006). Therefore, vasopressin fibers from these sources is well situated to modulate stress neurocircuitry.

In support of this idea, intracerebroventricular injection of vasopressin has been shown to inhibit basal corticosterone (CORT) levels (Chen & Herbert, 1995). Moreover, local infusion of antagonist targeting the predominant central vasopressin V1a receptor (V1aR) has shown that this subtype inhibits glucocorticoid output. Injections of V1aR antagonist into the vicinity of the hypothalamic paraventricular nucleus (PVN) or supraoptic nucleus increase basal adrenocorticotropic hormone (ACTH) and CORT levels (Kalsbeek et al., 2002). Similar local injections of antagonist also increase glucocorticoid elevations

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3 This chapter will be submitted for peer-review.
induced by osmotic stress and social defeat (Wotjak et al., 2002; Wotjak et al., 1996). However, the effects of V1aR activation have yet to be examined in the context of repeated psychological stress.

In addition to acting on the widely distributed V1aR, central vasopressin can also act on the V1b isoform. Although distribution of V1b receptor mRNA is markedly limited compared to V1aR, the V1b isoform is densely expressed in pituitary corticotropic cells where it facilitates ACTH release (Ostrowski et al., 1994; Vaccari et al., 1998; Volpi et al., 2004). Pituitary V1b receptors are well established as promoting glucocorticoid output (Stewart et al., 2008). Thus we suspected that upstream from the hypothalamus, that the more prominent V1aR might coordinate an opposite and inhibitory influence on activation of the hypothalamic-pituitary-adrenal (HPA) axis.

Previous work using an intracerebroventricular (icv) antagonist approach revealed that continuous icv blockade of V1aR had no effect on basal HPA output, or acute ACTH and CORT stress responses. Antagonism did, however, blunt HPA declines on the last day of repeated restraint (Gray et al., submitted). V1aRs have a wide neural distribution, thus the goal of this study was to begin mapping possible regions and receptor populations mediating these antagonist effects.

In experiment 1, we investigated the impact of icv antagonism on normal patterns of cellular activation using the immediate early gene Fos. Similar to glucocorticoid responses, Fos protein also shows temporarily stress-induced increases that habituate in response to repeated restraint (Melia et al., 1994). Here we examined potential treatment effects on normal Fos patterns to gauge which areas might be directly or indirectly influenced by V1aR antagonism. Having created a map of candidate regions, experiment 2 surveyed levels of V1aR binding following repeated restraint to test for potential stress-induced changes. Receptors are known to change in neuropsychiatric disorders (Kato & Ban, 1982). Therefore examining possible V1aR changes under normal stress conditions may also provide a useful reference for clinical investigations of post-mortem tissue and psychopathology.
4.2 Methods

4.2.1 Animals

Male Sprague-Dawley rats (Charles River, Canada) once received (28 days old) entered a three week quarantine period in our animal facility. Rats were pair housed under controlled temperature (23 ± 2°C) and lighting conditions (12:12-hour light:dark cycle, lights on at 0700 hours) with food and water available ad libitum (Labdiet; Rat diet 5012). All protocols were approved by the University of British Columbia Animal Care Committee.

4.2.2 Exp. 1: Effects of V1aR antagonism on stress-induced Fos and endocrine responses

To examine possible antagonist effects on stress-induced counts of Fos-positive cells, vehicle (n=6/group) and antagonist treated (n=8-9/group) rats were sacrificed during the first or last restraint exposure (acute vs. repeated stress), 90 min from stress onset to obtain tissue when Fos protein levels optimally peak in PVN neuroendocrine cells (Viau & Sawchenko, 2002). To control for possible treatment effects on basal Fos levels, a cohort of vehicle (n=6) and antagonist treated (n=8) rats were sacrificed under non-stressed basal conditions. An additional cohort of vehicle (n=6) and antagonist treated (n=8) rats received daily episodes of 3h restraint for 4 days then were sacrificed 24h later under basal conditions to control for possible treatment and/or stress effects on basal Fos.

4.2.3 Surgery

Rats (54-55 days old, 290-310g) were anesthetized with a combination of ketamine, xylazine and acepromazine maleate, then maintained with isoflurane once in the stereotaxic frame. Surgical prep included a local injection of carbocaine in the intended incision zone, subcutaneous metacam to facilitate post-operative recovery, and nail trimming to minimize post-operative scratching. Cannulae were implanted unilaterally into the lateral ventricle (DV -4.5mm from skull, LAT 1.5mm, AP -0.4mm) and the connecting osmotic pump (Alzet kit#2, model 2004) tucked subcutaneously above the shoulder blades. Superglue adhered the cannula to the skull, then 3 jeweler screws and dental acrylic further secured the cannula in place. One week of post-surgical recovery was allocated prior to testing.
4.2.4 Pharmacological blockade

Rats either received icv vehicle or antagonist (10ug/day, Sigma V2255, dissolved in saline). Continuous delivery was selected to avoid the stress of daily injections. The drug employed, d(CH$_2$)$_5$Tyr(Me)AVP, (Manning compound) is a potent and selective antagonist for the V1aR subtype (Kruszynski et al, 1980; Laszlo et al, 1991) and its physiological and behavioral effects have been reported to be maximal during the first seven hours when administered acutely, modest at 12h, and abolished at 24-48h (Ferris & Potegal, 1988). The possibility of blocked cannulae was tested during perfusion by injecting Evans Blue dye (50ul, 0.02%). As well during tissue slicing, animals not showing proper cannula placement as confirmed by blue ventricular staining were removed from the analysis.

4.2.5 Repeated restraint and endocrine sampling

Restraint occurred 0900-1200, and consisted of five episodes of daily 3h restraint. Blood samples were obtained during the first and last (day 5) restraint exposure. Plasma was collected from a scalpel nick over the lateral tail vein at 0, 30, 60, and 90 min from restraint onset. Samples (300ul) were collected into ice-chilled, EDTA- and aprotinin-treated tubes, centrifuged at 10,000 rpm at 4°C for 20 min, then stored at -20°C. Rats were returned to their home cages at the end of testing and left undisturbed until the next day. Non-stressed controls remained in the colony room during testing, and so as not to witness the stress conditions.

4.2.6 Plasma hormone assays

Plasma levels of ACTH and CORT were measured in duplicate using radioimmunoassay kits (MP Biomedicals) with [I$^{125}$] tracer. To ensure detection was above the minimal detection limit and within the linear part of the standard curve, samples were prepared as previously described (Gray et al., 2010). The intra- and inter-assay coefficients of variation for assays ranged from 1-7 and 2-13%, respectively. The standard curve ED$_{50}$ for ACTH and corticosterone was 89.12 pg/ml and 160.1 ng/ml, respectively. The detection limit for each was 8.1 pg/ml and 6.72 ng/ml, respectively.
4.2.7 Tissue collection for immunohistochemistry

Rats were anesthetized under basal or stress conditions with chloral hydrate (700 mg/kg, ip) and perfused with 0.9% saline then 4% paraformaldehyde (pH 9.5, 4°C), delivered over 5 and 20 min, respectively, at a flow rate of 20–25 ml/min. Brains were post-fixed 4h and cryo-protected overnight with 15% sucrose in 0.1M potassium phosphate buffered saline (KPBS, pH 7.3). Five one-in-five series of coronal sections (30 µm) were collected using a microtome and stored in antifreeze (30% ethylene glycol, 20% glycerol in 0.05 M sterile KPBS) at -20°C until processing.

4.2.8 Immunohistochemistry for Fos

Cells immunoreactive for Fos protein were localized using a primary antiserum (1:40,000, 48h incubation) raised against amino acids 4-17 of the human Fos protein (Ab-5 Cat#PC38, lot#D27025 Calbiochem). Free floating sections were prepared as previously described (Williamson & Viau, 2008).

4.2.9 Immunohistochemical analysis of Fos cell counts

Regions were defined by the morphology revealed by thionin staining of an adjacent series of tissue. Microscope images were captured using a Retiga 1300 CCD digital camera (Q-imaging) and analyzed with Openlab software (Quorum Technologies). Estimates of total cell number were generated by bilaterally counting the number of Fos-positive cells through each region of interest and averaging the unilateral counts for each section. Averages were then summed across the number of sections analyzed and multiplied by a factor of five to account for slice frequency (1 in 5 sections). Depending on the region of interest, the number of sections analyzed varied as followed: 1 section for divisions of the dorsal hippocampus and divisions of the PVN; 2 sections for divisions of the BST, dorsal medial preoptic area, suprachiasmatic nucleus; 3 sections for the ventrolateral septum; 4 sections for the dorsomedial hypothalamus, posterior paraventricular thalamus, and divisions of the amygdala.
4.2.10 **Exp. 2: Effects of repeated restraint on V1aR autoradiography**

To test if repeated restraint induces changes in V1aR binding, adult male rats (62 days old) were either exposed to 3h daily restraint for 5 days (n=6), or non-stress conditions (n=6). Restraint occurred daily between 0900-1200 (0700 lights on). Twenty-four hours following the last restraint session animals were sacrificed under basal conditions so steady state levels of receptor binding could be assessed.

4.2.11 **Receptor autoradiography**

After rapid decapitation brains were removed, frozen in -50°C isopentane, and stored at -80°C. Using a cryostat 20µm sections were collected over 5 series, mounted onto Superfrost slides (Fisher), and stored at -80°C. During processing, dried sections were fixed in 0.1% paraformaldehyde for 2 min, rinsed in 50nM Tris-HCl (pH 7.4), then incubated in a solution of 50nM Tris-HCl (pH 7.4), 10mM MgCl₂, 0.1% bovine serum albumin, 0.05% bacitracin, and 50pM ^125^I-lin-vasopressin, a V1aR antagonist (Perkin Elmer, Cat#NEX3100) for 1h. Tissue was incubated in this radioactive solution by lying slides flat, drawing a hydrophobic barrier around the perimeter (PAP pen, Abcam Cat#ab2601) then covering the sections with 400ul of solution. Non-specific binding was assessed by incubating sections with the radioactive specific ligand solution combined with 2uM of unlabelled V1aR ligand (Tocris, Cat#3377). Slides were rinsed in 4°C washing buffer, dipped in cold water, and left to dry. Slides were later placed in cassettes with BioMaxMR film (Amersham) and C-14 microscale standards (American Radiolabeled Chemicals) for 2 and 4 days. Relative optical density (OD) levels of binding were determined from films using ImageJ software (NIH), and specific binding determined by subtracting non-specific from total OD values. Relative levels of OD were then converted into activity units (uCi/g) according to the linear portion of a C-14 standard scale.

4.2.12 **Statistics**

Counts of Fos-positive cells were analyzed with a three-way ANOVA to determine the influence of treatment (vehicle vs. drug), stress (basal vs. stress) and repeated restraint exposure (acute vs. repeat). Hormone responses used a three-way within subject repeated
measures ANOVA to compare responses between treatment, restraint exposure (acute vs. repeat), and time of sampling. Receptor autoradiography data was analyzed using unpaired t-tests for each region of interest to compare the effects of repeated restraint between stressed and non-stressed groups. All data is represented as mean ± SEM, and when appropriate was followed by the Tukey-Kramer post hoc comparison test with the significance level set at P ≤ 0.5.

4.3 Results

4.3.1 Exp. 1: Effects of VlaR antagonism on stress-induced endocrine responses

The time courses for ACTH and corticosterone responses on the first and last day of repeated restraint are shown in Figure 4-1. Using a three-way repeated measures ANOVA with a within subject factor, both ACTH and corticosterone showed a main effect of restraint \([F(1,13) = 118.58, P < 0.001], [F(1,13) = 14.65, P = 0.002]\), time \([F(3,39) = 47.41, P < 0.0001], [F(3,39) = 129.08, P < 0.0001]\), and a restraint x time interaction \([F(3,39) = 34.16, P < 0.001], [F(3,39) = 24.89, P < 0.0001]\). Corticosterone additionally showed an interaction of restraint x treatment \([F(1,13) = 8.43, P = 0.012]\) and a restraint x time x treatment interaction \([F(3,39) = 3.73, P = 0.019]\). Post hoc comparisons found stress-induced ACTH and corticosterone increases were significantly lower during repeated restraint compared to acute responses, and that antagonism prevented full habituation of the 30 min ACTH response \((P = 0.015)\), as well as the corticosterone 60 min \((P = 0.028)\) and 90 min \((P = 0.008)\) response.

4.3.2 Exp. 1: Effects of VlaR antagonism on stress-induced Fos responses

Fos in the PVN:

Stress-induced changes in Fos cell counts were analyzed by three-way ANOVA comparing the effects of stress (basal vs. stress), repeated restraint (acute vs. repeat) and treatment (vehicle vs. drug) within individual brain regions (Figure 4-3, Table 4-1). A main effect of stress \([F(1, 47) = 37.36 – 288.37 range, P < 0.0001]\) and repeated restraint \([F(1, 47) = 11.26 – 61.86 range, P < 0.002 to 0.0001]\) was found in the medial parvocellular dorsal
(mpd), dorsal parvocellular (dp) and medial parvocellular ventral (mpv) compartments of the PVN (Figure 4-2). A main effect of stress was found in the PVN posterior magnocellular nucleus (pm) \[F(1, 47) = 37.36, P < 0.0001\]. A main effect of treatment was found in the mpd \[F(1, 47) = 4.51, P = 0.039\], and a stress x repeated restraint interaction was found in all four PVN regions \[F(1, 47) = 7.69 – 69.07 range, P < 0.008 to 0.0001\]. Post hoc analysis revealed this interaction was due to lower stress-induced Fos levels on the last day of restraint in all PVN compartments relative to acute stress-induced responses [mpd & mpv (P < 0.0001), dp (P < 0.002), pm (P < 0.042)]. Pairwise comparisons also revealed that the treatment effect was due to higher stress-induced Fos levels in the PVN mpd during repeated restraint in the antagonist group (P = 0.003).

**Fos in the remaining hypothalamus:**

A main effect of stress was found in the dorsomedial hypothalamus (DMH) \[F(1, 47) = 337.4, P < 0.0001\] and dorsal medial preoptic area (dMPOA) \[F(1, 55) = 211.0, P < 0.0001\] (Figure 4-3, dMPOA not shown). No effects were found in the suprachiasmatic nucleus (SCN) \[F(1, 47) = 0.19, P > 0.05\]. A main effect of repeated restraint was found in the DMH \[F(1, 47) = 6.81, P < 0.012\] and dMPOA \[F(1, 47) = 5.98, P < 0.018\]. A main effect of treatment was found in the DMH \[F(1, 47) = 3.79, P = 0.05\], and a stress x treatment interaction \[F(1, 47) = 4.52, P < 0.039\]. Post hoc tests found that antagonism blunted Fos increases during acute stress in the DMH (P = 0.011) relative to vehicle controls. Stress-induced DMH Fos increases during repeated restraint were also lower in vehicle controls (P = 0.005), relative to acute response. This habituation was blocked by antagonism (P > 0.05). Stress-induced Fos increases did not habituate in the dMPOA (P > 0.05) with no effect of treatment.

**Fos in the amygdala and bed nucleus:**

A main effect of stress was found in the MeA, central amygdala (CeA), anterior bed nucleus of the stria terminalis (aBST), and posterior BST (pBST) \[F(1, 47) = 14.83 – 92.7 range, P < 0.0001\] (Figure 4-3). A main effect of repeated restraint was found in the MeA,
aBST, and aBST \( [F(1, 47) = 5.47 - 8.46 \text{ range, } P < 0.024 \text{ to } 0.0001] \). No effect of repeated restraint was found in CeA \( [F(1, 47) = 0.6, P = 0.44] \) (Figure 4-3). An interaction of stress x repeated restraint was found in the MeA and pBST \( [F(1, 47) = 9.01 - 20.11 \text{ range, } P < 0.004 \text{ to } 0.0001] \), while a stress x treatment interaction was found in the aBST \( [F(1, 47) = 4.77, P < 0.034] \). A day x stress x treatment interaction was only found in the MeA \( [F(1, 47) = 4.27, P = 0.044] \). Post hoc tests found that relative to the acute response, MeA, aBST and pBST stress-induced Fos increases during repeated restraint were lower in vehicle controls \( (P = 0.0001, P = 0.021, P < 0.01, \text{ respectively}) \). This habituation was blocked by antagonism in the MeA and aBST \( (P > 0.005) \). Stress-induced Fos levels in the MeA during repeated restraint were also significantly higher relative to vehicle controls \( (P = 0.044) \). The effect of stress in the CeA was due to stress-induced Fos decreases, however this decrease was only significant during repeated \( (P < 0.04) \), not acute restraint.

**Fos in hippocampus:**

A main effect of stress was found in the CA1 region of the hippocampus \( [F(1, 47) = 13.94, P = 0.001] \) and the dentate gyrus, \( [F(1, 47) = 37.8, P < 0.0001] \) (Figure 4-4). Our survey of the dentate gyrus found similar effects within the lower and upper limbs, so our data represents the combined Fos expression of the two compartments. No effects were found in the CA3 region \( [F(1, 47) = 0.19 - 2.03 \text{ range, } P > 0.05] \). No effect of repeated restraint was found in the hippocampus (CA1, CA3, dentate) \( [F(1, 47) = 0.083 - 2.88 \text{ range, } P > 0.05] \). A stress x treatment interaction was found in the CA1 hippocampal region \( [F(1, 47) = 6.87, P = 0.012] \). Post hoc tests found that relative to the acute response, CA1 stress-induced Fos increases during repeated restraint did not habituate in vehicle controls \( (P > 0.05) \), and were significantly higher compared to the antagonist group during repeated restraint stress \( (P= 0.02) \). Although V1aRs are densely expressed in the dentate gyrus no effect of treatment was observed. Stress-induced Fos decreases in the dentate gyrus also did not change in response during repeated restraint.
**Fos in the septum and thalamus:**

A main effect of stress, and repeated restraint was found in the ventrolateral septum (vlSEPT) \( F(1, 47) = 130.5, P < 0.0001 \), \( F(1, 47) = 4.5, P < 0.039 \) and posterior paraventricular thalamus (pvTHAL) \( F(1, 47) = 63.8, P < 0.0001 \) \( F(1, 47) = 15.9, P < 0.0001 \) (Figure 4-3). A main effect of treatment was found in the vlSEPT \( F(1, 47) = 4.1, P < 0.049 \). An interaction of stress x repeated restraint was found in the vlSEPT \( F(1, 47) = 4.76, P < 0.034 \), and the pvTHAL \( F(1, 47) = 17.7, P < 0.0001 \). A stress x treatment interaction was also found in the vlSEPT \( F(1, 47) = 4.30, P < 0.043 \). Post hoc tests found that relative to the acute response, stress-induced Fos increases during repeated restraint in vehicle controls were lower in the posterior pvTHAL (P = 0.01), and vlSEPT (P = 0.002). Antagonism however blocked this habituation in the vlSEPT (P > 0.05). Antagonism also blunted Fos increases during acute stress in the vlSEPT (P < 0.001).

**4.3.3 Exp. 2: Effects of repeated restraint on V1aR autoradiography**

As expected \(^{125}\)I-V1aR labeling was strongly distinguished in the hippocampal dentate gyrus, CeA, anterolateral BST, thalamus, dorsal septum, vlSEPT, and SCN (Johnson et al., 1993). No group differences (stress vs. non-stressed) were found in the rostral extent of the CeA, but repeated restraint did lower binding levels in the caudal CeA (P = 0.023). Similar stress-induced decreases were found in the dentate gyrus (P = 0.017) and thalamus (P = 0.007). Conversely, repeated restraint increased receptor binding in the anterolateral BST (P = 0.039) and vlSEPT (P = 0.016). Although there was a trend for stress to increase binding levels in the dorsal septum (P = 0.079) and SCN (P = 0.071), no significant group differences were found (Figure 4-5).
Table 4-1. Mean ± SEM Fos positive cell counts during acute and repeated restraint, under basal and stress conditions for vehicle and antagonist (V1aR) groups.

<table>
<thead>
<tr>
<th>Hypothalamus</th>
<th>Acute</th>
<th>Repeat</th>
<th>Effect of antagonism on stress-induced Fos response</th>
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<tr>
<td></td>
<td>Basal</td>
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<td>PVN mpd</td>
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<tr>
<td>Vehicle</td>
<td>15 ± 3.5</td>
<td>363 ± 45.5</td>
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<td>17 ± 3.5</td>
<td>373 ± 35.7</td>
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<td>dp</td>
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<td>30 ± 2.7</td>
<td>107 ± 14.7</td>
<td>27 ± 4.2</td>
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<td>21 ± 2.6</td>
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<td>mpv</td>
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<td>42 ± 8.4</td>
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<td>pm</td>
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<td>V1aR</td>
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<td>DMH</td>
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<td>563 ± 79.1</td>
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<td>MeA</td>
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<td>3089 ± 528.6</td>
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<td>CeA</td>
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<td>217 ± 45.6</td>
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<td>aBST</td>
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<td>182 ± 27.6</td>
<td>517 ± 27.4</td>
<td>122 ± 22.1</td>
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<td>240 ± 23.1</td>
<td>432 ± 48.1</td>
<td>202 ± 40.0</td>
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<td>pBST</td>
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<tr>
<td>Vehicle</td>
<td>19 ± 2.6</td>
<td>140 ± 32.3</td>
<td>33 ± 12.2</td>
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<td>V1aR</td>
<td>33 ± 5.1</td>
<td>127 ± 27.3</td>
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<td>Hippocampus</td>
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<tr>
<td>CA1</td>
<td></td>
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<td>20 ± 5.3</td>
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<tr>
<td>V1aR</td>
<td>23 ± 8.3</td>
<td>76 ± 23.0</td>
<td>31 ± 7.3</td>
</tr>
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<td>CA3</td>
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<td></td>
<td></td>
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<td>Vehicle</td>
<td>37 ± 13.4</td>
<td>122 ± 9.4</td>
<td>43 ± 11.6</td>
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<td>75 ± 9.9</td>
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<td>Dentate</td>
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<td>Vehicle</td>
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<td>183 ± 38.2</td>
<td>235 ± 38.6</td>
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<tr>
<td>V1aR</td>
<td>299 ± 59.0</td>
<td>142 ± 33.8</td>
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<td>Thalamus</td>
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<td></td>
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<tr>
<td>pvTHAL</td>
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<tr>
<td>Vehicle</td>
<td>1234 ± 159.4</td>
<td>3033 ± 175.5</td>
<td>1178 ± 168.0</td>
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<td>vSEPT</td>
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<tr>
<td>V1aR</td>
<td>406 ± 89.6</td>
<td>3277 ± 513.5</td>
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</tr>
</tbody>
</table>

↑ = Antagonism increased normal Fos responses
↓ = Antagonism decreased normal Fos responses
--- = Antagonism had no effect on normal Fos responses
Figure 4-1. Mean ± SEM plasma (A) ACTH and (B) CORT responses within vehicle and V1aR antagonist treated rats during acute and repeated 3h restraint. *P<0.05 vs vehicle.
Figure 4-2. Mean + SEM counts of Fos-positive cells in male rats receiving continuous vehicle or V1a receptor antagonist under basal and stress conditions during acute and repeated 3h restraint. Compartments of the hypothalamic paraventricular nucleus (PVN) are shown; (A) medial parvocellular dorsal region, mpd, (B) posterior magnocellular region, pm, (C) dorsal parvocellular region, dp, and (D) the medial parvocellular ventral region, mpv. *P < 0.05 vs basal, **P < 0.05 vs acute, †P < 0.05 vs vehicle.
Figure 4-3. Mean ± SEM counts of limbic Fos-positive cells during acute and repeated restraint under basal and stress conditions. Regions include the: (A) the dorsal medial hypothalamus, DMH (B) anterior bed nucleus, aBST (C) posterior bed nucleus, pBST (D) ventrolateral septum, vlSEPT (E) medial amygdala, MeA and (F) the central amygdala (CeA). *P < 0.05 vs basal, **P < 0.05 vs acute, †P < 0.05 vs vehicle.
Figure 4-4. Mean ± SEM counts of hippocampal Fos positive cells during acute and repeated restraint under basal and stress conditions. Regions include the (A) CA1 and (B) CA3 hippocampal regions, and the (C) dentate gyrus. *P < 0.05 vs basal, †P < 0.05 vs vehicle.
Figure 4-5. Distribution of V1aR binding within coronal sections are shown from rostral (top) to caudal (bottom) to illustrate the regions surveyed in our comparison of repeatedly restrained male rats versus non-stressed controls. (A-F) Sections were processed with $[^{125}]$ labeled V1aR ligand in the absence (A-E) or presence (F) of 2uM unlabelled ligand to illustrate non-specific binding. Note the dense labeling of (A) the ventrolateral septum (vlSEPT), (B) anteriolateral bed nucleus (alBST), (C) superchiasmatic nucleus (SCN), (D) anterior thalamus (THAL), caudal central amygdala (CeA), (E) the dentate gyrus (DG), and the ventral anterior thalamus (THAL). (G & H) Mean + SEM levels of V1aR binding (uCi/g) within regions showing stress induced (G) decreases and (H) increases. N=6/group. *P< 0.05 non-stressed males.
4.4 Discussion

Here we confirm that central V1a receptors (V1aR) facilitate reductions in stress-induced hypothalamic-pituitary-adrenal (HPA) axis responses as measured by endocrine output and cellular activation. We also document that an important aspect of this process could entail stress-induced changes in V1aR binding levels, which could have functional consequences towards both dampening, or in some regions promoting vasopressin neuromodulation. Since antagonism altered HPA responses during repeated but not acute restraint, our findings suggest habituation of stress responses relies on the recruitment of V1aR signaling pathways. Our overlapping findings that antagonism blocked Fos habituation in the septum and bed nucleus of the stria terminalis (BST), paired with our observations that repeated restraint increases V1aR levels in these regions highlights the importance these regions might play in the process of habituation.

As is typically found in paradigms of repeated restraint, ACTH and CORT responses in vehicle treated controls declined across exposures. Consistent with our previous findings (Gray et al., submitted), this decline was blunted in rats receiving continuous infusion of V1aR antagonist. The occurrence of ACTH alterations demonstrates that the drug effects are mediated by the pituitary. However, the more prominent drug effect on CORT levels indicates autonomic circuits regulating CORT secretion and metabolism may also be affected (Engeland & Arnhold, 2005). Underscoring this preferential drug effect, continuous ventricular V1aR antagonism during 0.5h repeated restraint across 10 days also blunts habituation of CORT responses with minimal effect to normal ACTH responses (Gray & Viau, unpublished). Based on these findings, it appears V1aR-mediated habituation is applicable to different forms of repeated restraint, and highlights the ability of vasopressin to modulate HPA output through pituitary-dependent, and independent routes that have yet to be fully characterized (Kalsbeek et al., 2002). It should also be emphasized that antagonism did not completely block endocrine and Fos responses from showing some signs of habituation. This observation suggests that V1aR antagonism may only delay the onset of habituation, and that habituation appears to rely on a multitude of complementary processes including glucocorticoid negative feedback (Cole et al., 2000; Jaferi & Bhatnagar, 2006),
endocannabinoid signaling (Hill et al., 2010), and β-adrenergic signaling (Grissom & Bhatnagar, 2011).

4.4.1 Effects of restraint and antagonism on Fos expression

The immediate early gene Fos is widely used to study patterns of cellular activity. Although Fos by itself cannot indicate the phenotypic nature of a cell, be it inhibitory (GABAergic) or excitatory (glutamnergic), it is a reliable tool for mapping and detecting differences in activated pathways (Morgan & Curran, 1991). V1aRs are densely expressed throughout the rat brain including afferents to the hypothalamic paraventricular nucleus (PVN). Due to their wide distribution in sensory, cognitive and autonomic circuits this study focused on key limbic-HPA relays as a starting point (Herman et al., 2005). As expected stress-induced Fos increases in vehicle-treated rats declined across days in most regions, and this decrease was attenuated by antagonism in the dorsomedial hypothalamus, ventrolateral septum, medial amygdala (MeA), anterior BST, and PVNmpd. These findings suggest Fos habituation in these regions relies on V1aR activation. The dorsomedial hypothalamus (ter Horst & Luiten, 1986; Thompson et al., 1996), amygdala (Marchilhac & Siaud, 1997), anterior BST and septum (Elmquist et al., 1998) all have direct projections to the PVN. The anterior BST (Dong & Swanson, 2006), lateral septum (Elmquist et al., 1998), and MeA also densely innervate the PVN surround (Herman et al., 2004). Thus, in the absence of possible drug effects directly in the PVNmpd, attenuated Fos habituation in these PVN afferents could have contributed to the parallel lack of Fos habituation in the PVNmpd. Antagonism also blocked stress-induced Fos increases in the CA1 hippocampal region during repeated restraint. These findings complement previous lesion studies by suggesting that hippocampal activation facilitates HPA axis inhibition (Herman et al., 2005). This finding also suggests V1aR-mediate habituation may rely on hippocampal signaling.

To determine if these drug effects might be direct or indirect, acute responses were also examined. Interestingly, continuous antagonism only blunted stress-induced Fos increases in the dorsomedial hypothalamus and ventrolateral septum, suggesting drug effects in other regions were likely indirect. Both the dorsomedial hypothalamus and ventrolateral septum heavily express V1aR mRNA, so drug effects localized here are not surprising.
These findings suggest V1aR activation within the dorsomedial hypothalamus and ventrolateral septum does not contribute to acute stress responses, but may be critical for the development or expression of habituation later on.

Lastly, our Fos findings suggest that V1aR-mediated habituation might rely on the dorsomedial hypothalamus and anterior BST, two regions that send both excitatory (Boudada et al., 1997; Choi et al., 2007) and inhibitory projections to the PVNmpd (Boudada et al., 1996; Radley et al., 2009). Given the dual capacity of these regions, future studies should consider further investigating how V1aR signaling might act on these regions to modulate HPA axis activity. Also, having implicated the V1aR as a modulator of cellular activity throughout the limbic-HPA axis, continued work employing local antagonism should reveal the direct or indirect nature of the current findings.

4.4.2 Effects of repeated restraint on V1aR binding levels

Receptor autoradiography is an effective tool for visualizing and quantifying distribution of V1aRs. To our knowledge, this is the first study documenting changes in V1aR binding levels following repeated restraint exposure. However, we cannot specify if the observed changes are due to up- and/or down-regulation of receptors. Increases and/or decreases in receptor binding do not necessarily guarantee changes in receptor protein levels. Changes in receptor binding levels can also result from protein conformational changes that can increase or decrease the affinity a receptor has for its ligand.

V1aR binding was not detected in the PVN, MeA, or CA1 hippocampal region. These findings complement our Fos data by suggesting V1aR antagonism does not act directly in these regions. Failure to detect V1aR binding levels in these areas is also consistent with other rat studies (Philips et al., 1988) despite the presence of V1aR mRNA (Ostrowski et al., 1994). To the best of our knowledge, it has yet to be clarified if V1aR mRNA is specifically found in the PVNmpd. Therefore, our conservative interpretation is that the drug effects were mediated by receptor populations upstream from the PVNmpd. Consistent with other reports, detectable levels of V1aR binding were not found in the dorsomedial hypothalamus (Philips et al., 1988; Tribollet et al., 1999). This is however in contrast to other studies (Johnson et al., 1993). Our findings do not preclude the dorsomedial hypothalamus from
contributing to V1aR-mediated HPA habituation, but highlight the resolution limitations of our study and the benefits that other techniques like receptor immunohistochemistry might provide.

Repeated restraint decreased V1aR binding in the central amygdala, dentate gyrus, and thalamus, and V1aR antagonism had no effect on Fos patterns in these areas. On this basis, we suspect V1aR-mediated HPA habituation does not rely on vasopressin signaling in these nuclei. In contrast, we found an interesting convergence of Fos and binding data in the anterior BST and septum. The anterolateral region of the BST and the ventrolateral septum were the only regions showing stress-induced increases in V1aR binding levels. Since central V1aRs promote habituation of glucocorticoid responses, these nuclei could be excellent candidate targets for future studies employing local antagonism. The ability of the BST and septum to communicate with the amygdala, hippocampus, and hypothalamic nuclei also suggests that the observed drug effects in the BST and septum could be the catalyst for treatment effects observed elsewhere (Dong & Swanson, 2004; Risold & Swanson, 1997). Further, the complementary findings of our Fos and binding analysis emphasize V1aR-mediated HPA habituation may require vasopressin signaling within the BST and septum.

4.4.3 Considering the functional role of V1aR increases

Stress-induced V1aR increases in the BST and septum likely facilitate HPA inhibition by promoting activation of their inhibitory PVN projections (Herman et al, 2005; Dobrakova et al., 1982). Therefore these data suggest that stress-induced V1aR increases in the BST and septum could be localized to GABAergic cells targeting the PVN, or glutaminergic projections targeting GABAergic cells in the PVN surround. Lesion studies have shown that the posterior BST inhibits, and the anteroventral BST (including the dorsomedial and fusiform nuclei) increases HPA stress responses (Choi et al., 2007). Restraint studies however, have yet to selectively lesion the anterolateral BST region that encompasses the oval and juxtacapsular nuclei, where our observed increases in V1aR binding were found. The anterolateral BST is a key integration site for forebrain, amygdalar and visceral relays (Larriva-Sahd, 2006), therefore we suspect V1aRs in this zone assist in integrating and modulating descending input. Based on the GABAergic phenotype of cells in
this region, and their ability to directly project to the PVN, we suspect V1aRs in the anterolateral BST could have a direct inhibitory influence on endocrine output, or indirect influence via fusiform projections (Larriva-Sahd, 2006). If future work can confirm that V1aR populations in the BST and septum contribute to habituation it would be intriguing to test whether this process is glucocorticoid dependent. Glucocorticoids upregulate V1aR binding and mRNA levels in the BST and dorsolateral septum (Watters et al., 1996). Thus, our observed binding increases might be a product of CORT elevations experienced during repeated restraint. This relationship also suggests the contributions of V1aR signaling during habituation and glucocorticoid negative feedback could be converging mechanisms directing HPA declines.

4.4.4 Mechanism considerations

The basic mechanisms coordinating HPA habituation are thought to include changes in excitatory and/or inhibitory input to the HPA axis (Girotti et al., 2006). Habituation is associated with widespread decrements in stress-induced cellular activation, suggesting this process is mediated by decreased activation of the HPA axis. However, the fact that antagonists (Cole et al., 2000; Hill et al., 2010; Grissom & Bhatnagar, 2011) and contextual environmental changes can prevent or reverse habituated responses (Grissom et al., 2007), underscores a complex mechanism involving constant environmental processing and active inhibition.

4.4.5 Sensory processing and V1aR

Grissom et al., (2007) have shown that glucocorticoid habituation is blocked when familiar background odors are replaced with novel odors such as banana or peppermint. A vast amount of literature has also established that social recognition, learning and memory tasks, and general sensory processing depends on V1aR (Caldwell et al., 2008; Donaldson & Young, 2008; Choleris et al., 2009). While the neuroanatomical specificity of these effects is not fully understood, studies have isolated the hippocampus and septum as key regions mediating vasopressin enhancement of memory consolidation and retrieval (see Paban et al., 2003 for review). The olfactory bulb and septum are also implicated as key sites contributing
to V1aR-mediated social recognition (Tobin et al., 2010; Bielsky et al., 2005). Since V1aR antagonism blunts stress-induced Fos increases in the CA1 hippocampal region and ventrolateral septum, it is possible that the contribution of these regions, and the influence of V1aRs during habituation, correspond with a learning and memory component. Receptor increases in the BST may also contribute to this influence as the BST is regarded as a conduit of limbic information integrating both cognitive and sensory input targeting the hypothalamus (Herman et al., 2005). This proposed cognitive mechanism, which is independent of CORT feedback (Cole et al., 2000), would also account for reports that adrenalectomized rats show both ACTH and Fos habituation during repeated restraint (Melia et al., 1994; Jaferi & Bhatnagar, 2006).

4.4.6 Conclusions

Here we demonstrate that habituation of stress-induced glucocorticoid and Fos responses during repeated restraint depend on V1aR signaling. Interestingly, many psychiatric disorders display abnormal levels of central and/or peripheral vasopressin associated with hyperglucocorticoid secretion, suggesting that the current findings may be applicable to clinical states encompassing depression, obsessive compulsion (Egashira et al., 2009), schizophrenia (Goldman 2009), and cognitive disorders (Gold et al., 1981). With this perspective, future work may reveal in humans as well, that limbic V1aR activation plays a pivotal role in limiting hyperglucocorticoid secretion and promoting adaptive psychological responses in stressful scenarios.
Chapter 5: General discussion

5.1 Overview

The goal of this dissertation was to examine the basic mechanisms restricting glucocorticoid secretion during stress adaptation, with a focus on the neurotransmitter vasopressin. Previous studies have demonstrated that central vasopressin V1a receptors (V1aR) inhibit glucocorticoid secretion under basal conditions, and during acute physical and social stress (Kalsbeek et al., 1996a, 1996b; Wotjak et al., 2002; Wotjak et al., 1996). However, the contributions of V1aR during repeated stress had yet to be investigated.

Overall this work demonstrates that central V1aR signaling promotes the decline of glucocorticoid responses during repeated psychological stress. The data also suggests that dysregulation of limbic V1aR-circuitry might predispose individuals to mental illnesses that are associated with hyperglucocorticoid secretion.

5.1.1 Chapter 2

In chapter 2 we showed that habituation of endocrine responses during repeated restraint is associated with limbic increases in vasopressin mRNA in the posterior bed nucleus of the stria terminalis (BST) and medial amygdala. Based on this association it appeared that limbic vasopressin could be important to the process of endocrine habituation. Previous studies have documented similar increases in limbic mRNA or vasopressin-positive cell count (Gomez et al., 2004; Bingham et al., 2005), however our study was the first to confirm that this finding is consistent across different forms of repeated restraint. This study also showed that variations of repeated restraint induce different changes in central corticotropic-releasing hormone (CRH) and gonadotrophin releasing hormone (GnRH) mRNA. Given that various forms of repeated restraint are often used by different labs to study similar processes, these data show that variations in repeated restraint pose different physiological demands, which can evoke different neuronal changes.
5.1.2 Chapter 3

In this chapter intracerebroventricular (icv) antagonism was used to determine if inactivation of V1aR affects the process of endocrine habituation. Three hour repeated restraint was selected for this study since in chapter 2 it showed the most stress-induced changes. Hence this paradigm would have enabled us to also survey possible antagonist effects on normal stress-induced changes in CRH and GnRH as well. We found that relative to vehicle treated controls continuous icv antagonism had no effect on basal HPA tone or on steady state levels of vasopressin mRNA in non-stressed controls. Antagonism did however attenuate habituation of adrenocorticotropic hormone (ACTH) and corticosterone responses. Antagonism also increased the levels of stress-provoked vasopressin mRNA detected in the medial amygdala. These findings are the first to show that central V1aR activation can influence habituation of endocrine responses during repeated restraint. These findings also add to the existing literature that has shown that central V1aR activation has an inhibitory influence on endocrine output from the hypothalamic-pituitary-adrenal (HPA) axis (Kalsbeek et al., 1996a, 199b; Wotjak et al., 1996; Wotjak et al., 2002). Chen and Herbert (1995) were the first to show that central V1aR antagonism attenuates habituation of thermal responses during repeated immobilization stress. So, although this work complements and extends their findings, it should be emphasized that their study was pivotal for first demonstrating that habituation of normal stress responses depends on central V1aR activation, regardless of the measure used.

5.1.3 Chapter 4

In this chapter vehicle and antagonist treated male rats were again exposed to three hour repeated restraint, but this time brains were examined for changes in cellular activity using Fos protein. Here we replicated our previous findings and confirmed central V1aR antagonism significantly alters endocrine responses during repeated, but not acute restraint. We also showed that while antagonism had no effect on basal Fos levels, it did blunt normal stress-induced increases during acute and repeated restraint. As expected antagonism prevented the normal habituation of stress-provoked Fos responses within regions of the limbic-HPA axis. These findings show that normal cellular activity of stress-dedicated circuitry relies on V1aR signaling and has allowed us to begin mapping the extent of our
drug effects. A separate experiment found that repeated restraint induces region-specific decreases and increases in V1aR binding levels. Of particular interest, increases in the BST and septum were reported. This suggests increased V1aR signaling capacity in these regions may promote endocrine habituation. Although this has yet to be confirmed, our Fos data supports the hypothesis that V1aR-mediated habituation could rely on V1aR pathways involving the BST and septum. A follow-up study superimposing continuous vehicle or V1aR antagonist during half hour repeated restraint given daily for ten days was also undertaken (see Appendix). Similar to our previous observations, antagonism had no effect on basal HPA endocrine levels or acute endocrine stress response. Antagonism did however impede habituation of glucocorticoid responses with a noticeably greater influence on corticosterone relative to ACTH. Together these findings confirm habituation of endocrine responses depends on central V1aR signaling. This data also shows our findings can extend to different forms of repeated restraint.

5.2 Methodology considerations

5.2.1 Tail bleeding

To examine plasma glucocorticoid responses during restraint stress, repeated blood samples were taken from an approximately 2mm scapel nick over the lateral tail vein during the first and last restraint exposures. Blood sampling in this manner has the advantage of being quick and minimally invasive compared to other approaches relying on implanted catheters. Glucocorticoid concentrations obtained from repeated tail nick sampling have also been shown to be equivalent to those obtained during repeated cathether sampling (Vahl et al., 2005). This suggests that while catheter sampling might be perceived as less distressing to an animal, that in fact both methods are comparable in terms of their potential additive effect to overall glucocorticoid response. Although serial sampling from the tail vein has the potential to increase glucocorticoid levels, this effect has been shown to be minimal when rats are sampled repeatedly during a 3 hour period, using a protocol similar to that used in our studies (Fluttert et al., 2000). Therefore, although the experience of tail vein sampling could have contributed to the profile of glucocorticoid responses during our restraint studies, existing literature indicates this effect would have had a minor additive effect to restraint.
5.2.2 Vasopressin mRNA versus protein expression

The goal of chapter 2 was to survey potential stress-induced changes in neuropeptide mRNA levels following repeated restraint. This approach was chosen so we could compare our findings to previous work from our lab showing stress-induced vasopressin mRNA levels during half hour repeated restraint (Bingham et al., 2005). Transcript levels of vasopressin mRNA reliably correspond to increases in vasopressin synthesis (Murphy et al., 1998; Si-Hoe et al., 2000), making this approach informative for gauging possible changes in vasopressin signaling. However, this measure is indirect. Hence, future studies could consider validating our findings through the use of immunohistochemistry or microdialysis techniques. Our findings are however supported by the work of Gomez et al., (2004) that has demonstrated that three hour restraint repeated daily for three days increases vasopressin-positive cell counts in the BST.

5.2.3 Intracerebroventricular infusion using Alzet pumps

Based on the wide distribution of V1aRs throughout the limbic-HPA axis, we decided to target V1aR using an icv approach. Having confirmed a role for V1aR signaling during repeated restraint, future studies will need to confirm which V1aR populations are mediating our observed effects using local targeting. Previous literature has demonstrated that V1aR antagonism in the vicinity of the PVN increases basal and stress-induced ACTH and corticosterone levels during social and osmotic stress (Kalsbeek et al., 1996a, 1996b; Wotjak et al., 1996; Wotjak et al., 2002). Therefore, I was initially skeptic that our antagonist treatment was not altering basal HPA tone or acute HPA endocrine responses. This motivated a follow-up study examining the effects of antagonism on circadian tone in greater detail. Here we confirmed our icv approach using this selected dose does not alter basal HPA levels in different phases of the light cycle by sampling animals in the morning and evening across five consecutive days. Follow-up experiments testing the impact of antagonism on repeated three hour and half hour restraint have thus been important for replicating and confirming that: (1) this approach does not alter basal HPA tone, and (2) this approach does not alter normal acute endocrine responses. Together these findings suggest the antagonist approach we employed does not act on V1aR-circadian circuits regulating HPA rhythms or directly target V1aR populations in the vicinity of the PVN that can also
modulate HPA output. Importantly, by using this icv approach and specific dose we have been able to study the effects of V1aR antagonism on normal limbic activation, without causing a pre-existing disruption to circadian HPA tone that might have otherwise confounded our examination.

Our data also complements existing studies that have shown that icv approaches have a greater influence on regions proximate to the ventricles compared to more distant regions, including the hypothalamus. This has been demonstrated by Francis et al., (2006) who used nuclear immunoreactivity to examine the extent that icv administration of glucocorticoid receptor (GR) agonist (RU28362) induces central changes in GR translocation in adrenalectomized rats. They showed that one hour following a high-dose of GR agonist, that GR nuclear staining was only observed in brain regions adjacent to the lateral and third ventricle. These regions included the medial but not the lateral population of parvocells in the hypothalamic paraventricular nucleus (PVN). These findings suggest that our icv approach might have exerted a larger influence on limbic regions surrounding the ventricles compared to more distant hypothalamic regions regulating circadian tone.

5.2.4 Continuous versus daily drug injections

While this dissertation establishes a role for V1aR signaling during habituation, future studies will need to determine if V1aR activation facilitates the acquisition of habituation, the expression of habituation, or both to reveal the specific features of this mechanism. Our decision to use continuous administration with osmotic pumps has advantages. This approach avoided the added stress of daily icv injections. This approach also provided a consistent level of drug exposure that is difficult to achieve with daily injections. The tradeoff however, is that we cannot say when V1aR activation is most critical. Pharmacological studies have demonstrated that V1aR antagonism alters memory acquisition, consolidation, and retrieval (Caldwell et al., 2008), so V1aR signaling may be important at multiple phases during repeated restraint. In order to address this question, select drug exposure during the first exposure, last exposure, or days in between will be needed to delineate this mechanism further.
5.2.5 Potential effects on physiological responses

V1aR has been implicated in a multitude of biological processes ranging from osmotic, cardiovascular, metabolic to thermal regulation. These physical processes if disrupted have the capacity to alter endocrine responses by altering normal homeostasis. Therefore, an early consideration was that the effects of V1aR antagonism on HPA activity might be secondary to, or driven by possible physiological changes. To test this possibility, the work of chapter 4 included preliminary assessment of body temperature responses during restraint (see Appendix). We found that continuous V1aR antagonism had no effect on basal temperature and no effect on thermal responses during acute and repeated restraint. These findings are not consistent with previous reports by Chen and Herbert (1995), showing that V1aR antagonism blocks habituation of thermal responses during repeated immobilization. Supine immobilization is a different and more severe stress than the inescapable confinement or ‘restraint’ we used, so differences in protocol or methodology in temperature assessment may be a factor. It may also be the case that a higher dose is required to observe treatment effects on thermal response during repeated restraint. If central V1aR antagonism in itself posed a physical stress we would have expected an increase in basal glucocorticoid levels. We also would have expected that the stress response during acute restraint would have been potentiated by antagonism. Although these findings suggest that our antagonist approach did not affect thermal regulation, or pose a significant physical stress, future studies should consider maintaining an eye on thermal response, and additional physical measures.

5.3 Future considerations

5.3.1 V1aR-mediated behavior

It is likely that the full consequences of our antagonist approach include other behavioral and biological changes which future work could investigate. For instance, central V1aRs can enhance pain threshold (Oluyomi & Hart 1992; Schorscher-Petcu et al., 2010), increase central water content (Rosenberg et al., 1990), regulate glucose rhythms via direct liver projections (Kalsbeek et al., 2004), and increase blood pressure and heart rate (Stojicic et al., 2008). In terms of behavior, V1aRs can increase (Ferris et al., 2006: Bosch &
Neumann 2012) and decrease aggression (Newphew & Bridges, 2008; Veenema et al, 2010), increase motor behavior (Poulin et al., 1995), increase maternal memory (Nephews & Bridges, 2008), increase maternal behavior (Pedersen et al., 1994; Bosch & Neumann 2012 review), and enhance olfactory processing and memory retrieval (Tobin et al, 2010; Alescio-Lautier et al., 1995). Since our antagonist approach blocked acute stress-induced Fos increases in the septum, it would be of particular interest to gauge what other septal-mediated processes may have been altered. Pharmacological studies and selective knockout approaches have shown that septal-V1aRs enhance social memory and recognition (Dantzer et al., 1988; Engelmann & Landgraf, 1994; Bielsky et al., 2005), increase pair bonding and paternal behavior (Liu et al., 2001; Wang et al., 1993), increase open-arm activity in the plus-maze (Everts & Koolhaas, 1999), and augment intermale aggression (Veenema et al., 2010). Thus in a broader context, it could be the case that V1aR signaling not only promotes habituation of endocrine responses, but has an anxiolytic effect on behavior as well.

5.3.2 Neuroanatomy

V1aR antagonism increased HPA responses during repeated but not acute restraint. This suggests that the circuits mediating acute and repeated stress responses could be different, or alternatively that these processes use similar circuitry but different mechanisms. These findings also suggest that the circuitry coordinating repeated restraint responses utilizes V1aR-signaling pathways. Antagonism appears to have had a direct effect on septal cellular activation indicating that the septum may be a key region mediating our antagonist effects. Further, stress-induced V1aR increases were also found in the septum and BST highlighting these regions as potential focal points for our emerging model. Since V1aR signaling facilitates HPA axis habituation, we suspect increased V1aR activation in these areas promotes inhibition of the HPA axis. These data thus provide additional evidence, and complements existing lesion studies that have demonstrated an inhibitory septal influence on ACTH and corticosterone release (Dobrakovova, 1982; Singewald et al., 2011). This inhibitory role is also in agreement with behavioral studies that have shown septal lesions produce exaggerated and uninhibited defensive behaviors in rats, otherwise referred to as ‘septal rage’ (Risold & Swanson, 1997). The septum is positioned to modulate the HPA axis via direct projections to the PVN surround, but also through indirect hypothalamic
connections. Given this indirect and multi-synaptic design, it has been difficult for scientists to clarify and confirm that the septum provides an inhibitory influence on the HPA axis (Herman et al., 2003). Further complicating this issue, the septum also contains hippocampal fibers of passage. Therefore it has been suggested that the effects of septal lesions may in part also reflect the contributions of the hippocampus (Raisman, 1966).

Nonetheless, the findings of this dissertation provide additional evidence that cellular activation of the septum is associated with facilitating HPA axis habituation. Given that stress-induced V1aR increases were documented in the BST and septum, these data suggest that these two regions could be important relays mediating V1aR-dependent habituation. Vasopressin in the septum has an excitatory influence that is thought to facilitate the maintenance of long-term potentiation (LTP) within excitatory and inhibitory circuits (Jakab et al., 1991; Van den Hooff et al., 1989). The influence of V1aR signaling in the BST however has yet to be determined. Based on the preliminary findings of this dissertation, it appears that limbic V1aR increases likely facilitate the activation of inhibitory circuits targeting the PVN. Given the ability of the septum to directly communicate with the hippocampus, amygdala and dorsomedial hypothalamus (Raisman, 1966; Risold & Swanson, 1997), I suspect that direct antagonism of septal V1aRs may have triggered the observed antagonist effects on Fos responses in these other regions. Therefore, future studies might benefit by directing further attention to this circuit.

We have also presented new data indicating that repeated restraint is associated with decreased V1aR expression in the caudal central amygdala, thalamus, and dentate gyrus. These findings suggest that V1aR signaling in these regions does not contribute to HPA axis habituation, however this suspicion will need to be tested. If V1aRs in general promote HPA axis inhibition, decreases in these regions may be linked to maintaining HPA axis responsiveness. In particular the thalamus and central amygdala are implicated in the promotion of reflexive startle responses and autonomic responses (Kumari et al., 2007; Liang et al., 1992; Teves et al., 2004). Little is known about the actions of thalamic V1aRs, however the role of V1aR signaling in the amygdala has been investigated (Huber et al., 2005). V1aRs are found in the medial division of the central amygdala where they have an excitatory influence on the expression of physiological fear responses (Vivani & Stoop, 2008; Cassell et al., 1999). Its possible V1aR activation in this region coordinates a spectrum
of responses encompassing: increased acoustic startle, increased freezing, increased blood pressure, heart rate, respiration and intestinal motility (Vivani & Stoop, 2008; Stojicic et al., 2008). Towards this possibility, V1aR antagonism in the central amygdala has been found to reduce maternal aggression (Bosch & Neuman 2012 review). These data suggest that V1aR decreases in the central amygdala might promote the suppression of fear responses, and actually facilitate the habituation of behavioral responses.

The hippocampus and posterior paraventricular thalamus are inhibitory components in the limbic-HPA axis (Sapolsky et al., 1984; Bhatnagar et al., 2002), so receptor decreases in the dentate and thalamus may modulate the inhibitory tone of these regions. Vasopressin signaling in the hippocampus has been shown in vivo and in vitro to increase LTP in the dentate gyrus (Chen et al., 1993; Dubrosky et al., 2002). Conversely, microinjection of anti-AVP into the dentate gyrus decreases memory consolidation in behavioral tasks, which is thought to be due to suppression of LTP (Kovacs et al., 1982). Studies have yet to confirm if these effects are mediated by V1aR, so it can only be speculated as to whether V1aR decreases in the dorsal dentate gyrus might have cognitive consequences. Vasopressin in the dentate gyrus enhances serotonin release from nerve terminals (Auerback 1992 & Lipton), which is presumably one way that vasopressin is able to enhance serotonin-mediated neurogenesis (Radley & Jacobs, 2002). Thus, it seems likely that stress-induced V1aR decreases are not important to habituation, but could instead be linked to suppressed LTP, decreased neurogenesis, and the overall expression of stress-induced cognitive deficits.

Conversely, if V1aR decreases in the dentate gyrus do contribute to the process of habituation, I anticipate decreases could be beneficial by optimizing the modulation of other mechanisms. V1aR potentiation of β-adrenergic signaling in the hippocampus is thought to enhance cognitive processing, however there can be too much of a good thing. V1aR potentiation of β-adrenergic signaling does not occur when vasopressin levels are high (Brinton & McEwen, 1989). Therefore V1aR decreases may be a compensatory mechanism to maintain optimal hippocampal signaling during repeated restraint.
5.3.3 Mechanism

Habituation of endocrine responses during repeated restraint is associated with widespread declines in cellular activation within stress- and sensory- dedicated circuitry (Melia et al., 1994; Girrotti et al., 2006). Although declines in cellular activity and HPA output could reflect a process of desensitization, HPA responsiveness is not compromised when habituated animals are presented with a novel (or heterotypic) stress. Here we have provided additional evidence that the process of habituation entails reduced activation of the HPA axis, and not a reduced capacity to be stimulated. Although V1aR antagonism attenuated habituation, it did not completely block ACTH and corticosterone responses from declining across exposures. This suggests that other independent mechanisms facilitating habituation could have contributed to this decline, including mineralcorticoid receptor (MR) signaling, endocannabinoid signaling, and β-adrenergic signaling (Cole et al., 2000; Hill et al., 2010; Grissom & Bhatnagar, 2011).

A key feature of stress habituation is that habituation of responses only occurs when the same stimulus is presented repeatedly. This stimulus-specific nature of habituation suggests this process relies on a learning and memory component that enables animals and humans to recognize and discriminate familiar from novel stressors. As demonstrated by Grissom & Bhatnagar (2007) male rats show normal endocrine habituation during repeated restraint when stress exposure is paired with a background banana or peppermint odor. If that familiar background odor is replaced with a novel scent, habituated males show a reversal of glucocorticoid response and exhibit increases in endocrine magnitude that are similar to that provoked during acute stress. This study shows that slight changes in environmental context have the capacity to block HPA axis habituation. This study also highlights that habituation relies on constant processing of environmental cues and the ability to distinguish novel from familiar stimuli.

5.3.4 V1aR-mediated cognitive processing

The possibility that V1aR-mediated habituation entails a learning and memory component is supported by a culmination of pharmacological and knockout studies that have established V1aR signaling enhances cognition. In a variety of vertebrate V1aR activation
promotes social recognition, learning and memory tasks, and general sensory processing (Donaldson & Young, 2008; Choleris et al., 2009; Caldwell et al., 2008). The neuroanatomical specificity of these effects is not fully established, but studies have isolated the ventral hippocampus and lateral septum as key regions mediating vasopressin enhancement of memory consolidation and memory retrieval (Kovacs, 1979; Joels, 1984; Alescio-Lautier et al., 1995; Egashira et al., 2004). A cognitive mechanism such as this which is independent of corticosterone negative feedback, could explain why adrenalectomized rats still have the capacity to show habituation of stress-induced Fos protein levels and display habituation of ACTH responses during repeated restraint (Melia et al., 1994; Jaferi et al., 2006).

Vasopressin can also modulate other signaling pathways regulating cognition, and in particular β-adrenergic signaling which is implicated in the process of stress habituation. Recently Grissom & Bhatnagar (2011) showed that local antagonism of β-adrenergic receptors in the basolateral amygdala blocks habituation of endocrine stress responses during repeated restraint. V1aRs in the hippocampus potentiate β-adrenergic signaling (Brinton & McEwen, 1989), therefore we suspect that V1aR-mediated promotion of limbic β-adrenergic signaling might be one way these basic mechanisms converge.

5.3.5 V1aR-mediated negative feedback

Repeated restraint is documented to increase levels of hippocampal GR expression (Sapolsky et al., 1984; Makino et al., 1995), however the cause of this effect has only been examined in limited scenarios. One possibility is that stress-induced vasopressin increases during repeated restraint facilitate GR increases. In support of this hypothesis lower levels of GR hippocampal expression are found in Brattleboro rats that are genetically vasopressin deficient (De Kloet et al., 1980; Sapolsky et al., 1984). Although Brattleboro rats show normal levels of vasopressin mRNA, a genetic mutation prevents them from synthesizing vasopressin into protein (Schmale & Richter, 1984). Vasopressin treatment in Brattleboro rats increases corticosterone binding capacity in the hippocampus and pituitary (Veldhuis & de Kloet, 1982). Therefore, stress-induced GR increases might rely on vasopressin as well. To the best of our knowledge the closest study to test this has come from the lab of Michel
Aubert. Using an icv approach they showed that daily injections of exogenous vasopressin repeated daily for five days increases GR mRNA levels in the hippocampus and pituitary (Hugin-Flores et al., 2004). To confirm this increase was vasopressin-dependent they tested if GR increases induced during repeated restraint could be blocked by administering a non-specific V1a/b receptor antagonist (Hugin-Flores et al., 2004). This pharmacological blockade prevented stress-induced GR and MR increases in receptor binding in both the hippocampus and anterior pituitary. Together this work suggests V1aR activation could be facilitating HPA axis habituation by up-regulating central GR and MR levels, although a potential contribution of the V1b receptor cannot be ruled out. The paradigm used by Hugin-Flores et al., (2004) entailing three hour restraint repeated across five consecutive days is equivalent to that used for the studies of this thesis. Therefore these data suggest our observed antagonist effects may have included disruption of normal stress-induced changes in GR/MR signaling. After the implantation of cannulas our rodents were given a week of surgical recovery. Alzet pumps once implanted undergo a priming phase before they become fully activated. In the case of our experiments, the model we used (#2004) takes about forty hours to equilibrate or become primed. After accounting for this initial priming phase the remaining 5-6 days of drug exposure faced by the antagonist group might have been adequate to alter MR/GR expression prior to acute restraint testing. However, if this had occurred in a significant way we would have expected antagonism to increase HPA basal tone and increase HPA axis responses during acute restraint.

5.3.6 GR-mediated enhancement of V1aR signaling

In chapter 4 repeated restraint was found to induce V1aR increases in the BST and septum. In the periphery glucocorticoids promote upregulation of V1aR expression in the liver, which is thought to enhance glycogenolysis when increased energy demands arise (Watters et al., 1996a). Similarly, central V1aR populations in the BST and septum are up-regulated by corticosterone. This was first documented by Watters and Dorsa (1996b) who showed that adrenalectomy abolishes V1aR mRNA and binding expression in these regions, and that this effect can be reversed with corticosterone and dexamethasone replacement. Since V1aR expression in these regions is corticosterone-dependent, we suspect negative feedback signaling in the BST and septum is one mechanism leading to the enhancement, or
promotion of V1aR-mediated habituation of endocrine responses (see Figure 5-1 for a proposed model).
Figure 5-1. Schematic diagram of proposed signaling pathway facilitating V1aR-mediated habituation of HPA axis activity during repeated restraint. (1) V1aR activation in the septum during acute restraint is associated with increased stress-induced Fos responses in the CA1 hippocampal region during repeated restraint. (2) Stress-induced activation of the CA1 region during repeated restraint might facilitate inhibition of the PVN through indirect routes by recruiting the bed nucleus of the stria terminalis (BST), septum, and surrounding nuclei in the hypothalamus (i.e. dorsomedial nucleus, DMH) to promote PVN inhibition. (3) The BST is also likely involved in mediating direct inhibition of the medial amygdala (MeA) to indirectly promote HPA axis inhibition. (4) Stress-induced V1aR increases in the BST and septum during repeated restraint may also facilitate activation of inhibitory BST afferents to the PVN.
Although V1aR binding is found throughout the rodent brain it is interesting that glucocorticoid upregulation has only been found in these select regions. The stress-induced V1aR increases in the septum and BST we observed therefore could be due to the glucocorticoid elevations experienced during repeated restraint. The region-specificity of these effects further underscores that the BST and septum could be important regions for future investigation. It may also be the case that glucocorticoid dependent V1aR populations occur elsewhere in the brain, however to the best of our knowledge this has yet to be thoroughly examined.

5.3.7 Opposing roles for V1aR and V1bR

The findings of this dissertation support the perspective that central V1a and V1b receptors have opposing roles that could be important for maintaining tight regulation of the systems they influence. V1b receptors (V1bR) facilitate HPA activation during acute novel stress and during repeated restraint conditions (Spiga et al., 2009a, 2009b; Lolait et al., 2007). Conversely, V1aR reduces stress-induced HPA responses during both acute and repeated stress (Kalsbeek et al., 1996a, 1996b; Wotjak et al., 1996; Wotjak et al., 2002, Gray et al, unpublished). Although central V1aRs inhibit daily HPA rhythms, pharmacological and knockout studies suggest V1bRs do not influence basal ACTH and corticosterone levels, suggesting V1aR therapeutics may be more effective treatments for clinical states showing abnormal basal glucocorticoid secretion (Spiga et al., 2009b; Lolait et al., 2007). Overall, these studies show that therapeutics aimed at normalizing glucocorticoid secretion in clinical settings may need to consider both receptors as potential targets.

5.3.8 Clinical applications

This work joins the existing literature by suggesting that V1aR specific therapeutics have the potential to promote endocrine adaptation and the normalization of hyper-glucocorticoid secretion (Ring, 2005; Swaab et al., 2005). Clinical trials using vasopressin analogues have reported some improvement of depressive symptoms and cognition (Jaja et al., 1993; Zohar et al., 1985). However, even modest improvements run the risk of fluid and electrolyte complications (Iager et al., 1986). Mixed and inconsistent results are not
surprising since vasopressin analogues can elicit a range of effects. These examples importantly illustrate why current drug development is focused on receptor specific therapeutics.

The findings of this thesis suggest that clinical states showing low central vasopressin expression may be vulnerable to hyperglucocorticoid secretion and benefit from therapeutics agonizing V1aR. Reduced vasopressin concentrations in the cerebrospinal fluid (CSF) of schizophrenic, depressed patients, and individuals with dementia have been reported (Gold et al., 1981; Gjerris et al., 1985; Sorensen et al., 1983; De Wied, 2002). Post mortem tissue from the temporal lobe of schizophrenic patients also shows decreased vasopressin expression (Frederiksen et al., 1991). Lower vasopressin levels in the brains of Alzheimer patients have also been found in the hippocampus and basal nuclei (Mazurek et al., 1986). Sorensen et al. (1985) have reported that recovery from depression is also associated with increased CSF vasopressin levels. Thus, the data presented here suggests that compromised vasopressin signaling could be the basis, if not a contributing factor to disorders featuring hyperglucocorticoid secretion.

Given that V1aR-circadian and V1aR-limbic circuits promote HPA axis inhibition, disinhibition of either pathway could increase PVN activation leading to the release of increased CRH and vasopressin into the pituitary, and ultimately ACTH and corticosterone elevations. Plasma vasopressin originates from the PVN and supraoptic nucleus (SON). This point is important for understanding how increased vasopressin levels in some parts of the brain (PVN, SON) and increases in plasma concentrations might correspond with reduced CSF vasopressin levels, and reduced vasopressin content in other neural regions. Although reports of vasopressin CSF and plasma levels show a lack of consistency (Ring, 2005), vasopressin expression in the PVN and SON of depressed patients is generally increased, but decreased in the suprachiasmatic nucleus (SCN) (Purba et al., 1996; Zhou et al., 2001). Decreased vasopressin in the SCN is thought to increase basal HPA axis tone and lead to sleep disturbances (Swaab et al., 2005). Thus, there is a clear scientific basis as to why some depressed patients respond well to light-therapy. In these cases, light is thought to stimulate the synthesis and release of vasopressin from the SCN, triggering HPA axis inhibition, leading to the normalization of HPA tone and sleep patterns (Boa et al., 2008). However, only twenty-five percent of depressed patients show elevated basal cortisol levels (Young et
al., 2001). This suggests that the remaining patients with hyperglucocorticoid secretion likely display dysregulation of other HPA axis regulating circuits (limbic-V1aR, pituitary-V1bR, pituitary-CRH-R1).

The majority of V1aR drugs in development have focused on antagonizing the V1aR in order to reduce hypertension and improve cardiovascular health. The orally active non-peptide V1aR antagonists SR49059 and OPC-21268 decrease platelet aggregation and decrease blood pressure in animals and humans (Serradeil-Le Gal et al., 1993; Yamamura et al., 1991; Imaizumi et al., 1992). Similarly, the V1aR antagonist SRX251 can cross the blood brain barrier and has been shown to centrally facilitate decreases in aggressive behavior in male hamsters (Ferris et al., 2006). Based on our present findings, that central V1aR antagonism can increase glucocorticoid responses during repeated psychological stress, it appears that V1aR antagonists might be useful in the treatment of autoimmune disorders to reverse symptoms of hypogluocorticoid secretion (Kudielka & Kirschbaum, 2005).

Although V1aR antagonists present clinical benefits, it should be noted that V1aR review papers rarely discuss potential side-effects of such treatments. However, it appears V1aR antagonist therapies do pose the risk of increasing pain sensitivity (Oluyomi & Hart 1992; Schorscher-Petcu et al., 2010), dampening glucose mobilization (Kalsbeek et al., 2004) increasing inflammatory cytokines (Zhoa & Brinton, 2004), impairing memory (Caldwell et al., 2008), and possibly impairing gonadal status (Orcel et al., 2002).

Lastly, although not readily available yet, orally active non-peptide V1aR agonists may prove promising for the promotion of HPA axis inhibition, and as a behavioral anxiolytic. While therapeutics antagonizing CRH-R1 and V1bR appear promising treatments to reduce glucocorticoid elevations (Alonso et al., 2004; Griebal et al., 2003), this thesis presents new evidence that there may also be a place for therapeutic V1aR agonists.

### 5.4 Conclusions

Overall, this work has demonstrated that HPA axis habituation relies on central V1aR signaling and that an intrinsic aspect of habituation may include associated enhancement and downgrading of V1aR expression in the limbic-HPA axis. These findings suggest that compromised limbic V1aR signaling may be an underlying feature of psychiatric disorders.
leading to HPA axis hyperactivation, and an inability to successfully adapt during scenarios of chronic psychological stress.
References


Gray JM, Bingham B, Innala L, Viau V (2009) Central vasopressin (V1a) receptors and testosterone act, but do not interact on HPA habituation to repeated 3h restraint. Proc Annual Meeting of the Society for Neuroscience, Chicago, IL (Abstract).


Appendix A: Effects of V1aR antagonism during 0.5h repeated restraint

A.1 Methods

A.1.1 Animals

Adult male Sprague Dawley rats (Charles River, Canada) were used, weighing 250-260 g on arrival (50 days old). Rats were pair housed under controlled temperature (23 ± 2°C) and lighting conditions (12:12 hour light:dark cycle, lights on at 0700 hours) with food and water available ad libitum. All protocols were approved by the University of British Columbia Animal Care Committee.

A.1.2 Surgery and pharmacological blockade

Rats (54-55 days old, 290-310g) were anesthetized and implanted unilaterally with a cannula targeting the lateral ventricle. All surgeries were performed as previously described in chapter 3. Male rats either received icv vehicle or V1aR antagonist (10ug/day, Sigma V2255, dissolved in 0.9% NaCl). Proper cannula placement was verified at the end of the experiment, and cannulas were tested for potential blockages. One week of post-surgical recovery was allocated prior to testing.

A.1.3 Repeated restraint and endocrine sampling

Restraint occurred 0900-1200 (0700 lights on), and consisted of 10 episodes of daily 0.5h restraint (see Gray et al., 2010). Blood samples were obtained during the first and last (day 10) restraint exposure. Plasma was collected from a scalpel nick over the lateral tail vein at 0, 30, and 60 min from restraint onset. These time points were selected to minimize the physiological load of multiple sampling and optimize our ability to detect treatment effects on stress-induced glucocorticoid increases. Samples (300ul) were collected into ice-chilled, EDTA- and aprotinin-treated tubes, centrifuged at 10,000 rpm at 4°C for 20 min, then stored at -20°C. Rats were returned to their home cages at the end of testing and left undisturbed until the next day. Non-stressed controls remained in the colony room during
restraint so as not to witness the stress conditions. Final endocrine analysis included vehicle (n=5) and antagonist (n=7) group sizes.

A.1.4 Plasma hormone assays

Plasma levels of ACTH and corticosterone were measured in duplicate using radioimmunoassay kits (MP Biomedicals) with [\(^{125}\)I] tracer as discussed in chapter 3. The intra- and inter-assay coefficients of variation for all assays ranged from 1-7 and 2-13\%, respectively. The standard curve ED\(_{50}\) for ACTH and corticosterone was 89.12 pg/ml and 160.1 ng/ml, respectively. The detection limit for each was 8.1 pg/ml and 6.72 ng/ml, respectively.

A.1.5 Statistics

Three-way ANOVAs (between treatment, within subject) were used to analyze hormone responses using restraint (acute, repeat) and time as repeated measures. When appropriate, post hoc comparisons were made using the Tukey-Kramer test.

A.2 Results

The time courses for ACTH and corticosterone (CORT) responses on the first and last day of repeated restraint are shown in Figure A-1. Using a three-way repeated measures ANOVA with a within subject factor, both ACTH and corticosterone showed a main effect of restraint \([F(1,10) = 35.78, P < 0.001], [F(1,10) = 7.41, P = 0.021]\), time \([F(2,20) = 78.83, P< 0.0001], [F(2,20) = 67.97, P < 0.0001]\), and a restraint x time interaction \([F(2,20) = 29.33, P< 0.0001], [F(2,20) =4.39, P < 0.026]\). Corticosterone additionally showed an interaction of restraint x treatment \([F(1,10) = 7.72, P = 0.019]\). Post hoc comparisons found stress-induced ACTH and corticosterone increases in vehicle controls were significantly lower during repeated restraint at 30 min \((P = 0.001, P = 0.01)\) and 60 min \((P = 0.009, P = 0.046, \text{ respectively})\). ACTH responses at 30 min during repeated restraint also habituated in antagonist treated animals \((P = 0.004)\), however antagonism blocked habituation of the 60
min response. For corticosterone, antagonism prevented habituation of the 30 and 60 min responses. Antagonist treated animals also had significantly higher corticosterone levels at 30 min compared to vehicle controls (P = 0.008). At most, ACTH levels at 30 min during repeated restraint showed a trend towards an antagonist effect (P = 0.075), but overall no significant treatment effect was found for ACTH.

A.3 Conclusions

These data show that V1aR antagonism blocks habituation of corticosterone responses during daily 0.5h restraint repeated across 10 exposures. These findings support the view that activation of V1a receptors is important for mediating glucocorticoid declines across different types of repeated restraint.
Figure A-1. Mean ± SEM plasma (A) ACTH and (B) CORT responses within vehicle and V1aR antagonist treated rats during acute and repeated (day 10) 0.5h restraint. *P< 0.05 vs acute. †P<0.05 vs vehicle.
Appendix B: Effects of V1aR antagonism on stress-induced thermal responses during 3h repeated restraint

B.1 Methods

B.1.1 Animals

Male Sprague-Dawley rats (Charles River, Canada) once received (28 days old) entered a 3 week quarantine period in our animal facility. Rats were pair housed under controlled temperature (23 ± 2°C) and lighting conditions (12:12-hour light:dark cycle, lights on at 0700 hours) with food and water available ad libitum (Labdiet; Rat diet 5012). All protocols were approved by the University of British Columbia Animal Care Committee.

B.1.2 Surgery and pharmacological blockade

Rats (54-55 days old, 290-310g) were anesthetized and implanted with a cannulae targeting the right lateral ventricle that was connected to a subcutaneous Alzet osmotic pump. Surgical conditions were identical to that described in chapter 4. Rats were either implanted with pumps administering saline (n=8), or V1aR antagonist (n=8) (10ug/day, d(CH2)5Tyr(Me)AVP, Sigma V2255, dissolved in 0.9% NaCl). One week of post-surgical recovery was allocated prior to testing.

B.1.3 Repeated restraint and endocrine sampling

Restraint occurred 0900-1200 (0700 lights on), and consisted of five episodes of daily 3h restraint (see Gray et al., 2010). Thermal readings were taken at 0, 30, 60, 90 and 180 min from restraint onset using rectal thermometers liberally coated with vaseline lubricant. Rats were returned to their home cages at the end of testing and left undisturbed until the next day.
B.1.4 Statistics

Three-way ANOVAs (between treatment, within subject) were used to analyze hormone and thermal responses using restraint (acute, repeat) and time as repeated measures. When appropriate, post hoc comparisons were made using Tukey’s analysis.

B.2 Results

The time course of temperature response on the first and last day of repeated 3h restraint is shown in Figure B-1. Using a three-way repeated measures ANOVA with a within subject factor, thermal measures showed a main effect of restraint \([F(1,14) = 6.15, P = 0.026]\), and time \([F(4,56) = 63.41, P < 0.0001]\), and a restraint x time interaction \([F(1,14) = 5.99, P = 0.028]\). No significant restraint x treatment interaction \([F(1,14) = 2.02, P = 0.17]\), or time x treatment interaction \([F(4,56) = 0.46, P = 0.76]\) was found. Post hoc comparisons found stress-induced temperature increases were unchanged during repeated restraint, with no effect of treatment. Vehicle treated controls showed a significant decrease in basal temperature on the last day of restraint \((P = 0.023)\).

B.3 Conclusions

These data suggest that V1aR antagonism does not change stress-induced thermal responses during 3h repeated restraint.
Figure B-1. Mean ± SEM temperature (F) responses within vehicle and V1aR antagonist treated rats during acute and repeated (day 5) 3h restraint.*P < 0.05 vs 0 min. #P < 0.05 vs acute exposure.