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Chronic Stress Potentiates The Response To Intra-Bed Nucleus Of The Stria Terminalis (bnst) Pituitary Adenylate Cyclase Activating Peptide (pacap) Infusion.

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CHRONIC STRESS POTENTIATES THE RESPONSE TO INTRA-BED NUCLEUS OF THE STRIA TERMINALIS (BNST) PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE (PACAP) INFUSION.

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ABSTRACT

Chronic or repeated exposure to stressful stimuli can result in several maladaptive consequences, including increased anxiety-like behaviors and altered peptide expression in brain structures involved in emotion. Among these structures, the bed nucleus of the stria terminalis (BNST) has been implicated in emotional behaviors as well as regulation of hypothalamic-pituitary-adrenal (HPA) axis activity. In rodents, chronic variate stress (CVS) has been shown to increase BNST pituitary adenylate cyclase activating polypeptide (PACAP) and its cognate PAC1 receptor transcript, and BNST PACAP signaling may mediate the maladaptive changes associated with chronic stress. In order to determine whether chronic stress would potentiate the behavioral and/or endocrine response to subthreshold BNST PACAP infusion, rats were exposed to a 7 day CVS paradigm previously shown to upregulate BNST PAC1 receptor transcripts; control rats were not stressed. Twenty-four hours following the last stressor, stressed and control rats were bilaterally infused into the BNST with 0.5 µg PACAP. Startle response to intra-BNST PACAP infusion was assessed post-infusion in Experiment 1. In Experiments 2 and 3, blood was sampled via a tail nick 30 min following PACAP infusion to assess the corticosterone response to PACAP following CVS. We found an increase in startle amplitude and an increase in plasma corticosterone levels 30 minutes following BNST PACAP infusion only in rats that had been previously exposed to CVS. These results were likely mediated via PAC1 receptors, as equimolar infusion of the VPAC1/2 receptor ligand vasoactive intestinal polypeptide (VIP) had no effect on plasma corticosterone levels. These results suggest that repeated exposure to stressors sensitizes the neural circuits underlying the behavioral and endocrine responses to BNST PACAP infusion and BNST PACAP/PAC1 receptor signaling likely plays a critical role in mediating stress responses.

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Stressors activate several physiological and behavioral systems to promote homeostasis and survival. However, when stressor exposure becomes severe or repeated it places enhanced challenges on both physiological and psychological systems leading to the over activation of the physiological and behavioral systems typically recruited to reestablish homeostasis. Stressor exposure engages multiple response systems, including the sympathetic arm of the autonomic nervous systems, the hypothalamic—pituitary—adrenal (HPA) axis, and central nervous system (CNS) circuits responsible for fear and anxiety-like behavior. The sustained activation of these CNS nuclei in response to chronic stressor exposure has been argued to lead to the maladaptive morphological and functional changes that underlie fear- and anxiety-related affective states, including altered peptide expression in the brain structures involved in emotion and increased anxiety-like behaviors (Hammack and May, 2014; Pego et al, 2008; Schulkin et al, 1998; Vyas et al, 2003). This has led to the idea that chronic stress encourages and produces stress-related disorders by increasing neuronal plasticity within stress-responsive nuclei. Indeed, Herman and colleagues suggest that the connection between limbic structures, affective disorders and HPA axis dysfunction is likely associated with impaired integration of hippocampal, amygdalar and/or prefrontal cortical information at one or more of the key subcortical regulatory nodes such as the bed nucleus of the stria terminalis (BNST) (Herman *et al*, 2005).

As mentioned, multiple forebrain limbic regions, including the hippocampus, medial prefrontal cortex (mPFC) and amygdala, regulate HPA axis in response to threatening stimuli. The nature of these stressors determines which stress-related regions are activated and the contribution of these regions to the stress response. For instance, the central amygdala (CeA) may be selectively tuned to interoceptive or visceral stimuli (reviewed in Herman *et al*, 2005). Additionally, processive or extroceptive stressors or threats with an emotional component requiring limbic structures activate peripheral stress responses and also stimulate central (behavioral) stress responding via extrahypothalamic projections to brain nuclei associated with fear and anxiety. Importantly, these regions consistently show maladaptive changes in affective disorders. This highlights a critical need for a better understanding of the pathways by which forebrain limbic structures regulate HPA axis responses as dysfunction among these structures and circuitry have been highly implicated in the etiology of several stress-related disorders. For example, posttraumatic stress disorder (PTSD) and depression both typically manifest HPA axis abnormalities (Choi *et al*, 2007). For example, the hippocampus contains glucocorticoid receptors and plays a critical role in the regulation and termination of the HPA axis and has been suggested to be overactive in stress-related pathologies like hypercortisolemia (reviewed in Herman *et al*, 2005). In addition to the hippocampus, the medial prefrontal cortex (mPFC) is also a critical structure in regulating stress responses. Indeed, the mPFC has been shown to be critical for inhibiting the PVN; moreover, repeated stressor exposure has been shown to reduce mPFC neuron activity (Radley, Arias & Sawchenko,

2006; Radley and Sawchenko, 2011). The disinhibition of the PVN likely plays a role in increased stress responding and stress-induced anxiogenic phenotypes. Additionally, lesions of different mPFC subregions have also been shown to differentially effect HPA axis output (Radley and Sawchenko, 2011). Finally, the amygdala has also been implicated in regulating HPA axis activity. Indeed, stimulation of the CeA excites the HPA axis (Vyas *et al*, 2003), and repeated stressor exposure increases dendritic arborization in the basolateral amygdala (BLA) (Herman *et al*, 2005).

Importantly, almost every limbic region involved in regulating HPA activity does not project directly to the corticotropin releasing hormone (CRH) synthesizing PVN. Thus, information from these limbic and forebrain nuclei is relayed to the PVN via basal forebrain and hypothalamic structures, indicating that the effects on HPA axis are indirect and depend on the structural and functional integrity of these subcortical structures/relays. There is considerable opportunity for integration of limbic information at intermediary sites. Notably, the BNST receives projections from all of these limbic regions, and in turn, sends projections to the PVN (Dong and Swanson, 2004; 2006). Moreover, there is considerable overlap between the inhibitory (e.g., the hippocampus and prelimbic cortex) and excitatory (e.g., medial amygdala) inputs within the BNST, suggesting that limbic information from stress-associated brain regions may be summated in the BNST, and then sent to the PVN (Herman *et al*, 2005). This likely role in integrating and relaying information from stress-associated regions coupled with the observation's that BNST coordinates several behavioral response to stressor exposure (as will be discussed in more detail below), we and others have

argued that the BNST represents a critical site of confluence between stress responding and pathological affective states (Hammack and May, 2014), and the stress-induced morphological alteration in plasticity within the BNST likely underlie several stress-related diseases.

1.2. The Bed Nucleus of the Stria Terminalis (BNST)

1.2.1. The BNST and Regulation of the Stress Response

As discussed above, stressors result in the activation of several evolutionarily important homeostatic systems resulting in the mobilization of these adaptive responses, including the sympathetic arm of the autonomic nervous systems, the HPA axis, and the central nervous system CNS circuits responsible for fear and anxiety-like behavior. The BNST has been argued to modulate all three of the responses to sustained threats (Hammack and May, 2014; Herman *et al*, 2005; Radley and Sawchenko, 2011; Walker and Davis, 2008; Walker *et al*, 2003, 2009). For instance, the anterior and posterior BNST sub-regions receive projections from and project to the PVN to tightly regulate activity and mediate both the endocrine and autonomic response to stressors (Choi *et al*, 2007; Dunn, 1987; Radley, Gosselink and Sawchenko, 2009).

The BNST can be divided into cytoarchitecturally and anatomically distinct subregions. Within the anterior BNST, the dorsolateral region expresses CRH; within the posterior BNST, the principal nucleus contains GABAergic fibers which also project to the PVN (Dong and Swanson, 2004; 2006). HPA axis activity can be excited or inhibited, depending on which of these regions is targeted. Anterior or lateral

lesions decreases responses to stress and reduce PVN CRH mRNA; medial lesions have no effect; and posterior lesions increase resting CRH mRNA expression, suggesting that distinct nuclei of the BNST differentially regulate HPA axis activity (Choi *et al,* 2007).

Furthermore, the BNST contains functionally distinct subregions that play opposing roles in integrating and processing limbic information in response to stress. For instance, posterior BNST lesions elevated plasma adrenocorticotropic hormone (ACTH) and corticosterone in response to acute stress, increased stress-induced PVN c-fos, and elevated PVN CRH and vasopressin (AVP) mRNA expression; in contrast, anterior BNST lesions attenuated plasma CORT responses and decreased c-fos mRNA induction in the PVN but had no effect on CRH and AVP mRNA expression in the PVN, suggesting that posterior BNST nuclei inhibit HPA axis activity, whereas anteroventral nuclei excite the HPA axis (Choi *et al*, 2007).

1.2.2. The BNST and Regulation of Anxiety-like Behaviors

The BNST plays a critical role in the development of anxiogenic behaviors in response to stressful stimuli and experience, acting as a sort of relay site for the multiple CNS pathways regulating PVN activity. Consequently, the BNST appears to play a crucial role in regulating the physiological and behavioral responses to stressor exposure. Support for this notion comes from studies showing that BNST activity is heavily implicated in mediating anxiety-like behavioral responding; complimenting these findings, pharmacological studies have shown anxiogenic agents to increase the expression of fos and other activation markers in the BNST (Singewald *et al,*

2003). More specifically, activation of the BNST has been argued to mediate anxiety-like behavioral responding to diffuse, long-duration and/or unpredictable threats and may be responsible for mediating anxiety-like emotional states (Walker *et al*, 2003; 2009; Waddell *et al*, 2006). Lee and Davis' (1991) early lesion studies were the first to suggest that the BNST was critical for anxiety-like behavioral responses; whereas the CeA was more involved in fear-like responses. Consistent with this view, the BNST has been implicated in other anxiety-like behaviors including light-enhanced startle (Walker and Davis, 1997) as well as the stress-induced anxiogenic behaviors resulting from repeated stressor exposure (Hammack *et al*, 2004). Additionally, electrical stimulation of the anterolateral BNST produces many of the endocrine, cardiovascular and respiratory responses that are elicited by anxiogenic stimuli (Casada and Dafny, 1991). Moreover, anxiogenic pharmacological agents such as yohimbine and m-chlorophenylpiperazine (mCPP) increase BNST expression markers of neuronal activation (i.e., c-fos) and BNST inactivation blocks several anxiogenic behavioral responses (Singewald *et al*, 2003).

The data implicating altered BNST structure and function in response to stressors are not limited to rodent models, as BNST activity has been correlated with anxiety-like behaviors in nonhuman primates as well as humans (Fox *et al*, 2008; Kalin *et al*, 2005; Somerville *et al*, 2010; Straube *et al*, 2007). For example, Kalin and colleagues (2005) showed that increased BNST activity in rhesus monkeys was positively correlated with individual differences in freezing behavior in response to eye contact (i.e., a threatening stimulus) with an intruder. In humans with arachnophobia,

BNST activation increases in responses to the anticipation of being shown an artificial spider (Straube *et al*, 2007). In aggregate, these findings contend that the BNST mediates anxiety-like responding when cues predict temporally distant threats (Waddell *et al*, 2006) and/or when behavioral responses need to be maintained for long durations (Walker and Davis, 2008). Thus, BNST-dependent responding drives a behavioral response distinct from fear and more representative of anxiety in humans, providing impetus for the idea that maladaptive BNST responding due to stress induced alteration within this structure, likely underlie some forms of anxiety disorders in humans (Hammack *et al*, 2010; 2012).

1.2.3. Effects of Stress on BNST Circuitry

As discussed above, altered BNST functioning is likely crucial for the etiology of several anxiety disorders associated with repeated stressor exposure (Hammack *et al*, 2009; 2010; 2012; Pego *et al*, 2008; Schulkin *et al*, 1998; Vyas *et al*, 2003). This interpretation is supported by findings demonstrating that repeated exposure to stressors or stress hormones increases anxiety-like behavior which is correlated with enhanced neuroplasticity within the BNST, including increased neuronal peptide expression (Hammack *et al*, 2009; Roman *et al*, 2014; Stout *et al*, 2000). For example, Stout and colleagues (2000) showed that, within the BNST, elevations in CRH and neuroplasticity are associated with more anxiogenic behavior and anhedonia. Additional studies have shown that repeated exposure to stressors also enhances dendritic branching, length, and total BNST volume (Pego *et al*, 2008; Vyas *et al*, 2003) and enhances excitatory synaptic transmission (Dumont *et al*, 2005). Given

that stressor exposure is a key component in the development of several affective disorders and that the BNST is a site of confluence between stress and emotion, these findings argue that stress-induced alterations in BNST neuronal function and plasticity likely underlie several forms of chronic anxiety in humans. Thus, chronic exposure to stressors physically alters BNST neurochemistry, morphology and physiology to facilitate function and promote increases in fear- and anxiety-like behaviors [reviewed in Hammack and May, 2014). These findings suggest that BNST neuropeptides with neurotrophic properties likely act as key mediators of the anxiogenic and maladaptive effects of chronic stress; however, the mechanisms of BNST signaling/plasticity in stress-induced anxiety remains to be elucidated.

1.3. Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

The findings discussed above suggest that alterations within the BNST result from increased neuroplasticity to produce the maladaptive consequences seen following chronic stress, and implicate neuropeptides with neurotrophic properties as key mediators of the anxiogenic and maladaptive effects of chronic stress. Several neurohormones and neuropeptides have been implicated in mediating these effects; perhaps none more than CRH. Yet, despite its key role in signaling and regulating fear- and anxiety-like behaviors, CRH lacks neurotrophic effects. Pituitary adenylate cyclase activating polypeptide (PACAP) and its cognate G-protein coupled PAC1 receptor have also been implicated in mediating stress- and anxiety-like responses and unlike CRH, the PACAP system exhibits neurotrophic properties and enhances neuronal excitability, and activation of the BNST PACAP system alone has been shown to be

anxiogenic. Moreover, PACAP and its PAC1 receptor are highly expressed in the BNST, are selectively upregulated in the BNST following chronic variate stress (CVS), exhibits neurotrophic properties and enhances neuronal excitability, and activation of the BNST PACAP system alone has been shown to be anxiogenic. Thus, the BNST PACAP system emerges as a prime mediator in regulating stress-induced anxiety (Hammack and May, 2014).

1.3.1. The Role of PACAP in Stress and Anxiety

As mentioned, both PACAP and PAC1 have been implicated in regulating stress- and anxiety-like responses. Moreover, PACAP and PAC1 are highly expressed in the BNST and are selectively upregulated following chronic stress. For example, Hammack, May and colleagues (2009) exposed rats to a 7 day CVS paradigm where they received one of 5 different stressors each day for the 7 days (**Table 1**); 24 hours after the last stressors, 12 stress and fear-related brain regions were micropunched for quantitative RT-PCR in order to examine whether chronic stress could regulate PACAP expression in in brain regions implicated in stress-responding and/or anxiety-like behavior. Among the 12 neural regions examined, results showed that CVS selectively increased PACAP transcripts more than 10-fold in the dorsolateral BNST; a smaller but significant 2-fold increase in PACAP transcript was observed in the PVN [reviewed in Hammack *et al,* 2010). Among the remaining regions, including the CeA and basolateral amygdala (BLA), there was no difference in PACAP transcript levels between CVS and control tissue. CVS also induced an accompanying 2-fold increase in PAC1 R mRNA in the dlBNST (Hammack *et al,* 2009).

Table 1

Day	Stressor	Duration
1	Oscillation	30 min
2	Swim	5 min
3	Footshock	5 s (x2)
4	Restraint	60 min
5	Pedestal	30 min
6	Swim	5 min
7	Footshock	5 s (x2)

Additionally, the PACAP system exhibits neurotrophic properties and enhances neuronal excitability. Indeed, within the same tissue set, CVS augmented other stress-related transcripts in the PVN and amygdala, some of which likely result from PACAP signaling. For example, following CVS the dlBNST showed a 3-fold and 2-fold increase in brain-derived neurotrophic factor (BDNF) and TrkB mRNA levels, respectively (Hammack *et al*, 2009). PACAP can stimulate neuronal BDNF and/or TrkB expression and function in several paradigms (Braas *et al*, 2007). Furthermore, Zink et al and colleagues have shown CNS BDNF expression to be diminished in PAC1 receptor knockout animals (Zink *et al*, 2004). Chronic stress results in long term structural and functional changes in dlBNST neuronal plasticity and BDNF function has been associated with anxiety-related behaviors, implicating PACAP-stimulated BDNF expression as one of the mechanisms underlying stress-induced plasticity within this structure. In support of this, our lab has that PACAP-treatment of dlBNST explants in serum-free cultures can increase expression of BDNF transcripts (Hammack and May, unpublished observation). Further corroboration comes from studies showing that following chronic stress other CNS nuclei, including the PVN and dorsal raphe nucleus (DRN), show increases in BDNF expression (Hammack *et al*, 2010). The

changes in BDNF expression within these regions may also reflect stress-mediated PACAP upregulation and signaling from the BNST as both structures are targets of BNST projections. These data suggest that among stress-related CNS nuclei, the dlBNST is a critical target of stress-induced plasticity, and this plasticity may represent a primary mechanism underlying anxiety disorders in humans.

Further implicating the PACAP/PAC1 receptor system in stress responding, PACAP and PAC1 receptor null mice show reduced anxiety-like behavior and fear, including contextual fear conditioning (Girard *et al*, 2006; Hashimoto *et al*, 2001; Otto *et al*, 2001), diminished HPA axis activation and altered glucocorticoid responses after prolonged stressor exposure (Hashimoto *et al*, 2009; Stroth and Eiden, 2010).

1.3.2. Effects of Intra-BNST PACAP on Stress and Anxiety

Additionally, we have shown that intra-BNST PACAP infusion mimics, and intra-BNST PACAP receptor antagonism prevents, many of the consequences of chronic stress in the absence of any stressor.

We have shown that bilateral intra-BNST infusions increased acoustic startle responding, were anxiogenic on the elevated plus maze (EPM), attenuated weight gain, and increased plasma corticosterone (Hammack *et al*, 2009; Kocho-Schellenberg *et al*, 2014; Lezak *et al*, 2014; Roman *et al*, 2014). These effects were dose dependent, not due to ventricular leakage and not sexually dimorphic. Furthermore, these effects were likely mediated via BNST PAC1 receptors, as the effects were mimicked by intra-BNST infusion of maxadilan (selective PAC1 receptor agonist) but not the VPAC1/2 receptor ligand vasoactive intestinal polypeptide (VIP) (Roman *et al*, 2014).

Thus, these results show that BNST PACAP is sufficient to produce a stress and anxiety-like phenotype in rodents, but they don't answer the question of whether endogenous BNST PACAP signaling participates in the behavioral and endocrine consequences of chronic stress. To address this rats were bilaterally cannulated and chronically delivered PACAP6-38 (a PAC1/VPAC2 receptor antagonist) during the week of CVS. Results revealed that PACAP6-38 was able to block the CVS-induced weight loss and anxiety-like behavior (Roman *et al,* 2014). Perhaps more importantly, the results showed that chronic stress sensitized the corticosterone response in reaction to a subsequent novel stress environment (i.e., open field maze) and acute BNST PACAP6-38 administration immediately before exposure to the novel environment attenuated the sensitized portion, specifically, of the corticosterone response to levels approximating those prior to stressor exposure (Roman *et al,* 2014). That is, PACAP antagonism blunted the corticosterone response when the animal had been previously exposed to chronic stress; but this attenuation was weakened in animals who had only been exposed to the acute stressor, suggesting that BNST PACAP signaling underlies the potentiated corticosterone response following chronic stress; but not acute stress. These results also corroborate Lezak et al. (2014) and further suggest that this system is recruited in response to long duration stressors and/or multiple stressor exposures (Stroth and Eiden, 2010). Taken together, the results implicate BNST PACAP signaling at PAC1 and/or VPAC2 receptors as a necessary system in mediating both the behavioral and endocrine consequences of chronic stressor exposure (Hammack *et al,* 2009; Hashimoto *et al,* 2009; Roman *et al,* 2014; Stroth and Eiden, 2010).

1.3.3. BNST PACAP System in Human Affective Disorders

The PACAP/PAC1 receptor system is highly conserved across species and is not just important in regulating stress responses in rodents, but has also been implicated in higher order primates. Indeed, in humans PACAP dysregulation has been associated with several psychopathologies, including post-traumatic stress disorder (PTSD). Elevated circulating PACAP levels and a PAC1 receptor gene (ADCYAP1R1) single-nucleotide polymorphism (SNP) predict PTSD symptoms and diagnosis in women; methylation status was also associated with PTSD independent of gender (Ressler *et al*, 2011). Importantly for the current study, a PAC1 receptor SNP has also been linked to startle behavior in male and female children (Jovanovic *et al*, 2013). These studies further implicate alterations in BNST PACAP signaling as a critical system mediating the stress-induced behaviors related to fear and anxiety.

1.4. Aims of the Current Study

The possible upregulation of BNST PAC1 receptors suggests that following CVS, the BNST may be more sensitive to PACAP release. To address this, the current experiments examined whether prior chronic stress could potentiate the behavioral and/or corticosterone response to a subthreshold intra-BNST PACAP infusion. We observed an increases in startle amplitude and plasma corticosterone levels following BNST PACAP infusion in stressed rats only. The effects were likely mediated via PAC1 receptors, as vasoactive intestinal polypeptide (VIP) infusion had no effect on plasma corticosterone levels. The current results suggest that repeated stressor exposure sensitizes BNST PACAP signaling, and this sensitization may play an important

role in mediating the maladaptive consequences of stressor exposure, and may be an important target for the treatment of stress-related disease.

CHAPTER 2: CVS POTENTIATES THE STARTLE RESPONSE TO INTRA-BNST PACAP INFUSION

2.1. Introduction

As mentioned above, we have previously examined the roles of BNST PACAP via direct injection of PACAP38 into the BNST and have shown that this elicits anxiety-like responses that mimic chronic stress in the absence of any stressor exposure; moreover intra-BNST PACAP receptor antagonism blocks the stress-induced consequences (Casada and Dafny, 1991; Roman *et al,* 2014; Stout *et al,* 2010; Walker and Davis, 1997). Specifically, bilateral intra-BNST infusions increased acoustic startle responding (Walker and Davis, 1997), was anxiogenic on the EPM (Casada and Dafny, 1991) produced anorexia and weight loss (Roman *et al,* 2014), and increased plasma corticosterone (Stout *et al,* 2010). These effects were dose dependent, not due to ventricular leakage and not sexually dimorphic. Furthermore, these effects were likely mediated via BNST PAC1 receptors, as the effects were mimicked by intra-BNST infusion of maxadilan (selective PAC1 receptor agonist) but not the VPAC1/2 receptor ligand vasoactive intestinal polypeptide (VIP) (Roman *et al,* 2014). These results show that BNST PACAP is both necessary and sufficient to produce a stress and anxiety-like phenotype in rodents. Moreover, we have shown that BNST PACAP and PAC1 receptor transcripts (but not VPAC1/2 transcripts) are upregulated following chronic stress. These results suggest that the PACAP system may be upregulated and thus more sensitive to PACAP release following chronic stress. To test this hypothesis, the current experiment examined whether prior chronic stress (i.e., CVS) could potentiate the behavioral response to a subthreshold intra-BNST PACAP infusion.

2.2. Methods

2.2.1. Animals

Adult male (200-225 g) Sprague-Dawley rats (n=36) were obtained from Charles River Laboratories (Canada). Rats were single-housed, maintained on a 12h light/dark cycle (lights on at 0700 h) and food and water were available ad libitum. Rats were allowed at least one week of habituation in their home cages prior to any experimentation. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

2.2.2. Surgical Procedures

For intra-BNST infusions, rats were anesthetized with isoflurane vapor (1.5—3.5%), and secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with "blunted" earbars. A midline head incision was made to expose the skull. After cleaning and sterilizing the skull, four burr holes were made, and four screws were inserted into the burr holes to provide skullcap stability. Two stainless steel guide cannulae (26 gauge, PlasticsOne, Roanoke, VA) were lowered at a 20º angle toward the midline to a point just above the oval BNST, using the following coordinates from Bregma in mm; AP = -0.1, ML = +3.9, and DV = -5.3 from the surface of the dura (Lezak *et al*, 2014). Once the guide cannulae were lowered into place, stylettes were inserted to ensure cannulae patency and a skullcap was constructed with dental cement to secure cannulae and stylettes in place. Immediately following surgery, rats were subcutaneously administered Lactated Ringer's Solution to provide hydration, and Carprofen (5 mg/kg) (Pfizer) for analgesia before being returned to their home cages

for one week of post-surgery recovery. During recovery all rats were observed and weighed daily. Additionally, all rats received a second subcutaneous injection of Carprofen (5 mg/kg) 24h following surgery.

2.2.3. Chronic Variate Stress (CVS)

Following postsurgery recovery, rats were randomly assigned to control or chronic variate stress (CVS) groups. Control group rats were handled and weighed daily, and housed in their home cages until infusion. The chronically stressed group of rats underwent a CVS paradigm in which rats were exposed to one of 5 different stressors (oscillation, forced swim, footshock, restraint and pedestal) each day for 7 days, as previously described in detail (Hammack *et al*, 2009). All rats were exposed to the same order of stressors for the same duration (**Error! Reference source not found.**).

For the oscillation stressor, rats were placed inside a plastic chamber 28 cm x 17 cm x 13 cm (L x W x H), that was secured to a clinical rotator and oscillated at low to medium speed for 30 min.

For forced swim, rats were placed in a cylindrical container 29 cm x 37 cm (D x H) that was filled with room temperature water to a depth that prevented the rats tail from touching the bottom. After 5 mins of monitored swimming, rats were placed in a holding chamber for 30 min prior to being returned to their homecage.

Footshock involved placing the rats inside a Plexiglas conditioning chamber (Med Associates, St. Albans, VT) 30 cm x 25 cm x 35 cm (L x W x H). After a 5 min acclimation period, two 1.0 mA 5 s scrambled footshocks were delivered

through the grid floor with a 5 minute inter-trial interval. Following the second shock, rats remained in the chamber an additional 5 min and were then returned to their homecage.

Restraint consisted of placing each rat into a cylindrical restraining device 9 cm x 15 cm (D x H) for 60 min. Following the 60 min, rats were returned to their homecage.

For pedestal, rats were placed on an elevated platform 20 cm x 20 cm (L x W) that was 60 cm from the floor. Rats remained on the pedestal for 30 min and were then returned to the homecage.

2.2.4. Startle Apparatus

Each acoustic startle stabilimeter chamber consisted of an 8 cm x 15 cm x 15 cm acrylic and wire-mesh cage with four stainless steel floor bars spaced 18 mm apart. The cage was suspended between compression springs within an acrylic frame located within a 90 cm x 70 cm x 70 cm ventilated sound-attenuating cubical. Chamber movement resulted in the displacement of an accelerometer (Model U321AO2; PCB Piezotronics, Depew, NY), which was fixed to the bottom of the cage; the resulting voltage was proportional to the velocity of displacement. The analog output of the accelerometer was amplified (PCB Piezotronics, Model 483B21) and digitized on a scale of 0—10 V by an InstruNET analog to digital converter (GW Instruments, Model 100B; Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-trough voltage during the first 200 ms after the stimulus onset. Startle responses were evoked by 50 ms white-noise bursts

generated by a Macintosh G3 computer sound file, amplified by a Radio Shack Amplifier (100 W; Model MPA-200; Tandy, Fort Worth, TX), and delivered through a high frequency speaker (Radio Shack Super-Tweeter; Tandy, Fort Worth, TX) located 5 cm from the back of each cage.

2.2.5. Cannulae Verification

Upon completion of each experiment, rats were anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline containing 0.1% heparin (Sagent, Schaumburg, IL) followed by 10% formalin. Brains were then removed and postfixed for 24h in 10% formalin. Fixed tissue was sectioned at 60 µm on a cryostat and mounted on subbed slides for staining with cresyl violet. Following staining, slides were coverslipped and cannulae placements were verified using a 4x Nikon Objective on an Olympus light microscope.

2.2.6. Statistics

Statistical analyses were completed using SPSS version 21 (IBM Software, Armonk, NY) and all graphical representations were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Rats were eliminated from analysis for BNST cannulation experiments if the cannulae fell outside the histological boundary of the BNST. Additionally, rats were eliminated if their startle amplitude was more than two standard deviations away from the treatment mean. Two-way ANOVA or Repeated Measures ANOVA was used to calculate overall group differences and interactions followed by Tukey's post hoc comparisons.

2.2.7. Experimental Procedure

Rats were implanted bilaterally with guide cannulae aimed at the dorsal BNST. Postoperative care was completed as described above. Following recovery, rats were administered 2 baseline startle test on consecutive days in which they were placed into the startle chambers and allowed a 5 min acclimation to the chamber. The response to 33 randomized noise burst (described above) that varied in intensity (95, 100, or 105dB) with a 30 s intertrial interval was then determined as the average response across the session. After each baseline startle testing session, rats were returned to their home cages. Based on the average startle across all 3 noise burst intensities for the 2 days of baseline startle rats were "matched" on their average startle to 1 of 4 experimental groups: control/PACAP (n=10), control/vehicle (n=6), CVS/PACAP (n=10), or CVS/vehicle (n=8), so that baseline startle amplitude did not differ between groups. For the subsequent 7 days, stressed rats were treated with CVS as described above; control rats were handled and weighed but not otherwise stressed. On the 8th day, prior to infusions, all rats were returned to the startle chambers and received a 3rd startle test that was identical to the prior baseline testing sessions. Immediately after this third test, rats were removed from the startle chamber and bilaterally infused with 0.5 µl 0.05% bovine serum albumin (BSA)/vehicle (Santa Cruz Biotech., Santa Cruz, CA) or 0.5 µg PACAP38 (American Peptide Co., Sunnyvale, CA) in 0.5 µl BSA vehicle. The PACAP dose was chosen as we have previously shown it to be below the threshold required to produce anxiety-like behavior and corticosterone release when infused into the BNST (Hammack *et al*, 2009; Lezak *et al*, 2014). Infusions were

completed in the colony room under light restraint. For each infusion: the stylette was removed and an internal cannula that extended 1 mm beyond the end of the guide cannula was inserted. Injections were completed using a 10 µl Hamilton syringe connected to the internal cannula with PE50 plastic tubing. A volume of 0.5 µl was infused into the BNST over the course of 60 s. Following infusion, the internal cannula was left in place for an additional 60 s to allow for diffusion of the drug away from the infusion site. The internal cannula was then removed, and an identical infusion was completed for the opposite side. After infusions rats were returned to the home cage for 15 minutes until subsequent testing.

Fifteen minutes after infusion, the rats were returned to the startle chamber and after a 5 min acclimation period, rats were administered a 4th and final startle test that consisted of 72 startle stimuli that varied in intensity. After the final startle test, rats were returned to their home cages.

2.3. Results

The BNST of adult male rats was bilaterally cannulated and a subthreshold dose of PACAP was infused into the BNST of chronically stressed and control rats 15 min prior to startle testing. BNST injection sites are depicted in Figure 1. Based on histology, no animals were excluded from analysis; however, outlier analyses revealed 2 subjects with startle amplitudes more than 2 standard deviations outside the treatment mean, and thus these subjects were excluded from further analyses.

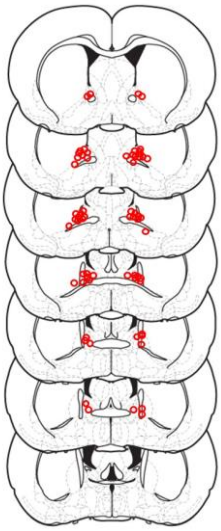

Figure 1: Experiment 1 BNST PACAP infusion sites. Placements of cannulae tips for rats that received 0.5 µg PACAP38 (0.5 µl/side) intra-BNST infusions. Open dots represent site(s) of drug infusion depicted on coronal sections as a distance (mm) from Bregma (Histological figures modified from Paxinos and Watson, 2009).

As can be seen in Figure 2, a typically subthreshold dose of PACAP38 bilaterally infused into the BNST increased startle amplitude later in the startle test session, but only in rats previously exposed to the CVS paradigm.

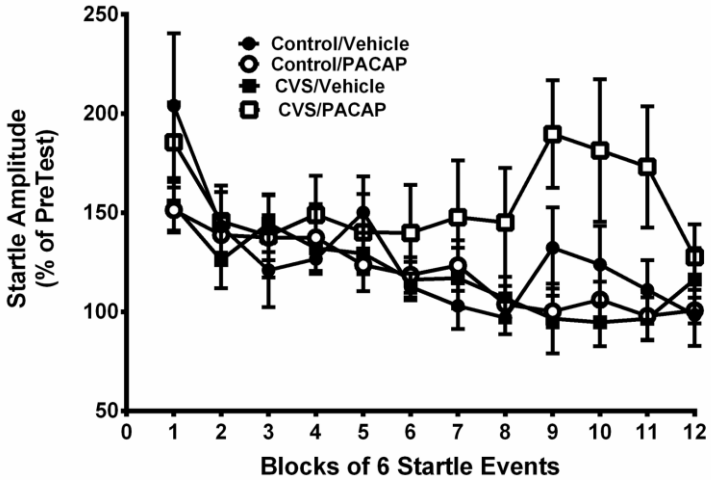

Figure 2: CVS Potentiates the Behavioral Response to Intra-BNST PACAP Infusion. Intra-BNST PACAP infusion increased startle amplitude but only in rats previously exposed to CVS. Data represent startle amplitude after infusion as a percent of pre-infusion Pretest startle. Significant 3-way Blocks x CVS x PACAP interaction, $p < 0.01$. The effects of PACAP vary across test blocks.

The data are presented as startle amplitude across the 12 3 minute blocks (i.e., 6 30s startle events) of test post-infusion as a percent of the last 9 minutes (18 startle events) of preinfusion/pretest startle. Repeated Measures Analysis of Variance revealed a significant 3-way Blocks x CVS x PACAP interaction across all test bins, F (13, 403) = 2.084, p = 0.01. However, there were no statistically significant main effects of CVS or PACAP, suggesting that the effects of PACAP vary across test blocks depending on whether the animal was chronically stressed or not.

To further investigate the effects of PACAP across test blocks, the 12 test blocks were broken into thirds and assessed with two-way ANOVA's (Figure 3). CVS nor PACAP had effects on startle amplitude in the first or second thirds of the test session (Figure 3A and B). In the last third of test (i.e., blocks 9-12), however, intra-BNST PACAP increased startle amplitude, but only in those rats exposed to CVS (Figure 3C). Two-way ANOVA revealed a significant CVS x PACAP interaction, F (1, 29) = 5.156, p = 0.03, but no statistically significant main effects of CVS or PACAP, again suggesting that stress potentiates the behavioral response to intra-BNST PACAP infusion.

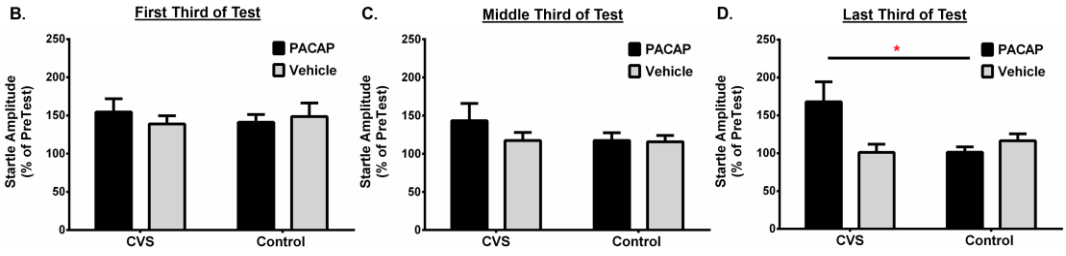

Figure 3: CVS nor PACAP had an effect on startle during the first (A) or second third (B) of the test session. (C) Intra-BNST PACAP increased startle amplitude only during the last third of the test session, and only in rats exposed to CVS, suggesting that stress potentiates the behavioral response to BNST PACAP infusion. Data represents mean +/- SEM. *Significantly different at p < 0.05.

2.4. Discussion

Results from experiment 1 showed that only those rats that had been previously exposed to CVS exhibited an increase in startle amplitude at approximately 30 minutes after a normally subthreshold dose (0.5 μg) of intra-BNST PACAP. These results suggest that CVS induces plasticity within the BNST, making it more sensitive to PACAP, resulting in a potentiated behavioral response. Because we have previously shown these effects to be mediated by PAC1 receptors, and PAC1 receptor transcripts are increased by CVS, this sensitivity is likely mediated via PAC1 receptor activation; however, this was not yet confirmed. The current results also further suggest that stress-induced sensitization in behavioral responses to PACAP likely plays a critical role in mediating the consequences of repeated stressor exposure.

In the current experiment, the potentiated startle effect was not observed until later in the startle test session at approximately 30 minutes post PACAP infusion. Walker, Davis and colleagues have shown a similar delayed onset in CRH-enhanced startle, with effects not becoming evident until 20 minutes after intra-BNST CRH infusion (Walker *et al*, 2003). They have argued that fear-potentiated startle represents conditioned fear; whereas CRH-enhanced startle more closely represents anxiety as it shows a gradual onset and prolonged effects (Lee and Davis, 1997; Walker *et al*, 2003). Thus, the current results suggest that intra-BNST PACAP infusion, like intra-BNST CRH infusion, also produces a sustained and long-lasting change in behavior and a persistent anxiety-like state, as opposed to a fear state, and this anxiety-like behavioral response becomes potentiated following chronic stress. This interpretation is

further supported by Hammack et al, who has shown a similar time effect with the initial PACAP-induced anxiogenic effects increasing over the test sessions as well as a long-lasting (7 day) intra-BNST PACAP effect on light-enhanced startle, which like CRH-enhanced startle is argued to be more akin to anxiety (Hammack *et al,* 2009; Walker *et al,* 2003). Thus, although we have shown the greatest effects of intra-BNST PACAP on the stress (i.e., corticosterone) response at 30 minutes post PACAP infusion, the current study in aggregate with previous studies suggest that the initial activation of these PACAP and/or CRH neuroendocrine systems (i.e., long-duration stimuli) is enough to activate and produce a delayed, yet long-lasting potentiation in anxiety-like behavior, representing a PACAP induced neuroplasticity within the BNST, resulting in a long-term increase in anxiety-like behavior.

There is a close relationship between the PACAP and CRH systems; especially among PVN and BNST neurons where PACAPergic fibers densely innervate CRH neurons (Kozicz *et al,* 1997; Missig *et al,* 2014). Notably, chronic stress has been shown to increase BNST CRH, mediating an increased anxiety-like behavioral phenotype (Fox *et al,* 2008). Furthermore, we have shown increased PACAP expression within these same brain regions following chronic stress, and intra-BNST PACAP infusion (current study) as well as intra-BNST CRH enhanced baseline startle. Together these results suggest that PACAP may mediate increased plasticity and function specifically within BNST CRH neurons. It could be argued that PAC1 receptors upregulate specifically on BNST CRH neurons. Future studies are needed to examine this possibility but given the advancements in genetics and viral

vectors, this hypothesis could be examined. For instance, a cre-animal genetically modified to express GFP in CRH neurons could be double-labeled with a PAC1 receptor antibody; if coexpression increased following chronic stress, this would suggest that the stress-induced changes in plasticity are mediated via upregulation of PAC1 receptors on BNST CRH neurons.

The results from Roman et al. (2014) suggest that BNST PACAP/PAC1 receptor signaling mechanisms may coordinate the behavioral and endocrine consequences of stress. Furthermore, chronic injection of stress levels of corticosterone fails to produce the chronic stress-induced increases in PACAP transcript (Lezak *et al*, 2014). These results suggest that the BNST PACAP system likely sits upstream of the corticosterone system as well as the other physiological outputs of stressor exposure. The current study further implicates the BNST PACAP system as an upstream mediator of the corticosterone as intra-BNST PACAP infusion was also able drive an enhanced startle response. Furthermore, taken with the circuitry laid out by Walker, Davis and colleagues (Lee and Davis, 1997; Walker and Davis, 1997; Walker *et al*, 2003), the results suggest that PAC1 receptors could be upregulated on CRH neurons in the BNST, leading to stronger activation of these CRH neurons projecting to the nucleus reticularis pontis caudalis (Lee and Davis, 1997), resulting in the enhanced anxiety-like startle response. These results also further implicate the BNST PACAP system as a good potential therapeutic target in stress and anxiety-related pathologies like PTSD.

CHAPTER 3: CVS POTENTIATES THE ENDOCRINE RESPONSE TO INTRA-BNST PACAP INFUSION

3.1. Introduction

Experiment 1 determined that intra-BNST infusion of a subthreshold dose of PACAP38 following CVS does produce an anxious phenotype, as measured by an increase in startle amplitude. Given that the behavioral response to PACAP was sensitized following chronic stress, the corticosterone response to PACAP may also be potentiated. Thus, experiment 2 examined the effects of a subthreshold dose of PACAP38 following CVS, on the corticosterone response, using procedures similar to experiment 1.

3.2. Methods

3.2.1. Animals

Adult male (200-225 g) Sprague-Dawley rats (n=39) were obtained from Charles River Laboratories (Canada). Rats were single-housed, maintained on a 12h light/dark cycle (lights on at 0700 h) and food and water were available ad libitum. Rats were allowed at least one week of habituation in their home cages prior to any experimentation. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

3.2.2. Surgical Procedures

All surgical and post-operative producers were identical to those used in experiment 1.

3.2.3. Chronic Variate Stress (CVS)

Following one week of postoperative recovery, rats were randomly assigned by weight into control (n=21) or CVS (n=18) groups. Control rats were handled and weighed daily, and housed in their home cages until infusion; stressed rats underwent the 7 days of CVS described above. Twenty-four hours after the last stressor, animals were weighed and bilaterally infused into the BNST with vehicle or PACAP38 as described above.

3.2.4. Corticosterone Enzyme-Linked Immunoassay

Following collection of plasma from tail nick blood samples, a corticosterone enzyme-linked immunoassay (CORT EIA, Enzo Life Sciences, Farmingdale, NY) was used to determine plasma corticosterone levels. Plasma samples were first diluted 1:30 and heated in a 75ºC water bath for approximately one hour to denature corticosterone binding globulin (Buck *et al,* 2011). The interassay coefficient of variation was X.X% and the sensitivity of the assay was 27.0 pg/ml.

3.2.5. Cannulae Verification

Using procedures identical to experiment 1, rats were anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline containing 0.1% heparin (Sagent, Schaumburg, IL) followed by 10% formalin. Brains were then removed and postfixed for 24h in 10% formalin. Fixed tissue was sectioned at 60 µm on a cryostat and mounted on subbed slides for staining with cresyl violet. Following staining, slides were coverslipped and cannulae placements were verified using a 4x Nikon Objective on an Olympus light microscope.

3.2.6. Statistics

Statistical analyses were completed using SPSS version 21 (IBM Software, Armonk, NY) and all graphical representations were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Rats were eliminated from analysis for BNST cannulation experiments if cannulae placement fell outside the histological boundary of the BNST. Additionally, rats were eliminated if their mean corticosterone response was more than two standard deviations away from the treatment mean. Two-way ANOVA was used to calculate overall group differences and interactions followed by Tukey's post hoc comparisons. Some comparisons were made using *t*-test.

3.2.7. Experimental Procedure

Male rats were bilaterally cannulated with guide cannulae aimed at the oval BNST as described above. Following one week of postoperative recovery, rats were matched by weight into control (n=21) or CVS (n=18) groups. Control rats were handled and weighed daily, and housed in their home cages until infusion; stressed rats underwent the 7 days of CVS described above. Twenty-four hours after the last stressor, animals were weighed and bilaterally infused into the BNST with vehicle or PACAP38 as described above. Following bilateral infusion, the rat was returned to the home cage and remained in the colony room for 30 minutes until blood sampling.

While blood sampling was conducted in a nearby room the time required to transport animals and obtain the blood sample was less than 3 min, in order to avoid contamination of the sample with the increase in corticosterone release associated with cage removal. Thirty minutes post-infusion, blood was collected via a small tail nick with the rat under light restraint. This time point was chosen because we have previously shown increased corticosterone following intra-BNST PACAP (1µg) at 30-60 min post infusion (Lezak *et al,* 2014). The sample was immediately placed into a refrigerated centrifuge and spun between 2000 and 4000 rpm for 15-20 minutes to separate the plasma. Plasma was then collected and stored at –20 ºC until corticosterone quantification via a CORT EIA (see above).

3.3. Results

Experiment 2 examined the effects of a subthreshold dose of PACAP38 following CVS, on the corticosterone response. Using procedures similar to experiment 1, adult male rats were bilaterally cannulated and infused with a subthreshold dose of PACAP38 following CVS, and plasma corticosterone levels were assessed. BNST injection sites are depicted in Figure 4. No subjects were removed for cannulae placements, however, outlier analysis revealed a single rat with unusually high plasma corticosterone (greater than 2 SD's above the mean) who was excluded from further analyses.

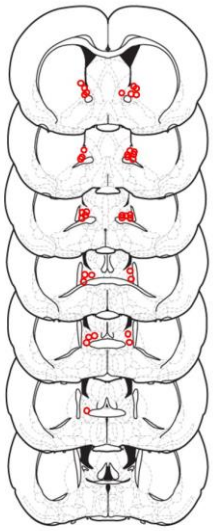

Figure 4: Experiment 2 BNST PACAP infusion sites. Cannulae placements for rats that received 0.5 µg PACAP38 (0.5 µl/side) intra-BNST infusions. Open dots represent site(s) of drug infusion depicted on coronal sections as a distance (mm) from Bregma (Histological figures modified from Paxinos and Watson, 2009).

There was an increase in plasma corticosterone 30 minutes after intra-BNST PACAP infusion, but only in the rats previously exposed to CVS (Figure 5). Two-way ANOVA revealed a significant main effect of CVS, F (1, 37) = 13.05, $p < 0.001$, and a significant main effect of PACAP, F (1, 37) = 7.016, $p = 0.01$, on plasma corticosterone levels. Additionally, results revealed a trending interaction between CVS and PACAP on plasma corticosterone levels, F (1, 37) = 3.401, $p = 0.07$. Further examination of the trending interaction with subsequent t-test revealed significant differences in plasma corticosterone levels between the CVS/PACAP and CVS/Vehicle groups, t (17) = 3.53, $p < 0.01$ as well as the CVS/PACAP and Control/PACAP groups, t (17) = 3.53, $p < 0.01$. There was no significant difference in corticosterone levels between the Control/PACAP and Control/Vehicle groups, t (20) = 0.54, *ns.*

The results of the t-tests further argue the interaction between CVS and PACAP, suggesting that, like the behavioral response in Experiment 1, CVS also sensitizes the corticosterone response to intra-BNST PACAP infusion.

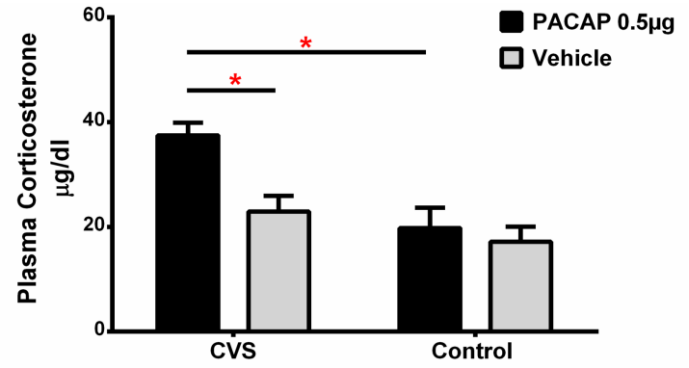

Figure 5: CVS potentiates the corticosterone response to intra-BNST PACAP infusion. Plasma corticosterone was elevated 30 m after BNST PACAP infusion, but only in rats exposed to CVS, suggesting that stress also potentiates the endocrine response to intra-BNST PACAP infusion. Data represents mean +/- SEM. *Significantly different at p < 0.05

3.4. Discussion

In experiment 2, rats that had been previously exposed to CVS exhibited an increase in plasma corticosterone at 30 minutes post PACAP infusion as compared to non-stressed/control PACAP-infused rats. These results suggest that CVS is capable of not only potentiating the behavioral response to subthreshold intra-BNST PACAP infusion, but also the corticosterone response, replicating and extending previous findings implicating increased plasticity within the BNST, resulting in a sensitizes response to PACAP signaling. Thus, like experiment 1, experiment 2 further supports a role for stress-induced plasticity within the BNST, sensitizing this arm of the stress response to subsequent PACAP release and signaling. This sensitized BNST PACAP system likely represents one of the primary mediators of the negative consequences of repeated stressor exposure.

We know that CRH and PACAP are both are highly expressed in close proximity, but form distinct populations within the oval nucleus of the BNST (Kozicz *et al,* 1997; Missig *et al,* 2014), and chronic stress increases endogenous PACAP expression selectively in the oval nucleus in patterns that are distinct from CRH patterns (Hammack and May, 20014; Roman *et al,* 2014). Thus, it seems likely that these distinct populations could interact within the dBNST to drive HPA axis activity and behavioral responding to stressful or emotional stimuli. Furthermore, the current study and others suggest that intra-BNST PACAP can mimic and intra-BNST PACAP receptor antagonism prevents, many of the consequences of chronic stress, including HPA axis responsiveness and increased levels of corticosterone (Lezak *et al,* 2014; Roman *et al,* 2014). These findings in aggregate suggest a critical BNST to PVN circuitry that is responsible for driving the enhanced corticosterone response. Thus, it seems likely that chronic stress produces an increase in endogenous PACAP expression and levels within the oval nucleus. This likely results in an upregulation of PAC1 receptors within the oval nucleus which could activate local oval nucleus CRH neurons to drive downstream corticosterone producing neurons in the PVN and a subsequent increase in corticosterone facilitating the stress response and anxiety-related behavior.

Again, both CRH and PACAP are highly expressed in the dBNST, and the interaction between these distinct populations are thought to drive PVN activity and subsequent corticosterone responses. However, it remains unclear as to whether the dBNST projects directly to the PVN to drive this response. Instead studies suggest that dBNST plays more of an integration and regulatory function, while the

ventrolateral BNST (vlBNST) sends direct projections to the PVN, driving its activity (Dong and Swanson, 2003; 2006). That is, stress acts on PACAPergic fibers and CRH neurons in the dBNST, where the information is integrated; this is then sent to the vlBNST, possibly via known dBNST-vlBNST GABAergic projections or possibly CRH or PACAPergic projections from dBNST. Within vlBNST, excitatory CRH, inhibitory GABA and possibly PACAP neurons project to and act on PACAP and/or CRH neurons within the PVN to produce the corticosterone response. Thus, dBNST regulates vlBNST, which then projects and drives PVN activity to produce the corticosterone response.

Despite the significant interaction and differences among the groups in experiment 2, the control animals still displayed unusually high levels of corticosterone. Given that this hormone is released in response to a stressor in order to bring the animal back into homeostasis, and that no other stressors were presented between the injection and blood collection, it seems like that the current studies injection procedures were inherently stressful. Indeed, we anticipated this and took precautions to minimize the natural stress response, however, it appears that the animals still experienced a stressful experience. This is unlikely to account for the differences observed among groups however, as experimental animals were exposed to the same infusion procedures controlling for this possibility.

CHAPTER 4: INTRA-BNST VIP INFUSION HAS NO EFFECT ON CIRCULATING CORTICOSTERONE

4.1. Introduction

Experiment 1 determined that intra-BNST infusion of a subthreshold dose of PACAP38 following CVS results in an anxious phenotype, and experiment 2 to showed that the endocrine response to PACAP was also potentiated following stress. Experiment 3 was designed to determine whether this sensitized response was mediated via PAC1 receptors. Recall that PACAP binds to PAC1R's and VPAC1&2 R's with similar affinities, whereas VIP binds selectively to VPAC receptors, but not PAC1 receptors. Thus, experiment 3 was designed to determine whether CVS could potentiate the response to BNST VIP infusion.

4.2. Methods

4.2.1. Animals

Adult male (200-225 g) Sprague-Dawley rats (n=33) were obtained from Charles River Laboratories (Canada). Rats were single-housed, maintained on a 12h light/dark cycle (lights on at 0700 h) and food and water were available ad libitum. Rats were allowed at least one week of habituation in their home cages prior to any experimentation. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

4.2.2. Surgical Procedures

All surgical and post-operative producers were identical to those used in experiment 1 and 2.

4.2.3. Chronic Variate Stress (CVS)

Following one week of postoperative recovery, rats were randomly matched by weight into control (n=16) or CVS (n=17) groups. Control rats were handled and weighed daily, and housed in their home cages until infusion; stressed rats underwent the 7 days of CVS described above.

4.2.4. Corticosterone Enzyme-Linked Immunoassay

Following collection of plasma from tail nick blood samples, a corticosterone enzyme-linked immunoassay (CORT EIA, Enzo Life Sciences, Farmingdale, NY) was used to determine plasma corticosterone levels. Plasma samples were first diluted 1:30 and heated in a 75ºC water bath for approximately one hour to denature corticosterone binding globulin (Buck *et al,* 2011). The interassay coefficient of variation was X.X% and the sensitivity of the assay was 27.0 pg/ml.

4.2.5. Cannulae Verification

Using procedures identical to experiment 1 and 2, rats were anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline containing 0.1% heparin (Sagent, Schaumburg, IL) followed by 10% formalin. Brains were then removed and postfixed for 24h in 10% formalin. Fixed tissue was sectioned at 60 µm on a cryostat and mounted on subbed slides for staining with cresyl violet. Following staining, slides were coverslipped and cannulae placements were verified using a 4x Nikon Objective on an Olympus light microscope.

4.2.6. Statistics

Statistical analyses were completed using SPSS version 21 (IBM Software, Armonk, NY) and all graphical representations were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Rats were eliminated from analysis for BNST cannulation experiments if cannulae placement fell outside the histological boundary of the BNST. Additionally, rats were eliminated if their mean corticosterone response was more than two standard deviations away from the treatment mean. Two-way ANOVA was used to calculate overall group differences and interactions followed by Tukey's post hoc comparisons. Some comparisons were made using *t*-test.

4.2.7. Experimental Procedure

All procedures were identical to those used in experiment 2. Twenty-four hours after the last stressor, animals were weighed and bilaterally infused into the BNST with 0.5 µl 0.05% BSA/vehicle (Santa Cruz Biotech., Santa Cruz, CA) or 0.73 µg VIP (American Peptide Co., Sunnyvale, CA) in 1.0 µl BSA vehicle. The VIP dose was chosen as it was determined to be equimolar (221µm) to the PACAP dose used in experiments 1 and 2, thereby limiting potential effects due to inherent pharmacodynamic differences in the ligands used. Following bilateral infusion, the rat was returned to the home cage and remained in the colony room for 30 minutes until blood sampling.

While blood sampling was conducted in a nearby room the time required to transport animals and obtain the blood sample was less than 3 min, in order to avoid contamination of the sample with the increase in corticosterone release associated with cage removal. Thirty minutes post-infusion, blood was collected via a small tail nick

with the rat under light restraint. This time point was chosen because we have previously shown increased corticosterone following intra-BNST PACAP (1µg) at 30-60 min post infusion (Lezak *et al*, 2014). The sample was immediately placed into a refrigerated centrifuge and spun between 2000 and 4000 rpm for 15-20 minutes to separate the plasma. Plasma was then collected and stored at –20 ºC until corticosterone quantification via a CORT EIA (see above).

4.3. Results

Using procedures identical to that of experiment 2, adult male rats were bilaterally cannulated (Figure 6) and infused with VIP following CVS. No subjects were removed from the analyses based on cannulae placements or outlier analysis.

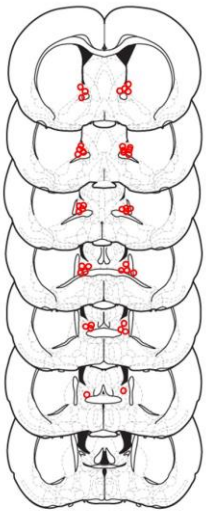

Figure 6: Experiment 3 BNST VIP infusion sites. Cannulae placements for rats that received 0.7 µg VIP (221µM) (0.5 µl/side) intra-BNST infusions. Open dots represent site(s) of drug infusion depicted on coronal sections as a distance (mm) from Bregma (Histological figures modified from Paxinos and Watson, 2009).

Following infusion of VIP into the BNST, plasma corticosterone levels were assessed. Two-way Analysis of Variance revealed no effect of CVS, F (1, 29) = 0.04, *ns*, and no effect of VIP, F (1, 29) = 0.001, *ns*, on plasma corticosterone levels (Figure 7). Furthermore, there was no significant interaction between CVS and VIP on corticosterone levels, F (1, 29) = 2.218, *ns*, suggesting that the potentiated endocrine response observed in experiment 2 was mediated via PAC1 receptors, not VPAC receptors.

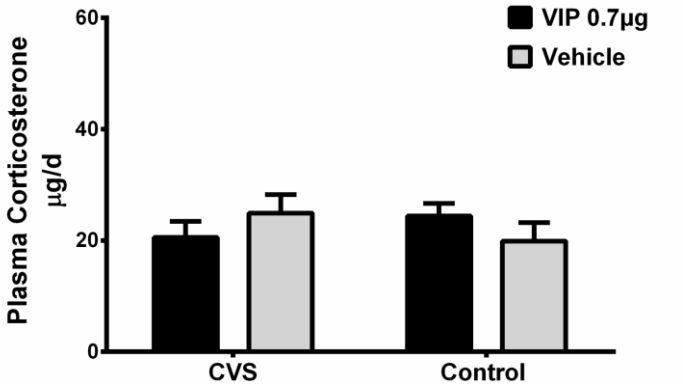

Figure 7: Intra-BNST VIP infusion has no effect on the corticosterone response following CVS. Intra-BNST VIP that was equimolar to the PACAP38 used in experiments 1 and 2 produced no effect on plasma corticosterone levels following CVS, suggesting that the sensitized corticosterone response observed in Experiment 2 was mediated via PAC1R's. Data represents mean +/- SEM.

4.4. Discussion

Results from experiments 1 and 2 provided evidenced for stress-induced plasticity and alterations in PACAP signaling within the BNST promoting maladaptive behavioral and endocrine responses. However, the results did not determine which receptors: PAC or VPAC1/2 were responsible for mediating these responses.

Recall that PACAP effects can be mediated via 3 receptor subtypes. Of these receptor subtypes, the PAC1 receptor is selective for PACAP while the VPAC1/2 receptors are demonstrate near equal affinities for PACAP and VIP. Thus, if the effects are mediated via PAC1 receptors then VIP infusion should have no effect on the potentiated responses.

Experiment 3 confirmed this hypothesis and showed that equimolar infusion (0.7 µg) of the VPAC1/2 agonist VIP had no effect on plasma corticosterone levels, even in stressed rats. Thus, the results seen in experiments 1 and 2 are appear to be driven via BNST PACAP/PAC1 receptor signaling.

These results are consistent with our previous finding (reviewed in Hammack and May, 2014) showing that it is PACAP/PAC1, not VPAC, activation and signaling which is responsible for the stress-induced changes in physiology and behavior.

It should be noted that VIP is found in the oval nucleus of the BNST and could have some role in the stressed induced plasticity. This doesn't seem likely, as studies have shown that PACAP and VIP display differential expression patterns; moreover, the 2 distinct systems appear non-compensatory in K.O. studies (Girard *et al*, 2006) suggesting distinct roles in biological activities. Our findings support this interpretation.

CHAPTER 5: GENERAL DISCUSSION

The upregulation of BNST PAC1 receptors suggests that following CVS, the BNST may be more sensitive to PACAP release and may be one of the critical mechanism involved in mediating the maladaptive consequences of repeated stressor exposure. The current set of experiments were designed to examine whether chronic stress produces these negative consequences by altering BNST plasticity via a potentiated PACAP system. Specifically, the experiments examined whether CVS could potentiate the behavioral and/or corticosterone response to a subthreshold intra-BNST PACAP infusion.

Results from experiment 1 showed a significant increases in startle amplitude at approximately 30 minutes after a normally subthreshold dose (0.5 μg) of intra-BNST PACAP; however these results were limited to those animals exposed to CVS. Correspondingly, experiment 2 showed a significant increase in plasma corticosterone levels at 30 minutes post subthreshold PACAP as compared to non-stressed PACAP-infused rats following BNST PACAP infusion; again, these results were only observed in those rats subjected to chronic stress. In addition, experiment 3 provided evidence that these sensitized responses were mediated via PAC1 receptors, as equimolar infusion (0.7 μg) of the VPAC1/2 agonist VIP had no effect on plasma corticosterone levels, even in stressed rats. The current results provide evidence for a potentiated BNST PACAP system and PACAP-signaling following chronic stress. Furthermore, the results provide a mechanism through which stress, via altered/sensitized neurotrophic peptide systems and subsequent modification and plasticity

within the BNST may promote the maladaptive consequences of stressor exposure. Additionally, the current study further argues that this system may be an important target for the treatment of stress-related disease.

Roman et al (2014) showed that PACAP antagonism could only block the sensitized portion of the corticosterone response to the open field maze (OFM). That is, PACAP antagonism blunted the corticosterone response only when the animal had been previously exposed to chronic stress, and thus, presumably had sensitized BNST PACAP signaling/activity. When the animal had only been exposed to an acute stressor and ostensibly lacked this sensitized system, PACAP antagonism had no effect on the corticosterone response, implicating BNST PACAP signaling as a key mediator of the sensitized endocrine response. The current results corroborate and extend these finding as a normally subthreshold dose of PACAP following chronic stress was able to sensitize both the behavioral and corticosterone response to intra-BNST PACAP, arguing for plastic alterations and/or increased signaling as a critical mechanism in facilitating the sensitized behavioral and endocrine response following chronic stress; but not acute stress.

We have argued that PACAP is neurotrophic and enhances plasticity, primarily within the BNST; moreover, we has suggested that chronic stress promotes stress- and anxiety-related affective states by modifying the BNST PACAP system (Hammack and May, 2014). This idea is supported by the current study as chronic stress presumably altered PACAP signaling (potentially via upregulated PAC1 receptors) within the BNST, making it more sensitive to subsequent PACAP release, resulting in a

potentiated behavioral and endocrine response.

Thus, the results of the current study, in combination with our previous findings suggest a model (Figure 8) in which the stress-induced plasticity responsible for the consequences of repeated stressor exposure result from BNST PACAP/PAC1 system interactions with the CRH system to produce both a potentiated behavioral (i.e., anxiety) and physiological (i.e., corticosterone) response. Specifically, chronic stress appears to upregulate PACAP and PAC1 receptors in the dBNST. The upregulated PAC1 receptors could be on CRH neurons, or PACAPergic fiber may interact with CRH neurons in the dBNST. This upregulation and signaling within the dBNST results in stronger activation of the CRH neurons that project to the nucleus reticularis pontis caudalis (Lee and Davis, 1997), resulting in the enhanced anxiety-like startle response. Similarly, the increased activation and signaling within the dBNST PACAP and CRH neurons could project directly to the PVN to drive the enhanced corticosterone response, but more likely, enhanced signaling within dBNST CRH and/or GABAergic (and possibly PACAP) projections to the vlBNST results in stronger activation of CRH neurons in the PVN and elevated corticosterone release.

PACAP/PAC1 signaling enhances neuronal activation and when combined with the previous results, suggest this system as a key mediator of the stress induced plasticity observed in the current study. This could drive subsequent neurochemical plasticity effects and resulting plasticity in BNST morphology (i.e., increased dendritic branching and overall BNST volume). For instance, we have shown that PACAP may be enhancing H-currents, thereby promoting high frequency firing and more

peptide release (Lezak and Hammack, unpublished). This could be one mechanism through which this system promotes multiple levels of plasticity.

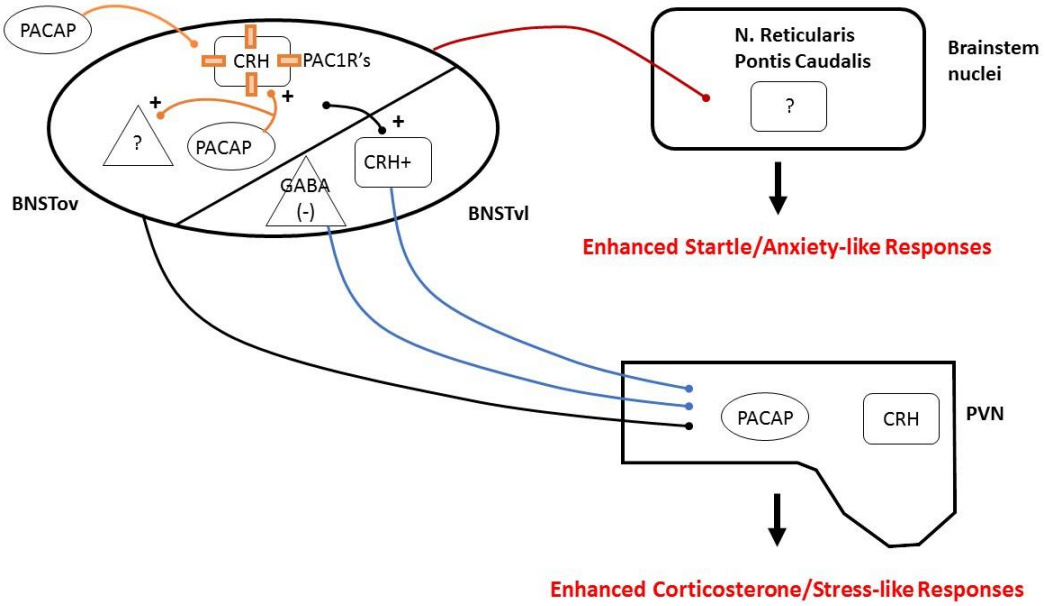

Figure 8: Proposed model of the stress-induced plasticity/alterations within the BNST circuits that mediate the downstream nuclei responsible for the sensitized physiological (corticosterone) and behavioral (startle) responses following chronic stress.

The results in aggregate support the hypothesis that repeated stressor exposure physically alters the neurochemistry, morphology and physiology associated with increases in fear- and anxiety-like behavior to inhibit function within the mPFC and facilitate function within BNST. Additionally, they emphasize the importance of altered neuropeptide expression in stress-related pathologies, and further implicate the PACAP system specifically as a critical system in integrating limbic information within the BNST to produce stress- and anxiety-related psychopathologies.

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