PACAP and PAC1 receptor signaling in chronic stress responses: Implications for anxiety-related disorders

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Dissertation Proposal
Specific Aims

Anxiety disorders are the most common neurological illnesses globally with a lifetime prevalence of over 15%. The healthcare costs associated with mental disorders surpass those of cardiovascular disease, cancer and diabetes, and yet despite the enormous societal burden, the neurobiological mechanisms underlying many of these conditions are unclear and the treatment options remain inadequate. Among factors, repeated exposure to stressors appears central to the etiology of anxiety-related disorders and contributes to a number of other related psychiatric conditions. Following stressor exposure, both central and peripheral systems become engaged in responses aimed at coping and restoring homeostasis. Corticotrophin releasing hormone (CRH) and adrenocorticotropin (ACTH) signaling in the hypothalamic-pituitary-adrenal (HPA) axis become activated centrally to stimulate adrenal glucocorticoid release into the systemic circulation to augment the availability/redistribution of energy stores. Parallel stress-mediated stimulation of the peripheral sympathetic nervous system increases adrenal epinephrine release to heighten “fight or flight” responses. Both of these systems are adaptive in nature and the responses typically dissipate after threat resolution. However, the continuous heightened activation of the same stress networks in modern society have been suggested to burden normal homeostatic mechanisms, and the resulting maladaptive neuroplasticity and disruptive responses appear causal to many neurological and physiological dysfunctions.

The bed nucleus of the stria terminalis (BNST) is one area of convergence for signals mediating emotional valence and metabolic homeostasis, and the stress-induced maladaptive neuroplasticity within the nucleus has been implicated as a contributing factor to HPA endocrine/stress circuit dysregulation and anxiety-like states. Consistent with this hypothesis, chronic stress-induced anxiety-like behaviors are correlated with changes in BNST neuronal morphology and electrochemical properties. Additionally, stimulation of the BNST can elicit endocrine and behavioral responses similar to those elicited by exposure to chronic stress. Behavioral studies suggest that the BNST mediates responses to threatening stimuli that are unpredictable, diffuse and/or of long-duration, suggestive of BNST roles in anxiety states. Increased activation of the BNST has even been demonstrated during anticipatory anxiety in humans with phobias. This contrasts with the anatomically related central nucleus of the amygdala (CeA), which mediates short-term responses to predictable stimuli more akin to “fear”.

Pituitary adenylate cyclase-activating peptides (PACAP) are members of the VIP/secretin/glucagon family of peptides and capable of binding to G protein-coupled PAC1, VPAC1 and VPAC2 receptors to mediate their neurotransmitter and neurotrophic activities. But aside from these well-described functions, many studies from independent laboratories have implicated PACAP signaling in stress-related responses. Central PACAP injections can increase activation of the HPA axis and sympathetic nervous system; PACAP knockout mice display decreased anxiety-like behavior and demonstrate attenuated endocrine and behavioral responses following stressor exposure. Notably, we have found that after chronic variate stress, a paradigm that induces anxiety-like behavior and decreased feeding in rats, PACAP and PAC1 receptor transcript levels are significantly increased within the dorsolateral BNST. Importantly, our lab has previously shown that PACAP injections into this area are sufficient to produce behavioral changes associated with repeated stressor exposure. Polymorphism in PACAP or PAC1 receptor gene has been linked to human behavioral disorders, including schizophrenia and major depressive disorder; more recently, our group has reported that serum PACAP levels and a single nucleotide polymorphism in the PAC1 receptor gene were associated with PTSD in women, while increased PAC1 receptor gene methylation correlated with PTSD in a sex-independent manner.

Yet despite these advances, many fundamental questions regarding the roles of the BNST and PACAP in stress-mediated anxiety behaviors, the specific BNST PACAP receptors mediating the stress-related responses, and the interactions of PACAP with hypothalamic stress pathways remain unclear. In addressing these issues, we hypothesize that: 1) chronic stress-induced PACAP signaling within the dorsolateral BNST is a primary mediator of anxiety-related behavioral responses, 2) BNST PACAP behavioral effects are mediated by PAC1/VPAC2 receptor subtypes; and 3) PACAP intersects with CRH pathways in HPA activation.

**AIM 1:** To determine if stress-induced BNST PACAP expression and signaling are necessary for the behavioral consequences of chronic stress. Q1. Does chronic variate stress increase PACAP expression in specific dorsolateral BNST and amygdala regions? Q2. Is the dorsolateral BNST one principal region for stress-induced anxiety-related responses? Q3. Are chronic variate stress-induced anxiety-related responses mediated by PACAP signaling?
AIM 2: To determine which PACAP receptor within the BNST mediates the behavioral responses to PACAP. Q1. Are the PACAP-mediated anxiety-like responses mediated by PAC1 receptors? Q2. Are VIP and VPAC receptors also implicated in some anxiety-related responses?

AIM 3: To investigate the effects of chronic variate stress and BNST PACAP receptor activation on HPA-axis function. Q1. Does PACAP signaling in the dorsolateral BNST activate the HPA axis? Q2. Does chronic variate stress alter serum PACAP levels?

Significance and Background

Anxiety disorders, including generalized anxiety disorder, post-traumatic stress disorder (PTSD), panic disorder, and phobias, are among the most prevalent neurological illnesses globally. From the World Health Organization (WHO), World Economic Forum (WEF), and Anxiety Disorders Association of America (ADAA), mental illness costs from healthcare and lost productivity have now exceeded those of cardiovascular disease, cancer, and diabetes with a global cost of nearly $2.5 trillion (Bloom et al. 2011). Over 65 million adults are affected by anxiety disorders in the US and EU alone and healthcare costs in the US exceed $42 billion a year, or nearly one-third of the country’s total mental healthcare bill (Smith 2011; Wittchen et al 2011; Greenberg et al. 1999). These exceptionally high costs reflect the many years or even decades long care required by the individuals afflicted, for which the treatment options are often inadequate. From these growing statistics, it is abundantly clear that any measure to identify individuals at risk for anxiety-related disorders, understand the causal and mechanistic underpinnings of the disease, or provide insights to new treatment options will have significant societal benefits. Among stress neuropeptides, several recent studies have implicated pituitary adenylate cyclase-activating polypeptide (PACAP) expression and function in stress-induced anxiety-related behaviors. Heightened PACAP expression and signaling in the BNST, a nexus of limbic stress pathways, appear anxiogenic. More recently, dysregulation of PACAP and its cognate G protein-coupled PAC1 receptor has been associated with PTSD. In this proposal we aim to delineate the roles of BNST PACAP in mediating the anxiogenic responses to chronic stress exposure, identify the PACAP receptor subtypes responsible for anxiety-related signaling, and investigate whether BNST PACAP intersects with HPA-axis function, a site of dysregulation for many anxiety disorders. In addressing these questions, we may identify potential new targets for therapeutics. These efforts are coupled with ongoing work to find novel small molecule PACAP receptor antagonists which may attenuate stress-induced signaling and plasticity that may contribute to anxiety disorder development. Together with the emergence of biomarkers for susceptibility genes to particular affective disorders, these antagonists may present alternative treatment options and offer prophylactic means to stem a growing behavioral disorder population. As stress signaling is also strongly associated with other physiological disorders including obesity/diabetes, cardiovascular disease, inflammation, gastrointestinal/urinary dysregulation and even tumor progression, the implications of these studies may be broad.

Chronic stress

Faced with a homeostatic threat, an organism’s stress-response systems become activated in an effort to redirect the physiological resources and respond to the challenge until the threat is resolved (Sapolsky 2000; Sapolsky et al. 2000; Ulrich-Lai and Herman 2009). The HPA-axis is the primary stress-response circuit; upon activation, parvocellular neurosecretory neurons within the paraventricular nucleus (PVN) of the hypothalamus release CRH from the median eminence into the hypophyseal portal system to induce anterior pituitary ACTH secretion into systemic circulation for adrenal corticosteroid (glucocorticoids and mineralocorticoids) release. The rise in glucocorticoids (cortisol in humans and corticosterone in most other species including rodents) functions to regulate homeostasis in cardiovascular, immunologic, and metabolic processes. In stimulating gluconeogenesis, inhibiting glucose uptake into muscle and adipose tissues, and facilitating lipolysis, glucocorticoid signaling redistributes the available energy to the brain and major muscle groups for “fight or flight” responses. Concomitantly, stressor exposure activates the sympathetic nervous system and causes increased adrenal medullary chromaffin epinephrine release to regulate heart rate, blood pressure, and respiratory function. Once the threat has terminated, the sympathetic outflow is decreased and parasympathetic activation is increased to help recover normal physiological functions. In classical feedback, elevated glucocorticoid levels inhibit hypothalamic CRH and corticotrope POMC gene transcription and peptide synthesis/secretion to diminish HPA activation. Hence, stress responses are adaptive - activated rapidly as necessary and terminated upon threat abatement. However, over-activation of these pathways as in chronic or repeated stress is increasingly appreciated to have damaging physiological and psychological consequences (Schulkin et al. 1998). Continuous activation of stress-response systems can result in maladaptive
neuroplasticity and dysregulated HPA/autonomic functioning leading to abnormal levels of hormones released, inappropriate neuroendocrine signaling, and inabilitys to terminate the stress-mediated responses. Without rectification, these abnormalities can produce not only long term metabolic, cardiovascular, and immunologic damages, but also manifest as behavioral pathologies such as generalized anxiety disorder, PTSD, panic disorder, or depression (McEwen and Stellar 1993; Sautter et al 2003; Marques et al. 2009).

**The BNST mediates stress responses**

Various limbic areas including hippocampus, prefrontal cortex, anterior thalamus, amygdala, and extended amygdala are known to mediate emotional responses as well as regulate hypothalamic activity. The BNST, a subregion of the extended amygdala, has been shown to play an important role in behavioral and HPA-axis responses to stress. Anatomical studies show that this area relays corticolimbic information regarding stressor exposure through direct and indirect projections to the hypothalamus (Dong, Petrovich et al. 2001; Dong, Petrovich et al. 2001). Stimulation of the BNST produces endocrine, cardiovascular, respiratory, and behavioral responses similar to those elicited by anxiogenic stimuli (Dunn 1987; Casada and Dafny 1991) and anxiogenic drugs activate a number of fear-related forebrain nuclei including the BNST (Singewald, Salchner et al. 2003). While the anatomically related CeA appears to mediate fear response systems that terminate quickly, behavioral studies suggest that the BNST mediates sustained responses to threatening stimuli that are unpredictable, diffuse, or of long-duration akin to anxiety-like states (Davis 1998; Walker, Toufexis et al. 2003; Waddell, Morris et al. 2006; Walker, Miles et al. 2009). However, this area has been subdivided into a number of separate regions, with evidence showing differential regulation of the HPA axis by different subnuclei (Dong, Petrovich et al. 2001; Choi, Furay et al. 2007). Anterior BNST lesions decrease PVN CRH mRNA and attenuate the ACTH/corticosterone responses to acute restraint stress; in agreement, stimulation of the anterolateral BNST increases plasma corticosterone in rodents, validating a role for the anterior BNST in HPA activation (Dunn 1987; Herman, Cullinan et al. 1994; Choi, Evanston et al. 2008). In contrast, posterior BNST lesions elevate CRH/ACTH/corticosterone levels following restraint stress implicating an inhibitory influence on HPA responding (Choi, Furay et al. 2008). Following chronic stress, however, the BNST undergoes neuroplasticity, reflected by changes in neuronal morphology and electrochemical properties (Vyas et al. 2003; Pego, Morgado et al. 2008; Joels, Karst et al. 2007), that has been suggested to cause dysregulation of normal stress responses and HPA-axis function leading to anxiety-like states. Like other limbic areas, the BNST expresses PACAP and CRH neurons (Gray and Magnuson 1992; Kozicz, Vigh et al. 1997; Kozicz, Vigh et al. 1998) and consistent with plasticity studies, our laboratory has shown that chronic variate stress increases PACAP and PAC1R transcripts within the dorsolateral BNST and that PACAP injections into this area produce dose-dependent increases in anxiety-like behavior accompanied by decreases in food intake and body weight (Hammack et al. 2009; Hammack et al. 2010). The BNST is therefore an integral site for stress related input and central to understanding stress regulation of behavioral responses and physiological homeostasis.

**PACAP and stress**

PACAP is a neurotransmitter and trophic peptide first identified for its potent abilities to activate anterior pituitary cyclic AMP production. The two alternatively processed peptides, PACAP27 and PACAP38, have widespread expression and actions both centrally and peripherally including stress pathways (for review see Vaudry et al. 2009). PACAP has been shown to stimulate CRH transcription via PKA and induce c-fos and CREB phosphorylation in a majority of PVN CRH neurons, and central PACAP administration can increase plasma corticosterone levels (Grinevich et al. 1997; Agarwal, Halvorson et al. 2005). PACAP is highly expressed in autonomic pathways and shown to be a preganglionic non-cholinergic regulator of sympathetic functions (May et al. 1998; Braas and May 1999). PACAP stimulates sympathetic neuron transmitter and peptide release, and similarly, PACAP release from preganglionic splanchnic nerves can stimulate adrenal medullary epinephrine secretion (Watanabe, Masuo et al. 1992; Hamelin, Tjurmina et al. 2002; Kuri, Chan et al. 2009). The roles of PACAP in stress signaling were reinforced with our observations that PACAP and PAC1R transcript are increased in the dorsolateral BNST following chronic variate stress exposure (Hammack et al. 2009). Acute injections of PACAP into the BNST dose dependently increase baseline startle amplitude as well as decrease exploration time in the open arm of an elevated plus maze, two measures of anxiety-like behavior. Stressed rats typically exhibit diminished weight gain and similarly, BNST PACAP injections also decreased body weight and food intake 24hours after injection (Hammack et al. 2010). Our more recent data has shown that circulating corticosterone is increased following intra-BNST PACAP injection (unpublished results), suggesting BNST PACAP regulation of HPA neurocircuits. These data implicate BNST PACAP as an
important regulator of stress-response systems, and in aggregate implicate heightened PACAP signaling to mediate limbic, HPA, and autonomic stress-induced responses.

The roles of PACAP in stress are further supported by data from PACAP and PAC1 receptor knockout mice. PACAP knockout mice not only exhibit decreased anxiety-like behaviors, but also show altered neuronal and endocrine responses to stress (Hashimoto, Shintani et al. 2001; Otto et al. 2001; Tanaka et al. 2006). From different stressor exposures, PACAP/- mice have an attenuated corticosterone response to novel environment exposure and immobilization stress, but not to cold exposure or ether inhalation, suggesting that PACAP is necessary for emotional stress responses that require limbic system activation. Further, CRH and ACTH administration to PACAP/- mice elicited normal corticosterone responses, demonstrating that the altered stress effects were central and not due to changes in pituitary or adrenal tissue responsiveness (Tsukiyama, Saida et al. 2011). Other studies demonstrated that hypothalamic CRH transcription and adrenal epinephrine biosynthetic enzymes tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) levels were impaired in PACAP/- mice compared with wild type controls after restraint stress (Stroth and Eiden 2010). Unlike wild-type mice, PACAP/- mice in forced-swim tests failed to induce c-fos expression in a number of stress-related brain areas with the greatest variance in the oval nucleus of the BNST (Gaszner et al. 2011). Hence in sum, PACAP/- are less anxious and demonstrate diminished stress responses upon emotional challenges; these observations are entirely consistent with roles for PACAP signaling in stress responding and behavioral phenotypes.

Crossing over to human studies, recent work has also linked VIP and/or PACAP signaling to human behavioral disorders. Just as genetic variations in the VPAC2 receptor gene confer risk for schizophrenia (Vacic et al. 2011), polymorphisms in the PACAP and PAC1 receptor genes have been associated with schizophrenia and major depressive disorder, two mental illnesses that are often linked to stressor exposure and have high comorbidity rates with anxiety disorders (Hashimoto et al. 2007; Hashimoto et al. 2010). Interestingly, serum levels of PACAP and a single nucleotide polymorphism (SNP) in the PAC1 receptor gene were associated with PTSD in women, but PAC1 receptor gene methylation was associated with PTSD in a sex-independent manner (Ressler et al. 2011). The association of PACAP with stress-related disorders in humans further strengthens the rationale to investigate PACAP mechanisms in stress and behavior.

**Innovation:** The most innovative aspect of the proposal is the study of PACAP expression and signaling in stress-related behaviors. PACAP is a pleiotropic peptide known to be involved in neuroprotection, development, and endocrine function, but the study of its roles in chronic, psychogenic stress and anxiety-related behaviors are relatively nascent. While PACAP deficient mice have been studied for alterations in various behaviors, few studies have evaluated endogenous and exogenous PACAP, or PACAP receptor agonist effects on behavior using diverse but complementary compendium of cellular, molecular and neurophysiological tools. Additionally, only a handful of studies have used targeted intracerebral injections of PACAP in vivo. In undertaking these studies, we further clarify roles for BNST PACAP signaling in anxiety-related behaviors; in the process, we also have the potential to identify new targets for affective disorder therapeutics.

**Approach:**

**Overview of proposal**

Using a rat model of chronic stress and in vivo pharmacological methods, we intend to manipulate activity within working neuronal BNST circuits and examine both behavioral and physiological responses. This will involve intra-cerebral targeted injections, multiple behavioral evaluations, and biochemical and molecular assays as end points. Using specific agonists we will determine which of the three PACAP receptors is responsible for the acute effects of PACAP injection as well as how HPA-axis functioning is affected by activation of specific PACAP receptors within the BNST.
Preliminary Data

Chronic variate stress increases BNST PACAP immunoreactivity and mRNA expression

To complement the QPCR data demonstrating enhanced PACAP and PAC1 receptor transcripts in the dorsolateral BNST of chronic variate stressed rats, we have performed immunocytological and in situ hybridization studies to better localize the specific BNST subregion(s) with PACAP expression. Following chronic variate stress, the rats were anesthetized and either perfused with 4% paraformaldehyde for immunocytochemistry or decapitated for in situ hybridization procedures (see methods below). As CRH was localized in previous work to the oval nucleus of the BNST and CeA, CRH antisera was also used in colocalization techniques for comparative distribution analyses. From these studies, both PACAP and CRH were localized to the oval nucleus of the BNST; the punctate PACAP staining patterns however appeared distinct from CRH and were not as dense as those for CRH immunoreactivity (Fig 1). The dichotomy was more evident in the CeA where CRH was localized to the medial CeA and PACAP in the lateral capsular division (Fig 2). In situ hybridization studies corroborated the localization of PACAP to the oval nucleus (Fig 3). From these data, BNST PACAP expression appeared enhanced following chronic stress, in agreement with QPCR results.

Fig 3. Chronic stress increases BNST PACAP mRNA expression.

Tissue sections containing the oval nucleus of the BNST from control and chronically stressed animals were processed using in situ hybridization techniques. 20μm cryosections were labeled using biotin-labeled UTP probes against PACAP mRNA and DAB as a substrate. Compared to control tissues, BNST PACAP transcripts were increased following exposure to chronic variate stress.
**BNST excitotoxic lesions attenuate stress-related weight change**

To demonstrate the importance of the BNST in mediating anxiety-related behaviors, we performed BNST excitotoxic lesions prior to chronic variate stress exposure. In these studies, rats received either vehicle (sham lesion) or NMDA infusions aimed at the oval nucleus of the BNST, and after postsurgical recovery, half of each group was then exposed to the chronic variate stress paradigm. Animal weight change was used as a rapid means of assessing stress-induced anxiety and unlike non-stressed control animals that gained weight steadily, weight gain in stressed animals was very much reduced. Notably, this stress-induced attenuation in weight gain was blunted in the stressed BNST excitotoxic lesioned animals (Fig 4). Among targeted BNST lesion studies, this is the first to show that the dorsolateral BNST is a principal region involved in chronic stress-induced anxiety-related behaviors.

**BNST PACAP signaling in anxiety-related behaviors is mediated by PAC1 receptors.**

PACAP can bind to all three PACAP/VIP receptor subtypes and although chronic stress enhanced PAC1 receptor transcript expression without augmenting either of the VPAC receptors, whether BNST PACAP signaling in anxiety-related behaviors is mediated by PAC1 receptors is still unclear. To examine this issue, we have initiated studies injecting maxadilan, a sandfly saliva PAC1 receptor selective peptide agonist, and VIP, which only binds to VPAC receptors with high potency, into the BNST. Similarly, using animal weight change as a proxy for anxiety-related responses, acute injections of various doses of maxadilan produced weight losses comparable in magnitude to changes observed for PACAP. By contrast, acute BNST injections of VIP had no apparent effects on animal weight (Fig 5). Hence these data implicate PAC1 receptor signaling for anxiety-like behaviors, but these studies will be amplified to include dose dependent effects on weight change and behaviors on elevated plus maze and open field tests.

**PAC1 receptor antagonist blocks chronic variate stress-mediated responses**

A key test to demonstrate BNST PACAP signaling in stress relies on the abilities for PACAP receptor antagonists to block the chronic stress-induced behavioral effects. However, there are few VIP/PACAP antagonists and the available compounds have been shown not to be selective for specific PACAP receptor subtypes. Among these, PACAP(6-38), the amino truncated form of PACAP38, has been widely used as an antagonist to PAC1 and VPAC2 receptors. Hence as a test in principle, we chronically infused PACAP(6-38) into the dorsolateral BNST in attempts to block stress-induced anxiety-like behavior and weight change in the chronic variate stress paradigm. In this study, rats received bilateral cannulae implants that were attached to osmotic minipumps containing vehicle or PACAP(6-38). Following postsurgical recovery, half the rats in each group (n = 8 per group) was exposed to chronic variate stress; changes in animal weight and food consumption were recorded daily. The non-stressed vehicle infused rats as well as non-stressed rats receiving PACAP antagonist, PACAP(6-38) exhibit a steady increase in body weight (Fig 6B; filled and open circles, respectively) demonstrating no effects of the antagonist alone on weight change. By contrast, repeated variate
stress over the last week of study attenuated weight gain in the vehicle-treated rats (closed triangles). Continuous BNST PACAP(6-38) infusions during the chronic stress period blocked the stress-induced changes in weight gain (open triangles); hence, unlike the plateau in weight change observed in the stress/vehicle group, stressed rats receiving PACAP(6-38) increased their body weight at rates comparable to those of non-stressed controls. Similarly in behavioral assessments, chronic variate stress produced anxiety-like behaviors on novelty and elevated plus-maze tests (Fig 6C,D); 2-way ANOVA analyses demonstrated that PACAP(6-38) was anxiolytic and capable of mitigating the anxiogenic consequences of repeated stressor exposure. In aggregate, these studies implicate enhanced BNST PACAP signaling for the maladaptive anxiety-related consequences of chronic stress.

**Fig. 6** Antagonism of PACAP signaling during chronic stress exposure attenuates stress-induced weight loss and anxiety-like behavior. A: Experimental timeline; rats with bilateral BNST cannulations were chronically infused with vehicle or PACAP(6-38) using osmotic minipumps. B: Average weight change by group over the course of the experiment. After recovery, two groups were exposed to chronic variate stress (start shown by grey box). Nontressed rats receiving vehicle or PACAP(6-38) gained weight steadily over time (filled and open circles, respectively). By contrast, chronically stressed animals receiving vehicle failed to gain as much weight from diminished food intake (filled triangles). However, chronically stressed animals infused with PACAP(6-38) gained weight over time (open triangle) comparable to levels in control nonstressed animals. C,D: Animals were also assessed for anxiety-like behaviors. Stressed rats receiving vehicle from the miniosmotic pumps demonstrated diminished open arm entry times (C) and novelty/exploratory behavior (D) compared to nonstressed animals. By contrast, stressed animals receiving PACAP(6-38) had attenuated anxiety-like behavior as shown by increased open arm entries and novelty times compared to stressed animals with vehicle.

**BNST PACAP signaling increases serum corticosterone**

Although the BNST has been suggested to have direct and indirect fiber connections to the hypothalamus and PACAP /-/- mice have impaired corticosterone responses to implicate PACAP signaling in HPA stress functions, whether BNST PACAP signaling affects HPA activities is still very much unclear. To address this, we have now acutely injected PACAP bilaterally into the dorsolateral BNST and after 60 minutes collected trunk blood from decapitated rats to assess serum corticosterone levels, using standard ELISA assays. Compared to vehicle injected animals, rats injected with PACAP demonstrated a 3-fold increase in serum corticosterone (Fig 7). These results implicate BNST PACAP signaling in HPA axis function and may be consistent with the blunted corticosterone responses observed in PACAP/-/- mice after restraint stress. Hence, alterations in PACAP-mediated BNST neuroplasticity may be involved in HPA axis dysregulation leading to anxiety-like states following exposure to chronic stress. From these results, we anticipate that hypothalamic CRH and anterior pituitary ACTH levels will be altered following BNST PACAP injections to reflect the increase in serum corticosterone; these experiments will also be pursued in the proposal.

**AIM 1:** To determine if stress-induced BNST PACAP expression and signaling are necessary for the behavioral consequences of chronic stress.

**Question 1:** Does chronic variate stress increase PACAP expression in specific BNST and amygdala regions? **Rationale:** We have shown previously that exposure of rats to a 7-day chronic variate stress paradigm increased PACAP and PAC1 receptor transcript levels more than 10- and 2-fold, respectively, in the dorsolateral BNST (Hammack et al. 2009). The dorsolateral BNST is composed of several distinct
nuclei; hence to investigate whether stress induces PACAP expression in specific regions, we will perform immunocytochemical and complementary in situ hybridization studies to identify and compare BNST PACAP expression sites with those for CRH, a stress peptide well expressed in the bed nucleus. Further, as PACAP and CRH are both highly expressed in other limbic tissues, we will also examine PACAP/CRH distribution patterns in the amygdala following stress for comparative analyses. The identification of BNST and amygdala PACAP expression sites may elucidate the downstream neuronal targets and help define stress neurocircuits with PACAP signaling.

**Methods and Approach:** For all studies outlined in this proposal, adult Sprague-Dawley rats (250-275g) will be allowed to habituate in their home cages for at least one week before experimentation. Rats will be single-housed and maintained on a 12-h light/dark cycle with food and water available ad libitum. Following habituation, each animal will be randomly assigned to either a control or chronically stressed group; control animals remain in their home cages. The chronically stressed group of animals will undergo a chronic variate stress paradigm in which the animals are exposed to one of 5 different stressors (oscillation, forced swim, restraint, pedestal standing and footshock) each day for 7 days, as described previously (Hammack, Cheung et al. 2009). All animals within the stress group will be exposed to the same order of stressors for the same duration. After the last stressor (day 8), the rats are anesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde; the brains are removed and protected in 30% sucrose for cryosectioning (30 um). For BNST staining, tissue sections from control and stressed animals will be matched using the anterior commissure and fornix as landmarks.

All immunocytochemistry reagents will be chosen to allow PACAP and CRH colocalization studies. The brain cryosections are permeabilized with 0.3% triton X-100, blocked with 1% BSA and incubated in a mouse monoclonal PACAP antibody (1:20; Jens Hannibal, Bisperg Hospital, Copenhagen, Denmark) and/or a rabbit affinity-purified CRH antibody (1:100; Phoenix Pharmaceuticals, Burlingame, CA). The monoclonal antibody is directed to the N-terminus of PACAP and does not discriminate between PACAP27 and PACAP38 (Hannibal, 2002). PACAP localizations will be performed using tyramide amplification as previously described (Fahrenkrug and Hannibal, 1998). After primary antibody incubation and washings, the cryosections are incubated in biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA); the sections are then washed, treated with strep-avidin horseradish peroxidase (HRP; 1:250), washed again and subsequently incubated in a tyramide-biotin reagent (1:100; Perkin Elmer, Boston, MA). After extensive buffer rinses, the PACAP immunoreactivity in the sections are localized using avidin-conjugated Cy2 (1:200; Jackson ImmunoResearch, West Grove, PA). For CRH localizations, the same cryosections are incubated in Cy3-conjugated donkey anti-rabbit IgG (1:250; Jackson ImmunoResearch, West Grove, PA). Omission of any one of the processing reagents is not expected to produce signals.

**In situ hybridization** for PACAP transcripts will be performed using previous methods (Brandenburg et al., 1997; Braas and May, 1999). After anesthesia, control and chronically stressed rats are euthanized by decapitation; the brains are frozen; and prepared cryosections (20 um) are fixed in 4% paraformaldehyde, acetylated and chloroform treated before probe hybridization (approximately 500 ng/slide) in a humidified chamber. Sense and antisense PACAP cRNAs are transcribed from linearized plasmids using biotin-labeled UTP. After high stringency washes in 20X SSC, and 0.2X SSC, and RNAse A digestion of residual probe, the cryosections are incubated in strep-avidin-HRP for tyramide-biotin amplification. The sections are finally incubated with ABC complex for signal detection with DAB.

Quantitative image analyses will be performed using NIH ImageJ (Rasband, 1997 - 2011). From the punctate nature of the immunocytochemical staining, the images, after background thresholding, will be examined by pixel analyses over uniform circumscribed areas. Labeled in situ labeled cells will be enumerated over comparable areas. Statistical Student’s t-tests will be performed using SigmaPlot/SigmaStat (Systat Software, San Jose, CA). p < 0.05 will be considered significant.

**Question 2: Is the dorsolateral BNST one principal region for stress-induced anxiety-related responses? Rationale:** As described previously, the BNST has been posited to mediate responses to unpredictable, diffuse and/or long-term stimuli which have been suggested to resemble anxiety-like behavior. However, the BNST has been subdivided into several distinct nuclei with separate subregions having differential effects on HPA-axis function and stress responses. While large anterior BNST lesions decrease CRH transcript levels in the PVN (Herman et al. 1994), and lateral BNST lesions attenuate stress-induced ACTH and corticosterone secretion (Gray et al. 1993), posterior lesions increase PVN CRH expression and corticosterone responses following chronic stress (Choi, Furay et al. 2008). In more recent work, lesions including either the dorsomedial/fusiform nucleus of the BNST or the posterior principal nucleus had differential
Effects on PVN CRH and corticosterone secretion after acute and chronic stress. However, in chronic variate stress paradigms, the same excitotoxic lesions in these areas failed to block the stress-induced weight loss (Choi, Evenson et al. 2008; Choi, Furay et al. 2008). Furthermore, behavioral testing was not assessed in any of these studies. Here we aim to test directly the role of the dorsolateral BNST in stress-mediated anxiety-like behaviors. We will perform BNST excitotoxic lesions with NMDA prior to chronic variate stress exposure and test the ability of these lesions to attenuate anxiety-related responses. The specificity of dorsolateral BNST function in stress-mediated anxiety-like behaviors will be assessed in similar paradigms with lesions in other limbic sites. We hypothesize that ablating the oval nucleus will blunt the behavioral and weight changes observed after chronic stress.

**Methods and Approach:** In these experiments we aim to assess if the dorsolateral BNST is an integral site in the circuitry causing stress-induced behavioral changes. We will produce lesions bilaterally in the dorsolateral BNST by injecting stereotaxically 4 ug of the excitotoxic agent NMDA (200 nl total volume at 50 nl/min). After injection, the syringe is left in place for 4 mins before removal to prevent upward spreading. Sham lesioned animals will undergo the same surgery with vehicle infusions (n=14 per group). After one week postsurgical recovery, half the rats in each group will be exposed to the 7-day chronic stress paradigm. The remaining half will serve as non-stressed controls. Animal weight and food intake will be recorded daily and 24 hours after the last stressor, all animals will be assessed for changes in exploratory behavior in open field and elevated plus maze tests. The open field test consists of an opaque white arena (55 cm x 55 cm with 50 cm high walls) illuminated using white light at 14 lux in which the animal is allowed to move freely for 7 minutes. Increased time spent in the perimeter of the arena (thigmotaxis) and decreased time spent in the center will be employed as indications of anxiety-like behavior. Anxiety-like behavior will also be assessed through each animal's propensity to explore the closed arms of a standard elevated plus maze. The elevated plus maze is made of black textured plastic, elevated 74 cm from the floor, and consists of two opposing open and two opposing closed arms (each arm 60 cm long and 9 cm wide) that extends perpendicularly from a central square platform (9 x 9 cm). The closed arms are walled by black opaque panels 30 cm in height providing a darkened corridor for the animal to conceal itself. Illumination of the maze with a red bulb at 4 lux in the center allows a camera to track all movement while the animal perceives nearly complete darkness. Animals will be placed in the center platform and allowed to move freely for 7 minutes. Time spent in the open and closed arms, and the total number of crosses into each arm will be measured. All behavioral testing will be digitally captured and measurements made using tracking software (EthoVision). Overall locomotor behavior (average velocity) will also be assessed using this software.

At the end of behavioral testing animals will be perfused transcardially with 4% paraformaldehyde and brains removed for sectioning. The extent of neuronal lesion will be verified in brain slices incubated with anti-NeuN (a neuron specific marker) followed by a fluorophore-conjugated secondary antibody. In a separate group of animals we will repeat the experiment but lesion the ventral BNST, or the amygdala to provide evidence of the specificity of the dorsolateral BNST in mediating stress-induced changes in behavior and weight.

**Question 3:** Are chronic variate stress-induced anxiety-related responses mediated by PACAP signaling? **Rationale:** Our laboratory has already shown that BNST PACAP is anxiogenic. As chronic variate stress increases BNST PACAP expression and facilitates anxiety-related behaviors, an immediate hypothesis posits that enhanced BNST PACAP expression and signaling during long term stress mediate stress-induced anxiety behaviors. Hence we will examine the concept directly by blocking BNST PACAP signaling during stress paradigms using the PACAP receptor antagonist PACAP(6-38), and assess whether anxiety-related behaviors can be attenuated. PACAP(6-38) is an amino terminal truncated form of PACAP38 that preserves the C-terminal alpha-helical receptor-binding domain without the N-terminal segment necessary for G-protein receptor activation. We will also test other more selective PACAP receptor antagonists as they become available.

**Methods and Approach:** Rats will be cannulated into the oval nucleus of the BNST and cannula will be attached to a bifurcation connector using catheter tubing that is then attached to an osmotic minipump (Alzet). The minipump will contain either vehicle or 75 uM PACAP(6-38) for bilateral infusions over 14 days (0.25 ul per side per hour) Again, each group is divided in half; one half will not be stressed (control) and the other half will be exposed to chronic variate stress (4 groups total, n = 8 per group). After post-surgical recovery, the stressed groups will received the 7-day chronic stress paradigm, during which the BNST will continuously be infused with either vehicle or antagonist. Twenty-four to 48 hours after the last stressor, rats will be tested for anxiety-like behavior through assessing novel object exploration in an open field and exploratory behavior on
the elevated plus maze using digital tracking software as described in the previous question. Throughout the study animals and their food and water will be weighed daily. Following completion of the behavioral tests, rats will be perfused transcardially and brains removed and sectioned for cannula verification. We expect to again replicate the finding that chronic variate stress increases anxiety-like behavior and decreases food intake and body weight over the 7-day stress paradigm by comparing vehicle treated animals exposed to the stress paradigm with non-stressed animals. In contrast, we hypothesize that stressed animals continuously infused with PACAP(6-38), to block BNST PACAP receptors during the week of stress, will show attenuated responses to the stressor exposure and have behavior and weight measures that more closely resemble non-stressed animals. These data would show that PACAP signaling within the BNST is necessary for the behavioral changes that occur following chronic stress. We will also control for effects of the antagonist infusion itself by including a non-stressed group implanted with PACAP(6-38) containing minipumps. As PACAP(6-38) blocks PAC1 and VPAC2 receptor activation, we plan to repeat the experiment as more specific PACAP receptor antagonists become available in order to assess the role each individual receptor has in the behavioral consequences of chronic stress. Development of these antagonists is currently in progress through collaboration with Dr. Mathias Brewer (Chemistry Dept. UVM).

**Anticipated results, interpretation and alternatives**

The immunocytochemical and in situ hybridization studies employ well characterized reagents and techniques in our laboratory and we do not anticipate difficulties. From our preliminary results, we anticipate that the data will correlate with previous transcript studies and demonstrate that PACAP expression levels will be enhanced following chronic stress. Furthermore, we expect that PACAP will be localized to the oval nucleus of the BNST and CeA in patterns distinct from CRH to suggest interactions within a common pathway. We have successfully performed dorsolateral BNST excitotoxic lesions as described in our preliminary studies for weight change and the proposed extended studies are anticipated to have similar effects on open field and plus maze behavioral tests. Hence we predict that for vehicle injected rats, we will replicate the finding that chronic variate stress increases anxiety-like behavior and decreases body weight over the 7-day stress paradigm upon comparisons with control non-stressed animals; BNST lesioned animals are expected to display less anxiety-like behavior after the chronic stress paradigm when compared to sham lesioned animals exposed to the same stressors. We anticipate no effect of lesion itself on these measures compared to control (sham lesioned) non-stressed animals. Although the procedures for dorsolateral BNST lesions are reliable, the coordinates for ventral BNST and amygdala lesions may have to be verified. The one important limitation of these studies is the confinement of the NMDA excitotoxin spread to confined regions. All lesions will be immunocytochemically verified as described above to survey the extent of neuronal damage and data from lesions found to be outside of targeted regions will be excluded from analyses.

We hypothesize that BNST PACAP signaling is necessary during exposure to chronic stress for the increase in anxiety-like behavior and associated changes in weight gain. We anticipate that antagonizing PACAP receptors within the oval nucleus of the BNST during exposure to stressors will block or blunt the effects of chronic stress and this is supported by our chronic infusion studies with PACAP(6-38). As described, PACAP(6-38) is a nonselective PAC1 and VPAC2 receptor antagonist and we are currently investigating whether other ligands may be used to complement these and the proposed studies in Aim 2. Some of the VPAC2 selective agonists in Aim 2 may be employed to refine our data and/or rule out possibilities; alternatively, as small molecule agonists/antagonists become available we may test the most promising compounds to support our work. As in the excitotoxin lesion studies, diffusional spread is a consideration and we can verify the targets by dye infusions at the termination of the experiments.

**AIM 2: To determine which PACAP receptor within the BNST mediates the behavioral responses to PACAP.**

**Question 1. Are the PACAP-mediated anxiety-like responses mediated by PAC1 selective receptors?**

**Rationale:** To date, all of the functions of PACAP can be ascribed to 3 G protein-coupled receptors. The two forms of PACAP (PACAP27 and PACAP38) bind with equal high affinity at the PAC1 receptor; both PACAP and VIP peptides bind with near equal affinities at the VPAC1 and VPAC2 receptors. Unlike VPAC receptors which activate only adenyl cyclase, the PAC1 receptor can engage multiple intracellular signaling cascades including adenyl cyclase, phospholipase C, MEK and Akt, with long term implications in neurodevelopment and plasticity. Hence the identity of the receptor subtype mediating the PACAP-mediated stress responses is important with respect to signaling mechanisms and pharmacological interventions. However, as PACAP(6-38) is a PAC1/VPAC2 receptor antagonist, the studies in Aim 1 will not have addressed this issue. There are no PAC1 receptor specific antagonists identified to date; however, the sandfly saliva protein maxadilan has been shown to be a PAC1 receptor selective agonist (Moro and Lerner
Methods and Approach: Rats will undergo surgery to have bilateral guide cannula implanted aimed at the oval nucleus of the BNST. After 7 days postsurgical recovery, a microinjector is inserted into each cannula to slowly infuse either 0.5 μl of maxadilan (80 μM) or vehicle into the BNST. Forty-five minutes after injection, the animals will be placed into an open field arena and exploratory behavior will be recorded and measured using motion tracking software (EthoVision). Two hours later, animals will also be tested for exploration on an elevated plus maze in order to examine either lasting or delayed effects of drug infusion on anxiety-like behavior. Body weight and food intake will be measured daily throughout the study. Maxadilan treated animals will be compared to matched age and weight vehicle treated controls. In previous research we have observed both increases in anxiety-like behavior and a decrease in food intake and subsequent weight loss following bilateral BNST PACAP injections. By mimicking these effects using the PAC1 receptor specific agonist, we will have supporting data that PACAP-PAC1 receptor signaling participates in the stress-related effects of intra-BNST PACAP.

Question 2. Are VIP and VPAC receptors also implicated in some anxiety-related responses?
Rationale: Although PACAP signaling is anxiogenic, whether the related VIP peptide also has behavioral effects is relatively unclear. While the work on VIP in behavior has not been extensive, comparative open field behavioral tests have suggested that VIP knockout mice also appear less anxious (Girard et al. 2006). To assess whether VIP signaling may participate in anxiety-related behaviors, we will perform VIP and VPAC receptor agonist infusions in studies complementary to Aim 2 Question 1. These studies may further clarify the coordinate or distinct roles of VIP and PACAP in stress-related anxiety and weight change behaviors.

Methods and Approach: In these experiments we will use the same methods as in Aim 2 Question 1. Animals will be cannulated for intra-BNST infusions and examined for changes in food intake, body weight, and anxiety-like behavior following injection of VIP or more specific VPAC receptor agonists. VPAC2 agonists BAY 55-9837, Ro 25-1392, Ro 25-1553 and the VPAC1 agonist Ala 11,22,28, VIP will first be tested for their ability to increase cAMP specifically in one of the two VPAC receptors using the Codex BioSolutions ActOne cAMP assay. This assay has been previously used by our laboratory in the screening of putative small molecule PACAP receptor antagonists. The assay utilizes 3 separate lines of HEK cells, each one over-expressing one of the human G protein-coupled PACAP receptors (PAC1, VPAC1, or VPAC2 receptor) and also expressing a cyclic nucleotide-gated ion channel. Changes in membrane potential caused by increases in cAMP are then measured using membrane potential dye and a fluorescence microplate reader. After incubating plated cells with lipophilic voltage-sensitive membrane indicator dye containing 25 μM phosphodiesterase inhibitor Ro20-1724, baseline fluorescence measurements will be taken using 530/560 excitation/emission wavelengths. After receptor agonist additions, the cAMP-activated fluorescence measurements will also be taken every 5 - 15 mins for a duration of 2 hours. This will allow for dose and kinetic measurements of cAMP changes in each cell type (PAC1, VPAC1, or VPAC2 receptor overexpression) as well as allow us to directly screen the extent of specificity of each agonist. Based on the results from the cAMP assay, we will choose the best agonists for in vivo infusions. Upon intra-BNST VIP receptor selective agonist infusion, we will evaluate effects on behavior, food intake, and weight change for comparisons to vehicle- and PACAP- treated animals (n = 8 per dose). These experiments will allow us to evaluate the role of PACAP and/or VPAC receptor signaling in the BNST on anxiety-related behaviors.

Anticipated results, interpretation and alternatives
We do not anticipate difficulties in executing these experiments. We have successfully performed acute BNST injections and in preliminary work, BNST injections with maxadilan have already elicited the expected weight loss associated with anxiety-related behaviors. The planned studies in this aim will follow the same procedures and as observed for weight change, we expect that injections of the specific PAC1 receptor agonist maxadilan into the BNST will elicit heightened anxiety-like responses on open field and elevated plus maze tests. One potential issue is maxadilan availability. Maxadilan is purified after bacterial expression and available through our colleague Ethan Lerner (Harvard Medical School). Accordingly, the supplies are limited and we may not have sufficient quantities for the evaluations at a wide range of doses. This may not be an issue unless the weight loss vs. open field/plus maze behavioral responses demonstrates differential maxadilan concentration optima. In this remote case, we may have to identify other PAC1 specific agonists or commercial sources for maxadilan.

As in our preliminary work, BNST VIP or VPAC receptor agonist injections are not expected to have significant weight change effects, and similarly, we may anticipate that the same VIP/VPAC agonists will not
induce anxiety-like behaviors on open field and elevated plus-maze tests. These results may correlate with our previous qPCR data in which VIP and VPAC1/2 receptor transcript levels were not changed following chronic variate stress. However, the effects of VIP on behavior have not been directly tested. In the proposed experiments, VPAC1 or VPAC2 selective agonists may present anxiety-like responses, which may be consistent with the behavioral phenotype of VIP/-/- mice; these observations may be key in delineating mechanisms underlying the feeding/weight change responses from the behavioral responses. The studies in Question 1 and 2 in aggregate may suggest for example, that PACAP activation of PAC1 receptors may have more prominent effects on feeding/weight change than behavior, and that PACAP/VIP activation of VPAC2 receptors has greater effects on behavior than feeding. Delineating these mechanisms can be important not only with respect to understanding stress neurocircuits but also in the search for specific receptor agonists for therapeutics.

AIM 3: To investigate the effects of chronic variate stress and BNST PACAP receptor activation on HPA-axis function. Question 1. Does PACAP signaling in the dorsolateral BNST activate the HPA axis? Rationale: While early acute stress studies failed to find PACAP associations with the HPA-axis (Hannibal et al. 1995) more recent work with chronic stress paradigms have shown unambiguously that PACAP signaling regulates stress-mediated serum corticosterone levels. Both PVN CRH transcription and secretion of corticosterone was impaired in PACAP/-/- mice compared with wild-type controls after 6 hours of restraint stress (Stroth and Eiden 2010) and central PACAP administration results in increased CRH transcription and plasma corticosterone levels (Agarwal et al. 2005). These results are supported by immunocytochemical data demonstrating PACAP-immunoreactive fibers apposed to CRH-expressing neurons, suggesting that PACAP is a component of HPA CRH/ACTH neurocircuit. Studies have shown that systemic or icv injection of PACAP can elicit increases in HPA-axis activation; however, even though the BNST can regulate the HPA-axis via direct or indirect fiber projection, whether PACAP signaling within the BNST can impact hypothalamic CRH and downstream anterior pituitary ACTH and serum corticosterone levels is still unclear. We will perform acute BNST PACAP and/or PAC1 receptor agonist infusions to address these issues. The BNST undergoes significant plasticity following chronic stress. It has been argued that changes in the BNST can affect HPA axis functioning and may be important to the etiology of behavioral disorders such as depression, anxiety disorders, PTSD, and panic disorder. Increases in PACAP production and signaling in this region following chronic stress may be a mechanism by which the BNST alters or dysregulates the HPA-axis. Changes in transcript levels will be assessed using qPCR, and changes in peptide levels will be assessed using immunoassays. These experiments will determine changes along the HPA-axis as a result of specific PACAP receptor activation within the BNST.

Methods and Approach: Rats will undergo surgery to implant bilateral BNST cannula aimed at the oval nucleus for bilateral PACAP (or related agonists) or vehicle injections. After infusion, the injected animal will then be placed into the home cage and brought into another separate, quiet room and left alone for a predetermined period of time (30 and 60 minutes). Subsequently, the animals will be rapidly decapitated, trunk blood collected and anterior pituitary and hypothalamus dissected. Blood will be centrifuged and serum collected. Dissected brain regions will be placed into individual tubes and frozen on dry ice. Tubes will be stored at -80°C until further processing.

Serum corticosterone measurements will be determined using colorimetric enzyme immunoassays (Arbor Assays). Serum from the vehicle and PACAP injected rats will be diluted 1:100 and 50 ul of each sample or a corticosterone standard will be added to 96-well plates coated with anti-sheep IgG. Subsequently, a corticosterone-peroxidase conjugate and sheep anti-corticosterone polyclonal antibody are added for assay competition with the serum samples or assay standards. The plates are then washed for peroxidase substrate development; the reaction is terminated upon acidification and assay absorbance determined at 450 nm. Sample serum corticosterone levels are calculated by regression from the standard curve and sample dilution correction.

Similarly, anterior pituitary ACTH and hypothalamic CRH levels will be measured by enzyme immunoassay. The anterior pituitary and hypothalamic tissues are homogenized in 5N acetic acid containing 30 gm/ml PMSF using a ground-glass/ground-glass homogenizer and subjected to three freeze/thawing cycles to lyse cellular vesicles. An aliquot of the homogenate is removed for protein assay and the remaining sample is centrifuged to pellet debris. The supernatant is recovered; 2 mg/ml BSA is added as carrier and the samples are lyophilized. The resulting pellet is solubilized in assay buffer for ACTH colorimetric immunoassay as described above. To verify ACTH response, complementary assays may be performed to determine serum ACTH levels. The same serum samples for corticosterone measurements will be prepared by concentrating
peptides on C18 Sep-Pak cartridges. The cartridges are washed in 0.1% TFA, and the peptides are eluted in 0.1% TFA containing 80% acetonitrile. The eluates are dried under vacuum and dissolved in assay buffer for ACTH assay.

Other assays may also be performed to examine BNST PACAP regulation of the HPA-axis. Different groups of vehicle or PACAP BNST infused rats may be prepared for total RNA extraction from anterior pituitary and hypothalamic tissues. After total RNA is extracted from each brain region, 2 ug of total RNA from each region will be used to synthesize cDNA using random hexamer primers. The cDNA will be subsequently diluted for quantitative PCR analyses using SYBR green detection for both CRH and POMC transcript. All measurements are normalized against ribosomal 18S levels in the same sample.

**Question 2. Does chronic variate stress alter serum PACAP levels? Rationale:** Our recent work has shown that serum PACAP levels are elevated in female patients with PTSD (Ressler et al. 2011). The human serum PACAP levels are positively correlated with the severity of total PTSD symptoms, and for each behavioral PTSD measure including intrusion, avoidance and hyperarousal or vigilance. What is unknown is whether serum PACAP levels can also reflect chronic stress states in rodents, and whether these parameters demonstrate gender dimorphism as in humans. We will examine these issues in chronically stressed male and female rats; the concordance of these data with previous human studies will enhance the validity of the results and foster applications for blood PACAP levels as measures of chronic stress. **Methods and Approach:** We have recently developed three new PACAP38 antisera for radioimmunoassays. The antisera were generated against the alpha-amidated carboxy-terminal end of PACAP38; all three antisera demonstrate a working assay dilution of 1:100,000 - 1:200,000 and an assay sensitivity of 9 - 20 fmol using an iodinated PACAP31-38 tracer. For these studies, control (non-stressed), acute (restraint, 1 day) and chronic variate stressed (7 - 14 days) rats will be euthanized by rapid decapitation and trunk blood will be collected for serum preparation. Aliquots of serum (0.5 ml) will be cycled over C18 Sep-Pak cartridges and bound peptides will be eluted from the resin with 0.1%TFA/80% acetonitrile. The eluates will be dried as before and reconstituted in assay buffer containing 30 mg/ml PMSF for double antibody radioimmunoassay using PACAP38 as standard. **Anticipated results, interpretation and alternatives**

We hypothesize that acute PACAP injections into the dorsolateral BNST will activate the HPA axis. These expectations are supported by our preliminary data demonstrating increased serum corticosterone levels following intra-BNST PACAP injection. Accordingly, we may expect to see small stimulated levels of hypothalamic CRH and anterior pituitary ACTH transcript expression with corresponding decreases in CRH and ACTH peptide levels in their respective tissues from heightened secretion. As an alternative method, immunohistochemical analysis using antibodies against c-fos or pCREB may be employed in conjunction with antibodies targeting hypothalamic CRH. These colocalization studies would assess activation of CRH cells as an indirect measure of increased secretion in the event that changes in transcript or peptide levels are not observed. We also anticipate that increased circulating corticosterone from PACAP injections will imply increased circulating ACTH levels as well. As a variation of these experiments, as in aim 2, we may assess injections of specific PAC1/VPAC receptor agonists into the BNST to determine which of the 3 PACAP receptors mediates the effects on the HPA axis. One consideration for this aim is the temporal aspects of the experiment. We found in the pilot studies that BNST PACAP injections increase circulating corticosterone 30 - 60 mins following injection; other temporal points may be considered to establish peak response time and duration of the response.

There are no anticipated difficulties in measuring PACAP from serum; we will employ double antibody radioimmunoassay techniques exactly like our human studies. Although the primary antisera are new, the assay midpoints of these newly generated antisera are comparable to that used previously; i.e., the assay sensitivities are comparable to detect serum peptide levels. There are a few uncertainties in this study; it is unclear whether changes in rodent serum PACAP levels will be detected after stress regardless of sex, whether serum PACAP changes can be detected after acute stress, or chronic stress, and the duration and intensity of the chronic stress necessary to detect changes. If the human condition serves as a model, then we might anticipate that female rats will demonstrate a stronger PACAP response to chronic stress compared to male rats. We may also anticipate that only chronic stress, and not acute stress, will result in altered blood PACAP levels. These data may establish the stress parameters by which blood PACAP levels will be altered. Extrapolation of these parameters may be important in understanding conditions and thresholds leading to human stress-related behavioral disorders.


Hammack, S. E., J. Cheung, et al. (2009). "Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of


