2016

Intra-Bed Nucleus of the Stria Terminalis Pituitary Adenylate Cyclase-Activating Peptide Infusion Reinstates Cocaine Seeeking in Rats

Olivia Miles
University of Vermont

INTRA-BED NUCLEUS OF THE STRIA TERMINALIS PITUITARY ADENYLATE CYCLASE-ACTIVATING PEPTIDE INFUSION REINSTATES COCAINE SEEKING IN RATS

A Thesis Presented

by

Olivia W. Miles

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Arts
Specializing in Psychology

January, 2016

Defense Date: October 6, 2015
Thesis Examination Committee:

Sayamwong (Jom) Hammack, Ph.D, Advisor
Victor May, PhD, Chairperson
Mark E. Bouton, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

The tendency of users to relapse severely hinders adequate treatment of addiction. Physical and psychological stressors often contribute to difficulties in maintaining behavior change, and may play a significant role in relapse. We have previously shown that the activation of pituitary adenylate cyclase activating peptide (PACAP) systems in the bed nucleus of the stria terminalis (BNST) mediates many consequences of chronic stressor exposure. Hence, chronic stress substantially increased BNST PACAP levels, intra-BNST PACAP infusions produced the behavioral and endocrine consequences of stressor exposure, and BNST PACAP antagonism blocked many of the consequences of chronic stress. In the present set of studies, we investigated the role of BNST PACAP in stress-induced reinstatement of cocaine seeking. Rats self-administered cocaine (3mg/ml; 0.5mg/ig/infusion, i.v.) for 1hr daily over 10 days, which was followed by extinction training in which lever pressing no longer resulted in cocaine delivery. In the first experiment we showed that intra-BNST PACAP infusion (1 μg; 0.5 μl per side) reinstated previously extinguished cocaine seeking behavior. In the second experiment intra-BNST infusions of the PAC1/VPAC2 antagonist, PACAP 6-38 (1 μg; 0.5 μl per side) blocked stress-induced reinstatement. Hence, stressor exposure (5 sec 2mA footshock) caused significant reinstatement of cocaine seeking behavior, which was blocked by intra-BNST PACAP6-38 infusion. Overall, these data suggest that BNST PACAP systems mediate stress-induced reinstatement to drug seeking. Understanding the neuropharmacology of BNST PACAP in stress-induced reinstatement and the role of PACAP systems may lead to viable targets for relapse prevention.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Substance abuse is costly to the United States with an estimated $700 billion spent annually in costs related to drug intoxication, withdrawal and relapse (Feit & Taylor, 2015), and the consequences of this chronic cycle, such as crime, health care, and lost work productivity. A better understanding of the mechanisms underlying substance abuse requires an investigation of the behavioral and physiological mechanisms that govern drug self-administration, withdrawal, and relapse (Nestler & Aghajanian, 1997).

In a clinical setting, a primary problem in the treatment of substance abuse is the tendency of users to relapse following acute or extended periods of abstinence. For example, in a study of cocaine users, Higgins & colleagues (1993, 1994, 2000) noted that there are several treatments available (i.e. contingency management and cognitive behavioral therapy; for review, see Higgins et al., 2000) that can help former drug users become abstinent; however, the tendency to relapse is a serious and ubiquitous problem. On average, 25-40 percent of substance abusers will return to drug use within one year of receiving treatment (Scott et al., 2011). While many factors may contribute to drug relapse, one critical factor may be exposure to a stressor (Sinha et al., 2006). Furthermore, stress may become a component of the contextual setting that cues drug use. Upon stressor exposure, several response systems are activated including the HPA axis and the sympathetic nervous system (SNS), both circuits associated with emotional components of fear and anxiety. Hence, brain areas involved in regulating these

emotional processes undergo neurochemical and morphological changes after stressor exposure. These changes may underlie several mental health disorders (such as PTSD and depression; Hammack et al., 2010) including relapse to drug use.

As will be discussed below, brain areas that modulate the stress-response system may be critical for stress-induced relapse of drug seeking behaviors. Our laboratory and others have demonstrated that neurochemical and morphological changes that occur as a consequence of stressor exposure likely play a role in several mental health disorders, such as PTSD, anxiety and affective disorders (Hammack et al., 2010). In this regard, the bed nucleus of the stria terminalis (BNST), a basal forebrain region that has been argued to represent the rostral extent of the central extended amygdala (Davis & Whalen, 2001) plays a key role in mediating responses to stressor exposure (Daniel & Rainnie, 2015; Hammack et al., 2010) and has been shown to mediate multiple physiological and behavioral responses to stress (Roman et al., 2014; Walker, Toufexis & Davis, 2003). If substance abuse triggers stress-related circuits, changes in the BNST during drug use may become part of the neurochemical context for relapse after a period of abstinence when exposed to a stressor. Furthermore, the BNST has been highly implicated in stress-induced reinstatement (Erb et al., 2001; see below), and may underlie potential treatment substrates for relapse to drug use.

1.2 Substance Abuse

While substance abuse creates a large financial burden on the United States, substance abuse disorders also have been implicated in altering household and family

dynamics (Orford et al., 2013), lost careers (Measham et al., 2011), and domestic violence and child abuse (Schumacher, 2012). The results of a 2009 survey conducted by the Substance Abuse and Mental Health Services Administration demonstrate that 23.5 million Americans sought drug abuse rehabilitation programs the previous year for multiple dependencies, including alcohol, nicotine, marijuana, cocaine, heroin, and opiates. While a predominant goal of substance abuse treatment is to help users stop abusing drugs, the tendency of users to relapse remains an important concern.

Characterization of Substance Abuse

Substance abuse is typically characterized by recurring episodes of relapse following extended drug-free periods (Erb, Shaham & Stewart, 1998). The debilitating condition represents a state whereby drug seeking and subsequent self-administration dominate the user's thoughts and behaviors. For some individuals, the tendency to abuse drugs continues despite detrimental side effects, such as health problems and lost work productivity (Feit & Taylor, 2015). While some argue that substance abuse represents a loss of self-control (Charbogne, Kieffer & Befort, 2014), others view addiction as a maladaptive neural plasticity caused by exposure to drugs of abuse (Feng & Nestler, 2013). Hence, multiple factors contribute to substance abuse, including the disordered regulation of reward systems, environmental stimuli, and vulnerable genes (Jasinska et al., 2014; Levran et al., 2015). As mentioned previously, the tendency of users to relapse severely hinders the adequate treatment of substance abuse disorders. Understanding the behavioral and physiological mechanisms of relapse processes in substance abuse disorders is critical to determine viable targets for relapse

prevention.

Behavioral processes associated with relapse

In a clinical scenario, many behavioral processes may be activated when an individual returns to drug use. Bouton and colleagues (1991, 2002; see also, Rescorla, 2004) have employed five distinct processes that may represent mechanisms underlying the relapse of drug-seeking behavior: *spontaneous recovery* describes the recovery of extinguished responding (i.e. drug-seeking behavior), after a period of extinction or abstinence; *rapid reacquisition* describes the condition when extinguished responding returns more quickly after the response is again reinforced; *resurgence* occurs when responding recovers when an alternate "second" response that has been reinforced during extinction is, itself, put on extinction; *renewal* describes the process in which responding reoccurs after a period of extinction when the behavior is removed from the extinction context; and finally, *reinstatement* occurs when a behavior is recovered after a period of extinction following exposure to the unconditioned stimulus (for a comprehensive review, see Bouton, Winterbauer & Vurbic, 2012). Importantly, many of these processes were first described in a Pavlovian conditioning scenario (for review, see Rescorla & Solomon, 1967; Pearce, 1987; Domjan, 2005); however, these processes have recently been characterized in operant conditioning paradigms. For example, while reinstatement procedures are now used to model the relationship between stress and relapse, the method was first derived as a means to study the return of drug-seeking after acute non-contingent exposure to a drug (Davis & Smith, 1976). Indeed, the term "reinstatement" has been used to describe several relapse paradigms

in the addiction literature (see Rescorla & Heth, 1975; Bouton & Bolles, 1979; Delamater, 1997). Hence, reinstatement of drug seeking behavior has been observed after re-exposure to the drug (drug priming), drug cues, or stressor exposure (Carroll & Comer, 1996; Ettenberg, MacConell, & Geist, 1996; Gerber & Stretch, 1975; Katner, Magalong & Weiss, 1999; Meil & See, 1996). While there are several methods to "reinstate" drug-seeking behavior, it is likely that these different treatments activate different neural pathways (Stewart, 2000). Furthermore, when recovering human addicts are exposed to drug stimuli or the drug itself, many report intense desire for the drug (Carter & Tiffany, 1999). These findings further validate the reinstatement model as a suitable method for studying relapse processes.

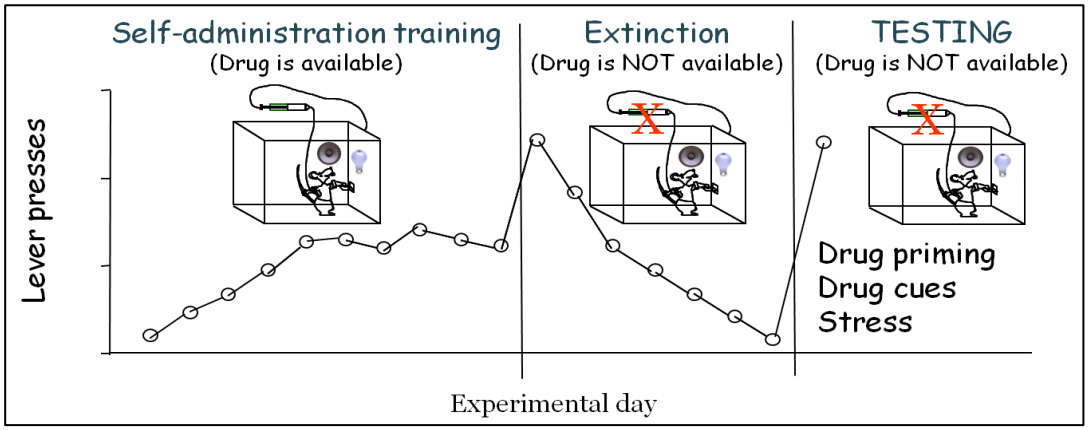

Figure 1. The Reinstatement Model of drug relapse. Representative self-administration paradigm, modified from a talk given by Yavin Shaham, PhD at the Neurobiology of Relapse Section, Behavioral Neuroscience Branch IRP/NIDA/NIH/DHHS, Baltimore. During acquisition, or self-administration training, animals are allowed to lever press for cocaine delivery for 10 days. During this period, lever press responses increase as animals continually seek the drug. Immediately following acquisition of drug-seeking, the behavior is extinguished in the Extinction phase. Here, lever press behavior is NOT followed by a drug stimulus and animals learn, over a 20-day extinction period, that lever presses no longer produce a drug reward. Finally, test day occurs one day following the termination of extinction. Reinstatement has been shown to occur with drug-priming, drug-cues and the presentation of a physical stressor.

Stress-induced reinstatement

Threats to an organism's homeostasis activate a cascade of behavioral changes associated with fear, anxiety, and emotional distress (Hammack et al., 2010). Stressor exposure activates the HPA axis, a central circuit that serves to maintain an organism's homeostasis by promoting necessary changes in physiological and behavioral responses to a stimulus. This action is dependent on stress hormone corticotropin releasing hormone (CRH) release (Tsigos & Chrousos, 2002). However, CRH activation in extra-hypothalamic brain regions may mediate some of the behavioral and physiological symptoms of stressor exposure, including stress-induced relapse. Notably, alterations in HPA axis function have been linked to anxiety and depressive disorders (Gold & Chrousos, 2002; Arborelius, Owens, Plotsky & Nemeroff, 1999), and may play a role in properties of addiction, such as relapse.

As noted above, drug-seeking can be accurately and reliably reinstated in response to stressor exposure (Erb, Shaham & Stewart, 1998). In a standard stress-induced reinstatement procedure (discussed in Chapter 2), rats self-administer cocaine via lever-press response, and then have the behavior extinguished, in which subsequent lever presses no longer deliver cocaine. After extinction, exposure to a stressor (i.e. intermittent unpredictable electrical footshock) can reinstate drug-seeking on the lever previously associated with drug delivery.

Many studies have investigated the neural circuits underlying stress-induced reinstatement (Kalivas & Volkow, 2014; McFarland et al., 2004; Sinha, 2001). For example, Erb & Stewart (1999) have implicated activation of CRH (see below)

in stress-induced relapse. Additionally, Shaham and colleagues (1997, 2000) demonstrated that an acute intracerebroventricular (ICV) injection of CRH mimicked stressor exposure and caused reinstatement, whereas the ICV injection of a CRH antagonist, α-helical CRH, blocked stress-induced reinstatement in heroin-trained rats. Furthermore, in a follow-up study done in cocaine-trained rats, Shaham, Erb, et al. (1998) showed that CRH receptor antagonists (D-Phe CRH and CP-154,526, respectively) also attenuated reinstatement to drug-seeking. When tested in drug-primed animals, these manipulations had no effect, indicating an important role of CRH specifically in the stress-induced reinstatement paradigm. Initially, the sites of CRH action were unclear; however, subsequent studies implicated the BNST as a critical site for stress-induced reinstatement of drug seeking behavior.

1.3 The Bed Nucleus of the Stria Terminalis (BNST)

The BNST has been linked to anxiety- and stress-responding in both clinical and laboratory settings (Avery et al., 2015; Walker, et al. 2003). Hence, the structure has been implicated in numerous neurochemical, morphological and physiological alterations after stressor exposure (Dumont, Rycroft, Maiz & Williams, 2008). A naturally heterogeneous structure, activity in the BNST may be critical for creating and maintaining these alterations long-term. The BNST has been subdivided into several different subregions based on neuronal morphology, afferent and efferent connections, and neurochemistry (Avery et al., 2015). Moreover, neurons within the BNST express numerous receptor subtypes that bind multiple neuropeptides, such as CRH, neurotensin, and encephalin. This heterogeneity, and the neuropeptides

present, suggests that the regulation of BNST activity is complex. Several researchers have implicated the BNST oval nucleus in the mediation of emotional (i.e. stressful) responses (Daniel & Rainnie, 2015). Indeed, the BNST oval nucleus receives CRH inputs from the central amygdala (CeA; an area responsible for some autonomic and behavioral components of emotion), receives projections from the basolateral amygdala (BLA; necessary for eliciting fear responses), hippocampus, and medial prefrontal cortex (mPFC), and projects to the paraventricular nucleus (PVN; in the hypothalamus), limbic structures, and brainstem nuclei (Crestani et al., 2013). The BNST is thought to serve as a relay station between limbic systems and emotional processing systems, as the structure projects to and receives projections from areas responsible for both the emotional and physiological responses to a stressor, and therefore may be prominent in mediating stress disorders (i.e. stress-induced relapse; Roman et al., 2014; Hammack & May, 2015).

Neurochemistry

As discussed above, CRH release from extrahypothalamic areas like the BNST may be an important component of the activation of anxiety- and fear-like behaviors. In several studies investigating fear responses, an ICV injection of CRH increased startle and caused a depressive-like state in rodents (Lee & Davis, 1997), an effect mediated by the BNST.

The BNST also receives projections from the ventral norepinephrine (NE) bundle, a cluster of neurons originating from the locus coeruleus and lateral tegmental nuclei. Projections from the lateral tegmental nuclei specifically target the

BNST, CeA, hypothalamus, and septum (Aston-Jones, et al., 1999; Aston-Jones et al., 1997; Delfs, et al., 1998) and are activated most prominently by stressor exposure (Brener, et al., 1996). Specifically, the BNST and CeA receive projections from the NE bundle, a connection that has been implicated in the stress responses associated with CRH (Delfs et al., 1998). Indeed, both extrahyothalamic sub-structures contain dense populations of CRH-immunoreactive cells (Shaham, Erb & Stewart, 2000). Furthermore, stressors have been shown to increase CRH mRNA in CeA (Hammack et al., 2009; Keifer, Hurt, Ressler & Marvar, 2015). The role of the BNST (and *not* the amygdala) is further supported by prior studies of CRH administration in the BNST and CeA of animals allowed to lever-press for cocaine (Erb & Stewart, 1999). In this set of studies, rats were injected with a CRH receptor antagonist, D-Phe CRH in the BNST or amygdala during tests of reinstatement, fifteen minutes prior to the administration of a physical footshock stressor. A different group of rats was injected with CRH itself into either the BNST or CeA to determine whether the presence of CRH in the system, even without stressor exposure, would induce reinstatement to drug-seeking. Notably, only injections (both antagonist and CRH itself) into the BNST had any effect – injections of either into the amygdala had no effect on reinstatement behaviors. That is, the injection of CRH receptor antagonist into the BNST caused an attenuation of stress-induced reinstatement when it occurred along with a footshock, and injection of CRH itself caused reinstatement-like behaviors when animals were tested in the context in which they had acquired self-administration behavior. This series of experiments suggests the BNST, but not the CeA, is a critical structure mediating the effects of stress-

induced reinstatement after the self-administration of drugs.

Stress & the BNST

A critical component of the stress circuit, the BNST receives projections from extra-hypothalamic regions including the amygdala, prefrontal cortex, and hypothalamus; the BNST projects to and regulates HPA axis activity in response to a stressor exposure. Indeed, the BNST is not only activated by stressor exposure, but may mediate some consequences of the stressor itself (Lezak et al., 2014). The BNST has also been shown to exhibit changes of neuroplasticity in response to chronic stress (Hammack et al., 2010; Pego et al., 2008).

While activation of the BNST mediates the behavioral and physiological responses to stressor exposure (Lezak, et al., 2014), its neurochemistry and morphology is also altered with repeated exposure to stress. Hence, in a study of unpredictable stress in rats, Pego and colleagues (2008) noticed an increase in BNST volume and behavioral manifestations of stress (i.e. anxiety-like behavior) after 28 days of chronic stressor exposure. However, it is important to note that certain sub-regions of the BNST may be more receptive to prolonged stressor exposure and have greater morphological and physiological changes than anatomically similar sub-regions. For example, our labotory has observed alterations in pituitary adenylate cyclase-activating peptide (PACAP) and PAC1 receptor mRNA levels in the anterior dorsolateral BNST (BNSTld), but not the ventral BNST (vBNST), following a repeated variate stress paradigm in rats (Hammack, Cheung et al., 2009). Additionally, several research teams have observed increases in CRH following the same stress paradigm in the BNSTld, but not

other regions of the BNST – changes that the researchers link to an increase in anxiety-like behavior (Lee & Davis, 1997; Schulkin, Gold & McEwen, 1998). Hence, stressor exposure and the activation of the HPA axis may be linked to PACAP and CRH signaling in the BNST.

1.4 Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

PACAP has been implicated as a key regulator of signaling in stress-related brain regions (Hammack & May, 2015; Stroth et al., 2011). First discovered in 1989 by Miyata and colleagues, the peptide is well-conserved, existing in both the 27- and 38-amino acid form. A neurotrophic factor, PACAP promotes cell survival of multiple neuron types, including progenitor cells, dorsal root ganglion cells, cerebellar granule cells, and peripheral sympathetic neurons (Stroth, et al., 2011). Importantly, PACAP has been shown to increase cell survival in response to stressor exposure (Stroth et al., 2013). The peptide belongs to the vasoactive intestinal peptide (VIP) – secretin – GHRH-glucagon family, and shares multiple characteristics with peptide VIP (Miyata et al., 1989; Miyata et al., 1990). PACAP38 predominates over PACAP27 in the nervous system, and PACAP-ergic fibers innervate multiple stress-related regions; large populations of these fibers are found in the hypothalamus, amygdala, and BNST. As mentioned previously, systemic and environmental stressors both activate the HPA axis and sympathetic nervous system (SNS), processes deeply connected to the hypothalamus. Indeed, researchers have postulated that PACAP regulates stress hormone synthesis in a chronic-stress paradigm (Stroth et al., 2011).

PACAP, because it has a similar morphology to peptide VIP, has been

shown to bind to multiple receptor subtypes: PAC1 (exclusively binds PACAP), VPAC1, and VPAC2 (both of which bind PACAP and VIP; for reference, see May, et al., 2010). By binding to these receptors, PACAP can have various effects on intracellular mechanisms (such as membrane depolarization and neurotransmitter release; see Harmar et al., 2012; Shioda, et al., 1996), including neurotrophic and neurotransmitter-like effects.

Role of PACAP in Stress-related Behavior

As mentioned previously, our laboratory and others have argued that PACAP plays a crucial role in stress responding (for review, see Hammack et al., 2010; Lezak et al., 2014; Mustafa et al., 2015; Missig et al., 2014). Hence, PACAP may also play an important role in addiction processes. Indeed, addiction, or substance abuse, is itself a chronic stressor, and as noted above, many addiction processes may be regulated by stressor exposure. PACAP and PAC1 receptors are highly expressed in areas that project to the HPA axis, and several studies have demonstrated the importance of PACAP in HPA signaling (Stroth et al., 2011). Furthermore, Stroth and colleagues (2011) demonstrated an increase in CRH transcription in response to PACAP in hypothalamic cells, and ICV administration of PACAP increased CRH mRNA in the PVN (Hashimoto et al., 2011). Finally, PACAP null mice have exhibited impaired long-term HPA axis activation in response to chronic stressors, an idea that supports the importance of PACAPs role in HPA axis regulation during a stressful situation (Vaudry et al., 2005; Hammack et al., 2010). Interestingly, mice deficient in PACAP, when exposed to ethanol, demonstrated a higher tendency to seek the substance than

control mice (Tanaka et al., 2010).

Our labotory has demonstrated that an intra-BNST infusion of PACAP mimics behavior and physiological responses to stressor exposure (see Hammack, Cheung, et al., 2009; Hammack et al., 2010). For example, the exposure of rats to a seven day repeated variate stress paradigm produces anxiety- and anorexic-like behaviors. These behaviors, when analyzed, were associated with a significant increase in PACAP and PAC1 receptors in the BNST. Bilateral intra-BNST infusion of PACAP38 on its own was sufficient to cause the same stress-related behaviors, including anorexic- and anxiety-like behaviors. Additionally, bilateral intra-BNST infusions of a PAC1 receptor antagonist reduced the stress-induced consequences of repeated variate stress, suggesting that PACAP in the BNST is necessary for the stress responses observed (Roman et al., 2014). Combined, these data implicate PACAP as an important regulator of stress-related pathologies (Hammack et al., 2012).

A growing literature has discussed PACAPs important role in the modulation of stress and anxiety. The present studies were designed to help further the understanding of PACAP's role in stress-induced reinstatement. Hence, we propose that PACAP may drive BNST CRH to produce stress-induced reinstatement. Data from these experiments will help elucidate PACAP's role in activation of the HPA axis and how stressor exposure triggers relapse behaviors after a period of abstinence. Additionally, we will gain a better understanding of how stress contributes to the development of stress-related pathologies, such as stress-induced relapse and PTSD.

CHAPTER 2: INTRA-BED NUCLEUS OF THE STRIA TERMINALIS (BNST) PITUITARY ADENYLATE CYCLASE-ACTIVATING PEPTIDE (PACAP) REINSTATES COCAINE SEEKING IN RATS

2.1 Introduction

PACAP is expressed in several brain regions associated with the activation of the HPA axis after stressor exposure, including the BNST (Vaudry et al., 2005). PACAP has also been argued to be a key regulator of stress responses (Stroth, Holighaus et al., 2011), and may therefore underlie some behavioral processes of stress-induced relapse. However, the brain circuits by which PACAP may regulate these behavioral responses are still under investigation. Combined, BNST PACAP's mediation of stressor exposure (Hammack et al., 2010) and the role of PACAP in regulation of the HPA axis in response to stressor exposure, suggest that BNST PACAP signaling may be important for stress-induced reinstatement to drug-seeking.

The first study in this set of experiments examined how a bilateral intra-BNST infusion of PACAP agonist (PACAP38) affects reinstatement to drug-seeking behavior after a period of extinction. Based on the proposed role of BNST PACAP in response to stressor exposure, we hypothesized that PACAP38 infusion would mimic stressor exposure and cause reinstatement to drug-seeking in rats who had previously undergone extinction of the drug-seeking behavior. This study helps further the understanding of BNST PACAP in stress-induced relapse and, combined with results from Experiment 2, may lead to viable targets for relapse prevention.

2.2 Methods

Animals

Adult male Sprague-Dawley rats (*n*=29; 225-250 g upon arrival), obtained from Charles River Laboratories (Canada), were individually housed and received water and rat chow ad libitum (except when specified). After delivery, rats were allowed to habituate to their home cage for one week before undergoing surgeries. Rats were maintained on a 12 hr light/dark cycle (lights on at 07:00 hours) and all experimentation occurred with lights-on (between 07:30-11:30). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Surgeries

All rats underwent a dual surgery. The first was a jugular vein catheter implantation. Rats were anesthetized using isofluorane vapor (1.5-4.0%) and all surgical procedures were conducted using aseptic techniques. Catheters were constructed using methods described by Fuchs et al., 2004 and consisted of external guide cannula with screw-type connectors (Plastic One Inc., Roanoke, VA, USA), silastic tubing (10 cm; SAI Infusion Technology, Lake Villa, IL, USA), and prolite polypropylene monofilament mesh (2 cm diameter; Biomedical Structures, Warwick, RI, USA). The end of the catheter was inserted into the right jugular vein, secured with suture, and exited on the rats back, posterior to the shoulder blades. To maintain patency, catheters were flushed once daily for 6 days after surgery with 0.2 ml heparinized saline. For the duration of the experiment, each animal received 0.1 ml

heparinized saline immediately following each self-administration session to ensure catheter patency.

Immediately following the jugular catheritization, all animals were re-sterilized and secured with blunted earbars in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) for intra-BNST cannulation. A midline incision was made and the skull was exposed and cleaned. Four burr holes were drilled and screws were fixed to help secure the cannula and dental cement skullcap. Guide cannula (22 guage, Plastics One Inc., Roanoke, VA, USA) were mounted onto each stereotaxic arm and aimed at an angle of 20 degrees just dorsal to the oval BNST (from bregma: AP= -0.1, ML= +3.8, DV= -5.3). Wire stylettes extending 1 mm past the tip were inserted into each guide cannula, and cemented in place. Animals received subcutaneous injections of lactated Ringer's solution and an analgesic (5 mg/kg Metacam, Boehringer Ingelheim, Vetmedica, St. Joseph, MO, USA; administered once immediately after the dual surgery and once the following day). Once awake, rats were returned to their home cages for 7 days post-surgery recovery, during which all rats were observed and weighed daily.

Cocaine Self-Administration/Acquisition

Seven days post-surgery, all rats were given the opportunity to self-administer cocaine (cocaine hydrochloride 3.0 mg/ml obtained from PENRO Specialty Compounding, Colchester, VT, USA) during daily 1-hr sessions for 10 days according to a fixed-ratio 1 schedule of reinforcement. At the start of each session, the catheter was connected to a swivel via polyethylene 20 tubing that was encased

in steel spring lashes (Plastics One Inc., Roanoke, VA, USA). Self-administration occurred in standard operant conditioning chambers (26x25x19 cm) linked to a computer data collection program (MED-PC, Med Associates Inc., St. Albans, VT, USA). The chambers were equipped with two levers (one active and one inactive for the purposes of this experiment), a stimulus light above each lever, and a house light. After the animals were placed in the chamber, a two minute period elapsed before the stimulus light came on above the active lever (signaling drug availability), allowing the animal time to orient to his surroundings. Lever presses on the active lever resulted in a 0.5-s cocaine infusion and a 0.5-s flashing presentation of the white stimulus light above the active lever. In an effort to help the animals acquire cocaine-seeking behavior, several manipulations were made during the 10 days of acquisition: all rats received mild food restriction (20 g rat chow per day) on days 1-2 of acquisition. During this two day mild food restriction period, home-cage rat chow was crushed and mixed with water to create a paste that was coated onto the active lever prior to the rats' self-administration session. One days 3-10 of acquisition, all animals received ad libitum food and water, and the lever was no longer baited. Moreover, the amount of drug delivered per infusion was decreased over the acquisition period, in order to increase cocaine-seeking behaviors, with the following schedule: days 1-3, 1.0 mg/kg/infusion; days 4-7, 0.75 mg/kg/infusion; days 8-10, 0.50 mg/kg/infusion.

Extinction

All rats then experienced daily 1-hr extinction sessions for 20 days during which active lever presses no longer resulted in drug delivery reward. At the completion

of extinction, lever press responses ~0-1 per session.

Reinstatement to cocaine seeking

On the day following the last extinction session, rats were infused into the BNST with 1.0 μg PACAP38 (American Peptide Co., Sunnyvale, CA, USA; 0.5 μl per side) or equivolume 0.05% BSA saline vehicle. For each infusion, the stylette was removed, and an internal cannula that extended 1 mm beyond the end of the guide cannula was inserted. This was connected to a 10ul Hamilton syringe via PE50 plastic tubing. Following the infusion, the internal cannula was kept in place for one minute to allow for diffusion of the drug away from the infusion site. Immediately following bilateral infusion, the rat was placed in the operant conditioning chamber and allowed to lever press (without cocaine reward delivery). Active and inactive lever presses were recorded for 1 hr.

Histology

One day after infusions, rats were anesthetized with Beuthanasia-D (sodium pentobarbital; Zoetis Inc., 333 Portage Street, Kalamazoo, MI, USA) solution and perfused transcardially with 200 ml saline (0.9%) with 0.1% heparin (Sagent, Schaumburg, IL, USA) followed by 100 ml of 10% formalin solution (Fisher Scientific, Atlanta, GA, USA). Brain tissues were post-fixed at 4°C for a minimum of 24 hr in 10% formalin. Fixed tissue was then cut using a cryostat, and sections were collected at 60 μm thickness. Sections were dry mounted, stained with cresyl violet stain, and coverslipped. Cannula placements were visualized using a 4x Nikon Objective on an Olympus microscope.

Data Analysis

Lever press responding during each phase of the experiment was analyzed using repeated measure ANOVAs. Pearson's correlation coefficients were calculated to examine relationships between average daily cocaine intake and reinstatement responding. All main effects were analyzed with appropriate post-hoc comparisons for significance. Effects were noted as significant at $p<0.05$. Rats that did not complete behavioral testing ($n=1$), or whose cannula were placed beyond the BNST ($n=2$) were not included in data analysis.

2.3 Results

Data from 29 animals were used in the analyses for the tests for reinstatement, as post-test histological examination of injection sites for these rats were found to be placed appropriately in the BNST. Two animals had improperly placed cannula and were excluded from analyses. Figure 2 portrays the distribution of cannula placements in analyzed animals.

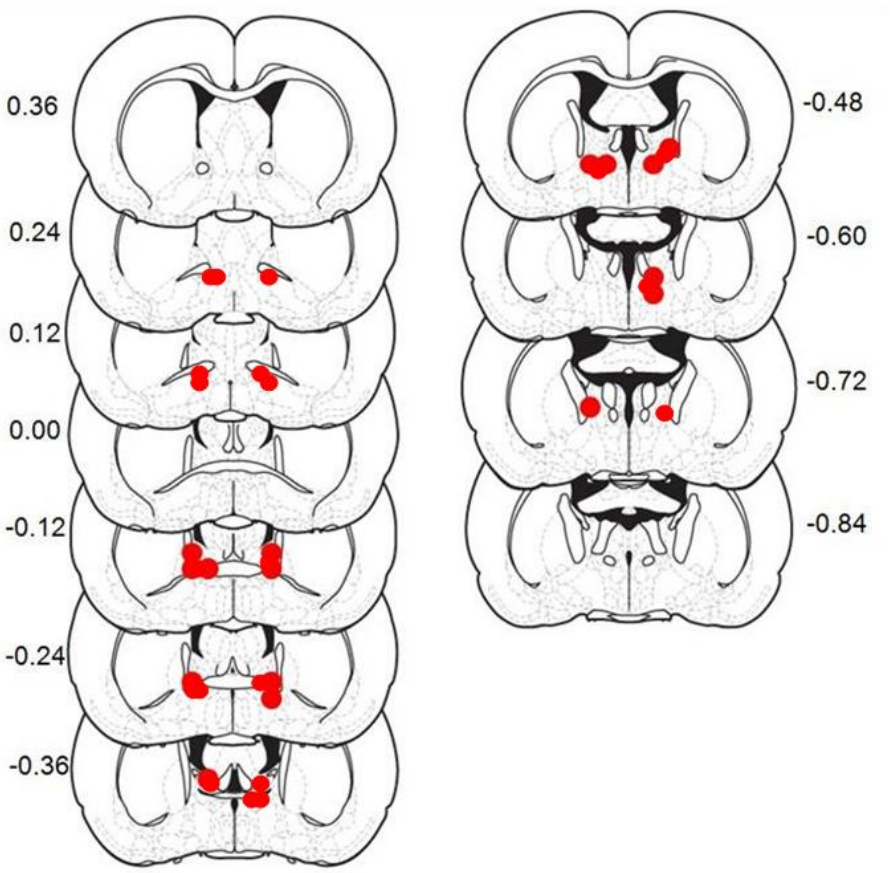

Figure 2: BNST Cannula Placements. Cannula placements for Experiment 1 are depicted above such that enclosed red dots represent a 1 mm extension beyond the tip of the cannula. Rats were included in the analysis if their placement resided within a portion of the BNST.

Self-Administration Training

Rats self-administered an average [mean (±SEM)] of 4.03 (±0.79) infusions of cocaine HCl per hour, 1.0 mg/kg/infusion, on days 1-3 of acquisition; 6.72 (±1.01) infusions per hour, 0.75 mg/kg/infusion, on days 4-7 of acquisition; and 10.36 (±0.68) infusions per hour, 0.5 mg/kg/infusion, on days 8-10 of acquisition (Figure 3). The mean (±SEM) number of responses made on the inactive lever on days 1-3 of acquisition was 3.58 (±1.94); 2.50 (±1.01) on days 4-7; and 0.96 (±0.75) on days 8-10 of acquisition. As demonstrated, by the last day of acquisition, responding on the inactive lever was uniformly low (Figure 3). Animals had an affinity for active lever presses, as responding solely on the active lever produced cocaine delivery; hence, active and inactive lever presses differed by the end of acquisition, ($F(2,17)=2.52$, $p<0.05$). The number of active lever presses was significantly higher ($p<0.05$) than inactive lever presses from Day5 of acquisition through Day10, the end of the self-administration period.

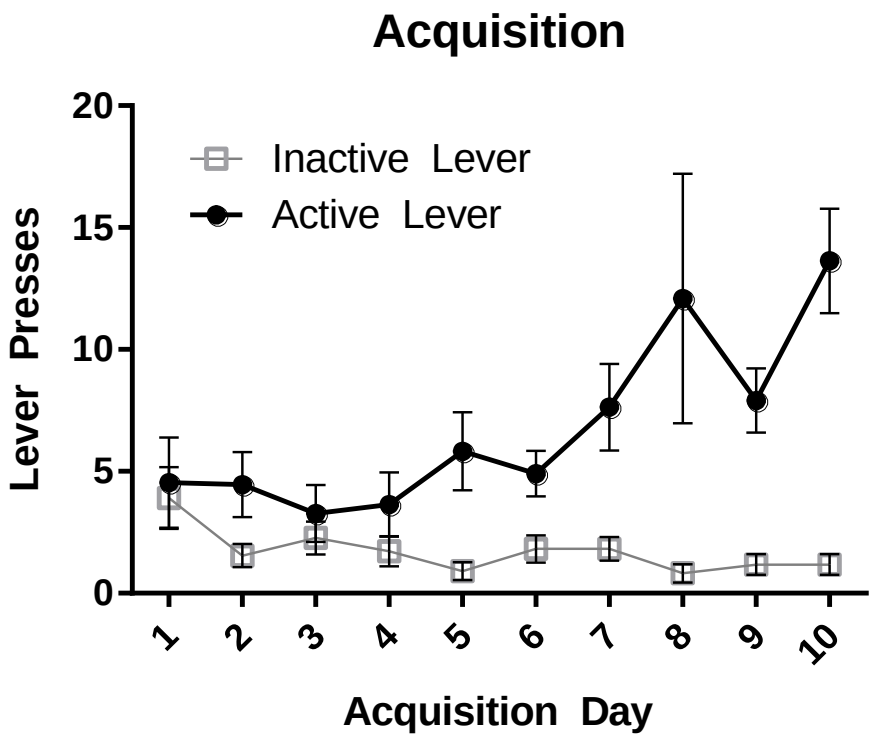

Figure 3: Self Administration training during Experiment 1.

Extinction

The mean (±SEM) number of responses made on the active lever on the first two days of extinction were 6.54 (±1.15) and 12.64 (±2.07), respectively. By the end of extinction, all rats had reached 5 or fewer responses (Figure 4). Inactive lever responses remained uniformly low throughout extinction. In contrast to the otherwise consistently decreasing lever press responses made over extinction, on Extinction Day 13, a 30-min fire alarm interrupted animals actively involved in extinction sessions (Figure 4; day 13). This interruption produced a noticeable effect solely on active lever press responses, perhaps demonstrating stress- induced reinstatement to drug seeking.

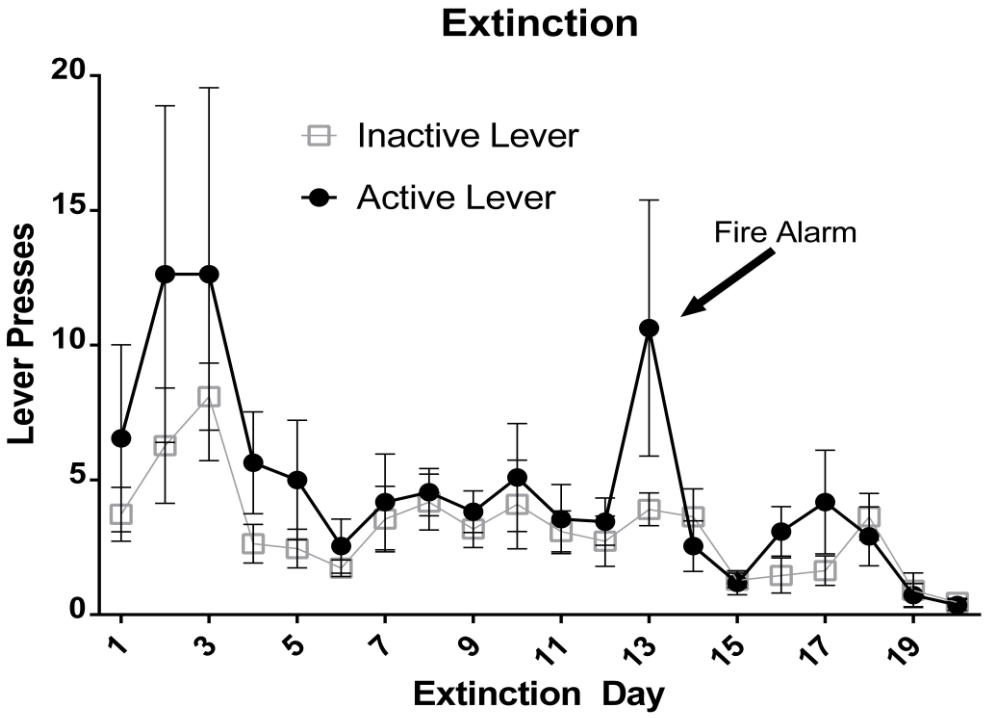

Figure 4: Extinction Training during Experiment 1. Extinction sessions occurred during the 20 days after self-administration training. Significant increases in responding on the first three days of extinction indicate awareness by the animals that the drug is no longer being delivered. On extinction day 13, a 30 min fire alarm interrupted extinction training, creating a stress-induced reinstatement-like response strictly on the active lever (previously associated with cocaine delivery).

Test for reinstatement

The mean (±SEM) number of lever presses made in the 1-hr session on the last day of extinction training was 0.53 (±0.11). The mean (±SEM) number of responses made on the active and inactive levers one day after the last day of extinction (during the test for reinstatement) is shown in Figure 5. No significant differences in responding were found between animals infused with BSA vehicle solution. However, bilateral intra-BNST infusion of PACAP agonist PACAP38 significantly reinstated drug-seeking behavior (13.81 (±2.68)23lever presses) on the lever previously

associated with cocaine delivery. A repeated measures ANOVA for responses on the active lever revealed a significant effect of day, $F(1, 27)=12.08$, $p<0.01$, where active lever press responses were significantly higher on test day than on the last day of extinction. Furthermore, there was a significant day by treatment interaction, $F(1,27)=9.25$, $p<0.01$, where only rats who received PACAP38 increased responding on the active lever during the test for reinstatement (Figure 5). It can be seen in Figure 5 that responding on the inactive lever during the test for reinstatement was very low. A repeated measures ANOVA for inactive lever press responses did not reveal significant effects.

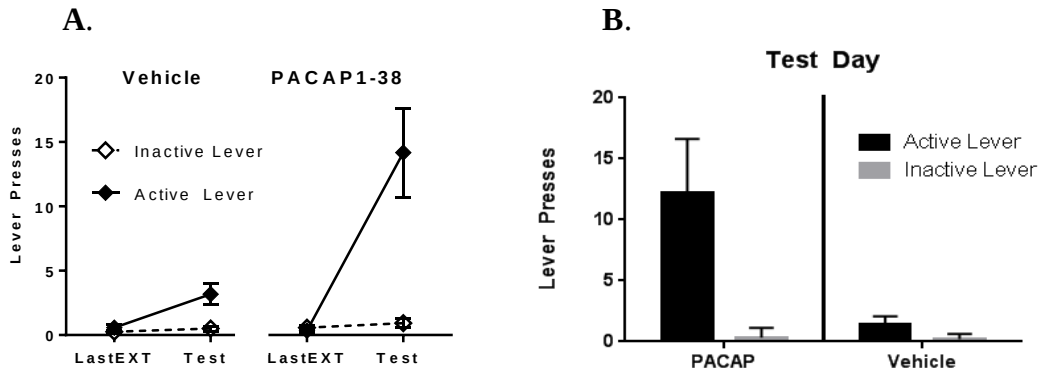

Figure 5: Intra-BNST PACAP38 causes reinstatement to drug-seeking. **A,B**. PACAP38 infusion causes significant reinstatement on the active lever (previously associated with cocaine delivery) during test for reinstatement compared to the last day of extinction (one day prior to test).

2.4 Discussion

Bilateral intra-BNST infusion of PACAP agonist PACAP38 substantially reinstated drug-seeking behavior on the lever previously associated with cocaine delivery. This finding suggests a role of BNST PACAP in stress-induced relapse

and demonstrates the potential of PACAP infusion to mimic stressor exposure. Hence, BNST PACAP is likely critical for behavioral manifestations of stress-induced relapse.

These data support findings from prior studies (Erb et al., 2001) implicating an important role of extra-hypothalamic CRH in stress-induced, but not drug-prime induced, reinstatement to drug seeking. Furthermore, our lab and others (Tsukiyama et al., 2011; Hammack & May, 2014; Roman et a., 2014) have argued that PACAP may be upstream of BNST CRH and therefore may play a prominent role in modulating an extra-hypothalamic CRH system implicated in relapse. Indeed, stressor exposure increases PACAP expression in the oval BNST (Roman et al., 2014), a factor that may facilitate CRH function in the PVN (associated with stress responses). Researchers have also recently shown that PACAP in the lateral parabrachial nucleus (PBn; Rouwette et al., 2012; Hammack & May, 2015) projects to the oval BNST, amygdala, and other stress-related regions. Hence, we suggest that continued drug-taking and the subsequent chronic stress facilitates PACAP expression in the oval BNST which activates local CRH function. This reaction then drives CRH activity in the PVN which facilitates stress responses. Our proposed model is supported by work conducted by Grinevich and colleagues (1997) observing the effects of intracerebroventricular (i.c.v) injections of PACAP38 and PACAP6-38 on CRH gene expression in the PVN in rats. In situ hybridization analysis showed an increase in the hybridization signal of CRH mRNA by PACAP injection that was completely ablated by injection of PACAP6-38. Hence, these data suggest PACAP is involved in the regulation of CRH gene expression and subsequently might be involved in regulating HPA axis activity

(Grinevich et al., 1997). As mentioned previously, we have argued that BNST PACAP may be upstream of CRH. Thus, when PACAP6-38 ablates the production of CRH mRNA, the role of BNST PACAP in CRH activity becomes clearer: upstream ovBNST PACAP may be necessary for the activity of PVN CRH. An alternative hypothesis suggests that drug-taking elicits a chronic stress response that activates PACAP in the PBn. Indeed, recent work has described the co-localization of PACAP and calcitonin gene related peptide (CGRP) in the PBn that extend to the BNST and amygdala (Hammack & May, 2015; Missig et al., 2014).

Other studies have also observed behavioral responses in PACAP-knockout (KO) mice to exogenous substances of abuse such as ethanol (Tanaka et al., 2004) and morphine (Abad et al., 2006). Tanaka and colleagues (2004) demonstrated decreased responding in PACAP-KO mice to the hypothermic and hypnotic effects of ethanol, while morphine withdrawal seemed to be augmented in PAC1 receptor-null mice (Abad et al., 2006). These studies further support the role of PACAP as a mediator of stressor-like responses elicited by substances of abuse.

PACAP plays key roles in the regulation of BNST activity and subsequent stress responding. Prior work in our lab has shown that a 1.0 μg PACAP38 infusion into the BNST increases corticosterone (CORT; a stress hormone) levels (Lezak et al., 2014). Importantly, corticosterone was elevated for about 1hr post-infusion, but not 4 h or 24h post-infusion. Increased corticosterone observed in this study after 1μg PACAP38 infusion suggests that BNST PACAP38 signaling promotes consequences related to stressor exposure (Hammack et al., 2010). Indeed, the 1μg PACAP38 dose utilized

facilitates behavioral and physiological responses in other stress-related paradigms (i.e. anorexic behavior; Lezak et al., 2014; Kocho-Schellenberg et al., 2014; Hammack et al., 2010).

Several other studies have implicated BNST PACAP38 in corticosterone activation that may mimic stressor exposure (Buck et al., 2011). Indeed, after 1μg PACAP38 administration, corticosterone levels are similar to those observed after 30 min of restraint stress (Choi et al., 2007; Radley et al., 2009). Hence, results from our research and others suggest that intra-BNST PACAP38 infusion mimics stress effects on corticosterone levels, potentially through activation of the HPA axis.

Finally, while PACAP can bind to multiple receptor subtypes (PAC1, VPAC1, VPAC2), work conducted in our lab has shown that PAC1 receptor activation likely mediates BNST PACAP effects. For example, when exposed to a repeated variate stress paradigm, anxious rats had an increase in PACAP and PAC1 receptor expression, but no elevation in VPAC1 or VPAC2 receptors, or VIP (Hammack, Cheung, et al., 2009). These results suggest that the anxiety-like behavior observed may be linked to an increase specifically in PACAP and PAC1 expression levels. Additionally, as mentioned below, infusion of a PAC1 receptor agonist, mazadillan, into the BNST blocked effects of stressor exposure while activation of PAC1 receptors caused anxiogenic responses (Roman et al., 2014; Missig et al., 2014). These results and others implicated an importance of BNST PACAP in the activation of downstream CRH and subsequent stress-responses.

Combined, these studies suggest an importance of BNST PACAP in stress

responding and the activation of the HPA axis. Indeed, in a clinical setting, Ressler and colleagues (2011; Andero & Ressler, 2012; Stevens et al., 2014) have shown an increase in PACAP expression in patients with post-traumatic stress disorder (PTSD). We therefore decided to observe the importance of BNST PACAP signaling in stress-induced reinstatement to drug seeking, as changes in PACAP signaling have been implicated in mental health disorders by HPA axis dysfunction.

CHAPTER 3: INTRA-BED NUCLEUS OF THE STRIA TERMINALIS (BNST) PITUITARY ADENYLATE CYCLASE-ACTIVATING PEPTIDE ANTAGONIST (PACAP6-38) ATTENUATES STRESS-INDUCED REINSTATEMENT IN RATS

3.1 Introduction

As demonstrated in the previous experiment, BNST PACAP may play an important role in stress-induced reinstatement to drug seeking, as a bilateral intra-BNST infusion of PACAP agonist PACAP38 substantially reinstated drug-seeking on the lever previously associated with cocaine delivery. In support of these findings, our laboratory has previously shown that the activation of BNST PACAP receptors with PACAP38 is sufficient to produce multiple behavioral manifestations of stressor exposure, including anorexic-like effects, weight loss, and heightened anxiety-like behaviors (Roman et al., 2014). Furthermore, when a PAC1 and VPAC2 receptor antagonist (PACAP6-38) was infused into the BNST, these stress-induced consequences were blocked, again implicating BNST PACAP in the behavioral manifestations of stressor exposure (Roman et al., 2014).

In the second study in this set of experiments, we hypothesized that injecting a PAC1/VPAC2 receptor antagonist, PACAP6-38, into the BNST directly prior to stressor exposure would attenuate drug-seeking behavior after stressor exposure. Indeed, Roman et al., 2014 demonstrated that blocking BNST PACAP signaling by continuously infusing PACAP6-38 into the BNST during a week of repeated variate stress attenuated anxiety-like behavior and accompanying weight alterations typically seen after stressor exposure. This chapter describes the effects of PACAP6-38 on

stress-induced reinstatement to drug seeking when rats are exposed to an intermittent unpredictable footshock (previously shown to cause stress-induced reinstatement; Erb et al., 2001). As mentioned in Chapter 1, stressor exposure has been shown to significantly increase drug-seeking behavior after a period of extinction; this was demonstrated in Experiment 1 (extinction day 13, during the fire drill). Furthermore, recent literature (Lezak et al., 2014; Hashimoto et al., 2011; Levran et al., 2015) has implicated PACAP as an important polypeptide in the modulation of stress circuitry. This set of experiments helps expand the understanding of how BNST PACAP may modulate stressor exposure, particularly in a stress-induced reinstatement paradigm. These data, and others, will aid in our understanding of how stress systems are modulated by BNST PACAP and how stress may contribute to stress-related pathologies, such as stress-induced relapse.

3.2 Methods

Animals

Adult male Sprague-Dawley rats (*n*=27; 225-250 g upon arrival), obtained from Charles River Laboratories (Canada), were individually housed and received water and rat chow ad libitum (except when specified). After delivery, rats were allowed to habituate to their home cage for one week before undergoing surgeries. Rats were maintained on a 12-hr light/dark cycle (lights on at 07:00 hours) and all experimentation occurred with lights-on (between 7:30-11:30 am). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Surgeries

All rats underwent a dual surgery, identical to the one described above (Chapter 2: Methods, *Surgeries*), in which they received bilateral intra-BNST cannulation and jugular vein catheterization. To maintain patency, catheters were flushed once daily for 6 days after surgery with 0.2 ml heparinized saline. For the duration of the experiment, each animal received 0.1 ml heparinized saline immediately following each self-administration session to ensure catheter patency. All animals received subcutaneous injections of lactated Ringer's solution and an analgesic (5 mg/kg Metacam, Boehringer Ingelheim, Vetmedica, St. Joseph, MO, USA; administered once immediately after the dual surgery and once the following day). Once awake, rats were returned to their home cages for 7 days post-surgery recovery, during which all rats were observed and weighed daily.

Cocaine Self-Administration/Acquisition

Seven days post-surgery, all rats were given the opportunity to self-administer cocaine (cocaine hydrochloride 3.0 mg/ml obtained from PENRO Specialty Compounding, Colchester, VT, USA) during daily 1-hr sessions for 10 days according to a fixed-ratio 1 schedule of reinforcement in manner identical to that described in Chapter 2. To review, lever presses on the active lever resulted in a 0.5-s cocaine infusion and a 0.5-s flashing presentation of the white stimulus light above the active lever. In an effort to help the animals acquire cocaine-seeking behavior, the same manipulations made in Experiment 1 were also made in this experiment: all rats received mild food restriction (20 g rat chow per day) on days 1-2 of acquisition,

and home-cage rat chow was coated lightly on the active lever prior to the rats' self-administration session. On days 3-10 of acquisition, all animals received ad libitum food and water, and the lever was no longer baited. Additionally, the amount of drug delivered per infusion was again decreased over the acquisition period, in order to increase cocaine-seeking behaviors, with the following schedule: days 1-3, 1.0 mg/kg/infusion; days 4-7, 0.75 mg/kg/infusion; days 8-10, 0.50 mg/kg/infusion.

Extinction

Extinction occurred exactly as described in Chapter 2. Rats experienced daily 1-hr extinction sessions for 20 days during which active lever presses no longer resulted in drug delivery reward. At the end of extinction, lever press responses ~0-1 per session.

Reinstatement to cocaine seeking

On the day following the last extinction session, rats were split into four groups, counterbalanced, that received different treatments. Group Shock-PACAP6-38 received a footshock along with a PACAP antagonist infusion; Group Shock-BSA received footshock and vehicle infusion; Group NoShock-PACAP6-38 received an infusion of PACAP antagonist before being re-exposed to the boxes on test day; and Group NoShock-BSA received a vehicle infusion and no stressor exposure. Rats were infused with 1.0 μg PACAP6-38 (a PAC1 receptor antagonist; American Peptide Co., Sunnyvale, CA; 0.5 μl per side) or equivolume 0.05% BSA vehicle. Infusions were carried out according to previously described methods. Immediately following infusion, animals were placed back in the operant conditioning chamber and administered

five 2.0-mA 5-s footshocks (averaging one shock every 12 min over 1 hr). Active and inactive lever presses were recorded during the 1 hr session.

Histology

One day after infusions, rats were anesthetized with Beuthanasia-D (sodium pentobarbital; Zoetis Inc., 333 Portage Street, Kalamazoo, MI, USA) solution and perfused transcardially as previously described. To summarize, brain tissues were post-fixed in 10% formalin, cut using a cryostat, dry mounted, stained with cresyl violet and visualized under a microscope. Animals with improper cannula placement were removed from data analysis.

Data Analysis

Lever press responding during reinstatement was analyzed using repeated measure ANOVAs. Pearson's correlation coefficients were calculated to examine relationships between average daily cocaine intake and reinstatement responding. All main effects were analyzed with appropriate post-hoc comparisons for significance. Effects were noted as significant at $p<0.05$. Rats that did not complete behavioral testing ($n=4$), or whose cannula were placed beyond the BNST ($n=5$) were not included in data analysis.

3.3 Results

Data from 27 animals were used in the analyses for the tests for reinstatement (Group Shock-PACAP6-38, $n=9$; Group Shock-BSA, $n=6$; Group NoShock-PACAP6-38, $n=6$; Group NoShock-BSA, $n=6$), as post-test histological examination of injector sites for these rats were found to be placed appropriately, and all completed

behavioral testing. Four animals did not complete behavioral testing due to gnawed-out neck sutures and subsequent jugular catheter exposure; five animals received improper cannula placements and were excluded from analysis. Figure 6 portrays the distribution of cannula placements in analyzed animals.

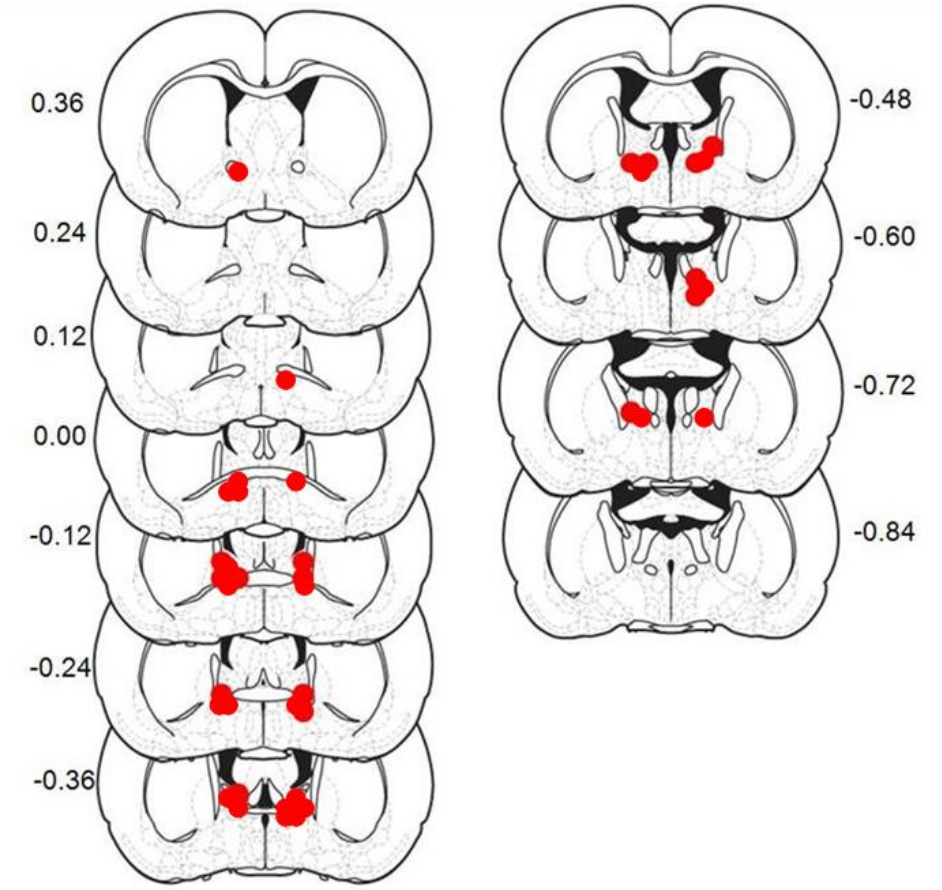

Figure 6: BNST Cannula Placements. Cannula placements for Experiment 2 are depicted above such that enclosed red circles represent a 1 mm extension beyond the tip of the cannula. Rats were included in analysis if their placement resided within a portion of the BNST.

Self-administration training

Rats lever pressed, on average, 3.86 (±0.64) times per hour, 1.0 mg/kg/infusion, of cocaine HCl on days 1-3 of acquisition; 3.39 (±0.56) times per hour, 0.75 mg/kg/infusion on days 4-7 of acquisition; and 7.11 (±0.52) times per hour, 0.5 mg/kg/infusion on days 8-10 of acquisition (Figure 7). The mean (±SEM) number of responses made on the inactive lever on the first day of acquisition was 3.61 (±0.75). By the last day of acquisition, responding on the inactive lever was uniformly low (0.76 (±0.22) lever presses per hour; Figure 7). As in Experiment 1, animals had an affinity for active lever presses, as responding solely on the active lever produced cocaine delivery; hence, active and inactive lever presses differed by the end of extinction. The number of active lever presses was significantly higher ($p<0.05$) than inactive lever presses from Day3 of acquisition through Day10, the end of the self-administration period.

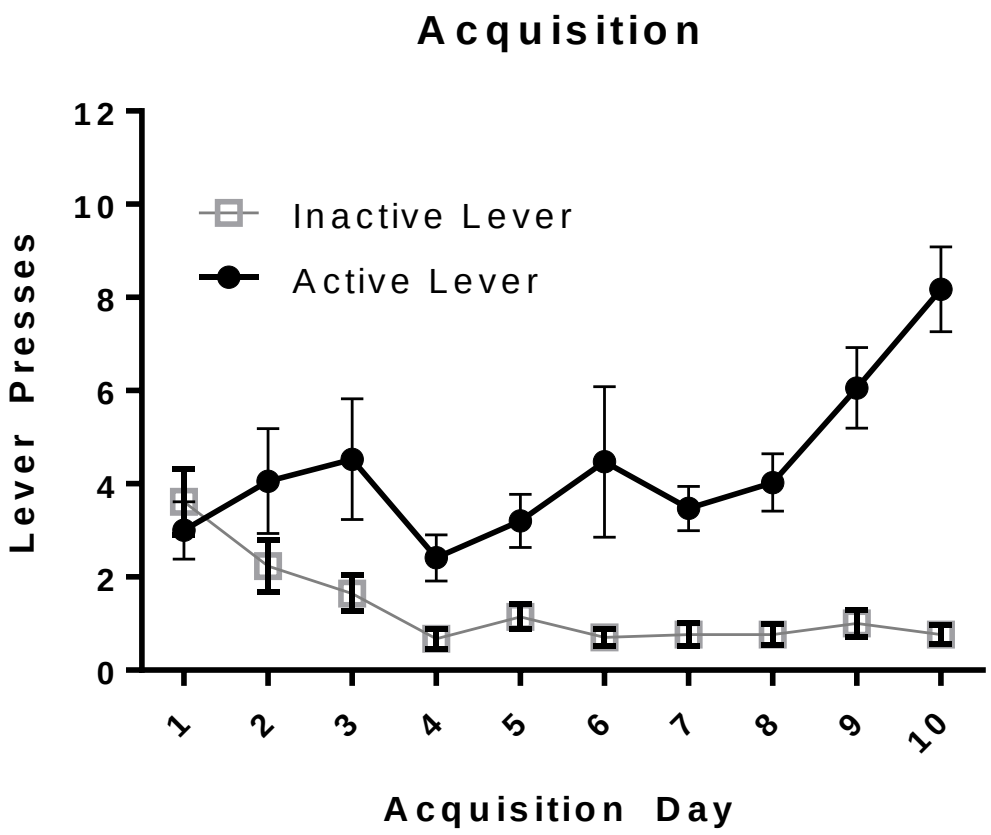

Figure 7: Self-Administration during Experiment 2.

Extinction

The mean (±SEM) number of responses made on the active lever on the first two days of extinction were 12.38 (±1.34) and 13.93 (±0.72), respectively, in a similar manner to the first two days of extinction in Experiment 1. Similarly, by the end of extinction, all rats had reached 5 or fewer responses (Figure 8). Inactive lever responses remained uniformly low throughout extinction.

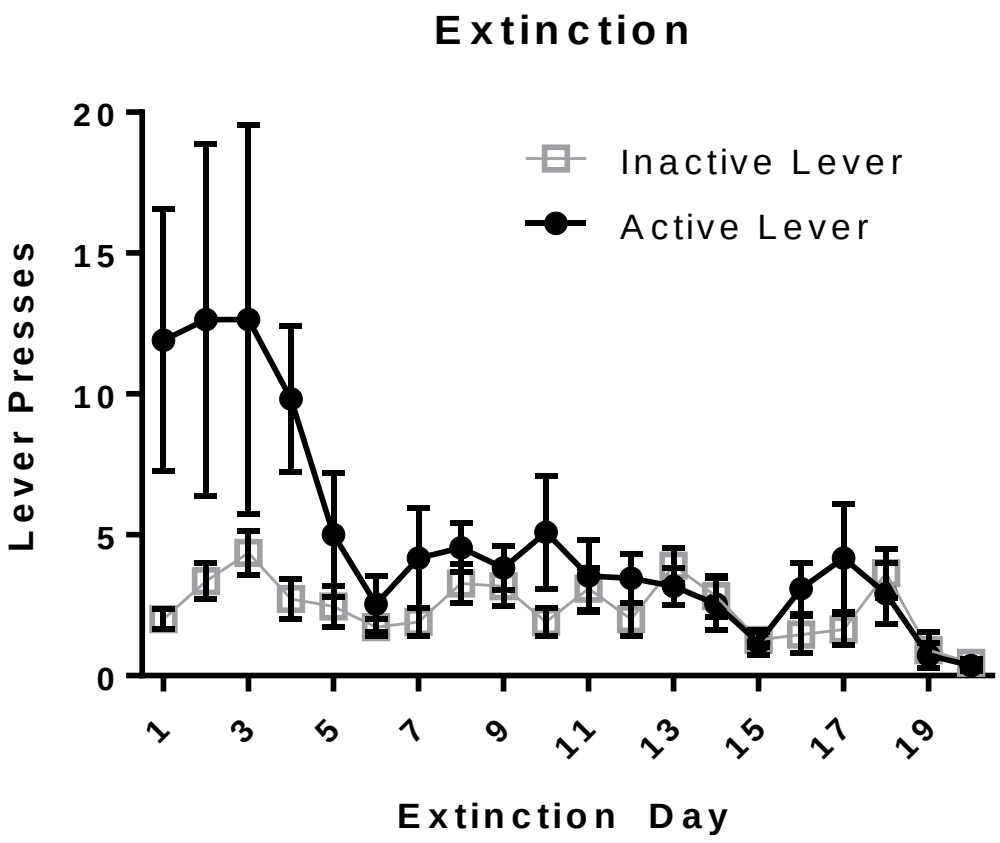

Figure 8: Extinction Training during Experiment 2.

Test for reinstatement

The mean (±SEM) number of lever presses made in the 1 hr session on the last day of extinction training was 0.91 (±0.21). The mean (±SEM) number of responses made on the active and inactive levers one day after the last day of extinction (during the test for reinstatement) is shown in Figure 9. Briefly, animals in the Shock-BSA condition lever pressed 9.04 (±0.77) times on the active lever, and 1.33 (±0.44) times on the inactive lever. The Shock-BSA group was the only group to demonstrate

stress-induced reinstatement behaviors. In contrast, animals in the Shock-PACAP6-38 group responded on average 1.11 (±0.77) times on the active lever, and 0.44 (±1.15) times on the inactive lever; the NoShock-PACAP6-38 group responded, on average, 1.16 (±1.16) times on the active lever, and 1.16 (±1.08) times on the inactive lever; and finally, animals in the NoShock-BSA group lever pressed 0.66 (±1.50) times on the active lever and 0.14 (±0.41) times on the inactive lever. Importantly, as will be discussed below, the Shock-PACAP6-38 group (Figure 9) demonstrated low levels of drug-seeking behavior even after stressor exposure.

A repeated measures ANOVA for inactive lever press responses did not reveal significant effects. Data from 27 animals were used in the analyses for the tests for reinstatement. There was a significant main effect of time on active lever press responses between the last day of extinction and test day, $F(1, 23)=9.47$, $p=0.005$. There was also a significant interaction between both time and shock, $F(1,23)=14.031$, $p=0.001$, and time and infusion treatment, $F(1,23)=7.89$, $p=0.010$, indicating an importance of both shock and the administration of the PACAP antagonist on lever pressing. Lastly, there was a significant time by shock by infusion treatment interaction, $F(1,23)=12.10$, $p=0.002$, indicating the necessity of the combined shock and treatment variables on lever press activity. As demonstrated in Figure 9, only animals who received the footshock paired with a vehicle solution infusion demonstrated stress-induced reinstatement; hence, PACAP6-38 blocked stress-induced reinstatement to cocaine seeking when the rats were exposed to a stressor. Post hoc analyses, comparing antagonist infusion with the vehicle condition, on total

responses on the active lever after footshock exposure revealed a significant effect of PACAP6-38 ($p<0.01$). The PAC1 receptor antagonist did not alter responding on the inactive lever ($F(1,23)=2.3$, NS) and responses on the inactive lever remained low (0-3 lever presses) in the 1 hr test period. Additionally, the number of responses on the active lever in the no-footshock condition after injections of PACAP6-38 did not differ from lever press activity after vehicle infusion (see Figure 9).

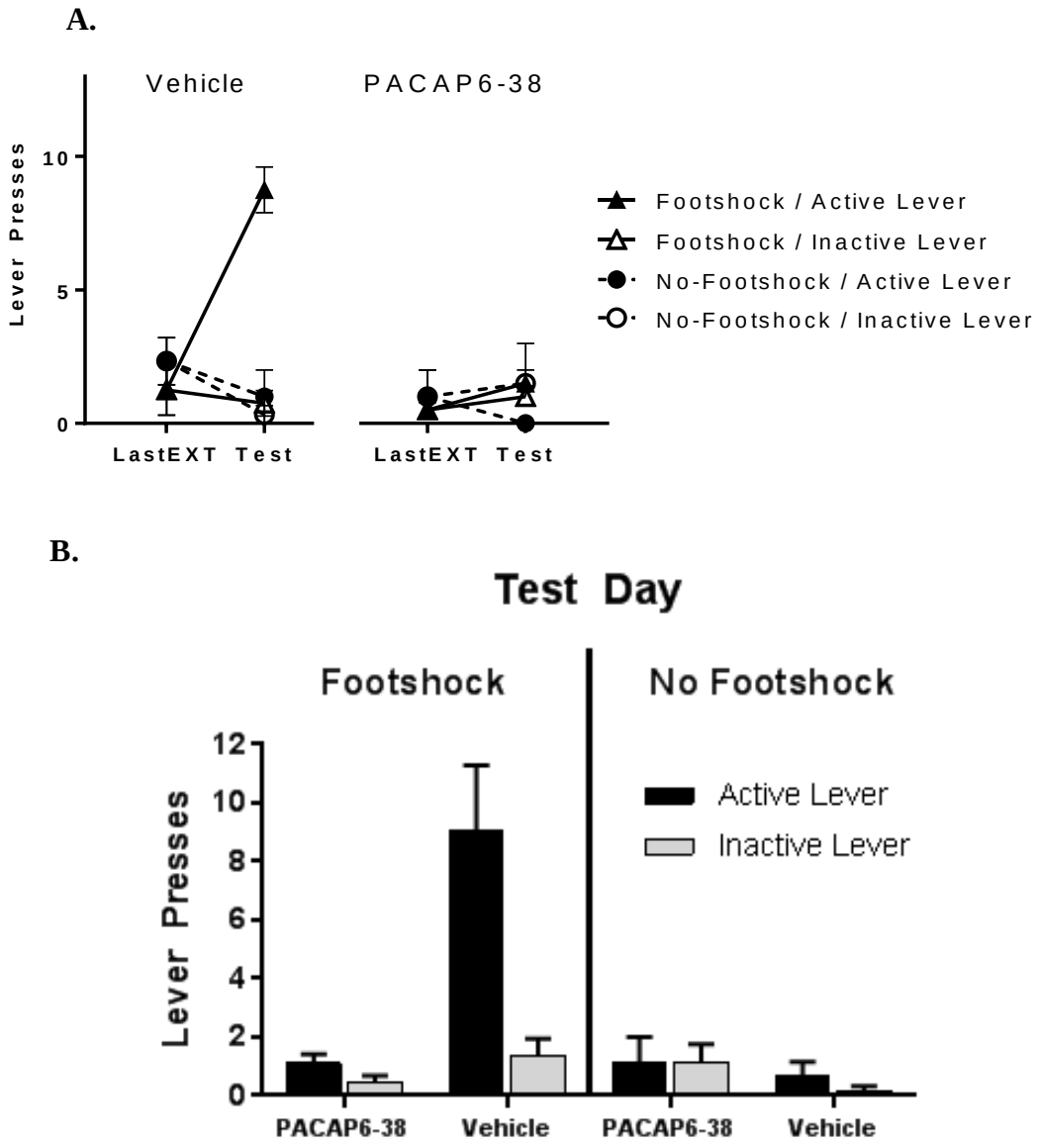

Figure 9: Intra-BNST PACAP6-38 attenuates stress-induced reinstatement when paired with intermittent, unpredictable footshock. **A**. Intra-BNST PACAP antagonist significantly attenuated stress-induced reinstatement when paired with a footshock when compared with the last day of extinction. Only animals who received intra-BNST vehicle treatment paired with a footshock demonstrated increased reinstatement to drug-seeking behaviors on the lever previously associated with cocaine delivery. **B.** Test for reinstatement by intermittent, unpredictable footshock.

3.4 Discussion

As mentioned previously, bilateral intra-BNST infusion of PACAP38 is sufficient to cause stress-related behaviors, including anorexic-like tendencies, and anxiety-like behaviors. Furthermore, bilateral intra-BNST infusion of PAC1/VPAC2 receptor antagonist PACAP6-38 has been shown to reduce consequences of stressor exposure (Roman et al., 2014). Together, these data suggest that BNST PACAP is necessary for stress responses, and is an important regulator of stress-related pathologies (such as PTSD and anxiety-like disorders; see Hammack et al., 2010; Ressler et al., 2011). The present experiment expanded upon this research and supports a role of BNST PACAP in stress-induced relapse. Hence, when PAC1/VPAC2 receptors are blocked, stressor exposure was no longer sufficient to reinstate drug-seeking behavior. Prior studies have shown that BNST PACAP/PAC1 receptor expression is elevated exposure to chronic stress (Hammack & May, 2015). BNST PACAP application mimics stressor exposure (see Experiment 1), PAC1/VPAC2 antagonist blocks consequences of stressor exposure. Combined with prior data, these results implicate BNST PACAP and/or PAC1/VPAC2 receptors as important mediators of behavioral outputs of stress.

As mentioned in Chapter 2, we hypothesize that drug-use activates PACAP upstream of CRH in the oval BNST, which then modulates CRH in the PVN to stimulate a stress response. Hence, administration of PAC1/VPAC2 antagonist PACAP6-38 into the BNST (see Experiment 3: Methods) may block receptors responsible for maintaining modulations of downstream PVN CRH (responsible

for stress responses). Support for this theory comes from work conducted by Stewart and colleagues (2000) describing the role of CRH in stress-induced reinstatement. As mentioned previously, CRH receptor antagonist D-Phe CRH was found to block footshock-induced reinstatement in animals that had been trained to lever press for heroin. Our model suggests that PACAP regulates CRH activity in the PVN and, subsequently, stress responses. Hence, blocking upstream PACAP would cause a reduction in reinstatement processes similar to those observed after the application of D-Phe CRH to the BNST.

As mentioned previously, promiscuous PACAP can bind to one of three receptors (PAC1, VPAC1, and VPAC2). While current research has not definitively stated that one receptor is more likely to mediate stress-induced relapse than another, several studies implicate PAC1 receptor activation as a key component of BNST PACAP effects. For example, following exposure to a repeated variate stress paradigm, rats demonstrated an increase in anxiety-like behavior and an increase in PACAP and PAC1 receptor expression, but no change in VPAC1 or VPAC2 receptors, or VIP expression (Hammack, Cheung, et al., 2009; Hammack et al., 2010). Furthermore, Roman et al., (2014), infused PAC1/VPAC2 receptor antagonist PACAP6-38 into the BNST following repeated variate stress exposure which blocked stress-related behaviors (i.e., anorexic effects). Combined, these data implicate a role of BNST PACAP38 and its PAC1 receptor on stress-related responding.

CHAPTER 4: GENERAL DISCUSSION

The present experiments were designed to examine the relationship between PACAP38, a polypeptide that has been shown to drive BNST CRH, and its receptor antagonist, PACAP6-38, in stress-induced reinstatement to drug seeking. We found that an infusion of PACAP38 into the BNST was sufficient to reinstate previously-extinguished cocaine seeking behavior. Furthermore, infusion of PAC1 receptor antagonist, PACAP6-38, immediately before stressor exposure, attenuated reinstatement to drug seeking when paired with that stressor.

Prior data (Hammack, Cheung, et al., 2009) has shown that repeated stressor exposure increases both PACAP and PAC1 receptors in the BNST. Additionally, preliminary studies in our lab have demonstrated that BNST PACAP expression may increase 24 hours after exposure to a single stressor. Hence, one stressor exposure may be sufficient to cause neurochemical changes in stress circuitry that manifest themselves in behavioral consequences.

One theory that may explain the results of this experiment and others (Erb et al., 2001; Carroll & Comer, 1996; Ettenberg, MacConell, & Geist, 1996; Gerber & Stretch, 1975; Katner, Magalong & Weiss, 1999; Meil & See, 1996) proposes that the activation of stress and anxiety circuits, and the subsequent neurochemical and morphological changes in stress circuitry that occurs following drug administration, become part of the context of drug taking that then cues drug-seeking (reinstatement) after extinction. Homeostatic threats (i.e. stressor exposure) have been shown to activate the HPA axis, a system that promotes the neurochemical, morphological, and physiological changes

needed to maintain homeostasis. Furthermore, drugs have been shown to activate the BNST, and several abusive substances elicit symptoms of anxiety and panic (Singewald, Salchner & Sharp, 2003). Acute and chronic drug self-administration has also been shown to activate the HPA axis (Garwin & Kleber, 1985; Ramos-Aliaga & Werner, 1982), and PACAP and PACAP receptors are highly expressed in brain areas that modulate HPA axis activity. Hence, PACAP is important in signaling HPA activity (Stroth, Holighaus, et al., 2011), and regulating behavioral responses to stressor exposure. Therefore, drugs that activate PACAP signaling, which then activates BNST CRH, during periods of self-administration may drive the behavioral and physiological stress response that becomes part of the context for drug taking. Our lab proposes that after a period of abstinence, or extinction, stressor exposure can then activate re-activate this circuit, an action that leads to increased BNST PACAP and subsequent CRH signaling (previously shown to reinstate drug-seeking behavior).

Some drugs (such as cocaine) have been reported to potentiate psychomotor stimulant effects, a reaction that may modify the user's metabolism and the activity of neurotransmitters (i.e. increases levels of acetylcholine, Wise & Kiyatkin, 2011) and inhibit re-uptake of serotonin, norepinephrine, and dopamine (Little et al., 2014, Economidou, Dalley & Everitt, 2011; Navarro et al., 2013). Prior research has implicated all these neurotransmitters in the stimulation of hypothalamic CRH secretion. Hence, drugs (i.e. cocaine) may activate the HPA axis by releasing hypothalamic CRH. Indeed, Calogero et al., (1989) demonstrated that cocaine stimulated CRH secretion from hypothalamic regions in vitro. However,

work done in our laboratory implicates a role of local BNST PACAP and its projection to the PVN in stress-related circuitries. Indeed, activation of the PVN results following activation of other extra-hypothalamic regions, such as the amygdala and hippomcapus, areas that also mediate emotional behavioral outputs (Herman & Cullinan, 2005).

PACAP and PAC1 receptor knock-out mice exhibit decreased anxiety-like behavior when compared to control mice (Hashimoto et al., 2001; Girard et al., 2006). PAC1 null mice also demonstrate reduced anxiety-like behavior in open field tests (used most commonly to determine anxiety-like states; Otto, Martin et al., 2001). In relation to the aforementioned proposed theory, PAC1 null mice also demonstrate deficits in contextual learning (Otto, Kovalchuk, et al., 2001). Hence, drug use (known to activate stress circuits) may activate BNST PACAP, which then cyclically cues for drug use after a period of abstinence when stressor exposure again activates the same system. Indeed, the activation of BNST PACAP has been shown to increase the excitability of CRH neurons in the BNST, which then activates the HPA axis (Choi et al., 2007). Moreover, our lab has proposed that PACAPergic fibers may increase CRH neuron excitability directly in the BNST oval nucleus (Lezak, et al., 2014), further implicating BNST PACAP as an important component of stress-induced reinstatement to drug seeking.

While our lab hypothesizes that drug administration activates PACAP signaling during drug-taking periods, thus driving BNST CRH, and that subsequent stressor exposure may re-activate this circuit and potentiate stress-induced reinstatement, stress systems may also be activated during withdrawal. In this scenario, stress system

activation may provide a contextual cue for withdrawal states. Stressor exposure may then provide a context cue that brings the user back to a state of withdrawal, thus producing stress-induced relapse. Hence, future experiments may strive to elucidate the initiation of PACAP signaling in stress-induced reinstatement. Based on this data and others, our lab postulates an increase in PACAP signaling to BNST CRH in response to stressor exposure that activates stress systems previously paired with drug use.

Sources of BNST PACAP

As mentioned previously, there are multiple pathways by which stress, drugs, and drug-associated stimuli are thought to induce reinstatement to drug-seeking. In short, stress and drug-associated stimuli have been shown to activate glutamate projections to the ventral tegmental area (VTA) from the prefrontal cortex and amygdala; in contrast, drugs of abuse themselves stimulate dopamine release from VTA dopamine neurons projecting to the nucleus accumbens. Indeed, relapse induced by priming injections of heroin and cocaine appears to activate mesolimbic dopaminergic pathways (Stewart, 2000). Stress-induced relapse, as mentioned previously, may utilize CRH, HPA axis activation, and subsequent corticosterone secretion to activate dopaminergic neurons in the VTA. Hence, CRH has been shown to induce relapse by actions in the BNST and blocked by CRH antagonists at this site (Stewart, 2000).

Several researchers have demonstrated that PACAP activity within the BNST originates from the PBn (Missig et al., 2014; Hammack & May, 2015). Conversely, PACAP and the mRNA encoding PACAP's precursor seem most abundant

in the hypothalamus (Ghatei et a., 1993). Indeed, PACAP within the BNST binds to one of at least two distinct receptors for PACAP, the PAC1 receptor (mentioned in Experiment 2), and the VPAC1 receptor (binds PACAP and VIP). However, it is important to note that while PACAP6-38 has been used as a PAC1 receptor antagonist, it does not discriminate between PAC1 and VPAC2 receptors. Thus, PACAPs role in stress-induced reinstatement may be directed toward either of these receptor subtypes.

Several studies have reported that during human abstinence from drug use, stress exposure often occurs in the presence of contextual cues that had previously been associated with drug use (Epstein et al., 2009); these cues may cause an initial lapse in abstinence. Additionally, in a similar manner to the drug-cue induced reinstatement mentioned above (Chapter 1), human studies have indicated that stressor exposure can lead to cue-induced drug craving (Moran-Santa Maria et al., 2015; Fox et al., 2014; Mantsch et al., 2015). Furthermore, Sinha and colleagues (1999; 2001) introduced a clinical trial based on the rodent stress-induced reinstatement paradigm. In these trials, stressor exposure (guided imagery stress and Trier Social Stress Task) caused increased drug craving. Additionally, researchers provided a systemic CRH injection to induce physiological (i.e. increased corticosl release) and psychological (i.e. increased anxiety) stress responses. Since then, many researchers have examined the effects of different drugs on stress-induced relapse (Sinha et al., 2007; Fox et al., 2012; McKee et al., 2014; Moran-Santa Maria et al., 2015), but none have observed the potential effect of PACAP on stress-induced relapse. In theory, PACAP treatments may locally target BNST PAC1/VPAC2 receptors vis systemic injection in an effort to

antagonize receptors and prevent PACAP binding that facilitates stress-responses and, subsequently, stress-induced relapse to drug seeking. By blocking BNST PACAP activation, PVN CRH activity may be modified, altering responses to stressor exposure. Continued research of mechanisms that underlie stress- and drug-cue- induced reinstatement after a period of abstinence may lead to the development of viable treatments for relapse prevention.

Finally, the limited data in human drug abusers indicate that there is a reasonable correlation between animal models of reinstatement and drug relapse and craving in humans. As research in the field of drug abuse continues, it will remain important to develop practical (medication development) and theoretical models to combat substance use. Future laboratory-based human models may mimic and complement existing laboratory-based rodent models. The potential use of PACAP antagonists as a method to prevent stress-induced relapse during times of known stressor exposure should be considered. Indeed, it will remain important throughout these developmental phases to investigate the concordance between human and animal data of stress-induced relapse.

Citations

Abad, C., Niewiadomski, P., Loh, D.H-W. & Waschek, J.A. (2006). Neurotransmitter and immunomodulatory actions of VIP and PACAP: Lessons from knockout mice. *International Journal of Peptide Research and Therapeutics, 12*(3): 297-310.

Andero, R., & Ressler, K. J. (2012). Fear extinction and BDNF: translating animal models of PTSD to the clinic. *Genes, Brain and Behavior*, *11*(5), 503-512.

Aston-Jones, G., Delfs, J.M., Druhan, J., Zhu, Y. (1999). The bed nucleus of the stria terminalis: a target site for noradrenergic actions in opiate withdrawal. *Annals of the New York Academy of Science, 877*:486-498.

Aston-Jones, G., Shipley, M.T., Grzanna, R. (1995). The locus coeruleus, A5 and A7 noradrenergic cell groups, in: G Paxinos (Ed.), The Rat Nervous System, Academic Press, San Diego, CA, pg. 183-213.

Avery, S. N., Clauss, J. A., & Blackford, J. U. (2015). The Human BNST: Functional Role in Anxiety and Addiction. *Neuropsychopharmacology*.

Bouton, M.E. & Bolles, R.C. (1979). Role of conditioned contextual stimuli in reinstatement of extinguished fear. *Journal of Experimental Psychology Animal Behavioral Processes, 5*: 368-378.

Bouton, M.E. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biological Psychiatry, 52*(10): 976-986.

Bouton, M.E. & Swartzentruber, D. (1991). Sources of relapse after extinction in Pavlovian and instrumental learning. *Clinical Psychology Review, 11*, 123-140.

Bouton, M.E., Winterbauer, N.E. & Vurbic, D. (2012). Context and extinction: Mechanisms of relapse in drug self-administration. *Clinical applications of learning theory*, 103-133.

Bremner, J.D., Krystal, J.H., Southwick, S.M., Charney, D.S. (1996). Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse, 23*: 28-38.

Brown, S.A., Vik, P.W., Patterson, T.L., Grant, I. & Schuckit, M.A. (1995). Stress, vulnerability and adult alcohol relapse. *Journal of Studies on Alcohol and Drugs, 56*, 538-545.

Buck, H.M., Hueston, C.., Bishop, C. & Deak, T. (2011). Enhancement of the hypothalamic-pituitary-adrenal axis but not cytokine responses to stress challenges imposed during withdrawal from acute acohol exposure in Sprague-Dawley rats. *Psychopharmacology (Berl), 218*(1): 203-215.

Carroll, M.E. & Comer, S.D. (1996). Animal models of relapse. *Experimenta and Clinical Psychopharmacololgy, 4*: 11-18.

Carter, B.L. & Tiffany, S.T. (1999). Meta-analysis of cue-reactivity in addiction research. *Addiction, 94*: 327-340.

Charbogne, P., Kieffer, B.L., Befort, K. (2014). 15 years of genetic approaches in vivo for addiction research: Opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology, 76*(B): 204-217.

Choi, D.C., Furay, A.R., Evanson, N.K., Ostrander, M.M., Ulrich-Lai, Y.M. & Herman, J.P. (2007). Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs. *Journal of Neuroscience, 27*(8): 2025-2034.

Cooper, M.L., Russell, M., Skinner, J.B., Frone, M.R. & Mudar, P. (1992). Stress and alcohol use: moderating effects of gender, coping and alcohol expectancies. *Journal of Abnormal Psychology, 101*, 139-152.

Conger, J.J. (1956). Reinforcement theory and the dynamics of alcoholism. *Quarterly Journal of Studies on Alcohol, 17*: 296-305.

Crestani, C.C., Alves, F.H.F., Gomes, F.V., Resstel, L.B.M., Correa, F.M.A. & Herman, J.P. (2013). Mechanisms in the bed nucleus of the stria terminalis involved in control of autonomic and neuroendocrine functions: A review. *Current Neuropharmacology, 11*(2): 141-159.

Daniel, S.E. & Rainnie, D.G. (2015). Stress modulation of opposing circuits in the bed nucleus of the stria terminalis. *Neuropsychopharmacology,* DOI: 10.1038/npp.2015.178

Davis, W.M. & Smith, S.G. (1976). Role of conditioned reinforcers in the initiation, maintenance and extinction of drug-seeking behavior. *Pavlovian Journal of Biological Science, 11*: 222-236.

Davis, M. & Whalen, P.J. (2001). The amygdala: vigilance and emotion. *Molecular Psychiatry, 6*(1): 13-34.

Delamater, A.R. (1997). Selective reinstatement of stimulus-outcome associations. *Animal Learning and Behavior, 25*: 400-412.

Delfs, J.M., Zhou, Y., Druhan, J.P., Aston-Jones, G. (1998). Origin of noradrenergic afferents to the shell subregion of the nucleus accumbens: anterograde and retrograde tract-tracing studies in the rat, *Brain Research, 806*: 127-140.

de Souza, E.B. (1995). Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. *Psychoneuroendocrinology, 20*, 789-819.

Domjan, M. (2005). Pavlovian conditioning: a functional perspective. *Annu. Rev. Psychol., 56*, 179-206.

Dong, H.W., Petrovich, G.D., Watts, A.G. & Swanson, L.W. (2001). Basic organization of projections from the oval and fusifor nuclei of the bed nuclei of the stria terminalis in adult rat brain. Journal of Comparative Neurology, *436*(4): 430-455.

Dumont, E.C., Rycroft, B.K., Maiz, J. & Williams, J.T. (2008). Morphine produces circuit-specific neuroplasticity in the bed nucleus of the stria terminalis. *Neuroscience, 153*(1): 232-239.

Dunn, A.J. & Berridge, C.M. (1990). Physiological and behavioral responses to corticotrophin-releasing factor: is CRF a mediator of anxiety or stress responses? *Brain Research Review, 15*, 71-100.

Economidou, D., Dalley, J. W., & Everitt, B. J. (2011). Selective norepinephrine reuptake inhibition by atomoxetine prevents cue-induced heroin and cocaine seeking. *Biological psychiatry*, *69*(3), 266-274.

Epstein, D.H., Wilner-Reid, J., Vahabzadeh, M., Mezghanni, M., Lin, J.L. & Preston, K.L. (2009). Real-time electronic diary reports of cue exposure and mood in the hours before cocaine and heroin craving and use. *Archives of General Psychiatry, 66*: 88-94.

Erb, S., Shaham, Y. & Stewart, J. (1996). Stress reinstates cocaine-seeking behavior after prolonged extinction and drug-free periods. *Psychopharmacology, 128*: 408-412.

Erb, S., Shaham, Y. & Stewart, J. (1998). The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. *Journal of Neuroscience, 14*: 5529-5536.

Erb, S & Stewart, J. (1999). A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *Journal of Neuroscience, RC35*: 1-6.

Erb, S., Salmaso, N., Rodaros, D & Stewart, J. (2001). A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology, 158*(4):360-365.

Ettenberg, A., MacConell, L.A. & Geist, T.D. (1996). Effect of haloperidol in a reponse-reinstatement model of heroin relapse. *Psychopharmacology, 124*: 205-210.

Feit, M.D. & Taylor, O.D. (2015). Contemporary substance use research. *Journal of Human Behavior in the Social Environment, 25*(4): 287.

Feng, J. & Nestler, E.J. (2013). Epigenetic mechanisms of drug addiction. *Current Opinion in Neurobiology, 23*(4): 521-528.

Fox, H.C., Seo, D., Tuit, K., Hansen, J., Kimmerling, A., Morgan, P.T. (2012). Guanfacine effects on stress, drug craving, and prefrontal activation in cocaine dependent individuals: preliminary findings. *Journal of Psychopharmacology, 26*: 958-972.

Fox, H.C., Mortan, P.T. & Sinha, R. (2014). Sex differences in guanfacine effects on drug craving and stress arousal in cocaine-dependent individuals. *Neuropsychopharmacology, 33*: 1402-1412.

Fuchs, R. A., Evans, K. A., Parker, M. P., & See, R. E. (2004). Differential Involvement of Orbitofrontal cortex Subregions in Conditioned Cue-induced and Cocaine-Primed Reinstatement of Cocaine Seeking in Rats. *The Journal of Neuroscience. 24*, 6600-6610

Gawin, F. H., & Kleber, H. D. (1985). Neuroendocrine findings on chronic cocaine abusers: A preliminary report. *The British Journal of Psychiatry*.

Gerber, G.J. & Stretch, R. (1975). Drug-induced reinstatement of extinguished self-administration behavior in monkeys. *Pharmacology, Biochemistry and Behavior, 3*: 1055-1061.

Ghatei, M.A., Takahashi, K., Suzuki, Y., Gardiner, J., Jones, P.M., Bloom, S.R. (1993). Distribution, molecular characterization of pituitary adenylate cyclase activating

polypeptide and its precursor encoding messenger RNA in human and rat tissues. *Journal of Endocrinology*, 163: 159-166.

Girard, B.A., Lelievre, V., Braas, K.M., Razinia, T., Vizzard, M.A., Ioffe, Y… (2006). Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP- and PACAP-deficient mice. *Journal of Neurochemistry, 99*(2): 499-513.

Grinevich, V., Fournier, A. & Pelletier, G. (1997). Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on corticotropin-releasing hormone (CRH) gene expression in the rat hypothalamic paraventricular nucleus. *Brain Research, 773*(1-2): 190-196.

Hamack, S.E., Cooper, M.A. & Lezak, K.R. (2012). Overlapping neurobiology of learned helplessness and conditioned defeat: implications for PTSD and mood disorders. *Neuropharmacology, 62*(2): 565-575.

Hammack, S.E., Cheung, J., Rhodes, K.M., Schutz, K.C., Falls, W.A., Braas, K.M. & May, V. (2009). Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. *Psychoneuroendrocrinology, 34*(6): 833-843.

Hammack, S. E., & May, V. (2014). Pituitary adenylate cyclase activating polypeptide in stress-related disorders: data convergence from animal and human studies. *Biological psychiatry*.

Hammack, S.E., Roman, C.W., Lezak, K.R., Kocho-Shellenberg, M., Grimmig, B., Falls, W.A., Braas, K. & May, V. (2010). Roles for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) in mediating the behavioral consequences of chronic stress. *Journal of Molecular Neuroscience, 42*(3): 327-340.

Harmar, A.J., Fahrenkrug, J., Gozes, I., Laburthe, M., May, V., Pisegna, J.R., Vaudry, D., Vaudry, H., Waschek, J.A. & Said, S.I. (2012). Pharmmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR Review 1. *British Journal of Pharmacology, 166*(1): 4-17.

Hashimoto, H., Shintani, N., Tanaka, K., Mori, W., Hirose, M., Matsuda… (2001). Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proceedings of the National Academy of Sciences of the United States of America, 98*(23): 13355-13360.

Hashimoto, H., Shintani, N., Tanida, M., Hayata, A., Hashimoto, R. & Baba, A. (2011). PACAP is implicated in the stress axes. *Current Pharmacology Des, 17*(10): 985-989.

Herman, J.P., Ostrander, M.M., Mueller, N.K. & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 29*(8): 1201-1213.

Higgins, S.T., Budney, A.J., Bickel, W.K., Foerg, F.E., Donham, R., Badger, G.J. (1994). Incentices improve outcome in outpatience behavioral treatment of cocaine dependence. *Archives of General Psychiatry, 51*(7): 568-576.

Higgins, S.T., Budney, A.J., Bickel, W.K., Hughes, J.R., Foerg, F., Badger, G.J. (1993). Achieving cocaine abstinence with a behavioral approach. *The American Journal of Psychiatry, 150*(5): 763-769.

Higgins, S.T., Wong, C.J., Badger, G.J., Ogden, D.E. H., Dantona, R.L. (2000). Contingent reinforcement increases cocaine abstinence during outpatient treatment and 1 year of follow-up. *Journal of Consulting and Clinical Psychology, 68*(1): 64-72.

Jasinska, A.J., Stein, E.A., Kaiser, J., Naumer, M.J. & Yalachkov, Y. (2014). Factors modulating neural reactivity to drug cues in addiction: A survey of human neuroimaging studies. *Neuroscience and Biobehavioral Reviews, 38*: 1-16.

Kalivas, P. W., & Volkow, N. D. (2014). The neural basis of addiction: a pathology of motivation and choice. *American Journal of Psychiatry*.

Katner, S.N., Magalong, J.G. & Weiss, F. (1999). Reinstatement of acohol-seeking behavior by drug-associated discriminative stimuli after prolonged extinction in the rat. *Neuropsychopharmacology, 20*: 471-479.

Keifer, O.P., Hurt, R.C., Ressler, K.J. & Marvar, P.J. (2015). The physiology of fear: Reconceptualizing the role of the central amygdala in fear learning. *Physiology, 30*: 389-401.

Kocho-Schellenberg, M., Lezak, K. R., Harris, O. M., Roelke, E., Gick, N., Choi, I., ... & Hammack, S. E. (2014). PACAP in the BNST produces anorexia and weight loss in male and female rats. *Neuropsychopharmacology*, *39*(7), 1614-1623.

Koob, G.F. Caine, S.B., Parsons, L., Markou, A., Weiss, F. (1997). Opponent process

model and psychostimulant addiction. *Pharmacology Biochemistry and Behavior, 57*(3): 513-521.

Koob, G.F. & Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. *Science, 278*: 52

Lee, Y. & Davis, M. (1997). Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. *Journal of Neuroscience, 17*(16): 6434-6446.

Levran, O., Peles, E., Randesi, M., Li, Y., Rotrosen, J., Ott, J., Adelson, M. & Kreek, M.J. (2015). Stress-related genes and heroin addiction: A role for functional fkbp5 variants. *Drug and Alcohol Dependence, 146*: 176.

Lezak, K. R., Roelke, E., Harris, O. M., Choi, I., Edwards, S., Gick, N., ... & Hammack, S. E. (2014). Pituitary adenylate cyclase-activating polypeptide (PACAP) in the bed nucleus of the stria terminalis (BNST) increases corticosterone in male and female rats. *Psychoneuroendocrinology, 45*, 11-20.

Little, K. Y., McLaughlin, D. P., Zhang, L., Livermore, C. S., Dalack, G. W., McFinton, P. R., ... & Cook, E. H. (2014). Cocaine, ethanol, and genotype effects on human midbrain serotonin transporter binding sites and mRNA levels.*American Journal of Psychiatry*.

Mantsch, J.R., Baker, D.A., Funk, D., Le, A.D. & Shaham, Y. (2015). Stress-induced reinstatement of drug seeking: 20 years of progress. *Neuropsychopharmacology Reviews*, 1-22.

Marlatt, G.A. & Gordon, J.R. (1985). Relapse prevention: maintenance strategies in the treatment of addictive behaviors. Guilford Press, New York.

May, V., Lutz, E., MacKenzie, C., Schutz, K.C., Dozark, K. & Braas, K.M. (2010). Pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1HOP1 receptor activation coordinates multiple neurotrophic signaling pathways: Akt activation through phosphatidylinositol 3-kinase gamma and vesicle endocytosis for neuronal survival. *Journal of Biological Chemistry, 285*(13): 9749-9761.

McFarland, K., Davidge, S. B., Lapish, C. C., & Kalivas, P. W. (2004). Limbic and motor circuitry underlying footshock-induced reinstatement of cocaine-seeking behavior. *The Journal of neuroscience, 24*(7), 1551-1560.

McKee, S.A., Potenza, M.N., Kober, H., Sofuoglu, M., Amsten, A.F., Picciotto, M.R.

(2014). A translational investigation targeting stress-reactivity and prefrontal cognitive control with guanfacine for smoking cessation. *Journal of Psychopharmacology*.

Meil, W.M. & See, R.E. (1996). Conditioned cued recovery of responding following prolonged withdrawal from self-administered cocaine in rats: an animal model of relapse. *Behavioral Pharmacology, 7*: 754-763.

Measham, F., Williams, L. & Aldridge, J. (2011). Marriage, mortgage, motherhood: What longitudinal studies can tell us about gender, drug 'careers' and the normalization of adult 'recreational' drug use. *International Journal of Drug Policy, 22*(6): 420-427.

Missig, G., Roman, C. W., Vizzard, M. A., Braas, K. M., Hammack, S. E., & May, V. (2014). Parabrachial nucleus (PBn) pituitary adenylate cyclase activating polypeptide (PACAP) signaling in the amygdala: Implication for the sensory and behavioral effects of pain. *Neuropharmacology, 86*, 38-48.

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L… Coy, D.H. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cycalse in pituitary cells. *Biochemical & Biophysical Research Communications, 164*(1): 567-574.

Miyata, A., Jiang, L., Dahl, R.D., Kitada, C., Hubo, K., Fujino, M…Arimura, A. (1990). Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochemical & Biophysical Research Communications, 170*(2): 643-648.

Moran-Santa Maria, M.., Baker, N.L., Ramakrishan, V., Brady, K.T., McRae-Clark, A. (2015). Impact of acuteguanfacine administration on stress and cue reactivity in cocaine-dependent individuals. *American Journal of Drug and Alcohol Abuse, 41*: 146-152.

Mustafa, T., Jiang, S. Z., Eiden, A. M., Weihe, E., Thistlethwaite, I., & Eiden, L. E. (2015). Impact of PACAP and PAC1 receptor deficiency on the neurochemical and behavioral effects of acute and chronic restraint stress in male C57BL/6 mice. *Stress*, (0), 1-11.

Navarro, G., Moreno, E., Bonaventura, J., Brugarolas, M., Farre, D., Aguinaga, D., ... & McCormick, P. J. (2013). Cocaine inhibits dopamine D2 receptor signaling via sigma-1-D2 receptor heteromers. *PLoS One, 8*(4), e61245.

Nestler, E.J. & Aghajanian, G.K. (1997). Molecular and cellular basis of addiction. *Science, 278*, 58-63.

Orford, J., Velleman, R., Natera, G., Templeton, L. & Copello, A. (2013). Addiction in the family is a major but neglected contributor to the global burden of adult ill-health. *Social Science & Medicine, 78*: 70-77.

Otto, C., Kovalchuk, Y., Wolfer, D.P., Gass, P., Martin, M., Zuschratter, W… (2001). Impairment of mossy fiber long-term potentiation and associative learning in pituitary adenylate cyclase activating polypeptide type 1 receptor-deficient mice. *Journal of Neuroscience, 21*(15): 5520-5527.

Otto, C., Martin, M., Wolfer, D.P., Lipp, H.P., Maldonado, R., Schultz, G. (2001). Altered emotional behavior in PACAP-type-I-receptor-decificient mice. *Brain Research, Molecular Brain Research, 92*(1-2) 78-84.

Pearce, J. M. (1987). A model for stimulus generalization in Pavlovian conditioning. *Psychological review, 94*(1), 61.

Pego, J.M., Morgado, P., Pinto, L.G., Cerqueira, J.J., Almeida, O.F.X. & Sousa, N. (2008). Dissociation of the morphological correlates of stress-induced anxiety and fear. *European Journal of Neuroscience, 27*: 1503-1516.

Radley, J.J., Gosselink, K.L. & Sawchenko, P.E. (2009). A discrete GABAergic relay mediates medial prefrontal cortical inhibition of the neuroendocrine stress response. *Journal of Neuroscience, 29:* 7330-7340.

Ramos-Aliaga,, R., & Werner, G. (1982). Pituitary-adrenocortical response in acutely and chronically cocaine-treated rats. *Research Communications in Substances of Abuse, 3*(1), 29-48.

Rescorla, R. A., & Solomon, R. L. (1967). Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychological review, 74*(3), 151.

Rescorla, R.A. (2004). Spontaneous recovery. *Learning and Memory, 11*:501-509.

Rescorla, R.A. & Heth, C.D. (1975). Reinstatement of fear to an extinguished conditioned stimulus. *Journal of experimental psychology animal behavioral processes. 104*: 88-96.

Ressler, K. J., Mercer, K. B., Bradley, B., Jovanovic, T., Mahan, A., Kerley, K., ... &

May, V. (2011). Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature*, *470*(7335), 492-497.

Roman, C.W., Lezak, K.R., Hartsock, M.J., Falls, W.A., Braas, K.M., Howard, A.B., Hammack, S.E., May, V. (2014). PAC1 receptor antagonistm in the bed nucleus of the stria terminalis (BNST) attenuates the endocrine and behavioral consequences of chronic stress. *Psychoneuroendocrinology, 47*: 151-165.

Rouwette, T., Vanelderen, P., Roubos, E.W., Kozicz, T. & Vissers, K., (2012). The amygdala, a relay station for switching on and off pain. *European Journal of Pain, 16*: 782-792.

Schulkin, J., Gold, P.W., McEwen, B.S. (1998). Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. *Psychoneuroendrocrinology, 23*(3): 219-243.

Schumacher, J.A. & Holt, D.J. (2012). Domestic violence shelter residents' substance abuse treatment needs and options. *Aggression and Violent Behavior, 17*(3): 188-197.

Scott, C.K., Dennis, M.L., Laudet, A., Funk, R.R. & Simeone, R.S. (2011). Surviving drug addiction: The effect of treatment and abstinence on Mortality. *American Journal of Public Health*, *101*(4): 737-744.

Shaham, Y., Erb, S. & Stewart, J. (2000). Stress-induced relapse to heroin and cocaine seeking in rats: a review. *Brain Research Reviews, 33*(1): 13-33.

Shaham, Y., Funk, D., Erb, S., Brown, T.J., Walker, C.D. & Stewart, J. (1997). Corticotropin-releasing factor, but not corticosterone, is involved in stress-induced relapse to heroin-seeking in rats. *Journal of Neuroscience, 17*: 2605-2614.

Shaham, Y., Erb, S., Leung, S., Buczek, Y., & Stewart, J. (1998). CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor1 receptor attenuates stress-induced relapse to drug seeking in cocaine-and heroin-trained rats. *Psychopharmacology*, *137*(2), 184-190.

Shiffman, S. (1982). Relapse following smoking cessation: a situational analysis. *Journal of Consulting and Clinical Psychology, 50*: 71-86.

Shioda, S., Shuto, Y., Somogyvari-Vigh, A., Legradi, G., Onda, H., Coy, D.H., Nakajo,

S. & Arimura, A. (1997). Localization and gene expression of the receptor for pituitary adenylate cyclase-activating polypeptide in the rat brain. *Neuroscience Research, 28*(4): 345-354.

Singewald, N., Salchner, P. & Sharp, T. (2003). Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. *Biological Psychiatry, 53*(4): 275-283.

Sinha, R., Catapano, D., O'Malley, S. (1999). Stress induced craving and stress response in cocaine dependent individuals. *Psychopharmacology, 142*: 343-351.

Sinha, R. (2001). How does stress increase risk of drug abuse and relapse? *Psychopharmacology, 158*: 343-359.

Sinha, R., Garcia, M., Paliwal, P., Kreek, M.J., Rounsaville, B.J. (2006). Stress-induced cocaine craving and hypothalamic-pituitary-adrenal responses are predictive of cocaine relapse outcomes. *JAMA Psychiatry, 63*(3): 324-331.

Sinha, R., Kimmerling, A., Doebrick, C., Kosten, T.R. (2007). Effects of lofexidine on stress-induced and cue-induced opioid craving and opioid abstinence rates: preliminary findings. *Psychopharmacology, 190*: 569-574.

Stevens, J. S., Almli, L. M., Fani, N., Gutman, D. A., Bradley, B., Norrholm, S. D., ... & Ressler, K. J. (2014). PACAP receptor gene polymorphism impacts fear responses in the amygdala and hippocampus. *Proceedings of the National Academy of Sciences*, *111*(8), 3158-3163.

Stewart, J. (2000). Pathways to relapse: the neurobiology of drug-and stress-induced relapse to drug-taking. *Journal of Psychiatry and Neuroscience, 25*(2), 125.

Stroth, N., Holighaus, Y., Ait-Ali, D. & Eiden, L.E. (2011). PACAP: a master regulator of neuroendocrine stress circuits and the cellular stress response. [Research Support, NIH, Intramural Review.] *Annals of the New York Academy of Sciences, 1220*: 49-59.

Stroth, N., Kuri, B.A., Mustafa, T., Chan, S.A., Smith, C.B. & Eiden, L.E. (2013). PACAP controls adrenomedullary catecholamine secretion and expression of catecholamine biosynthetic enzymes at high splanchnic nerve firing rates characteristic of stress transduction in male ice. *Endocrinology, 154*(1): 330-339.

Tanaka, K., Hashimoto, H., Shintani, N., Yamamoto, A., & Baba, A. (2004). Reduced

hypothermic and hypnotic responses to ethanol in PACAP-deficient mice. *Regulatory peptides, 123*(1), 95-98.

Tanaka, K., Kunishige-Yamamoto, A., Hashimoto, H., Shintani, N., Hayata, A. & Baba, A. (2010). Increased ethanol preference and serotonin 1A receptor-dependent attenuation of ethanol-induced hypothermia in PACAP-deficient mice. *Biochemical and Biophysical Research Communications, 391*(1): 773-777.

Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *Journal of psychosomatic research*, *53*(4), 865-871.

Tsukiyama, N., Saida, Y., Kakuda, M., Shintani, N., Hayata, A., Morita, Y., ... & Baba, A. (2011). PACAP centrally mediates emotional stress-induced corticosterone responses in mice. *Stress*, *14*(4), 368-375.

Vaudry, D., Hamelink, C., Damadzic, R., Eskay, R.L., Gonzalez, B. & Eiden, L.E. (2005). Endogenous PACAP acts as a stress response peptide to protect cerebellar neurons from ethanol or oxidative insult. *Peptides, 26*(12): 2518-2524.

Walker, D.L., Toufexis, D.J. & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology, 463*(1-3), 199-216.

Wise, R. A., & Kiyatkin, E. A. (2011). Differentiating the rapid actions of cocaine. *Nature Reviews Neuroscience*, *12*(8), 479-484.