BED NUCLEUS OF THE STRIA TERMINALIS PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE PROJECTIONS TO THE CENTRAL NERVOUS SYSTEM REGIONS

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ABSTRACT

Pituitary adenylate cyclase activating peptide (PACAP) is a pleiotropic neuropeptide that has vast functions, ranging from behavioral, to endocrine, cardiovascular, and cellular, in which often these effects are biphasic. This paper will focus on the effects of PACAP within the central nervous system (CNS), specifically in response to stress and anxiety. PACAP has been extensively researched in terms of its effect on the stress response and is well-known for its effects in inducing anxiety-like behaviors. PACAP induces such effects through working in regions of the CNS such as the amygdala, hypothalamus, and bed nucleus of the stria terminalis (BNST). Research has shown that injecting PACAP into the BNST produces anxiogenic effects; this is backed up by *in situ* hybridization and mRNA studies. While it is known that BNST-PACAP signaling produces such behavior, it is not fully clear how these processes are connected to other CNS regions that are implicated in the stress-response or have been shown to hold PACAP neurons.

Using Cre-recombinase technology, this study mapped BNST-PACAP projections to various CNS nuclei, specifically anterior projections to the nucleus accumbens, intrinsic BNST signaling, and posterior projections to the medial habenular nucleus (MHb), amygdala and hypothalamic regions. These projections occurred via three fiber pathways: stria terminalis, stria medullaris, and forebrain projections. The novel finding was of BNST-PACAP projections within the MHb via stria medullaris, as well as the conformation of one-directional BNST-PACAP projections to the amygdala via the stria terminalis and anterior projections to the nucleus accumbens.

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i. Background

Pituitary Adenylate Cyclase Activating Peptide

Pituitary adenylate cyclase activating peptide (PACAP; *ADCYAP1*) is a neuroendocrine peptide with broad and diverse physiological functions. PACAP participates in cardiovascular, respiratory, endocrine, gastrointestinal, reproductive, and central/peripheral nervous system (CNS/PNS) activities. In neural systems, PACAP can regulate neurodevelopment and neuroprotection through trophic survival, proliferation, differentiation/cell fate and plasticity decisions, and facilitate cell signaling to impact cellular transcription, translation/biosynthesis and secretion to modulate neurotransmission in a variety of processes, including endocrine/autonomic function, feeding and satiety, learning and memory, sensory processes and behavioral responses (Sherwood et al., 2000). Accordingly, maladaptive neural PACAPergic activities have been implicated in many disorders such as obesity/metabolic abnormalities, chronic pain, cognitive/memory deficits, and stress-related behavioral pathologies.

PACAP was isolated from ovine hypothalami based on its ability to stimulate anterior pituitary cell cAMP production (Miyata et al, 1990; Kimura et al., 1990) and from sequence homology, belongs to the VIP/glucagon/secretin family of related peptides. The precursor molecule allows for two bioactive isoforms of PACAP to be derived from alternative post-translational processing at dibasic endoproteolytic sites: PACAP-27 and PACAP-38. PACAP-38 is 38 amino acids long- and PACAP-27 is the 27

amino acid peptide from the N-terminus of PACAP-38. PACAP is evolutionarily well conserved among species and in all tissues examined to date, the levels of PACAP38 exceed those for PACAP27 nearly 10- to 100-fold (Arimura et al., 1991).

PACAP can bind to several G protein-coupled receptor subtypes. PAC-1R is a type I receptor, while VPAC1-R and VPAC2-R are type II receptors (Vaudry et al., 2009; Harmar et al., 2012; Blechman & Levkowitz, 2013). The PAC1 receptor is selective for PACAP and binds both PACAP-38 and PACAP-27 with high affinity; the VPAC1 and VPAC2 receptors bind PACAP and VIP with near equal high affinities. Whereas VPAC receptors are preferentially coupled to Gs for adenylyl cyclase (AC) activation and increased cyclic AMP (cAMP) production, PAC1 receptors belong to a special class of GPCRs that are dually coupled to Gs and Gq for AC and phospholipase C (PLC)-mediated signaling, respectively. In addition, PAC1 receptors are unique in that there are multiple variants depending on the absence or presence of two 84-bp Hip and/or Hop cassettes encoding inserts into the third cytoplasmic loop of the GPCR. Hence the PAC1 receptor may be Null (neither Hip nor Hop inserts), Hip, Hop1, Hop2 (short form of Hop1) or HipHop, and depending on the variant may show differential AC and PLC responses (Spengler et al., 1993). AC/cAMP signaling activates canonical downstream PKA/Epac pathways; PLC-mediated hydrolysis of PIP2 (phosphatidyinositol 4,5-bisphosphate) generates diacylglycerol to facilitate PKC activtion and IP3 for IP3R-mediated intracellular calcium release. Both the AC and PLC processes can stimulate MAPK/ERK pathways, but importantly PAC1 receptor β-arrestin-mediated internalization and effector scaffolding can generate long term endosomal ERK signaling

with high temporal and subcellular spatial resolution, capable of enhancing cellular excitability and changing transcriptional and translational profiles that can contribute to homeostatic or maladaptive neuroplasticity (Parsons and May, 2019).

Several studies have examined the distribution of PACAP and PAC1 receptor expression in the CNS using immunohistochemistry and in situ hybridization techniques (Hashimoto et al., 1996; Hannibal, 2002). Hashimoto et al. was the first study to look at the distribution of PACAP mRNA throughout the entire rat CNS. While this study did not look at the functional correlations of specific areas to PACAP and the receptors, it was able to generate a holistic image of the distribution of this neuroendocrine peptide. Areas that experienced the most intensely labeled PACAP receptor mRNA were the olfactory bulb, all cortical layers of the neocortical, perirhinal, entorhinal and subicular regions, the piriform cortex, the dentate gyrus, the lateral and medial septal nuclei, the bed nucleus of the stria terminalis (BNST), the striatum, the globus pallidus, the intercalated nucleus of the amygdala, the medial habenular nucleus, throughout the entire thalamus, the supraoptic nucleus of the hypothalamus and the lateral and medial mammillary nuclei (Hashimoto et al., 1996). This study also found that the distribution of PAC1 receptor mRNA was similar to previous data on PACAP binding sites (Masuo et al., 1991, 1992), however this data did not distinguish between the VIP-R and PAC-R subtypes. To further understand the distribution of PACAP throughout the rat brain, a study was done by Hannibal et al. in 2002 which specifically examined both PACAP mRNA and peptide expression, which had the potential of identifying cell bodies, and nerve fibers and/or cell bodies, respectively. This would allow for not only the

localization of PACAP-expressing cell bodies, but also their projections within the CNS. Areas that contained both PACAP expressing cells and nerve fibers were the rhinencephalon, all layers of the cerebral cortex, piriform cortex, hippocampus, amygdala and extended amygdala regions, and in the hypothalamic regions extending from the preoptic nucleus to the mammillary nuclei, with the highest accumulation of nerve fibers in the supraoptic nucleus. PACAP-expressing fibers were also found in the stria medullaris and other fiber pathways within the thalamus, epithalamus, and brainstem, but these regions did not have high levels of PACAP-expressing cells. PACAP-expressing nerve fiber projections could be observed descending to the reticular pontine and medullary reticular formation. Both fibers and cell bodies were found in all circumventricular organs, especially the area postrema, and all levels of the spinal cord (Hannibal et al., 2002). Among all areas, the hypothalamic regions had the highest levels of PACAP-expressing cells and nerve fibers.

For well understood reasons, there is not as much information regarding PACAP and receptor distribution throughout the human CNS. The highest concentrations of PACAP, found by radioimmunoassay, were found in the dorsal vagal complex, the BNST, the periventricular and paraventricular hypothalamic nuclei, brainstem nuclei, and spinal gray matters (Hirabayashi et al., 2018). PAC1 receptor mRNA has only been looked at in the human cerebellum during fetus development; mRNA was found in the external granule cell layer and internal granule cell layer (Hirabayashi et al., 2018).

The areas of most importance in the CNS for behavioral studies that express PACAP and PACAP receptors are the BNST, central amygdala (CeA), and basolateral

complex of the amygdala (BLA). The BNST will be discussed in further detail later in this chapter, so now the focus will be on the CeA and BLA. The CeA is important in the conditioned fear response and mediation of the emotional component of pain perception; regulation of CeA activity modifies nociceptive thresholds (Hammack & May, 2015). High levels of PACAP have been found in the lateral capsular division of the CeA (CeLC), which was dense with fiber elements. PACAP infusions into the CeA increased avoidance behaviors and reduced nociceptive thresholds, as well as produced anxiety-like behaviors on the elevated-plus maze (Hammack & May, 2015).

The BLA expresses both PACAP and PAC1 receptors, but not as highly as the BNST or CeA. The BLA is important in fear memories and an extensive amount of research has been done on the correlations between the BLA and posttraumatic stress disorders (PTSD). mRNA for PAC1receptors increased 1.5-fold during fear consolidation following fear conditioning and was correlated with increased freezing behavior, a common indicator of fear expression (Hammack & May, 2015). With the BNST studies (see below) the high expression and distribution of PACAP in the limbic areas all implicate PACAP/PAC1 receptor roles in fear memory and stress-induced anxiety-related behaviors which can include posttraumatic stress disorder (PTSD).

In sum, PACAP is seen to be widely distributed throughout the entire CNS, with the highest accumulation of nerve cells and fibers being in the hypothalamus, limbic areas, and nuclei receiving sensory information. These observations demonstrate that PACAP has many diverse roles especially autonomic functions, sensory and emotional processing.

Stress

Stress can broadly be defined as any change from body homeostasis, whether that change be positive or negative. An example of positive stress might be exercise, where the physical stresses are beneficial to body mass, bone strength and even mental well-being. Negative stresses can be physical and or mental/emotional; examples of negative physical stress can be bodily injuries or disorders, and emotional stresses can include mental pressures from daily challenges such as school examinations, public speaking, and unpaid bills. Stress can be either acute – one-time occurrences, or chronic – something that is sustaining or repeating which unfortunately is more commonplace today. Stress is important because physiologically, it is an evolutionarily conserved adaptive mechanism for organism survival. But regardless of the type or source of the stress, the physiological and sequence of events are the same. The responses are two-pronged but coordinate and integrated; one is autonomic and the other is neuroendocrine. Autonomic activation of the sympathetic systems results in the classic "fight or flight" response resulting in increased heart inotropism and chronotropism, redirection of blood supply to skeletal muscles, decreased salivation and pupil dilation. The neuroendocrine mechanism of stress results in hypothalamic-pituitary-adrenal axis (HPA) activation. The hypothalamus releases corticotropin releasing factor (CRF) into the portal system, which stimulates release of adrenocorticotropin hormone (ACTH) from the anterior pituitary corticotropes. ACTH causes the release of cortisol (corticosterone in rodents and most species) from the adrenal cortex to facilitate muscle glucose availability and usage, suppress immune responses, mediate cellular adaptive stress programs and in parallel,

serve as negative feedback to both the pituitary and hypothalamus to attenuate the responses as the stress events dissipate. The autonomic and neuroendocrine stress responses were designed for acute or transient stress insults, and not the chronic challenges that many people may experience. Chronic activation of the stress pathways have been well shown to have adverse consequences; the continuous release of stress hormones can cause maladaptive neuroplasticity in neuronal cytoarchitecture and signaling such that the body is in a continuous heightened stress response mode, or fails to accurately respond to stress at all. A continuous stress response implies that the body is constantly preparing for adversity and the resulting maladaptations can facilitate a variety of diseases including cardiovascular issues, cognitive and memory impairments, obesity/diabetes and related metabolic disorders, and stress-induced anxiety-related abnormalities such as depression, general anxiety disorder and posttraumatic stress disorders (PTSD).

Bed Nucleus of the Stria Terminalis

The bed nucleus of the stria terminalis (BNST) is a small but heterogeneous basal forebrain structure that acts as a relay, receiving a variety of sensory afferents for signal integration and transmission to diverse distal CNS targets important in the homeostatic regulation of stress-associated behaviors, including anxiety, pain, feeding/satiety, and sexual/reproductive responses (Lebow & Chen, 2016). Although the rodent BNST is no bigger than the head of a pin, it can be divided into approximately 18 subdivisions defined by cytoarchitecture, architecture and connectivity (Dong and Swanson, 2001; Hammack et al., 2010), populated by nearly 15 morphologically distinct neuronal cell

types (Larriva-Sahd, 2006). The BNST extends, from anterior to posterior, from the nucleus accumbens to the anterior hypothalamus and bounded by the anterior commissure, the lateral ventricles, and the internal capsule. There are several means to divide the BNST; broadly, the anterior and posterior BNST can be demarcated by the stria terminalis. The anterior BNST can be defined by the anterior commissure into dorsal and ventral BNST regions, and each region can be further subdivided into lateral and medial segments. Hence the anterior BNST has dorsolateral, dorsomedial, ventrolateral and ventromedial subdivisions. The BNST is part of the extended amygdala from cellular and functional similarities, and accordingly the BNST and amygdala are interconnected reciprocally via the stria terminalis, a fiber pathway that parallels the fornix, and the amgydalofugal pathway ventrally. The pathways and nuclei form a loop between the lateral thalamus and the caudate/globus pallidus. In addition to receiving afferents from the central and medial amygdala, the BNST also receives information from the hippocampus, medial prefrontal cortex, parabrachial nucleus and several hypothalamic nuclei (Crestani et al., 2013).

The best studied BNST efferent axonal projections include the ventral tegmental area (VTA), the lateral hypothalamus (LH), and the paraventricular nucleus (PVN). The BNST sends both GABAergic and glutamatergic projections to the VTA that can result in opposing behavioral responses; BNST GABAergic activation of VTA GABAergic terminals is rewarding and anxiolytic while BNST glutamatergic activation of VTA glutamatergic terminals is aversive and anxiogenic (Stamatakis et al., 2014). For the LH, pharmacological inactivation of the BNST resulted in an increase in *Fos* induction in

orexin neurons following a cocaine conditioned place preference test, and stimulation of BNST inputs to the LH resulted in behavioral anxiolysis without altering other reward behaviors (Stamatakis et al., 2014). The PVN receives numerous GABA and CRFergic projections from the BNST; the BNST GABAergic neurons synapse on PVN magnocellular cells, which project to the posterior pituitary, while BNST CRFergic neurons synapse on neurons in the parvocellular division, which projects to the median eminence to hormonally regulate anterior pituitary cells and HPA axis homeostasis (Stamatakis et al., 2014).

More than other regions, the posterior BNST is sexually dimorphic, expresses high levels of gonadal hormone receptors and aromatase enzymes, and participates in sexual responses and behaviors; these functions have been reviewed elsewhere and will not be summarized here. The anterior BNST has been best studied with respect to stress-related behaviors, although the circuits are integrated with the posterior BNST in modulating stress-associated sexual motivations and responses. From development and circuit intersections, the anterior BNST shares functional roles with the amygdala. However, in distinction from amygdala well-studied roles in coordinating immediate responses to predictable fearful threats, the BNST appears to mediate responses to long duration or temporally diffuse unpredictable stimuli similar to anxiety-related behaviors. These responses include hypervigilance, arousal and sensitivity to environmental dynamics, and understandably, maladaptations in BNST function from chronic stress-induced neuroplasticity – such as changes in neuronal morphology or cytoarchitecture,

synaptic density and phenotypic expression - may lead to behavior abnormalities such as depression, general anxiety disorders, and posttraumatic stress disorder (PTSD).

BNST and PACAP-Mediated Behavioral Responses

BNST PACAP signaling has been shown to have significant roles in stress-induced anxiety-like behaviors. Unlike other stress-regulated peptides, such as CRF (see below) which can be induced during acute stress, several studies have shown that acute single stressor events were not enough to increase PACAP mRNA levels (Hannibal et al., 1995; Lezak et al., 2014); by contrast, changes in limbic tissues expression or PACAP or the PAC1 receptor can be increased by chronic stress paradigms. Chronic variate stress in rats for 7 days, has been shown to selectively increase PACAP transcript levels approximately 8- to 10- fold within the dlBNST; these were large changes suggesting that intrinsic BNST PACAP signaling had roles in BNST-mediated behavioral responses (Hammack et al., 2010). The chronic stress-induced changes in BNST PACAP mRNA expression was confirmed by in situ hybridization studies, and in coherence, PACAP-immunoreactive cell bodies and fibers were identified in the BNST. In testing that hypothesis BNST PACAP has roles in behavior, PACAP (1 μg) infusions bi-laterally into the rat BNST increased anxiety-like responses in baseline startle, plus-maze and open field tests, and facilitated stress-induced weight loss (Roman et al., 2014). In agreement, the stress-related responses were mimicked by the PAC1 receptor – selective agonist Maxadilan, and not by VIP, suggesting that the PACAP stress responses were mediated by PAC1 rather than the VPAC receptors (Roman et al., 2014). Conversely, the PACAP and the chronic stress-induced anxiety-like responses were blocked with the

PAC1 receptor antagonist PACAP6-38 (Roman et al., 2014). PACAP/PAC1 receptor signaling also mediated chronic pain hypersensitivity and chronic pain-induced anxiety-related responses in the amygdala (Roman et al., 2014). These anxiogenic responses seen from PACAP infusions were consistent with data seen from PACAP and PAC1 receptor knock-out mice which exhibited diminished anxiety response to stressors (Roman et al., 2014).

PACAP has strong correlations with corticotropin releasing factor (CRF, also corticotropin releasing hormone or CRH) another neuropeptide implicated in stress responses. As described above, CRF from the PVN initiates the neuroendocrine stress response by binding to CRF receptors on anterior pituitary corticotrope cells following stressor exposure to initiate HPA activation. However, CRF is also highly and widely expressed in other limbic tissues and circuits related to physiological and behavioral stress responses, including the BNST. CRF levels can be increased following acute and chronic stress; some studies have found increased levels of BNST CRF following chronic stress paradigms (Miles & Maren, 2019). CRF has been extensively researched in correlation with PACAP and the BNST. PACAP fibers have been found to form axodendritic and axosomatic terminals on BNST CRF neurons (Kozicz et al., 1997, 1998), although whether they represented intrinsic and/or extrinsic BNST PACAP neuronal fibers remain unclear, and PACAP has been shown to regulate CRF production (Kozicz et al., 1997, 1998). From these and other observations, PACAP appeared to be an upstream regulator of BNST CRF activation in many CNS circuits. Hence, PACAP has both independent and CRF-dependent effects in many stress-related responses. The

normally heightened corticosterone response to chronic stress was blunted in these PACAP null mice suggesting in part diminished PACAP signaling onto CRF neurons (Stroth et al., 2011).

The expression of BNST PACAP, CRF and other bioactive peptides diversifies, complements, and amplifies the GABAergic and glutaminergic outputs from the BNST. As described above, PACAP cell bodies and fibers have been identified in the BNST. Some of the fibers represent afferents from other CNS regions; others can be short efferent projection fibers from intrinsic BNST neurons for local communication within the BNST, or long projection efferent fibers to distal CNS target sites. Not all of the long distance PACAP efferents from the BNST have been identified. Although BNST PACAP expression and function have clear and important roles in stress-related behaviors, without a clear mapping of the BNST PACAP axonal efferent projections, how PACAP may participate in other stress-related responses including addiction and reward mechanisms cannot be fully elucidated. Hence the current studies were undertaken to identify these projections and their distal target sites.

In sum, the BNST PACAP plays a vital role in the CNS response to stress-induced behaviors and maladaptations in PACAP BNST pathways may be contributory to psychopathologies. Not all of the BNST PACAP long distant efferent projections have been identified; a more complete accounting of these projections may clarity other PACAP roles in behavior.

Core Question

There is little research done on the whole-brain projections of PACAP neurons, including the efferents from BNST PACAP cells. Each brain mapping methodology has limitations. PCR methods are most sensitive but lack cellular resolution and identification of terminal sites. In situ hybridization techniques have cellular resolution in identifying where the peptide cells are located but as with PCR cannot reveal the efferent axonal fiber projections to distal targets. Immunocytochemical approaches have issues related to sensitivity and specificity, but more confoundingly preferentially label fibers than neuronal soma. Hence peptide immunocytochemistry can identify the PACAP targets but not the origin of the PACAP-expressing neurons. These limitations are important to overcome because the functions of PACAP are vast and the ability to trace the origins and projections of PACAP neurons will provide better understandings of the PACAP neurocircuits that regulate the many physiological processes. While much research has been done on BNST PACAP, not all of the BNST intrinsic PACAP neuronal projections have been identified which may reveal more comprehensively the extent of BNST PACAP roles in behavior. The availability of PACAP-Cre mice has availed new means to study CNS PACAP expression patterns and projections; the current work attempts to characterize some of the PACAP projection patterns from the BNST.

ii. Methods

Animals

PACAP-Cre breeding mice were obtained from Bradford Lowell (Deaconess Hospital, Tufts University). Adult male mice were selected and habituated in their home cages for at least one week before experimentation. Mice were group-housed, maintained on a 12 h light/dark cycle (lights on at 07:00 h), and food and water were available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Intra-BNST Stereotaxic Surgery

Mice were anesthetized by isoflurane inhalation, placed into a stereotaxic frame and the head was secured with ear bars. A small incision was made through the shaved scalp to expose the skull and locate Bregma for bilateral BNST syringe placements using coordinates: AP = +0.1 mm; ML= ±1 mm; and DV= -4.6 mm. A 1µl Hamilton 7001 KH syringe with needle size 25s gauge and needle length 70 mm was lowered into the BNST for 1µl infusion of the AAV2/Ef1a-DIO-mCherry reporter vector (UNC GTC Vector Lot # AV4375H). The infusion occurred for 1 minute and the syringe was left in place over a 2-minute time span. After syringe withdrawal, the scalp incision was closed, and the mice placed on a heating pad within a new covered cage for postsurgical recovery.

The PACAP-Cre mice have the Cre-recombinase gene tethered to the PACAP gene, hence all PACAP expressing neurons will produce the Cre-recombinase enzyme. This transgenic model takes advantage of the Cre-Lox system for neurocircuit tracing

using Cre-dependent reporter vectors. In the current studies, the adeno-associated virus (AAV) vector harbors sequences for the mCherry red fluorescent protein reporter in an anti-sense orientation. The mCherry cassette is flanked on both sides by double Lox sequences in the opposite orientation (DIO; double inverted open reading frame) and the transcription is controlled by the elongation factor 1a (EF1a) promoter. Upon AAV vector infusion and infection into CNS targets, only PACAP neurons with the Cre-recombinase enzyme will be able to loop and transform the anti-sense mCherry cassette sequences into the sense orientation to allow transcription and translation of the mCherry fluorescent protein. The mCherry protein is transported to all regions of the PACAP neuron (soma, dendrites, axons, and terminals) allowing the visualization of the entire neuronal architecture and tracing of the immediate axonal projections. Importantly, AAV infection of non-PACAP expressing neurons would not have Cre-recombinase enzyme expression and hence would not express the mCherry protein; hence, the labeling of PACAP neurons and circuits can be specific.

Microscopy

All euthanizations were performed approximately 21 days post-reporter vector infusions. All mice were deeply anesthetized by isoflurane inhalation and euthanized by transcardial 4% paraformaldehyde perfusions. The entire brain was removed and placed in a vial of 4% paraformaldehyde overnight, then transferred through a graded series of sucrose solutions (to 30% sucrose) for cryoprotection. The brain blocks were embedded in to TissueTek and 40 μm cryosections were prepared from the olfactory bulb to the posterior end of the cerebellum; the sections were stored in a 20 well tissue culture plate

containing 100 mM sodium phosphate buffer (pH 7.4) with 0.01% sodium azide. The sections were mounted onto gelatin-chrome alum subbed microscope slides, covered slipped using Citifluor antifade medium and stored until imaging occurred.

The slides were imaged on an Olympus BX51 Fluorescence Microscope using CY3 or TRITC filter sets. Micrographs were collected from sections slice using 4X – 20X objectives and processed using Image-Pro Plus 7.0. Brain regions were identified using "*The Mouse Brain in Stereotaxic Coordinates: Deluxe Edition of the Atlas*. 2nd Edition and *The Mouse Brain in Stereotaxic Coordinates* , Paxino and Franklin, 5th Edition".

iii. Results

The BNST is located in the basal forebrain and plays important roles in autonomic and neuroendocrine control, fear and aggression states, sexual responses, chronic pain and stress-related anxiety-related behaviors (Lebow & Chen, 2016). The BNST has reciprocal connections with the central amygdala (CeA) through the stria terminalis and amgydalofugal pathways that encircle the lateral thalamus and globus pallidus, and receives diverse projections from the hippocampus and medial prefrontal cortex (mPFC). Systemic stressors including pain have ascending inputs into relay nuclei that project to various regions of the BNST. Hence the BNST collects and integrates a variety of sensory signals and in turn coordinates the necessary physiological and behavioral responses through projections to distal regulatory areas such as the hippocampus, hypothalamic nuclei and brainstem regions. (Lebow & Chen, 2016). As described above, PACAP is expressed in the BNST and BNST PACAP/PAC1 receptor signaling participates in stress-induced anxiety-related behaviors. The BNST PACAP projections have not been well examined and with the recent availability of PACAP-Cre mice, the Cre-dependent expression of reporter vectors allows the identification of some BNST PACAP projections to further elucidate PACAP-mediated behavior responses. Among the ten male PACAP-Cre mice infused bilaterally with the mCherry reporter vector, 8 survived the procedure and despite correct placement of the infusion needle in these animals, 2 demonstrated minimal vector leak from the injection site. The most common leaks were along the superior edge of the anterior commissure resulting in vector expression in the median preoptic nucleus, and along the needle track resulting in cortical

neuron labeling. As neuronal projections from these unintended labeling sites could obfuscate BNST PACAP projections, the data below were derived primarily from the 2 brains with minimal vector leakage. For these 2 brains, the center of the viral infusions was directly into the BNST but just posterior to Bregma -0.01 mm (Figure 1), a bit posterior of the intended injection at Bregma +0.1 mm.

BNST-PACAP Neuron Morphology

As described previously, the BNST may contain as many as 15 morphologically distinct cell types (Larriva-Sahd, 2006). The morphology of neurons found within the BNST consists of interneurons and projecting neurons, each with distinct subtypes within each category. There are five distinct types of interneurons: neurogliaform (NG), spiny neurogliaform (SNG), spiny neurons with a chandelier-like axon (SCA), common spiny neurons (CSN) and basket cells (BC) (Larriva-Sahd, 2006). CSN are the most commonly found neuron in the BNSTov (oval nucleus of the BNST) and resemble other short-axon neurons found in the basal ganglia, the amygdala, and the juxtacapsular nucleus of the BNST. Most of the BNST interneurons are GABAergic suggesting that GABA might be directly involved in controlling the activity of BNSTov efferent neurons.

Projection neurons make up the efferent system of the BNSTov, and there are four distinct categories of projection neurons characterized by axon type: fusiform, heterodendritic, spiny, and stellate cells. These categories can be placed into two broad classes based on the shape and dimension of the dendritic field, either wide-range or constrained-range dendritic field. These wide-range neurons are likely to be involved in decoding inputs from amygdala and adjacent BNST inputs, while constrained-range

neurons are more likely to be involved in decoding inputs within focal areas of the neuropil and from restricted amygdala and nuclear domains (Larriva-Sahd, 2006).

In the current studies, the distribution and characterization of the BNST PACAP neurons and fibers are not meant to be comprehensive but to highlight the major observations. These include BNST PACAP neuronal projections to the nucleus accumbens, amygdala and to the medial habenula nucleus; some projections to the lateral hypothalamus were also apparent.

The BNST PACAP neurons can be both intrinsic interneurons or projection neurons and from the labeled PACAP-Cre mice micrographs, the BNST PACAP neurons are morphologically heterogeneous and both populations appear evident. The most distinct neuronal cell types appear to be the fusiform and heterodendritic bi-horn projection neurons (Figure 2) base on their large (~30 micron) fusiform or polygonal soma and apparent long parallel fiber structures with strong varicosities. In rats, these neurons together represent approximately 10% of BNST neurons, but whether all BNST fusiform and heterodendritic neurons express PACAP is still unclear. Both have ascending and descending projections, demonstrating that BNST PACAP neurons can signal to distant CNS nuclei. The current studies also labeled a smaller BNST PACAP neuron (~15 microns soma diameter) with more apparent localized fiber distribution (Fig. 2). These neurons are difficult to identify but may represent superficial and/or common spiny interneurons. As these interneurons represent nearly 50% of the BNST population, the PACAP interneurons likely represented only a fraction of the total BNST interneurons.

Nevertheless, these results also indicated that BNST PACAP neurons can participate in intrinsic BNST signaling.

The BNST can be divided into many subdivisions or subnuclei (see Introduction above) and many of these areas demonstrated red fluorescent for PACAP producing cells. In the anterior BNST, PACAP expressing neurons were identified in the dorsolateral, dorsomedial, ventrolateral and ventromedial BNST subnuclei. The dorsolateral BNST, which includes the BNSTov is an integration center of various inputs (Lebow & Chen, 2016) well studied with respect to mediating stress-related anxiety-like behaviors. In particular, the subdivision contains the oval nucleus, a heterogeneous region expressing a diverse array of peptides such as opioid peptides, NPY, somatostatin, oxytocin, vasopressin and urocortin, in addition to CRF and PACAP, in the intrinsic and long distance regulation of the stress-associated behaviors. The subdivision also includes the juxtacapsular nucleus, an important structure in shifts between positive and negative valence for correlating stress behaviors (Lebow & Chen, 2016). The dorsomedial BNST has been linked to the stress response, specifically through regulating autonomic control to affect the HPA (Crestani et al., 2013). It has the highest direct projections to the paraventricular nucleus (PVN) and CeA out of any area in the BNST and is important for regulating drinking and fluid homeostasis input to Barrington's Nucleus (Lebow & Chen, 2016). PACAP expressing cells were also identified in the ventrolateral and ventromedial BNST which have prominent projections to the hypothalamus.

The distribution of PACAP-expressing neurons extended into the posterior BNST (Figure 3). It is a sexually dimorphic region that deals with reproduction and social

defensive behavior. As with the ventral BNST, the posterior region also projects to the mammillary nuclei, PVN, and medial preoptic area (Lebow & Chen, 2016).

BNST PACAP Neuronal Fiber Projections

Three types of PACAP fibers can be present in the BNST: 1) PACAP afferent fibers from other CNS nuclei synapsing in the BNST; 2) short axonal efferent fibers intrinsic BNST PACAP neurons forming microcircuits within the BNST; and 3) long axonal efferent fibers from BNST PACAP neurons projecting to distal CNS sites outside of the BNST. The current PACAP-Cre studies would not have identified the PACAP afferents; the current studies target the PACAP efferent fibers and in particular, the long-distance projection fibers to identify novel BNST PACAP regulatory areas.

The principal fiber pathways from the BNST are the reciprocal projections with the amygdala through the stria terminalis and the amgydalofugal pathways, as discussed above. The stria terminalis is a fiber bundle pathway that connects the amygdala to the septal area, including the BNST in the basal forebrain (Mori et al., 2017). It travels traversely over the temporal horn of the lateral ventricle, continues laterally along the caudate border, and finally descends into the BNST; the fiber pathway travels in parallel with the fornix (Koller et al., 2019). One important behavioral processing that has been associated with the stria terminalis is fear processing; projections from the amygdala to the BNST via the stria terminalis are involved in the processing and selective responsiveness to individual fear stimuli. The ventral amgydalofugal pathway is more direct and shorter between the amygdala and BNST traversing the anterior hypothalamic area, just posterior to the optic chiasm. From the amygdala – BNST pathway loop, the

BNST has additional projections. There are anterior projections to the forebrain, local projection in the environs of the septal and hypothalamic regions and long distant posterior projections to brainstem areas. Hence, the current studies attempt to identify whether BNST PACAP neuronal fibers travel in any of these projection pathways. The BNST PACAP projections to these regions will be described in turn.

BNST PACAP Anterior Fiber Projections

With reporter injections into the BNST of PACAP-Cre mice, a sparse population of fibers were found in the nucleus accumbens. Considering the vector BNST injection site, these represented fairly long axonal projections (> 1mm). In the forebrain area of Bregma +1.2 mm, the axonal fibers had short stem-like branches with prominent bouton-like terminals in both the core and shell areas surrounding the anterior poles of the anterior commissure (Fig. 4). The nucleus accumbens participates in motivation, addiction and reward behaviors (see Discussion) and these observations implicate BNST PACAP signaling in these processes.

BNST PACAP Amygdala and Local Fiber Projections

Fiber Projections via the Stria Terminalis

As described previously, the stria terminalis carries reciprocal efferent connections between the BNST and amygdala. With vector injections into the BNST, PACAP neurons, predominantly those in the dorsolateral BNST, had projections that traveled superiorly into the stria terminalis. Portions of the linear fiber tracts could be

followed extensively through successive cryosections, from the lateral edges of the thalamus (Figure 5; approximately Bregma -0.55 to -1.31 mm) before turning ventrally to terminate into the amygdala (Figure 5; approximately Bregma -1.67 mm); the BNST PACAP axonal stria terminalis projections were extensive and appeared to terminate heavily in the lateral and medial divisions of the CeA, and extending ventrally to terminate apparently in the medial amygdala (MeA). Interestingly, PACAP fibers were not readily apparent in the amgydalofugal pathway suggesting that BNST PACAP may not use this fiber system in regulating amygdala function.

Fiber Projections to the Medial Habenula

From the same vector injections into the BNST, PACAP fibers were apparent from the dorsomedial BNST traveling in the stria medullaris pathway (Figure 3; approximately Bregma -0.23 mm). From successive coronal sections from Bregma -0.59 to -1.67 mm), the fibers coursed towards the third ventricle and traveled medially towards the medial habenula where the fibers terminated in large round synaptic endings (Figure 6). Interestingly, the terminals appeared restricted predominantly to regions immediately adjacent to the third ventricle; there were no apparent fibers to the lateral habenula.

Fiber Projections to the Hypothalamus

Within the posterior regions of the BNST, the labeled PACAP fibers could be observed to project ventrally to the hypothalamus (Figures 7). Some of the fibers appeared to project to the lateral hypothalamus which may participate in feeding and

aggression behaviors. These observations suggest that BNST PACAP signaling may directly mediate stress-induced feeding and aggression responses.

In these studies, no BNST PACAP long distance downstream projections to pontine or other brainstem structures were readily observed; only occasional sparse short fibers or terminal were evident. While these observations suggest that BNST PACAP neurons may not have direct brainstem projections, there may be other reasons for these results (see Discussion), but broadly there might have not been enough transport time nor optimal viral injections.

iv. Discussion

The bed nucleus of the stria terminalis (BNST) is located in the basal forebrain and plays important roles in autonomic, behavioral and neuroendocrine control, fear and aggression states and the resulting behavioral responses, sexually dimorphic behaviors and functions, and anxiety and stressful stimuli with the correlating behavioral responses (Lebow & Chen, 2016). The BNST has reciprocal connections with the central amygdala (CeA) and receives projections from the hippocampus and medial prefrontal cortex (mpFC). In addition, systemic stressors have ascending inputs into various regions of the BNST. Overall, the BNST is a relay center for limbic cognitive control, most importantly anxiety, reward and stress responses (Lebow & Chen, 2016).

With the availability of PACAP-Cre mice, the current studies examined the characteristics, distribution and projections of BNST PACAP neurons. Unlike previous in situ hybridization and immunocytochemical studies, which were able to identify BNST PACAP soma or fibers respectively, the advantage of using PACAP-Cre mice expressing reporter vectors is that the reporter elaborates both cell bodies and fibers. Accordingly, the technique allows the characterization of the BNST PACAP neurons based on their cell size, shape and dendritic arborization, and the tracing of BNST PACAP fibers to their distal targets. The latter has the potential of identifying new targets to implicate addition BNST PACAP functions. From the observed results, several outstanding issues were addressed. Firstly, although largely presumed, it was unclear whether BNST PACAP neurons represent intrinsic interneurons, projections neurons or both. These studies showed that the BNST PACAP neurons were structurally diverse but had the

morphology consistent with fusiform and heterodendritic bihorn projection neurons, and a smaller neuron that may have represented spiny interneurons. Hence these observations implicated BNST PACAP in long distance signaling and in the function of intrinsic BNST microcircuits. Secondly, PACAP neurons were found in both the anterior, posterior and ventral BNST suggesting that BNST PACAP can participate in a variety of BNST functions. Thirdly, although previous studies have shown PACAP immunoreactivity in the stria terminalis, given the bidirectional nature of the fiber pathway, it was unclear whether these PACAP fibers represented efferents from the amygdala and/or the BNST. The current studies demonstrate that BNST PACAP neurons can send projections through the stria terminalis to regulate amygdala function. Lastly, in addition to the stria terminalis, the current studies show that BNST PACAP neurons have anterior forebrain projections to reach the nucleus accumbens, and intermediate projections through the stria medullaris to the medial habenula. The latter appears to be a novel observation demonstrating BNST PACAP modulation of medial habenular function. Of note, prominent BNST PACAP descending projections to brainstem areas were not readily apparent. The most heuristic explanation is that BNST PACAP neurons do not have long distance descending projections but may regulate brainstem nuclei through other phenotypically distinct intermediate neurons, such as CRF neurons, in the circuit. Alternatively, the apparent absence of these projections may be technical; the reporter vector may not have infected the appropriate BNST subregions projecting to brainstem areas or the time of reporter expression was insufficient to allow mCherry reporter transport completely to the distal terminals. Additional studies in the future may

clarify these observations. As the present studies identified three major BNST PACAP neuronal projections, to the nucleus accumbens, the amygdala via the stria terminalis and the medial habenula via the stria medullaris, these areas will be discussed in turn.

BNST PACAP Anterior Projections to the Nucleus Accumbens (NAcc)

The parts of the accumbens nucleus (NAcc) that were significant in this study were the core of the accumbens nucleus (AcbC) and the shell of the accumbens nucleus (AcbSH). The AcbSH surrounds the AcbC, thus they have separate functions. The AcbC is important in motivation and addictive behaviors and has been linked to the reward system through dopamine release (Kupchik & Kalivas, 2017); specifically a different pattern of dopamine release than the shell, thus resulting in different cognitive processes (Saddoris et al., 2015). The AcbSH has been associated with circuit for chronic and acute pain with connections that include the mesencephalic nucleus, the hippocampus, and the amygdala, using dopamine as the key signaling mediator (Saddoris et al., 2015). Studies done that have focused on the NAcc as a whole have found a correlation with PACAP; microinjections of PACAP into the NAcc reduces hedonic drive-the reward of food is diminished to result in less consumption of food (Hurley et al., 2019).

The current studies suggest that a key source of nucleus accumbens PACAP may be derived from the BNST. Moderate densities of BNST PACAP fibers were observed in the core and shell regions of the NAcc and notably, the long axons appeared to have short branching terminals along its length. The identities of the post-synaptic neurons were not determined but most likely include dopaminergic cells that project to other limbic

structures. These observations implicate BNST PACAP in the modulation of hedonistic/reward responses and hence, may have roles in addiction processes.

BNST PACAP Stria Terminalis Efferents to the Amygdala

Described previously, the BNST is divided into many subdivisions and many of these regions appear to express PACAP neurons. Using the anterior commissure as a reference point, these areas included the lateral division of the dorsal part of the BNST (BSTLD), which is located in the anterolateral/dorsal region of the BNST and has a multitude of functions and signaling pathways, especially GABAergic signaling. The BSTLD includes the oval nucleus (BNSTov) and the juxtacapsular region. Both appear to mediate stress-related anxiety-like behavior which may intersect with CRF neurons, and are part of the negative feedback loop of the HPA; however the spectrum of the anxiety response depends on the inputs into the various nuclei subgroups and the resulting projections to various areas, such as the hypothalamic or raphe regions (Lebow & Chen, 2016). PACAP neurons were also found in the dorsomedial division of the anterior BNST (BSTMA, also BSTMD), which is located in the anteromedial part of the BNST and has been linked to the stress response, specifically through regulating autonomic control and the HPA (Crestani et al., 2013). The BSTMA has the highest direct projections to the paraventricular nucleus (PVN) and CeA out of any area in the BNST and is important for regulating drinking and fluid homeostasis input to Barrington's Nucleus (Lebow & Chen, 2016). PACAP neurons were also identified in the regions ventral to the anterior commissure. The lateral and medial divisions of the BNST (BSTLV and BSTMV) have not been well studied but have major projections to the

hypothalamus (Lebow & Chen, 2016). The ventral BNST has a feedback loop system and connective circuitry with various brainstem areas and holds the largest noradrenergic fiber projections and inputs in the brain (Lebow & Chen, 2016).

The posterior BNST (BSTLP also pBNST) is separated from the anterior BNST by the stria terminalis and is a sexually dimorphic region that deals with reproduction and social defensive behavior. The posterior region projects to several hypothalamic areas including the mammillary nuclei, PVN, and medial preoptic area (Lebow & Chen, 2016). Studies on sex differences in behavior and emotional processing focus on the posterior region, most commonly injecting peptides and transmitters directly into the region. Hence BNST PACAP may modulate sexual behaviors and responses.

As described previously, BNST PACAP fibers were observed to enter the stria terminalis. The stria terminalis is a bidirectional fiber pathway allowing reciprocal connections between the BNST and amygdala. The principal projection from the BNST is through the stria terminalis; from the BNST, the efferent fibers in the stria terminalis travel along the caudate border and follow a trajectory that parallels the fornix (Koller et al., 2019) to terminate in amygdaloid nuclei in the temporal lobe. The two structures are very important structures in fear and anxiety but have very different functions within this pathway. The CeA modulates the fear response while the BNST modulates the anxiety response. Having PACAP expression on this tract highlights the importance of PACAP for both fear and anxiety and how the specificity of the tissue and receptors impacts the resulting behavioral response. This points to the importance in distinguishing between the environmental threat present, whether it is long duration or not, for the appropriate

response to be generated. Having PACAP expression both in the amygdala and the BNST shows how the structures must work together in the pathway to generate the correct response. If the BNST role lies within the anxiety response, and anxiety can be thought of the fear of the fear, then it would make sense that these structures are connected via the stria terminalis; anxiety occurs because of fear so the BNST must be tuned to the amygdala response.

Stria Medullaris and Medial Habenular (MHb) Nucleus

From the posterior BNST, PACAP neuronal fibers were also observed to enter the stria medullaris pathway. The stria medullaris is a white matter tract that directly connects frontolimbic areas to the habenula in the epithalamus near the dorsal 3rd ventricle (D3V). It begins at the septal nuclei, arches over the anterior commissure and continues dorsally until it reaches the habenula (Roddy et al., 2018) and it serves to connect the septal nuclei, dorsal anterior cingulate, lateral hypothalamus and basal ganglia within a single unidirectional tract. The habenula then projects into the fasciculus retroflexus, a predominantly cholinergic fiber bundle (Valjakka et al., 1998) that projects directly and indirectly to the midbrain monoaminergic. There are two major subregions of the habenula – the lateral and medial habenula. Interestingly, the BNST PACAP projections through the stria medullaris could be followed through successive cryosections and the projections ended in large synaptic terminals only in the medial habenula (MHb) immediately adjacent to the D3V; no BNST PACAP fiber terminals were evident in the lateral habenula. The MHb has important roles in various behavioral functions, such as stress, fear, anxiety, depression, memory, nicotine withdrawal, and

mood disorders (Viswanath et al., 2014). It expresses one of the highest levels of GABAb receptors in the brain, implicating that the MHb is under strong inhibitory control; there are also lower levels of noradrenergic innervation. As seen with other structures, PACAP has important roles in PTSD presentation; in the MHb, PACAP has been shown to regulate the strength and persistence of fear memories, an important component of PTSD (Viswanath et al., 2014). As with the nucleus accumbens, these results are novel in demonstrating that one source of MHb PACAP fibers is from the BNST. The MHb has further been divided into the dorsal (dMHb) and ventral (vMHb) habenula. While the distinction was not made between the divisions of the MHb in these studies, the differences between the dMHb and vMHb may not be distinct - both regions appear important in fear and anxiety behaviors.

In addition to PACAP, the MHb is also innervated by other peptidergic systems such as substance P and NPY, and a variety of neurotransmitters including GABA, norepinephrine and acetylcholine. Cholinergic receptor signaling plays an important role in nicotine addiction; infusion of a nAChR antagonist, mecamylamine, into the MHb precipitated a withdrawal response in mice chronically treated with ethanol (McLaughlin et al., 2017). In some neuronal systems, PACAP has been shown to be co-localized with acetylcholine in the pre-synaptic terminal, and upon release, enhances the nAChR response. PACAP acts via PAC1 receptors to modulate synaptic function by increasing ACh release from the presynaptic terminals, ultimately resulting in recruitment of adenylate cyclase dependent signaling to control presynaptic transmitter output (Pugh et al., 2010). If the mechanisms were comparable, this could imply that PACAP modulates

nAChR activity within the MHb to effect nicotine addiction. Similarly, PACAP has been implicated facilitating in other addictive behaviors. Overall, the connections between the BNST and the MHb are complex and since these two structures are implicated in similar behavioral functions and PACAP signaling may be the common denominator in mediating these effects.

Hypothalamic Areas

The lateral hypothalamic area (LH) is an important area of the hypothalamic region whose functions range from sleeping and waking, to stress and feeding-specifically weight loss- and motivation (Stuber & Wise, 2016). Since the LH covers such a range of fundamentally crucial behaviors, it is no surprise that the neuronal population is vast. This diverse population of neurons makes it slightly more difficult to correlate with behavior and a specific cell type (Mickelsen et al., 2019), thus no specific studies have been done on the effect of stress within the LH; any change within these sub-functions would induce a change in homeostasis and thus incur stress. However, PACAP fibers have been found within the LH, and LH PACAP signaling has been linked to a reduction in food intake (Sekar et al., 2017). The LH PACAP fibers have been shown to be derived from the parabrachial nucleus (Missig et al., 2017) and in part, from current work, from the BNST. Stress alters feeding behaviors and BNST PACAP-mediated signaling in the LH may participate in that process.

Some sparse BNST PACAP fibers may have extended into hypothalamic mammillary bodies. The mammillary nuclei are divided into two separate nuclei with differing functions, the lateral part of the medial mammillary nucleus (ML) and the medial part of

the medial mammillary nucleus (MM); lesions of the ML and MM both produced an anxiolytic effect that resulted in a lower level of emotional arousal and decreased stimuli processing (Vann, 2010). More recently, PACAP signaling in the mammillary bodies have been associated with reproductive behaviors and function (Vann, 2010).

v. Conclusion and Future Directions

A principal finding of the current work is the identification of BNST PACAP projections through the stria medullaris pathway to the medial habenula. These observations were novel; a search for "PACAP" and "stria medullaris" in PubMed, Web of Science and PsycInfo databases failed to return meaningful results.

From what is known about the MHb role in PTSD, reward, decision making and mood disorders, and from what is well known about the BNST's role in these similar processes, it not surprising that the stria medullaris is a connecting pathway to modulate these resulting behaviors.

It is also understandable that BNST PACAP output dictates the response of the MHb, for the stria medullaris is a unidirectional fiber pathway that ends within the MHb. This means that if a stressor stimulated PACAP upregulation in the BNST, it would similarly affect the activity of the MHb.

One of the most significant pieces of data collected from this study is the robust PACAP reporter expression in the axons and terminal connections between the BNST and MHb through the stria medullaris. The MHb appears to be the only target of BNST PACAP neurons projecting through this pathway, i.e., no BNST PACAP fiber terminals were evident in the LHb. As described above, the release of PACAP from these terminals may act either pre- and/or postsynaptically to enhance neurotransmitter signaling on the postsynaptic cell.

Future directions should focus on the localization of PAC1 receptors within the MHb, as well as throughout the stria medullaris. This important structure and pathway

already have an established relationship through behavior and function, however little is known on the cellular relationship of signaling pathways. How exactly do projections from the BNST effect the MHb; what receptors are being targeted. If both the BNST and MHb are important in PTSD and various mood disorders, what exactly is their relationship and how can this relationship be leveraged for future understandings of the molecular foundation of such disorders.

There are several issues that should be considered in this study but importantly, the current work only used male mice, so one cannot draw the assumption that this relationship between the MHb and BNST through the stria medullaris pathway applies to both sexes. The BNST is sexually dimorphic and some studies have suggested that there is a sex difference within the habenular nucleus. Hence to fully understand the role of the BNST and MHb in anxiety, one would have to look at both sexes under identical conditions.

In sum, PACAP has diverse effects in maintaining physiological homeostasis. In the limbic system, including the BNST, amygdala, hypothalamus, parabrachial nucleus and hippocampus, PACAP has intersecting roles in stress responses, fear and anxiety-related behaviors, learning and memory, feeding, chronic pain and hedonistic/reward functions. How PACAP may coordinate these activities among the different limbic structures is still not well understood but the identification of the BNST PACAP interactions with the medial habenula adds another element into the circuit which may more fully elaborate understandings of our responses to mental and physical challenges. With this

identification, future work on PACAP MHb signaling and function may better define how the MHb PACAP system is integrated into these behavioral circuits.

vi. Figures

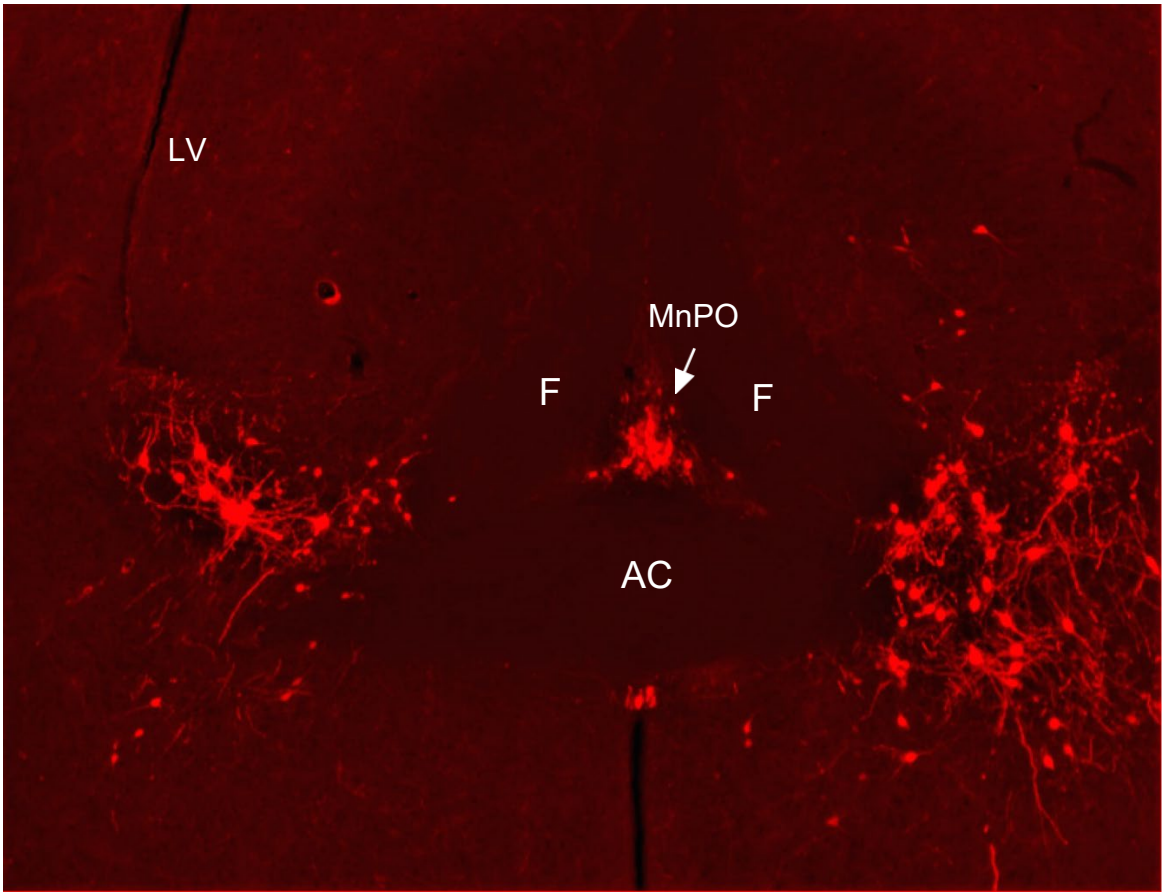

Figure 1. Viral mCherry reporter expression in the BNST. Representative micrograph of AAV-mCherry reporter injection and expression in the BNST; microinjection into BNST at Bregma +0.01 shown. Note high density of BNST PACAP expressing neurons dorsal to the anterior commissure (AC); PACAP expressing neurons were also present in the ventral BNST. PACAP expressing neurons in the median preoptic nucleus (MnPO) likely reflected vector diffusion along the superior surface of the AC. F, fornix

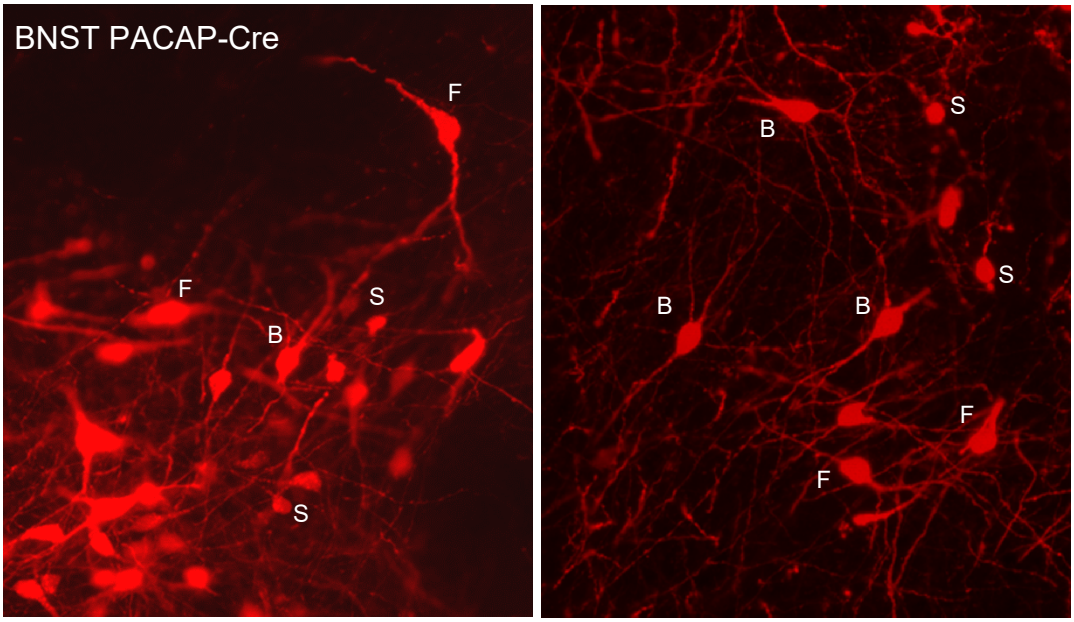

Figure 2. Morphology of BNST PACAP neurons. Representative micrographs of BNSTov PACAP-Cre neurons near Bregma +0.01 mm expressing mCherry reporter. The labeled neurons were very heterogeneous in cytoarchitecture. Labels indicate the presumptive neuronal types: F, fusiform projection neurons; B, heterodendritic bihorn projection neurons; and S, common or superficial spiny interneurons.

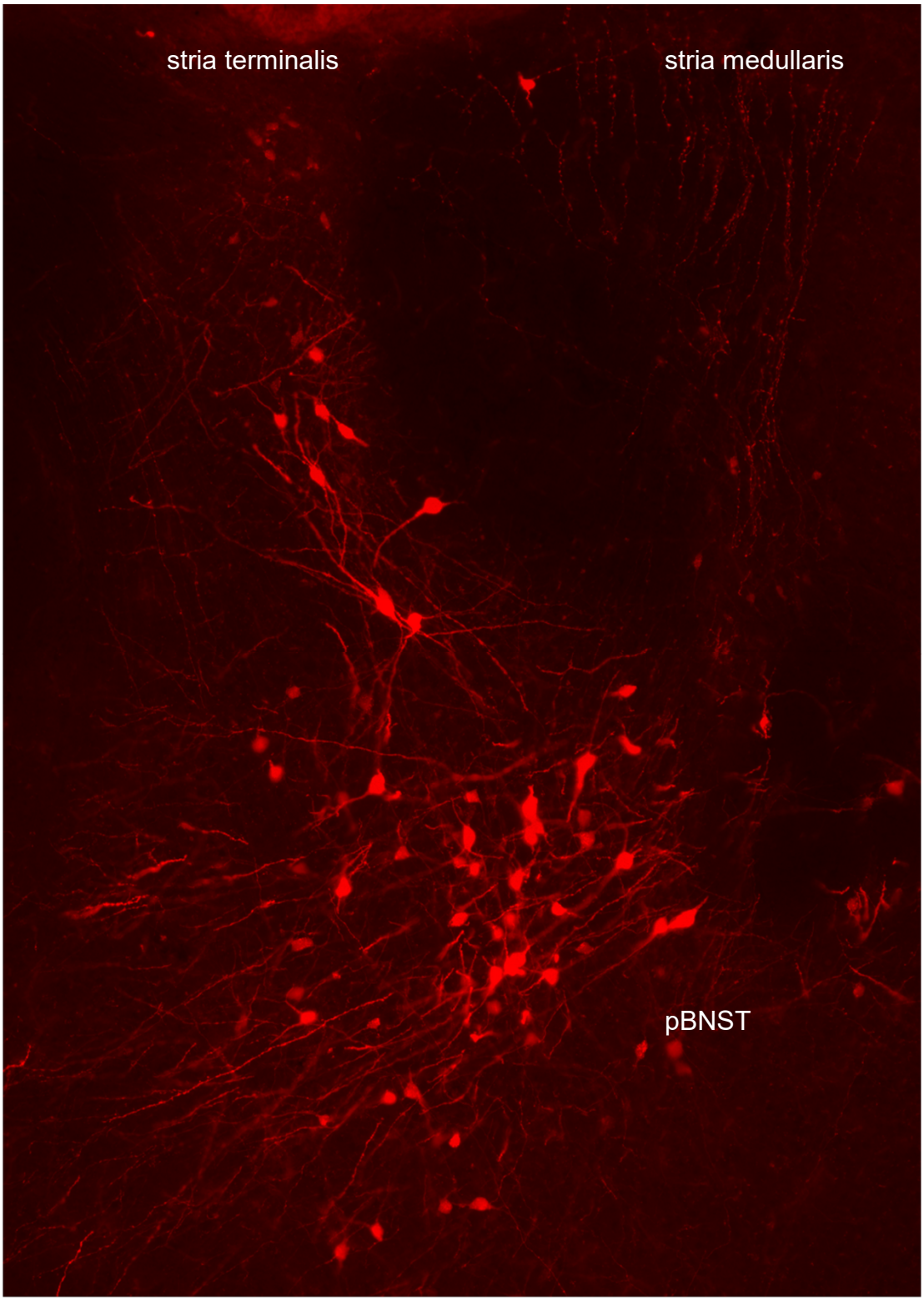
stria terminalis
stria medullaris
pBNST

Figure 3. Posterior BNST PACAP neuronal projections. mCherry labeled PACAP-Cre neurons in the right posterior BNST (pBNST) at approximately Bregma -0.35 mm. PACAP cells and fibers can be observed following to tracts. The lateral tract (left) represents the stria terminalis with fiber projections to the amygdala (see Figure 4). The medial tract (right) represents the stria medullaris with axonal projections to the habenula (see Figure 5).

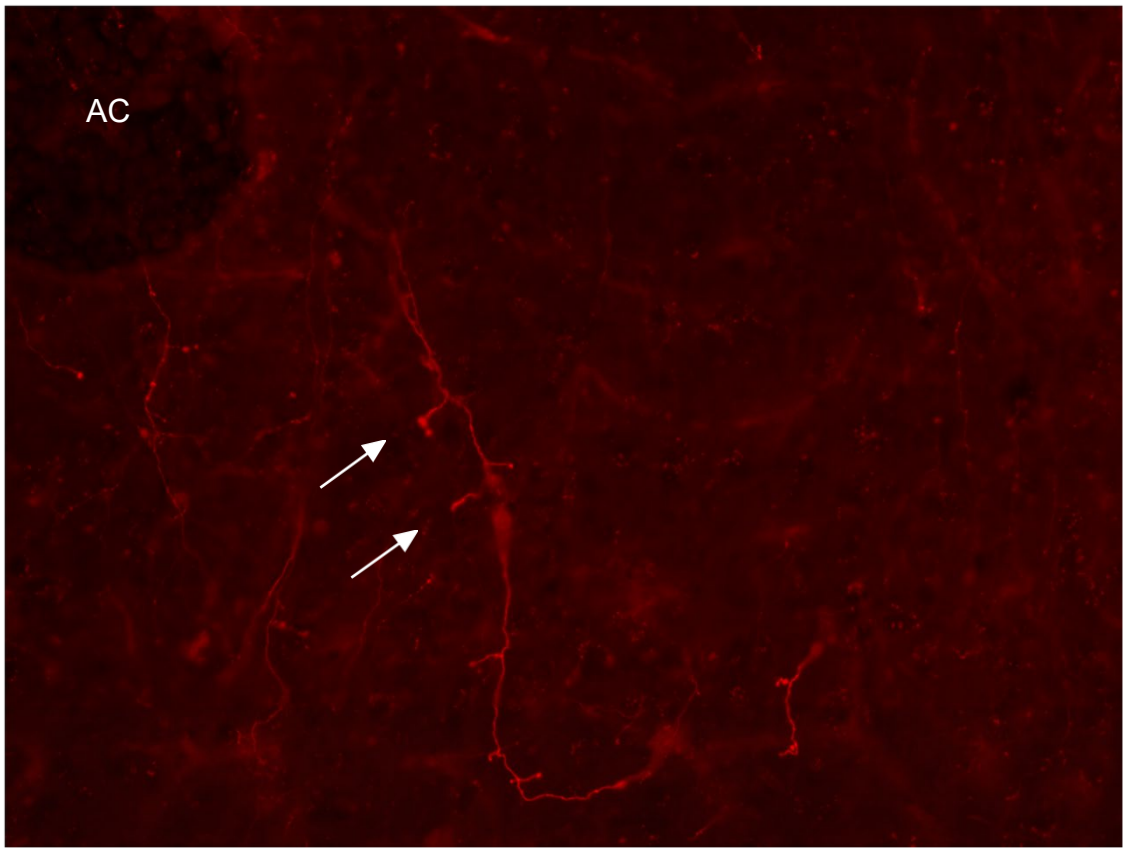

Figure 4. Anterior BNST PACAP projections. The BNST PACAP neurons have anterior projections to the forebrain. The mCherry-labeled BNST PACAP axonal fibers

can be identified in the core and shell regions of the nucleus accumbens. Notably, the long axons had numerous short branching terminals (arrows). The fiber density was moderate in these regions suggesting BNST PACAP regulation of nucleus accumbens functions.

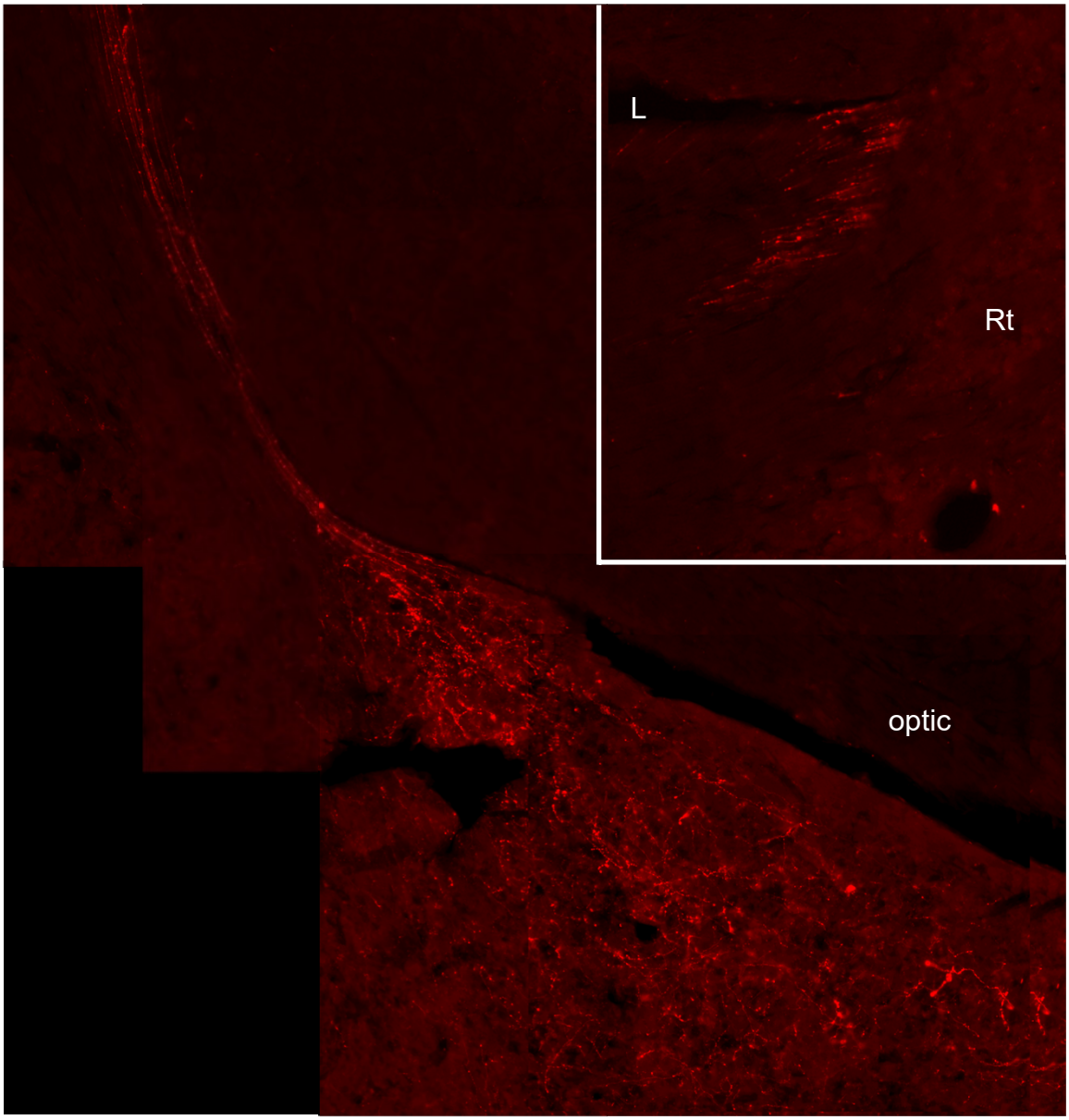

Figure 5. BNST PACAP projections via the stria terminalis. BNST PACAP projections through the stria terminalis, shown in Figure 2, can be followed coursing adjacent to the reticular prethalamic area (Rt; Bregma -0.59 mm, inset) before arcing posteriorly and inferiorly to project to the amygdala (Bregma -1.67 mm). The fiber terminals and varicosities were heaviest in the region of the central nucleus of the amygdala (CeA); fibers were also observed to extend and radiate broadly beyond the CeA and into the presumptive medial amygdala (MeA). Data produced in collaboration with Mahafuza Aktar. .

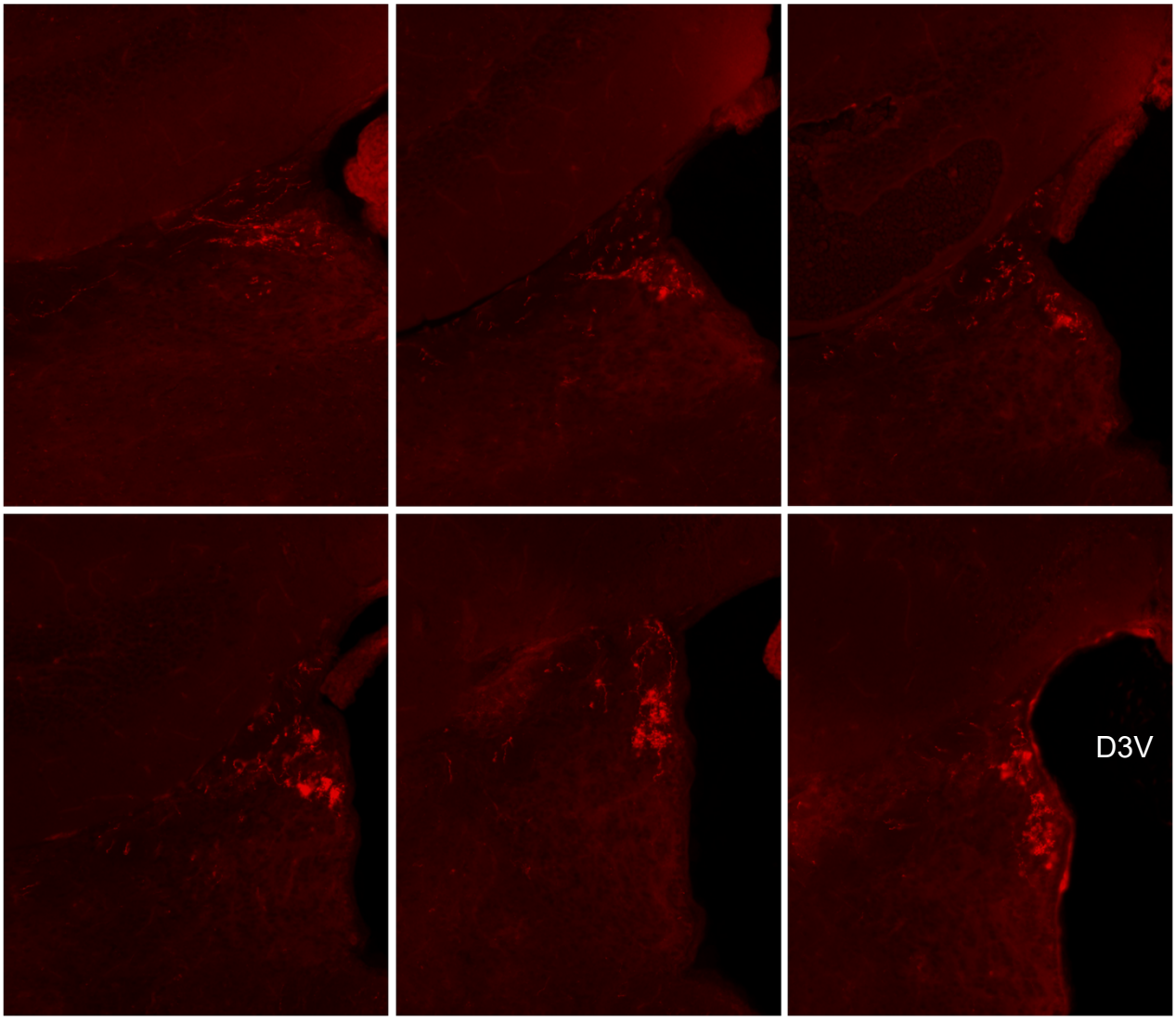

Figure 6. BNST PACAP stria medullaris projections to the medial habenula. BNST PACAP neuronal fibers in the stria medullaris (Figure 2) travel medially along the inferior border of the subfornical organ (A) and terminal in large synaptic terminals in the medial habenula (B – F), immediately adjacent to the dorsal third ventricle (D3V). Notably, the projection appears to be unique and highly localized; no projections were apparent in the lateral habenula.

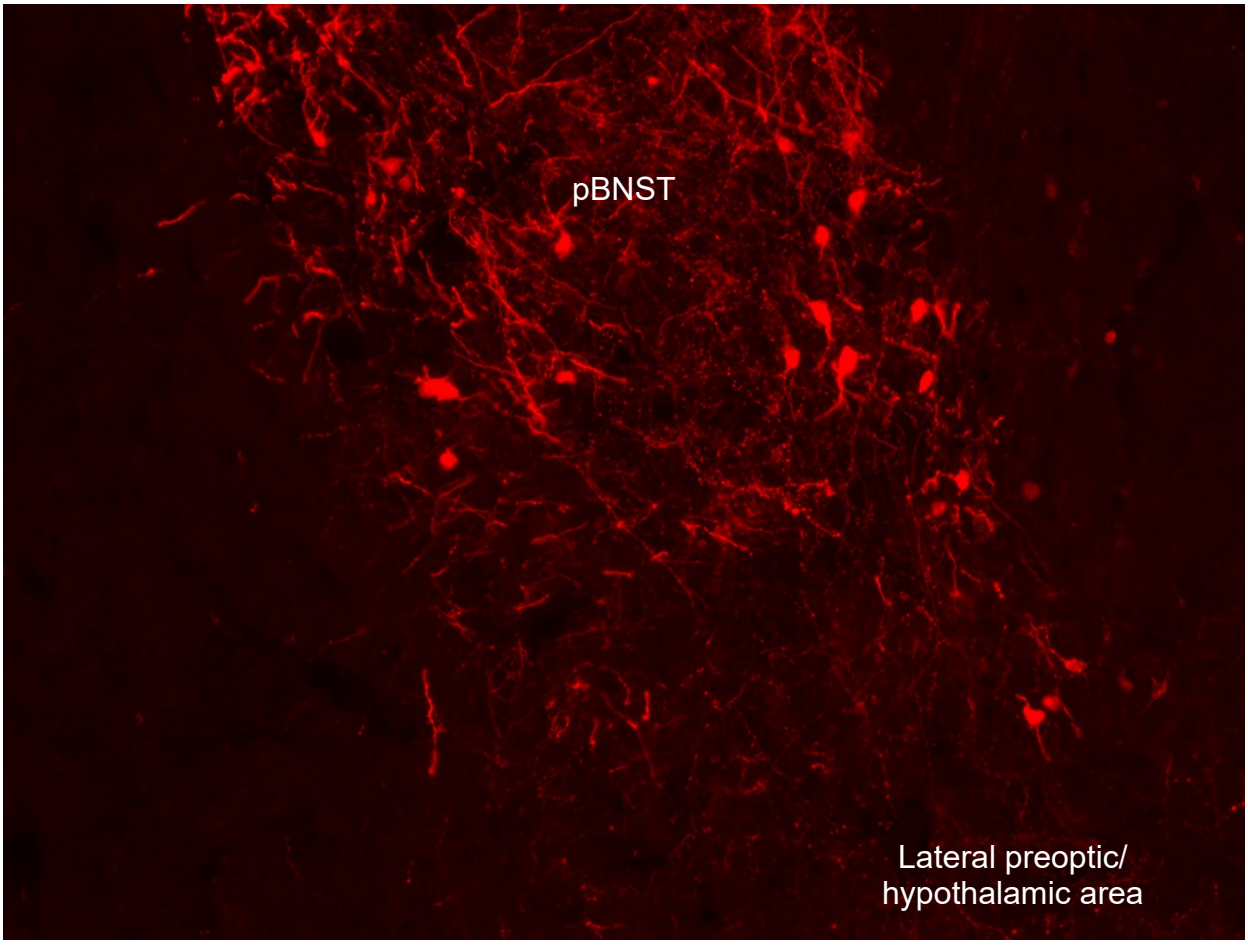

Figure 7. Posterior BNST PACAP projections to the hypothalamus. BNST PACAP expressing neurons in the posterior BNST were observed to project long distance inferiorly into the hypothalamus. Most of the axonal projections appeared to terminate in the lateral hypothalamus (LH).

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