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# Amygdala PACAP as a mediator of the emotional components of pain

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# AMYGDALA PACAP AS A MEDIATOR OF THE EMOTIONAL COMPONENTS OF PAIN

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## ABSTRACT

Chronic pain alters sensory responses and carries a strong emotional component. Persistent pain can heighten pain experiences, resulting in hyperalgesia and allodynia. Further, patients suffering from chronic pain are more prone to experience a range of affective disorders including depression, sleep dysregulation, panic disorders, anxiety abnormalities and stress-related disorders including post-traumatic stress disorder (PTSD). Hence while pain serves a protective function to prevent additional physiological harm by driving behavioral and cognitive responses, chronic or persistent pain can lead to maladaptive nociceptive responses and exacerbate psychopathologies. Among brain regions, the amygdala is centrally situated to integrate the many descending and ascending signals to modulate the sensory and emotional components of pain. The amygdala is well studied for its role in fear and stress-related behavioral processes. The central nucleus of the amygdala (CeA), and in particular the lateral capsular subdivision of the CeA (CeLC), receives prominent ascending pain neurotransmission via the spino-parabrachioamygdaloid tract. In this pathway, peripheral nociceptive signals carried via primary sensory Aδ- and C-fibers terminate in the dorsal horn where second order neurons send projections via the spino-parabrachial pathway to the lateral parabrachial nucleus (LPBn). Thus, the LPBn collects cutaneous (mechanical and thermal), deep (muscular and articular) and visceral nociceptive signals and relays the information in a highly organized manner principally to the CeLC for nociceptive processing. In pain, the CeA and the LPBn-CeLC projections have been shown to undergo plasticity in the forms of enhanced synaptic transmission and alterations in neurotransmitter and receptor expression. Accordingly, the neurocircuit intersections in the CeA can modulate the sensory and emotional responses to pain. Yet despite these associations, the mediators and mechanisms underlying the emotional consequences of pain are poorly understood.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neural and endocrine pleiotropic peptide important in the development and homeostatic regulation of many physiological systems. Recently, the expression of PACAP and its cognate PAC1 receptor has been shown to be upregulated in specific limbic regions by chronic stress. PACAP infusions into several limbic regions is anxiogenic, and altered blood PACAP levels and PAC1 receptor polymorphism have been associated with PTSD and other stress-related disorders. Here, we establish that CeLC PACAP originates from the LPBn as part of the spino-parabrachoamygdaloid pathway. Chronic pain enhanced PACAP expression along LPBn-CeLC projections, indicating it may be a component of pain-related plasticity. CeA PACAP signaling was sufficient to induce nociceptive hypersensitivity and anxiety-like behaviors. In a chronic neuropathic pain model, CeA PACAP signaling was found to contribute to heightened anxiety-like behaviors and nociceptive responses. Further, we characterized one prominent intracellular signaling mechanism through which CeA PACAP signaling influences these behaviors.

In these experiments we provide evidence that CeA PACAP signaling plays an important role in the emotional components of pain and that alterations in CeA PACAP signaling are part of pain-related plasticity. This work establishes novel molecular mechanisms that underlie the emotional component of pain and may contribute to the development of chronic pain and associated affective disorders.

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# Chapter 1.

## Literature Review

### 1.1. General Introduction

Chronic pain is one of the greatest medical health problems in the developed world affecting approximately 19% of the adult population (Breivik et al., 2006). From an economic standpoint, chronic pain presents an enormous burden. In 2010, the estimated additional health care costs due to pain ranged from $261-300 billion within the United States, and with the loss in productivity, the total costs increase to an estimated $560-635 billion (Gaskin & Richard, 2012). In relative terms, the annual cost of chronic pain is greater than that for heart disease ($309 billion), cancer ($243 billion), and diabetes ($188 billion) (Gaskin & Richard, 2012). While readily available and highly efficacious treatments for acute pain exist, successful treatment options for those suffering chronic pain still remain elusive, with current medications reducing pain severity by only 30-40% in fewer than 50% of patients treated (Turk, 2002).

Pain is an adverse sensory and emotional experience associated with real or potential tissue damage. Under normal conditions, pain serves a protective function, driving a set of responses that prevent the body from incurring additional harm. Pain is multidimensional, acting both as an immediate sensory-discriminatory indicator and as an emotional-affective drive that promotes defensive and vigilant behaviors. Poignantly illustrative of this protective function are the accounts from case reports of individuals with a congenital insensitivity to pain. Individuals with a set of rare nonsense mutations

in the SCN9A gene, encoding for the α-subunit of $Na_v1.7$ channel, display normal reactions to touch, warmth, cold, and pressure, but completely lack any kind of reaction to painful stimuli. These individuals live in constant threat of injury, displaying frequent bruises, cuts, damage to lips and tongue (from biting themselves during early years of life), and are at risk for early mortality from accidental injury (Cox et al., 2006).

While acute pain serves a clear protective function, pain can outlast the injury and the normal healing process and become chronic. In these cases, pain is detrimental to an individual's quality of life, without any physiological benefit. Clinically, chronic pain has often been defined as pain that persists for at least 3 to 6 months; however, very often chronic pain lasts much longer. One study reported that those seeking treatment at chronic pain treatment facilities did so for 7 years on average (Flor et al., 1992). There is a crucial need to determine both the mechanisms underlying the transition from acute to chronic pain, and the mechanisms that maintain and reinforce chronic pain. One of the key concepts to emerge from the efforts to understand the mechanisms of chronic pain is that of central sensitization. Sustained noxious input can result in the prolonged increase in excitability and synaptic efficacy in neurons along central nociceptive pathways. The enhancement of nociceptive transmission is manifested as pain hypersensitivity, in which pain can result from a normally non-painful stimulus (allodynia) or pain is enhanced from a painful stimulus (hyperalgesia). Since central sensitization results from changes occurring in central neurons, the increased responsiveness may become decoupled from the peripheral noxious stimulus and could result in the persistence of pain in the absence of injury (Latremoliere & Woolf, 2009). Thus, discovering the mechanisms underlying

the sensitization of nociceptive central circuits appears crucial to the understanding the pathogenesis of chronic pain and will provide opportunities to develop treatments for chronic pain.

Further increasing the burden of chronic pain sufferers is that pain often co-exists with psychiatric illness. One study found that 59% of those being treated for chronic back pain had at least one concurrent diagnosis of psychiatric illness compared to 15% in the general population, and 77% had at least one lifetime psychiatric diagnosis compared to 29-38% in the general population (Kroenke & Price, 1993). Epidemiological studies have found a strong association between chronic pain and anxiety disorders. A nationally representative sample (n=5877) of those who suffered chronic pain found that they were 2-3 times as likely to have an anxiety disorder compared to the general population. The rate of posttraumatic stress disorder (PTSD) was present 3.7 times more often in those with chronic pain than in the general population, and the rate of panic disorder (PD) was 4.3 times that of the general population (McWilliams et al., 2003). A large cross-national mental health survey (n=85,088) found similar results, with chronic back or neck pain being associated with PTSD, PD, and generalized anxiety disorder (GAD) 2-3 times the rate in the general population (Demyttenaere et al., 2007). This relationship appears to hold true across several different types of pain, as migraine, arthritis, and back pain sufferers were found to have 2-4 times the rate of anxiety disorders compared to the general population (McWilliams et al., 2004). Furthermore, pain may precipitate stress-related disorders, as it has been shown that the level of peritraumatic pain in patients admitted to a trauma center is highly predictive of the development of PTSD at 4 and 8

months following hospital admission (Norman et al., 2008). PTSD symptoms were significantly positively correlated with pain ratings, with PTSD sufferers having higher subjective pain and more pain-related disability (Phifer et al., 2011). Interestingly, PTSD sufferers also have altered reactions to acute pain, with higher pain thresholds to acute noxious stimulation, but greater intensity of pain with suprathreshold noxious stimulation (Defrin et al., 2008; Geuze et al., 2007).

Theoretical models have been proposed to explain the relationship underlying the concurrence of chronic pain and anxiety-related disorders. The mutual maintenance model holds that both disorders interact in a way that reinforces the persistence of the other (Asmundson & Katz, 2009). In the mutual maintenance model, the physiological, affective, and behavioral components of anxiety disorders interact to maintain or exacerbate symptoms of pain. Similarly, the various physiological and affective components of pain interact to maintain or exacerbate symptoms of anxiety disorders. For instance, pain sensations in a chronic pain sufferer could act as a persistent, conditioned reminder of trauma, resulting in increased anxiety. Alternatively, the shared vulnerability model posits that individual factors may predispose people to develop both anxiety disorders and chronic pain. These factors, such as feelings of loss of control or low threshold for alarm, may be genetically influenced (Asmundson & Katz, 2009). While these models offer explanations for how pain and stress interact, the biological mechanisms that underlie these relationships are still largely unknown.

In examining function of neuropeptide signaling within the nervous system, the laboratories of Dr. Victor May and Dr. Sayamwong Hammack have recently identified

pituitary adenylate cyclase activating polypeptide (PACAP) and its cognate PAC1 receptor as a mediator of the stress response system. Increased PACAP expression is found following a repeated variate stress (RVS) paradigm within the bed nucleus of the stria terminalis (BNST) and the paraventricular nucleus of the hypothalamus (PVH) (Hammack et al., 2009). Infusion of PACAP into the BNST is anxiogenic, increasing anxiety-like behaviors and hypothalamic pituitary axis (HPA) activation, and producing an anorexic response (Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Lezak et al., 2014). Blocking BNST PACAP signaling during RVS can significantly attenuate heightened anxiety-like behaviors and stress-induced anorexia (Roman et al., 2014). PACAP signaling may be relevant to human anxiety-disorders, as a single nucleotide polymorphism (SNP) in the PAC1 receptor gene, *ADCYAP1R1*, has been correlated with PTSD symptoms in women, and PAC1 receptor methylation has been found to be associated with PTSD symptoms in both sexes (Ressler et al., 2011). In aggregate, these findings implicate limbic PACAP signaling as a central mediator of the stress response system. In the course of our investigation, we noted dense PACAP immunoreactivity in the nerve terminals within the central amygdala (CeA). Subsequently, we found that CeA PACAP corresponded to nociceptive input originating from the parabrachial nucleus (PBn). Thus, PBn PACAP released in the CeA could serve as a mechanism linking nociceptive input to amygdala-mediated emotional responses.

To further understand the pathways described above, we investigated whether CeA PACAP signaling mediates the emotional components of pain, first, by establishing PACAP in the PBn-CeA projections, and next by examining whether CeA PACAP was

involved in pain-related emotional responses. Further, we characterized the potential molecular pathways through which CeA PACAP signaling may be acting. These investigations were aimed at understanding the molecular and anatomical substrates underlying the relationship of pain and emotional behaviors. The coexistence of pain with affective disorders may not only result in substantial disease burden, but also lead to the amplification and perpetuation of pain. The mechanisms linking these two systems may be particularly effective targets for the development of treatments for affective disorders comorbid with chronic pain.

In interest of clarity, the background and introduction are divided into three main sections: 1) mechanisms of nociception - reviewing the detection and transmission of nociceptive information; 2) amygdala and its functions in pain; and 3) PACAP signaling in pain. The subsequent two chapters are primary research studies in manuscript form, which is then concluded with a comprehensive discussion.

## 1.2. Neurobiology of Pain

*Detection of painful stimuli*

The detection of stimuli of a thermal, mechanical, or chemical nature is performed by a set of sensory afferent fibers in the body containing a set of specialized receptors that transduce sensory stimuli into electrical currents. For thermal stimuli, a clear demarcation between innocuous warmth and noxious heat exists and typically rests around 42.5 C. At this temperature lies the approximate thermal activation threshold for the transient receptor potential cation channel subfamily vanilloid member 1 (TRPV1)

receptor. A member of the transient receptor potential (TRP) ion channel family, TRPV1 receptor activation results in the perception of a burning sensation. Capsaicin, the pungent ingredient in "hot" chili peppers, is a TRPV1 agonist. On the other end of the thermal spectrum, TRPM8 and possibly TRPA1 receptors are sensitive to noxious cold stimuli and display an affinity to natural cooling agents such as menthol (Basbaum et al., 2009). Mechanical stimuli are detected through multiple mechanisms including high-threshold mechanoreceptors that terminate in free nerve-endings in the skin, low-threshold mechanoreceptors that terminate on hair fibers, as well as Merkel cells and Pacinian corpuscles, which detect texture, vibration, and light pressure. It is predicted that these structures contain ion channels that are activated directly by force underlie mechanotransduction; however the identity of these channels has been difficult to determine. Recently, piezo channels, piezo 1 and piezo 2, have been shown to be potential candidates for mechanotransduction (Coste et al., 2010). Piezo channels are extremely large proteins comprised of more than 2000 amino acids with 30 to 40 transmembrane segments, and exist in an even larger structural complex, as the functional channels appear to be tetramers. Initial evidence suggests that piezo1 might be particularly important in vascular architecture as a shear-stress-evoked ionic current (Li et al., 2014). Piezo 2 has been shown to be important in low-threshold mechanotransduction, mediating innocuous touch sensation (Ranade et al., 2014). The channels mediating high-threshold, noxious mechanosensation are still unknown. Noxious chemical stimuli can consist of environmental agents such as capsaicin, menthol, and isothiocyanates that bind to receptors that transduce noxious stimuli,

including TRPV1, TRPM8, and TRPA1 respectively. Additionally, noxious chemical stimuli can be substances that are endogenously released after tissue damage or physiological stress. These include signaling molecules, such as calcitonin gene related peptide (CGRP) and substance P, and factors released from mast cells and macrophages, such as bradykinin, prostaglandin E2, interleukin-6 (IL-6) and tumor necrosis factor – α (TNFα). These molecules bind to receptors on the cell surface to activate or sensitize nociceptors directly, thereby inducing pain or lowering the threshold for pain perception (Basbaum et al., 2009).

*Nociceptive pathways*

After detection of noxious stimuli and transduction into electrical currents, two main classes of fibers convey nociceptive information, medium diameter, lightly myleinated Aδ fibers and small diameter, unmyleinated C-fibers. Aδ-fibers range in diameter from 2-6 μm with a conduction velocity of 12-30 m/s, while C-fibers have a diameter of 0.4 to 1.2 μm with a conduction velocity between 0.5-2 m/s. Whereas Aδ afferents convey acute, well-localized pain, C-fibers are responsible for more diffuse, slow onset pain. Each fiber type can be further divided into subpopulations. Aδ-fibers can be divided into type I fibers that respond to mechanical stimuli but have a high heat threshold, and type II fibers that have a low heat threshold but high threshold for mechanical stimuli. Many C-fibers are polymodal, responding to both mechanical and thermal stimuli; however subsets of these fibers may have modality specificity. Based on molecular characterization, C-fibers consist of a peptidergic population that expresses

substance P and calcitonin-gene related peptide (CGRP), and a nonpeptidergic population that binds IB4 isolectin and express G protein-coupled receptors of the Mrg family (Basbaum et al., 2009). Although still a matter of debate, there might be modality specificity as it was recently found that selective ablation of these peptidergic fibers reduced sensation to noxious heat and capsaicin, without impairing mechanosensation (McCoy et al., 2013). Nociceptive fibers originate from pseudo-unipolar somatosensory neurons that have cell bodies residing in the dorsal root ganglion (DRG) or the trigeminal ganglion. The peripheral terminals of these neurons transduce nociceptive information and convey it to the central terminals that synapse in the outer layers of the dorsal horn of the spinal cord, specifically Rexed laminae regions I, II, and V. Subtypes of afferents synapse with regional specificity creating a distinct laminar organization. Projections from dorsal horn neurons within laminae I and III-VI form the main connections to brainstem and brain (Todd, 2010).

Within the dorsal horn, second order neuronal projections form multiple parallel pathways to convey nociceptive information to higher order central nervous system (CNS) regions. The afferent pain pathways can be separated on a phylogenetic basis into two different systems, ancient and more evolutionarily recent pathways. The evolutionarily ancient pathways run through the medial brainstem consisting of the paleospinothalamic, spinoreticular, spinomesencephalic, spinoparabrachio-amygdaloid, and spinohypothalamic tracts. In contrast, evolutionarily recent pathways traverse the lateral region of the brainstem and consist of the neospinothalamic and spinocervical tracts (Almeida et al., 2004). These tracts form the main projections from the superficial

dorsal horn to brainstem and brain. The main targets of dorsal horn projection neurons within the brainstem include the caudal ventrolateral medulla (CVLM), which is an integrative center of cardiovascular response and nociception, and a site of origin for many of the descending projections back to the dorsal horn. The nucleus of the solitary tract (NTS) is a second major target involved in cardio-respiratory integration, as well as a major target for visceral nociceptive information arriving via the vagus nerve. The periaqueductal grey (PAG) in the medulla is involved in the descending modulation of dorsal horn circuits, one of the key regions for the actions of analgesics, and critical in stress-induced analgesia through descending output from the amygdala (Butler & Finn, 2009). The lateral PBn (LPBn) is a major target of lamina I input, and LPBn neurons have axonal projections to the amygdala, hypothalamus, and BNST; these projections will be discussed further in the next section. Another major target of nociceptive lamina I projections neurons is the thalamus. In particular, several regions in the thalamus receive nociceptive information, including the ventral posterolateral nucleus (VPL), which receives nociceptive information from the body, and the ventral posteromedial nucleus (VPM), which receives nociception information from the face via the trigeminal nerve. The VPL and VPM have direct projections to the primary somatosensory cortex, and are involved in the sensory-discriminative aspects of pain. Another set of thalamic nuclei the posterior group and posterior triangular nucleus of the thalamus (PoT) also receive nociceptive information and project primarily to the insular cortex, secondary somatosensory cortex, and amygdala. These regions are thought to be involved the aversive emotional aspects of pain (Gauriau & Bernard, 2004). Studies in rodents have

examined the anatomical distribution for each spinal pathway by injecting retrograde tracers into each brain region and quantifying the number of lamina I neurons labeled (Spike et al., 2003; Todd, 2010). Upon examination, lamina I of the L4 segment of the rat spinal cord contains approximately 400 projection neurons, which is about 5% of the total number of neurons in lamina I. A majority of the neurons project to the contralateral side of the brain; however about 25% have bilateral projections. The vast majority of lamina I neurons exhibit extensive collateralization projecting to multiple regions. Hence, of all L4 lamina I projections neurons, an estimated 85% project to the LPBn, 85% project to the CVLM, 30% project to the PAG, 25% project to the NTS, and less than 5% project to the thalamus (Spike et al., 2003). The very small proportion of projections to the thalamus may be unique to the lumbar region, because the cervical spinal cord contains a greater number of spinothalamic projection neurons and fewer spinoparabrachial projection neurons (Al-Khater & Todd, 2009).

*Parabrachial nucleus (PBn) anatomy and connectivity*

The PBn is an anatomical area surrounding the superior cerebellar peduncle (SCP), or brachium conjunctivum, located in the dorsolateral rostral pons and caudal midbrain. The PBn can be divided into medial (MPBn) and lateral (LPBn) nuclei, with a third ventrolateral extension called the Kölliker-Fuse nucleus. The PBn can be further divided into 10 distinct subnuclei based on cytoarchitecture (Figure 1.1). Immediately ventromedial to the SCP is the MPBn subnucleus containing a heterogenous cell population. In contrast, the external MPBn subnucleus contains larger multipolar

neurons and is interposed between the MPBn and Kölliker-Fuse nucleus. The LPBn is made up of several homogeneous groups of cells, including the superior lateral, internal lateral, central lateral, ventral lateral, dorsal lateral, external lateral, and extreme lateral nuclei, which are delineated by morphology and spatial distribution (Fulwiler & Saper, 1984). Individual subdivisions can also be differentiated based on connectivity. The primary projections of the PBn include several hypothalamic regions (the medial preoptic hypothalamus (MPO), ventromedial hypothalamus (VMH), lateral hypothalamus, and paraventricular hypothalamus (PVH)), the nucleus of the solitary tract (NTS), several thalamic regions including the intralaminar nuclei and paraventricular nucleus of thalamus (PVT), and the extended amygdaloid complex including the BNST and CeA (Fulwiler & Saper, 1984). Within the amygdaloid complex, the central medial amygdala (CeM) receives projections mainly from the MPBn and ventral lateral subnucleus. The central lateral amygdala (CeL) and the BNST receives projections from the central LPBn and the outer portion of the external LPBn subnucleus. The central laterocapsular amygdala (CeLC) receives projections primarily from the external and dorsal LPBn (Bernard et al., 1993). Reconstruction of axonal branching patterns have found that, while the LPBn has projections that travel exclusively to either the BNST or CeA, the LPBn projections in passage to the CeA can also send collaterals to the BNST (Sarhan et al., 2005). After leaving the LPBn, the efferent fibers can travel through the dorsal and central tegmental tracts, and then join the medial forebrain bundle and ansa lenticularis. Here, the fibers can branch and course via dorsal or ventral pathways. In the ventral pathway, the fibers immediately turn laterally to reach the CeLC and CeL. In the dorsal

pathway, the fibers can continue to travel rostrally sending collaterals to the lateral BNST and traveling back around through the stria terminalis to reach the CeL and CeLC (Figure 1.2).

*Spino-parabrachio-amygdaloid tract*

The LPBn is a key site of convergence for nociceptive input. It is one of the largest targets of nociceptive dorsal horn projection neurons, relayed primarily through the dorsal lateral funiculus, and receives nociceptive information broadly from the body. Besides the substantial input from lamina I, the PBn also receives input from the trigeminal nucleus, carrying nociceptive information from the face, and from the NTS relaying visceral nociceptive inputs. Thus, the LPBn may integrate both peripheral and visceral nociceptive signals.

To examine the role of the parabrachio-amygdaloid projections in nociception, PBn neurons from anesthetized rats were examined using extracellular electrophysiological recordings (Bernard et al., 1996). Antidromic stimulation of the CeA was used to identify PBn-CeA projecting neurons and to examine the responsiveness of these neurons to mechanical thermal or visceral stimuli. Approximately 70% of PBn-CeA neurons were exclusively excited by noxious stimuli, whereas innocuous somatic or gustatory stimuli did not alter firing of these neurons. These neurons tended to have large excitatory receptive fields, often covering several areas of the body, suggesting that these neurons are likely not encoding specific spatial information to allow for sensory discrimination. A subpopulation (30%) of PBn-CeA

neurons had a smaller receptive field and were only excited when noxious stimuli were applied to a specific part of the body. Subthreshold or non-noxious stimuli were found to inhibit responses in the nociceptive-responsive PBn-CeA neurons. Noxious thermal stimuli tended to induce a stronger excitatory response than noxious mechanical stimuli. Morphine was found to have multiple effects blocking the excitatory response to thermal stimuli in PBn-CeA neurons and reducing c-fos expression in a subset of lamina I spino-parabrachial neurons following noxious stimulation (Huang et al., 1993; Jasmin, Wang, Tarczy-Hornoch et al., 1994).

*Central pain processing*

While this review primarily focuses on the role of the amygdala in pain processes, the experience of pain is multifactorial and utilizes a large distributed brain network commonly referred to as the pain matrix (Tracey & Mantyh, 2007). It can be divided into two main systems, one lateral sensory-discriminatory and the other medial affective-cognitive. The majority of the research on these systems has been based on neuroimaging studies to determine which brain regions are more or less active depending on the interplay of particular conditions and factors, including the type of injury, mood, and cognitive components. As such, the pain matrix is not precisely defined, nor is it always consistent as to which regions are included or excluded. Rather, the pain matrix may be more a pain signature, reflective of individual and subjective experiential differences (Tracey & Mantyh, 2007). During acute pain, the most common regions involved are the thalamus, primary and secondary somatosensory cortices, insular cortex,

anterior cingulate cortex, and prefrontal cortex (Apkarian et al., 2005). Within the pain matrix, the regions responsible for emotional components of pain, including those related to anxiety and depression, may act to further amplify the pain experience. Commonly associated regions in the processing of the emotional aspects of pain regions include the anterior cingulate cortex, insular cortex, hippocampus, and amygdala (Yalcin et al., 2014). Thus, in the context of the larger pain matrix, the amygdala might serve to impart an emotional context to pain.

## 1.3. Amygdala in Pain Processes

*Anatomy*

The amygdala refers to a group of nuclei deep within the temporal lobe, vital in the processing of emotion-related responses. The amygdala can be divided on the basis anatomy and function into several major divisions; the basolateral nuclei, cortical-like nuclei, and centromedial nuclei. The basolateral nuclei (BLA) consist of the lateral nucleus (LA) and the basal nucleus (BA). The BLA is bordered laterally by the external capsule and medially by the central amygdala (CeA). The cortical-like nuclei are the most superficial group consisting of the nucleus of the lateral olfactory tract, bed nucleus of the accessory olfactory tract, periamygdaloid cortex, anterior cortical nucleus, and posterior cortical nucleus. The centromedial group consists of the CeA and medial nucleus (MeA). The CeA can be further divided into central medial (CeM), central lateral (CeL), and central lateral capsular (CeLC) (Sah et al., 2003). Further, it has been argued on the basis of structural and functional homology that the centromedial group

should be extended rostrally and medially to include the bed nucleus of the stria terminalis (BNST) and the caudodorsal regions of the substantia innominata to form what is referred to as the extended amygdala complex (Alheid, 2003). This distinction is on the basis of similarities between efferent and afferent connections and histochemical architecture in these regions. Another amygdala group, the intercalated neurons, does not form a distinct nucleus but occurs as numerous dense clusters found in the external capsule on the lateral border of the BLA, and two clusters in the intermediate capsule between the BLA and CeA.

*Intrinsic and extrinsic connectivity*

The amygdala has fairly extensive intrinsic connections. In general, information in the amygdala tends to flow in a lateral to medial direction, with sensory and multimodal input from cortical association areas arriving in the LA. The main output is from the CeM, where the amygdala is strongly connected to autonomic and modulatory centers of the hypothalamus and brainstem, including the paraventricular hypothalamus (PVH), lateral hypothalamus (LH), the periaqueductal grey (PAG), PBn, NTS and dorsal vagal complex. Between the LA and CeM are few to no direct connections, instead projections to the CeL, CeLC and intercalated cells are thought to function to gate sensory input to modulate fear behavior under particular environmental conditions (Duvarci & Paré, 2014). The BLA is primarily composed (~80%) of large glutamatergic projection neurons with the remaining neurons belonging to a diverse set of GABAergic interneurons that form local circuits. These glutamatergic projections neurons synapse

primarily onto CeL, CeLC and intercalated neurons. The CeA is composed primarily of GABAergic neurons, with the CeM neurons having large soma and sparsely branching dendrites, and the CeL and CeLC neurons having smaller soma and dendritic trees that branch profusely (Ehrlich et al., 2009). Neurons within the CeL and CeLC project to the CeM, with few to no reciprocal projections (Petrovich & Swanson, 1997). Within the CeL and CeLC are microcircuits with GABAergic interneurons synapssing on a second GABAergic interneuron. In turn, these interneurons then project to the CeM and can result in the activation of the CeM through disinhibition. These two populations of interneurons have been defined genetically by the presence of protein kinase Cδ. During a fear-evoking stimulus, one CeL/CeLC interneuron population is selectively activated (CeL-On), whereas a second population is inhibited (CeL-Off). The CeL-Off population projects to the CeM, and the inhibition of CeL-Off neuron during fear results in disinhibition of the CeM and evokes fear-related behaviors (Ciocchi et al., 2010; Haubensak et al., 2010). In aggregate, this suggests that the CeL and CeLC form a complex inhibitory gate on the CeM, allowing for multiple points of modulation. One input to the CeLC and CeL is through the previously described projection from the LPBn to CeL and CeLC. These nociceptive inputs bypass the BLA completely. Additionally, while the CeM is the source of the largest output from the amygdala, projecting to multiple regions in the brainstem and hypothalamus, the CeL and CeLC also modulate behavior through a set of direct projections to the BNST (Dong et al., 2001).

*Amygdalar neuronal circuits for fear and anxiety*

Fear and and anxiety serve a protective function driving behavioral responses aimed at avoiding potential harm. The amygdala has been long held to be a key mediator of emotional behaviors. The pioneering studies in this area were of macaques with a temporal lobe lesion wherein the amygdala was ablated. Following the lesions, there were behavioral alterations including amnesia, inability to recognize familiar objects, docility and a striking lack of emotional responses. This was most apparent by the complete absence of fear responses in these macaques (Klüver & Bucy, 1937). Since then, the role of the amygdala in fear has become a focus of a substantial body of research. Due to relative simplicity and robustness of response, fear conditioning paradigms remain on the forefront of our ability to understand the brain at the level of neural circuits. The fundamental framework of the amygdalar fear conditioning network posits that information about both the unconditioned stimulus (US) and conditioned stimulus (CS) converge onto neurons in the LA. The LA then projects to the CeA, the main output, which then has projections to various brainstem regions that generate fear responses (Ledoux, 2000). In this model, the LA receives information related to the CS, such as context cues from the hippocampus or tone information from the auditory cortex. Concurrently, the LA is also receiving information related to the US, such as the aversive information from electrical shock via the thalamus. Hence, in this model there is a convergence of the US and CS in the LA, and synaptic plasticity within this region is thought to be critical for the formation of the association between the US and CS, or fear acquisition. The fundamentals of this model are still valid; however, numerous updates

and clarifications have been made surrounding the basic circuitry. Between LA neurons, which receive sensory input, and the CeA output neurons in the CeM, there are little to no direct connections. Rather, the LA projects to three intermediaries including the CeLC and CeL, ITC cells and the BLA, where each one in turn projects to the CeM (Duvarci & Paré, 2014; Tovote et al., 2015) (Figure 1.3). Additionally, while there is significant synaptic plasticity occurring in the LA following fear conditioning, synaptic plasticity in the CeA also appears important in the acquisition of fear (Paré et al., 2004). The infralimbic (IL) and prelimbic (PL) regions of the medial prefrontal cortex appear critical in the suppression of fear responses via projections of the IL to the ITC cells, and the PL to the BLA. These circuits appear particularily important in learned suppression of fear response, as occurs during fear extinction (Tovote et. al. 2015). Of particular relevance to the current work, is the recognition of direct nociceptive projections from the LPBn to the CeLC and CeL, which completely bypass the BLA and thalamus (Veinante et al., 2013). Further, it has emerged that the amygdala may play a central role in the response to short, phasic fear-evoking stimuli. However, in response to sustained sustained fear-evoking stimuli, more akin to anxiety, it is thought that the BNST becomes the primary mediator (Davis et al., 2010; Walker et al., 2009). The current work involving the amygdala in fear has begun to utilize novel genetic manipulations to dissect subpopulations of neurons that may represent circuits with specific functions. One of the ideas to emerge from this work is that within the BLA specific circuits might encode either a positive or negative valence, that either heightens or dampens the overall fear response (Namburi et al., 2015; Redondo et al., 2014).

*Nociceptive input*

Due to its integration of a wide range of emotionally salient sensory stimuli, extensive nociceptive input, and its prominent role in the production of emotional behaviors, the amygdala is thought to be a key region in the processing and production of the emotional components of pain. As a whole, the amygdala is thought to attach an emotional valence to sensory stimuli and initiate behavioral and affective responses. In the context of the pain matrix, the amygdala would likely function to attach a negative emotional valence to nociceptive stimuli, resulting in compensatory behavioral changes. Within the amygdala, the CeA is situated at the interface of two nociceptive pathways (Figure 1.3). The first pathway carries nociceptive information originating from the cerebral cortex and thalamus and relayed by the BLA. The BLA receives nociceptive information from the ventroposterior, posterior, triangular, and posterior intralaminar thalamic nuclei, the secondary somatosensory area, and the insular cortex (Sah et al., 2003; Shi & Davis, 1999). The BLA then projects to the CeLC and CeL or to the intercalated cell masses, which, in turn, project to the CeM. This nociceptive information has gone through the thalamus and cerebral cortex, where it can be integrated with other sensory, affective, and cognitive influences to become a highly polymodal and processed form of information. While the BLA receives a majority of its input from the cortex and thalamus, there is, however, a small projection from these cortical and thalamic areas that directly innervates the CeA (Shi & Davis, 1999). The second main nociceptive input is the spinoparabrachio-amygdaloid pathway that sends direct and less processed nociceptive information to the CeLC and CeL. Additionally, there is also a sparse

projection directly from lamina I in the dorsal horn to the CeA. Nociceptive information both directly through the PBn-CeA pathway and indirectly through the BLA-Ce A pathway converges in the CeLC and CeL (Neugebauer et al., 2004).

There is substantial evidence that neurons in CeA respond to nociceptive information. Using *in vivo* electrophysiology, CeA neurons of anesthetized rats were examined for their responsiveness to noxious stimuli, defined as stimuli that would be painful in an awake subject. The majority of responsive neurons were located in the CeLC with approximately 80% of neurons displaying responses exclusively or predominantly to noxious stimulation of superficial or deep body tissue (Bernard et al., 1992; Neugebauer & Li, 2002). Of the approximately 80% of nociceptive responsive neurons, 46% were excited by noxious stimulation and the remaining 34% of CeA neurons were inhibited in the presence of noxious stimulation (Bernard et al., 1992). The excited neurons tended to be located in the CeLC, and inhibited neurons tended to be located in the CeL and CeM. In light of recent understanding of CeA microcircuitry, in which a population of CeLC and CeL neurons have inhibitory projections onto neurons in the CeL and CeM, increased excitatory drive on these GABAergic CeLC neurons could be directly inhibiting CeM and CeL neurons and explain the heterogeneity of response. Three main types of neurons were described based on their responses to nociceptive stimulation. Nociceptive-specific neurons are activated exclusively by noxious stimulation. Multireceptive neurons respond to both nociceptive and innocuous stimuli, and nonresponsive neurons do not respond to noxious stimulation at all. The majority of responsive neurons have large, often bilateral, receptive fields that include large portions

of the body. In response to differing intensities of mechanical and nociceptive stimuli, responsive neurons display a sigmoidal response curve rather than increasing monotonically, suggesting poor resolution of intensity. Similar to the PBn, this suggests that CeA neurons are unlikely to encode a sensory-discriminative component of pain (Bernard et al., 1992; Neugebauer & Li, 2002; Neugebauer et al., 2004).

Human brain imaging supports involvement of the amygdala in pain. In experimental settings, application of an infrared laser thermal stimulus or colorectal distention leads to increased amygdala activity (Bonaz et al., 2002; Bornhovd, 2002). Although a few studies have reported either reduced activation or no change in the amygdala with noxious stimuli, a meta-analysis found that the majority of experiments applying noxious stimulation supported increased amygdala activation (Simons et al., 2012).

*Pain-related plasticity*

In states of persistent pain, the amygdala undergoes considerable synaptic, neurochemical, and transcriptional plasticity. Increases in the immediate early gene c-fos have been repeatedly found across different models of pain. Amygdala c-fos mRNA was increased one hour following intra-plantar injection with formalin or following intraperitoneal injection with acetic acid (Nakagawa et al., 2003). c-fos immunoreactivity increased in the amygdala with esophageal acid exposure, and increased c-fos immunoreactivity was found in the CeLC 4 hours following cyclophosphamide-induced cystitis (Bon et al., 1998; Suwanprathes et al., 2003).

Glutamatergic signaling is increased in the CeA, as demonstrated in models of arthritic pain or neuropathic pain where increased expression of the metabotropic glutamate receptors mGluR1 and mGluR5 were found, as well as increased phosphorylation of the NR1 subunit of the NMDA receptor (Bird et al., 2005; Neugebauer et al., 2003). A model of neuropathic pain was associated with increases in glucocorticoid receptor mRNA expression, and higher levels of corticotropin-releasing hormone (CRH) mRNA and peptide immunoreactivity in the CeA, suggesting involvement of several of these key mediators of the stress response system (Rouwette et al., 2012; Ulrich-Lai et al., 2006). Interestingly, one study raised the possibility that neurogenesis may be a component of pain-related plasticity in the amygdala. Two months following induction of neuropathic pain, bromodeoxyuridine (BrdU) incorporation was found in both BLA and CeA cells. While BrdU+ cells were found in astrocytes in both control and neuropathic pain conditions, the increase in BrdU+ cells under pain conditions also included cells that colocalized with neuronal markers, evidence suggesting that either enhanced neurogenesis or increased neuronal migration contributes to pain-related plasticity in the amygdala (Goncalves et al., 2008).

Electrophysiological recordings from rodent brain slices from models of persistent pain have demonstrated synaptic alterations. One of the most prominent changes is an enhancement of evoked PBn-CeA and BLA-CeA transmission. Using patch-clamp recordings of CeLC neurons, the regions of PBn or BLA afferents were electrically stimulated to characterize PBn-CeA or BLA-CeA transmission. An increase in PBn-CeA transmission was identified following neuropathic pain, arthritic pain, acid-

induced muscle pain, and visceral pain (Cheng et al., 2011; Han & Neugebauer, 2004; Ikeda et al., 2007; Neugebauer et al., 2003). The potentiation of these synapses heightened nociceptive input via the PBn and emotional-salience input via the BLA, leading to the amygdala being more reactive with chronic pain. This hypothesis has also been supported by human brain imaging studies, where changes in amygdala activity were identified in people suffering from arthritis, neuropathy, or irritable bowel syndrome (Bonaz et al., 2002; Kulkarni et al., 2007; Petrovic et al., 1999).

### 1.4. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

*PACAP and its receptors*

PACAP is a neuropeptide with diverse roles in neurotransmission, development, trophic support, and homeostatic function. This important neuropeptide was first identified in 1989 by Akira Arimura and colleagues, who were searching for undiscovered hypothalamic peptides capable of stimulating adenylate cyclase activity and cyclic AMP production in anterior pituitary cells (Miyata et al., 1989). In humans, the PACAP, *ADCYAP1,* gene is at chromosomal locus 18p11 and is comprised of five exons. The cDNA encodes for a 176-amino-acid prepro-protein, which is then endoproteolytically cleaved by prohormone convertases into either a 38-amino-acid or a 27-amino-acid form. Subsequently, peptidylglycine α-amidating monooxygenase converts the protein into a bioactive peptide. Within the central nervous system, PACAP38 peptide is approximately 10- to 100-fold more abundant than PACAP27 (Vaudry et al., 2009). The highest expression of PACAP transcript within the central

nervous system is within several hypothalamic nuclei, habenular nuclei, the pontine nucleus, the LPBn and the vagal complex (Hannibal, 2002).

PACAP is the most conserved member of the VIP/secretin/glucagon superfamily of peptides across animal species. Its amino acid sequence is well conserved across the mammals that have been studied. Among chicken and frogs, PACAP differs by only a single amino acid, and PACAP cDNA cloned from tunicates has 96% nucleotide identity with human cDNA. The closest related peptide, vasoactive intestinal peptide (VIP) shares 68% amino acid homology. It is thought that PACAP is the ancestral precursor to the VIP/secretin/glucagon family. The highly conserved nature of PACAP suggests that it might have functions essential for survival (Sherwood et al., 2000).

PACAP signals through three G-protein coupled receptor subtypes; PAC1, VPAC1 and VPAC2 receptors. Both the VPAC1 and VPAC2 receptors bind to PACAP and VIP with near equal affinity; however the PAC1 receptor has a much higher affinity for PACAP than VIP. The alternative splicing of the PAC1 receptor results in multiple variants to allow greater signaling diversity. Alterations in the N-terminal extracellular regions result in short receptor isoforms that can affect ligand-binding specificity. Alternative splicing also results in the presence or absence of two 84 base pair cassettes termed “hip” and “hop” within the PAC1 receptor corresponding to the third cytoplasmic loop resulting in the generation of at least 4 variants; PAC1-null (neither hip nor hop), PAC1-hip, PAC1-hop, and PAC1-hiphop. PAC1 receptor signaling can activate adenylate cyclase (AC) through Gs activation and phospholipase C (PLC) through Gq activation; variants in the third cytoplasmic loop of PAC1 receptors can result in the

differential engagement of AC and PLC signaling (Blechman & Levkowitz, 2013).

*PACAP expression in nociceptive pathways*

A variety of bioactive neuropeptides participate in the formation, transmission, modulation, and perception of pain. Substance P and neurokinin A of the tachykinin family of peptides and CGRP, for example, have expression patterns along nociceptive pathways and the ability to initiate and modulate nociceptive transmission (Basbaum et al., 2009). Although appreciated as a sensory peptide within a few years after discovery, the recent accumulation of evidence has generated renewed interest in PACAP as a nociceptive peptide critical in mediating the development of chronic pain and pain-related behavioral responses.

The initial evidence that PACAP plays a role in nociception stemmed from its distribution and expression patterns within the peripheral nervous system. Complementing other sensory peptides, PACAP expression has been identified in both DRG and trigeminal ganglion neurons through immunocytochemical and *in situ* hybridization histochemical studies (Moller et al., 1993; Mulder et al., 1994). Under normal physiological conditions, PACAP immunoreactivity in DRG neuronal soma and peripheral axons has been identified in small to medium-sized unmyelinated capsaicin-sensitive C-fiber nociceptor afferents, along with other sensory peptides, including CGRP and substance P. In addition, PACAP expression within a defined subset of peptidergic DRG neurons has been confirmed using single-cell RNA sequencing (Usoskin et al., 2015). In the spinal cord, the central axons of PACAPergic DRG neurons are largely

confined to lamina I and II of the dorsal horn, corresponding to projections important for nociceptive transmission (Jongsma et al., 2000; Moller et al., 1993; Zhang et al., 1996). In addition to DRG, there is also a population of PACAP-expressing neurons in lamina I and II of the spinal cord dorsal horn, raising the possibility that PACAP may be expressed in second order neurons in the nociceptive pathway (Beaudet et al., 1998; Pettersson et al., 2004). Based on *in vitro* receptor autoradiography and *in situ* hybridization, PAC1 receptors have been shown to be densely expressed in laminae I and II of the dorsal horn in correspondence with PACAP DRG central axon projections. While the distribution of PACAP fibers, PACAP neurons and PAC1 receptors in the superficial layers of the dorsal horn is suggestive, the potential functional 'PACAP to PACAP' connectivity between DRG and second order dorsal horn PACAPergic neurons is still unclear. Based on ultrastructural studies, PACAP signaling on PACAP expressing neurons has been suggested in the enteric nervous systems (Nagahama et al., 1998). Only a few isolated neurons in the ventral horn appear to express PAC1 receptors (Pettersson et al., 2004). PAC1 receptors are not apparent in DRG neurons implying that PACAP does not act in an autocrine or paracrine manner in the ganglion or presynaptically in the dorsal horn (Jongsma et al., 2000).

*Plasticity following injury*

Among several sensory peptides, PACAP demonstrates phenotypic plasticity in various peripheral models of injury- and inflammation-induced pain. Across different experimental paradigms, including axotomy, nerve compression and adjuvant treatments,

DRG PACAP transcripts, peptide levels and cell numbers can be dramatically induced (Jongsma et al., 2000; Jongsma Wallin et al., 2003; Mabuchi et al., 2004; Pettersson, et. al., 2004; Zhang et al., 1995; Zhang et al., 1998; Zhang et al., 1996). Notably, depending on the nature of insult, there appears to be an induction of PACAP within select DRG neuronal populations with concurrent changes in central and peripheral axon peptide immunoreactivity. PACAP is normally identified in a subpopulation of small and medium-sized nociceptive cells and following inflammatory insult, the induction of PACAP appears to be confined to the same small-sized neuronal population (Jongsma Wallin et al., 2003). Accordingly, inflammatory cyclophosphamide-induced cystitis augments DRG PACAP neuronal numbers and immunoreactive fiber density in the superficial layers of the dorsal horn, consistent with projections from DRG small neuron induction of PACAP (Vizzard, 2000). By contrast, axotomy shifts PACAP expression in different DRG populations, resulting in decreased peptide expression in small DRG neurons and increased peptide expression in the medium and large-sized DRG neurons (Jongsma et al., 2000; Zhang et al., 1996). Large DRG neurons project to deeper layers of the dorsal horn and in coherence with axotomy-mediated induction patterns, PACAP-immunoreactivity in fibers appear reduced in the superficial layers of the dorsal horn but enhanced in the deeper laminae. However, whether or not the decrease in PACAP fiber immunoreactivity in the superficial dorsal horn laminae reflects heightened C-fiber PACAP secretion has not been determined. Nerve compression increases PACAP levels in both small and large neuronal populations (Pettersson et al., 2004). The mechanisms underlying the various PACAP induction patterns to different injuries and the

consequences of the dynamics in fiber projections in pain remain unclear but are supported in recent transgenic animal studies (see below). Whether the second order PACAP neurons in laminae I and II of the dorsal horn also demonstrate plasticity under the different injury models is unknown, although no overt changes were observed following axotomy (Pettersson et al., 2004). In contrast to DRG PACAP inductions, PACAP binding in the dorsal horn after injury was diminished without apparent changes in PAC1 receptor transcript levels (Jongsma et al., 2000). Although the expression patterns for PACAP and PAC1 receptors exhibit an inverse relationship in some studies, the loss of PACAP binding may reflect higher PAC1 receptor internalization and turnover following heightened signaling (May et al., 2014; Merriam et al., 2013). Likewise, VPAC1 receptor expression is decreased but VPAC2 receptors are increased following neuropathic pain (Dickinson & Fleetwood-Walker, 1999). While the changes in PACAP expression in the multiple experimental models may be related to enhanced nociceptive neurotransmission, the interpretations are complicated by cellular stress-induced plasticity responses to the various injury challenges. PACAP/PAC1 receptor activation can engage neurotrophic pro-survival signals to promote regeneration (Vaudry et al., 2009); hence induction in DRG PACAP expression in the neuropathic and inflammatory pain paradigms may have distinct, dual or overlapping activities in nociception and trophic support.

*PACAP and PAC1 receptors in nociceptive signaling*

Based on PACAP and PAC1 receptor expression, distribution and plasticity observed in experimental injury models, the PACAPergic system was implicated in the facilitation of nociceptive responses. While seemingly straightforward, the results of PACAP infusion studies were equivocal as to whether PACAP was pro- or anti-nociceptive. At peripheral nerve terminals, the actions of PACAP appeared largely anti-nociceptive. While intraplantar PACAP injections alone had no effect on thermal or mechanical sensitivity in naïve animals, intraplantar PACAP injections proved anti-allodynic, anti-nociceptive and anti-hyperalgesic in experimental models of somatic and visceral inflammatory pain (Sándor et al., 2009). However, PACAP at knee joint afferents resulted in increased mechanical sensitivity (Sándor et al., 2009). Intrathecal PACAP injections was reported to inhibit spinal and inflammatory nociceptive responses (Yamamoto & Tatsuno, 1995; Zhang et al., 1996; Zhang et al., 1993), whereas PACAP administration was reported by others to be anti-nociceptive in the early phase of formalin induced pain, but transitioned to pro-nociception in the late phase of the inflammatory response (Shimizu et al., 2004).

The pro-nociceptive actions of PACAP, however, are compelling. Intrathecal PACAP infusions to naïve rats produced hyperalgesia in thermal hypersensitivity and tail flick latency tests, and amplified pain neurotransmission to the dorsal horn via NMDA mechanisms (Narita et al., 1996; Ohsawa et al., 2002). The intrathecal nociceptive effects of PACAP were gradual but long lasting, which were in contradistinction to the rapid and transient effects of substance P (Shimizu et al., 2004). Demonstrating a direct

effect of PACAP signaling, PACAP application to spinal cord neurons increased excitability of multireceptive cells in lamina III-V of the dorsal horn (Dickinson et al., 1997). Importantly, in comparable studies, blockade of PACAP signaling with the PAC1/VPAC2 receptor antagonist PACAP(6-38) or neutralizing PACAP antibodies attenuated the thermal hypersensitivity and nocifensive responses in a variety of neuropathic and inflammatory pain models (Davis-Taber et al., 2008; Ohsawa et al., 2002). Further, while PACAP(6-38) had no effects alone or upon non-noxious stimulation, the receptor antagonist blocked the increased excitation of dorsal horn neurons to noxious stimuli (Dickinson & Fleetwood-Walker, 1999). The effects of C-fiber stimulation on spinal nociceptive reflex responses were facilitated by PACAP administration and inhibited with a specific PAC1 receptor antagonist (Sakashita et al., 2001; Xu & Wiesenfeld-Hallin, 1996). The causes for the observed discrepancies in the PACAP nociceptive effects in the various experimental models are not well understood but may be related to dose and temporal parameters, and route or site of PACAP administration, especially after pain initiation. Under specific circumstances, PACAP may have activated autoregulatory or descending inhibitory pathways or stimulated anti-inflammatory responses by blocking immune cell cytokine release into the peripheral milieu of pain mediators to produce anti-nociceptive effects. Based on PACAP and PAC1 receptor expression and distribution in the sensory pathways, and the preponderance of electrophysiological and behavioral data, however, the central effects of PACAP in injury appear to result in system sensitization and are pro-nociceptive.

*Nociception studies in PACAP/PAC1 receptor knockout mice*

The most convincing evidence for PACAP involvement in pain stems from studies using transgenic PACAP (PACAP -/-) and PAC1 receptor (PAC1R -/-) knockout mice, which have been coherent in demonstrating the facilitatory roles of PACAP signaling in chronic pain (Table 1.1). PACAP-/- mice display a range of physiological and neuropsychiatric phenotypes, including decreased locomotor activities, decreased feeding behaviors, altered memory performance, and attenuated stress responses, reflecting the multifaceted roles of PACAP (Girard et al., 2006; Hitoshi Hashimoto et al., 2001; Hattori et al., 2012). In several experimental models, PACAP-/- mice exhibited important deficits in neuropathic pain development. Under control conditions, naïve PACAP-/- mice showed unaltered or slightly decreased sensitivity responses to thermal or mechanical stimuli (Mabuchi et al., 2004; May & Vizzard, 2010; Sándor et al., 2010). However, following chronic pain with intraplantar noxious stimulus, PACAP-/- mice displayed a marked loss in the induction of mechanical or thermal hypersensitivity, and nocifensive behaviors (Mabuchi et al., 2004; Sándor et al., 2010). Similarly, PACAP-/- mice failed to develop thermal or mechanical hypersensitivity in response to spinal nerve transection or sciatic nerve ligation, and demonstrated substantially attenuated writhing responses in response to intraperitoneal acetic acid injection (Botz et al., 2013; Mabuchi et al., 2004; Sándor et al., 2010). The diminished nociceptive responses in the PACAP-/- mice to either formalin or acetic acid treatments were accompanied by decreased c-fos expression in the somatosensory cortex and periaqueductal grey (PAG), indicating a tangible decrease in nociceptive transmission rather than an absence of behavioral

responses (Sándor et al., 2010). Interestingly, following intraplantar TRPV1 agonist resiniferatoxin injection into PACAP-/- mice, a reduction in mechanical sensitivity but an immediate enhancement of thermal nociception was observed, which suggests differential roles for PACAP in central versus peripheral nociceptive signaling.

PACAP activation of multiple different receptor subtypes and PAC1 receptor-mediated intracellular signaling appear central to nociceptive mechanisms. This was supported by studies where PACAP nociceptive responses were recapitulated with the PAC1 receptor selective agonist maxadilan and blocked by the specific receptor antagonist max.d.4 (Sakashita et al., 2001). Accordingly, as in PACAP -/- animals, mice with PAC1 receptor deficiency (PAC1R -/-) under naïve conditions also exhibited normal responses to acute thermal or mechanical stimuli, but demonstrated reduced nocifensive responses to intraplantar formalin administration and decreased abdominal responses to intraperitoneal acetic acid injection (Jongsma et al., 2001; Martin et al., 2003). The knockout studies conducted to date have not addressed the different potential sites of PACAP/PAC1 receptor action mediating the nociceptive responses; however, $PAC1^{CamKCre2}$ mice with forebrain-specific deletions of the PAC1 receptor (PAC1 receptor deletions in the forebrain cortical areas, hippocampus and olfactory bulb) did not demonstrate diminished chemical and visceral pain responses (Martin et al., 2003). Thus, the nociceptive actions of PACAP likely reside within the peripheral pathways, spinal cord and brainstem, or possibly in combinations these regions. The PACAP knockout studies do not exclude possible roles for VIP, or PACAP on VPAC1/VPAC2 receptor signaling in pain responses, as VIP is an important mediator of inflammatory processes,

and VIP administration is often potently anti-inflammatory (Delgado, Pozo, & Ganea, 2004). Nevertheless, these studies in aggregate implicated PACAP and PAC1 receptor involvement in the development of nociceptive hypersensitivity across several models of chronic pain.

*PACAP and PAC1 receptors in emotional behaviors*

In the peripheral and central nervous systems, PACAP and the PAC1 receptor are expressed in structures that orchestrate a diverse set of responses following stressor exposure. In autonomic pathways, PACAP appears to be one of the principal regulators of sympathetic function (Braas et al., 2007; May et al., 1998). In the brain, some of the highest levels of PACAP expression have been identified in hypothalamic and related limbic structures, and PACAP has been shown to regulate classical stress mediators (Piggins et al., 1996). PACAP stimulates hypothalamic CRH transcription, c-fos expression, and CREB phosphorylation, and can augment plasma corticosterone levels (Agarwal et al., 2005; Tsukiyama et al., 2011). Although previous work has shown that a variety of acute stress paradigms do not alter hypothalamic PACAP transcript levels, more recent studies have shown that chronic stress can increase PACAP and the PAC1 receptor transcript levels in the paraventricular nucleus (PVN) of the hypothalamus and the BNST (Hammack et al., 2009; Hannibal et al., 1995). Further, PACAP infusions into the BNST can mimic chronic stress-related responses by increasing startle and anxiety-like behavior on the elevated plus maze, decreasing weight gain and feeding (anorexia), and elevating circulating corticosterone levels (Hammack et al., 2009; Roman et al.,

2014; Kocho-Schellenberg et al., 2014; Lezak et al., 2014). A role for PACAP signaling in mediating these chronic stress responses was supported by the demonstration that PACAP receptor antagonists can attenuate all of these responses. To complement these observations, PACAP and PAC1 knockout mice exhibit decreased anxiety- like behaviors, have attenuated corticosterone responses, and show impairments in hypothalamus CRH regulation in response to stress (Girard et al., 2006; Hashimoto, 2006; Hattori et al., 2012). Evidence has also been found linking PACAP to disease. In humans, altered blood PACAP levels and PAC1 receptor polymorphism was associated with PTSD and other stress-related disorders (Ressler et al., 2011). In sum, these observations implicate PACAP/PAC1 receptor signaling in anxiety-related behaviors.

*Signaling through extracellular signaling regulated kinase (ERK) activation*

A signature of nociceptive signaling is extracellular signaling-regulated kinase (ERK) activation, which participates in the neuroplasticity that promotes the manifestation of chronic pain and stress-related disorders (Ji et al., 2009). Both inflammation and axotomy injury have been shown to increase pERK+ neurons in the DRG; following inflammatory or neuropathic pain, increased pERK levels are found in lamina I and II neurons of the spinal cord, and the ensuing development of hypersensitivity can be abrogated upon blockade of ERK phosphorylation by intrathecal application of a mitogen-activated protein kinase kinase (MEK) inhibitor (Ji et al., 1999; Obata et al., 2003). ERK signaling has been shown to contribute to pain-related

enhancement of PBn-CeLC synaptic neurotransmission and inhibition of CeA ERK activation attenuates pain-related behavioral hypersensitivity (Carrasquillo & Gereau, 2007; Cheng et al., 2011; Ji et al., 2009). PACAP and PAC1 receptor signaling can stimulate and sustain ERK activation potently and efficaciously (May et al., 2014; May et al., 2010). There are multiple intracellular PAC1 receptor effector mechanisms that activate ERK, including PKA and PKC (Barrie et al., 1997; Bouschet et al., 2003; May et al., 2014), but, more recently, it has been suggested that PAC1 receptor internalization and endosomal signaling provide a means to sustain cellular ERK levels (May et al., 2014; Merriam et al., 2013).

*PACAP and glutamate signaling*

In addition to stimulation of ERK-mediated neuroplasticity, PACAP signaling may also regulate postsynaptic neuronal function by modulating glutamatergic neurotransmission. PACAP is coexpressed with glutamate in a variety of systems, including retinal ganglion cells and the suprachiasmatic nucleus (Engelund et al., 2010; Hannibal et al., 2000). Furthermore, in the developing dorsal horn of the spinal cord, the same transcription factors that determine glutamatergic cell fate also appear to control PACAP expression (Guo et al., 2012). The co-release of PACAP with glutamate may function to modulate excitatory neurotransmission, since NMDA receptor blockade in the ventromedial hypothalamus (VMH) leads to diminished PACAP-induced hypophagia (Resch et al., 2014). The attenuation of fear conditioning by intra-BLA PACAP(6-38) administration was mediated through altered NMDA signaling (Schmidt et al., 2015).

Intrathecal PACAP-mediated pain resulted in a dose-dependent enhancement of NMDA-induced aversive behaviors and potentiated NMDA currents in dorsal horn neurons (Ohsawa et al., 2002). In addition, transgenic PACAP-/- mice failed to develop mechanical allodynia to NMDA, but allodynia could be restored by co-infusion of PACAP with NMDA (Mabuchi et al., 2004). There are multiple mechanisms by which PACAP could potentially modulate glutamatergic signaling. In the dorsal horn, there is evidence that PACAP may promote the functional coupling of nitric oxide synthase to NMDA receptors (Mabuchi et al., 2004). In the hippocampus, PACAP has been found to enhance synaptic NMDA trafficking and surface expression through Gq, PKC and Src signaling mechanisms (Chowdhury et al., 2013; Macdonald et al., 2005; Trepanier et al., 2012). In the amygdala, PACAP resulted in potentiation of BLA-CeA transmission through a postsynaptic mechanism involving synaptic targeting of GluR1 subunit-containing AMPA receptors. Alternatively, PACAP may enhance glutamate signaling by regulating mGluR function (Kammermeier, 2008).

### 1.5. Summary

Pain is a multidimensional experience comprised of both sensory-discriminative and emotional homeostatic components. Despite the high comorbidity between chronic pain and stress-related behavioral disorders, the neurocircuits, neurochemical mediators, and mechanisms underlying these responses are not well understood. The current work tests the hypothesis that PACAP expression, plasticity and signaling in nociceptive

pathways intersect with those in the amygdala and related limbic systems to drive the maladaptive behavioral responses.

The detection of noxious stimuli is performed by sensory nerve afferents that transduce noxious stimuli into electrical currents which are then relayed centrally. The second order projection neurons then relay the information via sets of spinal tracts to a wide range of brainstem and subcortical regions. The diffuse network of regions that process pain information is thought to result in the diverse sensory and emotional experiential components of pain. Maladaptive neurochemical and neuroplastic processes can produce central nociceptive network sensitization leading to the potentiation of nociceptive transmission and the amplification and persistence of pain.

In these studies, we identified PACAP expression in the spino-parabrachioamygdaloid nociceptive tract. As the amygdala is a critical structure for fear and anxiety-like behavior, the convergence of nociceptive input in the amygdala allows for the integration of pain with emotional information. We found that PACAP signaling in the amygdala produced both pain and anxiety-like behaviors. In a model of neuropathic pain, PACAP expression was found to be upregulated at multiple locations along the spinoparabrachio-amygdaloid tract. The increase in CeA PACAP signaling in neuropathic pain contributed to both heightened anxiety-like and hypersensitivity behaviors as the PAC1/VPAC2 receptor antagonist PACAP(6-38) attenuated the chronic pain-induced responses. Further, we demonstrated that PACAP signaling may modulate nociceptive hypersensitivity through ERK via the internalization of PACAP receptors. The adverse emotional consequences of chronic pain may result in the exacerbation and

perpetuation of both pain and anxio-depressive disorders. Understanding the mechanisms that link pain to its emotional consequences may offer novel approaches for the rational development of therapeutics to alleviate suffering.

The studies in this dissertation were divided into four main aims. The experiments in Aim 1 examined if PACAP is expressed in the PBn-CeA projections, as components of the spino-parabrachioamygdaloid tract. Aim 2 examined if CeA PACAP signaling is capable of altering pain or emotion-related behaviors. Aim 3 evaluated whether chronic pain heightened PACAP expression in the spino-parabrachioamygdaloid and whether these plasticity responses in CeA PACAP signaling contribute to heightened pain and anxiety-related behaviors. Lastly Aim 4, examined the potential downstream mechanism of CeA PACAP signaling. The results of these studies are presented in manuscript form in the following sections.

## 1.6. Figures

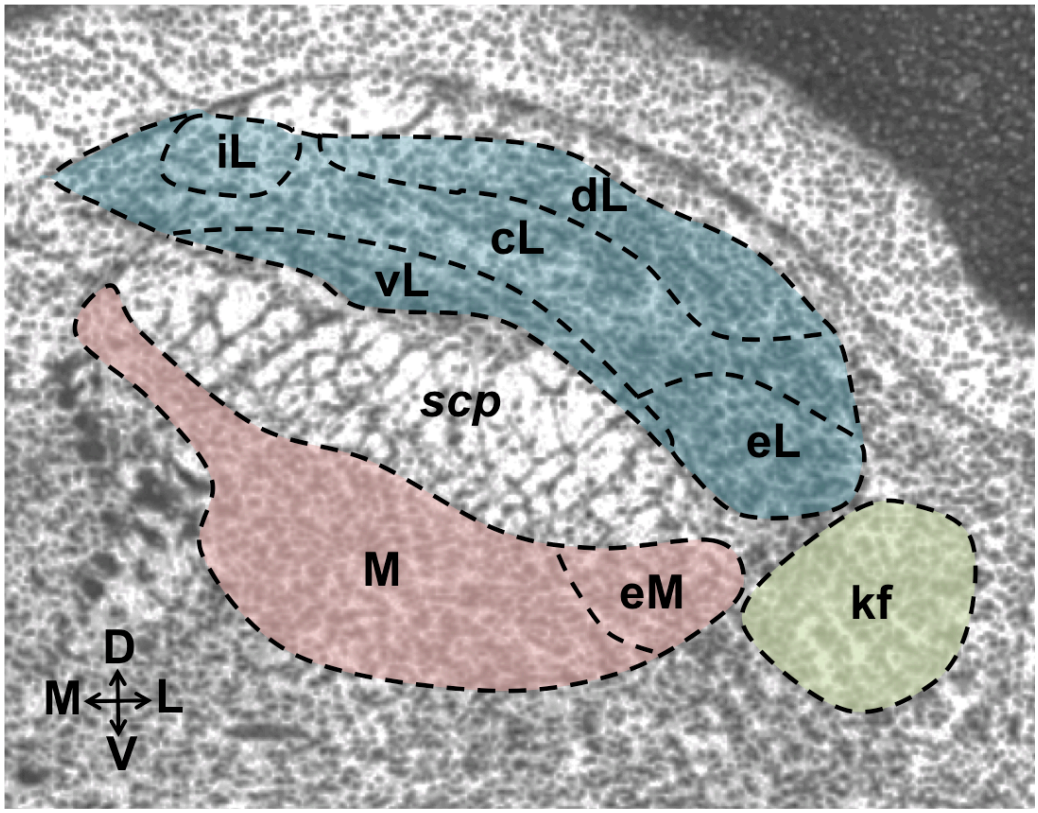


**Figure 1.1. Subnuclear organization of the parabrachial nucleus (PBn).** The PBn can be divided into the lateral PBn (LPBn, blue), medial PBn (MPBn, pink), and the Kölliker-fuse nucleus (kf, green). The MPBn consists of the medial (m) and external medial (em) subnuclei. The LPBn can be divided into the external (eL), ventral (vL), central (cL), dorsal (dL), internal (iL) lateral subnuclei, as well as the superior lateral subnucleus (*not shown)*. *scp*: superior cerebellar peduncle, *D:* dorsal, *V:* ventral, *M:* medial, *L:* lateral. Nomenclature adapted from (Fulweir et. al., 1985).

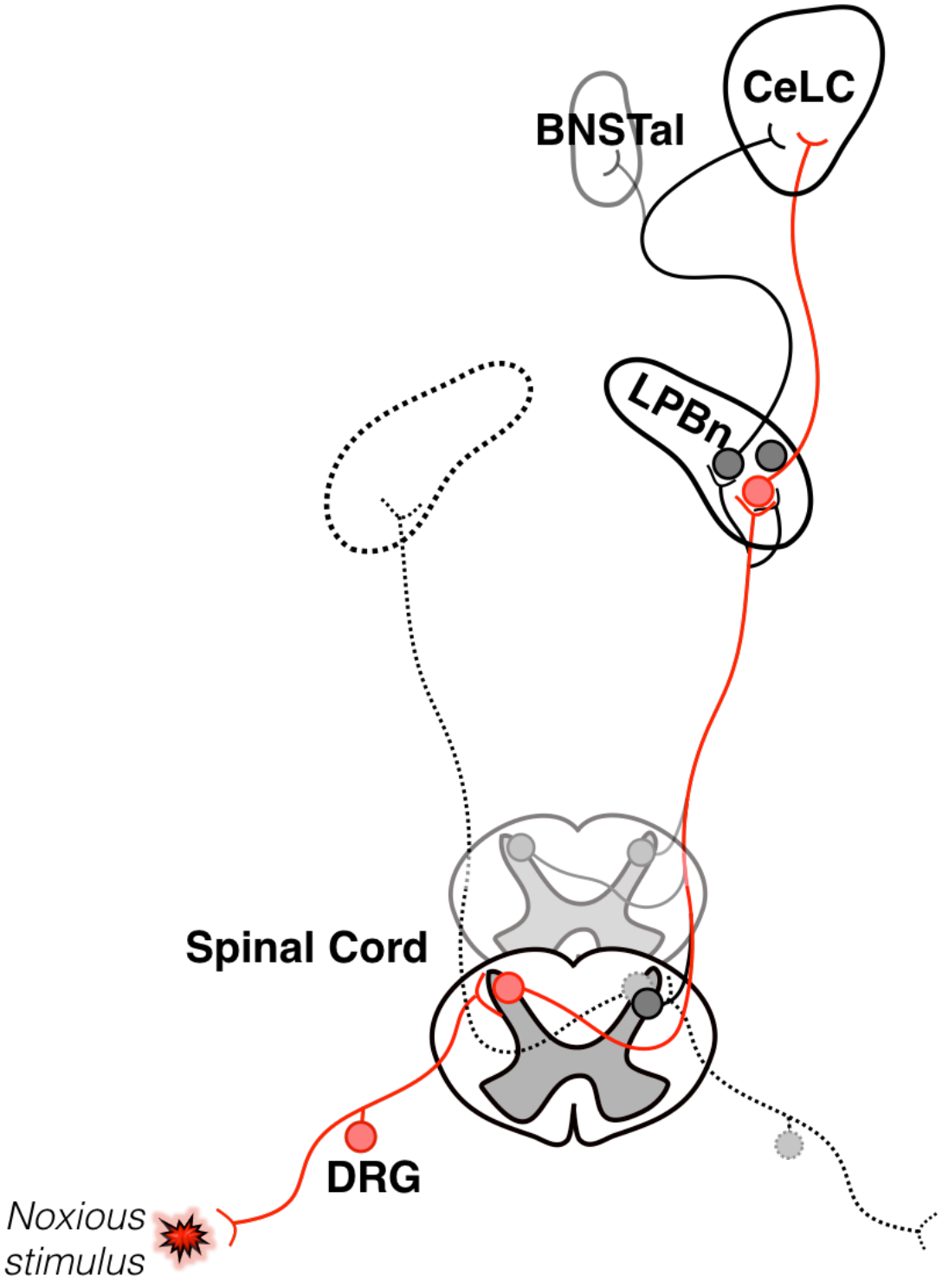
CeLC
BNSTal
LPBn
Spinal Cord
DRG
Noxious
stimulus

**Figure 1.2. Diagram of the spino-parabrachioamygdaloid tract.** Convergences and divergences of the spinoparabrachio-amygdaloid pathway are illustrated. In red is one particular pathway that nociceptive information travels, beginning at the detection of noxious stimulus and ending at the CeLC. The pathways in black show known alternative or variations on the pathway to the CeLC, including ipsilateral projections from the spinal cord, the convergence of projections multiple spinal cord segments in the LPBn, and an alternative LPBn-CeLC pathway that gives off collaterals to the anterolateral BNST (BNSTal) before returning to the CeLC. Dotted line illustrates contralateral projections. *DRG*: dorsal root ganglion.

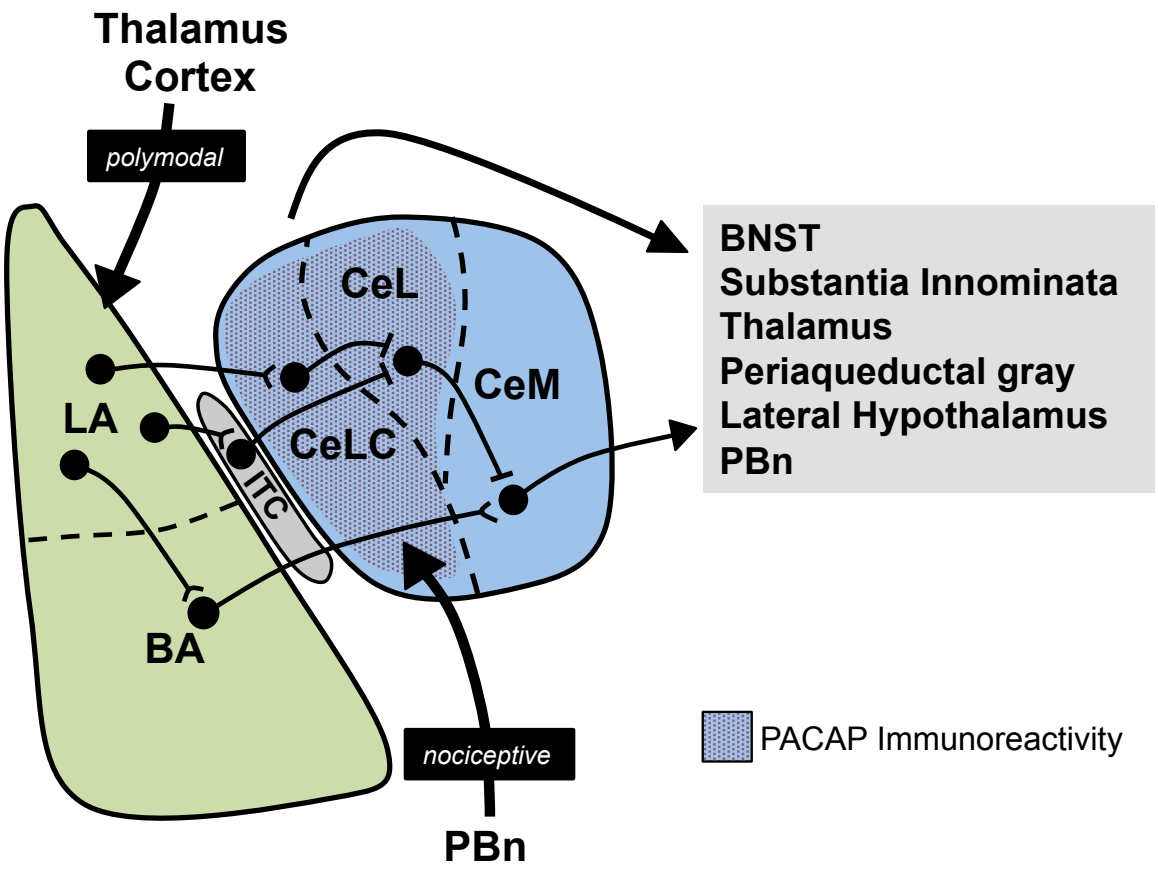
Thalamus
Cortex
polymodal
CeL
CeM
CeLC
LA
ITC
BA
nociceptive
PBn
BNST
Substantia Innominata
Thalamus
Periaqueductal gray
Lateral Hypothalamus
PBn
PACAP Immunoreactivity

**Figure 1.3. Afferent pathways and connections involved in pain processes in the amygdala.** The CeA (*blue region*) receives direct, highly processed, polymodal pain information from thalamus via the basolateral amygdala *(green region)* consisting of the basal (B) and lateral (LA) nuclei of the amygdala. Circuits from the LA known to enhance fear expression are shown, including BA to centromedial subdivision (CeM), via the inhibitory cells of the intercalated cell mass (ITC), and through interneurons in the centrolateral capsular (CeLC) and centrolateral (CeL) amygdala subdivisions. Direct, and less processed nociceptive information arrives in the CeLC and CeL as part of a spinal tract from the PBn. The main output is CeM, in a addition to some direct projections from the CeL/CeLC to a number of brain regions including the bed nucleus of the stria terminalis (BNST), periaqueductal gray (PAG) and a reciprocal projection back to the PBn.

| Gene Deleted | Phenotype |
|---|---|
| PACAP | *n.c.* Baseline thermal or mechanical sensitivity (Mabuchi et al., 2004)<br>⬇ Early and late nocifensive behaviors to formalin (Sandor et al., 2010)<br>⬇ Somatic sensitivity (May & Vizzard 2010)<br>⬇ Mechanical hypersensitivity following neuropathic pain (Mabuchi et al., 2004)<br>⬇ Acetic acid induced writhing (Sandor et al., 2010)<br>⬇ NMDA induced allodynia (Mabuchi et al., 2004)<br>⬇ c-fos expression in somatosensory cortex and brainstem following formalin or acetic acid pain (Sandor et al., 2010)<br>⬆ Thermal hypersensitivity to resiniferatoxin (immediate) (Sandor et al., 2010)<br>⬇ Mechanical hypersensitivity to resinferatoxin (delayed) (Sandor et al., 2010) |
| PAC1 R | *n.c.* Baseline thermal sensitivity (Jongsma et al. 2001)<br>⬇ Acetic acid induced writhing (no change in forebrain specific deletion) (Martin et al., 2003)<br>⬇ Morphine withdrawal symptoms (Martin et al., 2003)<br>⬇ Late phase of formalin induced nocifensive behaviors (Jongsma et al., 2001)<br>⬇ Galanin expression in DRG following nerve crush (Jongsma et al., 2001)<br>*n.c.:no change* |

**Table 1.1. Summary of pain-related behaviors in PACAP or PAC1 receptor gene knockout studies**

## 1.7. References


Agarwal, A., Halvorson, L. M., & Legradi, G. (2005). Pituitary adenylate cyclase-activating polypeptide (PACAP) mimics neuroendocrine and behavioral manifestations of stress: Evidence for PKA-mediated expression of the corticotropin-releasing hormone (CRH) gene. *Brain Res Mol Brain Res, 138*(1), 45-57.

Al-Khater, K. M., & Todd, A. J. (2009). Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. *J Comp Neurol, 515*(6), 629-646.

Alheid, G. F. (2003). Extended amygdala and basal forebrain. *Ann N Y Acad Sci, 985*, 185-205.

Almeida, T. F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Res., 1000*(1-2), 40-56.

Apkarian, A. V., Bushnell, M. C., Treede, R. D., & Zubieta, J. K. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *Eur J Pain, 9*(4), 463-484.

Asmundson, G. J. G., & Katz, J. (2009). Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. *Depress Anxiety, 26*(10), 888-901.

Barrie, A. P., Clohessy, A. M., Buensuceso, C. S., Rogers, M. V., & Allen, J. M. (1997). Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a Ras-independent, mitogen-activated protein Kinase/ERK kinase 1 or 2-dependent manner in PC12 cells. *J Biol Chem, 272*(32), 19666-19671.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell, 139*(2), 267-284.

Beaudet, M. M., Braas, K. M., & May, V. (1998). Pituitary adenylate cyclase activating polypeptide (PACAP) expression in sympathetic preganglionic projection neurons to the superior cervical ganglion. *J Neurobiol, 36*(3), 325-336.

Bernard, J. F., Alden, M., & Besson, J.-M. (1993). The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: Aphaseolus vulgaris leucoagglutinin (PHA-L) study in the rat. *J Comp Neurol, 329*(2), 201-229.

Bernard, J. F., Bester, H., & Besson, J. M. (1996). Involvement of the spino-parabrachio - amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. *Prog Brain Res, 107*, 243-255.

Bernard, J. F., Huang, G. F., & Besson, J. M. (1992). Nucleus centralis of the amygdala and the globus pallidus ventralis: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol, 68*(2), 551-569.

Bird, G. C., Lash, L. L., Han, J. S., & Zou, X. (2005). Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. *J Physiol, 564*(3), 907-921.

Blechman, J., & Levkowitz, G. (2013). Alternative Splicing of the Pituitary Adenylate Cyclase-Activating Polypeptide Receptor PAC1: Mechanisms of Fine Tuning of Brain Activity. *Front Endocrinol (Lausanne), 4*, 55.

Bon, K., Lantéri-Minet, M., Michiels, J. F., & Menétrey, D. (1998). Cyclophosphamide cystitis as a model of visceral pain in rats: a c-fos and Krox-24 study at telencephalic levels, with a note on pituitary adenylate cyclase activating polypeptide (PACAP). *Experimental Brain Research, 122*(2), 165-174.

Bonaz, B., Baciu, M., Papillon, E., Bost, R., Gueddah, N., Le Bas, J. F., . . . Segebarth, C. (2002). Central processing of rectal pain in patients with irritable bowel syndrome: an fMRI study. *Am J Gastroenterol, 97*(3), 654-661.

Bornhovd, K. (2002). Painful stimuli evoke different stimulus-response functions in the amygdala, prefrontal, insula and somatosensory cortex: a single-trial fMRI study. *Brain, 125*(6), 1326-1336.

Botz, B., Imreh, A., Sándor, K., Elekes, K., Szolcsányi, J., Reglodi, D., . . . Helyes, Z. (2013). Role of Pituitary Adenylate-Cyclase Activating Polypeptide and Tac1 gene derived tachykinins in sensory, motor and vascular functions under normal and neuropathic conditions. *Peptides, 43*, 105-112.

Bouschet, T., Perez, V., Fernandez, C., Bockaert, J., Eychene, A., & Journot, L. (2003). Stimulation of the ERK pathway by GTP-loaded Rap1 requires the concomitant activation of Ras, protein kinase C, and protein kinase A in neuronal cells. *J Biol Chem, 278*(7), 4778-4785.

Braas, K. M., Schutz, K. C., Bond, J. P., Vizzard, M. A., & Girard, B. M. (2007). Microarray analyses of pituitary adenylate cyclase activating polypeptide (PACAP)-regulated gene targets in sympathetic neurons. *Peptides, 28*(9), 1856-1870.

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., & Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur J Pain, 10*(4), 287-333.

Butler, R. K., & Finn, D. P. (2009). Stress-induced analgesia. *Prog Neurobiol., 88*(3), 184-202.

Carrasquillo, Y., & Gereau, R. W. (2007). Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J Neurosci, 27*(7), 1543-1551.

Cheng, S.-J., Chen, C.-C., Yang, H.-W., Chang, Y.-T., Bai, S.-W., Chen, C.-C., . . . Min, M.-Y. (2011). Role of extracellular signal-regulated kinase in synaptic transmission and plasticity of a nociceptive input on capsular central amygdaloid neurons in normal and acid-induced muscle pain mice. *J Neurosci, 31*(6), 2258-2270.

Cho, J.-H., Zushida, K., Shumyatsky, G. P., Carlezon, W. A., Meloni, E. G., & Bolshakov, V. Y. (2012). Pituitary Adenylate Cyclase-Activating Polypeptide Induces Postsynaptically Expressed Potentiation in the Intra-amygdala Circuit. *J Neurosci, 32*(41), 14165-14177.

Chowdhury, D., Marco, S., Brooks, I. M., Zandueta, A., Rao, Y., Haucke, V., . . . Perez-Otano, I. (2013). Tyrosine phosphorylation regulates the endocytosis and surface expression of GluN3A-containing NMDA receptors. *J Neurosci, 33*(9), 4151-4164.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B. E., Letzkus, J. J., Vlachos, I., . . . Lüthi, A. (2010). Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature, 468*(7321), 277-282.

Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., . . . Patapoutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science, 330*(6000), 55-60.

Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., . . . Woods, C. G. (2006). An SCN9A channelopathy causes congenital inability to experience pain. *Nature, 444*(7121), 894-898.

Davis, M., Walker, D. L., Miles, L., & Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology, 35*(1), 105-135.

Davis-Taber, R., Baker, S., Lehto, S. G., Zhong, C., Surowy, C. S., Faltynek, C. R., . . . Honore, P. (2008). Central Pituitary Adenylate Cyclase 1 Receptors Modulate Nociceptive Behaviors in Both Inflammatory and Neuropathic Pain States. *J Pain., 9*(5), 449-456.

Defrin, R., Ginzburg, K., Solomon, Z., Polad, E., Bloch, M., Govezensky, M., & Schreiber, S. (2008). Quantitative testing of pain perception in subjects with PTSD – Implications for the mechanism of the coexistence between PTSD and chronic pain. *Pain, 138*(2), 450-459.

Delgado, M., Pozo, D., & Ganea, D. (2004). The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev, 56*(2), 249-290.

Demyttenaere, K., Bruffaerts, R., Lee, S., Posada-Villa, J., Kovess, V., Angermeyer, M. C., . . . Von Korff, M. (2007). Mental disorders among persons with chronic back or neck pain: Results from the world mental health surveys. *Pain, 129*(3), 332-342.

Dickinson, T., & Fleetwood-Walker, S. M. (1999). VIP and PACAP: very important in pain? *Trends Pharmacol Sci.*, *20*(8), 324-329.

Dickinson, T., Fleetwood-Walker, S. M., Mitchell, R., & Lutz, E. M. (1997). Evidence for roles of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors in modulating the responses of rat dorsal horn neurons to sensory inputs. *Neuropeptides, 31*(2), 175-185.

Dong, H. W., Petrovich, G. D., & Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Brain Res Rev, 38*(1-2), 192-246.

Duvarci, S., & Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron, 82*(5), 966-980.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., & Lüthi, A. (2009). Amygdala inhibitory circuits and the control of fear memory. *Neuron, 62*(6), 757-771.

Engelund, A., Fahrenkrug, J., Harrison, A., & Hannibal, J. (2010). Vesicular glutamate transporter 2 (VGLUT2) is co-stored with PACAP in projections from the rat melanopsin-containing retinal ganglion cells. *Cell Tissue Res, 340*(2), 243-255.

Flor, H., Fydrich, T., & Turk, D. C. (1992). Efficacy of multidisciplinary pain treatment centers: a meta-analytic review. *Pain, 49*(2), 221-230.

Fulwiler, C. E., & Saper, C. B. (1984). Subnuclear organization of the efferent connections of the parabrachial nucleus in the rat. *Brain Res, 319*(3), 229-259.

Gaskin, D. J., & Richard, P. (2012). The economic costs of pain in the United States. *J Pain., 13*(8), 715-724.

Gauriau, C., & Bernard, J.-F. (2004). Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. *J Neurosci, 24*(3), 752-761.

Geuze, E., Westenberg, H. G. M., Jochims, A., de Kloet, C. S., Bohus, M., Vermetten, E., & Schmahl, C. (2007). Altered Pain Processing in Veterans With Posttraumatic Stress Disorder. *Arch Genl Psychiatry., 64*(1), 76-85.

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

Goncalves, L., Silva, R., Pinto-Ribeiro, F., Pego, J. M., Bessa, J. M., Pertovaara, A., . . . Almeida, A. (2008). Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. *Exp Neurol, 213*(1), 48-56.

Guo, Z., Zhao, C., Huang, M., Huang, T., Fan, M., Xie, Z., . . . Cheng, L. (2012). Tlx1/3 and Ptf1a Control the Expression of Distinct Sets of Transmitter and Peptide Receptor Genes in the Developing Dorsal Spinal Cord. *J Neurosci, 32*(25), 8509-8520.

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. *Psychoneuroendocrinology, 34*(6), 833-843.

Han, J. S., & Neugebauer, V. (2004). Synaptic plasticity in the amygdala in a visceral pain model in rats. *Neurosci Lett, 361*(1-3), 254-257.

Hannibal, J. (2002). Pituitary adenylate cyclase-activating peptide in the rat central nervous system: An immunohistochemical and in situ hybridization study. *J Comp Neurol., 453*(4), 389-417.

Hannibal, J., Mikkelsen, J. D., Fahrenkrug, J., & Larsen, P. J. (1995). Pituitary adenylate cyclase-activating peptide gene expression in corticotropin-releasing factor-containing parvicellular neurons of the rat hypothalamic paraventricular nucleus is induced by colchicine, but not by adrenalectomy, acute osmotic, ether, or restraint stress. *Endocrinology, 136*(9), 4116-4124.

Hannibal, J., Moller, M., Ottersen, O. P., & Fahrenkrug, J. (2000). PACAP and glutamate are co-stored in the retinohypothalamic tract. *J Comp Neurol, 418*(2), 147-155.

Hashimoto, H. (2006). New Insights into the Central PACAPergic System from the Phenotypes in PACAP- and PACAP Receptor-Knockout Mice. *Annal N Y Acad Sci., 1070*(1), 75-89.

Hashimoto, H., Shintani, N., Tanaka, K., Mori, W., Hirose, M., Matsuda, T., . . . Baba, A. (2001). Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proc Natl Acad Sci U S A., 98*(23), 13355-13360.

Hattori, S., Takao, K., Tanda, K., Toyama, K., Shintani, N., Baba, A., . . . Miyakawa, T. (2012). Comprehensive behavioral analysis of pituitary adenylate cyclase-activating polypeptide (PACAP) knockout mice. *Front Behav Neurosci, 6*, 58.

Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., . . . Anderson, D. J. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature, 468*(7321), 270-276.

Huang, G. F., Besson, J. M., & Bernard, J. F. (1993). Morphine depresses the transmission of noxious messages in the spino(trigemino)-ponto-amygdaloid pathway. *Eur J Pharmacol, 230*(3), 279-284.

Ikeda, R., Takahashi, Y., Inoue, K., & Kato, F. (2007). NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. *Pain, 127*(1-2), 161-172.

Jasmin, L., Wang, H., Tarczy-Hornoch, K., Levine, J. D., & Basbaum, A. I. (1994). Differential effects of morphine on noxious stimulus-evoked fos-like immunoreactivity in subpopulations of spinoparabrachial neurons. *J Neurosci, 14*(12), 7252-7260.

Ji, R. R., Gereau IV, R. W., Malcangio, M., & Strichartz, G. R. (2009). MAP kinase and pain. *Brain Res Rev., 60*(1), 135-148.

Ji, R. R., Baba, H., Brenner, G. J., & Woolf, C. J. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci, 2*(12), 1114-1119.

Jongsma, H., Danielsen, N., Sundler, F., & Kanje, M. (2000). Alteration of PACAP distribution and PACAP receptor binding in the rat sensory nervous system following sciatic nerve transection. *Brain Res., 853*(2), 186-196.

Jongsma, H., Pettersson, L. M., Zhang, Y., Reimer, M. K., Kanje, M., Waldenström, A., . . . Danielsen, N. (2001). Markedly reduced chronic nociceptive response in mice lacking the PAC1 receptor. *Neuroreport, 12*(10), 2215-2219.

Jongsma Wallin, H., Pettersson, L. M., Verge, V. M., & Danielsen, N. (2003). Effect of anti-nerve growth factor treatment on pituitary adenylate cyclase activating polypeptide expression in adult sensory neurons exposed to adjuvant induced inflammation. *Neuroscience, 120*(2), 325-331.

Kammermeier, P. J. (2008). Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. *J Neurosci, 28*(34), 8560-8567.

Klüver, H, Bucy, P.C. (1937). "Psychic blindness" and other symptoms following bilateral temporal lobectomy in rhesus monkeys. *Am. J. Physiol.*, *119*, 352-353.

Kocho-Schellenberg, M., Lezak, K. R., Harris, O. M., Roelke, E., Gick, N., Choi, I., . . . Hammack, S. E. (2014). Pituitary Adenylate Cyclase Activating Peptide (PACAP) in the Bed Nucleus of the Stria Terminalis (BNST) Produces Anorexia and Weight Loss in Male and Female Rats. *Neuropsychopharmacology, 39*(7), 1614-1623.

Kroenke, K., & Price, R. K. (1993). Symptoms in the community. Prevalence, classification, and psychiatric comorbidity. *Arch Intern Med, 153*(21), 2474-2480.

Kulkarni, B., Bentley, D. E., Elliott, R., Julyan, P. J., Boger, E., Watson, A., . . . Jones, A. K. (2007). Arthritic pain is processed in brain areas concerned with emotions and fear. *Arthritis Rheum., 56*(4), 1345-1354.

Latremoliere, A., & Woolf, C. J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain., 10*(9), 895-926.

Ledoux, J. E., (2000). Emotion circuits in the brain. *Annu. Rev. Neurosci., 23,* 155-184.

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.

Li, J., Hou, B., Tumova, S., Muraki, K., Bruns, A., Ludlow, M. J., . . . Beech, D. J. (2014). Piezo1 integration of vascular architecture with physiological force. *Nature, 515*(7526), 279-282.

Mabuchi, T., Shintani, N., & Matsumura, S. (2004). Pituitary adenylate cyclase-activating polypeptide is required for the development of spinal sensitization and induction of neuropathic pain. *J Neurosci., 24*(33), 7283-7291.

Macdonald, D. S., Weerapura, M., Beazely, M. A., Martin, L., Czerwinski, W., Roder, J. C., . . . MacDonald, J. F. (2005). Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires G alpha q, protein kinase C, and activation of Src. *J Neurosci, 25*(49), 11374-11384.

Martin, M., Otto, C., Santamarta, M. T., Torrecilla, M., Pineda, J., Schutz, G., & Maldonado, R. (2003). Morphine withdrawal is modified in pituitary adenylate cyclase-activating polypeptide type I-receptor-deficient mice. *Brain Res Mol Brain Res, 110*(1), 109-118.

May, V., Beaudet, M. M., Parsons, R. L., Hardwick, J. C., Gauthier, E. A., Durda, J. P., & Braas, K. M. (1998). Mechanisms of pituitary adenylate cyclase activating polypeptide (PACAP)-induced depolarization of sympathetic superior cervical ganglion (SCG) neurons. *Ann N Y Acad Sci., 865*, 164-175.

May, V., Buttolph, T. R., Girard, B. M., Clason, T. A., & Parsons, R. L. (2014). PACAP-induced ERK activation in HEK cells expressing PAC1 receptors involves both receptor internalization and PKC signaling. *Am J Physiol Cell Physiol, 306*(11), C1068-C1079.

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. *J Biol Chem, 285*(13), 9749-9761.

May, V., & Vizzard, M. A. (2010). Bladder dysfunction and altered somatic sensitivity in PACAP-/- mice. *J Urol., 183*(2), 772-779.

McCoy, E. S., Taylor-Blake, B., Street, S. E., Pribisko, A. L., Zheng, J., & Zylka, M. J. (2013). Peptidergic CGRPα primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. *Neuron, 78*(1), 138-151.

McWilliams, L. A., Cox, B. J., & Enns, M. W. (2003). Mood and anxiety disorders associated with chronic pain: an examination in a nationally representative sample. *Pain, 106*(1-2), 127-133.

McWilliams, L. A., Goodwin, R. D., & Cox, B. J. (2004). Depression and anxiety associated with three pain conditions: results from a nationally representative sample. *Pain, 111*(1-2), 77-83.

Merriam, L. A., Baran, C. N., Girard, B. M., Hardwick, J. C., May, V., & Parsons, R. L. (2013). Pituitary Adenylate Cyclase 1 Receptor Internalization and Endosomal Signaling Mediate the Pituitary Adenylate Cyclase Activating Polypeptide-Induced Increase in Guinea Pig Cardiac Neuron Excitability. *J Neurosci., 33*(10), 4614-4622.

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 cyclase in pituitary cells. *Biochem Biophys Res Commun., 164*(1), 567-574.

Moller, K., Zhang, Y. Z., Hakanson, R., Luts, A., Sjolund, B., Uddman, R., & Sundler, F. (1993). Pituitary adenylate cyclase activating peptide is a sensory neuropeptide: immunocytochemical and immunochemical evidence. *Neuroscience, 57*(3), 725-732.

Mulder, H., Uddman, R., Moller, K., Zhang, Y. Z., Ekblad, E., Alumets, J., & Sundler, F. (1994). Pituitary adenylate cyclase activating polypeptide expression in sensory neurons. *Neuroscience, 63*(1), 307-312.

Nagahama, M., Tsuzuki, M., Mochizuki, T., Iguchi, K., & Kuwahara, A. (1998). Light and electron microscopic studies of pituitary adenylate cyclase-activating peptide (PACAP)--immunoreactive neurons in the enteric nervous system of rat small and large intestine. *Anat Embryol (Berl), 198*(5), 341-352.

Nakagawa, T., Katsuya, A., Tanimoto, S., Yamamoto, J., Yamauchi, Y., Minami, M., & Satoh, M. (2003). Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. *Neurosci Lett, 344*(3), 197-200.

Namburi, P., Beyeler, A., Yorozu, S., Calhoon, G. G., Halbert, S. A., Wichmann, R., . . . Tye, K. M. (2015). A circuit mechanism for differentiating positive and negative associations. *Nature, 520*(7549), 675-678.

Narita, M., Dun, S. L., Dun, N. J., & Tseng, L. F. (1996). Hyperalgesia induced by pituitary adenylate cyclase-activating polypeptide in the mouse spinal cord. *Eur J Pharmacol., 311*(2-3), 121-126.

Neugebauer, V., & Li, W. (2002). Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. *J Neurophysiol., 87*(1), 103-112.

Neugebauer, V., Li, W., Bird, G. C., Bhave, G., & Gereau, R. W. (2003). Synaptic Plasticity in the Amygdala in a Model of Arthritic Pain: Differential Roles of Metabotropic Glutamate Receptors 1 and 5. *J Neurosci., 23*(1), 52-63.

Neugebauer, V., Li, W., Bird, G. C., & Han, J. S. (2004). The Amygdala and Persistent Pain. *Neuroscientist, 10*(3), 221-234.

Norman, S. B., Stein, M. B., & Dimsdale, J. E. (2008). Pain in the aftermath of trauma is a risk factor for post-traumatic stress disorder. *Psychol Med., 38*(4), 533-542.

Obata, K., Yamanaka, H., Dai, Y., Tachibana, T., Fukuoka, T., Tokunaga, A., . . . Noguchi, K. (2003). Differential activation of extracellular signal-regulated protein kinase in primary afferent neurons regulates brain-derived neurotrophic factor expression after peripheral inflammation and nerve injury. *J Neurosci, 23*(10), 4117-4126.

Ohsawa, M., Brailoiu, G. C., Shiraki, M., Dun, N. J., Paul, K., & Tseng, L. F. (2002). Modulation of nociceptive transmission by pituitary adenylate cyclase activating polypeptide in the spinal cord of the mouse. *Pain, 100*(1-2), 27-34.

Paré, D., Qurik, G. J., & Ledoux, J. E., (2004). New vistas on amygdala networks in conditioned fear. *J. Neurophysiol, 92,* 1-9.

Petrovic, P., Ingvar, M., Stone-Elander, S., Petersson, K. M., & Hansson, P. (1999). A PET activation study of dynamic mechanical allodynia in patients with mononeuropathy. *Pain, 83*(3), 459-470.

Petrovich, G. D., & Swanson, L. W. (1997). Projections from the lateral part of the central amygdalar nucleus to the postulated fear conditioning circuit. *Brain Res., 763*(2), 247-254.

Pettersson, L. M., Dahlin, L. B., & Danielsen, N. (2004). Changes in expression of PACAP in rat sensory neurons in response to sciatic nerve compression. *Eur J Neurosci, 20*(7), 1838-1848.

Pettersson, L. M. E., Heine, T., Verge, V. M. K., Sundler, F., & Danielsen, N. (2004). PACAP mRNA is expressed in rat spinal cord neurons. *J Comp Neurol, 471*(1), 85-96.

Phifer, J., Skelton, K., Weiss, T., Schwartz, A. C., & Wingo, A. (2011). Pain symptomatology and pain medication use in civilian PTSD. *Pain, 152*(10), 2233-2240.

Piggins, H. D., Stamp, J. A., Burns, J., Rusak, B., & Semba, K. (1996). Distribution of pituitary adenylate cyclase activating polypeptide (PACAP) immunoreactivity in the hypothalamus and extended amygdala of the rat. *J Comp Neurol, 376*(2), 278-294.

Ranade, S. S., Woo, S. H., Dubin, A. E., Moshourab, R. A., Wetzel, C., Petrus, M., . . . Patapoutian, A. (2014). Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature, 516*(7529), 121-125.

Redondo, R. L., Kim, J., Arons, A. L., Ramirez, S., Liu, X., & Tonegawa, S. (2014). Bidirectional switch of the valence associated with a hippocampal contextual memory engram. *Nature, 513*(7518), 426-430.

Resch, J. M., Maunze, B., Phillips, K. A., & Choi, S. (2014). Inhibition of food intake by PACAP in the hypothalamic ventromedial nuclei is mediated by NMDA receptors. *Physiol Behav, 133*, 230-235.

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., . . . May, V. (2014). PAC1 receptor antagonism 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., de Reus, M., Loohuis, N. O., Giele, J., van Egmond, J., . . . Kozicz, T. (2012). Experimental neuropathy increases limbic forebrain CRF. *Eur J Pain, 16*(1), 61-71.

Sah, P., Faber, E. S. L., Armentia, M. L. D., & Power, J. (2003). The Amygdaloid Complex: Anatomy and Physiology. *Physiol Rev., 83*(3), 803-834.

Sakashita, Y., Kurihara, T., Uchida, D., Tatsuno, I., & Yamamoto, T. (2001). Involvement of PACAP receptor in primary afferent fibre-evoked responses of ventral roots in the neonatal rat spinal cord. *Br J Pharmacol, 132*(8), 1769-1776.

Sándor, K., Bölcskei, K., McDougall, J. J., Schuelert, N., Reglodi, D., Elekes, K., . . . Helyes, Z. (2009). Divergent peripheral effects of pituitary adenylate cyclase-activating polypeptide-38 on nociception in rats and mice. *Pain, 141*(1-2), 143-150.

Sándor, K., Kormos, V., Botz, B., Imreh, A., Bölcskei, K., Gaszner, B., . . . Helyes, Z. (2010). Impaired nocifensive behaviours and mechanical hyperalgesia, but enhanced thermal allodynia in pituitary adenylate cyclase-activating polypeptide deficient mice. *Neuropeptides, 44*(5), 363-371.

Sarhan, M., Freund-Mercier, M.-J., & Veinante, P. (2005). Branching patterns of parabrachial neurons projecting to the central extended amgydala: single axonal reconstructions. *J Comp Neurol, 491*(4), 418-442.

Schmidt, S. D., Myskiw, J. C., Furini, C. R., Schmidt, B. E., Cavalcante, L. E., & Izquierdo, I. (2015). PACAP modulates the consolidation and extinction of the contextual fear conditioning through NMDA receptors. *Neurobiol Learn Mem, 118*, 120-124.

Sherwood, N. M., Krueckl, S. L., & McRory, J. E. (2000). The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev., 21*(6), 619-670.

Shi, C., & Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. *J Neurosci., 19*(1), 420-430.

Shimizu, T., Katahira, M., Sugawara, H., Inoue, K., & Miyata, A. (2004). Diverse effects of intrathecal pituitary adenylate cyclase-activating polypeptide on nociceptive transmission in mice spinal cord. *Regul Pept., 123*(1-3), 117-122.

Simons, L. E., Moulton, E. A., Linnman, C., Carpino, E., Becerra, L., & Borsook, D. (2014). The human amygdala and pain: Evidence from neuroimaging. *Hum Brain Mapp., 35*(2), 527-538.

Spike, R. C., Puskar, Z., Andrew, D., & Todd, A. J. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur J Neurosci, 18*(9), 2433-2448.

Suwanprathes, P., Ngu, M., Ing, A., Hunt, G., & Seow, F. (2003). c-fos immunoreactivity in the brain after esophageal acid stimulation. *Am J Med, 115 Suppl 3A*, 31S-38S.

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci., 11*(12), 823-836.

Tovote, P., Fadok, J. P., & Lüthi A. (2015). Neuronal circuits for fear and anxiety. *Nat Neurosci., 16,* 317-331.

Tracey, I., & Mantyh, P. W. (2007). The cerebral signature for pain perception and its modulation. *Neuron, 55*(3), 377-391.

Trepanier, C. H., Jackson, M. F., & MacDonald, J. F. (2012). Regulation of NMDA receptors by the tyrosine kinase Fyn. *FEBS J, 279*(1), 12-19.

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 (Amsterdam, Netherlands), 14*(4), 368-375.

Turk, D. C. (2002). Clinical effectiveness and cost-effectiveness of treatments for patients with chronic pain. *Clin J Pain, 18*(6), 355-365.

Ulrich-Lai, Y. M., Xie, W., Meij, J. T. A., Dolgas, C. M., Yu, L., & Herman, J. P. (2006). Limbic and HPA axis function in an animal model of chronic neuropathic pain. *Physiol Behav., 88*(1-2), 67-76.

Usoskin, D., Furlan, A., Islam, S., Abdo, H., Lonnerberg, P., Lou, D., . . . Ernfors, P. (2015). Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat Neurosci, 18*(1), 145-153.

Vaudry, D., Falluel-Morel, A., Bourgault, S., Basille, M., Burel, D., Wurtz, O., . . . Vaudry, H. (2009). Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev., 61*(3), 283-357.

Veinante, P., Yalcin, I., Barrot, M. (2013). The amygdala between sensation and affect: a role in pain. *J. Mol. Psychiatry, 1*, 9.

Vizzard, M. A. (2000). Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis. *J Comp Neurol, 420*(3), 335-348.

Walker, D. L., Miles, L. A., & Davis, M. (2009). Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 33*(8), 1291-1308.

Xu, X. J., & Wiesenfeld-Hallin, Z. (1996). Intrathecal pituitary adenylate cyclase activating polypeptide facilitates the spinal nociceptive flexor reflex in the rat. *Neuroscience, 72*(3), 801-804.

Yalcin, I., Barthas, F., & Barrot, M. (2014). Emotional consequences of neuropathic pain: insight from preclinical studies. *Neurosci Biobehav Rev., 47*, 154-164.

Yamamoto, T., & Tatsuno, I. (1995). Antinociceptive effect of intrathecally administered pituitary adenylate cyclase activating polypeptide (PACAP) on the rat formalin test. *Neurosci Lett., 184*(1), 32-35.

Zhang, Q., Shi, T.-J., Ji, R.-R., Zhang, Y.-t., Sundler, F., Hannibal, J., . . . Hökfelt, T. (1995). Expression of pituitary adenylate cyclase-activating polypeptide in dorsal root ganglia following axotomy: time course and coexistence. *Brain Res., 705*(1-2), 149-158.

Zhang, Y., Danielsen, N., Sundler, F., & Mulder, H. (1998). Pituitary adenylate cyclase-activating peptide is upregulated in sensory neurons by inflammation. *Neuroreport, 9*(12), 2833-2836.

Zhang, Y., Malmberg, A. B., Sjölund, B., & Yaksh, T. L. (1996). The effect of pituitary adenylate cyclase activating peptide (PACAP) on the nociceptive formalin test. *Neurosci Lett, 207*(3), 187-190.

Zhang, Y. Z., Hannibal, J., Zhao, Q., Moller, K., Danielsen, N., Fahrenkrug, J., & Sundler, F. (1996). Pituitary adenylate cyclase activating peptide expression in the rat dorsal root ganglia: up-regulation after peripheral nerve injury. *Neuroscience, 74*(4), 1099-1110.

Zhang, Y. Z., Sjolund, B., Moller, K., Hakanson, R., & Sundler, F. (1993). Pituitary adenylate cyclase activating peptide produces a marked and long-lasting depression of a C-fibre-evoked flexion reflex. *Neuroscience, 57*(3), 733-737.

## Chapter 2.

## Parabrachial nucleus (PBn) pituitary adenylate cyclase activating polpeptide (PACAP) signaling in the amygdala: Implication for the sensory and behavioral effects of pain

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(2014). *Neuropharmacology, 86*, 38-48.

## 2.1. Abstract

The intricate relationships that associate pain, stress responses and emotional behavior have been well established. Acute stressful situations can decrease nociceptive sensations and conversely, chronic pain can enhance other pain experiences and heighten the emotional and behavioral consequences of stress. Accordingly, chronic pain is comorbid with a number of behavioral disorders including depression, anxiety abnormalities and associated stress-related disorders including posttraumatic stress disorder (PTSD). The central nucleus of the amygdala (CeA) represents a convergence of pathways for pain, stress and emotion, and we have identified pituitary adenylate cyclase activating polypeptide (PACAP) immunoreactivity in fiber elements in the lateral capsular division of the CeA (CeLC). The PACAP staining patterns colocalized in part with those for calcitonin gene related peptide (CGRP); anterograde fiber tracing and excitotoxic lesion studies demonstrated that the CeLC PACAP/CGRP immunoreactivities represented sensory fiber projections from the lateral parabrachial nucleus (LPBn) along the spino-parabrachioamygdaloid tract. The same PBn PACAP/CGRP fiber system also projected to the BNST. As in the BNST, CeA PACAP signaling increased anxiety-like behaviors accompanied by weight loss and decreased feeding. But in addition to heightened anxiety-like responses, CeA PACAP signaling also altered nociception as reflected by decreased latency and threshold responses in thermal and mechanical sensitivity tests, respectively. From PACAP expression in major pain pathways, the current observations are novel and suggest that CeA PACAP nociceptive signaling and resulting neuroplasticity via the spino-parabrachio- amygdaloid tract may represent

mechanisms that associate chronic pain with sensory hypersensitivity, fear memory consolidation and severe behavioral disorders.

## 2.2. Introduction

Chronic neuropathic pain alters sensory responses and carries an emotional subtext that can have severe effects on behavior. Persistent pain can heighten pain experiences from hyperalgesia and allodynia (Rouwette et al., 2012; Veinante et al., 2013). Further, patients suffering from chronic pain are more prone to experience depression, sleep dysregulation, panic disorders, obsessive compulsive behavior, anxiety abnormalities and stress-related disorders including post-traumatic stress disorder (PTSD) (Asmundson and Katz, 2009). The intricate relationship between pain and behavior has been well studied and among brain regions, the amygdala is centrally situated to integrate the many descending and ascending signals to modulate the sensory and emotional components of pain. Highly processed descending polymodal nociceptive information is conveyed from the somatosensory cortex and thalamus to the basolateral amygdala (BLA) which in turn projects to the central nucleus of the amygdala (CeA). The resulting CeA efferents signals are relayed to other central nuclei, including those traveling with hypothalamic e periaqueductal grey projections for autonomic control and anti-nociception to dampen pain stimuli (Veinante et al., 2013). Among several ascending pathways carrying pain transmission to the CeA, the most prominent is the spino-parabrachioamygdaloid tract (Bernard et al., 1996; Gauriau and Bernard, 2002; Rouwette et al., 2012; Veinante et al., 2013). Peripheral nociceptive signals carried via primary

sensory Aδ- and C-fibers terminate in the dorsal horn where second order neurons send projections via the spino-parabrachial pathway to pontine lateral and external medial parabrachial nuclei (PBn) (Todd, 2010). Hence the PBn collects cutaneuous (mechanical and thermal), deep (muscular and articular) and visceral nociceptive signals and relays the information in a highly organized topographical manner principally to lateral capsular division of the CeA (CeLC). The roles of the CeA/CeLC in nociceptive processing have been examined from a number of vantages. In vivo electrophysiological studies have shown that noxious stimuli and chronic pain paradigms increase spontaneous and evoked CeA neuronal activity (Bernard et al., 1992; Ji and Neugebauer, 2009; Neugebauer and Li, 2003), and synaptic transmission at PBn-CeA and BLA-CeA synapses (Ikeda et al., 2007; Neugebauer et al., 2003). Visceral, inflammatory and chronic neuropathic pain can induce CeA neuron stress peptide and c-fos expression (Bon et al., 1998; Nakagawa et al., 2003; Suwanprathes et al., 2003; Ulrich-Lai et al., 2006; Rouwette et al., 2011) and increase glutaminergic NR1 receptor phosphorylation in CeA neurons (Bird et al., 2005). Further, human brain imaging studies have implicated the amygdala in pain (Simons et al., 2014). Hence the neurocircuit intersections in the CeA can modulate the sensory, emotional and affective responses to pain.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a well studied neural and endocrine pleiotropic peptide important in the development and homeostatic regulation of many physiological systems (reviewed in Vaudry et al., 2009). In the central and peripheral nervous systems, PACAP is neurotrophic to promote neuronal survival, proliferation and differentiation in development and regeneration, participates in

sensory and autonomic signaling, is important in hippocampal learning and memory processes and regulates a variety of hypothalamic/limbic stress-related behavioral responses. PACAP binds to several G protein-couple receptor subtypes (Braas and May, 1999; Harmar et al., 2012; Spengler et al., 1993). PACAP binds selectively at the PAC1 receptor; both PACAP and VIP bind the VPAC receptors with equal high affinity. Recently, the expression of PACAP and its cognate PAC1 receptor has been shown to be upregulated in specific limbic regions by chronic stress (Hammack et al., 2009). PACAP infusions into the bed nucleus of the stria terminalis (BNST) is anxiogenic, and altered blood PACAP levels and PAC1 receptor polymorphism have been associated with PTSD and other stress-related disorders (Almli et al., 2013; Chen et al., 2013; Ressler et al., 2011; Uddin et al., 2013; Wang et al., 2013). In sum, these observations have implicated limbic PACAP/PAC1 receptor signaling in stress- and anxiety-related behaviors.

In evaluating PACAP expression in other limbic structures, we noted high levels of PACAP immunoreactivity in fiber terminals and varicosities within the CeLC, suggesting that the CeLC may be a target of distant PACAP projections. The CeLC is heavily innervated by the lateral PBn (LPBn) and PACAP has been localized to many sensory pathways. From these observations, we have hypothesized that LPBn PACAP signaling to the CeLC has both sensory and behavioral consequences. In examining the localization and roles of PACAP to the CeLC, our current work demonstrates that PACAP is a component of the parabrachioamygdaloid pathway and that PACAP/PAC1 receptor signaling in the CeA elicits nociceptive and behavioral responses. The integration of these nociceptive and

emotion pathways may represent a set of neural circuits that mediate the adverse sensory and emotional consequences of chronic pain.

## 2.3. Methods

*Animals*

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were habituated to the animal facility for 1 week before experimentation. Rats were single-housed and maintained on a 12 h light/dark cycle (lights on at 0700 h). Food and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

*Chronic variate stress*

Following acclimation, each animal was randomly assigned to either a control or chronically stressed group. Control group animals were handled and remained in their home cages until euthanasia. The chronically stressed group of animals underwent a chronic variate stress paradigm in which rats were exposed to one of 5 different stressors (oscillation, forced swim, restraint, pedestal standing and foot- shock) each day for 7 days, as described previously (Hammack et al., 2009; Roman et al., 2012, 2014). All animals within the group were exposed to the same order of stressors for the same duration.

*Immunocytochemistry*

The brains from perfusion fixed animals were postfixed in 4% paraformaldehyde at 4C for 24 h, washed and equilibrated in 30% surcrose before embedding in Tissue-Tek OCT compound for cryosectioning. The sections (30 μm) were mounted onto subbed slides, permeabilized with 0.3% Triton X-100, blocked with 1% BSA and incubated in primary antibody for 48 h at 4C. CRH immunoreactivity was localized using an affinity purified rabbit antibody (1:100, No. G-019-06, Phoenix Pharmaceuticals, Burlingame, CA). CGRP immunoreactivity was examined using a polyclonal antibody raised against the full length CGRP(1-37) peptide (1:1500, Ian Dickerson, Univ Rochester) for visualization with AlexaFluor 488 conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch). PACAP immunoreactivity was detected using a mouse PACAP monoclonal antibody (1:10, Jens Hannibal, Bisperg Hospital, Copenhagen, Denmark) followed by tyramide signal amplification (Hannibal, 2002). Following primary PACAP antibody incubation, the tissues were incubated in biotinylated horse anti-mouse antibody (1:200, 2 h; Vector Laboratories, Burlingame, CA) and treated with streptavidin-HRP (1:200, 30 min) before application of tyramide-biotin reagent (1:100, 10 min; Perkin Elmer, Waltham, MA). After extensive washing, the PACAP immunoreactivity was localized with Cy3-conjugated streptavidin (1:200, 2 h; Jackson Immunoresearch, West Grove, PA). In dual localization studies, the sections were incubated in PACAP and CGRP or CRH antisera concurrently. Tissue sections from BDA anterograde tracing and

excitotoxic lesion studies were also processed for immunocytochemistry using the same procedures. Images from immunocytochemistry, excitotoxic lesion and anterograde tracing experiments were acquired sequentially with appropriate filter sets using a Nikon E800 point scanning confocal microscope. Image analyses were performed using NIH ImageJ (Schneider et al., 2012) to threshold, determine signal area (pixel number in staining area) and calculate Pearson's and Mander's correlation coefficients. In within subject excitotoxic lesion studies, the area of immunoreactivity on the side of the lesion was compared to the vehicle control contralateral side.

*Transcript analyses*

Quantitative PCR (QPCR) was performed exactly as described previously (Girard et al., 2002, 2006; Hammack et al., 2009). Briefly, after euthanasia by rapid decapitation, the coronal rat brain sections were prepared using a rodent brain matrix (Ted Pella, Inc. Redding, CA) and the micropunched amygdala tissues were quickly frozen on dry ice for total RNA extraction using STAT-60 RNA/mRNA isolation reagent (Tel-Test "B", Friendswood, TX). All RNA were reverse transcribed simultaneously using random hexamer primers with the SuperScript II Preamplification System (Invitrogen, Carlsbad, CA) to obviate variability. Real-time QPCR was performed as described using SYBR Green I detection (Girard et al., 2002, 2006; Hammack et al., 2009). Briefly, cDNA templates were diluted 5-fold to minimize the inhibitory effects of the reverse transcription reaction components and assayed on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green I JumpStartTM

Taq ReadyMix (Sigma, St. Louis, MO) containing 3.5 mM MgCl2, 200 µM dATP, dGTP, dCTP and dTTP, 0.64 U Taq DNA polymerase and 300 nM of each primer in a final 25 µl reaction volume. Oligonucleotide primer sequences were: PACAP (S) 5'-CATGTGTAGCGGAGCAAGGTT-3' (AS) 5'- GTCTTGCAGCGGGTTTCC-3'; CRH (S) 5'-TGGATCTCACCTTCCACCTTCTG-3' (AS) 5'-CCGATAATCTCCATCAGTTTCCTG-3'. The melting profiles for amplified DNA fragments were performed to verify unique product amplification in the quantitative PCR assays. For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence (Girard et al., 2002, 2006). The increase in SYBR Green I fluorescence intensity (DRn) was plotted as a function of cycle number and the threshold cycle (CT) was determined by the software as the amplification cycle at which the DRn first intersects the established baseline. The transcript levels in each sample were calculated from the CT by interpolation from the standard curve to yield the relative changes in expression. For each target sequence, all samples from the same brain region were amplified together in the same assay to minimize variability. All data were normalized to 18S RNA.

## *Surgical procedures*

### *Anterograde tracing*

Rats were anesthetized with isoflurane (1.5-3.5%), and secured into a stereo-tactic apparatus (David Kopf Instruments, Tunjunga, CA). The skull was exposed from a midline incision and a micropipette (30-50 µm tip diameter) filled with 10% biotinylated

dextran amine (BDA; 10 kDa) was lowered into the LPBn using coordinates (from bregma in mm) AP: -9.3, ML: ±2.3, DV: -8.0, for iontophoretic tracer application (5 μA, 7 s on and 7 s off, 20 min total). The process was repeated on the contralateral LPBn. After 14 days, the 4% paraformaldehyde perfusion-fixed rat brains were processed and the cryosections incubated in 1:200 streptavidin-Cy2 (Jackson Immunoresearch) for BDA tracer localization. The anterograde tracing studies were sometimes performed in conjunction with peptide immunocytochemistry for concurrent localizations (Section 2.3). As for most peptide antisera, the PACAP antibody preferentially labeled fibers than soma which precluded immunocytochemistry of retrogradely labeled LPBn neurons from the CeLC.

*Excitotoxic lesion*

Adult male rats were surgically prepared as above and a microsyringe (1 μl, Hamilton Co., Reno, NV) was unilaterally placed into the LPBn (from bregma in mm, AP: -9.3, ML: ±2.3, DV: -7.9) for automated pump infusion of 2 mg NMDA in 200 nl over 4 min. The syringe was left in place for an additional 4 min and following postsurgical recovery the rats were returned to their home cages and for 7 days. NMDA excitotoxic lesion at the targeted site was verified by processing the brain cryosections for neuron specific nuclear protein (anti-NeuN, 1:1500) immunoreactivity as visualized using Cy3-coupled secondary antisera (Roman et al., 2012). Only brains that displayed LPBn neuronal loss were used for further analyses.

*Intra-amygdalar PACAP infusion*

Rats were anesthetized and secured in a stereotactic apparatus as described above. Four screws were secured into the exposed skull and two stainless steel cannulae (22 GA, PlasticsOne, Roanoke, VA) were targeted to the CeA bilaterally using coordinates (from bregma in mm) AP: -2.6, ML: ±4.5, DV: -7.2. A dental cement skullcap was formed to secure the cannula and during the 7 day postsurgical recovery the rats were routinely wrapped in a towel to habituate handling. For treatments, the rats were similarly restrained in a towel and PACAP or vehicle (0.05% BSA in saline) was slowly infused (1 µg/0.5 µl each side) at 0.25 µl/min (Harvard Apparatus, Holliston, MA) through an internal cannula that projected 1 mm from the guide cannulae; the PAC1 receptor specific agonist maxadilan (from Ethan Lerner, Harvard/Massachusetts General Hospital) was similarly infused in some studies. The peptide concentrations and treatment procedures were similar to those described in previous work (Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Roman et al., 2014). The infusion cannula were left in place for an additional minute before removal. Animal body weights were determined before and 24 h after infusions for all experiments (Kocho-Schellenberg et al., 2014). At the end of each study, the rats were perfused with 4% paraformaldehyde and the brains cryosectioned for cresyl violet staining to confirm cannulae placement. Only data from correct CeA cannulae placements are described in Results. PACAP infusions into misplaced targets outside of the CeA, including the basolateral amygdala, had no effects

on stress-related behavior, body weight, food consumption and water intake.

## *Behavioral assessments*

### *Elevated plus maze*

The plus maze was elevated 75 cm from the floor and consisted of two opposing open and two opposing closed arms (each arm 50 cm long and 10 cm wide) that extended perpendicularly from a central square platform (10 x 10 cm). The length of the closed arms were walled with black opaque plastic panels 30 cm in height. Illumination using a red bulb was 6 lux at the center of the maze. The rats were first room habituated for 10 min and then individually placed in the center of the maze facing a closed arm for free exploration for 5 min. A ceiling mounted camera digitally captured all movements during each session for analyses.

### *Mechanical sensitivity testing*

Mechanical sensitivity assessment was performed using von Frey mono-filaments (Stoelting, Wood Dale, IL). All rats were first habituated in the clear acrylic testing chamber 20 min/day for 4 days with a fan to generate ambient noise. On day of testing, the rats were placed in the acrylic testing chamber on top of a metal mesh floor (IITC Life Science Inc., Woodland Hills, CA) and habituated again for 10 min before the application of von Frey filaments to the lateral plantar surface of the hindpaw. In ascending diameter thickness, each filament was applied until bent at 30° for 5-7 s. The smallest filament that evoked a paw withdrawal in at least 3 of 5 trials was used as the

mechanical threshold for that trial. Thresholds from both the left and the right hindpaws were measured.

*Thermal sensitivity testing*

Responses to thermal stimuli were tested using a Hargreave's apparatus (Plantar Analgesia Meter, IITC Life Science Inc., Woodland Hills, CA). Prior to behavioral testing, the rats were first habituated in the acrylic testing chamber for 4 days. On day of testing, the rats were placed in an elevated clear acrylic testing chamber on top of a glass floor with an internal heating element that heated the glass to a consistent 30 °C. Using a guide light to target the hindpaw, a beam of focused radiant light (4-6 mm, set to 25% of active intensity) from the apparatus beneath the glass floor was delivered to the plantar surface of the paw. Upon rat awareness of the heat stimuli, as indicated by withdrawal or licking of the hindpaw, the heat source was immediately terminated and the reaction time automatically recorded. An automatic cut-off timer set at 30 s was built into the system to prevent tissue damage. Each time point represented the latency average of 3 trials from both the left and right hindpaw separated by 5 min inter-trial intervals. The PACAP, maxadilan and vehicle treatment groups exhibited comparable average baseline latency scores (PACAP, 12.9 s; maxadilan, 12.5 s; vehicle, 12.3 s).

*Experimental treatment and testing procedures*

*Experiment 1 - behavioral effects of amygdala PACAP infusions on elevated plus maze*

Adult male rats were cannulated for amygdala infusions as described in Surgical procedures. The rats were handled daily for habituation and after 7 day postsurgery recovery, the rats were randomly assigned to vehicle or PACAP groups (n = 10 per group). On experimental day, the rats were weighed for baseline measures and bilaterally injected with vehicle or PACAP38 as described in random order. The injection needle was left in place for 1 min after which the rats were returned to their home cages for 30 min and habituated in the testing room (10 min) before evaluation on the elevated plus maze. The rats were allowed to freely roam the maze for 5 min and all data were captured digitally. At the same time the following day, the vehicle and PACAP-treated rats were re-weighed to assess weight change over 24 h; food and water consumption were also measured. All weight change measures in this and subsequent experiments were performed between 0900 and 1000 h. All behavioral tests were completed between 0900 and 1500 h; behavioral testing was randomized and counter balanced for order and time of testing.

*Experiment 2 - nociceptive effects of PACAP after amygdala infusions*

Adult male rats were surgically prepared and handled as described in Experiment 1 above. The rats received 2 days of baseline thermal and mechanical sensitivity testing,

and on experiment day, the rats were weighed and received either vehicle or PACAP38 amygdala infusions (n = 6 per group) as described in random order. After 30 min, the rats were tested for mechanical sensitivity using von Frey filaments and evaluated for thermal sensitivity on a Hargreave's apparatus at subsequent time points (1 h, 4 h and 24 h). As before, weight change in the vehicle and PACAP-infused rats was assessed after 24 h; food and water consumption was also determined. As robust PACAP-induced thermal sensitivity was noted at 1 h, a separate study was prepared to better establish amygdala PACAP thermal nociception onset and persistence (30 min and 72 h time points) using exactly the same procedures (n = 7-8 per group). The thermal sensitivity data at the different time points from the two cohorts were combined for analyses in a linear mixed model using an autoregressive covariate structure as described in statistical methods.

*Experiment 3 - the nociceptive effects of amygdala maxadilan infusions*

Adult male rats were surgically prepared, handled and treated exactly as described for the first study in Experiment 2 except for the application of maxadilan (n = 7 - 8 per group). Thirty min after amygdala maxadilan infusion, the rats were tested for mechanical sensitivity using von Frey monofilaments; at subsequent time points the rats were evaluated on a Hargreave's apparatus for thermal sensitivity.

*Statistics*

Statistical Student's t-tests were performed using GraphPad PRISM v.6. For

analyses of thermal withdrawal thresholds, a linear mixed model using an autoregressive covariate structure was employed to allow combined analysis of two cohorts with differing timepoints, followed by pairwise comparisons between groups using Sidak-Holmes correction for multiple comparisons (MIXED procedure of the SAS System for Windows version 9.2; SAS Institute Inc, Cary, NC). All values represent the mean change ± SEM. $P < 0.05$ was considered significant.

## 2.4. Results

*PACAP and CGRP are expressed in the CeA and BNST*

Our previous studies identified regulated PACAP expression in the BNST (Hammack et al., 2009). In evaluating PACAP expression in other limbic structures, we observed significant levels of PACAP immunoreactivity restricted to the lateral capsular division of the CeA (CeLC; Fig. 2.1). A number of neuropeptides have been identified in the CeA including corticotropin releasing hormone (CRH) which has been shown to regulated by psychological stressors (Makino et al., 1999). However, unlike PACAP in the CeLC, CRH immunoreactivity in the amydala was prominent in the adjacent lateral (CeL) and medial (CeM) subdivisions of the CeA, recapitulating the apparent dichotomy of PACAP and CRH peptidergic pathways in the limbic system (Roman et al., 2014). Further, the pattern of PACAP and CRH expression following repeated stress appeared converse of that in the BNST. Whereas BNST PACAP was augmented after stress (Hammack et al., 2009; Roman et al., 2014), chronic stress increased CRH immunoreactivity levels in the CeA approximately 2-fold without altering CeA PACAP

expression (Fig. 2.1A-C). The stress-mediated changes PACAP and CRH staining in the CeA mirrored transcript expression patterns (Fig. 2.1D) and in aggregate were suggestive of their distinct but complementary roles in stress pathways and behaviors.

A number of neuropeptides exhibit distinct expression patterns within the CeA (Cassell et al., 1986). From staining patterns the immunoreactivity for PACAP in the CeLC was largely punctate which appeared characteristic of terminals and varicosities of neuronal PACAP fiber projections from distal nuclei. As the CeLC is heavily innervated by the PBn in the spino-parabrachioamygdaloid tract (Bernard et al., 1996; Gauriau and Bernard, 2002; Rouwette et al., 2012; Veinante et al., 2013) and PACAP is highly expressed in sensory neurons in many pathways (Beaudet et al., 1998; Mulder et al., 1994; Pettersson et al., 2004b; Zhang et al., 1995), we examined whether the PACAP immunoreactivity in the CeLC reflected parabrachioamygdaloid projections. Further, as fibers in the CeLC have been described to contain CGRP immunoreactivity (Dobolyi et al., 2005), we also compared the relative distribution of PACAP and CGRP in the parabrachioamygdaloid tract.

In these studies, PACAP and CGRP immunoreactivities displayed considerable overlap in fiber elements (Fig. 2.2A-C) that appeared to form basket-like networks suggestive of axosomatic innervation of CeLC neurons. Given the heavy density of the peptide immunoreactivites, both Pearson's and Mander's correlation coefficients were determined for the acquired images to assess the extent of CeLC PACAP and CGRP colocalization. For both measures, scores closer to 1 represent greater degrees of overlap and from 4 independent studies, Pearson's r was >0.7 and Mander's coefficient was >0.6

(Mander's CGRP/PACAP ratio = 0.625; PACAP/CGRP ratio = 0.631).

Since the bed nucleus of the stria terminalis (BNST) is part of the central extended amygdala and has been described to display both PACAP and CGRP expression and function (Hammack et al., 2009; Sink et al., 2011), the relationship between PACAP and CGRP within the BNST was also investigated. BNST PACAP and CGRP expression was highest within the oval nucleus (BNSTov) and as in the CeLC, PACAP and CGRP immunoreactivities were coexpressed in a majority of the fiber elements (Fig. 2.2D-F). Image analyses were performed as before and from 3 independent experiments, Pearson's coefficient for PACAP and CGRP colocalization was approximately 0.7, and Mander's coefficient was approximately 0.6 (Mander's CGRP/PACAP ratio = 0.57; PACAP/CGRP ratio 1= 0.56). Hence the two statistical measures were in good agreement and suggested that more than half of the PACAP or CGRP neuronal fibers projecting to the CeLC and BNSTov expressed both peptides.

*PACAP and CGRP immunoreactives in the CeLC and BNST are localized to projection fibers from pontine parabrachial nucleus (PBn)*

From several considerations, our evaluations for the potential origins of the PACAP- and CGRP-expressing neurons projecting to the CeLC and BNSTov narrowed to the LPBn. The external LPBn contains a large population of PACAPergic neurons that may transmit signals to the amygdala (Das et al., 2007; Hannibal, 2002; Resch et al., 2013). Further, CGRP expression in the CeLC and BNSTov has been suggested previously to originate from PBn neurons (Dobolyi et al., 2005). Hence from these

observations, we examined whether PACAP- and CGRP-expressing fibers to the CeLC and BNSTov were components of the parabrachioamygdaloid tract.

For these studies, we first evaluated whether anterograde fibers from the LPBn to the amygdala and BNST expressed PACAP. From injection site analyses, the BDA infusions into the LPBn was confined to a small area (Fig. 2.3A). In the amygdala, the neuroanatomical tracer was confined to the CeLC and upon immunocytochemical processing, a subset of the BDA-labeled fibers in the CeLC expressed PACAP-immunoreactivity (Fig. 2.3B). Although these results provided evidence for CeLC PACAP immuonoreactivity originating from the LPBn, the small focal size of the PBn BDA injection resulted in a modest number of labeled fibers in the CeLC. Hence the number of BDA labeled fibers was not as extensive as that observed for PACAP-immunoreactivity which precluded estimations of the relative contribution of CeLC PACAP immunoreactive fibers originating from the PBn. From the same limitations, the BDA-labeled fibers from the PBn to the BNST appeared low (data not shown).

As an independent means of assessing peptide expression in LPBn projection fibers and to facilitate dual PACAP and CGRP immunocytochemistry in the same tissues, the LPBn was lesioned before amygdala and BNST immunocytochemistry (Fig. 2.4). As the BDA anterograde fiber labeling studies demonstrated that the external lateral PBn projected only to the ipsilateral amygdala, only one side of the PBn was lesioned so that the contralateral LPBn and limbic structures could remain intact and serve as vehicle controls. Accordingly, one side the LPBn was lesioned by excitotoxic NMDA injection (2 μg NMDA in 0.2 μl) and after postsurgery recovery for 7 days, coronal brain

cryosections were prepared to assess the extent of PBn lesion and altered peptide immunocytochemistry in the ipsilateral CeLC/BNST compared to staining patterns on the contralateral side. Only brain lesions with neuronal loss in the external LPBn as identified by diminished neu-N staining (Fig. 2.4A and B) were used in subsequent analyses.

Following external LPBn lesion, PACAP and CGRP immunoreactivities in both the CeLC and BNSTov were greatly reduced. The tissue sections were simultaneously processed for PACAP and CGRP immunoreactivities and within subjects, CeLC PACAP immunoreactivity was diminished 70% ± 5% on the side ipsilateral to the PBn lesion compared to staining levels in the contralateral CeLC in which the corresponding PBn received vehicle injection ($t(2) = 4.41$, $p = 0.048$; Fig. 2.4C-D and 2.5A). The same changes were observed in the BNSTov. PACAP staining levels in the BNSTov ipsilateral to the PBn lesion were diminished 59% ± 11% compared to the contralateral BNSTov with PBn vehicle injections ($t(2) = 5.77$, $p = 0.029$; Fig. 2.4G-H and 2.5B). As PACAP and CGRP demonstrated significant colocalization in these structures (Fig. 2.2), a similar change in CGRP staining was therefore anticipated. From analyses, LPBn lesions resulted in a 64% ± 8% loss in CGRP immunoreactivity in the CeLC ($t(2) = 7.49$, $p = 0.017$) and 72% ± 6% in the BNSTov ($t(2) = 8.90$, $p = 0.012$) compared to contralateral structures with vehicle injections into the PBn (Fig. 2.4E-F, 2.4I-J, 2.5A and 5B). Hence the anterograde labeling/lesion studies complement immunocytochemical data to demonstrate that PACAP and CGRP can be colocalized in the LPBn and that their projections are substantial components in the fibers innervating the CeLC and BNSTov.

*PACAP signaling in the amygdala alters emotional behaviors and pain responses*

Our previous work demonstrated that PACAP signaling in the BNST enhances anxiety-related responses including increased baseline startle responses, decreased open arm entries on the elevated plus maze, decreased open field crossings, decreased exploratory behavior in novelty tests and decreased weight gain (Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Roman et al., 2014). To examine whether PACAP expression and signaling in the central amygdala produced similar stress-related behavioral responses, we implanted bilaterally cannulae targeting the CeA for PACAP infusions (1 μg/0.5 μl) following previous treatment protocols (Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Roman et al., 2014; Experiment 1). Similar to PACAP-elicited responses in the BNST, amygdala PACAP infusions induced anxiety-like responses as shown by decreased open arm time (54.2 vs 88.3 s, $t(18) = 2.71$; $p = 0.01$) and open arm entries (5.9 vs 13.8; $t(18) = 4.39$, $p = 0.0003$) compared to vehicle-treated animals on the elevated plus maze (Fig. 2.6A). Unlike the BNST where PACAP had no apparent effects on locomotor activity, PACAP injections into the CeA appeared to produce a small but significant decrease in total distance traveled during the test period not attributed to spontaneous freezing behavior. To mitigate this potential confound, open arm preference (open : total arm entries) was calculated for each animal as this measure is less prone to locomotor vagaries. Whereas vehicle control animals had no preference for either open or closed arms (open : total arm entries = 0.51 ± 0.03), CeA PACAP- infused animals demonstrated diminished open arm preference (Fig. 2.6B, open : total arm entries = 0.32 ± 0.04; $t(18) = 3.70$, $p = 0.0015$). These PACAP-mediated

changes were comparable to those observed following BNST PACAP injections suggesting that PACAP signaling in the BNST and CeA can contribute to stress- related behaviors.

Similar to stress-mediated behaviors, BNST PACAP infusions were also capable of inducing anorexia-like responses resulting dramatic animal weight loss over the next 24 h which approximated 5-8% of body weight and was reflected by decreased food consumption. Accordingly, animal weight changes were also monitored during the CeA PACAP infusion studies (Experiments 1 and 2). After 24 h, animals with CeA PACAP injections demonstrated a small (~1%) but significant decrease in body weight compared vehicle treated animals ($t(45) = 2.63$, $p = 0.012$). Given the small weight changes, we sought to establish these observations using the PAC1 receptor selective agonist maxadilan (Experiment 3). CeA maxadilan infusions again produced a small decrease in body weight (1.5% decrease; $t(13) = 2.81$, $p = 0.014$) which was accompanied by diminished food intake (17.5% decrease; $t(13) = 2.66$, $p = 0.018$) without apparent changes in water consumption ($t(13) = 1.47$, $p = 0.163$). These changes largely reflected the propensity for vehicle treated animals to gain a small amount of weight during the 24 h period while the PACAP treated animals experienced a slight weight loss. Hence, in apparent contrast to the BNST, the effects of CeA PACAP signaling on stress related anxiety-like responses did not appear to be strongly associated with weight and feeding changes.

The fiber projections from the LPBn to the CeLC are part of the spino-parabrachial amygdaloid pathway conveying nociceptive information from the dorsal

horn to the amygdala. PACAP has been identified at many sensory pathway intersections including the dorsal root ganglion, layers 1 and 2 of the dorsal horn, and from previous and current work, the LPBn. The CeLC responds to noxious stimuli and in modulating pain perception may contribute to the affective component of the pain experience. Hence from its attributes as a sensory peptide and its localization in the CeLC, we examined whether PACAP signaling in the amygdala also altered spinal pain-associated reflexes. As before, cannulae were placed into the amygdala bilaterally and following recovery, the rats were habituated for Hargreave's thermal nociception tests (Experiment 2). A baseline latency for hindpaw thermal withdrawal was first determined for each rat; PACAP was subsequently infused into the CeA and the temporal changes in hindpaw withdrawal to the same thermal stimuli were examined over the next 72 h. Following PACAP infusion, there was a significant reduction in paw withdrawal latency at 30 min (35% decrease in latency; veh, 13.0 ± 1.0s vs PACAP 9.1 ± 0.9s, $p = 0.002$; Fig. 2.7A) and at 1h (31% decrease in latency; veh, 11.8 ± 0.9 s vs PACAP, 7.9 ± 0.6 s, $p = 0.011$). The PACAP-induced responses persisted at 4 h (21% decrease in latency; veh, 11.3 ± 0.8 s vs PACAP 8.9 ± 0.7 s; $p = 0.015$) and returned to baseline by 24 h. There was a small but significant decrease in latency at 72 h post injection compared to the corresponding vehicle control group ($p = 0.021$); whether this reflected any PACAP-mediated plasticity in the CeA remains to be examined. Again, the thermal sensitivity responses were recapitulated with the PAC1 receptor-specific agonist maxadilan (Experiment 3). CeA maxadilan infusions decreased paw withdrawal latency approximately 24% (veh, 11.7 ± 0.9 s vs maxadilan, 8.9 ± 0.6 s; $p = 0.002$; Fig. 2.7B) at 1 h which returned to baseline by

24 h. Overall, the PACAP and maxadilan results were robust and well reproducible across trials suggesting that intra-amygdalar PACAP signaling can facilitate thermal hyperalgesia.

To assess whether CeA PACAP infusion would elicit similar changes on mechanical threshold, the same animals were also evaluated using von Frey hair stimulation tests (Experiment 2). From baseline tests, all animals demonstrated decreases in mechanical threshold after repeated trials over time. Although mechanical threshold in the PACAP-and maxadilan-treated rats appeared decreased compared to vehicle control animals after 30 min, analyses revealed a trend rather than statistical difference (PACAP, $t(10) = 1.7$, $p = 0.11$; maxadilan, $t(13) = 1.65$, $p = 0.12$) which reflected in part the high variability within the assay. These apparent PACAP changes in mechanical threshold dissipated by 2 h post-peptide infusion. As thermal and mechanical pain are transduced by separate mechanisms, these differences may have contributed to the observed efficacy of PACAP between the two measures. Nevertheless, the ability for PACAP to modulate pain responses via amygdala signaling appears novel and suggests that it may carry nociceptive information to impact the behavioral and emotional aspects of pain.

## 2.5. Discussion

The central nucleus of the amygdala integrates nociceptive and stress-related signals that may be important for behavioral responses and the formation of emotional memory. In examining PACAP/PAC1 receptor expression and function in the limbic system, we identified high levels of fiber PACAP immunoreactivity in the CeLC. The

CeLC is innervated by LPBn neurons that form part of the spino-parabrachioamygdaloid pathway and although PBn PACAP expression was previously described, the targets of these PBn PACAP neurons were not identified. Our current work identified PACAP immunoreactivity in anterogradely labeled LPBn projection fibers to the CeLC, and importantly, LPBn lesions significantly abolished PACAP immunoreactivity in the CeLC and BNST. These studies were also revealing in demonstrating the relationships between PACAP and other CeA peptides. Both CRH and CGRP share functional similarities with PACAP in mediating pain, stress and anxiety-like behaviors (Hammack et al., 2002; Koob and Heinrichs, 1999; Lee and Davis, 1997; Sink et al., 2011). Yet the dual localization studies demonstrated a dichotomy in PACAP and CRH expression pattern; the localization of PACAP predominantly to the CeLC was distinct from CRH in the CeL which suggested separate but coordinate functions in intra-amygdalar neurocircuits. By contrast, PACAP and CGRP immunoreactivities in CeLC and BNST fibers were well colocalized from image analyses, and PBn lesions abolished much of the staining for both peptides in the CeLC and BNST to a comparable extent. Limbic PACAP and CGRP signaling share similarities in feeding and anxiety-like behaviors (Carter et al., 2013; Hammack et al., 2009; Kocho-Schellenberg et al., 2014; Sink et al., 2011); how their coordinate signaling modulates CeA and BNST functions however, remains to be evaluated.

Despite the extensive PACAP and CGRP colocalizations (60-70%), PACAP and CGRP may also exhibit independent CeLC and BNST functions. After LPBn lesions the remaining PACAP and CGRP immunoreactivities appeared largely dissociate (Pearson's

coefficient 0.3-0.4) which may have represented endogenous CeLC/BNST peptide expression or PBn subpopulations expressing one of the peptides not affected by the lesion procedures. The former may be consistent with the upregulation of BNST PACAP transcripts by chronic stress (Hammack et al., 2009). PACAP and CGRP immunoreactivities in subpopulations of dorsal root ganglion (DRG) neurons for example can be separate and overlapping (Mulder et al., 1994), and comparable expression patterns may be present in the PBn and limbic structures.

The presence of PACAP in the parabrachioamygdaloid pathway has prominent implications in its roles modulating the sensory and emotional consequences of pain. The ability for the amygdala to integrate pain processes and the emotional aspects of behavior has been well appreciated (Bernard et al., 1992; Gauriau and Bernard, 2002; Ulrich-Lai et al., 2006; Morano et al., 2008; Rouwette et al., 2011, 2012; Veinante et al., 2013) and among its many functions, the roles of PACAP as a sensory peptide are well recognized. PACAP and its PAC1/VPAC receptor subtypes are expressed in central and peripheral nervous system regions that mediate nociception. PACAP is found in small-diameter nociceptive DRG and in lamina I/II of the spinal cord neurons (Beaudet et al., 1998; Mulder et al., 1994; Pettersson et al., 2004a, 2004b), and neuropathic pain through axotomy, chemical induced cystitis or related models of nerve injury, can induce long-lasting upregulation of PACAP or PACAP receptor expression in these tissues (Dickinson et al., 1999; Mulder et al., 1994; Pettersson et al., 2004a; Vizzard, 2001). In the central nervous system, PACAP can be found in many regions such as the hypothalamus, limbic system, hippocampus, various brainstem nuclei including the PBn,

and a number of thalamic and cortical regions implicated in pain processing (Das et al., 2007; Hannibal, 2002; Resch et al., 2013).

However, the early investigations on PACAP in mediating pain were equivocal resulting in hyperalgesia in some experimental paradigms and hypoalgesia in others. These divergent responses likely reflected differences in the time course used in pain assessments in the different experimental models, and the peripheral vs central actions of PACAP. Peripheral intraplantar PACAP injections, for example, appeared to produce mechanical hypoalgesia in both the early and late stages of inflammatory pain (Sandor et al., 2009) whereas intrathecal injections were hyperalgesic (Ohsawa et al., 2002). In detailed studies, intrathecal PACAP administration resulted in an immediate analgesic response as measured by tail flick latencies, but transitioned into a long lasting hyperalgesia as demonstrated by increased aversive responses (Shimizu et al., 2004). By contrast, the studies using PACAP and PAC1 receptor knockout mice demonstrated unequivocally a role for PACAP signaling in the development of persistent pain. Mice deficient in PACAP or PAC1 receptor do not develop normal pain responses after arthritic pain or neuropathic pain (Jongsma et al., 2001; Mabuchi et al., 2004). PACAP knockout mice do not display thermal hyperalgesia or mechanical allodynia after intraplantar carrageenan injection or spinal nerve transection, but show normal acute nociceptive processes compared to wildtype mice (Mabuchi et al., 2004). In congruence, PAC1 receptor null mice exhibit dramatic decreases in thermal and mechanical nociceptive responses in the late phase of the formalin test, but preserve acute nociceptive processes in unchallenged states (Jongsma et al., 2001). Hence PACAP/PAC1 receptor

signaling and resulting neuroplasticity appear critical in the central sensitization and development of persistent pain states.

Fibers from the lamina I spinal cord neurons carry thermal and mechanical noxious stimuli and project heavily via the spino-parabrachioamygdaloid tract to the lateral and external medial PBn (Gauriau and Bernard, 2002; Todd, 2010). From the convergence of these projections onto the PBn, the sensory representations on the PBn neurons are therefore necessarily large, covering several areas of the body. The majority of PBn neurons then project onto the lateral division of the BNST, the ventral medial hypothalamus (VMH), and the CeA; interestingly, as in the dorsal horn, high levels of PACAP expression are found within all of these regions. The LPBn prominently innervates the CeLC and consistent with the modalities conveyed by the tract, in vivo electrophysiological studies demonstrate that these CeLC neurons are selectively activated by thermal and mechanical nociceptive stimuli with receptive fields that can encompass the entire body. Hence from the broad body areas capable of stimulating the PBn and CeLC, the stimulus-response profiles, and the demonstration that spino-parabrachioamygdaloid tract lesions in the dorsolateral funiculus does not modify noxious stimuli response latency/threshold, the amygdala does not appear to mediate sensory discrimination but the affective-emotional and behavioral consequences of pain.

Many models of visceral, inflammatory and neuropathic pain have been shown to increase not only CeA neuronal excitability and PBn-CeA transmission, but also CeA c-fos expression and ERK activation (Veinante et al., 2013) which may play roles in pain-related neuroplasticity. Among bioactive peptides, CeA infusions with oxytocin,

neurotensin and galanin have produced anti-nociceptive responses (Dobner, 2006; Jin et al., 2010; Robinson et al., 2002); interestingly, CRH and CGRP have been described to produce either nociceptive or antinociceptive processes which may have been related to dose and temporal parameters (Cui et al., 2004; Han et al., 2010; Ji et al., 2013; Xu et al., 2003). To facilitate understandings of PACAP roles in the CeLC, our current studies demonstrated that PACAP infusions into the CeA heightened noxious stimuli responses, especially in thermal reactivity tests. The effects of PACAP can be mediated by PAC1/VPAC receptors and notably, the PACAP-elicited CeA stress and nociceptive effects were recapitulated using maxadilan to implicate specific activation of the PAC1 receptor in these responses. Although the studies did not discriminate hyperalgesia from allodynia or spontaneous pain, the decrease in hindpaw withdrawal latency after CeA PACAP treatment was robust to clearly demonstrate altered sensory responses. The CeA PACAP effects in mechanical sensitivity assessments, however, appeared smaller which may have reflected assay variability in the testing protocol or neuronal responses to specific sensory modalities. The PBn responses to thermal stimuli are greater than those from mechanical stimuli (Bernard et al., 1996) and whether these mechanistic signals to the CeLC resulted in smaller PACAP-mediated mechanical responses remain to be established. The CeLC has major projections to the BNST, the dorsal substantia innominata and the medial CeA (CeM) which represents the major output of the CeA. The CeM has reciprocal projections to other nociceptive effector centers including thalamic nuclei, periaqueductal gray, lateral hypothalamus, ventromedial reticular formation, substantia nigra, rostral tegmental area, locus coeruleus, and dorsal raphe

complex; hence in aggregate, the CeA is well integrated within ascending and descending pathways to influence nociceptive signal processing and responses.

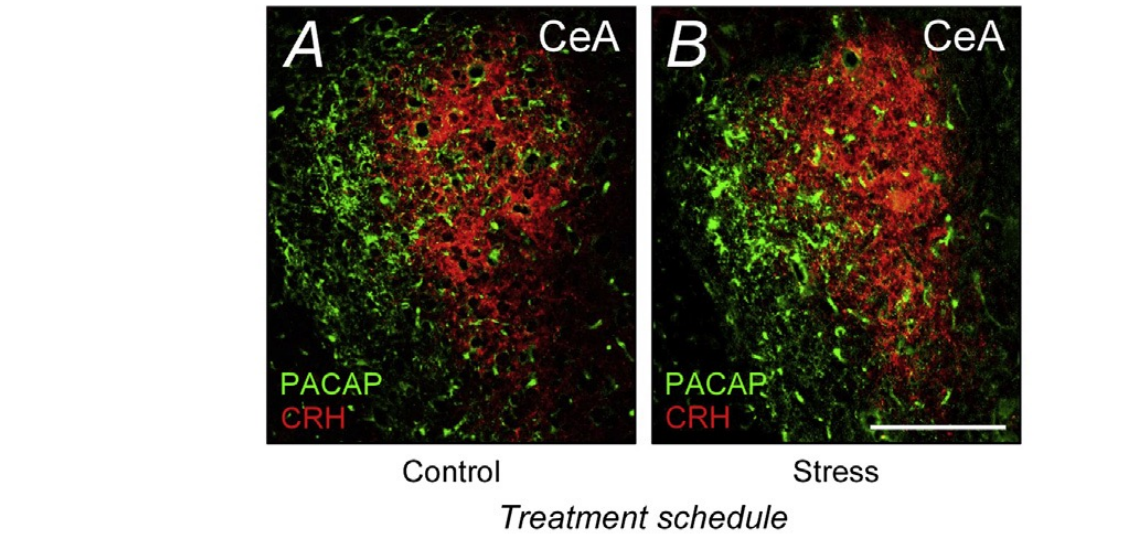
The amygdala assigns emotional valence to extrinsic challenges and has been well studied with respect to fear. The prominent nociceptive inputs to the CeLC and in particular the high levels of PACAP expression carrying nociceptive information in the spino-parabrachioamygdaloid tract provide important mechanistic insights on how chronic pain can initiate and/or amplify stress- related behavioral abnormalities, including depression and anxiety disorders. As in the BNST, PACAP signaling in the amygdala promoted anxiety-like responses. CeA PACAP infusions decreased open arm time, entries and preference on the elevated plus maze which appeared comparable in efficacy compared to that observed from BNST signaling. Although CeA PACAP infusions may have induced nociceptive sensitivity to decrease locomotion and affect behavior, mitigating the potential confound by open arm preference analyses still demonstrated PACAP-mediated increases in anxiety-like behaviors. Conversely, there is also a small possibility that CeA PACAP-induced stress- and anxiety-related behaviors may have contributed to the heightened nociceptive responses described above; this consideration is being pursued in ongoing studies. However, unlike the overt BNST PACAP-elicited anorexia that accompanied the stress-related behavioral responses, CeA PACAP signaling had modest effects on feeding and weight change. These observations suggested that the PACAP effects on stress-related behaviors and feeding may be not be strongly associated mechanisms or circuits; the small changes in weight, for example, may have reflected PACAP effects on thermogenesis (Hawke et al., 2009). Interestingly,

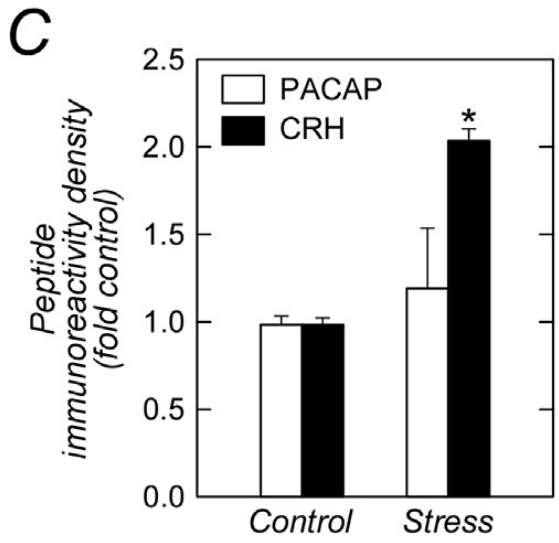
the PBn PACAP projections to the BNST also implicate direct nociceptive transmission to the BNST and in agreement, the anterolateral BNST has been shown to participate in pain and stress-induced nociceptive hypersensitivity (Morano et al., 2008; Rouwette et al., 2011; Tran et al., 2012). As in the BNST (Roman et al., 2014), preliminary experiments have shown that PACAP6-38, a PAC1/VPAC2 receptor antagonist, is capable of attenuating the effects of CeA PACAP signaling (data not shown). Although the neurocircuits and mechanisms underlying the CeA PACAP effects have not been examined extensively, one PACAP function has been suggested to potentiate excitatory transmission at the BLA-CeL synapse by enhancing post-synaptic AMPA receptor levels (Cho et al., 2012). The identities of PACAP targets in the CeLC, the functional mechanisms and consequences of PACAP CeLC signaling, and the functional relationships between PACAP and CGRP and CRH activities all remain to be investigated.

In summary, our results suggest that PACAP signaling via nociceptive fibers in the spino-parabrachioamygdaloid and associated tracts to the CeA and BNST may represent mechanisms that associate chronic pain with hypersensitivity and behavioral abnormalities including depression and anxiety-related disorders. Previous studies have shown that PACAP is a pleiotropic peptide with neurotransmitter, hormonal and neurotrophic functions which can facilitate neuroplasticity in development and regeneration after injury. PACAP signaling in chronic stress, fear and pain may facilitate the neuronal remodeling and plasticity in the limbic system that promote the maladaptive

behavioral responses, and transition short-term memory to long term forms that appear necessary for fear memory consolidation associated with PTSD.

## 2.6. Figures


A
CeA
PACAP
CRH
B
CeA
PACAP
CRH
Control
Stress
Treatment schedule


C
Peptide immunoreactivity density (fold control)
PACAP
CRH
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2.5
2.0
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Control
Stress

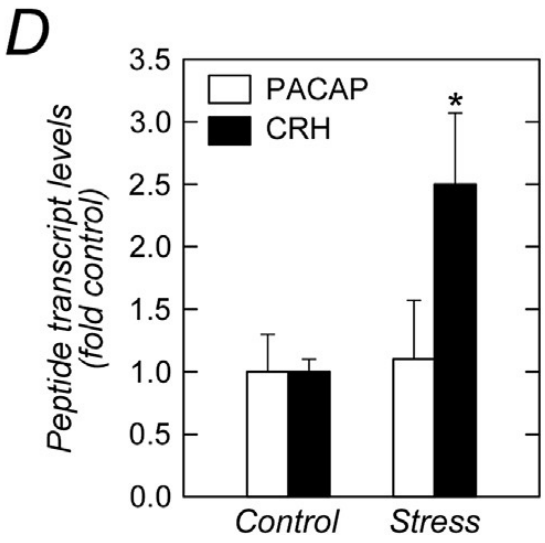
D
Peptide transcript levels (fold control)
PACAP
CRH
*
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Stress

**Figure 2.1. PACAP and CRH immunoreactivities are differentially distributed and regulated in the CeA**. Tissue sections from control (A) and chronically stressed (B) rats were examined for CeA PACAP (Cy2, green) and CRH (Cy3, red) staining patterns. In both groups, CeA fiber PACAP immunoreactivity was predominantly in the lateral capsular region (CeLC) with diffuse staining extending into the lateral division (CeL); CRH immunoreactivity was localized predominantly to the CeL. From quantitative image analyses, only CRH immunoreactivity was augmented by chronic variate stress (C, n = 3). These results complemented quantitative PCR measurements which also demonstrated increased CRH transcript expression after stress (D, n = 6). Data represent mean ± SEM. Asterisk, significantly different from control at $p < 0.05$. Scale bar, 250 μm.

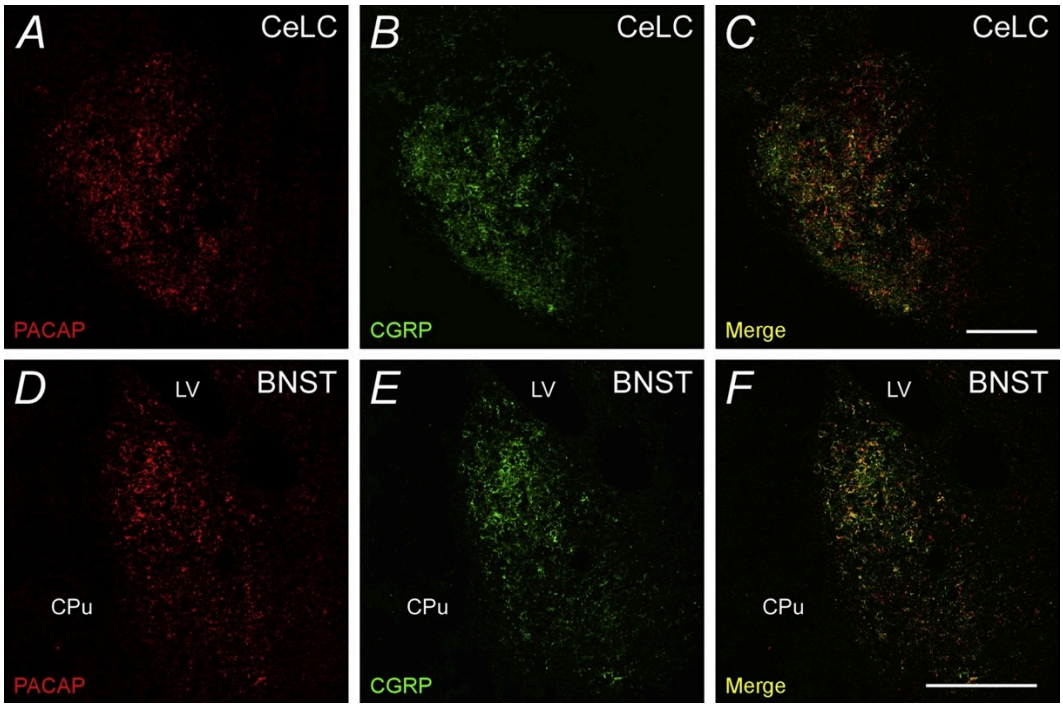


**Figure 2.2. PACAP and CGRP immunoreactivities can be colocalized in the CeLC and BNST.** Tissue sections for the amygdala (A-C) and BNST (D-F) were processed for dual PACAP (Cy3, red) and CGRP (Alexa488, green) immunocytochemical localization. The merged micrographs demonstrate that in both regions, PACAP and CGRP immunoreactivities were largely colocalized (yellow) in the same fiber structures. Amygdala, representative micrograph from 4 independent experiments; BNST, representative micrograph from 3 experiments. LV, lateral ventricle; CPu, caudate-putamen. Correlation coefficients described in text. Scale bar, 200 μm for corresponding tissues.

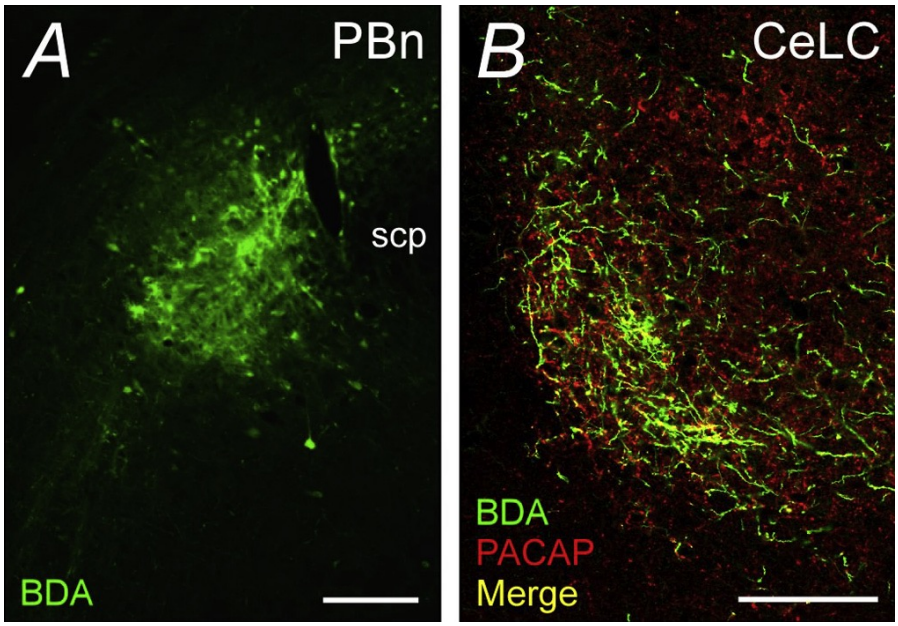


**Figure 2.3. PBn projection fibers to the CeLC demonstrate PACAP immunoreactivity.** Biotinylated dextran amine (BDA, 10 kD; 10%) was injected iontophoretically into the LPBn for anterograde transport into the CeLC over 14 days. BDA at the LPBn injection site (A) and in the projection fibers to the CeLC (B) were detected using streptavidin- conjugated Cy2 (green). Processing of the same CeLC sections for PACAP immunoreactivity (Cy3, red) demonstrated that the LPBn projection fibers can contain PACAP (B, merge in yellow). Representative data from 3 separate preparations. scp, superior cerebellar peduncle. Scale bar, 200 μm for corresponding tissues.

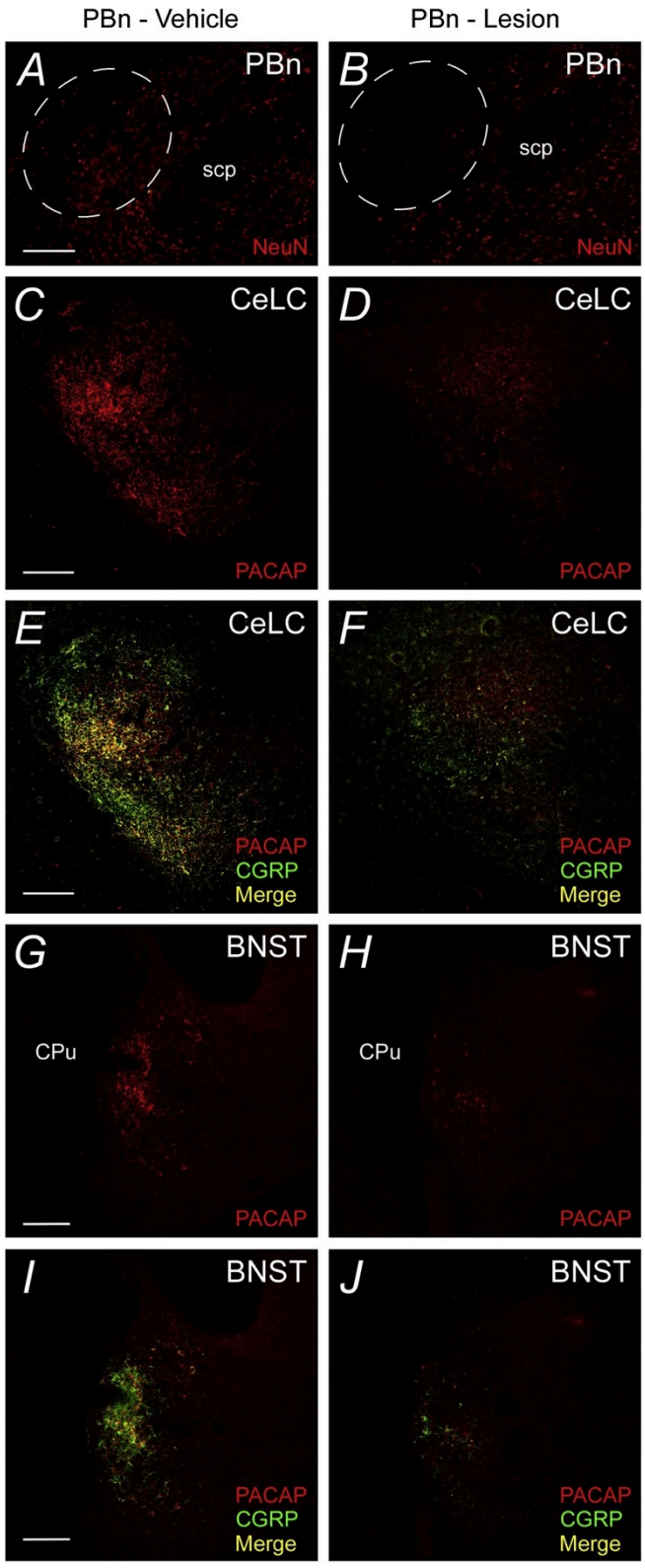
PBn - Vehicle
PBn - Lesion
A
PBn
scp
NeuN
B
PBn
scp
NeuN
C
CeLC
PACAP
D
CeLC
PACAP
E
CeLC
PACAP
CGRP
Merge
F
CeLC
PACAP
CGRP
Merge
G
BNST
CPu
PACAP
H
BNST
CPu
PACAP
I
BNST
PACAP
CGRP
Merge
J
BNST
PACAP
CGRP
Merge

**Figure 2.4. Excitotoxic LPBn lesions diminish PACAP and CGRP fiber immunoreactivities in the CeLC and BNST.** The LPBn was unilaterally lesioned with NMDA as described in Methods; the contralateral LPBn received vehicle. After 7 days, the PBn sections were processed for neuron-specific nuclear NeuN immunoreactivity (Cy3, red) to assess the specificity and extent of the lesion. Whereas vehicle injections had no apparent effects (A), NMDA injections produced substantial LPBn neuronal loss (B, dashed circled area). Representative vehicle treated and contralateral NMDA excitotoxic lesioned PBn in the same animal are shown; the lesioned image was flipped to facilitate comparison. CeA and BNST tissue sections from the NMDA excitotoxic lesioned animals were processed for dual PACAP and CGRP immunocytochemical localizations. Similar to Fig. 2.2, tissue sections ipsilateral to LPBn - vehicle injections (left panels) demonstrated substantial PACAP (Cy3, red) and CGRP (AlexaFluor 488, green) colocalization in the CeLC (C and E) and BNST (G and I); colocalization in merged micrographs illustrated in yellow. By contrast, the same CeLC and BNST regions in the contralateral half that received LPBn NMDA excitotoxic lesion (PBn - lesion) demonstrated marked decreases in both PACAP and CGRP immunoreactivities. Again, micrographs from the stained CeLC and BNST regions from the PBn - lesioned side were flipped for comparisons with the control vehicle e injected side from the same animals to facilitate comparisons. These data were consistent with the colocalization of PACAP and CGRP in Fig. 2.2 scp, superior cerebellar peduncle; CPu, caudate putamen. Representative figures from 3 separate animals. Scale bar, 200 μm.

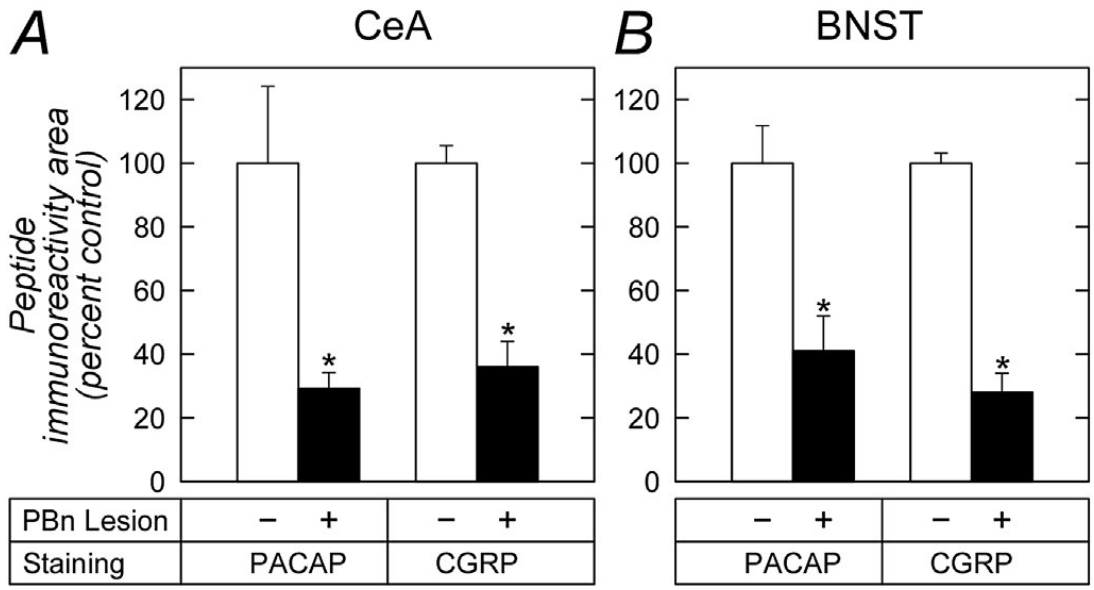


**Figure 2.5. CeA and BNST peptide immunoreactivities are diminished after PBn lesions.** PACAP and CGRP immunoreactivities in the CeA (A) and BNST (B) from studies described in Fig. 2.4 were subjected to image analyses as described in Methods. The PBn lesions decreased PACAP and CGRP immunoreactivities in the limbic regions to a comparable extent compared to levels on the contralateral hemisphere with PBn - vehicle injections. n = 3, data represent mean ± SEM. *, different from vehicle control at $p < 0.05$.

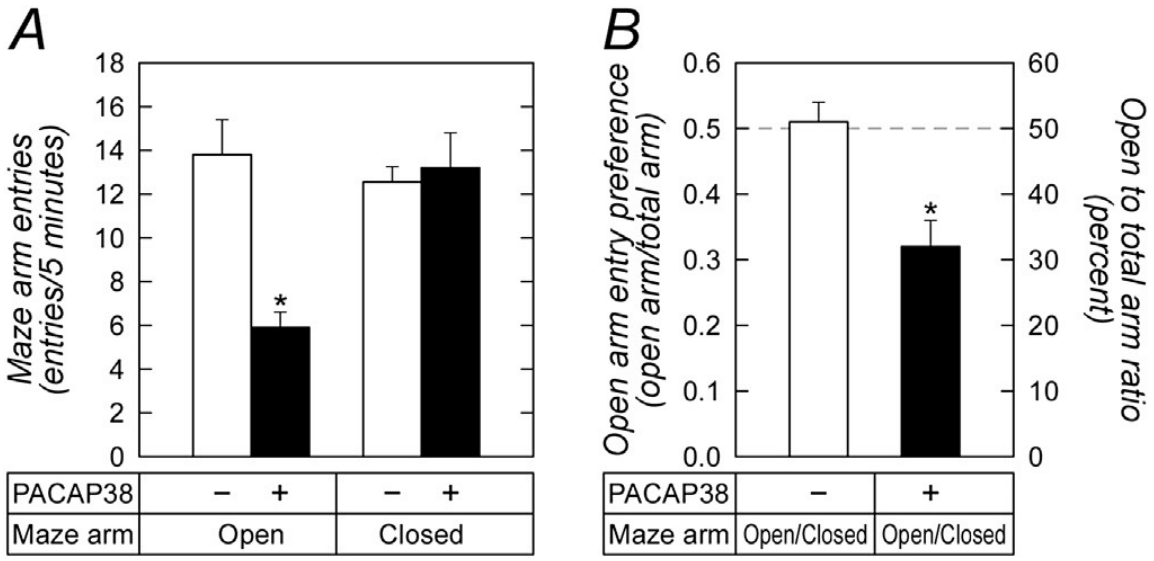


**Figure 2.6. PACAP infusions into the CeA decrease open arm entries on the elevated plus maze.** Adult rats were cannulated as described in Methods for CeA PACAP infusions. Thirty minutes after PACAP injection, the animals were placed in the center square of the elevated plus maze, facing a closed arm, for behavior testing during a 5 min period. All movements were tracked digitally for data analyses. Total open arm entries (A) and open arm preference (B, open arm entries/total arm entries) were calculated. CeA PACAP signaling significantly increased anxiety-like behavior reflected by decreased number of open arm entries and open arm preference. There were no changes in the number of closed arm entries and there were no indications of freezing behaviors. n = 10 per group, data represent mean ± SEM, *, different from vehicle control $p < 0.05$.

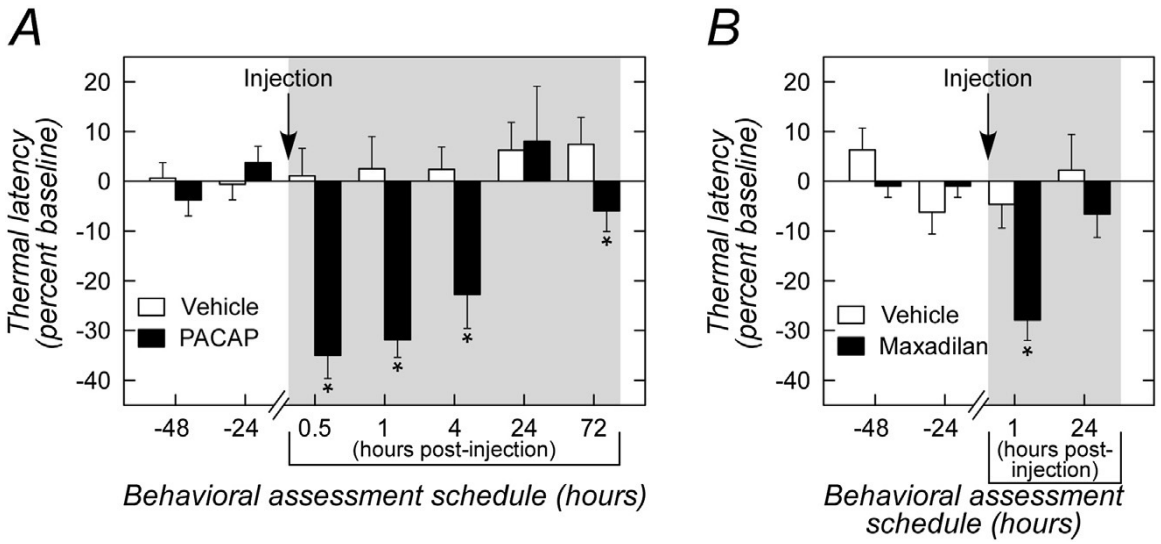


**Figure 2.7. CeA PACAP/PAC1 receptor signaling increases thermal sensitivity.** A, Rats were habituated in Hargreave's thermal sensitivity apparatus with 2 days of baseline assessments (24 and 48 h). PACAP was subsequently infused into the CeA (single injection) for thermal testing at the indicated time (shaded area). Whereas vehicle injection produced no apparent responses changes compared to baseline (white bars), CeA PACAP infusions consistently decreased thermal latency responses (black bars) up to 4 h post treatment. The responses dissipated by 24 h; the small but significant decrease in thermal latency at 72 h may reflect latent plasticity events. n = 6 - 8 per group, data represent mean response ± SEM, *, different from corresponding vehicle control, $p < 0.025$. B, the PACAP-induced decrease in thermal latency was mirrored in CeA infusions with the PAC1 receptor specific agonist maxadilan. The maxadilan responses observed at 1 h was again dissipated by 24 h n = 7 - 8 per group, data represent mean response ± SEM, *, different from corresponding vehicle control, $p = 0.002$.

## 2.7. References


Almli, L.M., Mercer, K.B., Kerley, K., Feng, H., Bradley, B., Conneely, K.N., Ressler, K.J., 2013. ADCYAP1R1 genotype associates with post-traumatic stress symptoms in highly traumatized African-American females. Am. J. Med. Genet. B Neuropsychiatr. Genet. 162B, 262-272.

Asmundson, G.J., Katz, J., 2009. Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. Depress Anxiety 26, 888-901.

Beaudet, M.M., Braas, K.M., May, V., 1998. Pituitary adenylate cyclase activating polypeptide (PACAP) expression in sympathetic preganglionic projection neurons to the superior cervical ganglion. J. Neurobiol. 36, 325-336.

Bernard, J.F., Bester, H., Besson, J.M., 1996. Involvement of the spino-parabrachio - amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog. Brain Res. 107, 243-255.

Bernard, J.F., Huang, G.F., Besson, J.M., 1992. Nucleus centralis of the amygdala and the globus pallidus ventralis: electrophysiological evidence for an involvement in pain processes. J. Neurophysiol. 68, 551-569.

Bird, G.C., Lash, L.L., Han, J.S., Zou, X., Willis, W.D., Neugebauer, V., 2005. Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. J. Physiol. 564, 907-921.

Bon, K., Lanteri-Minet, M., Michiels, J.F., Menetrey, D., 1998. Cyclophosphamide cystitis as a model of visceral pain in rats: a c-fos and Krox-24 study at telencephalic levels, with a note on pituitary adenylate cyclase activating poly-peptide (PACAP). Exp. Brain Res. 122, 165-174.

Braas, K.M., May, V., 1999. Pituitary adenylate cyclase-activating polypeptides directly stimulate sympathetic neuron NPY release through PAC1 receptor isoform activation of specific intracellular signaling pathways. J. Biol. Chem. 274, 27702-27710.

Carter, M.E., Soden, M.E., Zweifel, L.S., Palmiter, R.D., 2013. Genetic identification of a neural circuit that suppresses appetite. Nature 503, 111-114.

Cassell, M.D., Gray, T.S., Kiss, J.Z., 1986. Neuronal architecture in the rat central nucleus of the amygdala: a cytological, hodological, and immunocytochemical study. J. Comp. Neurol. 246, 478-499.

Chen, W., Boutaoui, N., Brehm, J.M., Han, Y.Y., Schmitz, C., Cressley, A., Acosta-Perez, E., Alvarez, M., Colon-Semidey, A., Baccarelli, A.A., Weeks, D.E., Kolls, J.K., Canino, G., Celedo_n, J.C., 2013. ADCYAP1R1 and asthma in Puerto Rican children. Am. J. Respir. Crit. Care Med. 187, 584-588.

Cho, J.H., Zushida, K., Shumyatsky, G.P., Carlezon, W.A.J., Meloni, E.G., Bolshakov, V.Y., 2012. Pituitary adenylate cyclase-activating polypeptide induces postsynaptically expressed potentiation in the intra-amygdala circuit. J. Neurosci. 32, 14165-14177.

Cui, X.Y., Lundeberg, T., Yu, L.C., 2004. Role of corticotropin-releasing factor and its receptor in nociceptive modulation in the central nucleus of amygdala in rats. Brain Res. 995, 23-28.

Das, M., Vihlen, C.S., Legradi, G., 2007. Hypothalamic and brainstem sources of pituitary adenylate cyclase-activating polypeptide nerve fibers innervating the hypothalamic paraventricular nucleus in the rat. J. Comp. Neurol. 500, 761-776.

Dickinson, T., Mitchell, R., Robberecht, P., Fleetwood-Walker, S.M., 1999. The role of VIP/PACAP receptor subtypes in spinal somatosensory processing in rats with an experimental peripheral mononeuropathy. Neuropharmacology 38, 167-180.

Dobner, P.R., 2006. Neurotensin and pain modulation. Peptides 27, 2405e2414. Dobolyi, A., Irwin, S., Makara, G., Usdin, T.B., Palkovits, M., 2005. Calcitonin gene-related peptide-containing pathways in the rat forebrain. J. Comp. Neurol. 489, 92-119.

Gauriau, C., Bernard, J.F., 2002. Pain pathways and parabrachial circuits in the rat. Exp. Physiol. 87, 251-258.

Girard, B.M., May, V., Bora, S.H., Fina, F., Braas, K.M., 2002. Regulation of neurotrophic peptide expression in sympathetic neurons: quantitative analysis using radioimmunoassay and real-time quantitative polymerase chain reaction. Regul. Pept. 109, 89-101.

Girard, B.M., Lelievre, V., Braas, K.M., Razinia, T., Vizzard, M.A., Ioffe, Y., El Meskini, R., Ronnett, G.V., Waschek, J.A., May, V., 2006. Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP- and PACAP-deficient mice. J. Neurochem. 99, 499-513.

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. Psychoneuroendocrinology 34, 833-843.

Hammack, S.E., Richey, K.J., Schmid, M.J., LoPresti, M.L., Watkins, L.R., Maier, S.F., 2002. The role of corticotropin-releasing hormone in the dorsal raphe nucleus in mediating the behavioral consequences of uncontrollable stress. J. Neurosci. 22, 1020-1026.

Han, J.S., Adwanikar, H., Li, Z., Ji, G., Neugebauer, V., 2010. Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. Mol. Pain 6, 10.

Hannibal, J., 2002. Pituitary adenylate cyclase activating peptide in the rat central nervous system: an immunocytochemical and in situ hybridization study. J. Comp. Neurol. 453, 389-417.

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. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. Br. J. Pharmacol. 166, 4-17.

Hawke, Z., Ivanov, T.R., Bechtold, D.A., Dhillon, H., Lowell, B.B., Luckman, S.M., 2009. PACAP neurons in the hypothalamic ventromedial nucleus are targets of central leptin signaling. J. Neurosci. 29, 14828-14835.

Ikeda, R., Takahashi, Y., Inoue, K., Kato, F., 2007. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 127, 161-172.

Ji, G., Fu, Y., Adwanikar, H., Neugebauer, V., 2013. Non-pain-related CRF1 activation in the amygdala facilitates synaptic transmission and pain responses. Mol. Pain 9, 2.

Ji, G., Neugebauer, V., 2009. Hemispheric lateralization of pain processing by amygdala neurons. J. Neurophysiol. 102, 2253-2264.

Jin, W.Y., Liu, Z., Liu, D., Yu, L.C., 2010. Antinociceptive effects of galanin in the central nucleus of amygdala of rats, an involvement of opioid receptors. Brain Res. 1320, 16-21.

Jongsma, H., Pettersson, L.M., Zhang, Y.Z., Reimer, M.K., Kanje, M., Waldenstrom, A., Sundler, F., Danielsen, N., 2001. Markedly reduced chronic nociceptive response in mice lacking the PAC1 receptor. Neuroreport 12, 2215-2219.

Kocho-Schellenberg, M., Lezak, K.R., Harris, O.M., Roelke, E., Gick, N., Choi, I., Edwards, S., Wasserman, E., Toufexis, D.J., Braas, K.M., May, V., Hammack, S.E., 2014. Pituitary adenylate cyclase activating peptide (PACAP) in the bed nucleus of the stria terminalis (BNST) produces anorexia and weight loss in male and female rats. Neuropsychopharmacology 39, 1614-1623.

Koob, G.F., Heinrichs, S.C., 1999. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res. 848, 141-152.

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. J. Neurosci. 17, 6434-6446.

Mabuchi, T., Shintani, N., Matsumura, S., Okuda-Ashitaka, E., Hashimoto, H., Muratani, T., Minami, T., Baba, A., Ito, S., 2004. Pituitary adenylate cyclase- activating polypeptide is required for the development of spinal sensitization and induction of neuropathic pain. J. Neurosci. 24, 7283-7291.

Makino, S., Shibasaki, T., Yamauchi, N., Nishioka, T., Mimoto, T., Wakabayashi, I., Gold, P.W., Hashimoto, K., 1999. Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. Brain Res. 850, 136-143.

Morano, T.J., Bailey, N.J., Cahill, C.M., Dumont, E.C., 2008. Nuclei- and condition-specific responses to pain in the bed nucleus of the stria terminalis. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 32, 643-650.

Mulder, H., Uddman, R., Moller, K., Zhang, Y.Z., Ekblad, E., Alumets, J., Sundler, F., 1994. Pituitary adenylate cyclase activating polypeptide expression in sensory neurons. Neuroscience 63, 307-312.

Nakagawa, T., Katsuya, A., Tanimoto, S., Yamamoto, J., Yamauchi, Y., Minami, M., Satoh, M., 2003. Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. Neurosci. Lett. 344, 197-200.

Neugebauer, V., Li, W., 2003. Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. J. Neurophysiol. 89, 716-727.

Neugebauer, V., Li, W., Bird, G.C., Bhave, G., Gereau, R.W., 2003. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J. Neurosci. 23, 52-63.

Ohsawa, M., Brailoiu, G.C., Shiraki, M., Dun, N.J., Paul, K., Tseng, L.F., 2002. Modulation of nociceptive transmission by pituitary adenylate cyclase activating polypeptide in the spinal cord of the mouse. Pain 100, 27-34.

Pettersson, L.M., Dahlin, L.B., Danielsen, N., 2004a. Changes in expression of PACAP in rat sensory neurons in response to sciatic nerve compression. Eur. J. Neurosci. 20, 1838-1848.

Pettersson, L.M., Heine, T., Verge, V.M., Sundler, F., Danielsen, N., 2004b. PACAP mRNA is expressed in rat spinal cord neurons. J. Comp. Neurol. 471, 85-96.

Resch, J.M., Maunze, B., Gerhardt, A.K., Magnuson, S.K., Phillips, K.A., Choi, S., 2013. Intrahypothalamic pituitary adenylate cyclase-activating polypeptide regulates energy balance via site-specific actions on feeding and metabolism. Am. J. Physiol. Endocrinol. Metab. 305, E1452-E1463.

Ressler, K.J., Mercer, K.B., Bradley, B., Jovanovic, T., Mahan, A., Kerley, K., Norrholm, S.D., Kilaru, V., Smith, A.K., J., M.A., Ramirez, M., Engel, A., Hammack, S.E., Toufexis, D., Braas, K.M., Binder, E.B., May, V., 2011. Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470, 492-497.

Robinson, D.A., Wei, F., Wang, G.D., Li, P., Kim, S.J., Vogt, S.K., Muglia, L.J., Zhuo, M., 2002. Oxytocin mediates stress-induced analgesia in adult mice. J. Physiol. 540, 593-606.

Roman, C.W., Lezak, K.R., Kocho-Schellenberg, M., Garret, M.A., Braas, K., May, V., Hammack, S.E., 2012. Excitotoxic lesions of the bed nucleus of the stria terminalis (BNST) attenuate the effects of repeated stress on weight gain: evidence for the recruitment of BNST activity by repeated, but not acute, stress. Behav. Brain Res. 227, 300e304.

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 antagonism 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., de Reus, M., Loohuis, N.O., Giele, J., van Egmond, J., Scheenen, W., Scheffer, G.J., Roubos, E., Vissers, K., Kozicz, T., 2011. Experimental neuropathy increases limbic forebrain CRF. Eur. J. Pain 16, 61-71.

Rouwette, T., Vanelderen, P., Roubos, E.W., Kozicz, T., Vissers, K., 2012. The amygdala, a relay station for switching on and off pain. Eur. J. Pain 16, 782-792.

Sandor, K., Bolcskei, K., McDougall, J.J., Schuelert, N., Reglodi, D., Elekes, K., Petho, G., Pinter, E., Szolcsanyi, J., Helyes, Z., 2009. Divergent peripheral effects of pituitary adenylate cyclase-activating polypeptide-38 on nociception in rats and mice. Pain 141, 143-150.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671-675.

Shimizu, T., Katahira, M., Sugawara, H., Inoue, K., Miyata, A., 2004. Diverse effects of intrathecal pituitary adenylate cyclase-activating polypeptide on nociceptive transmission in mice spinal cord. Regul. Pept. 123, 117-122.

Simons, L.E., Moulton, E.A., Linnman, C., Carpino, E., Becerra, L., Borsook, D., 2014. The human amygdala and pain: evidence from neuroimaging. Hum. Brain Mapp. 35, 527-538.

Sink, K.S., Walker, D.L., Yang, Y., Davis, M., 2011. Calcitonin gene-related peptide in the bed nucleus of the stria terminalis produces an anxiety-like pattern of behavior and increases neural activation in anxiety-related structures. J. Neurosci. 31, 1802-1810.

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P.H., Journot, L., 1993. Differential signal transduction by five splice variants of the PACAP receptor. Nature 365, 170-175.

Suwanprathes, P., Ngu, M., Ing, A., Hunt, G., Seow, F., 2003. C-fos immunoreactivity in the brain after esophageal acid stimulation. Am. J. Med. 115 (Suppl. 3A), 31S-38S.

Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat. Rev. Neurosci. 11, 823-836.

Tran, L., Wiskur, B., Greenwood-Van Meerveld, B., 2012. The role of the anterio-lateral bed nucleus of the stria terminalis in stress-induced nociception. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G1301-G1309.

Uddin, M., Chang, S.C., Zhang, C., Ressler, K., Mercer, K.B., Galea, S., Keyes, K.M., McLaughlin, K.A., Wildman, D.E., Aiello, A.E., Koenen, K.C., 2013. Adcyap1r1 genotype, posttraumatic stress disorder, and depression among women exposed to childhood maltreatment. Depress Anxiety 30, 251-258.

Ulrich-Lai, Y.M., Xie, W., Meij, J.T.A., Dolgas, C.M., Yu, L., Herman, J.P., 2006. Limbic and HPA axis function in an animal model of chronic neuropathic pain. Physiol. Behav. 88, 67-76.

Vaudry, D., Falluel-Morel, A., Bourgault, S., Basille, M., Burel, D., Wurtz, O., Fournier, A., Chow, B.K., Hashimoto, H., Galas, L., Vaudry, H., 2009. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol. Rev. 61, 283-357.

Veinante, P., Yalcin, I., Barrot, M., 2013. The amygdala between sensation and affect: a role in pain. J. Mol. Psychiatry 1, 9.

Vizzard, M.A., 2001. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. J. Chem. Neuroanat. 21, 125-138.

Wang, L., Cao, C., Wang, R., Qing, Y., Zhang, J., Zhang, X.Y., 2013. PAC1 receptor (ADCYAP1R1) genotype is associated with PTSD's emotional numbing symptoms in Chinese earthquake survivors. J. Affect Disord. 150, 156-159.

Xu, W., Lundeberg, T., Wang, Y.T., Li, Y., Yu, L.C., 2003. Antinociceptive effect of calcitonin gene-related peptide in the central nucleus of amygdala: activating opioid receptors through amygdala-periaqueductal gray pathway. Neuroscience 118, 1015-1022.

Zhang, Q., Shi, T.J., Ji, R.R., Zhang, Y.Z., Sundler, F., Hannibal, J., Fahrenkrug, J., Hokfelt, T., 1995. Expression of pituitary adenylate cyclase-activating poly-peptide in dorsal root ganglia following axotomy: time course and coexistence. Brain Res. 705, 149-158.

## Chapter 3.

## Parabrachial PACAP activation of amygdala endosomal ERK signaling regulates the emotional component of pain

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## 3.1. Abstract

The high coincidence of chronic pain and stress-related psychopathologies, such as depression, anxiety-associated abnormalities and posttraumatic stress disorder (PTSD) can aggravate the debilitating conditions of both disorders through neurocircuit intersections and mechanisms that are still not well understood. Pituitary adenylate cyclase activating polypeptide (PACAP; *Adcyap1*) and its cognate PAC1 receptor (*Adcyap1r1*) are expressed in peripheral nociceptive pathways, participate in anxiety-related responses and have been associated with stress-related disorders including PTSD. In a partial sciatic nerve ligation chronic constriction injury (CCI) model, we show that chronic neuropathic pain increases PACAP expression at multiple levels along the spino-parabrachioamygdaloid tract and bilaterally augments nociceptive amygdala (CeA) PACAP immunoreactivity, ERK phosphorylation and c-fos activation in parallel with heightened anxiety-like behavior and nociceptive hypersensitivity. Acute CeA infusions with the PACAP receptor antagonist PACAP(6-38) blocked CCI-induced behavioral responses; further, pretreatments with MEK or endocytosis inhibitors to block endosomal PACAP receptor ERK signaling attenuated PACAP-induced CeA neuronal activation and nociceptive responses. Accordingly, chronic pain-induced PACAP neuroplasticity and signaling in spino-parabrachioamygdaloid projections can impact CeA stress- and nociception-associated maladaptive responses, which can be ameliorated upon receptor antagonism even during disorder progression.

## 3.2. Introduction

Pain carries an aversive emotional component that can severely impact physiological and behavioral responses. Accordingly, chronic pain has been well associated with a number of stress-related psychopathologies, including depression, sleep dysregulation, panic disorders, obsessive compulsive behavior, anxiety abnormalities and post-traumatic stress disorder (PTSD) [1]. The high comorbidity between pain and stress-related behavioral disorders suggests that the two may be interrelated maladaptive processes [2]. Among brain regions, the amygdala is centrally situated to integrate the many descending and ascending signals to modulate the sensory and emotional components of pain. Among several direct ascending pathways carrying nociceptive transmission to the CeA, the most prominent is the spino-parabrachioamygdaloid tract [3-6]. Peripheral nociceptive signals carried via primary sensory Aδ- and C-fibers terminate on spinal projection neurons in lamina I/II and IV of the dorsal horn where the second order neurons send projections via the spino-parabrachial pathway to pontine lateral parabrachial nuclei (LPBn) [7]. In turn, the third-order LPBn neurons relay sensory information to the lateral (CeL) and lateral capsular (CeLC) subdivisions of the CeA. Hence the PBn collects cutaneous (mechanical and thermal), deep (muscular and articular) and visceral nociceptive signals and relays the information in a highly organized topographical manner principally to the nociceptive amygdala.

Although the integration of these inputs with amygdala circuits is a key mechanism underlying the emotional aspects of pain, the neurochemistry, neuroplasticity and regulatory events that drive the maladaptive responses are still not completely

understood. In the CeA, chronic pain upregulates mGluR1/mGluR5 levels and function, increases NMDA NR1 phosphorylation, enhances extracellular-regulated kinase (ERK) signaling and c-fos expression, and facilitates LPBn and basolateral amygdala (BLA) synaptic transmission to the CeLC[8-12] . Relatedly, the pathophysiology of pain and stress-related disorders has been attributed to the decrease or dysregulation of anti-nociceptive neuropeptide Y (NPY), opioid, endocannabinoid or neuroactive steroid actions on GABA signaling [2]. But in addition to diminished inhibitory neurocircuit function, persistent pain may also augment stimulatory CeA nociceptive neuropeptide levels including corticotropin releasing hormone (CRH) and calcitonin gene-related peptide (CGRP) as complementary means to facilitate the stress- and pain-induced changes in neural function [6,11,13].

Among brain peptides, there is accumulating evidence implicating pituitary adenylate cyclase activating polypeptide (PACAP) and its cognate PAC1 receptor in mediating the behavioral and physiological responses to a variety of homeostatic challenges [14]. Altered PACAP levels and a PAC1 receptor polymorphism have been associated with PTSD [15-19]. Mice that lack PACAP or the PAC1 receptor exhibit blunted anxiety-like behavior, show hypothalamic-pituitary-adrenal (HPA) axis and autonomic system dysregulation, and fail to develop hypersensitivity to nociceptive stimuli in inflammatory pain paradigms [20-27]. Furthermore, chronic but not acute stress leads to an upregulation of PACAP and PAC1 receptor transcript expression in the bed nucleus of the stria terminalis (BNST) [28-30]. BNST PACAP signaling increases anxiety-like behaviors and HPA axis activation, and mediates many of the behavioral consequences of

chronic stress [28-30]. The BNST and the CeA share similar circuit connectivity, architecture neurochemistry, and physiology, and may play complementary roles in emotional behavior processes. As in the BNST, dense PACAP immunoreactivity has been identified in the neuronal fibers of the CeLC/CeL which from previous work has shown to reflect LPBn PACAP projections in the spino-parabrachioamygdaloid tract [31]. Importantly, infusions of PACAP or a specific PAC1 receptor agonist directly into the CeA of naive rats produced both anxiety-like behaviors and nociceptive hypersensitivity, suggesting that LPBn PACAP activity via the spino-parabrachioamygdaloid circuit carries signals that may in part alter the emotional responses to pain. Using a partial sciatic nerve ligation chronic constriction injury (CCI) model, we examined whether persistent neuropathic pain alters PACAP transcript expression in the spino-parabrachioamygdaloid tract and whether PAC1 receptor antagonism can mitigate CCI-induced nociceptive hypersensitivity and anxiety-like behaviors. As PACAP signaling potently and efficaciously activates MAPK/ERK, a central mechanism in synaptic plasticity and CeA-dependent behaviors and pain hypersensitivity, we also assessed CeA PAC1 receptor mechanisms *in vivo*. The studies in aggregate suggest that endogenous PACAP signaling in the spino-parabrachioamygdaloid pathway and the resulting endosomal PAC1 receptor-stimulated activation of ERK in the CeA mediate the adverse emotional consequences of chronic pain, and may also explain comorbidities between chronic pain and other stress-related pathologies.

## 3.3. Results

*Neuropathic pain augments PACAP expression in the spino-parabrachioamygdaloid pathway.*

Our previous studies identified PACAP in neuronal projections from the LPBn to CeLC and demonstrated that CeA PACAP infusions resulted in heightened nociceptive sensitivity and anxiety-like behaviors [31]. As previous studies implicated PACAP phenotypic plasticity in sensory systems [32,33] we examined whether chronic neuropathic pain in a unilateral sciatic nerve CCI model regulated endogenous PACAP expression along the spino-parabrachioamygdaloid pathway. The partial sciatic nerve ligation procedure reliably heightened nociceptive sensitivity as reflected by decreased thermal latency responses and also induced anxiety-like behavior in open field tests 14 days post-surgery compared to sham controls without compromising locomotor activity (see below). Quantitative PCR analyses of micropunched PBn tissues demonstrated that CCI specifically elevated PBn PACAP transcript levels approximately 1.5 fold compared to tissues from sham animals ($t(12)=2.36$, $p=0.036$); CCI did not augment PACAP transcript levels in the CeA, anterolateral BNST, or the solitary nucleus (NTS)(figure S3.1). Additionally, no significant change was found for the PAC1 receptor transcripts in the LPBn or CeA (figure S3.1). As in other peptidergic systems, the immunocytochemical localization for PACAP in the nervous system preferentially identified bioactive peptides in fibers rather than soma to preclude corresponding analyses of neuronal LPBn PACAP peptide changes after CCI. But as an alternative means of evaluating injury-induced PACAP expression in the LPBn, the same unilateral

CCI was performed on PACAP-EGFP mice. LPBn PACAP-EGFP$^{+}$ cells were identified under basal conditions and CCI induced the number of PACAP neurons almost 2-fold compared to sham operated animals, main effect of CCI, $F(1,22)=7.99$, $p = 0.01$) (Figure 1A - 1C). The CCI-induced PACAP-EGFP$^{+}$ neurons appeared throughout the LPBn, with the majority confined to the external lateral and central lateral regions. Notably, the LPBn PACAP induction was observed both ipsilateral and contralateral to the injury, which reflected bilateral dorsal horn neuronal projections to LPBn (Figure 3.1C), no main effect of side main effect ($F(1,28) = 0.32$, $p = 0.6$).

In good correspondence to the increase LPBn PACAP transcripts and neurons, CCI also augmented CeLC fiber PACAP staining from parabrachioamygdaloid projections (Figure 3.1D - 1F). Consistent with previous studies, dense punctate PACAP-immunoreactivity characteristic of PACAP fiber terminals and varicosities was found primarily in the CeLC that extended into the CeL; image analyses after thresholding fluorescence intensity revealed a 1.4-fold increase in PACAP staining density in the CeLC of CCI animals compared to that in sham animals $F(1,28)=14.74$, $p=0.0006$). As anticipated from bilateral dorsal horn neuronal projections to the LPBn, the increase in CeLC PACAP immunoreactivity was also bilateral after unilateral CCI; however, there was a notable bias toward greater PACAP immunoreactivity in the right CeLC irrespective of the side of the CCI which appeared consistent with CeA lateralization described in previous studies [34,35] (Suppl Figure 3.2).

Given the role of PACAP in neuroplasticity, we also evaluated whether neuropathic pain from CCI similarly affected other PACAPergic neurons within the

spino-parabrachioamygdaloid tract in the PACAP-EGFP mice. While the sciatic nerve in sham operated and the contralateral leg of CCI animals demonstrated minimal PACAP-EGFP fluorescence, the sciatic nerve segment proximal to CCI ligation demonstrated pronounced fiber PACAP expression (condition*side $F(1,10)=57.22$, $p<0.0001$, post-hoc Sham-ipsilateral vs. CCI-ipsilateral, $p<0.0001$) (Figure 3.2D - 2F). In mouse, the sensory fibers in the sciatic nerve are predominantly peripheral axons from L3 - L5 dorsal root ganglia (DRG) with the largest contributions from L4 sensory neurons. In correspondence, CCI increased the number of PACAP-EGFP$^+$ L3 - L5 DRG neurons ipsilateral to the injury with the greatest increase in L4 DRGs compared to neurons from the same levels under all control conditions (L4 DRG condition*side $F(1,8)=93.12$, $p<0.0001$, post-hoc Sham-ipsilateral vs. CCI-ipsilateral, $p<0.0001$). (Figure 3.2A - 2C, L3, and L5 Suppl Figure 3.2). The CCI-induced increase in DRG PACAP expression was also reflected by a dramatic increase in DRG central axon EGFP fluorescence in laminae III-V of the ipsilateral dorsal horn and in the gracile fasciculus (Figure 3.2G) projecting to higher order central nuclei. There were no apparent changes in the number of second order PACAP-EGFP$^+$ dorsal horn neurons in CCI (data not shown). Interestingly, CCI also induced PACAP-EGFP in some ipsilateral ventral horn motor neurons which appeared consistent with previous nerve transection studies (Pettersson et al., 2004). Accordingly, these demonstrate that chronic neuropathic pain elevates PACAP expression levels along multiple neuronal elements in the spino-parabrachioamygdaloid pathway.

*CeA PACAP signaling facilitates neuropathic pain-related anxiety-like behaviors and thermal hypersensitivity*

We next examined if elevated CeA PACAP signaling in CCI contributes to heightened anxiety-like behaviors and nociceptive sensitivity. Our previous work demonstrated that CeA administration of PACAP or the PAC1 receptor specific agonist maxadilan was capable of producing both anxiety-like behaviors and thermal hypersensitivity[31]. But to evaluate the contribution of sustained endogenous CeA PACAP signaling in chronic neuropathic pain, the PAC1 receptor antagonist PACAP(6-38) was infused bilaterally into the CeA of CCI rats before assessing anxiety-like behavior in the open field and thermal nociception testing with the Hargreave's test (Figure 3.3A). Similar to chronic stress models, CCI attenuated weight gain over the course of observation; (Figure 3.3B, main effect of CCI, $F(10,280) = 80.80$, $p < 0.0001$). In open field tests 14 days post-surgery, CeA PACAP(6-38) infusions into the sham control group did not significantly change the number of center field entries compared to those receiving vehicle bonferroni's m.c. $t(22)=0.47$, $p = 0.9$) suggesting that the antagonist alone had no apparent behavior effects. Animals with CCI had fewer center field entries and these pain-associated stress responses were completely blocked upon CeA PACAP(6-38) administration (Figure 3.3C - 3D, bonferroni's m.c. $t(22)=3.12$, $p = 0.03$). These responses were paralleled by a trend for PACAP(6-38) to increase center field durations times in CCI (bonferroni's m.c. $t(22)=2.22$, $p = 0.07$). The CCI procedure did not impair locomotion or affect the total distance traveled ($F(1,20)=0.46$, $p = 0.6$), similarly PACAP(6-38) did not alter total distance travelled ($F(1,20)=1.22$, $p = 0.3$).

Concurrent with anxiety-related behaviors, CeA PACAP infusions also reliably facilitated nociceptive hypersensitivity in Hargreave's thermal assays that persisted for several hours[31]. CCI of the sciatic nerve has been well used to produce thermal nociceptive responses and comparable to previous work, CCI 14 days post-surgery typically decreased thermal latency 40 - 50% in the ipsilateral hindpaw compared to sham control groups or to the contralateral hindpaw (Figure 3.3E - 3G, $F(1,21)=14.13$, $p = 0.001$). In the same experimental paradigm, all of the CCI animal groups demonstrated ipsilateral hindpaw thermal sensitivity prior to treatments; however, 1 h following bilateral CeA PACAP(6-38) administration, the PAC1 receptor antagonist attenuated the heightened thermal nociceptive responses compared to baseline measures prior to antagonist treatments in the ipsilateral hindpaw in the CCI condition (Figure 3.3E, interaction of condition*treatment*day ($F(1,21) = 7.83$, $p = 0.009$). The effects were more marked when the responses in each animal were normalized to their own latency baseline immediately prior to the injections (Figure 3.3H, $F(1,21)=16.40$, $p = 0.001$, interaction of condition*treatment $F(1,21) = 15.49$, $p = 0.001$). There were no significant effects of CeA PACAP(6-38) on the uninjured contralateral hindpaw or in the sham operated condition (Figure 3.3F). Accordingly, these results mirrored previous PAC1 receptor antagonist studies, demonstrating that PACAP has no apparent behavioral effects under control sham handling conditions, but contributes to heightened anxiety-like behavior and nociceptive sensitivity in chronic neuropathic pain.

*CeLC PACAP-mediated ERK signaling in chronic neuropathic pain*

One of the most consistent amygdala responses to persistent pain is an increase in ERK activation in a subset of CeLC neurons. Enhanced amygdala ERK signaling can increase behavioral sensitivity in normal conditions and ERK signaling contributes to PBn - CeLC neurotransmission in persistent pain[10,36,37]. Conversely, MEK/ERK inhibition in inflammatory pain can decrease behavioral hypersensitivity[10]. Comparable to previous work and PACAP transcript/immunocytochemistry data above, unilateral CCI on either the right or left hind limb increased bilaterally the number of CeLC $pERK^+$ cells compared to sham (Figure 3.4A - 4C, main effect of CCI $F(1,26)=7.62$, $p = 0.01$); there was no apparent difference in response relative to the side of injury ((IL vs CL) no main effect of side $F(1,26) = 0.01$, $p = 0.9$). However, for all CCI (left or right hind limb), there was an apparent trend towards a greater number of $pERK^+$ cells in the right CeLC ((left vs right) $F(1,26) = 3.15$, $p = 0.09$) (Suppl Figure 3.2). These results signify that similar to other pain models, chronic neuropathic pain enhances ERK signaling in the CeLC.

But to evaluate whether CeA PACAP fibers can affect amygdala ERK activation in CCI, dual pERK and PACAP localization was performed (Figure 3.4D - 4E). Notably, the majority of the CCI $pERK^+$ cells ($84.5 \pm 5.0\%$) were in immediate apposition ($< 2$ μm) with CeLC PACAPergic fibers with a high occurrence of PACAP fibers forming perisomatic contacts. The fraction of $pERK^+$ cells with PACAP contacts is likely an underestimate given the limitations of section thickness and antibody penetration. Thus, PACAP is optimally situated to activate ERK in the CeLC. In good correspondence with

previous characterizations of PACAP neurons, the CeLC PACAP fibers are mainly glutamatergic from PACAP colocalization with vGlut2 immunoreactivity; there was little overlap with vGlut1 or glutamic acid decarboxylase (GAD) staining (Suppl Figures 3.3 and 3.5).

As the BNST displays structural and functional homology with the CeA and also receives LPBn PACAP projections[31], the effects of CCI on neuronal pERK were also examined in the BNST. As in the CeLC, CCI induced a robust increase in the number of $pERK^+$ cells in the anterolateral BNST, with almost no cellular pERK labeling under sham conditions (main effect of CCI, $F(1,8) = 15.04$, $p = 0.005$) (Suppl Figure 3.5). Similar to the CeLC, the majority of BNST pERK neurons ($83.1 \pm 0.5\%$) were in close contact with glutamatergic PACAP fibers (Suppl Figures 3.3 and 3.5). These results implied that BNST PACAP signaling may also have roles in the behavioral consequences of persistent pain, which complements previous work [38].

To establish whether CeA PACAP signaling via ERK can evoke thermal hypersensitivity, the MEK inhibitor PD98059 (20 μM) was infused into the CeA prior to PACAP38 injection. While infusions of PACAP38 alone resulted in marked increases in CeA c-fos and pERK immunoreactivity in the same neurons (Figure 3.5; co-incidence = 87%), pretreatment with the MEK inhibitor abolished the PACAP-stimulated responses, demonstrating that ERK activation is an essential component of PACAP signaling to instigate CeA neuronal activity (Figure 3.5A - 5L). CeA PACAP infusion and signaling within the same study heightened nociception sensitivity as shown by the decreases in thermal latency times; the PACAP responses were completely abolished by MEK

inhibition, corroborating that PACAP/PAC1 receptor signaling via ERK pathways is central to CeA nociception processes (Figure 3.5M - 5N, bonferroni m.c. $t(41)=3.59$, $p = 0.002$).

There are several potential mechanisms for PAC1 receptors to engage MEK/ERK pathways including PKA and/or PKC activation[39-42]; however, PAC1 receptor internalization into signaling endosomes has also been shown to be an alternative and efficacious means of ERK phosphorylation to potentially sustain cell stimulation [41,42]. Blocking PAC1 receptor internalization at ambient temperature conditions or with endocytosis inhibitors substantially attenuated ERK phosphorylation. Contiguous with the previous experiment, a separate experimental group was pretreated with Pitstop 2 (30 μM), an inhibitor of clathrin-mediated endocytosis, prior to PACAP infusion. Consistent with cell culture data [42], Pitstop 2 pretreatments markedly block PACAP-mediated c-fos expression and ERK phosphoryation in the CeA (Figure 3.5). Importantly, inhibition of clathrin-mediated endocytosis reduced PACAP-induced hypersensitivity (Figure 3.5M - 5N, bonferroni m.c. $t(41)=2.57$, $p = 0.03$). Neither PD98059 nor Pitstop 2 produced CeA damage or cellular apoptosis (Suppl Figure 3.6) The efficacy of Pitstop 2 in blocking the PACAP-mediated nociceptive responses appeared lower than that for MEK inhibition which may reflect in part drug potency *in vivo* vs *in vitro*, and ERK activation via PAC1 receptor PKA or PKC mechanisms. Nevertheless, these studies in aggregate provide the first in vivo evidence that GPCR PAC1 receptor internalization and downstream ERK signaling can modulate CeA nociception responses.

### 3.4. Discussion

The current studies establish roles for CeA PACAP signaling as an effector conveying the behavioral and sensory consequences of chronic neuropathic pain. Among several lines of evidence, CCI increased PACAP transcripts and neurons in the LPBn which correlated with enhanced LPBn PACAP projection fiber immunoreactivity in the CeLC and increased PACAP expression in the spino-parabrachioamygdaloid tract. In good agreement with previous studies demonstrating the anxiety-related and nociceptive hypersensitivity responses following CeA PACAP administration[31], blockade of endogenous PACAP signaling in CCI with PAC1 receptor antagonist PACAP(6-38) attenuated the CCI neuropathic pain-induced heightened anxiety-like behavior in the open field tests and nociceptive hypersensitivity in thermal assays. Importantly, both CCI and PACAP stimulated CeA ERK activation and c-fos expression which were diminished upon pretreatments with MEK or clathrin-mediated endocytosis inhibitors in parallel with diminished PACAP-induced nociceptive hypersensitivity. These results further our understandings of CNS PACAP mechanisms and functions, and how maladaptions in PACAP signaling in intersecting stress-related and pain circuits may negatively impact the course of psychopathologies.

Previous studies have shown PACAP neurophenotypic plasticity and demonstrated that central and peripheral neuronal PACAP expression can be upregulated in response to diverse homeostatic challenges. In a chronic stress paradigm, heightened PACAP and PAC1 receptor transcript expression was observed in the BNST and paraventricular nucleus of the hypothalamus[28]. In several nerve injury models, PACAP

was elevated in sensory, autonomic and motor neurons [32,33,43]. The recent availability of the PACAP-EGFP mice illustrated that plasticity; whereas basal endogenous PACAP levels appeared low in many neuronal systems, physiological challenges especially nerve injury significantly induced PACAP expression. Consistent with previous results, CCI increased DRG PACAP expression, which augmented dramatically PACAP levels in both peripheral sensory fibers in the sciatic nerve and central DRG axons in the dorsal horn and spinal pathways. Second order PACAP producing neurons were found in lamina I/II of the dorsal horn but notably CCI also increased PACAP expression centrally in the LPBn and CeA as a consequence of enhanced nociceptive signaling in the spino-parabrachioamygdaloid pathway. The injury mechanisms underlying the induction of phenotypically plastic peptides, including PACAP, are not well understood but may reflect inflammatory responses or cellular stress from diminished target tissue signaling. The same mechanisms may underlie the PACAP induction in the few ventral horn motor neurons in CCI; PACAP function in these neurons have not been studied but posited to be regenerative or neuroprotective. Uniquely, these studies demonstrate PACAP expression at all levels of the spino-parabrachio-amygdaloid pathway suggesting that PACAP is a common mediator at all levels of the nociceptive circuit.

The second order dorsal horn neurons project to the brain bilaterally; hence unilateral CCI produced bilateral increases in LPBn PACAP expression with corresponding increases in CeA PACAP immunoreactivity and pERK activation. However, when all data sets were analyzed with reference to tissues ipsilateral or contralateral to injury site, PACAP and pERK immunoreactivity was preferentially

heightened in the right CeA, irrespective of left or right sciatic nerve ligation. These observations agreed with studies suggesting that the CeA displays a degree of lateralized function with the right CeA displaying greater increases in pERK and synaptic potentiation in response to pain[34,35]. Accordingly, the lateralization of CeA PACAP may be consistent with the functional lateralization nociceptive processes in the CeA.

The evidence for PACAP functions as a nociceptive neurotransmitter is substantive. PACAP was identified initially as a sensory peptide[44] and in agreement with current work, other studies have shown that the low basal expression levels of PACAP in DRG neurons can be dramatically induced in sensory neurons and sciatic nerve fibers after injury. Furthermore, heightened DRG PACAP is likely released from C-fibers in the superficial layers of the dorsal horn, as capsaicin applications decreased dorsal horn PACAP immunoreactivity and increased PACAP levels in cerebral spinal fluid perfusate [44,45]. PACAP knockout mice develop significantly less thermal and mechanical hypersensitivity from both neuropathic and inflammatory pain models, and have decreased somatic sensitivity in normal conditions [26]. Consistent with these findings, mice that lack PAC1 receptors display reduced mechanical hypersensitivity during the late phase following formalin injection[27]. However, the nociception studies after PACAP infusion have been more variable depending on the route of peptide administration. In the periphery, direct PACAP injections into the hindpaw was largely anti-nociceptive reducing thermal and mechanical sensitization in inflammatory pain[46]. However, in parallel with our CeA studies, central and intrathecal PACAP administrations were pro-nociceptive capable of potentiating hypersensitivity under normal conditions, and the

responses could be blocked with PACAP(6-38) [31,47,48]. The reasons for the variable results are unclear but may be related to PACAP regulation of many homeostatic systems. In addition to expression and function in sensory systems, PACAP also regulates autonomic and immune functions; the anti-inflammatory and immunosuppressive attributes of PACAP for example, may be contributory to the peripheral anti-nociceptive effects.

Following CCI, a two week postsurgical recovery period was established to allow locomotor return from transient deficits, injury-induced PACAP expression and the development of chronic pain hypersensitivity and stress-related behaviors for multiple nociceptive and behavioral assessments. BNST PACAP expression was upregulated in a seven day chronic variate stress paradigm but not following one day of acute stress[29,30]; whether a similar time course is necessary for PACAP induction in chronic versus acute pain and whether PACAP levels in the spino-parabrachioamygdaloind pathway increase incrementally with chronic pain duration have not been established. As many weeks of CCI have been shown to gradually cause anxiodepressive-like disorders [49] and PACAP has been implicated in anxiety- and depression-related behaviors [15,29,50,51], the increase in PACAP expression and signaling may be a mechanism underlying the development of psychopathologies.

The current CCI paradigm produced anxiety-like responses in open field tests and thermal hypersensitivity in the ipsilateral hindpaw. To evaluate whether continued CeA PACAP signaling participates in these heightened pain and behavioral responses, the PAC1/VPAC2 receptor antagonist PACAP(6-38) was infused into the CeA before

testing. The infusion of PACAP(6-38) alone into sham control animals had no effects on either pain or stress-related behaviors, suggesting that PACAP signaling under basal conditions may be low not to significantly impact the normal course of CeA functions. The ability for acute PACAP(6-38) treatments to mitigate anxiety-like behavior and thermal hypersensitivity responses during chronic injury suggested that the increase in CeA PACAP levels and signaling was sustained during the course of CCI to facilitate the pain-related behavioral responses. The involvement of CeA PACAP only in a state of persistent pain and/or stress and not under normal conditions is comparable to observations for other CeA systems including CGRP, CRH and mGluR regulated-functions[8,52-54]. The mechanisms through which CCI-induced CeA PACAP may result in anxiety-like behaviors is not clear but may involve the potentiation of basolateral amygdala (BLA) excitatory postsynaptic transmission to the CeL [55]. Similarly LPBn PACAP projections to the BNST may not only have anxiogenic but hyperalgesic attributes by interactions with CRH systems [38]. Hence, CCI-induced LPBn PACAP expression and release could heighten nociceptive hypersensitivity and anxiety-like behaviors via multiple complementary mechanisms with projections facilitating BLA to CeL neurotransmission, modulating descending inhibitory signals, altering BNST function or enhancing CeLC nociceptive signals to the substantia innominata dorsalis for anxiety, aversion and fear responding[56].

Activation of the ERK pathway is a central means of nociceptive signaling in a variety of pain models. PACAP potently activates ERK through PAC1 receptors which may have contributed to the sustained levels of pERK in the CeLC during prolonged

CCI. Both CCI and acute CeA PACAP infusion increased ERK phosphorylation and levels of the neuronal activity marker c-fos. The increase in pERK and c-fos were colocalized to the same CeA neurons, and as c-fos stimulation could be abrogated concomitantly with pERK levels with MEK inhibitors, the increase in c-fos appeared downstream of PACAP signaling. This was supported by the observation that the majority of the pERK neurons was found to be in close apposition to PACAPergic fibers. Further, the ability for MEK inhibition to attenuate CeA PACAP-stimulated pERK and c-fos in parallel with blockade of PACAP-induced thermal sensitivity demonstrated that PACAP/PAC1 receptor-mediated ERK signaling is requisite for CeA nociceptive hypersensitivity responses. There are several routes of PAC1 receptor-mediated activation of MEK including adenylyl cyclase/cAMP and PLC/PKC. While these plasma membrane initiated cascades may be relatively short lived, the recent observations that PAC1 receptor endocytosis and recruitment of scaffolding proteins for endosomal MEK signaling may represent a key mechanism for prolonged intracellular ERK activation. As with MEK inhibitors, Pitstop 2, an inhibitor of clathrin mediated endocytosis also blocked PACAP-mediated CeA pERK and c-fos levels and attenuated PACAP-mediated nociceptive hypersensitivity responses. The internalization of several GPCR systems have been described to participate intracellular signaling; these results may be one demonstration of how GPCR internalization and endosomal signaling may relevant in a physiological mechanisms and in particular nociceptive mechanisms.

In summary, our results demonstrate that spino-parabrachioamygdaloid PACAP expression and signaling are augmented in neuropathic pain and that this heightened

expression may contribute to adverse pain- and stress-related behaviors. While clinical data have placed considerable emphasis on the dysregulation of inhibitory pathways as mechanisms underlying pain-associated psychopathologies, the maladaptations from ascending activating pathways including neurophenotypically plastic PACAPergic system may be contributory to that process. CeA PAC1 receptor antagonism or inhibition of downstream endosomal ERK signaling can blunt PACAP- and CCI-induced nociceptive hypersensitivity and associated anxiety-like responses. As PACAP receptor antagonism during CCI advancement can still ameliorate the adverse neuropathic pain and behavioral responses, these observations suggest that interventions in PACAP signaling during the progression of pain and associated behavioral responses may have therapeutic utility in improving disorder outcomes.

## 3.5. Methods

*Animals*

Adult male Sprague-Dawley rats were from Charles River Laboratories, Wilmington, MA. PACAP promoter-dependent EGFP BAC transgenic mice, generated by the GENSTAT (Gene Expression Nervous System Atlas) project were obtained from James Waschek (UCLA, Los Angeles, CA). All animals were housed under a 12-hour light/dark cycle (lights on 0700 h) with food and water available ad libitum, and habituated to the animal facility for at least one week prior to any experiments. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

*Neuropathic pain model*

Chronic constriction injury (CCI) of the sciatic nerve was performed in rats as described previously[57]. Rats were anesthetized with isoflurane and four loose ties (4-0 chromic gut sutures, Ethicon) were placed proximal to the trifurcation of the sciatic nerve. In sham surgeries, the sciatic nerve was briefly exposed before incision closing with wound clips. In some experiments with intra-amygdalar infusions, the stereotactic surgery for cannula implantation was performed concurrently with CCI. Only animals that developed thermal hypersensitivity in Hargreave's assay were used for testing and analyses. In PACAP-EGFP mice, the same CCI procedure was followed except only three chromic gut sutures were used.

*Intra-amygdalar infusion*

Rats were prepared as described previously[29,31] and two stainless steel cannulae (22GA, PlasticsONe, Roanoke, VA) were placed targeting the CeA using the coordinates (from bregma in mm) AP: -2.6, ML: ± 4.5, DV: -7.2. For CeA drug administration, rats were lightly restrained with a towel and infused with drug or vehicle (0.5 µl/min, Harvard Apparatus, Holliston, MA) through an internal cannula with a 1 mm projection beyond the end of the guide cannulae. Infused compounds included PACAP(6-38) (0.3 µg/0.5 µl), Pitstop 2 (30 µM/0.5 µl) and PD98059 (20 µM/0.5 µl).

*Immunohistochemistry*

Anesthetized rats were perfused transcardially with 4% paraformaldehyde and the brains were postfixed for 24 h, washed and equilibrated in 30% sucrose before embedding in OCT compound (ThermoFisher Scientfic, Waltham, MA) for cryosectioning (30 μm). The sections were mounted onto subbed slides, permabilized with 0.3% Triton X-100, blocked with 1% BSA and incubated in primary antibody. Immunocytochemical staining for PACAP (1:10, 48 h at 4 C, Jens Hannibal, Bisperg Hospital, Copenhagen, Denmark) was enhanced by tyramide signal amplification (Perkin Elmer, Waltham, MA) for visualization with Cy3-conjugated streptavidin (1:200, 2 h; Jackson Immunoresearch, West Grove, PA) as previously described[31]. Detection for phosphorylated ERK (1:1000, #4370 Cell Signaling Technology, Danvers, MA) and c-fos (1:300, sc-52 Santa Cruz Biotechnology, Dallas, TX) were performed using species specific AlexaFluor 488 or Cy3-conjugated secondary antibodies. Antibodies to vGlut1 (1:1000, AB5905), vGlut2 (1:1000, AB2251) and GAD (1:300, AB1511) were all from Millipore Billerica, MA.

*Image Analysis*

Micrographs were obtained using a Nikon E800 point scanning confocal microscope, except in analyses of PACAP immunoreactivity levels in which the images were captured using an Olympus fluorescence microscope captured using identical parameters. For quantification of CeA PACAP fiber immunoreactivity, the corresponding CeA fields in the different brains were identified using the hippocampus

and optic tracts as reference points; area of threshold was used as an indicator of relative fluorescence from same sized fields. For enumeration of CeA pERK-, c-fos-, and PACAP-EGFP$^{+}$ cells in fixed areas, a semi-autonomous cell counting method was performed in ImageJ. All data represent mean values ± SEM.

## *Behavioral Assessments*

### *Open Field*

Behavioral testing was performed 0.5 h following infusions. Rats were individually placed into the corner of a 75 cm x 75 cm opaque black open arena with 50 cm walls (United States Plastics Corp., Lima, OH) illuminated at 20 lux using a red bulb. Rat arena center entries and total distance traveled over 5 min test sessions were digitally captured with a ceiling mounted camera for analyses using EthoVision XT version 6.1.326 (Noldus Information Technology, The Netherlands).

### *Thermal Sensitivity Assessment*

A Hargreave's apparatus (Plantar Analgesia Meter, IITC Life Science, Inc., Woodland Hills, CA). was used to assess thermal stimuli responses. Following habituation in the acrylic testing chambers (30 min each day for 2 days), the rats were placed in the apparatus chamber with the glass floor maintained at 30 C with an internal heating element. A low intensity guide light (8% active intensity) was used to target the plantar surface of the each hindpaw from beneath the glass floor before a beam of focused radiant light (4 x 6 mm, 25% active intensity) was switched on. Upon animal

awareness of the heat stimulus, indicated by a withdrawal response or licking of the hindpaw, the heat source was terminated and the reaction time automatically recorded. An automated 30 sec cut-off was used to prevent tissue damage. The hindpaws were randomly selected at trial initiation and 3 trials separated by 5 min inter-trial intervals were performed on each of the left and right hindpaws .

*Transcript analysis*

Quantitative PCR (QPCR) was performed in the same manner as previously described[28,29] . Following brief isoflurane anesthesia and rapid decapitation, rat brains were quickly frozen in OCT compound (ThermoFisher Scientfic, Waltham, MA); 300 μm cryosections were prepared and 740 μm micropunches from each region were harvested. Total RNA extraction was performed using STAT-60 RNA/mRNA isolation reagent (Tel-Test "B", Friendswood, TX). Each set of brain regions was reverse transcribed simultaneously using random hexamer primers using SuperScript II Preamplification System (Invitrogen, Carlsbad, CA). The cDNA templates were diluted 10-fold and assayed on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green I JumpStart Taq ReadyMix (Sigma, St. Louis, MO) containing 5.0 mM MgCl2, 200 μM dATP, dGTP, dCTP and dTTP, 0.64 U Taq DNA polymerase and 300 nM of each primer in a 25 μl reaction volume. Oligonucleotide primers were as follows: PACAP (S) 5'-CATGTGTAGCGGAGCAAGGTT-3' (AS) 5'-GTCTTGCAGCGGGTTTCC-3', PAC1 (S) 5' -AACGACCTGATGGGACTAAAC-3' (AS) 5'-

CGGAAGCGGCACAAGATGACC-3'. Following amplification, melting profiles of amplicons were used to verify unique product generation. A standard curve constructed by amplification of serially 10-fold diluted plasmids containing the target sequence was used for analysis. Increase in SYBR Green I fluorescence intensity(ΔRn) was plotted as a function of cycle number and threshold cycle (CT) was determined using software as the amplification cycle at which the ΔRn intersects the established baseline. Transcript levels were calculated from the CT by interpolation from the standard curve. For each target sequence, all sample from the same brain region were amplified simultaneously. All data was normalized to 18s RNA and calculated as a fold change from control.

*Statistics*

All statistical tests were performed in SPSS (version 22) and GraphPad PRISM (version 6). Two-way analysis of variance (ANOVA) was performed to examine main effects and interactions, and Bonferrroni's multiple comparisons tests were used to compare different groups for all experiments, except for those indicated. A multifactorial ANOVA was used to examine PACAP6-38 treatment with CCI condition across side and day in tests of thermal sensitivity. Students T tests were performed to compare changes in average weight gain and post surgery weight loss.

### 3.6. Figures

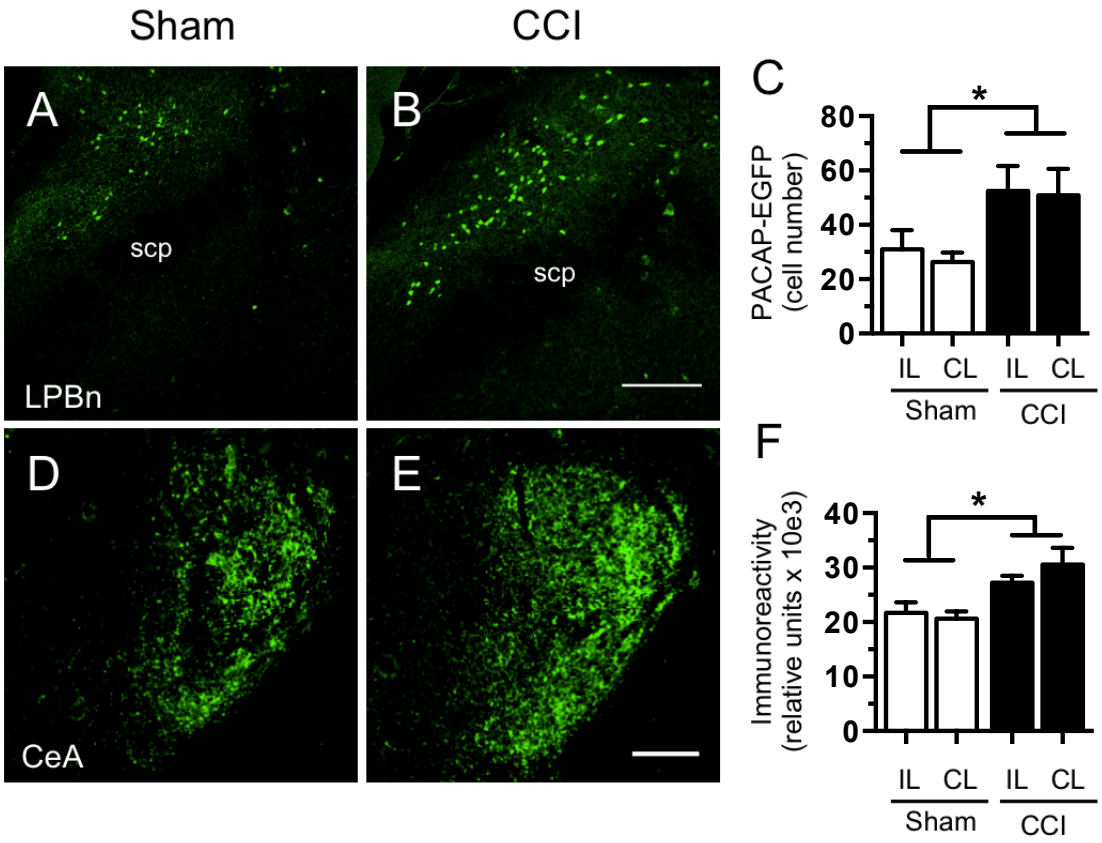

Sham
CCI
A
B
scp
scp
LPBn
D
E
CeA
C
PACAP-EGFP (cell number)
80
60
40
20
0
*
IL
CL
IL
CL
Sham
CCI
F
Immunoreactivity (relative units x 10e3)
40
30
20
10
0
*
IL
CL
IL
CL
Sham
CCI

**Figure 3.1. CCI increases LPBn and CeA PACAP levels.** Control sham surgery (A) or CCI (B) were performed on transgenic PACAP-EGFP mice and native EGFP fluorescence was examined in LPBn tissues 2 weeks following surgery. The number of LPBn PACAP-EGFP cells was increased bilaterally in CCI compared to sham with a main effect of condition (C; sham ipsilatera/contralateral = 31.0 ± 7.1 cells/26.3 ± 3.5 cells vs CCI ipsilateral/contralateral = 52.5 ± 9.2 cells/50.8 ± 9.7 cells; $F(1,22) = 7.99$, $p = 0.01$, n = 6-7 per group, 3 sections enumerated per side per animal). CeA PACAP immunoreactivity was also increased after CCI (E) compared to sham controls (D). From image analyses with thresholded area, there was a main effect of CCI (F; sham ipsilatera/contralateral = 21.7 ± 2.0 units/20.6 ± 1.3 units vs CCI ipsilateral/contralateral = 27.3 ± 0.4 units/ 30.6 ± 1.1, $F(1,28) = 14.74$, $p = 0.0006$; n = 8 per group) but no main effect ($F(1,28)=0.32$, $p=0.6$) or interaction ($F(1,28) = 1.17$, $p = 0.3$) with respect to side. Data represent mean cells/unit area or fluorescence units/unit area ± SEM; scp, superior cerebellar peduncle; IL, ipsilateral; CL, contralateral; Scale bar = 200 μm.

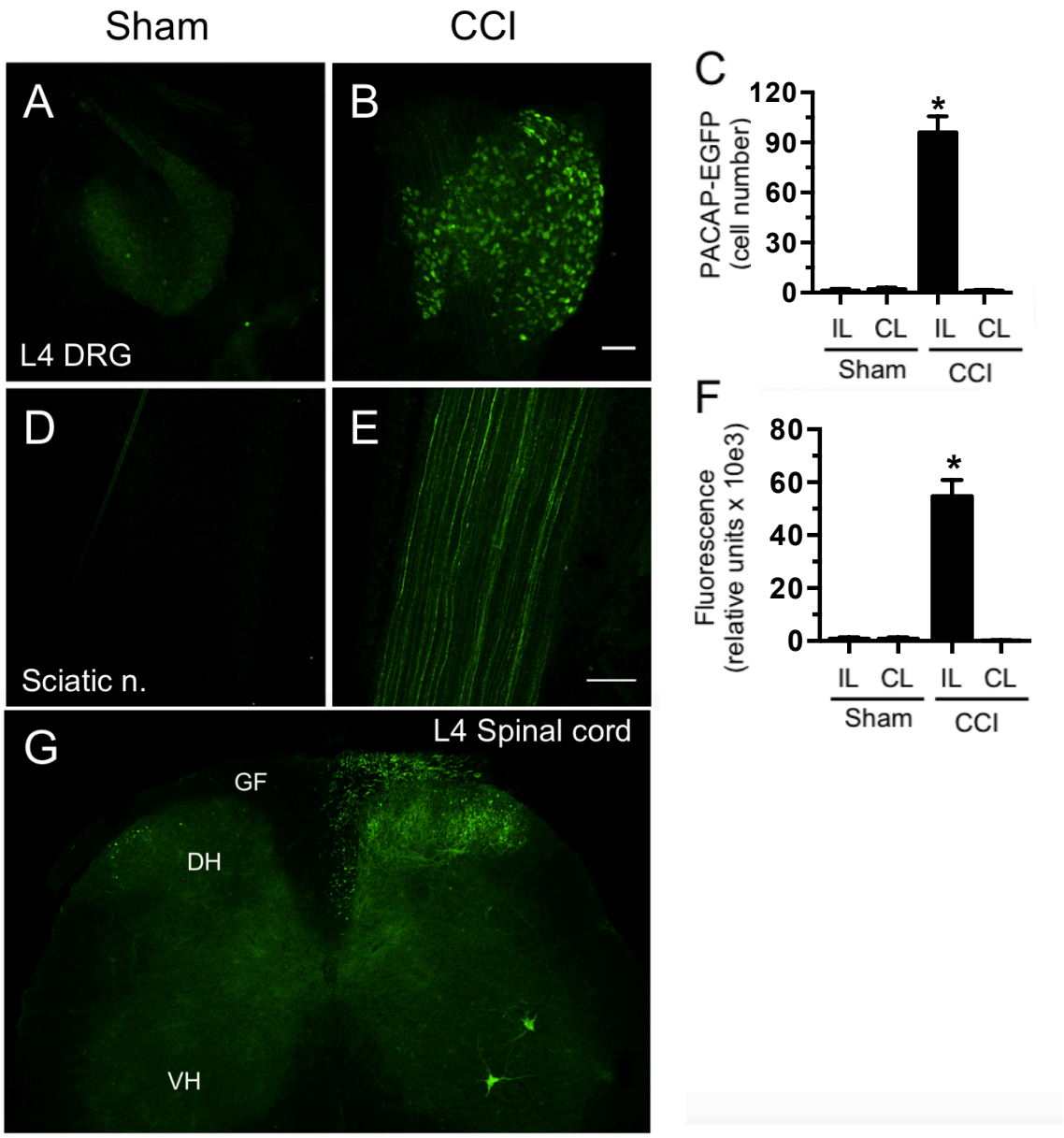
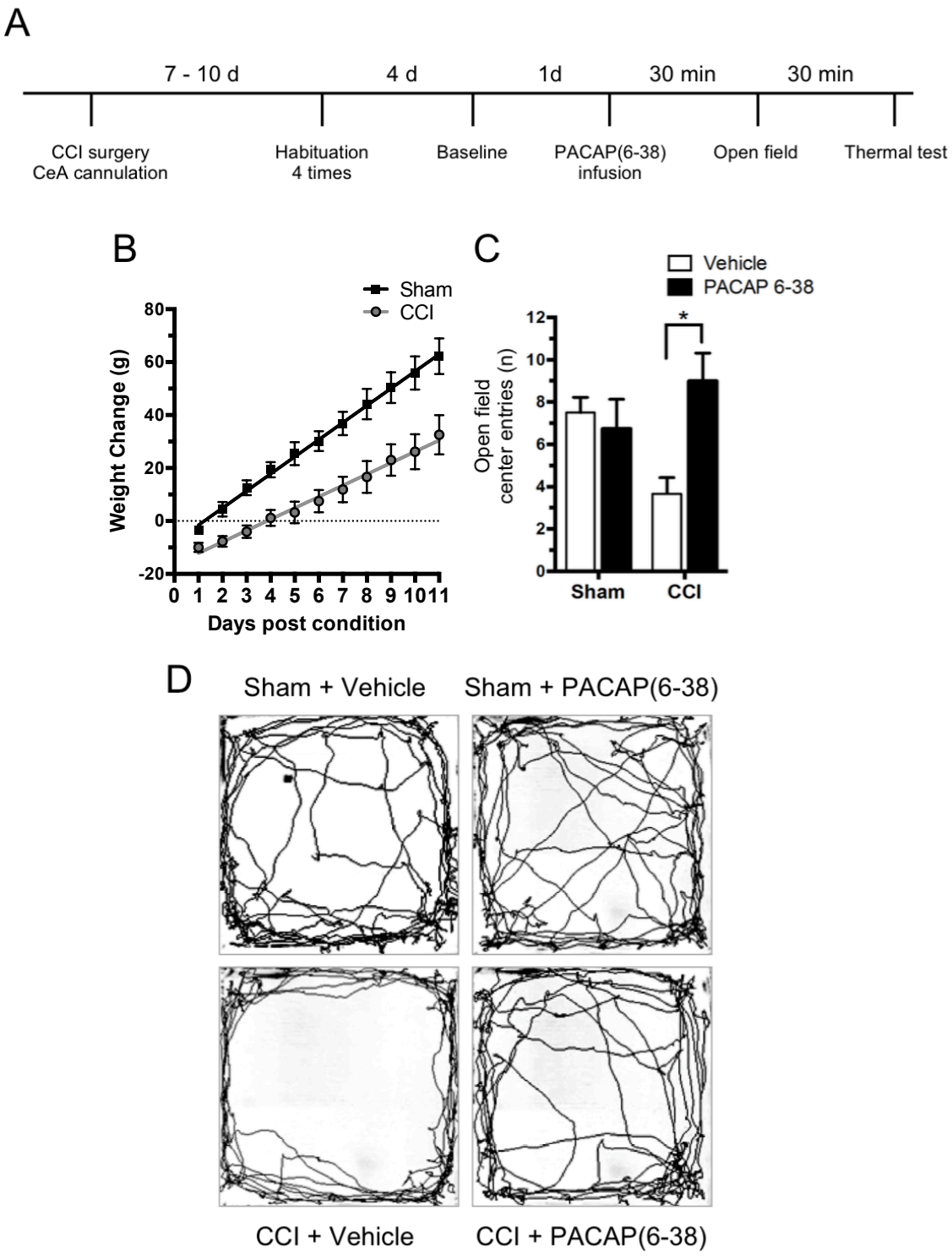

Sham
CCI
A
B
L4 DRG
C
PACAP-EGFP (cell number)
120
90
60
30
0
*
IL CL IL CL
Sham CCI
D
E
Sciatic n.
F
Fluorescence (relative units x 10e3)
80
60
40
20
0
*
IL CL IL CL
Sham CCI
G
L4 Spinal cord
GF
DH
VH

**Figure 3.2. Sensory pathway PACAP expression is enhanced by CCI.** Compared to sham surgery controls (A), unilateral partial sciatic nerve CCI (B) induced PACAP - EGFP expression in the ipsilateral L4 dorsal root ganglion (DRG) sensory neurons (C; sham ipsilateral/contralateral = 1.3 ± 0.9 cells/2.0 ± 1.0 cells vs CCI ipsilateral/contralateral = 96.0 ± 9.6 cells/1.3 ± 0.3 cells, condition*side $F(1,8) = 95.78$, $p<0.0001$), *p = 0.0001 Bonferroni's m.c; n = 3 per group). L4 DRG represents the major contributor to mouse sciatic nerve; similar PACAP-EGFP inductions were observed in L3 and L5 DRGs (Suppl Figure 3.3). The increase in CCI induced DRG PACAP expression was also reflected in peripheral and central DRG axons. The ipsilateral sciatic nerve fibers proximal to the ligation demonstrated pronounced PACAP-EGFP fluorescence (E) compared to sham (D) or contralateral control tissues (F; sham ipsilateral/contralateral = 0.8 ± 0.5 units/0.9 ± 0.4 units vs CCI ipsilateral/contralateral = 54.7 ± 6.1 units/0.2 ± 0.1 units; interaction side*condition $F(1,10)=57.22$, $p<0.0001$; n = 3 - 4). The CCI-induced PACAP-EGFP fluorescence in the central DRG axons were observed in the dorsal horn with prominent projections in the dorsal while matter tracts (G). Few PACAP-EGFP neurons were also observed in laminae I of the dorsal horn but there were no apparent differences between ipsilateral and contralateral dorsal horn PACAP neuronal number after sciatic nerve injury. CCI also induced PACAP-EGFP expression in the ipsilateral ventral horn motor neurons. Data represent mean cells/unit area or fluorescence units/unit area ± SEM; DH, dorsal horn; VH, ventral horn; GF, gracile fasciculus. Scale bars = 200 μm


A
7 - 10 d
4 d
1d
30 min
30 min
CCI surgery
CeA cannulation
Habituation
4 times
Baseline
PACAP(6-38)
infusion
Open field
Thermal test
B
Sham
CCI
Weight Change (g)
80
60
40
20
0
-20
0 1 2 3 4 5 6 7 8 9 10 11
Days post condition
C
Vehicle
PACAP 6-38
Open field
center entries (n)
12
10
8
6
4
2
0
*
Sham
CCI
D
Sham + Vehicle
Sham + PACAP(6-38)
CCI + Vehicle
CCI + PACAP(6-38)

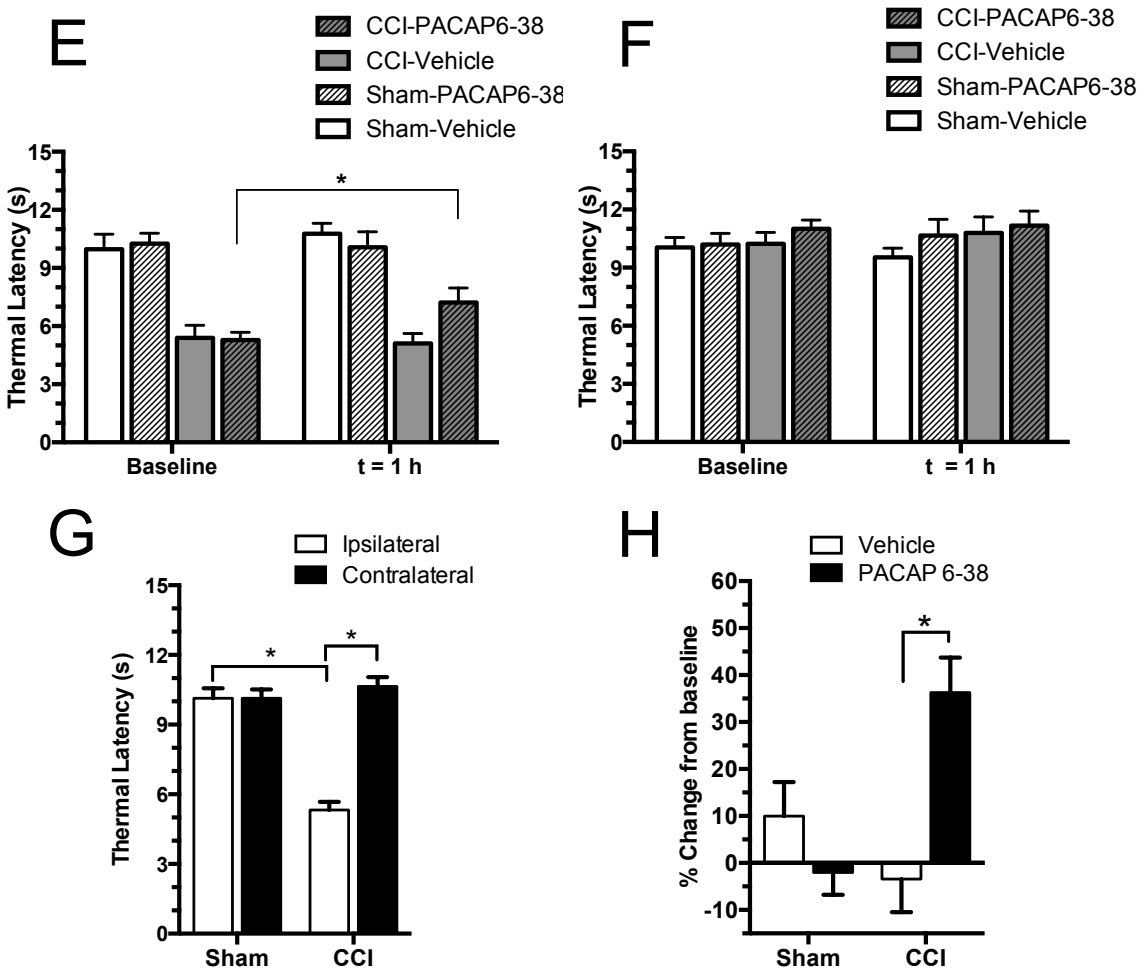

E
CCI-PACAP6-38
CCI-Vehicle
Sham-PACAP6-38
Sham-Vehicle
Thermal Latency (s)
15
12
9
6
3
0
*
Baseline
t = 1 h
F
CCI-PACAP6-38
CCI-Vehicle
Sham-PACAP6-38
Sham-Vehicle
Thermal Latency (s)
Baseline
t = 1 h
G
Ipsilateral
Contralateral
Thermal Latency (s)
*
*
Sham
CCI
H
Vehicle
PACAP 6-38
% Change from baseline
60
50
40
30
20
10
0
-10
*
Sham
CCI

**Figure 3.3. Blocking CeA PACAP signaling attenuates CCI-mediated anxiety-like behavior and thermal nociceptive hypersensitivity.** CCI and CeA cannulations were performed concurrently in rats for behavior and nociception studies in the experimental timeline shown (A). The CCI-mediated pain- and stress-related responses were associated with attenuated weight gain compared to sham control animals during the post-surgical recovery period (B). There was decreased weight gain in the CCI operated animals compared to sham, (main effect of CCI, $F(10,280) = 80.80$, $p < 0.0001$, n =8 per group).. The pain- and stress-related behavior in CCI was also reflected in decreased center entries in open field tests compared to sham controls (C; open bars). CeA infusions in sham operated animals with the PACAP receptor antagonist PACAP(6-38) had no effects on center field entries over the 5 min test period (sham-vehicle = 7.5 ± 0.7 vs sham-PACAP(6-38) = 6.75 ± 1.4, Bonferroni's m.c. $t(22) = 0.47$, $p = 0.9$) but blocked the stress- and anxiety-like open field responses in CCI (CCI-vehicle = 3.7 ± 0.8 vs CCI-PACAP(6-38) = 9.0 ± 1.3, Bonferroni's m.c. $t(22) = 3.12$, $p = 0.03$; condition*treatment $F(1,22) = 6.78$, $p = 0.02$, n = 5 - 8 per group). (D), Representative movement tracks in open field area for the 4 groups. There were no significant differences in total distance traveled for either condition or treatment. Data represent mean open field entry ± SEM. In Hargreave's thermal nociception assays, CCI increased thermal sensitivity as reflected by decreased baseline latency times in the ipsilateral hindpaw compared to the contralateral leg or in sham animals ($F(1,21) = 14.13$, $p = 0.001$). PACAP(6-38) infusions into the CeA attenuated the CCI-induced thermal hypersensitivity compared to baseline (E); simple effect of day in CCI-PACAP(6-38) on IL side (baseline: 5.3 ± 0.6s vs. 30

min: 7.2 ± 0.7s, $F(1,21) = 12.21$, $p = 0.002$) and interaction of condition*treatment*day ($F(1,21) = 7.83$, $p = 0.009$, n = 5 - 8 animals per group) and within group PACAP(6-38) ameliorated the nociceptive sensitivity. The effects were amplified when the responses of each animal were normalized to their own baseline measures prior to antagonist treatment (G; CCI-Vehicle: -3.4 ± 7.2% vs. CCI-PACAP(6-38): 36.2 ± 6.6%, simple effect of treatment $F(1,21) = 16.40$, $p = 0.001$, interaction of condition*treatment $F(1,21) = 15.49$, $p = 0.001$. (H); There were no effects of PACAP(6-38) on thermal latency in the contralateral leg (F).

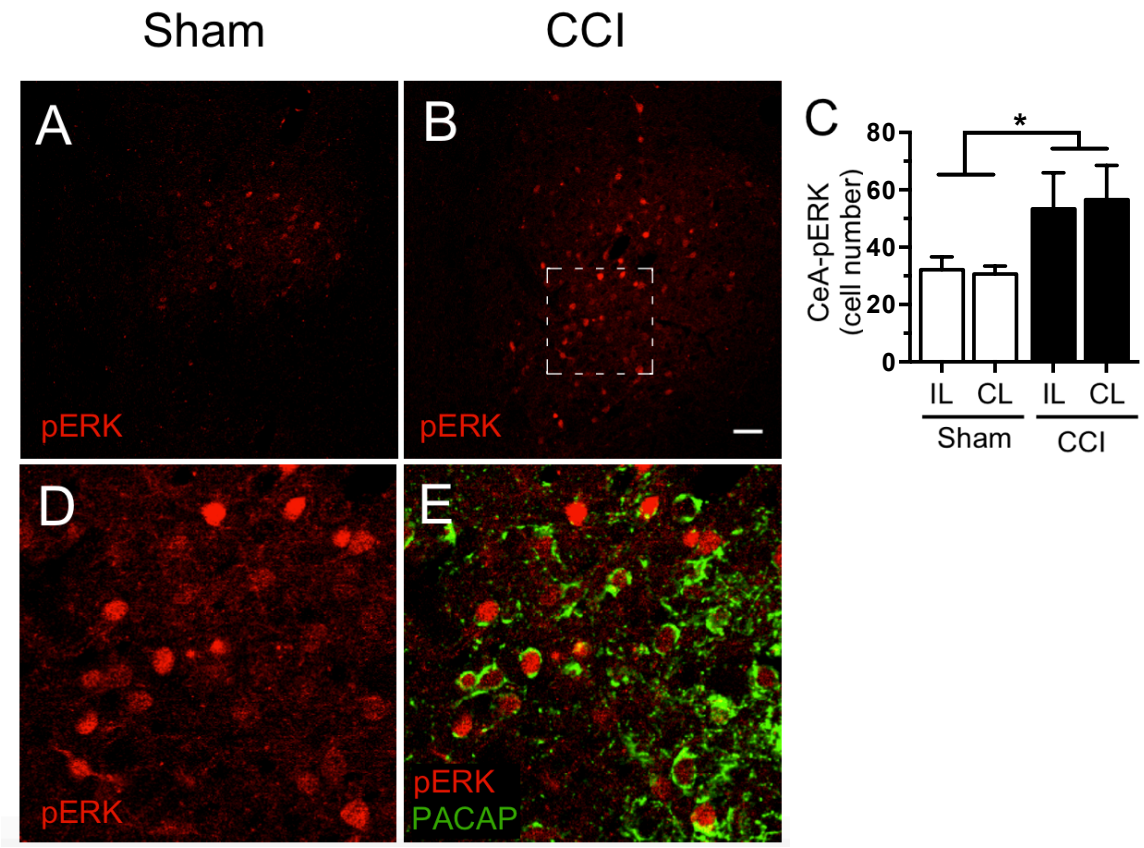
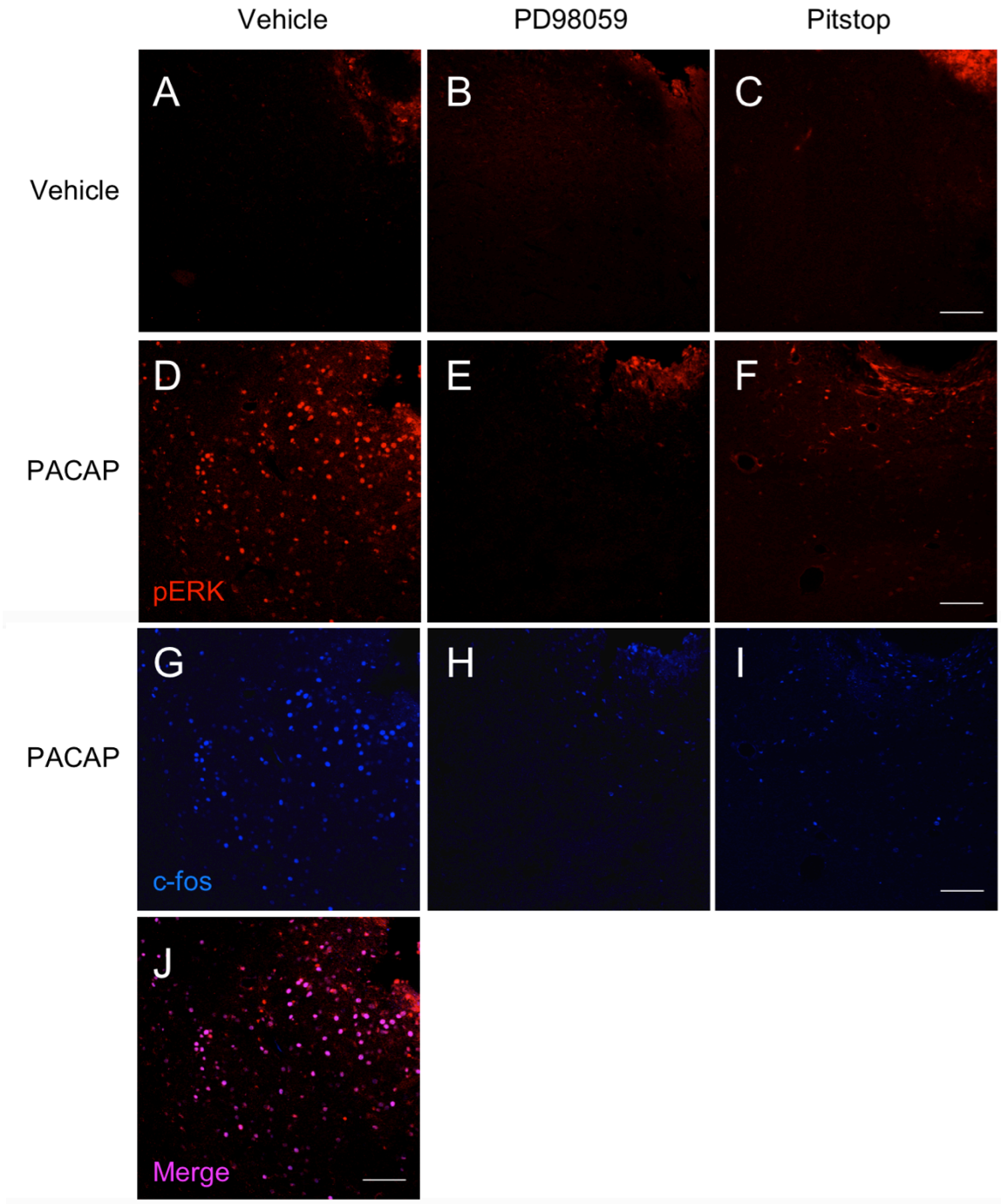



**Figure 3.4. PACAPergic fibers contact CeA activated ERK cells in CCI.**

CCI produced a bilateral increase in the number CeLC activated pERK$^+$ neurons (Cy3, red) compared to that in the sham condition (A - C; sham ipsilateral/contralateral = 32.1 ± 4.5 cells/30.6 ± 2.8 cells vs CCI ipsilateral/contralateral = 53.4 ± 12.6 cells/56.6 ± 11.9 cells, $F(1,26)=7.62$, $p = 0.01$, n = 7 - 8 animals per group). When the same sections were dually processed for PACAP immunoreactivity (AlexaFluor 488, green), a majority of the CeLC pERK$^+$ neurons were found in apposition to PACAP-immunoreactive fibers and varicosities (D - E). Data represent mean cells/unit area ± SEM; IL, ipsilateral; CL, contralateral. Scales bar = 50 µm


Vehicle
PD98059
Pitstop
Vehicle
PACAP
PACAP
A
B
C
D
E
F
G
H
I
J
pERK
c-fos
Merge

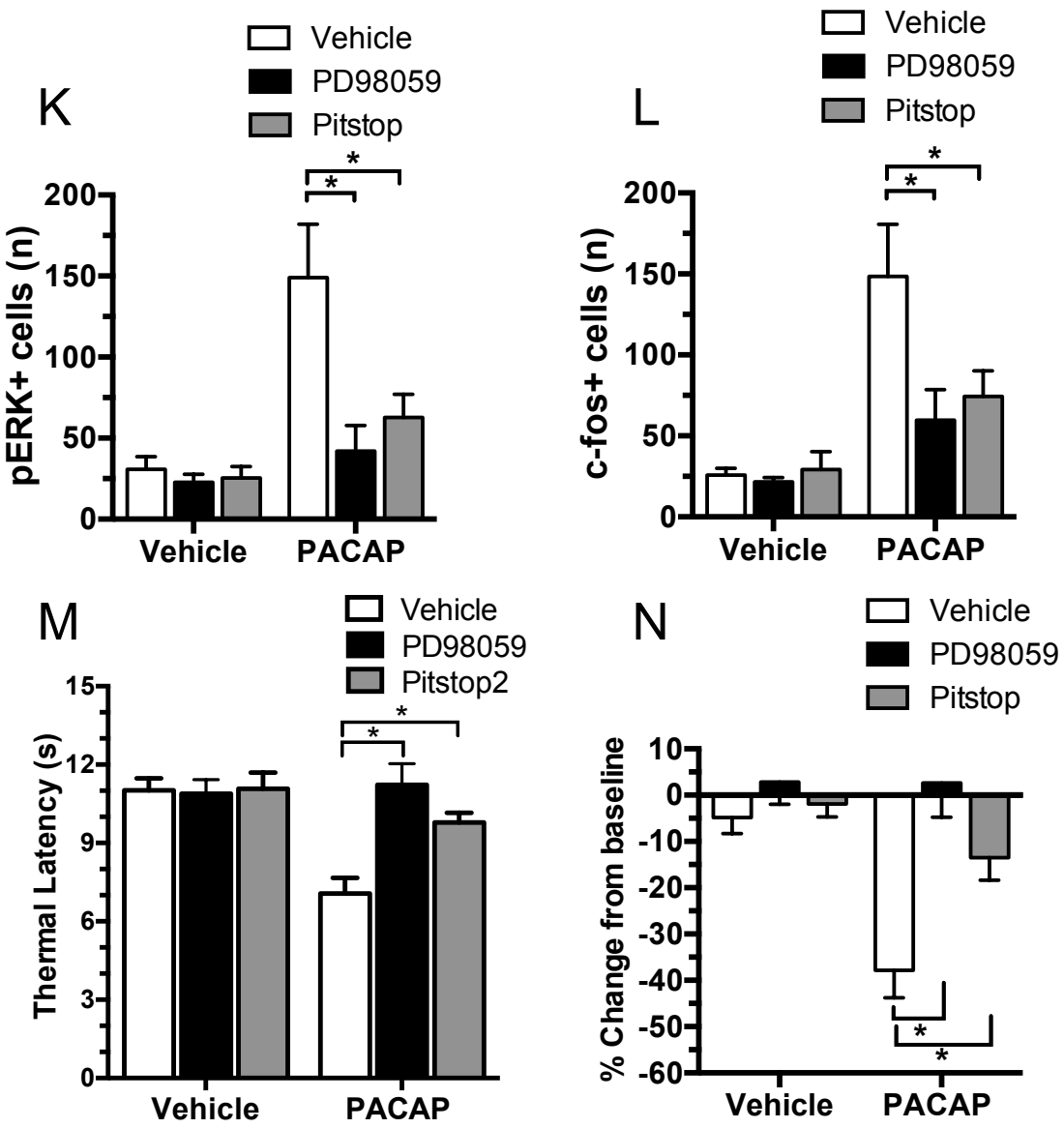
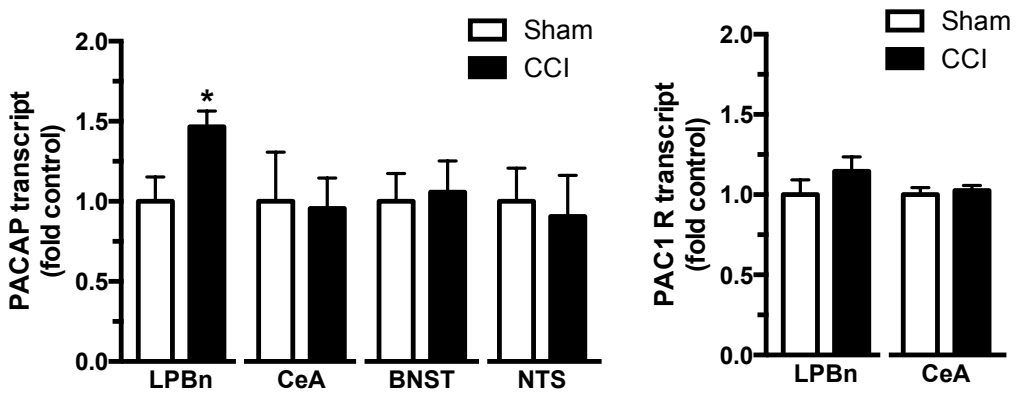

K
Vehicle
PD98059
Pitstop
pERK+ cells (n)
200
150
100
50
0
*
*
Vehicle
PACAP
L
Vehicle
PD98059
Pitstop
c-fos+ cells (n)
200
150
100
50
0
*
*
Vehicle
PACAP
M
Vehicle
PD98059
Pitstop2
Thermal Latency (s)
15
12
9
6
3
0
*
*
Vehicle
PACAP
N
Vehicle
PD98059
Pitstop
% Change from baseline
10
0
-10
-20
-30
-40
-50
-60
*
*
Vehicle
PACAP

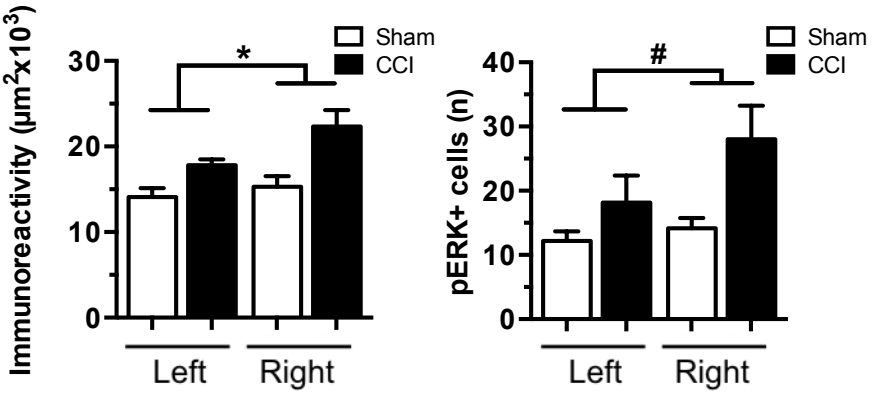
**Figure 3.5. PACAP receptor internalization and ERK activation participate in CeA-mediate nociceptive hypersensitivity.** Compared to vehicle (A), CeA PACAP infusion increased the number of activated phosphorylated ERK neurons (D, Cy3 red) which coincided with the increase in neuronal activity marker c-fos (G, J, blue). Pretreatments with MEK inhibitor PD98059 (B, E, H) or clathrin-mediated endocytosis inhibitor Pitstop 2 (C, F, I) blocked the ability of PACAP to induce ERK phosphorylation or c-fos in CeA neurons. K, The increase in PACAP-stimulated ERK activation was attenuated approximately 60 - 70% by PD98059 (vehicle + PACAP = 149.0 ± 33.1 cells vs PD98059 + PACAP = 41.9 ± 15.9 ± 6.7 cells, (bonferroni's m.c. $t(40) = 4.49$, $p = 0.0001$) and Pitstop 2 (veh + PACAP = 149.0 ± 33.1 cells vs Pitstop2 + PACAP = 62.7 ± 14.2 cells, bonferroni's m.c. $t(40) = 3.50$, $p = 0.002$), pretreatment*treatment($F2,40) = 4.67$, $p = 0.02$. L, Similarly, the increase in PACAP-stimulated c-fos levels activation was attenuated approximately 50 - 60% by PD98059 (cells/unit area, vehicle + PACAP = 148.5 ± 32.3 cells vs. PD98059 + PACAP = 59.8 ± 18.7 cells, bonferroni's m.c. $t(40) = 3.62$, $p = 0.002$) and Pitstop 2 (vehicle + PACAP = 148.5 ± 32.3 cells vs. Pitstop2 + PACAP = 74.4 ± 15.8 cells, $t(40) = 2.92$, $p = 0.02$) pretreatment*treatment ($F(2,40) = 3.46$, $p = 0.04$). Scale bar: 100 μm. Data represent mean cell number ± SEM; n = 7 - 8 per group. Commensurate with ERK activation, CeA PACAP injection induced nociceptive hypersensitivity in decreasing thermal latency; both MEK and endocytosis inhibition blocked the PACAP-induced thermal sensitivity (M; latency in sec, Veh + PACAP = 7.1 ± 0.6 sec vs PD98059 + PACAP = 11.2 ± 0.8, bonferroni's m.c. $t(41) = 5.05$, $p < 0.0001$; Veh + PACAP = 7.1 ± 0.6 vs

Pitstop2 + PACAP = 9.8 ± 0.4, bonferroni's m.c. $t(41) = 3.31$, $p = 0.004$) (pretreatment*treatment $F(2,41) = 6.64$, $p = 0.003$). Expressed as percent change from baseline measures of each animal before drug administration, both MEK inhibition (% latency change from vehicle control; PACAP = -37.8 ± 5.9% vs. PD98059 + PACAP = 2.6 ± 4.8%, bonferroni's m.c. $t(41) = 5.58$, $p=0.0001$) and endocytosis inhibitor Pitstop 2 (PACAP = -37.8 ± 5.9% vs Pitstop + PACAP = -13.5 ± 4.9%, bonferroni's m.c. $t(40) = 3.36$, $p = 0.003$) attenuated nociceptive hypersensitivity. Data represent mean ± SEM, n = 7 - 8 per group.

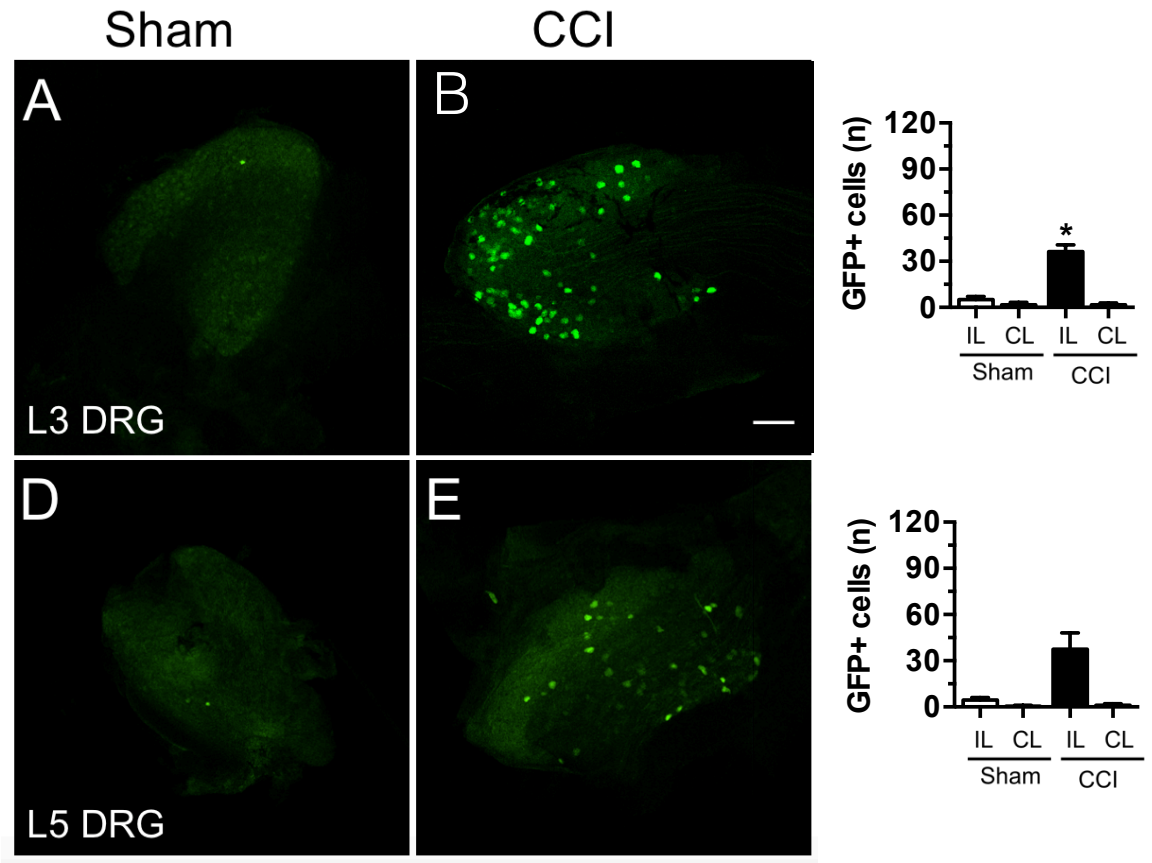



**Supplementary Figure 3.1. CCI increases PACAP transcript in the LPBn.**

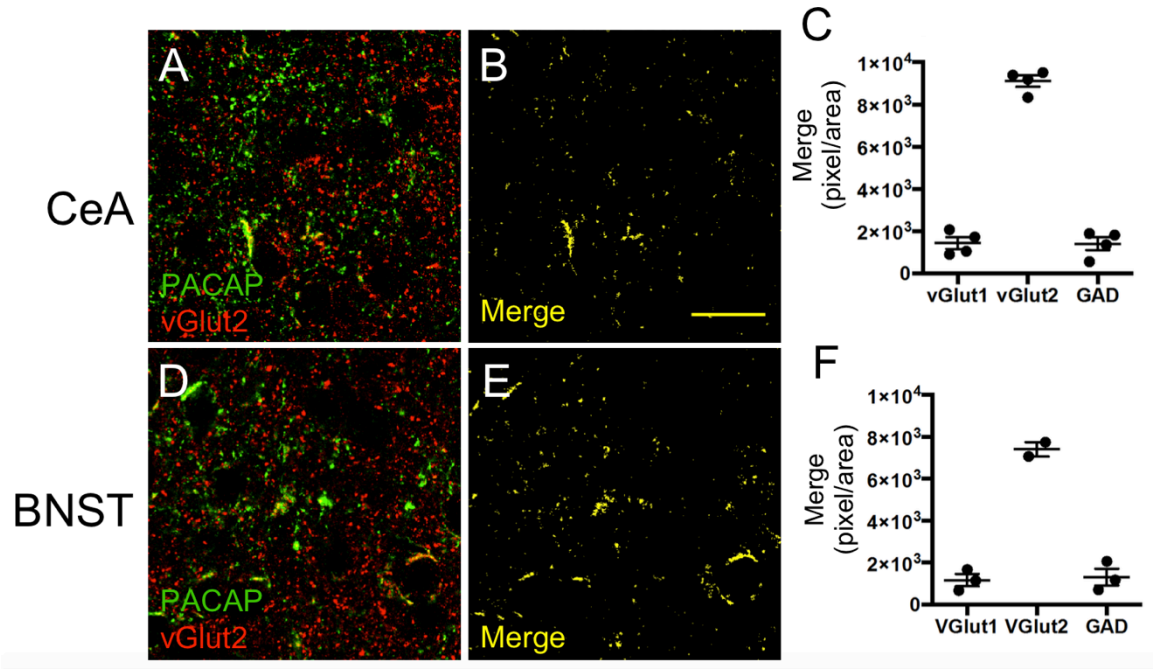
Adult male rats underwent either CCI or sham surgery as described in text and 14 days following the indicated brain regions were harvested for quantitative PCR analysis. Tissue samples for each region were reverse transcribed and normalized against 18s RNA. In the LPBn was a significant increase in PACAP transcript (1.47 ± 0.1) fold change SEM) compared to tissues from sham animals ((1.00 ± 0.2), $t(12) = 2.36$, $p = 0.036$). Demonstrating that this effect may be specific to the LPBn, there were no significant changes in PACAP transcript in the CeA (CCI: 0.96 ± 0.2 vs. sham: 1.00 ± 0.3, $t(12) = 0.12$, $p = 0.9$), anterolateral BNST (CCI:1.06 ± 0.2 vs. sham: 1.00 ± 0.2, $t(13) = 0.21$, $p = 0.8$), or the solitary nucleus (NTS)(CCI: 1.00 ± 0.2 vs. sham: 0.90 ± 0.3, $t(14) = 0.29$, $p = 0.8$). There were no significant changes in PAC1 R transcript in the LPBn (CCI:1.15 ± 0.1 vs. Sham 1.00 ± 0.1, $t(12) = 1.09$, $p = 0.3$) or CeA (CCI:1.03 ± 0.1 vs. Sham: 1.00 ± 0.1, $t(12) = 0.45$, $p = 0.7$). n = 6-8 per group, dependent on viability of tissue sample during processing. Data represent fold change normalized to 18s; ± SEM.



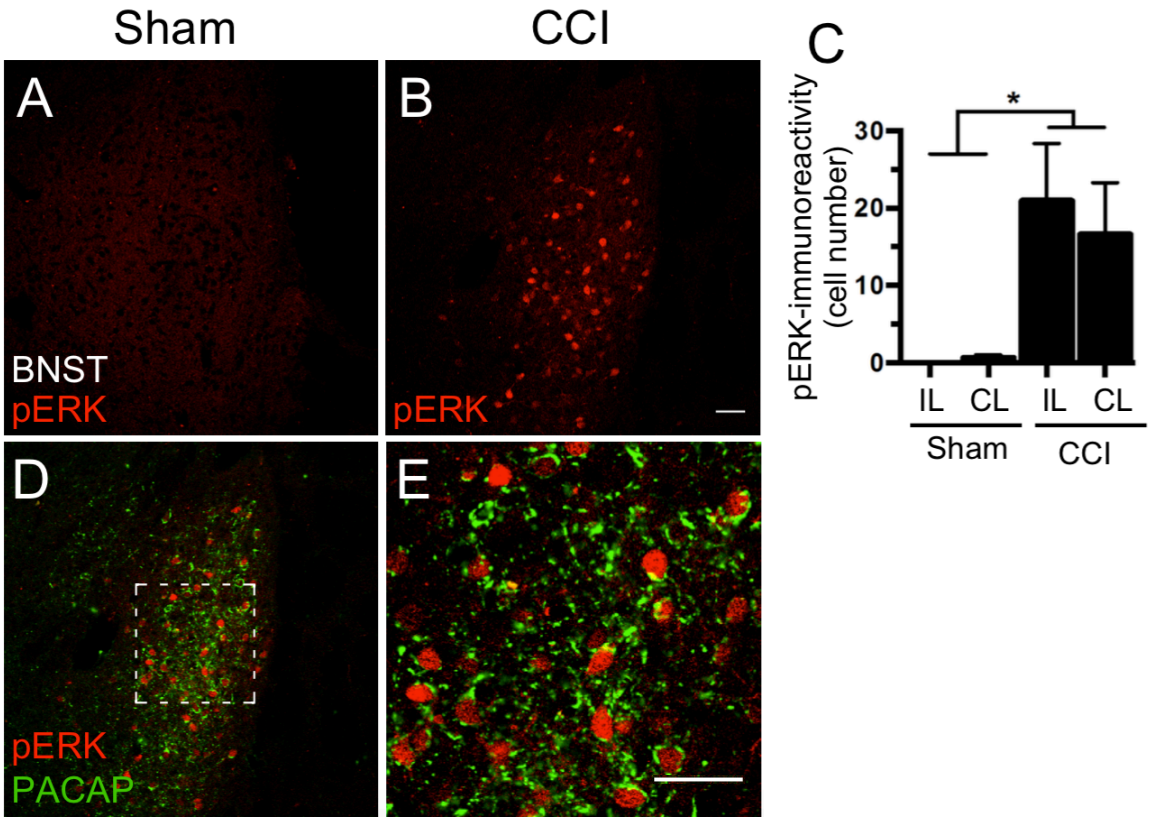

**Supplementary Figure 3.2. The CeA demonstrates lateralization in CCI-induced increases in PACAP and pERK immunoreactivity.** CCI (14 days) preferentially increased PACAP immunoreactivity and $pERK^+$ cells in the right CeA. When thresholded PACAP immunoreactivity from Figure 3.2 was analyzed with respect to right or left CeA, there was a significant main effect of side (A; $F(1,28) = 4.87$, $^*p = 0.04$), but no interaction between side and condition ($F(1,28) = 1.63$, $p = 0.2$), with greater PACAP immunoreactivity in the right CeA. There was a significant main effect of CCI for increased PACAP immunoreactivity ($F(1,28) = 17.24$, $p = 0.0003$). There was a similar bias in $pERK^+$ cells in the right CeA with a trend for the effect of side (B; $F(1,26) = 3.15$, $p = 0.09$). There was also a main effect of CCI for increased pERK+ cells ($F(1,26) = 8.85$, $p = 0.006$). These results appear consistent with the lateralization of CeA pERK shown previously in persistent pain, and implicate PACAP in the lateralization of the nociceptive process.



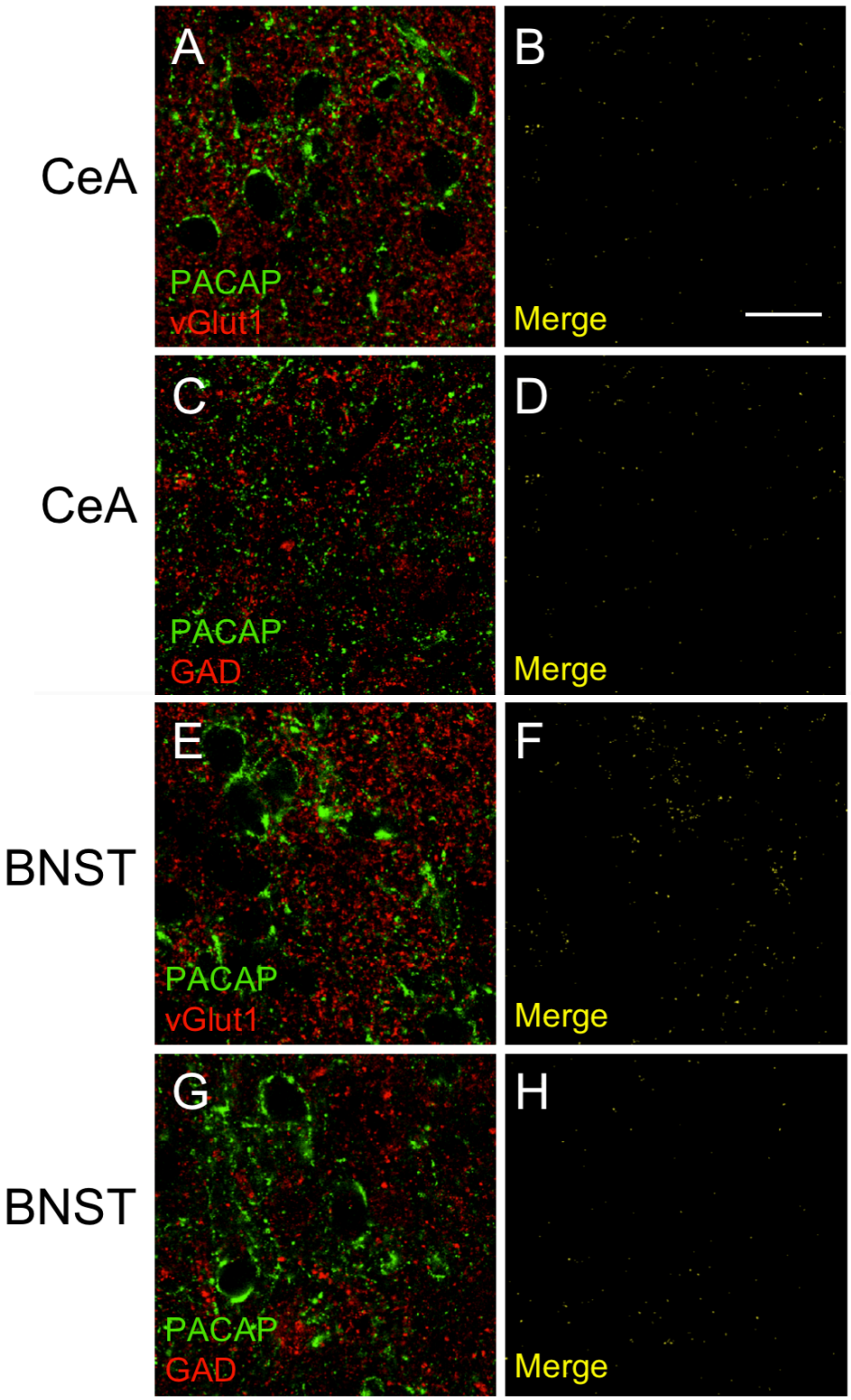

**Supplementary Figure 3.3. CCI increases PACAP-EGFP expressing L3 and L5 DRG neurons.** Similar to the L4 DRG (Figure 3.2), unilateral CCI increased the number of L3 and L5 DRG PACAP-EGFP$^{+}$ neurons 14 days postsurgery (B, E) compared to sham controls (A, D). L3 - L5 DRG peripheral sensory axons travel in the sciatic nerve with major contributions from L4. The increase in CCI-induced PACAP-EGFP+ neuron expression in L3 DRG (C; sham ipsilateral = 5.0 ± 2.1 cells vs CCI ipsilateral = 36.3 ± 4.3 cells, *p = 0.0002, n = 3 per group) and L5 DRG (F; sham ipsilateral = 4.5 ± 1.5 cells vs CCI ipsilateral = 37.5 ± 10.5 cells, n = 2 per group) was not as robust as that in L4 DRG. Data represent mean cells/unit area ± SEM.



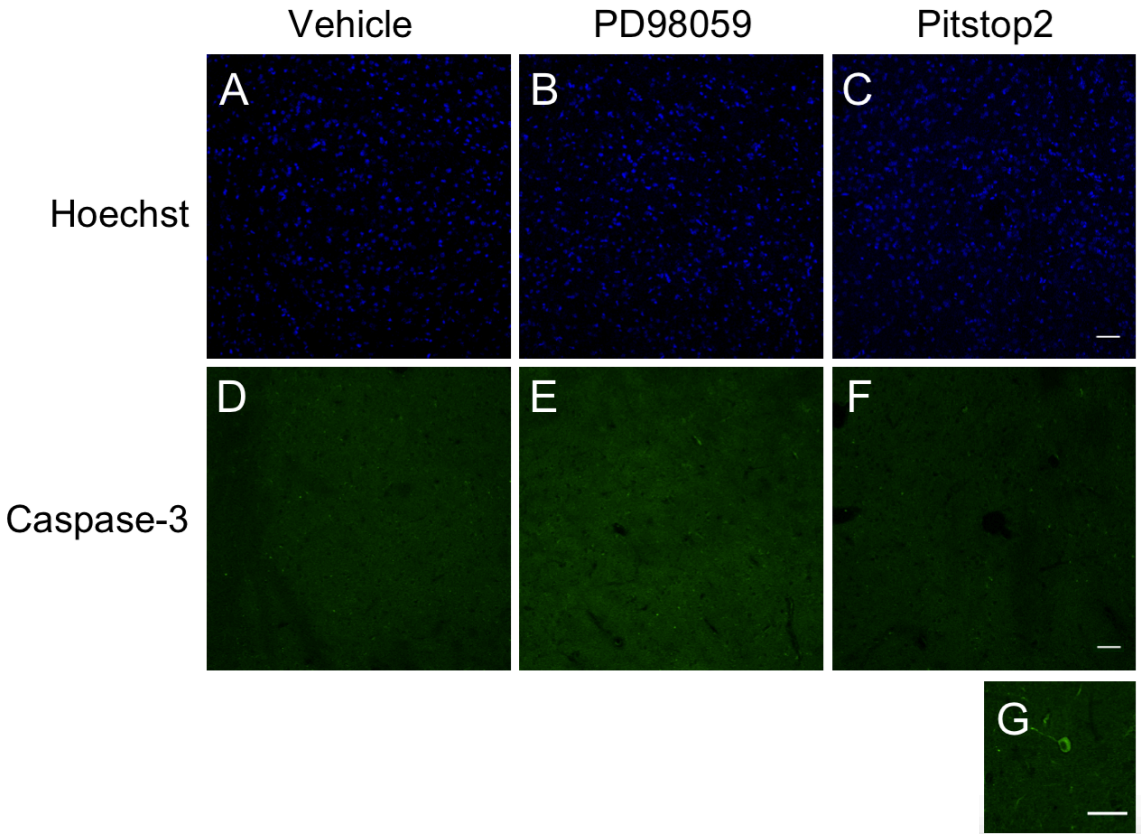

**Supplementary Figure 3.4. CeA and BNST PACAP fibers colocalize predominantly with vGlut2 immunoreactivity.** CeA (A) and BNST (D) tissues were dually processed for PACAP (Alexa Fluor 488, green) and vGlut2 (Cy3, red) to help establish neuronal transmitter identity. CeA and BNST PACAP colocalized with glutaminergic marker vGlut2 as shown in their respective isolated merged signals (B, E; yellow). From quantitative image analyses, there was minimal overlap between PACAP and vGlut1 or GAD (C, F; see Suppl. Figure 3.5). Scale bar = 25 µm




**Supplementary Figure 3.5. PACAPergic fibers contact BNST pERK$^+$ neurons in CCI.** As in the CeLC, CCI increased bilaterally the number of pERK$^+$ neurons in the anterolateral BNST compared to sham controls (A - C). The BNST pERK$^+$ cells (Cy3, red) were in close contact with PACAP fibers (D,E; Alexa Fluor 488, green), implicating PACAP as a potential mechanism of CCI-induced nociceptive ERK signaling. There is a main effect of CCI (C; $F(1,8) = 15.3$ $p = 0.005$, $n = 3$ per group). Scale bar = 50µm.


A
B
CeA
PACAP
vGlut1
Merge
C
D
CeA
PACAP
GAD
Merge
E
F
BNST
PACAP
vGlut1
Merge
G
H
BNST
PACAP
GAD
Merge

**Supplementary Figure 3.6. CeA and BNST PACAP immunoreactivity does not colocalize with vGlut1 or GAD.** CeA (A, B) and BNST (E, G) tissues were dually processed for PACAP (Alexa Fluor 488, green) and vGlut (Cy3, red) or GAD (Cy3, red). Unlike vGlut2 (Supplementary Figure 3.4), there was little overlap with the glutamatergic marker vGlut1 or GABAergic marker GAD in both regions as shown by the paucity of merged signals (B, D, F and H; yellow). Quantitative analyses in Suppl. Figure 3.4. Scale bar = 25 μm




**Supplementary Figure 3.7. Acute CeA infusions with inhibitors does not induce apoptosis.** To verify that the CeA infusions with drugs to block MEK (PD98059) or endocytic mechanisms (Pitstop 2) did not cause overt neurotoxicity and apoptosis to impact results, the treated tissues were also processed for nuclear Hoechst staining (A - C) and apoptotic marker cleaved caspase-3 immunoreactivity (D - F). Hoechst nuclear staining confirmed there were no apparent signs of substantial cell loss in any of the treatment conditions; further, there were no signs of any ongoing apoptosis in the CeA. Cleaved caspase $3^+$ cells were found sporadically throughout the brain; G, an example of a cleaved caspase $3^+$ hippocampal neuron at the same magnification. Scale bar = 50µm

### 3.7. References


1. Asmundson, G. J. & Katz, J. Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. *Depress Anxiety.* **26**, 888-901. (2009).

2. Scioli-Salter, E. R. *et al.* The shared neuroanatomy and neurobiology of comorbid chronic pain and PTSD: therapeutic implications. *Clin J Pain.* **31**, 363-374. (2015).

3. Veinante, P., Yalcin, I. & Barrot, M. The amygdala between sensation and affect: a role in pain. *Journal of Molecular Psychiatry* **1**, 9 (2013).

4. Bernard, J. F., Bester, H. & Besson, J. M. Involvement of the spino-parabrachio - amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. *Prog Brain Res.* **107**, 243-255. (1996).

5. Gauriau, C. & Bernard, J. F. Pain pathways and parabrachial circuits in the rat. *Exp Physiol.* **87**, 251-258. (2002).

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

7. Todd, A. J. Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci.* **11**, 823-836. (2010).

8. Neugebauer, V., Li, W., Bird, G. C., Bhave, G. & Gereau, R. W. t. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. *J Neurosci.* **23**, 52-63. (2003).

9. Bird, G. C. *et al.* Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. *J Physiol.* **564** 907-921 (2005).

10. Carrasquillo, Y. & Gereau, R. W. t. Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J Neurosci.* **27**, 1543-1551. (2007).

11. Lu, Y. C. *et al.* Neurochemical properties of the synapses between the parabrachial nucleus-derived CGRP-positive axonal terminals and the GABAergic neurons in the lateral capsular division of central nucleus of amygdala. *Mol Neurobiol* **51**, 105-118. (2015).

12. Ren, W. & Neugebauer, V. Pain-related increase of excitatory transmission and decrease of inhibitory transmission in the central nucleus of the amygdala are mediated by mGluR1. *Mol Pain.* **6**, 93. (2010).

13. Han, J. S., Adwanikar, H., Li, Z., Ji, G. & Neugebauer, V. Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. *Mol Pain.* **6**, 10. (2010).

14. Vaudry, D. *et al.* Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev.* **61**, 283-357. (2009).

15. Ressler, K. J. *et al.* Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* **470**, 492-497. (2011).

16. Almli, L. M. *et al.* ADCYAP1R1 genotype associates with post-traumatic stress symptoms in highly traumatized African-American females. *Am J Med Genet B Neuropsychiatr Genet.* **162B**, 262-272. (2013).

17. Jovanovic, T. *et al.* PAC1 receptor (ADCYAP1R1) genotype is associated with dark-enhanced startle in children. *Mol Psychiatry* **18**, 742-743 (2013).

18. Uddin, M. *et al.* Adcyap1r1 genotype, posttraumatic stress disorder, and depression among women exposed to childhood maltreatment. *Depress Anxiety.* **30**, 251-258. (2013).

19. Pohlack, S. T. *et al.* Neural mechanism of a sex-specific risk variant for posttraumatic stress disorder in the type I receptor of the pituitary adenylate cyclase activating polypeptide. *Biol Psychiatry.* **doi: 10.1016/j.biopsych.** (2015).

20. Hashimoto, H. *et al.* Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proc Natl Acad Sci USA* **98**, 13355-13360. (2001).

21. Girard, B. A. *et al.* Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP- and PACAP-deficient mice. *J Neurochem* **99**, 499-513. (2006).

22. Hattori, S. *et al.* Comprehensive behavioral analysis of pituitary adenylate cyclase-activating polypeptide (PACAP) knockout mice. *Front Behav Neurosci* **6**, 58. (2012).

23. Otto, C. *et al.* Altered emotional behavior in PACAP-type-I-receptor-deficient mice. *Brain Res Mol Brain Res* **92**, 78-84. (2001).

24. Stroth, N. & Eiden, L. E. Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling. *Neuroscience* **165**, 1025-1030. (2010).

25. Tsukiyama, N. *et al.* PACAP centrally mediates emotional stress-induced corticosterone responses in mice. *Stress* **14**, 368-375. (2011).

26. Botz, B. *et al.* Role of pituitary adenylate-cyclase activating polypeptide and Tac1 gene derived tachykinins in sensory, motor and vascular functions under normal and neuropathic conditions. *Peptides* **43**, 105-112. (2013).

27. Jongsma, H. *et al.* Markedly reduced chronic nociceptive response in mice lacking the PAC1 receptor. *Neuroreport* **12**, 2215-2219. (2001).

28. Hammack, S. E. *et al.* 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. *Psychoneuroendocrinology* **34**, 833-843. (2009).

29. Roman, C. W. *et al.* PAC1 receptor antagonism in the bed nucleus of the stria terminalis (BNST) attenuates the endocrine and behavioral consequences of chronic stress. *Psychoneuroendocrinology* **47**, 151-165. (2014).

30. Lezak, K. R. *et al.* Regulation of bed nucleus of the stria terminalis PACAP expression by stress and corticosterone. *J Mol Neurosci.* **54**, 477-484. ( 2014).

31. Missig, G. *et al.* 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. (2014).

32. Zhang, Y. Z. *et al.* Pituitary adenylate cyclase activating peptide expression in the rat dorsal root ganglia: up-regulation after peripheral nerve injury. *Neuroscience* **74**, 1099-1110. (1996).

33. Pettersson, L. M., Dahlin, L. B. & Danielsen, N. Changes in expression of PACAP in rat sensory neurons in response to sciatic nerve compression. *Eur J Neurosci.* **20**, 1838-1848. (2004).

34. Carrasquillo, Y. & Gereau, R. W. t. Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. *Mol Pain.* **4**, 24. (2008).

35. Ji, G. & Neugebauer, V. Hemispheric lateralization of pain processing by amygdala neurons. *J Neurophysiol.* **102**, 2253-2264. (2009).

36. Ji, R. R., 4th, G. R. W., Malcangio, M. & Strichartz, G. R. MAP kinase and pain. *Brain Res Rev* **60**, 135-148. (2009).

37. Cheng, S. J. *et al.* Role of extracellular signal-regulated kinase in synaptic transmission and plasticity of a nociceptive input on capsular central amygdaloid neurons in normal and acid-induced muscle pain mice. *J Neurosci.* **31**, 2258-2270. (2011).

38. Tran, L., Schulkin, J. & Greenwood-Van Meerveld, B. Importance of CRF receptor-mediated mechanisms of the bed nucleus of the stria terminalis in the processing of anxiety and pain. *Neuropsychopharmacology* **39**, 2633-2645. (2014).

39. Barrie, A. P., Clohessy, A. M., Buensuceso, C. S., Rogers, M. V. & Allen, J. M. Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a Ras-independent, mitogen-activated protein kinase/ERK kinase 1 or -dependent manner in PC12 cells. *J Biol Chem* **272**, 19666-19671. (1997).

40. Bouschet, T. *et al.* Stimulation of the ERK pathway by GTP-loaded Rap1 requires the concomitant activation of Ras, protein kinase C, and protein kinase A in neuronal cells. *J Biol Chem* **278**, 4778-4785. (2003).

41. May, V. *et al.* Pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1HOP1 receptor activation coordinates multiple neurotrophic signaling pathways: Akt activation through phosphatidylinositol 3-kinase and vesicle endocytosis for neuronal survival. *J Biol Chem* **285**, 9749-9761. (2010).

42. May, V., Buttolph, T. R., Girard, B. M., Clason, T. A. & Parsons, R. L. PACAP-induced ERK activation in HEK cells expressing PAC1 receptors involves both receptor internalization and PKC signaling. *Am J Physiol Cell Physiol.* **306**, C1068-C1079. (2014).

43. Moller, K. *et al.* The effects of axotomy and preganglionic denervation on the expression of pituitary adenylate cyclase activating peptide (PACAP), galanin and PACAP type 1 receptors in the rat superior cervical ganglion. *Brain Res.* **775**, 166-182. (1997).

44. Moller, K. *et al.* Pituitary adenylate cyclase activating peptide is a sensory neuropeptide: immunocytochemical and immunochemical evidence,. *Neuroscience* **57**, 725-732. (1993).

45. Zhang, Y. *et al.* Capsaicin-evoked release of pituitary adenylate cyclase activating peptide (PACAP) and calcitonin gene-related peptide (CGRP) from rat spinal cord in vivo. *Regul Pept.* **69**, 83-87. (1997).

46. Sandor, K. *et al.* Divergent peripheral effects of pituitary adenylate cyclase-activating polypeptide-38 on nociception in rats and mice. *Pain* **141**, 143-150. (2009).

47. Davis-Taber, R. *et al.* Central pituitary adenylate cyclase 1 receptors modulate nociceptive behaviors in both inflammatory and neuropathic pain states. *J Pain* **9**, 449-456. (2008).

48. Shimizu, T., Katahira, M., Sugawara, H., Inoue, K. & Miyata, A. Diverse effects of intrathecal pituitary adenylate cyclase-activating polypeptide on nociceptive transmission in mice spinal cord. *Regul Pept.* **123**, 117-122. (2004).

49. Alba-Delgado, C. *et al.* Chronic pain leads to concomitant noradrenergic impairment and mood disorders. *Biol Psychiatry.* **73**, 54-62. (2013).

50. Hammack, S. E. & May, V. Pituitary adenylate cyclase activating polypeptide in stress-related disorders: data convergence from animal and human studies. *Biol Psychiatry.* **doi: 10.1016/j.biopsych.2014.12.003** (2014).

51. Lehmann, M. L., Mustafa, T., Eiden, A. M., Herkenham, M. & Eiden, L. E. PACAP-deficient mice show attenuated corticosterone secretion and fail to develop depressive behavior during chronic social defeat stress. *Psychoneuroendocrinology* **38**, 702-715. (2013).

52. Han, J. S., Li, W. & Neugebauer, V. Critical role of calcitonin gene-related peptide 1 receptors in the amygdala in synaptic plasticity and pain behavior. *J Neurosci.* **25**, 10717-10728. (2005).

53. Fu, Y. & Neugebauer, V. Differential mechanisms of CRF1 and CRF2 receptor functions in the amygdala in pain-related synaptic facilitation and behavior. *J Neurosci.* **28**, 3861-3876. (2008).

54. Adwanikar, H. *et al.* Spinal CGRP1 receptors contribute to supraspinally organized pain behavior and pain-related sensitization of amygdala neurons. *Pain* **132**, 53-66 (2007).

55. Cho, J. H. *et al.* Pituitary adenylate cyclase-activating polypeptide induces postsynaptically expressed potentiation in the intra-amygdala circuit. *J Neurosci.* **32**, 14165-14177. (2012).

56. Bourgeais, L., Gauriau, C. & Bernard, J. F. Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. *Eur J Neurosci.* **14(2):**, 229-255. (2001).

57. Bennett, G. J. & Xie, Y. K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**, 87-107. (1988).

# Chapter 4.

## General Discussion

The studies in this dissertation were aimed to investigate the role of CeA PACAP signaling in mediating the emotional components of pain. Severe emotional dysregulation often co-exists in patients with chronic pain, as evidenced by the high rates of comorbid affective disorders including post-traumatic stress disorder (PTSD), generalized anxiety disorder (GAD), and panic disorder (PD). Chronic pain carries an enormous personal, societal, and economic burden and in the presence of comorbid affective disorders, the degree of disability and suffering in these individuals becomes greatly amplified. Moreover, the presence of an affective disorder may not only exacerbate pain, but may also act to reinforce the underlying mechanistic processes of chronic pain in a self-perpetuating cycle. As these mechanisms are not well understood, studies elucidating the key signaling molecules and neural circuits in this system may offer insights to the pathogenesis of these disorders and provide therapeutic approaches to break the cycle of chronic pain and affective disorders. To this end, the studies in this dissertation find evidence that PACAP signaling within the parabrachio-amygdaloid tract may be a key mediator of the emotional components of pain.

## 4.1. Insights into PACAP neurocircuits and plasticity

*PACAP expression in the spino-parabrachioamygdaloid tract*

In the course of ongoing investigation, our laboratory found dense PACAP fiber immunoreactivity in the CeLC and CeL regions of the central amygdala (CeA). From *in situ* hybridization data, there appeared to be little endogenous PACAP expression within the CeA, indicating that the observed immunoreactivity reflected axonal fiber projections of undetermined external origins (Piggins et al., 1996). Using anterograde tracing with 10 kDa BDA and excitotoxic lesion studies, we showed that the vast majority CeA PACAP immunoreactivity originated from the LPBn (ure 2.3, 2.4). This finding is of particular interest because sensory input converges on the LPBn before projecting to the CeLC. Nociceptive information from the entire body and face are relayed by the spinal cord and sensory trigeminal system, respectively, via second order sensory afferents onto LPBn neurons. Additionally, the LPBn also receives visceral input from the vagal nerve via relays from the NTS. Given the involvement of the LPBn in sensory systems, the expression of PACAP in the LPBn is suggestive of a role in the processing of nociceptive stimuli.

The LPBn has major projections to the CeLC, anterolateral BNST (BNSTal), and the VMH. Interestingly, LPBn-BNST projections are either direct or via collaterals from axons ultimately projecting to the CeLC (Sarhan et al., 2005). We found that lesioning the LPBn resulted in a substantial loss (~70%) of PACAP expression within the ipsilateral BNST, similar to the findings within the CeA (Figure 2.4). Although the source of the remaining BNST PACAP (~30%) was not investigated, the residual

PACAP could have originated from the PVH or dorsal vagal complex, or represented endogenous BNST expression (Hammack et al., 2009; Kozicz et al. 1998). Although not examined directly in this work, PACAP in the VMH was previously found to originate from the LPBn (Resch et al., 2013). In projecting to the CeLC, BNST and VMH, the population of LPBn PACAP neurons may be components of a much enlarged network and behave as a sensory distribution hub, relaying discrete information to these three regions to coordinate the behavioral and physiological responses to aversive sensory input. Along this line, the effect of PACAP signaling in each of these regions has now been investigated. PACAP infusion directly into the VMH resulted in hypophagia and increased thermogenesis (Resch et al., 2011). BNST PACAP infusion produces anxiety-like behaviors, hypophagia, weight loss, and HPA axis activation (Hammack et al., 2009; Roman et al. 2014; Kocho-Schellenberg et al., 2014; Lezak et al., 2014). Whereas, CeA PACAP was demonstrated in the current studies to produce nociceptive hypersensitivity and anxiety-like behavior, CeA PACAP signaling has also recently been reported to produce a delayed hypophagia and weight loss (Figure 2.6; Iemolo et al., 2015). Interestingly, PACAP signaling in CeA, BNST, and VMH appear to initiate various combinations of hypophagia, anxiety-like behavior, and nociceptive hypersensitivity. Hence, these responses may represent a behavioral and physiological phenotype that is characteristic of sustained or enhanced LPBn activity, as might occur following prolonged nociceptive input with chronic pain.

Further, PACAP expression in nociceptive pathways is not restricted to LPBn-CeLC projections, as it is found all along the spino-parabrachio-amygdaloid pathway

(Figure 4.1). PACAP is found within peripheral afferent terminals and within a subset of peptidergic DRG cells that also express CGRP and the precursor of substance P (Mulder et al., 1994; Usoskin et al., 2015). PACAPergic fibers from these DRGs project to the dorsal horn, and dense PACAP immunoreactivity is found in lamina I/II of the spinal cord (Vizzard, 2000). At the next step of this pathway, neurons in lamina I/II have been reported to express PACAP, and although yet to be established, these neurons may represent the second-order spinal projection neurons that relay nociceptive information to the LPBn (Pettersson et al., 2004). In our experiments utilizing PACAP-EGFP mice, we confirmed PACAP-EGFP expression in a subset of DRG neurons and consistently found PACAP-EGFP expressing cells within lamina I/II of the spinal cord (Figure 3.2). Our findings are in agreement with prior work and demonstrate that PACAP is expressed at all levels of the spino-parabrachio-amygdaloid pathway. These PACAP expression patterns raise the possibility of PACAP-expressing neurons synapsing onto other PACAP neurons (PACAP to PACAP projections) all along the spino-parabrachio-amygdaloid tract. Mechanistically this system would appear plausible, as PACAP signaling was found to exhibit positive autoregulation in the sympathetic system, with PACAP receptor activation driving more PACAP expression (Braas et al., 2007). Additionally, the existence of PACAPergic fibers synapsing on PACAPergic neurons has been suggested in the enteric nervous system (Nagahama et al., 1998). Further, infusion of PACAP appears pro-nociceptive at several levels of the spino-parabrachio-amygdaloid pathway (Table 4.1). Potentially this system could also involve visceral sensory input, as there is a substantial population of PACAP neurons within the NTS, corresponding to the

location of the primary relay of visceral information to the LPBn. The possibility of PACAP expression at every level of the spino-parabrachio-amygdaloid pathway is significant, as it would identify a signaling molecule used along an entire pathway. Further, it might indicate that PACAP expression could mark a set of neural circuits within the CNS that integrate aversive sensory information with emotional salience. Intriguingly, PACAP is the mostly highly conserved peptide in its family and appears to be present along one of the more phylogenetically ancient spino-parabrachial nociceptive pathway, in comparison to the more evolutionarily recent neospinothalamic pathway (Almeida et al., 2004; Sherwood et al., 2000). Given what is known about its function, PACAP-expressing neural circuits may function in the generation of a primitive, whole-body response to particularly averse and long-lasting challenges, such as prolonged pain.

*CEA PACAP is coexpressed with CGRP*

In addition to PACAP, the CeA also expresses diverse neuropeptides and markers, including somatostatin (SST), CRH, parvalbumin (PV), cholecystokinin (CCK), calbindin, calretinin and VIP, all of which display characteristic unique or overlapping expression patterns (Ehrlich et al., 2009; Kemppainen & Pitkanen, 2000). The CeA PACAP fiber immunoreactivity is confined to the CeLC and CeL, and the PACAP terminals form perisomatic basket-type innervations of amygdala neurons. We found no overlap between PACAP and somatostatin or CRH immunoreactivity in the CeA; the distribution of each peptide appeared to display non-overlapping, but intermingled expression patterns (Figure 2.1). However, co-labeling with CGRP and PACAP resulted

in fairly extensive colocalization in the CeA (Figure 2.2). CGRP expression in the CeLC has been previously shown to originate from the LPBn; thus PACAP and CGRP appear to demonstrate high levels of coexpression within LPBn-CeLC projections (Dobolyi et al., 2005). Similarly, within the BNSTal, there is a substantial overlap of PACAP and CGRP immunoreactivity, suggesting that this too is part of the LPBn projections. This latter finding was confirmed, as LPBn lesions produced a concomitant loss of CGRP immunoreactivity with PACAP in both the BNST and CeLC (Figure 2.2).

CGRP signaling could play a similar or complementary role to PACAP in the generation of stress-related behavioral responses in the limbic system. CGRP signaling can promote unconditioned fear, as CGRP infusions into the amygdala produced an unconditioned freezing response before any aversive stimulus was presented (Kocorowski & Helmstetter, 2001). Further, pretreatment with the CGRP receptor antagonist, CGRP(8-37) in the amygdala disrupted cued but not contextual fear conditioning. In the BNST, infusion of CGRP induced anxiety-like responses on the elevated plus maze and produced a dose-dependent enhancement of startle (Kelly et al., 2011). This effect appeared to be dependent on CRH signaling, since either pretreatment with CRHR1 antagonist or virally-mediated siRNA knockdown of CRH expression, blocked the ability of BNST CGRP to enhance startle (Sink et al., 2013). CGRP is well known as a peripheral modulator of nociceptive transmission and this role may hold true within the brain. Application of CGRP to amygdala sections increases excitatory postsynaptic currents (EPSCs) on PBn-CeLC synapses, increasing amplitude but not the frequency of miniature EPSCs (Han et al., 2010). Further, CeA administration of CGRP

into awake rats was found to increase audible and ultrasonic vocalizations and produce mechanical hypersensitivity. CeA CGRP may also play a role in feeding behavior. Using optogenetic and pharmacogenetic manipulation of CGRP-expressing PBn-CeLC projections, activation of these projections strongly suppressed appetite. Conversely, inhibition of CGRP LPBn-CeLC projections increased food intake in situations when mice normally do not eat, and prevented starvation after agouti-related peptide (AgRP) neuronal ablation, implicating CGRP LPBn-CeLC signaling may be connected to the principal feeding circuits within the hypothalamus (Carter et al., 2013). Interestingly, activation of CGRP LPBn neurons was sufficient to induce conditioned taste aversion in the absence of an anorexigenic substance, and inhibition of these same neurons attenuated conditioned taste aversion to lithium chloride (Carter et al., 2015). From these studies, CGRP LPBn-CeLC projections are thought to encode a type of visceral malaise signal. In sum, the effects of CGRP in the CeA bare some striking similarities to those of PACAP, inducing anxiety-like behaviors, nociceptive hypersensitivity, and hypophagia.

Given the overlap in expression and functional similarities of PACAP and CGRP in the CeLC, these two peptides might play complementary roles. In mammalian brains it has been found that generally when two or more neuropeptides are coexpressed within the same neuronal population, they are also co-stored within the same large dense core vesicles (Merighi, 2002). Co-storage would have functional implications; first, it would necessitate co-release of both neuropeptides, allowing for interactions between the different peptides, and second, it would suggest that regulation of these systems would be most readily accomplished through altering rates of synthesis. Neuropeptides can often

work in a synergistic manner. One of the best-known examples of this is the potentiation of CRH by vasopressin in the pituitary gland, where the effect of vasopressin greatly increases the amount of adreno-corticotropin releasing hormone (ACTH) that is released by CRH binding (Merighi, 2002). Studies of the ophthalmic artery, suggest the possibility of a synergistic interaction between PACAP and CGRP. In the porcine ophthalmic artery, both PACAP and CGRP each induced a concentration dependent vasorelaxation, but when both peptides where administered together the amount of relaxation substantially increased, beyond what would be predicted individually (Elsas & White, 1997).

*Pain-related plasticity of PACAPergic neural circuits*

To examine if there is increased PACAP signaling during persistent pain, we performed a set of experiments using a CCI model of neuropathic pain. At 14 days following CCI surgery, PACAP transcript was increased in the LPBn (Figure s3.1). This effect appeared to be specific to the LPBn, as no other CNS regions examined had significant alterations in expression. Increased PACAP transcript levels at 14 days corresponded to an increase in PACAP immunoreactivity in the CeA, indicating increased PBn PACAP biosynthesis and increased axonal transport of PACAP peptide to CeA terminals (Figure 3.1). Complementary to these studies, tissue from transgenic PACAP-EGFP mice was analyzed following CCI. In agreement, 14 days following CCI there was a bilateral increase number of PACAP-EGFP+ cells in the LPBn, compared to sham surgery (Figure 3.1). These results provide strong evidence that CCI increases

PACAP signaling in LPBn-CeLC circuits and support PACAP involvement in chronic pain-related plasticity.

In addition to the LPBn-CeLC, CCI induced PACAP expression along multiple neural sites within the spino-parabrachial amygdaloid pathway. Following CCI, PACAP-EGFP mice displayed marked PACAP-EGFP expression within the proximal sciatic nerve and in L3-L5 DRG ipsilateral to the injury (Figure 3.2). This is consistent with a number of previous studies reporting enhanced PACAP expression in peripheral nerve and DRG following injuries including axotomy, injection of complete Freund's adjuvant, L5 nerve transection, or capsaicin treatment (Jongsma et al., 2000; Mabuchi et al., 2004; Mulder et al., 1999; Nemeth et al., 2006; Pettersson et al., 2004). In the spinal cord, we identified prominent PACAP-EGFP fibers in the dorsal columns/medial lemnisicus tract that give off collaterals into lamina III-V (Figure 3.2). This pathway likely corresponds to Aβ fibers conveying non-noxious sensory information to the gracile nucleus. The presence of PACAP within this pathway may be a consequence of its role as a prosurvival/injury response factor. Interestingly, it has been proposed that during neuropathic pain, the sprouting of these collateral Aβ fibers from lamina III-V into lamina I-II may explain the presence of allodynia (Mannion et al., 1996; Woolf et al., 1992). In this model, as a consequence of pain-related sprouting, lamina I neurons would now receive non-noxious sensory input, and result in innocuous sensory stimuli leading to the perception of pain to normally non-noxious stimuli. However, this interpretation has been questioned because of the development of new more precise techniques, raising questions about peripheral sprouting of Aβ lamina III-V fibers as a mechanism of

sensitization (Hughes et al., 2003; Zhang et al., 2015). In our studies, the lack of PACAP-EGFP fibers in lamina I in either normal conditions or following CCI is surprising and might suggest a specific role in non-noxious sensory transmission in the periphery. However, under normal conditions PACAP immunoreactivity has been repeatedly found primarily within lamina I and not in lamina III-V (Hannibal, 2002; Vizzard, 2000). The lack of PACAP within lamina I could simply be a result of native EGFP detection limits or differences in cellular mechanisms between different sized neuronal fibers and causing EGFP to be found only in larger neuronal fibers. The increased expression of PACAP-EGFP along multiple levels of the spino-parabrachio-amygdaloid pathway suggests PACAP signaling might contribute to pain-related neural transmission and plasticity within distinct nociceptive pathways.

All experiments that examined alterations in PACAP expression were performed at 14 days following CCI surgery to allow comparisons across different experiments. There were several factors in the determination of this time point. The first is that it allowed sufficient time for recovery from surgery, such that the cutaneous incision would be healed, and motor deficits could be largely resolved. This time point also corresponds to a time following CCI when hypersensitivity behaviors are fully developed. Finally, given the nature of stress stimuli required in our previous studies, several days of persistent pain may be required for the regulation of PACAP in this system. Upregulation of PACAP and PAC1R transcript in the BNST was found following 7 days of stress, but no change following one acute stressor exposure (Hammack et al., 2009; Lezak et al., 2014). Further, although PAC1R deficient mice have normal stress response

to acute restraint stress, longer periods of stress (14-21 days) resulted in a significant attenuation of HPA axis activation and stress-induced hypophagia (Mustafa et al., 2015). Thus, the induction of PACAP expression and signaling might occur at later time points and require a prolonged stimulus. Future characterization at different time points is necessary to determine whether longer durations of pain are required to induce PACAP expression in the PBn-CeLC. Interestingly, the development of anxiodepressive behaviors in rats with CCI follows a very gradual timeline, appearing over the course of a number of days, in comparison to hypersensitivity which has more immediate development in the hours following surgery (Alba-Delgado et al., 2013). The biological mechanisms that could be governing these changes occurring in the timeframe of several days and weeks following initial onset are not well understood. Given the delayed onset of many pharmacological antidepressant treatments (Lam, 2012), the factors that mediate neural circuit plasticity over longer time courses might be those most valuable for treating psychiatric disease.

### 4.2. The role of PACAP in emotional behaviors

*PACAP as a regulator of anxiety-like behaviors*

The amygdala plays a principal role in assigning emotional salience to external stimuli and coordinating the behavioral and physiological responses to these triggers. As PACAP expression corresponds to a direct nociceptive input into the amygdala, this suggests that CeA PACAP could have a role in modifying the attachment of emotional salience to nociceptive stimuli. In agreement with this idea, we found CeA PACAP

infusion produced an aversive emotional response, as reflected by an increase in anxiety-like behaviors on the elevated plus maze (Figure 2.6). These findings are consistent with two prior studies suggesting CeA PACAP signaling can produce negative/defensive emotional behaviors. In the shock-probe fear/defensive burying task, rats are placed in an arena with an electrified probe and allowed to explore freely. After freely encountering the probe and receiving an electric shock, the resulting behavioral responses are recorded and classified into stereotypical categories. CeA PACAP infusion created a shift towards passive coping strategies characterized by increased immobility time and avoidance, in contrast to active behavioral strategies like burying the probe with bedding (Legradi et al., 2007). Another study, found that CeA PACAP signaling may also regulate feeding behavior. Infusion of PACAP in the CeA produced a dose-dependent decrease in food intake and resulted in weight loss through mechanisms that required melanocortin and TrkB signaling (Iemolo et al., 2015). In aggregate, these data suggest that CeA PACAP signaling appears produce an emotional state characterized by increased passive behaviors, decreased exploratory behaviors, and hypophagia.

The role of CeA PACAP signaling should be interpreted in the context of the neural circuitry of the larger extended amygdala complex that includes the BLA, CeA, BNST, and other less studied regions, such as the substantia innominate. While the terms fear and anxiety are often used interchangeably, on the basis of neural circuitry there may be rationale for the separation of these into two distinct entities (Davis et al., 2010; Walker et al., 2009). Within the extended amygdala, the CeA is thought to a have a greater role in fear responses, which are short phasic responses, and likely best

recapitulated in cued fear conditioning and fear-potentiated startle paradigms. On the other hand, the BNST is thought to mediate responses primarily to longer-duration, diffuse, or unpredictable threats, and which are more akin to anxiety. Paradigms such as light or CRH-enhanced startle, and learned helplessness were found to be dependent on BNST activity (Hammack et al., 2004; Davis et al., 2010; Walker et al., 2009). With this interpretation it might suggest that the emotional responses found following CeA PACAP might be related to ongoing fear behaviors or a decreased threshold in the generation of the fear response. Detailed analysis of the behaviors of CeA PACAP on elevated plus maze revealed that total locomotor activity was reduced; however this was not the result of increased spontaneous freezing responses (a fear response), but rather a selective decrease in the choice to enter the open arms. The traditional extended amygdala model is complicated by the fact that previous studies have relied largely on lesion techniques, where often the entire CeA was lesioned. In phasic fear responses, the CeM appears critical as an output to brainstem targets to drive fear responses. The role CeLC and CeL is less straightforward, although both areas have prominent projections to the CeM which are thought to be important in the release of inhibitory signals on the CeM, to allow the expression of fear behaviors through CeM to brainstem projections (Ciocchi et al., 2010; Haubensak et al., 2010). Additionally prominent projections of the CeL and CeLC to the BNST have been postulated to be important in the transition from phasic to sustained fear (Walker et al., 2009). A population of CRH neurons within the CeL is the source of the majority of CRH fiber immunoreactivity within the BNSTal, and CeA-BNST CRH signaling within is thought to be a mediator of conditioned anxiety-like behaviors. As

such, CeA PACAP could produce anxiety-like responses through enhanced neuronal activation or CRH release in the BNST (Beckerman et al., 2013). Previous studies within the PVH demonstrate that PACAP can drive CRH expression (Agarwal et al., 2005). In the future, determining which neurons are being activated in the CeLC and CeL by PACAP, and defining their projections will be crucial to understanding the role of PACAP within these neural circuits and the extended amygdala.

One intriguing possibility is that CeA PACAP signaling could lead to plasticity within fear circuitry that conveys the unconditioned stimulus (US). Compared to the BLA, the contribution of the PBn in fear conditioning has been relatively unexplored; however it has been recently found that following fear conditioning, there is a synaptic potentiation of both BLA-CeLC and LPBn-CeLC pathways (Watabe et al., 2013). This appears to be accomplished by both pre- and postsynaptic mechanisms. In addition, there was a correlation between BLA-CeLC and LPBn-CeLC synaptic potentiation suggesting a heterosynaptic interaction between these two pathways. In a follow up study, inactivation of the LPBn during acquisition of fear conditioning decreased freezing to the conditioned stimulus (CS) during testing. Further, optogenetic activation of LPBn-CeLC projections could be paired with a tone and resulted in increased freezing to the presentation upon presentation of the tone, suggesting that LPBn-CeLC activation could effectively act as a US (Sato et al., 2015).

Given the presence of PACAP within LPBn-BNST projections, BNST PACAP signaling might also have a role in the emotional components of pain, similar to CeA PACAP. Our previous work has extensively characterized BNST PACAP as it relates to

the behavioral and physiological consequences to chronic stress (Hammack et al. 2009; Roman et al., 2014; Kocho-Schellengberg et al., 2014, Lezak et al., 2014). Situations of chronic stress can potentiate pain experience, such as in stress-induced hyperalgesia (McEwen & Kalia, 2010). Thus, during chronic stress the BNST might enhance pain experience through direct or indirect influences on descending pathways to modulate pain. Further, the existence of the ascending LPBn-BNST projections and that some of these projections as collaterals of nociceptive LPBn-CeLC projections, suggests a possible role for the BNST in ascending nociceptive modulation. Unlike the LPBn-CeA projections, there have been sparse investigations into the contribution of LPBn-BNST projections as they relate to nociception. An electrophysiological study of anesthetized rats found that over a quarter of BNST neurons were excited by noxious stimulation, and that this afferent pathway was not a result of indirect input from the amygdala (Casada & Dafny, 1992).

*PACAP signaling in pain-related behaviors*

Since neuropathic pain increased PACAP expression in the CeLC, we wanted to determine if this heightened PACAP signaling in the CeLC contributed to pain-related behaviors. To examine this, the PACAP receptor antagonist PACAP6-38, a PAC1 and VPAC2 receptor antagonist, was injected into the CeA following CCI to examine its abilities to attenuate pain-induced hypersensitivity or emotional behaviors. The CCI model has been shown to heighten anxio-depressive behaviors including anxiety-like behaviors on the elevated plus maze and increased depressive behaviors in open field and

forced swim tests (Roeska et al., 2008; Zeng et al., 2008). Fourteen days following surgery, CeA infusion of PACAP6-38 was able to block heightened anxiety-like behaviors in the open field in the CCI condition (Figure 3.3). There was no effect of PACAP6-38 alone on rats with sham surgery, suggesting that ongoing CeA PACAP signaling has a role in modulating behavior in the setting of pain, but does not modulate behaviors under normal conditions. Similarly, CeA infusion of PACAP6-38 resulted in an attenuation of thermal hypersensitivity in the CCI afflicted hindpaw, but did not alter response latency for either the contralateral hindpaw, or for either hindpaws in the sham condition (Figure 3.3). A lack of thermal sensitivity in either condition implies that CeA PACAP signaling may modulate sensitivity in situations of persistent pain, but may not alter thresholds under normal conditions. The selective involvement of PACAP in states of persistent pain mimics the involvement of several neurotransmitter systems in the CeA that selectively contribute to increased CeA activity during pain. Arthritic pain increased expression of metabotropic glutamate receptor 1 (mGluR1) in the CeA; a selective mGluR1 antagonist reduced synaptic transmission in the CeA from arthritic animals but had no effect in the CeA of control animals (Neugebauer et al., 2003). Blockade of the CGRP1 receptor in the CeA attenuated enhanced synaptic transmission from arthritic animals, reducing EPSC amplitude and spike frequency, as well as attenuating heightened spinal reflexes and ultrasonic vocalizations, but had no effect in control animals (Han, et al., 2005). Thus, enhanced PACAP signaling might be one of a collection of molecular and synaptic changes in the CeA during pain that influences pain-related behaviors.

*A mechanism of CeA PACAP signaling via ERK*

Among intracellular signaling cascades, enhanced ERK signaling within the CeLC appears to have prominent roles in pain-related plasticity. At 4 hours following formalin injection into the hindpaw or 2 hours following acid-induced muscle pain, an induction of pERK+ cells was found in the CeLC (Carrasquillo & Gereau, 2007; Cheng et al., 2011). Consistent with these observations we found an increase in pERK+ cells in the CeLC 14 days following CCI surgery (Figure 3.4). The induction of CeLC ERK signaling in several different pain models signifies that ERK signaling is likely a pain signature, and not a response to any one model. Additionally, the presence of increased ERK signaling at 14 days following CCI would suggest that ongoing ERK signaling in the CeA may be a component of persistent pain, rather than just part of the initial plasticity.

Further, we found evidence that PACAP signaling may mediate CeLC ERK activation. In cell culture, PACAP signaling in primary neuronal or HEK EGFP-PAC1R cells results in potent and sustained ERK activation (May et al., 2010; May et al., 2014). In examining the CeA from CCI rats, a majority of pERK+ cells were immediately apposed to PACAP-immunoreactive fibers. CeA PACAP administration resulted in a robust induction of pERK+ cells, demonstrating that CeA PACAP signaling activates ERK in the amygdala neurons (Figure 3.5). Further, ERK activation was found to be necessary for CeA PACAP to alter nociception, as pretreatment with a MEK inhibitor abolished PACAP-induced thermal hypersensitivity. One remaining question is whether

PACAP signaling is the sole mediator of pain-related activation of ERK. This appears unlikely as several other candidates, including signaling through NMDA, mGluR, and reactive oxygen species (ROS) have been found to contribute to ERK activation in the CeLC (Cheng et al., 2011; Li, Ji, & Neugebauer, 2011). In this view, ERK signaling appears to be a site of convergence for multiple signaling systems in the CeLC to allow for diverse modulation and the dynamic regulation of amygdalar circuits.

One prominent mechanism through which PACAP may activate ERK is through the internalization of PACAP receptors. Internalization of G-protein coupled receptors (GPCRs) was once thought to be primarily a means of receptor desensitization; however, more recent studies have suggested GPCR internalization may play a role in receptor resensitization and even act as an alternative form of intracellular signaling (Ferguson, 2001; Sorkin & von Zastrow, 2009). The most common form of GPCR internalization of signaling endosomes is dependent on the binding of β-arrestin scaffolds and the formation of clathrin-coated pits, to result in a signaling endosome (Ferguson, 2001; Sorkin & von Zastrow, 2009). Using an EGFP-PAC1 receptor cell line, PACAP/PAC1 receptor binding and signaling was shown to induce PAC1 receptor internalization, which was inhibited by blocking either clathrin (Pitstop) or dynamin I/II (Dynasore) (Merriam et al., 2013). Blocking internalization of the PAC1 receptor was found to strongly reduce PACAP-mediated ERK activation. However, the induction of ERK signaling was not completely blocked by inhibiting internalization, as a further reduction of ERK signaling was affected by blocking phospholipase C/diacylglycerol/protein kinase C signaling. These results demonstrate that PAC1 receptor-mediated ERK activation is accomplished

via multiple mechanisms through both internalization/cytosolic signaling and plasma membrane signaling (May et al., 2014). The current work extends these findings *in vivo* and suggests that receptor internalization may occur with CeLC PACAP nociceptive signaling. Blocking endocytosis by using the clathrin inhibitor, Pitstop, attenuated the ability of PACAP to activate ERK in the CeA in parallel with a reduction in CeA PACAP- induced thermal hypersensitivity (Figure 3.5). This is the first piece of evidence to suggest that PACAP receptors may internalize *in vivo* and demonstrates a possible functional role of this process. However, an important limitation in this line of studies is that Pitstop is not selective to the PAC1 receptor and results in the global inhibition of clarthrin-mediated endocytosis. However, as there were no changes between vehicle and Pitstop-treated control animals that did not receive PACAP, it can be reasonably concluded that clathrin-mediated endocytosis is required for CeA PACAP signaling to fully induce pERK and behavioral hypersensitivity. To determine conclusively if this was a direct effect of the PAC1 receptor would likely require the genetic modification to generate a PAC1 receptor incapable of internalization. One possible role for PAC1 receptor internalization is that it may provide a mechanism to allow sustained ERK activation. This would be consistent with the findings that in comparison to some other neuropeptides, PACAP mediated effects have a more gradual onset but are much longer in duration (Shimizu et al 2004) . As such, PACAP released in the CeA during chronic pain might result in a prolonged excitability and heightened plasticity of CeA neurons and lead to the strengthening of amygdalar nociceptive and emotional-salience circuits.

One fundamental remaining question is whether CeA PACAP signaling is specific to nociception. Recent experiments utilizing genetic circuit manipulations have resulted in the hypothesis that specific subsets of amygdala neurons and their connections may not encode specific modalities but rather encode a positive or negative emotional valence (Namburi et al., 2015; Redondo et al., 2014). In this view, CeA PACAP signaling would likely be a circuit carrying negative emotional valence, and nociceptive stimuli would be one of numerous stimuli that result in PACAP release. Given that the vast majority of CeA PACAP originates from the LPBn, the decisive factor would be determining the modality of stimuli that activates the LPBn. Prior studies which suggest the existence of amygdalar circuits for positive and negative valence have been focused on the BLA, a region which receives highly polymodal and processed nociceptive information (Namburi et al., 2015; Neugebauer et al., 2004; Redondo et al., 2014). The LPBn, in contrast, receives direct nociceptive information from lamina I of the spinal cord and visceral inputs from the NTS. The contrast between the input of BLA compared to the LPBn is illustrated by its role in fear conditioning. The BLA is thought to receive afferents related to both the US (electric shock) in combination with a host of other environmental sensory information (light, tone, etc.). The PBn-CeLC projection is only thought to convey the only the US (electric shock), likely due to aversive/nociceptive nature (Paré et al., 2004; Sato et al., 2015). However, a recent study found that the CGRP expressing LPBn-CeLC projections (which overlap with PACAP projections) can convey a visceral malaise signal to strongly inhibit feeding behavior, such as those induced by lithium chloride (Carter et al., 2015). Even though these results were argued not to reflect nociceptive

activation, this was not tested directly (Carter et al., 2013). Regardless, CGRP LPBn-CeLC activation can be regarded as a highly aversive interoceptive stimulus. Further, it remains to be determined if inflammatory factors could also result in the activation of LPBn-CeA circuits via the NTS. Although much remains to be determined, CeA PACAP signaling may be encoding a negative valence that is associated with a subset of highly aversive sensory stimuli.

### 4.3. Summary

PACAP has been well established as neuropeptide that regulates homeostatic function. Several recent lines of research have demonstrated that PACAP signaling potently activates both physiological and behavioral responses to stressors and that these responses are likely due to PACAP signaling in limbic regions. Additionally, it had been previously established that PACAP might have important roles in nociceptive transmission and sensitization in peripheral systems. In this work, the role of PACAP in nociceptive processes was found to extend centrally into limbic regions via the spino-parabrachio-amgydaloid pathway. Through this pathway, PACAP functions to potentiate many of the emotional components of pain.

This work has several key limitations. The PBn was found to be a substantial source of CeA PACAP, thus the effects of infusions of PACAP or PACAP(6-38) on both pain and anxiety-related behaviors were attributed to nociceptive PBn-CeA projections. However, it cannot be ruled out that PACAP signaling from sources other than the PBn or locally within the amygdala could be contributing to these effects. Additionally, all

infusions were performed bilaterally**;** however our results and others have suggested that there may be a lateralization of the CeA in pain processing. Thus, comparisons of injections into the left and right CeA could be performed to explore this area. A second limitation comes from the use of hypersensitivity assessments to measure pain-related behaviors. While hypersensitivity assessments may provide a well-used indicator of evoked pain-responses, often a larger problem in chronic pain sufferers is the presence of spontaneous pain. However, spontaneous pain often been particularily difficult to model in rodents; hence careful design of experiments assessing spotaneous pain behaviors may be needed to address these questions. Finally, while the Pitstop experiments suggest that receptor internalization may be required for CeA PACAP-induced thermal hypersentivity, the studies did not specifically address whether PAC1 receptor internalization was the primary driver of the nociceptive effects or whether the response was a consequence of other interacting receptor systems. Future experiments that directly interfere with PAC1 receptor internalization would address this possibility.

The results of this work raise a number of important new questions. Heightened levels of PACAP expression were observed 14 days following CCI in the LPBn-CeLC pathway. The time course of induction and the exact nature of the challenge, whether it is specific to pain or aversive stimuli, all remain to be determined. We found that the use of PACAP-EGFP mice could offer a powerful tool for investigating these questions. The recent creation of PACAP-Cre mice allows for a whole new set of investigations examining circuit specific functions using optogenetic and pharmacogenetic manipulations. The behavioral and physiological role of PACAPergic projections from

the LPBn to the CeLC, BNST, and VMH can now be individually stimulated and inhibited to allow characterization. PACAPegeric PBn-CeA projections can be directly examined by injecting a virus containing a floxed channelrhodopsin directly into the PBn of PACAP-Cre mice. By optogentically stimulating the terminals of CeA fibers, the effects of activating only PACAP containing PBn-CeA projections can be examined for its effects on pain and anxiety-related behaviors. In a similar manner, by inhibiting these same fibers in a model of chronic pain, the contributions of the PBn-CeA projections can be determined in pain-related behavioral changes. The use of PACAP-specific viral tracting will allow for the deciphering of PACAP pain and stress circuits with better prescision and resolution in future functional studies.

## 4.4. Figures

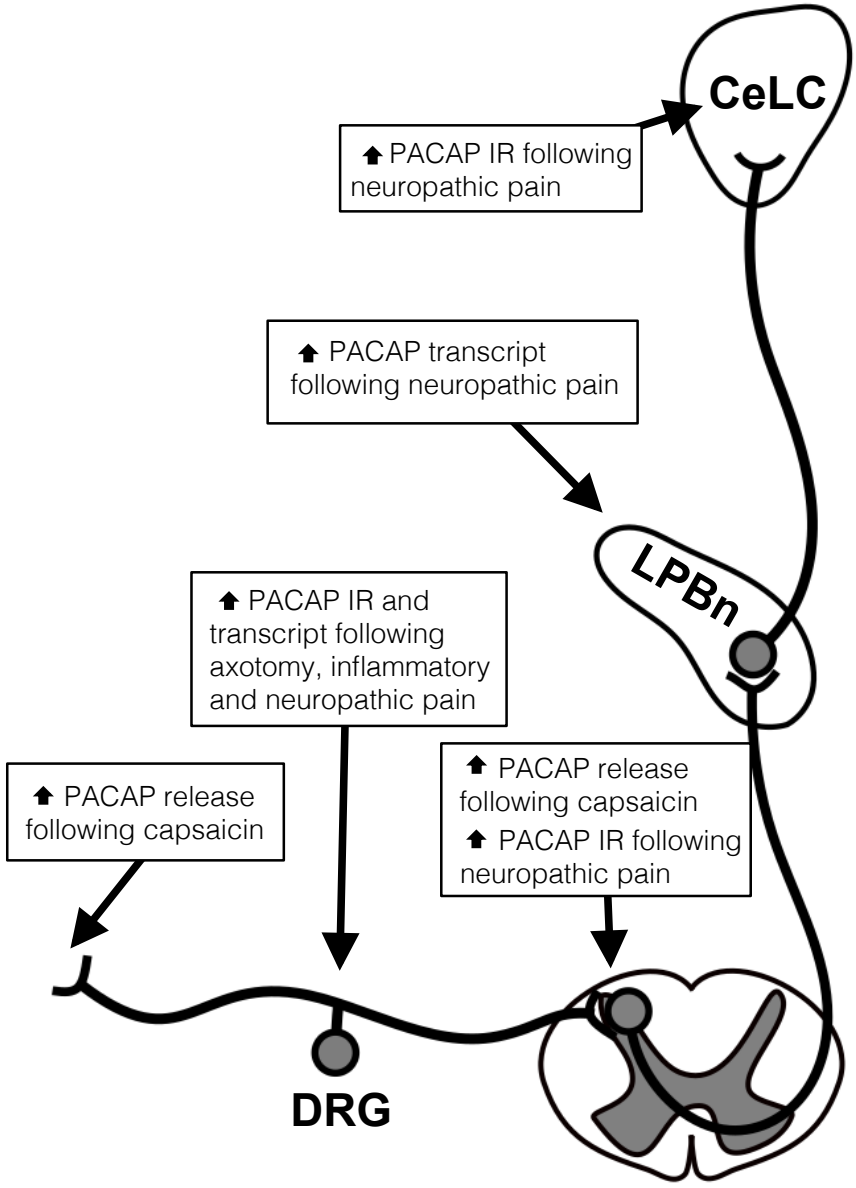



**Figure 4.1. Schematic of known pain-related plasticity of PACAP expression within the spino-parabrachioamygdaloid pathway**

| Region | Compound | Behavioral / physiological result |
|---|---|---|
| Amygdala | PACAP | ⬆ Thermal sensitivity (Missig et al., 2014)<br>⬆ Anxiety-like behavior (Missig et al., 2014)<br>⬇ Food intake (Iemolo et al., 2015)<br>⬆ Passive-behavior responding (shock-probe) (Legradi et al., 2007) |
| | PACAP(6-38) | ⬇ Pain-induced thermal hypersensitivity (unpublished observations)<br>⬇ Pain-induced anxiety-like behavior (unpublished observations |
| Spinal Cord | PACAP | ⬆ Thermal sensitivity (Ohsawa et al. 2002)<br>⬆ Aversive licking/scratching behavior (Ohsawa et al. 2002)<br>⬆ NMDA-induced nocifensive responses (Ohsawa et al., 2002)<br>⬆ Tail flick sensitivity *(late phase)* (Narita et al., 1996)<br>⬆ Multireceptive cell excitability (Dickinson et al. 1997)<br>⬆ NMDA currents (Ohsawa et al., 2002) |
| | PACAP(6-38) | ⬇ Nocifensive responses to formalin (Ohsawa et al. 2002) |
| Sensory Afferent Fibers | PACAP | ⬇ Nocifensive responses to formalin (Sandor et al., 2009)<br>⬇ Heat-injury induced thermal sensitivity (Sandor et al., 2009)<br>⬇ Acetic acid-induced writhing behaviors (Sandor et al., 2009)<br>*n.c.* Thermal / mechanical sensitivity *(baseline)* (Sandor et al. 2009)<br>⬆ Activity of knee joint (Sandor et al. 2009)<br>*n.c.: no change* |

**Table 4.1. Summary of site-specific actions of PACAP or PACAP(6-38)**

## Comprehensive Bibliography


Adwanikar, H., Ji, G., Li, W., Doods, H., Willis, W. D., & Neugebauer, V. (2007). Spinal CGRP1 receptors contribute to supraspinally organized pain behavior and pain-related sensitization of amygdala neurons. *Pain, 132*(1-2), 53-66.

Agarwal, A., Halvorson, L. M., & Legradi, G. (2005). Pituitary adenylate cyclase-activating polypeptide (PACAP) mimics neuroendocrine and behavioral manifestations of stress: Evidence for PKA-mediated expression of the corticotropin-releasing hormone (CRH) gene. *Brain Res Mol Brain Res, 138*(1), 45-57.

Al-Khater, K. M., & Todd, A. J. (2009). Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. *J Comp Neurol, 515*(6), 629-646.

Alba-Delgado, C., Llorca-Torralba, M., Horrillo, I., Ortega, J. E., Mico, J. A., Sanchez-Blazquez, P., . . . Berrocoso, E. (2013). Chronic pain leads to concomitant noradrenergic impairment and mood disorders. *Biol Psychiatry, 73*(1), 54-62.

Almli, L.M., Mercer, K.B., Kerley, K., Feng, H., Bradley, B., Conneely, K.N., Ressler, K.J. (2013). ADCYAP1R1 genotype associates with post-traumatic stress symptoms in highly traumatized African-American females. *Am. J. Med. Genet. B Neuro- psychiatr. Genet. 162B*, 262-272.

Alheid, G. F. (2003). Extended amygdala and basal forebrain. *Ann N Y Acad Sci, 985*, 185-205.

Almeida, T. F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Research*(1000), 40-56.

Apkarian, A. V., Bushnell, M. C., Treede, R. D., & Zubieta, J. K. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *Eur J Pain, 9*(4), 463-484.

Asmundson, G. J. G., & Katz, J. (2009). Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. *Depress Anxiety., 26*(10), 888-901.

Barrie, A. P., Clohessy, A. M., Buensuceso, C. S., Rogers, M. V., & Allen, J. M. (1997). Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a Ras-independent, mitogen-activated protein Kinase/ERK kinase 1 or 2-dependent manner in PC12 cells. *J Biol Chem, 272*(32), 19666-19671.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell, 139*(2), 267-284.

Beaudet, M. M., Braas, K. M., & May, V. (1998). Pituitary adenylate cyclase activating polypeptide (PACAP) expression in sympathetic preganglionic projection neurons to the superior cervical ganglion. *J Neurobiol, 36*(3), 325-336.

Beckerman, M. A., Van Kempen, T. A., Justice, N. J., Milner, T. A., & Glass, M. J. (2013). Corticotropin-releasing factor in the mouse central nucleus of the amygdala: ultrastructural distribution in NMDA-NR1 receptor subunit expressing neurons as well as projection neurons to the bed nucleus of the stria terminalis. *Exp Neurol, 239*, 120-132.

Bennett, G. J., & Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain, 33*(1), 87-107.

Bernard, J. F., Bester, H., & Besson, J. M. (1996). Involvement of the spino-parabrachio - amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. *Prog Brain Res, 107*, 243-255.

Bernard, J. F., Huang, G. F., & Besson, J. M. (1992). Nucleus centralis of the amygdala and the globus pallidus ventralis: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol, 68*(2), 551-569.

Bird, G. C., Lash, L. L., Han, J. S., & Zou, X. (2005). Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. *J Physiol, 564*(3), 907-921.

Blechman, J., & Levkowitz, G. (2013). Alternative Splicing of the Pituitary Adenylate Cyclase-Activating Polypeptide Receptor PAC1: Mechanisms of Fine Tuning of Brain Activity. *Front Endocrinol (Lausanne), 4*, 55.

Bon, K., Lantéri-Minet, M., Michiels, J. F., & Menétrey, D. (1998). Cyclophosphamide cystitis as a model of visceral pain in rats: a c-fos and Krox-24 study at telencephalic levels, with a note on pituitary adenylate cyclase activating polypeptide (PACAP). *Experimental Brain Research, 122*(2), 165-174.

Bonaz, B., Baciu, M., Papillon, E., Bost, R., Gueddah, N., Le Bas, J. F., . . . Segebarth, C. (2002). Central processing of rectal pain in patients with irritable bowel syndrome: an fMRI study. *Am J Gastroenterol, 97*(3), 654-661.

Bornhovd, K. (2002). Painful stimuli evoke different stimulus-response functions in the amygdala, prefrontal, insula and somatosensory cortex: a single-trial fMRI study. *Brain, 125*(6), 1326-1336.

Botz, B., Imreh, A., Sándor, K., Elekes, K., Szolcsányi, J., Reglodi, D., . . . Helyes, Z. (2013). Role of Pituitary Adenylate-Cyclase Activating Polypeptide and Tac1 gene derived tachykinins in sensory, motor and vascular functions under normal and neuropathic conditions. *Peptides, 43*, 105-112.

Bourgeais, L., Gauriau, C., & Bernard, J. F. (2001). Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. *Eur J Neurosci, 14*(2), 229-255.

Bouschet, T., Perez, V., Fernandez, C., Bockaert, J., Eychene, A., & Journot, L. (2003). Stimulation of the ERK pathway by GTP-loaded Rap1 requires the concomitant activation of Ras, protein kinase C, and protein kinase A in neuronal cells. *J Biol Chem, 278*(7), 4778-4785.

Braas, K.M., May, V. (1999). Pituitary adenylate cyclase-activating polypeptides directly stimulate sympathetic neuron NPY release through PAC1 receptor isoform activation of specific intracellular signaling pathways. J. Biol. Chem. 274, 27702-27710.

Braas, K. M., Schutz, K. C., Bond, J. P., Vizzard, M. A., & Girard, B. M. (2007). Microarray analyses of pituitary adenylate cyclase activating polypeptide (PACAP)-regulated gene targets in sympathetic neurons. *Peptides*.

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., & Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur J Pain, 10*(4), 287-333.

Butler, R. K., & Finn, D. P. (2009). Stress-induced analgesia. *Prog Neurobiol., 88*(3), 184-202.

Carrasquillo, Y., & Gereau, R. W. (2007). Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J Neurosci, 27*(7), 1543-1551.

Carrasquillo, Y. & Gereau, R. W. (2008) Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. *Mol Pain.*, *4*, 24.

Carter, M. E., Han, S., & Palmiter, R. D. (2015). Parabrachial calcitonin gene-related peptide neurons mediate conditioned taste aversion. *J Neurosci, 35*(11), 4582-4586.

Carter, M. E., Soden, M. E., Zweifel, L. S., & Palmiter, R. D. (2013). Genetic identification of a neural circuit that suppresses appetite. *Nature, 503*(7474), 111-114.

Casada, J. H., & Dafny, N. (1992). Evidence for two different afferent pathways carrying stress-related information (noxious and amygdala stimulation) to the bed nucleus of the stria terminalis. *Brain Res, 579*(1), 93-98.

Cassell, M.D., Gray, T.S., Kiss, J.Z. (1986). Neuronal architecture in the rat central nucleus of the amygdala: a cytological, hodological, and immunocytochemical study. *J. Comp. Neurol., 246*, 478-499.

Chen, W., Boutaoui, N., Brehm, J.M., Han, Y.Y., Schmitz, C., Cressley, A., Acosta-Perez, E., Alvarez, M., Colon-Semidey, A., Baccarelli, A.A., Weeks, D.E., Kolls, J.K., Canino, G., Celedo_n, J.C. (2013). ADCYAP1R1 and asthma in Puerto Rican children. *Am. J. Respir. Crit. Care Med., 187*, 584-588.

Cheng, S.-J., Chen, C.-C., Yang, H.-W., Chang, Y.-T., Bai, S.-W., Chen, C.-C., . . . Min, M.-Y. (2011). Role of extracellular signal-regulated kinase in synaptic transmission and plasticity of a nociceptive input on capsular central amygdaloid neurons in normal and acid-induced muscle pain mice. *J Neurosci, 31*(6), 2258-2270.

Cho, J.H., Zushida, K., Shumyatsky, G.P., Carlezon, W.A.J., Meloni, E.G., Bolshakov, V.Y. (2012). Pituitary adenylate cyclase-activating polypeptide in- duces postsynaptically expressed potentiation in the intra-amygdala circuit. *J. Neurosci. 32,* 14165-14177.

Chowdhury, D., Marco, S., Brooks, I. M., Zandueta, A., Rao, Y., Haucke, V., . . . Perez-Otano, I. (2013). Tyrosine phosphorylation regulates the endocytosis and surface expression of GluN3A-containing NMDA receptors. *J Neurosci, 33*(9), 4151-4164.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B. E., Letzkus, J. J., Vlachos, I., . . . Lüthi, A. (2010). Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature, 468*(7321), 277-282.

Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., . . . Patapoutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science, 330*(6000), 55-60.

Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., . . . Woods, C. G. (2006). An SCN9A channelopathy causes congenital inability to experience pain. *Nature, 444*(7121), 894-898.

Cui, X.Y., Lundeberg, T., Yu, L.C. (2004). Role of corticotropin-releasing factor and its receptor in nociceptive modulation in the central nucleus of amygdala in rats. Brain Res. 995, 23-28.

Das, M., Vihlen, C.S., Legradi, G. (2007). Hypothalamic and brainstem sources of pituitary adenylate cyclase-activating polypeptide nerve fibers innervating the hypothalamic paraventricular nucleus in the rat. *J. Comp. Neurol., 500*, 761-776.

Davis-Taber, R., Baker, S., Lehto, S. G., Zhong, C., Surowy, C. S., Faltynek, C. R., . . . Honore, P. (2008). Central Pituitary Adenylate Cyclase 1 Receptors Modulate Nociceptive Behaviors in Both Inflammatory and Neuropathic Pain States. *J Pain., 9*(5), 449-456.

Davis, M., Walker, D. L., Miles, L., & Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology, 35*(1), 105-135.

Defrin, R., Ginzburg, K., Solomon, Z., Polad, E., Bloch, M., Govezensky, M., & Schreiber, S. (2008). Quantitative testing of pain perception in subjects with PTSD – Implications for the mechanism of the coexistence between PTSD and chronic pain. *Pain, 138*(2), 450-459.

Delgado, M., Pozo, D., & Ganea, D. (2004). The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev, 56*(2), 249-290.

Demyttenaere, K., Bruffaerts, R., Lee, S., Posada-Villa, J., Kovess, V., Angermeyer, M. C., . . . Von Korff, M. (2007). Mental disorders among persons with chronic back or neck pain: Results from the world mental health surveys. *Pain, 129*(3), 332-342.

Dickinson, T., & Fleetwood-Walker, S. M. (1999). VIP and PACAP: very important in pain? *Trends Pharmacol Sci.*, *20*(8), 324-329.

Dickinson, T., Fleetwood-Walker, S. M., Mitchell, R., & Lutz, E. M. (1997). Evidence for roles of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors in modulating the responses of rat dorsal horn neurons to sensory inputs. *Neuropeptides, 31*(2), 175-185.

Dickinson, T., Mitchell, R., Robberecht, P., Fleetwood-Walker, S.M. (1999). The role of VIP/PACAP receptor subtypes in spinal somatosensory processing in rats with an experimental peripheral mononeuropathy. *Neuropharmacology, 38*, 167-180.

Dobner, P.R., 2006. Neurotensin and pain modulation. Peptides 27, 2405-2414.

Dobolyi, A., Irwin, S., Makara, G., Usdin, T. B., & Palkovits, M. (2005). Calcitonin gene-related peptide-containing pathways in the rat forebrain. *J Comp Neurol, 489*(1), 92-119.

Dong, H. W., Petrovich, G. D., & Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Brain Res Rev, 38*(1-2), 192-246.

Duvarci, S., & Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron, 82*(5), 966-980.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., & Lüthi, A. (2009). Amygdala inhibitory circuits and the control of fear memory. *Neuron, 62*(6), 757-771.

Engelund, A., Fahrenkrug, J., Harrison, A., & Hannibal, J. (2010). Vesicular glutamate transporter 2 (VGLUT2) is co-stored with PACAP in projections from the rat melanopsin-containing retinal ganglion cells. *Cell Tissue Res, 340*(2), 243-255.

Elsas, T., & White, L. R. (1997). Evidence for a possible synergism between pituitary adenylate cyclase activating polypeptide and calcitonin gene-related peptide in porcine ophthalmic artery. *Acta Ophthalmol Scand, 75*(2), 159-161.

Ferguson, S. S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev, 53*(1), 1-24.

Flor, H., Fydrich, T., & Turk, D. C. (1992). Efficacy of multidisciplinary pain treatment centers: a meta-analytic review. *Pain, 49*(2), 221-230.

Fu, Y. & Neugebauer, V. Differential mechanisms of CRF1 and CRF2 receptor functions in the amygdala in pain-related synaptic facilitation and behavior (2008). *J Neurosci. 28*, 3861-3876.

Fulwiler, C. E., & Saper, C. B. (1984). Subnuclear organization of the efferent connections of the parabrachial nucleus in the rat. *Brain Res, 319*(3), 229-259.

Gaskin, D. J., & Richard, P. (2012). The economic costs of pain in the United States. *J Pain., 13*(8), 715-724.

Gauriau, C., Bernard, J.F., (2002). Pain pathways and parabrachial circuits in the rat. *Exp. Physiol*. *87*, 251-258.

Gauriau, C., & Bernard, J.-F. (2004). Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. *J Neurosci, 24*(3), 752-761.

Geuze, E., Westenberg, H. G. M., Jochims, A., de Kloet, C. S., Bohus, M., Vermetten, E., & Schmahl, C. (2007). Altered Pain Processing in Veterans With Posttraumatic Stress Disorder. *Arch Genl Psychiatry., 64*(1), 76-85.

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

Goncalves, L., Silva, R., Pinto-Ribeiro, F., Pego, J. M., Bessa, J. M., Pertovaara, A., . . . Almeida, A. (2008). Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. *Exp Neurol, 213*(1), 48-56.

Guo, Z., Zhao, C., Huang, M., Huang, T., Fan, M., Xie, Z., . . . Cheng, L. (2012). Tlx1/3 and Ptf1a Control the Expression of Distinct Sets of Transmitter and Peptide Receptor Genes in the Developing Dorsal Spinal Cord. *J Neurosci, 32*(25), 8509-8520.

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. *Psychoneuroendocrinology, 34*(6), 833-843.

Hammack, S. E. & May, V. (2015) Pituitary adenylate cyclase activating polypeptide in stress-related disorders: data convergence from animal and human studies. *Biol Psychiatry, 78*(3): 167-177.

Hammack, S. E., Richey, K. J., Watkins, L. R., & Maier S. F. (2004). Chemical lesion of the bed nucleus of the stria terminalis blocks the behavioral consequences of uncontrollable stress. *Behav Neurosci, 118*(2), 443-448.

Han, J. S., Adwanikar, H., Li, Z., Ji, G., & Neugebauer, V. (2010). Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. *Mol Pain, 6*, 10.

Han, J. S., Li, W., & Neugebauer, V. (2005). Critical Role of Calcitonin Gene-Related Peptide 1 Receptors in the Amygdala in Synaptic Plasticity and Pain Behavior. *J. Neurosci., 25*(46), 10717-10728.

Hannibal, J. (2002). Pituitary adenylate cyclase-activating peptide in the rat central nervous system: An immunohistochemical and in situ hybridization study. *J Comp Neurol., 453*(4), 389-417.

Hannibal, J., Mikkelsen, J. D., Fahrenkrug, J., & Larsen, P. J. (1995). Pituitary adenylate cyclase-activating peptide gene expression in corticotropin-releasing factor-containing parvicellular neurons of the rat hypothalamic paraventricular nucleus is induced by colchicine, but not by adrenalectomy, acute osmotic, ether, or restraint stress. *Endocrinology, 136*(9), 4116-4124.

Hannibal, J., Moller, M., Ottersen, O. P., & Fahrenkrug, J. (2000). PACAP and glutamate are co-stored in the retinohypothalamic tract. *J Comp Neurol, 418*(2), 147-155.

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). Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. *Br. J. Pharmacol.,* 166, 4-17.

Hashimoto, H. (2006). New Insights into the Central PACAPergic System from the Phenotypes in PACAP- and PACAP Receptor-Knockout Mice. *Annal N Y Acad Sci., 1070*(1), 75-89.

Hashimoto, H., Shintani, N., Tanaka, K., Mori, W., Hirose, M., Matsuda, T., . . . Baba, A. (2001). Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proc Natl Acad Sci U S A., 98*(23), 13355-13360.

Hattori, S., Takao, K., Tanda, K., Toyama, K., Shintani, N., Baba, A., . . . Miyakawa, T. (2012). Comprehensive behavioral analysis of pituitary adenylate cyclase-activating polypeptide (PACAP) knockout mice. *Front Behav Neurosci, 6*, 58.

Hawke, Z., Ivanov, T.R., Bechtold, D.A., Dhillon, H., Lowell, B.B., Luckman, S.M. (2009). PACAP neurons in the hypothalamic ventromedial nucleus are targets of central leptin signaling. *J. Neurosci., 29,* 14828-14835.

Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., . . . Anderson, D. J. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature, 468*(7321), 270-276.

Huang, G. F., Besson, J. M., & Bernard, J. F. (1993). Morphine depresses the transmission of noxious messages in the spino(trigemino)-ponto-amygdaloid pathway. *Eur J Pharmacol, 230*(3), 279-284.

Hughes, D. I., Scott, D. T., Todd, A. J., & Riddell, J. S. (2003). Lack of evidence for sprouting of Abeta afferents into the superficial laminas of the spinal cord dorsal horn after nerve section. *J Neurosci, 23*(29), 9491-9499.

Iemolo, A., Ferragud, A., Cottone, P., & Sabino, V. (2015). Pituitary Adenylate Cyclase-Activating Peptide in the Central Amygdala Causes Anorexia and Body Weight Loss via the Melanocortin and the TrkB Systems. *Neuropsychopharmacology, 40*(8), 1846-1855.

Ikeda, R., Takahashi, Y., Inoue, K., & Kato, F. (2007). NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. *Pain, 127*(1-2), 161-172.

Jasmin, L., Wang, H., Tarczy-Hornoch, K., Levine, J. D., & Basbaum, A. I. (1994). Differential effects of morphine on noxious stimulus-evoked fos-like immunoreactivity in subpopulations of spinoparabrachial neurons. *J Neurosci, 14*(12), 7252-7260.

Ji, G., Fu, Y., Adwanikar, H., Neugebauer, V. (2013). Non-pain-related CRF1 activation in the amygdala facilitates synaptic transmission and pain responses. Mol. Pain 9, 2.

Ji, G., Neugebauer, V. (2009). Hemispheric lateralization of pain processing by amygdala neurons. *J. Neurophysiol., 102,* 2253-2264.

Ji, R. R., Gereau IV, R. W., Malcangio, M., & Strichartz, G. R. (2009). MAP kinase and pain. *Brain Res Rev., 60*(1), 135-148.

Ji, R. R., Baba, H., Brenner, G. J., & Woolf, C. J. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci, 2*(12), 1114-1119.

Jin, W.Y., Liu, Z., Liu, D., Yu, L.C. (2010). Antinociceptive effects of galanin in the central nucleus of amygdala of rats, an involvement of opioid receptors. *Brain Res., 1320*, 16-21.

Jongsma, H., Danielsen, N., Sundler, F., & Kanje, M. (2000). Alteration of PACAP distribution and PACAP receptor binding in the rat sensory nervous system following sciatic nerve transection. *Brain Research, 853*(2), 186-196.

Jongsma, H., Pettersson, L. M., Zhang, Y., Reimer, M. K., Kanje, M., Waldenström, A., . . . Danielsen, N. (2001). Markedly reduced chronic nociceptive response in mice lacking the PAC1 receptor. *Neuroreport, 12*(10), 2215-2219.

Jongsma Wallin, H., Pettersson, L. M., Verge, V. M., & Danielsen, N. (2003). Effect of anti-nerve growth factor treatment on pituitary adenylate cyclase activating polypeptide expression in adult sensory neurons exposed to adjuvant induced inflammation. *Neuroscience, 120*(2), 325-331.

Jovanovic, T. *et al.* PAC1 receptor (ADCYAP1R1) genotype is associated with dark-enhanced startle in children (2013). *Mol Psychiatry, 18*, 742-743.

Kammermeier, P. J. (2008). Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. *J Neurosci, 28*(34), 8560-8567.

Kemppainen, S., & Pitkanen, A. (2000). Distribution of parvalbumin, calretinin, and calbindin-D(28k) immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. *J Comp Neurol, 426*(3), 441-467.

Klüver, H, Bucy, P.C. (1937). "Psychic blindness" and other symptoms following bilateral temporal lobectomy in rhesus monkeys. *Am. J. Physiol.*, *119*, 352-353.

Kocho-Schellenberg, M., Lezak, K. R., Harris, O. M., Roelke, E., Gick, N., Choi, I., . . . Hammack, S. E. (2014). Pituitary Adenylate Cyclase Activating Peptide (PACAP) in the Bed Nucleus of the Stria Terminalis (BNST) Produces Anorexia and Weight Loss in Male and Female Rats. *Neuropsychopharmacology, 39*, 1614-1623.

Kocorowski, L. H., & Helmstetter, F. J. (2001). Calcitonin gene-related peptide released within the amygdala is involved in Pavlovian auditory fear conditioning. *Neurobiology of Learning and Memory, 75*(2), 149-163.

Koob, G.F., Heinrichs, S.C. (1999). A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res., 848,* 141-152.

Kozicz, T., Vigh, S., & Arimura, A. (1998). The source of origin of PACAP- and VIP-immunoreactive fibers in the laterodorsal division of the bed nucleus of the stria terminalis in the rat. *Brain Research, 810*(1-2), 211-219.

Kroenke, K., & Price, R. K. (1993). Symptoms in the community. Prevalence, classification, and psychiatric comorbidity. *Arch Intern Med, 153*(21), 2474-2480.

Kulkarni, B., Bentley, D. E., Elliott, R., Julyan, P. J., Boger, E., Watson, A., . . . Jones, A. K. (2007). Arthritic pain is processed in brain areas concerned with emotions and fear. *Arthritis Rheum., 56*(4), 1345-1354.

Lam, R. W. (2012). Onset, time course and trajectories of improvement with antidepressants. *Eur Neuropsychopharmacol, 22 Suppl 3*, S492-498.

Latremoliere, A., & Woolf, C. J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain., 10*(9), 895-926.

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. *J. Neurosci., 17,* 6434-6446.

Ledoux, J. E., (2000). Emotion circuits in the brain. *Annu. Rev. Neurosci., 23,* 155-184.

Lehmann, M. L., Mustafa, T., Eiden, A. M., Herkenham, M. & Eiden, L. E. PACAP-deficient mice show attenuated corticosterone secretion and fail to develop depressive behavior during chronic social defeat stress (2013) *Psychoneuroendocrinology, 38*, 702-715.

Legradi, G., Das, M., Giunta, B., Hirani, K., Mitchell, E. A., & Diamond, D. M. (2007). Microinfusion of pituitary adenylate cyclase-activating polypeptide into the central nucleus of amygdala of the rat produces a shift from an active to passive mode of coping in the shock-probe fear/defensive burying test. *Neural plasticity, 2007*, 79102.

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.

Lezak, K. R., Roman, C. W., Braas, K. M., Schutz, K. C., Falls, W. A., Schulkin, J., . . . Hammack, S. E. (2014). Regulation of Bed Nucleus of the Stria Terminalis PACAP Expression by Stress and Corticosterone. *J Mol Neurosci*., *54*(3), 477-484.

Li, J., Hou, B., Tumova, S., Muraki, K., Bruns, A., Ludlow, M. J., . . . Beech, D. J. (2014). Piezo1 integration of vascular architecture with physiological force. *Nature, 515*(7526), 279-282.

Li, Z., Ji, G., & Neugebauer, V. (2011). Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. *J Neurosci, 31*(3), 1114-1127.

Lu, Y. C., Chen, Y. Z., Wei, Y. Y., He, X. T., Li, X., Hu, W., . . . Dong, Y. L. (2015). Neurochemical properties of the synapses between the parabrachial nucleus-derived CGRP-positive axonal terminals and the GABAergic neurons in the lateral capsular division of central nucleus of amygdala. *Mol Neurobiol, 51*(1), 105-118.

Mabuchi, T., Shintani, N., & Matsumura, S. (2004). Pituitary adenylate cyclase-activating polypeptide is required for the development of spinal sensitization and induction of neuropathic pain. *J Neurosci., 24*(33), 7283-7291.

Macdonald, D. S., Weerapura, M., Beazely, M. A., Martin, L., Czerwinski, W., Roder, J. C., . . . MacDonald, J. F. (2005). Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires G alpha q, protein kinase C, and activation of Src. *J Neurosci, 25*(49), 11374-11384.

Makino, S., Shibasaki, T., Yamauchi, N., Nishioka, T., Mimoto, T., Wakabayashi, I., Gold, P.W., Hashimoto, K. (1999). Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. *Brain Res., 850,* 136-143.

Mannion, R. J., Doubell, T. P., Coggeshall, R. E., & Woolf, C. J. (1996). Collateral sprouting of uninjured primary afferent A-fibers into the superficial dorsal horn of the adult rat spinal cord after topical capsaicin treatment to the sciatic nerve. *J Neurosci, 16*(16), 5189-5195.

Martin, M., Otto, C., Santamarta, M. T., Torrecilla, M., Pineda, J., Schutz, G., & Maldonado, R. (2003). Morphine withdrawal is modified in pituitary adenylate cyclase-activating polypeptide type I-receptor-deficient mice. *Brain Res Mol Brain Res, 110*(1), 109-118.

May, V., Beaudet, M. M., Parsons, R. L., Hardwick, J. C., Gauthier, E. A., Durda, J. P., & Braas, K. M. (1998). Mechanisms of pituitary adenylate cyclase activating polypeptide (PACAP)-induced depolarization of sympathetic superior cervical ganglion (SCG) neurons. *Ann N Y Acad Sci., 865*, 164-175.

May, V., Buttolph, T. R., Girard, B. M., Clason, T. A., & Parsons, R. L. (2014). PACAP-induced ERK activation in HEK cells expressing PAC1 receptors involves both receptor internalization and PKC signaling. *Am J Physiol Cell Physiol, 306*(11), C1068-C1079.

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. *J Biol Chem, 285*(13), 9749-9761.

May, V., & Vizzard, M. A. (2010). Bladder dysfunction and altered somatic sensitivity in PACAP-/- mice. *J Urol., 183*(2), 772-779.

McEwen, B. S., & Kalia, M. (2010). The role of corticosteroids and stress in chronic pain conditions. *Metabolism, 59*, S9-S15.

McCoy, E. S., Taylor-Blake, B., Street, S. E., Pribisko, A. L., Zheng, J., & Zylka, M. J. (2013). Peptidergic CGRPα primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. *Neuron, 78*(1), 138-151.

McWilliams, L. A., Cox, B. J., & Enns, M. W. (2003). Mood and anxiety disorders associated with chronic pain: an examination in a nationally representative sample. *Pain, 106*(1-2), 127-133.

McWilliams, L. A., Goodwin, R. D., & Cox, B. J. (2004). Depression and anxiety associated with three pain conditions: results from a nationally representative sample. *Pain, 111*(1-2), 77-83.

Merighi, A. (2002). Costorage and coexistence of neuropeptides in the mammalian CNS. *Prog Neurobiol, 66*(3), 161-190.

Merriam, L. A., Baran, C. N., Girard, B. M., Hardwick, J. C., May, V., & Parsons, R. L. (2013). Pituitary Adenylate Cyclase 1 Receptor Internalization and Endosomal Signaling Mediate the Pituitary Adenylate Cyclase Activating Polypeptide-Induced Increase in Guinea Pig Cardiac Neuron Excitability. *J Neurosci., 33*(10), 4614-4622.

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 cyclase in pituitary cells. *Biochem Biophys Res Commun., 164*(1), 567-574.

Moller, K., Reimer M., Ekblad, E., Hannibal J., Fahrenkrug, J., Kanje M., Sundler, F. (1997) The effects of axotomy and preganglionic denervation on the expression of pituitary adenylate cyclase activating peptide (PACAP), galanin and PACAP type 1 receptors in the rat superior cervical ganglion. *Brain Res., 775*(1-2), 166-182.

Moller, K., Zhang, Y. Z., Hakanson, R., Luts, A., Sjolund, B., Uddman, R., & Sundler, F. (1993). Pituitary adenylate cyclase activating peptide is a sensory neuropeptide: immunocytochemical and immunochemical evidence. *Neuroscience, 57*(3), 725-732.

Morano, T.J., Bailey, N.J., Cahill, C.M., Dumont, E.C. (2008). Nuclei- and condition-specific responses to pain in the bed nucleus of the stria terminalis. Prog. Neuro-Psychopharmacol. *Biol. Psychiatry, 32,* 643-650.

Mulder, H., Jongsma, H., Zhang, Y., Gebre-Medhin, S., Sundler, F., & Danielsen, N. (1999). Pituitary adenylate cyclase-activating polypeptide and islet amyloid polypeptide in primary sensory neurons. *Mol. Neurobio., 19*(3), 229-253.

Mulder, H., Uddman, R., Moller, K., Zhang, Y. Z., Ekblad, E., Alumets, J., & Sundler, F. (1994). Pituitary adenylate cyclase activating polypeptide expression in sensory neurons. *Neuroscience, 63*(1), 307-312.

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*, 1-11.

Nagahama, M., Tsuzuki, M., Mochizuki, T., Iguchi, K., & Kuwahara, A. (1998). Light and electron microscopic studies of pituitary adenylate cyclase-activating peptide (PACAP)--immunoreactive neurons in the enteric nervous system of rat small and large intestine. *Anat Embryol (Berl), 198*(5), 341-352.

Nakagawa, T., Katsuya, A., Tanimoto, S., Yamamoto, J., Yamauchi, Y., Minami, M., & Satoh, M. (2003). Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. *Neurosci Lett, 344*(3), 197-200.

Namburi, P., Beyeler, A., Yorozu, S., Calhoon, G. G., Halbert, S. A., Wichmann, R., . . . Tye, K. M. (2015). A circuit mechanism for differentiating positive and negative associations. *Nature, 520*(7549), 675-678.

Narita, M., Dun, S. L., Dun, N. J., & Tseng, L. F. (1996). Hyperalgesia induced by pituitary adenylate cyclase-activating polypeptide in the mouse spinal cord. *Eur J Pharmacol., 311*(2-3), 121-126.

Nemeth, J., Reglodi, D., Pozsgai, G., Szabo, A., Elekes, K., Pinter, E., . . . Helyes, Z. (2006). Effect of pituitary adenylate cyclase activating polypeptide-38 on sensory neuropeptide release and neurogenic inflammation in rats and mice. *Neuroscience, 143*(1), 223-230.

Neugebauer, V., & Li, W. (2002). Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. *J Neurophysiol., 87*(1), 103-112.

Neugebauer, V., Li, W., (2003). Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. *J. Neurophysiol., 89,* 716-727.

Neugebauer, V., Li, W., Bird, G. C., Bhave, G., & Gereau, R. W. (2003). Synaptic Plasticity in the Amygdala in a Model of Arthritic Pain: Differential Roles of Metabotropic Glutamate Receptors 1 and 5. *J Neurosci., 23*(1), 52-63.

Neugebauer, V., Li, W., Bird, G. C., & Han, J. S. (2004). The Amygdala and Persistent Pain. *Neuroscientist, 10*(3), 221-234.

Norman, S. B., Stein, M. B., & Dimsdale, J. E. (2008). Pain in the aftermath of trauma is a risk factor for post-traumatic stress disorder. *Psychol Med., 38*(4), 533-542.

Obata, K., Yamanaka, H., Dai, Y., Tachibana, T., Fukuoka, T., Tokunaga, A., . . . Noguchi, K. (2003). Differential activation of extracellular signal-regulated protein kinase in primary afferent neurons regulates brain-derived neurotrophic factor expression after peripheral inflammation and nerve injury. *J Neurosci, 23*(10), 4117-4126.

Ohsawa, M., Brailoiu, G. C., Shiraki, M., Dun, N. J., Paul, K., & Tseng, L. F. (2002). Modulation of nociceptive transmission by pituitary adenylate cyclase activating polypeptide in the spinal cord of the mouse. *Pain, 100*(1-2), 27-34.

Paré, D., Quirk, G. J., & Ledoux, J. E. (2004). New vistas on amygdala networks in conditioned fear. *Journal of Neurophysiology, 92*(1), 1-9.

Petrovic, P., Ingvar, M., Stone-Elander, S., Petersson, K. M., & Hansson, P. (1999). A PET activation study of dynamic mechanical allodynia in patients with mononeuropathy. *Pain, 83*(3), 459-470.

Petrovich, G. D., & Swanson, L. W. (1997). Projections from the lateral part of the central amygdalar nucleus to the postulated fear conditioning circuit. *Brain Res., 763*(2), 247-254.

Pettersson, L. M., Dahlin, L. B., & Danielsen, N. (2004). Changes in expression of PACAP in rat sensory neurons in response to sciatic nerve compression. *Eur J Neurosci, 20*(7), 1838-1848.

Pettersson, L. M. E., Heine, T., Verge, V. M. K., Sundler, F., & Danielsen, N. (2004). PACAP mRNA is expressed in rat spinal cord neurons. *J Comp Neurol, 471*(1), 85-96.

Phifer, J., Skelton, K., Weiss, T., Schwartz, A. C., & Wingo, A. (2011). Pain symptomatology and pain medication use in civilian PTSD. *Pain, 152*(10), 2233-2240.

Piggins, H. D., Stamp, J. A., Burns, J., Rusak, B., & Semba, K. (1996). Distribution of pituitary adenylate cyclase activating polypeptide (PACAP) immunoreactivity in the hypothalamus and extended amygdala of the rat. *J Comp Neurol, 376*(2), 278-294.

Pohlack, S. T., Nees, F., Ruttorf, M., Cacciaglia, R., Winkelmann, T., Schad, L. R., . . . Flor, H. (2015). Neural Mechanism of a Sex-Specific Risk Variant for Posttraumatic Stress Disorder in the Type I Receptor of the Pituitary Adenylate Cyclase Activating Polypeptide. *Biol Psychiatry*. doi: 10.1016/j.biopsych.2014.12.018

Ranade, S. S., Woo, S. H., Dubin, A. E., Moshourab, R. A., Wetzel, C., Petrus, M., . . . Patapoutian, A. (2014). Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature, 516*(7529), 121-125.

Redondo, R. L., Kim, J., Arons, A. L., Ramirez, S., Liu, X., & Tonegawa, S. (2014). Bidirectional switch of the valence associated with a hippocampal contextual memory engram. *Nature, 513*(7518), 426-430.

Ren, W. & Neugebauer, V. (2010). Pain-related increase of excitatory transmission and decrease of inhibitory transmission in the central nucleus of the amygdala are mediated by mGluR1. *Mol Pain., 6*, 93.

Resch, J. M., Boisvert, J. P., Hourigan, A. E., Mueller, C. R., Yi, S. S., & Choi, S. (2011). Stimulation of the hypothalamic ventromedial nuclei by pituitary adenylate cyclase-activating polypeptide induces hypophagia and thermogenesis. *Am J Physiol Regul Integr Comp Physiol, 301*(6), R1625-1634.

Resch, J. M., Maunze, B., Gerhardt, A. K., Magnuson, S. K., Phillips, K. A., & Choi, S. (2013). Intrahypothalamic pituitary adenylate cyclase-activating polypeptide regulates energy balance via site-specific actions on feeding and metabolism. *American journal of physiology. Endocrinology and metabolism, 305*(12), E1452-1463.

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.

Robinson, D.A., Wei, F., Wang, G.D., Li, P., Kim, S.J., Vogt, S.K., Muglia, L.J., Zhuo, M. (2002). Oxytocin mediates stress-induced analgesia in adult mice. *J. Physiol., 540,* 593-606.

Roeska, K., Doods, H., Arndt, K., Treede, R.-D., & Ceci, A. (2008). Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. *Pain, 139*(2), 349-357.

Roman, C. W., Lezak, K. R., Hartsock, M. J., Falls, W. A., Braas, K. M., Howard, A. B., . . . May, V. (2014). PAC1 receptor antagonism 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., de Reus, M., Loohuis, N. O., Giele, J., van Egmond, J., . . . Kozicz, T. (2012). Experimental neuropathy increases limbic forebrain CRF. *Eur J Pain, 16*(1), 61-71.

Sah, P., Faber, E. S. L., Armentia, M. L. D., & Power, J. (2003). The Amygdaloid Complex: Anatomy and Physiology. *Physiol Rev., 83*(3), 803-834.

Sakashita, Y., Kurihara, T., Uchida, D., Tatsuno, I., & Yamamoto, T. (2001). Involvement of PACAP receptor in primary afferent fibre-evoked responses of ventral roots in the neonatal rat spinal cord. *Br J Pharmacol, 132*(8), 1769-1776.

Sándor, K., Bölcskei, K., McDougall, J. J., Schuelert, N., Reglodi, D., Elekes, K., . . . Helyes, Z. (2009). Divergent peripheral effects of pituitary adenylate cyclase-activating polypeptide-38 on nociception in rats and mice. *Pain, 141*(1-2), 143-150.

Sándor, K., Kormos, V., Botz, B., Imreh, A., Bölcskei, K., Gaszner, B., . . . Helyes, Z. (2010). Impaired nocifensive behaviours and mechanical hyperalgesia, but enhanced thermal allodynia in pituitary adenylate cyclase-activating polypeptide deficient mice. *Neuropeptides, 44*(5), 363-371.

Saper, C. B., Romanovsky, A. A., & Scammell, T. E. (2012). Neural circuitry engaged by prostaglandins during the sickness syndrome. *Nat Neurosci, 15*(8), 1088-1095.

Sarhan, M., Freund-Mercier, M.-J., & Veinante, P. (2005). Branching patterns of parabrachial neurons projecting to the central extended amgydala: single axonal reconstructions. *J Comp Neurol, 491*(4), 418-442.

Sato, M., Ito, M., Nagase, M., Sugimura, Y. K., Takahashi, Y., Watabe, A. M., & Kato, F. (2015). The lateral parabrachial nucleus is actively involved in the acquisition of fear memory in mice. *Mol Brain, 8*(1), 22.

Schmidt, S. D., Myskiw, J. C., Furini, C. R., Schmidt, B. E., Cavalcante, L. E., & Izquierdo, I. (2015). PACAP modulates the consolidation and extinction of the contextual fear conditioning through NMDA receptors. *Neurobiol Learn Mem, 118*, 120-124.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods, 9*, 671-675.

Scioli-Salter, E. R., Forman, D. E., Otis, J. D., Gregor, K., Valovski, I., & Rasmusson, A. M. (2015). The shared neuroanatomy and neurobiology of comorbid chronic pain and PTSD: therapeutic implications. *Clin J Pain, 31*(4), 363-374.

Sherwood, N. M., Krueckl, S. L., & McRory, J. E. (2000). The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev., 21*(6), 619-670.

Shi, C., & Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. *J Neurosci., 19*(1), 420-430.

Shimizu, T., Katahira, M., Sugawara, H., Inoue, K., & Miyata, A. (2004). Diverse effects of intrathecal pituitary adenylate cyclase-activating polypeptide on nociceptive transmission in mice spinal cord. *Regulatory Peptides, 123*(1-3), 117-122.

Simons, L. E., Moulton, E. A., Linnman, C., Carpino, E., Becerra, L., & Borsook, D. (2014). The human amygdala and pain: Evidence from neuroimaging. *Hum Brain Mapp., 35*(2), 527-538.

Sink, K. S., Chung, A., Ressler, K. J., Davis, M., & Walker, D. L. (2013). Anxiogenic

effects of CGRP within the BNST may be mediated by CRF acting at BNST CRFR1 receptors. *Behavioural Brain Research, 243*, 286-293.

Sink, K. S., Walker, D. L., Yang, Y., & Davis, M. (2011). Calcitonin gene-related peptide in the bed nucleus of the stria terminalis produces an anxiety-like pattern of behavior and increases neural activation in anxiety-related structures. *J Neurosci, 31*(5), 1802-1810.

Sorkin, A., & von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol, 10*(9), 609-622.

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P.H., Journot, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature, 365*, 170-175.

Spike, R. C., Puskar, Z., Andrew, D., & Todd, A. J. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur J Neurosci, 18*(9), 2433-2448.

Stroth, N., & Eiden, L. E. (2010). Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling. *Neuroscience, 165*(4), 1025-1030.

Suwanprathes, P., Ngu, M., Ing, A., Hunt, G., & Seow, F. (2003). c-fos immunoreactivity in the brain after esophageal acid stimulation. *Am J Med, 115 Suppl 3A*, 31S-38S.

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci., 11*(12), 823-836.

Tovote, P., Fadok, J. P., & Lüthi A. (2015). Neuronal circuits for fear and anxiety. *Nat Neurosci., 16,* 317-331.

Tracey, I., & Mantyh, P. W. (2007). The cerebral signature for pain perception and its modulation. *Neuron, 55*(3), 377-391.

Tran, L., Wiskur, B., Greenwood-Van Meerveld, B. (2012). The role of the anterio-lateral bed nucleus of the stria terminalis in stress-induced nociception. Am. J. Physiol. Gastrointest. *Liver Physiol., 302*, G1301-G1309.

Trepanier, C. H., Jackson, M. F., & MacDonald, J. F. (2012). Regulation of NMDA receptors by the tyrosine kinase Fyn. *FEBS J, 279*(1), 12-19.

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 (Amsterdam, Netherlands), 14*(4), 368-375.

Turk, D. C. (2002). Clinical effectiveness and cost-effectiveness of treatments for patients with chronic pain. *Clin J Pain, 18*(6), 355-365.

Uddin, M., Chang, S.C., Zhang, C., Ressler, K., Mercer, K.B., Galea, S., Keyes, K.M., McLaughlin, K.A., Wildman, D.E., Aiello, A.E., Koenen, K.C. (2013). Adcyap1r1 genotype, posttraumatic stress disorder, and depression among women exposed to childhood maltreatment. *Depress Anxiety, 30,* 251-258.

Ulrich-Lai, Y. M., Xie, W., Meij, J. T. A., Dolgas, C. M., Yu, L., & Herman, J. P. (2006). Limbic and HPA axis function in an animal model of chronic neuropathic pain. *Physiol Behav., 88*(1-2), 67-76.

Usoskin, D., Furlan, A., Islam, S., Abdo, H., Lonnerberg, P., Lou, D., . . . Ernfors, P. (2015). Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat Neurosci, 18*(1), 145-153.

Vaudry, D., Falluel-Morel, A., Bourgault, S., Basille, M., Burel, D., Wurtz, O., . . . Vaudry, H. (2009). Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev., 61*(3), 283-357.

Veinante, P., Yalcin, I., Barrot, M. (2013). The amygdala between sensation and affect: a role in pain. *J. Mol. Psychiatry, 1*, 9.

Vizzard, M. A. (2000). Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis. *J Comp Neurol, 420*(3), 335-348.

Walker, D. L., Miles, L. A., & Davis, M. (2009). Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 33*(8), 1291-1308.

Watabe, A. M., Ochiai, T., Nagase, M., Takahashi, Y., Sato, M., & Kato, F. (2013). Synaptic potentiation in the nociceptive amygdala following fear learning in mice. *Mol Brain, 6*, 11.

Xu, W., Lundeberg, T., Wang, Y.T., Li, Y., Yu, L.C. (2003). Antinociceptive effect of calcitonin gene-related peptide in the central nucleus of amygdala: activating opioid receptors through amygdala-periaqueductal gray pathway. *Neuroscience, 118*, 1015-1022.

Xu, X. J., & Wiesenfeld-Hallin, Z. (1996). Intrathecal pituitary adenylate cyclase activating polypeptide facilitates the spinal nociceptive flexor reflex in the rat. *Neuroscience, 72*(3), 801-804.

Yalcin, I., Barthas, F., & Barrot, M. (2014). Emotional consequences of neuropathic pain: insight from preclinical studies. *Neurosci Biobehav Rev., 47*, 154-164.

Yamamoto, T., & Tatsuno, I. (1995). Antinociceptive effect of intrathecally administered pituitary adenylate cyclase activating polypeptide (PACAP) on the rat formalin test. *Neurosci Lett., 184*(1), 32-35.

Woolf, C. J., Shortland, P., & Coggeshall, R. E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature, 355*(6355), 75-78.

Zeng, Q., Wang, S., Lim, G., Yang, L., Mao, J., Sung, B., . . . Mao, J. (2008). Exacerbated mechanical allodynia in rats with depression-like behavior. *Brain Res, 1200*, 27-38.

Zhang, Q., Shi, T.-J., Ji, R.-R., Zhang, Y.-t., Sundler, F., Hannibal, J., . . . Hökfelt, T. (1995). Expression of pituitary adenylate cyclase-activating polypeptide in dorsal root ganglia following axotomy: time course and coexistence. *Brain Res., 705*(1-2), 149-158.

Zhang, Y., Chen, Y., Liedtke, W., & Wang, F. (2015). Lack of evidence for ectopic sprouting of genetically labeled Abeta touch afferents in inflammatory and neuropathic trigeminal pain. *Mol Pain, 11*, 18.

Zhang, Y., Danielsen, N., Sundler, F., & Mulder, H. (1998). Pituitary adenylate cyclase-activating peptide is upregulated in sensory neurons by inflammation. *Neuroreport, 9*(12), 2833-2836.

Zhang, Y., Malmberg, A. B., Sjölund, B., & Yaksh, T. L. (1996). The effect of pituitary adenylate cyclase activating peptide (PACAP) on the nociceptive formalin test. *Neurosci Lett, 207*(3), 187-190.

Zhang, Y. Z., Hannibal, J., Zhao, Q., Moller, K., Danielsen, N., Fahrenkrug, J., & Sundler, F. (1996). Pituitary adenylate cyclase activating peptide expression in the rat dorsal root ganglia: up-regulation after peripheral nerve injury. *Neuroscience, 74*(4), 1099-1110.

Zhang, Y. Z., Sjolund, B., Moller, K., Hakanson, R., & Sundler, F. (1993). Pituitary adenylate cyclase activating peptide produces a marked and long-lasting depression of a C-fibre-evoked flexion reflex. *Neuroscience, 57*(3), 733-737.