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A Pain in the Brain: How PACAP CeA Infusion Alters Mechanical and Thermal Sensitivity

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1.0 Introduction

1.1 Prevalence of Pain

Pain is part of the human experience. Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (International Association for the Study of Pain (IASP), 2011). Acute and chronic pain affects large numbers of individuals around the world, including the U.S. In 2011, over 1.5 billion people worldwide were burdened by chronic pain (Global Industry Analysts, Inc., 2011), including 100 million U.S. adults, more than the number affected by heart disease, diabetes, and cancer combined (Institute of Medicine, 2011). The effects of pain are extremely expensive not only in terms of health care costs, but also in rehabilitation and lost worker productivity. Pain also places a huge financial and emotional burden on patients and their families. The total national annual economic cost associated with pain ranges from $560 billion to $635 billion. This estimate includes the incremental cost of health care ($261-300 billion) and the cost of lost productivity ($297-336 billion) attributable to pain (Institute of Medicine, 2011).

For many patients, the treatment of pain is inadequate. In 2006, the National Center for Health Statistics reported that over one-quarter of Americans (26%) age 20 years and over reported that they have had a problem with pain that persisted for more than 24 hours in duration. Another 2006 study conducted by the American Pain Foundation evaluated chronic pain in patients who had been treated by a physician and were using an opioid for treatment. They reported that 51% of patients felt that they had little or no control over their pain and 60% experienced pain one of more times daily, despite being treated. These episodes of pain were severely impacting their quality of life. 59% reported an impact of their overall enjoyment of life, 77% felt depressed, 70% had trouble concentrating, 74% said their energy level was
decreased, and 86% reported an inability to sleep well. Furthermore, in 2009, Asmundson and Katz discovered that patients suffering from chronic pain were more prone to experience psychological problems including depression, panic disorders, compulsive behavior, anxiety abnormalities, and stress-related disorders like post-traumatic stress disorder (PTSD). A first step in addressing the worldwide burden of pain is understanding the underlying mechanisms that lead to the perception of pain, and how these may be altered in pathological pain states. This understanding will facilitate the proper development and use of pain treatments.

1.2 Pain Processing

Pain, like all other somatic sensory modalities, serves an important protective function. Pain alerts individuals to injuries and leads them to seek out treatment. Not being able to feel pain can be dangerous because severe injuries often go unnoticed and can lead to permanent tissue damage. Congenital insensitivity to pain (CIP) is a condition that inhibits the ability to feel physical pain. CIP is caused by a mutation in the sodium channel gene SCN9A that leads to the loss of function of NaV1.7, a voltage gated sodium channel type IX alpha subunit (Drenth & Waxman, 2007). NaV1.7 sodium channels are found in nociceptors, the neurons responsible for the transmission of pain signals to the spinal cord and brain (Wang et al., 2011). CIP is an extremely rare disorder, and as of 2012 only an estimated 20 cases had been reported in the scientific literature (Genetics Home Reference). The case of Miss C exemplifies what can happen when people are born with insensitivity to pain (Melzack & Wall, 1988). As a child, Miss C suffered from many childhood injuries that resulted from her inability to experience pain, including burning herself on a radiator and biting her tongue while eating. This lack of awareness of pain led to an accumulation of bruises, wounds, broken bones, and other health
issues that went undetected. As an adult, she developed joint problems as a result of a lack of discomfort from staying in one position for too long. She died at age 29 from infections that probably would have been prevented if she could have perceived pain and was alerted to injury risk (Melzack & Wall, 1988).

Pain differs from nociception. Nociception refers to the neurophysiologic manifestations generated by noxious stimuli, while pain is the perception of an aversive stimulus, which requires abstraction and the elaboration of sensory impulses (Millan, 1999). Pain is not the direct expression of a sensory event, but rather the product of elaborate processing by the brain of multiple incoming signals. The perception of pain is subjective and influenced by many factors. An identical sensory stimulus can elicit different responses in distinct individuals as well as in the same individual under different conditions (Kandel et al., 2013).

Pain comes in two major forms, acute and chronic. Acute pain is defined by a limited period of time and disappears upon the resolution of the pathological process. Chronic pain is pain that persists for an extended period of time and is associated with chronic pathological processes (Merskey & Bogduk, 1994).

The experience of pain begins with the activation of nociceptors, free nerve endings of primary sensory neurons that respond to various forms of tissue damage, to bodily processes that signal damage, such as inflammation, and to stimuli that have the potential to harm tissues (including extreme temperatures below 5 C and above 45 C) (Kandel et al., 2013; Purves et al., 2012). Because nociceptive axons terminate in unspecialized endings, they are categorized by the properties of their nerve fibers. Aδ fibers are lightly myelinated that respond to intense mechanical or to mechanothermal stimuli. C fibers are unmyelinated fibers that respond to thermal, mechanical, and noxious chemical stimuli. There are three main classes of nociceptors:
thermal, mechanical, and polymodal. Thermal nociceptors are activated by extreme temperatures and are the peripheral endings of small diameter A\(\delta\) axons that conduct potentials at speeds of 5 to 30 m/s. Mechanical nociceptors are activated by intense pressure on the skin and are also the nerve endings of A\(\delta\) axons. Polymodal nociceptors are activated by high intensity mechanical, chemical, or thermal stimuli and are the endings of small-diameter, unmyelinated C axons that conduct action potentials at speeds less that 1m/s (Supplemental Image 1). The receptor fields of nociceptors are relatively large and are widely distributed in skin and deep tissues, meaning they are often coactivated (Kandel et al., 2013; Purves et al., 2012).

The activation of nociceptors eventually leads to the perception of pain. There are two major categories of pain perception: a sharp first pain and a more delayed and longer lasting second pain. A\(\delta\) fibers propagate specific information, with high intensity and short latency. They are responsible for the quick, sharp first pain that triggers a withdrawal response. C fibers propagate more slowly. These slow potentials induce aching and sometimes a burning pain, referred to as second pain (Kandel et al., 2013; Purves et al., 2012).

Nociceptors are the free nerve endings of dorsal root ganglia and trigeminal ganglia (Kandel et al., 2013; Purves et al., 2012). When these axons reach the dorsal horn, they branch into ascending and descending collaterals, creating the dorsolateral tract of Lissauer. Axons in this tract run up or down for one or two spinal cord segments before innervating the dorsal horn in several of Rexed’s laminae. These dorsal root ganglia carrying nociceptive information innervate laminae I, II, and V. Laminae I and V contain projection neurons whose axons travel to the brainstem and thalamus. C fibers terminate exclusively in laminae I and II, while A\(\delta\) fibers synapse in layers I and V. Laminae I and II are the outermost layers of the dorsal horn of the spinal cord and are known as the marginal zone (layer I) and substantia gelatinosa (layer II).
Many neurons in lamina I respond to noxious stimuli carried by Aδ and C fibers, and are known as nociception-specific neurons. A second group of lamina I neurons receives input from C fibers that are selectively activated by extreme cold. Other classes of lamina I neurons respond to both noxious and innocuous mechanical stimulation and are called wide-dynamic-range neurons. Lamina II, the substantia gelatinosa, is filled with both excitatory and inhibitory interneurons, some of which respond selectively to nociceptive inputs, while others respond to both nociceptive and innocuous stimuli. Lamina V contains neurons that respond to a variety of noxious stimuli. These neurons receive direct inputs from Aδ fibers, as well as from non-nociceptive Aβ fibers, which communicate crude touch. The dendrites of these neurons also extend into laminae IV, III, and II and are innervated by C fibers in lamina II. In summary, neurons in lamina I receive direct input from Aδ fibers and direct and indirect (via interneurons of lamina II) from C fibers. Lamina V neurons receive low threshold input from non-nociceptive Aβ fibers of mechanoreceptors and inputs from nociceptive Aδ and C fibers (Supplemental Image 2). The axons of the second order neurons in laminae I and V cross the midline and ascend into the brainstem and thalamus in the anterolateral fascicle of the contralateral spinal cord. These fibers form anterolateral tract (Kandel et al., 2013; Purves et al., 2012).

1.3 Pain Pathways

There are six major ascending pathways that convey nociceptive information: The spinothalamic, spinoreticular, spinomesencephalic, spinoparabrachial, spinocervical, and spinohypothalamic tracts, but this thesis will focus on the spinothalamic and the spinoparabrachioamygdaloid (SPA) division of the spinoparabrachial tract (Almeida et al., 2004; Kandel et al., 2013). The spinothalamic tract is the most prominent ascending nociceptive
pathway (Supplemental Image 3). It includes the axons of nociceptive-specific, thermosensitive, and wide-dynamic-range neurons in laminae I and V, which carry noxious potentials that are related to pain, temperature, touch, and itching. These axons cross the midline and travel up the anterolateral white matter of the contralateral spinal cord. The fibers of the spinothalamic tract project to the thalamus where they form synapses in the thalamic ventral posterior nucleus (VPN). Neurons of the VPN project to the primary and secondary somatosensory cortex (S1, S2), where the perception of pain begins to be processed. The sensory-discriminative aspects of pain: the location, intensity, and quality of the noxious stimuli are thought to depend on information relayed through the spinothalamic tract into S1 and S2 (Kandel et al., 2013; Kenshalo & Insensee, 1983; Purves et al., 2012).

Other divisions of the pain system are responsible for the affective-motivational aspects: the unpleasant feeling, fear, anxiety, and the autonomic activation that accompany exposure to noxious stimuli. Targets of these systems include the superior colliculus, the reticular formation, the hypothalamus, the periaqueductal gray matter, the septal nucleus, the anterior cingulate cortex, the insula, and the amygdala (Purves et al., 2012; Willis & Westlund, 1997). Evidence from functional imaging studies has shown that different brain regions mediate the sensory-discriminative and affective-motivational aspects of pain. Painful stimuli activate both the primary somatosensory cortex and anterior cingulate cortex. Using hypnotic suggestion to selectively increase or decrease unpleasantness or intensity of pain, it was discovered that changes in unpleasantness were accompanied by changes in the activity of neurons in the anterior cingulate cortex (Rainville, 1997), while changes in intensity were highly correlated with changes in the activity of neurons in the somatosensory cortex (Hofbauer, 2001). The SPA
pathway has been implicated as an important pathway for the affective-motivational aspects of pain.

1.4 The CeA in Pain Processing

Over the last 20 years, the amygdala, especially its central nucleus (CeA), has emerged as a key element of the pain matrix. The amygdala is centrally located to integrate the many ascending and descending signals to modulate both the emotional and sensory aspects of pain. It possesses connections that influence the descending pain control systems and is also connected to other brain regions involved in emotional, affective, and cognitive functions. The CeA receives nociceptive information from the brainstem and receives highly processed descending polymodal nociceptive information from the cerebral cortex and the thalamus. This descending information is conveyed to the basolateral amygdala (BLA), which then projects to the CeA. The CeA in turn projects to other brain nuclei. The CeA efferents include those that travel with antinociception hypothalamic-periaqueductal grey projections that dampen pain (Veinante et al., 2013). The most prominent ascending pathway carrying nociceptive information to the CeA is the SPA pathway. In the SPA pathway, primary Aδ and C fibers terminate in laminae I and V, where second order neurons project to the parabrachial nuclei (PBn) (Todd, 2010) (Supplemental Image 4). The PBn collects nociceptive information, including both mechanical and thermal nociceptive signals, and relays the information in a highly organized topographical manner to the lateral capsular division of the CeA (CeLC). This ascending pathway does not require the conveyance of information through the BLA. In addition, spinal neurons in the deep dorsal horn form monosynaptic connections with amygdala neurons and may provide sensory, including nociceptive, input to the amygdala (Burstein & Potrebic, 1993).
The CeA is the output nucleus for major amygdala functions. It modulates various systems involved with emotional response through widespread, reciprocal connections with the forebrain and brainstem, including the bed nucleus of the stria terminalis (BNST), frontal cortical areas, hippocampus, septal nuclei, lateral hypothalamus, parabrachial area, solitary tract nucleus and brain stem areas involved in endogenous pain control (as reviewed by Neugebauer & Li, 2002).

The role of the CeA in pain processing and the modulation of pain behavior has been highly investigated. Nociceptive stimuli have been shown to increase several markers of CeA activation (Rouwette et al., 2012). In vivo electrophysiological studies have shown that chronic pain and noxious stimuli increase spontaneous and evoked CeA neuronal activity (Bernard et al., 1992; Neugebauer & Li, 2002; Neugebauer & Li, 2003). Neugebauer and Li (2002) discovered that mechanical and thermal cutaneous nociceptive stimulation as well as joint and muscular deep tissue nociception provoked excitability in neurons of the CeA. Further studies demonstrated that most of these neurons were located in the CeLC, while few neurons in the central (CeL) and medial (CeM) division of the CeA responded to nociceptive stimulation. This gave rise to the name “nociceptive amygdala” to define the CeLC (Neugebauer et al., 2004).

In vivo electrophysiological studies have also revealed that noxious stimuli and chronic pain increase synaptic transmission at PBn-CeA and BLA-CeA synapses (Ikeda et al., 2007; Neugebauer et al., 2003) Neugebauer et al. (2003) noted that CeA neurons of arthritic rats developed an increased excitability compared with control CeA neurons. Synaptic plasticity was accompanied by upregulation of presynaptic group I metabotropic glutamate receptors (mGluR1 and mGluR5) and increased presynaptic mGluR1 function, demonstrating a physiological response to pain at the level of the synapse. Studies have also shown that visceral, inflammatory,
and chronic pain can induce c-Fos expression in the CeA. Specifically, intraperitoneal or esophageal acetic acid injection (Nakagawa et al., 2003; Suwanprathes, 2003), colorectal distension (Traub et al., 1996) and experimental cystitis (Bon et al., 1998) were discovered to induce c-fos expression in the CeA.

Human brain neuroimaging studies have implicated the amygdala in pain. Painful stimuli increase blood oxygen level dependent (BOLD) signals in the amygdala (Bornhövd, 2002). Furthermore, Bornhövd and colleagues (2002) found that repeated thermal nociceptive stimulations of increasing intensity led to an activation of the amygdala that was correlated with the pain perception rating. Behavioral studies in animals have also revealed that the CeA plays a role in pain perception. Electrical stimulation of the amygdala elicited vocalizations accompanied by emotional responses in monkeys (Jurgens et al., 1967). Lesions or temporary inactivation of the CeA decreased tonic pain responses (Manning, 1998) as well as emotional pain reactions, without altering normal behavior or baseline nociceptive responses (as reviewed by Neugebauer & Li, 2002). Chronic pain was also shown to induce anxiety in mice with concomitant changes in opiodergic function in the amygdala (Narita et al., 2006). The amygdala, specifically its CeA, appears to modulate the behavioral and emotional responses to pain.

1.5 PACAP and Pain

Pituitary adenylate cyclase activating polypeptide (PACAP) is a well-studied, widely expressed neural and endocrine pleiotropic peptide. PACAP has been found to exert pleiotropic effects, participating in control of neurotransmitter release, vasodilation, bronchodilation, stimulation of cell proliferation and/or differentiation, promotion of neuronal survival, sensory and autonomic signaling, hippocampal learning and memory processes, and stress-related
behavioral responses (as reviewed by Vaudry et al., 2009). PACAP was originally isolated from
the hypothalamus based on its ability to stimulate anterior pituitary adenylyl cyclase activity
(Miyata et al., 1989). PACAP arises from a prohormone that can be cleaved into two formations,
the bioactive α-amidated PACAP 38 or PACAP 27 (Miyata et al., 1990). PACAP38 appears to
be the more abundant version, with 10-fold to 100-fold more PACAP38 in most tissues including
the central nervous system (CNS) (Arimura et al., 1991; Miyata et al., 1990). PACAP binds to
three G-protein receptors; to PAC1 selectively and to VPAC1 and VPAC2, which bind PACAP
and VIP with equal affinities (Harmar et al., 2012).

PACAP systems have been shown to be dysregulated in emotional-related processes.
There is a PACAP single-nucleotide polymorphism (SNP) associated with PTSD (Ressler et al.,
2011), a SNP associated with schizophrenia (Hashimoto et al., 2007) and a SNP associated with
major depression (Hashimoto et al., 2010). The SNPs can occur on the PAC1 receptor gene or on
the PACAP gene itself. Furthermore, our laboratory recently demonstrated that the expression of
PACAP and its PAC1 receptor were upregulated in specific limbic regions by chronic stress and
that PACAP infusion into the BNST was anxiogenic (Hammack et al., 2009).

PACAP has also been shown to alter pain responses at various levels of the nervous
system. Studies have demonstrated that intrathecal injections of PACAP induced mechanical
hyperalgesia in mice (Ohsawa et al., 2002), intrathecal injections of PACAP produced
hyperalgesia in tail-flick assays (Narita et al., 1996), and PACAP knockout animals did not
display neuropathic mechanical sensitivity after spinal nerve transection (Mabuchi et al., 2004;
Sándor, 2010). These results suggest an important involvement of PACAP in pain and
nociception.

Using the knowledge that the SPA pathway projects from the PBn to the CeLC ,
PACAPergic fibers project from the PBn (Bernard et al., 1993; as reviewed by Hammack & May, 2014), and PACAP can alter pain, our laboratory investigated the presence of PACAP in the CeLC. Missig et al. (2014) identified PACAP immunoreactivity in fiber elements of the CeLC and used anterograde tracing to demonstrate that the CeLC PACAP immunoreactivity represented sensory fiber projects from the lateral PBn (LPBn). In addition, Missig et al. (2014) provided evidence that the LPBn was the PACAP source of both the CeLC as well as the BNST through excitotic lesion studies. Excitotic lesions of the LPBn led to a significant decrease in PACAP immunoreactivity in both the CeLC and the BNST (Missig et al., 2014). Evidence that PACAP cells in PBn project to the CeA and that the CeA contains PACAP suggests that PACAP release may be critical for the perception of pain.

With the discovery of PACAP within the CeA and the knowledge that the CeA plays a central role in the emotional process of pain, we investigated the effects of PACAP on pain processing. In this study, we examined the effects of CeA PACAP infusion on thermal and mechanical nociception and found that bilateral PACAP infusions into the CeA reduced nociceptive thresholds on Hangreaves thermal sensitivity tests, but not on von Frey mechanical sensitivity assessments.

2.0 Methods

2.1 Animals

16 Adult (250-350g), male, Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and were habituated in their home cages in the animal facility for at least one week before experimentation. Rats were single-housed, maintained on a 12 h light/dark cycle (lights on at 07:00 h), and food and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University
of Vermont.

2.2 Apparatuses

2.2.1 Mechanical Apparatus

Von Frey Filaments: A set of 20 Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL) were used with a target force between 2 and 26 grams. However, filaments were not used that had a target force greater than 10% of the rat’s body weight as they could raise the hindpaw in absence of a paw withdrawal.

2.2.2 Thermal Apparatus

Hargreave’s apparatus (Plantar Analgesia Meter, IITC Life Science Inc., Woodland Hills, CA) is a heating apparatus that measures response to infrared heat stimulus, applied to the plantar surface. A guide light was used to allow the experimenter to target the hindpaw. A beam of focused radiant light (4x6 mm, set to 25% active intensity) from the apparatus beneath the glass of the testing chamber was delivered to the plantar surface of the paw. An automatic cut-off timer set at 30 seconds was built into the system to prevent tissue damage.

2.2.3 Testing Chamber

The testing chamber was a clear, acrylic chamber placed on top of a wire mesh for the mechanical threshold testing and a glass platform for thermal threshold testing with an internal heating element that heated to 30°C. (IITC Life Science Inc., Woodland Hills, CA).

2.3 Surgical Procedure

To implant indwelling cannulae, rats were anesthetized with isoflurane vapor (1.5 - 3.5%), and secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with “blunt” earbars. A midline incision was made and the skull was exposed and cleaned. Four
screws were then inserted to provide skullcap stability. Two stainless steel cannulae (22 GA, PlasticsOne, Roanoke, VA) were lowered into the CeA, using the following coordinates from bregma in mm, AP = -2.6, ML = + 4.5, and DV = - 7.2 at a 0 degree angle. Once in place, the cannulae were held in place using dental cement (Hammack et al., 2009).

2.4 Post-Operative Procedure

Once awake, rats were returned to their home cages for one week of post-surgery recovery, during which the animals received post-operative analgesia (Carprofen 5mg/kg) and were routinely wrapped in a towel to habituate handling. The animals were also observed and weighed daily. After the post-operative week the rats were habituated to the testing chamber for 20 minutes a day for 4 days with a fan to generate ambient noise. Following habituation, rats were then assessed for baseline withdrawal thresholds to both thermal and mechanical stimuli for two days.

2.5 Testing Procedures

Following baseline withdrawal assessment, rats were loosely restrained in a towel and the CeA was infused with sterile saline (control) or PACAP (1µg in 0.5µl each side) over two minutes (.25 µl/min) (Harvard Apparatus, Holliston, MA), through an internal cannula that projected 1mm from the guide cannulae (Hammack et al., 2009). The infusion needle was left in place for a minute following infusion. Animal body weights were determined before and 24 hours after infusions.

2.5.1 Mechanical Sensitivity Testing

Following infusion, rats were placed into the testing chamber and mechanical sensitivity was tested using von Frey Fiber testing at 30 minutes, 2 hours, 24 hours and 48 hours after
infusion. On the day of testing rats were placed in the testing chamber on top of a metal mesh and habituated for 10 minutes before von Frey filament testing. Following habituation, von Frey filament testing occurred. In ascending diameter thickness, each filament was applied to the lateral plantar surface of the hindpaw until bent at 30 degrees for 5-7 seconds. A positive response was defined as a swift withdrawal of the hindpaw. The mechanical threshold was defined as the force of the smallest filament that resulted in 3 out of 5 hindpaw withdrawals to the von Frey hair stimulation. If a negative response occurred the next von Frey hair was tested. Thresholds from both the right and left hindpaws were measured and the average mechanical threshold from the left and right hindpaw was recorded. One animal was excluded due to ceiling effect on baseline.

2.5.2 Thermal Sensitivity Testing

Latency to hindpaw withdrawal to thermal stimuli was measured at 1, 4, 24, and 72 hours after infusion. On the day of testing rats were placed in the testing chamber on the glass platform and habituated for 10 minutes before von Frey filament testing. Rats were placed in the testing chamber and a Hargreave’s apparatus (Plantar Analgesia Meter, IITC Life Science Inc., Woodland Hills, CA) was used to measure withdrawal latency to a thermal stimulus. A focused radiant beam of light (4x6 mm, set to 25% active intensity) was placed on the hindpaw. The point at which the hindpaw was withdrawn or the hindpaw was licked, the heat source was immediately terminated and the reaction time was recorded. For measurements to thermal stimuli, each time point was the average of 3 paw withdrawal latencies from both the left and right hindpaw separated by 5 minute intertrial intervals. The PACAP and vehicle treatment groups exhibited similar average baseline latency scores (PACAP, 11.6±0.7s; vehicle, 11.1±0.6s).
2.6 Analyses of Data

Statistics were calculated using a repeated measures 2-way ANOVA comparing Vehicle to PACAP treatment. The data was analyzed using GraphPad PRISM. Bonferroni’s multiple comparisons tests were used to compare treatment effects at all time points and adjusted P values were calculated.

2.7 Cannula Verification

To verify cannula placements in the CeA, rats were anesthetized and underwent transcardial perfusion with 4% paraformaldehyde. Brains were then removed, fixed in 4% paraformaldehyde, equilibrated in a 30% sucrose solution, embedded in Tissue-Tek OCT compound, frozen, and sectioned on a cryostat at 50µm. The sections were then mounted on slides and stained with a Cresyl Violet solution. Cannula verifications were conducted under a light microscope. Only data from correct CeA cannula placements were included in the results.

3.0 Results

3.1 Histological Verification

Only data from correct CeA cannula placements were included in the analysis (Image 1). One animal was excluded due to incorrect cannula placement.
3.2 Mechanical and Thermal Sensitivity

![Mechanical Sensitivity graph](image)

**Figure 1**: CeA PACAP infusion had no effect on mechanical threshold. B1 and B2 demonstrate baseline values. Mean +/- SEM.

Figure 1 displays the mechanical threshold of hindpaw withdrawal and Figure 2 (see below) displays the latency of hindpaw withdrawal to a thermal stimulus for each treatment over time. Treatments groups were assigned to have matching baseline scores prior to testing, (Mechanical: PACAP: 16.4 g, Vehicle: 14.6 g, Thermal: PACAP: 11.7 s, Vehicle: 11.6 s). Statistics were calculated using a 2-way ANOVA comparing Vehicle to PACAP treatment.

Infusion of PACAP into the CeA had no effect on mechanical threshold. No significant difference in threshold was observed after the infusion of PACAP into the CeA (F(5, 60) = 0.412, p > 0.05). There was a significant main effect of time point (F(5, 60) = 12.28, p < 0.0001), with a gradual increase in mechanical sensitivity over time when compared to the initial baseline. Post hoc analyses revealed a significant difference between B1 and all post infusion time points and a
There was no significant main effect of PACAP treatment ($F(1,12)=0.9726, p>0.05$) and no significant interaction ($F(5,60)=0.412, p>0.05$).

**Figure 2:** CeA PACAP infusion increased thermal sensitivity. B1 and B2 are baseline values. Mean +/- SEM.

Infusion of PACAP into the CeA had a significant effect on paw withdrawal latency to a thermal stimulus. There was a significant main effect of time point ($F(5,60)=3.358, p<0.05$), but no significant main effect of treatment ($F(1,12)=0.9726, p>0.05$). There was a significant interaction between treatment and time point ($F(5,60)=4.021, p<0.05$). Bonferroni corrected post-hoc tests revealed that PACAP significantly reduced withdrawal latency at 1 hour that diminished by 4 hours. Withdrawal responses remained non-significant at 24 hours and 72 hours and had returned to near baseline values. These results suggest that CeA PACAP infusion led to thermal hyperalgesia at one hour after infusion that dissipated by 4 hours, but had no effect on mechanical sensitivity.
4.0 Discussion

CeA PACAP infusion had no effect on mechanical sensitivity, but led to thermal hyperalgesia that dissipated after 4 hours. We discovered a reduction in paw withdrawal latency in response to thermal stimuli at 1 hour among rats that had received CeA PACAP infusion.

The CeA is a brain region of converging pathways involving pain, stress, and emotion and plays an important role in mediating the emotional elements of pain. It modulates both ascending and descending nociceptive signals. The CeLC is innervated by LPBn neurons that form part of the SPA pathway, one of the major pathways that convey nociceptive information to the brain and that is particularly important for modulating the emotional components of pain (Veinante et al., 2013). Previous studies in our laboratory demonstrated that PACAP is present in the CeLC and that a major source of PACAP to the CeLC is the LPBn (Missig et al, 2014). The presence of PACAP within the parabrachioamygdaloid pathway suggests that PACAP may be a critical mediator in emotional aspects of pain. To facilitate the effects of PACAP in the CeLC, our studies demonstrated the CeA PACAP infusions increased noxious stimulus responses in thermal reactivity tests.

CeA PACAP infusion had no effect on mechanical sensitivity, but led to thermal hyperalgesia that began to dissipate after 4 hours. It was hypothesized that infusion of PACAP into the CeA would decrease the mechanical and thermal thresholds, leading to a reduction in paw withdrawal latency in response to thermal and mechanical stimulation. Because of its potential role in mediating the emotional components of pain, we expected that the microinfusion of PACAP into the CeA might potentiate pain responses. This hypothesis was based on the prediction that PACAP released by the PBn is potentiating CeA synapses as part of the PBn-CeA nociceptive pathway. Previous research had shown that other neuropeptides, including calcitonin
gene-related peptide (CGRP) receptor ligands (Han et al., 2010) and mGluRs ligands (Crock et al., 2012), play this role in the amygdala and when injected into the CeA increased mechanical sensitivity.

Although mechanical threshold in PACAP-treated animals appeared lower compared to vehicle controls after 30 minutes, analyses revealed a trend rather than statistical difference. There was no significant difference between PACAP treated animals and controls at any time point. Rather, there was only a main effect of time, with a gradual decrease in mechanical threshold with repeated testing over time. The simplest explanation for this result is animals’ sensitization to the von Frey hairs over time, independent of PACAP.

There was, however, a reduction in paw withdrawal latency in response to thermal stimuli at 1 hour among rats that had received CeA PACAP infusion. Thermal and mechanical pain are transduced by separate fibers and mechanisms, these distinctions may have contributed to the observed differences in the efficacy of PACAP. The PBn demonstrates greater responses to thermal stimuli than to mechanical stimuli (Bernard et al., 1996) and it is possible that the transfer of these signals to the CeLC resulted in smaller PACAP-mediated mechanical responses. The variance in results between mechanical and thermal nociception may also be explained by a difference in the intensity of the stimulus. From the results we can speculate that the von Frey hairs were bothersome rather than painful, which explains why both the PACAP infused rats and controls became more sensitive to mechanical stimulation from 30 minutes to 24 hours. A-Delta nociceptive fibers respond to both dangerously intense mechanical or to mechanothermal stimuli, whereas C fibers respond to thermal, mechanical, and chemical stimuli (Purves et al., 2012). In this case, the von Frey hairs may not have activated nociceptors, but rather may have activated rapidly adapting mechanotransducers (A-Beta fibers). Hargreaves Test, on the other hand,
allowed for intense thermal stimulation of the hindpaw. Therefore, due to either the intensity or modality of the stimulus Hargreaves Test was more likely to activate nociceptors than von Frey testing. If PACAP in the CeA alters pain circuits involving nociceptive fibers and pathways then we would only expect to see a change in thresholds among stimuli that are transmitted via nociceptors.

Furthermore, even if von Frey hairs do activate nociceptors, neuropeptides, including PACAP, require high frequency stimulation to be released. The differential release of small-molecule transmitters and neuropeptides is probably based on the distribution of Ca\(^{2+}\) and vesicles in the presynaptic terminal. Small-molecule transmitters are found in vesicles docked to the presynaptic membrane before Ca\(^{2+}\) entry whereas vesicles containing neuropeptides are further from the presynaptic membrane. At low frequency stimulation, the increase in Ca\(^{2+}\) appears to remain close to the Ca\(^{2+}\) channels limiting release to small-molecules because their vesicles neighbor these channels. Higher levels of stimulation increase the concentration of Ca\(^{2+}\) throughout the presynaptic terminal, leading to the release of neuropeptides (Purves et al., 2012). Given that PACAP is a neuropeptide it is likely that PACAP is only released when nociceptive stimuli are of high intensity.

It is possible that infused PACAP synergizes with endogenous PACAP that is only released in response to high intensity stimulation, such as thermal stimulation. If endogenous PACAP is only released in response to Hargreaves test and not in response to von Frey hair testing and endogenous and infused PACAP synergize, then one would only expect to see a change in hindpaw withdrawal in response to thermal stimulation. This could explain why only thermal and not mechanical stimulation led to a change in hindpaw withdrawal.

In congruence with this idea, Stroth et al. (2013) argued that PACAP is the main
neurotransmitter during periods of high firing rates. In their study, they demonstrated that catecholamine (CA) secretion evoked by direct high-frequency stimulation of the splanchnic nerve is abated from male PACAP-deficient mice and found that PACAP is both necessary and sufficient for CA secretion ex vivo during stimulation protocols that mimic stress. This may explain why thermal stimulation, but not mechanical stimulation led to a shift in latency of hindpaw withdrawal. It is possible that Hargreaves test reached the high frequency necessary for PACAP to become the main neurotransmitter in the implicated SPA pathway whereas von Frey testing did not. Therefore, a significant decrease in hindpaw withdrawal latency would only be expected in response to thermal stimulation.

Afferents to the CeA do not only release PACAP, but also contain glutamate and contain other neuropeptides including CGRP (Missig et al., 2014). Therefore, glutamate may be released at low levels of stimulation, while PACAP may only be released alongside glutamate in the presence of a very salient stimulation. PACAP, then, would augment the effects of glutamate. In concordance with this idea, Cho et al. (2012) demonstrated that PACAP increases synaptic excitability in the CeA. Specifically, they found that PACAP augmented glutamatergic input in the CeA, leading to an increase in excitatory postsynaptic potentials (EPSPs). Enhancement of synaptic transmission by PACAP would explain PACAP’s ability to alter pain processing in the CeA.

PACAP receptors may also play a role in modulation of emotional aspects of pain in the CeA. Following our research, Missig et al. (2014) found that the thermal and mechanical sensitivity responses were recapitulated with the PAC1 receptor-specific agonist maxadilan. These results implicated specific activation of the PAC1 receptor in these mechanical and thermal responses.
The signal transduction of PACAP is important to understanding the effects that PACAP has on the emotional aspects of pain. PACAP can be coupled to multiple G-protein systems (as reviewed by Hammack & May, 2014). One of the downstream mediators of central pain processing is phosphorylated extracellular signal-regulated kinase (pERK) (Polgar et al., 2007). ERK phosphorylation is observed in the CeA in acid induced muscle pain (Cheng et al., 2011) and after interplantar formalin (Carrasquillo & Gereau, 2007). ERK has been demonstrated to be a downstream molecule for PACAP (May et al., 2014) and our laboratory recently demonstrated that pERK increases as a result of PACAP stimulation (Missig et al., unpublished). Furthermore, Missig and colleagues (unpublished) recently demonstrated that inhibition of ERK blocked the induction of thermal sensitivity by PACAP. PACAP appears to be activating plasticity pathways that involve ERK.

Carrasquillo and Gereau (2007) demonstrated that activation of ERK in the amygdala was both necessary for and sufficient to provoke long-lasting peripheral tactile hypersensitivity. In contrast to our results, inhibiting ERK in the CeA decreased mechanical, but not thermal hypersensitivity. Similarly, the direct pharmacological activation of ERK induced mechanical, but not thermal hypersensitivity, in the absence of peripheral inflammation. These results demonstrated that molecular pathways in the amygdala might modulate thermal and mechanical hypersensitivity in distinct ways.

One explanation for these conflicting results is that the exact neurons being activated in our study and the Carasquillo and Gereau study may be distinct. Our study only activated a subset of neurons that contain PACAP receptors (mostly receiving input from the PBn), which may have resulted in distinct hypersensitivities. Also, there may be different pathways activated by PACAP in addition to ERK that affect sensitivity. PACAP signals through $G_s/cAMP$ and
Gq/phospholipase C (PLC) pathways, which have a variety of downstream targets including ERK (as reviewed by Hammack & May, 2014). These other downstream targets and their interactions may also be important in generating a specific sensitivity response. Similarly, the mechanism by which ERK is activated could be important. Unique to PACAP/PAC1 receptor signaling, the PAC1 receptor is able to signal through internalization from the cell membrane into the cytosol, forming a signaling endosome complex that activates ERK (May et al., 2014; Merriam et al., 2013). This unique receptor signaling mechanism of PACAP/PAC1 could lead to distinct ERK activation.

Although this study found no change in mechanical sensitivity, the possibility still exists that CeA PACAP infusion results in mechanical hyperalgesia to stronger stimuli. To evaluate this, additional behavioral tests should be performed to measure responses to more intense mechanical stimuli. One possibility is a Randall-Selitto test, a test that applies increasing amounts of pressure to a rat’s hindpaw. Another possibility is that PACAP in the amygdala might selectively alter sensitivity to thermal and not mechanical stimuli. This could be examined by using other forms of mechanical and thermal stimulation to see if PACAP effects are specific to modality.

Pain is a universal experience. Acute and chronic pain affect large numbers of individuals around the world and cost economies billions of dollars. For many patients, treatment of pain is inadequate; over 1.5 billion people worldwide report suffering from chronic pain (Global Industry Analysts, Inc., 2011). The first step in addressing the worldwide burden of pain is discovering the underlying neurological mechanisms that lead to the perception of pain so that appropriate treatments can be developed. Understanding the role of PACAP in the modulation of pain will further our comprehension of pain as a whole and the intersection between pain and
other psychopathologies, which in turn will allow us to identify new targets for the treatment of pain.
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Supplemental Image 1: Nociceptive Fiber Types  
(Kandel et al., 2013)

Supplemental Image 2: Rexed's Laminae and Projections  
(Kandel et al., 2013)

Supplemental Image 3: Spinothalamic Tract  
(Purves et al., 2012)
Supplemental Image 4: Spinoparabrachioamygdaloid Tract (Almeida et al., 2004)