

NEUROCHEMICAL STUDIES OF REWARD FROM PAIN RELIEF

by

Diana S. Meske

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Diana S. Meske, titled Neurochemical Studies of Reward from Pain Relief and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

\_\_\_\_\_  
Edward French Date: (Enter Date)

\_\_\_\_\_  
Frank Porreca Date: (Enter Date)

\_\_\_\_\_  
Edita Navratilova Date: (Enter Date)

\_\_\_\_\_  
Bonnie La Fleur Date: (Enter Date)

\_\_\_\_\_  
Greg Dussor Date: (Enter Date)

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

\_\_\_\_\_  
Dissertation Director: Edward French Date: (Enter Date)

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Signed: Diana S. Meske

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## DEDICATION

I dedicate this dissertation to my parents Jean and Robert Meske and sister Shannon. Without your love and support I would not be here today.

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## Abstract

Chronic pain has been estimated to impact the economy of the United States by an annual cost of \$635 billion per year and to affect approximately 100 million Americans (1). Pain is the primary reason patients seek medical attention yet physicians have few options for therapies and there remains a vast unmet medical need for effective and safe analgesics. Most of the drugs clinically available today either have limited efficacy or a variety of unwanted side effects. Discovery of novel therapeutics has been challenging with scientists struggling to find ways to better translate research from the bench-top to the bedside. One impediment in this process has been differences in preclinical and clinical assessment of pain. Preclinical models have historically relied heavily on evoked or reflexive endpoints in non-verbal animals while clinical measures of pain have the advantage of assessing changes in self-reported pain ratings. It is likely, and data from the studies reported in this dissertation show, that mechanisms that underlie threshold responses to evoked stimuli differ from those mediating affective (i.e., aversive) qualities of pain. A further confound is that many effective analgesics are narcotics that carry risk of addiction. Fear of addiction and possibly misuse for chronic treatment of pain may result in undertreatment in many patients. The most clinically relevant question in the management of pain is whether or not a treatment improves the patient's quality of life. Here, we demonstrate that the aversiveness of ongoing pain can be assessed using motivated behavior (conditioned place preference; CPP) and neurochemical

output (*in vivo* NAc microdialysis). Additionally, we assessed the mechanistic effects of three clinically relevant analgesics. Our results show that: (1) pain relief is rewarding and activates reward circuitry that differs from circuits mediating addictive qualities of opiates, and (2) that drugs that mimic the consequences of engagement of descending inhibitory systems act by increasing spinal norepinephrine (NE) levels. These studies provide much needed information that helps build a platform from which more effective analgesics can be discovered and characterized in the preclinical setting and that may help in the introduction of new therapies for patients.

## Chapter 1: Neurobiology of Pain

Pain is a complex, subjective, multidimensional experience that is critical for survival of all species. The International Association for the Study of Pain defines pain as an “unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Melzack and Casey described the human experience of pain in terms of three proposed dimensions: sensory-discriminative (i.e., sense of the intensity, location, quality and duration), affective-motivational (i.e., pain unpleasantness and the urge to escape) and cognitive-evaluative (i.e., cognitions such as appraisal, cultural value, distraction and hypnotic suggestion) (2). This chapter will initially focus on the sensory aspects of pain, descending inhibitory mechanisms and end with integration of these processes with affective features of pain to elicit the human pain experience.

Pain can be broadly categorized as nociceptive (i.e., early-warning and physiologically protective), inflammatory (i.e., adaptive and protective), and maladaptive or dysfunctional (i.e., not protective) (3). Nociceptive pain functions as a early-warning system to help prevent or reduce tissue damage and occurs when a stimuli of high intensity (i.e., noxious; sufficient to cause tissue damage) activates nociceptors, a specific class of primary afferent nerve fibers (3). In the periphery, nociceptors are pseudounipolar afferent fibers that are either thinly myelinated or non-myelinated that are termed A-delta and C

fibers respectively (4). Larger diameter myelinated fibers termed A-alpha or A-beta are primarily responsible for proprioception and touch, respectively (4-6). Small diameter, myelinated A-delta fibers carry the pain signal at a greater conduction speed (5-40 m/s versus 0.5-2 m/s) than the even smaller diameter, unmyelinated C fibers (5). When the pain signal reaches the spinal cord it synapses at second order neurons in the spinal dorsal horn that generally cross to the contralateral side of the spinal cord and project rostrally to the brain via multiple ascending tracts including the well-studied spinothalamic tract (4). Additionally, these primary afferent nociceptive fibers are preferentially activated by specific stimulus modalities such as thermal, mechanical (A-delta and C) or chemical (C only) stimuli (4, 6).

Inflammatory pain is also protective but is adaptive and occurs as a result of an acute inflammatory process that involves immune system mediators.

Sensitization is an adaptive process in which nociceptors, and post-synaptic ascending pain transmission cells, adapt in the setting of tissue injury to amplify the pain signal; this process can occur at various levels of nociceptive pathways (i.e., peripheral, central) (3, 7, 8). The result of peripheral nociceptor sensitization and central sensitization is hyperalgesia (i.e., when a noxious stimulus is enhanced) and/or allodynia (i.e., when an innocuous stimulus becomes noxious) (7, 8) and this net effect is critical to inflammatory pain as it promotes healing in the sensitized area by dissuading movement or contact at

the injury site. Peripheral and central sensitization will be discussed in greater detail in the next section of this chapter.

Lastly, pain that becomes chronic and ceases to be protective is referred to as “maladaptive” (i.e., chronic neuropathic pain) and peripheral and central sensitization are crucial in this transition, as will be discussed in the next section of this chapter. Pain that occurs in the absence of tissue injury is “dysfunctional” (i.e., fibromyalgia, irritable bowel syndrome, etc.). The cause of these abnormal pain conditions is largely unknown and warrants further study (3).

### **Peripheral and central sensitization**

As mentioned in the previous section, nociceptive pain is experienced as the ‘ouch’ pain associated with injuries such as touching a hot stove, needle prick, etc. (3). In contrast, clinical pain usually presents an ongoing or “spontaneous” pains (3). Clinical pain can occur due to damage to the nervous system (neuropathic pain), tissue injury and inflammation (inflammatory pain) or alterations in the normal function of the nervous system (functional pain) and each of these are associated with hypersensitivity to peripheral stimuli (3) referred to as allodynia and hyperalgesia (3, 8). Allodynia describes the phenomenon that occurs when thresholds are lowered so that normally non-noxious stimuli become noxious (3, 9). Similarly, hyperalgesia is an enhanced responsiveness to a noxious stimuli that results in exaggerated or prolonged pain (3, 9). Pain hypersensitivity plays a functional role to promote the healing

of damaged tissue but when pain hypersensitivity persists after the injury is healed this manifestation represents a pathological change in the nervous system (10). Peripheral and central sensitization are two mechanisms that are known to contribute to pain hypersensitivity and the concept of sensitization refers to an increase in the excitability of neurons in both the peripheral and central nervous systems making them more responsive and excitable (3, 7, 8).

Peripheral sensitization occurs when the peripheral terminals of nociceptors (i.e., high-threshold peripheral sensory neurons) demonstrate an increased responsiveness and reduction in threshold to activation (10). Two major ways in which peripheral sensitization occurs is by (i) directly activating nociceptors via release of inflammatory mediators arising from invading immune cells or damaged resident cells; such sensitizing substances include prostaglandins, bradykinin, ATP, serotonin or changes in pH and/or (ii) by changing response thresholds of ion channels such as temperature-sensitive transient receptor potential channels, ATP-gated channels, acid-sensitive ion channels and voltage-gated sodium, potassium, calcium or HCN channels via intracellular signaling molecules including, for example, protein kinases that can phosphorylate these channels (3, 11, 12). A good example of this net effect is the change in heat sensitivity experienced following a sunburn; a warm water shower can feel burning hot on sunburned areas due to peripheral sensitization occurring at the site of the sunburn.

Central sensitization was first described by Prof. Clifford Woolf and represents the phenomenon by which a stimulus produces an increased response due to an amplification of signaling in the central nervous system (CNS) (7). Similar to peripheral sensitization, the net effect of this process is amplification that manifests as allodynia and/or hyperalgesia (3). Sensitization can be described as a non-associative learning process in which a repeated stimulus manifests as a progressive amplification of a response, in nociceptive neurons in the spinal dorsal horn as a result of peripheral tissue damage (13, 14). In other words, nociceptive neurons enter a state of facilitation, potentiation, augmentation or amplification due to recruitment of previously subthreshold synaptic inputs to these neurons generating an increased or augmented action potential output (15). Central sensitization represents the fundamental contribution of the CNS to pain hypersensitivity associated with acute and chronic pain conditions (8). Importantly, changes to the properties of these neurons in the CNS provokes an uncoupling so that they no longer function as they would to acute nociceptive pain stimuli (i.e., sensing duration, quality or location) but rather create a scenario in which normal sensory inputs (i.e., those that usually evoke innocuous sensations) produce pain hypersensitivity (15).

The early phase of central sensitization is generally characterized by changes in synaptic connections within the spinal cord by release of a host of signal molecules from central terminals of nociceptors (3). These signaling molecules consist of excitatory amino acids, glutamate, neuropeptides (e.g., substance P

and calcitonin gene-related peptide [CGRP]) and synaptic modulators such as brain-derived neurotrophic factor (BDNF) (10). Once released these signaling molecules act at specific receptors on neurons of the spinal dorsal horn resulting in activation of intracellular signaling pathways which culminate in phosphorylation of membrane receptors, like NMDA or AMPA to enhance glutamatergic tone, and channels (8, 10). The final result of these processes are post-translational changes that result in hyperexcitability of neurons by a lowering of thresholds and opening characteristics of these channels (10). Finally, there is a late-phase transcription dependent phase in which changes in protein expression such as dynorphin or Cox-2 lead to persistent hyperexcitability of the CNS (13, 15). These changes in both central and peripheral responsiveness play a major role in the production of clinical abnormal or dysfunctional pain (3).

### **Ascending and descending pain pathways**

In the absence of injury, nociceptors respond only to noxious, or high intensity, stimuli. The specificity of these fibers to respond to certain stimuli (i.e., thermal, mechanical or chemical) is dependent on expression of specific transducers, generally ion channels, on their peripheral terminals. Nociceptive sensory fibers can be classified as A-delta or C fibers. A-delta fibers are medium diameter, myelinated fibers that send the pain signal relatively quickly to the spinal cord; these fibers mediate the initial, sharp pain we feel (5, 16). In contrast, C fibers are slow conducting, small diameter, unmyelinated fibers that are associated

with the dull, burning, aching pain of an injury (5, 16). These first order neurons are pseudounipolar, meaning that they have a bidirectional axonal branch, with cell bodies residing in the dorsal root ganglion (DRG) for afferents that innervate the body, and in the trigeminal ganglion for afferents originating in the face and head (4). A-delta and C fibers synapse on second order neurons of the dorsal horns that cross the cord via the anterior white commissure and ascend to the brain via the spinothalamic, and other, tracts including the spinoparabrachial, spinoreticulohalamic, and in some instances spinocerebellar, for processing (4, 16). The spinal dorsal horn is organized into distinct laminae based on anatomical and electrophysiological properties (4). Neurons contained within lamina I are generally responsive to noxious stimuli (A-delta and C fibers) while those within lamina III and IV are primarily responsive to nonnoxious stimuli (A-beta) (4). Finally neurons within laminae V are referred to as wide dynamic range neurons due to their ability to respond to a broad range of nonnoxious and noxious stimuli intensities via direct input from A-beta and A-delta fibers and indirect input from C fibers (4). The evolutionary significance of these ascending nociceptive pathways is that they allow the brain to locate and identify the source of the potentially tissue damaging stimulus. A fundamental feature of nociceptive activation is that this results in a sensation that is emotionally unpleasant and that promotes protective avoidance behavior.

In 1965 Melzack and Wall published their gate control theory of pain that proposed a gating mechanism within the dorsal horn of the spinal cord that

could prevent pain signals from reaching the brain (17). More specifically, they proposed a gate control system comprised of large- and small-diameter fibers that projected to the substantia gelatinosa (SG) and a central transmission cell (T cells). They suggested that the SG's inhibitory effect on afferent fiber terminals was enhanced by large-diameter fibers and diminished by activity of small-diameter fibers. Further, that a central control trigger existed between the large-fiber system and the central control mechanism, which in turn, projected back to the gate control system. Finally, they suggested that the T cells projected onto entry cells of an "action system", so that these mechanism functioned together to act as an endogenous control mechanism of pain (17). Presently, this theory has been shown to be oversimplified as well as having flaws in the now understood spinal neural networks. However, this theory was revolutionary and marked the beginning of the era of modern pain research in which pain was conceived of something that did not result from a simple unprocessed input to the brain. Pain was more than a primitive alarm system and has led to the current understanding of processing of nociceptive signals at the level of the spinal cord and in other areas of the brain as well as resulting from descending modulatory controls that are now known to arise in the brainstem (17).

Nociceptive information (i.e., pain signal) from the skin, viscera or other organs is subject to a diversity of mechanisms that have the ability to enhance or inhibit this signal prior to reaching higher centers in the brain (18). This network of descending projections from higher cerebral structures of the brain to the dorsal horn play a complex role in pathways that suppress (descending inhibition) or

potentiate (descending facilitation) pain transmission. Analgesics such as opiates, serotonin and/or norepinephrine reuptake inhibitors and adrenergic ( $\alpha$ -2) agonists do, or are, implicated to exploit descending inhibitory mechanisms to provide analgesia (18). Conversely, it is likely that many abnormal pain conditions (i.e., fibromyalgia, irritable bowel syndrome, etc.) result from maladaptation of descending pain control mechanisms: either decreased inhibition or increased facilitation (19, 20).

Descending pain control mechanisms originating in midbrain and medullary sites provides bidirectional control over nociceptive information. Specifically, the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) act as relay points through which cortical and subcortical sites can influence nociceptive information. Activation of the PAG by either electrical stimulation or microinjection of opioids elicits powerful analgesia in both animals (21, 22) and humans (23-25) that is naloxone-reversible (25-29). This suggests, and is widely accepted, that the PAG is a source of opioidergic descending projections that are highly involved in descending pain inhibitory mechanisms (27, 30, 31). Additionally, the PAG receives ascending nociceptive inputs from the dorsal horn via the parabrachial nuclei (32), reciprocal connections to the amygdala (33) and RVM (34). Human imaging studies also suggest that connections between the rostral anterior cingulate cortex (rACC) and the PAG play an important role in descending inhibition; these connections will be discussed in greater detail later in this chapter (35, 36).

As important as the PAG, the RVM is the final common relay in descending modulation of nociceptive inputs and together these two areas form the key nexus of pain modulation (34). The RVM contains 3 categories of neurons: on-cells, off-cells and neutral cells each of which are characterized by their responses to nociceptive input (37). Immediately before nociceptive input off-cells show a transitory decrease in firing rate while simultaneously on-cells show a burst of activity, neutral cells show no response to input of this nature (37, 38). Pharmacological activation of off-cells, usually through disinhibition, produces antinociception indicating that these cells play an important role in descending pain mechanisms (38). In addition to reciprocal connections to the PAG, the RVM also receives inputs from the thalamus, parabrachial region and locus coeruleus and has projections to the spinal dorsal horns and trigeminal nucleus caudalis (34, 39, 40). Together, the PAG/RVM system provides the basic neuronal system for negative and positive pain modulation and it is likely that maladaptation to this system underlies many abnormal pain conditions as is evident by the plethora of clinically effective pharmacological agents that are effective due to exploitation of this system. Some of these agents and the crucial role for norepinephrine (NE) in descending pain control will be discussed in greater detail in Chapter 2.

### **Brain processing of pain**

Modulation of pain occurs in a top-down fashion in which descending pain pathways can act to inhibit or facilitate afferent nociceptive traffic, shaping the

overall experience of pain. Disruption of these descending pain modulatory circuits can lead to amplification of facilitatory pathways sometimes leading to chronic pain (41). This ability to modulate (i.e., enhance or inhibit) the experience of pain results in a nonlinear relationship between the pain experience and activation of nociceptors (42, 43). The evolutionary importance of pain is to avoid tissue injury, further, the role of negative (pain) and positive (relief) affective states is to elicit motivation (i.e., escape/avoidance or approach behaviors) and facilitate learning of how to predict danger or reward (44). The ability of an organism to analyze competing or conflicting motivations (i.e., pain versus danger from a predator) is encoded in higher brain regions (45). The basal ganglia (including the caudate-putamen, subthalamic nucleus, globus pallidus and nucleus accumbens) and other limbic structures such as the hypothalamus, amygdala and the hippocampus (6) play a crucial role in evaluating competing motivations and these regions are highly conserved across vertebrate evolution (45). In the case of an abnormal state, such as chronic pain, suppression of emotion and natural rewards can lead to diminished quality of life in patients (46).

It has only been in recent years that neuroimaging technology has advanced to the point that researchers have been able to start unraveling how pain is encoded in the human brain, though to date this remains unknown. During acute pain the regions most commonly activated include the thalamus, insula, somatosensory cortices (S1, S2; primary and secondary) and the anterior

cingulate cortex (47). Each of these regions receive direct afferent nociceptive information from the spinal cord so it is no surprise that these regions are activated by noxious stimuli in the periphery (48). Additionally, regions associated with reward/motivation, both cortical and subcortical, are activated during anticipation of pain or expectation of relief (i.e., placebo analgesia) (49). In the context of pain, these corticolimbic structures associated with reward/motivation are hypothesized to integrate internal and external signals facilitating valuation, action selection and learning (50).

Activation of mesolimbic reward pathways culminating in release of dopamine (DA) in the nucleus accumbens (NAc) is associated with natural reward (i.e., sex, chocolate, etc.) and reward of drugs of abuse (6). The mesolimbic reward-valuation pathway extends from the ventral tegmental area (VTA), a major source of many DA pathways in the brain, via the medial forebrain bundle to the NAc (51). DA acts on D1 and D2 receptors to respectively increase or inhibit the production of cAMP which modulates neuronal function. The net consequence of DA neurotransmission is relevant to motivation, alertness and learning (51, 52). The removal of aversive states (e.g., thirst, hunger, etc.) is also rewarding and relief of pain falls into this category. The reward of pain relief has been observed in human studies (53, 54) but only recently in animal studies, including data presented in later chapters (55-57). Traditional pathways through which natural reward and drugs of abuse act to promote DA neurotransmission in the striatum, more specifically the NAc, involve activation of dopaminergic neurons

with cell bodies that reside in the VTA and have projections to the NAc. As previously mentioned, these circuits play a fundamental role in survival (i.e., reward, motivation and learning), the experience of pain in all vertebrate animals and remain highly conserved across species (50) making these mechanisms likely targets for preclinical models that better assess ongoing pain (58). In other words, relief of ongoing pain in humans produces a positive affective state (i.e., reward) and this similar state can be assessed in rodents based on their motivated or learned behavior (59).

Conditioned place preference (CPP) is a form of Pavlovian conditioning in which learning can be measured. Historically, the CPP paradigm has been used to assess the rewarding qualities of addictive drugs. More recently our laboratory, and others, have used it to assess the rewarding effects of pain relief in rodents (60, 61). Additionally, when CPP is accompanied with direct measurement of activity of brain reward/motivational circuit (i.e, *in vivo* microdialysis to measure NAc DA efflux) this technique allows assessment of ongoing pain that may have improved translation to the human experience of pain (55).

In addition to mesolimbic areas such as the VTA and NAc, reward-related information is also encoded in areas of the frontal lobe; specifically the anterior cingulate cortex (ACC), lateral orbitofrontal cortex (OFC), and ventromedial and anterior prefrontal cortices (PFC) (62, 63). This is not surprising as these regions, perhaps most importantly the ACC, receive direct dopaminergic inputs

from mesolimbic sites (64). The ACC is of particular interest in pain as it has been most frequently linked to the experience of pain in that it appears to be highly involved in the emotional reaction to pain (65). More specifically, the ACC plays a critical role in encoding learning both appetitive and aversive outcomes, reward value of a chosen action and therefore is important in future decision making (62, 66). Together these regions (mesolimbic and cortical) integrate signals that enable an organism to predict future outcomes based on past and present experience and to select an optimal action in the moment (67). These circuits are greatly influenced by pain and pain relief, thus, pain and the relief (reward) of pain can greatly influence decision making (68).

### **The anterior cingulate cortex**

The ACC comprises the frontal part of the cingulate cortex (Brodmann areas 24, 32 and 33) and resembles a “collar” that surrounds a portion of the corpus callosum. It has been indicated to play an important role in autonomic function but is also involved in rational cognitive functions such as reward anticipation, decision-making, empathy, impulse control and emotion (69-71). The ACC can be anatomically divided into areas associated with emotional (ventral) or cognitive (dorsal) functions (72). The dorsal region of the ACC acts as a central processing center for both top-down (i.e., signals originating in the brain) and bottom-up (i.e., signals originating in the periphery) stimuli, assigning appropriate control to other brain regions (73). This is not surprising as the dorsal ACC is connected to both the prefrontal and parietal cortex, the motor

system and frontal eye fields (73). In contrast, the ventral area of the ACC is involved in assessing emotion, motivational information as well as learning and decision-making. It is likely that these functions arise from connections between the ACC and the amygdala, NAc, hypothalamus and anterior insula (74).

The ACC also plays a crucial role in registering pain affect that is supported by numerous human imaging studies as well as animal studies (48, 75). Electrophysiological recordings in rats and rabbits identified nociceptive neurons in the ACC with properties, large and bilateral receptive fields, consistent with a role in affective and/or motivational processing (76-78). Additionally, a similar electrophysiological study in awake and freely moving rats found that neuronal responses in the ACC were suggestive of a role in associative processes related to the affective-motivational component of pain (79). Interestingly, this same study also found that neurons in the ACC were not primarily involved in precise pain discrimination (79). Along these lines, microinjection of excitatory amino acids in the rostral ACC (rACC) of rats has no effect on sensory threshold but does produce conditioned place aversion (CPA) (75). Further, lesion of the rACC in rats with experimental neuropathic pain is sufficient to abolish pain-induced aversive behavior yet has no effect on sensory thresholds (80, 81). Human studies also demonstrate a similar role for the ACC. One such study shows that hypnotic suggestion during a constant painful stimulus can alter the unpleasantness of the pain and simultaneously elicits activation of the ACC (82). Collectively, these data suggest that the ACC plays a

crucial role in the affective-motivational aspects of pain but likely has little or no role in sensory-discriminative aspects of pain.

### **Opioid and dopamine neurosignaling and pain**

As previously discussed, top-down regulation of nociceptive traffic is facilitated in higher brain regions via projections from prefrontal regions, including the ACC, to the PAG and the RVM which results in modulation (inhibit or facilitate) of nociceptive traffic at the level of the spinal cord in animal models (41, 83). This intrinsic control appears to be consistent in humans; Blood-oxygen-level dependent functional magnetic resonance imaging (BOLD-fMRI) has demonstrated that expectation of pain relief (i.e., placebo analgesia) reduces nociceptive signal at the level of the spinal dorsal horn (84). Further, anticipation of pain relief causes increased neuronal activity in cortical regions (ACC, PFC, OFC) and in the PAG suggesting that descending modulatory mechanisms are being engaged by higher brain regions (49, 85). Emotional control of pain has also been demonstrated to engage descending modulatory systems via prefrontal cortices, the ACC and PAG, to reduce pain in humans (43, 86).

Central endogenous opioid signaling is highly involved in supraspinal and spinal control of pain. In this section I will discuss the placebo and nocebo effects in pain modulation. Placebo is a phenomenon in which medically ineffective treatments (e.g., sugar pill, saline injection, etc.) produce analgesia in patients

predominately through expectation. In contrast, the nocebo effect involves negative expectation and increases pain. PET studies using opioid radiotracers in humans demonstrated activation of  $\mu$ -opioid receptor mediated neurotransmission in higher-order and sub-cortical regions (including the ACC, PFC insular cortex and NAc) response to placebo analgesia (87). Reduction in sensory and affective qualities and lower ratings of pain intensity paralleled activation of these brain areas (87). Additional PET imaging studies have identified increased opioid neurotransmission in similar regions and also increased DA neurotransmission in the ventral basal ganglia, most interestingly the NAc (88, 89). Changes in DA and opioid neurotransmission were associated with anticipation of pain relief as well as reductions in continuous pain scores (89). Specifically, the greater the placebo effect in the patient the greater the observed DA and opioid activity was in the NAc while nocebo responses were associated with deactivation of DA and opioid signaling (89). These data combined indicate that DA and opioid signaling are functionally interconnected in brain areas involved in pain modulation, reward responses and motivated behavior.

### **Opioid mechanism of action**

Opiates act at a family of G-protein coupled receptors (GPCRs) to exert their effects. There are three known opiate receptors mu ( $\mu$ ), delta ( $\delta$ ), kappa ( $\kappa$ ) and a structurally related but less understood receptor termed ORL-1 or nociceptin receptor (90). Activation of the  $\mu$ -opiate receptor provides the most efficacious

analgesia but is also associated with significant side-effects. Exogenous drugs activating the  $\mu$ -opioid receptor have high abuse liability (i.e., heroin, morphine, etc.) (90, 91).  $\mu$ -opioid receptors can be found throughout the central and peripheral nervous system as well as the gastrointestinal (GI) tract.  $\mu$ -opioid receptor agonists can act in the periphery to diminish the nociceptive signal being projected for central processing, they also have similar action in the spinal cord. However, the effects of opiates on pain affect (i.e., the aversiveness of pain or pain unpleasantness) are often said to provide the greatest degree of analgesia to the patient, and it is likely that this occurs at doses lower than those required for antinociceptive effects (92, 93). Opiates also have a long list of side effects that range from unpleasant (constipation, sedation, depression, euphoria, etc.) to deadly (respiratory depression), the latter accounts for the vast majority of deaths associated with opiate use.

The  $\mu$ -opiate receptor is located both pre- and post-synaptically throughout the central and peripheral nervous system. These receptors are dispersed through a variety of brain regions including the striatum, thalamus, cortex, locus coeruleus, hippocampus and, importantly, the ventral tegmental area (VTA) and NAc.  $\mu$ -opioid agonists, both exogenous (i.e., an agent introduced from outside the organism) and endogenous (i.e., produced internally by the organism), can mediate changes in neuronal excitability via inhibition of presynaptic release of GABA as well as many other neurotransmitters. GABA is the inhibitory neurotransmitter of the CNS. It is well understood that the addictive properties

of  $\mu$ -opioid agonists occur via a disinhibition of GABAergic neurons located in the VTA. These GABAergic neurons further project onto dopaminergic neurons with projections to the NAc resulting in DA efflux. The role DA in the NAc plays in addiction and pain will be discussed in greater detail in the following section.

The distribution of the  $\mu$ -opioid receptor is found in circuits that are closely associated with pain modulation. This receptor is expressed throughout the ascending pain transmission system, in nociceptors and at both pre- and post-synaptic sites in the spinal dorsal horn, the brain stem, thalamus and cortex (94, 95). Additionally, this receptor can be found in many structures that make up the descending inhibitory system, such as the periaqueductal grey (PAG), thalamus and locus coeruleus (95-97). Opioid receptors at these locations function to directly inhibit transmission of nociceptive information via ascending tracts or indirectly by activating descending inhibitory mechanism (these topics are discussed in greater detail in this chapter and chapter 3) (95). At the cellular level, opioids produce analgesia by decreasing presynaptic transmitter release, hyperpolarization of postsynaptic elements or via disinhibition (95)

## **Addiction**

Compulsive, out-of-control drug use despite serious negative consequences is the defining characteristic of addiction (98). These uncontrollable compulsive actions are the hardest thing for clinicians, addicts and family members of addicts to understand. The most problematic issue is that these compulsive

urges often never cease, even in abstinent addicts, meaning that there is a high risk of relapse long after withdrawal symptoms have abated and often for the lifetime of the addict (98-101). These facts justifiably classify addiction as a chronic medical illness with serious societal concerns, including an estimated economic cost to society of \$559 billion a year (including illegal drugs, alcohol and tobacco) (102, 103). A 33-year follow-up study was conducted with 581 male patients admitted between 1962 and 1964 to a California-state drug treatment program for heroin use. This study found that at the time of follow-up 284 patients had died, most of these deaths reported to be from drug overdose or violence (104). Of the remaining 241 subjects that could be located 40.5% admitted to heroin use in the past year, 20.7% had a positive urine test for heroin during the follow-up interview and 23.5% were either incarcerated or refused to provide a urine sample (104). Like many chronic neurological diseases, treatment for addiction is insufficient at this point.

### **Addictive brain reward pathways**

The mesolimbic DA system is generally referred to as the “reward circuit” and in the case of addictive drugs it has been well established that dopaminergic projections from cell bodies in the ventral tegmental area (VTA) to the NAc are critical (99). The VTA expresses an abundance of  $\mu$ -opioid receptors, including on inhibitory GABAergic neurons. When activated there is a disinhibition of the DA neurons that project to the NAc with a net effect of increased release of DA into the NAc (6). For many years it was widely accepted that the role for DA in

the NAc or prefrontal cortex was to directly mediate the hedonic effect of drugs of abuse (i.e., the experience of pleasure from these drugs) (98). However, there is a growing body of evidence that suggest that the role of DA has a much more complex role than simply mediating pleasure (99). For instance, it has been demonstrated that increased firing of midbrain dopamine neurons can occur in rats in response to noxious stimuli, such as tail pinch (105). Additionally, animals trained to understand cues that predict reward results in DA neuron firing and subsequent release of DA into the NAc during reward anticipation, prior to consumption of the actual reward (106-109). This result is consistent with the role of DA in learning and not as a mediator of hedonic experience (106-109). Further, when dopamine is depleted in the NAc and dorsal striatum in rats with the neurotoxin 6-hydroxydopamine the animals still show normal hedonic responses to sucrose and even have the ability to learn about new hedonic stimuli (110). Collectively, these data suggest a role for DA in learning the motivational significance of a stimulus. However, the exact circuitry and neurotransmitters involved in the hedonic experience of drugs of abuse will require further investigation.

Dopaminergic cell bodies in the VTA also have projections to other limbic structures besides the NAc including the amygdala, ventral pallidum, and the hippocampus, together these connections make up the mesolimbic pathways (111). Additionally, there is the mesocortical pathway which includes dopaminergic projections from the VTA to cortical areas such as the PFC, OFC

and ACC (111). Collectively, these circuits make up the corticostriatolimbic system which all act in parallel but have somewhat different roles in addiction (112). As mentioned above, the NAc and also the ventral pallidum are involved in the reinforcing effects of drugs of abuse (113). The hippocampus and amygdala play an important role in conditioned learning (114, 115) while the PFC, OFC and ACC regulate emotional responses, cognitive control and executive function (116). Repeated drug exposure has been demonstrated to result in cellular adaptations in glutamatergic pathways between the PFC and NAc, these changes likely contribute to persistent addictive behaviors including diminished cognitive control and hyper-responsiveness to drug-associated stimuli (117).

In general, all drugs of abuse enhance extracellular DA levels in the NAc (118, 119) to a greater extent than natural rewards and this effect of drugs of abuse does not undergo habituation as is the case with natural rewards (120). Further implicating the mesolimbic DA system in the reinforcing effects of drugs of abuse is the finding that lesions of the NAc, VTA or ventral pallidum is sufficient to attenuate self-administration of cocaine and heroin in rodents (121-123). Systemic administration of DA synthesis inhibitors (124, 125) and DA antagonists (126-128) have also been shown to reduce self-administration of a variety of drugs including opiates, cocaine, amphetamine and ethanol. Further supporting the role of DA in reinforcement, *in vivo* microdialysis experiments in the NAc shell have successfully demonstrated increases in extracellular DA in response to self-administration of morphine (129), heroin (130, 131), ethanol

(132-134), cocaine (129, 135, 136), amphetamines (129, 137), cannabinoids (138, 139) and nicotine (139, 140). Additionally, microinjection of a variety of drugs of abuse into various brain regions in the mesocorticolimbic system results in rewarding effects in animals, while injection into other brain sites does not have these effects (141, 142). Collectively, these data, and others, demonstrate the crucial role the mesocorticolimbic DA system plays in the reinforcing properties of drugs of abuse, and these effects are likely to initiate the addiction cycle. Certainly, GABA, glutamate, endogenous opioid peptide and the endocannabinoid systems also play an important role in the acute reinforcing effects of drugs, but it is likely to a lesser degree than DA (111). In the case of chronic addiction, it is likely that DA plays less of a role in the unconditioned reinforcing effects of drugs (i.e., liking) and has more to do with the persistence of drug-seeking behaviors by enhancing incentive salience to reward-associated stimuli (i.e., wanting) (143).

### **Clinical concerns for treatment of chronic non-malignant pain with opiates**

Opiates are the most potent analgesic in use today, and have been for centuries (90). They are the mainstay of post-surgical and cancer pain. However, their use in chronic non-malignant pain is more controversial and opponents of their use argue that the risk of abuse and/or addiction to these patients supersedes any usefulness in treating pain. Opponents also point out that the widespread use of opioids for treatment of chronic pain over recent decades has contributed to the increase in opiate abuse and dependence throughout the country (144).

Conversely, proponents argue that the risk of addiction, when taken as prescribed, is minimal and that the benefit to control patients' chronic pain outweighs the risk (145). The fact of the matter is that there is not sufficient controlled clinical trial data to establish whether or not opioid use for the long-term treatment of chronic non-malignant pain is ultimately beneficial to patients or to some, but not all, patients. It is likely that there is a subpopulation of patients that can achieve some degree of analgesia from long-term opioid therapy without the occurrence of aberrant drug-related behaviors or intolerable side effects.

One prospective study followed 15,000 veterans that began opiate use for the first time to control pain, the study reported that only 2% developed opioid abuse, however, this study only followed patients for 3 months (145). This finding is consistent with the estimated 2.6% of the US population reported to abuse prescription opioids by a study conducted in 2012 by the National Survey of Drug Use and Health (146). Other studies have reported that the risk of abuse is closer to 6% in pain patients but, again, these studies lack the duration needed to come to a true conclusion (147). In contrast, a large health care institution reported that the rate of opioid abuse among out-patients on long-term opioids to be as high as 26% (148). Additionally, the 2010 TROUP study reported rates as high as 24% of recipients in a commercially insured sample and 20% in a sample of patients with Medicaid (149). Another important study of note assessed the prevalence of abuse between tramadol (a weak  $\mu$ -opioid agonist

with SNRI activity), nonsteroidal anti-inflammatory drugs (NSAIDs; not intrinsically rewarding) and hydrocodone (powerful  $\mu$ -opioid receptor agonist) in patients with chronic non-malignant pain (150). A total of 11,352 patients were enrolled with 72% of patients completing all 9 interviews over the 12-month period (150). The percent of patients that scored positive for abuse once were 2.5% for NSAIDs, 2.7% for tramadol and 4.9% for hydrocodone, however, these numbers dropped considerably when measured for persistence of abuse (0.5%, 0.7% and 1.2%, respectively) (150). In either case, patients receiving hydrocodone had significantly higher abuse rates than either NSAIDs or tramadol (150).

Another important aspect in the controversy of whether to prescribe opioids for management of chronic non-malignant pain is whether they improve patient quality of life and/or patient function. If their usefulness to these patients can be affirmed, the argument of whether to prescribe, despite risk of addiction, is much stronger. A review article by Devulder et al. in 2005 reviewed all published trials to date that met the pre-defined criteria (specifying study design, population, intervention and outcome measures) and assessed chronic non-malignant pain patients' quality of life to long-term opioid treatment for their pain (151). Nine total articles were included in the final analysis; 4 randomized trials and 5 observational studies. Three of the four randomized trials and 4 of the 5 observational studies found a significant improvement in patient quality of life (151). Conversely, a Danish epidemiological matched

cohort study of 1,906 patients assessed pain relief, quality of life and functional capacity between opioid and non-opioid users with chronic non-malignant pain (152). This study found that opioid users reported significantly more pain, poorer self health ratings and lower quality of life than nonusers (152). So, not unlike studies assessing risk of addiction, the results are varied when it comes to outcomes of patient quality of life.

Duration of opioid regimen in patients with chronic non-malignant pain can last decades due to the nature of the disease. Long-term use of this class of drugs raises another issue of whether analgesic efficacy can realistically be maintained in patients. There is accumulated evidence to suggest that there is a loss of efficacy in these patients over time (153-157), opioid refractoriness in opioid treated patients and failure to overcome tolerance with dose escalation (158-163). Of those, tolerance is possibly the main clinical concern as the net effect to the patient is a decrease in analgesic efficacy and while dose escalation works well in the short term it is less effective during long-term treatment. Additional side effects include opioid induced-hyperalgesia and withdrawal. Opioid-induced hyperalgesia is well documented to occur during withdrawal, however, it can also occur during treatment (156-158, 160, 164-166), though it will resolve once opioid therapy is discontinued (167-169). Withdrawal also poses a major clinical concern as it is not only unpleasant for the patient but can also lead to physical and psychological symptoms that could lead to opioid-seeking behaviors (112, 170, 171).

## **Issues with clinical trials to evaluate usefulness of opioids for chronic pain**

Overall, the usefulness of long-term opioid regimens for treatment of chronic non-malignant pain remains controversial and to date there is not sufficient clinical data to assess their usefulness and long-term risks. Even though this is a well-known fact designing, implementing and analyzing well thought out studies are incredibly difficult for these drugs in a chronic pain setting. An ideal trial would assess risk of addiction, patient outcomes (pain scores, functional outcomes, quality of life, etc.) and analgesic efficacy (tolerance, opioid-induced hyperalgesia, etc.) over multiple years of treatment. Randomized control trials (RCT) with parallel study design are generally accepted to provide the best evidence of data, however, in the case of long-term trials such as these there are many factors that limit their usefulness. Some factors are intrinsic to RCTs while others are specific to this question. The most notable are that RCTs do poorly over time as patients tend to drop out, long-term studies are incredibly expensive, they have sensitivity issues (likely due to therapeutic misconceptions), narrowing of study question (i.e., one or very few outcomes can be assessed), as well as ethical dilemmas (i.e., long-term placebo group). Crossover trial designs mitigate some of these issues: cost (greatly reduced sample size requirements) and reduced variability (i.e., with-in patient comparison versus inter-patient variability). But they have other deterrents (nonrandom drop-outs, carryover effects, same ethical dilemma, not ideal for long term studies, etc.) that exclude them as a practical alternative.

Failure of clinical trials evaluating opioid analgesics for chronic pain (i.e., indistinguishable difference between analgesic effect and placebo) is a significant problem, even when there is established clinical efficacy of the drug (172). Katz outlined a list of potential reasons for trial failure that are particularly relevant to opioid trials including factors intrinsic to opioid analgesics: high drop-out rates (primarily due to side effects), tolerance over time to analgesic effects, heterogeneity of patients with regard to opioid responsiveness, and variability of pharmacodynamic and pharmacokinetic factors (172). Additionally, Katz outlined factors related to study design for opioid trials; inclusion of patients unlikely to respond to opioids, outcome measures that do not adequately reflect benefit of the treatment, inappropriate analytic methods, prolong duration of treatment, rapid titration, fixed-dose versus flexible dosing, concomitant medications, rescue dosing, etc. (172). Consequently, it is no surprise why there is a lack of trial data evaluating the long-term usefulness and abuse liability of opioids for the treatment of chronic pain. Much of the data, in this regard, is limited to observational (retrospective and prospective) and epidemiologic cohort studies. And, while these reports are useful in this debate they show conflicting results likely due to some of the methodological issues described above. Ideally, a large-scale prospective matched cohort study that takes into consideration these concerns will be used to better evaluate these questions.

## **Screening, monitoring and evaluating patients for opioid treatment as a means to reduce risk of aberrant drug-related behaviors**

Consistent, efficient and routine monitoring of individual patients for aberrant drug-related behaviors, pain relief, patient functioning and adverse events is necessary to effectively manage treatment and potentially prevent or reduce the occurrence of abuse or addiction in chronic pain patients. In 2012-2013, the Federation of State Medical Boards of the United States (FSMB) updated their Model Policy for the use of Opioid Analgesics in the Treatment of Chronic Pain (173). The FSMB sets clear guidelines for physicians prescribing opiates including, but not limited to; understanding the pain, developing clear treatment goals, written informed consent and treatment agreement between patient and physician, ongoing monitoring of the patient and periodic drug testing (173). Further, the FSMB dictates that medical records should reflect the nature and intensity of the pain, current and past treatments for pain, underlying or coexisting diseases or conditions, the effect of the pain on physical and psychological function and history of substance abuse (174). Shockingly, a review of 300 chronic pain patients' charts revealed that 61% had no documentation of a treatment plan (175). Another study found that only 43% of historical findings and 28% of physical examination findings were recorded during initial consultations of 513 patients with acute musculoskeletal pain (176). Five-hundred and twenty single visits to an outpatient oncology practice were randomly selected and reviewed, of those <1% had any quantitative pain scores and only 60% had any mention of qualitative assessments of pain (177).

Finally, when medical records at a cancer center were reviewed from 111 patients that underwent urine toxicology screens only 37.8% listed a reason for the test and 89% did not include the results of the test (178). These findings suggest that there is a need for more efficient and consistent tools for physicians to adequately monitor chronic pain patients receiving long-term opioid treatments.

Several screening strategies and instruments have been introduced in the past decade or so to help identify patients that would benefit from opioid therapy, pose a risk of opioid abuse or addiction or that provide a framework for monitoring patients routinely throughout the course of treatment. However, there is still no single validated test or instrument widely accepted that can reliably and accurately predict patients that are not suitable for opioid therapy or that need increased monitoring during treatment (179). Ideally, a single screening method can be validated to assess aberrant drug-behaviors, pain relief, patient functioning and adverse events. Passik et al. developed the Pain Assessment and Documentation Tool (PADT) that focused on these 4 key outcome domains (aberrant drug-behaviors, pain relief, patient functioning and adverse events) (174). While the initial report indicated that it was useful in evaluating these outcome measures and offered a simple means of documenting patient care it is yet to be validated on a large scale (174). Further, a 2012 review by Sehgal et al. reviewed all published assessment tools to date and concluded that none were sufficient and/or validated to be used on a wide scale

at the time of publication (179). At least for the foreseeable future, prescription opioids will continue to be used for the treatment of chronic non-malignant pain. Until there is more evidence to deter physicians from prescribing opiates or better alternative analgesics are developed adequate screening tools are needed to help combat the growing problem of opioid abuse and addiction.

### **Dissertation objectives**

In Chapter 1, I presented background information for the purpose of providing context for the experimental data that is presented throughout the dissertation. Following descriptions of experimental methods in next chapter, Chapter 3 describes the assessment of three clinically effective drugs; duloxetine (serotonin and norepinephrine reuptake inhibitor), morphine ( $\mu$ -opioid agonist) and tapentadol (dual acting  $\mu$ -opioid agonist and norepinephrine reuptake inhibitor). My work assesses how each of these drugs acts by modulation of descending spinal inhibitory pathways including the release of norepinephrine (NE) in animals with and without chronic neuropathic pain. I attempt to answer the following questions in Chapter 3: (1) Does systemic administration of duloxetine, morphine or tapentadol modulate spinal NE levels in uninjured rats? And (2) Does a chronic neuropathic injury (spinal nerve ligation; SNL) alter the effect of these drugs on spinal NE levels. The goal is to understand whether neuronal changes occur in the descending modulatory system that alters the effects of these drugs. In order to accomplish this I used a rat model of chronic

neuropathic pain (SNL) and a direct cerebrospinal fluid (CSF) collection method from the cisterna magna with HPLC analysis to evaluate changes in NE levels.

In chapter 4, I address whether the reward of pain relief is separable from reward of addiction using *in vivo* rat models of chronic neuropathic pain. In this chapter, I asked if (1) the effects of morphine on sensory and affective/emotional dimensions of pain are separable; (2) the rewarding effects of pain relief separable from those of the reward of addiction? (3) opioid signaling in the anterior cingulate cortex (ACC) mediates the reward of pain relief? and whether (4) it is possible to develop a method that can measure endogenous opioid release in the ACC? In order to address these questions, I used *in vivo* microdialysis and conditioned place preference (CPP) output measures in injured and uninjured rats.

## **Chapter 2: Experimental Methods**

### ***Animals***

Male, Sprague-Dawley rats (Harlan Laboratories Inc., Indianapolis, IN, USA) weighing 300 - 325 g at the time of testing, were housed in a climate-controlled room on a 12-hour light/dark cycle. Food and water were available at all times ad libitum. All experiments were performed in accordance to policies and procedures set forth by the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals. Approval was obtained from the Institutional Animal Care and Use Committee of the University of Arizona prior to all experimentation. Every effort was made to minimize animal pain and distress as well as to minimize the number of animals used. Experimenters were blinded to the treatment group in all behavioral experiments.

### ***Surgical techniques***

#### ***Spinal nerve ligation***

As previously described by Kim and Chung, the L5/L6 surgical procedure was used to produce experimental chronic neuropathic pain (180). Rats were anesthetized with isoflurane (2% mixed with room air; 2L/min) and the lumbar vertebrae were exposed. The L5 and L6 spinal nerves were identified and tightly ligated with 4-0 silk suture and the wound was closed. Sham-operated rats were prepared in the same manner as the SNL rats except the L5/L6 spinal nerves

were not ligated. All rats were monitored for any visual signs of motor deficits, as well as for general health and weight maintenance.

*Intracranial ACC and NAc cannulation for CPP and microdialysis (Chapter 4)*

All stereotaxic surgeries were performed in rats anesthetized with a ketamine/xylazine mixture (80/12 mg/kg; Western Medical Supply/Sigma) and cannulas were implanted according to the brain atlas.

For conditioned place preference (CPP) experiments; a single piece containing two 26-gauge guide cannulas spaced 1.2 mm apart (Plastics One) were directed towards the ACC using the following coordinates at anteroposterior (AP), bregma +2.6 mm; dorsoventral (DV), skull -1.8 mm). Appropriate stainless steel dummy cannulas were inserted into each guide for the purpose of keeping cannulas free of debris. Surgeries were done immediately prior to SNL or sham surgeries. Following surgery all animals were housed individually and allowed 14-16 days to recover.

For NAc microdialysis experiments (i.e., morphine and gabapentin); a single piece containing two 26-gauge guide cannulas spaced 1.6mm apart (Plastics One) were directed towards the ACC using the following coordinates at a 25° forward facing angle : anteroposterior (AP), bregma -4.1 mm; dorsoventral (DV), skull -3.0 mm). For the NAc, a single guide cannula (AG-8; Eicom Corp.) was implanted vertically into the left NAc shell: (AP, bregma +1.7 mm; mediolateral (ML), midline  $\pm$ 1.0 mm; DV, skull -6.0 mm). Appropriate stainless steel dummy

cannulas were inserted into each guide for the purpose of keep cannulas free of debris. Following surgery all animals were housed individually and were allowed to recover 7-9 days prior to experiments.

For ACC microdialysis experiments (i.e., endogenous opioid release); a single piece containing one 26-guage guide cannula (AZ-08; Eicom) was directed towards the ACC using the following coordinates: anteroposterior (AP), bregma +2.6 mm; mediolateral (ML), midline +0.8 mm; dorsoventral (DV), skull -2.0 mm. Appropriate stainless steel dummy cannulas were inserted into each guide for the purpose of keep cannulas free of debris. Following surgery all animals were housed individually and were allowed to recover 7-9 days prior to experiments.

### ***Drug administration***

#### *Intraperitoneal injections*

For experiments presented in Chapter 3, animals received an intraperitoneal (i.p.) injection of morphine (10 mg/kg) or tapentadol (10 and 30 mg/kg) dissolved in sterile saline or duloxetine (30 mg/kg) in distilled H<sub>2</sub>O. Normal saline was used as the vehicle. Duloxetine was purchased from ChemPacific Corporation (Baltimore, MD, USA), morphine was provided by the NIDA Drug Supply Program and tapentadol was provided by Grunenthal GmbH (Aachen, Germany).

### *Intravenous injections*

For experiments presented in Chapter 4, animals received an intravenous injection of vehicle (sterile saline), morphine (0.25, 0.5, 1.0, 2.0 or 4.0 mg/kg; NIDA drug supply program) or gabapentin (50 mg/kg; Spectrum Chemical) dissolved in sterile saline for all experiments. Injections were done in awake animals that were minimally restrained by experimenter 10 min prior to start of experimental dialysate fraction collection for microdialysis experiments. For CPP experiments, i.v. morphine or saline was administered in lightly anesthetized animals using isoflurane (2% mixed with room air, 2 L/min) and animals were allowed to wake from anesthesia in paired chamber. For evoked studies, i.v. morphine or saline was administered in animals using a restraining device.

### *Brain microinjection*

Bilateral intracranial microinjections of  $\beta$ -Funaltrexamine hydrochloride ( $\beta$ -FNA) (3  $\mu$ g/ $\mu$ l per side; Tocris), morphine (1  $\mu$ g/0.5  $\mu$ l per side; NIDA drug supply program) or vehicle (sterile saline) into the ACC was done using an injector that extended 1 mm beyond guide cannula (Plastics One). Following each experiment, all animals were euthanized via CO<sub>2</sub> overdose and ink was injected (0.5 ml per side) into ACC cannula and placement of the guide cannulas/injection sites were verified with histology methods. Data from animals with misplaced cannulas were removed from analyses.

## ***Behavioral testing***

### *Tactile hypersensitivity*

Tactile withdrawal thresholds were determined prior to SNL or sham surgery (pre-surgery baseline) and 14-16 (i.e., immediately prior to start of experiment) days following sham or SNL surgery (post-surgery baseline). The rats were placed in suspended plastic chambers with wire mesh bottoms for 0.5 hr prior to testing. A series of calibrated von Frey filaments was applied perpendicular to the plantar aspect of the ipsilateral hindpaw until the filament buckled (61, 181, 182). The up-down method was used to determine the 50% withdrawal threshold with the Dixon nonparametric test as previously described (61, 181, 182). For experiments presented in Chapter 3, behavioral testing was performed approximately 10 minutes prior to initiation of CSF collection (i.e.; 20, 50 and 80 minutes after injection). For experiments presented in Chapter 4, tactile hypersensitivity was tested 20, 40, 60 and 90 min after i.v. morphine or vehicle administration.

### *Conditioned Place Preference (CPP)*

Experiments were conducted according to previously described methods [5; 6]. Rats were handled for 2-3 days prior to the experiment in order to acclimate the animals to the experimenter. The day before the experiment, tactile withdrawal thresholds were determined and only rats that displayed withdrawal thresholds lower than 6 g were included in the study. On pre-conditioning day (Day 1), rats were placed in the CPP box (*San Diego Instruments*) with free access to all

chambers for 15 min and time spent in each chamber was automatically calculated using the included software. Rats that spent more than 720 sec or less than 180 sec in either testing chamber were excluded from the study as this represents a natural preference/aversion for one chamber. Pretreatment with  $\beta$ -FNA (3  $\mu$ g/ $\mu$ L) or vehicle (sterile saline) was done 20-24 hours prior to i.v. morphine administration, and after preconditioning measures, by injecting 1ml/side bilaterally into guide cannulas implanted into the ACC. On conditioning day (Day 2 of CPP, 14 to 16 days after SNL or sham surgery), rats were intravenously administered saline and immediately placed into the conditioning chamber for 30 min. Four hours later, rats were intravenously administered morphine and placed into the opposite conditioning chamber for 30 min. On the test day (Day 3), rats were placed in the CPP box with free access to all chambers for 15 min and time spent in each chamber was automatically measured using provided software. Difference scores were calculated by subtracting the time spent in the drug-paired chamber of Day 1 from that of Day 3.

### ***Cerebrospinal fluid collection from cisterna magna (Chapter 3)***

#### *CSF collection catheter preparation*

The day before the surgery, 2-inch segments of PE-60 tubing (Scientific Commodities Inc., Lake Havasu, AZ, USA) were cut. Needle tips were removed from 23G syringes (BD Precision Glide, Franklin Lakes, NJ, USA). Using super glue, syringe needle, PE-60 tubing, and a gel loading pipette tip (Fisher Scientific,

Pittsburgh, PA, USA) were securely fastened together to form a catheter. Catheters were allowed to dry overnight. On the day of the collection a P200 Pipettman (Rainin, Columbus, OH, USA) was used with the prepared catheter to collect CSF.

#### *CSF collection technique*

CSF was collected from naïve animals, or following sham- or SNL surgery. Rats were anesthetized with isoflurane (2% in air, 2 L/min) and placed in a stereotaxic frame. A 1.5 cm longitudinal incision from the back ridge of the skull to C1 was made and the muscles were retracted to expose the atlanto-occipital membrane. A prepared catheter and micropipette was used to puncture the membrane and collect the CSF (70-150  $\mu$ L), free of blood, from the cisterna magna. The CSF was combined with an antioxidant cocktail (6.0 mM 1-cysteine, 2.0mM oxalic acid, and 1.3% glacial acetic acid) and kept on ice in order to prevent the breakdown of catecholamines (183).The samples were centrifuged (14,000 rpm) at 4°C for 5 min. The amount of CSF collected was measured and added to a single catecholamine extraction tube. Catecholamine extraction kits were purchased from ESA, Inc (Chelmsford, MA, USA) and the protocol was followed in full as per the provided manual.

### ***In vivo microdialysis techniques***

#### *NAC microdialysis for collection of dopamine*

Microdialysis experiments were done in awake and freely moving animals. Microdialysis probes (AZ-8-02; Eicom) were inserted into the guide cannula so the 2 mm semi-permeable membrane protrudes from the guide into the NAc shell. The microdialysis probe was perfused with artificial cerebrospinal fluid (aCSF: 147.0 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl<sub>2</sub> and 1.2 mM CaCl<sub>2</sub>) at a rate of 2.0 µl/min using a gastight syringe, syringe pump drive and hive syringe pump controller (MDN-0250, MD-1001, MDN-1020; BASi) set-up. Following a 75-90 minute washout period 1 baseline and 1 experimental fraction (90 min/fraction) were collected into pre-chilled (4 °C) amber Eppendorf tubes containing 1.5 µl of 40x antioxidant solution (6.0 mM L-cysteine, 2.0 mM oxalic acid and 1.3% glacial acetic acid) (184). All experimental treatments/manipulations were done 10 min prior to the start of the experimental fraction collection to account for the time needed for the sample to move through the microdialysis set-up tubing. Following the experimental fraction collection rats were injected with cocaine (20 mg/kg, i.p.) and dialysate was collected for an additional 60 min in the same fashion as previous samples.

#### *ACC microdialysis for collection of methionine-enkephalin (YGGFM) and leucine-enkephalin (YGGFL)*

Microdialysis experiments were done in awake and freely moving animals. Microdialysis probes (AZ-8; Eicom) were inserted into the guide cannula so the 2

mm semi-permeable membrane protrudes from the guide into the ACC. The microdialysis probe was perfused with artificial cerebrospinal fluid (aCSF: 147.0 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl<sub>2</sub> and 1.2 mM CaCl<sub>2</sub>) at a rate of 0.5 µl/min using a gas tight syringe, syringe pump drive and hive syringe pump controller (MDN-0250, MD-1001, MDN-1020; BASi) set-up. Following a 105-120 minute washout period 3 baselines, 1 high KCl infusion period and 3 experimental fractions (20 min/fraction) were collected into pre-chilled (4 °C) 0.5 mL siliconized eppendorf tubes after on-line acetic acid mixture was combined. High KCl (147.0 mM NaCl, 75 mM KCl, 1.2 mM MgCl<sub>2</sub> and 1.2 mM CaCl<sub>2</sub>) infusion was started 10 minutes prior to the 20 min high KCl infusion period to account for the time needed for the sample to move through the microdialysis set-up tubing. High KCl was perfused for a total of 20 min through the microdialysis probe into the ACC. Perfusion of high KCl was limited to 20 mins to avoid any serious consequence to the rats such as seizure or death. Samples were immediately frozen on dry ice as soon as the total volume was collected.

***Online preservation system for microdialysate collected from the ACC for analysis of Met- and Leu-enkephalin***

An online preservation system was developed to increase recovery of Met and Leu enkephalin (YGGFM and YGGFL). Figure 4.4 A and B shows the set-up of the in-house constructed online-peptide-preservation system (i.e., mixing tee); it consists of a Micro T connector (CMA000043; CMA Microdialysis), FEP tubing (840 9501; CMA Microdialysis) and tubing adaptors (340 9500; CMA

Microdialysis). An aqueous acetic acid mix (10% (v/v) acetic acid, 40  $\mu$ M methionine, 1  $\mu$ M bestatin and 1  $\mu$ M thiorphan) was spiked with 100 pM of non-endogenous d2-alanine, d5-leucine-enkephalin (DADLE), an analog type internal standard and the mixture was combined with dialysate via the mixing tee. Online-preservation mixing tee occurs after the dialysate is recovered from the implanted microdialysis probe so there is no concern of the mix diffusing across the microdialysis membrane and having a deleterious effect on the animal or influence the integrity of the experiment.

***ACC microdialysate preparation for analysis of Met- and Leu-enkephalin via mass spectrometry***

Microdialysate was frozen and left on dry ice immediately after sample was collected, once all samples were collected they were transferred and stored at -80°C until analysis (< 24 hours from collection). Fractions were thawed in batches of 4 and were desalted using a C18 zip-tip (ZTC18S096; EMD Millipore). The zip-tip was briefly equilibrated with 3- 10  $\mu$ L volumes of acetonitrile (ACN) followed by 3- 10  $\mu$ L volumes of 0.1% trifluoroacetic acid (TFA). Next, the 10  $\mu$ L sample fraction is loaded onto the zip tip with 10 passes. The sample is then washed with 2 volumes of 0.1% TFA and then eluted in 10  $\mu$ L of 60% ACN, 40% water with 0.1% TFA. Finally, using a speed vac the sample is dried to < 1  $\mu$ L to ensure proper loading on the capillary chromatography system.

***Analytical methods for catecholamine and endogenous opioid peptide detection***

*HPLC with EC detector analysis of catecholamines*

The HPLC system consisted of an Agilent 1100 quaternary pump and thermostated autosampler (Agilent Technologies, Palo Alto, CA, USA) coupled to an in-line Coulochem III electrochemical detector with model 5011A analytical cell (E1 -150mV and E2 +250mV) and model 5020 guard cell (+350mV) (ESA Inc., Chelmsford, MA, USA). Using MD-TM mobile phase (ESA Inc.; 10% acetonitrile [ACN] for NE and diluted in-house to 9% ACN for DA), at a flow rate of 0.400 ml/min, catecholamines were separated in samples using a MD-150 (2 x 150mm; 3mm) column (ESA Inc., Chelmsford, MA, USA). Agilent ChemStation data acquisition software was used to analyze the chromatograms.

For samples collected from the cisterna magna (Chapter 3) and analyzed for NE; each sample of CSF was then spiked with a known amount of NE and re-injected into the HPLC system. The chromatograms were overlaid to confirm that the correct peak, indicated by a retention time of approximately 2.6 minutes, was collected (Figure 3.1 A). Additionally, a minimum of 3 separate series of sequentially varying amounts of NE in artificial CSF (aCSF) was injected into the HPLC system in order to generate a standard curve ( $y = 0.8588x + 1.203$ ,  $r^2 = 0.9998$ ). The lower limit of detection (LOD; 0.5 pg) and lower limit of quantification (LOQ; 1.5 pg) were determined (Figure 3.1 B).

For microdialysis samples analyzed for DA (Chapter 4); samples were injected on HPLC 2 times per sample. DA concentration was determined using a standard curve with seven serial dilutions of DA in aCSF. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the formulas  $LOD = 3.3 (SDr/S)$  and  $LOQ = 10 (SDr/S)$ , where the SD of the response  $SDr$  (SD of  $y$  intercepts of regression lines) and the slope of the standard curve  $S$  was determined from the measurement of 10 independent standard curves. Data from all rats that failed to generate DA efflux in response to cocaine injection were excluded. DA concentrations were expressed as percent of their corresponding baseline level.

*Mass spectrometry analysis of microdialysate for YGGFL and YGGFM*

Immediately prior to injection on the capillary-LC system samples are reconstituted in 0.1% TFA. Samples are separated using a Proxeon nanoLC II (Thermo Scientific) with a 2 cm pre column (Easy-column, ID 100  $\mu$ m, 5  $\mu$ m particle, C18-A1) and a 10 cm analytical column (Easy-column ID 75  $\mu$ m, 3  $\mu$ m particle, C18-A2). Electrospray ionization of the samples was achieved using a chip-based ESI system (Triversa Nanomate, Advion). An Orbitrap Velos Pro (Thermo Scientific) hybrid ion trap-orbitrap mass spectrometer. Mass analysis is conducted solely in the linear ion trap (LIT) with radial ejection of ions for sensitive detection. Multistage MS is carried out with two fragmentation steps ( $MS^3$ ) for all enkephalin species. YGGFL and YGGFM are analyzed through initial ion-trap isolation of  $m/z$  556 and 574, respectively and then fragmented at a

collision energy sufficient to produce a maximum intensity for  $a_4$  ion ( $m/z$  397). The  $a_4$  ion is then isolated and fragmented to produce a characteristic MS<sup>3</sup> spectrum which is dominated by the fragment ions with  $m/z$  380, 323, 279. Fragmentation pathways are analogous for met-enkephalin sulphoxide and for DADLE.

#### *LC-MS<sup>3</sup> description and concentration calculations*

Our approach utilized chromatography and ionization systems using nano-LC column and pump (Thermo Proxeon) and a chip-based nanospray ionization source (Advion). The mass spectrometer (MS) used a linear-ion trap instrument with radial ejection of ions and detection at dual electron multipliers. We used a multistage MS with two fragmentation steps. Each stage of mass spectrometry decreases both chemical (background) noise but also decrease signal intensity, however, chemical noise decreases faster than the signal intensity (185). This resulted in an increase in the signal-to-noise ratio giving us greater detection of Met- and Leu-enkephalin at low *in vivo* concentrations. MS was coupled with a liquid chromatography (LC) system that utilized a 75 micron diameter C-18 column.

LC forces sample, using a liquid (mobile phase), at high pressure through a tightly packed column containing a stationary phase of irregularly or spherically shaped particles to achieve a separation of compounds in the sample. Chromatography can be described as a mass transfer process involving

absorption. Data from LC is represented as a chromatogram, where the x-axis represents time and the y-axis represents peak height (i.e., intensity). MS measures mass-to-charge ratio of charged particles. Data from MS is also represented as a chromatogram, however, the x-axis represents the mass divided by charge number of ions ( $m/z$ ) and the y-axis represents signal intensity. The charge number of ions ( $z$ ) is almost always 1, so the  $m/z$  value is often considered to simply be mass.

A standard curve was created by running known amounts on LC-MS<sup>3</sup> with concentration on the x-axis and peak area on the y-axis. A standard curve based on the external calibration was produced (Figure 4.6 B) with the equation  $y = 178.09x + 1$ ,  $R^2 = 0.99944$  and  $y = 175.05x + 2.6667$ ,  $R^2 = 0.99913$  for Met- and Leu-enkephalin respectively. A standard curve was also created based on the internal standard calibration (Figure 4.6 A) with the equation  $y = 0.0457x + 0.0028$ ,  $R^2 = 0.99999$  and  $y = 0.0464x + 0.0028$ ,  $R^2 = 0.99994$  for Met- and Leu-enkephalin. Unknown concentrations from microdialysate samples were calculated from these standard curves for quantification purposes.

### ***Statistical analysis***

#### *Chapter 3*

Evoked pain behaviors were collected prior to surgery (baseline), after sham or SNL surgery (SNL) and 10 min prior to CSF collection (20 or 50 min). Data (20 and 50 min) were analyzed for significant changes from post-surgery baseline

(SNL) values with one-way ANOVA followed by the *post-hoc* Dunnett test. Spinal NE levels are expressed as the % of the mean spinal NE concentration obtained from naïve animals ( $0.82 \pm 0.08$  pg/ $\mu$ l; N=42). Significant changes in NE levels from naïve, representing 100%, were determined with ANOVA followed by the *post-hoc* Dunnett test. All evaluations were obtained using GraphPad Prism 5.00 for Windows (Graphpad Software, San Diego, CA, USA; <http://www.graphpad.com>).

#### *Chapter 4*

For evoked response experiments (i.e., paw withdrawal thresholds), evoked pain behaviors were collected prior to surgery (baseline), after sham or SNL surgery (SNL) and at 20, 40, 60 and 90 min after systemic drug administration. Differences over time between treatment groups were determined using two-way ANOVA for repeated measures followed by Student-Neuman-Keuls *post-hoc* test. For CPP experiments, difference scores were calculated for each rat by: test time in chamber – preconditioning time spent in chamber. The difference from baseline scores for the drug-paired chamber was analyzed using Students t-test. For microdialysis experiments, NAc DA levels are expressed as percent of their corresponding baseline levels for individual rats. Two-way ANOVA was used followed by Tukey's *post-hoc* test to determine differences from baseline (0%) or between sham and SNL groups. All evaluations were obtained using GraphPad Prism 5.0.

### **Chapter 3: Opioid and noradrenergic contributions of tapentadol in experimental neuropathic pain**

#### **Abstract**

Tapentadol is a dual action molecule with mu opioid agonist and norepinephrine (NE) reuptake blocking activity that has recently been introduced for the treatment of moderate to severe pain (186). The effects of intraperitoneal (i.p.) morphine (10mg/kg), tapentadol (10 or 30 mg/kg) or duloxetine (30 mg/kg), a norepinephrine/serotonin (NE/5HT) reuptake inhibitor, were evaluated in male, Sprague-Dawley rats with spinal nerve ligation (SNL) or sham surgery. Additionally, the effects of these drugs on spinal cerebrospinal fluid (CSF) NE levels were quantified. Response thresholds to von Frey filament stimulation decreased significantly from baseline in SNL, but not sham, operated rats. At the doses previously mentioned, duloxetine, tapentadol and morphine produced significant and time-related reversal of tactile hypersensitivity. Duloxetine significantly increased spinal CSF NE levels in both sham and SNL rats and no significant differences were observed in these groups. Tapentadol (10 mg/kg) produced a significant increase in spinal NE levels in SNL, but not in sham, rats. At a higher dose (30 mg/kg), tapentadol produced a significant increase in spinal CSF NE levels in both SNL and sham groups; however, spinal NE levels were elevated for an extended period in the SNL rats. This could be detected 30 min following tapentadol (30 mg/kg) in both sham and SNL groups. Surprisingly,

while morphine (10 mg/kg) reversed tactile hypersensitivity in nerve-injured rats, CSF NE levels were significantly reduced in both sham- and SNL rats. The data suggest that tapentadol elicits enhanced elevation in spinal NE levels in a model of experimental neuropathic pain offering a mechanistic correlate to observed clinical efficacy in this pain state.

### **Introduction**

Pain is a complex experience with sensory, emotional and cognitive components and the context in which nociceptors are activated plays an important role in the overall experience of pain (187-189). Contextual modulation of pain is thought to occur in a “top-down” fashion as a result of activation of multiple brain regions and results in facilitation or inhibition of nociceptive inputs at the level of the spinal and trigeminal dorsal horn (190). Human imagining studies in healthy volunteers with a variety of experimental noxious conditions has been shown to activate brain areas known to process emotional responses, mood and attention in addition to regions involved in “top-down” modulation (i.e., PAG and RVM) (189, 191).

As discussed in Chapter 1, the RVM is the final common relay in the descending modulation of nociceptive inputs and together with the PAG these regions form the key nexus of pain modulation (34). These regions also have reciprocal connections with the locus coeruleus (A6) and the Kolliker-Fuse nucleus (A7)

that combine to make up the pontine nuclei and are the main source of noradrenergic projections to the spinal cord (192-194). Activation of these noradrenergic projections and subsequent spinal release of norepinephrine (NE) is sufficient to produce analgesia and plays a major role in descending inhibition (34, 195-197). Accordingly, a variety of clinically effective drugs, including reuptake blockers, appear to engage descending inhibition or mimic the consequence of enhanced spinal NE (e.g.,  $\alpha_2$ -agonists) to elicit pain relief. Additionally, the endogenous noradrenergic inhibitory system plays a crucial role in the development of chronic pain and this system has been shown to be protective against the development of signs of neuropathic pain in nerve-injured rats (198-200).

Descending noradrenergic projections have also been implicated to play an important role in opiate analgesia and early animal studies demonstrate that the antinociceptive effect of supraspinal, but not spinal, morphine is dependent on activation of spinal  $\alpha_2$ -adrenergic receptors (201). Similarly, both systemic and spinal administration of  $\alpha_2$ -adrenergic agonists has been shown to enhance the antinociceptive effects of opiates while antagonists diminish or block their effects (201-205). In line with this, numerous pharmacological studies have shown that a synergistic relationship between  $\mu$ -opioid and  $\alpha_2$ -adrenergic agonists exist at the spinal level (203, 206). Based on these combined data, tapentadol was developed to exploit this synergistic relationship between  $\mu$ -opioids and  $\alpha_2$ -adrenergic agonists (207). This compound has similar analgesic

efficacy as morphine despite its weak affinity for the  $\mu$ -opioid receptor (i.e., 50-fold lower than morphine) suggesting that its action as a NE reuptake inhibitor (NRI) has a mechanistic interaction with its opioid activity, especially in neuropathic pain states (207).

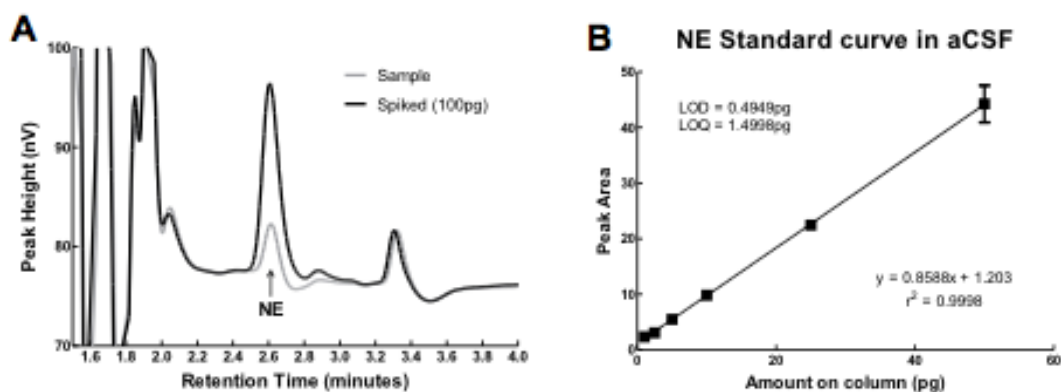
Interestingly, few studies have directly measured the effects of opioids, NRIs or serotonin-NE reuptake inhibitors (SNRIs) at analgesic doses on spinal NE levels. This chapter will compare the ability of tapentadol, morphine and duloxetine (SNRI) to reverse nerve-injury induced tactile hypersensitivity and to modulate spinal NE in rats with experimental neuropathic pain or in sham-operated controls.

## **Results**

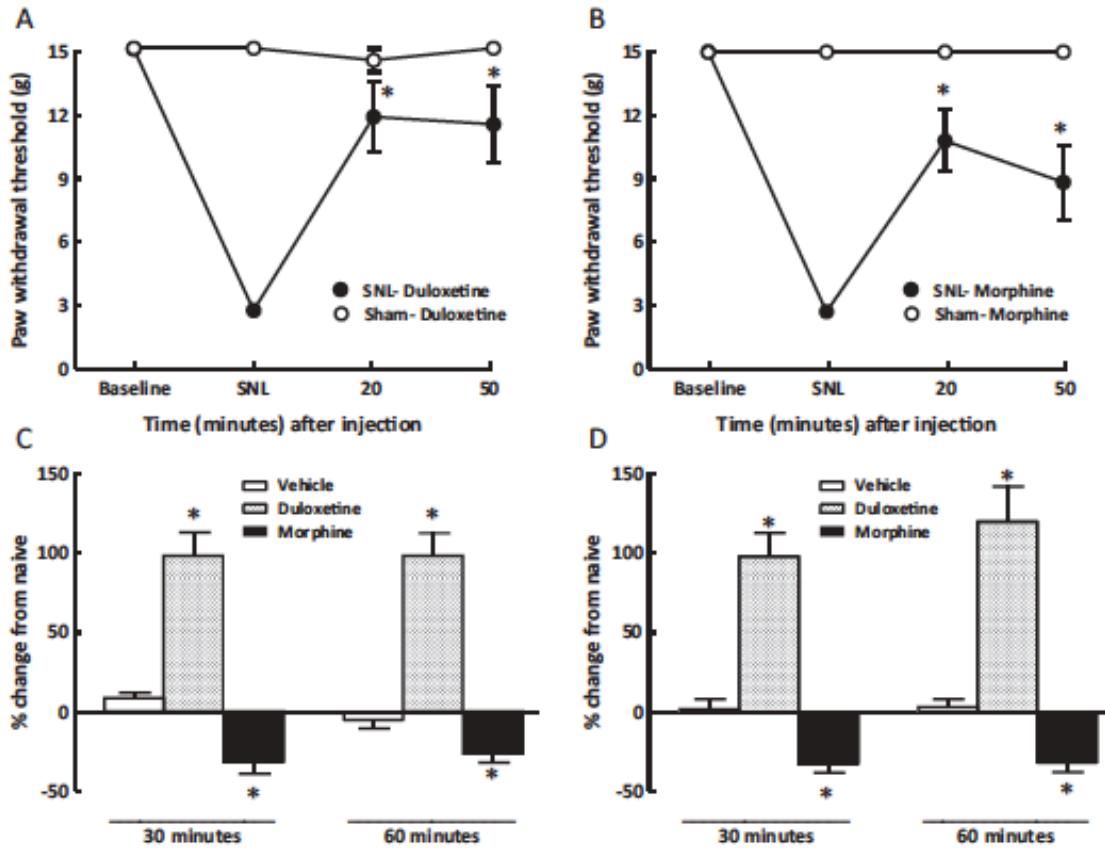
### ***Reversal of tactile hypersensitivity in rats with SNL***

Tactile hypersensitivity was measured in animals 10-14 days following sham- or SNL surgery (i.e., baseline) and again at 20 and 50 min after drug injection. These time points were approximately 10 minutes prior to collection of CSF (i.e., at 30 and 60 min post-injection). SNL, but not sham, surgery produced tactile hypersensitivity that was significantly ( $p < 0.05$ ) reversed by duloxetine (30 mg/kg, i.p.) or morphine (10 mg/kg, i.p.) (Figure 3.2 A,B). Neither duloxetine nor morphine produced any change in sham-operated animals (Figure 3.2 A,B). Vehicle injection did not alter paw withdrawal thresholds of sham-operated or SNL rats. The paw withdrawal thresholds of vehicle-injected, sham-operated rats

ranged between  $14.4 \pm 0.64$  g and  $15 \pm 0$  g and those of the SNL rats ranged between  $2.2 \pm 0.29$  g and  $2.6 \pm 0.17$  g. Similarly, administration of tapentadol (10 or 30 mg/kg, i.p.) reversed tactile hypersensitivity in SNL rats (Figure 3.3 A,B). No effect of tapentadol was observed in sham-operated rats (Figure 3.3 A,B).



**Figure 3.1.** (A) Representative chromatogram of CSF sample and of a sample with 100 pg of NE added. The chromatograms are overlaid to confirm the location of the NE peak. (B) Standard curve was generated from HPLC and electrochemical detection of known amounts of NE in artificial cerebrospinal fluid. The lower limit of detection was found to be 0.5 pg and the and lower limit of quantification was found to be 1.5 pg.



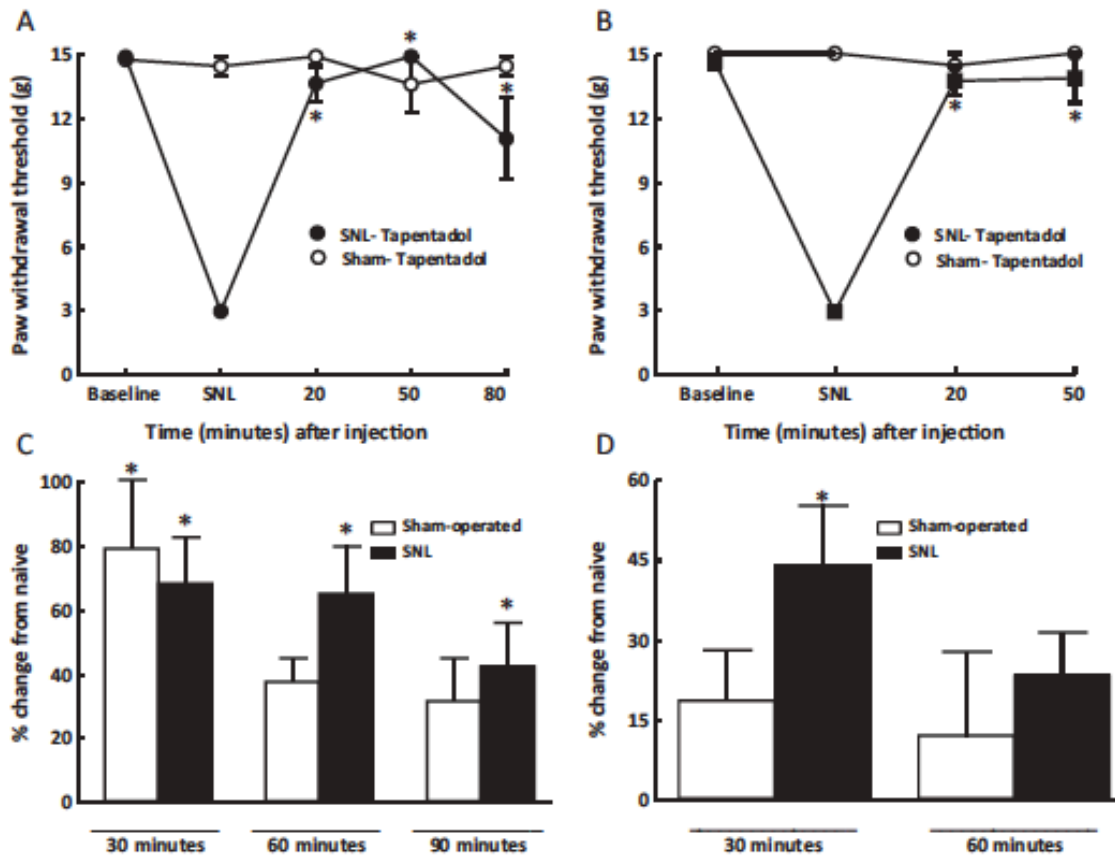
**Figure 3.2.** Rats with sham surgery or SNL received duloxetine (30 mg/kg, i.p.) or morphine (10 mg/kg, i.p.). SNL surgery significantly lowered paw withdrawal thresholds of rats, and duloxetine (A) or morphine (B) attenuated tactile hypersensitivity, as shown by the significant ( $p < 0.05$ ) increases in paw withdrawal thresholds. Vehicle injection did not alter CSF NE levels of sham-operated (C) or SNL (D) rats 30 or 60 minutes after injection. Duloxetine (30 mg/kg, i.p.) produced a significant ( $p < 0.05$ ) elevation of CSF NE at both time points. The increases in NE were similar for sham-operated (C) and SNL (D) rats. Morphine produced a significant ( $p < 0.05$ ) reduction of CSF NE levels in both sham-operated (C) or SNL (D) rats 30 or 60 minutes after injection. \* $p < 0.05$ ; N = 7 to 12 rats per group.

### ***Modulation of NE levels in spinal CSF of sham-operated and SNL rats***

Within 10 min of behavioral testing, sham-operated and SNL rats were anesthetized and the cisterna magna was exposed and punctured in order to extract CSF. CSF NE levels from sham- or SNL rats were measured with HPLC/electrochemical detection (Figure 3.1) and compared to levels from naïve animals in order to determine possible effects of prior surgery. Naïve animals that were not subjected to surgery or behavioral testing showed a mean NE concentration of  $0.82 \pm 0.08$  pg/ $\mu$ l. The CSF concentration of NE of vehicle-treated sham-operated (Figure 3.2 C) and SNL (Figure 3.2 D) rats did not differ from that of naïve rats. Duloxetine produced a significant, approximately, 2-fold increase ( $p < 0.05$ ) of CSF concentration of NE, relative to naïve rats, at 30 and 60 minutes after injection in both sham-operated (Figure 3.2 C) and SNL (Figure 3.2 D) rats; no significant differences were observed between sham and SNL rats. In contrast, morphine (10 mg/kg, i.p.) significantly ( $p < 0.05$ ) reduced NE concentration by approximately 30%, relative to naïve rats, in both sham-operated (Figure 3.2 C) and SNL (Figure 3.2 D) rats.

Tapentadol (10 mg/kg i.p.), produced a significant elevation in CSF concentration of NE only in SNL rats at 30 min after injection (Figure 3.3 D). At the higher dose, tapentadol (30 mg/kg, i.p.) produced significant ( $p < 0.05$ ) increases in spinal CSF levels of NE 30 min after administration to sham-operated or SNL rats (Figure 3.3 C). However, the CSF concentration of NE

remained significantly elevated at 60 or 90 min after injection only in the SNL rats (Figure 3.3 C).



**Figure 3.3.** Rats with sham surgery or SNL received either 30 mg/kg, i.p. (A) or 10 mg/kg, i.p. (B) of tapentadol. Both doses of tapentadol produced a full reversal of tactile hypersensitivity in SNL rats, indicated by the significant ( $p < 0.05$ ) elevations in paw withdrawal thresholds. No effect was observed in sham-operated rats. Tapentadol (30 mg/kg) (C) produced a significant ( $p < 0.05$ ) elevation of CSF NE levels 30 minutes after injection in sham-operated rats, and 30, 60 and 90 minutes after administration to SNL rats. The administration of 10 mg/kg, i.p. (D) of tapentadol produced significantly elevated CSF levels of NE at 30 minutes in SNL rats. Tapentadol did not significantly increase NE in sham-operated rats at either time point, nor was NE elevated 60 minutes after injection in SNL rats.  $*=p < 0.05$ ;  $N = 7$  to 12 rats per group.

## Discussion

Electrical stimulation or opioid microinjection into the PAG or RVM promotes release of NE into the cerebrospinal fluid (CSF) resulting in antinociception that is reversed by application of spinal adrenergic antagonists (184, 208-210). While these regions do not contain any noradrenergic neurons, they both communicate with the A5-A7 noradrenergic nuclei that comprise the major source of noradrenergic projections into the spinal cord (211). Spinal release of NE can act both pre- and post- synaptically to inhibit nociceptive transmission by impeding afferent inputs and the response of second-order neurons of the spinal dorsal horns (191, 193). This inhibitory effect of spinal NE is mediated through  $\alpha_2$ -adrenergic receptor and is likely the mechanism through which  $\alpha_2$ -adrenergic agonists, such as clonidine, produce antinociceptive effects (194, 205, 207). Additionally, it is this mechanism that is exploited with NE reuptake inhibitors, such as duloxetine, in patients with neuropathic pain such as diabetic neuropathy and fibromyalgia for which duloxetine is currently approved (212).

Genetically altered mice lacking dopamine  $\beta$ -hydroxylase, and thus do not produce NE, show a marked attenuation of the antinociceptive effects of systemic morphine, indicating that descending noradrenergic systems act to enhance the effect of opioids (210). Additionally, spinal pretreatment with the  $\alpha_2$ -adrenergic antagonist yohimbine attenuates the analgesic effects of systemic (202) or spinal morphine (204). Along these lines, co-activation of  $\alpha_2$ -

adrenergic receptors and  $\mu$ -opioid receptors has a strong antinociceptive synergy (203). Microinjection of morphine into the PAG and locus coeruleus produces an antinociceptive synergy in rats with acute pain suggesting a spinal/supraspinal synergy that is likely dependent on descending noradrenergic inhibition (213). This overall synergy seen between opioidergic and noradrenergic systems is likely the culprit to the favorable analgesic effects of tapentadol, a dual action  $\mu$ -opioid agonist and NE reuptake blocker, observed clinically (207, 214).

For our studies, the dose of duloxetine (30 mg/kg, i.p.) was chosen based on previous reports of efficacy in experimental neuropathic pain (215, 216) and, while a larger dose, was an equi-analgesic dose to that of morphine (10 mg/kg, i.p.). One possible reason duloxetine needed a 3-fold higher dose than morphine could be due to the pro-nociceptive effects at 5-HT, meaning a greater increase in spinal NE is required to overcome these effects (217, 218). The “high” and “low” dose of tapentadol was chosen to mirror the doses used for morphine and duloxetine; all three of these drugs at each dose were sufficient to reverse tactile hyperesthesia. Additionally, we demonstrated that duloxetine and tapentadol elevated CSF concentration of NE. However, tapentadol (~50-60%) produced less of an increase in CSF concentration of NE relative to duloxetine (~100%). This increase in NE concentration that we demonstrate with tapentadol is similar to the elevations (~70%) reported by another study employing microdialysis of spinal thoracic CSF with similar (10 mg/kg and 21.5 mg/kg)

doses of tapentadol (219). Tapentadol was able to completely block tactile hyperesthesia in the same manner as duloxetine, even though it produced less reuptake inhibition than duloxetine. This suggests that the efficacy of tapentadol is likely a result of the opioid/adrenergic synergistic profile, as its affinity for the  $\mu$ -opioid receptor is approximately 0.02 times that of morphine (207, 219).

Another interesting finding is that tapentadol produced a greater increase in spinal NE concentrations in animals with nerve injury as compared with sham-operated rats. This observation is consistent with the literature in that several studies have suggested that alterations of the noradrenergic system occur in conditions of nerve injury. One such study shows that electrical stimulation of the locus coeruleus produces enhanced antinociceptive effects and increased spinal NE in nerve-injured rats (220). Another demonstrated elevated biosynthesis of NE in the locus coeruleus and an increase in noradrenergic terminals in the spinal cord following nerve injury (221). Importantly, a study using *in vivo* electrophysiological analysis shows that while the inhibitory effects of tapentadol are effectively blocked by a  $\mu$ -opioid antagonist in sham rats,  $\alpha_2$ -adrenergic blockade is more effective in nerve-injured rats (222). These observations combined with the results from the current study suggest that tapentadol may be most effective in patients suffering from neuropathic pain conditions.

As has been discussed previously, opioid-induced analgesia has long been attributed, in part, to engagement of descending noradrenergic inhibition that results in increased spinal NE release. However, this assumption is based on pharmacological intervention and has only been directly tested 2 times previously. The first study was done using microdialysis of the spinal dorsal horn of sheep, i.v. morphine (1 mg/kg) produced a 5-fold increase in NE measured in the microdialysate (223). This study also reported a 4-fold increase in NE in CSF collected from a single patient following an intravenous injection of morphine (10 mg/kg) (223). However, in a more recent study with rats, morphine (1, 3 and 10 mg/kg, i.p.) produced a significant and dose-dependent decrease in NE levels in dialysate collected from the thoracic spinal cord (219). Based on the latter study, we chose the “high” morphine dose (10 mg/kg, i.p.) and demonstrate results that are consistent with this recent study; an approximate 30% reduction in CSF concentrations of NE (219). Both of these observations appear to contradict with pharmacologic evidence for a role of spinal noradrenergic activity in morphine-induced antinociception, however altered levels of NE within spinal microcircuitry may underlie noradrenergic contributions to opioid-induced antinociception. This issue remains to be elucidated.

In summary, we found that tapentadol produces an elevation of spinal NE that is likely through blocking neuronal reuptake of NE. It was also found that the NE release was greater in animals with nerve-injury than sham-operated rats,

suggesting that tapentadol may be most effective in patients with neuropathic pain. A surprising finding was that morphine reduced, rather than increased, spinal NE concentrations, this observation warrants further investigation.

## **Chapter 4: Neurochemical and behavioral evaluation of the reward of pain relief and the brain circuits that mediate it**

### **Abstract**

Relief of ongoing pain is a natural reward that activates the mesolimbic dopamine (DA) reward pathway. Opiates can modulate sensory nociceptive transmission however they preferentially act by alleviating pain affect (i.e., unpleasantness). Opiates can also directly activate DA reward pathways making them a clinical liability due to fear of addiction. Using a rat model of experimental neuropathic pain we assessed motivated behaviors (conditioned place preference; CPP) and performed neurochemical (*in vivo* microdialysis) analyses to show that morphine's effects on modulation of pain affect (i.e., the anti-aversive effects) occur at doses that do not alter sensory (i.e., evoked) responses. Additionally, the anti-aversive effects of morphine were also separable from mechanisms that are associated with addictive effects in uninjured animals that occur only at doses an order of magnitude higher. Importantly, the anti-aversive effects were abolished by pharmacological blockade of opioid receptors in the rostral anterior cingulate cortex (ACC) while the rewarding effects were not dependent on the ACC. Microinjection of morphine in the ACC was sufficient to alleviate pain unpleasantness without affecting evoked hypersensitivity and without activating reward circuits in uninjured states. Additionally, the anti-aversive effects of the non-opioid

analgesic gabapentin also appear to be dependent on opioid signaling in the ACC. Finally, we discuss the methodological development of an *in vivo* microdialysis technique that allows for the measurement of endogenous opioid peptide, Met- and Leu-enkephalin, in the ACC.

## **Introduction**

Opiates, including morphine, are the mainstay therapy for many patients with severe acute postsurgical or cancer pain. However, the usefulness of opiates for the treatment of chronic non-malignant pain remains unclear, mainly due to their addictive liability. Pain is a multidimensional subjective experience comprised of sensory, affective and cognitive dimensions. Unpleasantness of pain (the affective dimension) is what patients find most bothersome (92, 93). While it is understood that opiates can modulate sensory nociceptive transmission, clinical evidence suggests that the most critical action these drugs have is alleviating pain affect (i.e., unpleasantness). However, risk of addiction with chronic opiate use is a major clinical concern to which activation of mesolimbic dopamine (DA) reward pathways comprised of dopaminergic neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc) are mechanistically critical. Interestingly, these same reward circuits are activated by offset of an acute noxious stimulus (224, 225) or placebo analgesia (226) as demonstrated by human functional imaging studies. Additionally, we have demonstrated that in rodents relief of ongoing

postsurgical pain produces conditioned place preference (CPP) and activates mesolimbic dopaminergic reward circuits (55). Together these data suggest that the relief of pain is a natural reward that is sufficient to activate DA reward pathways.

Activation of the rostral anterior cingulate cortex (rACC) has been consistently demonstrated by human neuroimaging studies in response to acute noxious stimulation in healthy volunteers and in chronic pain patients (68, 227). Importantly, PET studies using opioid radiotracers demonstrate positive correlation between pain induced endogenous opioid release and/or receptor engagement in the ACC and reduction in pain-specific MPQ (McGill Pain Questionnaire) affective scores (228). Further, molecular imaging techniques in humans show significant placebo-induced activation of  $\mu$ -opioid receptor-mediated neurotransmission in various cortical regions including the rACC (87). In rodent models rACC lesion abolishes formalin-induced conditioned place aversion without affecting evoked hypersensitivity (81). Additionally, rACC lesion blocks reward elicited by pain relief following RVM lidocaine without affecting tactile hypersensitivity in a rat chronic neuropathic pain model (229). Collectively, these data suggest that endogenous  $\mu$ -opioid signaling within the rACC plays a crucial role in processing the aversiveness of pain.

### ***Opioid analgesics and their role in the affective dimensions of pain***

Opioid receptors are distributed throughout the peripheral and central nervous system and stimulation of receptors by exogenously administered opiate drugs provides analgesia in patients and animal models (230). Recent clinical studies demonstrated that a peripherally restricted  $\mu$ -opioid receptor antagonist, methylnaltrexone, was insufficient to attenuate analgesia of a systemic opiate agonists for the treatment of pain in chronic non-malignant, acute postsurgical and advanced illness patients highlighting the critical role of the central nervous system in the analgesic actions of exogenous opiates (231-233). Rodent models also suggest that the doses of morphine needed to selectively reduce the aversiveness (i.e., affective/emotional dimension) of pain are lower than doses needed to provide relief of sensory aspects of pain (234-237). Similarly, in a human fMRI study, areas of the brain that are highly involved in processing emotion and/or the unpleasantness of pain are activated at lower doses of opiates than regions associated with processing the sensory intensity of pain (238).

Based of these data, we hypothesized that brain circuits mediating the reward of relief of ongoing pain and those mediating addictive qualities of opiates may be separable. We also hypothesized that opiate effects on modulation of pain affect may occur at doses that do not affect sensory (i.e., evoked) pain responses. We tested our hypotheses in a rat model of chronic neuropathic pain (spinal nerve

ligation; SNL) using behavioral (CPP) and neurochemical (*in vivo* microdialysis) analyses. These data are presented first in this chapter.

### ***The role of the ACC and endogenous opioids in non-opiate analgesics***

As discussed above, and in Chapter 1, central processing of the affective dimension of pain (i.e., pain aversiveness) occurs in multiple brain regions including the insula, prefrontal cortex (PFC), anterior cingulate cortex (ACC), amygdala and striatum (dorsal and ventral) (48, 68). The ACC receives nociceptive input from the spinal cord by way of the thalamus (77) and is activated by acute noxious stimulation (47). This region of the brain is also correlated with processing the unpleasantness of pain (82). In humans, the ACC is activated by relief of neuropathic pain in patients with painful mononeuropathy of a lower extremity using a peripheral nerve block (239), removal of a noxious stimulus (56) and during placebo analgesia (49). Additionally, emotional states have the ability to alter pain unpleasantness indicating the ACC's involvement in pain modulation (43, 48). Collectively, these findings indicate a crucial role for the ACC in pain modulation and processing of the affective component (i.e., pain aversiveness) of pain and it is likely that both opiate and non-opiate analgesics exploit this mechanism.

Gabapentin is used clinically for the treatment of neuropathic pain and is effective in about one-third of patients with a number needed to treat (NNT) of 5.8 (240). However, gabapentin is far less effective for the treatment of acute

post-surgical pain (NNT is 11) (240). Despite its common use for the treatment of neuropathic pain the mechanism through which it provides analgesia is unknown. Our lab has previously published that a high oral dose (300 mg/kg), that is consistent with other preclinical studies (241-243), of gabapentin produces DA release in the NAc in animals with spinal nerve ligation (SNL) (244). Additionally, we have published that oral ketorolac, a non-steroidal anti-inflammatory (NSAID), also produces NAc DA release and CPP in injured animals (244).

Based on these data, and results from systemic morphine experiments presented in the first part of this chapter, we hypothesized that intravenous gabapentin (50 mg/kg) would cause DA efflux in animals with nerve injury only. Further, that this rewarding effect requires endogenous opioid neurotransmission in the ACC. We tested these hypotheses using *in vivo* microdialysis techniques in rats with neuropathic pain (SNL) or sham surgery.

***Methodological development of an in vivo microdialysis technique for the evaluation of endogenous opioid release in the ACC***

Relief of an aversive state, such as pain, is rewarding and is mediated by dopaminergic signaling in the nucleus accumbens (NAc) (55, 244). In the first two sections of this chapter I presented data that shows pharmacologic blockade of  $\mu$ -opioid signaling (i.e.,  $\beta$ -FNA pretreatment) in the anterior cingulate cortex (ACC) is sufficient to block the rewarding effects of both opioid and non-opioid

analgesics. This was demonstrated using the conditioned place preference (CPP) paradigm that was used to assess the motivational drive of rats with ongoing neuropathic pain to seek relief (negative reinforcement) (55, 59). Additionally, it was assessed by neurochemical output (i.e., DA release in the NAc) using *in vivo* microdialysis techniques. In the first section of this chapter I will demonstrate that a systemic low dose (0.5 mg/kg, i.v.) of morphine preferentially induces CPP and NAc DA release in SNL but not sham rats. This low dose was not sufficient to reverse tactile hypersensitivity in SNL rats, suggesting it was only effective against the aversiveness of pain and did not activate addictive reward pathways (i.e., VTA). In contrast, the high dose (4.0 mg/kg, i.v.) of morphine was sufficient to produce CPP and NAc DA release in both sham and SNL animals as well as reverse tactile hypersensitivity in SNL rats. In the second part of this chapter I will present data that shows that gabapentin (50 mg/kg, i.v.), a drug that is not intrinsically rewarding, is also capable of eliciting NAc DA release preferentially in SNL animals, this effect has been shown with other non-opioid analgesics previously in our lab (55, 244). Additionally, I will present data showing that the rewarding effects of pain relief to both opioid and non-opioid analgesics was attenuated by pharmacological blockade of  $\mu$ -opioid signaling in the rACC but this blockade had no effect in uninjured animals to a high dose of morphine that was likely sufficient to activate addictive pathways. Further, I will show that microinjection of morphine into the ACC was sufficient to elicit reward preferentially in SNL animals by relieving pain affect. Collectively, these data support our hypothesis

that opioid signaling in the ACC and subsequent downstream activation of DA neurotransmission in the NAc (mediates reward of pain relief) is required for the relief of ongoing pain.

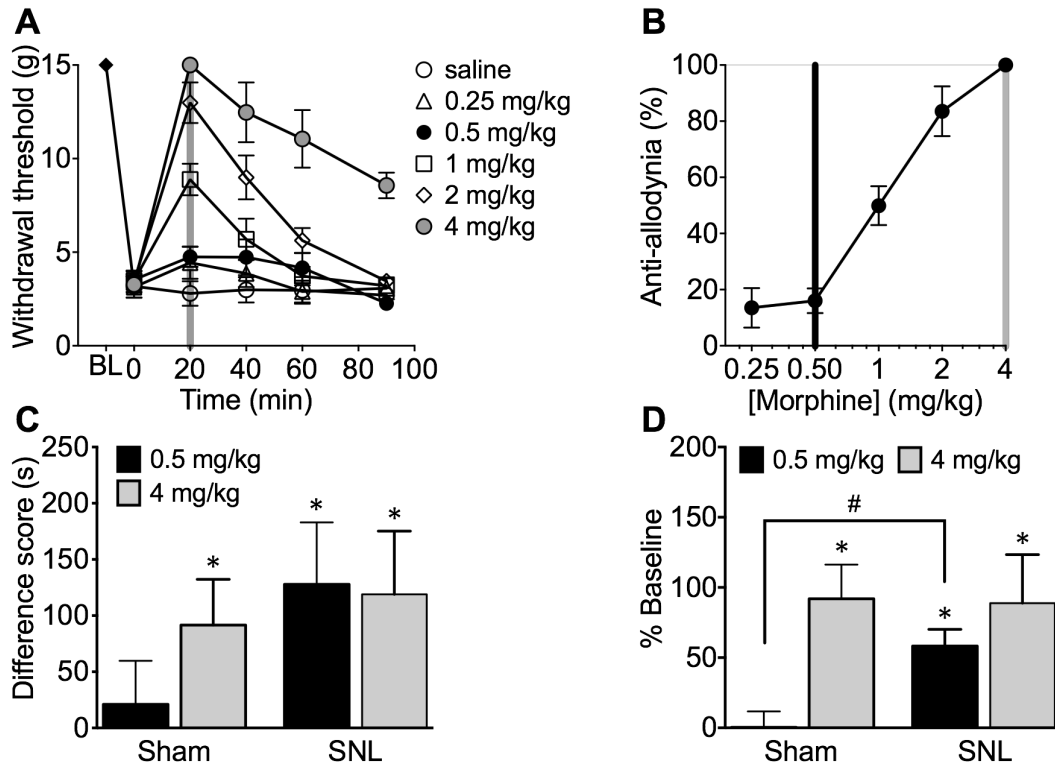
While these data are compelling, the results can only be implied through pharmacological interventions, leading us to ask the question: can we directly measure release of endogenous opioids in the ACC? More specifically, can changes in methionine and leucine enkephalin (YGGFM and YGGFL) be detected in microdialysate from the ACC? The later sections of this chapter will focus on the methods developed to successfully measure release of endogenous opioid enkephalins in the ACC from similar methods used for microdialysate from rat globus pallidus (245) and striatum (246). I will also discuss the implications of this method as well as future biological questions one can ask with an established method in place.

## **Results**

### ***Effect of intravenous morphine dose response curve on tactile hypersensitivity in SNL animals***

Tactile hypersensitivity was measured using von Frey filaments in animals prior to SNL surgery (i.e., baseline), 12-16 days following surgery (i.e., SNL) and again at 20, 40, 60 and 90 min after i.v morphine injection. SNL surgery produced tactile hypersensitivity that was significantly ( $p < 0.05$ ) reversed by 1.0, 2.0 and

4.0 mg/kg doses of morphine 20 min post-injection (Figure 4.1 A). 0.25 and 0.5 mg/kg doses had no significant effect on tactile hypersensitivity at any time point. 4.0 mg/kg dose was chosen for the high dose of morphine for subsequent experiments based on its ability to fully reverse tactile hypersensitivity and 0.5 mg/kg dose was chosen as the low dose as it had no effect on tactile responses.



**Figure. 4.1. Morphine relieves pain aversiveness at doses that are not effective to reverse evoked hypersensitivity.** (A) Paw withdrawal thresholds were significantly reduced 12-16 days following SNL but not sham surgery. Administration of morphine (0.25, 0.5, 1.0, 2.0 or 4.0 mg/kg, i.v.) dose dependently reversed tactile hypersensitivity: 4.0 mg/kg, i.v. morphine attenuated tactile hypersensitivity at 20, 40, 60 and 90 minutes, but 0.25 or 0.5 mg/kg, i.v. morphine did not, as shown by the significant increase in paw withdrawal thresholds (Mean  $\pm$  SEM,  $n = 5-7$ ,  $*P < 0.05$ , One-way ANOVA with Tukey's post-hoc). (B) The dose-response curve was calculated 20 min post morphine. Morphine (0.5 mg/kg, i.v.) produced (C) CPP and (D) NAc DA release in SNL animals but not sham. Morphine (4.0 mg/kg, i.v.) produced (B) CPP and (C) NAc DA release in both sham and SNL animals. Mean  $\pm$  SEM,  $n =$  (B) 21-22

and (C) 6-11,  $^{*}P < 0.05$ , Student's paired t-test. CPP work done by Dr. Kozo Morimura.

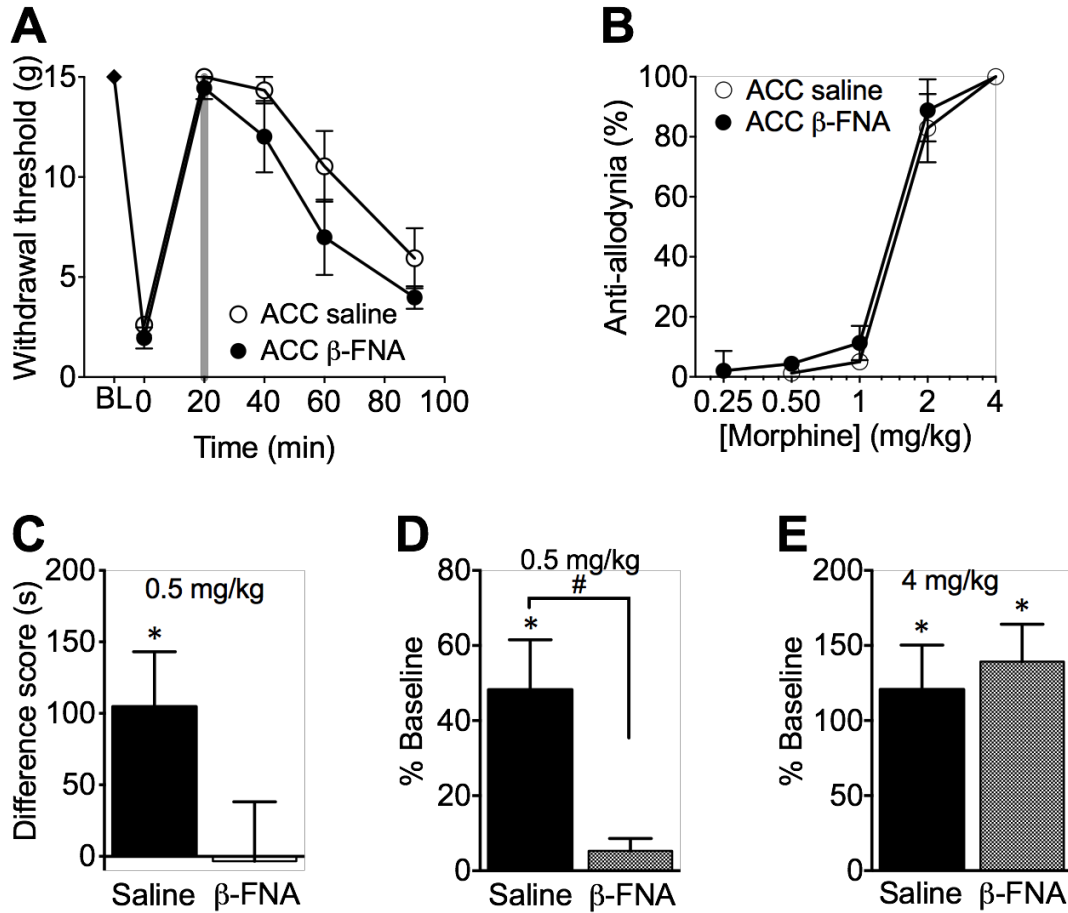
***Low dose morphine induces CPP and NAc DA release in SNL but not sham animals.***

To determine whether the effects of morphine on sensory and affective/emotional dimensions of pain are separable and/or if these effects are separable from addictive effects, we administered a high dose of morphine (4.0 mg/kg, i.v.) that fully reversed tactile hypersensitivity in SNL animals and a low dose (0.5 mg/kg, i.v.) that was ineffective at reversing tactile responses (Figure 4.1 A) and evaluated CPP and NAc DA release in both sham and SNL animals. High (4.0 mg/kg, i.v.) or low (0.5 mg/kg, i.v.) dose of morphine was administered to both sham and SNL animals and paired with a context. Twenty-four hours later (test day) both sham and SNL animals showed significant ( $p < 0.05$ ) preference over baseline ( $91.6 \pm 40.7$  and  $118.9 \pm 56.3$  sec) to the high dose morphine-paired chamber. However, only SNL animals and not sham showed significant preference ( $127.8 \pm 55.2$  versus  $21.1 \pm 38.8$  sec) to low dose morphine-paired chamber as compared with baseline (Figure 4.1 B). Additionally, the same high and low dose of morphine was given to both sham and SNL animals and *in vivo* NAc microdialysis was performed and DA release was assessed (Figure 4.1 C). Both sham and SNL animals showed a significant ( $p < 0.05$ ) increase ( $92.0 \pm 24.3$  % and  $88.8 \pm 34.6$  %) in NAc DA release as compared with baseline in response to high dose morphine. However, in-line with our CPP findings, SNL but not sham animals had significant NAc DA release ( $49.7 \pm 16.1$  versus  $-2.8 \pm 12.8$  %) as compared with baseline (0%) (Figure 4.1 C). These results confirm that morphine at 4 mg/kg dose is capable of activating

reward circuits in both uninjured and injured rats. Additionally, these results indicate that a low dose of morphine, that is ineffective in reversing tactile hypersensitivity, is rewarding in SNL, but not sham rats. The latter may be due to relief of pain unpleasantness.

***Pretreatment with  $\beta$ -FNA in the ACC has no effect on intravenous morphine dose response curve to tactile hypersensitivity in SNL animals***

Tactile hypersensitivity was assessed in animals prior to SNL surgery (i.e., baseline), 12-16 days post SNL surgery and 20-24 hours after pretreatment with ACC  $\beta$ -FNA or saline (i.e., SNL) and again 20, 40, 60 and 90 minutes post-intravenous morphine (Figure 4.2 A,B). SNL surgery produced tactile hypersensitivity which was significantly ( $p < 0.05$ ) reversed by 4.0 mg/kg, i.v. morphine in both groups (saline: N = 6,  $p < 0.001$ ;  $\beta$ -FNA: N = 5,  $p < 0.001$ ). This suggests that blockade of  $\mu$ -opioid signaling in the ACC with  $\beta$ -FNA has no effect on the sensory analgesic mechanisms to morphine.



**Figure 4.2. ACC  $\beta$ -FNA pre-treatment blocks morphine's effects on affective but not sensory aspects of pain.** (A) Rats with SNL were pre-treated 20-24 hours prior to experiment with  $\beta$ -FNA (3  $\mu$ g/1  $\mu$ l) or saline in the ACC and paw withdrawal thresholds were determined following administration of morphine (4.0 mg/kg, i.v.). ACC saline or  $\beta$ -FNA had no effect on morphine's anti-hyperalgesic effects. (Mean  $\pm$  SEM,  $n = 6, 5$ ,  $*P < 0.001$ , One-way ANOVA with Tukey's post-hoc). (B) The dose-response curves were calculated 20 min following morphine. Pre-treatment with  $\beta$ -FNA in the ACC, but not saline, abolished (C) CPP and (D) NAc DA release in SNL animals receiving 0.5 mg/kg, i.v. morphine. Pre-treatment with  $\beta$ -FNA or saline had not effect on (E) NAc DA

release in sham animals to 4.0 mg/kg, i.v. morphine. Mean  $\pm$  SEM,  $n =$  (B) 16-19, (D) 11 and (E) 7-9,  $^{*}P < 0.05$ , Student's paired t-test.

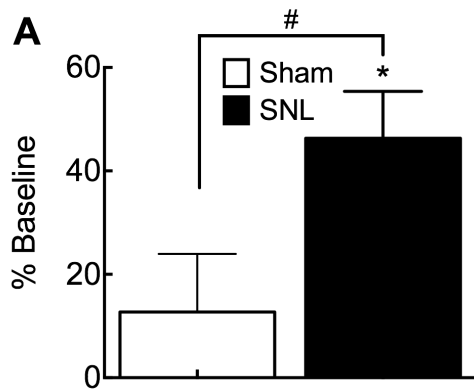
***Pretreatment with  $\beta$ -FNA in the ACC abolishes CPP and NAc DA release in SNL animals***

To determine if opioid signaling within the ACC is critical to morphine's effects on the affective/emotional dimension of pain; SNL animals were pretreated with the irreversible  $\mu$ -opioid antagonist,  $\beta$ -funaltrexamine ( $\beta$ -FNA), or saline and CPP and NAc microdialysis to low dose (0.5 mg/kg, i.v.) morphine was evaluated. Pretreatment with ACC  $\beta$ -FNA or saline was done 20-24 hours prior to i.v. morphine administration. Animals were given low dose morphine (0.5 mg/kg, i.v.) and paired with a context. On the following day, time spent in the morphine-paired chamber was assessed. Consistent with uncannulated animals (Figure 4.1 B), animals receiving ACC saline spent significantly ( $p < 0.05$ ) more time in the morphine-paired chamber as compared with baseline ( $104.8 \pm 38.3$  sec). In contrast, animals receiving ACC  $\beta$ -FNA showed no preference ( $-3.4 \pm 41.4$  sec) for morphine-paired chamber (Figure 4.2 B), demonstrating that ACC pretreatment with  $\beta$ -FNA blocks the anti-aversive effects of low dose morphine. Next, NAc DA release was assessed in animals receiving the same treatment. Similarly to uncannulated rats (Figure 4.1 C), animals receiving ACC saline pre-treatment had a significant ( $p < 0.05$ ) increase in DA release ( $48.2 \pm 13.3$  %) in response to low dose (0.5 mg/kg, i.v.) morphine as compared with baseline (Figure 4.2 C), while animals receiving ACC  $\beta$ -FNA demonstrated no increased release in DA ( $5.3 \pm 11.0$  %). The difference between the ACC saline and  $\beta$ -FNA pre-treatment was statistically significant indicating that  $\beta$ -FNA blocked the effects of low dose morphine on DA efflux in SNL rats. In addition, NAc DA

release was evaluated in sham animals to high dose (4.0 mg/kg, i.v.) morphine following ACC pretreatment with either  $\beta$ -FNA or saline; both groups demonstrated significant ( $p < 0.05$ ) NAc DA release (saline:  $120.7 \pm 29.5$  %;  $\beta$ -FNA:  $139.2 \pm 24.9$  %) as compared with baseline (0 %) suggesting that blockade of  $\mu$ -opioid signaling has no effect on DA release in animals without pain (Figure 4.2 D).

***Microinjection of morphine into the ACC produces NAc DA release in SNL but not sham rats***

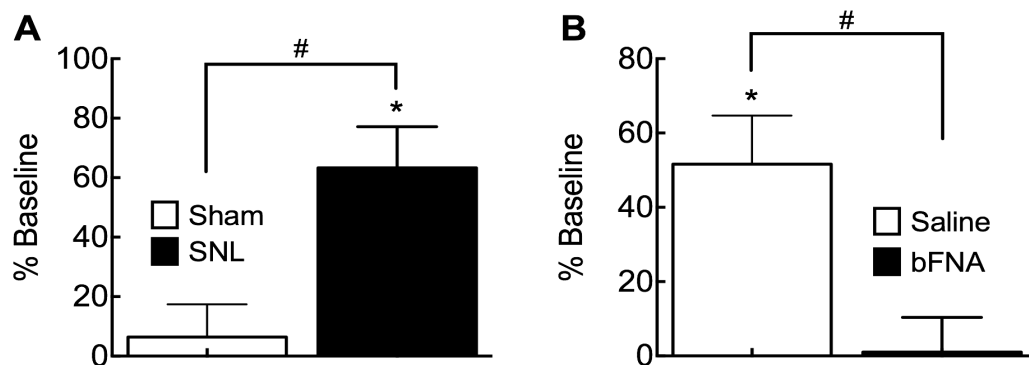
Morphine (1  $\mu$ g per side) was bilaterally injected into the ACC of sham and SNL animals and NAc DA release was evaluated to further investigate the role of opioid signaling within the ACC. In line with our previous findings; ACC morphine elicited significant DA release in SNL ( $46.3 \pm 9.1$  %) animals as compared with baseline and sham ( $12.7 \pm 11.3$  %) animals (Figure 4.3). These results are consistent with other findings in this study and further emphasize the role the ACC plays in modulating pain aversiveness.



**Figure. 4.3. ACC morphine relieves aversiveness of pain but does not directly activate mesolimbic reward pathways.** (A) Rats with SNL or sham surgery received 1 $\mu$ g bilateral intracranial ACC morphine and increased NAc DA release was observed in SNL rats and not sham. Mean  $\pm$  SEM,  $n = 8-9$ , \*  $P < 0.05$ , Student's paired t-test.

***Reward from pain relief of intravenous gabapentin is dependent on opioid receptors in the rACC***

Intravenous (i.v.) gabapentin (50 mg/kg) produced reward of pain relief in animals with experimental neuropathic pain as demonstrated by NAc DA efflux. Administration of i.v. gabapentin caused an increase ( $63.3\% \pm 13.8$ ;  $n = 12$ ) in DA efflux in the NAc which was significantly ( $p < 0.05$ ) increased from sham animals ( $6.4\% \pm 11.0$ ;  $n = 11$ ) (Figure 4.4 A). Next, animals with experimental neuropathic pain (SNL) were pretreated with microinjection of the irreversible  $\mu$ -opioid antagonist  $\beta$ -FNA ( $3 \mu\text{g} / 1\mu\text{l} / \text{per side}$ ) or sterile saline ( $1 \mu\text{l}$  per side) in the rACC 20-24 hours prior to i.v. gabapentin administration. Saline microinjection into the ACC had no effect on DA release in SNL animals ( $51.6\% \pm 13.06$ ;  $n = 8$ ), however, microinjection of  $\beta$ -FNA attenuated DA release in SNL animals ( $1.0\% \pm 9.4$ ;  $n = 9$ ) (Figure 4.4 B).



**Figure 4.4. Pre-treatment with rACC  $\beta$ -FNA prevents pain relief induced DA release in injured rats by intravenous gabapentin.** (A) i.v. gabapentin (50 mg/kg) produces pain relief induced DA release in the NAc preferentially in SNL rats but not sham. Mean  $\pm$  SEM,  $p < 0.05$ ,  $n = 11, 12$ . (B) rACC pretreatment with  $\beta$ -FNA (3  $\mu$ g/1  $\mu$ L per side) and not saline (1  $\mu$ L per side) abolished pain relief induced DA release in the NAc. Mean  $\pm$  SEM,  $p < 0.05$ ,  $n = 8, 9$ .

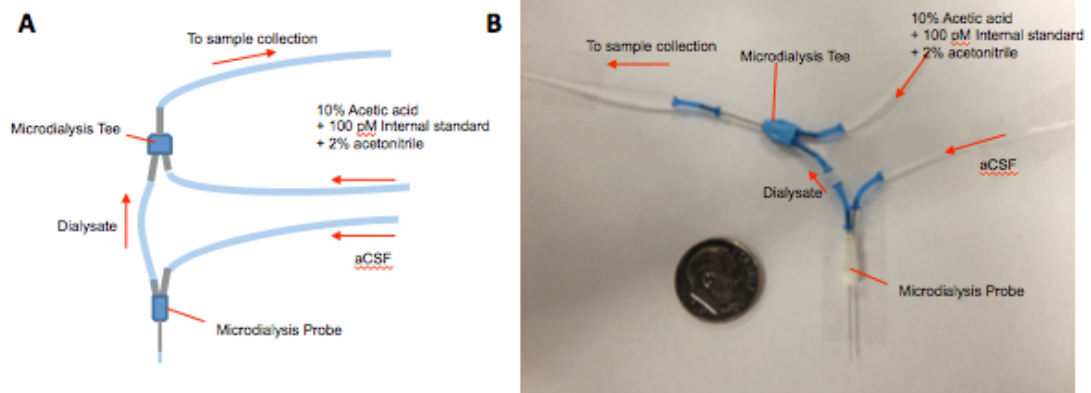
***Capillary liquid chromatography coupled with multistage mass spectrometry (LC-MS) allows for successful separation and quantification of YGGFM and YGGFL***

Microdialysate samples were collected and frozen immediately. Immediately prior to mass spectrometry analysis samples were thawed and underwent a “zip-tip” clean up (details can be found in methods section). Using LC-MS methods outlined in Chapter 2, YGGFM, YGGFL and DADLE internal standard were successfully quantified from microdialysate collected from the ACC. An internal standard calibration (Figure 4.6 A; YGGFM:  $y = 0.0464x + 0.0028$ ;  $R^2 = 0.99994$ ; YGGFL:  $y = 0.457x + 0.0223$ ;  $R^2 = 0.99999$ ) and external calibration (Figure 4.6 B; YGGFM:  $y = 178.09x + 1$ ;  $R^2 = 0.99944$ ; YGGFL:  $y = 175.05x + 2.667$ ;  $R^2 = 0.99913$ ) were created and from those a lower limit of quantification (LOQ) was calculated to be 10 attomoles, which corresponds to a 10  $\mu$ L sample volume with a 1 pM concentration limit of quantification. This represents the first time opioid enkephalins have been successfully measured in the ACC of rats using *in vivo* microdialysis and a LC-MS approach.

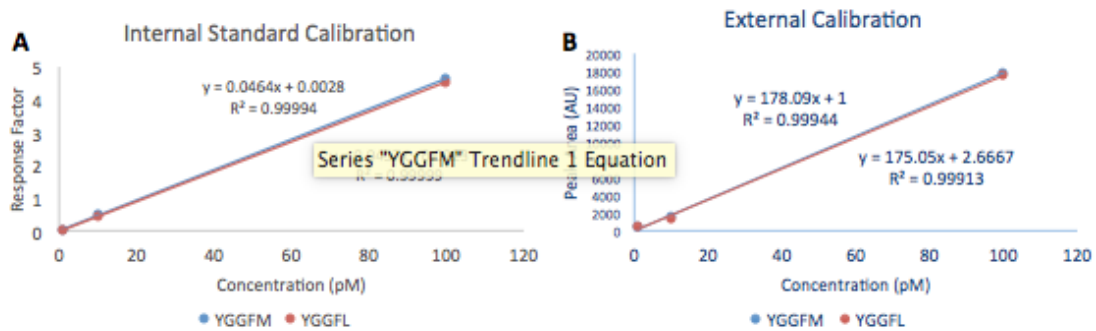
***Online-peptide-preservation system achieves significant increases in absolute signal (peak area in LC-MS experiments)***

Acetic acid mix was used in all experiments to inhibit the degradation of YGGFM and YGGFL by reducing pH, acetic acid is also an ideal choice as it works well in LC-MS analyses (246). In the “standard method” for analysis, microdialysate from the ACC was collected into chilled (4°C) siliconized tubes containing the

acetic acid mix. Artificial cerebrospinal fluid (aCSF) was perfused at a rate of 0.5  $\mu\text{L}/\text{min}$  meaning, in the standard method, it took approximately 10 min for the microdialysate containing YGGFM and YGGFL to reach the collection tube and the acetic acid mix. The half-life of YGGFM and YGGFL is approximately 15 min in preliminary experiments in mouse serum, indicating it is likely that substantial degradation in opioid peptides occurs before the sample has even reaches the collection tube with this approach. In order to decrease degradation of YGGFM and YGGFL an online preservation system was designed to allow for mixing of the acetic acid mix as quickly as possible after the dialysate moved past the microdialysis membrane. The online preservation system was designed and manufactured in house as described in the methods section and shown in Figure 4.5 A and B. The online preservation system significantly (YGGFM:  $p = 0.02$ ; YGGFL:  $p = 0.05$ ) increases the absolute signal (peak area in LC-MS experiments) versus the standard method for both YGGFM ( $4656.4 \pm 1116.0$  versus  $281.2 \pm 112.4$ ;  $n = 3, 3$ ) and YGGFL ( $7446.4 \pm 2358.5$  versus  $913.7 \pm 254.5$ ;  $n = 3, 3$ ).



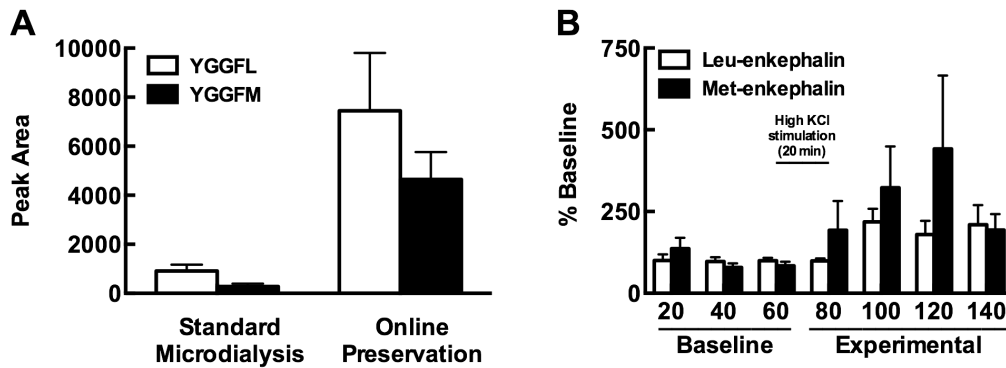
**Figure 4.5. Online-peptide preservation system.** Diagram (A) and picture (B) of online preservation system set up for use in measuring endogenous opioid system.



**Figure 4.6.** LC-MS method can successfully quantify enkephalins in the anterior cingulate cortex. Representative LC-MS trace of YGGFM, YGGFL and the internal standard DADLE (A). Standard curves were generated to quantify unknown sample amounts to the internal standard (B) and an external standard (C), from that the lower limit of detection was calculated at 10 attomoles which corresponds to a 10  $\mu$ L sample volume with a 1 pM concentration limit of quantification.

***High KCl perfusion into the ACC through the microdialysis membrane causes a significant increase in YGGFM and YGGFL that is detectable by our LC-MS method***

Following baseline fraction collection a high KCl (75mM) solution was perfused through the microdialysis probe into the ACC of awake and freely moving rats for 20 mins. High KCl was perfused to cause a depolarization of the neurons in the ACC resulting in release of neurotransmitters, including YGGFM and YGGFL. 3 more fractions were collected following high KCl perfusion and analyzed for changes in YGGFM and YGGFL. High KCl perfusion produced a significant increase in YGGFM and YGGFL over time (Figure 4.7 B;  $p = 0.0049$ ;  $n = 6$ ).



**Figure 4.7. Online preservation system increases absolute signal intensity and allows for detectable changes in YGGFM and YGGFL levels in the ACC.**

Online peptide preservation system achieves significant increases in absolute signal (peak area in LC-MS experiments) through prevention of degradation pathways (B). Stimulation with high KCl (75mM) buffer in the ACC shows a significant increase in YGGFM and YGGFL levels (C). YGGFM signal is calculated from a sum of the met-enkephalin and the related sulphoxide metabolite/degradation product.

## Discussion

The affective dimension (i.e., unpleasantness of pain) is a crucial component to the human experience of pain. However, it is difficult to capture in nonverbal animal models and most preclinical models rely on sensory (i.e., reflexive) measures. Previous work from our laboratory demonstrates that the removal of an aversive state promotes motivated behavior (measured by CPP) and activates mesolimbic reward circuits (i.e., NAc DA release measured using *in vivo* microdialysis) (55, 229, 247-249). Through the use of these behavioral (CPP) and neurochemical (*in vivo* microdialysis) analyses we are able to unmask ongoing (i.e., spontaneous) pain by demonstrating that its removal is rewarding (i.e., CPP and NAc DA release) allowing us to better assess the affective dimension of pain.

### ***The effects of morphine on sensory and affective/emotional dimensions of pain are separable***

We hypothesized that it is possible to discriminate between the pharmacological effect of morphine on sensory and affective dimensions of pain by assessing the effects of varying doses on evoked (i.e., sensory; von Frey) versus affective (i.e., CPP and *in vivo* microdialysis) analyses in a rat model of experimental neuropathic pain (SNL). Our findings show that a low dose of morphine (0.5 mg/kg, i.v.) is sufficient to relieve the unpleasantness of pain as demonstrated by the significant CPP and NAc DA release seen in SNL but not sham animals (Figure

4.1 B and C) while having no effect on tactile hypersensitivity (Figure 4.1 A). Interestingly, it took a dose of morphine almost an order of magnitude higher (4 mg/kg, i.v.) to fully reverse tactile hypersensitivity in SNL rats. Additionally, this dose produced rewarding effects (i.e., CPP and NAc DA release) in both sham and SNL animals (Figure 4.1 B and C) likely due to this dose's ability to increase DA release to a greater extent (~125%) than low dose (~50%). This also suggests that high dose morphine is likely sufficient to directly activate addictive reward pathways. These results are consistent with previous preclinical studies that also demonstrate that a dose of morphine that is ineffective at reversing tactile hypersensitivity can still attenuate pain unpleasantness (i.e., pain affect) (234-237). In addition, these findings also corroborate what is observed clinically in that treatment of pain affect (i.e., unpleasantness) with low doses of morphine is often sufficient to provide relief to patients (92, 93, 250). Further, a recent study demonstrated that morphine dose-dependently increases CPP in sham animals but in animals with experimental neuropathic injury the dose-response has a bell-shaped curve in which a low dose morphine (2 mg/kg, i.p.) produces greater CPP than a high dose (8 mg/kg) (251). The results from this paper are in line with our study in that they indicate that a low dose of morphine is sufficient to alleviate neuropathic pain in injured animals resulting in CPP, further that motivation for opioid-induced reward is different in injured and uninjured states (251).

***The effects of morphine on pain are separable from addictive effects***

Morphine directly activates mesolimbic dopamine (DA) reward pathways comprising dopaminergic neurons in the VTA and their projections to the NAc. Robust activation of this circuitry in uninjured state is associated with the addictive qualities of morphine. In addition to morphine and other positive reinforcing drugs; natural rewards and reward-predicting cues all consistently demonstrate activation of mesolimbic dopaminergic neurons using electrophysiological (252), *in vivo* microdialysis (253, 254) and behavioral measures (255-257). Additionally, there are distinct patterns of neuronal activity associated with positive reinforcement and learned appetitive behaviors that further highlights the role of the VTA in these forms of reward (258). However, the role of the VTA in pain relief associated reward is less clear.

Our laboratory previously demonstrated that following termination of an aversive state (i.e., peripheral nerve block (PNB) in rat postsurgical model of pain) VTA dopaminergic neurons are activated and DA efflux in the NAc occurs which indicates a population of midbrain DA neurons are also activated (55). Interestingly, VTA microinjection of naloxone (nonselective opioid receptor antagonist) is insufficient to attenuate PNB-induced CPP (55). This is particularly interesting in that endogenous opioids in the VTA are known to underlie the positive reinforcing effects of addictive drugs (259). Together, this suggests that while reward of pain relief share similarities with appetitive

rewards the apparent lack of involvement of the endogenous opioid system within the VTA indicates there are likely important differences as well.

Functional imaging studies in humans have indicated that the ACC is highly involved in processing the unpleasant affective aspects of pain (68, 224, 225, 227) as well as placebo analgesia (87). Similarly, preclinical rodent models have also implicated the involvement of the ACC in processing the affective, but not sensory, dimensions of pain (81, 229). Activation of the ACC through the spinothalamic pathway via nociceptive inputs from neurons located in the thalamus is likely responsible for processing the unpleasantness of pain in humans (260). Accordingly, the ACC projects, either directly or via other limbic regions in a similar fashion as the amygdala, to the mesolimbic reward system (261, 262). Collectively, these data implicate an important role of the ACC in pain affect processing and, potentially, the reward of pain relief.

Our findings further implicate the role of the ACC, specifically endogenous opioid signaling in this region, in processing of pain affect and the reward of pain relief. Pretreatment with  $\beta$ -FNA, an irreversible  $\mu$ -opioid antagonist, in the ACC does not alter morphine's effects on sensory (i.e., evoked) measures of pain (Figure 4.2 A) in SNL animals or NAc DA release in sham animals (~125%) receiving high dose morphine (Figure 4.2 D). Interestingly, pretreatment with  $\beta$ -FNA in the ACC is sufficient to abolish CPP and NAc DA release in SNL animals to low dose morphine (Figure 4.2 B and C). Moreover, microinjection of morphine into

the ACC produces NAc DA release in SNL but not sham animals (Figure 4.3). These results indicate that distinctive circuits, including mesolimbic reward pathways, and opioid signaling within the ACC play a crucial role in modulation of the affective/emotional dimension of pain and it is independent from circuits that process the sensory dimensions of pain. Further, these results demonstrate a dose-dependent spectrum of morphine where low doses are sufficient to alleviate the aversiveness of pain but do not induce positive reinforcing effects while higher doses will directly activate “addictive” reward pathways.

These data suggest that the effects of morphine on sensory and affective/emotional dimensions of pain are separable. This finding is consistent with previous preclinical studies that also suggest that morphine has differential effects on sensory and affective dimensions of pain (234, 237, 238, 251). Additionally, the effect of morphine on affective dimensions of pain (i.e., the anti-aversive effect) is also separable from mechanisms that are associated with addictive effects in uninjured animals that occur only at doses an order of magnitude higher. Cahill et al. reported that the CPP dose-response to morphine is linear in uninjured animals but bell-shaped in animals with chronic neuropathic pain, suggesting that motivation for opioid induced reward is different in animals with injury (251). We demonstrated that the rewarding effects of morphine are not dependent on the ACC while the anti-aversive effects were abolished by blockade of opioid receptors in the ACC. Finally, microinjection of morphine in the ACC is sufficient to alleviate pain

unpleasantness without affecting evoked hypersensitivity and without activating reward circuits in uninjured state. Our results suggest that it is possible to pharmacologically modulate pain aversiveness and to produce pain relief without the need to activate addictive pathways. Ultimately, non-opioid analgesics that specifically target anti-aversive (i.e., ACC) circuits may be developed without risk of addiction.

### ***The role of endogenous opioid neurotransmission in the rACC in non-opioid analgesia***

Results from the first section of this chapter indicated that opioid signaling in the rACC is required for morphine to relieve the affective dimension of pain. Next, we investigated whether endogenous opioid signaling in the rACC is also required for reward of pain relief produced by a non-opioid analgesic. Unlike opioids, gabapentin has no intrinsically rewarding properties, yet in the setting of ongoing neuropathic pain i.v. administration produced reward in SNL animals but not in sham animals (Figure 4.4 A). Our group and others have demonstrated that the relief of pain is rewarding, including oral gabapentin (48, 55, 244), so it is no surprise that i.v. gabapentin in a rat experimental neuropathic pain model elicited DA release in the NAc. Interestingly, we show that pretreatment of the ACC with microinjection of  $\beta$ -FNA, an irreversible  $\mu$ -opioid antagonist, prior to i.v. administration of gabapentin (50 mg/kg) is sufficient to block NAc DA release in injured animals (Figure 4.4 B). These data

shows that endogenous opioid release in the rACC is likely to be necessary for relief of the aversiveness of ongoing pain.

***Methodological development of an in vivo microdialysis technique for the evaluation of endogenous opioid release in the ACC***

A literature review indicates that the data presented in this chapter is the first instance that opioid enkephalin peptides, YGGFM and YGGFL, have been successfully measured in the ACC using an LC-MS approach. The method presented here was adapted, and improved for our purposes, from previous reports of successful analysis of microdialysate for YGGFM and YGGFL from the rat GP and striatum (245, 246, 263). The major improvement in our method was including an online-peptide-preservation system (Figure 4.5 A and B). This addition significantly increased the absolute signal (peak area) of both YGGFM and YGGFL through prevention of various degradation pathways. This improvement occurs through the addition of the acetic acid mix that inhibits enzymatic degradation processes and reduces adsorption to the interior walls of the microdialysis lines by reducing the pH of the microdialysate. Researchers have previously shown that the introduction of acetic acid solution can preserve enkephalins for up to 5 days without significant degradation (246). Additionally, introduction of the antioxidant species ascorbic acid or D,L-methionine can reduce the extent of oxidative degradation during sample collection.

Another advantage of the online preservation system is that it allows for constant delivery of an internal standard agent during microdialysis collection. The use of DADLE as an analog type internal standard represents an improvement over other reported techniques that use no internal standard to account for variation (245, 246, 263). DADLE is particularly useful as it is non-endogenous so we can assume 100% of the signal comes from the introduction of the internal standard. Further, because the online preservation-mixing tee occurs after the dialysate is recovered from the implanted probe, there is no danger of DADLE or any of the preservation agents diffusing across the microdialysis membrane and having a deleterious effect on the animal or the integrity of the experiment.

The ability to measure changes in endogenous opioids using *in vivo* microdialysis in the ACC is a significant methodological advancement in general and also in terms of the project presented earlier in this chapter. Prior to the development of this technique it was only possible to infer, through pharmacologic manipulations, the role endogenous opioids in the ACC play in relieving the affective dimension of pain. While these experiments are useful they do have their own limitations, such as potential for unknown off target effects of the drug, uneven spread and simply that they lack the ability to directly assess the local physiology. This advancement allows us to directly monitor real-time changes in YGGFM and YGGFL in the ACC.

### ***Biological significance and experimental potential***

Development of a technique that allows for the accurate measurement of endogenous opioid peptides YGGFL and YGGFM in the ACC allows us to directly measure release. This complements previous pharmacological techniques. The first question to ask would be: are there any differences in baseline levels of YGGFM and/or YGGFL in the rACC between injured and uninjured animals? It has been proposed that changes in the descending inhibitory system are likely upregulated with persistent functional pain (18) and that over the course of abnormal chronification of pain these systems begin to fail (18, 264, 265). Likewise, multiple human studies have indicated lower endogenous opioid levels and diminished endogenous opioid analgesic activity in chronic pain patients compared to healthy individuals (265-270). Specifically, two of these studies have shown an inverse correlation between endogenous opioid levels in cerebrospinal fluid with duration of chronic pain indicating that progressive dysfunction of the endogenous opioid system is linked to chronification of pain (266, 268). Additionally, human PET studies using nonselective opioid receptor radiotracers suggest there is endogenous opioid release, down-regulation of opioid receptors or both in patients with persistent painful conditions that have been studied before and after treatment (271-273). Collectively, these data suggest that there is likely down-regulation of the endogenous opioid system in chronic pain, meaning that there are likely to be measureable differences in baseline YGGFM and YGGFL levels between rats with chronic pain and rats without. Further, it is possible to explore any changes in tonic release of YGGFM

and YGGFL before (baseline) and at various time points following nerve injury. Any observed changes could give us a better understanding of the maladaptions that occur in the endogenous opioid system with chronic pain on a timeline.

Lastly, this technique can be used to evaluate the effect both systemic opioid and non-opioid analgesics have on endogenous opioid enkephalin release in the ACC. As previously mentioned, pharmacologic antagonist studies in the ACC are strongly suggestive of this system playing a crucial role in relief of pain affect and this new method will further confirm these findings. Overall, this method is highly valuable, very unique and has the ability to provide answers to many unanswered questions in regards to the involvement of the endogenous opioid system in pain chronification and central analgesic action.

## Chapter 7: General Discussion

The sensory dimension of pain relays information about the intensity, location, source (i.e., epidermal versus visceral) and quality of pain (i.e., pinch versus burn). The sensory dimension of pain is closely linked to activation of nociceptors by mechanical, thermal or chemical noxious stimuli (92). Until recently, most preclinical models of pain focus on sensory (i.e., reflexive) endpoints. These models can be useful for certain types of acute pain, however they fail to mimic aspects the human experience of pain, especially for ongoing pain-type categories (i.e., chronic neuropathic pain). Nociceptor activation has a nonlinear relationship with the experience of pain, indicating the ability of an organism to modulate (i.e., enhance or inhibit) pain (42, 43). Further, pain can occur without identifiable nociceptor activation (i.e., chronic neuropathic pain, fibromyalgia, irritable bowel syndrome, etc.), conversely, nociceptive activation can fail to produce pain (274). The affective dimension of pain, or aversiveness of pain, is crucial to survival as it promotes withdrawal from a harmful stimulus and also facilitates learning to avoid certain stimuli in the future (45). Clinically, it is the aversiveness of pain (i.e., spontaneous or ongoing pain) that causes most patients to seek treatment, meaning, that in order to successfully treat pain one must alleviate the aversiveness of pain (275, 276). In the preclinical realm, this may suggest that in order to improve translation from the bench-top to the bedside one needs preclinical models that capture the affective dimension of pain (i.e., ongoing pain). The work presented in this dissertation attempts to not

only provide insight to scientific questions but also to demonstrate that we have developed preclinical models with greater translation.

***Potential reasons for lack of translation in preclinical pain models***

Over the past few decades there have been hundreds of novel targets identified for the treatment of pain, yet to date only a handful of these targets have resulted in clinically successful analgesics. It is likely that lack of translation from the bench-top to bedside is, at least partly, to blame for these failures. There are a number of factors that might contribute including (i) lack of robust translational assays across species and relevant pain conditions, (ii) difficulties in preclinical target assessment, (iii) few clinically qualified biomarkers and (iv) small effect sizes in clinical trials compared to placebo groups (277).

Human populations used in clinical testing are largely heterogenous in terms of genetic similarities, previous and concurrent medication use and mechanism underlying pain conditions (278). This is in contrast to the largely homogenous animal populations used in preclinical testing and could likely confound differences between conclusions drawn from each group and adds to the lack of translation. Additionally, most preclinical animal models rely heavily on reflexive (i.e., withdrawal) endpoints while human clinical pain is largely ongoing (i.e., spontaneous). Human pain also involves emotional and cognitive modulatory factors and human measures generally rely heavily on quality-of-life outcomes; preclinical models often fail to capture these measures. It has not

been until recently that researchers have begun to accept that preclinical assessments such as hot plate, tail flick, von Frey or Hargreaves provide little real-world translation to the human experience of pain and should be limited to appropriate situations such as pharmacokinetic/pharmacodynamic studies or acute settings in which humans also develop allodynia and/or hyperalgesia (277). These factors combined are a likely culprit behind the lack of translation between preclinical and clinical trial findings. Luckily, researchers, including our lab, have been making strides by developing more robust preclinical models and output measures that more closely relate to the human experience of pain, these models will be discussed in greater detail later in this chapter.

Two additional caveats of preclinical models that could account, in part, for some of the failure to find robust preclinical assays is that (i) most animal models of chronic pain are tested days or weeks post-injury while human chronic pain lasts for years and (ii) the etiology of injury models may not be adequate. The latter two issues are harder to address; first, most preclinical models test 14 days or less post-injury. There is evidence to suggest that the quality of the pain is the same at day 14 as it is at much later dates. Conversely, it has been reported that there are anatomical changes (i.e., gray matter volume changes in multiple brain regions) that occur for months, in a similar fashion to humans, following a neuropathic injury in rats and could likely effect the validity of these models (279). Additionally, most preclinical injury models are chosen because they are effective at creating pain in almost every animal and not based on a

similar etiology to human injury. For example, in the case of chronic neuropathic pain, only a very small percentage of nerve injuries result in long-term chronic neuropathic pain in humans, while rodent models (i.e., chronic constriction injury, SNL, etc.) produce neuropathic pain in almost 100 percent of the animals. This suggests that there may be a disconnect between animal models of chronic neuropathic pain and human incidence. Other models, such as post-surgical incisional or inflammatory, may have better translation to the human experience as they more closely mimic the injury.

Preclinical target assessment for analgesic drugs is another major hurdle owed consideration in this discussion. Pain is a complex biological process with the primary purpose being survival, meaning that there is ample redundancy and overlap in the biological workings of the system. This redundancy makes drug discovery efforts increasingly more difficult as the most common approaches focus on single protein modulation resulting in drugs that are likely to be insufficient in patient populations (277). Many successful analgesics on market today act on multiple pathways, targets and/or receptors that further highlights the need for pharmacological agents with multiple mechanisms of action. Preclinical data can be obtained from a variety of assays including *in vitro* and *in vivo* assays that can assess anything from function to confirmation of efficacy in pain models, however, ultimately validation only truly occurs in humans. However, validating targets in humans is often unrealistic due to ethical concerns, making this approach to drug discovery unreliable at best.

Preclinical research is not solely to blame for the failure to bring successful analgesics to market, as discussed in greater detail in Chapter 1, analgesic clinical trials have their own set of limitations. Effects sizes in analgesic clinical trials are generally very small making successful trials increasingly difficult to accomplish. There are great efforts by groups such as Analgesic, Anesthetic, and Addiction Clinical Trial Translations, Innovations, Opportunities, and Networks (ACTTION), a public-private partnership with the United States Food and Drug Administration (FDA), to find ways to improve assay sensitivity and efficiency by analyzing research methods. However, to date there is still tremendous failure rates in analgesic clinical trials, even with drugs that are known to be efficacious.

Despite all of these issues, drug discovery efforts are still greatly needed in an attempt to meet unmet needs to pain patients worldwide. Much has been learned from preclinical efforts about disease mechanisms, fundamental adaptive changes in the periphery and CNS, identification of molecular targets, etc. Similarly, advances in the methodology of analgesic clinical trials have made trials more successful. As preclinical and clinical researchers continue to collaborate to improve translation from the bench-top to the bedside each of these issues should continue to improve translational outcomes.

## **Preclinical models that more accurately portray the human occurrence of pain**

The three most common models of chronic neuropathic pain in rodents are chronic constriction injury (CCI), spinal nerve ligation (SNL) model and partial sciatic ligation model (280). The model discussed throughout this dissertation and most commonly used in our lab is the SNL model that consists of a tight ligation of L5/L6 spinal nerves and results in allodynia and hyperalgesia in the associated hind paw. Sprague-Dawley (SD) rats are the most commonly used strain for pain studies and these animals develop SNL-induced tactile allodynia in 85% or more following this surgery (198). Interestingly, in patients peripheral nerve injury only produces chronic pain in a very small subset of patients. However, in a related strain of rats, Holtzman (HZ), only about 50% of these animals develop SNL-induced tactile allodynia (198). Using these rats in this model represents an opportunity to more closely mimic what is observed in humans. Likewise, it provides a chance to better assess the differences between those that do and do not develop neuropathic pain. Recently our lab demonstrated that engagement of descending inhibition from the rostral ventromedial medulla (RVM) protects against the development of chronic neuropathic pain in HZ rats that do not have SNL-induced allodynia (209). These results corroborate what is already suggested in the literature; that a disruption of descending modulatory pathways likely plays a crucial role in the development of chronic neuropathic pain (41, 281).

These findings in HZ rats highlights the importance of descending inhibition and its' protective nature in the development (or lack) of chronic neuropathic pain. Many current therapies on market today exploit the descending inhibitory system including  $\alpha_2$  agonists (e.g., clonidine, tizanidine, etc.) (282), norepinephrine reuptake inhibitors (NRI; e.g., duloxetine, etc.) (283), or combination  $\mu$ -opioid agonists/NRI (e.g., tapentadol) (186). Chapter 3 investigated how three clinically effective analgesics (duloxetine, tapentadol and morphine) exploited endogenous mechanisms of descending inhibition to increase noradrenergic tone (measured as an increase in NE concentration in spinal CSF). The use and availability of drugs that mimic or enhance descending inhibitory mechanisms speaks to the highly successful research completed in this area over the past few decades. Interestingly, drugs that interfere specifically with descending facilitation are not clinically available (18) though morphine and other opiates diminish descending facilitation and enhance descending inhibition. While a lot is known about descending inhibitory mechanisms this area is far from exhausted, though a shift towards understanding affective/emotional dimensions of pain has begun in many laboratories around the world.

***Preclinical models to assess ongoing pain that have better clinical translation***

In recent years, our lab and others have adapted the rodent conditioned place preference (CPP) model, that was traditionally used for addiction studies to

assess rewarding effects of drugs of abuse, to study the reward from pain relief (60, 61). Relief of pain is rewarding, so analgesic agents that are not intrinsically rewarding will only become rewarding in the setting of ongoing pain. Additionally, activation of mesolimbic reward pathways by the relief of pain underlies the motivational drive for all organisms to seek pain relief (55). Further, this motivational drive to seek relief facilitates learning to avoid injury or behaviors that cause harm. Thus, reinforcement of behavior occurs not only to maximize benefit (positive reinforcement) but also by the removal of an aversive stimulus (i.e., reduce loss or injury; negative reinforcement). The CPP learning approach can be used to exploit pain-induced motivation (i.e., negative reinforcement) allowing evaluation of (i) the time-dependent nature of ongoing pain in rodent pain models, (ii) the neural mechanisms underlying pain relief-motivated behavior, and (iii) the efficacy of molecular targets for relieving ongoing pain (50, 284). This approach is unique in that it allows us to assess relief of ongoing, aversive, pain and not reflexive (i.e., sensory) endpoints. The development of this approach for our purposes is a crucial step towards better translation of preclinical output measures.

Complementary to CPP assays, *in vivo* microdialysis allows for direct measurement of neurochemical outputs. Using microdialysis techniques one can measure neurotransmitter release in real-time including, but not limited to, DA in any number of specific brain regions or spinal cord. To date, we have most commonly used this technique to assess DA release in the NAc to analgesic

drugs, but, as mentioned in Chapter 4, we have also adapted this method to look at release of endogenous opioids. Increases in NAc DA release has been demonstrated with relief of ongoing pain in a variety of rodent pain models; neuropathic (244), incisional (55, 244) and cancer pain (unpublished). Historically, this technique was used to establish that drugs of abuse elicit NAc DA release (118). In addition to use as a neurochemical assay to complement CPP, this technique provides a preclinical model that evaluates activation of mesocorticolimbic reward circuits (i.e., NAc DA efflux) in a variety of animal pain models and has the potential to represent an objective neurochemical assay that could serve as a biomarker of efficacy for novel analgesic mechanisms. This approach will allow preclinical researchers to rely less on tricky behavioral and reflexive models, or use them as a complement to these models, which will reduce the likelihood of an efficacious analgesic agent failing in preclinical models due to issue intrinsic to animal models. This development is a major milestone in the fight to address translational issues between preclinical and clinical models.

***Using CPP and in vivo microdialysis to understand the brain circuits involved in the reward of pain relief***

Previous work in our lab, and others, has provided an abundance of data to support the hypothesis that relief of ongoing pain (incisional, neuropathic, osteoarthritic and inflammatory pain) produces CPP and confirms that pain relief elicits reward (55, 244, 247-249). Using an incisional model of ongoing

post-surgical pain our lab has shown that peripheral nerve block (PNB) induced CPP requires activation of dopaminergic neurons from the ventral tegmental (VTA) to the nucleus accumbens (NAc) (55). Specifically, microinjection of lidocaine (blocks neuronal activity including fibers of passage) or baclofen (a GABA<sub>B</sub> receptor agonist known to inhibit dopaminergic neurons and reduce NAc DA release) into the VTA blocked CPP in animals with post-surgical pain. Interestingly, microinjection of naloxone in the VTA to block endogenous opioid signaling had no effect on CPP even though this mechanism is well established to underlie the positive reinforcing effects of drugs of abuse, we will come back to this point later in this chapter (259). Additionally, we reported that blockade of dopaminergic activity in the NAc with either lidocaine or flupenthixol (non-selective DA receptor antagonist) is sufficient to abolish CPP in the aforementioned model (55). These data provide direct evidence for a causal relationship between negative reinforcing effects on pain relief and mesostriatal circuit activation.

As mentioned previously, blockade of endogenous opioid signaling in the VTA with microinjection of naloxone had no effect on CPP (55). This is contrary to what most would speculate as it has been well established that endogenous opioid signaling in the VTA is required for the reinforcing effects of drugs of abuse (259). These results sparked new speculation about what brain circuits might underlie the reward of pain relief. Various brain regions have been identified through human and animal studies in processing the affective

dimensions of pain (i.e., pain aversiveness) including the prefrontal cortex (PFC), insula, amygdala, striatum (dorsal and ventral) and anterior cingulate cortex (ACC) (48, 68). The ACC receives nociceptive inputs from the spinal cord by way of the thalamus and is consistently activated in response to acute noxious stimulation (47). However, even though increased activity in the ACC has been correlated with noxious stimuli, rostral ACC (rACC) lesion fails to alter evoked behavioral pain responses, but it does abolish pain-induced aversive behavior (i.e., effects the affective and not the sensory dimensions of pain) (80, 81, 229). Human imagining studies have also identified activation of the ACC during termination of a noxious stimulus (56), placebo-analgesia (49) and following relief of neuropathic pain (239, 285). More specifically, activation of endogenous opioid signaling in the ACC has been implicated during sustained experimental pain (228) but most importantly during placebo analgesia (226, 286). Collectively, this indicates an important role for endogenous opioid signaling in the ACC in processing and relieving affective dimensions of pain. We hypothesized that dopamine neurotransmission in the NAc in response to the relief of ongoing pain (i.e., reward of pain relief) requires endogenous opioid signaling in the ACC.

An incisional model of post-surgical pain with PNB and spinal nerve ligation (SNL) model of chronic neuropathic pain with intrathecal (i.th.) clonidine are both sufficient to preferentially produce CPP and NAc DA release in animals with ongoing pain (287). Neither i.th. clonidine or PNB have any intrinsically

rewarding properties, indicating that these rewarding effects are due solely to the relief of ongoing pain. Interestingly, we show that this reward from pain relief is dependent on opioid receptors in the ACC by demonstrating that microinjection of naloxone or ablation of MOR expressing neurons with dermorphin-saporin (DERM-SAP) in the ACC is sufficient to attenuate CPP and NAc DA release in both models (287). Further, opioid signaling in the rACC preferentially activates reward circuits in injured animals (SNL and incision) as shown by CPP and NAc DA release in injured animals to microinjection of morphine into the rACC (287). Further, NAc pretreatment with flupenthixol (non-selective DA receptor antagonist) is sufficient to abolish CPP produced by microinjection of morphine in the rACC in neuropathic rats, further suggesting the role of the rACC on reward circuits in injured states (287). These results are consistent with human imaging studies that indicate an essential role of endogenous opioid signaling in the ACC. Further, these results show that endogenous opioid release in the rACC is sufficient and required for relief of ongoing (i.e., aversiveness) pain.

***Importance of distinguishing pain relief circuits from addictive effects of morphine***

In Chapter 1 I discussed the clinical implications of widespread use of opioids for the treatment of chronic non-malignant pain. Prescription opioid abuse, misuse and dependence had an estimated societal cost in the United States alone of \$55.7 billion in 2007, and this number is on the rise (288). Further, the 2007

National Survey on Drug Use and Health (NSDUH) estimated that 12.5 million Americans had used prescription pain medication for nonmedical purposes, which is up from the estimated 11 million in 2002, and is likely still growing (288). Opioids are the most effective class of drugs for the treatment of pain, and have been regarded as such for millennia. And while most clinicians would not argue their importance in the treatment of acute post-surgical or cancer pain, their long-term administration for chronic non-malignant pain is very controversial. These compounds are widely feared in society due to their association with abuse, misuse, dependence and diversion. This concern is so great that it is speculated to have contributed to the under treatment of disorders (i.e., acute, cancer and end-of-life pain) that are considered to be widely appropriate for opioid therapy (289-291). Despite this, the use of long-term opioids for the treatment of chronic non-malignant pain is on the rise, and has been consistently since the 1990's (292). Yet, researchers and clinicians have yet to come to a clear decision of what types of conditions can be safely and effectively treated, what patient-type responds well or what the clinical goals should be with their use (144, 292, 293). Further complicating the issue, clinical trial and epidemiological study data is very inconsistent and, for the most part, poorly executed.

To explore this dilemma further, we hypothesized that it is possible to distinguish pain relief circuits from addictive effects of morphine in rodent models (data presented in Chapter 3). Excitingly, we show that a low dose of

morphine preferentially elicits CPP and NAc DA release in SNL rats and not sham. This indicates that there are doses, at least in rat models, that have the ability to alleviate the aversiveness of pain but do not directly activate addictive reward pathways. Further, we show that this effect is dependent on opioid signaling in the rACC using pharmacological blockade of  $\mu$ -opioid signaling ( $\beta$ -FNA). Lastly, we show that the intrinsically rewarding effects of morphine are not dependent on  $\mu$ -opioid signaling in the rACC. These findings are particularly exciting as they suggest that opiates have anatomically and pharmacologically separate mechanisms that facilitate pain relief and addiction.

Endogenous opioid signaling in the ACC plays a crucial role in alleviating the aversiveness of pain and the reward of pain relief, which is demonstrated with downstream NAc DA release, in opioid and non-opioid analgesics. Further, the data presented here would suggest that pain relief brain circuits are distinguishable from circuits mediating the addictive effects of morphine. And that it is possible to find doses of morphine that can alleviate the aversiveness of pain without activating addictive reward circuits. The clinical significance of this work would indicate that it is possible to treat chronic pain patients, assuming of course other concerns are satisfied (i.e., tolerance, withdrawal, analgesia, etc.), with opiates without fear of addiction. However, to truly understand whether these findings would relate to the clinical setting it would be necessary to use an experimental paradigm that more closely translated to the clinical setting of chronic pain management (i.e., long-term, repeated dosing studies). Further,

that it is possible to create unique analgesics that specifically target the aversiveness of pain.

In summary, this work was completed not only to address specific clinically relevant questions but also to provide further evidence for the use of preclinical models with better translation to the bedside. We used experimentally unique methods to attempt to capture the important human dimensions of pain. By doing this we feel confident that the results hold strong implications and form a basis for future work in our laboratory, as well as the field of pain moving forward. We have shown that endogenous opioid signaling in the rACC is sufficient and necessary for the relief of ongoing aversive pain states. Additionally, that the reward of pain relief (i.e., NAc DA release) is likely dependent on opioid signaling in the ACC. Moreover, we provide evidence to support that opiate mechanisms promoting pain relief and addiction are anatomically and pharmacologically separable. With the development of a method to directly measure changes in endogenous opioid levels in the rACC (and other brain regions) the room for expansion on this work is limitless.

### ***Conclusions***

At the end of Chapter 1 I presented a series of questions that I would answer throughout this work. The first two questions were: (1) what effect does systemic administration of duloxetine, morphine or tapentadol have on spinal NE levels in rats? (2) Does chronic neuropathic injury (SNL) alter the effect of

these drugs on spinal NE levels (i.e., do changes occur in an injury state to the descending modulatory system that alters how effective each of these drugs are)? In Chapter 3 I presented data that shows systemic administration of tapentadol or duloxetine produces an elevation of NE in spinal CSF, likely through blocking neuronal reuptake of NE. Interestingly, tapentadol produced NE release that was greater in nerve-injured rats relative to sham-operated or naïve rats. This enhanced noradrenergic activity after nerve injury suggests that tapentadol could have enhanced benefit in neuropathic pain patients, possibly due to the opioid- $\alpha_2$ -adrenergic antinociceptive synergy. A surprising finding was that morphine appeared to reduce, rather than increase, spinal NE concentrations which is contradictory to other pharmacological data that suggests the opposite would be true. These findings are significant in that they not only provide greater insight into the mechanism of action of three clinically relevant drugs but also into the understanding of changes of endogenous descending inhibitory mechanisms following nerve injury. Future work is needed to better understand why morphine failed to produce an increase in NE concentrations.

Chapter 4 addressed the following questions: (1) Are the effects of morphine on sensory and affective/emotional dimensions of pain separable? (2) Are the rewarding effects of pain relief separable from those of the reward of addiction? (3) Does opioid signaling in the ACC mediate the reward of pain relief? (4) Is it possible to develop a method that can measure endogenous opioid release in the

ACC? Using 2 doses of morphine, separated by almost an order of magnitude (0.5 and 4.0 mg/kg, i.v.), I presented data that shows that the effects of morphine on sensory and affective/emotional dimensions of pain are separable (i.e., low dose morphine does not alter sensory thresholds but does produce CPP and NAc DA release). Additionally, using these same doses I was able to show that the rewarding effects of pain relief and addiction are separable (i.e., low dose only produces reward in animals with nerve injury and not sham-operated). Further, this low dose of systemically (i.v.) administered morphine, that is not reinforcing in sham-operated animals, acts in the ACC to relieve pain aversiveness and facilitate reward. Microinjection of morphine in the ACC is sufficient to elicit NAc DA efflux in injured but not uninjured animals, promoting reward of pain relief. Lastly, that opioid signaling in the ACC is also required for relief of ongoing pain produced by systemic non-opioid treatments. These findings not only anatomically and pharmacologically separate opiate mechanisms promoting pain relief and addiction but also represent a general mechanism of pain modulation that could potentially serve as a biomarker for analgesic efficacy and help facilitate analgesic drug discovery efforts.

Finally, we asked ourselves the next logical question – is it possible to directly measure Met- and Leu- enkephalin release in the ACC? The data discussed in the last paragraph strongly suggests a role of these endogenous opioids in relief of pain aversiveness and facilitating reward, however they do not directly measure physiological changes occurring in the ACC. In the last part of Chapter 4 I

presented methods that our lab developed to directly measure these enkephalins. Development of this method will allow us to answer a multitude of questions moving forward including: (1) does tonic release of endogenous Met- and Leu-enkephalin differ in the ACC between injured and uninjured animals? (2) Does tonic release vary over the progression of a nerve injury from acute to chronic? (3) Does endogenous enkephalin release in the ACC vary between injured and uninjured animals to systemic opioid and non-opioid analgesics? Answers to these questions will not only allow us to understand more about relevant mechanisms to analgesics but will also allow us to learn about changes in endogenous opioid signaling through the chronification of pain.

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