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DYNORPHIN PROMOTES OPIOID-INDUCED ABNORMAL PAIN AND ANTINOCICEPTIVE TOLERANCE

by

Luis Roberto Gardell

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Luis Roberto Gardell entitled Dynorphin promotes opioid-induced abnormal pain and antinociceptive tolerance and recommend that it be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

Frank Porreca, Ph.D. — 8/7/02 — Date

Josephine Lai, Ph.D. — 8/7/02 — Date

Todd W. Vanderah, Ph.D. — 8/7/02 — Date

Edward French, Ph.D. — 8-7-02 — Date

Robert Sloviter, Ph.D. — 8-7-02 — Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy 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.

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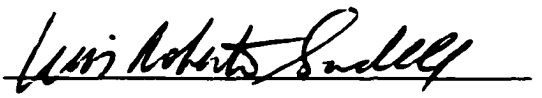
Signed: 

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CHAPTER I

ABSTRACT

Consequences of injury to peripheral nerves and opioid tolerance share features including tactile and thermal hypersensitivity, decreased spinal opioid antinociception and upregulation of spinal dynorphin. Dynorphin may normally produce antinociception (opioid effect) but may be pronociceptive in pathological states (through direct or indirect actions at NMDA receptors). Paradoxical opioid-induced pain has been repeatedly demonstrated in humans and animals. The mechanisms of such pain are unknown but may relate to opioid-induced activation of descending pain facilitatory systems and enhanced expression and pronociceptive actions of spinal dynorphin.

Sustained morphine, but not placebo, exposure elicited a time-related onset of both tactile and thermal hypersensitivity along with antinociceptive tolerance to i.th. morphine. Spinal dynorphin levels were significantly increased following morphine exposure. Treatment with either i.th. MK-801 or antiserum to dynorphin (Dyn A/S) reversed both morphine-induced abnormal pain and antinociceptive tolerance to spinal morphine. Control pre-immune control serum had no effect.

The possibility that opioid-induced abnormal pain, antinociceptive tolerance and dynorphin up-regulation are mediated through activation of spinal μ opioid receptors was tested. Sustained administration of either DAMGO or (-)-oxymorphone (active form), but not (+)-oxymorphone (inactive form) or vehicle, produced both tactile and thermal hypersensitivity along with antinociceptive tolerance to i.th. agonist challenge. Spinal dynorphin levels were significantly increased following treatment with either DAMGO or

(-)-oxymorphone. Treatment with Dyn A/S reversed DAMGO-induced abnormal pain and antinociceptive tolerance to spinal morphine. Control pre-immune control serum had no effect. A comparison of μ opioid receptor immunoreactivity in tissues taken from either morphine- or DAMGO-infused rats demonstrated a time-related decrease in μ opioid receptor immunoreactivity.

The possibility that opioid-induced central changes might mediate increased excitability to the spinal cord was tested. Tactile and thermal hypersensitivity was observed at 7, but not 1, day after subcutaneous morphine pellet implantation; placebo pellets produced no effects. Basal and capsaicin-evoked release of CGRP was measured in minced spinal cord tissues taken from naive rats or from rats on post-pellet days 1 and 7. The content and evoked release of CGRP was significantly increased in tissues from morphine-exposed rats at 7, but not 1, day after implantation. Morphine increased spinal dynorphin content on day 7 in rats with sham bilateral lesions of the dorsolateral funiculus (DLF), but not in rats with DLF lesions. Pharmacological application of dynorphin $A_{(2-13)}$, a non-opioid fragment, to tissues from naive rats enhanced the evoked release of CGRP. Enhanced evoked release of CGRP from morphine-pelleted rats was blocked by Dyn A/S or by prior lesions of the DLF.

Prodynorphin "knock-out" (KO) and wild-type (WT) mice were studied for changes in sensitivity to non-noxious mechanical and noxious radiant heat after morphine or placebo pellet. After 5 days of treatment, WT, but not KO, mice developed antinociceptive tolerance. Morphine, but not placebo pellet produced a time-related increased sensitivity to non-noxious and noxious stimuli in WT, but not in KO, mice.

Spinal dynorphin levels were significantly increased by morphine in WT mice. Tissues taken from morphine-treated WT mice demonstrated a marked potentiation of capsaicin-evoked CGRP release as compared to WT placebo-treated mice. Capsaicin-evoked release of CGRP was not altered by morphine-treatment in the KO mice. Total CGRP content was elevated in tissues taken from morphine-exposed WT and KO mice as compared to placebo controls. Administration of i.th. MK-801 or Dyn A/S reversed morphine-induced abnormal pain and antinociceptive tolerance in WT mice. Control pre-immune serum had no effect.

These data suggest that sustained morphine induces plasticity in both primary afferents and the spinal cord, including increased CGRP and dynorphin content. Morphine-induced elevation of spinal dynorphin content depends on descending influences and enhances stimulated CGRP release. Taken together, these data support the hypotheses of this dissertation, specifically, a) opioid-induced abnormal pain manifests, behaviorally, as antinociceptive tolerance such that, manipulations that block opioid-induced pain will restore the potency of spinal opioids; b) descending pain facilitation arising from the RVM will result in plasticity at the spinal cord level including the upregulation of dynorphin; and c) pathological levels of spinal dynorphin act to promote opioid-induced abnormal pain by potentiating the release of CGRP from the central terminals of primary afferent neurons.

CHAPTER I: INTRODUCTION

Tolerance can be demonstrated to virtually all opioid effects, but is clinically most important in relation to pain relief. Analgesic tolerance is demonstrated by a reduced or lost analgesic effect of a given opioid dose administered repeatedly or the diminution of effect seen with continuous administration of an opioid over a period of time, usually ranging from several days to weeks. Opioid analgesic tolerance is well recognized experimentally and clinically (Foley 1993; Foley 1995; Way et al. 1969). Despite abundant research, however, the mechanisms responsible for the development of this phenomenon remain largely unknown. At the physiological level, opioid antinociceptive tolerance can be blocked or prevented by inhibition of many diverse endogenous transmitters and receptor systems (for a summary see Table 1.1). These include, among others, calcitonin gene-relate peptide (CGRP) antagonists (Menard et al. 1996; Powell et al. 2000), nitric oxide synthase (NOS) inhibitors (Powell et al. 1999), calcium channel blockers (Aley and Levine 1997), cholecystokinin (CCK) antagonists (Xu et al. 1992), cyclooxygenase (COX) inhibitors (Powell et al. 1999) and protein kinase C (PKC) inhibitors (Mao et al. 1995c). The fact so many systems have been shown to modulate opioid antinociceptive tolerance makes it difficult to implicate a common cellular mechanism for the actions of all these substances. For this reason, I have focused on possible commonalities in the endogenous mechanisms, which promote, rather than block, opioid antinociceptive tolerance.

Table 1.1. A sampling of compounds reported to block opioid antinociceptive tolerance.

Compounds	References
Benzodiazepine antagonists	(Raghavendra and Kulkarni 1999)
Calcium channel antagonists	(Aley and Levine 1997; Contreras et al. 1997)
CGRP antagonists	(Menard et al. 1996; Powell et al. 2000)
Cholecystokinin antagonists	(Tang et al. 1984; Xu et al. 1992)
Cyclooxygenase inhibitors	(Powell et al. 1999)
Glutamate transporter activator	(Nakagawa et al. 2001)
I_2 Imidazoline agonists	(Boronat et al. 1998; Kolesnikov et al. 1996)
Nitric oxide synthase inhibitors	(Powell et al. 1999)
NMDA antagonists	(Trujillo and Akil 1991a)
Protein kinase A inhibitors	(Bernstein and Welch 1997)
Protein kinase C inhibitors	(Mao et al. 1995c)
Orphanin FQ/nociceptin	(Lutfy et al. 2001)
Substance P antagonists	(Gardell & Porreca, unpublished)

Opioids produce paradoxical pain both clinically and experimentally

The remarkable ability of opioids to alleviate moderate to severe pain in many conditions underlies the continued therapeutic significance of these drugs. Paradoxically, however, opioids have also been shown to produce pain in clinical and experimental settings. A clinical review of 750 cases where patients received spinal (epidural or intrathecal) morphine, across an average period of 128 days, demonstrated the development of hyperesthesia (increased sensitivity to stimuli) and allodynia (pain elicited by normally innocuous sensory stimulation) along with tolerance to the analgesic effect of morphine (Arner et al. 1988). Long-term intrathecal infusion of morphine, which originally alleviated cancer pain, has spontaneously elicited abnormal pain

syndromes unrelated in quality or dermatomal distribution from the original pain complaint (Ali 1986; De Conno et al. 1991). The fact that the secondary onset of pain was unrelated to the initial pain indicated that it was not simply a matter of increased pain due to progression of the disease, but rather because novel mechanisms were invoked after opioid administration (Ali 1986; De Conno et al. 1991). Paradoxical intense pain also occurred after intrathecal morphine in cancer patients (Stillman et al. 1987). Similar observations were reported in patients with non-cancer pain. For example, intrathecal sufentanil infusion in a patient with neuropathic pain secondary to arachnoiditis and laminectomy originally alleviated the pain but then evoked hyperesthesias in the back, abdomen and legs, and was of a different quality than the original pain (Devulder 1997).

Many animal studies have confirmed the fact that opioids may produce abnormal pain, even after short-term exposures (Celerier et al. 2000; Woolf 1981; Yaksh et al. 1986). Bolus doses of spinal morphine have provoked paroxysmal bouts of biting and scratching behavior, indicative of pain, in rats (Woolf 1981; Yaksh et al. 1986). Both repeated injections and a constant infusion of morphine elicit thermal hyperalgesia in rats (Mao et al. 1994; Mao et al. 1995e; Trujillo and Akil 1991a). In more recent studies, it has been reported that even a single systemic dose of either heroin or fentanyl produced hyperalgesia evident after the initial opioid-induced antinociceptive action had subsided (Celerier et al. 1999; Celerier et al. 2000; Larcher et al. 1998). It has been suggested that opioid-induced hyperalgesia simply results from an unmasking of a compensatory neuronal hyperactivity in response to morphine-induced inhibition of neuronal function (Gutstein 1996). This hyperresponsiveness may become evident after the opioid is

removed or occurs intermittently between injections such that opioid-induced hyperalgesia might be interpreted as a result of repeated episodes of "miniwithdrawals" (Gutstein 1996). Studies of abnormal pain consequent to long-term opioid administration that depends on paradigms of repeated injection are also subject to this criticism (Mao et al. 1994; Mao et al. 1995e; Trujillo and Akil 1991a).

Organization of the pain transmission system

Cutaneous primary afferent fibers can be classified into three major types on the basis of diameter, structure, and conduction velocity. The largest afferent fibers have diameters ranging from 6-22 μM; they include Aα or Aβ fibers and are heavily myelinated and fast conducting (30-100 M/sec). Aα fibers have the largest diameter of the myelinated primary afferents and include muscle proprioceptors (i.e, muscle spindles and Golgi tendon organs). Both Aα and Aβ fibers are activated by innocuous stimuli, thus transmitting information related to muscle stretch, light touch and position. The second type of fiber is medium sized having a diameter of 2-6 μM. These are designated as Aδ fibers and are also myelinated and of intermediate conduction velocity (12-30 M/sec). The third type of fiber is small sized having a diameter of 0.4-1.2 μM. These are designated as C fibers and are unmyelinated and of slow conduction velocity (0.5-2.0 M/sec). Both Aδ and C fibers become activated following exposure to noxious thermal, chemical and mechanical stimuli. Selective activation of these fibers by noxious, but not non-noxious, stimuli defines their roles as nociceptors. For the purpose of this dissertation, focus will be primarily on fibers of the Aβ and C type.

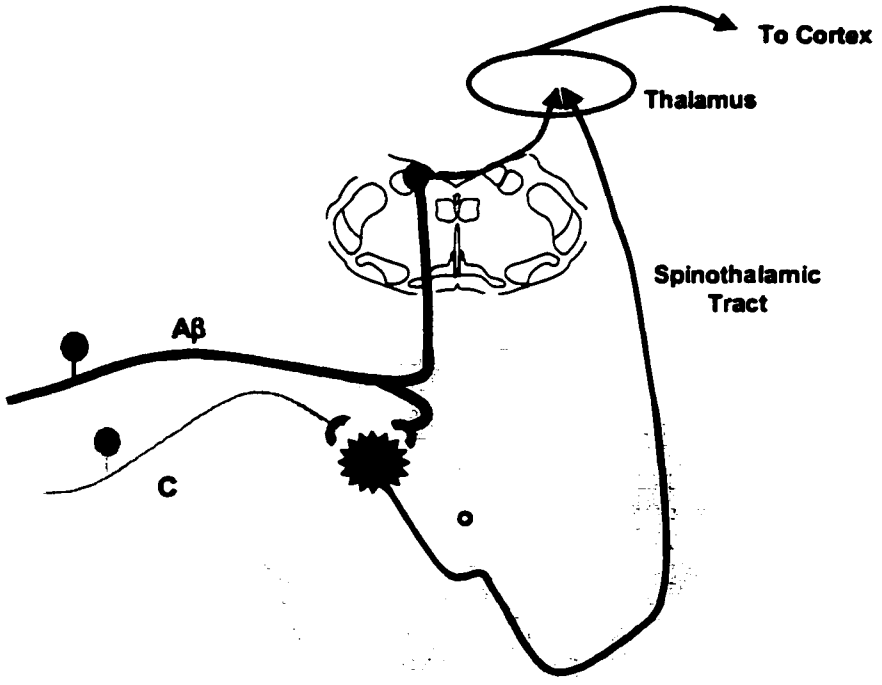

Figure 1.1. Ascending sensory fiber pathways. Depicted are the primary afferent Aβ and C fibers entering the spinal cord and ascending via the ipsilateral dorsal column to the dorsal column nuclei (n. gracilus or n. cuneatus) or contralateral spinothalamic tract to the thalamus, respectively. From the thalamus, these fibers project to the somatosensory cortex, and to other centers.

All primary afferent neurons that enter the spinal cord have their cell bodies in the dorsal root ganglion (DRG). These cell bodies are psuedounipolar sending axons out to the periphery and also have axons that enter into the spinal cord (Figure 1.1). Upon entering the spinal cord, Aβ fibers ascend the dorsal column to the nucleus gracilus, or the nucleus cuneatus, in the brain stem and also send collateral projections to lamina III-IV of the spinal dorsal horn. These fibers synapse onto their 2nd order neuron in the dorsal column nuclei and then this cell then crosses the midline, and projects via the medial lemniscus to the thalamus. Aβ fibers also send collateral fibers that synapse in the dorsal horn, where they can modulate the activity of wide-dynamic range neurons. C fibers, in contrast, enter the dorsal horn and synapse onto their 2nd order neuron, which

crosses the midline, and ascend via the spinothalamic tract to the thalamus 3rd order cells project to cortical areas (Figure 1.1). In the thalamus, input from spinothalamic tract and medial lemniscus neurons are relayed to the somatosensory cortex, and other sites, where the perception of pain occurs.

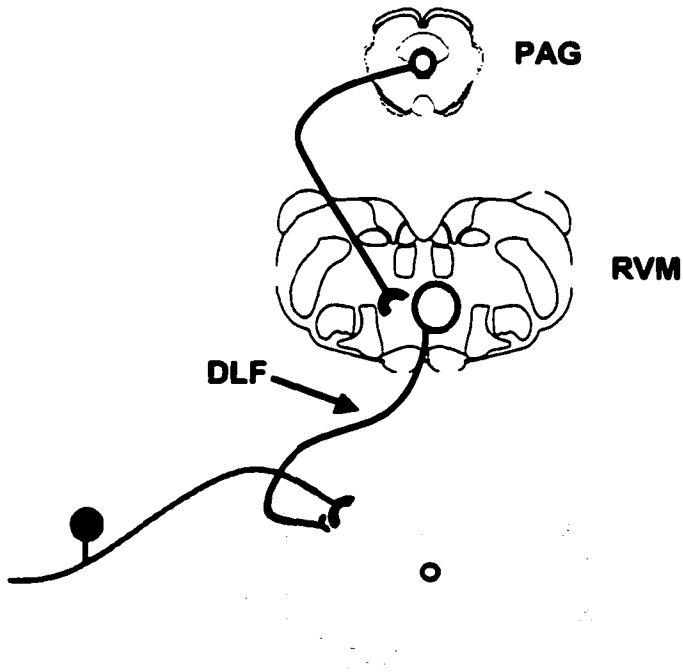

Figure 1.2. Descending pain modulatory pathways. Depicted are some of the major structures of the descending pain modulation system. Neurons from the periaqueductal gray (PAG) project to the rostral ventromedial medulla (RVM). Neurons from the RVM project to the spinal cord via the dorsolateral funiculus (DLF).

In addition to the ascending pain pathway, endogenous descending pain pathways can either inhibit or facilitate the processing of pain. One of the major sites in the brain known to play a role in pain modulation is the periaqueductal gray (PAG) of the midbrain. Neurons project from the PAG to a number of areas including the rostral ventromedial medulla (RVM) (Figure 1.2). The RVM serves as a relay station in the descending modulation of pain. Neurons from the RVM project to different laminae in

the spinal cord via the dorsolateral funiculus (DLF). Lesions of the DLF have been shown to block both PAG- and RVM-induced inhibition of spinal nociceptive reflexes (i.e., tail-flick).

Localization of opioids receptors and mechanism of action

Figure 1.3 summarizes the localization of μ opioid receptors in sites known to be important in pain transmission, both in the peripheral and central nervous systems. It is widely accepted that, at the level of the spinal cord, the action of morphine in acute pain is predominantly mediated through μ opioid receptors localized on the central terminals of small diameter primary afferent fibers (or C fibers) (Lombard and Besson 1989; Yaksh et al. 1995). Studies utilizing dorsal rhizotomies have reported marked reductions in μ opioid binding in the superficial lamina of the dorsal horn ranging from approximately 60-75% (Besse et al. 1992; Hohmann et al. 1999). The remaining 25-40% of these μ opioid receptors are likely post-synaptic and expressed by interneurons and spinothalamic tract neurons (Hohmann et al. 1999) (Figure 1.3). Additionally, immunostaining for μ opioid receptors has been shown in DRG cell bodies (Li et al. 1998; Mansour et al. 1995; Mansour et al. 1994a) as well as, in peripheral axon terminals of C fibers (Stein et al. 1990) (Figure 1.3).

Descending pathways originating in the brainstem that act to inhibit spinal nociceptive reflexes and reduce spinal neuronal activity elicited by noxious stimuli can also influence nociception. One important supraspinal site in antinociception is the PAG, where microinjection of opioid agonists elicits a marked reduction in pain behaviors (Yaksh 1997). Electrical stimulation of the PAG has also been shown to elicit

antinociception (Baskin et al. 1986; Hosobuchi et al. 1977; Richardson and Akil 1977). Additionally, studies have demonstrated the presence of μ opioid binding sites (Mansour et al. 1987; Tempel and Zukin 1987) as well as the mRNA encoding μ opioid receptors in this region (Mansour et al. 1994a; Peckys and Landwehrmeyer 1999; Wang and Wessendorf 2002).

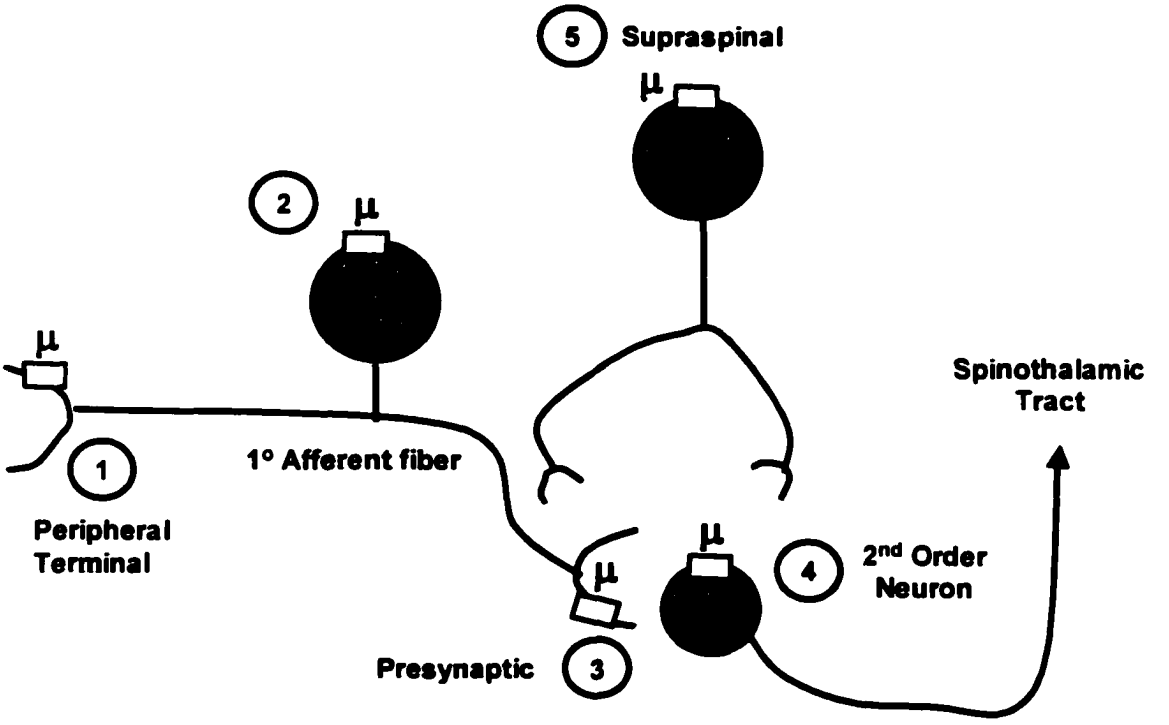

Figure 1.3. The five major sites of endogenous or exogenous opioid analgesia. These sites include 1) peripheral terminals of small diameter unmyelinated primary afferent neurons; 2) dorsal root ganglion (DRG) cells; 3) central terminals of small diameter unmyelinated primary afferent neurons; 4) second-order neurons; and 5) supraspinally in the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM).

Since the PAG is thought to have very limited direct connections with the spinal cord, it has been suggested that PAG neurons modulate the spinal cord dorsal horn through an indirect mechanism via projections to antinociceptive circuits located in the RVM (Basbaum and Fields 1984; Fields et al. 1991). The mechanism through which

opioids produce antinociception in the PAG is thought to be via disinhibition of tonic release of GABA from afferents to PAG-RVM neurons (Roychowdhury and Fields 1996; Vaughan et al. 1997; Yaksh et al. 1976) (Figure 1.4). Activation of μ opioid receptors results in a decreased release of GABA, relieving enkephalinergic projection neurons of inhibitory input, thus leading to an increased release of enkephalins in the RVM and locus ceruleus (LC).

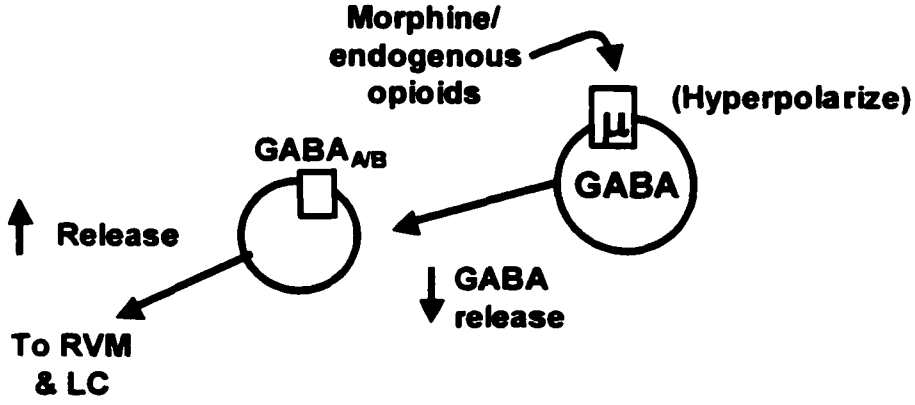

Figure 1.4. Opioids produce analgesia in the periaqueductal gray (PAG) via disinhibition. Opioids act to hyperpolarize GABAergic neurons resulting in net decrease of GABA release. A reduction in GABA release removes the inhibitory tone on PAG projection neurons allowing them to depolarize and release excitatory transmitters in other regions of the brain including the rostral ventromedial medulla (RVM) and locus ceruleus (LC).

Another key supraspinal site in the modulation of nociception is the RVM, where either microinjection of opioid agonists (Fields and Basbaum 1978; Fields et al. 1991; Jensen and Yaksh 1989) or electrical stimulation (Zorman et al. 1981) elicits analgesia. Additional studies have demonstrated the presence of mRNA encoding μ opioid receptors (Bowker et al. 1988; Bowker and Dilts 1988; Mansour et al. 1994a; Mansour et al. 1994b; Peckys and Landwehrmeyer 1999; Porreca et al. 2001). Depicted in Figure 1.5 is one proposed circuitry scheme for pain modulation in the RVM, based on

electrophysiological responses to a noxious thermal stimulus. This model identifies three types of RVM neurons (Fields and Basbaum 1999; Fields et al. 1983; Fields and Heinricher 1985). The "OFF"-cells pause, and the "ON" cells accelerate, in their firing immediately prior to a withdrawal response to noxious stimuli occurs, while "neutral" cells show no electrophysiological responses to nociception.

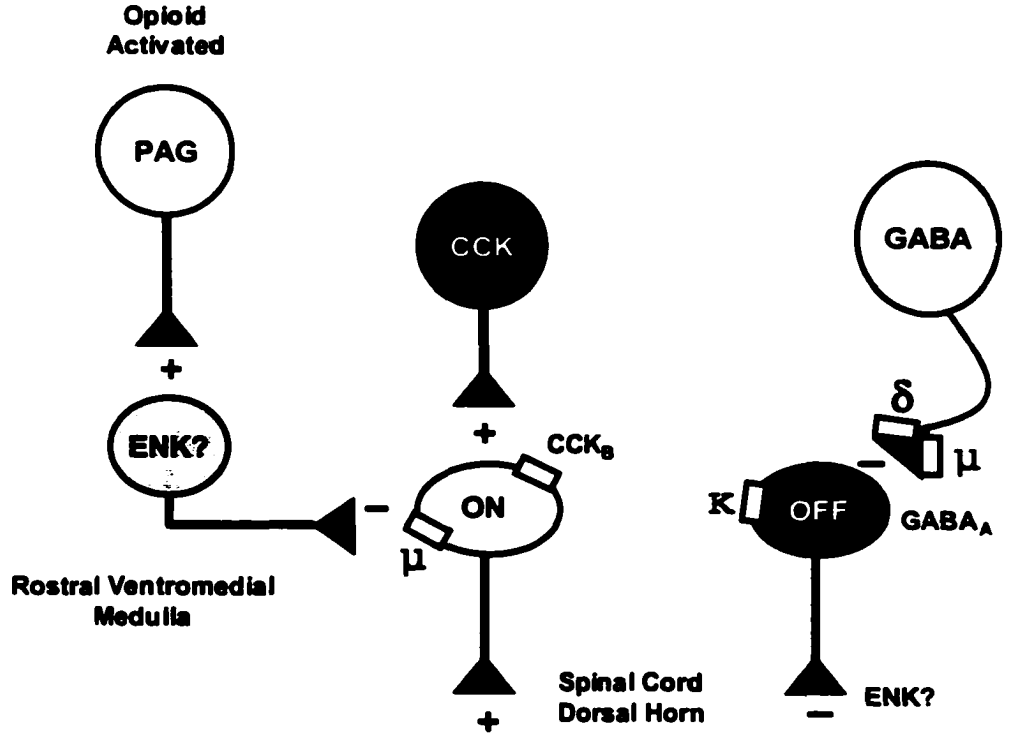

Figure 1.5. A proposed circuit in the rostral ventromedial medulla (RVM) for pain modulation. "OFF"-cells pause immediately prior to a withdrawal response to noxious stimulus and "ON" cells accelerate their firing. Neurons activated by opioids in the PAG may stimulate enkephalinergic projection neurons, which inhibit the firing of "ON"-cells.

The activation of "OFF"-cells (through disinhibition) is associated with an inhibition of nociceptive input and nocifensive responses (Fields and Basbaum 1999; Fields et al. 1983; Fields and Heinricher 1985; Heinricher et al. 1992). In contrast, however, the "ON"-cells increase their firing rate just prior to the reflex, and these

neurons have been found to facilitate nociceptive processing through both local interactions within the RVM and descending systems projecting to the spinal cord (Fields et al. 1983; Fields and Heinricher 1985; Fields et al. 1991; Heinricher et al. 1992; Heinricher and Roychowdhury 1997). The terminals of projection neurons from the RVM, which comprise the DLF, are concentrated in dorsal horn laminae (I, II, and V) and contain both terminals of C fibers and cell bodies of spinothalamic tract neurons (Fields 1987). It is believed that opioids produce antinociception in the RVM through the disinhibition of "OFF"-cells (see Fields and Basbaum 1999 for review). Inhibition of "ON"-cells appears to be unnecessary for the antinociceptive effect of opioids administered in the RVM (Heinricher et al. 1994). Recent work from our laboratory has demonstrated using saporin conjugated dermorphin to destroy RVM cells that express the μ opioid receptor: presumably these cells include facilitatory "ON"-cells (Porreca et al. 2001), which are known to express μ opioid receptors (Heinricher et al. 1994). Although rats treated with the dermorphin-saporin conjugate, either before or after ligation of the L_5 and L_6 spinal nerves, did not show neuropathic pain behaviors, their normal nociceptive responses remained intact (Porreca et al. 2001).

Mechanisms of opioid antinociceptive tolerance

A. Desensitization and down-regulation

At the cellular level, opioid receptors have been shown to consistently undergo internalization upon agonist activation (Gaudriault et al. 1997; Sternini et al. 1996). This redistribution of the receptors from the cell surface to intracellular compartments occurs within minutes of exposure to agonists, and is considered to be a major cellular

mechanism for the refractoriness of the cell to further agonist challenge. Such refractoriness at the receptor level is termed desensitization, and is a common phenomenon among members of the superfamily of G protein coupled receptors (GPCR) (Koenig and Edwardson 1997). Receptor desensitization appears to be initiated by agonist-induced phosphorylation of the receptor (Inglese et al. 1993), which uncouples the receptor from further interactions with G proteins, and promotes the binding of the receptors with arrestins, which recruit the receptors to a dynamin-dependent endocytosis process (Ferguson et al. 1996). Agonist-induced desensitization may be an effective negative feedback mechanism to regulate the duration of signal transduction via the GPCR. As receptor internalization is a rate-limiting step for the recycling of the receptors and their resensitization after agonist exposure, this process may be necessary for maintaining receptor function (Mundell et al. 1999; von Zastrow and Kobilka 1992).

Prolonged desensitization at the receptor level in the continuous presence of agonist has been proposed to be the underlying molecular mechanism of tolerance to drugs, which is defined as a rightward shift in the dose-response function to agonists. In the case of the opioid receptor types, the relationship between agonist induced internalization and the development of tolerance is still not well understood, despite much research effort. It is known that prolonged exposure to agonists also leads to a phenomenon of receptor down-regulation, which is defined as a reduction of receptor expression by the cell (Chakrabarti et al. 1995). The onset of receptor down-regulation takes longer, involving hours instead of minutes. In this regard, the temporal relationship of down-regulation appears to be closer to that of opioid tolerance in vivo. The cellular

processes that mediate receptor down-regulation are complex, and the factors that drive this specific process are not well understood, but nevertheless, prolonged agonist exposure does result in this compensatory down-regulation in numerous GPCRs, including opioid receptors, and is therefore a common phenomenon (Tsao and von Zastrow 2000). Receptor down-regulation is a result of an enhanced rate of degradation of the receptor protein and may be coupled with a reduction in receptor synthesis, such that there are fewer functional receptors at the cell surface at any one time. Internalized GPCRs can also be trafficked by a recycling pathway to the plasma membrane, which promotes resensitization of receptor-mediated signal transduction (Claing et al. 2002; Lefkowitz et al. 1998; Trejo and Coughlin 1999; Whistler et al. 2002).

B. NMDA receptors

A number of convergent studies provide support for the idea that the development of antinociceptive tolerance may be linked to sensitization of sensory neurons through activation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate (Baranauskas and Nistri 1998; Ma and Woolf 1995). This suggests that opioid-induced abnormal pain may be dependent upon the facilitation of pain produced by increased NMDA receptor activity (Celerier et al. 2000; Larcher et al. 1998; Laulin et al. 1998; Mao et al. 1994; Mao et al. 1995c). Repeated daily injections of spinal morphine to rats produced tolerance to the antinociceptive effect of morphine along with thermal hyperalgesia of the hindpaws, and both of these effects were prevented by concurrent injections of the NMDA receptor antagonist MK-801 (Mao et al. 1994). In another study, the systemic coadministration of morphine with MK-801 to rats prevented, in a dose-dependent manner, the development

of tolerance to the antinociceptive effect of morphine (Trujillo and Akil 1991a). In these studies, MK-801 did not produce antinociception alone, nor did it increase antinociceptive action of morphine in non-tolerant rats. Continuous spinal infusion of morphine also resulted in tolerance to its antinociceptive effect, and the development of tolerance was prevented by co-infusion with either of the NMDA antagonists, MK-801 or dextromethorphan (Manning et al. 1996). The presence of presynaptic NMDA receptors on central terminals of primary afferent fibers has been demonstrated, and these receptors are believed to provide a positive feedback mechanism allowing further release of glutamate and co-localized neurotransmitters from primary afferent neurons (Liu et al. 1997; Liu et al. 1994). Since these sites also contain μ-opioid receptors, an anatomical link between these two systems exists (Liu et al. 1997).

Recent work by Mao and colleagues (Mao et al. 1995c) has proposed a model of opioid tolerance based on possible interactions between NMDA receptors and opioid receptors linked through the activity of protein kinase C (PKC). Agonist-induced activation of μ opioid receptors results in the activation of G-proteins that, among numerous other effectors, can activate PKC. PKC can then phosphorylate NMDA receptor channels, resulting in a removal of the Mg^{2+} block, allowing activation of the NMDA receptor with lower concentrations of glutamate (Mao et al. 1995c; Smart and Lambert 1996). Activation of the NMDA receptor would result in Ca^{2+} influx, which could have a variety of effects including, enhanced activation of PKC, altered gene expression, or activation of nitric oxide production, all of which would act to maintain the cell in a hyperexcitable state (Mao et al. 1995c; Mayer et al. 1999). Additionally, it has

been speculated that PKC can phosphorylate μ opioid receptors, thus resulting in the uncoupling of GPCR and decreased agonist efficacy and potency (Mao et al. 1995c).

C. Calcitonin Gene-related Peptide

Calcitonin gene-related peptide (CGRP) is expressed in a subset of small diameter DRG cells (Traub et al. 1990), is colocalized with substance P and glutamate in central terminals of primary afferent fibers and is thought to promote nociceptive processing in the spinal cord (Gibson et al. 1984). Studies have shown that evoked CGRP release is blocked by opioids suggesting that the μ-opioid receptor is expressed on primary afferents containing this excitatory transmitter (Collin et al. 1993; Del Bianco et al. 1994). CGRP has been associated with increased glutamate release and enhanced NMDA receptor activity, both of which would oppose the antinociceptive action of opioids (Kangrga et al. 1990; Murase et al. 1989). Exogenously administered CGRP also elicits significant rightward shifts in the antinociceptive dose-response curves for morphine (Welch et al. 1989). Tolerance produced by spinal morphine infusion was accompanied by increased spinal expression of CGRP, while spinal levels of substance P, galanin, neurotensin and neuropeptide Y remained unchanged (Menard et al. 1995). Coadministration of morphine along with the CGRP antagonist, $CGRP_{(8-37)}$, prevents the development of antinociceptive tolerance to morphine and the elevations in endogenous spinal CGRP levels (Menard et al. 1996). It was concluded that a critical interaction may develop between opioidergic systems and CGRP to promote the development of tolerance to opioids (Menard et al. 1996; Powell et al. 2000). It is believed that CGRP, by increasing the release of glutamate and of substance P from primary afferents, may

potentiate nociceptive input by amplification of excitatory amino acid activity on the postsynaptic second order neurons of the dorsal horns of the spinal cord (Kangrga et al. 1990; Oku et al. 1987).

D. Descending Facilitation

Recent studies have indicated that opioid-induced pain and antinociceptive tolerance may result from activation of a descending pain facilitatory pathway (Vanderah et al. 2001b). Noxious inputs to the spinal cord are known to be modulated by supraspinal sites. One region that is critical to the integration of nociceptive processing and descending modulation is the RVM (Fields and Basbaum 1999; Fields and Heinricher 1985). Sustained exposure to a noxious thermal stimulus produced increased "ON" cell discharge along with a facilitation of nociceptive reflexes, and these effects were blocked by RVM lidocaine microinjection (Morgan and Fields 1994). The microinjection of CCK_8 into the RVM attenuated morphine-induced activation of "OFF" cell activity (Heinricher et al. 2001) and elicited enhanced sensitivity to normally innocuous mechanical (Kovelowski et al. 2000) and noxious thermal stimuli (Burgess, Lai & Porreca, unpublished). Electrical stimulation of the RVM facilitated dorsal horn neuronal activity and the spinal nociceptive tail flick reflex (Zhuo and Gebhart 1997). Spontaneous "ON"-cell activity increases along with sensitivity to noxious stimuli during naloxone-precipitated withdrawal (Kaplan and Fields 1991). These actions were blocked by microinjection of lidocaine into the RVM (Kaplan and Fields 1991). In spite of these investigations, the state of "ON"-cell or "OFF"-cell firing during sustained morphine administration, in the absence of withdrawal, has not been directly studied. As sustained

opioid exposure elicits enhanced pain, and enhanced pain can result from increased "ON" cell activity, we have hypothesized opioid-induced abnormal pain and antinociceptive tolerance may be the result of increased activity of pain facilitation cells arising in the RVM.

Continuous morphine exposure by subcutaneous pellet implantation or osmotic minipump produces enhanced sensitivity to normally innocuous mechanical and noxious thermal stimuli that is reversibly blocked by lidocaine in the RVM (Vanderah et al. 2001b). The ability of RVM lidocaine to block opioid-induced abnormal pain supports the concept of tonic or evoked discharge of pain facilitation cells in the RVM. Spinopetal projections from RVM neurons make up the majority of fibers of the DLF (Fields and Heinricher 1985). DLF lesions blocked the antinociception produced by manipulations in the RVM and other supraspinal nuclei, indicating that inhibition of spinothalamic tract neurons in the dorsal horns of the spinal cord may be meditated through this pathway (Cho and Basbaum 1989a). Electrical stimulation of the DLF produced excitation of neurons in the superficial dorsal horn, demonstrating a clear descending facilitation through this pathway (McMahon and Wall 1988). Consistent with these observations, disruption of the DLF blocked the enhanced sensitivity to normally innocuous mechanical stimuli and thermal hyperalgesia resulting from sustained opioid delivery by subcutaneous pellets or osmotic minipump (Vanderah et al. 2001b). Such findings are also consistent with the reversal of nerve injury-induced abnormal pain by either RVM lidocaine (Kovelowski et al. 2000) or DLF lesion (Ossipov et al. 2000). These observations suggest that tonic activation of descending facilitation may represent a

mechanism of chronic pain resulting from nerve injury or opioid exposure (see Porreca et al. 2002 for review).

E. Spinal dynorphin

A number of studies have suggested that sustained morphine exposure elicits neuroplasticity, either in the RVM or in structures communicating with the RVM, resulting in bulbospinal facilitation (Vanderah et al. 2001b). Such descending facilitation might also play a significant role in spinal plasticity resulting from sustained exposure to opioids. A consistent observation is the increase in expression of dynorphin in the spinal dorsal horn following opioid administration. Evidence suggests that spinal dynorphin is an important mediator of sustained abnormal pain (Malan et al. 2000; Wang et al. 2001). Although dynorphin was originally identified as an endogenous κ-opioid agonist (Goldstein et al. 1979) and may act as an endogenous antinociceptive agent under certain conditions (Ossipov et al. 1996), this peptide also has significant non-opioid activity. Considerable evidence now supports the conclusion that enhanced expression of spinal dynorphin is pronociceptive (Caudle and Isaac 1988a; Cho and Basbaum 1989b; Draisci et al. 1991; Dubner and Ruda 1992; Nahin et al. 1992; Stanfa and Dickenson 1995; Wang et al. 2001).

Non-opioid actions of dynorphin may promote opioid-induced abnormal pain and antinociceptive tolerance

Substantial in vivo evidence exists which indicates that dynorphin $A_{(1-17)}$, and/or its fragments, may act as a "functional" NMDA agonist. Both i.th. dynorphin $A_{(1-13)}$ and non-opioid dynorphin fragments produce hindlimb paralysis (Faden and Jacobs 1984;

Przewlocki et al. 1983; Stevens et al. 1987) and depletion of neuronal cell bodies in the spinal cord (Long et al. 1988). Both NMDA antagonists or agents that modulate the NMDA-glycine receptor complex have been reported to protect against dynorphin-induced hindlimb paralysis (Bakshi and Faden 1990; Bakshi et al. 1990; Long et al. 1994; Long and Skolnick 1994), loss of tail-flick reflex (Caudle and Isaac 1988a; Stewart and Isaac 1991) or loss of neuronal cell bodies (Long and Skolnick 1994). Additional studies have shown that a single i.th. injection of a subparalytic, but relatively high dose of dynorphin $A_{(1-17)}$, dynorphin $A_{(1-13)}$ or of the *des*-Tyr derivative dynorphin $A_{(2-17)}$, which is devoid of binding affinity to opioid receptors (refer to Table 1.2), has the ability to produce a remarkable long-lasting, and apparently irreversible, mechanical and thermal hypersensitivity in rats (Vanderah et al. 1996). These effects, also seen in mice (Laughlin et al. 1997), were blocked by the prior i.th. administration of MK-801, but not naloxone (Laughlin et al. 1997; Vanderah et al. 1996). Finally, pre-emptive i.p. administration of an antibody to dynorphin $A_{(1-17)}$ did not block allodynia produced by sciatic cryoneurolysis, but produced an increase in spinal glutamate immunoreactivity (Wagner and Deleo 1996), suggesting a link between dynorphin and glutamate activity and raising the possibility of indirect dynorphin influences with the NMDA receptor.

Data obtained from in vitro electrophysiological studies are contradictory, suggesting both an inhibitory (Chen et al. 1995) and excitatory (Lai et al. 1998) action of dynorphin at the NMDA receptor complex. Recent work has demonstrated a direct, high affinity inhibitory binding site for dynorphin at the NMDA receptor (Tang et al. 1999). In other studies, dynorphin induced the influx of calcium into isolated cortical cells by a

mechanism not mediated through opioid or NMDA receptors, suggesting a novel excitatory action (Tang et al. 2000). The precise mechanism by which dynorphin modulates the NMDA receptor remains therefore to be elucidated. Additional evidence for an excitatory action of spinal dynorphin has been supported by observations demonstrating that pharmacologically administered dynorphin $A_{(1-13)}$ evokes an increased release of glutamate and aspartate into the extracellular fluid of the spinal cord (Skilling et al. 1992). These effects of dynorphin were blocked by pretreatment with MK-801, suggesting an NMDA-receptor mediated action (Laughlin et al. 1997; Skilling et al. 1992; Vanderah et al. 1996). Further, infusion of dynorphin $A_{(1-17)}$ and the *des*-Tyr derivative dynorphin $A_{(2-17)}$ into hippocampal tissue through a microdialysis probe produces dose-dependent release of glutamate and aspartate, and this effect was not blocked by opioid antagonists (Faden 1992). Dynorphin $A_{(1-17)}$ has also been shown to potentiate capsaicin-evoked release of substance P from caudal trigeminal nuclear slices. This effect was not blocked by μ, δ or κ opioid receptor antagonists but was blocked by NMDA antagonists (Arcaya et al. 1999). Furthermore, neonatal capsaicin, which selectively destroys substance P containing primary afferent neurons, blocked the ability of dynorphin to facilitate K^+-evoked release of substance P in trigeminal slices (Arcaya et al. 1999). It was concluded that dynorphin enhances the release of substance P from primary afferent C fibers through an NMDA-related mechanism to promote exaggerated pain (Arcaya et al. 1999). Hargreaves and colleagues have recently demonstrated enhanced capsaicin-evoked CGRP release by dynorphin $A_{(2-13)}$ from naive rat spinal cord sections (Claude et al. 1999a).

Table 1.2. Binding affinities of dynorphin A peptides at cloned human opioid receptors.

Dynorphin Fragment	$_h$KOR[A] K_i (nM)	$_h$DOR[B] K_i (nM)	$_h$MOR[C] K_i (nM)
Dynorphin $A_{(1-17)}$	5.3	182	6.9
Dynorphin $A_{(1-13)}$	6.4	141	16.4
Dynorphin $A_{(2-17)}$	>10,000	>10,000	>10,000
Dynorphin $A_{(2-13)}$	>10,000	>10,000	>10,000

KOR = κ opioid receptor; DOR = δ opioid receptor; MOR = μ opioid receptor. [A]KOR were labeled with 1.8 nM [^{3}H]U69,593 (Kd = 1.4 nM); [B]DOR were labeled with 1.5 nM [^{3}H]pCl-DPDPE (Kd = 1.3 nM); and [C]MOR were labeled with 0.45 nM [^{3}H]DAMGO (Kd = 370 pM). Data were mean values from two independent experiments.

Summary of research goals

As previously discussed, dynorphin was originally identified as an endogenous opioid peptide, although it does possess significant non-opioid pharmacology. In general opioids tend to be inhibitory. These compounds interact with their respective opioid receptor to limit either neurotransmission or neuronal excitability. Dynorphin, however, can elicit excitatory effects in vivo that are not mediated through opioid receptors. The fact that NMDA receptor antagonists can block these excitatory effects of dynorphin suggests there may be an increased release of excitatory amino acid and other transmitters. Therefore, the focus of this dissertation will be to explore the role of dynorphin as a pronociceptive mediator in opioid-induced paradoxical pain and antinociceptive tolerance. Specifically, three hypotheses will be tested. First, opioid-induced abnormal pain manifests, behaviorally, as antinociceptive tolerance such that, manipulations that block opioid-induced pain will restore the potency of spinal opioids.

Second, descending pain facilitation arising from the RVM will result in plasticity at the spinal cord level including the upregulation of dynorphin. Finally, pathological levels of spinal dynorphin act to promote opioid-induced abnormal pain by potentiating the release of CGRP from the central terminals of primary afferent neurons.

CHAPTER II. MATERIALS AND GENERAL METHODS

Animals Used

Unless otherwise indicated the experiments contained in this dissertation were carried out using male Sprague-Dawley rats (225-300g; Harlan; Indianapolis, IN) and male ICR mice (20-30g; Harlan; Indianapolis, IN). All animals were maintained on a 12/12 hr light/dark cycle and provided food and water *ad libitum* except during the experimental procedures. All experiments, involving animals, were performed under an approved protocol in accordance with institutional guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Prodynorphin "Knock-out" Mice

The mice used in this study were progeny of progenitor 129/SvEv-Tac mice that were heterozygous at the prodynorphin gene with one wild type allele (+) and one null allele (-). In the null allele (-), the coding region and 1 kb of the 3' untranslated region of the prodynorphin gene were deleted by the replacement of exons 3 and 4 with a neomycin cassette pAB5 (Sharifi et al. 2001). Male mice that were homozygous prodynorphin knock-out (-/-), and littermates which were homozygous wild-type (+/+) were used. Both genotypes were viable and showed normal growth and reproduction. Genotyping of litters was initially performed with PCR and confirmed by Southern transblot. Subsequently, mice were routinely genotyped by PCR using a set of prodynorphin primers (5'-CAG GAC CTG GTG CCG CCC TCA GAG-3', 5'-CGC TTC

TGG TTG TCC CAC TTC AGC-3'; these yield a 500 bp product) and *neo* primers (5'-ATC CAG GAA ACC AGC AGC GGC TAT-3', 5'-ATT CAG ACA CAT CCC ACA TAA GGA CA-3'; these yield a 1200 bp product). Each mouse was genotyped twice using DNA from two separate extractions from the tail tissue samples.

Drugs and Doses Used

The opioid μ agonists used in the experiments contained in these pages were morphine sulfate (Sigma, St. Louis, MO), [D-Ala^2,N-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) (Sigma, St. Louis, MO) and the (+/-) enantiomers of oxymorphone (courtesy of Dr. Kenner Rice).

The doses of dynorphin $A_{(1-13)}$ antiserum (Bachem, San Carlos, CA) used were and 50, 100, 150 and 200 μg; these doses were chosen on the basis of previous work (Gardell et al. 2002a; Vanderah et al. 2000). According to the manufacturer, the antiserum to dynorphin $A_{(1-13)}$ demonstrates 100% cross-reactivity with dynorphin $A_{(1-17)}$ and dynorphin $A_{(1-12)}$ fragments. Pre-immune (control) serum served as a control for the dynorphin $A_{(1-13)}$ antiserum, and was given in a dose of 150 or 200 μg. This control serum was collected, in the absence of antigen, from the same species of rabbits. However, this serum was not taken from the same rabbit used to generate the dynorphin $A_{(1-13)}$ antiserum.

The vehicle for morphine sulfate, DAMGO and (-)-oxymorphone HCl was distilled water. The vehicle for the (+)-oxymorphone was equimolar HCl. The vehicles for the dynorphin $A_{(1-13)}$ antiserum and for MK-801 were distilled water and physiological saline, respectively.

Intrathecal Drug Administration

Intrathecal (i.th.) injections (5.0 μl) were made into the subarachnoid space (L_5-L_7) in unanesthetized mice using a modified version (Porreca and Burks 1983) of a previously described method (Hylden and Wilcox 1980). While under halothane anesthesia, rats were implanted with i.th. catheters (PE-10, 7.5 cm) as described previously (Yaksh and Rudy 1976) for drug administration at the level of the lumbar spinal cord. Test compounds were injected through the i.th. catheter in a volume of 5.0 μl followed by a 9.0 μl saline flush.

Sustained drug administration

For sustained drug administration, 75 mg morphine pellets or placebo pellets were implanted subcutaneously. For mice, one of each pellet was used whereas, for rats, two of each type of pellet was used. Chronic spinal infusions were performed in rats with osmotic mini-pumps (Durect Corporation, Cupertino, CA). The osmotic pumps have the capacity to deliver test compounds at a rate of 1.0 μl/hour for up to 7 days. The minipumps were attached to the indwelling i.th. catheters and placed in the subcutaneous space. On test day the mini-pumps were disconnected under light surgical anesthesia (halothane), removed and the i.th. catheter exteriorized. As before, test compounds were injected through the i.th. catheter in a volume of 5.0 μl followed by a 9.0 μl saline flush.

Spinal DLF lesions

Bilateral spinal lesions at the T_8 level were performed in naive rats under halothane anesthesia. A laminectomy was made at the T_8 level to expose the spinal cord. Lesions of the dorsolateral funiculus (DLF) were performed by crushing the area with

fine forceps. Sham DLF surgery was performed by exposing the vertebrae and performing the laminectomy, but without cutting neuronal tissue. Hemostasis was confirmed and the wound over the exposed spinal cord was packed with gelfoam and closed. All lesions were verified histologically at the termination of the experiment. Only rats with appropriately placed DLF lesions were included in the subsequent data analysis.

Tissue extraction and preparation

Rats were deeply anesthetized with halothane and decapitated. The spinal column was cut through at the pelvic girdle. Hydraulic extrusion was performed by inserting a 16-gauge needle (22-gauge needle for mice) into the sacral vertebral canal and expelling with ice-cold saline. The spinal cord was immediately placed on ice in a glass petri dish and the dorsal half of the lumbar cord was dissected. Tissue samples to be used for dynorphin quantification were immediately frozen in liquid nitrogen and stored at -70°C until assayed. Tissues for use in the CGRP release assay, were weighed and chopped into 0.2 mm cubes with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall UK).

Dynorphin immunoassay

Tissues that were stored at -70°C were thawed and placed in 1.0 N acetic acid, disrupted using a Polytron homogenizer and incubated for 30 min at 95°C. After centrifugation at 14,000 x g for 20 min (4°C) the supernatant was lyophilized and then stored at -70°C. Protein concentrations were determined using the bichinchoninic acid method with bovine serum albumin as a standard. Immunoassay was performed with a

commercial enzyme immunoassay system using an antibody specific for dynorphin $A_{(1-17)}$ (Bachem, San Carlos, CA). Standard curves were constructed and the dynorphin content determined using GraphPad Prizm (GraphPad Inc, San Diego, CA).

CGRP release assays

After chopping the fresh tissue, these samples were then placed in a 1.0 cc chamber and continually superfused with oxygenated modified Kreb's buffer (135 mM NaCl, 3.5 mM KCl, 1.0 mM $MgCl_2$, 20 mM NaHCO, 1.0 mM NaHPO, 2.5 mM $CaCl_2$, 3.3 mM dextrose, 0.1 mM ascorbate, 10 mM thiorphan and 0.1 % bovine serum albumin) maintained at 37°C, pH 7.4, at a rate of 0.5 ml/min with a Brandel Superfusion Pump (Brandel, Gaithersburg, MD). The tissue was allowed to equilibrate for 45 minutes. Superfusate was collected in 3-minute intervals into test tubes by using a Gilson FC203B fraction collector (Gilson, Middleton, WI). A total of 5 fractions (15 min) were collected prior to adding capsaicin. Capsaicin was then added for a perfusion concentration of 1.0 μM for 6 minutes (2 fractions). Superfusate was then collected for an additional 27 minutes (9 fractions).

Radioimmunoassay for CGRP in perfusate

The superfusate obtained from the release assay was pre-incubated with 100 μl of anti-CGRP antibody (courtesy of Dr. Michael Iadorola; or obtained from a commercial source, Bachem, San Carlos, CA) for 24 hr at 4°C. Both anit-CGRP antibodies bind CGRP equally well. Then, the samples each received 100 μl of [^{125}I]-$CGRP_{28-37}$ or [^{125}I]-CGRP (Bachem, San Carlos, CA) (at 20,000-30,000 cpm) and 50 μl of goat anti-rabbit antisera coupled to ferric beads and were subjected to an additional incubation for 24 hr.

Both [^{125}I]-CGRP$_{28-37}$ and [^{125}I]-CGRP demonstrated similar binding to the primary anti-CGRP antibody. At that time, the [^{125}I]-CGRP bound to the CGRP antibody was separated from the free tracer through immunomagnetic separation (PerSeptive Diagnostics, Cambridge, MA). The liquid was aspirated and the immunoprecipitated product was subjected to a gamma-counter. Standard curves were generated and CGRP content was determined through logit-log analysis. This assay yielded a minimal detection limit of 1.0 to 3.0 fmol/tube. The CGRP antiserum used in these experiments binds near the C-terminal end of CGRP and does not cross-react with cholecystokinin, neuropeptide Y, substance P, dynorphin $A_{(1-17)}$, nociceptin (orphanin F/Q), or other peptides with similar C-terminal residues. The CGRP concentrations are plotted against time, and the area under the time-effect curve above the baseline CGRP levels and obtained during the capsaicin infusion provide capsaicin-evoked CGRP release.

Total CGRP content

Tissues that were stored at -70°C were thawed and placed in 2.0 ml of 0.01 N HCl and disrupted using a Polytron homogenizer. The tissue homogenate was then centrifuged at 2500 x g for 20 min and the supernatant was diluted 1:400 in modified Kreb's buffer and then assayed for total CGRP content using the radioimmunoassay described above.

Immunostaining for the μ opioid receptor

The naive, saline-infused, morphine-infused and DAMGO-infused rats were deeply anesthetized with ketamine and perfused transcardially with 200 ml of PBS, pH 7.4, containing heparin (1500 IU/ l), followed by 500 ml of cold 4% paraformaldehyde.

After perfusion the spinal cords were isolated and post-fixed for 4 hr in 4% paraformaldehyde and then cryoprotected with 30% sucrose in PBS overnight at 4°C. Frontal frozen sections (40 μm) were prepared from the lumbar enlargement of the spinal cord. These sections were immunolabeled with either a guinea pig antiserum against prodynorphin or with a rabbit antiserum against the rat μ-opioid receptor (courtesy of Dr. Robert Elde). Briefly, the spinal cord sections were rinsed twice for 5 min each in PBS and then preincubated with PBS containing 4% normal goat serum, 0.3% Triton X-100, and 1% bovine serum albumin for 30 min at room temperature. The sections were then incubated with the primary antiserum diluted in the preincubation buffer overnight at 4°C (prodynorphin antiserum at 1:40,000 dilution; μ opioid receptor antiserum at 1:20,000 dilution). The sections were washed three times for 10 min each in PBS, followed by incubation with a biotinylated secondary antibody (goat anti-guinea pig IgG or goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA) at 1:1000 in PBS with 0.25% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) for 60 min at room temperature. Sections were washed three times for 10 min each in PBS and stained with the avidin–biotin complex (ABC kit; Vector Laboratories); sections were incubated with an avidin-biotinylated horseradish peroxidase complex diluted 1:500 in PBS-BT for 2 hr at room temperature, washed three times for 10 min each in PBS, and developed with a solution of diaminobenzidine and H_2O_2 (FAST DAB SETS; Sigma, St. Louis, MO) maintained uniformly throughout the experiments. Sections from control and treated animals were processed in parallel under identical experimental conditions. The sections were washed

and mounted on glass slides, air-dried overnight, rinsed in histological clearing solvent, and coverslipped with DPX.

Immunofluorescence of CGRP

Following the anesthesia with ketamine HCl/Xylazine (1.0 ml/kg, Sigma, St. Louis, MO), the rats were perfused transcardially with 0.1 M PBS until the exudate ran clear, and fixed with 10% formalin and 0.05% glutaraldehyde in 0.1 M PBS (pH 7.4) for approximately 15 min. Lumbar spinal cords were harvested and postfixed in 10% formalin overnight and cryoprotected with 20% sucrose in 0.1 M PBS. Frozen frontal sections (20 μm) were washed in 0.1 M PBS, then incubated with a rabbit anti-CGRP antiserum (1:40,000, Bachem, San Carlos, CA) in 0.1 M PBS with 5% normal goat serum overnight at 4°C, followed by washing and secondary incubation with a Cy3-conjugated goat anti-rabbit IgG (1:500, Jackson Labs, Bar Harbor, ME) for 2 hrs. The sections were rinsed and mounted in VectorShield. Fluorescence digital images were captured using a Nikon E800 fluorescence microscope outfitted with a Hamamatsu C5810 color CCD camera that output to a Pentium microcomputer.

Assessment of sensitivity to non-noxious mechanical stimuli

Mice or rats were allowed to acclimate within plexiglass enclosures for approximately 20 min. A series of calibrated (0.02-2.34 g for mice and 1.56-15.0 g for rats, logarithmically spaced) von Frey filaments were applied to the plantar aspect of a hindpaw until a response was elicited. Paw withdrawal thresholds to probing were determined according to the method previously described (Chaplan et al. 1994). These data were represented as mean paw withdrawal threshold ± SEM. Paw withdrawal

thresholds were determined to the nearest 0.1 g before treatment (baseline) and again, either daily during, or after the termination of, the treatment regimen.

Assessment of sensitivity to noxious thermal stimuli

Radiant heat paw flick test

Mice or rats were allowed to acclimate within plexiglass enclosures, on a surface maintained at 30°C, for approximately 20 min. A radiant heat source (i.e., high intensity projector lamp) was activated with a timer and focused onto the plantar surface of the hindpaw. A motion detector that halted both lamp and timer when the paw was withdrawn determined paw withdrawal latency. A maximal cut-off of 40 sec for rats and 30 sec for mice was employed to prevent tissue damage. A significant reduction in paw withdrawal latency from baseline value was interpreted as thermal hypersensitivity. Pairwise comparisons were performed with Student's t-test and significance was set at $p \leq 0.05$.

Hot plate test

Mice were placed in a plexiglass rectangle on a heated plate with temperature controlled to 55°C unless otherwise noted. The latency to licking of a hindpaw indicated the nociceptive response. The cut-off time was set at 30 sec in order to prevent tissue injury.

Tail flick test

Nociceptive testing was also performed using the tail-flick test with water at 52°C employed as the noxious stimulus. Tail flick latencies were determined to the nearest 0.1 s before treatment and in response to a challenge dose of test compound. A cut-off

latency of 10 sec for rats and 15 sec for mice was employed in order to prevent tissue injury.

CHAPTER III: SYSTEMIC MORPHINE STUDIES IN MICE

Introduction

Although compounds that are μ opioid receptor agonists remain the primary choice for the treatment of acute pain, the use of opioids in chronic neuropathic pain states often results in inadequate analgesia. It has been observed clinically that repeated spinal administration of μ opioids produces abnormal pain states that are similar to states of neuropathic pain (Ali 1986; Arner and Meyerson 1988; De Conno et al. 1991; Devulder 1997; Stillman et al. 1987). These findings have also been replicated in experimental animals models (Celerier et al. 2000; Larcher et al. 1998; Mao et al. 1998; Mao et al. 1994; Mao et al. 1995e; Vanderah et al. 2000; Vanderah et al. 2001a; Vanderah et al. 2001b; Woolf 1981; Yaksh and Harty 1988; Yaksh et al. 1986). Clinical studies involving chronic spinal morphine administration have reported that paradoxical novel pain can occur in areas other than the site of the original insult (Ali 1986; De Conno et al. 1991; Devulder 1997; Stillman et al. 1987). In addition, the reduction in analgesic potency following sustained spinal μ opioids is similar to the diminished effect of spinal μ opioids in animal models of neuropathic pain (Ossipov et al. 1995). Therefore, both opioid tolerance and neuropathic conditions share features of diminished μ opioid analgesic potency and efficacy with the development of abnormal pain including thermal and mechanical hypersensitivity. These common features have led to the idea that there exists a possible common mechanism in the post-nerve injury state and in spinal μ opioid tolerance (Mao et al. 1994; Mao et al. 1995a; Mao et al. 1995c).

There are a number of recent reports that provide evidence for the contribution of *N*-methyl-D-aspartate (NMDA) receptors to opiate tolerance as well as other phenomena involving plasticity of the nervous system, particularly neuropathic pain (Marek et al. 1991; Trujillo and Akil 1991a). The systematic co-administration of morphine with an NMDA receptor antagonist, MK-801, to rats prevents the development of tolerance in a dose-dependent manner (Trujillo and Akil 1991a). MK-801 alone does not produce analgesia, nor does it increase the effects of morphine in non-tolerant rats (Trujillo and Akil 1991a). Furthermore, administration of MK-801 to mice made tolerant to opioids restores the antinociceptive effects of spinal opioids (Gardell et al. 2001). Basbaum and colleagues (Liu et al. 1997; Liu et al. 1994) have reported the presence of NMDA receptors on central terminals of primary afferent fibers, sites known to express opioid receptors. For this reason, an anatomical basis exists for modulation of opioid tolerance by altering NMDA receptor activity. However, the source of activation of the NMDA receptor complex during the development of opioid tolerance is uncertain.

A key feature of pain induced by nerve-injury (or inflammation) is the increase in expression of spinal dynorphin. While dynorphin is an endogenous opioid receptor ligand which acts acutely to produce antinociception at the kappa opioid receptor (Ossipov et al. 1996), this peptide may also have a pronociceptive role in chronic pain states such as the neuropathic pain elicited by injury to peripheral nerves. Several investigators have shown that peripheral nerve injury results in an elevation of spinal dynorphin content (Cho and Basbaum 1989b; Draisci et al. 1991; Dubner 1991; Dubner and Ruda 1992; Kajander et al. 1990). Bian and colleagues (Bian et al. 1999)

demonstrated a significant increase in multisegmental levels of dynorphin after L_5/L_6 or S_2 spinal nerve ligation which was associated with mechanical and thermal hypersensitivity of the hindpaw. Functional evidence supporting the pronociceptive role of spinal dynorphin is demonstrated by the findings that (a) dynorphin antiserum blocks the hyperalgesia associated with nerve injury (Wegert et al. 1997), (b) restores the antinociceptive effectiveness of spinal morphine in the nerve-injured rat (Nichols et al. 1997; Wegert et al. 1997), (c) elicits an antiallodynic action of spinal morphine, and (d) restores the expected synergy of spinal/supraspinal morphine administration which is lost in the nerve-injury state (Bian et al. 1999). The effects of antiserum to dynorphin $A_{(1-13)}$ parallel those seen with MK-801 (Bian et al. 1999; Wegert et al. 1997). These observations and others suggest that dynorphin may interact directly or indirectly with the NMDA receptor to promote some aspects of nerve-injury associated pain. Elevated levels of dynorphin in the spinal cord may be a key factor in the maintenance of sensory hypersensitivity, resulting in an enhancement of excitatory amino acid release leading to an increase in the subsequent activation of excitatory amino acid receptors.

Given the known ability of MK-801 to block and to reverse opioid tolerance, and the evidence of direct and indirect interaction of dynorphin with the NMDA receptor, these data also suggest that increases in dynorphin expression may represent a common spinal mechanism, which promotes tolerance and abnormal pain. The purpose of this Chapter is to further explore the role of dynorphin in opioid tolerance and in the accompanying paradoxical pain that develops following sustained spinal opioids.

Results of experiments with sustained morphine in an outbred strain of ICR mice

Non-noxious mechanical thresholds

Paw withdrawal thresholds to probing with von Frey filaments were obtained before (baseline) and across the 5 day period after animals were implanted with either morphine or placebo pellets (Figure 3.1). Paw withdrawal thresholds to probing with von Frey filaments did not change significantly ($p > 0.05$) following placebo pellet implantation in ICR mice. The mean pooled baseline paw withdrawal threshold for these mice prior to receiving any treatments was 1.42 ± 0.10 g. The paw withdrawal thresholds on day 5 for the placebo pelleted mice were 1.40 ± 0.16 g. Sustained subcutaneous administration of morphine significantly reduced ($p \leq 0.05$) paw withdrawal thresholds across the 5 day period of testing in these mice. Paw withdrawal thresholds were significantly ($p \leq 0.05$) reduced to 0.36 ± 0.08 g by Day 2 and progressively diminished to 0.05 ± 0.01 g by Day 5 (Figure 3.1).

In addition, two groups of 20 mice each received either placebo or morphine pellets for 5 days. The paw withdrawal thresholds for the placebo-pelleted mice were 1.40 ± 0.16 g, whereas for the morphine-pelleted mice the paw withdrawal thresholds were 0.05 ± 0.01, on day 5 (Figure 3.2). Each of these 2 groups was then further subdivided into 3 groups containing either 6 or 7 mice each. The groups were treated with either an i.th. injection of MK-801 (n=7), dynorphin $A_{(1-13)}$ antiserum (n=7) or control serum (n=6). The spinal administration of MK-801 (3.4 μg) and of dynorphin antiserum (150 μg) both reversed the increased sensitivity to normally innocuous mechanical stimuli in the mice treated with morphine pellets. Paw withdrawal thresholds

were significantly ($p \leq 0.05$) increased to 1.46 ± 0.26 g and 1.42 ± 0.27 g, respectively. Administration of i.th. MK-801 or dynorphin $A_{(1-13)}$ antiserum did not significantly ($p > 0.05$) alter the paw withdrawal thresholds of the placebo treated mice, achieving paw withdrawal thresholds of 1.63 ± 0.20 g and 1.44 ± 0.24 g, respectively (Figure 3.2). The control serum (150 μg) produced no significant ($p > 0.05$) effects, in any of the groups tested (Figure 3.2).

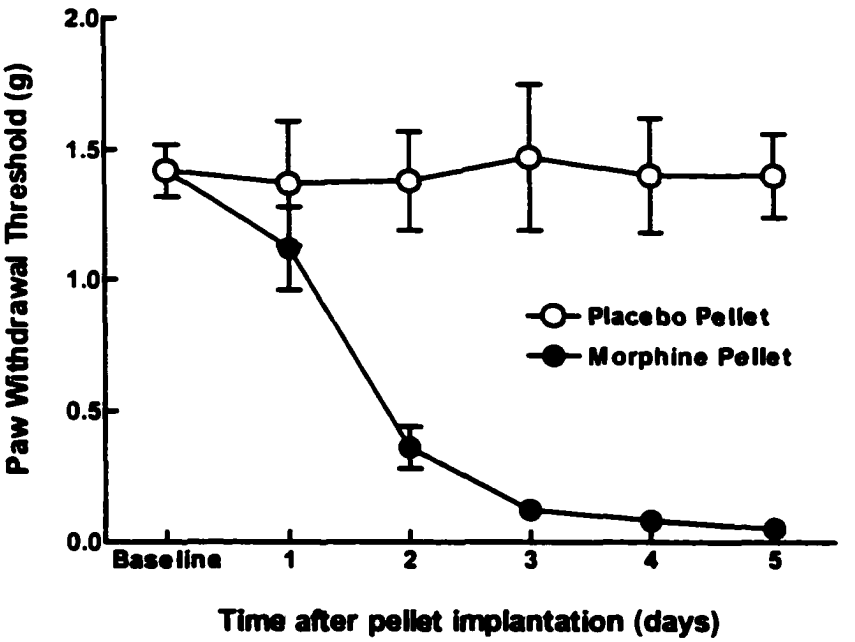

Figure 3.1. The time course of paw withdrawal response thresholds to non-noxious mechanical stimuli (von Frey filaments) across a 5 day period following implantation of a single placebo or morphine pellet (75 mg, s.c.) are represented. Sustained morphine, but not placebo, administration produced an increased sensitivity to response thresholds from von Frey filaments by Day 2 that progressed with time.

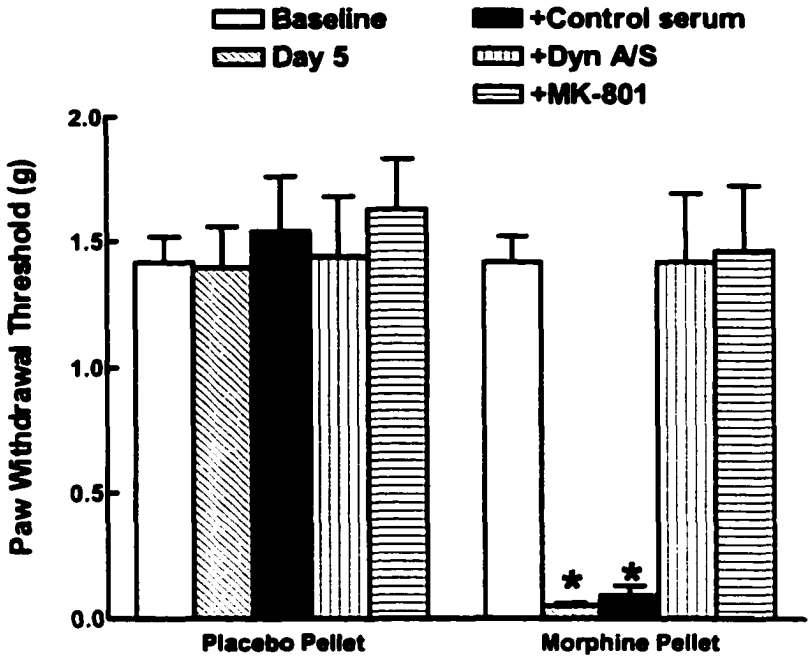

Figure 3.2. A single dose of MK-801 (3.4 μg, i.th.) or antiserum to dynorphin $A_{(1\text{-}13)}$ (150 μg i.th.) reversed the increased responsiveness to probing with von Frey filaments seen following sustained morphine administration. MK-801 or antiserum to dynorphin $A_{(1\text{-}13)}$ did not produce any significant alterations in the placebo treated mice. Control serum did not alter paw withdrawal thresholds in any of the mice. * indicates significant ($p \leq 0.05$) difference from baseline values.

Noxious thermal thresholds

Response latencies to noxious thermal stimuli were obtained before (baseline) and across the 5 day period after animals were implanted with either morphine or placebo pellets (Figure 3.3). Paw withdrawal latencies to noxious radiant heat did not change significantly ($p > 0.05$) following placebo pellet implantation for these mice. The mean pooled baseline paw withdrawal latency for these mice prior to receiving any treatments was 11.03 ± 0.12 sec. The mean paw withdrawal latency on day 5 for the placebo pelleted mice was 11.07 ± 0.20 sec. Sustained subcutaneous administration of morphine significantly reduced ($p \leq 0.05$) paw withdrawal latencies across the 5 day period of

testing in these mice. Paw withdrawal latencies were significantly ($p \leq 0.05$) reduced to 8.38 ± 0.16 sec by day 2 and progressively diminished to 5.97 ± 0.18 sec by day 5 (Figure 3.3).

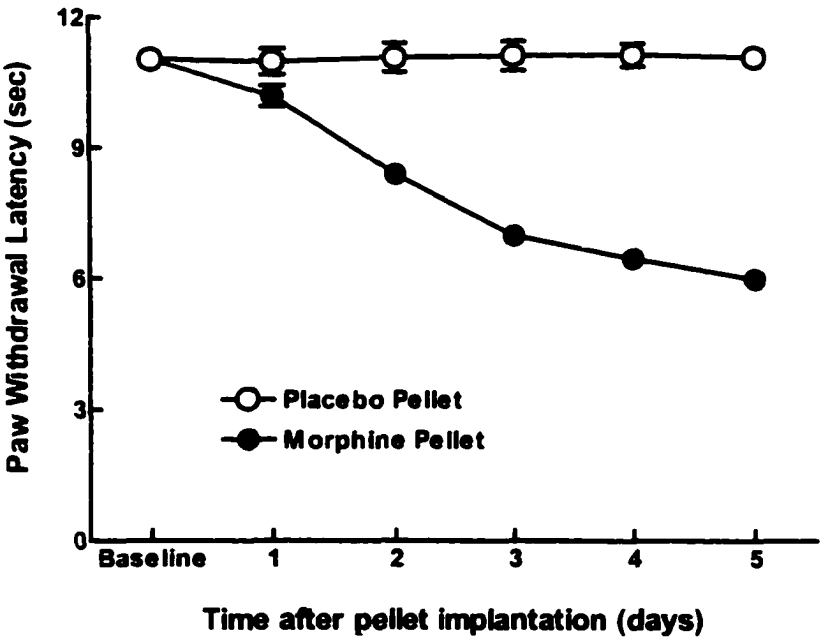

Figure 3.3. The time course of paw withdrawal latencies to noxious thermal stimuli across a 5 day period following implantation of a single placebo or morphine pellet (75 mg, s.c.) are represented. Sustained morphine, but not placebo, administration produced an increased sensitivity to the thermal stimulus by Day 2 that progressed with time.

In addition, 2 groups of 20 mice each received either placebo or morphine pellets for 5 days. The mean paw withdrawal latencies for these mice were 11.07 ± 0.20 sec and 5.97 ± 0.18 sec, respectively, on day 5 (Figure 3.4). Each of these 2 groups was then further subdivided into 3 groups containing either 6 or 7 mice each. The groups were treated with either an i.th. injection of MK-801 (n=7), dynorphin $A_{(1\text{-}13)}$ antiserum (n=7) or control serum (n=6). The spinal administration of MK-801 (3.4 μg) and of dynorphin antiserum (150 μg) both reversed the increased sensitivity to noxious thermal stimuli in

the mice treated with morphine pellets. Paw withdrawal latencies were significantly ($p \leq 0.05$) elevated to 11.33 ± 0.49 sec and 11.05 ± 0.33 sec, respectively. Administration of i.th. MK-801 or dynorphin $A_{(1-13)}$ antiserum did not significantly ($p > 0.05$) alter the mean paw withdrawal latency of the placebo-treated mice, achieving latencies of 11.26 ± 0.35 g and 10.46 ± 0.49 sec, respectively (Figure 3.4). The control serum (150 μg) produced no significant ($p > 0.05$) effects, in any of the groups tested (Figure 3.4).

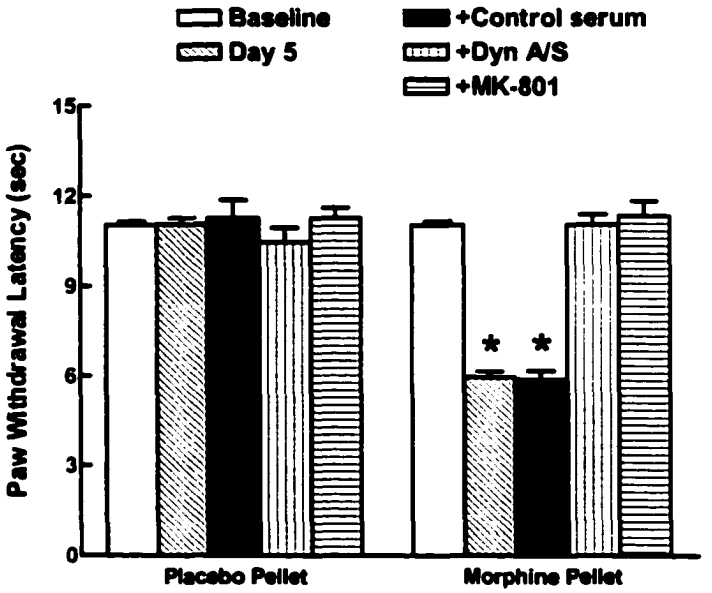

Figure 3.4. A single dose of MK-801 (3.4 μg, i.th.) or antiserum to dynorphin $A_{(1-13)}$ (150 μg, i.th.) reversed the increased sensitivity to the thermal stimulus seen following sustained morphine administration. MK-801 or antiserum to dynorphin $A_{(1-13)}$ did not produce any significant alterations in the placebo-treated mice. Control serum did not alter paw withdrawal latency in any of the mice. * indicates significant ($p \leq 0.05$) difference from baseline values.

Dynorphin Immunoassay

Dorsal lumbar spinal cord halves were processed for dynorphin content in these mice using an enzyme immunoassay. Data obtained was normalized to the placebo-pelleted control group. Sustained morphine administration for 5 days produced a

significant ($p \leq 0.05$) elevation of dorsal lumbar spinal dynorphin content in these mice. Dynorphin peptide content increased from a level of 100.0% ± 3.7% in placebo pelleted mice to 135.0% ± 3.5% in morphine pelleted mice (Figure 3.5).

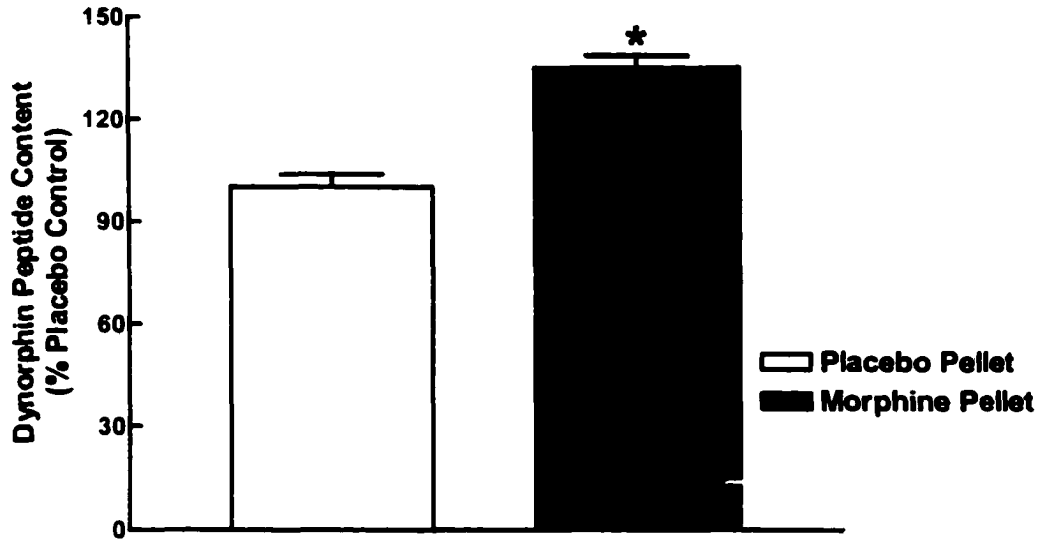

Figure 3.5. Mice that were implanted with a morphine, but not a placebo, pellet demonstrated a significant ($p \leq 0.05$) elevation of dorsal lumbar spinal dynorphin peptide content. * indicates a significant difference ($p \leq 0.05$) relative to the placebo treated group.

Antinociceptive Tolerance

Antinociceptive dose-response curves for i.th. morphine were generated in naive ICR mice and in ICR mice that received either sustained administration of subcutaneous morphine or placebo for 5 days (Figure 3.6). The dose-response curve for i.th. morphine in naive ICR mice was chosen as the benchmark against which all subsequent comparisons were made. Morphine demonstrated an A_{50} value of 3.4 μg (3.2–3.7; 95% C.I.) in these mice (Figure 3.6). However, 5 days after morphine pellet implantation these mice demonstrated antinociceptive tolerance to i.th. morphine as indicated by a

significant ($p \leq 0.05$) 11-fold rightward shift of the i.th. morphine dose-response curve, having an A_{50} value of 37.1 μg (30.9–44.5; 95% C.I.) (Figure 3.6). In contrast, placebo-pelleted mice failed to demonstrate antinociceptive tolerance to i.th. morphine 5 days after placebo pellet implantation, achieving an A_{50} value of 3.4 μg (2.8–4.1; 95% C.I.) as compared to naive ICR mice which had an A_{50} value of 3.4 μg (3.2–3.7; 95% C.I.) (Figure 3.6).

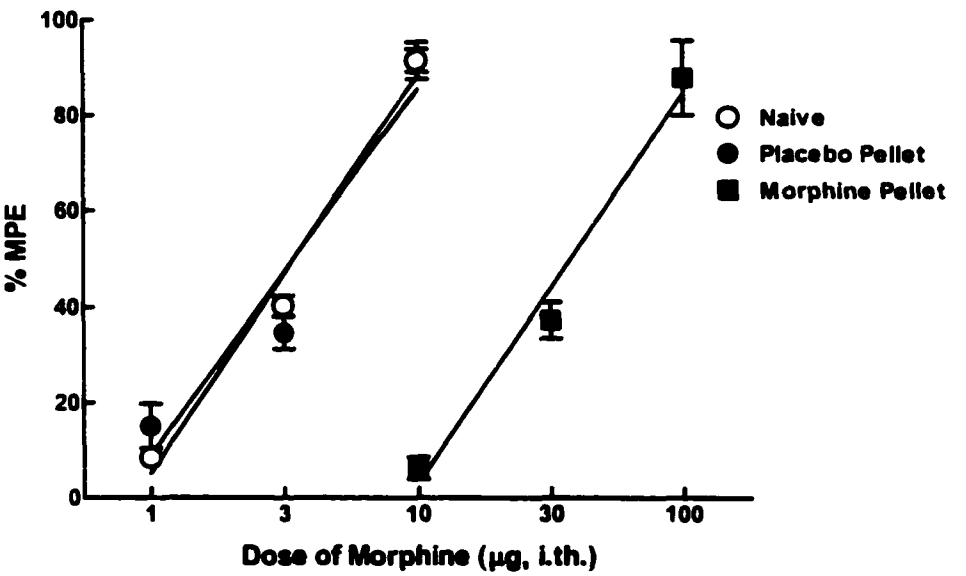

Figure 3.6. The antinociceptive dose-response curves for i.th. morphine in (a) naïve mice (open circles), (b) mice 5 days after placebo pellet implantation (filled circles), and (c) mice 5 days after morphine pellet implantation (filled squares). Sustained morphine administration produced a significant ($p \leq 0.05$) 11-fold shift to the right in the antinociceptive dose-response curve for i.th. morphine. Placebo pellet did not alter the antinociceptive effect of i.th. morphine.

In an additional 2 groups of mice, which received morphine pellets for 5 days, dose-response curves for i.th. morphine were constructed in the presence of either MK-801 (3.4 μg) or dynorphin $A_{(1\text{-}13)}$ antiserum (150 μg) pretreatment (-20 min) (Figure 3.7). Both pretreatment with MK-801 and dynorphin $A_{(1\text{-}13)}$ antiserum completely restored the

potency of i.th. morphine, as indicated by significant ($p \leq 0.05$) 12.4-fold and 12.0-fold leftward shifts of the i.th. morphine dose-response curve, in morphine-pelleted ICR mice. The A_{50} values for i.th. morphine in morphine-pelleted ICR mice were 3.0 μg (2.6–3.4; 95% C.I.) and 3.1 μg (2.7–3.4; 95% C.I.), respectively, compared with an A_{50} value of 3.4 μg (3.2–3.7; 95% C.I.) achieved in naive ICR mice (Figure 3.7). Pretreatment with control serum did not significantly ($p > 0.05$) alter the potency of i.th. morphine in any of the pelleted ICR mice.

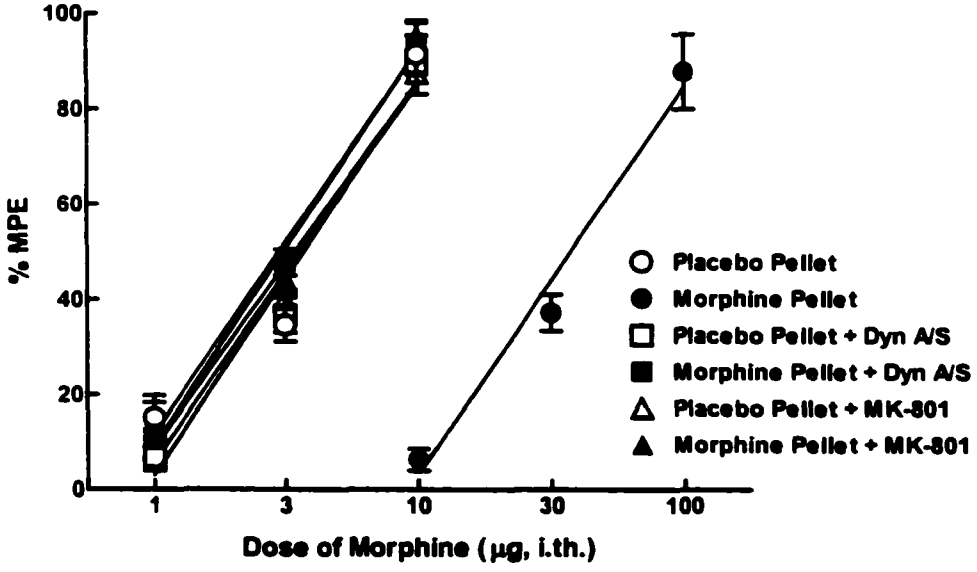

Figure 3.7. The antinociceptive dose-response curves for i.th. morphine in (a) mice 5 days after placebo pellet implantation that were pretreated (-20 min) with i.th. saline (open circles), dynorphin $A_{(1\text{-}13)}$ antiserum (150 μg, open squares) or MK-801 (3.4 μg, open triangles) and in (b) mice 5 days after morphine pellet implantation that were pretreated (–20 min) with i.th. saline (filled circles), dynorphin $A_{(1\text{-}13)}$ antiserum (150 μg, filled squares) or MK-801 (3.4 μg, filled triangles). Sustained administration of morphine produced an 11-fold shift to the right in the antinociceptive dose-response curve for morphine in the animals that received saline prior to morphine challenge. In contrast, pretreatment with MK-801 or antiserum to dynorphin $A_{(1\text{-}13)}$ prior to challenge with morphine produced a significant block of the right shift in the i.th. morphine antinociceptive dose-response curve.

SUMMARY

The data from the present Chapter support the hypothesis of mechanistic similarities at the spinal level between states of opioid tolerance and peripheral nerve injury. A novel concept introduced here is that spinal dynorphin acts as a common mediator promoting these two conditions. Both sustained systemic administration of morphine and peripheral nerve injury elicit mechanical and thermal hypersensitivity, decreased antinociceptive responsiveness to spinal morphine (opioid tolerance) and increases in spinal dynorphin content. The fact that antiserum to dynorphin $A_{(1-13)}$ and MK-801 can acutely reverse morphine tolerance under conditions of desensitized opioid receptors on the primary afferent terminals or spinal cord neurons suggests that homologous receptor desensitization (and perhaps down-regulation) may not be the primary mediator of morphine tolerance in vivo. These data suggest that dynorphin $A_{(1-17)}$ acts as an endogenous mediator promoting some aspects of nerve-injury and opioid-induced abnormal pain as well as opioid antinociceptive tolerance.

The current Chapter suggests an important role for spinal dynorphin in abnormal pain since direct blockade of the effects of dynorphin $A_{(1-17)}$ with antiserum or indirectly with MK-801 clearly attenuated both mechanical and thermal hypersensitivity. The observation of a block of mechanical hypersensitivity by dynorphin $A_{(1-13)}$ antiserum and MK-801 might appear to be inconsistent with the failure of the dynorphin A antiserum to block nerve-injury elicited mechanical hypersensitivity (Malan et al. 2000; Nichols et al. 1996). However, an important aspect of the experiments contained in this Chapter was the evaluation of abnormal pain while morphine was present in these mice. This

procedure insured that the observed abnormal pain was not due to the development of a state of opioid withdrawal. Further, when antiserum to dynorphin and MK-801 were administered spinally, the possible antiallodynic, antihyperalgesic and antinociceptive actions of morphine (at the spinal level) were unmasked. In this regard, the antiallodynic, antihyperalgesic and antinociceptive effects of spinal morphine were increased in the presence of antiserum to dynorphin $A_{(1-13)}$ and MK-801 in the nerve-injured rat (Bian et al. 1999; Nichols et al. 1997; Wegert et al. 1997). These effects of antiserum to dynorphin and MK-801 were specific as (a) control serum had no effect on either placebo- or morphine-pelleted and (b) dynorphin antiserum and MK-801 had no effects on placebo-pelleted mice.

In addition to its role in abnormal pain, spinal dynorphin appears to promote opioid tolerance. In the present study, construction of an i.th. morphine dose-response curve 6 days following morphine pellet implantation resulted in the expected rightward shift with decreased maximum effect, typical of states of opioid tolerance. However, the efficacy and potency of spinal morphine to suppress the tail-flick response was restored by spinal administration of antiserum to dynorphin or MK-801 but not by control serum. Again, these data suggest that the effects of antiserum to dynorphin are specific to dynorphin as (a) control serum had no effect in mice implanted with placebo- or morphine-pellets and (b) dynorphin antiserum and MK-801 had no effects in mice implanted with placebo pellets. These data again demonstrate the parallel actions of antiserum to dynorphin $A_{(1-13)}$ and MK-801, though some reports suggest the need for multiple doses of MK-801 to reverse established opioid tolerance (Trujillo and Akil

1991b). It should be noted that in spite of the ability of MK801 or dynorphin antiserum to block hyperalgesia associated with nerve-injury, neither substance has been shown to modulate tactile allodynia in rats with L_5/L_6 SNL (Nichols et al. 1997; Wegert et al. 1997) or cyroneurolysis of the sciatic nerve (Wagner and Deleo 1996). Blockade of tactile allodynia in rats with spinal nerve injury was achieved with very high doses of MK-801 that produced motor dysfunction (Chaplan et al. 1997). The mechanisms of tactile allodynia appear to differ significantly from those of hyperalgesia in terms of (a) their sensitivity to spinal antagonists, (b) the sensitivity of allodynia, but not thermal hyperalgesia, to spinal transection (Bian et al. 1998; Sun 1999; Sung et al. 1998), and (c) the types of fibers likely mediating the afferent signal (Ossipov et al. 1999). While i.th. morphine does not block allodynia in nerve-injured animals when given alone, it produces dose-dependent antiallodynia in the presence of spinal MK-801 or antiserum to dynorphin $A_{(1-13)}$, suggesting a restoration of the actions of spinal opioids following normalization of the sensitized state of the spinal cord (Bian et al. 1999; Nichols et al. 1997).

While it has long been recognized that NMDA antagonists can block the development of, and reverse established, opioid tolerance, the source of activation of the NMDA receptor under such circumstances is unknown (Trujillo and Akil 1991b). Since opioids hyperpolarize neurons and inhibit neurotransmitter release, the modulation of opioid tolerance by NMDA antagonists has been suggested to occur through intracellular mechanisms in cells that express both opioid and NMDA receptors (Mao et al. 1995b). The present results offer an alternative possibility by which both nerve-injury and spinal

opioids regulate the expression of spinal levels of dynorphin, which may act directly, or indirectly, through the NMDA receptor to promote and maintain a state of spinal sensitization, leading to abnormal pain and opioid tolerance.

CHAPTER IV: SPINAL MU OPIOID RECEPTOR AGONIST STUDIES

Introduction

The previous Chapter demonstrated that sustained systemic morphine results in the development of abnormal pain characterized by the presence of mechanical and thermal hypersensitivity, tolerance to the antinociceptive effects of spinal morphine, and an upregulation of spinal dynorphin. Interference with the actions of spinal dynorphin, either directly using antiserum to dynorphin $A_{(1-13)}$, or indirectly using the NMDA antagonist MK-801, blocks opioid-induced abnormal pain and restores the potency of spinal morphine.

The purpose of the present Chapter is to further explore the mechanism of opioid-induced abnormal pain, antinociceptive tolerance and upregulation of spinal dynorphin, by administering selective μ opioid receptor agonists intrathecally. A second goal of this Chapter is to explore the role of receptor down-regulation in opioid-induced abnormal pain and antinociceptive tolerance.

Results of experiments with sustained delivery of DAMGO in rats

Non-noxious mechanical thresholds

The paw withdrawal threshold in response to probing with von Frey filaments was significantly ($p \leq 0.05$) reduced in rats receiving spinal infusion of DAMGO (1.0 nmol/μl/hr, for 7 days). When tested on the 6th day of DAMGO infusion (i.e., while the infusion was continuing), the paw withdrawal threshold decreased significantly ($p \leq 0.05$) from a baseline of 14.1 ± 0.6 g (before initiation of infusion) to 4.1 ± 0.5 g (Figure 4.1),

indicating the presence of mechanical hypersensitivity during the DAMGO infusion. Rats receiving spinal infusion of saline showed no significant ($p > 0.05$) difference between their preinfusion baseline response (i.e., 14.1 ± 0.6 g) and the paw withdrawal threshold obtained on the 6th day of infusion (i.e., 13.6 ± 0.6 g).

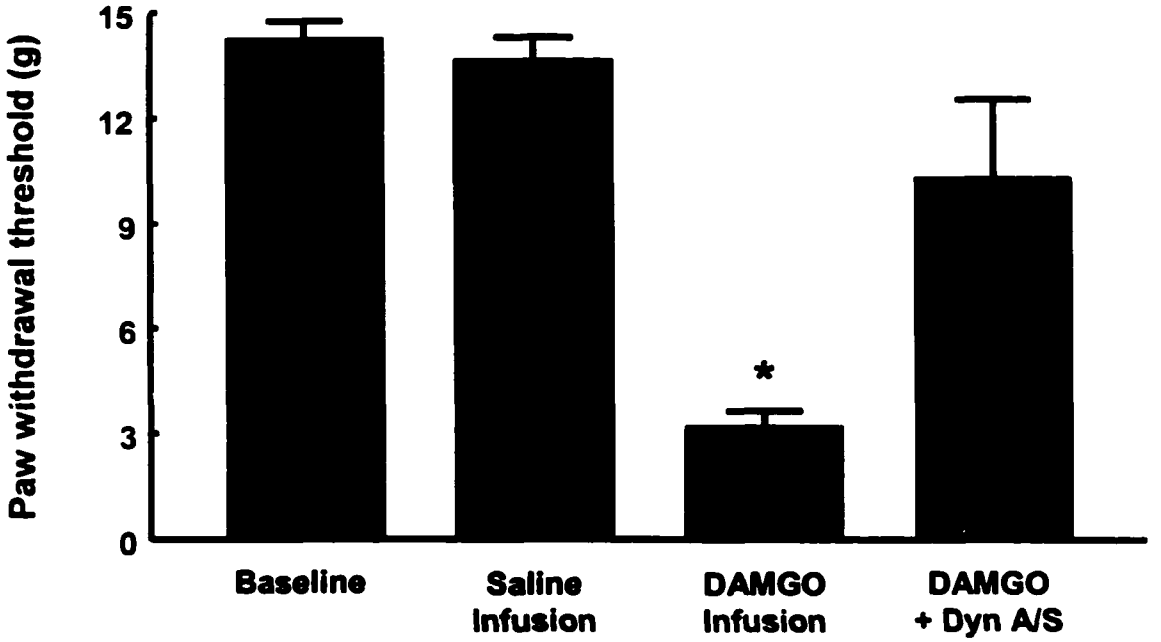

Figure 4.1. Rats received i.th. infusions of saline or DAMGO (1 nmol/μl/hr) for 7 days. Withdrawal thresholds to probing with von Frey filaments were determined prior to infusion (baseline) and on the 7th day after the start of infusion. Mechanical hypersensitivity was indicated by a significant (*; $p \leq 0.05$) decrease in paw withdrawal thresholds when compared to pooled baseline values. Rats that received a single injection of antiserum to dynorphin $A_{(1\text{-}13)}$ (200 μg, i.th.) following spinal DAMGO infusion demonstrated a blockade of mechanical hypersensitivity, as indicated by the elevated paw withdrawal thresholds that were not significantly different from those of the pooled baseline values or the saline-infused control group.

The administration of control serum (200 μg, i.th.) at the termination of the spinal DAMGO infusion on Day 7 had no effect on mechanical hypersensitivity; the paw withdrawal threshold after control serum was 4.2 ± 0.5 g, a value not significantly ($p > 0.05$) different from the response determined on Day 6 during the DAMGO infusion (4.1

± 0.5 g, data not shown). The administration of dynorphin $A_{(1-13)}$ antiserum (200 μg, i.th.) at the termination of the DAMGO infusion on Day 7, however, significantly ($p \leq 0.05$) reversed mechanical hypersensitivity; the paw withdrawal threshold was elevated to 11.5 ± 2.2 g from 4.1 ± 0.5 g (Figure 4.1). Neither control serum nor antiserum to dynorphin $A_{(1-13)}$ alone had any effect on the paw withdrawal thresholds following saline infusion (baseline and post-serum values were 14.1 ± 0.6 g).

Noxious thermal thresholds

The paw withdrawal latencies in response to a noxious thermal stimulus were significantly ($p \leq 0.05$) reduced in rats receiving i.th. infusion of DAMGO. When tested on the 6th day of DAMGO infusion (i.e., while the infusion was continuing), the paw withdrawal latency decreased from a pre-infusion baseline of 17.3 ± 1.5 sec to 10.8 ± 1.2 sec (Figure 4.2), indicating the presence of thermal hypersensitivity during the DAMGO infusion. Rats receiving saline infusion showed no change between their preinfusion baseline paw withdrawal latency (i.e., 18.9 ± 1.1 sec) and their response latency on the 6th day of infusion (i.e., 18.7 ± 0.9 sec).

The administration of control serum (200 μg, i.th.) at the termination of the DAMGO infusion on Day 7 had no effect on thermal hypersensitivity; the mean paw withdrawal latency obtained after the injection of control serum was 12.7 ± 0.8 sec (data not shown), which was not significantly ($p > 0.05$) different from the response latency determined on Day 6 during the infusion of DAMGO (see Figure 4.2). The administration of dynorphin $A_{(1-13)}$ antiserum (200 μg, i.th.) at the termination of the DAMGO infusion on Day 7, however, significantly ($p \leq 0.05$) reversed thermal

hypersensitivity; the paw-flick response after dynorphin antiserum was 35.6 ± 3.6 sec, which was significantly greater than the pre-infusion baseline latency (Figure 4.2). This response indicates not only a reversal of thermal hypersensitivity but also the production of antinociception. Neither control serum nor antiserum to dynorphin $A_{(1-13)}$ had any effect on paw withdrawal latencies following saline infusion of (i.e., pre-infusion baseline values were 20 ± 0.7 and post control serum or dynorphin $A_{(1-13)}$ antiserum values were 20.5 ± 0.7 sec and 19.7 ± 0.6 sec, respectively).

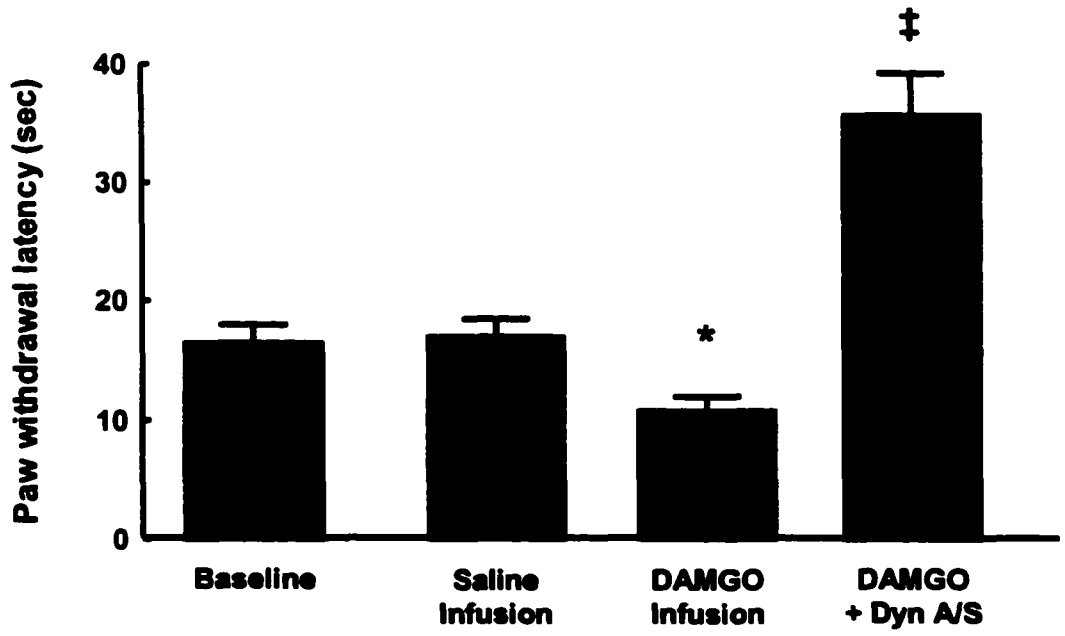

Figure 4.2. Rats received i.th. infusions of saline or DAMGO (1 nmol/μl/hr) for 7 days. Withdrawal latencies of the hindpaws to radiant heat were determined prior to infusion (baseline) and 7 days after the infusion began. Thermal hypersensitivity was indicated by a significant (*; $p \leq 0.05$) decrease in paw withdrawal latencies compared to pooled baseline values. Rats receiving a single injection of antiserum to dynorphin $A_{(1-13)}$ (200 μg, i.th.) showed not only a blockade of thermal hypersensitivity, but unmasked the antinociceptive effect of the infused DAMGO, indicated by the significant elevation in latencies above those of baseline or of saline-infused controls (‡; $p \leq 0.05$).

Dynorphin Immunoassay

Dynorphin content in the dorsal half of the lumbar spinal cord was significantly ($p \leq 0.05$) elevated (1700 ± 120 pg/mg protein) in rats receiving i.th. infusion of DAMGO over a 7 day period as compared to rats receiving infusion of saline over the same period (1297 ± 95 pg/mg protein) (Figure 4.3).

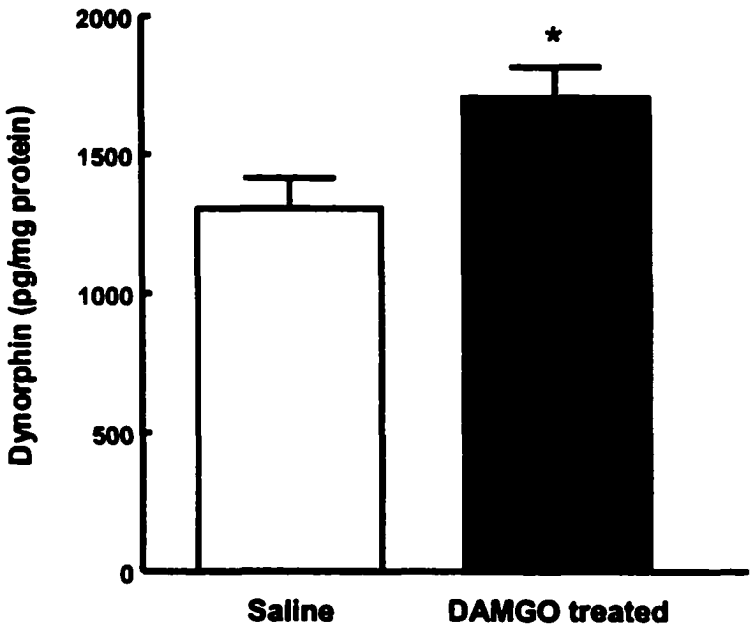

Figure 4.3. Rats received i.th. infusions of saline or of DAMGO (1.0 nmol/μl/hr) for 7 days. The spinal cords were removed and assayed for dynorphin $A_{(1\text{-}17)}$ content with enzyme immunoassay. The rats with DAMGO infusions showed a significant ($p \leq 0.05$) increase in spinal dynorphin content when compared to saline-infused rats.

Antinociceptive tolerance

Spinal infusion of DAMGO over a 7 day period elicited antinociceptive tolerance to a subsequent challenge with DAMGO (1.0 μg, i.th.) and cross-tolerance to challenge with morphine (10 μg, i.th.) (Figure 4.4). The effect produced by DAMGO in the tail-flick test before the DAMGO infusion was 92 ± 4% MPE. The antinociceptive response of the same dose of DAMGO given on Day 7 was significantly ($p \leq 0.05$) reduced to 12 ±

4% MPE, indicating the development of antinociceptive tolerance. Similarly, spinal morphine given before the DAMGO infusion produced a 91 ± 6% MPE, whereas the same dose given to rats receiving DAMGO infusion over a 7 day period resulted in a significant ($p \leq 0.05$) reduction of the antinociceptive effect to 25 ± 6% MPE, indicating cross-tolerance between DAMGO and morphine. The antinociceptive effect of spinal morphine was unaltered in rats receiving saline infusion; morphine (10 μg, i.th.) produced an 88 ± 8.6 and 89 ± 11.4 % MPE before and after saline infusion, respectively.

Dose-response curves for the antinociceptive effect of spinal morphine were generated in naive, saline-infused and DAMGO-infused rats. The A_{50}s for i.th. morphine were 3.1 (2.4-4.1; 95% C.I.), 3.3 (2.9-3.8; 95% C.I.) and >10 μg, respectively (Figure 4.4). Administration of control serum (200 μg, i.th.) did not alter the antinociceptive effect of a single morphine dose (10 μg, i.th.) in rats infused with saline; this dose of morphine elicited an 91.6 ± 6 and 93.5 ± 5 % MPE before and after control serum, respectively. Similarly, dynorphin $A_{(1-13)}$ antiserum (200 μg, i.th.) did not alter the effect of i.th. morphine in rats infused with saline. The A_{50} for i.th. morphine was 3.7 μg (3.1-4.4; 95% C.I.) after dynorphin $A_{(1-13)}$ antiserum. However, the decreased morphine antinociceptive effect seen in rats infused with DAMGO was reversed by antiserum to dynorphin $A_{(1-13)}$. Following dynorphin $A_{(1-13)}$ antiserum, the A_{50} for i.th. morphine in DAMGO-infused rats was 3.6 μg (2.9-4.3; 95% C.I.) (Figure 4.4), a value which was not significantly ($p > 0.05$) different from saline infused rats (see above). Control serum had no effect on the antinociceptive effect of i.th. morphine in rats infused with DAMGO.

The effect of a single dose of morphine (10 μg, i.th.) in DAMGO infused rats before and after control serum was 3.3 ± 2.1 and 2.8 ± 1.9 %MPE, respectively.

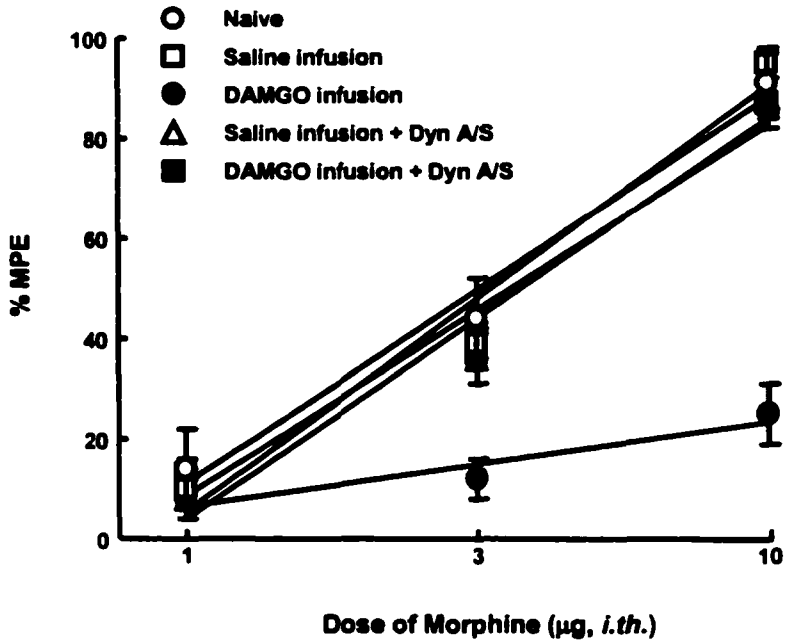

Figure 4.4. Rats received i.th. infusions of saline or of DAMGO (1.0 nmol/μl/hr) for 7 days. Dose response curves for i.th. morphine were generated in the 52°C water tail flick test for several groups of rats receiving different treatments. Morphine dose response curves were generated in naive rats (open circles), rats infused with saline (open squares), and rats infused with DAMGO (filled circles). Additional spinal morphine dose response curves were generated for rats infused with saline (open triangles) or with DAMGO (filled squares) when morphine was injected 20 min after pretreatment with antiserum to dynorphin $A_{(1-13)}$ (200 μg, i.th.). The DAMGO-infused rats showed tolerance to spinal morphine, as indicated by the downward and rightward shift of the morphine dose response curve. Reversal of tolerance by antiserum to dynorphin $A_{(1-13)}$ is shown by the restoration of the dose response curve in the presence of sustained spinal DAMGO such that the curve is superimposed on that of the naive and the saline-infused control groups.

Immunohistochemistry

To further characterize the apparent elevation of spinal dynorphin in DAMGO-infused rats the distribution of its precursor peptide prodynorphin was also examined by immunohistochemistry using an antiserum specific for prodynorphin. Prodynorphin immunoreactivity was found mainly in the superficial laminae, laminae IV and V, and

laminae X of the dorsal horn of the lumbar spinal cord (Figure 4.5). There is an apparent decrease in prodynorphin content in the spinal cord obtained from DAMGO-infused rats when compared to the saline-infused control (Figure 4.5), based on visual inspection of the spinal sections. This apparent decrease in prodynorphin content may reflect the increased production of dynorphin from this precursor. In the superficial laminae, discrete cell bodies and numerous fibers were labeled (Figure 4.5, Panels A & B), whereas in laminae V and VI (Figure 4.5, Panels C & D) and lamina X (Figure 4.5, Panels E & F), prodynorphin immunoreactivity was predominantly associated with fibers. Lumbar spinal sections from DAMGO-infused rats exhibited a reduction of prodynorphin immunoreactivity in all laminae stated above when compared with the saline-infused control (Figure 4.5). This reduction appeared to be primarily caused by a loss of fiber staining in these laminae (Figure 4.5, Panels B, D & F). In the superficial laminae, staining was still clearly present in the cell bodies; the density of immunolabeled cell bodies was similar between control and DAMGO-infused rats (Figure 4.5, Panels A & B).

Lumbar spinal cord sections from control and DAMGO-infused rats were also labeled with μ opioid receptor-specific antibodies. Immunoreactivity for the μ opioid receptor was associated predominantly with the superficial laminae of the dorsal horn of the spinal cord (Figure 4.6). When compared with tissue sections from control rats, sections from the DAMGO-infused rats exhibited a significant reduction in μ opioid receptor immunoreactivity in the superficial laminae (Figure 4.6). This observation is

consistent with a down-regulation of the μ opioid receptors in the spinal cord following the induction of tolerance to a μ opioid agonist.

Additional spinal cord sections from rats were analyzed for μ opioid receptor immunoreactivity in order to explore the time-course of DAMGO-induced down-regulation and to determine whether sustained administration of morphine (by spinal infusion or s.c. pellet) was capable of down-regulating μ opioid receptors (Figure 4.7). Immunoreactivity for the μ opioid receptor in spinal cord tissues taken from naïve rats was compared to immunoreactivity observed in sections from saline, morphine-infused (10 nmol/hr, i.th.) or pelleted (150 mg, s.c.), and DAMGO-infused (1.0 nmol/hr, i.th.) for 7 days. These doses of opioids were chosen as they produce an equivalent degree of antinociception (i.e., approximately 90%). Additional groups of rats were infused with DAMGO for 4 hours, 1 day or 3 days. The spinal cord sections obtained from naïve or saline-infused rats show that μ opioid receptor immunoreactivity is located in the superficial laminae of the dorsal horn (Figure 4.7, Panels A & B). Immunoreactivity for the μ opioid receptor observed in spinal cord sections taken from morphine-treated rats (Figure 4.7. Panels C & D) is similar in distribution and intensity as those from naïve or saline-treated rats when processed in parallel. However, DAMGO infusion produced a time-related reduction in μ opioid receptor immunoreactivity by day 3 of infusion and was clearly evident by the 7th day of DAMGO infusion (Figure 4.7, Panels G & H).

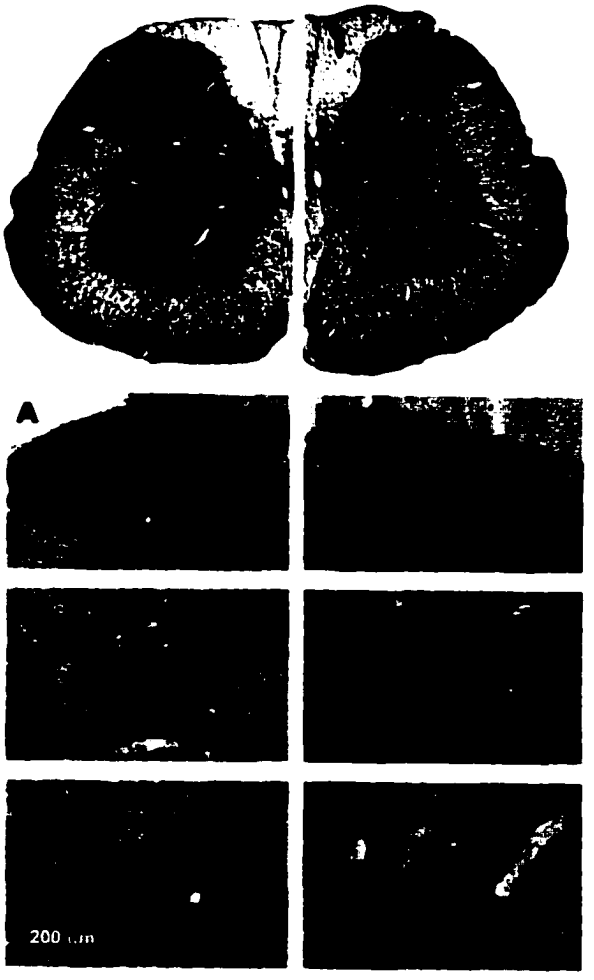

Figure 4.5. Immunohistochemical analysis of prodynorphin in lumbar spinal sections. Rats were infused with either saline (control) or DAMGO for 7 days. Lumbar cross sections (40 µm) were labeled with the antiserum for prodynorphin (1:40,000) and processed for DAB staining by the ABC method. Spinal cord halves are represented in close juxtaposition to allow visual comparison. Top, the left half is obtained from the saline-infused control, and the right half is obtained from the DAMGO-infused rat. Bottom, As seen under higher magnification, the superficial laminae (panels A & B), discrete cell bodies, and numerous fibers were labeled, whereas in laminae V and VI (panels C & D) and laminae X (panels E & F), prodynorphin immunoreactivity was predominantly associated with fibers. Lumbar spinal sections from DAMGO-infused (panels B, D & F) rats exhibited a reduction of prodynorphin immunoreactivity in all the laminae stated above when compared with the saline-infused control (panels A, C & E). This reduction appears to be primarily caused by loss of fiber staining in these laminae. In the superficial laminae, staining was still clearly present in cell bodies; the density of immunolabeled cell bodies was similar between control and DAMGO-infused rats.

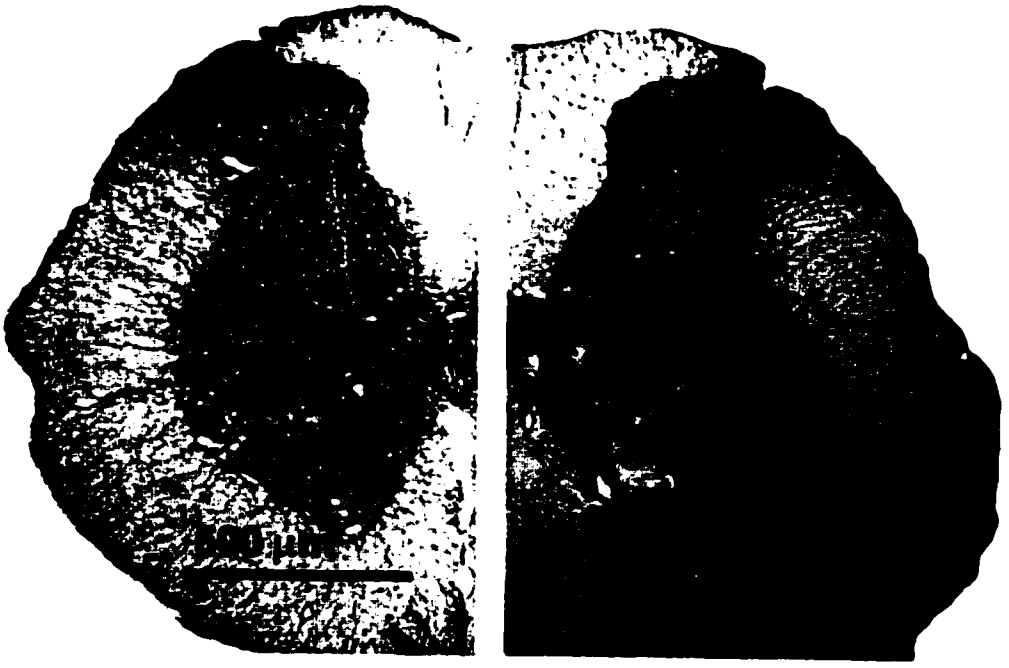

Figure 4.6. Immunohistochemical analysis of the μ opioid receptor in lumbar spinal cord sections. Rats were infused with either saline (control) or DAMGO for 7 days. Lumbar spinal cross sections (40 μm) were labeled with the antiserum for rat μ opioid receptor (1:20,000) and processed for DAB staining by the ABC method. Spinal cord halves are represented in close juxtaposition to allow visual comparison. The left half is obtained the saline-infused control, and the right half is obtained from the DAMGO-infused rat. Substantially higher μ opioid receptor immunoreactivity is observed in the superficial laminae (I and II) of the control cord, indicating a greater concentration of μ opioid receptors, than in that of the DAMGO-treated group.

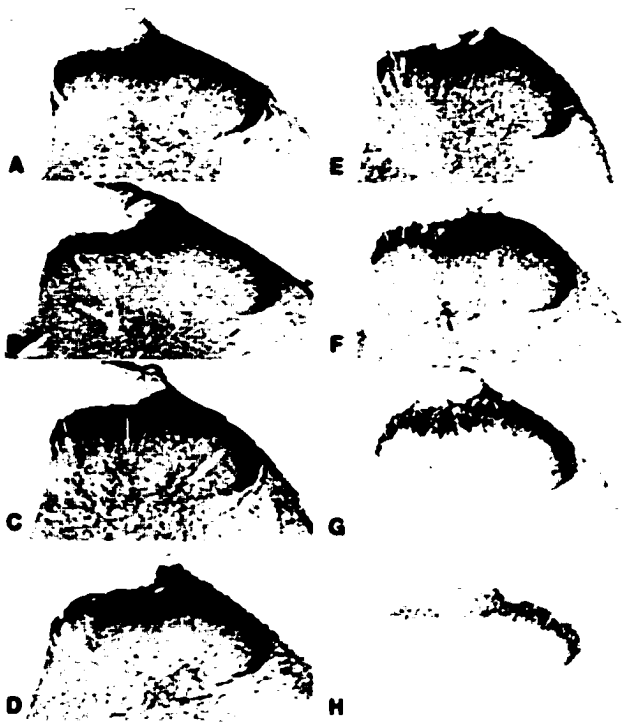

Figure 4.7. A time-course of immunohistochemical analysis of the μ opioid receptor in lumbar spinal cord sections. Lumbar spinal cross sections (40 μm) were labeled with the antiserum for rat μ opioid receptor (1:20,000) and processed for DAB staining by the ABC method. Panels A-D show μ opioid receptor immunoreactivity in naïve rats; 7 days after saline infusion; 7 days after morphine infusion; and 7 days after morphine pellet implantation, respectively. Panels E-H show μ opioid receptor immunoreactivity in rats that received DAMGO infusion for 4 hours; 1 day; 3 days and 7 days, respectively. No differences are apparent in μ opioid receptor immunoreactivity between naïve, placebo-infused, morphine-infused or pelleted rats, consistent with the idea that morphine-treatment does not result in μ opioid receptor down-regulation. In contrast, DAMGO-treated tissues showed a time-related decrease in μ opioid receptor immunoreactivity that is apparent by day 3 of infusion and is maximal after 7 days of infusion, which is consistent with μ opioid receptor down-regulation.

Results of experiments with sustained delivery of (+/-)-oxymorphone

Non-noxious mechanical thresholds

In order to determine whether opioid-induced mechanical hypersensitivity is mediated through the activation of the μ opioid receptors, rats received sustained i.th. vehicle, (-)-oxymorphone (59.2 nmol/μl/hr), or its inactive enantiomer (+)-oxymorphone (59.2 nmol/μl/hr), across a 7 day period. Baseline paw withdrawal thresholds were compared with paw withdrawal thresholds obtained on Day 6 after vehicle-, (-)-oxymorphone-, and (+)-oxymorphone-treatment, (Figure 4.8). Treatment with (-)-oxymorphone, but not vehicle or (+)-oxymorphone, resulted in the development of mechanical hypersensitivity. Paw withdrawal thresholds did not significantly ($p > 0.05$) change as a result of vehicle- (15.0 ± 0.00 vs. 15.0 ± 0.00 g) or (+)-oxymorphone-treatment (15.0 ± 0.00 vs. 15.0 ± 0.00 g). However, for (-)-oxymorphone-treated rats, paw withdrawal thresholds were significantly ($p \leq 0.05$) reduced (15.0 ± 0.00 vs. 4.40 ± 0.55 g), indicating the presence of mechanical hypersensitivity.

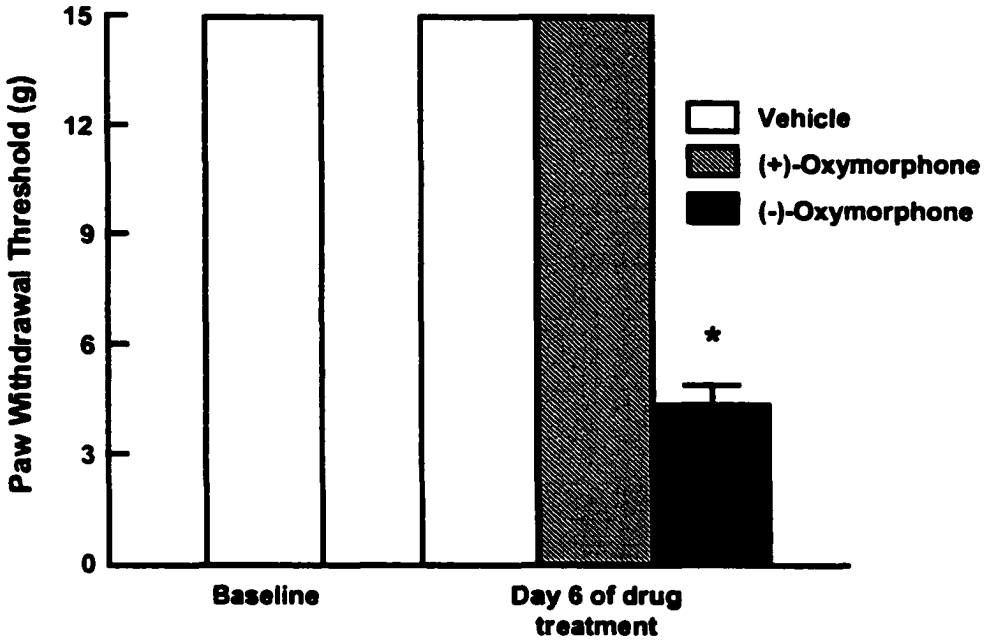

Figure 4.8. Rats received either sustained spinal infusions of vehicle, (+)-oxymorphone or (-)-oxymorphone across a 7 day period. In these rats, paw withdrawal thresholds in response to probing with von Frey filaments were determined prior to infusion (baseline) and after 6 days of sustained i.th. infusion. Sustained i.th. (-)-oxymorphone, but not vehicle or (+)-oxymorphone, administration produced an increased sensitivity to response thresholds from von Frey filaments. * indicates significant ($p \leq 0.05$) difference from baseline values.

Noxious thermal thresholds

In order to determine whether opioid-induced thermal hypersensitivity is mediated through the activation of the μ opioid receptors, rats received sustained i.th. vehicle, (-)-oxymorphone or its inactive enantiomer, (+)-oxymorphone, across a 7 day period. Baseline paw withdrawal latencies to radiant heat were compared with paw withdrawal latencies obtained after vehicle-, (-)-oxymorphone-, and (+)-oxymorphone-treatment, (Figure 4.9). Treatment with (-)-oxymorphone, but not vehicle or (+)-oxymorphone, resulted in the development of thermal hypersensitivity. Paw withdrawal latencies did not significantly change as a result of vehicle- (21.03 ± 0.62 vs. 21.78 ±

0.37 sec, $p > 0.05$) or (+)-oxymorphone-treatment (22.00 ± 0.58 vs. 21.78 ± 0.37 sec, $p > 0.05$). However, for (-)-oxymorphone-treated rats, paw withdrawal latencies were significantly reduced (16.89 ± 0.36 vs. 21.78 ± 0.37 sec, $p \leq 0.05$), indicating the presence of thermal hypersensitivity.

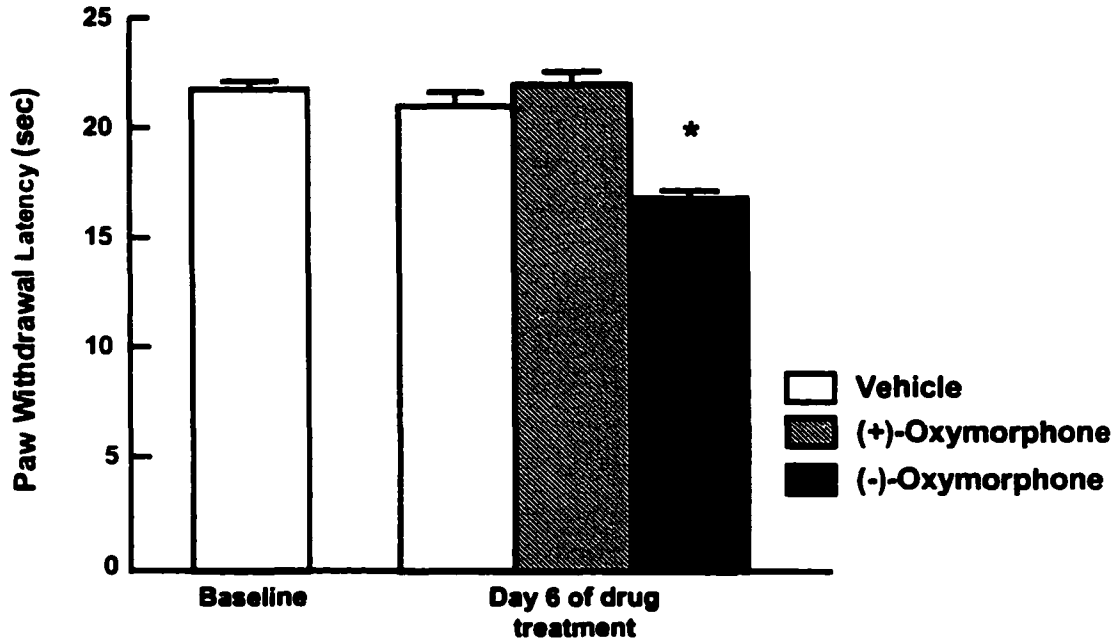

Figure 4.9. Rats received either sustained spinal infusions of vehicle, (+)-oxymorphone or (-)-oxymorphone across a 7 day period. In these rats, paw withdrawal latencies in response to a noxious thermal stimuli were determined prior to infusion (baseline) and after 6 days of sustained i.th. infusion. Sustained i.th. (-)-oxymorphone, but not vehicle or (+)-oxymorphone, administration produced an increased sensitivity to noxious thermal stimuli. * indicates significant ($p \leq 0.05$) difference from baseline values.

Dynorphin immunoassay

Sustained i.th. (-)-oxymorphone, but not vehicle or (+)-oxymorphone, across a 6-day period results in a state of abnormal pain indicated by the presence of both mechanical and thermal hypersensitivity. Next, we determined whether this opioid-induced abnormal pain state was accompanied by an elevation of spinal dynorphin peptide. To accomplish this, dorsal lumbar spinal cord sections were removed on Day 6

and were subjected to an enzyme immunoassay for spinal dynorphin peptide content (Figure 4.10). Data obtained were normalized to the level of the vehicle treated controls, 100% ± 4.9% pg dynorphin/mg total protein. (-)-Oxymorphone-treatment resulted in a significant elevation ($p \leq 0.05$) of spinal dynorphin peptide content, in the dorsal half of lumbar spinal cord, as compared to vehicle- and (+)-oxymorphone-treated groups (127.8% ± 7.6% vs. 100% ± 4.9% and 110.1% ± 7.8% pg dynorphin/mg protein, respectively).

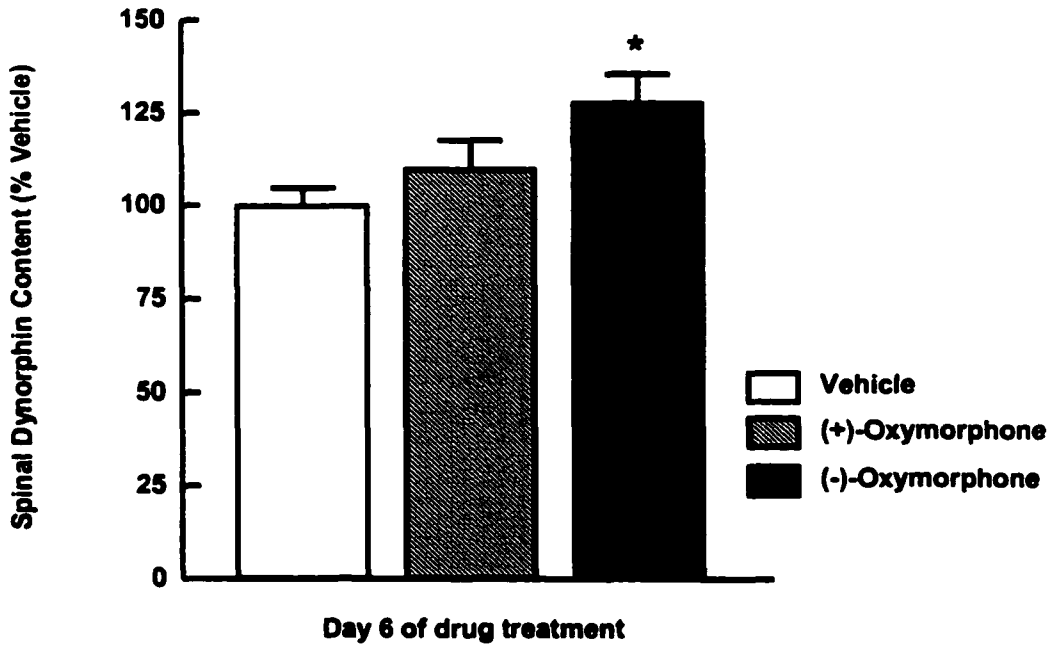

Figure 4.10. Rats received either sustained spinal infusions of vehicle, (+)-oxymorphone or (-)-oxymorphone across a 7 day period. On the 7th day, dorsal lumbar spinal cord tissue was taken from these rats and assayed for spinal dynorphin content using and enzyme immunoassay. Sustained i.th. (-)-oxymorphone, but not vehicle or (+)-oxymorphone, treatment produced an significant increase in dynorphin levels as compared to levels obtained from tissues treated with vehicle or (+)-oxymorphone. Data were normalized to values obtained in vehicle-treated tissues. * indicates significant ($p \leq 0.05$) difference from the vehicle-treated group.

Antinociceptive tolerance

Rats received sustained i.th. infusion of either (-)-oxymorphone or (+)-oxymorphone (59.2 nmol/μl/hr) for 6 days. They were then challenged with a single dose of (-)-oxymorphone (100 μg, i.th.). The antinociceptive effect obtained for (-)-oxymorphone in rats receiving 6 days of spinal (+)-oxymorphone treatment was 87.3 ± 5.0 % MPE and while the response for (-)-oxymorphone in naïve rats was 91.2 ± 2.8 % MPE (Figure 4.11). In contrast, the response of the (-)-oxymorphone pretreated rats was 37.7 ± 2.4 % MPE, which was significantly ($p \leq 0.05$) diminished compared to that of the naive and (+)-oxymorphone-treated groups (Figure 4.11).

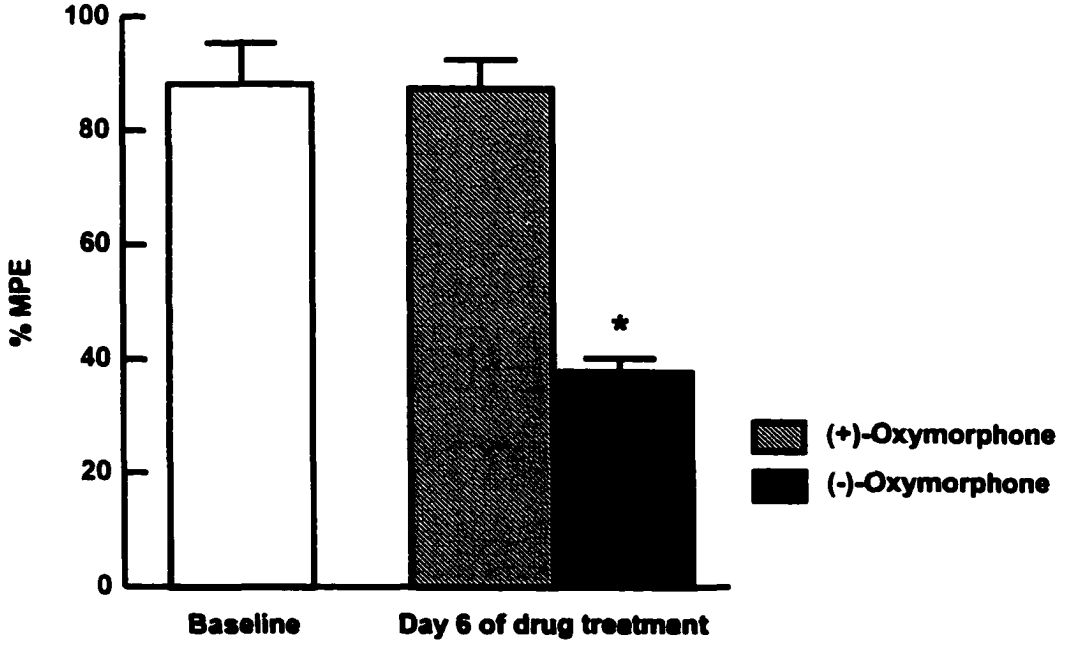

Figure 4.11. Rats received either sustained spinal infusions of (+)-oxymorphone or (-)-oxymorphone across a 7 day period. In these rats, the antinociceptive effect of a spinal A_{90} dose of (-)-oxymorphone (100 μg, at –20 min) was evaluated in response to a noxious thermal stimuli (52°C water) prior to infusion (baseline) and after 6 days of sustained i.th. infusion. Sustained i.th. (-)-oxymorphone, but not (+)-oxymorphone, administration resulted in tolerance to the antinociceptive effects of (-)-oxymorphone. * indicates significant ($p \leq 0.05$) difference from baseline values.

SUMMARY

Results from the present Chapter demonstrate that the continuous intrathecal infusion of selective μ opioid receptor agonists results in tactile and thermal hypersensitivity along with antinociceptive tolerance. These effects were accompanied by a significant elevation in spinal dynorphin content. Data obtained with enantiomeric pairs of oxymorphone suggest that elevation of spinal dynorphin results from μ opioid receptor activation. Additionally, intrathecal administration of antiserum to dynorphin $A_{(1-13)}$ to rats receiving DAMGO infusions not only blocked the manifestations of abnormal pain, but also unmasked the antinociceptive action of the DAMGO infusion. Further, dynorphin $A_{(1-13)}$ antiserum also reversed antinociceptive tolerance evoked by DAMGO infusion. The role of abnormally elevated levels of spinal dynorphin appears to be a common denominator in many circumstances promoting abnormal pain and antinociceptive tolerance. For example, prolonged exposure to spinal or systemic morphine is associated with elevated spinal dynorphin, and the abnormal pain and antinociceptive tolerance so produced is blocked by dynorphin $A_{(1-13)}$ antiserum given intrathecally (refer to Chapter III).

A significant distinction between prolonged exposure to morphine and DAMGO is that sustained administration of DAMGO is associated with a decrease (down-regulation) of μ opioid receptor from the dorsal horn of the spinal cord, whereas morphine is not (refer to Figures 4.6 and 4.7). Because of this observation, abnormal pain and antinociceptive tolerance produced by morphine may be dependent upon an activation of tonic descending facilitation. In contrast, however, DAMGO-induced pain

and tolerance is more likely to be the result of increased excitatory neurotransmitter release from primary afferent neurons in response to the diminished inhibitory tone following μ opioid receptor down-regulation.

In support of possible mechanistic differences between morphine- and DAMGO-induced abnormal pain and antinociceptive tolerance is the fact that manipulations that produce down-regulation of the μ opioid receptor appear to mimic DAMGO-induced abnormal pain and tolerance. One key example is the observation that "knock-down" of μ opioid receptor by treatment with antisense oligodeoxynucleotides to mRNA for μ opioid receptor produced tactile and thermal hypersensitivity, along with a loss of antinociceptive potency of spinal DAMGO (Gardell and Porreca, unpublished observations). These behavioral changes were accompanied by elevated spinal dynorphin content and a loss of μ opioid receptor immunostaining in the spinal dorsal horn. Furthermore, the spinal administration of antiserum to dynorphin $A_{(1\text{-}13)}$ blocked the manifestations of abnormal pain, as did MK-801. These observations are extremely similar to those seen with prolonged DAMGO administration. Similarly, mice lacking the gene coding for the μ opioid receptor also demonstrate abnormal pain mimicking the effects of antisense knock-down of μ opioid receptor and an insensitivity to the antinociceptive effects of morphine (Gardell and Porreca, unpublished observations). These observations all suggest that excitatory inputs to the spinal cord that drive abnormal pain and tolerance arise, in part, from primary afferents that increase release of pronociceptive transmitters, including CGRP and most likely glutamate and substance P, leading to spinal sensitization.

Increased excitatory input may be derived from disinhibition of primary afferents, such as when presynaptic μ opioid receptors are reduced or eliminated, or by excitation of the primary afferent by dynorphin, glutamate at presynaptic excitatory NMDA receptors, and as yet unknown mechanisms of tonic descending facilitation, or both. Thus, regardless of the underlying mechanisms initiating this, the net effect is the same, namely, increased pain and behavioral manifestation of antinociceptive "tolerance". Such mechanisms may represent an important part of the process of antinociceptive tolerance though, clearly, other mechanisms may also contribute.

CHAPTER V: CGRP RELEASE STUDIES

Introduction

It is well documented that morphine elicits paradoxical abnormal pain in both clinical and preclinical settings. Abnormal pain has been described in patients after spinal morphine was administered for chronic pain (Arner and Meyerson 1988). Such abnormal pain differs in presentation, location and quality from the original pain complaint (Ali 1986; De Conno et al. 1991; Devulder 1997; Stillman et al. 1987). Opioid-induced abnormal pain has also been demonstrated in animal experiments (Celerier et al. 2000; Larcher et al. 1998; Mao et al. 1998; Mao et al. 1994; Mao et al. 1995e; Vanderah et al. 2000; Vanderah et al. 2001a; Vanderah et al. 2001b; Yaksh and Harty 1988; Yaksh et al. 1986). The mechanisms underlying such pain are unknown. Recent investigations have demonstrated that morphine-evoked abnormal pain may result from neuroplastic changes in the rostral ventromedial medulla (RVM) (Vanderah et al. 2001b). The rostral ventromedial medulla (RVM) has been well characterized as a source of spinopetal inhibitory and facilitatory modulation of nociceptive inputs (Fields 1992; Heinricher et al. 1989; Morgan et al. 1992). Manipulations performed in the RVM that block its activity are known to block pain in a number of settings including inflammation (Mansikka and Pertovaara 1997) and nerve-injury (Kovelowski et al. 2000). Hyperalgesia resulting from naloxone-precipitated opioid withdrawal was blocked by RVM lidocaine (Kaplan and Fields 1991) and associated with increased

discharge of cells thought to mediate facilitation (i.e., "ON" cells) (Bederson et al. 1990; Kim et al. 1990).

Similarly, tactile and thermal hypersensitivity resulting from sustained delivery of subcutaneous morphine was abolished by RVM microinjection of lidocaine and by bilateral lesions of the dorsolateral funiculus (DLF), suggesting the importance of descending facilitatory influences in opioid-induced abnormal pain (Vanderah et al. 2001b). Manipulations which blocked opioid-induced pain also blocked the behavioral manifestations of opioid-antinociceptive tolerance. Opioid-induced abnormal pain and antinociceptive tolerance are also characterized by an upregulation in spinal dynorphin content (Vanderah et al. 2000). Spinal administration of dynorphin has been shown to induce pain (Laughlin et al. 1997; Vanderah et al. 1996) and spinal MK-801 or dynorphin antiserum block morphine-induced abnormal pain and antinociceptive tolerance (Vanderah et al. 2000). These studies have suggested a pronociceptive role for upregulated spinal dynorphin as a mediator of opioid-induced pain.

Morphine-induced abnormal pain and antinociceptive tolerance is known to be blocked by NMDA receptor antagonists (Celerier et al. 1999; Larcher et al. 1998; Laulin et al. 1999; Mao et al. 1995c; Mao et al. 1995e). While a common interpretation of these findings is an interaction between opioid and NMDA receptors via intracellular mechanisms (Mao et al. 1995a; Mao et al. 1995d; Mao et al. 1995e), an alternate possibility might be the blockade of opioid-induced excitation. However, the source of possible opioid-induced excitation is not known. Recently, Hargreaves and colleagues have suggested that non-opioid fragments of dynorphin can enhance evoked released

CGRP-immunoreactivity in isolated spinal cord tissues (Claude et al. 1999b). The present study was undertaken to test the hypothesis that the upregulation of spinal dynorphin resulting from sustained exposure acts to enhance the evoked release of an excitatory transmitter from primary afferent fibers. Additionally, the possible relationship between opioid-induced descending facilitation and spinal dynorphin was explored.

Results

Evaluation of tactile and thermal thresholds following placebo or morphine pellets

The mean pretreatment (i.e., baseline) paw withdrawal threshold to probing of the hindpaw with von Frey filaments was 15 ± 0 g and the paw withdrawal latency to noxious radiant heat applied to the plantar aspect of the hindpaw was 20.8 ± 0.26 sec. Rats with morphine pellets demonstrated a significantly reduced mean paw withdrawal threshold of 4.53 ± 0.74 g ($p \leq 0.05$) 7 days after pellet implantation. Similarly, these animals also showed a significant reduction in the mean paw withdrawal latency of 13.9 ± 1.02 sec ($p \leq 0.05$). Previous studies have shown that the development of tactile and thermal hypersensitivity after morphine pellet implantation is time-related. Vanderah et al. (2001x) showed that the response thresholds to tactile and thermal stimulation were modestly reduced from pretreatment values within 1 day of morphine exposure but did not achieve a maximal level of change until day 7. In the present studies, the paw withdrawal threshold was reduced from a baseline of 15.0 ± 0 to 13.1 ± 1.4 g at day 1 after morphine pellets. Similarly, paw withdrawal latencies were essentially unchanged with baseline values of 21.0 ± 0.6 and 20.15 ± 0.6 sec at day 1 following morphine

pellets. These tactile and thermal threshold values at day 1 post-morphine pellets were not significantly different from the pre-pellet baseline ($p > 0.05$, Student's t test). In control rats that received placebo pellets, the mean paw withdrawal threshold (15 ± 0 and 14.5 ± 0.5 g at 1 and 7 days, respectively), and the mean paw withdrawal latency (21.2 ± 0.51 and 20.6 ± 0.66 sec at 1 and 7 days, respectively) were not significantly different from the respective pre-pellet values ($p > 0.05$). These results suggest that exposure to subcutaneous morphine pellets elicits enhanced pain which is firmly established by post-pellet day 7, with earlier time points likely representing a time-related transitional period of sensory thresholds (Vanderah et al. 2001b).

Morphine-induced upregulation of spinal dynorphin is blocked by DLF lesions

Rats were prepared with bilateral DLF, or sham-DLF, lesions and were implanted with either placebo or morphine pellets 2 days after the surgical procedures. On the 7th day after pellet implantation, the dorsal halves of the lumbar spinal cords were removed and assayed for dynorphin content. The sustained exposure to morphine in rats with sham DLF lesions produced a significantly greater ($p \leq 0.05$) spinal dynorphin content relative to the placebo-pelleted control group (Figure 5.1). The dynorphin levels were 384 ± 18 fmol/mg protein and 296 ± 12 fmol/mg protein in the morphine- and placebo-treated sham-DLF groups, respectively (Figure.5.1). In rats that had bilateral DLF lesions prior to morphine pellet implantation, however, the dynorphin level was 292 ± 8 fmol/mg protein, which was not significantly different ($p > 0.05$) from that of the placebo-pelleted, sham-lesioned control group (Figure 5.1). Lesions of the DLF alone did not alter spinal dynorphin expression. Rats implanted with placebo pellets that also

received DLF lesion had a mean dynorphin content of 282 ± 8 fmol/mg protein. which was not significantly different ($p > 0.05$) from that of the placebo pelleted, sham-lesioned control group (Figure 5.1). Thus, sustained morphine exposure induced an enhanced level of spinal dynorphin that could be prevented by bilateral lesions of the DLF.

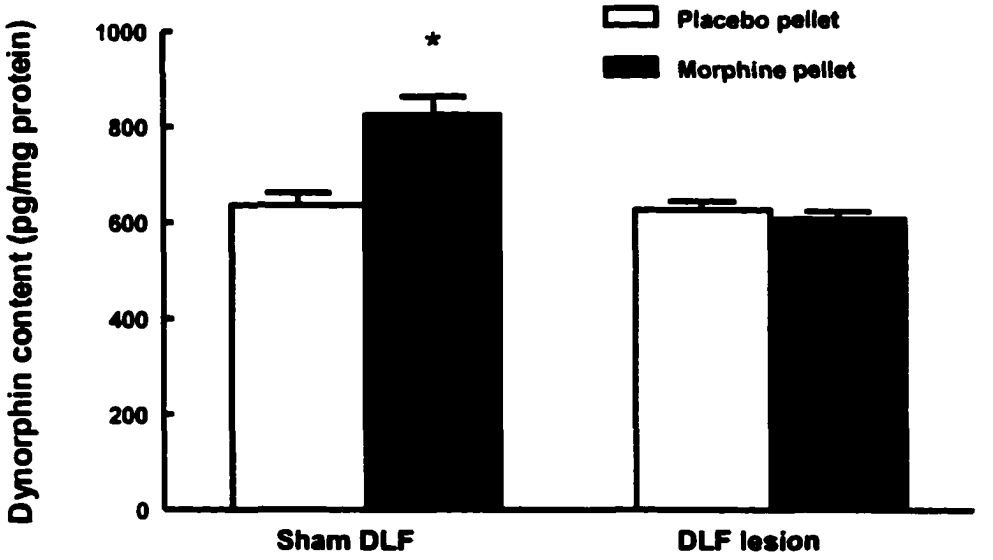

Figure 5.1. Rats received bilateral lesions of the DLF or sham surgery at T_8 and were allowed to recover for 2 days. The rats were then implanted with two subcutaneous placebo or morphine (75 mg) pellets. After 7 days, the spinal cords were removed and the dorsal lumbar halves of the lumbar cords were assayed for dynorphin content by enzyme immunoassay. The dorsal lumbar cords of rats with morphine pellets and sham DLF lesions showed significantly greater levels of dynorphin (*; $p \leq 0.05$) than tissues from rats with placebo pellets and sham-DLF lesions. The levels of spinal dynorphin from the rats with placebo pellets and DLF lesions were not different from those seen in tissues from rats with placebo pellets and sham-DLF lesion ($p > 0.05$). Morphine pellets failed to significantly increase the levels of dynorphin in spinal tissues taken from rats with DLF lesions. These levels were not different from those seen with placebo pellets and DLF lesion or placebo pellets and sham-DLF lesion. Each treatment group consisted of 6 rats.

Sustained morphine exposure enhances CGRP content

Sustained morphine exposure also produced an upregulation of immunoreactivity for CGRP in the lumbar dorsal horn of the spinal cord (Figure 5.2) in a time-dependent manner. Enhanced fluorescence labeling for CGRP was seen in the dorsal horn 7 days

(Figure 5.2, panel D), but not 1 day (Figure 5.2, panel B) following morphine pellet implantation when compared with that seen in tissues from placebo-pelleted rats at post-implantation days 1 and 7 (Figure 5.2, panels A & C, respectively) (preabsorbed control not shown). CGRP was confined bilaterally to the outer laminae (I and II) of the spinal dorsal horn in placebo rats and rats that were exposed to morphine for 1 day (Figure 5.2, panels A, B & C). Tissues taken from rats after 7 days of morphine pellet implantation, however, showed that the CGRP was more intense in the outer laminae of the dorsal horn, and that immunolabeling could be seen to extend into deeper laminae (Figure 5.2, panel D).

CGRP in the dorsal horn was quantified by radioimmunoassay of spinal cord extracts in separate parallel assays from placebo- and morphine-pelleted rats after day 1, and again after day 7, of pellet implantation. CGRP content was determined to be 1120 ± 58 and 1336 ± 81 pmol/g tissue in spinal tissues from 1 day placebo- and morphine-pelleted rats, respectively (n=6 rats per group). When compared with the day 1 placebo-pelleted control group, the morphine-pelleted group on day 1 showed a 19 ± 7.5% increase in CGRP; this difference was small but statistically significant ($p \leq 0.05$). CGRP content was determined to be 2455 ± 309 and 6451 ± 595 pmol/g tissue in spinal tissues from 7 day placebo- and morphine-pelleted rats, respectively (n=8 rats per group). When compared with the 7 day placebo-pelleted control group, the morphine-pelleted group on day 7 showed a 163 ± 24% increase in CGRP, a difference which was statistically significant ($p \leq 0.05$). These data are in good agreement with the immunohistochemical analysis of CGRP after 1 day or 7 days of morphine exposure.

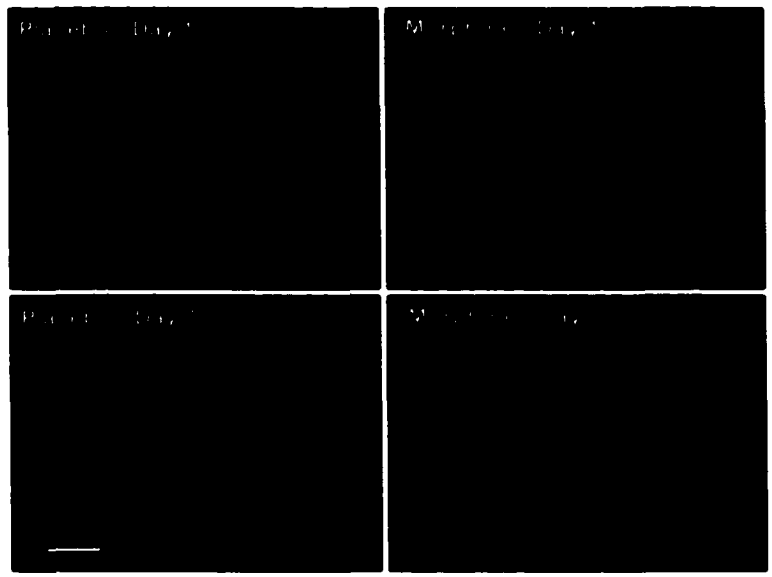

Figure 5.2. CGRP-immunoreactivity in the spinal cord at approximately L_5. Tissue from placebo-treated rats is shown in the left panels and that from morphine-treated rats is shown on the right panels. No apparent difference in CGRP immunoreactivity is seen between the spinal sections obtained from placebo-treated and morphine-treated rats 1 day after pellet implantation (Panels A and B, respectively). The staining intensity of CGRP in tissues from morphine-treated rats on day 7 is enhanced when compared with the placebo-treated rats on day 7 (Panels D and C, respectively). The enhanced staining is apparent in both laminae I/II and in deeper laminae. Scale bar represents 400 μm.

Sustained morphine exposure enhances capsaicin-evoked release of CGRP

Basal CGRP release was measured in tissues taken from rats 1 or 7 days after implantation of placebo or morphine pellets. No differences were observed in unstimulated release in these groups (Figure 5.3, Panel A). Capsaicin-stimulated release of CGRP in tissues taken from rats 1 day after placebo-pellets did not differ significantly from that observed in tissues taken from rats 7 days after placebo pellets (Figure 5.3, Panels A & B). Similarly, the capsaicin-stimulated release of CGRP observed in tissues taken from rats 1 day after morphine pellets did not differ significantly from that

observed from tissue taken from placebo-pelleted rats at either time point (Figure 5.3, Panels A & B).

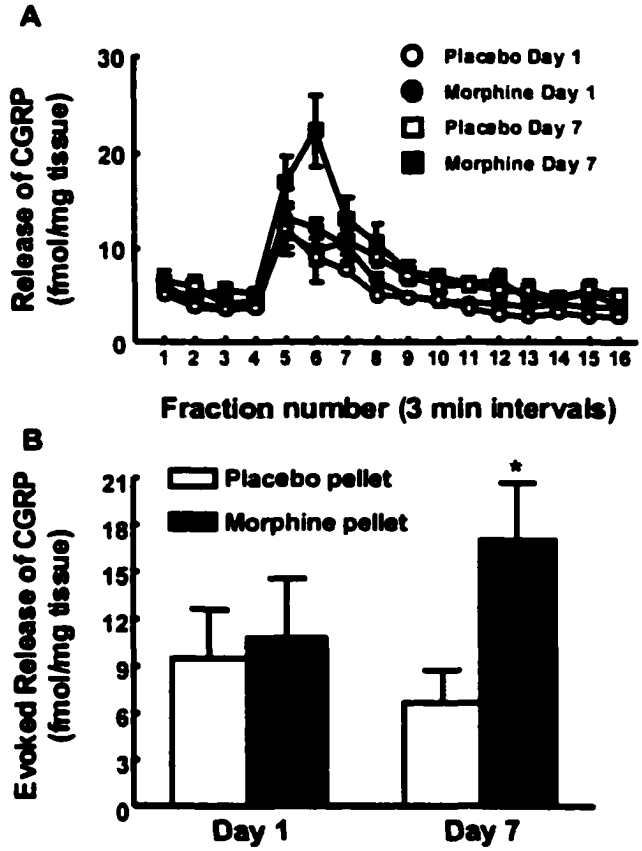

Figure 5.3. Rats were implanted with two placebo pellets or morphine pellets. Separate groups were sacrificed 1 day and 7 days after pellet implantation and dorsal lumbar spinal cord tissues were isolated, minced, and placed in perfusion chambers. Panel A shows the basal and evoked CGRP in the perfusate collected at 3 minute intervals. The horizontal bar represents the period where capsaicin (1μM) was added to the perfusate in order to evoke CGRP release. The capsaicin-evoked release of CGRP from the spinal tissues in vitro is demonstrated in panel B, and represents the amount of CGRP above the baseline release for each individual group. Basal levels of CGRP release did not differ among the treatment groups. Evoked CGRP release was not different between tissues from placebo- and morphine-treated groups at day 1 after pellet implantation ($p > 0.5$). However, tissues from the 7-day morphine treated group showed a significantly greater level of capsaicin-evoked release of CGRP (*; $p \leq 0.05$). Each treatment group consisted of 6 rats.

In contrast, capsaicin-evoked release of CGRP in tissues taken from rats 7 days after morphine-pellets was significantly greater than that seen in either the placebo- or

morphine-pellet day 1 tissues (Figure 5.3, Panels A & B). For comparison purposes, evoked post-placebo pellet day 1 CGRP release was normalized to 100 ± 27%. Under the same conditions, the evoked CGRP released from spinal dorsal horn tissue harvested 1 day after morphine pellet implantation was 112 ± 38% of this control group (difference not significant). Similarly, evoked post-placebo pellet day 7 CGRP was normalized as 100 ± 31%. Under the same conditions, the evoked CGRP released from spinal dorsal horn tissues harvested 7 days after morphine pellets was 270 ± 49% of this control ($p \leq 0.05$).

Enhanced evoked released CGRP in tissues from morphine-exposed rats is blocked by prior bilateral DLF lesions

Tissues were taken 7 days after placebo or morphine pellet implantation from rats with prior sham-DLF or DLF lesions for evaluation of basal and evoked CGRP release. No differences were observed in unstimulated release in these groups (Figure 5.4, Panel A). Capsaicin-stimulated release of CGRP in tissues from placebo-pelleted sham-DLF rats did not differ significantly from that observed in tissues from placebo-pelleted DLF rats (Figure 5.4, Panel A). Evoked CGRP release in tissues taken from sham-DLF morphine pelleted rats was significantly higher than that observed from tissues from either of the placebo-pelleted groups ($p \leq 0.05$, Figure 5.4, Panel B).

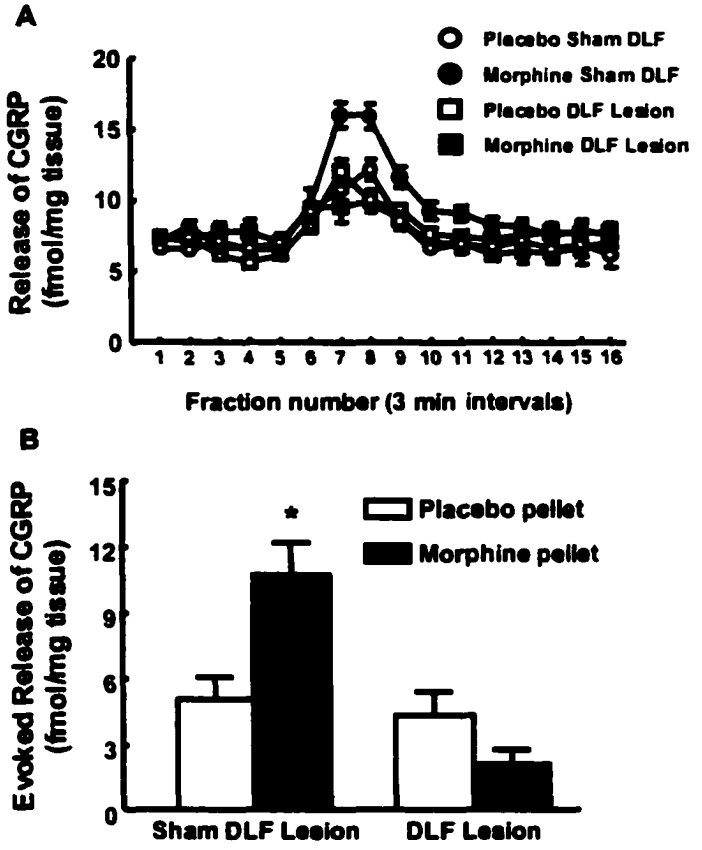

Figure 5.4. Rats received bilateral lesions of the DLF or sham surgery at T_8 and were allowed to recover for 2 days. The animals were then implanted with two subcutaneous placebo or morphine (75 mg) pellets. Seven days after pellet implantation, the spinal cords were removed and the dorsal halves of the lumbar cords were assayed for basal and evoked CGRP release. The horizontal bar in Panel A represents the period where capsaicin (1μM) was added to the perfusate in order to evoke the release of CGRP. Basal CGRP release was not different among the treatment groups. In tissues from the sham DLF-lesioned and morphine-treated rats (n=11), capsaicin-evoked release of CGRP was significantly (*; $p \leq 0.05$) greater when compared to sham DLF, placebo treated group (n=11). Tissues from rats with DLF lesion and placebo pellets (n=10) did not show significantly different levels of evoked CGRP release when compared with tissues from sham-DLF and placebo pellets. However, tissues from rats with bilateral DLF lesion and morphine pellets (n=8), showed levels of evoked CGRP which were not significantly different from the DLF-lesioned placebo group or the sham DLF-lesioned placebo group.

In contrast, evoked CGRP release in tissues taken from DLF morphine-pelleted rats was not significantly different from that observed in tissues from the placebo-pelleted groups and was significantly lower than that seen in tissues from the sham-DLF

morphine pelleted group ($p \leq 0.05$) (Figure 5.4, panel B). For comparison purposes, evoked CGRP release in sham-DLF placebo pelleted tissues was normalized to 100 ± 20%. The evoked CGRP release in sham-DLF morphine-pelleted tissues was 210 ± 28% of this control group ($p \leq 0.05$). Similarly, evoked CGRP release in DLF placebo-pelleted tissues was normalized to 100 ± 25%. The evoked CGRP release in DLF morphine-pelleted tissues was 78 ± 16 of this control group (difference not significant).

Enhanced capsaicin-evoked released CGRP in tissues from morphine-exposed rats is blocked by anti-dynorphin antiserum

Neither control serum nor anti-dynorphin antiserum (1 mg/100 ml) produced any change in basal release of CGRP in tissues taken 7 days after placebo or morphine pellets (n= 10 per group, data not shown). As noted previously, no differences were observed in unstimulated CGRP release in tissues taken 7 days after placebo or morphine pellet implanation (Figure 5.5, panel A). Evoked CGRP release in tissues taken from placebo-pelleted rats did not differ significantly from that observed from tissues taken from placebo-pelleted rats in the presence of control serum or dynorphin antiserum (Figure 5.5, panel A). Evoked CGRP release in tissues taken from morphine-pelleted rats did not differ significantly from that observed in tissues taken from morphine-pelleted rats in the presence of control serum (Figure 5.5, panel A) but evoked release in both of these groups was significantly greater than that observed in the placebo-pelleted groups in the absence or presence of control serum.

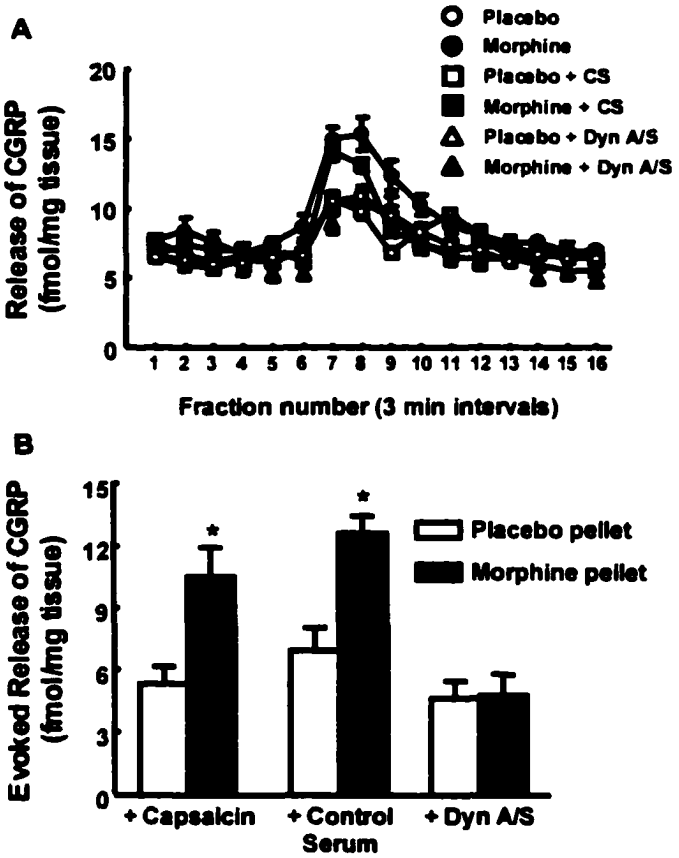

Figure 5.5. Rats were implanted with two subcutaneous placebo or morphine pellets and sacrificed 7 days after implantation. The dorsal lumbar spinal cord tissue was dissected, minced, and placed in perfusion chambers. Basal and evoked CGRP released into the perfusate was measured at 3 minute intervals (A), and capsaicin-evoked CGRP release was indicated by the amount of CGRP present above the baseline release levels (B). The horizontal bars in panel A represents the period where capsaicin (1μM) and dynorphin antiserum (1 mg/100 ml) (Dyn A/S) or a rabbit control serum (1 mg/100 ml) (CS) were added to the perfusate. Basal levels of CGRP release were not significantly different among the treatment groups. In separate experiments, neither Dyn A/S or CS altered basal CGRP release (data not shown). Capsaicin-evoked release of CGRP was significantly greater in tissues from morphine-treated rats 7 days after pellet implantation when compared to the placebo-treated group (*; $p \leq 0.05$). Administration of capsaicin with CS to the perfusion medium did not alter the capsaicin-evoked release of CGRP from tissues obtained from either placebo- or morphine-treated rats; evoked CGRP release in the morphine-treated tissues were significantly greater than that seen in the placebo-pelleted group. Administration of capsaicin with Dyn A/S to the perfusion medium prevented the enhanced capsaicin-evoked release of CGRP in tissues from morphine treated rats such that the level of release was not different from the placebo control group. Each treatment group consisted of 10 rats.

In contrast, evoked CGRP release in tissues taken from morphine-pelleted rats in the presence of anti-dynorphin antiserum was significantly lower than the evoked CGRP release observed in tissues taken from morphine-pelleted rats in the absence or presence of control serum ($p \leq 0.05$) and not significantly different from that observed in placebo-pelleted tissues in the absence or presence of control serum (Figure 5.5, Panels A & B). For comparison purposes, evoked CGRP release in placebo-pelleted tissues was normalized to 100 ± 15%. Evoked CGRP release in morphine-pelleted tissues was found to be 197 ± 26% of this control group ($p \leq 0.05$). Similarly, evoked CGRP release in tissues taken from placebo-pelleted rats in the presence of control serum was normalized to 100 ± 12%. Evoked CGRP release in the presence of control serum in tissues taken from morphine-pelleted rats was 182 ± 12% ($p \leq 0.05$). Evoked CGRP release in the presence of dynorphin antiserum in tissues from placebo-pelleted rats was normalized to 100 ± 17%. Evoked CGRP release in the presence of anti-dynorphin antiserum in tissues from morphine-pelleted rats was found to be 102 ± 22% of control, a value that was not significantly different.

Dynorphin $A_{(2-13)}$, a non-opioid dynorphin fragment, enhances capsaicin-evoked released CGRP in naive tissue

The possibility that pharmacological application of dynorphin $A_{(2-13)}$ might mimic the apparent facilitatory effects of upregulated endogenous spinal dynorphin elicited by sustained morphine treatment, was tested. Basal and capsaicin-evoked CGRP release from spinal tissues harvested from naive rats was measured in the absence and presence of dynorphin $A_{(2-13)}$. Dynorphin $A_{(2-13)}$ (1 μM) applied to the superfusate in the absence

of capsaicin did not evoke significant release of CGRP above the basal value (data not shown). In contrast, application of dynorphin $A_{(2-13)}$ (1 μM) significantly enhanced capsaicin-evoked CGRP release (Figure 5.6, panels A & B). For comparison, the mean evoked release of CGRP by 1μM capsaicin was normalized to 100 ± 18%. Superfusion of capsaicin with dynorphin $A_{(2-13)}$ resulted in an evoked CGRP release of 336 ± 96% ($p \leq 0.05$) of this control group (Figure 5.6).

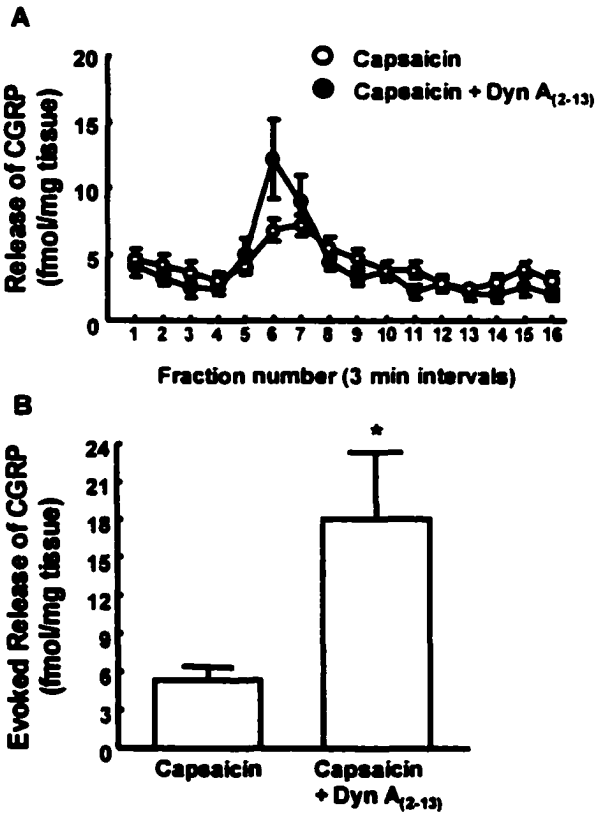

Figure 5.6. Capsaicin-evoked (1 μM) release of CGRP from the dorsal lumbar spinal cord slices harvested from rats was significantly enhanced in the presence of dynorphin $A_{(2-13)}$ a non-opioid fragment. CGRP release was measured at 3 minute intervals (A), and capsaicin-evoked CGRP release was indicated by the amount of CGRP present above the baseline release levels (B). The horizontal bars in panel A represents the period where capsaicin (1μM) and dynorphin $A_{(2-13)}$ (1μM) were added to the perfusate in order to evoke release of CGRP. CGRP released from the tissues induced by capsaicin was significantly enhanced by the co-administration of 1 μM of a non-opioid fragment, dynorphin $A_{(2-13)}$ (*; $p \leq 0.05$). Each treatment group consisted of 10 rats.

SUMMARY

The results of the present Chapter demonstrate the remarkable plasticity induced in the nervous system following sustained exposure to morphine. The present data confirm and extend previous findings indicating that morphine exposure significantly upregulates spinal dynorphin (Vanderah et al. 2000) and CGRP (Ma et al. 2000; Ma et al. 2001; Menard et al. 1996; Powell et al. 2000). The increase in expression of these substances appears to be of functional significance, providing a physiological basis for increased stimulus-induced excitation to the spinal cord that may underlie paradoxical opioid-induced pain. Several novel insights can be obtained from these data, which impact our understanding of opioid-induced paradoxical pain and possibly the behavioral manifestation of antinociceptive tolerance. First, the enhanced expression of spinal dynorphin produced by sustained morphine exposure requires descending influences via the DLF. Second, sustained morphine exposure results in enhanced content and evoked release of CGRP. Third, pharmacological application of non-opioid fragments of dynorphin enhances capsaicin-evoked release of CGRP, confirming the observations of Hargreaves and colleagues and providing a possible basis for the increased evoked CGRP release measured in tissues from morphine-pelleted rats. Fourth, the increased evoked CGRP release from spinal tissues taken from morphine-exposed rats is blocked by antiserum to dynorphin, but not by a control serum, implicating endogenous dynorphin in this effect. Finally, manipulations which block the upregulation of spinal dynorphin resulting from morphine pellets, namely bilateral DLF lesions, also block the enhanced evoked CGRP release observed in tissues taken from morphine-pelleted rats. Taken

together, these data support the hypothesis that morphine-induced upregulation of spinal dynorphin results secondary to plasticity in the RVM and that dynorphin acts in a non-opioid fashion to enhance the evoked release of excitatory transmitters from primary afferent fibers. As DLF lesions and dynorphin antiserum are manipulations which have also been shown to block opioid-induced pain and antinociceptive tolerance (Vanderah et al. 2000; Vanderah et al. 2001b), it seems reasonable to suggest that enhanced evoked excitatory transmitter release may be an important factor which underlies opioid-induced paradoxical pain and the subsequent expression of antinociceptive tolerance.

The supraspinally mediated facilitation of nociceptive inputs at the level of the spinal cord is well recognized (Fields 1992; Porreca et al. 2002; Urban and Gebhart 1997; Urban et al. 1996; Zhou et al. 1992). Based on observations made following microinjection of lidocaine and lesions of the DLF, an exaggerated persistence of such tonically active descending facilitation from the RVM has been suggested to be critical for the expression of morphine-induced paradoxical pain and antinociceptive tolerance (Tortorici et al. 2001; Vanderah et al. 2000; Vanderah et al. 2001b). Spinopetal projections from the RVM are known to descend through the DLF (Fields and Basbaum 1999), suggesting the possibility that such descending influences may elicit changes at the spinal level. While descending facilitation may promote nociceptive inputs directly, such influences may also promote pain indirectly perhaps by invoking the upregulation of pronociceptive substances, such as dynorphin, in the spinal cord (Ossipov et al. 2001; Vanderah et al. 2001a). Studies have repeatedly demonstrated that persistent exposure to morphine strongly upregulates spinal dynorphin content (Nylander et al. 1995; Rattan and

Tejwani 1997; Vanderah et al. 2000; Vanderah et al. 2001a). The present study extends these previous observations by further demonstrating the regulatory influences of descending projections as disruption of the DLF blocked opioid-induced upregulation of spinal dynorphin. Importantly, lesions of the DLF also prevented the development of enhanced capsaicin-evoked release of CGRP observed in spinal tissues taken from morphine-treated rats. It therefore appears that the same neuroplasticity of the RVM that has been suggested to result in descending facilitation also elicits the upregulation of spinal dynorphin, and subsequently increased evoked neurotransmitter release, suggesting a strong functional link among these three phenomena and ultimately the behavioral consequences of morphine exposure.

The role of spinal dynorphin as a possible mediator of pain resulting from sustained morphine exposure is also supported by these, and previous, findings. Mice with deletions of the gene coding for prodynorphin, fail to develop antinociceptive tolerance or behavioral signs of abnormal pain in response to repeated intrathecal injections of DAMGO (Gardell and Porreca, unpublished observations). Furthermore, the spinal injection of MK-801 or of antiserum to dynorphin blocked enhanced pain and antinociceptive tolerance in the wild-type littermates (Gardell and Porreca, unpublished observations), as well as in morphine-exposed rats (refer to Chapter IV). In spite of these observations, the precise mechanisms by which spinal dynorphin might mediate these effects is not clear. Evidence for an excitatory action of spinal dynorphin has been supported by the observation of pharmacologically-induced pain (Laughlin et al. 1997; Vanderah et al. 1996) and by observations demonstrating that pharmacological dynorphin

$A_{(1-13)}$ evoked an increased release of glutamate and aspartate into the extracellular fluid of the spinal cord (Skilling et al. 1992). These dynorphin effects were blocked by MK-801, suggesting an NMDA-receptor mediated action (Laughlin et al. 1997; Skilling et al. 1992; Vanderah et al. 1996). Furthermore, dynorphin $A_{(1-17)}$ and the *des*-Tyr derivative dynorphin $A_{(2-17)}$, which is devoid of binding affinity to opioid receptors, infused into hippocampal tissue through a microdialysis probe produced dose-dependent release of glutamate and aspartate, and this effect was not blocked by opioid antagonists (Faden 1992). Dynorphin $A_{(1-17)}$ was found to potentiate the capsaicin-evoked release of substance P (SP) from caudal trigeminal nuclear slices and this effect was not blocked by μ, δ or κ opioid receptor antagonists but was blocked by NMDA antagonists (Arcaya et al. 1999). Furthermore, neonatal capsaicin, which selectively destroys SP-containing primary afferents, blocked the ability of dynorphin to facilitate K^+-evoked release of SP in trigeminal slices (Arcaya et al. 1999). It was concluded that dynorphin enhances the release of SP from primary afferent C-fibers through an NMDA-related mechanism to promote exaggerated pain (Arcaya et al. 1999). Hargreaves and colleagues have recently demonstrated enhanced capsaicin-evoked release of CGRP by dynorphin $A_{(2-13)}$ from spinal cord sections (Claude et al. 1999c). This observation was confirmed in the present investigation, and extended to encompass the role of endogenous dynorphin upregulated in the spinal cord following morphine treatment.

A possible basis for the enhanced evoked release of CGRP seen in tissues from morphine-exposed rats is the increased content of CGRP evident in both the spinal cord and in the dorsal root ganglia. Increased spinal CGRP appeared to extend into deeper

lamina of the spinal dorsal horn following morphine treatment. The reasons for this are not clear. Preliminary evidence suggests that the apparent number of CGRP positive cells in the DRG is increased following morphine treatment increased. However, the nature of these cells (i.e., size, phenotype) awaits further characterization. Dynorphin can pharmacologically enhance the evoked release of CGRP from naive tissue suggesting that upregulated content is not necessary. It is not clear whether the enhanced evoked release of CGRP seen in tissues from morphine-exposed rats, in which there is enhanced expression of endogenous dynorphin, results from the actions of dynorphin alone or depends also on the upregulation of CGRP. Nevertheless, the critical importance of spinal dynorphin is supported by the blockade by dynorphin antiserum of enhanced evoked release in morphine-exposed tissues. Dynorphin $A_{(1\text{-}13)}$ antiserum did not block evoked CGRP release in naive or placebo-pelleted tissues. Additionally, no enhanced evoked CGRP release was observed in tissues taken from morphine-exposed rats with prior lesions of the DLF. DLF or sham-DLF lesion did not alter evoked CGRP release in placebo-pelleted rats. This finding also demonstrates that the increased release of CGRP seen in tissues from morphine-exposed rats was not the result of an in vitro expression of opioid withdrawal. This conclusion is supported by the fact that tissues taken from rats with prior DLF lesions had been exposed to morphine in exactly the same way as those without DLF lesions. Also supporting this conclusion is the failure to demonstrate differences in basal (i.e., unstimulated) CGRP release in tissues from morphine- or placebo-pelleted rats. The latter observation also argues against possible "sensitization" of the release mechanisms in the afferent fibers following morphine treatment.

The molecular mechanisms by which dynorphin may enhance evoked release from primary afferents remains to be elucidated (see Lai et al. 2001 for review). However, the actions of dynorphin, upregulated after sustained opioid exposure appear to promote excitation, abnormal pain and antinociceptive tolerance. Blockade of dynorphin actions or expression has been shown to block opioid abnormal pain and antinociceptive tolerance. In this regard, these findings are consistent with most of the actions of many other classes of substances, which have been reported to block opioid antinociceptive tolerance (refer to Table 1.1). These substances most commonly block endogenous substrates that promote excitation and pain (see Vanderah et al. 2001a for review). As pain can be regarded as a "physiological antagonist" of opioid antinociception, the present findings provide at least one possible basis for increased pain following sustained exposure to opioids and may allow new approaches for preventing the expression of opioid antinociceptive tolerance.

CHAPTER VI: SYSTEMIC MORPHINE STUDIES IN PRODYNORPHIN KNOCKOUT MICE

Introduction

A number of studies have suggested that sustained morphine exposure elicits neuroplasticity, either in the RVM or in structures communicating with the RVM, resulting in tonic bulbospinal facilitation (Vanderah et al. 2001b). Such descending facilitation might play a significant role in spinal plasticity resulting from sustained exposure to opioids. A consistent observation is the increase in expression of dynorphin in the spinal dorsal horn following opioid administration (Chapters III, IV and V). Evidence suggests that spinal dynorphin is an important mediator of sustained abnormal pain (Malan et al. 2000; Wang et al. 2001). Although dynorphin was originally identified as an endogenous κ-opioid agonist (Goldstein et al. 1979) and may act as an endogenous antinociceptive agent under certain conditions (Ossipov et al. 1996), this peptide has significant non-opioid activity. Considerable evidence now supports the conclusion that enhanced expression of spinal dynorphin is pronociceptive (Caudle and Isaac 1988a; Cho and Basbaum 1989b; Draisci et al. 1991; Dubner and Ruda 1992; Nahin et al. 1992; Stanfa and Dickenson 1995; Wang et al. 2001).

Evidence for an excitatory action of spinal dynorphin has been supported by the observation of mechanical and thermal hypersensitivity following spinal administration (Laughlin et al. 1997; Vanderah et al. 1996) and by observations demonstrating that pharmacologically administered dynorphin $A_{(1-13)}$ evokes an increased release of

glutamate and aspartate into the extracellular fluid of the spinal cord (Skilling et al. 1992). These effects of dynorphin were blocked by pretreatment with MK-801, suggesting an NMDA-receptor mediated action (Laughlin et al. 1997; Skilling et al. 1992; Vanderah et al. 1996). Additionally, neonatal capsaicin, which selectively destroys substance P containing primary afferent neurons, blocked the ability of dynorphin to facilitate K^+-evoked release of substance P in trigeminal slices (Arcaya et al. 1999). It was concluded that dynorphin enhances the release of substance P from primary afferent C fibers through an NMDA-related mechanism to promote exaggerated pain (Arcaya et al. 1999). Hargreaves and colleagues have recently demonstrated enhanced capsaicin-evoked CGRP release by dynorphin $A_{(2-13)}$ from naive rat spinal cord sections (Claude et al. 1999a), an effect that was replicated in Chapter V. Further, experiments in Chapter V have demonstrated that capsaicin-evoked CGRP release is potentiated in spinal cord tissues taken from morphine-pelleted rats. The potentiation of CGRP release was blocked by the addition of antiserum to dynorphin $A_{(1-13)}$ to the perfusate or by producing bilateral lesions of the DLF prior to morphine pellet implantation. Moreover, both MK-801 and antiserum to dynorphin $A_{(1-13)}$ completely block opioid-induced abnormal pain and, as a consequence, restore the potency of spinal opioids.

The purpose of this Chapter is to further explore the role of dynorphin in opioid-induced abnormal pain and antinociceptive tolerance utilizing mice lacking the prodynorphin gene and its products.

Results

Characterization

These mice have been characterized in a previous report from our laboratory (Wang et al. 2001). To briefly summarize, initial analysis of spinal cord tissues from wild-type mice showed that prodynorphin immunoreactivity is primarily located in the superficial laminae I/II of the dorsal horn, but is entirely absent in spinal cord tissues from the prodynorphin knock-out mice, and there were also no detectable changes in the expression of the kappa opioid receptor (Wang et al. 2001). When these mice were evaluated for their nociceptive responsivity, the wild-type and knock-out mice displayed similar latency to innocuous tactile stimulation, as well as latencies to noxious input based on radiant heat and hot-plate tests (Wang et al. 2001). However, the knock-out mice consistently showed a small, but significant decrease in tail-flick response latency when compared with the wild-type mice (Wang et al. 2001).

Furthermore, when the wild-type and knock-out mice were tested using a model of tonic nociceptive input by an injection of 2% formalin to the right hindpaw, both the wild-type and knock-out mice displayed a biphasic flinching response over a 75 min period (Wang et al. 2001). No differences were detected in the amplitude and duration of the first phase of flinching between the wild-type and knock-out mice, however, the knock-out mice showed a small, but significant ($p \leq 0.05$) enhancement of the second phase of flinching, indicated by the total number of flinches during the second phase compared with that exhibited by the wild-type mice over the same period (Wang et al. 2001). The knock-out mice exhibited normal growth and development, feeding, motor

function and weight that were indistinguishable from their wild-type littermates, as was observed in the previos report (Wang et al. 2001).

Results of experiments with sustained morphine in prodynorphin knock-out mice

Non-noxious mechanical thresholds

Paw withdrawal thresholds to probing with von Frey filaments were obtained before (baseline) and across the 5 day period after animals were implanted with either morphine or placebo pellets (Figure 6.1). Paw withdrawal thresholds did not change significantly ($p > 0.05$) following placebo pellet implantation for either wild-type of knock-out mice. The mean pooled baseline paw withdrawal threshold for the wild-type and the knock-out mice prior to receiving any treatments was 1.35 ± 0.11 g and 1.37 ± 0.12 g, respectively. The mean paw withdrawal thresholds on Day 5 for the placebo-pelleted wild-type and knock-out mice were 1.27 ± 0.13 g and 1.28 ± 0.15 g, respectively.

In addition, 4 groups (2 wild-type and 2 knock-out groups) of 15 mice each either received placebo or morphine pellets for 5 days. The paw withdrawal thresholds for the wild-type mice were 1.27 ± 0.13 g and 0.06 ± 0.02 g, respectively, whereas for the knock-out mice the paw withdrawal thresholds were 1.28 ± 0.15 g and 1.20 ± 0.09 g, respectively, on Day 5 (Figure 6.2). Each of these 4 groups was then further subdivided into 3 groups of 5 mice each. The groups were treated with either an i.th. injection of MK-801, dynorphin antiserum or control serum. The spinal administration of MK-801 (3.4 μg) and of dynorphin $A_{(1-13)}$ antiserum (150 μg) both reversed the increased sensitivity to normally innocuous mechanical stimuli in the wild-type mice treated with

morphine pellets. Paw withdrawal thresholds were significantly ($p \leq 0.05$) increased to 1.36 ± 0.30 g and 1.26 ± 0.22 g, respectively. Administration of MK-801 or dynorphin $A_{(1-13)}$ antiserum did not significantly ($p > 0.05$) alter the paw withdrawal thresholds of any of the remaining groups (Figure 6.2). The control serum produced no significant ($p > 0.05$) effects in any of the groups tested (Figure 6.2).

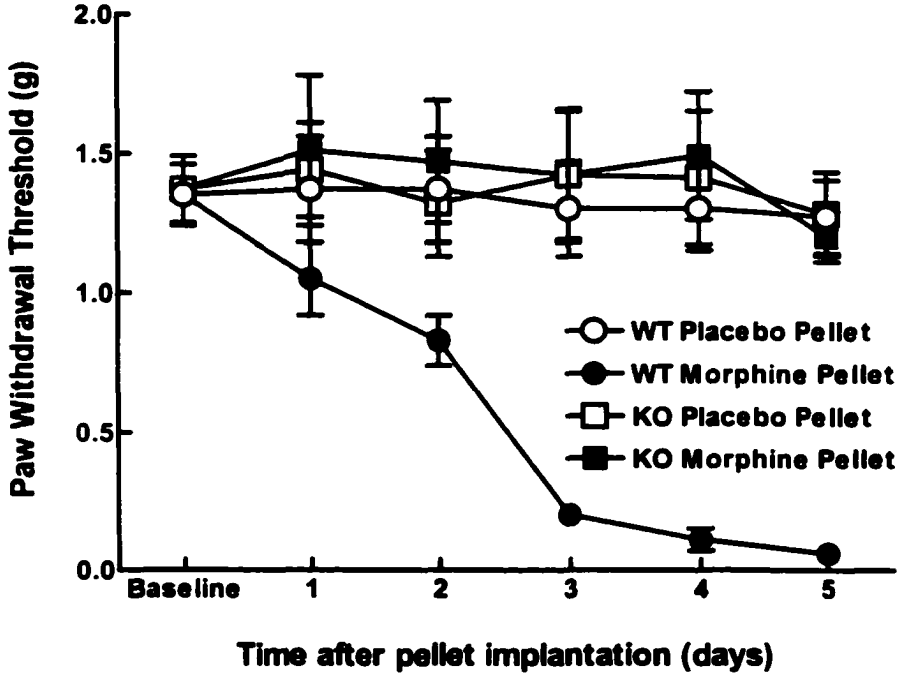

Figure 6.1. The time course of paw withdrawal response thresholds to non-noxious mechanical stimuli (von Frey filaments) across a 5 day period following implantation of a single placebo or morphine pellet (75 mg, s.c.) in prodynorphin knock-out (KO, circles) and wild-type (WT, squares) mice are represented. Sustained morphine (filled symbols), but not placebo (open symbols), produced an increased tactile sensitivity by Day 2 that progressed with time in the WT mice. In contrast, morphine-pelleted KO mice failed to demonstrate an increased tactile sensitivity. Placebo administration did not alter the paw withdrawal response thresholds in any of the mice tested.

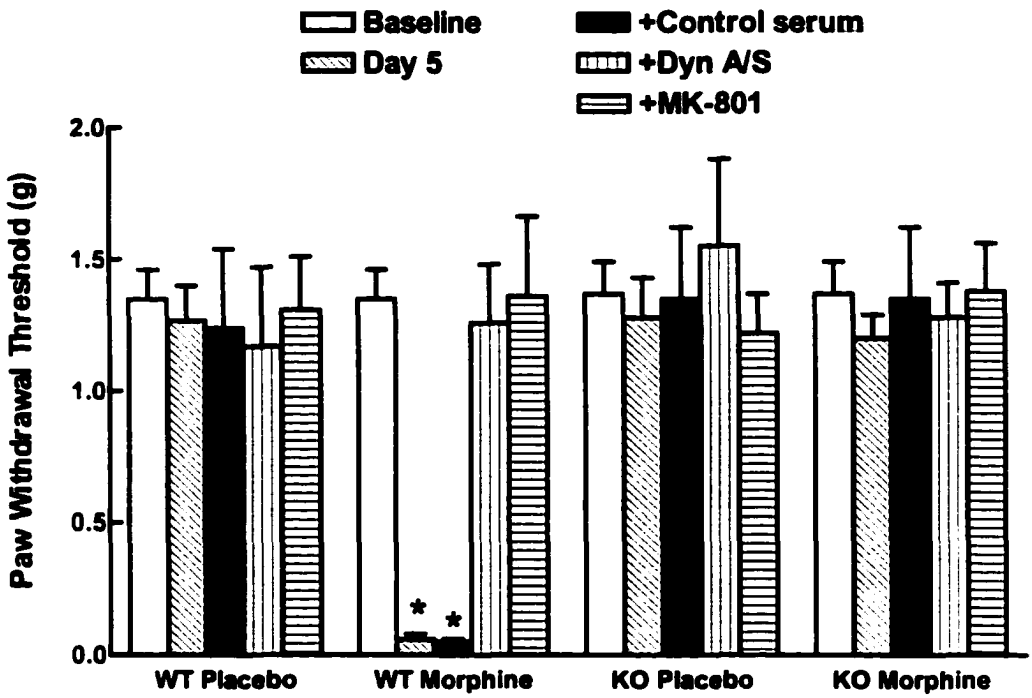

Figure 6.2. A single dose of MK-801 (3.4 μg, i.th.) or antiserum to dynorphin $A_{(1-13)}$ (150 μg i.th.) blocked the mechanical hypersensitivity seen following sustained morphine administration in the wild-type mice (WT). MK-801 or antiserum to dynorphin $A_{(1-13)}$ did not produce any significant ($p > 0.05$) alterations in the placebo-treated mice or any of the knock-out mice (KO). Control serum did not alter paw withdrawal thresholds in any of the mice tested. * indicates significant ($p \leq 0.05$) difference from baseline values.

Noxious thermal thresholds

Response latencies to noxious thermal stimuli were obtained before (baseline) and across the 5 day period after animals were implanted with either morphine or placebo pellets (Figure 6.3). Paw withdrawal latencies to noxious radiant heat did not change significantly ($p > 0.05$) following placebo pellet implantation for either wild-type or knock-out mice. The mean pooled baseline paw withdrawal latencies for the wild-type and the knock-out mice prior to receiving any treatments was 11.21 ± 0.23 sec and 11.13 ± 0.17 sec, respectively. The paw withdrawal latencies on Day 5 for the placebo pelleted

wild-type and knock-out mice were 11.02 ± 0.19 sec and 10.87 ± 0.22 sec, respectively. Sustained subcutaneous administration of morphine significantly reduced ($p \leq 0.05$) paw withdrawal latencies across the 5 day period of testing in the wild-type mice. Paw withdrawal latencies were significantly ($p \leq 0.05$) reduced to 9.32 ± 0.32 sec by Day 2 and progressively diminished to 6.14 ± 0.21 sec by Day 5 (Figure 6.3). In contrast to the wild-type mice, sustained morphine did not significantly alter ($p > 0.05$) the paw withdrawal latencies of the knock-out mice across the 5 day period of testing, demonstrating a baseline latency of 11.13 ± 0.17 sec compared to 11.01 ± 0.31 sec on day 5 (Figure 6.3).

In addition, 4 groups (2 wild-type and 2 knock-out groups) of 15 mice each either received placebo or morphine pellets for 5 days. The paw withdrawal latencies for the wild-type mice were 11.02 ± 0.19 sec and 6.14 ± 0.21 sec, respectively, whereas for the knock-out mice the paw withdrawal thresholds were 10.87 ± 0.22 sec and 11.01 ± 0.31 sec, respectively, on day 5 (Figure 6.4). Each of these 4 groups was then further subdivided into 3 groups of 5 mice each. The groups were treated with either an i.th. injection of MK-801, dynorphin $A_{(1-13)}$ antiserum or control serum. The spinal administration of MK-801 (3.4 μg) and of dynorphin $A_{(1-13)}$ antiserum (150 μg) both reversed the increased sensitivity to noxious thermal stimuli in the wild-type mice treated with morphine pellets. Paw withdrawal latencies were significantly ($p \leq 0.05$) elevated to 10.80 ± 0.25 sec and 10.63 ± 0.39 sec, respectively. Spinal administration of MK-801 or dynorphin $A_{(1-13)}$ antiserum did not significantly ($p > 0.05$) alter the paw withdrawal

thresholds of any of the remaining groups (Figure 6.4). The control serum produced no significant ($p > 0.05$) effects in any of the groups tested (Figure 6.4).

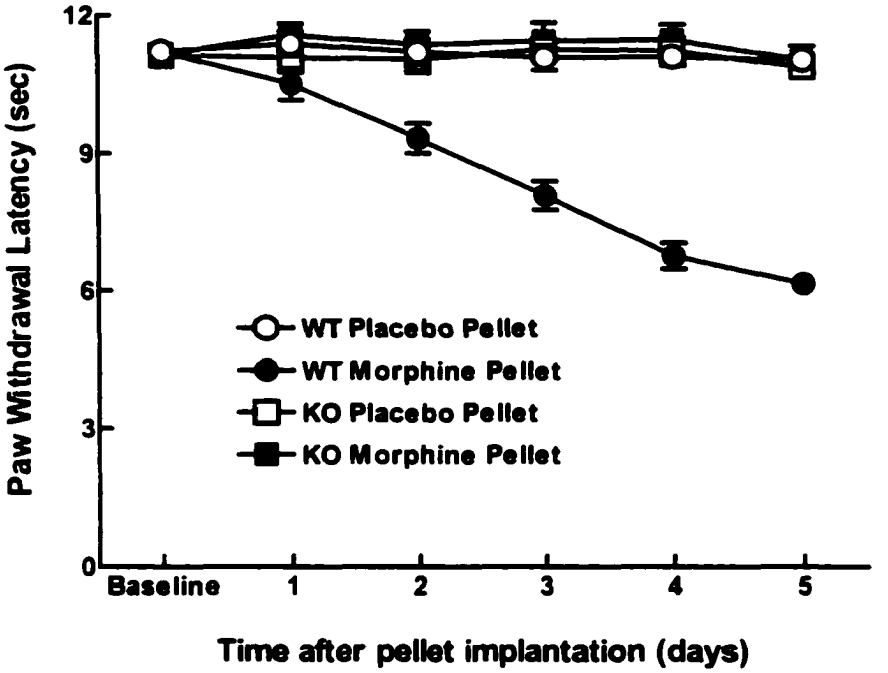

Figure 6.3. The time course of paw withdrawal latencies to noxious thermal stimuli across a 5 day period following implantation of a single placebo or morphine pellet (75 mg, s.c.) in prodynorphin knock-out (KO, circles) and wild-type (WT, squares) mice are represented. Sustained morphine (filled symbols), but not placebo (open symbols), administration produced an increased sensitivity to noxious radiant heat by Day 2 that progressed with time in the WT mice. In contrast, morphine pelleted KO mice failed to demonstrate an increased sensitivity to noxious radiant heat. Placebo administration did not alter the paw withdrawal latencies in any of the mice tested.

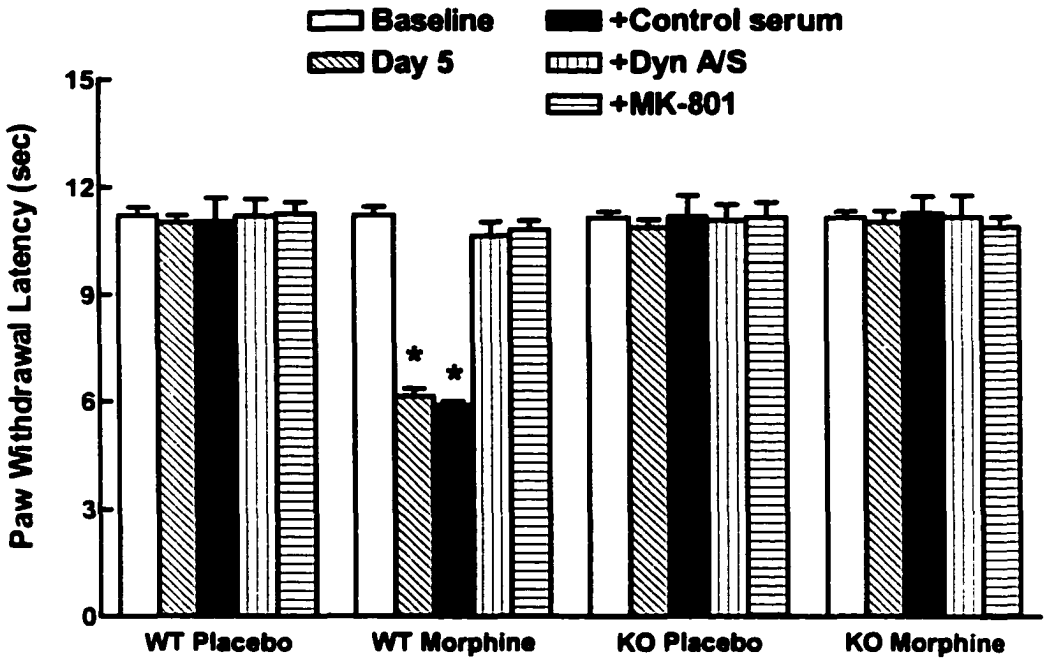

Figure 6.4. A single dose of MK-801 (3.4 μg, i.th.) or antiserum to dynorphin $A_{(1-13)}$ (150 μg i.th.) blocked the increased responsiveness to noxious thermal stimuli seen following sustained morphine administration in the wild-type mice (WT). MK-801 or antiserum to dynorphin $A_{(1-13)}$ did not produce any significant alterations in the placebo treated mice or any of the knock-out mice (KO). Control serum did not alter paw withdrawal latencies in any of the mice tested. * indicates significant ($p \leq 0.05$) difference from baseline values.

Dynorphin Immunoassay

Dorsal lumbar spinal cord halves were processed for dynorphin content in wild-type mice using an enzyme immunoassay. Data obtained were normalized to the wild-type placebo pelleted group. Sustained morphine administration for 5 days produced a significant ($p \leq 0.05$) elevation of lumbar spinal dynorphin content in the wild-type mice. Dynorphin peptide content increased from a level of 100.0% ± 6.8% in placebo pelleted mice to 129.2% ± 7.2% in morphine pelleted wild-type mice (Figure 6.5). As a negative

control, tissues from naïve knock-out mice were also analyzed. These tissues yielded a value of 9.0% ± 0.6% (Figure 6.5), likely representing non-specific binding.

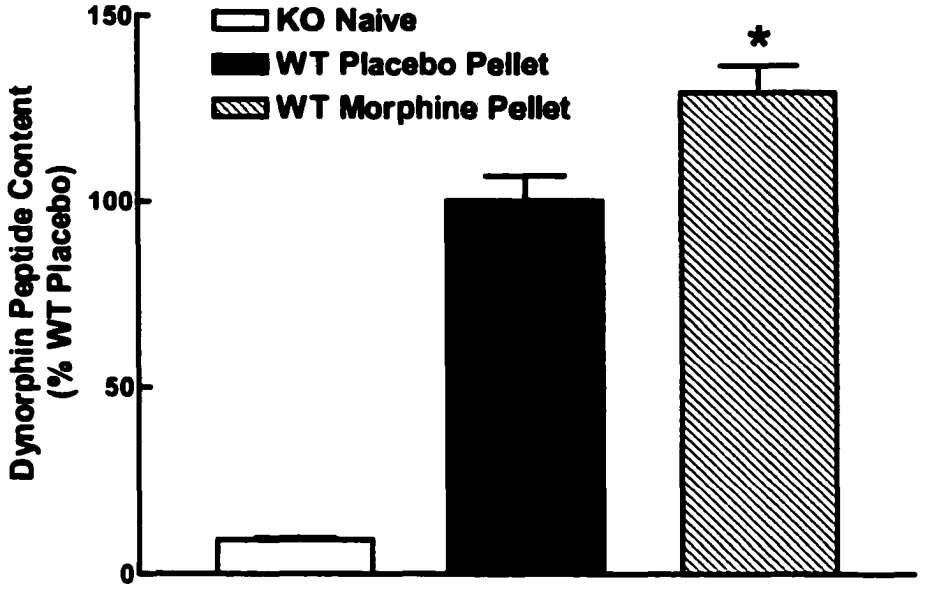

Figure 6.5. Five days following implantation of either morphine or placebo pellets, dorsal halves of lumbar spinal cord were removed from wild-type mice and assayed for dynorphin peptide content. Tissues from naïve knock-out mice were also analyzed. * indicates significant ($p \leq 0.05$) difference from placebo pelleted group.

Antinociceptive Tolerance

Antinociceptive dose-response curves for i.th. morphine were generated in naive wild-type and knock-out mice, and in wild-type and knock-out mice that received either sustained administration of subcutaneous morphine or placebo for 5 days (Figure 6.6). The dose-response curves for morphine in naive wild-type and knock-out mice were chosen as benchmarks against which all subsequent comparisons were made. Morphine was equipotent in both strains of mice, having A_{50} values of 2.9 μg (2.5-3.2; 95% C.I.) in the wild-type mice and 3.0 μg (2.6-3.4; 95% C.I.) in the knock-out mice (Figure 6.6).

However, 5 days after morphine pellet implantation, wild-type mice demonstrated antinociceptive tolerance to spinal morphine as indicated by a significant ($p \leq 0.05$) 9-fold rightward shift of the morphine dose-response curve, having an A_{50} value of 25.5 μg (22.2-29.2; 95% C.I.) (Figure 6.6). In contrast, knock-out mice failed to demonstrate antinociceptive tolerance to spinal morphine 5 days after morphine pellet implantation, achieving an A_{50} value of 3.6 μg (2.9-4.6; 95% C.I.) as compared to naive knock-out mice which had an A_{50} value of 3.0 μg (2.6-3.4; 95% C.I.) (Figure 6.6).

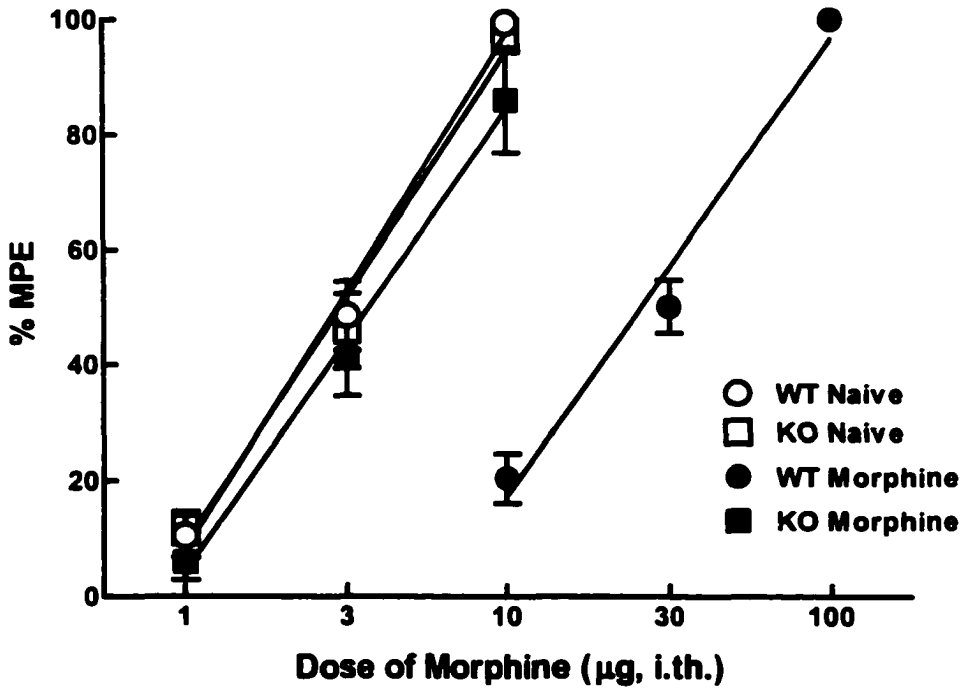

Figure 6.6. The antinociceptive dose-response curves for i.th. morphine in naïve mice (open symbols) and in mice 5 days after morphine pellet implantation (filled symbols). Sustained morphine produced a significant ($p \leq 0.05$) 9-fold shift to the right in the dose-response curve for i.th. morphine in the wild-type mice (circles). In contrast, sustained morphine did not alter the dose-response curve for i.th. morphine in the prodynorphin knock-out mice (squares).

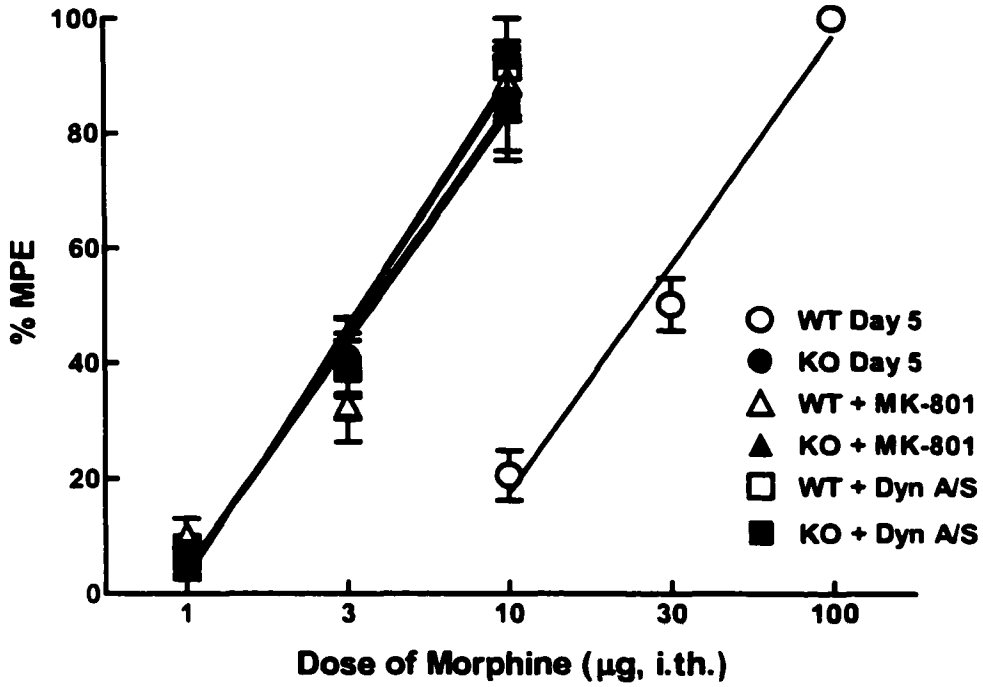

Figure 6.7. The antinociceptive dose-response curves for i.th. morphine in (a) wild-type (WT, open symbols) and knock-out (KO, filled symbols) mice 5 days after morphine pellet implantation (circles) and in (b) WT and KO mice 5 days after morphine pellet implantation that were pretreated (–20 min) with i.th. MK-801 (3.4 μg, triangles) or dynorphin $A_{(1-13)}$ antiserum (150 μg, squares). Sustained administration of morphine produced a 9-fold shift to the right in the dose-response curve for morphine in WT mice that received morphine pellets. In contrast, pretreatment with MK-801 or antiserum to dynorphin $A_{(1-13)}$ prior to challenge with morphine in WT mice produced a significant block of the rightward shift in the i.th. morphine dose-response curve. Pretreatment with MK-801 or antiserum to dynorphin $A_{(1-13)}$ did not alter the effect of i.th. morphine in the KO mice.

In an additional 4 groups of mice (2 wild-type and 2 knock-out groups), which received morphine pellets for 5 days, dose-response curves for spinal morphine in the presence of MK-801 (3.4 μg) and dynorphin $A_{(1-13)}$ antiserum (150 μg) pretreatment (-20 min) were constructed (Figure 6.7). Both MK-801 and dynorphin $A_{(1-13)}$ antiserum pretreatment completely restored the potency of morphine, as indicated by a significant

($p \leq 0.05$) 7-fold leftward shift of the spinal morphine dose-response curve, in morphine-pelleted wild-type mice. The A_{50} values for morphine in morphine-pelleted wild-type mice were 3.7 μg (3.0-4.6; 95% C.I.) and 3.5 μg (2.9-4.3; 95% C.I.), respectively, compared with an A_{50} value of 2.9 μg (2.5-3.2; 95% C.I.) achieved in naive wild-type mice (Figure 6.7). Pretreatment with MK-801 and dynorphin $A_{(1-13)}$ antiserum did not significantly ($p > 0.05$) alter the potency of spinal morphine in morphine-pelleted knock-out mice. The A_{50} values for morphine in morphine-pelleted knock-out mice were 3.4 μg (2.8-4.2; 95% C.I.) and 3.7 μg (3.0-4.7; 95% C.I.), respectively, compared with an A_{50} value of 3.0 μg (2.6-3.4; 95% C.I.) achieved in naive knock-out mice (Figure 6.7).

CGRP Release

To ascertain whether dynorphin has the ability to modulate the release of pronociceptive transmitters in the dorsal horn of the lumbar spinal cord, we utilized an *in situ* spinal cord perfusion model that uses capsaicin to evoke release of CGRP from a subset of intact primary afferent terminals (C fibers), which express VR_1 receptors. Wild-type and prodynorphin knock-out mice were pelleted subcutaneously with either a morphine or placebo pellet. Dorsal lumbar spinal cord halves were taken from these mice 7 days following pellet implantation and capsaicin evoked CGRP release was quantified by radioimmunoassay (Figure 6.8).

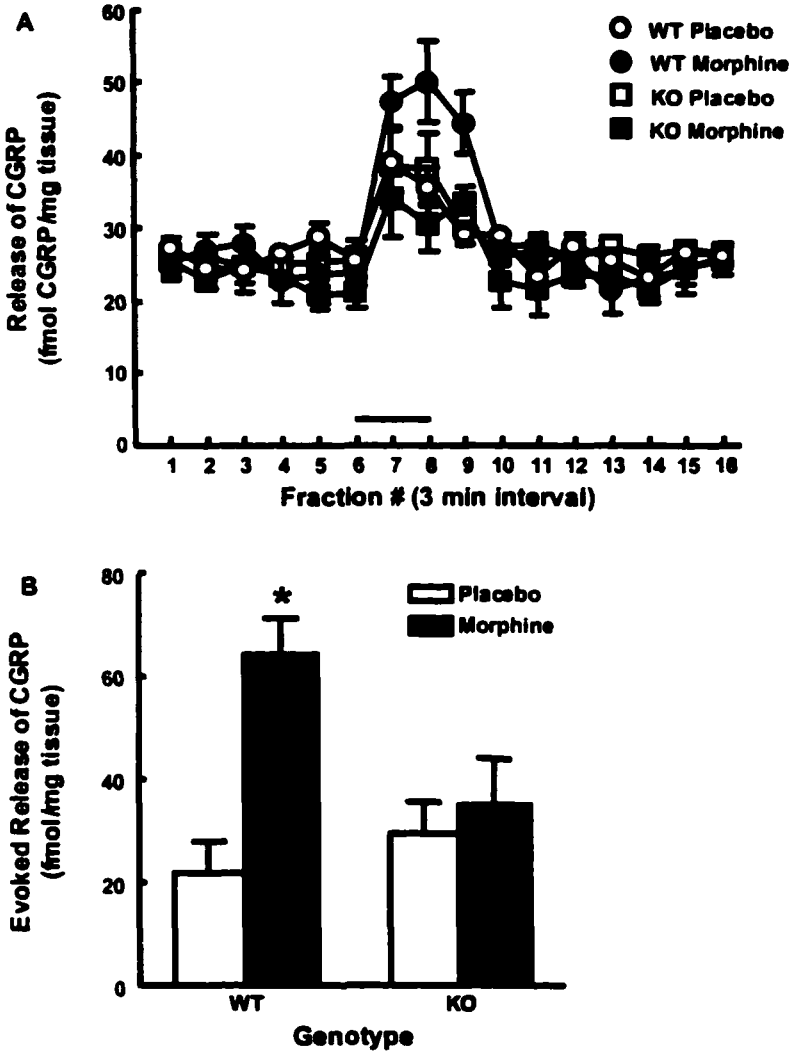

Figure 6.8. Wild-type (WT) and prodynorphin knock-out (KO) mice were implanted with a placebo pellet or a morphine pellet. Separate groups were sacrificed 7 days after pellet implantation and dorsal lumbar spinal cord tissues were isolated, minced, and placed in perfusion chambers. Panel A shows the basal and evoked CGRP in the perfusate collected at 3 minute intervals. The horizontal bar represents the period where capsaicin (1μM) was added to the perfusate in order to evoke CGRP release. The capsaicin-evoked release of CGRP from the spinal tissues in vitro is demonstrated in panel B, and represents the amount of CGRP above the baseline release for each individual group. Basal levels of CGRP release did not differ among the treatment groups. Evoked CGRP release was not different between tissues from placebo-treated groups at Day 7 after pellet implantation ($p > 0.05$). However, tissues from the morphine-treated WT mice showed a significantly greater level of capsaicin-evoked release of CGRP (*; $p \leq 0.05$). In contrast, morphine treatment failed to potentiate capsaicin evoked CGRP release, above levels obtained in placebo-treated controls, for the KO mice ($p > 0.05$). Each treatment group consisted of a minimum of 5 mice.

In the presence of capsaicin, there is a significant ($p \leq 0.05$) increase in the evoked release of CGRP from tissues taken from all groups of mice, regardless of their genotype (Figure 6.8). In tissues taken from wild-type mice there is a significant ($p \leq 0.05$) potentiation of capsaicin evoked CGRP release from the morphine-treated tissues as compared to placebo-treated tissues. The level of capsaicin evoked CGRP release from wild-type placebo-treated tissues was 21.9 ± 6.2 fmol CGRP/mg tissue whereas, in the morphine-treated tissues the level of evoked release was 64.3 ± 6.9 fmol CGRP/mg tissue (Figure 6.8). In tissues taken from knock-out mice the morphine-induced potentiation of capsaicin evoked CGRP release is completely absent. The level of capsaicin evoked CGRP release from knock-out placebo-treated tissues was 29.5 ± 6.0 fmol CGRP/mg tissue whereas, in the morphine treated tissues the level of evoked release was 34.9± 9.1 fmol CGRP/mg tissue (Figure 6.8).

SUMMARY

The results of the present Chapter demonstrate the necessity of spinal dynorphin upregulation for the plasticity induced in the nervous system following sustained exposure to morphine. The present data confirm and extend previous findings indicating that morphine exposure significantly upregulates spinal dynorphin (Vanderah et al. 2000) and CGRP (Ma et al. 2000; Ma et al. 2001; Menard et al. 1996; Powell et al. 2000). The increase in expression of these substances appears to be of functional significance, providing a physiological basis for increased stimulus-induced excitation to the spinal cord that may underlie paradoxical opioid-induced pain. Several insights can be obtained from these data, which impact our understanding of opioid-induced paradoxical pain and possibly the behavioral manifestation of antinociceptive tolerance. First, morphine-exposed wild-type mice developed time-related tactile and thermal hypersensitivity that accompanied morphine antinociceptive tolerance. Second, sustained morphine exposure in wild-type mice results in enhanced content and evoked release of CGRP. Third, administration of either intrathecal MK-801 or antiserum to dynorphin $A_{(1-13)}$ restored the pain thresholds of the morphine-exposed wild-type mice and restored the potency of spinal morphine. Finally, morphine-exposed prodynorphin knock-out mice failed to show opioid-induced abnormal pain, antinociceptive tolerance and a marked increase of capsaicin-evoked CGRP release, implicating endogenous dynorphin in these effects.

It is important to note that opioid-induced tactile and thermal hypersensitivity was evident in the wild-type mice during the course of morphine delivery, minimizing possible pharmacokinetic concerns about sustained opioid delivery. No signs of opioid

withdrawal syndrome were detected in any of the mice tested despite careful monitoring; suggesting that the opioid-induced tactile and thermal hypersensitivity observed in the wild-type mice was not the result of abstinence or states of "mini-withdrawal" (Gutstein 1996). This conclusion is reinforced by the time-related onset of opioid-induced pain, which was clearly detectable in wild-type mice by day three of morphine administration. The consequences of opioid-induced pain were significant in determining subsequent opioid antinociceptive potency. Clinical studies have repeatedly shown the need for increased opioid dosage to achieve equivalent pain relief during periods of enhanced pain (e.g.; Cherney and Portenoy 1999). Similarly, the presence of opioid-induced abnormal pain would be expected to require increased opioid to overcome the elevated nociceptive state and such requirement for increased opioid would manifest behaviorally as decreased antinociceptive potency (i.e., tolerance). Previous studies from our laboratory have shown that manipulations that block opioid-induced pain, such as RVM lidocaine or DLF lesions, also restore morphine antinociceptive potency (Vanderah et al. 2001b). These manipulations revealed no apparent alteration of antinociceptive signal transduction in morphine-pelleted rats. Based on such observations, the central hypothesis of this dissertation is that tonic activation of descending pain facilitation arising in the RVM may represent a mechanism of chronic pain and that such descending facilitation is a primary mechanism of opioid-induced pain and the behavioral manifestation of antinociceptive tolerance.

A potential variable complicating the interpretation of the mechanisms underlying the resistance of prodynorphin knock-out mice to opioid-induced abnormal pain and

antinociceptive tolerance is the genetic background of these mice. The prodynorphin knock-out mice were derived from a background containing the 129S6 strain (formerly known as 129/SvEv; (Festing et al. 1999)) (Sharifi et al. 2001). It is quite possible that the deficiency of opioid-induced pain and antinociceptive tolerance in these mice may be an inheritable trait from the 129S6 background, and is independent of the function of dynorphin. It should be noted, however, that the wild-type mice used in this study were littermates, such that the genetic background should be identical to that of the prodynorphin knock-out mice.

The underlying deficits in the 129S6 mice that confer a resistance to opioid-induced pain are deserving of further investigation as a possible genetic key to the fundamental mechanisms by which such pain is expressed. Previous findings suggest that 129S6 mice do not develop opioid antinociceptive tolerance (Kolesnikov et al. 1998), and the authors suggest that it may be due to a deficiency in the nitric oxide signaling pathway resulting from an inability of the NMDA receptors to activate NOS. Previous work from this laboratory has demonstrated that the 129S6 mice do not demonstrate opioid-induced abnormal pain, antinociceptive tolerance, or an up-regulation of spinal dynorphin following repeated injections of DAMGO (Gardell & Porreca, unpublished observations), or tactile and thermal hypersensitivity following injury to peripheral nerves (Gardell et al. 2002c). Such a deficit may also impact on the expression of opioid-induced pain as seen here because NMDA receptor blockade has been shown to inhibit opioid-induced pain and restore the potency of spinal opioids (Chapter III). Similarly, a lack of spinal dynorphin upregulation in the 129S6 mice is also consistent with this

strain's behavioral response to prolonged opioid exposure and injury to peripheral nerves. A critical issue to consider here, therefore, is that the potential contribution of the 129S6 background to the genotype of the prodynorphin knock-out mice may give rise to two important characteristics that may act alone or in concert to inhibit neuropathic pain in these mice: a lack of spinal dynorphin upregulation (or dynorphin at all) and the possibility of deficits in NMDA receptor sensitivity.

Such observations call for caution in the interpretation of data obtained from animals with specific gene deletions. Specifically, these data highlight the potential bias in the interpretation of cause and effect when using transgenic animal models, and reinforce the concept that multiple observations through multiple experimental approaches are needed to validate a potential mechanism. The present data show that upregulation of spinal dynorphin A consistently correlates with the expression of opioid-induced pain and antinociceptive tolerance in multiple strains of mice, as previously observed in rats, and continue to support the hypothesis that the upregulation of spinal dynorphin A is a crucial factor in opioid-induced pain and antinociceptive tolerance.

The critical importance of spinal dynorphin is supported by the blockade by dynorphin antiserum of enhanced evoked release in morphine-exposed tissues (Chapter V). Dynorphin $A_{(1\text{-}13)}$ antiserum did not block evoked CGRP release in naive or placebo-pelleted tissues. Additionally, no enhanced evoked CGRP release was observed in tissues taken from morphine-exposed rats with prior lesions of the DLF (Chapter V). DLF or sham-DLF lesion did not alter evoked CGRP release in placebo-pelleted rats. This finding also demonstrates that the increased release of CGRP seen in tissues from

morphine-exposed rats was not the result of an *in vitro* expression of opioid withdrawal. This conclusion is supported by the fact that tissues taken from rats with prior DLF lesions had been exposed to morphine in exactly the same way as those without DLF lesions. Also supporting this conclusion is the failure to demonstrate differences in basal (i.e., unstimulated) CGRP release in tissues from morphine- or placebo-pelleted rats (Chapter V) or in wild-type and prodynorphin knock-out mice. These latter observations also argue against possible "sensitization" of the release mechanisms in the afferent fibers following morphine treatment.

As DLF lesions and dynorphin antiserum are manipulations which have also been shown to block opioid-induced pain, antinociceptive tolerance (Vanderah et al. 2001b), Chapter IV) and increased CGRP release (Chapter V), it seems reasonable to suggest that enhanced evoked excitatory transmitter release may be an important factor which underlies opioid-induced paradoxical pain and the subsequent expression of antinociceptive tolerance. Taken together, these data support the hypothesis that dynorphin acts, in a non-opioid fashion, to enhance the evoked release of excitatory transmitters from primary afferent fibers to promote abnormal pain which manifests behaviorally as antinociceptive tolerance.

CHAPTER VII: CONCLUSIONS

The focus of this dissertation is to explore the role of dynorphin as a pronociceptive mediator in opioid-induced paradoxical pain and antinociceptive tolerance. Specifically, three hypotheses were tested. First, opioid-induced abnormal pain manifests, behaviorally, as antinociceptive tolerance such that, manipulations that block opioid-induced pain will restore the potency of spinal opioids. Second, descending pain facilitation arising from the RVM will result in plasticity at the spinal cord level including the upregulation of dynorphin. Finally, pathological levels of spinal dynorphin act to promote opioid-induced abnormal pain by potentiating the release of CGRP from the central terminals of primary afferent neurons.

Recent work from our laboratory has shown that the microinjection of cholesystokinin (CCK) into the RVM produces a short-lasting tactile and thermal hypersensitivity, which is blocked by prior bilateral lesions of the DLF (Xie and Porreca, unpublished observations). RVM administration of the CCK_B antagonist L365,260 blocks both tactile and thermal hypersensitivity produced by ligation of the L_5 and L_6 spinal nerves (Kovelowski et al. 2000). Others have found that CCK infused into the RVM blocked the antinociceptive effect of systemic morphine (Heinricher et al. 2001). Although the circuitry is not fully understood, it appears to do so by blocking the morphine-induced increase in firing of RVM "OFF"-cells (Heinricher et al. 2001). Additional work from our laboratory has shown that CCK is elevated in the RVM following sustained morphine administration (Gardell and Porreca, unpublished

observations). Further, administration of L365,260 into the RVM blocks opioid-induced pain and restores the potency of intrathecal morphine (Xie and Porreca, unpublished observations). Finally, following sustained morphine exposure there is an apparent increase in the basal release of CCK in the RVM (Vanderah and Porreca, unpublished observations). Taken together these data support a role for CCK as a key component in the activation of descending pain facilitation. Depicted in Figure 7.1 below is a proposed mechanism of paradoxical pain and antinociceptive tolerance induced by sustained administration of systemic morphine.

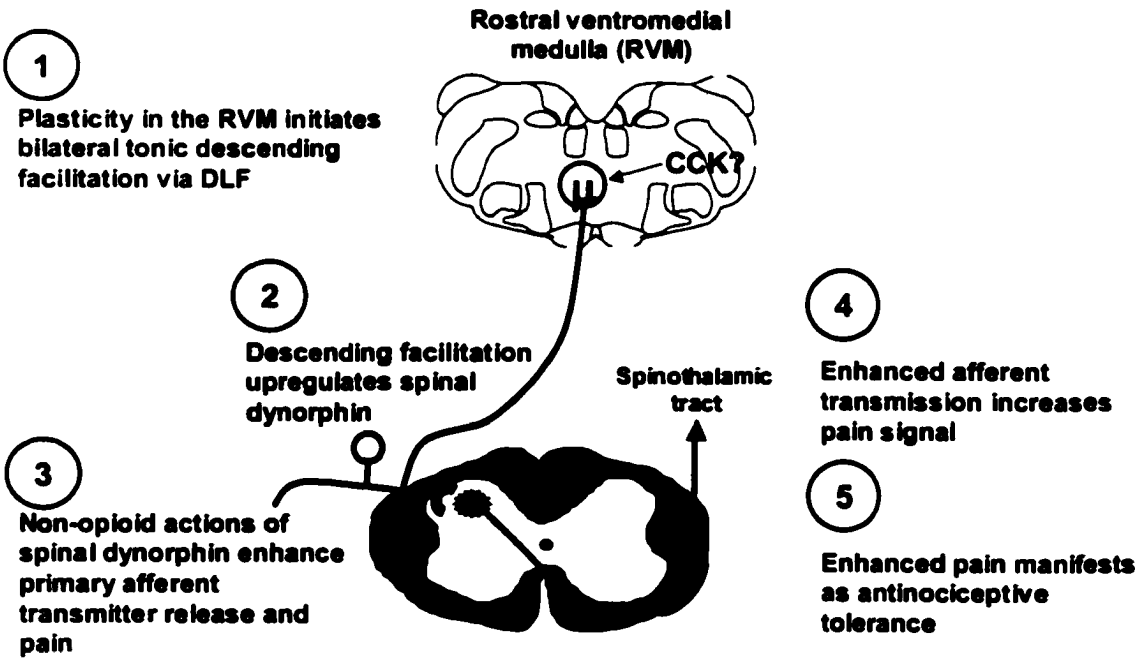

Figure 7.1. Proposed scheme of morphine-induced abnormal pain and antinociceptive tolerance. Sustained administration of systemic morphine results in plasticity at supraspinal sites including the RVM. Elevated levels of CCK may drive bilateral descending pain facilitation via the DLF. Descending pain facilitation drives the upregulation of spinal dynorphin. Dynorphin can act in a non-opioid manner to potentiate the release of excitatory neurotransmitters from capsaicin-sensitive primary afferent terminals. The enhanced release of pronociceptive transmitters enhances the pain signal. This enhanced pain signal requires the escalation of drug dose, in order to achieve adequate analgesia, thus manifesting as antinociceptive tolerance.

Sustained administration of morphine results in tonic activation of descending pain facilitation from the RVM, perhaps mediated by elevated levels of CCK, results in plasticity at the level of the spinal cord, specifically the up-regulation of dynorphin. This is supported by the data of Chapter V which demonstrates that prior bilateral lesions of the DLF blocks the up-regulation of spinal dynorphin that is observed 7 days following morphine pellet implantation. Pathological levels of dynorphin can act, in a non-opioid manner, to promote a state of abnormal pain via its ability to potentiate the release of excitatory transmitters from the central terminals of primary afferent fibers. This is supported by the data from Chapters III, V and VI which show that interfering with pathological levels of dynorphin, either indirectly with MK-801, directly with dynorphin $A_{(1-13)}$ antiserum, or completely using prodynorphin knock-out mice, can restore sensory thresholds and evoked release of CGRP to levels observed in placebo-treated controls. Finally, opioid-induced abnormal pain manifests behaviorally as antinociceptive tolerance. This is supported by the data from Chapters III, V and VI, which show that manipulations that block opioid-induced abnormal pain restore the antinociceptive potency of spinal morphine.

Summarized in Figure 7.2 below is a proposed mechanism of paradoxical abnormal pain and antinociceptive tolerance induced by sustained spinal opioids. Data from Chapter IV has shown that increased pain, upregulation of spinal dynorphin and spinal antinociceptive tolerance can also result from sustained intrathecal delivery of opioids including DAMGO. These findings suggest the possibility of a local, spinal site of opioid-induced pain, perhaps through modulation of release of excitatory transmitters

from primary afferents without the need for activation of descending facilitatory pathways. Supporting this idea is additional data, which show that intrathecal delivery of antisense oligodeoxynucleotides (ODNs) to the μ opioid receptor elicits abnormal pain that is indistinguishable from that seen with spinal DAMGO (Gardell and Porreca, unpublished observations). The effects of antisense knock-down of μ opioid receptors also produces a rightward shift in the antinociceptive dose-response curve for i.th. DAMGO, due to loss of opioid receptor protein; this rightward shift in the antinociceptive dose-response curve is analogous to the antinociceptive "tolerance" seen with spinal DAMGO administration (Gardell and Porreca, unpublished observations).

Such observations point to possible differences between the mechanisms of action of morphine and DAMGO in inducing pain and opioid tolerance. An important distinction is the observation that DAMGO and μ opioid receptor antisense ODNs, but not morphine, produces down-regulation of the μ opioid receptor in the spinal dorsal horn (Chapter IV and Gardell and Porreca, unpublished observations). Thus, while morphine-induced pain and antinociceptive tolerance may require activation of descending pain facilitatory pathways (which might be engaged even after sustained spinal administration through retrograde transport to supraspinal sites), DAMGO and μ opioid receptor antisense knock-down of μ opioid receptors may produce pain and expression of antinociceptive tolerance locally at the spinal level through a mechanism not available to morphine (Figure 7.2). The observation of differential actions of morphine and DAMGO to produce receptor down-regulation, in spite of common ability to elicit antinociceptive tolerance, have led many investigators to conclude that receptor down-regulation is not

an essential aspect of opioid tolerance. This conclusion, however, may not be correct as manipulations, which lead to receptor down-regulation, using μ opioid receptor antisense ODN, clearly produce increased pain. Such increased pain is also observed in mice that lack the μ opioid receptor (Gardell and Porreca, unpublished observations).

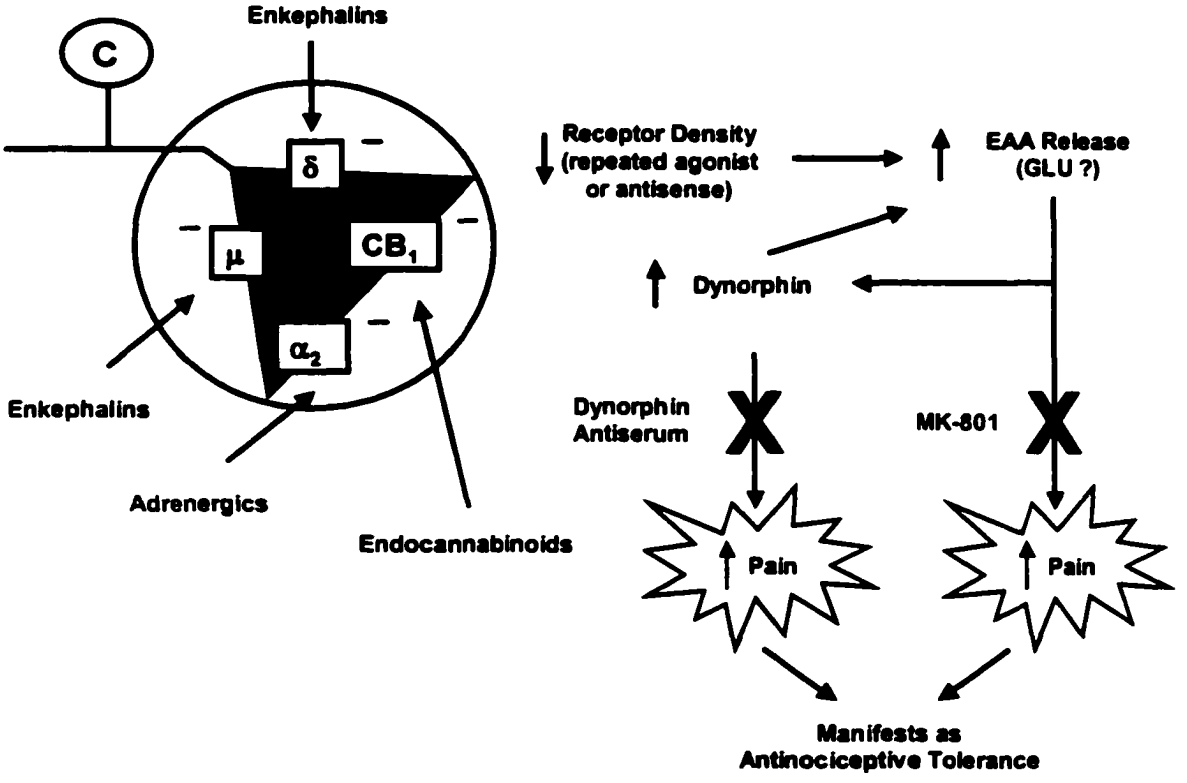

Figure 7.2. Proposed scheme of DAMGO- or μ opioid receptor antisense-induced abnormal pain and antinociceptive tolerance. A reduction in receptor density either through agonist-induced down-regulation or by interference with protein production (using antisense ODNs) compromises the efficacy of an inhibitory receptor system. Loss of endogenous inhibitory tone results in a net increase in the release of excitatory transmitters from primary afferent terminals. Increased excitatory input results in the upregulation of spinal dynorphin. Dynorphin, which can act through a non-opioid mechanism, to further facilitate the release of excitatory transmitters from primary afferent terminals to promote an enhanced state of pain. Manipulations that block the enhanced pain restore the potency of spinal antinociceptive agents.

There are a number of different inhibitory GPCRs expressed on the central terminals of C fibers including, but not limited to, μ and δ opioid, CB_1 cannabinoid, and

α_2 adrenergic receptors. Repeated i.th. administration of antisense ODNs directed at μ and δ opioid (Gardell and Porreca, unpublished observations) or the CB_1 cannabinoid (Dogrul et al. 2002) receptors results in a state of abnormal pain (characterized by the presence of tactile and thermal hypersensitivity), the expected reduction of agonist potency reminiscent of antinociceptive tolerance, as well as a significant up-regulation of spinal dynorphin. Administration of MK-801 or antiserum to dynorphin $A_{(1-13)}$ blocks both mechanical and thermal hypersensitivity and restores the spinal potency of the agonist. It also appears that repeated administration of either CB_1 cannabinoid agonist WIN 55-212,2 (Gardell et al. 2002b) or the α_2 adrenergic agonist clonidine (Malan and Porreca, unpublished observations) results in tactile and thermal hypersensitivity, the expected rightward displacement of the spinal agonist dose-response curve indicative of antinociceptive tolerance, as well as a significant up-regulation of spinal dynorphin. Administration of MK-801 or antiserum to dynorphin $A_{(1-13)}$ blocks both tactile and thermal hypersensitivity and restores the spinal potency of the agonist. These findings demonstrate the generality of dynorphin to promote abnormal pain across a number of inhibitory GPCR systems.

Shown in Figure 7.3 below is a proposed mechanism for the pronociceptive actions of pathological levels of spinal dynorphin. Dynorphin when administered alone in a pharmacological experiment using spinal cord tissues from naïve rats in the release assay is not sufficient to evoke CGRP release, however, when co-administered with capsaicin there is a significant potentiation of evoked CGRP release. These data indicate that activation of primary afferent fibers must occur that, in turn, leads to the subsequent

activation of post-synaptic cells, which include 2nd order projections neurons as well as dynorphinergic interneurons. The subsequent activation of dynorphinergic interneurons must occur in order to release dynorphin, and then dynorphin can act through a yet unknown non-opioid mechanism to facilitate the release of excitatory transmitters including CGRP.

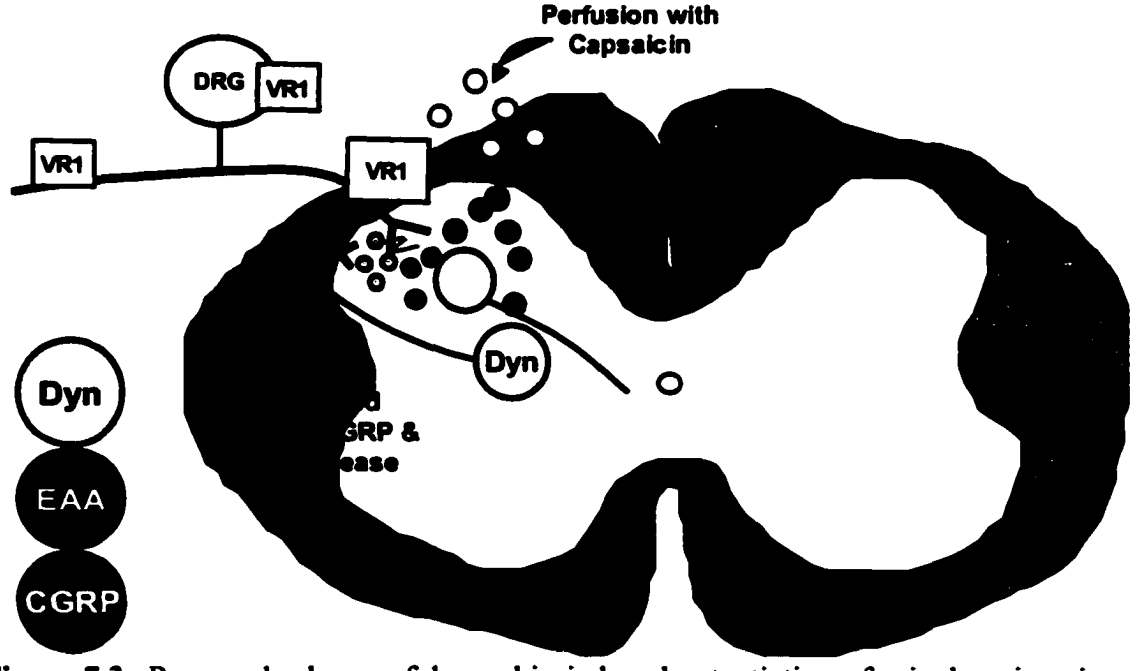

Figure 7.3. Proposed scheme of dynorphin-induced potentiation of spinal excitatation.

One future study is to examine dynorphin levels in fractions collected following capsaicin treatment. Although dynorphin levels in the spinal cord are elevated following sustained opioid exposure, whether there is an increased release of dynorphin remains to be demonstrated. Another planned study is to examine the role of the post-synaptic neurons on the ability of dynorphin to potentiate transmitter release. We can examine this possibility using substance P conjugated with saporin. Substance P-saporin should selectively destroy those cells that express the NK1 receptor (i.e., post-synaptic neurons).

The findings contained in this dissertation are consistent with a large body of data supporting non-opioid, net excitatory activity of dynorphin in vivo (Bakshi and Faden 1990; Bakshi et al. 1992; Caudle and Isaac 1988b; Long et al. 1994; Stewart and Isaac 1991; Vanderah et al. 1996). The mechanism by which dynorphin promotes excitatory activity is uncertain though our laboratory has recently demonstrated a direct, high affinity inhibitory binding site for dynorphin at the NMDA receptor (Tang et al. 1999) and dynorphin induced accumulation of calcium in isolated cortical cells (Tang et al. 2000). The latter effect was not mediated through either opioid or NMDA receptors, suggesting a novel excitatory action (Tang et al. 2000). Work is currently in progress to identify and subsequently clone the receptor through which dynorphin elicits its pronociceptive effects.

APPENDIX - LIST OF PUBLICATIONS

1. Gardell LR, Hubbell CL, & Reid LD. Naltrexone persistently reduces rats' intake of a palatable alcoholic beverage. **Alcoholism: Clinical and Experimental Research, 20,** 584-588, 1996.

2. Reid LD, Gardell LR, Chattopadhyay S, & Hubbell CL. Periodic naltrexone and propensity to take alcoholic beverage. **Alcoholism: Clinical and Experimental Research, 20,**1329-1334, 1996.

3. Gardell LR, Whalen CA, Chattopadhyay S, Cavallaro CA, Hubbell CL, & Reid LD. The combination of fluoxetine and naltrexone as pharmacotherapy for alcohol abuse. **Alcoholism: Clinical and Experimental Research, 21,** 1435-1439, 1997.

4. Gardell LR, Reid LD, Boedeker KL, Liakos TM, & Hubbell CL. Isradipine and naltrexone in combination with isradipine interact with a period of abstinence to reduce rats' intake of an alcoholic beverage. **Alcoholism: Clinical and Experimental Research, 21,** 1592-1598, 1997.

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