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SPINAL CORD MECHANISMS OF
OPIOID TOLERANCE AND DEPENDENCE
by

Dana Sharon Rohde

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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This dissertation is dedicated to my parents who encouraged me to pursue my intellectual interests and never blinked even when I turned 30 and continued to be a student.

SPINAL CORD MECHANISMS OF OPIOID TOLERANCE AND DEPENDENCE

Dana S. Rohde

Abstract

Morphine tolerance is characterized by a decrease in responsiveness of neurons to the acute effects of morphine. This may result in part from the development of a compensatory response in neurons that express the opioid receptor or of neural circuits in which those neurons participate. Since morphine analgesia and tolerance have been demonstrated spinally, we proposed that some of the signs and symptoms of withdrawal from morphine is due in part to activation of spinal cord neurons that are altered during the development of morphine tolerance. To this end, we monitored expression of Fos, a nuclear protein that is a marker of neuronal activity, after naltrexone injection in opiate-naïve and morphine-pelleted rats. After daily implantation of morphine or placebo pellets, the rats received a subcutaneous injection of saline or naltrexone and behavior was monitored for one hour. Some rats were injected with formalin into the hindpaw, instead of subcutaneous

naltrexone, to determine the effect of a noxious stimulus on the "activity" of spinal neurons in morphine-tolerant rats. One hour later, the rats were anesthetized, perfused, and the tissue was prepared for Fos immunocytochemistry. To determine the source of input to the induced upregulation of Fos expression in the spinal cord, we repeated the withdrawal studies in rats that were previously treated to eliminate peripheral or supraspinal input.

Morphine withdrawal was associated with increased Fos-like immunoreactivity in the dorsal horn of the spinal cord. This increase was further enhanced after elimination of peripheral or supraspinal input. Formalin injection induced greater total Fos-like immunoreactivity in morphine-tolerant rats compared to non-tolerant rats.

These data suggest that spinal cord dorsal horn neurons are sensitized during the development of morphine tolerance. Supraspinal and possibly peripheral input inhibit spinal cord neurons during withdrawal, effectively attenuating the magnitude of the withdrawal syndrome and the neuronal activity related to it. We believe that the sensitization that develops in spinal cord dorsal horn neurons during the development of morphine tolerance may underlie the hyperalgesia that is demonstrated in morphine-tolerant rats and humans.

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Chapter 1

Introduction

Morphine analgesia results from the activation of brainstem and spinal antinociceptive circuits that inhibit the firing of dorsal horn nociceptive neurons (Basbaum and Fields 1978; Basbaum and Fields 1984; Guilbaud et al. 1973). At the cellular level, opiates activate K⁺ channels (North et al. 1987) which causes neuronal hyperpolarization. Opiates also inhibit adenylyl cyclase activity (Griffin et al. 1985; Makman et al. 1988; Sharma et al. 1975). Since pertussis toxin blocks the adenylyl cyclase inhibition (Griffin et al. 1985; Makman et al. 1988) as well as morphine analgesia (Parolaro et al. 1990; Sanchez-Blazquez and Garzon 1988), these changes are believed to be mediated by an inhibitory G protein (G_i).

Morphine tolerance is defined as a decrease in the effectiveness of a drug after repeated administration or a requirement for an increase in drug dosage to maintain the same effect. Morphine dependence is defined as a change in function produced by the long-term administration of morphine such that further administration is necessary to avoid the physical signs of withdrawal. Once the drug is terminated, a withdrawal syndrome follows.

It is commonly believed that morphine tolerance results from an uncoupling of the G_i protein from the opioid receptor with chronic exposure. Uncoupled receptors may be internalized; however, receptor downregulation (decrease in receptor affinity or number of binding sites) is not evident when tolerance is first present (Holtt et al. 1975). The lack of evidence for receptor downregulation gives credence to the idea that receptors are desensitized by functional uncoupling of the G_i protein from

the opioid receptor (Werling et al. 1989). It is puzzling, however, that naltrexone can precipitate withdrawal, as it is simply removing an agonist that is purportedly not coupled to downstream events. That naltrexone precipitates abstinence suggests that in the tolerant state, morphine *is* functioning at some level, although it is no longer capable of producing analgesia.

Compensatory response in cAMP system leads to morphine tolerance and dependence

An alternative hypothesis is that chronic morphine produces tolerance due to neuronal adaptations that counteract the inhibitory effects of morphine (Aghajanian 1988; Christie et al. 1987a). In fact, chronic morphine administration is associated with *increases* in adenylyl cyclase activity in neuroblastoma x glioma (NG-108) cells (Sharma et al. 1975) and in dorsal root ganglion explants (Makman et al. 1988). This upregulation in the cAMP system is postulated to be a compensatory response that counteracts the continued presence of morphine (Sharma et al. 1975). The cells are now tolerant and dependent on morphine; the dependence is masked, however, and becomes apparent only when the opiate is removed, for example with naltrexone, an opioid antagonist, that, by itself is without intrinsic activity. Upon naltrexone administration, cAMP rises to abnormally high levels, presumably contributing to the hyperexcitability of neurons during withdrawal. In fact, signs of withdrawal are usually opposite to the acute actions of the drug. Thus, morphine withdrawal is manifest as salivation, rhinorrhea, diarrhea, increase in excitability of the animal, and hyperalgesia.

That chronic morphine administration leads to a compensatory upregulation of cAMP in neurons exposed to the opiate receptor is supported by a study of Fry et al. (1980). In that report, naloxone was microiontophoresed onto single neurons in the frontal cerebral cortex and striatum in rats treated with morphine pellets. In naive rats, naloxone had no effect on glutamate or acetylcholine-evoked activity, but in tolerant/dependent rats, the activity evoked by glutamate or acetylcholine was increased after naloxone. The authors postulated that this excitatory response represents an opioid withdrawal response at the single neuron level and hypothesized that the postsynaptic membrane is in a state of "latent hyperexcitability."

Rasmussen et al. (1990) have also demonstrated that chronic morphine leads to an upregulation in the G protein/cAMP system in the locus coeruleus *in vitro* and demonstrated that this upregulation is inhibited *in vivo* by chronic morphine administration. Specifically, they demonstrated increased G protein levels, adenylyl cyclase activity and cAMP-dependent protein kinase activity in isolated locus coeruleus subcellular fractions. These biochemical parameters correlate well with behavioral and electrophysiological parameters of withdrawal.

It is possible, then, that these compensatory responses underlie the mechanism of tolerance- and/or dependence-associated sensitization or hyperalgesia. In fact, there are several biochemical changes that occur in both tolerance-associated sensitization and injury-evoked central sensitization.

Correlates of morphine tolerance and injury-evoked central sensitization

Sensitization is defined as: 1) a decrease in the threshold of nociceptors for activation such that they now respond to non-noxious stimuli; 2) an increase in the magnitude of response of neurons to suprathreshold (noxious) stimuli; and 3) an increase in spontaneous activity of neurons. Central sensitization is manifest as a prolonged decrease in threshold and expansion of the receptive field size of dorsal horn neurons secondary to changes in the spinal cord. Woolf (1983) demonstrated a central component of post-injury pain hypersensitivity in his study of the flexion reflex in decerebrate rats after a thermal injury to the foot. Electrical recordings from the nerve to the biceps femoris demonstrated an increase in spontaneous activity, decrease in mechanical threshold for evoking responses from the ipsilateral and contralateral receptive fields, and an increase in response amplitude to standard pinch. This suggests that after tissue injury, increased excitability of the flexion reflex is due, at least in part, to changes in the activity of spinal cord neurons.

Importantly, Woolf and Thompson (1991) have shown that the induction and maintenance of central sensitization by peripheral afferent nerve fibers is dependent on N-methyl-D-aspartate (NMDA) receptor activation. NMDA controls a cation channel which is highly permeable to calcium, sodium and potassium. With the entry of calcium through these channels, calcium-dependent second messenger cascades are activated. These, in turn, trigger biochemical changes that contribute to certain forms of long-lasting synaptic modification. Like central sensitization, the hyperalgesia that results from intense stimulation and in neuropathic-like pain states produced by nerve injury can be blocked by NMDA antagonists (Coderre and

Melzack 1991; Davar et al. 1991; Mao et al. 1992a; Tal and Bennett 1993; Yamamoto and Yaksh 1992). Like these forms of neural plasticity, morphine tolerance and dependence are also blocked by NMDA antagonists (Ben et al. 1992; Marek et al., 1991a, 1991b; Tanganelli et al. 1991; Trujillo and Akil 1991).

NMDA receptor activation is believed to contribute to neuronal plasticity by a calcium-mediated activation of protein kinase C (PKC) (Alkon and Rasmussen 1988; Nishizuka 1989) and production of nitric oxide (NO) (Bredt and Snyder 1992). PKC, in fact, has been implicated in spinal cord mechanisms of hyperalgesia (Coderre 1992; Hayes et al. 1992; Kawamoto and Hidaka 1984; Mao et al. 1992b, 1994) as well as the development of tolerance (Mayer et al. 1995). Moreover the same central loci (spinal cord laminae I and II) are believed to be where translocation and activation of PKC occurs in mediating these phenomena (Mao et al. 1992b; Mayer et al. 1995). NO is also involved in hyperalgesia (Malmberg and Yaksh 1993; Meller et al. 1992) and the development of morphine tolerance (Elliott et al. 1994; Kolesnikov et al. 1993). Possibly the changes in PKC activation and NO production may enhance post-synaptic excitability by altering the efficacy of receptor-ion channel complexes (Chen and Huang 1992; Numann et al. 1991; West et al. 1991) leading to central hyperactive states (hyperalgesia or morphine tolerance). These data suggest that similar biochemical mechanisms, i.e. NMDA receptor-mediated translocation and activation of PKC and NO production, underlie the development of hyperalgesia and morphine tolerance.

Central sensitization is due to a long-term change in postsynaptic neurons that results in greater response to a subsequent stimulus, as well as increased spontaneous activity and lowered threshold to peripheral inputs (Woolf et al. 1983). In the case of morphine tolerance and dependence, it is unknown if

the postsynaptic expression of neural plasticity is induced by presynaptic or postsynaptic changes. It is of interest, however, that we have recently demonstrated that the NMDAR1 receptor, the essential subunit of the receptor, is located both pre- and postsynaptically in the spinal cord dorsal horn of the rat (Liu et al. 1991). This raised the possibility that the NMDA receptor is an autoreceptor which, when acted upon by glutamate released from primary afferents, could significantly enhance and prolong transmitter release from the primary afferent. Thus, it is reasonable to hypothesize that the tolerance-induced changes originate in the primary afferent and that noxious stimuli evoke greater responses because there is greater release of neurotransmitter from primary afferents. However, the role of substance P and glutamate release during tolerance and withdrawal is controversial. A more complete understanding of which subpopulations of neurons are at the foundation of the neural changes that occur during the development of morphine tolerance is warranted. Furthermore, an understanding of how the system interacts to influence the changes that may occur at the cellular level is warranted.

Cellular vs systems effects on tolerance and withdrawal

In addition to the controversies surrounding the mechanism of the development of opioid tolerance/dependence, there are also controversies as to whether or not tolerance and dependence are a cellular phenomenon or a systems phenomenon. To some extent, these arguments are based, not on different experimental results, but primarily on interpretation of the results. In other words, often the same data lead different authors to come to different conclusions.

There is evidence, for example, that tolerance and dependence occur at the cellular level. Sharma et al. (1975) demonstrated tolerance and dependence in NG108 cells and Fry et al. (1980) and Aghajanian (1978) have demonstrated dependence at the single neuron level. North and Karras (1978) demonstrated that guinea pig ileum becomes tolerant and dependent after prolonged exposure of myenteric neurons to opioids *in vitro*. The myenteric plexus of guinea pig ileum becomes tolerant to the inhibition of morphine even under conditions of synaptic blockade, and also becomes hyperactive with antagonists. The dependence persists even with the ganglionic blocking drug hexamethonium, which suggests that dependence arises from changes in the post-ganglionic region, where opioid sensitive neurons are located. Furthermore, the response to naloxone increases with the duration of exposure to opioids and opioid concentration, which suggests that the opioid-sensitive neuron is the site of neuronal plasticity. Finally, since the amount of naloxone necessary to precipitate withdrawal in the whole animal is very low, it is presumed that naloxone is acting only at opioid receptors on these neurons.

Williams and North (1984) however, proposed that tolerance results from a collective response and multicellular interactions. For example, in order to make guinea pig ilea tolerant, tolerance must be established in the whole animal. The same is true for the mouse vas deferens; tolerance must develop in the whole animal and then the tolerant organs can be isolated (Wuster et al. 1982). Also, although *in vivo* locus coeruleus firing rates increase after withdrawal, withdrawal-induced activation of the locus coeruleus is *not* evident in isolated slice preparations (Andrade et al. 1983; Christie et al. 1987b). It was later demonstrated that the locus coeruleus needs afferent

connections from the nucleus paragigantocellularis (Rasmussen and Aghajanian 1989) to reveal withdrawal-induced increased firing.

Roerig et al. (1991) demonstrated that tolerance may develop in the central nervous system without the development of tolerance at a given single site. They showed that the concurrent administration of morphine supraspinally and spinally produced a multiplicative interaction when analgesia was tested by the tail-flick test in control mice. However, in morphine-tolerant mice, this multiplicative interaction decreased to an additive interaction. It appears that the decrease in the multiplicative interaction allows tolerance to develop from systemic interactions without the necessity of tolerance at a specific site spinally or supraspinally. Thus, morphine may continue to have an effect at the cellular level even though analgesic tolerance exists in the "whole" animal.

Do morphine tolerance and morphine dependence develop together?

Theoretically, a cellular site of drug dependence is in line with the consideration that tolerance and dependence occur in a unitary manner. Collier (1980) suggested that morphine tolerance is accompanied by morphine dependence but the extent of the decrease in response to opioids (tolerance) may be less than the extent of the increased response to antagonists (dependence). However, in some organ preparations, tolerance and dependence appear to be dissociated from each other. For example, in the *in vitro* locus coeruleus preparation, one can demonstrate tolerance, but not dependence (Christie et al. 1987a, 1987b); this suggests that dependence, but not tolerance requires systems or synaptic interactions. Similarly, Wuster et al. (1982) demonstrated increased tolerance to opioid administration *in vivo* but not withdrawal with naloxone in isolated mouse

vas deferens. It has been proposed that the dissociated mouse vas deferens has no network of interconnecting interneurons, which may be necessary for the expression of dependence. Importantly, the isolated guinea pig ileum shows tolerance and dependence, and has an extensive neuronal network (Schulz 1978). However, careful analysis (Schulz and Wuster 1984) shows that tolerance and dependence do not appear in parallel. That the intensity of the withdrawal contracture reaches maximum levels at a low degree of tolerance suggests that two different states are involved in the development of tolerance and dependence.

This review illustrates that two different conclusions can be reached from the same data. It is difficult to ascertain whether or not tolerance and dependence occur together. By definition, to measure tolerance, one needs to administer the drug and to measure dependence one needs to take the drug away. Thus the two cannot be measured in the same preparation at the same time. Furthermore, the parameters to establish tolerance (a shift in the dose- response curve) are different from the parameters to measure dependence (behavioral indices) so the degree of tolerance may be different from the degree of dependence. This has been interpreted to mean that tolerance and dependence either occur together or are dissociated.

Use of Fos to monitor neuronal activity

c-fos is an immediate early gene that is activated by extracellular stimuli. *c-fos* mRNA levels increase within minutes after synaptic activation of a secondary neuron by neurotransmitter release from a primary afferent neuron. After transcription and translation of the gene, the Fos protein is translocated back into the nucleus of the activated neurons and can be identified by immunocytochemistry. Although basal expression of Fos in the

spinal cord is very low (Presley et al. 1990), it increases markedly in dorsal horn neurons with noxious stimulation (Hunt et al. 1987) and thus can be used to reveal large populations of presumed nociresponsive neurons, with resolution at the single cell level. While noxious stimulation in the rat hindpaw evokes increases in Fos in the ipsilateral lumbar cord, particularly in lamina I and outer II, acute morphine decreases its expression (Presley et al. 1990). It should be noted, however that the use of Fos to mark neuronal activity has its limitations. Some neurons, although stimulated, do not show Fos induction (Hunt et al. 1987; Morgan et al. 1987).

Hayward et al. (1990) demonstrated that *c-fos* is induced in the locus coeruleus during opioid withdrawal. Rats that were withdrawing from morphine had increases in *c-fos* mRNA levels and Fos protein levels in the locus coeruleus, as compared to rats given acute or chronic morphine, without precipitating abstinence. The increase in Fos expression correlated with the activity of neurons in the locus coeruleus, which increases during withdrawal (Aghajanian 1978; Christie et al. 1987b). Similar results were seen in the amygdala, ventral tegmentum, nucleus accumbens, neostriatum, and cerebral cortex, but not in several other regions in the brain. Thus, *c-fos* can be used to detect neuronal activity during acute morphine administration, chronic morphine administration, and during abstinence.

The present study is prompted by reports that withdrawal from morphine is associated with hyperalgesia in animals (Yaksh et al. 1977) and by the possibility that the compensatory response observed in tolerant animals contributes to the hyperalgesia observed with central sensitization. Since opioids have direct actions on the spinal cord (Yaksh and Rudy 1976), we hypothesize that dependence on opioids and the ensuing withdrawal upon

removal of morphine is mediated by changes in the spinal cord. Previous work in our laboratory has established a correlation between acute administration of opiates, pain behavior, and *c-fos* expression in the spinal cord. It is possible that these same neurons compensate for the effects of chronic morphine. The present series of studies examined the organization of spinal cord neurons involved in the development of tolerance and dependence as well as the expression of withdrawal. It also examined possible sources of input to the spinal cord that may modulate the neuronal responses of spinal cord neurons. These data may shed light on whether or not morphine tolerance/dependence is a cellular or systems phenomenon.

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Chapter 2

The development of opioid tolerance in the formalin test in the rat

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Abstract

The formalin test produces persistent pain in animals and is believed to better model the pain experienced by humans than do tests that measure reflex nociceptive thresholds. The present study evaluated whether tolerance to morphine develops in the formalin test in the rat. Morphine (75 mg) or placebo pellets were implanted subcutaneously for five consecutive days. On the sixth day, a subcutaneous dose of either morphine (10 mg/kg) or saline was given thirty minutes prior to the injection of 50 μ l of 5% formalin into the left hindpaw of the rats. In placebo-pelleted rats administered saline, formalin evoked characteristic pain behavior consisting of licking, biting and flinching of the affected hindpaw. The pain behavior in morphine tolerant rats given morphine did not differ significantly from the saline control; i.e., tolerance to the analgesic effect of morphine was demonstrable in the formalin test. These results do not agree with previous published reports. Rather, these results suggest that the mechanisms involved in morphine analgesia in the formalin test and in reflex nociceptive tests are similar and subject to the same problem of tolerance with chronic opioid administration.

Introduction

The formalin test was introduced in 1977 (Dubuisson and Dennis 1977), and has continued to gain popularity as a model of tonic inflammatory pain. The pain produced in the formalin tests differs significantly from that of acute nociceptive tests. Specifically, formalin creates a tonic pain secondary to tissue injury, inflammation and central sensitization. As a result, pain behavior in the formalin test has been postulated to better reflect the pain commonly experienced by humans.

Morphine is antinociceptive in both acute reflex nociceptive tests and in the formalin test (Abbott et al. 1982a,b; Coderre et al. 1993). On the other hand, although repeated administration of morphine produces a tolerance to its antinociceptive effects in reflex nociceptive tests (Stevens and Yaksh 1989; Kissin et al. 1991; Paronis and Holtzman 1992), Abbott et al. (1981) reported that rats do not become tolerant to the analgesic effects of systemic morphine in the formalin test. Thus, in rats that received daily injections of morphine for 7 days, these authors found that, although a subsequent acute injection of morphine no longer increased reflex nociceptive thresholds, it was still able to suppress the pain behavior recorded in the formalin test. The authors proposed that morphine acts at different sites in the CNS to produce analgesia in the formalin and reflex nociceptive tests. Whereas morphine has been found to activate descending inhibitory systems to produce antinociception in reflex nociceptive tests (Basbaum and Fields 1984), they postulated that the analgesia produced by morphine in the formalin test is entirely supraspinal. Recent studies in rats have provided support for this hypothesis (Matthies and Franklin 1992).

In the course of studies that evaluated noxious stimulation evoked expression of the *c-fos* proto-oncogene in opioid-tolerant rats, we found evidence that rats, in fact, do become tolerant to morphine in the formalin test. Because the issue of tolerance in the morphine test has been a key finding upon which investigators have postulated that morphine works via a different mechanism in reflex nociceptive tests and the formalin test, and because of the clinical implications of tolerance not occurring in tonic pain conditions, we have re-examined this question. a preliminary report of these studies has appeared previously (Rohde et al. 1993).

Methods

The experiments received approval from the Institutional Care and Animal Use Committee at the University of California at San Francisco. Twelve adult, male Sprague-Dawley rats (300 g) were randomly assigned to three groups, with four rats in each group. Animals were housed in groups of three under a standard 12h light-dark cycle with food and water available ad libitum. Each animal was used only once. the first group was implanted for five consecutive days with 75 mg morphine pellets, kindly supplied by the National Institute on Drug Abuse (NIDA). The other two groups received vehicle pellets in the same paradigm. Implantation of the pellets was accomplished through a small, 1.0 cm incision in the skin over the dorsum of the rat using halothane (Fluothane™) anesthesia. The skin was closed with silk sutures and the pellets were never removed.

On the 6th day, the morphine group and one vehicle pellet group received a subcutaneous injection of 10 mg/kg of morphine (Sigma™), a dose that we have previously demonstrated suppresses the pain behavior produced in the

formalin test (Presley et al. 1990; Jasmin et al. 1994). The other vehicle group received a s.c. injection of saline. Thirty minutes after morphine or saline, we injected the plantar surface of the left hindpaw with 50 μ l 5% formalin, using a 30 gauge needle, while manually restraining the animals. Pain behavior was quantitated by an observer blinded to the treatment. We counted the number of flinches per 5 min period for a total of 60 min as recently described (Wheeler et al. 1990; Wheeler and Cowan 1991). A flinch was recorded when the paw was rapidly withdrawn causing a shudder or rippling movement across the back. Counting of flinches began immediately after injection. The number of flinches between the groups was compared using a repeated measures one way ANOVA followed by Newman-Keuls post hoc test. A $P < 0.05$ was considered statistically significant.

Results

Placebo-pelleted rats given s.c. saline prior to the injection of the formalin displayed pain behavior that included licking, biting and flinching of the affected hindpaw. Quantitation of the number of flinches revealed the previously described first phase followed by a quiescent period and then resumption of spontaneous pain behavior during the second phase (Fig. 1). Acute morphine (10 mg/kg) administration in vehicle-pelleted rats almost completely abolished flinching and other pain behaviors normally produced by the formalin test. This was true for both the first and second phases. Conversely, acute administration of 10 mg/kg of morphine in the morphine-pelleted rats was without effect on pain behavior. The pain behavior displayed by rats in this group was not significantly different from that recorded in the vehicle group that received subcutaneous saline. This

behavior was, however, significantly different from the vehicle-pelleted rats that received acute morphine ($p < 0.05$). Although the average number of flinches in the morphine tolerant group given morphine was slightly less on average than the vehicle group given saline, this difference was not statistically significant.

Discussion

Our results demonstrate that rats do become tolerant to the analgesic effect of morphine in the formalin test. Although we did not assess the magnitude of the tolerance by determining rightward shift in the ED₅₀, the fact that 10 mg/kg, an ~ED₉₅ in normal rats was without antinociceptive effect, argues strongly that a rightward shift, i.e. tolerance had indeed occurred. Our results do not support the suggestion that the mechanisms of opiate analgesia in reflex nociceptive tests and in the formalin test are different.

Several factors may account for the differences between the present results and those of previous studies. First, Abbott and colleagues (1981) injected formalin into the dorsal aspect of the forepaw of the rat. Since plantar injections of formalin into the hindpaw produce an inflammation that makes weight bearing more uncomfortable than do injections of the forepaw, the rat spends more time with the paw elevated and is more likely to withdraw the paw quickly when weight bearing is attempted. This could result in a higher pain score that might be more difficult to suppress by morphine. By contrast, morphine may maintain its analgesic efficacy when the noxious stimulus is applied to the dorsal aspect of the paw. Since the intrinsic activity of opioids has been shown to vary with the intensity of the

noxious stimulus (Millan 1989), it is possible that the intrinsic activity of morphine in the formalin test varies with the locus of injection of formalin.

For similar reasons, the different results may be explained by the differences in dose and concentration of formalin that were used in the different studies. Abbott et al. (1981) used 30 μ l of 2.5% formalin; we used 50 μ l of 5.0% formalin. Although a smaller volume and concentration of formalin may be sufficient to provoke pain behavior, this behavior may be suppressed by lower doses of morphine. Conceivably, the dose of acute morphine chosen by Abbott et al. was much greater than what is necessary to suppress pain behavior, even in tolerant animals. By either 1) increasing the intensity of the noxious stimulus, with an increased volume or concentration of formalin or 2) by using a smaller dose of acute morphine, the previous studies might have been able to detect a rightward shift in the dose-response curve. In fact, a follow up study from this group (Abbott et al. 1982a) concluded that the dose-response curve in their initial study was incomplete. In the second study they used doses of acute morphine from 0.5-32 mg/kg and demonstrated a 2.75 fold shift in the dose response curve in the formalin test in morphine-tolerant animals. Although this shift was actually larger than the 1.8 fold rightward shift observed in the tail-flick test in the same study, the authors stated that this small shift is consistent with their previous failure to detect any tolerance in the formalin test. They concluded that to demonstrate tolerance, "it is necessary to administer high doses of morphine chronically but challenge with low doses." Clearly, our challenge dose of 10 mg/kg is much greater than the 0.5- 4 mg/kg with which they found some tolerance, yet we were still able to show tolerance.

A third confounding variable is that the measured behaviors used in our study were different from those used in the other studies. We scored

behavior in the formalin test by counting the number of flinches; Abbott et al. (1981) measured the duration of time that the forepaw was elevated, with the nails barely touching the floor. Their analysis did *not* include time spent either flinching, licking or biting the paw, because of their impression that chronic morphine alone produces persistent chewing and licking parts of the rats' bodies. Despite this concern, quantitative controls to assess the effect of chronic morphine alone on pain behavior were not presented (Abbott et al., 1981). We suggest that the biting and licking that they observed may have represented pain behavior, which might explain why they were unable to show a difference between the chronic morphine and saline groups. In fact, the increased licking or flinching of the paw most likely occurred at the expense of time spent in a paw lift position; the more time that an animal licks or flinches its hindpaw, the less time it spends with its paw elevated. This might artifactually decrease the pain score.

Although the method that we used to quantitate pain behavior also differs from the original scoring system of Dubuisson and Dennis (1977), Wheeler-Aceto and Cowan and colleagues (1990, 1991) demonstrated that flinches alone were as predictable as the more complex scoring system in determining the antinociception produced by various pharmacologic interventions. Furthermore, even in the original scoring system, flinching and licking of the affected paw received the highest score. Since our results demonstrate such dramatically different results between the tolerant and non-tolerant animals, while measuring a behavior that receives the highest score in the traditionally scored formalin test, it is likely that a more complex scoring system would have only served to further substantiate tolerance to morphine in our study.

There, of course, is a very different interpretation to the failure of morphine to produce analgesia. Specifically, there is recent evidence that chronic administration of morphine can lead to a hyperalgesic state (Mao et al. 1994). A rightward shift in the morphine dose-response curve could thus result from a combination of tolerance development and the fact that noxious stimuli evoke more 'pain' in these animals. We, in fact, have found that the pattern of formalin-evoked fos expression is increased in rats that had daily morphine pellet implantation compared to placebo-pelleted rats. However, we did *not* find a parallel increase in the pain behavior evoked by the formalin stimulus in the morphine-pelleted rats. This suggests that the loss of morphine efficacy in the formalin test that we report in the present study is indeed due to the development of tolerance.

Based in part on the absence of tolerance to morphine in the formalin test, Abbott et al. (1981) have argued that morphine produces antinociception by a different mechanism in the formalin test than in reflex nociceptive tests. It is well established that morphine produces antinociception in reflex nociceptive tests by activating descending inhibitory pathways that travel in the dorsolateral funiculus (DLF) (Basbaum and Fields 1978; Barton et al. 1980). However, studies that have tried to determine whether descending antinociceptive controls are involved in morphine antinociception in the formalin test have met with mixed results. Ryan et al. (1985) reported that DLF lesions did not affect the antinociception produced by systemic morphine in the formalin test and Abbott et al. found that lesions of the medullary raphe nuclei disrupted morphine analgesia only in the tail flick but *not* in the formalin test (Abbott and Melzack 1982; Abbott et al. 1982b). The authors concluded that morphine, whether given systemically or supraspinally, does not block pain of the formalin test by inhibiting spinal

cord nociresponsive neurons. Based on these results, they hypothesized that different neural substrates mediate the morphine blockade of pain in phasic (e.g. tail flick) and tonic (e.g. formalin) pain models and that only the former neural substrate shows tolerance to morphine.

In contrast to these studies, we demonstrated that systemic morphine, in fact, decreases spinal cord fos expression in the formalin test (Presley et al. 1990), which suggests that the inhibition of spinal cord nociresponsive neurons contributes to morphine analgesia in the formalin test. Furthermore, we found that DLF lesions blocked both the decrease in fos expression and the antinociceptive effects produced by i.c.v. DAMGO, a mu-opioid agonist, in the formalin test (Gogas et al. 1991). Taken together with a recent study in mice that also documented tolerance to morphine in the formalin test (Connell et al. 1994), these studies provide strong evidence that descending pathways are, in fact, involved in morphine antinociception in the formalin test and that tolerance to morphine in the formalin test results from loss of these descending inhibitory controls. To what extent other supraspinal sites (e.g. amygdala) that may *not* activate descending pathways are also involved, and how they integrate with controls that are exerted at the spinal cord level need to be studied.

In conclusion, our results indicate that the pain induced by the subcutaneous injection of formalin is subject to the same problems of tolerance to morphine as are the reflex nociceptive tests. Taken together with studies demonstrating that opioids block pain in the formalin test by activating descending controls, these results do not support the hypothesis that tonic pain evoked in the formalin test is regulated differently from pain produced in reflex nociceptive tests.

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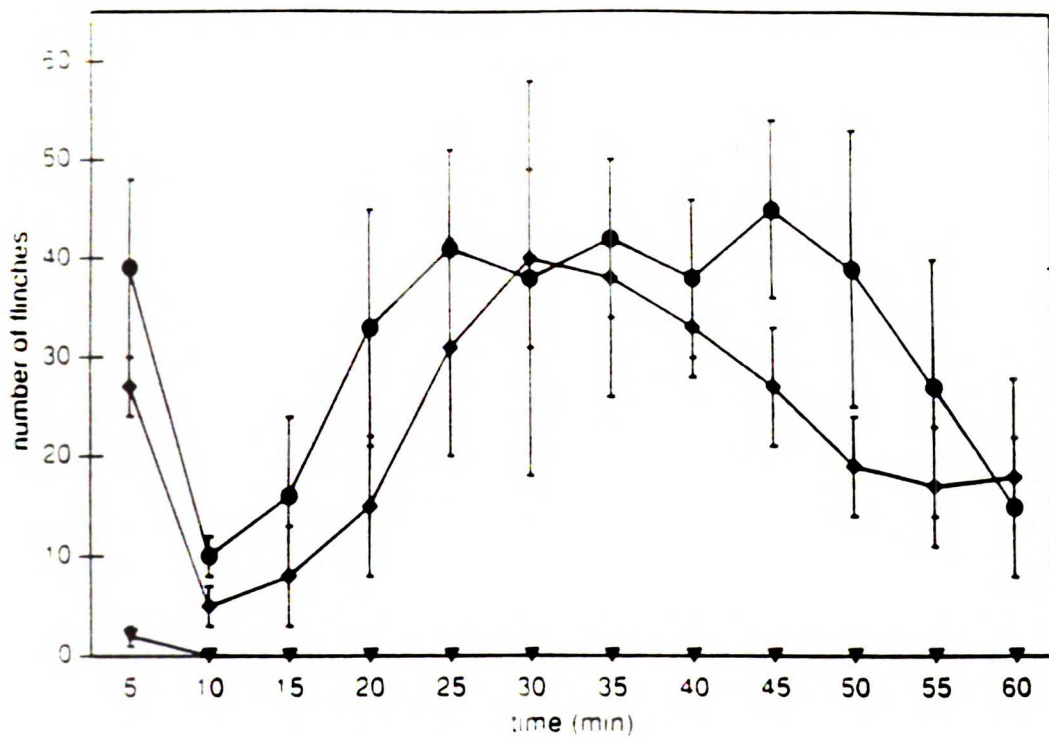
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Figure 1: This figure illustrates the number of flinches per five minute block after hindpaw injection of formalin for each of the three groups. The acute morphine group (placebo-pelleted rats administered morphine; triangles) differ from the control group (placebo-pelleted rats administered saline; diamonds) and the chronic morphine group (morphine-pelleted rats administered morphine; circles). The latter two groups do not differ from each other indicating that tolerance to the effects of morphine had occurred ($p < 0.05$ compared by a one way ANOVA with Newman-Keuls post hoc tests).



Chapter 3

Spinal cord mechanisms of opioid tolerance and dependence: Fos expression increases in subpopulations of spinal cord neurons during withdrawal

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Abstract

Tolerance to the analgesic effects of morphine results in part from the development of a compensatory response in neurons that express the opioid receptor or of neural circuits in which those neurons participate. According to this formulation, withdrawal from morphine results in an overshoot of several neuronal properties because of the unopposed action of the compensatory response system. To identify the population of spinal cord neurons that underlies this state, we monitored expression of fos-like-immunoreactivity, after naltrexone-precipitated abstinence in normal and morphine-tolerant rats. After daily (five days) implantation of morphine or placebo pellets, the rats received an injection of saline or naltrexone and behavior was monitored for one hour. The rats were then killed, their spinal cords removed and 50 μm transverse sections of the lumbar cord were immunostained with a rabbit polyclonal antiserum directed against fos.

Naltrexone injection in the placebo group did not increase spinal cord fos expression. Naltrexone-precipitated abstinence resulted in an increase in fos expression at all levels of the spinal cord; the greatest increase and densest staining was in laminae I through VI. Importantly, when withdrawal was precipitated in anesthetized rats, we recorded a significant reduction in fos expression, particularly in laminae III through VI, but there was persistent expression in the superficial dorsal horn, particularly in lamina I.

These results suggest that spinal cord nociresponsive neurons are sensitized during the development of tolerance. This sensitization is unmasked by the administration of naltrexone and is manifested by fos induction in laminae I/II in awake or anesthetized withdrawing animals.

Introduction

Several hypotheses have been proposed to explain the development of tolerance and dependence that results from chronic morphine administration. One view holds that tolerance results from decreased coupling of the opioid receptor to an inhibitory G protein (Christie et al. 1987a, 1987b; Cox 1991; Smith et al. 1988, Werling et al. 1989) that regulates many downstream effects, including inhibition of adenylyl cyclase (Sharma et al. 1975) and increased potassium conductance (North et al. 1987). The hypothesis that the basis of tolerance lies proximal to the signalling cascade, i.e. in the coupling of the opioid receptor to the G protein, however, cannot account for the induction of the acute withdrawal/abstinence syndrome that is produced by injection of an opiate antagonist, such as naltrexone, into morphine-tolerant animals. With the exception of a recent report indicating that opiate antagonists can develop negative intrinsic activity (Wang et al 1994), it is generally held that naltrexone exerts no effect in the absence of opiate agonists. As a pure antagonist, it can only displace morphine from the opioid receptor. Consistent with its lack of intrinsic activity, naltrexone injection in morphine-naive rats, regardless of dose, does not evoke a withdrawal syndrome. Rather, these observations suggest that the state of tolerance is one in which morphine remains functionally bound to an opioid receptor; not only does morphine function in the tolerant state, but it also "prevents" the induction of a withdrawal syndrome.

To account for the rebound phenomena that are observed when naltrexone is injected into morphine-tolerant animals, an alternative hypothesis has been proposed, namely that tolerance to morphine results from the development of a compensatory response in neurons that express

the opioid receptor, or of neural circuits in which those neurons participate (Nester and Tallman 1988; Nestler et al. 1992; Sharma et al. 1975). In the tolerant state, the opioid receptor remains functionally coupled to the inhibitory G proteins, and the compensatory responses balance the inhibitory effects of morphine. Withdrawal from morphine or precipitated abstinence results in an overshoot of several neuronal properties because of the unopposed action of the compensatory response system. For example, there is a dramatic increase in activity of adenylyl cyclase, an increase in cAMP (Duman et al. 1988, Nestler and Tallman 1988; Sharma et al. 1975) and an increase of cAMP-dependent protein kinase activity, (Nestler and Tallman 1988). The spontaneous firing of neurons, notably in cerebral cortex, (Fry et al. 1980) locus coeruleus (LC) (Aghajanian 1978) and spinal cord (Johnson and Duggan 1981) is also significantly increased during withdrawal. There is also a significant and relatively localized induction of the immediate early gene, *c-fos*, in LC neurons (Hayward et al. 1990). Conceivably, many features of the withdrawal syndrome, including the increased spontaneous activity of LC neurons and increased *c-fos* induction in LC neurons are related to the increased levels of cAMP that are produced. In fact, Collier et al. (1981) demonstrated that phosphodiesterase inhibitors, which increase cAMP levels, produce a morphine withdrawal-like behavior in rats.

Fry et al. (1980) aptly referred to the tolerant state as one of latent sensitization; the sensitization is only manifested when the opiate antagonist is injected. Latent sensitization in the tolerant animal has features in common with the sensitization of spinal cord neurons that occurs after prolonged or intense noxious stimulation. Specifically, noxious stimulation at C-fiber intensities results in long term changes in the firing of dorsal horn neurons, including decreased threshold for firing, increased

spontaneous activity and enlarged peripheral receptive fields (Dubner and Ruda 1992; Hylden et al. 1989). These neuronal changes may, in fact, contribute to the behavioral hypersensitivity and irritability that is produced when an opiate antagonist is administered to an animal whose spinal cord is made tolerant by continuous intrathecal injection of morphine (Yaksh et al. 1977). The parallel between the tolerant- and pain-associated sensitized state is further demonstrated by studies that found that N-methyl-D-aspartate (NMDA) receptor mechanisms contribute both to the development of tolerance to and dependence on morphine (Ben et al. 1992; Elliott et al. 1994; Trujillo and Akil 1991) and to the central sensitization of dorsal horn neurons produced by C-fiber stimulation (Dickenson and Sullivan 1987; Woolf and Thompson 1991).

The objective of the present study was to pursue the relationship between pain-evoked and tolerance-associated sensitization. In particular, we examined the locus of tolerance-associated changes in spinal cord neurons. Our studies were prompted by those of Hayward et al. (1990) which demonstrated a relatively restricted induction of fos expression in the LC when withdrawal was precipitated in morphine-tolerant rats. In the present study we made rats tolerant to morphine and then monitored the expression of the Fos protein in the spinal cord after withdrawal was precipitated. We report that the predominant changes occur in the superficial laminae of the spinal cord dorsal horn. A preliminary report of these studies has been published (Rohde et al. 1993).

Methods

Experiments were performed on male Sprague-Dawley rats (260-340gm; Bantin and Kingman, Fremont, CA), with the approval of the Institutional Animal Care and Use Committee at UCSF. Efforts were made to minimize the numbers of animals used and to minimize animal discomfort. The rats were housed two in a cage with free access to food and water. The rats were randomly assigned into six different treatment groups. Three of the groups were rendered tolerant to morphine by implantation of morphine pellets. The experimental groups of rats were administered either (1) morphine pellets followed by naltrexone injection (MN) (n=7) or (2) morphine pellets followed by naltrexone injection while under Halothane anesthesia for the one hour withdrawal period (MHN) (n=4). The control groups of rats were administered either (1) placebo pellets followed by saline injection (PS) (n=3) or (2) morphine pellets followed by saline injection (MS) (n=5) or (3) placebo pellets followed by naltrexone injection (PN) (n=3) or (4) placebo pellets followed by naltrexone injection while under Halothane anesthesia for one hour (PHN) (n=4).

Pellets were implanted subcutaneously (75 mg morphine sulfate or placebo pellet kindly provided by National Institute on Drug Abuse, Rockville, MD), at the rate of one per day for five days, under light Halothane anesthesia. The five day morphine pellet protocol leads to the development of morphine tolerance as determined by electrophysiological (Aghajanian 1978) and behavioral (Beitner et al. 1989; Hayward et al. 1990) criteria. Additionally, we have demonstrated that all of the morphine-pelleted rats displayed tolerance in the formalin test (5% formalin, 50 μ l injected into the plantar hindpaw). That is, the behavioral response of morphine-pelleted rats

to formalin after an acute dose of morphine (10 mg/kg) was similar to the response of placebo-pelleted animals given an acute injection of saline, followed by the formalin injection. Naïve animals given an acute dose of morphine (10 mg/kg) showed significant analgesia in the formalin test (Detweiler et al. 1995).

The pellets were not removed at any time during the study. On day 6, naltrexone (10 mg/kg or 100 mg/kg) diluted in 800 μ l physiological saline, or saline alone, were injected subcutaneously in awake or Halothane-anesthetized rats. We chose a dose of 100 mg/kg naltrexone to follow the protocol used in the studies of Hayward et al. (1990) Since this dose is much greater than what is required to precipitate abstinence, in 3 of the 7 morphine-pelleted animals, we precipitated abstinence by injection of 10 mg/kg naltrexone. There was no significant difference between the fos expression at these doses, and thus we combined the results from these two groups.

The animals were monitored for signs of withdrawal after the injection on day 6. After one hour, the rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) followed immediately by intracardiac perfusion through the ascending aorta with 100 ml 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 300 - 400 ml 4% paraformaldehyde fixative in 0.1 M phosphate buffer (PB).

Two hours after perfusion, the lumbar spinal cord was removed and postfixed in 4% paraformaldehyde for an additional two hours and then cryoprotected for 40 hours in 30% sucrose in 0.1 M PB. Fifty micron frozen sections were cut in the transverse plane and collected in 0.05 M PBS for immunocytochemical analysis. The sections were then immunostained for the Fos protein by the avidin-biotin-peroxidase method of Hsu et al. (1981).

The sections were washed with a solution of 0.05 M PBS with 1% normal goat serum and 0.3% Triton-X and then incubated for 1 hr at room temperature in a blocking solution of 3% normal goat serum in 0.05 M PBS with 0.3% Triton-X. The blocking solution was aspirated from the tissue, and the sections were incubated for 40 hours at room temperature in the primary antiserum, which is a rabbit polyclonal antiserum directed against an *in vitro* translated product of *c-fos* (kindly provided by Dr. D. Slamon, UCLA). The antiserum was diluted 1:21,000 and preabsorbed against acetone-dried liver powder for 1 hr at 37°C and for 1 hr at 4°C prior to use. After the primary antiserum, the sections were washed and then incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Elite Kit; Vector Labs, Burlingame, CA). To visualize the *fos*-like immunoreactivity as a black reaction product we used a nickel-diaminobenzidine (DAB) glucose-oxidase reaction following a protocol adapted from Llewellyn-Smith and Minson (1992).

To quantitate the numbers of *fos*-like-immunoreactive (FLI) neurons, the L4/5 sections were first examined under dark-field illumination to identify the appropriate segmental level. We then took photographs of the selected sections under light-field illumination at low (4X) power with Kodak technical pan film on a Nikon Microphot-FXA microscope. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. Individual sections of the spinal cord were then printed at 60X enlargement. The photographs were next divided into four segments: (i) the superficial laminae (laminae I, IIo, IIi), (ii) the nucleus proprius (laminae III and IV), (iii) the neck of the dorsal horn (laminae V and VI) and (iv) the ventral cord (laminae VII, VIII, IX, and X). FLI neurons were identified by a person blinded to the treatments. Up to six

spinal cord sections per rat were examined and the number of FLI cells in each of these regions was counted and averaged so that each animal had a mean value for regional FLI. The numbers of labeled cells per 50 μ m section were compared using 2-way analysis of variance (ANOVA) for treatment and laminae and within laminae one-way ANOVA's for treatment. Fisher's Protected Least Significant Difference was used for *post hoc* comparisons.

Results

Signs of withdrawal

Neither the placebo-pelleted groups that received naltrexone, nor the morphine-pelleted group that received saline demonstrated any behavioral signs of withdrawal. By contrast, the awake morphine pelleted group that received naltrexone displayed clear signs of withdrawal that included increase in respiration, ptosis, diarrhea, jumping, burrowing in the sawdust, teeth chattering, salivation, weight loss, and marked generalized hyperactivity.

Fos expression

The placebo-saline, morphine-saline, and placebo-naltrexone groups showed only low levels of fos expression in awake animals (Figs. 1a, 2, 3; Table 1). Since the three control groups were not statistically different from one another, they were grouped together as the awake control group (Fig. 1a). By contrast, we recorded a significant increase in fos expression in the morphine-pelleted group that received naltrexone (MN), i.e. in withdrawing animals, when compared to the control groups (Figs. 1a, 2, 3; Table 1). This increase was significant in laminae I through VI. The most striking

increase in fos expression (181%) in withdrawing vs. control animals occurred in laminae I/II. We also recorded a 79% increase in laminae III/IV and a 94% increase in laminae V/VI. Although we recorded a 62% increase in the ventral horn of withdrawing animals as compared to the control groups, this was not statistically significant.

To determine whether the increase in FLI in the withdrawing animals was due to the increase in motor activity that accompanies withdrawal, we repeated the experiments under Halothane anesthesia. Rats remained anesthetized during the naltrexone injection and for the one hour withdrawal period. The placebo-pelleted group that received naltrexone under Halothane anesthesia showed a large reduction in fos expression (45% in laminae I/II, 65% in laminae III/IV, 71% in laminae V/VI, and 31% in the ventral horn) when compared to the awake control groups, suggesting that basal fos expression in the awake control animals is due, in part, to normal locomotor activity (Figs. 1b, 2, 3; Table 1). Halothane also caused a significant and widespread decrease in fos expression in the anesthetized withdrawing group (MHN) when compared to the awake withdrawing group (MN): 52% in laminae I/II, 81% in laminae III/IV and 84% in laminae V/VI (Fig. 1b, Table 1). Although we recorded a decreased fos expression (41%) in the ventral horn, this was not statistically significant (Fig. 1b, Table 1). In the anesthetized withdrawal group (MHN), fos expression was reduced to the extent that there were no statistical differences between this group and the control anesthetized group (PHN) in laminae III/IV, V/VI, or the ventral horn. On the other hand, there was still a statistically significant *increase* in fos expression in the superficial dorsal horn (146%) in the anesthetized withdrawing group (MHN) when compared to the anesthetized control group (PHN) (Figs. 1b, 2, 3; Table 1). Indeed, although the absolute number

of FLI neurons in laminae I/II of awake withdrawing animals was less than in laminae III/IV, this reversed in anesthetized withdrawing animals; the absolute numbers of FLI neurons in laminae I/II was greater than in laminae III/IV (Fig. 1b). Although our analysis did not distinguish lamina I from lamina II, the residual fos expression in the anesthetized withdrawing animals was largely confined to lamina I (Fig. 3).

Although we only analyzed the data for the L4/5 level, we recorded comparable changes in fos expression throughout the cord. Figure 3 illustrates fos expression at L2 in the various groups. As at the L4/5 segment, there are low levels of basal fos in the control rats (PN), increased fos expression in the awake withdrawal animals (MN) and decreased fos expression in the deeper dorsal horn of rats that withdrew under Halothane (MHN), with preservation of fos expression in the superficial laminae.

Discussion

The present study demonstrates that large populations of spinal neurons are modified when rats are made tolerant to morphine. Naltrexone-precipitated abstinence resulted in an increase in fos expression at all levels of the spinal cord; the greatest increase was in laminae I through VI. Importantly, when withdrawal was precipitated in anesthetized rats, we recorded a significant reduction in fos expression, particularly in laminae III-VI, and persistent expression in the superficial dorsal horn. In some cases, the residual fos expression in these rats was restricted to lamina I. These results suggest that the labeling in laminae III and IV, which contain neurons that respond to non-noxious stimuli, is secondary to the increased afferent input associated with withdrawal behavior. In contrast, we suggest

that the fos expression in the superficial dorsal horn is a naltrexone-precipitated manifestation of a compensatory response that occurs in these neurons, or in the input to these neurons, as a result of chronic exposure to morphine.

Basal fos expression

In our earlier studies, we found that basal fos expression in awake, unstimulated rats was restricted to laminae III and IV, presumably because of inputs associated with movement (Jasmin et al. 1994; Presley et al. 1990). In the present study we found basal expression in neurons throughout the spinal gray matter. Three factors probably contributed to these differences. First, in the Presley et al (1990) study, we used an antiserum from an earlier bleed of the same rabbit, the titer of which was much lower than that of the bleed used in this study. Second, in the present study we used the Vector Elite kit, rather than the standard kit, which produces significantly greater labeling, with lower background. Third, our use of a nickel-enhanced DAB as the chromogen provided greater sensitivity. Importantly, the basal labeling that we recorded in this study was typically light, suggesting a low level of fos expression. Since the density of labeling in the rats that withdrew from morphine was significantly greater than that recorded in the different groups of control rats, none of which differed significantly from one another, we believe that the distribution of fos expression that we have described is indicative of increased neuronal activity, a conclusion consistent with electrophysiological studies that have found increased spontaneous activity of several neuronal groups during withdrawal from morphine (Aghajanian 1978). We and others have previously addressed the controversy concerning the relationship between fos expression and

neuronal activity (Basbaum 1994; Gogas et al. 1991; Hunt et al. 1987; Jasmin et al. 1994; Zimmerman and Herdegen 1994).

Withdrawal-induced fos expression

The intense motor behavior that occurs during precipitated abstinence almost certainly is associated with significantly increased activity of large diameter primary afferent fibers from skin, muscle and joints. In a previous report, we demonstrated that fos is selectively increased in neurons of laminae III, IV, medial V and VI in rats that walked for one hour on a rotating rod (Jasmin et al. 1994). That pattern differs considerably from what is seen after noxious stimulation. Specifically, the latter is associated with significantly increased labeling in superficial laminae (Bullitt 1990; Hunt et al. 1987; Presley et al. 1990). Since neurons in laminae III and IV respond to non-noxious stimulation, we believe that the large (81%) decrease in fos expression in laminae III and IV in rats that withdrew under anesthesia resulted from decreased large diameter, primary afferent input. Since neurons in lamina VI are predominantly activated by joint afferent input, the comparable (84%) decrease in fos expression in this region in anesthetized, withdrawing rats, was also expected. On the other hand, neurons of lamina V receive both small and large diameter inputs; thus a decrease in fos expression in lamina V was not readily predicted. Had we been able to reliably distinguish the borders of laminae V and VI, we may have been able to differentiate an effect of anesthetic on fos expression in these two regions. In other words, it is possible that "activity" would have persisted in laminae V, as it did in lamina I and II in the rats that withdrew under Halothane.

Despite the uncertainty about the factors that contribute to fos expression in lamina V, our results indicate that the increased fos expression in the superficial dorsal horn of tolerant rats involves factors other than, or in addition to, inputs associated with withdrawal behavior. This conclusion follows from analysis of the effects of anesthetic on fos expression in *both* experimental and control groups of rats. Specifically, although Halothane decreased fos expression in the deeper dorsal horn (laminae III through VI) by about 68% in the anesthetized control (PHN) vs. the awake control groups (PS, MS, PN) and by 82% in the anesthetized withdrawal group (MHN) vs. the awake withdrawal group (MN), we only recorded an approximate 52% decrease of fos expression in laminae I and II in the anesthetized withdrawal (MHN) vs. the awake withdrawal (MN) groups. That is, the number of FLI neurons in the superficial dorsal horn of awake or anesthetized withdrawal animals was still significantly (more than two-fold) greater than it was in awake or anesthetized placebo animals. Based on these results, we conclude that the long term effects of exposure to morphine, as manifested by fos expression during withdrawal, are most pronounced in neurons of the superficial dorsal horn.

As described above, in many sections we noted that the fos expression was concentrated in lamina I. This was particularly true in tissue from rats that withdrew under anesthesia. Since many neurons in lamina I are at the origin of major ascending projection pathways (Hylden et al 1986; Jasmin et al. 1994), it follows that some of increased "activity" that occurs in spinal neurons during withdrawal is transmitted to supraspinal sites. This raises the possibility that the withdrawal-induced fos expression that has been reported previously, for example in the LC (Hayward et al. 1990), is mediated, in part, by changes that originate at the spinal level. Furthermore, since

neurons of lamina I transmit information that could lead to the perception of pain, it is possible that the pain associated with withdrawal (see below) involves changes at the level of the spinal cord.

What factors contribute to the induction of fos expression in the superficial dorsal horn during withdrawal from morphine?

Based on *in vitro* studies that demonstrated an upregulation of basal adenylyl cyclase activity in cells made tolerant to morphine (Makman et al. 1988; Sharma et al. 1975), and a significant increase beyond basal when tolerant cells are "withdrawn" with naloxone (Sharma et al. 1975), it has been proposed that tolerance results from the development of a compensatory response in neurons that express the opioid receptor, i.e., in neurons that are chronically exposed to morphine (Nestler 1992). The increased fos expression during withdrawal is presumed to be a manifestation of this compensatory response.

That morphine analgesia and tolerance can be induced spinally (Yaksh et al. 1977; Yaksh and Rudy 1976) supports the notion that local spinal elements in the superficial dorsal horn are the locus of the compensatory response. Two major neuronal elements in the spinal cord express opioid receptors. There are presynaptic receptors on the terminals of small diameter primary afferents that arborize in the superficial dorsal horn and there are postsynaptic receptors on interneurons and projection neurons throughout the spinal gray matter (Duggan et al. 1976; LaMotte et al. 1976; Mudge et al. 1979; Yaksh et al. 1980; Zimmerman and Herdegen, 1994). Since Halothane anesthesia blocked almost all fos expression, except that in the superficial dorsal horn, we suggest that what is unique to the superficial dorsal horn may be critical to the induction of fos in this region during withdrawal.

Specifically, the peptide-containing small diameter primary afferents, which express opioid receptors (Atweh and Kuhar 1977; Gamse et al. 1979; LaMotte et al. 1976), and which terminate almost exclusively in the superficial dorsal horn (DeBiasi and Rustioni 1988; Sugiura et al. 1986), may be the locus of the compensatory response that develops with chronic exposure to opioids.

To test this hypothesis we are studying the effect of eliminating the C-fiber population, by treatment with neonatal capsaicin, on the pattern of fos expression induced by withdrawal in the adult. In related studies we are evaluating the effect of dorsal rhizotomy. Finally, we are directly addressing the question of the location of the opioid receptor, by staining for the fos protein during withdrawal and then labeling the tissue for opioid receptor message with *in situ* hybridization. If fos is induced in neurons that express the receptor, it would suggest that the compensatory changes also occur in the postsynaptic neurons that "experience" the chronic morphine. It is, of course, possible that the increased fos expression occurs in neurons that are downstream of the opioid receptor.

Significance of fos expression in laminae I/II: central sensitization of superficial dorsal horn neurons during the development of morphine tolerance

The fact that the focus of the increased fos expression during withdrawal occurred in the superficial dorsal horn, taken together with the evidence that increased pain responsiveness (hyperalgesia) occurs during withdrawal (Bederson et al. 1990; Wilcox et al. 1979; Yaksh et al. 1977), suggests that common elements underlie these two phenomena. Hyperalgesia results in part from noxious stimulation-evoked long-term changes (central sensitization) of dorsal horn neurons. Central sensitization is manifested at

the cellular level as a decreased threshold of firing of the spinal cord nociceptive neuron, increased receptive field size and increased spontaneous activity (Woolf, 1983). At the behavioral level, the stimulus threshold for evoking pain behavior decreases and noxious stimuli evoke exaggerated pain responses. Conceivably, changes comparable to those that underlie the compensatory response (i.e. latent sensitization) (Fry et al. 1980) that develops during chronic exposure to opioids contribute to the central sensitization produced by noxious stimuli. Interestingly, not only is the latent sensitization manifested as an increase in fos expression during withdrawal, but hyperalgesia commonly develops (Bederson et al. 1990; Wilcox et al. 1979; Yaksh et al. 1977). Importantly, blockade of the NMDA receptor blocks *both* the noxious stimulus-evoked central sensitization (Woolf and Thompson, 1991) and the development of tolerance at the spinal cord level (Ben et al. 1992; Elliott et al. 1994; Mao et al. 1994; Trujillo and Akil, 1991). We recently demonstrated that the NMDA receptor is located on the terminals of small diameter primary afferents in the superficial dorsal horn (Liu et al. 1994). It is, thus, conceivable that central sensitization and the manifestations of withdrawal are blocked because the compensatory response that develops in the primary afferent involves a glutamate action on an NMDA presynaptic autoreceptor. This could result in enhanced release of primary afferent neurotransmitter when subsequent noxious stimuli are presented or during naltrexone-precipitated withdrawal, both of which would increase the expression of fos in dorsal horn neurons. Consistent with this formulation, we have recently demonstrated that the spinal cord distribution of fos expression produced by hindpaw injection of formalin in the rat is significantly increased in rats that are tolerant to morphine (Rohde et al. 1993). In this case, the latent sensitization is manifested as

enhanced fos expression in response to a noxious stimulus. Taken together these results support the hypothesis that central sensitization in response to noxious stimuli has features in common with the sensitization that occurs with chronic exposure to morphine. This is further supported in studies by Mao et al. (1994), which demonstrate that thermal hyperalgesia develops in association with the development of morphine tolerance and that co-administration of NMDA antagonists with morphine prevents the development of both hyperalgesia and tolerance.

It is also possible that the opioid receptor-bearing neurons located supraspinally are the primary site of tolerance-induced compensatory changes and that withdrawal-induced activity in these regions secondarily activates neurons in the superficial dorsal horn. Significant changes at supraspinal sites have, in fact, been described. For example, Aghajanian (1978) has shown that the activity of LC neurons, which express opioid receptors (Moyse et al. 1986), is increased during withdrawal. Furthermore, Nestler and colleagues demonstrated that tolerance to morphine alters the properties of LC neurons, including increased adenylyl cyclase activity (Duman et al. 1988), cAMP and cAMP-dependent protein kinase activity (Nestler and Tallman 1988), and of *c-fos* mRNA and Fos protein during withdrawal (Hayward et al. 1990). Although increased activity in LC neurons could drive the increased fos expression in neurons of the superficial dorsal horn, most studies indicate that the LC exerts an inhibitory control in normal animals (Hodge et al. 1983). This would tend to suppress, rather than increase activity of spinal neurons. In other unpublished studies, we found an *increase* in fos expression in laminae I and II in spinalized withdrawing rats compared to intact withdrawing rats. Conceivably this results from loss of the increased LC inhibitory activity that would occur during withdrawal.

An alternative supraspinal source is the rostral ventral medulla (RVM). Fields and colleagues characterized several classes of neurons in this region, including the so-called "on" cells which are activated by noxious stimulation (Fields et al. 1983) and inhibited by morphine (Barbaro et al. 1986). Other evidence suggests that the activity of on-cells facilitates the firing of spinal cord neurons; during periods of on-cell activity the latency for eliciting spinal nociceptive reflexes is decreased (Heinricher et al. 1989). Furthermore, an overshoot of on-cell activity is produced when an acute injection of morphine is followed by naloxone (i.e., withdrawal is precipitated even after a single injection of the antagonist); concurrently, tail flick latencies decreased (Bederson et al. 1990; Kaplan and Fields 1991). Since some on-cells project to the dorsal horn (Vanegas et al. 1984), it is conceivable that increased firing of on-cells during withdrawal contributes to the increased fos expression in the superficial dorsal horn. If this were the case, however, one would expect that spinalized rats would show decreased fos expression in the spinal cord during withdrawal when compared to intact withdrawing animals. By microinjecting naloxone at supraspinal sites, we are evaluating the contribution of these loci to the pattern of spinal cord FLI that we observed.

Summary

There are many steps in the development of opioid tolerance that remain to be explained. In this report, we provide evidence that the focus of changes in the spinal cord that occur during tolerance, and which are manifested during withdrawal, involve a relatively limited number of neurons and occur in a spatially restricted region, namely the superficial dorsal horn. This makes the analysis of the factors that induce and maintain

changes more amenable to study. To understand the mechanism of "activation" of dorsal horn neurons during withdrawal, we have initiated studies that address the contribution of peripheral input, descending input, as well as intrinsic spinal cord circuitry. Of particular interest is the contribution of the NMDA receptor to the changes that occur during withdrawal. It is likely that a better understanding of the mechanisms underlying the reorganization that takes place during the development of tolerance will contribute to our understanding of the general principles of plasticity that operate under other conditions, including those that contribute to the hyperalgesic states that follow injury.

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Table 1. Distribution of fos-immunoreactive neurons in different treatment conditions

Placebo/Saline (n=3)	21.6 +/- 5.5	44.3 +/- 9.7	39.0 +/- 14.2	28.3 +/- 5.8
Placebo/Naltrex (n=3)	25.3 +/- 7.0	49.0 +/- 3.6	44.0 +/- 6.5	32.3 +/- 3.2
Morphine/Saline (n=5)	21.6 +/- 4.9	49.8 +/- 12.5	41.8 +/- 8.5	51.2 +/- 14.6
Morphine/Naltrex (n=7)	63.4 +/- 6.2 ****	86.0 +/- 6.4 **	80.8 +/- 8.2 **	64.5 +/- 10.0
Morphine/Naltrex under halo (n=4)	30.7 +/- 3.9 *	16.3 +/- 3.8	12.8 +/- 3.0	38.3 +/- 9.2
Placebo/Naltrex under halo (n=4)	12.5 +/- .6	16.8 +/- 3.8	12.0 +/- 5.5	27.3 +/- 4.8

Numbers (+/-SEM) of Fos-immunoreactive neurons per 50 μ m section in different laminae of the L4/5 spinal cord. With the exception of the ventral horn, naltrexone-precipitated withdrawal in awake rats was associated with a significant increase in fos expression in all laminae compared to control awake animals (placebo-saline, placebo-naltrexone, and morphine-saline). Naltrexone-precipitated withdrawal in Halothane-anesthetized rats was only associated with a significant increase in fos expression in laminae I/II compared to control anesthetized rats.

(*p < .05, **p < .01, ****p < .0001)

Figure 1. Numbers of Fos-immunoreactive neurons per 50 μ m section in each laminae of the spinal cord A: after naltrexone-precipitated withdrawal. There is a significant increase in fos expression in laminae I-VI in withdrawing rats (morphine/naltrexone) compared to control animals (morphine/saline, placebo/saline and placebo/naltrexone) and B: after naltrexone-precipitated withdrawal under Halothane anesthesia. There is a significant increase in fos expression in laminae I-VI in awake, withdrawing rats (morphine/naltrexone) compared to anesthetized animals that withdrew under Halothane (morphine/halo/naltrexone). Halothane decreased the expression of fos in deeper laminae; there were no longer significant differences between withdrawal and control (placebo/halo/naltrexone) rats. However, there was still a significant increase in fos expression in laminae I/II of rats that withdrew under anesthesia compared to control, anesthetized rats. (* $p < .05$, *** $p < .001$, **** $p < .0001$)

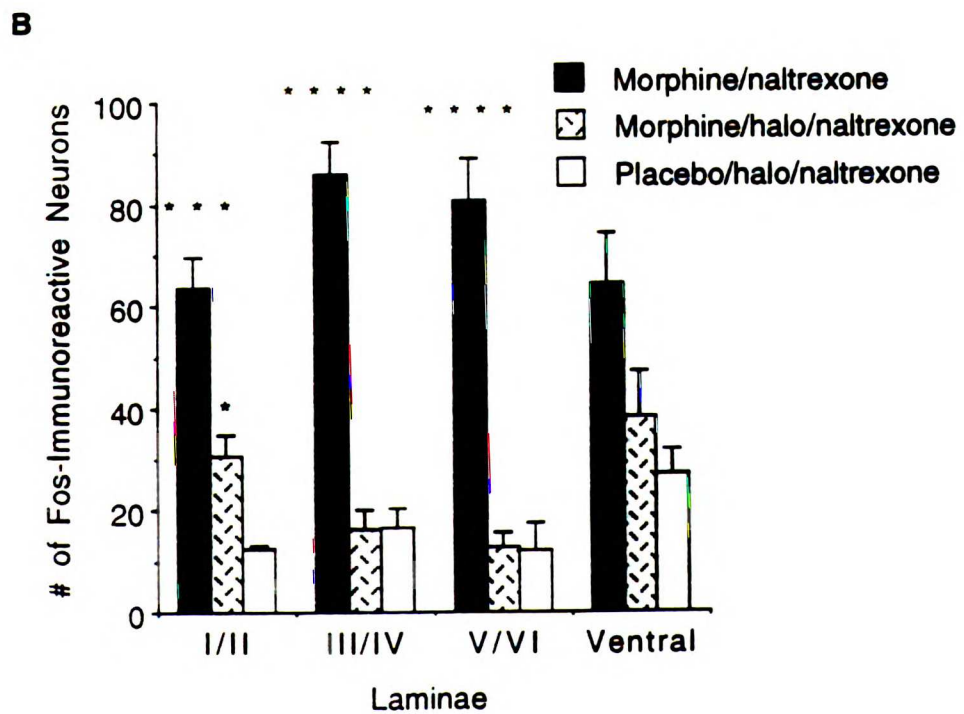
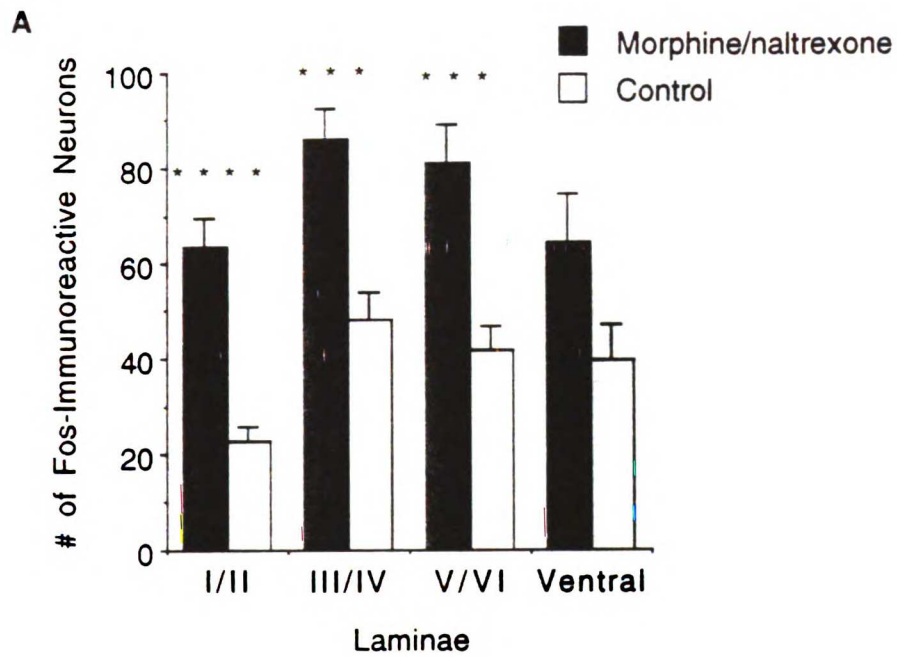


Figure 2. These photomicrographs illustrate the distribution of fos-like-immunoreactive neurons in the L4/5 segments of the spinal cord in three groups of control rats: A: placebo-pelleted rats that received saline injection; B: morphine-pelleted rats that received saline; and C: placebo-pelleted rats that received naltrexone under anesthesia. There is very low fos expression in all three groups. Arrowheads point to fos-like-immunoreactive neurons in laminae III/IV. Calibration bar equals 250 μ m in A,B,C.

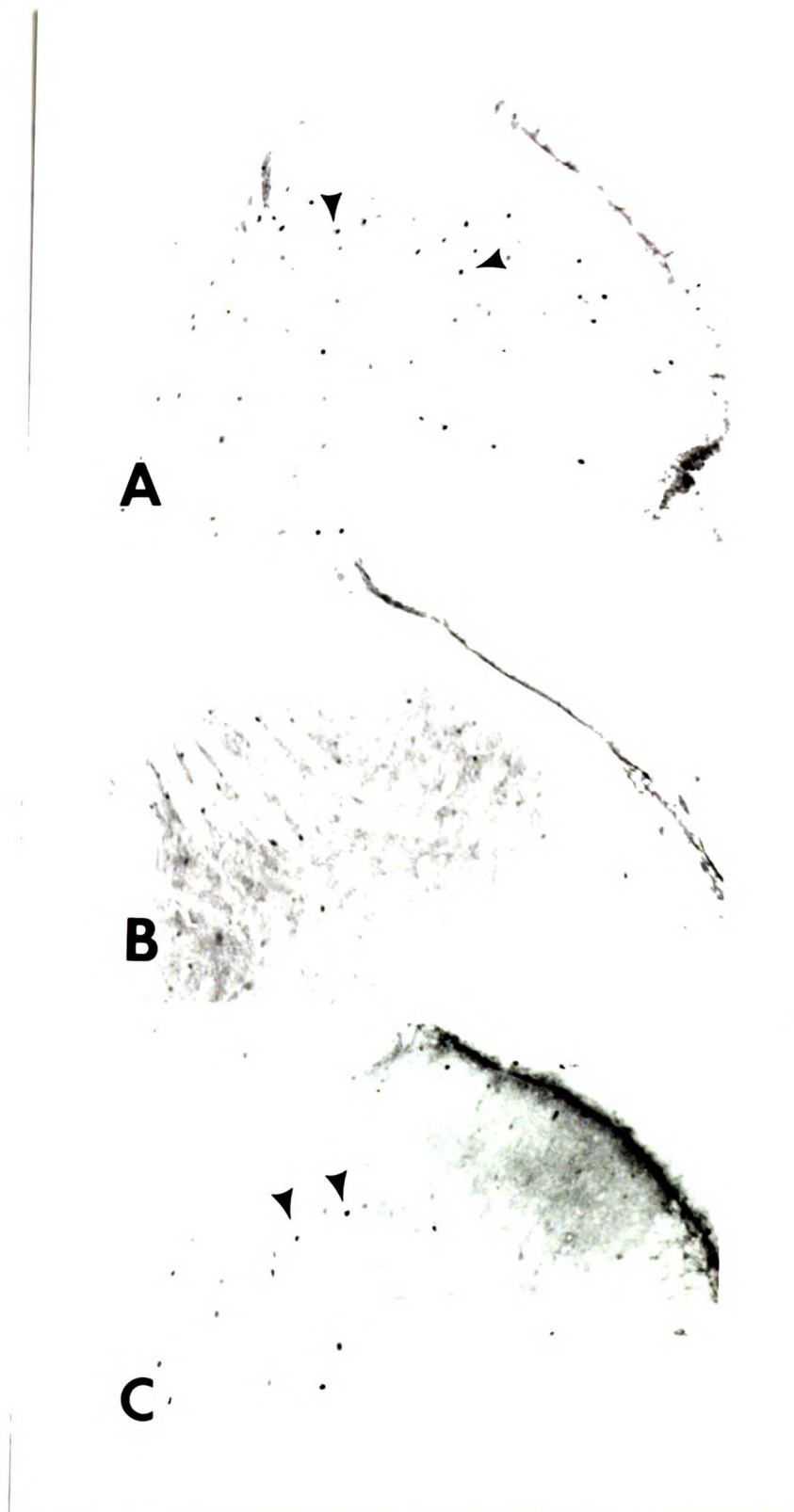
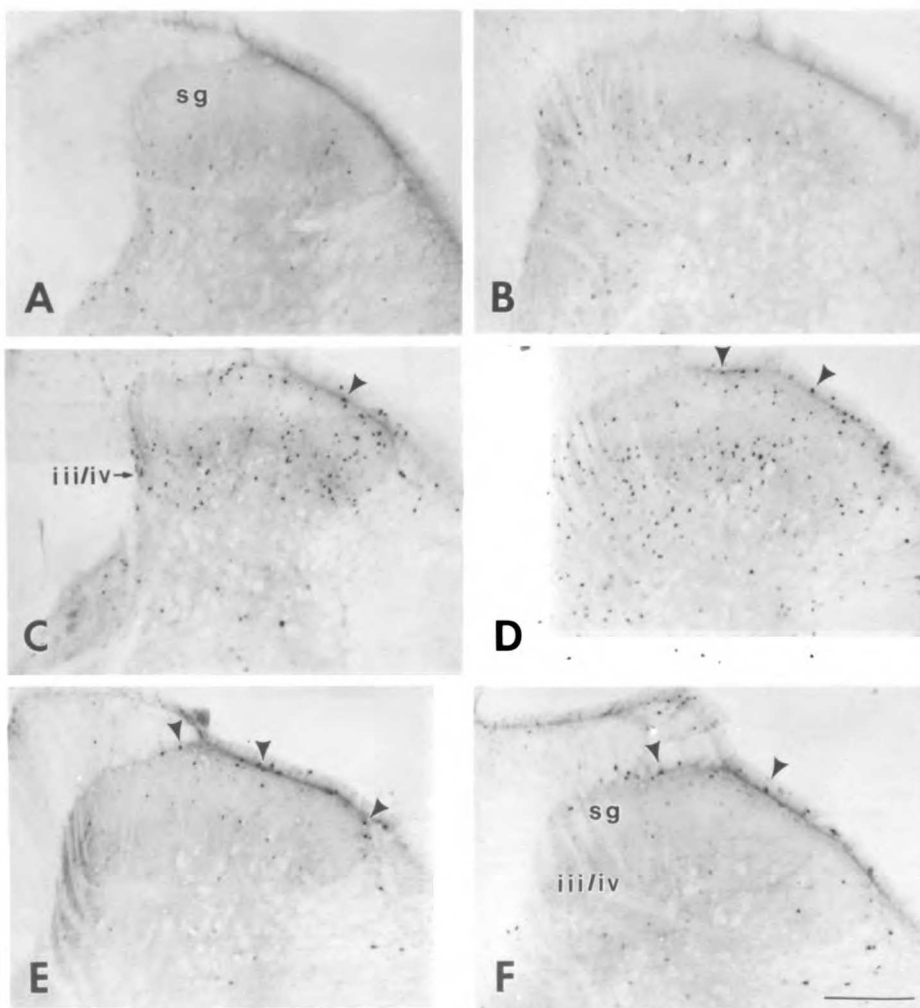


Figure 3. These photomicrographs illustrate the distribution of fos-like-immunoreactive neurons in the L2 (A,C,E) and L4/5 (B,D,F) segment of the spinal cord from different groups of rats. A and B: placebo-pelleted rats that received naltrexone; C and D: morphine-pelleted rats that received naltrexone; E and F: morphine-pelleted rats that received naltrexone but were anesthetized for the one hour withdrawal period. The pattern of fos expression at all spinal segments is comparable within treatment groups. There is very low basal expression of fos in the placebo group (A,B). When withdrawal occurs in the awake rat (C,D), we recorded a large number of densely labeled neurons throughout the dorsal horn. When withdrawal occurred under anesthesia, there was a significant (80%) reduction in the number of fos-like-immunoreactive neurons in laminae III/IV. However, significant fos expression persisted in the superficial laminae, notably in lamina I (E,F). Arrowheads point to fos-like-immunoreactive neurons in lamina I. sg: substantia gelatinosa. iii/iv: laminae III/IV.

Calibration bar equals 250 μ m in A-F.



Chapter 4

Morphine Tolerance Enhances Nociceptive Processing in Rat Lumbar Spinal Cord

Dana S. Rohde, Dawn J. Detweiler and Allan I. Basbaum

Abstract

It has been hypothesized that tolerance to the analgesic effects of morphine results from the development of a compensatory response in neurons that express the opioid receptor or in neural circuits in which those neurons participate. The compensatory response establishes a sensitized state in these neurons. According to this hypothesis, withdrawal from morphine produces an abstinence syndrome and increases neuronal firing because the sensitized state is unmasked. We have previously demonstrated increased Fos-like immunoreactivity, a marker of neuronal activity, in superficial dorsal horn neurons of the lumbar spinal cord after naltrexone-precipitated withdrawal in morphine-tolerant rats. To determine if administration of a noxious stimulus can similarly unmask a sensitization of dorsal horn neurons in morphine-pelleted rats, we injected morphine-tolerant and control rats with 100 μ l 5% formalin into the plantar surface of the left hindpaw and counted the number of flinches for one hour. The rats were then killed, their spinal cords removed and 50 μ m transverse sections of the lumbar cord were immunostained with a rabbit polyclonal antiserum directed against the Fos protein.

Formalin injection in the placebo-pelleted rats induced Fos-like immunoreactivity in the ipsilateral L4/5 segment. There was very little labelling on the contralateral side or in rostral (L2) segments. Although there was no significant difference in flinching behavior between the morphine-tolerant and control groups, we recorded significantly increased total Fos-like immunoreactivity at the L4/5 and L2 segments both ipsilateral and contralateral to the site of formalin injection in the morphine-tolerant

rats compared to the control rats. The percentage increases in Fos-like immunoreactivity for the morphine-tolerant vs. the control animals was greatest in spinal cord regions contralateral and rostral to those directly innervated by primary afferents from the hindpaw. These results suggest that lumbar spinal cord neurons are sensitized during the development of tolerance, that the sensitization can be unmasked by the administration of a noxious stimulus and that it is manifested as increased expression of the Fos protein in the lumbar cord. The mechanisms that underlie the development of tolerance to the analgesic action of morphine may thus be similar to those that underlie injury-induced central sensitization and hyperalgesia.

Introduction

Acute administration of opioids produces analgesia via activation of brainstem and spinal antinociceptive circuits that inhibit the firing of dorsal horn nociresponsive neurons (Basbaum and Fields 1978; Basbaum and Fields 1984; Guilbaud et al. 1973). Chronic administration of opioids produces a state of tolerance in which subsequent doses of opioids have a decreased antinociceptive effect; i.e. there is a rightward shift in the dose-response curve (Johnstone and Smith 1992). Studies at the cellular level indicate that the acute effects of opioids results from a G-protein mediated activation of K⁺ channels (Aghajanian and Wang 1986; North et al. 1987) that cause neuronal hyperpolarization. Morphine also reduces cAMP levels (Griffin et al. 1985; Makman et al. 1988; Sharma et al. 1975). Although there is evidence that tolerance results from a decoupling of the opioid receptor from an inhibitory G protein (Christie et al. 1987; Cox 1991; Werling et al. 1989), other studies suggest that it results, in part, from the development of a compensatory response in neurons that contain the opioid receptor and/or in the neural circuits that participate in antinociception (Nestler 1992, Nestler and Tallman 1988; Sharma et al. 1975). This compensatory response, which brings neuronal firing rates and cAMP levels to control levels, counteracts the inhibition produced by morphine, thereby diminishing its antinociceptive effect. Opioid tolerant neurons are said to be in a state of "latent hyperexcitability" (Fry et al. 1980), which is revealed only when the opioid is displaced from the opioid receptor leaving the upregulated cAMP pathway unopposed. This is manifested as an overshoot in neuronal firing and cAMP levels as well as symptoms of withdrawal in the intact animal.

Hayward et al. (1990) demonstrated increased Fos mRNA and protein in certain brain regions of the rat, particularly the locus coeruleus, after withdrawal from morphine. In a comparable study, we recently reported increased Fos-like-immunoreactivity (Fos-LI) in laminae I through VI of the spinal cord (Rohde et al. 1996). Moreover, when we precipitated withdrawal under halothane anesthesia, to block movement-induced increases in Fos-LI, there was still heightened Fos-LI in neurons of the superficial dorsal horn (lamina I/II). Fos-LI in laminae III/IV was reduced to control levels, which suggested that it was largely related to the movements associated with withdrawal.

Neurons of laminae I/II receive small diameter nociceptive primary afferent input, and most respond to noxious stimulation (Christensen and Perl 1970; Light and Perl 1979; Fitzgerald and Wall 1980). Furthermore, noxious stimulation evokes a long term hyperexcitable state in these neurons that is manifested as an increase in spontaneous activity, decrease in nociceptive threshold and increase in receptive field size (Cook et al. 1987; Hylden et al. 1989; Woolf 1983). The behavioral correlate of these changes is a persistent hyperalgesia and allodynia (Sluka et al. 1993; Meller et al. 1994; Woolf 1983). In many respects, therefore, the sensitization of superficial dorsal horn neurons that occurs in response to noxious stimulation is comparable to that which we believe occurs in the setting of morphine tolerance.

Consistent with this hypothesis, similar biochemical mechanisms underlie the neuronal changes that develop in the superficial dorsal horn after painful stimulation or after the administration of chronic morphine (Mao et al. 1992a; Mao et al. 1994; Mayer et al. 1995). For example, NMDA receptor antagonists block both noxious stimulus-evoked central sensitization (Woolf

and Thompson 1991) and the development of tolerance at the spinal cord level (Ben et al. 1992; Elliott, et al. 1994). Taken together, these data led to the hypothesis that the latent sensitization that characterizes the morphine-tolerant state can also be manifest as an increased activity of superficial dorsal horn neurons in response to a noxious stimulus.

To test this hypothesis, we injected formalin into the hindpaw of morphine-tolerant and control rats and then monitored behavior and spinal cord neuronal activity using Fos immunocytochemistry as a marker of the activity. We report that noxious stimulation produces significantly increased total Fos-LI in spinal cord neurons of morphine-tolerant animals compared to controls. Surprisingly, the increased Fos-LI was not only detected in regions of the cord that receive direct input from the injected paw, but also in distant sites, contralateral and rostral to the level of input from the noxious stimulus. A preliminary report of these studies has been published (Rohde et al. 1993).

Methods

Subjects

Experiments were performed on male Sprague-Dawley rats (240-260g.; Bantin and Kingman, Fremont, CA), with the approval of the Institutional Animal Care and Use Committee at UCSF. Animals were housed in groups of three under a standard 12h light-dark cycle with food and water available ad libitum.

Tolerance and formalin protocol

The rats were randomly assigned to two groups. Rats were implanted subcutaneously with 75 mg morphine sulfate (n = 9) or placebo (n = 8) pellets kindly provided by National Institute on Drug Abuse, Rockville, MD at the rate of one per day for five days, under light halothane anesthesia. This protocol leads to the development of morphine tolerance as determined by electrophysiological (Aghajanian 1978) and behavioral criteria (Detweiler et al. 1995; Hayward et al. 1990). On day six, each rat was injected with 100 μ l 5% formalin into the plantar surface of the left hindpaw. The number of flinches was recorded every five minutes for the next 60 minutes by an observer blinded to the treatment. A flinch was defined as a sudden withdrawal of the paw producing a rippling movement over the back (Wheeler-Aceto and Cowan 1991).

Fos immunocytochemistry

Sixty minutes after the formalin injection, the rats were deeply anesthetized with pentobarbital (100 mg/kg i.p.) and perfused transcardially with 200 ml of 0.05 M phosphate-buffered saline (PBS) followed by 300-400 ml of phosphate buffered 4% paraformaldehyde. Two hours after perfusion, the lumbar spinal cord was removed and postfixed in 4% paraformaldehyde for an additional two hours and then cryoprotected for 40 hours in 30% sucrose in 0.1 M phosphate buffer. Fifty micron frozen sections were cut in the transverse plane and collected in 0.05 M PBS for immunocytochemical analysis. The sections were then immunostained for the Fos protein by the avidin-biotin-peroxidase method of Hsu et al. (Hsu et al. 1981). Briefly, sections were washed with a solution of 0.05 M PBS with 1% normal goat

serum and 0.3% Triton-X and then incubated for 1 hr at room temperature in a blocking solution of 3% normal goat serum in 0.05 M PBS with 0.3% Triton-X. The blocking solution was aspirated from the tissue, and the sections were incubated for 40 hours at room temperature in the primary antiserum, which is a rabbit polyclonal antiserum directed against an in vitro translated product of *c-fos* (kindly provided by Dr. D. Slamon, UCLA). The antiserum was diluted 1:21,000 and preabsorbed against acetone-dried liver powder for 1 hr at 37°C and for 1 hr at 4°C prior to use. After the primary antiserum, the sections were washed and then incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Elite Kit; Vector Labs, Burlingame, CA). To visualize the Fos-LI as a black reaction product we used a nickel-diaminobenzidine glucose-oxidase reaction following a protocol adapted from Llewellyn-Smith and Minson (1992).

Data collection and analyses

To quantitate the numbers of Fos-LI neurons, sections through the lumbar spinal cord were first examined under dark-field illumination to identify the L2 and L4/5 segmental levels. We then took photographs of the selected sections under light-field illumination at low (4X) power with Kodak technical pan film and a Nikon Microphot-FXA microscope. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. Individual sections of the spinal cord were then printed at 60X enlargement. The photographs were next divided into four segments: (i) the superficial laminae (laminae I, Ilo, Ili), (ii) the nucleus proprius (laminae III and IV), (iii) the neck of the dorsal horn (laminae V and VI) and (iv) the ventral horn (laminae VII, VIII, IX, and X). Three to five spinal cord sections per rat were examined and the number of

Fos-LI cells in each were counted and averaged to generate a mean value for Fos-labeled cells for each animal.

Total Fos-LI (all laminae, ipsilateral plus contralateral sides) was first compared with a one-way ANOVA for treatment (morphine or placebo pellets). Since significant differences were detected, we next independently assessed changes in total Fos-LI on the ipsilateral or the contralateral sides with a one-way ANOVA for treatment. *Post-hoc* analyses (Fisher's Protected Least Significant Difference) for laminar differences were then performed. The L4/5 and L2 lumbar segments were analyzed independently in all ANOVAs. The number of flinches was compared using a one-way repeated measures ANOVA for the overall number of flinches.

Results

Placebo-pelleted rats

In a previous study we reported that the pelleting procedure itself does not alter the normally very low basal expression of Fos protein (Rohde, et al. 1996). Thus, the pattern of Fos-LI that we recorded in formalin-injected rats, with the exception of some lightly labelled neurons in laminae III and IV, can be attributed almost exclusively to the noxious stimulus. Consistent with this conclusion, the pattern of Fos-LI evoked by hindpaw formalin injection in placebo-pelleted rats was comparable to that which we have described in several previous studies (Gogas et al. 1996; Presley et al. 1990). Thus, the most concentrated labelling was found in the medial half of the ipsilateral superficial dorsal horn, laminae I and II. This region corresponds to the zone of termination of small diameter afferents from the rat hindpaw (Light and Perl 1979). There was additional labelling in regions that contain

neurons that respond to noxious stimulation, including laminae V, VII and VIII and the region around the central canal, lamina X. The increased Fos-LI in the ipsilateral laminae III and IV is presumed to result from the non-nociceptive inputs associated with the shaking of the injected hindpaw (Presley et al. 1990). As reported previously (Presley et al. 1990), the superficial dorsal horn labelling is concentrated in segments L4 and L5. Rostral and caudal to these segments, there is much less Fos-LI and it is located in the intermediate gray and in the ventral horn.

Morphine-pelleted rats

Total Fos-LI (all laminae, ipsilateral and contralateral sides) was significantly increased (48%) at the L4/5 segment in the formalin-injected morphine-pelleted rats compared to the placebo-pelleted rats (Figs. 1A, 3, 4). This was also true when we separately compared the ipsilateral and contralateral sides. Thus, there was a 40% increase on the ipsilateral side and a 70% increase on the contralateral side for total Fos-LI at the L4/5 segment in the morphine-tolerant rats compared to the placebo-pelleted rats (Figs. 1B, 3, 4).

Rostrally, at the L2 segment, we also recorded significant increases in total Fos-LI (64%) in the formalin-injected morphine-pelleted rats vs. the placebo-pelleted rats (Figs. 1A, 3, 5). Again, this increase was seen when the two sides were analyzed independently; there was a 53% increase on the ipsilateral side and a 78% increase on the contralateral side at the L2 segment (Figs. 1C, 5, 7).

Although the Fos-LI was increased in the morphine-pelleted rats vs. the placebo-pelleted rats in all laminae both ipsilateral and contralateral to the injected paw at both the L4/5 and L2 segments, the greatest increase in Fos-

LI was found in spinal cord regions that do not receive a major direct input from the hindpaw, namely regions contralateral and rostral to L4/5. Thus, we only recorded a 24% increase in Fos-LI in laminae I/II of the ipsilateral L4/5 lumbar segment, and this difference was not statistically significant. However, we recorded significantly increased Fos-LI in laminae V/VI (42%) and in the ventral horn (66%) (Figs. 2A, 3, 4) of the ipsilateral L4/5 segment. Contralateral to the injected paw, we also recorded significantly increased Fos-LI in laminae V/VI (83%) (Figs. 2B, 3, 4). In more rostral regions of the spinal cord, the L2 segment, we recorded significantly increased Fos-LI in the ipsilateral laminae III/IV (80%), V/VI (51%), and the ventral horn (51%) (Figs. 2C, 3, 5) as well as in the contralateral laminae V/VI (96%) and ventral horn (96%) (Figs. 2D, 3, 5) in the morphine-pelleted rats vs. the placebo-pelleted rats. Despite the significant increases in Fos-LI in the morphine-pelleted vs. the control rats, we did not record a statistically significant difference in the number of flinches between the two groups of animals in the formalin test (Fig. 6).

Discussion

The present study demonstrates that the pattern of spinal cord Fos-LI evoked by a noxious stimulus is significantly altered when the stimulus is presented to rats that are rendered tolerant to the analgesic effects of morphine. Not only did we record a difference in the number of Fos-labelled neurons in spinal segments that receive a direct small-diameter primary afferent input from the injected hindpaw (namely, the L4/5 segment), but there was also a significant increase in Fos-LI in segments of the cord contralateral and rostral (L2) to the level of afferent input. The increased

Fos-LI in the setting of morphine tolerance is opposite to what is produced by an acute injection of morphine in non-tolerant rats. Specifically, acute morphine blocks formalin-evoked pain behavior and decreases Fos-LI in all laminae of the cord (Gogas et al. 1996; Presley et al. 1990; Tölle et al. 1990). Since we have previously demonstrated that chronic treatment with morphine does not, by itself, increase Fos-LI (Rohde et al. 1996), we believe that the enhanced Fos-LI is a consequence of the noxious stimulus being presented in the setting of opioid tolerance.

Our study supports the view that tolerance results from the development of a compensatory response that establishes a sensitized state in neurons that express the opioid receptor, or in neural circuits of which that neuron is a component. The compensatory response counterbalances the inhibitory effects of the opiates, resulting in an apparent normalization of neuronal firing and cAMP levels. However, the normalization can be readily disrupted by administration of an opioid antagonist, such as naloxone, which removes the opioid regulation, increasing adenylyl cyclase activity (Duman et al. 1988) and cAMP levels (Sharma et al. 1975) in cells and producing a behavioral withdrawal syndrome in animals. We hypothesize that the expansion of Fos-LI, a marker of neuronal activity, in the spinal cord, ipsilateral, as well as contralateral and rostral to the level of input, is also a manifestation of a sensitized state that occurs in the spinal cord in the setting of opioid tolerance.

Since the enhanced Fos-LI was not restricted to the primary zone of termination of the small diameter primary afferent from the formalin-injected hindpaw, we cannot identify the locus of the hypothesized compensatory response. We do not believe that it corresponds to the entire population of neurons that express the Fos protein in response to noxious

stimulation in the tolerant rat. Rather, we hypothesize that there is a relatively discrete population of neurons that are sensitized by chronic opioid administration, in much the same way that a noxious stimulation sensitizes populations of dorsal horn neurons to subsequent stimuli. For example, Cook et al. (1987) reported that the receptive field properties of neurons in lamina I are significantly altered by a conditioning stimulus to C fibers; receptive fields expand, spontaneous activity increases and the threshold for firing of lamina I neurons decreases significantly. The same changes can be demonstrated in the setting of persistent inflammation (Ren et al. 1992). In the setting of tolerance the threshold for activation of these neurons by a noxious stimulus would be lowered and the magnitude of the response for a given stimulus would be enhanced, resulting in an expansion of the distribution of Fos-LI induced by formalin injection. Based on our previous studies which demonstrated that increased Fos-LI induced by naloxone in tolerant rats is concentrated in the superficial dorsal horn (in some cases exclusively in lamina I), we hypothesize that chronic morphine induces changes in neurons of lamina I and II and that these changes are at the basis of the enhanced Fos-LI produced by noxious stimulation.

Although we did not find a significant increase in Fos expression in laminae I/II of the morphine-tolerant rats, we believe that laminae I/II may still be a critical locus for the neuronal plasticity that occurs during the development of morphine tolerance. Importantly, in placebo-pelleted rats, formalin injection induces Fos expression in the majority of cells in laminae I/II. Thus, the failure to reach a statistically significant increase may have resulted from a ceiling effect for Fos expression being reached. Had we been able to measure changes in the density of staining of individual neurons, significant changes in laminae I/II may have been detected.

Although we previously recorded a very high correlation between the number of formalin-evoked neurons that express the Fos protein in the spinal cord and the magnitude of pain behavior (Gogas et al. 1991), in the present study, there was no significant difference in pain behavior between the placebo- and morphine-pelleted rats. There are several possible explanations for this result, which in light of other evidence demonstrating hyperalgesia in the setting of morphine tolerance (Mao et al. 1994; Sjogren et al. 1994), was unexpected. First, as for the failure to find increased Fos-LI in laminae I/II, we believe that a ceiling effect of behavior in response to 100 ul 5% formalin limited our ability to detect increased pain behavior. This is consistent with a recent study of Abbott et al. (1996), which evaluated the effect of dorsolateral funiculus lesions on formalin-evoked pain behavior. Second, it is possible that populations of neurons that do not contribute to pain behavior express the Fos protein in the tolerant rats. It is of interest in this regard that our preliminary double label studies reveal that few of the neurons that express Fos in withdrawing rats project to supraspinal sites. Since spinothalamic tract neurons can be sensitized by noxious stimulation (Dougherty and Willis 1991; Hylden et al. 1989; Schaible et al. 1987), it may be the case that the populations of dorsal horn neurons sensitized by noxious stimulation or chronic opioid administration are not completely overlapping. Finally, it is possible that tests of behavior that measure changes in sensitivity/hyperalgesia rather than overt pain behavior would reveal a behavioral correlate of the increased Fos expression. For example, changes in nociceptive thresholds in regions *outside* of the area injected with formalin, may correlate with the increased Fos expression observed at the L2 level and contralateral to the injection site.

The fact that the sensitization produced by continuous morphine can be manifest both by naloxone and by noxious stimulation is consistent with the evidence for a comparable pharmacology and biochemical profile underlying noxious stimulus-induced central sensitization and morphine tolerance. For example, NMDA and nitric oxide synthase antagonists block both noxious stimulus-induced central sensitization of dorsal horn neurons (and the associated hyperalgesia) as well as morphine tolerance, including that induced at the level of the spinal cord (Bredt and Snyder 1992; Kolesnikov et al. 1993; Malmberg and Yaksh 1993; Mao et al. 1992b; Trujillo and Akil 1991; Woolf and Thompson 1991). Furthermore, there is evidence that translocation and activation of PKC in lamina I and II contributes to hyperalgesia in the setting of morphine tolerance (Mao et al. 1994, Mayer et al. 1995), which supports the view that the locus of the compensatory response is in the superficial dorsal horn.

Since opioid receptors are located on the terminals of small-diameter primary afferents and on projection neurons and interneurons of the superficial dorsal horn (Atweh and Kuhar 1977; LaMotte et al. 1976), the primary afferent and/or the postsynaptic neuron may be the primary locus of the compensatory response to chronic opioid administration. Previous studies from our laboratory indicate that the enhanced Fos-LI produced when withdrawal is precipitated in opioid-tolerant rats may derive predominantly from neuronal circuits intrinsic to the superficial dorsal horn (Rohde, in press, Neuroscience), rather than from enhanced primary afferent activity. It is of interest, however, that an essential subunit of the N-methyl-D-aspartate (NMDA) receptor is also located on primary afferent terminals (Liu et al. 1994). We have speculated that the presynaptic NMDA receptor is an autoreceptor, which when acted upon by glutamate released

from primary afferents, could significantly enhance and prolong transmitter release from the primary afferent. Conceivably, the tolerance-induced changes originate in the opioid and NMDA receptor-laden primary afferents such that noxious stimuli evoke a more widespread pattern of Fos-LI because there is greater release of neurotransmitter from primary afferents.

In summary, our results indicate that morphine tolerance induces a profound change in subpopulations of central nervous system neurons that can be manifested as increased neuronal expression of the Fos protein, either when withdrawal is precipitated or by noxious stimulation. The predominant locus of the changes may be in neurons of the superficial dorsal horn, or in the the primary afferent fibers that terminate there. Since common neural substrates underlie morphine tolerance and hyperalgesia and since the biochemical changes occur in the same central loci (spinal cord superficial dorsal horn), an interaction between these intracellular mechanisms is likely. That is, morphine tolerance may lead to hyperalgesic states and hyperalgesia may lead to the development of morphine tolerance (Inoki et al. 1990; Mao et al. 1994). Knowledge of the biochemical changes that occur during the development of morphine tolerance as well as the locus of these changes may be beneficial for future clinical therapies.

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Figure 1. Total number ($\pm$ S.E.M.) of Fos-LI neurons in A: the L4/5 and L2 lumbar segments; B: the ipsilateral and contralateral sides of the L4/5 lumbar segment.; C: the ipsilateral and contralateral sides of the L2 lumbar segment. All rats were injected with formalin. We recorded significantly increased Fos-LI in the morphine-pelleted (n=9) vs. the placebo-pelleted (n=8) for total fos and for total ipsilateral or total contralateral fos. * $p < 0.05$, * $p < 0.01$.

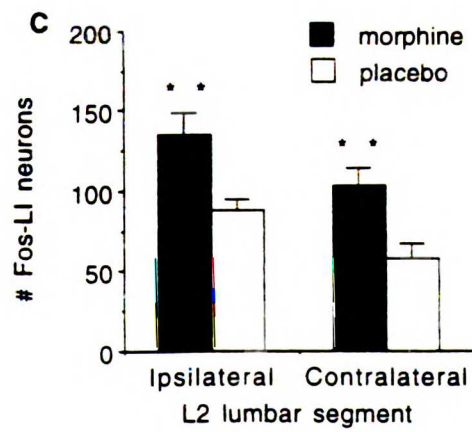
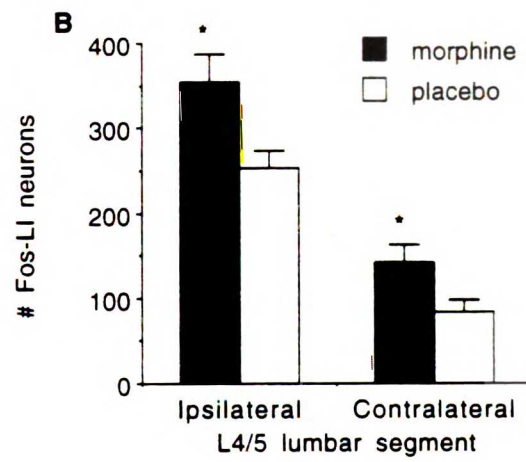
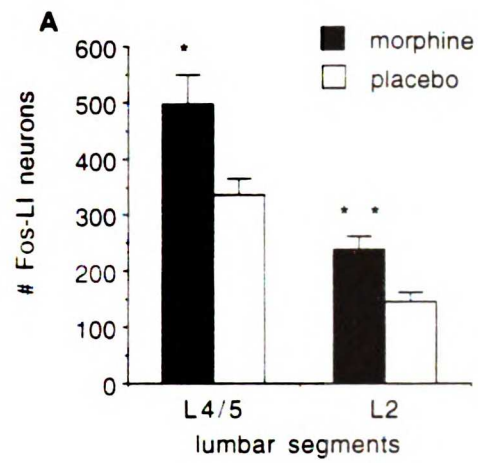


Figure 2. Number (+/- S.E.M.) of Fos-LI neurons in the different spinal cord laminae in A: ipsilateral L4/5; B: contralateral L4/5; C: ipsilateral L2; and D: contralateral L2 . All rats were injected with formalin. We recorded significantly increased Fos-LI in the morphine-pelleted (n=9) vs the placebo-pelleted (n=8) rats in several laminae ipsilateral and contralateral to the hindpaw formalin injection at both the L4/5 and L2 segments. * $p < 0.05$, * $p < 0.01$.

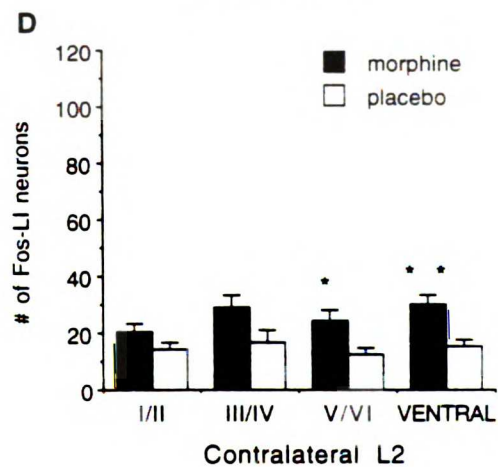
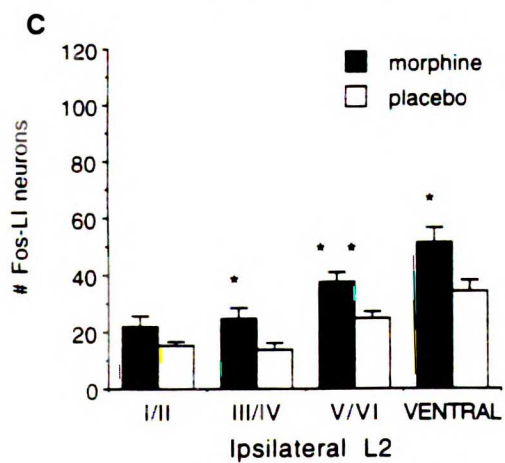
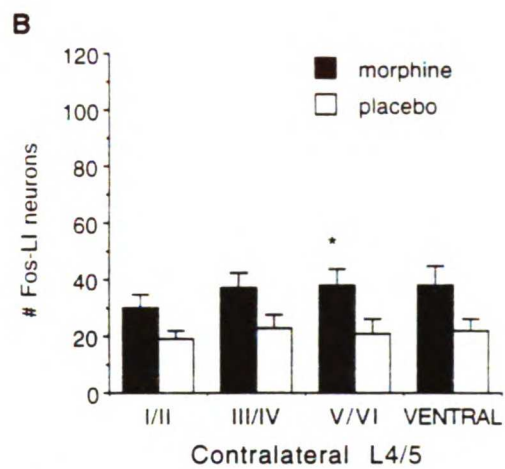
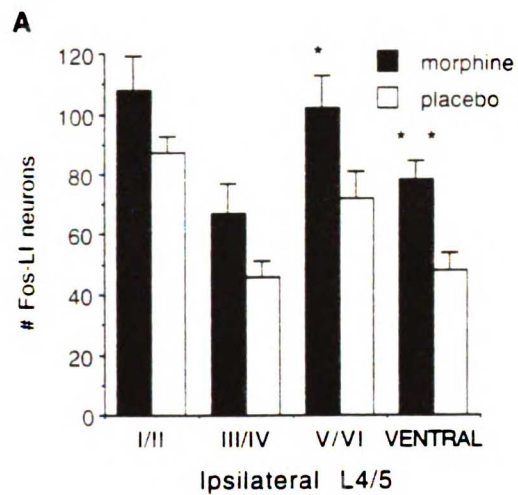


Figure 3. Low-power photomicrograph of A: an L4/5 section of a placebo-pelleted rat; B: an L4/5 section of a morphine-pelleted rat; C: an L2 section of a placebo-pelleted rat; and D: an L2 section of a morphine-pelleted rat. All rats were injected with formalin. Note the increased Fos-LI in the morphine-pelleted rats in all regions of the cord, on the ipsilateral and contralateral sides. Arrowheads indicate increased Fos-LI in the deeper dorsal horn of the morphine-pelleted rats. Calibration bar = 500 μ m

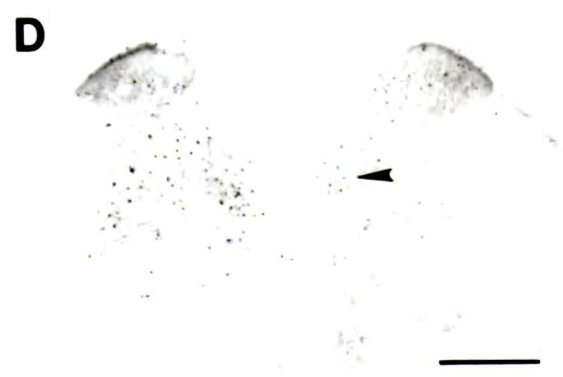
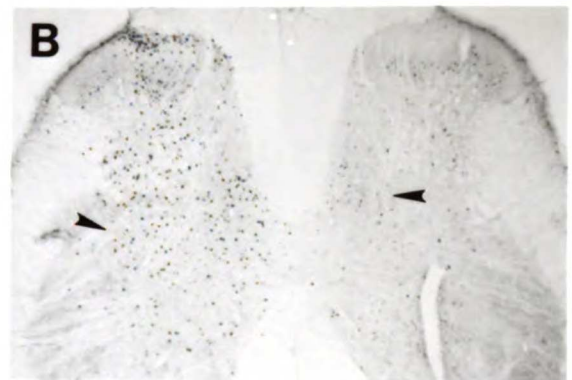
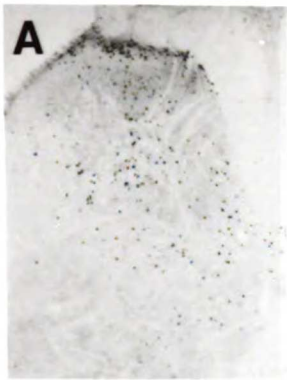


Figure 4. These high-power photomicrographs better illustrate the increased staining in the L4/5 dorsal horn of morphine-pelleted vs. placebo-pelleted rats injected with formalin. A: ipsilateral side of a placebo-pelleted rat; B: contralateral side of a placebo-pelleted rat; C: ipsilateral side of a morphine-pelleted rat; D: contralateral side of a morphine-pelleted rat. Arrow indicates increased Fos-LI lamina I, contralateral to the formalin injection. Arrowheads indicate increased Fos-LI in laminae V/VI, ipsilateral and contralateral to the formalin injection. Calibration bar = 250 μ m

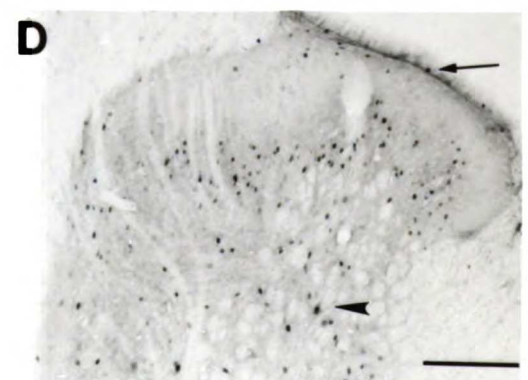
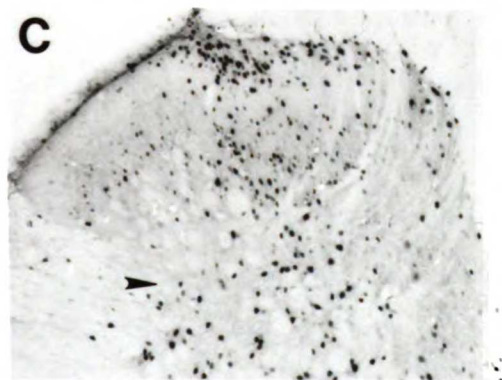


Figure 5. These high-power photomicrographs better illustrate the increased staining in the L2 dorsal horn of morphine-pelleted vs. placebo-pelleted rats injected with formalin. A: ipsilateral side of a placebo-pelleted rat; B: contralateral side of a placebo-pelleted rat; C: ipsilateral side of a morphine-pelleted rat; D: contralateral side of a morphine-pelleted rat. Arrow indicates increased Fos-LI in the contralateral superficial dorsal horn. Arrowhead indicates increased Fos-LI in ipsilateral laminae V/VI. Calibration bar = 250 μ m

A



B



C



D

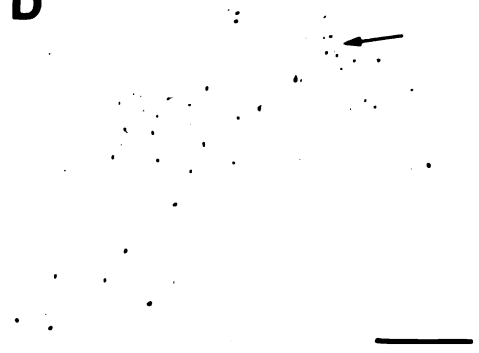
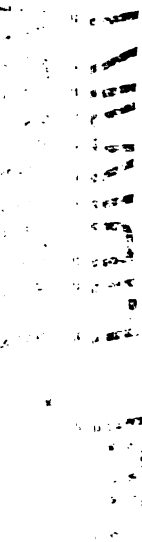
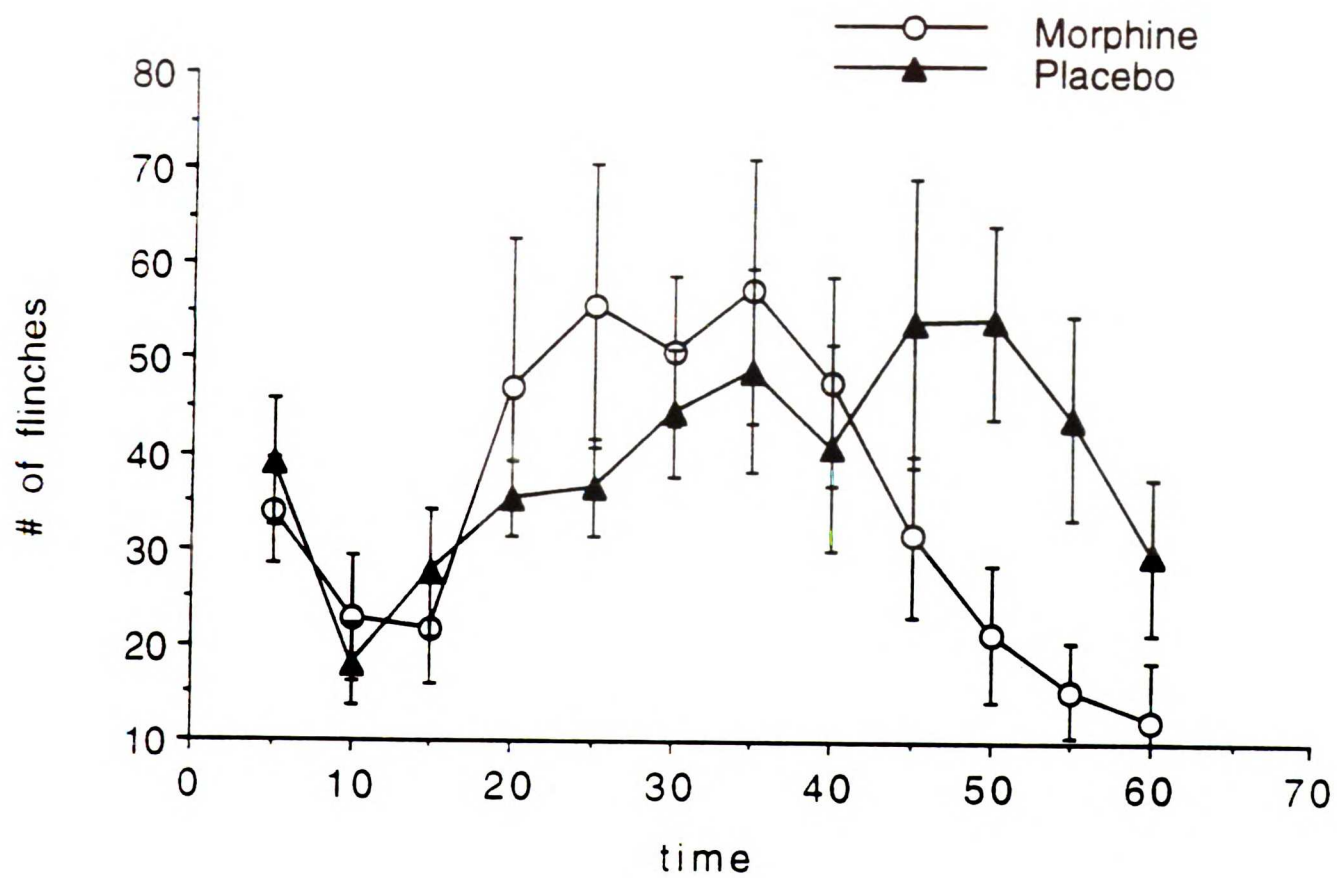


Figure 6. This graph plots the number ($\pm$ S.E.M.) of flinches in the morphine-pelleted (n=9) vs the placebo-pelleted (n=8) rats injected with formalin. There are no significant differences between the two groups.





Chapter 5

The contribution of supraspinal, peripheral and intrinsic spinal circuits to the pattern and magnitude of fos-like immunoreactivity in the lumbar spinal cord of the rat withdrawing from morphine

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Abstract

Withdrawal from morphine evokes increases in Fos-like immunoreactivity in the spinal cord, particularly in the superficial dorsal horn, laminae I/II. To determine the origin of the increased Fos-like immunoreactivity, we selectively targetted central or peripheral opioid receptors with naloxone methiodide, an antagonist that does not cross the blood brain barrier, or induced withdrawal after eliminating possible sources of input to the superficial dorsal horn. To induce tolerance, we implanted rats with morphine or placebo pellets (75mg, 6 pellets over 3 days). On day 4, withdrawal was precipitated and after one hour, the rats were killed, their spinal cords removed and 50 um transverse sections of the spinal cord immunoreacted with a rabbit polyclonal antiserum directed against the Fos protein. In placebo-pelleted rats, none of the different procedures, viz. spinal transection, unilateral dorsal rhizotomy (L4 - S2), neonatal capsaicin treatment or direct intrathecal opioid antagonist injection, induced expression of the Fos protein. However, both spinally transected and rhizotomized withdrawing animals showed significant *increases* in Fos-like immunoreactivity in laminae I/II, compared to intact withdrawing rats. Neonatal treatment with capsaicin, which eliminates C-fibers, did not alter Fos-like-immunoreactivity. Selective withdrawal of morphine from peripheral opioid receptors by naloxone-methiodide did not induce Fos-like immunoreactivity in the lumbar spinal cord greater than that recorded in non-withdrawing rats. However, intrathecal injection of naloxone-methiodide increased Fos-like immunoreactivity in laminae I/II and the ventral horn to a greater extent than did subcutaneous injection of naloxone.

We hypothesize that the increased Fos expression after systemic withdrawal in spinally-transected rats results from a loss of descending inhibitory control that is activated during withdrawal. The increase in withdrawal-induced Fos-like immunoreactivity after rhizotomy may be secondary to loss of inhibitory controls exerted by large diameter primary afferents or to deafferentation-induced reorganization in the dorsal horn. Since capsaicin did not alter the magnitude of Fos-like immunoreactivity in withdrawing rats, we conclude that hyperactivity of opioid receptor-laden C-fibers is not a necessary contributor to the withdrawal-induced increase in Fos-like immunoreactivity in laminae I and II. Taken together with the results recorded after intrathecal injection of naloxone-methiodide in tolerant rats, we conclude that the pattern of lumbar spinal cord Fos expression following systemic withdrawal is primarily a consequence of increased activity in opioid receptor-containing circuits intrinsic to the dorsal horn and that the magnitude of Fos expression is normally dampened by supraspinal and primary afferent-derived inhibitory inputs.

Introduction

Opiates, such as morphine, and opioid peptides hyperpolarize neurons and decrease adenyl cyclase through an inhibitory G protein (Griffin et al. 1985; Makman et al. 1988; Sanchez-Blazquez and Garzon 1988; Sharma et al. 1975). Although uncoupling of the opioid receptor from an inhibitory G protein is thought to underlie the development of tolerance to morphine (Christie et al. 1987; Cox 1991; Werling et al. 1989), recent studies indicate that other mechanisms are also involved. In particular, since the opioid antagonist naloxone induces a withdrawal syndrome in tolerant animals or increases firing of single neurons (Aghajanian 1978), it has been hypothesized that the tolerant state is characterized by a compensatory response that develops in neurons that express the opioid receptor or in neural circuits in which those neurons participate (Nestler and Tallman 1988; Nestler et al. 1992; Sharma et al. 1975). This compensatory response is hypothesized to counteract the inhibitory effects of the opiate agonist. The state of tolerance, therefore, is one in which there is a latent sensitization or hyperexcitability of neurons (Fry et al. 1980), which can be unmasked upon injection of an opioid antagonist.

With a view to identifying subpopulations of neurons in which the compensatory response can be unmasked, several laboratories have evaluated changes in expression of the Fos protein, a marker of neuronal activity (Basbaum 1994; Hunt et al. 1987; Presley et al. 1990), when withdrawal is precipitated in tolerant rats (Chieng et al. 1995; Hayward et al. 1990; Rohde et al. 1996). Because of our interest in the effects of opioids on spinal nociceptive processing, and because hyperexcitability/sensitization is a hallmark of persistent pain conditions, (Woolf 1983; Woolf and Thompson

1991), we have studied the changes that occur in the spinal cord dorsal horn after antagonist-precipitated withdrawal (Rohde et al. 1996). We found extensive expression of the Fos protein in neurons of the superficial dorsal horn (laminae I/II) and in the nucleus proprius (laminae III/IV). When rats withdrew under anesthesia, which eliminated the movement associated with withdrawal, we found that the focus of Fos-like immunoreactivity was in the superficial dorsal horn; in some cases, the labeled neurons were restricted to lamina I. Our results raised the possibility that the development of latent sensitization/hyperexcitability is concentrated in neurons of the superficial dorsal horn, which we hypothesized could underlie withdrawal-induced hyperalgesia.

The objective of the present study was to determine the contribution of primary afferent, supraspinal and local spinal circuits to the increase in Fos-like immunoreactivity in lumbar spinal cord neurons during precipitated withdrawal from morphine. To this end, we studied the pattern of Fos-like immunoreactivity during withdrawal in rats that underwent surgical or pharmacological manipulations that selectively targeted or eliminated input from these different sources. Based on these studies we conclude that withdrawal-induced expression of the Fos protein is largely attributed to opioid receptor-containing circuits within the spinal cord dorsal horn, although it can be modulated from supraspinal and peripheral sites.

Methods

Animals

Experiments were performed on male Sprague-Dawley rats (260-400 gm; Bantin and Kingman, Fremont, CA) with the approval of the Institutional

Animal Care and Use Committee at UCSF. Animals were housed two to a cage in sawdust bedding in a temperature-controlled room with a 12 h light and dark cycle. They were given free access to food and water. All efforts were made to minimize the number of animals used and to minimize animal suffering.

Experimental Groups

Spinal transection

The first set of experiments was designed to determine the contribution of descending inputs to withdrawal-induced behavior and to the lumbar spinal cord pattern of Fos-like immunoreactivity. To eliminate supraspinal inputs, we performed spinal transections at the T3 level, under pentobarbital anesthesia (60 mg/kg). After laminectomy from T2 - T4 and incision and retraction of the dura, we pinched the exposed spinal cord with a fine-tip forceps and then cut the dorsal cord through the pinched region of cord with iridectomy scissors. We then lifted the spinal cord from the ventral bone to avoid ventrally located blood vessels and completed the transection. To minimize postoperative bleeding, we placed a piece of Gelfoam^R between the cut ends of the retracted spinal cord. After the overlying muscle and skin were sutured, the animals were returned to their cages and allowed to recover from the anesthetic. All rats were monitored for weight loss and the bladder was expressed at least four times per day. Neurological examination confirmed complete lack of sensory and motor function below T3. One day after the cord transection, we started implantation of pellets. We randomly assigned the rats into three different treatment groups (n = 4 for each group): (i) transected rats implanted with morphine pellets followed by

precipitated withdrawal by naltrexone injection or (ii) intact rats implanted with morphine pellets followed by precipitated withdrawal by naltrexone injection or (iii) transected rats implanted with placebo pellets followed by naltrexone injection.

Lumbosacral dorsal rhizotomy

The second set of experiments was designed to determine the contribution of primary afferent input to the pattern of withdrawal-induced behavior and Fos-like immunoreactivity. To this end we performed unilateral dorsal rhizotomy (L4 through S2) under pentobarbital anesthesia (60 mg/kg). After laminectomy, the dura was incised and reflected laterally to expose the dorsal roots. After identifying individual dorsal root ganglia, each root was cut with iridectomy scissors, avoiding the underlying ventral roots. A sheet of Gelfilm^R was placed over the exposed spinal cord. We then sutured the overlying muscle and skin and returned the rats to their cages to recover from the anesthetic. Neurological examination confirmed the deafferentation. Although the rats used the limb to ambulate, there was no reflex withdrawal to pinch. One day after the surgery we began implantation of the pellets. In this set of experiments, we randomly assigned the rats into three treatment groups: (i) rhizotomized rats implanted with morphine pellets followed by precipitated withdrawal by naltrexone injection (n=10) or (ii) intact rats implanted with morphine pellets followed by precipitated withdrawal by naltrexone injection (n=7) or (iii) rhizotomized rats implanted with placebo pellets followed by naltrexone injection (n=7).

Capsaicin pretreatment

The third set of experiments specifically addressed the contribution of small diameter primary afferents to the pattern of withdrawal-induced behavior and expression of the Fos protein. To destroy small-diameter afferents, we administered capsaicin (8-methyl-N-vanillyl-6-nonenamide, Sigma) in a 1:1 Tween80: ethanol solution, 100 mg/kg, 2.0 ul/gm, s.c. within 24 hours of birth. The rats were implanted with morphine pellets at the age of nine to eleven weeks. In this set of experiments, we randomly assigned rats into three different treatment groups: (i) capsaicin-pretreated rats implanted with morphine pellets followed by precipitated withdrawal by naltrexone injection (n=8) or (ii) age-matched non-capsaicin-treated adult rats implanted with morphine pellets followed by precipitated withdrawal by naltrexone injection (n=7) or (iii) capsaicin-pretreated rats implanted with placebo pellets followed by naltrexone injection. The age-matched controls did not differ significantly in weight from the capsaicin-pretreated rats.

Selective targetting of *peripheral* opioid receptors by subcutaneous injection with naloxone-methiodide

The fourth set of experiments addressed the contribution of peripheral opioid receptors to the changes in behavior and Fos-like immunoreactivity during morphine withdrawal. To selectively displace morphine from peripheral opioid receptors, we precipitated withdrawal by s.c. administration of naloxone-methiodide, a hydrophilic form of naloxone that does not readily cross the blood brain barrier (Brown and Goldberg 1985; Schroeder et al. 1991). Since the affinity of quaternary naloxone for the opiate receptor is reported to be approximately 5% of the opiate antagonist

activity of naloxone (Manara et al. 1982; Valentino et al. 1983) and since its potency is also less than naloxone (Brown et al. 1983; Valentino et al. 1983), we performed pilot studies with doses of naloxone-methiodide that were up to 10 - 20 x greater than the dose of naloxone (1.0 mg/kg) used in these experiments. We used naloxone instead of naltrexone because the affinity and potency of naloxone-methiodide has only been compared to naloxone. We found that 1.0 mg/kg naloxone induced behavior and Fos-like immunoreactivity comparable to that produced by 10 mg/kg naltrexone. In preliminary studies, we found that at 1.0 mg/kg, naloxone-methiodide injected in dependent rats did not induce any signs of withdrawal. Ten mg/kg precipitated withdrawal diarrhea and at 20 mg/kg, other withdrawal signs were recorded, including teeth chattering. Since teeth chattering is assumed to be centrally mediated (Bozarth 1994, Maldonado, et al. 1992), we used 10 mg/kg naloxone-methiodide to precipitate withdrawal at peripheral sites only. We randomly assigned rats into three different treatment groups: (i) morphine-pelleted rats injected s.c. with naloxone-methiodide, 10 mg/kg (n = 6) or (ii) placebo-pelleted rats injected s.c. with naloxone-methiodide, 10 mg/kg (n = 5) or (iii) morphine-pelleted rats injected s.c. with naloxone, 1.0 mg/kg (n = 5). This last group allowed us to compare the pattern of Fos-like immunoreactivity produced after peripherally- and systemically-induced withdrawal. These three groups of rats also received direct intrathecal injections of 20 ul of saline so that the Fos-like immunoreactivity could be compared and analyzed with the groups of animals described below. However, only the morphine-pelleted rats injected s.c. with naloxone were analyzed with the groups of animals described below.

Selective targetting of *spinal* opioid receptors by intrathecal injection with naloxone-methiodide

The fifth set of experiments addressed the contribution of spinal opioid receptors to the changes in behavior and Fos-like immunoreactivity seen after withdrawal. To selectively target spinal opioid receptors we precipitated withdrawal by intrathecal injection of naloxone-methiodide, 10 μ g. Although 6 - 10 μ g has been used to precipitate withdrawal by spinal injection of naloxone (Marshall and Buccafusco 1985; Yaksh et al. 1977), the affinity and potency of *intrathecal* naloxone-methiodide is not known relative to naloxone. Our pilot studies demonstrated seizure-like activity in normal rats with intrathecal injections of naloxone-methiodide at doses of 20 μ g or greater. Ten μ g did not induce seizures, but did evoke withdrawal behavior, including hyperactivity of the hindlimbs and tail. To determine if there is a difference between intrathecally injected naloxone-methiodide and naloxone, we injected an additional group of rats with intrathecal naloxone, 10 μ g.

In this experiment, we randomly assigned rats into five treatment groups: (i) rats implanted with morphine pellets followed by direct intrathecal injection of naloxone-methiodide, 10 μ g (n = 6) or (ii) rats implanted with placebo pellets followed by direct intrathecal injection of naloxone-methiodide, 10 μ g (n = 5) or (iii) rats implanted with morphine pellets followed by direct intrathecal injection of naloxone, 10 μ g (n = 6) or (iv) rats implanted with placebo pellets followed by direct intrathecal injection of naloxone, 10 μ g (n = 6) or (v) rats implanted with morphine pellets followed by direct intrathecal injection of saline and s.c. injection of naloxone, 1.0 mg/kg (n=5) to precipitate systemic withdrawal (these are the same rats as in the fourth set of experiments). This last group allowed us to

compare the pattern of Fos-like immunoreactivity produced after spinal or systemic withdrawal. We administered the opioid antagonists *directly* into the intrathecal space at the level of the L5/L6 vertebral junction using a 1.0 ml syringe and 27G needle, with the rat lightly anesthetized under 2% halothane with a 2:1 flow ratio of N₂O: O₂, as modified from the procedure by Mestre et al.(1994) Proper placement in the intrathecal space was confirmed by observation of a tail flick prior to, upon administration of, and after the injection.

Tolerance and withdrawal protocol

In our previous studies (Detweiler et al. 1995; Rohde et al. 1996) we used a tolerance protocol that involved single pellet implantation for five consecutive days. In the present study we used increasing numbers of pellets over a three day period. To demonstrate that the three-day protocol also produces significant tolerance to the analgesic effects of morphine, we evaluated the effect of an acute dose of morphine on hindpaw formalin-evoked pain behavior in these rats. Naive rats given an acute dose of morphine (10 mg/kg) showed significant analgesia in the hindpaw formalin test (5%, 100 μ l). However, the 3 day morphine-pelleted animals injected with an acute dose of morphine (10 mg/kg) displayed pain behavior (flinching and licking) in the formalin test comparable to that of untreated rats injected with formalin (data not shown). Thus, tolerance had indeed developed with the 3 day protocol.

We implanted the pellets subcutaneously (75 mg morphine sulfate pellet or placebo pellet kindly provided by National Institute on Drug Abuse, Rockville, MD) under anesthesia with a mixture of 5% halothane and a 2:1 flow ratio of N₂O: O₂, according to the following schedule: day 1 - one pellet,

day 2 - two pellets, day 3 - three pellets. We did not remove the pellets at any time during the study. On day 4, we injected either s.c. naltrexone (10 mg/kg), intrathecal saline plus s.c. naloxone-methiodide (10 mg/kg), intrathecal saline plus s.c. naloxone (1.0 mg/kg), intrathecal naloxone-methiodide (10 μ g) or intrathecal naloxone (10 μ g). The s.c. drugs were diluted in 800 μ l physiological saline and injected into the nape of the neck. The intrathecal injections were diluted in 20 μ l saline. The opiate antagonists were purchased from Research Biochemicals International (Natick, MA).

Behavior and immunocytochemistry protocol

We monitored the animals for signs of withdrawal (ptosis, teeth chattering, diarrhea, jumping, weight loss, salivation, burrowing in the sawdust, and generalized hyperactivity) for 1 h following the injection on day 4. After 1 h, the rats were deeply anesthetized with pentobarbital (200 mg/kg, i.p.) followed immediately by intracardiac perfusion through the ascending aorta with 100 ml 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 500-600 ml 10% formalin in 0.1 M phosphate buffer (PB). We removed the lumbar spinal cord 1 h later, postfixed it in 10% formalin for an additional 3 h and then cryoprotected it overnight in 30% sucrose in 0.1 M PB. Fifty μ m frozen sections were cut in the transverse plane and collected in 0.05 M PBS for immunocytochemical analysis. We then immunostained the sections for the Fos protein by the avidin-biotin-peroxidase method of Hsu et al. (Hsu, et al. 1981). The sections were washed with a solution of 0.05 M Tris PBS with 1% normal goat serum and 0.3% Triton-X and then incubated for 1 h at room temperature in a blocking solution of 3% normal goat serum in 0.05 M Tris PBS with 0.3% Triton-X. We then removed the blocking solution from

the tissue, incubated the sections overnight at room temperature in the primary antiserum, which is a rabbit polyclonal antiserum directed against an *in vitro* translated product of *c-fos* (kindly provided by Dr. D. Slamon, UCLA). We diluted the antiserum 1:21,000 and preabsorbed it against acetone-dried liver powder for 1 h at 37°C and for 1 h at 4° C prior to use. After the primary antiserum, we washed the sections and incubated them in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Elite Kit; Vector Labs, Burlingame, CA). To visualize the Fos-like immunoreactivity as a black reaction product we used a nickel-diaminobenzidine (DAB) glucose oxidase reaction following a protocol adapted from Llewellyn-Smith and Minson (1992).

Data analysis

To quantitate the numbers of Fos-like-immunoreactive neurons, we photographed sections from L4/5 at low (4X) power with Kodak technical pan film on a Nikon Microphot-FXA microscope. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. We printed individual sections of the spinal cord at 60X enlargement. The photographs were divided into four segments: (i) the superficial laminae (laminae I, Ilo, Ili), (ii) the nucleus proprius (laminae III and IV), (iii) lamina V/VI, and (iv) the ventral horn. A person blinded to the treatments identified and plotted the Fos-like immunoreactive neurons. Four to six spinal cord sections were counted per rat and averaged so that each animal had a mean value for regional Fos-like immunoreactivity. We compared the numbers of labeled cells using two-way analysis of variance (ANOVA) for treatment and laminae and performed *post hoc* comparisons with Fisher's Protected Least Significant Difference.

Results

Behavioral signs of withdrawal

The placebo-pelleted groups did not demonstrate any behavioral signs of withdrawal. By contrast, the intact, morphine-pelleted rats that received systemic naltrexone displayed signs of withdrawal, including ptosis, teeth chattering, diarrhea, jumping, salivation, burrowing in the sawdust and marked generalized hyperactivity. The morphine-pelleted group of rats that had undergone prior spinal cord transection also displayed clear signs of withdrawal, which included periodic movements of their lower body and legs. The capsaicin-treated and rhizotomized animals also displayed signs of withdrawal comparable to those in intact withdrawing animals. Of note, however, the rats in the capsaicin-treated group had more severe diarrhea and lost significantly more weight than did the non-capsaicin-treated animals withdrawing from morphine (Rohde, in press, Brain Research).

Although the tolerant rats that received a s.c. injection of naloxone-methiodide appeared agitated, and 50% of these rats had diarrhea, we did not record other signs of withdrawal in this group. Importantly, unlike rats withdrawing from systemically or centrally administered opiate antagonists, the rats injected peripherally with naloxone-methiodide were not hyperreactive to touch. Rats withdrawing from intrathecal naloxone demonstrated withdrawal signs similar to systemically withdrawing rats (ptosis, chattering, burrowing and generalized hyperactivity), but only one had diarrhea. Finally, rats withdrawing after intrathecal naloxone-methiodide demonstrated considerable movement in the hind part of the body and some tail quivering, but no other signs of withdrawal.

Fos-like immunoreactivity

Spinal Transection

When examined four days after surgery, spinal transection in placebo-pelleted animals did not increase Fos-like immunoreactivity in the lumbar cord. Presumably any change induced by the surgical procedure had disappeared because of the short half-life of the Fos protein. By contrast, compared to the intact withdrawing rats, the transected withdrawing group had significantly increased Fos-like immunoreactivity in laminae I/II (73%) and in the ventral horn (246%) (Figs. 1A, 1B, 2, and 3). We also recorded significantly increased Fos-like immunoreactivity in all regions of the spinal gray in transected withdrawing rats compared to the placebo-pelleted transected rats.

Lumbosacral dorsal rhizotomy

When examined four days after surgery, rats that underwent dorsal rhizotomy and then received placebo-pellets followed by naltrexone did not show increased Fos-like immunoreactivity in the lumbar spinal cord. This is consistent with an earlier report by Abbadie et al. (1992) and indicates that injury-induced Fos disappeared by 4 days. However, in withdrawing rats, the number of Fos-like immunoreactive neurons in laminae I/II ipsilateral to the rhizotomy was significantly greater (29%) than on the contralateral (non-rhizotomized) side (Figs. 1C, 4, and 5). Importantly, ipsilateral to the rhizotomy, we also recorded a significant *decrease* in Fos-like immunoreactivity in laminae III/IV (39%) and laminae V/VI (34%), compared to the contralateral side. No differences between the ipsilateral

and contralateral sides were recorded in the ventral horn. Similar changes were noted when we compared the rhizotomized side to either side of an intact withdrawing rat. In other words, intact rats demonstrated Fos-like immunoreactivity comparable to that of the contralateral (non-rhizotomized) side of a rhizotomized rat. Thus, rhizotomized withdrawing animals demonstrated significant increases in Fos-like immunoreactivity on the rhizotomized side in laminae I/II (37%), and decreases in Fos-like immunoreactivity in laminae III/IV (42%) and V/VI (51%) compared to intact withdrawing rats. Again, there were no differences between the two groups in the ventral horn. Finally, rhizotomized rats that underwent withdrawal had significantly increased Fos-like immunoreactivity in laminae I/II, ipsilateral to the rhizotomy, compared to the rhizotomy control group (placebo-pelleted rats that underwent unilateral rhizotomy).

Capsaicin pretreatment

As expected, we recorded significantly increased Fos-like immunoreactivity in laminae I/II in the capsaicin-treated withdrawing rats compared to the capsaicin-treated control group (capsaicin-treated placebo-pelleted rats). Surprisingly, there were no differences between these two groups in the deeper laminae. Furthermore, the capsaicin-treated withdrawing rats did not differ significantly in Fos-like immunoreactivity in any laminae compared to intact withdrawing rats (Figs. 1A, 1D, and 6).

Peripherally-precipitated withdrawal

Peripheral withdrawal (tolerant rats injected s.c. with naloxone-methiodide) induced significantly less Fos-like immunoreactivity in laminae I/II (35%) compared to the systemically withdrawing rats (Figs. 1A, 1E, and

7). The peripheral withdrawal group did not differ from the control group (placebo-pelleted rats injected s.c. with naloxone-methiodide).

Intrathecally-precipitated withdrawal

Intrathecal injections of naloxone-methiodide in placebo-pelleted animals did not induce Fos-like immunoreactivity in the lumbar cord. By contrast, intrathecally-precipitated withdrawal with naloxone-methiodide resulted in significantly increased Fos-like immunoreactivity in laminae I/II (60%) and the ventral horn (112%) compared to systemically withdrawing rats (tolerant rats injected intrathecally with saline and s.c. with naloxone), and increased Fos-like immunoreactivity in laminae I/II (68%), III/IV (71%), and V/VI (111%) compared to tolerant rats injected with intrathecal naloxone (Figs. 1A, 1F, 8 and 9). Surprisingly there were no differences in Fos-like immunoreactivity between the groups of rats injected either intrathecally or subcutaneously with naloxone. Finally, intrathecal injections of naloxone-methiodide in morphine-pelleted rats induced significant increases in Fos-like immunoreactivity in all regions of the cord compared to the control rats (placebo pelleted rats injected intrathecally with naloxone-methiodide).

Discussion

In a previous study, we reported that opiate antagonist-induced withdrawal from chronic morphine treatment in the rat results in increased Fos-like immunoreactivity in the spinal cord, with a focus of labelling in the superficial dorsal horn (laminae I and II) (Rohde et al. 1996). In the present study, we performed a variety of manipulations to identify the origin of this increased Fos-like immunoreactivity. Although we hypothesized that

removing these inputs would, if anything, reduce the numbers of Fos-like immunoreactive neurons, in many cases we found the opposite. For example, after spinal transection, which removes descending inputs from the brainstem, we recorded an even larger increase in the number of withdrawal-induced Fos-like immunoreactive cells in both the superficial dorsal horn and in the ventral horn, compared to intact, withdrawing rats. Similarly, after unilateral dorsal rhizotomy, which removes input from both large- and small-diameter primary afferent fibers, we found enhanced Fos-like immunoreactivity (compared to intact withdrawing rats) in the superficial dorsal horn ipsilateral to the deafferentation. By contrast, neonatal capsaicin treatment, which selectively eliminates small-diameter primary afferent input to the spinal cord, did not produce a statistically significant increase in the magnitude of withdrawal-induced Fos-like immunoreactivity compared to intact rats. This suggests that peripheral inputs do not contribute significantly to the *induction* of the Fos protein. Consistent with this conclusion, we found that selective withdrawal at peripheral opioid receptors, with naloxone methiodide, did not significantly increase lumbar Fos-like immunoreactivity. By contrast, when withdrawal was precipitated by spinal injection of naloxone-methiodide, we recorded even greater numbers of Fos-like immunoreactive neurons in laminae I/II and in the ventral horn compared to that produced by systemic injection of naloxone. Based on these results, we conclude that the pattern of Fos expression in the lumbar spinal cord of rats that withdraw from morphine is primarily a consequence of increased activity in opioid receptor-containing circuits *intrinsic* to the dorsal horn and that the magnitude of Fos expression is normally dampened by supraspinal and primary afferent-derived inhibitory inputs.

Although it is possible that some of the differences in behavior and Fos-like immunoreactivity that we observed were due to differences in the potency or affinity of the opiate antagonist used rather than to the site targetted, we do not believe that this is the case. Thus, when we precipitated withdrawal systemically, with doses 10 or 100 times that reported in the present study (i.e. 10 or 100 mg/kg naloxone), we found comparable withdrawal behavior and a comparable magnitude and distribution of Fos-like immunoreactivity. Furthermore, 1.0 - 2.0 mg/kg naloxone is typically used to block morphine analgesia (Yaksh and Rudy 1976) or to induce withdrawal (Yaksh et al. 1977). Most importantly, the fact that intrathecal injection of naloxone-*methiodide* induced behavioral withdrawal restricted to the tail and hindpaws not only indicates that its distribution was limited to the spinal cord but also that an effective dose had been used.

Spinal transection

As previously reported (Berge and Hole 1981; Marshall and Buccafusco 1985), although rats with spinal cord transection are paralyzed, they still show significant movement of the hindlimbs during withdrawal from morphine. However, since this movement was still considerably less than the movement that is associated with withdrawal in the intact rat, we do not believe that the increased Fos-like immunoreactivity after spinal transection resulted from movement alone. Rather, since descending inhibitory controls that regulate the firing of dorsal horn neurons are eliminated by spinal cord transection, we suggest that the increased expression of the Fos protein reflects a loss of inhibitory controls that are normally "activated" during systemic withdrawal. Importantly, since we did not detect increased Fos-like immunoreactivity in the placebo-pelleted transected animals, it is unlikely

that the controls are tonically active. Rather, they are manifested when withdrawal is induced; this would tend to counteract the increase in Fos-like immunoreactivity (i.e. a marker of neuronal activity) that is induced at the level of the spinal cord during withdrawal.

Several supraspinal sources, including the locus coeruleus (LC), rostral ventral medulla (RVM) and periaqueductal gray (PAG) exert descending inhibitory influences on the spinal cord dorsal horn and ventral horn (Fields et al. 1977; Gray and Dostrovsky 1983; Hodge et al. 1981; Jones and Gebhart 1986). Since these sites have high concentrations of opiate receptors (Pert et al. 1975), and are implicated in opiate analgesia (Azami et al. 1982; Barton et al. 1980; Basbaum et al. 1976; Basbaum et al. 1978; Fields et al. 1977; Levy and Proudfit 1979) and withdrawal (Aghajanian 1978; Chieng et al. 1995; Hayward et al. 1990; Maldonado et al. 1992; Wilcox et al. 1979), it is possible that withdrawal-induced increased activity in each of these areas enhances descending control. This could explain the increased Fos-like immunoreactivity seen in withdrawing rats after transection. Since LC neurons are activated during withdrawal (Aghajanian 1978; Hayward et al. 1990) and inhibit the firing of dorsal horn neurons (Headley et al. 1978; Hodge et al. 1981; Jones and Gebhart 1986), it is likely that a noradrenergic descending inhibition is activated during withdrawal; transection would eliminate this component. The fact that the LC also projects to the ventral horn (Clark and Proudfit 1992; Holstege and Kuypers 1982; Nygren and Olson 1977; Westlund and Coulter 1980) may explain the large increase in ventral horn Fos-like immunoreactivity in the transected withdrawing rats. To determine the contribution of LC activity during withdrawal, we are currently evaluating the effects of lesions of the LC on the magnitude of withdrawal-induced Fos-like immunoreactivity.

Since acute injection of morphine activates descending inhibitory controls, particularly those arising from the midbrain PAG and medullary nucleus raphe magnus (Azami et al. 1982; Basbaum et al. 1976; Levy and Proudfit 1979), it is paradoxical that increased inhibition is generated both during the production of analgesia and during withdrawal from morphine. If anything, one might have predicted that withdrawal is associated with *decreased* inhibitory controls, particularly in light of studies of Fields and colleagues, who described a population of so-called "on" cells, which are activated during naloxone-precipitated withdrawal and which facilitate the firing of dorsal horn nociresponsive neurons (Bederson et al. 1990; Kaplan and Fields 1991). Spinal cord transection would, of course, eliminate any enhancement of Fos-like immunoreactivity that resulted from increased on-cell activity during withdrawal. Taken together these results suggest that the caudal raphe is not the primary locus of the *increased* descending controls that we believe are activated during systemic withdrawal from morphine.

Deafferentation by dorsal rhizotomy

We recorded greater numbers of Fos-like immunoreactive neurons in laminae I/II ipsilateral to multiple dorsal rhizotomy in withdrawing rats than on the contralateral side, and also greater numbers compared to that recorded in intact withdrawing rats. Since we found no difference between the numbers of Fos-like immunoreactive neurons in intact rats and on the side *contralateral* to the rhizotomy, we conclude that the difference between the ipsilateral and contralateral sides of the deafferented rats resulted from an increase in Fos-like immunoreactivity ipsilateral to the rhizotomy, rather than from a decrease contralateral. This increase occurred despite there being a significant *decrease* in the magnitude of Fos-like immunoreactivity

in laminae III/IV and V/VI ipsilateral to the rhizotomy. The reduction in laminae III/IV, which contains neurons that receive large diameter primary afferent inputs, was expected (Rohde et al. 1996) and argues for the effectiveness of the rhizotomy. This result also provides evidence that a significant component of the pattern of Fos-like immunoreactivity is secondary to the increased large-diameter primary afferent input associated with withdrawal behavior. The decrease in Fos-like immunoreactivity in laminae V/VI on the ipsilateral side of the rhizotomized rats compared to the contralateral side or to intact withdrawing rats may also be due to decreased afferent input on the rhizotomized side. A loss of large-diameter proprioceptive input to lamina VI neurons may be relevant.

Several factors may contribute to the rhizotomy-induced increase in Fos-like immunoreactivity in laminae I/II during withdrawal. First, since large diameter primary afferents exert an indirect inhibitory effect on the firing of dorsal horn nociresponsive neurons (Abram et al. 1980; Lundberg 1983), including those in laminae I and V, it is possible that the enhanced Fos-like immunoreactivity ipsilateral to the rhizotomy resulted from a loss of withdrawal-associated inhibition. That is, movement associated with withdrawal would not only activate neurons in laminae III and IV, but also *suppress* the firing of neurons in the superficial dorsal horn. After rhizotomy, this would be manifested as increased Fos-like immunoreactivity, due to loss of inhibition.

In addition to altering some properties of dorsal horn neurons (Basbaum and Wall 1976), there is evidence that deafferentation leads to a reorganization of descending control systems (Hodge et al. 1983). Although there is conflicting evidence as to whether dorsal rhizotomy alters the distribution of descending monoaminergic pathways (Colado et al. 1988;

Marlier et al. 1991; Weil-Fugazza et al. 1989), which mediate a significant part of the inhibitory controls, Hodge et al. (Hodge et al. 1983) demonstrated that rhizotomy changes LC and RVM descending input from inhibitory to facilitatory. Thus, if a descending excitatory component is unmasked, then withdrawal-induced increases in LC and RVM activity could contribute to the rhizotomy-induced enhancement of withdrawal-related Fos-like immunoreactivity.

Capsaicin

There is considerable disagreement in the literature concerning the contribution of primary afferent C-fibers to the magnitude of the morphine withdrawal syndrome. Neonatal capsaicin treatment, which destroys C-fibers (Nagy 1982; Nagy et al. 1983) has either no effect on (Chahl 1988), decreases (Sharpe and Jaffe 1986), or increases (Sziksay et al. 1983) the signs of withdrawal. In the present study, we did not find a significant effect of neonatal capsaicin treatment on withdrawal-induced behavior, except for more severe weight loss (Rohde, in press, Brain Research). Although the capsaicin-treated withdrawing animals appeared to be more hyperexcitable than the intact withdrawing animals, this was difficult to quantitate. Importantly, capsaicin treatment did not significantly alter the number of Fos-like immunoreactive neurons in the lumbar cord of withdrawing rats compared to intact withdrawing rats. Surprisingly, we recorded increased numbers of Fos-immunoreactive neurons in laminae III through VI in the placebo-pelleted capsaicin-treated rats. Conceivably, sprouting of large fibers contributed to these changes.

We conclude that C-fiber activity is not a major contributor to the pattern of withdrawal-induced Fos-like immunoreactivity in the lumbar spinal cord.

Since noxious stimulation-induced activity of C fibers is a powerful stimulus to the induction of dorsal horn Fos-like immunoreactivity (Presley et al. 1990), our results also suggest that the syndrome of withdrawal is not associated with "painful" input from the periphery. Furthermore, since opioid receptors are concentrated on capsaicin-sensitive small-diameter afferents (Nagy 1982), our results indicate that withdrawal of morphine from "tolerant" primary afferent terminals is not a necessary source of the hyperactivity that drives the increase in Fos-like immunoreactivity in lamina I. The different results recorded in rhizotomized and capsaicin-treated rats are consistent with the hypothesis that the effects of dorsal rhizotomy on the pattern of Fos expression result from a loss of inhibitory controls exerted by large diameter primary afferent fibers.

Peripheral withdrawal

With the exception of diarrhea, we did not find significant signs of withdrawal when morphine-tolerant rats were injected with the peripherally acting opiate antagonist, naloxone-methiodide. Consistent with the lack of behavioral signs of withdrawal, the magnitude of Fos-like immunoreactivity in these rats did not differ from that in non-withdrawing rats. This provides support for the conclusion that the characteristic naloxone-induced withdrawal syndrome is largely a result of displacement of morphine from CNS opioid receptors. Taken together with our previous study (Rohde et al. 1996) that showed that lamina I neurons are labelled even when rats withdraw under anesthesia, which eliminates withdrawal-associated movements, we conclude that the enhanced Fos-like immunoreactivity in lamina I that occurs when withdrawal is precipitated by naloxone is not driven by peripheral afferent inputs.

Spinal withdrawal

In contrast to the minimal changes produced by peripheral withdrawal, we recorded marked *increases* in Fos-like immunoreactivity in laminae I/II and the ventral horn when naloxone-methiodide was injected intrathecally. Importantly, the withdrawal syndrome produced by intrathecal naloxone-methiodide was characteristic of a selective action at the lumbar and sacral spinal cord; motor activity was limited to the hindlimbs and tail. This differed significantly from the effect of intrathecal naloxone, which rapidly induced ptosis, but no signs of diarrhea. Based on these withdrawal signs, we conclude that intrathecal naloxone spreads rapidly to supraspinal sites, but does not cross the blood brain barrier. The fact that the pattern and magnitude of Fos-like immunoreactivity produced by systemic and intrathecal naloxone were comparable is consistent with this conclusion. This result contradicts Yaksh et al. (Yaksh et al. 1977) but is comparable to another report (Marshall and Buccafusco 1985) that found no difference in the withdrawal syndrome produced by systemic or intrathecal injection of naloxone. It follows that intrathecal naloxone, like systemic naloxone, can activate supraspinal inhibitory systems, which would explain why intrathecal naloxone-methiodide induced greater numbers of Fos-like immunoreactive neurons in laminae I through VI than did intrathecal naloxone. In other words, in the absence of supraspinal or peripheral withdrawal, spinal withdrawal alone leads to greater induction of the Fos protein when compared to systemic withdrawal. Thus, the increased Fos-like immunoreactivity in laminae I/II and the ventral horn in spinally-withdrawing rats may be due to lack of withdrawal-induced descending or peripheral inhibition and supports our results seen after spinal transection

or dorsal rhizotomy wherein *elimination* of supraspinal or peripheral input led to *increased* expression of the Fos protein.

Conclusions

This study provides evidence that the expression of Fos protein in the lumbar spinal cord during withdrawal from morphine is largely a result of neuronal activity generated within local circuits of the spinal cord dorsal horn. The fact that the focus of the increased Fos-like immunoreactivity during withdrawal occurs in the superficial dorsal horn, taken together with the evidence that increased pain responsiveness (hyperalgesia) occurs during withdrawal (Bederson et al. 1990; Wilcox et al. 1979; Yaksh et al. 1977) suggests that common elements underlie these two phenomena. Hyperalgesia results in part from noxious stimulation-evoked long term changes (central sensitization) of dorsal horn neurons (Woolf 1983). Conceivably changes comparable to those that underlie the compensatory response (i.e. latent sensitization) (Fry et al. 1980) that develops during chronic exposure to opioids also contribute to the central sensitization produced by noxious stimuli. Consistent with this formulation, we recently demonstrated that the number of spinal cord Fos-like immunoreactive neurons produced by hindpaw injection of formalin in the rat is significantly increased in rats that are tolerant to morphine (Rohde et al. 1993).

It is of interest that antagonism at the NMDA receptor blocks both noxious stimulus-evoked central sensitization (Woolf and Thompson 1991) and the development of tolerance at the spinal cord level (Ben et al. 1992; Elliott et al. 1994; Mao et al. 1994; Trujillo and Akil 1991). Since the dorsal horn contains high concentrations of NMDA receptors (Liu et al. 1994), and since NMDA is implicated in both central sensitization (Woolf and Thompson 1991) and

hyperalgesia (Coderre and Melzack 1992; Davar et al. 1991; Mao et al. 1992), it is possible that changes that occur in the dorsal horn contribute to withdrawal-induced neuronal activity and hyperalgesia. This proposal is supported by studies of Mao et al. (1994) which demonstrated that thermal hyperalgesia develops in association with the development of morphine tolerance and that co-administration of NMDA antagonists with morphine prevents the development of both hyperalgesia and tolerance. Thus, although supraspinal and peripheral input appear to modify the magnitude of Fos-like immunoreactivity, as evidenced by the enhancing effect of transection or dorsal rhizotomy, the pattern of Fos-like immunoreactivity is largely accounted for by activity within local spinal circuits.

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Figure 1. These low-power photomicrographs illustrate the distribution of Fos-like-immunoreactive neurons in the L4/5 segment of the spinal cord in: A: morphine-pelleted animals injected with naltrexone; B: morphine-pelleted spinally- transected animals injected with naltrexone; C: morphine-pelleted rhizotomized animals injected with naltrexone (left is rhizotomized side; for high power see Fig. 4); D: capsaicin-pretreated rats, morphine-pelleted and then injected with naltrexone; E: morphine-pelleted rats injected s.c. with naloxone-methiodide; and F: morphine-pelleted rats injected intrathecally with naloxone-methiodide. Arrowheads point to Fos-like immunoreactive neurons in laminae I/II. Arrows point to Fos-like immunoreactive neurons in the ventral horn. Calibration bar equals 500 μm in A-F.

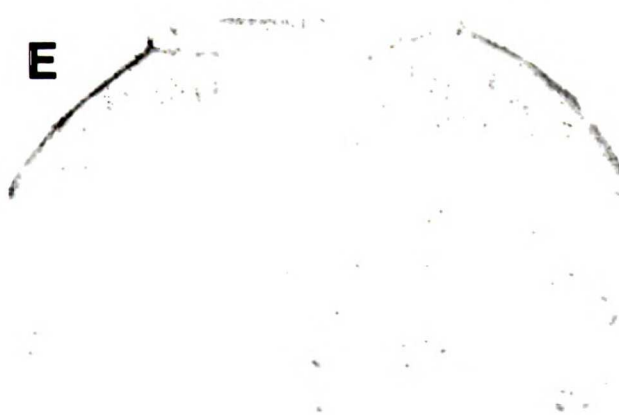


Figure 2. These high-power photomicrographs illustrate the distribution of Fos-like-immunoreactive neurons in the L4/5 segment of the spinal cord in A: morphine-pelleted intact rats injected with naltrexone; B: morphine-pelleted transected rats injected with naltrexone; and C: placebo-pelleted transected rats injected with naltrexone. There is increased Fos-like immunoreactivity in laminae I/II (arrowheads) in the transected withdrawing rats. Calibration bar equals 250 μ m in A-C.

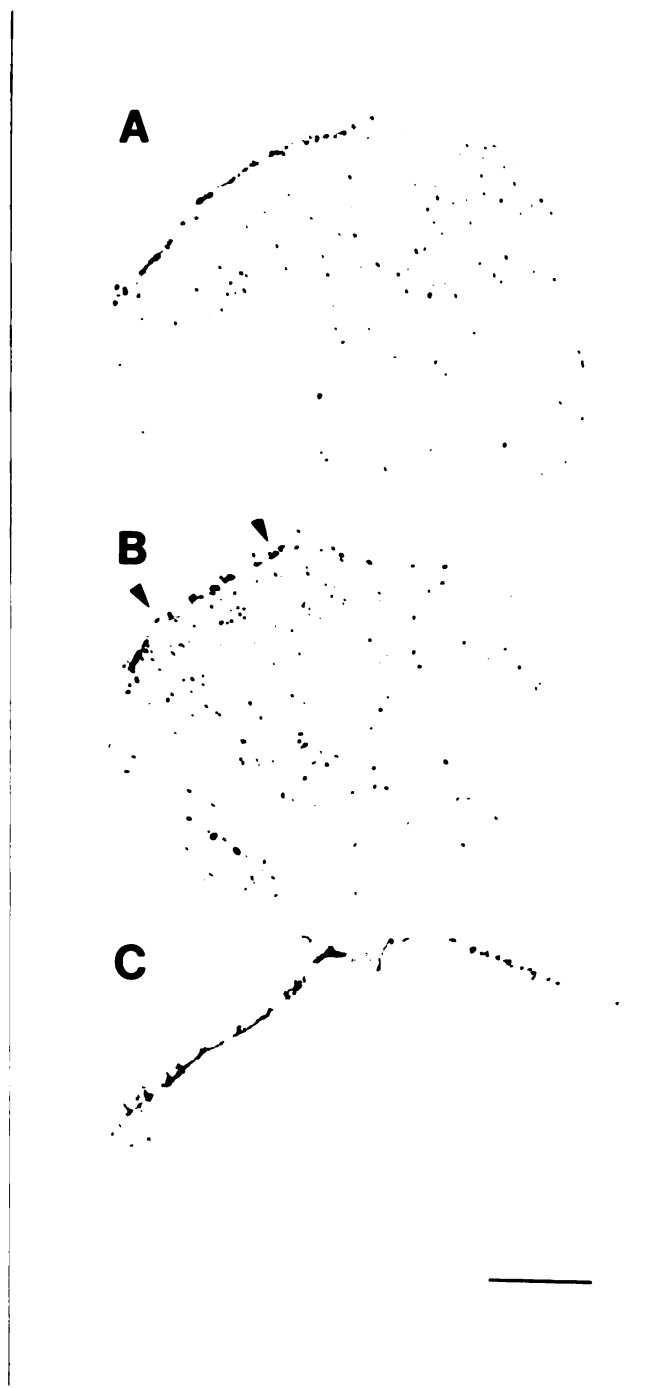


Figure 3. Number of Fos-like immunoreactive neurons per 50 μ m section in L4/5 in spinally-transected and intact withdrawing rats. The transected withdrawing rats (trans/mor/naltrexone) demonstrated significantly increased Fos-like immunoreactivity in laminae I/II and the ventral horn compared to the intact withdrawing rats (mor/naltrexone) and significantly greater Fos-like immunoreactivity in all laminae compared to the transected placebo-pelleted rats. Asterisks are compared to the transected/mor/naltrexone group. ** $p < .01$

Abbreviations - mor: morphine, plac: placebo; trans: transected

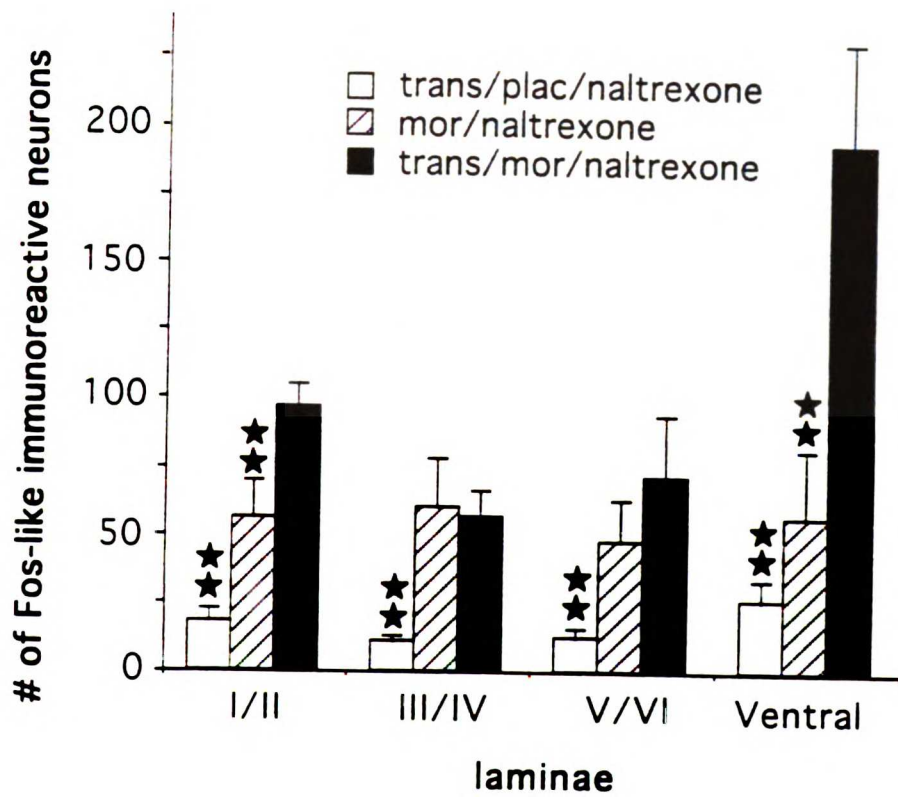


Figure 4. These high-power photomicrographs illustrate the distribution of Fos-like-immunoreactive neurons in the L4/5 segment of the spinal cord in A: morphine-pelleted rhizotomized rats injected with naltrexone (ipsilateral side); B: morphine-pelleted rhizotomized rats injected with naltrexone (contralateral side); C: morphine-pelleted intact rats injected with naltrexone; and D: placebo-pelleted rhizotomized rats injected with naltrexone (ipsilateral side). Note the increased Fos-like immunoreactivity in laminae I/II (arrowheads) and decreased Fos-like immunoreactivity in laminae III/IV (arrow) in the rhizotomized withdrawing rats on the ipsilateral side compared to the contralateral side or to intact withdrawing rats. Calibration bar equals 250 μm in A-D.

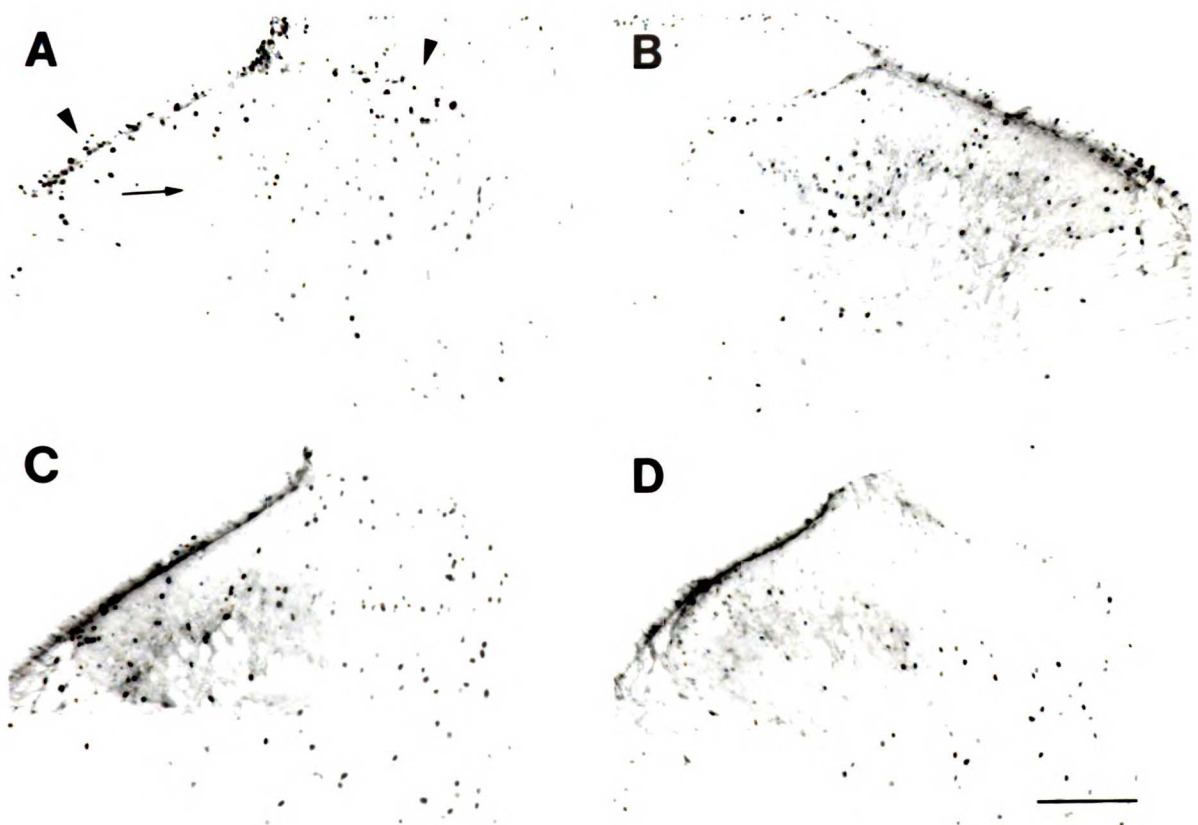


Figure 5. Number of Fos-like immunoreactive neurons *per side* of a 50 μ m section in L4/5 of rhizotomized and intact rats. *Ipsilateral* to the dorsal rhizotomy, withdrawing rats demonstrated significantly greater Fos-like immunoreactivity (rhizotomy, ipsi/mor/naltrexone) in laminae I/II, and significantly less Fos-like immunoreactivity in laminae III/IV and V/VI, compared to either the contralateral side of withdrawing rhizotomized (rhizotomy, contra/mor/naltrexone) rats or to either side of intact withdrawing rats (mor/naltrexone). We also recorded significantly more Fos-like immunoreactivity ipsilateral to the rhizotomy in laminae I/II compared to the ipsilateral side of rhizotomized placebo-pelleted rats. Note that since the data were tabulated for only one side of the cord in order to analyze the ipsilateral vs. contralateral sides of the spinal cord, the numbers of labelled cells are lower than in the other graphs. Asterisks are compared to the rhizotomy (ipsi)/mor/naltrexone group.

* $p < .05$, ** $p < .01$ For abbreviations, see Fig. 3, ipsi: ipsilateral to rhizotomy, contra: contralateral to rhizotomy

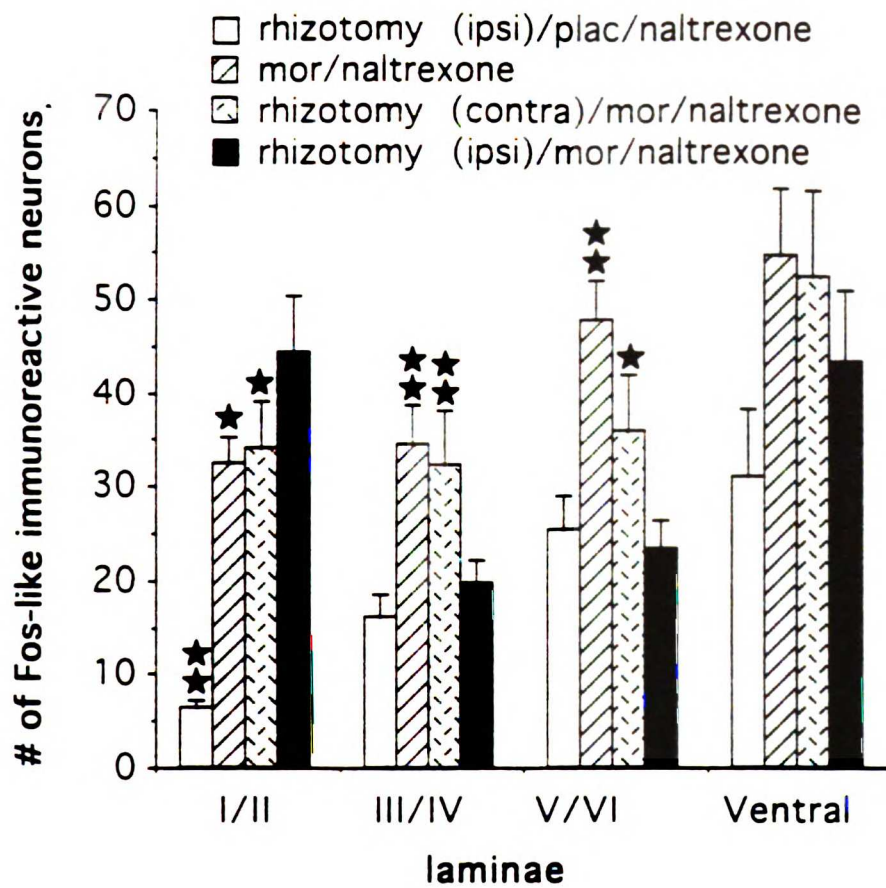


Figure 6. Number of Fos-like immunoreactive neurons per 50 μ m section in L4/5 in neonatal capsaicin-treated and non-treated withdrawing rats. There were no significant differences between the capsaicin-treated (capsaicin/mor/naltrexone) and the non-treated withdrawing rats (mor/naltrexone) in any laminae. As expected, the capsaicin-treated withdrawing rats demonstrated significantly greater Fos-like immunoreactivity in laminae I/II compared to capsaicin-treated placebo-pelleted rats. Asterisks are compared to the capsaicin/mor/naltrexone group.

** $p < .01$ For abbreviations, see Fig. 3

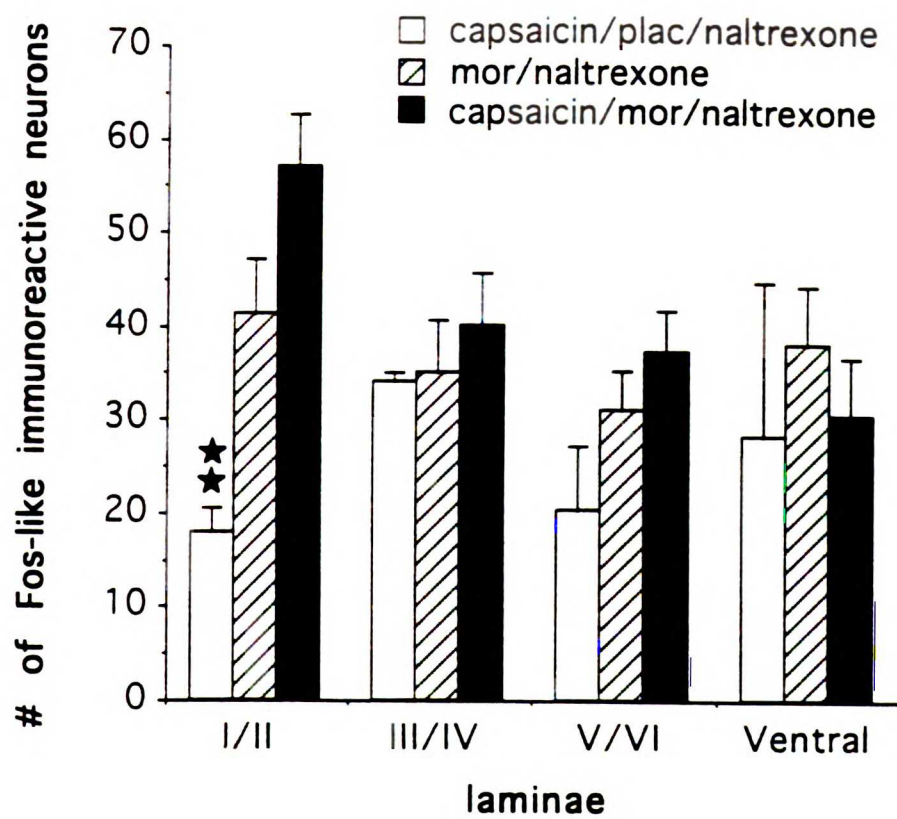


Figure 7. Number of Fos-like immunoreactive neurons per 50 μm section in L4/5 in peripherally- (mor/s.c. nal-methiodide) and systemically-induced (mor/s.c. naloxone) withdrawing rats. The peripherally withdrawing rats demonstrated significantly less Fos-like immunoreactivity in laminae I/II compared to the systemically withdrawing rats, but did not differ from the control, non-withdrawing rats (plac/s.c. nal-methiodide). Asterisks are compared to the mor/s.c. nal-methiodide group.

* $p < .05$ For abbreviations, see Fig. 3, nal: naloxone

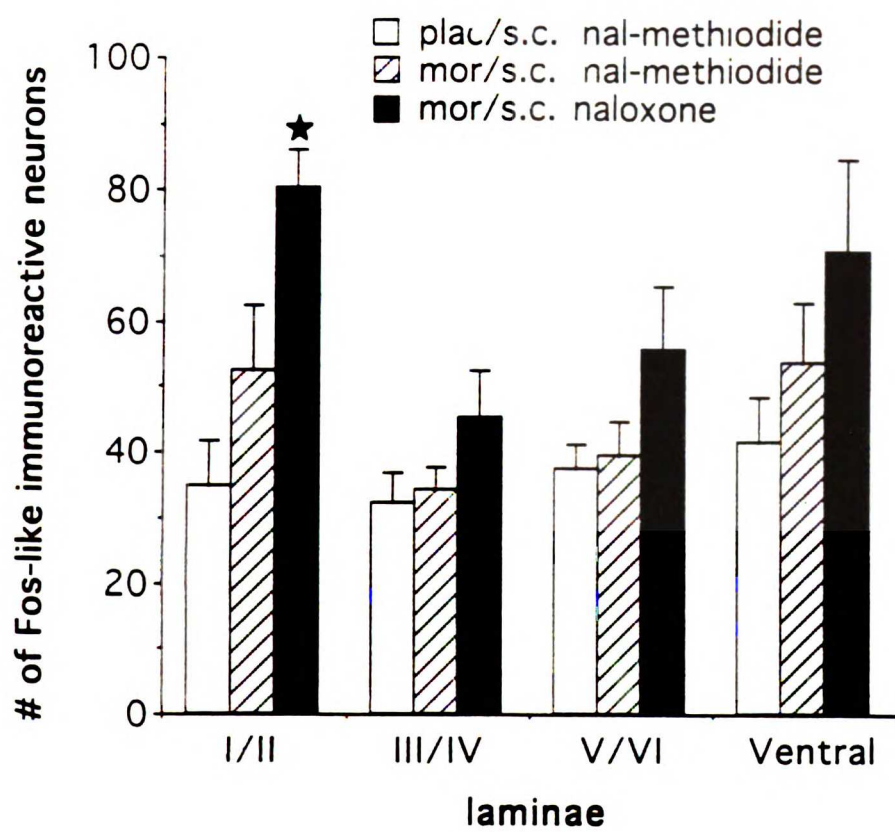
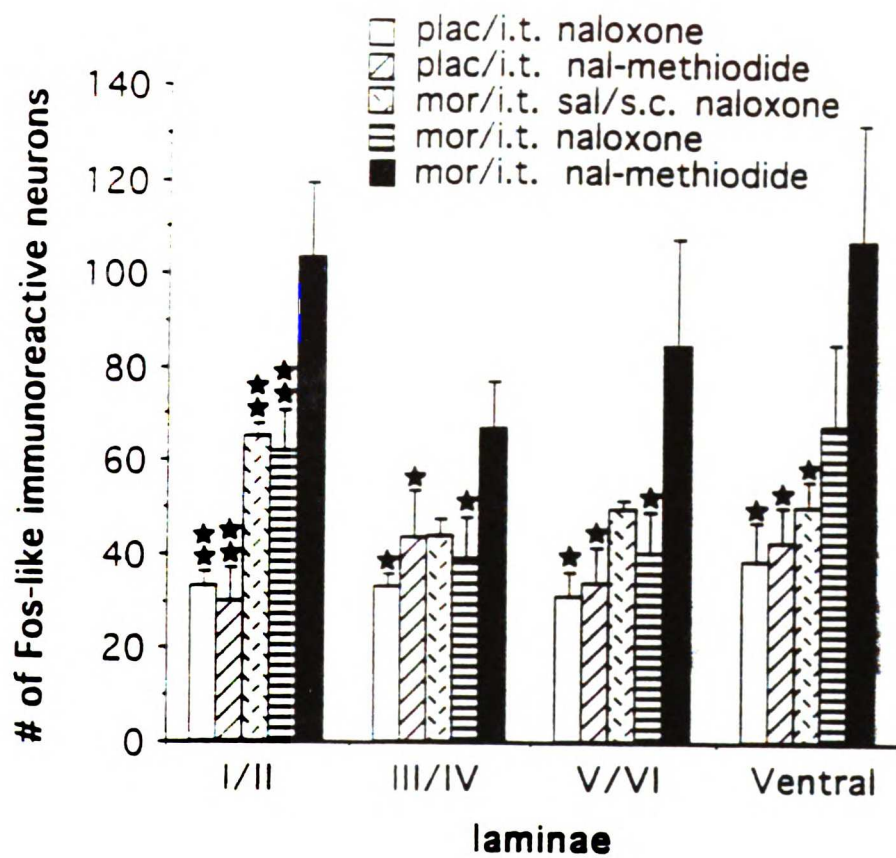


Figure 8. These high-power photomicrographs illustrate the distribution of Fos-like-immunoreactive neurons in the L4/5 segment of the spinal cord in A: morphine-pelleted rats injected intrathecally with naloxone-methiodide; B: morphine-pelleted rats injected intrathecally with naloxone; C: morphine-pelleted rats injected intrathecally with saline and s.c. with naloxone; and D: placebo-pelleted rats injected intrathecally with naloxone-methiodide. There is increased Fos-like immunoreactivity in laminae I/II (arrowheads) in the morphine-pelleted rats injected intrathecally with naloxone-methiodide. Calibration bar equals 250 μm .



Figure 9. Number of Fos-like immunoreactive neurons per 50 μ m section in L4/5 in intrathecally- and systemically-induced withdrawing rats. Fos-like immunoreactivity in intrathecally-induced withdrawing animals (mor/i.t. nal-methiodide) was significantly greater in laminae I/II and the ventral horn than in systemically-induced withdrawing rats (mor/i.t. sal/s.c. naloxone). The intrathecal *naloxone-methiodide* withdrawing rats also demonstrated significantly increased Fos-like immunoreactivity in laminae I - VI compared to the intrathecal *naloxone* group. In fact, the latter group (mor/i.t. naloxone) did not differ from the group that withdrew after systemic naloxone. Finally, Fos-like immunoreactivity in withdrawing animals injected intrathecally with naloxone-methiodide was significantly increased in all laminae when compared with intrathecally injected placebo-pelleted rats (plac/i.t. naloxone or plac/i.t. nal-methiodide). Note that the absolute numbers of Fos-like immunoreactive neurons in the morphine-pelleted rats injected intrathecally with saline and s.c. with naloxone in the peripheral and spinal withdrawal studies are different (Fig. 7 vs. 9). In these experiments, the tissue was immunoreacted separately, but importantly, all tissue from animals *within* a single study, including controls, were immunoreacted at the same time. Asterisks are compared to the mor/i.t. nal-methiodide group.

* $p < .05$, ** $p < .01$ For abbreviations, see Figs. 3, 7, sal: saline



Chapter 6

Contribution of Sacral Spinal Cord Neurons to the Autonomic and Somatic Consequences of Withdrawal from Morphine in the Rat

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Abstract

In this study, we monitored Fos-like immunoreactivity in the sacral spinal cord to identify neurons that are likely to contribute to the autonomic manifestations of opioid antagonist-precipitated withdrawal in morphine-tolerant rats. Injection of systemic antagonist increased the Fos-like immunoreactivity throughout the first sacral segment, particularly in laminae I/II, X, and in the sacral parasympathetic nucleus. Selective peripheral withdrawal, with a hydrophilic antagonist that does not cross the blood brain barrier, induced diarrhea, but no other withdrawal signs were evident. Compared to rats that withdrew systemically, peripheral withdrawal evoked significantly less Fos-like immunoreactivity in laminae V/VI, X and the sacral parasympathetic nucleus. By contrast, selective spinal withdrawal, by intrathecal injection of an opioid antagonist that does not cross the blood brain barrier, provoked hyperactivity of the hindlimbs and tail, but no diarrhea. These animals demonstrated significantly *increased* Fos-like immunoreactivity in laminae I/II, V/VI, the sacral parasympathetic nucleus, and the ventral horn compared to rats that withdrew systemically. Animals treated neonatally with capsaicin, to eliminate C-fiber input, demonstrated withdrawal behavior similar to intact withdrawing rats, except that the capsaicin-pretreated rats had significantly greater weight loss. However, this group had less Fos-like immunoreactivity in laminae V/VI, X and the sacral parasympathetic nucleus compared to the intact withdrawing rats.

These data suggest that withdrawal from morphine evokes hyperactivity of sacral neurons, particularly those involved in regions that process

nociceptive and autonomic information. Peripheral withdrawal is sufficient to induce diarrhea, but it does not fully explain the associated weight loss. Unmyelinated primary afferents may contribute a tonic peripheral inhibition to circuits that regulate gut motility and intestinal fluid transport. Taken together, these data suggest that chronic exposure to opioids induces a latent sensitization in sacral cord neurons that can be manifested as neuronal hyperactivity during withdrawal; this mechanism may underlie withdrawal-induced hyperalgesia and gut hypermotility.

Introduction

Opiates, such as morphine, and opioid peptides activate K⁺ channels (Aghajanian and Wang, 1986; North et al. 1987) and decrease adenyl cyclase through an inhibitory G protein (Griffin et al. 1985; Makman et al. 1988; Sharma et al. 1975). Although uncoupling of the opioid receptor from the inhibitory G protein is thought to underlie the development of tolerance to morphine (Christie et al. 1987; Cox 1991; Smith et al. 1988; Werling et al. 1989), recent studies indicate that other mechanisms are involved. In particular, since the opioid antagonist naloxone induces a withdrawal syndrome in tolerant animals and increases neuronal firing (Aghajanian 1978), it has been hypothesized that in the tolerant state, a compensatory response develops in neurons that express the opioid receptor or in neural circuits in which those neurons participate (Nestler 1992; Nestler and Tallman 1988; Sharma et al. 1975). This compensatory response is hypothesized to counteract the inhibitory effects of the opiate. The state of tolerance is, therefore, an equilibrium state that masks a latent hyperexcitability of neurons (Fry et al. 1980), which can be manifest by injection of an opioid antagonist.

With a view to identifying subpopulations of neurons in which the compensatory response can be unmasked, several laboratories have evaluated the distribution of the Fos protein, a marker of neuronal activity, after withdrawal is precipitated with an opioid antagonist in morphine-tolerant rats (Chieng et al. 1995; Hayward et al. 1990; Rohde et al. 1996; Stornetta et al. 1993). In previous studies, we found extensive expression of the Fos protein in neurons of the lumbar superficial dorsal horn (Rohde et al. 1996) after withdrawal from morphine. Our results raised the possibility

that the development of latent sensitization/hyperexcitability in neurons of the superficial dorsal horn contributes to withdrawal-induced hyperalgesia.

Autonomic symptoms, including diarrhea, are also a major feature of withdrawal. Given the high concentration of opioids in the sacral cord (de Groat et al. 1983; Glazer and Basbaum 1980) and the gastrointestinal system (Bu'Lock et al. 1983; Schultzberg et al. 1980), as well as the involvement of the sacral cord (de Groat et al. 1979; de Groat and Krier 1978) and opioids (Manara et al. 1980; Porreca et al. 1986; Stewart et al. 1978) in control of the colon, we wished to specifically address the changes that occur in the sacral cord during withdrawal, and to examine the contribution of peripheral and central opioid receptors to withdrawal-induced behavior and Fos-like immunoreactivity. We hypothesized that withdrawal-associated changes in sacral parasympathetic outflow and gastrointestinal activity are influenced by compensatory responses of opioid-responsive spinal cord neurons and that withdrawal patterns of Fos-like immunoreactivity in these areas can be used to identify populations of relevant neurons.

Thus, in the present study, we made rats tolerant to morphine and then monitored the expression of the Fos protein in the sacral cord one hour after withdrawal was precipitated systemically (subcutaneous injection with an opioid antagonist that acts in the central and peripheral nervous system), peripherally (subcutaneous injection with an opioid antagonist that does not cross the blood brain barrier), or spinally (intrathecal injection with an opioid antagonist that does not cross the blood brain barrier). In some experiments, we used naloxone-methiodide, an antagonist that does not cross the blood brain barrier, to selectively target either central or peripheral opioid receptors. We report that systemic withdrawal is associated with significant increases in Fos-like immunoreactivity in the sacral spinal cord,

particularly in regions that process nociceptive and autonomic information, viz. laminae I/II, X, and the sacral parasympathetic nucleus (SPN). The results after selective withdrawal at peripheral and central sites, however, suggest that there are differences between the central and peripheral opioid receptor contribution to withdrawal-induced behavior, control of gastrointestinal motility and Fos-like immunoreactivity.

Methods

Animals

Experiments were performed on male Sprague-Dawley rats (260-400 gm, Bantin and Kingman, Fremont, CA) with the approval of the Institutional Animal Care and Use Committee at UCSF. The animals were housed two to a cage in sawdust bedding in a temperature-controlled room with a 12h light/dark cycle. The rats had free access to food and water.

Experimental Treatment Groups

Systemic withdrawal

In the first set of experiments, we randomly assigned rats into five different treatment groups. Rats were administered either (1) daily morphine pellets followed by naltrexone injection (n=5), (2) daily morphine pellets followed by naltrexone injection, while under gaseous anesthesia with a mixture of halothane, to eliminate withdrawal-related movements (5% for induction and 1% for maintenance and a 2:1 flow ratio of N₂O: O₂ throughout the one hour withdrawal period) (n=4), (3) daily placebo pellets

followed by naltrexone injection, (n=4), (4) daily morphine pellets followed by saline injection (n=4) or (5) daily placebo pellets followed by naltrexone injection, while under halothane anesthesia for the one hour withdrawal period (n=4). All naltrexone injections were 10 mg/kg.

Peripheral withdrawal

The second set of experiments was designed to distinguish the contribution of central and peripheral opioid receptors to withdrawal behavior and the pattern and extent of withdrawal-induced Fos-like immunoreactivity. To selectively displace morphine from peripheral opioid receptors, we precipitated withdrawal with naloxone-methiodide, a quaternary form of naloxone that does not readily cross the blood brain barrier (BBB) (Bianchi et al. 1982; Brown and Goldberg 1985; Schroeder et al. 1991) and which has been used in numerous studies to distinguish central from peripheral actions of opiates (Bozarth 1994; Milne et al. 1990; Parsons and Herz 1990; Russell et al. 1982). In preliminary studies we found that at 1.0 mg/kg, naloxone-methiodide injected in dependent rats did not induce any signs of withdrawal. At 10 mg/kg, it precipitated withdrawal-diarrhea and at 20 mg/kg, other withdrawal signs were recorded including teeth chattering. Since teeth chattering is assumed to be centrally mediated (Bozarth 1994; Maldonado et al. 1992), we used 10 mg/kg naloxone-methiodide to precipitate withdrawal of opiates from peripheral receptors. Since the affinity and potency of naloxone-methiodide are only known relative to naloxone, to compare the effects of naloxone-methiodide with a systemically acting antagonist, we precipitated withdrawal in the control group with naloxone, instead of naltrexone. We used a dose of 1.0 mg/kg naloxone, which produces signs of withdrawal and levels of Fos-like immunoreactivity comparable to

those seen with 10 mg/kg of naltrexone. Thus, in this set of experiments, we randomly assigned rats into three different treatments groups. Rats were administered either (1) daily morphine pellets followed by naloxone-methiodide, 10 mg/kg (n=6), (2) daily morphine pellets followed by naloxone, 1.0 mg/kg (n= 5) or (3) daily placebo pellets followed by naloxone-methiodide, 10 mg/kg (n=5). These three groups of animals were also injected intrathecally with saline so they could be compared to animals in the next set of experiments. In separate experiments, we also injected some tolerant rats with 10 or 100 mg/kg of naloxone to determine if the effects of systemic withdrawal are due to the dose administered or due to the location of opioid receptors targeted.

Spinal withdrawal

The third set of experiments extended the analysis of the contribution of peripheral vs. central opioid receptors in the withdrawal syndrome. Six to ten μ g intrathecal naloxone has previously been used to induce withdrawal from systemic morphine (Marshall and Buccafusco 1985; Yaksh et al. 1977), but the potency of intrathecal naloxone-methiodide is not known relative to intrathecal naloxone. However, preliminary experiments showed that while doses of 1 μ g naloxone-methiodide did not induce withdrawal signs in morphine-pelleted rats, doses of 20 μ g of naloxone-methiodide produced seizure-like activity (intense tail whipping, squeaking) in placebo-pelleted rats. Thus, we used a dose of 10 μ g naloxone-methiodide to precipitate withdrawal. We randomly assigned rats into five different treatment groups. Rats were administered either (1) daily morphine pellets followed by direct intrathecal injection of naloxone-methiodide, 10 μ g (n=6), (2) daily placebo pellets followed by direct intrathecal injection of naloxone-

methiodide, 10 μ g (n=5), (3) daily morphine pellets followed by direct intrathecal injection of naloxone, 10 μ g (n=6) (4) daily placebo pellets followed by direct intrathecal injection of naloxone, 10 μ g (n=6), or (5) daily morphine pellets followed by direct intrathecal injection of saline followed immediately by a subcutaneous injection of naloxone, 1.0 mg/kg (n=5). The last group is the same group of animals noted above. All intrathecal injections were in 20 μ l saline and were injected directly into the intrathecal space at the L5/L6 vertebral space (cauda equina) using a 1 ml syringe, 27G needle, with the rat lightly anesthetized under 2% halothane with 2%N₂O: 1% O₂. These procedures were modified from those of Mestre et al. (1994).

Withdrawal after neonatal capsaicin treatment

The fourth set of experiments was designed to determine the contribution of primary afferent C-fibers, which express opioid receptors on their cell bodies and axons (Nagy 1982), to withdrawal-induced changes in behavior and Fos-like immunoreactivity. We administered 100 mg/kg capsaicin (8-methyl-N-vanillyl-6-nonenamide) on day one of birth, to destroy the majority of primary afferent C-fibers (Jancso et al. 1977; Nagy et al. 1983). In this set of experiments, we assigned rats into three different treatment groups. Two of the groups of rats were administered capsaicin as neonates; nine to eleven weeks later, they received either (1) daily morphine pellets followed by naltrexone, 10 mg/kg (n=8) or (2) daily placebo pellets followed by naltrexone, 10 mg/kg (n=3). The third group was made up of weight-matched non-capsaicin-treated adult rats that received daily morphine pellets followed by naltrexone injection (n=7). Capsaicin (Sigma, St. Louis, MO) was diluted in a 1:1 Tween 80: ethanol mixture. We injected the capsaicin

subcutaneously into the nape of neonates (100 mg/kg, 2 μ l/g) within 24 hours of birth with a 100 μ l Hamilton syringe attached to a 30G needle.

Protocol for the development of morphine tolerance

We implanted pellets (75 mg morphine sulfate pellet or placebo pellet, kindly provided by National Institute on Drug Abuse, Rockville, MD) subcutaneously on the lower back of the rats under gaseous anesthesia with a mixture of 5% halothane and a 2:1 flow ratio of N₂O: O₂, according to the following schedule: day 1 - one pellet, day 2 - two pellets, day 3 - three pellets. This protocol produces tolerance to the analgesic effects of morphine; although naive rats injected with acute morphine, 10 mg/kg, showed significant analgesia in the hindpaw formalin test (5%, 100 μ l), the three-day morphine-pelleted rats injected with an acute dose of morphine displayed pain behavior (flinching and licking) comparable to that of untreated rats injected with formalin. On day 4, we injected either s.c. naltrexone (10 mg/kg), intrathecal saline plus s.c. naloxone (1.0 mg/kg), intrathecal saline plus s.c. naloxone-methiodide (10 mg/kg), intrathecal naloxone (10 μ g) or intrathecal naloxone-methiodide (10 μ g). The s.c. drugs were diluted in 800 μ l physiological saline and injected into the nape of either awake or halothane-anesthetized rats. The opiate antagonists were purchased from Research Biochemicals International (Natick, MA).

Withdrawal behavior and immunocytochemical protocol

We monitored the animals for both qualitative (ptosis, diarrhea, teeth chattering, salivation, burrowing in the sawdust, and generalized hyperactivity) and quantifiable (weight loss) signs of withdrawal after the injection on day 4. After one hour, the rats were deeply anesthetized with

pentobarbital (200 mg/kg, i.p.) followed immediately by intracardiac perfusion with 100 ml 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 500-600 ml 10% formalin in 0.1 M phosphate buffer (PB). The sacral cord was removed one hour later, postfixed in 10% formalin for an additional three hours and then cryoprotected overnight in 30% sucrose in 0.1 M PB. Fifty μ m frozen sections were cut in the transverse plane and collected in 0.05 M PBS for immunocytochemical analysis. The sections were then immunostained for the Fos protein by the avidin-biotin-peroxidase method of Hsu et al. (Hsu, et al. 1981). The sections were washed with a solution of 0.05 M Tris PBS with 1% normal goat serum and 0.3% Triton-X and then incubated for 1 hr at room temperature in a blocking solution of 3% normal goat serum in 0.05 M Tris PBS with .3% Triton-X. The blocking solution was removed from the tissue, and the sections were incubated overnight at room temperature in the primary antiserum, which is a rabbit polyclonal antiserum directed against an *in vitro* translated product of *c-fos* (kindly provided by Dr. D. Slamon, UCLA). The antiserum was diluted 1:21,000 and preabsorbed against acetone-dried liver powder for 1 hr at 37°C and for 1 hr at 4° C prior to use. After the primary antiserum, the sections were washed and then incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Elite Kit; Vector Labs, Burlingame, CA). To visualize the Fos-like immunoreactivity as a black reaction product we used a nickel-diaminobenzidine glucose oxidase reaction following a protocol adapted from Llewellyn-Smith and Minson (1992).

Data Analysis

To quantify the number of Fos-like immunoreactive neurons, we first selected sections of the first sacral segment under dark-field illumination according to the shape of the gray matter. We then took photographs of these sections under light-field illumination at low (4X) power with Kodak technical pan film on a Nikon Microphot-FXA microscope. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. We printed individual sections of the spinal cord at 60X enlargement.

The photographs were divided into six segments: (i) the superficial laminae (laminae I, IIo, IIi), (ii) the nucleus proprius (laminae III and IV), (iii) lamina V/VI (iv) lamina X and the surrounding gray matter (v) the region containing the SPN, and (vi) the ventral spinal cord (laminae VII, VIII, and IX) (Fig 1). However, the number of cells in laminae V/VI may be overestimated because its border with lamina X and the SPN, which contain large numbers of labelled neurons, is difficult to identify. Since substance P (SP), serotonin and enkephalin immunoreactivity extends approximately 300 μm around the central canal, and since nociceptors terminate in this same area and cells within 300 μm of the central canal respond to noxious stimulation, (Nahin et al. 1983) we defined lamina X as the gray matter dorsal to the central canal forming a semicircle with a diameter of 350 μm (Fig 1).

Fos-like immunoreactive neurons were counted by a person blinded to the treatments. Three to four spinal cord sections were counted per rat and averaged so that each animal had a mean value for regional Fos-like immunoreactivity. The numbers of labeled cells per 50 μm section were compared using two-way analysis of variance (ANOVA) for treatment and

laminae; *post hoc* comparisons were performed using Fisher's Protected Least Significant Difference. The extent of weight loss was analyzed with a one-way ANOVA for treatment, with the same *post hoc* test.

Results

Signs of Withdrawal

Neither the rats in the placebo-pelleted groups nor those in the morphine-pelleted group that received saline demonstrated behavioral signs of withdrawal. Furthermore, these control groups did not show increased signs of mobility. By contrast, the awake morphine-pelleted group that received naltrexone or naloxone displayed clear signs of withdrawal including ptosis, diarrhea and weight loss, teeth chattering, salivation, burrowing in the sawdust and marked generalized hyperactivity. The tolerant animals injected intrathecally with naloxone showed signs of withdrawal similar to the systemically withdrawing rats, except that the former group did not have diarrhea. By contrast, the only sign of withdrawal displayed by morphine-pelleted rats that were injected subcutaneously with naloxone-methiodide was diarrhea. On the other hand, intrathecal injection of naloxone-methiodide in morphine-pelleted rats evoked hyperactivity and mild quivering of the hindlimbs and tail, but salivation, ptosis, and diarrhea were not evident. Finally, the capsaicin-treated rats that received morphine pellets followed by naltrexone displayed withdrawal signs similar to those in the intact withdrawing animals, except that the capsaicin-treated rats had more severe diarrhea and weight loss.

Weight Loss

Intact withdrawing animals lost significantly more weight than rats in each of the following groups: placebo-pelleted animals (18 g. vs. 2.5 g.) (Fig. 2A), tolerant rats injected with subcutaneous naloxone-methiodide (18 g. vs. 6.8 g.) (Fig. 2B), tolerant rats injected intrathecally with naloxone (18 g. vs. 8.7 g.) (Fig. 2C) and tolerant rats injected intrathecally with naloxone-methiodide (18 g. vs. 6.3 g.) (Fig. 2C). The latter three groups did not differ significantly in weight loss from one another, but lost significantly more weight than did the placebo-pelleted animals (about 7.3 g. vs. 2.5 g.). Capsaicin-treated animals that withdrew from morphine showed significantly *greater* weight loss (32.3 g. vs. 18 g.) than did intact withdrawing rats (Fig. 2D). The intact withdrawing rats include the morphine-pelleted animals that were injected with naltrexone or naloxone. The placebo-pelleted rats include animals injected with s.c. naltrexone, s.c. naloxone, intrathecal naloxone, intrathecal naloxone-methiodide, or the capsaicin-pretreated placebo-pelleted rats.

Effect of withdrawal on Fos-like immunoreactivity in the sacral cord

Spinal cord sections from the placebo-naltrexone, morphine-saline, and placebo-halothane-naltrexone groups contained only low levels of Fos-like immunoreactivity. Since the three control groups were not statistically different from one another in any of the laminae (Table 1), they were grouped together as the "control" group (Fig. 4). Compared to this control group, we recorded a significant increase in Fos-like immunoreactivity in the morphine-pelleted animals that received naltrexone (morphine/naltrexone) (Table 1, Figs. 3A,B and 4). This increase in Fos-like immunoreactivity was significant in all laminae (187% in laminae I/II, 169% in laminae III/IV, 354% in lamina V/VI, 800% in lamina X, 306% in the SPN,

and 104% in the ventral horn). The anesthetized withdrawing rats (morphine/halothane/naltrexone) demonstrated significantly less Fos-like immunoreactivity, in laminae III/IV (64%) compared to the awake withdrawal group (morphine/naltrexone), bringing the Fos-like immunoreactivity to the same levels seen in the control group. Although Fos-like immunoreactivity in the SPN decreased 41% in the anesthetized withdrawing rats, there was still a significant increase in Fos-like immunoreactivity in the SPN of the anesthetized withdrawing animals compared to the control animals. In the remaining laminae, however, we did not record a significant difference in Fos-like immunoreactivity between the anesthetized and the awake withdrawing animals. That is, Fos-like immunoreactivity in the anesthetized withdrawal animals was still significantly greater than that recorded in the control animals in these regions.

Effect of naloxone-methiodide-induced withdrawal on Fos-like immunoreactivity

Peripheral withdrawal induced by naloxone-methiodide evoked significantly less Fos-like immunoreactivity in laminae V/VI (43%), X (42%), and the SPN (37%), when compared to animals that were injected with naloxone and underwent systemic withdrawal (Figs. 3C and 5). The number of labelled neurons was, however, significantly greater in laminae I/II, X and the SPN compared to placebo-pelleted animals injected with s.c. naloxone-methiodide. Intrathecal injections of naloxone-methiodide, which limits withdrawal to the spinal cord, significantly increased Fos-like immunoreactivity in laminae I/II (65%), V/VI (52%), the SPN (43%) and the ventral horn (139%) compared to systemically withdrawing rats (tolerant

animals injected intrathecally with saline and then subcutaneously with naloxone) (Figs. 3E and 6). Although we recorded increased Fos-like immunoreactivity in the rats that withdrew after intrathecal naloxone-methiodide compared to those injected with intrathecal naloxone, there were no significant differences between these two groups (Fig. 3E,F). We recorded significantly increased Fos-like immunoreactivity in both intrathecally injected groups in all laminae (except III/IV for the intrathecal naloxone group) compared to their placebo controls. Neither the withdrawal behavior nor the magnitude of Fos-like immunoreactivity differed in rats that received 1.0, 10 or 100 mg/kg naloxone (data not shown).

Effect of neonatal capsaicin treatment on withdrawal-induced Fos-like immunoreactivity

The capsaicin-treated withdrawing rats displayed significantly less Fos-like immunoreactivity than intact withdrawing rats in laminae V/VI (52%), X (53%), and the SPN (42%) (Figs. 3A,D and 7), but significantly greater Fos-like immunoreactivity than in placebo-pelleted capsaicin-treated rats in laminae I/II, X and the SPN.

Discussion

In the present study, we addressed the possibility that sacral cord neurons are activated during opioid antagonist-precipitated withdrawal and evaluated the relationship between increased Fos protein expression (a marker of neuronal activity) and somatic and autonomic signs of withdrawal. We demonstrate that distinct behavior and patterns of Fos-like immunoreactivity

are produced when withdrawal is induced in morphine-tolerant rats by opioid antagonist injections targetted to different sites. Importantly, since the withdrawal behavior and magnitude of Fos-like immunoreactivity did not differ in rats that received 1, 10 or 100 mg/kg naloxone and since Yaksh et al. (1977) demonstrated that withdrawal-induced hyperreflexia did not vary from 10 μ g up to 40 μ g of intrathecal naloxone, we believe that our results after spinal, peripheral, or systemic withdrawal reflect the location of the opioid receptors targetted, not the dose of antagonist used.

Selective peripheral withdrawal produced the mildest syndrome, the only sign of withdrawal being diarrhea. By contrast, selective spinal withdrawal, with intrathecal naloxone-methiodide, produced intense withdrawal hyperactivity and the most extensive pattern of Fos-like immunoreactivity in the sacral cord. Since peripheral and spinal withdrawal induced different behavioral signs and since spinal withdrawal in the *absence* of supraspinal or peripheral withdrawal induced the most Fos-like immunoreactivity, we conclude that spinal and peripheral opioid receptors differentially contribute to the somatic and autonomic signs of withdrawal.

Fos expression in the superficial dorsal horn: effects of systemic withdrawal

In the sacral cord, we recorded a significant increase in Fos-like immunoreactivity in laminae I/II during withdrawal. This concurs with our earlier study on Fos-like immunoreactivity in the lumbar spinal cord (Rohde et al. 1996) and suggests that neurons in the superficial dorsal horn of the spinal cord are activated during naltrexone-precipitated withdrawal. As in the lumbar cord (Rohde et al. 1996), anesthetized withdrawing rats still demonstrated significantly greater Fos-like immunoreactivity in laminae

I/II than in control rats, but significantly *decreased* Fos-like immunoreactivity in laminae III/IV in the sacral cord, to levels seen in non-withdrawing rats. This supports our previous conclusion that it is only in lamina I and II that withdrawal-induced neuronal activation is independent of the movements associated with withdrawal.

Fos expression in the superficial dorsal horn: effects of peripheral withdrawal

Selective withdrawal of morphine from peripheral opioid receptors with naloxone-methiodide also induced significant increases in Fos-like immunoreactivity in the sacral superficial dorsal horn. This is very different from our study in the lumbar spinal cord (Rohde et al., in press, Neuroscience), in which peripheral withdrawal did *not* induce increased Fos-like immunoreactivity compared to non-withdrawing rats. These different results suggest that peripheral afferent input to the spinal cord during withdrawal differs between the lumbar and sacral cord. Since the sacral spinal cord receives input from the bladder and colon, and since withdrawal is associated with profound changes in gut motility and fluid transport, it is likely that activation of visceral afferents is an important contributor to the induction of Fos-like immunoreactivity in the sacral cord. In fact, activation of primary afferents in the pelvic nerve leads to increased Fos-like immunoreactivity in laminae I/II, X, and the SPN of the sacral spinal cord (Birder et al. 1991; Cruz et al. 1994; Lanteri-Minet et al. 1993; Traub et al. 1993). Since there are no comparable visceral inputs to the lumbar cord, peripheral withdrawal would have less impact in this region.

Fos expression in the superficial dorsal horn: effects of neonatal capsaicin treatment

Since the capsaicin-treated withdrawing rats did not show a decrease in laminae I/II Fos-like immunoreactivity compared to intact withdrawing animals, it appears that withdrawal-induced increased Fos-like immunoreactivity in this region is not mediated via C-fibers, a conclusion that we arrived at in our lumbar cord studies. Importantly, the pelvic nerve contains small myelinated as well as unmyelinated fibers (Hulsebosch and Coggeshall 1981; Langley and Anderson 1896). For example, although colonic afferents are exclusively unmyelinated (de Groat and Krier 1975), bladder afferents include A δ and C-fibers (de Groat and Ryall 1969). Together, these provide a mixed termination of visceral afferent A δ - and C-fiber projection to lamina I (Morgan et al. 1981). It is thus possible that the lack of effect of neonatal capsaicin treatment is due to a relatively large contribution of A δ visceral afferent input to the sacral cord during peripheral withdrawal.

Although some authors have described a contribution of C-fibers to visceral stimulation-induced Fos-like immunoreactivity in sacral laminae I/II (Cruz et al. 1994), the importance of C-fibers may depend on whether the stimulus is chemical or electrical. For example, capsaicin pretreatment did not block the response to electrical stimulation but almost completely blocked the response to chemical irritation of the bladder (Birder et al. 1991). Presumably, the A δ component of the bladder afferents maintained the response to electrical stimulation. Another possibility for the lack of effect of neonatal capsaicin on spinal cord Fos-like immunoreactivity is that there was an incomplete loss of C-fibers after neonatal capsaicin treatment. Compensatory sprouting of surviving C-fibers (Cervero et al. 1984) and reappearance of CGRP and SP immunoreactive axons in the dorsal horn

(Hammond and Ruda 1991) may also be relevant. Importantly, even when capsaicin has eliminated up to 95% of C-fibers (Jancso et al. 1977; Nagy et al. 1980; Scadding 1980) dorsal horn neuronal responses to C-fiber input may only decrease by 50% in the adult (Cervero et al. 1984).

Fos expression in the superficial dorsal horn; effects of spinal withdrawal

Rats injected with intrathecal naloxone-methiodide showed significantly greater Fos-like immunoreactivity in laminae I/II compared to systemically withdrawing rats. Similar increases in laminae I/II Fos-like immunoreactivity were seen in the lumbar cord when spinal withdrawal was induced with naloxone-methiodide or when morphine withdrawal occurred in rats that had undergone spinal transection or dorsal rhizotomy (Rohde et al. 1995). In the latter study, we found that the intrathecal naloxone group did not differ in Fos-like immunoreactivity from the systemic withdrawal group; both groups demonstrated significantly less Fos-like immunoreactivity than the intrathecal naloxone-methiodide group. We hypothesized that the naloxone spread supraspinally and released descending inhibitory controls while the naloxone-methiodide remained localized in the spinal cord. In the present study, the intrathecal naloxone and intrathecal naloxone-methiodide withdrawing rats did not differ significantly from each other; both groups demonstrated significantly greater Fos-like immunoreactivity than the systemically withdrawing group. Conceivably, the different results in the lumbar and sacral cords reflect the fact that descending inhibitory controls that we hypothesized are activated during systemic withdrawal are counterbalanced by the greater contribution of peripheral input in the sacral cord. Taken together, these

data suggest that laminae I/II neurons are activated by either local or peripheral input and that C-fiber input is not necessary for this activation.

Fos expression in lamina X and the SPN

We recorded an eight-fold increase in lamina X Fos-like immunoreactivity and a 300% increase in SPN Fos-like immunoreactivity after withdrawal compared to non-withdrawing rats. Since numerous studies have shown large increases in Fos-like immunoreactivity in lamina X and the SPN after distention and/or noxious stimulation of the bladder (Birder and de Groat 1993; Cruz et al. 1994), or colorectal distention (Lanteri-Minet et al. 1993; Traub et al. 1993), it is possible that withdrawal-induced changes in colon and/or bladder pressures induced the changes in Fos-like immunoreactivity recorded in these regions.

That systemic withdrawal increased lamina X and SPN Fos-like immunoreactivity to a much greater extent than did peripheral withdrawal supports the notion that induction of Fos is secondary to long-term changes/sensitization of intrinsic spinal cord neurons that express the opioid receptor. However, in contrast with our results in laminae I/II, where we did not find any effect of capsaicin treatment, we recorded a significant decrease in Fos-like immunoreactivity in lamina X and the SPN in the capsaicin-treated rats. This is consistent with the fact that peripheral withdrawal induced increased Fos-like immunoreactivity in these two regions compared to non-withdrawing rats. The loss of Fos-like immunoreactivity in lamina X and the SPN of capsaicin-treated withdrawing rats vs. non-treated withdrawing rats is comparable to the loss seen in these regions after bladder stimulation in capsaicin-treated rats (Cruz et al. 1994) and suggests that C-fiber input, most likely from pelvic nerve visceral

afferents, contributes to neuronal activity in these regions. Cruz et al. (1994) recorded a smaller, but still significant, capsaicin-induced loss of Fos-like immunoreactivity in laminae I/II after bladder stimulation. Importantly, visceral afferent input from the pelvic nerve, which is composed of small myelinated and unmyelinated fibers (Hulsebosch and Coggeshall 1981; Langley and Anderson 1896), may differ between these laminae. Specifically, the entering visceral afferents split into a lateral and medial collateral pathway which terminate in distinct parts of laminae I/II, X and the SPN.

Taken together, these studies suggest that Fos-like immunoreactivity in lamina X and the SPN during withdrawal results both from changes in the properties of spinal cord neurons that express the opioid receptor, or which are part of opioid receptor local circuits, and from increased primary afferent C-fiber input. These changes may contribute to withdrawal-induced hyperalgesia and/or increased autonomic activity.

Contribution of central and peripheral opioid receptors, and primary afferent neurons to withdrawal-induced gastrointestinal activity

There is evidence that acute and chronic opioids regulate gut activity by both central (Gold and Pottash 1981; Manara et al. 1980; Schreier and Burks 1980; Stewart 1982) and/or peripheral mechanisms (Bianchetti et al. 1985; Bozarth 1994; Koob, et al. 1992). In the present study, we observed diarrhea in more than 90% of the rats injected with naltrexone or naloxone, both of which cross the BBB, and in more than 50% of the rats injected systemically with naloxone-methiodide, which does not cross the BBB, but in none of the rats that received intrathecal naloxone-methiodide. These results suggest that withdrawal of opioids from peripheral receptors is both necessary and

sufficient for the induction of withdrawal diarrhea. On the other hand, since intrathecal naloxone-methiodide induced greatly increased Fos-like immunoreactivity in the SPN of animals that did not develop diarrhea, activation of SPN neurons is not sufficient for the induction of diarrhea. By contrast, since peripheral naloxone-methiodide also increased SPN Fos-like immunoreactivity, activation of the SPN may be necessary for diarrhea to occur. This conclusion is supported by the study of Vaught et al. (Vaught et al. 1983) which found that intrathecal opioids produce analgesia but do not affect gastrointestinal motility in the rat. Surprisingly, although peripheral, but not spinal, withdrawal is sufficient to induce diarrhea, there was comparable weight loss in these two groups. Since opioids are believed to initiate changes in gut *motility* at both central and peripheral sites, but to alter gut fluid *transport* only at central sites (Brown and Miller 1983; Jiang et al. 1990; Primi et al. 1986), the additional weight loss recorded in systemically withdrawing rats may be due to the effect of supraspinal systems on fluid transit. Some studies (Bozarth 1994) that did not report peripherally-induced withdrawal diarrhea observed the rats for less than 20 minutes after antagonist injection. Since diarrhea often appears after 20 minutes, it may be significant that we monitored the rats for 1 hour after the injection of opioid antagonist.

Despite the *decrease* in Fos-like immunoreactivity in the SPN in capsaicin-treated withdrawing rats, these rats had significantly *greater* weight loss and diarrhea when compared to intact withdrawing animals. This contrasts with Sharpe and Jaffe's (1986) report that neonatal rats treated with capsaicin displayed weight loss similar to intact withdrawing rats. Methodological differences between our two studies may be relevant. In the latter study, the rats received a smaller dose of capsaicin on day two and

were on a different tolerance and withdrawal protocol that induced fewer withdrawal signs and less weight loss than did the protocol used in this study. Since the C-fiber neuropeptide, calcitonin-gene-related peptide (CGRP) primarily inhibits gastrointestinal motility and transit (Lenz 1988) and has been shown to inhibit colonic inflammation (Bernstein et al. 1993), it is possible that capsaicin-induced loss of CGRP (Gibson et al. 1984; Jancso et al. 1977; Nagy et al. 1980; Pohl et al. 1990) underlies the increase in diarrhea and weight loss that we observed in the withdrawing capsaicin-treated rats. Loss of the protective effects of CGRP on the gut may contribute to increased gastrointestinal motility, transit and inflammation during withdrawal, leading to greater diarrhea and weight loss. Since the central effects of morphine's antidiarrheal and anti-propulsive actions are believed to be vagally-mediated (Ewart and Wingate 1983), it is also possible that capsaicin's effects on the vagus nerve, which is 90% unmyelinated (Douglas and Ritchie 1962) affects withdrawal-induced weight loss. Taken together, we conclude that withdrawal-induced diarrhea is dependent upon withdrawal of morphine from peripheral opioid receptors but that diarrhea accounts for only a part of the weight loss seen during withdrawal.

Summary and Conclusions

Systemic withdrawal induces Fos-like immunoreactivity throughout the sacral cord, particularly in areas of the cord that transmit and regulate nociceptive and autonomic functions, laminae I/II, X and the SPN. It has been proposed that tolerance results from the development of a compensatory response in neurons that express the opioid receptor (Nestler 1992; Sharma et al. 1975). The fact that the focus of the increased Fos-like immunoreactivity during withdrawal occurs in the superficial dorsal horn,

lamina X and the SPN, taken together with the evidence that increased pain responsiveness (hyperalgesia) and gut hypermotility occur during withdrawal (Bederson et al. 1990; Bianchetti et al. 1985; Schreier and Burks 1982; Wilcox et al. 1979; Yaksh et al. 1977) suggests that common elements underlie these phenomena. Conceivably, changes comparable to those that underlie the compensatory response, i.e. latent sensitization (Fry et al. 1980) that develops during chronic exposure to opioids, contribute to the central sensitization produced by noxious stimuli. According to this formulation, neurons in the sacral cord and/or in the gut are also sensitized during the development of morphine tolerance; opiate antagonists unmask this sensitization, which leads to the withdrawal syndrome, a major feature of which is diarrhea.

We conclude that spinal and peripheral withdrawal activate different populations of laminae I/II neurons; peripheral withdrawal may selectively activate laminae I/II neurons that receive visceral afferent input. Although SPN neuronal activity is induced by both peripheral and spinal withdrawal, the induction of the Fos protein in the SPN is not sufficient to induce diarrhea; in fact, diarrhea appears to be peripherally-mediated. The increased weight loss observed in the capsaicin-treated withdrawing animals may have resulted from the loss of primary afferent CGRP inhibitory control in the gut. A better understanding of the central and peripheral opioid receptor sites that are targeted should provide new approaches to minimizing some of the clinical discomforts of withdrawal.

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Table 1. Fos-like immunoreactive neurons in different treatment conditions.

laminae	placebo/ naltrexone	morphine/ saline	placebo/halo/ naltrexone	morphine/ naltrexone
I/II	30.0 +/- 9.5	34.0 +/- 7.8	30.3 +/- 8.0	89.4 +/- 10.0 **
III/IV	54.5 +/- 11.6	50/3 +/- 15.2	19.8 +/- 7.2	113.2 +/- 25.0 *
V/VI	31.0 +/- 9.5	30.0 +/- 10.8	12.0 +/- 5.6	109.4 +/- 14.9 * *
SPN	35.5 +/- 12.8	48.0 +/- 11.9	16.0 +/- 5.6	134.0 +/- 18.6 * *
X	16.0 +/- 5.4	17.8 +/- 5.8	8.5 +/- 4.2	125.8 +/- 15.5 * *
Ventral	23.0 +/- 5.3	34.5 +/- 10.8	20.0 +/- 8.4	52.8 +/- 8.6 *

Number of Fos-like immunoreactive neurons (+/-SEM) in different laminae of the first segment of the sacral cord (S1). Naltrexone-precipitated withdrawal (morphine/naltrexone) induced a significant increase in Fos-like immunoreactivity in all laminae compared to the control animals (placebo/naltrexone, morphine/saline, and placebo/halo/naltrexone); the latter three did not differ significantly from each other.

Abbreviations: halo = halothane *P < .05, **P < .01

Figure 1. Diagram of an S1 segment showing specific regions that were analyzed for Fos-like immunoreactivity. The region enclosing lamina X was defined by a semicircular template, of radius 350 μm , placed with its ventral border crossing through the central canal. The sacral parasympathetic nucleus was also defined with a template.

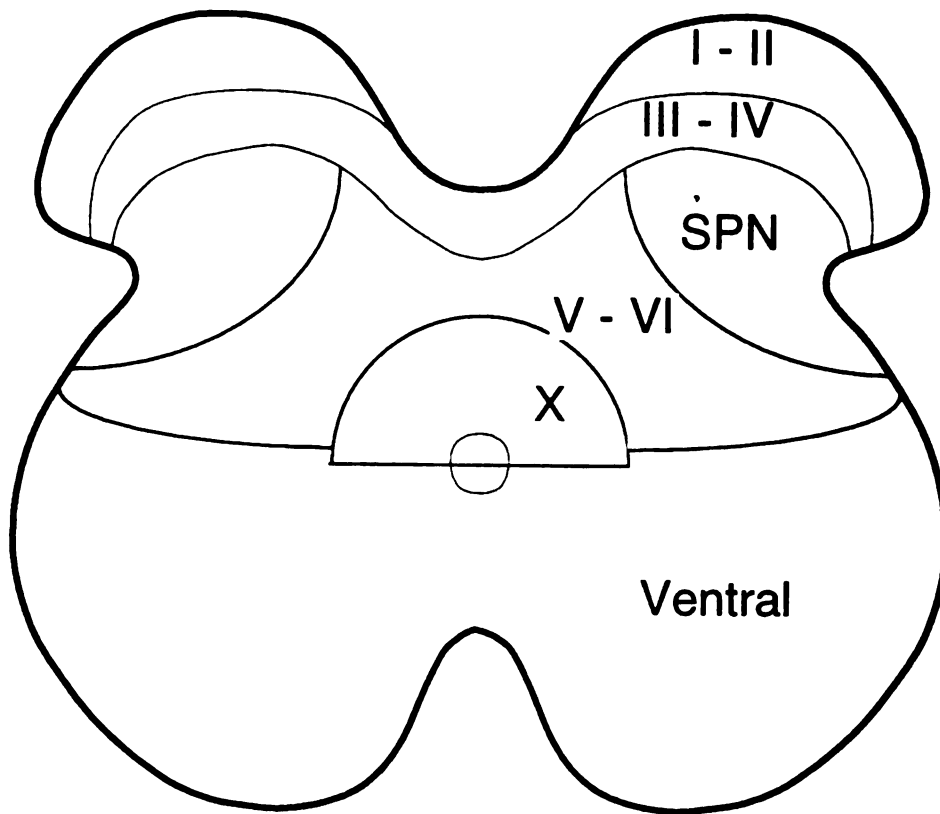


Figure 2. Percent weight loss in the one hour observation period in A: systemically withdrawing (morphine/naltrexone) vs the placebo-pelleted rats; B: systemically withdrawing (morphine/s.c. naloxone) vs. peripherally withdrawing (morphine/s.c. naloxone-methiodide) vs. the placebo-pelleted rats; C: systemically withdrawing (morphine/s.c. naloxone) vs. the intrathecal naloxone- or intrathecal naloxone-methiodide-injected withdrawing rats vs. the placebo-pelleted rats; and D: systemically withdrawing (morphine/naltrexone) vs. capsaicin-pretreated systemically withdrawing vs. the placebo-pelleted rats (D). Although the ANOVA was calculated with all of the groups together, they are graphed separately for ease of observation and interpretation. The withdrawing rats lost significantly more weight than the non-withdrawing rats (A), the peripherally withdrawing rats (B), or the intrathecally-injected withdrawing rats (C). However, the capsaicin-treated withdrawing rats lost significantly more weight than the intact systemically withdrawing rats (D). All of the withdrawing rats lost significantly more weight than the placebo-pelleted rats, but for clarity, asterisks were omitted. * $P < .05$, ** $P < .01$

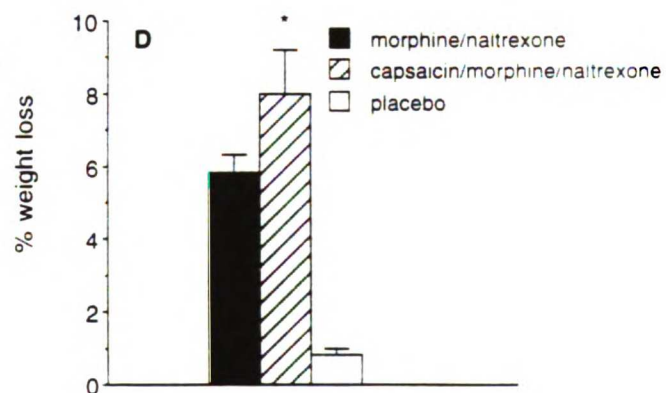
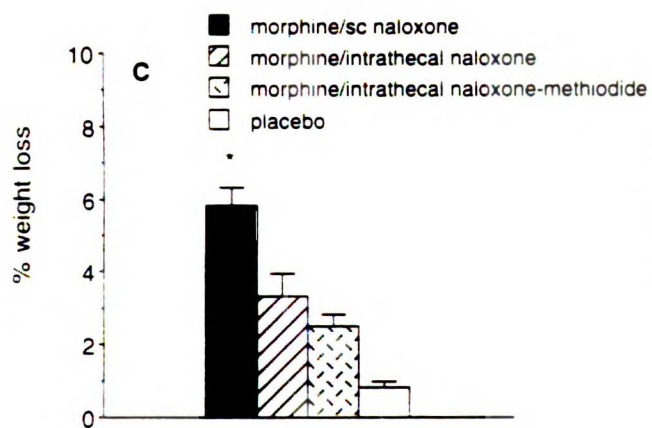
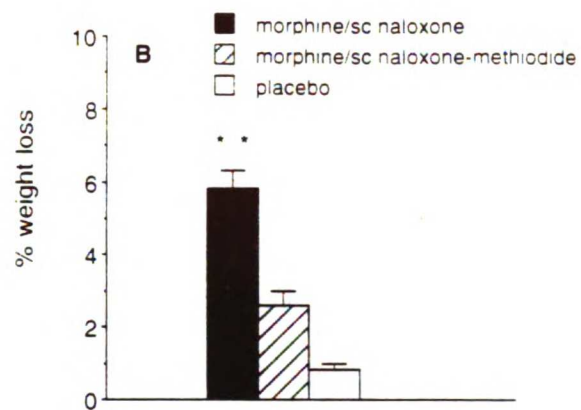
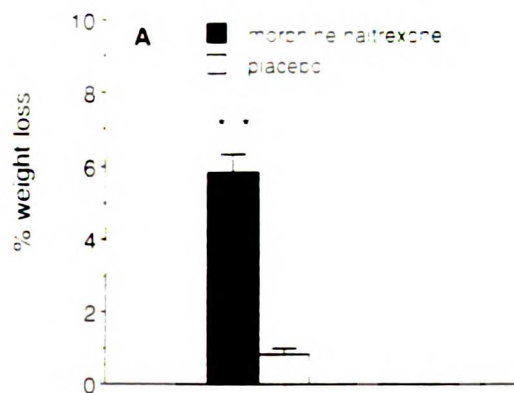


Figure 3. These photomicrographs illustrate the distribution of Fos-like-immunoreactive neurons in the S1 segment of the spinal cord in A: morphine-pelleted rats injected with naltrexone; B: placebo-pelleted rats injected with naltrexone; C: morphine-pelleted rats injected subcutaneously with naloxone-methiodide; D: capsaicin-pretreated morphine-pelleted rats injected with naltrexone; E: morphine-pelleted rats injected intrathecally with naloxone-methiodide; and F: morphine-pelleted rats injected intrathecally with naloxone. Asterisks point to Fos-labelled neurons in laminae I/II. Arrowheads point to Fos-like-immunoreactive neurons in the SPN. Arrows point to Fos-like-immunoreactivity in lamina X. Note the increased Fos-like immunoreactivity in the withdrawing animals (A) throughout the section, particularly in laminae I/II, X and the SPN, the decreased FLI in lamina X and the SPN in the peripherally withdrawing rats (C) and in the capsaicin-pretreated withdrawing rats (D), and the increased Fos-like immunoreactivity in laminae I/II in the intrathecally injected rats (E and F). Calibration bar = 500 μ m

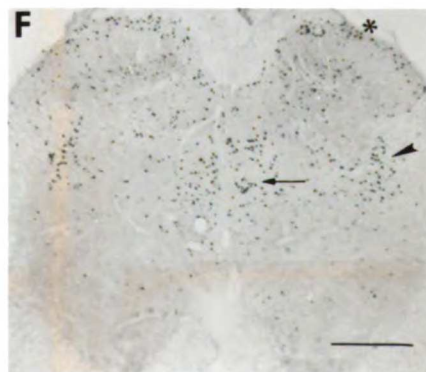
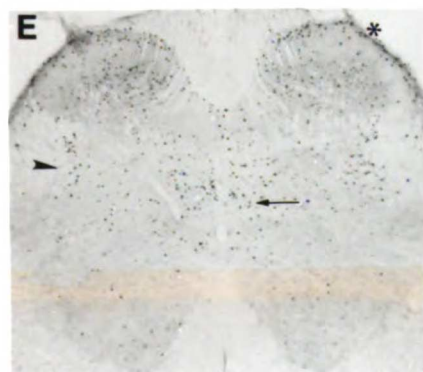
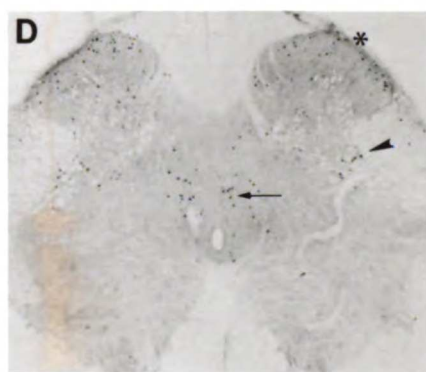
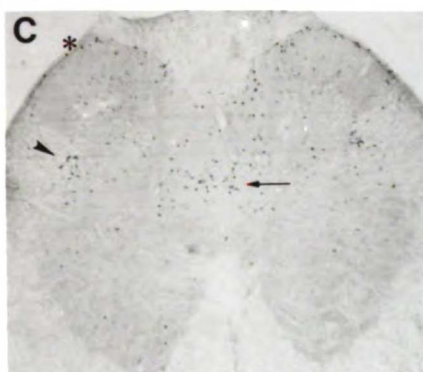
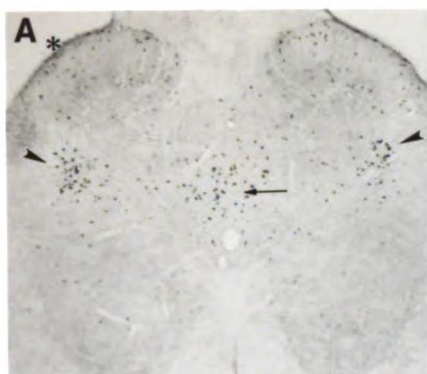


Figure 4. Induction of Fos-like immunoreactivity in the sacral spinal cord after naltrexone-precipitated withdrawal. Number of labeled cells per 50 μ m section. There is a significant increase in Fos-like immunoreactivity in all laminae in systemically withdrawing rats (morphine/naltrexone) (n=5) compared to control animals (morphine/saline, placebo/naltrexone, placebo/halothane/naltrexone) (n=12). Halothane significantly reduced Fos-like immunoreactivity in withdrawing rats (morphine/halothane/nalt) (n=4) in laminae III/IV, to levels seen in awake non-withdrawing rats. Importantly, with the exception of laminae III/IV, Fos-like immunoreactivity was still greater in all laminae in anesthetized withdrawing rats compared to non-withdrawing rats. F *P < .05, **P < .01

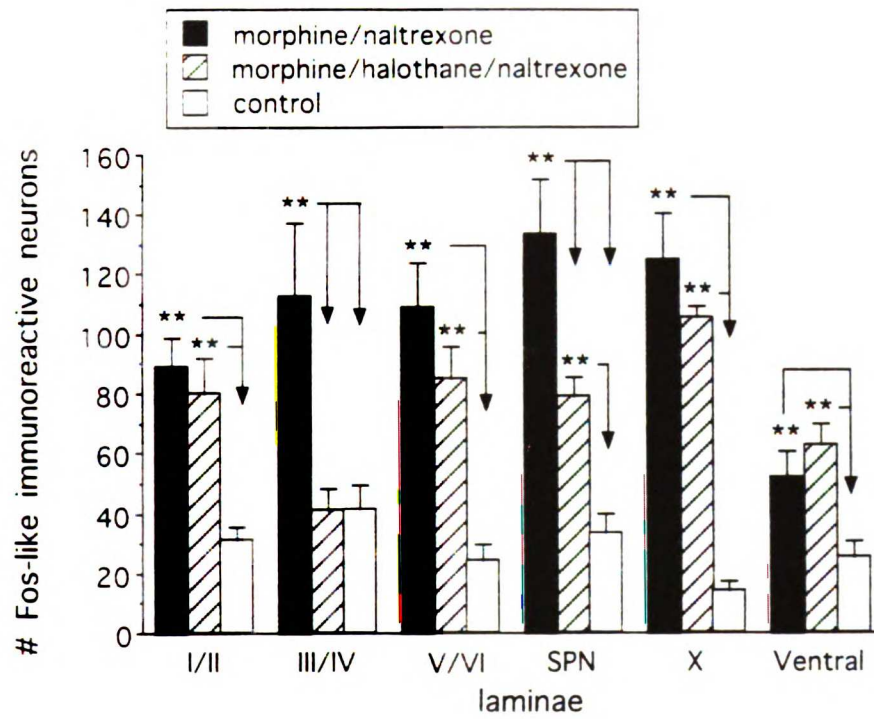


Figure 5. Induction of Fos-like immunoreactivity in the sacral cord after systemic (morphine/sc naloxone) and peripheral (morphine/sc naloxone-methiodide) withdrawal. Number of labeled cells per 50 μ m section. Systemically withdrawing rats (n=5) showed significantly increased Fos-like immunoreactivity in laminae I/II, V/VI, X and the SPN compared to peripherally withdrawing rats (n=5). There was, however, a significant increase in Fos-like immunoreactivity in laminae I/II, X, and the SPN in peripherally withdrawing rats vs. placebo-pelleted animals that received naloxone-methiodide (n=5). The withdrawing group injected with s.c. naloxone also demonstrated significantly increased Fos-like immunoreactivity in all laminae compared to the placebo-pelleted group, but for clarity, asterisks were omitted. **P < .01

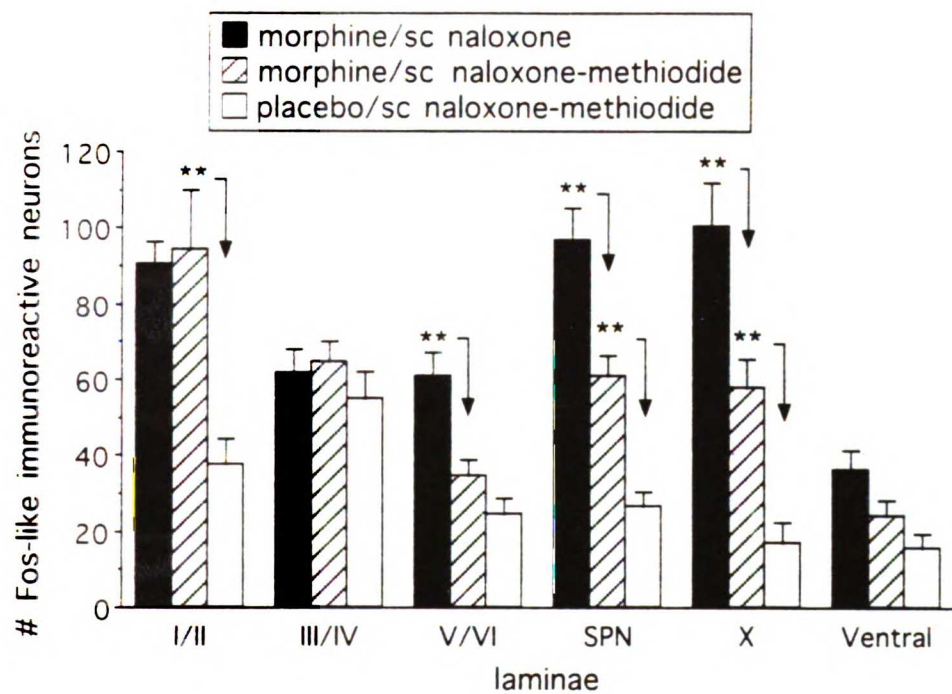


Figure 6. Induction of Fos-like immunoreactivity in the sacral cord after spinal (morphine/intrathecal naloxone-methiodide) and systemic (morphine/sc naloxone) withdrawal. Numbers of labeled cells per 50 μ m section. Spinally withdrawing rats (morphine/intrathecal naloxone-methiodide) (n=6) showed significantly increased Fos-like immunoreactivity in laminae I/II, V/VI, the SPN and the ventral horn compared to the systemically withdrawing rats (morphine/s.c. naloxone) (n=5). and increased Fos-like immunoreactivity in all laminae compared to the placebo-pelleted group (placebo/intrathecal naloxone-methiodide) (n=6), but for clarity, asterisks were omitted. Although the tolerant rats injected with intrathecal naloxone (n=6) did not differ in Fos-like immunoreactivity from those injected with intrathecal naloxone-methiodide, the former group only demonstrated increased Fos-like immunoreactivity in laminae I/II and V/VI compared to systemically withdrawing rats.

*P < .05, **P < .01

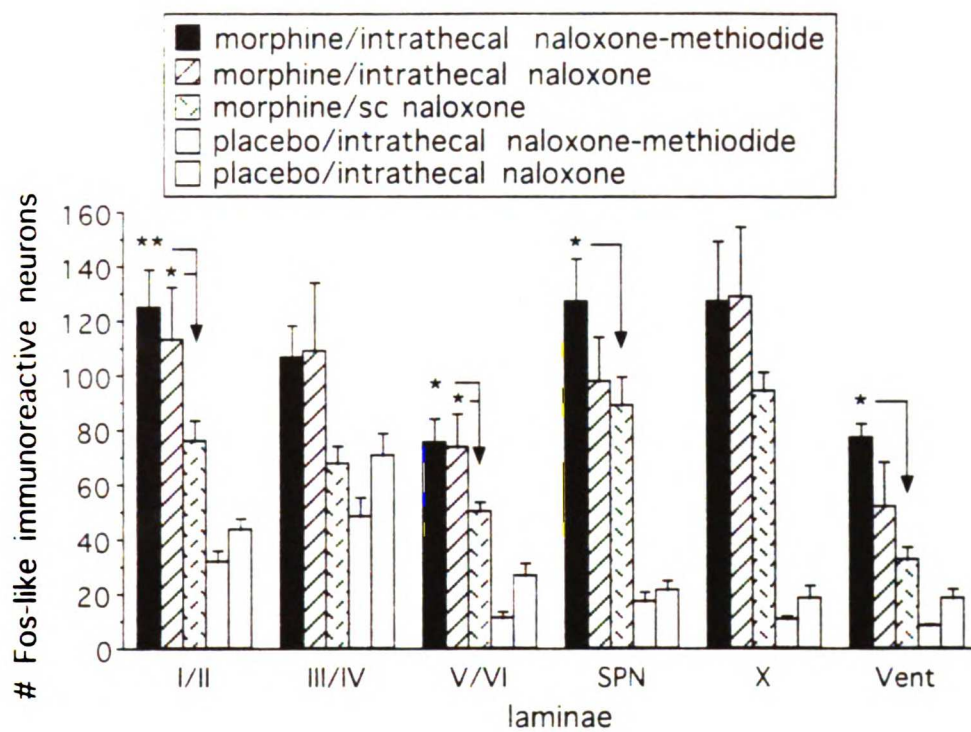
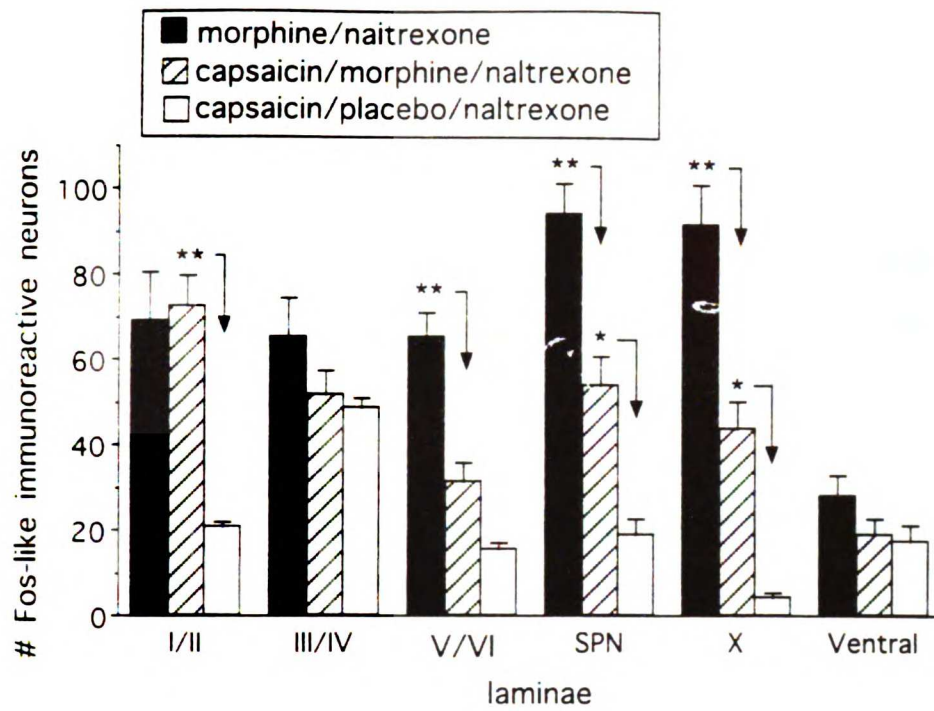


Figure 7. Induction of Fos-like immunoreactivity in the sacral cord in capsaicin and weight-matched non-capsaicin-treated rats withdrawing from morphine. Number of labeled cells per 50 μ m section. Intact withdrawing animals (morphine/naltrexone) (n=7) have significantly increased Fos-like immunoreactivity in laminae V/VI, X, and the SPN compared to the capsaicin-treated withdrawing animals (n=8). The capsaicin-treated withdrawing animals show significantly increased Fos-like immunoreactivity in laminae I/II, X, and the SPN compared to capsaicin-treated placebo-pelleted rats (n=3). There was no difference in laminae I/II between the capsaicin and non-capsaicin withdrawing rats. The intact withdrawing animals also demonstrated significantly increased Fos-like immunoreactivity in all laminae except laminae III/IV and the ventral horn compared to the capsaicin-pretreated placebo-pelleted rats (n=3), but for clarity, the asterisks were omitted.

* $P < .05$, ** $P < .01$



Chapter 7

Destruction of the locus coeruleus enhances withdrawal behavior and increases Fos-like immunoreactivity in the spinal cord of the rat.

Dana S. Rohde and Allan I. Basbaum

Abstract

Withdrawal from morphine evokes increases in Fos-like immunoreactivity in the spinal cord, particularly in the superficial dorsal horn, laminae I/II. We have previously recorded increased Fos expression in spinally transected withdrawing rats compared to intact withdrawing rats and have proposed that there is a supraspinal descending inhibition to spinal cord neurons during withdrawal. In order to determine the source of the supraspinal inhibition, we injected rats i.c.v. with saline or anti-dopamine- β -hydroxylase-saporin, a toxin that destroys the locus coeruleus. Eleven days after the injection of the toxin or saline, we implanted rats with morphine or placebo pellets. On day four, we precipitated withdrawal with naltrexone and after one hour, the rats were killed, their brains and spinal cords removed and 50 μ m transverse sections of the brains and spinal cords were immunoreacted with a rabbit polyclonal antiserum directed against the Fos protein.

In placebo-pelleted rats, the toxin injection did not alter behavior or induce expression of the Fos protein. However, compared to saline injected withdrawing rats, the toxin-treated rats withdrawing from morphine demonstrated increased behavioral activity including marked forepaw flutter. They also demonstrated significantly *increased* Fos-like immunoreactivity in all laminae in the cervical cord and in laminae I/II and the ventral horn of the lumbar cord. Sacral cord Fos expression was not altered by the toxin treatment. We hypothesize that the increased withdrawal hyperactivity and the increased Fos-like immunoreactivity in withdrawing rats injected with the toxin that destroyed the locus coeruleus results from a loss of descending inhibitory control that is activated by the locus coeruleus

Introduction

Chronic administration of opioids produces a state of tolerance in which subsequent doses of opioids have a decreased antinociceptive effect. Some studies suggest that opioid tolerance results from a compensatory response in the adenylyl cyclase system that develops in neurons that express the opiate receptor or in neural circuits in which those neurons participate (Nestler 1992; Nestler and Tallman 1988; Sharma et al. 1975). This compensatory response, which brings neuronal firing rate and cAMP levels to control levels, counteracts the inhibitory effects of morphine on cAMP levels. Thus, opioid tolerant neurons are said to be in a state of "latent hyperexcitability" (Fry et al. 1980) which is revealed only when the opioid is displaced from the opioid receptor leaving the upregulated cAMP pathway unopposed. The cellular changes are then manifest by increased adenylyl cyclase activity and behavioral signs of withdrawal.

We have previously found extensive expression of the Fos protein in neurons of the superficial dorsal horn of withdrawing rats and have hypothesized that the development of latent hyperexcitability or sensitization is concentrated in these neurons and may underlie withdrawal-induced hyperalgesia (Rohde et al. 1996). To determine the factors that regulate the increased Fos-like immunoreactivity (Fos-LI), we evaluated the effect of blocking descending input from the brain to the spinal cord by spinal transection at the T3 level. In these rats, we found *increased* Fos-like immunoreactivity in the lumbar spinal cord dorsal and ventral horns of withdrawing rats compared to intact withdrawing rats. Since descending inhibitory controls that regulate the firing of dorsal horn neurons are

eliminated by spinal cord transection, we hypothesized that the increased expression of the Fos protein reflects a loss of inhibitory controls that are normally activated during systemic withdrawal and/or loss of tonic inhibitory control during the development of morphine tolerance (Rohde et al. in press, Neuroscience).

There are several possible supraspinal sources of descending inhibition to the spinal cord, including the rostral ventral medulla (RVM) (Fields et al. 1977; Gray and Dostrovsky 1983) and the locus coeruleus (LC) (Hodge et al. 1981; Jones and Gebhart 1986). Although the RVM is believed to inhibit dorsal horn neuronal activity in normal rats, there is evidence that some RVM neurons facilitate dorsal horn neuronal activity in withdrawing rats (Bederson et al. 1990; Kaplan and Fields 1991). However, LC neurons inhibit dorsal horn neurons (Hodge et al. 1981; Jones and Gebhart 1986). and importantly, are activated during withdrawal (Aghajanian 1978; Hayward et al. 1990). Thus, we hypothesized that the LC may be a source of descending inhibition to the spinal cord and may explain the increased Fos-LI that we detected in spinally transected withdrawing rats.

The objective of the present study was to determine the contribution of the LC to behavior and spinal cord neuronal activity during precipitated withdrawal from morphine. To this end, we studied the pattern of Fos-LI during withdrawal in rats that were pretreated with saline or anti-D β H-saporin, a toxin that selectively destroys noradrenergic neurons of the LC and its projections. We demonstrate that destruction of the LC leads to *enhanced* withdrawal behavior and *increased* Fos-LI in the spinal cord dorsal and ventral horns. We conclude that withdrawal-induced LC neuronal activity normally inhibits spinal cord neuron activity.

Methods

Experiments were performed on male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA) with the approval of the Institutional Animal Care and Use Committee at UCSF. The rats were housed two in a cage on a 12 h light: 12 h dark cycle with free access to food and water. The rats were randomly assigned into four different treatment groups. One group was injected with the anti-D β H-saporin toxin into the lateral ventricle and then subdivided into placebo-pelleted (n = 4) and morphine-pelleted (n = 7) groups. Another group was injected with saline into the lateral ventricle and then subdivided into placebo-pelleted (n = 4) and morphine-pelleted (n = 4) groups. The toxin was kindly provided by Dr. Ron Wiley, VA Medical Center, Nashville, TN.

Anti-dopamine- β -hydroxylase-saporin (anti-D β H-saporin) is a toxin that is taken up by LC nerve terminals and selectively destroys LC cell bodies. The "immunotoxin" is composed of an antibody to the noradrenergic specific enzyme, dopamine- β -hydroxylase (D β H), that is coupled to saporin, a ribosomal inactivating protein. D β H is an intravesicular enzyme that catalyzes the conversion of dopamine to norepinephrine. Upon release of norepinephrine, D β H is temporarily membrane-bound; the immunotoxin thus can bind the membrane-bound subunit of D β H and upon endocytosis, the toxin undergoes retrograde axonal transport to the neuronal cell body. There, it arrests protein synthesis at the level of polypeptide chain elongation, by targeting an adenine that is responsible for the interaction of elongation factor 2 with the ribosome. This toxin is more selective than other toxins used previously to destroy noradrenergic nerve terminals in that it

targets the LC and its projections, while sparing non-coerulear noradrenergic neurons, serotonergic neurons, and dopaminergic neurons.

Rats weighed 240-260 g on the day of surgery. We anesthetized the rats with .15 cc xylazine/.3 cc ketamine and placed them in stereotaxic ear bars. A hole was drilled into the lateral ventricle using the following coordinates: from bregma: AP: -1.0; ML: -1.5; from skull: DV: -4.3. Next, we lowered a 10 μ l Hamilton syringe into the lateral ventricle and slowly injected either toxin (5.25 μ g toxin/5 μ l saline) or saline (5 μ l) over 2 minutes. Gel foam was placed over the hole in the skull and wound clips were used to close the skin.

Eleven days later, we implanted pellets subcutaneously (75 mg morphine sulfate or placebo pellet kindly provided by National Institute on Drug Abuse, Rockville, MD) according to the following schedule: one on day 1, two on day 2, three on day 3. We have previously demonstrated that rats become tolerant and dependent on morphine on this schedule (Rohde et al., in press, Neuroscience). On day four (two weeks after the surgery), we injected all of the rats with 10 mg/kg naltrexone, subcutaneously into the nape of the neck. The naltrexone was diluted in 800 μ l physiological saline.

We monitored the animals for signs of withdrawal for one hour after the naltrexone injection and then the rats were deeply anesthetized with pentobarbital (200 mg/kg, i.p.) followed immediately by intracardiac perfusion with 100 ml 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 500-600 ml 10% formalin in 0.1 M phosphate buffer (PB). We removed the brains and spinal cords one hour later and postfixed them in 10% formalin for an additional three hours. After cryoprotection overnight in 30% sucrose in 0.1 M PB, fifty μ m frozen sections were cut in the

transverse plane (spinal cord) or coronal plane (brain) and collected in 0.05 M PBS for immunocytochemical analysis.

The sections were immunostained for Fos, D β H, or tyrosine hydroxylase (TH) by the avidin-biotin-peroxidase method of Hsu et al. (1981) as described below. We washed the sections with a solution of 0.05 M Tris PBS with 1% normal goat serum and 0.3% Triton-X and then incubated them for 1 hr at room temperature in a blocking solution of 3% normal goat serum in 0.05 M Tris PBS with .3% Triton-X. The blocking solution was removed from the tissue, and the sections were incubated overnight at room temperature in the primary antiserum in the above buffer (Tris PBS/1%NGS/Triton). The Fos antibody (kindly provided by Dr. D. Slamon, UCLA) was diluted 1:30,000, the anti-D β H antibody (EugeneTech) was diluted 1:5,000, and the anti-TH antibody (EugeneTech) was diluted 1:1000. The primary antibody was removed and the sections were washed and then incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Elite Kit; Vector Labs, Burlingame, CA). To visualize the D β H immunoreactivity as a brown reaction product we used a non-nickel diaminobenzidine (DAB) glucose oxidase reaction following a protocol adapted from Llewellyn-Smith and Minson (1992). To visualize the Fos-LI as a black reaction product we used a nickel-DAB reaction. Next, the brain and spinal cord sections were mounted and coverslipped.

To quantitate the numbers of Fos-LI neurons, the cervical (C7/8), lumbar (L4/5) and sacral (S1) sections were photographed at 4x (low) power with Kodak technical pan film on a Nikon Microphot-FXA microscope. For analysis of the LC, the sections were first examined under dark-field to identify the appropriate levels for counting. We selected sections that contained the superior cerebellar peduncle and the motor trigeminal nucleus. We then

switched to light-field and photographed the sections. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. For the spinal cord analysis, individual sections of the cervical and lumbar cord were then divided into four regions: (i) the superficial laminae (laminae I, IIo, Iii); (ii) the nucleus proprius (laminae III and IV); (iii) the neck of the dorsal horn (laminae V and VI); and (iv) the ventral horn (laminae VII, VIII, IX, and X). The sacral cord was divided into six regions as described earlier (Rohde, in press, Brain Research): (i) laminae I/II; (ii) laminae III/IV (iii) laminae V/VI; (iv) the sacral parasympathetic nucleus (SPN); (v) lamina X; and (vi) the ventral horn. Fos-LI neurons were identified by a person blinded to the treatments. Up to six spinal cord or brain sections were examined per rat and the number of Fos-LI cells in each of these regions was counted and averaged so that each animal had a mean value for regional Fos-LI. We first performed a two-way ANOVA for total Fos for surgical pretreatment (toxin or saline injection) and drug treatment (morphine/naltrexone or placebo/naltrexone). The numbers of labeled cells per 50- μ m section were compared using two-way ANOVAs for treatment and laminae and within laminae one-way ANOVAs for treatment. Fisher's Protected Least Significant Difference was used for *post hoc* comparisons.

Results

Withdrawal Behavior

The toxin treatment did not affect the normal weight gain of the rats and all rats appeared healthy throughout the two-week period. Neither the saline-injected nor the toxin-injected rats that received placebo pellets

demonstrated unusual behavior and did not respond to naltrexone. By contrast, the morphine-pelleted rats that had received an i.c.v. saline injection demonstrated signs of withdrawal that included ptosis, teeth chattering, hyperactivity, diarrhea, and salivation. The withdrawing rats that were previously injected with the toxin, however, showed much more severe signs of salivation as well as stereotypic movements of the forepaws (forepaw flutter) that we have not previously recorded in rats undergoing withdrawal. There was no change in the percentage weight loss in the toxin-injected compared to the saline-injected withdrawing rats.

Verification of Toxin-induced destruction of the LC

Neither morphine nor placebo pellets affected the pattern or density of D β H-immunoreactivity. However, we noted a marked depletion in D β H immunoreactivity in the LC (Fig. 1) and spinal cord dorsal horn (Fig. 2); some staining persisted in the ventral horn. We did not quantitate these differences, as there was, in fact, an almost complete loss of staining in the toxin-treated rats. By contrast, the D β H-immunoreactivity in A5, A7, and the thoracic intermediolateral cell columns did not differ between the groups (Fig. 3).

Fos-expression in the LC of non-withdrawing rats is normally extremely low (Hayward et al. 1990). In this study, we, too, did not detect many Fos-labelled cells in the placebo-pelleted rats that received naltrexone (Fig 4). The toxin treatment did not significantly alter Fos-LI in the LC in placebo-pelleted rats compared to the placebo-pelleted rats injected i.c.v. with saline as demonstrated by the two way ANOVA for total Fos using pretreatment and drug treatment. The withdrawing rats demonstrated significantly increased Fos-LI in the LC compared to non-withdrawing rats, which is in accordance

with a previous study (Hayward et al. 1990). Importantly, the toxin-treated withdrawing rats demonstrated significantly less Fos-LI (67%) in the LC compared to the saline-treated withdrawing rats (Figs. 1 and 4).

Fos-LI in the spinal cord

We have previously demonstrated that withdrawal from morphine induces significant increases in Fos expression in the spinal cord. These increases have been demonstrated in the lumbar dorsal horn (Rohde et al., 1995) and in the sacral dorsal horn, lamina X and sacral parasympathetic nuclei (Rohde et al., in press, Brain Research). Naltrexone injection in placebo-pelleted rats does not induce Fos expression in the spinal cord.

The toxin treatment did not significantly alter Fos-LI in any regions of the spinal cord in placebo-pelleted rats as demonstrated by the two way ANOVA for total Fos using pretreatment and drug treatment. However, withdrawal-induced total Fos expression was significantly *increased* in the spinal cord after toxin treatment (Figs. 4 and 5). Thus, in the cervical cord, the toxin-treated withdrawing rats demonstrated significantly increased Fos-LI in all laminae when compared to the saline-treated withdrawing rats (68% in laminae I/II, 86% in laminae III/IV, 109% in laminae V/VI, and 153% in the ventral horn). In the lumbar cord, the toxin-treated withdrawing rats also demonstrated significantly increased Fos-LI in the superficial dorsal horn (65%) and the ventral horn (83%) compared to the saline-treated withdrawing rats. Although we recorded increased Fos-LI in the toxin-treated placebo-pelleted rats in laminae I/II in the cervical and lumbar cord compared to the saline-treated placebo-pelleted rats, these differences were not significant. In the sacral cord, we did not record any difference between the toxin-treated and the saline-treated withdrawing rats.

Discussion

In the present study, we have demonstrated increased withdrawal behavior and increased Fos-LI in the spinal cord of withdrawing rats that have a substantial depletion of the noradrenergic cell bodies in the LC and of their projections. Since activity of LC neurons is associated with decreased firing of dorsal horn neurons (Hodge et al. 1981; Jones and Gebhart 1986), our data suggest that the LC normally inhibits spinal cord neurons during withdrawal. Thus, the activity of LC neurons during withdrawal may serve to counteract the increased activity of spinal neurons and the withdrawal syndrome to which these neurons contribute. This conclusion is in contrast to the role designated to the LC by others (Nestler 1992; Rasmussen and Aghajanian; 1989 Rasmussen et al. 1990) who have suggested that withdrawal-induced LC activity drives many of the signs and symptoms seen during withdrawal.

Anti-D β H-saporin immunotoxin

Our conclusion concerning the contribution of the LC in part derives from the selectivity of the immunotoxin treatment which selectively targeted and destroyed the LC and its projections, while sparing non-coerulear noradrenergic nuclei and their projections. Specifically, we noted a decrease in D β H-immunoreactivity in the LC as well as projections of the LC, particularly in the cortex, cerebellum and the spinal cord dorsal horn. There was a smaller loss of staining in the spinal cord ventral horn. It should be noted that it is controversial as to whether or not the LC projects to the spinal cord dorsal horn (Fritschy et al. 1987) or ventral horn (Proudfit and

Clark 1991). Importantly, the toxin did not destroy the other major noradrenergic nuclei of the medulla and pons, A5 and A7, and we did not detect a decrease in D β h-immunoreactivity in targets of these nuclei such as the intermediolateral gray cell columns of the thoracic spinal cord. Furthermore, in the toxin-treated rats, we detected a loss of TH-immunoreactivity in the LC, but did not detect any loss of TH-immunoreactivity in dopaminergic neurons of the substantia nigra. This distinction illustrated the selectivity of the toxin for noradrenergic projection systems.

The analysis of Fos-LI provided further evidence for the efficacy of this toxin in destroying the LC. Previous studies demonstrated increased Fos-LI in rats withdrawing from morphine (Hayward et al. 1990; Stornetta et al. 1993) and we recorded similar results in this study in withdrawing rats that did not receive the toxin injection. However, there was a significant decrease in Fos-LI in the LC of the toxin-treated withdrawing rats compared to the non-treated withdrawing rats. Since Fos is a somatic marker, its reduction is consistent with withdrawal-induced Fos having occurred in the noradrenergic cell bodies of the LC.

Since the toxin targets noradrenergic nerve terminals, it is not clear why this toxin selectively targets the LC; the terminals of A5 and A7 neurons should be affected. Accessibility to the toxin may be critical. That is, when injected into the lateral ventricle, periventricular structures, many of which are targets of the LC, would have the greatest access to the toxin. These targets include the bed nucleus of the stria terminalis (Aston-Jones et al. 1995) and the paraventricular nucleus of the hypothalamus (Cunningham and Sawchenko 1988). In fact, we detected a loss of D β H-immunoreactivity in these regions. Furthermore, since the LC has extensive projections

throughout the brain, these projections may take up more of the toxin. Studies of Wiley, et al. (in press, Brain Research) indicate that at higher doses of toxin injected into the lateral ventricle, the A5 and A7 cell groups show some degeneration. This does not, however, explain the extensive and selective loss of D β H-immunoreactivity in the spinal cord. It is doubtful that the toxin diffused through the cerebrospinal fluid to reach the nerve terminals in the spinal cord. Conceivably, the loss of LC neurons due to brain structures that took up the toxin led to loss of spinal cord noradrenergic innervation via collateralization of the LC neurons. In fact, single LC neurons are highly branched with projections often leading to distant and functionally different target sites, including the cortex and spinal cord. This was demonstrated by double retrograde labelling studies and electroanatomical studies (Guyenet 1980; Nagai et al. 1981; Room et al. 1981).

Withdrawal behavior and Fos-LI

The marked forepaw stereotypy that we recorded in the toxin-treated rats that withdrew from morphine has not been previously described in rats. Comparable behavior, however, has been described in rats withdrawing from cannabinoids. For example, Tsou et al. (1995) described "forepaw fluttering," a tremor-like movement characterized by rapid repetitive medial-lateral movements of the forepaws. They proposed that alterations in circuits in the basal ganglia may have led to such behavior. Although alterations in LC projections to the basal ganglia may explain this behavior, an alternative mechanism is described below.

It is of interest that the flutter occurred only in the forepaws. In fact, in the cervical cord, we saw increased Fos-LI in all laminae, including laminae III/IV which is likely due to the increased forepaw movements. We have

previously demonstrated that movements of the limbs induces Fos-LI in laminae III/IV of the cervical or lumbar cord (Jasmin et al. 1994; Presley et al. 1990; Rohde et al. 1996). The extent to which the LC projects to both the dorsal and ventral horns is unclear (Fritschy et al. 1987; Proudfit and Clark 1991). In the lumbar cord, we only detected an increase in the superficial dorsal and ventral horns, the same regions of the cord that we detected increased Fos-LI after spinal transection (Rohde et al., in press, Neuroscience). These data suggest that the LC projects to both the dorsal and ventral horns and normally plays an inhibitory role during withdrawal.

It is of interest that we did not see an effect of the toxin on withdrawal-induced occurrence of diarrhea, weight loss or sacral cord Fos-LI. These data suggest that LC innervation of the sacral cord does not alter withdrawal induced gastrointestinal motility which is in accord with our previous studies (Rohde et al., in press, Brain Research) which provided evidence that withdrawal-induced diarrhea is a peripheral phenomenon that in turn contributes to the Fos expression seen in the sacral cord of withdrawing rats and contributes to withdrawal-induced weight loss. It is also possible that withdrawal in non-toxin treated rats induces maximal Fos expression in the sacral cord and that enhancement of this Fos expression can not be detected. In fact, we did record increased Fos-LI in the sacral cord of the toxin-treated withdrawing rats compared to the non-treated withdrawing rats but the differences were not significant.

Contribution of the LC in morphine analgesia and withdrawal

The contribution of the LC in morphine analgesia is controversial (Hammond and Proudfit 1980; Sasa et al. 1977) and in fact paradoxical. Activation of the LC or noradrenergic agonists applied spinally are

antinociceptive (Jones and Gebhart 1986; Kuraishi et al. 1979; Reddy and Yaksh 1980). There are clearly direct coeruleospinal projections in the rat, cat, and primate (Commissiong et al. 1979; Olson and Fuxe 1972; Westlund et al. 1983). It is hypothesized that LC-mediated antinociception results from a release of norepinephrine from coeruleospinal terminals (Mokha and Iggo 1987; Yaksh 1985). These act on an α_2 receptor to inhibit the firing of noceresponsive neurons. Both pre-and postsynaptic inhibitory noradrenergic controls have been identified (Bird and Kuhar 1977; Guyenet and Aghajanian 1977). The paradox arises because of the fact that opioids which also inhibit the firing of dorsal horn nociresponsive neurons inhibit the firing of LC neurons (Bird and Kuhar 1977; Guyenet and Aghajanian 1977; Korf et al. 1974). It follows, then, that opioid-induced LC inhibition should activate dorsal horn neurons.

In a similar vein, there is disagreement concerning the contribution of the LC to the withdrawal syndrome. Nestler and colleagues have suggested that LC activity during withdrawal causes some of the withdrawal signs (Nestler 1992; Rasmussen et al. 1990), but LC activity should inhibit spinal cord neurons. Previous studies that destroy the LC are not clear. For example rats with electrolytic lesions of the LC have demonstrated decreased withdrawal behavior (Maldonado and Koob 1993), but these lesions may destroy fibers of passage as well as cell bodies. Studies have used drugs to selectively destroy noradrenergic neurons, but these also have significant limitations. For example, 6-hydroxydopamine (6-OHDA) causes a loss of noradrenergic axons, but also destroys dopaminergic neurons (Bloom, et al. 1969 Breese and Traylor 1971). Another drug, N-2 chloroethyl-N-ethyl-2-bromobenzylamine (DSP4) preferentially interacts with noradrenergic terminals arising from the LC (Fritschy and Grzanna 1991) but also interacts

with non-LC terminals to a smaller extent. DSP4 also damages serotonergic neurons to a small degree (Jonsson et al. 1981). Finally, although degeneration of LC nerve terminals occurs within two weeks (Fritschy and Grzanna 1991; Grzanna et al. 1989), the cell bodies remain intact for up to six months (Fritschy and Grzanna 1991). The anti-D β H-saporin toxin by contrast, results in rapid cell body loss.

Results from electrophysiological (Aghajanian 1978), immunocytochemical (Hayward et al. 1990) and behavioral (Koob et al. 1992; Maldonado and Koob 1993; Rasmussen et al. 1990) studies suggest that LC neuronal hyperactivity during morphine withdrawal drives many of the behavioral signs of withdrawal. Furthermore clonidine suppression of LC activity is correlated with a suppression of withdrawal-induced increase in *c-fos* mRNA (Rasmussen et al. 1995), suppression of withdrawal behavior (Aghajanian 1978; Redmond 1981; Taylor et al. 1988; Tseng et al. 1975) and suppression of withdrawal-induced increase turnover of norepinephrine in the cerebral cortex (Zigun et al. 1981). Finally, lesions of the LC have led to a loss of withdrawal behavior (Maldonado and Koob 1993), while electrical stimulation of the LC in primates causes behavior similar to those in opiate withdrawal (Grant et al. 1988; Redmond 1981).

There are several caveats to these studies. First, the connection between the LC and withdrawal behavior is correlative, not causative. Furthermore, although clonidine suppresses LC neurons, the LC is not necessarily the major site of action in decreasing the severity of withdrawal (Britton et al. 1984; Starke and Altmann 1973; Zhao and Duggan 1987). Clonidine is an α_2 agonist and acts centrally (Starke and Montel 1973) on post synaptic α_2 receptors, but also decreases sympathetic outflow and acts in the periphery on presynaptic receptors (Starke and Altmann 1973). In fact, autoradiographic studies of

ligand binding suggest a similar distribution in the brain and spinal cord of $\alpha 2$ and opioid receptors (Unnerstall et al. 1984), which suggests that clonidine could substitute for opiates. Importantly, clonidine only suppresses a subset of withdrawal symptoms (Charney et al. 1982; Jasinski et al. 1985; Taylor et al. 1988). Opioid withdrawal is characterized by sympathetic hyperactivity and spinal sympathetic neurons may be the site of action of Clonidine (Franz et al. 1982). In fact, it is not surprising that spinal actions of clonidine suppress withdrawal, since opioid withdrawal occurs in the spinal cord (Delander et al. 1984; Delander and Takemori 1983). Finally, the increased Fos in the LC (Hayward et al. 1990; Stornetta et al. 1993) was not seen in anesthetized rats (Chieng et al. 1995) although PAG Fos expression persisted under anesthesia.

Other studies suggest that the LC is not involved in the withdrawal syndrome. Thus, Chieng and Christie (1995) reported that the behavioral withdrawal syndrome is not altered in rats treated with saline as compared to those treated with DSP4. They confirmed a loss of noradrenaline via cortical noradrenaline concentration, inhibition of decapitation hindpaw reflex, and D β H immunocytochemistry. However, noradrenaline concentrations decreased by only 50%, leaving substantial residual noradrenaline that could mediate the signs of withdrawal. Finally, some studies, like ours, suggest that noradrenergic systems inhibit withdrawal. For example, 6-OHDA lesions exacerbate abstinence signs (Elchisak and Rosecrans 1979; Friedler et al. 1972). Spontaneous withdrawal after abrupt cessation of morphine after 32 days was increased in 6-OHDA-induced catecholamine-depleted rats. This effect was not due to loss of dopamine as it could not be repeated in rats that were permanently depleted of only dopamine (Elchisak and Rosecrans 1979). Finally, morphine abstinence in mice undergoing naloxone-induced

withdrawal demonstrated increased jumping and weight loss if pre-treated with 6-OHDA (Friedler et al. 1972).

Summary

In this study, the decreased D β H-immunoreactivity and Fos-LI in the LC of the toxin-treated rats demonstrate there is a large loss of LC neurons after toxin treatment. This loss includes a loss of D β H-immunoreactivity in the spinal cord, but not of other non-coerulear noradrenergic neurons or targets. That we saw increased withdrawal behavior and Fos-LI in the spinal cord of toxin-treated withdrawing rats suggests that withdrawal-induced LC activity normally inhibits spinal cord neurons.

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Figure 1. These photomicrographs illustrate A: D β H immunocytochemistry in the LC of a saline-injected withdrawing rat; B: D β H immunocytochemistry in the LC of a toxin-injected withdrawing rat; C: Fos-LI in the LC of a saline-injected withdrawing rat; and D: Fos-LI in the LC of a toxin-injected withdrawing rat. Arrows indicate the decrease in D β H immunoreactivity and Fos-LI in the toxin-treated rats.

A



B



C



D



Figure 2. These photomicrographs illustrate D β H immunoreactivity in the A: lumbar dorsal horn of a saline-injected rat; B: lumbar dorsal horn of a toxin-injected rat; C: lumbar ventral horn of a saline-injected rat; and D: lumbar ventral horn of a toxin-injected rat. Note the complete loss of D β H immunoreactivity in the dorsal horn of the toxin-treated rats and the extensive loss of D β H immunoreactivity in the ventral horn of the toxin-treated rats.

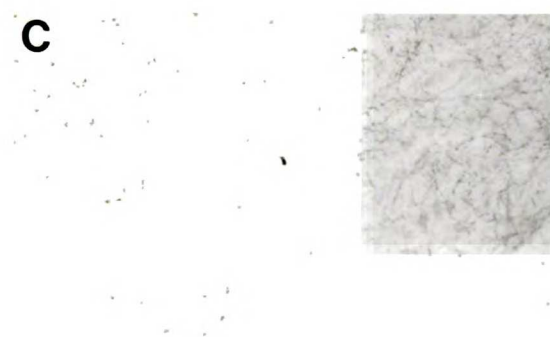
A



B



C



D



Figure 3. These photomicrographs illustrate D β H immunoreactivity in A: the thoracic spinal cord of a saline-injected rat; B: A5 of a saline-injected rat C: the thoracic spinal cord of a toxin-injected withdrawing rat (low power, 4x); C: thoracic spinal cord of a toxin-injected rat; and D: A5 of a toxin-injected rat. Note the complete loss of DC: A5 of a saline-injected withdrawing rat. The A5 cell group and the intermediolateral cell columns of the thoracic cord in the toxin-treated rats appear to retain their D β H staining, although there is loss of D β H-immunoreactivity in the thoracic dorsal horn (see arrow).



Figure 4. These graphs illustrate the number of Fos-labelled neurons per 50 μm sections of the A: LC. There is a significant loss of Fos-LI in the toxin-treated withdrawing rats compared to the saline-injected withdrawing rats; B: cervical cord. The toxin-treated rats demonstrated significantly increased Fos-LI in all laminae compared to the saline-injected withdrawing rats; C: lumbar cord. We recorded significantly increased Fos-LI in the superficial dorsal horn and ventral horn of the toxin-treated withdrawing rats compared to the saline-injected withdrawing rats; and D: sacral cord. We did not record any difference between the toxin- and saline-injected withdrawing rats. Fos expression remained extremely low at all levels of the brain and spinal cord in the placebo-pelleted rats that were injected with saline or toxin.

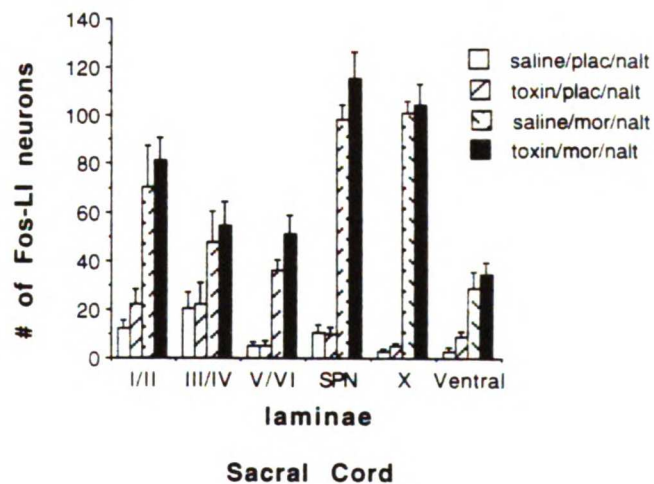
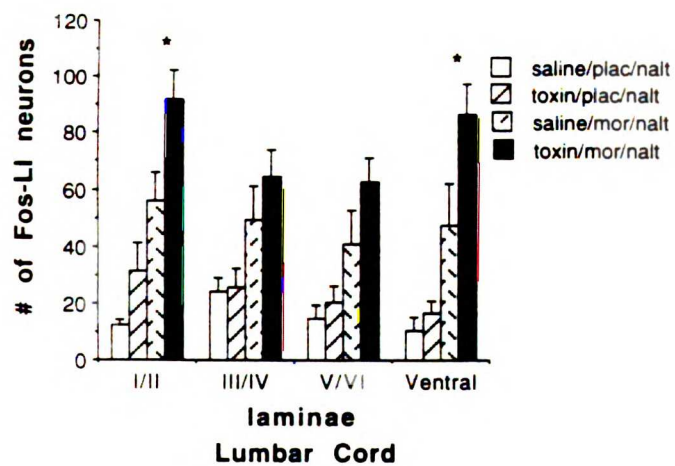
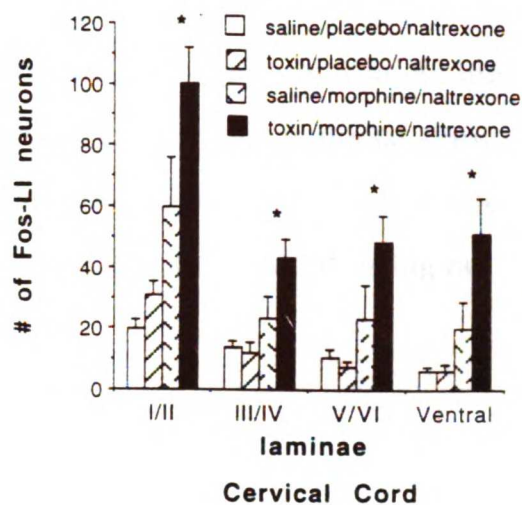
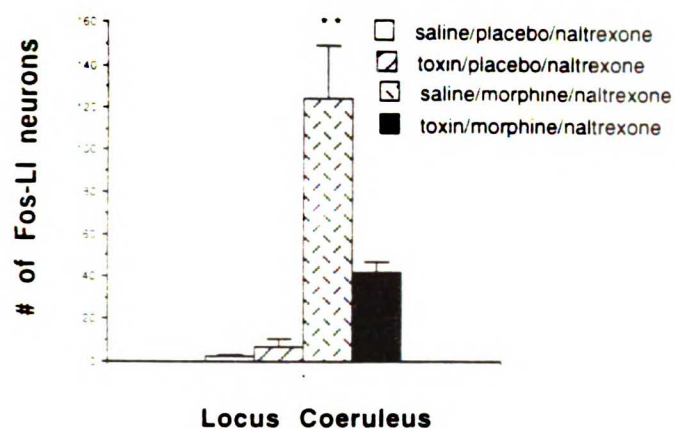


Figure 5. These photomicrographs illustrate the Fos-LI in the A: lumbar dorsal horn of a saline-injected withdrawing rat; B: lumbar dorsal horn of a toxin-injected withdrawing rat; C: cervical dorsal horn of a saline-injected withdrawing rat; D: cervical dorsal horn of a toxin-injected withdrawing rat.

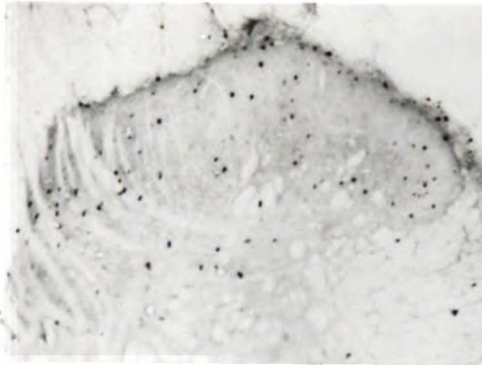
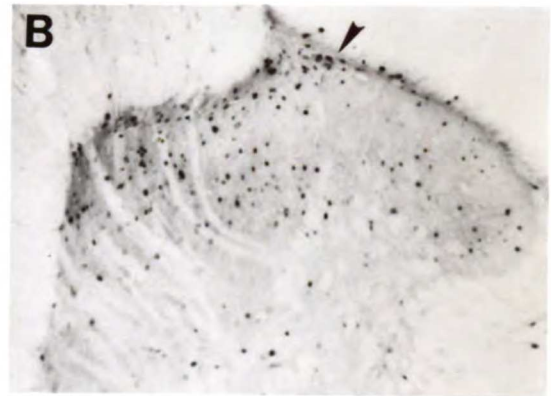
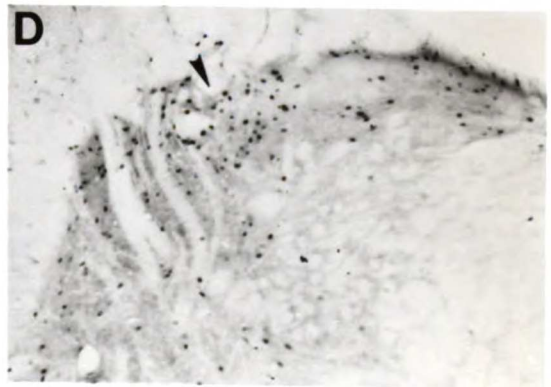
A**B****C****D**

Figure 6. These photomicrographs illustrate the Fos-LI in the A: sacral cord of a saline-injected withdrawing rat; B: sacral cord of a toxin-injected withdrawing rat. There were no differences in Fos-LI in these two groups.

A



B



Chapter 8

Summary

This thesis provides evidence for a contribution of the spinal cord in the development of opioid tolerance and dependence. The data suggest that neurons in the spinal cord are sensitized during the development of tolerance and that the sensitization can be unmasked by the administration of an opioid antagonist or a noxious stimulus. The sensitization supports the view that tolerance results from the development of a compensatory response in the neuron or in the neural circuits that contain the opioid receptors. This idea was put forth by Fry et al. (1980) who demonstrated a latent hyperexcitability of cortical and striatal neurons in rats withdrawing from morphine. It is also supported by Nestler et al. (1988, 1989) who have demonstrated upregulation of the G protein/cAMP system in tolerant/dependent rats.

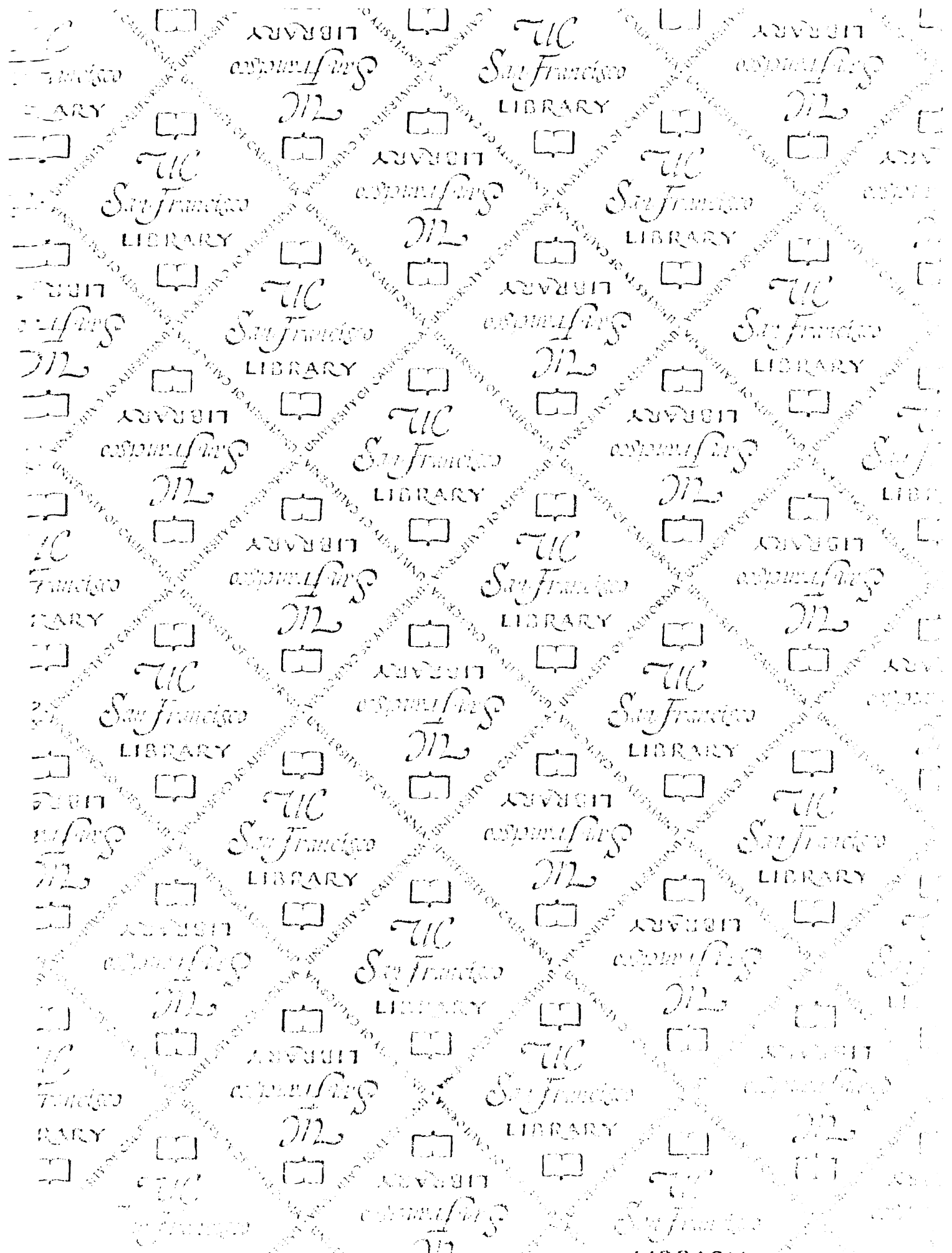
The results are consistent with the idea that tolerance and dependence can occur at the single cell level, but are modulated by systems input. Thus, on the one hand, the Fos-labelled neurons represent neurons that are sensitized due to the development of tolerance. On the other hand, the modulation of spinal cord Fos expression is apparent after dorsal rhizotomy, spinal transection or destruction of the locus coeruleus. This suggests that peripheral and/or supraspinal input can modulate the spinal cord neuronal circuits that are engaged during the development of tolerance and after precipitation of withdrawal.

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For reference

Not to be taken
from the room.

