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Studies on the contribution of tachykinin and opioid neuropeptides to spinal cord modulation of nociceptive input

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Studies on the contribution of tachykinin and opioid neuropeptides to spinal cord modulation of nociceptive input

by

Jodie Anne Trafton

DISSERTATION

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Dedication

This thesis is dedicated to Dr. Marion Trafton

Whose example and support undeniably drove my interest in science.

I only wish neuroscience could have helped preserve her mind long enough to see that Dr. Jodie is a perfectly good name for one who never aspired to seriousness.

Preface and Acknowledgements

This thesis would not have been possible without the support, assistance, and encouragement provided by a large number of wonderful people who have so generously graced my life.

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The text "Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation of the hindpaw" of chapter 1 is a reprint of the material as it appears in the Journal of Neuroscience. Catherine Abbadie initiated and directed the research that forms the basis of this portion of the thesis. The text "Spinal opioid analgesia: How critical is the regulation of SP release?" of chapter 3 is a reprint of the material as it appears in the Journal of Neuroscience. Jodie

Trafton contributed to the studies of the changes in NK-1 receptor response under conditions of inflammation in chapter one. The studies of morphine's effects on NK-1 receptor signaling in chapter 3 were designed, implemented, and analyzed by Jodie Trafton and Catherine Abbadie cooperatively. Jodie Trafton completed the studies of the correlation between NK-1 receptor internalization and NK-1 receptor calcium signaling, all alone, with no one to talk to, in a series of dark little rooms with microscopes.

Studies on the contribution of tachykinin and opioid neuropeptides to spinal cord modulation of nociceptive input

Jodie Anne Trafton

Abstract

The perception of pain following a potentially damaging stimulus depends not only on the qualities of the stimulus, but greatly upon the behavioral state and previous experience of the perceiver. These factors modulate the transmission of pain signals in the nervous system, amplifying or dampening them to increase or decrease their salience. Much of this modulation takes place in the spinal cord dorsal horn, the first processing center for nociceptive input. Several classes of peptide neurotransmitters have been implicated in spinal cord modulation of sensory input; tachykinins have been shown to sensitize, while opioids have been shown to reduce responses to noxious stimulation.

Here, I examine how and when these peptide neurotransmitters act in the spinal cord dorsal horn. Using internalization of the receptors for these peptides as a marker for their activation, I attempt to determine when and which neuropeptides are released, the populations of neurons they activate, and the consequences of their interactions with their receptors. I demonstrate that tachykinin mediated neurokinin-1 and opioid mediated mu opioid receptor activation is limited to very specific neuronal populations and environmental

circumstances. The various tachykinin and opioid peptides act selectively upon specific sub-populations of neurokinin and opioid receptors.

Neurokinin-1 receptor signaling occurs only in response to tissue damaging stimuli, in an intensity dependent manner and is restricted to neurons in lamina I. Under inflammatory conditions, neurokinin-1 receptor internalization is seen at lower intensities and neurokinin-1 receptors in lamina III-VI are also activated. Multiple tachykinin peptides act at the neurokinin-1 receptor, although they bind the receptor differently and produce different effects in the dorsal horn.

Despite the expression of mu opioid receptors on tachykinin containing terminals, mu opioids do not modulate signaling via the neurokinin-1 receptor following noxious stimulation. Tachykinins appear to be released in significant excess to make opioid receptor mediated reductions in their release irrelevant. Thus, presynaptic inhibition of tachykinin release is not a major mechanism by which opioids reduce nociceptive responsiveness. Post-synaptic lamina II mu opioid receptors do respond to exogenously applied opioids and their activation correlates with the behavioral analgesia produced. Never the less, these post-synaptic MORs do not appear to be involved in effects observed after noxious stimulation, stress or activation of descending modulatory systems, or stimulation of enkephalinergic lamina II interneurons.

These studies provide insight into the functioning of both neuropeptides and the spinal cord dorsal horn, and have significant implications for the understanding and treatment of pain.

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GENERAL INTRODUCTION

The sensation of pain produced by a given noxious stimulus is not a consistent experience. Rather, the same stimuli can produce very different sensations, both qualitatively and quantitatively, depending on the circumstances preceding or surrounding the stimulus. For example, under injury conditions noxious stimuli may cause greater pain than normal, a state referred to as hyperalgesia, and non-noxious stimuli may begin to evoke pain, referred to as allodynia. Alternatively, under highly stressful conditions, noxious stimuli may be noticed little if at all.

The nervous system has developed complex circuits that regulate how noxious sensory information is perceived. There is evidence that considerable modulation of sensory information occurs in the spinal cord dorsal horn, filtering sensory information immediately as it is relayed by sensory neurons from the periphery. Although numerous mechanisms have been proposed and probably contribute to this sensory processing, several key players have been identified in the form of the tachykinin and opioid families of neuropeptide transmitters.

The pro-nociceptive neuropeptides substance P and neurokinin A, members of the tachykinin family, have long been known to sensitize the spinal cord to noxious input, and have thus been hypothesized to underlie states of hyperalgesia and allodynia. These two peptides are produced from the same gene, the pre-protachykinin A gene, and act upon neurokinin-1 and 2 receptors. They are coexpressed in primary sensory nociceptors in the dorsal root ganglia (DRG) and in a small population of spinal cord interneurons and are released following noxious stimulation. Although these tachykinin peptides have for

many years been implicated in the transmission of nociceptive messages and in the process of sensitization the details of their endogenous function are still unknown.

In contrast to the tachykinins, opioid peptides, including the endomorphins and enkephalins, are potently analgesic when administered at the level of the spinal cord. These actions are thought to occur mainly through the mu and delta opioid receptors (MOR & DOR), both of which are expressed on primary afferent sensory neurons and on interneurons in the dorsal horn. Mechanisms underlying both pre and post-synaptic inhibition of transmitted "pain" signals by opioids have been proposed, however, the relevance and occurrence of these processes in relationship to opioid analgesia have not been confirmed.

Both tachykinins and opioids have been studied intensively in the spinal cord, but research has been limited by the technical difficulties inherent in the study of neuropeptides *in vivo*. The recent discovery that most G-protein coupled receptors internalize into endosomes as part of the recycling process, has provided researchers with a new tool for studying neuropeptide actions in vivo. As receptor internalization is readily observable and occurs when the receptor is activated by ligand, this technique has allowed for determination of when and to what extent neuropeptides are active *in vivo*.

Using these new methods, in combination with other neuroanatomical, pharmacological, behavioral and *in vitro* techniques, the studies outlined below examine how the tachykinin and opioid peptides function in the spinal cord to modulate the perception of potentially damaging stimuli. By addressing the

following questions, I hope to bring us closer to understanding how spinal cord neuropeptides regulate the pain that is produced by noxious stimulation.

THESIS OBJECTIVES

Under what conditions do tachykinins act in the spinal cord? Which cell populations do they influence?

Rational: It is now well established that tachykinins are expressed (synthesized) by primary afferent nociceptors and released following noxious stimulation. Nevertheless, the precise stimulus dependency of their release and the targets of their action are still unknown. To address these outstanding questions, in the first series of experiments we examined the populations of spinal neurons activated by tachykinins following noxious stimulation, the stimulus dependency of this NK-1 receptor activation and the changes that occur in these responses under injury conditions.

Which of the tachykinin peptides are responsible for NK-1 receptor activation in the spinal cord? Do NKA and SP have different effects in the spinal cord dorsal horn?

Rational: SP and NKA are coreleased from primary afferent fibers. Both can activate neurons via the NK-1 receptor, but there is *in vitro* evidence that they trigger somewhat different signaling cascades. In the second series of experiments we attempt to determine to what degree each peptide is responsible for NK-1 receptor activation after noxious stimulation. Additionally, although

only NK-1 and NK-3 receptors have been identified in the spinal cord dorsal horn NKA is classically thought to be an NK-2 receptor agonist. Despite lack of anatomical evidence, several pharmacological studies have suggested the presence of NK-2 receptor-mediated effects in the spinal cord. As part of this analysis we attempted to determine whether spinal tachykinin-generate effects are solely NK-1 receptor mediated or whether NK-2 and NK-3 receptors are also involved.

Do opioids inhibit the actions of tachykinins in the spinal cord? Is this process responsible for their analgesic effects?

Rational: Inhibition of substance P release by opioids has long been hypothesized to be a major mechanism underlying the analgesic action of spinally administered opioids. Thus, numerous studies have demonstrated the ability of opioids to reduce tachykinin release. On the other hand, the functional consequences of this inhibition of neuropeptide release has only been inferred (it has, in fact, never been directly assessed. Here, we studied the effect of opioids on the signaling induced by a noxious stimulus that evokes the release of tachykinins in the spinal cord; the functional endpoint that we monitored was the internalization of the neurokinin-1 receptor, a marker of the activation of dorsal horn nociresponsive neurons.

Under what conditions are post-synaptic lamina II MORs activated? Is this related to opioid-mediated analgesia? Under what conditions are they activated endogenously?

What is the source of the endogenous opioids (primary afferents, spinal interneuron, or supraspinal descending modulation)?

Rational: A population of presumed excitatory interneurons in lamina II expresses the MOR. Activation of the MOR on these neurons hyperpolarizes these neurons, perhaps preventing the transmission of nociceptive messages. While this is a plausible mechanism by which spinal opioids could produce analgesia, it is unclear how and when these MORs are activated *in vivo*. To address this question, we examined whether these MORs are activated by exogenous opioids, in a manner consistent with their functioning to produce antinociceptive effects. We also attempted to determine how these receptors are activated endogenously. Three potential local sources of endogenous opioids were examined: endomorphins from primary afferent fibers, enkephalins from inhibitory lamina II interneurons, and supraspinally-triggered spinal opioid release.

Chapter 1

Activation of the NK-1 receptor in the dorsal horn of the spinal cord

GENERAL INTRODUCTION

Although it has long been known that noxious stimulation induces release of tachykinins in the dorsal horn of the spinal cord, only recently has it been possible to determine the neurons and sites at which these tachykinins act. The neurokinin-1 (NK-1) receptor is the major tachykinin receptor expressed in the dorsal horn of the spinal cord. Because the NK-1 receptor internalizes into easily observable endosomes upon agonist activation, it is now possible to determine where and when tachykinins affect neurons through activation of this receptor. The NK-1 receptor is highly expressed in a population of lamina I projection neurons, on occasional large lamina III neurons, a population of smaller but more numerous lamina V-VI neurons and by a cluster of large neurons in lamina X (Brown et al, 1995; Ding et al, 1995) Mantyh et al (1995) demonstrated that noxious stimulation selectively activates NK-1 receptors located on the plasma membrane of cell bodies and dendrites of neurons of lamina I, and on the dorsally-directed dendrites of lamina III-VI neurons, some of which arborize in lamina I. These studies implicate this lamina I population in the tachykinin-mediated behaviors induced by noxious stimulation. These studies, however, did not address the stimulus response relationship or modality specificity of this NK-1 receptor activation nor did they shed light on the contribution, if any of tachykinins, to nociceptive processing by NK-1 receptor expressing neurons located in deeper laminae.

Inflammatory injury induces profound changes in spinal tachykinin systems. Inflammation results in an upregulation of both substance P and the NK-1 receptor in the spinal cord dorsal horn (Donnerer et al, 1993; Hanesch et al,

1993; Schafer et al, 1993; Abbadie et al, 1996), and changes in the spread of released tachykinins have been reported. (Shaible et al, 1992) Again, however, it is unknown how these changes in protein expression affect activation of tachykinin receptors in the spinal cord. In the following series of experiments we addressed these outstanding questions.

Abbadie C, Brown J, Mantyh P, Basbaum A. (1996) Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. Neurosci 70: 201-209.

Brown JL, Liu H, Maggio JE, Vigna SR, Mantyh PW, Basbaum AI. (1995) Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. J Comp Neurol 356: 327-344.

Ding YQ, Takada M, Shigemoto R, Mizumo N. (1995) Spinoparabrachial tract neurons showing substance P receptor-like immunoreactivity in the lumbar spinal cord of the rat. Brain Res 674 :336-40.

Donnerer J, Schuligoi R, Stein C, Amann R. (1993) Upregulation, release and axonal transport of substance P and calcitonin gene-related peptide in adjuvant inflammation and regulatory function of nerve growth factor. Reg Pept 46: 150-4.

Hanesch U, Pfrommer U, Grubb B, Heppelmann B, Schaible H. (1993) The proportion of CGRP-immunoreactive and SP mRNA-containing dorsal root

ganglion cells is increased by a unilateral inflammation of the ankle joint of the rat. Reg Pept 46: 202-3.

Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE, Simone DA (1995) Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268: 1629-1632.

Schafer M, Nohr D, Krause J, Weihe E. (1993) Inflammation-induced upregulation of NK1 receptor mRNA in dorsal horn neurons. Neuroreport 4: 1007-1010.

Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation of the hindpaw

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ABSTRACT

Although the neurokinin-1 (NK-1)/substance P (SP) receptor is expressed by neurons throughout the spinal dorsal horn, noxious chemical stimulation in the normal rat only induces internalization of the receptor in cell bodies and dendrites of lamina I. Here we compared the effects of mechanical and thermal stimulation in normal rats and in rats with persistent hindpaw inflammation. Electron microscopic analysis confirmed the upregulation of receptor that occurs with inflammation and demonstrated that in the absence of superimposed stimulation, the increased receptor was, as in normal rats, concentrated on the plasma membrane. In general, noxious mechanical was more effective than noxious thermal stimulation in inducing NK-1 receptor internalization, and this was increased in the setting of inflammation. Although a 5 sec noxious mechanical stimulus only induced internalization in 22% of lamina I neurons in normal rats, after inflammation, it evoked near maximal (98%) internalization in lamina I, produced significant changes in laminae III-VI and expanded the rostrocaudal distribution of neurons with internalized receptor. Even non-noxious (brush) stimulation of the inflamed hindpaw induced internalization in large numbers of superficial and deep neurons. For thermal stimulation, the percent of cells with internalized receptor increased linearly above 45°C, but in normal rats, these were restricted to lamina I. After inflammation, however, the 52°C stimulus also induced internalization in 25% of laminae III-IV cells. These studies provide a new perspective on the reorganization of dorsal horn circuits in the setting of persistent injury and demonstrate a critical contribution of SP.

INTRODUCTION

Numerous studies have implicated substance P (SP) in the transmission of nociceptive messages at the level of the spinal cord. SP is synthesized by small diameter, primary afferent fibers, many of which respond to noxious stimulation and terminate in regions of the spinal dorsal horn that contain neurons responsive to noxious stimulation, including the superficial laminae I and II and to a much lesser extent lamina V (Besson et al, 1969; Hökfelt et al, 1975; Menétrey et al, 1977) Noxious stimulation also evokes the release of substance P into the spinal cord (Duggan et al, 1988; Kuraishiet al, 1989) and spinal CSF (Tiseo et al, 1990; Yaksh et al, 1980) and iontophoretic application of SP excites nociresponsive neurons in the dorsal horn (De Koninck & Henry, 1991; Henry, 1976). Finally, intrathecal injection of SP evokes behaviors indicative of pain (Cridland & Henry, 1988; Hylden & Wilcox, 1981) (however, see (Frenk, 1988)).

Despite this evidence numerous questions remain. It has been surprisingly difficult to block noxious stimulus-evoked pain behavior with selective antagonists of the receptor at which SP acts, the neurokinin 1 (NK-1) receptor (Munro et al, 1993; Parker et al, 1993; Yamamoto et al, 1993; Yamamoto & Yaksh, 1992). To some extent this may reflect there being a preferential release of SP by noxious mechanical stimuli (Duggan et al, 1988; Kuraishi et al, 1989). Another paradox concerns the significant mismatch between the distribution of the NK-1 receptor, and the location of SP (Brown et al, 1995; Liu et al, 1994). In particular, although the NK-1 receptor is located throughout the dorsal horn, when we monitored internalization of the NK-1 receptor, which occurs when SP binds the receptor, we only found changes in the

superficial dorsal horn, in cell bodies and dendrites of lamina I neurons and in the dorsally-directed dendrites of neurons in lamina III (Mantyh et al, 1995). Although there is evidence that SP activates neurons located in deeper parts of the dorsal horn (De Koninck et al, 1992) our results suggested that when SP is released in the normal animal, it predominantly targets neurons in the superficial dorsal horn.

In the present study we evaluated NK-1 receptor internalization in response to different modalities of noxious stimulation and compared the pattern of internalization in normal rats and in rats with an inflamed hindpaw, a condition associated with significant upregulation of the NK-1 receptor in the dorsal horn (Abbadie et al, 1996) Importantly, inflammation induces a central sensitization of dorsal horn neurons that can be reduced by NK-1 receptor antagonists (Thompson et al, 1994) . Since, the sensitization is manifest as an increase in spontaneous activity, increased excitability and enlarged receptive fields of neurons in laminae I and VI and since SP is more readily detected in deep dorsal horn under conditions of inflammation (Schaible et al, 1990), we hypothesized that noxious stimulus-evoked internalization of the NK-1 receptor might occur in a wider distribution of neurons in the setting of inflammation.

METHODS

Experimental animals

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at UCSF. Experiments were performed on male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA), weighing 230-270g. Inflammation

was induced by subcutaneous injection, in the left hindpaw, of 50 µl of complete Freund's adjuvant (CFA, killed *mycobacterium butyricum* suspended in mineral oil, solution at 10 mg/ml). Three days after the injection, the rats were stimulated. Control groups of rats were "intact" rats that received no injection.

All experiments were performed 10-15 minutes after the rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). This dose blocked all flexor reflex responses to hindpaw stimulation. The hindpaw of the rats was stimulated 10-15 minutes after anesthesia was induced.

Hindpaw stimulation

Both non-noxious and noxious mechanical stimuli were used. The non-noxious mechanical stimulus was brush applied to the dorsal surface of the left hindpaw (approximately one brush per second for 2 min.; n=5 in each group). Noxious mechanical stimulation (pinch) was applied to the distal part of one hindpaw with a hemostat for 5, 15, 30 sec. or 2 min (n=4 in all groups except n=5 for the intact group stimulated for 2 min and n=6 for the CFA group stimulated for 30 sec). In 3 rats with inflammation, we applied the 2 min noxious stimulus to the hindpaw contralateral to the CFA injection. For all stimulus conditions in these groups, the rats were perfused 5 min after the stimulation ended.

For thermal stimulation, the rat's hindpaw (to just below the ankle) was dipped into a water bath heated to either 45, 48, 50 or 52°C (n=4 in all groups except n=5 for the 48°C stimulus in the group of rat with inflammation). The duration of the stimulus was 2 min. All rats were perfused 5 min after the end of the stimulation.

In a previous report we found that the receptor recycled to the plasma membrane within 60 min of stimulation (Mantyh et al, 1995). To compare the temporal pattern of recycling in inflamed and intact groups of rats, in a different experiment we perfused rats 30, 60 min or 2 hours after the stimulation (n=4 in all groups). For this study, we used a noxious pinch applied for 2 min; this stimulus produces maximal internalization of the NK-1 receptor in lamina I. In another group of rats we evaluated the functional state of the recycled NK-1 receptor by applying a second stimulus, at different times after the first. The first stimulus was a 15 sec pinch of the paw. The same stimulus was then applied one hour after the first. We chose a 15 sec stimulus because it produced profound, but less than maximal internalization of the NK-1 receptor in the intact rat; thus, we could detect both increases and decreases in the magnitude of internalization after the second stimulus. The rats were perfused 5 min after the second stimulus.

Immunocytochemistry

At the appropriate time, the animals received an additional injection of sodium pentobarbital (100 mg/kg, i.p.) and were perfused intracardially with 50 ml of phosphate-buffered saline 0.1 M (PBS) followed by 500 ml of 4% formaldehyde in 0.1 M phosphate buffer (PB). The time between the end of the stimulation and the beginning of the fixative flow was approximately 7-8 min. After the perfusion, the lumbar spinal cord was removed, postfixed for four hours in the same fixative and then cryoprotected overnight in 30% sucrose in 0.1 M PB. Immunostaining was performed on 30 µm lumbar (from L2 to L6 segments) spinal cord sections cut in the sagittal plane on a freezing microtome. The tissue sections were incubated for 60 min

at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST). The sections were then incubated overnight at 4°C in the primary antiserum, diluted to 1:5,000. The characteristics of the antiserum, which was directed against the C terminal of the NK-1 receptor, have been described previously (Vigna et al, 1994) After the primary antiserum, the sections were washed 3 times in 1% NGST and then incubated in indocarbocyanine Cy-3™ conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 1: 600) for 2 hours at room temperature. Finally, the sections were washed 3 times in PB, mounted on gelatin-coated slides, dried, and coverslipped with DPX (Electron Microscopy Science).

For electron microscopic analysis of the NK-1 receptors in the setting of inflammation, we studied 3 rats that had received a hindpaw injection of CFA. Three days after the CFA injection, the rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused through the ascending aorta with 100 ml of 0.1 M PBS (pH 7.4) followed by a 0.1 M PBS fixative solution containing 4% formaldehyde, 1% glutaraldehyde and 0.1% picric acid. After the perfusion, the lumbar spinal cord was removed and postfixed in the same solution for 2-4 hr, and washed in 0.1 M phosphate buffer for several hours.

We used a pre-embedding immunogold method (Liu et al, 1994) to localize the receptor. Briefly, 40 μm vibratome sections were incubated in 50% ethanol to improve penetration of the NK-1 receptor antibody. The latter was localized with a 1.0 nM colloidal gold conjugated secondary antibody. After the immunoreaction, the sections were silver-enhanced and then dehydrated and embedded in plastic. From each animal we analyzed at least 9 grids containing 2 thin sections each. These were collected from 3 different plastic-embedded vibratome sections through lamina I.

Neuronal profiles were considered to be positive for NK-1 receptors when the density of silver particles was at least three times greater than in surrounding neuroropil. To quantify the distribution of NK-1 receptor labeling, we collected photomicrographs through lamina I and counted silver particles. The results are expressed as number of particles per unit length of the plasma membrane or per area of cell bodies and dendrites.

Quantification of NK-1 receptor immunoreactivity and statistical analysis

We only quantified internalization in cell bodies. Dendrites were not analyzed in this study because the extensive overlap of labeled profiles made it difficult to evaluate endosomal labeling in dendrites, even with confocal analysis. It is particularly difficult to distinguish endosomes in the thin distal dendrites that arborize within lamina I. Based on our previous studies, however, we believe that internalization in cell bodies and dendrites follows the same time course (Mantyh et al, 1995). To analyze internalization in cell bodies we used 20X and 60X objectives on a Nikon microscope equipped for fluorescence. We counted all NK-1 receptor-like immunoreactive (LI) cell bodies in laminae I, III-IV and V-VI of the dorsal horn, ipsilateral to the side of stimulation, from segments L2 to L6. In cell bodies that do not contain internalized receptors, the immunoreactivity is uniformly distributed on the cell surface, but in the neurons that have internalized the NK-1 receptors, the cytoplasm contained bright, immunofluorescent structures. Unstimulated cells contained less than five endosomes per cell. In the present study we considered a cell to have internalized receptor if it contained more than 20 endosomes. Importantly, since we did not count the number of endosomes if fewer than 20 were present (i.e.

the categorization was all-or-none), it is possible that we missed subtle changes in the magnitude of internalization.

Since we found no difference in the magnitude of internalization along the mediolateral extent of the superficial dorsal horn, we counted all the cells within one segment, without taking into account the mediolateral position of the cells. All counts were then expressed as the percentage of NK-1 receptor-immunoreactive neurons that contained internalized receptor. The investigator who counted the NK-1 receptor-LI cells was unaware of the treatment of the animal.

For statistical analysis, we used a three-way analysis of variance for treatment condition (intact vs. inflammation), for temperature intensity or for duration of the stimulation (for pinch), and for spinal segment (L2-L6 lumbar segments). For multiple comparisons, we used the PLSD Fisher's test; $p<0.05$ was considered statistically significant.

In some cases the ANOVA was inappropriate either because of the heterogeneity of variances or because there was a clear difference between the two groups in NK-1 receptor internalization induced by mechanical stimulation (intact and inflamed). For the analysis, therefore, we specifically addressed the shape of the curve of the response of NK-1 receptor internalization in lamina I of the L4 spinal segment, which is the main target of primary afferent fibers from the hindpaw. To evaluate the contribution of duration of the mechanical stimulation (5 to 120 secs), we performed a two way ANOVA that compared the differences between the intact rats and the rats with chronic inflammation. Because of differences in responses (percent of NK-1 receptor internalization as a function of stimulus duration) in the two groups of rats, we modeled the responses independently. For the inflamed rats, because the response seems constant, we tested for equality in responses by comparing the one-

way (duration) ANOVA to the corresponding linear model. For the intact animals, the data were fitted to the Hill equation using weighted nonlinear regression to account for variance non-homogeneity in the data (Boeynaems, 1980). This approach takes into account differences in the variances and the ordered relationship of stimulus duration (5, 15, 30 and 120 sec). In a second step, the model was tested against a linear model using a Fisher test based on the residual deviance (i.e., variability of residuals corrected for differences in variances seen in the data). In the study that examined noxious heat induced internalization, we first performed a two-way ANOVA for effect of treatment condition (i.e. inflamed vs. intact) and then for the effect of temperature (45 to 52°C). To test for linearity of the relationship, we used a generalized linear model (McCullagh et al, 1989) with temperature as the continuous independent variable and treatment condition as the categorical variable.

Confocal images

Although our quantitative analysis was performed on tissue observed with epi-illuminated fluorescence, to better illustrate the patterns of receptor internalization that were induced in the different treatment conditions, we examined some sections by confocal microscopy. The confocal images described below were collected with an MRC 600 confocal microscope (Biorad). Images were then transferred in NIH-Image (version 1.60) and montages were created in Photoshop (Adobe, version 3.0).

RESULTS

NK-1 receptor-like immunoreactivity in intact and inflamed rats without superimposed stimulation

As we reported previously (Brown et al, 1995), there is a very distinct pattern of NK-1 receptor-immunoreactive neuronal staining in the dorsal horn of the rat. The densest staining is found in cell bodies and dendrites of lamina I. The immunoreactivity is best viewed in sagittal section because the dendrites, which express the bulk of the immunoreaction product, arborize in the rostrocaudal plane (Fig. 1). Lamina II (the substantia gelatinosa) contains very few NK-1 receptor-LI neurons, except for some dorsally-directed dendrites of relatively large NK-1 receptor-LI neurons located in laminae III-IV (Fig. 3). Smaller neurons with round cell bodies are also located in laminae III-VI; dendrites of these neurons arborize in all directions and in all planes (Fig. 3). A few labeled neurons in lamina V also have dorsally-directed dendrites that extend into lamina I. Finally, densely stained, large, round cell bodies are clustered around the central canal. (Fig. 4). In all regions, the NK-1 receptor immunoreactivity is concentrated on the plasma membrane of cell bodies and dendrites.

As previously reported, the density of NK-1 receptor-LI increases significantly in rats with an inflamed hindpaw. This was found in lamina I, not only in the L4 and L5 segments, which receive primary afferent input from the injected hindpaw, but also rostrally, in L1 and caudally, into sacral segments. We also observed an increase in lamina III-V; however, changes in these areas were not analyzed quantitatively. Although afferent input from the hindpaw targets the medial part of the dorsal horn,

the upregulation of the NK-1 receptor after inflammation occurs across the mediolateral extent of the dorsal horn. In our previous study (Abbadie et al, 1996) we used an HRP detection method, which is not ideal for light microscopic distinction of membrane and cytoplasmic labeling. In the present fluorescence analysis, we established that the increased receptor labeling after inflammation (in the absence of superimposed stimulation) remains on the membrane. Counts of labeled immunofluorescent neurons also confirmed that the number of SPR-immunoreactive neurons did not increase in the rats with inflammation. For example, in lamina I of L4 segment we found 86.32±5.3 (mean per rat) NK-1 receptor-LI neurons in rats with inflammation, and 82.08±2.1 in intact rats. This result indicates that the increase in SPR immunoreactivity in the rats with inflammation did not arise from receptor upregulation in neurons that previously did not express the receptor. Rather it appears to be an increased NK-1 receptor expression in the *existing* population of NK-1 receptor-immunoreactive neurons.

Quantitative analysis of the electron micrographs from the dorsal horn ipsilateral and contralateral to the CFA injection further established that although there was a 3.1 fold increase in the density per unit area of NK-1 receptor immunoreactivity (Fig. 2), the percentage of total labeling found on the membrane vs. the percentage found in the cytoplasm did not differ on the two sides of the cord. In cell bodies, 70.4 % of particles were distributed on the membrane in the ipsilateral side vs. 75.6 % in the contralateral side; in dendrites, 79.7 % of particles were distributed on the membrane in the ipsilateral side vs. 78.2 % in the contralateral side.

Effects of stimulation in normal rats

The number of immunoreactive neurons in sagittal dorsal horn sections varies somewhat along the mediolateral extent of the dorsal horn. In a 30 μm section through the L4 segment of the intact rat, we counted 10±4 cells in lamina I on one side, 0-5 cells in laminae III-IV, and 20±7 cells in laminae V-VI. Since we never found stimulation-evoked internalization of the NK-1 receptor in neurons of the dorsal horn contralateral to the stimulated hindpaw (even in the setting of inflammation and regardless of modality of the stimulus), the following quantitative analysis is limited to ipsilateral segments.

Mechanical stimulation - Noxious mechanical stimulation of the hindpaw (pinch) was a particularly effective stimulus for evoking NK-1 receptor internalization in dorsal horn neurons. We found that the magnitude of receptor internalization (% of cells showing internalization) increased with the duration of the mechanical stimulus; the relationship, however, was not linear (Fig. 5). For example, we recorded a significant difference ($p<0.001$) between the 5 and the 15 sec stimulus (22 % and 81 %, respectively in lamina I of the L4 segment), but no significant difference ($p=0.68$) between the 15 and 30 sec stimulation (81 % and 88 %, respectively), or between the 30 and the 120 sec stimulation (88 % and 98 %, respectively; $p=0.26$).

Thermal stimulation - Noxious thermal stimulation, produced by dipping the hindpaw in hot water, evoked a temperature-dependent increase in NK-1 receptor internalization in neurons of lamina I ipsilateral to the stimulus, however, in intact rats, we never found noxious heat-induced NK-1 receptor internalization in neurons located ventral to lamina I. Using the criterion of 20 endosomes per cell to define internalization, we found that the threshold for detecting significant increases in NK-

1 receptor internalization of lamina I was greater than 45°C. In fact, even 2 min at 45°C had no effect on the receptor distribution (<1 % in lamina I of L4). When the temperature was increased to 48°C approximately 50-55% of neurons in lamina I of the L4 segment contained internalized NK-1 receptor; the percentage of cells increased to 70-75 % at 50°C and to 95-100 % at 52°C. Statistical analysis revealed that there was a significant ($p<0.001$) effect of the intensity of the stimulus on NK-1 receptor internalization and that the temperature-internalization relationship was linear in the range 45 to 52°C. (Fig. 7). We also recorded a significant difference in the magnitude of internalization at different spinal segments ($p<0.001$). Specifically, more neurons with internalized receptor were found in segments L4 and L5 than at more rostral or caudal segments (Fig. 7B).

Effects of stimulation in rats with hindpaw inflammation

Mechanical stimulation - Noxious mechanical stimulation of the paw induced both a greater number of internalized cells in rats with inflammation than in intact rats and a shift in the dose effect curve for stimulus duration (Fig. 5). The difference between the magnitude of receptor internalization in the intact and rats with inflammation was significant ($p<0.001$). In the rats with inflammation, the 5 sec stimulation induced near maximal NK-1 receptor internalization; 95.5 % of NK-1 receptor immunoreactive neurons in lamina I cells of the L4 segment contained internalized receptor. At longer duration, the magnitude of internalization in the rats with inflammation was comparable, 96, 92 and 99 % for 15, 30 and 120 sec respectively. Not surprisingly, we found that there was a significant difference ($p<0.001$) in lamina I, between the magnitude of internalization in the two groups for the 5, 15 and 30 sec stimulation, but not for the 120 sec stimulation.

As noted above, for intact rats, although the relationship between the magnitude of internalization and intensity of the stimulus was linear for temperature, this was not the case for the duration of the mechanical stimulus. Rather, we found that the curve for magnitude of internalization vs. stimulus duration could be fit by the Hill equation (Fig. 5). The duration of stimulus that corresponded to 50% of internalization was estimated to be 7.2 seconds and the maximal value of internalization was estimated to be 95.5%. The maximal was not different from the percentage in the CFA-treated animals (t-test). For rats with inflammation, we obtained a response that plateaued (Fig. 5).

The laminar distribution of cells that contained internalized NK-1 receptor after noxious mechanical stimulation also differed in intact rats and in rats with an inflamed hindpaw. Thus in intact rats, we rarely found changes in laminae III-VI; less than 5% of NK-1 receptor-immunoreactive neurons responded to noxious mechanical stimulation by internalizing the receptor. In rats with inflammation, however, between 15 and 40% of the NK-1 receptor-immunoreactive neurons contained internalized receptor. The high variability that we recorded reflects the fact that there are relatively few NK-1 receptor immunoreactive neurons in laminae III-IV (Fig. 6). Although the large cells with dorsally-directed dendrites are easily recognized, they are not common (Figs. 3 and 4). Finally, with the most prolonged noxious mechanical stimulus (2 min) we also found changes in neurons of laminae V-VI. Thus in the L4 segment, this stimulus induced approximately 50% of the neurons to internalize the NK-1 receptor. Even the most prolonged noxious mechanical stimulus was without effect on the NK-1 receptor immunoreactive neurons in lamina X (Fig. 4). This was also true for noxious thermal stimulation).

Non-noxious mechanical stimulation - In intact rats, non-noxious stimulation of the paw (brush for 2 min) never evoked NK-1 receptor internalization in neurons of the lumbar spinal cord (Fig. 6A). In contrast, when the same stimulus was applied to the inflamed hindpaw, we recorded considerable numbers of neurons that contained internalized NK-1 receptor. Specifically, brush stimulation evoked NK-1 receptor internalization in approximately 75% of neurons in lamina I (Fig. 6B), and in about 20% of neurons in laminae III-IV (Fig. 6C) of the L4 spinal segment. Brush stimulation also induced receptor internalization in lamina I neurons of the L2 and L6 segments, 39% and 29.6%, respectively.

Thermal stimulation - As for mechanical stimulation, we found that internalization in response to noxious thermal stimuli increased in the setting of inflammation. The most striking change was observed with the 52°C stimulus, which after inflammation not only evoked internalization in 98-100% of lamina I neurons, but also in approximately 25% of neurons in laminae III-IV. In contrast, to mechanical stimulation, however, neither the threshold for evoking internalization nor the slope of the curve relating magnitude of internalization and temperature changed in intact and in rats with inflammation ($p=0.6$; Fig. 7A). Furthermore, the rostrocaudal distribution of internalized receptor at different spinal segments was the same in the two groups of rats (Fig. 7B).

Temporal pattern of NK-1 receptor recycling in intact and rats with inflammation

In our previous studies, we found that the receptor recycled to the plasma membrane within 60 min of stimulation. To evaluate whether there are differences in the temporal pattern of receptor recycling in normal rats and in rats with inflammation we assessed the magnitude of NK-1 receptor internalization at

different times after noxious mechanical stimulation. To produce maximal internalization in the different groups of rats, we used the 2 min stimulus. As described above the magnitude of internalization at 5 min after the stimulus was comparable in the two groups of rats (98 % in L4 of intact rats, 99.75 % in rats with inflammation). When we evaluated the spinal cords 30 min after the stimulus, however, we recorded a significantly greater magnitude of internalization of the receptor in neurons of lamina I cells ($p<0.0001$) in the rats with inflammation compared to controls, 62% vs. 40%, respectively in L4 (Fig. 8A). Sixty minutes after the stimulation, there was still significantly greater numbers of cells with internalized receptor in the rats with inflammation compared to the intact rats (16.5 %; $p<0.005$). At two hours the distribution of NK-1 receptor-LI in the rats with inflammation did not differ from stimulated controls or from unstimulated rats that received the CFA injection.

The preceding results establish that the receptor recycles to the membrane, but they do not establish that the receptor that is reinserted in the membrane is functional. Thus to assess the functional integrity of the membrane receptor at different times after stimulation, we also examined whether the receptor could be re-internalized by a second noxious stimulus. These studies were performed 60 min after the first stimulus, at which point most of the receptor recycles. The rats were perfused 5 min after the second stimulus, so that we could compare the magnitude of internalization to that produced by the same stimulus administered for the first time. In these studies, we used a stimulus of shorter duration (15 sec), so that we could increase the detectability of differences in the response of the receptor at the 60 min. time point. In rats with inflammation, we found no difference ($p=0.7$) in the magnitude of internalization between the rats stimulated once for 15 sec and those

stimulated twice (Fig. 8B). We observed a tendency for a reduced magnitude after the second stimulus in intact rats, but only in the L5 segment did we find a statistically significant difference ($p=0.015$, Fig. 8B). These results indicate that the recycled receptor is, in fact, equally responsive to the stimulus conditions and will internalize upon repeated stimulation.

DISCUSSION

In the present study we demonstrate that inflammation is associated with an upregulation of the NK-1 receptor, an increase in the number and distribution of dorsal horn neurons that internalize the NK -1 receptor in response to mechanical stimulation, and a decrease in the mechanical threshold for inducing internalization. The latter result implicates SP in the development of mechanical allodynia, whereby non-noxious stimulation can provoke withdrawal responses and pain behavior in the awake animal (Calvino et al, 1987; Colpaert, 1987). We found less change in the magnitude and distribution of neurons that contained internalized NK-1 receptor in response to noxious thermal stimulation and no change in the thermal threshold for triggering the internalization. These results not only provide new evidence for a contribution of SP to the reorganization of dorsal horn circuits in the setting of persistent injury, but also indicate that the processing of nociceptive mechanical and thermal information is differentially modified in the setting of injury.

NK-1 receptor internalization in normal rats: mechanical vs. thermal stimulation

Noxious pinch was the most potent stimulus for evoking internalization of the NK-1 receptor. Even the shortest duration noxious mechanical stimulus evoked near maximal internalization after inflammation. These results strongly suggest that SP is a neurotransmitter of high threshold mechanoreceptors. Since we did not detect internalization at 45°C, a temperature that activates polymodal nociceptors (Croze et al, 1976; Lynn et al, 1982) our results further suggest that this afferent is not the predominant source of SP, or that the level of SP release at this temperature is not high enough to induce internalization. It is also possible that the release of SP depends on the pattern or frequency of firing of the SP-containing primary afferent fiber and that these vary with mechanical and thermal noxious stimulation.

These results do not agree with those of Henry and colleagues, who found that NK-1 receptor antagonists block the response of deep dorsal horn neurons to noxious thermal stimulation (Radhakrishnan et al, 1995). One possibility is that the population of neurons from which they made recordings is not representative of the majority of wide dynamic range neurons in the deep dorsal horn. Importantly, the electrophysiological analysis detects short latency responses, which are probably driven by the direct synaptic inputs to the neurons. These may not be readily detected when the endpoint is internalization, as only a few receptors may internalize in response to a brief noxious heat pulse. It is also possible that the sensitivity of our assay does not allow us to detect the functional consequences of small increases of SP release.

Despite these potential methodological explanations for the discrepancies, our results are consistent with several other studies that more directly monitored the release of SP. For example, using antibody coated microelectrodes, Duggan and

colleagues found, in the cat, that only at temperatures that produced clear inflammation (>50°C) was there significant release of SP into the superficial dorsal horn (Duggan et al, 1988). In the rat these authors found that 48°C stimulation was sufficient (Lang & Hope, 1994). The latter results, and ours, are also consistent with those of Kuraishi *et al.*, who reported that although a 45°C stimulus evoked the release of somatostatin into rat dorsal horn, SP was only detected when the temperature was at least 48°C (Kuraishi et al, 1985; Kuraishi et al, 1989).

NK-1 receptor internalization in the setting of inflammation

The most important results in this paper concern the profound changes that occurred in the setting of inflammation. In our previous report that described an upregulation of the NK-1 receptor at different times after CFA induced inflammation of the hindpaw, we suggested that the upregulation receptor occurred in neurons that normally express the receptor. The use of fluorescence immunocytochemistry in the present study allowed a more accurate characterization of numbers of labeled neurons, and confirmed that the number of NK-1 receptor-immunoreactive neurons per section does not differ in the different groups of rats. Furthermore, using electron microscopy we established that in the absence of stimulation, the receptor remained concentrated on the plasma membrane, even in the rats with an inflamed hindpaw and greatly upregulated receptor. Unless there is incredibly rapid turnover of the receptor, this result suggests that a SP-NK-1 receptor interaction does not come into play in the basal firing of spinal cord neurons. This is true despite the fact that SP levels and the affinity of the NK-1 receptor increase during the development of inflammation (Stucky et al, 1993). We conclude that the upregulation of the NK-1

receptor is only functionally manifested when a stimulus is superimposed upon a background of inflammation-induced alterations in the dorsal horn.

In the setting of inflammation we found that noxious mechanical stimulation induced NK-1 receptor internalization to a much greater extent in neurons of the deep dorsal horn and we recorded greater numbers of lamina I neurons in segments outside of the primary terminal region of afferents from the hindpaw, i.e. in L2 and L6. The most striking observation in the rats with inflammation of the hindpaw, however, was that even non-noxious stimulation induced NK-1 receptor internalization in dorsal horn neurons; this was never seen in intact rats. This provides the strongest evidence to date that the altered properties of dorsal horn neurons that occur in the setting of injury (Calvino et al, 1987; Hylden et al, 1987; Hylden et al, 1989; Menétrey et al, 1982) involve release of substance P and interaction with dorsal horn neurons that express the NK-1 receptor. Since mechanical allodynia is a characteristic feature of persistent injury states (Calvino et al, 1987), our results further suggest that SP contributes to the clinical manifestations of chronic inflammation.

There are several mechanisms through which these results could have been generated. In the normal animal, SP is concentrated in small diameter, nociceptive afferents and when there is inflammation, there is a cyclooxygenase-dependent sensitization of the terminals of the afferents, such that non-noxious stimuli can activate the afferents (Martin et al, 1988; Taiwo and Levine, 1986). Inflammation also induces a central sensitization of dorsal horn nociresponsive neurons, in which the receptive field of the dorsal horn neuron is increased, as is spontaneous activity, and the threshold for evoking activity in these neurons decreases (Calvino et al, 1987;

Hylden et al, 1987; Hylden et al, 1989; Menétrey et al, 1982). Since C fibers do not project directly to neurons of the deep dorsal horn, an alternative explanation for the appearance of significant SP-mediated internalization is required. As noted previously, based on studies of Duggan and colleagues (Schaible et al, 1990), it is likely that diffusion of SP from superficial laminae to deep laminae is an important contributor; the fact that this is exacerbated in the setting of inflammation provides additional evidence in favor of this hypothesis. In the absence of significant diffusion of substance P, it is likely that large diameter, A-beta fibers provide the non-nociceptive input to neurons that have undergone central sensitization. Although these large diameter afferents do not synthesize SP in the normal rat, they do so in the setting of peripheral nerve injury (Noguchi et al, 1989; Noguchi et al, 1994) and inflammation (Neumann, 1996). This could provide a monosynaptic input to deep neurons that express the NK-1 receptor. Large fiber-mediated polysynaptic activation of SP-containing interneurons may also be involved. Thus, both small and large diameter afferents may be the source of the SP that induces NK-1 receptor internalization by non-noxious stimulation, the former to cell bodies and dendrites of marginal neurons and to the dorsally-directed dendrites of lamina III neurons, the latter to neurons of the deep dorsal horn. Distinguishing between the contribution of peripheral and central sensitization is difficult because any treatment that reduces peripheral sensitization of C fiber afferent would concurrently reduce the inflammation. It may be possible to use electrical stimulation to selectively activate fibers of different caliber, however, the surgical procedure required to place the stimulating electrodes, could itself induce internalization of the receptor.

Finally, despite there being a significant shift in the mechanical threshold for evoking NK-1 receptor internalization in the rats with hindpaw inflammation, we found little change in the response to noxious thermal stimulation. Only at the highest temperature (52°C), was the magnitude of the response increased in rats with persistent inflammation, and only at this temperature did the distribution of neurons that contained internalized receptor change. Specifically at 52°C, we observed NK-1 receptor internalization in about 25 % of laminae III-IV neurons. These data suggest that a SP-mediated thermal allodynia does not develop in the setting of inflammation. Consistent with this conclusion, Liu and Sandkuhler (1995) found enhanced responses of lamina I neurons to mechanical, but not to thermal skin stimuli during superfusion with SP.

Summary

We have proposed that the pattern of neurons that internalize the NK-1 receptor in response to natural stimulation provides a functional measure of release of SP from primary afferent fibers (and possibly from SP-containing interneurons) and a "picture" of the distribution of neurons that are activated by SP. The results of the present study provide strong evidence that the population of neurons with which SP interacts changes dramatically in the setting of inflammation. We also demonstrate that mechanical stimulation is particularly effective in evoking the release of SP and inducing NK-1 receptor internalization. We suggest that the SP-NK-1 receptor mediated changes that we have identified are critical contributors to the central sensitization of dorsal horn circuits that occurs in the setting of inflammation and thus to the allodynia and hyperalgesia that ensue after injury.

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Figure 1: Confocal images illustrating the dorsoventral pattern of internalization of the neurokinin-1 receptor (NK-1 R) after mechanical stimulation of the hindpaw. These images were generated by superimposition of 3 optical sections taken at 2.5 μm in sagittal sections of L4. A: rat with inflamed hindpaw with no additional stimulation; the receptor is distributed on the plasma membrane of neurons in laminae I-III. B: intact rat after mechanical stimulation of the hindpaw (pinch for 30 sec); cell bodies and dendrites in lamina I contain internalized NK-1 receptor, however, no changes were recorded in lamina III. C: rat with inflamed hindpaw after mechanical stimulation of the hindpaw (pinch for 30 sec). There is extensive receptor internalization, in cell bodies and dendrites of neurons throughout the dorsal horn.

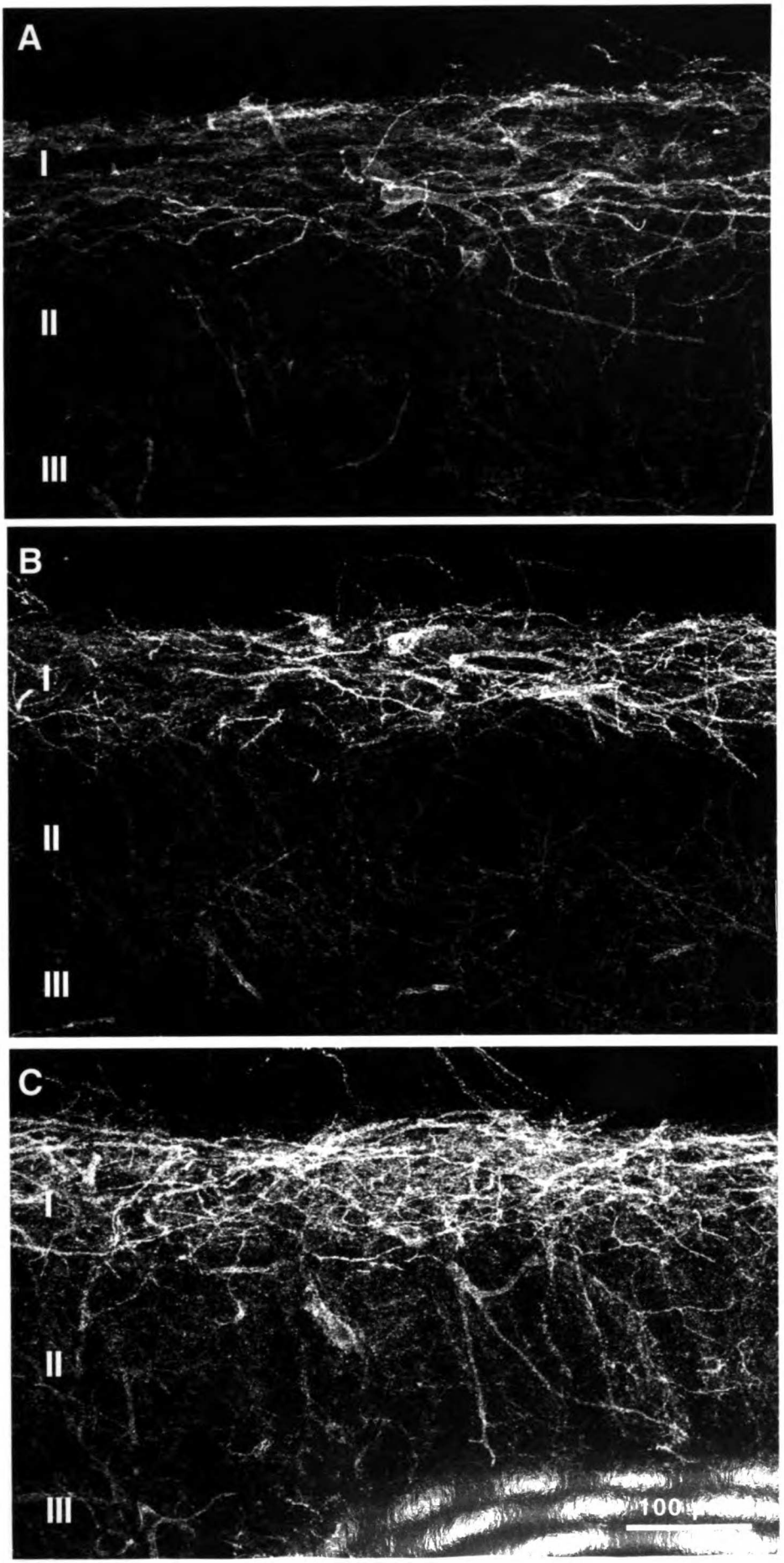

A
I
II
III
B
I
II
III
C
I
II
III
100

Figure 2: These electron micrographs illustrate the distribution of NK-1 receptor immunoreactivity in neurons of lamina I of the dorsal horn of an intact rat (A,C) and a rat 3 days after inflammation of the hindpaw was induced with CFA (B,D). In the intact rat the receptor labeling (arrowheads) is concentrated on the plasma membrane of both cell bodies (A) and dendrites (C). After inflammation there is a significant increase in NK-1 receptor immunoreactivity, however, the receptor labeling (arrowheads) is still concentrated on the plasma membrane of cell bodies (B) and dendrites (D). In both cases, there is some cytoplasmic label present. Calibration bars equal 2 μm in A, B and 0.25 μm in C, D.

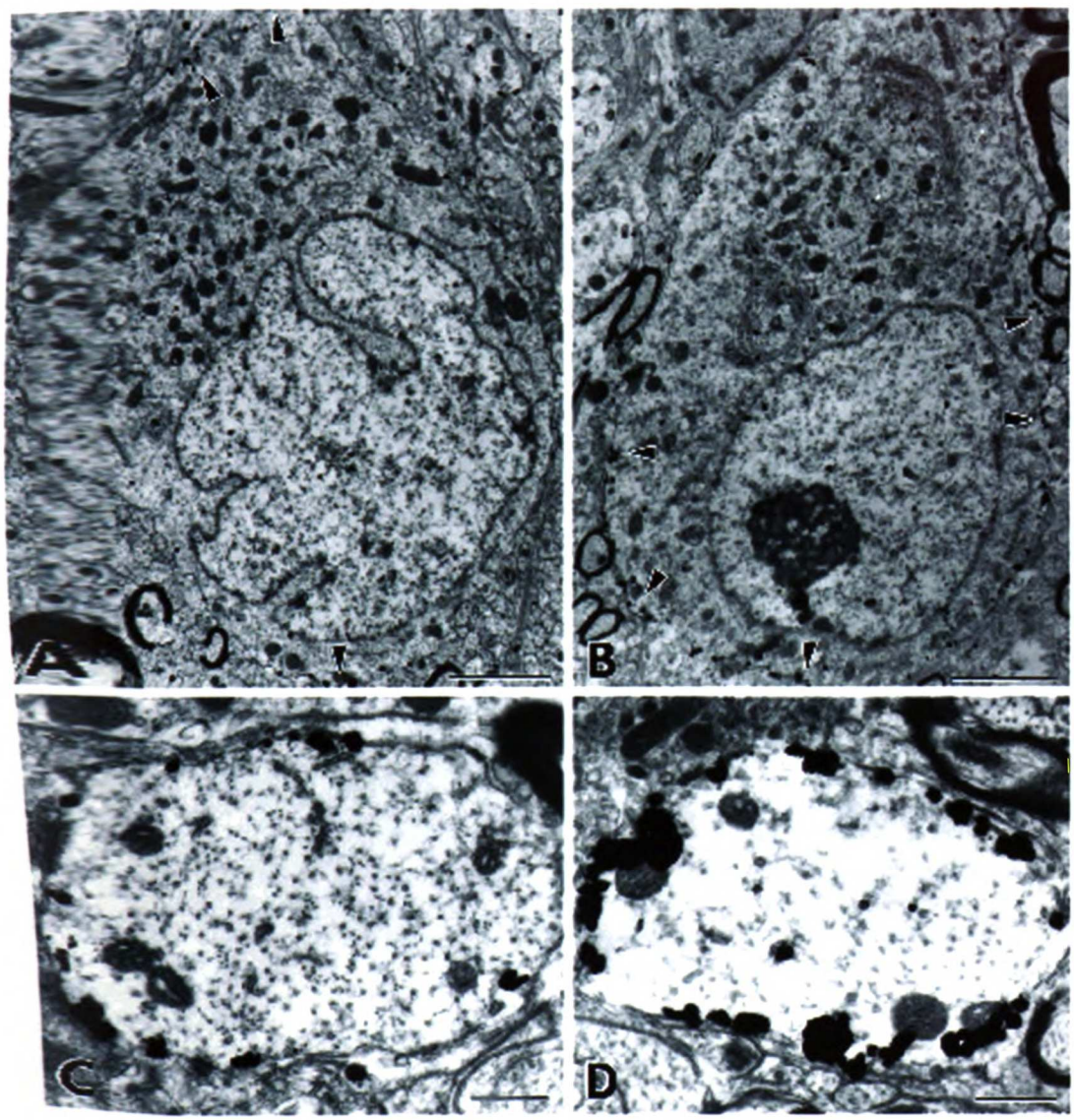

A
B
C
D

Figure 3: These confocal images illustrate internalization of the NK-1 receptor in neurons of laminae III and V after mechanical stimulation of the hindpaw (pinch for 30 sec). A, C and D were produced by superimposition of 3 optical sections taken at 2.5 µm in sagittal sections of L4; B, D and E were produced by superimposition of 7 optical sections taken at 0.6 µm. A-B: intact rat after mechanical stimulation of the hindpaw (pinch for 30 sec); there is no internalization in this lamina III neuron. C-D: rat with inflamed hindpaw after mechanical stimulation of the hindpaw (pinch for 30 sec); there is extensive internalization of NK-1 receptors in this lamina III neuron. E-F: same experimental condition as for C-D; note internalization of NK-1 receptors in this lamina V neuron.

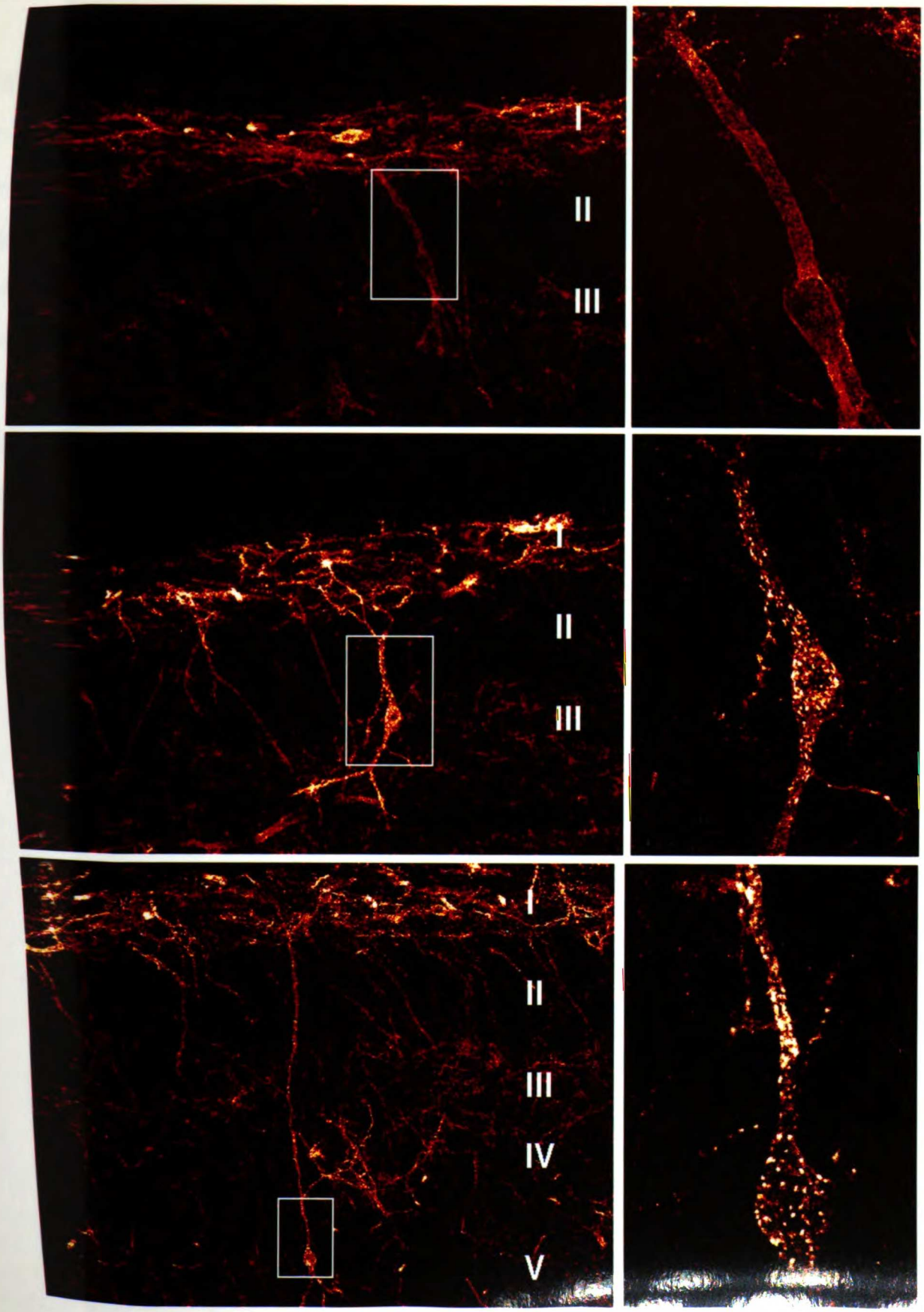

I
II
III
I
II
III
I
II
III
IV
V

Figure 4: These confocal images illustrate internalization of the NK-1 receptor in neurons of laminae III, V and X. A-F: rat with inflamed hindpaw after mechanical stimulation of the hindpaw (pinch for 2 min). A, C and D were produced by superimposition of 3 optical sections taken at 2.5 μm in sagittal sections of L4; B, D and E were produced by superimposition of 7 optical sections taken at 0.6 μm. There is internalization of NK-1 receptors in laminae III (A,B) and V (C,D) after mechanical stimulation of rats with persistent inflammation. In intact rats this stimulus only induces internalization in lamina I neurons. Neither mechanical nor thermal stimulation induced internalization in neurons of lamina X (F).

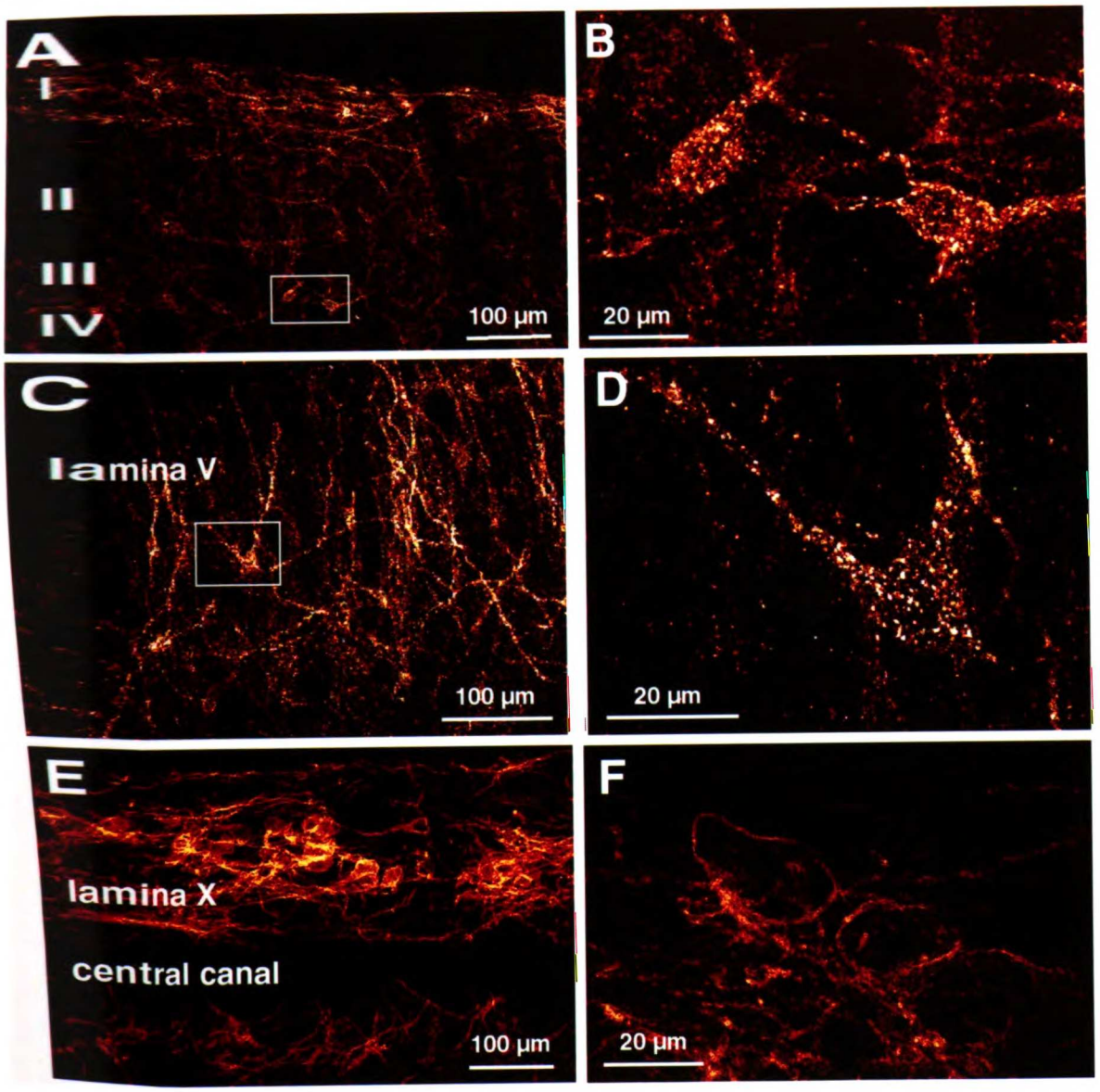

A
I
II
III
IV
100 µm
B
20 µm
C
lamina V
100 µm
D
20 µm
E
lamina X
central canal
100 µm
F
20 µm

Figure 5: This histogram illustrates the percentage of cells showing internalization of the NK-1 receptor in lamina I of the L4 segment after different durations of mechanical stimulation (pinch) of the hindpaw in normal rats and in rats 3 days after inflammation of the hindpaw was induced with CFA. Results are expressed as means ± S.E.M. per group. In contrast to the effects of temperature, which produced a linear relationship between intensity stimulation and number of neurons having internalized NK-1 receptors (see Fig. 7), the relationship between the duration of the mechanical stimulation and the degree of internalization was not linear. For the intact animals, the Hill equation fit the data (see insert). In the setting of inflammation, the response plateaued.

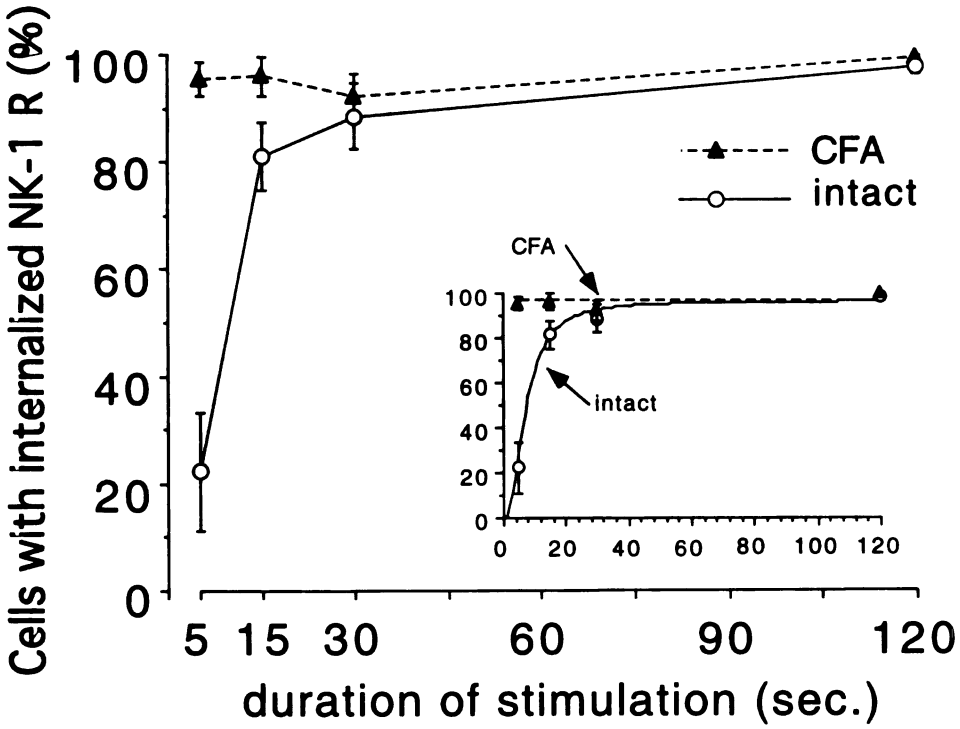

Cells with internalized NK-1 R (%)
100
80
60
40
20
0
5 15 30 60 90 120
duration of stimulation (sec.)
CFA
intact
CFA
intact
100
80
60
40
20
0
0 20 40 60 80 100 120

Figure 6: These histograms illustrate the percentage of cells in different laminae of the lumbar spinal cord and in different lumbar segments that contained internalized NK-1 receptor after mechanical stimulation of the hindpaw. Results are expressed as means ± S.E.M. per group. Note that: (1) a non-noxious mechanical stimulus (brush for 2 min) did not induce internalization in intact rats (A), but in rats with persistent inflammation (3 days after CFA injection) this stimulus induced internalization in neurons of laminae I and in laminae III-IV (B,C); (2) the magnitude of receptor internalization in rats with persistent inflammation did not differ with noxious mechanical stimuli (pinch) of different duration (B,C). This contrasts with intact rats, in which the number of internalized cell bodies increases with the duration of the stimulus (A).

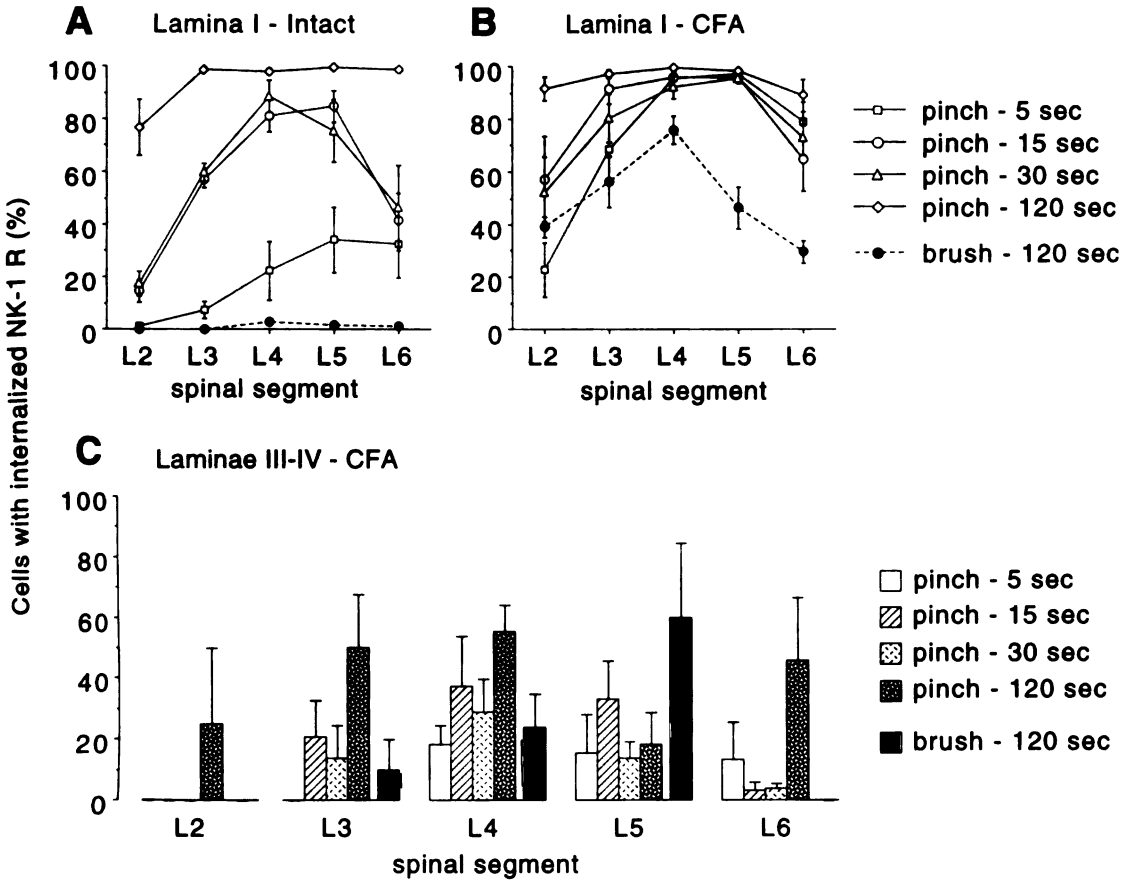

A
Lamina I - Intact
B
Lamina I - CFA
C
Laminae III-IV - CFA
Cells with internalized NK-1 R (%)
100
80
60
40
20
0
L2
L3
L4
L5
L6
spinal segment
pinch - 5 sec
pinch - 15 sec
pinch - 30 sec
pinch - 120 sec
brush - 120 sec

Figure 7: These histograms illustrate the percentage of spinal cord cells with internalized NK-1 receptor after thermal stimulation of the hindpaw in intact rats and in rats with an inflamed hindpaw (3 days after CFA injection). Rats were stimulated for 2 min at either 45, 48, 50 or 52°C. The results are expressed as means ± S.E.M. per group. A: percent of internalized cells in lamina I of L4; B: rostrocaudal (L2-L6) distribution of neurons with internalized receptor. Note that (1) in neither intact rats nor in rats with inflammation did the 45°C stimulus induce NK-1 receptor internalization; (2) the number of internalized cells increased with temperature; (3) there was no significant difference between the number of lamina I neurons with internalized receptor in the two groups of rats, regardless of temperature.

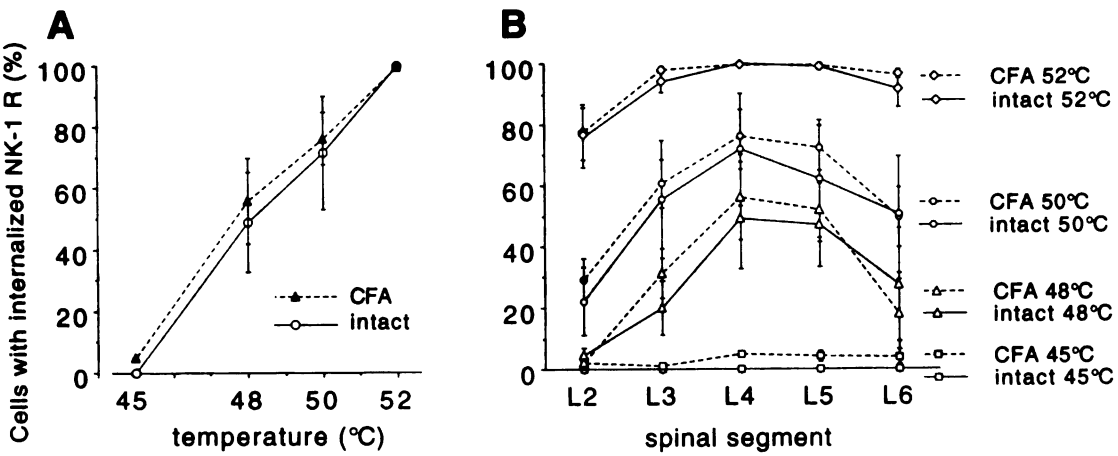
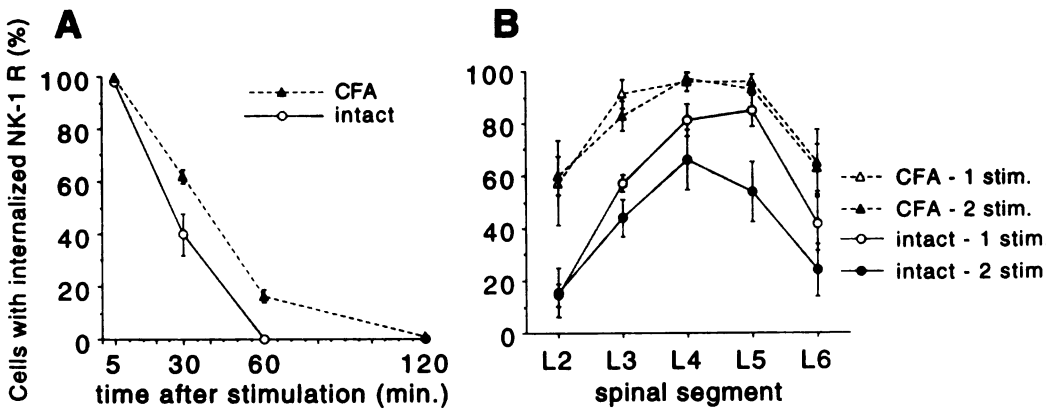

A
Cells with internalized NK-1 R (%)
100
80
60
40
20
0
45
48
50
52
temperature (°C)
CFA
intact
B
100
80
60
40
20
0
L2
L3
L4
L5
L6
spinal segment
CFA 52°C
intact 52°C
CFA 50°C
intact 50°C
CFA 48°C
intact 48°C
CFA 45°C
intact 45°C

Figure 8: These histograms illustrate the percentage of cells with internalized NK-1 receptor in lamina I of the L4 segment. Results are expressed as means ± S.E.M. per group. A: Time course of NK-1 receptor internalization from rats perfused at different times after hindpaw stimulation (pinch for 2 min). The number of internalized cells is significantly greater in rats with an inflamed hindpaw than in intact rats, at both 30 and 60 min. B: Results of two successive mechanical stimuli: the second stimulus (pinch for 15 sec) was applied one hour after the first, and the rats were perfused 5 min later. In the inflamed groups of rats, there is no difference ($p=0.7$) in the magnitude of receptor internalization between the rats stimulated once and those stimulated twice. In intact rats there is a smaller number of cells with internalized receptor after the second stimulus, but the difference is only significant in the L5 segment ($p=0.015$).


A
Cells with internalized NK-1 R (%)
100
80
60
40
20
0
5
30
60
120
time after stimulation (min.)
CFA
intact
B
100
80
60
40
20
0
L2
L3
L4
L5
L6
spinal segment
CFA - 1 stim.
CFA - 2 stim.
intact - 1 stim.
intact - 2 stim.

FURTHER CONCLUSIONS, DISCUSSION AND QUESTIONS

We conclude that NK 1 receptor-mediated tachykinin activity in the spinal cord dorsal horn of normal rats only occurs with highly noxious stimulation. Above this high threshold, NK-1 receptor internalization in lamina I is graded with respect to stimulus intensity . In the setting of inflammation, noxious stimulation additionally induces NK-1 receptor internalization in the cell bodies of neurons in deeper lamina, namely III-VI. The stimulus intensity dependence of NK-1 receptor internalization is strongly shifted for mechanical stimuli, with noxious stimuli inducing NK-1 receptor internalization in significantly more lamina I neurons than in normal animals. Additionally non-noxious mechanical stimuli (brushing of the inflamed paw) now induce tachykinin-mediated postsynaptic activity, something that is never seen in normal animals. Interestingly, the stimulus intensity dependence of lamina I NK-1 receptor internalization was not altered for thermal stimuli,.

These studies raise some interesting questions for further study. First, the mechanisms underlying the increases in NK-1 receptor internalization seen with inflammation are still unknown. Our initial hypothesis was that the spread of internalization could be explained by the previously described induction and upregulation of tachykinin expression in large and small diameter primary afferent fibers (Neumann et al, 1996) in the setting of inflammation. Additional SP/NKA release from a population of low threshold mechanically responsive/thermally insensitive A beta afferents might thus explain the increased NK-1 receptor internalization we observed. However, Allen et al. (1999) recently reported that electrical stimulation of A beta fibers during

inflammation was unable to induce any NK-1 receptor internalization. Furthermore, although A delta fiber stimulation induced some NK-1 receptor internalization in lamina I, this was not greater that what is observed under normal conditions. Only with electrical stimulation at intensities high enough to recruit C-fibers were the authors able to induce increased NK-1 receptor internalization in laminae III-VI. This suggests that increases in NK-1 receptor internalization seen with inflammation still require the activation of C-fibers. Furthermore, agents that produce a more rapid inflammation (carrageenin, formalin) produce the same changes in NK-1 receptor internalization within 3 hours (Honoré et al, 1999). The time course of these changes suggest that they reflect sensitization of small diameter SP containing neurons and increased spread of tachykinins rather than changes in gene expression. In this regard Schaible et al (1992) suggested that CGRP can prevent the degradation of tachykinins by competing for endopeptidases,. leading to increased spread of tachykinins in the spinal cord. The observed changes may thus reflect a peripheral sensitization of SP-containing DRG neurons (presumably via their peripheral afferent terminals), resulting in tachykinin release in response to normally non-noxious stimuli. As this would occur concurrently with a sensitization of CGRP-containing neurons (which coexist with SP in DRG neurons), there would also be a spread of tachykinins into deeper lamina. Together the combined peripheral and central changes in the setting of injury could dramatically alter the impact of non-noxious and noxious peripheral stimuli.

The significance of the spread of NK-1 receptor activation to neurons of the deeper dorsal horn is far from obvious. In the case of the NK-1 receptor

expressing neurons of lamina III, it is a very small number of neurons in which we see internalization following a very intense stimulus. The function of these neurons is unknown, and thus it is difficult to formulate hypotheses as to the function or consequences of this observed change. As noted above an important feature of some of these lamina III neurons is that their dorsal dendrites arborize in lamina I, and can be observed to internalize the NK-1 receptor following noxious stimulation. This occurs in normal animals, i.e. in the absence of ongoing injury.. Is there a difference between activating NK-1 receptors on a neuron's distal dendrites versus its cell body? Just because the difference is observable does not mean that it is functionally important. Further study of both the cell biology of NK-1 receptor induced cellular signaling and the neuroanatomy and physiology of the NK-1 receptor expressing neuronal population is necessary before the importance of these observations to nociceptive processing can be fully appreciated.

Finally, the source of the tachykinins that activate cells in deeper lamina is unknown. It is possible that all the NK-1 receptor activation that we observed results from release of tachykinins from primary afferent sensory neurons. Although the C fibers terminate exclusively in the superficial dorsal horn, where all of the release must occur, it is possible that in the setting of inflammation, neuropeptides diffuse ventrally, into the deep dorsal horn.. Although this proposal is consistent with the previously described changes in CGRP inhibition of endopeptidase degradation (Shaible et al, 1992), several observations suggest that this may not fully explain the changes that we observed. First, we found that high doses of intrathecally injected tachykinins still activated only lamina I NK-1 receptors, even in the presence of inflammation (unpublished

observations). Thus, spread of tachykinins into deeper lamina is not facilitated under basal conditions during inflammation. On the other hand, because we did not coinject CGRP , we may not have sufficiently facilitated ventral diffusion. In other words, tachykinin spread may only occur with noxious stimulation because such stimuli would concurrently induce CGRP release.

The pattern of overall NK-1 receptor staining in the spinal cord dorsal horn after noxious stimulation in the presence of inflammation is suggestive. Thus, although scattered NK-1 receptor positive endosomes can be observed throughout the tissue just dorsal horn to most lamina III neurons that contain internalized NK-1 receptor, this is often not the case with lamina V-VI neurons that contain NK-1 receptor internalization. Indeed we have observed lamina V neurons with internalized receptor directly below other lamina V neurons that did not contain internalized receptor. Instead, small "clouds" of NK-1 receptor positive endosomes were sometimes observed surrounding lamina V-VI neurons with internalized NK-1 receptor ,with the normal non-speckled background labeling located dorsal to and and around the patch. This staining pattern seems more consistent with the idea of diffusion of primary afferent-derived tachykinin into lamina III-IV and/or local release of interneuron-derived tachykinins in lamina V-VI. Clearly, neither observation unequivocally can pinpoint the source of the tachykinins, which underscores the need for further to resolve this question.

Allan BJ, Li J, Menning PM, Rogers SD, Ghilardi J, Mantyh PW, Simone DA. (1999) Primary afferent fibers that contribute to increased substance P receptor internalization in the spinal cord after injury. J Neurophy 81: 1379-1390.

Honore P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM, Mantyh PW. (1999) Spinal substance P receptor expression in internalization in acute, short-term, and long-term inflammatory pain states. J Neurosci 19: 7670-7678.

Neumann S, Doubell TP, Leslie T, Woolf C. (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384: 360-364.

Schaible HG, Hope PJ, Lang CW, Duggan AW. (1992) Calcitonin gene-related peptide causes intraspinal spreading of substance P released by peripheral stimulation. Eur J Neurosci 4: 750-757.

Chapter 2

Targets and differential effects of SP and NKA in the spinal cord dorsal horn

GENERAL INTRODUCTION

The tachykinins substance P and neurokinin A are both products of the preprotachykinin-A gene and share similar N-terminal sequences. Both can activate the neurokinin-1 and neurokinin-2 receptors in similar concentration ranges. Given that their expression is thus jointly regulated and their effects occur via the same target receptors, the question arises as to the extent to which they are indeed functionally identical

It has been suggested that because the two neuropeptides are differentially degraded by neutral endopeptidase, the extent to which the two tachykinins diffuse after release differs. Perhaps more interestingly, the two peptides have been shown to bind distinct sites on the neurokinin-1 receptor (Wijkhuisen et al, 1999) and there has been speculation and some evidence that this might result in different downstream signaling in response to activation by the different tachykinin peptides (Sagan et al, 1996).

Little is known about the *in vivo* actions of these two peptides in the spinal cord. Differences in their actions have been assumed to be mediated via an NKA action on NK-2 receptors. The problem with that formulation is that to date there is no evidence for NK-2 receptor message or protein in the spinal cord.

In the following series of studies we used NK-1 receptor internalization and Fos expression to begin to examine how the actions of these two tachykinin peptides differ in the spinal cord. Our studies focused on the extent to which each peptide contributes to the pattern of noxious stimulus evoked neurokinin-1 receptor activation that we have observed and to possible differences in the downstream effects produced by the two tachykinins.

Sagan S, Chassaing G, Pradier L, Laville S. (1996) Tachykinin peptides affect differently the second messenger pathways after binding to CHO-expressed human NK-1 receptors. J Phamacol Exp Therapeutics 276: 1039-1048.

Wijkhuisen A, Sagot MA, Frobert Y, Creminon C, Grassi J, Boquet D, Couraud JY. (1999) Identification in the NK1 tachykinin receptor of a domain involved in recognition of neurokinin A and septide but not of substance P. FEBS letters 447:155-159.

Differential Contribution of Substance P and Neurokinin A to Spinal Cord Neurokinin-1 Receptor Signaling

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ABSTRACT

The tachykinins substance P and neurokinin A are co-expressed in primary afferent nociceptors and are co-released following noxious stimulation. Both peptides act upon neurokinin receptors, but little is known about the extent to which the two tachykinins have differential actions *in vivo*. Although there is pharmacological evidence that NKA selectively acts upon spinal NK-2 receptors, this receptor has not been identified in the cord. For this reason our studies addressed the extent to which SP and NKA contribute to spinal nociceptive processing via the neurokinin-1 receptor.

We found that SP and NKA induce NK-1 receptor internalization *in vivo* and *in vitro* with identical dose dependence. Both tachykinins also induce increases in intracellular calcium levels at the same concentrations, suggesting that SP and NKA are equally capable of activating the NK-1 receptor in the spinal cord. We found, however, that +NK-1 receptor activation and internalization by the two tachykinins were differentially inhibited by the compound GR 205171. Thus, GR 205171 blocked NKA, but not SP-induced NK-1 receptor internalization *in vivo*. This selectivity of the GR 205171inhibition of the NK-1 receptor for NKA induced activation was confirmed in vitro.

Using the selectivity of GR 205171 for NKA-induced NK-1 receptor activation, we next examined the extent to which noxious stimulus-induced NK-1 receptor activation in the spinal cord was NKA-mediated. We estimate that NKA contributes to at least 50% of the NK-1 receptor activation in lamina I following noxious mechanical or thermal stimulation of the hindpaw. Moreover, under inflammatory conditions, all of the NK-1 receptor internalization induced

by noxious stimulation in neurons of the deep dorsal horn was eliminated by pretreatment with GR 205171, which suggests that it is all mediated by NKA. These studies suggest that NKA has robust and functionally significant effects via the NK-1 receptor *in vivo* and that the NK-1 receptor may be the predominant target of NKA in the CNS.

INTRODUCTION

The tachykinins, substance P (SP) and neurokinin A (NKA) are produced from a single precursor, the preprotachykinin A gene, by a population of small diameter primary afferent neurons. Both SP and NKA are transported to the peripheral and central terminals of these afferents, where they are costored in dense core vesicles and can be released in response to noxious stimulation. Furthermore, there is electrophysiological evidence that both SP and NKA can activate dorsal horn neurons (Henry, 1976; De Koninck and Henry, 1991; Radhakrishnan and Henry, 1997), and induce pain behaviors after intrathecal injection (Hylden and Wilcox, 1981; Gamse and Saria, 1986). Despite these apparent comparable effects in the spinal cord, it is not clear whether the actions of one completely mimics the other, (i.e. whether there is redundancy) or whether different receptors, located on different populations of dorsal horn neurons, mediate the actions of these peptides.

The tachykinins target three neurokinin receptors (NK-1, NK-2, and NK-3). Early binding studies indicated that each of the cloned receptors has a higher affinity, (i.e. show preferential binding) for a different tachykinin. Thus, it was proposed that SP, NKA and NKB (a product of the preprotachykinin B gene, which is not found in DRG neurons) are endogenous ligands for the NK-1, NK-2 and NK-3 receptors, respectively. Although pharmacological support for this view has been provided in studies of peripheral tachykinin actions, the categorization is more difficult to sustain in the CNS. Specifically, with the exception of forebrain and some limited glial expression, there is no evidence that NK-2 mRNA or protein exists in the CNS. Because of this, it has been

postulated that the conclusions about NK-2 receptor function that were based on binding studies were incorrect.

On the other hand, pharmacological studies using selective antagonists have provided considerable support for the existence of functional and distinct NK-2 receptors. This is particularly true in studies of spinal cord nociceptive processing. For example, the NK-2 receptor selective antagonist SR 48968 blocks C-fiber induced long term potentiation in the spinal cord, reduces the responses of dorsal horn neurons to mechanical stimulation of the knee and the hyperexcitability of these responses after inflammatory injury, and reduces the hyperalgesic effects of intrathecal NKA in the tail flick test (Yashpal et al, 1996; Neugebauer et al, 1996; Liu et al, 1997).

The paradox may in part be explained by a growing literature that suggests that NKA may, in fact, target NK-1 receptors at concentrations similar to that of SP. Indeed, Hastrup and Schwartz (1996), using homologous rather than heterologous binding studies, found that the affinity of NKA for the NK-1 receptor was much higher than previously claimed. Furthermore, both SP and NKA increase IP3 production and induce inward currents via the NK-1 receptor at similar concentrations (Akasu et al, 1996; Sagan et al, 1996). These findings highlight the possibility that NKA could act as an NK-1 receptor ligand *in vivo*. Consistent with this view, electrophysiological studies in the spinal cord demonstrate that exogenously applied NKA activates wide dynamic range neurons in the dorsal horn via the NK-1 receptor (Radhakrishnan and Henry, 1997). To date, however, no studies have directly addressed the extent to which the functional consequences of endogenously released NKA are mediated via the NK-1 receptor.

In the present study, we used an in vivo and in vitro analysis of SP and NKA induced functional effects that are exerted via the NK-1 receptor. As a functional marker of NK-1 receptor activation, we monitored NK-1 receptor internalization, which we have previously shown occurs in response to substance P and correlates quantitatively with NK-1 receptor-mediated increases in intracellular calcium (Trafton et al, 1999). Additionally, we describe an NK-1 receptor antagonist, GR 205171, that selectively inhibits NKA as opposed to SP-mediated NK-1 receptor activation. Making use of the unusual pharmacological properties of this compound to only inhibit NKA-induced NK-1 receptor activation, we examined the contribution of NKA to noxious stimulus induced NK-1 receptor activation in the dorsal horn of the spinal cord.

METHODS

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at UCSF.

Cell culture experiments

Spinal Cord cultures

Spinal cord cultures were prepared from E19 Sprague-Dawley rats using a modification of the method of Yu et al. (1984). Spinal cords were dissected out, washed and treated with a standard 0.25% trypsin/versene mixture (STV, cell culture facility, UCSF) for 12 minutes. Cords were again washed and mechanically dissociated by titruation. Cells were next diluted into 24 ml of

MEM-PAK buffer (cell culture facility, UCSF) supplemented to contain 5% normal horse serum, 5% fetal calf serum and penicillin/streptomycin, 30 mM glucose and 2 mM glycine. Cells were plated on glass coverslips (Carolina, Burlington, NC) or 8 well coverglass (Fisher, Santa Clara, CA) and incubated at 37°C in a humidified incubator with 5% CO2/95% O2. Cultures were used after 5-6 days in culture.

Calcium Imaging

Spinal cord cultures plated on 8 well coverglass were incubated in CI buffer (in mM: 130 NaCl,.3 KCl, 2.5 CaCl2, 0.6 MgCl2, 1.2 NaHCO3, 10 glucose, 10 HEPES, pH 7.4) containing 10 μM Fura-2 acetoxymethyl ester and 0.02% pleuronic acid (Molecular Probes) for 25 minutes. Fura was then removed and replaced with 150 μl/well of fresh CI buffer. We performed ratiometric calcium imaging with a Nikon Diaphot fluorescence microscope equipped with a variable filter wheel (Sutter Instruments) and an intensified CCD camera (Hamamatsu). Dual images (340 and 380 nm excitation, 510 nm emission) were collected every 4 sec. For each well, we recorded five baseline images and then 150 μl of SP in CI buffer at twice the desired final concentration was pipetted into the well. Responses were recorded for the following 40 seconds.

The average 340/380 ratio was calculated for each of the cells in the field and for each of the time points. All cells that showed an average increase in 340/380 ratio of greater than twice the average baseline 340/380 ratio for that cell were considered responders. Only responders were considered in the subsequent analysis. The total increase in intracellular calcium was calculated

for all responders in each well by taking the sum of the 340/380 ratios over the 40 secs after SP application with the average baseline values measured over the first 20 secs subtracted from those values.

In vitro internalization

Spinal cord cultures plated on coverglass were incubated in culture media containing the indicated concentration of tachykinin. After 15 minutes, media was removed and the cells were fixed in 10% formalin for 20 minutes. NK-1 receptor was labeled for immunofluorescent analysis as described below for tissue slices. Confocal images (75X 4.0 iris diameter) were taken of 5 NK-1 receptor positive neurons per coverslip and the number of endosomes in each cell was counted and averaged for neurons over the coverslip. Images were collected on a Biorad MRC 1024 confocal microscope. The investigator taking images and counting endosomes was unaware of the treatment that the cultures received. All neurons containing greater than 10 endosomes were considered to have internalized the NK-1 receptor, and from the endosome counts described above, for each coverslip we calculated the percentage of NK-1 receptor positive neurons that were internalized.

Statistical analysis

The % of maximal observed effect was calculated using the following formula:

(Average value with tachykinin dose (x) - Average value with 0 mM tachykinin)/ Highest observed average value with any tachykinin dose

The % of maximal observed responders was calculated using the following formula:

(% of responders with tachykinin dose (x) - % of responders with 0 mM tachykinin) / Highest % of responders observed with any tachykinin dose

The threshold values that we chose for counting cells as responders were based on the baseline and maximal response values. Similar results were obtained when we performed the analysis using 15 endosomes and 1.5 times baseline as thresholds. EC50's were calculated with Prism (GraphPad Software, Inc). For statistical analysis, we used a two-way analysis of variance for measurement of intracellular calcium concentration or NK-1 receptor internalization, and for tachykinin dose.

In Vivo experiments

Experimental animals

Experiments were performed on male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA), weighing 230-270g. Noxious mechanical and thermal stimulation and i.t. injections were performed 10-15 minutes after the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). This dose blocked flexor reflex responses to hindpaw stimulation. In some rats, inflammation was induced by subcutaneous (s.c.) injection of 100 µl of complete Freund's adjuvant (CFA, killed mycobacterium butyricum suspended in mineral oil, solution at 10

mg/ml, Sigma, St. Louis, MO) in the left hindpaw. Rats were stimulated two days after the inflammation was induced.

Injections

The NK-1 receptor antagonist GR 205171 (kindly provided by Glaxo-Wellcome) in 1.0 ml of saline was injected subcutaneously (s.c.) in the back of the neck 20 minutes prior to treatment.

Intrathecal injections were made directly between the S1-S2 vertebrae with a 27 1/2-gauge needle in a 20 µl saline. Control animals received an equal volume of saline. Placement of the needle in the intrathecal space was verified by a lateral flick of the tail.

Hindpaw stimulation

Noxious mechanical stimulation (pinch) was applied to the distal part of one hindpaw with a hemostat for 15 sec. For thermal stimulation, the rat's hindpaw (to just below the ankle) was dipped for 2 min into a water bath heated to 51°C. The rats were perfused 5 min after the stimulation ended.

Tissue preparation and immunofluorescent labeling

At the appropriate time, the animals received an additional injection of sodium pentobarbital (100 mg/kg, i.p.) and were perfused intracardially with 50 ml of phosphate-buffered saline 0.1 M (PBS) followed by 500 ml of 10% formalin in 0.1 M phosphate buffer (PB). After the perfusion, the lumbar spinal cord was removed, postfixed for four hours in the same fixative and then cryoprotected overnight in 30% sucrose in 0.1 M PB. Immunostaining was performed on 30 µm

lumbar spinal cord sections (from L2 to L6 segments) cut in the sagittal plane on a freezing microtome. The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST).

For immunofluorescent staining of the NK-1 receptor, the sections were incubated overnight in the primary antiserum, diluted to 1:5,000. The characteristics of the antiserum, directed against the C terminal tail of the NK-1 receptor, have been described previously (Vigna et al, 1994). After the primary antiserum, the sections were washed 3 times in 1% NGST and then incubated in indocarbocyanine Cy-3™ conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1: 600) for 2 hours at room temperature.

Quantification of internalization

Quantification of NK-1 receptor internalization was performed as previously described (Abbadie et al, 1997). Briefly, to analyze internalization in cell bodies we used a 20X objective on a Nikon FXA microscope equipped for fluorescence. We counted NK-1 receptor-like immunoreactive cell bodies in laminae I, III-IV and V-VI of the dorsal horn, ipsilateral to the side of stimulation, from segments L2 to L6. NK-1 receptor-like immunoreactivity is uniformly distributed on the surface of cell bodies that do not contain internalized receptors, but in the neurons that have internalized NK-1 receptors, the cytoplasm contains bright, immunofluorescent endosomes (Fig. 1). Neurons were considered internalized if they contained more than 20 endosomes in the cell

body. All counts are expressed as the percentage of NK-1 receptor immunoreactive neurons that contain internalized receptor.

Because we found no difference in the magnitude of internalization along the mediolateral extent of the superficial dorsal horn, we counted all the neurons within a section, without taking into account the mediolateral position of the cells. Neurons from five sagittal sections were counted from each rat.

Hot plate

To determine if the NKA-mediated activation of NK-1 receptors contributed to endogenous tachykinin-mediated behaviors, we tested rats treated with GR 205171 in a behavioral paradigm in which mice lacking the PPT-A gene, i.e. lacking SP and NKA have been shown to respond differently. These tachykinin knock out mice respond normally on a 52.5 or 58.5°C degree hot plate test, but show significantly longer response latencies at 55.5°C. The hot plate test consists of a plexiglass container with a temperature-controlled floor. The animals are placed on the heated floor and the latency to hindpaw licking or jumping is recorded, at which point the animals are immediately removed. Rats were tested on the hot plate, injected with GR 205171 and then retested on a 52.5, 55.5 or 58.5 degree hot plate.

RESULTS

SP and NKA activate the NK-1 receptor at similar concentrations in the spinal cord

To determine if NKA could affect the NK-1 receptor *in vivo*, we first compared the abilities of exogenously applied NKA and SP to produce NK-1 receptor internalization in the lumbar spinal cord. Increasing doses of NKA and SP were injected intrathecally, and the extent of the resulting NK-1 receptor internalization was quantified over the lumbar cord. Both NKA and SP induced NK-1 receptor internalization in the cell bodies and dendrites of lamina I neurons in a dose-dependent fashion (Figure 1). Intrathecal injection of neither SP nor NKA resulted in internalization of NK-1 receptors in lamina III-VI, even at the highest doses tested. We found that NKA and SP were equally effective at inducing NK-1 receptor internalization in the spinal cord, there being no difference between the dose response curves (two-way ANOVA, P=0.9538). The EC50's for NK-1 receptor internalization were 171μM (95% C.I.: 66.2 - 441 μM) (3.42 nmol : 1.32 - 8.82 nmol) and 210 μM (95% C.I.: 87.8 - 518 μM) (4.2 nmol: 1.75- 10.4 nmol) for SP and NKA, respectively.

The response of a receptor to an exogenously applied peptide *in vivo* depends not only on the receptor's response to the peptide but also on the peptide's access to the receptor. To be sure that the similar responses seen with NKA and SP *in vivo* were due to similar activity at the NK-1 receptor and not the result of differences in peptide diffusion or degradation, we next compared the abilities of NKA and SP to induce NK-1 receptor internalization in primary cultures of spinal cord neurons. As in the *in vivo* experiment, both NKA and SP induced NK-1 receptor internalization with an identical dose-dependence. There was no difference between the number of NK-1 receptor positive endosomes observed in neurons following incubation with NKA or SP (two-way

ANOVA: P = 0.6292). The EC50's were also identical (SP: 14.28 nM (95% C.I.: 2.35 - 87.79 nM); NKA: 26.7 nM (95% C.I.: 1.39 - 515.00 nM). Additionally, there was no difference in the percentage of neurons that internalized greater than 10 endosomes following incubation with NKA or SP (two way ANOVA, P= 0.9773 (treatment alone P=0.6191); SP EC50: 15.7 nM (95% C.I.: 13.60 -18.17 nM), NKA EC50: 21.5 nM (95% C.I.: 14.8 - 31.2 nM).

Although NK-1 receptor internalization has been shown to correlate consistently with other indicators of NK-1 receptor activity, receptor internalization is not necessarily a direct marker of receptor-mediated cell signaling. To ensure that the results obtained using NK-1 receptor internalization indeed reflect neurokinin activation and signaling via the NK-1 receptor, we assessed the effects of NKA and SP on intracellular calcium levels in primary spinal cord cultures. The NK-1 receptor is known to signal through Gq/11, activating phospholipase C and thus inducing an IP3 mediated release of intracellular calcium stores (Roush & Kwatra, 1998). As we have previously reported for SP, application of NKA to primary spinal cord cultures induced a dose-dependent increase in intracellular calcium concentration in a subpopulation of neurons and glia.

As with NK-1 receptor internalization, we found that the dose response curves for NKA and SP-induced calcium increases did not differ. We found no difference in the percentage of neurons responding with an increase in intracellular calcium (two way ANOVA, P= 0.8994; SP EC50: 16.1 nM (95% C.I.: 4.27 - 60.5 nM) NKA EC50: 20.53 nM (95% C.I.: 10.9 - 37.0 nM)), demonstrating that the two peptides not only induce NK-1 receptor internalization at equivalent concentrations, but also induce equivalent signaling.

Finally, as we previously reported for SP (Trafton et al, 1999), the dose response relationships for NKA-induced NK-1 receptor internalization and intracellular calcium increases were identical (data not shown). Thus, NKA-induced signaling occurs at the same doses as receptor internalization, further supporting the use of NK-1 receptor internalization as a marker of tachykinin-mediated activity.

GR 20571 antagonizes NKA but not SP induced NK-1 receptor internalization

We next tested the ability of an NK-1 receptor antagonist, the compound GR 205171, to prevent the NK-1 receptor internalization produced by an intrathecal injection of SP or NKA. We injected rats with 10 mg/kg GR 205171 s.c., 20 minutes prior to intrathecal injection of 100 μg of either SP or NKA and then quantified NK-1 receptor internalization over lamina I of the lumbar spinal cord. To our surprise, GR 205171 had no effect on NK-1 receptor internalization induced by intrathecal injection of SP, but it completely prevented NKA-induced NK-1 receptor internalization (SP: P=0.4968, n= 5-6; NKA: P=0.0001, n=4-6) (Figure 3). We repeated the experiment using 1.0 μg of SP, the lowest dose that we have found to reliably produce observable NK-1 receptor internalization. Even at this low dose of SP, 10 mg/kg GR 205171 s.c. could not reduce i.t. SP induced NK-1 receptor internalization (P=.6394, n=3-5). Recognizing the possibility that the 1.0 μg dose was too low to observe a reduction in NK-1 receptor internalization, we again repeated the experiment, this time using 10 μg of SP against a 10 mg/kg dose of GR 205171. Still, we found no decrease in NK-1

receptor internalization in lamina I of the lumbar spinal cord (P=0.8681, n=5).

To examine this apparent difference in the ability of GR 205171 to block activation of the NK-1 receptor by NKA versus SP, we next looked at the ability of GR 205171 to inhibit NK-1 receptor internalization in primary spinal cord cultures. In this system, the doses of SP, NKA and the antagonist could be better controlled. Spinal cord cultures were incubated in a mixture of 100 nM SP or NKA with varying doses of GR 205171 and then NK-1 receptor internalization was quantified. As described above, we found that 100 nM of either SP or NKA produced maximal NK-1 receptor internalization. GR 205171 was effective in reducing NKA-induced internalization at significantly lower concentrations than were required to inhibit SP-induced NK-1 receptor internalization. Thus although 10 - 100 nM GR 205171 effectively inhibited NKA induced NK-1 receptor internalization, no inhibition of SP induced NK-1 receptor internalization was observed at these concentrations. At higher concentrations, however, GR 205171 did prevent SP induced NK-1 receptor internalization, demonstrating that, in sufficient excess, the compound is capable of antagonizing SP-mediated NK-1 receptor activation.

GR 205171 reduces noxious stimulus-evoked NK-1 receptor internalization

As we found that 10 mg/kg GR 205171 selectively inhibits NKA induced NK-1 receptor internalization *in vivo*, we used this dose to determine the relative contribution of NKA and SP to noxious stimulus-induced NK-1 receptor activation. We have previously shown that noxious thermal, mechanical or chemical stimulation of the hindpaw induces NK-1 receptor internalization in

lamina I neurons of the ipsilateral spinal cord (Abbadie et al, 1997). In the setting of inflammation of the hindpaw, noxious mechanical stimulation produces an even greater magnitude of NK-1 receptor internalization in lamina I neurons of the spinal cord, and neurons in lamina III-VI also contain internalized receptor. We have previously reported that 10 mg/kg GR 205171 reduces NK-1 receptor internalization induced by noxious mechanical stimulation in both normal animals and in animals with an inflammatory injury (Trafton et al, 1999). Here, we reexamined these data in the context of the NKA selectivity of GR 205171, and additionally assessed the ability of preadministration of 10 mg/kg GR 205171 s.c. to prevent NK-1 receptor internalization evoked by a noxious thermal stimulus, namely immersion of the hindpaw in 51° C water for 2 minutes.

With all stimuli GR 205171 significantly reduced NK-1 receptor internalization (Thermal, $P<0.0001$: 45 % fewer neurons showing receptor internalization (55% decrease from saline) mechanical, $P<0.0001$: 55% fewer (80% decrease); mechanical with inflammation, $P<0.0001$: lamina I 45% fewer (55% decrease), lamina III 24% fewer (94% decrease), lamina V 8% fewer (100% decrease)). GR 205171 was less effective in preventing NK-1 receptor internalization in lamina I following noxious mechanical stimulation in the CFA-treated animals (compare SP components in normal rats versus rats with inflammation). However, GR 205171 almost completely prevented NK-1 receptor internalization in lamina III-VI neurons in these same rats.

When we replotted the data to show the relative contributions of NKA (the difference in internalization between saline and GR 205171 groups) and SP

(the remaining internalization in the GR 205171 group) we recognized several trends. In lamina I, we estimate that the largest SP contribution is seen around L4-L5, the lumbar segments most densely innervated by primary afferents neurons projecting to the hindlimb. In lumbar segments L2, L3 and L6, which receive a much weaker direct primary afferent input from the site of stimulation, we estimate that NKA was responsible for a much larger proportion of the NK-1 receptor internalization. Additionally, we estimate that NKA was nearly entirely responsible for the NK-1 receptor internalization in deeper lamina of the spinal cord (i.e. when the stimulus was applied to the inflamed hindpaw). Of note, however, is the observation that although NK-1 receptor internalization in the cell bodies of lamina III-VI neurons was completely prevented by GR 205171, occasionally we observedNK-1 receptor internalization in the dendrites of these neurons that extend dorsally into lamina *I.*

Effect of GR 205171 on nociceptive behaviors

Mice lacking the gene for preprotachykinin-A, the precursor protein from which both NKA and SP are derived, show very specific deficits in nociceptive behaviors (Cao et al, 1998). As these mice lack both SP and NKA, a behavioral analysis in these mice does not provide information on the relative contribution of SP and NKA or different neurokinin receptors. Because, GR 205171 blocks only NKA mediated NK-1 receptor activation, we, therefore, asked whether administration of this compound could mimic the behavioral phenotypes seen in the PPT-A mutant mice, thereby implicating NKA and the NK-1 receptor in these effects.

PPT-A mutant mice and their wild type controls respond with identical latencies on both a 52.5 and 58.5°C degree hot plate. On a 55. 5-degree hot plate, however, there is a striking difference between the mice. Normal mice respond as rapidly as they do on a 58.5 degree hot plate, but the PPT-A mutant mice respond at a longer latency, comparable to seen on the 52.5 degree hot plate. We tested rats given 10 mg/kg GR 205171 s.c and their saline controls on a 52.5, 55.5 and 58.5 degree hot plate. Saline-treated rats behaved similarly to the wild type mice with a significant decrease in latency to hindpaw licking at the 55.5 degree temperature, which was maintained at 58.5 degrees. Rats treated with GR 205171 showed an intermediate latency on the 55.5 degree hot plate, a behavior mid-way between that seen in the normal animals and that seen in the animals that lacked PPT-A gene products.

DISCUSSION

Identical effects of SP and NKA

These results confirm previous suggestions that SP and NKA have NK-1 receptor mediated effects at similar concentrations. Both NK-1 receptor internalization and calcium mobilization occur in vivo and in vitro at the same doses, demonstrating that both peptides are apt to work at NK-1 receptors in vivo.

It is clear, however, that despite the similar effects seen with the two peptides, the two tachykinins do not bind the NK-1 receptor identically. Homologous binding studies (where only SP or only NKA was used) found

similar binding affinities for SP and NKA while heterologous binding studies show a much higher affinity for SP. (Hastrup and Schwartz, 1996) In fact, numerous studies have demonstrated a higher affinity binding site for SP, that has never been observed for NKA (see Maggi, 1995 for review). Recently, a domain near the end of the second extracellular loop of the NK-1 receptor was identified that is necessary for the binding and biological activity of NKA but not SP through this receptor. (Wijkhuisen et al, 1999) This result suggests that the two neurokinins bind distinct sites on the NK-1 receptor, and thus SP and NKA may act on the NK-1 receptor in unique manners. Regardless of their differences in binding characteristics, SP and NKA have been shown to activate aspects of NK-1 receptor signaling at the same doses, demonstrating that they share some functional characteristics. (Akasu et al, 1996; Sagan et al, 1996)

Difference in GR 205171 effects on SP vs. NKA induced activation

If a receptor has two distinct binding domains, it is theoretically possible to devise compounds that selectively block one site versus another. We believe that this accounts for the ability of GR 205171 to selectively decrease NKA mediated NK-1 receptor trafficking. Most likely, GR 205171 more directly inhibits the NKA binding portion of the NK-1 receptor. In support of this idea, many other NK-1 receptor antagonists have been shown to have similar properties. For example, CP 96345, FK 888, GR 82, 334, RP 67, 580, CP-99994 and MEN-10581 have been shown to better inhibit NKA or septide activation than SP activation (Maggi, 1995; Jenkinson et al, 1999). As such, we believe that the selective inhibition of NKA-mediated activity by GR 205171 seen in this study is readily explainable and real. The selectivity of NK-1 receptor antagonists for

NKA versus SP at this single receptor brings into question the purported selectivity of some of the many NK-1 and NK-2 receptor antagonists. A number of these compounds have been characterized based on their ability to inhibit the actions of SP-like versus NKA-like tachykinins on the assumption that NKA effects were NK-2 receptor mediated and SP effects were NK-1 receptor mediated. By this criterion, compounds like GR 205171, which relatively selectively inhibit NKA mediated effects *through the NK-1 receptor,* would be mistakenly characterized as NK-2 receptor selective. Evidence that such mischaracterization have occurred exists. For example, Sagan et al (1996) demonstrate that the "NK-2 receptor antagonist" GR 94800 selectively blocks NKA induced activity in NK-1 receptor transfected CHO cells. As CHO cells do not express neurokinin receptors basally, this antagonist clearly acts at the NK-1 receptor. This same antagonist had been used previously to argue for the presence of NK-2 receptors in the spinal cord (Lepre et al, 1994). Additionally, SR 48968, the NK-2 receptor antagonist with which the most functional evidence for spinal NK-2 receptors has been obtained, has been shown to be selective for the NK-2 receptor over the NK-1 receptor by several orders of magnitude. However, the doses chosen for spinal cord studies were characterized as NK-2 receptor selective solely on the basis of their selectivity for inhibiting NKA versus SP effects (Yashpal et al, 1996; Neugebauer et al, 1996; Liu and Sandkuhler, 1997). If this antagonist shows similar selectivity for antagonism of NKA over SP effects at the NK-1 receptor as GR 205171, one would expect that a dose could be found that would give "NK-2 receptor selective" effects by the criteria used via action at the NK-1 receptor. Thus, care should be taken in

interpretation of the pharmacological literature on NK-1 versus NK-2 receptors in light of new understanding of NK-1 receptor properties.

While we clearly demonstrate a difference in GR 205171's antagonism of NKA versus SP effects, our in vivo experiments rely on the assumption that the dose of GR 205171 that we use only inhibits NKA mediated NK-1 receptor activation. For the following reasons we believe that this is indeed the case. Ten mg/kg GR 205171 did not reduce NK-1 receptor internalization produced by a dose of SP that induces less than 25% of maximal internalization. In other words, our results cannot be explained by arguing that the SP concentration was supramaximal and therefore not blockable. Moreover, diffusion of SP from an intrathecal injection at the cauda aquina should result in a gradient of SP concentrations over the lumbar cord. As only 25% of neurons in the lumbar cord internalize the NK-1 receptor at the 1μg dose, it must be the case that the substance P concentration at the lumbar cord drops below the range in which SP can activate the receptor. If GR 205171 could inhibit SP induced NK-1 receptor internalization at low SP concentrations, we should see it at this dose. Thus, it seems that this dose of GR 205171 cannot prevent SP-mediated NK-1 receptor internalization produced by even minimal doses of SP at the threshold for causing NK-1 receptor internalization. Given our intrathecal SP results, we do not believe that the difference in GR 205171's ability to prevent NK-1 receptor internalization in L2, L3 and L6 versus in L4/L5 is due to antagonism of SP-induced NK-1 receptor internalization in regions containing lower concentrations of SP. Additionally, we have previously shown that 1 mg/kg GR 205171 does not reduce noxious stimulus-induced NK-1 receptor internalization and 10 mg/kg produces a partial reduction. (Trafton et al, 1999) Thus, the dose

of antagonist that we used in these studies is no more than 10 times greater than an ineffective dose and therefore clearly in a range in which strictly NKA selective effects would be expected, based on our *in vitro* data.

Extent of NKA induced NK-1 receptor activity

We demonstrate that GR 205171, at doses that do not block the action of SP, can prevent the majority of noxious stimulus-induced NK-1 receptor internalization. This suggests that under normal, physiological conditions, neurokinin A mediate a large proportion of NK-1 receptor activation. Despite the evidence that SP and NKA can activate the NK-1 receptor at similar doses, given the well documented ability of SP to outcompete NKA for binding of the receptor in heterologous binding studies, this result is surprising. (Hastrup and Schwartz, 1996) As the peptides are coreleased in vivo, one might expect that SP would be the predominant NK-1 receptor agonist, simply due to its higher affinity for the NK-1 receptor. There are numerous possible explanations for the greater effect of NKA on dorsal horn NK-1 receptors. Several observations have been made that might lend an answer. Bakhle and Bell (1995) observed that SP and NKA were not evenly distributed throughout sensory neurons, with there being a greater proportion of SP expressed in the peripheral terminals and dorsal root ganglion and a greater proportion of NKA in the spinal cord terminals. Thus, it is possible that primary afferent fibers release more NKA than SP into the dorsal horn during noxious stimulation. Additionally, it has been shown that NKA is less susceptible to degradation by endogenous endopeptidases. (Nyberg et al, 1984; Theodorsson-Norheim et al, 1987) Thus, SP may be degraded too

rapidly to have full effects, leaving ample receptor upon which the less degradable NKA could act.

This difference in peptide degradation may also contribute to the preferential activation of NK-1 receptors by NKA in deeper lamina of the spinal cord during inflammatory injury. We show that nearly all of the NK-1 receptor internalization seen in lamina III-VI is blocked by GR 205171 and thus induced by NKA, suggesting that SP does not act below lamina II. This is supported by results of Hope et al. (1990) who demonstrated that NKA can be detected more ventrally in the spinal cord gray matter than SP following joint stimulation during a peripheral inflammation.

Functional relevance

Differences in signal transduction via the NK-1 receptor: SP vs. NKA

Although SP and NKA appear to have similar effects on most aspects of NK-1 receptor signaling (electrophysiological, calcium/IP3, internalization) qualitative differences in SP and NKA signaling through the NK-1 receptor have been reported. Of note, SP but not NKA has been shown to increase cAMP levels in CHO cells expressing the NK-1 receptor (Sagan, 1996). This suggests that SP and NKA may exert different effects on target neurons *through the same receptor.*

As the two peptides are differentially degraded, this could allow for different signaling through the NK-1 receptor in different target regions. Microprobe studies have shown that following noxious stimulation NKA is found more evenly throughout the dorsal horn than SP (Duggan et al, 1990) Our data support this result, as the proportion of NK-1 receptor internalization

induced by NKA is greater over the rostral/caudal extent of the lumbar cord. Based on the residual effects in the presence of GR 205171. We reasoned that the SP contribution is focused at the site of stimulated primary afferent input, namely L4/L5. Based on the pattern of NK-1 receptor internalization induced by NKA versus SP and the observations on the diffusion of the two peptides, it follows that the neurons proximal to the site of release would be selecively activated by SP while those more distant from the release site would be activated by NKA. This might not only produce different signaling regionally in the spinal cord, but there may also be differences in NK-1 receptor signaling in different domains of individual neurons depending on their proximity to synaptic terminals that release the tachykinins. This is most easily demonstrated in the case of deeper dorsal horn neurons, as shown in figure 5, however it is likely that this also occurs in lamina I. This is difficult to assess, however, as the dendritic trees of lamina I neurons overlap extensively and are thus difficult to trace back to their cell bodies. Analysis of the responses of the NK-1 receptor neuron population (fig. 4) with GR 205171 also suggests that this may be occurring.

The functional relevance of differential activation of NK-1 receptors by SP versus NKA is not clear. However, given the differences in signal transduction pathways stimulated, one might expect the two peptides to have qualitatively different effects on signaling and gene transcription in the same neurons.

Behavior

Behaviorally, we found that rats given GR 205171 showed thermal nociceptive responsiveness midway between that seen in null mutant PPT mice and their wild type controls. This suggests that both SP and NKA contribute to

nociceptive behaviors at the NK-1 receptor but that SP cannot produce fully normal behavior in the absence of NKA. A more extensive study of the behavioral effects of GR 205171 are necessary to fully distinguish the contribution of SP and NKA at the NK-1 receptor (changes in inflammation, different modalities, spread/extent of hyperalgesia, receptive field changes). But these could potentially bring insight into the differences in the two peptides actions at the NK-1 receptor and help differentiate the effects of tachykinin actions in lamina I versus lamina III-VI during inflammation.

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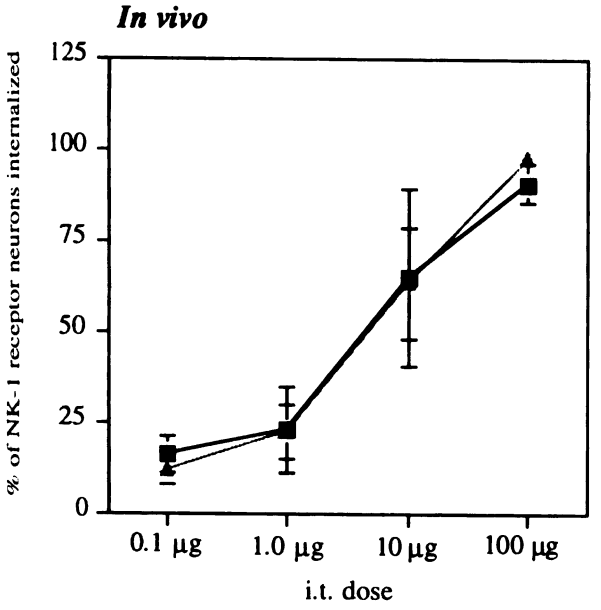
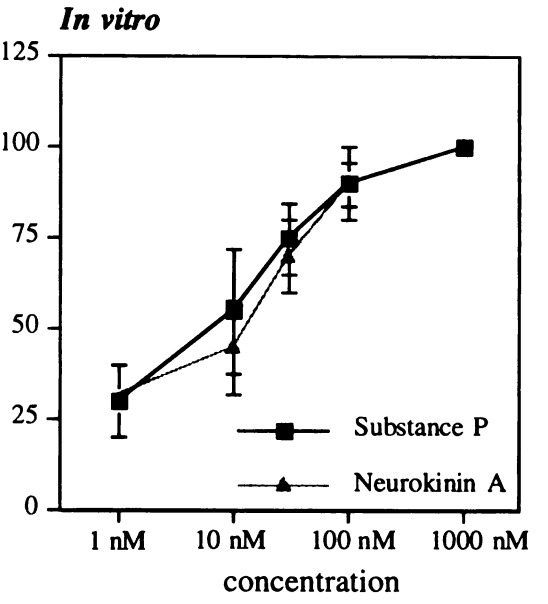
Figure 1: SP and NKA act at the NK-1 receptor at the same concentrations. A: Intrathecal injection of SP or NKA produces equivalent amounts of NK-1 receptor internalization in the lumbar spinal cord. Percentage of NK-1 receptor expressing neurons in lamina I of the lumbar spinal cord (L2-L6) showing internalized receptor after i.t. injection of various doses of SP or NKA. (n=4-5 rats) B: Incubation of primary spinal cord cultures with SP or NKA results in identical quantities of NK-1 receptor activation. Percentage of NK-1 receptor expressing neurons in primary spinal cord cultures showing internalization after incubation for 15 minutes in varying concentrations of SP or NKA. (n=4 coverslips) C. Administration of SP or NKA to primary spinal cord cultures produces indistinguishable increases in intracellular calcium. Percentage of the maximal percentage of neurons from primary spinal cord cultures showing an increase of greater than 2 times baseline values after application of varying concentrations of SP or NKA. (n=5-11 coverslips; maximal percent responders SP=50.66, NKA=50.38)


In vivo
% of NK-1 receptor neurons internalized
125
100
75
50
25
0
0.1 µg
1.0 µg
10 µg
100 µg
i.t. dose


In vitro
125
100
75
50
25
0
Substance P
Neurokinin A
1 nM
10 nM
100 nM
1000 nM
concentration

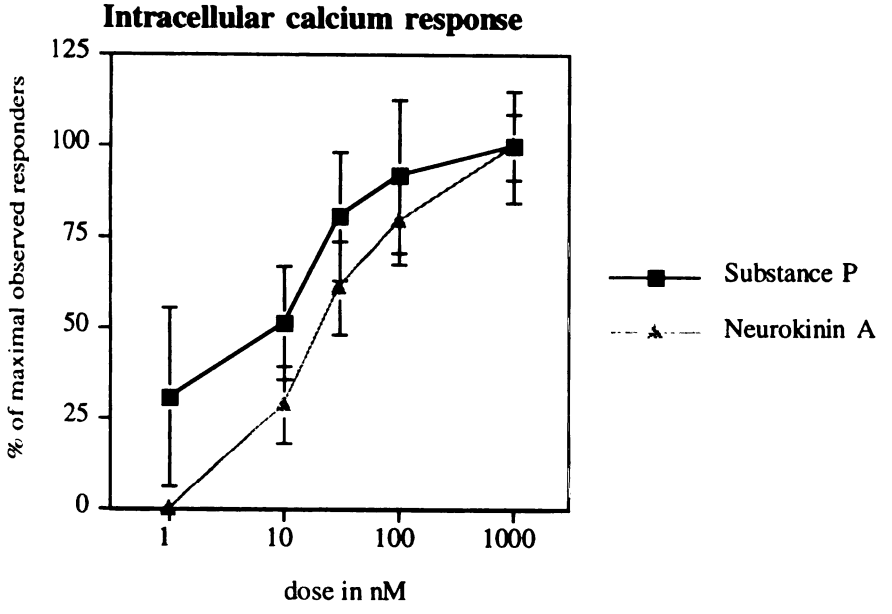
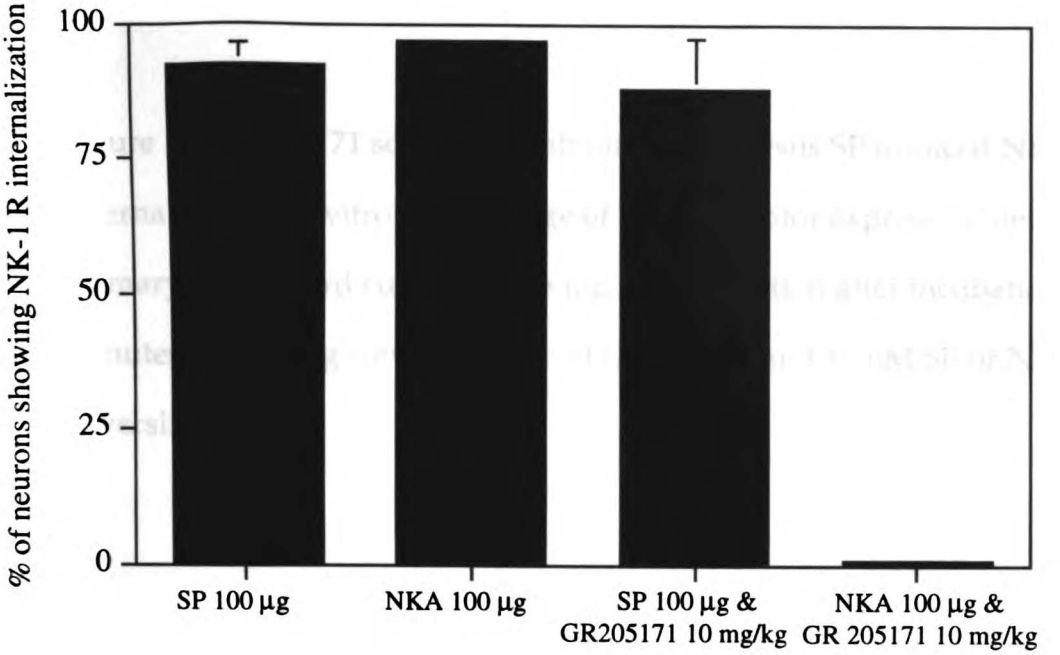

Intracellular calcium response
% of maximal observed responders
125
100
75
50
25
0
1
10
100
1000
dose in nM
Substance P
Neurokinin A

Figure 2: GR205171 prevents NKA but not SP induced NK-1 receptor internalization in vivo. Percentage of neurons in lamina I of the lumbar spinal cord (L2-L6) showing internalized NK-1 receptor following intrathecal injection of various doses of SP or NKA in the presence and absence of the NK-1 receptor antagonist. (n=3-6 rats)


% of neurons showing NK-1 R internalization
100
75
50
25
0
SP 100 µg
NKA 100 µg
SP 100 µg & GR205171 10 mg/kg
NKA 100 µg & GR 205171 10 mg/kg

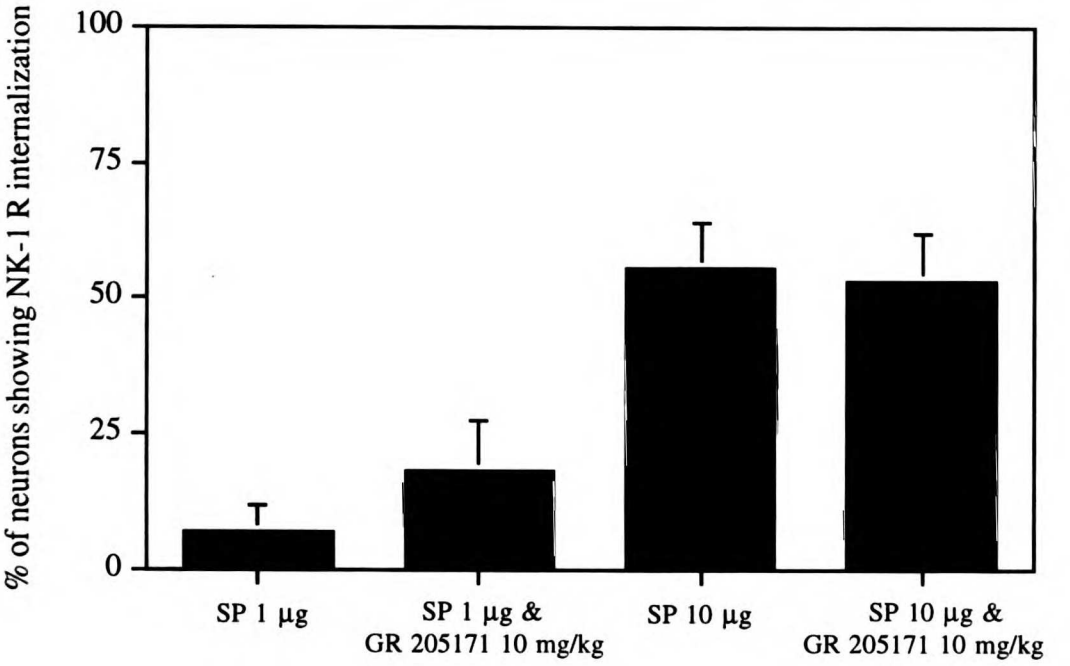

% of neurons showing NK-1 R internalization
100
75
50
25
0
SP 1 µg
SP 1 µg & GR 205171 10 mg/kg
SP 10 µg
SP 10 µg & GR 205171 10 mg/kg

Figure 3: GR 205171 selectively inhibits NKA versus SP induced NK-1 receptor internalization in vitro. Percentage of NK-1 receptor expressing neurons in primary spinal cord cultures showing internalization after incubation for 15 minutes in varying concentrations of GR 205171 and 10 nM SP or NKA (n= 4-5 coverslips).

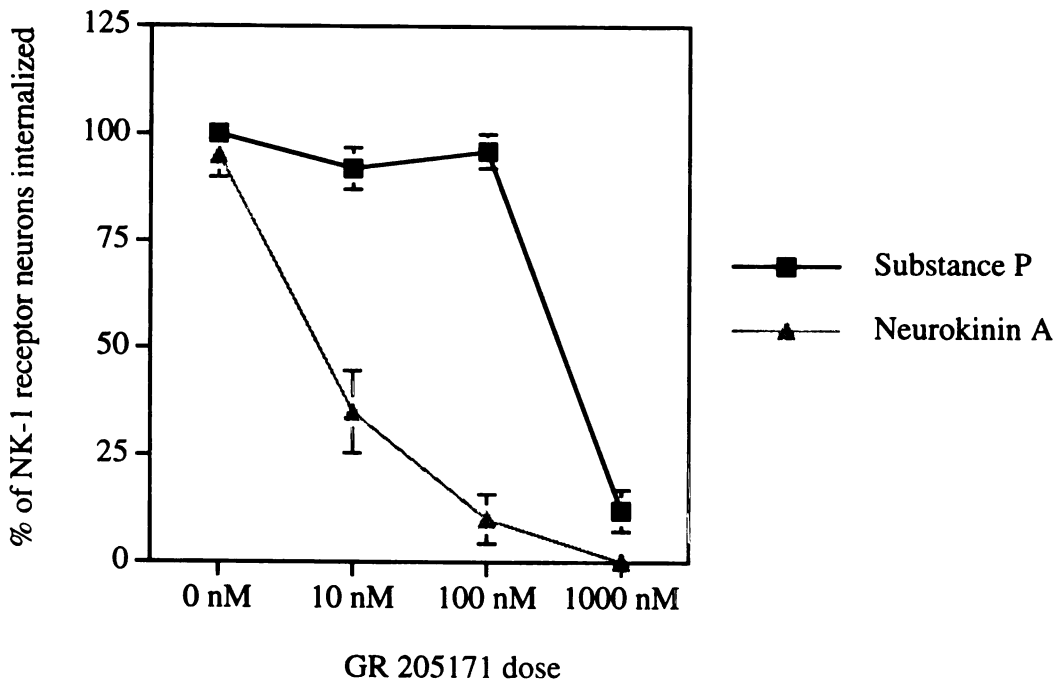
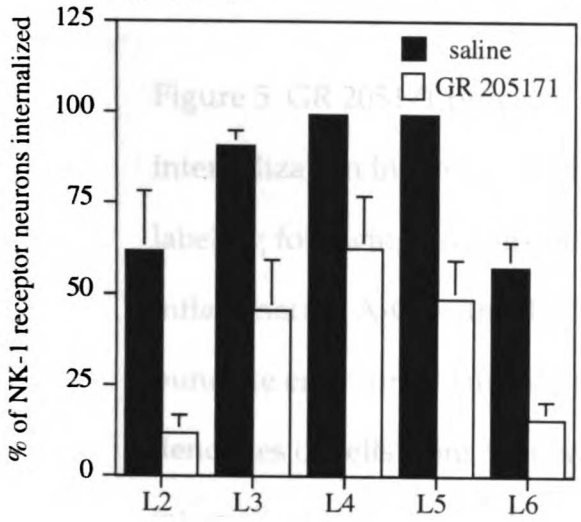

% of NK-1 receptor neurons internalized
125
100
75
50
25
0
0 nM
10 nM
100 nM
1000 nM
GR 205171 dose
Substance P
Neurokinin A

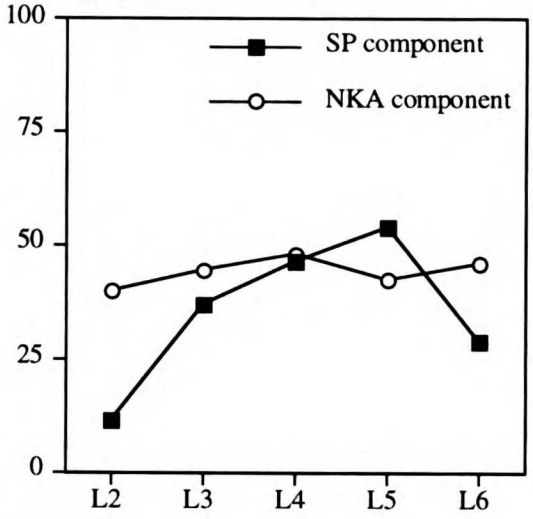
Figure 4: GR 205171 decreases noxious stimulus induced internalization. A: Percentage of NK-1 receptor expressing neurons showing receptor internalization across the lumbar spinal cord (L2-L6) after a two minute immersion of the hindpaw in a 50 degree celcius water bath in the presence and absence of GR 205171 (10 mg/kg s.c.) (n= rats) B-F: Percentage of NK-1 receptor internalization attributable to SP (values in the presence of 10 mg/kg GR 205171) or NKA (saline values - values with 10 mg/kg GR 205171) across the lumbar spinal cord (L2-L6) following various noxious stimuli. B: Lamina I neurons following noxious thermal stimulation. C: Lamina I neurons following noxious mechanical stimulation D: Lamina I neurons following noxious mechanical stimulation in the presence of inflammation. E: Lamina III-IV neurons following noxious mechanical stimulation in the presence of inflammation F: Lamina V-VI neurons following noxious mechanical stimulation in the presence of inflammation.

Thermal



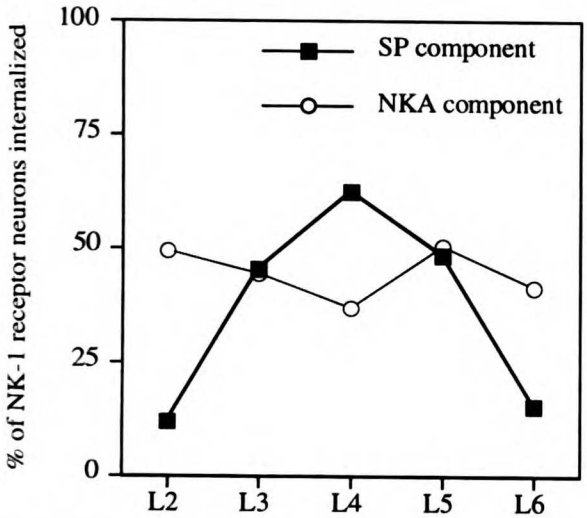
Mechanical with Inflammation

Lamina I



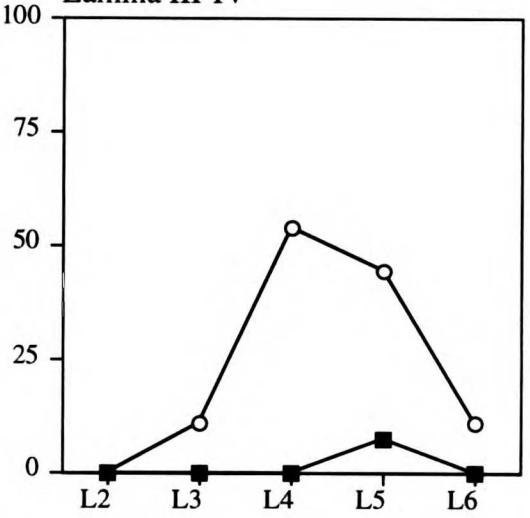
% NKA: 78 54 51 44 61

Thermal



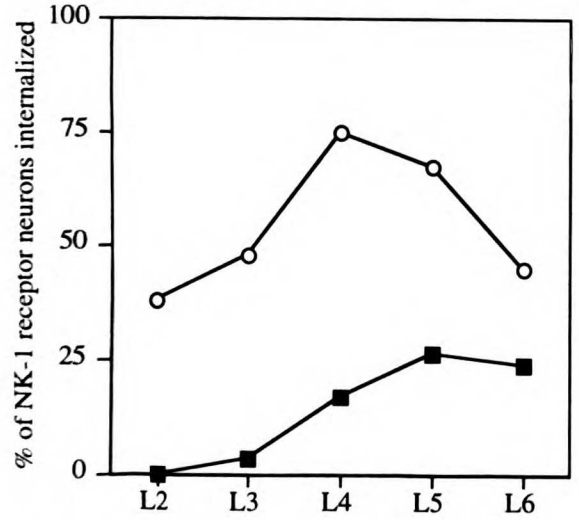
% NKA: 80 49 37 51 72

Lamina III-IV



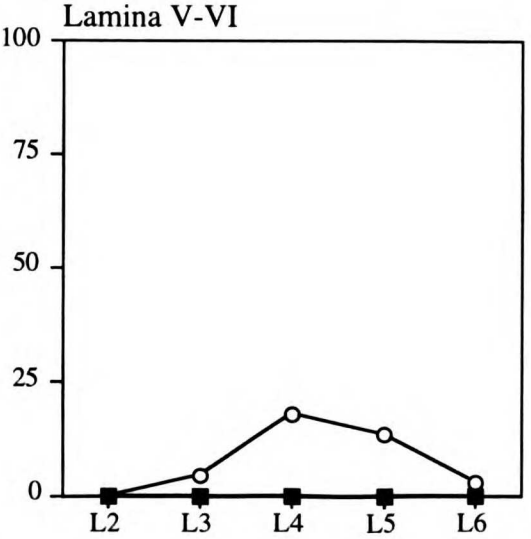
% NKA: N/A 100 100 85 100

Mechanical



% NKA: 100 92 81 68 65

Lamina V-VI



% NKA: N/A 100 100 100 100

Figure 5: GR 205171 prevents noxious stimulus evoked NK-1 receptor internalization in laminae below lamina I during inflammation. NK-1 receptor labeling following noxious mechanical stimulation in the presence of inflammation A-C: with GR 205171 (10 mg/kg s.c.) D-F: with saline. Note that punctate endosomal labeling ends abruptly at the lamina I-II border. Only the dendrites of cells from deeper lamina that project to lamina I show internalization and receptor internalization does not spread down dendrites.

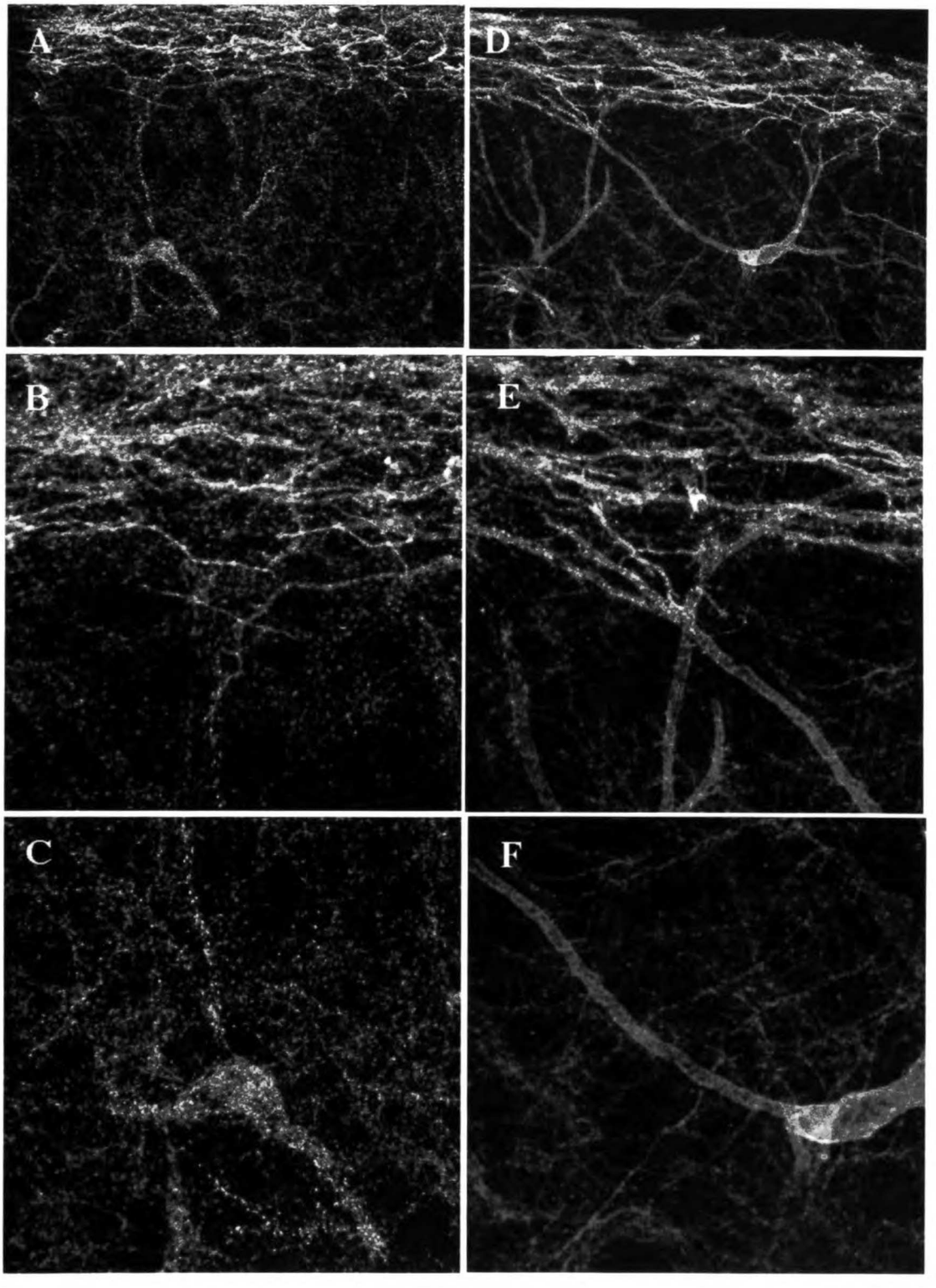
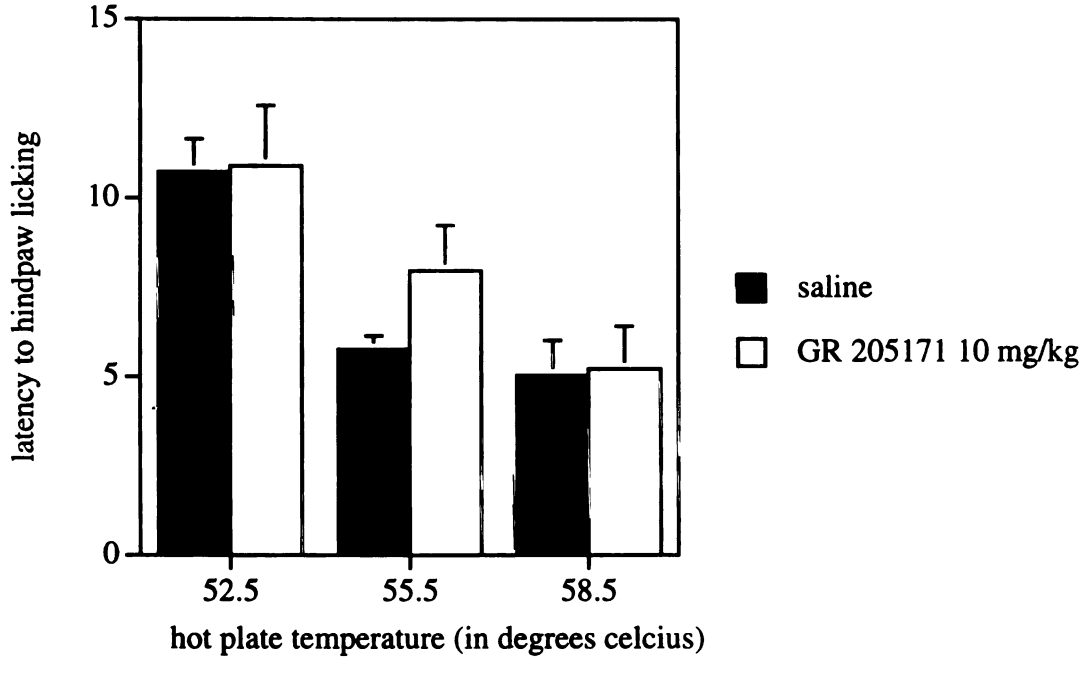

A
D
B
E
C
F

Figure 6: GR 205171 only effects thermal latencies at 55.5 degrees, the same stimulus to which PPTA KO mice show increased latencies. Latencies at 55.5 degrees celcius are equivalent to those at 58.5 degrees in saline treated animals, but intermediate to those at 52.5 and 58.5 degrees in animals treated with GR 205171.


15
10
5
0
latency to hindpaw licking
52.5
55.5
58.5
hot plate temperature (in degrees celcius)
saline
GR 205171 10 mg/kg

Do SP and NKA have similar effects in the dorsal horn of the spinal cord? : Studies of immediate early gene induction.

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San Francisco, CA 94143

ABSTRACT

While SP and NKA are coreleased into the spinal cord dorsal horn and may act on similar receptors, the extent to which they have similar effects is unknown. Here we attempt to address this question by examining the expression of the immediate early gene protein Fos in response to the two tachykinin ligands. We find that while SP and NKA induce similar Fos expression in NK-1 receptor expressing lamina I neurons, intrathecal injection of NKA results in expression of Fos protein in nearly twice as many lamina I non-NK-1 receptor expressing neurons as does SP. Neither the NK-1 receptor antagonist GR 205171 nor the NK-2 receptor antagonist MEN 11420 reduced the Fos expression produced by intrathecal NKA, at doses of the antagonists that blocked the redness and salivation or defection responses seen following i.t. injection of the tachykinin.

These results suggest that the two tachykinins do not have identical actions in the spinal cord. In addition to actions at NK-1 and NK-2 receptors, NKA appears to have excitatory actions at other sites in the superficial dorsal horn.

INTRODUCTION

The tachykinins SP and NKA are highly expressed in primary afferent nociceptors and released into the spinal cord with noxious stimulation. Numerous functions for and responses to these peptides in the dorsal horn of the spinal cord have been described or hypothesized, ranging from presumably nociceptive biting and scratching behaviors (Gamse and Saria, 1986; Seybold et al, 1982), and sensitization of nociceptive behaviors and reflexes (Moochhala & Sawynok, 1984; Cridland and Henry, 1986) to potentiation of NMDA receptor responses (Rusin et al, 1993), and even opioid receptor mediated antinociceptive responses (Krumins et al, 1989; Goettl & Larson, 1994).

Most of these effects have been assumed to be mediated through one of the cloned neurokinin receptors, the NK-1, NK-2 or NK-3 receptors. NK-1 and NK-3 receptors are both highly expressed in the dorsal horn, NK-1 receptor in neurons in lamina I, III-VI and X (Brown et al, 1995); and NK-3 receptor in neurons in lamina II (Zerari et al, 1997; Shughrue et al, 1996). Additionally, there is some evidence that both NK-1 and NK-2 receptors may be expressed on primary afferent neurons, although this may be limited to special cases during development and injury. (Weinreich et al, 1997; Li and Zhao, 1998) However, it is less clear which tachykinin actions are mediated by which receptors and cell populations.

Additionally, however, other non-NK receptor mediated effects of tachykinins have been described. Several breakdown products of substance P have been demonstrated to have effects in the spinal cord, presumably independent of the neurokinin receptors due to the sensitivity of the effects to

opioid antagonists and the observed binding of substance P fragments to opioid receptors (Krumins et al, 1989; Skilling et al, 1990; Larson & Sun, 1993; Goettl & Larson, 1994). Recently, however, Michael-Titus et al (1999) have demonstrated that these same breakdown products, namely SP(1-7) or SP(5-11) can induce NK-1 receptor internalization, suggesting that they too might act through NK-1 receptor sites.

SP and NKA have been shown to have somewhat different affinities and actions on the various NK receptors and the two are differentially degraded. Thus, it is unclear how similar the actions of these two tachykinins are in the spinal cord. We attempt to probe this question, by examining the effects of intrathecal SP and NKA on autonomic function and behaviors and Fos expression.

METHODS

Injections

Intrathecal injections, performed under halothane anesthesia, were made directly between the S1-S2 vertebrae with a 30 gauge needle in a 20 ml volume of saline. Control animals received an equal volume of saline.

GR 205171A dihydrochloride salt, an NK-1 receptor antagonist (kindly provided by Glaxo-Wellcome) was given s.c. (10 mg/kg) at the base of the neck, 20 minutes prior to intrathecal injections. This dose completely blocks NK-1 receptor internalization in response to intrathecal injection of 100 µg NKA. MEN 11420 (kindly provided by Carlos Maggi at Menarini Research), an NK-2

receptor antagonist, was given intrathecally immediately before injection of tachykinins.

Tissue Preparation and Immunocytochemistry

Ninety minutes following the intrathecal injections, the rats received an additional injection of sodium pentobarbital (100 mg/kg, i.p.) and were perfused intracardially with 50 ml of 0.1 M phosphate-buffered saline (PBS) followed by 500 ml of 10% formalin in 0.1 M phosphate buffer (PB). After the perfusion, the lumbar spinal cord was removed, postfixed for four hours in the same fixative and then cryoprotected overnight in 30% sucrose in 0.1 M PB. Immunostaining was performed on 30 µm lumbar spinal cord sections (from L2 to L6 segments) cut in the sagittal plane, or 40 µm lumbar and sacral spinal cord sections cut in the transverse plane, on a freezing microtome. The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST).

Sections were incubated in the Fos antisera (1: 30,000; kindly given by Dr. Dennis Slamon, UCLA) overnight. Immunostaining was performed according to the avidin-biotin peroxidase method of Hsu et al. (1981) To localize the HRP immunoreaction product for Fos, we used a nickel-intensified diaminobenzidine protocol with glucose oxidase. For double labeling studies, sections were then incubated in NK-1 receptor antisera (Advanced Targeting Systems, Carlsbad, CA) (1: 20,000) and a diaminobenzidine protocol with glucose oxidase without nickel was used. Finally, the sections were washed 3 times in PB, mounted on

gelatin-coated slides, dried, and coverslipped with DPX (Electron Microscopy Science, Gibbstown, NJ).

Quantification of Immunoreactivity

In studies that analyzed only Fos expression, the number of Fos positive nuclei was counted for each of five 40 μm transverse L4/L5 sections of spinal cord. We counted the number of Fos positive nuclei labeled in each of the following regions: lamina I/II, lamina III/IV, lamina V/VI for each side of the spinal cord, as well as lamina X.

To analyze double labeling for Fos and the NK-1 receptor, we only counted neurons in lamina I in saggital sections of lumbar spinal cord (L2-L6). We recorded the number of neurons that were (1) only positive for Fos, (2) only positive for the NK-1 receptor and (3) double-labeled for both NK-1 receptor and Fos.

In all experiments, the investigators who quantified labeling were not aware of the treatment that the animal received. For statistical analysis, we used a two-way analysis of variance for treatment condition and for spinal segment (L2-L6 lumbar segments) or lamina (I/II, III/VI, V/VI and X). For multiple comparisons, we used Fisher's PSLD test; $p<0.05$ was considered statistically significant.

RESULTS

Responses produced by intrathecal injection of SP and NKA in the anesthetized rat

Intrathecal injection of either SP or NKA resulted in immediate profuse salivation, a deep reddening observed in the ears and paws, an increase in breathing rate and less reliably urination. These responses were similar between the two peptides, although the reddening produced by NKA was generally both deeper and of shorter duration that that observed after SP. Additionally, NKA but not SP triggered a prolonged period of increased defecation which lasted for over an hour. Feces were normal; only the rate and thus amount of defecation was increased.

Fos expression induced by intrathecal injection of SP and NKA

Intrathecal injection of either SP or NKA produced extensive Fos expression in the superficial dorsal horn. Both tachykinins induced Fos expression in lamina I neurons that did and did not express NK-1 receptor. While the extent of the Fos expression produced in NK-1 receptor expressing neurons was similar in response to equivalent doses of SP and NKA, there was a large difference in Fos expression produced in other lamina I neurons. Nearly twice as many Fos positive nuclei were found in lamina I non-NK-1 receptor expressing neurons in response to intrathecal NKA as compared to SP.

Effects of GR 205171 on responses and Fos expression following intrathecal NKA

To determine if the effects observed following intrathecal injection of NKA were mediated via the NK-1 receptor, we repeated the intrathecal injections in the presence of the NK-1 receptor antagonist GR 205171. This antagonist selectively inhibits NK-1 receptor internalization produced by NKA but not SP in vivo. We administered a dose that we have shown eliminates NK-1 receptor

internalization produced 10 minutes after intrathecal injection of 100 mg NKA but does not alter NK-1 receptor internalization produced by SP. Unexpectedly, GR 205171 had no effect on Fos expression evoked by intrathecal NKA, even in NK-1 receptor expressing neurons.

Despite its ineffectiveness preventing tachykinin induced Fos expression, GR 205171 did inhibit responses to injection of NKA. GR 205171 completely prevented the salivation and reduced the redness seen following injection of NKA. It also reduced the NKA induced increase in defecation rate, although not as effectively as MEN 11420.

Effects of MEN 11420 on responses and Fos expression following intrathecal NKA

We made use of the NK-2 receptor antagonist MEN 11420 to test the involvement of NK-2 receptors in intrathecal NKA evoked Fos expression and behavior. Intrathecal MEN 11420 also had no effect on NKA induced Fos expression in lamina I neurons. It did, however, completely prevent the increase in defecation rate seen with i.t. NKA.

DISCUSSION

Here we show that the endogenous tachykinins SP and NKA, despite having similar effects on NK-1 receptor internalization (see previous paper), induce different patterns of Fos expression in lamina I of the spinal cord dorsal horn. This could be due to SP and NKA acting on different receptor targets, or a result of differential downstream signaling produced by activation of the same, presumably NK-1, receptors. Our inability to block NKA induced Fos expression

with GR 205171 favors the first possibility, however, further study is needed to properly address the question.

Other possible sites of tachykinin action

Other tachykinin receptors

While the neurokinin-1 receptor is the most obvious and highly expressed target for tachykinins in the spinal cord dorsal horn, there are numerous other potential sites at which tachykinins might act. The neurokinin-3 receptor is abundantly expressed on a population of inhibitory interneurons in lamina II of the spinal cord and can be activated by both SP and NKA, although with significantly lower affinity than is seen with the endogenous neuropeptide NKB. This receptor has been shown to have anti-nociceptive actions in the spinal cord, potentially by inducing release of endogenous opioid peptides (Laneuville et al, 1988). Thus, activation of these receptors might serve to modulate or balance the pro-nociceptive effects of NK-1 receptor activation.

Neurokinin-2 receptors have not been found to be expressed on neurons in the spinal cord, despite pharmacological evidence for their presence. Interpretation of these studies is made all the more difficult due to the complicated pharmacology of the NK-1 receptor and the characterization of the antagonists on isolated peripheral tissue, which may have somewhat mixed neurokinin receptor populations. NK-2 receptors are highly expressed in peripheral tissues and there is evidence that they may be expressed in primary afferent neurons, especially during inflammation (Weinrich et al, 1997). In these studies we find that NK-2 receptor antagonists block the increase in defecation rate seen with intrathecal NKA but have few other effects on tachykinin

mediated behaviors. Given the lack of evidence for spinal receptors, it is most likely that this NK-2 receptor mediated response occurs via primary afferent derived NK-2 receptors. Alternatively, recent immunohistochemical labeling has localized NK-2 receptors to astroglia in the spinal cord, predominantly in the medial aspect of lamina I and lamina X (Zerari et al, 1998). NKA could act at these astroglial sites, possibly releasing glutamate and other amino acids, prostaglandins, or cytokines which could act upon dorsal horn neurons to produce effects such as the increased Fos expression and defecation behavior seen in this study.

Substance P degradation products

There is evidence that some of the peptide fragments produced by endopeptidase breakdown of substance P have actions on their own. It has been suggested that SP (1-7) binds and activates opioid receptors in the spinal cord dorsal horn (Krumins et al, 1989; Goettl & Larson, 1994). Thus, degradation of substance P might act to inhibit activity in the dorsal horn by triggering opioidergic signaling. Assuming degradation of NKA does not produce peptides that are active at opioid receptors, this might explain the difference in expression of Fos following intrathecal injection of the two tachykinins. Opioidergic inhibition produced by SP (1-7) might prevent substance P induced Fos expression, resulting in less overall expression than is produced by NKA mediated activation of the same neurokinin receptors. *Nicotinic receptors*

There is recent evidence that tachykinins may act to modulate activity of nicotinic acetylcholine receptors through a non-competitive action directly at the receptor (Lukus and Eisenhour, 1996). Nicotinic receptors are expressed in the spinal cord and have been shown to be involved in nociceptive processing (Khan

et al, 1998; Damaj et al, 1998). Although it is not clear whether SP and NKA differentially regulate activity at this receptor and it is unknown whether endogenous tachykinins actually have actions at nicotinic sites, this is another potential site at which SP and NKA could produce different effects in the spinal cord.

Endopeptidase competition

In addition to receptor mediated effects, it is possible that tachykinins could produce effects in the spinal cord by *competing* for endopeptidases, which are involved in the degradation and thus regulation of signaling of multiple neuropeptides found in the spinal cord. Most endopeptidases are involved in the degradation of several endogenous neuropeptides and thus may be partially responsible for interactions between neuropeptide systems. For example, SP, CGRP and enkephalin have all been shown to share common degradative enzymes (Suzuki et al, 1994).

There is evidence that release of CGRP can increase the spread and activity of tachykinins by competing for an endopeptidase and thus slowing their degradation. (Schiable et al, 1992; Saleh et al, 1996) It is likely that tachykinins have similar effects in reverse; their presence would slow the degradation of not only CGRP, but also opioids and other peptide substrates. Thus, it is possible that tachykinins might trigger signaling through other neuropeptide systems, by causing the levels of other neuropeptides to increase to the point that they further activate their own receptors. As SP and NKA have been shown to be differentially degraded by endopeptidases (Nyberg et al, 1984; Theodorsson-Norheim et al, 1987) one would expect that they would have different effects through such endopeptidase competition.

Differential actions of tachykinins through the same receptor

Significant and ever accumulating evidence suggests that substance P and neurokinin A have distinct and distinguishable actions upon the NK-1 receptor. Both tachykinins clearly bind the receptor, however, recent evidence suggests that they may have separate binding sites. Mutational analysis has uncovered sites necessary for NKA but not SP binding, and pharmacological observations have suggested that two separate classes of agonists and antagonists can be identified for the receptor (Maggi, 1995; Wijkhuisen et al , 1999). Substance P like agonists, bind the receptor with high affinity, activate both PLC and cAMP signaling effectively at low nanomolar concentrations, have unusually slow offset kinetics, and will displace the binding of the NKA/septide like agonists. The NKA/septide like agonists will also bind the receptor at low nanomolar concentrations, but only effectively activate PLC signaling at these doses and have fast offset kinetics. Additionally, NK-1 receptor antagonists have been shown to antagonize binding and activation by these two classes of agonists in qualitatively different manners. For example, RP 67,580 is a competitive antagonist for the SP class but non-competitive antagonist for the NKA/septide like ligands (Pradier et al, 1994)

The observation that these two tachykinins differentially stimulate second messenger signaling cascades, presumably via differences in receptor activation of Gq/11 and Gs, is particularly intriguing, as it suggests that the two neuropeptides could have very different effects upon signaling and gene transcription on the same cell via the same receptor (Sagan et al. , 1996).

Inability of GR 205171 to inhibit Fos induction by NKA

Our finding that a dose of the NK-1 receptor antagonist GR 205171 that block i.t. NKA induced NK-1 receptor internalization did not reduce i.t. NKA induce Fos expression is very surprising. As GR 205171 reduced the redness and salivation produced by intrathecal NKA in these same rats, we believe that the drug was acting as an effective NK-1 receptor antagonist.

Thus result thus suggests that the Fos expression resulting from intrathecal injection of NKA, even that within NK-1 receptor expressing neurons, is not NK-1 receptor dependent. As discussed prior, there are numerous other targets at which NKA could produce its effects. Never the less, assumptions to date favor the NK-1 receptor as being the primary site of tachykinin action in the spinal cord.

It is always possible that the lack of effect on Fos expression was due to pharmacological limitations or detection problems. The GR 205171 may have been cleared faster than the NKA resulting in delayed NK-1 receptor mediated effects, although we have no behavioral evidence of this occurring. Alternatively, little NK-1 receptor activation might be necessary to trigger expression of Fos in the dorsal horn. The remaining NK-1 receptor activation in the presence of GR 205171 might be sufficient to produce Fos expression, despite greatly reduced NK-1 receptor activation. If so, Fos expression would require significantly less NK-1 receptor activation than the redness or salivation behaviors. To address these possibilities, studies might be undertaken in mice genetically lacking the NK-1 receptor, where pharmacological concerns are absent.

While the mechanisms underlying the effects are still not clear, these data suggest that SP and NKA have qualitatively different effects in the dorsal horn of the spinal cord. Though not conclusive, the antagonist data suggests that these differences might reflect actions on non-neurokinin receptor targets. Identification of these sites will be helpful in understanding the various effects of tachykinins in the spinal cord.

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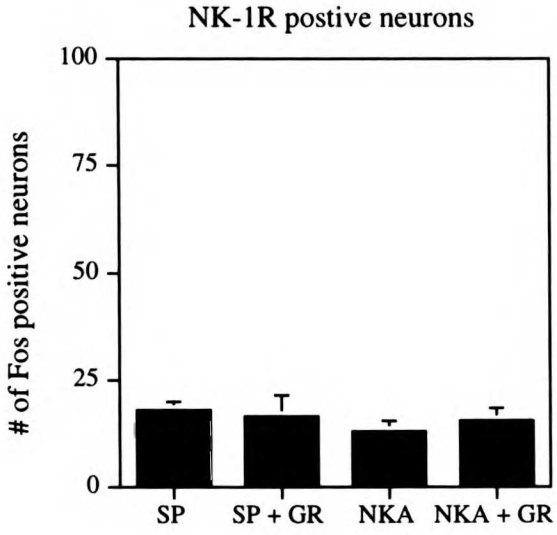
Figure 1: Tachykinins induce Fos expression in both neurokinin-1 receptor positive and negative neurons. Substance P and NKA induce Fos expression in NK-1 receptor positive neurons equivalently. This Fos expression is not prevented by the NK-1 receptor antagonist GR 205171. Intrathecal NKA produces significantly more Fos expression in NK-1 receptor negative neurons than SP ($p=0.0346$). In NK-1 receptor negative neurons as well, GR 205171 has no effect on either SP or NKA evoked Fos expression.


NK-1R postive neurons
# of Fos positive neurons
100
75
50
25
0
SP
SP + GR
NKA
NKA + GR

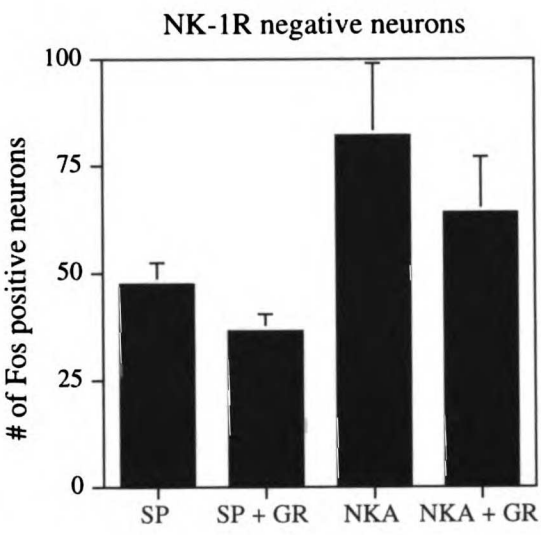

NK-1R negative neurons
# of Fos positive neurons
100
75
50
25
0
SP
SP + GR
NKA
NKA + GR

Figure 2: Intrathecal injection of the NK-2 receptor antagonist MEN 11420 does not reduce the expression of Fos in the superficial dorsal horn evoked by intrathecal injection of NKA.

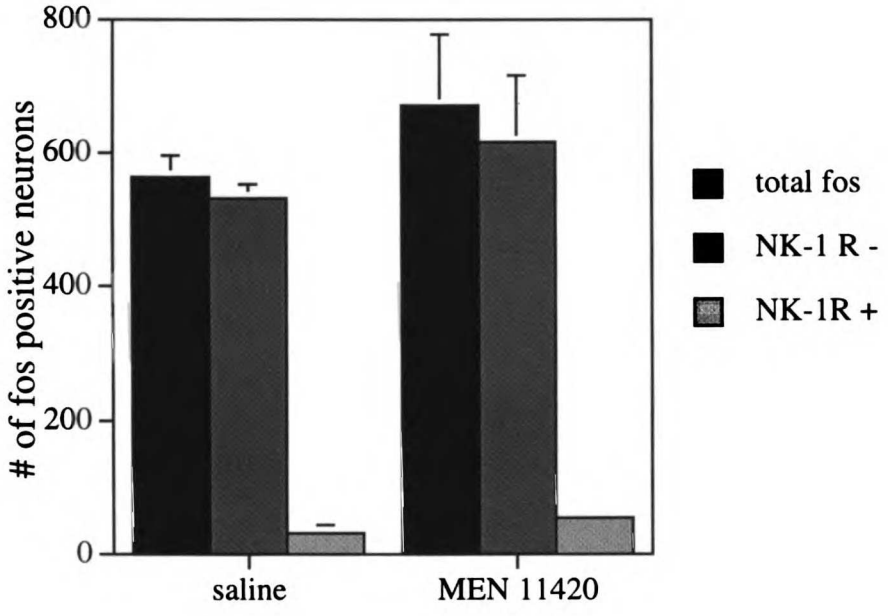
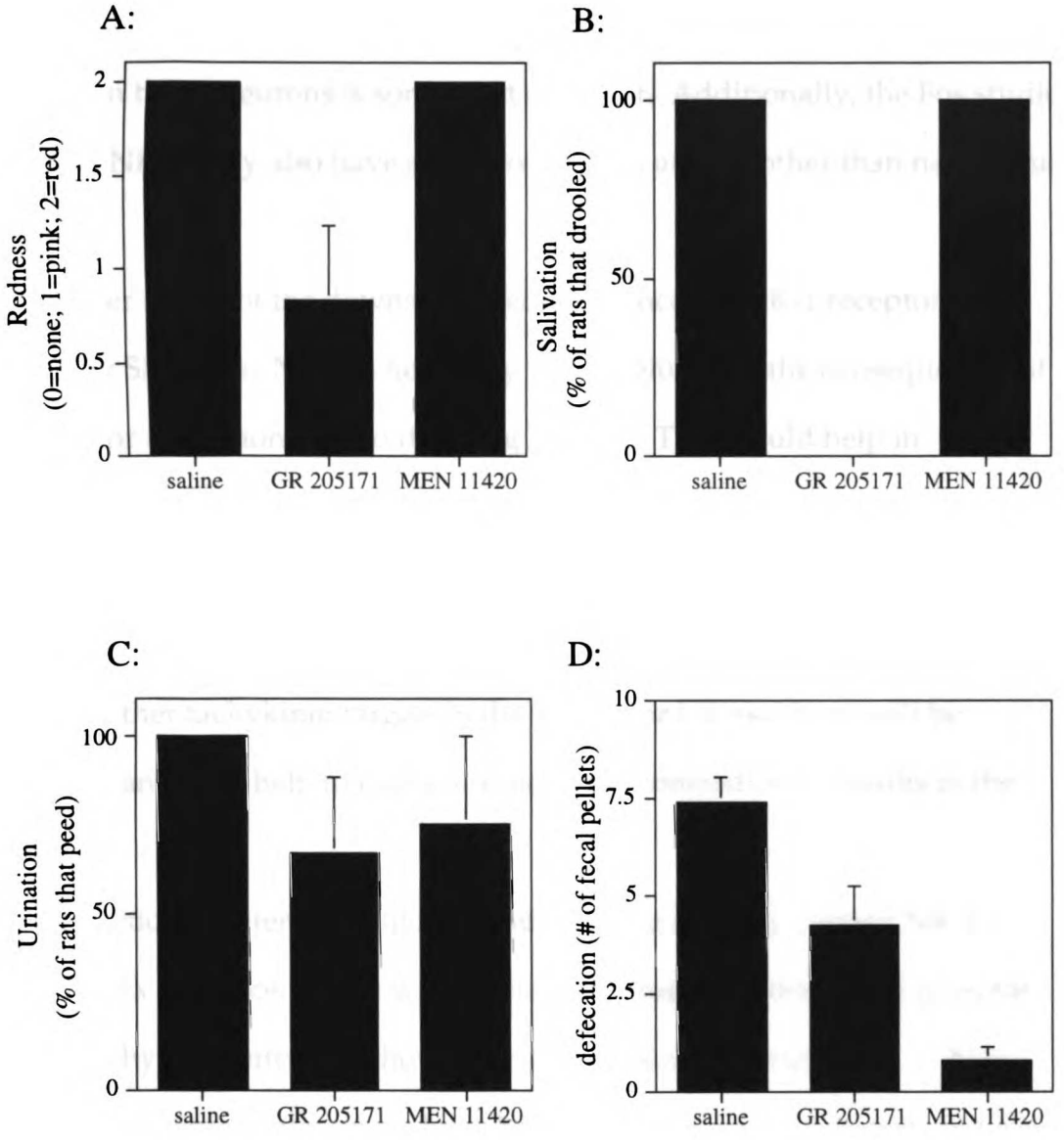

800
600
400
200
0
# of fos positive neurons
saline
MEN 11420
total fos
NK-1 R -
NK-1R +

Figure 3: Systemic GR 205171 and intrathecal MEN 11420 differentially prevent some behaviors produced by intrathecal injection of NKA. A) GR 205171 but not MEN 11420 reduces i.t. NKA evoked reddening of the ears and paws. B) GR 205171 but not MEN 11420 prevents i.t. NKA induced salivation. C) Neither GR 205171 nor MEN 11420 reduces i.t. NKA induced urination. D) MEN 11420 reduces i.t. NKA induced defection to a significantly greater extent than does GR 205171.


A:
Redness
(0=none; 1=pink; 2=red)
2
1.5
1
0.5
0
saline
GR 205171
MEN 11420
B:
Salivation
(% of rats that drooled)
100
50
0
saline
GR 205171
MEN 11420
C:
Urination
(% of rats that peed)
100
50
0
saline
GR 205171
MEN 11420
D:
defecation (# of fecal pellets)
10
7.5
5
2.5
0
saline
GR 205171
MEN 11420

FURTHER CONCLUSIONS, DISCUSSION AND QUESTIONS

These results suggest that while SP and NKA clearly both act at NK-1 receptor sites following noxious stimulation, the neurons that they target and their effect on these neurons is somewhat different. Additionally, the Fos studies suggest that NKA may also have additional effects at sites other than neurokinin receptors.

Further study of the downstream consequences of NK-1 receptor activation by SP versus NKA is necessary to help elucidate the consequences of NK-1 receptor activation by two differing ligands. This would help in understanding the relevance of having SP mediated NK-1 receptor activation in lamina I neurons near the site of input and NKA mediate NK-1 receptor activation in deeper lamina and more distant lamina I regions. Additionally isolation of other tachykinin targets in the spinal cord dorsal horn will be illuminating and may help to explain some of the contradictory results in the literature.

Of particular interest would be studies of the recently created NK-1 receptor knock out mice. These would allow for studies of non-NK-1 receptor mediated tachykinin effects without the confounds of pharmacology. New neurokinin-3 receptor antagonists have been and are being developed, and these might be used to determine if any of the tachykinin's effects are mediated through these receptors. Studies with endopeptidase inhibitors or neutral endopeptidase (NEP) knock out mice should be helpful in determining if differences in the degradation of SP and NKA are responsible for some of their differing effects in the spinal cord. Additionally, it would be helpful to know

how induction of Fos gene expression is regulated by the two neuropeptides, as the lack of knowledge about the immediate cause of Fos expression in a given neuron limits conclusions that can be made from the data obtained. Finally, studies of mice expressing only SP or NKA, as are currently being generated in the lab, should also help to clarify some of these questions.

Chapter 3

Pre-synaptic MOR regulation of neuropeptide release

GENERAL INTRODUCTION

Several theories have been proposed to explain the analgesic effects of opioids applied at the spinal level. All predict the same final outcome, namely a reduction in firing of spinal cord neurons that project to the brain, an effect that has been well documented in electrophysiological studies. (Willcockson et al, 1984; Wilcockson et al, 1986; Hylden & Wilcox, 1986) One of these theories suggests that opiates reduce the release of neurotransmitters from primary afferent nociceptors, the peripheral neurons that respond to potentially tissue damaging stimuli. In this way, opiates would prevent noxious information from ever reaching spinal cord neurons, thus reducing their response. Supporting this idea, opioid receptors have been found on the terminals of primary afferent nociceptors (Ding et al, 1995a), and opiates decrease calcium conduction in DRG neurons in vitro (Schroeder & Mcclesky, 1993), making the theory a plausible one. Functional evidence for the idea has been limited, however. A major focus has been on opiate regulation of release of SP, the neuropeptide whose contribution to the transmission of nociceptive information in the spinal cord we examined in the last two chapters. A number of studies have found a decrease in substance P release with opiates in in vitro DRG cultures (Suarez-Roca & Maixner, 1995) and slice preparations (Lembeck & Donnerer, 1985) and with dialysis of spinal CSF (Aimone & Yaksh, 1989, Hirota et al, 1985; Yaksh et al, 1980). An in vivo study of substance P release using antibody microprobes in spinal cord dorsal horn (Morton et al, 1990) found no effect of opiates, however, and no studies have convincingly demonstrated evidence of decreased activity in

spinal cord neurons stemming from this proposed reduction in released substance P. As such, this remains an unproven hypothesis.

To elucidate the relevance of this mechanism to analgesia in vivo, here we examine the effects of opioids on tachykinin signaling in the spinal cord. By understanding the interactions between these two neuropeptide modulators in the spinal cord, we should gain insight into how the amplification or dampening of nociceptive input is controlled.

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Spinal opioid analgesia: How critical is the regulation of SP release?

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ABSTRACT

Although opioids can reduce stimulus-evoked efflux of SP from nociceptive primary afferents, the consequences of this reduction on spinal cord nociceptive processing has not been studied. Rather than assaying SP release, in the present study we examined the effect of opioids on two postsynaptic measures of SP release, Fos expression and neurokinin-1 (NK-1) receptor internalization, in the rat. The functional significance of the latter was first established in *in vitro* studies which showed that SP-induced Ca^{2+} mobilization is highly correlated with the magnitude of SP-induced NK-1 receptor internalization in dorsal horn neurons. Using an *in vivo* analysis, we found that morphine had little effect on noxious stimulus-evoked internalization of the NK-1 receptor in lamina I neurons. However, internalization was reduced when we coadministered morphine with a dose of an NK-1 receptor antagonist that by itself was without effect. Thus, although opioids may modulate SP release, the residual release is sufficient to exert maximal effects on the target NK-1 receptors. Morphine significantly reduced noxious stimulus-induced Fos expression in lamina I, but the Fos inhibition was less pronounced in neurons that expressed the NK-1 receptor. Taken together, these results suggest that opioid analgesia predominantly involves postsynaptic inhibitory mechanisms and/or presynaptic control of non-SP containing primary afferent nociceptors.

INTRODUCTION

Although spinal administration of opioids produces a profound antinociceptive effect, the mechanisms underlying this action are not fully understood. Because there is considerable evidence that primary afferent-derived SP contributes to the transmission of nociceptive messages in the spinal cord (Hökfelt et al, 1975; Hylden and Wilcox, 1981; Duggan et al, 1988; DeKoninck and Henry, 1991) and because opioid receptors are located on primary afferents, many studies have focused on the ability of opioids to presynaptically control SP release. In fact, opioids reduce K^+ or noxious stimulus-evoked release of SP (Jessell and Iversen, 1977; Yaksh et al, 1980; Hirota et al, 1985; Aimone and Yaksh, 1989), leading to the hypothesis that inhibition of SP release by opioids is a major mechanism by which opioids produce analgesia. However, none of these studies have demonstrated functional consequences of this reduction in SP release. In the present study, we specifically examined the functional impact of such presynaptic inhibitory controls on SP-mediated signaling in the spinal cord dorsal horn.

SP binds preferentially to the NK-1 receptor, a G-protein coupled receptor that is expressed in the spinal cord dorsal horn (Brown et al, 1995). Because the NK-1 receptor internalizes when it binds agonist (Bunnett et al, 1995), we have used NK-1 receptor internalization as a marker of neurons that are activated by SP. We demonstrated that intense noxious peripheral stimulation induces internalization of the NK-1 receptor in lamina I neurons (Mantyh et al, 1995; Abbadie et al, 1997). During inflammation, NK-1 receptor positive neurons

located more ventrally, in laminae III-VI, also internalize the NK-1 receptor in response to noxious stimulation (Abbadie et al, 1997). This technique thus provides a simple and reliable method for visualizing signaling produced by SP.

Our aim, therefore, was to gauge the effects of opioid receptor agonists on the functional consequences of noxious stimulation-induced release of substance P from primary afferent nociceptors. As studies have reported a greater potency of morphine during inflammatory injury (Colpaert, 1979; Kayser and Guilbaud, 1983), we examined the effects of morphine in both normal rats and in rats with persistent hindpaw inflammation. To ensure that the endpoint of internalization indeed correlates with NK-1 receptor-mediated signaling, we performed *in vitro* studies in which we compared SP-mediated increases in intracellular calcium and NK-1 receptor internalization. Because opioids likely have other effects on neurotransmitter release and interneuronal signaling in the spinal cord, we also evaluated the effect of morphine on a more general marker of neuronal activity, namely Fos expression , in dorsal horn neurons that express the NK-1 receptor. Finally, to discriminate between pre and postsynaptic opioid effects, we measured the effect of morphine on NK-1 receptor internalization and Fos expression produced by direct intrathecal injection of SP.

MATERIALS AND METHODS

Experimental animals

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at UCSF. Experiments were performed on male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA), weighing 230-270g. In

some rats, inflammation was induced by subcutaneous (s.c.) injection of 100 ml of complete Freund's adjuvant (CFA, killed mycobacterium butyricum suspended in mineral oil, solution at 10 mg/ml, Sigma, St. Louis, MO) in the left hindpaw. Rats were stimulated two days after the inflammation was induced.

Drug treatments

Morphine sulfate was given s.c. (10 mg/kg) at the base of the neck 25-30 min or intrathecally (i.t., 10 or 30 mg) 20-25 min prior to stimulation. Selective opioid receptor agonists were given i.t. 20 min prior to stimulation: DAMGO (1.0 mg, Sigma, St. Louis, MO), DPDPE (30 mg, Sigma, St. Louis, MO) or U-50488H (100 mg, RBI, Natick, MA). The doses of these opioids were chosen because these are established antinociceptive doses (Miaskowski et al, 1991). GR 205171A dihydrochloride salt, an NK-1 receptor antagonist (kindly provided by Glaxo-Wellcome) was given s.c. (1.0 or 10 mg/kg) at the base of the neck, 20-25 min prior to the stimulation. In control experiments, we established that neither saline, nor any of these drugs induced internalization of the NK-1 receptor without additional stimulation (see Figure 1). Subcutaneous injections were made in a 1.0 ml volume of saline. Intrathecal injections, performed under halothane anesthesia, were made directly between the S1-S2 vertebrae with a 30 gauge needle in a 20 ml volume of saline. Control animals received an equal volume of saline. In some experiments, naloxone (1.0 mg/kg, s.c.) was given 5 min prior to the morphine.

To assess the possible postsynaptic effects of morphine on neurons that express the NK-1 receptor, we injected SP i.t. (100 mg, diluted in 20 ml of saline, Sigma, St. Louis, MO). This dose induces internalization of the NK-1 receptor in

100% of neurons in the lumbar cord. In these studies we injected morphine (10 mg i.t. or 10 mg/kg s.c.) 20-25 min before the SP injections.

Hindpaw stimulation

All experiments were performed 10-15 minutes after the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). This dose blocked flexor reflex responses to hindpaw stimulation. Noxious mechanical stimulation (pinch) was applied to the distal part of one hindpaw with a hemostat for 15 sec. For thermal stimulation, the rat's hindpaw (to just below the ankle) was dipped for 2 min into a water bath heated to 50°C. To study noxious stimulus-induced internalization of the NK-1 receptor, the rats were perfused 5 min after the stimulation ended. For experiments that examined double-labeling of NK-1 receptor and Fos, the rats were perfused 90 min after the stimulation.

Immunocytochemistry

At the appropriate time, the animals received an additional injection of sodium pentobarbital (100 mg/kg, i.p.) and were perfused intracardially with 50 ml of phosphate-buffered saline 0.1 M (PBS) followed by 500 ml of 10% formalin in 0.1 M phosphate buffer (PB). After the perfusion, the lumbar spinal cord was removed, postfixed for four hours in the same fixative and then cryoprotected overnight in 30% sucrose in 0.1 M PB. Immunostaining was performed on 30 mm lumbar spinal cord sections (from L2 to L6 segments) cut in the sagittal plane on a freezing microtome. The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST).

For immunofluorescent staining of the NK-1 receptor, the sections were incubated overnight in the primary antiserum, diluted to 1:5,000. The characteristics of the antiserum, directed against the C terminal tail of the NK-1 receptor, have been described previously (Vigna et al, 1994). After the primary antiserum, the sections were washed 3 times in 1% NGST and then incubated in indocarbocyanine Cy-3™ conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1: 600) for 2 hours at room temperature. For double-labeling studies, sections were first incubated in the Fos antisera (1: 30,000; kindly given by Dr. Dennis Slamon, UCLA) overnight. Immunostaining was performed according to the avidin-biotin peroxidase method of Hsu et al. (1981) To localize the HRP immunoreaction product for Fos, we used a nickel-intensified diaminobenzidine protocol with glucose oxidase. Sections were then incubated in the NK-1 receptor antisera (1: 20,000) and a diaminobenzidine protocol with glucose oxidase without nickel was used. Finally, the sections were washed 3 times in PB, mounted on gelatin-coated slides, dried, and coverslipped with DPX (Electron Microscopy Science, Gibbstown, NJ).

Quantification of immunoreactivity and statistical analysis

Quantification of NK-1 receptor internalization was performed as previously described (Abbadie et al, 1997). Briefly, to analyze internalization in cell bodies we used a 20X objective on a Nikon FXA microscope equipped for fluorescence. We counted NK-1 receptor-like immunoreactive cell bodies in laminae I, III-IV and V-VI of the dorsal horn, ipsilateral to the side of stimulation, from segments L2 to L6. NK1 receptor-like immunoreactivity is uniformly distributed on the surface of cell bodies that do not contain internalized

receptors, but in the neurons that have internalized NK-1 receptors, the cytoplasm contains bright, immunofluorescent endosomes (Fig. 1). Neurons were considered internalized if they contained more than 20 endosomes in the cell body. All counts are expressed as the percentage of NK-1 receptor immunoreactive neurons that contain internalized receptor.

Because we found no difference in the magnitude of internalization along the mediolateral extent of the superficial dorsal horn, we counted all the neurons within a section, without taking into account the mediolateral position of the cells. Neurons from five sagittal sections were counted from each rat for both NK-1 receptor internalization and Fos double labeling.

To analyze double labeling for Fos and the NK-1 receptor, we only counted neurons in lamina I. We recorded the number of neurons that were (1) only positive for Fos, (2) only positive for the NK-1 receptor and (3) double-labeled for both NK-1 receptor and Fos. In all experiments, the investigators who quantified internalization or double labeling were not aware of the treatment that the animal received. For statistical analysis, we used a two-way analysis of variance for treatment condition (saline vs. drug), and for spinal segment (L2-L6 lumbar segments). For multiple comparisons, we used Fisher's PSLD test; $p<0.05$ was considered statistically significant.

Confocal images

Although most quantitative analysis was performed on tissue observed with epi-illuminated fluorescence, to demonstrate that morphine did not cause a decrease in the number of endosomes in each individual neuron, we examined some sections by confocal microscopy. The confocal images described below (Fig.

1) were collected with an MRC 600 confocal microscope (Bio-Rad, Hercules, CA) with a 60X objective. Images were reformatted in NIH-Image (version 1.60) and montages were created in Photoshop (Adobe, version 3.0). Optical sections of about 1.0 μm were taken through the center of 20 neurons in lamina I of the L4 segment for each animal. The first 5 neurons found on each of 4 sections were imaged. Bright puncta within the limits of the cell body were counted as endosomes. The investigator taking images and counting endosomes was unaware of the treatment of the animal.

Spinal cord cultures

Spinal cord cultures were prepared from E19 Sprague-Dawley rats using a modification of the method of Yu et al. (1984). Spinal cords were dissected out, washed and treated with a standard 0.25% trypsin/versene mixture (STV, cell culture facility, UCSF) for 12 minutes. The cord were again washed and mechanically dissociated with a large bore pipette. Cells from each cord were diluted into 24 ml of MEM-PAK buffer (cell culture facility, UCSF) supplemented to contain 5% normal horse serum, 5% fetal calf serum and penicillin/streptomycin, 30 mM glucose and 2 mM glycine. Cells were plated on glass coverslips (Carolina, Burlington, NC) or 8 well coverglass (Fisher, Santa Clara, CA) and incubated at 37°C in a humidified incubator with 5% CO_2/95% O_2. Cultures were used at 5-6 days of age.

Calcium imaging

Spinal cord cultures plated on 8 well coverglass were incubated in CI buffer (in mM: 130 NaCl,.3 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 1.2 $NaHCO_3$, 10 glucose, 10 HEPES, pH 7.4) containing 10 mM Fura-2 acetoxymethyl ester and 0.02% pleuronic acid (Molecular Probes, Eugene, OR) for 25 minutes. The Fura was then removed and replaced with 150 ml/well of fresh CI buffer. Ratiometric calcium imaging was performed with a Nikon Diaphot fluorescence microscope equipped with a variable filter wheel (Sutter Instruments, Novato, CA) and an intensified CCD camera (Hamamatsu, Bridgewater, NJ). Dual images (340 and 380 nm excitation, 510 nm emission) were collected every 4 sec. For each well, five baseline images were recorded and then 150 ml of SP in CI buffer at twice the desired final concentration was pipetted into the well. Responses were recorded for the following 40 seconds.

For every image that was collected, we calculated the average 340/380 ratio for each of the cells in the field. All cells that showed an average increase of the 340/380 ratio that was greater than twice the average baseline 340/380 ratio for that cell were considered responders. Only responders were considered in the subsequent analysis. In each well, we calculated the total increase in intracellular calcium for all responders in each well by taking the sum of the 340/380 ratios over the 40 secs after SP application (10 images). From each value we subtracted the average baseline 340/380 ratio of the cell, which was measured over the 20 secs prior to application of SP.

In vitro internalization

Spinal cord cultures plated on coverglass were incubated in culture media containing SP. After 15 minutes, media was removed and cells were fixed in 10% formalin for 20 minutes. NK-1 receptor was labeled for immunofluorescent analysis as described above for the tissue sections. Confocal images (75X 4.0 iris diameter) were taken of 5 NK-1 receptor positive neurons per coverslip and the number of endosomes in each cell was counted and averaged for neurons over the coverslip. Images were taken on a Bio-Rad MRC 1024 confocal microscope. The investigator taking images and counting endosomes was unaware of the treatment that the cultures received. All neurons with greater than 10 endosomes in their cytoplasm were considered responders.

Statistical analysis

To normalize the responses of the neurons, we first determined the maximal response to SP and then determined the percent of this response generated by different doses of SP. Percent maximal possible effect (MPE) produced by a given dose was calculated as follows:

*Percent MPE = ((Average response produced by the particular SP dose) – (Average response produced in the absence of SP)) / (Average maximal effect) * 100.*

We also assessed the response after correcting for the fact that not all neurons in a given dish will respond (because not all express the NK-1 receptor). For this calculation we first determined the maximal number of responsive cells (responders) and then determined the percent of this response generated by different doses of SP. Percent MPR produced by a given dose was calculated as follows:

Percent MPR = ((Average percent of responders produced by the particular dose of SP) – (Average percent of responders produced in the absence of SP)) / (Average maximal percent responders)* 100.

The thresholds for considering a cell a responder were chosen arbitrarily based upon variation observed in untreated cultures. Analysis was also performed using 15 endosomes and 1.5 times baseline as thresholds. The two approaches produced similar results. EC_{50}'s were calculated with Prism (GraphPad Software, Inc). For statistical analysis, we used a two-way analysis of variance for measure (intracellular calcium concentration or NK-1 receptor internalization) and for SP dose.

RESULTS

As we reported previously (Brown et al, 1995), there is a very distinct pattern of NK-1 receptor staining in the dorsal horn of the rat. The densest staining is found in cell bodies and dendrites of lamina I. The immunoreactivity is best viewed in sagittal section because the dendrites, which express the bulk of the immunoreaction product, arborize in the rostrocaudal plane. Lamina II (the substantia gelatinosa) contains very little NK-1 receptor-LI, except for dorsally-directed dendrites of relatively large NK-1 receptor-LI neurons located in laminae III-IV. Smaller neurons with round cell bodies are also located in laminae III-VI; dendrites of these neurons arborize in all directions and in all planes. Although dendrites of the majority of lamina V neurons that express the NK-1 receptor arborize locally, a few also have dorsally-directed dendrites that

extend into lamina I. Finally, densely stained, large, round cell bodies are clustered around the central canal. In all regions, the NK-1 receptor immunoreactivity is concentrated on the plasma membrane of cell bodies and dendrites (Fig. 1 A and D).

Effects of opioids on internalization of the NK-1 receptor induced by peripheral stimulation

Systemic morphine

As we previously reported (Abbadie et al, 1997), noxious mechanical stimulation of the hindpaw (pinch) is a particularly effective stimulus for evoking NK-1 receptor internalization in dorsal horn neurons. In the present study we used a 15 sec stimulus, which induces internalization in 80-100% of NK-1 receptor immunoreactive lamina I neurons in the L4 segment of normal rats. The percentage of neurons that internalized the receptor decreased rostrally and caudally, to 10-30% in L2 and 30-50% in L6 (Fig. 2A). In normal rats, pinch-evoked internalization only occurred in lamina I cell bodies and dendrites, whereas in rats with inflammation the same stimulation also induced internalization in deeper laminae III-IV and V-VI neurons. In this latter group, we also found an increase in the number of lamina I neurons that internalized the NK-1 receptor in response to pinch, to 60-70% in L2, 85-95% in L3, 95-100% in L4 and L5 and 70-80% in L6 (Fig. 2B).

Morphine produced a small, but statistically significant reduction of the percentage of neurons showing internalization after mechanical stimulation in normal rats ($p=0.04$, Fig. 2A). We observed a 17% reduction in the L4 segment,

but found no difference in more rostral or caudal segments. In rats with inflammation, morphine had no significant effect (p=0.25, Fig 2B). If anything morphine slightly increased the percentage of cells showing NK-1 receptor internalization. In laminae III-IV, we found no difference in the magnitude of internalization between morphine-treated and saline-treated CFA injected rats. The combined injection of morphine and naloxone or of naloxone alone had no effect on NK-1 receptor internalization.

Noxious thermal stimulation produced by dipping the hindpaw in hot water (50°C for 2 min) also evoked NK-1 receptor internalization, in 80-85% of neurons of lamina I of L4-L5. This is less than that induced by mechanical stimulation (Fig. 2C). In contrast to mechanical stimulation, we found no difference in evoked internalization between normal rats and those with inflammation (Fig. 2C and D). In normal rats, morphine had no effect on NK-1 receptor internalization (p=0.6, Fig. 2C), but in rats with inflammation, morphine decreased the number of cells with internalized receptor (p=0.04, Fig. 2D). The magnitude of this effect was 20-25% in L4-L5 segments. Because thermal stimulation did not induce internalization in deep dorsal horn laminae, we could not study the effect of morphine in this region. Although these small differences in the effect of opioids on the consequences of mechanical versus thermal stimulation may indicate modality specific differences in susceptibility to morphine, the inhibition in all cases was very small.

Intrathecal injection of morphine

In light of the minimal reduction of NK-1 receptor internalization after systemic injection of morphine, we also evaluated the effect of intrathecal (i.t.)

injection. Compared to saline, intrathecal morphine produce a small, albeit significant (p=0.012) reduction in the number of NK-1 receptor internalized cells (Fig. 3) induced by the 15 s pinch stimulus. However, there was no difference between the two doses of morphine; the 10 and 30 μg morphine reduced the magnitude of internalization by 20 and 19%, respectively. Finally, we evaluated the effect of i.t. morphine on NK-1 receptor internalization induced by direct i.t. injection of SP, at a dose (100 μg) that evoked internalization of the receptor in 100% of the NK-1 positive lamina I neurons (data not shown). Although these doses of morphine are antinociceptive in most acute pain tests, we found that i.t. morphine had no effect (p=0.7) on i.t. SP-induced NK-1 receptor internalization.

The results above are based on estimates of internalization in populations of neurons using an all or none criterion. It is conceivable that this approach missed a small reduction of internalization in individual neurons, which if present in large numbers of neurons that express the NK-1 receptor, could have significant functional consequences. To address this possibility, we counted the number of endosomes internalized in *individual* L4 lamina I neurons from the rats in the 30 mg i.t morphine group and in their i.t. saline controls (Fig. 1). In fact, there was no difference in endosome numbers between the groups (ANOVA, p=0.6626). Saline animals had 78 +/- 4 (s.e.m.) endosomes/neuron and the animals that received 30 mg i.t. morphine had 71.7 +/- 15 endosomes/neuron.

Intrathecal injection of receptor selective opioid agonists

Because there is evidence for a differential effect of receptor selective opioid ligands on SP release (Mauborgne et al., 1987; Suarez-Roca and Maixner,

1993; Zachariou and Goldstein, 1996a; Zachariou and Goldstein, 1996b; see Bourgoin et al, 1994 for review) and because recent anatomical evidence indicates that there is a differential localization of the different receptor subtypes, including a remarkable association of the delta opioid receptor with vesicles that store SP (Zhang et al, 1998), we repeated the experiment using selective agonists for the mu, delta and kappa opioid receptors. Again, we used mechanical stimulation of the hindpaw to induce receptor internalization. We found that a very high concentration (1.0 mg, i.t.) of DAMGO (a selective mu opioid receptor agonist) significantly decreased the number of neurons with internalized NK-1 receptor, by 31% in L4 (p=0. 004; Fig. 4). Neither DPDPE (a selective delta opioid receptor agonist, 30 mg i.t.) nor U-50488H (a selective kappa opioid receptor agonist, 100 mg i.t.) had a significant effect: DPDPE and U-50488H produced a 6.0 and 1.0% reduction, respectively (Fig. 4).

Effects of an NK-1 receptor antagonist, GR 205171, on internalization of the NK-1 receptor induced by peripheral stimulation

Normal Rats

As we previously observed (Abbadie et al, 1997) the selective NK-1 receptor antagonist, GR 205171 significantly reduces noxious stimulus-induced internalization of the NK-1 receptor in spinal cord neurons, indicating that internalization requires ligand binding. In the present study we found that the effect of GR 205171 increased with increasing dose. Thus, 1.0 mg/kg slightly decreased the number of cells that internalized the NK-1 receptor (<10%), but this decrease was not significantly (p=0.19) different from the saline-treated

group (Fig. 5). At 10 mg/kg, GR 205171 significantly ($p<0.0001$, Fig. 5) decreased the number of cells that internalized the NK-1 receptor. In the L4 segment the decrease was 77.5% (Fig. 5). Qualitatively, we observed that after GR 205171, endosomal and membrane labeling often coexisted in neurons; i.e. internalization was rarely complete. Moreover, endosomes appeared smaller than in saline-treated rats (Fig. 6). In contrast to the morphine animals in which no change in extent of endosomal labeling was found, this labeling pattern is suggestive of decreased NK-1 receptor activation of individual neurons, which raises the possibility that we are underestimating the effects of GR 205171.

Our inability to reduce NK-1 receptor internalization with systemic morphine alone may have resulted from a saturation of the noxious stimulus-induced response. To test this possibility we coadministered morphine with a low dose of an NK-1 receptor antagonist. We found that the combined administration of morphine (10 mg/kg s.c.) and GR 205171 (1.0 mg/kg s.c.) at doses that were ineffective when administered alone, significantly ($p<0.0001$) decreased the number of neurons that internalized NK-1 receptor in response to noxious stimulation (Fig. 5). Internalization in the group that received both morphine and the NK-1 receptor antagonist was decreased by 45% in the L4 segment compared to the saline-treated group. The rats that received the two drugs concurrently also showed a significant reduction in the percentage of cells showing internalization compared to the group that received morphine alone ($p<0.01$) or to the group that received GR 205171 at 1.0 mg/kg ($p<0.01$). On the other hand, the combination was significantly less effective in reducing internalization of the NK-1 receptor ($p<0.05$) than was the NK-1 receptor antagonist at the highest dose (10 mg/kg).

Rats with an inflamed hindpaw

Because there is an increase in levels of SP and its precursor mRNA in DRG cells and an upregulation of NK-1 receptor in the dorsal horn (Hanesch et al, 1993; Donnerer et al, 1993; Mapp et al, 1993; Schafer et al, 1993; Abbadie et al, 1996) in the setting of inflammation, we next evaluated the effect of NK-1 receptor antagonists and morphine in this context. In animals with inflammation we again found a dose-related effect of GR 205171. Thus, although 1.0 mg/kg GR 205171 had no effect on the percentage of cells that internalized the NK-1 receptor in lamina I through VI (Fig. 3), 10 mg/kg GR 205171 significantly ($p<0.0001$, Fig. 7) decreased the number of cells that internalized the NK-1 receptor. In lamina I of the L4 segment we observed a decrease of 50 % (Fig. 7) and in laminae III-IV and V-VI, GR 205171 (10 mg/kg) completely blocked internalization of the NK-1 receptor (Figs 7 B and C). We next found that the combined administration of morphine (10 mg/kg s.c.) and GR 205171 (1.0 mg/kg s.c.) decreased the number of NK-1 receptor internalized cells. However, this effect was only significant in lamina I of L2-L3. The two drugs were more potent in normal rats (56 % decrease of the area under the curve) than in the CFA-treated group (21.5 % decrease).

Correlation of NK-1 receptor-induced "activity" and its internalization

Although we assume that NK-1 receptor internalization provides a measure of the functional consequences of SP release (e.g. increased neuronal activity), this relationship has not been demonstrated in neurons. To make useful conclusions about neuronal activity in the dorsal horn based on the preceding

data, we needed to establish that there is a precise relationship between NK-1 receptor internalization and NK-1 receptor-mediated signaling. To address this question, we evaluated tachykinin-induced increases in intracellular calcium (Heath et al, 1994; Garland, 1996) and correlated these responses with the magnitude of NK-1 receptor internalization in primary cultures of spinal cord. Figure 8A illustrates that addition of SP to the bathing medium of primary spinal cord cultures rapidly increased intracellular calcium levels and induced NK-1 receptor internalization in neurons and glia in a dose-dependent fashion. The EC_{50}'s for total calcium influx and number of NK-1 receptor positive endosomes were 8.74 nM (95% C.I: 2.58 - 29.6 nM) and 14.28 nM (95% C.I: 2.35 - 86.8 nM), respectively, and the overall dose response curves did not differ; 2-way ANOVA for dose and measure; P=0.861; (Figure 8b).

Interestingly, not only were the dose response curves for the magnitude of SP-induced calcium signaling and NK-1 receptor internalization virtually identical, but similar curves were obtained when we measured the percentage of neurons that responded over a threshold value for either calcium signaling or internalization (Fig. 8c). The EC_{50}'s for the percent of neurons responding were 8.48 nM (95% C.I.: 5.23 - 13.73 nM) for increases in intracellular calcium concentration and 15.74 nM (95% C.I.: 13.64 - 18.17 nM) for NK-1 receptor internalization. There was no difference between the dose response curves for percent of neurons responding with a calcium increase and percent responding with NK-1 receptor internalization (2-way ANOVA for dose and measure; P=0.978).

Morphine regulation of Fos expression in NK-1 receptor expressing neurons

Although we found that morphine had a marginal effect on NK-1 receptor internalization, inhibition of SP release is only one of many mechanisms through which morphine could alter the activity of SP-responsive neurons. Postsynaptic inhibition of these neurons or of excitatory interneurons that activate lamina I cells is also likely to occur. To test this hypothesis, we used a double-labeling method to visualize noxious stimulus-evoked Fos protein and the NK-1 receptor simultaneously, so that we could evaluate the effect of morphine on the activity (i.e. postsynaptic response) of NK-1 receptor positive neurons. This allowed us to look at differences in opioid regulation of lamina I neuron activity that correlate with NK-1 receptor expression and activation by SP. Of particular interest was whether Fos could be blocked in neurons in which NK-1 receptor internalization persists. We examined both the effect of morphine (1) on neuronal activity directly induced by intrathecal injection of SP and (2) on neuronal activity induced by the noxious mechanical stimulation described above.

Morphine modulation of the central effects of i.t. SP

First, we found that i.t. SP (100 μg) was a very effective stimulus for the induction of Fos in dorsal horn neurons. In fact, almost all NK-1 receptor-LI neurons expressed Fos after i.t. SP, but, interestingly, Fos was also expressed in NK-1 receptor negative cells (Table 1), including many in lamina II. We presume that these neurons lie downstream of the NK-1 receptor-LI neurons of lamina I. Because lamina II is almost devoid of NK-1 receptor expressing neurons we only quantified the effect of morphine in lamina I neurons. In saline-injected rats, we counted 57.1 +/- 4.3 Fos-LI nuclei per 30 μm sagittal section in the lumbar

enlargement (L2-L6). Morphine (10 mg/kg s.c.) had no effect on the number of lamina I Fos-LI neurons induced by SP (Table 1, p=0.93). This was true for both NK-1 receptor-positive and negative cells (Table 1).

Effects of morphine on noxious mechanical stimulation-induced Fos expression

After mechanical stimulation, the number of neurons labeled for both Fos and NK-1 receptor differed according to the segmental level. The largest number was in the segments that receive greater innervation from the stimulated area, namely L4-L5,(i.e. where the Fos induction is greatest). In the L4-L5 segments, 75-80% of the NK-1 receptor-LI cells were also Fos-LI, but only 18% in L2 and 50-55% in L3 or L5 were double labeled. By contrast, the percentage of Fos-LI cells that were NK-1 receptor-LI was constant over the lumbar cord; 15-25% of Fos-LI neurons were NK-1 receptor-LI in segments from L2-L6.

In contrast to the lack of effect of morphine on SP-induced Fos expression, we found that morphine (10 mg/kg s.c.) significantly ($p<0.001$) decreased Fos-LI expression in lamina I neurons evoked by mechanical stimulation. On the other hand, we found a difference in the ability of morphine to prevent Fos-LI expression in NK-1 receptor positive vs. NK-1 receptor negative neurons (Figs. 9 and 10). In L4-L5, morphine produced a 60% decrease of Fos expression in NK-1 negative neurons, but only a 20-40 % decrease in cells that were NK-1 receptor-LI (Fig. 10). This result suggests that morphine was not as effective upon cells activated by SP.

DISCUSSION

Opioid regulation of the central effects of SP release from primary afferents:

With some exception (Lang et al, 1991, Kuraishi et al, 1993) opioid inhibition of SP release from primary afferent fibers has been demonstrated in both an *in vitro and in vivo* setting. In spite of these results, there is no information on the functional significance of the opioid reduction of SP release. In the present study we used a measure of the postsynaptic response to a neuron's interaction with SP, namely internalization of the NK-1 receptor, to address this question. We found a modest reduction in NK-1 receptor internalization with opioids, but the internalization that persisted was more striking. We estimate that at least 80% of the tachykinin signaling is intact after morphine administration, *at doses that produce analgesia in awake animals.* The fact that other compounds, such as baclofen, can greatly reduce noxious stimulus-evoked NK-1 receptor internalization further underscores the ineffectiveness of opioids (Marvizon et al, 1999). We conclude that NK-1 receptor signaling is only slightly reduced under conditions of profound opioid analgesia.

Importantly, we provide new evidence that NK-1 receptor internalization is indeed a reliable and quantifiable indicator of the extent of NK-1 receptor activation. Thus, SP-induced changes in intracellular calcium concentration, which provide a direct measure of the second messenger signaling that is thought to underlie NK-1 receptor actions, were highly correlated with the magnitude of NK-1 receptor internalization. This was the case whether the number of NK-1 receptor-containing endosomes per neuron or the percentage of cells containing greater than a threshold number of endosomes was quantified.

Although this internalization cannot discriminate between the effects of SP and NKA (Maggi and Schwartz, 1997), it allows measurement with cellular resolution and can be performed without prior surgical manipulation, a procedure that itself must induce tachykinin release.

In light of the extensive literature demonstrating decreases in SP release, the minimal effects of opioids on dorsal horn tachykinin signaling that we observed were surprising. These differing results, however, are readily reconciled. Because bound SP is internalized along with the NK-1 receptor (Bunnett et al, 1995), those studies that assayed extracellular SP concentrations only measured SP that was not receptor-bound. In other words, studies of the extracellular concentrations of SP monitor only peptide that is in excess of that necessary for receptor activation and internalization. Similarly, the magnitude of NK-1 receptor internalization does not provide a measure of the total amount of SP released. Instead, and in contrast to traditional release studies, NK-1 receptor internalization provides a measure of the amount of SP that interacts with an NK-1 receptor (Fig. 11a).

According to this reasoning opioid effects might have the following consequences: if morphine were to reduce SP release to a level just sufficient to activate all nearby receptors, then NK-1 receptor internalization would be saturated and thus SP effects on target neurons would still be maximal. However, concurrently, the amount of excess extracellular SP would decrease tremendously. Thus, small alterations of the amount of SP that is released might have little effect on the resulting activation of lamina I neurons, but could greatly affect the levels of SP collected from CSF (See Fig. 11b). In support of this hypothesis, we found that a combination of a low dose of the NK-1 receptor

antagonist GR 205171 and morphine decreased NK-1 receptor internalization to a greater extent than did either drug alone. We suggest that addition of an ineffective dose of NK-1 receptor antagonist uncovered the relatively large decrease in total SP released.

Opioid regulation of SP release during inflammation

Behavioral studies have shown that morphine is more effective in rats with a persistent inflammation of the hindpaw (Colpaert, 1979; Kayser and Guilbaud, 1983). This inflammation is associated with an increase in SP and NK-1 receptor levels in primary afferents and in spinal cord (Hanesch et al, 1993; Donnerer et al, 1993; Mapp et al, 1993; Schafer et al, 1993; Abbadie et al, 1996). We have also observed increased noxious stimulus-evoked internalization of the NK-1 receptor in lamina I neurons in the setting of persistent injury (Abbadie et al, 1997). Together these results suggest that opioid regulation of SP release may be more effective and relevant under inflammatory conditions. To address this possibility, we repeated the NK-1 receptor internalization studies in CFA-treated animals. Rather than having an increased efficacy in this condition, morphine was even less able to decrease NK-1 receptor internalization during inflammation. The NK-1 receptor antagonist GR 205171 also produced less of a reduction.

Although this result suggests that the increased analgesic efficacy of opioids in an inflammatory model is not due to enhanced regulation of tachykinin signaling, the decreased effect of morphine and GR 205171 may be explained by the upregulation of SP and the increase in SP release that characterizes this model. Because GR 205171 is a competitive antagonist,

increased release of SP from primary afferents should reduce the activity of a given dose of NK-1 receptor antagonist. It would also make opioid-induced decreases in release more difficult to detect. That is, if the response had saturated, a small opioid effect would be lost. Finally, an inflammation-induced expression of SP in large diameter sensory neurons, which do not express mu opioid receptors (Neumann et al, 1996), would also make detection of opioid inhibitory effects less likely. Based on our results, we conclude that although opioids can reduce SP release, this is probably not a major mechanism by which opioids produce analgesia. We suggest that an opioid action on lamina II interneurons or non-SP containing primary afferent terminals is likely to be more important for production of spinal opioid analgesia.

Functional consequences of activation of NK-1 receptor-containing neurons

We previously reported that morphine does not decrease noxious stimulus-evoked Fos expression in lamina I spinoparabrachial neurons (Jasmin et al, 1994) and suggested that signaling via the spinoparabrachial pathway is largely unchanged under conditions of opioid analgesia. It is thus of interest that a large percentage (70%) of lamina I neurons that project to the parabrachial nucleus express the NK-1 receptor (Ding et al, 1995). Given the extensive overlap between these populations, our results provide a possible explanation for the fact that this pathway is refractory to opioid inhibition. Specifically, it is possible that the preservation of Fos expression in lamina I projection neurons reflects the fact that opioids do not sufficiently reduce the SP input to these neurons. Thus, continued release of SP (and probably other neurotransmitters) could maintain

activity in this pathway, even under conditions in which other neurons are inhibited.

Clinical relevance

In a recent study, we reported that mice lacking the preprotachykinin gene showed behavioral deficits in tests of nociception only when intense mechanical, thermal or chemical test conditions were used (Cao et al, 1998). Previous studies that detected opioid-mediated decreases in SP release also used extreme stimuli, for example 47 mM KCl in slices (Jessell and Iversen, 1977), bilateral sciatic nerve stimulation or i.t. capsaicin (Yaksh et al, 1980). Our finding that only highly noxious stimuli induce NK-1 receptor internalization in the spinal cord of the normal rat suggests that tachykinins are only released under such conditions (Abbadie et al, 1997). Because morphine is not particularly effective in blocking pain produced by the highly noxious acute stimuli that we found are required to promote SP release, but is effective against most clinically encountered pains, we suggest that SP is not involved in pain conditions most sensitive to morphine treatment. It follows that the inability of morphine to prevent NK-1 receptor signaling induced by the stimuli that release SP may underlie its ineffectiveness against these types of pain. This hypothesis may also explain the ineffectiveness of NK-1 receptor antagonists as analgesics in clinical trials; the pain conditions tested may not involve significant SP-induced activity.

Given that lamina I NK-1 receptor neurons contribute to the transmission of nociceptive messages and that morphine is relatively ineffective at reducing their activity, NK-1 receptor antagonists may be useful as adjunct therapies with morphine, to control severe acute pain conditions that are refractory to morphine

treatment. This possibility is supported by the observation of increased potency of opioids in SP/NKA KO mice in tests of nociception in which SP was shown to be required (Cao et al, 1998). Of course, any compound that would sufficiently inhibit NK-1 receptor mediated activity in lamina I would also be useful. As noted above, the GABA B receptor agonist, baclofen, and NMDA receptor antagonists, respectively reduce NK-1 receptor internalization under *in vivo* and *in vitro* conditions (Riley et al, 1997; Marvizon et al, 1997, Marvizon et al, 1999). Although the mechanism through which these compounds regulate the release of SP from primary afferents terminals differs (Teoh et al, 1996; Liu et al, 1997) the result on the magnitude of NK-1 receptor internalization is similar, indicating that these compounds should also prevent tachykinin-mediated "pain" transmission and thus improve opioid analgesia.

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Table 1. Effects of morphine on double-labeled cells (Fos, NK-1 receptor)

groups	n	total Fos	Fos in NK-1 R negative cells	Fos in NK-1 R positive cells	NK-1 R positive and Fos negative cells
SP i.t. + saline	5	57.1 ± 4.3	41.4 ± 3.3	15.7 ± 1.6	4.3 ± 3.1
SP i.t. + morphine	4	57.9 ± 9.9	39.9 ± 11.7	19.9 ± 4.5	2.2 ± 1.3

Figure 1: These confocal images (A-F) illustrate the effect of morphine on noxious stimulus-evoked internalization of the NK-1 receptor in NK-1 receptor immunoreactive neurons. Each figure is from sagittal sections through lamina I of the L4 segment of the spinal cord. The confocal images were taken through the center of neurons that express the NK-1 receptor. In all examples, the noxious stimulus was a 15 s pinch of the hindpaw. A-C: saline pretreatment; D-F: morphine pretreatment.

A: Contralateral to the noxious stimulus in a rat that received i.t. saline. The NK-1 receptor-LI is localized to the plasma membrane, indicating that internalization had not occurred.

D: Contralateral to the noxious stimulus in a rat that received i.t. morphine. There is no NK-1 receptor internalization. By contrast, in B, C, E and F, there is extensive NK-1 receptor-LI in endosomes in the cytoplasm indicating extensive internalization. B: ipsilateral in a rat that received i.t. saline; C: ipsilateral in a rat that received s.c. saline; E: ipsilateral in a rat that received i.t. morphine (30 µg); F: ipsilateral in a rat that received s.c. morphine (10 mg/kg). Note that the magnitude of NK-1 receptor internalization in individual cells (number or brightness of endosomes) was not altered by morphine treatment. Calibration bar in D equals 20 µm.

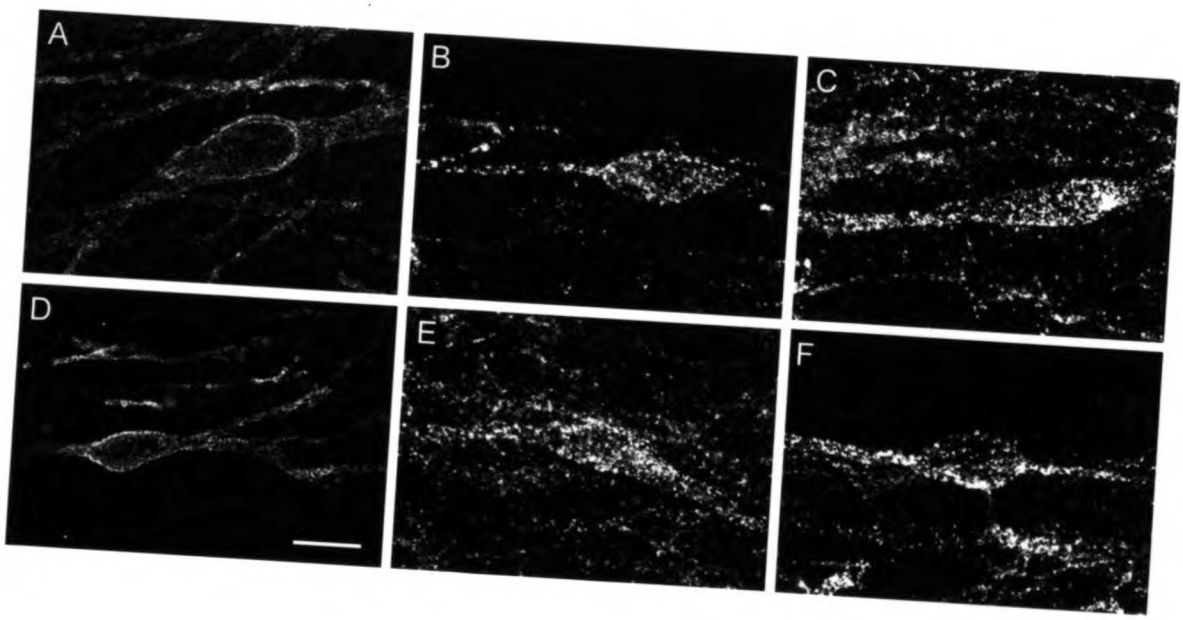

A
B
C
D
E
F

Figure 2: These graphs illustrate the effects of morphine on the percentage of neurons that contain internalized NK-1 receptor in lumbar segments L2-L6 after mechanical (A, B) or thermal (C, D) stimulation of the hindpaw in normal rats (A, C) and in rats with inflammation (B, D). Saline or morphine (10 mg/kg s.c.) was administered prior to the stimulation. Results are expressed as mean +/- S.E.M. for each group. Significance is expressed with reference to the saline group, using PLSD Fisher's test (*$p<0.05$), n=4. Note that: (1) morphine did not affect the number of internalized neurons induced by mechanical stimulation in normal rats (A), or CFA-treated rats (B). (2) morphine had no effect on the number of internalized neurons induced by thermal stimulation in normal rats (C), but significantly decreased the number of internalized neurons in CFA-treated rats at the L2 and L3 segments (D).

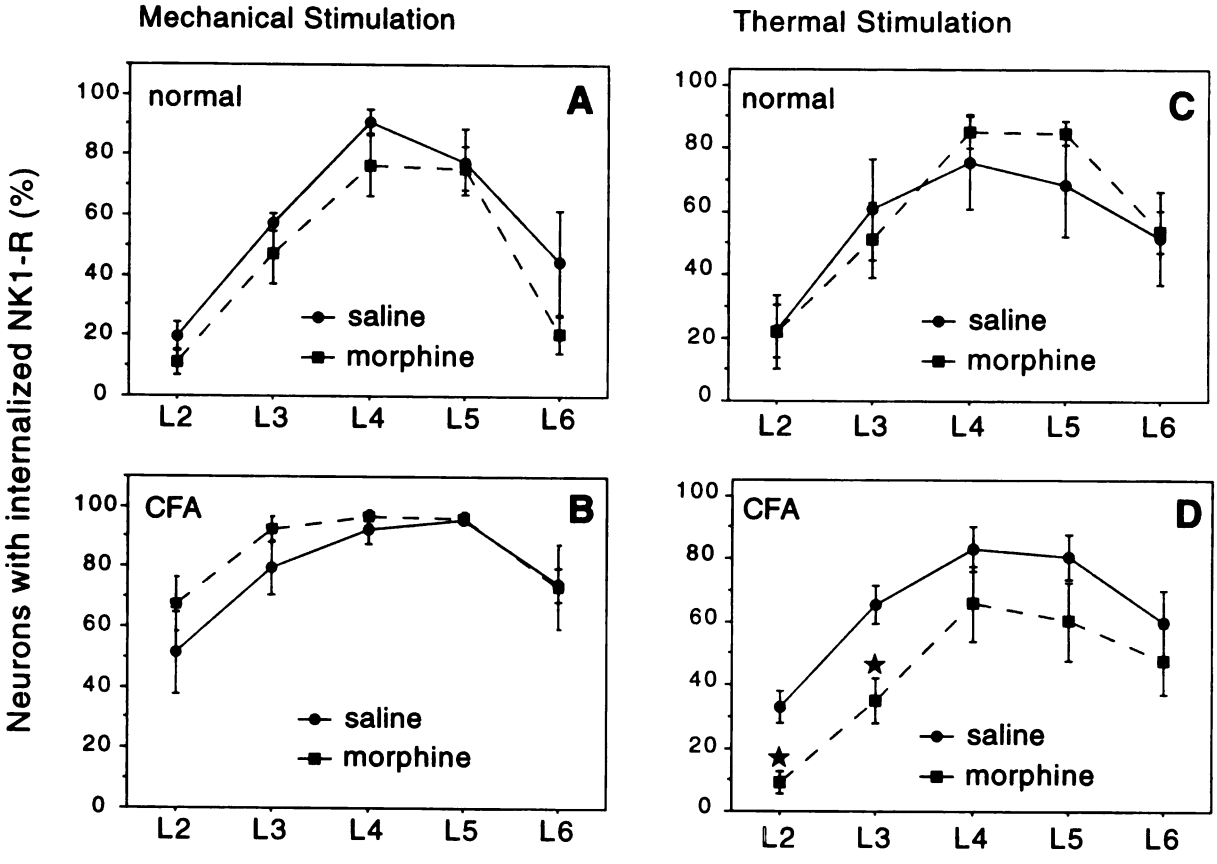

Mechanical Stimulation
Thermal Stimulation
Neurons with internalized NK1-R (%)
normal
A
saline
morphine
CFA
B
normal
C
CFA
D
100
80
60
40
20
0
L2
L3
L4
L5
L6

Figure 3: This graph illustrates the effects of intrathecal morphine on the percentage of neurons that contain internalized NK-1 receptor in lumbar segments (L2-L6) after mechanical stimulation of the hindpaw in normal rats. Saline (n=8), 10 μg i.t. (n=4) or 30 μg i.t. (n=5) morphine was administered prior to the stimulation. Results are expressed as mean +/- S.E.M. for each group. Morphine slightly but significantly decreased (by about 20%) the number of NK-1 receptor internalized cells, but there was no difference between the 10 or 30 mg dose.

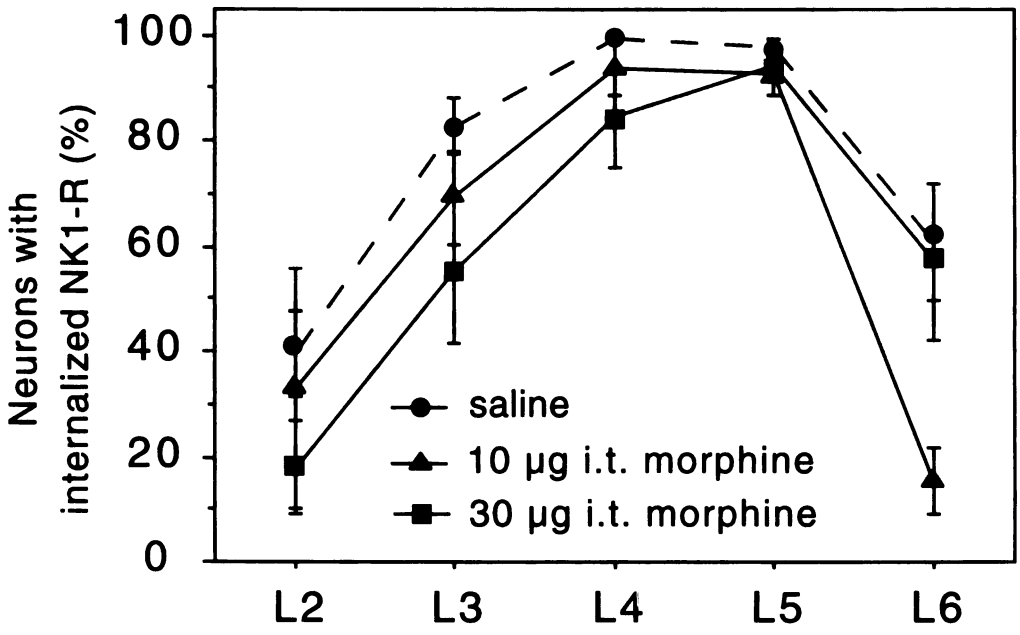

Neurons with internalized NK1-R (%)
100
80
60
40
20
0
L2
L3
L4
L5
L6
saline
10 µg i.t. morphine
30 µg i.t. morphine

Figure 4: This graph illustrates the effects of selective mu, delta or kappa opioid receptor agonists on the percent of neurons that contain internalized NK-1 receptor in lumbar segments L2-L6 after mechanical stimulation of the hindpaw in normal rats. Saline or a selective opioid agonist (1.0 mg i.t. DAMGO, mu opioid receptor agonist; 30 mg i.t. DPDPE, delta opioid receptor agonist; 100 mg i.t. U50488H, kappa opioid receptor agonist) were administered prior to the stimulation; n=5 in all groups. Results are expressed as mean +/- S.E.M. for each group. Significance is expressed with reference to the saline group, using PLSD Fisher's test (*$p<0.05$). Only DAMGO significantly decreased the number of NK-1 receptor internalized neurons.

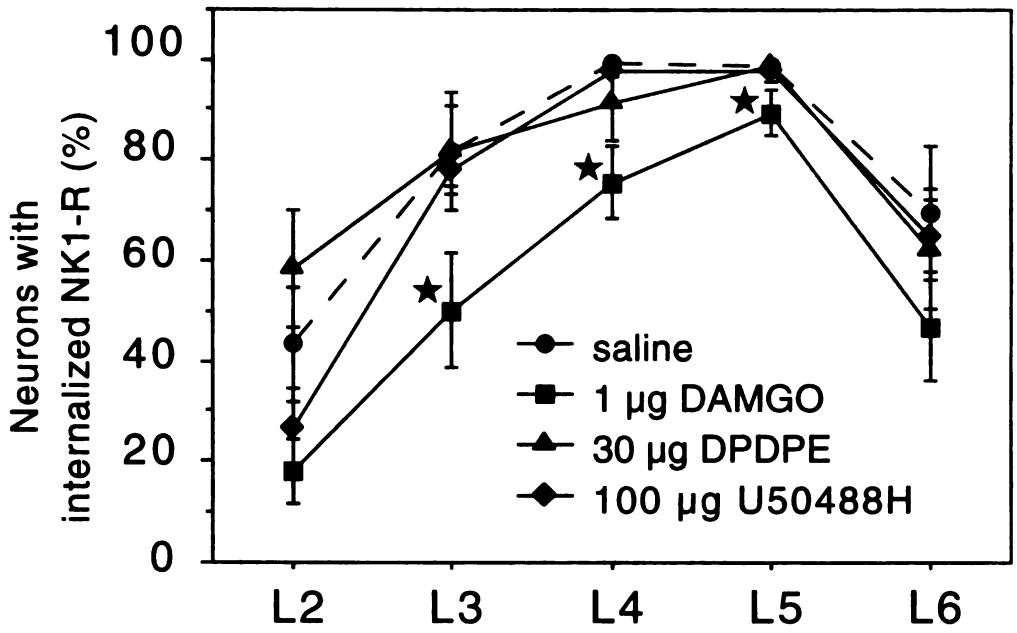

Neurons with internalized NK1-R (%)
100
80
60
40
20
0
L2
L3
L4
L5
L6
saline
1 µg DAMGO
30 µg DPDPE
100 µg U50488H

Figure 5: This graph illustrates the effects of the NK-1 receptor antagonist GR 205171 alone or in combination with morphine on the percentage of neurons that contain internalized NK-1 receptor in lumbar segments L2-L6 after mechanical stimulation (pinch for 15 sec) of the hindpaw in normal rats. Saline or GR 205171, with or without morphine, was administered prior to the stimulation; n=5 in all groups. Results are expressed as mean +/-S.E.M. for each group. Significance is expressed with reference to the saline group, using PLSD Fisher's test (*$p<0.05$). GR 205171 (10 mg/kg) significantly reduced the number of internalized cells. A lower dose of GR 205171 (1.0 mg/kg) or morphine (10 mg/kg) given alone had no significant effect. However, GR 205171 (1.0 mg/kg) in combination with morphine (10 mg/kg) significantly reduced the number of internalized neurons evoked by the mechanical stimulation.

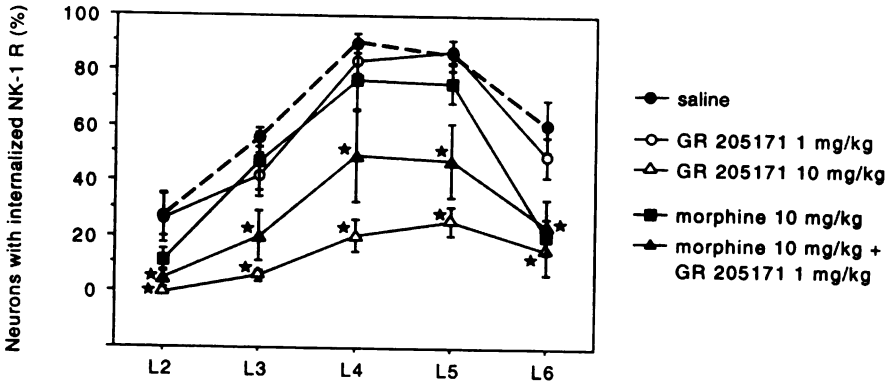

Neurons with internalized NK-1 R (%)
100
80
60
40
20
0
L2
L3
L4
L5
L6
saline
GR 205171 1 mg/kg
GR 205171 10 mg/kg
morphine 10 mg/kg
morphine 10 mg/kg +
GR 205171 1 mg/kg

Figure 6: These confocal images of NK-1 receptor labeling in lamina I illustrate the decrease in internalization after systemic injection of the NK-1 receptor antagonist GR 20517: A,D) no stimulus; B,E) pinch 15 sec; C,F) pinch with 10 mg/kg s.c. GR 205171. White arrows and white arrowheads indicate membrane and endosomal labeling, respectively. Note the qualitative difference in NK-1 receptor labeling in neurons showing internalization in the presence of GR 205171; there is a decrease in endosome size and number and there is residual membrane labeling. Calibration bars: in A equals 50 μm; (for A-C); in D equals 20 μm (for D-F).

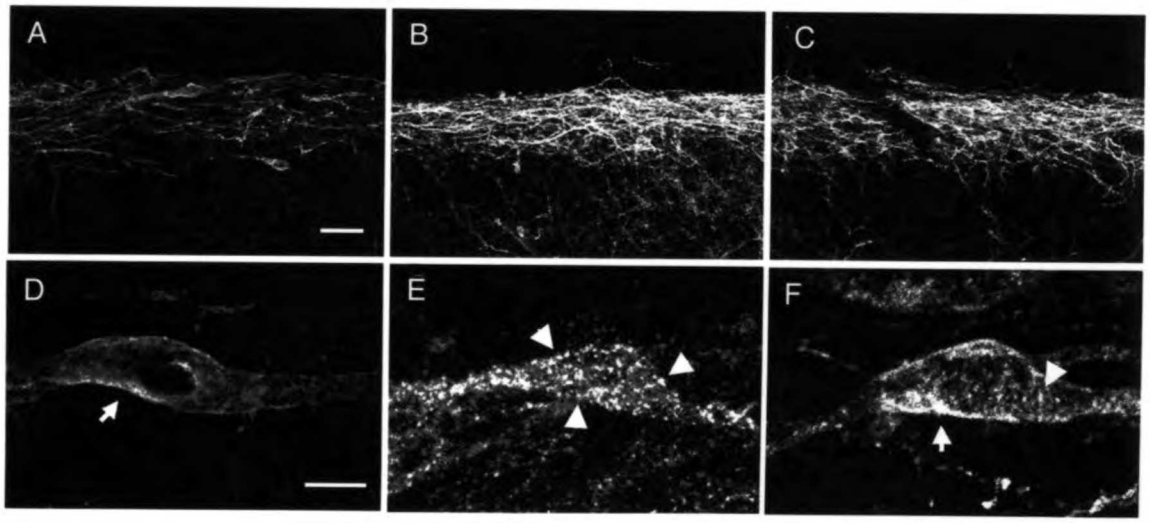

A
B
C
D
E
F

Figure 7: This graph illustrates the effects of the NK-1 receptor antagonist, GR 205171 alone or in combination with morphine on the percent of neurons that contain internalized NK-1 receptor in lumbar segments L2-L6 after mechanical stimulation (pinch for 15 sec) of the hindpaw in CFA-treated rats. Saline or GR 205171, with or without morphine, was administered prior to the stimulation; n=5 in all groups. Results are expressed as mean +/- S.E.M. for each group. Significance is expressed with reference to the saline group, using PLSD Fisher's test (*$p<0.05$). GR 205171 (10 mg/kg) significantly decreased the number of internalized cells in lamina I (A) In laminae III-VI (B-C) it completely blocked the internalization. Neither GR 205171 (1.0 mg/kg) nor morphine (10 mg/kg) given alone had a significant effect on the number of internalized cells. However, the combination of GR 205171 (1.0 mg/kg) and morphine (10 mg/kg) significantly decreased internalization in neurons of the L2 and L3 segments.

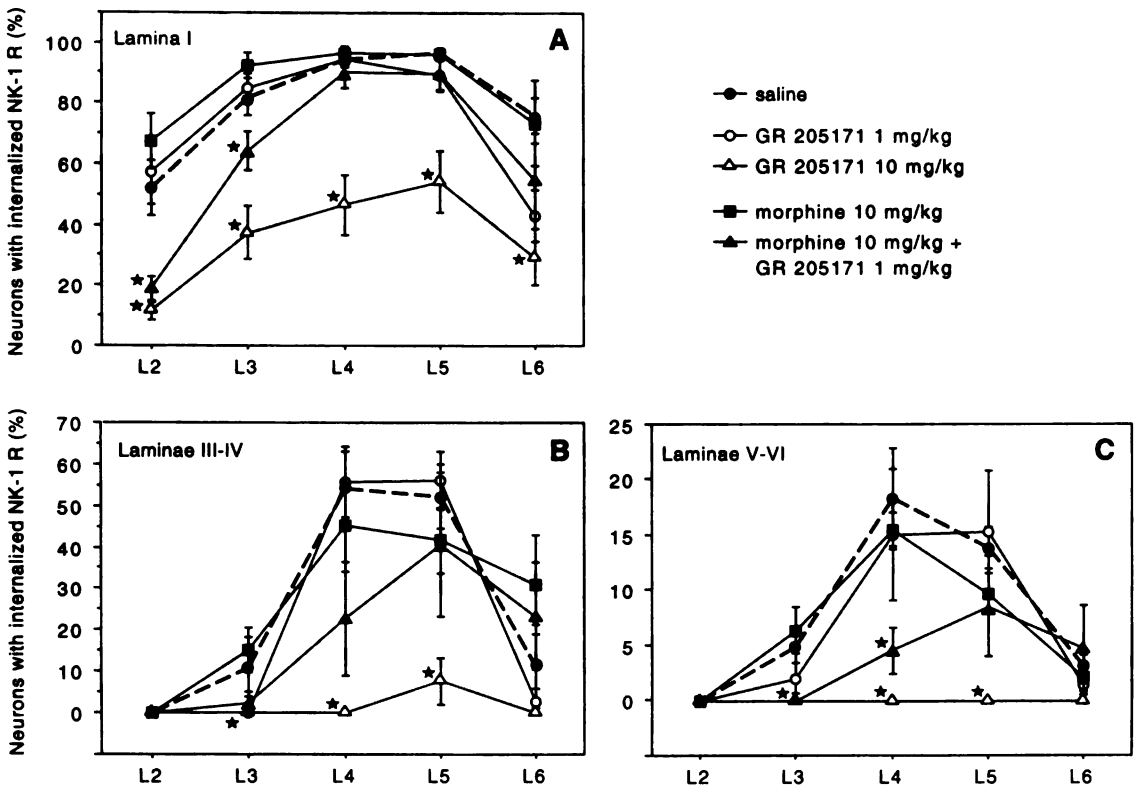

Lamina I
A
Neurons with internalized NK-1 R (%)
100
80
60
40
20
0
L2
L3
L4
L5
L6
saline
GR 205171 1 mg/kg
GR 205171 10 mg/kg
morphine 10 mg/kg
morphine 10 mg/kg +
GR 205171 1 mg/kg
Laminae III-IV
B
70
60
50
40
30
20
10
0
Laminae V-VI
C
25
20
15
10
5
0

Figure 8: These graphs illustrate the dose response relationship between SP concentration and increases in intracellular calcium concentration and NK-1 receptor internalization in primary spinal cord cultures. A) Average 340/380 nm fluorescence ratio in Fura-2 AM-loaded primary spinal cord cultures during the application of varying doses of SP. Only cells showing average increases in 340/380 ration that were at least twice the average baseline value are included in this graph; n=4-8 coverslips. There is a significant effect of SP dose on the 340/380 ratio (ANOVA: p= 0.0258). B) Percent of maximal possible effect for both the number of NK-1 receptor positive endosomes observed and the total calcium influx observed in the first 40 sec with application of varying concentrations of SP. The maximal number of endosomes/neuron observed was 22.65; untreated cultures contained 6.95 endosomes/neuron (n=4 coverslips). The maximal total increase in 340/380 ratio was 23.78; untreated cultures showed a total increase of 0. C) Percent of maximal possible responders for calcium changes and increases in endosome number. Thresholds were set at 10 endosomes for NK-1 receptor internalization and twice baseline for increases in intracellular calcium. The maximal number of responders for NK-1 receptor internalization was 100%; untreated cultures showed 25% of neurons responding. The maximal number of responders for increases in intracellular calcium was 50.6%; untreated cultures showed 0% responding.

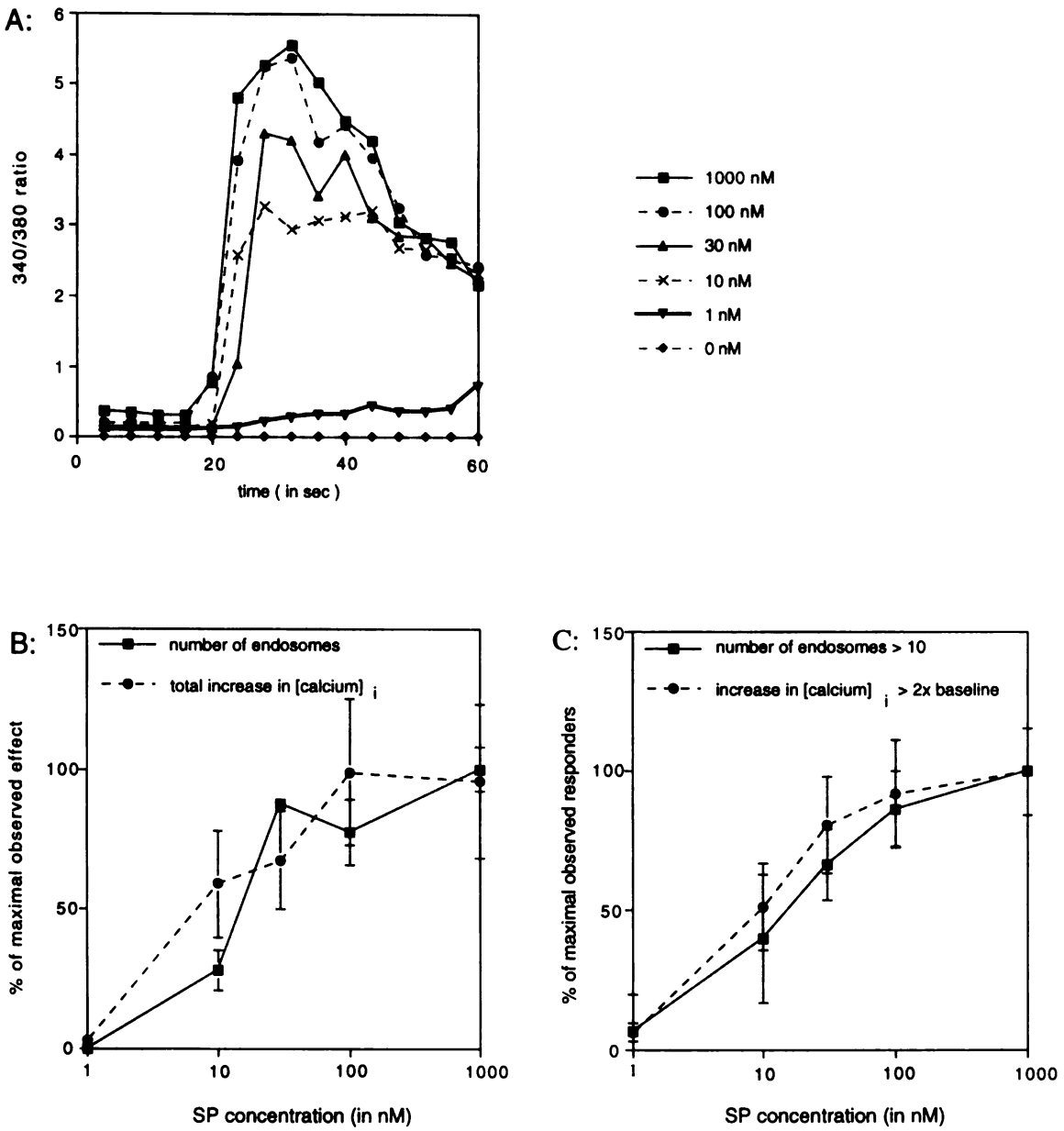

A:
340/380 ratio
time (in sec)
1000 nM
100 nM
30 nM
10 nM
1 nM
0 nM
B:
number of endosomes
total increase in [calcium] i
% of maximal observed effect
SP concentration (in nM)
C:
number of endosomes > 10
increase in [calcium] i > 2x baseline
% of maximal observed responders
SP concentration (in nM)

Figure 9: These photomicrographs illustrate the effect of morphine on noxious stimulus-evoked expression of Fos-like immunoreactivity in NK-1 receptor immunoreactive neurons in lamina I. Each figure is from sagittal sections of the lumbar spinal cord. In all examples, the noxious stimulus was a 15 s pinch of the hindpaw.

A-F: Double labeling for Fos-LI (black nuclei) and for NK-1 receptor-LI (gray cytoplasm in cell bodies and dendrites). A, B and C: rats that received s.c. saline; D, E and F: rats that received s.c. morphine. After morphine, the number of Fos-LI neurons decreased significantly (D). The effect of morphine on the number of double-labeled (Fos and NK-1 receptor-LI) neurons was less pronounced (E,F). Calibration bars: in D equals 50 μm; (for A,B,D,E); in F equals 20 μm (for C and F).

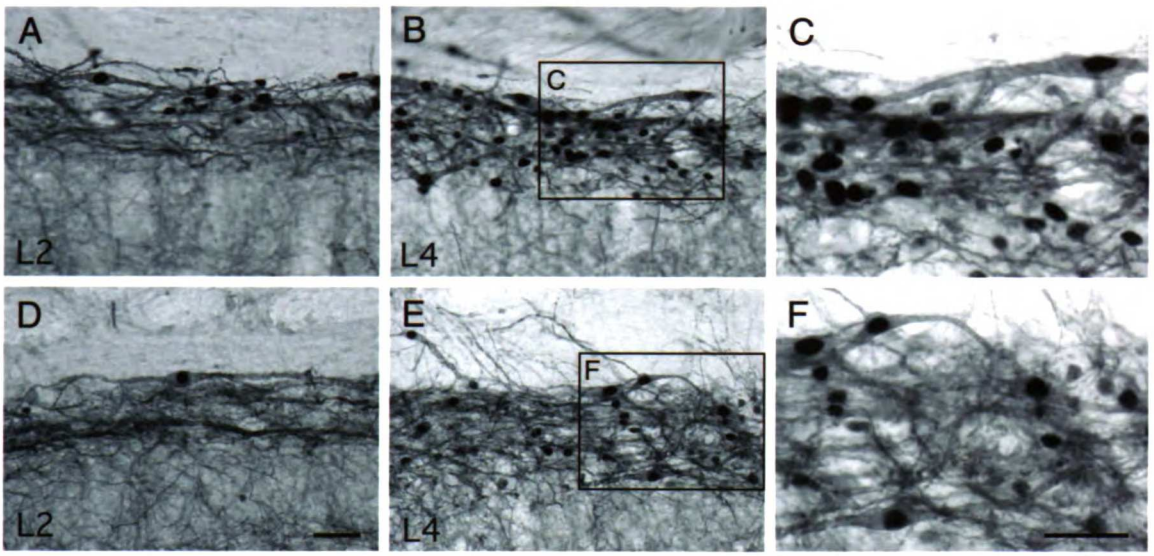

A
L2
B
C
L4
C
D
L2
E
F
L4
F

Figure 10: These graphs illustrate the effects of morphine on Fos-like immunoreactivity in neurons of lamina I in lumbar segments L2-L6 after mechanical stimulation of the hindpaw in normal rats. Saline or morphine was administered prior to the stimulation; n=5 in all groups. Results are expressed as mean +/- S.E.M. for each group. Significance is expressed with reference to the saline group, using PLSD Fisher's test (*$p<0.05$). A: number of Fos-LI nuclei in neurons that are not NK-1 receptor-LI. B: number of Fos-LI nuclei in neurons that are NK-1 receptor-LI. Note that there is a greater decrease in Fos-LI neurons that are not NK-1 receptor-LI in the lumbar segments L4-L5. These segments also contain a high percentage of neurons with NK-1 receptor internalization after mechanical stimulation.

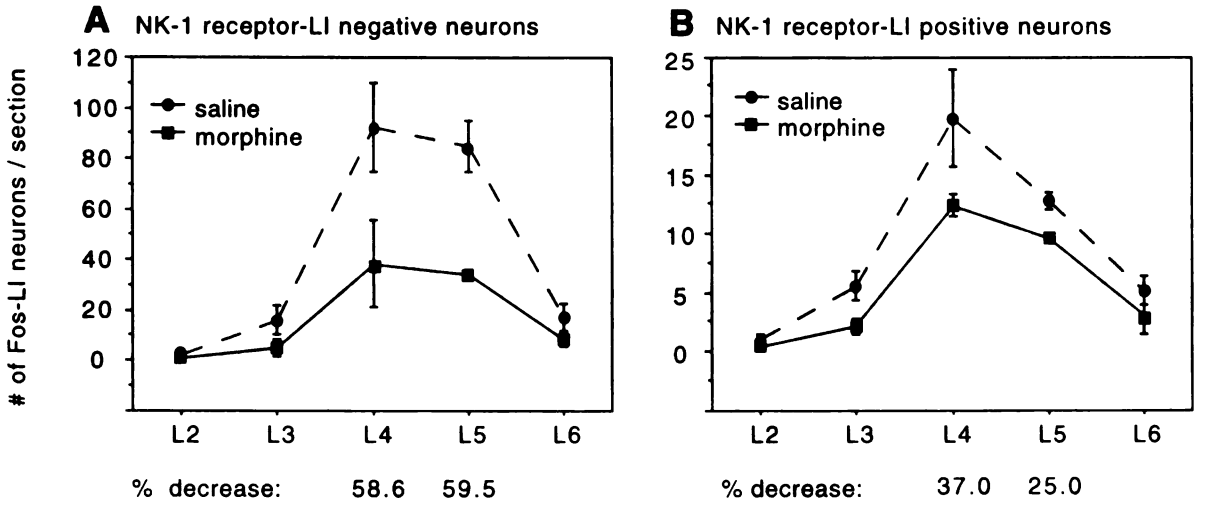
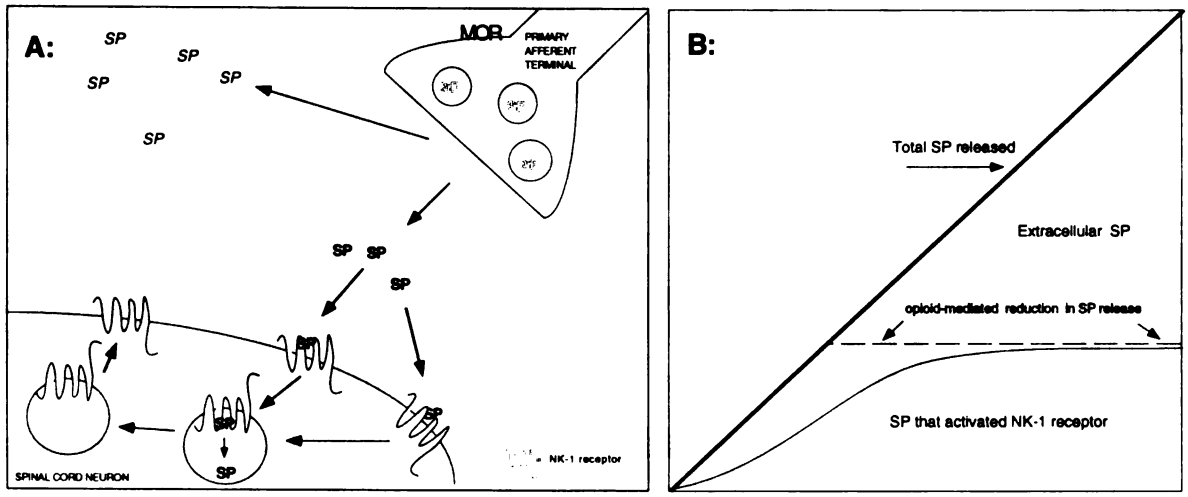

A
NK-1 receptor-LI negative neurons
# of Fos-LI neurons / section
120
100
80
60
40
20
0
saline
morphine
L2
L3
L4
L5
L6
% decrease:
58.6
59.5
B
NK-1 receptor-LI positive neurons
25
20
15
10
5
0
saline
morphine
L2
L3
L4
L5
L6
% decrease:
37.0
25.0

Figure 11: A) Substance P (SP) is contained in dense core vesicles in terminals of small diameter primary afferents and in some spinal cord interneurons. Following noxious stimulation, SP is released. Some percent of the released substance P (**SP**) diffuses to target cells, where it interacts with and activates NK-1 receptors. This substance P is internalized along with the NK-1 receptor into endosomes. Acidification of these endosomes dissociates the **SP** from the NK-1 receptor. The **SP** is degraded, and the NK-1 receptor is recycled to the membrane. Unbound Substance P (*SP*) diffuses into the extracellular space and eventually into the CSF where it may be broken down by endopeptidases.

Previous release studies measured the amount of substance P in the CSF or extracellular space (*SP*). Because the **SP** that binds and activates the NK-1 receptor is degraded intracellularly, these studies measured essentially the overflow of substance P, i.e. the released content of the peptide that did *not* have a postsynaptic effect. In contrast, we take advantage of the fact that activated NK-1 receptors internalize with their associated ligand. This provides a measure of the amount of released peptide that has a functional, postsynaptic effect via the NK-1 receptor (**SP**).

B) The total amount of SP released can be divided into two measurable pools: SP that diffuses extracellularly and SP that binds the NK-1 receptor and is internalized. The amount of SP that enters each of these two pools for any given amount of SP released is not known. However, the NK-1 receptor binding SP component is saturable; the extracellular SP pool is not. The consequences of this difference are depicted here. Changes in extracellular SP are greatest and most easily detectable when the NK-1 receptor binding pool is saturated. If there are

no further NK-1 receptor sites to activate, however, these changes in SP release will have no effect on SP-mediated postsynaptic signaling in the spinal cord dorsal horn. We have illustrated a hypothetical opioid-mediated reduction of the extracellular contect of SP that can occur (dashed line) without an observable effect on NK-1 receptor-mediated signaling.


A:
SP
SP
SP
SP
SP
MOR
PRIMARY AFFERENT TERMINAL
SP SP
SP
SP
SP
SP
SP
SPINAL CORD NEURON
= NK-1 receptor
B:
Total SP released
Extracellular SP
opioid-mediated reduction in SP release
SP that activated NK-1 receptor

FURTHER CONCLUSIONS, DISCUSSION AND QUESTIONS

We conclude that despite the ability of opioids to partially reduce the release of tachykinins from primary afferents, they do not decrease tachykinin signaling via the NK-1 receptor in the dorsal horn. This conclusion holds for both normal and inflammatory conditions. This inability to reduce tachykinin signaling is supported by the persistence of noxious stimulus-induced Fos expression in NK-1 receptor-positive lamina I neurons in the presence of opioids. We conclude that inhibition of tachykinin signaling at the level of the spinal dorsal horn is not the mechanism by which opioids produce analgesia and that the persistent tachykinin signaling may even underlie the fact that opioids do not provide adequate analgesis for certain pain conditions

Several questions remain. Although we found that tachykinin activation of lamina I NK-1 receptor expressing neurons is persists during opioid analgesia, the functional relevance of this ongoing signaling is not established. Huang et al (1993) reported that morphine inhibits noxious stimulus-evoked activity in the lateral parabrachial nucleus, which is the major target of NK-1 receptor expressing lamina I projection neurons. It is possible that opioids simply inhibit the residual nociceptive signaling at a more rostrad point in the along the spinoparabrachial pathway; this could occur in the parabrachial nucleus or more rostrally, in the amygdala . Indeed analgesia has been produced by direct opioid injections into the amygdala (Rodgers, 1997; Rodgers, 1978). If this is true then the persistence of NK-1 receptor activity is irrelevant under conditions of systemic opioid analgesia. Such residual tachykinin mediated activity may, however, be of importance during endogenous opioid or spinal opioid analgesia.

The increased effectiveness of morphine as an analgesic seen in mice lacking the preprotachykinin-A gene suggests that this remaining signaling may be of importance behaviorally, yet further study is necessary to determine the precise consequences.

It is also of interest that release of dense core vesicles may be regulated differentially from general synaptic vesicles (Tandon et al, 1998). This raises the possibility that opioids, despite their inability to inhibit tachykinin release to a relevant extent, might reduce glutamate release sufficiently to significantly dampen signaling from these same tachykinin -containing primary afferents. Marvizon et al (1997; 1999) have found evidence for strict regulation of neuropeptide release from substance P containing primary afferents by both NMDA and GABA B receptors. They suggest that NMDA receptor activation is necessary to overcome tonic inhibition of neuropeptide release at these terminals by GABA B receptors. It is possible that opioids are also not capable of inhibiting neurotransmitter release mediated via NMDA receptor activation. In this case, opioids might greatly reduce the glutamate release that occurs to more normal low intensity, low frequency activation of these primary afferents, but be of little use against the high intensity/frequency activation that brings into play NMDA receptors. Such a mechanism might function to keep an animal from becoming so analgesic as to become a danger to itself; still feeling the pain of tissue damage, but unaware of the unrealized danger of stimuli which are only potentially damaging. Further study is necessary to determine if opioids indeed preferentially reduce glutamatergic mediated nociceptive processing.

Huang GF, Besson JM, Bernard JF. (1993) Morphine depresses the transmission of noxious messages in the spino(trigemino)-ponto-amygdaloid pathway. Eur J Pharmacol 230:279-84.

Marvizon JC, Martinez V, Grady EF, Bunnett NW, Mayer EA. (1997) Neurokinin-1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. J Neurosci 17: 8129-8136.

Marvizon JC, Grady E, Stefani E, Bunnett N, Mayer E. (1999) Substance P release in the dorsal horn assessed by receptor internalization: NMDA receptor counteract a tonic inhibition by GABA(B) receptors. Eur J Neurosci 11: 417-426.

Rodgers RJ. (1977) Elevation of aversive threshold in rats by intra-amygdaloid injection of morphine sulphate. Pharmacol Biochem Behav 6:385-390.

Rodgers RJ. (1978) Influence of intra-amygdaloid opiate injections on shock thresholds, tail-flick latencies and open field behaviour in rats. Brain Res 153: 211-216.

Tandon A, Bannykh S, Kowalchyk JA, Banerjee A, Martin TF, Balch WE. (1998) Differential regulation of exocytosis by calcium and CAPS in semi-intact synaptosomes. Neuron 21:147-54.

Chapter 4

Post-synaptic MOR activation in lamina II of the spinal cord dorsal horn

Another hypothesis to explain the analgesic action of spinal opioids has been built around a population of interneurons in lamina II inner which have been shown to express the mu opiate receptor. Electrophysiological studies have shown that iontophoresis of opiates into the substantia gelatinosa can selectively inhibit firing of lamina V projection neurons excited by noxious stimulation to a greater extent than seen with direct application of opioids on the lamina V neuron itself. (Duggan, 1976; Duggan, 1977). This result is consistent with the hypothesis that opioids may inhibit projection neurons through the action of a MOR containing lamina II interneuron, although, as stated by the authors, it is equally consistent with an action on primary afferent fibers that terminate there. Unfortunately, due to the difficulty in recording from small interneurons and the lack of anatomical mapping of the substantia gelatinosa, little is known about the inputs, projections, or neurochemistry of these MOR containing neurons and thus one may only speculate as to how they might modulate activity driven by noxious stimulation. A study by Price et al. (1979) showed that some lamina II interneurons receive nociceptive input from primary afferent fibers, and fire previous to lamina I neurons whose larger receptive fields overlap those of the lamina II interneurons. Based on these data, it was speculated that lamina II cells might act as excitatory interneurons that relay noxious information to lamina I cells. Given the ability of opioids to inhibit some substantia gelatinosa neurons (Grudt & Williams, 1994; Yoshimura, 1983), the simplest hypothesis would predict that MOR containing neurons excite lamina I

projection neurons and are inhibited by opiates. This remains to be shown, however.

Despite significant study of the effects of exogenous opioids on this neuronal population, little is known about when and how these lamina II MORs are activated in vivo. There are numerous sources of endogenous opioids, all of which have been hypothesized to contribute to nociceptive processing in the spinal cord. The three most likely potential sources of endogenous opioid ligands for these receptors are from 1) release of primary afferent derived endomorphins 2) supraspinally triggered spinal opioid release from an unspecified source or 3) release of enkephalins from lamina II inhibitory interneurons. Evidence to date is consistent with any of these mechanisms being involved in anti-nociception. In the following studies, the contribution of each of these sources to lamina II MOR activation is examined.

Using MOR internalization to visualize the activation of MORs on this population of lamina II interneurons, I have attempted to determine under what conditions these receptors function in vivo. Using stimulation paradigms suggested to result in opioid release from the above mentioned sites, I have examined the contribution of post-synaptic MOR activation to anti-nociception under each of these conditions. By gaining knowledge of the endogenous function of these lamina II MORs, we should gain a better understanding of not only how opioids produce analgesia in the spinal cord, but also how nociceptive input is modulated in vivo.

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Post-synaptic Activity of the MOR in the Spinal Cord: Response to Exogenous Opioids and Noxious Stimulation

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ABSTRACT

While both pre- and post-synaptic mechanisms have been proposed to explain how mu opioids produce analgesia at the spinal cord, it is not known under what conditions these each come into play. As the mu opioid receptor can be visualized in individual lamina II excitatory interneurons and is known to internalize into endosomes upon ligand binding, we tested whether MOR internalization could be monitored and used to indicate the extent of post-synaptic MOR activity that had recently occurred. Subsequently, we examined post-synaptic MOR internalization following nociceptive events to test whether endogenous opioids modulate these lamina II interneurons during noxious stimulation.

Here we show that MOR internalization occurs in response to opioid ligands in the spinal cord as observed in other systems. Moreover, MOR internalization is dose dependent with a similar dose response to that observed for opioid-induced potassium conductance. We demonstrate that MOR internalization in lamina II neurons correlates precisely with the extent of analgesia produced by intrathecal DAMGO. These results suggest that MOR internalization provides a good marker for MOR activity in the spinal cord, and that post-synaptic MORs on lamina II interneurons likely participate in the analgesia produced by exogenous opioids. We found, however, that noxious stimuli, under normal or inflammatory conditions, did not induce MOR internalization. Thus, endogenous enkephalins and endomorphins, thought to be released during noxious peripheral stimuli, do not modulate nociceptive messages via MORs on lamina II interneurons under these circumstances.

INTRODUCTION

Although the ability of spinally-administered mu opioids to produce analgesia is well documented, the mechanisms through which these opioids act are not fully understood. The mu opioid receptor (MOR) is expressed by primary afferent nociceptors that terminate in lamina I and II of the dorsal horn and glutamatergic interneurons in lamina II (Arvidsson et al, 1995a). Based on this distribution and on functional studies of opioid induced activity, two major mechanisms for producing opioid analgesia have been proposed, namely presynaptic inhibition of neurotransmitter release from primary afferent nociceptors and postsynaptic hyperpolarization of excitatory interneurons. Unclear, however, are the conditions when these two mechanisms come into play.

Like other G-protein coupled receptors, the MOR internalizes into endosomes in vitro and in vivo upon exposure to receptor agonists (Arden et al, 1995; Sternini et al, 1996). Because receptor internalization is agonist dependent, it has been suggested that internalization of seven-transmembrane domain receptors can be used as a marker of their activity (Mantyh et al, 1995). For example, MOR internalization has been used to document opioid activity induced by estrogen priming and exogenous opioids in hypothalamus and enteric neurons, respectively (Sternini et al, 1996; Eckersell et al, 1998). Due to the small size and punctate appearance of synaptic terminals, light microscopic examination of MOR internalization cannot be used to monitor presynaptic MOR activity. However, it should be possible to observe internalization of the post-synaptic MOR in interneurons in lamina II where membrane and intracellular

labeling are easily distinguishable. Although the relationship between opioid receptor internalization and function is not well known, if MOR internalization is correlated with MOR mediated postsynaptic inhibition, MOR internalization could be used to monitor MOR activation of these interneurons in vivo.

The conditions under which lamina II MORs are activated in vivo are not defined. Nevertheless, there is evidence which suggests that MORs may be endogenously activated in the spinal cord by painful nociceptive stimuli. Neurochemical studies have demonstrated that endogenous enkephalins are released into spinal CSF following repeated noxious stimulation; these could target and activate the MOR in the spinal cord (Le Bars et al, 1987; Bourgoin et al, 1988; Cesselin et al, 1989; Bourgoin et al, 1990). Additionally, the newly discovered mu opioid peptides, endomorphin-1 and endomorphin-2 have been localized in the spinal cord dorsal horn in the terminals of small diameter neuropeptide (SP/CGRP) containing primary afferents (Pierce et al, 1998; Martin-Schild et al, 1998). As these are probably nociceptors, they could provide a source of pain-induced endogenous opioid activity at postsynaptic MORs. To address this question, in the present study, we examined the relationship between MOR internalization in lamina II interneurons and MOR-related activity and behavior. Then, using MOR internalization as a marker of opioid induced activity, we addressed the question of whether postsynaptic MOR activity modulates spinal nociceptive processing under normal and inflammatory conditions.

METHODS

Internalization of lamina II MORs in vivo

To establish that MOR internalization occurs and can be observed in lamina II interneurons, we first looked at the ability of various exogenously applied mu opioid receptor agonists to internalize the MOR in the spinal cord. The following compounds were injected as described:

Agonist application

Intrathecal injection - Rats were anesthetized with halothane and then either DAMGO, morphine or endomorphin-1 was injected in 20 µl of saline with a Hamilton syringe. A 27.5 gauge needle was inserted between the L4/L5 vertebrae above the cauda equina. Intrathecal placement was verified by a flick of the tail upon needle entry.

Systemic injection - morphine was injected subcutaneously (s.c.) at the nape of the neck. In other rats, we injected remifentanil, a short-acting, potent alkaloid mu opioid agonist, into one hindpaw.

Morphine pellet implantation – we implanted 75 mg morphine pellets or equivalently-sized vehicle pellets s.c. at the rear of the flank under halothane anesthesia. We implanted one pellet on day 1, two additional pellets on day 2, and another 3 pellets on day 3. These rats were perfused on day 4.

Perfusion and tissue preparation

Following treatment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and then intracardially perfused with 50 ml 0.1 M

phosphate buffered saline followed by 500 ml 10% formalin. The brain and spinal cord were dissected out, post-fixed for 4 hours in the same fixative and then transferred to a 30% sucrose solution. Thirty micron sagittal sections of lumbar cord were cut on a freezing microtome.

Immunofluorescence labeling

Sections were blocked in 5% normal goat serum in 0.1 M phosphate buffered saline with 0.3% triton x-100 for 30 minutes and then incubated in a rabbit anti-MOR antibody (Incstar, Stillwater, MN) at a 1:5000 dilution overnight. Sections were washed and then incubated in a Cy-3 conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA) at a 1:600 dilution for 2 hours. Tissue was then washed and mounted on gelatin coated slides.

Time Course of MOR recycling

To be certain that stimulus-induced MOR internalization that occurred was detected, we first established the temporal parameters of internalization, namely, the length of time after ligand binding that MOR internalization could be observed. To determine this we injected 1.0 μg of DAMGO intrathecally and then assayed for analgesia 10 minutes following injection using the hot plate test. Only rats showing maximal analgesia (60 sec latency) were used for the study. This ensured that mu opioid receptors were activated by the injection. Immediately following testing the rats were injected with 1.0 mg/kg naloxone s.c., to prevent further activation of the mu opioid receptor and thus mark the latest time at which mu opioid receptor internalization could be induced. Five minutes after the naloxone injection, analgesia was again assayed on the hot

plate test to confirm that naloxone had reversed the increase in latency. Rats were then anesthetized with pentobarbital and perfused intracardially with 10% formalin at 7.5, 15, 30, 60, or 120 minutes after naloxone injection. Tissue was processed for MOR immunoreactivity as above.

Hot plate test

Rats were placed in a plexiglass container in which the floor was heated to 52.5°C. Behavior was monitored and the latency until hindpaw licking was determined. Rats were removed from the hot plate as soon as a hindpaw was licked. Sixty seconds was used as a cut off value.

Quantification of MOR internalization

For the *in vivo* studies, we collected confocal images (Nikon 60 X plan apo (1.40 oil) objective, 2X zoom, 3.0 iris setting on a Biorad MRC 1024) through the optical center of 20 MOR-LI lamina II neurons in the L4/L5 segments of each rat. The number of endosomes in each cell was counted from the images by an investigator who was unaware of the treatment that the rat received. The average number of endosomes per neuron was calculated for each rat. Values are presented as mean +/- standard error of the mean.

Correlation between MOR internalization and MOR-induced hyperpolarization

Although MOR internalization has been shown to require agonist binding, the relationship between MOR internalization and MOR induced activity has not been clearly demonstrated. Because it is difficult to directly measure opioid-

induced internalization and hyperpolarization together, we mimicked the conditions that have been used to study opioid induced hyperpolarization in interneurons of lamina II recorded in slice preparations of the dorsal horn (specifically the medullary dorsal horn) (Grudt and Williams, 1994). We determined dose response curves for MOR internalization using both DAMGO and met-enkephalin, which were the agonists tested in the Grudt and Williams study. EC50s were calculated using PRISM software (GraphPad Software, San Diego, CA) The curve was a sigmoidal dose-response.

P15-23 Sprague Dawley rats were anesthetized with halothane and the lumbar and sacral spinal cord were dissected out. The spinal cord was placed in a bath of carbogenated sucrose buffer (in mM: sucrose 240; KCl 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1.0; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.5) and the dura and dorsal and ventral roots were removed under a dissecting microscope. The cut surface of the lumbar cord was superglued to the stage of a vibratome (Pelco 101 series 1000), placed against an 5% agar block and immersed in carbogenated sucrose buffer. Transverse sections of spinal cord were cut at 500-600 μm and transferred to carbogenated incubation solution (in mM: NaCl 124; KCl 5; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) for 2 hours. After 2 hours, slices were transferred to carbogenated recording solution at 37$^{\epsilon}$C (in mM: NaCl 127; KCl 1.9; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) containing the designated concentration of opioid for 15 minutes. Slices were then fixed overnight in 10% formalin and then transferred to a 30% sucrose solution for several hours. They were next cut at 40 μm on a freezing microtome.. Immunohistochemistry and quantification of internalization were performed as

for the *in vivo* studies except that confocal images were taken and quantified for only 6 MOR-LI lamina II neurons from each slice.

Correlation between MOR internalization and behavioral analgesia

We wished to test the hypothesis that activation and internalization of MORs on lamina II interneurons is necessary for the analgesia produced by intrathecal mu opioids. If this hypothesis is correct, then the extent of MOR internalization in lamina II at the lumbar segments innervated by the stimulated dermatome (in this case L4/L5 for the plantar surface of the hindpaw) should be correlated with the extent of analgesia seen at the dermatome itself. To test this hypothesis, we examined this relationship in individual rats injected intrathecally with various doses of the mu opioid agonist DAMGO.

Rats were tested on the hot plate test as described above. DAMGO was injected intrathecally in 20µl of saline at doses of 0, 10, 100 or 1000 ng. Fifteen minutes after the injection the rats were again tested on the hot plate test to determine the extent of analgesia produced by the injection. At 25 minutes post-injection, rats were anesthetized with pentobarbital and perfused for immunocytochemistry to visualize and quantify MOR internalization as described above.

Correlation coefficients and 95% confidence intervals were computed using Statview 4.02 (Abacus Concepts, Inc., Berkeley, CA). A Fisher's r to z transformation was used to test the statistical significance of the correlation.

Nociceptive Stimuli

To determine if nociceptive stimuli release mu opioids that act upon lamina II interneurons, we examined MOR internalization in lamina II interneurons throughout the lumbar and cervical spinal cord following exposure of the rat to various noxious stimuli. Stimuli were chosen to mimic those used in studies that detected enkephalin release in spinal CSF (Le Bars et al, 1987; Bourgoin et al, 1988; Cesselin et al, 1989; Bourgoin et al, 1990) or that demonstrated release of neurokinins from dense core vesicles of primary afferent C-fibers (Liu, et al, 1997; Abbadie et al, 1997; Honore et al, 1999; Trafton et al, 1999). As endomorphins have been observed in small diameter primary afferents, we assume that these neurokinin-releasing stimuli would also release these endogenous opioids.

Rats were anesthetized with pentobarbital (50 mg/kg) and exposed to one of the following stimuli to the left hindpaw: 2 minute pinch with a hemostat, 2 minute immersion in a 52°C water bath, alternate immersion and removal from a 52°C water bath every 10 seconds for 10 or 20 minutes, interplantar injection of capsaicin (100µg in 20 µl), and interplantar injection of 50 µl of 5% formalin. Pinch and waterbath stimuli were also assessed 2 days after injection of 100 µl of Complete Freund's Adjuvant (CFA) into the hindpaw, which produces a persisent inflammation. Rats were perfused 5-15 minutes after the end of the stimulation. We also evaluated the effect of intrathecal injection of NMDA (6.8 µg in 20 µl) and direct electrical stimulation of the sciatic nerve (10 Hz, 0.5 mS, 10 mA for 1 minute). Additional time points were tested for the pinch stimulus, with normal rats being perfused at 30 minutes and CFA treated rats being perfused at 30 minutes and at 1, 2, and 3 hours post stimulus.

RESULTS

Exogenous opioid-evoked MOR internalization

To establish that agonist-induced internalization of the MOR occurs in the spinal cord in vivo, we tested a variety of opioid receptor agonists in rats and localized the MOR in lamina II neurons in the spinal cord dorsal horn. We performed immunofluorescent labeling with an antibody against the C-terminal tail of the MOR and examined sagittal sections of lumbar spinal cord. We found that in untreated, saline injected or vehicle pelleted rats MOR-LI was confined to the plasma membrane with fewer than 5 MOR-LI containing endosomes per lamina II neuron. (fig 1a) In rats injected intrathecally with the opioid peptides DAMGO (1.0 μg in 20 μl) or endomorphin-1 (25 μg in 20 μl) 25 minutes before perfusion, MOR-LI was depleted from the plasma membrane and was observed in numerous endosomes within the neuron. (fig 1b) Systemic injection of remifentanil (10 μg in 50 μl interplantar) resulted in a comparable pattern of internalization of MOR-LI. (fig 1c) On the other hand, when we administered morphine systemically (10 mg/kg s.c), intrathecally (30 μg in 20 μl) or via s.c. pellets, we never observed internalization of the MOR-LI above control levels. (fig 1d)

Time course of MOR recycling

To ensure that MORs did not recycle before we could detect their internalization in response to a stimulus, we needed to assess how long after opioid binding MOR internalization could be observed. Thus, we determined the

extent of MOR-LI internalization at various times after agonist application, thus providing a time course of this receptor recycling. Rats were intrathecally injected with DAMGO and allowed 10 minutes for the drug to spread and activate MORs. At this time rats were given a systemic injection of naloxone to displace DAMGO from the receptor and to prevent further action of any DAMGO that persisted in the CSF. Rats were perfused at various times post-naloxone injection and the average number of endosomes per lamina II neuron was determined for 20 neurons of the L4 segment. We found that internalization of the MOR peaked at 15 minutes (fig 2). By 60 minutes the MOR immunoreactivity was indistinguishable from that seen in untreated animals, suggesting that MOR had been recycled to the plasma membrane. Because some MOR internalization was still observed at 30 minutes, it appears that there is an approximate half hour window following activation during which MOR internalization can be detected immunocytochemically.

Correlation between MOR internalization and MOR-induced hyperpolarization

Although the phenomenon of MOR internalization is well documented, the relationship between MOR internalization and opioid function is less clear. Mu opioids have been shown to hyperpolarize a subpopulation of lamina II interneurons in the dorsal horn in a dose dependent manner. Thus, Grudt and Williams (1994) demonstrated that the mu opioid agonists DAMGO and met-enkephalin induce potassium currents in substantia gelatinosa neurons; the EC50 for DAMGO-induced effects was 72 nM +/- 12 nM (s.e.m.). To determine the relationship between MOR internalization and this previously described postsynaptic inhibition of MOR containing interneurons, we studied MOR

internalization in a spinal cord slice preparation. We determined the dose response relationship for DAMGO-induced MOR internalization in these cells, mimicking the conditions used in the Grudt and Williams study. We found that bath application of DAMGO resulted in a dose dependent internalization of the MOR in these neurons (EC50: 90.96 nM (*95% C.I. 2.7 - 3058 nM*). (fig 3a, c1-c3) This dose response relationship is similar to that published for inducing potassium currents in lamina II interneurons. (Grudt and Williams, 1994) We also examined the dose response relationship for met-enkephalin-induced MOR internalization. As previously described, fairly high concentrations of met-enkephalin were necessary to activate the MOR (fig 3b). We found that maximal MOR internalization occurred at a concentration of 50 μM. This is slightly higher than would be predicted based on the single dose (3μM) tested in the Grudt and Williams study. However, that study employed peptidase inhibitors and we did not.

Correlation between MOR internalization and opioid-induced behavioral analgesia

Although MOR internalization in lamina II interneurons clearly correlated with hyperpolarization of these same cells, the relationship between MOR internalization and the behavioral effects of opioids had not been demonstrated. To determine if the internalization of the MOR in lamina II of the spinal cord correlates with spinal opioid-induced analgesia, we compared the magnitude of MOR internalization in lamina II neurons with the magitude of behavioral analgesia on the hot plate test following intrathecal injection of DAMGO. We found that the dose response relationship for internalization of the MOR in

lamina II of the lumbar spinal cord was remarkably similar to that which produced analgesia in the hot plate test. (fig 4a) Furthermore, there was a high correlation between the magnitude of analgesia and the average number of endosomes per lamina II neuron of the lumbar spinal cord seen in individual rats (fig 4b) (correlation coeffIicient: 0.913 (95% CI: 0.807 - 0.962) p = <0.0001.).

Noxious stimuli

Several studies have demonstrated that endogenous opioids are released in the spinal cord following noxious stimuli. Here we examined whether noxious stmulation also induced MOR internalization in lamina II interneurons. To this end we monitored MOR-LI in lamina II of lumbar spinal cord following noxious stimuli of various modalities. We chose stimulus paradigms that modeled those used in studies that demonstrate release of enkephalins or primary afferent neuropeptides in the spinal cord.. In none of these experiments did we detect significant MOR internalization (fig 5).

All of these stimuli induced internalization of the NK-1 receptor in lamina I of the spinal cord in the same animals (data not shown, but see Liu et al, 1997; Abbadie et al, 1997; Honore et al, 1999; Trafton et al, 1999), demonstrating that the stimuli were sufficient to release dense core vesicles from at least a subpopulation of small diameter primary afferents. As non-segmental enkephalin release has been reported (Le Bars et al, 1987), sagittal sections of both lumbar and cervical spinal cord were examined. MOR localization in lamina II spinal cord neurons from animals treated as described (see methods) was indistinquishable from that in untreated animals.

Because there is evidence for enhanced release of opioids during peripheral inflammation, we looked for MOR internalization under these conditions. Again, none of the stimuli tested induced any detectable MOR internalization in the spinal cord (fig 6). Among the stimuli used was intraplantar injection of 5% formalin, a stimulus that produces both inflammation and pain behavior. Noxious stimuli in the presense of fully developed inflammation were also ineffective. No MOR internalization was observed after induction of profound inflammation of the hindpaw and ankle with CFA. Even superimposed on these inflammatory conditions, noxious pinch of the hindpaw for 2 minutes (at 5, 30, 120, or 180 min) or immersion of the hindpaw in a 52 degree water bath for 2 minutes (at 5 min) did not induce MOR internalization.

DISCUSSION

In the present study we show that MOR internalization occurs in the spinal cord in a manner comparable to that observed in the hypothalamus, enteric nervous system and in cultured cells. We could detect significant MOR internalization following administration of opioids locally or systemically. However, as previously described, morphine did not induce comparable internalization of the MOR, even with long exposures or when high doses were used (Keith et al, 1996). Importantly, all endogenous opioids as well as most non-morphine alkaloid agonists (such as remifentanil) tested in this and other studies (Burford et al, 1998; Keith et al, 1998; McConalogue et al, 1999) induced internalization of MORs. Thus, although it is possible for an opioid (specifically

morphine) to induce MOR signalling without internalization, it is unlikely that this would occur in response to endogenous MOR ligands.

We found that the MOR is internalized rapidly and recycles to the plasma membrane within approximately 60 minutes. This time course is similar to that observed for other G-protein coupled receptors (Mantyh et al, 1995). This suggests that there is about a 30 minute time window in which receptor activation can be detected. Importantly, this relatively prolonged window of recycling time ensures that we did not miss any significant MOR internalization that might have occurred following release of endogenous opioids. The animals were perfused long before the receptor would have completely recycled.

Of additional note, we found that the behavioral analgesia produced by intrathecal DAMGO injection is maintained for at least 30 minutes yet can be reversed by naloxone within mintues. Recycling of the MOR, however, is not completed for greater than 30 minutes after naloxone treatment. The fact that naloxone can reverse the analgesia produced by DAMGO much more quickly than the receptors appear to recycle suggests that this continuing analgesia requires ongoing activation of MOR, and is not the result of extended second messenger signaling or activation of a circuit triggered by an initial activation of MORs. This suggests that all MOR receptors on a given neuron are not functionally desensitized during the period in which most of the immunoreactivity appears to be endosomal. Thus, despite the internalization of a large population of MORs, a significant number of non-desensitized receptors likely remain at the plasma membrane even in the presence of high doses of a high efficacy agonist.

Relationship between MOR internalization and MOR activity

The similarity between the dose response curves obtained here for DAMGO and enkephalin induced MOR internalization and those previously described for MOR activated potassium currents suggests that MOR internalization can be used accurately as a marker of MOR induced activity. Based on comparison with the dose response curves obtained for MOR induced hyperpolarization (Grudt and Williams, 1994), MOR internalization is as good a marker for MOR activity as intracellular recordings and can indicate whether postsynaptic inhibition is occuring by the mechanisms proposed. It is, of course, possible that some MOR activity does occur that we cannot detect, however this activity is at least below the threshold for inducing potassium conductance and thus should not affect the membrane potential of the neuron. Recently, Marvizon et al (1999) reported a similar dose reponse for DAMGO induced MOR internalization in the spinal cord slice (EC50=30 nM), using a slightly different quantification method (counting percentage of neurons with MOR internalization rather than number of MOR positive endosomes). As they point out, the EC50s for MOR internalization in the spinal cord slice are nearly identical to those obtained for adenyl cyclase inhibition or [g-35S]GTP binding in cell culture systems (Keith et al, 1996; Keith et al, 1998; Yabaluri and Medzihradsky, 1997), again suggesting that MOR internalization can be used to indicate MOR activation using either method of quantification.

DAMGO induced MOR internalization correlated exceptionally well with DAMGO induced hot plate analgesia. Indeed only when rats were rendered analagesic by intrathecal DAMGO did we observe MOR internalization. This result suggests that post-synaptic MOR mediated hyperpolarization of excitatory

interneurons does occur and is likely involved in the behavioral analgesia produced by exogenous opioids. Of course, primary afferent MORs are nearby and thus would probably also be exposed to DAMGO that accessed this region of the cord. In fact, if the primary afferent opioid receptor terminals were critical to the induction of spinal analgesia, then MOR internalization in lamina II neurons might correlate with, but not be critical to the behavioral analgesia. The presence of internalization would merely indicate that sufficient drug reached the appropriate region of the spinal cord.

The close corellations between G-protein coupled receptor internalization and functional activity found here are not unprecedented. Keith et al (1998) found similar dose response relationships for DAMGO and etorphine-induced MOR internalization and inhibition of forskolin-stimulated cAMP accumulation in stably transfected HEK-293 cells. We also found a tight correlation between neurokinin-induced NK-1 receptor internalization and neurokinin-induced increases in intracellular calcium in cultured spinal cord neurons (Trafton et al, 1999). Additionally, it has been suggested that MOR internalization is linked to some aspects of its signaling, specifically the activation of MAP kinase (Ignatova et al, 1999; Polakiewicz et al, 1998), demonstrating that internalization and activity may be functionally related, not just temporally correlated.

Opioid consequences of noxious stimulation

Despite the literature suggesting that endogenous opioids are released during noxious stimulation, we found no indication that MOR's on interneurons in lamina II are internalized/activated after noxious stimulation. If endomorphins are released from primary afferent fibers during noxious stimuli,

it seems that their activity is at least restricted to MORs on primary afferent terminals. Because MORs have been observed on the terminals of small diameter fibers that contain endomorphins (Martin-Schild et al, 1998), it follows that these endomorphins may act in an autocrine fashion to restrict further C-fiber activity once dense core vesicles are released. Whatever their function, however, these endomorphins do not appear to have postsynaptic effects in lamina II.

These studies also suggest that the met-enkephalin release that has been detected following noxious stimuli (Le Bars et al, 1987; Bourgoin et al, 1988; Cesselin et al, 1989; Bourgoin et al, 1990), does not evoke post-synaptic effects via the MOR. Importantly, enkephalins have been shown to have higher affinity for the delta opioid receptor (DOR) (Corbett et al, 1993). DOR is also found in the superfical dorsal horn, (Arvidsson et al, 1995b) and it is possible that released enkephalins selectively activate this opioid receptor subtype. Alternatively, it is possible noxious stimulation does not release opioid peptides in sufficient quantity to activate any opioid receptors. This possibility is consistant with the lack of effect of the general opioid receptor antagonist naloxone on nociceptive behaviors or pain sensation in a number of acute pain models (El-Sobky et al, 1976; Grevert and Goldstein, 1978; Stacher et al, 1988).

Opioids have been shown to be more effective under inflammatory conditions, (Neil et al, 1986; Millan et al, 1988) and there is some evidence that levels of enkephalin in the dorsal horn increase with peripheral inflammation (Millan et al, 1986; Iadarola et al, 1988). These results suggested the possibility that greater amounts of enkephalin may be released and/or activate opioid receptors under inflammatory conditions (But see, Pohl et al, 1997). However, we found no indication of basal or noxious stimulus evoked MOR activity

following inflammatory stimuli. It appears that even under these conditions of hypersensitivity, spinal post-synaptic MORs are not activated enodgenously.

Given the profound analgesia that we found associated with MOR internalization in lamina II interneurons, it makes sense that this does not occur during normal or injury-related nociceptive responses. Were MORs activated to such an extent in vivo, responses to noxious stimuli would be virtually absent, preventing the withdrawal from and attendance to stimuli which are potentially damaging. This would clearly be undesireable and potentially life-threatening were it to occur during noxious stimulation regularly.

The question remains as to when activation of MORs on lamina II interneurons occurs in vivo. While it is clearly associated with the analgesia produced by exogenous opioids, one presumes that the receptors are there to perform some more natural function. Given their hypothesized role in behavior, a function in stress or sex-induced analgesia might be promising candidates, as in these conditions an inattention to pain might be beneficial to survival or reproduction.

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Figure 1: Exogenous opioids but not morphine internalize the MOR in lamina II interneurons. Confocal images of lamina II MOR immunoreactivity in the L4/L5 segments of the spinal cord in animals treated with the following: A) placebo pellets, B) intrathecal endomorphin-1, C) intraplantar remifentanil D) morphine (picture 1 is at 35X, picture 2 at 105X) In A & D, MOR immunoreactivity is seen predominantly on the cell membranes of neurons and their dendrites. In B & C, numerous MOR immunoreactive endosomes can be seen in the cell bodies and dendrites of neurons.

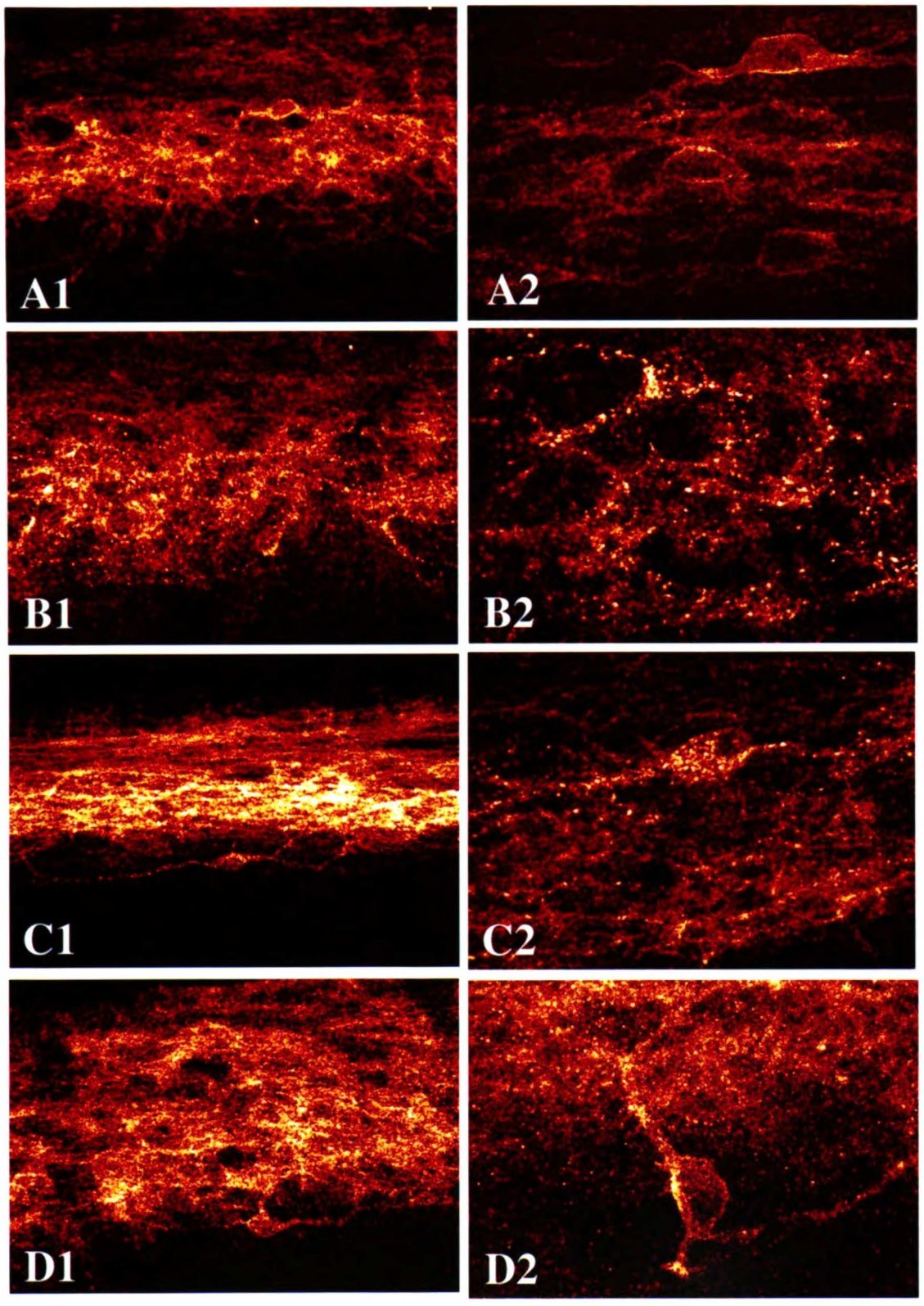

A1
A2
B1
B2
C1
C2
D1
D2

Figure 2: Time course of MOR recycling. Average number of MOR immunoreactive endosomes found in L4/L5 lamina II neurons in rats injected first with 1 μg intrathecal DAMGO and then 1 mg/kg naloxone s.c. 10 minutes after. Quantification was performed at various times following injection of naloxone (n=4).

Confocal images of lamina II MOR immunoreactivity in the L4/L5 segments of the spinal cord at the indicated times following naloxone injection (0, 7.5 15, 30 and 60 minutes) MOR internalization can be detected for up to 30 minutes following agonist application (Note numerous punctate inclusions in neurons). MOR immunoreactivity has returned to the plasma membrane by 60 minutes.

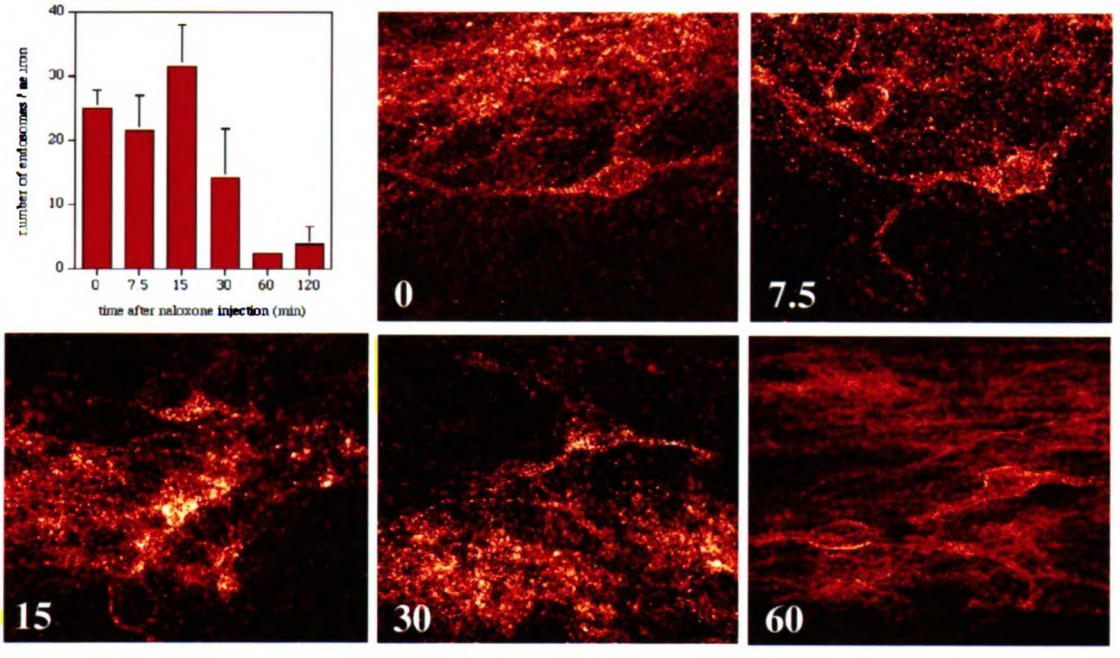

number of endosomes / neuron
40
30
20
10
0
0
7.5
15
30
60
120
time after naloxone injection (min)
0
7.5
15
30
60

Figure 3: Opioid receptor internalization occurs in a dose dependent manner. Slices of spinal cord were incubated in various doses of A) DAMGO or B) met-enkephalin for 15 minutes, and the average number of MOR immunoreactive endosomes per lamina II neuron was determined. Untreated control slices had 6.53 +/- 1.16 endosomes per neuron for the DAMGO experiments and 9.17 +/- 0.76 endosomes per neuron for the enkephalin experiments (n=4-9 for DAMGO; n=5 for met-enkephalin).

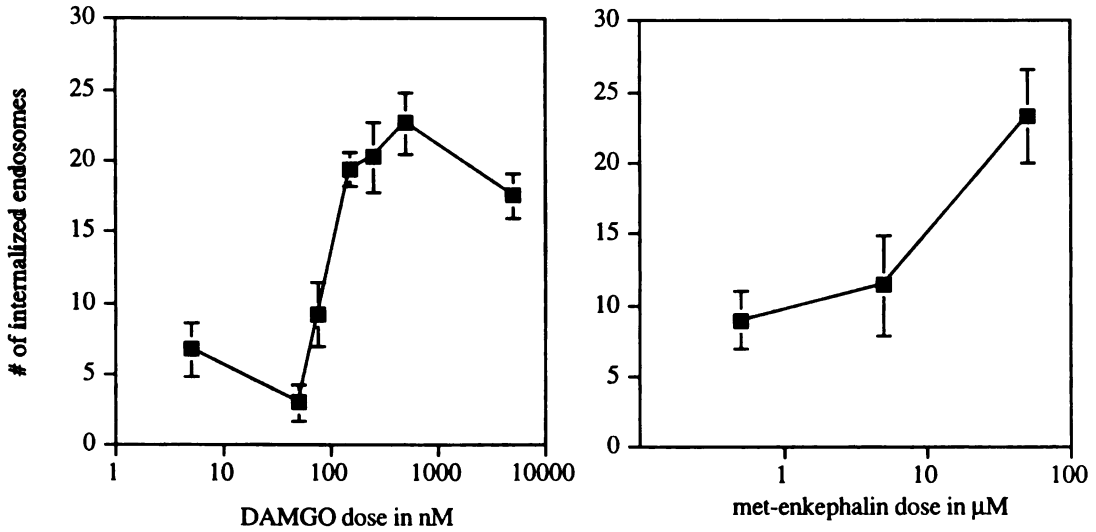

# of internalized endosomes
30
25
20
15
10
5
0
1
10
100
1000
10000
DAMGO dose in nM
30
25
20
15
10
5
0
1
10
100
met-enkephalin dose in μM

Figure 4: Lamina II MOR internalization correlates with intrathecal DAMGO induced hot plate analgesia. Rats were injected with various doses of intrathecal DAMGO, tested for analgesia on the hot plate test and fixed. MOR internalization was quantified in lamina II neurons from the L4/L5 segment of the spinal cord. A) Dose response curves for changes in hot plate latency and extent of MOR internalization. DAMGO produces spinal MOR internalization and behavioral analgesia at the same doses. Saline injected rats had 5.58 +/- 1.17 endosomes per neuron (n=6) B) Graph of the extent of analgesia (hot plate latency in seconds) versus the extent of MOR internalization (number of endosomes per neuron) in lamina II of the L4/L5 segment in individual rats. No rats showed significant MOR internalization without being profoundly analgesic. C) Confocal images of MOR immunoreactivity in lamina II from rats injected intrathecally with 1) saline 2) 100 ng DAMGO or 3) 1000 ng DAMGO. MOR labeling is observed on the plasma membrane in vehicle treated rats. Increasing doses of DAMGO produced increases in the amount of MOR immunoreactivity that appeared as punctate inclusions in the cytoplasm.

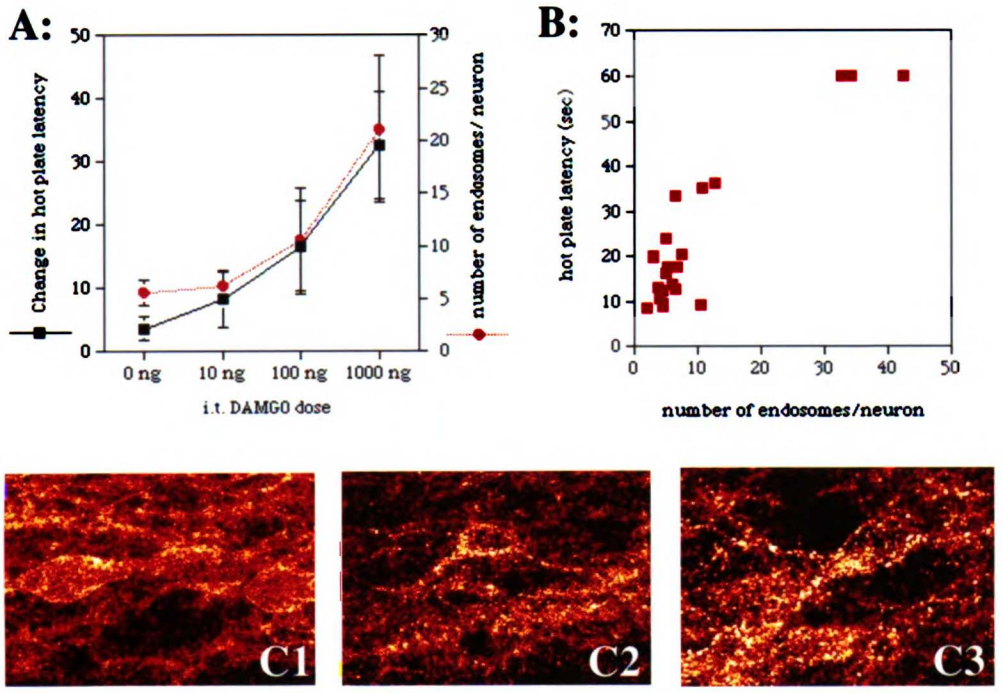

A:
Change in hot plate latency
number of endosomes/ neuron
0 ng
10 ng
100 ng
1000 ng
i.t. DAMGO dose
B:
hot plate latency (sec)
number of endosomes/neuron
C1
C2
C3

Figure 5: Noxious stimuli do not internalize the MOR. Noxious stimuli were presented to a hindlimb of an anesthetized rat. Rats were perfused 5-30 minutes later. Confocal images of MOR immunoreactivity in lamina II of the L4/L5 segments of the spinal cord ipsilateral to the stimulus. A) from a rat that received a noxious pinch of the hindpaw. B) from a rat that was injected with capsaicin intraplantar C) from a rat whose hindpaw was alternatively dipped and removed from 52 degree water every 10 seconds for 10 minutes. (Picture 1 is at 35X; picture 2 is at 105X)

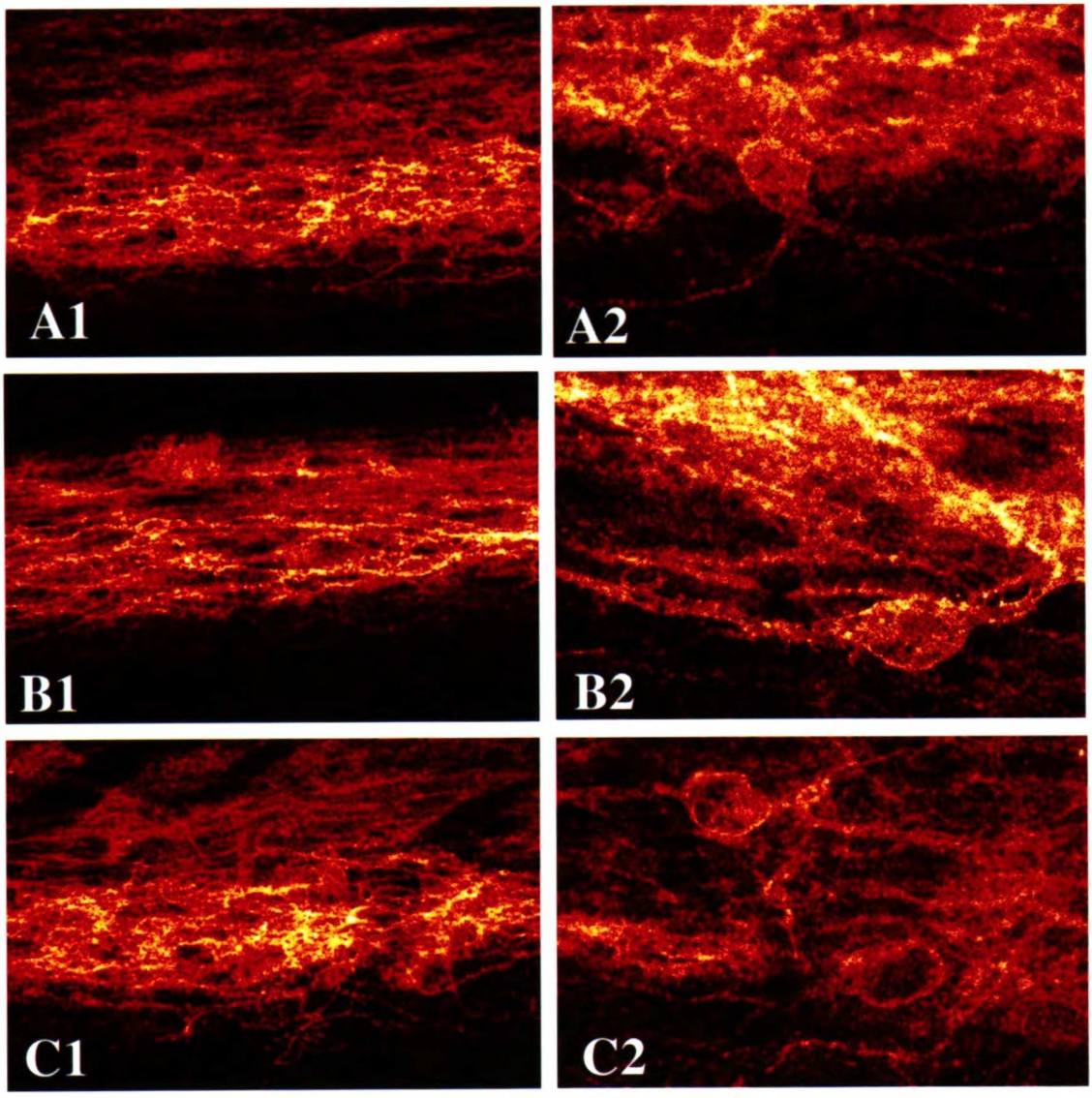
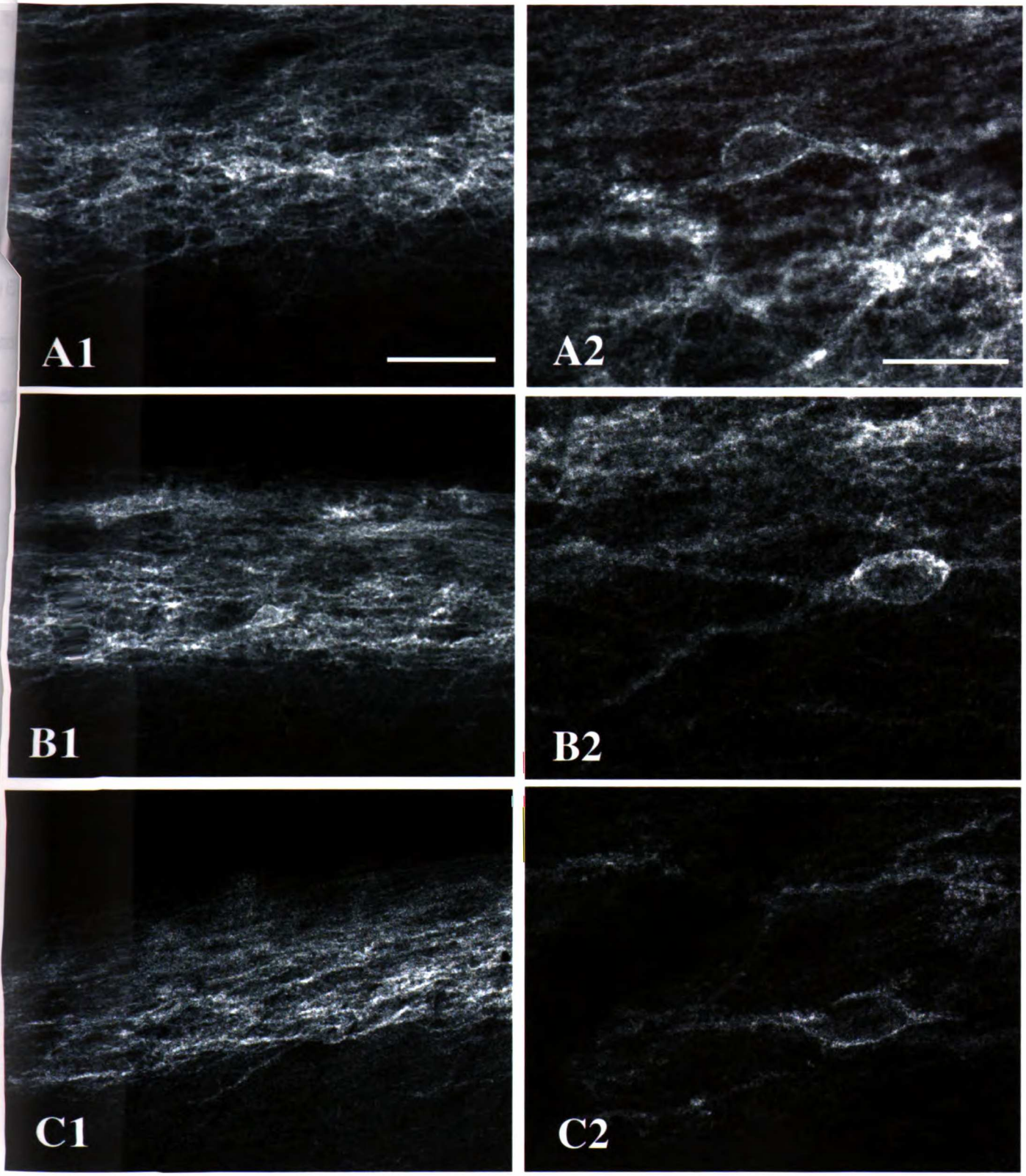

A1
A2
B1
B2
C1
C2

Figure 6: Noxious stimuli do not internalize the MOR under conditions of inflammation. Confocal images of MOR immunoreactivity in lamina II of the L4/L5 segments of the spinal cord ipsilateral to the inflammation and/or stimulus A) from a rat that received and intraplantar injection of 5% formalin, B) from a rat that received a noxious pinch to an hindpaw inflamed due to injection of CFA two days prior. C) from a rat that whose hindpaw, inflamed due to injection of CFA two days prior, was dipped in a 52 degree water bath for 2 minutes.


A1
A2
B1
B2
C1
C2

Post-synaptic Activity of the MOR in the Spinal Cord: Response to Stress and Activation of Descending Inhibitory Control Pathways

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ABSTRACT

Supraspinal inputs to the dorsal horn of the spinal cord have been known to modulate nociceptive transmission for years. While a number of different projections and mechanisms likely underlie this descending control system, in some cases the anti-nociception produced is mediated by an opioid at the spinal level. Nevertheless, the exact circuitry, peptides and receptors underlying these observed opioid effects are not defined. Here we examine four examples of descending inhibition for which evidence for spinal opioid actions exist to determine if post-synaptic activation of dorsal horn MORs is involved in these forms of anti-nociception.

Using a combination of MOR internalization and pharmacology, we found no evidence for a post-synaptic MOR component to the antinocicecption produced by i.c.v. injection of opioids, PAG injection of bicucculine, swim stress or spinal serotonin or norepinephrine release. None of these paradigms induced MOR internalization in lamina II interneurons of the lumbar spinal cord. We suggest that activation of presynaptic MORs on primary afferent nociceptors or activation of DORs underlie the antinociception produced by these systems.

GENERAL INTRODUCTION

There is considerable evidence that nociceptive processing at the level of the spinal cord is subject to powerful descending inhibitory controls. The most studied system originates in the midbrain periaqueductal gray and includes connections in the rostrventral medulla with descending axons that arise from the nucleus raphe magnus. This inhibitory control system can be activated by electrical stimulation of the PAG or by exogenous administration of opioids. One of the more provocative features of the circuitry through which descending controls regulate nociceptive processing at the level of the spinal cord is that it contains multiple opioid receptor links. This feature no doubt underlies the fact that microinjection of opioids at several levels within the descending inhibitory control pathway, including the PAG, raphe magnus and spinal cord can produce a profound antinociception. It is also presumed that the analgesia produced by systemic injection of opioids, notably morphine, not only results from the activation of descending controls that originate in the PAG, but also from a synergistic antinociceptionsecondary to the concurrent activation of multiple opioid receptor links within the circuit. Importantly, because microinjection of the opioid antagonist naloxone at any level of the circuit can block the effect of PAG injection of morphine or brain stimulation regions (Zorman et al, 1982; Tseng & Tang, 1989; Pan & Fields, 1996; Roychowdhury & Fields, 1996; Tortorici et al, 1996; Budai & Fields, 1998)., it appears that the opioid receptor links in the descending pathway are serially connected.

It is generally presumed that the antinociception produced by microinjection of opioids at multiple levels of the descending circuit mimics a physiologically relevant circuit that is mediated by release of a local endorphin. Indeed enkephalins and dynorphin are found in high concentration in the PAG, RVM and spinal cord and several studies have implicated release of endorphins in various manipulations that produce analgesia (e.g. stress, etc...). The nature of the endorphin-opioid receptor circuitry that is engaged by this manipulations is, however, not well understood. Furthermore, although microinjection of an opioid antagonist points to the general location of the critical opioid receptors (e.g. in the superficial dorsal horn), it does not reveal their precise location and thus the relevant circuit cannot be determined.

For example, many neurons in the dorsal horn of the spinal cord and dorsal root ganglion express MOR and DORs (Arvidsson et al, 1995a; Arvidsson et al, 1995b; Zhang et al, 1998). Activation of either of these receptors is thought to produce antinociceptive effects via inhibition of neurotransmitter release from primary afferent neurons or hyperpolarization of excitatory interneurons in lamina II of the spinal cord (Jessel & Iversen, 1977; Yoshimura & North, 1983). Here we attempt to determine if activation of descending control systems produces spinal antinociception via endorphin-mediated circuits that involve post-synaptic MORs. To this end we used internalization of the MOR receptor to monitor the conditions under which MOR's are brought into play. We previously showed that the magnitude of the antinociception produced by intrathecal opioids is highly correlated with the extent of MOR internalization in interneurons in lamina II. In the present study we examined a number of paradigms that have implicated release of endogenous opioids following

activation of descending controls and assessed the extent to which they induce internalization of the MOR in lamina II interneurons.

GENERAL METHODS

Perfusion and tissue preparation

Following treatment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and then intracardially perfused with 50 ml 0.1 M phosphate buffered saline followed by 500 ml 10% formalin. The brain and spinal cord were dissected out, post-fixed for 4 hours in the same fixative and then transferred to a 30% sucrose solution. Thirty micron sagittal sections of lumbar cord were cut on a freezing microtome.

Immunofluorescent labeling

Sections were blocked in 5% normal goat serum in 0.1 M phosphate buffered saline with 0.3% triton x-100 for 30 minutes and then incubated in a rabbit anti-MOR antibody (Incstar, Stillwater, MN) or anti-NK-1 receptor antibody (Advanced Targeting Systems, Carlsbad, CA) at a 1:5000 dilution overnight. Sections were washed and then incubated in a Cy-3 conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA) at a 1:600 dilution for 2 hours. Tissue was then washed and mounted on gelatin coated slides.

Analysis of MOR labeling

Coded slides with spinal cord sections from a single animal were closely examined by an observer who was unaware of the treatment the rat received. Lamina II neurons in lumbar segments L2-L6 and sometimes cervical segments C5 - C8, were examined on a NIKON Axiophot fluorescent microscope with a 60X oil objective. The extent of MOR internalization, judged by the presence and number of punctate inclusions, was noted and scored for each slide. Alternatively, the endosome counting method described in the previous section was employed.

SWIM STRESS INDUCED ANALGESIA

INTRODUCTION

Cold water swimming, like numerous other stressors, produces a robust analgesia in rodents. Depending on the duration of the swim and the water temperature, this analgesia has been shown to be either opioid or non-opioid mediated. Antinociception produced by the former manipulations was reversed by naloxone (Terman et al, 1986; Tierney et al, 1991). Although multiple mechanisms and opioid receptor links are likely involved in the anti-nociception produced by such stressors, it has been suggested that at least some of the opioid-mediated forms involve spinal cord opioid receptors. Thus, intrathecal injection of opioid receptor antagonists prevents the antinociception produced by some stressors, such as brief forepaw shock (Watkins & Mayer, 1982) or longer duration swims (Terman et al, 1986).

As neither the opioid receptor subtype nor the dorsal horn location of the opioid receptor could be determined from the previous studies , in the present study we used internalization of the MOR to determine whether MOR located on lamina II interneurons come into play in the setting of swim-stress induced analgesia.

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METHODS

Rats were tested on the 52.5° degree hot plate test to determine baseline thermal latencies. Immediately following testing, the rats were swum in a bath of 15 °C water for 5 or 10 minutes. The rats were then removed from the bath and towel dried for 1 minute, before being retested on the 52.5° hot plate. After testing, the rats were anesthetized with pentobarbital (100 mg/kg) and within 5-10 minutes of the end of the swim, transcardially perfused with 10% formalin.

RESULTS

Although we found that cold water swim produced a significant analgesia in rats, we found no evidence for a concurrent MOR internalization in lamina II interneurons in the lumbar spinal cord. Consistent with this observation, we were unable to reverse this analgesia with 1.0 mg/kg s.c. naloxone, a dose of the general opioid receptor antagonist that we have shown is sufficiently high to completely block systemic or intrathecal opioid-induced MOR internalization in lamina II interneurons.

DISCUSSION

These results suggest that swim stress induced analgesia does not require or involve significantactivation of post-synaptic MORs in the spinal cord dorsal horn. Our inability to reverse the analgesia with 1 mg/kg naloxone suggests that pre-synaptic MORs are also not involved. While this result seems initially incongruous with other findings, closer inpection of the literature reveals that this is not really the case. While studies of fore-limb shock induced analgesia were consistent with opioid mediated stress analgesia involving a spinal mu opioid receptor, demonstrating reversal of anti-nociception by 1 mg/kg naloxone s.c. or 1 ug naloxone i.t. (Watkins and Mayer, 1982) , subsequent studies using other stressors have found evidence favoring the involvement of delta rather than mu receptors. Studies demonstrating the naloxone sensitivity ot swim stress induced analgesia used 5-10 mg/kg naloxone, doses that will inhibit not only the MOR, but also DORs and KORs (Terman et al, 1986; Tierney et al, 1991). More convincingly, Mizoguchi et al (1997) have demonstrated that a 3 min 4 degree celcius swim produced an opioid analgesia that was blocked by i.t. pretreatment with met-enkephalin antiserum or the delta 2 receptor antagonist naltriben. Furthermore, only delta 2 antagonists would block analgesia produced by i.t. met-enkephalin and neither analgesia from swim stress or i.t. met-enkephalin was blocked by the selective mu opioid receptor antagonist CTOP

The possibility that swim stress induced analgesia only involves a delta and not a mu opioid component in the spinal cord is completely consistent with our observations. While our paradigm produced a profound stress induced

analgesia, it neither was reversed by 1 mg/kg naloxone nor produced MOR internalization in lamina II interneurons in the spinal cord, thus suggesting that it did not require mu opioids or activate post-synaptic MORs in the spinal cord. As other labs have shown that analgesia produced by an identical stress paradigm is prevented by high doses (5-10 mg/kg) of naloxone, it is likely that DOR or KOR are involved. While further study is clearly required, these results demonstrate that swim stress induced analgesia does not require or involve lamina II MORs, and thus other opioidergic mechanisms obviously exist for production of endogenous opioid analgesia.

OPIOID ACTIVATION OF THE PERIAQUEDUCTAL GREY - icv DAMGO AND MOPRHINE

INTRODUCTION

Evidence suggests that an endogenous spinal opioid may contribute to anti-nociception produced by activation of the much studied periaqueductal grey(PAG)/rostroventral medulla(RVM)/spinal cord decending control system(Zorman et al, 1982; Tseng & Tang, 1989; Budai & Fields, 1998). This pathway can be activated by injection of opioids into the PAG or intracerebroventricularly, presumably via inhibition of inhibitory interneurons in the PAG (see Basbaum and Fields, 1984 for review) To test whether the antinociception produced by activation of this pathway involved activation of spinal post-synaptic MORs, we injected morphine or DAMGO

intracerebroventricularly and looked for MOR internalization in the PAG and spinal cord.

METHODS

240-260 g rats were pentobarbital anesthetized (55 mg/kg i.p.) and mounted on a stereotax. The skull was exposed and 1 µl injections were made at the following coordinates from lambda: AP +4.45 mm, DV -6.0 mm on the midline. Either 0.6 µg DAMGO or 10 µg morphine was injected. Injections were made over 5 minutes and the syringe was left in for 5 minutes after the injection. Rats were then removed from the stereotax and transcardially perfused with 10% formalin 30 minutes after the start of the injection.

RESULTS

As expected, DAMGO, but not morphine, which does not trigger MOR internalization, induced extensive MOR internalization in neurons and dendrites throughout the PAG. However, in neither case was any MOR internalization observed in lamina II MOR expressing neurons in either the lumbar or cervical spinal cord.

DISCUSSION

These results suggest that activation of MORs in the PAG does not result in activation of post-synaptic MORs in the spinal cord dorsal horn. This is surprising given the evidence for the involvement of spinal mu opioids in the production of icv opioid induced analgesia, however, as we cannot visualize MOR activation in pre-synaptic terminals, it is possible and perhaps likely that these MOR are involved instead.

One potential caveat to this experiment is the use of pentobarbital anesthesia during the injection and perfusion. The antinociceptive projection from the PAG is known to be subject to GABAergic inhitibition. Pentobarbital increases currents through the GABA A receptor and thus might reduce the activity produced in the PAG by i.c.v. opioids. Indeed, the anti-nociception produced by microinjection of morphine into the PAG has been shown to be attenuated by pentobarbital. Thus, PAG activation by i.c.v. opioids produced by this study may not be maximal despite extensive activation of MORs in the PAG. This caveat may be overcome by using a different paradigm to activate this circuit. Injection of bicucculine into the PAG has been shown to be a more reliable and intense method of producing analgesia via the same circuit; thus we reexamined this question using this stimulus paradigm.

BICUCCULINE ACTIVATION OF THE PERIAQUEDUCTAL GREY

INTRODUCTION

Budai & Fields (1998) have demonstrated that intra-PAG injection of bicucculine, which disinhibits projection neurons by antagonizing the inhibitory

actions of GABA A receptor activation, can reduce noxious stimulus evoked activity of lamina V cells via an opioidergic mechanism at the spinal cord. This is presumed to occur via the previously described PAG-RVM-spinal cord pathway, and is assumed to involve MORs in the spinal cord, as the inhibition observed is blocked by selective MOR antagonists. The precise circuitry underlying this effect is not yet known; presynaptic MORs on small diameter primary afferents or post-synaptic MORs on lamina II interneurons are both obvious potential mu opioid targets.

To determine if these effects are mediated by post-synapttic MORs in lamina II, we looked at MOR internalization in lamina II interneurons in the lumbar spinal cord following intra-PAG injection of bicucculine. We have previously shown that morphine in combination with a low dose of the NK-1 receptor antagonist GR205171 can reduce noxious stimulus evoked NK-1 receptor internalization in lamina I neurons of the lumbar spinal cord. We hypothesized that it might be possible to observe endogenous mu opioid mediated pre-synaptic inhibition of transmitter release by studying noxious stimulus evoked NK-1 receptor internalization in the presence of 1 mg/kg GR 205171. To test this, we also examined NK-1 receptor internalization in the lumbar spinal cord following noxious pinch of the hindpaw after intra-PAG bicucculine injection.

METHODS

Rats were nembutal anesthetised, mounted on a stereotaxic and a 1,0 µl Hamilton syringe was lowered to the following coordinates from lambda: AP

+1.0 mm, LM -0.6 mm, DV -6.8 mm. Rats were then given a subcutaneous injection of saline or 1 mg/kg GR 205171. Ten minutes later, 0.4 µl of saline or 1.0 mM bicucculine methiodide was injected in the PAG. Ten minutes after the injection, the left hindpaw was pinched for 15 seconds. Rats were transcardially perfused 7 minutes after the pinch. Tissue was prepared as described and half was labeled for NK-1 receptor immunoreactivity and half was labeled for MOR immunoreactivity.

RESULTS

To determine if the anti-nociceptive actions of intra-PAG bicucculine occured via mu opioid action on lamina II MORs, we microinjected bicucculine in the PAG of anesthetized rats and perfused 15 minutes later to look at MOR internalization in the spinal cord. Intra-PAG bicucculline, as previously reported, induced whisker whisking and increases in breathing rates in the anesthetized rats. Nevertheless, no MOR internalization was observed in lamina II of the lumbar spinal cord in any of the animals. The number of MOR positive endosomes per lamina II interneuron was the same in saline and bicucculine injected rats and injection of GR 205171 had no effect ($p=0.3816$; $n=3$-4).

Additionally, we examined the ability of intra-PAG injection of bicucculine to reduce noxioux stimulus evoked NK-1 receptor internalization. To increase the chance of seeing an endogenous opioid mediated reduction, half the rats were given injections of a low dose of the NK-1 receptor antagonist GR 205171. We found no effect of intra-PAG injection of bicucculine on the extent of NK-1 receptor internalization evoked by noxious pinch. While GR 205171 did

slightly reduce NK-1 receptor internalization on its own, there was no further reduction upon injection of bicucculine in the PAG. Injection sites were verified by identifying needle tracks in 50 μM coronal sections of the PAG. All injections were within the PAG in the ventrolateral quadrent.

DISCUSSION

These studies suggest that post-synaptic MORs are not activated during PAG disinhibition induced spinal anti-nociception. Additionally, we found no evidence for presynaptic inhibition of tachykinin-expressing primary afferents. These results suggest that the opioid mediated anti-nociception observed by Budai & Fields (1998) , may involve presynaptic inhibition of transmitter release from non-SP containing primary afferents, or selective inhibition of glutamate release from SP expressing nociceptors. This is consistent with data from Budai's own work, in which he found that PAG bicucculine reduced noxious stimulus evoked activity, but not that induced by iontophoresis of glutamate receptor agonists. The lack of inhibition seen in response to exogenously applied agonists suggests that pre- rather than post- synaptic inhibitory mechanisms are involved (Budai & Fields, 1998).

The fact that no presynaptic inhibition of SP release was detected is curious but not as suprising as it might initially appear. While MORs have been found on tachykinin expressing primary afferents, these opioid receptors are by no means restricted to this primary afferent population. Immunohistochemical and in situ hybridization results estimate that only 28% or 42% of MOR containing DRG neurons express SP and thus, clearly other nociceptor

populations express MORs (Minami et al, 1995; Lu et al, 1997; Li et al, 1998). Thus, activation of presynaptic MOR on the terminals of these primary afferents may underly the anti-nociceptive behavior.

SEROTONERGIC AND ADRENERGIC PROJECTIONS TO THE SPINAL CORD

INTRODUCTION

Studies have suggested that descending activation of spinal opioid analgesia may involve serotonergic or adrenergic projections from the brainstem. Destruction of spinal serotonergic terminals or medullary 5-HT containing neurons blocks the analgesic action of systemic opiates and the analgesia produced by icv injection of morphine can be prevented by intrathecal injection of a 5-HT antagonist and an alpha adrenergic antagonist. (Vogt, 1974; Yaksh, 1979) The spinal cord dorsal horn receives numerous serotonergic and adrenergic projections from brain stem nuclei which have been shown to be involved in anti-nociception or inhibition of dorsal horn neurons (Kwiat & Basbaum, 1992). These projections course through the dorso-lateral funiculus (DLF), the integrity of which is required for descending modulation of nociceptive responses (Basbaum et al, 1976). Thus, it has been hypothesized that these serotonergic or adrenergic projections may trigger the release of spinal opioids which act to reduce nociceptive transmission in the dorsal horn of the spinal cord (Fields et al, 1991).

We looked at whether application of these agonists to the dorsal horn of the spinal cord would induce MOR internalization in lamina II interneurons. If release of serotonin or norepinephrine from supraspinal projections to the dorsal horn resulted in release of endogenous opioids which acted upon post-synaptic MORs to decrease nociceptive transmission, we would expect to see MOR internalization upon application of these transmitters to the spinal cord. For these studies, we made use of a spinal cord slice preparation in which we have previously demonstrated clear and dose dependent increases in MOR internalization with application of the MOR agonists DAMGO or met-enkephalin.

METHODS

P15-23 Sprague Dawley rats were anesthetized with halothane and the lumbar and sacral spinal cord were dissected out. The spinal cord was placed in a bath of carbogenated sucrose buffer (in mM: sucrose 240; KCl 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1.0; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.5) and the dura and dorsal and ventral roots were removed under a dissecting microscope. The cut surface of the lumbar cord was superglued to the stage of a vibratome (Pelco 101 series 1000), placed against a 5% agar block and immersed in carbogenated sucrose buffer. Transverse sections of spinal cord were cut at 500-600 μm and transferred to carbogenated incubation solution (in mM: NaCl 124; KCl 5; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) for 2 hours. After 2 hours, slices were transferred to carbogenated recording solution at 37$^{\epsilon}$C (in mM: NaCl 127; KCl

1.9; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) containing the designated concentrations of serotonin, norepinephrine, glutamate, or DAMGO for 15 minutes. Slices were then fixed overnight in 10% formalin and then transferred to a 30% sucrose solution for several hours. They were next cut at 40 μm on a freezing microtome. Standard immunofluorescent labeling of the MOR was performed and sections were examined at 40x on a Nikon axiophot microscope and at 120x on a Biorad MRC 1024 confocal microscope.

RESULTS

Bath application of high doses of serotonin or norepinephrine to spinal cord slices did not induce internalization of lamina II MORs in the slice. MOR labeling in these sections was indistinguishable from that seen in sister slides incubated in buffer alone. This was true even when 5-HT and NE were applied in combination with 500 μM glutamate to further excite the slice. In all cases, sister slices incubated in DAMGO showed profound internalization of the MOR in lamina II interneurons, demonstrating the slices and MORs were healthy and functioning.

DISCUSSION

These results suggest that neither serotonin or norepinephrine release in the spinal cord is sufficient to trigger the release of endogenous opioids which act on post-synaptic MORs. It is quite possible that these neurotransmitters

release endogenous opioids, which act on presynaptic MORs or DORs selectively, as we cannot monitor activation of these receptors in this system. Such an action would be perfectly consistent with data suggesting a spinal opioid component to the descending control systems that make use of serotonin and nor-epinephrine. Nevertheless, our data argue that post-synaptic mu sites likely do not contribute.

Behavioral studies looking at the antinociceptive effects of activation of descending control systems have been mixed in their conclusions as to the role of endogenous spinal opioids. This study would suggest that post-synaptic MOR activation is not direct result of release of biogenic amines from descending fibers.

One caveat to this conclusion stems from our use of an in vitro system for the experiment. While much of the local circuitry is intact in a slice preparation, segmental and supraspinal connections are obviously damaged and absent. If these elements were required for serotonergic or adrenergic mediated activation of post-synaptic MORs, then no internalization would be observed. Similarly, if simultaneous activation of descending control systems and nociceptive sensory neurons or the like were necessary, one would also expect a falsely negative result. Finally, immature rats were used for the spinal cord slices and it is possible that the circuitry involved was not fully developed.

GENERAL DISCUSSION

In none of the paradigms studied did we find any evidence of activation of post-synaptic MORs. This inability to find any stimulus which produced

analgesia in lamina II interneurons of the spinal cord dorsal horn raises concern about the sensitivity of our internalization assay. However, we have found that MOR internalization occurs in these neurons at the same concentrations as and correlated with spinal opioid induced-analgesia. In no case have we been able to demonstrate analgesia in response to spinal administration of opioids in the absense of MOR internalization (with the exception of morphine which poorly internalizes the MOR.). This suggests that any undetectable MOR internalization is not capable of producing significant analgesia. The lack of post-synaptic lamina II MOR internalization is consistent with the pharmacological inability to block some of these analgesias with low doses of naloxone and the ineffectiveness of PAG activation for inhibition of glutamate induced currents in WDR cells. Thus, we believe that the lack of MOR internalization seen in our studies is indicative of the inactivity of this receptor population in these analgesic states.

It thus appears that opioidergic inhibition of these lamina II interneurons is not a common mechanism by which supraspinal circuits modulate dorsal horn nociceptive processing. Even during procedures shown to produce analgesia via a spinal MOR, no MOR internalization was observed, suggesting that presynaptic primary afferent MOR are the relevant mediator of this opioidergic inhibition. Additionally, it is clear that non-opioid circuitry/mechanisms exist in the spinal cord, which participate in and can produce analgesia. These studies would suggest that they may be of greater importance in endogenous analgesia and support their usefulness as drug targets. These studies also suggest that spinal opioid receptors are likely selectively targeted, not diffusely activated by supraspinal inputs, as evidenced by the probable activation by PAG bicucculine

of presynaptic MOR on primary afferent terminals in lamina II in the absence of post-synaptic MOR activation of nearby lamina II interneurons.

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Table 1. Effect of intra-PAG bicucculine on spinal cord MOR and NK-1 R internalization

Treatments		MOR Internalization	NK-1 R Internalization
PAG injection	s.c. injection	endosomes/neuron	% neurons internalized
saline	saline	9.85 +/- 0.65	61.89 +/- 7.8
saline	GR 205171	9.24 +/- 0.78	32.42 +/- 9.12
bicucculine	saline	11.57 +/- 1.13	62.22 +/- 7.38
bicucculine	GR 205171	9.69 +/- 0.95	49.55 +/- 6.44

Figure 1: Swim stress produces a thermal antinociception which is not prevented by MOR antagonists (10 μg i.t. naloxone methiodide or 1 mg/kg s.c. naloxone) n= 2-4.

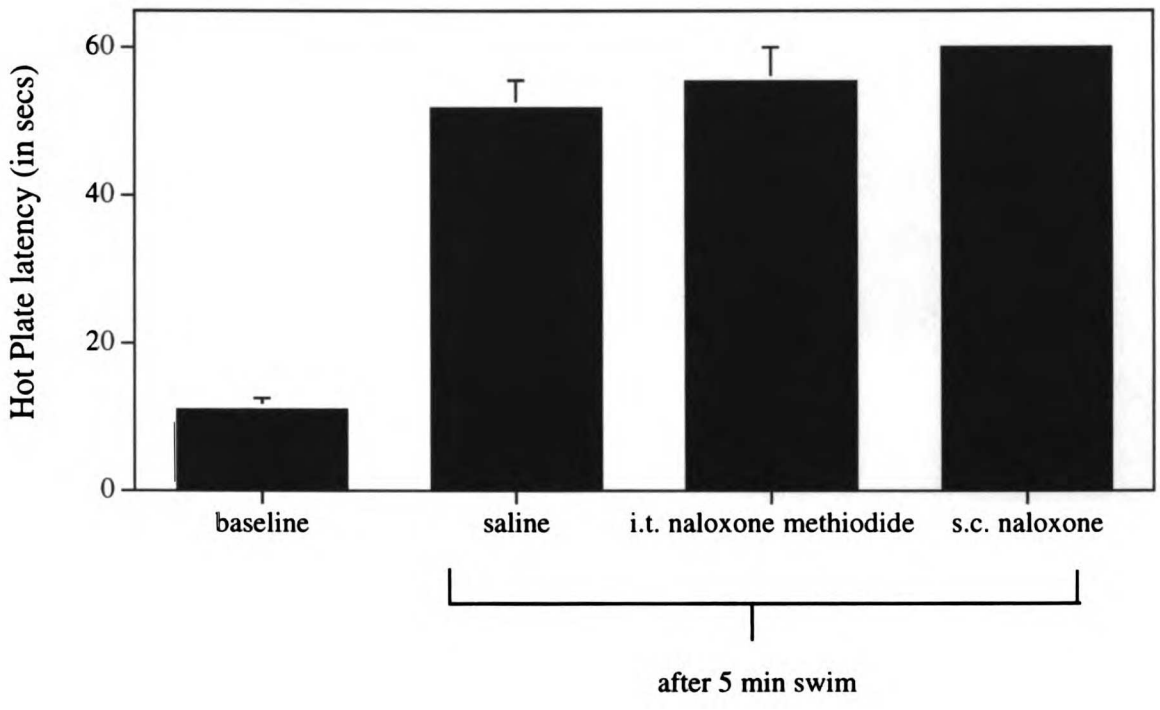
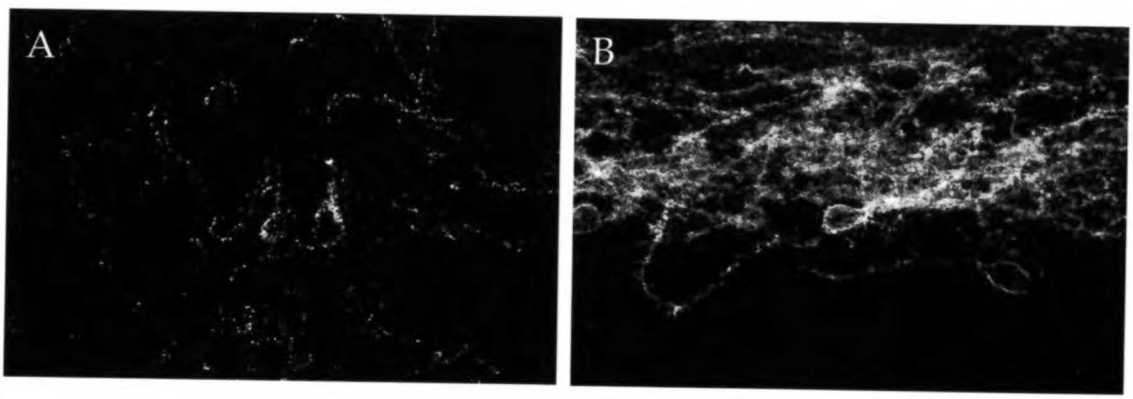

Hot Plate latency (in secs)
60
40
20
0
baseline
saline
i.t. naloxone methiodide
s.c. naloxone
after 5 min swim

Figure 2: I.c.v. injection of DAMGO results in MOR internalization in the PAG but not the spinal cord dorsal horn. A) MOR immunoreactivity in the PAG following icv DAMGO. Note the punctate endosomal labeling. B) MOR immunoreactivity in the dorsal horn of the lumbar spinal cord in the same rat. Labeling is restricted to the cell membrane as seen in untreated controls. Pictures are at 120X.


A
B

Post-synaptic Activity of the MOR in the Spinal Cord: Response to Release of Endogenous Enkephalins

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ABSTRACT

While endogenous opioid analgesias have long been hypothesized to include a spinal component, the circuitry underlying the spinal opioidergic effects are still relatively undefined. The endogenous opioid peptide enkephalin has been shown to be expressed in a population of GABAergic non-glycinergic, NOS expressing lamina II interneurons in the dorsal horn. NK-3 receptor has been similarly localized to such a lamina II interneuron population.

Here we show that intrathecal injection of neurokinin-3 receptor agonists results in a series of stereotyped behaviors including a thermal anti-nociception. We show that this anti-nociception is likely delta opioid receptor mediated, as it is reversed by high doses of systemic naloxone or intrathecal injection of the selective DOR antagonist naltrindole. Mu opioid receptors were not involved, as evidenced by the lack of MOR internalization observed in lamina II interneurons and the insensitivity of the anti-nociceptive behavior to doses of systemic naloxone shown to block mu opioid receptor mediated effects. We suggest that spinal enkephalins, contained in neurokinin-3 receptor expressing GABAergic lamina II interneurons, act exclusively upon delta opioid receptors to produce a robust thermal analgesia.

INTRODUCTION

Endogenous opioid analgesia has long been thought to have a spinal component. However, the exact circuitry and mechanisms underlying such opioid effects are not clear.

Lamina II of the spinal cord dorsal horn contains a large population of enkephalinergic interneurons. As such, these neurons constitute an obvious source of endogenous opioids that could be involved in endogenous modulation of nociceptive input. Using immunohistochemical double labeling techniques, some of these neurons have been shown to be GABAergic, non-glycineric islet cells, suggesting they act as inhibitory interneurons. (Todd et al, 1992; Todd & Spike, 1993) Additionally, a large percentage of these inhibitory interneurons have been shown to express nitric oxide synthase (NOS). (Laing et al, 1994; Spike et al, 1993) Of interest, the neurokinin-3 receptor has been shown to co-localize almost completely with the lamina II NOS expressing neuronal population (Seybold et al, 1997).

The NK-3 receptor is a member of the tachykinin family of G-protein coupled receptors. It is thought to be activated predominantly by the tachykinin ligand NKB and to induce G protein mediated signaling events including activation of phospholipase C and adenylate cyclase (Nakajima et al, 1992). NK-3 receptor agonists have been shown to be antinociceptive when injected spinally, and it has been suggested that this is via an opioidergic mechanism based on its naloxone sensitivity. (Laneuville et al, 1988; Couture et al, 1993)

These pharmacological observations in conjunction with the described anatomy suggest the following possible mechanism for producing a spinal

endogenous opioid analgesia. NK- 3 receptor agonists in lamina II could excite the GABAergic opioidergic interneurons that express the NK- 3 receptor leading to release of these transmitters in the spinal cord dorsal horn. These released opioids may then act upon spinal opioid receptors to produce behavioral analgesia. The specific targets of these opioid peptides, however, are unclear.

Although able to act at both receptors, the enkephalins (met and leu) are known to have a higher affinity for DORs rather than MORs. The implications of this observation for endogenous activation of the opioid receptors are still a matter of speculation. Recently, a new family of opioid peptides, the endomorphins, was discovered which preferentially activate the MOR (Zadina et al, 1997). This suggested the hypothesis that the enkephalins function as the endogenous ligands for DOR and the endomorphins act as the endogenous MOR ligands. However attractive that simplicity may be, it remains possible that there is significant promiscuity among the opioid peptides and receptors. Given the proximity between enkephalinergic interneurons and excitatory MOR expressing interneurons in lamina II, it seems likely that these enkephalins would activate the nearby MORs. The selectivity of NK-3 receptor expression on enkephalinergic interneurons in the spinal cord dorsal horn allows us to examine this issue in vivo, while probing the nature of this opioidergic antinociceptive circuit in the cord.

METHODS

Behavioral Experiments

Rats were injected intrathecally with 20 µl of 0.5 mg/ml senktide under halothane anesthesia. At -2, 7, 15, 22, 30, and 40 minutes, rats were tested to determine nociceptive sensitivity on the hot plate test. In this test, rats were placed in a plexiglass container in which the floor was heated to 52.5°C. Behavior was monitored and the latency until hindpaw licking was determined. Rats were removed from the hot plate as soon as a hindpaw was licked. Sixty seconds was used as a cut off value. For studies of the naloxone sensitivity of the behavioral analgesia, naloxone (1 or 10 mg/kg) or saline was injected subcutaneously at the nape of the neck 15 minutes before i.t. injection of senktide. For studies with selective DOR and KOR antagonists, naltrindole HCl (30 nmol (14 µg) in 20 µl saline), nor-binaltorphimine HCl (30 nmol (25 µg) in 20 µl saline) or saline was injected intrathecally under halothane anesthesia immediately prior to the i.t. injection of senktide. For MOR internalization studies, rats were perfused at 27 minutes post-injection.

Perfusion and Tissue Preparation

Following treatment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and then intracardially perfused with 50 ml 0.1 M phosphate buffered saline followed by 500 ml 10% formalin. The brain and spinal cord were dissected out, post-fixed for 4 hours in the same fixative and then transferred to a 30% sucrose solution. Thirty micron sagittal sections of lumbar cord were cut on a freezing microtome.

Immunofluorescent labeling

Sections were blocked in 5% normal goat serum in 0.1 M phosphate buffered saline with 0.3% triton x-100 for 30 minutes and then incubated in a rabbit anti-MOR antibody (Incstar, Stillwater, MN) at a 1:5000 dilution overnight. Sections were washed and then incubated in a Cy-3 conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA) at a 1:600 dilution for 2 hours. Tissue was then washed and mounted on gelatin coated slides.

For the *in vivo* studies, we collected confocal images (Nikon 60 X plan apo (1.40 oil) objective, 2X zoom, 3.0 iris setting on a Biorad MRC 1024) through the optical center of 20 MOR-LI lamina II neurons in the L4/L5 segments of each rat. The number of endosomes in each cell was counted from the images by an investigator who was unaware of the treatment that the rat received. The average number of endosomes per neuron was calculated for each rat. Values are presented as mean +/- standard error of the mean.

Spinal Cord Slice Preparation

P15-23 Sprague Dawley rats were anesthetized with halothane and the lumbar and sacral spinal cord were dissected out. The spinal cord was placed in a bath of carbogenated sucrose buffer (in mM: sucrose 240; KCl 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1.0; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.5) and the dura and dorsal and ventral roots were removed under a dissecting microscope. The cut surface of the lumbar cord was superglued to the stage of a vibratome (Pelco 101 series 1000), placed against a 5% agar block and immersed in carbogenated sucrose buffer. Transverse sections of spinal cord were cut at 500-600 μm and transferred to

carbogenated incubation solution (in mM: NaCl 124; KCl 5; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) for 2 hours. After 2 hours, slices were transferred to carbogenated recording solution at 37°C (in mM: NaCl 127; KCl 1.9; $MgSO_4$ 1.3; KH_2PO_4 1.2; glucose 10; $NaHCO_3$ 26; $CaCl_2$ 2.4) containing the designated concentrations of senktide, serotonin, norepinephrine, glutamate, or DAMGO for 15 minutes. Slices were fixed overnight in 10% formalin and then transferred to a 30% sucrose solution for several hours. They were next cut at 40 µm on a freezing microtome. Standard immunofluorescent labeling of the MOR was performed and sections were examined at 40x on a Nikon axiophot microscope and at 120x on a Biorad MRC 1024 confocal microscope.

RESULTS

i.t. NK-3 receptor agonists produce analgesia and other behaviors

Intrathecal injection of the NK-3 receptor agonist senktide produced a series of stereotyped behaviors including extension of the hind limbs, whipping of the tail, wet dog shakes, belly crawling, genital licking and freezing. The behaviors were observed to occur over the course of an hour after injection of senktide. Generally, the hind limb extension and tail whipping, if it occurred, was observed early after injection (0-15 minutes), and the more complex behaviors, wet dog shakes, belly crawling, genital licking and freezing were observed at after a short delay (from about 10 - 40 minutes). This may have been complicated by the fact that the intrathecal injections were done under halothane

anesthesia; although the rats regained conciousness quickly, they were obviously recovering from the anesthesia for the first 5 minutes of observation.

In addition to these behaviors, intrathecal senktide was significantly anti-nociceptive. Treated animals displayed increased latencies on the 52.5 ° C hot plate test for over 30 minutes post-injection (Figure 2). While the analgesia consistently accompanied the other behaviors in male rats, this was not always the case in females. Of the 8 females tested, 5 showed no increase in hot plate latency, despite displaying the other behaviors described above. The remaining three female animals showed analgesic responses equivalent to or even greater than seen in the males. When the same animals and their saline controls were reinjected with i.t. senktide 8 days later to look for MOR internalization, anti-nociception was observed in 8 of the animals (although the extent of the analgesia varied greatly), while 3 showed no change in latency. The behavior of the female rats on the second trial was not predicted by their behavior on the first. Notably, however, the hot plate responses of cage-mates (rats were housed 2/cage) were almost always similar. Due to the unexplained variability observed in the female rats, male rats were used for all further experiments in which we attempted to prevent or reverse the anti-nociception produced by i.t. senktide.

Opioid receptor selectivity of the anti-nociception

Spinal administration of NK-3 receptor agonists has been reported to produce anti-nociception via an opioidergic mechanism. To determine which of the three cloned opioid receptors mediated this effect, we attempted to reverse

i.t. senktide induced anti-nociception with opioid receptor antagonists. Rats were injected with a low or high dose of naloxone (1 or 10 mg/kg) subcuataneously prior to i.t. injection of senktide. While we have shown that 1 mg/kg s.c. naloxone is sufficient to completely reverse analgesia and MOR internalization produced by intrathecal injection of a high dose (1 μg) of the selective MOR agonist DAMGO, this dose of naloxone is not apt to effectively antagonize opioid activity at the DOR or KOR. In contrast, 10 mg/kg naloxone should be adequate to prevent activity at all three opioid receptors. Neither dose of naloxone had an effect on hot plate latencies on its own. We found that intrathecal senktide anti-nociception was unaltered in rats pretreated with 1 mg/kg naloxone, but completely abolished by 10 mg/kg naloxone. This suggested that MORs were not required for producing the analgesic effects, but did not distinguish between DOR or KORs. To determine which of these receptors was necessary for the behavior, we gave rats an intrathecal dose of either the DOR selective antagonist naltrindole or the KOR selective antagonist nor-binaltorphimine (nor-BNI) immediately prior to injection with senktide. Nor-BNI had no effect on senktide induced hot plate analgesia at either a 5 or 25 μg dose. Naltrindole, however, completely prevented the senktide induced increase in hot plate latency, suggesting that DORs were responsible for the analgesic effect. Again, despite reversing i.t. senktide induced analgesia, i.t. naltrindole did not have similar effects on other senktide associated behaviors (fig 4 A-D)

Senktide effect on MOR internalization in vivo

Eleven male and eleven female rats were injected intrathecally with senktide, tested for analgesia on the hot plate test and then perfused at 20-30 minutes post-injection. Sagittal sections of spinal cord from the L4/L5 segments were labeled for MOR immunoreactivity and examined for MOR internalization. Sections from senktide treated rats were indistinguishable from sections from 4 male saline treated controls. Labeling was observed predominantly on the plasma membrane of the cell body and dendrites of lamina II neurons. Endosomal labeling was not observed

Senktide effects on MOR internalization in spinal cord slices

To be sure that adequate senktide reached the receptors, we looked at the ability of bath applied senktide to internalize lamina II MORs in a spinal cord slice preparation. At all doses tested (0- 1000 nM) no MOR internalization was detected. Slices were indistinguishable from buffer treated controls. To further excite the slice, senktide was co-applied in combination with 500 μM glutamate, 100 μM serotonin, or 100 μM norepinephrine in hopes of increasing enkephalin release. Even under these conditions, no MOR internalization was observed. In all experiments, sister slices were treated with DAMGO in the bath as a positive control for slice health and receptor function. In all cases, DAMGO produced extensive MOR internalization in lamina II.

DISCUSSION

While not directly confirmed, evidence suggests that the NK-3 receptor is expressed on enkephalinergic lamina II neurons. NK-3 receptors are found on 86% of NOS expressing lamina II cells (Seybold et al, 1997) and NOS has been found to be expressed in many lamina II GABAergic non-glycinergic and glycinergic neurons (Spike et al, 1993). Similarly, enkephalin has been shown to be expressed in the lamina II GABAergic non-glycinergic neuron population (Todd et al, 1992). While we were unable to demonstrate that these neuronal populations overlap due to technical incompatibility between the labeling protocols, the anatomical evidence is at least consistent with NK-3 receptor and enkephalin being co-expressed in some lamina II neurons. The behavioral data that indicate that intrathecal NK-3 receptor agonists release an endogenous opioid are consistent with this proposed distribution and provide further support for the model.

More importantly perhaps, we show that expression of the NK-3 receptor is restricted to lamina II. We found no primary afferent labeling with the NK-3 receptor antibody. The expression of endomorphins has been shown to be restricted to small diameter primary afferents in the spinal cord (Martin-Schild et al, 1998; Pierce et al, 1998). Given their exclusive distributions, it is unlikely that NK-3 receptor agonists would act to release these endomorphins in the spinal cord. Similarly, while enkephalinergic neurons are expressed in other regions of the spinal cord, the overlap between NK-3 receptor and enkephalin expression is restricted to lamina II, suggesting that these lamina II neurons and endogenous enkephalins are responsible for the effects observed.

We show that intrathecal senktide anti-nociception is blocked only by high doses of naloxone and selective DOR antagonists. Doses of opioid antagonists sufficient to block MOR and KOR activation had no effect on senktide-induced analgesia. Thus, it appears that the anti-nociceptive effects of the presumed enkephalin release require only activation of DORs. Whether the DORs involved are expressed on primary afferent terminals or post-synaptic neurons is not known, however, activation of either is consistent with models of anti-nociceptive mechanisms in the spinal cord. The anti-nociception observed in response to i.t. senktide is modest in comparison to that which can be produced by intrathecal injection of exogenous opioids. This might be explained by the specificity of these endogenous opioid actions; selective activation of DORs might produce a more mild analgesia than is seen with activation of MORs or DORs and MORs in combination.

Despite their regional proximity and the well documented activity of enkephalins at MORs, we found no evidence that the enkephalins released by NK-3 receptor activation had any activity at MORs in the cord. A systemic dose of naloxone (1 mg/kg) which we have shown to completely antagonize the intrathecal effects of a high dose of the selective MOR agonist DAMGO (1 μg) (see chapter 4 part 1), had no effect on senktide induced antinociception. Additionally, senktide produced no detectable internalization of MORs in lamina II both in vivo and with bath application in an in vitro slice preparation. Thus, lamina II enkephalins appear to target spinal DORs in a highly selective manner. Activity at nearby MORs, if it occurred, was negligible. This suggests that, at least in the spinal cord, there may be specificity of opioid ligands for their

preferred receptors, with enkephalins targeting DORs, endomorphins targeting MORs and dynorphins targeting KORs.

We found that despite displaying indistinguishable motor effects in response to intrathecal senktide (wet dog shakes, freezing, hind limb extension), female rats showed much more variation in the extent of analgesia produced by the drug, with some animals displaying no anti-nociceptive effects and others demonstrating considerably greater analgesia than seen in the males. Notably, the anti-nociception observed in female animals was generally consistent between female animals in the same cage. As co-housed female animals tend to synchronize their reproductive cycles, this suggests that the differences may be related to the female's ovarian cycles.

Enkephalin expression is known to be regulated by the estrogen receptor and enkephalin levels in the spinal cord of female rats have been shown to be modulated greatly with natural changes in estrogen levels (Amandusson et al, 1999). As at least the majority of the antinociception produced by senktide is mediated through or requires activation of the DOR, it is not surprising that variation in the extent of behavioral antinociception would be observed in animals with varying enkephalin expression levels. While numerous other factors could be responsible for the differences seen in the anti-nociception observed in the male versus the female rats, it is comforting that simple plausible mechanisms for these differences seem capable of explaining the behavior. More study of these gender differences may be useful for explaining the myriad of observations of male/female differences in pain sensitivity and tolerance, susceptibility to pathological pain states and analgesia.

Interestingly, it has been suggested that opioid mediated stress induced analgesia involves met-enkephalin mediated activation of delta opioid receptors in the spinal cord. Intrathecal antiserum against met-enkephalin prevents anti-nociception produced by a cold water swim (4 degrees C; 3 minutes), and the delta 2 receptor antagonist naltriben antagonizes analgesia from either this forced swim or intrathecal met-enkephalin (Mizoguchi et al, 1997) Notably, similar to our observations with i.t. senktide induced analgesia, a selective mu opioid receptor antagonist, CTOP, did not affect swim stress induced antinociception. It is thus possible that the anti-nociceptive circuit examined in this paper is the same as that activated during opioid mediated stress analgesia. It would be interesting to determine if intrathecal NK-3 receptor antagonists could reduce this form of stress induced analgesia, as the parallels between the pharmacology of cold water swim stress and i.t. senktide induced analgesia suggest they may share spinal mechanisms.

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Figure 1: Intrathecal senktide produces a thermal anti-nociception that is more variable in females. Intrathecal injection of senktide produces an increase in latency to hindpaw licking on the hot plate in male and some female rats (n= 5 males; n= 19 females (11 responders, 8 non-responders).

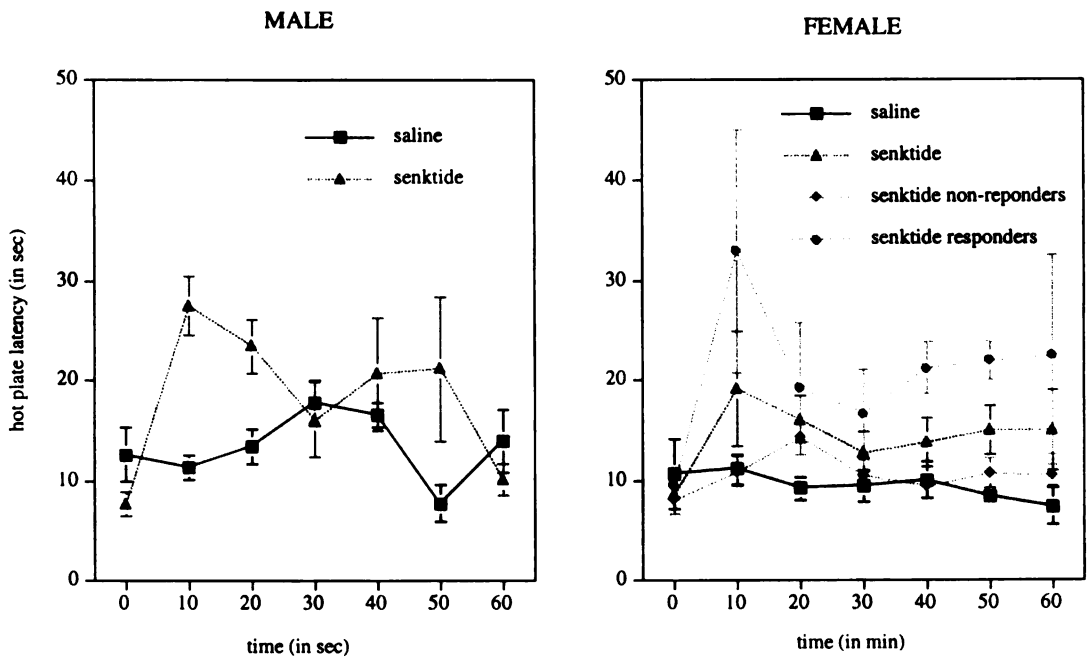

MALE
FEMALE
hot plate latency (in sec)
time (in sec)
time (in min)
saline
senktide
senktide non-reponders
senktide responders
0
10
20
30
40
50
60

Figure 2: Intrathecal senktide analgesia is prevented by antagonists that block DOR, but not MOR or KOR activation. A) Ten mg/kg systemic naloxone but not 1 mg/kg systemic naloxone prevents the anti-nociception produced by intrathecal senktide ($p=0.0188$; $n= 5$). B) Intrathecal naltrindole, a DOR antagonist, but not intrathecal nor-binaltorphamine, a KOR antagonist prevents the anti-nociception produced by intrathecal senktide ($p=0.0353$; $n=6-9$).

hot plate latency (in sec)
A:
30
20
10
0
saline saline
saline senktide
naloxone (1) saline
naloxone (1) senktide
naloxone (10) saline
naloxone (10) senktide
B:
30
20
10
0
saline saline
saline senktide
NOR BNI (25) saline
NOR BNI (5) senktide
NOR BNI (25) senktide
Naltrindole (25) saline
Naltrindole (25) senktide

Figure 3: Other i.t. senktide associated behaviors are not similarly altered by 10 but not 1 mg/kg naloxone. A) Systemic naloxone increases the tendency for rats to jump during the hot plate test. B) One mg/kg naloxone reduces wet dog shake behavior produced by intrathecal senktide. Ten mg/kg naloxone has no added effect. C) Naloxone increases and senktide potentiates the propensity for genital licking. D) Both intrathecal senktide and systemic naloxone increase the incidence of freezing behavior, however intrathecal senktide limits the extent of freezing behavior seen in naloxone injected rats.

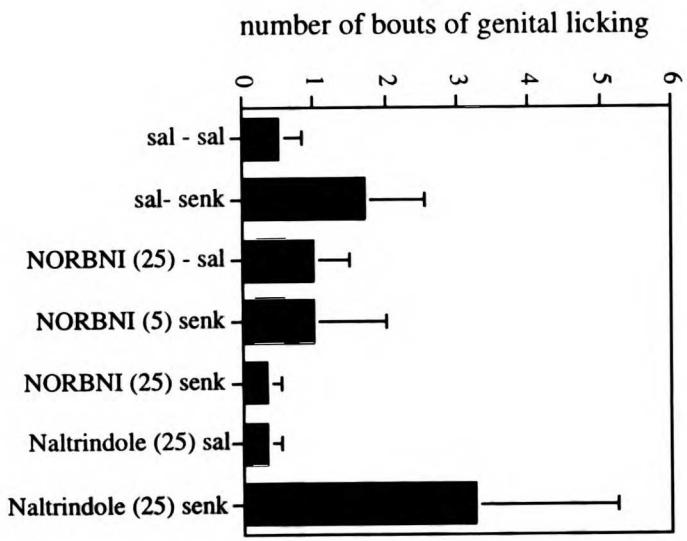
A:
1
0.5
0
sal - sal
sal - senk
nal(1) - sal
nal(1) - senk
nal(10) - sal
nal(10) - senk

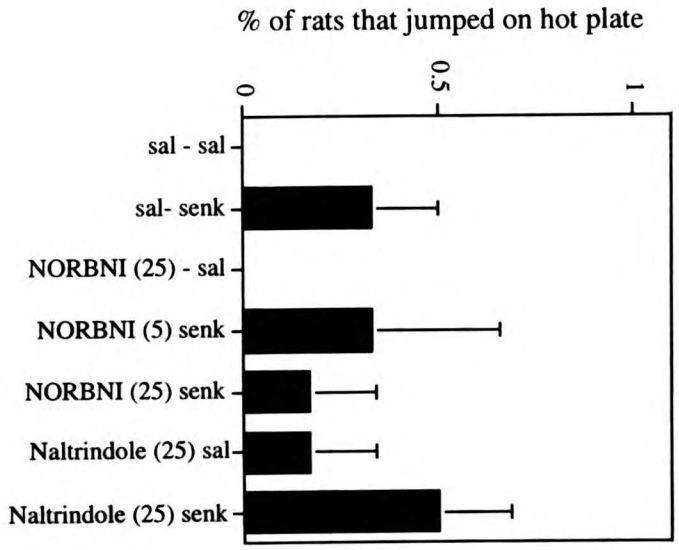
B:
number of wet dog shakes
40
30
20
10
0
sal - sal
sal - senk
nal(1) - sal
nal(1) - senk
nal(10) - sal
nal(10) - senk

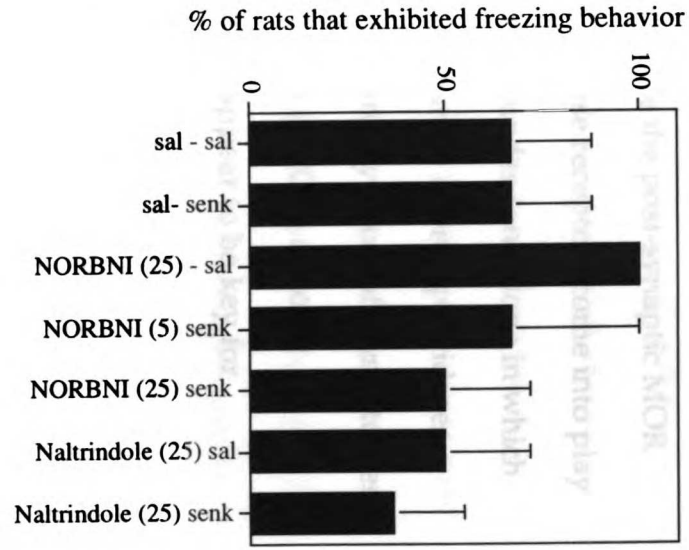
C:
number of bouts of genital licking
5
4
3
2
1
0
sal - sal
sal - senk
nal(1) - sal
nal(1) - senk
nal(10) - sal
nal(10) - senk

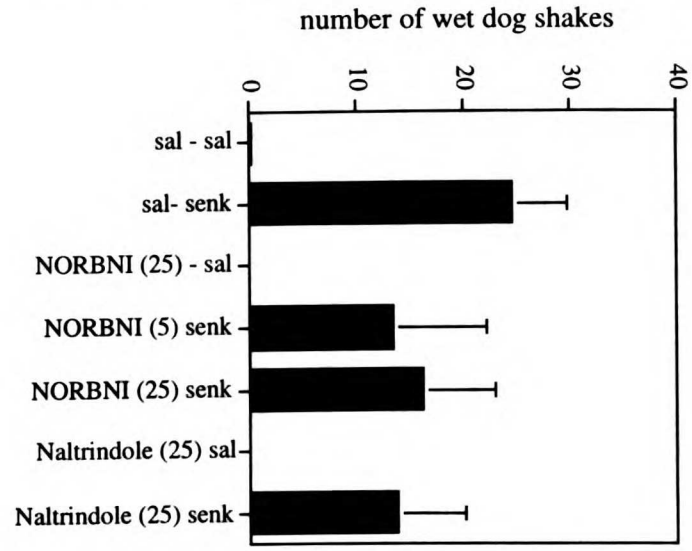
D:
% of rats showing freezing behavior
1
0.5
0
sal - sal
sal - senk
nal(1) - sal
nal(1) - senk
nal(10) - sal
nal(10) - senk

Figure 4: Other i.t. senktide associated behaviors are not similarly altered by intrathecal DOR but not KOR antagonists. A) Intrathecal DOR and KOR antagonists do not affect the tendency for rats to jump on the hot plate. B) Intrathecal DOR and KOR antagonists do not decrease senktide induced wet dog shake behaviors. C) Intrathecal KOR and DOR antagonists have no effects on genital licking behaviors. D) No effects of senktide or KOR or DOR antagonists on freezing behaviors were noted. However, a large increase in freezing behavior was observed in this experiment in all groups, most likely due to the halothane anesthesia used during intrathecal injections.


number of bouts of genital licking
0
1
2
3
4
5
6
sal - sal
sal- senk
NORBNI (25) - sal
NORBNI (5) senk
NORBNI (25) senk
Naltrindole (25) sal
Naltrindole (25) senk


% of rats that jumped on hot plate
0
0.5
1
sal - sal
sal- senk
NORBNI (25) - sal
NORBNI (5) senk
NORBNI (25) senk
Naltrindole (25) sal
Naltrindole (25) senk


% of rats that exhibited freezing behavior
0
50
100
sal - sal
sal- senk
NORBNI (25) - sal
NORBNI (5) senk
NORBNI (25) senk
Naltrindole (25) sal
Naltrindole (25) senk


number of wet dog shakes
0
10
20
30
40
sal - sal
sal- senk
NORBNI (25) - sal
NORBNI (5) senk
NORBNI (25) senk
Naltrindole (25) sal
Naltrindole (25) senk

FURTHER CONCLUSIONS, DISCUSSION AND QUESTIONS

Although detectable MOR internalization appears to occur in response to behaviorally relevant stimulation by opioids, we were unable to find any natural stimulus which induced MOR internalization in lamina II interneurons in vivo. Release of neuropeptide containing vesicles from primary afferent c-fibers thought to express endomorphins, stress analgesia and activation of descending modulatory pathways, and excitation of lamina II inhibitory interneurons all failed to produce MOR internalization in post-synaptic MORs. While none of these results is at odds with the current literature (PAG disinhibition induced spinal analgesia is likely mediated through presynaptic MORs and primary afferent endomorphins are also apt to work at these receptors, and stress induced analgesia and lamina II enkephalin release likely activate spinal DORs) it is still curious that none of these opioidergic effects involve the post-synaptic MOR population. Indeed, the conditions under which these receptors come into play remain a mystery. Perhaps they are only activated under conditions in which endogenous opioids are highly upregulated (pregnancy) or endopeptidases involved in their breakdown are inhibited. Alternatively, most of these studies were done exclusively in male rats. Perhaps lamina II MORs are only behaviorally relevant in females. Lamina II MORs appear to be key for production of spinal analgesia in response to exogenous opioids, however, further study is necessary to determine their endogenous function.

OVERALL CONCLUSIONS

These studies shed light on both specific aspects of neuropeptide modulation of nociception and neuropeptide function in general. Spinal cord tachykinin and opioid signaling appears even more regulated and specific then previously thought, and the extent, downstream signaling and occurrence of receptor activation is both controlled and limited.

This work provides evidence that neuropeptides can have selective localized effects on a given subtype of receptor in the spinal cord as well as different effects upon a single receptor. For example, we show that senktide induced enkephalin release specifically activates DORs, despite the close proximity of MORs. Whether this is due to limited release, which keeps enkephalin concentrations too low to activate the MORs, or to localized release near DORs and rapid degradation by endopeptidases is not known. Regardless, it is clear that opioids are not acting in a global endocrine-like fashion in the spinal cord; release of a given pool of opioids in the spinal cord activates only a selected population of the many spinal opioid receptors. Additionally, we demonstrate that tachykinins may differentially activate the NK-1 receptor. Despite being co-released from the same dense core vesicles and acting upon the NK-1 receptor at similar concentrations, SP and NKA activate NK-1 receptor on different neuron populations. As others have provided evidence for triggering of different signaling cascades through the NK-1 receptor by the two tachykinins, this allows for different receptor effects

depending on receptor localization and expression of degradative enzymes starting with a single peptide source and a single receptor population.

We also demonstrate that neuropeptide action is rare and associated with specific environmental or behavioral states. Tachykinins and opioids are not used for general neurotransmission in the spinal cord dorsal horn. Activation of these neuropeptide receptors occurs only under special conditions and is associated with significant behavioral modulation. No basal or MOR or NK-1 receptor activation was detected, nor did we find occasions during which receptor activation was not associated with behavioral effects.

Tachykinins signal the presence of tissue damage, not nociception per se. NK-1 receptors are activated not at intensities at the pain threshold, but only following stimuli that will lead to inflammation. They directly activate spinal cord lamina I projection neurons that excite nociceptive responsive parabrachial neurons projecting to limbic nuclei such as the amygdala and hypothalamus. Thus, they are apt to be more important for promoting behaviors that contribute to acute injury response and recovery.

Opioids dampen pain sensation but do not shut down tachykinin signaling. This might allow them to shut off conscious awareness of pain without turning off protective autonomic and emotional responses. It also means that tachykinin mediated sensitization will occur despite the animal being in an analgesic state.

Numerous opioid mechanisms exist in the spinal cord that produce analgesia. These may be activated individually endogenously under different behavioral conditions, to produce qualitatively different opioid mediated anti-nociception. Which are activated when and by what is not completely determined, nor are the differences or consequences of activating one versus another obvious. It is unclear when post-synaptic MORs are employed. It is likely that activation of these MORs leads to analgesia, but when they are used has not been identified. Pre-synaptic MORs on primary afferent sensory neurons appear important for behavioral anti-nociception induced by periaqueductal gray activation, and spinal cord DORs mediate at least some forms of stress induced analgesia, as well as the anti-nociception produced by intrathecal NK-3 receptor agonists. It will be interesting to investigate how these different anti-nociceptive mechanisms alter nociceptive processing in the dorsal horn.

Though tachykinin and opioid systems in the dorsal horn are the most studied of spinal cord neuropeptide systems, there is still much that we do not know. These studies make it clear that both systems are not globally regulated, nor are receptor effects constant. Further study of the dorsal horn circuitry underlying specific tachykinin and opioid effects will greatly enhance our understanding of how pain is modulated.