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Title Page

Resting and Evoked Spinal Substance P Release during Chronic Intrathecal Morphine Infusion: Parallels with Tolerance and Dependence

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Abbreviations:

AP-5: 2-amino 5-phosphonovaleric acid

IT: intrathecal

NK1r: neurokinin 1 receptor

SP: substance P

L 703,606: *cis*-2-(Diphenylmethyl)-N-[(2-iodophenyl)methyl]-1 azabicyclo[2.2.2]octan-3-amine

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Abstract

Spinal opiate analgesia is associated with presynaptic inhibition of release of excitatory neurotransmitters/neuromodulators, e.g., substance P (SP), from primary afferent terminals. Chronic intrathecal (IT) administration of opiates such as morphine results in an initial analgesia followed by tolerance and a state of dependence. In this study, we examined the resting and evoked neurokinin 1 receptor (NK1r) internalization, indicative of endogenous SP release, in dorsal horn neurons of the lumbar spinal cord by immunocytochemistry during chronic IT infusion of morphine in rats. Noxious mechanical stimulation (compression) applied to unilateral hind paw evoked a significant increase in NK1r internalization in lamina I neurons in the ipsilateral dorsal horn. IT morphine infusion (40 nmol/μl/h) for 1 day possessed similar analgesic efficacy as acute morphine, and blocked compression-induced spinal NK1r internalization. After 5 days of morphine infusion, thermal escape latencies were the same as in preinfusion animals or saline-infused controls, and compression-evoked NK1r internalization was no longer suppressed. Systemic administration of naloxone to rats on day 6 of morphine infusion resulted in prominent withdrawal behaviors and a concomitant increase in NK1r internalization in dorsal horn. The naloxone-induced internalization was blocked by NK1r antagonist L-703,606 or pretreatment with capsaicin, confirming that the internalization is due to the endogenous SP release from the primary afferents. We conclude that inability to suppress release of excitatory neurotransmitters/neuromodulators from primary afferents by morphine after chronic exposure is an important component in spinal morphine tolerance, and excessive release from these afferents contributes to the spinal morphine withdrawal syndrome.

Introduction

Acute spinal delivery of morphine yields a dose dependent analgesia, primarily mediated by the activation of μ opioid receptors in the dorsal horn. These receptors are localized both presynaptically on small afferent terminals and post synaptically on neurons in lamina I-II of the dorsal horn (Fields et al., 1980; Abbadie et al., 2001). Inhibition of voltage-gated calcium channels (Schroeder et al., 1991) via the activation of presynaptic μ opioid receptors, which may serve to reduce the release from primary afferents of excitatory neurotransmitters or neuromodulators, such as SP (Jessell and Iversen, 1977; Chang et al., 1989; Yaksh et al., 1980), has been implicated as one of the potential mechanisms in spinal opiate analgesia.

With continuous IT administration of μ agonists such as morphine or DAMGO, a progressive time-dependent loss of analgesia has been observed. This loss of effect with the continued presence of drug, defined as tolerance, can be overcome by increasing the agonist dose. Thus, following persistent exposure, there is a right shift in the IT morphine or DAMGO dose response curve (Stevens and Yaksh, 1989a). In this model, the phenomenon of spinal dependence is revealed by the appearance of agitation and hyperalgesia when the spinal opiate is discontinued or a pharmacological antagonist administered, reflecting withdrawal (Stevens and Yaksh, 1989b).

The cellular and molecular mechanism of opioid tolerance and dependence has been long a subject of interest (Mao et al., 1995; Waldhoer et al., 2004). A variety of mechanisms have been posited including: i) changes in receptor number, function or coupling (Harrison et al., 1998; Bohn et al., 2000); ii) changes in system-level function wherein there is an increase in the neuronal excitability secondary to increases in for example NMDA receptor functions (Inoue et al., 2003); iii) phosphorylating activity of protein kinase C (Mao et al., 1995; Granados-Soto et al., 2000), and/or iv) the expression of "anti-opiate " systems such as dynorphin or cholecystokinin (Harrison et al., 1998; Ossipov et al., 2004). Given the well-defined loss of effect with long term exposure of spinal morphine and the evident effect of spinal μ agonists on SP release, we were interested in determining if persistent spinal opioid receptor activation modulates primary afferent SP release, and whether this modulation correlates with changes in analgesia and withdrawal observed with continuous spinal opiate exposure. Quantification of NK1r internalization in spinal dorsal horn neurons by immunocytochemistry has been used as a measurement for endogenous SP release, a method that has been widely reported (Abbadie et al., 1997; Trafton et al., 1999; Marvizon et al., 1997). We recently demonstrated that intrathecal (IT) morphine and DAMGO, at doses which produce analgesia, prevents afferent stimulation-induced NK1r internalization (Kondo, et al 2005). Accordingly, in the present study, we aimed to investigate: i) if there are changes in resting or noxious stimulation-evoked spinal SP release during chronic IT morphine infusion in association with the development of tolerance; and ii) whether

naloxone-precipitated morphine withdrawal would evoke spinal SP release, a reflection of terminal dependence and withdrawal.

Methods

Animals

The protocols performed in the present study were approved by the Animal Care Committee of the University of California San Diego, in accordance with the guidelines of the National Institute of Health and United States Department of Agriculture. Male Holzman Sprague-Dawley rats (300-400 g) were obtained from Harlan (Indianapolis, IN, USA) and housed in individual cages on a 12:12 light/dark cycle (light on at 07:00h). Food and water were available *ad libitum.*

Animal Preparation

IT catheter implantation and morphine infusion. Rats were implanted with a single IT catheter for morphine delivery. Briefly, rats were anesthetized by induction with 4% isoflurane in a room air/oxygen mixture (1:1), and the anesthesia maintained with 2% isoflurane delivered by mask. The animal was placed in a stereotaxic head holder with the head flexed forward. A midline incision was made on the back of the occipital bone and the neck to expose the cisternal membrane. The membrane was carefully opened with a stab blade and a single lumen polyethylene (PE-5: OD = 0.36 mm) catheter (8.5 cm) inserted and passed into the intrathecal space surrounding L3-L4 spinal segments. The

other end of the catheter was connected to an Alzet osmotic mini-pump (model 2001, flow rate 1 μl/h, Palo Alto, CA) pre-filled with drug solution and inserted into a subcutaneous pouch between medial borders of the scapulas. In studies of dependence, groups of rats were implanted as described above with a double lumen intrathecal catheter (OD 0.36mm x 0.71mm, 8 cm) as previously described (Hayes et al., 2003). Following the procedures of IT catheter implantation described above, one of the lumens was connected to an osmotic mini-pump. The other free end was externalized on the forehead, and used for drug administration. Rats showing motor weakness or signs of paresis upon recovery from anesthesia were eliminated from the experiment and euthanized immediately.

Assessment of thermal nociception

To determine the development of morphine tolerance, the latency of the hind paw withdrawal evoked by thermal stimulation was evaluated using a modified Hargreaves Box (Dirig et al., 1997) on day 0, 1, 3, and 5 (day 0 refers to the day of IT implantation). Briefly, animals were placed on a 30˚C temperature glass surface inside plexiglass cages. A radiant lamp stimulus was focused on the plantar surface of the hind paw. The lamp and timer were immediately turned off by an abrupt withdrawal of the hind paw to the stimulus by a photocell sensor. In the absence of withdrawal, the lamp and timer automatically turned off at 20 seconds (cut-off time) after stimulus onset. Thermal escape latency data were

expressed as % maximal possible effect (%MPE), where %MPE is defined as: (postdrug latency-baseline) x 100 / (cutoff time – baseline).

Study paradigms

Chronic morphine–induced analgesic tolerance. Groups of rats were implanted with IT catheters linked to osmotic pumps as described above. Pumps were preloaded to deliver saline (1 µl/h) or morphine sulfate (40 nmol/µl/h). Rats were tested at day 0, 1, 3, and 5 after implant for their thermal escape latency.

Paw compression evoked NK1r internalization in IT morphine-infused animals. Rats were prepared as described above with intrathecal catheters and subcutaneous minipumps filled to deliver saline or morphine sulfate (40 nmol/µl). On day 1 or day 6 after IT infusion, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). This dose blocked the flexor reflex to hind paw stimulation. The left hind paw was positioned perpendicularly across the mid-point of jaws of a 6' mosquito forceps. The non-serrated jaws were closed to the third click and the compression applied for 60 seconds. Five minutes later, animals were perfusion-fixed, and the spinal cords harvested for NK1r immunostaining (see Immunocytochemistry).

Morphine withdrawal-induced NK1r internalization. Rats received 6-day IT infusion of saline or morphine sulfate (40 nmol/µl). On day 6, morphine withdrawal was induced by a single injection of the opioid antagonist, naloxone (0.6 mg/kg, i.p.). Thirty minutes prior to injection of naloxone, animals were

placed in Plexiglas cylinder for acclimation. Withdrawal signs were scored at 10, 20, 30 and 60 min after the injection of naloxone. Those signs included allodynia to light touch, spontaneous vocalization, abnormal body posture, ejaculation, urination, diarrhea, exophthalmos, piloerection, headshakes, hindlimb extension, chewing/licking, and tremor. The scores ranged from 0-3 (0=none; 1=minor, 2=moderate, 3=severe). In general, minor withdrawal behavior means noticeable change in the parameters being evaluated with occasional episodes; moderate withdrawal shows obvious changes in these clinical observations with frequent episodes; and severe means significant changes with constant occurrence. For example, allodynia was rated as: minor, 1-3 vocalizations during 10 gentle strokes with a light brush; moderate, 4-6 out of 10 strokes, and severe, 7-10 out of 10 strokes. Animals were sacrificed at 10, 30, and 60 min after naloxone, and their spinal cords were dissected out for the examination of NK1r internalization.

In addition, we carried out pharmacological studies to characterize morphine withdrawal-induced NK1r internalization. In these animals prepared with double lumen catheters, spinal morphine was continuously delivered through one of the lumens, and the other lumen was used for the following purposes: i) spinal NK1r antagonism: a group of rats were pretreated with an NK1r antagonist, L703606 (50 nmol/10μl, IT) (Marvizon et al 1997), 15 min prior to naloxone; ii) Depletion of SP in small sensory afferent by capsaicin pretreatment: a group of animals was pretreated with capsaicin (75 μg/10μl, IT) 3 days prior to i.p. naloxone injection (Jessell et al., 1978; Gamse et al., 1981) and iii) NMDA receptor antagonism: a group of rats were pretreated with a competitive NMDA receptor antagonist, AP-5

(10 μg/10μl, IT) (Hua et al, 2004), 15 min prior to naloxone. All animals were sacrificed 10 min after naloxone for the examination of NK1r internalization.

Immunocytochemistry

Tissue preparation. Anesthetized animals were perfused transcardially with 50-100 ml 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). Lumbar spinal cord was removed by laminectomy and postfixed in the same fixative overnight. After cryoprotection in 20% sucrose, 30-micron transverse (coronal) or 35-micron sagittal sections were cut through the lumbar cord using a Leica cryostat. Immunofluorescence staining was performed to examine NK1r expression in the spinal dorsal horn. Briefly, sections were incubated in a rabbit anti-NK1r polyclonal antibody (Advanced Targeting Systems, San Diego, CA) overnight at room temperature. The antibody was diluted to the concentration of 1:3,000 in 0.01 M PBS containing 10% normal goat serum and 0.3% Triton X-100. After rinses in PBS, sections were then incubated for 90 min at room temperature in a goat anti rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Eugene, OR) diluted at 1:1,000 in 0.01 M PBS containing 5% normal goat serum and 0.5% Triton X-100. All sections were finally rinsed and mounted on silane-coated glass slides (Sigma, St. Louis, MO) and coverslipped with ProLong mounting medium (Molecular Probes, Eugene, OR).

Quantification of NK1r internalization. NK1r internalization was counted under an Olympus fluorescence microscope at the magnification of 40 x and followed the standard of the previous reports (Mantyh et al., 1995; Abbadie et al., 1997; Marvizon et al., 1997; Trafton et al., 1999). The total number of NK1r immunoreactive neurons in lamina I and II, with or without NK1r internalization was counted, and presented as the percentage of NK1r positive neurons with internalization versus total NK1r immunoreactive cells. Neuronal profiles that have ten or more endosomes in their soma and the contiguous proximal dendrites were considered to have internalized NK1r receptors. NK1r neurons in both sides of the dorsal horn, i.e. ipsilateral and contralateral to the noxious stimulation, in each section were counted. The person counting the neurons was blinded to the treatment given to the animals, and to the side of the dorsal horns. Three to five sections per segment of the lumbar spinal cord were counted and at least 3 animals were used for each experiment.

Confocal microscopy and image processing. Confocal images of representative NK1r cells were acquired by a Leica TCS SP2 confocal system equipped with AOBS or a Bio-Rad MRC 1024 system at the magnification of 100 x (N.A. 1.40). Images were acquired at a digital size of 1024 x 1024 pixels. Five to ten adjacent optical sections (approximately 0.5 micron-thick) along the Z-axis were projected together for demonstration. Images were processed with Adobe Photoshop software and plotted using Freehand software (Macromedia, San Francisco, CA).

Drugs

Drugs used included morphine (morphine sulfate, Merck, Rahway, NJ); naloxone hydrochloride (DuPont Pharmaceuticals, Wilmington, DE); L-703,606 (Sigma, St. Louis, MO) and capsaicin (Sigma, St. Louis, MO). Morphine, naloxone and AP-5 were freshly prepared in sterile 0.9% NaCl solution. Capsaicin and L-703, 606 were prepared in a solution of 10% ß-hydroxypropylcyclodextrin (Wacker Chemie, Neuperlach, Germany).

Statistical analysis

Data acquired were analyzed using Prism 3.0 for Macintosh or 4.01 for PC (GraphPad, San Diego, CA). All data presented are presented as mean with SEM. The statistical significance was calculated using one-way ANOVA with Bonferroni's post-hoc test. The p value less than 0.05 is defined to be significant. Graphs were plotted using Prism software.

Results

Behavioral effects of intrathecal morphine infusion

Chronic morphine-induced analgesic tolerance. Continuous IT infusion of morphine (40 nmol /µl/h) produced a significant increase in thermal escape latencies, compared with saline-infused animals on day 1, with a decline on day 3 and returning to baseline on day 5. There was no statistically significant

difference between morphine- and saline-infused animals on day 5 (Fig.1A). These observations indicate the development of analgesic tolerance produced by chronic intrathecal morphine infusion.

Naloxone precipitated morphine withdrawal. Naloxone administration (i.p.) to morphine-tolerant rats on day 6 produced profound withdrawal behaviors, which include agitation, vocalization, biting and grooming of the flanks. Few signs were noted in saline-infused animals. The withdrawal signs began shortly after injection of naloxone (<2-3 min) and lasted for approximately 60 min, with the peak effect at 10 min (Fig.1B).

Noxious stimulation-induced NK1r internalization in chronic morphine-infused rats

Under non-stimulated conditions (naïve), NK1r receptors undergo minimal internalization in the spinal dorsal horn (less than 10%). In saline-infused rats, 5 min after a unilateral mechanical paw compression, NK1r internalization was observed in neurons in lamina I of the ipsilateral, but not the contralateral side of the lumbar spinal cord (Figs.2A, B, 3A). Moreover, NK1r neurons with internalization were more numerous in the lower lumbar segments (e.g., L5-6) (Fig.3A). The profile of internalization evoked by paw compression in the saline-infused rats resembled that of NK1r internalization observed in rats without chronic IT infusion (see Kondo, et al, 2005).

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In contrast to the saline-infused animals, continuous IT infusion of morphine (40 nmol/μl/h) for 1 day significantly decreased paw compression-induced NK1r internalization in the ipsilateral dorsal horn (Figs. 2C,D, 3B,D). This effect is in parallel with the full expression of morphine analgesia on day 1, indicating that after 24 hours morphine infusion, the ability of morphine to suppress spinal SP release is intact. However, once analgesic tolerance to morphine has been developed on day 6 (Fig.1A), the paw compression evoked spinal NK1r internalization was no longer blocked by morphine (Figs.2E,F, 3C,D), indicating that after 6 days of morphine infusion and at a time when there was no observable antinociception, morphine also failed to inhibit noxious stimulation-induced SP release. Importantly, examination of the percentage of the overall NK1r internalization (total numbers of cells with internalized NK1r versus total numbers of NK1r cells without internalization) in the dorsal horn of morphine-infused rats ipsilateral to the paw compression revealed that the degree of internalization was not different from that observed in saline-infused rats ($p > 0.05$). Unexpectedly, the percentage of the NK1r internalization in the dorsal horn contralateral to the compression in 6-day morphine-infused rats was slightly but significantly higher than that in rats which had received IT saline ($p < 0.05$) (Table 1).

NK1r internalization in response to morphine withdrawal

Concomitant with the withdrawal behaviors described above, naloxone-precipitated morphine withdrawal produced an increase in NK1r internalization in

lamina I neurons of the lumbar dorsal horn (66.6 ± 3.5%, at 10 min), that is significantly higher than that observed in saline-infused animals received naloxone (i.p.) (27.5 ± 2.4%, $P < 0.05$), or morphine-infused animals which received saline (i.p.) injection (26.3 ± 3.9%, $P<0.05$) (Figs.4A,B,C, 5A). The time course of naloxone-evoked NK1r internalization paralleled to the time course of naloxone-induced withdrawal signs, i.e., peaking at 10 min, and declining after 30 min (Fig.5A).

The NK1r internalization evoked by naloxone in morphine tolerant animals was blocked by IT pretreatment with L 703,606 (50 nmol) (Figs.4D, 5B), a selective NK1r antagonist, which has been shown to block the NK1r internalization evoked by afferent stimulation or direct application of SP to spinal cord (Marvizon et al, 1997). Intrathecal capsaicin pretreatment (75 µg, 3 day prior), a well-known technique to deplete afferent SP (Gamse et al., 1981) which was also confirmed in the present study (SP fmol/mg dorsal cord tissue extract: vehicle 68±16 vs. capsaicin 26±5, $p < 0.05$, N=4), blocked naloxone-evoked spinal NK1r internalization (Fig. 5B). These two observations indicate that morphine withdrawal-induced NK1r internalization is due to an increase in endogenous SP release from the primary afferents and reflects the activation of NK1r. However, AP-5, a competitive NMDA receptor antagonist, at a given dose (10 µg, IT) which is potently antihyperalgesic (Hua et al, 2004) and effectively blocks withdrawal symptoms (data not shown, see Jhamandas et al., 1996), did not alter the evoked NK1r internalization (Fig. 5B), suggesting that presynaptic NMDA

receptors on primary afferents are not involved in morphine withdrawal-induced spinal SP release.

Discussion

Continuous exposure of the spinal cord to a fixed concentration of morphine chronically resulted in a progressive loss of spinal opiate receptor-mediated antinociception and a right shift in the dose-effect curves generated with bolus doses of the toleragen (Stevens and Yaksh,1989a; Dunbar and Yaksh, 1996). Our recent work has shown that the intrathecal delivery of μ and ∂ opioid agonists produces a dose-dependent, naloxone-reversible suppression of dorsal horn NK1r internalization evoked by a noxious stimulus (Kondo, et al, 2005). We set out here to examine three questions with regard to the effects of chronic morphine exposure.

1) Is the suppression of the stimulus-evoked SP release by acute morphine lost after continuous morphine exposure, e.g. does it show tolerance? During the initial 24 hrs of IT infusion, morphine (40 nmol/hr) elevated thermal escape latencies and inhibited the noxious stimulus-evoked release of SP. This finding is consistent with the effects produced by acute bolus intrathecal delivery of morphine. It indicates that the infusion paradigm was indeed associated with a behaviorally effective analgesic dose. After 6 days of morphine exposure, animals developed analgesic tolerance, i.e., they showed baseline escape

latencies which were not different from pre-infusion control or from animals receiving intrathecal saline infusion. At this time, the magnitude of the noxious stimulus-evoked NK1r internalization was not different from that noted in control or IT saline-infused animals. Thus, coincident with the loss of behavioral effect, chronic morphine had no effect upon the evoked afferent terminal release. These observations indicate that these terminals display a prominent tolerance to the inhibitory effects of opioids.

2) Does naloxone-evoked spinal SP release reflect terminal dependence? In animals exposed to chronic morphine, naloxone evoked prominent agitation, a mild hyperalgesia and importantly, a significant increase in NK1r internalization in the dorsal horn in the absence of other stimulation. These results suggest that small afferent terminals indeed display a state of opioid dependence and a large SP release in the presence of naloxone, e.g. withdrawal.

3) Does chronic opiate exposure enhance the excitability of the SP releasing terminals? If chronic exposure leads to enhanced terminal excitability, then there might be an increase in resting SP release and/or an enhanced release in response to a given noxious stimulus in spinal morphine tolerant animals, indicating exaggerated reactivity to a peripheral stimulus. In the present work, after 6 days of spinal morphine infusion, baseline thermal escape latencies were not different from either pre-infusion baseline or from the escape latency of IT saline-infused controls. Hence these animals were not hyperalgesic. Thus, at

this time, the paw compression yielded a comparable increase of NK1r internalization in either 6-day morphine- or saline-infused animals. Examination of the basal NK1r internalization in the contralateral spinal dorsal horn at the 6th day after morphine exposure revealed that there was a slight increase in constitutive NK1r internalization over that in IT saline-infused animals. The principal observation, however, is that in the chronic morphine-infused animals, the magnitude of SP release evoked by the stimulus is not different from that in IT saline-infused animals. These observations suggest that in the chronic morphine-infused animal there was an evident failure of opiate mediated inhibition. Of equal importance is that there was no apparent facilitated excitability in primary afferent terminals.

Opiate regulation of primary afferent release of SP.

In agreement with previous reports (Jessell and Iversen, 1977; Yaksh et al., 1980; Aimone and Yaksh, 1989), as well as with our recent work (Kondo et al., 2005), data in the present study indicate that short term (24 hrs) exposure to IT morphine at doses which are behaviorally analgesic reduces the stimulus-evoked NK1r internalization. This direct effect is considered to be an important component of the drug action leading to the potent analgesia initiated by IT μ opioids. While other investigators did not see such an intrathecal block (Trafton et al., 1999), this difference is believed to reflect use of sub-analgesic doses in those studies (Kondo et al, 2005). The mechanism of the opiate inhibition is by block of the opening of voltage-gated calcium channels necessary to mobilize

transmitter release. This inhibitory action is thought to be mediated via activation of the Gi/Go protein and the subsequent interaction of its β/γ subunits with the calcium channel (Herlitze et al., 1996).

Morphine tolerance and spinal SP release

The present studies emphasize that in the continuous presence of opiate agonists, there is a loss of regulatory effect. Based on the role of SP-releasing terminals in nociception, it is plausible that when morphine loses its inhibitory effect on afferent evoked release during chronic treatment, its analgesic action is correspondingly attenuated. This loss of effect with chronic exposure does not appear to be associated with an enhanced response to afferent stimuli. Hence, for the primary afferent, we do not believe that the loss of opiate effect is related to a physiological antagonism, secondary to a facilitation of terminal release. A number of alternatives have been suggested, including the increased expression of anti-opioid peptides such as CCK and dynorphin (Ossipov et al., 2004) or altered signaling involving the appearance of opiate coupling with stimulatory G protein (Gintzler and Chakrabarti, 2004). These results are consistent with the absence of hyperalgesia at day 6. However, our observations are in distinction to other reports (Mayer et al., 1999). We note that in such cases, once or twice daily bolus deliveries are often administered. Given the half life of the intrathecal morphine, we believe that this hyperalgesia is possibly a reflection of periodic withdrawal in which, as shown in these studies, spontaneous increases in C fiber release are in fact observed. Such increases in afferent terminal release of SP

or glutamate would initiate a variety of down stream events which by local effects (such as the release of prostaglandins) (see Jhamandas, et al., 1996; Yaksh, et al., 1999), or by extra spinal circuitry (Vanderah et al., 2001) that would lead to a facilitated state of spinal nociceptive processing. Given the opioid blockade of voltage dependent calcium channel function in regulating terminal release, it is plausible to assume that it is the failure of this coupling which accounts for the failure of inhibition. Such failure of coupling may occur in a number of ways. Previous work has shown that PKC phosphorylates receptor-coupled G protein, thereby suppressing its ability to mediate receptor-evoked inhibition of adenylyl cyclase (Katada et al., 1985; Nestler, 1993) and to regulate calcium channels (Connor et al., 1999). IT infusion of PKC inhibitors has indeed been shown to attenuate tolerance (Granados-Soto et al., 2000).

Withdrawal evoked release of SP

Our data clearly suggest that morphine withdrawal-induced NK1r internalization is due to the induction of SP release presynaptically from primary afferents and activation on the NK1r, since the internalization was blocked by the pretreatment of capsaicin that depletes afferent SP and by NK1r antagonist L-703606. Although the mechanism underlying the induction of SP release by morphine withdrawal is not clear, many factors may contribute to this phenomenon. An increase in excitability of NMDA receptors has been considered to play a primary role in the development of tolerance as well as withdrawal behavior. Blockade of spinal NMDA receptors effectively attenuate withdrawal symptoms (Jhamandas

et al., 1996; Dunbar & Yaksh 1996; Mao et al 1995). Enhancement of spinal release of excitatory amino acids including glutamate during morphine withdrawal has been observed (Jhamandas et al., 1996; Ibuki et al., 2003). NMDA receptors are located on a variety of spinal neurons and dendrites, and also on presynaptic terminals of SP-containing afferents (Liu et al., 1997). Thus, glutamate could activate presynaptic NMDA receptors that stimulate SP release (Liu et al., 1997; Marvizon et al., 1997). In the present study, however, AP-5, a competitive NMDA antagonist given at a dose adequate to block nociceptive transmission, did not affect withdrawal-evoked NK1r internalization. Our data may indicate that withdrawal-evoked SP release is not regulated via the activation of NMDA receptors. Moreover, the presynaptic NMDA effect may itself be complicated. Activation of presynaptic NMDA receptors could initiate primary afferent depolarization and inhibit glutamate release (Bardoni et al, 2004).

Although it was not investigated in the present study, other work has suggested that protein kinases such as PKC could facilitate SP release from primary sensory afferents (Frayer et al., 1999). Expression and/or phosphorylation of this enzyme are elevated in spinal cord after chronic opiate exposure (Granados-Soto et al., 2000). Moreover, morphine-induced “superactivation” of cAMP in the sensory neurons could also be involved in the withdrawal SP release (Waldhoer et al., 2004). An increase of CREB in the DRG in chronic morphine treated animals has been reported (Ma et al., 2001). Upon removal of morphine,

elevated cAMP may promote the release of SP (Hingtgen et al., 1995). Further studies are needed to test the hypotheses.

In conclusion, inability to suppress endogenous SP release from the primary sensory afferents appears to be an important component in spinal morphine tolerance, and excessive SP release from the primary afferents is an important component in spinal morphine withdrawal.

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Footnotes

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Legends for Figures

Fig. 1. Changes of thermal withdrawal latencies and withdrawal scores after chronic IT morphine infusion. **A**. IT morphine (40 nmol/μl/h), but not IT saline infusion (1μl/h), significantly increases hindpaw thermal withdrawal latencies on day 1 and 3 but loses its effect after 5 days; **B**. In rats receiving IT infusion of morphine for 6 days, naloxone (0.6 mg/kg, i.p.) results in a time-dependent increase in the withdrawal signs. Few changes are noted in IT saline-infused rats. One-way ANOVA and Bonferroni's post hoc-test indicates significant differences between morphine- and saline-infused animals (*** $p < 0.001$).

Fig. 2. Effect of morphine on NK1r internalization induced by unilateral paw compression in the lumbar spinal cord. Confocal images showing NK1r internalization in lamina I at the L5 lumbar spinal level in animals that received unilateral noxious hindpaw compression. Remarkable NK1r internalization is observed in neurons ipsilateral **(A)** to hindpaw compression but not on the contralateral side **(B)** of a 6-day saline-infused rat. However, the ipsilateral NK1r internalization **(C)** is abated after 1 day (1D) morphine infusion, with profiles being similar to those observed on the contralateral side **(D)**. After 6-day (6D) morphine infusion, NK1r internalization appears in neurons in the ipsilateral dorsal horn (E), in contrast to the contralateral side (F). Scale bar = 20 μM. Arrows in **(B)** indicate an NK1r positive neuron without NK1r internalization.

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Fig. 3. Quantification of paw compression-induced NK1r internalization in the lumbar spinal dorsal horn in animals that received chronic saline or morphine IT infusion. **A**. Percentage of NK1r internalization (numbers of cells with internalized NK1r versus numbers of NK1r cells without internalization) in lamina I of the dorsal horn contralateral and ipsilateral to the hindpaw compression at different segments after 6 days' IT saline infusion; **B**. Percentage of NK1r internalization in lamina I of the dorsal horn contralateral and ipsilateral to the hindpaw compression at different segments after 1 day's IT morphine infusion; **C**. Percentage of NK1r internalization in lamina I of the dorsal horn contralateral and ipsilateral to the hindpaw compression at different segments after 6 days' IT morphine infusion; **D**. Summary of morphine effect on NK1r internalization in lamina I of the dorsal horn contralateral and ipsilateral to the hindpaw compression. Bar represents the percentage of NK1r neurons in all segments of the lumbar cord. One-way ANOVA and Bonferroni's post hoc-test indicate significant differences in paw compression-induced NK1r internalization between ipsi- and contralateral sides of the dorsal horn in morphine- and saline-infused animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NK1r internalization in neurons in the contralateral dorsal horn in 6-D morphine-infused rats is also significantly higher than that in the contralateral dorsal horn in 6-D saline-infused rats. † $p < 0.05$.

Fig. 4. Confocal images of NK1r immunoreactive cells in the lamina I of the lumbar spinal dorsal horn in rats which were **(A)** infused for 6 days with saline and received naloxone (NLX, 0.6 mg/kg, i.p.) 10 min prior to sacrifice; **(B)** infused for 6 days with morphine followed by i.p. saline; **(C)** infused for 6 days with morphine followed by naloxone (0.6 mg/kg, i.p.); and **(D)** infused for 6 days with morphine followed by L703606 (50 nmol/10μl, IT) and then naloxone (0.6 mg/kg, i.p.) 10 min prior to sacrifice. Significant NK1r internalization is only seen in rats infused for 6 days with morphine followed by naloxone. Scale bar = 20 μM.

Fig. 5. A. Quantification of naloxone-induced spinal NK1r internalization in morphine- and saline-infused rats. Rats received intrathecal infusion of morphine for 6 days and then received an injection of naloxone (NLX, 0.6 mg/kg, i.p.). Animals were sacrificed after 10, 30, and 60 min and the incidence of NK1r internalization was assessed. As shown, naloxone evoked a significant increase in NK1r internalization in lamina I of the lumbar spinal cord in morphine-infused group at 10 and 30 min, but not in the control rats, i.e., the rats infused with saline and then injected with naloxone (Saline+NLX), or infused with morphine and then injected with saline (Mor+Saline), and sacrificed after 10 min. ** $p < 0.01$, *** $p < 0.001$, morphine+NLX vs. the control groups; B. Naloxone-induced NK1r internalization was blocked by pretreatment of L703606 or capsaicin, but not by NMDA antagonist AP-5. L703606 (50 nmol/10μl, IT) was administered 15 min prior to naloxone. Capsaicin (Caps, 75 μg/10μl, IT) was injected 3 days prior to i.p. naloxone injection. AP-5 (10 μg/10μl, IT) was administered 15 min prior to

naloxone. (Sal 6D: Saline infusion for 6 days) *** $p < 0.001$, Morphine 6D / Morphine 6D + AP-5 vs. the other three groups. There is no significant difference between Morphine 6D and Morphine 6D + AP-5 ($p > 0.05$). NK1r internalization was examined 10 min after naloxone injection.

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Tables

Table 1. NK1r internalization (%) in 6-day saline or morphine-infused rats

IT infusion (6 days)	Spinal segments					
	Ipsilateral			Contralateral		
	L1-2	L3-4	L5-6	L1-2	L3-4	L5-6
Saline	14.4±3.9	19.6±2.6	37.4±6.4	2.6±1.2	0.8±0.6	1.3±0.5
Morphine	23.0±8.2	29.6±8.0	34.9+6.0	4.9±2.3	3.3±1.3	3.8±1.5

Figure 1

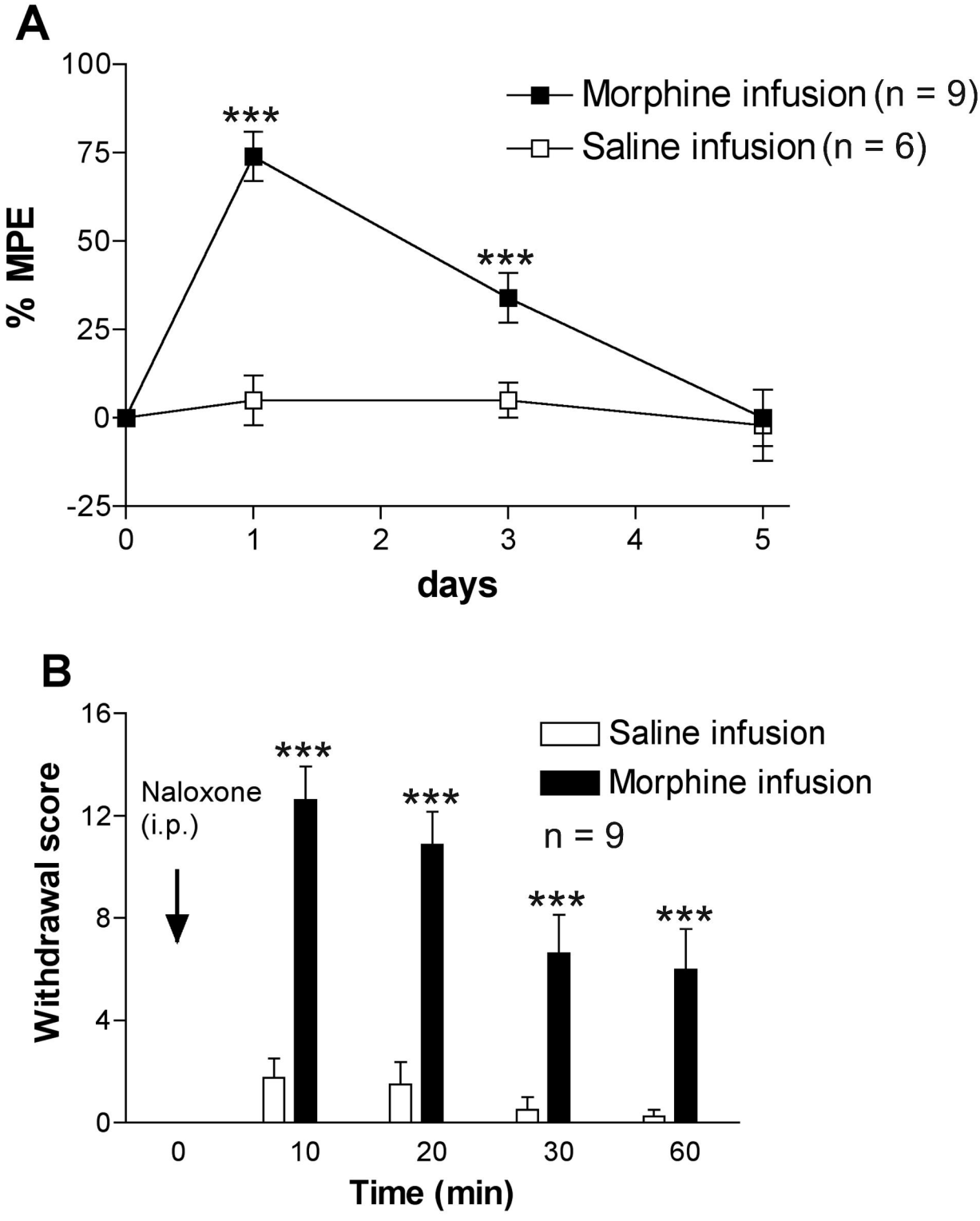

A
100
75
50
25
0
-25
% MPE
***
***
Morphine infusion (n = 9)
Saline infusion (n = 6)
0
1
2
3
4
5
days
B
16
12
8
4
0
Withdrawal score
Naloxone
(i.p.)
***
***
***
***
Saline infusion
Morphine infusion
n = 9
0
10
20
30
60
Time (min)

Figure 2

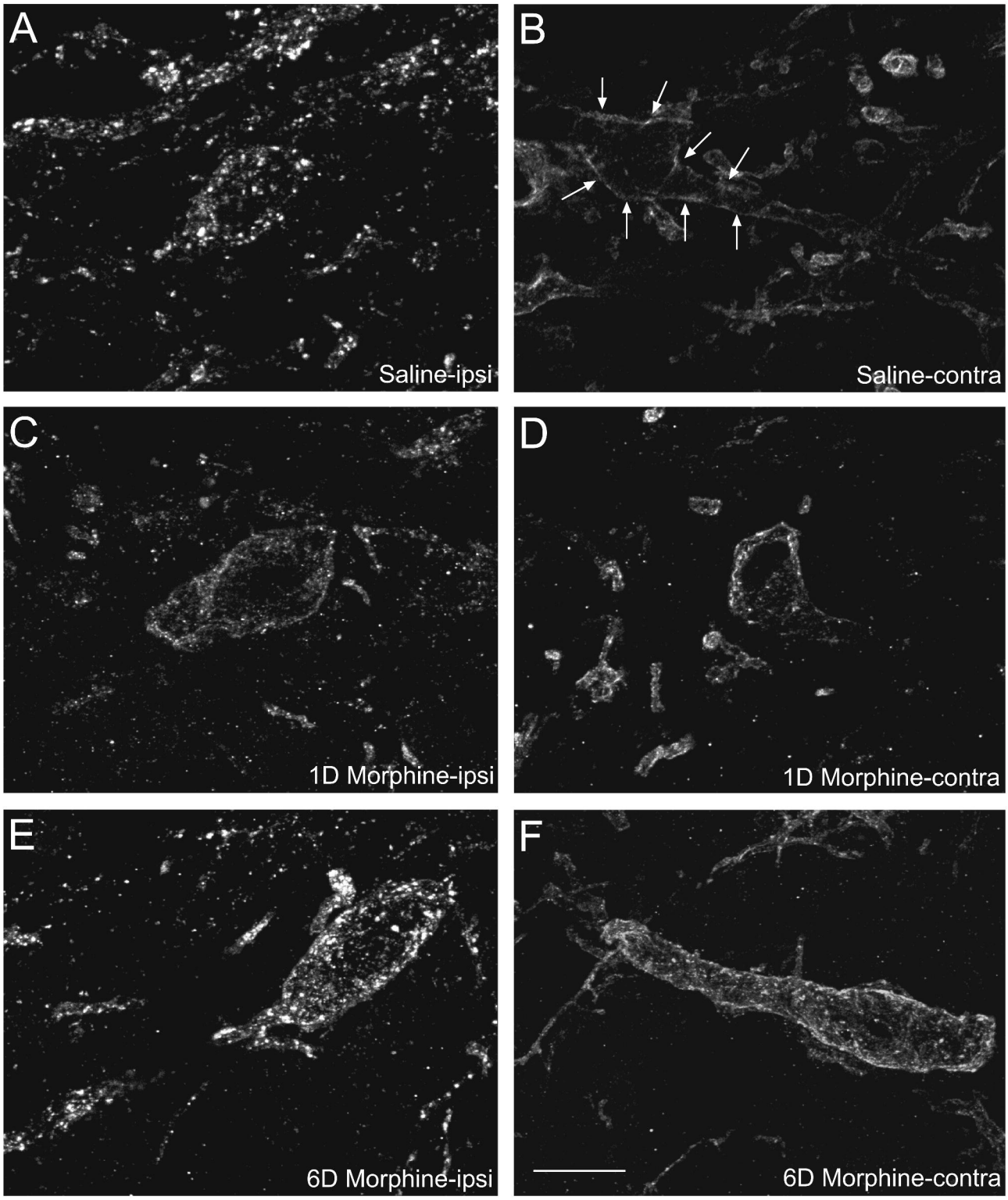

A
Saline-ipsi
B
Saline-contra
C
1D Morphine-ipsi
D
1D Morphine-contra
E
6D Morphine-ipsi
F
6D Morphine-contra

Figure 3

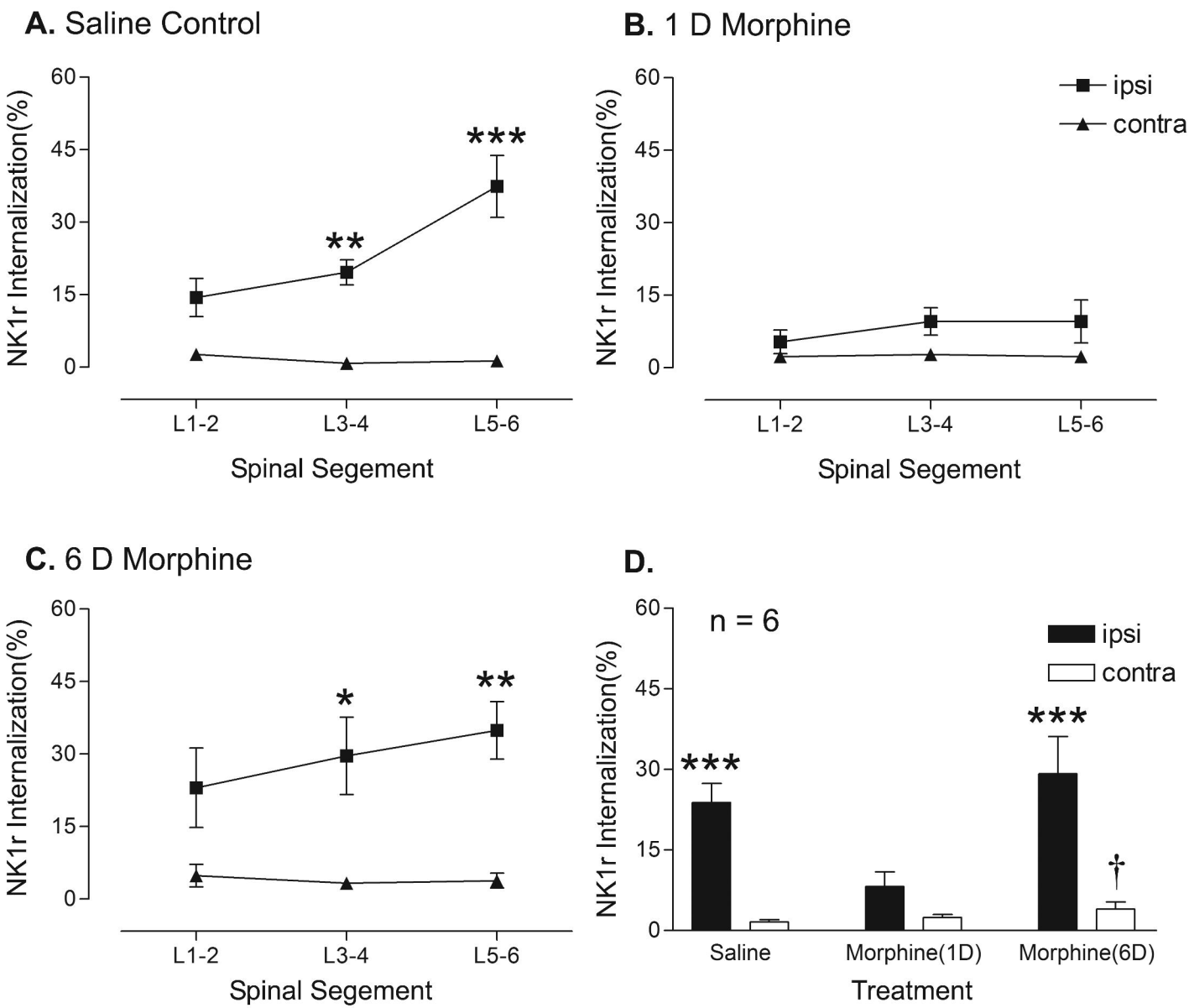

A. Saline Control
B. 1 D Morphine
C. 6 D Morphine
D.
NK1r Internalization(%)
60
45
30
15
0
L1-2
L3-4
L5-6
Spinal Segement
ipsi
contra
*
**
***
n = 6
Saline
Morphine(1D)
Morphine(6D)
Treatment
†

Figure 4

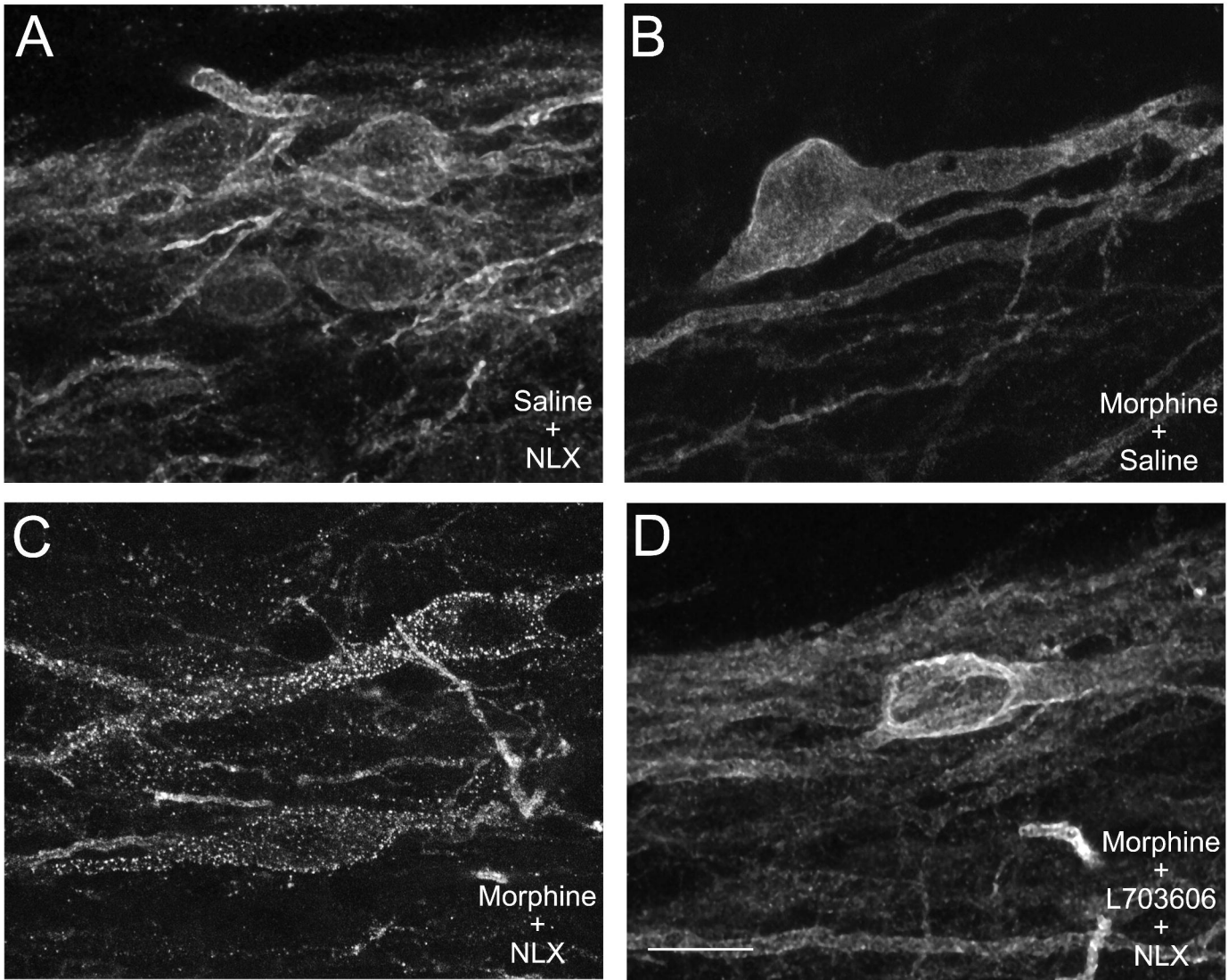
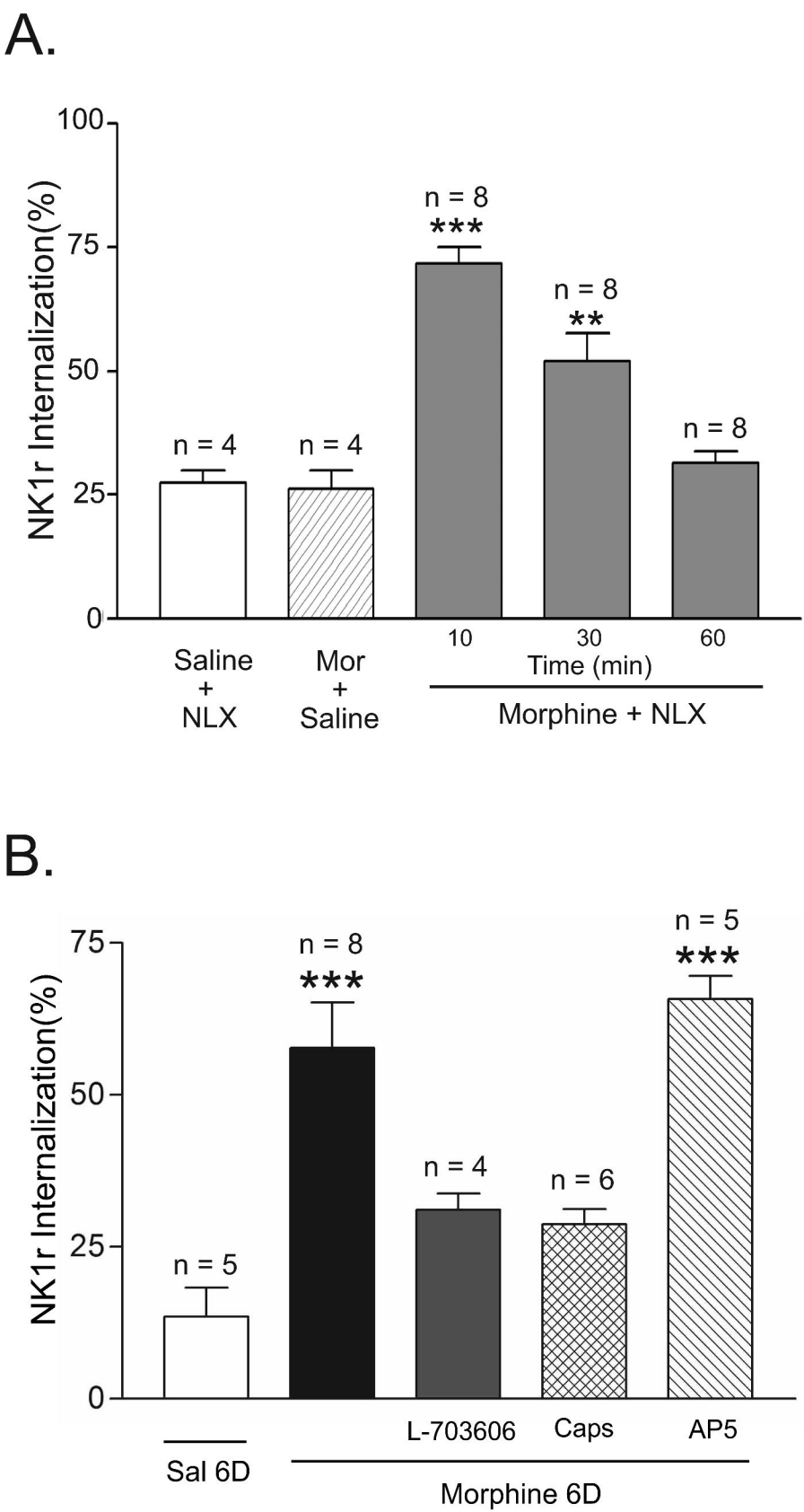

A
Saline
+
NLX
B
Morphine
+
Saline
C
Morphine
+
NLX
D
Morphine
+
L703606
+
NLX

Figure 5


A.
NK1r Internalization(%)
100
75
50
25
0
n = 4
n = 4
n = 8
***
n = 8
**
n = 8
10
30
60
Time (min)
Saline + NLX
Mor + Saline
Morphine + NLX
B.
NK1r Internalization(%)
75
50
25
0
n = 5
n = 8
***
n = 4
n = 6
n = 5
***
L-703606
Caps
AP5
Sal 6D
Morphine 6D