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Decreased Substance P and NK1 Receptor Immunoreactivity and Function in the Spinal Cord Dorsal Horn of Morphine-Treated Neonatal Rats

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Abstract

Opiate analgesic tolerance is defined as a need for higher doses of opiates to maintain pain relief following prolonged opiate exposure. Though changes in the opioid receptor undoubtedly occur during conditions of opiate tolerance, there is increasing evidence that opiate analgesic tolerance is also caused by pronociceptive adaptations in the spinal cord. We have previously observed increased glutamate release in the spinal cord dorsal horn of neonatal rats made tolerant to the opiate morphine. Here we investigate whether spinal substance P (SP) and its receptor, the neurokinin 1 (NK1) receptor, are also modulated by prolonged morphine exposure. Immunocytochemical studies show decreased SP- and NK1-immunoreactivity in the dorsal horn of morphine-treated rats, whereas SP mRNA in the dorsal root ganglia is not changed. Electrophysiological studies show that SP fails to activate the NK1 receptor in the morphine-treated rat. Taken together, the data indicate that chronic

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morphine treatment in the neonatal rat is characterized by a loss of SP effects on the NK1 receptor in lamina I of the neonatal spinal cord dorsal horn. The results are discussed in terms of compensatory spinal cord processes that may contribute to opiate analgesic tolerance.

Perspective—This article describes anatomical and physiological changes that occur in the spinal cord dorsal horn of neonatal rats following chronic morphine treatment. These changes may represent an additional compensatory process of morphine tolerance and may represent an additional therapeutic target for the retention and restoration of pain relief with prolonged morphine treatment.

Keywords

Opiate tolerance; Substance P; NK1 receptor; Spinal cord; Whole-cell voltage clamp

Opiate analgesic tolerance is a laboratory and clinical phenomenon defined as a need for increasingly higher levels of opiates to maintain pain inhibition following repeated drug exposure. Several mechanisms of opiate analgesic tolerance have been proposed, including desensitization or internalization of the opioid receptor (see 10 for review). An additional possible mechanism of opiate tolerance is the development of an increased sensitivity to pain that results from long-term exposure to opiates^{25,31}. Such compensatory (opponent) processes described for opiate analgesic tolerance include the activation of multi-level pain facilitatory systems^{15,21}, as well as spinal mechanisms, including the down-regulation of spinal glutamate transporters^{26,43} and increased NMDA receptor activity⁴⁷.

We have recently demonstrated an increase in glutamate release, as measured by frequency of spontaneous (s) and miniature (m) excitatory postsynaptic currents (EPSCs), in lamina I of the spinal cord of rats who had previously received repeated injections of the opiate morphine⁴⁷. This increase in glutamate release appeared to be dependent on presynaptic NMDA receptors (NMDARs). To test this further, we conducted electron microscopy studies that showed an increase in NMDARs on substance P (SP)-containing primary afferent terminals from morphine-tolerant rats. These rats also demonstrated behavioral tolerance to an analgesic dose of morphine following the treatment regimen we used, which was identical to the protocol used in the present study.

The neuropeptide, SP, plays a key role in the transmission of pain signals, especially those of moderate to severe intensity⁸. Since SP is synthesized by small diameter primary afferent fibers⁶ and its receptor, the neurokinin 1 (NK1) receptor, is densely distributed in spinal cord lamina I nociceptive-specific neurons¹, both SP and its receptors are well-situated to send and receive nociceptive input from the periphery. Indeed, both have been implicated in the heightened nociception that accompanies inflammatory^{1,13,14,17,29,30,32,37,39}, neuropathic,^{1,7,14,18,24,27,29,33,35,38,41,44} and experimentally-induced states of central sensitization (e.g., capsaicin or mustard oil application, repeated afferent stimulation)^{2,19,45}.

There is evidence to suggest that SP-NK1 neurotransmission is also involved in morphine tolerance^{4,21,23,34}. Increased levels of SP have been observed following sustained morphine administration in cultured dorsal root ganglia^{4,23}, as well the spinal cord dorsal horn²¹. Furthermore, antagonism of the NK1 receptor reverses the development of morphine analgesic tolerance *in vivo*³⁴. We previously showed that both glutamate release and presynaptic NMDARs are increased in lamina I of morphine-tolerant rats⁴⁷. Since SP and glutamate are co-localized in primary afferents⁹, we asked whether SP-NK1 neurotransmission in the spinal cord dorsal horn might also be modulated by prolonged morphine exposure. The following anatomical and electrophysiological experiments were performed to test whether prolonged morphine exposure alters SP-NK1 expression and function in lamina I of the spinal cord dorsal horn.

Materials and Methods

Animals

Sprague-Dawley time-mated dams were purchased (Taconic, Germantown, NY) and gave birth in our animal care facility. Veterinary care was provided by the Department of Comparative Medicine, fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and licensed by the United States Department of Agriculture for rat care. Anatomical and quantitative real-time polymerase chain reaction (QRT-PCR) studies were carried out when rat pups were 16-17 days of age. Electrophysiological studies were conducted when rat pups were 12-17 days of age. All studies were in accordance with the guidelines for animal research published by the International Association for the Study of Pain and the Society for Neuroscience. Morphine injections, QRT-PCR, and electrophysiological studies were conducted at the University of Washington and were in compliance with the regulations of the University of Washington Institutional Animal Care and Use Committee (IACUC). Immunocytochemistry and confocal analysis was performed at the Oregon Health & Science University. For the immunocytochemical studies, age-matched, saline-injected rats were used as controls. Animals were shipped from Seattle, WA to Portland, OR for perfusion and processing as approved by the Oregon Health & Science University IACUC.

Morphine Injections

Subcutaneous injections of morphine were administered twice daily for 3 days (15, 30 and 45 mg/kg on days 1, 2 and 3, respectively). All studies of morphine-treated animals were conducted within 48 hours after the last morphine injection, on day 4 or 5. Control animals used for anatomical studies were age-matched and saline-injected. Control animals used for electrophysiological studies were tested at ages equivalent to days 1 -5 of the treatment regimen, but were untreated.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

RNA was harvested from the dorsal root ganglia of control and morphine-treated rats using Qiashredder homogenizer columns and the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). One step quantitative real-time PCR was performed on a Stratagene Mx3000 machine using the Brilliant SYBR Green QPCR Master Mix with StrataScript Reverse Transcriptase (Stratagene, La Jolla, CA). Substance P (SP) and a control endogenous housekeeping gene, ribosomal protein ARBP, were amplified in separate reactions starting with 20ng of total RNA for each sample and run in duplicate. The oligo sequences used for amplification of SP were Forward (ACCAAATCAAGGAGGCAATG) and Reverse (AGCCTTTAACAGGGCCACTT), and for ARBP, were Forward (AAAGGGTCCTGGCTTTGTCT) and Reverse (CTTCCTTTGCTTCGACCTTG). Standard curves were generated using serial dilutions of RNA and the PCR efficiencies were found to be similar for both oligo sets. Dissociation curves for the products of each oligo set had a single peak indicating specific amplification. For each sample, the cycle at which the fluorescence measured reached the threshold to be above the ambient background signal (Ct), was determined. For each sample, the ΔC_t was calculated by subtracting the Ct for the endogenous control gene, ARBP, from the Ct for SP. Relative mRNA quantity was measured using the ΔC_t calculation²², and is expressed as the fold change over the levels in dorsal root ganglia from control rats.

Dual-label Fluorescent Immunocytochemistry

Rats were overdosed with pentobarbital sodium (150 mg/kg) and perfused transcardially through the ascending aorta. Each rat was perfused with the following sequence of solutions: (1) 10 ml of heparinized saline (1000 units/ml), (2) 10 ml of 3.8% acrolein in 2%

paraformaldehyde, and (3) 50 – 100 ml of 2% paraformaldehyde (in 0.1 M phosphate buffer (PB), pH 7.4). The lumbar spinal cord was removed and then cut into 40 μ m sections on a vibrating microtome. Tissue sections were stored at -20°C in storage solution (30% sucrose, 30% ethylene glycol in 0.1 M phosphate buffer) until they were processed for appropriate immunocytochemical procedures.

Spinal cord sections were removed from storage solution and rinsed thoroughly in 0.1 M phosphate buffer before incubating in 1% sodium borohydride in 0.1 M phosphate buffer for 30 minutes. Following immersion in 0.5% BSA in 0.1 M Tris-saline, tissue sections were incubated in an antibody cocktail consisting of rat anti-SP (Accurate Chemical & Scientific Corporation, 1:1000) and rabbit anti-NK1 antisera (Novus Biologicals, 1:1000) in 0.1% BSA in 0.1 M Tris-saline for 40 h at 4°C. The antibodies to SP and NK1 were visualized with a 2 h incubation in a cocktail containing goat anti-rat IgG conjugated to Alexa Fluor 647 (Invitrogen - Molecular Probes, 1:800) and donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen - Molecular Probes, 1:800) in 0.1% BSA in 0.1 M Tris-saline. All incubations, except the primary antibody incubation, were carried out at room temperature with continuous agitation and sections were rinsed between incubations in 0.1 M Tris-saline. Sections were mounted onto gelatin-coated slides, coverslipped with Prolong Gold™ Antifade Media (Invitrogen-Molecular Probes) and stored at -20°C.

Confocal Analysis

The distributions of SP- and NK1-immunoreactivity (ir) within the medial dorsal horn were examined with a Zeiss LSM 510 scanning confocal microscope. Images were captured with a 63x/1.4 oil objective on coded sections from all animals. A single pass, multi-tracking format was utilized to allow the two tracers to be individually excited with different lasers, and the emitted spectra collected separately. A 488 nm (Argon/2) laser and a 633 nm (HeNe2) laser were used to excite the Alexa Fluor 488 and Alexa Fluor 647 fluorophores, respectively. The excitation of the Alexa Fluor 488 fluorophores produced emission wavelengths that passed through a 500-550 nm band pass filter before image collection. The emitted wavelengths from the excited Alexa Fluor 647 fluorophores passed through a 650-710 nm band pass filter before image collection. Images from all animals were collected with identical scanning parameters, as Z-stacks bounded vertically by the visible extent of both fluorescent tags. Consecutive optical sections within Z-stacks were separated by an interval of 0.28 μ m. Scans were performed on both the left and right medial dorsal horns of spinal cord sections until at least 8 NK1-ir cells were identified within each animal. Using the LSM Examiner Program, each NK1 labeled cell and its immediate vicinity was traced with the curved drawing tool, and the absolute frequencies (number of pixels / pixel intensity (0-255)) of SP- and NK1-ir within that defined area were measured. The weighted intensities of SP- and NK1-ir were then calculated for each cell as [sum of (number of SP or NK1 pixels x pixel intensity)]. A threshold intensity of 100, on a scale from 0 to 255, was set to eliminate the influence of background fluorescence on the data. The average weighted intensity ($\pm$ SEM) for each group was then calculated.

Electrophysiological Recordings

Spinal slice preparation—Methods in preparing viable spinal slices were adapted from the methods of Berger and Takahashi⁵ as previously described⁴². Briefly, a laminectomy was performed while rats were anesthetized with isoflurane. Rats were then euthanized by decapitation and the spinal cord quickly removed and bathed in an ice-cold dissecting buffer (composed of NaCl, 113 mM; KCl, 3 mM; NaH₂PO₄, 1 mM; NaHCO₃, 25 mM; glucose, 11 mM; MgCl₂, 10 mM; previously bubbled with 95%-5% O₂-CO₂ for at least an hour). The isolated spinal cord was then placed in a small Petri dish and embedded in low melting point agar (2%, w/v in modified artificial cerebrospinal fluid (aCSF; NaCl, 113 mM; KCl, 3 mM; NaH₂PO₄, 1 mM; NaHCO₃, 25 mM; glucose, 11 mM; CaCl₂, 2 mM; MgCl₂, 1 mM)) and

cooled to 34° C. A block of agar containing the lower lumbar cord (L4-L6) was cut and glued to the cutting chamber of a Vibratome with cyanoacrylate glue. The chamber was filled with ice cold bubbled dissecting buffer and 300-400 µm transverse sections of the spinal cord were cut. Slices were transferred using a Pasteur pipette to a Plexiglas experimental chamber, attached to a fixed stage Axioskop FS microscope equipped with a band-pass optical filter (770 +/-40 nm) in the transilluminating light path and an attached CCD camera and monitor. Additional slices, not currently being studied, were placed in a chamber containing bubbled room temperature aCSF. Slices placed in the experimental chamber were held in place with a platinum wire harp and were perfused continuously with aCSF (1-2 ml/min). Bicuculline (10 µM) and strychnine (1µM) were added to the aCSF in all experiments to isolate EPSCs.

Electrophysiological recordings—The temperature of the bath in the recording chamber was heated to 34 degrees Celsius using an in-line heater (Warner) with feedback control. A glass recording electrode (4-6 MΩ) filled with intracellular pipette solution (125 mM KMeSO₄, 8 mM NaCl, 10 mM Hepes, 2 mM MgATP, 0.3 mM NaGTP, and 5 mM EGTA (pH 7.3 and approximately 285 mOsm)) was advanced into the recording chamber using direct vision and a micro-manipulator (Sutter). Lamina I cells were visually targeted for whole cell voltage clamp recording using DIC-IR imaging. Once established, whole cell voltage clamp was maintained at -70 mV using an Axopatch 200B amplifier (Axon) and telegraphed software (HEKA's Pulse) running on a Macintosh computer. Initial series resistance and capacitance were noted at the beginning of each experiment and at intervals throughout the study (series resistance or capacitance changes >20% were exclusionary criteria).

Electrophysiology Drug Application—L732,138 (L732), a selective NK1 receptor antagonist was purchased from Tocris Cookson Ltd. Tetrodotoxin was purchased from Calbiochem. Bicuculline, strychnine, and substance P acetate salt hydrate were purchased from Sigma-Aldrich. All drugs were applied by gravity fed superfusion from a drug reservoir containing bubbled aCSF in which the drug was dissolved. The combined volume of the recording chamber and tubing from the drug reservoir was less than 2 ml to minimize latencies of drug effects at a flow rate of 1-2 ml/min.

Electrophysiological Data Analysis—All electrophysiological data were digitized and collected using Pulse (HEKA) on a Macintosh based computer. From Pulse, data were exported into a spreadsheet for further data manipulation and graphing (Microsoft's Excel) or statistics (GB-Stat for Macintosh). Analysis of spontaneous EPSCs (sEPSCs) was accomplished with Minianalysis (Synaptosoft). Minianalysis allows a threshold-based event detection algorithm enabling events to be analyzed by frequency and amplitude, as well as grouped and plotted. Holding current and current-voltage (IV) curves were analyzed in 2-minute epochs, whereas sEPSCs were recorded in 5-minute epochs with the last pre-treatment epoch serving as baseline. Consistent with our previous observations⁴⁷, we did not see any differences in spinal cord lamina I cells of morphine-treated and control rats across a range of parameters (capacitance, resistance, holding current; see ⁴⁷ for tabular summary).

Results

Decreased SP- and NK1-immunoreactivity in spinal cord dorsal horn of morphine-treated rats

To determine if chronic morphine treatment leads to a change in SP, we used immunocytochemistry to examine the intensity of SP-ir in the lumbar spinal dorsal horn. Tissue sections from 3 saline-treated (control) and 3 morphine-treated rats were examined, and regions of dorsal horn containing both SP- and NK1-ir were analyzed. The fluorescence intensity for NK1 was examined in selected neurons, and SP-ir was examined in the region immediately

surrounding each neuron. For the morphine-treated rats, 38 identified NK1-ir cells were examined; and in control rats, 34 NK1-ir cells were examined. Using confocal microscopy, significant decreases were found in the amount of both SP-ir (73% decrease) and NK1-ir (56% decrease) in morphine-treated neonatal rats as compared to age-matched control rats (Figure 1). To ensure that any observed differences were not due to a sampling bias, the cross-sectional diameter of each cell was measured through the region of the nucleus. NK1-ir cells were of similar size in both groups (morphine-treated: $8.5 \pm 0.3 \mu\text{m}$; control: $8.8 \pm 0.3 \mu\text{m}$). In addition, the extent of NK1 receptor internalization was scored by observers blind to experimental condition. Although the extent of membrane NK1-ir appeared to be less extreme in the neonate than we and others have seen previously in the adult spinal cord, no change in the extent of receptor internalization was found between groups (data not shown).

Equal SP mRNA in the dorsal root ganglia of control and morphine-treated rats

The decreased SP-ir observed in the dorsal horn of morphine-treated rats could have been due to a change in the synthesis of SP in the dorsal root ganglia of morphine-treated rats. To address this possibility, we used QRT-PCR to determine relative mRNA levels of SP. We found no difference in the quantity of SP mRNA in the DRG of control and morphine-treated rats (control = 1.01 ± 0.06 fold-change, $n = 4$ rats; morphine = 1.04 ± 0.06 fold-change, $n = 4$ rats). These results suggest that the decreased SP-ir that was observed in the spinal cord dorsal horn of the morphine-treated rats was not attributable to a change in SP mRNA production.

Substance P reversibly potentiates sEPSC frequency in spinal cord slices from control, but not morphine-treated rats

Though no difference in SP mRNA was observed between control and morphine-treated rats, decreased SP-ir could indicate a role for increased SP release in central sensitization³⁶ in the spinal cord of morphine-treated rats. To test this hypothesis using electrophysiological methods, we applied the NK1 receptor antagonist L732 ($4 \mu\text{M}$) to spinal cord slices from control and morphine-treated rats. L732 caused no change in sEPSC frequency (Figure 2C) or amplitude (data not shown) in lamina I cells from either control or morphine-treated rats. This is supportive of findings by ourselves and others that sEPSCs in these cells are completely abolished by the AMPA glutamate receptor antagonist CNQX^{28,42,46}. In contrast, application of SP ($1 \mu\text{M}$) induced a potentiation of sEPSC frequency (Figures 2A and B) but not amplitude (data not shown) in lamina I cells from control rats which was reversed by L732 ($n=3$, data not shown) or by removing SP from the perfusate (Wash, Figure 2B). Notably, SP did not cause a similar increase in mEPSC frequency in lamina I cells from control rats in the presence of TTX ($n = 3$, data not shown). This suggests that the potentiating effects of SP on sEPSC frequency in Lamina I cells is not due to direct effects on synaptic glutamate release but is indirect via intrinsic neurons of the spinal cord which then synapse on lamina I neurons.

Surprisingly, though cells from morphine-treated rats trended toward a higher baseline sEPSC frequency, no reversible effect (i.e., change over baseline) of SP was observed in slices from morphine-treated rats (Figure 2B). Not only does this observation argue against a role for SP in the increased excitation we have previously seen in slices from morphine-tolerant rats⁴⁷, but it also suggests a decrease in SP receptor (NK1) function in the morphine-treated slice.

To look more specifically at the effects of SP on lamina I NK1 receptors in control and morphine-treated rats, we measured the current required to voltage clamp our cells at -70 mV in both groups following the application of SP. SP induced a rapid inward current (approximately $150\text{-}200 \text{ pA}$) in lamina I cells from control, but not morphine-treated rats (Figure 3). The SP-induced inward current observed in control lamina I cells was similar to that seen in other areas of the brain³, sensory afferents,⁴⁶ and spinal cord²⁸ and indicates a

depolarizing effect of SP on lamina I cells from control rats that was not present in lamina I cells from morphine-treated rats.

The failure of SP to affect currents at -70 mV in lamina I cells from morphine-treated rats suggests that the NK1 receptors on these cells were either differentially expressed or activated different ionic currents following SP application, compared to cells from control rats. To address the question of differential activation of ionic currents, we performed current-voltage (IV) curves on lamina I cells from control and morphine-treated rats following SP application. Whereas SP produced an inward current with a reversal potential of approximately -60 mV in cells from control rats, an effect that is consistent with other studies looking at SP effects on dorsal horn neurons²⁸, it caused no change in the IV curve at any voltage step (-140 to 20 mV) in lamina I cells from morphine-treated rats (Figure 4). From these data, it appears that the absence of an effect on holding current by SP on lamina I cells from morphine-treated rats was due, not to a change in the ionic conductance of the NK1 receptor, but rather to general failure of SP to activate lamina I NK1 receptors.

The absence of an effect of SP on the holding current and IV curve of lamina I cells from morphine-treated rats is consistent with our finding of reduced NK1 receptor immunoreactivity in the spinal cord dorsal horn of morphine-treated rats. The results from these studies suggest that fewer NK1 receptors were available to bind SP in the spinal cord dorsal horn of the morphine-treated rat.

Discussion

The primary finding of this report is a loss of SP-NK1 function in lamina I of the spinal cord dorsal horn in rats exposed to repeated, escalating doses of morphine. Immunoreactivity for SP was decreased in the dorsal horn of morphine-treated neonatal rats. The observation of relatively equal amounts of SP mRNA in the dorsal root ganglia of both control and morphine-treated rats suggests that the decrease in SP-ir in the morphine-treated rats is not attributable to a down-regulation of SP production. In contrast, the decreased SP-ir suggests a possible increase in SP release in the spinal cord of the morphine-treated rats, though other possibilities exist, including epitope or translational changes. At the functional level, SP both excited and depolarized lamina I cells in the control spinal cord slice, but had no effect on lamina I cells from morphine-treated rats. Anatomical data showing a reduction of NK1-ir in the spinal cord dorsal horn of morphine-treated rats correlated with these functional changes, suggesting that SP does not affect lamina I cells in the morphine-treated spinal cord slice because its receptor was down-regulated. Although morphine analgesic tolerance was not assessed in the present study, these results suggest that repeated morphine exposure, a prerequisite for the development of morphine analgesic tolerance, profoundly alters SP and NK1 expression and function in the spinal cord dorsal horn.

Data suggest that SP acting at NK1 receptors underlies heightened pain states resulting from inflammatory^{1,13,14,17,29,30,32,37,39} and neuropathic^{1,7,14,18,24,27,29,33,35,38,41,44} pain conditions, as well as experimentally-induced states of central sensitization^{2,19,45}. However, less is known regarding the role of SP and its receptor in morphine analgesic tolerance, a condition that has been characterized by an increased sensitivity to pain^{25,31}. Of the few studies that have looked at the role of SP and NK1 in morphine tolerance, perhaps the most relevant is the observation by King and colleagues who observed increased NK1 receptor internalization (upon noxious stimulation) and expression in morphine-tolerant rats²⁰. Two significant differences between these studies are the protocols used to induce morphine tolerance and the age of the animals. Although the distribution¹² and density¹¹ of spinal cord SP has been reported to be similar in adult and two week-old neonates, we cannot rule out developmental factors in explaining the difference between our results and those reported by

King and colleagues²⁰. Possible developmental changes aside, however, the different effects of morphine on SP and its receptor may be due to differences in tolerance induction protocols. Whereas King *et al.*²⁰ tested the effects of six days of sustained morphine administration (implanted subcutaneous pellet), we tested the effects of three days of intermittent morphine administration (twice daily subcutaneous injections). It is highly conceivable that intermittent morphine, which is, perhaps, more consistent with the clinical (morphine pain therapy) and recreational (morphine abuse) conditions of morphine exposure⁴⁰, has different effects on NK1 receptor expression than uninterrupted morphine exposure. Indeed, the NK1 receptor has been shown to be relatively quiescent during steady-state conditions, but highly labile, undergoing phosphorylation, internalization, and either recycling or destruction, during periods of sustained ligand activation¹⁶. Therefore, we believe that the up-regulation of the NK1 receptor reported by King *et al.*²⁰ and the down-regulation of the receptor reported here reflect different stages of a similar phenomenon, namely, increased signaling by the receptor's ligand, SP.

Increased SP signaling may represent an important mechanism of the morphine-induced hyperalgesia that underlies morphine analgesic tolerance. Indeed, Powell and colleagues have demonstrated that the administration of an NK1 receptor antagonist can both block and reverse the development of morphine tolerance³⁴. We have previously implicated other mechanisms in the increased spinal excitatory neurotransmission observed in spinal cord slices from morphine-tolerant neonatal rats⁴⁷. For example, we see an increase in glutamate release that is at least partially dependent on increased pre-synaptic NMDA receptors⁴⁷ as well as down-regulation of spinal cord glutamate transporters^{26,43}. Increasingly, morphine tolerance is emerging as a condition that depends on compensatory or opponent processes in addition to desensitization and/or internalization of the opioid receptor. Although contributing to the mechanistic complexity of morphine tolerance, these various compensatory systems, including SP and glutamate, may also represent important therapeutic targets for the retention and restoration of pain relief with prolonged morphine treatment.

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References

1. Abbadie C, Brown JL, Mantyh PW, Basbaum AI. Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neuroscience* 1996;70:201–9. [PubMed: 8848125]
2. Afrah AW, Fiska A, Gjerstad J, Gustafsson H, Tjolsen A, Olgart L, Stiller CO, Hole K, Brodin E. Spinal substance P release in vivo during the induction of long-term potentiation in dorsal horn neurons. *Pain* 2002;96:49–55. [PubMed: 11932060]
3. Aosaki T, Kawaguchi Y. Actions of substance P on rat neostriatal neurons in vitro. *J Neurosci* 1996;16:5141–53. [PubMed: 8756443]
4. Belanger S, Ma W, Chabot JG, Quirion R. Expression of calcitonin gene-related peptide, substance P and protein kinase C in cultured dorsal root ganglion neurons following chronic exposure to mu, delta and kappa opiates. *Neuroscience* 2002;115:441–53. [PubMed: 12421610]
5. Berger AJ, Takahashi T. Serotonin enhances a low-voltage-activated calcium current in rat spinal motoneurons. *J Neurosci* 1990;10:1922–8. [PubMed: 2355258]
6. Besson JM, Chaouch A. Peripheral and spinal mechanisms of nociception. *Physiol Rev* 1987;67:67–186. [PubMed: 3543978]
7. Cahill CM,Coderre TJ. Attenuation of hyperalgesia in a rat model of neuropathic pain after intrathecal pre- or post-treatment with a neurokinin-1 antagonist. *Pain* 2002;95:277–85. [PubMed: 11839427]

8. Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI. Primary afferent tachykinins are required to experience moderate to intense pain. *Nature* 1998;392:390–4. [PubMed: 9537322]
9. De Biasi S, Rustioni A. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci U S A* 1988;85:7820–4. [PubMed: 2459717]
10. Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* 2004;27:107–44. [PubMed: 15217328]
11. Gibbs LM, Kendig JJ. Substance P and NMDA receptor-mediated slow potentials in neonatal rat spinal cord: age-related changes. *Brain Res* 1992;595:236–41. [PubMed: 1281736]
12. Ho RH. Widespread distribution of substance P- and somatostatin-immunoreactive elements in the spinal cord of the neonatal rat. *Cell Tissue Res* 1983;232:471–86. [PubMed: 6192926]
13. Honore P, Kamp EH, Rogers SD, Gebhart GF, Mantyh PW. Activation of lamina I spinal cord neurons that express the substance P receptor in visceral nociception and hyperalgesia. *J Pain* 2002;3:3–11. [PubMed: 14622848]
14. Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. *Neuroscience* 2000;98:585–98. [PubMed: 10869852]
15. Hsu MM, Wong CS. The roles of pain facilitatory systems in opioid tolerance. *Acta Anaesthesiol Sin* 2000;38:155–66. [PubMed: 11125691]
16. Hua XY, Chen P, Polgar E, Nagy I, Marsala M, Phillips E, Wollaston L, Urban L, Yaksh TL, Webb M. Spinal neurokinin NK1 receptor down-regulation and antinociception: effects of spinal NK1 receptor antisense oligonucleotides and NK1 receptor occupancy. *J Neurochem* 1998;70:688–98. [PubMed: 9453563]
17. Ishigooka M, Zermann DH, Doggweiler R, Schmidt RA, Hashimoto T, Nakada T. Spinal NK1 receptor is upregulated after chronic bladder irritation. *Pain* 2001;93:43–50. [PubMed: 11406337]
18. Jang JH, Nam TS, Paik KS, Leem JW. Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. *Neurosci Lett* 2004;360:129–32. [PubMed: 15082150]
19. Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA. Spinal neurons that possess the substance P receptor are required for the development of central sensitization. *J Neurosci* 2002;22:9086–98. [PubMed: 12388616]
20. King T, Gardell LR, Wang R, Vardanyan A, Ossipov MH, Malan TP Jr, Vanderah TW, Hunt SP, Hruby VJ, Lai J, Porreca F. Role of NK-1 neurotransmission in opioid-induced hyperalgesia. *Pain* 2005;116:276–88. [PubMed: 15964684]
21. King T, Ossipov MH, Vanderah TW, Porreca F, Lai J. Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? *Neurosignals* 2005;14:194–205. [PubMed: 16215302]
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8. [PubMed: 11846609]
23. Ma W, Zheng WH, Kar S, Quirion R. Morphine treatment induced calcitonin gene-related peptide and substance P increases in cultured dorsal root ganglion neurons. *Neuroscience* 2000;99:529–39. [PubMed: 11029544]
24. Mansikka H, Sheth RN, DeVries C, Lee H, Winchurch R, Raja SN. Nerve injury-induced mechanical but not thermal hyperalgesia is attenuated in neurokinin-1 receptor knockout mice. *Exp Neurol* 2000;162:343–9. [PubMed: 10739640]
25. Mao J, Price DD, Mayer DJ. Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. *Pain* 1995;62:259–74. [PubMed: 8657426]
26. Mao J, Sung B, Ji RR, Lim G. Chronic morphine induces downregulation of spinal glutamate transporters: implications in morphine tolerance and abnormal pain sensitivity. *J Neurosci* 2002;22:8312–23. [PubMed: 12223586]
27. Meert TF, Vissers K, Geenen F, Kontinen VK. Functional role of exogenous administration of substance P in chronic constriction injury model of neuropathic pain in gerbils. *Pharmacol Biochem Behav* 2003;76:17–25. [PubMed: 13679213]

28. Murase K, Ryu PD, Randic M. Tachykinins modulate multiple ionic conductances in voltage-clamped rat spinal dorsal horn neurons. *J Neurophysiol* 1989;61:854–65. [PubMed: 2470866]
29. Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA, Mantyh PW. Transmission of chronic nociception by spinal neurons expressing the substance P receptor. *Science* 1999;286:1558–61. [PubMed: 10567262]
30. Ohtori S, Takahashi K, Ino H, Chiba T, Yamagata M, Sameda H, Moriya H. Up-regulation of substance P and NMDA receptor mRNA in dorsal horn and preganglionic sympathetic neurons during adjuvant-induced noxious stimulation in rats. *Ann Anat* 2002;184:71–6. [PubMed: 11876485]
31. Ossipov MH, Lai J, King T, Vanderah TW, Porreca F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers* 2005;80:319–24. [PubMed: 15795927]
32. Palecek J, Paleckova V, Willis WD. Postsynaptic dorsal column neurons express NK1 receptors following colon inflammation. *Neuroscience* 2003;116:565–72. [PubMed: 12559111]
33. Pitcher GM, Henry JL. Nociceptive response to innocuous mechanical stimulation is mediated via myelinated afferents and NK-1 receptor activation in a rat model of neuropathic pain. *Exp Neurol* 2004;186:173–97. [PubMed: 15026255]
34. Powell KJ, Quirion R, Jhamandas K. Inhibition of neurokinin-1-substance P receptor and prostanoid activity prevents and reverses the development of morphine tolerance in vivo and the morphine-induced increase in CGRP expression in cultured dorsal root ganglion neurons. *Eur J Neurosci* 2003;18:1572–83. [PubMed: 14511336]
35. Robertson B, Schulte G, Elde R, Grant G. Effects of sciatic nerve injuries on delta -opioid receptor and substance P immunoreactivities in the superficial dorsal horn of the rat. *Eur J Pain* 1999;3:115–129. [PubMed: 10700341]
36. Ruscheweyh R, Sandkuhler J. Opioids and central sensitisation: II. Induction and reversal of hyperalgesia. *Eur J Pain* 2005;9:149–52. [PubMed: 15737805]
37. Schaible HG, Jarrott B, Hope PJ, Duggan AW. Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. *Brain Res* 1990;529:214–23. [PubMed: 1704282]
38. Shimoyama M, Tatsuoka H, Ohtori S, Tanaka K, Shimoyama N. Change of dorsal horn neurochemistry in a mouse model of neuropathic cancer pain. *Pain* 2005;114:221–30. [PubMed: 15733648]
39. Sluka KA, Westlund KN. Behavioral and immunohistochemical changes in an experimental arthritis model in rats. *Pain* 1993;55:367–77. [PubMed: 7510059]
40. Taylor BK. Insights into morphine-induced plasticity and spinal tolerance. *Pain* 2005;114:1–2. [PubMed: 15733624]
41. Taylor BK, McCarron KE. Neurokinin-1 receptor gene expression in the mouse dorsal horn increases with neuropathic pain. *J Pain* 2004;5:71–6. [PubMed: 15042514]
42. Terman GW, Eastman CL, Chavkin C. Mu opiates inhibit long-term potentiation induction in the spinal cord slice. *J Neurophysiol* 2001;85:485–94. [PubMed: 11160487]
43. Thomson LM, Zeng J, Terman GW. Differential effect of glutamate transporter inhibition on EPSCs in the morphine naive and morphine tolerant neonatal spinal cord slice. *Neurosci Lett* 2006;407:64–9. [PubMed: 16949209]
44. Vachon P, Masse R, Gibbs BF. Substance P and neurotensin are up-regulated in the lumbar spinal cord of animals with neuropathic pain. *Can J Vet Res* 2004;68:86–92. [PubMed: 15188951]
45. Weng HR, Mansikka H, Winchurch R, Raja SN, Dougherty PM. Sensory processing in the deep spinal dorsal horn of neurokinin-1 receptor knockout mice. *Anesthesiology* 2001;94:1105–12. [PubMed: 11465604]
46. Wu ZZ, Guan BC, Li ZW, Yang Q, Liu CJ, Chen JG. Sustained potentiation by substance P of NMDA-activated current in rat primary sensory neurons. *Brain Res* 2004;1010:117–26. [PubMed: 15126124]
47. Zeng J, Thomson LM, Aicher SA, Terman GW. Primary afferent NMDA receptors increase dorsal horn excitation and mediate opiate tolerance in neonatal rats. *J Neurosci* 2006;26:12033–42. [PubMed: 17108177]

Figure 1.

SP- and NK1-ir are significantly reduced in morphine-treated rats. **A.** Confocal micrographs of SP- and NK1-ir in lumbar dorsal horn of morphine-treated and control rats. SP- (purple) and NK1- (green) immunoreactivity were reduced in the outer laminae of the spinal cord in morphine-treated rats. The overlapping distribution of both markers is shown in the bottom panels. Scale bars = 10 μ m. **B.** The average weighted intensity of SP- (left) and NK1-ir (right) in control (black bars) and morphine-treated (gray bars) rats (mean $\pm$ SEM). *,*** represent significant differences from control rats ($P < 0.05$, $P < 0.01$, respectively; Mann-Whitney rank sum test; averaged weighted intensities were calculated for 38 cells from morphine-treated rats and 34 cells from control rats; $n = 3$ rats).

Figure 2.

SP induces a reversible potentiation of sEPSC frequency in lamina I spinal cord dorsal horn cells from control, but not morphine-treated rats. **A.** Representative recordings from a lamina I cell from a control (top) and a morphine-treated (bottom) rat following the application of SP (1 μ M). **B.** Average effect of SP on the rate of spontaneous excitatory postsynaptic currents (sEPSCs) in the spinal cord dorsal horn of control and morphine-treated rats (mean $\pm$ SEM). SP (black bars) induces a reversible potentiation of sEPSC rate in lamina I cells from control ($n=10$ cells for SP and Wash), but not morphine-treated ($n=11$ cells for SP and Wash) rats. * indicates significant difference from washout of SP (Wash, gray bars, $P < 0.05$, Repeated Measures ANOVA with Fisher's LSD *post-hoc* comparisons). **C.** Application of L732,138 (L732, 4 μ M, filled bars), a selective antagonist of the NK1 receptor, does not affect sEPSC rate in lamina I spinal cord dorsal horn cells of either control ($n=5$, 4 cells for L732, Wash (gray bars) respectively) or morphine-treated rats ($n=7$ cells for L732 and Wash) ($P > 0.05$, Repeated Measures ANOVA).

Figure 3.

SP depolarizes lamina I cells in the spinal cord dorsal horn of control, but not morphine-treated rats. **A.** Representative trace of holding current of a lamina I cell from a control (top) and morphine-treated (bottom) rat. SP (1 μ M) causes an inward shift in holding current in a lamina I cell from a control rat (approximately 1 1/2 to 2 min from SP application), but causes no change in holding current from a lamina I cell from a morphine-treated rat. **B.** The average changes in holding current (pA; mean $\pm$ SEM) from baseline show that SP (black bars) induces a reversible increase in negative holding current in lamina I cells from control rats ($n=9$, 8 cells for SP and Wash, respectively). ^^ indicates significant difference from morphine-treated cells following SP application ($P < 0.01$, Repeated Measures ANOVA with Fisher's LSD *post-hoc* comparisons); ** indicates significant difference from washout of SP (Wash, gray bars, $P < 0.01$, Repeated Measures ANOVA with Fisher's LSD *post-hoc* comparisons).

Figure 4.

SP does not activate NK1 receptors on lamina I cells of morphine-treated rats across a range of voltage steps. **A.** Protocol used to evaluate the effect of SP ($1\mu\text{M}$) on current across a range of voltage steps (-140 to 20 mV). **B.** Representative IV curve before (baseline, filled diamonds) and 2 min after SP application (open squares) in a lamina I cell from a control rat. SP augments inward currents at hyperpolarized potentials (-140 to -80 mV) and outward currents at depolarized potentials (-60 to 20 mV). **C.** Representative IV curve before (baseline, filled diamonds) and 2 min after SP application (open squares) in a lamina I cell from a morphine-treated rat. SP causes no change in current at any tested voltage step. **D.** Grouped data showing the effect of SP on current in lamina I cells from control and morphine-treated rats. The SP effect was measured by subtracting the current after SP application from the baseline current before SP application at each voltage step for all recorded cells. *, ** indicate significant difference from lamina I cells from morphine-treated rats ($P < 0.05$ and $P < 0.01$ respectively, Repeated Measures ANOVA with Fisher's LSD *post-hoc* comparisons, $n = 4$ control cells (filled diamonds), 6 morphine-treated cells (open squares)).