Published in final edited form as:

Neuroscience. 2009 June 16; 161(1): 157–172. doi:10.1016/j.neuroscience.2009.03.021.

Acute inflammation induces segmental, bilateral, supraspinally mediated opioid release in the rat spinal cord, as measured by μ -opioid receptor internalization

Wenling Chen and Juan Carlos G. Marvizón

Center for the Neurobiology of Stress, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, Veteran Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073

Abstract

The objective of this study was to measure opioid release in the spinal cord during acute and long-term inflammation using μ -opioid receptor (MOR) internalization. In particular, we determined whether opioid release occurs in the segments receiving the noxious signals or in the entire spinal cord, and whether it involves supraspinal signals. Internalization of neurokinin 1 receptors (NK1Rs) was measured to track the intensity of the noxious stimulus. Rats received peptidase inhibitors intrathecally to protect opioids from degradation. Acute inflammation of the hindpaw with formalin induced moderate MOR internalization in the L5 segment bilaterally, whereas NK1R internalization occurred only ipsilaterally. MOR internalization was restricted to the lumbar spinal cord, regardless of whether the peptidase inhibitors were injected in a lumbar or thoracic site. Formalin-induced MOR internalization was substantially reduced by isoflurane anesthesia. It was also markedly reduced by a lidocaine block of the cervical-thoracic spinal cord (which did not affect the evoked NK1R internalization) indicating that spinal opioid release is mediated supraspinally. In the absence of peptidase inhibitors, formalin and hindpaw clamp induced a small amount of MOR internalization, which was significantly higher than in controls. To study spinal opioid release during chronic inflammation, we injected Complete Freund's Adjuvant (CFA) in the hindpaw and peptidase inhibitors intrathecally. Two days later, no MOR or NK1R internalization was detected. Furthermore, CFA inflammation decreased MOR internalization induced by clamping the inflamed hindpaw. These results show that acute inflammation, but not chronic inflammation, induce segmental opioid release in the spinal cord that involves supraspinal signals.

Keywords

Descending pathways; dorsal horn; dynorphin; enkephalin; formalin; neurokinin 1 receptor

The release of endogenous opioid peptides (henceforth “opioids”) in the spinal cord is an important pain modulation mechanism (Zorman et al., 1982; Jensen and Yaksh, 1984; Morgan et al., 1991; Budai and Fields, 1998). Neurophysiological states that induce spinal opioid

Corresponding author: Juan Carlos G. Marvizón, VA Greater Los Angeles Healthcare System, building 115, 11301 Wilshire Blvd., Los Angeles, CA 90073; phone: 310-478 3711 extension 41850; fax: 310-312 9289; e-mail: marvizon@ucla.edu.
Current addresses: Chen, Marvizon: VA Greater Los Angeles Healthcare System, building 115, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

release may include stress and pain. Although there is evidence that opioids are released by acute noxious stimuli (Le Bars et al., 1987a; Le Bars et al., 1987b; Cesselin et al., 1989; Bourgoin et al., 1990; Lao et al., 2008), it is important to establish whether opioid release is maintained during inflammation and chronic pain (Przewlocki et al., 1986; Ballet et al., 2000), or whether its decline contributes to hyperalgesia.

Spinal opioid release appears to be driven by neural pathways that originate supraspinally (Basbaum et al., 1976; Basbaum and Fields, 1984; Fields et al., 1991; Mason, 1999). Thus, opioid receptor antagonists applied to the spinal cord reverse the analgesia induced by stimulation of the rostroventral medulla (Zorman et al., 1982) or the periaqueductal gray (Budai and Fields, 1998). Moreover, some forms of stress-induced analgesia are blocked by spinal opioid antagonists (Watkins et al., 1982; Terman et al., 1983) and by lesions in brainstem nuclei or the dorsolateral funiculus (Watkins et al., 1983). Other evidence, however, suggests that spinal opioid release may be driven by several pathways, some of them restricted to the spinal cord (Yaksh and Elde, 1981; Cruz and Basbaum, 1985; Harlan et al., 1987).

Spinal opioid release was also studied by measuring opioids in spinal superfusates during stimulation with different noxious stimuli. The position of the superfusion catheter was used to determine whether the release was “segmental”, i.e., limited to the spinal segment receiving the stimulus, or “heterosegmental”, i.e., occurring over the whole spinal cord, which would suggest the involvement of supraspinal signals. Noxious mechanical stimulation produced heterosegmental Met-enkephalin release (Le Bars et al., 1987a; Le Bars et al., 1987b), whereas thermal stimulation (Cesselin et al., 1989) or subcutaneous formalin (Bourgoin et al., 1990) produced segmental release. This suggests that different pain modalities involve different opioid-releasing pathways. However, this approach has several problems: 1) determining the origin of opioid release based on the position of the catheter is uncertain because of the unknown diffusion of opioids out of the spinal cord and inside the spinal canal; 2) opioids other than Met-enkephalin are released (Yaksh et al., 1983); 3) the use of anesthesia can affect opioid release.

A way to overcome these limitations is to use MOR internalization as an in situ measure of opioid release (Trafton et al., 2000; Song and Marvizon, 2003b; 2003a; Mills et al., 2004; Song and Marvizon, 2005). An initial attempt to elicit MOR internalization with noxious stimuli produced negative results (Trafton et al., 2000). However, this can be accomplished by inhibiting three peptidases (aminopeptidase, dipeptidyl carboxypeptidase and neutral endopeptidase) that avidly degrade enkephalins and dynorphins in the spinal cord (Song and Marvizon, 2003b; Chen et al., 2007; Lao et al., 2008). Here, we use this approach to study spinal opioid release during peripheral inflammation.

Experimental Procedures

Animals

Experiments were performed in adult male Sprague Dawley rats (300-450 g, Harlan, Indianapolis, IN). Rats were housed together, three rats to a cage, and had access to food and water at all times. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

Intrathecal injections

Rats were surgically implanted with chronic intrathecal catheters, which were used to deliver peptidase inhibitors to the lumbar or thoracic spinal cord. For the lidocaine spinal block experiment, rats were outfitted with two catheters: one terminating over the lumbar spinal cord

(used to deliver peptidase inhibitors) and another terminating over the cervical-thoracic spinal cord (used to deliver lidocaine). Different surgical procedures were used to implant catheters terminating over the lumbar and the thoracic spinal cord.

For intrathecal injections onto the lumbar spinal cord, the catheter was inserted between the L5 and L6 lumbar vertebrae (Storkson et al., 1996). The catheter consisted of 20-25 mm of polyethylene PE-5 tube heat-fused to 150 mm of PE-10 tube. The rat was anesthetized (2-3% isoflurane in O₂) and placed on an aluminum platform maintained at 37 °C. The skin of the lumbar region was shaved and cut at the lumbar midline. The point where vertebra L5 overlaps vertebra L6 was exposed by cutting the muscle. Raising the spine, a blunted 20 G needle was inserted between the L5 and L6 vertebrae to puncture the dura mater. The PE-5 end of the catheter was inserted into the subdural space and pushed rostrally to terminate over the L5-L6 spinal segments. Placement of the catheter under the dura mater was inferred from a reflex flick of the tail or paw and the backflow of spinal fluid, and it was confirmed post-mortem. The bead of plastic at the junction of the PE-5 and PE-10 tubes was sutured to the muscle to immobilize the catheter. The catheter was tunneled through the subcutaneous space and externalized over the head. The catheter was flushed with 10 µl saline and closed with an electric cauterizer.

For intrathecal injections onto the cervical-thoracic spinal cord, the catheter was implanted from the cisterna magna (Yaksh and Rudy, 1976; LoPachin et al., 1981). The back of the head and neck of the rat were shaved, and the head was clamped flexed forward using the ear bars of a stereotaxic holder. A midline incision was made in the skin at the back of the neck, and the muscle was cut at its juncture with the edge of the cranium. The atlanto-occipital cisternal membrane was exposed and punctured with a needle to insert the catheter, which was passed into the intrathecal space to end at the C7-T1 segments. The other end of the catheter was tunneled under the skin over the cranium and externalized.

Rats that showed motor weakness or signs of paresis were euthanized. This never happened with the first procedure (lumbar insertion) and rarely with the second (cervical insertion). Rats were housed separately and allowed to recover for 5-7 days. Unless stated otherwise, injection volume was 10 µl of injectate plus 10 µl saline flush. Solutions were preloaded, in reverse order of administration, into a PE-10 tube, and delivered with a 50 µl Hamilton syringe within 1 min. The position of the catheter was examined postmortem, and if it terminated inside the spinal cord or its tip was occluded the animal was excluded.

Formalin injection

Formalin injection was used as a model of short-term inflammation. Five to seven days before the experiment, rats were implanted with chronic intrathecal catheters terminating onto the lumbar spinal cord (except experiment 4, where they terminate onto the cervical-thoracic spinal cord, C7-T1). The day of the experiment, at time 0 min the intrathecal catheter was used to inject saline or peptidase inhibitors (100 nmol amastatin, captopril and phosphoramidon). At time 5 min, rats were injected subcutaneously with 50 µl of 2.5% formalin (37% formaldehyde, Sigma; 2.5% refers to the actual concentration of formaldehyde injected) into the plantar surface of the left hindpaw (Yamamoto and Yaksh, 1992; Traub, 1996). Control rats received a similar injection of 50 µl saline. Rats were put back in their cages and their behavior was observed. At time 20 min, the rats were euthanized with pentobarbital (100 mg/kg) and fixed immediately by aortic perfusion. This 20 min interval extended into the second phase of formalin effect (Dubuisson and Dennis, 1977; Tjolsen et al., 1992). Waiting 20 min does not affect the protective effect of the peptidase inhibitors, which lasts for at least 25 min (Chen et al., 2007), or the evoked MOR internalization, which persists for 60 min (Trafton et al., 2000). Anesthesia (2.5% isoflurane in oxygen) was used only in some experiments: in experiment 1, rats were anesthetized for the intrathecal injection and allowed to wake up

immediately afterward; in experiment 2 (studying the effect of anesthesia), one group of rats was not anesthetized at all, and another group was anesthetized throughout the procedure. In experiments 3-5, rats were not anesthetized.

Mechanical stimulation

Five to seven days after implanting the intrathecal catheters, rats were anesthetized with isoflurane (2-3%) and given an intrathecal injection of saline or peptidase inhibitors (100 nmol amastatin, captopril and phosphoramidon). After minutes, a noxious mechanical stimulus was delivered, consisting in clamping one paw with a hemostat (closed to the first notch) for 30 sec (Le Bars et al., 1987a). Ten minutes later, rats were euthanized with pentobarbital (100 mg/Kg).

Complete Freund's adjuvant (CFA) injection

Five days after implanting the intrathecal catheters, rats were anesthetized (2-3% isoflurane) and injected subcutaneously with 150 µl of CFA (Sigma) into the plantar surface of the left hindpaw. Two days later, the rats received one of the following treatments: 1) intrathecal injection of peptidase inhibitors; 2) intrathecal peptidase inhibitors followed 5 min later by clamping the inflamed left hindpaw with a hemostat for 30 sec (under isoflurane anesthesia). Rats were euthanized with pentobarbital (100 mg/kg) 10 min after the intrathecal injection or the hindpaw clamp, and fixed immediately by aortic perfusion.

Immunohistochemistry

Spinal cord sections were double-labeled for MORs and NK1Rs (Song and Marvizón, 2003a). Rats were euthanized with pentobarbital (100 mg/kg) and fixed immediately by aortic perfusion of 100 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) containing 0.01% heparin, followed by 400 ml of ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in phosphate buffer). Spinal segments L5, T10 and C2 were dissected based on root identification, and post-fixed, cryoprotected in 20% sucrose, embedded in Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA) and frozen on dry ice. Free-floating transversal sections (25 µm thick) throughout each segment were cut with a cryostat. Sections were washed twice with PBS and twice with PBS containing 0.5% Triton X-100, 0.01% thimerosal (PBS/Triton) and 5% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA), and then incubated overnight with a mixture of antibodies against the MOR (1:6000) and the NK1R (1:4000) in PBS/Triton. The anti-MOR rabbit antiserum was raised against amino acids 384-398 of rat MOR-1 (ImmunoStar, Hudson, WI). It has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). The anti-NK1R guinea pig antiserum (Millipore, Billerica, MA) was raised against amino acids 393-407 of the rat NK1R (Song and Marvizón, 2003a). Double labeling with the guinea pig NK1R antiserum and a rabbit NK1R antiserum (# 94168, CURE: Digestive Disease Research Center), previously characterized (Grady et al., 1996), resulted in co-localization of the staining at the cellular and sub-cellular levels. After three washes with PBS, sections were incubated for two hours with two secondary antibodies: Alexa Fluor-488 goat anti-rabbit IgG (1:2000, Invitrogen-Molecular Probes, Eugene, OR), and rhodamine red-X donkey anti-guinea pig IgG (1:1000, Jackson ImmunoResearch). After four more washes, sections were mounted in Prolong Gold (Invitrogen-Molecular Probes). All incubations and washes were done at room temperature.

Confocal microscopy

Confocal images were acquired using a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Excitation was provided by the 488 nm line of an argon laser for Alexa Fluor 488 and the 561 nm line of a diode laser for rhodamine red-X. Corresponding emission windows were 500-530 nm and 600-670 nm. We used a pinhole of

1.0 Airy unit and objectives of 20× (numerical aperture [NA] 0.7), 63× oil (NA 1.32) and 100× oil (NA 1.4), resulting in estimated optical section thicknesses (full width at half maximum) of 2.53 μm , 0.69 μm and 0.62 μm , respectively. Images were acquired as confocal stacks (5–32 optical sections) with an optical section separation (z-interval) of 1.180 μm for the 20× objective and 0.285 μm for the 100× objective. Optical sections were acquired at a digital size of 1024×1024 pixels and averaged 3–4 times to reduce noise.

Deconvolution and image processing

The antibodies used produced high quality confocal images, with low background and noise. Images were further improved using deconvolution to reduce blur (Wallace et al., 2001; Cannell et al., 2006). Adaptive point spread function deconvolution (Holmes et al., 2006) of the entire confocal stacks was performed in 5 iterations using AutoQuant X2.0.1 (Media Cybernetics, Inc., Bethesda, MD). The resulting 32-bit three dimensional image file was imported into Imaris (x64, 6.1.5, Bitplane AG, Zurich, Switzerland), which was used to crop the image (without changing the resolution), adjust the brightness and gamma, and select the color for each channel: green for MOR/Alexa Fluor-488 and magenta for NK1R/rhodamine red-X (instead of red, to make it visible to colorblind readers). A two-dimension projection picture was imported into Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA), which was used to compose the multi-panel figures, and to add text, arrows and scale bars.

Quantification of MOR and NK1R internalization

Previously described procedures were used to quantify MOR internalization (Marvizón et al., 1999a; Song and Marvizón, 2003b, 2003a, 2005) and NK1R internalization (Mantyh et al., 1995; Abbadie et al., 1997; Marvizón et al., 1997; 1999b; Trafton et al., 1999; Trafton et al., 2001; Marvizón et al., 2003a). MOR or NK1R neurons were visually counted while classifying them as with or without internalization, using an objective of 63× (NA 1.40) and a Zeiss Axio-Imager A1 microscope (Carl Zeiss, Inc., Thornwood, NY). Somata with >5 (MOR) or >10 (NK1R) endosomes were considered with internalization, as in previous studies. Counting was done blind to the treatment. Four ipsilateral half-sections and four contralateral half-sections were counted for each spinal segment. In every half-section, all MOR neurons in laminae I–II and all NK1R neurons in lamina I were counted. With this strategy, the average number of MOR cells counted per rat was (mean $\pm$ standard error of a sample of 17 experiments): L5 ipsilateral 87 ± 4 , L5 contralateral 82 ± 3 , T10 ipsilateral 29 ± 3 , T10 contralateral 28 ± 2 , C2 ipsilateral 51 ± 3 , C2 contralateral 52 ± 3 . The average number of NK1R cells counted per rat was: L5 ipsilateral 32 ± 2 , L5 contralateral 20 ± 2 , T10 ipsilateral 5 ± 0.4 , T10 contralateral 5 ± 0.4 , C2 ipsilateral 8 ± 1 , C2 contralateral 7 ± 1 . The C2 and T10 segments have a lower density of MOR and NK1R neurons than the L5 segment.

An endosome was defined as a small region of bright MOR or NK1R staining separated from the cell surface. In each neuron, endosomes were counted by changing the focus to scan the soma (not the dendrites) in the z-axis. Whereas high concentrations of exogenous opioids elicit the appearance of numerous endosomes and the almost complete disappearance of MOR immunoreactivity from the cell surface (Marvizón et al., 1999a; Song and Marvizón, 2003b; Chen et al., 2007), endogenously released opioids produce a more subtle pattern (Song and Marvizón, 2005; Chen et al., 2008a; Chen et al., 2008b; Lao et al., 2008). Endosomes are observed, but a large part of the MOR immunoreactivity remains at the cell surface forming clusters (Supplementary Fig. 1, available online). In contrast, neurons without MOR internalization have practically no endosomes and less clustering of MORs at the cell surface (Supplementary Fig. 2, available online). MOR clusters can be mistaken as endosomes when several confocal sections are superimposed in an image. However, clusters and endosomes can usually be distinguished visually by slightly focusing up and down. Consistent results were obtained when the same sample was counted by two different investigators blinded to the

treatment and to each other's results. Counting criteria were applied consistently to all the sections and all the animals.

Statistical analysis

Data were analyzed and plotted using Prism 5 (GraphPad Software, San Diego, CA). Data from each rat was considered as one replicate for statistical purposes. Significance was set at 0.05. Statistical analysis consisted of two-way ANOVA. In all rats we measured MOR internalization and NK1R internalization in several spinal cord segments. MOR internalization and NK1R internalization data were analyzed separately. One variable of the ANOVA was the treatment given to the animals (i.e., formalin, CFA, anesthesia, lidocaine block, etc.). The second variable was the spinal cord region where the internalization was measured, i.e., the spinal cord segment and side (ipsilateral or contralateral to the stimulus). Bonferroni's post-hoc tests were used to study differences produced by the first variable (treatment) in each spinal cord region, and occasionally to study differences between spinal cord regions.

Results

Experiment 1: MOR internalization induced by formalin

Our first objective was to determine whether formalin injection induces MOR internalization in the spinal cord. To increase the amount of MOR internalization induced by the released opioids we injected peptidase inhibitors (amastatin, captopril and phosphoramidon, 100 nmol) onto the lumbar spinal cord using chronic intrathecal catheters. Rats were anesthetized to facilitate the intrathecal injection, but allowed to recover from anesthesia 5 min before the formalin injection. Rats injected with formalin (2.5%) protected the injected left paw and licked it repeatedly throughout the 20 min observation period, whereas saline-injected rats only licked the paw immediately after the injection.

NK1R internalization provided a measure of the effectiveness of formalin (Abbadie et al., 1997; Honore et al., 1999). Formalin induced NK1R internalization in lamina I neurons of ipsilateral L5 (Fig. 1 A), but not in contralateral L5 (Fig. 1 B) or in T10 (Fig. 2 B). These results were confirmed quantitatively by counting NK1R neurons (Fig. 3). A two-way ANOVA revealed a significant effect of formalin ($p=0.0001$) and significant differences between spinal cord segments ($p<0.0001$), as well as the interaction between these two variables ($p<0.0001$). In saline-injected rats, NK1R internalization in ipsilateral L5 was significantly lower than in formalin-injected rats ($p<0.001$). Some NK1R internalization was detected in ipsilateral L5 of the control rats, however, probably because the saline injection is moderately noxious. In formalin-injected rats, NK1R internalization in ipsilateral L5 was significantly higher than in contralateral L5 or other spinal cord segments ($p<0.001$), showing that substance P release was restricted to the dorsal horn receiving innervation from the injured paw.

MOR internalization was measured in spinal segments L5, T10 and C2 to determine whether opioid release was segmental or heterosegmental. MOR internalization (Fig. 1) was less pronounced than NK1R internalization, both in terms of number of endosomes and the amount of immunoreactivity remaining at the cell surface (supplementary Figs. 1 and 2). MOR neurons with internalization were found mostly in the medial dorsal horn and were rare in the lateral dorsal horn (Fig. 1 A), as we found in previous studies (Song and Marvizón, 2003,2005; Lao et al., 2008). Quantitatively, MOR internalization in the formalin-injected rats was significantly higher than in saline-injected controls (Fig. 3 A). Two-way ANOVA revealed a significant effect of formalin ($p=0.007$), significant differences between spinal cord regions ($p<0.0001$), and significant interaction between the two variables ($p=0.001$). Surprisingly, in formalin-injected rats MOR internalization in L5 occurred both ipsilaterally and contralaterally to the formalin injection, and was not significantly different between these two sides ($p>0.05$, Fig. 3

A). Fig. 1 B shows examples of MOR neurons with internalization in contralateral L5 (insets a, b, h). MOR internalization was negligible in segments T10 and C2 (Fig. 2 B). Fig. 2 A shows the lack of MOR internalization in ipsilateral L5 of saline-injected rats. In formalin-injected rats, MOR internalization in ipsilateral and contralateral L5 was significantly higher than in the other spinal cord segments ($p < 0.001$), showing that the opioid release was segmental.

Experiment 2: Effect of anesthesia on formalin-induced MOR internalization

Since anesthesia can affect neuronal pathways involved in opioid release, we investigated its effect on formalin-evoked MOR internalization. One group of rats was anesthetized with isoflurane just before the intrathecal injection of peptidase inhibitors and kept under anesthesia throughout the experiment. Another group of rats was not anesthetized at all. Both groups received a formalin injection in the left hindpaw. Formalin induced considerably more MOR internalization in the non-anesthetized rats (Fig. 4 A), both in ipsilateral and contralateral L5. Two-way ANOVA revealed a significant effect of the anesthesia ($p = 0.0004$), spinal cord region ($p < 0.0001$), and their interaction ($p = 0.001$). Increased MOR internalization in the absence of anesthesia was apparent in both ipsilateral L5 ($p < 0.001$) and contralateral L5 ($p < 0.05$). MOR internalization in ipsilateral L5 was higher than contralateral L5 ($p < 0.001$) in the non-anesthetized rats, but not in the anesthetized rats ($p > 0.05$). In contrast, anesthesia did not affect NK1R internalization evoked by formalin (Fig. 4 B, two-way ANOVA: $p = 0.4$ for anesthesia, $p < 0.0001$ for spinal cord region), showing that it did not interfere with primary afferents signals, substance P release or NK1R activation.

Experiment 3: Effect of a lidocaine spinal block on formalin-induced MOR internalization

One key issue is whether formalin-evoked opioid release involves propriospinal or supraspinal circuits. We did not use spinal cord transection (Watkins et al., 1984; Hutchison et al., 1990) to address this issue because post-surgery pain produced by it could be a confounding variable when studying nociception-evoked opioid release. Moreover, spinal transection increases dynorphin and enkephalin gene expression (MacArthur et al., 1999). Therefore, we opted for a less invasive procedure: using lidocaine to block descending signals at the cervical-thoracic level (Ren and Dubner, 1996). Rats were implanted with two intrathecal catheters. One, used to inject peptidase inhibitors, was inserted between the lumbar vertebrae to terminate over L5-L6. Another, used to inject lidocaine, was implanted from the cisterna magna to terminate over C7-T1. Rats were not anesthetized. The timeline was: 1) peptidase inhibitors onto L5-L6 at 0 min; 2) lidocaine or saline onto C7-T1 at 2 min; 3) formalin in the hindpaw at 5 min.

Several pilot experiments were performed to optimize the lidocaine injection. Two problems needed to be avoided: 1) if the volume or dose of lidocaine was too large, it would spread into the lumbar spinal cord and block opioid release there; 2) if its dose was too small, the spinal block would be incomplete. To control these two problems, we observed the presence of motor deficits in the paws, and also used NK1R internalization to assess whether lidocaine blocked action potentials in the dorsal roots (Lao et al., 2003). Injecting 1 μ l of 10% lidocaine plus a 3 μ l saline flush was found to be optimal because it produced paralysis in the forepaws. It also eliminated NK1R internalization in the cervical-thoracic spinal cord induced by forepaw formalin (2.5% in 50 μ l). Thus, NK1R internalization values in control rats ($N = 3$) and in lidocaine-injected rats ($N = 5$) were, respectively: $79 \pm 6\%$ and $0 \pm 0\%$ ($p < 0.001$) in ipsilateral T1; $58 \pm 7\%$ and $6 \pm 6\%$ ($p < 0.001$) in ipsilateral C7, and $27 \pm 22\%$ and $0 \pm 0\%$ ($p < 0.01$) in ipsilateral C2. This shows that lidocaine blocked action potentials in the T1-C2 dorsal roots and thus likely in the adjacent spinal cord segments. Importantly, there was not lidocaine spillover to L5, because NK1R internalization in L5 induced by hindpaw formalin was the same in lidocaine-injected and saline-injected rats (Fig. 5 B; two-way ANOVA: $p = 0.17$ for lidocaine, $p < 0.0001$ for spinal cord region, $p = 0.8$ for interaction).

The lidocaine spinal block produced a substantial decrease in the MOR internalization evoked by formalin, both in ipsilateral and contralateral L5 (Fig. 5 A). Two-way ANOVA revealed significant effects of lidocaine block ($p=0.0002$), spinal cord region ($p<0.0001$) and their interaction ($p=0.0007$). The lidocaine block significantly decreased MOR internalization in ipsilateral L5 ($p<0.001$) and contralateral L5 ($p<0.01$). However, it did not completely abolish the evoked MOR internalization. Therefore, supraspinal pathways drive or facilitate the formalin-induced spinal opioid release, although some spinal opioid release may occur in the absence of supraspinal signals.

Experiment 4: Effect of the site of injection of peptidase inhibitors on formalin-induced MOR internalization

Since the injection of peptidase inhibitors was given onto the lumbar spinal cord, it could be that MOR internalization did not occur in the T10 and C2 spinal segments because the peptidase inhibitors did not reach them. This is unlikely, however, because intrathecal injections of the same volume (10 μ l plus 10 μ l flush) of Leu-enkephalin and peptidase inhibitors produced MOR internalization over the entire length of the spinal cord (Chen et al., 2007). The goal of this experiment was to confirm that the site of the intrathecal injection was not responsible for MOR internalization being segmental. MOR internalization was evoked by injecting formalin into the left hindpaw, but this time peptidase inhibitors were injected onto the cervical-thoracic (C7-T1) spinal cord using an intrathecal catheter implanted from the cisterna magna. Formalin-induced MOR internalization was still negligible in segments C2 and T10 (Fig. 6 A), confirming that opioid release was segmental. In the L5 segment, there was a small decrease in MOR internalization in the contralateral side ($p<0.05$) with the thoracic injection of peptidase inhibitors. Two-way ANOVA revealed a marginally significant effect of the site of intrathecal injection ($p=0.03$), a significant effect of spinal cord region ($p<0.0001$) and no significant interaction ($p=0.2$). Therefore, the segmental nature of MOR internalization could not be attributed to the site of injection of the peptidase inhibitors.

Experiment 5: Formalin-induced MOR internalization without peptidase inhibitors

We wondered whether it would be possible to detect formalin-induced MOR internalization in the absence of peptidase inhibitors. In the experiment shown in Fig. 7, rats received intrathecal saline instead of peptidase inhibitors, and saline or 2.5% formalin in the hindpaw. Formalin induced a small amount of MOR internalization in the L5 segment, both the ipsilaterally and contralaterally (Fig. 7 A), which was significantly higher than in saline-injected rats. Two-way ANOVA revealed a significant effect of formalin ($p=0.0002$), no significant differences between ipsilateral and contralateral L5 ($p=0.054$) and no significant interaction ($p=0.9$). Grouping the MOR internalization data for ipsilateral L5 in Fig. 7 A with those in Fig. 3 A for a two-way ANOVA revealed significant effects of formalin ($p=0.0097$) and peptidase inhibitors ($p=0.0027$), but not of their interaction ($p=0.08$). A similar two-way ANOVA of data in contralateral L5 yielded $p=0.006$ for formalin and $p=0.004$ for peptidase inhibitors. Therefore, although peptidase inhibitors increase formalin-evoked MOR internalization, the effect of formalin could be readily detected in the absence of peptidase inhibitors.

Experiment 6: MOR internalization induced by hindpaw clamp without peptidase inhibitors

We also determined whether MOR internalization evoked by an acute mechanical stimulus (hindpaw clamp with a hemostat) could be detected in the absence of peptidase inhibitors. We previously reported (Lao et al., 2008) that, after intrathecal peptidase inhibitors, hindpaw clamp induced MOR internalization in the L5 and L6 spinal cord segments. Rats under isoflurane anesthesia received intrathecal peptidase inhibitors or saline; 5 min later their left hindpaw was clamped with a hemostat for 30 sec, and after 10 min more they were euthanized. In the presence

of peptidase inhibitors, hindpaw clamp produced MOR internalization in ipsilateral L5 (Fig. 8 A). This is lower than what we reported previously (Lao et al., 2008), probably because in that study we used a different stimulus (hindpaw clamp 10 s on and 10 s off for 10 min). Two-way ANOVA yielded $p < 0.0001$ for spinal cord region and $p = 0.016$ for interaction, but no overall significant effect for peptidase inhibitors ($p = 0.07$), probably because the analysis included data in C2 and T10 where there was no MOR internalization. Indeed, the Bonferroni's post-test revealed a significant effect of peptidase inhibitors in ipsilateral L5 ($p < 0.001$), but not in contralateral L5 ($p > 0.05$). MOR internalization was significantly lower in contralateral L5 than in ipsilateral L5 ($p < 0.001$). MOR internalization was negligible in the C2 and T10 segments, confirming that this mechanical stimulus also induces segmental opioid release. In the absence of peptidase inhibitors, MOR internalization in ipsilateral L5 was still significantly higher ($p < 0.05$) than in C2 and T10 (Fig. 8 A). Since in naïve rats MOR internalization is negligible in the L1-L6 segments (Lao et al., 2008), hindpaw clamp induced some MOR internalization in L5 in the absence of peptidase inhibitors.

NK1R internalization (Fig. 8 B) was high in ipsilateral L5 and negligible in contralateral L5, C2 and T10 ($p < 0.0001$ for spinal cord region), in agreement with previous studies (Mantyh et al., 1995; Abbadie et al., 1997; Lao et al., 2008). Peptidase inhibitors did not significantly affect NK1R internalization ($p = 0.2$, two-way ANOVA), but a trend towards an increase was noted in ipsilateral L5.

Experiment 7: MOR internalization after CFA

We hypothesized that long-term inflammation produced by CFA would induce a sustained release of opioids in the spinal cord. CFA was injected in one hindpaw. Two days later peptidase inhibitors were injected intrathecally, and the rats were euthanized 10 min afterward. MOR internalization was negligible in segments C2, T10 and L5 (Fig. 9 A), indicating that CFA-induced inflammation did not evoke opioid release in the spinal cord at this time point. NK1R internalization was also negligible (Fig. 9 B), in agreement with a previous study (Honore et al., 1999). Fig. 10 A shows examples of MOR and NK1R neurons without internalization in the L5 dorsal horn ipsilateral to the CFA injection.

Because Honore et al. (1999) found that CFA inflammation increases the amount of NK1R internalization induced by noxious mechanical stimulation, we wondered whether CFA would similarly increase MOR internalization. We found the opposite: CFA injected two days earlier produced a significant decrease in MOR internalization in ipsilateral L5 induced by hindpaw clamp (Fig. 9 A). Two-way ANOVA of data in Fig. 9 A revealed significant effects of the treatment (CFA, clamp and CFA + clamp, $p < 0.0001$), spinal cord region ($p < 0.0001$) and their interaction ($p < 0.0001$). The Bonferroni's post-test yielded significant differences between 'clamp' and 'CFA + clamp' for ipsilateral L5 ($p < 0.05$), but not for contralateral L5. Examples of MOR neurons after hindpaw clamp in naïve and CFA-injected rats are shown in Fig. 10.

In lamina I, NK1R internalization induced by hindpaw clamp showed a trend towards increasing in CFA-injected rats compared with naïve rats (Fig. 9 B). This increase was not significant, probably because NK1R internalization was near its maximum. However, in lamina III, where it is usually not found, NK1R internalization was noted after clamping the CFA-inflamed hindpaw (inset e of Fig. 10 C). This suggests that in the CFA-treated rats the amount of released substance P spilled over into the deeper laminae of the dorsal horn, as previously reported by Honore et al. (1999).

Discussion

The main findings of this study are: 1) acute inflammation with formalin released opioids (measured as MOR internalization) in the spinal cord; 2) MOR internalization was segmental;

3) it was bilateral; 4) it involved supraspinal signals; 5) long-term inflammation (CFA) did not induce MOR internalization, and 6) long-term inflammation decreased MOR internalization induced by acute noxious stimuli.

Involvement of spinal and supraspinal circuits in formalin-induced spinal opioid release

Opioids are not directly released by primary afferents, which contain no enkephalins (Pohl et al., 1994) and only limited amounts of dynorphins (Botticelli et al., 1981; Basbaum et al., 1986). Immunohistochemistry studies (Martin-Schild et al., 1998; Pierce et al., 1998; Spike et al., 2002; Nydahl et al., 2004) suggested that the putative endogenous MOR agonists endomorphins are present in substance P-containing primary afferents. However, we found that electrical stimulation of the dorsal root did not induce MOR internalization, while it produced abundant NK1R internalization (Song and Marvizón, 2003a). In contrast, electrical stimulation of the entire dorsal horn did induce abundant MOR internalization. These findings rule out that endomorphins are co-released with substance P from primary afferents. Instead, the opioids whose release induces MOR internalization are most likely enkephalins and dynorphins, which are expressed by two different populations of dorsal horn neurons (Cruz and Basbaum, 1985; Standaert et al., 1986; Harlan et al., 1987; Todd et al., 1992; Todd and Spike, 1993).

MOR internalization induced by formalin was segmental, since it was detected in L5 and not in T10 or C2. Although we did not measure MOR internalization in lumbar segments other than L5, we previously found that MOR internalization induced by hindpaw clamp occurred only in the L5 and L6 segments, i.e., the ones that receive innervation from the paw (Lao et al., 2008). It is likely that MOR internalization induced by formalin, likewise, is low in the L1-L4 lumbar segments. We previously reported (Song and Marvizón, 2003a) that dorsal root stimulation did not induce MOR internalization in dorsal horn neurons, but those experiments were done in coronal spinal cord slices where most spinal circuits are severed. Hence, while that study showed that opioids are not released from primary afferent terminals, it could not rule out that they are released by complex spinal circuits processing primary afferent signals. Formalin-induced opioid release was also bilateral, i.e., it occurred both ipsilaterally and contralaterally to the formalin injection. In a previous study (Lao et al., 2008) we missed this contralateral effect because hindpaw clamp produces less MOR internalization contralaterally than formalin (Fig. 8 A). The occurrence of opioid release contralaterally could be explained by the existence of axons connecting the ipsilateral and the contralateral dorsal horns (Fitzgerald, 1983).

The fact that a lidocaine spinal block substantially decreased formalin-induced MOR internalization demonstrates that the spinal opioid release involves supraspinal signals. Thus, it is likely that supraspinal axons make excitatory synapses with the opioidergic dorsal horn neurons, so that formalin-induced spinal opioid release is mediated by a spinal cord-brainstem-spinal cord loop. The lidocaine block appears to be complete, because it abolished NK1R internalization in the cervical-thoracic spinal cord induced by formalin injection in the forepaw. This indicates that lidocaine suppressed action potentials in the dorsal roots and likely also in descending spinal cord axons. However, we cannot rule out that lidocaine did not block completely the descending signals. The idea that spinal opioid release is driven supraspinally has been considered for some time (Basbaum et al., 1976; Basbaum and Fields, 1984; Fields et al., 1991; Mason, 1999). It is based on indirect evidence: opioid receptor antagonists applied to the spinal cord reversed the analgesia induced by stimulation of the rostroventral medulla (Zorman et al., 1982) or the periaqueductal gray (Budai and Fields, 1998). Tambeli et al. (2003) found that noxious stimuli applied to the hindpaw produced antinociception (measured as an attenuation of the trigeminal jaw-opening reflex), which was also blocked by spinal opioid antagonists.

However, the facts that formalin-induced opioid release was both segmental and supraspinally-mediated are hard to reconcile. One possible explanation is that spinally-projecting areas in the brainstem map to the spinal cord, so that a signal from one spinal segments elicits a brainstem signal directed to the same segment. Indeed, it has been suggested that the nucleus raphe magnus (Watkins et al., 1980) and other spinally-projecting regions (Watkins et al., 1981) have such a somatotopic organization, but supporting evidence is rather weak. Another explanation is that spinal opioid release requires the convergence of primary afferents signals and supraspinal signals. According to this idea, the supraspinal signals are not targeted to particular spinal segments but reach the entire spinal cord, whereas the primary afferents signals reach only a few spinal segments and confer opioid release its segmental specificity. Only where these two signals converge opioid release occurs in detectable amounts. At the cellular level, it is possible that the opioidergic dorsal horn neurons receive excitatory synapses from both primary afferents and descending axons, which synergize to drive opioid release.

Anesthesia decreases spinal opioid release

We found that formalin-induced MOR internalization was substantially decreased by isoflurane anesthesia. In contrast, anesthesia had no effect on formalin-induced NK1R internalization. The most likely explanation for the inhibition of the evoked MOR internalization by anesthesia is that the supraspinal brain areas involved in spinal opioid release are suppressed by inhalation anesthetics. On the other hand, substance P release and NK1R internalization is driven by signals in primary afferents, which are not affected by inhalation anesthetics.

Most previous studies on spinal opioid release were carried out in anesthetized animals (Yaksh and Elde, 1981; Yaksh et al., 1983; Hutchison et al., 1990; Tseng et al., 1997). This could explain why some of these studies [for example (Yaksh and Elde, 1981)] did not detect supraspinal modulation of spinal opioid release. Recognizing the possible confounding effect of anesthesia, the group of Cesselin conducted several studies on spinal opioid release (Cesselin et al., 1985; Le Bars et al., 1987a; Le Bars et al., 1987b; Cesselin et al., 1989; Bourgoin et al., 1990) in which the rats were paralyzed with gallamine and anesthetized with a very low dose (0.5%) of halothane. However, we could not achieve stable anesthesia with doses of isoflurane lower than 2%. Like us, these investigators found that subcutaneous formalin produced segmental Met-enkephalin release (Bourgoin et al., 1990), but they found that mechanical stimuli produced heterosegmental Met-enkephalin release (Le Bars et al., 1987a; Le Bars et al., 1987b) in contrast to what we found in fully anesthetized rats (Lao et al., 2008). It is unclear whether this disagreement stems from the level of anesthesia or from other experimental variables, like the method used to measure opioid release.

Requirement for peptidase inhibitors

In contrast with our results, a study by Trafton et al. (2000) found that formalin injection in the paw, among other noxious stimuli, did not induce MOR internalization in the rat spinal cord. This disagreement is explained by several key differences with our study: 1) Trafton et al. did not use peptidase inhibitors; 2) they anesthetized the rats; 3) they quantified MOR internalization by counting endosomes, whereas we counted neurons with and without internalization. We were able to detect MOR internalization without using peptidase inhibitors, induced by formalin injection or by hindpaw clamp. In the later case, MOR internalization was detected even though the rat was anesthetized. Therefore, our discrepancy with the study by Trafton et al. is only partially explained by the use of peptidase inhibitors and the absence of anesthesia. It is likely that Trafton et al. missed some of the MOR neurons with internalization because they sampled only 20 neurons per rat in the L4-L5 segments, whereas we sampled all the neurons in laminae I-II in four sections, which amounted to 80-90 neurons per rat in each half of the L5 segment.

It has been known for some time that enkephalins and dynorphins are highly susceptible to peptidase degradation (Guyon et al., 1979; Hiranuma et al., 1998). Peptidase inhibitors dramatically increase the potency of enkephalin to induce MOR internalization (Song and Marvizón, 2003b; Chen et al., 2007) and analgesia (Kishioka et al., 1994; Chen et al., 2007). For this reason, peptidase inhibitors are usually required to detect MOR internalization evoked by spinal opioid release (Song and Marvizón, 2003a, 2003b, 2005; Lao et al., 2008). Still, one may wonder how opioids released in the spinal cord produce analgesia (Zorman et al., 1982; Jensen and Yaksh, 1984; Morgan et al., 1991; Budai and Fields, 1998) if peptidases prevent them from activating MORs.

The key issue is how many of the MORs in a neuron need to be occupied by agonist to produce an effect. To detect MOR internalization we need to activate a substantial fraction of the MORs in each neuron, otherwise there will not be enough endosomes to meet our internalization criterion. However, much less agonist occupancy of MORs may be sufficient to produce an analgesic signal. Thus, low doses of intrathecal enkephalin produced analgesia while inducing only marginal MOR internalization (Chen et al., 2007). This small amount of MOR internalization is similar to that induced in the absence of peptidase inhibitors, *in vivo* by formalin injection (Fig. 7) or hindpaw clamp (Fig. 8), and in spinal cord slices by high K^+ (Chen et al., 2008b).

An alternative possibility is that, in fact, noxious stimuli do not release enough endogenous opioids to overcome their degradation by peptidases and produce analgesia. If this is the case, then opioid release of physiological significance may require additional supraspinal signals caused by states like stress (Watkins et al., 1982, 1983, 1984). Indeed, early studies (El-Sobky et al., 1976; Grevert and Goldstein, 1978) found that systemic injections of naloxone did not change pain perception in humans, and intrathecal injections of naloxone did not change tail-flick responses in rats (Zorman et al., 1982). This suggests that noxious stimuli do not produce enough opioid release to induce analgesia. However, naloxone produces excitatory actions on dorsal horn neurons (Henry, 1979; Budai and Fields, 1998) that may counter the analgesic action of opioids. Still, MOR antagonists that do not have the excitatory effects of naloxone did not increase responses of dorsal horn neurons to noxious heat, either (Budai and Fields, 1998). Note, however, that Zorman et al. and Budai and Fields used noxious heat in their experiments, while we found that noxious heat does not induce MOR internalization in the dorsal horn (Lao et al., 2008). Therefore, it is possible that other noxious stimuli, like formalin and paw clamp, produce enough spinal opioid release to induce antinociception.

Spinal opioid release decreases during long-term inflammation

The results we obtained with CFA are important because they show that chronic inflammation does not produce a sustained release of opioids in the spinal cord. Instead, spinal opioid release seems to be short-term and driven by acute noxious stimuli. It is also noteworthy that CFA does not produce substance P release, either (Honore et al., 1999). This indicates that the chronic inflammation produced by CFA does not induce sustained firing of primary afferents, which would result a continuous release of substance P. Instead, CFA sensitizes the primary afferents, so that subsequent acute stimuli produce a greater amount of substance P release (Honore et al., 1999). This sensitization is one of the hallmarks of inflammatory pain. Surprisingly, a noxious stimulus applied to the inflamed paw did not increase spinal opioid release, as it did substance P release, but rather it decreased it. Since this decrease in opioid release cannot be attributed to less firing of primary afferents, it is probably caused by changes in spinal or supraspinal circuits. Depletion of spinal opioids needs to be ruled out, however, because inflammation upregulates the synthesis in the spinal cord of dynorphins (Ruda et al., 1988; Weihe et al., 1989; MacArthur et al., 1999) and, to a lesser extent, enkephalins (Draisci et al., 1991; Dubner and Ruda, 1992). It is possible that this decrease in spinal opioid release

contributes to hyperalgesia during long-term inflammation. In any case, there is no compensatory increase in spinal opioid release during chronic inflammation.

To summarize, we found that acute inflammatory pain (formalin), but not chronic inflammation (CFA), induces the release of opioids in the spinal cord. The opioid release is at least partly dependent on supraspinal signals, and is decreased by inhalation anesthetics. The opioid release is also segmental and bilateral. This could be explained by a somatotopic projection of brainstem regions onto the spinal cord, or by the induction of opioid release by the convergence of supraspinal signals with primary afferent signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by grant 2 R01-DA012609 to J.C.M. from NIDA. We thank Dr. Guohua Zhang and Dan Ha Phan for their help.

References

- Abbadie C, Trafton J, Liu H, Mantyh PW, Basbaum AI. Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. *J Neurosci* 1997;17:8049–8060. [PubMed: 9315923]
- Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R. Distribution and targeting of a mu-opioid receptor MOR1 in brain and spinal cord. *J Neurosci* 1995;15:3328–3341. [PubMed: 7751913]
- Ballet S, Mauborgne A, Hamon M, Cesselin F, Collin E. Altered opioid-mediated control of the spinal release of dynorphin and met-enkephalin in polyarthritic rats. *Synapse* 2000;37:262–272. [PubMed: 10891863]
- Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci* 1984;7:309–338. [PubMed: 6143527]
- Basbaum AI, Clanton CH, Fields HL. Opiate and stimulus-produced analgesia: functional anatomy of a medullospinal pathway. *Proc Natl Acad Sci U S A* 1976;73:4685–4688. [PubMed: 1070018]
- Basbaum AI, Cruz L, Weber E. Immunoreactive dynorphin B in sacral primary afferent fibers of the cat. *J Neurosci* 1986;6:127–133. [PubMed: 2418172]
- Botticelli LJ, Cox BM, Goldstein A. Immunoreactive dynorphin in mammalian spinal cord and dorsal root ganglia. *Proc Natl Acad Sci U S A* 1981;78:7783–7786. [PubMed: 6121324]
- Bourgoin S, Le Bars D, Clot AM, Hamon M, Cesselin F. Subcutaneous formalin induces a segmental release of Met-enkephalin-like material from the rat spinal cord. *Pain* 1990;41:323–329. [PubMed: 2388769]
- Budai D, Fields HL. Endogenous opioid peptides acting at mu-opioid receptors in the dorsal horn contribute to midbrain modulation of spinal nociceptive neurons. *J Neurophysiol* 1998;79:677–687. [PubMed: 9463431]
- Cannell, MB.; McMorland, A.; Soeller, C. Image Enhancement by Deconvolution. In: Pawley, JB., editor. *Handbook of Biological Confocal Microscopy*. Vol. Third. New York, NY: Springer; 2006. p. 488-500.
- Cesselin F, Bourgoin S, Clot AM, Hamon M, Le Bars D. Segmental release of met-enkephalin-like material from the spinal-cord of rats, elicited by noxious thermal stimuli. *Brain Res* 1989;484:71–77. [PubMed: 2713703]
- Cesselin F, Le Bars D, Bourgoin S, Artaud F, Gozlan H, Clot AM, Besson JM, Hamon M. Spontaneous and evoked release of methionine-enkephalin-like material from the rat spinal cord in vivo. *Brain Res* 1985;339:305–313. [PubMed: 2992702]

- Chen W, Song B, Marvizon JC. Inhibition of opioid release in the rat spinal cord by α_2C adrenergic receptors. *Neuropharmacology* 2008a;54:944–953. [PubMed: 18343461]
- Chen W, Song B, Zhang G, Marvizon JC. Effects of veratridine and high potassium on μ -opioid receptor internalization in the rat spinal cord: stimulation of opioid release versus inhibition of internalization. *J Neurosci Methods* 2008b;170:285–293. [PubMed: 18377995]
- Chen W, Song B, Lao L, Perez OA, Kim W, Marvizon JCG. Comparing analgesia and μ -opioid receptor internalization produced by intrathecal enkephalin: Requirement for peptidase inhibition. *Neuropharmacology* 2007;53:664–667. [PubMed: 17845806]
- Cruz L, Basbaum AI. Multiple opioid peptides and the modulation of pain: immunohistochemical analysis of dynorphin and enkephalin in the trigeminal nucleus caudalis and spinal cord of the cat. *J Comp Neurol* 1985;240:331–348. [PubMed: 2907522]
- Draisci G, Kajander KC, Dubner R, Bennett GJ, Iadarola MJ. Up-regulation of opioid gene expression in spinal cord evoked by experimental nerve injuries and inflammation. *Brain Res* 1991;560:186–192. [PubMed: 1684729]
- Dubner R, Ruda MA. Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* 1992;15:96–103. [PubMed: 1373925]
- Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 1977;4:161–174. [PubMed: 564014]
- El-Sobky A, Dostrovsky JO, Wall PD. Lack of effect of naloxone on pain perception in humans. *Nature* 1976;263:783–784. [PubMed: 186714]
- Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci* 1991;14:219–245. [PubMed: 1674413]
- Fitzgerald M. Influences of contralateral nerve and skin stimulation on neurones in the substantia gelatinosa of the rat spinal cord. *Neurosci Lett* 1983;36:139–143. [PubMed: 6306518]
- Grevert P, Goldstein A. Endorphins: naloxone fails to alter experimental pain or mood in humans. *Science* 1978;199:1093–1095. [PubMed: 343250]
- Guyon A, Roques BP, Guyon F, Foucault A, Perdisot R, Swerts JP, Schwartz JC. Enkephalin degradation in mouse brain studied by a new H.P.L.C. method: further evidence for the involvement of carboxy dipeptidase. *Life Sci* 1979;25:1605–1611. [PubMed: 522617]
- Harlan RE, Shivers BD, Romano GJ, Howells RD, Pfaff DW. Localization of preproenkephalin mRNA in the rat brain and spinal cord by in situ hybridization. *J Comp Neurol* 1987;258:159–184. [PubMed: 3584538]
- Henry JL. Naloxone excites nociceptive units in the lumbar dorsal horn of the spinal cat. *Neuroscience* 1979;4:1485–1491. [PubMed: 553244]
- Hiranuma T, Kitamura K, Taniguchi T, Kanai M, Arai Y, Iwao K, Oka T. Protection against dynorphin-(1-8) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. *J Pharmacol Exp Ther* 1998;286:863–869. [PubMed: 9694943]
- Holmes, TJ.; Biggs, D.; Abu-Tarif, A. Blind Deconvolution. In: Pawley, JB., editor. *Handbook of Biological Confocal Microscopy*. Vol. Third. New York, NY: Springer; 2006. p. 468–487.
- Honore P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM, Mantyh PW. Spinal cord substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J Neurosci* 1999;19:7670–7678. [PubMed: 10460273]
- Hutchison WD, Morton CR, Terenius L. Dynorphin A: in vivo release in the spinal cord of the cat. *Brain Res* 1990;532:299–306. [PubMed: 1980853]
- Jensen TS, Yaksh TL. Spinal monoamine and opiate systems partly mediate the antinociceptive effects produced by glutamate at brainstem sites. *Brain Res* 1984;321:287–297. [PubMed: 6149792]
- Kishioka S, Miyamoto Y, Fukunaga Y, Nishida S, Yamamoto H. Effects of a mixture of peptidase inhibitors (amastatin, captopril and phosphoramidon) on Met-enkephalin-, beta-endorphin-, dynorphin-(1-13)- and electroacupuncture-induced antinociception in rats. *Jpn J Pharmacol* 1994;66:337–345. [PubMed: 7869621]
- Lao L, Song B, Marvizon JCG. Neurokinin release produced by capsaicin acting on the central terminals and axons of primary afferents: relationship with NMDA and GABA_B receptors. *Neuroscience* 2003;121:667–680. [PubMed: 14568027]

- Lao L, Song B, Chen W, Marvizon JC. Noxious mechanical stimulation evokes the segmental release of opioid peptides that induce μ -opioid receptor internalization in the presence of peptidase inhibitors. *Brain Res* 2008;1197:85–93. [PubMed: 18207137]
- Le Bars D, Bourgoin S, Clot AM, Hamon M, Cesselin F. Noxious mechanical stimuli increase the release of Met-enkephalin-like material heterosegmentally in the rat spinal cord. *Brain Res* 1987a;402:188–192. [PubMed: 3828785]
- Le Bars D, Bourgoin S, Villanueva L, Clot AM, Hamon M, Cesselin F. Involvement of the dorsolateral funiculi in the spinal release of met-enkephalin-like material triggered by heterosegmental noxious mechanical stimuli. *Brain Res* 1987b;412:190–195. [PubMed: 3607456]
- LoPachin RM, Rudy TA, Yaksh TL. An improved method for chronic catheterization of the rat spinal subarachnoid space. *Physiol Behav* 1981;27:559–561. [PubMed: 7335798]
- MacArthur L, Ren K, Pfaffenroth E, Franklin E, Ruda MA. Descending modulation of opioid-containing nociceptive neurons in rats with peripheral inflammation and hyperalgesia. *Neuroscience* 1999;88:499–506. [PubMed: 10197770]
- Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE. Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* 1995;268:1629–1632. [PubMed: 7539937]
- Martin-Schild S, Gerall AA, Kastin AJ, Zadina JE. Endomorphin-2 is an endogenous opioid in primary sensory afferent fibers. *Peptides* 1998;19:1783–1789. [PubMed: 9880085]
- Marvizon JC, Grady EF, Waszak-McGee J, Mayer EA. Internalization of μ -opioid receptors in rat spinal cord slices. *Neuroreport* 1999a;10:2329–2334. [PubMed: 10439458]
- Marvizon JC, Martinez V, Grady EF, Bunnett NW, Mayer EA. Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J Neurosci* 1997;17:8129–8136. [PubMed: 9334388]
- Marvizon JC, Grady EF, Stefani E, Bunnett NW, Mayer EA. Substance P release in the dorsal horn assessed by receptor internalization: NMDA receptors counteract a tonic inhibition by GABA(B) receptors. *Eur J Neurosci* 1999b;11:417–426. [PubMed: 10051742]
- Marvizon JC, Wang X, Matsuka Y, Neubert JK, Spigelman I. Relationship between capsaicin-evoked substance P release and neurokinin 1 receptor internalization in the rat spinal cord. *Neuroscience* 2003a;118:535–545. [PubMed: 12699788]
- Marvizon JCG, Wang X, Lao L, Song B. Effect of peptidases on the ability of exogenous and endogenous neurokinins to produce neurokinin 1 receptor internalization in the rat spinal cord. *Br J Pharmacol* 2003b;140:1389–1398. [PubMed: 14623771]
- Mason P. Central mechanisms of pain modulation. *Curr Opin Neurobiol* 1999;9:436–441. [PubMed: 10448161]
- Mills RH, Sohn RK, Micevych PE. Estrogen-induced μ -opioid receptor internalization in the medial preoptic nucleus is mediated via neuropeptide Y-Y1 receptor activation in the arcuate nucleus of female rats. *J Neurosci* 2004;24:947–955. [PubMed: 14749439]
- Morgan MM, Gold MS, Liebeskind JC, Stein C. Periaqueductal gray stimulation produces a spinally mediated, opioid antinociception for the inflamed hindpaw of the rat. *Brain Res* 1991;545:17–23. [PubMed: 1860042]
- Noble F, Smadja C, Valverde O, Maldonado R, Coric P, Turcaud S, Fournie-Zaluski MC, Roques BP. Pain-suppressive effects on various nociceptive stimuli (thermal, chemical, electrical and inflammatory) of the first orally active enkephalin-metabolizing enzyme inhibitor RB 120. *Pain* 1997;73:383–391. [PubMed: 9469529]
- Nydahl KS, Skinner K, Julius D, Basbaum AI. Co-localization of endomorphin-2 and substance P in primary afferent nociceptors and effects of injury: a light and electron microscopic study in the rat. *Eur J Neurosci* 2004;19:1789–1799. [PubMed: 15078552]
- Pierce TL, Grahek MD, Wessendorf MW. Immunoreactivity for endomorphin-2 occurs in primary afferents in rats and monkey. *Neuroreport* 1998;9:385–389. [PubMed: 9512376]
- Pohl M, Collin E, Bourgoin S, Conrath M, Benoliel JJ, Nevo I, Hamon M, Giraud P, Cesselin F. Expression of preproenkephalin-A gene and presence of Met-enkephalin in dorsal root ganglia of the adult rat. *J Neurochem* 1994;63:1226–1234. [PubMed: 7931276]

- Przewlocki R, Lason W, Silberring J, Herz A, Przewlocka B. Release of opioid peptides from the spinal cord of rats subjected to chronic pain. *NIDA Research Monograph* 1986;75:422–425. [PubMed: 3123959]
- Ren K, Dubner R. Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. *J Neurophysiol* 1996;76:3025–3037. [PubMed: 8930252]
- Ruda MA, Iadarola MJ, Cohen LV, Young WS III. In situ hybridization histochemistry and immunocytochemistry reveal an increase in spinal dynorphin biosynthesis in a rat model of peripheral inflammation and hyperalgesia. *Proc Natl Acad Sci U S A* 1988;85:622–626. [PubMed: 2893375]
- Song B, Marvizón JCG. Dorsal horn neurons firing at high frequency, but not primary afferents, release opioid peptides that produce μ -opioid receptor internalization in the rat spinal cord. *J Neurosci* 2003a; 23:9171–9184. [PubMed: 14534251]
- Song B, Marvizón JC. Peptidases prevent μ -opioid receptor internalization in dorsal horn neurons by endogenously released opioids. *J Neurosci* 2003b;23:1847–1858. [PubMed: 12629189]
- Song B, Marvizón JCG. NMDA receptors and large conductance calcium-sensitive potassium channels inhibit the release of opioid peptides that induce μ -opioid receptor internalization in the rat spinal cord. *Neuroscience* 2005;136:549–562. [PubMed: 16203108]
- Spike RC, Puskar Z, Sakamoto H, Stewart W, Watt C, Todd AJ. MOR-1-immunoreactive neurons in the dorsal horn of the rat spinal cord: evidence for nonsynaptic innervation by substance P-containing primary afferents and for selective activation by noxious thermal stimuli. *Eur J Neurosci* 2002;15:1306–1316. [PubMed: 11994125]
- Standaert DG, Watson SJ, Houghten RA, Saper CB. Opioid peptide immunoreactivity in spinal and trigeminal dorsal horn neurons projecting to the parabrachial nucleus in the rat. *J Neurosci* 1986;6:1220–1226. [PubMed: 3012010]
- Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. *J Neurosci Methods* 1996;65:167–172. [PubMed: 8740594]
- Tambeli CH, Quang P, Levine JD, Gear RW. Contribution of spinal inhibitory receptors in heterosegmental antinociception induced by noxious stimulation. *Eur J Neurosci* 2003;18:2999–3006. [PubMed: 14656295]
- Terman GW, Lewis JW, Liebeskind JC. The sensitivity of opioid mediated stress analgesia to narcotic antagonists. *Proc West Pharmacol Soc* 1983;26:49–52. [PubMed: 6684295]
- Tjolsen A, Berge OG, Hunnskaar S, Rosland JH, Hole K. The formalin test - An evaluation of the method. *Pain* 1992;51:5–17. [PubMed: 1454405]
- Todd AJ, Spike RC. The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. *Prog Neurobiol* 1993;41:609–645. [PubMed: 7904359]
- Todd AJ, Spike RC, Russell G, Johnston HM. Immunohistochemical evidence that Met-enkephalin and GABA coexist in some neurones in rat dorsal horn. *Brain Res* 1992;584:149–156. [PubMed: 1515935]
- Trafton JA, Abbadie C, Basbaum AI. Differential contribution of substance P and neurokinin A to spinal cord neurokinin-1 receptor signaling in the rat. *J Neurosci* 2001;21:3656–3664. [PubMed: 11331395]
- Trafton JA, Abbadie C, Marek K, Basbaum AI. Postsynaptic signaling via the μ -opioid receptor: Responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J Neurosci* 2000;20:8578–8584. [PubMed: 11102461]
- Trafton JA, Abbadie C, Marchand S, Mantyh PW, Basbaum AI. Spinal opioid analgesia: how critical is the regulation of substance P signaling? *J Neurosci* 1999;19:9642–9653. [PubMed: 10531466]
- Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. *Pain* 1996;67:151–161. [PubMed: 8895243]
- Tseng LF, Wang HQ, Xu JY. Inhibition of spinal nitric oxide synthase by N omega -nitro-L-arginine blocks the release of Met-enkephalin and antinociception induced by supraspinally administered beta-endorphin in the rat. *Neuroscience* 1997;78:461–467. [PubMed: 9145802]
- Wallace W, Schaefer LH, Swedlow JR. A workingperson's guide to deconvolution in light microscopy. *BioTechniques* 2001;31:1076–1078. [PubMed: 11730015]
- Watkins LR, Cobelli DA, Mayer DJ. Classical conditioning of front paw and hind paw footshock induced analgesia (FSIA): naloxone reversibility and descending pathways. *Brain Res* 1982;243:119–132. [PubMed: 7116148]

- Watkins LR, Griffin G, Leichnetz GR, Mayer DJ. The somatotopic organization of the nucleus raphe magnus and surrounding brain stem structures as revealed by HRP slow-release gels. *Brain Res* 1980;181:1–15. [PubMed: 7350948]
- Watkins LR, Griffin G, Leichnetz GR, Mayer DJ. Identification and somatotopic organization of nuclei projecting via the dorsolateral funiculus in rats: a retrograde tracing study using HRP slow-release gels. *Brain Res* 1981;223:237–255. [PubMed: 6169400]
- Watkins LR, Young EG, Kinscheck IB, Mayer DJ. The neural basis of footshock analgesia: the role of specific ventral medullary nuclei. *Brain Res* 1983;276:305–315. [PubMed: 6627011]
- Watkins LR, Drugan R, Hyson RL, Moye TB, Ryan SM, Mayer DJ, Maier SF. Opiate and non-opiate analgesia induced by inescapable tail-shock: effects of dorsolateral funiculus lesions and decerebration. *Brain Res* 1984;291:325–336. [PubMed: 6697193]
- Weihe E, Millan MJ, Holtt V, Nohr D, Herz A. Induction of the gene encoding prodynorphin by experimentally induced arthritis enhances staining for dynorphin in the spinal cord of rats. *Neuroscience* 1989;31:77–95. [PubMed: 2570379]
- Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. *Physiol Behav* 1976;17:1031–1036. [PubMed: 14677603]
- Yaksh TL, Elde RP. Factors governing release of methionine enkephalin-like immunoreactivity from mesencephalon and spinal cord of the cat in vivo. *J Neurophysiol* 1981;46:1056–1075. [PubMed: 7299446]
- Yaksh TL, Terenius L, Nyberg F, Jhamandas K, Wang JY. Studies on the release by somatic stimulation from rat and cat spinal cord of active materials which displace dihydromorphine in an opiate-binding assay. *Brain Res* 1983;268:119–128. [PubMed: 6860955]
- Yamamoto T, Yaksh TL. Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. *Anesthesiology* 1992;77:757–763. [PubMed: 1416174]
- Zorman G, Belcher G, Adams JE, Fields HL. Lumbar intrathecal naloxone blocks analgesia produced by microstimulation of the ventromedial medulla in the rat. *Brain Res* 1982;236:77–84. [PubMed: 6279238]

Abbreviations

ANOVA	analysis of variance
CFA	Complete Freund's Adjuvant
MOR	μ-opioid receptor
NK1R	neurokinin 1 receptor

Fig. 1. Images of MOR and NK1R dorsal horn neurons after formalin injection

Rats received peptidase inhibitors intrathecally and formalin in the left hindpaw. Spinal cord sections were double-labeled with antibodies against the MOR (green) and the NK1R (magenta). Cells with internalization are labeled “*”, cells without internalization “o”. **A:** L5 dorsal horn ipsilateral to the formalin-injected paw; NK1R internalization is extensive in all 3 NK1R neurons sampled, and MOR internalization is present in 3 out of 7 MOR neurons. **B:** L5 dorsal horn contralateral to the formalin-injected paw in the same section; NK1R internalization is absent in all 3 NK1R neurons, but MOR internalization is present 4 out of 8 MOR neurons. Main panels: composite images of two confocal stacks (5-7 optical sections) taken with a 20× objective. Insets: images taken with a 100× objective of the cells indicated by the frames and arrows in the main panels; images consist of 3-6 optical sections taken through the middle of the cells. Scale bars are 40 μm for the main panels and 4 μm for the insets.

Fig. 2. Images of MOR and NK1R dorsal horn neurons after saline or formalin injection
Rats received peptidase inhibitors intrathecally and saline (**A**) or formalin (**B**) in the left hindpaw. Spinal cord sections were double-labeled with antibodies against the MOR (green) and the NK1R (magenta). Cells with internalization are labeled “*”, cells without internalization “o”. **A:** L5 dorsal horn ipsilateral to the paw injected with saline; some internalization is present in only 1 out of 5 NK1R neurons and in 1 out of 8 MOR neurons. **B:** T10 dorsal horn ipsilateral to the formalin-injected paw with; there is no internalization in any of the NK1R and MOR neurons sampled. Main panels: composite images of two confocal stacks (6 optical sections) taken with a 20× objective. Insets: images taken with a 100× objective of the cells indicated by the frames and arrows in the main panels; images consist of

3-4 optical sections taken through the middle of the cells. Scale bars are 40 μm for the main panels and 4 μm for the insets.

Fig. 3. Quantification of MOR and NK1R internalization produced by formalin injection in the hindpaw

Anesthetized rats were given intrathecal peptidase inhibitors and then injected in the left hindpaw with 50 μ l of saline (control) or 2.5% formalin. Rats were euthanized 20 min later. MOR internalization (A) or NK1R internalization (B) were measured in the ipsilateral and contralateral dorsal horns (laminae I-II) of spinal segments C2, T10 and L5. Bonferroni's post-hoc tests: ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. Effect of anesthesia on MOR internalization induced by formalin

Rats were anesthetized with 2.5% isoflurane (n=4) or not anesthetized (n=4). They received intrathecal peptidase inhibitors followed by 50 μ l formalin (2.5%) in the left hindpaw. MOR internalization (A) or NK1R internalization (B) were measured ipsilaterally and contralaterally in laminae I-II of spinal segments C2, T10 and L5. Bonferroni's post-hoc tests: * $p<0.05$, *** $p<0.001$.

Fig. 5. Lidocaine spinal block decreases MOR internalization produced by formalin

Rats were implanted with two intrathecal catheters, one terminating at L5-L6 and the other at C7-T1. Peptidase inhibitors were injected onto the L5-L6 spinal cord. Two minutes later the second catheter was used to inject onto the C7-T1 spinal cord 1 μ l of saline (n=3) or 10% lidocaine (n=4), followed by a 3 μ l flush with saline. Three minutes after that, 50 μ l formalin (2.5%) was injected in the left hindpaw. Rats were not anesthetized for the experiment, and were euthanized 20 min after formalin. MOR internalization (A) or NK1R internalization (B) were measured in spinal segments C2, T10 and L5. Bonferroni's post-hoc tests: ** p<0.01, *** p<0.001.

Fig. 6. The site of intrathecal injection of peptidase inhibitors does not affect MOR internalization produced by formalin

Rats (not anesthetized) were given an intrathecal injection of peptidase inhibitors (PI) onto the lumbar (L6) or the thoracic (C7-T1) spinal cord. Five minutes afterwards, the rats received 50 μ l formalin (2.5%) in the left hindpaw. After 20 min more, rats were euthanized. MOR internalization was measured in laminae I-II of spinal segments C2, T10 and L5. Data in the “PI lumbar” group are the same as the “formalin” group in Fig. 3 (included for comparison). Bonferroni's post-hoc tests: * $p < 0.05$.

Fig. 7. MOR internalization produced by formalin in the absence of peptidase inhibitors
 Rats (not anesthetized) were given an intrathecal injection of saline onto the lumbar spinal cord. Five minutes later, the rats received 50 μ l of saline (control) or 2.5% formalin in the left hindpaw. After 20 min more, rats were euthanized. MOR internalization was measured in laminae I-II of the L5 spinal segment. Bonferroni's post-hoc tests: *** $p < 0.001$, ** $p < 0.01$.

Fig. 8. Effect of peptidase inhibitors on MOR internalization produced by hindpaw clamp
Rats (n=4) were anesthetized with isoflurane and injected intrathecally with saline or peptidase inhibitors. Five minutes later, the left hindpaw was clamped with a hemostat for 30 sec, and after 10 min more the rats were euthanized. MOR internalization (A) or NK1R internalization (B) were measured in laminae I-II of spinal segments C2, T10 and L5. Bonferroni's post-hoc tests: *** $p < 0.001$.

Fig. 9. MOR and NK1R internalization after CFA injection in the hindpaw

Rats received three treatments (n=4 per group). CFA: 150 μ l CFA subcutaneously in the left hindpaw, followed two days later by an intrathecal injection of peptidase inhibitors. Clamp: intrathecal peptidase inhibitors, followed 5 min later by clamping of the left hind paw with a hemostat for 30 sec. CFA + clamp: CFA was injected in the left hindpaw, followed two days later by intrathecal peptidase inhibitors; 5 min later the inflamed left hindpaw was clamped with a hemostat for 30 sec. The CFA injection and clamping of the paw were done under isoflurane anesthesia. Rats were euthanized 10-15 min after the intrathecal injection. MOR internalization (A) and NK1R internalization (B) were measured in laminae I-II of spinal segments C2, T10 and L5. Bonferroni's post-hoc tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 10. Images of MOR and NK1R dorsal horn neurons after CFA and hindpaw clamp

Spinal cord sections were double-labeled for MOR (green) and NK1R (magenta). Images are of the L5 dorsal horn ipsilateral to the hindpaw injected with CFA and/or clamped. Cells with internalization are labeled “*”, cells without internalization “o”. **A:** CFA in the hindpaw followed two days later by intrathecal peptidase inhibitors; internalization is absent in all 8 MOR neurons, and there is slight internalization in 1 out of 2 NK1R neurons. **B:** intrathecal peptidase inhibitors followed 5 min later by hindpaw clamp; internalization is present in 3 out of 6 MOR neurons sampled, and in the two NK1R neurons. **C:** CFA in the hindpaw followed two days later by intrathecal peptidase inhibitors and, after 5 min, by hindpaw clamp; there is internalization in 2 out of 7 MOR neurons, and in two NK1R neurons in lamina I and one NK1R neuron in lamina III with dendrites crossing lamina II. Main panels: composite images of two confocal stacks (7-8 optical sections) taken with a 20× objective. Insets: images taken with a 100× objective (63× objective in inset e of panel C) of the cells indicated by the frames and arrows in the main panels. Images consist of 3-9 optical sections taken through the middle of the cells; 13 optical sections for inset e of panel C. Scale bars: 50 μm (main panels), 5 μm (insets) and 16 μm (inset e of C).