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Noxious mechanical stimulation evokes the segmental release of opioid peptides that induce μ -opioid receptor internalization in the presence of peptidase inhibitors

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Abstract

The internalization of μ -opioid receptors (MORs) provides an ideal way to locate areas of opioid peptide release. We used this method to study opioid release in the spinal cord evoked by noxious stimuli in anesthetized rats. Previous studies have shown that opioids released in the spinal cord produce MOR internalization only when they are protected from peptidase degradation. Accordingly, rats were implanted with chronic intrathecal catheters that were used to inject a mixture of peptidase inhibitors (amastatin, captopril and phosphoramidon) onto the lumbar spinal cord. Five minutes later, a noxious stimulus was delivered to the paw. Lumbar spinal segments were double-stained with antibodies against MORs and neurokinin 1 receptors (NK1Rs) using immunofluorescence. Mechanical stimulation of the hindpaw consisted of repeated 10 sec clamps with a hemostat for 10 min. In the ipsilateral dorsal horn, the stimulus produced abundant NK1R internalization in segments L3–L6, and a more modest but significant MOR internalization in segments L5 and L6. In the contralateral dorsal horn, NK1R was substantially lower and MOR internalization was negligible. The same mechanical stimulus applied to a forepaw did not produce NK1R or MOR internalization in the lumbar spinal cord. Thermal stimulation consisted of immersing a hindpaw in water at 52 °C for 2 min. It produced substantial NK1R internalization ipsilaterally in segment L6, but no MOR internalization. These results show that mechanical stimulation induces segmental opioid release, i.e., in the dorsal horn receiving the noxious signals and not in other spinal segments.

Keywords

Dorsal horn; Enkephalin; Internalization; Mu-opioid receptor; Neurokinin 1 receptor; peptidase

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1. Introduction

Opioid receptors in the spinal cord play a key role in pain modulation (Budai and Fields, 1998; Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982). However, little is known about the neuronal circuitry in the spinal cord that drives the release of endogenous opioid peptides (henceforth “opioids”). Elucidating these neural pathways is critical to understand the role of opioids in conditions that produce analgesia, such as stress (Yamada and Nabeshima, 1995), acupuncture (Han, 2003) or pain (Gear et al., 1999).

Opioids detected in spinal cord superfusates after electrical stimulation of the sciatic nerve or the hindpaw included enkephalins and dynorphins of different lengths, but not β -endorphin (Yaksh et al., 1983), which is not present in the dorsal horn (Tsou et al., 1986). The most extensive studies on spinal opioid release were conducted by the group of Cesselin, who measured Met-enkephalin in spinal cord superfusates after stimulation with different pain modalities. An important goal of those studies was to determine whether Met-enkephalin was released from the same spinal segment that received the noxious stimulus (‘segmental’ release), or from other spinal segments (‘heterosegmental’ release). The first case would indicate that the opioids are released from local neuronal circuits in the dorsal horn, whereas the second would suggest that opioid release is driven supraspinally by ‘diffuse noxious inhibitory controls’ (DNIC) (Le Bars et al., 1987b). These investigators found that noxious mechanical stimulation produced heterosegmental Met-enkephalin release (Le Bars et al., 1987a; Le Bars et al., 1987b), whereas subcutaneous formalin (Bourgoin et al., 1990) or noxious thermal stimulation (Cesselin et al., 1989) produced segmental Met-enkephalin release. However, they estimated the origin of the released enkephalin based on the position of the superfusion catheter. Given the unknown extent of diffusion of peptides in the subdural space, doubts remain about determining the locus of release of Met-enkephalin using this technique. Moreover, it is also unknown whether opioid receptors in the spinal cord are activated by Met-enkephalin or by other opioid peptides (Yaksh et al., 1983), in which case the physiological relevance of measuring solely Met-enkephalin release would also be in doubt.

The internalization of μ -opioid receptors (MORs) has been used to measure in situ opioid release in tissues including brain (Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001), intestine (Patierno et al., 2005) and spinal cord (Song and Marvizon, 2003a; Song and Marvizon, 2003b; Song and Marvizon, 2005; Trafton et al., 2000). This approach provides an ideal way to locate the areas of opioid release, because MORs serve as opioid detectors located in close proximity to opioid-releasing terminals and able to detect all naturally-occurring MOR agonists (Song and Marvizon, 2003a). Previously, neurokinin 1 receptor (NK1R) internalization had been used to measure substance P release (Abbadie et al., 1997; Allen et al., 1997; Honore et al., 1999; Kondo et al., 2005; Mantyh et al., 1995; Marvizon et al., 1997; Marvizon et al., 2003). Importantly, the magnitude of NK1R internalization increased with the intensity of the stimulus used to evoke substance P release, both when a noxious stimulus was used in vivo (Allen et al., 1997), or a chemical stimulus in vitro (Marvizon et al., 2003). Likewise, MOR internalization increased with the intensity of the stimulus delivered to spinal cord slices to evoke opioid release (Song and Marvizon, 2003b).

Surprisingly, an initial attempt to use a wide variety of noxious stimuli to induce MOR internalization produced negative results (Trafton et al., 2000). A possible explanation is that enkephalins and dynorphins are rapidly degraded by peptidases located in close proximity to the receptors. Indeed, we found that inhibitors of three peptidases (aminopeptidase, dipeptidyl carboxypeptidase and neutral endopeptidase) increased a hundred times the apparent potency of Leu-enkephalin to induce MOR internalization in spinal cord slices (Song and Marvizon, 2003a). In vivo, intrathecal Leu-enkephalin was unable to produce not only MOR

internalization, but also analgesia, unless it was co-injected with inhibitors of these three peptidases (Chen et al., 2007).

We hypothesized that noxious stimuli would induce MOR internalization in the spinal cord if the released opioids were protected from peptidase degradation. Importantly, in these conditions MOR internalization could be used to determine the locus of release of opioids evoked by different pain modalities, and in particular whether it was segmental or heterosegmental.

2. Results

Our initial goal was to determine whether acute noxious stimuli induce MOR internalization in dorsal horn neurons when opioids are protected from peptidase degradation. To this end, a mixture of peptidase inhibitors (amastatin, captopril and phosphoramidon, 100 nmol each) was injected intrathecally onto the lumbar spinal cord. Five minutes later, a noxious mechanical or thermal stimulus was applied. The rats were then fixed and MOR internalization was measured in the lumbar spinal cord. Control rats received intrathecal injections of saline and no noxious stimulation. In them the percentage of MOR neurons with internalization in lumbar spinal segments was negligible: L1, 0.4 ± 0.4 %; L2, 1.8 ± 1.1 %; L3, 1.0 ± 0.5 %; L4, 2.3 ± 1.8 %; L5, 3.5 ± 2.2 %; L6, 1.5 ± 1.5 % ($n=3$ rats). We have previously shown that peptidase inhibitors by themselves do not produce MOR internalization (Chen et al., 2007). In other experiments we also measured the internalization of NK1Rs in lamina I neurons in order to assess the intensity of the noxious stimuli. MORs and NK1Rs are present in different dorsal horn neurons (Song and Marvizon, 2003b; Spike et al., 2002).

Noxious mechanical stimulation consisted of repeated clamping of the hindpaw, 10 sec on and off, for a total duration of 10 min. A similar procedure, although of longer total duration (30 min), was found to increase the amount of Met-enkephalin in spinal cord superfusates (Le Bars et al., 1987a; Le Bars et al., 1987b). This stimulus did not produce bleeding, puncture the skin or break the bones. It did, however, induce a noticeable swelling of the hindpaw by the end of the stimulus, indicating injury to the dermis and other soft tissues. In agreement with a previous study (Abbadie et al., 1997), this stimulus produced abundant NK1R internalization in lamina I neurons of the ipsilateral dorsal horn (Fig. 1 A). NK1R internalization increased towards the caudal end of the lumbar spinal cord, reaching its maximum in segments L4–L6. This roughly parallels the extent of sciatic innervation, as is to be expected given that substance P was released from primary afferents originating in the hind leg (Allen et al., 1999). A two-way ANOVA revealed significant differences ($p<0.0001$) in NK1R internalization between the ipsilateral and the contralateral sides and between spinal segments ($p<0.01$). However, NK1R internalization in the contralateral side was still quite high (Fig. 1 A), probably because the presence of peptidase inhibitors increased the effect of substance P released in basal conditions (Lao et al., 2003). It does not seem that NK1R internalization in the contralateral side was caused by diffusion of substance P from the ipsilateral side, because it appeared to be lower precisely in those segments (L4 and L5) where ipsilateral NK1R internalization was higher (although differences between segments in the contralateral side were not statistically significant).

The mechanical stimulation also induced MOR internalization, although to a much lesser extent than NK1R internalization (Fig. 1 B). Importantly, MOR internalization was induced only ipsilaterally to the stimulus, and only in spinal segments L5 and L6, i.e., the segments receiving the nociceptive signals from the stimulated paw. MOR internalization was negligible in the contralateral dorsal horn and very low in the other lumbar segments. A two-way ANOVA revealed significant differences in MOR internalization between the ipsilateral and the contralateral sides ($p<0.0001$) and between spinal segments ($p<0.01$).

Fig. 2 shows examples of NK1R (Fig. 2 A, C) and MOR (Fig. 2 B, D) dorsal horn neurons in the L5 spinal segment, where mechanical noxious stimulation produced maximal internalization of both receptors (Fig. 1). Only the medial and central parts of the dorsal horn are shown in Fig. 2, because the field of the 20x objective could not span the entire dorsal horn, NK1R were more abundant in these areas, and MOR internalization was rarely found in the lateral dorsal horn. Ipsilateral to the stimulated paw, NK1R neurons in lamina I show extensive NK1R internalization (Fig. 2 A). The completeness of the NK1R internalization can be appreciated by the absence of receptors at the cell surface and the abundance of NK1R-positive endosomes throughout the cytoplasm. MOR internalization in the ipsilateral dorsal horn was less robust. This can be appreciated in Fig. 2 B, which shows staining for MOR in the same section as Fig. 2 A. Of the seven MOR neurons in lamina II sampled, four show MOR internalization and three do not. The four cells with MOR internalization have a fair amount of MOR-positive endosomes, but also abundant MOR staining at the cell surface, indicating that the internalization was not as complete as in the case of the NK1R. MOR internalization was largely absent in cells in the lateral dorsal horn, hence when these MOR cells were included in the count we obtained the lower percentage of MOR cells with internalization given in Fig. 1 B. In the contralateral dorsal horn, NK1R internalization is absent in the three cells shown in Fig. 2 C. MOR cells in the same contralateral section (Fig. 2 D) do not show MOR internalization, although some clustering of MORs at the cell surface can be observed occasionally.

To further investigate whether noxious stimuli are able to produce heterosegmental opioid release, we applied the same noxious mechanical stimulus to the forepaw. Peptidase inhibitors were still injected onto the lumbar spinal cord (L2–L4), where NK1R and MOR internalization were assessed. Both NK1R internalization and MOR internalization were very low in the lumbar spinal cord, ipsilaterally and contralaterally (Fig. 3).

Finally, we explored whether MOR internalization could be evoked by thermal noxious stimulation, consisting of immersing the hindpaw in water at 52 °C for 2 min. The same stimulus (Abbadie et al., 1997), or one of shorter duration (30 sec) (Allen et al., 1997), have been used to evoke NK1R internalization. However, a thermal stimulus of longer duration was used to evoke Met-enkephalin release from the spinal cord (Cesselin et al., 1989): repeated (10 sec on and off) immersion of the rat's muzzle or tail in water at 52°C for 30 min. We used a short stimulus because MOR internalization occurs in relatively short times (5 min) (Marvizon et al., 1999b). This stimulus induced NK1R internalization ipsilaterally in segments L4–L6, being maximal and significantly different from the contralateral side at L6 (Fig. 4 A). The extent of NK1R internalization was lower than that reported by Abbadie et al. (1997), but similar to that reported by Allen et al. (1997). Thermal stimulation produced less NK1R internalization than mechanical stimulation ($p < 0.0001$, two-way ANOVA). MOR receptor internalization after this thermal stimulus was negligible (Fig. 4 B).

3. Discussion

This study shows that mechanical noxious stimulation induces opioid release in the dorsal horn, and that the released opioids produce MOR internalization when the peptidases that degrade them are inhibited. However, judging by both the amount of MOR neurons with internalization and the completeness of the internalization in these neurons, the extent of opioid release was much less than the extent of substance P release produced by the same stimulus.

In contrast, a previous study by Trafton et al. (2000), conducted that in the absence of peptidase inhibitors, found that a variety of noxious stimuli did not induce MOR internalization. Therefore, it seems that even though noxious stimuli do evoke the release of opioids, in most conditions they are cleaved by peptidases before they can activate MORs. Indeed, we

previously showed in spinal cord slices (Song and Marvizon, 2003a) that peptidase inhibitors increased the apparent potencies of dynorphin A and Leu-enkephalin to induce MOR internalization by one and two orders of magnitude, respectively. Moreover, opioids released from the slices by electrical or chemical stimulation produced MOR internalization only in the presence of peptidase inhibitors (Song and Marvizon, 2003a; Song and Marvizon, 2003b; Song and Marvizon, 2005; Trafton et al., 2000). Importantly, the inability of opioids to produce MOR internalization in the absence of peptidase inhibitors does reflect their inability to activate them. Thus, intrathecal Leu-enkephalin failed to produce analgesia in the tail-flick test, even at high doses (100 nmol), but it produced analgesia at low very doses (0.3 nmol) when it was co-injected with peptidase inhibitors (Chen et al., 2007). Another study (Kishioka et al., 1994) found that peptidase inhibitors substantially increased the analgesia produced by Met-enkephalin, dynorphin or electroacupuncture. The high susceptibility of enkephalins (Guyon et al., 1979; Hiranuma et al., 1998b) and dynorphins (Hiranuma et al., 1998a; Numata et al., 1988) to peptidase degradation is well-known, and prompted the development of peptidase inhibitors as analgesics (Noble et al., 1992a; Noble et al., 1992b; Noble et al., 1997).

However, the idea that opioids cannot activate opioid receptors unless peptidases are inhibited conflicts with studies showing that intrathecal injections of opioid antagonists block the analgesia produced by stimulation of the periaqueductal gray or the rostro-ventral medulla (Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982), or by noxious stimuli (Tambeli et al., 2003). Indeed, it seems futile to release of opioids if they are going to be degraded by peptidases before they can activate their receptors. The most likely explanation for this paradox is that there are endogenous peptidase inhibitors that enable the action of opioids. In fact, three endogenous inhibitors of aminopeptidase and neutral endopeptidase have been found: spinorphin - LVVYPWT (Nishimura and Hazato, 1993), sialorphin - QHNPR (Rougeot et al., 2003) and opiorphin - QRFSR (Wisner et al., 2006). The release of these peptidase-inhibiting peptides appears to play a role in pain control, since sialorphin and opiorphin have analgesic properties, while spinorphin increased the antinociception produced by Leu-enkephalin (Honda et al., 2001). Hence, the instances of analgesia mediated by spinal opioid receptors mentioned above may involve the simultaneous release of opioids and peptidase-inhibiting peptides.

A key finding in this study was that the opioid release evoked by noxious mechanical stimulation was segmental, i.e., it occurred only in spinal segments that receive innervation from the stimulated hindpaw, and only ipsilaterally to the stimulus. This conclusion was confirmed by delivering noxious stimulation to the forepaw, which did not produce MOR internalization in the lumbar spinal cord. The lack of MOR internalization in other spinal segments was not caused by a limited diffusion of the peptidase inhibitors, because then MOR internalization would have occurred contralaterally. Moreover, the intrathecal canula terminated over spinal segments L2–L4, whereas MOR internalization occurred in segments L5–L6. The finding that opioid release was segmental argues against the idea that it was driven by brainstem descending signals affecting the entire spinal cord (DNIC). Rather, the opioid release appears to be driven by a neuronal circuit in the dorsal horn. We have shown that opioids are not released directly from primary afferents or from descending axons, but from intrinsic dorsal horn neurons (Song and Marvizon, 2003b). Here we provide additional evidence that descending axons do not release opioids, because it is unlikely that they could target opioid release exclusively to the spinal cord segment receiving a noxious signal. It is far more likely that opioids are released by intrinsic dorsal horn neurons, probably by integrating descending signals from the brainstem with noxious signals in primary afferents.

In this issue our results conflict with the conclusion of Le Bars et al. (1987a; 1987b): that noxious mechanical stimulation produced heterosegmental Met-enkephalin release. These authors deduced the site of enkephalin release based on the position of their superfusion

catheters, whereas in the present study MOR internalization provides a more reliable and accurate way to locate the sites of release. In addition, opioids other than Met-enkephalin may have induced MOR internalization, since most of the opioids peptides released from the spinal cord are not the Met and Leu-enkephalin pentapeptides, but longer enkephalins, dynorphins and α -neoendorphin (Yaksh et al., 1983). A critical factor in these experiments is anesthesia, which may block some neural pathways. Le Bars et al. performed their experiments in rats paralyzed with gallamine and anesthetized with a low concentration (0.5 %) of halothane, which probably allowed them to work below the anesthesia level that we used. Although these investigators gave assurances that their animals did not experience pain, we could not use their anesthesia procedure because it is not allowed by the standards for the ethical treatment of animals at our institution. Another variable is the duration of the noxious stimulus: Le Bars et al. applied it for 30 min, and we for 10 min. We used a shorter duration because the stimulus was strong enough to produce a large amount of NK1R internalization, and MOR internalization does not require exposing the receptor to opioids for more than 10 min. However, it is still possible that a prolonged stimulus is necessary to recruit brainstem descending pathways that release opioids.

Unlike mechanical stimulation, thermal stimulation did not induce MOR internalization at all. Again, our results differ from those of Cesselin et al. (1989), who found that thermal stimulation induced segmental release of Met-enkephalin. As discussed above, Cesselin et al. used a longer stimulus duration and low level anesthesia with gallamine paralysis. Since our thermal stimulus produced less NK1R internalization than the mechanical stimulus, its inability to induce MOR internalization may be related to its intensity and not to its modality.

In summary, this study shows that when released opioids are protected from peptidase degradation, mechanical stimulation induces MOR internalization in the dorsal horn receiving the noxious signals, but not in other spinal segments.

4. Experimental Procedures

4.1. Animals

Experiments were performed in male Sprague Dawley rats, 2 month old (Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed in standard cages on a 12 h light/dark cycle and allowed free access to food and water. 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.

4.2. Intrathecal injections

Rats were implanted with chronic intrathecal catheters for drugs injection following established procedures (LoPachin et al., 1981; Yaksh and Rudy, 1976). Rats were anesthetized with isoflurane using an induction box, and anesthesia was maintained throughout the procedure with a vaporizer delivering isoflurane (2–4 %) in oxygen through a nose cone. The back of the head and neck was shaved and the rat was placed on an aluminum platform maintained at 37° C. 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 an 8.0 cm polyethylene (PE-5 or PE-10) catheter, which was and passed into the intrathecal space so that it ended at the L3–L4 spinal segments. The other end of the catheter was tunneled through the subcutaneous space over the cranium and externalized. Muscle and skin were sutured. The catheter was flushed with 10 μ l saline and shut with an electrical cauterizer. Rats that showed motor weakness or signs of paresis were

ethanized. The rats were housed separately and allowed to recover for 5–7 days prior to the experiment. The day of the experiment the catheter was cut open and used for the intrathecal injections, which consisted of 10 μ l injectate (peptidase inhibitors) plus 20 μ l of saline to flush the catheter. Solutions were preloaded, in reverse order of administration, into a tube, and delivered with a 50 μ l Hamilton syringe within 1 min. In all the rats, the position of the catheter was examined when the spinal cord was extracted after fixation. If the catheter terminated inside the spinal cord or its tip was otherwise occluded, the animal was excluded from the study.

4.3. Noxious stimulation

The day of the experiment, the rats were anesthetized with 40–50 mg/kg pentobarbital i.p., the lowest dose found to provide an appropriate level of anesthesia. The intrathecal catheter was used to inject a mixture of peptidase inhibitors (at doses of 100 nmol each) onto the lumbar spinal cord. Peptidase inhibitors were amastatin, captopril and phosphoramidon, which inhibit, respectively, aminopeptidases, dipeptidyl carboxypeptidase and neutral endopeptidase, the three enzymes that cleave opioids in the spinal cord (Guyon et al., 1979; Hiranuma et al., 1998a; Hiranuma et al., 1998b; Numata et al., 1988; Song and Marvizon, 2003a). Noxious stimuli were applied 5 min after the intrathecal injection. For mechanical stimulation, a hindpaw or a forepaw was positioned perpendicularly across the jaws of a hemostat and it was closed to the first click, 10 sec on and 10 sec off, for 10 min (Le Bars et al., 1987a). For thermal stimulation, one hindpaw was dipped to just below the ankle in water at 52°C for 2 min (Trafton et al., 2000). Rats were euthanized and fixed for immunohistochemistry 10 min after the end of the noxious stimulation, and their lumbar spinal cord was extracted for immunohistochemistry.

4.4. Immunohistochemistry

Histological sections from the spinal cord of the rats were double-labeled for MORs and NK1Rs to assess the internalization of both receptors, as previously described (Song and Marvizon, 2003b). Rats were euthanized with an overdose of isoflurane and fixed by aortal perfusion of 100 ml 0.1 M sodium phosphate buffer (pH 7.4) at room temperature, followed by 400 ml of ice-cold fixative (4 % paraformaldehyde, 0.18 % picric acid in 0.1 M sodium phosphate buffer). Immediately after fixation, a laminectomy was performed to extract the lumbar spinal cord (L1–L6), which was cleaned of dura matter, cut into one-segment blocks, post-fixed and cryoprotected in 20 % sucrose. The spinal cord segments were embedded in a drop of Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA), and frozen on dry ice. Free-floating coronal sections (25 μ m thick) were prepared 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 at room temperature overnight with a mixture of antibodies against the MOR (1:7000 dilution) and the NK1R (1:1000 dilution) in PBS/Triton. The anti-MOR antibody was a rabbit antiserum raised against amino acids 384–398 of the cloned rat MOR-1 (DiaSorin, Stillwater, MN, catalog no. 24216), which has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). Pre-absorption of the MOR antibody with its immunizing peptide (10 μ g/ml) abolished the staining. The anti-NK1R antibody was a NK1R guinea pig antiserum (1:1000 dilution) purchased from Chemicon (Temecula, CA, catalog # AB5800), raised against amino acids 393–407 of the rat NK1R (Song and Marvizon, 2003b). After three washes with PBS, sections were incubated for two hours at room temperature with a mixture in PBS/Triton of the two secondary antibodies: Alexa Fluor-488 goat anti-rabbit IgG and Alexa Fluor-568 goat anti-guinea pig IgG (Molecular Probes, Eugene, OR) at 1:2000 and 1:1000 dilutions, respectively. After four more washes, sections were mounted on glass slides and coverslipped in Prolong or Prolong Gold with DAPI (Molecular Probes).

4.5. Quantification of MOR and NK1R internalization

Previously described procedures were used to quantify MOR internalization (Marvizon et al., 1999b; Song and Marvizon, 2003a; Song and Marvizon, 2003b; Song and Marvizon, 2005) and NK1R internalization (Abbadie et al., 1997; Mantyh et al., 1995; Marvizon et al., 1997; Marvizon et al., 1999a; Marvizon et al., 2003; Trafton et al., 1999; Trafton et al., 2001). MOR or NK1R neurons were counted visually, classifying them as with or without receptor internalization. This was done using a Zeiss Axiovert 135 (Carl Zeiss, Inc., Thornwood, NY) microscope with an objective of 100x (numerical aperture 1.40). Counting was done blind to the treatment. The percentage of MOR neurons with internalization was calculated in relation to the total number of MOR neurons in laminae I-II. Similarly, the percentage of NK1R neurons with internalization was calculated relative to the total number of NK1R neurons in lamina I. MOR neuronal somas with >5 endosomes and NK1R somas with >10 endosomes were considered to have internalization. At least three sections per spinal segment (chosen randomly) were counted.

4.6. Confocal microscopy

Confocal images were acquired at the UCLA's Carol Moss Spivak Cell Imaging Facility 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 the green channel and the 561 nm line of a diode laser for the red channel. Corresponding emission windows were 500–570 nm and 600–670 nm. We used a pinhole of 1.0 Airy unit and objectives of 20x (NA 0.7) and 100x oil (NA 1.4), resulting in an estimated optical section thickness (full width at half maximum) of 2.53 μm and 0.62 μm , respectively. Images were acquired as confocal stacks with an optical section separation (z-interval) of 1.180 μm for the 20x objective and 0.285 μm for the 100x objective. Optical sections were averaged 3–4 times to reduce noise. Images were acquired at a digital size of 1024 $\times$ 1024 pixels, and were later cropped to the relevant part of the field without changing the resolution. Confocal stacks consisted of 9–29 optical sections. Images were processed using Imaris 5.7.1., 64-bit version (Bitplane AG, Saint Paul, MN) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). With Imaris the confocal stacks were examined, cropped in three dimensions, and combined into a two-dimensional image. This image was then exported to Photoshop, which was used to adjust the gamma, add text, and compose the multi-paneled figure.

4.7. Data analysis

Data were analyzed and graphics drawn using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical analyses consisted of two-way ANOVA and Bonferroni's post-test, with significance set at 0.05. The first variable of the two-way ANOVA, to which the Bonferroni's post-test was applied, was "stimulus", consisting of comparing the dorsal horns ipsilateral and contralateral to the paw receiving the noxious stimulus. The second variable was spinal cord segment. MOR internalization and NK1R internalization data were analyzed separately. The absence of asterisks in the figure indicates that the difference between the ipsilateral (stimulated) and contralateral sides are not significant.

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Glossary

Abbreviations

ANOVA

analysis of variance

DNIC

diffuse noxious inhibitory controls

MOR

μ -opioid receptor

NK1R

neurokinin 1 receptor

Fig. 1. NK1R and MOR internalization produced by noxious mechanical stimulation of the hindpaw

Anesthetized rats received an intrathecal injection of peptidase inhibitors (amastatin, captopril and phosphoramidon, 100 nmol). Five minutes later, a hindpaw was clamped with a hemostat, 10 sec on and 10 sec off, for 10 min. Rats were fixed 10 min after the noxious stimulus. Spinal segments L1–L6 were double-labeled for NK1Rs and MORs. **A**, NK1R internalization measured in lamina I neurons. **B**, MOR internalization measured in lamina I–II neurons. Data points represent the mean $\pm$ SE of 6 rats. A two-way ANOVA was performed for each panel, the two variables being stimulus (stimulated vs. contralateral) and spinal segment. Overall p values indicated significant effects of stimulus ($p < 0.0001$) and segment ($p < 0.05$) for both NK1R and MOR internalization. Bonferroni's post-tests comparing ipsilateral and contralateral sides: *** $p < 0.001$, ** $p < 0.01$.

Fig. 2. Images of MOR and NK1R neurons in the dorsal horn after noxious mechanical stimulation
 Confocal images were taken from the L5 spinal cord segment of a rat that received an intrathecal injection of peptidase inhibitors and noxious mechanical stimulation (clamp with a hemostat) of one hindpaw. Histological sections were double-labeled with antibodies against the NK1R (red channel, A, C) and the MOR (green channel, B, D). Main panels: images taken with a 20x objective of the medial-central portion of the dorsal horn, consisting of 5 (B), 6 (A) or 8 (C, D) optical sections. Insets: images taken with a 100x objective of the cells indicated by the frames and arrows in the main panels; images consist of 4–6 optical sections taken through the middle of the cells. Asterisks (*) and “o” indicate cells with and without receptor internalization, respectively. **A**, NK1R staining of the dorsal horn ipsilateral to the stimulated

paw; NK1R internalization is found in all the cells sampled. **B**, MOR staining of the ipsilateral dorsal horn (same section as in A); MOR internalization is found in four of the seven cells sampled. **C**, NK1R staining of the contralateral dorsal horn; no NK1R internalization is observed in the three cells sampled. **D**, MOR staining of the contralateral dorsal horn (same section as in C); no MOR internalization is observed in the five cells sampled. Scale bars are 50 μm (main panels) and 3 μm (insets).

Fig. 3. NK1R and MOR internalization after noxious mechanical stimulation of the forepaw
Anesthetized rats received an intrathecal injection of peptidase inhibitors (amastatin, captopril and phosphoramidon, 100 nmol). Five minutes later, a forepaw was clamped with a hemostat, 10 sec on and 10 sec off, for 10 min. **A**, NK1R internalization in lamina I neurons. **B**, MOR internalization in lamina I–II neurons. Data points represent the mean $\pm$ SE of 3 rats. Two-way ANOVA for NK1R internalization revealed a marginally significant effect of stimulus (ipsilateral vs. contralateral, $p=0.02$), but no significant differences were detected in the Bonferroni's posttest. MOR internalization was negligible.

Fig. 4. NK1R and MOR internalization produced by noxious thermal stimulation of the hindpaw Anesthetized rats received an intrathecal injection of peptidase inhibitors (amastatin, captopril and phosphoramidon, 100 nmol). Five minutes later, a hindpaw was immersed in water at 52 °C for 2 min. **A**, NK1R internalization measured in lamina I neurons. **B**, MOR internalization measured in lamina I–II neurons. Data points represent the mean $\pm$ SE of 3 rats. Two-way ANOVA for the NK1R internalization data revealed significant effects of the variables stimulus ($p=0.002$) and segment ($p=0.03$); Bonferroni's post-test: *, $p<0.05$. MOR internalization was negligible.