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Substance P release and neurokinin 1 receptor activation in the rat spinal cord increases with the firing frequency of C-fibers

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Abstract

Both the firing frequency of primary afferents and neurokinin 1 receptor (NK1R) internalization in dorsal horn neurons increase with the intensity of noxious stimulus. Accordingly, we studied how the pattern of firing of primary afferent influences NK1R internalization. In rat spinal cord slices, electrical stimulation of the dorsal root evoked NK1R internalization in lamina I neurons by inducing substance P release from primary afferents. The stimulation frequency had pronounced effects on NK1R internalization, which increased up to 100 Hz and then diminished abruptly at 200 Hz. Peptidase inhibitors increased NK1R internalization at frequencies below 30 Hz, indicating that peptidases limit the access of substance P to the receptor at moderate firing rates. NK1R internalization increased with number of pulses at all frequencies, but maximal internalization was substantially lower at 1–10 Hz than at 30 Hz. Pulses organized into bursts produced the same NK1R internalization as sustained 30 Hz stimulation.

To determine whether substance P release induced at high stimulation frequencies was from C-fibers, we recorded compound action potentials in the sciatic nerve of anesthetized rats. We observed substantial NK1R internalization when stimulating at intensities evoking a C-elevation, but not at intensities evoking only an A δ -elevation. Each pulse in trains at frequencies up to 100 Hz evoked a C-elevation, demonstrating that C-fibers can follow these high frequencies. C-elevation amplitudes declined progressively with increasing stimulation frequency, which was likely caused by a combination of factors including temporal dispersion. In conclusion, the instantaneous firing frequency in C-fibers determines the amount of substance P released by noxious stimuli.

Keywords

A δ -fiber; neurokinin A; neuropeptide; pain; presynaptic terminal; tachykinins

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Recent discoveries have underscored the importance of substance P and its receptor, the neurokinin 1 receptor (NK1R), in chronic pain. The tachykinins substance P and neurokinin A (collectively termed “substance P” henceforth) are present in 50% of C-fibers and 20% of A δ -fibers (McCarthy and Lawson, 1989; Lawson et al., 1993). Neurons expressing the NK1R are found throughout the dorsal horn except in lamina II (Brown et al., 1995) and often project to the brain (Marshall et al., 1996; Todd et al., 2000). These neurons play a key role in the induction and maintenance of chronic pain states (Traub, 1996; Mantyh et al., 1997; De Felipe et al., 1998; Laird et al., 2000). Thus, hyperalgesia may involve an increase in neuronal excitability produced by activation of the phospholipase C pathway by NK1Rs (Rusin et al., 1993) and long-term potentiation of synapses between substance P-containing fibers and NK1R neurons (Liu and Sandkuhler, 1998; Todd et al., 2002; Ikeda et al., 2003).

A crucial question is what type of activity in primary afferents induces substance P release. While every action potential in primary afferents releases glutamate (Randic et al., 1993), release of substance P probably obeys different mechanisms (Muschol and Salzberg, 2000). Substance P release correlates with the intensity of noxious stimuli (Abbadie et al., 1997; Allen et al., 1997; Honore et al., 1999; Honore et al., 2002), probably because nociceptors encode the intensity of noxious stimuli in their firing pattern. As the intensity of the noxious stimulus increases, so does the firing rate of nociceptors, until at sufficiently high noxious intensities the firing transitions to a bursting pattern. For example, brief exposure of the splanchnic nerve to hydrogen peroxide evoked an abrupt transition from low level irregular discharge to ongoing bursting activity (Adelson et al., 1996, 1997). A similar pattern of burst discharge was evoked from C-fibers in inflamed skin in response to mechanical stimulation (Andrew and Greenspan, 1999) and in rabbit corneal C-fibers in response to volatile anesthetics (MacIver and Tanelian, 1990). Importantly, release of brain-derived neurotrophic factor (BDNF) was induced by burst stimulation, but not sustained stimulation, of the dorsal root (Lever et al., 2001).

In view of this, we hypothesized that substance P release occurs when primary afferents fire at high frequency or in bursts. There is some evidence for this. Go and Yaksh (1987), found that substance P release in the cat spinal cord evoked by sciatic nerve stimulation increased with the frequency of stimulation up to 20 Hz. Likewise, Marvizon et al. (1997; 1999) found that NK1R internalization evoked by dorsal root stimulation was higher at 100 Hz than at 10 Hz or 1 Hz. However, Duggan et al. (1995) found that substance P release in the cat spinal cord was not affected by the frequency of stimulation, although it increased with a bursting stimulation pattern. Moreover, the idea that substance P is released by C-fibers firing at frequencies as high as 100 Hz is difficult to reconcile with evidence suggesting that C-fibers do not follow high frequency stimulation (Raymond et al., 1990; Waddell and Lawson, 1990). It is possible that the C-fibers that contain substance P fire at high frequency, or that high frequency stimulation releases substance P from A δ -fibers. Here, we studied the effect of different modes of primary afferent firing on substance P release by stimulating spinal cord slices at the dorsal root or live rats at the sciatic nerve. Substance P release was measured in situ with NK1R internalization (Trafton et al., 1999; Trafton et al., 2001; Marvizon et al., 2003a). To assess whether C-fibers can follow high frequency stimulation, we recorded compound action potentials (CAPs) in the sciatic nerve.

Experimental Procedures

Animals

All 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 used and their suffering.

Media for spinal cord slices

Artificial cerebrospinal fluid (aCSF) contained (in mM) 124 NaCl, 1.9 KCl, 26 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂ and 10 glucose. K⁺-aCSF was the same medium with 5 mM KCl. Sucrose-aCSF was the same medium with 5 mM KCl and 215 mM sucrose instead of NaCl (iso-osmotic replacement). In some experiments CaCl₂ was omitted from the aCSF to obtain a nominally Ca²⁺-free medium. These media were bubbled with 95% O₂/5% CO₂ for a pH of 7.4.

Spinal cord slices

Spinal cords were prepared from 3–4 weeks old Sprague-Dawley rats (Harlan, Indianapolis, IND) under isoflurane anesthesia (Halocarbon Laboratories, River Edge, NJ). Coronal slices (400 µm) with one dorsal root were cut with a Vibratome (Technical Products International, St. Louis, MO) from a lumbar spinal cord segment (L2–L4), as previously described (Randic et al., 1993; Marvizon et al., 1997; Lao et al., 2003; Lao and Marvizon, 2005). Briefly, the spinal cord segment was rapidly extracted, cleaned of dura mater and ventral roots in ice-cold sucrose-aCSF, and glued vertically to a block of agar on the stage of the Vibratome. Slices (3–6 per rat) were cut in ice-cold sucrose-aCSF using minimum forward speed and maximum vibration. To ensure fiber continuity between the dorsal root and the dorsal horn, this was done with the help of a stereo microscope. Fiber continuity between the root and the dorsal funiculus was assessed by examining the dorsal root and the dorsal surface of the slice with the stereo microscope: at least 80% of the dorsal funiculus should be continuous with dorsal root fibers, and these should not have cuts or compression damage. Slices that did not meet these criteria were discarded. Slices were kept at 35 °C in K⁺-aCSF for one hour, and in aCSF up to three hours after their preparation.

Dorsal root stimulation

The spinal cord slice with attached dorsal root was placed in a custom-made superfusion chamber, where it was stimulated electrically (Lao et al., 2003; Song and Marvizon, 2003; Lao and Marvizon, 2005). Slices were superfused at 3–6 ml/min with aCSF at 35 °C. A bipolar platinum stimulation electrode (0.5 mm wire diameter, 1 mm separation) was located in a compartment separated from the superfusion chamber by a movable partition. The dorsal root was drawn into the electrode compartment through a hole in the partition (sealed with vacuum grease) and placed on top of the platinum wires. The electrode compartment was then emptied of aCSF and filled with mineral oil. Contact between the root and the electrode wires and the thickness of the sheet of aCSF surrounding the root was monitored with a stereo microscope, and any excess aCSF short-circuiting the electrode was suctioned away. This ensured that electrical current circulated through the root, and that the stimulus was consistent between preparations. Electrical stimulation was generated by a Master-8 stimulator and a Iso-Flex stimulus isolating unit in constant voltage mode (A.M.P. Instruments, Jerusalem, Israel), and consisted of square pulses of 1–30 V intensity, 0.4 ms duration. Before placing the slice in the chamber, the integrity of the circuit and the accuracy of the programmed stimulus were verified by monitoring the output across the bipolar electrode with an oscilloscope (which was disconnected during the actual stimulation). Following electrical stimulation, slices were kept in the chamber for 10 min and then fixed in ice-cold fixative (4 % paraformaldehyde, 0.18 % picric acid). In experiments testing the effects of peptidase inhibitors, these were added to the aCSF beginning 5–10 min before root stimulation, and were superfused continuously thereafter. To recognize the side ipsilateral to the stimulus in the histological sections, a hole was punched in the ipsilateral ventral horn of the slice. Treatments were randomized between slices, and no more than two slices from the same animal received the same treatment.

Nerve stimulation and recording *in vivo*

We used a modification of methods previously described (Go and Yaksh, 1987; Allen et al., 1999). Rats were anesthetized with 25% urethane (i.p., 1.5 ml/kg total, divided into two injections), followed by additional doses up to 0.2 ml i.p., if needed, to render the animal areflexic to strong paw-pinch. EKG electrodes were affixed to the fore- and hind-paws and EKG signals were amplified using a differential amplifier (Model 1700, A-M Systems, Carlsborg WA) to monitor pseudoaffective reflex changes in heart rate at noxious intensities of nerve stimulation. A dorsal incision was made in the left thigh to expose the sciatic nerve from the mid-thigh to the level of the popliteal fossa. This incision was either continued down the leg to the mid-calf, or a second incision was made in the calf area. The calf muscle was reflected to expose the tibial branch of the sciatic nerve, approximately midway between ankle and knee. Skin flaps were sutured to an overlying steel ring, forming basins that were then filled with warm mineral oil to protect the nerve from desiccation and to provide electrical isolation. The epineurium surrounding the tibial trunk proximal to the popliteal fossa (mid-thigh) was dissected free of the trunk but enough of the epineurium was left attached to provide support to the nerve and protect it against damage. The perineurium was carefully opened for a length of approximately 2 mm, and the desheathed portion of the trunk was hung on a hook bipolar platinum-iridium recording electrode (0.25 mm wire diameter, 1 mm separation, FHC Inc. Bowdoin, ME) to record the compound action potential (CAP) as it propagated centrally. All sciatic branches other than the tibial branch (sural, peroneal, articular) were cut near the popliteal fossa. Distally, the tibial nerve was similarly de-sheathed, hung on a second platinum-iridium electrode used to stimulate the nerve, and then was cut distal to this stimulating electrode. The approximate conduction distance between the stimulating and recording electrodes (20–45 mm) was determined by laying a silk suture along the course of the nerve and measuring its length. This conduction distance permitted reliable resolution of A β -, A δ - and C-fiber elevations, yielding records comparable to those observed previously in the saphenous nerve of the cat (Douglas and Ritchie, 1957). The CAP signal was amplified 10,000-fold with the differential amplifier and digitized, along with EKG and stimulus monitor traces, using a Micro-1401 mk II A-D interface and Spike-2 acquisition software (Cambridge Electronic Design, Cambridge, England). The differential amplifier filter passband was typically set to 1Hz-1KHz, and 60 Hz notch filtering was avoided. This filtering arrangement avoided ringing or distortion of the stimulus artifact and/or A β elevation(s) that might lead to the appearance of excursions that either obscure or be mistaken for the A δ elevation.

Electrical stimulus pulses were generated using a Grass S-88 stimulator and stimulus isolation unit (Grass Instruments, SIU5). To insure that differences in the stimulating conditions (i.e., electrical contact between each electrode and the nerve, nerve diameter, etc) between preparations did not alter the population of fibers excited in each experiment, the thresholds for A δ - and C-fiber activation were determined for each preparation. This was done by stimulating the nerve at 1 Hz with increasing pulse intensities, using a constant pulse duration of 0.4 ms. The polarity of the stimulus was set to avoid anodal block of the centrally propagating CAP. However, due to the pronounced excitability and rapid conduction velocity of large diameter A fibers, the rising and falling edges of the square wave pulse function effectively as a pair of stimuli, of opposite polarity, each capable of firing A β -fibers. Due to the phenomenon of anodal block, the conducted waves evoked by the rising and falling edge are typically of differing amplitudes, and thus their superposition may yield unexpectedly complex waveforms, particularly due to the use of a bipolar recording electrode, and spreading of waves due to stray capacitances. The dual triggering of A-fiber elevations by the rising and falling edge of the stimulus square pulse could be unambiguously discriminated by 1) increasing the pulse duration in steps up to 5 ms, which caused the two A waves or complexes (of typically differing amplitude) to separate by a corresponding distance, and 2) reversing the polarity of the stimulus for several pulses to reverse the positions of the two separated waveforms of similar shape but

differing amplitude. Although the conduction distances used in most experiments were such that the separation between the A β and A δ elevations rendered this complication irrelevant, we nonetheless performed this test as a pro-forma matter to be certain we did not confuse artifacts of a complex waveform with the A δ elevation.

Following determination of the threshold and saturation stimulation intensities for the A δ and C elevations, the rat was left undisturbed for 1–2 hr, to allow any NK1Rs internalized during threshold determination to cycle back to the cell surface (Wang and Marvizon, 2002). Following this rest period, the nerve was stimulated with 300 pulses of an intensity of either 2.5 times the A δ -fiber activation threshold (typically corresponding to ~40% of the C-fiber threshold) or 2.5 times the C-fiber threshold, at a frequency of 30 Hz. The centrally propagating CAP was recorded during this stimulus train to be certain that any possible changes in electrical contact during the intervening hour of recovery did not alter the fiber population recruited, and to verify that each stimulus pulse evoked the expected CAP. The rat was fixed 10 min after stimulation by aortic perfusion of 150 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) at room temperature and 400 ml of ice-cold fixative (4 % paraformaldehyde, 0.2 % picric acid, 0.1 M sodium phosphate, pH 7.4). The L3-L5 segment of the spinal cord was extracted, post-fixed overnight and processed for NK1R immunohistochemistry to quantify NK1R internalization.

In a separate set of experiments to examine the ability of C-fibers to follow high frequency stimulation, the nerve was stimulated with pulses of an intensity 7 times the C-fiber threshold at frequencies between 1 and 100 Hz to examine the relationship between continuous stimulation frequency and CAP amplitude. These animals were not used to measure NK1R internalization.

Immunohistochemistry

Histological sections from spinal cord slices or lumbar spinal cord (L3-L5) were labeled with an antibody recognizing the NK1R as previously described (Marvizon et al., 1997; Marvizon et al., 1999; Lao et al., 2003; Song and Marvizon, 2003; Lao and Marvizon, 2005). Briefly, the tissue was fixed, cryoprotected, frozen on dry ice, and sectioned with a cryostat at 25 μ m in the coronal plane. Sections were washed four times and then incubated overnight at room temperature with the NK1R antibody diluted 1:3000 in phosphate-buffered saline containing 0.3 % Triton X-100, 0.001 % thimerosal and 5 % normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). The NK1R antibody was rabbit antiserum # 94168 (a gift from Dr. Nigel Bunnett, UCSF), raised against amino acids 393–407 of the rat NK1R, previously characterized (Grady et al., 1996). After three washes, the secondary antibody (1:2000, Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes-Invitrogen, Eugene, OR) was applied at for 2 hours at room temperature. Sections were washed four more times, mounted on glass slides, and coverslipped with Prolong Gold (Molecular Probes-Invitrogen).

Confocal microscopy

Confocal images were acquired with a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). We used a pinhole of 1.0 Airy units and objectives of 20x (numerical aperture [NA] 0.70), 63x oil (NA 1.32) and 100x oil (NA 1.4), resulting in an estimated optical section thickness (full width at half maximum) of 2.53 μ m, 0.69 μ m and 0.62 μ m, respectively. Excitation was provided by an argon laser (488 nm line), and the emission window was 500–550 nm. Images were acquired as confocal stacks (5–18 optical sections) with optical section separations of 0.57 μ m, 0.61 μ m or 1.22 μ m for the 100x, 63x and 20x objectives, respectively. Optical sections were acquired at a digital size of 1024 $\times$ 1024 pixels and averaged four times to reduce noise. A zoom factor of 2 was used with some images.

Deconvolution and image processing

The NK1R antibody used in this study produced high quality confocal images, with low background and noise. Nevertheless, some of the images were improved further by reducing the amount of blur using deconvolution. The program AutoQuant X2.0.1 (Media Cybernetics, Inc., Bethesda, MD) was used to perform adaptive point spread function (PSF) or “blind” deconvolution (Holmes et al., 2006) of the whole confocal stack, using 10 iterations. The resulting three dimensional image file was imported into Imaris (x64, 6.0.0, Bitplane AG, Zurich, Switzerland), which was used to crop the image in three dimensions (without changing the resolution), adjust the brightness and obtain a two-dimension projection picture. This picture was imported into Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA), which was used to compose the multi-panel figures.

Quantification of NK1R internalization

The amount of NK1R internalization was quantified using a standard method (Mantyh et al., 1995; Abbadie et al., 1997; Riley et al., 2001; Trafton et al., 2001) with minor modifications (Marvizon et al., 1997; Marvizon et al., 1999; Lao et al., 2003; Lao and Marvizon, 2005). It consisted of visually counting NK1R immunoreactive neurons in lamina I with and without NK1R internalization. Zeiss Axiovert 135 or Axio-Imager A1 (Carl Zeiss, Inc., Thornwood, NY) fluorescence microscopes fitted with 100x and 63x objectives were used. The person counting the neurons was blinded to the treatment given to the slice or the animal. The criterion for having internalization was the presence of ten or more NK1R endosomes in the neuronal soma. At least three sections per slice or rat were used, and all lamina I NK1R neurons in each section were counted. NK1R internalization was quantified in the dorsal horns ipsilateral and contralateral to the stimulated root or nerve, and expressed as the percentage of the NK1R neurons in lamina I that had NK1R internalization.

Data analysis

Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical analyses consisted of two-way ANOVA followed by Bonferroni's post-hoc test. Statistical significance was set at 0.05. In some experiments, a sigmoidal dose-response function,

$$Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{-(\text{Log EC}_{50} - \text{Log [X]})})$$

was fitted to the data points by non-linear regression. The “top” and “bottom” parameters were constrained to values <100% and >0%, respectively. EC₅₀ is the effective dose for 50% of the response. The statistical error associated with the EC₅₀ was expressed as 95% confidence interval (95% C.I.).

Results

Effect of the intensity of stimulation of the dorsal root

To determine how primary afferent firing affect substance P release, we stimulated spinal cord slices at the dorsal and measured NK1R internalization in lamina I neurons (Marvizon et al., 1997; Marvizon et al., 1999; Wang and Marvizon, 2002; Marvizon et al., 2003a; Marvizon et al., 2003b; Lao and Marvizon, 2005). We first examined the effect of the intensity (voltage) of the electrical pulses delivered to the dorsal root (Fig. 1). We used square pulses with a fixed duration at 0.4 ms, which is sufficient to recruit C-fibers provided that the intensity is high enough (Koslow et al., 1973; Li and Bak, 1976). We delivered 300 pulses to the dorsal root at 30 Hz in a single train. In the dorsal horn contralateral to the stimulated root, NK1R internalization was consistently low and unaffected by pulse intensity (Figs. 1, 2A). In the

ipsilateral dorsal horn, NK1R internalization in lamina I neurons was negligible up to 5 V (Figs. 1, 2B), became significantly higher than the contralateral side at 10 V (Figs. 1, 2C, 2D), and was maximal at 20 V (Figs. 1, 2E). NK1R internalization in laminae III–IV was negligible, even with the higher intensities of stimulation (Fig. 2F). Therefore, substance P release sufficient to activate NK1Rs took place with pulse intensities higher than 5 V. With 20 V, most of the substance P-containing axons in the root were recruited. Since pulses of 20 V evoke EPSPs with C-fiber latencies (Randic et al., 1993; Liu and Sandkuhler, 1995; Liu and Sandkuhler, 1997; Sandkuhler et al., 1997), these results suggest that recruitment of C-fibers is necessary to evoke enough substance P release to induce NK1R internalization. In practical terms, this experiment served to determine the optimal stimulation intensity to be used in subsequent experiments: 20 V.

NK1R internalization evoked by dorsal root stimulation is caused by substance P release

Next, we confirmed that NK1R internalization induced by dorsal root stimulation was caused by substance P release. To that end, we stimulated the dorsal root with 1000 pulses (20 V, 0.4 ms) delivered at 100 Hz while superfusing the slices with a NK1R antagonist, low Ca^{2+} aCSF, or lidocaine, a local anesthetic and Na^+ channel blocker. In control slices, which were superfused with normal aCSF, NK1R internalization was induced in about 65% of the NK1R neurons in lamina I (Fig. 3). The NK1R antagonist L-703,606 (10 μM , Fig. 3) abolished the internalization, showing that it was produced by agonist binding to the receptor. The internalization was also abolished in aCSF with a low Ca^{2+} concentration (0.2 mM instead of 2.4 mM). This is consistent with the idea that it was caused by substance P release, which is Ca^{2+} -dependent. We have previously shown that NK1R internalization induced by adding substance P to spinal cord slices is not Ca^{2+} -dependent (Lao et al., 2003); therefore, the low Ca^{2+} could not have inhibited NK1R internalization itself instead of substance P release. Finally, NK1R internalization was abolished by the Na^+ channel blocker lidocaine (1 mM, Fig. 3), which we used to block action potentials in the dorsal root entering the slices. Hence, the substance P release that elicited NK1R internalization was driven by the firing of primary afferents.

Effect of the frequency of stimulation of the dorsal root

To determine whether SP release and NK1R activation increases with the firing frequency of primary afferents, we stimulated the dorsal root at frequencies from 1 Hz to 500 Hz (Fig. 4A), using continuous trains of 1000 pulses (20 V, 0.4 ms). Since the same number of pulses was given at the different frequencies, the train duration varied between 2 sec (for 500 Hz) and 1000 sec (or 16.7 min, for 1 Hz). We did not maintain constant the train duration because then the number of pulses would have increased with the frequency, and the number of pulses has a prominent effect on NK1R internalization (see below). However, note that the extent of NK1R internalization depends on the peak concentration of substance P (Marvizon et al., 2003b), so spreading the release of substance P over longer periods of time produces less internalization for the same total amount of substance P released. Still, since NK1R internalization reflects its activation, this experiment provides information on how effectively the NK1R is activated at different frequencies of firing of primary afferents, regardless of the total amount of substance P released. This is important because NK1R activation is what determines the physiological effects, and not the amount of substance P released.

The frequency of stimulation of the dorsal root had a substantial effect on the evoked NK1R internalization (Fig. 4A). As in previous experiments, NK1R internalization in the contralateral dorsal horn was negligible and unaffected by the stimulation frequency, indicating that no substance P release was evoked in it. Ipsilaterally, NK1R internalization increased very slightly (if at all) between 1 Hz and 20 Hz and then suddenly at 30 Hz (Figs. 4A, 5A, 5B, 5E). It stayed high up to 100 Hz, and then decreased abruptly at 200 Hz and 500 Hz to values not significantly

different from the contralateral side. The latter effect was probably due to the inability of C-fibers to follow these high frequencies of stimulation. Two-way ANOVA revealed significant effects of both frequency ($p<0.0001$) and ipsilateral vs. contralateral ($p<0.0001$). Curiously, NK1R internalization seemed to dip at 50 Hz, although this decrease was not statistically significant when compared with 30 Hz or 70 Hz (Bonferroni's post-hoc test). Note that, even though NK1R internalization evoked at low frequencies was low (Fig. 5A, B), it was still significantly higher than in the contralateral dorsal horn, indicating that substance P release was evoked to some extent at low frequencies. Fig. 5B shows one neuron with many NK1R endosomes after 10 Hz stimulation, but note that some NK1R immunoreactivity remained at the cell surface. In contrast, after 30 Hz stimulation (Fig. 5E) NK1R immunoreactivity not only appears in endosomes, but it also disappears from the cell surface.

Effect of the frequency of stimulation in the presence of peptidase inhibitors

Degradation by peptidases limits the ability of the substance P and neurokinin A to bind to the NK1R (Marvizon et al., 2003b). Since stimulation at low frequencies spreads the release of substance P over longer period of times than stimulation at high frequencies, the extent of degradation of substance P by peptidases would be expected to be higher at the lower frequencies. This could be responsible for the lower NK1R internalization observed at these frequencies. Indeed, we have already reported (Marvizon et al., 2003b) that peptidase inhibitors increase NK1R internalization evoked by root stimulation at 1 Hz, but not at 30 Hz. The question, then, is whether peptidase degradation is solely responsible for the frequency-dependence of NK1R internalization evoked by root stimulation. If not, then NK1R internalization would increase with the frequency even when peptidases are inhibited.

The experiment shown in Fig. 4B studies NK1R internalization evoked by dorsal root stimulation at different frequencies in the presence of peptidase inhibitors. These were thiorphan (10 μ M), an inhibitor of neutral endopeptidase (EC 3.4.24.11), and captopril (10 μ M), an inhibitor of dipeptidyl carboxypeptidase (EC 3.4.15.1). We did not use aminopeptidase inhibitors because, unlike thiorphan and captopril, they did not increase NK1R internalization evoked by exogenous substance P (Marvizon et al., 2003b). Peptidase inhibitors were superfused to the slices starting 5 min before the onset of root stimulation, which again consisted of 1000 pulses (20 V, 0.4 ms). Comparing Figs. 4A and B reveals that peptidase inhibitors increased NK1R internalization evoked at frequencies of 1–20 Hz, but not at frequencies of 30–100 Hz. A two-way ANOVA comparing data in the ipsilateral side in Fig. 4A and Fig. 4B revealed a significant effect of both peptidase inhibitors and frequency ($p<0.0001$). Bonferroni's post-hoc tests found significant differences ($p<0.05$) between the data with and without peptidase inhibitors at 5 Hz, 10 Hz, 20 Hz and 50 Hz. Like in the experiment without peptidase inhibitors, NK1R internalization decreased sharply at 200 Hz, likely because substance P-containing primary afferents did not follow this frequency. These results show that, during low frequency firing of primary afferents, peptidases decrease NK1R activation by degrading some of the substance P being released, whereas during high frequency firing the peptidases appear to be saturated.

Nevertheless, the increase by peptidase inhibitors of the NK1R internalization evoked at low frequencies did not eliminate its frequency-dependence. ANOVA (one-way) of the data in the ipsilateral side in Fig. 4B (200 Hz point excluded) revealed a significant effect of frequency ($p<0.001$). Therefore, substance P release from primary afferents is frequency-dependent, and maximal at frequencies of 30–100 Hz. In addition, peptidase degradation of substance P released at low firing frequencies contributes to make NK1R activation maximal at these frequencies.

Effect of the number of pulses

We wondered whether the frequency-dependence of NK1R internalization would disappear if the number of pulses was increased. Accordingly, we studied the effect of the number of pulses on NK1R internalization evoked at frequencies of 1 Hz, 10 Hz and 30 Hz, using continuous trains of synchronous stimulation. We did not use more than 1000 pulses with 1 Hz stimulation, because this would entail an excessively long (>16 min) train duration. With the other frequencies, up to 10000 pulses were given. NK1R internalization increased with the number of pulses at all three frequencies, following sigmoidal dose-response curves (Fig. 6). Representative lamina I neurons after stimulation at 30 Hz with 30, 300 and 1000 pulses are shown in panels C, D and E of Fig. 5, respectively. The evoked NK1R internalization saturated at a maximum value as the number of pulses increased. This maximum value increased with the frequency: it was 29 ± 4 % at 1 Hz, 54 ± 8 % at 10 Hz and 78 ± 5 % at 30 Hz, as calculated from the non-linear regression fitting of the data (Fig. 6). To determine whether these differences were statistically significant, we compared these values across the three different curve fittings using an extra sum-of-squares F-test (Motulsky and Christopoulos, 2003), which revealed that these differences were indeed significant ($p=0.0078$). Therefore, the frequency-dependence of the evoked NK1R internalization persisted when the number of pulses was increased to saturation.

Effect of burst firing of primary afferents on NK1R internalization

Although we used synchronous stimulation in the experiment discussed above, primary afferents do not usually fire synchronously but in bursts, particularly during intense noxious stimulation (Adelson et al., 1996, 1997; Lever et al., 2001). This appears to have important consequences, because BDNF is released only by these bursting patterns (Lever et al., 2001). We wondered whether the pattern of primary afferent firing (i.e. synchronous or bursting) could similarly influence substance P release. To investigate this, we stimulated the dorsal root using a bursting pattern (theta burst stimulation, TBS) consisting of bursts of 4 pulses delivered at 100 Hz, with an inter-burst frequency of 5 Hz (i.e., burst were delivered every 0.2 s). As done with synchronous stimulation, the number of pulses was increased by increasing the number of bursts (Fig. 6). The resulting curve for TBS was identical to that obtained using synchronous stimulation at 30 Hz, showing that these two firing patterns were equally efficacious in activating NK1Rs.

Determination of thresholds for activation of A δ - and C-fibers

Our finding that NK1R internalization evoked by dorsal root stimulation is maximal at high frequencies (30–100 Hz) contradicts some current ideas. Thus, substance P is believed to be released mostly from C-fibers, and it is also believed that C-fibers do not follow these high frequencies of stimulation (Raymond et al., 1990; Waddell and Lawson, 1990). Our results suggest either that C-fiber do follow up to 100 Hz, or that the substance P released at high frequencies is not from C-fibers but from A δ -fibers, which also contain substance P (McCarthy and Lawson, 1989; Lawson et al., 1995). To discriminate between these two possibilities, we used sciatic nerve stimulation *in vivo* to separate the A β , A δ - and C-elevations of the CAP. This allowed us to record C-fiber activity during high frequency stimulation, and also to find a stimulation intensity that recruited A δ -fibers but not C-fibers.

We determined the thresholds to recruit A δ - and C-fibers in each rat by stimulating the sciatic nerve at 1 Hz and increasing the intensity of the pulses in a stepwise fashion (Fig. 7, see Methods). A δ -thresholds varied between preparations from 0.2 to 0.8 V, while C-thresholds ranged between 2 and 4.5 V (Table 1). In preliminary experiments the range of these thresholds was even greater. Possible causes of such variability included differences in nerve desheathing, efficiency of the contact between the nerve and the electrode, and other factors difficult to standardize.

At a stimulation rate of 1 Hz, sciatic nerve stimulation at intensities higher than the C threshold (typically by 10%–30%) produced an increase in the rat's heart rate (Fig. 7B), suggesting recruitment of nociceptive pseudoaffective reflexes. Of interest, while 1 Hz stimulation during threshold determination did not evoke heart rate changes until intensities above the C-threshold were reached, during A δ stimulation at 30 Hz, heart rate did increase (typically by ~ 12 beats per minute), suggesting that higher frequency stimulation of nociceptive A δ fibers could drive this response.

Do C-fibers follow high frequency stimulation?

Next, we investigated whether C-fibers are able to follow high frequency stimulation (up to 100 Hz). In three rats, the sciatic nerve was stimulated above the C-fiber threshold at frequencies ranging from 1 Hz to 100 Hz (Fig. 8, see Table 1 for A δ - and C-fiber thresholds and conduction speed). At all frequencies tested, a C-fiber elevation was evoked by every pulse, showing that, as a population, C-fibers followed the stimulation. Note that at frequencies above 40 Hz the latency of the C-elevation is such that one or more stimulus artifacts appear in the trace between the C-elevation and the stimulus artifact of the pulse that evoked it.

In the experiment shown in Fig. 8, the frequency was increased from 1 Hz to 50 Hz by steps of 9–10 Hz every 5–7 s. The peak amplitude of the C-elevation stayed relatively constant at any given frequency, but it decreased as the frequency was increased, and its width increased. This phenomenon is probably due to a combination of the following factors: a) temporal dispersion, with peaks and troughs of impulses in fibers conducting at different speeds interfering with each other; b) failure to fire of a progressively larger subpopulation of fibers at higher frequencies of stimulation, or c) increases in the probability of failure in each fiber for each stimulus pulse at higher frequencies.

To further investigate this, single identical pulses were delivered before (1 Hz trace, Fig. 8) and after the 100 Hz train (separate pulse at the end of the 100 Hz trace, Fig. 8). Pulses evoked during the 100 Hz stimulation train had peak amplitudes lower than the test pulse, but interference between elevations evoked by consecutive pulses and between impulses in individual fibers evoked by the same stimulus pulse is almost certainly responsible for part of this reduction. The test pulse after the train had similar amplitude to the one evoked before the train, indicating that high frequency stimulation did not inactivate C-fibers for more than 100 ms. Clearly, any decrease in the amplitude of the C elevation during the train was not caused by a persistent reduction in the excitability of the C-fibers. However, the latency of the test pulse after the 100 Hz train was substantially increased (by 25%) compared to that of the test pulse before the train (Fig. 8), demonstrating the well-established phenomenon of progressive slowing of C-fiber conduction velocity with high frequency stimulation.

NK1R internalization produced by sciatic nerve stimulation at A δ - or C-fiber intensity

This last experiment was performed to determine whether evoking substance P release and subsequent NK1R internalization required the recruitment of C-fibers. Substance P release was evoked *in vivo* by stimulating the sciatic nerve (tibial branch) at either C-fiber or A δ -fiber intensity. In addition to the stimulating electrode, a recording electrode was placed proximal to the spinal cord and at least 20 mm from it. This allowed a good separation of the A δ - and C-elevations of the CAP. The cathode of the stimulating electrode was proximal to the spinal cord, in order to avoid anodal block. The pulse intensity thresholds to recruit A δ - and C-fiber were determined as shown in Fig. 7 and are given in Table 1. After threshold determination, the preparation was left undisturbed for one hour or more. This time is sufficient to allow any NK1Rs internalized during the threshold determination trials to recycle back to cell surface (Wang and Marvizon, 2002).

Stimulation to induce NK1R internalization consisted in 300 pulses at 30 Hz. Three rats were used as a sham control: their sciatic nerves were exposed and mounted on electrodes, but no current was delivered. Six rats received pulses at A δ -fiber intensity: 1.5 times the threshold for A δ -fibers, which was still below the C-fiber threshold. Four rats received pulses of 1.5 times the C-fiber threshold. Note that, because the A δ - and C-fiber thresholds varied between rats, the actual pulse intensities (in volts) delivered to different rats were not the same. NK1R internalization was measured in lamina I neurons of the L3-L5 spinal segments, both ipsilaterally and contralaterally to the stimulated nerve. As shown in Fig. 9, sciatic nerve stimulation above the C-fiber threshold produced a substantial amount of NK1R internalization in the ipsilateral, but not the contralateral, dorsal horn. In contrast, stimuli that recruited A δ -fibers but not C-fibers produced very little NK1R internalization, which was not significantly different from the sham controls or the contralateral dorsal horn. To assess whether stimulating A δ -fibers with more pulses or at higher frequency could induce NK1R internalization, two additional rats received sciatic nerve stimulation at A δ -fiber intensity: one received 900 pulses at 30 Hz, and the other 300 pulses at 100 Hz. In both rats NK1R internalization was low (<30 %).

The relative position of the stimulating and recording electrodes had no effect on the NK1R internalization evoked at C-fiber intensity. Four more rats ("C-fiber*" in Fig. 9) received the same C-fiber stimulus, but in them the position of the electrodes was reversed: the stimulating electrode was proximal and the recording electrode distal to the spinal cord. In this case, the polarity of the stimulating electrode was with the cathode distal to the spinal cord during threshold determination, and was then reversed during stimulation of NK1R internalization. This ensured that there was no anodal block during threshold determination or induction of NK1R internalization. Stimulating the sciatic nerve this way produced similar amounts of NK1R internalization that with our regular electrode arrangement. This indicates that the ability of the stimulus to produce NK1R internalization was not affected by the placement of the nerve on the recording electrode, or by the distance between the stimulating electrode and the spinal cord.

Fig. 10 shows images of representative NK1R neurons in lamina I in a sham-operated rat (Fig. 10A), a rat stimulated at A δ -fiber intensity (Fig. 10B), and the ipsilateral (Fig. 10C) and contralateral (Fig. 10D) dorsal horns of a rat stimulated at C-fiber intensity. NK1R internalization is observed only ipsilaterally to the nerve stimulated at C-fiber intensity.

We concluded that most of the substance P released in the dorsal horn during high frequency stimulation comes from C-fibers and not A δ -fibers. The experiment also confirms that NK1R internalization can be evoked by high frequency nerve stimulation in physiological conditions.

Discussion

This study made several important observations concerning the relationship between the firing patterns of primary afferent fibers and the activation of NK1Rs in the superficial dorsal horn. NK1R activation is higher when primary afferent fibers fire at high frequencies or in bursts than when they fire at low frequency. This is caused by both an increase in substance P release and a decrease in its degradation by peptidases at the higher frequencies. Substance P is released primarily from C-fibers, which are able to follow stimulation frequencies up to 100 Hz.

Frequency dependence of substance P release and NK1R activation

We found that NK1R internalization evoked by dorsal root stimulation increases with the stimulation frequency. NK1R internalization is a measure of NK1R activation, as evidenced by its correlation with NK1R-mediated increases in intracellular Ca²⁺ (Trafton et al., 1999; Trafton et al., 2001). NK1R internalization was induced by substance P released from primary

afferents, because it was blocked by an NK1R antagonist, low Ca^{2+} and lidocaine. The release was not from second order neurons, because it was not affected by blocking synaptic transmission from primary afferents with a glutamate receptor antagonist (Marvizon et al., 1997).

In agreement with our results, Go and Yaksh (1987) reported that substance P release from the cat spinal cord evoked by sciatic nerve stimulation increased with the frequency up to 20 Hz and stayed high up to 200 Hz. In contrast, Duggan et al. (1995) found that substance P release in the cat spinal cord did not change with the frequency of stimulation of the tibial nerves, from 0.5 Hz to 20 Hz. However, it increased when the nerves were stimulated with short bursts of high frequency pulses. We did find that a bursting pattern evoked a high level of substance P release, but this was the same as the release evoked by high frequency stimulation.

It has been known for some time that the release of neuropeptides is elicited by specific firing patterns (Bartfai et al., 1986; Brezina et al., 2000). A similar frequency dependence of substance P release was found in the substantia nigra (Diez-Guerra et al., 1988) and the enteric nervous system (Baron et al., 1983). Likewise, burst stimulation or high frequency stimulation ($>20\text{Hz}$) were most effective at eliciting the release of neuropeptide Y from peripheral sympathetic terminals (Allen et al., 1984; Donoso et al., 1997), while noradrenalin was released efficiently at low stimulation frequencies. Importantly, different neurotransmitters can be released from the same terminal depending on the firing pattern. Thus, some C-fiber terminals contain glutamate, substance P and BDNF (De Biasi and Rustoni, 1988; Michael et al., 1997), but BDNF is only released by bursting firing whereas glutamate is released with every action potential (Lever et al., 2001). Our results show that substance P release takes place at all firing frequencies, but substantially increases at frequencies of 30–100 Hz. Glutamate is contained in light synaptic vesicles, whereas neuropeptides like substance P are contained in dense-core synaptic vesicles. Release from light vesicles depends on the rapid raise in Ca^{2+} produced by individual action potentials, while release from dense-core vesicles is driven by long-lasting Ca^{2+} levels produced by the cumulative effect of high frequency firing (Muschol and Salzberg, 2000).

Effect of peptidases on the frequency dependence of NK1R activation

The second factor that determines the frequency dependence of NK1R activation is the degradation of substance P by peptidases. We found that peptidase inhibitors did not affect NK1R internalization evoked by stimulating the root at high frequencies. This suggests that high firing frequencies lead to the rapid build up of substance P in the extracellular space, saturating the peptidases. In contrast, peptidase inhibitors increased NK1R internalization evoked by low frequencies, showing that peptidases effectively degrade substance P when it is released at slower rates.

Relative contribution of A δ - and C-fibers to substance P release

Substance P is present in 50% of the C-fibers and 20% of A δ -fibers, but not in A β -fibers (McCarthy and Lawson, 1989; Lawson et al., 1993). Therefore, substance P release from A δ -fibers might be responsible for the greater NK1R internalization observed with high frequency stimulation. However, we found that stimulation with pulses that recruit A δ -fibers but not C-fibers produced negligible NK1R internalization, which argues against this possibility. The same stimulation with pulses supra-threshold for C-fibers evoked abundant NK1R internalization. Similarly, in spinal cord slices NK1R internalization occurred only when the dorsal root was stimulated with pulse intensities larger than 10 V, commonly used to recruit C-fibers (Randic et al., 1993; Liu and Sandkuhler, 1995; Liu and Sandkuhler, 1997; Sandkuhler et al., 1997). Since pulses of C-fiber intensity also recruited A δ -fibers, we cannot rule out the possibility that NK1R internalization was induced by the combined release of substance P from

A δ - and C-fibers. While it is possible that some high-threshold A δ -fibers may have not been recruited with stimulus pulses at 2.5 times the A δ threshold, we think it unlikely that these few A δ -fibers contribute enough substance P release to alter the outcome of the experiment.

It should be noted that our findings are at variance with those of Allen et al. (1999), who reported that stimulating the sciatic nerve at A δ -fiber intensity produced NK1R internalization in 66 % of the NK1R neurons in lateral lamina I. Perhaps this difference is not real: if lateral lamina I is assumed to be one third of the entire lamina I and there was no internalization in central and medial lamina I, there could be as few as 22 % NK1R neurons with internalization, a value similar to what we found (20 $\pm$ 6%). Otherwise, it is possible that the stimuli used by Allen et al. recruited some C-fibers. While we determined the A δ and C thresholds in every rat and adjusted the pulse intensity accordingly, Allen et al. determined the thresholds in a separate group of rats. We observed that the thresholds were quite sensitive to nerve de-sheathing and electrode contact, and that these conditions were difficult to replicate between rats. In any case, it seems clear that C-fibers contribute much more than A δ -fibers to the activation of NK1Rs in lamina I neurons.

Do C-fibers follow high frequency stimulation?

Taken together, our results indicate that NK1Rs are activated by substance P released from C-fibers, and that NK1R activation is maximal when these C-fibers are stimulated at frequencies of 30–100 Hz. However, it has been argued that C-fibers do not follow frequencies higher than 10–20 Hz (Raymond et al., 1990; Waddell and Lawson, 1990). We re-examined this idea by recording C-fiber CAPs while stimulating the sciatic nerve at frequencies up to 100 Hz. At all these frequencies, every pulse in the stimulus train evoked a clear C-elevation, showing that at least some of the C-fibers are able to follow high frequencies. However, the fact that the amplitude of the C-elevation decreased as the frequency increased suggests that some C-fibers failed to follow. It is unlikely that different C-fibers failed at every pulse, because once a C-fiber fails it also fails for all subsequent pulses in the train (Raymond et al., 1990). This decrease in the C-elevation amplitude with frequency could also be explained by a widening of the CAP caused by temporal dispersion resulting from different slowing of the conduction velocity in different classes of C-fibers (Raymond et al., 1990). Indeed, we observed systematic changes in conduction velocity of the C-elevation with increasing frequency of stimulation, particularly in the transition from 1 Hz to 10 Hz. At 100 Hz, interference between the peaks and troughs of C-elevations evoked by successive pulses can also contribute to the decrease in their amplitude, as suggested by the fact that a single pulse given 500 ms after the end of the 100 Hz train had an amplitude similar to the C-elevations evoked at 1 Hz (Fig. 8).

Previous studies concluding that C-fibers do not follow high frequencies sampled only a small number of fibers. For example, Raymond et al. (1990), using single fiber-recording, found that only two out of seven nociceptors fired throughout a 20 s stimulus train at 10 Hz. In an *in vitro* study, Waddell and Lawson (1990) measured a following frequency of 16 $\pm$ 17 Hz in eight C-fibers. This high dispersion in following frequencies suggests that some C-fibers may have followed frequencies of 30 Hz or more. In all, our results indicate that some C-fibers are able to follow stimulation up to 100 Hz. It is likely that these C-fibers contribute most of the substance P released during intense noxious stimulation.

Physiological relevance

Nociceptive fibers likely encode the intensity of noxious stimuli in their firing pattern, not just their firing rate. The fact that high frequency stimulation and TBS were equally effective indicates that a critical factor governing SP release is the interspike interval, i.e. the time between the arrival of successive impulses at the central terminal. In fact, the mean instantaneous frequency may be more important than the mean firing rate in determining the

extent of substance P release. Consistent with this idea, a number of authors have observed a predisposition of C-fibers to fire in bursts of a few action potentials with instantaneous frequencies approaching 100 Hz in response to intense noxious stimulation (Adelson et al., 1996; Puig and Sorkin, 1996; Adelson et al., 1997; Lever et al., 2001). NK1R activation in the spinal cord also increases with the intensity of noxious stimuli (Abbadie et al., 1997; Allen et al., 1997; Honore et al., 1999; Honore et al., 2002). Our results demonstrate the link between these phenomena: increasing the intensity of noxious stimuli causes C-fibers to fire at high frequency or in bursts, which increases substance P release and minimize its degradation by peptidases, leading to greater activation of NK1Rs.

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Abbreviations

aCSF	artificial cerebrospinal fluid
K+-aCSF	artificial cerebrospinal fluid containing 5 mM KCl
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
NA	numerical aperture
NK1R	neurokinin 1 receptor
sucrose-aCSF	artificial cerebrospinal fluid containing 5 mM KCl and 215 mM sucrose

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]
- Adelson DW, Wei JY, Kruger L. H₂O₂ sensitivity of afferent splanchnic C fiber units in vitro. *J Neurophysiol* 1996;76:371–380. [PubMed: 8836231]
- Adelson DW, Wei JY, Kruger L. Warm-sensitive afferent splanchnic C-fiber units in vitro. *J Neurophysiol* 1997;77:2989–3002. [PubMed: 9212251]
- Allen BJ, Li J, Menning PM, Rogers SD, Ghilardi J, Mantyh PW, Simone DA. Primary afferent fibers that contribute to increased substance P receptor internalization in the spinal cord after injury. *J Neurophysiol* 1999;81:1379–1390. [PubMed: 10085363]
- Allen BJ, Rogers SD, Ghilardi JR, Menning PM, Kuskowski MA, Basbaum AI, Simone DA, Mantyh PW. Noxious cutaneous thermal stimuli induce a graded release of endogenous substance P in the spinal cord: imaging peptide action in vivo. *J Neurosci* 1997;17:5921–5927. [PubMed: 9221788]
- Allen JM, Bircham PM, Bloom SR, Edwards AV. Release of neuropeptide Y in response to splanchnic nerve stimulation in the conscious calf. *J Physiol* 1984;357:401–408. [PubMed: 6150993]

- Andrew D, Greenspan JD. Mechanical and heat sensitization of cutaneous nociceptors after peripheral inflammation in the rat. *J Neurophysiol* 1999;82:2649–2656. [PubMed: 10561434]
- Baron SA, Jaffe BM, Gintzler AR. Release of substance P from the enteric nervous system: direct quantitation and characterization. *J Pharmacol Exp Ther* 1983;227:365–368. [PubMed: 6195327]
- Bartfai T, Iverfeldt K, Brodin E, Ogren SO. Functional consequences of coexistence of classical and peptide neurotransmitters. *Prog Brain Res* 1986;68:321–330. [PubMed: 2882558]
- Brezina V, Church PJ, Weiss KR. Temporal pattern dependence of neuronal peptide transmitter release: models and experiments. *J Neurosci* 2000;20:6760–6772. [PubMed: 10995819]
- Brown JL, Liu H, Maggio JE, Vigna SR, Mantyh PW, Basbaum AI. Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. *J Comp Neurol* 1995;356:327–344. [PubMed: 7642798]
- De Biasi S, Rustoni A. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci USA* 1988;85:7820–7824. [PubMed: 2459717]
- De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJ, Laird JM, Belmonte C, Cervero F, Hunt SP. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* 1998;392:394–397. [PubMed: 9537323]
- Diez-Guerra FJ, Sirinathsinghji DJ, Emson PC. In vitro and in vivo release of neurokinin A-like immunoreactivity from rat substantia nigra. *Neuroscience* 1988;27:527–536. [PubMed: 2905788]
- Donoso MV, Brown N, Carrasco C, Cortes V, Fournier A, Huidobro-Toro JP. Stimulation of the sympathetic permesenteric arterial nerves releases neuropeptide Y potentiating the vasomotor activity of noradrenaline: involvement of neuropeptide Y-Y1 receptors. *J Neurochem* 1997;69:1048–1059. [PubMed: 9282927]
- Douglas WW, Ritchie JM. Nonmedullated fibres in the saphenous nerve which signal touch. *J Physiol* 1957;139:385–399. [PubMed: 13492231]
- Go VL, Yaksh TL. Release of substance P from the cat spinal cord. *J Physiol* 1987;391:141–167. [PubMed: 2451003]
- Grady EF, Baluk P, Bohm S, Gamp PD, Wong H, Payan DG, Ansel J, Portbury AL, Furness JB, McDonald DM, Bunnett NW. Characterization of antisera specific to NK1, NK2, and NK3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. *J Neurosci* 1996;16:6975–6986. [PubMed: 8824334]
- Honore P, Kamp EH, Rogers SD, Gebhart GF, Mantyh PW. Activation of lamina I spinal cord neurons that express the substance P receptor in visceral nociception and hyperalgesia. *J Pain* 2002;3:3–11. [PubMed: 14622848]
- 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]
- Ikeda H, Heinke B, Ruscheweyh R, Sandkuhler J. Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. *Science* 2003;299:1237–1240. [PubMed: 12595694]
- Kondo I, Marvizon JC, Song B, Salgado F, Codeluppi S, Hua XY, Yaksh TL. Inhibition by spinal mu- and delta-opioid agonists of afferent-evoked substance P release. *J Neurosci* 2005;25:3651–3660. [PubMed: 15814796]
- Koslow M, Bak A, Li CL. C-Fiber excitability in the cat. *Exp Neurol* 1973;41:745–753. [PubMed: 4759544]
- Laird JM, Olivar T, Roza C, De Felipe C, Hunt SP, Cervero F. Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience* 2000;98:345–352. [PubMed: 10854767]
- Lao L, Marvizon JCG. GABA A receptor facilitation of neurokinin release from primary afferent terminals in the rat spinal cord. *Neuroscience* 2005;130:1013–1027. [PubMed: 15652997]
- 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]
- Lawson SN, Perry MJ, Prabhakar E, McCarthy PW. Primary sensory neurones: Neurofilament, neuropeptides and conduction velocity. *Brain Res Bull* 1993;30:239–243. [PubMed: 7681350]

- Lawson, SN.; Nyberg, F.; Sharma, HS.; Wiesenfeld-Hallin, Z. Progress in Brain Research. Elsevier Science BV; 1995. Neuropeptides in morphologically and functionally identified primary afferent neurons in dorsal root ganglia: substance P, CGRP and somatostatin; p. 161-173.
- Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, Marvizon JC, Malcangio M. Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. *J Neurosci* 2001;21:4469–4477. [PubMed: 11404434]
- Li CL, Bak A. Excitability characteristics of the A- and C-fibers in a peripheral nerve. *Exp Neurol* 1976;50:67–79. [PubMed: 1248547]
- Liu X, Sandkuhler J. Characterization of long-term potentiation of C-fiber-evoked potentials in spinal dorsal horn of adult rat: essential role of NK1 and NK2 receptors. *J Neurophysiol* 1997;78:1973–1982. [PubMed: 9325365]
- Liu XG, Sandkuhler J. Long-term potentiation of C-fiber-evoked potentials in the rat spinal dorsal horn is prevented by spinal N-methyl-D-aspartic acid receptor blockage. *Neurosci Lett* 1995;191:43–46. [PubMed: 7659287]
- Liu XG, Sandkuhler J. Activation of spinal N-methyl-D-aspartate or neurokinin receptors induces long-term potentiation of spinal C-fibre-evoked potentials. *Neuroscience* 1998;86:1209–1216. [PubMed: 9697127]
- MacIver MB, Tanelian DL. Volatile anesthetics excite mammalian nociceptor afferents recorded in vitro. *Anesthesiology* 1990;72:1022–1030. [PubMed: 2350017]
- 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]
- Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 1997;278:275–279. [PubMed: 9323204]
- Marshall GE, Shehab SA, Spike RC, Todd AJ. Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. *Neuroscience* 1996;72:255–263. [PubMed: 8730722]
- 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* 1999;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]
- McCarthy PW, Lawson SN. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* 1989;28:745–753. [PubMed: 2469036]
- Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestley JV. Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J Neurosci* 1997;17:8476–8490. [PubMed: 9334420]
- Motulsky, H.; Christopoulos, A. Fitting models to biological data using linear and nonlinear regression. San Diego, CA: GraphPad Software, Inc; 2003.
- Muschol M, Salzberg BM. Dependence of transient and residual calcium dynamics on action-potential patterning during neuropeptide secretion. *J Neurosci* 2000;20:6773–6780. [PubMed: 10995820]
- Puig S, Sorkin LS. Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. *Pain* 1996;64:345–355. [PubMed: 8740613]
- Randic M, Jiang MC, Cerne R. Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. *J Neurosci* 1993;13:5228–5241. [PubMed: 8254370]

- Raymond SA, Thalhammer JG, Popitz-Bergez F, Strichartz GR. Changes in axonal impulse conduction correlate with sensory modality in primary afferent fibers in the rat. *Brain Res* 1990;526:318–321. [PubMed: 2257488]
- Riley RC, Trafton JA, Chi SI, Basbaum AI. Presynaptic regulation of spinal cord tachykinin signaling via GABA(B) but not GABA(A) receptor activation. *Neuroscience* 2001;103:725–737. [PubMed: 11274791]
- Rusin KI, Bleakman D, Chard PS, Randic M, Miller RJ. Tachykinins potentiate N-methyl-D-aspartate responses in acutely isolated neurons from the dorsal horn. *J Neurochem* 1993;60:952–960. [PubMed: 7679730]
- Sandkuhler J, Chen JG, Cheng G, Randic M. Low-frequency stimulation of afferent Aδ-fibers induces long-term depression at primary afferent synapses with substantia gelatinosa neurons in the rat. *J Neurosci* 1997;17:6483–6491. [PubMed: 9236256]
- Song B, Marvizon 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* 2003;23:9171–9184. [PubMed: 14534251]
- Todd AJ, McGill MM, Shehab SA. Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *Eur J Neurosci* 2000;12:689–700. [PubMed: 10712649]
- Todd AJ, Puskas Z, Spike RC, Hughes C, Watt C, Forrest L. Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. *J Neurosci* 2002;22:4103–4113. [PubMed: 12019329]
- 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, 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]
- Waddell PJ, Lawson SN. Electrophysiological properties of subpopulations of rat dorsal root ganglion neurons in vitro. *Neuroscience* 1990;36:811–822. [PubMed: 2234413]
- Wang X, Marvizon JC. Time-course of the internalization and recycling of neurokinin 1 receptors in rat dorsal horn neurons. *Brain Res* 2002;944:239–247. [PubMed: 12106686]

Fig. 1. Effect of intensity of stimulation

The dorsal root of spinal cord slices was stimulated with 300 pulses of the intensities indicated (0.4 ms duration) delivered at 30 Hz. NK1R internalization was measured in lamina I ipsilateral and contralateral to the stimulated root. Points are the mean $\pm$ SEM of three slices. Two-way repeated measures ANOVA indicated a significant effect ($p < 0.0001$) of the variables “intensity” and “side” (ipsilateral vs. contralateral), as well as their interaction ($p = 0.0004$). Bonferroni’s post-hoc test: ** $p < 0.01$, *** $p < 0.001$, compared with contralateral.

Fig. 2. Confocal images of NK1R neurons after dorsal root stimulation with different intensities
 The dorsal root of spinal cord slices was stimulated with pulses of 0.4 ms and 5–20 V delivered at 30 Hz. Images were taken in the coronal plane, and are from neurons in lamina I (except F, lamina IV) ipsilateral to the stimulated root (except A, contralateral). Dorsal side is up. **A.** Contralateral dorsal horn (stimulus was 1000 pulses of 20 V); there is no NK1R internalization. **B.** Stimulus was 300 pulses of 5 V; there is little NK1R internalization. **C, D.** Stimulus was 300 pulses of 10 V; there is NK1R internalization, but more marked in D than in C. **E.** Stimulus was 300 pulses of 20 V; there is abundant NK1R internalization. **F.** Lamina IV neurons ipsilateral to a root stimulated with 300 pulses of 20 V; there is no NK1R internalization. Image in F was taken with a 63x objective and consist of 7 optical sections separated 0.61 μ m. Other

images were taken with a 100x objective and consist of 3 (A, B) or 4 (C–E) optical sections separated by 0.57 μm . All images were deblurred using deconvolution. Scale bars are 10 μm .

Fig. 3. NK1R internalization evoked by dorsal root stimulation was abolished by a NK1R antagonist, low Ca^{2+} or lidocaine

The dorsal root of spinal cord slices was stimulated with 1000 pulses (0.4 ms, 20 V) at 100 Hz while the slices were superfused with aCSF alone (control), 10 μM L-703,606 (NK1R antagonist), low Ca^{2+} (0.2 mM CaCl_2) or 1 mM lidocaine. Superfusion with L-703,606 started 40 min before stimulation. N is indicated by the numbers next to the bars. Two-way ANOVA showed significant effects ($p < 0.0001$) for the variables “drugs” and “side” (ipsilateral vs. contralateral) and their interaction. Bonferroni’s post-hoc test: *** $p < 0.001$ compared to control ipsilateral; differences between ipsilateral and contralateral were significant ($p < 0.001$) for control, not significant for L-703,606, low Ca^{2+} and lidocaine.

Fig. 4. Effect of stimulation frequency

The dorsal root of spinal cord slices was stimulated with 1000 pulses of 0.4 ms and 20 V delivered at the frequencies indicated. The slices were superfused with aCSF alone (A) or with peptidase inhibitors (10 μ M thiorphan and 10 μ M captopril, B), starting 5 min before and ending 10 after stimulation. NK1R internalization was measured in lamina I ipsilateral and contralateral to the root. Points are the mean $\pm$ SE of 3–5 slices. For each panel, a two-way ANOVA yielded significant effects of the variables “frequency” ($p < 0.0003$) and “side” (ipsilateral vs. contralateral, $p < 0.0001$), as well as their interaction ($p < 0.0001$). Bonferroni’s post-hoc tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the contralateral side.

Fig. 5. NK1R neurons after dorsal root stimulation with different frequencies and number of pulses
 The dorsal root was stimulated with 30, 300 or 1000 pulses (0.4 ms, 20 V) delivered at the indicated frequencies. Images are from lamina I neurons ipsilateral to the stimulated root (coronal plane). Dorsal side is up. **A.** Stimulus was 1000 pulses at 1 Hz; the rightmost neuron in this cluster shows some NK1R internalization. **B.** Stimulus was 1000 pulses at 10 Hz; this neuron shows both NK1R internalization and surface staining. **C.** Stimulus was 30 pulses at 30 Hz; these three neurons do not have NK1R internalization. **D.** Stimulus was 300 pulses at 30 Hz; all the neurons show extensive NK1R internalization and little surface staining. **E.** Stimulus was 1000 pulses at 30 Hz; the two neurons show abundant NK1R internalization and little surface staining. **F.** Stimulus was 300 pulses at 100 Hz; this neuron shows extensive NK1R internalization. Images were taken with a 100x objective and consist of 4 (A, B, D, F) or 5 (C, E) optical sections separated 0.57 μm . All images were deblurred using deconvolution. Scale bars are 10 μm .

Fig. 6. Dependence of NK1R internalization on the number of pulses at different frequencies and stimulation patterns

The dorsal root was stimulated with 10–1000 pulses (0.4 ms, 20 V) delivered using four different stimulation patterns: 1) single train at 1 Hz, 2) single train at 10 Hz, 3) single train at 30 Hz, 4) “theta burst stimulation” (TBS), consisting in multiple trains (bursts) of 4 pulses at 100 Hz, with inter-burst frequency of 5 Hz (every 0.2 sec). NK1R internalization was measured in lamina I of the ipsilateral dorsal horn. In the contralateral dorsal horn, NK1R internalization was low (<15% NK1R neurons with internalization, not shown). Points are the mean $\pm$ SE of 3 slices. Curves represent fitting by non-linear regression of a sigmoidal dose-response function to the data points, which produced the following parameter values: 1) 1 Hz: top = 29 ± 4 %, EC_{50} = 115 pulses (95% CI, 16–800 pulses), 2) 10 Hz: top = 47 ± 6 %, EC_{50} = 171 pulses (95% CI, 39–747 pulses), 3) 30 Hz: top = 78 ± 5 %, EC_{50} = 280 pulses (95% CI, 162–481 pulses), 4) TBS: top = 79 ± 5 %, EC_{50} = 217 pulses (95% CI, 115–411 pulses). The ‘bottom’ parameter was assumed to be equal to the average value obtained contralaterally (6.35%) and kept constant for non-linear regression.

Fig. 7. Determination of thresholds to evoke Aδ- and C-fiber CAPs

CAPs were recorded from the tibial branch of the sciatic nerve of a rat. They were evoked with a stimulating electrode placed 29 mm distal from the recording electrode. Stimuli were applied at 1 Hz. In each panel, the upper traces plot the heart rate during the stimulation and the lower traces the amplitude of electrical stimuli. Insets show examples of CAPs evoked by the stimuli indicated by the arrows and the corresponding roman numerals. **A.** Threshold for the Aδ-fiber CAP. A well-developed Aβ-fiber CAP (truncated) was evoked with the lower stimulation intensity (0.1 V, i). The Aδ-fiber CAP (peak at a conduction velocity of 6.5 m/s) appeared with pulses of 0.4 V and increased with pulses up to 1.0 V. **B.** Threshold for the C-fiber CAP. The C-fiber CAP (conduction velocity centered around 0.86 m/s) appeared with pulses of 3 V and increased with pulses up to 10 V. At 1 Hz stimulation, heart rate increased with pulse intensities higher than 6 V.

Fig. 8. C-fiber CAPs evoked by sciatic nerve stimulation at different frequencies

The tibial branch of the sciatic nerve was stimulated at the indicated frequencies with pulses of a constant intensity of 21 V, 7 times the C-fiber threshold. All traces are from the same rat and were taken a few minutes apart. The latencies of the CAPs are indicated under the arrows linking each pulse with the CAP it evoked. For each series, the upper trace shows the CAP and the lower trace the stimulus monitor output. Traces at 1–50 Hz were recorded by increasing the frequency every 5–7 s. The amplitude of the C-fiber elevation remained stable during stimulation at each rate, but decreased as the frequency was increased. At 40 Hz and higher frequencies, the CAP was recorded after the stimulus artifact of the next pulse. At 100 Hz, successive CAPs interfere with each other. Because of that, a CAP evoked by a single pulse

was recorded 500 ms after the end of the 100 Hz train in order to measure its latency and amplitude.

Fig. 9. NK1R internalization evoked by sciatic nerve stimulation

The tibial branch of the left sciatic nerve of a rat was mounted on recording and stimulating electrodes, and the Aδ- and C-fiber thresholds were determined as shown in Fig. 7. The stimulating electrode was placed distal and the recording electrode proximal to the spinal cord (in “C-fiber*” the electrode position was reversed). One hour after threshold determination, the following stimuli were delivered: “sham”, no stimulation; “Aδ-fiber”, 300 pulses of intensity 1.5 times the Aδ-fiber threshold (but sub-threshold for C-fibers); “C-fiber” and “C-fiber*”, 300 pulses of intensity 1.5 times the C-fiber threshold. Pulse duration was 0.4 ms, and the frequency was 30 Hz. The rat was fixed 10 min afterwards, and the L3-L5 spinal segment was processed for NK1R immunohistochemistry. NK1R internalization was measured in lamina I. Data are the mean $\pm$ SEM. The number of rats (N) is given inside the bars. Two-way

ANOVA revealed overall significance ($p < 0.0001$) for the variables “pulse intensity” and “side”. Bonferroni’s post-hoc test: *** $p < 0.001$ compared with “sham” ipsilateral, ††† $p < 0.001$ (as indicated).

Fig. 10. NK1R neurons after sciatic nerve stimulation

Images from coronal sections of the lumbar spinal cord (L3-L5) showing NK1R neurons in lamina I after sciatic nerve stimulation. **A.** Sham (electrodes placed on the nerve, but no current passed) produced no NK1R internalization. **B.** Stimulation at 1.5x A δ -fiber threshold but sub-threshold for C-fibers (300 pulses at 30 Hz) produced no NK1R internalization. **C.** Stimulation at 1.5x C-fiber threshold (300 pulses at 30 Hz) produced NK1R internalization. **D.** Dorsal horn contralateral to the sciatic nerve stimulated at C-fiber intensity; no NK1R internalization is observed. Main panels images were taken with a 20x objective (scale bar is 50 μ m), and consist of three optical sections separated 1.22–1.91 μ m. Inset images were taken with a 100x objective (scale bars are 10 μ m) and consist of 6 (A), 5 (B, C) or 4 (D) optical sections separated 0.61 μ m.

Table 1
Thresholds and conduction speeds for A δ - and C-fibers in the rat sciatic nerve

Exp.	A δ TH, V	C TH, V	distance, mm	A δ latency, ms	A δ speed, m/s	C latency, ms	C speed, m/s
Fig. 7	0.30	3.0	36	3.1	12	29	0.8
Fig. 8	0.35	3.0	29	ND	ND	25	0.9
Fig. 9 A δ -fiber	0.25	2.2	ND	7.6	ND	34	ND
Fig. 9 A δ -fiber	0.30	2.0	43	2.2	20	35	0.8
Fig. 9 A δ -fiber	0.50	3.2	28	1.6	18	24	0.9
Fig. 9 A δ -fiber	0.35	2.0	ND	2.1	ND	16	ND
Fig. 9 C-fiber	0.80	4.5	45	2.5	18	39	0.9
Fig. 9 C-fiber	0.40	3.0	39	2.5	16	34	0.9
Fig. 9 C-fiber	0.40	2.1	38	2.8	14	29	0.8
Fig. 9 C-fiber	0.20	4.5	ND	2.8	ND	30	ND
Mean	0.38	3.0	37	3.0	16	29.4	0.83
SD	0.17	0.9	6	1.8	3	6.7	0.04

The tibial branch of the sciatic nerve of a rat was mounted on recording and stimulating electrodes, and the A δ - and C-fiber thresholds ("TH") were determined as shown in Fig. 7. The conduction speed ("speed") of each fiber group was determined by dividing the separation between the stimulating and the recording electrodes ("distance") by the latency from the stimulus to the A or C peak ("latency"). The leftmost column indicates the experiments ("Exp.") where the preparation was used and whether the sciatic nerve was stimulated at A δ -fiber or C-fiber intensity to evoke NKIR internalization in the experiment in Fig. 9. "SD": standard deviation. "ND": not determined.