NK(1) receptor activation leads to enhancement of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse

NK_1 receptor activation leads to enhancement of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse

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Abstract

Substance P (SP) is a well-established pain messenger in the spinal cord, although its role in substantia gelatinosa (lamina II) still remains elusive. We carried out patch-clamp recordings on lamina II neurons from transverse mouse spinal cord slices (P8–12), using the selective NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]-SP (SM-SP, 3–5 μM) in the presence of NBQX. Activation of NK_1 receptors was confirmed after pre- incubation with selective NK_1 antagonist L732,138 (4 μM) that consistently blocked the effects of SM-SP (nine neurons). After SM-SP challenge and spontaneous inhibitory post-synaptic current (sIPSC) analysis, 50% of recorded neurons (15 out of 30) were found to display a transient increase in frequency; in five neurons this was also associated with increase of peak amplitude. Five out of eight neurons displayed pure $GABA_A$ receptor-mediated sIPSCs, whereas the remaining ones showed mixed GABAergic/glycinergic events. After miniature IPSC analysis, a significant increase in frequency was observed in three out of 14 SM-SP responsive neurons. At least four different morphological types were apparent among NK_1-responsive neurons after filling with Lucifer Yellow/biocytin: fusiform with dorso-ventral dendritic arbors (i); round-to-oval with dendritic arborization mainly directed to lamina I (ii) or III (iii), and round-to-oval with dendrites sparsely distributed all around the cell body (iv). Thus, there was no correlation between morphology and electrophysiological properties of responsive neurons. Our observations provide new insights on the processing of sensory neurotransmission in spinal cord, and indicate that activation of NK_1 receptors is involved in the maintenance of the inhibitory tone of substantia gelatinosa interneurons.

Keywords: NK_1 receptors; substantia gelatinosa neurons; spontaneous inhibitory post-synaptic current; substance P

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1. Introduction

Several lines of evidence have implicated substance P (SP) as a major neurotransmitter of pain (see Afrah et al., 2001; Basbaum, 1999; Mantyh, 2002; Saria, 1999; Urban and Gebhart, 1999; Woolf et al., 1998). SP is synthesized in nociceptors of the dorsal root ganglia which send their axons (C and Ad fibers) to dorsal horn projection neurons in laminae I and IV–V and to nocispecific interneurons in laminae II–III (see Millan, 1999), and is released in vivo upon activation of C and Ad fibers (Afrah et al., 2001; Duggan and Furmidge, 1994; Hua et al., 1986; Teoh et al., 1996). Administration of morphine or opioid peptides specifically blocks this release (Jessell and Iversen, 1977; Takano et al., 1993; Yaksh et al.,

1980), while in hyperalgesic conditions release of SP is increased (Coderre and Yashpal, 1994; Garry and Hargreaves, 1992; Meller and Gebhart, 1994; Oku et al., 1987a,b). SP evokes slow excitatory post-synaptic potentials (EPSPs) in second-order sensory neurons in the dorsal horn (De Koninck and Henry, 1991; De Koninck et al., 1992; Otsuka and Yoshioka, 1993), and mediates central sensitization (Xu et al., 1992), leading to hyperalgesia and allodynia (McMahon et al., 1993; Woolf and Costigan, 1999; Yaksh et al., 1999). These altered conditions of pain sensitivity are attenuated by blocking of the SP preferred receptor NK_1 (Honor et al., 1999; Mantyh et al., 1997; Nichols et al., 1999).

Despite the wealth of data, mechanisms of SP action remain elusive in the substantia gelatinosa (SG—lamina II), which is traditionally considered to play a pivotal role in modulating nociceptive transmission (Ribeiro-Da-Silva, 2003; Willis and Coggeshall, 1991; Yoshimura, 1996). Different studies have concluded that SP was responsible for the generation of slow EPSPs elicited by C and Ad fiber stimulation in the dorsal horn (De Koninck and Henry, 1991; Otsuka and Yoshioka, 1993; Yoshimura and Jessell, 1989, 1990; Yoshimura and Nishi, 1993; Yoshimura et al., 1993). However, administration of SP or synthetic agonists of the SP NK_1 receptor evoked an excitatory response in a very limited subset of SG neurons in other studies (Bleazard et al., 1994; Yoshimura et al., 1993), and it was thus proposed that the peptide does not play a role in lamina II. This lack of effect has been considered resulting from the low density of NK_1 receptor-immunoreactive neurons in the SG (Ribeiro-Da-Silva et al., 2001) but this explanation is weakened by the observation that SP activates more than a single intracellular pathway: it displays maximal affinity for its preferred NK_1 receptor (Ikeda et al., 2003), but also binds other NK receptors (Severini et al., 2002), and modulates N-methyl-D-aspartate (NMDA) neurotransmission (Budai and Larson, 1996; Chizh et al., 1995; Cumberbatch et al., 1995; Rusin et al., 1993).

To further analyze the effects of SP in SG, we made electrophysiological recordings from mouse spinal cord slices. We found that an NK_1 receptor-specific agonist enhances inhibitory neurotransmission in lamina II neurons.

2. **Methods**

2.1. Animals

Studies were performed on 35 neonatal mice (8–12 days old). All experimental procedures were approved by the Committee of Bioethics and Animal Welfare of the University of Torino. After deep sodium penthobarbital anesthesia (30 mg/kg), animals were decapitated, a laminectomy was performed and the spinal cord quickly removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1 $NaHPO_4$, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, saturated with 95% O_2:5% CO_2. Transverse vibratome slices 350 mm thick were kept in ACSF at 35 8C for 30 min. Slices were subsequently maintained at room temperature until used for the experiments.

2.2. Electrophysiological recordings

All experiments were performed at room temperature by placing a slice in a recording chamber constantly perfused at 2 ml/min with oxygenated ACSF. Neurons were visually identified using a fixed-stage microscope (Axioskop 1, Zeiss, Germany), equipped with infrared gradient contrast optics (Luigs and Neumann, Germany) and a 63x insulated water immersion objective (Achroplan, Zeiss). Patch-clamp whole cell recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, USA). Patch pipettes were pulled from borosilicate glass tubing (WPI, USA) and had a resistance of 4–7 MU when filled with the intracellular solution. To study spontaneous excitatory post-synaptic currents (sEPSCs), pipettes were filled with an intracellular solution containing (in mM): 145 Kgluconate, 5 EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATPNa, and 0.2 GTPNa, pH 7.2 (with KOH).

To study spontaneous inhibitory post-synaptic currents (sIPSCs), the intracellular solution contained (in

mM): 145 KCl, 5 EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATPNa, and 0.2 GTPNa, pH 7.2 (with KOH). Under these conditions sIPSCs were detected as inward currents. In some cases, 0.5% biocytin (Sigma, USA) and 0.1% Lucifer Yellow (LY, Sigma, USA) were added.

Lamina II neurons were patched under visual control, and only cells with membrane potential more negative than K55 mV were considered for the recordings. Currents were sampled at 10 kHz, filtered at 2 kHz, and analyzed with Minianalysis (Synaptosoft, USA) and pClamp 8 (Axon Instruments, USA) software. On an average, 30–80 IPSCs were analyzed from each cell in order to obtain the mean kinetic and amplitude parameters. From the average of these events, we measured the rise time, calculated from 10 to 90% peak amplitude, and the values of decay time constants (expressed as t_1 and t_2) by fitting the 10–90% region of the decay phase with a biexponential function. All electrophysiological values were expressed as mean GSEM with n, indicating the number of cells.

2.3. Drugs

All drugs were bath applied. [Sar^9,$Met(O_2)^{11}$]-substance P (SM-SP), an analog of SP that shows greater selectivity for NK_1 receptors and greater potency and duration of action than SP in vivo (Regoli et al., 1988; Sakurada et al., 1994; Tousignant et al., 1989) was used to study the effects of NK_1 activation in slices. SM-SP was delivered continuously during the recording in all experiments. N-Acetyl-L-tryptophan-3,5-bistrifluoromethyl benzyl ester (L732,138), a potent selective antagonist of the NK_1 receptor (Cascieri et al., 1994) was also used in certain experiments to block the effects of SM-SP and thus assess the specificity of NK_1 receptor activation. 2,3-Dioxo- 6-nitro1,2,3,4tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX), SM-SP and L732,138 were from Tocris (UK) and tetrodotoxin (TTX), bicuculline methiodide, and strychnine methiodide from Sigma (USA).

2.4. Histological procedures

After recording, slices were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 2.5% glutaraldehyde overnight at 4 8C, and subsequently rinsed several times in phosphate-buffered saline (PBS, pH 7.4). Slices were then mounted in a fluorescence-free medium or processed further for the visualization of intracellular biocytin by the ABC method (Vector, USA). Briefly, slices were incubated in PBS containing 10% methanol and 3% hydrogen peroxide for 30 min to suppress endogenous peroxidase activity, thoroughly rinsed in PBS, and then incubated in ABC for 2.5 h at room temperature. After extensive washing in PBS, the biocytin-bound peroxidase was revealed using nickel ammonium sulphate-intensified 3,3′ diaminobenzidine (DAB) for 60 min at room temperature (0.025% DABC1% nickel ammonium sulfateC0.04% H_2O_2 in PBS).

Labeled neurons were observed with a light microscope (Axiophot 1, Zeiss) under bright field or fluorescence illumination and photographed with a high-resolution digital camera (Coolpix 995, Nikon, Japan). Digital images were further processed using Adobe Photoshop 5 (USA).

The immunocytochemical visualization of NK_1 receptor distribution within the dorsal horn was performed on free- floating neonatal mouse spinal cord sections incubated in PBS containing 10% methanol and 3% hydrogen peroxide for 30 min to suppress endogenous peroxidase activity, and then blocked in 0.02 M PBS containing 6% normal goat serum (NGS) for 30 min at room temperature, before treatment with an affinity-purified polyclonal rabbit anti- NK_1 receptor antibody (Sigma, USA; Mantyh et al., 1995), diluted 1:30,000 in PBS containing 1% NGS. After washing in PBS, sections were incubated in 1:250 biotinylated anti-rabbit IgG (Vector, USA) and then in 1:100 avidin– biotinylated-peroxidase complex (Vector, USA). The peroxidase reaction was developed using 0.025% (w/v) DAB (Sigma, USA) and 0.04% (v/v) hydrogen peroxide. Sections were then transferred onto glass slides and mounted in PBS–glycerol.

3. **Results**

3.1. Electrophysiology

Seventy one neurons in lamina II were voltage-clamped at a holding potential (V_h) of K63 mV and challenged with SM-SP (3–5 μM). Since both sEPSCs and sIPSCs were evident after SM-SP superfusion in the absence of specific receptor antagonists, we employed different intracellular solutions to better discriminate between the two types of current.

Using the low Cl^- intracellular solution, bath-application of SM-SP induced a transient increase in both amplitude and frequency of sEPSCs (from 1.5 to 5 Hz) in two out of 18 cells recorded (Fig. 1).

sIPSCs were recorded using the high Cl^- intracellular solution in the presence of 10 μM NBQX to block AMPA/kainate neurotransmission (illustrative traces are shown in Fig. 2A–C).

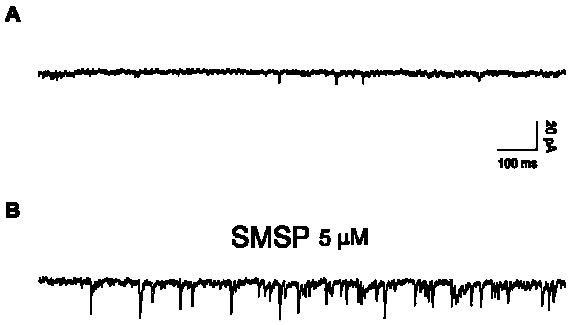

Fig. 1. Representative traces of sEPSCs recorded from a SG neuron under effects of SM-SP. Whole cell recordings of sEPSCs from a SG neuron in control (A), and under bath-applied SM-SP (B). Upon SM-SP perfusion this cell displayed an increase in both frequency and amplitude of sEPSCs. The holding potential (V_h) was K63 mV.

In 15 out of 30 neurons recorded (Fig. 2F), activation of NK_1 receptors elicited a strong increase in sIPSC frequency (from 0.6±0.2 to 1.6±0.3 Hz, P!0.05). However, this was not observed over the entire SM-SP application: increase in the sIPSC frequency lasted only for about 60–120 s, as shown in Fig. 2A. This transitory effect was likely due to internalization of NK_1. Studies in vivo (Mantyh et al., 1995) have indeed shown that NK_1 receptors are internalized after noxious stimulation, and that, as internalization proceeds, there are parallel changes in pain-related behaviors (Mantyh, 2002).

Rise and decay time constants of sIPSCs were not affected by application of SM-SP. The mean rise time for the control was 1.1±0.3 and 1.2±0.4 ms in the presence of SM-SP (n=15, P>0.05). The mean decay values were: t_1=6.6±1.1ms, t_2=40±5ms for the control and t_1=5.9±1.2ms, t_2=42±5ms in the presence of SM-SP (n=15, P>0.05). Average events calculated from one neuron recorded under control conditions and in the presence of SM-SP are shown in D and E, respectively.

In five neurons, the presence of SM-SP also led to an increase in sIPSC peak amplitude. In these cells, the mean amplitude values were 37.4±1.4 pA in controls and 52.8±1.7 pA in the presence of SM-SP (P<0.05, Fig. 2G). This change was unlikely to arise via a post-synaptic effect, as SM-SP still increased the frequency in the presence of TTX, without affecting the amplitude (see below).

To document that sIPSCs were indeed generated by specific NK_1 receptor signaling, slices were superfused with 4 μM of the specific NK_1 receptor antagonist L732,138 for at least 8 min before SM-SP challenge (Kombian, 2003a,b; Yang et al., 2000). Nine cells were successfully recorded and their outputs statistically analyzed (Fig. 3). In these cells, the frequency of sIPSCs passed from 0.26±0.04 Hz upon L732,138 superfusion to 0.29±0.06 Hz following SM-SP (5 μM; P>0.05), and amplitude from 41.08±6.77 to 41.31±5.48 pA (P>0.05). This lack of statistical significance demonstrates that the effects of SM-SP on inhibitory neurotransmission are specifically due to NK_1 receptor activation; it

also rules out concerns about

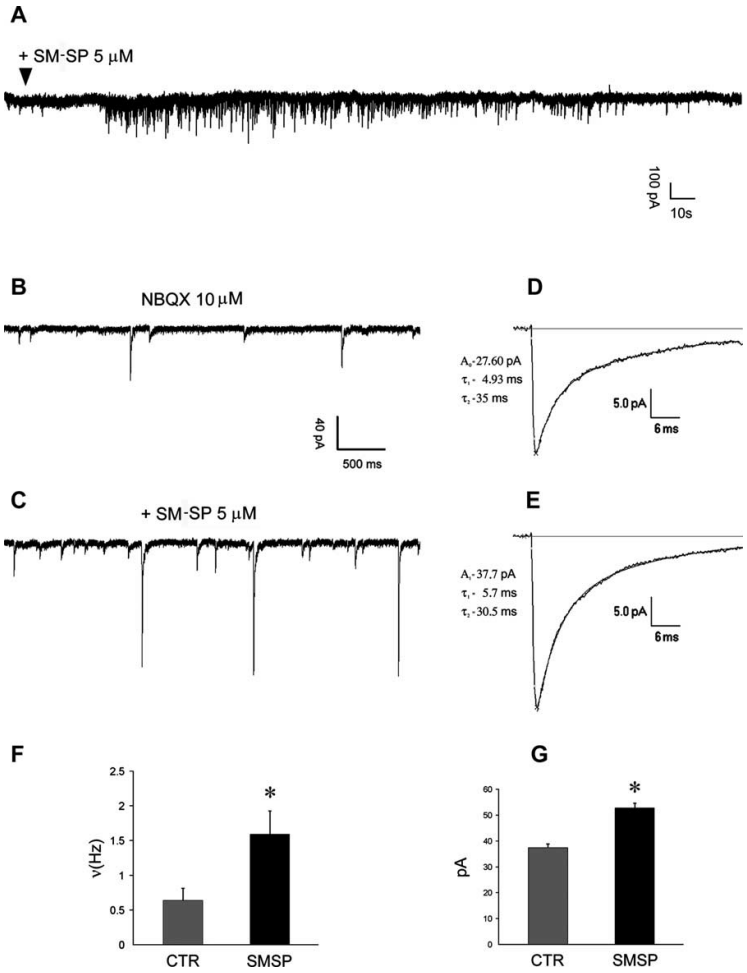

Fig. 2. Effects of SM-SP on sIPSCs recorded from SG neurons. (A–C) Whole cell recordings of sIPSCs. (A) In the presence of 10 μM NBQX, SM-SP (5 μM) evoked a transient increase in frequency and amplitude of sIPSCs which returned to control values after about 100 s still in the presence of the agonist. The arrowhead indicates the start of SM-SP application. V_hZK63 mV. (B and C) Effect of SM-SP on the frequency and amplitude of sIPSCs recorded from another SG neuron (B, control; C, SM-SP). (D and E) Averages of sIPSCs detected in control (D) and in presence of SM-SP (E); same neuron as in B and C. Note that superfusion with SM-SP elicited an increase in both amplitude and frequency without affecting sIPSC kinetics. (F) Pooled data from 15 lamina II neurons reveal a significant increase in sIPSC frequency upon SM-SP superfusion. The mean frequency passed from 0.6±0.2 Hz in control to 1.6±0.3 Hz in the presence of the agonist (n=15, P<0.05). (G) In five neurons, the increase in sIPSC frequency was accompanied by a significant increase in amplitude (from 37.4±1.4 pA in control to 52.8±1.7 pA under SM-SP) (n=Z5, P<0.05).

selectivity of the agonist at the relatively high concentration employed here in comparison with most electrophysiological studies (1–4 μM) on rat brain/spinal cord slices or isolated neurons (King et al., 1997; Kombian, 2003a, b; Li and Zhao, 1998). Nonetheless it should be noted that up to 20 μM, SM-SP has been used to study substance P-mediated excitation and expression of the transcription factor FOS in rat dorsal horn neurons in vitro without any noticeable loss of agonist specificity (Badie-Mahdavi et al., 2001). The possible existence of interspecies differences in NK receptor agonist affinities should also be considered (Nsa Allogho et al., 1997).

In order to further study the sIPSCs, 10 μM bicuculline and/or 1 μM strychnine was added to the bath solution at the end of each experiment performed on eight SM-SP responsive neurons (Fig. 4). In five of these cells, sIPSCs were completely blocked by bicuculline, revealing that they were mediated by

$GABA_A$ receptor activation only (Fig. 4B). The remaining cells (3/8) displayed mixed GABAergic and glycinergic events, although the latter were very rare (Fig. 4D). This was not surprising since: (i) co-existence of GABA/glycine has been widely reported in the superficial dorsal horn (Keller et al., 2001; Maxwell et al., 1995; Todd et al., 1996); and (ii) functionally active

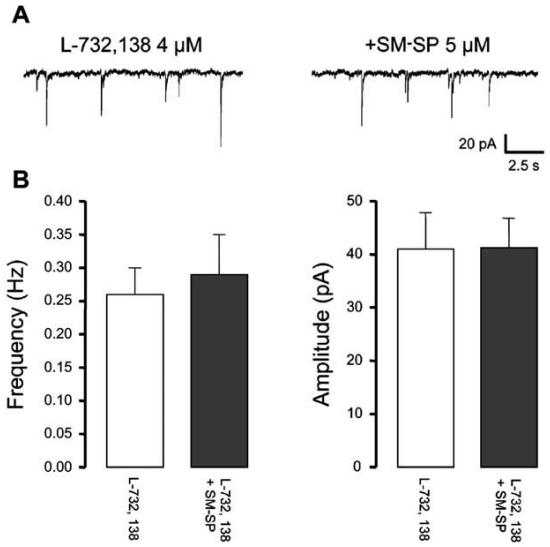

Fig. 3. SM-SP effects on sIPSCs are blocked by the selective NK_1 antagonist L732,138. (A) sIPSCs recorded from a lamina II neuron in the presence of 4 μM L732,138 (left) and 4 μM L732,138C5 μM SM-SP (right). V_hZK63 mV. When slices were pre-treated with the selective NK_1 receptor antagonist, SM-SP no longer elicited a significant increase in sIPSC frequency and amplitude. (B) Pooled data from nine lamina II neurons. The frequency of sIPSCs was 0.26±0.04 Hz upon L732,138 superfusion and 0.29±0.06 Hz during SM-SP administration ($P>0.05$), and mean amplitude passed from 41.08±6.77 to 41.31±5.48 pA ($P>0.05$).

GABA/glycine co-synapses exist in the dorsal horn, at least in certain stages of development (Jonas et al., 1998; Keller et al., 2001).

To better characterize the nature of NK_1 activation in lamina II neurons, we recorded miniature IPSCs (mIPSCs) in the presence of 1 μM TTX. The mean mIPSC frequency was 0.2±0.1 Hz (n=14), and a clear effect of SM-SP was only observed in three out of 14 neurons recorded (21.4%). In these cells, a significant increase (150±20%) in mIPSC frequency was detected, compared to the control (Fig. 5). Moreover one of these three neurons responded to SM-SP application by a slow inward current that peaked at 30 pA and was associated with an increase in amplitude (from 18±7 to 34±12 pA).

3.2. Morphological characterization of recorded SG neurons

Fourteen of the recorded neurons, were successfully injected with LY or biocytin to reveal their morphology. All labeled neurons were located in the SG (Fig. 6A–C), and exhibited different dendritic arrangements and orientations. On this basis, we observed several different morphological types: (i) fusiform neurons with two funnel-shaped dorso-ventrally oriented dendritic arbors; (ii) rounded neurons with a funnel-shaped dendritic arbor originating from a main dendritic trunk primarily directed to lamina I;

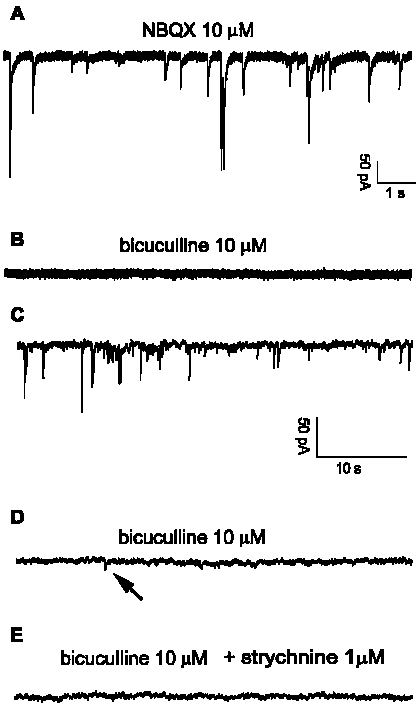

Fig. 4. sIPSCs of SM-SP-responsive neurons are mainly mediated by $GABA_A$ receptor. (A and B) Recordings from a lamina II neuron in the presence of 10 µM NBQX and SM-SP challenge. Block of $GABA_A$ receptors with 10 µM bicuculline (B) totally suppressed activity, V_h= - 63 mV. (C–E) Recordings from another lamina II neuron in the presence of 10 µM NBQX and SM-SP challenge. The block of $GABA_A$ receptors with 10 µM bicuculline (D) did not completely suppress inhibitory activity. V_h= - 63 mV. Note a single glycinergic event of small amplitude (arrow) in about 30 s. The frequency of glycinergic events in this cell was 0.01 Hz. (E) Co-application of bicuculline and strychnine completely suppressed sIPSCs.

(iii) rounded neurons with a funnel-shaped dendritic arbor originating from a main dendritic trunk primarily directed to lamina III; and (iv) oval-to-rounded neurons with an overall spherical distribution of the dendritic arborization that originated from several independent dendrites. The fusiform neurons (three in all) were located in lamina II_O (Fig. 6A). Only one of them was SM-SP-responsive. All had a long dorso-ventrally oriented axis with primary beaded dendrites arising from the two opposite poles of the cell body (Fig. 6A and B). Dendritic branches were mainly restricted to lamina II, and displayed sparse spines. Of the seven rounded neurons, four were located in lamina II_O, and three in II_i (Fig. 6C). Three rounded neurons

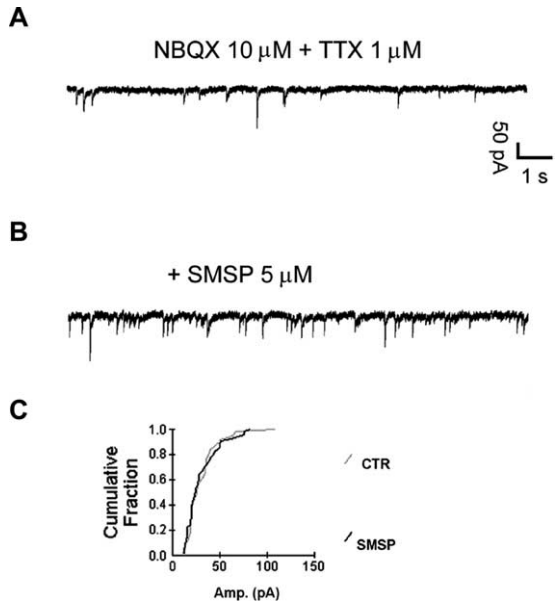

Fig. 5. mIPSCs recorded from SG neurons. (A) mIPSCs recorded from a lamina II neuron in the presence of 1 μM TTX. (B) Activation of NK_1 receptors induced an increase in mIPSC frequency; $V_{h=}$ - 63 mV. (C) Cumulative amplitude plot of mIPSCs recorded from a lamina II neuron in control (gray line) and in the presence of 5 μM SM-SP (black line). Note that the mean peak amplitude was not significantly affected by perfusion of the NK_1 agonist.

were SM-SP-responsive. Irrespective of their sublaminar location these cells had two or more primary beaded dendrites projecting to lamina I (Fig. 6D) or lamina III (not shown). The dendrites had no spines, and had a rather thin constant diameter along their whole length (Fig. 6D). The axon, when visible, projected to lamina III.

Of the four oval-to-rounded neurons, three were located in lamina II_o, and one in lamina II_i. Two oval-to-rounded neurons were SM-SP-responsive. Their spherical dendritic tree was confined within the SG, and the axon, when filled by the tracer, was seen to project ventrally for a short distance.

3.3. Distribution of NK_1 receptor immunoreactivity in the dorsal horn

Labeling of mouse spinal cord sections with the anti-NK_1 receptor antibody (Fig. 6E and F) resulted in intense staining within the superficial dorsal horn, particularly in lamina I, where a dense meshwork of neuronal processes, mainly oriented on a transversal plane, was detected at all levels of the cord. The superficial dorsal horn also contained numerous immunoreactive fusiform cell bodies with bipolar dendritic arborization.

Staining in lamina II was far less prominent, revealing a looser network of processes, mainly oriented on a longitudinal plane and more densely packed in lamina II_o. Some NK_1-immunoreactive cell bodies were also detected in this lamina; they were oval-to-fusiform, and gave rise to positive dorso-ventrally oriented dendrites.

In deeper laminae, immunostaining was limited to scattered processes and isolated neurons of larger size. These cells usually displayed an irregularly polygonal perykarion, with few long dendrites traveling across to more superficial or ventral laminae of the dorsal horn.

4. **Discussion**

4.1. Effects of SM-SP administration

Whereas anatomical, functional, and recombinant DNA studies have shown that SP is a fundamental messenger of certain types of pain conveyed to supraspinal centers by lamina I and III–V projection neurons (Basbaum, 1999; De Felipe et al., 1998;Honor et al., 1999;Ikeda et al., 2003; Mantyh, 2002; Mantyh et al., 1997; McLeod et al., 1999; Millan, 1999; Nichols et al., 1999; Todd, 2002; Todd et al., 2002; Woolf et al., 1998), the role of SP in lamina II has remained unclear. In this study, about 50% of recorded neurons in mouse SG responded to NK_1 receptor stimulation with a SP synthetic analog by showing a transient increase in inhibitory spontaneous activity. Such an effect was mainly due to $GABA_A$ receptor activation. Nonetheless, in neurons displaying mixed GABAergic/glycinergic activity, activation of NK_1 receptors was likely to enhance the release of glycine also, but because of the very low frequency of isolated glycinergic events we did not pursue this issue.

Although an inhibitory role of SP might be surprising in light of its well-known role as a positive pain modulator, numerous reports in an array of different experimental contexts and species support our present findings. When the release of amino acids evoked by SP in neonatal rats was examined in the isolated spinal cord (Sakuma et al., 1991) or in a hemisected spinal cord preparation (Maehara et al., 1995), it was demonstrated that 10 μM SP evoked a significant increase in the basal release of GABA and glycine. Moreover in cats, SP is released from primary nociceptive afferent terminals, and activates second-order GABAergic interneurons in the dorsal horn (Wei and Zhao, 1996). Similarly, SP facilitated the strychnine-sensitive glycine response in neurons acutely dissociated from the rat sacral dorsal commissural nucleus, leading to conclude that SP may suppress nociception in the spinal cord (Wang et al., 1999). Finally, in CNS areas other than the spinal cord, SP was also shown to stimulate GABA-mediated inhibitory synaptic transmission in vitro (Kombian, 2003a, b; Kouznetsova and Nistri, 1998; Maubach et al., 2001; Ogier and Raggenbass, 2003; Stacey et al., 2002). Taken together, these studies demonstrate that release of GABA/ glycine by SP is relevant in vivo, and our observations show that SG is a further CNS site in which such a release is also relevant.

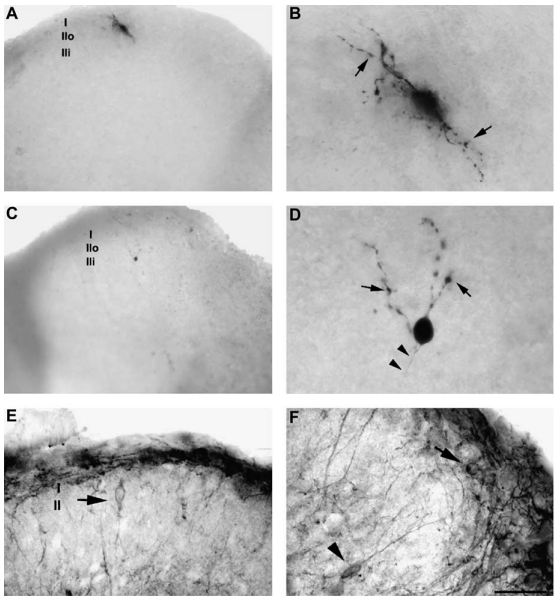

Fig. 6. Morphology of SG neurons recorded, and immunocytochemical distribution of the NK_1 receptor. (A–D) Biocytin-injected neurons in lamina II of transverse spinal cord slices. A SM-SP-responsive neuron in lamina II_o (A) is shown at higher magnification in (B). This neuron, fusiform in shape, had a long axis extended dorso-ventrally with primary dendrites arising from the two poles of the cell body. Arrows point to beaded dendrites. An SM-SP-responsive neuron in lamina II_i (B) is shown at higher magnification in (C). This neuron, round in shape, had a dendritic arborization (arrows) mainly directed to lamina I. An axon process, emerging from the soma, projected to lamina III (arrowheads). (E and F) Distribution of NK_1 receptor immunoreactivity. A dense meshwork of immunoreactive neuronal processes is spread throughout in lamina I. An NK_1-positive fusiform neuron in

lamina II displays an immunoreactive dendrite directed to deeper laminae (E, arrow). A fusiform lamina III neuron, with a long dendrite projecting to lamina II, is intensely labeled for NK_1 (F, arrowhead). Note that lamina I neurons (F, arrows) and processes are also NK_1-immunoreactive. Scale bars: A, C=200 μm; B, D=50 μm; E, F=40 μm.

4.2. Morphological characterization of SM-SP responsive neurons in SG

Numerous studies have examined the morphology of neurons in the SG of the spinal cord (Beal and Bice, 1994; Beal et al., 1988; Bennett et al., 1980; Bicknell and Beal, 1984; Gobel, 1975, 1978; Gobel et al., 1980; Todd and Lewis, 1986). Several different types and differences have been reported among species (see Ribeiro-Da-Silva, 2003). The two most common neuronal types described by Cajal (1952), i.e. the central cells, which are widely distributed throughout the lamina, and the marginal cells, which are concentrated at the border with lamina I, have been referred to, respectively, as islet and stalked cells in cats (Gobel, 1975) and rats (Todd and Lewis, 1986). Of the SM-SP- responsive neurons in this study, three could be classified as stalked cells, two as islet cells, and one did not belong to any of these categories. This lack of structure-to-function correlation may be due to the fact that maturation of lamina II neuron dendrites occurs after birth, with extensive rearrangement until the adult pattern is attained (Bicknell and Beal, 1984; Falls and Gobel, 1979). However, in keeping with our findings, numerous other studies have failed to show a correlation between morphology and electrophysiological properties of SG neurons (see Ribeiro-Da-Silva, 2003).

4.3. Circuitry involved in the response of SG neurons to SM-SP

SP, neurokinin A (NKA) and neurokinin B (NKB) belong to a family of peptides, the tachykinins, all implicated in nociception, and they, respectively, act upon preferred receptors, namely NK_1, NK_2 and NK_3, and cross-talk between different tachykinins at different receptors is known to occur (Regoli et al., 1987, 1994; Saria, 1999; Severini et al., 2002). Existence of NK_1 receptor subtypes has been proposed: two types of NK_1 receptors were originally described with preference for either septide or Sar9/-SP-sulphone (Fox et al., 1996). More recently, a septide-sensitive and a 'classical' conformation of NK_1 receptor have been proposed to explain different tachykinin affinities and signal transduction pathways (Maggi and Schwartz, 1997). Since SM-SP selectively targets the NK_1 receptor in its classical conformation, a proportion of SP-sensitive SG neurons might have been missed in this study. Nonetheless this does not invalidate, but rather reinforces, the functional significance of our findings in vivo where it seems possible that SP's effect on inhibitory neurotransmission is greater than in our experimental conditions.

As to the circuitry involved in the response of SG neurons to SM-SP, one can hypothesize that: (i) responsive cells receive a direct synaptic input from GABAergic (and/or glycinergic) neurons expressing NK_1 receptors at their membrane; and/or (ii) a polysynaptic chain is activated consisting (in its simplest configuration) of a first synapse between a pre-synaptic excitatory NK_1-positive element and a post-synaptic GABAergic neuron, that, in turn acts at a second synapse upon the SG-responsive cell.

One is also led to ask what is (are) the neuronal domain(s) in which pre-synaptic NK_1 receptors are located. Our results show that they are mainly (but not exclusively) located at cell soma and/or dendrites, since blockade of Na^+-dependent action potentials with TTX was largely effective in blocking the effects of SM-SP (11 out of 14 neurons). This observation is in agreement with localization studies, which failed to show NK_1 receptor immunoreactivity in axons (McLeod et al., 1998; Naim et al., 1997; Ribeiro-Da-Silva et al., 2001, and this study). However, the increase in the frequency of mIPSCs in a few neurons indicates that pre-synaptic NK_1 receptors could also be expressed in the axonal domain.

Co-expression of NK_1 and GABA has been reported in brain areas other than the spinal cord (Echevarria et al., 1997). However, in rat, most spinal neurons which possessed NK_1 receptor-immunoreactivity were not GABA- or glycine-immunoreactive (Littlewood et al., 1995). Nonetheless,

these authors described a few GABA (and glycine)-immunoreactive neurons in lamina III and, to a lesser extent, lamina IV (see Fig. 4 in Littlewood et al., 1995). NK_1/GABA-positive neurons of the deep dorsal horn have dorsally oriented dendrites branching in laminae I–II, where they receive an input from tachykinin-releasing primary afferent fibers (PAFs – Brown et al., 1995; Littlewood et al., 1995; Liu et al., 1994; Naim et al., 1997). It is therefore conceivable that mouse laminae III–IV neurons expressing NK_1 receptors at dendrites are capable of dendritic release of GABA (and/or glycine) upon SM-SP challenge. Anatomically, dendritic spines filled with GABA-immunoreactive vesicles have been observed to contact other vesicle-containing dendrites (Carlton and Hayes, 1991; Powell and Todd, 1992). Functionally, dendritic release of GABA has not been described in the spinal cord, but is relevant in other CNS areas such as the olfactory bulb (Halabisky and Strowbridge, 2003; Isaacson and Vitten, 2003).
Putative dendro-dendritic synapses between NK_1 expressing GABAergic (and glycinergic) neurons and SM-SP-responsive post-synaptic targets may be an important means to regulate the activity of the SG neurons expressing $GABA_A$ (and $glycine_A$) receptors. In addition, considering that PAFs release a mixture of SP and NKA, and that selectivity of the NK receptor subtypes is not absolute for naive peptides, one cannot exclude that enhancement of inhibitory neurotransmission in SG could also be due in vivo to NKA release (Li and Zhuo, 2001; Trafton et al., 2001). It is also possible that in the intact spinal cord SP activates other NK receptor subtypes besides NK_1, considering that both NK_2 and NK_3 receptors are expressed in the dorsal horn, albeit at lower levels than NK_1 (Ding et al., 2002; Fleetwoodwalker et al., 1990; Fox et al., 1996; Ribeiro-Da-Silva et al., 2001).

4.4. Functional implications

Inhibitory interneurons in SG regulate the flow of nociceptive information to supraspinal centers. They probably do this by directly targeting neurons of the wide dynamic range and nocispecific projection types, and/or primary afferent terminals. The overall organization involved allows for both pre- and post-synaptic inhibitory modulation (Basbaum, 1999; Millan, 1999). Inhibitory interneurons are themselves targeted by C and Ad fibers (Bernardi et al., 1995; Todd and Spike, 1993; Todd et al., 1994; Yoshimura and Nishi, 1995). This suggests a direct C and Ad counter-regulatory inhibitory feed-back control upon their parallel excitation of neurons of the wide dynamic range and nocispecific projection types. Our results indicate that SP is involved in such a feed-back circuit: thus, pain elicited upon direct activation of projection neurons by C and Ad fibers may be limited by simultaneous recruitment of inhibitory interneurons, at least when the stimulus is of short duration and sub-maximal intensity.
A further element which adds to the complexity of spinal modulation of pain processing by SP is the notion that the peptide can also be released from sources other than PAFs, such as intersegmental neurons and descending fibers (Ribeiro-da-Silva and Hökfelt, 2000). Independently from this, loss of physiological inhibitory tone in the dorsal horn following injuries and/or experimental manipulations has serious effects, altering pain perception and leading to conditions such as hyperalgesia and allodynia (Basbaum, 1999).

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