Vanilloid receptor-1 (TRPV1)-dependent activation of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse

UNIVERSITÀ DEGLI STUDI DI TORINO

Vanilloid receptor-1 (TRPV1)-dependent activation of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse

Francesco Ferrini [a], Chiara Salio [a], Angela Maria Vergnano [a], Adalberto Merighi [a,b,*]

[a] Department of Veterinary Morphophysiology, Via Leonardo da Vinci 44, 10095 Grugliasco, Italy
[b] Rita Levi-Montalcini Center for Brain Repair, Via Leonardo da Vinci 44, 10095 Grugliasco, Italy

Abstract
Inhibitory neurotransmission in spinal cord dorsal horn is mainly mediated by g-aminobutiric acid (GABA) and glycine. By patch clamp recordings and correlative immunocytochemistry, we studied here the effect of 2 μM capsaicin-induced vanilloid receptor-1 (TRPV1) activation on IPSCs in spinal lamina II neurons from post-natal mice. Specificity was confirmed after pre-incubation with the competitive antagonist SB366791 (10 μM). After a single capsaicin pulse, an intense increase of spontaneous IPSC (sIPSC) frequency was observed in the presence of NBQX 10 μM (62/81 neurons; ~76%) or NBQX 10 μM + AP-5 20–100 μM (27/42 neurons; ~64%). Only a subpopulation (~40%) of responsive neurons showed a significant amplitude increase. Seventy-two percent of the neurons displayed pure $GABA_A$ receptor-mediated sIPSCs, whereas the remaining ones showed mixed GABAergic/glycinergic events. After two consecutive capsaicin pulses, frequency rises were very similar, and both significantly higher than controls. When the second pulse was given in the presence of 4 μM L732,138, a selective antagonist of the substance P (SP) preferred receptor NK1, we observed a significant loss in frequency increase (63.90% with NBQX and 52.35% with NBQX + AP-5). TTX (1 μM) largely (~ 81.5%) blocked the effect of capsaicin. These results show that TRPV1 activation on primary afferent fibers releases SP. The peptide then excites inhibitory neurons in laminae I, III and IV, leading to an increased release of GABA/glycine in lamina II via a parallel alternative pathway to glutamate.

Keywords: TRPV1, NK1 receptor; Substantia gelatinosa; Inhibitory post-synaptic currents; Capsaicin; GABA; Inhibitory Neurotransmission; Substance P; Glycine; Spinal cord; Pain; Electrophysiology; Anatomy

*Corresponding author: Tel. +390116709118
E-mail address: adalberto.merighi@unito.it (A. Merighi).

1. Introduction

Inhibitory synaptic transmission in spinal cord dorsal horn (DH) is mainly mediated by c-amino butyric acid (GABA) and glycine (Malcangio and Bowery, 1996; Lynch, 2004). These amino acids are expressed by about one-third of DH interneurons (Todd and Sullivan, 1990) and are co-released at the same inhibitory synapses (Chéry and De Koninck, 1999). Behavioral studies following partial or total suppression of GABAergic/glycinergic neurotransmission (Hao et al., 1994; Sherman and Loomis, 1994; Malan et al., 2002; Drew et al., 2004) suggested that modulation of inhibitory tone at the DH level has an important role in the processing of nociceptive information relayed to supraspinal centers. Notwithstanding, there is still an incomplete knowledge about the neurotransmitters and circuitry involved in the release of GABA/glycine from DH neurons. Previous observations have demonstrated a sub- stance P (SP)-induced release of the two amino acids from an isolated spinal cord preparation (Sakuma et al., 1991; Maehara et al., 1995), leaving however the question open as to the site(s) of action of this well-known pain-related neuropeptide. We have recently shown that a synthetic analog of SP induces a significant increase of spontaneous inhibitory post-synaptic currents (sIPSCs) in lamina II (substantia gelatinosa) neurons (Vergnano et al., 2004), by acting upon the preferred SP receptor NK1 (Mantyh et al., 1997; Nichols et al., 1999). This strongly indicates that SP acts as a modulator of inhibitory neurotransmission in lamina II. Primary afferent fiber (PAF) terminals are known to be the major source of SP in DH. Numerous studies have shown that capsaicin, the pungent vanilloid of hot chili pepper (Caterina et al., 1997; Tominaga et al., 1998), induces a strong release of the peptide (and other neurotransmitters) from these terminals (Kuraishi et al., 1991; Okano et al., 1997; Garry et al., 2000; Afrah et al., 2001; Lever et al., 2001; Lao et al., 2003). Release follows activation of the vanilloid receptor-1 (TRPV1), a cation-channel receptor belonging to the superfamily of transient receptor potential (Caterina and Julius, 2001; Gunthorpe et al., 2002), which is highly expressed in nociceptive sensory neurons of dorsal root ganglia (DRGs) giving rise to PAFs of the C and Ad types (Michael and Priestley, 1999; Guo et al., 2001).

A significant amount of work has been carried out to characterize the effect of capsaicin on glutamatergic excitatory post-synaptic currents (EPSCs) from PAFs (Urban and Dray, 1992; Yang et al., 1998, 1999, 2000; Yang and Li, 2001; Baccei et al., 2003). However, these studies have not analyzed the TRPV1-dependent modulation of inhibitory neurotransmission, or failed to demonstrate that such a modulation occurs (Yang et al., 1998; Baccei et al., 2003), in spite of the morphological evidence for GABA depletion (Wei and Zhao, 1996) and increased Fos reactivity (Zou et al., 2001) in GABAergic DH neurons following capsaicin treatment.

We investigated here the effect of TRPV1 activation on IPSCs recorded from lamina II neurons. We aimed primarily to ascertain whether or not endogenous SP released by capsaicin was capable of affecting inhibitory neurotransmission in substantia gelatinosa.

Materials and methods

Animals and spinal cord slice preparation

All experimental procedures were approved by the Italian Ministry of Health and the Committee of Bioethics and Animal Welfare of the University of Torino.

Eight-(P8) to 12-day-old (P12) mice (n = 64) were deeply anesthetized with sodium pentobarbital (30 mg/kg). Dissection of the spinal cord was performed in 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. While submerged in ACSF, the thoracic and lumbar regions of the cord were isolated and transversally cut at 350 μm with a vibratome (Electron Microscopy Sciences, Hatfield, PA). Slices were then transferred into a chamber with oxygenated ACSF at 35 °C for 30 min and

further maintained at room temperature until used for experiments.

Electrophysiology

After recovery, slices were placed in a recording chamber constantly perfused (2 ml/min) with oxygenated ACSF at room temperature. The chamber was mounted on a fixed-stage microscope (Axioskop 1, Zeiss, Oberkochen, Germany) equipped with infrared gradient contrast optics (Luigs and Neumann, Ratingen, Germany). Under these conditions the substantia gelatinosa was easily identified as a translucent band by a 10 long-distance working objective, and recordings from visually identified lamina II neurons were subsequently carried out with a 40x water immersion objective (Achroplan, Zeiss). Patch pipettes were obtained from single-filament borosilicate capillaries (1.5 mm OD, 0.84 mm ID; WPI, Sarasota, FL) using a two-stage vertical puller (PC-10; Narishighe, Tokyo, Japan) or a horizontal puller (P-97; Sutter, Novato, CA). Pipette resistances ranged from 5 to 7 MΩ once filled with the intracellular solution.

To study IPSCs, voltage-clamp experiments were performed using an intracellular solution containing (in mM): 145 KCl, 5 EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATPNa, 0.2 GTPNa and 0.1% Lucifer Yellow (LY; Sigma, St. Louis, MO) pH 7.2 (with KOH) for a post hoc morphological analysis of recorded cells.

Patch-clamp whole cell recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Sunnyvale, CA). IPSCs were recorded at a holding potential of -63 mV and isolated via the application of 2,3-Dioxo-6-nitro-1,2,3, 4-tetrahydro-benzo[f]quinoxaline-7-sulfonamide disodium salt 10 μM (NBQX; Tocris, London, UK) to block a-amino-3-hy-droxy-5-methyl-4-isoxazolepropionate (AMPA)/kainite receptor-mediated neurotransmission. Under the above conditions, the reversal potential for Cl^- ions was +2 mV, and IPS-Cs were thus detected as inward currents. Access resistance was continuously monitored during experiments. Recordings were included for subsequent analysis only if: (i) membrane potential was more negative than -55 mV; (ii) access resistance was lower than 22 MΩ and changed less than 20% throughout the recording session. Currents were sampled at 10 kHz, filtered at 2 kHz and stored on a personal computer using Digidata 1322A with pClamp 8.0 software (Axon Instruments).

Data analysis

IPSCs were visually detected and analyzed off-line with Mini Analysis software (Synaptosoft, Decatur, GA). Amplitude and frequency of sIPSCs in control traces and after capsaicin application were compared within 200 s time intervals. When the event started to rise before the previous event returned to baseline, the baseline of the second event was estimated by extrapolating the decay time of the first event; then, the second peak amplitude was calculated as the difference between the baseline of the first peak and the extrapolated baseline of the second peak. Cumulative probability histograms of both amplitude and inter-event interval were compared using the Kolmogorov–Smirnov test for statistical significance. Neurons were classified as responsive when the inter-event interval difference after capsaicin administration was statistically significant ($P < 0.05$).

In order to compare decay and rise time of miniature IPSCs (mIPSCs), mean kinetic parameters were calculated by considering at least 30 mIPSCs per cell. Detection threshold for mIPSCs was set at 8 pA. The rise time was obtained from 10% to 90% peak amplitude. Because of the presence of mixed GABA/glycine mIPSCs in young animals (Keller et al., 2001), decay time was calculated by fitting the 10–90% region of the decay phase with a bi-exponential function and was expressed as time constants τ_1 and τ_2.

As previously observed (Baccei and Fitzgerald, 2004), IPSC electrophysiological data sampled from different lamina II neurons fail to show a gaussian distribution. Since this observation was confirmed by the Shapiro–Wilk test, non-parametric tests were used for subsequent statistical analysis. Precisely, the Wilcoxon matched-pairs test was used: (i) to compare IPSC electrophysiological values before and after capsaicin administration; (ii) to compare IPSC frequencies (m) between two consecutive capsaicin pulses (CAP1 and CAP2) and the respective controls (CTR1 and CTR2; see Section 3) after plotting

$\Delta\nu_{CAP1\text{-}CTR1}$ against $\Delta\nu_{CAP2\text{-}CTR2}$. Differences among repeated measures from the same neuron were studied with a non-parametric ANOVA analog (Friedman test), and multiple comparisons between groups were performed post-hoc with the Student–Newman–Keuls test (SNK). Unpaired data were compared with Kruskal–Wallis test.
Statistics were performed with Origin Microcal software (OriginLab Corporation, Northampton, MA) and with a soft- ware by S. Glantz (McGraw-Hill Companies; Columbus, OH) for non-parametric analysis.
Electrophysiological values were reported as means ±SEM, with n indicating the number of neurons. Values of $P < 0.05$ were considered statistically significant.

Immunocytochemistry and confocal microscopy

At the end of patch-clamp recordings, slices were fixed at room temperature for 30 min with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). After several washing in phosphate buffered saline (PBS; 0.02 M, pH 7.4), slices were preincubated in PBS with 1% normal goat serum for 30 min and then incubated overnight at 4 °C in rabbit anti-NK1 1:10000 (Sigma; Mantyh et al., 1995; Vergnano et al., 2004) or goat anti-TRPV1 1:100 (Santa Cruz, Biotechnology, Santa Cruz, CA; Doly et al., 2004) with 0.1% Triton X-100. Slices were then washed in PBS and incubated with appropriated second layer antibodies [3 h with anti-rabbit AlexaFluor 495 1:300 (Molecular Probes, Carlsbad, CA) or 3 h with biotinylated anti-goat 1:250 (Vector Lab., Burlingame, CA) followed by 1 h Extravidin-Cy3 1:1000 (Sigma)]. Slices were finally mounted in Vectashield H-1000 mounting medium (Vector Lab.). Confocal laser scanning microscopy was performed by using a Zeiss LSCM with a 63 oil lens. LY green fluorescence was acquired using 475 nm excitation and 458 nm emission filters and AlexaFluor 594 or Cy3 red fluorescence using 560 nm excitation and 543 nm emission filters. To reconstruct the overall morphology of recorded neurons and their relationship with immunoreactive structures computer-aided z-series reconstructions were carried out by analyzing successive confocal optical sections 153.5 153.5 μm (area size) at 0.5 μm intervals. Series were processed with LSM Image program (Zeiss). Ten representative optical sections cut through the cell body and the main processes of LY injected neurons were randomly chosen per cell. Green and red fluorescence were merged with Photoshop 5.0 (Adobe, San Jose, CA) to visualize yellow spots of colocalization, corresponding to contact points between red NK1 or TRPV1 immunoreactive processes and LY injected neurons. The mean cell area (MCA; LY-green) and the mean area of dual fluorescence superimposition (DFS; yellow) were calculated from reconstructed images with the ImageJ software (NIH, Bethesda, MD). The ratio of DFS/MCA 100 was plotted against the degree of electrophysiological response by performing a linear regression analysis. The degree of electrophysiological response was expressed as the normalized sIPSC frequency increase after capsaicin administration or as the per cent loss of capsaicin effect following blockade of NK1 receptor.

Drugs

Capsaicin, NBQX, DL-2-Amino-5-phosphonopentanoic acid (AP-5), Tetrodotoxin (TTX), N-Acetyl-L-tryptophan 3,5-bis-(trifluoromethyl)benzyl ester (L732,138), 4'-Chloro-3-meth-oxycinnamanilide (SB366791) were from Tocris; bicuculline methiodide and strychnine methiodide were from Sigma. Capsaicin was used at 2 μM final concentration because previous studies indicated that SP is released from TRPV1-expressing PAFs in a dose-dependent manner at concentrations higher than 1 μM (Afrah et al., 2001; Lao et al., 2003).
All drugs were prepared from at least 1000 times concentrated stock solutions.

Results

The effect of capsaicin on IPSCs was studied in a total of 144 lamina II neurons from P8-P12 mice. Recordings were performed in the steady presence of: (i) NBQX 10 μM; (ii) NBQX 10 μM and AP-5 20-100 μM; (iii) NBQX 10 μM and TTX 1 μM. Morphological characterization was carried out on 37

neurons (one neuron/ slice). Seventeen slices were simply studied for the overall morphology of responsive LY injected neurons. Ten slices with responsive neurons were processed for immunocytochemical detection of the SP-preferred receptor NK1. Other 10 slices containing a total of 6 capsaicin- responsive and 4 unresponsive neurons were processed for detection of TRPV1 immunoreactivity.

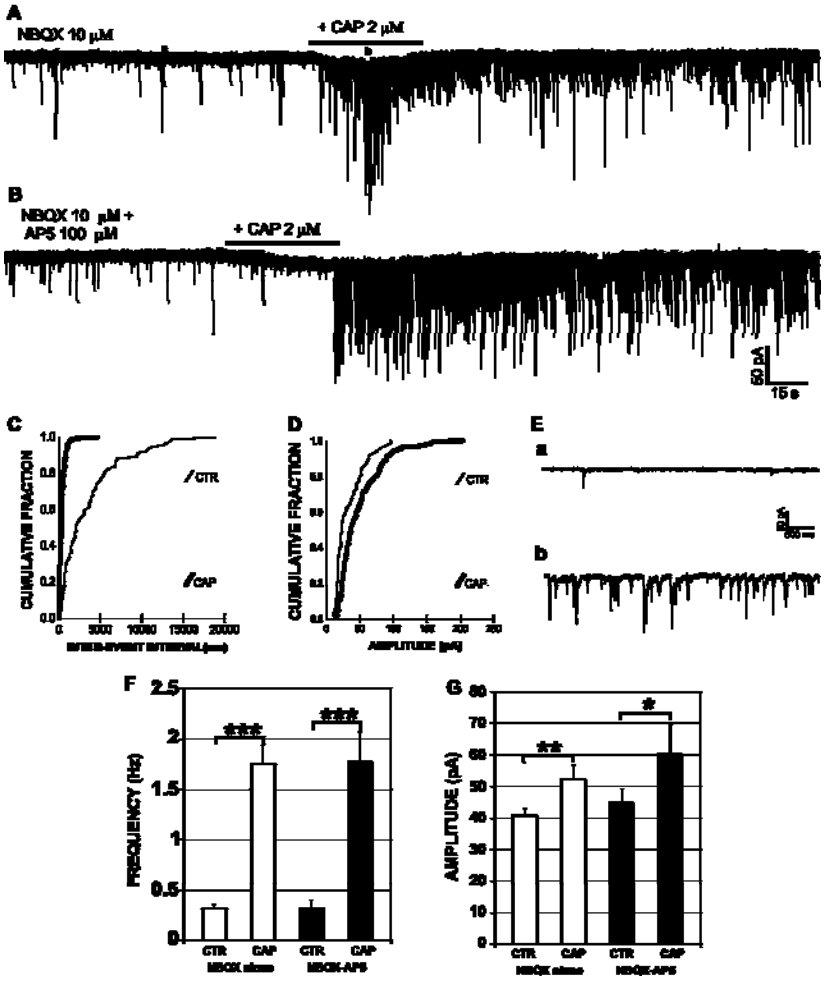

Fig. 1. Effect of a single capsaicin pulse (1 min, 2 μM) on sIPSCs. (A and B) Representative traces of sIPSC frequency and amplitude increases in steady presence of NBQX 10 μM (A) or NBQX 10 μM and AP-5 100 μM (B). A slow inward current was observed during capsaicin administration. V_h = -63 mV. (C and D) Cumulative plots (referred to traces A) showing the shifting in sIPSC inter-event interval (C) and amplitude distribution (D) after the capsaicin pulse (Kolmogorov–Smirnov test, $P < 0.05$). (E) Enlargements of the trace in A corresponding to a five second recording before (a) and after (b) capsaicin administration. (F) sIPSC frequency increase in the presence of NBQX (white bars; n = 62, Wilcoxon matched-pairs test, $^{***}P < 0.001$) or NBQX and AP-5 (black bars; n = 27, Wilcoxon matched-pairs test, $^{***}P < 0.001$). (G) sIPSC amplitude increase in the presence of NBQX alone (white bars; n = 62, Wilcoxon matched-pairs test, $^{**}P < 0.01$) or NBQX and AP-5 (black bars; n = 27, Wilcoxon matched-pairs test, $^{*}P < 0.05$). Abbreviations: CAP, capsaicin; CTR, control.

Effect of a single capsaicin pulse (2 μM for 1 min) on sIPSCs

Upon blockade of AMPA/kainate neurotransmission (Fig. 1A and E), capsaicin elicited in 62 out of 81 neurons (~76%), a strong increase of sIPSC frequency (from 0.32 ± 0.04 Hz to 1.75 ± 0.18 Hz; Wilcoxon matched-pairs test, $P < 0.001$; Fig. 1C and F, white bars) and amplitude (from 40.76 ± 2.23 pA to 52.36 ±4.40 pA; Wilcoxon matched-pairs test, $P < 0.01$; Figs. 1D and G, white bars). Noteworthy, there were considerable variations in the amplitude response to capsaicin: only 26 out of the 62 neurons that responded to capsaicin with a significant increase of their sIPSC frequency also displayed a significant increase in sIPSC amplitude (Kolmogorov–Smirnov test, $P < 0.05$), shifting in aver- age from 37.13 ± 2.50 pA to 68.19 ± 8.73 pA (Wilcoxon matched-pairs test, $n = 26$, $P < 0.001$), whereas the remaining 36 cells showed almost no changes (43.37 ±3.35 pA in control; 40.93 ± 3.16 pA; $P > 0.05$). The effect of capsaicin lasted in most cases from 10 to 30 min and then sIPSC frequency and amplitude returned to control levels. A slow inward current (Fig. 1A) with an average amplitude of 15.78 ± 3.03 pA was observed in about one-third of the responsive neurons (19 out of 62).

This current appeared at the beginning of capsaicin administration before the sIPSC frequency increase, and disappeared quickly after capsaicin wash-out. In 19 out of 81 neurons (rv24%) capsaicin administration did not induce significant changes of sIPSC frequency (from 0.24 ± 0.06 Hz to 0.22 ± 0.05 Hz; paired t-test, $P > 0.05$) or amplitude (from 45.16 ± 3.83 pA to 43.20 ± 4.05 pA; Wilcoxon matched-pairs test, $P > 0.05$).

Under blockade of AMPA/kainate and N-Methyl-D-Aspartate (NMDA) neurotransmission (Fig. 1B) a single pulse of capsaicin produced a significant increase of sIPSC frequency (from 0.32 ± 0.08 Hz to 1.78 ±0.29 Hz; Wilcoxon matched-pairs test, $P < 0.001$; Fig. 1F, black bars) and amplitude (from 45.03 ±4.28 pA to 60.37 ± 9.20 pA; Wilcoxon matched-pairs test; $P < 0.05$; Fig. 1F, black bars) in 27 out of 42 neurons (~64%). Also in this series of experiments, the amplitude increase after capsaicin pulse was significant at the Kolmogorov–Smirnov test in only 13 out of 27 responsive neurons (~48.10%; Fig. 1B). A slow inward current was seen in 7 out of 27 neurons (25.92%; Fig. 1B) with an average amplitude of 14.06 ± 2.34 pA.

To better investigate the nature of the sIPSCs elicited by capsaicin we blocked the currents mediated by $GABA_A$ and glycine receptors with bicuculline 10 μM and strychnine 1 μM in 36 responsive neurons (Fig. 2). In 26 of these cells (~72%), sIPSCs were completely blocked by bicuculline (Fig. 2A and B). In the remaining 10 cells (~28%) sIPSCs were only partially blocked by bicuculline, but the residual bicuculline-resistant synaptic activity was completely abolished by further addition of strychnine to the perfusion bath (Fig. 2C–E).

In brief these results demonstrated that under complete blockade of ionotropic glutamate neurotransmission, capsaicin significantly increased the release of GABA/glycine upon lamina II neurons.

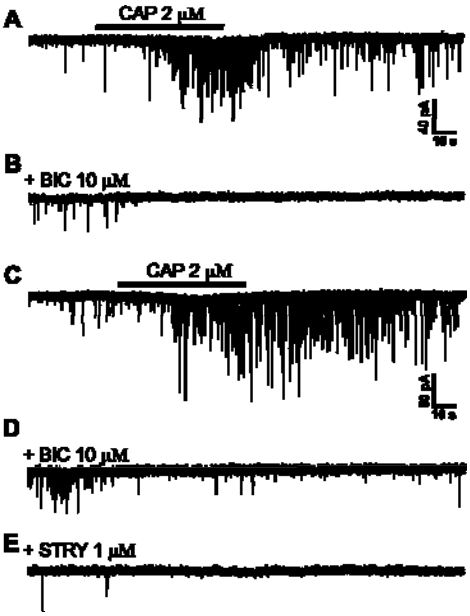

Fig. 2. Nature of sIPSCs in lamina II neurons. (A and B) Illustrative traces of the complete block of sIPSCs (A) by application of the $GABA_A$ receptor antagonist bicuculline 10 μM (B). (C–E) Illustrative traces of the partial block of sIPSCs in another neuron in bicuculline. The residual bicuculline resistant activity was completely blocked by the glycine receptor antagonist strychnine 10 μM. Note the slow inward current during capsaicin administration (A and C). Recordings were carried out in steady presence of NBQX 10 μM. Abbreviations: BIC, bicuculline; CAP, capsaicin; STRY, strychnine.

Effect of two consecutive capsaicin pulses (2 μM for 1 min) on sIPSC frequency

TRPV1 desensitization (Caterina et al., 1997; Bhave et al., 2002) has often been reported as limiting factor for evoking comparable electrophysiological responses after subsequent capsaicin pulses during the same recording session (Yang et al., 1998, 2000; Tominaga et al., 2001).

In this series of experiments (Fig. 3A), sIPSC frequency was sequentially sampled upon: (i) perfusion in ACSF + NBQX (CTR1); (ii) after the first pulse of capsaicin (CAP1); (iii) after returning to values comparable to control level (CTR2 – usually at least 25 min of washing with ACSF + NBQX); and, (iv) after the second capsaicin pulse (CAP2).

sIPSC frequency increased from 0.19 ± 0.05 Hz in CTR1 to 1.57 ± 0.36 Hz after CAP1 (Wilcoxon matched-pairs test, $n = 10$, $P < 0.02$), and from 0.26 ± 0.06 Hz in CTR2 to 1.71 ± 0.48 Hz after CAP2 (10 μM, 10 min starting from the arrowhead) was given between CAP1 and CAP2. The effect of CAP2 was completely prevented by the antagonist.

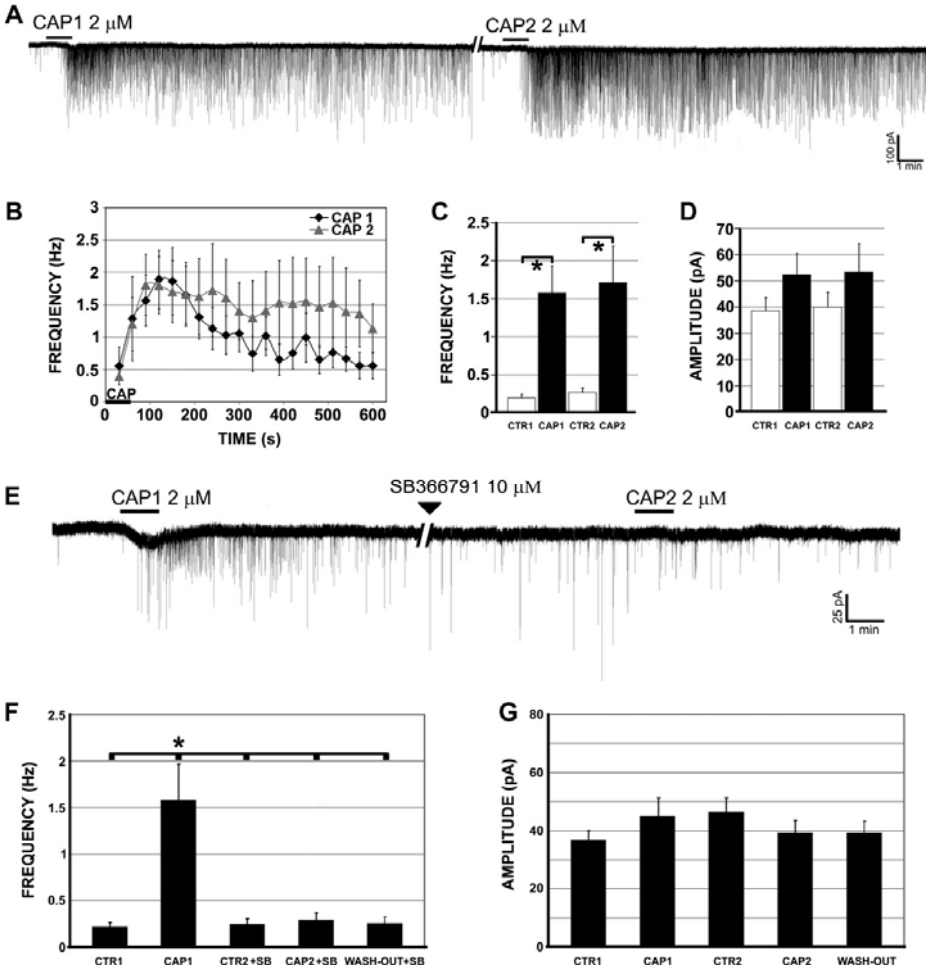

Fig. 3. Effect of two consecutive capsaicin pulses (1 min to 2 μM) on sIPSCs and specificity of TRPV1 receptor activation. (A) Illustrative trace showing the effect of CAP1 and CAP2, given 25 min after CAP1. A segment of the trace corresponding to 10 min recording between the two pulses has been cut away as indicated by //. Note that the slow inward current was more evident at CAP1. Recording was carried out in steady presence of NBQX 10 μM. (B) Superimposition of frequency variations versus time after CAP1 (black squares) and CAP2 (grey triangles). Time 0 corresponds to the beginning of capsaicin administration, and the thick line on x-axis indicates the duration of the two capsaicin pulses. Each marker represents the mean frequency of 30 s traces sampled from 10 neurons. (C) Multiple comparisons after Friedman test ($n = 10$, $x^2 = 25.92$, $P < 0.001$) showed that sIPSC frequency increases after CAP1 and CAP2 (black bars) were significantly higher than that of CTR1 and CTR2 (white bars; SNK test $^*P < 0.05$). (D) The slight amplitude increase after CAP1 and CAP2 was not significant (Friedman test, $n = 10$, $x^2 = 4.44$, $P = 0.29$). (E) SB366791

Ten minutes of the trace were cut away where indicated by //. Recordings were carried out in steady presence of NBQX 10 μM. (F) sIPSC frequency differences among control groups (CTR1 and CTR2), capsaicin groups (CAP1 and CAP2) and the wash-out groups ($n = 12$, $x^2 = 25.8$, $P < 0.001$).
CTR2, CAP2 and wash-out were recorded in steady presence of the TRPV1 antagonist. Post-hoc multiple comparisons showed that CAP1 frequency was significantly higher than that of each other group (SNK test, $^*P < 0.05$). (G) The sIPSC amplitude fluctuations were not significant ($n = 12$, $x^2 = 8.5$, $P > 0.05$). Note the amplitude increase during CAP1 administration. Abbreviations: CAP1, first capsaicin pulse; CAP2, second capsaicin pulse; SB, SB366791.

(Wilcoxon matched-pairs test, n = 10, P < 0.02; Fig. 3B and C). Analysis of ranks difference with Friedman test (n = 10, x^2 = 25.92, P < 0.001) and multiple comparison through SNK test showed that CAP1 and CAP2 frequencies were both significantly higher (P < 0.05) than the control frequencies, whereas no significant differences were observed between those of CTR1 and CTR2 or CAP1 and CAP2 (Fig. 3C). In particular, by normalizing frequency values after the pulses and their respective controls, CAP1 frequency became 11.68 ± 2.62 times that of CTR1, whereas CAP2 frequency was 9.81 ± 2.47 times that of CTR2. When the differences between the frequencies sampled at CAP1/CTR1 and CAP2/CTR2 were compared to each other there was no statistical difference (Wilcoxon matched-pairs test, n = 10, P > 0.05). A slight amplitude increase was also observed after each pulse, but it was not statistically significant (CAP1: from 38.51 ± 5.03 pA to 52.22 ± 8.08 pA; CAP2: from 39.91 ± 5.76 pA to 53.18 ± 10.90 pA; Friedman test, n = 10, x^2 = 4.44, P = 0.29; Fig. 3D). The slow inward current, previously described after a single capsaicin pulse, was also detected in 5 neurons during both capsaicin administrations, even though its amplitude was usually lower at CAP2 than CAP1 (26.79 ± 7.69 pA vs. 15.23 ± 4.39 pA). Amplitude reduction at CAP2, as already reported by others (Yang et al., 1998; Baccei et al., 2003), is likely an effect of TRPV1 desensitization. From these results we concluded that the effects of two consecutive capsaicin pulses on sIPSC frequency were highly comparable, and that receptor desensitization has a minor impact on our experimental paradigm, if not for reduction of the slow inward current amplitude at the second vanilloid pulse.

Capsaicin effects on sIPSCs are specifically mediated by TRPV1

SB 366791, a competitive highly selective TRPV1 antagonist (Gunthorpe et al., 2004), was used in these experiments (Fig. 3E). The antagonist was applied for at least 5 min before CAP2, in neurons that had previously shown to be responsive at CAP1.

Pre-administration of SB366791 10 μM for at least 10 min (12 neurons; Fig. 3E) produced an intense reduction of the effect of the second capsaicin pulse (CAP2 + SB366791) compared with CAP1. Frequency shift was significant after CAP1 (from 0.21 ± 0.05 Hz to 1.57 ± 0.40 Hz; Wilcoxon matched-pairs test, $n = 12$, $P < 0.02$), but not after CAP2 + SB366791 (from 0.24 ± 0.06 Hz to 0.29 ± 0.08 Hz, Wilcoxon matched-pairs test, $n = 12$, $P > 0.05$). Multiple group comparison showed that frequency values after CAP1 were statistically different compared with each other group, whereas no significant differences were detected among other groups (Friedman test with SNK post- hoc, $n = 12$, $x^2 = 25.8$, $P < 0.001$; Fig. 3F). sIPSC frequency after CAP1 was 9.47 ± 1.80 times CTR1, while after CAP2 + SB366791 it was 1.15 ± 0.17 times CTR2, showing an almost complete block of the capsaicin effect. As shown in Fig. 3G, no statistically significant changes were observed among amplitude values (Friedman test, $n = 12$, $x^2 = 8.5$, $P > 0.05$), even though we noticed an amplitude increase after CAP1 (from 36.55 ± 3.53 pA to 44.86 ± 6.51 pA) and a slight decrease after CAP2 + SB36679 (from 46.22 ±5.12 pA to 39.06 ± 4.41 pA). Five neurons showed a slow inward current at CAP1 (13.48 ± 4.79 pA), but not at CAP2 + SB366791 (Fig. 3E).
These experiments demonstrated that changes in sIPSC frequency and slow inward currents were specifically due to TRPV1 activation.

Effect of L732,138 on sIPSC frequency increase

In some experiments after CAP1 and washing-out, L732,138, a selective NK1 receptor antagonist, was added to bath solution (4 μM) for at least 8 min in the presence of NBQX (Fig. 4A–D). After CAP1, frequency increase was from 0.35 ± 0.08 Hz to 1.87 ± 0.24 Hz (Wilcoxon matched-pairs test, $n = 13$, $P < 0.02$) whereas after CAP2 + L732,138 the frequency shifted from 0.45 ± 0.10 Hz to 0.92 ± 0.18 Hz (Wilcoxon matched- pairs test, $n = 13$, $P < 0.02$; Fig. 4B and C), showing a significant loss of the effect of capsaicin in the presence of the NK1 receptor antagonist (Wilcoxon matched- pairs test, $n = 13$, $P < 0.02$). As reported in Fig. 4C, multiple comparisons with SNK test following Friedmann test ($n = 13$, $x^2 = 31.15$, $P < 0.001$) showed that CAP1 frequency was significantly higher compared with frequencies from each other group, including CAP2 + L732,138 ($P < 0.05$). sIPSC frequency after CAP1 normalized to CTR1 was 9.34 ± 2.25,

whereas after CAP2 + L732,138 was 2.27 ± 0.31, resulting in a loss of effect of 63.90% ± 8.46%. A limited and not statistically significant increase of sIPSC amplitude was observed at both capsaicin administrations (CAP1: from 42.66 ± 4.38 pA to 56.95 ± 7.82 pA; CAP2 + L732,138 from 48.14 ± 5.33 pA to 52.98 ± 4.43 pA; Friedman test, n = 13, x^2 = 1.15, P > 0.05; Fig. 4D). In 3 neurons a slow inward current was observed during both capsaicin administrations, with an average amplitude of 9.85 ± 3.09 pA at CAP1 and 4.36 ± 1.33 pA at CAP2+L732,138.

After pre-incubation with NBQX 10 μM and AP-5 20–100 μM (Fig. 4E–H), the sIPSC frequency increase was from 0.22 ± 0.04 Hz to 1.90 ± 0.42 Hz following CAP1 (Wilcoxon matched-pairs test, n = 11, P < 0.02), and from 0.28 ± 0.05 Hz to 1.23 ± 0.36 Hz after CAP2 + L732,138 (Wilcoxon matched-pairs test, n = 11, P < 0.02; Fig. 4F and G). The loss of effect at CAP2 + L732,138 compared with the effect at CAP1 was significant (n = 11, Wilcoxon matched-pairs test, P < 0.05). Also the differences among sIPSC frequencies of each group became statistically significant (Friedman test, n = 11, x^2 = 26.89, P < 0.001). Furthermore, multiple comparisons with SNK test showed that, while

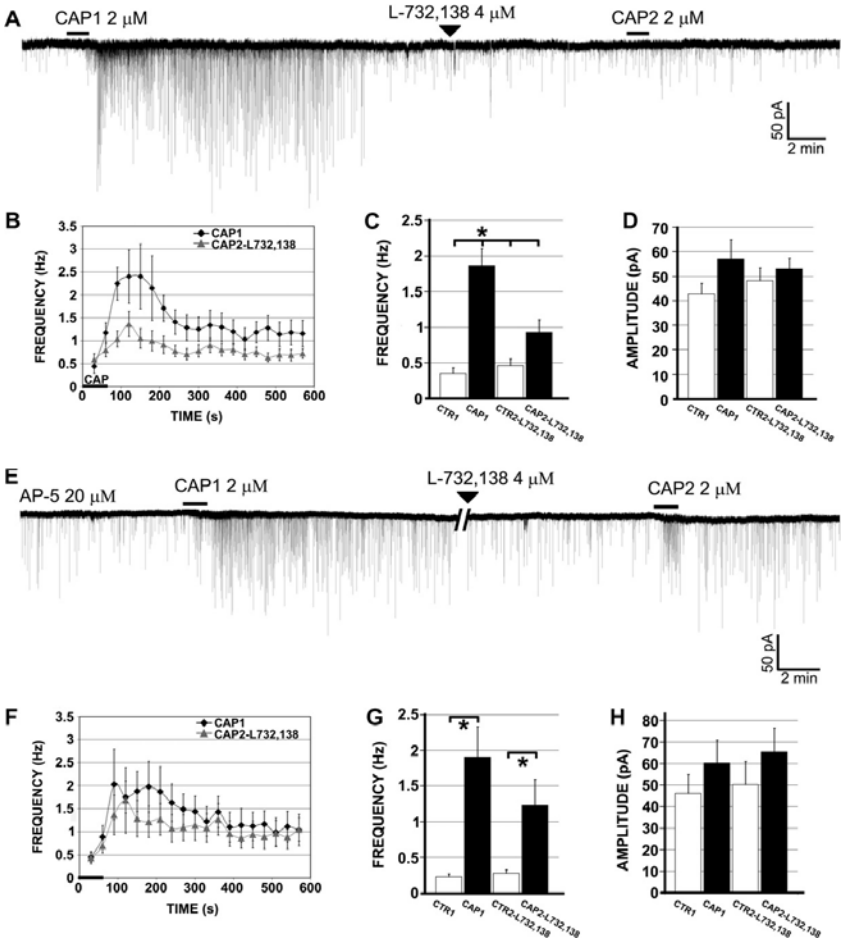

Fig. 4. Effect of the NK1 antagonist L732,138 on capsaicin-induced sIPSC frequency increase. (A and E) illustrative traces of the effect of L732,138 (4 μM for 8–10 min starting from the arrowhead) given between CAP1 and CAP2 in the presence of NBQX alone (A), or NBQX and AP-5 (E). Fifteen minutes of trace E were cut away where indicated by //. (B and F) Superimposition of frequency variations versus time after CAP1 (black squares) and CAP2-L732,138 (grey triangles) in presence of NBQX (B) or NBQX and AP-5 (F). Time 0 is fixed at the start of each capsaicin administration and the thick line on x-axis indicates the duration of pulse. Each marker represents the mean frequency of 30 s traces sampled from 13 (B) or 11 (F) neurons, respectively. In both graphs note the reduction of the effect of capsaicin, and the more rapid frequency recovery after CAP2- L732,138 than after CAP1. (C and G) sIPSC frequency differences among control groups (CTR1 and CTR2-L732,138, white bars) and capsaicin groups (CAP1 and CAP2-L732,138, black bars) were checked with Friedman test (C, NBQX: $n = 13$, $x^2 = 31.15$, $P < 0.001$; G NBQX and AP-5: $n = 11$, $x^2 = 26.89$, $P < 0.001$). Post-hoc multiple comparisons showed that: (i) in C, CAP1 frequency was significantly higher than that of CTR1, CTR2-L732,138 and CAP2-L732,138 (SNK test, $^*P < 0.05$); (ii) in G, CAP1 frequency was significantly higher than that of CTR1, and CAP2-L732,138 frequency was significantly higher than that of CTR1 (SNK test, $^*P < 0.05$), but no difference was found between those of CAP1 and CAP2-L732,138 (SNK test, $P > 0.05$). (D and H) sIPSC amplitude differences among controls (white bars) and CAP groups (black bars) were not significant (D, NBQX: Friedman test $n = 13$, $x^2 = 1.15$, $P > 0.05$; H, NBQX and AP-5: Friedman test, $n = 11$, $x^2 = 3.98$, $P > 0.05$). Abbreviations: CAP1, first capsaicin pulse; CAP2-L732,138, second capsaicin pulse in the presence of L732,138; CTR1, first control; CTR2, second control.

CAP1 and CAP2 + L732,138 groups were both significantly different compared with CTR1 and CTR2 + L732,138 ($P < 0.05$), differences between CAP1 and CAP2 + L732,138 or CTR1 and CTR2 + L732,138 were not significant (Fig. 4G). sIPSC frequencies after CAP1 and after CAP2 + L732,138 were 11.90 ± 4.55 and 5.55 ± 2.34 times their respective control, indicating a loss of effect of about 52.35% ± 12.32%. As shown in Fig. 4H, amplitude fluctuations observed among these groups were not statistically significant (CAP1: from 46.02 ± 8.81 pA to 60.15 ± 10.69 pA; CAP2 +L732,138: from 50.13 ± 10.80 pA to 65.33 ± 11.04 pA; Friedman test, $n = 11$, $x^2 = 3.98$, $P > 0.05$; Fig. 4H). The slow inward current was observed in 3 of these neurons with a mean amplitude of 18.19 ± 3.32 pA at CAP1 and 9.41 ± 3.37 pA at CAP2. These observations demonstrated that the effect of capsaicin on lamina II neurons was substantially due to activation of NK1 receptor and occurred independently from blockade of NMDA-mediated neurotransmission.

Effects of capsaicin after isolation of mIPSCs

Twenty-seven neurons incubated with TTX were analyzed (Fig. 5). Six of them were previously treated with capsaicin 2 μM for 1 min in presence of NBQX alone to check their responsiveness to TRPV1 activation. The mean frequency and amplitude of mIPSC in controls were 0.13 ± 0.03 Hz and 35.19 ± 3.74 pA, respectively, and thus lower than those of sIPSCs. Only 5 out of 27 neurons (~18.5%) showed a significant mIPSC frequency increase after capsaicin application (from 0.25 ± 0.16 Hz to 0.34 ± 0.19 Hz; Wilcoxon matched-pairs test, $n = 5$, $P < 0.05$; Fig. 5A and B, E), without significant changes in mean amplitude (from 39.77 ± 5.97 pA to 39.86 ± 8.04 pA; Wilcoxon matched-pairs test, $n = 5$, $P > 0.05$; Fig. 5F). Wilcoxon matched-pairs Wilcoxon matched-pairs 5.97 pA to 39.86 ± 8.04 pA; Wilcoxon matched-pairs Wilcoxon matched-pairs

test, n = 5, P > 0.05; Fig. 5F).

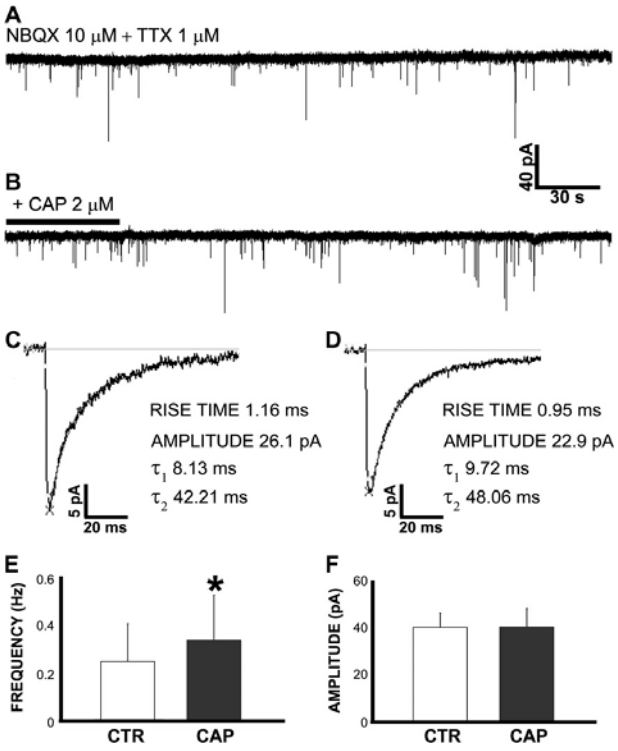

Fig. 5. Effect of capsaicin on mIPSCs. (A and B) Representative traces of mIPSCs under control conditions (A) and after a single capsaicin pulse (B). V_h = -63 mV. In this neuron capsaicin produced only a slight but significant frequency increase (from 0.095 Hz to 0.175 Hz, Kolmogorov–Smirnov test, P < 0.05). (C and D) Average of mIPSC events during control (C) and after capsaicin administration (D). No significant changes were observed in mean amplitude or kinetics. (E) Only 5 out of 27 neurons showed a significant mIPSC frequency increase after capsaicin administration (black bar) compared with control (white bar; Wilcoxon matched-pairs test, *P < 0.05). (F) No amplitude changes were observed (P > 0.05).

An inward current was seen during capsaicin administration in 7 neurons (~26%) with a mean amplitude of 14.90 ± 3.72 pA. A TRPV1-dependent modulation of mIPSC kinetics was excluded by comparing rise and decay time before and after capsaicin administration (Fig. 5C and D). Namely, no significant changes were observed either in rise time (1.34 ± 0.15 ms in control and 1.27 ± 0.11 ms after capsaicin; Wilcoxon matched-pairs test, n = 17, P > 0.05) or in decay time constants (τ_1 = 17.58 ± 2.48 ms and τ_2 = 90.97 ± 14.99 ms in control; τ_1 = 17.35 ± 2.61 ms and τ_2 = 87.11 ± 13.91 ms after capsaicin; Wilcoxon matched-pairs test, n = 17, P > 0.05). From these experiments we concluded that the effect of capsaicin on inhibitory neurotransmission was mainly driven by an action potential-dependent mechanism.

Histology

According to previous classifications (Grudt and Perl, 2002), 33 capsaicin-responsive lamina II neurons were subdivided into five morphological classes after being injected with LY: islet/central cells (30.3%), radial cells (21.2%), medio-lateral cells (12.1%), stalked cells (9.1%), and unclassified cells (27.3%). However, there was not a significant correlation between cell morphology and sIPSC frequency increase (Kruskal–Wallis test, P = 0.14; not shown).

Nonetheless, a total of twenty additional slices, each containing one electrophysiologically characterized neuron in lamina II, were immunolabeled with the anti-TRPV1 (n = 10; Fig. 6A) or the anti-NK1 receptor (n = 10; Fig. 6B) antibody. Control experiments were performed on acutely dissected spinal cord slices by omitting the primary antibodies. In these experiments, the background brought by the

secondary antibody or extravidin-Cy3 was negligible.
As expected (see for example Guo et al., 2001), TRPV1 immunoreactive processes were primarily observed in lamina I, whereas they were sparser and/ or only appeared in a punctate form in laminae II-III (in red – Fig. 6A). All the 10 recorded neurons subjected to subsequent immunocytochemical analysis for TRPV1 localization were not immunoreactive. However, it was common to detect the presence of variable numbers of spots of double fluorescence superimposition (DSF) at their somatodendritic domains. When the shift in sIPSC frequency after a single pulse of capsaicin was plotted against the percent cell area occupied by TRPV1 contacts (DFS/MCA 100, Fig. 7A), not only the linear regression between the two variables was not significant ($P = 0.4$), but also the correlation coefficient was negative ($R = -0.32$). Description of NK1 immunoreactivity in mouse spinal cord was previously reported from our laboratory (Vergnano et al., 2004). All neurons previously recorded before and after L732,138 incubation and then filled with LY were not NK1 immunoreactive. However, spots of DSF (yellow), indicative of the presence of contacts between immunoreactive processes (red) and the LY filled neurons (green), were often observed on somata and larger dendrites (Fig. 6B). Comparing the normalized sIPSC frequency after CAP2 + L732,138 with the normalized frequency after CAP1, the per cent loss of effect between the two capsaicin pulses was correlated with the percent cell area contacted by immunolabeled processes (DFS/ MCA 100; Fig. 7B). The correlation was not statistically significant ($R = 0.52$, $P = 0.11$). From these experiments we can conclude that the anatomical distribution of TRPV1 and NK1 immunoreactivities within lamina II does not correlate with the functional effects of capsaicin on inhibitory neurotransmission.

Discussion

We show here that capsaicin, by acting through TRPV1, leads to an intense increase of inhibitory spontaneous neurotransmission in lamina II neurons of mouse spinal cord. We also demonstrate that this effect is largely mediated by the activation of the SP-preferred receptor NK1, thus providing strong evidence that release of endogenous SP may be capable of affecting the inhibitory tone of DH interneurons. The most interesting observation arising from this study is that, although it is well established that capsaicin induces a strong glutamate release after binding to TRPV1, its ability to enhance inhibitory neurotransmission persists after blockade of ionotropic glutamate receptors. This demonstrates the existence of a SP-mediated parallel alternative pathway to glutamate in the transfer of nociceptive information to inhibitory lamina II neurons.

TRPV1 activation leads to enhancement of inhibitory neurotransmission

Several electrophysiological studies (Urban and Dray, 1992; Yang et al., 1998, 1999, 2000; Yang and Li, 2001; Baccei et al., 2003) have shown that activation of TRPV1 at PAF terminals produces a strong glutamate release, and, consequently, a significant increase of EPSCs in lamina II and other

DH neurons. Some of these studies, performed in young and adult rats, did not report any effect of capsaicin on mIPSCs (Baccei et al., 2003) or sIPSCs (Yang et al., 1999). In spite of this, increased fos expression in GABAergic DH neurons (Zou et al., 2001) after peripheral capsaicin, and up-regulation of inhibitory neurotransmission in acute and chronic capsaicin inflammation models (Castro- Lopes et al., 1994; Rees et al., 1995) have been described in the same species. Moreover, morphological evidence for a capsaicin-dependent GABA-depletion of DH neurons has been provided in cat (Wei and Zhao, 1996). All these data are in full accord with those presented here, as well as with the electrophysiological observations demonstrating a capsaicin-evoked SP-mediated IPSC increase in mouse lamina V neurons (Nakatsuka et al., 2005).

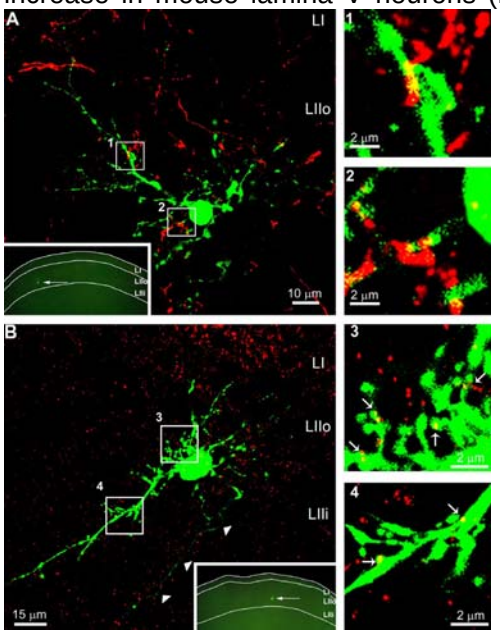

Fig. 6. Dual fluorescence confocal microscope reconstructions of recorded neurons after TRPV1 or NK1 immunolabeling. (A) Illustrative 0.5 µm-thick optical section of a capsaicin-responsive radial neuron injected with LY (green channel) after TRPV1 receptor immunostaining (red channel). The neuron showed a slight sIPSC frequency and amplitude increase and a large slow inward current after CAP1. The slow inward current was also observed after CAP2 in presence of TTX 1 µM. Some TRPV1 positive processes contacted the LY filled dendrites and, rarely, the cell soma (see yellow contacting areas in the inserts 1 and 2). The insert at the bottom left corner shows the localization of the recorded neuron (white arrow). (B) Illustrative 0.5 µm-thick optical section of a capsaicin-responsive stalked cell injected with LY (green channel) after NK1 receptor immunostaining (red channel). The axon is indicated by the arrowheads. About 94% of the effect of capsaicin on sIPSC frequency increase was lost after CAP2- L732,138 compared with CAP1. NK1 immunoreactivity is mainly represented by a punctate staining of neuronal processes in lamina I and, to a lesser extent, lamina II. NK1 positive processes contact LY-filled dendrites and the cell soma (yellow) as shown by the arrows in the inserts 3 and 4. The insert at the bottom right corner shows the localization of the recorded neuron (white arrow). Abbreviations: CAP1, first capsaicin pulse; CAP2, second capsaicin pulse; CAP2-L732,138, second capsaicin pulse in the presence of L732,138; LY, lucifer yellow.

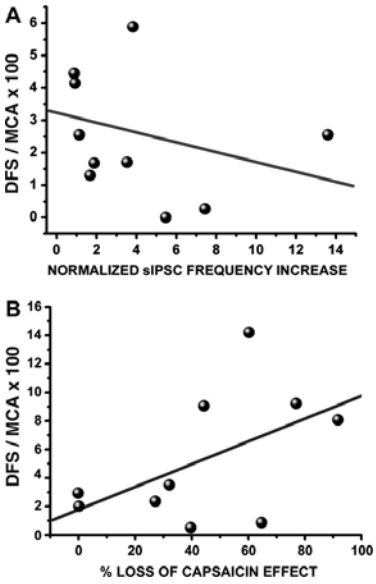

Fig. 7. Correlation between the degree of electrophysiological response and the mean area of DFS. (A) The normalized sIPSC frequency after capsaicin in responsive and unresponsive neurons is plotted against the percentage of MCA contacted by TRPV1 immunoreactive processes. No correlation was found after linear regression analysis (n = 10, R = -0.32, P = 0.4). (B) The percent loss of capsaicin effect in the presence of L732,138 is plotted against the percentage of MCA contacted by NK1 immunoreactive processes. A positive correlation between these two variables was found (R = 0.52), but it was not statistically significant (n = 10, P = 0.11). Abbreviations: DFS, dual fluorescence superimposition; MCA, mean cell area.

Increase in sIPSC frequency after capsaicin administration is likely due to the activation of inhibitory inter- neurons pre-synaptic to the recorded cell. This likely reflects augmented release of both GABA/glycine, in keeping with the widely established notion of their co-existence/co-release in superficial DH (Todd et al., 1996; Chéry and De Koninck, 1999; Keller et al., 2001). A significant amplitude increase was also observed, likely consequent to an action potential-dependent mechanism (Kawasaki et al., 2004). The latter, however, was significant in only 40% of responsive neurons, although the effects of capsaicin were blocked by TTX in the majority of cells. Several explanations can be sought for this apparent discrepancy (Jang et al., 2002; Maccaferri and Dingle- dine, 2002; Jeong et al., 2003). Specifically regarding inhibitory spinal cord neurons, it was demonstrated that activation of pre-synaptic $GABA_A$/glycine autoreceptors causes a pre-synaptic depolarization depending upon chloride effux, membrane depolarization and subsequent activation of TTX-sensitive sodium channels, eventually leading to calcium influx through voltage-dependent channels (VDCCs – Jang et al., 2002; Jeong et al., 2003). It is thus possible that in those neurons where sIPSC amplitude was not significantly changed after capsaicin, the increase in calcium levels within the pre-synaptic terminals was small and not sufficient to cause marked increases in GABA/glycine release. Alternatively or in addition to, GABA/glycine may be asynchronously released in relation to different types of VDCCs

present on pre-synaptic terminals (Hestrin and Galarreta, 2005) and this could also account for the lack of an amplitude increase.
Finally it remains to explain why a small number of neurons (18.5%) still showed a slight increase of frequency, but never of amplitude, under TTX. It is thus possible that: (i) capsaicin elevates intracellular calcium in PAF terminals, causes a release of SP, and this, in turn, elevates intracellular calcium in inhibitory interneurons, hence increasing the frequency of mIPSCs; (ii) since

TRPV1 is present in dendrites of a few intrinsic spinal neurons (Valtschanoff et al., 2001; Doly et al.,

2004), capsaicin may directly elevate calcium in these neurons leading to augmented transmitter release. Also, a capsaicin-induced slow inward current was observed irrespective from TTX blockade of sodium-mediated action potential, as previously reported by others (Yang et al., 1998, 2000).

Based on previous histological findings, it is conceivable that the effect of capsaicin on IPSCs was

mainly due to activation of TRPV1 localized on PAF terminals (Guo et al., 2001), even though the activation of dendritic TRPV1s in lamina II interneurons cannot be excluded. However, in keeping with the results of most TTX experiments, there was not a statistically significant correlation between the degree of response to capsaicin and the cellular area occupied by presumptive contacts with TRPV1 immunoreactive processes.

NK1 contribution to sIPSC frequency increase evoked by capsaicin

Administration of capsaicin in the presence of L732,138 resulted in a significant loss (nearly 70%) of effect, demonstrating that endogenous SP largely contributed to the effect of the vanilloid (Fig. 8A and B), in agreement with our recent observation that [Sar^{9},- $Met(O_2)^{11}$]-SP, a synthetic analog of SP, produced a sig- nificant increase of sIPSCs in lamina II neurons (Vergnano et al., 2004). Although PAFs are not the sole source of SP in DH, a capsaicin-induced SP release from PAFs has previously been reported in many in vitro studies (Go and Yaksh, 1987; Afrah et al., 2001; Lao et al., 2003), and morphological evidence for TRPV1 + SP colocalization in DRG neurons and PAF central terminals has been provided (Michael and Priestley, 1999; Guo et al., 2001).

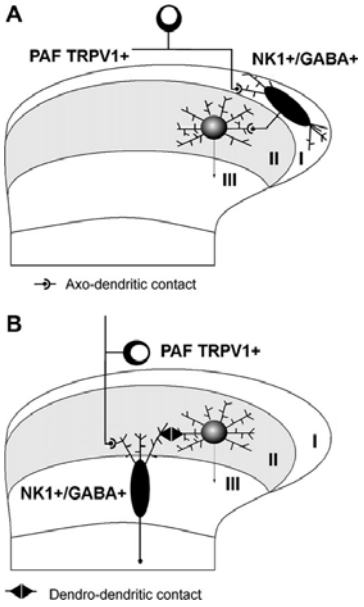

Fig. 8. Neuronal circuits involved in the enhancement of IPSCs by capsaicin. Neuronal circuits compatible with electrophysiological and morphological observations on lamina II capsaicin responsive neurons expressing functional $GABA_A$ and/or glycine receptors. Results of experiments carried out under blockade of ionotropic glutamatergic neurotransmission indicate that the effect of capsaicin is unlikely to be mediated by a polysynaptic chain linking the responsive neuron with those releasing the inhibitory neurotransmitter(s). The activation of TRPV1-NK1-$GABA_A$/glycine receptors involves the existence of a population of NK1+/GABAergic neurons at different laminar locations in DH (Littlewood et al., 1995). NK1-immunoreactive neurons are found predominantly in laminae I and III–IV, but only rarely in lamina II (Ribeiro-Da-Silva et al., 2001). Circuits in A and B are further supported by: (i) the existence of SP-containing PAFs monosynaptically linked to laminae I, III–IV NK1-positive neurons; (ii) histological/immunocytochemical data on distribution of the dendritic arbors/laminar localization of NK1 immunoreactive DH neurons. Both configurations may involve the existence of dendro-dendritic contacts (as shown in B) as the sites of inhibitory neurotransmitter release (see Vergnano et al., 2004, for discussion of this issue). The putative implication of such synapses in capsaicin effect does not necessarily exclude a TTX sensitive mechanism. For the sake of simplicity, axo-dendritic contacts between PAFs expressing TRPV1 and dendrites expressing NK1 have been indicated, although it is well-known that SP also acts via non-synaptic volumetric transmission.

Nonetheless, there was no a significant correlation between the degree of NK1 expression and the loss of effect after receptor blockade. It is thus unlikely (also in agreement with the lack of effect in TTX) that inhibitory neurotransmitter release is consequent to a direct SP-dependent depolarization of NK1-positive GABAergic/glycinergic terminals contacting the responsive cells. Rather, it is conceivable that the site(s) of SP release and NK1 activation, was(were) not so close to the cell body of the recorded neuron. Such a release can occur in lamina I or lamina III/IV, since these are the laminae with more intense NK1 immunoreactivity (Ribeiro-Da-Silva et al., 2001).
In L732,138 + AP-5 experiments, blockade of NMDA receptors resulted in a reduction of the NK1-dependent contribution to the enhancement of sIPSCs elicited by capsaicin. This finding is consistent with the notion that NMDA and NK1 receptors cooperatively interact in producing central sensitization (Thompson et al., 1994; Cumberbatch et al., 1995; Harris et al., 2004). Moreover, NMDA acts as an autoreceptor in PAFs, thereby facilitating SP release (Liu et al., 1997; Afrah et al., 2001; Lao et al., 2003).

Are laminae I and III–IV interneurons activated in vivo by SP to modulate lamina II inhibitory neurotransmission?

A substantial body of observations arising from the present study converge to indicate that enhancement of IPSCs evoked by capsaicin in lamina II neurons follows the activation of laminae I and III–IV inhibitory interneurons by SP released from TRPV1-expressing PAF endings (Fig. 8A and

B). In keeping, physiological data have shown a functional relationship between capsaicin-sensitive PAFs and the spinal cord neurons expressing NK1 receptors (Lao et al., 2003), that are likely to be monosynaptically linked to each other (Labrakakis and MacDermott, 2003). Moreover, SP is released from TRPV1-expressing PAFs in a dose-depen- dent manner (Afrah et al., 2001; Lao et al., 2003), and a SP-mediated loss of GABA-immunoreactivity in lamina II neurons was demonstrated after capsaicin treatment in intact animals (Wei and Zhao, 1996). The role of TRPV1 in peripheral inflammation has been fully clarified (Carlton and Coggeshall, 2001), and studies in TRPV1 knock out mice have demonstrated that these animals still respond to thermal stimuli, but do not develop inflammatory pain behaviors (Caterina et al., 2000; Davis et al., 2000). In central nervous system, the biological effects of TRPV1 are still poorly understood. The present demonstration that upon activation of central TRPV1s SP acts as a relevant parallel pathway independent from glutamate to transfer nociceptive information to lamina II neurons opens the way to speculate about possible therapeutic implications of these findings. It is well-known for example that NK1 antagonists per se are poor analgesics in man (Hill, 2000), and thus it seems conceivable that the poor therapeutic efficacy of NK1 blockers reflects the fact that SP can stimulate the release of GABA/glycine in lamina II thereby affecting the inhibitory tone in DH. On the other hand, the strongest glutamate antagonists, such as ketamine, have psychomimetic effects (Griebel, 1999; Sang, 2000; Hocking and Cousins, 2003; Annetta et al., 2005). Therefore it might be of benefit to combine NK1 blockers and weak glutamate antagonists for a more effective control of certain painful conditions.

Acknowledgements

This work was supported by grants of the University of Torino, Regione Piemonte (Ricerca Sanitaria Finalizzata Bando, 2004 and Ricerca Scientifica fondi CIPE, 2004), Compagnia di San Paolo, and Fondazione CRT Torino. We are greatly indebted to Prof. Rita Bardoni for her critical reading of the manuscript and suggestions, and to Prof. Fiorella Biasi and Dr. Barbara Vizio for help with confocal microscope Z series reconstructions.

References

Afrah AW, Stiller CO, Olgart L, Brodin E, Gustafsson H. Involvement of spinal N-methyl-D-aspartate receptors in capsaicin-induced in vivo release of substance P in the rat dorsal horn. Neurosci Lett 2001;316:83–6.

Annetta MG, Iemma D, Garisto C, Tafani C, Proietti R. Ketamine: new indications for an old drug. Curr Drug Targets 2005;6:789–94. Baccei ML, Fitzgerald M. Development of GABAergic and glycinergic transmission in the neonatal rat dorsal horn. J Neurosci 2004;24:4749–57.

Baccei ML, Bardoni R, Fitzgerald M. Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. J Physiol 2003;549:231–42.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 2002;35:721–31.

Carlton SM, Coggeshall RE. Peripheral capsaicin receptors increase in the inflamed rat hindpaw: a possible mechanism for peripheral sensitization. Neurosci Lett 2001;310:53–6.

Castro-Lopes JM, Tavares I, Tolle TR, Coimbra A. Carrageenan-induced inflammation of the hind foot provokes a rise of GABA-immunoreactive cells in the rat spinal cord that is prevented by peripheral neurectomy or neonatal capsaicin treatment. Pain 1994;56:193–201.

Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 2001;24:487–517.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816–24.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, et al. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000;288:306–13.

Chéry N, De Koninck Y. Junctional versus extrajunctional glycine and $GABA_A$ receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. J Neurosci 1999;19:7342–55.
Cumberbatch MJ, Chizh BA, Headley PM. Modulation of excitatory amino acid responses by tachykinins and selective tachykinin receptor agonists in the rat spinal cord. Br J Pharmacol 1995;115:1005–12.
Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, et al. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 2000;405:183–7.
Doly S, Fischer J, Conrath M. The vanilloid receptor-1 (TRPV1) is expressed in some rat dorsal horn NK1 cells. Brain Res 2004;1004:203–7.
Drew GM, Siddall PJ, Duggan AW. Mechanical allodynia following contusion injury of the rat spinal cord is associated with loss of GABAergic inhibition in the dorsal horn. Pain 2004;109:379–88.
Garry MG, Walton LP, Davis MA. Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from the spinal cord is mediated by nitric oxide but not by cyclic GMP. Brain Res 2000;861:208–19.
Go VL, Yaksh TL. Release of substance P from the cat spinal cord. J Physiol 1987;391:141–67.
Griebel G. Is there a future for neuropeptide receptor ligands in the treatment of anxiety disorders? Pharmacol Ther 1999;82:1–61.
Grudt TJ, Perl ER. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J Physiol 2002;540:189–207.
Gunthorpe MJ, Benham CD, Randall A, Davis JB. The diversity in the vanilloid (TRPV) receptor family of ion channels. Trends Pharmacol Sci 2002;23:183–91.
Gunthorpe MJ, Rami HK, Jerman JC, Smart D, Gill CH, Soffin EM, et al. Identification and characterisation of SB-366791, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. Neuropharmacology 2004;46:133–49.
Guo A, Simone DA, Stone LS, Fairbanks CA, Wang J, Elde R. Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons. Eur J Neurosci 2001;14:293–304.
Hao JX, Xu XJ, Wiesenfeld-Hallin Z. Intrathecal gamma-aminobutyric acidB (GABAB) receptor antagonist CGP 35348 induces hypersensitivity to mechanical stimuli in the rat. Neurosci Lett 1994;182:299–302.
Harris J, Joules C, Stanley C, Thomas P, Clarke RW. Glutamate and tachykinin receptors in central sensitization of withdrawal reflexes in the decerebrated rabbit. Exp Physiol 2004;89:187–98.
Hestrin S, Galarreta M. Synchronous versus asynchronous transmitter release: a tale of two types of inhibitory neurons. Nat Neurosci 2005;8:1283–4.
Hill R. NK1 (Substance P) receptor antagonists–Why are they not analgesic in humans? Trends Pharmacol Sci 2000;21:244–6.
Hocking G, Cousins MJ. Ketamine in chronic pain management: an evidence-based review. Anesth Analg 2003;97:1730–9.
Jang IS, Jeong HJ, Katsurabayashi S, Akaike N. Functional roles of presynaptic GABA(A) receptors on glycinergic nerve terminals in the rat spinal cord. J Physiol 2002;541:423–34.
Jeong HJ, Jang IS, Moorhouse AJ, Akaike N. Activation of presynaptic glycine receptors facilitates glycine release from presynaptic terminals synapsing onto rat spinal sacral dorsal commissural nucleus neurons. J Physiol 2003;550:373–83.
Kawasaki Y, Fujita T, Kumamoto E. Enhancement of the releases of GABA and glycine during ischemia in rat spinal dorsal horn. Biochem Biophys Res Commun 2004;316:553–8.
Keller AF, Coull JA, Chery N, Poisbeau P, de Koninck Y. Region-specific developmental specialization of GABA-glycine cosynapses in laminas I–II of the rat spinal dorsal horn. J Neurosci 2001;21:7871–80.
Kuraishi Y, Minami M, Satoh M. Serotonin, but neither noradrenaline nor GABA, inhibits capsaicin-evoked release of immunoreactive somatostatin from slices of rat spinal cord. Neurosci Res 1991;9:238–45.
Labrakakis C, MacDermott AB. Neurokinin receptor 1-expressing spinal cord neurons in lamina I

and III/IV of postnatal rats receive inputs from capsaicin sensitive fibers. Neurosci Lett 2003;352:121–4.
Lao LJ, Song B, Marvizon JC. Neurokinin release produced by capsaicin acting on the central terminals and axons of primary afferents: relationship with N-methyl-D-aspartate and GABA(B) receptors. Neuroscience 2003;121:667–80.
Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, et al. Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. J Neurosci 2001;21:4469–77.
Littlewood NK, Todd AJ, Spike RC, Watt C, Shehab SA. The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience 1995;66:597–608.
Liu H, Mantyh PW, Basbaum AI. NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 1997;386:721–4.
Lynch JW. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 2004;84:1051–95.
Maccaferri G, Dingledine R. Complex effects of CNQX on CA1 interneurons of the developing rat hippocampus. Neuropharmacology 2002;43:523–9.
Maehara T, Suzuki H, Yoshioka K, Otsuka M. Characteristics of substance P-evoked release of amino acids from neonatal rat spinal cord. Neuroscience 1995;68:577–84.
Malan TP, Mata HP, Porreca F. Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. Anesthesiology 2002;96:1161–7.
Malcangio M, Bowery NG. GABA and its receptors in the spinal cord. Trends Pharmacol Sci 1996;17:457–62.
Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, et al. Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. Proc Natl Acad Sci USA 1995;92:2622–6.
Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, et al. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 1997;278:275–9.
Michael GJ, Priestley JV. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 1999;19:1844–54.
Nakatsuka T, Chen M, Takeda D, King C, Ling J, Xing H, et al. Substance P-driven feed-forward inhibitory activity in the mammalian spinal cord. Mol Pain 2005;1:20.
Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, et al. Transmission of chronic nociception by spinal neurons expressing the substance Preceptor. Science 1999;286:1558–61.
Okano K, Ueda M, Kuraishi Y, Satoh M. Effect of repeated cold stress on capsaicin-evoked release of glutamate from rat spinal dorsal horn slices. Neurosci Res 1997;29:319–24.
Rees H, Sluka KA, Westlund KN, Willis WD. The role of glutamate and GABA receptors in the generation of dorsal root reflexes by acute arthritis in the anaesthetized rat. J Physiol 1995;484:437–45.
Ribeiro-Da-Silva A, McLeod AL, Krause JE. Neurokinin receptors in the CNS. In: Björklund A, Hökfelt T, editors. Handbook of chemical neuroanatomy. Amsterdam: Elsevier; 2001. p. 195–240.
Sakuma M, Yoshioka K, Suzuki H, Yanagisawa M, Onishi Y, Kobayashi N, et al. Substance P-evoked release of GABA from isolated spinal cord of the newborn rat. Neuroscience 1991;45:323–30.
Sang CN. NMDA-receptor antagonists in neuropathic pain: experimental methods to clinical trials. J Pain Symptom Manage 2000;19:S21–5.
Sherman SE, Loomis CW. Morphine insensitive allodynia is produced by intrathecal strychnine in the lightly anesthetized rat. Pain 1994;56:17–29.
Thompson SW, Dray A, Urban L. Injury-induced plasticity of spinal reflex activity: NK1 neurokinin receptor activation and enhanced A- and C-fiber mediated responses in the rat spinal cord in vitro. J Neurosci 1994;14:3672–87.
Todd AJ, Sullivan AC. Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J Comp Neurol 1990;296:496–505.

Todd AJ, Watt C, Spike RC, Sieghart W. Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. J Neurosci 1996;16:974–82.
Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998;21:531–43.
Tominaga M, Wada M, Masu M. Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proc Natl Acad Sci USA 2001;98:6951–6.
Urban L, Dray A. Synaptic activation of dorsal horn neurons by selective C-fibre excitation with capsaicin in the mouse spinal cord in vitro. Neuroscience 1992;47:693–702.
Valtschanoff JG, Rustioni A, Guo A, Hwang SJ. Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. J Comp Neurol 2001;436:225–35.
Vergnano AM, Salio C, Merighi A. NK1 receptor activation leads to enhancement of inhibitory neurotransmission in spinal substantia gelatinosa neurons of mouse. Pain 2004;112:37–47.
Wei F, Zhao ZQ. Blockade of capsaicin-induced reduction of GABA-immunoreactivity by spantide in cat spinal superficial dorsal horn. Neuroscience 1996;71:277–83.
Yang K, Li YQ. Origins of spontaneous and noxious stimuli-evoked miniature EPSCs in substantia gelatinosa. Neuroreport 2001;12:39–42.
Yang K, Kumamoto E, Furue H, Yoshimura M. Capsaicin facilitates excitatory but not inhibitory synaptic transmission in substantia gelatinosa of the rat spinal cord. Neurosci Lett 1998;255:135–8.
Yang K, Kumamoto E, Furue H, Li YQ, Yoshimura M. Action of capsaicin on dorsal root-evoked synaptic transmission to substantia gelatinosa neurons in adult rat spinal cord slices. Brain Res 1999;830:268–73.
Yang K, Kumamoto E, Furue H, Li YQ, Yoshimura M. Capsaicin induces a slow inward current which is not mediated by substance P in substantia gelatinosa neurons of the rat spinal cord. Neuropharmacology 2000;39:2185–94.
Zou X, Lin Q, Willis WD. NMDA or non-NMDA receptor antagonists attenuate increased Fos expression in spinal dorsal horn GABAergic neurons after intradermal injection of capsaicin in rats. Neuroscience 2001;106:171–82.