Modulation of inhibitory neurotransmission by the vanilloid receptor type 1 (TRPV1) in organotypically cultured mouse substantia gelatinosa neurons

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Abstract

The vanilloid receptor type 1 (TRPV1) plays a pivotal role in modulating thermal, chemical and inflammatory pain. TRPV1s are expressed in some dorsal horn (DH) neurons, but their contribution, if any, to central pain processing still remains unclear. We studied the effects of 2 μM capsaicin-induced TRPV1 activation in organotypically cultured substantia gelatinosa neurons from post-natal (8–12) mice. Capsaicin affected sIPSC frequency (272 ± 60% of control, n = 14, P < 0.02), but not amplitude (131 ± 12% of control, n = 14, P > 0.05) in patch clamp recordings, also in the presence of 50 μM AP-5 (frequency: 265 ± 69% of control; n = 8, P < 0.05; amplitude: 156 ± 28% of control; n = 8, P > 0.05). The frequency increase was reduced by TTX (181 ± 21% of control; n = 12, P < 0.05). Pre-administration of I-RTX (1 μM), a TRPV1 antagonist, prevented the capsaicin effect (frequency: 149 ± 28% of control, P > 0.05, n = 12; amplitude: 97 ± 4% of control, P > 0.05, n = 12). NADA (1 μM), an endovanilloid/endocannabinoid agonist of TRPV1, induced a significant increase of sISPC frequency (191 ± 40% of control; n = 8, P < 0.05) without affecting the amplitude (102 ± 6% of control; n = 8, P > 0.05), and the co-application of two naturally occurring N-acyldopamines, PALDA (5 μM) and STEARDA (5 μM) that facilitate the effect of TRPV1 agonists, also induced a significant increase of sIPSC frequency (278 ± 67% of control, n = 6, P < 0.05). The presence of TRPV1 protein and mRNA in DH neurons was confirmed by histological (immunocytochemistry, in situ PCR) and biochemical (Western blotting, PCR) procedures. These data show that TRPV1 modulates inhibitory neurotransmission in cultured substantia gelatinosa neurons, and suggest that endogenous agonists can activate the spinal receptors in vivo.

Key words: TRPV1 receptor, substantia gelationsa, inhibitory post-synaptic currents, capsaicin, organotypic cultures, endovanilloids

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1. Introduction

The vanilloid receptor type 1 (TRPV1) is a cation channel play- ing a central role in the transduction of thermal, chemical, and inflammatory pain [7,14]. Not only TRPV1 is strongly expressed in small-sized neuronal cell bodies of dorsal root ganglia (DRGs) but also in their peripheral and central projections, the primary afferent fibers (PAFs) of the C and Ad types [21,28,35]. Therefore, noxious heat and inflammatory mediators can directly interact with the receptor at the site of pain generation. Specifically, the peripheral activation of TRPV1 is primarily associated with the transmission of thermal nociception and induction of thermal hyperalgesia [10,14]. Indeed, intradermal or intraplantar injections of its selective agonist capsaicin induce a burning painful sensa- tion, and the lack of the receptor in knock-out mice [4,6], the silencing of TRPV1 mRNA [9], or the selective ablation of TRPV1-expressing terminals [8] produces a reduction of inflammatory heat hyperalgesia. TRPV1-expressing central PAF endings terminate in laminae I–II of the spinal cord dorsal horn (DH) [21,28], which are known to play a critical role in the transmission and modulation of pain information. Capsaicin activation of central TRPV1s induces a strong release of glutamate and neuropeptides [1,32,47] that, in turn, activate excitatory [31,37] and inhibitory pathways [19,36] in DH. Even though most of these effects can be explained by the activation of the receptor on PAFs, two inde- pendent electron microscopy studies have shown that partof TRPV1 immunoreactivity within the superficial DH is due to TRPV1 expression in the dendrites and, to lesser extent, cell bodies of spinal neurons [18,48]. In keeping with this observation, TRPV1 immunoreactivity was recently shown to partly survive dorsal rhi- zotomy and, under these conditions, the receptor appears to be able to modulate excitatory neurotransmission [51]. These results converge to demonstrate the existence of a population of DH inter- neurons expressing functional TRPV1s. However, a true involve- ment of TRPV1s expressed other than at PAF endings in the central processing of pain modulation still needs to be proven beyond reasonable doubt. To do so we have employed an organotypic approach where the spinal cord is cultured after deafferentation and made amenable to experiments at a stage in vitro where PAFs, and hence the TRPV1s they bear, are degenerated. By this approach we have been able to isolate the role of spinal TRPV1s in the modulation of inhibitory neurotransmission.

2. Materials and methods

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

2.1. Animals and tissue sampling

P8–P12 mice (n = 58) were deeply anaesthetized with an intra- peritoneal dose of sodium pentobarbital (30 mg/kg). Additional electrophysiological experiments (see Sections 3.2 and 3.3) were also carried on in more mature animals (P21–P23, n = 10). After decapitation, the lumbar spinal cord was exposed by performing a dorsal laminectomy and dissected out 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.

For protein or RNA extraction, the dorsal half of the lumbar spinal cord, the lumbar dorsal root ganglia (DRGs), or the organotypic cultures (obtained from P8 mice and maintained for 10 days in vitro – DIV; see below) were deep frozen in liquid nitrogen and subsequently ground into powder in a mortar.
For in situ RT-PCR, immediately after dissection the lumbar spinal cord and DRGs were fixed by immersion in 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4) for 4 h at room temperature. Spinal cord organotypic cultures (as mentioned above) were fixed by simply transferring them in fixative for 30 min. Samples were subsequently embedded in paraffin wax according to standard procedures. Paraffin sections (7 lm) were mounted on Superfrost slides (Thermo Fisher Scientific, Waltham, MA, USA). All procedures were carried under RNAse-free conditions by using diethyl-pyrocarbonate treated double distilled water.

2.2. Spinal cord slice preparation

Pieces of the lumbar spinal cord were glued to an agar block that was positioned on the stage of a vibrating microtome (Leica VT 1200, Leica Microsystems, Weitzlar, Germany). While submerged in ice-cold ACSF, 350 lm-thick transverse slices were obtained, and allowed to recover in oxygenated ACSF at 35 °C for at least 30 min. Slices were either transferred in the recording cham- ber for patch clamp experiments or used for the preparation of organotypic cultures. Developmental changes occur in the post-natal spinal cord and appear to be somehow paralleled by rearrangements in organotypic DRG-spinal cord co-cultures in vitro [34]. Given that TRPV1 expression in DH is developmentally regulated [21] and that in our cultures the spinal DH is disconnected from DRGs (see Section 4), capsaicin experiments in acute slices were carried on in P8–P12 and P21–P23 animals to compare the response at these develop- mental stages.

2.3. Spinal cord organotypic culture preparation

Since the capsaicin response was unchanged in acute slices explanted within the P8–P23 interval (see Section 3), we decided to carry out all experiments on organotypic cultures obtained from P8–P12 mice that allow for an easier

explantation of the spinal cord.

Individual slices were placed onto a Millicel-CM filter (Millipore, Billerica, MA, USA) under strict sterile conditions. The filter was then placed inside a 35 mm Petri dish containing 1.1 ml cul- ture medium. The medium was composed as follows: 50% Eagle Basal Medium (Sigma, St. Louis, MO, USA), 25% horse serum (Gibco, Carlsbad, CA, USA), 25% Hanks balanced salt solution (Sigma), 0.5% glucose, 0.5% 200 mM L-glutamine, 1% antibiotic/antimycotic solu- tion (Sigma). Cultures were incubated at 34 °C in 5% CO_2 for at least 7 days. Medium was changed every three days. Cultures suitable for further analysis were selected according to previous described morphological criteria [17]

2.4. Patch clamp recordings

Patch clamp recordings were performed on 7–21 DIV cultures or in acute slices (0 DIV). Only one neuron per organotypic culture or slice was studied. No significant correlations were observed between the age of the cultures and the physiological effect on IPSCs ($P = 0.4$, $R2 = 0.066$, not shown), thus demonstrating that there were no differences in physiological properties within the in vitro time interval considered in this work. Culture filters were placed in oxygenated ASCF and the membrane around the cord was cut with a razor blade. The slice and its attached membrane portion were then transferred into the recording chamber and constantly perfused (2 ml/min) with oxygenated ACSF at room temperature (RT). Neurons were visualized using a fixed stage microscope (Axioskop 1, Zeiss, Oberkochen, Germany) equipped with infrared gradient contrast optics (Luigs and Neumann, Ratingen, Germany) and a 40x water immersion objective (Achroplan, Zeiss). All acute slice recordings were obtained from lamina II (substantia gelatinosa), easily identified as a translucent band in the superficial DH. Recordings from organotypic cultures were also taken from a re- gion corresponding to lamina II. Patch pipettes were prepared using a horizontal puller (P-97; Sutter, Novato, CA). Pipette resis- tances ranged from 3 to 5 MX once filled with the intracellular solution. Patch-clamp whole cell recordings were obtained with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Currents were sampled at 10 kHz and filtered at 2 kHz. Recordings were included for the subsequent analysis only if: (i) membrane potential was more negative than -55 mV; (ii) access resistance changed less than 20% throughout the recording session.

Passive and active membrane properties of substantia gelatinosa neurons were studied with the following intracellular solution (in mM): 125 K-gluconate, 20 KCl, 5 EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATP- Na, 0.2 GTPNa, pH 7.2 (with KOH). Membrane voltage was cor- rected off-line for the liquid junction potential (13.7 mV). A high chloride intracellular solution [containing (in mM): 145 CsCl, 5

EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATPNa, 0.2 GTPNa, pH 7.2 (with CsOH)] and a low-chloride intracellular solution [containing (in mM): 145 $CsMeSO_4$, 5 EGTA, 2 $MgCl_2$, 10 Hepes, 2 ATPNa, 0.2 GTPNa, pH 7.2 (with CsOH)] were used for studying spontaneous inhibitory (sIPS- Cs) and excitatory post-synaptic currents (sEPSCs), respectively. Both IPSCs and EPSCs were isolated at -63 mV. sIPSCs were recorded in the presence of 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo[f]quinoxaline-7-sulfonamide (NBQX, 10 lM; Tocris, London, UK) to block AMPA/Kainate-mediated neurotransmission. Lucifer Yellow (LY – 0.1%, Sigma) was added to the Cs^+ based solutions for the subsequent morphological analysis. The distance of the injected neurons from the utmost dorsal border of the slice was mea- sured in all histological samples.

Events were visually detected and analyzed off-line with Mini Analysis software (Synaptosoft, Decatur, GA, USA). According to the mean duration of capsaicin effect in organotypic slices (see Sec- tion 3), amplitude and frequency of IPSCs and EPSCs were com- pared within 120 s time intervals. Neurons were classified as responsive if the inter-event interval differences were statistically significant (Kolmogorov–Smirnov test, $P < 0.01$) and if there were >20% increases in sIPSC frequency. Decay time was calculated by fitting the decay phase of at least 50 sIPSCs per cell with either a mono- or a bi-exponential function: when the addition of one exponential significantly improved the fitting (change of $R^2 > 1\%$), the decay was considered bi-exponential and expressed as time constants s_{fast} and s_{slow}; otherwise, the decay time was considered mono-exponential and expressed as s_{mono}.

Wilcoxon matched-pairs test was used for paired data analysis, Mann–Whitney test for unpaired data analysis (unless otherwise stated). Tests were performed on rough values. Electrophysiological data are expressed as a percentage of the pre-drug control value ± SEM, with n indicating the number of neurons. Data were considered significantly different when $P < 0.05$.

All drugs were bath-applied. Capsaicin, NBQX, DL-2-amino-5-phosphonopentanoic acid (AP-5), tetrodotoxin (TTX), iodoresinf- eratoxin (I-RTX), 4^0 -chloro-3-methoxycinnamanilide (SB366791), N-arachidonoyldopamine (NADA), N-palmitoyldopamine (PALDA), N-stearoyldopamine (STEARDA), and AM251 were from Tocris. Bicuculline methiodide and strychnine methiodide were from Sigma. Capsaicin was used at 2 lM final concentration [19]. All drugs were prepared from at least 1000 times concentrated stock solutions.

2.5. **Immunohistochemistry**

Acute slices or organotypic cultures were fixed for 30 min with 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). For immunohistochemistry, after several washings in phosphate buf- fered saline (PBS; 0.02 M, pH 7.4), slices were pre-incubated in PBS containing 6% bovine serum albumin for 30 min, incubated overnight at 4 °C in goat anti-TRPV1 raised

against a peptide map- ping near the N-terminus of TRPV1 of rat origin (1:500; Santa Cruz, Biotechnology, Santa Cruz, CA) [18] with 0.1% Triton X-100, then incubated for 3 h with secondary biotinylated anti-goat 1:500 (Vector Laboratories, Burlingame, CA, USA), and for 1 h with Extravidin-Cy3 1:1000 (Sigma). Control experiments were per- formed by omitting the primary antibody. Immunofluorescence was acquired using a Leica TCS SP5 confocal microscope with 20x or 63x oil lens. Z-series reconstructions were obtained by acquiring sequential confocal optical sections at 1 lm intervals.

2.6. **Western blotting**

Proteins were extracted with standard procedures in general lysis buffer containing protease inhibitors (1 mM PMSF, 1 mg/ml leupeptin and 5 mg/ml aprotinin) from homogenates of acutely dissected mouse spinal cord or 8 DIV spinal cord organotypic cultures. After gel loading (30 lg per lane), they were separated by SDS–PAGE in 8% acrylamide gels and blotted onto nitrocellulose paper. Blots were blocked with Blotto and probed with a rabbit anti-TRPV1 antibody raised against the N-terminus sequence (res idues 4–21) of the receptor (1:1000; Abcam, Cambridge, MA, USA; [22]), followed by peroxidase-labeled anti-rabbit antibody (1:15,000; Promega Corporation, Madison, WI, USA,). Protein bands were detected by an ECL western blotting system (Amersham Biosciences, Piscataway, NJ, USA). Specificity controls were obtained by omitting the primary antibody.

2.7. **RT-PCR**

For the tube procedure, total RNA was extracted by RNAWiz (Ambion, Inc., Austin, TX, USA) with a standard protocol. RNA (0.5 lg) was reverse transcribed with Reverse Transcription System (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. HPRT-1 (hypoxanthine phosphoribosyl-transferase 1) was used as a normalization gene to correct for RNA concentration and reverse transcription efficiency. Diluted cDNAs (1:10) were used for PCR with PCR Master MIX (Promega). TRPV1 primers (mouse – Accession No. AY445519) were: forward 5' -CGAGGATGGGAAGAATAACTCACTG-3' (nucleotides 1800–1824), reverse 5' -GGATGATGAAGACAGCCTTGAAGTC-3' (nucleotides 1987–1963). HPRT-1 primers were: forward: 5' -CGAGGATGGGAAG AATAACTCACTG-3' ; reverse: 5' –GGATGATGAAGACAGCCTTGAAG TC-3'. Amplification was as follows: cycle 1, 95 aC for 5 min; cycle 2, 95 aC 1 min, 62 aC 40 s, 72 aC 40 s for 35 cycles; cycle 3, 72 aC for 5 min. PCR products were visualized with ethidium bromide after electrophoresis on 2% agarose gel. The size of the amplified cDNAs for TRPV1 and HRPT-1 was 181 and 154 bp, respectively.

The in situ procedure was performed on de-paraffinized sections after pretreatments with proteinase K and triethanolamine. Reverse transcription was performed by the Reverse Transcription System with Oligo(dT)15 primer

(Promega), amplification with PCR master mix (Promega), 11-digoxigenin-D-uridine 5' -triphosphate (1 mM), and the TRPV1 or HPRT-1 primers used above. Amplification was the same as the tube procedure. To reveal the amplification products, slices were then incubated in goat anti-digoxigenin antibodies conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany), followed by the nitro blue tetrazolium/5-bromo- 3-indolylphosphate-p-toluidine salt.

3. Results

3.1. Electrophysiological properties and morphology of substantia gelatinosa neurons in organotypic cultures

Ninety-eight neurons were recorded from the superficial DH of 7–21 DIV organotypic cultures. Twenty-one of them were success- fully injected with LY during the recording sessions. LY injected neurons (Fig. 1) were localized in a band of the superficial DH between 75 and 200 μm from the utmost superficial border and showed comparable morphological aspects to those described for substantia gelatinosa neurons in mouse acute slices [19] or rat organotypic cultures [34]. Passive and active membrane properties of these neurons are summarized in Table 1. The firing pattern was studied in 12 neurons. Most of the firing patterns described in con- ventional electrophysiological preparations of lamina II [20,23,40] were easily identified. Seven neurons exhibited a delayed firing (Fig. 2A), 1 neuron an initial bursting pattern (Fig. 2B), 3 neurons a gap firing pattern (Fig. 2C) and 1 neuron a tonic firing (Fig. 2D). In keeping with previous observations [40], delayed (60%) and gap firing (25%) were the most common patterns observed, while tonic firing, usually encountered in lamina I and III neurons, was observed in one cell only.

Spontaneous excitatory post-synaptic currents (sEPSCs) in cultures were recorded with a low-chloride intracellular solution at -63 mV. Mean frequency and amplitude were, respectively, 15.45 ± 4.01 Hz and 21.91 ± 1.90 pA (n = 13; Fig. 2E). The frequency was considerably higher than sEPSC frequency recorded in acute slices with the same intracellular solution (1.13 ± 0.37 Hz, n = 11; Mann–Whitney test, $P < 0.001$), whereas no differences were ob- served for the amplitude (18.20 ± 1.76 pA, n = 11; Mann–Whitney test, $P > 0.05$). As recently described in a different model of spinal cord organotypic cultures [34], large inward currents (named ''giant sEPSCs") with relative slow kinetics and complex waveforms were recorded in 10 of 13 neurons in coincidence with bursts of fast sEPSCs (frequency: 0.14 Hz ± 0.03 Hz; amplitude: 206.16 ±42.95 pA; Fig. 2E and F). Since the bursts of fast sEPSCs were fre- quently superimposed to the slow waveforms (Fig. 2F), it is conceivable, in keeping with the hypothesis in [34], that a large release of glutamate activates a presynaptic mechanism stimulat ing the transmitter release and a post-synaptic mechanism that generates the large

inward currents. Both ‘‘giant” and fast sEPSCs were blocked by the AMPA/Kainate receptor antagonist NBQX (10 μM; n = 7; Fig. 2G).

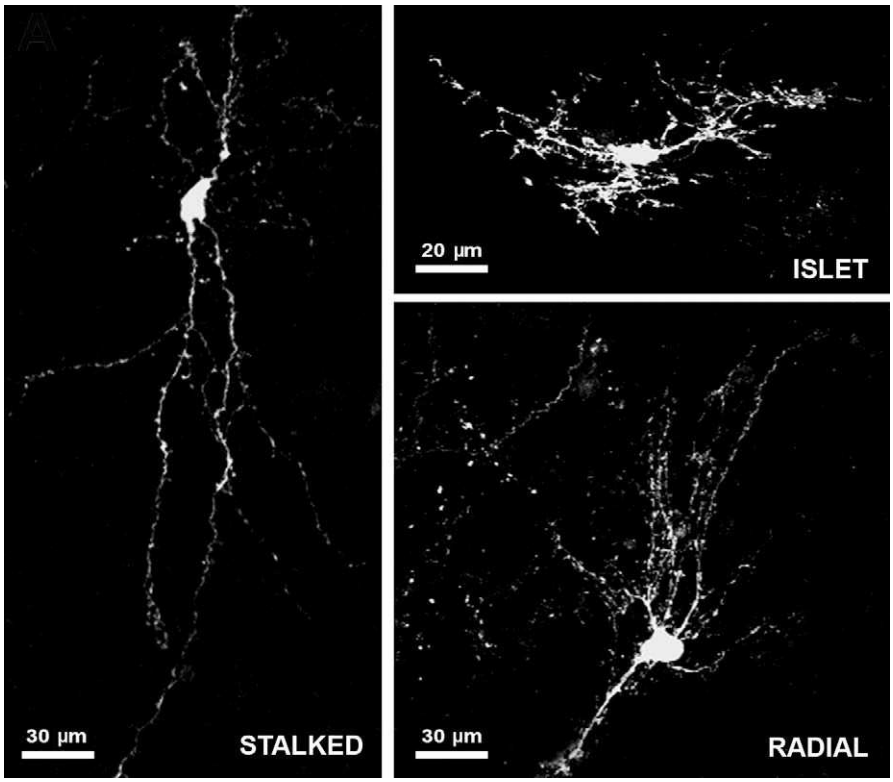

Fig. 1. Cell types and morphology of substantia gelatinosa neurons in spinal cord organotypic cultures. Three representative cells after recording and LY filling are classified as stalked, islet and radial cells according to morphological criteria [19]. Top of each panel is dorsal.

Spontaneous inhibitory post-synaptic currents (sIPSCs) in cultures were recorded using a high chloride intracellular solution at -63 mV in the presence of 10 lM NBQX. The frequency and amplitude of sIPSCs were 1.15 ± 0.24 Hz and 45.30 ± 4.93 pA (n = 18; Fig. 2H and I), respectively. Also in this case, we noticed a significantly higher sIPSC frequency in organotypic cultures than in acute slices (0.41 ± 0.10 Hz, n = 11; Mann–Whitney test, $P < 0.05$). No differences were observed concerning the sIPSC amplitude (42.82 ± 3.88, n = 11; Mann–Whitney test, $P > 0.05$).

The nature of sIPSCs was determined by bath-application of the $GABA_A$ receptor antagonist bicuculline (10 lM) or the glycine receptor antagonist strychnine (1 lM; Fig. 2J). In line with our pre- vious results in acute slices [19], bicuculline alone abolished all
ongoing fast events in 5 of 9 neurons. In the remaining, sIPSCs were blocked by the co-application of bicuculline and strychnine. How- ever, in the presence of $GABA_A$ and glycine receptor antagonists we observed the recurrence of slow and large inward waveforms (823.80 ± 97.27 pA, n = 5), likely corresponding to the previously described ‘‘giant sEPSCs” (Fig. 2J).

Table 1
Passive and active membrane properties of substantia gelatinosa neurons in organo- typic spinal cord cultures (>6 DIV). Numbers in parentheses indicate the number of cells.

Resting membrane potential (mV) (11)	-69.0 ± 2.1
Membrane resistance (MX) (13)	543.2 ± 60.4
Membrane capacitance (pF) (13)	35.3 ± 3.3
Action potential threshold (mV) (12)	44.5 ± 1.2
Action potential width (at the threshold, ms) (12)	2.5 ± 0.1
Action potential height from the base (mV) (12)	85.4 ± 4.0
Action potential height from the threshold (mV) (12)	78.6 ± 3.9
sEPSC frequency (Hz) (13)	15.4 ± 4.0
sIPSC frequency (Hz) (18)	1.2 ± 0.2

These currents had a mean amplitude of 764.04 ± 99.39 pA (n = 5), a relative fast rise time (125.42 ± 40.88 ms; n = 4), and a slow decay time (1247.22 ± 166.77 ms; n = 4). In line with the effect of strychnine and bicuculline on rhythmic bursting activity in organotypic spinal networks [5], under these experimental conditions, ‘‘giant sEPSCs” showed a regular pattern of activity. Bath-applied AP-5 (50 lM), a NMDA receptor antagonist, completely blocked all ongoing large inward currents (n = 3; Fig. 2K).

3.2. Effect of capsaicin on sEPSCs – acute slices and organotypic cultures

A first series of experiments were performed in order to com- pare the capsaicin effect on lamina II excitatory neurotransmission in acute slices and organotypic cultures. sEPSCs were recorded with the ‘‘low chloride” intracellular solution. Administration of capsaicin (2 lM – 1 min) on acute mouse spinal cord slices obtained from P8–P12 mice elicited a strong and long lasting sEPSC frequency increase (1437 ± 578% of the control; n = 6, $P < 0.05$), without affecting amplitude (109 ± 11%; n = 6, $P > 0.05$; Fig. 3A and B). In more mature animals (P21–P23), capsaicin induced a similar frequency increase (1576 ± 384% of control; n = 9, $P < 0.01$; Fig. 3B – light bars). A slow inward current was usually

detected at the onset of the effect. Conversely, capsaicin (2 μM – 1 min) did not significantly change sEPSC frequency (126 ± 30% of the control; n = 13, P > 0.05) or amplitude (95 ± 2% of the control; n = 13 P > 0.05) in cultured substantia gelatinosa neurons (Fig. 3C and D). About 50% of recorded neurons showed a slow inward current during administration of the drug (n = 6; 11.82 ± 3.25 pA).

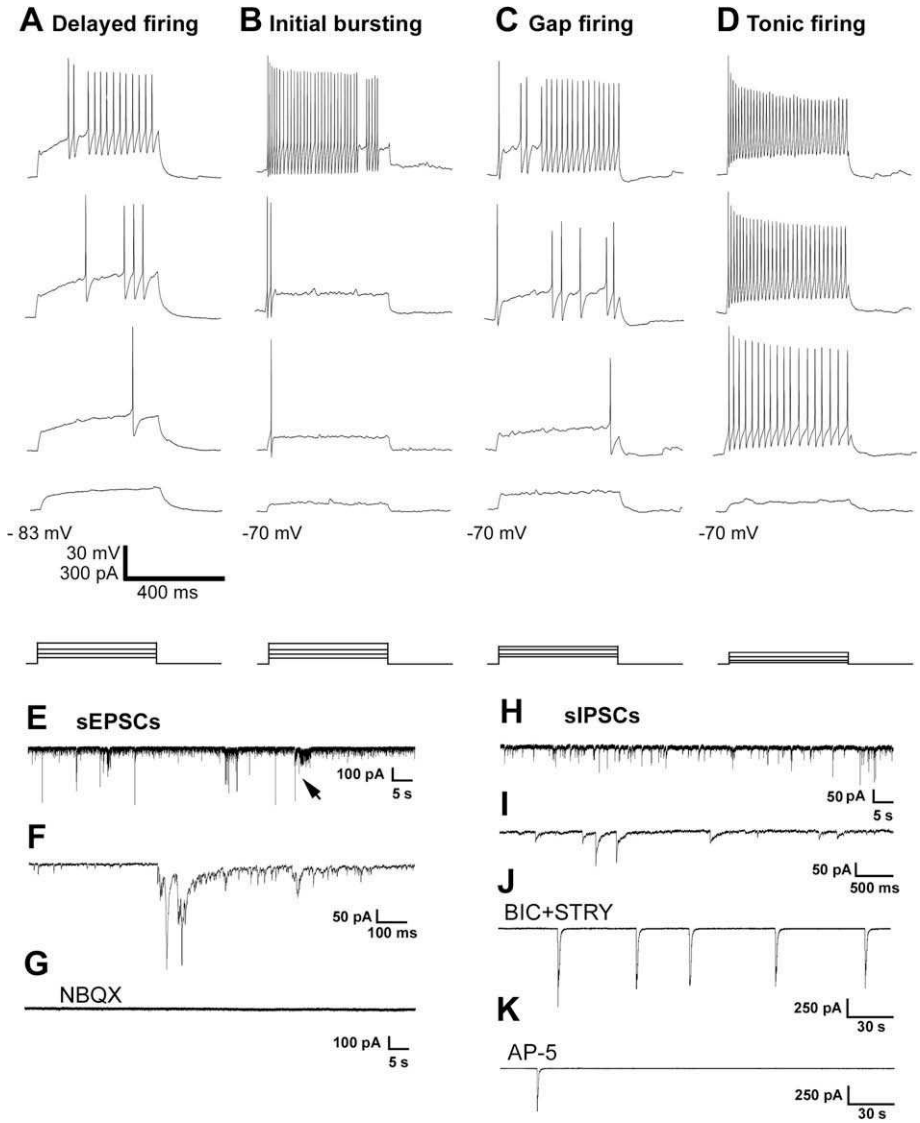

Fig. 2. Firing patterns and spontaneous activity of superficial DH neurons from spinal cord organotypic cultures. (A–D) Firing patterns are obtained in response to injections of depolarizing currents (25 pA steps), hold at different membrane potentials (between -55 and -60 mV, -65 and -70 mV, and -70 and -80 mV) to investigate their dependence on the holding potential. From left to right, a delayed firing neuron (A), an initial bursting neuron (B) a gap firing neuron (C) and a tonic firing neuron (D). (E) A representative trace showing sEPSCs recorded with a 0 mM Cl^- intracellular solution at VH = -63 mV; the arrow indicates a large ‘‘giant” sEPSCs. (F) Particular of the ‘‘giant” sEPSCs indicated by the arrow in (E). (G) NBQX (10 lM) completely blocks both ‘‘giant” and small sEPSCs. (H) A representative trace showing sIPSCs recorded with a 145 mM Cl^- intracellular solution at

VH = -63 mV in the presence of NBQX 10 lM. (I) Enlargement of the trace in (H). (J) sIPSCs are blocked by bicuculline (10 lM) and strychnine (2.5 μM); however, large and slow inward currents quickly appear in the presence of the antagonists. (K) A further addition of the NMDA receptor antagonist AP-5 (50 lM) blocks the large inward currents. Abbreviations: sEPSCs, spontaneous excitatory post-synaptic currents; sIPSCs, spontaneous inhibitory post-synaptic currents; BIC, bicuculline; Stry, strychnine.

3.3. Effect of capsaicin on IPSCs – acute slices

To investigate the capsaicin effect on inhibitory neurotransmission, sIPSCs were recorded with the ‘‘high chloride” intracellular solution in the presence of NBQX (10 μM; Fig. 4A). According to our previous data [19], bath-applied capsaicin on acute slices induced a strong (rv5-fold) and long lasting (>5 min) increase of sIPSC frequency in either P8–P12 or P21–P23 mice (567 ± 142% of the control, n = 7, P < 0.02 and 574 ± 225% of the control, n = 10, P < 0.01, respectively), without affecting the overall mean amplitude (115 ± 7% of the control, n = 7 and 114 ± 11% of the con- trol, n = 10, respectively, P > 0.05; Fig. 4A and B).

3.4. Effect of capsaicin on IPSCs – organotypic cultures

In organotypic cultures also, capsaicin induced a strong increase of mean sIPSC frequency (272 ± 60% of the control, n = 14, P < 0.02), without significantly affecting the amplitude (131 ± 12% of the control, n = 14, P > 0.05; Fig. 4C and D). Frequency increase was sig- nificant in 10 of 14 cells (411 ± 51% of the control; P < 0.02), whereas a significant amplitude increase was observed in 5 of these cells (144 ± 17% of the control; P < 0.05). However, the duration of capsaicin effect was shorter than in acute slices (119.40 ± 21.95 s, n = 10; Fig. 4A and C), and frequency quickly re- turned to control values after removal of the vanilloid (Fig. 4C). In 11 neurons, a slow inward current was observed during capsaicin administration (28.16 ± 5.18 pA). Interestingly, when capsaicin was applied twice to the same neuron (after a wash-out of at least 10 min), no effects due to receptor desensitization were observed: the frequency increase was similar or even stronger after the second capsaicin administration (486 ± 97% of the control) compared to the first one (227 ± 25% of the control, n = 4) and the amplitude of the slow inward currents was unchanged (23.44 ± 3.49 pA after the first pulse and 24.51 ± 5.02 pA after the second, n = 4; data not shown).

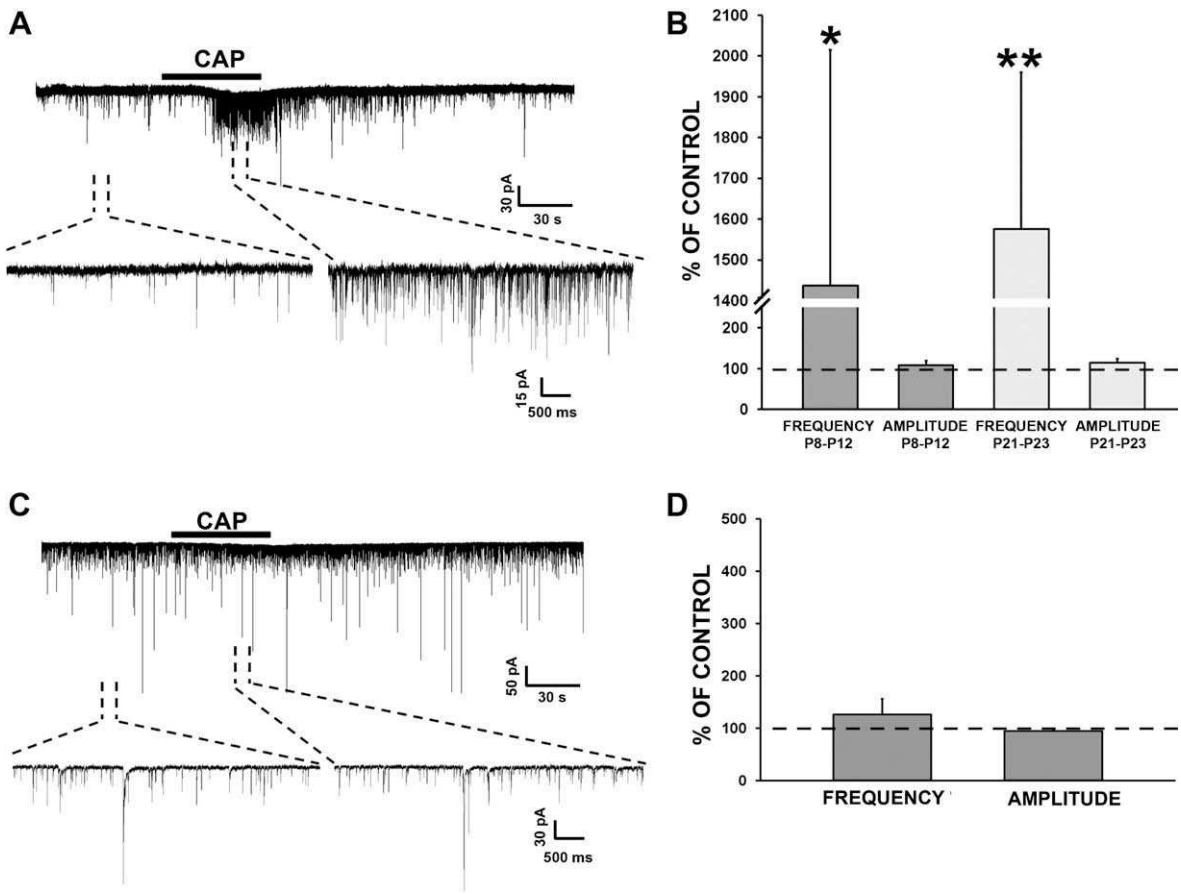

Fig. 3. Capsaicin effect on sEPSCs in spinal cord mouse acute slices and organotypic cultures. (A). Representative trace recorded from a lamina II neuron in an acute slice. Capsaicin (2 μM – 1 min) induced a strong and long lasting increase of sEPSC frequency (see the relative enlargements of control and capsaicin effect below the main trace) and a slow inward current. (B) Pooled data of frequency and amplitude from P8–P12 (dark bars) or P21–P23 (light bars) mouse lamina II neurons, showing a significant frequency increase under capsaicin; values are normalized to the control (dashed line). (C) Representative trace recorded from a lamina II neuron in a 10 DIV organotypic culture. Capsaicin (2 lM – 1 min) did not induce any effect on sEPSC frequency (see the relative enlargements of control and capsaicin effect below the main trace), but only a slow inward current. (D) Pooled data of frequency and amplitude from 13 organotypically cultured substantia gelatinosa neurons; values are normalized to the control (dashed line). Abbreviations: CAP, capsaicin; *P < 0.05, **P < 0.01.

These data suggest that TRPV1 in dorsal horn neurons may have different desensitization properties compared to TRPV1 in sensory neurons [3,30]. The increase of sIPSC frequency was not accompanied by a change in their kinetics. In particular, no significant changes were observed in the decay time of both mono-exponential (s_{mono}: 105 ± 5.31 ms, capsaicin 20.79 ± 4.79 ms, P = 0.8, n = 8) and bi-exponential (s_{fast}: 105 ± 11% of the control, P = 0.9; s_{slow}: 96 ± 7% of the control, P = 0.6, n = 8) sIPSCs (Fig. 4E and F). According to previous studies, bi-exponential decaying IPSCs are mixed GABAergic/glycinergic events [29]. Mixed events under control condition were 47% of the sIPSCs, while after capsaicin the proportion was slightly smaller (42%; Fig. 4G). However, this difference was not significant at the v^2 test (P = 0.06). To determine whether the activation of NMDA receptor was involved in the TRPV1-

induced increase of inhibitory neurotransmitter release in organotypic cultures, the effect of capsaicin was also studied in the presence of AP-5 (50 lM; Fig. 5A). Under this condition, capsaicin still elicited a significant increase of sIPSC frequency (265 ± 69% of the control; n = 8, P < 0.05), without affecting the amplitude (156 ± 28% of the control; n = 8, P > 0.05) (Fig. 5B). No significant differences were found between the mean frequency increases with and without AP-5 (Mann–Whitney test, P > 0.05). Pre-administration of I-RTX (1 μM), a specific TRPV1 antagonist, prevented the capsaicin effect (frequency: 149 ± 28% of the control, P > 0.05, n = 12; amplitude: 97 ± 4% of the control, P > 0.05, n = 12; Fig. 5C and D).

An additional series of experiments was carried out in the pres- ence of TTX (1 μM) to block voltage dependent sodium channels (Fig. 5E). A significant but slighter effect of capsaicin on miniature IPSC (mIPSC) frequency was observed (181 ± 21% of the control; n = 12, P < 0.05; Fig. 5F). However, in keeping with our previous data on acute slices [19], the effect was seen only in 4 of 12 neurons (266 ± 15% of the control; Kolmogorov–Smirnov test, P < 0.01). No significant amplitude changes wereobserved (110 ± 6% of the control; n = 12, P < 0.05; Fig. 5F). These data suggest that the effect of capsaicin is largely mediated by action potentials, and therefore TRPV1 receptors are unlikely to be localized on inhibitory axon terminals in substantia gelatinosa neurons.

3.5. Effect of the putative endovanilloid NADA on inhibitory neurotransmission – organotypic cultures

The endovanilloid/endocannabinoid NADA has been proposed as endogenous agonist of TRPV1 [25,41]. Since the cannabinoid 1 (CB1) receptor is also expressed in DH [42,43], experiments to test the responsiveness of organotypically cultured substantia gelatinosa neurons to NADA were carried out in the presence of AM251 (10 μM), an antagonist of CB1.

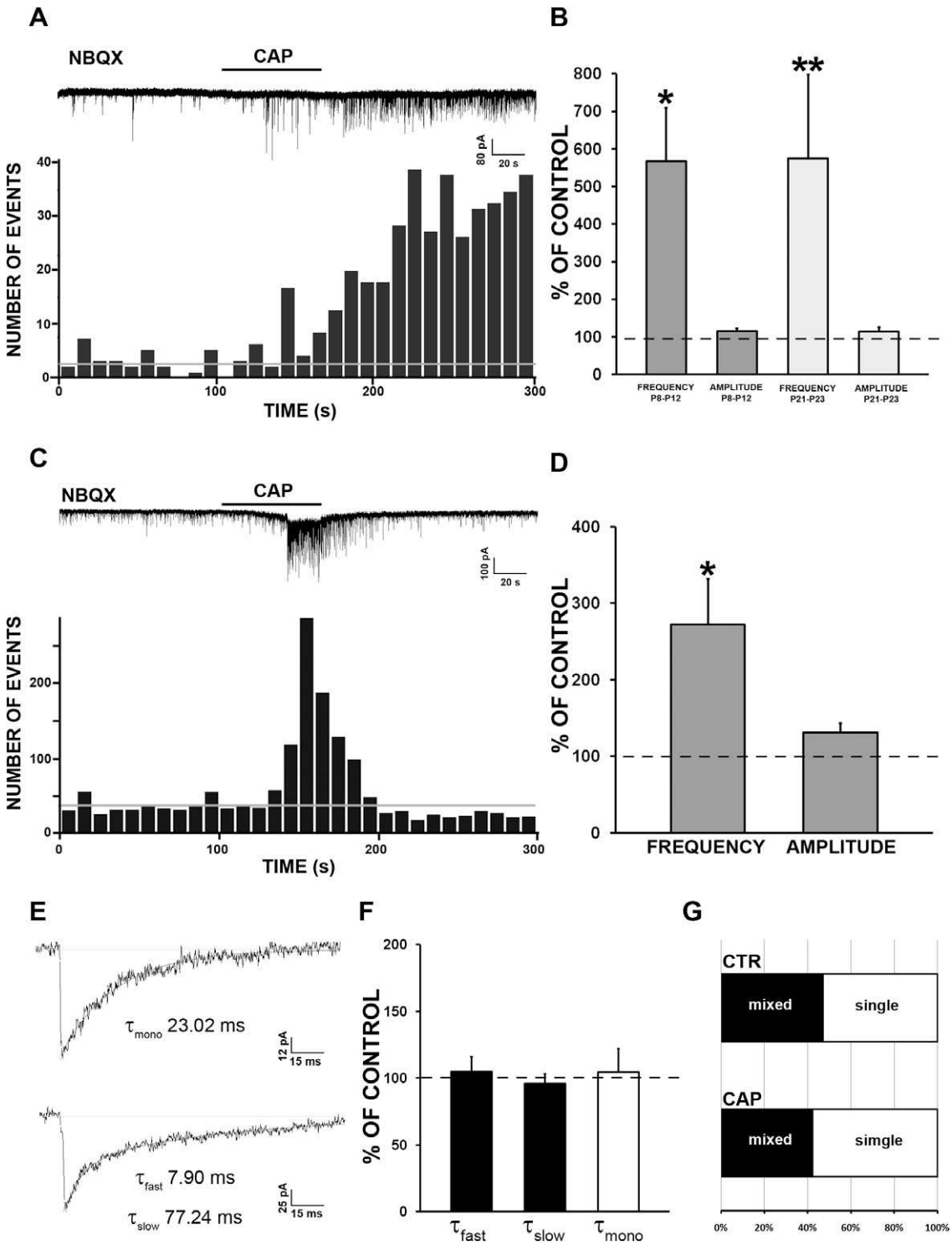

Fig. 4. Capsaicin effect on sIPSCs in spinal cord mouse acute slices and organotypic cultures. (A) Representative trace recorded from a lamina II neuron in an acute slice. Capsaicin (2 lM – 1 min) in the presence of NBQX (10 lM) induced a strong and long lasting increase of sIPSC frequency and a slow inward current. Below, the time course of the recording; note that the frequency does not return to the control level (grey line) after capsaicin wash-out. (B) Pooled data of frequency and amplitude from P8–P12 (dark bars) or P21–P23 (light bars) mouse lamina II neurons, showing a significant frequency increase under capsaicin; values are normalized to the control (dashed line). (C)

Representative trace recorded from a lamina II neuron in a 10 DIV organotypic culture. Capsaicin (2 μM – 1 min) in the presence of NBQX (10 μM) induced a strong increase of sIPSC frequency and a slow inward current. Below, the time course of the recording; note that frequency quickly returns to the control level (grey line) after capsaicin wash- out. (D) Pooled data of frequency and amplitude from 14 organotypically cultured neurons, showing a significant frequency increase under capsaicin; values are normalized to the control (dashed line). (E) Representative sIPSCs with a decay phase better fit by a mono-exponential (up) or a bi-exponential function (down). (F) Pooled data of decay time constants from 7 organotypically cultured neurons, showing no significant changes under capsaicin. (G) Diagram showing the proportion of mono-exponential vs. bi-exponential (likely mixed GABAergic/glycinergic) decaying events. Abbreviations: CAP, capsaicin; *P < 0.05, **P < 0.01.

Under these conditions, NADA (1 μM – 1 min) induced a significant increase of sISPC frequency (191 ± 40% of the control; n = 8, P < 0.05) without affecting the amplitude (102 ± 6% of the control; n = 8, P > 0.05; Fig. 6A and B– grey bars). However, NADA-induced frequency increase was about 70% of the frequency increase after capsaicin and only 50% of recorded neurons were responsive (4 of 8).

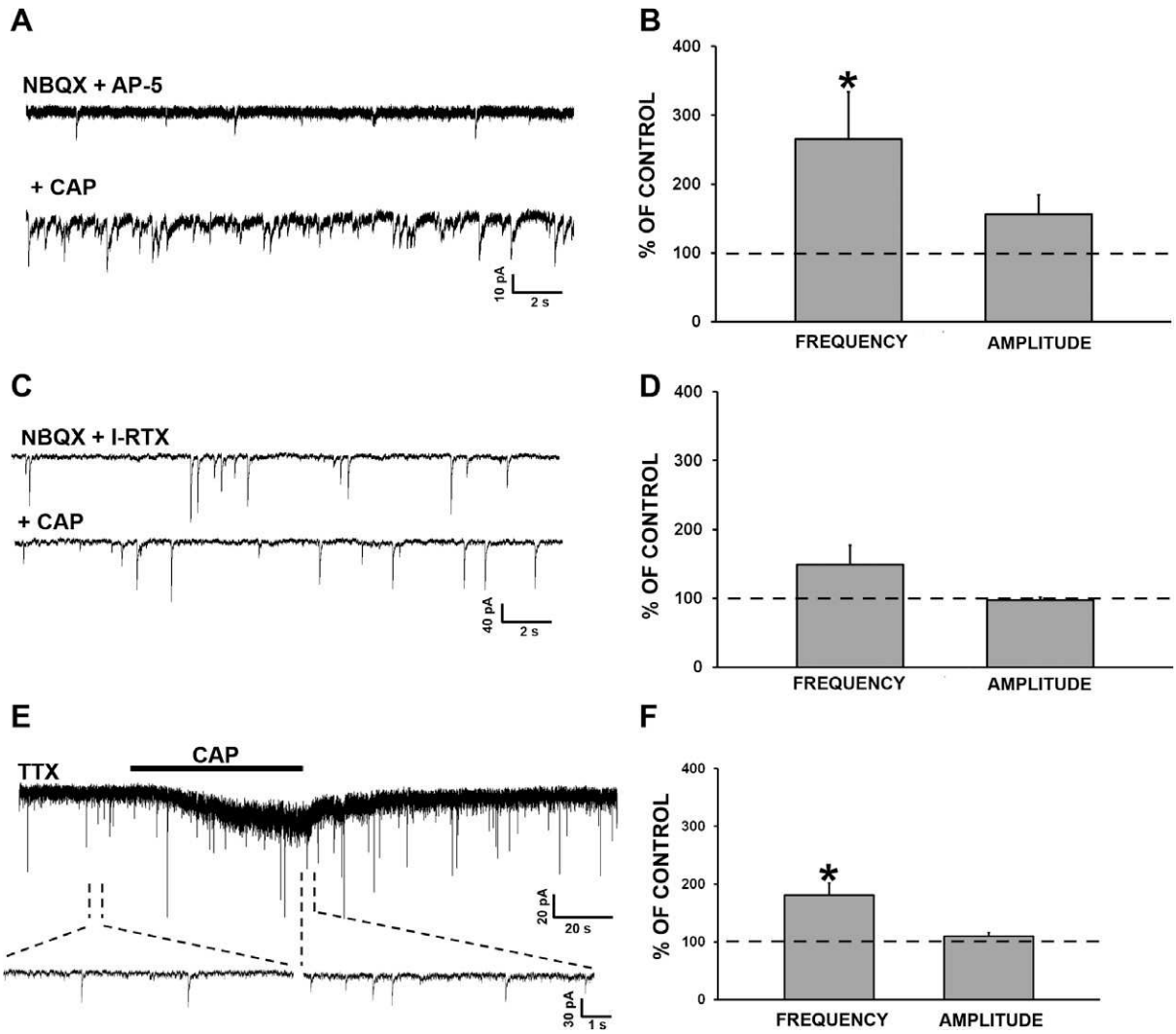

Fig. 5. Specificity and pharmacology of capsaicin effect. (A) Representative trace recorded from a substantia gelatinosa neuron in a 8 DIV organotypic culture. Capsaicin (2 μM– 1 min) in the presence of NBQX (10 μM) and AP-5 (50 μM) induced a strong increase of sIPSC frequency. (B) Pooled data of frequency and amplitude from 8 organotypically cultured neurons, showing a significant frequency increase under capsaicin; values are normalized to the control (dashed line). (C) Representative trace recorded from a substantia gelatinosa neuron in a 10 DIV organotypic spinal cord slice. Capsaicin (2 lM – 1 min) in the presence of NBQX (10 μM) and I-RTX (1 μM) had no effect on sIPSC frequency. D. Pooled data of frequency and amplitude from 12 organotypically cultured neurons, in the presence of I-RTX. (E) Capsaicin (2 μM – 1 min) in the presence of NBQX (10 μM) and TTX (1 μM) induced a slight increase of mIPSC frequency (see the relative enlargements of control and capsaicin effect below the main trace) and a slow inward current. (F) Pooled data of frequency and amplitude from 12 organotypically cultured neurons, showing a mild frequency increase under capsaicin; values are normalized to the control (dashed line). Abbreviations: CAP, capsaicin; I-RTX, iodoresinferatoxin; TTX, tetradotoxin; *$P < 0.05$.

These observations are consistent with a lower efficacy of NADA compared to capsaicin, at least under basal conditions [38]. The effect was prevented by pre-incubation with the TRPV1 antagonist I-RTX (1 μM; frequency: 89 ± 24% of the

control, n = 4, P > 0.05; amplitude of the control: 93 ± 2%, n = 4, P > 0.05; Fig. 6B – black bars).

Interestingly, the co-application for 5 min of two naturally occurring N-acyldopamines, PALDA (5 lM) and STEARDA (5 lM) [10,15], also induced a significant increase of sIPSC frequency (278 ± 67% of the control, n = 6, P < 0.05; Fig. 6C and D). Since PALDA and STEARDA are not supposed to directly activate TRPV1, but are likely to facilitate the effect of endogenous TRPV1 agonists (‘‘entourage compounds”) [15], the present data suggest that these molecules may increase the ability of endogenous agonists, such as NADA, to facilitate TRPV1 opening. The effect on sIPSC frequency was prevented in the presence of the TRPV1 antagonist SB366791 (10 lM; 143 ± 49% of the control, n = 4, P > 0.05; Fig. 6D – black bar). In the presence of PALDA and STEARDA we also noticed a reduction of about 20% of sIPSC amplitude (79 ± 9% of the control, n = 6, P < 0.05; Fig. 6D – grey bar). However a similar reduction was also observed in the presence of SB366791 (80 ± 15% of the control, n = 4; Fig 6D – black bar), and it is therefore related to a non-specific effect of TRPV1.

3.6. Activation of TRPV1 receptors in lamina II neurons and cell morphology

The effect of TRPV1 activation on inhibitory neurotransmission in organotypic cultures was correlated to the cell morphology. Fourteen LY injected neurons were analyzed and classified as islet (n = 5), radial (n = 4) and stalked cells (n = 5), according to previous criteria [20]. As previously reported [19], there was not significant correlation between cell morphology and sISPC frequency increase (Kruskal–Wallis test, P = 0.6).

3.7. TRPV1 expression in organotypic cultures

It is well known that TRPV1 is strongly expressed in the superficial laminae of the intact spinal cord in vivo, as well as in acute spinal cord slices (Fig. 7A), as described by others [22].

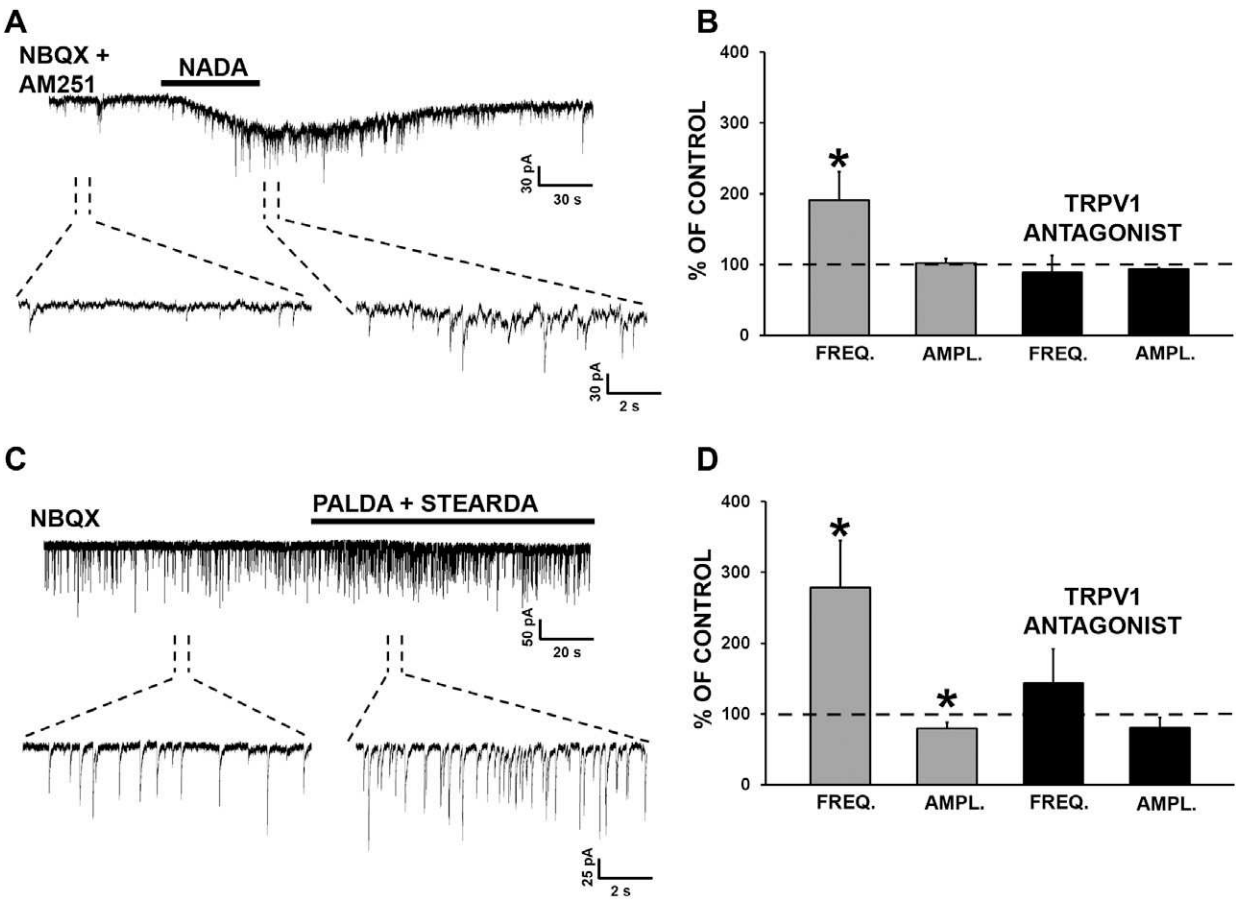

Fig. 6. Effect of putative endogenous vanilloid/cannabinoid molecules on TRPV1 organotypically cultured substantia gelatinosa neurons. (A) Representative trace recorded from a neuron at 8 DIV. NADA (1 μM – 1 min) in the presence of NBQX (10 μM) and AM251 (10 μM) increased sIPSC frequency (see the relative enlargements of control and NADA effect below the main trace) and induced a slow inward current. (B) Pooled data of frequency and amplitude under NADA alone (grey bars, n = 8) or under NADA in the presence of I-RTX (1 μM; black bars; n = 4); values are normalized to the control (dashed line). (C) Representative trace recorded from a neuron at 8 DIV. Steady bath- application of PALDA (5 μM) and STEARDA (5 μM) increased sIPSC frequency (see the relative enlargements of control and effect below the main trace). (D) Pooled data of frequency and amplitude under PALDA/STEARDA only (grey bars, n = 6) or PALDA/STEARDA in presence of SB366791 (10 μM; black bars; n = 4); values are normalized to the control (dashed line). Abbreviations: AMPL., amplitude; FREQ., frequency.

In keeping with the functional demonstration deriving from our electrophysiological results, we have obtained evidence for the expression of the TRPV1 protein and its mRNA in organotypic cultures. After immunofluorescence, the TRPV1 protein is detected in a delicate mesh of processes within the superficial DH (Fig. 7B). The protein is also present in extracts from acute slices and organotypic cultures probed by Western blotting (Fig. 8A). After RT-PCR procedures, the TRPV1 mRNA was shown to be present in the spinal cord DH both in situ (Fig. 7C–F) and after biochemical extraction (Fig. 8B). Specifically, the in situ procedure carried out on organotypic

cultures has demonstrated that mRNA was localized to a pop- ulation of small-sized neurons in the superficial DH (Fig. 7E and F). As expected, there were strong levels of expression of the TRPV1 mRNA in the DRGs used as positive controls (Figs. 7D and 8B).

4. Discussion

Here we studied the effects of TRPV1 activation on DH neurotransmission by using an organotypic spinal cord culture model. Since all long-distance travelling axons are cut, our model appears to be suitable for studying neurons having their axonal arbors confined within the extension of the spinal cord slice that is put in culture, i.e. the substantia gelatinosa neurons. Our data demonstrate that, under our experimental conditions, TRPV1 activation modulates inhibitory but not excitatory neurotransmission in mouse substantia gelatinosa.

4.1. Morphological and functional characterization of substantia gelatinosa neurons in mouse post-natal organotypic spinal cord cultures

For periods as long as several weeks in vitro, CNS organotypic cultures [17,24,34] have been proven to maintain cytoarchitectonic boundaries, laminar layouts and neuronal phenotypes in a fashion similar to the in vivo situation. Nonetheless they are subjected to a certain degree of morphological and functional plasticity as a consequence of the interruption of afferent and efferent projections that inevitably occurs during the slicing procedure [33]. Lu et al. [34] have shown that the fundamental development of substantia gelatinosa neuron phenotypes in spinal cord cultures obtained from rat foetuses with attached DRGs is comparable to age-related acute slices. Interestingly, they did not report significant differences between the physiological properties of DH neurons in cultures vs. acute slices, with the exception of ‘‘giant” sEPSCs and increased network excitability. Although our organotypic cultures were obtained from another species and by the use of a remarkably different approach, the morphological and functional characterization of the substantia gelatinosa neurons performed here yielded striking similar results.

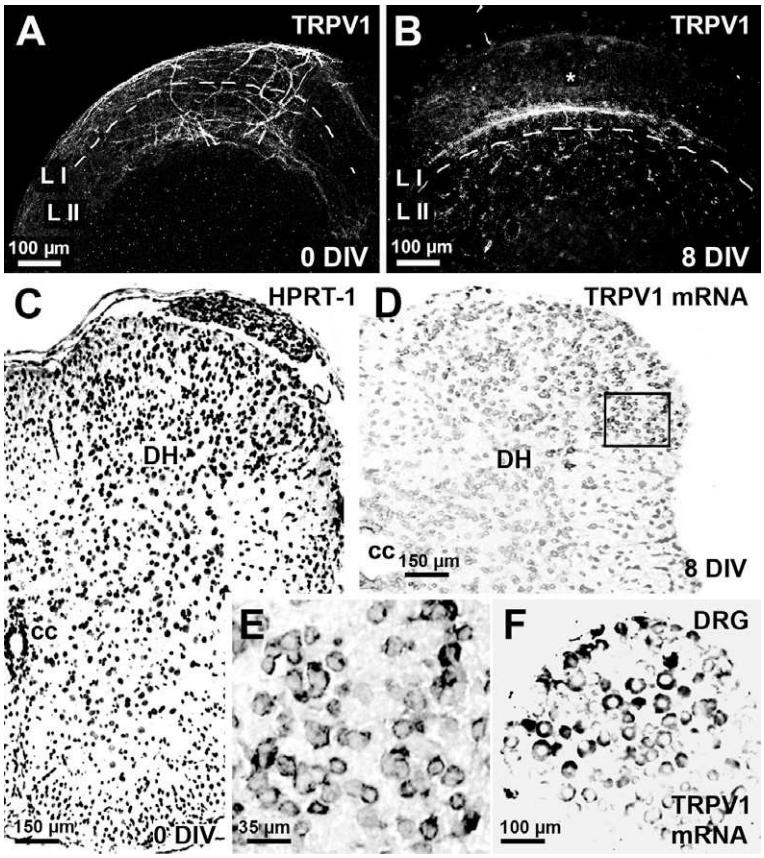

Fig. 7. Expression of TRPV1 protein and mRNA in histological samples. (A and B) Expression of TRPV1 protein in acute slices and organotypic cultures of mouse spinal cord. (A) In acute slices, TRPV1 immunoreactivity is detected in fibers within laminae I–II. (B) TRPV1 expression in an organotypic culture at 8 DIV appears in the form of a delicate mesh of processes within laminae I–II. (C–F) Localization of TRPV1 mRNA after in situ RT-PCR. (C) Ubiquitous expression of HPRT-1 mRNA as a positive control. (D and E) Expression of TRPV1 mRNA in an organotypic culture after 8 DIV. Neurons in the superficial laminae of DH are intensely labeled. The area of the rectangle in (D) is shown at higher magnification in (E). (F) Expression of TRPV1 mRNA in DRG neurons of heterogeneous sizes. Abbreviations: cc, central canal; DH, dorsal horn; DIV, days in vitro; *, culture outgrowth area; LI, lamina I; LII, lamina II.

It is generally accepted that some properties of organotypic cultures with regard to neuronal morphology and functional connections substantially differ from the characteristics acquired during the physiological brain maturation in vivo [33]. For example, fluorescent dye tracing of single neurons in organotypic hippocampal slices showed a more complex pattern of dendritic branching, and, consistently with this, a significant increase in the frequency of glutamatergic miniature synaptic currents, probably reflecting an increased number of total synapses [16]. Therefore, cutting of projection axons may lead to a compensatory response over the course of the culture period, so that damaged axons have a chance to recover and reroute their processes to form new neural connections. In this respect, ‘‘giant” sEPSCs and increased network excitability in our spinal cord cultures are likely to reflect an increased number

of synaptic inter- actions rather than altered properties of individual neurons as observed previously by others in similar preparations [2,33]. Irrespective of this, one can safely come to the conclusion that substantia gelatinosa neurons in our organotypic preparations maintain their morphology as well as most of their physiological properties. Cells recorded in organotypic cultures showed passive and active membrane properties consistent with healthy superficial DH neurons as reported previously by many other investigators [20,23,40].

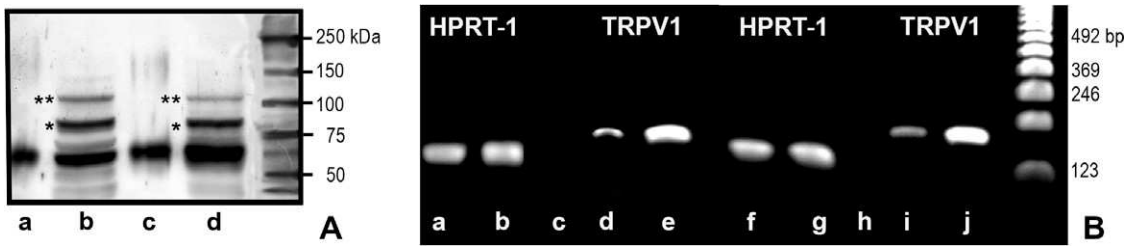

Fig. 8. Expression of TRPV1 protein and mRNA in tissue extracts. (A) Western blots of TRPV1 protein in whole extracts obtained from acutely dissected P21 spinal cords (lanes a and b) and 8 DIV organotypic cultures explanted at P8 (lanes c and d). In lanes a and c the primary antibody was omitted as a negative control. After immunodetection the primary antibody recognized a major band (*) of about 90 kDa and an additional band (**) around 100 kDa. This latter band has been related to possible post-translational modifications (e.g. glycosylation) of the TRPV1 protein. The large band around 64 kDa in all lanes is due to non-specific recognition of IgG heavy chains by the secondary antibody. See [22] for further discussion on antibody specificity. (B) RT-PCR amplified products from total RNA extracts of spinal cord (lanes a, d, f, and i) and P21 DRGs (lanes b, e, g, and j). In lanes c and h samples were substituted by double distilled water (negative controls). Spinal cord samples were from 8 DIV organotypic cultures (lanes a and d) or P21 mice (lanes g, and i). Note that the level of expression in spinal cord is lower than in DRGs. Abbreviations: DIV, days in vitro.

In addition, the overall morphology of LY injected neurons, together with the firing patterns encountered, lead to conclude that recorded cells were mostly located in lamina II.

4.2. Expression of TRPV1 in organotypically cultured substantia gelatinosa neurons

Valtschanoff et al. [48] showed at the EM level that part of TRPV1 immunoreactivity in DH is due to expression of the receptor in dendrites of lamina II neurons of intact animals. We have demonstrated here that the TRPV1 protein and its mRNA are present in extracts and tissue samples of the mouse spinal cord. In our organotypic cultures where PAFs that are the main source of TRPV1s in DH are degenerated, we still detected the TRPV1 protein and mRNA after biochemical and histological procedures. Specifically, we have shown that a population of cultured neurons in the superficial DH express the TRPV1 mRNA and synthesize

the TRPV1 protein. Our biochemical and histological data, together with the reduced phys- iological effect in the presence of TTX, suggest a main distribution of the TRPV1 protein at dendritic level in cultured substantia gelatinosa neurons. In addition, it seems possible that at least part of functional TRPV1s are directly expressed on recorded neurons, considering the occurrence of the slow inward current during capsaicin administration in most of the recorded cells.

4.3. TRPV1 activation increases the inhibitory tone in substantia gelatinosa neurons with a different timing compared to acute slices

Consistently with the notion that capsaicin activates TRPV1 expressed on PAFs and induces an intense glutamate release from PAF endings onto lamina II neurons [32,50], we here observed a strong increase of sEPSC frequency in acute slices, as previously reported [50]. Since PAFs degenerate in the type of cultures used here, but the effects of capsaicin are still observed, one can conclude that the substantia gelatinosa neurons expressing TRPV1 are activated by the vanilloid [48]. Differently from what reported by Zhou et al. [51], we have been unable to detect an effect of capsaicin on sEPSCs. Nonetheless this discrepancy is likely a consequence of the profound differences in the preparations employed in the two studies. Zhou et al. used dorsal rhizotomy or I-RTX to destroy TRPV1-expressing PAFs. Under both circumstances there is the possibility that part of the fibers survive and/or that their terminal field is invaded by sprouting from nearby segments of the spinal cord [12,27,45]. On the other hand, the modifications in neuronal circuitry that occur in our type preparation may account for lack of the excitatory response to TRPV1 stimulation. As mentioned, rewiring of connections in organotypic slices eventually leads to increased glutamatergic activity [33]. Therefore, it seems unlikely that our negative results on sEPSCs are hampered by the organotypic approach. In keeping with this assumption, the inhibitory response of substantia gelationsa neurons to capsaicin shows remarkably similar features in acute slices and organotypic cultures (Fig. 9). We observed that about 70% of recorded neurons in cultures responded to capsaicin with a significant increase of sIPSC frequency. As shown by the analysis of decay time, activation of TRPV1s equally increases the frequency of single exponential sIPSCs as well as mixed GABA/glycinergic sIPSCs. The response was also observed upon complete blockade of fast glutamatergic neurotransmission, suggesting that GABA/glycine release was likely due to a direct excitatory action of the vanilloid onto substantia gelatinosa inhibitory neurons, rather than to the activation of a neuronal chain (Fig. 9). These observations support our previous data in acute slices [19], specifically with regard to the existence of a subpopulation of lamina II neurons responding to the drug in the presence of TTX. Interestingly, acute and organotypic

cultures differ in the timing of the capsaicin effect: while in slices the effect lasted for several minutes after wash-out (>5 min), in cultures the sIPSC

frequency quickly returned at the control level. These data suggest that the activation of TRPV1 on PAFs is involved in the long lasting effects of capsaicin on sIPSCs in acute preparations, while the activation of spinal TRPV1s triggers the short-lasting effects, as shown in culture.

4.4. NADA as putative endogenous vanilloid activating substantia gelatinosa TRPV1s

Several putative endogenous agonists of TRPV1, commonly referred to as ‘‘endovanilloids”, have been identified [44,49], including molecules that are shown to activate both the endovanilloid and endocannabinoid systems. The endovanilloid/endocannabi- noid NADA seems to have the better profile in terms of activity and potency as endogenous TRPV1 agonist [25,46]. In the spinal cord and primary sensory neurons, NADA induces TRPV1-depen- dent effects [25,26,41]. Our data demonstrate that NADA is able per se to induce a significant increase of GABA/glycine spontaneous

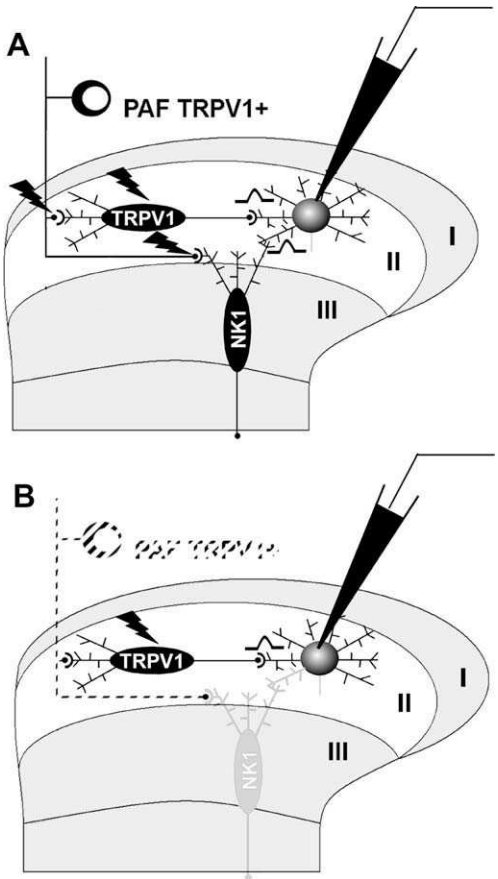

Fig. 9. Neuronal circuits underlying the capsaicin effect on DH inhibitory neuro- transmission. Schematic diagram illustrating the putative mechanisms that lead to an increased inhibitory input onto lamina II neurons upon capsaicin administration. (A) Acute slices (modified from [19]): capsaicin (black arrows) increases sIPSC frequency of lamina II neurons (grey + recording pipette) by two different mech- anisms. The vanilloid acts on

TRPV1s expressed at PAF endings that, in turn, may depolarize the inhibitory neurons (in black) in DH by direct and indirect mechanisms (e.g. through the activation of NK1 receptors). Capsaicin can also activate TRPV1-expressing neurons in DH (see [19] for further discussion). (B) Organotypic cultures. After PAF degeneration (dashed lines), the increased release of inhibitory neurotransmitters after capsaicin/endovanilloids is due to the activation of spinal TRPV1s expressed by neurons in the superficial DH (in black) . In this context, the role of NK1 expressing neurons in DH (shadowed) still needs to be addressed.

activity in organotypic slices, similarly to capsaicin albeit at lower degree, given that only 50% of the cells were responsive to NADA. Although on purely speculative basis, such a difference can be re- lated to the functional state of TRPV1s since it has been shown that NADA can act as a potent agonist when TRPV1 is in a PKC-mediated phosphorylation state [38]. NADA is synthesized in primary sensory neurons [25], but little is known concerning the regulation of its synthesis and activity in the spinal cord. However, we were also able to reproduce a TRPV1-dependent increase of inhibitory neurotransmission by using PALDA and STEARDA [15], that do not activate TRPV1 directly, but are supposed to facilitate the activity of TRPV1 agonists. Altogether these data strongly support the idea that endogenous agonists of TRPV1 may be of relevance in vivo for the regulation of inhibitory neurotransmission in substantia gelatinosa.

5. Conclusions

Current research in the pharmacological treatment of pain shows an increasing interest in the development of TRPV1 antagonists (for review: [11,39]). The ability of these molecules to reach adequate concentrations in the spinal cord has been recently pro- ven to be important for establishing their efficiency in pain treatment [13]. Our study demonstrates that the activation of spinal TRPV1 increases the inhibitory tone in substantia gelatinosa neurons in vitro, and suggests that such an effect may also be relevant in vivo, since endovanilloids are locally present in DH. Under this perspective, it would be of interest for the future to investigate the site of production of NADA and its regulation in the sensory system under different experimental conditions.

6. Summary

Capsaicin/endovanilloid activation of spinal TRPV1 in vitro increases the inhibitory tone in substantia gelatinosa neurons. The effect of endovanilloids may be also relevant in vivo.

Acknowledgements

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