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Fos and pERK immunoreactivity in spinal cord slices: comparative analysis of *in vitro* models for testing putative antinociceptive molecules

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Abstract

To detect central neuron activation, expression of the transcription factor Fos and phosphorylation of the protein kinase ERK (pERK) can be visualized by immunocytochemistry. These approaches have been extensively used to quantify the activation of nociceptive neurons in the spinal dorsal horn (DH) following peripheral stimulation *in vivo*. Here we propose an alternative and simplified *in vitro* model to investigate Fos and pERK expression based on the stimulation of acutely dissected spinal cord slices to mimic acute inflammatory changes in DH. Transverse slices were obtained from postnatal (P8-P12) CD1 mice and were treated for 5 min with capsaicin (CAP, 2 μ M). CAP induces a strong release of glutamate from primary afferent terminals which, in turn, excites spinal DH neurons. Since ERK phosphorylation and Fos expression occur following different time frames, two distinct protocols were used to detect their activation. Thus, for studying Fos immunoreactivity CAP-treated slices were left for 3 hours in Krebs solution after stimulation. Instead, for studying pERK immunoreactivity slices were maintained in Krebs solution for only 15 min after stimulation. Both Fos and pERK were significantly up-regulated following CAP challenge. To validate our model we tested the efficacy of octreotide (OCT, 1 μ M) in preventing the CAP effect on Fos and pERK expression. OCT is a synthetic antinociceptive analogue of somatostatin, one of the neuropeptides involved in the negative modulation of pain signals in DH. After CAP, OCT reduced the response to both Fos and pERK. Our data validate the use of Fos and pERK immunoreactivity *in vitro* to investigate the activation of spinal nociceptive pathways and testing potentially antinociceptive molecules.

Key words: mouse spinal cord, Fos, pERK, nociception, capsaicin, octreotide

1.Introduction

Nociceptive neurons in the spinal dorsal horn (DH) are physiologically activated in response to high threshold stimuli. In pharmacological studies, an important indication of the antinociceptive efficacy of a drug can be found in its capacity to prevent or block neuronal transmission in nociceptive pathways. Unfortunately, directly measuring neuronal activity by means of electrophysiological techniques is invasive, time consuming and technically demanding. Although electrophysiological approaches are still essential to properly investigate the mechanisms of synaptic transmission, the question whether a neuronal pathway is activated or inhibited by a given molecule can be more easily addressed by immunohistochemical techniques targeting markers of neuronal activation (Hoffmann and Lyo, 2002; Gao and Ji, 2009). Indeed, membrane excitation (i.e. neuronal firing) is typically followed by an increase in the cytoplasmatic calcium concentration which in turns promotes the activation of numerous intracellular pathways and the transcription of new proteins. Thus, neuronal excitation leaves a molecular signature which makes an activated neuron phenotypically identifiable.

The most common marker of neuronal activation is the transcription factor Fos (Dragunow and Faull, 1989). Fos is the product of the early oncogene *c-fos* which is rapidly transcribed following stimulation (Greenberg,et al., 1986; Hunt et al., 1987; Herdegen and Leah, 1998). The extensive use of Fos as a neuronal marker in spinal nociceptive pathways has been minutely reviewed by Coggeshall (2005). More recently, the development of phospho-specific antibodies (Patton et al., 1991) has allowed the visualization of proteins which are highly phosphorylated after neuronal excitation (Rosen et al., 1994; Mao et al., 1999; Wu et al., 2001; Knight et al., 2012). In particular the activation of nociceptive neurons in spinal DH has been directly associated with the phosphorylation of the extracellular signal-regulated kinase (pERK; Ji et al., 1999). Under normal conditions, both Fos and pERK are expressed at low level in spinal DH and their expression is specifically induced by high threshold stimulation, thus supporting their use as markers of neuronal activation in nociceptive pathways (Gao and Ji, 2009).

A typical experimental approach to activate nociceptive neurons consists in peripheral injections of algogenic chemicals, such as capsaicin, the pungent ingredient of hot chili pepper (Jinks et al., 2002). The characteristic burning pain associated with capsaicin application is gated by the transient receptor potential vanilloid type 1 (TRPV1; Caterina et al., 1997). The latter is a cation channel sensitive to hot temperature and low pH expressed by most peptidergic nociceptors, as well as by a subset of non-peptidergic nociceptors (Michael and Priestley, 1999; Bencivinni et al., 2011; Cavanaugh et al., 2011; Barabas and Stucky, 2013). Peripheral application of capsaicin mimics the sustained activation of nociceptors observed in inflammatory pain. Intracutaneous and visceral

injections of capsaicin stimulate a dose-dependent increase in Fos in the ipsilateral spinal DH one to two hours after vanilloid administration (Zou et al., 2001; Jinks et al., 2002; Kalezic et al., 2004; Seki et al., 2005). Similarly, pERK is highly increased in spinal neurons following peripheral capsaicin injection, but increase in the phosphorylated protein can be already detected within few minutes after the stimulation (Ji et al., 1999; Kawasaki et al., 2004). TRPV1-expressing terminals can also be activated by capsaicin in acutely dissected spinal cord slices (Yang et al., 1998; Ferrini et al., 2007, 2010), and consequently Fos and pERK expression could, in theory, also be analyzed in DH neurons *in vitro* (Kawasaki et al., 2004; Vergnano et al., 2008; Bencivinni et al., 2011).

Here, we addressed the question of whether the increase in Fos and pERK expression in spinal cord slices after capsaicin stimulation is a good tool for screening antinociceptive molecules. To do so, we tested the response of slices to octreotide (OCT), a synthetic analogue of somatostatin which produces pain relief in humans (Dahaba et al., 2009) by inhibiting the activation of primary afferent fibers (PAFs; Carlton et al., 2004; Bencivinni et al., 2011).

2. Materials and methods

All experimental procedures were approved by the Italian Ministry of Health and the Committee of Bioethics and Animal Welfare of the University of Torino. Animals were maintained according to NIH Guide for the Care and Use of Laboratory Animals.

2.1 Acute spinal cord slices preparation and capsaicin stimulation

Postnatal CD1 mice (P8-P12; $n=11$) were given a lethal dose of sodium pentobarbital (30 mg/Kg, intraperitoneal).

Spinal cord dissection was performed by constantly maintaining the tissues in ice-cold artificial cerebrospinal fluid (ACSF) containing: NaCl 125 mM, KCl 2.5 mM, NaHCO₃ 25 mM, NaH₂PO₄ 1 mM, glucose 25 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, saturated with 95% O₂-5% CO₂. The lumbar spinal cord was removed by performing a dorsal laminectomy and the dorsal and ventral roots were cut. After removal of the dura mater, spinal cord was glued to an agar block and positioned on the stage of a vibrating microtome (Leica Microsystems, Nussloch, Germany). Transverse slices (350 μ m-thick) were obtained in ice-cold ACSF and then allowed to recover in oxygenated ACSF at room temperature (RT). After 1 hour recovery, slices were: *i*- incubated with normal ACSF; *ii*- incubated with capsaicin (2 μ M for 5 min; Tocris, Bristol, United Kingdom); or *iii*- pre-incubated with OCT (1 μ M for 10 min; Tocris), followed by capsaicin + OCT for 5 min. Slices were then washed in normal ACSF and further processed for immunocytochemistry.

2.2 Fos staining procedure

Acute spinal cord slices were kept in ACSF for 3 hours after capsaicin stimulation (see 2.1), then fixed for 30 min with 4 % paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB) and placed

overnight in 80% ethanol. Slices were subsequently dehydrated in increasing ethanol concentrations, clarified in xylene and wax embedded. Seven μm -thick sections were obtained with a microtome (Leica Microsystems) and placed onto polylysine-coated slides. Sections were then deparaffinized in decreasing ethanol concentrations and incubated for 30 min with methanol (3% v/v; Sigma, St. Louis, MO, USA) to block endogenous peroxidase. Rehydrated sections were pre-incubated with normal goat serum (NGS 1/100; Sigma) and then incubated overnight at RT with a rabbit anti-Fos antibody (1/100; Abcam, Cambridge, UK). After washing in PBS, sections were incubated with a biotinylated anti-rabbit IgG (1/1000 for 1 hour at RT; Life Technologies, Paisley, UK) and then in avidin-biotinylated-peroxidase complex (1/100, 1 hour at RT; Vector, Burlingame, CA, USA). Immunoreactivity was revealed by 0.025% (w/v) 3,3'-diaminobenzidine (Sigma) and 0.04% (v/v) hydrogen peroxide. Sections were then mounted in DPX (Sigma). Images of the DH were acquired in bright field with a 20x objective (Leica) and stored in a personal computer for subsequent analysis.

2.3 pERK staining procedure

Acute spinal cord slices were kept in ACSF for 15 min after capsaicin stimulation and then fixed as described above (see 2.2). Slices were directly processed for the detection of pERK immunoreactivity without re-sectioning. Briefly, 350 μm free-floating slices were pre-incubated in NGS 1/100 in PBS and then incubated overnight at 4°C with a rabbit anti-pERK1/2 antibody (Thr 202/Tyr 204; 1:100; Santa Cruz Biotech., Santa Cruz, CA, USA). After washing in PBS, slices were incubated 3 hours with an anti-rabbit secondary antibody IgG AlexaFluor 488-conjugated (1/1000, Life Tech.) and then mounted in anti-fade mounting medium (Fluoroshield, Sigma). In a subset of experiments, a double fluorescence staining was performed by using a mouse anti-NeuN antibody (1/100, Merck Millipore, Billerica, MA, USA) to confirm that the expression of pERK specifically occurred in neurons (not shown). Fluorescent images were acquired using a Leica TCS SP5 confocal laser scanning microscope (20x objective) with appropriate filter settings and, gain, offset, laser intensity and pinhole unchanged for all stacks. Spinal DHs were sequentially scanned along the z axis in 2 μm steps. Five consecutive optical sections in a stack with the highest signal intensity were superimposed and the output image stored for subsequent analysis. A minimum of 3 slices per condition were processed for each animal.

2.4 Image analysis and statistics

Analysis was performed blind to the treatment using the ImageJ software (NIH, Bethesda, Maryland, USA). The boundaries of the DH laminae were delineated according to previous anatomical criteria (Molander et al. 1984; Paxinos and Watson, 1998).

All images were converted to a 8-bit grayscale format and the signal was selected by setting a gray threshold value. Fos nuclear staining within laminae I-II was expressed as number of cells per mm^2

(cell density). pERK cytoplasmatic staining was expressed as the percentage of laminae I-II area occupied by the signal (signal area/lamina I-II area x 100). As pERK staining was directly performed on 350 μm -thick slices without re-sectioning (see 2.3), and only few slices were obtained from individual mice, statistics was performed by pooling data per animal (with n = number of mice). Differences between groups were tested by Mann-Whitney test or Kruskal-Wallis test with Dunn test post-hoc).

Data were expressed as mean \pm SEM and differences were considered significant for $P < 0.05$.

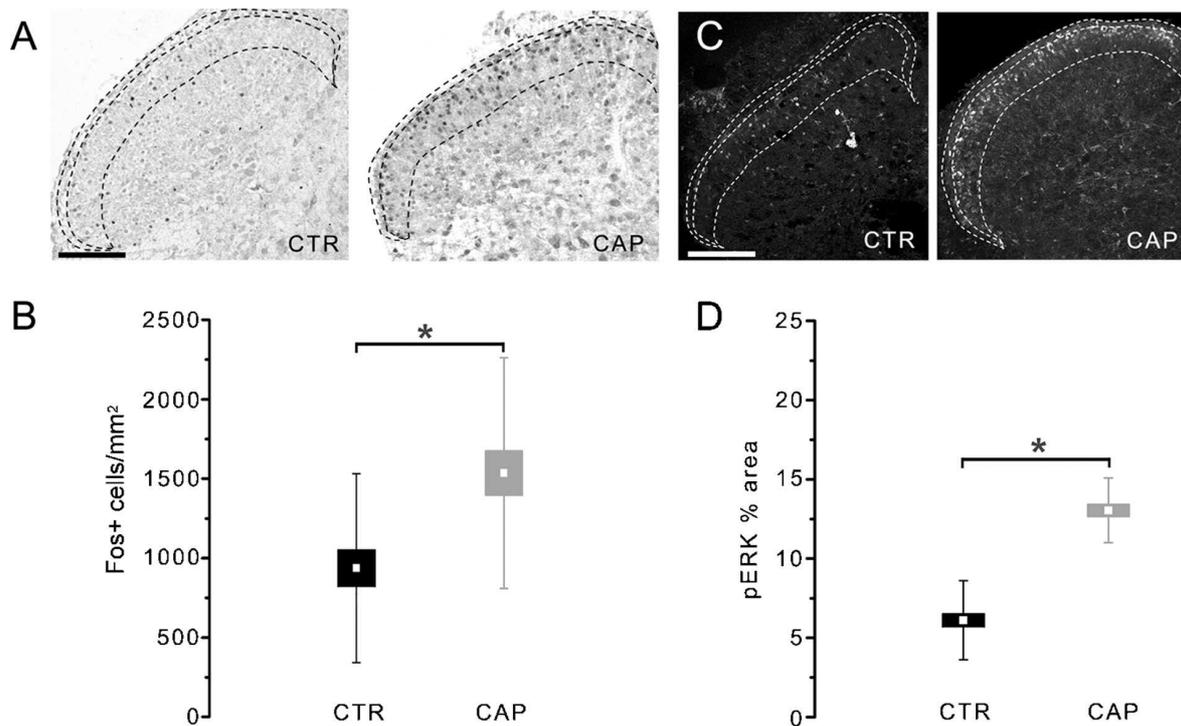


Figure 1. Capsaicin-induced increase in Fos and pERK immunoreactivity in the spinal DH. (A) Fos immunoreactivity in the spinal DH of representative control and capsaicin-treated slices. Dashed lines outline lamina I and II borders. (B) Pooled data of Fos-positive cell density in control (black, $n=14$ sections) and capsaicin-treated slices (gray, $n=27$ sections; Mann-Whitney test, $*P<0.05$). (C) pERK immunoreactivity in the spinal DH of representative control and capsaicin-treated slices. (D) Pooled data of the relative pERK immunoreactivity area in control (black, $n=4$ mice) and capsaicin treated slices (gray, $n=4$; Mann-Whitney test, $*P<0.05$). In the box plots, the central square is the mean, the vertical box width is s.e.m and whiskers represent s.d.; scale bars, 100 μm ; abbreviations: CTR = control, CAP = capsaicin.

2.5 Light and electron microscopy

For immunohistochemical studies, four CD1 mice (P8-12) were used for light microscopy (LM) and eight for electron microscopy (EM; $n=4$ post-embedding protocol; $n=4$ pre-embedding FluoroNanogold protocol).

Under deep pentobarbital anesthesia (30 mg/Kg), mice were perfused with Ringer solution followed by cold fixative. The latter consisted of 4% PFA in PB for LM, 1% PFA + 2% glutaraldehyde for

post-embedding EM and 4% PFA + 0.01% glutaraldehyde for pre-embedding FluoroNanogold EM. After perfusion, the lumbar spinal cord was dissected out, cut in segments and post-fixed for 2 additional hours in the same aldehyde mixture. For LM, coronal sections were cut on a vibratome at a thickness of 70 μm . For EM, spinal cord segments were cut with a vibratome (200 μm for post-embedding; 70 μm for pre-embedding) and embedded in Araldite (Merighi and Polak, 1993). Ultrathin sections were then cut with an EM UC6 ultramicrotome (Leica Microsystems) and collected onto uncoated nickel grids (200 mesh).

Antibodies and controls. The following primary antibodies have been used in this study: goat-anti-somatostatin receptor type 2a (SSTR2a;1:100 -LM and EM; Santa Cruz Biotechnology), rabbit anti-somatostatin (SST-28; 1:2000 - LM, 1:100 - EM; Merighi et al., 1989), rabbit anti-CGRP (1:1000 - LM, 1:100 - EM; Merighi et al., 1991), rabbit anti-TRPV1 (1:1000 -LM; Alomone Labs, Jerusalem, Israel).

Lectin *Griffonia simplicifolia* (IB4), biotin conjugated (1:250 - LM, 1:20 -EM; Sigma) was also used in immunohistochemical procedures (Hunt and Mantyh, 2001). Immunohistochemical controls consisting in omission of primary antibodies were routinely performed (Salio et al., 2005).

Double immunofluorescence staining. Free-floating spinal cord sections were double immunostained following a classical immunofluorescence protocol (Salio et al., 2005). Images were acquired using a Leica TCS SP5 confocal laser scanning microscope with appropriate filter settings. Green and red fluorescence were then merged using Photoshop 7.0.1 (Adobe Systems, San Jose, CA, USA).

EM Post-embedding immunostaining. Ultrathin sections were immunostained following a conventional post-embedding immunostaining protocol (Merighi and Polak, 1993). Sections were observed with a transmission electron microscope (CM10, Philips, NL).

EM Pre-embedding FluoroNanogold immunostaining. Free-floating spinal cord sections were immunostained for SSTR2a visualization at the electron microscope with the Fluoronanogold™-Streptavidin protocol (Nanoprobes, Yaphank, NY, USA) as described previously (Salio et al., 2011). Some ultrathin sections, immunolabeled for SSTR2a with the pre-embedding FluoroNanogold protocol were also stained for IB4 following the post-embedding immunostaining protocol.

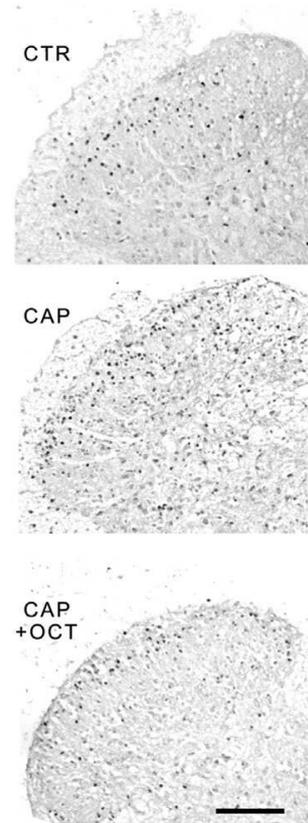
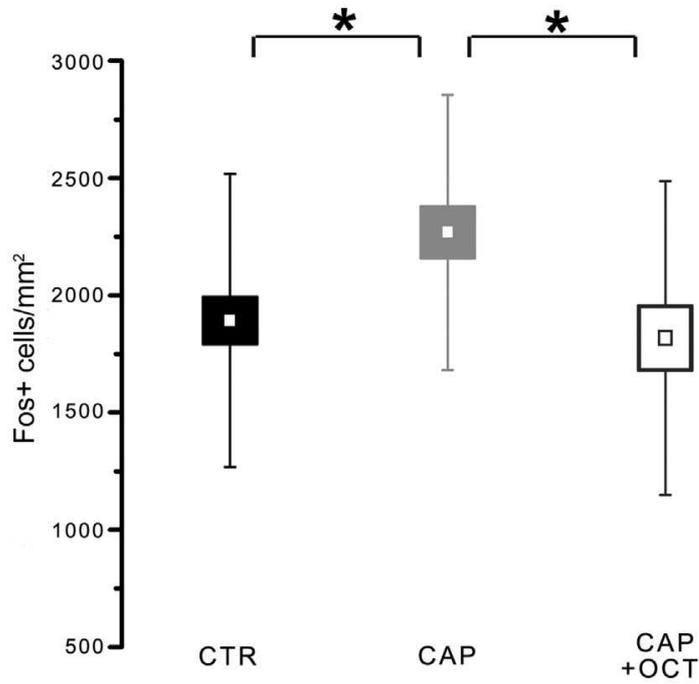
3.Results

3.1 In vitro capsaicin stimulates both Fos and pERK expression in the spinal DH

As previously shown (Vergnano et al., 2008; Kawasaki et al., 2004), the application of capsaicin on isolated spinal cord slices is an effective model to stimulate spinal nociceptive pathways. In our model, the application of capsaicin (2 μM) for 5 minutes induced a significant increase in Fos immunoreactivity in laminae I and II (Fig. 1A). The density of Fos-positive neurons was 968 ± 159 cells/ mm^2 ($n=14$) in control and 1577 ± 140 cells/ mm^2 ($n=27$) in capsaicin treated slices (Mann-Whitney test, $P < 0.05$; Fig. 1B). Similarly, the percentage area of laminae I-II occupied by pERK

immunoreactivity was almost doubled in capsaicin treated slices ($6.1 \pm 1.2\%$, $n=4$ in control and $13.0 \pm 1.0\%$, $n=4$ in capsaicin; Mann-Whitney test, $P < 0.05$, Fig. 1C-D).

A



B

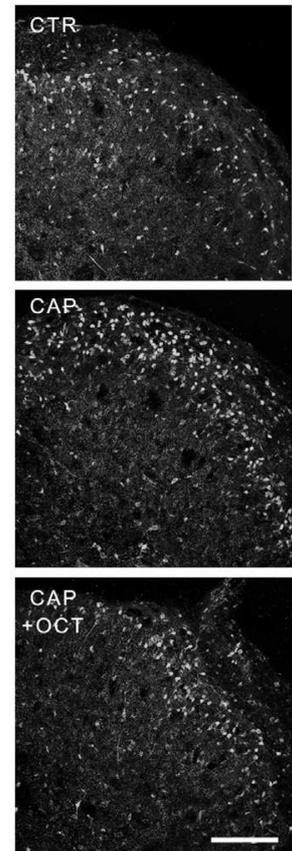
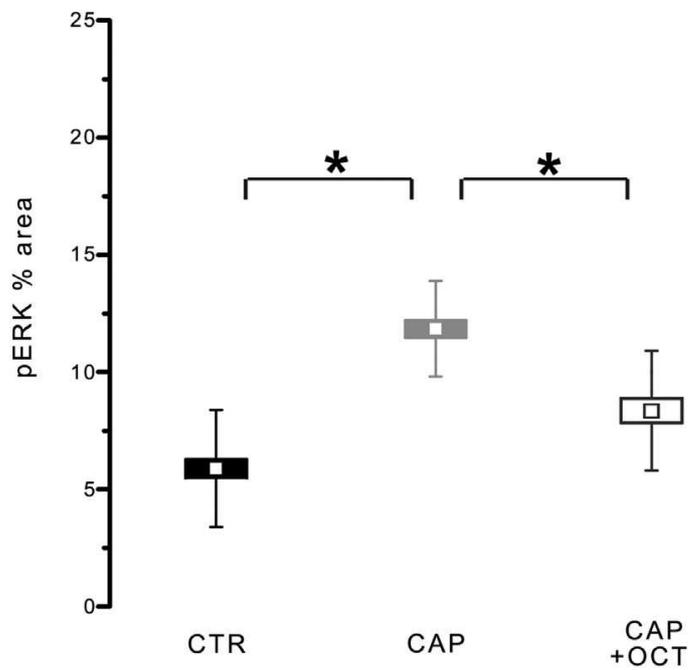


Figure 2. Effect of OCT on capsaicin induced increase of Fos and pERK in the spinal DH. (A) Fos- positive cell density in control (*black*, $n=38$ sections), capsaicin (*gray*, $n=28$ sections) and capsaicin+OCT (*empty box*, $n=23$ sections; Kruskal-wallis test with Dunn post-hoc , $P<0.05$). On the right panel, representative Fos staining in the spinal DH for each experimental condition. (B) relative pERK immunoreactivity area in control (*black*, $n=5$ mice), capsaicin (*gray*, $n=5$ mice), and capsaicin+OCT (*empty box*, $n=5$; Kruskal-wallis test with Dunn post-hoc , $P<0.05$). On the right panel, representative pERK staining in the spinal DH for each experimental condition. In the box plots, the central square represents the *mean*, the vertical box width represents *s.e.m* and whiskers represent *s.d.*; scale bars, 100 μm ; abbreviations: CTR = control, CAP = capsaicin, OCT = octreotide.

3.2 Effect of the somatostatin synthetic analogue OCT on capsaicin-induced Fos and pERK expression

In a second set of experiments, we investigated the effect of OCT on capsaicin-induced activation of spinal laminae I-II neurons by carrying out Fos and pERK staining on control sections, CAP sections, and on sections pre-incubated with OCT. In line with our previous observations (Bencivinni et al., 2011), pre-incubation with OCT (1 μM - 10 min) and subsequent capsaicin stimulation in presence of OCT (capsaicin+OCT) was effective in preventing capsaicin-induced Fos increase in laminae I-II (1893 ± 101 cells/ mm^2 , $n=38$ in control, 2269 ± 111 cells/ mm^2 , $n=28$ in capsaicin, 1818 ± 140 cells/ mm^2 , $n=23$ in capsaicin+OCT; Kruskal-Wallis test, $P<0.05$; Fig. 2A). The same protocol was then used to test the effect of OCT on capsaicin-induced pERK expression. Also in this case, OCT significantly reduced the percentage area of laminae I-II occupied by pERK immunoreactivity ($5.7\pm 1.0\%$, $n=5$ in control, $12.24\pm 1.2\%$, $n=5$ in capsaicin; $7.9\pm 1.1\%$, $n=5$ in capsaicin+OCT; Kruskal-Wallis test, $P<0.05$; Fig. 2B). These data show that in slices OCT also blocks the activation of nociceptive pathways, thus supporting the notion that it is acting *in vivo* as an antinociceptive molecule. In addition, they indicate that both Fos and pERK upregulation following capsaicin stimulation *in vitro* are sensitive tools for testing drug effects on nociceptive pathways.

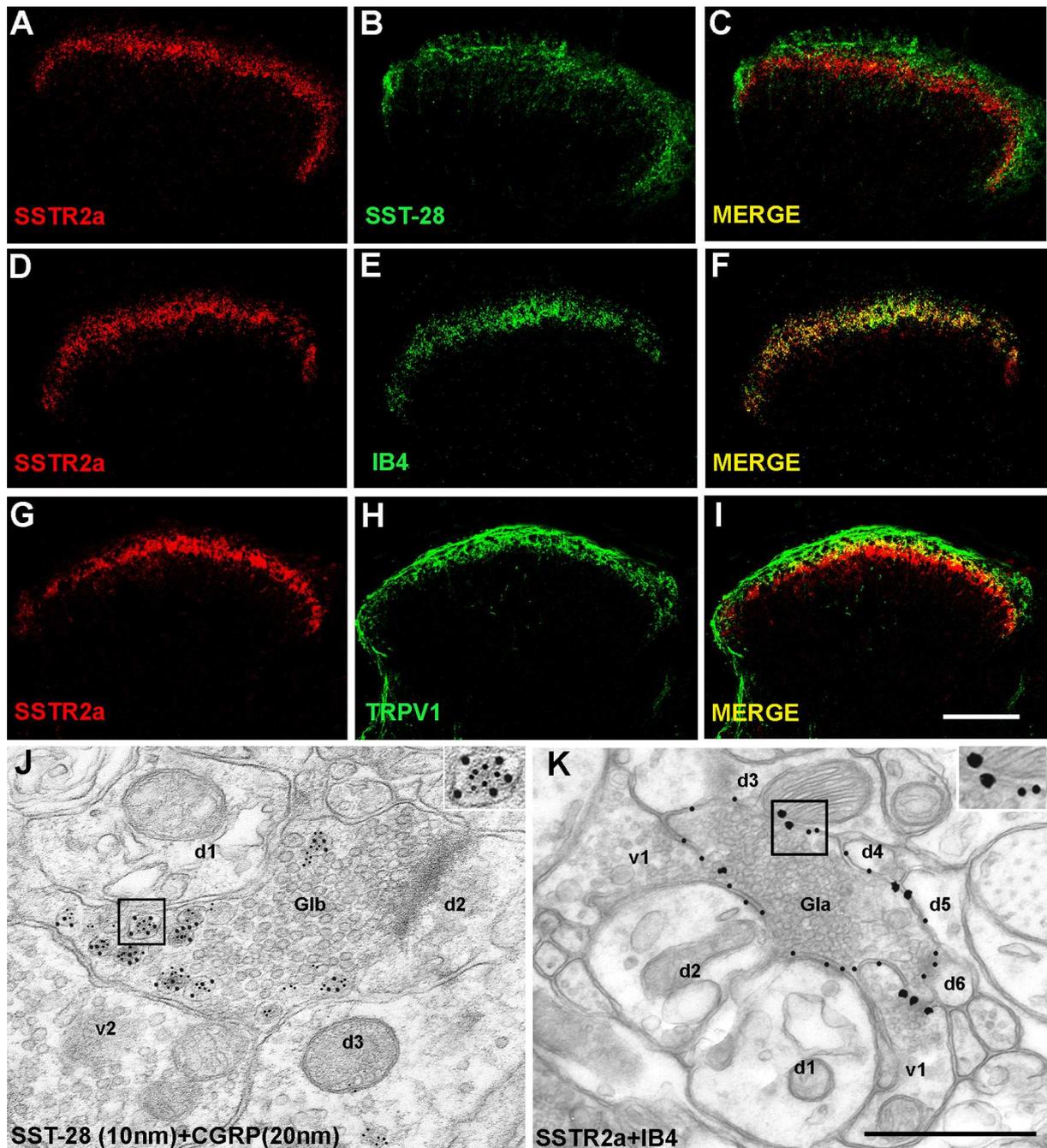


Figure 3. Immunohistochemical localization of SSTR2a at the light and electron microscopic level. (A) SSTR2a immunoreactive (IR) nociceptive PAFs in lamina Iii of the spinal DH (red). (B) SST-28 IR peptidergic nociceptive PAFs in laminae I-Iio (green). (C) SSTR2a and SST-28 are distributed in two different populations of nociceptive PAFs in laminae I-II (merge). (D) SSTR2a IR nociceptive PAFs in lamina Iii of the spinal DH (red). (E) IB4 IR non-peptidergic nociceptive PAFs in lamina Iii (green). (F) SSTR2a and IB4 are co-expressed in nociceptive non-peptidergic PAFs of lamina Iii (merge). (G) SSTR2a IR nociceptive PAFs in lamina Iii of the spinal cord (red). (H) TRPV1 IR nociceptive fibers in laminae I-Iio (green). (I) SSTR2a and TRPV1 are co-expressed in a population of PAFs distributed at the border between lamina Iio and lamina Iii (merge). (J) Post-embedding immunogold labeling for SST-28+CGRP in spinal lamina II. SSTR2a (10 nm) and CGRP (20 nm) are selectively localized in DCVs of a peptidergic type Ib glomerular terminal (Glb). The SSTR2a+CGRP IR Glb contacts three unlabeled dendrites (d1-d3) and a peripheral axonal bouton (v2; see Ribeiro da Silva, 2004). (K) Combined pre-embedding FluoroNanogold SSTR2a and post-embedding IB4 IRs in non-peptidergic type Ia glomeruli (Gla) of lamina II. SSTR2a receptors have been localized by gold intensified, irregular particles (25-30 nm; arrowhead in the insert), while IB4 immunostaining was characterized by gold particles of regular round shape and constant size (20 nm, arrow in the insert). The SSTR2a+IB4-IR Gla terminal is surrounded by several unlabeled dendrites (d1-d6) and two vesicle-containing dendrite (v1; see Ribeiro da Silva, 2004). Scale bars: A-I: 250 μ m; J-K: 500nm.

3.3 Somatostatin and its receptors in spinal nociceptive pathways

The inhibition of capsaicin-induced activation of DH neurons by OCT, as evidenced by the reduction of capsaicin-induced Fos and pERK immunoreactivity, is consistent with the localization of somatostatin (SST-28) and its receptors in spinal nociceptive pathways. Indeed, somatostatin was expressed by a subpopulation of peptidergic nociceptive PAFs prevalently localized in laminae I-II outer (Ilo; Fig. 3B-C). Conversely, SSTR2a, the most characterized somatostatin receptor subtype in nociceptive pathways (Schulz et al., 1998; Carlton et al., 2004; Bencivinni et al., 2011), was mainly localized on IB4-positive non-peptidergic nociceptive PAFs and principally distributed in lamina II inner (Ili; Fig 3D-F), while no colocalization was observed with somatostatin (Fig 3C). A subset of SSTR2a-immunolabeled fibers, in addition, co-expressed the capsaicin receptor TRPV1 (Fig. 3G-I). At the subcellular level, somatostatin was localized in peptidergic Glb glomeruli, together with CGRP (Fig. 3J), while SSTR2a expression was confirmed in non-peptidergic GlA glomeruli, together with IB4 (Fig. 3K). Therefore, the release of somatostatin from peptidergic PAFs, or the exogenous application of OCT, are likely to inhibit capsaicin-induced activation of spinal nociceptive pathways by binding SSTR2a expressed by non-peptidergic PAFs.

4. Discussion

Testing antinociceptive molecules *in vivo* is a necessary step to ascertain their effectiveness in combating pain. However, a first screening step can be more conveniently performed by using *in vitro* models mimicking the central activation of nociceptive pathways rather than carrying out a series of time consuming experiments *in vivo*. In the present study, we stimulated spinal nociceptive pathways by applying capsaicin on an acutely dissected mouse spinal cord slice preparation and investigated whether or not Fos and pERK could be sensitive markers to detect neuronal activation *in vitro*. Capsaicin-induced central stimulation is based on the activation of the vanilloid receptor TRPV1 which is highly expressed on the central terminals of C fibers (Guo et al., 1999) and elicits a strong release of neurotransmitters onto DH neurons (Yang et al., 1998; Garry et al., 2000; Lao et al., 2003). Compared to C fiber electrical stimulation (Jennings and Fitzgerald, 1998; Ji et al., 1999), capsaicin-induced C fiber activation provides several advantages. First, it is technically easier, as it does not require intact nerves or dorsal roots attached to the spinal cord slices or electrical equipment to deliver the stimulus. Second, since the stimulus intensity (capsaicin concentration) can be minutely and uniformly graded (Marvizon et al., 2003), experimental conditions are more efficiently kept under control. Finally, at nano-to-micromolar working concentrations, capsaicin-induced activation of nociceptors is specific (Caterina et al., 1997), thus reducing the risk of recruiting non-nociceptive spinal pathways. Moreover, although TRPV1 is also expressed by a subpopulation of DH interneurons (Valtschanoff et al., 2001; Doly et al., 2004), these neurons are inhibitory (Ferrini

et al., 2010; Kim et al., 2012) and are therefore not directly involved in the capsaicin-induced excitatory drive onto DH neurons, at least under physiological conditions. In summary, capsaicin stimulation *in vitro* represents a simple and specific tool to activate the central terminals of nociceptive PAF, thus mimicking the effect of inflammatory pain on nociceptors. Capsaicin has been previously shown to induce Fos (Vergnano et al., 2008; Bencivinni et al., 2011) and pERK activation (Kawasaki et al., 2004) in spinal cord preparations. The most obvious difference between these neuronal activation markers lies in their temporal dynamics (Gao and Ji, 2009). Although Fos expression starts early after stimulation, it requires the synthesis of sufficient levels of the protein before being detected, which typically peaks in a few hours (Vergnano et al., 2008; Presley et al., 1990; Curran and Morgan, 1995). Conversely, ERK phosphorylation reaches detectable levels within a few minutes after induction (Ji et al., 1999) making such an approach more suitable for analysis of rapid synaptic neuronal response studies (Gao and Ji, 2009). Importantly, the slow onset of Fos expression is also paralleled by a longer time to recover to control level as compared to pERK: while the former typically takes more than 5 hours to return to control level (Sharp et al., 1991), the latter is already decayed within 3 hours (Gao and Ji, 2009). A major consequence of such difference is that the capsaicin-induction of neuronal excitation during slice preparation procedure can steadily affect the basal level of Fos immunoreactivity which, unlike pERK, might not fully recover. Indeed, the control level of Fos immunoreactivity in our experiments was higher compared to the level typically reported *in vivo* (Birder and de Groat, 1992; Rashid and Ueda, 2005). Notwithstanding, capsaicin induced a significant increase in Fos immunoreactivity in the DH as compared to control slices and similarly induced a significant increase in pERK signal. Thus, both Fos and pERK represent sensitive tools to detect capsaicin-induced activation *in vitro* and this model can be easily adopted to test the antinociceptive effects of putative analgesic molecules. In this study, we tested OCT, a synthetic analogue of somatostatin which binds preferably to SSTR2 (Lesche et al., 2009) and has been suggested to have antinociceptive effects (Penn et al., 1992; Schmidt et al., 1993; Dahaba et al., 2009). In our previous study (Bencivinni et al., 2011), we demonstrated that OCT reduces the capsaicin-induced release of glutamate from PAFs and the subsequent activation of DH neurons. Consistently, here we show that the administration of OCT on spinal cord slices *in vitro* inhibits the capsaicin-induced expression of Fos and pERK. Our anatomical study suggests that SSTR2s are mostly expressed on non-peptidergic C fiber terminals. Since a subpopulation of these fibers also express the capsaicin receptor TRPV1, OCT is likely to act pre-synaptically, thus reducing the capsaicin-induced release of transmitters onto DH neurons. Interestingly, our data also show that endogenous somatostatin is expressed in peptidergic PAFs, which also express TRPV1; thus, it can be supposed that the activation of peptidergic fibers *in vivo* might control the activity of non-peptidergic PAFs via somatostatin-SSTR2. Nevertheless, somatostatin is also expressed by intrinsic spinal neurons (Mizukawa et al., 1988) or descending fibers (Krisch, 1981), and the peptide may also induce neuronal inhibition by activating G-protein-

coupled inwardly rectifying K⁺ (GIRK) channels in spinal neurons (Nakatsuka et al., 2008). Therefore, the involvement of these alternative pathways cannot be totally ruled out.

In summary, our data support the use of capsaicin-induced Fos and pERK expression to test the antinociceptive effects of target molecules on spinal cord slices. The use of an *in vitro* approach, when possible, may substantially shorten the screening phase for the identification of molecules with putative analgesic properties, reducing the number of animals required and contain the experiment costs.

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The authors have no conflicts of interest with regard to the studies described in this manuscript.

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