DISPARATE CHANGES IN THE EXPRESSION OF TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 RECEPTOR mRNA AND PROTEIN IN DORSAL ROOT GANGLION NEURONS FOLLOWING LOCAL CAPSAICIN TREATMENT OF THE SCIATIC NERVE IN THE RAT

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Abstract—*In situ* hybridization, quantitative reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and Western blot analysis were applied to study the changes in expression of the major nociceptive ion channel transient receptor potential vanilloid type 1 receptor (TRPV1) after the perineural application of capsaicin or nerve transection. In control rats, quantitative morphometric and statistical analyses of TRPV1 protein and mRNA expression in L5 dorsal root ganglion cells revealed distinct populations of small (type C) and small-to-medium (type B) neurons, which showed very high and moderate levels of TRPV1, whereas larger (type A) neurons mostly did not express this receptor. After either transection or capsaicin treatment of the sciatic nerve, immunohistochemistry and Western blotting demonstrated a massive (up to 80%) decrease in the proportion of TRPV1-immunoreactive neurons and TRPV1 protein at all postoperative survival times. *In situ* hybridization indicated marked decreases (up to 85%) in the proportion of neurons that expressed TRPV1 mRNA after sciatic nerve transection. In contrast, although perineural treatment with capsaicin resulted in similar substantial decreases in the proportions of type B and C neurons of the L5 dorsal root ganglia 3 days postoperatively, a clear-cut tendency to recovery was observed thereafter. Hence, the proportions of both type B and C neurons expressing TRPV1 mRNA reached up to 70% of the control levels at 30 days postoperatively. In accord with these findings, quantitative RT-PCR revealed a marked and significant recovery in TRPV1 mRNA after

perineural capsaicin but not after nerve transection. These observations suggest the involvement of distinct cellular mechanisms in the regulation of the TRPV1 mRNA expression of damaged neurons, specifically triggered by the nature of the injury. The present findings imply that the antinociceptive and anti-inflammatory effects of perineurally applied capsaicin involve distinct changes in neuronal TRPV1 mRNA expression and long-lasting alterations in (post)translational regulation. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: capsaicin, perineural, TRPV1 mRNA, pain, nerve transection, *in situ* hybridization.

Chemosensitive primary sensory neurons, which are sensitive to capsaicin (Jancsó, 1968; Jancsó et al., 1977) and express the transient receptor potential vanilloid type 1 receptor (TRPV1) (Caterina et al., 1997; Caterina and Julius, 2001), play a fundamental role in pain mechanisms. By virtue of their dual functional character, these particular nociceptive neurons comprise a unique population of primary afferent neurons, which transmit impulses generated by noxious stimuli and release neuropeptides from their peripheral and central terminals in response to stimulation (Maggi and Meli, 1988; Holzer, 1991; Jancsó, 2009). The chemosensitive primary afferent neurons, which are selectively sensitive to the stimulatory and neurotoxic effects of capsaicin, account for around 50% of the dorsal root ganglion (DRG) cells and 95% of the unmyelinated dorsal root fibers in the rat (Jancsó et al., 1977, 2011; Nagy and Hunt, 1983). Previous studies have demonstrated that selective elimination of these nociceptive afferents either from the whole animal or from selected regions of the body by the systemic (neonatal) or localized (perineural) administration of capsaicin and related vanilloids leads to profound antinociceptive and anti-inflammatory effects (Jancsó et al., 1977, 1980; Fitzgerald and Woolf, 1982; Gamse et al., 1982). The perineural application of vanilloid compounds that results in highly selective regional thermal and chemical analgesia has attracted much interest because of the promising therapeutic relevance of this intervention. Local application of capsaicin or resiniferatoxin has been shown to induce long-lasting increases in the thresholds of nociceptive responses elicited by chemical irritants and intense heat stimuli (Jancsó et al., 1980; Gamse et al., 1982; Chung et al., 1985; Kissin et al., 2002). Local treatment with capsaicin or resiniferatoxin also reduces inflammatory

thermal and mechanical hyperalgesia and ischemic reactive hyperemia (Kissin et al., 2002; Domoki et al., 2003; Pospisilova and Palecek, 2006; Holzer, 2008; Jancsó et al., 2008; Oszlács et al., 2009) and arthritis (Donaldson et al., 1995). Antidromic vasodilatation and neurogenic inflammation, the cardinal local vascular responses of chemosensitive afferent endings brought about through stimulation with chemical irritants or antidromic stimulation of sensory nerves, are completely abolished by such treatment (Jancsó and Király, 1980; Oszlács et al., 2009). Although the antinociceptive and anti-inflammatory effects of locally applied vanilloid compounds have been repeatedly demonstrated, the mechanisms of these unique antinociceptive/analgesic effects are still unclear. Electrophysiological studies have revealed a selective and long-lasting reduction of impulse conduction in unmyelinated, but not in myelinated sensory axons (Jancsó and Such, 1983; Baranowski et al., 1986; Pini et al., 1990), associated with a reduction of polymodal nociceptor units in rat (Welk et al., 1983; Pini et al., 1990), guinea pig, and rabbit (Baranowski et al., 1986) peripheral nerves. Similar findings have been reported in monkeys following the treatment of peripheral nerves with capsaicin (Chung et al., 1993). Morphological investigations have disclosed a substantial, but partial reduction in the number of unmyelinated sensory (Baranowski et al., 1986; Jancsó and Lawson, 1990), but not autonomic (Jancsó et al., 1987) axons in capsaicin-treated peripheral nerves and in skin areas innervated by a capsaicin-treated peripheral nerve (Jancsó et al., 1980; Dux et al., 1999). Recent findings indicated that the application of resiniferatoxin to peripheral nerves induced lasting analgesia without noticeable fine structural alterations in the rat (Kissin et al., 2002, 2007). Histochemical and immunohistochemical studies have revealed the marked depletion of sensory neuropeptides from the spinal ganglia and the dorsal horn of the spinal cord relating to the peripheral nerve treated with a vanilloid agent (Gamse et al., 1982; Jancsó and Lawson, 1988; Oszlács et al., 2009). However, changes in the expression of the TRPV1, a molecular integrator of nociception (Winter et al., 1988; Caterina et al., 1997; Tominaga et al., 1998), which confers capsaicin (vanilloid) sensitivity on chemosensitive primary afferent neurons (Winter et al., 1988; Caterina et al., 1997; Michael and Priestley, 1999) have not been investigated so far after perineural treatment with vanilloid compounds. The present experiments were therefore initiated in an attempt to make use of in situ hybridization, quantitative reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and Western blot analysis to reveal possible changes in the expression of the TRPV1 following perineural capsaicin treatment and, for comparison, peripheral nerve transection.

EXPERIMENTAL PROCEDURES

Adult male Wistar rats weighing 240–260 g at the start of the experiments were used in this study. The animal house was maintained under a 12-h light/dark cycle. All experimental procedures were approved by the Ethical Committee for Animal Care of the University of Szeged and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Perineural capsaicin treatment

The rats were anesthetized with chloral hydrate (400 mg/kg, i.p., Reanal, Budapest, Hungary). The sciatic nerves were exposed high in the thigh on both sides, and small pieces of gelfoam moistened with 0.1 ml of a 1% solution of capsaicin (Fluka, Buchs, Switzerland) or the same volume of the vehicle (6% ethanol, 8% Tween 80 in saline) were wrapped around the right and left nerves, respectively. After 20 min, the gelfoam pieces were removed, the wounds were closed, and the rats were returned to the animal house. After 3, 14, or 30 days, the animals were again anesthetized and sacrificed for immunohistochemical and in situ hybridization analyses.

Peripheral nerve transection

The rats were anesthetized with chloral hydrate (400 mg/kg, i.p., Reanal, Budapest, Hungary). The right sciatic nerve was exposed high in the thigh and transected distal to a ligature. Sham-operated animals served as controls. After 3, 14, or 30 days, the animals were again anesthetized and sacrificed for immunohistochemical and in situ hybridization analyses.

In situ hybridization

The synthesis of the cRNA probe and in situ hybridization were carried out as described by Maniatis et al. (1982), with slight modifications. To generate TRPV1 mRNA-specific probes, total mRNA was isolated from rat trigeminal ganglia and was reverse transcribed by using the universal dT17-adapter primer (5'-GACTCGAGTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT-3', M-MuLV reverse transcriptase; Fermentas, Vilnius, Lithuania) according to the manufacturer's recommendations. This cDNA template was used to perform RT-PCR with the following primer combination: forward 5'-AACCATGGAACAACGGGCTAGC-3'; reverse 5'-AACTCGAGTTAGAACAGAGCTGACA-3'. The amplified 255 bp length product was cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The identity of the amplified product was confirmed by DNA sequencing and Northern blotting. After linearization of the vectors, sense and antisense digoxigenin-11-UTP-labeled cRNA probes were transcribed with T7 or SP6 polymerases, using a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol.

For in situ hybridization, DRGs were quickly removed, embedded in Cryomatrix embedding material (Shandon Scientific, Pittsburgh, PA, USA), and frozen immediately at −70 °C. Serial frozen sections of DRGs (15 μm in thickness) were cut on a cryostat and thaw-mounted onto 3-aminopropyltriethoxysilane-coated glass slides. Sections were air-dried and stored at −20 °C until further processing. The specimens were fixed for 5 min in 2× sodium chloride–sodium citrate (SSC) buffer (0.3 M NaCl and 0.03 M Na-citrate, pH 7.0) containing 4% formaldehyde, washed twice in 2× SSC buffer for 2 min, permeabilized with 0.1% Triton X100, washed again as before, and then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride at room temperature for 5 min. Hybridization was performed in 50 μl hybridization solution (50% formamide, 5× sodium chloride–sodium phosphate–EDTA buffer, 1× Denhardt's reagent, 10% dextran sulfate, 50 mM dithiothreitol, 100 μg/ml salmon sperm DNA, and 100 μg/ml yeast tRNA containing 200 nmol/ml labeled probe) under parafilm cover slips in a humidified chamber at 56 °C for 20 h. The sections were extensively rinsed in 2× SSC buffer supplemented with 50% formamide at 50 °C for 15 min, treated with RNase A at 37 °C for 30 min, and washed again in 2× SSC–50% formamide solution at 50 °C. To block nonspecific antibody binding, sections were incubated with

buffer 1 (100 mM Tris–HCl and 150 mM NaCl, pH 7.5) containing 5% normal goat serum for 1 h at room temperature, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2500, Boehringer Mannheim GmbH, Mannheim, Germany) in buffer 1 at 4 °C overnight. Sections were washed in buffer 1 for 3×5 min, rinsed in buffer 2 (100 mM Tris–HCl, 100 mM NaCl, and 50 mM MgCl$_2$, pH 9.5) for 10 min, and developed in buffer 2 containing 340 μg/ml nitro blue tetrazolium and 180 μg/ml 5-bromo-4-chloro-3-indolyl phosphate for 12 h in a dark chamber. The reaction was terminated by rinsing the slides in a buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) for 10 min. The sections were covered with glycerol.

Quantitative RT-PCR measurements

To measure changes in the total TRPV1 mRNA expression in DRGs affected by the transection or capsaicin treatment of the sciatic nerve, quantitative RT-PCR was used. Rats were terminally anesthetized 3, 14, and 30 days after surgery, and the L5 DRGs were excised and transferred into 1 ml ice-cold Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total mRNA was isolated by Trizol solution according to the protocol of the manufacturer. The extracted total mRNA was reverse transcribed by using BioRad iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Specific primers were designed to amplify TRPV1 and beta-2-microglobulin (B2-MG, reference gene) by using the Primer-Blast open source software (http://www.ncbi.nlm.nih-.gov/tools/primer-blast). The sequences of the primers were as follows: B2-MG (NM_012512; reference gene): 5′-TCTCCG-GTGGATGGCGAGAGT-3′ (reverse); 5′-GCTCGCTCGGT-GACCGTGATC-3′ (forward); TRPV1 (NM_031982.1): 5′-TGTCTTCCGGGCAACGTCCA-3′ (reverse); 5′-AAGCGCCT-GACTGACAGCGA-3′ (forward). Primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). These primers produced distinct PCR amplification products with length of 129 bp for TRPV1 and 106 bp for B2-MG, as confirmed by gel-electrophoresis. Quantitative RT-PCR was performed in triplicates utilizing SYBR Green technique (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA, USA) and BioRad MyiQ5 Real Time Detection System running the following amplification protocol: 10 min on 95 °C (hot start) followed by 40 amplification cycles (denaturation: 10 s on 95 °C, annealing: 30 s on 56 °C; elongation and detection: 20 s on 72 °C). At the end of the amplification, melt-curve analysis was also applied to exclude nonspecific fluorescent signals. Relative quantities of target (TRPV1) mRNAs as compared with the housekeeping reference gene B2-MG were calculated by using the Pfaffl-method (Pfaffl, 2001).

TRPV1 immunohistochemistry

The animals were deeply anesthetized and perfused transcardially with an aldehyde fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L5 DRG was removed and postfixed in the same fixative for 2 h and then placed into a phosphate-buffered 30% sucrose solution. Representative serial sections of L5 DRGs 15 μm in thickness were cut on a cryostat and mounted on gelatin-coated glass slides. Sections were rinsed twice in phosphate-buffered saline (PBS) and incubated overnight with the primary antibody (1:1000; rabbit anti-TRPV1 IgG, ACC030, Alomone Labs, Jerusalem, Israel) with 0.3% Triton X100 added. After rinsing in PBS, the sections were incubated for 2 h with the secondary antibody (1:500 biotin-conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in PBS containing 0.3% Triton X100. To visualize the biotin-conjugated antibody, the sections were rinsed and treated with the Vectastain ABC Elite staining kit (Vector laboratories, Burlingame, CA, USA) according to the instructions of the manufacturer. The sections were dehydrated and covered with DPX mounting medium (Fluka, Buchs, Switzerland).

Semiquantitative densitometry

The sections cut from the DRGs and processed for visualization of the TRPV1 mRNA by *in situ* hybridization or the TRPV1 protein by immunohistochemistry were examined under bright-field illumination with a DMLB microscope (Leica, Wetzlar, Germany) equipped with a Nikon Coolpix (Nikon, Tokyo, Japan) digital camera. Under identical conditions, microphotographs were taken of DRGs relating to control sciatic nerves and sciatic nerves transected or treated perineurally with capsaicin following a systemic random sampling method. The optical density of DRG neurons with clear-cut nuclei was measured by means of the NIH Scion Image analysis program. In sections processed for the demonstration of TRPV1 mRNA, many neurons exhibited granular staining of different intensities in the perikaryon. In contrast, in labeled neurons the TRPV1 immunoreactivity displayed diffuse staining throughout the cell bodies and sometimes in their axons. Gray values (GVs) between 0 and 255 were assigned to each neuron with a clearly visible nucleus, and their cross-sectional areas (CSAs) were measured. Relative optical densities (RODs) were determined according to the equation $ROD=\log_{10}(255/(255-GV))$. The CSA and ROD for each cell were determined and plotted as distribution histograms or scatter plots.

Classification of DRG neurons

The DRG neurons were classified into different subpopulations by using a statistical approach. Pilot experiments suggested the existence of three distinct neuronal subpopulations in the control DRGs, with different levels of mRNA signal and TRPV1 immuno-staining. Discriminant analysis was performed to define the ROD classification effect among the different subpopulations of DRG neurons. To determine the threshold values of ROD for the separation of the neuronal subpopulations, the receiver operating characteristic (ROC) method was applied pairwise (Armitage, 2001; Armitage and Colton, 2005).

Western blot analysis

L5 DRGs were removed from rats 3, 14, and 30 days after perineural capsaicin treatment or transection of the sciatic nerves and were homogenized immediately in ice-cold radio immuno precipitation assay (RIPA) buffer containing 50 mM Tris (pH 8), 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxy-cholate, 0.1% sodium dodecyl sulfate (SDS), 2 μg/ml leupeptin (Sigma), and 1 μg/ml pepstatin (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 15000 g for 10 min. The pellet was discarded, and protein concentrations from the supernatant were determined according to the method of Lowry et al. (1951). Protein samples (60 μg/well) were separated through a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and blocked for 12 h in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. The membranes were incubated for 2 h with rabbit anti-TRPV1 (1:500, Chemicon, Temecula, CA, USA) and mouse anti-β-actin primary antibody (1:20000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% nonfat dry milk in 0.1% TBS–Tween 20. After three washes in 0.1% TBS–Tween 20, the membranes were incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies (1:2000, Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK), and washed five times as before. The enhanced chemiluminescence method (ECL Plus Western blotting detection reagent; Amersham Biosciences, Little Chalfont, UK) was used to reveal immunoreactive bands according to the manufacturer's protocol. The films were scanned at 600×600 dpi resolution, and the densitometric quantification was performed by the ImageJ public domain image processing and analysis software (NIH, Bethesda, MD, USA). After subtracting background, TRPV1 band

densities were normalized to β-actin. The ratio of the TRPV1 to β-actin band density was used to calculate the changes in TRPV1 expression. Results of three independent experiments are shown as means±SD.

Statistics

The experimental data are shown as means±SD. Statistical analyses were performed with ANOVA and Holm-Sidak, Brown-Forsythe, or Bonferroni correction methods for *post hoc* comparisons by using SPSS (v.18, Statistical Software package, IBM Corporation, NY, USA). Differences between groups were considered statistically significant if $P<0.05$.

RESULTS

Localization of TRPV1 mRNA and protein in the L5 DRG of the rat

In the control DRGs, three types of neurons could be distinguished with different levels of TRPV1 mRNA expression and TRPV1-immunostaining. Small- to medium-sized neurons displayed intense and moderate expression levels, whereas particularly the larger neurons were mostly devoid of TRPV1 mRNA and protein (Fig. 1A–D). The optimal cut-off point for the TRPV1 mRNA ROD to distinguish between group C and the remaining population was 0.40, which provided a specificity of 96% and a sensitivity of 90%. Similarly, a cut-off value of 0.24 provided the optimal differentiation between groups A and B (Fig. 1E, G). Type C and B neurons were characterized by their small (CSA range: 0–400 μm^2) and medium sizes (range: 410–900 μm^2), and high (0.41–1) and moderate (0.25–0.40) RODs, respectively. The population of type A neurons was composed of cells of various sizes with low RODs (0–0.24), which hardly exceeded the background ROD. The type C and B neurons were regarded as expressing high and moderate levels of TRPV1 mRNA, whereas type A cells were classified as TRPV1-negative neurons. The *in situ* hybridization experiments revealed that around half of the DRG cells expressed TRPV1 mRNA in control ganglia. The type C cells accounted for around 19% and the type B cells approximately 29% of the total neuronal population. About half (51%) of the cells in the DRGs were clearly negative for TRPV1 mRNA. Although the majority of the TRPV1 mRNA-negative neurons were large, some small neurons also exhibited low RODs.

Statistical analysis of the TRPV1-immunopositive neurons revealed three subpopulations of DRG neurons with respect to their TRPV1 protein content (Fig. 1F, H): the type C and B neurons were mainly small to medium-sized, with strong or moderate staining intensity, respectively, whereas the TRPV1-negative neurons were mostly large.

Effects of perineural capsaicin treatment or transection of the sciatic nerve on the expression of the TRPV1 in the L5 DRG of the rat

In the rat, the sensory fibers of the sciatic nerve originate from the fourth, the fifth, and (to a much lower extent) the sixth lumbar DRGs (Green, 1968). Up to 85% of the neurons in the fifth lumbar DRG project their axons into the sciatic nerve (Yip et al., 1984; Aldskogius et al., 1988). In

the present study, therefore, the fifth lumbar DRG was chosen to study possible changes in the expression of the TRPV1 following two types of nerve injury: nerve transection, a physical injury resulting in neurotmesis, damage to all types of axons of the sciatic nerve (Seddon, 1943), and perineural treatment with capsaicin, which produces a selective chemodenervation of C-fiber afferents, but leaves the continuity of the nerve intact.

Perineural treatment with capsaicin resulted in a rapid decrease in the expression of TRPV1 mRNA in the neurons of the fifth lumbar DRG, with reductions by about 50% and 75% in type B and C cells 3 days after the treatment. However, this initial decrease in TRPV1 expression was followed by a distinct recovery and the proportion of TRPV1 mRNA-expressing neurons gradually increased up to 70% of the control levels toward the end of the study (Table 1). The experiments using quantitative RT-PCR confirmed these findings by showing an early and marked reduction in TRPV1 mRNA expression already 3 days after perineural capsaicin treatment. However, at later survival times quantitative RT-PCR measurements revealed a clear-cut tendency to recovery toward control expression levels resulting in a marked and statistically significant increase in TRPV1 mRNA at 30 days (Fig. 2A). Study of the localization of the TRPV1 protein by means of immunohistochemistry revealed that the proportion of TRPV1-positive ganglion cells had decreased markedly (to about 30% of the control level) 3 days after perineural capsaicin treatment, and it remained at that low level throughout the entire period of the study (Table 1). The reduction in the proportion of type C cells was especially pronounced, by about 85%. The analysis of the experimental data clearly showed the time-dependent and cell type-specific changes in the expression of TRPV1 mRNA and protein, respectively (Figs. 3 and 4). Western blot analysis of the TRPV1 protein supported the immunohistochemical findings. The TRPV1 protein was markedly and significantly reduced at all time points after perineural treatment with capsaicin (Fig. 2B, C).

Similarly to perineural treatment with capsaicin, peripheral nerve transection resulted in rapid and marked reductions in both TRPV1 mRNA expression and TRPV1 protein in the type B and C cells of the related fifth lumbar DRG 3 days after surgery. However, in contrast with capsaicin treatment, the TRPV1 mRNA expression did not recover, but remained at a low level for the entire remainder of the study period. In accord with this, the proportion of TRPV1-immunoreactive neurons dropped to about 30% of the control level and then remained low throughout the study. Again, the decreases in TRPV1 mRNA expression and TRPV1 protein (by about 80%) were especially marked in the type C cells (Table 1, Fig. 5). In accordance with the results obtained with *in situ* hybridization, quantitative RT-PCR measurements revealed marked and significant reductions in the TRPV1 mRNA expression 3 and 14 days after nerve transection. TRPV1 mRNA expression showed some increase after 30 days, but that did not reach significance (Fig. 2A).
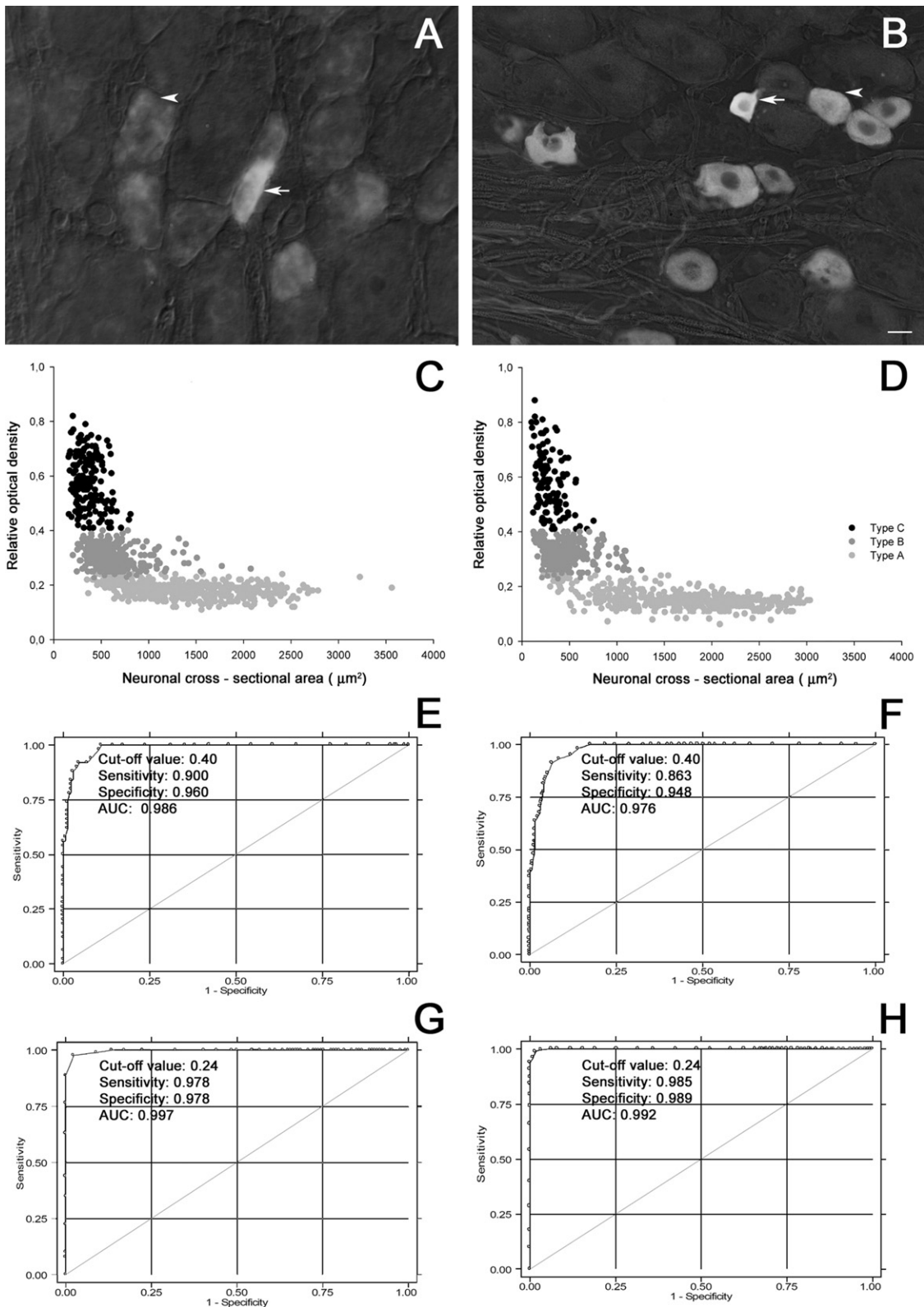
Fig. 1. (A, B) In control ganglia, *in situ* hybridization (A) and immunohistochemistry (B) revealed small- to medium-sized neurons with intense (arrow) and moderate (arrowhead) levels of TRPV1 mRNA and protein, respectively. Larger neurons were usually devoid of both TRPV1 mRNA and protein. Inverse microphotographs; scale bar indicates 25 μm. (C, D) Scatter plots of DRG cells, showing the cell sizes and the three separate populations of neurons with intense, moderate, and very low RODs. (E, G) ROC analysis of TRPV1 mRNA RODs revealed the cut-off values for the separation of type C and B (E) and type B and A (G) neurons, respectively, and disclosed the high sensitivity and specificity of the analysis involving the use of ROD. (F, H) ROC analysis of the RODs of TRPV1-immunopositive neurons revealed the cut-off values for the separation of type C and B (F) and type B and A (H) neurons, respectively, and disclosed the high sensitivity and specificity of the analysis using ROD.

Table 1. Percentage distribution of TRPV1-expressing (type B, C) and TRPV1-negative (type A) L5 DRG neurons 3, 14, and 30 d after perineural capsaicin treatment and nerve transection

Neuron type	TRPV1 mRNA expression				TRPV1 immunohistochemistry			
	Control	3 d	14 d	30 d	Control	3 d	14 d	30 d
Perineural capsaicin								
C	19±1.28	5±0.74*	9±0.62*#	12±1.4*#	17±1.73	2±0.56*	4±0.30*	3±0.43*
B	29±2.33	15±1.03*	15±0.91*	20±1.2*#	36±1.00	9±2.23*	11±0.72*	12±0.82*
A	51±3.13	79±1.76*	75±1.51*	69±4.7*#	46±1.00	89±2.06*	84±0.45*	84±0.44*
Nerve transection								
C	18±1.37	3±0.36*	2±0.03*	2±0.20*	15±1.73	2±0.60*	5±1.03*	4±0.26*
B	28±3.10	15±1.03*	16±1.72*	16±1.46*	37±4.70	16±1.90*	16±1.20*	18±0 65*
A	53±1.85	82±1.33*	82±1.69*	81±1.26*	50±0.60	80±2.35*	78±0.80*	77±1 00*

Data are expressed as means±SD.
* Significantly different from the control, $P<0.05$.
# Significantly different from the 3 d value, $P<0.05$.

DISCUSSION

Chemosensitive primary sensory neurons which express the TRPV1 play a fundamental role in the transmission of nociceptive impulses (Jancsó et al., 1977; Caterina et al., 1997; Julius and Basbaum, 2001). The level of expression of the TRPV1 is an important determinant of the nociceptor function. Increases in TRPV1 mRNA expression and in peripherally directed axonal transport of TRPV1 protein have been demonstrated to be associated with neuropathic pain states and inflammation (Tohda et al., 2001). Conversely, knockdown of the TRPV1 gene prevents the development of inflammatory hyperalgesia in the rat (Caterina et al., 2000; Davis et al., 2000; Kasama et al., 2007). Hence, TRPV1 antagonism or procedures, which inhibit the activation of the receptor may produce significant antinociception. Indeed, the local application of capsaicin and some other vanilloids directly onto peripheral nerve trunks has been shown to provide long-lasting and selective chemical and thermal analgesia, confined to the region innervated by the affected nerve (Jancsó et al., 1980, 2008, 2011; Gamse et al., 1982; Fitzgerald and Woolf, 1982; Kissin et al., 2002; Knotkova et al., 2008). Despite numerous investigations that have made use of perineural capsaicin treatment (Gamse et al., 1982; Gibson et al., 1982; Chung et al., 1985; Jancsó and Lawson, 1987, 1990; Jancsó et al., 1987; Pini et al., 1990; Jancsó and Ambrus, 1994; Kissin et al., 2002), the mechanism of analgesia induced by perineural capsaicin remained unclear.

In the present study, the cell size and the ROD of the mRNA signal and the immunostaining were measured, and a statistical approach was applied to classify subpopulations of DRG neurons which express the TRPV1. In agreement with the findings of a previous radioactive *in situ* hybridization study (Michael and Priestley, 1999), the present findings revealed two subpopulations of small- and medium-sized neurons that exhibited moderate and high intensities of TRPV1 mRNA expression and TRPV1 immunoreactivity. The two populations of DRG neurons that expressed TRPV1 mRNA or TRPV1 protein could be clearly distinguished through a statistical approach involving ROC analysis based on two characteristic traits of

TRPV1-positive neurons: the cell size and the ROD of the mRNA signal or the immunostaining for TRPV1. The quantitative data demonstrated that a distinct subpopulation of small DRG neurons displayed a significantly higher TRPV1 mRNA expression than did a larger population of small- and medium-sized TRPV1-expressing neurons, which accounted for around 19% and 30% of the total neuronal population, respectively, in the L5 DRGs of the rat.

The main finding of the present study is the demonstration of disparate changes in the expression of TRPV1 mRNA and protein in DRG neurons after selective chemical denervation by perineural capsaicin treatment. Further, the findings also indicate differences in the regulation of TRPV1 expression following selective chemical and physical injuries inflicted upon primary sensory neurons.

In accord with previous reports, peripheral nerve transection resulted in a substantial reduction in the proportion of TRPV1 mRNA-expressing neurons, which was already evident 3 days after surgery and persisted for at least 4 weeks in the L5 DRGs. This was closely paralleled by a significant and persistent decrease in the proportions of TRPV1-immunoreactive neurons in the L5 DRGs. These findings corroborate and extend previous reports of parallel reductions in TRPV1 mRNA expression and protein level in axotomized DRG neurons (Michael and Priestley, 1999). The present study further supported these observations by measurements of TRPV1 mRNA and protein using quantitative RT-PCR and Western blotting, respectively. The results indicated marked, significant, and permanent reductions in TRPV1 protein confirming the immunohistochemical analysis. TRPV1 mRNA expression was markedly reduced 3 and 14 days after nerve transection, but it showed a moderate increase after 30 days, which did not reach significance.

In sharp contrast, following perineural treatment with capsaicin, neurons in the L5 DRG exhibited distinct changes in TRPV1 mRNA and protein expression and TRPV1 immunostaining. Although the expression of TRPV1 mRNA in type C neurons was markedly decreased 3 days after the treatment, there was a clear-cut tendency toward recovery after 2 weeks, and a statistically significant recovery to about 60%
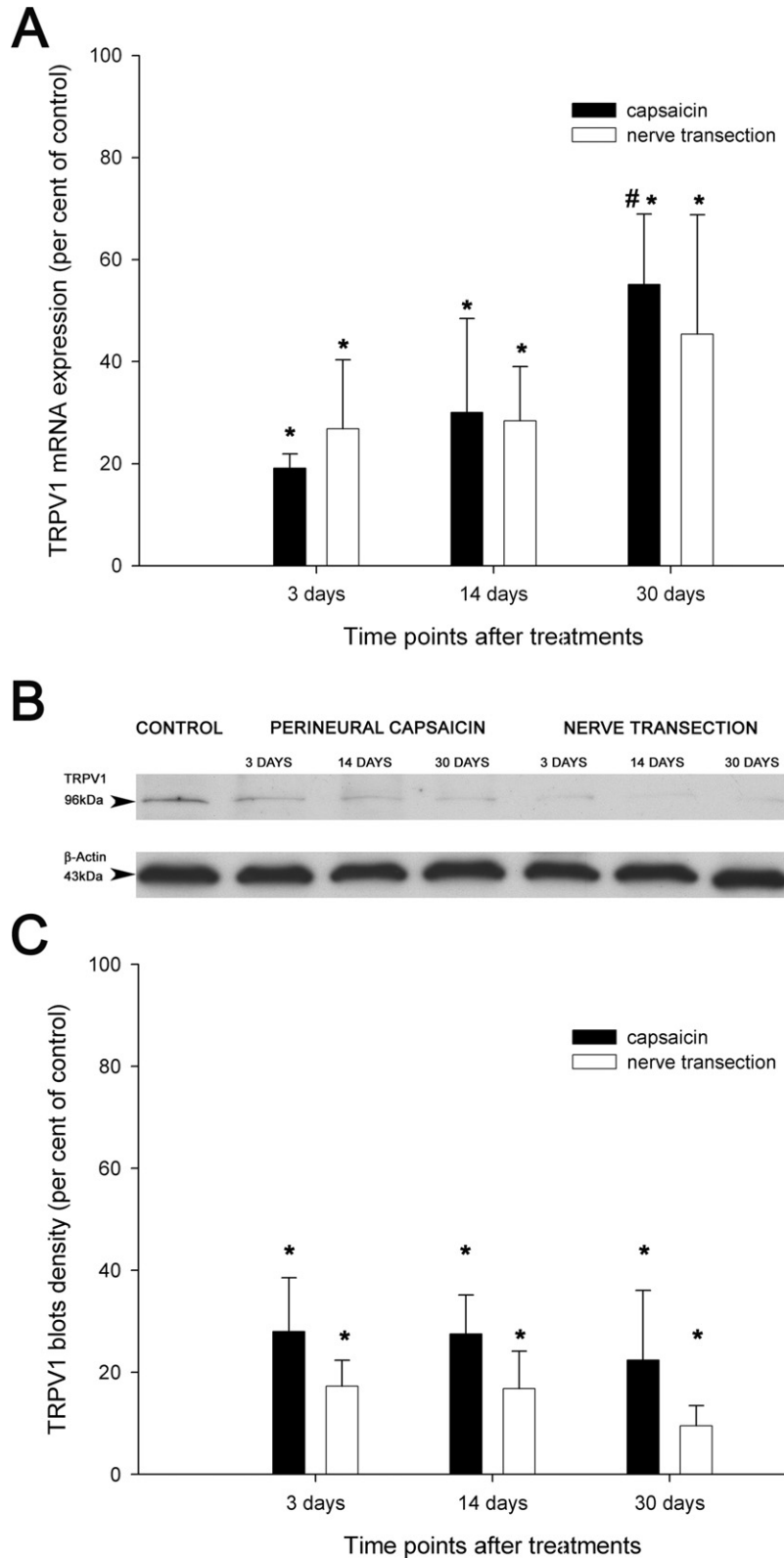
Fig. 2. Quantitative RT-PCR and Western blot analyses of the TRPV1 mRNA and protein expression. (A) Results of three to six independent experiments demonstrate the time course of changes in TRPV1 mRNA expression measured with quantitative RT-PCR in L5 DRGs following perineural capsaicin treatment and transection of the sciatic nerve. Note the marked time-dependent increase in TRPV1 mRNA expression following perineural capsaicin treatment. (B) Representative immunoblots of TRPV1 and β-actin proteins in L5 DRGs 3, 14, and 30 d after perineural capsaicin treatment and transection of sciatic nerve. (C) Results of three independent experiments demonstrate the time course of changes in TRPV1 protein. Note the marked decreases in the TRPV1 protein at all time points after perineural capsaicin treatment and nerve transection. * Significantly different from the control, $P<0.05$. # Significantly different from the 3-day value, $P<0.05$.
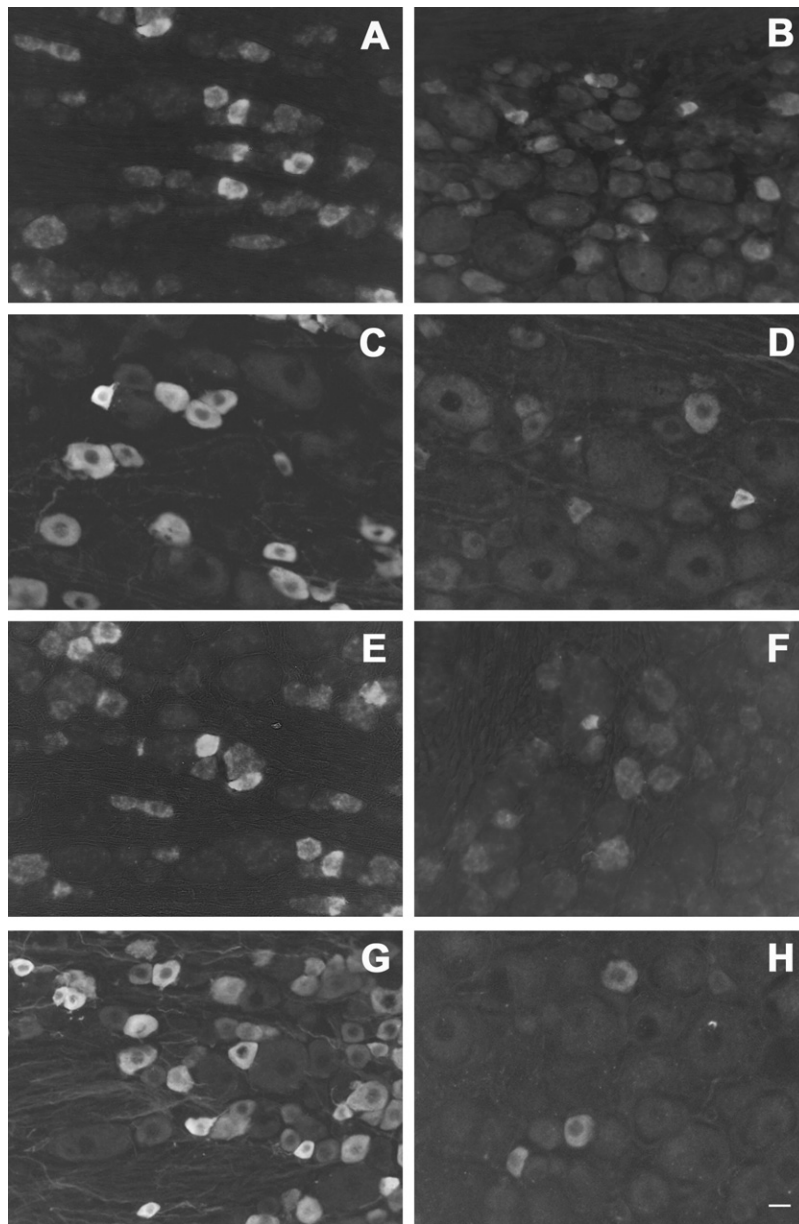
Fig. 3. Representative inverse microphotographs of the L5 DRGs, illustrating the effects of perineural capsaicin treatment (A–D) or sciatic nerve transection (E–H) on the TRPV1 mRNA expression (A, B and E, F) or TRPV1 immunoreactivity (C, D and G, H). Microphotographs illustrate the corresponding control (A, C, E, G) DRGs and DRGs ipsilateral to the sciatic nerve treated with capsaicin (B, D) or peripheral nerve transection (F, H) after 14 d. Scale bar indicates 25 μm and applies to all microphotographs.

of the control value was evident after a survival period of 4 weeks. In type B neurons, the TRPV1 mRNA expression already displayed a significant reduction by 3 days, with a significant recovery at the end of the study period. The measurements of total TRPV1 mRNA with quantitative RT-PCR in DRGs relating to the capsaicin-treated sciatic nerve confirmed these findings. An early profound decrease in TRPV1 mRNA expression was followed by a clear-cut tendency to recovery resulting in a significant increase in TRPV1 mRNA expression to about 60% of the control at the end of the study. Interestingly, however, when the TRPV1 immunoreactivity was investigated, a

tendency to recovery was not observed. The proportions of TRPV1-immunoreactive type C and type B DRG neurons decreased to about 12% and 25% of the total control neuronal population after 3 days and remained at these low levels even after a survival period of 4 weeks. It should be noted that these changes in the proportions of affected TRPV1 mRNA-expressing and TRPV1-immunoreactive neurons should be considered in light of the fact that about 20% of the neurons in the L5 DRGs are not affected by the lesions for their axons run in nerves other than the sciatic nerve (Yip et al., 1984; Aldskogius et al., 1988). These immunohistochemical findings were strongly supported by measurements of the
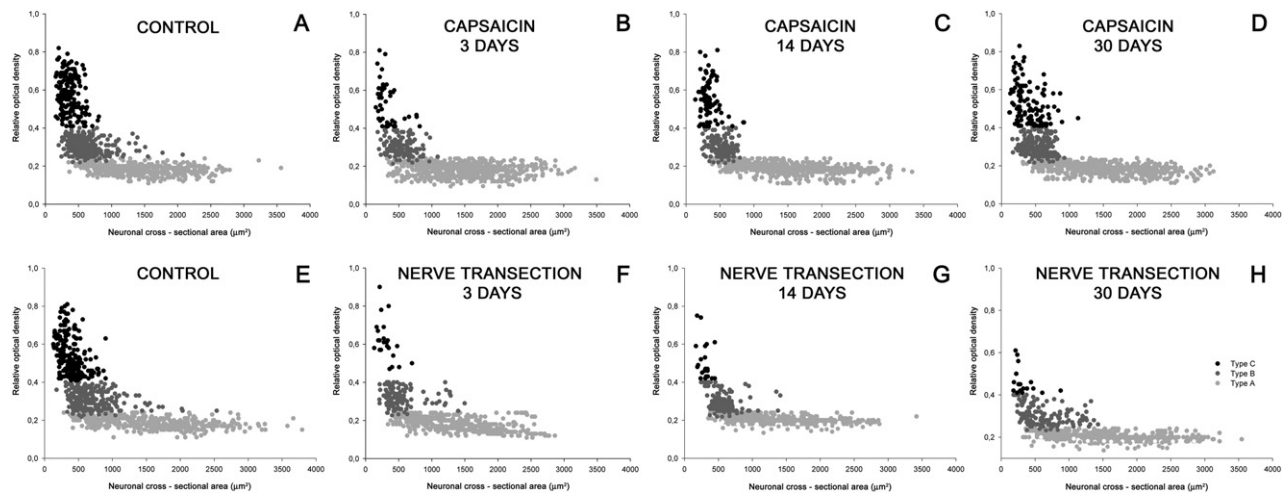
Fig. 4. Scatter plots showing the time course of changes in the populations of TRPV1 mRNA-expressing L5 DRG neurons following perineural capsaicin treatment (A–D) and transection of the ipsilateral sciatic nerve (E–H). Symbols of decreasing graytone intensities denote type C, B, and A neurons, respectively.

TRPV1 protein with Western blotting of the L5 DRGs relating to the capsaicin-treated sciatic nerves. The TRPV1 protein was markedly decreased already 3 days after the capsaicin treatment and remained at that low level amounting about 30% of the control throughout the entire period of the study. The long-lasting, apparently irreversible functional impairments observed after perineural capsaicin treatment, such as the abolition of chemogenic pain and neurogenic inflammation, elevated latencies of thermal nociceptive reflexes, and reduced thermal hyperalgesia, are in accord with the down-regulation of TRPV1 protein in the DRG neurons.

Several factors must be considered in the interpretation of the disparate changes brought about by the two types of nerve injuries, which differ substantially in their nature, that is, nerve transection and perineural capsaicin treatment. Nerve transection, classified as neurotmesis (Seddon, 1943), results in complete severance of the nerve. In contrast, although leading to a selective chemodenervation of nociceptive afferents which express the TRPV1 by a mechanism which involves a slowly progressing dying-back type of degeneration process (Jancsó and Lawson, 1990; Jancsó, 1992), perineural treatment with capsaicin leaves the nerve fibers continuous. The exact nature of this denervation process is still unclear, but it has been demonstrated that, although practically all capsaicin-sensitive C-fiber afferents are functionally inactivated, only about half of this population undergo degeneration, the number of unmyelinated axons in capsaicin-treated nerves decreasing by only some 30% (Jancsó and Lawson, 1990; Pini et al., 1990; Jancsó, 1992). This may imply that after perineural capsaicin, unlike after nerve transection, the surviving axons may provide some trophic support for the chemically injured neurons, which may be sufficient to promote the transcription, but not the translation of TRPV1 mRNA. This assumption is supported by find-
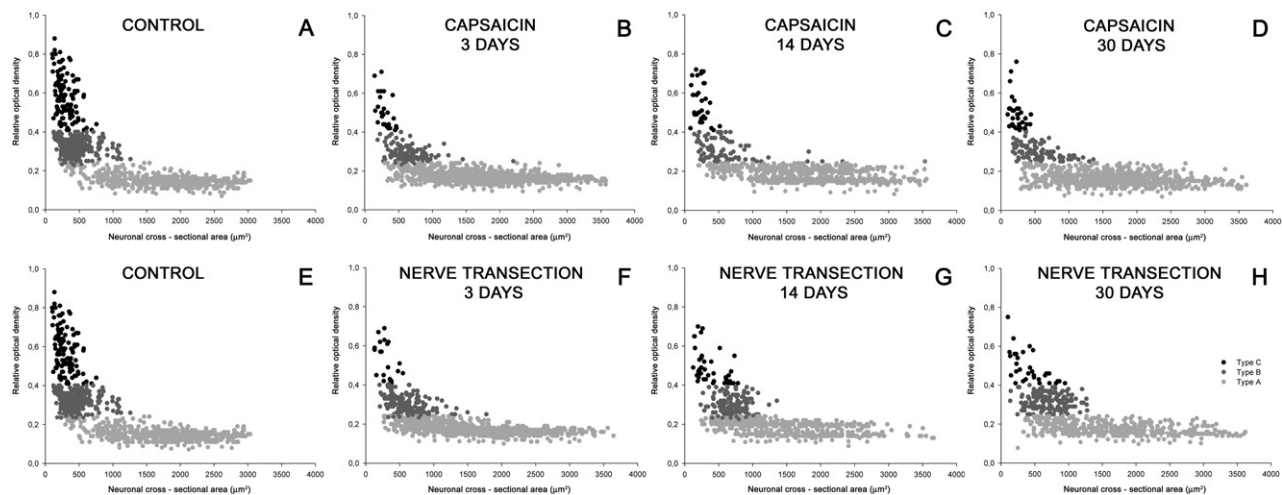

Fig. 5. Scatter plots showing the time course of changes in the populations of TRPV1-immunoreactive L5 DRG neurons following perineural capsaicin treatment (A–D) and transection of the ipsilateral sciatic nerve (E–H). Symbols of decreasing graytone intensities denote type C, B, and A neurons, respectively.

ings indicating that perineural capsaicin treatment exerts a profound selective, but transient blockade of axonal transport processes in C-fiber primary afferent neurons (Gamse et al., 1982; Sántha and Jancsó, 2003). Nerve growth factor (NGF) reaching the perikarya of the DRG neurons through retrograde axonal transport has been shown to play a pivotal role in the regulation of the expression of TRPV1 mRNA and protein in DRG neurons. Indeed, deprivation of DRG neurons of NGF under either *in vivo* or *in vitro* conditions has been shown to lead to a downregulation of TRPV1 mRNA expression and a loss of sensitivity to capsaicin (Winter et al., 1988; Aguayo and White, 1992; Jancsó and Ambrus, 1994; Jancsó et al., 1997; Michael and Priestley, 1999).

Similar phenomena involving a mismatch of mRNA and protein expressions have been reported, depending on the developmental and/or functional state of the DRG neurons. Peripherin mRNA and protein have been shown to be expressed in parallel in developing DRG neurons. However, in mature DRGs, large neurons express peripherin mRNA, but not the protein. This was attributed to changes in the availability of peripherally derived trophic factors such as NGF (Goldstein et al., 1996).

Although the distinct changes in the availability of trophic factors probably best explain, at least in part, the findings of the present study, other mechanisms may also be considered. The replacement of chemically injured neurons by proliferating DRG cells may offer an alternative possibility for the partial restitution of the neuron populations which express TRPV1 mRNA. Indeed, recent findings demonstrated a restoration of viscerosensory innervation by neurogenesis following a systemic injection of capsaicin (Czaja et al., 2008), which results in the degeneration of large populations of nodose and DRG neurons (Jancsó et al., 1977, 1980, 1985; Ritter and Dinh, 1988; Jancsó and Lawson, 1990; Jancsó, 1992; Hiura et al., 2002). However, this possibility seems unlikely, since little if any functional recovery was demonstrated after perineural treatment with capsaicin (Jancsó et al., 1980, 2011; Fitzgerald and Woolf, 1982; Jancsó and Lawson, 1990; Jancsó, 1992; Dux et al., 1999; Sántha and Jancsó, 2003).

The present study suggests that the regulation of the expression of TRPV1 after nerve injury is dependent on the type of the injury and not on the type of the DRG neuron. Whereas nerve transection resulted in an apparently long-lasting downregulation of TRPV1 mRNA expression, the selective chemodenervation of capsaicin-sensitive DRG neurons produced a transient and largely reversible downregulation of TRPV1 mRNA expression as shown by both *in situ* hybridization and quantitative RT-PCR. However, both treatments induced a seemingly irreversible inhibition of TRPV1 translation and/or changes in post-translational processing, resulting in a massive and permanent loss of TRPV1 protein from DRG neurons, as assessed by immunohistochemistry and Western blotting following perineural capsaicin treatment or nerve transection. The present findings may have important implications as concerns the mechanism(s) of chemically induced selective analgesia. The results point to the possibility that interfering with the translation and/or post-translational

processing of nociceptive ion channels, such as the TRPV1, by using specific siRNAs, for example, may offer a novel approach to the production of antinociception by employing molecular biological tools.

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