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Effect of genetic deletion of the vanilloid receptor TRPV1 on the expression of Substance P in sensory neurons of mice with adjuvant-induced arthritis

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Abstract

The neuropeptide Substance P (SP), expressed by nociceptive sensory afferents in joints, plays an important role in the pathogenesis of arthritis. Capsaicin causes neurons in the dorsal root ganglia (DRG) to release SP from their central and peripheral axons, suggesting a functional link between SP and the capsaicin receptor, the transient receptor potential vanilloid 1 (TRPV1). The expression of both TRPV1 and SP have been reported to increase in several models of arthritis but the specific involvement of TRPV1-expressing articular afferents that can release SP is not completely understood. We here wanted to ascertain whether the increase in the number of SP-positive primary afferents in arthritis may be affected by genetic deletion of TRPV1. For this, we used immunohistochemistry to quantify the expression of SP in primary afferent neurons in wild type mice (WT) vs. TRPV1-knockout (KO) mice with adjuvant-induced arthritis (AIA). We found that the expression of SP in DRG 1) increased significantly over naïve level in both WT and KO mice 3 weeks after AIA, 2) was significantly higher in KO mice than in WT mice in naïve mice and 2-3 weeks after AIA, 3) was significantly higher on the side of AIA than on the contralateral, vehicle-injected side at all time points in WT mice, but not in KO mice, and 4) increased predominantly in small-size neurons in KO mice and in small- and medium-size neurons in WT mice. Since the size distribution of SP-positive DRG neurons in arthritic TRPV1-KO mice was not significantly different from that in naïve mice, we speculate that the increased expression of SP is unlikely to reflect recruitment of A-fiber primary afferents and that the higher expression of SP in KO mice may represent a plastic change to compensate for the missing receptor in a major sensory circuit.

Keywords

Substance P; TRPV1; Dorsal root ganglion; Adjuvant-induced arthritis; Mouse

1. Introduction

Substance P (SP), a member of the tachykinin family of neuropeptides, is synthesized by sensory neurons in the dorsal root ganglion (DRG) and released peripherally in the skin, viscera, and joints, and centrally in the superficial laminae of the spinal dorsal horn (Ribeiro-

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da-Silva and Hokfelt, 2000). In DRG neurons, SP colocalizes with the amino acid transmitter glutamate (Battaglia and Rustioni, 1988) and the neuropeptide calcitonin gene-related peptide (CGRP, Wiesenfeld-Hallin et al., 1984).

Peripheral axons containing SP have been reported in healthy and arthritic joint tissues, including synovium, joint capsule, epiphyseal cartilage, menisci, intraarticular disc, and subchondral bone (Bjurholm et al., 1988; Goto et al., 1998; Abramovici et al., 1991; Lunam and Gentle, 2004; Danielson et al., 2006); most of these studies found colocalization of SP with CGRP in peripheral sensory fibers in the joints and periarticular bone (Gajda et al., 2005). It is generally accepted that SP is involved in the pain and inflammation of arthritis, although the exact mechanisms are not fully understood (Keeble and Brain, 2004). SP is involved in the spinal processing of nociceptive input from joints (Graven-Nielsen and Arendt-Nielsen, 2002) and is required for maintenance of hyperexcitability of spinal cord neurons during arthritis (Neugebauer et al., 1995)..

SP is expressed by C- and A δ -fiber primary afferents, the majority of which express the vanilloid receptor TRPV1 (Guo et al., 1999). TRPV1, a key peripheral integrator of pain stimuli, was originally described on C-type nociceptive neurons as a molecular target for capsaicin, the pungent vanilloid ingredient of hot peppers (Caterina et al., 1997; Holzer, 1991; Szallasi and Blumberg, 1996). In mice, knocking out the TRPV1 gene prevented the occurrence of thermal hyperalgesia in a model of hindpaw inflammation (Caterina et al., 2000; Davis et al., 2000) and TRPV1-KO mice have been used to study the involvement of capsaicin-sensitive sensory afferents in several inflammatory models, including arthritis (Bolcskei et al., 2005). In particular, we and others have found that the severity of adjuvant-induced arthritis (AIA) is significantly reduced in TRPV1-KO mice (Barton et al., 2006; Chen et al., 2009; Szabo et al., 2005).

SP is released from terminals of capsaicin-sensitive sensory neurons (Geppetti et al., 2008) and systemic capsaicin treatment in neonatal rats leads to selective elimination of SP-containing primary afferent fibers (Liu et al., 1997), suggesting a functional link between SP and TRPV1, the receptor for capsaicin (Premkumar and Sikand, 2008). Capsaicin can attenuate the inflammatory response in experimental arthritis and the increase in SP content in peripheral nerves, DRG, and spinal cord in rats with AIA (Colpaert et al., 1983), suggesting that the role of SP in the peripheral mechanisms of arthritis may be mediated by TRPV1. Moreover, pretreatment with capsaicin was found to mitigate the upregulation of SP in the rat model of AIA (Ahmed et al., 1995b), supporting previous observations that capsaicin reduces the expression of neuropeptides in AIA (Levine et al., 1986).

The mechanism through which TRPV1 participates in the pathogenesis of arthritis and the specific involvement of TRPV1-expressing articular afferents that can release neuropeptides is not fully understood. We showed previously that the fraction of CGRP-immunopositive DRG neurons is higher in TRPV1-KO mice than in wild-type (WT) mice with AIA, and that this increase is bilateral but significantly greater on the inflamed side than on the control side (Chen et al., 2008). In the present study, we wanted to ascertain whether the increase in the number of SP-positive primary afferents during AIA is similarly affected by the genetic deletion of TRPV1. For this, we used immunohistochemistry to quantify the expression of SP in primary afferent neurons in TRPV1-KO mice vs. WT mice with AIA.

Changes in phenotype of nociceptive primary afferent neurons, mostly caused by injury to their peripheral axons, are accompanied by changes in expression of neurotransmitters, and involve the recruitment of larger (A-fiber) DRG neurons (Obata et al., 2004; Peters et al., 2005). In a recent study of a rat model of osteoarthritis, Henry argued that there is no expectation that SP expression will occur in DRG neurons that do not normally express SP, but given its implication

in nociceptive mechanisms it would be important to establish whether there is such a change; interestingly, he reported that the biggest changes of firing patterns in this model occurred not in the small, high-threshold afferents but in large, low-threshold A-fiber afferents (Henry, 2009). Therefore, we also wanted to investigate whether the increased expression of SP in DRG of arthritic mice involves recruitment of larger primary afferent neurons and whether this phenomenon is affected by the genetic deletion of TRPV1.

2. Materials and methods

2.1. Animal model, euthanasia, and tissue processing

A total of 29 male mice, ages 16-20 weeks and weighing 28-30 g, were used in this study. These included 14 TRPV1-KO and 15 wild-type C57BL/6 mice (Jackson Labs, Bar Harbor, ME). All experimental procedures involving mice were carried out in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals, and according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Carolina.

AIA was induced as described previously (Chen et al., 2009): mice anesthetized with a mixture of ketamine and xylazine (90 mg/kg and 10 mg/kg, i.p.) were injected with 10 μ l of complete Freund's adjuvant (CFA, 20 mg/ml suspension of heat-killed *Mycobacterium tuberculosis* in vehicle; Difco Lab, Detroit, MI) in each of two sites, front and back of the left ankle, using a 30-gauge needle attached to a 10 μ l Hamilton syringe. The right ankle of each mouse was injected with the same volume of vehicle (paraffin oil containing mannide monooleate; incomplete Freund's adjuvant, IFA; Difco). Three-four each of TRPV1-KO and WT mice were sacrificed before (naïve) or 7, 14, and 21 days after induction of arthritis.

For tissue collection, mice anesthetized with sodium pentobarbital (80 mg/kg, i.p.) were perfused intracardially with 30 ml freshly prepared solution of 1% paraformaldehyde (PF) in 0.1 M phosphate buffer, pH 7.4 (PB), followed by 100 ml solution of 4% PF in PB. The L4-L5 DRG were dissected out bilaterally, postfixed in 4% PF for 4 hours, cryoprotected in 30% sucrose in PB for 48 hours, and sectioned on a cryostat at 10 μ m.

2.2. Immunohistochemistry

For immunohistochemistry, sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) for 15 minutes, blocked with 5% normal donkey serum in 0.1% Triton X-100 in PBS (NDS; Jackson Immunoresearch, West Grove, PA) for 30 minutes, and incubated overnight with a guinea pig anti-SP antibody (1:1,000; Neuromics, Edina, MN, Cat# GP14103) in NDS. After several rinses with PBS and incubation with 5% NDS for 30 minutes, sections were incubated with donkey anti-guinea pig antibody conjugated to Cy3 (1:200; Jackson) for 2 hours, rinsed, and coverslipped with Vectashield (Vector, Burlingame, CA). Digital micrographs were obtained with a Retiga EX cooled CCD camera (Q-Imaging, Surrey, CA) attached to a Leitz DMR fluorescent microscope (Leitz, Wetzlar, Germany) and saved as TIFF files; contrast and brightness were adjusted with Photoshop CS2 (Adobe Systems, San Jose, CA). The primary antibody employed here was characterized and its selectivity was confirmed using Western blots and preadsorption with a blocking peptide (Neuromics, Edina, MN, Cat# P14103), and is in common use in our laboratory. As a routine control, we processed sections according to the above protocol, except that primary or secondary antibodies were omitted; omission of primary or secondary antibodies completely abolished specific staining. We also immunostained sections of all mice for TRPV1 (1:250; goat anti-TRPV1, Santa Cruz #SC-12498, Lot #L1406) to verify the lack of immunostaining in KO mice.

2.3. Data collection and statistics

Digital images were analyzed using Image J (NIH, Bethesda, MD). All counts and measurements were performed by an investigator blinded to the source material, including the genotype of the mouse and the side. To determine the percentage of SP-positive neurons, a total of 96,971 neuronal profiles (NP) with visible nuclei, identified by their typical cellular morphology, were counted in 653 DRG sections taken at 60 μm intervals (Table 1). To determine the size distribution of SP-positive vs. SP-negative DRG neurons, 2,529 NP in 34 sections from 7 naïve and 6 arthritic mice (21 days of AIA), were outlined on an Intuos graphics tablet (Wacom, Vancouver, WA) and their areas were measured using Image J.

To distinguish SP-positive from SP-negative NP, the cut-off brightness level (labeling density threshold) was determined by averaging the integral brightness of three NP per section that were judged to be minimally positive. All profiles whose mean labeling density exceeded this threshold were counted as positive. The “background” immunofluorescence was high enough to allow visualization of immunonegative NP; in some cases these were verified with DIC optics. The fraction of immunopositive NP in each section were expressed as a percentage of total counted NP [% = (positive NP/total counted NP) \times 100]. Data were analyzed with SPSS 11.5x (SPSS, Chicago, IL) and graphed using Kaleidagraph (Synergy Software, Reading, PA).

Differences between animal groups were studied for significance with one-way analysis of variance (ANOVA), which assessed the overall influence of genotype, side (left, CFA-injected or right, IFA-injected), and time after induction of arthritis as factors, followed by a post hoc general contrast comparison using Tukey's test. Significance was set at $p < 0.05$.

3. Results

In DRG from all mice, multiple NP and axons were immunostained for SP (Fig. 1). Counts revealed that in naïve WT mice, $12.6 \pm 0.5\%$ (mean value \pm standard error of mean) of all NP were SP-positive while in naïve KO mice this percentage was $13.6 \pm 0.5\%$ (Fig. 2). In arthritic mice, the expression of SP was significantly greater on the CFA-injected than on the IFA-injected side in WT mice ($13.5 \pm 0.5\%$ vs. $10.6 \pm 0.5\%$ after 7 days; $10.5 \pm 0.7\%$ vs. $8.9 \pm 0.6\%$ after 14 days; and $14.4 \pm 0.6\%$ vs. $11.5 \pm 0.7\%$ after 21 days), however in TRPV1-KO mice, the expression was not significantly different between the two sides ($13.3 \pm 0.5\%$ vs. $12.5 \pm 0.8\%$ after 7 days; $13.6 \pm 0.6\%$ vs. $14.4 \pm 0.6\%$ after 14 days; and $16.1 \pm 0.5\%$ vs. $17.4 \pm 0.9\%$ after 21 days). The expression of SP in WT mice decreased significantly, compared to naïve values, at 14 days of AIA, followed by a significant increase at 21 days; the expression in KO increased significantly at 21 days. KO mice had a significantly greater expression than WT mice at 0, 14, and 21 days on both the CFA- and IFA-injected sides.

To ascertain whether the increased expression of SP after CFA was associated with phenotypic changes, we studied the size distribution of SP-positive NP in DRG in naïve mice vs. 21 days after CFA (Fig. 3). Data showed a shift towards larger cells between naïve and arthritic mice, particularly in the 300-600 μm^2 area range, suggesting a recruitment of medium-size neurons (presumed A-fiber type, Tan et al., 2008) in WT but not in KO mice; there were no apparent changes in size distribution of SP-negative NP.

4. Discussion

The main findings of this study are that the expression of SP in DRG 1) increased significantly over naïve level in both WT and KO mice 21 days after CFA, 2) was significantly higher in KO than in WT naïve mice 14-21 days after CFA, 3) was significantly higher on CFA-injected side than on the contralateral, vehicle-injected side at all time points in WT mice, but not in

KO mice, and 4) increased predominantly in small-size neurons in KO mice and in medium-size neurons in WT mice.

In naïve animals, depending on the species and ganglion level (Vizzard, 2001), and perhaps more importantly on the criteria for counting, counts of SP-expressing neurons may vary up to ~20% of the total DRG neuronal population (Battaglia and Rustioni, 1988; Hokfelt et al., 1975). Thus, SP-positive neurons constitute 6-21% of all DRG neurons in the rat (Price and Flores, 2007; Tuchscherer and Seybold, 1985). In mice, the expression of SP in articular afferents has been found to increase with age: Salo et al. (2002) reported that 1.8% of knee afferents in young mice expressed SP, in older mice, this number increased to 3.3% at 52 weeks and to 2.5% by 96 weeks. We found that the percentage of SP-positive DRG neurons in naïve mice was higher in TRPV1-KO mice than WT mice, similar to that of CGRP (Chen et al., 2008).

In mice with AIA, the expression of SP increased bilaterally but was more pronounced on the CFA-injected than on the IFA-injected side. The level of increase was similar to the reported changes in the tissue concentrations of SP in a rat model of AIA (5.93 and 5.63 pmoles/gm for the right and the left DRG, respectively, in arthritic rats vs. 3.71 and 3.88 pmoles/gm in naive rats, Ahmed et al., 1995a) and was significantly higher in TRPV1-KO than in WT mice, similar to that of CGRP (Chen et al., 2008). Since the TRPV1/SP population of DRG cells is smaller than the TRPV1/CGRP population, the effect of deletion of TRPV1 on the expression of SP may have been underestimated.

Although the increased expression of SP in articular afferents is common to various forms of experimental arthritis, its time course may depend on the particular model: it has been reported that in the AIA model, SP increased rapidly (Bulling et al., 2001), while in the kaolin and carrageenan models, it initially decreased, followed by a slower increase (Sluka and Westlund, 1993). In our model of AIA, the expression of CGRP increased as early as 3 days post CFA-injection (Chen et al., 2008), compared to the delayed changes of SP, including a decrease in the WT mice at 14 days followed by an increase at 21 days. The decreased number of SP-positive cell bodies may be due to the increased release of SP peripherally and/or centrally (Maggi, 1995; Sluka and Westlund, 1993).

The bilateral increase of the expression of SP in mice with AIA may reflect the arthritogenic potential of IFA and/or the ability of CFA, when injected in one joint, to evoke arthritis in the contralateral joint (Cannon et al., 1993; Szabo et al., 2005). Upregulation of SP in AIA may occur in parallel with the increased expression of TRPV1 in DRG neurons (Chen et al., 2009) or in the peripheral nerve fibers at the site of inflammation (Carlton and Coggeshall, 2001; Yiangou et al., 2001). Also in parallel with TRPV1, the changes in SP expression may depend on the particular model of arthritis, survival time, and animal species (Bar et al., 2004).

Upregulation of SP has been observed in joints with AIA (Garrett et al., 1995) although a reduction in the SP-containing fibers in the synovium has also been reported (Kontinen et al., 1992), particularly in joints with heavy inflammatory infiltration, suggesting that the articular expression of SP may be regulated differently at different stages of arthritis (Keeble and Brain, 2004). The synovial release of SP in the course of arthritis (Lotz et al., 1987; Saito and Koshino, 2000) may be triggered by depolarization of articular afferents via TRPV1. In support of this possibility, the proinflammatory role of TRPV1-expressing sensory afferents in pancreatitis has been shown to be mediated by SP (Hutter et al., 2005; Nathan et al., 2001).

We showed in the rat that TRPV1-positive primary afferents contact ascending nociceptive neurons in the spinal dorsal horn that express the preferred receptor for SP, neurokinin-1 (NK-1), and are thus likely to be targets for centrally-released SP (Hwang et al., 2003).

However, based on the virtual lack of colocalization of TRPV1 and SP in the central axons of DRG neurons (Hwang and Valtschanoff, 2003), we also argued that the TRPV1-positive afferents provide a separate, largely SP-independent, input onto the NK-1-expressing spinal neurons that relies on glutamate as neurotransmitter (Hwang et al., 2004). Whether these two afferent input systems are separate also in the mouse remains to be established.

The increased expression of SP after CFA involved medium-size neurons (presumed A-fiber type) in WT but not in KO mice. We conclude that the increased expression of SP in KO mice is unlikely to reflect a phenotypic switch of primary afferents from C- to A-fiber type, and speculate that it may represent a plastic change to compensate for the missing receptor in a major sensory circuit. Alternatively, the lack of TRPV1 may decrease the release of SP so that more DRG neurons in KO than in WT mice retain SP above the level of detection with immunohistochemistry.

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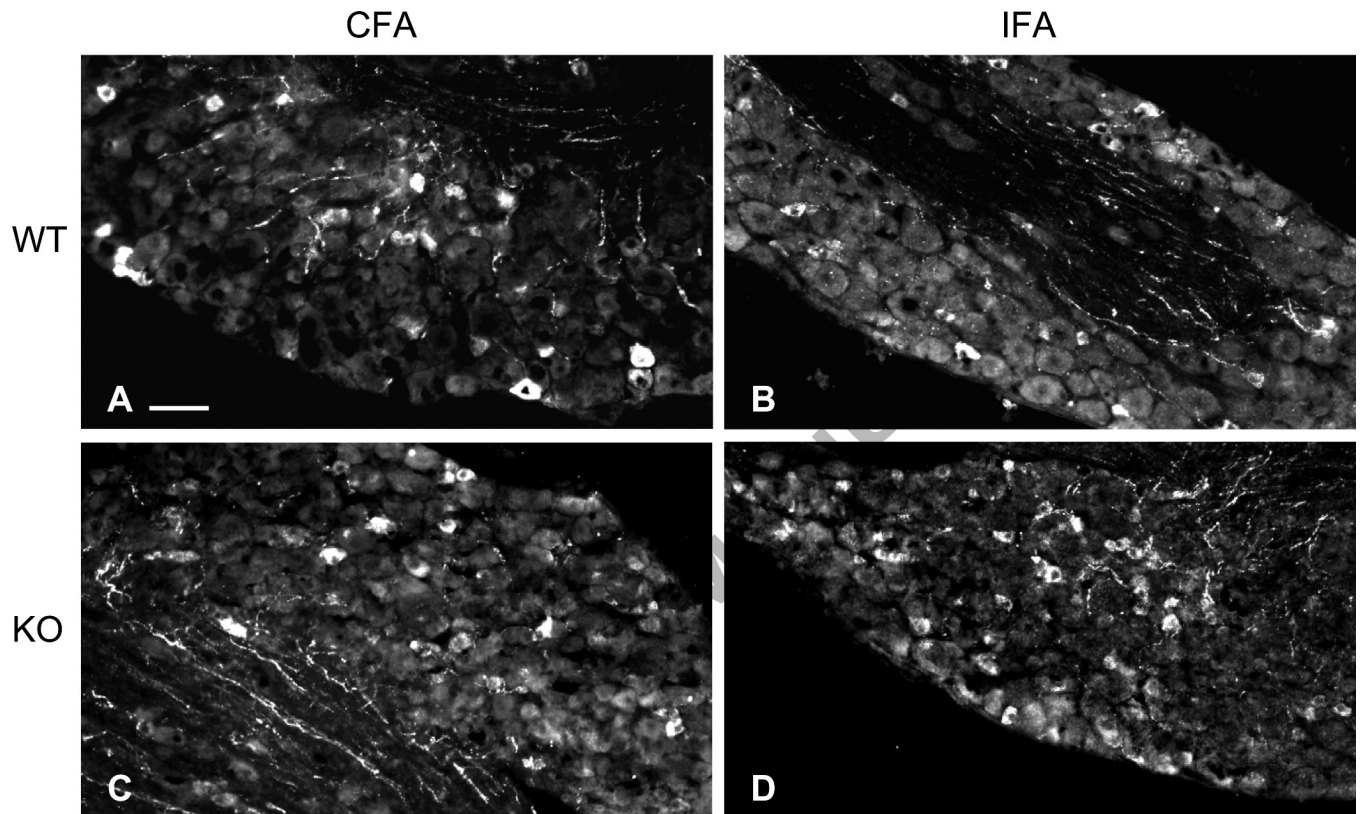


Fig. 1. Immunofluorescent staining for SP in DRG sections of wild type (WT) and TRPV1-KO (KO) mice 21 days after induction of AIA. In each group (WT and KO), the sections are from the left and right L5 DRG of the same mouse. Scale bar, 100 μ m.

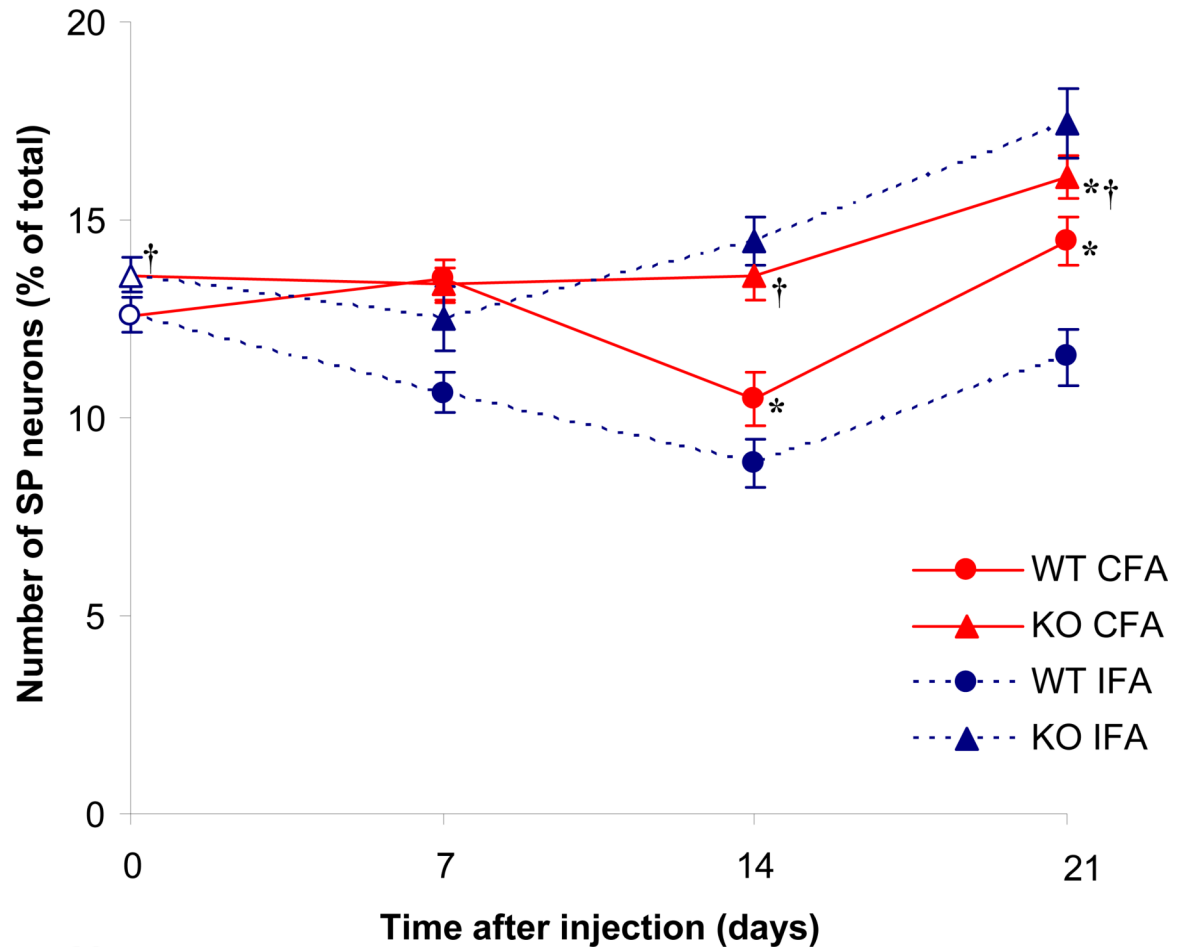


Fig. 2.

The expression of SP was higher in TRPV1-KO (KO) than in wild type (WT) mice: the fraction (mean \pm standard error of mean) of immunostained neuronal profiles was higher in KO mice than WT naïve mice (time point “0”) and 14-21 days after induction of AIA. It was also significantly higher on the side of CFA than on the contralateral, IFA-injected side at all time points in WT mice, but not in KO mice. *significantly different compared to naïve values for the same genotype; †significantly different compared to WT values for the same time point; $p < 0.05$, ANOVA.

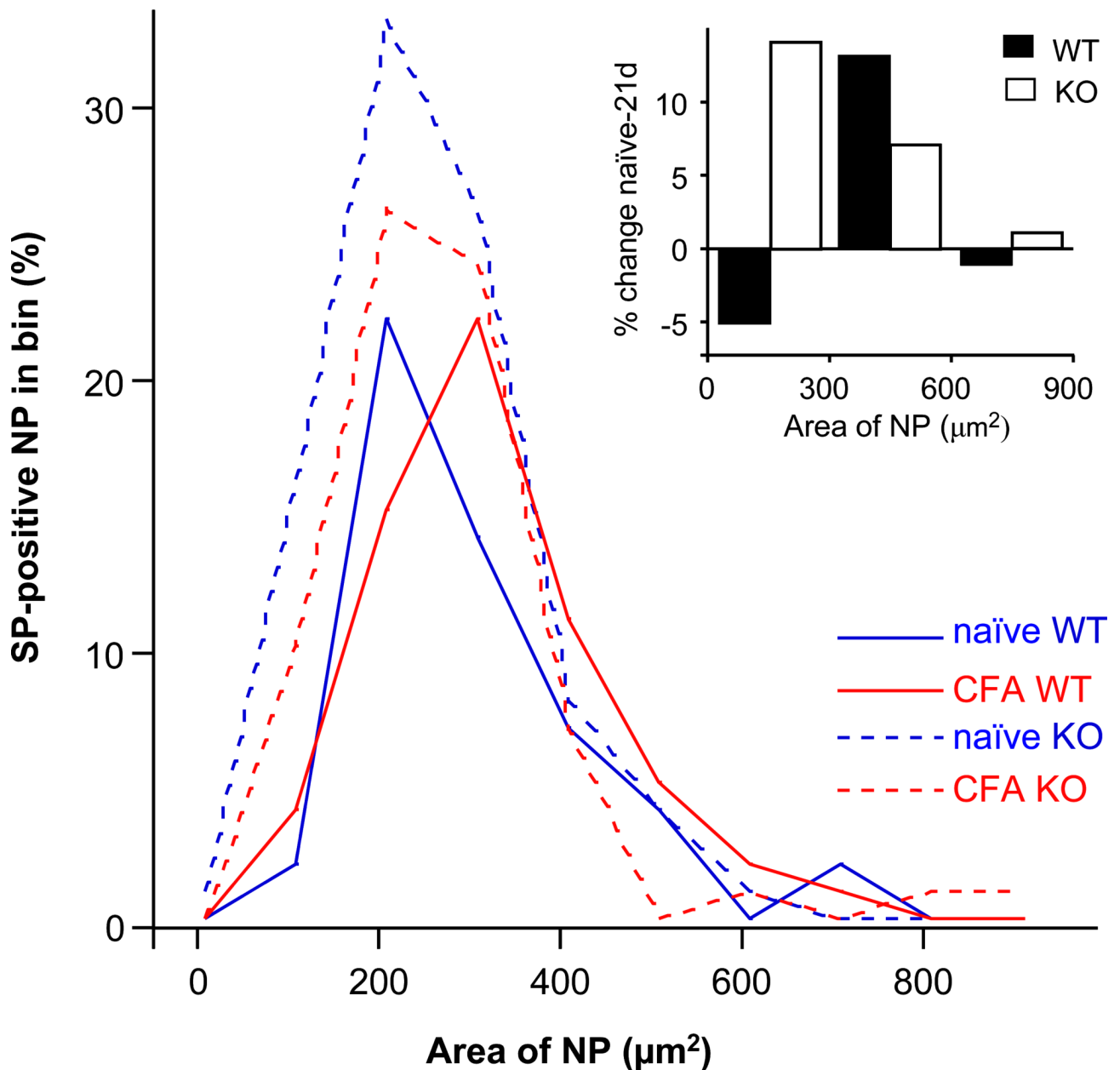


Fig. 3. Size distribution of SP-positive neuronal profiles (NP) in DRG of wild type (WT) and TRPV1-KO (KO) mice before (naïve) and 21 days after injection of CFA. Data suggest a shift towards larger cells, particularly in the 200-400 μm^2 bins, between naïve and arthritic mice for WT but not for KO mice. In the insert, histogram suggests a pronounced increase in the number of medium-size neurons (300-600 μm^2) in WT mice (solid bars), whereas in KO mice (open bars), the increase occurred mostly in small neurons (<300 μm^2). There were no significant changes in size distribution of SP-negative NP (not shown for clarity).

Table 1

Material used for counts

	Days post injection			
	0	7	14	21
WT CFA	865/7226 (48)	934/6959 (41)	425/4143 (34)	846/6048 (43)
KO CFA	1176/8213 (52)	1175/8833 (45)	451/3051 (29)	1403/8828 (52)
WT IFA	591/4631 (34)	829/7771 (45)	351/4162 (40)	590/5110 (37)
KO IFA	771/6170 (44)	831/6202 (34)	529/3775 (29)	1028/5849 (39)

For each treatment group and time point, the number of neuronal profiles (NP) in L4-5 DRG are shown as SP-positive NP/total NP; the number of sections is given in parentheses.