HHS Public Access

Author manuscript

Brain Struct Funct. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Brain Struct Funct. 2016 May ; 221(4): 1985–2004. doi:10.1007/s00429-015-1019-6.

Axotomy of tributaries of the pelvic and pudendal nerves induces changes in the neurochemistry of mouse dorsal root ganglion neurons and the spinal cord

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Abstract

Using immunohistochemical techniques, we characterized changes in the expression of several neurochemical markers in lumbar 4-sacral 2 (L4-S2) dorsal root ganglion (DRG) neuron profiles (NPs) and the spinal cord of BALB/c mice after axotomy of the L6 and S1 spinal nerves, major tributaries of the pelvic (targeting pelvic visceral organs) and pudendal (targeting perineum and genitalia) nerves. Sham animals were included. Expression of cyclic AMP-dependent transcription factor 3 (ATF3), calcitonin gene-related peptide (CGRP), transient receptor potential cation channel subfamily V, member 1 (TRPV1), tyrosine hydroxylase (TH) and vesicular glutamate transporters (VGLUT) types 1 and −2 was analysed seven days after injury. L6-S1 axotomy induced dramatic de novo expression of ATF3 in many L6-S1 DRG NPs, and parallel significant downregulations in the percentage of CGRP-, TRPV1-, TH- and VGLUT2-immunoreactive (IR) DRG NPs, as compared to their expression in uninjured DRGs (contralateral L6-S1-AXO; sham mice); VGLUT1 expression remained unaltered. Sham L6-S1 DRGs only showed a small ipsilateral increase in ATF3-IR NPs (other markers were unchanged). L6-S1-AXO induced de novo expression of ATF3 in several lumbosacral spinal cord motoneurons and parasympathetic preganglionic neurons; in sham mice the effect was limited to a few motoneurons. Finally, a moderate decrease in CGRP- and TRPV1-like-immunoreactivities was observed in the ipsilateral superficial dorsal horn neuropil. In conclusion, injury of a mixed visceral/non-visceral nerve leads to considerable neurochemical alterations in DRGs matched, to some extent, in the spinal cord. Changes in these and potentially other nociception-related molecules could contribute to pain due to injury of nerves in the abdominopelvic cavity.

Keywords

CGRP; Glutamate; Nerve injury; TRPV1; Visceral pain

Introduction

Entrapment, tumors, trauma and iatrogenesis during various surgical procedures have all been identified as potential causes of pelvic and/or lower abdominal nerve injury (Viswanathan et al. 2009) and are associated with chronic pelvic pain. Typical symptomatic manifestations of pelvic or abdominal nerve injury are chronic paresthesias and pain referred to the area of distribution of the compromised nerve(s), and also to pelvic viscera (Viswanathan et al. 2009).

Injury of two types of peripheral nerves may contribute to the mechanisms of chronic pelvic pain: visceral and non-visceral (parietal) nerves. The former are represented in the pelvis by the pelvic and hypogastric nerves. Of particular, relevance to the present study, the pelvic nerve conveys afferent information from bladder, colon, prostate, vagina, vaginal cervix and uterine cervix (see Van der Horst and Holstege 1998). Purely non-visceral nerves such as the ilioinguinal, iliohypogastric or genitofemoral convey afferent input from the abdominopelvic wall or the skin of genitals and lower limbs and are located in the abdominopelvic wall or

within the abdominopelvic cavity itself (genitofemoral) (Shoja et al. 2013). Finally, mixed visceral and non-visceral nerves such as the pudendal, convey afferent information from visceral (vagina, part of the uterine cervix and the mucosa of the urethra and anus) and non-visceral (skin of the penis, clitoris and perineum and the striated pelvic muscles) structures (see Van der Horst and Holstege 1998).

One of the initial consequences of peripheral nerve injury is the occurrence of dramatic neurochemical changes in dorsal root ganglion (DRG) neurons. Most studies thus far have analysed changes in DRG neurons producing non-visceral nerves (commonly, the sciatic nerve). Thus, since the initial descriptions of altered neurochemical expression of substance P (Jessell et al. 1979), vasoactive intestinal polypeptide (VIP) (Shehab and Atkinson 1986) or galanin (Hökfelt et al. 1987) in primary afferent neurons after axotomy of the sciatic nerve, a plethora of complex changes have been demonstrated for a variety of molecules in DRG neurons, more recently also through use of global gene array methodology (Brumovsky et al. 2013; Costigan et al. 2002; Hökfelt et al. 2013; Xiao et al. 2002). Importantly, such alterations, reflecting maladaptive changes in neurons along the nociceptive pathway, participate in the mechanisms of neuropathic pain (Cohen and Mao 2014).

The most relevant model replicating iatrogenic nerve injury and acute postoperative pain is the rat model of incisional pain (Brennan et al. 1996). Recently, its impact on the neurochemistry of non-visceral DRG neurons has been addressed (Hill et al. 2010; Li et al. 2008; Spofford et al. 2011; Yalamuri et al. 2013). However, this model is performed in the hindpaw skin, and the actual changes in DRG somata giving rise to non-visceral abdominopelvic nerves have yet to be addressed. The same applies to analysis of peripheral nerve injury in DRG neurons projecting axons through mixed (pudendal) or visceral (pelvic, hypogastric) nerves. In fact, only one study, now 20 years old, has been published, where it was shown that unilateral excision of the major pelvic ganglion (MPG) in the rat (with axotomy of the pelvic nerve as a consequence) results in a dramatic upregulation of neuronal nitric oxide synthase (nNOS) in DRG neurons innervating pelvic organs (Vizzard et al. 1995).

In contrast to DRG somata, the impact of peripheral nerve injury on sympathetic and parasympathetic neurons projecting to lower abdominal and pelvic visceral organs and genitalia has been extensively studied in rodents, most commonly through cavernous (penile) nerve axotomy (Karakiewicz et al. 1996). Using this model, the consequences of the deafferentation of autonomic pelvic ganglia on their neurochemistry and nerve regeneration (Calenda et al. 2012; Girard et al. 2013; Keast 2006; Song et al. 2014), on erectile dysfunction (Calenda et al. 2012; Karakiewicz et al. 1996; Song et al. 2014; Yamashita et al. 2009) or bladder and urethral functions (Lee et al. 2013) have been examined. Axotomy of the pelvic nerve or its tributaries [lumbar 6 (L6) and sacral 1 (S1) spinal nerves axotomy] was introduced for the study of the neural and behavioral effects of vaginocervical stimulation (Pfaus et al. 2006), neurochemical changes in the lumbar sympathetic chain (LSC) (see Brumovsky et al. 2011b), changes in colonic transit (Ridolfi et al. 2011), or the physiology of parturition (Martinez-Gomez et al. 1998; Tovar et al. 2013). Even a model of uterine extirpation (naturally resulting in axotomy of uterine nerves) was developed to study

changes in the expression of molecules in the MPG of pig (Wasowicz 2003). Virtually none of these studies has been dedicated to the analysis of pain after nerve injury or of neurochemical changes in primary afferent neurons.

In the present study in mouse, we address the potential changes in the immunohistochemical expression of a number of neurochemical markers, most of them associated with pain mechanisms (see below), in DRG neurons and the spinal cord, after axotomy of the L6-S1 spinal trunk, giving rise to the pelvic and pudendal nerves in the rat (McKenna and Nadelhaft 1986; Pacheco et al. 1989; Pastelin et al. 2008), and presumably also in the mouse.

Materials and methods

Animals

The experiments were performed using twenty-four male BALB/c mice (b. wt. 22–28 g). Animals were maintained under standard conditions on a 12-h day/night cycle, with water and food available ad libitum.

All experiments in this study have been performed according to the Society for Neuroscience guidelines for the use of animals in research, and were approved by the Regulatory Committee of Experimental Animals of the Faculty of Biomedical Sciences at Austral University.

Axotomy of the L6-S1 nerve trunk (L6-S1-AXO)

The procedure was performed as described previously (Brumovsky et al. 2011a, b). Briefly, in twenty-four isoflurane-anesthetized mice (5 % induction, 2.5 % maintenance, 1 l/min O_2 flow rate, Baxter Health Care Laboratories, Guayama, Puerto Rico), and after protecting their corneas using a 2 % polyacrylic acid sterile ophthalmic gel (Poen Laboratories, Buenos Aires, Argentina), the ventral branches of the left 6th lumbar (L) and 1st sacral (S) spinal nerves, both major contributors to the pelvic and pudendal nerves, were exposed by aseptic laparotomy and dissection. In thirteen mice, these two spinal nerves were tightly ligated and distally axotomized; in the remaining eleven mice the same spinal nerves were exposed as described, but left intact (sham group). After suturing of the abdominal wall, a subcutaneous injection of dexketoprophen (5 mg/kg, Menarini Laboratories, Buenos Aires, Argentina), and topical application of 2 % lidocaine hydrochloride gel (AstraZeneca, Buenos Aires, Argentina), mice recovered in a warm environment.

Immunohistochemistry

Seven days after axotomy ($n = 13$) or sham surgery ($n = 11$), mice were deeply anaesthetized with chloral hydrate (350 mg/kg; Biopack, Buenos Aires, Argentina) and perfused via the ascending aorta with 20 ml warm (37 °C) Tyrode's buffer, followed by 20 ml of a mixture of 4 % paraformaldehyde and 0.2 % picric acid in 0.16 M phosphate buffer (pH 6.9, 37 °C) and 50 ml of the same fixative at 4 °C, the latter passed in approximately, 5–7 min, as previously described (Brumovsky et al. 2012a, b). The ipsilateral and contralateral L4-5, L6-S1 and S2 DRGs, as well as the corresponding levels of the spinal cord, were removed and postfixed in

the same fixative for 90 min at 4 °C. The tissue was finally immersed in 10 % sucrose in phosphate-buffered saline (PBS) (pH 7.4) for 24 h and in 20 % sucrose in PBS for an additional 24 h. After embedding in Cryoplast OCT compound (Biopack, Argentina) and deep-freezing, all tissues were sectioned in a cryostat (HM 505E, Microm, Heidelberg, Germany) at 12- or 20-μm thickness for DRGs and spinal cords, respectively.

Sections were mounted onto gelatin-coated slides for immunohistochemical processing following the avidin-biotin complex (ABC) protocol as previously described (Brumovsky et al. 2004). After two rinses in PBS (10 min each) and dehydration using increasing concentrations of ethanol (ETOH) followed by xylene (1 min each), endogenous peroxidase was inactivated by hydrogen peroxidase (H_2O_2; 0.5 %) diluted in methanol (20 min). This was followed by rehydration using, in sequence, 96° and 70° ETOH (1 min each) and two rinses in PBS (10 min each). Tissue sections were incubated with primary antibodies diluted in 0.01 M PBS [containing 0.2 % bovine serum albumin (w/v), 0.03 % Triton X-100 (v/v) and 0.1 % sodium azide (w/v)] for 24 h at 4 °C. Antibodies included rabbit anti-ATF3 (1:4000; sc-188, Santa Cruz, Dallas, TX, USA), anti-CGRP (1:12,000; C8198, Sigma, Saint Louis, MO, USA), anti-TRPV1 (1:4000; ACC-030, Alomone Labs, Jerusalem, Israel), anti-TH (1:4000; AB152, Millipore, Temecula, CA, USA), anti-VGLUT1 (1:4000; Brumovsky et al. 2007, 2011a, b; Kawamura et al. 2006) or guinea pig anti-VGLUT2 (1:4000; Brumovsky et al. 2007, 2011a, b; Miyazaki et al. 2003). Next, sections were washed twice in PBS, incubated at room temperature (RT) for 60 min with biotinylated donkey anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA, USA) or anti-guinea pig IgG (1:100, Vector Laboratories) secondary antibodies, rinsed twice in PBS, and incubated in ABC Elite Kit reagents (Vector Laboratories) for 30 min at RT. Peroxidase activity was demonstrated by reaction with H_2O_2 using a nickel-intensified diaminobenzidine protocol for enhancement of the immunoreaction product. Following development, sections were incubated for 40 min with 40,6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml), for identification of the nuclear profiles of all cells present per section (Kubista et al. 1987). Finally, sections were washed, dehydrated and coverslipped using Canada balsam (Biopack).

Microscopy and image processing

Sections were examined using a Nikon Eclipse E-800 photomicroscope equipped with appropriate objectives and filters for bright-field and fluorescence visualization, and a DS U2 Nikon digital camera (Nikon, Tokyo, Japan). DAPI staining was visualized using an UV filter and fluorescence illumination, whereas all other markers were examined using bright-field illumination.

Resolution, brightness and contrast of the images were optimized using the Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA, USA).

Quantification and statistical analysis

Quantification was performed in DRGs processed for immunohistochemistry from L6-S1-axotomy (AXO) and sham mice, comparing contra- and ipsilateral sides. Every fourth DRG section was used, from the first to the last cut section, and a total of 5–7 sections per DRG was analysed (equivalent to about a fifth of the whole DRG) per each marker. First, the total

number of neuron profiles (NPs) present in each section was quantified, under fluorescence illumination and using an UV filter, counting only NPs containing clearly visible DAPI-stained neuronal nuclei (Brumovsky et al. 2007, 2011a, b). This was followed by quantification of the number of NPs positive for each immunohistochemical marker, under bright-field illumination. Subsequently, percentages of NPs expressing different markers were obtained by comparing their number with the total number of DAPI-stained NPs. The quantification method utilized in this study may have resulted in an overestimation of large-diameter NPs and a reduction in the opportunity of small NPs to be counted (Guillery 2002). Thus, it is possible that the percentages presented here underestimated the actual number of small DRG NPs. A neurostereological approach would avoid this error (Mayhew and Gundersen 1996), but this study only aimed to reveal the relative expression of different markers, and an estimate of the degree of change after AXO.

ATF3-immunoreactive (IR) NPs were counted in L4-5, L6-S1 and S2 DRGs, revealing scarcity of changes in expression in the ipsilateral L4-5 or S2 DRGs. Accordingly, analysis of CGRP-, TRPV1-, VGLUT1 or VGLUT2-IR NPs was only performed in L6-S1 DRGs. In other words, priority was given to the directly affected DRGs (L6-S1).

Neuronal size distribution of VGLUT2-IR NPs was performed by the analysis of cell body diameters of a representative sample of nucleated NPs expressing VGLUT2 (4195 NPs) in contralateral or ipsilateral L6-S2 DRGs of sham ($n = 3$) or L6-S1-AXO ($n = 3$) mice using the public domain NIH program ImageJ (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nihimage/). All values were pooled into histograms parcelled either in bins of 200 μm^2 (maximum of 1600 μm^2), or based on a NP size distribution categorized as follows (Brumovsky et al. 2007): (1) small, <400 μm^2; (2) medium, 400–1225 μm^2; and (3) large, >1225 μm^2.

Changes in the expression of CGRP and TRPV1 in the lumbosacral dorsal horn after PNA ($n = 4$), comparing the contralateral and ipsilateral sides, were assessed by analysis of the area occupied by CGRP- and TRPV1-like-immunoreactive (LI) fibers (other markers tested in this study appeared not to change after injury, and received no further analysis in the spinal cord). Sham mice were also tested ($n = 4$). High resolution micrographs of the contralateral and ipsilateral dorsal horns were taken with a 20× objective plus additional 2× zooming using side lenses available in the E-800 Nikon microscope tower. This was followed by the selection of three areas within each superficial dorsal horn (lateral, mid and medial) using 1–1.5 cm squares by means of the selection tool in Photoshop (such selection approach allowed the specific analysis of laminae I–III; it also allowed standardizing the total area against which the area occupied by stained nerve fibers was compared). Each photographic triplet was merged in a single file, converted to binary in Photoshop (threshold = 128) and saved for analysis using ImageJ. After being opened in ImageJ, the total area plus the area occupied by stained nerve fibers in each triplet was measured and recorded. Such procedure was performed in 5–7 spinal cord sections separated by 10–20, 20-μm thick sections, in all tested animals. The data thus recorded was then transferred to an Excel sheet and arranged per animal. Subsequently, percentages of stained area were obtained by comparison with the total evaluated area, per animal, and later per group.

Data are presented as mean ± SEM and were analysed statistically using the unpaired student's *t* test; $P < 0.05$ was considered significant.

Results

L6-S1-AXO induces expression of ATF3 in a large proportion of L6-S1 DRG neuron profiles

Whenever present, ATF3 immunostaining was observed in the form of intense and clearly localized nuclear staining, commonly associated with weaker cytoplasmic staining (Fig. 1a – d). Only a very small percentage of nuclear ATF3-IR NPs was detected in contralateral L6-S1 DRGs of L6-S1-AXO or sham mice (Table 1; Fig. 1a, c). In contrast, L6-S1-AXO resulted in a dramatic upregulation in the percentage of ATF3-IR NPs in ipsilateral L6-S1 DRGs that appeared to span all cell-body sizes (Table 1; Fig. 1b). Sham-injury resulted in a significant but small upregulation in the percentage of L6-S1 ATF3-IR DRG NPs (Table 1; Fig. 1d). Only small-sized DRG NPs appeared to be affected in sham mice (Fig. 1d). In many DRG NPs in which ATF3 was upregulated, nuclear lateralization could be observed, independent of cell body size (Fig. 1b, d) or ganglionic level.

Analysis of the neighboring L4-5 and S2 DRGs revealed a small percentage of ATF3-IR NPs in the contralateral side of sham and L6-S1-AXO mice. This pattern of expression was not significantly altered in the ipsilateral side (Table 1).

The presence of ATF3-IR NPs in L4-5 and S2 DRGs could be due to the manipulation of tissues within the abdominal cavity during the induction of PNA or sham-injury, and the accidental injury of an axon supplied by those DRGs. However, because the percentage of ATF3-IR NPs was generally very low at these DRG levels and not statistically significant between contralateral and ipsilateral sides (Table 1), other neurochemical changes induced by L6-S1-AXO were subsequently studied only in L6-S1 DRGs as described in the following section.

L6-S1-AXO downregulates the expression of CGRP, TRPV1, TH and VGLUT2, but not VGLUT1, in L6-S1 DRG neuron profiles

Many small, medium-sized and some large CGRP-IR NPs were detected in the contralateral L6-S1 DRGs of L6-S1-AXO and sham mice (Table 1; Fig. 1e, g). In contrast, L6-S1-AXO, but not sham injury, resulted in a significant downregulation in the percentage of CGRP-IR NPs in ipsilateral DRGs (Table 1; Fig. 1f, h).

A large number of small and medium-sized TRPV1-IR NPs were observed in the contralateral L6-S1 DRGs of L6-S1-AXO and sham mice (Table 1, Fig. 2a, c). L6-S1-AXO resulted in a significant reduction in the percentage of TRPV1-IR NPs in ipsilateral DRGs (Table 1; Fig. 2f). This was in contrast to observations made in sham mice, where no change in the percentage of TRPV1-IR NPs was noticed (Table 1; Fig. 2h).

A number of small and medium-sized TH-IR NPs was detected in the contralateral L6-S1 DRGs of L6-S1-AXO mice (Table 1; Fig. 2e, g), whereas L6-S1-AXO resulted in a significant decrease in the percentage of TH-IR NPs in ipsilateral DRGs (Table 1; Fig. 2f).

The percentage of TH-IR NPs did not change in ipsilateral DRGs of sham mice (Table 1; Fig. 2h).

Several medium-sized and large VGLUT1-IR NPs were detected in the contralateral L6-S1 DRGs of L6-S1-AXO and sham mice (Table 1; Fig. 3a, c). Unlike observed for CGRP, TRPV1 and TH, neither sham-injury nor L6-S1-AXO induced any significant change in the percentage of VGLUT1-IR NPs in ipsilateral DRGs (Table 1; Fig. 3b, d). VGLUT2-IR NPs spanning all cell body sizes were normally observed in high numbers in the contralateral L6-S1 DRGs of L6-S1-AXO and sham mice (Table 1; Fig. 3e, g, i). L6-S1-AXO resulted in a small but significant down-regulation of the percentage of DRG NPs expressing this transporter (Table 1; Fig. 3f, h). In addition, an apparent increase in VGLUT2-like-immunoreactivity (Li) was observed in several DRG NPs of small size (Fig. 3f, h). In contrast to L6-S1-AXO, sham mice did not show any noticeable change in the percentage or signal intensity of VGLUT2-IR NPs (Table 1; Fig. 3j).

Analysis of cell body size of VGLUT2-IR NPs showed a tendency towards a reduced representation of medium-sized NPs in AXO mice (Fig. 4a, b), both when comparing contralateral vs. ipsilateral L6-S1 DRGs from AXO mice, or ipsilateral L6-S1 DRGs between sham vs. AXO mice.

L6-S1-AXO induces the upregulation of ATF3 in lumbosacral spinal motoneurons and in neurons in the intermediolateral cell column

L6-S1-AXO resulted in the upregulation of ATF3 in a considerable number of motoneurons in the ipsilateral spinal cord (Fig. 5a–c). ATF3-IR motorneuron clusters could be observed in the lateral as well as the medial aspects of the ipsilateral ventral horn in L6-S1-AXO mice (Fig. 5a, b), and also at both the lumbosacral (Fig. 5a, c) and sacral spinal cord levels (Fig. 5b). In contrast, ATF3-IR neurons were only infrequently observed in the contralateral ventral horn of L6-S1-AXO mice (data not shown).

L6-S1-AXO also induced an increase of ATF3 in neurons in the ipsilateral intermediolateral cell column of the lumbosacral spinal cord (Fig. 5a, d), but not contralateral to AXO. ATF3-IR motoneurons and neurons in the intermediolateral cell column were infrequently observed in the same section (Fig. 5a, c). In addition, one L6-S1-AXO mouse exhibited two ATF3-IR neurons in area X, ipsilateral to the lesion (Fig. 5e).

In sham mice, ATF3-IR motoneurons in the ipsilateral ventral horn ranged from none (Fig. 5f) to a few (Fig. 5g), even though the magnitude of upregulation was considerably lower when compared to L6-S1-AXO mice (Fig. 5a–c). In contrast, none of the sham mice showed ATF3-IR neurons in the intermediolateral cell column of the lumbosacral spinal cord or area X (Fig. 5f).

Finally, occasional ATF3-IR motoneurons were detected throughout the thoracic, thoracolumbar and the lumbar enlargement, usually on the ipsilateral side of sham and L6-S1-AXO mice and very rarely on the contralateral side (data not shown).

L6-S1-AXO induces a modest downregulation of CGRP- and TRPV1-, but not of VGLUT1- and VGLUT2-LI in the lumbosacral spinal cord

An intense CGRP-LI was observed in the superficial dorsal horn of the spinal cord of sham and L6-S1-AXO mice (Fig. 6a–d). However, compared to sham mice (Fig. 6d, h), L6-S1-AXO induced a modest reduction in CGRP-LI, noticeable in the ipsilateral laminae I–II of the dorsal horn (Fig. 6b, f). This downregulation was apparent respect to both the density of CGRP-IR boutons and the intensity of immunoreactivity (compare Fig. 6f with e, g, h). Image analysis confirmed a reduction in the area occupied by CGRP-IR fibers in the ipsilateral dorsal horn of PNA mice, as compared to the contralateral side (26.9 ± 1.8 vs. 35.8 ± 1.4, respectively; $P = 0.0085$). A difference was not detected in sham animals (37.2 ± 3.4 vs. 36.5 ± 2.7, respectively; $P = 0.8652$).

TRPV1-LI was also detected in the superficial dorsal horn of the spinal cord of sham and L6-S1-AXO mice (Fig. 7a–d). As with CGRP-LI, a modest decrease of TRPV1-LI was observed in the ipsilateral superficial dorsal horn of L6-S1-AXO mice (Fig. 7b, f) compared to their sham counterparts (Fig. 7d, h). The downregulation seemed to be due mostly to a reduction in the density of TRPV1-IR boutons, particularly noticeable in lamina II (compare Fig. 7f with e, g, h). Image analysis confirmed the reduction in the area occupied by TRPV1-IR fibers in the ipsilateral dorsal horn of PNA mice, as compared to the contralateral side (25.6 ± 1.6 vs. 34.2 ± 2.1, respectively; $P = 0.0196$). A difference was not detected in sham animals (34.7 ± 1.7 vs. 36.4 ± 2.2, respectively; $P = 0.5724$).

Finally, variable VGLUT1-LI (denser in laminae I, III–VII of the dorsal horn; less dense in the ventral horns; nonexistent in lamina II; Fig. 8a, c) and widespread VGLUT2-LI (virtually all gray matter; Fig. 8b, d) was observed in sham and L6-S1-AXO mice. However, neither L6-S1-AXO nor sham-injury induced any noticeable change in VGLUT1- or VGLUT2 bouton density or intensity of immunoreactivity (Fig. 8a, b).

Discussion

Alterations in the expression of several molecules in non-visceral afferent neurons, particularly after various types of injury of the sciatic nerve, have been previously analysed in detail, contributing to our understanding of the mechanisms underlying chronic pain associated with peripheral neuropathy (Brumovsky et al. 2013; Cohen and Mao 2014; von Hehn et al. 2012). In contrast, the neuro-chemical consequences of injury of nerves innervating perineal and/or pelvic structures has received little attention, the only exception being the work by Vizzard et al. (1995) who reported upregulation of nNOS in visceral DRG neurons after axotomy of the pelvic nerve in rat. Here, we addressed the neurochemical changes occurring in DRG neurons and the spinal cord as a consequence of axotomy of a mixed visceral and non-visceral nerve innervating pelvic, perineal and genital structures. We show considerable alterations in the expression of several markers, some directly linked to nociception. The observed changes are in line with those evoked by injury of non-visceral DRG neurons, and may open the discussion about their involvement in chronic pelvic pain after injury of intrapelvic nerves.

Methodological considerations

Before discussing the findings, we would like to address the issue of neuronal death after axotomy of peripheral nerves. Previous careful neurostereology studies, comparing sham-operated and sciatic nerve axotomy mice, have reported that more than 20 % of all L4-5 DRG neurons are lost 7 days after axotomy (Shi et al. 2001b). Interestingly, proximity of the site of injury to the affected ganglia and neuronal death have been proposed as directly related (Shi et al. 2001b), since axotomy of the sciatic nerve at the mid-thigh level in the rat does not result in neuron loss in L5 DRGs (Tandrup et al. 2000), as compared to an injury 7 mm distal to the ganglion (Vestergaard et al. 1997). In the present study, PNA was performed by injury of the ventral branches of L6 and S1, close to their exit from the intervertebral foramen. Thus, it could be possible that neuronal death, and/or severe atrophy occurred after PNA, and this in turn affected the absolute number of DRG neurons, as well as the quantification presented here. However, the focus of our study was not to demonstrate neuronal death after PNA, and its consequence on absolute numbers. Therefore, all results and the following discussion should be read as based on the extent of relative change in the expression of various neurochemical markers.

Effects of L6-S1-AXO in the neurochemistry of mouse DRG neurons

Cyclic AMP-dependent transcription factor 3 is a member of the ATF/CREB family of basic leucine zipper domain transcription factors (see Patodia and Raivich 2012). Sciatic nerve axotomy (Tsujino et al. 2000), postganglionic axotomy of the superior cervical ganglion (Boeshore et al. 2004) and optic nerve crushing (Takeda et al. 2000), all induce de novo expression of ATF3 in the cell bodies of rodent sensory neurons and motoneurons (Tsujino et al. 2000), autonomic neurons (Boeshore et al. 2004; Brumovsky et al. 2011b) and in retinal ganglion cells (Takeda et al. 2000). Here we show that axotomy of nerves targeting pelvic organs and perineal structures in the mouse also induce a dramatic upregulation of ATF3 in a large proportion of L6-S1 DRG neurons. In agreement, it was recently shown in rats that inferior rectal nerve compression by means of retrouterine balloon inflation results in de novo expression of ATF3 in S1 DRG neurons (Peirce et al. 2011). The present results also complement a previous study showing that L6-S1-AXO induces ATF3 upregulation in many lumbar sympathetic chain (LSC) neurons (Brumovsky et al. 2011b). ATF3 has long been proposed, along with other molecules, to participate in mechanisms of nerve regeneration and neuronal survival (Nakagomi et al. 2003; Patodia and Raivich 2012; Seijffers et al. 2007). Interestingly, an association between postsurgical pain, peritoneal adhesions (see Moro et al. 2014; ten Broek et al. 2013) and growth of nerve fibers within the adhesions (Sulaiman and Gordon 2013) has been recently proposed. Similarly, it has been proposed that chronic pelvic pain after particularly difficult vaginal deliveries develops as a consequence of injury and a series of aberrant reinnervations by uterine nerves (Pastelin et al. 2012; Quinn 2004, 2011). Therefore, it could be hypothesized that the upregulation of ATF3 in visceral and non-visceral DRG (present study) and LSC (Brumovsky et al. 2011b) neurons after AXO of the L6-S1 trunk participated in the mechanisms of aberrant innervation of peritoneal adhesions or reinnervation of tissues and organs deafferented during surgical procedures or trauma of the lower abdominal/pelvic cavities, potentially contributing to pain sensations.

CGRP is probably the most abundant constitutively expressed neuropeptide in small, medium-sized and (some) large non-visceral DRG neurons (see McMahon and Priestley 2005) and, along with substance P, it is considered a key neuropathic pain-signaling neurotransmitter (see Nitzan-Luques et al. 2013). Axotomy (see Brumovsky et al. 2013; Hökfelt et al. 1994), chronic constriction (Garry et al. 1991; Ma and Bisby 2000; Nahin et al. 1994) and partial sciatic nerve injury (Ma and Bisby 2000) have been shown to downregulate CGRP protein and transcript expression in non-visceral primary afferent neurons. CGRP is also a predominant neuropeptide in visceral DRG neurons (see Robinson and Gebhart 2008), although the effects of nerve injury on its expression have to date only been analysed in relation to cranial nerves. Thus, axotomy of the glossopharyngeal and carotid sinus nerves in the rat (innervating head and neck structures) results in down-regulation in the number of neurons expressing CGRP protein (Helke and Rabchevsky 1991) and transcript (Huang et al. 1994). Axotomy of the vagus nerve, innervating the entire length of the gut (Grundy 2006), including the urinary bladder and the distal colon and rectum (Herrity et al. 2014), has been shown to reduce the number of nodose ganglion neurons expressing CGRP transcript (Huang et al. 1994), but not protein (Helke and Rabchevsky 1991). Here, we demonstrate that axotomy of the L6-S1 trunk results in downregulation of the number of CGRP-IR DRG neurons in the mouse.

Another molecule strongly linked with nociceptive signaling and normally expressed by small and medium-sized visceral (see Robinson and Gebhart 2008) and non-visceral (see Caterina 2007) DRG neurons is TRPV1. Peripheral nerve injury of non-visceral neurons, as shown by means of axotomy of the sciatic nerve (Michael and Priestley 1999) and L4-5 spinal nerves (Hudson et al. 2001; Michael and Priestley 1999), L4 spinal nerve ligation (Fukuoka et al. 2002), partial sciatic nerve transection (Hudson et al. 2001) and transection of various branches of the trigeminal nerve (Biggs et al. 2007; Kim et al. 2008; Zakir et al. 2012), result in downregulation of TRPV1 transcript (Fukuoka et al. 2002; Michael and Priestley 1999) and protein (Biggs et al. 2007; Hudson et al. 2001; Kim et al. 2008; Zakir et al. 2012) in DRG and trigeminal ganglion neurons. Interestingly, the decrease observed for TRPV1 after trigeminal nerve injury is followed by its progressive upregulation (Biggs et al. 2007; Kim et al. 2008; Zakir et al. 2012), in uninjured (Biggs et al. 2007; Kim et al. 2008) or regenerating severed neurons (Zakir et al. 2012), suggesting roles of TRPV1 in regeneration after injury. Here, we show that axotomy of an intrapelvic nerve also downregulates the expression of TRPV1 in L6-S1 DRG neurons. It remains to be established if TRPV1 downregulation in severed axons of L6-S1 DRG neurons is followed by its progressive upregulation in neighboring uninjured neurons or during regeneration of spared nerves. If so, it could be speculated that while contributing to regeneration of damaged nerves, such upregulation could also facilitate the mechanisms of pain after pelvic surgeries or trauma.

Visceral (Brumovsky et al. 2012a) and non-visceral (Brumovsky et al. 2006a; Li et al. 2011) DRG neurons also express the noradrenergic marker TH. Previously, we observed that axotomy of the sciatic nerve results in a tendency towards a reduction in the number of neurons expressing TH in mouse L4-5 DRGs (Brumovsky et al. 2006a). Chronic constriction injury in rats (Herradon et al. 2008) and L5 spinal nerve ligation in transgenic mice expressing EGFP under the TH promoter (Xie et al. 2011) also induce a decrease in TH transcript (Herradon et al. 2008) or EGFP (Xie et al. 2011). In the present study in

mouse, we show that L6-S1-AXO induces a significant downregulation in the number of TH-IR DRG neurons projecting through the pelvic and perineal nerves. In agreement, axotomy of the visceral organ-targeting vagus and glossopharyngeal nerves also results in the down-regulation of TH protein (Helke and Rabchevsky 1991) and its enzymatic activity (Katz and Black 1986) in the nodose and petrosal ganglia, respectively. The functional role of TH in visceral and non-visceral DRGs and cranial primary afferent neurons remains elusive, although several lines of evidence support a role in sensation and pain (see Brumovsky et al. 2006a, b, 2012a).

The fact that rodent visceral and non-visceral DRG neurons are largely glutamatergic (Brumovsky 2013; Keast and Stephensen 2000) has been recently confirmed by demonstration of their expression of VGLUTs. Thus, rodent sciatic nerve- (Brumovsky et al. 2007; Oliveira et al. 2003; Seal et al. 2009), as well as urinary bladder- (Brumovsky et al. 2012b) or colorectum-projecting DRG neurons (Brumovsky et al. 2011b) express the three known VGLUTs (types 1, 2 and 3). VGLUT2 is by far the most widely expressed VGLUT in visceral and non-visceral DRG neurons of all sizes, followed by VGLUT1 in large and medium-sized neurons, and VGLUT3 primarily in small ones (see Brumovsky 2013). Axotomy of the sciatic nerve in mouse reduces the number of VGLUT1- and to some extent also VGLUT2-IR DRG neurons (Brumovsky et al. 2007). In contrast, only a modest decrease in the number of VGLUT3 transcript expressing DRG neurons of mouse has been demonstrated after sciatic nerve axotomy (Malet et al. 2013), suggesting that transcription and translation of VGLUTs may be differentially modulated after injury (Brumovsky 2013; Malet et al. 2013). In the present study, L6-S1-AXO resulted in a modest, but significant downregulation of the number of VGLUT2-IR neurons, leaving VGLUT1 unaffected. Such downregulation of VGLUT2-IR DRG neurons may occur at the expense of medium-sized neurons because large or small DRG neurons appeared largely unaffected. In addition, we detected what appears to be an increase in the immunoreactive signal of VGLUT2 in small DRG neurons, in agreement with a similar observation in small non-visceral neurons after axotomy of the mouse sciatic nerve (Brumovsky et al. 2007). The lack of effect on VGLUT1 is intriguing, as injury of a non-visceral nerve clearly downregulates its protein expression (Brumovsky et al. 2007). However, it could be speculated that since L6-S1 DRGs contain a mixture of visceral and non-visceral neurons, the effect of peripheral nerve injury may not apply to VGLUT1 with the same impact in both populations, resulting in a “dilution effect” and an apparent lack of change.

Effects of L6-S1-AXO in the mouse spinal cord

Axotomy, and other types of injury, of the sciatic nerve (Hu et al. 2007; Kataoka et al. 2007; Linda et al. 2011; Tsujino et al. 2000) or ventral root avulsion (Linda et al. 2011) induce a marked increase in the expression of ATF3 in motoneurons in the lumbar enlargement of the rat (Hu et al. 2007; Kataoka et al. 2007; Linda et al. 2011; Tsujino et al. 2000) and human (Linda et al. 2011) spinal cord. Here, we show in mouse that injury of an intrapelvic nerve also induces de novo expression of ATF3 in lumbar and lumbosacral motoneurons and in neurons in the intermediolateral cell column at lumbosacral levels. This observation is in line with previous studies in rat, where axotomy of the pelvic or hypogastric nerves induces the expression of ATF3 in lumbar and sacral parasympathetic preganglionic neurons (Peddie

and Keast 2011). At least a proportion of the motoneurons affected by L6-S1-AXO presumably produce the efferent innervation supplied by the pudendal nerve to various striated pelvic muscles (Van der Horst and Holstege 1998). Upregulated ATF3 in the intermediolateral cell column of the lumbosacral spinal cord likely occurs in preganglionic parasympathetic neurons projecting by way of the pelvic and pudendal nerves towards the MPG (De Groat 2006; Keast 2006; Peddie and Keast 2011). On the contrary, the nature of area X neurons showing de novo upregulation of ATF3 remains to be established. As with DRGs, the upregulation of ATF3 in spinal cord neurons may reflect the cellular efforts for survival and axonal regeneration after injury (Nakagomi et al. 2003; Patodia and Raivich 2012; Seijffers et al. 2007).

We observed moderate decreases in CGRP- and TRPV1-LIs in the dorsal horn of the spinal cord, ipsilateral to the L6-S1-AXO. The decreases were mostly seen as reductions in the density of immunoreactive boutons and the intensity of immunoreactive signal, in laminae I–III (CGRP) or exclusively in lamina II (TRPV1). Such modest changes contrasted with the more dramatic downregulation of CGRP or TRPV1 observed in DRG neurons after L6-S1-AXO. In fact, more pronounced effects were expected, considering the important contribution of the central projections of DRG neurons to the spinal expression of CGRP (see Shi et al. 2001a) and TRPV1 (Corder et al. 2010; Valtschanoff et al. 2001). However, even a 14-day axotomy of the sciatic nerve in rat only results in a modest reduction (even if more noticeable than in the present study) in the density of CGRP-IR boutons and in fluorescence intensity in laminae I–III of the dorsal horn, despite the massive downregulation observed in DRG neurons (Zhang et al. 1993). Contributions of fibers from levels above and below the injury, as suggested by the considerable but not complete reduction in CGRP expression after dorsal rhizotomy in rat (Chung et al. 1988; Gibson et al. 1984), cat (Gibson et al. 1984; Traub et al. 1989) and mouse (Brumovsky et al. 2005; Shi et al. 2001a), may also in part explain the modest changes we noted. Finally, even if still debated for TRPV1 (see Spicarova et al. 2014), discrete numbers of spinal CGRP- (Shi et al. 2001a) and TRPV1-synthesizing neurons (Doly et al. 2004a, b; Ferrini et al. 2010; Kim et al. 2012; Valtschanoff et al. 2001; Zhou et al. 2009) or glial cells (Doly et al. 2004a, b) have been described in mouse (Ferrini et al. 2010; Kim et al. 2012; Shi et al. 2001a) and rat (Doly et al. 2004a, b; Valtschanoff et al. 2001; Zhou et al. 2009) and could mask the effects of peripheral nerve injury. Alternatively, because neuro-transmitter depletion at central terminals of DRG neurons may show a delay as compared to the effect observed in their cell bodies, another possibility is that survival times longer than 7 days revealed greater magnitude changes.

Finally, L6-S1-AXO failed to exert alterations in the expression of VGLUT1 and VGLUT2 in the spinal cord. In a previous study in mouse, we show that axotomy of the sciatic nerve in mouse does not induce changes in the spinal immunohistochemical expression of the above-mentioned VGLUTs (Brumovsky et al. 2007). Interestingly, in the first study in rats showing VGLUT1 down-regulation in the spinal cord after sciatic nerve axotomy, the effect was pronounced in the ventral horns and lamina V, especially from 2 weeks of survival and onwards; a moderate effect was also observed in the superficial dorsal horn, at survival times longer than 1 week (Hughes et al. 2004). Thus, it could be speculated that longer survival times after L6-S1-AXO may again result in more pronounced alterations in VGLUT1-LI, at

least in the ventral horn. As for VGLUT2, the failure to detect changes after L6-S1 trunk axotomy could be explained by the rather modest transport of VGLUT2 from DRG neurons to the spinal cord (Brumovsky et al. 2007), the presence of abundant VGLUT2-expressing local dorsal horn neurons (Alvarez et al. 2004; Brumovsky et al. 2007; Malet et al. 2013; Morris et al. 2005; Oliveira et al. 2003; Todd et al. 2003), and even the contribution of descending pathways (Du et al. 2012). In support of this, we observed a similar lack of change after axotomy of the sciatic nerve for the neuropeptide tyrosine receptor type 1 (Y1R) in the superficial layers of the dorsal horn (Brumovsky et al. 2004), even when the receptor is actively transported by the central projections of DRG neurons (Brumovsky et al. 2002). Also for the Y1R, its abundant local expression in interneurons and projection neurons (see Brumovsky et al. 2006a) is a likely reason for the lack of changes after injury.

Nerve injury and chronic pelvic pain: potential implications

Chronic pelvic pain and nerve injury seem to be intimately related, at least in a proportion of patients undergoing surgical procedures in the lower abdominal/pelvic cavities, having obstetric interventions or experiencing trauma. Thus, pudendal nerve entrapment due to use of transvaginal meshes for reconstructive surgery in women suffering pelvic organ prolapse (Gyang et al. 2014) or to bone and muscle remodeling in athletes (Antolak et al. 2002) are recognized causes of chronic pelvic pain. Entrapment of the ilioinguinal, iliohypogastric and/or pudendal nerves has also been associated with postoperative pelvic pain in female patients treated for stress incontinence with minimally invasive, tension-free, midurethral sling (see Fisher and Lotze 2011). Pelvic and acetabular fractures (automobile accidents being the principal cause) result in chronic persistent pelvic pain in a proportion of the affected patients, and an association with nerve injury has been suggested (Báča et al. 2013; Gerbershagen et al. 2010). Finally, chronic pelvic pain after hysterectomy (Brandsborg et al. 2007), cesarean section (Nikolajsen et al. 2004), difficult deliveries (Eisenach et al. 2013; Hannah et al. 2004; Pastelin et al. 2012; Quinn 2004) and even excessive straining during defecation (Quinn 2011) have also been linked to nerve injury. Therefore, nerve injury remains a relevant factor associated with development of chronic pelvic pain. However, the cellular and molecular mechanisms involved remain unknown.

Three main questions have been posed, particularly in relation to the participation of injured nerves in chronic postsurgical pain: "…first, what level of nerve injury is required to induce the changes that result in neuropathic pain? Second, can damage to tissues other than nerves cause neuropathic pain? Last, what is the relative contribution of central and peripheral changes in the nervous system?" (Macrae 2008). The results obtained in the present study in mouse address the last question, and support the hypothesis that at least in the peripheral nervous system the profound neurochemical changes observed after injury in ganglia containing mixed visceral and non-visceral afferent neurons, also supporting alterations in afferent transmission, could be relevant in generation and maintenance of chronic pelvic pain after surgical interventions. As with non-visceral pain, more research will be needed to better characterize these phenotypic changes and to explain if and what is the relationship between them and the generation and maintenance of chronic pelvic pain.

Another relevant question is, are both visceral and non-visceral nerves/sensory neurons involved in chronic pelvic pain? In particular, when analyzing chronic postsurgical pain, most of the emphasis seems to be put on the neuropathic pain detected in the incisional area and its association with the injury of parietal non-visceral nerves (Brandsborg et al. 2011; Bruce and Krukowski 2006; Burke and Shorten 2009; Dualé et al. 2014; Kehlet et al. 2006; Possover and Lemos 2011; Reddi and Curran 2014). In fact, most analgesic strategies designed to prevent chronic postsurgical pain used today are employed assuming that nerve damage at the surgical incision and associated pain are the most important components (Bouman et al. 2014; Collins et al. 2014; Ducic et al. 2006; Gyang et al. 2014; Kehlet et al. 2006; Possover and Lemos 2011; Reddi and Curran 2014). However, injury of visceral nerves may also be of importance to chronic postsurgical pain (Cervero and Laird 1999). This is suggested by observations in patients reporting chronic postsurgical pain after gastrointestinal surgery not only at, or near, the operative site, but also in the central or lower abdominal area (deep pain) (Bruce and Krukowski 2006). Further supporting altered visceral afferent processing, it was observed that visceral pain, but not incisional and referred pain, during the first week after laparoscopic cholecystectomy is associated with chronic unexplained pain of more than 12 months duration (Blichfeldt-Eckhardt et al. 2014). Finally, neuropathic pain associated with the incisional scar, as well as visceral pain traits reflected by presence of deep abdominal and pelvic pain (often related to uterine contractions), have been suggested to develop and even coexist in patients undergoing obstetric interventions (Landau et al. 2013; Lavand'homme 2013). Interestingly, afferent transmission modulation by epidural analgesia (Bouman et al. 2014) or sacral nerve stimulation (Martellucci et al. 2012) efficiently reduces the incidence of chronic postsurgical pain, including deep pain. However, it remains to be established what is the relative effect of epidural analgesia or sacral nerve stimulation over visceral vs. non-visceral afferents.

In conclusion, injury of a mixed visceral/non-visceral nerve leads to significant neurochemical alterations in DRGs and the spinal cord. Changes in these and potentially other nociception-related molecules could contribute to pain due to injury of parietal and intrapelvic nerves. We did not study changes in DRG neurons contributing to the pelvic and pudendal nerves in the mouse after axotomy of the L6-S1 trunk, relative to their identity as being visceral or non-visceral, even though the proximity of the nerve cut to the cell bodies naturally implies affecting a good proportion of both populations. Future experiments should include evaluation of changes in identified visceral and non-visceral DRG neurons, e.g. by means of selective axonal tracing, and analysis after more specific injuries of the pelvic, pudendal and also parietal nerves. This will allow establishing the degree of participation of each neuronal population in the mechanisms of chronic pelvic pain.

Acknowledgments

We acknowledge the generous donation of VGLUT1 and VGLUT2 antibodies by Dr. Masahiko Watanabe, Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan. We also thank Mr. Guillermo Gastón and Juan Miguel Bayo Fina, Faculty of Biomedical Sciences, Austral University, Buenos Aires, Argentina, for valuable technical assistance. This study was supported by an Austral University Grant and CONICET (P.R.B.) and NIH award R01 DK093525 (GFG).

Abbreviations

ATF3	Cyclic AMP-dependent transcription factor 3
CGRP	Calcitonin gene-related peptide
DRG	Dorsal root ganglion
IR	Immunoreactive
L	Lumbar
LI	Like-immunoreactivity
LSC	Lumbar sympathetic chain
MPG	Major pelvic ganglion
nNOS	Neuronal nitric oxide
NP	Neuron profile
PBS	Phosphate buffered saline
RT	Room temperature
S	Sacral
TH	Tyrosine hydroxylase
TRPV1	Transient receptor potential cation channel subfamily V, member 1
VGLUT	Vesicular glutamate transporter

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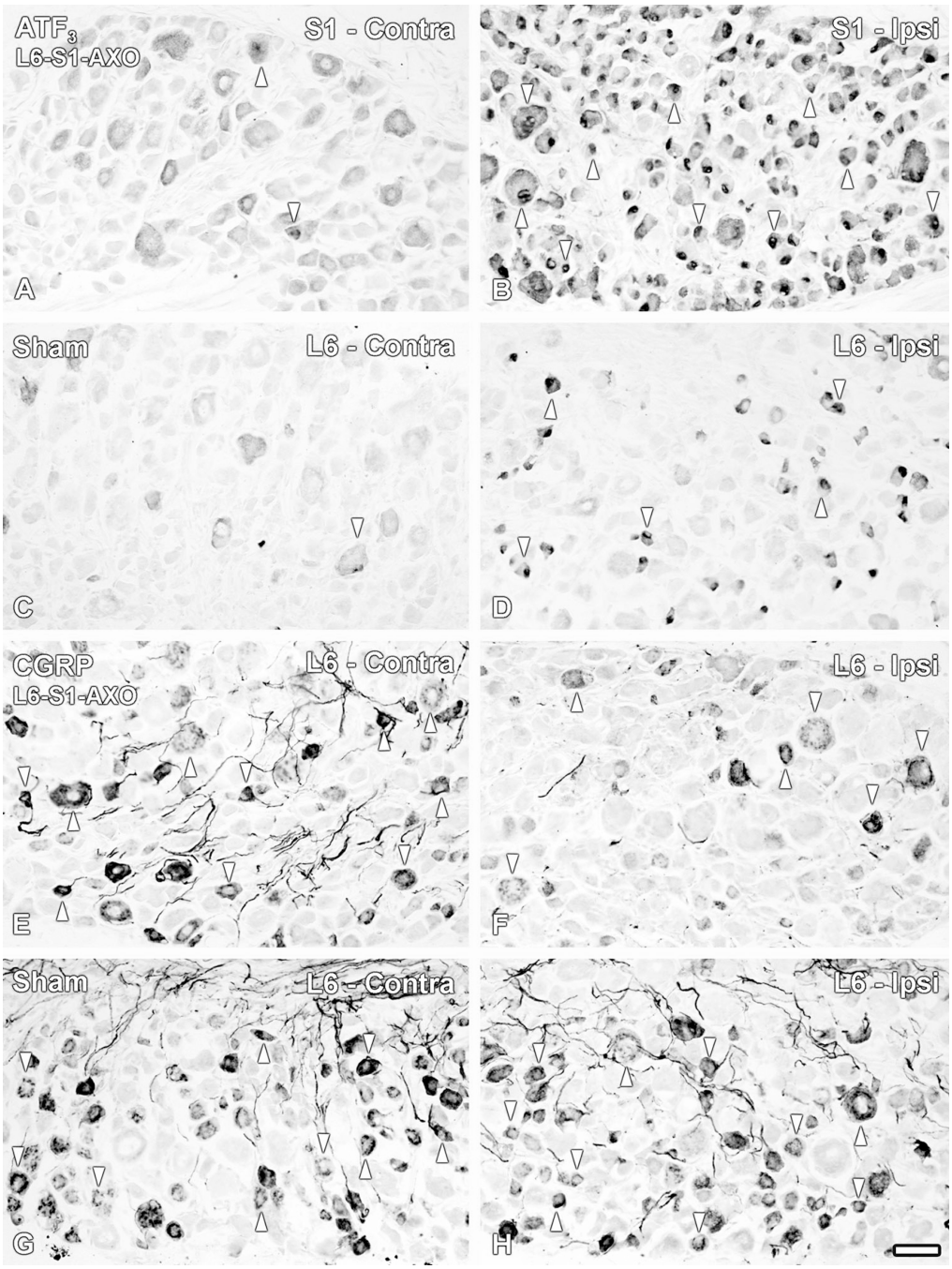

Fig. 1.

Immunohistochemical visualization of ATF3 and CGRP in L6 and S1 DRG neurons of L6-S1-AXO and sham mice. Bright-field photomicrographs of sections of contralateral (**a, c, e, g**) and ipsilateral (**b, d, f, h**) S1 (**a, b**) and L6 (**c–h**) DRGs of L6–S1-AXO (**a, b, e, f**) or sham (**c, d, g, h**) mice, after incubation with ATF3 (**a–d**) or CGRP (**e–h**) antisera. (*Arrowheads* show ATF3-(**a–d**) or CGRP- (**e–h**) IR NPs). **a–d** Only rare ATF3-IR NPs are observed in contralateral S1 or L6 DRGs from L6-S1-AXO (**a**) or sham (**c**) mice. In contrast, a dramatic upregulation in the number of ATF3-IR NPs is observed in the

ipsilateral DRGs of L6-S1-AXO mice, encompassing various cell body sizes (**b**). Note the lateralization of the nucleus of several of the ATF3-IR NPs. Sham mice show a much smaller increase in the number of ATF3-IR NPs, mostly of small size (**d**). **e–h** Many CGRP-IR NPs of different sizes and a noticeable CGRP-IR neuropil are detected in contralateral L6 DRGs from L6-S1-AXO (**e**) or sham (**g**) mice. In contrast, L6-S1-AXO induces a considerable downregulation of the number of CGRP-IR NPs in the DRG of L6-S1-AXO mice, and only a few small, medium-sized and large NPs expressing the peptide remain (**f**). In sham mice, the number of CGRP-IR NPs and the presence of CGRP-IR nerve fibers remain unaltered (**h**). *Scale bar* 50 μm (**h** = **a–g**)

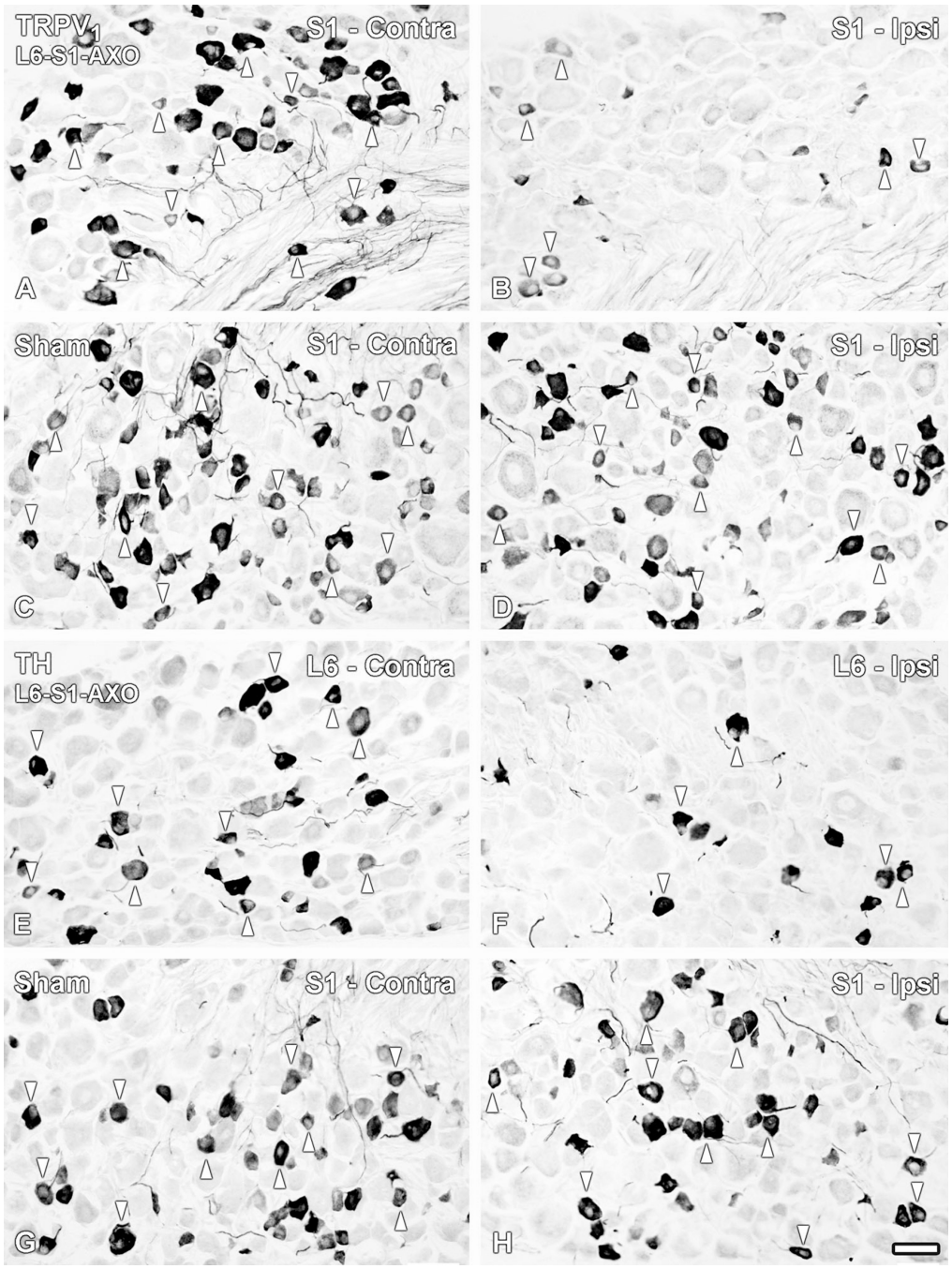

Fig. 2.

Immunohistochemical visualization of TRPV1 and TH in L6 and S1 DRG neurons of L6-S1-AXO and sham mice. Bright-field photomicrographs of sections of contralateral (**a, c, e, g**) and ipsilateral (**b, d, f, h**) S1 (**a–d, g, h**) and L6 (**e, f**) DRGs of L6-S1-AXO (**a, b, e, f**) or sham (**c, d, g, h**) mice, after incubation with TRPV1 (**a–d**) or TH (**e–h**) antisera. (*Arrowheads* show TRPV1- (**a–d**) or TH- (**e–h**) IR NPs). **a–d** Several small and medium-sized TRPV1-IR NPs are observed in contralateral S1 DRGs from L6-S1-AXO (**a**) or sham (**c**) mice. Several TRPV1-IR nerve fibers within these ganglia are also apparent (**a**). In

contrast, a pronounced decrease in the number of TRPV1-IR NPs is observed in the ipsilateral DRGs of L6-S1-AXO mice, and only a few immunoreactive NPs remain (**b**). The number of TRPV1-IR NPs and the presence of TRPV1-IR nerve fibers in sham mice DRGs show no apparent change (**d**). **e–h** A number of small and medium-sized TH-IR NPs are detected in contralateral L6 and S1 DRGs from L6-S1-AXO (**e**) or sham (**g**) mice, respectively. L6-S1-AXO results in the downregulation of the number of TH-IR NPs (**f**). L6-S1-AXO also seems to reduce the number of TH-IR nerve fibers (**f**). In sham mice, neither the number of TH-IR NPs nor the presence of TH-IR nerve fibers appears to be altered (**h**). *Scale bar* 50 μm (**h** = **a–g**)

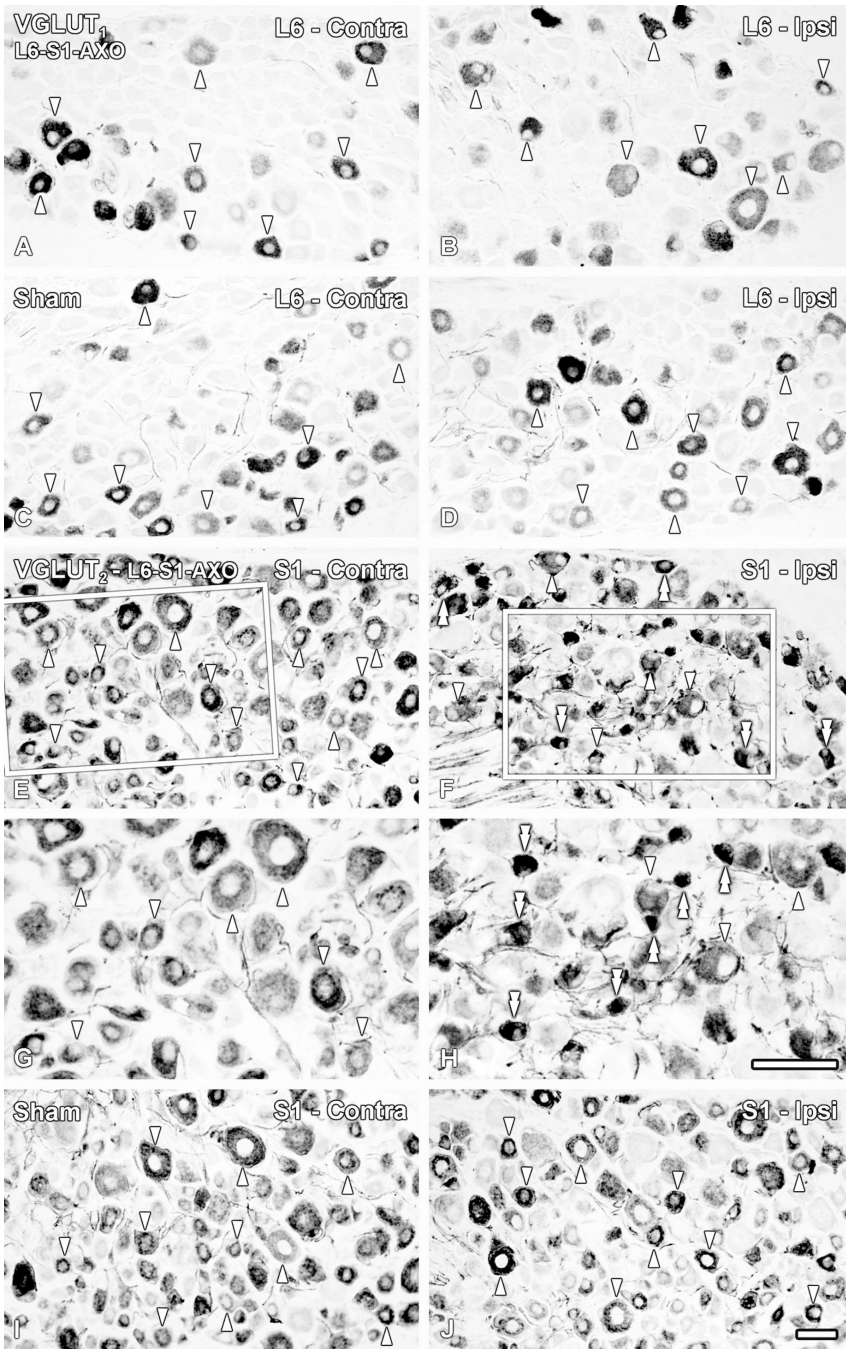

Fig. 3.

Immunohistochemical visualization of VGLUT1 and VGLUT2 in L6 and S1 DRG neurons of L6-S1-AXO and sham mice. Bright-field photomicrographs of sections of contralateral (**a, c, e, g, i**) and ipsilateral (**b, d, f, h, j**) L6 (**a–d**) and S1 (**e–j**) DRGs of L6-S1-AXO (**a, b, e–h**) or sham (**c, d, i, j**) mice, after incubation with VGLUT1 (**a–d**) or VGLUT2 (**e–j**) antisera. (*Arrowheads* show VGLUT1-(**a–d**) or VGLUT2- (**e–j**) IR NPs. *Double arrowheads* show intensely VGLUT2-IR NPs. **g, h** High power magnifications of the *boxes* in **e** and **f**, respectively). **a–d** A number of medium-sized and large VGLUT1-IR NPs are observed in

contralateral L6 DRGs from L6-S1-AXO (**a**) or sham (**c**) mice. The number of VGLUT1-IR NPs remains unaltered in ipsilateral DRGs of L6-S1-AXO (**b**) or sham (**d**) mice. **e–j** Abundant VGLUT2-IR NPs of all sizes are observed in contralateral S1 DRGs from L6-S1-AXO (**e, g**) or sham (**i**) mice. L6-S1-AXO induces a modest downregulation of the number of VGLUT2-IR NPs (**f, h**). Some small and medium-sized DRG NPs remain, while several other small NPs apparently exhibiting a stronger VGLUT2-LI, normally obscuring the observation of the nucleus, are also observed (**f, h**). In sham mice, neither the number nor the appearance of VGLUT2-IR NPs varied noticeably (**j**). *Scale bars* 100 μm (**h** = **g**), 50 μm (**j** = **a**–**f, i, j**)

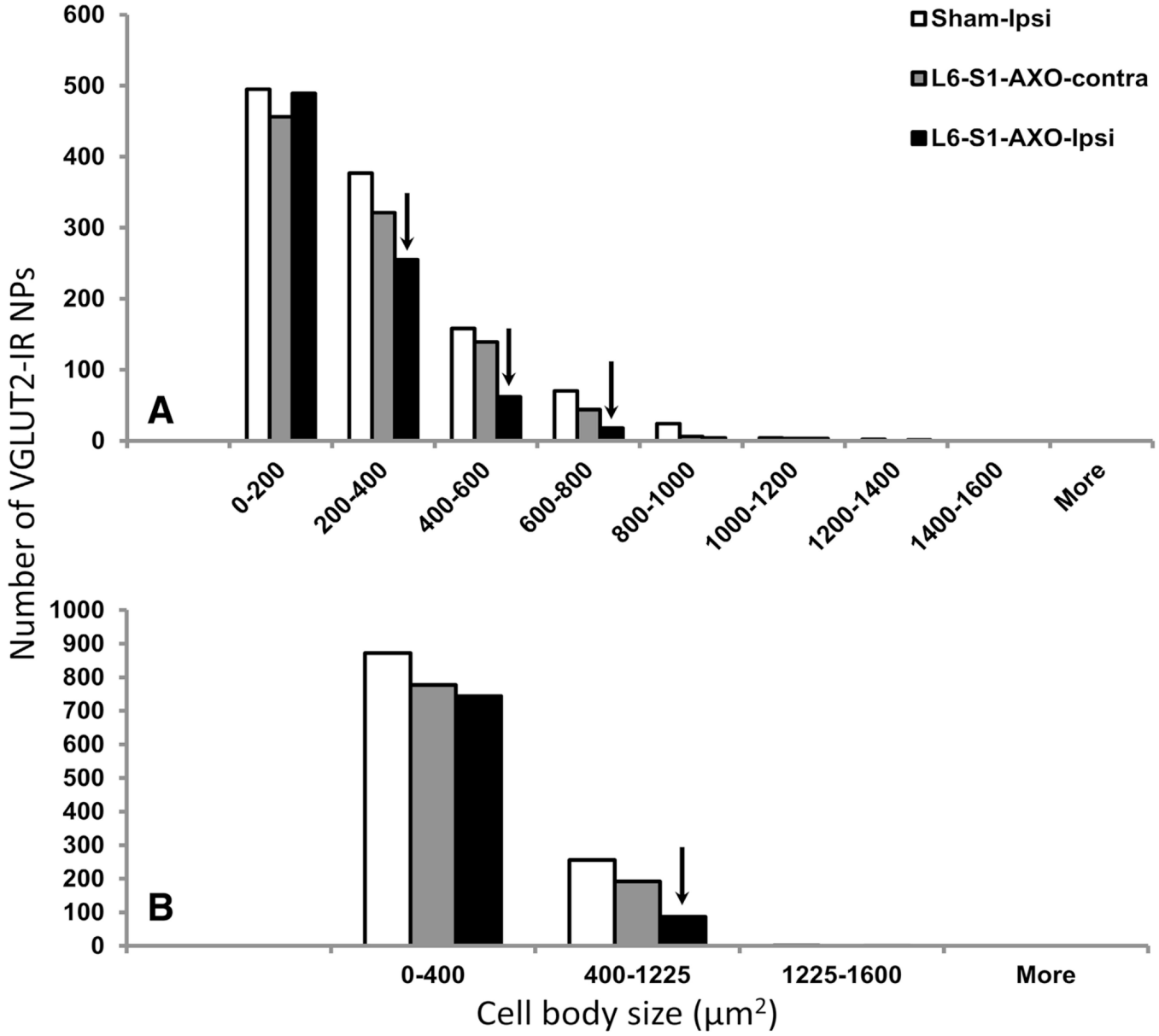

Fig. 4.

Cell body size distribution of VGLUT2-IR NPs in L6-S1 DRGs of L6-S1-AXO and sham mice. **a** Histogram shows VGLUT2-IR NPs size distribution, parceled into 200-µm^2 bins. **b** Data in (**a**) are grouped based on the regular distribution of mouse DRG NPs in small (0–400 µm^2), medium-sized (400–1225 µm^2) or large (1225-more) categories (*arrows* show the tendency of decrease in the numbers of medium-sized VGLUT2-IR NPs)

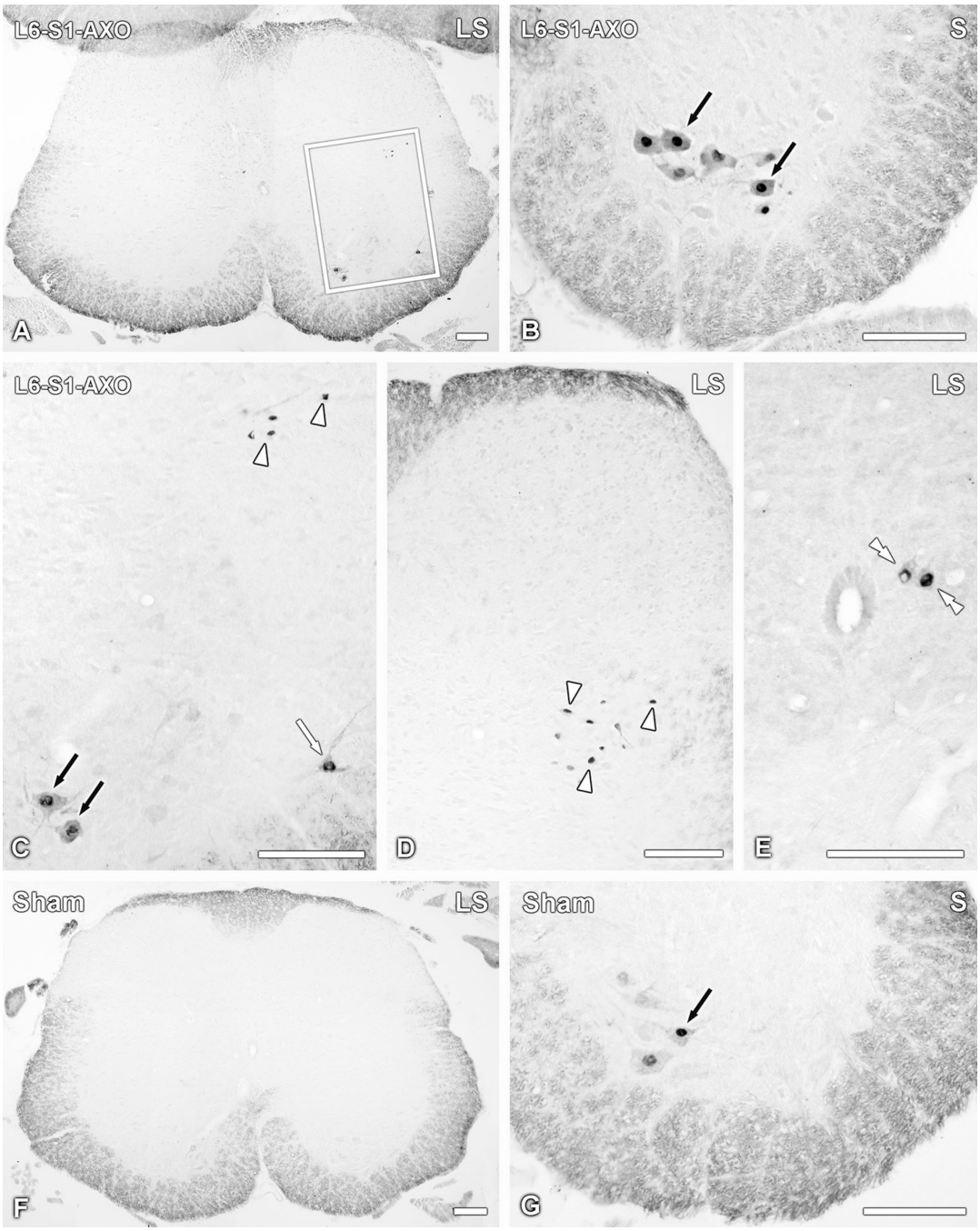

Fig. 5.

Immunohistochemical visualization of ATF3 in the LS or S spinal cord of L6-S1-AXO and sham mice. Bright-field photomicrographs of transverse sections of the mouse LS (**a, c–f**) or S (**b, g**) spinal cord after incubation with ATF3 antiserum (**c** is a high power magnification of the *box* in **a**). **a–d** L6-S1-AXO results in upregulation of ATF3 in motoneurons (*black* and *white arrows* in **a–c**) and smaller neurons in the intermediolateral cell column (*arrowheads* in **a, d**). **c, d** A high power magnification of **a** highlights the presence of ATF3-IR neurons in the medial (*black arrows*) and lateral (*white arrows*) aspects of the ventral

horn, as well as in neurons in the intermediolateral cell column (*arrowheads*). More abundant neurons in this location are also shown in **d** (*arrowheads*). **e** Very rarely, ATF3-IR neurons are detected in area X (*double arrowheads*) towards the ipsilateral side of the spinal cord in an L6-S1-AXO mouse. Expression of ATF3 in the spinal cord of sham mice was never detected in neurons in the intermediolateral cell column (**f**) and was limited to a few motoneurons in the medial aspects of the ventral horn (*black arrow* in **g**). *Scale bars* 100 μm

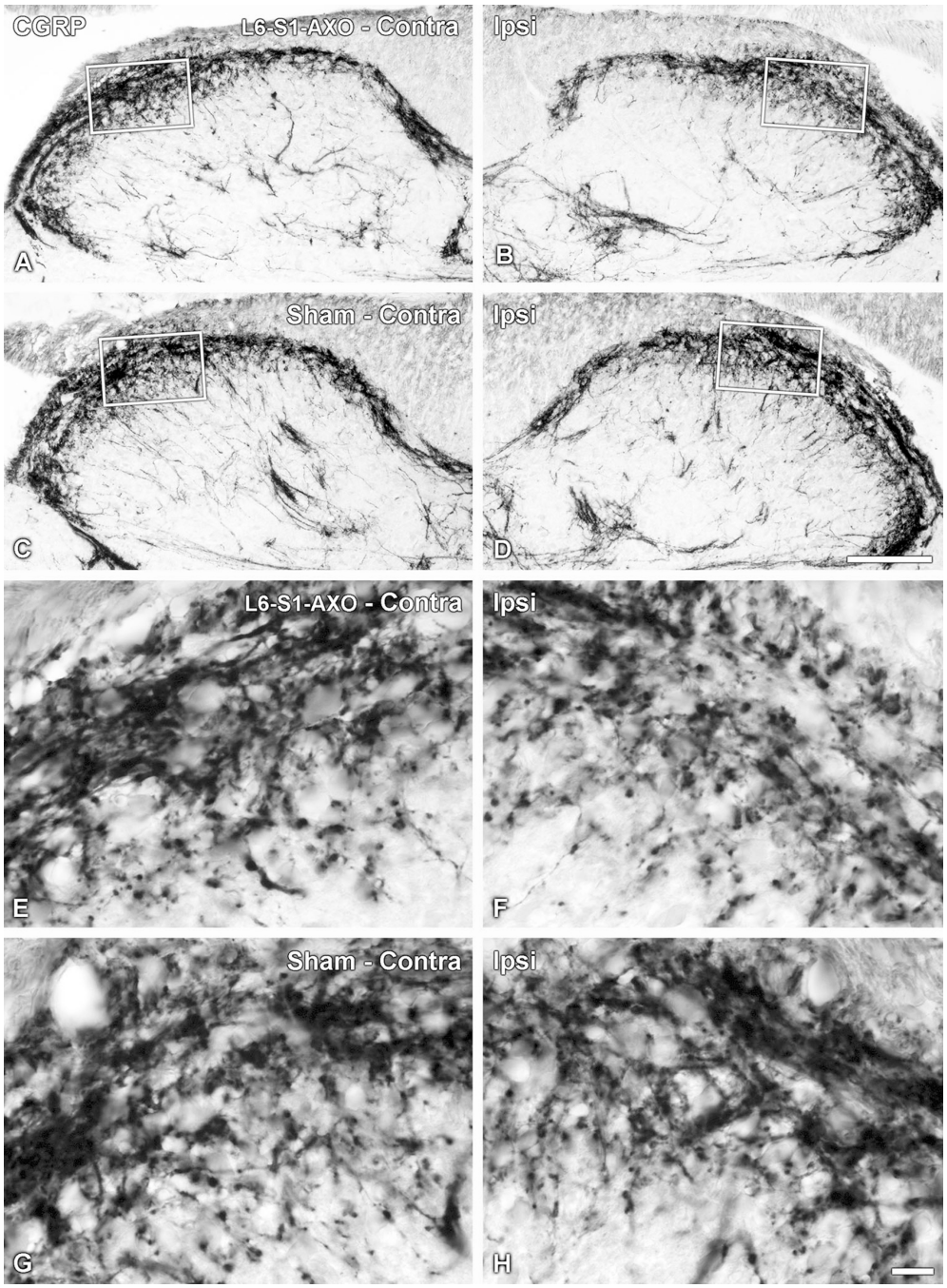

Fig. 6.

Immunohistochemical visualization of CGRP in the LS dorsal horn of the spinal cord of L6-S1-AXO and sham mice. Bright-field photomicrographs of transverse sections of the mouse LS dorsal horn of the spinal cord of L6-S1-AXO (**a, b, e, f**) or sham (**c, d, g, h**) mice after incubation with CGRP antiserum (**e–h** are high power magnifications of the *boxes* in **a–d**, respectively). **a–h** Dense CGRP-LI is detected in the contralateral superficial dorsal horn of L6-S1-AXO and sham mice (**a, c, e, g**). L6-S1-AXO induces a modest decrease in the density and staining intensity of CGRP-IR nerve fibers and boutons in the superficial dorsal

horn (**b, f**). In contrast, sham mice do not show a noticeable change in CGRP-LI in the dorsal horn. *Scale bars* 100 μm (**d** = **a–c**); 10 μm (**h** = **e–g**)

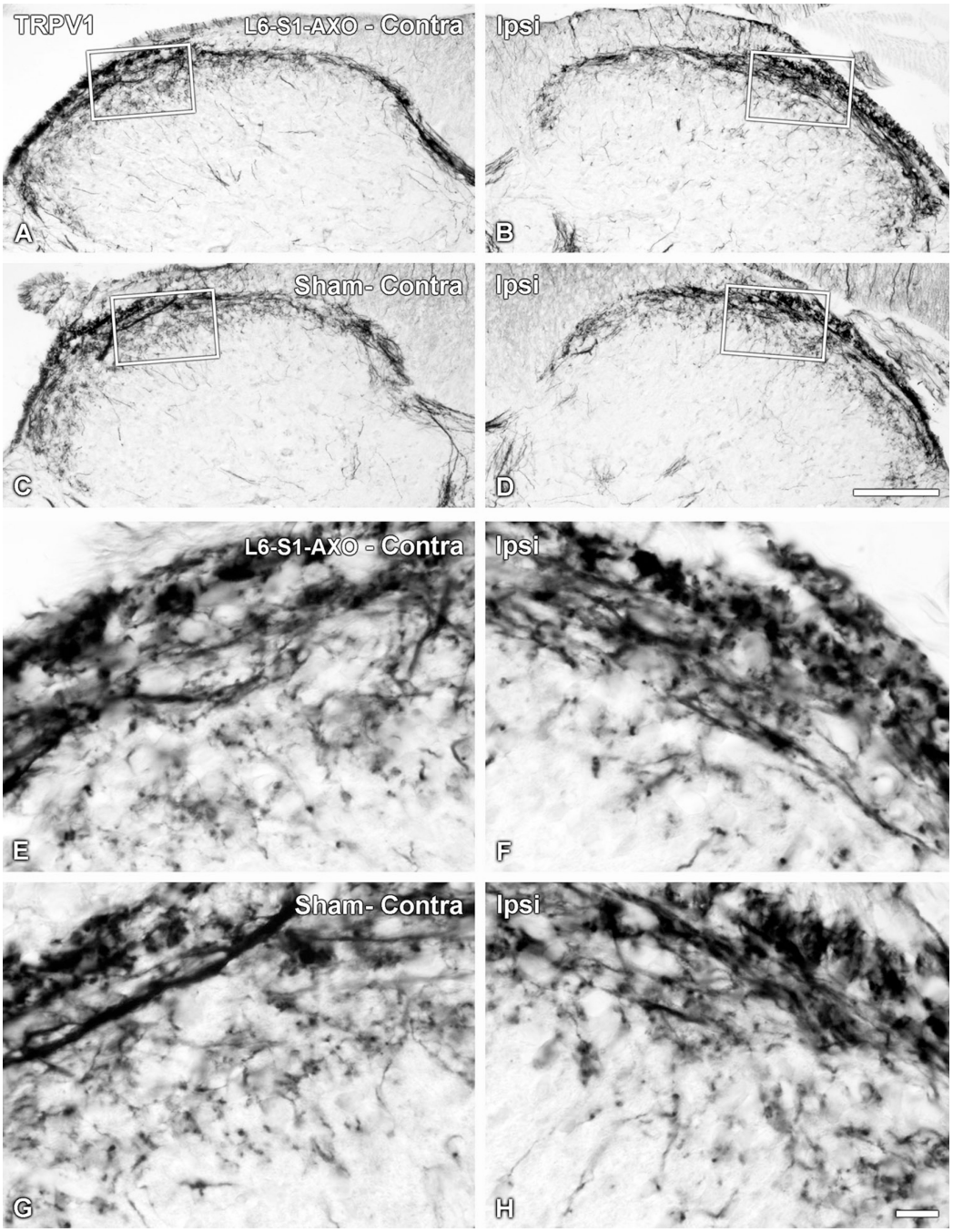

Fig. 7.

Immunohistochemical visualization of TRPV1 in the LS dorsal horn of the spinal cord of L6-S1-AXO and sham mice. Bright-field photomicrographs of transverse sections of the mouse LS dorsal horn of the spinal cord of L6-S1-AXO (**a, b, e, f**) or sham (**c, d, g, h**) mice after incubation with TRPV1 antiserum (**e–h** are high power magnifications of the *boxes* in **a–d**, respectively). **a–h** TRPV1-LI is detected in the contralateral superficial dorsal horn of L6-S1-AXO and sham mice (**a, c, e, g**). L6-S1-AXO induces a decrease in the density of TRPV1-IR nerve fibers and boutons in the superficial dorsal horn, especially in lamina II (**b,**

f). In contrast, sham mice do not show a noticeable change in TRPV1-LI in the dorsal horn. *Scale bars* 100 μm (**d** = **a–c**); 10 μm (**h** = **e–g**)

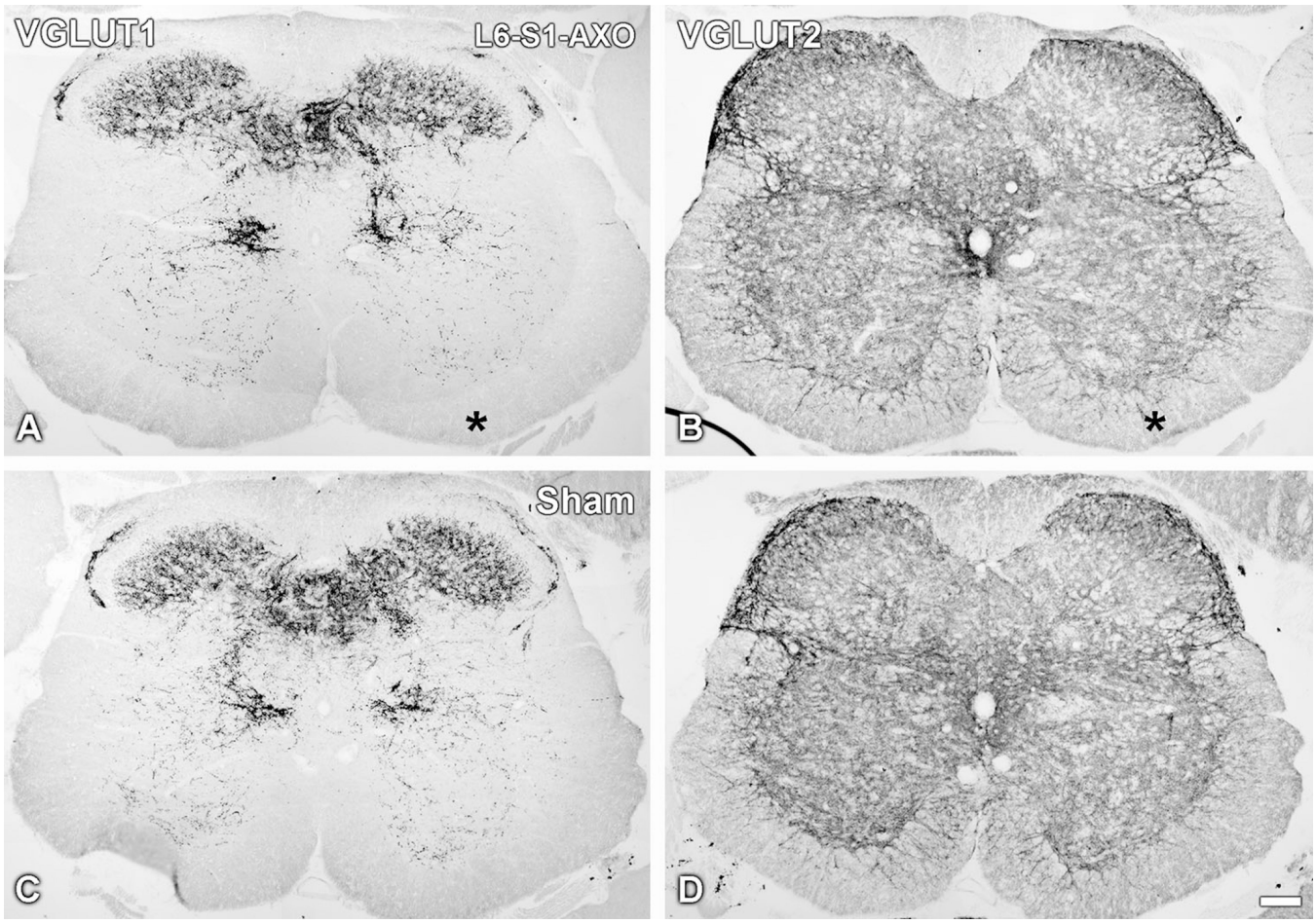

Fig. 8.

Immunohistochemical visualization of VGLUT1 and VGLUT2 in the LS dorsal horn of the spinal cord of L6-S1-AXO and sham mice. Bright-field photomicrographs of transverse sections of the mouse LS dorsal horn of the spinal cord of L6-S1-AXO (**a, b**) or sham (**c, d**) mice after incubation with VGLUT1 (**a, c**) or VGLUT2 (**b, d**) antisera (*asterisks* show injury side). **a–d** VGLUT1-IR neuropil is detected in laminae I, III–VII, the outer aspects of area X and the ventral horn of L6-S1-AXO (**a**) and sham (**c**) mice. Such distribution shows different densities, being lesser in the ventral horns and nonexistent in lamina II (**a, c**). In contrast, very abundant VGLUT2-IR neuropil is detected in the gray matter of the LS spinal cord of L6-S1-AXO (**b**) and sham (**d**) mice. The neuropil appears to be slightly denser in the superficial dorsal horn and around the central canal (**b, d**). Neither the distribution nor intensity of VGLUT1- or VGLUT2-LIs appears to differ when comparing L6-S1-AXO and sham mice (**a, b** vs. **c, d**). *Scale bars* 100 μm (**d** = **a–c**)

Table 1

Effects of L6-S1-AXO on the expression of ATF3, CGRP, TRPV1, TH, VGLUT1 and VGLUT2 in L6-S1, L4-5 and S2 mouse DRGs and comparison with Sham-treated mice

	L6-S1-AXO			Sham		
	Contra		**Ipsi**	**Contra**		**Ipsi**
L6-S1						
ATF3	**0.7 – 0.3**	(*n* = 7)	**79.9 – 1.1** ***	**0.5 – 0.2**	(*n* = 6)	**3.5 – 0.8** **
L4-L5						
ATF3	**0.1 – 0.1**	(*n* = 7)	**2.0 – 0.6**	**1.7 – 1.1**	(*n* = 6)	**3.8 – 2.6**
S2						
ATF3	**0.1 – 0.1**	(*n* = 7)	**0.3 – 0.2**	**1.3 – 1.3**	(*n* = 6)	**0.2 – 0.2**
L6-S1						
CGRP	**26.1 – 0.9**	(*n* = 7)	**12.9 – 1.2** ***	**26.0 – 2.0**	(*n* = 6)	**24.7 – 1.5**
TRPV1	**21.9 – 1.5**	(*n* = 7)	**9.4 – 1.0** ***	**21.2 – 1.0**	(*n* = 5)	**22.4 – 0.7**
TH	**13.8 – 1.1**	(*n* = 6)	**7.0 – 1.4** **	**11.8 – 1.4**	(*n* = 5)	**11.2 – 1.7**
VGLUT1	**12.2 – 2.8**	(*n* = 5)	**10.4 – 2.6**	**10.8 – 2.0**	(*n* = 5)	**11.4 – 2.0**
VGLUT2	**56.2 – 2.5**	(*n* = 6)	**48.0 – 2.0** *	**53.0 – 3.2**	(*n* = 5)	**53.6 – 2.8**

Statistically significant differences are shown between contralateral (Contra) and ipsilateral (Ipsi) sides within each group by means of asterisks (unpaired student *t* test,

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$)

Brain Struct Funct. Author manuscript; available in PMC 2017 May 01.