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Peripheral axotomy induces only very limited sprouting of coarse myelinated afferents into inner lamina II of rat spinal cord

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Abstract

Peripheral axotomy-induced sprouting of thick myelinated afferents (A-fibers) from laminae III–IV into laminae I–II of the spinal cord is a well-established hypothesis for the structural basis of neuropathic pain. However, we show here that the cholera toxin B subunit (CTB), a neuronal tracer used to demonstrate the sprouting of A-fibers in several earlier studies, also labels unmyelinated afferents (C-fibers) in lamina II and thin myelinated afferents in lamina I, when applied after peripheral nerve transection. The lamina II afferents also contained vasoactive intestinal polypeptide and galanin, two neuropeptides mainly expressed in small dorsal root ganglion (DRG) neurons and C-fibers. In an attempt to label large DRG neurons and A-fibers selectively, CTB was applied four days before axotomy (pre-injury-labelling), and sprouting was monitored after axotomy. We found that only a small number of A-fibers sprouted into inner lamina II, a region normally innervated by C-fibers, but not into outer lamina II or lamina I. Such sprouts made synaptic contact with dendrites in inner lamina II. Neuropeptide Y (NPY) was found in these sprouts in inner lamina II, an area very rich in Y1 receptor-positive processes. These results suggest that axotomy-induced sprouting from deeper to superficial layers is much less pronounced than previously assumed, in fact it is only marginal. This limited reorganization involves large NPY immunoreactive DRG neurons sprouting into the Y1 receptor-rich inner lamina II. Even if quantitatively small, it cannot be excluded that this represents a functional circuitry involved in neuropathic pain.

Introduction

Rat dorsal root ganglion (DRG) neurons can be grossly divided into large and small neurons, the former giving rise to A-fibers and the latter to C-fibers. In the spinal cord, C-fibers terminate in laminae I–II, thin myelinated nociceptive afferents in laminae I and V, and A-fibers in laminae III–IV (Maxwell & Rethelyi, 1987; Willis & Coggeshall, 1991; Grant, 1995). Peripheral nerve injury can cause neuropathic pain, and peripheral nerve transection has therefore been used as one model to study the underlying mechanisms (Wall *et al*., 1979). Many studies have shown dramatic neuronal plasticity at spinal level after nerve injury (Dray *et al*., 1994; Hökfelt *et al*., 1994; Zigmond *et al*., 1996; Woolf & Salter, 2000).

It is known that, after peripheral nerve injury, neurons in laminae I–II can be activated by selective electrical stimulation of A-fibers, both using the proto-oncogene *c-fos* as a functional marker (Bester *et al*., 2000; Molander *et al*., 1992; Chi *et al*., 1993; Shortland & Molander, 1998; Catheline *et al*., 1999) and electrophysiology (Dalal *et al*., 1999; Ikeda *et al*., 1999; Kohama *et al*., 2000; Pitcher & Henry, 2000). However, the structural and molecular mechanisms for the functional reorganization are still poorly understood. Peripheral nerve injury has been reported to induce a prominent sprouting of A-fibers into laminae I–II (Woolf *et al*., 1992; Woolf *et al*., 1995; Coggeshall *et al*., 1997). The demonstration of the sprouting has mainly been based on the use of two retrograde and transganglionic neuronal tracers, CTB and CTB conjugated to horseradish peroxidase (Cuatrecasas, 1973; Stoeckel *et al*., 1977). Normally, they are mainly taken up by large neurons and label A-fibers (Trojanowski *et al*., 1981; Rivero-Melian & Grant, 1990; LaMotte *et al*., 1991). Therefore, the marked increase in CTB-labelled fibers of laminae I–II has been interpreted as sprouting of A-fibers, and suggested to be an anatomical basis for the development of chronic pain (Woolf & Mannion, 1999; Woolf & Salter, 2000). Our previous study shows that peripheral axotomy apparently causes a phenotypic change in small DRG neurons, i.e. an up-regulation of the binding site for CTB, resulting in a marked increase in the capacity of DRG neurons to take up and transport CTB, so that in addition to large neurons, almost all small neurons are labelled (Tong *et al*., 1999). These findings strongly suggest that CTB-labelled fibers in lamina II mainly represent C-fibers, and seriously question the role of anatomic sprouting as a basis for neuropathic pain, as recently discussed (Blomqvist & Craig, 2000). Our previous results did, however, not rule out the possibility that the injured C-fibers transport CTB to DRG but not further into the dorsal horn. Therefore, further studies

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are needed to re-evaluate the bearing of A-fiber sprouting and the role of CTB transport in C-fibers for the CTB labelling that appears in laminae I–II after nerve injury.

To determine the extent of injury-induced sensory sprouting, we tried to anatomically distinguish the sprouting A-fibers by studying the CTB-labelled C-fibers and A-fibers in parallel. To directly observe the sprouting A-fibers, we injected CTB into the sciatic nerve before axotomy to achieve a selective CTB labelling of A-fibers, and observed only a few A-fibers that sprout into inner lamina II. Double-labelling with peptide markers for C- or A-fibers and CTB provided evidence that sprouting of A-fibers is only very limited.

Materials and methods

Nerve transection and tracer injection

Fifty adult male Sprague–Dawley rats (b.wt. 200–250 g; Shanghai Center of Experimental Animals, Chinese Academy of Sciences, China; B & K Universal, Stockholm, Sweden) were used in the present study. The experiments were carried out in accordance with the policy of the North American Society for Neuroscience on the use of animals in neuroscience research.

Post-injury application of CTB

Under deep anaesthesia, the left sciatic nerve of 20 rats was exposed and transected at mid thigh level. A portion (approximately 5 mm) of the proximal part of the transected nerve was resected. The rats were, following post-lesion times of 2, 7, 14, 28 and 56 days (four rats for each time interval), anaesthetized as above, and the sciatic nerves on both sides exposed at mid thigh level. Two microlitres of 1% CTB (List Biological Laboratories, Campbell, CA, USA), dissolved in 0.1% Fast Green in distilled water, were slowly injected into the proximal part of the transected left sciatic nerve (approximately 4 mm proximal to the stump) and into the right intact nerve at the same level using a 33-gauge Hamilton syringe. All of these rats were allowed to survive for another 3 days after CTB injection. For control, four rats were carefully injected with CTB into left sciatic nerve and survived for another 3 days.

Pre-injury application of CTB

Under deep anaesthesia, 12 rats were subjected to slow injections of 2 μL of 1% CTB bilaterally into the sciatic nerve at mid thigh level four days prior to left sciatic nerve transections. The operations and injections were carried out with the aim of minimizing damage to the nerve. Transection of left sciatic nerve was made at least 5 mm above the CTB injection site, and a portion (approximately 5 mm) of the proximal part of the transected nerve was resected. These animals were allowed to survive for another 2, 7 and 14 days (four rats for each time interval), respectively, after the sciatic nerve transection. For control, unilateral injections were made into the sciatic nerve in four additional, nonaxotomized rats and allowed to survive for 14 days.

In addition, three rats were unilaterally axotomized 4 days after receiving CTB injection and were allowed to survive for another 14 days. Three unilaterally CTB injected and nonaxotomized rats were used as control. All six rats were prepared for electron microscopy.

Fixation and tissue preparation for immunohistochemistry

The animals were deeply anaesthetized and perfused *via* the ascending aorta with 50 mL warm (37 °C) saline followed by 50 mL warm fixative composed of 4% paraformaldehyde and 0.2% picric acid in 0.16 M phosphate buffer at pH 6.9. This was immediately followed by 200 mL of the same, but ice-cold fixative for another 5 min. The lumbar spinal cord and DRGs were dissected out, post-fixed in the same fixative for 90 min at 4 °C, and immersed in 10% sucrose in 0.01 M phosphate buffered saline (PBS) overnight. For all cases, 20 μm thick, transverse sections from the lumbar 4 and 5 spinal cord segments, and 14 μm thick, longitudinal sections from the DRGs, were cut in series in a cryostat and mounted on gelatin-coated slides.

CTB immunohistochemistry

The slide-mounted sections were processed by indirect immunofluorescence histochemistry. All antisera used were diluted in PBS with 1% bovine serum albumin and 0.3% Triton X-100. Briefly, the sections were incubated overnight with a goat antiserum against CTB (1 : 1000) (List) at 4 °C. After several rinses in PBS, the sections were incubated for 30 min at 37 °C with fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (1 : 100) (Jackson ImmunoResearch, West Grove, PA, USA), rinsed again, mounted with a mixture of glycerol/PBS (3 : 1) containing 0.1% para-phenylenediamine and examined under a Leica SP2 confocal microscope (Leica Microsystem, Heidelberg, Germany). Slide-mounted sections processed for the immunoperoxidase method were first incubated with goat anti-CTB (1 : 1000), and then in biotinylated anti-goat IgG (1 : 200) (Vector ABC Kit, Vector, Burlingame, CA, USA) and avidin-biotin-peroxidase complex (1 : 100) (Vector). The sections were then incubated in a medium containing 60 mg of 3,3′-diaminobenzidine tetrahydrochloride, 200 mg of glucose, 40 mg of ammonium chloride, 3 g of nickel ammonium sulphate and 0.7 mg glucose oxidase in 100 mL of 0.1 M acetate buffer. Afterwards, they were dehydrated, mounted with coverslips and examined under a Leica DMRA microscope.

CTB-galanin, -VIP, -NPY or -NPY Y1 receptor double-labelling immunohistochemistry

Cryostat sections of rat spinal cord and DRGs were incubated with a mixture of goat anti-CTB (1 : 1000) and rabbit antiserum against galanin (1 : 400) (Peninsula Laboratories, Belmont, CA, USA), or rabbit antiserum against vasoactive intestinal polypeptide (VIP) 1 : 1000 (Fahrenkrug & Schaffalitzky de Muckadell, 1978), or mouse monoclonal antibodies against neuropeptide Y (NPY) 1 : 3000 (Grouzmann *et al*., 1992), or rabbit antiserum against the Y1 receptor (1: 4000) (Zhang *et al*., 1994) for 18–24 h at 4 °C. After rinsing in PBS, the sections were incubated with a mixture of FITC-conjugated donkey anti-goat IgG and lissamine rhodamine (LRSC)-conjugated donkey anti-rabbit or donkey anti-mouse IgG (1 : 100) (Jackson) for 30 min at 37 °C, rinsed in PBS, mounted and examined under the confocal microscope.

B4 binding and CTB immunohistochemistry

Cryostat sections of the L4 and L5 spinal cord segments were incubated with rabbit anti-CTB (1 : 500) (List) for 18–24 h at 4 °C. After rinsing in PBS, the sections were incubated with a mixture of LRSC-conjugated donkey anti-rabbit IgG (1 : 40) (Jackson) and FITC-conjugated isolectin B4 from *Griffonia simplicifolia* I (B4) (0.1 mg/mL) (Vector) for 1 h, rinsed in PBS, mounted and examined under the confocal microscope.

B4-NPY Y1 receptor double-labelling immunohistochemistry

Sections of control and axotomized rat spinal cord were incubated with B4 (1 μg/mL) (Vector) at room temperature for 1 h. After rinsing in PBS, the sections were incubated with a mixture of goat

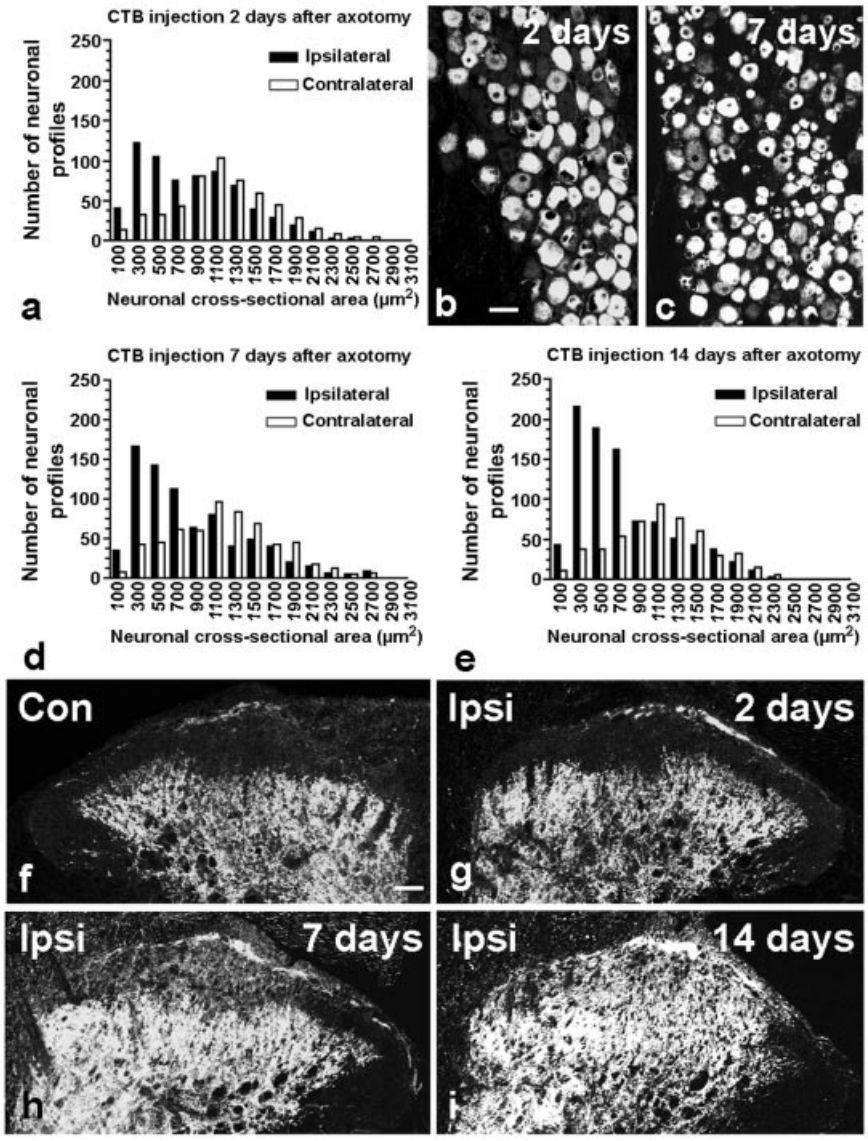

FIG. 1. Correlation between the number of CTB-labelled small DRG neurons and CTB-labelled afferent fibers in laminae I–II. CTB is bilaterally injected into the sciatic nerve 2 (a, b, f and g), 7 (c, d and h) or 14 (e and i) days after unilateral sciatic nerve transection and examined in L4 and L5 DRGs and spinal cord 17 days after axotomy. (a) The histogram shows that in the ipsilateral DRG the peaks of the size distribution of CTB-labelled NPs are located at 300–700 μm^2, representing small NPs, and another one for larger NPs at 900–1500 μm^2, 2 days after axotomy. In contrast, in the contralateral DRG, the size of CTB-labelled NPs is located in the range of large NP. Immunofluorescence micrographs show that CTB-labelled small NPs appear in the ipsilateral DRG 2 days (b) after axotomy, and the number is increased 7 days (c) after axotomy. Histograms show that the shift of CTB-labelled NPs to small NPs is more pronounced 7 (d) and 14 (e) days after axotomy. (f) In the contralateral dorsal horn, intensive CTB labelling is observed in all dorsal horn laminae except lamina II, where only a few scattered, labelled fibers are seen. (g) There is no marked change in the distribution of CTB labelling in ipsilateral lamina II 2 days after axotomy. However, CTB labelling is increased in laminae I–II 7 (h) and 14 (i) days after axotomy. Scale bar in (b), 50 μm (b and c); scale bar in (f), 50 μm (f–i).

anti-*Griffonia simplicifolia* I antiserum (1 : 1000) (Vector) and rabbit anti-Y1 receptor (1 : 4000) (Zhang *et al*., 1994) for 18–24 h at 4 °C. They were then incubated in a mixture of LRSC-conjugated donkey anti-goat IgG (1 : 100) (Jackson) and FITC-conjugated donkey anti-rabbit IgG (1 : 100) (Jackson) for 30 min at 37 °C, rinsed in PBS and mounted.

Electron microscopy

The rats used for electron microscopy were perfused with 50 mL warm (37 °C) saline, followed by 50 mL warm fixative composed of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, immediately followed by 200 mL of the same, but ice-cold fixative for another 5 min. The lumbar spinal cord was dissected out and post-fixed in the same fixative for 2 h at 4 °C. For all cases, 50 μm thick, transverse sections from the L4 and L5 spinal cord segments were cut on a Vibratome™. The sections were processed for the immunoperoxidase procedure similar to the one described above, except that the CTB antibody was diluted in 0.01 M PBS and 1% BSA. The sections were then post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812. The middle portion of laminae I–III of the spinal cord that contained CTB-labelled fibers in lamina II was selected under a microscope and mounted on blank

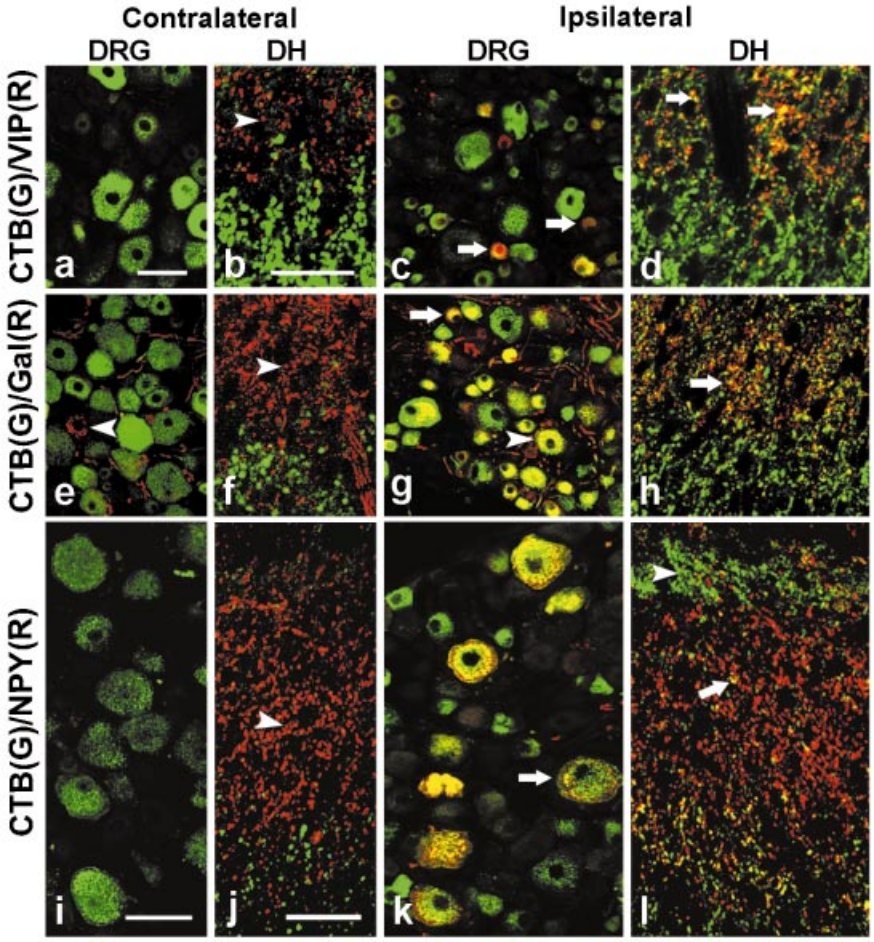

FIG. 2. Neuropeptides in post-nerve injury CTB-labelled DRG neurons and afferent fibers in laminae I and II. Micrographs of double-immunofluorescence-labelled contralateral (a, b, e, f, i and j) and ipsilateral (c, d, g, h, k and l) L5 DRGs (a, c, e, g, i and k) and spinal dorsal horn (b, d, f, h, j and l) 17 days after unilateral nerve cut. CTB is shown with green (G) and neuropeptides with red (R). VIP is not expressed in the contralateral DRGs (a), but in the nerve fibers of spinal neurons in lamina II (arrowhead) (b). After axotomy, VIP is expressed in many CTB-labelled small neurons (arrows) (c), and their fibers (arrows) in lamina II (d). (e) A few small neurons express galanin (Gal) (arrow) in the contralateral DRG. (f) Gal-positive fibers (arrowhead) in the contralateral lamina II are not labelled by CTB. After axotomy, many small neurons (arrow) and some large neurons (arrowhead) express Gal (g) that is found simultaneously with an increase in CTB and Gal-positive afferent fibers (arrows) in lamina II (h). (i) NPY is not expressed in contralateral DRGs and NPY-positive fibers (arrowhead) in lamina II are not labelled by CTB (j). After axotomy, NPY is expressed in many large neurons (arrow) (k), and some CTB-labelled fibers in lamina II are NPY-positive (arrow) (l). NPY is not seen in CTB-labelled fibers in lamina I (arrowhead). A few yellow structures are seen in lamina I, because two separate small structures are apparently overlapping. Scale bar in (a), 50 μm (a, c, e, and g); scale bar in (b), 50 μm (b, d, f and h); scale bar in (i), 50 μm (i and k); scale bar in (j), 50 μm (j and l).

resin stubs. Ultrathin sections were cut on an LKB V ultratome, counter-stained with uranyl acetate and lead citrate, and examined under a Hitachi 600 electron microscope.

Control for immunohistochemistry

Control experiments were conducted by pre-absorption of antisera with CTB (List) or galanin, VIP, NPY (Peninsula Laboratories) or NPY Y1 receptor peptide, respectively, at concentrations of 100 μg/mL (CTB) and 10^{-6} M (peptides) for 24 h at 4 °C.

Quantification

The DRG sections used for quantification were all processed for immunoperoxidase histochemistry, slightly counter-stained by toluidine blue and examined under bright-field illumination in the microscope using a 20× objective lens.

To determine the percentage of CTB-labelled neuronal profiles (NPs), counting was made in all ipsilateral and contralateral L5 DRGs in the axotomized rats and the left L5 DRG of four control rats. Every 15th serial section of the rat DRG (five sections per ganglion) was selected for the counting. Both labelled and unlabelled NPs were selected randomly and counted. A total number of 1149–1285 NPs from each DRG was counted. All data were processed for ANOVA and the unpaired two-tailed *t*-test. Results were presented as mean ± standard error of mean (SEM).

To determine the size of CTB immunoreactive NPs, the square area of NPs was measured ipsi- and contralaterally in post-injury CTB-labelled L5 DRGs 2, 7 and 14 days after axotomy, and in pre-injury CTB-labelled L5 DRGs 14 days after axotomy. Only NPs with a clear nucleus were included in the quantification. In three sections at 280 μm intervals from each L5 DRG of three rats, at each time interval, 400 NPs, including CTB-labelled NPs, were selected randomly and measured. A Leica Q550 image system (Leica) connected to a microscope was used to collect and analyze the data. The data were presented as total number of labelled NPs in the

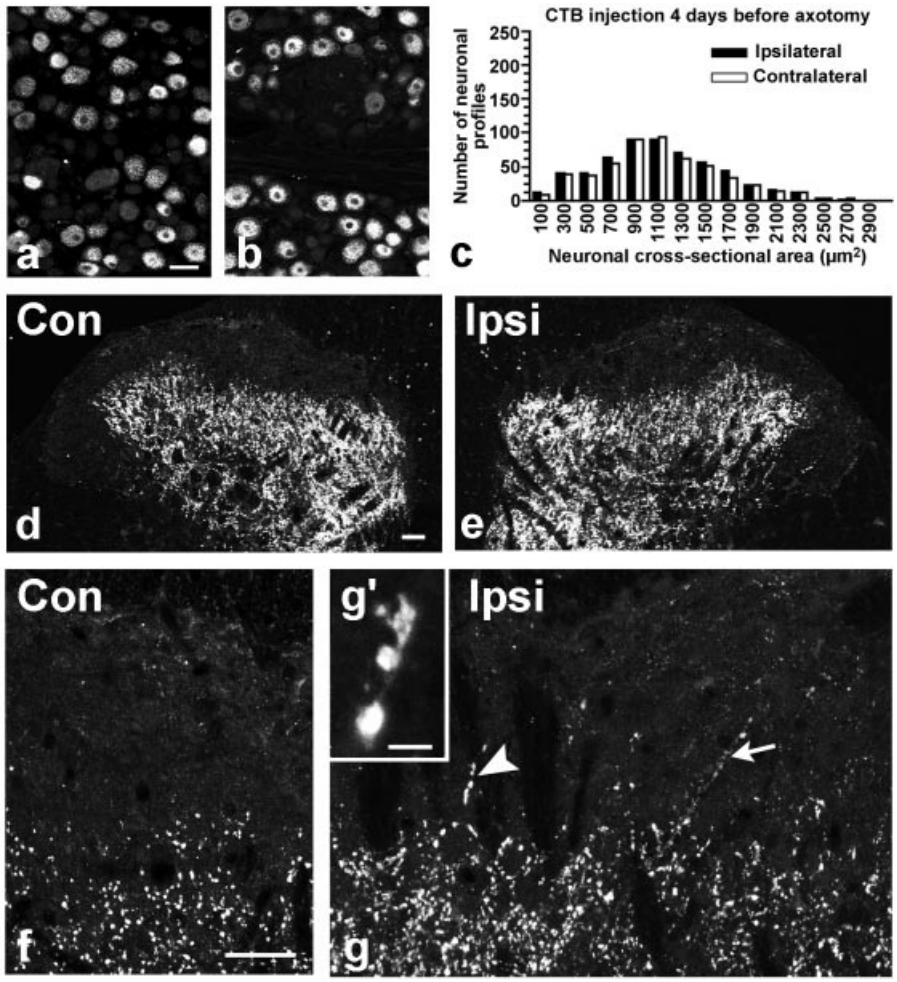

FIG. 3. The sprouting of A-fibers distinguished by pre-injury CTB labelling. Immunofluorescence micrographs showing CTB-labelled contralateral (a) and ipsilateral (b) L5 DRGs and a histogram of size distribution of CTB-labelled NPs (c), following CTB injection into the sciatic nerve 4 days before unilateral axotomy and a post-operative survival of 14 days. There is no marked increase in CTB labelling in ipsilateral lamina II (d and e). With the confocal microscope to Z-scan the dorsal horn at high magnification, only a few, scattered CTB-positive fibers can be seen in laminae I–II of the contralateral dorsal horn (projection of 18 1 μm thick optic sections) (f). However, some CTB-labelled afferent fibers (arrow and arrowhead) are observed in lamina II of the ipsilateral dorsal horn, especially in the inner lamina II (projection of 18 1 μm thick optic sections) (g). Higher magnification of the arrowhead-pointed CTB-labelled fiber in g is shown in g′. Scale bar in (a), 50 μm (a and b); scale bar in (d), 50 μm (d and e); scale bar in (f), 10 μm (f and g); scale bar in (g′), 1 μm.

ipsilateral and contralateral L5 DRGs of three animals at each time interval vs. NP cross-sectional area indexes with 200 μm^2 intervals.

Semi-quantification of the CTB-labelled fibers was carried out on both sides of the spinal cord, performing camera lucida drawings of the middle portion of laminae I–II and the dorsal portion of lamina III. Seven 20-μm-thick sections of the spinal cord of three rats were studied at each time interval. These rats had been subjected to pre-injury CTB injections and were examined 2, 7 and 14 days after unilateral axotomy. Every fifth serial section of the caudal, approximately 1 mm-thick, portion of the fourth lumbar segment was selected, processed for immunoperoxidase histochemistry, and analyzed.

Culture of CTB-labelled DRG neurons and CTB/neurofilament 200 double labelling

Under deep anaesthesia, the sciatic nerves of three rats were exposed on both sides at mid thigh level. Two microlitres of 1% CTB were slowly injected into the intact nerve. All of these rats were allowed to survive for another 3 days after CTB injection. After anaesthesia, the L4 and L5 DRGs of the rats were dissected and incubated in 2.5 mL L-15 medium (Gibco BRL, Rockville, ML, USA) containing 3 mg/mL collagenase (Sigma Type 1 A; Sigma-Aldrich, St. Louis, MO, USA) and 1 mg/mL trypsin (Sigma Type I) at 37 °C for 45 min. After enzymatic digestion, cells were triturated in the Dulbecco's modified Eagle's medium (Gibco BRL) with 10% fetal bovine serum (Gibco BRL) and plated on coverslips. Cells were fixed in 4% paraformaldehyde and 0.02% picric acid for 20 min after culture for 1, 7 or 14 days. Then the cells were incubated with a mixture of goat anti-CTB (1 : 1000) and mouse antiserum against neurofilament 200 (Nf 200) (1 : 500) (Sigma-Aldrich) for 24 h at 4 °C. After rinsing in PBS, the cells were incubated with a mixture of FITC-conjugated donkey anti-goat IgG and LRSC-conjugated donkey anti-mouse IgG (1 : 100) (Jackson) for 30 min at 37 °C, rinsed in PBS, mounted and examined under the confocal microscope.

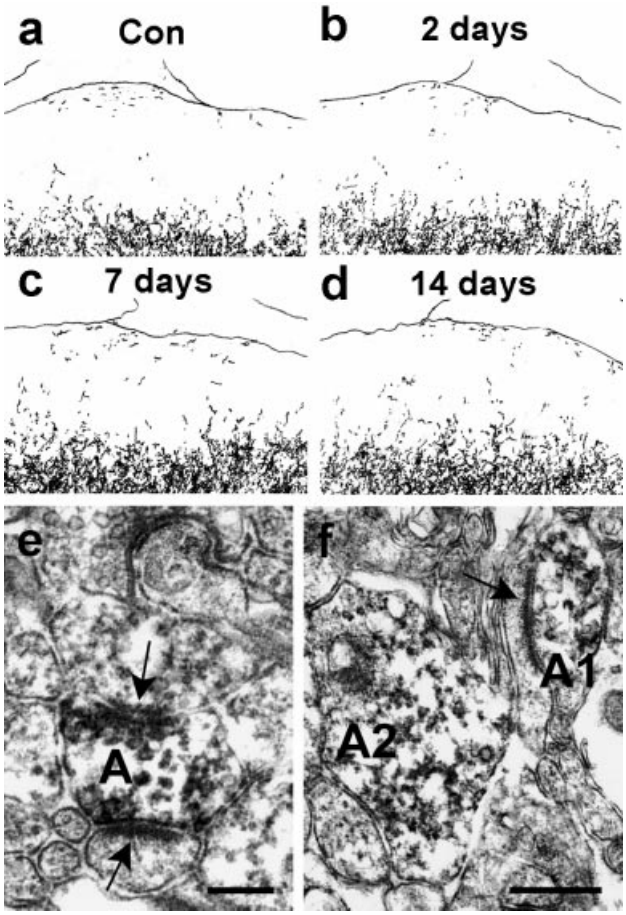

FIG. 4. The distribution and ultrastructure of the sprouting of CTB-labelled A-fibers in lamina II. Camera lucida drawings, each based on seven 20-μm-thick sections showing the distribution of CTB-labelled afferent fibers in the middle portion of laminae I, II and lamina III (the dorsal part) of the contralateral (a) and ipsilateral (b–d) dorsal horn, following CTB injections 4 days before axotomy and processing for immunoperoxidase labelling 2 (b), 7 (c) and 14 (a and d) days post-operatively. A small increase in the number of CTB-labelled afferent fibers is seen in the inner lamina II of the ipsilateral dorsal horn 7 and 14 days after axotomy. Electron microscopy shows that in the middle portion of the inner lamina II of ipsilateral dorsal horn 14 days after axotomy (e), a CTB-labelled fine afferent fiber containing several synaptic vesicles (A) makes synapses with two dendrites (arrows). (f) A CTB-labelled small nerve terminal (A1) forms a synapse with a dendrite (arrow) and a CTB-labelled fiber (A2) contains many microtubules and vesicles. Scale bars, 0.2 μm (e) and 0.5 μm (f).

Results

Post-injury neuronal tracing by CTB labels both unmyelinated and myelinated afferent fibers in the dorsal horn

The number of NPs labelled by CTB was increased to 60.9 ± 2.7%, 66.75 ± 3.1%, and 78.5 ± 2.6% for all NPs in the ipsilateral L5 DRGs 2, 7, and 14 days, respectively, after unilateral sciatic nerve transection ($P < 0.01$, compared with the controls). These CTB-positive NPs were found both among small and large NPs (Fig. 1a–e). In contrast, 45.4 ± 1.5% of the counted NPs were labelled by CTB in control DRGs, and the corresponding percentage in contralateral DRGs were 45.5 ± 2.0%, 43.9 ± 2.8%, and 45.8 ± 2.2%, respectively. These NPs were mainly large (Fig. 1a, d and e). We define small neurons (diameter less than 32 μm) as having an area of less than 900 μm^2 (Giuffrida & Rustioni, 1992). Therefore, there was a shift in size of ipsilateral CTB-labelled NPs towards smaller ones, and this shift increased with time.

Also, the CTB-labelling of nerve fibers in laminae I–II of the ipsilateral dorsal horn became more intense as CTB was injected at later time intervals after axotomy (Fig. 1f–i). In the contralateral dorsal horn and in normal rats, an intensely stained network of CTB-positive fibers was localized in laminae III–V, with few scattered CTB-positive fibers in lamina II and several labelled fibers in lamina I (Fig. 1f). No certain increase was seen in the amount of CTB-positive fibers in lamina II 2 days after axotomy (Fig. 1g), but there was a marked increase at 7 days (Fig. 1h) and a very marked increase at 14 days (Fig. 1i) after lesion. This paralleled the ipsilateral increase in the number of CTB-labelled small NPs in the L5 DRG. Furthermore, the intensity of the ipsilateral CTB-labelling of laminae III, IV and V also increased with time (Fig. 1h and i).

In L4 and L5 DRGs of control rats and in contralateral L4 and L5 DRGs, VIP was not detected in CTB-labelled DRG neurons 17 days after axotomy (Fig. 2a). Low levels of VIP are seen in a few primary afferents and several axonal terminals from local neurons in lamina II of the spinal cord in the normal rat (Zhang *et al*., 1995b). Using the advanced confocal microscopy, more VIP-positive fibers were seen in the contralateral and control laminae I–II (Fig. 2b). VIP was not colocalized with CTB in the fibers (Fig. 2b). In the ipsilateral L4 and L5 DRGs, 14 days after axotomy, VIP was exclusively expressed in small neurons, and CTB was present in 90.9 ± 3.1% of VIP-positive small NPs (Fig. 2c). Colocalization of VIP and CTB was now observed in many afferent fibers in lamina II of the ipsilateral dorsal horn (Fig. 2d).

In control DRGs, galanin was expressed only in a few small CTB-negative neurons (Fig. 2e), and only occasionally colocalization was observed. Galanin-positive, CTB-negative fibers were mainly seen in laminae I–II, and occasionally in lamina III (Fig. 2f). After axotomy, galanin was expressed in many small and some large neurons, and almost all (95.3 ± 2.7%) galanin-positive small neurons were labelled by CTB (Fig. 2g). Also, most large galanin-positive NPs were CTB-labelled (Fig. 2g). Ipsilaterally, 14 days after sciatic nerve transection, a moderate increase was detected in the number of galanin-positive nerve fibers in laminae I–II, with a very limited expansion into lamina III, and CTB-labelling was seen in many galanin-positive fibers (Fig. 2h).

NPY was detected neither in the contralateral, nor in control DRGs (Fig. 2i). In laminae I–II of the contralateral dorsal horn, NPY was expressed in many local neurons and their processes (Fig. 2j), with some fibers also in lamina III (Fig. 2j). These fibers did not contain CTB (Fig. 2j). After axotomy, NPY was expressed in many large and a few small ipsilateral neurons. As many as 81.5 ± 5.6% of the CTB-labelled large NPs were NPY-positive and 93.6 ± 3.7% of the NPY-positive large NPs were CTB-labelled (Fig. 2k). Only 4.7 ± 1.2% of the total number of counted CTB- and NPY-positive NPs were small. Many small neurons labelled by CTB were only seen in addition (Fig. 2k). In agreement with many published studies, an increase in the number of NPY-positive primary afferent fibers was observed in laminae III–IV after axotomy. In addition, CTB-labelling in lamina I was also markedly increased. However, NPY was not present in these CTB-labelled afferent fibers in lamina I (Fig. 2l). Some CTB-positive fibers in lamina II were also NPY-immunoreactive (Fig. 2l). There were no further distinct changes in the correlation of the distribution of CTB with the studied neuropeptides in laminae I–II 28 and 56 days after peripheral axotomy (data not shown). These data demonstrate that most C-fibers could be labelled by CTB applied after axotomy. Thus, post-injury neuronal tracing with CTB cannot be used to selectively identify sprouting of A-fibers into laminae I–II.

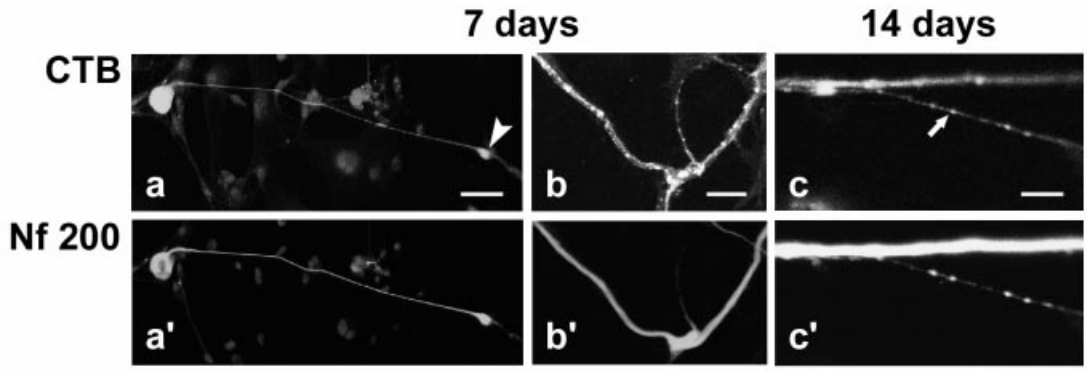

FIG. 5. Distribution of CTB in the culture of CTB-labelled DRG neurons. CTB-labelled DRG neurons are cultured for 7 (a–b′) and 14 (c and c′) days, and double-labelled with CTB (a–c) and neurofilament (Nf) 200 (a′–c′) that is used to identify the fine neurites. (a) Seven days after culture, CTB is present in both the cell body, the newly emerged neurite, including a spheroid enlargement (arrowhead). (b) CTB is seen in a fine branch (arrow) arising from the main neurite. (c) The fine neurite is still labelled by CTB (arrow) after 14 days in culture. Scale bar in (a), 25 μm (a and a′); scale bar in (b), 1 μm (b and b′); scale bar in (c), 0.5 μm (c and c′).

Pre-injury neuronal tracing by CTB selectively labels myelinated primary afferents after axotomy

To obtain selective labelling of large DRG neurons and thick myelinated primary afferent fibers, CTB was injected into the sciatic nerve bilaterally 4 days before unilateral sciatic nerve cut. This resulted in labelling mainly of large neurons in both contralateral (Fig. 3a) and ipsilateral (Fig. 3b) DRGs. The cell-size measurements showed a similar cell-size distribution in ipsi- and contralateral DRGs (Fig. 3c), indicating that no marked shift in size of the labelled NPs had occurred. In ipsilateral DRGs, 44.47 ± 4.13% of NPs were labelled by CTB, which did not differ from the contralateral DRGs (43.96 ± 3.51%).

At low magnification, CTB-labelled fibers in the dorsal horn showed a similar distribution pattern ipsilaterally and contralaterally (Fig. 3d and e). The border between laminae II and III appeared distinct, due to the intense CTB-labelling in lamina III. However, when the sections were analyzed at high magnification under the confocal microscope (Fig. 3f and g), a few CTB-labelled afferent fibers were clearly observed 14 days after axotomy in the region innervated by C-fibers in lamina II (Fig. 3g and g′). In general, however, the CTB-labelling of these fibers was relatively weaker and finer than seen in the afferents in the deeper laminae, and the fibers were varicose and often with small branches (Fig. 3g). Occasionally, CTB-labelled fibers in lamina III could, in fact, be followed into lamina II (Figs 3g and 6b, c, e and f). In Fig. 4a–d, a semi-quantitative analysis of sprouting CTB-labelled afferent fibers in lamina II is shown, based on camera lucida drawings of seven 20-μm-thick sections from control and axotomized rats (axotomized 2, 7 and 14 days before they were killed), showing the middle portion of laminae I, II and lamina III (the dorsal part only). Numerous afferent fibers in lamina III were labelled by CTB and formed a relatively clear border, with only a few CTB-labelled fibers in control lamina II and some in lamina I (Fig. 4a). There was no marked increase in the number of ipsilateral CTB-labelled afferent fibers in laminae I and II 2 days after axotomy (Fig. 4b), but a slight increase in lamina II, mainly in its inner part, 7 and 14 days after axotomy (Fig. 4c and d). The number of CTB-labelled fibers in lamina I was not markedly changed.

The ultrastructure of CTB-labelled sprouting fibers was examined in the ipsilateral, inner part of lamina II 14 days after axotomy. Under the electron microscope, lamina I could be distinguished from outer lamina II by a high density of thin myelinated fibers; inner lamina II was characterized by a very low density of such fibers, which again became abundant in lamina III. Outer lamina II was defined as the area between lamina I and inner lamina II, as described above. Lamina I was virtually devoid of glomeruli, but a few fibers, which are from thin myelinated afferents, were CTB-positive. In ipsilateral, inner lamina II, several CTB-labelled fine afferent fibers were observed, often with varicosities containing a small amount of synaptic vesicles, mitochondria and some microtubules. Some CTB-labelled fibers made synaptic contact with dendrites (Fig. 4e and f). Terminals of unmyelinated fibers in lamina II (not shown) could be identified as central terminals of glomeruli (not shown). Axonal labyrinths composed of a central terminal, winding, flattened, sheet-like neuronal profiles and glial expansions were also seen. CTB-labelling was not localized in these structures, and was rarely seen in fibers in the contralateral lamina II.

Culture of CTB-labelled DRG neurons and CTB/neurofilament 200 double labelling

To know whether pre-injury injected CTB could be centrifugally transported in sprouting DRG neurons, we cultured CTB pre-labelled DRG neurons for 7 and 14 days. When the pre-labelled DRG neurons were examined after one day in culture, CTB-labelled neurons were generally seen without or with only short processes, which were labelled with Nf 200. After seven days in culture, CTB-labelled neurons gave rise to long neurites which were labelled by both CTB and Nf 200 (Fig. 5a and a′). CTB appeared in the finest branches from the main neurites; in fact, it could occasionally be seen even in fibers without detectable Nf 200 (Fig. 5b and b′). The CTB labelling had a dot-like distribution (Fig. 5b). The same pattern of CTB and Nf 200 could be still seen after 14 days in culture (Fig. 5c and c′).

Neuropeptides: focus on NPY and Y1 receptors

VIP-LI was not detected 14 days after axotomy in pre-injury CTB-labelled small DRG neurons (Fig. 6a), nor in CTB-labelled inner lamina II-afferents (Fig. 6b–c″). Although galanin was expressed in some large DRG neurons, it was not observed in CTB-labelled sprouting fibers in inner lamina II (data not shown). NPY was expressed in many ipsilateral CTB-labelled large neurons (Fig. 6d), and almost all CTB-labelled sprouts contained NPY-LI (Fig. 6e–f″).

CTB-labelled A-fibers and B4-labelled C-fibers formed a relatively distinct border in the superficial dorsal horn of control spinal cord (Fig. 7a). Many neurons expressed NPY Y1 receptor at high level in

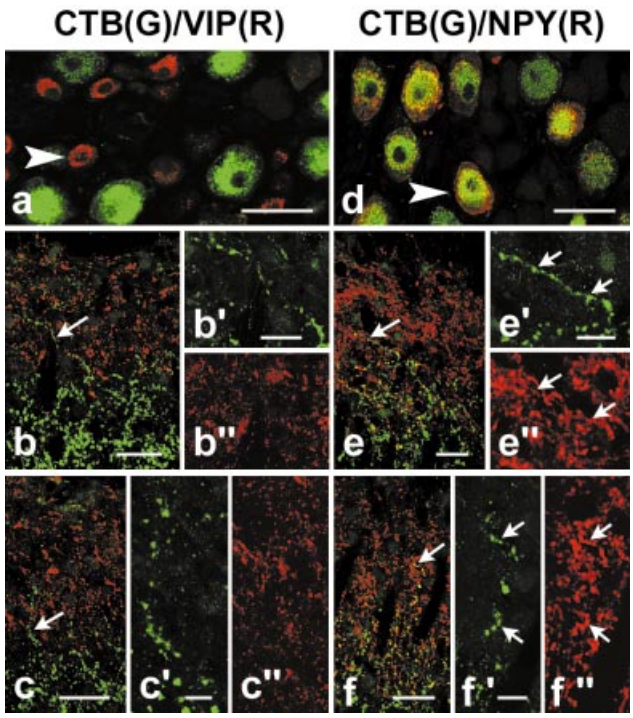

FIG. 6. Neuropeptide in sprouting A-fiber neurons 14 days after axotomy. Confocal micrographs of ipsilateral L5 DRG, laminae I, II and dorsal portion of lamina III of ipsilateral dorsal horn 14 days after axotomy and pre-injury-labelling with CTB and immunofluorescence staining for VIP (a–c″) or NPY (d–f″). CTB is shown with green (G) and neuropeptides with red (R). (a) VIP is expressed in many small neurons (arrow), which are not labelled by CTB, and two CTB-labelled afferent fibers extending into the inner lamina II do not contain VIP (arrow) (b–b″; c–c″; projections of 18 1 μm thick optic sections). (d) NPY is expressed in many CTB-labelled large neurons (arrow) after axotomy. In contrast to VIP, NPY is localized in CTB-positive afferent fibers in the inner lamina II (e–e″; f–f″; projections of 18 1 μm thick optic sections). Scale bars, 50 μm (a, b, c, d, e and f); scale bar in (b′), 25 μm (b′ and b″); scale bar in (e′), 25 μm (e′ and e″); scale bar in (c′), 10 μm (c′ and c″); scale bar in (f′), 10 μm (f′ and f″).

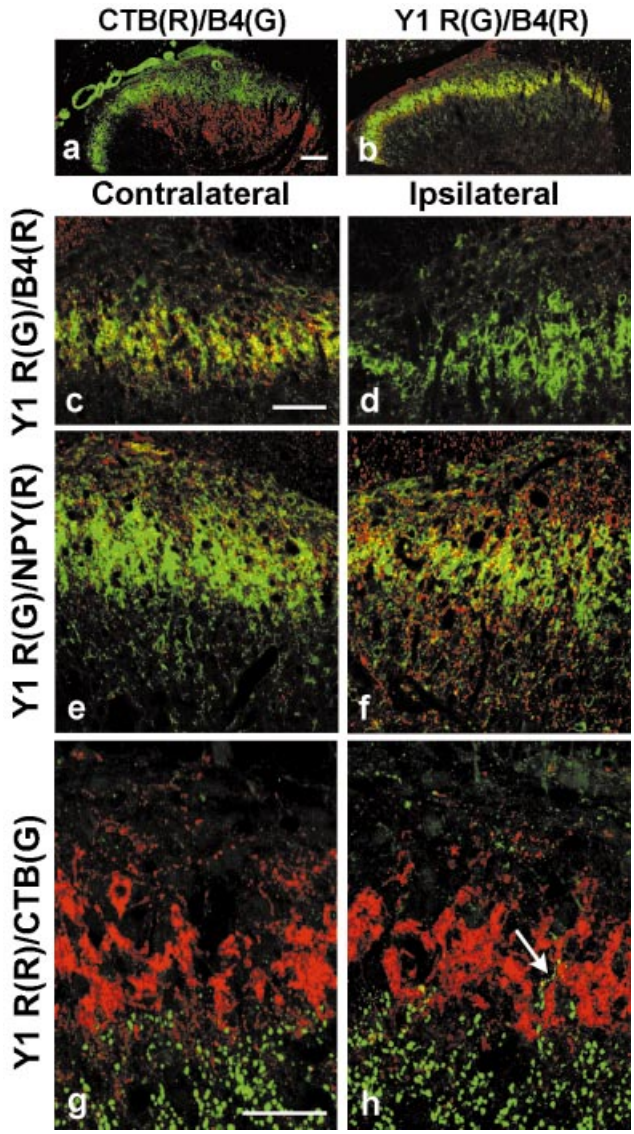

FIG. 7. The relationship between NPY containing afferent fibers and NPY Y1 receptor in the dorsal horn. Confocal micrographs of dorsal horn of the spinal cord of the pre-injury CTB injected rat 14 days after unilateral axotomy. (a) A distinct border between B4 binding C-fibers and CTB-labelled A-fibers is observed in the superficial dorsal horn. Note the absence of CTB labelling laterally, i.e. the area not supplied by sciatic nerve. (b and c) Very similar distribution of Y1 receptor and B4 binding are seen in laminae I and II of contralateral dorsal horn with a higher intensity and patchy distribution in the inner lamina II. (d) In the ipsilateral dorsal horn, B4 binding is almost completely absent, whereas strongly Y1 receptor-stained patches are still seen in the inner lamina II. (e) NPY is present in many nerve fibers in laminae I and II of the contralateral dorsal horn. Only a few NPY-positive fibers are seen in lamina III, where many Y1 receptor-positive neurons and fibers are seen ventral to the heavily labelled Y1R area in lamina II. (f) In the ipsilateral dorsal horn, intense NPY-positive fibers are distributed in laminae I–III to cover all Y1 receptor-distributed area. (g) CTB-labelled afferent fibers are located in lamina III of the contralateral dorsal horn ventral to the strongly labelled Y1R-positive interneurons (projection of 18 1 μm thick optic sections). (h)Arrow points to a CTB-labelled afferent fiber penetrating into the ipsilateral inner lamina II (projection of 14 1 μm thick optic sections). Scale bar in (a), 100 μm (a and b); scale bar in (c), 50 μm (c–f); scale bar in (g), 50 μm (g and h).

lamina II (Fig. 7b–d). Double immunofluorescence labelling showed that Y1 receptor-positive neurons and B4-positive afferents formed a distinct, strongly overlapping band in inner lamina II (Fig. 7b and c). The ventralmost part of this intensely Y1 receptor-positive band was the border of CTB-labelled afferents, with a limited overlap (Fig. 7g). Under normal circumstances, NPY was expressed in lamina II neurons, with their processes mainly in lamina II, without forming a distinct band in inner lamina II. Y1 receptor-LI was, in addition, expressed in some neurons in lamina III and in a few neurons in lamina IV, where only a few NPY-positive fibers were seen in control and contralateral dorsal horn (Fig. 7e). After axotomy, B4-binding sites were reduced in the afferent fibers, but the pattern of distribution and intensity of Y1 receptor remained (Fig. 7d). In addition, NPY-LI was present not only in lamina II but also in many afferents in laminae III and IV, corresponding to the distribution of Y1 receptors (Fig. 7f). Furthermore, CTB- and NPY-positive sprouts of afferents were also found in the Y1 receptor-enriched band in inner lamina II (Figs 4d, 6e and f, and 7h).

Control for immunohistochemistry

Pre-absorption of antiserum with an excess of CTB, VIP, galanin, NPY and NPY Y1 receptor peptide, respectively, abolished all immunostaining patterns described above.

Discussion

The superficial layers of the dorsal horn of the spinal cord represent the area involved in modulation of nociceptive information (Willis &

Coggeshall, 1991). It is well established that C-fibers terminate in laminae I and II, and form synaptic circuits conveying distinct nociceptive inputs (Maxwell & Rethelyi, 1987; Willis & Coggeshall, 1991). Many studies on the mechanisms underlying neuropathic pain have demonstrated extensive structural and molecular plasticity of the neurons in the pain pathway following nerve injury (Dray *et al*., 1994; Hökfelt *et al*., 1994; Zigmond *et al*., 1996; Woolf & Mannion, 1999; Woolf & Salter, 2000) including a peripheral axotomy-induced marked increase in the number of CTB-labelled afferent fibers in laminae I and II of rat spinal cord following injection of the tracer into a peripheral nerve (Bennett *et al*., 1996; Woolf *et al*., 1992; Woolf *et al*., 1995; Lekan *et al*., 1996; Mannion *et al*., 1996; Coggeshall *et al*., 1997; Doubell & Woolf, 1997; Doubell *et al*., 1997; Eriksson *et al*., 1997). This has been interpreted as evidence for a prominent reorganization of the dorsal horn circuitry and proposed to represent an important anatomic basis for neuropathic pain. However, in a previous study we have demonstrated that the number of CTB- and CTB-HRP-labelled DRG neurons is markedly increased after peripheral axotomy, especially with regard to the subpopulation of small neurons (Tong *et al*., 1999), suggesting that CTB-labelled fibers in laminae I and II may be C-fibers. Even if interpreted differently by Siri *et al*. (2001), their recent demonstration that application of NGF to a previously transected nerve can reverse CTB labelling in lamina II would be compatible with this, if, for example, NGF reverses the injury evoked increased affinity of C-fiber afferents to CTB. The trkA receptor, which binds NGF, is located predominantly on a subpopulation of C-fiber afferents. Taken together, these results therefore raise several questions: (i) can sprouting of myelinated afferent fibers be specifically demonstrated at all with neuronal tracing methods; (ii) is there any marker that selectively labels A-fibers after axotomy; and (iii) to what extent does sprouting of A-fibers occur after peripheral axotomy? One approach is intra-axonal tracing of single A-fibers (Woolf *et al*., 1992; Koerber *et al*., 1994; Kohama *et al*., 2000), but it cannot directly illustrate the extent of sprouting.

To address these questions, we carried out several experiments, focusing on sensory sprouting. In a first series of experiments, we used the post-injury paradigm, i.e. made a unilateral sciatic nerve transection, which was followed by bilateral nerve injections of CTB. Firstly, in these experiments, we observed a parallel increase in CTB-labelling of the fibers in laminae I and II, and of small DRG neurons, when the injection of CTB was made in the axotomized nerve 7 and 14 days after peripheral axotomy. This supports our previous findings (Tong *et al*., 1999) that the increase in CTB-labelled primary afferents in laminae I and II is essentially related to the increased uptake of CTB in small neurons. In the present study we have used the method of profile counts for quantification, only providing estimates of percentages of labelled vs. total neurons, and the distribution of the area of the neuronal profiles (Giuffrida & Rustioni, 1992). Thus, we have not attempted to calculate total numbers of neurons, because here the axonal transection was made at mid thigh level, which has been shown not to cause a significant cell death and changes in the mean perikaryal volumes for all DRG neurons during the first four weeks (Tandrup *et al*., 2000). Therefore, cell loss and shrinkage should not influence the principal conclusions. Secondly, VIP, which is expressed exclusively in small neurons after axotomy (Shehab & Atkinson, 1986), and galanin, which after axotomy is mainly expressed in small neurons (Hökfelt *et al*., 1987; Villar *et al*., 1989), was observed in many CTB-labelled afferent fibers in lamina II. As a consequence, most CTB-labelled fibers in lamina II most likely originate from small neurons.

In the same experiments, we tried to use NPY as a marker for A-fibers, which is expressed in large neurons after axotomy (Wakisaka *et al*., 1991). In agreement with this, some CTB-labelled fibers in lamina II contained NPY, and they could therefore represent the sprouts of A-fibers. However, this interpretation is not conclusive, as they could alternatively represent C-fibers from a small population of small neurons that express NPY after axotomy (Zhang *et al*., 1995a; Zhang *et al*., 1995b). Furthermore, NPY was not present in post-injury CTB-labelled afferent fibers in lamina I, indicating that the increase of such fibers in this thin layer may result from increased uptake of CTB in thin myelinated afferents, and not from sprouting of thick myelinated A-fibers from deeper laminae, as proposed in many previous studies (see above). It has been suggested that CTB-HRP-labelled cutaneous afferents to lamina I are thin myelinated fibers (Rivero-Melian & Grant, 1990, 1991). Taken together, our results provide evidence that many of the CTB-labelled afferent fibers in lamina II are C-fibers and that the increase in CTB-labelled afferent fibers in lamina I does not represent sprouting of thick myelinated A-fibers.

Our results clearly show that the post-injury CTB tracing method is not suitable to demonstrate sprouting of A-fibers, which requires selective labelling of large neurons and A-fibers even after peripheral axotomy. We have therefore used a modification of the CTB tracing method, which normally labels large and medium-sized neurons and A-fibers (Robertson & Grant, 1985, 1989; Rivero-Melian & Grant, 1990, 1991; LaMotte *et al*., 1991). Thus, we have pre-stored CTB in DRG neurons 4 days prior to peripheral axotomy, here termed, pre-injury CTB tracing, and expected to detect any sprouting from the CTB-labelled A-fibers. Under these circumstances, CTB should not label C-fibers, because small neurons are only rarely labelled by CTB normally (Robertson & Grant, 1989). There are three essential factors that may influence the results of this experiment. First, the uptake of CTB by large neurons should have been accomplished, and there should be no CTB available to be taken up by C-fibers in the proximal end of transected sciatic nerve. Our data show that CTB injections made 4 days prior to peripheral nerve cut, and with the nerve lesion site at least 10 mm above the injection site, can meet this demand. Second, the storage of CTB should be lasting without a marked decrease in labelling intensity. In our hands, CTB can remain in DRG neurons for at least 18 days without marked degradation. We have also tested longer post-lesion periods and observed a decrease in the CTB-labelling three weeks after axotomy, and a pronounced decrease after 4 weeks (data not shown). Therefore, we used a 2-week post-lesion period, which is also the period showing marked sprouting in many published papers (see above). Third, pre-stored CTB should, in fact, label the sprouts. CTB binds to cell membrane glycoconjugates, especially to the monoganglioside GM1 (Cuatrecasas, 1973; Holmgren *et al*., 1973). The CTB is, however, internalized following application peripherally and then transported retrogradely to the DRGs, and from there anterogradely into the spinal cord and dorsal column nuclei. Thus, it is not unlikely that CTB would also label the sprouts. As with anterograde tracing methods in general, however, one cannot without reservation claim that all of the finer ramifications are demonstrated. To evaluate these possibilities, we studied pre-injury CTB labelled neurons in culture and were able to show that CTB is, indeed, transported into the new neurites and that the labelling remained during the 14 days in culture, indicating that newly emerging axons do contain CTB that come from the pre-labelled cell bodies. The present results show that pre-injury CTB-labelling is well

restricted to the population of DRG neurons that is normally labelled by CTB, that is, large and medium-sized neurons, suggesting that this method can be used to selectively label A-fibers and their sproutings after axotomy.

For a proper analysis of sensory sprouting it is essential to identify the border between the regions of termination of C-fibers and A-fibers in the dorsal horn. In the present study, we therefore defined the border between the afferent terminations by a line between the ventralmost border of B4-binding C-fibers/NPY Y1 receptor staining in the inner lamina II, and the dorsal-most termination of CTB-labelled A-fibers. CTB-labelled afferents crossing this line and penetrating into lamina II have been considered as evidence for sprouting. Only a small number of A-fibers was found to sprout into inner lamina II, sharply contrasting the very prominent increase of CTB-labelled afferents in laminae I and II of the spinal cord after peripheral axotomy reported here and in previous studies (Woolf *et al*., 1992; Woolf *et al*., 1995; Bennett *et al*., 1996; Lekan *et al*., 1996; Mannion *et al*., 1996; Coggeshall *et al*., 1997; Doubell & Woolf, 1997; Doubell *et al*., 1997; Eriksson *et al*., 1997; Mannion *et al*., 1998; Siri *et al*., 2001). With the pre-injury tracing method, we cannot address the issue of the late sprouting that has been shown by intra-axonal injection of individual afferents in rats in which nerves were sectioned 6–9 weeks earlier (Koerber *et al*., 1994; Woolf *et al*., 1992; Kohama *et al*., 2000). However, we could not find further changes in the patterns of the correlation between VIP, Gal, NPY and post-injury-injected CTB in laminae I–II 28 and 56 days after peripheral axotomy, suggesting that sprouting at long survival time may also be limited. It has been reported that following nerve injury regenerative synaptoneogenesis occurs in the superficial dorsal horn (Csillik & Knyihar-Csillik, 1981; Knyihar-Csillik *et al*., 1985). We observed that CTB-labelled axonal terminals made synaptic contacts with dendrites in the deeper lamina II, indicating that even if quantitatively limited, the sprouting may be of functional significance, as indicated in several electrophysiological and *c-fos* studies (see Introduction).

At the spinal level, NPY is involved in the modulation of pain transmission (White, 1997). Under normal circumstances, NPY is only expressed in local dorsal horn neurons in lamina II, and their processes are mainly distributed in this lamina (Gibson *et al*., 1984). In contrast, NPY receptors have a more extensive distribution, being present both in DRG neurons and spinal cord neurons. The Y1 receptor is mainly a somatic receptor present in small DRG neurons containing substance P and calcitonin gene-related peptide, and it is also expressed in many neurons in laminae I–III, often colocalized with somatostatin in inner lamina II neurons (Zhang *et al*., 1994; Zhang *et al*., 1999). After nerve injury, NPY is expressed in large neurons (Wakisaka *et al*., 1991) and is spontaneously released (Mark *et al*., 1998; Colvin & Duggan, 2001). In agreement with this, almost all detectable sprouts of A-fibers contained NPY. Thus, NPY-positive sprouts in lamina II from NPY-positive A-fibers in lamina III could influence many post-synaptic Y1 receptors on lamina II neurons, thus forming a modified, functional NPY circuitry in the dorsal horn after peripheral nerve injury. It is known that most NPY neurons in the dorsal horn are GABAergic (Rowan *et al*., 1993), and it is thus possible that this inhibitory transmitter could also be involved in the new circuitry.

In conclusion, the present results support and extend our previous findings, suggesting that A-fiber sprouting into superficial layers only provides a very modest contribution to the lamina II labelling seen after CTB injection into an axotomized nerve.

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Abbreviations

A-fibers, thick myelinated afferents; CTB, cholera toxin B subunit; C-fiber, unmyelinated afferent; DRG, dorsal root ganglion; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; FITC, fluorescein isothiocyanate; B4, isolectin B4 from *Griffonia simplicifolia* I; LRSC, lissamine rhodamine; NP, neuronal profile; Nf 200, neurofilament 200.

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