

Research report

Effect of a graded single constriction of the rat sciatic nerve on pain behavior and expression of immunoreactive NPY and NPY Y1 receptor in DRG neurons and spinal cord

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Abstract

In the present study, the rat sciatic nerve was constricted to varying degrees using only one ligature with a very thin polyethylene sheath placed between nerve and ligature thread. Complete nerve transection was studied for comparison. With a 40–80% constriction of the nerve we observed allodynia to a similar extent as in the so-called Bennett model based on four loose ligatures. We also monitored changes in the expression of neuropeptide Y (NPY) and the NPY Y1 receptor (Y1R) in the lumbar 4–5 dorsal root ganglia (DRG) and dorsal horn and found upregulation of NPY and downregulation of the Y1R in DRG neurons after injury. These results indicate that similar peptide and receptor changes occur in this model as after axotomy and in other nerve injury models, although the immunohistochemical and behavioral changes seem to be dependent on the degree of constriction of the nerve. Thus, it seems relevant to monitor the degree of constriction when evaluating pain and other post-injury events. The possibility that some of the changes in NPY-ergic neurotransmission are related to the generation of allodynia is discussed; as well as the possibility to use this mononeuropathic model based on a single ligature nerve constriction (SLNC) as a complementary approach to other widely used pain models.

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1. Introduction

Chronic constriction injury (CCI) of the sciatic nerve [7] and complete nerve transection [71,72] represent two animal models for the study of pain triggered by peripheral nerve injury. In these models, there are dramatic phenotypic

changes in primary sensory neurons which may be involved in the mechanisms underlying pain after a lesion to the nerve.

Neuropeptide tyrosine (Y) (NPY) is one of the most widely distributed neuropeptides in the mammalian central nervous system (CNS) [4,15,18,19], and is also present in many parts of the peripheral nervous system (PNS) [3,36,47]. NPY like-immunoreactivity (-LI) cannot be detected in dorsal root ganglion (DRG) neurons in normal animals [24,48,53,68,69,82], with the exception of a few small primary sensory neurons and some primary afferent nerve endings in the rat dorsal horn as seen with immunoelectron microscopy [85,87]. In the dorsal horn of the spinal cord, instead, a rich plexus of fibers containing NPY-LI originating from local neurons is observed, especially in laminae I–II [24]. A supraspinal supply of NPY has also been described [26].

Abbreviations: ATF-3, activating transcription factor 3; CCI, chronic constriction injury; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; KO, knock-out; -IR, immunoreactive; -LI, like-immunoreactivity; NP, neuron profile; NPY, neuropeptide tyrosine (Y); PBS, phosphate buffered saline; RT, room temperature; S.E.M., standard error of the mean; SLNC, single ligature nerve constriction; Y1R; NPY Y1 receptor; Y2R; NPY Y2 receptor

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The NPY Y1 receptor (Y1R) is expressed in almost 25% of all DRG neurons, principally in small and some medium-sized neurons [83,84,90]. Most of these neurons also express calcitonin gene-related peptide (CGRP) and often also substance P [83,84]. The NPY Y2R (Y2R) is present mainly in large CGRP-positive DRG neurons [88].

Transection of the sciatic nerve induces increased expression of NPY-LI in neurons in the corresponding lumbar DRGs [23,53,68,69] with parallel changes in mRNA levels both in rat [51] and mouse [16,57]. The increase in NPY-LI occurs principally in medium-sized and large cells, both in rat and mouse, with subsequent increased centrifugal transport of the peptide to the terminals in deeper laminae of the dorsal horn [68,69,85] and the gracile nucleus [38,82]. There is also, to some extent, an increase in NPY-LI in laminae I–II due to upregulation in a population of small DRG neurons [85]. Dorsal rhizotomy prevents the increase of NPY-LI in the dorsal horn [53].

The principal effect of axotomy on Y1R mRNA levels in the DRGs is a marked decrease in small neurons [84,86], whereas an increase in the number of large and small neurons expressing Y2R mRNA has been reported [35,88]. No significant changes in Y1R-LI were seen in the inner part of laminae II of the lumbar dorsal horn 12 days after dorsal rhizotomy [12]. Moreover, peripheral axotomy had little, if any effect on Y1R mRNA [84] or Y1R protein [50] expression in lamina II interneurons [50,84]. Very recent evidence indicates, however, presence of Y1R-LI in axons of DRG neurons, including afferents to the dorsal horn [12].

Using the so-called Bennett model [7], it has been reported that NPY mRNA levels [48,58] as well as NPY-LI [38,45,58,69] increase in large and medium-sized DRG neurons. At the spinal cord level, there is a parallel increase of NPY-LI in laminae I–V [38,40,58,69].

The placement of loose chromic gut sutures around the sciatic nerve of a rat results in thermal and mechanical hyperalgesia, mechano- and cold allodynia, with onset 2 days after surgery and lasting approximately 80 days before a slow recovery occurs [5,7,33,52,56]. Similar results were obtained in mice [59]. Most neurochemical changes in DRGs and spinal cord have been studied 7–28 days after CCI [13,37,38,45,48,62], but at least some changes persist beyond that period [46].

At present, there is some debate about the role of NPY and its receptors in the generation and/or maintenance of pain after peripheral nerve injury. Thus, an analgesic role for the Y1R has been proposed in studies on Y1R knock-out (KO) mice [49]. In contrast, induction of pain in neuropathic rats [74], or biphasic effects in normal and axotomized rats after intrathecal injection of NPY [80] have also been reported.

In the present study, we describe a modified peripheral nerve injury model based on a single constriction of the sciatic nerve. We have analyzed pain behavior and changes of NPY and the Y1R, two markers of mainly large and small

DRG neurons, respectively, both of which have been shown to undergo robust changes after axotomy. For comparison total nerve transection was studied. Changes were monitored both in L4–5 DRG neurons and at the corresponding levels of the dorsal horn of the spinal cord.

2. Methods

2.1. Animals

Experiments were performed on 102 male Wistar and 155 male Sprague–Dawley rats (250–300 g body weight), used for immunohistochemical and behavioral experiments, respectively. The animals were kept under standard conditions on a 12 h day/night cycle (light on 6:00 a.m.) with free access to food and water. All the rats were deeply anaesthetized with pentobarbital (Mebumal; 60 mg/kg., ip.), both before any kind of lesion and transcardiac perfusion.

The experiments performed in this study have been approved by the local Ethical Committee from the Department of Bioethics of the Faculty of Biomedical Sciences of the Austral University (#24/03) and by the local ethical committee in Stockholm (Stockholms Norra Djurförsökseiska Nämnd) (#N206/99).

2.2. Nerve injury model

In 224 rats, the diameter of the right sciatic nerve was reduced by a permanent constriction. The sciatic nerve was exposed at midhigh level and dissected free from the surrounding tissue with sharp microscissors in a 5- to 8-mm-long segment. Then the nerve was wrapped with a thin strip (5 mm long and 5 mm wide) of polyethylene and constricted to varying degrees with a tie around the strip using a 3.0 silk suture (Barbour Threads, Lisburn, Ireland). All constrictions were performed under a dissection microscope (40 × magnification). The animals were divided in three groups according to the degree of the single ligature nerve constriction (SLNC) (Fig. 1): (1) ‘strong’ SLNC with a reduction of around 90% of the original diameter of the nerve, produced by an extreme constriction ($n=51$); (2) ‘medium’ SLNC with a reduction of 40–80% ($n=126$) and (3) ‘light’ SLNC with a 10–30% constriction of the nerve, only producing retardation of the epineural blood supply ($n=47$). Finally, the degree of constriction for each nerve after sacrifice and fixation was estimated under the dissection microscope using a 10 mm ruler, and also by microscopical observation of immunostained sections of the nerve.

In 23 rats, the right sciatic nerve was exposed, strongly ligated and transected distal to the ligation. In all cases, a 5-mm-long segment was resected distal to the transection.

In addition, in some rats the strip of polyethylene and the silk suture were placed surrounding the sciatic nerve without constriction.

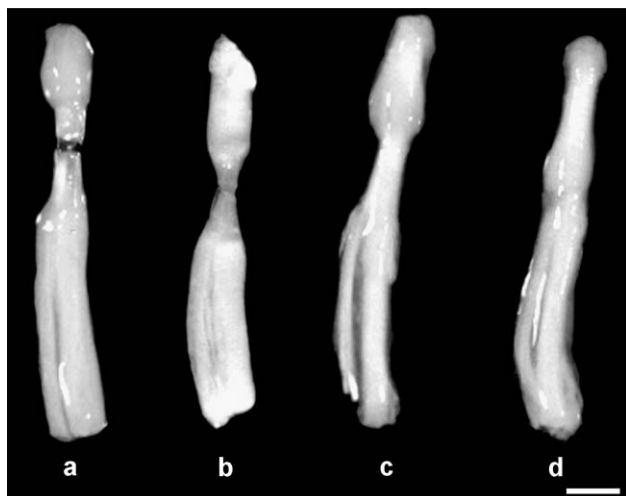


Fig. 1. Photography showing different degrees of constriction of the sciatic nerve (distal side down). From left to right: (a) 'strong' (90%) SLNC; (b) 'medium' (70–80%) SLNC; (c) 'medium' (40–80%) SLNC; and (d) 'light' (10–30%) SLNC. Note that the nerve appears translucent only when the SLNC is close to 90% (b). After 'strong' SLNC, complete transparency at the site of constriction can be seen (a). Calibration bar: 2 mm.

2.3. Immunohistochemistry

2.3.1. Tissue preparation

After different survival times (1, 3, 7, 14, 28 days, 2 and 6 months), lesioned ($n=92$) and control ($n=10$) animals destined for immunohistochemical experiments were re-anesthetized and perfused through the heart with 50 ml of warm (37 °C) Tyrode's buffer, followed by 50 ml of a mixture of 4% paraformaldehyde and 0.2% picric acid in 0.16 M phosphate buffer (PB) (pH 6.9) [54,81] at 37 °C, and 300 ml of the same fixative at 4 °C. The ipsilateral and contralateral sciatic nerve and L4–5 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 then rinsed in 20% sucrose in phosphate buffered saline (PBS) (pH 7.2) containing 0.01% sodium azide (Sigma, St. Louis, MO, USA) and stored until immunohistochemical processing. The tissue was embedded with OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA) and cut in a cryostat (Microm, Heidelberg, Germany) at 16 or 20 μm thickness for the DRGs and the spinal cords, respectively.

2.3.2. Incubation protocol

The sections were mounted onto chrome alum-gelatin-coated slides for immunohistochemical processing following the avidin–biotin complex (ABC) protocol [27]. After rinsing twice in PBS and dehydration, peroxidase was inactivated with hydrogen peroxidase (H_2O_2) (0.5%) diluted in methanol, and this was followed by rehydration and two rinses in PBS. Alternate sections were then incubated in a humid chamber at 4 °C for 24 h with rabbit NPY (Peninsula Laboratories, Belmont, CA, USA) or Y1R [83] polyclonal

antiserum diluted 1:4000 and 1:8000, respectively, in PBS [containing 0.2% bovine serum albumin (w/v), 0.03% Triton X-100 (v/v) and 0.1% sodium azide (w/v)], rinsed twice in PBS, incubated at room temperature (RT) for 60 min with biotinylated goat anti-rabbit secondary antibodies (1:100, Vector Laboratories, Burlingame, CA, USA), rinsed twice in PBS, and incubated in ABC Elite Kit reagents (Vector Laboratories) for 1 h at RT. Peroxidase activity was demonstrated by reaction with H_2O_2 using a nickel-intensified diaminobenzidine protocol for enhancement of the immunoreaction product. After dehydration, the sections were mounted in Permount (Fisher Scientific, Fair Lawn, NJ, USA) and coverslipped.

All sections were examined under bright-field illumination in a Nikon Eclipse E-800 photomicroscope. Technical Pan film (Eastman Kodak, Rochester, NY, USA) was used for photography.

2.3.3. Controls

For control purposes, parallel incubations of sections with NPY and Y1R antiserum (dilutions as above) preadsorbed with, respectively, NPY (Peninsula Laboratories) and Y1R peptide [83] at 10^{-6} M were performed. In addition, sections were incubated with only primary or secondary antibodies and processed as described before.

2.4. Behavioral assessment

Behavioral testing was performed during daytime (9.00–18.00) in 155 Sprague–Dawley rats 1 ($n=23$), 3 ($n=16$), 7 ($n=16$) and 14 ($n=100$) days after unilateral sciatic nerve constriction of varying degree ('strong' SLNC, $n=22$; 'medium' SLNC, $n=106$; 'light' SLNC, $n=27$). The animals were placed in transparent plastic domes (8 × 8 × 18 cm) on a metal mesh floor with a hole size of 3 × 3 mm. After 15–30 min of adaptation, a series of von Frey filaments (0.88, 1.28, 2.7, 5.1, 7.5, 8.8, 13.5, 23 g) (Stoelting, WoodDale, IL, USA) were applied in ascending manner from below to the center of the plantar surface of both hindpaws [14]. Each filament was delivered three times with approximately 5-s intervals. The lowest force, at which each of the three applications of the filament elicited a paw withdrawal, was taken as the mechanical response threshold. A paw withdrawal reflex obtained with 5.1 g force or less was considered as an allodynic response. Autotomy was scored according to a scale from 1 to 11 as described by Wall et al. [72]. After the experiments, the animals were deeply anesthetized using pentobarbital and sacrificed by decapitation.

2.5. Quantification

The number of neurons exhibiting either NPY- or Y1R-LI in L4–5 DRGs was determined by counting immunostained neuron profiles (NPs) under bright-field illumination using a 20 × objective (total magnification,

200×) in randomly, systematically sampled sections throughout the DRG (every 12th section, five to eight sections per ganglion). The total number of NPY- and Y1R-IR NPs counted for each rat were later correlated to the sum of cross-sectional area of the sampled sections (only areas containing cell bodies were considered, i.e. nerve fiber bundles within the ganglia were excluded). The area calculations were performed using a Macintosh II computer (Apple Computer, Cupertino, CA, USA) equipped with a Quick Capture frame grabber board (Data Translation, Marlboro, MA, USA) and a Cagemti 72 CCD series camera (DAGE-MTI, Michigan City, IN, USA) connected to a Nikon microscope. Computerized quantification was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Thus, the number of positive NPs per 100,000 μm^2 area in contralateral and ipsilateral DRGs for each rat was used to obtain a mean \pm standard error (S.E.M.) per group for final statistical analysis.

2.6. Statistics

Differences in the percentage of NPY- and Y1R-IR NPs between the contralateral and ipsilateral side for each treatment group (Axo, ‘strong’, ‘medium’, and ‘light’ SLNCs) at each survival time were tested using the paired Student’s *t*-test. Also, comparison between the different groups at each survival time was performed using repeated measures ANOVA, followed by the Tukey HSD post hoc test, when the latter gave a significant result.

Nonparametric statistics were employed to study the effect on pain behavior. The comparison between contralateral and ipsilateral side in the lesioned rats was analyzed using the Wilcoxon Matched Pairs Test. Treatment effects at each survival time were statistically tested using the Kruskal–Wallis Rank Sum Test. When this test gave a significant result, comparisons among treatments were performed with the Mann–Whitney *U*-test.

The level of significance in all cases is presented as follows: **P*-value (*P*) < 0.05, ***P* < 0.01, ****P* < 0.001.

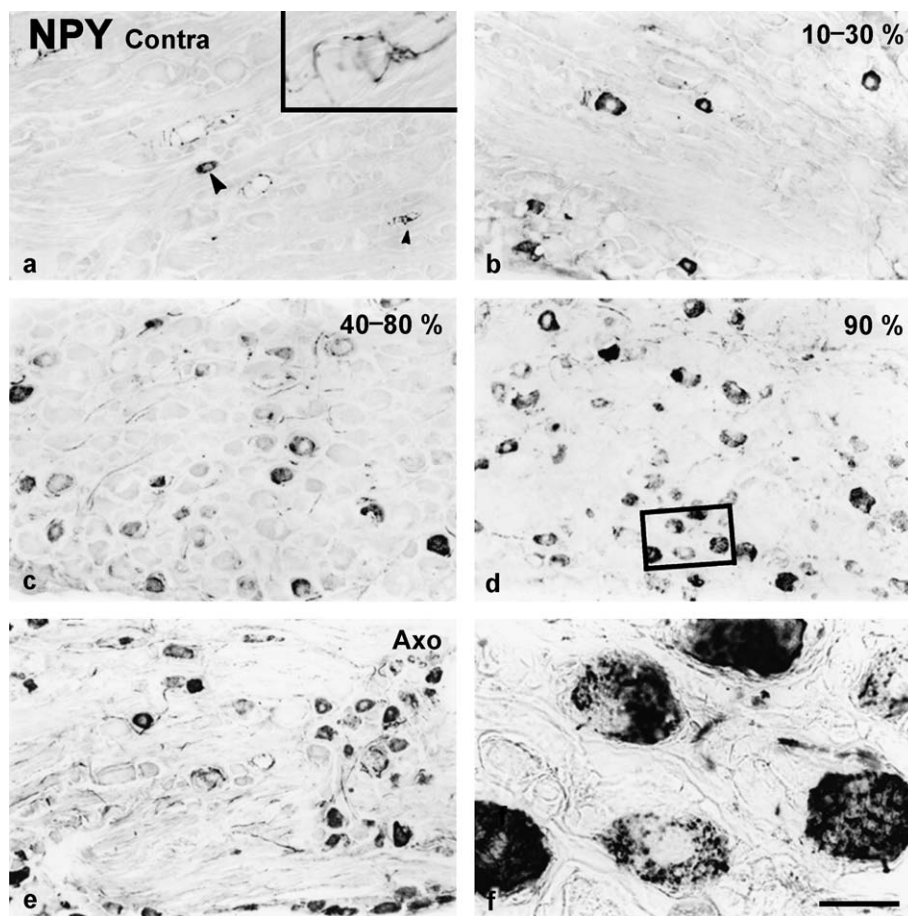


Fig. 2. Photomicrographs of sections immunostained with NPY antiserum showing a contralateral (a) and ipsilateral DRGs 14 days after ‘light’ (b), ‘medium’ (c), or ‘strong’ (d, f) SLNC or axotomy (e). Single NPY-IR neurons can be seen in the contralateral DRG (a, large arrowhead), and several blood vessels are surrounded by NPY-IR nerve fibers (small arrowhead and inset) of probable sympathetic origin. Note the progressive increase in the number of NPY-IR NPs with the higher degrees of nerve constriction. Boxed area in (d) is shown at higher magnification in (f). Calibration bars: 100 μm for (a–e); 25 μm for inset in (a) and for (f).

3. Results

3.1. Sciatic nerve

All constricted nerves showed a reduction in their diameter with a direct correlation to the degree of constriction (Fig. 1). Proximal to the site of constriction swelling was observed (Fig. 1). Only with the ‘strong’ SLNC, and at the site of constriction, complete transparency of the nerve could be seen (Fig. 1a). Interestingly, the “translucence” described for the Bennett model in the zone of multiple constrictions was present in our model when the ‘medium’ SLNC was close to 80% of the diameter of the nerve (Fig. 1b). Lighter SLNCs showed a decrease in the diameter of the nerve without marked translucence (Fig. 1c, d), even after long survival times. All the animals, including those that received the polyethylene and silk thread in a non-constrictive fashion, presented fibrous tissue formation at the site of application, starting at 3 days of survival and onwards.

3.2. L4–5 dorsal root ganglia

Microscopical analysis (Figs. 2 and 3) and subsequent quantification (Fig. 4) of positive NPY- and Y1R-IR NPs showed extensive changes in the expression of these two markers in a lesion degree-dependent manner in different populations of DRG neurons.

3.2.1. NPY-LI

In normal control and contralateral ganglia of operated rats NPY-LI was virtually absent in ganglion neurons, but few immunoreactive NPs could still be identified (Fig. 2a). However, NPY-immunoreactive (IR) perivascular fibers were regularly observed both in contra- and ipsilateral ganglia (Fig. 2a and inset).

All animals subjected to SLNC exhibited an ipsilateral increase in the number of NPY-IR NPs paralleling the degree of constriction, mostly in medium-sized and large neurons but also in a few small ones (Figs. 2b–d, f and 4a). The signal varied from lightly stained cells with a cytoplasmic

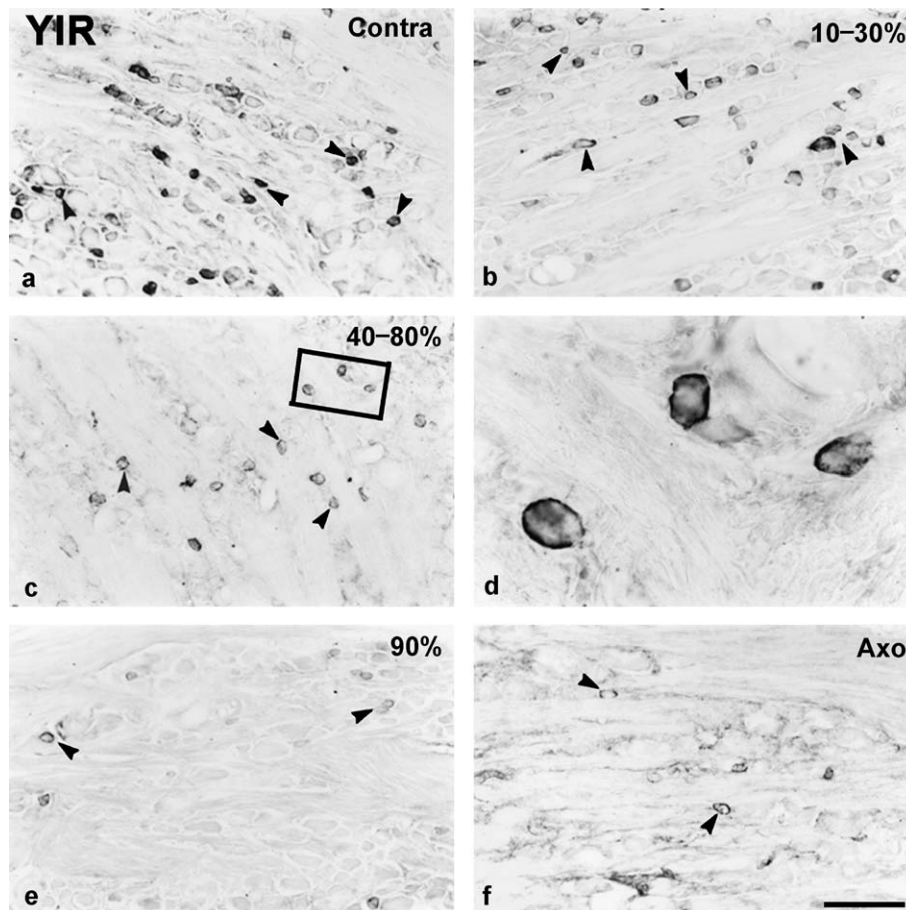


Fig. 3. Photomicrographs of sections immunostained with Y1R antiserum, showing contralateral (a) and ipsilateral DRGs 14 days after ‘light’ (b), ‘medium’ (c) or ‘strong’ (e) SLNC or axotomy (f). Note the decrease in number of Y1R-IR NPs with progressively increased degrees of constriction. Only a few positive cells are seen after axotomy or ‘strong’ SLNC (arrowheads). Boxed area in (c) is shown at higher magnification in (d). Calibration bars: 100 μ m for (a–c) and (e, f); 25 μ m for (d).

mic granular, Golgi-like distribution, to cells completely filled with NPY-LI (Fig. 2f). The increase was noticeable after the first day, especially after axotomy and 90% SLNC (Fig. 4a). From the third day (data not shown) and onwards, the increase was dramatic. Axotomy and the ‘strong’ SLNC reached the highest numbers of NPY-IR NPs at all survival times (Figs. 2d–f and 4a). ‘Medium’ SLNC appeared to induce a considerable increase in positive NPY NPs, with amounts that were similar to axotomy or ‘strong’ SLNC only after 14 days of survival (Figs. 2c, e and 4a). ‘Light’ SLNC was also capable of inducing a small but significant increase in NPY-IR NPs, observed even after 28 days of survival (Fig. 4a).

With long survival times, axotomy and ‘strong’ SLNC maintained increased numbers of NPY-IR NPs, whereas ‘medium’ and ‘light’ SLNCs showed decreasing values. Even after 2 and 6 months, the longest survival time studied, rats with axotomy and ‘strong’ SLNC had consistently higher number of NPY-positive NPs than controls (data not shown).

3.2.2. Y1R-LI

Several Y1R-IR NPs were observed in control L4–5 DRGs as well as in contralateral DRGs of lesioned animals (Fig. 3a). The immunostaining was mainly related to the plasmalemma of small and some medium-sized neurons

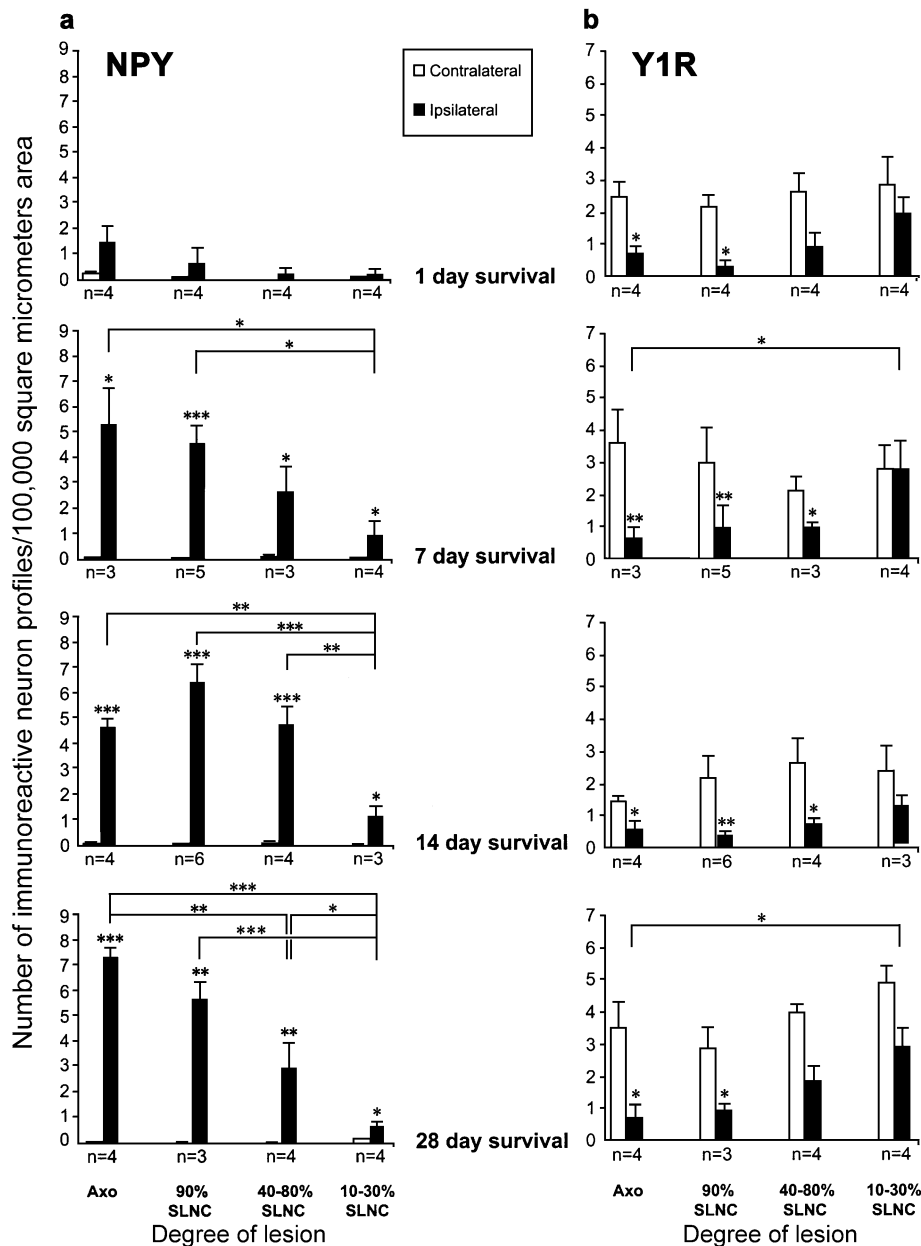


Fig. 4. Number of NPY- (a) and Y1R- (b) IR NPs per unit area in contra- and ipsilateral L4–5 DRGs 1, 7, 14 and 28 days after a ‘light’, ‘medium’ or ‘strong’ SLNC or axotomy. Statistically significant differences are shown at a P -value < 0.05 (*), < 0.01 (**) or < 0.001 (***)

(Fig. 3a). Different SLNCs induced a decrease in the number of Y1R-IR NPs ipsilateral to the affected nerve as compared to the contralateral side at all survival times tested (Fig. 4b), being most pronounced after ‘strong’ SLNC (Figs. 3e and 4b). The ‘medium’ SLNC showed a significant Y1R-LI decrease between 7 and 14 days of survival (Figs. 3c and 4b), whereas ‘light’ SLNC induced the smallest changes compared to the contralateral side (Figs. 3b and 4b). Axotomy and the ‘strong’ SLNC showed a similar decrease in the number of Y1R-IR NPs (Figs. 3f and 4b), and only few immunoreactive NPs persisted after 14 and 28 days of survival (Fig. 3e, f and 4b).

3.3. Lumbar 4–5 dorsal horn

3.3.1. NPY-LI and Y1R-LI

Changes in NPY-LI were detectable in animals subjected to axotomy or a ‘strong’ SLNC, showing an ipsilateral increase in NPY-IR fibers occupying the middle regions of laminae I–IV and starting at 7 days of survival (Fig. 5a, b). With ‘light’ SLNC no changes were observed, whereas ‘medium’ SLNC only produced a small increase after longer survival times (data not shown).

Around half of the rats with ‘strong’ SLNC showed an ipsilateral decrease of Y1R-LI in the entire inner lamina II, especially after 14 days and longer survival times (Fig. 5c, d). No changes were seen after ‘medium’ or ‘light’ SLNCs (data not shown). Axotomy induced a decrease of Y1R-LI in laminae II of the lumbar dorsal horn, comparable to the ‘strong’ SLNC (Fig. 5e, f).

3.4. Controls

None of the staining patterns described above were seen after preadsorption with NPY or Y1R peptide or after omitting either the primary or secondary antibodies.

3.5. Behavioral testing

Signs of spontaneous pain were analyzed at 1, 3, 7 and 14 days of survival. All rats subjected to ‘strong’, several to ‘medium’ but only a few to ‘light’ SLNC showed guarding behavior and changes in the posture of the affected hindpaw, including plantar flexion and toe-clenching. Only rats with axotomized sciatic nerves had signs of autotomy. A single rat exceeded an autotomy score of 8 at 14 days after

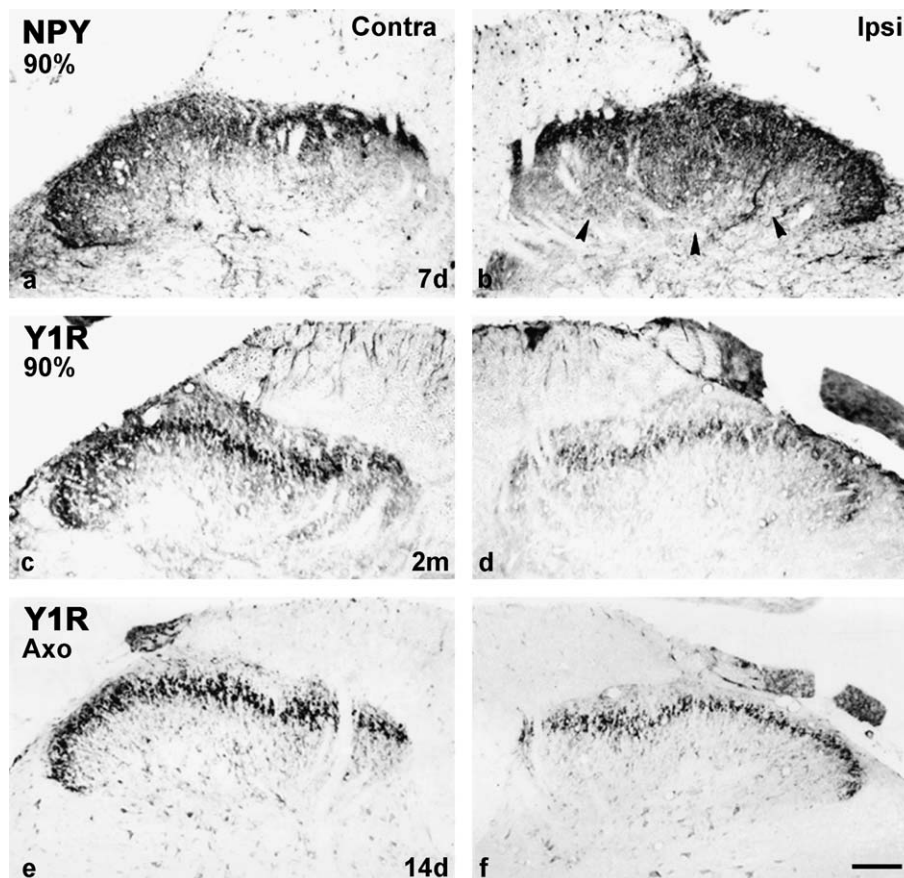


Fig. 5. Photomicrographs of sections immunostained with NPY (a–b) and Y1R (c–f) antiserum, showing the L4–5 levels of contralateral (a, c, e) and ipsilateral (b, d, f) dorsal horn 7 days (a, b) or 2 months (c, d) after a 70–90% constriction or after 14 days of axotomy (e, f). An increase in NPY-LI in the neuropil in the middle region of the deep layers is observed ipsilaterally (arrowheads in b). In contrast, a certain decrease of Y1R-LI can be seen 2 months after ‘strong’ SLNC (c, d) as well as 14 days after axotomy (e, f). Calibration bar: 100 μ m for (a–f).

axotomy, whereas the rest of the axotomized rats showed scores varying from 1 to 8.

Using the von Frey hair test 14 days after lesion, rats subjected to ‘strong’ SLNC showed no allodynia, except for 1 out of 22 rats (Fig. 6a). Virtually all rats with ‘medium’ SLNC ($n=51$) were significantly affected ($p<0.001$). In this group, 67% of the rats developed allodynia (≤ 5.1 g), 25% had a consistent decrease in paw withdrawal threshold without reaching allodynic level (7.5–13.5 g) and 8% showed no reduction in paw withdrawal threshold (≥ 23 g) (Fig. 6a). Only four rats (15%) subjected to ‘light’ SLNC ($n=27$) developed allodynia, while 41% showed a decrease in paw withdrawal threshold and 44% no reduction at all

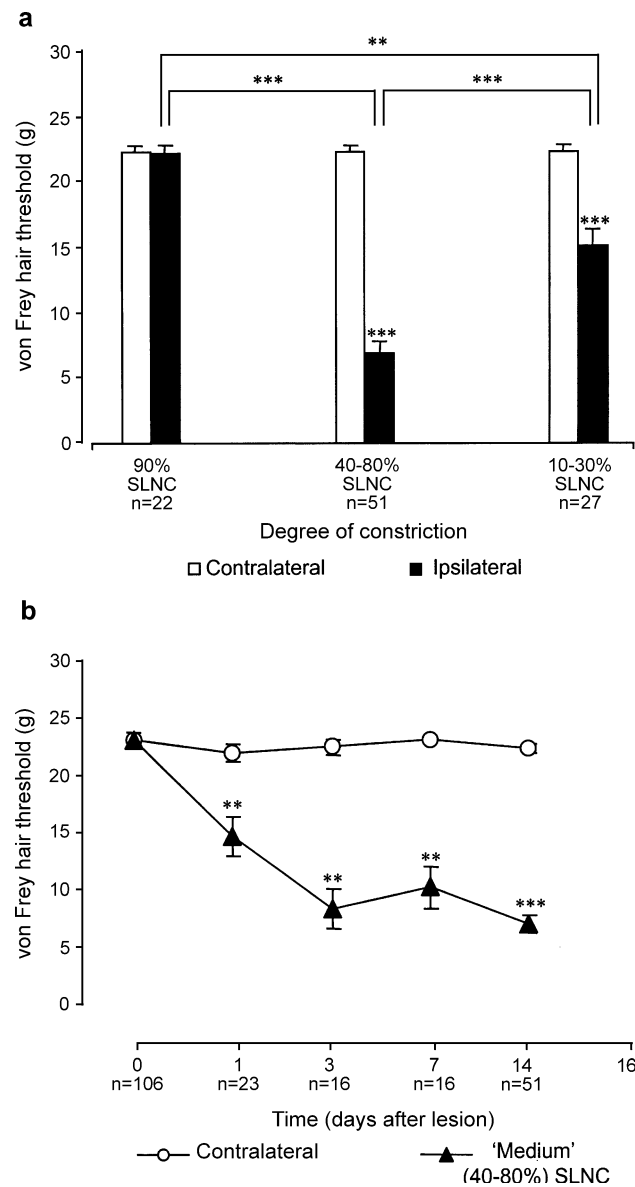


Fig. 6. Effect of SLNC of the sciatic nerve on the development of mechanical allodynia 14 days after different degrees of constriction (a) or on the development of pain behavior 1, 3, 7 and 14 days after a 40–80% constriction (b). Values show mean \pm S.E.M. P -values: <0.01 (**) and <0.001 (***).

(Fig. 6a). Comparison between groups showed statistical significance for ‘strong’ versus ‘medium’ or ‘light’ SLNC ($p<0.001$; $p<0.01$, respectively) and ‘medium’ versus ‘light’ SLNC ($p<0.001$).

Analysis of the effect of the ‘medium’ SLNC on pain behavior over time showed a significant decrease in pain threshold already on the first day ($p<0.01$), with allodynic levels of high significance on day three and onwards (Fig. 6b).

In most rats, the contralateral side, used as a control, did not show signs of affection by the surgical procedures, except for 7 out of 155 rats (5%) that developed a slight decrease in withdrawal threshold and 1 that showed consistent signs of contralateral allodynia (5.1 g).

4. Discussion

The aim of the present study was to explore if a single sciatic nerve constriction can produce similar neurochemical and behavioral changes as seen with four loose ligatures in the widely used Bennett neuropathic pain model [7]. We tested constrictions of varying degrees, with a very thin polyethylene sheath between nerve and ligature. The results show that, especially after a ‘medium’ SLNC (40–80%), mechanical allodynia develops in a similar fashion with regard to time and intensity, as described for the Bennett model in rats [5,7,33,56]. This degree of constriction is, in fact, fairly similar to the 25–75% constriction obtained with the four loose ligatures employed by Bennett and Xie [7]. We also found similar changes in NPY expression in the DRGs, as previously described for CCI (see below). It is therefore possible that the present model, based on a single ‘medium’ constriction (40–80%), can be used as a complementary tool to study various aspects on injured DRG neurons. To what extent changes in the expression of the Y1R (down) and its ligand NPY (up) in the lumbar DRGs and dorsal horn after this kind of lesion are related to a possible involvement of spinal (and perhaps ganglionic) NPY in pain behavior will be discussed below. It should, however, be remembered that NPY and the Y1R only are two of a very large group of molecules which change their expression after peripheral nerve injury [17,73,78].

4.1. Methodological aspects

Many studies on the Bennett model indicate a possible chemical influence of the chromic gut sutures in the generation of pain [41], perhaps by producing proinflammatory changes [41,42,60]. Interestingly, Mosconi and Kruger [44] could not evoke peripheral sensitization when placing very loose polyethylene cuffs around, or polyethylene tubing beside the sciatic nerve, not even after placement of chromic gut suture beside the nerve. However, they did observe generation of pain, when the above-mentioned or other materials such as silk or dextran suture resulted in induction

of CCI [44]. We performed a graded mononeuropathy of the rat sciatic nerve with a single silk 3.0 thread, including a very thin polyethylene sheath surrounding the nerve, mainly in order to avoid rupture of the axons at the site of constriction, especially after ‘strong’ SLNC. The lack of allodynic effects after a ‘strong’ SLNC in comparison to the two other levels of constriction suggests that in this case there is no conduction of mechanical pain information.

We also placed the strip of polyethylene and the silk suture around the nerve without constriction in some animals, but no substantial changes in the expression of the studied markers were observed at 14 days of survival (unpublished data), although fibrous tissue formation was seen as previously described [7,44,56].

An important aspect for our quantitative analysis is to what extent the single ligation causes DRG neuron loss. This has not been calculated; in fact not for any of the mononeuropathic pain models, to our knowledge. However, using the stereology method developed by the Aarhus group [10], it has now been shown that complete sciatic nerve transection at midhigh level in the rat does not cause significant neuronal loss during the first 4 weeks after the lesion [63]. This does not assure that it is the case also in our model. In fact, this may occur, but if so probably only at the longer time periods studied (2–6 months) [cf. 63].

4.2. Effects of different SLNCs on NPY and Y1R expression in primary afferent neurons

In L4–5 DRGs, a distinct increase of NPY-IR NPs was seen after all types of constrictions performed, in accordance with previous studies on axotomy/CCI [38,68,69,82]. The increase was directly related to the intensity of the nerve constriction, with progressively larger numbers of NPY-positive neurons induced by higher degrees of constriction. Only a slight increase in NPY-IR NPs could be detected after a ‘light’ SLNC.

The progressive increase of NPY-LI observed in this study with higher degrees of SLNCs, mainly in large- and medium-sized neurons, may reflect the number and type of affected sciatic nerve fibers. Mosconi and Kruger [44], using polyethylene cuffing with increasing inner diameter to constrict the sciatic nerve, have described that a higher degree of constriction results in a correspondingly higher numerical depletion of myelinated fibers known to arise from large diameter DRG neurons [see 77].

Previous studies have shown a robust decrease in the intensity of the Y1R mRNA signal in small lumbar DRG neurons after sciatic nerve axotomy in the rat [36,84,86], as well as an increase in the number of small neurons expressing the Y1R mRNA at low levels and the appearance of this receptor transcript in some large neurons [84,86]. In contrast, our present results only show a decrease in the number and intensity of Y1R-IR NPs after lesion. It is possible that the Y1R mRNA changes described [84,86] are not translated into protein, or that the immunohistochemical technique

used here is not sensitive enough to show the probably low levels of Y1R protein. Taken together it seems likely that the functionally relevant overall effect of peripheral nerve injury on DRG neurons is a markedly reduced transmission through the Y1R [see 1]. At the same time, the Y2R normally present in small DRG neurons is upregulated in large and small NPs after axotomy [88].

4.3. Axotomy and ‘strong’ SLNC induce changes in the superficial dorsal horn

In the dorsal horn of the spinal cord, we observed an increase of NPY-LI, particularly in the middle regions of layers I–IV, in agreement with several studies based on CCI [38,40,45]. We also observed a decrease of Y1R-LI in the inner part of laminae II, especially after axotomy and ‘strong’ SLNC. To what extent this decrease is related to local Y1R dorsal horn neurons [83,90] remains to be analyzed. However, sciatic nerve cut induced a decrease in μ -opioid receptor-LI in the lamina II of the dorsal horn, probably a transganglionic effect [89]. Also, a decrease in GABA-LI [30] as well as in the GABA synthesizing enzyme glutamic acid decarboxylase (GAD-65 but not GAD-67) was seen in the superficial dorsal horn after CCI of the sciatic nerve [43]. In fact, even transneuronal degeneration has been demonstrated in the superficial layers of the dorsal horn of adult rats after peripheral axotomy [6] or CCI [43,62,75]. Therefore, it is possible that the increased primary afferent activity as well as a reduction of local inhibitory influences induced by a peripheral nerve lesion contribute to an excited state of dorsal horn neurons. Here also a decrease in Y1R-LI could play a role.

Recently the Y1R has been localized at prejunctional sites in several systems [34,55,61,65], and there is evidence that the Y1R is also transported into the axons of DRG neurons [12]. Moreover, peripheral nerve injury induced a significant decrease in [¹²⁵I]NPY binding in the superficial layers of the lumbar dorsal horn, proposed to reflect decreased transport of an NPY receptor [32]. However, the decrease in dorsal horn Y1R-LI observed here after ‘strong’ SLNC and axotomy still seems mainly to reflect an effect on the Y1R interneurons, i.e. a transganglionic effect. Whether this represents neuronal loss or a more functional process, e.g. receptor downregulation or internalization, remains to be analyzed.

4.4. Functional significance

The significance of lesion-induced changes in expression of NPY and its receptors in DRG neurons and the dorsal horn is still obscure, as reflected by the diversity of reports that propose analgesic, biphasic or even pronociceptive functions for NPY. Thus, upregulation of NPY in DRG neurons after peripheral nerve injury may counteract pain, since intrathecal application of high doses of NPY and also of Y1R agonists was able to inhibit the nociceptive flexor

reflex in normal and axotomized rats after initial low-dose facilitation of this reflex [28,79,80]. Furthermore, injection of a Y2R agonist in axotomized rats depressed the flexor reflex [80], possibly through inhibition of Ca^{2+} currents and of depolarization-induced release of substance P, as shown in cultured rat DRGs [9,22,70], and with microprobe technique *in vivo* after direct microinjection of NPY in lamina II [21,25].

In agreement with an analgesic role of NPY, Y1R knockout mice have a markedly reduced mechanical pain threshold [49], suggesting a tonic antinociceptive role for this receptor. Thus, the situation in the Y1R KO mouse is somewhat similar to the present model. However, since the receptor has been deleted at all sites in the KO mouse, including dorsal horn interneurons and presumably projection neurons, this effect may not be exerted only at the level of the DRGs.

There is also evidence for a pronociceptive role of NPY. White [74] has shown that long-term intrathecal administration of NPY or a Y1 agonist, but not a Y2 agonist, exacerbates hyperalgesia induced by nerve injury (Seltzer model), indicating a pronociceptive role for the Y1R. Moreover, there is increased peripheral hyperalgesia after subcutaneous injection of NPY [64], and Y1R dependent enhancement of Ca^{2+} influx in vagal sensory neurons could possibly induce neurotransmitter release [76].

An important question is if the NPY upregulated in large DRG neurons after peripheral nerve lesion mainly has a role in the dorsal horn, or if it can modulate excitability of neuronal cell bodies within the DRGs. Local application of NPY on the cell soma increases the excitability of control DRG neurons via Y2Rs, an effect due to attenuation of Ca^{2+} -sensitive K^{+} -conductance, secondary to the suppression of N-type Ca^{2+} channel conductance [1,2]. In contrast, binding to Y1Rs potentiates L-type Ca^{2+} channel currents, without altering neuronal excitability [1,2]. After axotomy (2 to 7 weeks), the effect on Y2Rs is in fact enhanced and the Y1R activation reduced [1,2], in agreement with the axotomy-induced upregulation of Y2Rs [88] and downregulation of Y1Rs [84,86]. In fact, after axotomy the strong upregulation of NPY [38,68,69] and of Y2Rs [88], along with changes in excitability in large DRG neurons, may lead to NPY release from cell bodies and activation of somatic auto- and/or hetero-Y2 receptors, as suggested by Mantyh et al. (1994). Indeed, release of peptides from DRG [29] and trigeminal [66] cell bodies has been demonstrated, as well as chemically mediated cross-excitation between DRG neurons [20,67], probably mostly related to A-type neurons [20].

5. Concluding remarks

Several previous studies have compared changes in peptide expression in DRG neurons [39,48] and the spinal cord [13,45,46] with pain generation after sciatic nerve

constriction. A similar analysis was performed for the trigeminal ganglia after constriction of the infraorbital nerve [8]. Here we demonstrate that intensity-graded SLNCs of the rat sciatic nerve cause changes of different magnitudes in the expression of NPY and the Y1R in DRG neurons. Moreover, also the pain behavior was dependent on the degree of nerve constriction, ‘medium’ SLNC being the most effective in inducing allodynia-like pain behavior. Both chemical changes and pain behavior were similar to those seen in Bennett model rats, although the effect on the Y1R has not previously been studied using this model.

Our results also suggest that some of the discrepancies in behavior reported in different studies using the Bennett model could be due to the different degree of constriction, as already discussed by others [44,56]. However, the relation between peripheral nerve lesions, the changes generated in primary afferents and the manifestation of pain in rats must be analyzed with caution. Thus, intense pain without important changes in the number of affected large myelinated fibers [11] or hypoalgesia derived from the intense loss of myelinated fibers [31] have been shown in groups of diabetic patients displaying peripheral neuropathy.

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