

The effect of treatment with BRX-220, a co-inducer of heat shock proteins, on sensory fibers of the rat following peripheral nerve injury

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Abstract

In this study, we examined the effect BRX-220, a co-inducer of heat shock proteins, in injury-induced peripheral neuropathy. Following sciatic nerve injury in adult rats and treatment with BRX-220, the following features of the sensory system were studied: (a) expression of calcitonin gene-related peptide (CGRP); (b) binding of isolectin B4 (IB4) in dorsal root ganglia (DRG) and spinal cord; (c) stimulation-evoked release of substance P (SP) in an in vitro spinal cord preparation and (d) nociceptive responses of partially denervated rats.

BRX-220 partially reverses axotomy-induced changes in the sensory system. In vehicle-treated rats there is a decrease in IB4 binding and CGRP expression in injured neurones, while in BRX-220-treated rats these markers were better preserved. Thus, $7.0 \pm 0.6\%$ of injured DRG neurones bound IB4 in vehicle-treated rats compared to $14.4 \pm 0.9\%$ in BRX-220-treated animals. Similarly, $4.5 \pm 0.5\%$ of DRG neurones expressed CGRP in the vehicle-treated group, whereas $9.0 \pm 0.3\%$ were positive in the BRX-220-treated group. BRX-220 also partially restored SP release from spinal cord sections to electrical stimulation of primary sensory neurones. Behavioural tests carried out on partially denervated animals showed that BRX-220 treatment did not prevent the emergence of mechanical or thermal hyperalgesia. However, oral treatment for 4 weeks lead to reduced pain-related behaviour suggesting either slowly developing analgesic actions or enhancement of recovery processes. Thus, the morphological improvement seen in sensory neurone markers was accompanied by restored functional activity. Therefore, treatment with BRX-220 promotes restoration of morphological and functional properties in the sensory system following peripheral nerve injury.

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Introduction

A series of anatomical, morphological and functional changes occur following damage to the peripheral axons of sensory neurones. These changes include the down-regulation of specific markers in different cell populations of the dorsal root ganglia (DRG) and in the axon terminals of these cells in the dorsal horn of the spinal cord. For example,

more than half of the small-diameter cells in the DRG down-regulate the expression of substance P (SP) and calcitonin gene-related peptide (CGRP) soon after injury (Xu et al., 1990). Similarly, another subset of small-diameter DRG cells that can be identified by binding of the lectin isolectin B4 (IB4) from *Griffonia simplicifolia* down-regulates lectin binding because of peripheral nerve injury (Bradbury et al., 1998). The same altered sensory neuron marker expression has also been described in laminae I and II of the spinal cord dorsal horn where these primary afferents terminate (Bennett et al., 1998).

Following peripheral nerve injury, the neuropeptide SP gets down-regulated in small neurones (C fibers) and expressed “de novo” in large sensory neurones (Aβ fibers;

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Noguchi et al., 1995). Thus, we have previously demonstrated “de novo” release of SP in the isolated dorsal horn following electrical stimulation of the attached dorsal roots at A fiber strength and impaired SP release from stimulation of C fibers (Malcangio et al., 2000). These changes in SP release from sensory neurones may contribute towards the development of mechanical allodynia in models of neuropathic pain.

Functional changes that occur following peripheral nerve injury also include increased spontaneous activity of the injured sensory nerve (Boucher et al., 2000), reduced A fiber-conduction velocity (Laird and Bennett, 1992), prolonged electrical response upon stimulation and lowered thresholds to external stimuli (Laird and Bennett, 1993). These electrophysiological changes observed in animals with partially transected sciatic nerve are also accompanied by thermal hyperalgesia, cold and mechanical allodynia, spontaneous pain and dysesthesia (Bennett and Xie, 1988). Partial transection of a spinal nerve is widely used as a model of neuropathic pain (Kim and Chung, 1992). There is some evidence that neuropathic pain is caused by the interaction between degenerating and intact axons. These remaining intact axons of the sciatic nerve transmit afferent inputs to the spinal cord from the receptive field of the injured nerves, causing a pain sensation, which is characteristic for neuropathic pain (Li et al., 2002).

Peripheral neuropathy and neuropathic pain occur in humans as a complication in a number of pathological conditions such as diabetes or traumatic nerve injury (Hansson and Kinnman, 1996). Recently, chronic treatment with Bimoclolmol (Biorex R&D Co., Hungary), a hydroxylamine derivative, has been shown to improve some of the symptoms of peripheral neuropathy in diabetic rats, such as impaired nerve conduction velocities (Biro et al., 1997). There is evidence that Bimoclolmol and its analogues act in conjunction with general cellular defense mechanisms in injured cells by enhancing the synthesis of various cellular chaperones such as hsp70 and hsp90, thereby enabling injured cells to restore normal functions and improving their survival (Vigh et al., 1997). BRX-220 (Biorex R&D Co., Hungary) is a Bimoclolmol analogue that we have successfully used in a recent study of motoneurone degeneration. Following axotomy of the sciatic nerve in neonatal rats, treatment with BRX-220 improved long-term survival and functional recovery of injured motoneurons while enhancing levels of hsp70 and hsp90 in injured spinal cord (Kalmar et al., 2002b). Up-regulation of certain hsp's such as hsp27 and hsp70 has been previously observed following injury to the nervous system (Costigan et al., 1998; New et al., 1989) and has been proven to be protective against injury-induced cell death (Houenou et al., 1996; Kalmar et al., 2002a; Lewis et al., 1999).

In this study, we investigated the potential neuroprotective effect of BRX-220. In a model of sciatic nerve axotomy, we evaluated whether the expression of CGRP and/or the binding of IB4 that are known to down-regulate following

injury in the affected L4 and L5 DRG neurones and in the lumbar spinal cord are altered because of treatment with BRX-220. In addition, in a model of neuropathic pain, in which the L5 spinal nerve is ligated and transected, we investigated whether treatment with BRX-220 ameliorates neuropathic pain and affects the expression of hsp70 in DRG neurones. In addition, using both injury models, the effects of treatment with BRX-220 on the pattern of substance P release was investigated from isolated spinal cord preparations.

Methods

Surgical procedures

Male Sprague–Dawley rats (approximately 200 g body weight) were used in these experiments. All experimental animals were cared for in accordance with guidelines issued by the Home Office, UK. All surgical procedures were carried out under halothane anaesthesia and sterile conditions. In one group of animals, the right sciatic nerve was exposed and cut in the mid-thigh region (axotomy). In the other group, the left L5 spinal nerve was ligated and transected (SNL). All animals recovered without incident.

Treatment with BRX-220

BRX-220 was dissolved in distilled water (2 mg/ml). Animals were treated once daily by gavage (10 mg/kg) for up to 4 weeks. The administered volume did not exceed 2 ml volume. Vehicle-treated animals received sterile distilled water at the same volume and route as drug-treated animals.

Immunostaining for sensory neurone markers

Following axotomy and treatment with either BRX-220 or water, the animals were examined 2 or 4 weeks later. The rats were anaesthetized with pentobarbitone and perfusion-fixed through the aorta first with saline followed by 4% paraformaldehyde (PFA). The lumbar region of the spinal cord and the L4 and L5 DRGs were removed, postfixed in 4% PFA for 3 h. Tissues were then kept overnight in cryoprotectant containing 30% sucrose in 0.1% phosphate buffer. Serial transverse spinal cord sections (20 μ m) were cut on a cryostat. In order to visualize both IB4 binding and CGRP in each section, the sections were first stained for isolectin B4 (IB4) using biotinylated IB4 (Sigma, UK; 5 μ g/ml) and then immunostained for calcitonin gene-related peptide (CGRP) using a polyclonal antibody raised in sheep (Affiniti Research Products Ltd, UK; 1:2000). DRG samples were cryostat cut serially at 10 μ m and double stained for either IB4/ β III tubulin or CGRP/ β III tubulin. β III tubulin staining was carried out using a mouse monoclonal antibody (Promega, Southampton, UK; 1:500) to visualize neurons in DRGs. All reagents were diluted in 0.1 M phosphate-buffered saline

(PBS) containing 0.2% Triton X-100 and 0.1% sodium azide. The same solution was used for washes. After incubation in 10% normal donkey serum, sections were exposed to the primary antibodies overnight. After washing, secondary antibodies were applied for 2 h as follows: for spinal cord sections, FITC-conjugated Extra-Avidin (Sigma; 1:500) and a TRITC-conjugated anti-sheep antibody (host: donkey, Jackson ImmunoResearch, West Grove, PA; 1:400) were used. In DRG sections, an AMCA-conjugated anti-mouse antibody (host: donkey, Jackson ImmunoResearch; 1:200) was combined with either FITC-labeled Extra-Avidin or TRITC-conjugated anti-sheep antibody. After a final wash, the slides were mounted in glycerol–PBS, coverslipped and viewed with a Leica fluorescence microscope with a standard filter set at 10× (spinal cord sections) and 20× (DRG sections) objective magnification.

Analysis of immunostained sections

In each group, tissues obtained from at least four animals were analyzed. For each animal, at least six sections were counted with at least 50- μ m separation between analyzed sections. The number of DRG neurons was established by counting the number of cells stained for β III tubulin. In the same sections, the number of cells with strong CGRP or IB4 staining was also counted. Only cells with strong staining were included in the counts. Thus, bright, intensely fluorescent cells, which were easy to distinguish from background staining, were counted. In each experimental group, the mean number of IB4 or CGRP positive cells is expressed as a percentage of the total number of DRG cells.

Spinal cord sections from the L4–L5 segmental level of vehicle and BRX-220-treated, axotomized animals were examined. Sections were viewed using a 10× objective under a Leica fluorescent microscope and images were captured using a JVC color digital camera. Images were analyzed using a Sigma Scan program for measuring pixel densities across dorsal laminae I–IV in the dorsal horns. In each section, two areas were analyzed for optical density measurements. A “box” measuring 100 pixel width and 300 pixel long was drawn around (a) the area of the dorsal horn in which sciatic sensory fibers normally terminate and (b) a control area of the same dorsal horn in which non-sciatic fibers terminate. An example of these areas is shown in Fig. 1A. The same areas (a and b) were selected for each section analyzed for optical density measurement. All data were normalized against the background pixel density. In each section, approximately 200 optical density measurements were made along the length of each box, into the dorsal horn laminae I and II. This is the area that IB4 and CGRP staining is specifically present (see Fig. 1A). For both vehicle- and BRX-220-treated animals, in each section, these optical density measurements obtained from the sciatic area (a) were expressed as a ratio of the maximal optical density measurement taken from non-sciatic area (b)

of the dorsal horn. Staining in the non-sciatic area acts as a control as it will not be affected by injury to the sciatic nerve.

Release of endogenous substance P from the dorsal horn of the rat spinal cord

The release of SP from the dorsal horn of the spinal cord was assessed in the following groups of animals: (a) unoperated control, (b) vehicle- or BRX-220-treated, axotomized animals 2 weeks post-injury and (c) vehicle- or BRX-220-treated, spinal nerve-lesioned (SNL) animals 4 weeks post-injury. Rats were euthanized and the lumbar enlargement of the spinal cord excised for SP release experiments. The lumbosacral segment of the spinal cord was longitudinally hemisected using a Vibratome (Camden Instruments, UK), as previously described (Malcangio and Bowery, 1993; Malcangio et al., 1997a,b). Dorsal horn slices (400 μ m thick) with two dorsal roots attached (L4 and L5) were mounted in three-compartment chambers. The slices were continuously superfused with oxygenated (95% O₂ and 5% CO₂) Krebs' solution (in mM: NaCl, 118; KCl, 4; MgSO₄·7H₂O, 1.2; KH₂PO₄ 1.2; NaHCO₃ 25; CaCl₂ 2.5 and glucose 11) at 1 ml/min at room temperature. The dorsal roots were sealed across a leak-proof partition of high vacuum grease (Dow Corning Corp., Midland, MI, USA) into the lateral compartments. The spinal roots were placed onto pairs of bipolar platinum electrodes and the compartments filled with mineral oil to avoid dehydration (Aldrich, Milwaukee, WI, USA). After 1-h equilibration period, normal Krebs' solution was substituted with modified Krebs' solution containing 0.1% bovine serum albumin (BSA), 100 μ M captopril, 1 μ M phosphoramidon, 20 μ g/ml bacitracin and 6 μ M dithiothreitol (Sigma) to prevent SP enzymatic degradation and oxidation. After 15 min, samples of perfusates (8-ml fractions) were collected in acetic acid (0.1 M) as follows: three fractions to assess SP basal outflow before electrical stimulation of the dorsal roots; one fraction during stimulation of the dorsal roots; three fractions after stimulation to assess SP recovery to basal values. The effect of injury and treatment with BRX-220 on the evoked SP release was measured by stimulating the injured nerves in the ipsilateral side. Thus, when spinal cords from axotomized rats were used, ipsilateral L4 and L5 dorsal roots were stimulated. When using spinal cords of SNL animals, only the ipsilateral L5 dorsal root was stimulated. Roots were stimulated at A β fiber strength (5 V, 0.1 ms, 1 Hz for 8 min) during the first stimulation period. After a 24-min interval, the same roots were stimulated at C fiber strength (20 V, 0.5 ms, 1 Hz for 8 min) (Malcangio et al., 2000). Samples were partially purified and desalted using 100 mg Sep-Pak C₁₈ reverse-phase silica gel cartridges (Waters Associates, UK). Cartridges were first washed with 3 ml acetonitrile (100% HPLC grade) followed by 2 ml of trifluoroacetic acid 0.1% (TFA). Samples were then loaded into the column followed by 2 ml TFA.

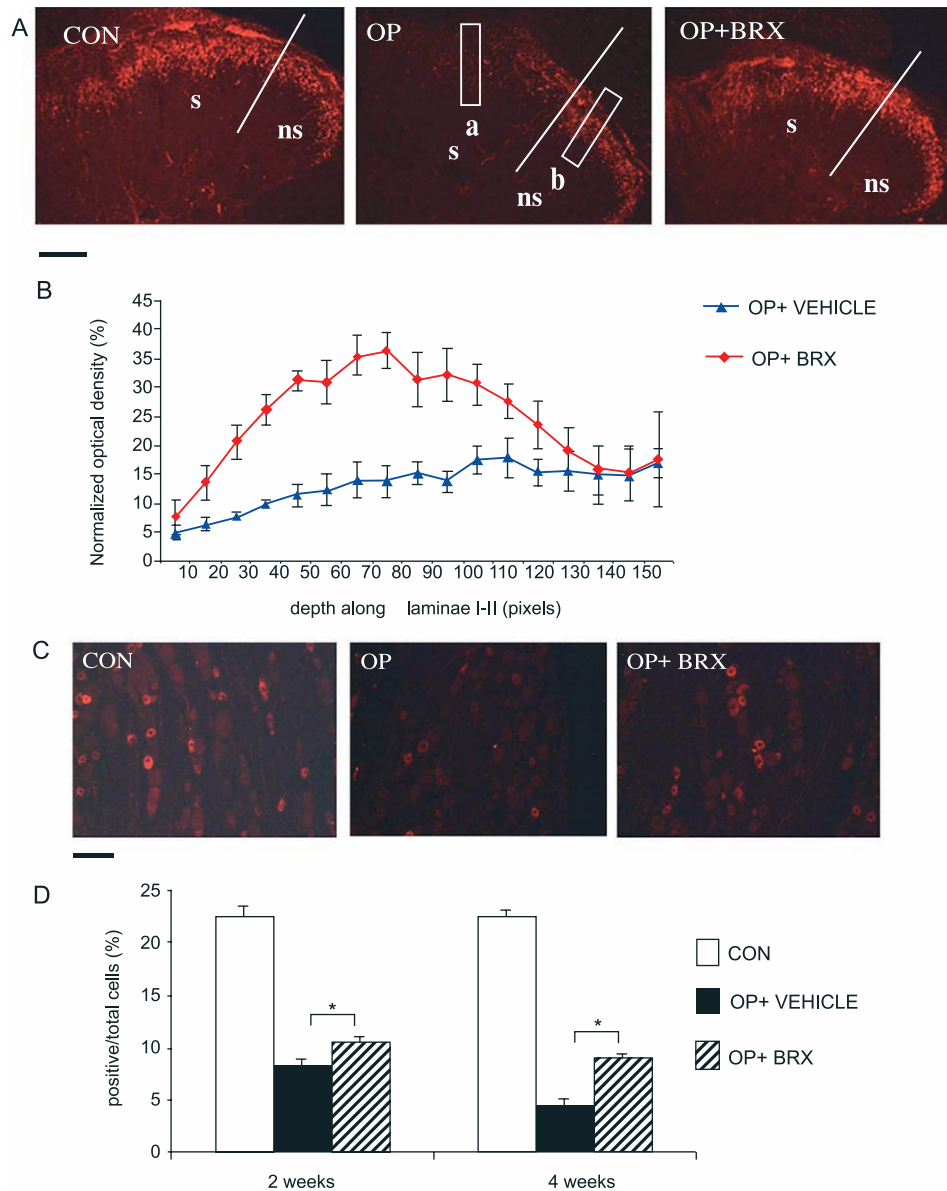


Fig. 1. Calcitonin gene-related peptide (CGRP) immunoreactivity in spinal cord and DRG following axotomy of the sciatic nerve and treatment with BRX-220. (A) Sections of the dorsal horn stained for CGRP immunoreactivity obtained from control (CON), axotomized, vehicle-treated (OP) and axotomized, BRX-220-treated rats (OP + BRX). The sciatic (s) and non-sciatic (ns) areas are delineated in each image by the line. The territory of the sciatic nerve is represented by box “a” and the non-sciatic, uninjured nerve terminal region by box “b”. Scale bar = 200 μ m. (B) The intensity of CGRP immunoreactivity within the dorsal horns of operated, vehicle-treated (OP + VEHICLE, $n = 4$) and operated, BRX-220-treated (BRX-220, $n = 6$) animals 4 weeks after injury was quantified. Optical density measurements taken from unoperated controls was taken as 100%. The graph summarizes the mean (\pm SEM) optical density readings obtained from sections such as those shown in (A), from operated, vehicle-treated (blue trace) and operated, BRX-220-treated (red trace) rats. In each case, optical density readings were taken from the operated sciatic region (box “a”) and the non-sciatic region (box “b”) as delineated in (A). Optical density measurements were taken from each box at 140–200 points into the dorsal horn. Each measurement taken from the sciatic region (box a) was normalized against the maximal optical density reading obtained from the non-sciatic region (box b) in the same section and plotted as a function of depth in the dorsal horn shown in pixels. Error bars = SEM ($P < 0.05$, two-way ANOVA on repeated measures). (C) Sections of L4 DRG from control (CON), operated, vehicle-treated (OP) and operated, BRX-220-treated (OP + BRX) rats 4 weeks after axotomy and stained for CGRP immunoreactivity. Scale bar = 100 μ m. (D) From sections such as those shown in (C), the total number of CGRP immunoreactive DRG neurones in DRGs from control (CON, $n = 5$), operated, vehicle-treated (OP, $n = 4$) and operated, BRX-220-treated (OP + BRX, $n = 6$) rats was established 2 and 4 weeks after injury. In each case, at least six sections from each DRG were analyzed. In all sections, only bright, strongly immunoreactive neurones were counted ($*P < 0.01$, Mann–Whitney test).

Peptides were eluted using 1 ml of 80% acetonitrile in 0.1% TFA (recovery 90%). The eluates were dried by evaporation at 55 $^{\circ}$ C under nitrogen and stored at -70° C until they could be assayed for SP-LI content by radioim-

unoassay (1 fmol/tube sensitivity) using the scintillation proximity assay (Amersham, UK) as described previously (Lewis et al., 1999; Li et al., 2002; Malcangio and Bowery, 1993). All samples of an experiment were processed

simultaneously in the same assay. Rabbit antiserum against SP was reactive against the whole SP undecapeptide but showed no reaction with N-terminal fragments and neurokinin A or B (Amersham).

Behavioural testing

Six groups of rats including control, unoperated, sham-operated and spinal nerve-lesioned (SNL) animals, treated with either BRX-220 or vehicle, were used. In each group, at least five animals were included. Behavioural tests were conducted every third day over a 25-day period. Prior to surgery, all animals were trained three times for each test. At the time of testing, the experiments were carried out blind in regard to the treatment and surgery.

Hargreaves test

The latency of hind paw withdrawal to a noxious thermal stimulus was quantified using the method of Hargreaves et al. (1988). Briefly, before testing, each rat was placed in a clear plastic chamber with a glass bottom and allowed to acclimatize for 30 min. A radiant heat source was then placed directly beneath each hind paw in turn, and the latency of paw withdrawal was calculated to the nearest 0.1 s (Plantar test, Ugo Basile, Italy). The stimulus was applied to each paw three times, each trial being separated by at least 5 min. The average paw withdrawal latency for each animal across trials was noted for each day of testing.

Von Frey test

Mechanical threshold was ascertained using a mechanical plantar test apparatus (Ugo Basile). Animals were placed in Perspex enclosures over a mesh floor. Mechanical force (max: 30 g, ramp 20 s) was exerted in the middle of the hind paw using a fine metal filament. Time and force were measured when the withdrawal reflex could be observed. On each experimental day, both hindlimbs were tested three times with at least 3-min intervals between measurements.

Noxious cold

The response to noxious cold was assessed by placing the animal in a 1-cm-deep, 1°C water bath (adapted from Fisher et al. (1998)). In order to habituate to water, animals were first placed in a Perspex chamber with perforated floor into 1-cm-deep, 28°C water for 1 min. The test chamber was then placed into same amount of 1°C water. On the operated hindlimb the frequency of reactions (lifting, shaking) was recorded over a period of 60 s.

Western blot analysis

Western blot analysis of hsp70 was carried out in control, unoperated and injured DRGs from operated, saline-treated rats ($n = 6$) and operated, BRX-220-treated

rats ($n = 6$) 2 weeks following sciatic axotomy. Each experimental group was divided into two sets of samples containing pooled tissue obtained from three animals. Each blot was repeated at least twice.

The rats were deeply anaesthetized (4% chloral hydrate, ip) and the L4 and L5 DRGs were removed from both unoperated and operated sides and collected separately. Tissue samples were pooled from three animals in each group. Groups included control, unoperated untreated DRGs, operated untreated DRGs, unoperated, BRX-220-treated control DRGs and operated, BRX-220-treated DRGs. Tissue samples were homogenized in homogenizing buffer (5 mM Tris, 2% SDS, 2 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonylfluoride (PMFS), 10 μ M leupeptin pH 6.8). Samples were spun at 14000 rpm for 10 min and the supernatant was collected. Protein concentration was determined using a BIO-Rad assay system (Cat#: 500-0116) and a series of BSA standards. Samples were boiled 5 min before SDS polyacrylamide gel electrophoresis (PAGE). Equal amounts of 15 μ g protein was loaded and separated at 200 V for 55 min. Proteins were electrophoretically transferred to Hybond™ ECL™ nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dried milk and 0.1% Triton X-100 in PBS for 1 h at room temperature. A primary antibody for hsp70 was used that recognizes both constitutive hsc70 and inducible hsp72 (Santa Cruz Biotechnology, mouse monoclonal, Cat#:sc-24) at a dilution of 1:1000 in PBS containing 5% dried milk, 0.1% Triton X-100 for 1 h at room temperature. After three washes in PBS containing 0.1% Triton X-100 for 15 min each, membranes were incubated in HRP-conjugated anti-mouse (Dako Cat#: P0260) and anti-rabbit (Dako Cat#: P0217) immunoglobulines for staining for hsp70 and hsp90, respectively. These antibodies were used at a dilution of 1:1000 in PBS containing 5% dried milk and 0.1% Triton X-100 for 1 h. After three washes in PBS containing 0.1% Triton-X100 for 15 min each, membranes were treated with a chemiluminescent detection reagent (ECL™, Amersham Pharmacia Biotech). Blots were visualized using Kodak Films and fixation. Each western blot contained samples from three animals in each group and each sample was run on at least two blots. On each blot, equal protein quantities of brain homogenates were run next to the samples as standards.

Data calculation and statistical analysis

Statistical analysis on the data of markers in the spinal cord was performed using two-way ANOVA on repeated measures. Substance P release and behavioural test data were analyzed using the Mann–Whitney *U* test for comparison of independent samples. Two-tailed tests were used in all instances, and significance level was set at $P < 0.05$. Significance levels are marked as follows: * $P < 0.05$; ** $P < 0.05$; *** $P < 0.001$.

Results

CGRP immunoreactivity in L4 or L5 DRG and spinal cord sections

CGRP is normally strongly expressed within laminae I and II of the dorsal horn, where some primary afferent fibers terminate (see Fig. 1A). Following axotomy of the sciatic nerve, CGRP immunoreactivity sharply declines within the nerve termination territory in the dorsal horn (Fig. 1A). Thus, because of sciatic nerve injury, peak staining intensity is only about 10–20% of the staining intensity of CGRP immunoreactivity in the non-sciatic area of the dorsal horn (see Fig. 1B, blue trace). However, following treatment with BRX-220, the pattern and intensity of CGRP staining closely resembled that observed in the normal unoperated dorsal horn (Fig. 1A). This effect was rather modest 2 weeks after nerve injury but became significant at 4 weeks. A comparison was made between the maximal normalized optical density readings obtained from vehicle-treated and BRX-220-treated axotomized rats. In rats treated with BRX-220, the maximal value of the normalized optical density of CGRP staining within the region of the dorsal horn where primary afferents of the sciatic terminate was $36.4 \pm 3.1\%$ of the same region in the control, non-sciatic area of the spinal cord (see Fig. 1B, red trace). In vehicle-treated rats, the maximal normalized staining intensity in the sciatic area of the dorsal horn on the operated side was only $13.9 \pm 2.6\%$ of that of the control area of the dorsal horn (see Fig. 1B, blue trace). Comparing the complete traces obtained from readings along the selected areas it becomes apparent that in spinal cords of vehicle-treated animals the characteristic peak in CGRP staining that marks the specifically stained spinal cord layer is completely abolished, while it is partially restored in BRX-220-treated animals (Fig. 1B). This improved staining pattern was significantly different from that of vehicle-treated animals ($P < 0.05$ two-way ANOVA on repeated measures on the whole series of optical density measurements).

These changes in dorsal horn were reflected in the protein levels seen immunohistochemically in the cell bodies of sensory neurones in the DRG. We established the total number of DRG neurones present in our sections by counting the β III tubulin positive cells. The number of strongly CGRP immunoreactive DRG neurones was also counted and the proportion of CGRP positive neurones was calculated. We found that CGRP was clearly observed in a subset of small-diameter cells with $22.5 \pm 0.8\%$ of the total number of DRG neurones staining strongly positive (L4 and L5 DRGs; Figs. 1C and 1D). In those animals in which the sciatic nerve was axotomized, significantly fewer DRG neurones were CGRP positive and only $8.2 \pm 0.7\%$ of DRG cells stained for CGRP 2 weeks after injury (Fig. 1D). Treatment with BRX-220 after axotomy prevented some of the axotomy-induced loss of CGRP immunoreactivity. Two weeks after injury, in BRX-220-treated animals, $10.6 \pm$

0.4% of DRG neurones were CGRP positive ($P < 0.01$). Furthermore, in BRX-220-treated animals there was no further significant loss of CGRP staining so that 4 weeks after injury $9.0 \pm 0.3\%$ of cells were strongly CGRP positive compared to only $4.5 \pm 0.5\%$ in vehicle-treated DRGs ($P < 0.003$; see Figs. 1C and 1D).

IB4 isolectin binding in L4 and L5 DRGs and spinal cord sections

As shown in Fig. 2A, in the spinal cord, IB4 binding is normally found within the inner lamina II of the dorsal horn. However, 2 weeks after axotomy to the sciatic nerve, IB4 staining is markedly decreased in the sciatic nerve territory of the dorsal horn and remains so for at least 4 weeks after injury (Fig. 2A). Treatment with BRX-220 prevents this reduction in IB4 binding in the dorsal horn (Fig. 2A). Optical density measurements of IB4 binding in the sciatic nerve territory of the operated dorsal horn were taken at various points into the dorsal horn laminae I and II and each measurement was normalized against the optical density of the non-sciatic area of the dorsal horn. The results are shown in Fig. 2B. As can be seen in Fig. 2B, in contrast to normal, unoperated control animals, the normalized optical density trace of vehicle-treated animals does not display a peak that is normally seen because of specific staining for IB4 present in the inner lamina II of the spinal cord opposing nonspecific and lighter staining intensities in the surrounding non-IB4 positive areas. This lack of staining and peak intensity seen in injured vehicle-treated animals implies a complete abolition of specific staining in the sciatic region of the dorsal horn (blue trace). Although 2 weeks after injury BRX-220 treatment did not significantly increase IB4 staining in the operated dorsal horn, by 4 weeks, the IB4 staining profile in BRX-220-treated spinal cords was significantly more intense than in vehicle-treated rats, with a peak in the normalized optical density measurements. These results indicate that BRX-220 treatment restores specific IB4 staining within the sciatic region (Fig. 2B, red trace; $P < 0.01$ two-way ANOVA on repeated measures on the whole series of optical density measurements). In normal, unoperated animals, the relative optical density of the sciatic area to non-sciatic area is 100%. A comparison of the maximum optical density measurement obtained in BRX-220- and vehicle-treated rats shows that maximal staining intensity of the sciatic region in BRX-220-treated rats is $43.7 \pm 5\%$ of that in the non-sciatic area of the dorsal horn, compared to only $19.5 \pm 5.4\%$ in vehicle-treated rats.

In DRG sections obtained from operated, vehicle-treated and operated, BRX-220-treated animals, we established the total number of DRG neurones present in our sections by counting the β III tubulin positive cells. The number of strongly IB4 positive DRG neurones was also counted and the proportion of IB4 positive neurones was calculated. Following axotomy of the sciatic nerve, in vehicle-treated rats, the proportion of DRG neurones that stain intensely for

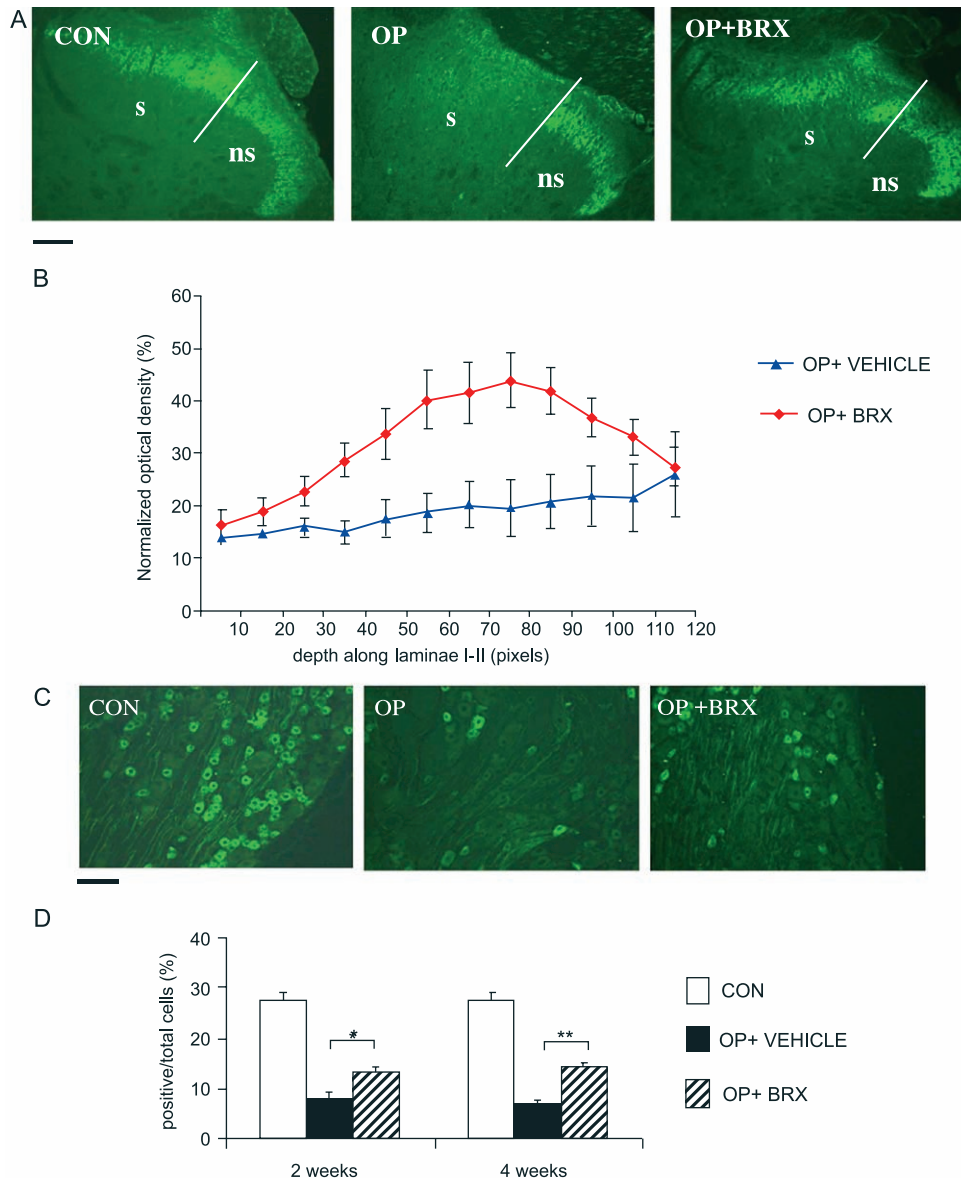


Fig. 2. Isolectin B4 (IB4) binding in spinal cord and DRG following axotomy and treatment with BRX-220. (A) Dorsal horn sections stained for IB4 binding in control (CON), axotomized, vehicle-treated (OP) and axotomized, BRX-220-treated rats (OP + BRX). In each case, the sciatic (s) and non-sciatic (ns) areas are delineated by the dotted line. Scale bar = 200 μ m. (B) From sections such as those shown in (A), the intensity of IB4 staining in the sciatic and non-sciatic regions of the dorsal horn of operated, vehicle-treated ($n = 4$) and operated, BRX-220-treated rats ($n = 6$) 4 weeks after injury was quantified. Optical density measurements taken from unoperated controls was taken as 100%. Analysis was carried out as described in Fig. 1D. Thus, boxes “a” (sciatic) and “b” (non-sciatic) as shown in Fig. 1C were applied in each section to determine control and operated pixel density values for each section. The graph shows mean \pm SEM of normalized intensity of IB4 staining as a function of depth in the dorsal horn ($P < 0.01$, two-way ANOVA on repeated measures). (C) Sections of L4 DRG from control (CON), operated, vehicle-treated (OP) and operated, BRX-220-treated (OP + BRX) rats 4 weeks after axotomy and stained for IB4. Scale bar = 100 μ m. (D) From sections such as those shown in (C), the number of DRG neurons that stain for IB4 in DRGs from operated, vehicle-treated ($n = 4$) and operated, BRX-220-treated ($n = 6$) animals 2 and 4 weeks after injury was established ($*P < 0.05$; $**P < 0.001$, Mann–Whitney test).

IB4 falls from $27.8 \pm 3.1\%$ to $8.1 \pm 1.5\%$ within the first 2 weeks of injury (Figs. 2C and 2D). By 4 weeks, the number of IB4 positive cells drops slightly further, to $7.0 \pm 0.6\%$. Treatment with BRX-220 partially restores the normal IB4 cell profile by maintaining IB4 binding, and 2 weeks after injury $13.2 \pm 1.1\%$ of DRG neurones are strongly IB4 positive ($P < 0.05$; Figs. 2C and 2D). This significant improvement in IB4 positive cell profiles in BRX-220-treated rats is maintained in the long-term and 4

weeks after injury $14.4 \pm 0.9\%$ of DRG cells are IB4 positive ($P < 0.001$; Fig. 2D).

Effect of treatment with BRX-220 on the release of endogenous SP in the dorsal horn following dorsal root stimulation

In both normal, unoperated control and axotomized rats, stimulation of the dorsal roots at A fiber strength did not

evoke SP release above basal levels in the dorsal horn (Fig. 3A). Furthermore, basal SP release, which ranged from 10 to approximately 25 fmol between animals, was not altered by BRX-220 treatment (Fig. 3A). In addition, our results also confirmed previous observations (Malcangio et al., 2000) that 4 weeks after L5 spinal nerve lesion, stimulation of L5 dorsal root at A fiber strength induces a significant increase in SP release above basal levels, which is 1.72 ± 0.12 times greater than basal levels (Fig. 3B). However, this “de novo” release of SP following A fiber stimulation was significantly inhibited by treatment with BRX-220, and SP release was only 1.2 ± 0.09 times above basal SP outflow (Fig. 3B). The results summarized in Figs. 3C and 3D show that high voltage stimulation of the dorsal roots, recruiting both A and C fibers, in sham-operated control animals results in an elevation in SP release that is 2.78 ± 0.6 times greater than basal SP release (Figs. 3C and 3D). Following axotomy and treatment with vehicle for 2 weeks, high voltage stimulation of the injured L4 and L5 spinal nerves caused only a slight increase in SP release, which

was only 1.3 ± 0.2 times greater than basal SP outflow (Fig. 3C). Treatment with BRX-220 partially restored the responsiveness of injured dorsal horns to A + C fiber stimulation and 2 weeks after axotomy, evoked SP release was 1.96 ± 2.5 times greater than basal values (Fig. 3C). This is a significant improvement compared to vehicle-treated spinal cords ($P < 0.05$; Fig. 3C). A similar significant functional improvement was also observed in spinal cords of L5 spinal nerve-lesioned, BRX-220-treated animals. Thus, 4 weeks after injury, in BRX-220-treated spinal nerve-lesioned rats, SP release was 1.46 ± 0.07 times greater than basal levels, whereas in vehicle-treated rats, SP release did not reach basal levels and was only 0.9 ± 0.15 times the basal outflow ($P < 0.05$; Fig. 3D).

Effect of treatment with BRX-220 on nociceptive responses following L5 spinal nerve ligation and transection

Following unilateral L5 spinal nerve ligation and daily treatment with either vehicle or BRX-220, changes in

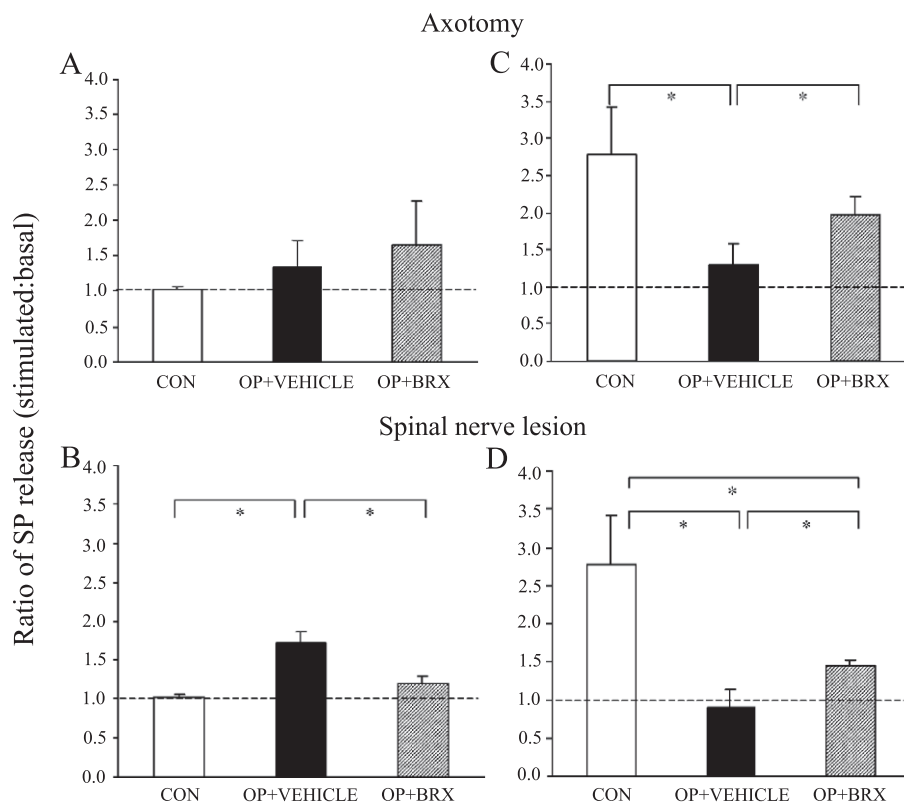


Fig. 3. Effect of treatment with BRX-220 on the release of SP from dorsal horn sections of the spinal cord following electrical stimulation of low-threshold A fibers and low- and high-threshold C fibers. Vehicle or BRX-220 (10 mg/kg) was administered daily by gavage ($n = 5$ in each group). The histograms show the ratio between SP content in superfusates collected during stimulation and superfusates collected in three fractions before stimulation. In each histogram, the basal levels of SP release are represented by dotted line (ratio 1.0). (A) The ratio of SP release in dorsal horn of sham-operated, untreated animals (CON), axotomized rats treated with vehicle (OP) and axotomized treated with BRX-220 (OP + BRX) upon stimulation of A fibers of the injured L4 and L5 spinal nerve at 2 weeks after injury. (B) The ratio of SP release in the dorsal horn of sham-operated (CON), L5 spinal nerve-lesioned, vehicle-treated (OP) or BRX-220-treated rats (OP + BRX) upon stimulation of A fibers in the injured L5 spinal nerve 4 weeks after surgery ($*P < 0.05$; Mann–Whitney test). (C) The ratio of SP release in the dorsal horn of sham-operated, untreated animals (CON) and axotomized animals treated with vehicle (OP) or BRX-220 (OP + BRX) upon stimulation of the A + C fibers in the injured L4 and L5 spinal nerve ($*P < 0.05$; Mann–Whitney test) 2 weeks after injury. (D) The ratio of SP release in the dorsal horn of sham-operated (CON), spinal nerve-lesioned animals treated with either vehicle (OP) or BRX-220 (OP + BRX) 4 weeks after spinal nerve lesion and stimulation of the A + C fibers of the injured L5 spinal nerve ($*P < 0.05$; Mann–Whitney test).

thermal and mechanical nociceptive thresholds were monitored for 4 weeks after the injury. Nociceptive thresholds of vehicle- and BRX-220-treated, sham-operated animals were no different from that of unoperated, untreated animals. However, both vehicle- and BRX-220-treated spinal nerve-lesioned rats developed significant thermal hyperalgesia as well as mechanical and cold allodynia within 3 days after surgery.

Following L5 spinal nerve ligation, cold stimuli induced a clear increase in the mean number of responses in the injured hindlimb during the observation period. Thus, the number of responses observed over a 60-s period increased from 2.2 ± 0.4 to 8.2 ± 0.7 in the first 2 weeks after injury (Fig. 4A). This cold allodynia persisted throughout the whole

period of the experiment so that in the second phase, between days 17 and 25, as many as 10 ± 0.5 actions were observed on the ipsilateral hindlimb. Treatment with BRX-220 did not affect cold allodynia behaviour for the first 15 days after surgery since in BRX-220-treated animals 7.5 ± 0.9 actions were observed during the 60-s exposure to cold water (Fig. 4A). However, in the second phase after the injury, between 17 and 25 days, there was a significant improvement in cold allodynia behaviour in BRX-220-treated animals, which only responded with 5.9 ± 0.8 actions over the 60-s period ($P < 0.01$; Fig. 4A).

Similarly, mechanical allodynia developed very quickly after nerve injury and within the first 15 days of injury, mechanical threshold dropped significantly in vehicle-trea-

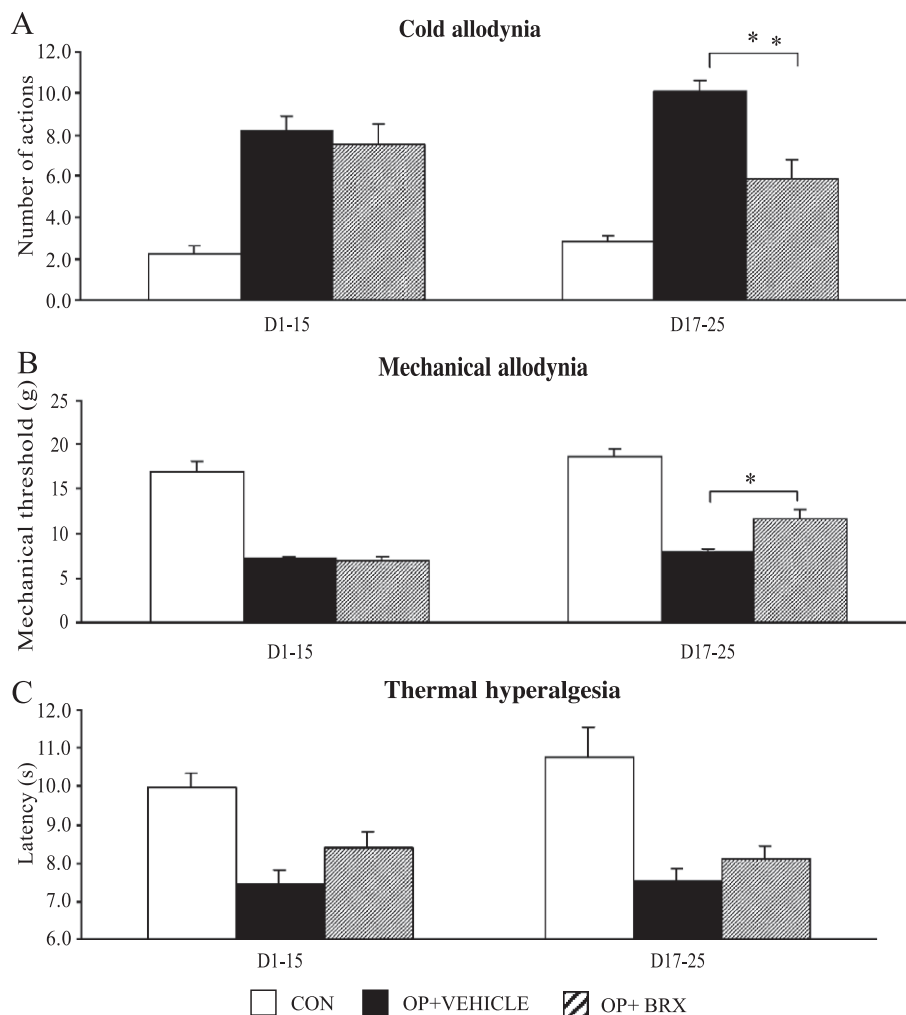


Fig. 4. The effect of treatment with BRX-220 on nociceptive responses following L5 spinal nerve injury was examined. In all cases, the mean results from control, sham-operated, vehicle-treated animals (open bars), vehicle-treated, spinal nerve-lesioned animals (black bars) and BRX-220-treated, spinal nerve-lesioned animals (striped bars) are shown. Measurements were made during two phases following injury, days 1–15 and 17–25. (A) Responsiveness of neuropathic rats to cold stimuli (cold allodynia). The number of reactions (shaking, lifting) of the injured limb on exposure to cold water (1°C) was counted over a 60-s period ($**P < 0.01$). (B) Changes in mechanical threshold following spinal nerve lesion and the effect of BRX-220 treatment (mechanical allodynia). Mechanical force of increasing intensity (max: 30 g) was exerted in the middle of the hind paw and the force at which the withdrawal reflex could be observed was recorded ($*P < 0.05$). (C) Thermal threshold to noxious heat (thermal hyperalgesia). The latency of hindlimb withdrawal to a noxious thermal stimulus applied to the hind paw was measured.



Fig. 5. Western blot analysis for hsp70 levels in L4 and L5 DRGs following sciatic axotomy and treatment with BRX-220. Western blot analysis of hsp70 levels in unoperated control and axotomized L4 and L5 DRGs was carried out 2 weeks after sciatic nerve axotomy and treatment with either vehicle or BRX-220. The figure shows an example of such a blot where several DRG samples were pooled ($n = 3$). Operated, saline-treated DRGs appear to contain slightly higher levels of hsp70 compared to controls, whereas the operated, BRX-220-treated spinal cords contained markedly higher levels of hsp70.

ted rats, from 17.0 ± 1 to 7.1 ± 0.3 g (Fig. 4B). During the same period, the mean mechanical threshold in BRX-220-treated rats was no different from vehicle-treated animals (6.9 ± 0.4 g; Fig. 4B). In the second phase, between 17 and 25 days after injury, mechanical threshold of vehicle-treated rats slightly increased to 7.9 ± 0.3 g. At this time, between 17 and 25 days after injury, there was a significant improvement in mechanical thresholds in BRX-220-treated neuropathic rats compared to vehicle-treated animals, with 11.6 ± 1 g ($P < 0.05$; Fig. 4B).

As can be seen in Fig. 4C, following L5 spinal nerve lesion, the mean latency to noxious heat stimuli decreased throughout the study period, from 10.0 ± 0.4 to 7.4 ± 0.4 s. In BRX-220-treated animals, the mean latency times were slightly higher than the latency in vehicle-treated injured animals, although this increase was not statistically significant (8.1 ± 0.3 s; Fig. 4C).

Effect of treatment with BRX-220 on the expression of hsp70 in the injured sensory system

The expression of hsp70 following sciatic axotomy and treatment with either saline or BRX-220 was examined in DRG samples 2 weeks after injury. The level of hsp70 expression was examined in control, untreated and unoperated BRX-220-treated, as well as operated, saline-treated and operated BRX-220-treated L4 and L5 DRG samples using Western blot analysis. An example of such a blot is shown in Fig. 5. Axotomy induced a slight increase in vehicle-treated DRG samples, but following treatment with BRX-220, this increase was markedly enhanced (Fig. 5).

Discussion

In this study, the effect of treatment with a co-inducer of heat shock proteins in two models of neuropathic pain was examined. The results showed that treatment with BRX-220 (a) partially reversed the decline in sensory neurone marker expression in both DRG and spinal cord that otherwise occurs following injury, (b) restored the responsiveness of sensory fibers to afferent stimulation as measured by SP

release and (c) with time, improved sensory function following spinal nerve lesions. We also showed using Western blot analysis that BRX-220 induces the expression of hsp70 in the injured sensory system as a possible mechanism of action.

BRX-220 is an analogue of the non-peptidergic hydroxylamine compound Bimoclolmol. Bimoclolmol has been shown to have a neuroprotective action in a rat model of diabetic neuropathy, restoring reduced conduction velocities in the damaged nerve (Biro et al., 1997, 1998). Bimoclolmol and its analogues are thought to exert their protective effect by enhancing cellular defense mechanisms in injured cells, in particular by inducing an up-regulation in the expression of certain heat shock proteins (hsps) that are responsible for maintaining normal homeostasis during stress conditions (Vigh et al., 1997). Hsps are part of a phylogenetically old cellular defense system present in virtually all cell types and for example serve as chaperones for cellular proteins. The expression of many hsps is induced by a variety of stress conditions. Bimoclolmol analogues act as co-inducers of heat shock proteins, in particular hsp60, hsp70 and hsp90 (Vigh et al., 1997). Thus, under nonstress conditions these analogues do not induce any hsp response, although following cellular stress they enhance the production of the stress-induced hsps and thus, the ability of cells to cope with the stressful insult.

There are several lines of evidence that support the protective role of enhanced hsp expression in promoting cellular survival and adaptation. In vitro evidence demonstrates that overexpression of hsp70 in cell lines provides protection against ischaemic and thermal injury (Amin et al., 1996). Moreover, primary neurone cultures of dorsal root ganglia are also more resistant to heat when transfected with hsp70 protein (Uney et al., 1994). Similar results were obtained when neural cells were transfected with the hsp27 protein. Cells infected with a virus containing the hsp27 gene were more resistant to apoptotic stimuli than noninfected cells (Wagstaff et al., 1999). The findings of these studies were confirmed in vivo experiments using models of peripheral nerve injury in the rat (Kalmar et al., 2002a). Exogenously applied hsp70 protein onto the spinal cord has been shown to enhance survival of sensory neurones after neonatal nerve injury (Houenou et al., 1996). Injured DRG neurones were also more likely to survive the injury if they were able to up-regulate hsp27 expression (Lewis et al., 1999).

The DRG neurone population can be divided into several groups according to the type of axons they possess and specific markers they express (Bradbury et al., 2000). Half of the small-diameter DRG neurones that contain high levels of specific peptidergic neuromodulators such as SP and CGRP normally express the nerve growth factor (NGF) specific tyrosine kinase receptor, trkA (Averill et al., 1995). Another subset of small-diameter DRG cells can be identified by binding of the lectin isolectin B4 from *G. simplicifolia* (IB4) and these cells express GFR α and Ret, receptors for glial cell line-derived neurotrophic factor

(GDNF) and the purinergic P2X3 receptor. Following injury to their peripheral axons, both of these DRG cell types down-regulate the expression these markers (Bennett et al., 1998; Bradbury et al., 1998). It has been shown by other authors that this loss of staining is not due to loss of neurones since neuronal death does not occur in DRG cells until a later stage (Tandrup et al., 2000). The same pattern of altered sensory neuronal marker expression has been described in laminae I and II of the spinal cord dorsal horn, areas corresponding to the nerve terminals of injured DRG neurones (Bennett et al., 1998). Recent studies have shown that exogenously applied neurotrophic factors can promote sensory nerve regeneration in nerve injury models in vivo (Bradbury et al., 2000; Ramer et al., 2000). However, treatment with different neurotrophic factors resulted in a selective rescue of different subtypes of sensory axons. Treatment with NGF appears to rescue axons of CGRP-containing neurones and cells that express the trkA NGF receptor but does not have any effect in other subgroups (Bradbury et al., 2000). Similarly, GDNF only acts on cells that bind IB4 and express the GDNF receptor, RET, and GFR α 1/2, whereas NT3 is effective on cells with trkC receptors (Bennett et al., 1998; Bradbury et al., 2000; Ramer et al., 2000). In the present study, we showed that treatment with BRX-220 significantly improves the cellular marker profiles that are usually strongly down-regulated following nerve injury. Moreover, this effect is not limited to one specific marker. Both IB4 and CGRP labeling showed significant improvement because of daily BRX-220 treatment. The effect of treatment with BRX-220 is more pronounced in the long-term experiments carried out 4 weeks after injury. Thus, BRX-220 treatment seems to improve sensory neurone profiles progressively, exerting general beneficial effects in more sensory neurone types. This effect of BRX-220 treatment may be explained by the proposed mechanism of BRX compounds in that they mobilize a rather unspecific recovery mechanism through the increased induction of hsp expression rather than targeting specific neuronal groups through specific receptors.

In addition to improving sensory marker profiles in injured sensory neurones, BRX-220 treatment also restored SP responsiveness to electrical stimuli. Thus, BRX-220 treatment has beneficial effects on the recovery of stimulation-evoked SP release in injured sensory fibers. SP is normally found in small-diameter A δ and C fibers where it is stored in dense-core vesicles and is released from their terminals upon nociceptive stimuli. Following peripheral axotomy, while SP content is down-regulated in these small-diameter sensory cells and axons, it is expressed de novo in A β fibers (Noguchi et al., 1995). We have previously reported that spinal nerve lesion results in a significant decrease in the release of SP from high-threshold sensory neurones in the dorsal horn and “de novo” release of the peptide from low-threshold fibers (Malcangio et al., 2000). It has been established that SP release is highly dependent on

the site of nerve injury (Malcangio et al., 2000). Thus, distal peripheral axotomy causes substantial decrease in SP release from nerve terminals in the spinal cord upon high- and low-threshold stimuli (A β + A δ + C fiber strength) but not upon stimulation with high-threshold stimuli alone (A β strength; Malcangio et al., 2000). An injury affecting more proximal spinal nerves results in further decrease in SP release upon high- and low-threshold stimuli and also causes a substantial increase in SP release following stimulation with A β strength (Malcangio et al., 2000). In the present study, we have shown that oral treatment with BRX-220 partially restores the impaired release upon A + C fiber stimulation while not influencing the low-threshold response. In contrast, lesion to the L5 spinal nerve, a model of proximal nerve lesion, results in a further decrease in SP release in response to A + C fiber strength stimuli and an abnormal evoked SP release upon A β strength stimuli. In this animal model, BRX-220 treatment significantly reversed the effect of nerve injury on evoked SP release in both conditions.

In order to test the effect of treatment with BRX-220 on functional changes in sensory processing, we carried out behavioural tests on neuropathic rats treated daily with BRX-220. We found that BRX-220 did not alter mechanical and cold allodynia or thermal hyperalgesia behaviour in the first phase after injury (between postoperative days 1–15). However, during later stages after the injury (postoperative days 15–25), mechanical and cold allodynia behaviour profiles improved significantly compared to vehicle-treated animals. This profile of drug action implies that BRX-220 does not prevent the development of neuropathy after partial denervation. However, the behavioural improvement seen with daily treatment suggests either a slow and progressive analgesic action or enhancement of the recovery processes.

The results of this study therefore show that treatment with BRX-220 can be beneficial in pathological conditions such as peripheral neuropathies, promoting restoration of normal morphological and functional properties of sensory functions. These effects of BRX-220 are likely to be the consequence of the ability a BRX-220 to enhance hsp expression as already established in the injured motor system (Kalmar et al., 2006).

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