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ABLATION OF A POPULATION OF NEURONS IN THE DORSAL HORN OF THE SPINAL CORD IS INSUFFICIENT TO INDUCE SPROUTING OF TOUCH RESPONSIVE MYELINATED AFFERENTS INTO THE INNERVATION TERRITORY OF PAIN SENSITIVE UNMYELINATED AFFERENTS

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RESEARCH ARTICLE

ABSTRACT. IT HAS BEEN PROPOSED THAT the sprouting of touch responsive Aβ-fibers into the innervation territory of pain sensitive C-fibers in the spinal cord contributes to pain behaviors following peripheral nerve injury. The stimulus for Aβ sprouting is unclear, but it has been proposed that the death of dorsal horn neurons may result in "vacant synapses" that could act as a sufficient stimulus to induce sprouting. In order to test this hypothesis, we have investigated whether selectively ablating a population of cells in laminas I and II, in the absence of peripheral nerve injury, would induce sprouting of Aβ-fibers as identified using the selective transganglionic tracer, cholera toxin-β. We demonstrate that intrathecal injection of a substance P and saporin conjugate caused a statistically significant reduction of neurokinin 1 immunostaining in the rat spinal cord. However, this ablation of dorsal horn neurons did not result in the presence of cholera toxin-β in the dorsal part of lamina II, as was observed following axotomy of the sciatic nerve. These data suggest that the death of dorsal horn neurons, in the absence of peripheral nerve damage, does not necessarily induce sprouting of Aβ-fibers into lamina II.

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1. INTRODUCTION

The spinal cord of the rat is topographically organized into laminas. Myelinated afferents (Aβ-fibers) terminate in lamina I, the ventral part of lamina II and laminas III-VI, while unmyelinated afferents (C-fibers) terminate predominately in lamina II. The spinal termination pattern of Aβ-fibers can be demonstrated using transganglionic transport of the β subunit of cholera toxin (CTB). CTB selectively binds GM1 gangliosides that are present on myelinated afferents but absent from unmyelinated afferents. Therefore, when injected into the sciatic nerve CTB is transganglionically transported to lamina I, the ventral part of lamina II and laminas III-VI and is not found in the dorsal part of lamina II. Woolf and colleagues [1] demonstrated that axotomy of the sciatic nerve results in the presence of CTB in the dorsal part of lamina II. This result was interpreted as sprouting of touch responsive Aβ-fibers into the innervation territory of pain sensitive C-fibers (i.e., the dorsal part of lamina II). Furthermore, as allodynia (defined as pain due to a non-painful stimuli, such as touch) can develop following nerve injury, sprouting was proposed as a possible cause. Subsequent to this observation, sprouting has been demonstrated in a number of animal models of chronic neuropathic [2-5] and inflammatory pain [6]. Degeneration of C-fibers in lamina II has been proposed as the stimulus that initiates sprouting from deeper laminas. In support of this hypothesis, it has been shown that axotomy-induced sprouting can be prevented or reversed by application of trophic factors that prevent C-fiber degeneration [7-10]. In addition, Mannion and colleagues found that capsaicin-mediated destruction of C-fibers led to Aβ-fiber sprouting [11]. In contrast, a more recent study by Santha and Jancso [12] did not find evidence of Aβ-fiber sprouting following capsaicin-mediated destruction of C-fibers. Furthermore, dorsal rhizotomy, which will also cause degeneration of C-fibers, does not lead to sprouting [13]. More recently, the extent of sprouting has been further challenged, and Tong et al. [14] have demonstrated that axotomy leads to increased uptake of CTB by C-fibers. This would also explain the presence of CTB in the dorsal part of lamina II. In a follow-up study, Bao and colleagues [15] pre-labeled the Aβ-fibers to avoid axotomy-induced uptake of CTB by C-fibers and concluded that a limited quantity of sprouting does occur and that it could be physiologically relevant.

An alternative hypothesis is that it is the death of spinal neurons (not damage to primary afferent fibers) that stimulates the sprouting of Aβ-fibers. The death of dorsal horn interneurons has been observed in neonates following axotomy [16-18], in adults following axotomy [19] and in a number of neuropathic pain models [20-23]. To test this hypothesis we have selectively ablated a population of neurons in lamina I and II of the dorsal horn of the rat spinal cord using a substance P and saporin conjugate (SP-SAP) which, following intrathecal administration, is toxic to neurons that express the neurokinin-1 (NK-1) receptor [24,25]. This technique is well-established in the literature as a method for eliminating this population of neurons and immunohistochemical staining for NK-1 has been previously used to confirm ablation [26]. The ablation of dorsal horn NK-1 receptor expressing neurons were chosen as a target as firstly, they represent a substantial population of neurons in laminas I and II of the dorsal horn and secondly, they have a direct physiological significance [24,25]. We then measured the transganglionic transport of CTB following injection into the sciatic nerve. This approach also avoids any complications that may arise due to uptake of CTB by C-fibers following nerve injury [14].

2. MATERIALS AND METHODS

2.1. ANIMALS

All animal procedures were approved by the Purdue Pharma Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats (90-120 g; Taconic, NY), 8-10 per group, were used in all experiments. Animals were housed in groups of 3 and had free access to food and water at all times. Animals were on a 12 hour light-dark cycle.

2.2. SURGERY

Prior to axotomy, rats were anesthetized with 2% isofluorane in O_2 and the skin of the left hind limb prepared in a sterile manner. The skin and biceps femoris were incised at mid-thigh level, the sciatic nerve was exposed, tightly ligated and

transected distal to the ligature. In sham animals the sciatic nerve was exposed but not ligated. The wound was closed using 4-0 vicryl (Ethicon) and the animals were allowed to recover. Fourteen days post-axotomy or sham operation, the animals were re-anesthetized, the sciatic nerve exposed and CTB (2 μL, 2%; SigmaAldrich, MO) was injected into the nerve via a 10 μL Hamilton syringe fitted with a 30-gauge needle. The wound was closed and the rats were allowed to recover.

Separate groups of rats were anesthetized as above and intrathecal injection of SP-SAP (10 μL, 5 μM in PBS as a single bolus; Advanced Targeting Systems, CA) was performed using a 10 μL Hamilton syringe fitted with a 27-gauge needle. Control animals received an intrathecal injection of PBS (10 μL). Fourteen days post SP-SAP or PBS the sciatic nerve was exposed and CTB injected as described above.

Three days following CTB injection animals were sacrificed then trans-cardially perfused via the left ventricle with 400 ml of 0.1 M phosphate buffered saline (PBS) followed by 400 mL of 4% formalin in 0.1 M PBS. The spinal cord was removed, post-fixed overnight in 4% formalin and stored at 4ºC in 30% sucrose with 0.02% sodium azide.

2.3. IMMUNOHISTOCHEMISTY

Transverse sections (60 μm) of the lumbar spinal cord (L4-L6) were cut on a freezing microtome and stored in 5% sucrose with 0.02% sodium azide. Sections were washed (PBS), blocked by incubation with blocking solution (2% normal donkey serum for NK-1 or 2% normal horse serum for CTB) for 30 minutes and incubated overnight in primary antisera against either NK-1 (Advanced Targeting Systems, CA) at 1:1000 dilution or CTB (List Biological Laboratories, CA) at 1:40000 dilution. All antibodies were diluted in blocking solution. The presence of CTB was visualized using the avidin-biotin immunohistochemical procedure. This involved incubation in biotinylated anti-goat IgG (1:200, Jackson ImmunoResearch, PA) for 2 h followed by avidin peroxidase (1:200. Vector, CA) for 1 h. Bound peroxidase was visualized by reaction with hydrogen peroxide in the presence of 3,3-diaminobenzidine (DAB, Vector, CA). Staining for NK-1 involved incubation in Cy3 conjugated anti-rabbit IgG (1:200, 1 h). Sections were washed between steps in 0.1 M PBS (30 min) and following the final wash were dehydrated through a series of ascending alcohols, cleared in histoclear and mounted in DPX (Sigma, MO). Digital images of the sections stained for NK-1 were examined for fluorescence intensity utilizing ImagePro Plus software (Media Cybernetics, Inc, Silver Spring, MD). Total fluorescence intensity was measured from lamina I and the dorsal part of lamina II (as indicated in FIG. 2B, 2D). Ten sections, selected randomly across the lumbar spinal cord (L4-L6), were analysed for each animal. Untransformed data were analysed using the student's t-test. The level of significance was set at $P < 0.05$. Data are shown as mean ± the standard error of the mean.

3. RESULTS

3.1. CTB IS PRESENT IN THE DORSAL PART OF LAMINA II FOLLOWING SCIATIC AXOTOMY

Spinal cords from rats that had received a sham surgery followed by intraneural injection of CTB displayed prominent CTB immunostaining in the motor neurons (data not shown), lamina I, the ventral part of lamina II and laminas III-VI (Fig. 1A) on the side ipsilateral to the injection. The dorsal part of lamina II was devoid of positive immunostaining for CTB (FIG. 1A). The boundaries between lamina I and the dorsal part of lamina II and between the dorsal and ventral parts of lamina II were easily discernable (FIG. 1A).

Spinal cord sections from rats that received an axotomy of the sciatic nerve followed by intraneural CTB displayed an obvious presence of CTB in the dorsal part of lamina II in addition to the motor neurons, lamina I, the ventral part of lamina II and laminas III-VI (FIG. 1C). In these sections the borders of the dorsal part of lamina II were not discernable, similar to previous reports [1]. In all cases no immunostaining for CTB above background was observed on the contralateral side of sham or axotomized animals (FIG. 1B, 1D).

3.2. INTRATHECAL SP-SAP RESULTS IN THE ABLATION OF NK-1 IMMUNOSTAINING

Spinal cords from non-axotomized rats that received an intrathecal injection of PBS displayed

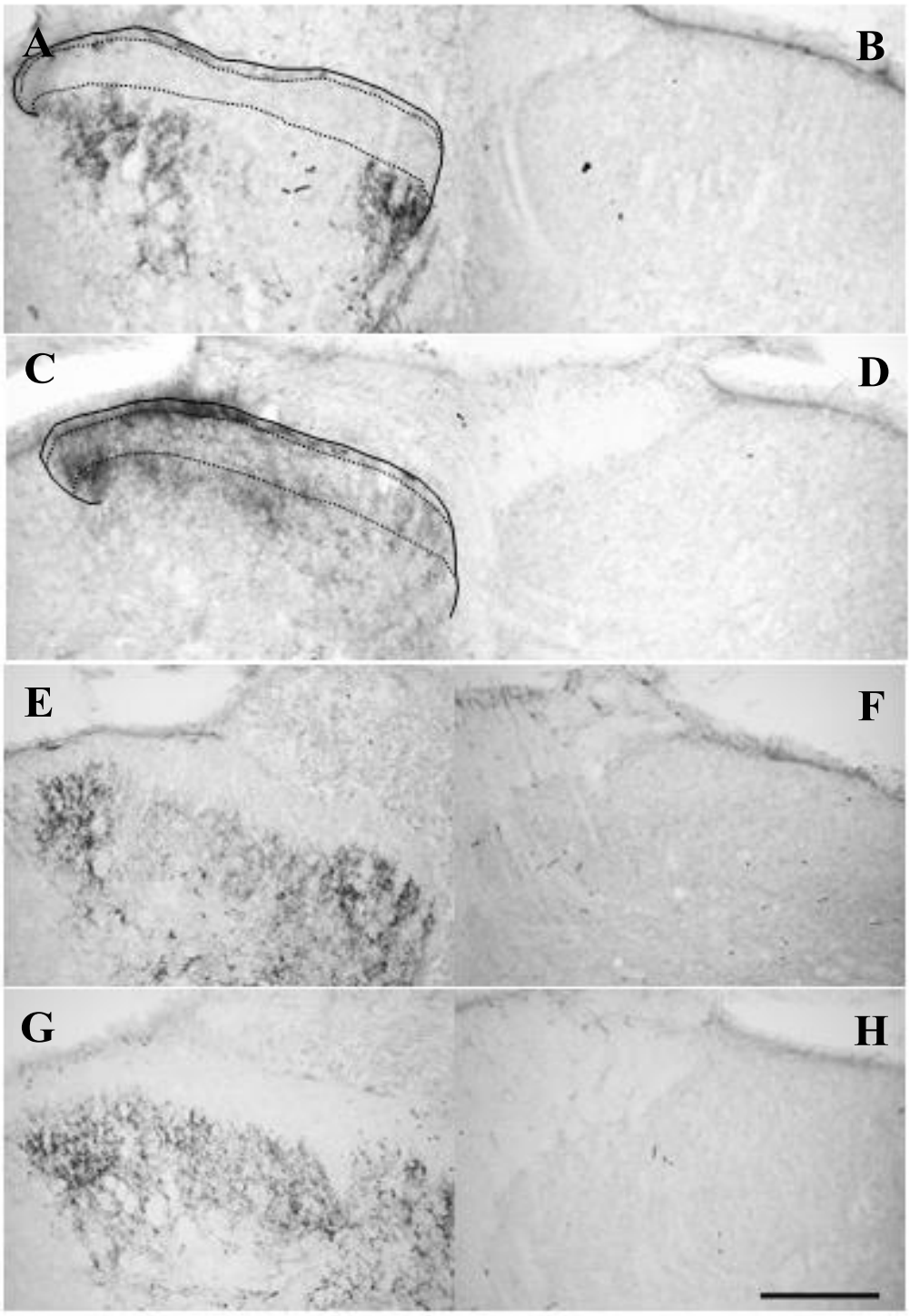

FIGURE 1. BRIGHTFIELD PHOTOMICROGRAPHS SHOWING IMMUNOHISTOCHEMICAL STAINING FOR TRANSGANGLIONICALLY-TRANSPORTED CHOLERA TOXIN-B (CTB) IN THE DORSAL HORN OF THE LUMBAR SPINAL CORD. A, B: Rats received a sham operation followed two weeks later by intraneural cholera toxin-β (CTB). There was no immunostaining in the dorsal part of lamina II of the ipsilateral (ips) side (A) or on the contralateral side (B). C, D: Following sciatic axotomy obvious CTB staining was observed in the dorsal part of lamina II (C) while the contralateral side remained devoid of staining (D). E–H: Following intrathecal (i.t.) phosphate buffered saline (E) or i.t. substance P and saporin conjugate (SP-SAP; G) there was no CTB immunostaining present in the dorsal part of lamina II. No staining above background was seen on the contralateral side (F and H). Fine dashed lines show the approximate position of the dorsal part of lamina II. Scale bar = 380 μm.

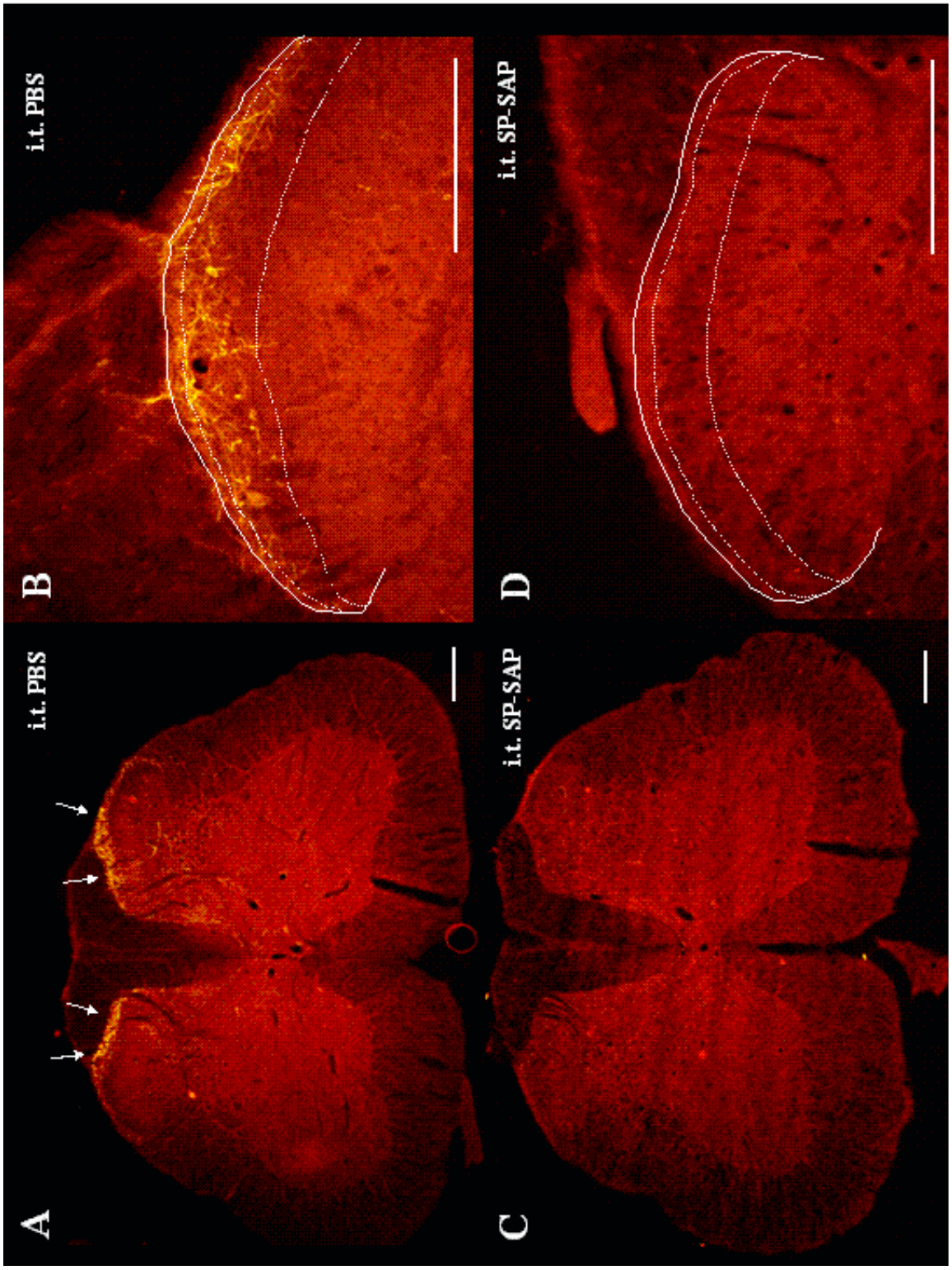

FIGURE 2. EPIFLUORESCENCE PHOTOMICROGRAPHS SHOWING THE CYTOTOXICITY OF A SUBSTANCE P AND SAPORIN CONJUGATE (SP-SAP) 17 DAYS AFTER INTRATHECAL (I.T.) ADMINISTRATION. A: In rats that received phosphate buffered saline (PBS), NK-1 (the substance P receptor) immunostaining (arrows) is seen in the spinal cord. B: A higher powered image showing that substantial staining is observed in laminas I and II. C: Following intrathecal SP-SAP a significant reduction in NK-1 immunostaining is seen in laminas I and II. D. A higher powered view of C. Fine dashed lines show the approximate position of the dorsal part of lamina II. Scale bars = 400 µm.

prominent NK-1 immunostaining. NK-1-positive neuronal cell bodies were most abundant in laminas I and II (FIG. 2A, 2B), and they were also observed, albeit to a lesser extent, in the deeper laminas. Intense staining was often seen around the central canal and occasionally these cells extended processes into the deeper laminas (data not shown).

Sections of spinal cords from non-axotomized rats that received an intrathecal injection of SP-SAP displayed a marked reduction in NK-1 immunostaining in laminas I and II, as compared to PBS injected controls. The surviving lamina I immunoreactive neurons displayed shrunken cell bodies and shortened processes. In the majority of sections the reduction was complete (FIG. 2C, 2D). NK-1 positive cell bodies were observed in the deeper laminas and around the central canal as described above. These results confirm those of previous studies [24,26] reporting a marked depletion of NK-1 expressing neurons.

SP-SAP treatment resulted in a statistically significant reduction of total fluorescent intensity (304,720 ± 130,183 intensity units) as compared to PBS treated controls (3,255,469 ± 444,853 intensity units). This constitutes a reduction of over 90% of the immunofluorescence.

3.3. CTB IS NOT PRESENT IN THE DORSAL PART OF LAMINA II FOLLOWING INTRATHECAL SP-SAP TREATMENT

When non-axotomized rats received an intrathecal injection of SP-SAP followed by an intraneural injection of CTB an absence of staining was observed in the dorsal part of lamina II (Fig. 1G). There was no difference in the pattern of staining observed in these rats and either rats that had received an intrathecal injection of PBS followed by intraneural CTB (FIG. 1E) or animals that had received a sham operation followed by intraneural CTB (FIG. 1E, 1A). In all cases there was prominent immunostaining in the motor neurons, lamina I, the ventral part of lamina II and laminas III-VI. The boundaries between lamina I and the dorsal part of lamina II and between the dorsal and ventral parts of lamina II were easily discernable.

In all cases no immunostaining for CTB was observed on the contralateral side of SP-SAP- or PBS-treated rats (FIG. 1F, 1H).

4. DISCUSSION

Since the discovery of primary afferent sprouting in the dorsal horn following peripheral nerve axotomy by Woolf and colleagues [1], it has been postulated that this phenomenon is involved in the pathology of nerve injury-related chronic pain. Intense study has attempted to understand the neurobiological mechanisms that mediate this event. One avenue of investigation has centred on the stimulus that initiates the sprouting of Aβ-fibers into the dorsal part of lamina II [6-8,13-15]. Initially it was postulated that degeneration of the central terminals of primary afferent C-fibers, in lamina II, initiated sprouting [7-11]. However, more recent studies have questioned this hypothesis [12,13]. An alternative hypothesis is that the death of dorsal horn interneurons could be the stimulus that initiates Aβ-sprouting. Indeed, neuronal cell death has been demonstrated in the dorsal horn of the spinal cord in response to axotomy [19] and in a number of models of neuropathic pain [20-23]. We have investigated this hypothesis by, first confirming the original discovery of Woolf and colleagues that sciatic axotomy induces the transganglionic transport of CTB into the dorsal part of lamina II of the dorsal horn [1]. Next, we investigated whether the ablation of a population of neurons in the dorsal horn, in the absence of peripheral axotomy, would stimulate Aβ-fiber sprouting. NK-1 expressing dorsal horn neurons were chosen as a target for ablation as firstly, they represent a significant population of physiologically relevant dorsal horn neurons and secondly, they can be specifically deleted using SP-SAP [24-26]. We have used immunohistochemical staining for the NK-1 receptor to confirm results from previous studies [24,26] reporting a marked depletion of NK-1 expressing neurons. As C-fibers make functional connections with NK-1 cells [24] this procedure creates “vacant synapses”, which we hypothesized may act as a stimulus for neuronal sprouting. Mechanistically it is possible that this occurs via the release of growth factors. Ablation of the NK-1 expressing cells in lamina I and II did not, however, induce sprouting of Aβ-fibers into the dorsal part of lamina II. Therefore, we believe that the death of NK-1 expressing neurons in the dorsal horn of the spinal cord is not a sufficient stimulus to induce primary afferent sprouting. We cannot rule out the

possibility that ablation of a different population of neurons would stimulate Aβ-fiber sprouting. This second possibility could be investigated using alternative conjugated toxins that ablate other neuronal populations. Alternatively, nerve injury associated alterations in gene or protein expression in primary afferents neurons (phenotypic switch), release of trophic factors by spinal neurons or a combination of these events may be the stimulus responsible for reorganization of primary afferent fibers.

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