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# B-50 (GAP-43) in the rat spinal cord caudal to hemisection: lack of intraspinal sprouting by dorsal root axons

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**Abstract**

The controversial hypothesis that intraspinal sprouting by dorsal root axons promotes reinnervation of partially denervated neurons caudal to a low thoracic cord hemisection was re-investigated in rats using quantitative immunohistochemical analysis of the neural specific growth-associated protein B-50 (GAP-43) at postoperative survival times of 3, 10, 21, 42, and 90 days. The lack of increase in B-50-immunoreactivity in all segments below the hemisection at all survival times does not support the concept of intraspinal sprouting following the removal of supraspinal descending pathways.

Keywords: Sprouting; Dorsal columns; Synaptic plasticity; Degeneration; Regeneration; Pyramidal tract; Spinal cord injury

In many regions of the central nervous system (CNS), reinnervation of partially denervated neurons occurs by collateral sprouting of undamaged systems [5,7,8,19,21]. It has been proposed that sprouting of dorsal root axons distal to a low thoracic cord hemisection is responsible for the reinnervation of partially denervated regions of spinal gray [8,10,15,16]. In the absence of any functionally significant regeneration of interrupted pathways, such dorsal root sprouting has been suggested to be involved in postlesional motor recovery in experimental animals [7,16] and also in human spasticity [15]. The observation that dorsal rhizotomy ipsilateral and caudal to a hemisection permanently abolishes any motor recovery [8,16] clearly demonstrates the postlesional involvement of primary afferent systems in the return of motor function. This concept has been confirmed by more recent locomotor and reflex analyses [10]. However, the hypothesis of dorsal root sprouting distal to a spinal cord hemisection has become a matter of controversy, partly due to technical limitations of the silver degeneration and HRP tracing methods employed in earlier investigations [7,8,20]. Recently, this issue has been examined in the cat using immunohistochemical techniques for the identification of B-50 (GAP-43) [10,18], a nervous-tissue specific phosphoprotein widely considered to be involved in developmental and regenerative axon growth as well as in synaptic plasticity [2,6,13,22–24). However, this approach has led to conflicting observations. Helgren and Goldberger [10] reported a postlesional increase in the area of superficial dorsal horn laminae occupied by GAP-43 (B-50) immunoreactivity (B-50-IR), which was interpreted as an indication for dorsal root sprouting. In contrast, Nacimiento et al. [18] found no evidence of alterations in the distribution or intensity of B-50 expression in any segment caudal to the hemisection.

The discrepancies concerning B-50-IR in the dorsal horn after hemisection could be explained by the use of different antibodies, which may recognize different epitopes of the protein. However, this is unlikely since the antibody used in our study clearly revealed a transient unilateral increase in B-50 expression in the rat lumbar dorsal horn during synaptic remodelling following a sciatic nerve lesion [13]. Alternatively, the discrepancies may have been due to the use of an inappropriate dilution

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of primary antibody [2,4]. A recent study [2] employing three different antibodies against B-50 (GAP-43), has demonstrated that each antibody is capable of detecting an increase of immunoreactivity in the lumbar dorsal horn after sciatic nerve lesion, provided that a sufficiently low concentration of primary antibody was used. The use of relatively high concentrations of primary antibody partially impedes the detection of postlesional changes of B-50 (GAP-43) due to the intense labelling of the constitutive protein [2,4]. Finally, it is possible that the use of a relatively small number of cats in our previous study [18] precluded the opportunity of detecting subtle changes in B-50-IR following spinal cord hemisection. Therefore, in an attempt to clarify this issue, we have quantitatively reexamined B-50 expression in the lumbosacral spinal gray of adult rats following a low thoracic spinal cord hemisection using several dilutions of the primary antibody in a larger population of experimental animals. Although, species differences must be considered in this approach, thoracic spinal cord hemisection in the adult rat is also followed by a high degree of locomotor recovery in which segmental afferent input has been implicated [14].

These experiments were conducted in 18 adult female rats, 250–300 g in body weight. Under deep chloral hydrate anesthesia (375 mg/kg, i.p.) a laminectomy was performed at the level Th11, the dorsal and lateral surfaces of the low thoracic spinal cord were exposed, and hemisection of the left side of the spinal cord was performed with a straight-edged scalpel blade. Rats were sacrificed at postoperative (p.o.) survival times of 3, 10, 21, 42, and 90 days (3 rats per group). Additionally, control tissue from two unoperated animals and one sham operated animal was also processed for B-50-IR.

Under terminal anesthesia, rats were perfused through the left ventricle with 0.9% saline solution, followed by 200 ml of fixative (0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) The spinal cord and brainstem were removed and each segment from Th10 (including the lesion) to S2 identified using the dorsal root entry zones as landmarks. Tissue blocks were postfixed for 24 h in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cryoprotected overnight (30% sucrose) prior to being frozen. Immunohistochemistry for B-50 was performed on free-floating transverse cryostat sections (20 $\mu$m) as described previously [17,18]. Four dilutions (1:3600, 1:5400, 1:7600 and 1:11 600) of primary antibody were used. A primary antibody dilution of 1:5400 gave optimum conditions for the B-50 staining of rat spinal cord. Although the intensity of B-50-IR became slightly weaker with increasing dilutions of primary antibody, all dilutions revealed the same result, i.e. bilaterally symmetrical staining.

The accuracy of each hemisection was assessed in cresyl violet stained serial sections (30 $\mu$m) taken at the site of injury. In all rats, the ipsilateral ventral, lateral and dorsal funiculi were completely destroyed, whereas the contralateral hemicord was intact. An automated image analysis system was used to quantify density and spatial area of B-50-IR in the spinal gray of segments L2, L4, L6, and S2 in all operated and unoperated rats, as described previously [18].

The lumbosacral spinal gray of all operated and unoperated animals displayed a bilaterally symmetrical distribution pattern and intensity of B-50-IR (Figs. 1A–C). Immunoreactivity was confined to the neuropil, neuronal cell bodies being unstained. The intensity of labeling varied greatly between the Rexed's laminae [3,12,17],

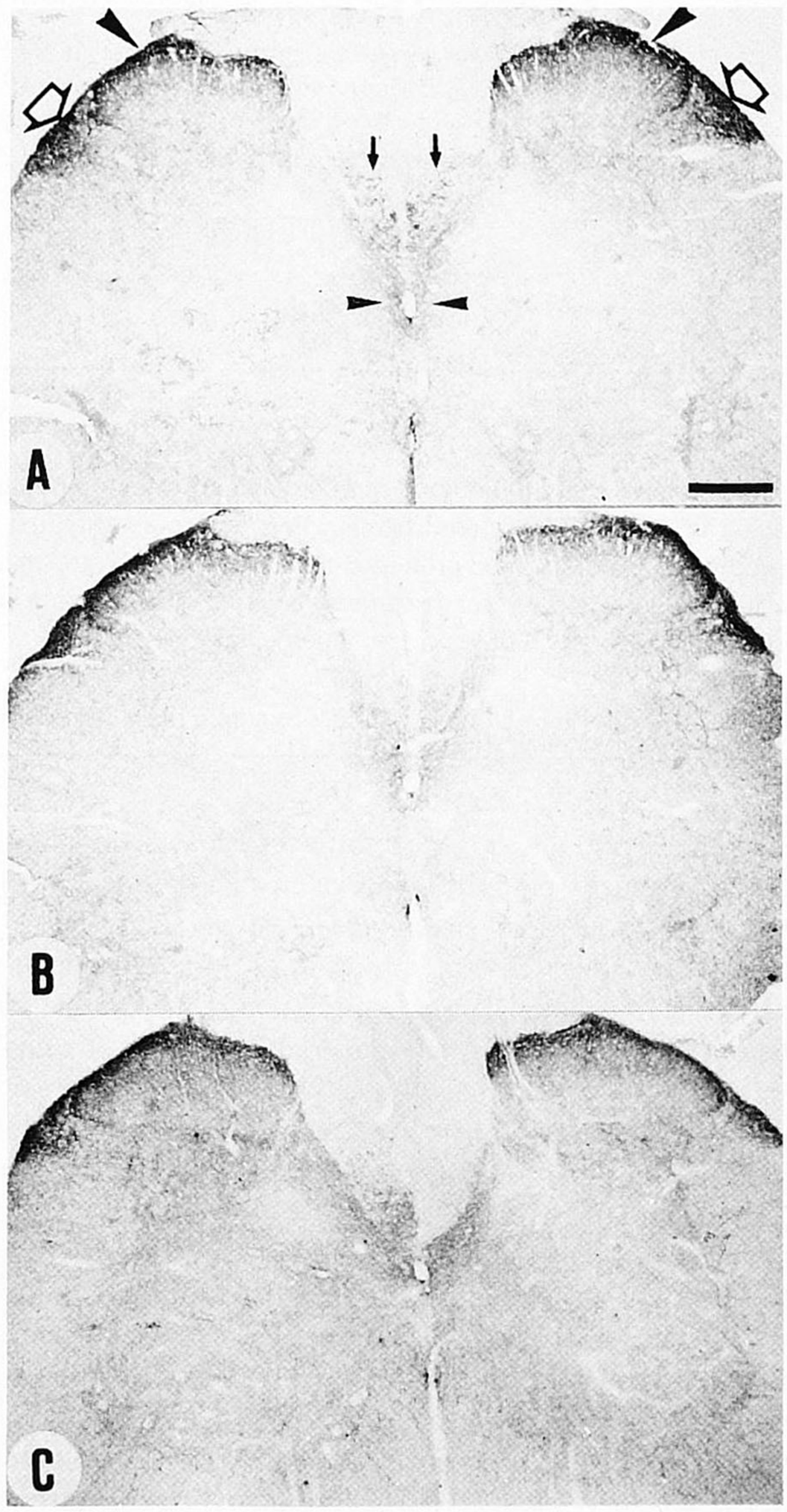


Fig. 1. B-50-IR in the spinal gray of segment L3, 10 days (A), 21 days (B), and 42 days (C) after cord hemisection. The operated side is shown on the right. Note the bilaterally symmetrical staining in superficial laminae of the dorsal horn (large arrowheads) and also very slightly in lamina X (small arrowheads), whereas the remaining laminae are unstained. In the white matter, B-50-IR is also present in the pyramidal tract (arrows) and in Lissauers tract (open arrows). Scale bar: 300 $\mu$m.

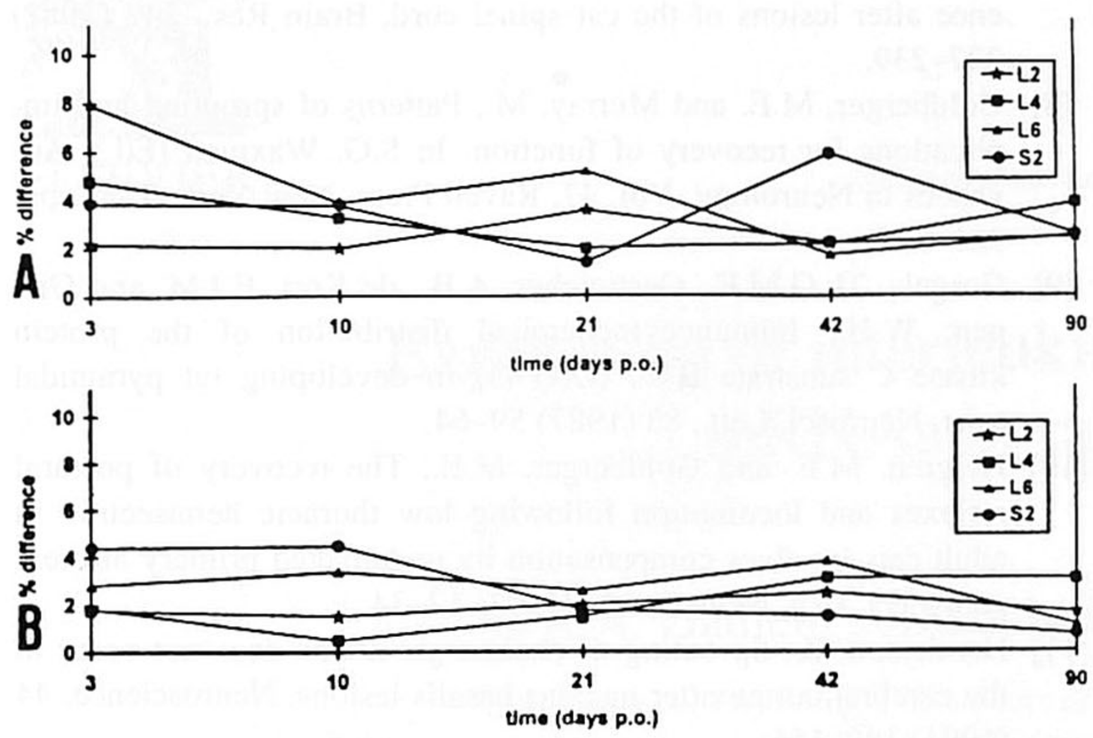


Fig. 2. Quantitative results: percentages of side to side differences in the spinal gray (A) in area occupied by B-50-IR and (B) mean optical density of staining. There are no significant differences in both parameters at all survival times and segmental levels studied. Wilcoxon-test was used for statistical analysis.

being most conspicuous in the superficial laminae (I–II) of the dorsal horn. A slight immunostaining was also present in the central portion of the intermediate zone (lamina X). Conversely, the lateral portion of the intermediate gray (laminae VI–VII) and the ventral horn (laminae VIII–IX) were almost devoid of immunostaining. Quantitative analysis in the spinal gray confirmed that, in all spinal segments caudal to the hemisection, the extent and intensity of B-50-IR were bilaterally symmetrical (Figs. 2A,B), the staining being primarily localized in the dorsal horn.

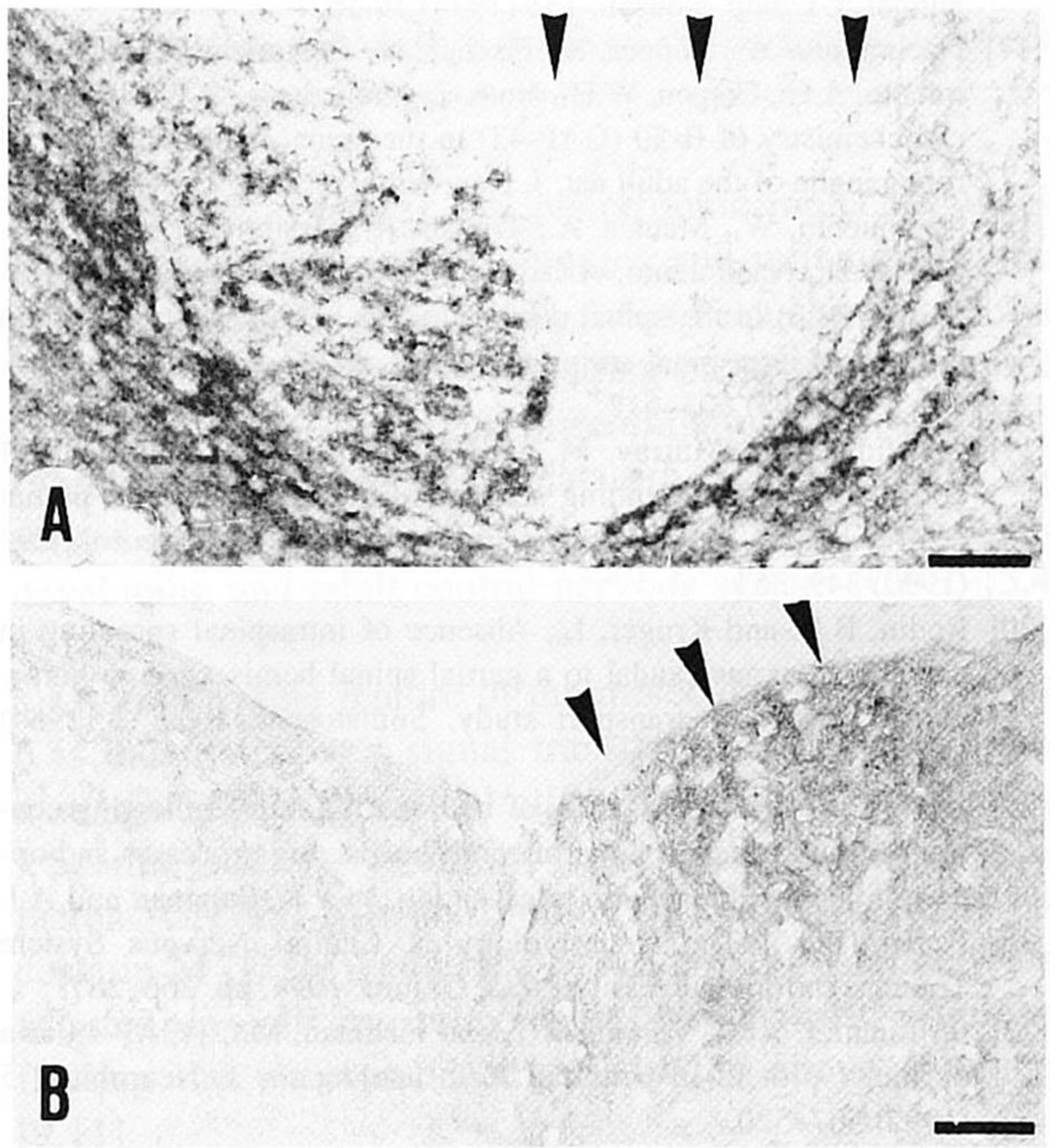


Fig. 3. (A) B-50-IR in the pyramidal tract of segment L3, 42 days after hemisection. The operated side is on the right. Note that the pyramidal tract of the operated side is virtually unstained (arrowheads), compared to the intense B-50-IR on unoperated side. Scale bar: 50 $\mu$m. See also Fig. 1C. (B) B-50-IR in the nucleus gracilis, 90 days after hemisection. The operated side is on the right. There is a conspicuous labelling of the neuropil on the operated side (arrowheads), whereas the unlesioned side is only slightly stained. Scale bar: 60 $\mu$m.

In the pyramidal tract, ipsilateral and caudal to the lesion, there was a marked decrease of the normally intense [3,9,12] B-50-IR by 42 days p.o. and a complete loss by 90 days p.o. (Figs. 1C and 3A). This finding is in keeping with the degeneration of this tract after hemisection and provided an additional indication of the accuracy of surgery. Intense B-50-IR was also detected in Lissauers tract, which was not altered by the hemisection.

We were also able to obtain preliminary data on postlesional changes of B-50-IR in the nucleus gracilis. In the unoperated rat, this nucleus is virtually devoid of staining [12], whereas after hemisection, an ipsilateral biphasic increase of perineuronal B-50-IR was detected, the peaks of which occurred at 10 and 90 days p.o. (Fig. 3B). A much smaller biphasic increase of B-50-IR was also noted in the neuropil of the nucleus gracilis on the contralateral (unoperated) side, which followed the same temporal dynamics. To the best of our knowledge, this is the first demonstration of an increase of B-50-IR in the nucleus gracilis after spinal cord hemisection, presumably in response to deafferentation from ascending input. These data support previous electron microscopic evidence of synaptic remodelling in the nucleus gracilis following bilateral dorsal column section [1,5]. Further quantitative light microscopic studies and ultrastructural analyses on postlesional changes of B-50-IR in this region are underway.

Although the immunohistochemical technique used here was sufficiently sensitive to detect small changes in the expression of B-50 (GAP-43) rostral to the hemisection, we found no evidence of altered B-50-IR caudal to the lesion. There was no postlesional increase in the constitutive level of B-50-IR, nor was there B-50 (GAP-43) expression in the normally unstained areas of the lumbosacral spinal gray distal to the lesion (i.e. in those regions which were disconnected from supraspinal descending pathways). Therefore, as in the previous study on the hemisected cat spinal cord [18], the concept of intraspinal sprouting and reinnervation of lumbosacral neurons in response to removal of descending tracts is not supported by the present data. However, sprouting of B-50 (GAP-43) negative fibers cannot be excluded. Therefore, quantitative analysis of tracing studies, employing DiI are currently being performed in this model. An alternative interpretation of the present results is that a lesion induced loss of descending B50-IR profiles is temporally and spatially matched by a corresponding increase of B-50-IR in dorsal root afferents and/or intrinsic spinal cord neurons. However, this is unlikely, since in the rat, the vast majority of the constitutive B-50-IR in the spinal gray derives from primary afferents and not from descending systems [2].

In a recent light microscopic study in cat, using low thoracic cord hemisection, Helgren and Goldberger [10] described a transient ipsilateral increase in the area of B-50-IR in superficial laminae of the lumbar dorsal horn and a permanent increase in the immunohistochemical staining for a specific antigen of primary afferents (revealed by the monoclonal antibody RAT 102) throughout the lumbar spinal gray. The authors regarded these data as indications for intraspinal sprouting of dorsal root axons and postulated that this mechanism was responsible for the postlesional recovery of motor function. However, the morphological data were inconclusive since the increased spread of RAT 102 labelling was not mirrored by a similar increase in B-50-IR. Indeed, the reported alterations of B-50-IR were restricted to superficial laminae of the dorsal horn. The apparent expansion of RAT 102 staining in the lumbar spinal gray ipsilateral to the lesion [10] may reflect an increased metabolic activity of already existing afferent fibres rather than the formation of new sprouts. This interpretation was already considered by Helgren and Goldberger [10] and is compatible with the functional enhancement of ipsilateral primary afferents which clearly contribute to the recovery of locomotion and postural reflexes after cord hemisection [8,10,14]. Therefore, the postlesional enhancement of the segmental afferent input may be induced by mechanisms not related to sprouting, such as alterations in the amount of neurotransmitters released and/or in the number of receptors in the target neurons for dorsal root input [25].

In conclusion, the differential expression of B-50-IR in target regions of supraspinal descending and ascending systems after low thoracic cord hemisection suggests that sprouting and reinnervation by unlesioned systems may be a regionally specific phenomenon rather than a generalized response to partial denervation in the CNS [11].

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