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SHORT COMMUNICATION
Sprouting and regeneration after pyramidotomy and blockade of the myelin-associated neurite growth inhibitors NI 35/250 in adult rats

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Abstract

After a selective unilateral lesion of the corticospinal tract (CST) at the level of the brainstem (pyramidotomy) and neutralization of the myelin associated neurite growth inhibitors NI-35/250 with the monoclonal antibody (mAb) IN-1, we had previously observed a strong behavioural recovery in parallel with an enhanced structural plasticity of the lesioned as well as the unlesioned CST. The present study focuses on the regenerative response of the cut CST axons at the lesion site in these adult rats. The results show a strong enhancement of regenerative sprouting of CST fibres by treatment with the mAb IN-1. Successful elongation of these sprouts through the pyramidal decussation and into the cervical spinal cord was also dependent on the presence of this antibody. In the spinal cord, regenerating fibres were rarely found in the position of the former CST; most of the fibres were distributed seemingly randomly over the entire lateral extent of the spinal cord.

Introduction

After large CNS lesions in adult higher vertebrates, spontaneous recovery is limited in contrast to the immature CNS. For example, after unilateral sensorimotor cortex removal or after transection of the pyramidal tract in neonatal rodents, considerable anatomical and functional plasticity has been demonstrated (e.g. see Leong & Lund, 1973; Barth & Stanfield, 1990). In addition to plastic changes, regeneration of lesioned fibres also occurs in the neonatal rodent spinal cord after corticospinal tract (CST) lesions (Bates & Stelzner, 1993).

In many regions of the developing rat CNS, the postnatal decrease of the neuronal growth associated protein-43 (GAP-43) and of the capacity for lesion-induced plasticity and regeneration closely correlate with the pattern and time course of myelin formation (Kapfhammer & Schwab, 1994). Disrupting myelin with an immunological treatment or by focal X-irradiation (e.g. see Keirstead *et al*., 1995; Vanek *et al*., 1998) can extend the growth-permissive period. Several myelin-associated proteins and proteoglycans have strong inhibitory properties for neurite growth *in vitro*. Neutralization of two of them, the neurite growth inhibitors NI-35/250 by the monoclonal antibody (mAb) IN-1 greatly decreases the inhibitory activity of oligodendrocytes and myelin *in vitro* (Caroni & Schwab, 1988) as well as *in vivo*, allowing long-distance regeneration and recovery of specific reflex and locomotor functions (Schnell & Schwab, 1990; Bregman *et al*., 1995).

The purpose of the present study was to investigate the response of CST fibres to a selective unilateral lesion at the brainstem level (pyramidotomy) and neutralization of the myelin-associated neurite growth inhibitors NI-35/250 in adult rats.

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Materials and methods

In this study a total of 18 Lewis rats of either sex, mean age 2.5 months (± 15 days) and mean weight 230 g (± 100 g) at the time of the lesion were included. All animals received unilateral lesions of the CST at the level of the medulla oblongata (pyramidotomy, PT). The animals were randomly divided into three groups: animals that underwent only a lesion (PT only, $n = 6$); lesioned animals treated with antibodies against myelin-associated neurite growth inhibitors (PT + mAb IN-1, $n = 6$); and animals with lesion and treatment with control antibodies (antibodies against horseradish peroxidase (PT + ant-HRP, $n = 6$). All animals were also part of a behavioural study (Z'Graggen et al., 1998) and were perfused 16 weeks after the lesion. All animal experiments were approved by the Veterinary Department of the Canton of Zurich.

Pyramidotomy and antibody application

A unilateral left pyramidotomy was performed under deep anaesthesia with a combination of ketamine and midazolam, at the level of the caudal medulla oblongata by using a ventral approach as described by Z'Graggen et al. (1998). To achieve a constant antibody supply, hybridoma cells secreting either the mAb IN-1 or anti-HRP as a control antibody (Schnell & Schwab, 1990) were implanted into the hippocampal region contralateral to the lesion as described in a

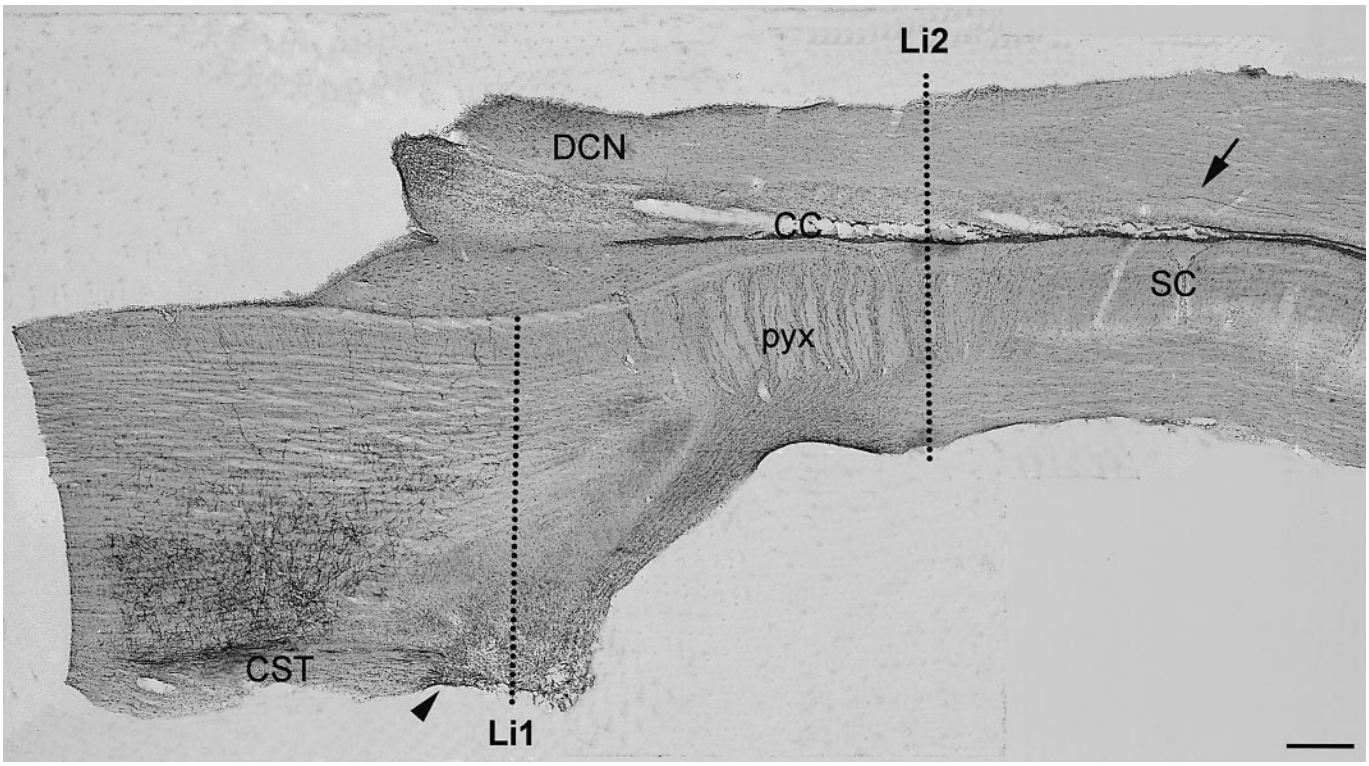

FIG. 1 Scheme of the position of the two lines (Li1 and Li2) used for quantitative analysis of sprouting and regenerating CST fibres (Li1 for sprouting index, Li2 for regeneration index). The black arrow points to a BDA-labelled CST fibre running ventrally in the dorsal funiculus of the cervical spinal cord of an IN-1-treated animal. Large arrow head shows the lesion site. CC,: central canal; CST, corticospinal tract; DCN, dorsal column nuclei; Pyx, pyramidal decussation; SC, spinal cord. Scale bar, 350 μm.

previous report (Z'Graggen et al., 1998). The animals were immune suppressed by cyclosporin A (10 mg/kg, i.p., Sandimmun, Novartis, Basel, Switzerland) for 8 days.

Tracing of the corticospinal tract

In all animals, the caudal forelimb area of the sensorimotor cortex (Neafsey *et al*., 1986) of the hemisphere corresponding to the lesioned pyramidal tract was pressure injected stereotaxically with the anterograde tracer biotin dextran amine (BDA; 10′000 MW, Molecular Probes, Eugene, OR, USA; 0.5 μL of a 10% solution; Z'Graggen et al., 1998). Two weeks later, all animals were killed with an overdose of pentobarbital and fixed by perfusion with a solution of 4% paraformaldehyde/5% sucrose in 0.1 M phosphate buffer. Sagittal frozen sections of the lesion site and the rostral 1.5 cm of the spinal cord were cut at 50 μm and reacted for BDA according to the semifree floating technique as described by Herzog & Brösamle (1997). Briefly, the sections were incubated overnight with an avidin-peroxidase complex (ABC elite, Vector Labs, Burlingame, CA, USA) and the colour reaction was achieved by using a nickel-enhanced diaminobenzidine protocol (DAB; Sigma, Buchs, Switzerland). The sections were air-dried, lightly counterstained with cresyl violet and coverslipped.

Analysis of axonal sprouting and regeneration

Sprouting of lesioned fibres was analysed by counting the intersections of BDA-labelled corticospinal fibres with a vertical (dorso-ventral) line positioned at the level of the lesion site (Li1 as shown in Fig. 1) on every section of the series. For the few missing sections an average of the values of the two preceding and of the two following sections was taken. The sum of all values was divided by the total number of sections to reveal the average number of crossing fibres and normalized as described below.

To quantify regenerating fibres, intersections of BDA-labelled CST fibres with a line positioned 1250 μm caudal to the posterior part of the inferior olive, i.e. ≈ 2 mm from the lesion site (Li2 in Fig. 1) were counted. The exact position of each BDA-labelled CST fibre was recorded on all sections of the complete uninterrupted section series.

In order to normalize for differences in the tracing efficiency of the individual animals, the fibre counts were divided by the total number of labelled corticospinal fibres estimated in the cerebral peduncle at the level of the pontine nuclei. For each animal, the same midpontine level was chosen on cross sections, and two consecutive sections were analysed. Electronic images were acquired with a Xillix microimager slow-scan, high-resolution CCD camera attached to a Zeiss axiophot microscope. First, the total cross-sectional area of the cerebral peduncle was measured by using a 10 × objective and the MCID-program (M2 Analysing Program, Imaging Research Inc., Ontario, CA). Then a square of 2975.5 μm^2 was placed four times in a systematic way over the area of the peduncle, and the BDA-positive fibres within these squares were counted at a magnification of 320 ×. This area corresponded to ≈ 3% of the total cerebral peduncle cross-section. The four values were averaged and the total number of BDA-positive fibres was extrapolated. The values obtained from two consecutive sections were averaged.

As the number of fibres present in the cerebral peduncle at the level of the pontine nuclei is higher than the number of fibres present in the cervical spinal cord, the sprouting and regenerative index obtained must be considered as relative values.

Results and discussion

The inhibitor-neutralizing antibody IN-1 enhances sprouting of lesioned CST axons

The lesion area, clearly visible by the cresyl violet staining, was always located at the level of the rostral part of the inferior olivary nucleus. The lesion transected the left CST completely, with little damage to the olive and no visible damage to other deeper structures. Animals with incomplete lesions (i.e. those where typical straight BDA-labelled fibres could be seen across the lesion site) were excluded from the 'regeneration analysis'. These were two animals in the mAb IN-1-treated group and two animals in the anti-HRP-treated group.

A qualitative comparison of the normal morphology of the CST and the lesioned tract at the lesion level revealed clearly that a large amount of sprouting occurred in all lesioned groups. These sprouts projected from the lesioned tract dorsally to the caudal brainstem, running around the inferior olivary nucleus as a dense network of fibres with numerous branch points and arborizations (Fig. 1). Some entered the scar region, running for limited distances before forming a bulbous end. The majority of these fibres grew for < 1 mm, and stopped before entering the decussation area. The quantitative analysis of the sprouts showed that treatment with the mAb IN-1 significantly enhanced the number of lesioned CST collaterals and sprouts at the lesion site. This enhancing effect was specific to the monoclonal IN-1 antibody since no significant difference was seen between the two control groups (lesion only versus lesion and control anti-HRP antibody) (Fig. 2A).

Elongation of regenerating CST fibres is dependent on the mAb IN-1

In the IN-1-treated rats, but not in the two control groups, fibres were frequently seen growing through the decussation and down the cervical spinal cord (Fig. 3). Quantification of the number of regenerating fibres was performed by counting fibres crossing a line positioned 2 mm caudal to the lesion site (Fig. 1). This distance was sufficient to distinguish regenerative elongation from local sprouting which rarely exceeded 1 mm. In the mAb IN-1-treated rats the number of fibres found 2 mm caudal to the lesion was significantly higher in all animals in comparison with the two control groups (Fig. 2B). A significant difference could also be seen between the two control groups. This hybridoma-dependent effect is thought to be due to acute and/or chronic release of factors secreted be the hybridoma cells and/or the surrounding tissue following the implantation. However, the position and morphology of the fibres in the IN-1-treated rats was often ectopic (see below) and clearly different from the occasional single fibres seen in the control groups. The position of these fibres in the normal CST and their straight course suggest that these were unlesioned fibres. The present observations were done 4 months after the lesion and 3.5 months after termination of the antibody treatment. Therefore, these regenerated fibres represent stable new axons and connections.

Regenerative fibres are randomly distributed across the spinal cord in mAb IN-1-treated animals

The position of all fibres present in the spinal cord at the Li2 level was recorded. This analysis allowed a three-dimensional reconstruction of the fibre distribution across the spinal cord. Despite a large variation between individual animals, the results clearly showed a seemingly random distribution of the regenerated CST fibres in the mAb IN-1 treated animals. For the statistical analysis, those fibres running

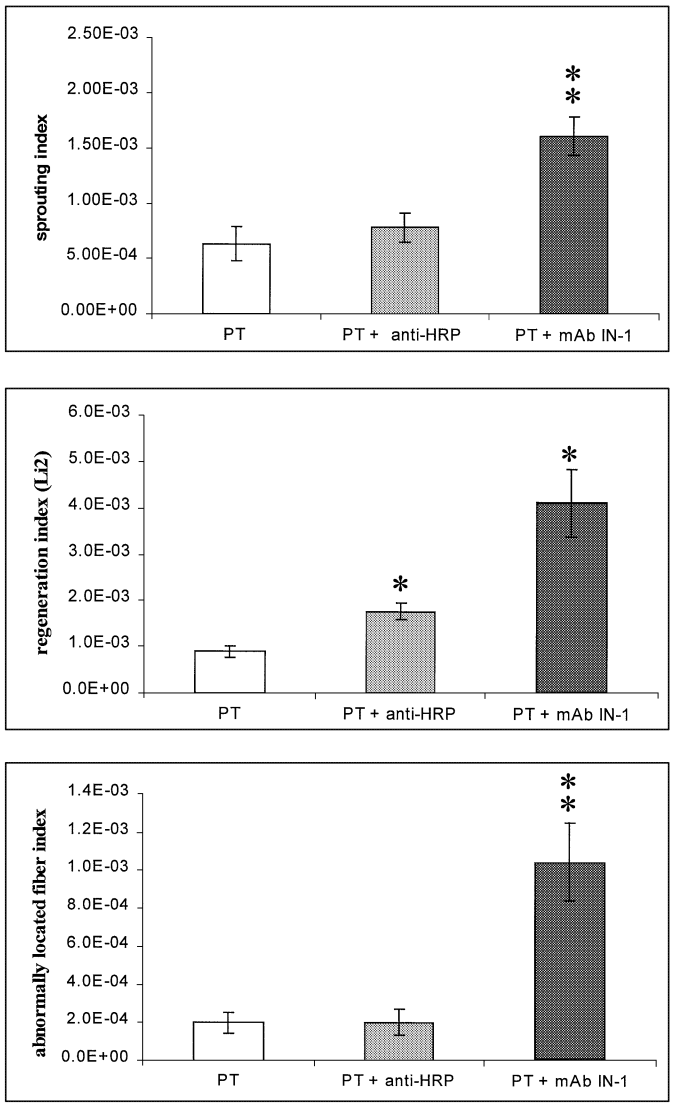

FIG. 2 (A) Sprouting index of CST fibres at the lesion site. The average number of fibres crossing level Li1 (see Fig. 1) per section was divided by the total number of BDA-labelled CST fibres as determined at the level of the pons for each animal. (B) Regeneration index given by the average number of fibres crossing level Li2 divided by the total number of BDA-labelled CST fibres at the level of the pons. (C) Index of abnormally located corticospinal fibres at the Li2 level. Abnormally distributed fibres were defined as fibres positioned outside the main, dorsal crossed and the minor ventral uncrossed CST. To normalize for differences in staining efficiency between individual animals, these values were divided by the total number of BDA-labelled CST fibres counted at the level of the pons. Means ± SEM are shown. $*P < 0.05$; $**P < 0.01$, ANOVA. PT, Pyramidotomy only; PT + anti-HRP, lesioned, control antibody-treated animal; PT + mAb IN-1, lesioned, mAb IN-1-treated animals.

ventrally contralateral to the lesion or dorsally ipsilateral to the lesion were defined as aberrant. Fibres with abnormal positions, but too close to the midline (< 200 μm), were not considered in the analysis to avoid false positive counts due to small aberrations in the section plane. In control groups only zero or one aberrant fibre per rat was detected. In contrast, all mAb IN-1-treated animals showed fibres in abnormal locations (4–9 fibres per rat). These fibres coursed dorsally to the inferior olive to project either ipsi- or contralaterally to the dorsal or the ventral part of the spinal cord, seemingly without particular preference (Fig. 3A and B). Quantification and statistical analysis of these results confirmed the high incidence of randomly distributed fibres in the animals treated with the mAb IN-1 (Fig. 2C).

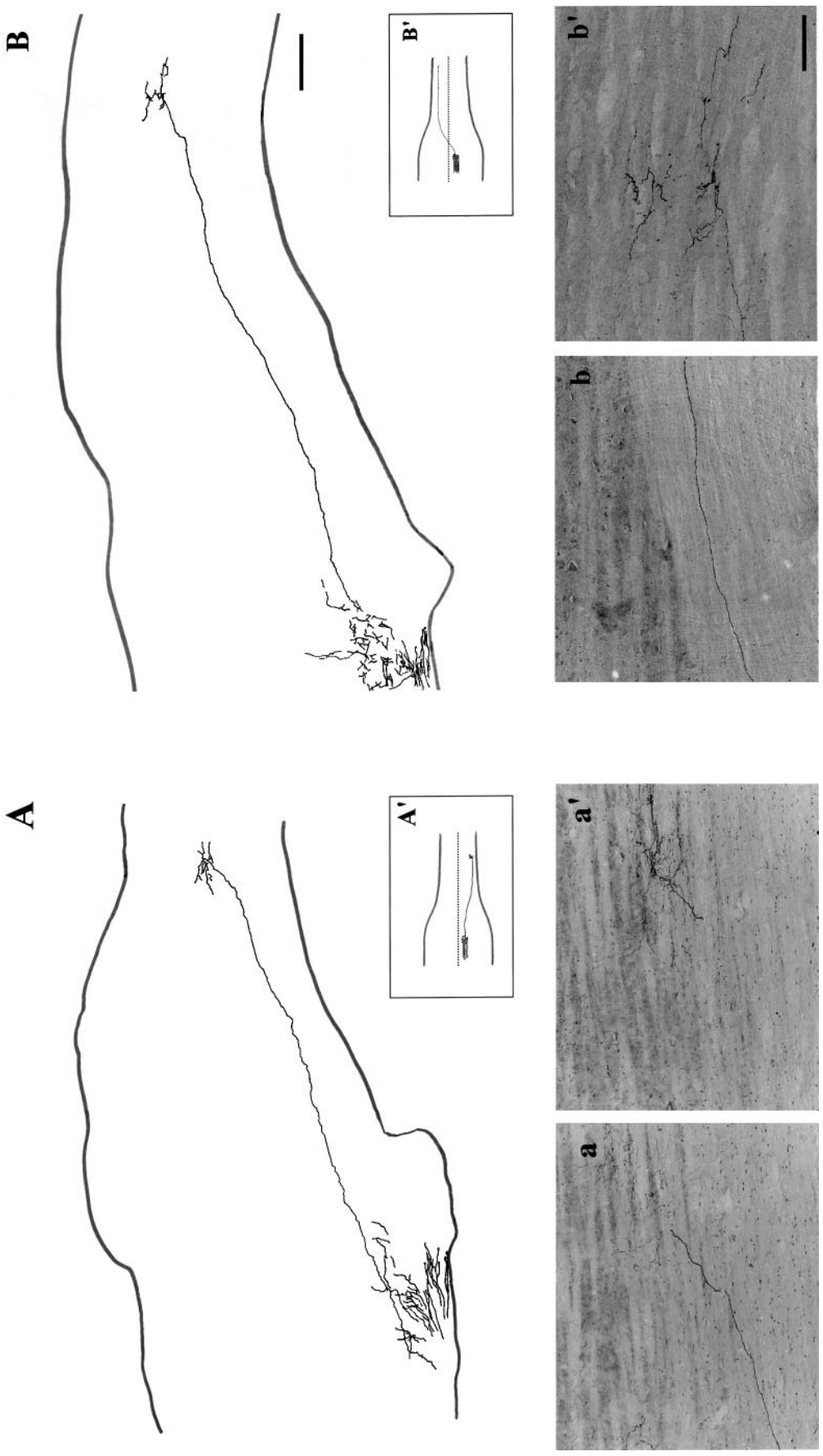

FIG. 3 Regenerated fibres in mAb IN-1-treated rats. (A) Camera lucida reconstruction (sagittal plane) of the brainstem and cervical spinal cord of a lesioned, mAb IN-1-treated animal. A regenerated fibre projects from the lesioned CST to the ipsilateral ventral spinal cord (A, dorsal view; dotted line represents the midline). (B) regenerated fibre projecting from the lesioned CST to the contralateral ventral spinal cord (B, dorsal view). (a, a′, b and b′) Photomicrographs of the course and the terminal arborization of the reconstructed regenerated fibres. Scale bar, 350 μm (A and B); 100 μm (a, a′, b and b′).

The ability of corticospinal axons to growth and regenerate after lesioning decreases dramatically during the first postnatal weeks (e.g. Kalil & Reh, 1982; Bates & Stelzner, 1993). Different mechanisms probably contribute to this developmental change. Nevertheless, the present results show that treatment with the mAb IN-1 can partially restore the regenerative ability of CST axons in the adult CNS. However, the numbers of regenerated fibres in the presence of the mAb IN-1 were low. This, together with the often abnormal localization of these fibres suggests that signals for pathfinding may no longer be present in the adult medulla oblongata and pyramidal decussation. Changes of the local tissue properties in the context of the lesion (scar formation and inflammation) have also to be considered, in addition to the intrinsic properties of adult neurons (for review, see Schwab & Bartholdi, 1996).

Studies of the red nucleus and the pons following unilateral pyramidotomy showed sprouting of cortico-bulbar fibres resulting in crossed, bilateral projections (Z'Graggen et al., 1998). As the innervation occurred in a topographically correct pattern, the persistence or re-expression of guidance or target-recognition cues at these anatomical sites is suggested. A similar phenomenon was observed for the intact, remaining CST at spinal levels (Thallmair *et al*., 1998). Behavioural studies showed a high degree of functional recovery of precision movements, grid walk and rope climbing in these rats (Z'Graggen et al., 1998) after mAb IN-1 treatment. The fact that there was no loss of food-pellet grasping abilities in these animals following a second lesion 1 mm rostral to the first one suggests that for this given behaviour, the regenerated CST axons do not play a significant role in the functional recovery. It is more likely that plastic reactions of the unlesioned CST (Thallmair *et al*., 1998) and of the lesioned CST at a subcortical level (Z'Graggen et al., 1998), or of other fibre systems, may underlie the functional recovery observed in the mAb IN-1-treated animals.

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Abbreviations

BDA, biotin dextran amine; CNS, central nervous system; CST, corticospinal tract; Li1, line positioned at the level of the lesion site; Li2, line positioned 1250 µm caudal to the posterior part of the inferior olive, i.e. ≈ 2 mm from the lesion site; GAP-43, growth associated protein-43; HRP, horseradish peroxidase; mAb, monoclonal antibody; NI, neurite growth inhibitor; PT, pyramidotomy.

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