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Increased lesion-induced sprouting of corticospinal fibres in the myelin-free rat spinal cord

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Abstract

Myelin contains potent inhibitors of neurite growth which have been implicated in the failure of long-distance regeneration of nerve fibres within the CNS. These myelin-associated neurite growth inhibitors may also be involved in the stabilization of neural connections by suppressing sprouting and fibre growth. After lesions of the CNS in neonatal animals, extensive rearrangements of the remaining fibre systems have been observed. In the rat, this plasticity of neuronal connections is severely restricted following the first few weeks of postnatal life, coincident with the progression of myelination of the nervous system. A well-studied example of postnatal plasticity is the sprouting of one corticospinal tract (CST) into the denervated half of the spinal cord after unilateral motor cortex or pyramidal lesions. In the hamster and rat, significant CST sprouting is restricted to the first 10 postnatal days. Here we show that very extensive sprouting of corticospinal fibres occurs after deafferentations as late as P21 if myelination is prevented by neonatal X-irradiation in the rat lumbar spinal cord. Sprouted fibres from the intact CST cross the midline and develop large terminal arbors in the denervated spinal cord, suggesting the establishment of synaptic connections. Our results suggest that myelin and its associated neurite growth inhibitors play an important role in the termination of neurite growth permissive periods during postnatal CNS development. Corticospinal sprouting subsequent to lesions early in life, i.e. in the absence of myelin-associated neurite growth inhibitors may explain the frequent occurrence of mirror movements in patients with hemiplegic cerebral palsy.

Introduction

After lesions of the developing nervous system, the remaining nerve fibres show a remarkable capability for rearranging their connections. Following early deletions of the central visual target nuclei, for example, optic fibres were induced to terminate in auditory centres (Metin & Frost, 1989; Pallas & Sur, 1994). After unilateral lesions of the superior colliculus in newborn hamsters, retinal fibres can be induced to recross the midline and terminate in the inappropriate remaining colliculus (Schneider, 1973). Typically, these major changes in fibre projections are limited to a critical period which ends at or shortly after birth in rodents.

One factor that might contribute to the termination of these critical periods is myelination of CNS tissue, since CNS myelin contains proteins that strongly inhibit neurite growth (Caroni & Schwab, 1988a,b; Savio & Schwab, 1990; Schnell & Schwab, 1990; reviewed in Schwab *et al.*, 1993). After neutralization of the inhibitory properties of CNS myelin by application of the monoclonal antibody IN-1, long-distance regeneration of lesioned corticospinal tract (CST) fibres has been demonstrated in the adult CNS (Schnell & Schwab, 1990, 1993; Bregman *et al.*, 1995). Myelin-associated neurite growth inhibitors could contribute to the limitation of the critical period by rendering the CNS microenvironment inhibitory for growing and sprouting nerve fibres (Kapfhammer & Schwab, 1994a,b; Kapfhammer, 1996). Evidence in support of this hypothesis comes from experiments in which the critical period for regeneration of lesioned descending fibre tracts in the chick or opossum spinal cord could be extended by eliminating oligodendrocytes and preventing myelination or by the antibody IN-1, respectively (Keirstead *et al.*, 1992; Varga *et al.*, 1995a,b). Similarly, increased sprouting of regenerating retinal fibres was found in the optic tectum of young hamsters in the presence of the IN-1 antibody (Kapfhammer *et al.*, 1992). When myelination in the lumbar part of the spinal cord is prevented by neonatal X-irradiation, the expression of the growth-associated protein GAP-43 is strongly increased (Kapfhammer & Schwab, 1994b). This increase in GAP-43 expression is correlated with a significant increase in collateral sprouting of primary afferents in myelin-free spinal cords after dorsal root lesions (Schwegler *et al.*, 1995).

In the case of the CST, extensive sprouting of corticospinal fibres across the midline into the denervated spinal cord has been demonstrated in hamsters and rats after unilateral pyramidal or cortical lesions in the first postnatal week (Leong & Lund, 1973; Hicks & D'Amato, 1975). Sprouting then declines and becomes very sparse

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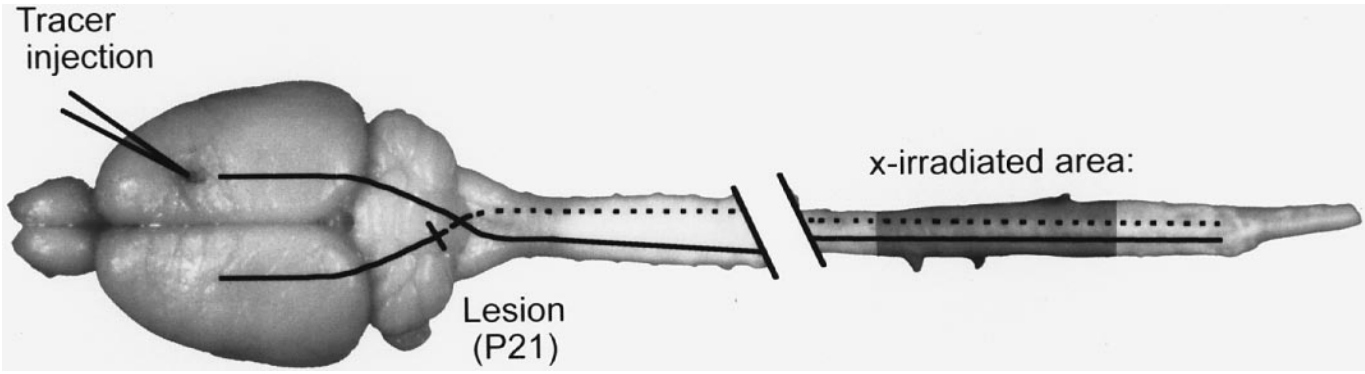

FIG. 1. Schematic illustration of the experimental procedures. Newborn rats received X-irradiation of the lower thoracic and lumbar spinal cord in order to suppress myelination. Unilateral lesions of the corticospinal tract (CST) were performed at the level of the pyramid at P21. The tracer wheat germ agglutinin–horseradish peroxidase or biotinylated dextran amine (WGA-HRP or BDA) was injected into the motor cortex contralateral to the lesion site. After a survival time of 2 weeks from the day of the lesion animals were perfused at P35 and sprouting of CST fibres was analysed in the myelinated cervical and upper thoracic, and in the X-irradiated lower thoracic and lumbar area of the spinal cord.

after lesions made after the second postnatal week (Hicks & D'Amato, 1970, 1975; Leong, 1976; Kuang & Kalil, 1990). This time course corresponds to the increase of myelin in the spinal cord grey matter (Schwab & Schnell, 1989; Kapfhammer & Schwab, 1994a). Sprouting of CST-fibres into the denervated half of the spinal cord is of particular clinical interest because it is likely to occur in humans after early acquired brain damage. In subjects with hemiplegic cerebral palsy due to perinatal injury after preterm deliveries, mirror movements which may correlate with sprouting of the CST are a common finding. In contrast, these bilateral movements rarely occur after brain lesions acquired in later life (Carr *et al.*, 1993; Cao *et al.*, 1994).

We have now directly tested whether myelination contributes to the termination of the critical period for CST sprouting after unilateral lesions. After prevention of myelination by neonatal X-irradiation, pronounced sprouting of CST fibres into the denervated half of the spinal cord was found well after the end of the critical period. Our results strongly suggest that myelin-associated neurite growth inhibitors contribute to the termination of the growth-permissive period for the CST in postnatal life.

Materials and methods

Lesions

Unilateral lesions of the CST were performed at postnatal day 21 (P21) in Lewis rats. Rats were anaesthetized by an intraperitoneal injection of Hypnorm (Janssen; 0.3 mg/kg body weight) and Dormicum (Roche; 0.6 mg/kg body weight). The medullary pyramids were exposed by a ventral approach through an opening of the occipital bone as described by Kalil & Reh (1982). The left CST was transected rostral to the decussation using a fine tungsten needle with the basilar artery serving as a landmark for the midline. CST fibres were traced with wheat germ agglutinin–horseradish peroxidase (WGA-HRP) or biotinylated dextran amine (BDA) as described below and killed 2 weeks after the lesion (Fig. 1).

The animals were divided into the following groups: (i) unlesioned, traced with WGA-HRP ($n = 4$); (ii) unlesioned, X-irradiated, traced with WGA-HRP ($n = 7$); (iii) lesioned, traced with WGA-HRP ($n = 10$); (iv) lesioned, X-irradiated, traced with WGA-HRP ($n = 9$); (v) lesioned, traced with BDA ($n = 9$) lesioned, X-irradiated, traced with BDA ($n = 6$). All animal experiments were carried out under supervision of the cantonal veterinary department.

Neonatal X-irradiation

Rats were irradiated at day of birth (P0) and at P3 as described by Savio & Schwab (1990). After anaesthesia by hypothermia the animals were placed on their sides: The lower thoracic and lumbar spinal cord was exposed to a dose of 55 GY of 50 kV X-rays (Phillips), whereas the rest of the body was protected by a lead shield.

Anterograde tracing of the corticospinal tract

WGA-HRP

The right motor cortex (contralateral to the lesion) was injected with WGA-HRP (Sigma) at P33, i.e. 12 days after the CST lesion (Fig. 1). Two microlitres of 5% WGA-HRP were injected into the motor area (3–4 injection sites). After a survival time of 36–48 h animals were killed by an overdose of pentobarbital (450 mg/kg body weight) and perfused transcardially with Ringer's solution containing 0.25% $NaNO_2$ and 100 000 units/L heparin, followed by 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The brain and spinal cord were dissected, postfixed for 2 h at 4 °C and stored in a 30% sucrose solution for 36 h at 4 °C for cryoprotection. The spinal cord was then divided into five parts of equal length, embedded in Tissue Tek and frozen by immersion in isopentane at –40 °C. Cross sections of 40 μm were cut on a cryostat. Sections were mounted on superfrost/plus slides (Menzel-Gläser, Germany) and processed for peroxidase activity using tetramethylbenzidine (TMB; Sigma, St Louis, MO) as a substrate and sodium nitroferricyanide as stabilizing agent (Mesulam, 1978).

BDA

The right motor cortex was injected with BDA (Molecular Probes, Eugene, OR, USA) at the day of the lesion: 2.5 μL 10% BDA were distributed over 4–5 injection sites. Animals were perfused after 14 days as described above but with 4% paraformaldehyde, 5% sucrose in 0.1 M PB at pH 7.4. Cryostat sections were collected in cold 0.1 M PB, rinsed 3 × 30 min in TBS-X (50 mM Tris; 0.9% NaCl; 0.5% Triton X-100; pH 8.0) and incubated overnight with an avidin–biotin–peroxidase complex (Vectastain ABC Elite Kit, Vector Burlingame, CA, USA; 1 : 100 in TBS-X) at room temperature. After 3 × 30 min washing in TBS-X the sections were rinsed with 50 mM Tris-HCl (pH 8.0) and preincubated in 0.4% nickel ammonium sulphate in 50 mM Tris-HCl for 10 min. Sections were further

preincubated for 10 min in the nickel ammonium sulphate solution to which 0.015% of 3,3′diaminobenzidine tetrahydrochloride (DAB; Sigma) was added and finally reacted in a nickel ammonium/DAB mixture containing 0.004% H_2O_2. After 10–30 min the reaction was stopped with 50 mM Tris-HCl and the sections were rinsed 3 × 10 min in 50 mM Tris-HCl. Sections were mounted on slides, dehydrated and embedded in Eukitt (Kindler, Germany).

Immunohistochemistry

For antibody staining against MBP, the IN-1 antigen and GAP-43, 25 μm cryostat sections of formalin-fixed spinal cord tissue were cut, dried and pretreated with 95% ethanol/5% acetic acid for 25 min at 4 °C. Sections were rehydrated, rinsed in phosphate-buffered saline (PBS) for 5 min, blocked with 5% bovine serum albumin (BSA, Sigma) and incubated with antibody solution overnight at 4 °C. Antibodies were diluted in 5% BSA in PBS to the following concentrations: monoclonal antibody against myelin basic protein (mouse anti-MBP, Boehringer Mannheim, Germany) 1 : 500 and monoclonal antibody directed against GAP-43 (antibody 10E8, Meiri *et al.*, 1991) 1 : 10 with 0.1% digitonin. For the monoclonal antibody IN-1, hybridoma supernatant of cells grown in Iscove medium supplemented with 10% foetal calf serum was used undiluted. After several rinses with PBS, sections were incubated with either rat adsorbed biotinylated antimouse immunoglobulin (1 : 100 in 5% BSA, Vector) for MBP or biotinylated antimouse immunoglobulin (1 : 200 in 5% BSA, Vector) for IN-1 and GAP-43. Sections were further processed for peroxidase activity as described above using DAB as a chromogen. Some sections were stained for general histology with 0.125% cresyl violet. Immunohistochemical staining procedures were always performed in the same batch under identical conditions for sections of normal spinal cord or X-irradiated spinal cord. Micrographs were taken at identical exposure times for X-irradiated and control sections (Fig. 2).

Quantitative analysis of CST sprouting in spinal cord cross sections

Only spinal cords that fulfilled the following criteria were selected for quantitative analysis: (i) complete lesion of the right CST (checked after dissection and histologically in a series of animals), and (ii) absence of myelin in the irradiated part of the spinal cord. Sprouting was quantified in transverse sections of spinal cords at the lumbar level, either traced with WGA-HRP (Fig. 6) or traced with BDA (Fig. 7).

WGA-HRP

Three to four sections per animal were selected blindly from coded section series for quantification. The dorsal half of the spinal cord grey matter was divided into three sectors on each side: A, B, C and A', B', C' (see Fig. 6). In each of these sectors grains of HRP reaction product were counted under darkfield illumination with polarized light at a magnification of 400×. Grains of labelled fibres were easily distinguishable from stained catalase-positive erythrocytes and occasional artefacts of the HRP reaction. Counting was performed in the region showing the densest labelling in each sector within a square of 0.06 × 0.06 mm. The counts for each sector were averaged over the three to four sections evaluated, and the relative amount of labelling in all the sectors was calculated as a percentage of the labelling in sector A (= 100%). This eliminated differences between individual animals due to the inherent variability of the tracing. These relative percentage values were then compared for the two lesioned groups (X-irradiated vs. control) and tested for statistical significance using the Mann–Whitney test.

BDA

Two to three sections were evaluated for each animal by counting the intersections of BDA-labelled CST fibres with four lines placed on the sections as shown in Fig. 7, upper half). A and A' were placed near the midline, the lateral border of the CST serving as a landmark. Line B and B' were placed 1.3 mm lateral to the midline. BDA-labelled CST fibres crossing the lines were counted under brightfield illumination at a magnification of 100×. The averaged values for the denervated side were related to the values of the normal side (A'/A and B'/B), thus eliminating differences in the tracing efficiency of the individual animals. Counts for X-irradiated and control animals were compared and tested for statistical significance using the Mann–Whitney test.

Results

X-irradiation generates myelin-free areas in the spinal cord

The capacity of X-ray irradiation for generating myelin-free areas of the spinal cord with the loss or strong reduction of myelin antigens and the myelin-associated neurite growth inhibitors has been demonstrated previously (Gilmore, 1963; Savio & Schwab, 1990; Kapfhammer & Schwab, 1994b; Schwegler *et al.*, 1995). In addition, it has been shown that the growth-associated protein GAP-43 is strongly increased in the myelin-free spinal cord (Kapfhammer & Schwab, 1994b). In this study we have used immunostaining of transverse spinal cord sections at P35 for myelin basic protein (MBP), the IN-1 antigen and the growth-associated protein GAP-43 to determine the efficacy of the X-irradiation. MBP was used as a marker for myelin proteins, the IN-1 antibody detects the myelin-associated neurite growth inhibitors NI35/250 (Caroni & Schwab, 1988b; Rubin *et al.*, 1994). As shown in Figure 2, X-irradiation resulted in a strong reduction of MBP staining (Fig. 2A,B) and a virtual loss of IN-1 antigen (Fig. 2C,D). Only few MBP-immunoreactive fibres were seen in the ventral and dorsal funiculi. GAP-43 protein was strongly upregulated in the myelin-free spinal cord (Fig. 2E,F). The X-irradiation therefore was effective in reducing the myelin content and the presence of myelin-associated neurite growth inhibitors in the lumbar spinal cord

The corticospinal tract projects predominantly contralateral in the normal and myelin-free spinal cord in non-lesioned rats

In the rat, the CST projects predominantly to the contralateral half of the spinal cord. After tracing with WGA-HRP, the CST contralateral to the injection site was heavily labelled and terminal labelling was mainly limited to the spinal cord grey matter contralateral to the injection site (Fig. 3A,B). The heaviest labelling was found in the dorsal horn, but a projection to the ventral horn was also present. Little label was present in the ipsilateral half of the spinal cord. In the myelin-free spinal cord the labelling density in the grey matter appeared to be slightly increased compared to control spinal cord. Nevertheless, the vast majority of the label was found contralateral to the injection site, with few labelled terminals on the ipsilateral side (Fig. 3C,D). The termination pattern of the CST was thus rather normal in the myelin-free spinal cord.

After unilateral CST lesions at P21, sprouting to the ipsilateral side is sparse and restricted to the medial third of the spinal cord in control rats

After unilateral lesions of the CST at P21 and survival to P35, a small increase in CST fibres on the ipsilateral side of the spinal

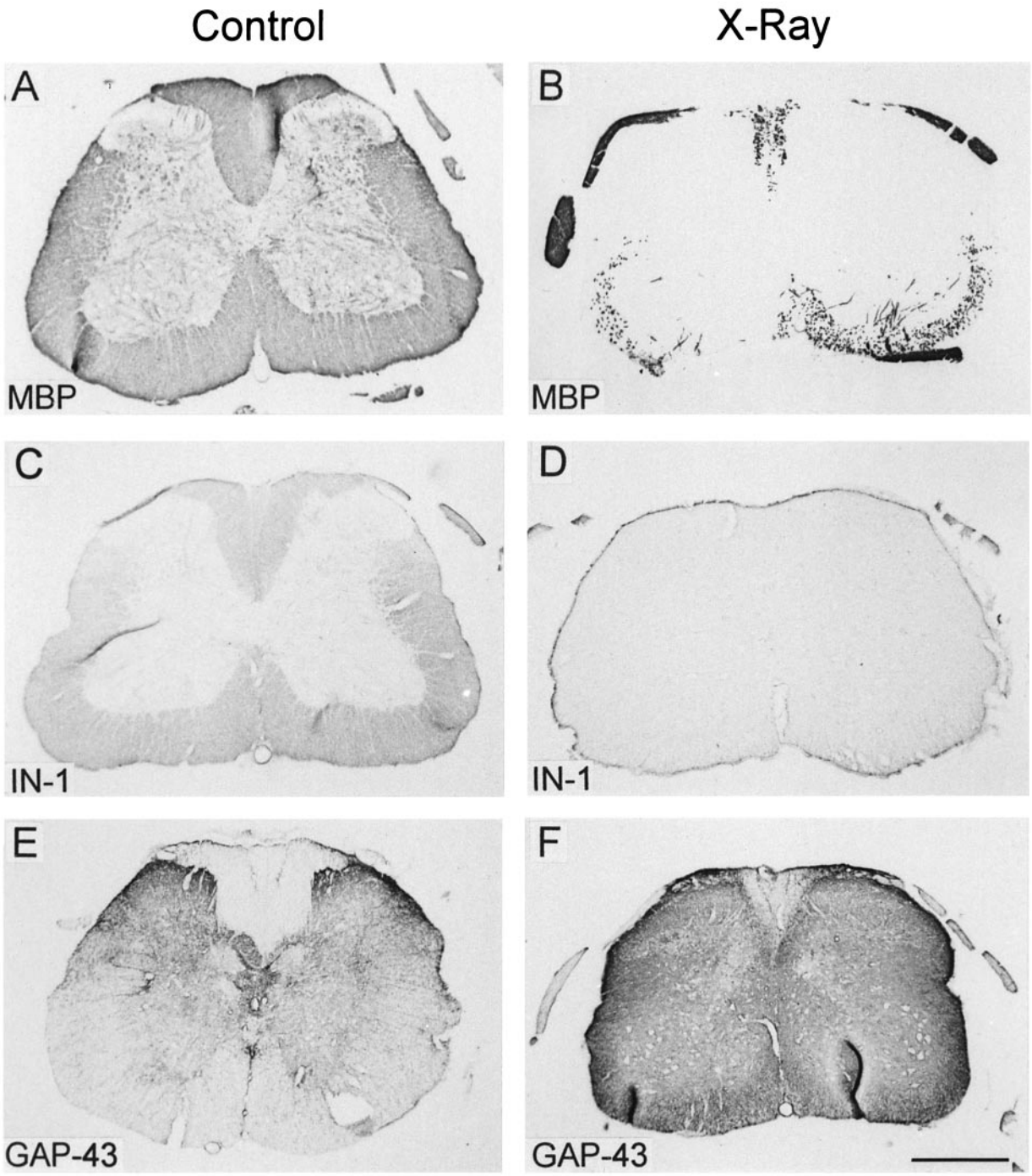

FIG. 2. Immunohistochemical stainings for evaluation of myelin suppression by the X-irradiation. Transverse sections of the normal (A, C, E) and X-irradiated (B, D, F) lumbar spinal cord at P35 reacted with antibodies against myelin basic protein (MBP), myelin-associated neurite growth inhibitors (IN-1 antigens NI35/210), and GAP-43. MBP-staining shows myelination in spinal cord grey and white matter (A). In the X-irradiated spinal cord MBP expression (B) was strongly reduced with a few remaining myelinated fibres in the ventral and ventro-lateral funiculus. Immunoreactivity for the myelin-associated neurite growth inhibitors as detected by staining with the monoclonal antibody IN-1 was virtually absent in the X-irradiated spinal cord (C, D). Immunoreactivity for the growth associated protein GAP-43 was strongly increased in the X-irradiated spinal cord (E, F). Scale bar = 500 μm.

cord could be found (camera lucida drawings Fig. 4A–C, original micrographs Fig. 5A–C). These fibres most likely represent sprouts from contralateral corticospinal fibres because they were restricted to the medial third of the ipsilateral spinal cord. Profiles of fibres crossing the midline of the spinal cord could be seen (Fig. 4C). These ipsilateral fibres appeared more frequently in the lumbar level of the spinal cord as compared to the cervical level. Sprouting from intact corticospinal fibres after unilateral CST lesions at P21 therefore appeared to take place, but it was very sparse and restricted to the medial third of the ipsilateral spinal cord grey matter.

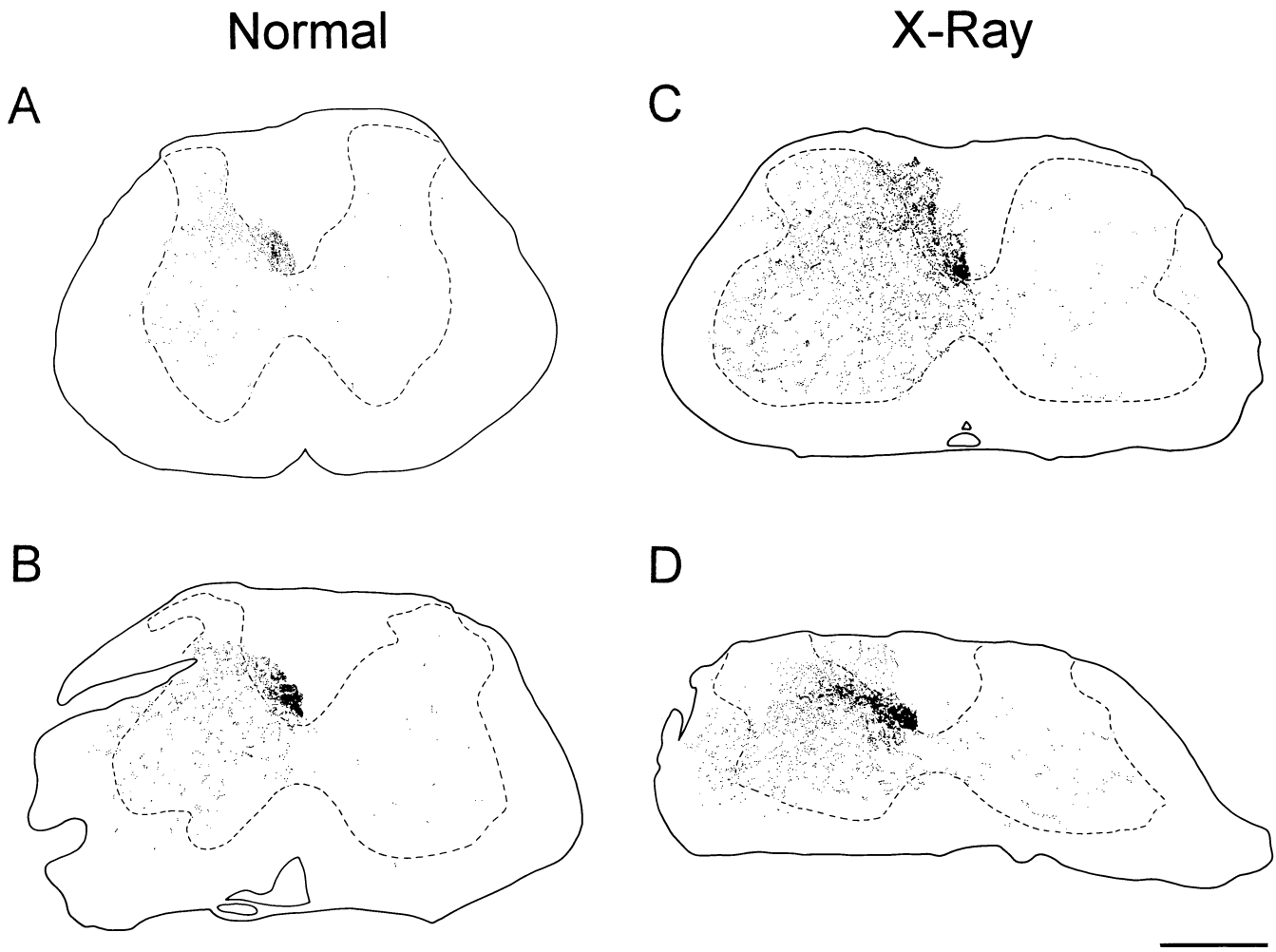

FIG. 3. Termination pattern of corticospinal fibres in normal and X-irradiated spinal cord without a corticospinal tract (CST) lesion.. Camera lucida drawings of the CST after wheat germ agglutinin–horseradish peroxidase (WGA-HRP) tracing on transverse sections of the lumbar spinal cord of normal and X-irradiated rats without a CST lesion. In the normal and myelin-free spinal cords CST fibres and terminals are confined to the side of the spinal cord contralateral to the tracer injection. The few labelled fibres on the ipsilateral side represent the very minor uncrossed portion of the CST. Scale bar = 500 μm.

In the myelin-free spinal cord, sprouting of CST fibres was increased and extended into the lateral third of the spinal cord

Collateral sprouting after unilateral CST-lesion at P21 was greatly enhanced in the myelin-free spinal cord. The numbers of labelled CST fibres on the denervated side were clearly increased compared with control animals and were distributed over the whole extent of the spinal cord grey matter (camera lucida drawings Fig. 4D–F, original tracing micrographs Fig. 5D–F). Fibres crossing the midline were seen frequently indicating that the sprouted fibres most likely crossed the midline at the level of the spinal cord. As in normal spinal cord, the terminal labelling was more dense in the dorsal than in the ventral horn. The majority of the sprouted fibres were present in the medial third of the spinal cord grey matter (Figs 4 and 5). In the myelin-free spinal cord labelled fibres were also present in the middle and lateral third of the spinal cord, regions where fibres were very rarely observed in control animals (Fig. 4).

Quantitative analysis of sprouting of CST fibres

In order to quantify the sprouting response of CST fibres we have quantified the amount of labelled fibres on the denervated half of the spinal cord both in animals traced with WGA-HRP and BDA. Grains of WGA-HRP reaction product reflecting labelled CST fibres were counted in different sectors of the spinal cord, the values of three–four sections averaged and expressed as the percentage of the label counted in sector A (intact side). Therefore, the variability in the labelling intensity from animal to animal was corrected. The results are shown in Fig. 6. On the intact side of the spinal cord labelling intensity was similar for myelin-free and control animals. In contrast, the labelling intensity was strongly increased on the denervated side in myelin-free animals compared with myelinated controls. Values were increased approximately twofold in the central third and two to threefold in the middle and lateral third of the spinal cord grey matter. These differences were statistically significant (Mann–Whitney Test, P-value < 0.001 central third; P-value < 0.05 middle and lateral third). Very similar results were obtained using BDA as a tracer for the CST. The density of BDA-labelled fibres in the denervated half of the spinal cord was greatly increased in the X-irradiated part of the spinal cord (Fig. 7). In the area near the midline, the number of fibres

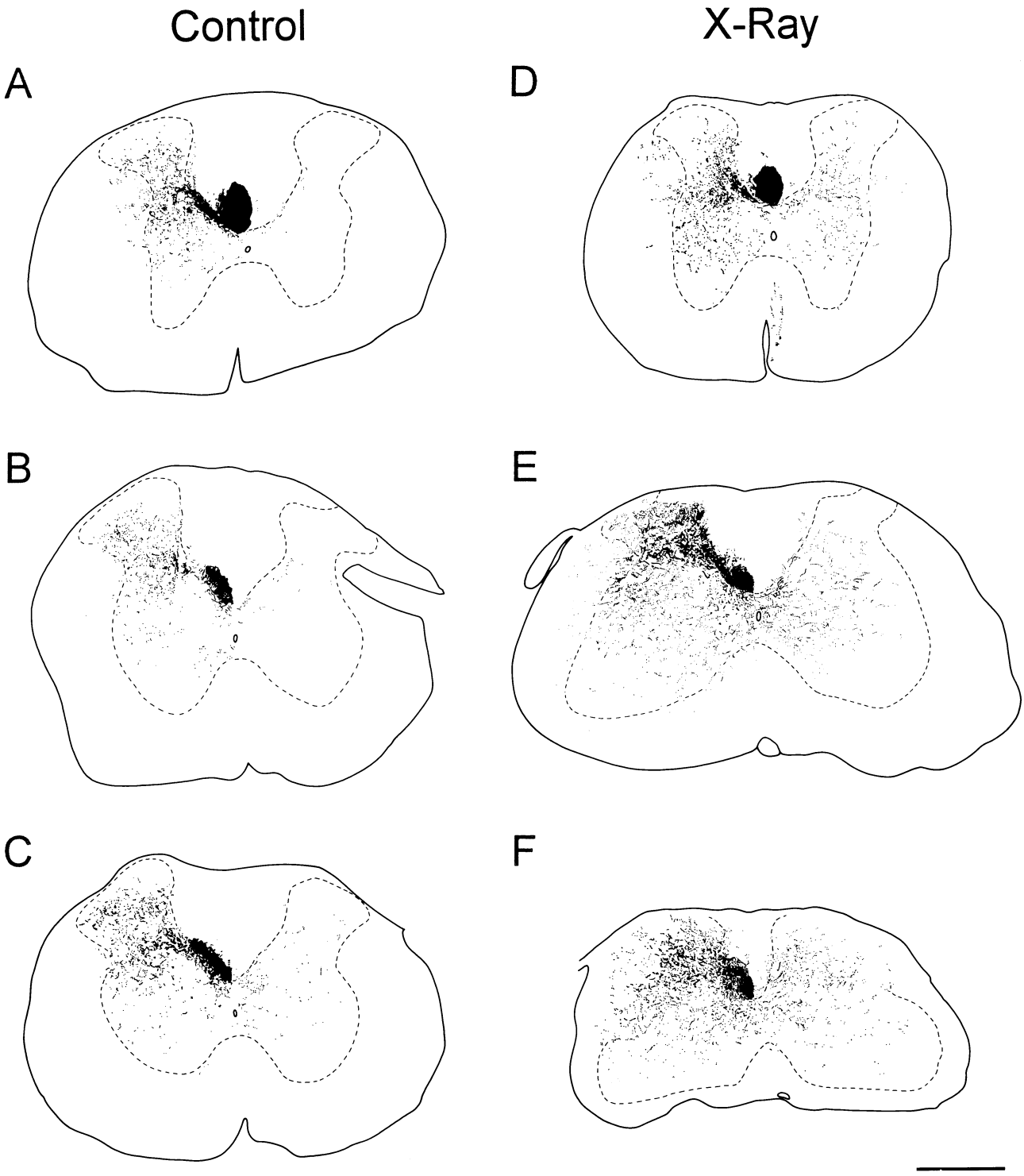

FIG. 4. Termination pattern of corticospinal fibres in control and X-irradiated spinal cord two weeks after a corticospinal tract (CST) lesion. Examples of camera lucida drawings of the CST after wheat germ agglutinin–horseradish peroxidase (WGA-HRP) tracing on transverse sections of the lumbar spinal cord of lesioned control and X-irradiated, myelin-free rats (lesion at P21, survival time 14 days). In the control spinal cord (A–C) only few labelled profiles are seen on the denervated side. Some of these labelled profiles probably represent sprouts arising from the intact CST. In the X-irradiated spinal cord (D–F) CST fibres on the denervated side of the spinal cord are abundant and extent over the entire dorsal and ventral horn. Scale bar = 500 μm.

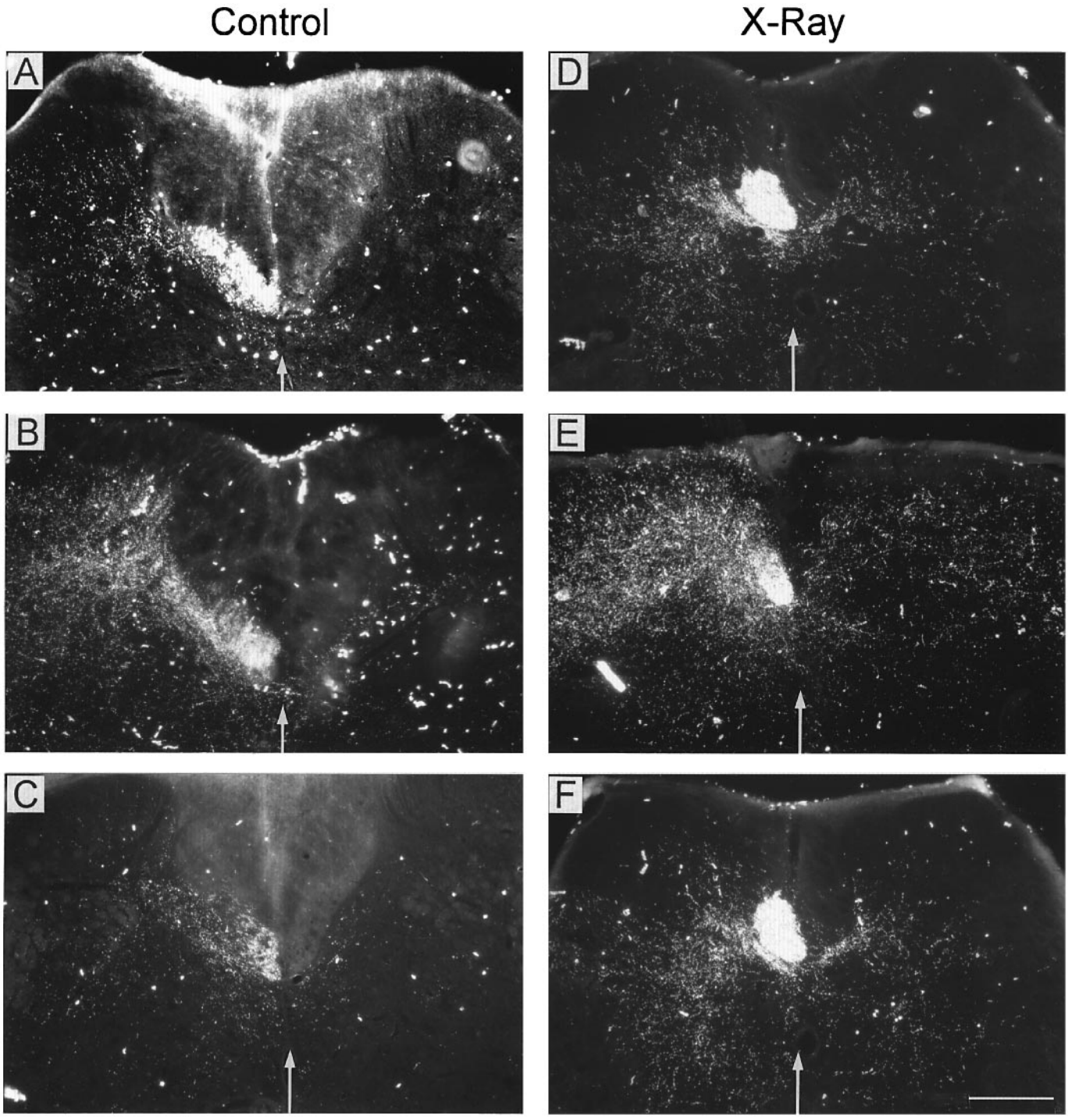

FIG. 5. Wheat germ agglutinin–horseradish peroxidase (WGA-HRP) tracing of corticospinal fibres in control and X-irradiated spinal cord two weeks after a corticospinal tract (CST) lesion. Darkfield micrographs of transverse sections showing WGA-HRP traced corticospinal tracts 14 days after a unilateral CST lesion. In control spinal cords (A–C) labelled fibres are mainly confined to the intact side, contralateral to the tracer injection. In the X-irradiated spinal cords (D–F) many labelled fibres are present on the denervated side of the spinal cord, ipsilateral to the tracer injection, indicating sprouting of corticospinal fibres. In some cases (e.g. F) fibre bundles crossing the midline of the spinal cord can be seen. The position of the midline is indicated by a white arrow. Scale bar = 200 μm.

found in the denervated half of myelin-free spinal cords was close to 90% of those detected on the intact side, compared with about 60% in the myelinated controls (P-value < 0.01). In the lateral part of the denervated spinal grey matter, BDA-labelled CST fibres were threefold more frequent in myelin-free animals (P-value < 0.05). These results show that sprouting from the intact CST into the denervated side of the spinal cord is greatly increased in the absence of myelin.

Terminal arbors of sprouted CST fibres

The better resolution of BDA as a tracer for the CST allowed analysis of the terminal arbors of the sprouted corticospinal fibres.

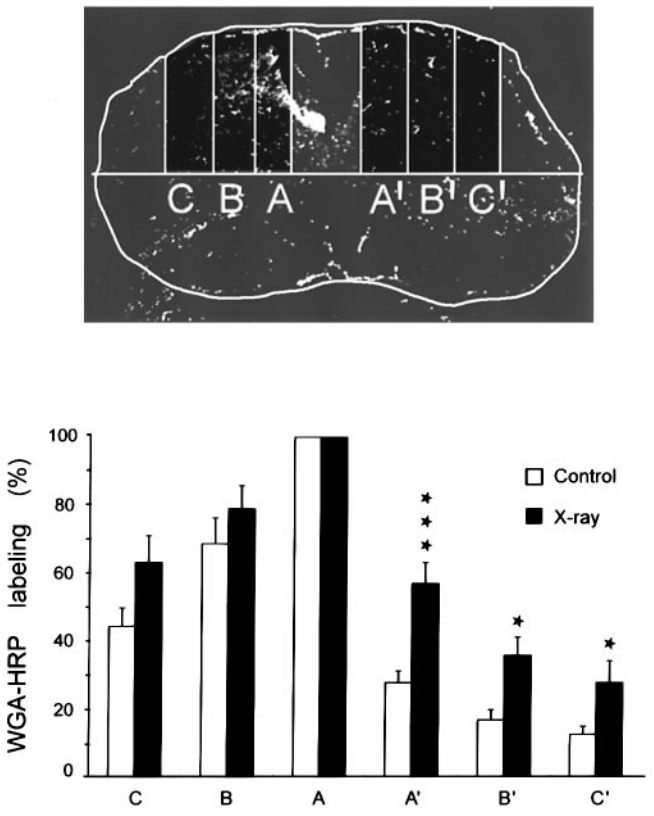

FIG. 6. Quantitative analysis of sprouting from wheat germ agglutinin–horseradish peroxidase (WGA-HRP) labelled corticospinal tract (CST) fibres after unilateral lesions in the control and X-irradiated spinal cord. Labelled fibres (WGA-HRP-grains) were counted on transverse sections of control and X-irradiated spinal cords after unilateral CST lesions. The spinal cord grey matter adjacent to the dorsal columns was divided into three parts contralateral (A, B, C) and ipsilateral (A', B', C') to the side of the tracer injection (upper part). The value for part A was set to 100% and the remaining counts were expressed as percentages of this value in order to control for general differences in labelling intensity between the animals. Counts from three to four sections were averaged. Results (mean value + SEM) are shown in the lower part of the figure. White bars represent counts from control spinal cords ($n = 10$), black bars those from X-irradiated spinal cords ($n = 9$). The intensity of labelling is similar on the intact side for control and X-irradiated spinal cords. On the denervated side labelling is much more intense in the X-irradiated spinal cords indicating an increased sprouting of CST fibres. Differences in labelling intensity between control and X-irradiated spinal cords on the denervated side were statistically significant with $P < 0.001$ (indicated by ***) or $P < 0.005$ (indicated by *) in the Mann–Whitney test.

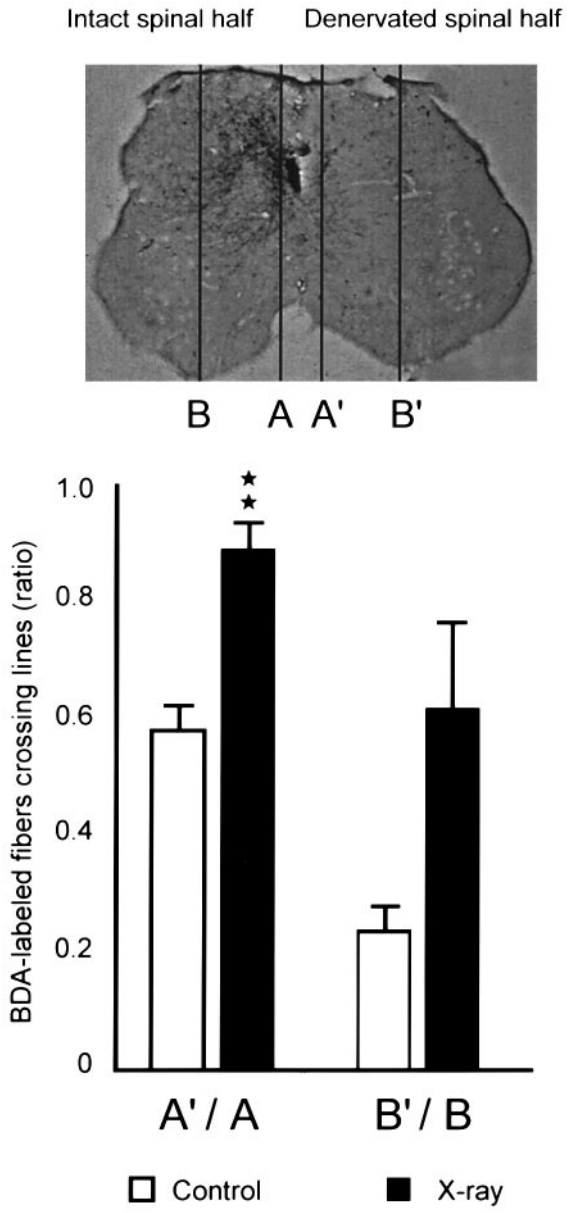

FIG. 7. Quantitative analysis of sprouting from biotinylated dextrane amine (BDA)-labelled corticospinal tract (CST) fibres after unilateral lesions in the control and X-irradiated spinal cord. Intersections of BDA-labelled CST fibres with four lines placed on the sections are shown in the upper part. A and A' were placed near the midline, the lateral border of the CST serving as a landmark. Line B and B' were placed 1.3 mm lateral to the midline. BDA-labelled CST fibres crossing the lines were counted under brightfield illumination at a magnification of 100x. The averaged values for the denervated side were related to the values of the normal side (A'/A and B'/B). In the area near the midline, the number of fibres found in the denervated half of myelin-free spinal cords was close to 90% of those detected on the intact side, compared to about 60% in the myelinated controls (**P-value < 0.01, Mann–Whitney test). In the lateral part of the denervated spinal grey matter, BDA-labelled CST fibres were threefold more frequent in myelin-free animals (P-value < 0.05) indicating an increased sprouting of CST fibres

Figure 8 shows two examples of CST fibres on the denervated side of the myelin-free spinal cord. The fibres are derived from the intact CST. They cross the midline, either through the dorsal commissure (Fig. 8A) or sometimes through the territory of the lesioned, degenerated CST (Fig. 8A). The shapes of the terminal arbors of the sprouted CST fibres resemble those of normal CST axons. This termination pattern and the occurrence of bouton-like structures indicate that the sprouted fibres might make specific contacts to denervated target cells.

Discussion

The capacity of CNS axons to sprout in response to lesions is typically high in the early postnatal time period and declines with increasing maturation of the nervous system. The factors contributing to the reduced sprouting in the mature nervous system are largely unknown. We have now shown that the permissive period for sprouting of the CST can be extended by preventing myelination and, thereby, the expression of myelin-associated neurite growth inhibitors. This

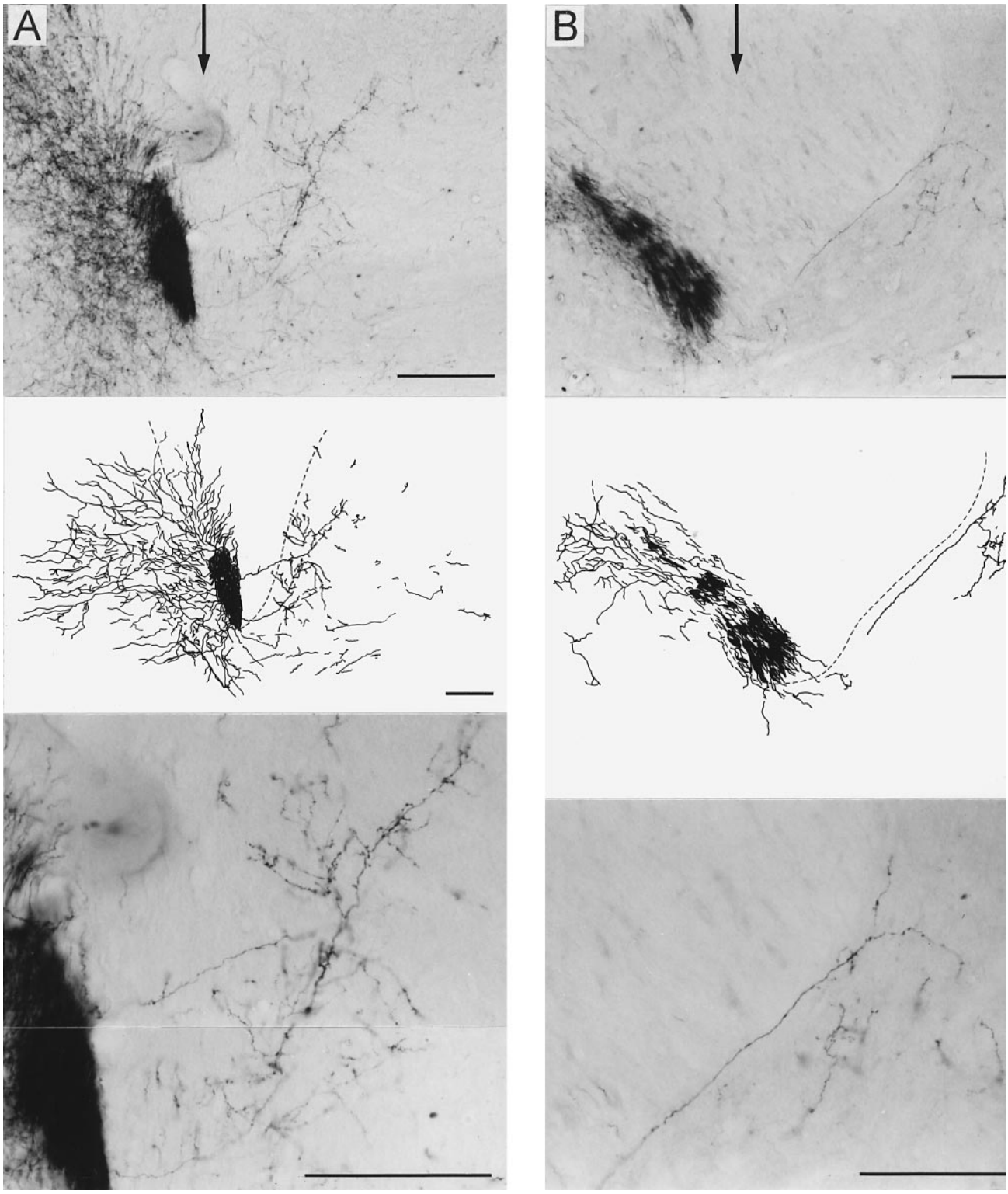

FIG. 8 Morphology of sprouted corticospinal tract (CST) fibres after tracing with biotinylated dextrane amine (BDA). Two examples (A, B) of the morphology of CST fibres sprouted to the denervated side of the spinal cord as seen after tracing with BDA. Original micrographs at intermediate magnification (upper row), low magnification camera lucida drawings and high magnification (lower row) of the same sections. Note that the sprouted fibres develop extensive and apparently normal terminal arbors and terminal boutons, suggesting the establishment of synaptic connections to target cells in the spinal cord grey matter. Scale bars = 100 μm.

strongly suggests that the growth-inhibitory environment provided by oligodendrocytes is a major factor regulating the plasticity of the corticospinal tract.

Collateral sprouting of the CST is negatively correlated with the expression of myelin-associated neurite growth inhibitors

In the hamster, a time course for sprouting of the intact corticospinal tract after unilateral denervation has been established previously (Kuang & Kalil, 1990). Sprouting is greatest after lesions up to P5 and then gradually declines until very little sprouting is seen after lesions done at P19 and P23. One possible explanation for this decrease of sprouting may be the emergence of myelin-associated neurite growth inhibitors. For the hamster, no detailed analysis of the time course of appearance of these growth inhibitors in the spinal cord is available. In the rat, immunoreactivity for myelin antigens is limited to some fibre tracts at P5 (Schwab & Schnell, 1989). Only by P16 does the grey matter show substantial myelination and expression of myelin-associated neurite growth inhibitors. By P28 the pattern of myelination in grey and white matter is rather complete in the rat spinal cord (Kapfhammer & Schwab, 1994a). We have lesioned the rat CST at P21 and have found that in control animals few sprouts extend to the denervated side of the spinal cord, suggesting that in the rat the decrease of sprouting is similar to the hamster. The decline in sprouting of the CST is thus well correlated with the increasing myelination in the spinal cord grey matter.

The increase of labelled fibres on the denervated half of the spinal cord reflects an increased sprouting of CST fibres

Labelled fibres were only very rarely detected in the lateral half of the denervated spinal cord both in WGA-HRP or BDA traced control animals, but were commonly found in the myelin-free spinal cord. This indicates that sprouted fibres in the myelin-free were able to reach areas of the denervated spinal cord which they do not reach in the presence of myelin. In addition to this qualitative difference, we have quantified the number of sprouted fibres. Due to limitations of the available tracing methods which do not label all fibres with equal intensity, such a quantification cannot yield a completely true picture of the actual number of sprouted fibres. However, we have taken care to correct for all evident variables associated with the tracing procedures. In particular, variances in labelling intensity from animal to animal were fully accounted for by relating counts on the denervated side to those of the intact side of the same spinal cord. In order to further strengthen the validity of our results we have quantified sprouting using two independent tracing methods in two independent sets of experimental animals. Both groups gave very similar results, i.e. a two- to threefold increase in the number of sprouted fibres in the myelin-free spinal cords.

The increase in sprouting is most likely due to the absence of myelin-associated neurite growth inhibitors

We have prevented myelination in the lumbar spinal cord by neonatal X-irradiation (Gilmore, 1963; Hirayama *et al.*, 1984), which results in a strong reduction of myelin antigens and the almost complete absence of myelin-associated neurite growth inhibitors (Savio & Schwab, 1990; Kapfhammer & Schwab, 1994b; Schaeren-Wiemers *et al.*, 1995). The increased sprouting of CST fibres is likely to be due, at least in part, to the absence of myelin-associated neurite growth inhibitors. Similar results were obtained recently in adult, myelinated rats by the application of a monoclonal antibody (IN-1) against the inhibitory myelin proteins NI-35/250 (Thallmair *et al.*, 1996). The role of a recently identified repulsive molecule collapsin-1/semaphorin III remains to be analysed. Sema III mRNA is expressed in subsets of motoneurones in the adult spinal cord and its role for CST development is unknown (Giger *et al.*, 1996). Neonatal X-irradiation certainly will have effects in addition to preventing myelination. However, neuronal differentiation proceeds and astrocytes develop rather normally in the myelin-free spinal cords (Kapfhammer & Schwab, 1994b; Schwegler *et al.*, 1995). Although the neurones in the X-irradiated area are already postmitotic and survive well after X-irradiation, we cannot exclude changes of the synaptic environment in the X-irradiated area which might then retrogradely affect, for example, the growth status of the corticospinal neurones (see below). The cell somata of the sprouting corticospinal fibres are well outside the field of X-irradiation.

Myelin-associated neurite growth inhibitors and the termination of critical periods during development

Interestingly, in other systems a relation between myelination and fibre growth phenomena has also been observed. The permissive period for fibre regeneration after lesions either in the spinal cord of the chick or the opossum ends with the onset of myelination and the expression of myelin-associated neurite growth inhibitors (Hasan *et al.*, 1993; Varga *et al.*, 1995a), and it can be extended by either preventing myelination or by neutralizing the myelin-associated neurite growth inhibitors (Keirstead *et al.*, 1992; Varga *et al.*, 1995b). In the hamster, after lesions of one superior colliculus sprouting retinal fibres grow to the contralateral colliculus but avoid the myelinated stratum opticum. They can be induced to invade it by neutralization of myelin-associated neurite growth inhibitors (Kapfhammer *et al.*, 1992). In the cat visual cortex, the end of the critical period for ocular dominance shifts coincides with the onset of myelination and the expression of myelin-associated neurite growth inhibitors (LeVay *et al.*, 1980). Therefore, in a variety of systems myelin-associated neurite growth inhibitors have been identified as a major factor contributing to the termination of growth permissive critical periods during development.

The increased sprouting of the CST correlates with an increased expression of GAP-43 in the spinal cord

In the myelin-free spinal cord, expression of the neuronal growth-associated protein GAP-43 (Skene, 1989; Strittmatter *et al.*, 1992) is strongly increased (Kapfhammer & Schwab, 1994b). Recently, it has been shown that in transgenic mice overexpressing GAP-43, sprouting of spinal cord primary afferents is increased (Aigner *et al.*, 1995). Similarly, sprouting of spinal cord afferents can be induced by peripheral nerve lesion, which results in an induction of GAP-43 expression in the dorsal root neurones (Schreyer & Skene, 1991; Woolf *et al.*, 1992; Florence *et al.*, 1993). Lesions of the dorsal roots, which do not induce a strong increase of GAP-43 in dorsal root neurones, are followed only by little sprouting of the injured fibres (Chong *et al.*, 1994). The increased sprouting observed in the myelin-free spinal cord may therefore be due to an increased expression of GAP-43 in this myelin-free environment. For the CST, this issue may be difficult to resolve, since the CST even in the normal adult rat shows a rather strong immunoreactivity for GAP-43 (Gorgels *et al.*, 1987; Schreyer & Skene, 1991; Curtis *et al.*, 1993; Kapfhammer & Schwab, 1994a). In a less inhibitory environment created by the absence of oligodendrocytes, sprouting fibres would be able to extend over larger distances. This improved growth may in turn stimulate the expression of intrinsic growth determinants such as GAP-43. Molecules in the environment of the neurite or nerve terminal that affect neurite growth could thus indirectly influence the expression

of GAP-43, resulting in a long-lasting suppression or stimulation of neurite growth. The increased sprouting seen in our experiments may thus be the combined result from a reduced inhibition of neurite extension in a myelin-free environment and an improved capacity to generate sprouts and extend neurites by the increased levels of GAP-43.

Mechanisms involved in the sprouting of the CST

It is obvious that the sprouting of CST fibres after lesions is a complex phenomenon involving a variety of different mechanisms. In addition to the growth inhibitors discussed above and intrinsic molecules such as GAP-43, growth-promoting factors of the neurotrophin type or growth-promoting substrate molecules may play a role. This is reflected by the fact that despite the increased sprouting found in this study the innervation of the denervated half of the spinal cord does not reach a normal density. An important aspect for the induction of the sprouting response was the unilateral denervation of the spinal cord by the CST lesion; without a lesion no sprouting was detectable in the myelin-free spinal cord. Likely candidates for inducing the sprouting response are soluble growth factors which could be released by the denervated target neurones. The observed outgrowth of branches at right angles from the CST across the midline suggests the presence of potent chemoattractants for these fibres. After spinal cord lesions, local sprouting of CST fibres at the lesion site could be increased by the application of neurotrophin-3 (NT-3) (Schnell *et al.*, 1994). The capacity of adult CST fibres to sprout in response to strong growth promoting conditions is also shown by experiments in which Schwann cells were transplanted into the adult CNS (Li & Raisman, 1994). Effects of NT-3 or similar growth-promoting compounds may normally be blunted by the presence of growth inhibitory factors. The cues and molecules mediating the recognition of specific synaptic targets for CST axons in the dorsal and ventral horn of the spinal cord are not known. In the present experiment the neuroanatomical distribution of the sprouted CST fibres in the denervated hemicord closely resembled the normal CST innervation pattern. However, the tracing techniques used did not allow conclusions about the precision or possible errors in the connections formed.

Neurite growth inhibitors are likely to influence plasticity and functional restoration after lesions of the CNS

Hemiplegic cerebral palsy is a rather common complication of irregular and premature deliveries due to damage in one cerebral hemisphere (Volpe, 1994). This type of lesion is similar to the CST lesions performed in our study by resulting in the absence or reduction of one CST. In affected individuals, magnetic stimulation experiments have provided evidence that a direct pathway to motoneurones on the ipsilateral side of the spinal cord exists (Carr *et al.*, 1993; Cao *et al.*, 1994). This new pathway is most likely the result of either an enlarged ipsilateral component of the CST, or sprouting of the crossed intact CST to the denervated side. In either case, a reorganization of CST terminals with sprouting from uninjured corticospinal fibres is required. A possible behavioural correlate of these bilateral corticospinal connections is the occurrence of mirror movements in the brain damaged individuals.

A well-known clinical finding is that the outcome of cortical injury is dependent on the age at which the injury was acquired. In agreement with the Kennard principle (Kennard, 1936), motor performance of affected subjects is better when the lesion is acquired early in life as compared to subjects who acquire cortical lesions at later stages (Carr *et al.*, 1993; Cao *et al.*, 1994). Typically, mirror movements are more frequent in subjects with early acquired brain lesions. These findings correlate well with the decrease of corticospinal sprouting with increasing age at the time of the lesion as reported by Kuang and Kalil in a hamster (1990). Together with the results presented in this study, which show that this permissive period can be extended in the absence of myelin and myelin-associated neurite growth inhibitors, it becomes likely that the determinants of sprouting of corticospinal fibres are similar in rodents and humans. Myelination of the human spinal cord grey matter becomes evident between the 35th and 40th week of pregnancy (Tanaka *et al.*, 1995). Human spinal cord contains neurite growth inhibitors (IN-1 antigens) with biochemical properties very similar to those of rat or bovine myelin (Spillmann *et al.*, 1997). Means to affect the activity of myelin-associated neurite growth inhibitors might therefore be useful to improve the prognosis and the recovery of patients affected by brain lesions.

Abbreviations

BDA	biotinylated dextran amine
BSA	bovine serum albumin
CNS	central nervous system
CST	corticospinal tract
DAB	3,3′ diaminobenzidine tetrahydrochloride
GAP-43	growth associated protein 43
HRP	horseradish peroxidase
IN-1	inhibitor neutralizing protein 1
MBP	myelin basic protein
P	postnatal day
WGA	wheat germ agglutinin

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