Diss. ETH No. 13043

Structural plasticity following unilateral lesions of the corticospinal tract and neutralization of myelin-associated neurite growth inhibitors

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree
of Doctor of Natural Sciences

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1999

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Summary

Regenerative and plastic growth following lesions is restricted to short distances in the adult mammalian central nervous system (CNS). The problem of growth failure in the mature CNS of higher vertebrates is outlined in the introduction (Chapter 1) and is compared to the immature CNS and to the nervous system of lower vertebrates where regrowth and structural plasticity are commonly seen. The factors and mechanisms that may be responsible for the poor growth potential in the adult CNS are discussed. Chapter 2 describes lesion-induced sprouting of intact corticospinal tract (CST) fibers after a complete unilateral lesion of the contralateral CST in the medulla oblongata. Sprouting was investigated in the thoraco-lumbar segments of normally myelinated and partially non-myelinated spinal cord of adolescent rats. The compensatory growth of CST fibers was strongly increased in the absence of myelin showing that myelin and its components are crucially involved in the termination of the growth-permissive period. We directly investigated the role of the myelin-associated neurite growth inhibitors in restricting structural plasticity in adult rats by application of a neutralizing antibody (mAb IN-1) after lesioning one CST (Chapter 3). Here we examined not only the intact CST in the cervical spinal cord, but included a study on the lesioned CST in the red nucleus and the basilar potine nuclei. Both tracts, the lesioned and the unlesioned CST, showed impressive collateral sprouting across the brainstem or spinal cord midline, respectively. The newly grown fibers terminated in their novel targets in a topographic pattern. The structural changes were paralleled by a functional recovery in several behavior tests. Chapter 4 describes first results obtained with a recombinant, humanized Fab (hrIN-1 Fab) fragment after the same unilateral CST lesion in adult rats. Application of such neutralizing Fab fragments may be an important step toward a therapeutic approach to neutralize the myelin-associated neurite growth inhibitors after CNS lesion. This pilot project demonstrates that the hrIN-1 fragment has similar effects on plasticity as the mAb IN-1 although less impressive. Combinations with neurotrophin-3 (NT-3), however, enhanced lesion-induced sprouting significantly. Chapter 5 outlines a clinically important lesion paradigm, a unilateral cortical destruction, and the effects of mAb IN-1 on the spared CST.

Also with this lesion, a significant increase in structural plasticity was found. A short summarizing discussion leads to final conclusions and an outlook.

Zusammenfassung

Regenerative und plastische Wachstumsprozesse finden im adulten Zentralnervensystem (ZNS) höherer Wirbeltiere nur in beschränktem Mass statt. Die Einleitung (Kapitel 1) stellt das Problem des Faserwachstums im erwachsenen ZNS dar und zeigt Beispiele für erfolgreiches Faserwachstum (Regeneration und strukturelle Plastizität) nach Verletzungen des sich entwickelnden ZNS und des Nervensystems niederer Vertebraten. Die Faktoren und Mechanismen, die an der Einschränkung des Wachstumsvermögens adulter ZNS-Nervenbahnen beteiligt sein könnten, werden diskutiert. Das Kapitel 2 beschreibt kompensatorisches Wachstum intakter Fasern des Kortikospinaltraktes nach einer einseitigen Verletzung der Kortikospinalbahn auf der Höhe der Medulla oblongata in adoleszenten Ratten. Das Aussprossen von Kortikospinalfasern wurde in thorako-lumbalen Segmenten des Rückenmarks untersucht, die entweder experimentell unmyelinisiert oder normal myelinisiert waren. Starkes kompensatorisches Faserwachstum war vor allem in den nicht-myelinisierten Segmenten zu beobachten. Diese Befunde liessen den Schluss zu, dass Myelin und seine assoziierten wachstums-inhibierenden Proteine eine wichtige Rolle bei der Beendigung der wachstums-permissiven Periode des Rückenmarks spielen. Wir untersuchten deshalb den Einfluss eines spezifischen Inhibitors (NI-250/Nogo-A) auf die strukturelle Plastizität in erwachsenen Ratten durch Anwendung des neutralisierenden Antikörpers mAk IN-1 (Kapitel 3). In dieser Studie untersuchten wir nicht nur den intakten Kortikospinaltrakt (im zervikalen Rückenmark), sondern auch den verletzten Trakt (im Nucleus ruber und in den basilaren, pontinen Kernen). Beide Trakte zeigten deutliches kollaterales Aussprossen in den mAk IN-1 behandelten Tieren: Die neuen Faserzweige kreuzten im Rückenmark bzw. im Hirnstamm auf die gegenüberliegende Seite und re-innervierten die deafferentierten Regionen in topographischer Weise. Diese strukturellen Veränderungen wurden von einer funktionellen Erholung der lädierten Ratten in verschiedenen Verhaltenstests begleitet. Das Kapitel 4 beschreibt erste Versuche zur Plastizität des Kortikospinaltraktes nach Gabe eines humanisierten, rekombinanten IN-1 Fab (hrIN-1 Fab) Fragments. Die Verwendung eines derartigen Fab Fragments könnte ein erster Schritt zu einer therapeutischen

Anwendung der Neutralisierung der myelin-assoziierten Inhibitoren nach einer ZNS-Verletzung im Menschen sein. Dieses Projekt zeigte, dass hrIN-1 Fab auf plastisches Faserwachstum ähnliche Effekte wie mAk IN-1 hat, wenngleich weniger konsistent. Eine Kombination des hrIN-1 Fab mit Neurotrophin-3 zeigte jedoch eine signifikante Zunahme an aussprossenden Fasern im zervikalen Rückenmark von derart behandelten Tieren. Das Kapitel 5 beschreibt ein Läsionsmodell, das klinisch sehr relevant ist: Eine einseitige Verletzung des motorischen Kortex. Wir untersuchten die Effekte von mAk IN-1 auf verletzungsbedingtes Auswachsen von Kollateralen des intakten Kortikospinaltrakt im Nucleus ruber und der Pons. Auch in diesem Läsionsmodell war die strukturelle Plastizität nach Neutralisierung der myelin-assoziierten Inhibitoren signifikant erhöht. Eine kurze zusammenfassende Diskussion führt zu einem Ausblick auf weitere Projekte dieses Gebiets.

Chapter 1

Introduction

Introduction

In the adult mammalian central nervous system (CNS) regenerative and compensatory growth processes are very restricted. Therefore, mechanical or ischemic lesions lead to dramatic and often irreversible functional impairments. The consequences of a lesion to the mature CNS have been described already 4500 years ago: „When you examine a man with a dislocation of a vertebra of his neck, you find him unable to move his arms, and his legs. His penis is erect; urine drips from his penis unknowingly. Then you have to say: A disease one cannot treat." (Edwin Smith Surgical Papyrus, in Schwab and Bartholdi, 1996). In contrast to the poor growth capacity of the CNS, the peripheral nervous system (PNS) retains a high regenerative capability throughout life. Comparing the growth ability of the mature CNS in higher vertebrates (mammals, birds) to that of lower vertebrates (fish, amphibia), one finds that CNS fibers of lower vertebrates can grow over long distances and that the newly grown fibers can lead to functional recovery. Interestingly, the same processes – regenerative growth, structural plasticity and functional recovery – can take place in higher vertebrates if the CNS lesion occurs very early in life or during embryogenesis.
The abortive growth reaction following a lesion in the mature CNS as compared to lesions in the immature CNS was described by Ramón y Cajal (1928, 1959) already: „Once development is completed, the sources of growth and regeneration of axons and dendrites are irrevocably lost. In the adult brain, nervous pathways are fixed and immutable; everything may die, nothing may be regenerated." A lot of evidence has been gained since then showing that the adult brain is not "immutable", but in fact plastic; changes of dendrites and axonal arborizations in the adult CNS do occur and are most likely a common biological process in the mature brain (Purves et al., 1986; Rossi et al., 1991; Woolf et al., 1992). The distance of such growth, however, is very limited and rarely exceeds 1 mm. Ramón y Cajal further described the formation of growth cones and sprouts following a lesion in the spinal cord. These sprouts, however, were "abortive" and regeneration was absent. We know now that many types of adult CNS neurons survive axotomy and show a transient sprouting response before the axon is finally retracted. Atrophy of the cell body may eventually

follow and some cells may die. Understanding the reasons for the lack of regeneration and structural plasticity in the mature CNS would offer the opportunity to intervene with the normal process following lesions, and thereby would allow to increase the sprouting response and the regenerative attempt to improve the functional outcome eventually.

To elucidate why growth processes in the lesioned CNS are limited I will first give an overview on spontaneous regeneration processes. In the following I will describe our present understanding of the mechanisms and molecules that are involved in CNS growth processes or its failure in higher vertebrates.

Spontaneous axon regrowth and structural plasticity

In the following sections and chapters the term "structural plasticity", which is equal to "reactive collateral sprouting" or "compensatory growth" will be used for the growth of axons/collaterals from *intact* neuronal cells into denervated areas, which are not necessarily their natural targets. In contrast, the term "regeneration" describes the regrowth of *injured* axons toward their original target.

Axonal regrowth occurs in the PNS and in the CNS of lower vertebrates

Damage to the PNS is often reversible: In the PNS regeneration occurs after lesions and leads frequently to restoration of function. Upon nerve lesion and axonal degeneration Schwann cells produce a variety of factors, many of which are involved in growth processes, e.g. extracellular matrix molecules, adhesion molecules or trophic factors. After lesioning a dorsal root, fibers will regrow to the dorsal root entry zone but will not enter the CNS tissue. Sometimes a few growing axons may grow into the CNS but only over very short distances and along blood vessels or invading astrocytes (Nathaniel and Nathaniel, 1973). These observations show that adult, lesioned neurons are able to regenerate their axons, but the growth seems to be controlled by environmental factors.

Regeneration of CNS fibers is commonly found in lower vertebrates (fish, amphibia). The classical experiments by Sperry in the 1940ies and 1950ies

(Sperry, 1963) investigated regeneration of the retinotectal projection in the frog. After transection of the optic nerve fibers regrew to the tectum and specifically innervated their original target cells. The retinotopic re-innervation occurred even after rotation of the eye by 180°.

Regeneration and structural plasticity in the immature mammalian CNS

In the 1930ies Kennard and her colleagues reported that monkeys with motor cortex lesions in infancy developed motor skills that were lost permanently in monkeys undergoing similar lesions in adulthood (Kennard, 1936; Kennard, 1938). The results of these experiments influenced our ideas about the restitution of function after lesions and are known as the "Kennard Principle" (Teuber, 1974) which states that the earlier the brain damage, the better the functional recovery. Recently, this principle came under some discussion (e.g. Passingham et al., 1983) as this difference of behavioral restitution is not always that pronounced. However, although the outcome after early CNS injuries is not always complete, the functional recovery is remarkable if compared with adult lesions (for review see Kolb and Whishaw, 1989).

The best studied system with regard to structural plasticity in mammals is the corticobulbar and corticospinal tract (pyramidal tract). When the pyramidal tract is cut unilaterally at the level of the medulla oblongata in the adult hamster, fibers degenerate in both anterograde and retrograde directions from the lesion (Kalil and Reh, 1979). In the immature hamster, however, regrowth of severed axons via a new brainstem pathway to their appropriate targets in the medulla oblongata and spinal cord has been found (Kalil and Reh, 1982). The regrowing corticospinal fibers did not cross the lesion site. Instead, there was a new axonal pathway arising from the severed corticospinal tract some millimeters rostral to the lesion. Most of these fibers crossed to the contralateral brainstem and grew caudally for 6-7 mm to reach the cervical spinal cord. Although the trajectory of the regrowing axons was abnormal, their pattern of termination in the dorsal column nuclei and dorsal horn of the cervical spinal cord seemed normal. These newly growing fibers formed synapses within their appropriate target areas. The regrowth of corticospinal fibers was maximal with lesions at 4-

8 days of age. The growth capacity then declined sharply and after 20 days of age, unilateral pyramidal tract lesions elicited no new growth but instead axon degeneration. Some years later the group of Kalil investigated the intact corticospinal tract (CST) and its reaction to the unilateral pyramidal tract lesion in early postnatal rats. They found that fibers of the CST contralateral to the lesion crossed at spinal cord levels to terminate in the denervated spinal half (Kuang and Kalil, 1990b). The terminations were specific concerning their origin: corticospinal axons arising from the somatosensory cortex projected primarily to the dorsal horn, whereas those from the motor cortex terminated mainly in the ventral horn (Kuang and Kalil, 1990a). The time course for this lesion-induced compensatory growth was comparable to the time window for regrowth: Lesions performed at 19 days of age or later did not elicit any structural plasticity of the intact CST any more. This postnatal growth-permissive time window is rather unique for the CST as it is a late-developing tract; other tracts like the rubrospinal tract fail to regrow at that time. Regrowth and structural plasticity depend on the developmental stage of a given part of the nervous system rather than on the age of the animal.

The problem of fiber growth in the mature CNS of higher vertebrates

To understand the mechanisms that regulate and restrict structural plasticity and regeneration in the adult CNS has been a challenge for many scientists until today. The results of these studies have been controversial, however, and the discussion about processes and factors involved in the failure of CNS regeneration and structural plasticity is still continuing.

One of the first ideas to explain the limited fiber growth was the hypothesis that CNS neurons cannot regenerate after completion of development – the poor growth capability would be an intrinsic property of CNS nerve cells. This hypothesis was almost a "law of nature" for some decades. At the beginning of this century already, and then extensively again in the early 1980ies scientists tried to examine this hypothesis experimentally, and it became obvious that the

limited growth ability is not only due to intrinsic properties of the neurons but depends also on environmental cues.

The microenvironment of the nerve fiber influences its growth ability

Since it was well known that lesioned PNS fibers regenerate over long distances, implantation of peripheral nerve tissue into various areas of the adult CNS was a direct approach to examine the growth ability of CNS neurons into a permissive, peripheral nerve graft. The first successful transplantation experiments by Tello (Tello, 1911) showed that fibers from rabbit cortex were able to grow into implants of sciatic nerve tissue. Ramón y Cajal (1928, 1959), based on these transplantation studies, postulated that the lack of trophic and tropic support might be the reason for the poor outgrowth in the adult CNS. Later it was shown that Schwann cells indeed produce a variety of those factors following denervation. Several other experiments finally indicated that the scar could be the major obstacle for regenerating fibers (Reier et al., 1983; Windle et al., 1952). In many lesions, however, a bridge of intact tissue survives and regenerating fibers could grow around the lesion site and avoid the scar tissue. Nevertheless, in the adult CNS fibers usually stop at the lesion site and retract (die back) after a transient sprouting response. In the early 1980ies, Aguayo and co-workers finally confirmed the earlier transplantation studies by showing that many CNS neurons are indeed capable of growing into a peripheral nerve transplant. They showed regeneration through a peripheral nerve graft in the spinal cord and optic nerve of adult mammals by retrograde horseradish peroxidase (HRP) tracing. When CNS tissue of the optic nerve was replaced with segments of peripheral sciatic nerve in adult rats. Lesioned fibers of the retinal ganglion cells were able to regrow into the graft and to re-innervate their target, the superior colliculus. The regrowing fibers penetrated the tectum for up to 500 µm and branched into the superficial layers, their main normal target (Carter et al., 1989). Ultrastructural analysis showed that normal synaptic structures were formed (Carter et al., 1991; Carter et al., 1989). These connections were functional as typical postsynaptic potentials could be recorded in the deep tectal layers upon stimulation of the retina using light flashes (Keirstead et al., 1989). The synapses were still found after more than

one year suggesting that the connections to the superior colliculus were permanently restored (Carter et al., 1994). Schwann cells within the peripheral nerve graft were shown to be the crucial elements for the growth of the lesioned fibers. Similar regeneration experiments were done in the medulla oblongata and thoracic spinal cord where a PNS graft was implanted to bridge the lesion site (David and Aguyao, 1981; Richardson et al., 1984; Richardson et al., 1980). Many fibers originating from different nuclei in the brainstem and from spinal cord neurons entered the graft but failed to reenter the CNS tissue – fibers never invaded the CNS tissue for more than 0.6 mm (David and Aguyao, 1981). Corticospinal fibers, however, did not enter the peripheral nerve implant (Richardson et al., 1982) indicating that the growth-permissive PNS environment was not sufficient to induce regrowth of this fiber tract. Aguayos findings illustrated that many adult neurons not only retain and are able to reactivate their intrinsic axonal growth program but are also able to recognize guidance cues and to establish functional synapses. On the other hand these studies have shown that guidance cues are retained or re-expressed after injury in the mature CNS (Carter et al., 1989; Wizenmann et al., 1993).

Recently it has been shown, that transplantation of Schwann cells alone can induce regeneration of some fiber tracts in the mature CNS (Li and Raisman, 1994; Paino et al., 1994; Stichel et al., 1996).

All these grafting-experiments showed that many types of lesioned adult CNS neurons are capable to reactivate their cellular growth machinery when a permissive PNS microenvironment is provided. Adult CNS tissue, however, seems to be unfavorable for growth. The idea that the microenvironment of a CNS fiber is strongly influencing its growth capacity became finally accepted.

Lesion-induced collateral sprouting in some regions of the CNS

Lesions of CNS fibers or dorsal roots result in denervation of their original target areas. In some CNS areas reactive collateral sprouting by intact fibers has been observed and provided further evidence showing that the CNS is not a "hard-wired" system. After fimbria fornix lesions, Raisman and Field showed sprouting in the denervated septum by serotonergic fibers (Raisman and Field, 1973). Sprouting was found after perforant path lesions in the hippocampus by several

types of axons (Gage et al., 1983). Partial lesions of the inferior olive and their climbing fibers to the cerebellum evoked sprouting of the spared fibers which innervated the denervated Purkinje cells (Rossi et al., 1991). In the adult rat, lesions of the lumbar dorsal roots evoked some sprouting of primary afferents from the neighboring roots (Schwegler et al., 1995). Finally, structural plasticity in the cortex can be induced by functional alterations (Darian-Smith and Gilbert, 1994).

Neuronal growth is influenced by many factors

As briefly summarized above, axon growth is a complex process in which intrinsic properties of the neuron and the properties of the microenvironment influence the ability and extent of regeneration or structural plasticity. Different neuronal cell populations show a different growth potential, which is further modulated by environmental factors. In general, the growth capacity declines with age in all neuronal populations (for review see Fawcett, 1992).

Growth-associated genes are expressed after axotomy

Studies of the development of the nervous system have provided some important clues on the mechanisms of regrowth and structural plasticity. Many genes that are expressed during the initial outgrowth in development are re-expressed upon axotomy and during successful outgrowth in the adult CNS. The cellular changes following axotomy were termed "cell body response" (Barron, 1989; Lieberman, 1971). After injury the neuron changes the rate of transcription and translation of cytoskeletal proteins e.g. Tα1 tubulin mRNA (Fawcett, 1992), of regeneration- or growth-associated proteins such as GAP-43 (Benowitz and Routtenberg, 1997; Skene, 1989) and of certain immediate early genes.

Cytoskeletal proteins such as tubulin, neurofilament protein and microtubule-associated proteins are synthesized in a very similar pattern during regenerative growth as during development (for review see Fawcett, 1992). However, some cytoskeletal differences also exist: MAP1x, for example, is expressed in

embryos, but not during regeneration (Woodhams et al., 1989), and E-NCAM, the embryonic form of the cell adhesion molecule NCAM does not cover the whole axon during regrowth, but during initial outgrowth (Daniloff et al., 1986).

The growth-associated protein GAP-43 is highly expressed during development in growing neurons of the PNS and CNS (Skene, 1989) and is downregulated after synaptogenesis. Strong GAP-43 expression is retained in the adult CNS in regions known for their plastic potential like the olfactory bulb, the hippocampus and the substantia gelatinosa of the spinal cord (Kapfhammer and Schwab, 1994). GAP-43 is re-expressed at high levels after peripheral nerve injury in dorsal root ganglion cells (Chong et al., 1992), in motoneurons (Linda et al., 1992) and in retinal ganglion cells (Doster et al., 1991). Upregulation of GAP-43 mRNA was also found in spinal motoneurons contralateral to a peripheral lesion indicating that GAP-43 levels are not only changed as a direct consequence following a lesion but also after altered synaptic input.

Immediate early genes (IEGs) are rapidly induced following an injury and many IEGs serve as inducible transcription factors. C-Jun, JunB, JunD, c-fos and Zif268 are likely candidates as regulators of the initial steps of plasticity and regeneration as they are strongly induced in several growth paradigms, e.g. during mossy fiber sprouting in the hippocampus (Morgan and Curran, 1991) and dorsal ganglion cell regeneration (Broude et al., 1997). Olivocerebellar axons (Bravin et al., 1997; Rossi et al., 1995), cerebellar mossy fibers (Armengol et al., 1989) and axons from the deep cerebellar nuclei (Dooley and Aguayo, 1982) can sprout and regenerate when provided with a growth-permissive environment, in contrast to Purkinje cell axons (Buffo et al., 1997; Dooley and Aguayo, 1982; Dusart et al., 1997; Rossi et al., 1995). The cell body response of these neuronal populations showed that only those neurons that induce c-Jun, JunD and GAP-43 can regrow their axon (Buffo et al., 1998; Zagrebelsky et al., 1998). In contrast, almost all Purkinje cells failed to upregulate these IEGs (Bravin et al., 1997; Dusart et al., 1997; Rossi et al., 1995). Thus, the strength of the cell body response correlates with the growth capacity of the neuron and differs among various neuronal populations.

The precise role of these IEGs and the growth-associated genes is not clear yet – for some genes the function is quite controversial as, for example, c-Jun has

been related also to cell death processes (Herdegen et al., 1997; Isenmann and Baehr, 1997).

In the adult CNS, the cell body response following axonal lesions is only transitory, and this response seems not to be sufficient to permit regeneration in the absence of a growth-permissive environment.

The cell body response is modulated by environmental factors

As described in the preceding section, Purkinje cells did not express any IEGs after axotomy. However, a few Purkinje cells in close vicinity to the lesion induced IEGs after some days (Zagrebelsky et al., 1998). Rubrospinal neurons express regeneration-associated genes after axotomy at cervical levels but not after thoracic lesion. This observation correlates with their ability to regrow axons into peripheral grafts in the cervical spinal cord, whereas such regeneration is not observed when such transplants were placed in the thoracic cord (for review see Tetzlaff et al., 1994). These results indicate that the distance between the lesion site and the soma influences the cellular reaction of the neuron to the injury.

In search for a signal that might influence the cell body response, Zagrebelsky and colleagues found that blocking the axonal flow using colchicin evoked an increased cell body response in Purkinje cells (Zagrebelsky et al., 1998). Interestingly, a neutralizing antibody against the myelin-associated neurite growth inhibitors - those will be described in one of the following sections - also elicited these cellular changes and increased the expression of some growth-associated genes.

In some neuronal populations the expression of growth-associated genes can be increased by the presence of a growth-permissive transplant (peripheral nerve or embryonic tissue; Broude et al., 1997; Chong et al., 1996; Hüll and Bähr, 1994; Vaudano et al., 1995).

Growth-promoting factors

Molecules that exhibit growth-promoting properties in vitro like neurotrophic factors and their receptors, cell adhesion molecules and extracellular matrix

molecules are present in the adult CNS. Upon a lesion many of this positive growth-regulators are upregulated, but their role in vivo is not entirely clear.

Neurotrophic factors

Neurotrophic factors comprise a family of small, secreted proteins (NGF, BDNF, NT-3, NT-4/5, NT-6, CNTF, LIF, TGFβ, GDNF, neurturin, persephin, FGF) that regulate the survival and differentiation of developing neurons in the PNS (Barde, 1989; Davies and Lumsden, 1990; Oppenheim et al., 1991; Oppenheim et al., 1992; Sendtner et al., 1997; Sendtner et al., 1992; Sendtner et al., 1990) and CNS: The survival of neonatal rat corticospinal motor neurons in vitro can be promoted by ciliary growth factor (CNTF), neurotrophin-4/5 (NT-4/5) and glial cell line-derived neurotrophic factor (GDNF), but not by nerve growth factor (NGF), neurotrophin-3 (NT-3) or brain-derived growth factor (BDNF) (Junger and Varon, 1997). The expression of neurotrophic factors and especially of their receptors increases rapidly after a brain insult (for review see Lindvall et al., 1994) and might protect against neuronal damage and stimulate sprouting and synaptic reorganization in the case of the CST. Many corticospinal neurons die following axotomy if the lesion site is close to the cell body, for example in the internal capsule. The surviving neurons undergo severe atrophy. GDNF, BDNF and NT-3, but not NGF, can fully prevent the lesion-induced death and abolish the atrophy of adult rat corticospinal neurons in vivo (Giehl et al., 1997; Giehl and Tetzlaff, 1996). Similarly, rubrospinal neurons show a severe atrophy following a cervical axotomy. BDNF or NT-4/5 treatment stimulate the expression of GAP-43 and Tα1-tubulin mRNA and promote regeneration into a peripheral nerve graft, whereas NGF or NT-3 treatment have no significant effect (Kobayashi et al., 1997). Axotomized CST fibers show increased sprouting after local application of NT-3, but not BDNF (Schnell et al., 1994). These findings indicate that certain neuronal populations need certain neurotrophic factors that exert specific effects on these neurons.

In some cases the expression of neurotrophins is regulated by neuronal activity: NGF and BDNF mRNA levels are upregulated after seizure activity in the hippocampus (Ernfors et al., 1991; Isackson et al., 1991), and eliciting long-term potentiation (LTP) increases BDNF and NT-3 mRNAs (Patterson et al., 1992).

These findings suggest a role for neurotrophic factors in regulating synapse strength and plasticity. Recently, more and more evidence accumulated suggesting that neurotrophins are involved in neuronal plasticity (Lo, 1995; Thoenen, 1995). BDNF knock-out mice, for example, show a reduction of long-term potentiation (LTP) (Korte et al., 1995) and BDNF-gene transfer into CA1 neurons of those mice could restore LTP (Korte et al., 1996). NGF allows synaptic sprouting in the cerebral cortex of lesioned primate brains (Burgos et al., 1995) and plays a role in the collateral sprouting of nociceptive fibers (Diamond et al., 1987; Mearow and Kril, 1995). During regeneration of retinal ganglion cells of fish the expression of the high-affinity NGF receptor, trkA, is increased (Vecino et al., 1998). Antibodies against basic growth factor (bFGF, FGF-2) decrease cholinergic sprouting in the denervated hippocampus, whereas application of FGF-2 enhanced this sprouting (Fagan et al., 1997). FGF-2 might also be involved in the enhanced sprouting of the intact hemisphere after unilateral focal cerebral infarction (Kawamata et al., 1997).
The potential of neurotrophic factors to promote survival and to enhance sprouting and regeneration is of great interest concerning therapeutic interventions after stroke and other CNS insults. However, as proteins do not cross the blood-brain barrier and the neurotrophins' penetration into the tissue is rather limited, neurotrophic factors have to be delivered directly into the tissue or into the ventricle, a procedure that can lead to complications and additional risks (e.g. Giehl et al., 1998). Improvements of the in vivo application of these factors and a better understanding of their mode of action after CNS lesions could lead to a treatment after neuronal injuries.

Cell adhesion and extracellular matrix molecules

Extracellular matrix molecules (ECM molecules) are involved in regulating many aspects of neural development and have been shown to influence regeneration after neural injuries (for review see Venstrom and Reichardt, 1993). Over the last two decades many ECM components (laminin, fibronectin, collagen, tenascin, proteoglycans: versican, perlecan, brevican) and neuronal surface receptors for ECM molecules like the integrins have been identified. On the molecular level, some domains promote neuronal adhesion, whereas other

ECM components or domains repel growth cones (anti-adhesive molecules). ECM molecules that promote axonal elongation include fibronectins, laminins, tenascins, F-spondin and several sulfated proteoglycans (Hynes and Lander, 1992; Venstrom and Reichardt, 1993). However, several chondroitin sulfate proteoglycans and other sulfated proteoglycans as well as tenascins can also inhibit axonal elongation (Letourneau et al., 1992; Davies and Silver, 1998). Major neuronal receptors for ECM components are the integrins that mediate cell-cell adhesion and mediate cell-matrix interactions (Reichardt and Tomaselli, 1991).

PSA-NCAM and L1 are developmentally regulated and promote axonal outgrowth. These molecules are re-expressed during regeneration and sprouting of certain systems (for review see Aubert et al., 1995; Rutishauser and Landmesser, 1996) and might play an important role for neurite elongation. Recently it has been shown that the E587 antigen, a L1-like cell adhesion molecule, is upregulated by goldfish oligodendrocytes and by retinal ganglion cell axons after optic nerve lesions and supports regeneration (Ankerhold et al., 1998). L1 and PSA are expressed during sprouting and regeneration in the hippocampus after complete fimbria fornix lesions and grafting of NGF- or β-galactosidase producing fibroblasts in adults (Aubert et al., 1998).

The precise mechanisms and roles of all these components during regeneration and sprouting in vivo remain to be determined.

Growth-inhibiting factors

Various cell culture experiments in the 1980ies gave evidence for the existence of specific growth inhibiting factors in the mammalian CNS (e.g. Walter et al., 1987). Schwab and Thoenen (Schwab and Thoenen, 1985) used a compartmentalized culture system to investigate the role of neurotrophic factors in the failure of growth processes in the CNS. Using optimal trophic factor conditions they offered explants of sciatic and optic nerve from adult rat as substrates to cultured central or peripheral neurons. Whereas fibers grew heavily into the sciatic nerves, no fibers invaded the optic nerve explants. This experiment indicated that the failure of regeneration and structural sprouting in the adult CNS cannot be completely explained by a lack of trophic factors and

led to the concept of neurite growth inhibitors that are associated specifically with CNS tissue. This hypothesis was supported by several other experiments: When neurons were grown on frozen CNS sections, no cell adhesion or neurite growth was seen on white matter areas (Carbonetto et al., 1987; Crutcher, 1989; Savio and Schwab, 1989; Tuttle and Matthew, 1991; Watanabe and Murakami, 1989). To further analyze the inhibitory substrate effect, Schwab and Caroni (Schwab and Caroni, 1988) investigated the growth potential of neurons on cultures of dissociated optic nerves of young rats. Neurons adhered and grew well on most of these cells and formed a dense neurite network. Differentiated oligodendrocytes and their processes, however, were avoided. Time-lapse video-microscopy showed that differentiated oligodendrocytes induced a long-lasting growth cone collapse of rat dorsal root ganglion cells upon contact with the oligodendrocyte processes (Bandtlow et al., 1990; Fawcett et al., 1989; Vanselow et al., 1990). The inhibitory effect depended on direct contact with the oligodendrocyte as shown by the very localized action – other axons of the same neuron are not affected.

When small patches of central and peripheral myelin were adsorbed to a polylysine coated culture dish and peripheral or central neurons were seeded on the dish, neurites strictly avoided the central myelin whereas dense neurite outgrowth was found on peripheral myelin. These experiments showed that the growth inhibitory activity is highly enriched in central nervous system white matter, is associated with mature oligodendrocytes, leads to a long-lasting growth arrest and is strictly dependent on physical contact with the oligodendrocyte. Partial purification and biochemical characterization of the inhibitory constituent of CNS myelin revealed two protein fractions with molecular weights of 35 and 250 kDa. These very potent neurite growth inhibitors were called NI-35 and NI-250. Those growth inhibiting factors were also found in other mammals like opossum (Varga et al., 1995b), chicken (Steeves et al., 1994), bovine (Spillmann et al., 1998) and human material (Spillmann et al., 1997). The full length cDNA of rat NI-250 has been cloned recently (Chen et al., 1997). It codes for a novel membrane protein that inhibits neurite growth as a recombinant protein, an effect which is neutralized by specific antibodies (incl. mAb IN-1). The mAb IN-1, a monoclonal antibody was

raised against the inhibitory fractions from rat material (Caroni and Schwab, 1988). This antibody neutralized the inhibitory activity of CNS myelin and cultured, differentiated oligodendrocytes. After injection of mAb IN-1 into explants of optic nerve, rat dorsal root ganglion cells now extended numerous fibers into these explants over long distances (Caroni and Schwab, 1988). Immunohistochemical stainings with mAb IN-1 showed a myelin staining comparable to that with myelin basic protein (MBP) or myelin-associated glycoprotein (MAG) (Rubin et al., 1994). After a bilateral transection of the dorsal spinal cord in adult rats fibers of the corticospinal tract usually retract from the lesion site after initial sprouting. Application of the neutralizing antibody IN-1 by implanted hybridoma cells, however, led to long-distance regeneration of a portion of CST axons (Schnell and Schwab, 1990; Schnell and Schwab, 1993). Application of a control antibody never allowed any long-distance regeneration; fiber growth very rarely exceeded more than 1mm. Neonatal X-irradiation of the thoraco-lumbar spinal cord generates myelin-free segments. In such animals a similar regeneration was seen after a dorsal bilateral transection in the young adult rat (Savio and Schwab, 1990). Similarly, the growth permissive period in the chick spinal cord can be prolonged by prevention of myelination (Keirstead et al., 1992). All these findings indicate that myelin and the IN-1 antigens play an important role in restricting regenerative fiber growth in the CNS.

In Xenopus a very interesting situation is found: lesioned fibers regenerate in the optic nerve but not in the spinal cord. Immunohistochemical stainings with mAb IN-1 showed that the myelin-associated neurite growth inhibitors are present in the spinal cord myelin after metamorphosis but not in Xenopus optic nerve myelin. In vitro assays confirmed the growth permissiveness of Xenopus optic nerve in contrast to Xenopus spinal cord (Lang et al., 1995).

Recently, MAG, an immunglobulin-family member, was shown to have growth inhibiting properties in vitro (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and to evoke growth cone collapse when coated onto beads (Li et al., 1996). Depending on the age and the type of the neuron MAG can either act as an inhibitor or as a promotor of neurite outgrowth (Mukhopadhyay et al., 1994). The finding that MAG is a potent neurite growth inhibitor is unexpected as MAG

is present not only in the CNS, but also in the PNS that is known to be permissive for axon growth. In vivo studies with MAG-deficient mice led to contradictory results: Li and colleagues (Li et al., 1996) observed more regenerating fibers and longer axons in MAG-deficient mice as compared to wildtype mice, whereas in a study performed by Martin Schwab's and Melitta Schachner's groups (Bartsch et al., 1995) no improvement in regeneration of the lesioned CST or the optic nerve was found in MAG-/- mice. Application of the mAb IN-1, however, promoted regeneration in wildtype and MAG-deficient mice (Bartsch et al., 1995).

Other inhibiting factors include ECM molecules such as chondroitin sulfate proteoglycans (Snow et al., 1990) and cytotactin/tenascin-C which inhibit neurite outgrowth under certain conditions in vitro (Faissner and Kruse, 1990). Tenascin is highly expressed during development in PNS and CNS by glial cells, particularly by immature astrocytes, and is downregulated in the adult nervous system (Faissner and Kruse, 1990). Following an injury, tenascin is re-expressed in areas around the lesion in the adult CNS especially by reactive astrocytes (Brodkey et al., 1995; Laywell et al., 1992) and in the regenerating peripheral nerve (Martini et al., 1990). The bifunctional effect of tenascin (growth-promoting or inhibiting) depends on the type and age of the neurons and on the presence of other substrate components. Embryonic DRG neurons, for example, are inhibited by purified tenascin after one week in culture, whereas 4d old embryonic DRG neurons can grow (Wehrle-Haller and Chiquet, 1993).

Repulsive guidance cues

Semaphorins: Biochemical purification of a growth cone collapsing activity from membranes of embryonic chick brain resulted in the molecular characterization of collapsin-1/Semaphorin III (Luo et al., 1993). Collapsin is a 100kD glycoprotein that elicits growth cone collapse of sensory ganglion cells but not of retinal ganglion cell axons. This collapse-inducing molecule can steer developing neuronal growth cones and prevents mature neurites from regeneration. A close homology was found to fascilin IV (Sema I) (Kolodkin et al., 1992), the first member of the semaphorin family of repulsive guidance

molecules identified in the grasshopper. Semaphorins are a large group of secreted and transmembrane molecules that are highly conserved from invertebrates to mammals (Kolodkin et al., 1993). Bioassay experiments led to the conclusion that some semaphorins prevent neurite defasciculation and branching rather than acting as an absolute inhibitor of axon outgrowth (Kolodkin et al., 1992). Sema III acts as a chemorepellent in patterning the sensory projections in the spinal cord (Messersmith et al., 1995). Interestingly, it was found recently that semaphorins can also act as attractors via the receptor neuropilin-2 (Takahashi et al., 1998). A role for Sema III and its receptor neuropilin-1 in the regenerative failure of olfactory neurons after bulbectomy as well as for the successful re-innervation of the target after axotomy was suggested by a recent study in Verhaagen's laboratory (Pasterkamp et al., 1998).

Netrins are a small family of secreted, highly conserved guidance molecules (Culotti and Kolodkin, 1996; Kennedy and Tessier-Lavigne, 1995). The first identified member of the netrin family was UNC-6 in Caenorhabditis elegans (Ishii et al., 1992); the vertebrate homologues netrin-1 and netrin-2 were identified in the floor plate of the neural tube where they act as diffusible chemotropic factors for commissural neurons in early development (Serafini et al., 1994). In vivo and in vitro data showed that trochlear axons can be repelled by netrin-1 suggesting that netrin-1 acts as a bifunctional molecule in vivo (Colamarino and Tessier-Lavigne, 1995; Messersmith et al., 1995). Unc-5 and members of the DCC subfamily are implicated to be receptors for the attractive and repulsive effects of netrins (Culotti and Merz, 1998).

Eph receptor tyrosine kinases and their ligands constitute the largest known family of receptor tyrosine kinases and have been shown to play an important role in patterning the CNS during embryonic development. The first members have been identified in studies searching for guidance signals in the development of the visual system (Tessier-Lavigne, 1995). Ephrin-A5 (AL-1; RAGS) and ephrin-A2 (ELF-1) are expressed in overlapping gradients in the posterior part of the tectum and form part of the posterior repulsive guidance activity (Drescher et al., 1997). The Eph-related tyrosine kinase receptor, EphA5 (REK7) mediates the effects of ephrin-A5 and related ligands and plays

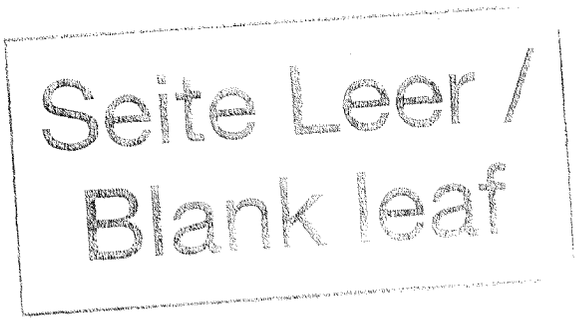
a role in the guidance of retinal, cortical, thalamo-cortical and hippocampal axons during development (Winslow et al., 1995; Meima et al., 1997; Drescher et al., 1997; Castellani et al., 1998; Gao et al., 1998a). The continued expression of EphA5 in the adult brain, in particular in areas associated with a high degree of synaptic plasticity, suggests a role for this receptor tyrosine kinase in plastic events. Indeed, antagonists to EphA5 impaired the induction of LTP in the hippocampus (Gao et al., 1998b) implicating that Ephrin-EphA5 system is needed for LTP establishment in the mature hippocampus. In the adult olfactory bulb a moderate level of Ephrin-B1 (LERK-2) is retained, whereas Ephrin-B1 is downregulated in all other regions of the adult brain (Carpenter et al., 1995). The specific role of these guidance cues in the adult CNS during regeneration and structural plasticity has still to be elucidated.

Aim of the present work

Myelin-associated inhibitors have been shown to be involved in limiting axonal regeneration in the adult CNS (Schwab et al., 1993). Some findings also suggested that the IN-1 antigens may play a role in the termination of the growth-permissive period during postnatal life: Collateral sprouting declined with age and coincided in time and location with the onset and progression of myelination (for review see Kapfhammer, 1997). Suppression of myelination was shown to prolong the critical period for axon growth (Savio and Schwab, 1990; Keirstead et al., 1992; Schwegler et al., 1995; Varga et al., 1995a; Varga et al., 1995b). The inverse regional distribution of GAP-43 and myelin in most parts of the adult CNS suggests a role for the myelin-associated neurite growth inhibitors in restricting plastic fiber growth (Kapfhammer, 1994). The myelin-associated inhibitors NI-35/250 are relatively abundant in CNS white matter and, thus, could inhibit axon growth almost completely in these areas, whereas in CNS gray matter these inhibitors are present to various degrees and their relative abundance might influence the extent of plastic growth of axons and/or dendrites in this region.

To address the question if myelin-associated inhibitors are involved in termination of the critical, growth-permissive period and if they are candidates for stabilizing the mature CNS, we wanted to investigate an easily accessible

fiber system. The CST of the rat seems to be a good experimental target as it is a very well studied tract. At the decussation the tract crosses almost completely to the contralateral side (Rouiller et al., 1991) allowing a quite clear separation of the two "halfs" of the CST. A unilateral transection of the CST at the level of the medulla oblongata rostral to the decussation allows the investigation not only of the lesioned, but also of the intact tract. The CST is a rather late developing tract and, thus, can be manipulated during its development after birth. Plasticity occurs in the CST after unilateral lesions in the newborn and is well described in the literature (e.g. Kuang and Kalil, 1990b; Leong and Lund, 1973), but was so far never observed following adult lesions (Kuang and Kalil, 1990b). We wanted to study the reaction of the CST to adult lesions using different experimental approaches, either by suppressing myelination (Chapter 2) or by neutralizing the inhibitory properties of myelin NI-35/250 with specific antibodies or Fab fragments (Chapter 3 and 4). After finding an astonishing amount of plasticity after a precise, unilateral lesion of the pyramid of the brainstem and application of the neutralizing antibody IN-1, we also used another, clinically very important lesion model, the unilateral destruction of the motor cortex, to further evaluate the effects of the IN-1 antibody in evoking plastic fiber growth in the adult CNS (Chapter 5).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Chapter 2

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

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European Journal of Neuroscience 10:45-56 (1998)

Abstract

Myelin contains potent inhibitors of neurite growth which have been implicated in the failure of long distance regeneration of nerve fibers within the CNS. These myelin-associated neurite growth inhibitors may also be involved in the stabilization of neural connections by suppressing sprouting and fiber growth. After lesions of the CNS in neonatal animals, extensive rearrangements of the remaining fiber 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 fibers occurs after deafferentations as late as P21 if myelination is prevented by neonatal X-irradiation in the rat lumbar spinal cord. Sprouted fibers 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 fibers show a remarkable capability for rearranging their connections. Following early deletions of the central visual target nuclei, for example, optic fibers were induced to terminate in auditory centers (Metin and Frost, 1989; Pallas and Sur, 1994). After unilateral lesions of the superior colliculus in newborn hamsters, retinal fibers can be induced to recross the midline and terminate in the inappropriate remaining colliculus (Schneider, 1973). Typically, these major changes in fiber 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 and Schwab, 1988a,b; Savio and Schwab, 1990; Schnell and 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) fibers has been demonstrated in the adult CNS (Schnell and 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 fibers (Kapfhammer and Schwab, 1994a, b; Kapfhammer, 1996). Evidence in support of this hypothesis comes from experiments in which the critical period for regeneration of lesioned descending fiber 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 fibers 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 and 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 fibers 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 and Lund, 1973; Hicks and D'Amato, 1975). Sprouting then declines and becomes very sparse after lesions made after the second postnatal week (Hicks and D'Amato, 1970; 1975; Leong, 1976; Kuang and Kalil, 1990). This time course corresponds to the increase of myelin in the spinal cord gray matter (Schwab and Schnell, 1989; Kapfhammer and Schwab, 1994a). Sprouting of CST-fibers into the denervated half of the spinal cord is of particular clinical interest since it is likely to occur in humans after early acquired brain damage. In subjects with hemiplegic cerebral palsy due to perinatal injury after pre-term 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 fibers 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 anesthetized 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 and 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 fibers were traced with wheat germ agglutinin-horseradish peroxidase (WGA-HRP) or biotinylated dextran amine (BDA) as described below and sacrificed two weeks after the lesion (Fig. 1).

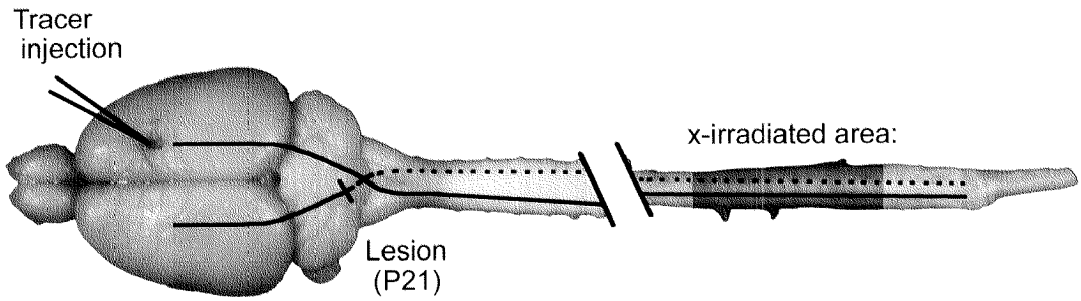

Fig.1: Schematic illustration of the experimental procedures.
Newborn rats received X-irradiation of the lower thoracic and lumbar spinal cord in order to suppress myelination. Unilateral lesions of the CST were performed at the level of the pyramid at P21. The tracer wheatgerm agglutinin-horseradish 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 fibers was analyzed in the myelinated cervical and upper thoracic, and in the X-irradiated lower thoracic and lumbar area of the spinal cord.

The animals were divided into the following groups: 1) unlesioned, traced with WGA-HRP (n=4); 2) unlesioned, X-irradiated, traced with WGA-HRP (n=7); 3) lesioned, traced with WGA-HRP (n=10); 4) lesioned, X-irradiated, traced with WGA-HRP (n=9); 5) 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 and Schwab (1990). After anesthesia 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). 2 µl of 5% WGA-HRP were injected into the motor area (3-4 injection sites). After a survival time of 36 to 48 hours 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 0.1M phosphate buffer (PB) at pH 7.4. The brain and spinal cord were dissected, postfixed for 2 hours at 4°C and stored in a 30% sucrose solution for 36 hours at 4°C for cryoprotection. The spinal cord was then divided into 5 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) as a substrate and sodium nitroferricyanide as stabilizing agent (Mesulam, 1978).

BDA. The right motor cortex was injected with BDA (Molecular Probes, Eugene, OR) at the day of the lesion: 2.5 µl 10% BDA were distributed over 4-5 injection sites. Animals were perfused after 14 d 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 3x30 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, 1:100 in TBS-X) at room temperature. After 3x30 min washing in TBS-X the sections were rinsed with 50 mM TRIS-HCl (pH 8.0) and preincubated in 0.4% nickel

ammonium sulfate in 50 mM TRIS-HCl for 10 min. Sections were further preincubated for 10 min in the nickel ammonium sulfate 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 3x10 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 re-hydrated, 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% fetal calf serum was used undiluted. After several rinses with PBS, sections were incubated with either rat adsorbed biotinylated anti-mouse immunoglobulin (1:100 in 5% BSA, Vector) for MBP or biotinylated anti-mouse 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: (1) complete lesion of the right CST (checked after dissection and

histologically in a series of animals), and (2) 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: Three to four sections per animal were selected blindly from coded section series for quantification. The dorsal half of the spinal cord gray 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 400x. Grains of labeled fibers were easily distinguishable from stained catalase-positive erythrocytes and occasional artifacts of the HRP reaction. Counting was performed in the region showing the densest labeling in each sector within a square of 0.06 x 0.06 mm. The counts for each sector were averaged over the three to four sections evaluated, and the relative amount of labeling in all the sectors was calculated as a percentage of the labeling in sector A (=100%). This eliminated differences between individual animals due to the inherent variability of the tracing. These relative percent 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-labeled CST fibers 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 1.3mm lateral to the midline. BDA-labeled CST fibers 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), 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 and Schwab, 1990; Kapfhammer and 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 and 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 and Schwab, 1988b; Rubin et al., 1994). As shown in Fig. 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 fibers 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

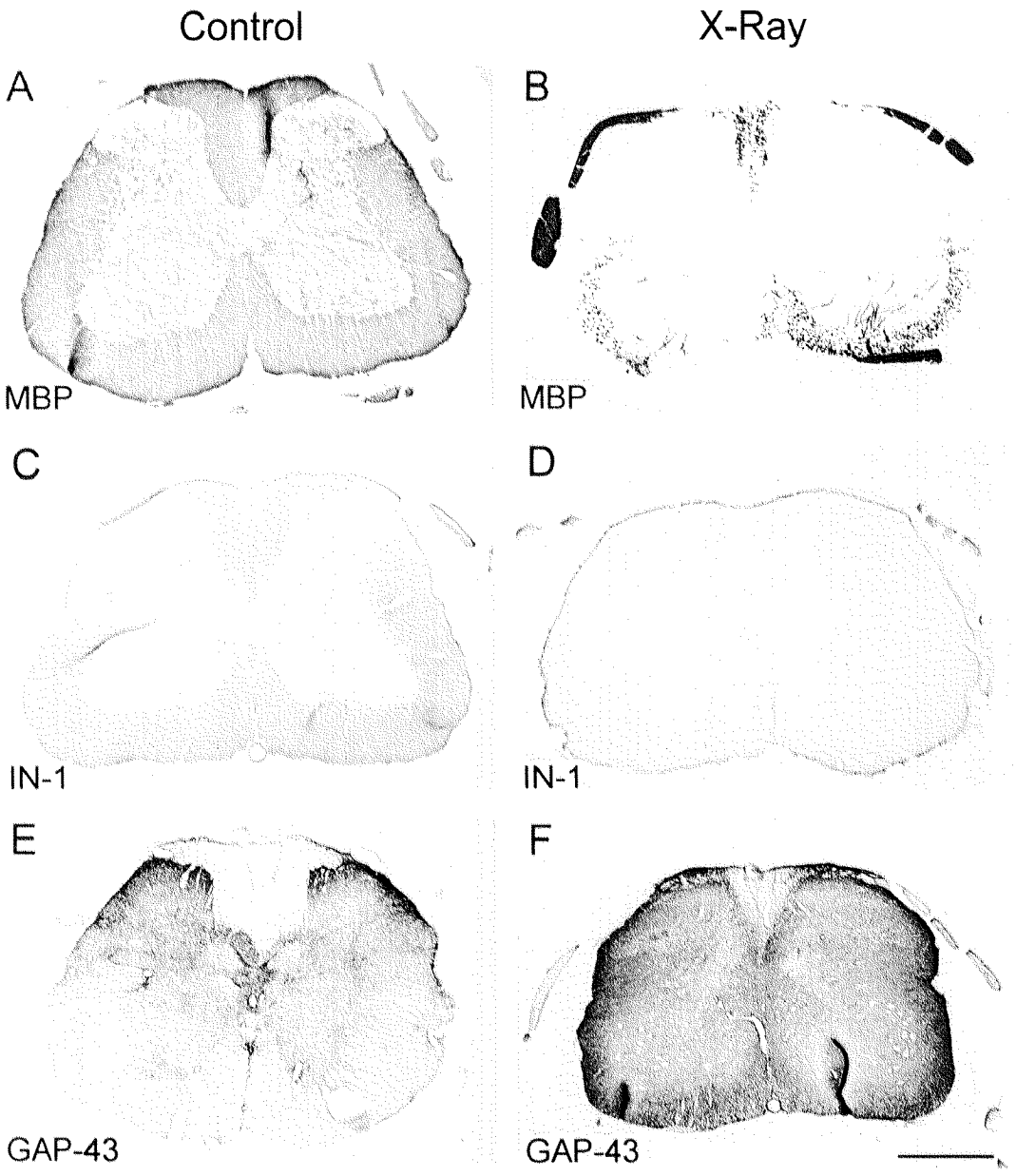

Fig. 2: Immunohistochemical stainings for evaluation of myelin suppression by the X-irradiation Transverse sections of the normal (A, C, E) and X-irradiated (B, D, F) lumbar spinal cord at P35 reacted with antibodies against myelin basic protein (MBP), myelin-associated neurite growth inhibitors (IN-1 antigens NI35/210), and GAP-43. MBP-staining shows myelination in spinal cord gray and white matter (A). In the X-irradiated spinal cord MBP expression (B) was strongly reduced with a few remaining myelinated fibers 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.

The corticospinal tract projects predominantly contralateral in the normal and myelin-free spinal cord in unlesioned 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 labeled and terminal labeling was mainly limited to the spinal cord gray matter contralateral to the injection site (Fig. 3A, B). The heaviest labeling was found in the dorsal horn, but a projection to the ventral horn was also present. Only little label was present in the ipsilateral half of the spinal cord. In the myelin-free spinal cord the labeling density in the gray 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 labeled terminals on the ipsilateral side (Fig. 3C,D). The termination pattern of the CST was thus rather normal in the myelin-free spinal cord.

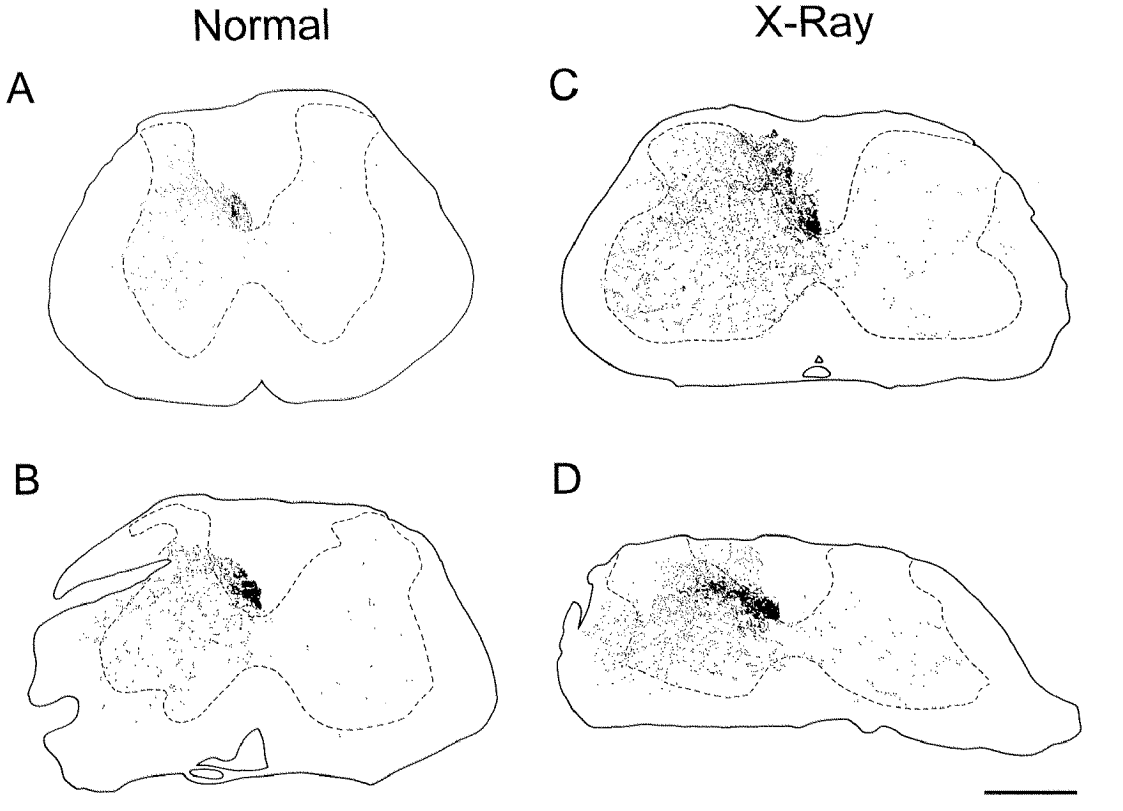

Fig. 3: Termination pattern of corticospinal fibers in normal and X-irradiated spinal cord without a CST lesion.Camera lucida drawings of the CST after WGA-HRP tracing on transverse sections of the lumbar spinal cord of normal and X-irradiated rats without a CST lesion. In the normal and myelin-free spinal cords CST fibers and terminals are confined to the side of the spinal cord contralateral to the tracer injection. The few labeled fibers on the ipsilateral side represent the very minor uncrossed portion of the CST. Scale bar = 500 µm.

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 fibers on the ipsilateral side of the spinal cord could be found (camera lucida drawings Fig. 4A - C, original micrographs Fig. 5A - C). These fibers most likely represent sprouts from contralateral corticospinal fibers because they were restricted to the medial third of the ipsilateral spinal cord. Profiles of fibers crossing the midline of the spinal cord could be seen (Fig. 4C). These ipsilateral fibers appeared more frequently in the lumbar level of the spinal cord as compared to the cervical level. Sprouting from intact corticospinal fibers 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 gray matter.

In the myelin-free spinal cord, sprouting of CST fibers 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 labeled CST fibers on the denervated side were clearly increased compared to control animals and were distributed over the whole extent of the spinal cord gray matter (camera lucida drawings Fig. 4D - F, original tracing micrographs Fig. 5D - F). Fibers crossing the midline were seen frequently indicating that the sprouted fibers most likely crossed the midline at the level of the spinal cord. As in normal spinal cord, the terminal labeling was more dense in the dorsal than in the ventral horn. The majority of the sprouted fibers were present in the medial third of the spinal cord gray matter (Fig. 4, 5). In the myelin-free spinal cord labeled fibers were also present in the middle and lateral third of the spinal cord, regions where fibers were very rarely observed in control animals (Fig. 4).

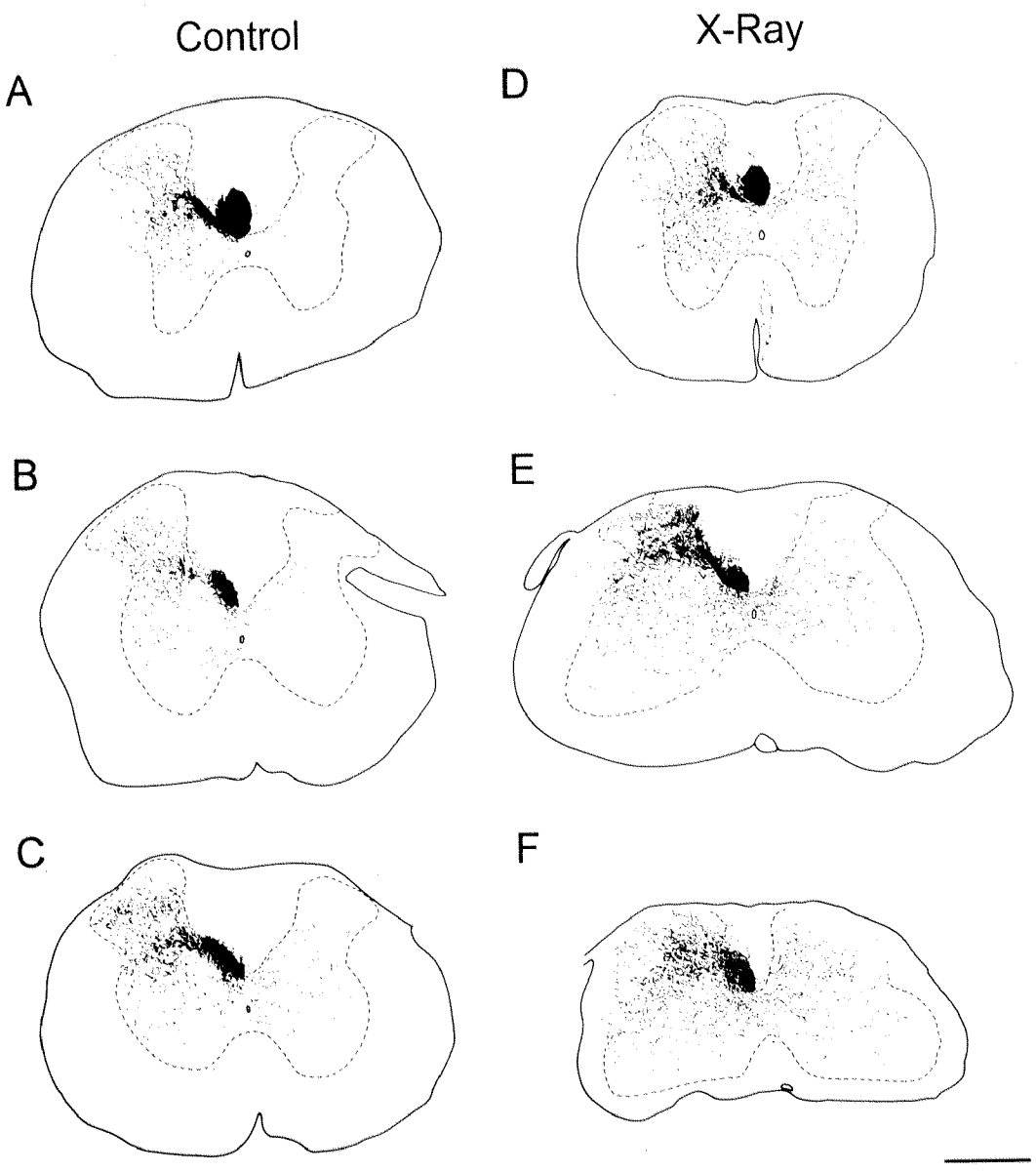

Fig. 4: Termination pattern of corticospinal fibers in control and X-irradiated spinal cord two weeks after a CST lesion.
Examples of camera lucida drawings of the CST after WGA-HRP tracing on transverse sections of the lumbar spinal cord of lesioned control and X-irradiated, myelin-free rats (lesion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

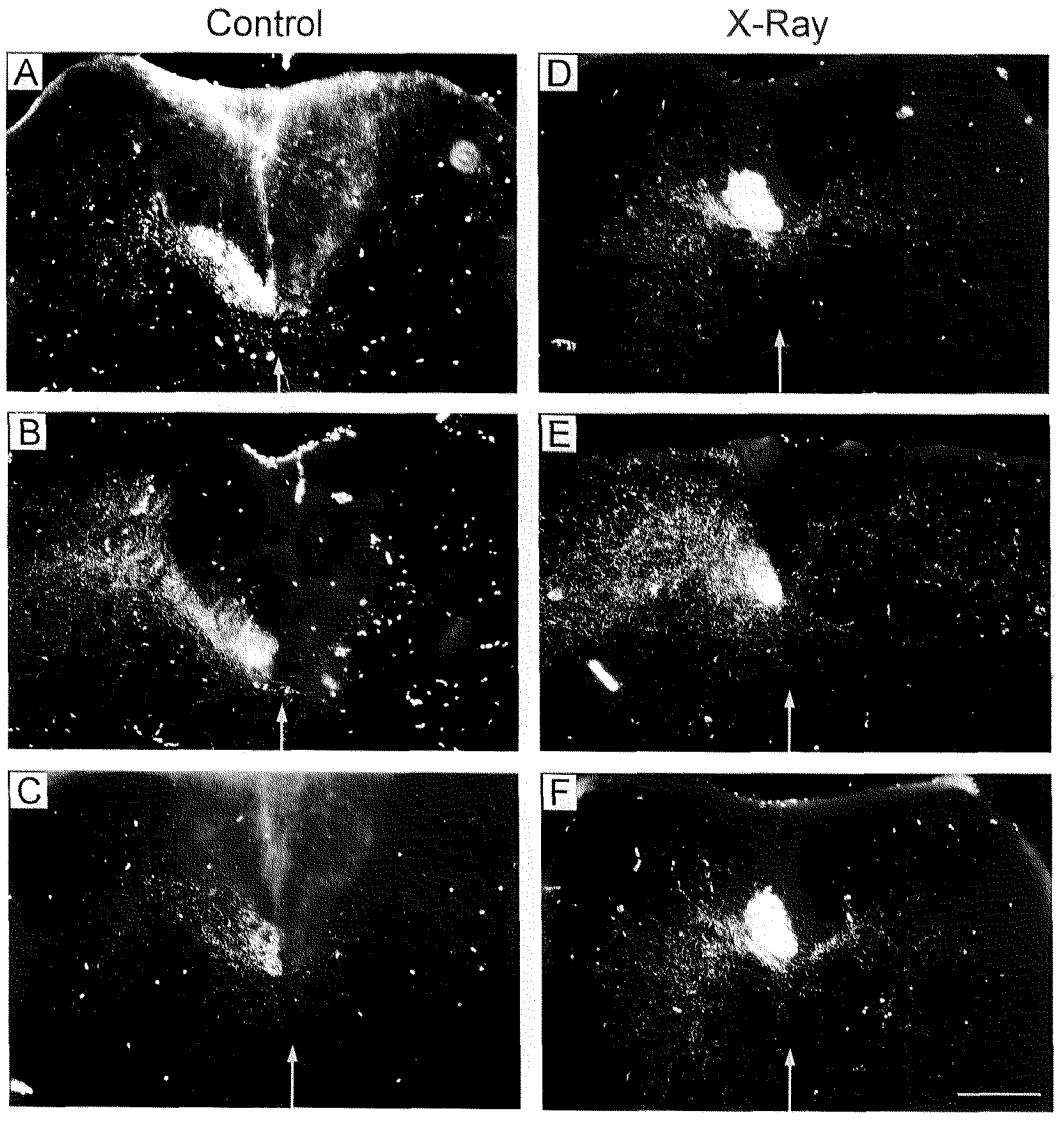

Fig. 5: WGA-HRP tracing of corticospinal fibers in control and X-irradiated spinal cord two weeks after a CST lesion.
Darkfield micrographs of transverse sections showing WGA-HRP traced corticospinal tracts 14 days after a unilateral CST lesion. In control spinal cords (A - C) labeled fibers are mainly confined to the intact side, contralateral to the tracer injection. In the X-irradiated spinal cords (D - F) many labeled fibers are present on the denervated side of the spinal cord, ipsilateral to the tracer injection, indicating sprouting of corticospinal fibers. In some cases (e.g. F) fiber 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.

Quantitative analysis of sprouting of CST fibers

In order to quantify the sprouting response of CST fibers we have quantified the amount of labeled fibers on the denervated half of the spinal cord both in animals traced with WGA-HRP and BDA. Grains of WGA-HRP reaction product reflecting labeled CST fibers were counted in different sectors of the spinal cord, the values of 3-4 sections averaged and expressed as the percentage of the label counted in sector A (intact side). Therefore, the variability in the labeling intensity from animal to animal was corrected. The results are shown in Fig. 6. On the intact side of the spinal cord labeling intensity was similar for myelin-free and control animals. In contrast, the labeling intensity was strongly increased on the denervated side in myelin-free animals compared to 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 gray 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-labeled fibers 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 fibers 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). In the lateral part of the denervated spinal gray matter, BDA-labeled CST fibers 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.

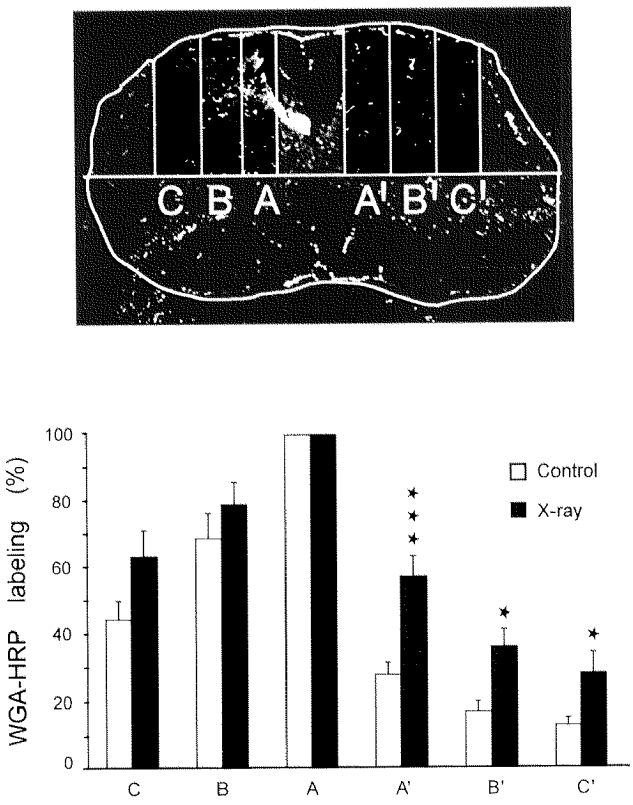

Fig. 6: Quantitative analysis of sprouting from WGA-HRP labeled CST fibers after unilateral lesions in the control and X-irradiated spinal cord.
Labeled fibers (WGA-HRP-grains) were counted on transverse sections of control and X-irradiated spinal cords after unilateral CST lesions. The spinal cord gray 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 labeling 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. Gray bars represent counts from control spinal cords (N=10), black bars those from X-irradiated spinal cords (N=9). The intensity of labeling is similar on the intact side for control and X-irradiated spinal cords. On the denervated side labeling is much more intense in the X-irradiated spinal cords indicating an increased sprouting of CST fibers. Differences in labeling 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.

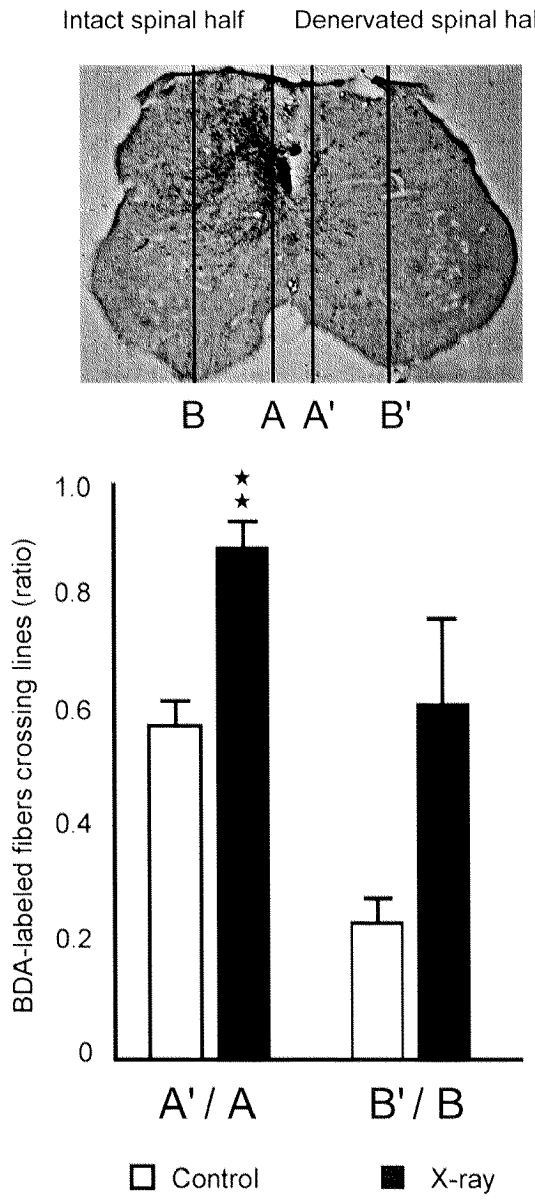

Fig. 7: Quantitative analysis of sprouting from BDA labeled CST fibers lesions in the control and X-irradiated spinal cord.
Intersections of BDA-labeled CST fibers with four lines placed on the sections as shown in the upper part. A and A' were placed near the midline, the lateral border of the CST serving as a landmark. Line B and B' were placed 1.3mm lateral to the midline. BDA-labeled CST fibers 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 fibers 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 gray matter, BDA-labeled CST fibers were threefold more frequent in myelin-free animals (p-value < 0.05) indicating an increased sprouting of CST fibers.

Terminal arbors of sprouted CST fibers

The better resolution of BDA as a tracer for the CST allowed analysis of the terminal arbors of the sprouted corticospinal fibers. Fig. 8 shows two examples of CST fibers on the denervated side of the myelin-free spinal cord. The fibers 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 fibers resemble those of normal CST axons. This termination pattern and the occurrence of bouton-like structures indicate that the sprouted fibers might make specific contacts to denervated target cells.

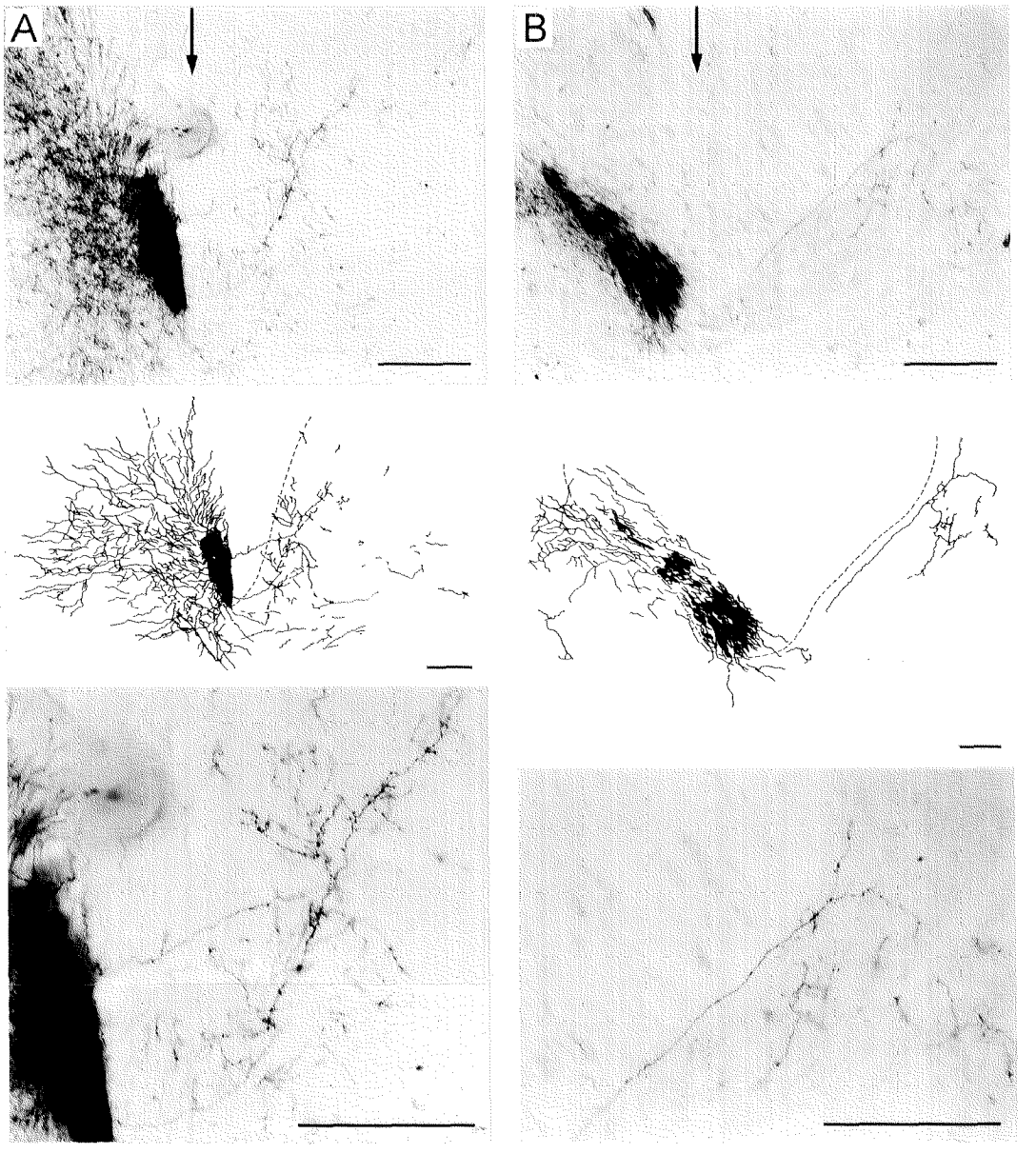

Fig. 8: Morphology of sprouted CST fibers after tracing with BDA.
Two examples (A, B) of the morphology of CST fibers sprouted to the denervated side of the spinal cord as seen after tracing with BDA. Original micrographs at intermediate magnification (upper row), low magnification camera lucida drawings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

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 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 and 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 fiber tracts at P5 (Schwab and Schnell, 1989). Only by P16 does the gray matter show substantial myelination and expression of myelin-associated neurite growth inhibitors. By P28 the pattern of myelination in gray and white matter is rather complete in the rat spinal cord (Kapfhammer and 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 gray matter.

The increase of labeled fibers on the denervated half of the spinal cord reflects an increased sprouting of CST fibers

Labeled fibers 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 fibers 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 fibers. Due to limitations of the available tracing methods which do not label all fibers with equal intensity, such a quantification cannot yield a completely true picture of the actual number of sprouted fibers. However, we have taken care to correct for all evident variables associated with the tracing procedures. In particular, variances in labeling 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 fibers 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 and Schwab, 1990; Kapfhammer and Schwab, 1994b; Schaeren-Wiemers et al., 1995). The increased sprouting of CST fibers 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 analyzed. Sema III mRNA is expressed in subsets of motoneurons 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 and Schwab, 1994b; Schwegler et al., 1995). Although the neurons 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 neurons (see below). The cell somata of the sprouting corticospinal fibers 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 fiber growth phenomena has also been observed. The permissive period for fiber 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 fibers sprout 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 and 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 neurons (Schreyer and 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 neurons, are followed only by little sprouting of the injured fibers (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 and Skene, 1991; Curtis et al., 1993; Kapfhammer and Schwab, 1994a). In a less inhibitory environment created by the absence of oligodendrocytes, sprouting fibers 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 fibers 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 neurons. The observed outgrowth of branches at right angles from the CST across the midline suggests the presence of potent chemoattractants for these fibers. After spinal cord lesions, local sprouting of CST fibers at the lesion site could be increased by the application of neurotrophin-3 (NT-3) (Schnell et al., 1994). The capacity of adult CST fibers 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 and 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 fibers 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 motoneurons 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 fibers is required. A possible behavioral 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 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 fibers are similar in rodents and humans. Myelination of the human spinal cord gray 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.

Chapter 3

Neurite growth inhibitors restrict plasticity and functional recovery following corticospinal tract lesions

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Nature Neuroscience 2: 124-131(1998)

Abstract

Anatomical plasticity and functional recovery after lesions of the rodent corticospinal tract (CST) decrease postnatally in parallel with myelin formation. Myelin-associated neurite growth inhibitory proteins prevent regenerative fiber growth, but whether they also prevent reactive sprouting of unlesioned fibers is less clear. Here we show that after unilateral CST lesion in the adult rat brainstem, both intact and lesioned tracts show topographically appropriate sprouting after treatment with a monoclonal antibody that neutralizes these inhibitory proteins. Antibody-treated animals showed full recovery in motor and sensory tests, whereas untreated, lesioned rats exhibited persistent severe deficits.

Neutralization of myelin-associated neurite growth inhibitors thus restores in adults the structural plasticity and functional recovery normally found only at perinatal ages.

Introduction

The adult mammalian central nervous system (CNS) has a very limited capacity for functional and anatomical repair after lesions. Myelin and the myelin-associated neurite growth inhibitors NI-35/250 seem be for preventing regenerative fiber growth (Schwab and Bartholdi, 1996). A monoclonal antibody (IN-1) that neutralizes the inhibitory effect of myelin enhances long-distance regeneration of adult corticospinal axons (Schnell and Schwab, 1990; Schnell and Schwab, 1993) and recovery of locomotory functions (Bregman et al., 1995).In contrast to the adult CNS, lesions of the perinatal CNS can cause both regeneration of the lesioned fibers and plastic sprouting of unlesioned fibers, which may account for the high degree of functional recovery seen at that age (Barth and Stanfield, 1990; Carr et al., 1993; Farmer et al., 1991; Kennard, 1936; Reh and Kalil, 1982; Whishaw and Kolb, 1988). For the rodent corticospinal tract, the capacity for anatomical and functional plasticity declines postnatally (Barth and Stanfield, 1990; Kalil and Reh, 1979; Reh and Kalil, 1982; Whishaw and Kolb, 1988) with the onset and progression of myelination (Kapfhammer and Schwab, 1994; Kuang and Kalil, 1990b). If myelin formation in the spinal cord is experimentally prevented, unilateral section of the CST at the level of the pyramid (a part of the brainstem) in young adult rats causes sprouting of the intact corticospinal tract across the midline of the myelin-free spinal cord into the denervated areas (Vanek et al., 1998).

There are many advantages to using pyramidotomy to investigate structural plasticity. A CST lesion in the brainstem is more likely to spare the other descending and ascending fiber systems than a large spinal cord lesion. The CST runs very superficially in the pyramid with the basilar artery as a landmark between the two CSTs, thus allowing a specific unilateral lesion. Many studies describe lesion-induced sprouting of the CST and other descending tracts in neonatals animals and the absence of sprouting in adults after such a lesion (Barth and Stanfield, 1990; Hicks and D'Amato, 1970; Kalil and Reh, 1982; Kuang and Kalil, 1990b). The normal CST innervation pattern in the spinal cord is very specific and well described (Brösamle and Schwab, 1997; Casale et al.,

1988; Kuang and Kalil, 1990a; Liang et al., 1991; Valverde, 1966), and the CST projection that originates in the forelimb area of the motor cortex topographically innervates the red nucleus (Flumerfelt, 1980; Naus et al., 1985a) and the pontine nuclei (Mihailoff et al., 1978; Panto et al., 1995; Rouiller et al., 1993; Wiesendanger and Wiesendanger, 1982), which lie rostral to the pyramid.

Here we investigated the plastic fiber growth and functional recovery of the mature rodent CST in response to a complete unilateral section at the lower brainstem (pyramidotomy): In one set of experiments we anterogradely labeled the unlesioned CST and examined its behavior in the cervical spinal cord. In another set of experiments, we labeled the forelimb brainstem projection of the lesioned CST and studied its reaction rostral to the lesion site in the red nucleus and the pontine nuclei, as well as its innervation of the dorsal column nuclei. Our experimental animals were tested for recovery of motor function in a food pellet reaching task (Whishaw et al., 1993), because the integrity of the CST is believed to be necessary for skilled forelimb use. We also used a forelimb footprint analysis to reveal deficits in forelimb placement and rotation. In the sticky-paper test, we assessed the loss and recovery of sensory function. Lesions of the CST led to functional impairment on each of these tasks, and the anatomical studies revealed only limited anatomical changes in response to the lesion. In contrast, animals treated with the IN-1 antibody showed full functional recovery on all behavioral assays, and this was accompanied by extensive plastic sprouting of both the lesioned and the contralateral unlesioned CST.

Results

The monoclonal antibody IN-1 was raised against a rat NI-250 myelin protein, a major neurite growth inhibitory component. IN-1 also neutralizes rat NI-35, as well as myelin inhibitory activity of bovine and human spinal cord (Rubin et al., 1994; Spillmann et al., 1997). Unilateral lesions of the CST were performed at the level of the medulla oblongata (see Fig. 4) in adult Lewis rats of either sex at two to three months of age. At the time of surgery, 10^5 hybridoma cells secreting either mAb IN-1 or a control antibody directed against horseradish peroxidase (Schnell and Schwab, 1990) were injected into the cortex contralateral to the lesion. Cyclosporin A (1 mg per 100 g body weight) given postoperatively allowed the growth of small, antibody secreting aggregates of hybridoma cells. Additional control groups included rats with lesion but no hybridoma cells, and sham-operated rats treated with either anti-HRP or IN-1 hybridomas (antibody-only). For anatomical studies, the sensorimotor cortex on either the intact or the lesioned side was injected on the day of the surgery with the anterograde tracer biotin dextran amine, and the animals were sacrificed by perfusion two weeks later. In one group of experiments, we investigated the effects of lesions and antibody treatment on the intact (contralateral) CST in the cervical spinal cord. In a second group, we examined the projections from the ipsilateral (lesioned) fibers to the contralateral red nucleus and pons. A third group of animals was analyzed behaviorally. In these animals, the dorsal column nuclei of the brainstem were analyzed after the testing period.

Lesion-induced sprouting in the spinal cord

In the rat, the CST decussates almost completely at the pyramidal decussation within the brainstem, and thus most fibers project, via the dorsal funiculus, to the contralateral half of the spinal cord (Rouiller et al., 1991). A small number of fibers does not cross at the pyramidal decussation, but instead continues ipsilaterally in the ventral and dorsal funiculi (Brösamle and Schwab, 1997; Rouiller et al., 1991). In the control groups, after two weeks we found a small increase in the number of labeled fibers in the denervated spinal cord gray

matter contralateral to the lesion (lesion only or antibody only; data not shown). Lesioned animals treated with control antibodies showed a similar sparse sprouting into the denervated areas (Fig. 1A, D). In contrast, lesioned rats treated with IN-1 antibody showed a significant increase in sprouting into the deafferented areas (Fig. 1B, D). Fibers grew out of the intact CST running in the dorsal column, crossed the spinal cord midline through the dorsal commissure or through the area of the degenerating CST (Fig. 1C) and extended branches into the denervated dorsal, intermediate and ventral horn (mainly laminae VI and VII). These newly formed collaterals showed bouton-like structures along the length and at their tips (Fig. 1C). In the lesioned group treated with IN-1, the number of fibers crossing the midline was increased more than twofold as compared to animals treated with anti-HRP antibodies (Fig. 1D, $p<0.001$, Mann-Whitney test). Lesioned animals treated with IN-1 were also significantly different from lesion-only and sham-operated, antibody-treated animals (data not shown), whereas there was no significant difference between the lesioned animals treated with anti-HRP antibodies and either of the other control groups. The normalized values shown in figure 1D correspond to about 3.2 fibers per section in the IN-1-treated group (n=10) and about 1.8 fibers per section in the anti-HRP-treated animals (n=10). Pilot experiments using a recombinant, humanized IN-1 Fab fragment infused by osmotic minipumps for 2 weeks over the thoracic spinal cord showed a similar lesion-induced sprouting (Bandtlow et al., 1996; Brösamle et al., 1996).

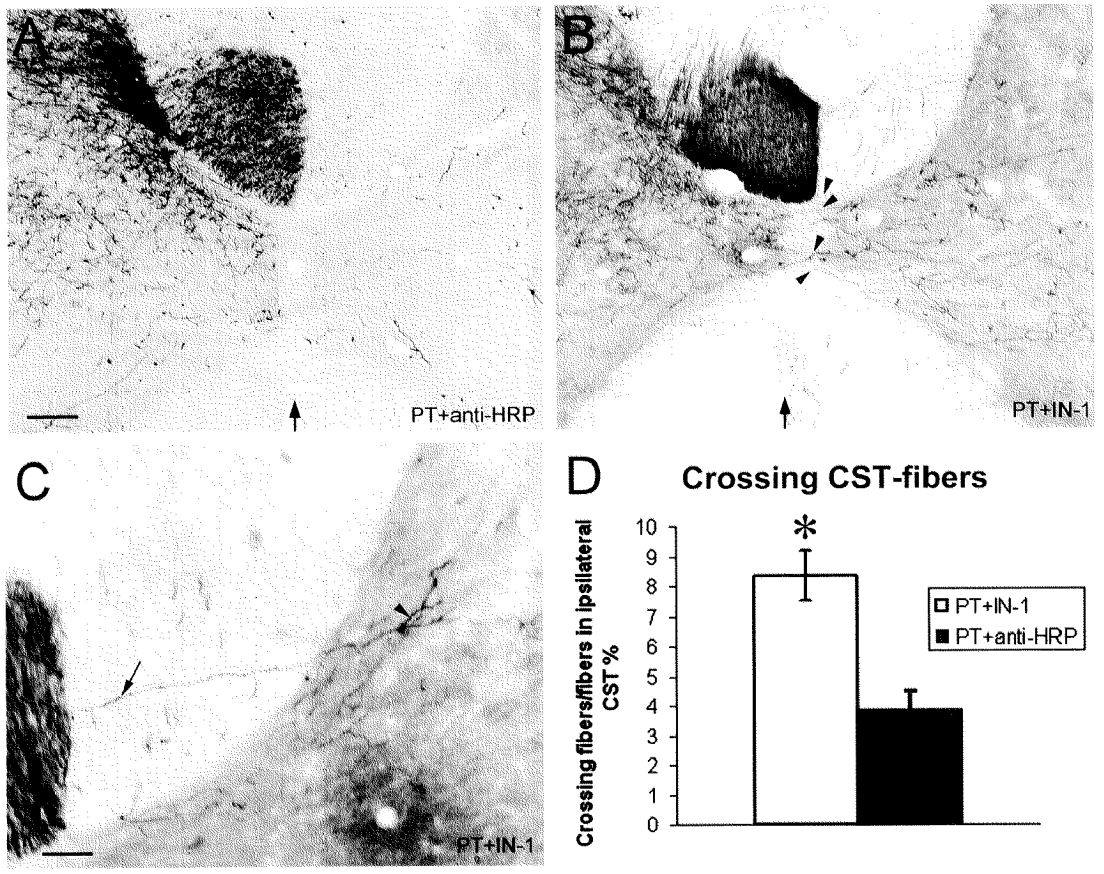

Fig. 1 Treatment with IN-1 increases lesion-induced sprouting of the intact CST, as revealed by the increased number of fibers crossing the spinal cord midline. The sensorimotor cortex of the unlesioned CST was injected with the anterograde tracer biotin dextran amine. A, B Photomicrographs of cross sections through the cervical spinal cord of lesioned animals treated either with control antibody (PT+ anti-HRP; A) or IN-1 (PT+IN-1, B). The position of the midline is indicated by an arrow. In the lesioned group treated with IN-1, many labeled CST axons cross the midline (arrowheads) and branch into denervated regions of the gray matter. In lesioned animals treated with anti-HRP antibodies, only a very small portion of fibers sprouts into the denervated half. Note also the few ipsilateral uncrossed CST fibers in the dorsal and ventral funiculi. Scale bar for *A* and B, 120µm. C Occasionally, fibers from the labeled, intact CST cross through the area of the degenerated, contralateral CST (arrow) and branch into the denervated region (arrowhead). Scale bar, 60µm. D Fibers crossing the midline were counted blind to treatment, and the counts were averaged per animal (n=10 per group). Mean values were normalized to the labeled ipsilateral fibers (see methods). The difference is highly significant (*$p<0.001$, Mann-Whitney). Error bars indicate standard error.

A new contralateral projection in IN-1-treated animals

In the brainstem, we examined the red nucleus and the basilar pontine nuclei. Both structures receive a strong input from the primary motor cortex, i.e. direct corticorubral or corticopontine projections and collaterals of the CST axons (Akintunde and Buxton, 1992; Naus et al., 1985a; Panto et al., 1995). These nuclei are involved in motor control via the cerebellum. Cortical fibers end mainly in the parvocellular part of the red nucleus; in the basilar pons each cortical area has a distinct termination pattern (Fig. 2C and D; left half). These projections are unilateral, and only a very small number of fibers cross the midline to project to the contralateral nuclei. In lesion-only rats (data not shown) and in lesioned animals treated with control antibody treated animals, these so-called corticobulbar projections were almost indistinguishable from normal, except for a slight increase in contralateral terminations in the animals treated with control antibody (Fig. 2A, C). In contrast, in animals treated with the IN-1 antibody, the number of fibers crossing the midline was increased, both in the region of the parvocellular red nucleus (Fig. 2B) and in the caudal regions of the pons. In the basilar pontine nuclei, a relatively large increase in the fiber density of the terminal plexus was found on the contralateral side in the group treated with IN-1 antibody (Fig. 2D). Densitometry of these terminal fields showed a contralateral component of 8.3% (± 2.3, n=5) in normal animals as compared to 26.3% (± 2.6, n=5, $p<0.01$, ANOVA) in the lesioned group treated with IN-1 antibody. These fibers innervated the appropriate contralateral regions (forelimb areas of the pons).

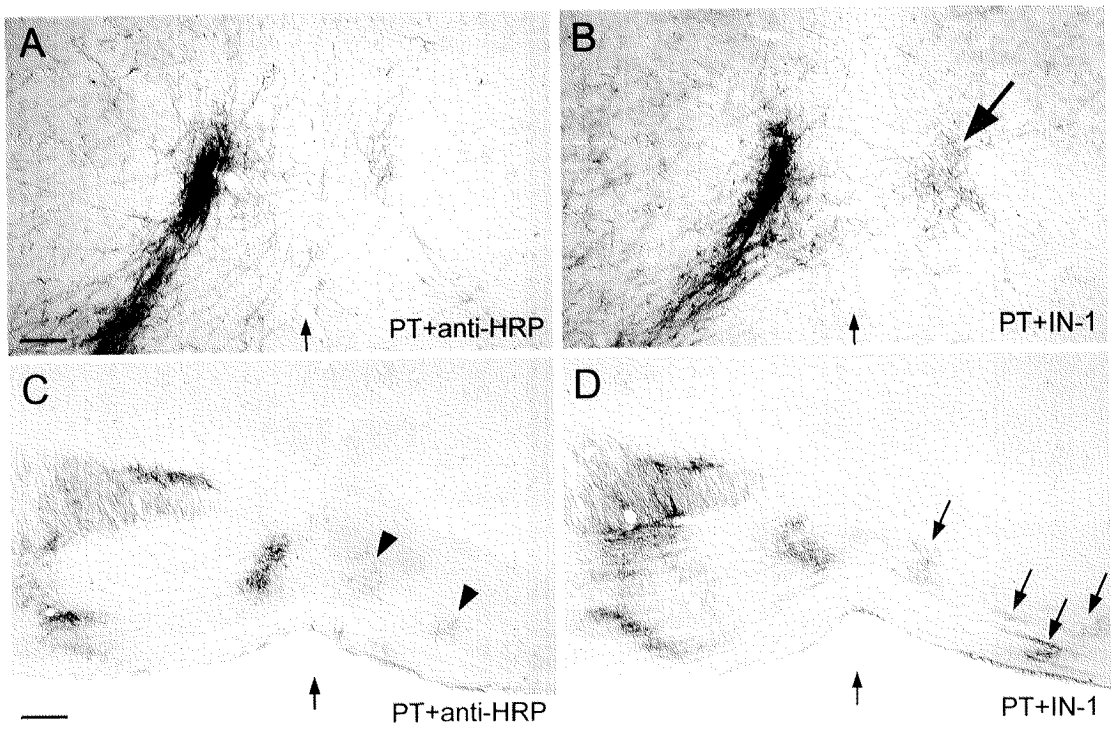

Fig. 2 Corticobulbar axons establish a bilateral projection in the red nucleus and pons after unilateral pyramidotomy and treatment with IN-1. The lesioned tract was traced with biotin dextran amine. A Photomicrograph of the innervation pattern of the rostral part of the red nucleus of an animal treated with control antibody (anti-HRP). B Corticorubral projections of an IN-1 treated animal. The extent of the termination area is enlarged (arrow). Scale bar (A, B), 120µm. C Typical projection pattern of corticopontine fibers originating from the forelimb motor cortex at the mid-pontine level of an animal treated with control antibody. Note the small contralateral projection (arrowheads). D Projection fields of corticopontine fibers at mid-pontine level of an IN-1 treated animal, showing a large increase of contralateral projections and their arborization in the typical forelimb areas (arrows). The position of the midline is indicated by an arrow. Scale bar (C, D), 240µm.

IN-1 increases dorsal column nuclei reinnervation

In rodents, CST collaterals projecting to the dorsal column nuclei (DCN; the nucleus cuneatus and nucleus gracilis) originate at the level of the decussation and spread into rostral direction to innervate the contralateral DCN (Fig. 3A; (Antal, 1984). Because of this innervation pattern, a lesion of the CST at brainstem level induces a complete denervation of the DCN. Innervation of the DCN was qualitatively compared by labeling the lesioned CST in all animal groups that had undergone behavioral tests. Lesion-only as well as lesioned, anti-HRP treated animals showed little or no DCN reinnervation (Fig. 3B; B'). In contrast, two thirds of the animals treated with IN-1 had a dense reinnervation of the DCN (Fig. 3C and D, D'). The anatomical results are summarized in figure 4.

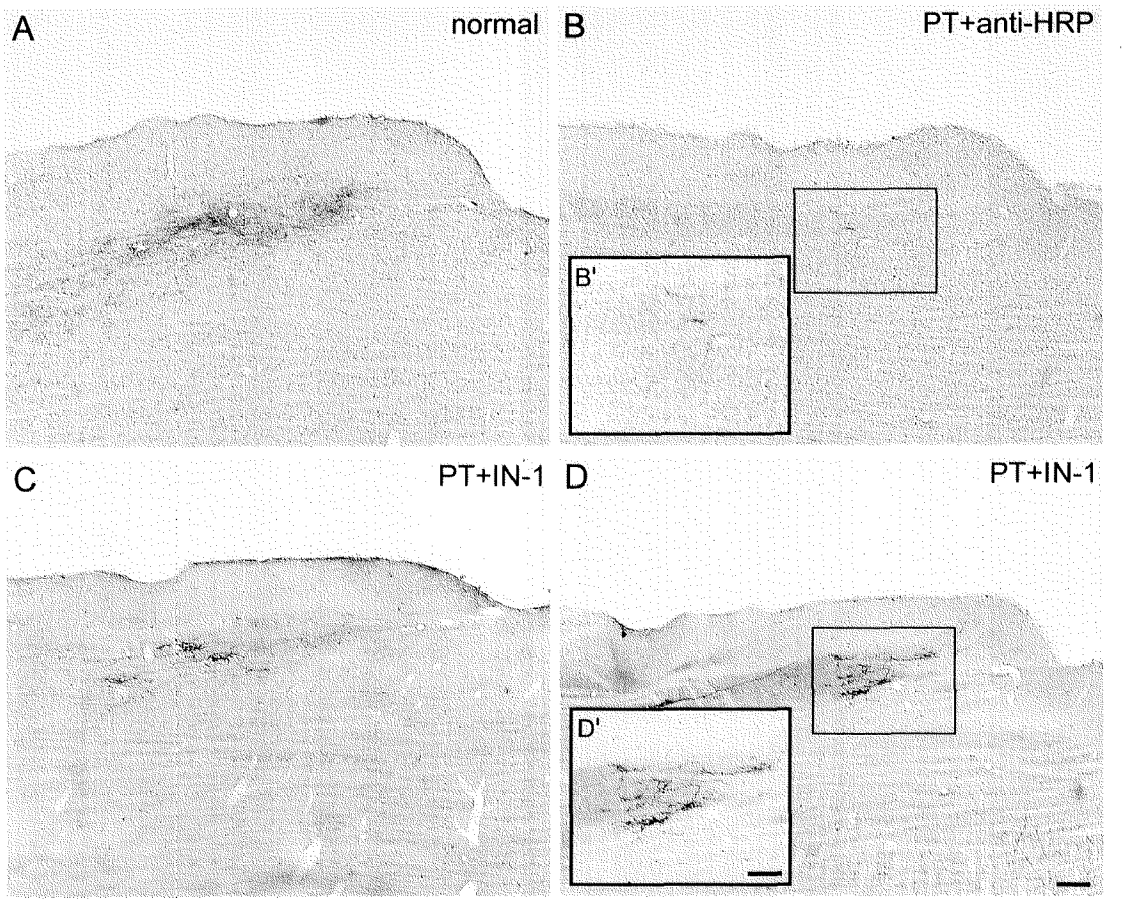

Fig. 3 Treatment with IN-1 induces a partial reinnervation of the dorsal column nuclei after pyramidotomy. All longitudinal sections are taken at the level of the solitary nucleus and counterstained with cresyl violet. A Photomicrograph of CST innervation of the DCN in a normal animal, traced with biotin dextran amine. B In lesioned animals treated with control antibodies, only a few fibers re-enter the DCN at 16 weeks postoperatively. B' Close-up of the CST fibers in the DCN. C and D Two examples of animals treated with IN-1, 16 weeks after the lesion. Notice the increase in labeling in the DCN. D' Close-up of the reinnervating fibers and their arborization. Scale bars (A,B,C,D), 200µm; B' and D', 50µm.

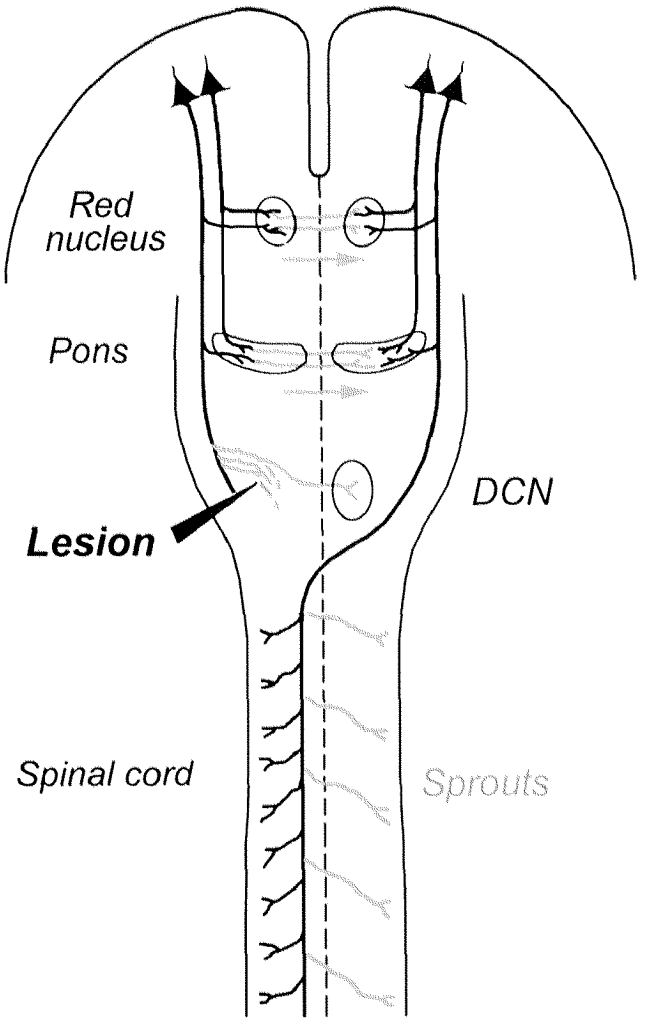

Fig.4 Schematic representation of the lesion site and the corticospinal projections that were examined in this study. Gray lines represent newly sprouted fibers after the unilateral CST lesion and the IN-1 treatment. The arrows indicate the growth direction of the sprouts. The intact CST showed collaterals that recrossed the midline at spinal cord levels to branch into the denervated hemicord. The lesioned tract increased its innervation to the contralateral red nucleus and pontine nuclei and reinnervated the dorsal column nuclei (DCN).

Functional recovery parallels structural plasticity

The remarkable structural plasticity observed in the rats treated with IN-1 antibody led to the question of whether functional improvements parallel these anatomical changes. Adult Lewis rats were trained daily for two to three weeks in a food pellet reaching task (Whishaw et al., 1993) (testing set-up in Fig. 5A) and then operated (five experimental groups: lesion only, sham operation and anti-HRP, sham operation and IN-1, lesion and anti-HRP, lesion and IN-1). Animals were allowed to recover from the operation and treatment for two weeks and were then tested for the next four weeks. On the first day of postoperative training, most lesioned animals reached with the ipsilateral, unimpaired forelimb. During postoperative training, the animals were forced to switch to the contralateral, impaired forelimb. The animals were required to grasp food pellets from a smooth surface with the impaired forelimb. The first test measured the time required to eat 20 stabilized food pellets (quantitative analysis in Fig. 5B). The preoperative baseline measurement showed that the animals needed about 75 seconds to grasp and eat the 20 pellets. Forty-two days after the operation, lesion-only animals and lesioned animals treated with anti-HRP needed significantly more time, about 150 seconds, as compared to the preoperative baseline or to sham-operated animals. In contrast, sham-operated animals and lesioned animals treated with IN-1 did not differ from the preoperative baseline (75 seconds). In a second task, the success rate to obtain 10 unstabilized pellets from the shelf was recorded (Fig. 5C). Compared to sham-operated animals, which grasped 7 pellets, lesion-only animals did grasp significantly fewer pellets (4.5) on postoperative day 42. The success rate of lesioned animals treated with IN-1 was similar to that of sham-operated animals. An increased success rate was observed in the lesioned animals treated with IN-1 antibody and in sham-operated animals as compared to the preoperative values, which was probably due to the postoperative training (Fig. 5C). In the lesion only and the lesioned, anti-HRP treated animals, the lower success rate reflects the well known permanent deficits that normally follow CST lesion (Castro, 1972b; Whishaw et al., 1993).

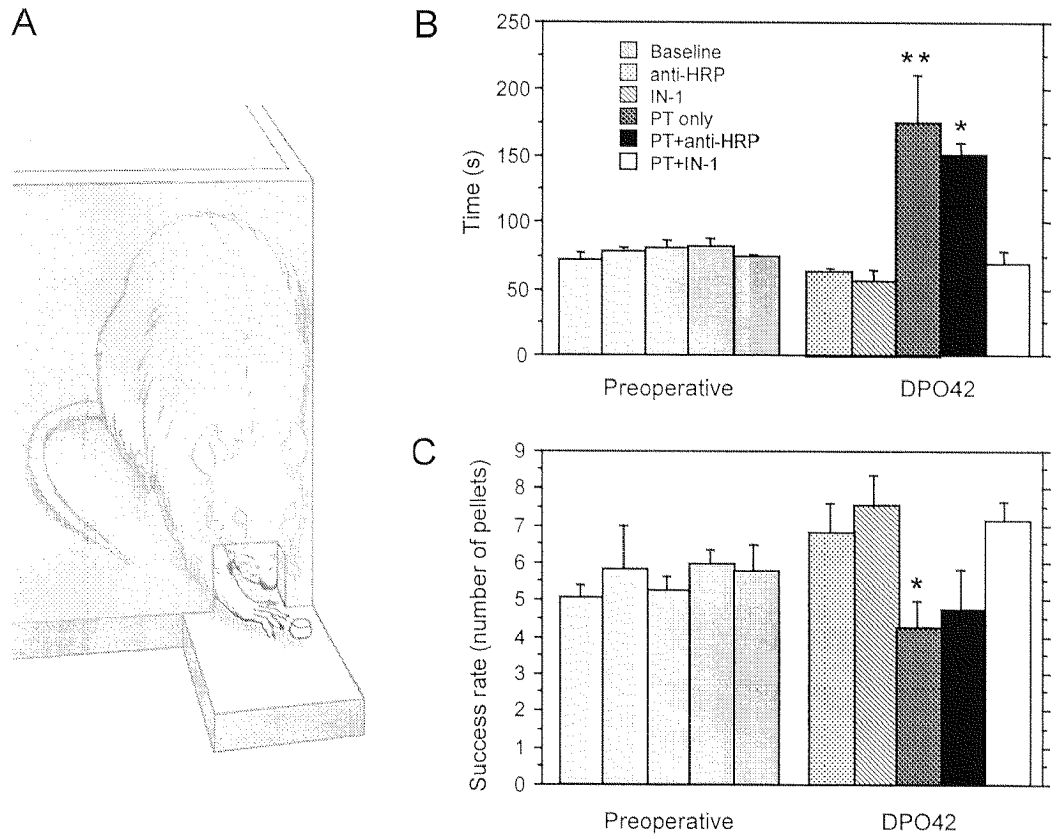

Fig. 5 The monoclonal antibody IN-1 leads to functional recovery in the food pellet reaching task. A Testing set-up; B Time (sec.) needed to grasp 20 stabilized pellets (s). C Quantitative analysis of the number of pellets grasped and eaten in the 10-pellet-test (success rate), where pellets were presented without stabilization; preoperative baseline and 6 weeks after operation. These results reveal improvements in mAb IN-1 treated animals in quantitative measurements to nearly normal performance levels. Sham operated, antibody treated animals (anti HRP N=4 or IN-1 only N=4); pyramidotomy (PT only; N=10); PT+anti-HRP (N=10); PT+IN-1 (N=10). Error bars indicate ± S.E.M. Asterisks indicate significance in reference to AB only animals: * $p<0.05$; ** $p<0.01$; Kruskal-Wallis.

Motor function was also assessed with a rope climbing task. Foot slips on the impaired side were counted while rats were climbing up a rope (Fig. 6A), and the number of slips per step was calculated (Fig. 6B). This test was done exclusively with female rats (see methods). At 42 days after surgery, lesion-only and lesioned, anti-HRP treated animals made a mean of 4.8 or 4.5 slips respectively per 10 steps (Fig. 6B), as compared to 2.5 slips per 10 steps in the sham-operated groups. Lesioned animals treated with IN-1 antibody performed as well as sham-operated animals, which were significantly better than the lesioned control groups. Compared to the preoperative performance, all animals made more footslips, which might be due to the increased body weight.

Recovery of sensory function was assesses using the sticky paper test, known to be particularly sensitive to somatosensory deficits (Hernandez and Schallert, 1988). At 42 days postoperatively, the time the rats needed to remove adhesive tapes from the palm of the forepaws was recorded for the unimpaired and the impaired side (Fig. 6C). Sham-operated animals removed the paper within 15 seconds from either forelimb. After CST lesions, animals needed 30 seconds to remove the paper from the unimpaired side (Fig. 6D). Removal of the paper sticking to the impaired forepaw was significantly prolonged to a mean of 110 seconds ($p<0.05$ for lesion only, Scheffe test). When initiated, the movements leading to paper removal were fast, showing that the motor component of this behavior was not visibly affected. Lesioned animals treated with IN-1 showed no statistical difference from preoperative values or sham-operated rats. The lesioned animals treated with IN-1 removed the papers from both the impaired and unimpaired sides within the same time (20 seconds; Fig. 6D). Interestingly, re-innervation of the DCN correlated with fast removal of the sticky paper of the impaired forepaw in the lesioned animals treated with IN-1.

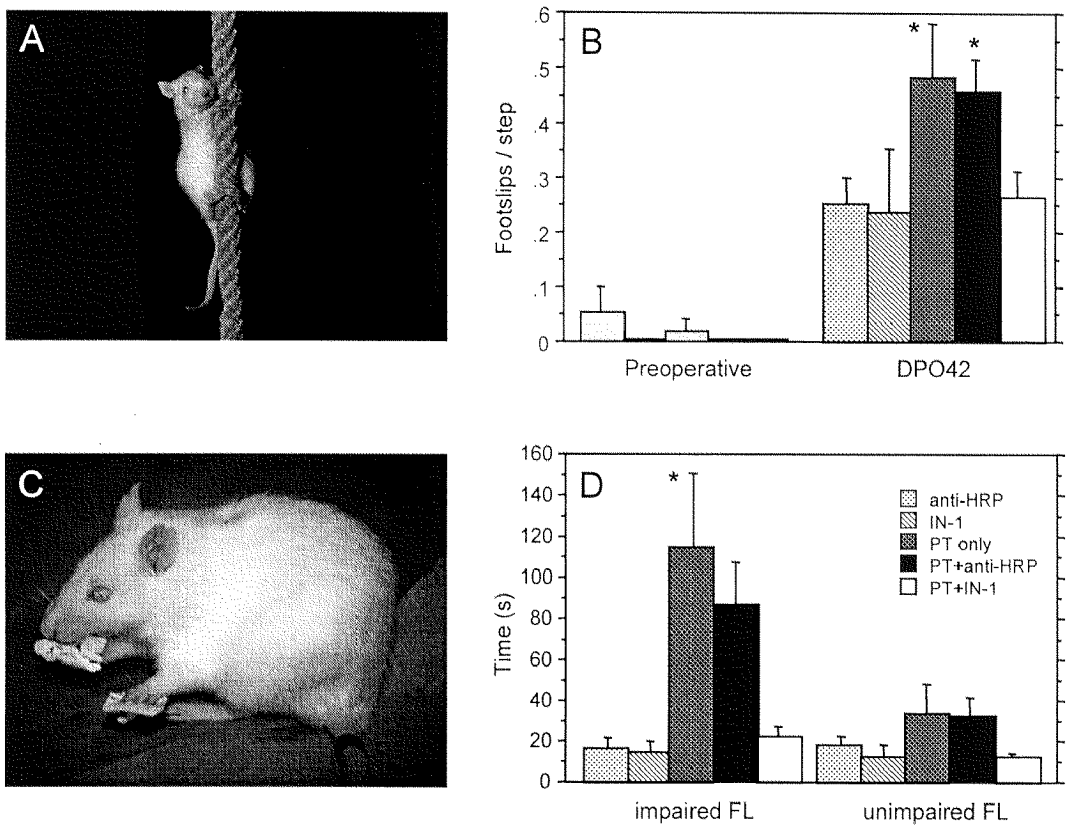

Fig. 6 Treatment with mAb IN-1 leads to improvements in the sticky paper and rope climbing test. A Rope climbing. The performance when climbing a vertical rope was recorded. B The number of foot slips per step was measured preoperatively (baseline) and 42 days after the lesion (DPO42) for female animals. This test showed improved climbing ability in mAb IN-1 treated animals as compared to lesioned control groups. Anti-HRP, sham operated with anti-HRP treatment (N=2); IN-1, sham operated with mAb IN-1 treatment (N=3); PT only, lesion only (N=4); PT+anti-HRP, lesion with control antibody treatment (N=5); PT+IN-1, lesion with mAb IN-1 treatment (N=8). Error bars indicate ± S.E.M. *$p<0.05$, Kruskal-Wallis. C Sticky paper test. The time to remove self-adhesive paper from the palm of the forelimb was measured. D Time (s) was recorded for all experimental groups on day 42 postoperatively for the contralateral (impaired) and ipsilateral (unimpaired) side. MAb IN-1 treated animals removed the paper as rapidly as sham operated animals. Anti-HRP, sham operated with anti-HRP treatment (N=4); IN-1, sham operated with mAb IN-1 treatment (N=4); PT only, lesion only (N=10); PT+anti-HRP, lesion with control antibody treatment (N=10); PT+IN-1, lesion with mAb IN-1 treatment (N=10). Error bars indicate ± S.E.M. *$p<0.05$, Scheffe.

We also used a forelimb footprint analysis to examine forelimb placement and rotation. Forelimb exorotation, i.e. an outward rotation of the impaired side, was significantly increased at 42 days postoperatively in the lesion-only group and lesioned animals treated with anti-HRP (27 and 25 degrees, respectively, compared to 20 degrees in sham-operated and lesioned animals treated with IN-1 antibody; Fig. 7). Base of support; stride length and toe spreading did not differ between the experimental groups.

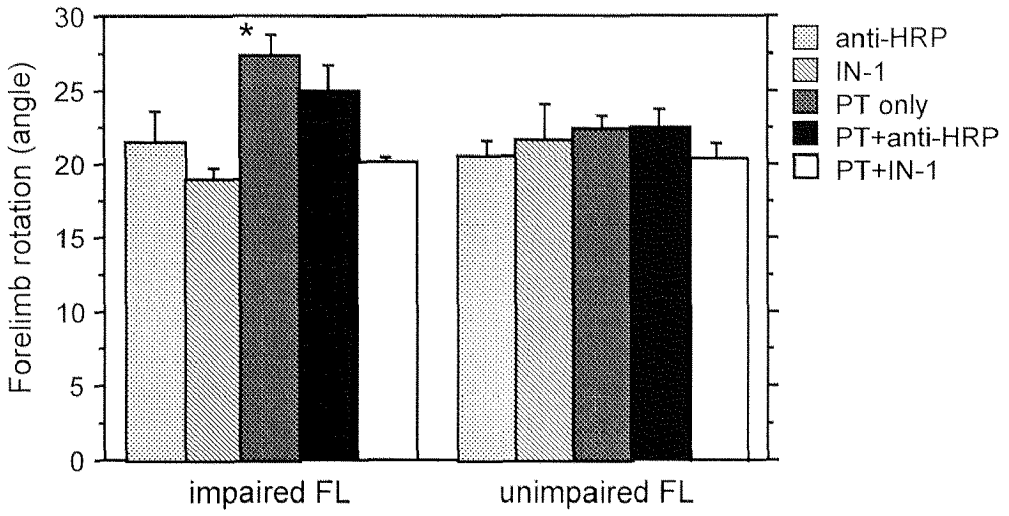

Fig. 7 Footprint analysis: Angle of forelimb rotation. Compared to preoperative values the forelimb rotation of the impaired side was increased at DPO 42 in the lesion only and the lesioned, anti-HRP treated animals. The lesioned, mAb IN-1 treated animals showed no significant change in forelimb rotation. Sham operated, antibody treated animals (anti HRP N=4 or IN-1 only N=4); pyramidotomy (PT only; N=10); PT+anti-HRP (N=10); PT+IN-1 (N=10). Error bars indicate ± S.E.M. Asterisks indicate significance's in reference to AB only animals: * $p<0.05$; ** $p<0.01$; Kruskal-Wallis.

Discussion

This study shows that the monoclonal antibody IN-1, which neutralizes the inhibitory properties of myelin, increases lesion-induced structural plasticity in adult rats after unilateral lesion of the corticospinal tract, resulting in a bilateral innervation of the cervical spinal cord, red nucleus and pons. Interestingly, the innervation of the dorsal column nuclei that was lost due to the lesion was also re-established in animals treated with IN-1. Moreover, IN-1 treatment resulted in the functional recovery of precise forelimb movements and sensory function.

IN-1 enhances plasticity of corticofugal pathways

The monoclonal antibody IN-1 recognizes a high-molecular-weight novel protein (NI-250), with very potent neurite growth inhibitory activity in rat, bovine and human CNS myelin (Caroni and Schwab, 1988b; Spillmann et al., 1997). Our results show that substantial structural plasticity (fiber growth from intact and lesioned axons; Fig.4) can occur in the adult CNS if myelin-associated neurite growth inhibitory activity is neutralized. This fiber growth is very precise and topographically specific in both the spinal cord and in the brainstem. Fibers sprouted from the unlesioned CST and crossed the spinal cord midline to arborize in the denervated half of the spinal cord. Bouton-like structures on the new collaterals suggest the presence of synaptic terminals. Thus, although the denervated half of the spinal cord has lost its normal cortical input following the degeneration of the ipsilateral CST, the observed structural plasticity may allow it to be controlled by the intact contralateral CST, and hence by the other cortical hemisphere. Some regeneration of the lesioned CST axons across or around the lesion site does occur in rats treated with IN-1, but is quantitatively weak, possibly due to the axons' difficulty in navigating through the pyramidal decussation (O. Raineteau, in press). Our findings agree with earlier observations after neonatal lesions where a similar "aberrant ipsilateral pathway" by recrossing of CST fibers at spinal cord levels was described (Barth and Stanfield, 1990; Kuang and Kalil, 1990b; Rouiller et al., 1991). We also observed sprouting of corticobulbar axons, which originate from the lesioned

CST rostral to the lesion site. These sprouts which innervate the contralateral red nucleus and pons, might have similar consequences as the sprouting found in the spinal cord: By enhancing the contralateral projection to these nuclei, the cortical hemisphere that has lost its access to the spinal cord might (for example, via the cerebellum) obtain an indirect influence on its former target regions. Interestingly, similar projections develop following unilateral lesions of one sensorimotor cortex in neonatal animals: the intact cortex sends bilateral projections to the red nucleus (Leong and Lund, 1973; Naus et al., 1985b) and the basilar pontine nuclei (Castro and Mihailoff, 1983; Leong and Lund, 1973). Thus, neutralization of the inhibitory properties of myelin results in compensatory structural plasticity which is normally only found after neonatal lesions.

Underlying mechanisms

In the spinal cord, sprouting occurred into a denervated area. Denervation may induce an upregulation of neurotrophic factors and chemoattractants (Fagan et al., 1997), which may guide the newly growing collaterals across the midline and to appropriate target regions. The nature of these factors in the spinal cord remains to be determined. A different mechanism would presumably be required to explain the sprouting that occurred in the contralateral pons and red nucleus, which are rostral to the lesion site and are not themselves denervated by the lesion. Possibly this form of plasticity might be induced by factors expressed as a consequence of the functional imbalance of the system, for example by neurotrophic factors that are regulated by neuronal activity (Thoenen, 1995). The anatomical specificity of the sprouting suggests that mechanisms for target recognition are present, or can be re-expressed, in the adult lesioned CNS.

Functional recovery

Skilled forelimb use is strongly and permanently impaired after unilateral lesion of the CST at the level of the brainstem in adult rodents (Kalil and Schneider,

1975; Reh and Kalil, 1982), and there is only limited spontaneous recovery. The very high degree of functional recovery of forelimb reaching in the rats treated with IN-1, like the anatomical plasticity, is normally observed only after lesions at perinatal ages (Castro, 1972a; Whishaw et al., 1993). It remains to be determined to what extent this functional recovery is due to the newly grown CST collaterals in the spinal cord or to the new bilateral corticobulbar projection. Under the growth-permissive conditions established by IN-1, other tracts such as the rubrospinal tract may also react by increased and adaptive plasticity. Plasticity may also occur in sensory systems (see below) and in the cortex. To confirm that the observed functional recovery is mediated by structural plasticity and not by regeneration of the lesioned CST fibers, we introduced a second lesion rostral to the first one, thus cutting the CST again, including the regenerating fibers. This procedure did not abolish the functional recovery in the food pellet reaching task of the IN-1 treated animals (Z'Graggen et al., 1998). Forelimb footprints showed an increased exorotation on the impaired side, an effect, that was also reversed in the rats treated with IN-1.

The dorsal column nuclei, the most important relay station of the ascending somatosensory system, are densely innervated by the CST. This input was lost following the lesion and partially re-established by sprouts of the lesioned axons in the IN-1 rats. After IN-1 treatment, the sticky-paper test revealed a high degree of sensory recovery. Our data thus support former suggestions that the CST modulates sensory information (Endo et al., 1973) and imply that reinnervation of the DCN might influence the sensory recovery after such lesions. The increased reinnervation of the DCN might at least partially account for the sensory recovery in the sticky-paper and reaching tests.

Neutralization of myelin-associated neurite growth inhibitors with IN-1 induced a very high degree of structural plasticity and functional recovery in adult rats. In humans, the outcome of CNS injury depends strongly on the age at which the injury was sustained; affected patients show a much better motor performance when the lesion occurs very early in life (Cao et al., 1994; Carr et al., 1993; Kennard, 1936). 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). Therefore, the presented results suggest a new possibility for therapeutic approaches after CNS lesions or stroke, for which tools like recombinant and humanized IN-1 Fab fragments are now becoming available (Bandtlow et al., 1996; Brösamle et al., 1996).

Methods

Pyramidotomy. Unilateral lesions of the CST were performed in two- to three-month-old Lewis rats. Rats were anesthetized by an intraperitoneal injection of Hypnorm (0.3 mg/kg body weight, i.p.; Janssen, Buckinghamshire, England) and Dormicum (0.6 mg/kg body weight, i.p.; Roche, Basel, Switzerland). The medullary pyramids were exposed by a ventral approach through an opening of the occipital bone as described (Kalil and 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. At the time of the surgery, hybridoma cells secreting either monoclonal antibody IN-1 (Caroni and Schwab, 1988b; Schnell and Schwab, 1990) (n=25) or anti-HRP as control antibody (Schnell and Schwab, 1990) (n=25) were injected into the cortex or the hippocampal region contralateral to the lesion. 6µl of cell suspension, containing 10^5 cells, was injected. Cyclosporin A (10mg/kg body weight, i.p.; Sandimmun, Novartis, Basel, Switzerland) was given daily during the first eight days postoperatively to allow the transplants to grow. To prevent infections Co-trimoxazol (0.83ml/kg, i.p.; Bactrim, Roche, Basel, Switzerland) was given with cyclosporin A.

Tracing. In one group of animals, the sensorimotor cortex of the unlesioned CST was pressure injected at the day of operation with the anterograde tracer biotin dextran amine (10% BDA in 0.1M phosphate buffer; Molecular Probes, Eugene; 2.5 µl into 3-4 injection sites). To examine the topography of the corticorubral and corticopontine projections in the second group of animals, we wanted to trace exclusively the forelimb area of the motor cortex, but without allowing the tracer to diffuse too far, so we chose a iontophoretic injection. The ipsilateral motor cortex was mapped by intracortical microstimulation and five points of the caudal forelimb motor area were iontophoretically injected with the anterograde tracer BDA (7s pulse, 14s pause, 5µA; duration 15min). The animals which underwent intracortical microstimulation were pretreated with atropine (0.025mg, i.p., Sintetica S.A., Mendrisio, Switzerland) and

anaesthetized with ketamine (100mg/kg body weight; i.p., Ketalar, Parke-Davis, New Jersey). After a survival time of two weeks, the corticospinal projections of the non-lesioned CST and the corticorubral and corticopontine projections of the lesioned CST were examined. After the behavioral tests, the dorsal column nuclei were investigated after tracing of the ipsilateral motor cortex using BDA (pressure injection).

Tissue handling. After a survival time of 14 days, the animals were killed by an overdose of pentobarbital (450 mg/kg body weight; Nembutal, Abbott Laboratories, Cham, Switzerland) and perfused transcardially with Ringer's solution containing 0.25% $NaNO_2$ and 100 000 units/l heparin, followed by 4% paraformaldehyde, 5% sucrose in 0.1 M phosphate buffer (PB) at pH 7.4. The brain and spinal cord were dissected, postfixed for 2 hours at 4°C and stored in a 30% sucrose solution for 36 hours at 4°C for cryoprotection. The spinal cord or the brainstem were embedded in a gelatin-chicken albumin solution polymerized with 25% glutaraldehyde, covered with Tissue Tek and frozen by immersion in isopentane at -40°C. Cross sections of 50µm were cut on a cryostat. The BDA was detected by immunohistochemistry as described (Herzog and Brösamle, 1997). Briefly, sections were collected in cold 0.1 M PB, rinsed 3x30 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, 1:100 in TBS-X) at room temperature. After 3 x 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 sulfate in 50 mM TRIS-HCl for 10 min. Sections were further preincubated for 10 min in the nickel ammonium sulfate 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 x 10 min in 50 mM TRIS-HCl. Sections were mounted on slides, dehydrated and embedded in Eukitt (Kindler, Germany). Sections of the brainstem were counterstained with cresyl violet.

Measurement of CST sprouting. Fibers crossing the midline were counted blind to treatment at an Olympus microscope. Fibers were counted at a 200x magnification in six to eight sections per animal and the counts were averaged per animal. To compensate for tracing efficiency in individual animals (due to variances in tracer uptake and transport) we counted labeled ipsilateral fibers (ventromedial and dorsoventral funiculus) and calculated the ratio to crossing fibers for each individual animal. (The proportion of the main, crossed CST in relation to the ipsilateral CST fibers is very stable and was determined in normal animals, n=4). Statistical analysis was performed using the Mann-Whitney-test.

Food pellet reaching task. Tests were performed as described (Kartje-Tillotson and Castro, 1980) in a transparent Plexiglas box (30 x 36 x 30cm) with a rectangular opening (1.5 x 3cm) in the front wall adjacent to the left side wall (Fig. 3A). The floor was constructed as a mesh that ensured that dropped food pellets were lost to the rats. A smooth Plexiglas shelf was attached to the wall underneath the rectangular opening. Small food pellets (dustless precision pellets, 45mg, Bioserv, Frenchtown, NJ) were placed one after the other onto the shelf. A plastic bar between the shelf and the opening prevented scooping of pellets. By the placement of the opening and the pellets at a distance of 1.5cm from the opening animals were biased to use the impaired forelimb. After weight reduction to about 95% of their initial weight, animals underwent a training phase and five days preoperative baseline measurements. Postoperatively, the animals were trained daily, starting two weeks after the lesion and ending six weeks after the lesion. Statistical significance was tested using the Kruskal-Wallis test. In both pre- and postoperative sessions the time to obtain 20 stabilized pellets was measured, starting from when they first touched a pellet. In the 10-pellet test, the animals received ten pellets without stabilization by placing them one after the other onto the shelf, which required the rats to reach accurately and carefully. The number of pellets grasped and eaten (success rate) was recorded in each session. If animals used the ipsilateral limb for reaching or did not start to reach at all, a maximum time of five minutes was given and the session was ended.

Rope climbing. To examine grip strength, the rope climbing test was performed. Animals had to climb a 160cm long vertical rope of to reach a platform (Carlini et al., 1967); the number of footslips of the affected side (fore- and hindlimb) was counted and reported as total number of footslips per total number of steps. As error rates increase with body weight, the pre- and 42-days-postoperative values are given only for females (n=22).

Sticky paper test. Self adhesive labels (1.3 x 2.6cm) were placed onto the palm of the forepaws as described (Hernandez and Schallert, 1988). The time the rats needed to remove the paper was recorded for each side. This test was originally developed to assess somatosensory asymmetry and sensory function after sensorimotor cortex lesions.

Footprint analysis. Footprint analysis was modified from de Medinaceli et al., (1982). The forepaws were inked and footprints were made on paper covering a 7cm wide, 50cm long runway that forced the rats to walk in line in a given direction. A series of at least ten sequential steps, recorded in two trials, was used to determine mean values of limb rotation, stride length, base of support and toe spread. Limb rotation was estimated by the angle formed by the intersection of the line through the print of the third digit and the print representing the metatarsophalangeal joint with the line through the metatarsophalangeal print parallel to the walking direction. Stride length was measured between two consecutive prints on each side. The base of support was determined by measuring the core-to-core distance of the print representing the planar cushion underlying the metatarsophalangeal joint. Toe spread was measured as the distance between toe one and toe four.

Statistical analysis of behavioral data. Analysis of behavioral data was performed with StatView 4.53 statistical package (Abacus Concepts, Inc., Berkeley, CA). For parametric data (time measurements, sticky paper test and footprint analysis), the Scheffe test was used. For non-parametric data (success rates) the Kruskal-Wallis test or the Wilcoxon signed rank test was used. A p-value less than 0.05 per number of samples was chosen as significance level.

All data are presented with standard error. As there were no differences among sham-operated rats, anti-HRP treated rats and the sham-operated rats treated with IN-1, they were combined into one group (antibody only) to increase the power of the statistical analysis.

All protocols are approved by the cantonal veterinary department of Zürich.

Acknowledgement

We thank Regula Schneider, Ruedi Kägi and Martina Weber for technical assistance. Roland Schöb helped with photography and Eva Hochreutener with the graphics. This work was supported by the Swiss National Science Foundation, the International Research Institute of Paraplegia (Zürich) and the American Paralysis Association (Springfield, New Jersey).

Chapter 4

Structural plasticity of corticospinal fibers in the adult rat spinal cord is induced by the monoclonal antibody IN-1 and the corresponding hIN-1 Fab fragment

Abstract

Myelin of the central nervous system (CNS) contains proteins that inhibit neurite growth among which the very potent inhibitory protein NI-220/250/Nogo-A that is highly conserved amongst several species. Previously it was shown that the monoclonal IgM antibody IN-1 is able to neutralize this myelin-associated neurite growth inhibitor effectively. In vivo application of the mAb IN-1 enhanced long-distance regeneration of the corticospinal tract (CST) after spinal cord lesions in adult rats and allowed the recovery of certain aspects of locomotor function. Local injections of neurotrophin-3 (NT-3) enhanced sprouting of lesioned CST fibers at the lesion site but only IN-1 increased the number of regenerating fibers. In a different lesion paradigm, a unilateral transection of the CST at brainstem level, the neutralization of the IN-1 antigens allowed compensatory growth of lesioned and unlesioned corticofugal fibers in the spinal cord and the brainstem. In the present experiments we investigated if a partially humanized recombinant IN-1 Fab (hrIN-1 Fab) fragment derived from the original mAb IN-1 influences the structural plasticity of unlesioned CST fibers following a unilateral pyramidotomy. We infused either hrIN-1 Fab or bovine serum albumin in buffer subdurally at thoracic spinal cord level. In one experiment we combined the hrIN-1 Fab with a high concentration of NT-3. A significant increase of sprouting fibers at the infusion site of hrIN-1 Fab fragment was found only after combinations with NT-3 whereas the hrIN-1 Fab alone did not lead to consistent results. Further experiments have to be done to clarify the effect of hrIN-1 Fab fragment and to elucidate the role of NT-3.

Introduction

The growth capacity of lesioned fibers in the mature CNS is rather limited in comparison to the immature CNS or the CNS of lower vertebrates. Different experimental approaches have been taken to induce or enhance growth processes in the adult CNS. Most of these approaches tried to alter the microenvironment of neurites offering a more favorable substrate than the CNS tissue, for example by grafting Schwann cells, embryonic or olfactory ensheathing cells, peripheral nerve grafts or genetically modified fibroblasts or Schwann cells to deliver neurotrophic factors (e.g. Xu et al., 1994; Li and Raisman, 1994; Paino et al., 1994; Xu et al., 1995; Cheng et al., 1996; Bravin et al., 1997; Davies et al., 1997; Grill et al., 1997a; Grill et al., 1997b; Li et al., 1997; Li et al., 1998; Menei et al., 1998; Ramon-Cueto et al., 1998; Tuszynski et al., 1998). Many of these grafting studies were successfully evoking fiber growth into the graft, but the fibers often failed to re-enter the host tissue. Adult CNS myelin was shown to contain potent inhibitory proteins that are crucially involved in restricting fiber growth in vitro and in vivo (Caroni and Schwab, 1988a; Schnell and Schwab, 1990). A monoclonal antibody, the mAb IN-1, was raised against a gel-purified inhibitory rat myelin fraction (rat NI-35/250; Caroni and Schwab, 1988b). This antibody was able to neutralize the inhibitory components and allowed axon growth in vitro (Caroni and Schwab, 1988b) and in vivo (Schnell and Schwab, 1990; Schnell and Schwab, 1993; Schnell et al., 1994). The monoclonal antibody IN-1 belongs to the IgM/κ subclass and, thus, is a very large molecule (>900kD MW, Caroni and Schwab, 1988b). The penetration into the CNS tissue is therefore rather limited. In addition to the low penetration ability, the mode of delivery is another disadvantage. As the mAb IN-1 shows only a very low stability it cannot be concentrated or stored. Antibody-producing hybridoma cells have therefore to be implanted into the CNS parenchyma relatively close to the ventricle system to insure a continuous and sufficient antibody delivery and to allow its distribution over large regions of the CNS. The implanted animals have to be immunesuppressed to allow the growth of these cells. It is not possible, however, to control the growth rate of the hybridoma

implant, and a large variability in the hybridoma xenograft size is often observed. Exuberant division of the cells can lead to a fast expansion of the graft and eventually to a compression of CNS structures. The variability in xenograft size also implicates variable antibody concentrations. In addition, subpopulations of cells might stop antibody-production and then rapidly overgrow the rest of the cell population. By using encapsulated hybridoma cells one can circumvent the need for immunsupression and the danger of exuberant growth, but only a small number of cells and thus a small amount of antibody can be applied and, again, a control of the antibody concentration is not possible. Recently, an *Escherichia coli*-derived, partially humanized Fab fragment of the IN-1 was shown to have similar neutralizing activity as the mAb IN-1 in vitro, although a tenfold higher concentration was needed (Bandtlow *et al.*, 1996). The monovalent hrIN-1 Fab fragment can be produced in a purified form in large quantities and at appropriate concentrations. In vivo, osmotic minipumps allow the controlled release of a defined amount of the antibody fragment over a defined time period. In addition, the small Fab fragment can penetrate the CNS tissue much better than the original mAb IN-1, as recently shown by using a fluorescein-tagged (Alexa 488) Fab fragment (C. Brösamle, personal communication). In vivo experiments infusing the hrIN-1 Fab into a spinal cord lesion site promoted long-distance regeneration of the CST. The regenerated fibers formed large arbors within the spinal cord gray matter showing terminal-like boutons (Brösamle *et al.*, 1996).

Our laboratory is recently focusing on the role of myelin-associated neurite growth inhibitors in structural plasticity. The application of mAb IN-1 to adult rats that underwent a unilateral lesion of the CST at the level of the medulla oblongata allowed compensatory growth of the unlesioned and lesioned CST into the denervated half of the cervical spinal cord or into contralateral brainstem targets, respectively. An impressive functional recovery was observed in parallel with these structural changes (Thallmair *et al.*, 1998; Z'Graggen *et al.*, 1998). In this chapter, I report the first results using the hrIN-1 Fab fragment after such a lesion and show that the Fab fragment has some effects on compensatory fiber growth. This sprouting was further enhanced by neurotrophin-3 (NT-3).

Materials and methods

Animals and treatment

The following animal groups were investigated: (1) *normal anatomy* (N=9). Animals of this group did not receive any treatment; four of these animals were used to evaluate the ratio between labeled fibers in the main CST (crossed component in the dorsal funiculus) and ipsilateral fibers of the uncrossed CST component running in the ventral and dorsal funiculi. (2) *lesion only* animals (N=5); these rats received a lesion as described below without any further treatment. (3) *lesioned, vehicle treated animals*: lesioned animals treated with saline or bovine serum albumin (BSA) in Fab buffer: The Fab buffer was PBS (2.6mM KCl, 136mM NaCl, 6.4mM Na_2HPO_4, 2.6mM KH_2PO_4 with 0.01M EDTA) for the first experiments (N=9) and was then changed into a carbonate buffer which is more similar to the cerebrospinal fluid (110mM NaCl, 1.5mM $CaCl_2$, 1.4mM $MgCl_2$, 4mM KCl, 30mM $NaHCO_3$, 1mM citric acid (pH 7.2); N=4). The results are presented separately for each of these control groups and the appropriate Fab fragment group. (4) *lesioned, hrIN-1 Fab treated animals* received the partially humanized, recombinant IN-1 Fab fragment (5mg/ml in PBS/EDTA, N=9; or in carbonate buffer, N=9). (5) *lesioned, hrIN-1 Fab/NT-3 treated animals* received a combination of hrIN-1 Fab treatment (5mg/ml in PBS/EDTA) and NT-3 (15.7mg/ml in PBS/EDTA; N=5; rHu NT-3, Regeneron, Amgen).

Lesion and Fab fragment application

Unilateral lesions of the CST were performed in adult Lewis rats of either sex at 8-10 weeks of age (groups 2-5). Rats were anesthetized 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 previously described (Kalil and Reh, 1982; Thallmair *et al.*, 1998). The left CST was transected using a fine tungsten needle rostral to the decussation with the basilar artery serving

as a landmark for the midline. Animals belonging to groups 3-5 were implanted with an osmotic minipump (Alzet 2002, 200µl volume, 0.5µl/h for 14 days; Charles River, France). The pumps were filled either with BSA in the same buffer as the Fab fragment, with hrIN-1 Fab fragment or a combination of hrIN-1 Fab and NT-3 the day before implantation and were stored overnight at 4°C. After the CST lesion, a laminectomy was performed at T9/T10, the dura was opened by a small incision and a thin polyethylene tubing connected to the pump was inserted under the dura with the opening in the rostral direction. The catheter was fixed by sutures to the muscles at the vertebrae and the pump was placed under the skin of the back of the rat.

At the day of the surgery, the sensorimotor cortex of the unlesioned CST (i.e. the right motor cortex, also in unlesioned control groups) was pressure injected with the anterograde tracer biotin dextran amine (10% BDA in 0.1M phosphate buffer; Molecular Probes, Eugene; 2.5 µl into 3-4 injection sites) using a Hamilton syringe. Two weeks after the lesion animals were killed by an overdose of pentobarbital (450mg/kg body weight) and perfused transcardially with Ringer's solution containing 0.25% $NaNO_2$ and 100'000 units/l heparine, followed by 4% paraformaldehyde, 5% sucrose in 0.1M phosphate buffer (PB) at pH 7.4. The brain and spinal cord were dissected and postfixed overnight at 4°C. The spinal cord was then divided into 5 parts of equal length and embedded in a glutaraldehyde-polymerized protein matrix (Herzog and Brösamle, 1997).

Tissue processing and quantitative analysis of CST sprouting in the spinal cord

Cross sections of 50 µm were cut on a vibratome and sections were collected for semifree-floating treatment (Herzog and Brösamle 1997).

For determination of the ratio of the main CST (crossed component running at the ventral base of the dorsal funiculus) to the ipsilateral, minor CST components at the level C4 we detected the tracing in four normal animals with fluorescein avidin DCS (Vector Burlingame, CA; 1:200). Sections were incubated overnight at 4°C, washed and embedded with glycerol/Mowiol (Calbiochem). Electronic images were taken with the MCID-program (M2

Analyzing Program; Imaging Research Inc., Ontario, Canada). Labeled fibers in the main CST were counted on these images with help of Paint Shop Pro (JASC Inc., Eden Prairie, MN, USA) and the Image Tool program (University of Texas, Health Science center, San Antonio, TX, USA). A 100x100 pixel field was measured within the area of the main CST and all stained fibers above background signal were counted within this field. The area of the main CST was calculated by measuring the perimeter of the dorsal funiculus comprising labeled fibers. The total number of CST fibers was then evaluated by extrapolating the number of labeled fibers of the 100x100 pixel field to the whole CST area. Then, the fibers in all the ipsilateral tracts were counted. For each animal 12 sections were evaluated and the ratio between ipsilateral CST component and crossed CST portion was expressed in percent.

In the other animals, BDA was detected by binding to an avidin-horseradish-peroxidase complex (Vectastain ABC Elite Kit, Vector Burlingame, CA, 1:100) followed by a nickel-enhanced diaminobenzidine (DAB; Sigma) horseradish peroxidase reaction to visualize the tracing.

Sprouting was quantified in transverse sections of spinal cords at the cervical level C4 and at the thoracic level (approximately T8), i.e. immediately rostral to the end of the tubing connected to the pump. All evaluations were done blindly to treatment on coded slide series. Fibers crossing the midline were counted on 6-10 sections (every third section per slide) under brightfield illumination at a 200x magnification on an Olympus microscope with the help of the Neurolucida program (MicroBrightField Inc., USA). The counts per section were averaged per animal and individual tracing variances were compensated for by calculating the ratio of crossing fibers to the number of labeled fibers in the ipsilateral tracts for each individual animal as described before (Thallmair *et al.*, 1998). This value, which is a correlate for the number of midline crossing fibers was called sprouting index. The counts of the different experimental groups were tested for statistical significance using the Student's t-test or the Mann-Whitney test.

Results

In the unlesioned rat the corticospinal tract projects mainly contralaterally and fibers crossing at spinal levels are rare

In the rat, most CST fibers cross at the pyramidal decussation and run down in the ventral part of dorsal funiculus in the contralateral half of the spinal cord (Rouiller *et al.*, 1991). Only a minor part of the fibers remains ipsilaterally and joins the ventral and dorsal funiculi (Brösamle and Schwab, 1997). Quantification of these ipsilateral fibers after BDA tracing and detection with ABC-Fluorescein in normal rats showed an average of 9.8% ± 0.66 (S.E.M.; N=4; Fig. 1) CST fibers in relation to the labeled fibers of the contralateral main CST at cervical level.

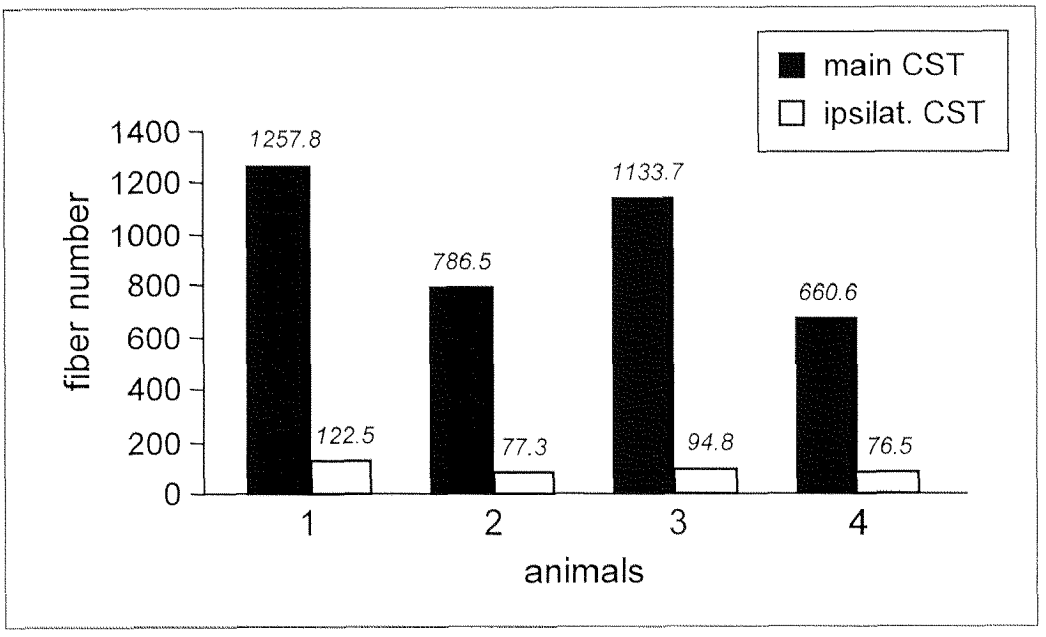

Fig.1 Determination of labeled fibers in the main, crossed CST component running at the base of the dorsal funiculus in comparison to the minor, uncrossed CST components (ipsilateral dorsal and ventral funiculus) in four normal animals at spinal level C4. The average number of ipsilateral fibers equals 9.8% of the labeled fibers in the main CST.

The labeled terminal arbors of the CST as analyzed at the cervical spinal level C4 were mainly found in the spinal gray matter contralateral to the BDA injection site. Most of the labeling was seen in the dorsal horn and intermediate gray matter, but projections to the ventral horn also existed. Only little labeling was found in the gray matter ipsilateral to the tracer injection. These terminals may originate from uncrossed ipsilateral ventral or dorsal CST fibers. Occasionally but rarely, single fibers crossing the midline at spinal levels could be found (see next section and Fig. 2).

Sprouting is very restricted after unilateral CST lesions in adult rats

In animals that received a unilateral lesion of the CST at brainstem levels without further treatment (lesion only) the midline crossing fiber ratio (sprouting index) was 3.3 ± 0.5 (S.E.M.; N=5). These fibers originated from the intact, normally innervated spinal cord, and grew into the spinal half that was devoid of its CST input due to the lesion. Statistical evaluation showed a significant increase compared to the number of midline crossing fibers in normal animals (sprouting index 1.3% ± 0.3 S.E.M.; N=5; $p \leq 0.5$; Mann-Whitney; Fig. 1).

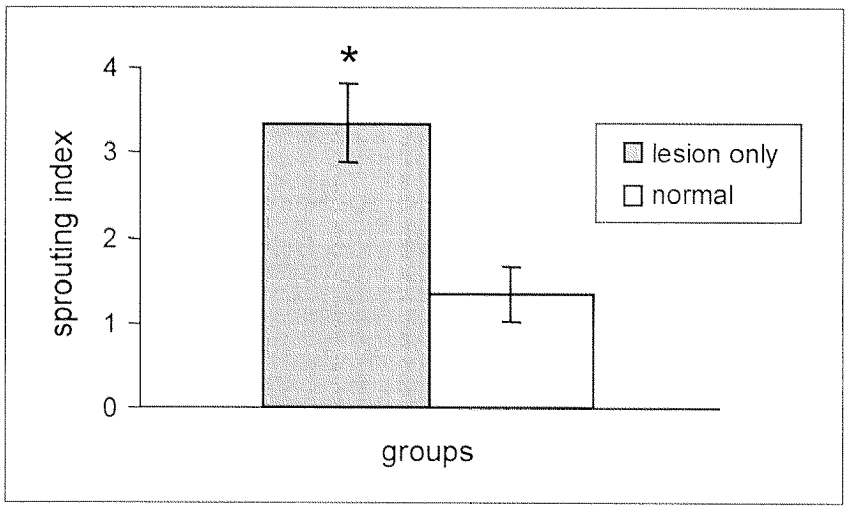

Fig. 2 The number of midline crossing fibers (sprouting index) was significantly increased at the cervical spinal cord level C4 after a unilateral lesion of the CST at the level of the medulla oblongata (rostral to the decussation). The sprouting index was 2.5x higher after the lesion compared to normal, untreated animals.

Lesion-induced sprouting after two weeks of hrIN-1 Fab infusion

In our experiments we used two different batches of hrIN-1 Fab fragment where the buffer system in which the Fab was diluted and applied was different (also in the control treated groups). Therefore, I will present these data separately.
In the first series of experiments we used four control rats (saline treatment) and four hrIN-1 Fab fragment treated rats (Fab in PBS/EDTA). To our surprise we found an increase in midline crossing fibers at cervical levels in hrIN-1 Fab treated rats but no difference at the infusion site in the thoracic spinal cord (mean values are given in Fig. 3, left half: hrIN-1 Fab group cervical 5.1 ± 1.0, thoracic 1.5 ± 0.3 S.E.M.; control group cervical 2.3 ± 0.5, thoracic 1.6% ± 0.8 S.E.M.). Two control rats showed even a higher sprouting index than all Fab infused rats of this experiment (Fig. 3, right half) at the infusion site. No significant differences were reached though.

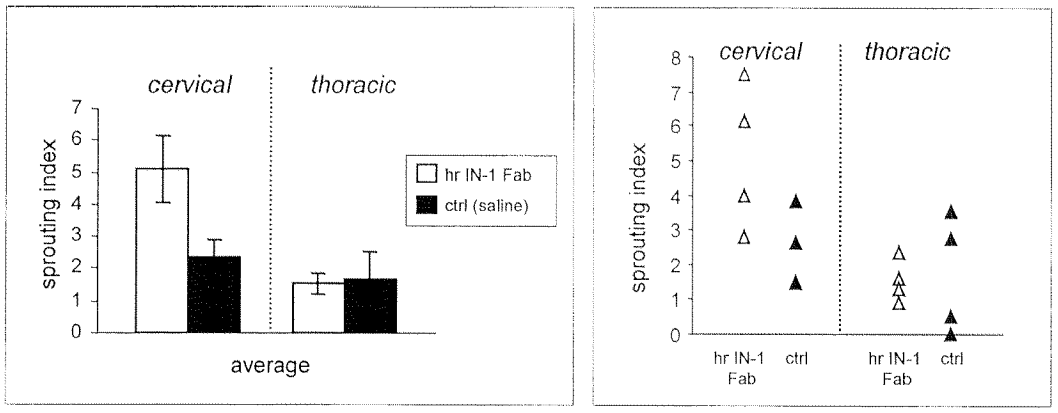

Fig. 3 Infusion of the hrIN-1 Fab fragments in PBS/EDTA and determination of the lesion-induced sprouting. No differences were seen at the infusion site (thoracic level). At cervical levels, lesioned, hrIN-1 Fab treated animals showed an increased sprouting index, but the difference was not significant. Left half, mean group values; right half, averaged individual values.

In the second series of experiments four control rats (BSA in carbonate buffer) and nine hrIN-1 Fab treated rats (Fab fragments in carbonate buffer) were included into the counting. Some animals (N=5) were discarded from further evaluation as either their tracing was too weak or the tubing had caused a large compression of the spinal cord. In contrast to the previous results with the

PBS/EDTA diluted Fab fragment, we observed a small increase in the number of midline crossing fibers in the hrIN-1 treated rats at the infusion site (hrIN-1 Fab group 4.8 ± 1.2 S.E.M.; control group 2.9 ± 0.8 S.E.M.). In contrast, at cervical levels an increase of the average sprouting index was found in control animals (hrIN-1 Fab group 4.1 ± 0.6 S.E.M.; control group 6.6 ± 0.8 S.E.M.; Fig. 4). Both groups, however, showed an increased cervical sprouting index compared to lesion only animals. None of the changes was statistically significant.

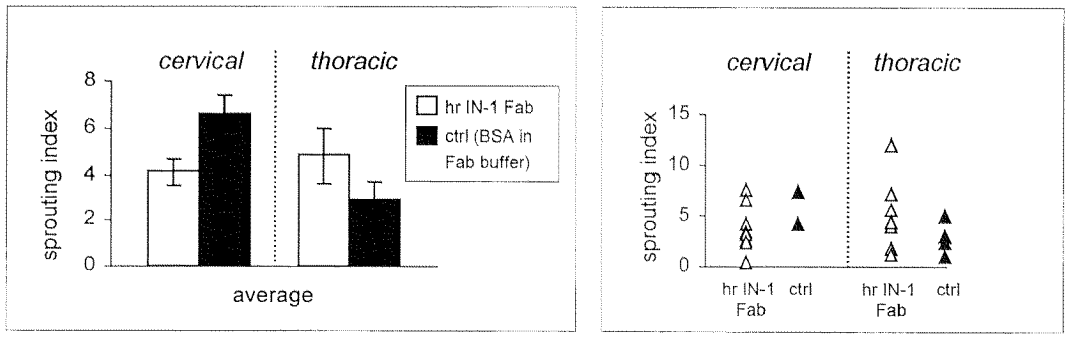

Fig. 4 Infusion of the hrIN-1 Fab fragment in carbonate buffer and determination of lesion-induced sprouting at C4 and at the infusion site. No statistical differences were seen at these levels. A small increase of sprouting fibers in the Fab treated animals compared to the control group was found at the infusion site (thoracic level). At cervical levels, control animals showed an increased sprouting index. Left half, mean group values; right half, averaged individual values.

In the last experiment rats survived for six weeks (N=5 for each group) after the surgery with 14 days Fab (in PBS/EDTA) or saline infusion. In this experiment we wanted to check effects of the Fab infusion on the weight of the rats over a longer time period. Weight gain was not altered by the Fab infusion compared to normal rats. After the survival time of six weeks, evaluation of the sprouting index showed no differences in the cervical cord (hrIN-1 Fab group 4.2 ± 1.0 S.E.M.; control group 3.9 ± 1.1 S.E.M.). However, a slight increase of the midline crossing fiber number at the thoracic infusion site (hrIN-1 Fab group 2.7 ± 0.9 S.E.M.; control group 1.3 ± 0.3 S.E.M.; Fig. 5) was observed.

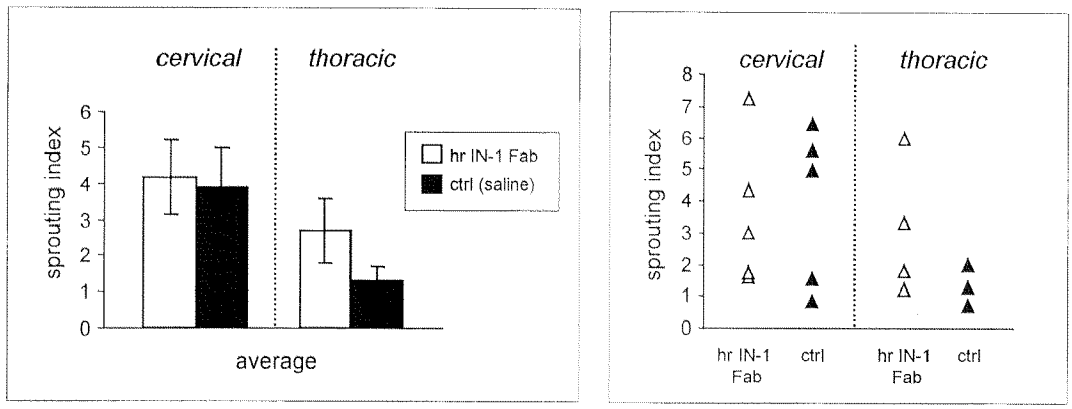

Fig. 5 Infusion of the hrIN-1 Fab fragment in PBS/EDTA for 14 days and determination of the lesion-induced sprouting after six weeks survival. A small increase of midline crossing fibers was seen at the infusion site (thoracic level). At cervical levels no difference was found. Left half, mean group values; right half, averaged individual values.

Lesion-induced plasticity is significantly increased after application of the neutralizing hr IN-1Fab fragment and NT-3

Animals receiving a combined treatment of hrIN-1 Fab and NT-3 (in PBS/EDTA) showed a significant increase of the sprouting index at the infusion site (thoracic level) in comparison to control animals (hrIN-1 Fab/NT-3: 2.2 ± 0.6 S.E.M.; control 0.8 ± 0.2 S.E.M.; $p>0.5$; Student's t-test). At cervical level, however, no difference was seen between control group and hrIN-1 Fab/NT-3 treated group (hrIN-1 Fab/NT-3: 4.1 ± 1.9 S.E.M.; control 2.5 ± 0.5 S.E.M.).

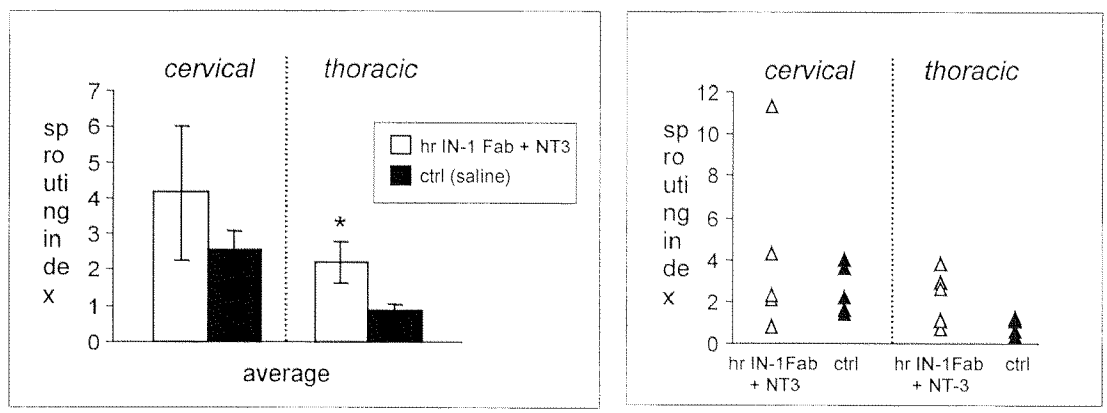

Fig. 6 Infusion of the hrIN-1 Fab fragment and NT-3 in PBS/EDTA and determination of the lesion-induced sprouting. A significant increase in the number of midline crossing fibers was seen at the infusion site (thoracic level; t-test). At cervical levels no differences were found. Left: mean group values; right:: averaged individual values.

Discussion

In the mature CNS fibers usually show only a very limited capacity for structural plasticity. Two weeks treatment with a recombinant, humanized IN-1 Fab fragment infused subdurally into the thoracic spinal cord was shown to promote lesion-induced compensatory sprouting of intact CST fibers.

Methodological considerations

The present experiments represent a series of pilot studies investigating the growth-promoting properties of a new recombinant, humanized Fab fragment on structural plasticity in the spinal cord after a unilateral pyramidotomy.

Several methodological problems became obvious during the study and were partially solved during these experiments. Many aspects, however, need to be checked in further experiments. In addition, some important control groups are missing, like an appropriate control for the hrIN-1 Fab/PBS/EDTA group or a NT-3 only group.

A PBS/EDTA buffer was used as a vehicle for the hrIN-1 Fab in most of these experiments. In a series of subsequent experiments spinal cord lesioned animals were infused with the Fab fragment (in PBS/EDTA) and the functional recovery (e.g. the BBB score, a locomotor score) of these animals was compared to animals treated with saline. Surprisingly, the Fab treated group showed a lower BBB score than the control animals, although the animals eventually recovered to the same BBB level than the control animals (K. Fouad, G. Metz, personal communication). As such a finding was never made with the mAb IN-1, we assumed that the drop in BBB score level was due to some side effects of the infused Fab-solution probably due to the EDTA-containing buffer, increasing for example secondary tissue damage. This may partially explain the inconsistent results using the Fab/PBS/EDTA in our studies on plasticity.

The experiments using the new Fab buffer (carbonate buffer) showed an increased number of midline crossing fibers, resulting in the highest sprouting index of all experiments presented here. Further experiments with the new Fab solution and the appropriate controls are needed to confirm these results.

The sprouting index established in the cervical spinal cord showed a wide range of values within each experimental group, resulting in large standard errors. This may point to the fact that small variations in the counting site within the C4 segment may lead to large variations in the number of midline crossing fibers.
Another problem with the infusion of the Fab fragment or the vehicle solution is the compression of the spinal cord by the catheter. In some animals no compression was found, in other animals, however, compression lesions of different severity occurred. We cannot exclude influences by this additional compression lesion on the sprouting of fibers.

Does the increased sprouting correlate with the neutralization of the myelin-associated neurite growth inhibitors?
In the present study we have tested whether lesion-induced sprouting can be enhanced by application of the hrIN-1 Fab fragment. In all experiments we found at least a small increase in the number of midline crossing fibers after addition of the neutralizing Fab fragment. The variability between the individual animals might be due to several reasons. First, the pump and catheter placement may influence the flow of the delivered substance resulting in a different availability of the Fab fragment. Second, it is known from regeneration studies that not every animal "responds" to the mAb IN-1 (Schnell and Schwab, 1990) or hrIN-1 Fab fragment treatment (C. Brösamle, personal communication). Only in about 60% of the lesioned animals the neutralizing agent was able to promote regeneration. This might be due also to additional factors present in the scar around the lesion site. The Fab concentration at cervical level might be rather low as Fab fragments usually stay close to their infusion site if there are not taken up and transported. Therefore, the finding that sprouting at cervical level was not greatly altered is not suprising.

NT-3 and lesioned-induced sprouting
In regeneration studies NT-3 was shown to promote CST sprouting at the lesion site (Schnell et al., 1994; Grill et al., 1997). We investigated sprouting after a combined treatment of hrIN-1 Fab fragment and a high concentration of NT-3 and found a significant increase of midline crossing fibers at the infusion site.

These results have to be interpreted with caution, however, as lesiond controls like NT-3 only and hrIN-1 Fab only in the same batch of experiments are missing. These experiments need to be done to elucidate the role of NT-3 in this lesion paradigm.

The hrIN-1 Fab fragment – a potential therapeutic tool?

This report and previous studies (Thallmair *et al.*, 1998; Z'Graggen *et al.*, 1998) demonstrated that neutralization of the myelin-associated neurite growth inhibitor Ni-250 is able to increase structural plasticity in adult lesioned rats. In agreement with the Kennard principle (Kennard, 1936; Kennard, 1938), the outcome of CNS injury is dependent on the age at which the injury was acquired. Like rats, hamsters or cats humans with unilateral brain lesions show a better motor performance when the lesion is acquired early in life (Woods and Teuber, 1978; Carr et al., 1993; Cao et al., 1994) e.g. during preterm deliveries. Interestingly, these subjects - in addition to an often very high degree of functional compensation – can show so-called mirror movements. An involvement of the intact hemisphere has been shown using focal transcranial magnetic stimulation (TMS). The data obtained by TMS and EMG recordings suggested that homologous motoneuron pools on both sides of the human spinal cord a innervated by the same descending fibers, implicating a bilateral innervation of the spinal cord by CST fibers (Farmer et al., 1991; Carr et al., 1994). 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; Spillmann *et al.*, 1998). Together with the results presented in this and earlier studies showing that the permissive period can be prolonged when myelin-associated neurite growth inhibitors are neutralized by mAb IN-1/ IN-1 Fab fragments, it becomes likely that a similar treatment may be used in the future to ameliorate the outcome after CNS insults. The route of antibody (e.g. hrIN-1 Fab) application could be similar to that of drugs, e.g. baclofen, which are given by intrathecal or intraventricular pumps in human patients routinely (Becker et al., 1997; Penn et al., 1997; Gerszten et al., 1998).

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Chapter 5

Increased corticofugal plasticity after unilateral cortical lesions in adult rats and neutralization of the IN-1 antigen

Christian A. Wenk, Michaela Thallmair, Gwendolyn L. Kartje and Martin E. Schwab

Journal of Comparative Neurology, in press

Abstract

If damage to the central nervous system (CNS) occurs early in life, extensive rearrangements of the remaining fiber systems as well as regeneration of lesioned fibers take place. In the rat or hamster, newly grown projections have been described only if the lesion occurred within the first two weeks postnatally. This decreasing growth ability correlates with CNS maturation and the progression of myelination. Myelin contains the potent neurite growth inhibitors NI-35/250 that are crucially involved in the failure of long-distance regeneration and the lack of compensatory structural plasticity after adult CNS lesions. In this study we show that extensive remodeling occurs well after the termination of the growth permissive period in the adult rat if we neutralize the inhibitory properties of myelin with the monoclonal antibody IN-1. After ablation of one motor cortex and treatment with the antibody IN-1 we observed that the remaining corticospinal tract (CST) from the spared hemisphere sprouted into the denervated, contralateral red nucleus and pons. In the pons these fibers terminated in a typical somatotopic pattern. For comparison with neonatal plasticity we performed the same lesion in two day old rats (no antibody) which as well led to sprouting of the remaining CST into denervated brainstem nuclei, resulting in a bilateral corticofugal projection. Our results show that neutralization of myelin-associated neurite-growth inhibitors after CNS lesions leads to a structural remodeling of the spared corticofugal fibers in adult rats, a process which is normally restricted to a short postnatal period.

Introduction

Brain damage early in life evokes a variety of plastic changes within the CNS which can lead to new neuronal connections that might differ from the normal developmental pattern (Kuang and Kalil, 1990; for review see Kolb and Whishaw, 1989). A high degree of functional recovery is usually seen after these neonatal CNS lesions (Whishaw and Kolb, 1988; Barth and Stanfield, 1990). In the case of unilateral cortical damage the formation of an "aberrant" ipsilateral CST from the spared hemisphere was described that might be the anatomical correlate for the sparing of skilled forelimb reaching (Hicks and D'Amato, 1970; Leong and Lund, 1973; Castro, 1975; Leong, 1976; Kartje-Tillotson et al., 1985; 1987; Gomez-Pinilla et al., 1986; Whishaw and Kolb, 1988; Barth and Stanfield, 1990; Kuang and Kalil, 1990). Additionally, an increased crossed projection from the intact hemisphere to the contralateral basilar pontine nuclei, red nucleus and striatum have been described (Leong and Lund, 1973; Nah and Leong, 1976; Mihailoff and Castro, 1981; Villablanca et al., 1982; Kartje-Tillotson et al., 1986; Murakami and Higashi, 1988; Kolb et al. 1992). In contrast to the flexible "rewiring" and functional recovery after lesions in the immature CNS, functional and anatomical repair is very limited in the adult brain and spinal cord (Kennard, 1936; 1938; Kuang and Kalil, 1990). Interestingly, the decrease of lesion-induced sprouting within the first two weeks postnatally correlates in time and location with the progression of myelination in white and gray matter (Kapfhammer and Schwab, 1994a). Myelin contains factors that may contribute to the termination of the growth permissive period, the myelin-associated neurite growth inhibitors NI-35 and NI-250. These proteins induce long-lasting growth cone collapse and inhibit neurite growth *in vitro* (Caroni and Schwab, 1988a; Bandtlow et al., 1990; Spillmann et al., 1998). The monoclonal antibody IN-1 that was raised against these inhibitory proteins allowed neurite outgrowth on CNS myelin or cultured oligodendrocytes *in vitro* (Caroni and Schwab, 1988b). *In vivo* application of mAb IN-1 led to long-distance regeneration after CST lesions in adult rats (Schnell and Schwab, 1990; Schnell and Schwab, 1993; Schnell et al., 1994) and to functional recovery of some locomotor functions (Bregman et al., 1995). In addition,

neutralization of the myelin-associated neurite growth inhibitors allows compensatory structural plasticity and restoration of function in response to an adult focal CST lesion (Thallmair et al., 1998; Z'Graggen et al., 1998).

In the present study we examined remodeling of the corticorubral and corticopontine projections from the spared hemisphere in adult rats after a unilateral motor cortex ablation and mAb IN-1 treatment. To compare these results with the structural plasticity seen after neonatal cortical ablations a group of rats underwent cortical lesion at 2 days of age. The anterograde tracer biotin dextran amine (BDA) (Brandt and Apkarian, 1992; Veenman et al., 1992) was used to trace the projections of the intact forelimb motor area to the red nucleus and the basilar pontine nuclei in all animal groups. Our results show topographically specific structural plasticity comparable to that seen after neonatal lesions in the red nucleus and the basilar pontine nuclei following unilateral lesions of the caudal motor cortex in adult, mAb IN-1 treated rats.

Materials and methods

All animal experiments were carried out under the supervision of the veterinary department of the Canton of Zurich, Switzerland. Institutional guidelines for animal care and safety were adhered to. Animals were housed in groups and had free access to food and drinking water. Figure 1 illustrates the experimental procedures.

Animals

The study was performed on 35 male Lewis rats from our own breeding colony. Twenty-four animals underwent a unilateral aspiration lesion of the caudal motor cortex at an age of 50 - 55 days. Six animals of the same age served in the anatomy control group and five animals underwent cortical lesion at postnatal day 2 (P2). The animals were divided into the following five groups:

- Unlesioned, untreated animals (n=6; Anatomy)
- Adult cortical lesion (n=6) without further treatment (Lesion only)
- Adult cortical lesion (n=8) receiving a treatment with a control antibody against horseradish peroxidase (anti-HRP)
- Adult cortical lesion (n=10) receiving a treatment with the monoclonal antibody against the myelin associated neurite growth inhibitor (mAb IN-1)
- Neonatal lesioned animals (n=5) without any further treatment used for comparison with the adult lesioned mAb IN-1 treated animals (P2-lesion)

Surgery of adult rats: tracing, lesion, and tumor application

The animals were pretreated with atropine (0.025 mg, i.p., Sintetica S.A., Mendrisio, Switzerland) and anesthetized with ketamine (Ketalar®, Parke-Davis, New Jersey; i.p., initial dose 100 mg/kg body weight followed by supplemental doses of 40 mg/kg injected i.m. whenever necessary depending on the reflex status of the animal). The animals were placed in a stereotaxic frame, laying on a heating pad (37 °C), allowing the forelimbs to hang free for a

better observation of movements during intracortical microstimulation (ICMS). The skull was exposed with a midline skin incision and the bone overlying the right caudal motor cortex area was removed (stereotaxic coordinates: 1 mm to 4 mm lateral / 2.5 mm rostral to 1 mm caudal relative to Bregma). The craniotomy was made without damaging the dura and the brain was protected with mineral oil. The cisterna magna was opened and drained to reduce swelling of the cortex.

For ICMS five points in the caudal forelimb motor cortex were chosen corresponding to the map of Neafsey et al. (1986): 2 mm lateral, 0 mm rostral / 2.5mm lateral, 0.5 rostral / 3mm lateral, 0.5mm rostral / 2.5mm lateral, 1mm rostral / 3mm lateral, 1mm rostral relative to Bregma. Using a thin low-impedance tungsten microelectrode, forelimb movements were evoked with low currents (to prevent cortical damage maximum current was 20 μA), 60 ms train, 0.2 ms cathodal pulses, 330 Hz at a depth of 1.7 to 1.9 mm. A point was accepted for iontophoresis of the BDA tracer when the threshold for forelimb movement stimulation was lower than 14 μA. Higher thresholds or blood vessels on the cortex surface caused in some cases a small shift of the coordinates.

For iontophoresis, the lesion and the hybridoma-cell implantation, ketamine treatment was stopped and a single dose of midazolam (4 mg/kg body weight, Dormicum®, Roche, Basel, Switzerland) was injected intraperitoneally. To visualize the projection originating in the caudal forelimb area, 10% BDA (10 kD MW, Molecular Probes, Eugene, OR) in phosphate buffer (10 mM, pH 7.2) was iontophoretically injected (Graybiel and Devor, 1974): a glass micropipette (tip-diameter 20 μm) was inserted perpendicular to the surface of the cortex at the five points determined by microstimulation and BDA was injected iontophoretically at the depth of lowest threshold (5 μA, pulse duration 7 s, interval 14 s) for 15 minutes at each point. The cortex was then covered with gelfoam and the skull closed with dental cement. The skin of the anatomy animals was sutured, whereas all the other animals underwent a unilateral aspiration lesion of the contralateral, left caudal motor cortex. For that reason, a second craniotomy was made and the dura was removed using forceps and a

scalpel blade. The exposed cortex was aspirated with a small glass pipette to a depth of 2 mm (1 mm to 5 mm lateral and 3 mm rostral to 3.5 mm caudal relative to Bregma).

In the two groups (n=18) receiving a hybridoma-cell implantation a small craniotomy 3 mm lateral and 5 mm caudal to Bregma was made with the dental drill and 6 µl of a cell suspension containing a total number of 10^5 living hybridoma cells was slowly injected into the lesioned hemisphere at a depth of 3 mm using a 10 µl Hamilton syringe (Schnell and Schwab, 1990; Thallmair et al., 1998). One group (n=10) received cells secreting the monoclonal antibody IN-1 against the rat neurite growth inhibitory protein NI-250 (Caroni and Schwab, 1988b) and the other group (n=8) received control cells producing an antibody against horseradish peroxidase (anti-HRP) generated from the same parent myeloma cell line P3U (Schnell and Schwab, 1990). Cultured hybridoma cells were regularly checked for their IN-1 or anti-HRP antibody production before they were implanted. The skin was sutured and the animals were returned to their cages after giving another dose of midazolam (2 mg/kg, i.p.). The day before the surgery and during the entire survival period of 14 days, the animals received a daily injection of cyclosporin A (10 mg/kg body weight, i.p., Sandimmun®, Novartis, Basel, Switzerland) to prevent an immune reaction against the implanted cells. The non-lesioned and lesion only animals received the same cyclosporin treatment. An antibiotic treatment with Co-trimoxazol (0.83 mg/kg body weight, i.p., Bactrim®, Roche, Basel, Switzerland) was started at the same time in all animals. After the survival period of 14 days all animals were killed by an overdose of pentobarbital (450 mg/kg body weight, i.p., Nembutal®, Abbott Laboratories, Cham, Switzerland) and perfused through the heart with a Ringer solution containing 20'000 U/l heparin (Liquemin®, Roche, Basel, Switzerland) and 0.25% $NaNO_2$ followed by the fixative of 4% paraformaldehyde in 0.1 M phosphate buffer and 5% sucrose. The brains were removed and postfixed in 4% paraformaldehyde for 1 day before being immersed in 30% sucrose at 4°C for cryoprotection.

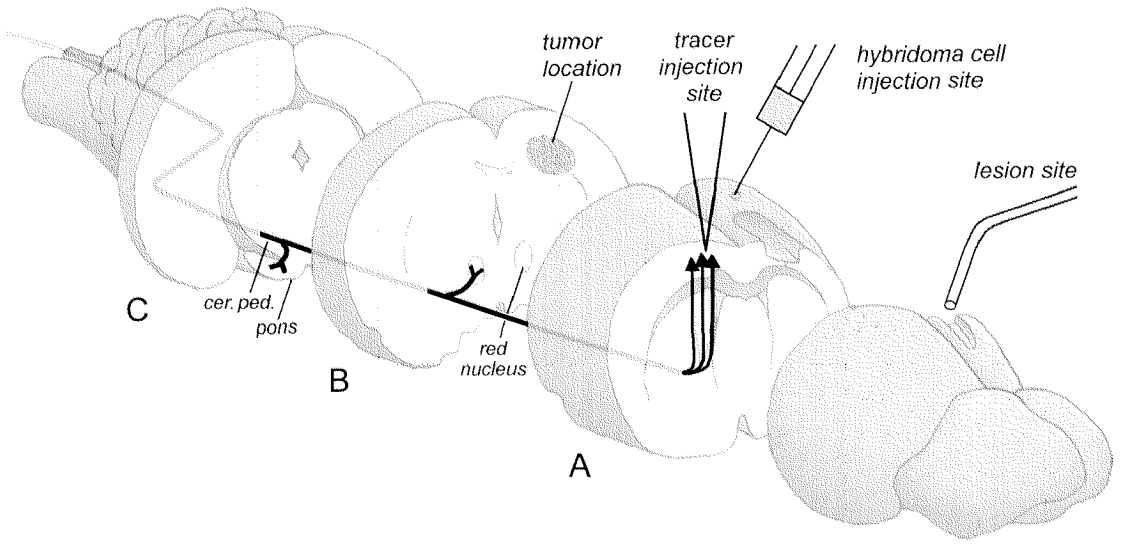


Fig. 1 Schematic illustration of the experimental procedures. Cortico-fugal fibers from the right forelimb motor cortex to the termination areas in the red nucleus and in the basilar pontine nuclei are represented. Cross sections: *A,* BDA injection region; *B* at the level of the red nucleus and *C* at the level of the cerebral peduncle (*cer. ped.*) and the basilar pontine nuclei. The aspiration lesion in the left hemisphere, the BDA tracer injection sites, the hybridoma cell implantation site and the location of the tumor are also schematically shown.

Neonatal surgery

Pups at two days of age (P2) were anesthetized by hypothermia. The cranium was exposed with a midline skin incision and the skull overlying the left frontal cortex was removed with forceps. The dura was opened using small forceps and a scalpel blade, and the sensorimotor cortex was aspirated by mild suction with a small glass pipette as previously described (Kartje-Tillotson et al., 1985). The wound was packed with gelfoam and the skin sutured. The pups were warmed under an incandescent lamp, returned to their mothers until weaning and then housed together until they were one year old. The ICMS and iontophoresis procedures were performed as described for the adult lesioned animals with the exception that in these animals the caudal forelimb motor cortex of the intact side and consequently the tracer injection points were shifted rostrally as a result of the neonatal lesion (Papathanasiou et al., personal communication). The survival time after iontophoretic BDA injection was 14 days.

Tissue processing

After immersion in sucrose for 3 days the brains were cut into two parts at the level of the thalamus. The brainstem was separated from the spinal cord just caudal to the CST decussation. The dura was carefully taken off and the cerebellum was removed. Then the tissue was embedded in gelatin-chicken albumin solution polymerized with 25% glutaraldehyde (Herzog and Brösamle, 1997) and immediately frozen by immersion in -40°C isopentane. Cross sections of 50 μm were cut on a freezing microtome. Every second section of the brain and each section of the brainstem were serially mounted on Superfrost® slides (Menzel-Gläser, Germany) and reacted for BDA using the semifree-floating method (Herzog and Brösamle, 1997). Briefly, the slides were washed three times for 30 minutes in 50 mM Tris buffered saline (0.9%, pH 8.0) with 0.5% Triton X-100 (TBST-X) and incubated over night at 4°C with an avidin-biotin-peroxidase complex (ABC elite, Vector Labs, Burlingame, CA) diluted in TBST-X. The next day slides were washed again three times for 10 minutes in TBST-X, rinsed in 50 mM Tris-HCl buffer (pH 8.0) and preincubated

in 0.4% nickel ammonium sulfate (Sigma, St. Louis, MO) in 50 mM Tris-HCl for 10 minutes. Sections were further preincubated for 10 minutes in the nickel ammonium sulfate solution to which 0.015% of 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma, Buchs, Switzerland) was added and finally reacted in a nickel ammonium / DAB mixture containing 0.004% H_2O_2. After 10-20 minutes the reaction was stopped with 50 mM Tris-HCl and the sections were rinsed three times for 10 minutes in 50 mM Tris-HCl. Sections were air dried, dehydrated and embedded in Eukitt (Kindler, Freiburg, Germany).

Neuroanatomical analysis

All anatomical structures were identified with help of the atlas of Paxinos and Watson (1982). The BDA injection site and the cortical lesion were analyzed for their localization, extent and depth (Fig. 3A, B). The hybridoma cell injection region was examined for the presence of the hybridoma xenografts. When an antibody producing tumor - or necrotic tissue in the case where the tumor was already resorbed - was found close to the hippocampal formation and the third ventricle (Fig. 3C) the brains were further analyzed. Brains that showed no signs of a tumor or brains showing enlarged tumor invasion to deeper regions resulting in a compression of brainstem structures were not included in the study.

In the brainstem the red nucleus (parvocellular part) and the basilar pontine nuclei (dorso-lateral, dorso-medial, lateral, intermedial and medial pontine nucleus; Mihailoff et al., 1978), were qualitatively and quantitatively analyzed ipsi- and contralaterally to the injection site.

For the quantitative, densitometric analysis, electronic images were acquired with a Xillix Microimager slow-scan, high resolution CCD camera attached to a Zeiss axiophot microscope. All analyses of these images were performed with the MCID-program (M2 Analyzing Program; Imaging Research Inc., Ontario, Canada): we measured the optical density over an area A (integrated optical density = iOD) which represented a value for the number of labeled fibers contained in this area. To test for linearity of the iOD to the number of labeled fibers, measurements of the iOD and counts of labeled fibers (over the same

area) were made for 11 regions of different fiber density in the cerebral peduncle (Fig. 2A) and in a pontine projection area (Fig. 2B). The results showed a linear, significant relationship with a very high correlation between iOD and actual fiber numbers in both regions (Fig. 2A, B).

Background corrections were done by subtracting the optical density (extrapolated to the area A) of surrounding tissue (containing no labeled fibers):

$$iOD = iOD_A - ((A / B) \times iOD_B)$$

iOD = calculated integrated optical density (of labeled fibers only) in a projection area (A); iOD_A = measured integrated optical density over area A (inclusive background); B = area of surrounding tissue including no labeled fibers; iOD_B = measured integrated optical density over area B (background only)

Quantification of CST labeling in the cerebral peduncle

The numbers of labeled CST axons in the cerebral peduncle were determined and used to normalize the inter-animal variations in the BDA tracing. The ipsilateral labeled cerebral peduncle on five consecutive sections of the intermediate part of the pons was measured (iOD_A), and the contralateral unlabeled cerebral peduncle served as iOD_B for background subtraction. The resulting iOD values and the calibration (iOD vs. number of labeled CST axons, Fig. 2A) were used to extrapolate the total number of labeled CST fibers (Fig. 2C). Statistical significance of differences of the labeled CST-axon numbers was assessed with the unpaired t-test assuming unequal variances.

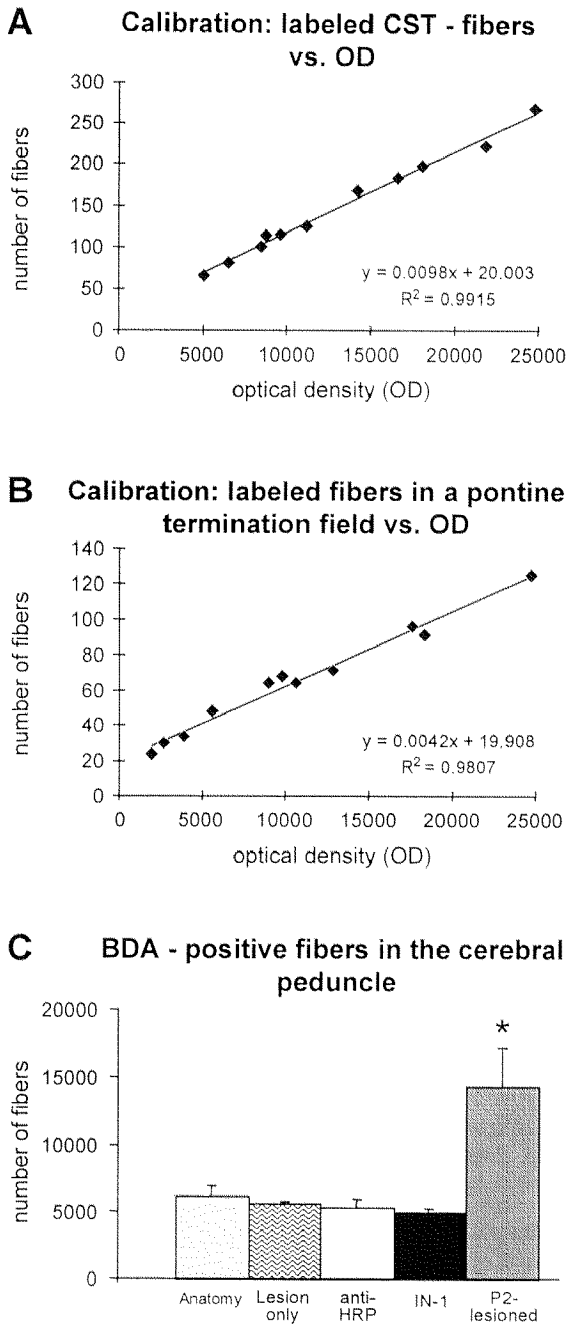


Fig. 2 Calibration and normalization of fiber density determinations.
A, Linear relationship between the number of labeled CST axons in the midpontine cerebral peduncle and their optical density (*OD*). *B*, Same procedure as in *A* for fibers in a pontine termination field. *C*, Total number of labeled CST-axons in the cerebral peduncle at midpontine level. These numbers were used as normalization values for the inter-animal tracing differences. Significantly more CST fibers are found in neonatally lesioned animals. Mean values ± SEM. The *asterisk* indicates a significant difference between *P2-lesioned* and adult *lesion only* animals, *$p < 0.05$, t-test, two tailed.

Quantification of the corticorubral projection

The crossed corticorubral projection originating in the tracer-injected forelimb motor cortex contralateral to the lesion was analyzed by counting all BDA-positive fibers crossing the midline on 15 sections containing the parvocellular part of the red nucleus. In each animal the values of the 15 sections through the red nucleus were added up for the total number in each animal. To correct for the inter-animal tracing differences, the values were divided by the number of labeled CST fibers in the cerebral peduncle and expressed as fibers crossing the midline per thousand labeled CST axons. Statistical significance was assessed using the unpaired t-test assuming unequal variances. The course and termination fields of fibers projecting to the red nucleus were qualitatively analyzed.

Quantification of the corticopontine projection

All sections comprising the pons (30-35 sections corresponding to a rostrocaudal distance of 1.5 mm to 1.75 mm) were included in the analysis. On each section the labeling of all basilar pontine nuclei ipsi- and contralaterally to the injection site was densitometrically determined, resulting in the iOD_A values. For background subtraction the optical density of unlabeled neighboring areas (iOD_B) was measured. The calculations of iOD were made as described above, separately for the ipsilateral and the contralateral side. The contralateral labeling was then expressed as a percentage of the ipsilateral innervation (ratio iOD contralateral/iOD ipsilateral). Furthermore, all pontine fibers crossing the midline were counted and normalized for inter-animal tracing differences, resulting in values expressing numbers of midline crossing fibers per thousand labeled axons in the CST. The contralateral innervation density of labeled fibers was further brought in relation to the number of midline crossing fibers (iOD of the contralateral side per midline crossing fiber). The *innervation index* given in the figures 6 and 7 represents the normalized iOD values.

For analysis of innervation specificity, the pons was divided into three parts:

- rostral part of the pons containing one forelimb-related projection field
- intermediate part showing five columns: dorso-lateral, lateral, intermedial, medial and dorso-medial pontine nucleus (Mihailoff et al., 1978; Wiesendanger and Wiesendanger, 1982); in the analysis the values of the dorso-lateral and lateral pontine nucleus and of the dorso-medial and the medial nucleus were combined
- caudal part with one large central projection area

The same analysis as described above for the entire pons was made seperately for each part of the pons. As the values of the non-lesioned, the lesion only and the anti-HRP treated animals did not differ significantly they were taken together into one control group and compared to the mAb IN-1 treated animals and to the neonatal lesioned animals, respectively (Fig. 6F, 7). Values for the rostral, intermedial and caudal part were each expressed as a percentage of the values of the entire pons. Thus, the total of the three values of one group in figure 6F and figure 7 always added up to 100%.

In addition to the analysis of the rostrocaudal fiber distribution, we also studied the mediolateral distribution of the corticopontine innervation. Electronic images were acquired with a Xillix CCD camera attached to a Zeiss axiophot microscope using a 4x objective. With the MCID-program the optical density (OD) was measured and integrated over a bar shaped field (50µm x 2300µm) placed horizontally over the densest areas of the labeled projection on the sections, thus, resulting in integrated optical density values (iOD). 500 pixels in the images corresponded to a distance of about 900 µm on the sections (Fig. 8).

Differences were tested for statistical significance between lesioned, mAb IN-1 and lesioned, anti-HRP treated animals and between neonatally lesioned and lesion only animals (exceptions are mentioned in the figure legends) using the unpaired t-test (unequal variances).

Figure preparation

Electronic images were acquired with a Xillix Microimager slow-scan, high resolution CCD camera attached to a Zeiss axiophot microscope. Images were assembled in Photoshop 4.0 (Adobe). Contrast was adjusted, when necessary.

Results

Lesion site and antibody application

Histological examination of all lesions in the left motor cortex showed that there were only small variations in the extent or placement of the lesion (maximum 0.5 mm shift from the reference lesion site; see Material and Methods). All cortical layers of the motor cortex were removed without damaging subcortical structures. A representative lesion can be seen in figure 3A.

Hybridoma cells were implanted close to the hippocampal region and the third ventricle ipsilateral to the lesion site (Fig. 3C). In some cases, the cells invaded the lesion site or an increased cell division rate led to a contusion of neighboring brain areas. Animals with tumors of this extent were excluded from further analysis. In vivo mAb secretion was tested by staining with FITC-labeled anti-mouse antibody in analogous experiments. A high level of mouse antibodies in the tumors, the ventricles and the brain surfaces could be seen (data not shown; Schnell and Schwab, 1990). A weak staining could be detected in the parenchyma of the brainstem (Z'Graggen et al., 1998).

Tracing of the forelimb corticofugal axons

BDA injection sites showed most of the tracer localized in layer V (Fig. 3B) with only small amounts in other layers and without affecting the underlying white matter. The distribution of BDA positive fibers corresponding to the forelimb corticofugal pathway in cross sections of the cerebral peduncle was similar in all animal groups, except for the neonatally lesioned group. In the latter group the distribution of labeled fibers was homogenous across the entire cerebral peduncle with only a slight decrease of labeling laterally. In all other groups, however, most BDA positive fibers were found in the medial half of the peduncle, with only a minor portion situated more laterally (Mihailoff et al., 1978; Kosinski et al., 1986). The average numbers of BDA labeled fibers in the cerebral peduncle at midpontine level were similar in the different adult treatment groups: 4998 (± 318 SEM, n=10) in lesioned, mAb IN-1 treated animals, 5347 (± 675 SEM, n=8) in lesioned, anti-HRP-treated animals, 5635 (± 179 SEM, n=6) in animals with lesion only and 6204 (± 780 SEM, n=6) in

normal, unlesioned animals (Fig. 2C). Interestingly, the neonatally lesioned animals showed a greatly increased number of fibers in the cerebral peduncle compared to the other groups: 14'384 (± 2886 SEM, n=5, Fig. 2C).

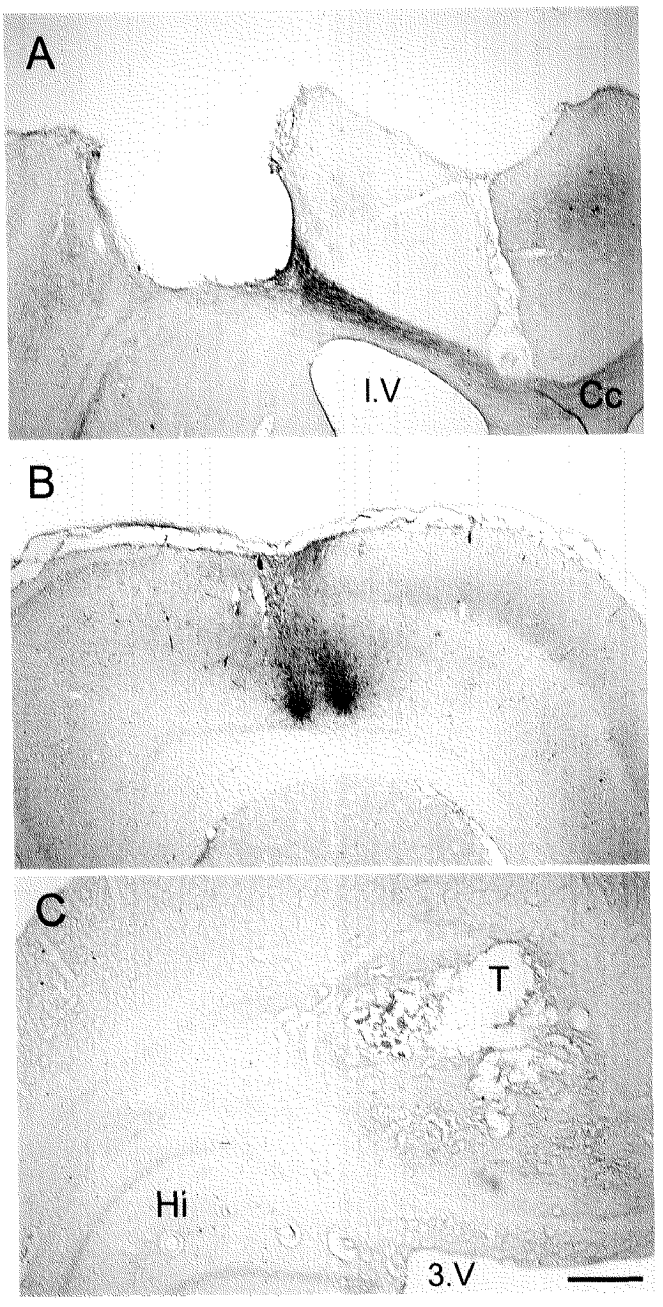


Fig. 3 Photographic illustrations of the experimental procedures.
A, Photomicrograph of a cross section through a lesioned, BDA-traced rat motor cortex, 2 mm rostral to Bregma (*I.V = lateral Ventricle, Cc = Corpus callosum*). *B,* Section through the intact hemisphere with two BDA tracer injection sites 0.5 mm rostral to Bregma. *C,* Tumor (*T*) location close to the Hippocampus (*Hi*) and the third ventricle (*3.V*). Lesion and tumor are localized in the left, the tracer injection site in the right cortical hemisphere. Scale bar = 640 µm and magnification 25x.

Plasticity of the corticorubral projection

The ipsilateral corticorubral projection originating in the caudal cortical forelimb area showed a similar pattern in all animal groups. At midbrain levels the labeled fibers left the cerebral peduncle from its dorsal aspect, passed the substantia nigra, where some fibers terminated, and then turned sharply medial and dorsal, towards the red nucleus, projecting mainly to the parvocellular part (Fig. 4A) in a similar fashion as described by others (Fig. 4A; Brown, 1974; Flumerfelt, 1980; Naus et al., 1985a). In addition, a few fibers terminated in the magnocellular part (Z'Graggen et al., 1998). In normal animals, none or only few BDA labeled fibers crossed the midline and terminated in the contralateral red nucleus, primarily in the parvocellular region.

Rats with a unilateral cortical lesion without any antibody treatment, and lesioned anti-HRP treated animals were indistinguishable from these normal animals (Fig. 4A). In lesioned, mAb IN-1 treated animals the ipsilateral corticorubral projection was similar to the control groups, but significantly more BDA-positive fibers crossed the midline and terminated in the deafferented, contralateral red nucleus (Fig. 4B,D). Neonatally lesioned animals also showed an increase in midline-crossing fibers resulting in a very dense innervation of the contralateral, denervated red nucleus, where the fiber distribution precisely mirrored the ipsilateral projection (Fig. 4C). In the mAb IN-1 treated animals and in the P2 group many fibers bypassed the ipsilateral parvocellular red nucleus ventrally to project directly to the corresponding contralateral area, some of them showing bouton-like endings. Other midline crossing fibers seemed to be branches of axons also terminating ipsilaterally or coursing through the ipsilateral parvocellular red nucleus to reach the contralateral parvocellular red nucleus.

Counting of the midline crossing fibers in the parvocellular region of 15 sections after correction for the inter-animal tracing variations resulted in 26.75 $\pm$ 3.38 fibers per thousand labeled CST-axons in normal, 27.9 $\pm$ 2.75 fibers in lesion only, 27.35 $\pm$ 2.64 fibers in lesioned, anti-HRP treated animals and 45.24 $\pm$ 2.19 fibers in the lesioned, mAb IN-1 treated animals (Fig. 4D). The mAb IN-1 treatment in lesioned, adult rats thus resulted in a 1.7-fold increase of midline crossing fibers as compared to the control groups. Neonatally lesioned animals

showed 32.4 ± 4.01 labeled midline crossing fibers per thousand CST-axons (Fig. 4D), a value which is only slightly higher than that of normal or lesion only animals.

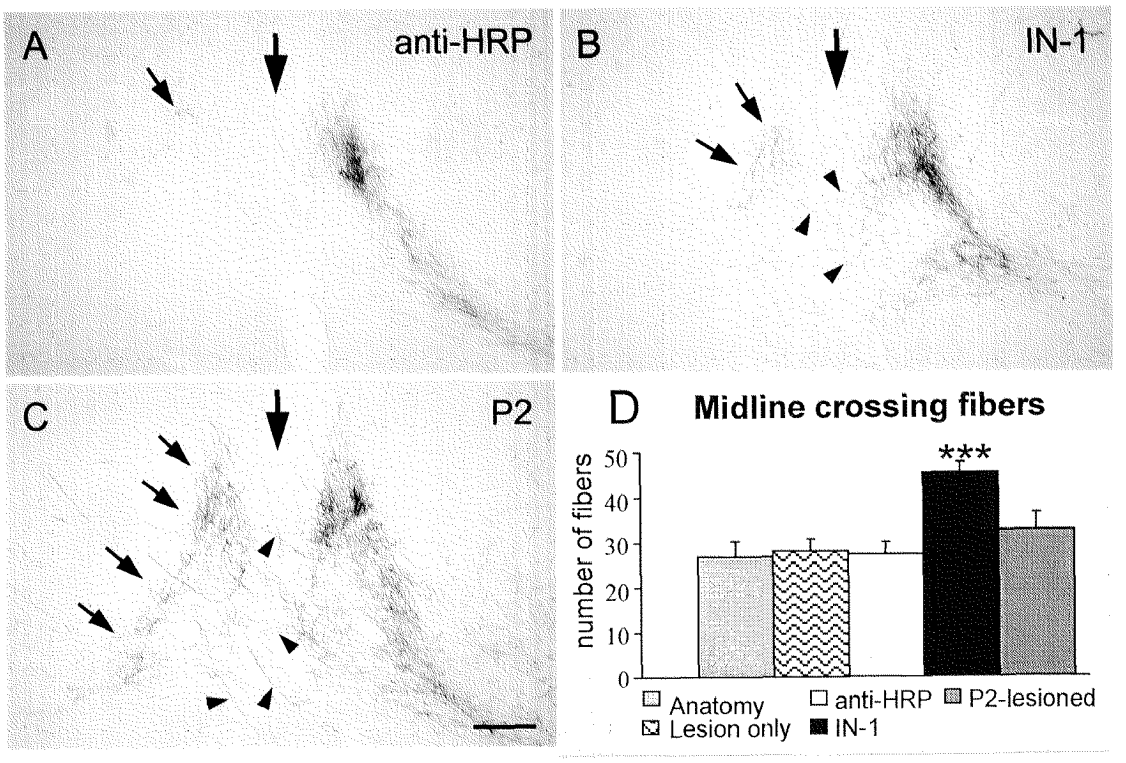


Fig. 4 Corticorubral projection; cross sections through the parvocellular red nucleus (*A-C*) and quantification of midline crossing fibers (*D*). *A,* Corticorubral projection after treatment with the control, anti-HRP antibody during two weeks (survival time after lesion). A very small termination area of labeled fibers crossing the midline (*large arrow*) to the denervated side (*small arrows*) is visible. *B,* Termination pattern in a mAb IN-1 treated animal. The photomicrograph shows many midline crossing fibers (*arrowheads*) and a denser innervation contralateral to the tracer injection. *C,* Neonatally lesioned animals show a very dense projection to the denervated side and, as in the mAb IN-1 treated animals, the contralateral termination pattern mirrors the ipsilateral one. *D,* Relative numbers of labeled fibers (per thousand labeled CST axons) crossing the midline in the parvocellular red nucleus (15 sections). In mAb IN-1 treated animals significantly more corticorubral fibers per thousand labeled CST-axons cross the midline, whereas P2 lesioned animals show only a small increase. *A-C*, The *large arrow* shows the midline, the *small arrows* the contralateral projection and the *arrowheads* the midline crossing fibers. Scale bar = 160 µm and magnification 100x. *D, Error bars* indicate mean values ± SEM. *Asterisks* indicate significance (*IN-1* compared with *anti-HRP*), ***p < 0.001, t-test, two tailed.

Plasticity of the corticopontine projection

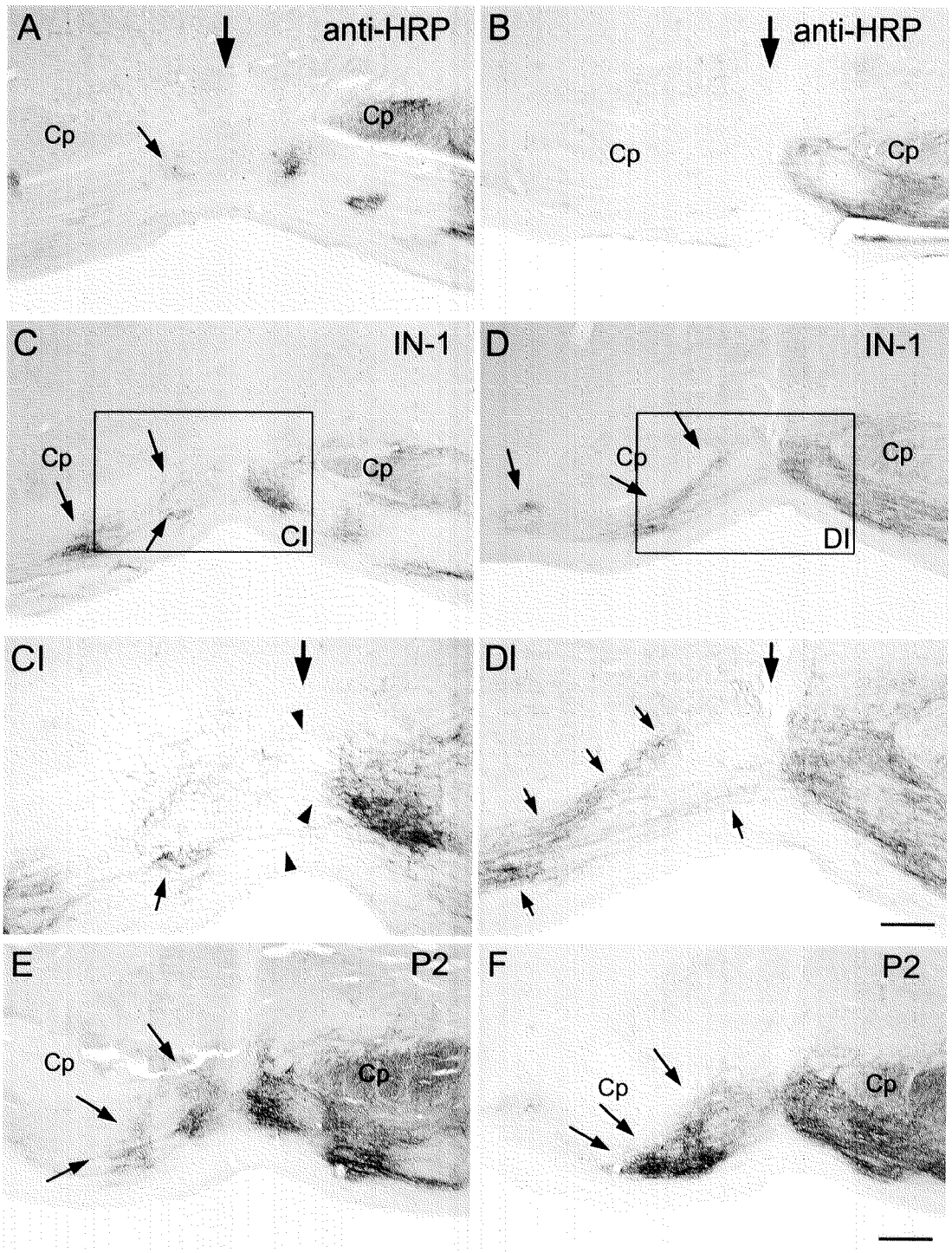
In all animal groups cortical fibers innervated the basilar pontine nuclei ipsilaterally in a topographic pattern typical for their origin in the forelimb motor cortex (Mihailoff et al., 1978; Wiesendanger and Wiesendanger, 1982; Panto et al., 1995). At rostral levels one dense termination field was observed in the center of the ipsilateral pons. At midpontine levels the termination field split into three longitudinal columns (medial, intermedial and lateral column). In addition, dorsomedial and dorsolateral termination zones were present. At caudal levels all these termination zones fused again, and a dense terminal plexus was observed in the medial, intermedial and lateral pons and around the ventral and dorsal aspect of the cerebral peduncle. This ipsilateral projection pattern to the basilar pontine nuclei was similar in all experimental groups with no observable difference in fiber distribution. However, quantification of the ipsilateral projection showed significant differences in the lesioned, mAb IN-1 treated and in the P2 lesioned group: Lesioned, mAb IN-1 treated animals showed an increased innervation index, whereas after neonatal cortical lesions these values were slightly lower than in rats (Fig. 6A).

Greater differences among the experimental groups were seen for the contralateral projections. In normal rats and in the control animals (lesion only and lesioned, anti-HRP treated) only a very minor contralateral projection, restricted to the midpontine, caudal level and to regions close to the midline was found (Figs. 5A, B, 6B). In lesioned, mAb IN-1 treated animals the contralateral pons showed a strong innervation, again mainly at midpontine and caudal pontine levels (Figs. 5C, D, 6B). A very pronounced increase of fibers projecting to the contralateral pons was seen in animals lesioned at P2 (Fig. 5E, F, 6B). The innervation index of the contralateral pons was 0.504 ± 0.126 for normal, 0.593 ± 0.119 for lesion only and 0.679 ± 0.175 for anti-HRP treated rats, in comparison to 2.106 ± 0.407 for mAb IN-1 treated and 5.845 ± 1.646 for neonatally lesioned animals (Fig. 6B). Relating the contralateral to the ipsilateral innervation index showed a significant increase of that ratio in lesioned, IN-1 antibody treated and P2 lesioned animals (Fig. 6E).

Counting of midline crossing fibers in the pons showed a highly significant increase in lesioned animals receiving mAb IN-1 treatment: 157.4 ± 18.3 fibers

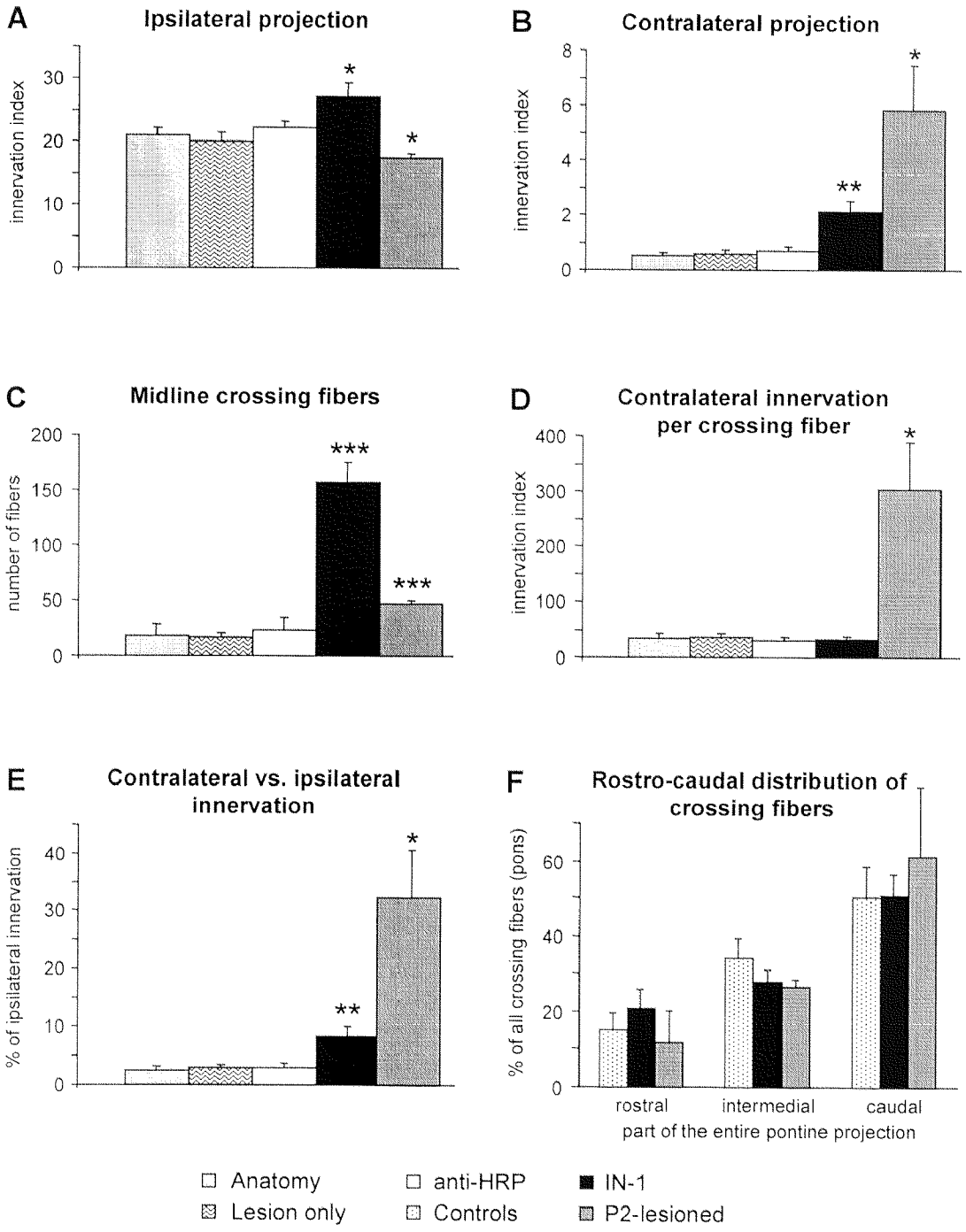
per thousand labeled CST axons compared to 23.47 ± 4.56 in anti-HRP treated animals (Fig. 6C). Figures 5CI and 5DI show these crossing fibers of mAb IN-1 treated animals at midpontine (Fig. 5CI) and caudal pontine level (Fig. 5DI). Neonatally lesioned animals showed a more than 2.5-fold increase of midline crossing fibers (46.95 ± 2.6) compared to lesion only animals (16.86 ± 1.43; Fig. 6C).

Fig. 5 (next page)
Cross sections through the basilar pontine nuclei. Photomicrographs on the left side (*A, C, CI, E*) show the column structure in the intermediate part, whereas on the right side sections through the caudal part of the pons are shown (*B, D, DI, F*).*A and B,* Animals treated with the antibody anti-HRP show only very few fibers terminating contralaterally (left half of the photographs). Clearly visible is only a small innervation in the medial column of the intermediate part (*small arrow, A*). *C and D,* Highly increased contralateral projection after two weeks of treatment with the mAb IN-1. The innervation pattern in the denervated pons (left) mirrors the innervation pattern in the intact pons (right), although most of the contralateral projection is found in the medial and intermedial column. *CI,* Higher magnification of the insert in graph C (100x, Scale bar = 160 µm), to visualize corticorubral fibers (*arrowheads*) crossing the midline (*large arrow*) at midpontine level. *Cp* stands for cerebral peduncle. *DI,* Higher magnification of the insert in graph D (100x, Scale bar = 160 µm) showing the dense termination field (*small arrows*) in the denervated basilar pontine nuclei of the caudal pons after two weeks treatment with the mAb IN-1. *E and F,* Sections through the pons in neonatal lesioned animals showed a similar pattern as mAb IN-1 treated rats (ipsi- and contralaterally), but the innervation density is much higher. In these animals, new sprouting was also observed mainly in the medial parts of the denervated side. *A-F, large arrows* indicate the midline, *small arrows* the contralateral projection and *Cp* stands for cerebral peduncle. Scale bar, 320 µm and magnification 50x (A, B, C, D, E, F).


A
anti-HRP
Cp
Cp
B
anti-HRP
Cp
Cp
C
IN-1
Cp
Cp
CI
D
IN-1
Cp
Cp
DI
CI
DI
E
P2
Cp
Cp
F
P2
Cp
Cp

To check if the increased contralateral pontine projection was due to the greater number of midline crossing fibers or an increase of terminal arborization, we related the iODs of the contralateral side to the numbers of midline crossing fibers. Figure 6D shows that the increased labeling in the denervated, contralateral pons in the mAb IN-1 treated rats exactly paralleled the enhanced number of midline crossing fibers. In contrast, neonatally lesioned animals showed a significantly increased contralateral labeling per crossing fiber (Fig. 6D).

Fig. 6 (next page)
Quantification of the labeled ipsi- and contralateral pontine projection fields, their innervation pattern and the midline crossing fibers. *A,* Ipsilateral pontine projection (*innervation index* = optical density of all ipsilateral termination fields divided by the number of labeled CST-fibers; see *Fig. 2C* and *Materials and Methods*). The mAb IN-1 treated animals show a small but significant increase in the ipsilateral innervation index. Neonatally lesioned animals show a small decrease. *B,* Contralateral, denervated side. The innervation index of the mAb IN-1 treated animals is three-fold higher than in control animals, that of neonatally lesioned animals shows a nine-fold increase. The ratio of the contralateral to the ipsilateral innervation is shown in *E. C,* Number of pontine midline crossing fibers per thousand labeled CST-axons. In mAb IN-1 treated animals six-fold more corticopontine fibers cross the midline than in controls, whereas in neonatally lesioned animals two-fold more fibers cross the midline. *D,* When the contralateral innervation density is expressed in relation to midline crossing fibers, only neonatally lesioned animals show an increased value. *F,* Rostro-caudal distribution of midline crossing fibers in the three main parts of the pons (rostral, intermedial and caudal part). In each group most of the crossing fibers are localized in the caudal pons. *Controls* include anatomy, lesion only and anti-HRP treated animals, which show comparable values. Mean values ± SEM. *Asterisks* indicate significance (*IN-1* compared with *anti-HRP* and *P2-lesioned* compared with *lesion only*. Exception: in graphs *B* and *C*, *P2-lesioned* is compared with *IN-1*), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, t-Test, two tailed.


A
Ipsilateral projection
innervation index
0
10
20
30
*
*
B
Contralateral projection
innervation index
0
2
4
6
8
**
*
C
Midline crossing fibers
number of fibers
0
50
100
150
200
***
***
D
Contralateral innervation per crossing fiber
innervation index
0
100
200
300
400
*
E
Contralateral vs. ipsilateral innervation
% of ipsilateral innervation
0
10
20
30
40
**
*
F
Rostro-caudal distribution of crossing fibers
% of all crossing fibers (pons)
0
20
40
60
rostral
intermedial
caudal
part of the entire pontine projection
Anatomy
Lesion only
anti-HRP
Controls
IN-1
P2-lesioned

Due to the anatomical subdivision of the pontine nuclei and their complex cortical input pattern we analyzed the rostrocaudal distribution of midline crossing fibers and their ipsilateral and contralateral terminations (Figs. 6F, 7A,B). The distribution of the ipsilateral projection to the rostral, intermediate and caudal part of the pons was similar in all groups: Slightly more labeling was found in the caudal part than in the rostral and intermediate part of the pons (Fig.7A). The contralateral projection, however, showed some differences between individual compartments in the P2 group (Fig. 7B): The intermediate pons of the P2 lesioned rats received a smaller, and the caudal pons a larger proportion of cortical axons. The control and mAb IN-1 treated groups were not significantly different. A similar effect was found for the number of crossing fibers in the neonatally lesioned animals, but with a less clear manifestation (Fig. 6F)

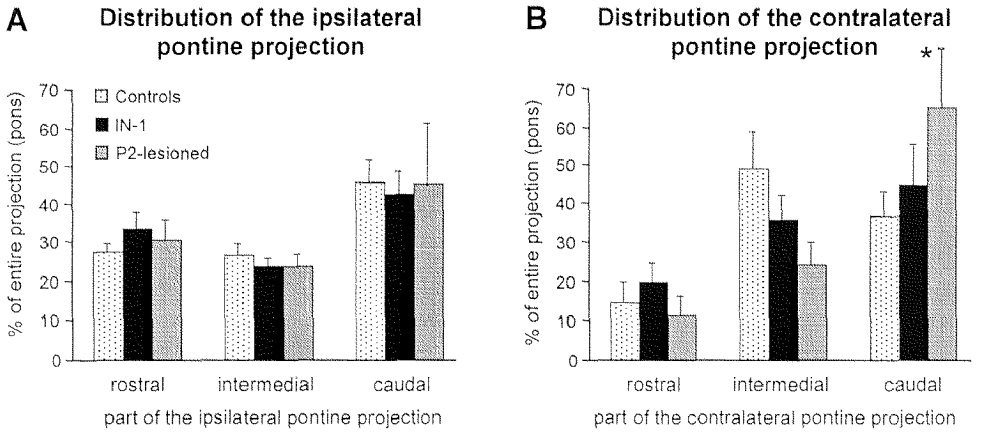


Fig. 7 *A,* The rostro-caudal distribution of the ipsilateral projection to the pontine nuclei does not alter after treatment. About 50% of the ipsilateral labeling is found in the caudal pons in all groups. *B,* On the contralateral side neonatally lesioned animals show a relative increase of this caudal projection. *Controls* include anatomy, lesion only and anti-HRP treated animals. Mean values ± SEM.

To further analyze the topographic specificity of the contralateral pontine innervation the mediolateral distribution of the corticopontine fibers in the intermediate and caudal part of the ipsilateral and contralateral pons was assessed. Figure 8 shows the medio-lateral innervation density profile of a lesioned, anti-HRP, a lesioned, mAb IN-1 treated and a neonatally lesioned, untreated animal. Whereas the very minor contralateral innervation in the lesioned, control antibody treated rats is restricted to the region close to the midline, the lesioned, mAb IN-1 treated and neonatally lesioned animals show nearly symmetrical bilateral innervation patterns. The histograms show that the new, contralateral innervation of the pontine nuclei is topographically organized and specific for fibers originating in the forelimb motor area.

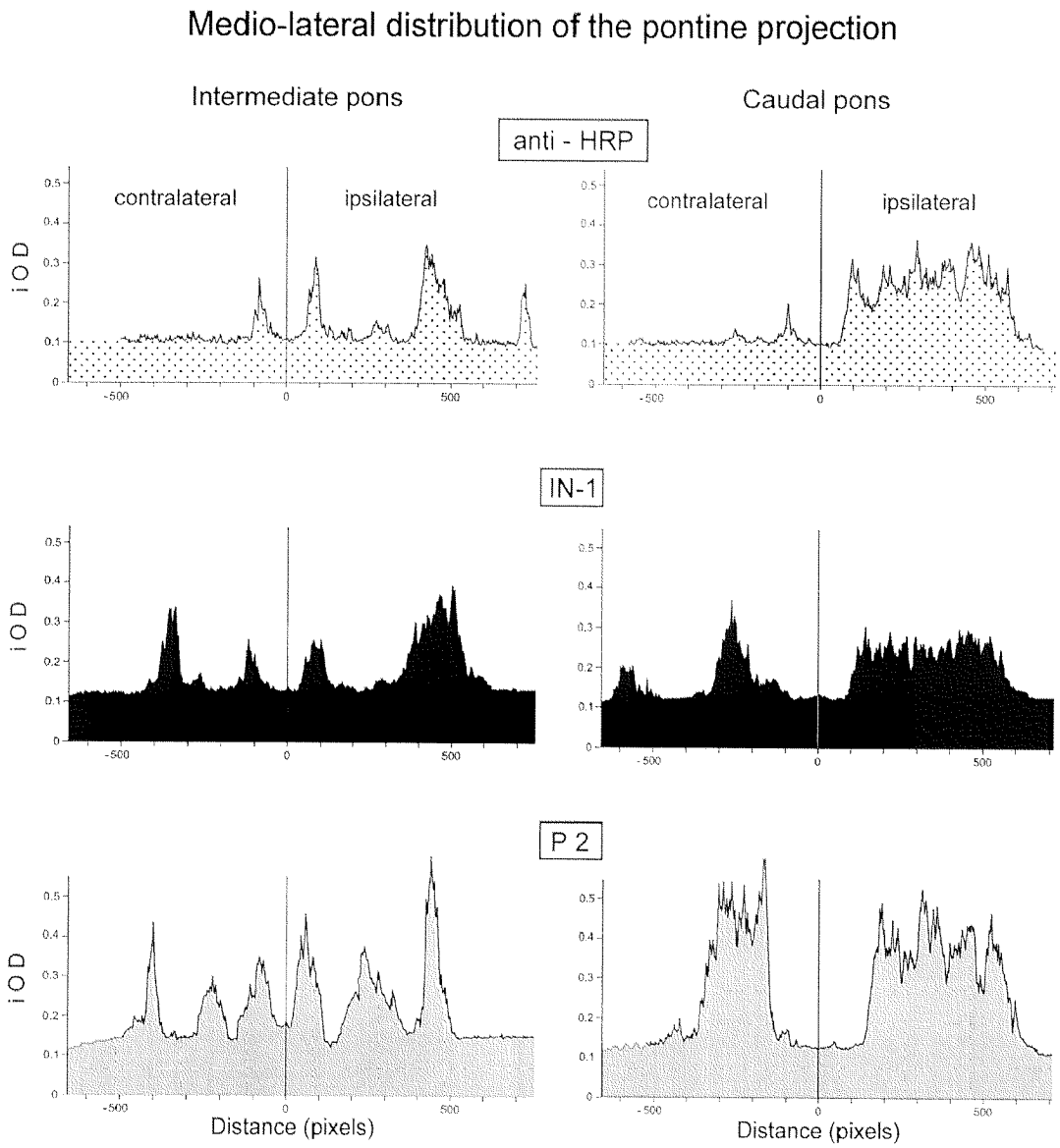


Fig. 8 Density profiles of the mediolateral distribution of the cortical forelimb projection to the basilar pontine nuclei. In both parts, intermediate and caudal pons, the localization of the new contralateral forelimb projection fields (columns) mirror the ipsilateral projection fields showing topographic specificity of these crossed projections. *500 pixels* correspond to a distance of about 900 µm. The optical density (OD) was measured and integrated over a bar shaped field (50 µm x 2300 µm) placed over the areas of the densest labeling on the section, thus resulting in an integrated optical density value *(iOD)*.

Discussion

The present results show the occurrence of sprouting and plastic remodeling of corticorubral and corticopontine fibers in the adult brain after neutralization of the myelin-associated neurite growth inhibitors by the monoclonal antibody IN-1. Following removal of one caudal motor cortex fibers originating in the forelimb area of the intact side crossed over the midline and terminated in the denervated contralateral red nucleus and pons. The anatomical distribution of these sprouted fibers mirrored the normal ipsilateral somatotopic projection. Similar lesion-induced sprouting was found after neonatal cortical lesions.

Increased fiber number in the cerebral peduncle in P2 lesioned rats

Counting of BDA-labeled fibers in the cerebral peduncle revealed no significant differences between the intact, the lesion only, the lesioned, anti-HRP treated and the lesioned, mAb IN-1 treated groups. Although the injection site (caudal forelimb area of the motor cortex) and the parameters for the iontophoretic injection of BDA did not differ in animals that sustained lesions as neonates, a large, almost threefold increase of labeled fibers in the cerebral peduncle was observed and the labeling pattern was different: In the P2 group fibers were distributed homogenously over the cerebral peduncle whereas the other groups showed only little labeling laterally. The increase probably reflects a survival effect - due to the lack of fibers in the contralateral pathway. Target derived factors from the enlarged target area may have decreased the normally occurring postnatal reduction of CST fibers (Nah et al., 1980; Mihailoff et al. 1984) from the intact hemisphere. This may fit with the observation in earlier studies (Kolb and Tomie, 1988; Kolb et al., 1992) that after early cortical lesions, the remaining hemisphere has an increased thickness of the cortex.

Topographic sprouting of corticorubral fibers

Studies of the corticorubral projections in the normal rat reported a predominantly ipsilateral innervation of the parvocellular part of the red nucleus (Brown, 1974; Gwyn and Flumerfelt, 1974; Flumerfelt, 1980; Naus et al.,

1985a). Only few fibers crossed the midline and terminated in the contralateral red nucleus (Murakami and Higashi, 1988; Z'Graggen et al., 1998). Our results showed an increase of midline crossing corticorubral fibers originating in the forelimb area of the intact hemisphere after unilateral motor cortex ablation and mAb IN-1 treatment in adult rats. The corticorubral axons reached the contralateral parvocellular red nucleus and formed a dense innervation plexus. Such corticorubral sprouting after motor cortex ablation has so far only been described after neonatal lesions in cats and rats (Leong, 1976; Nah and Leong, 1976; Villablanca et al., 1982; Naus et al., 1985b; Murakami and Higashi, 1988; Fisher et al., 1988). The examination of the rats that underwent the cortical lesion at P2 confirmed these earlier findings. In the P2 lesioned as well as in the lesioned, mAb IN-1 treated adult rats the crossing fibers terminated in a topographically correct pattern, i.e. in the most rostral, parvocellular part of the red nucleus. The new, contralateral termination mirrored the normal, ipsilateral projection.

Topographic sprouting of corticopontine fibers

In normal rats, forelimb motor corticopontine projections terminate somatotopically within the ipsilateral pontine nuclei. Only very few fibers cross the midline and terminate within the contralateral pontine gray mostly close to the midline (Mihailoff et al., 1978; Wiesendanger and Wiesendanger, 1982; Castro and Mihailoff, 1983; Kartje-Tillotson et al., 1986). Adult lesioned, mAb IN-1 treated animals showed an increased number of midline crossing fibers and a relatively dense contralateral innervation. The crossed projection formed a termination pattern specific for fibers originating in the forelimb area mirroring the projections of the ipsilateral side. This finding suggests that positional cues and targeting signals may be present or re-expressed that can be recognized by the sprouting axons. Our findings after neonatal lesions coincide with previous studies (Leong and Lund, 1973; Leong, 1976; Mihailoff and Castro, 1981; Castro and Mihailoff, 1983; Kartje-Tillotson et al., 1986): Unilateral ablation of the motor cortex in P2 lesioned rats also resulted in an enhanced contralateral innervation and an increase of midline crossing fibers as compared to normal animals. Mihailoff and Castro (1981) found bouton-like structures after such

neonatal lesions in the contralateral pons suggesting that sprouted corticopontine fibers established synapses. Whether sprouted fibers formed synapses after mAb IN-1 treatment is unclear although we found terminal-like boutons at some fibers. Electron microscopic examinations should be done to answer this question.

In the mAb IN-1 treated animals more fibers cross the midline than in the animals lesioned as neonates. In the developing CNS specific factors are expressed at the midline that attract or repulse fibers in order to establish the correct pattern. If these guidance cues are still expressed in the mature CNS or if their re-expression is only triggered by a lesion is not clear yet (e.g. in the visual system lesions induce guidance cues; Wizenmann et al., 1993; Bähr et al., 1995).

Interestingly, in the neonatally lesioned rats the contralateral labeling per crossing fiber is increased in comparison to the control animals. In contrast, the ratio between contralateral labeling and crossing corticopontine fibers is unchanged in lesioned, mAb IN-1 treated rats. These observations suggest that the midline crossing fibers increase their terminal arborization after neonatal lesions, but not after an adult lesion and mAb IN-1 treatment. This might be due to the different environmental cues in the developing versus the mature CNS. For example, only little or no myelin is present in the pontine nuclei in the neonatally lesioned group at the time of the lesion whereas in the adult rats myelin is present and only the myelin-associated neurite growth inhibitors (NI-35/250) are neutralized by mAb IN-1. In addition, the P2 lesioned rats had a much longer survival time, one year, as compared to the lesioned, mAb IN-1 treated animals that survived only for 14 days.

Interestingly, the ipsilateral corticopontine projection changed in the P2 lesioned and the lesioned, mAb IN-1 treated animals. The ipsilateral innervation index was slightly reduced after neonatal unilateral cortical ablation as compared to normal animals. Chemoattractive cues might guide a portion of the corticopontine fibers directly to the contralateral, denervated pontine nuclei. We observed, however, that the innervation index of the ipsilateral pontine nuclei was increased after lesion and mAb IN-1 treatment in adult rats. The possibility that lesion-induced sprouting leads not only to re-innervation of the

contralateral, deafferented pons but also affects the ipsilateral pontine nuclei seems likely. Other reports support the idea that the application of mAb IN-1 can have an effect on areas other than the ones deafferented by the lesion (e.g. Z'Graggen et al., 1998; G.L. Kartje, personal communication).

Possible mechanisms

After removal of one sensorimotor cortex in neonatal rats, the remaining hemisphere innervates the red nucleus, the pontine nuclei and the spinal cord bilaterally (Leong and Lund, 1973; Leong, 1976; Nah and Leong, 1976; Mihailoff and Castro, 1981; Villablanca et al., 1982; Castro and Mihailoff, 1983; Naus et al., 1985b; Kartje-Tillotson et al., 1986; Murakami and Higashi, 1988; Kuang and Kalil, 1990). Earlier studies showed that these bilateral connections are not surviving pre-existing, normally transient connections but that they are newly formed as a consequence of the neonatal cortex lesion (Nah et al., 1980; Mihailoff, 1984). These findings suggest the induction of factors by target denervation that may induce sprouting and guide the newly grown axons. Neurotrophins or chemoattractants but also extracellular matrix or surface molecules are likely candidates (Thoenen, 1995; Fagan et al., 1997). The role of the very few pre-existing crossing fibers in guiding newly growing fibers is not clear. As we found midline crossing fibers after the lesion preferentially where we found the few, crossing fibers in normal animals, a guidance function of those pre-existing fibers is possible. In the case of neonatally lesioned rats, Naus and colleagues used two retrograde tracers to determine if the crossing fibers in the red nucleus following neonatal lesions are collaterals of the ipsilaterally projecting fibers. They did not find any double-labeled cortical neurons, suggesting that at least after neonatal cortical lesions the new, contralateral corticorubral projection does not consist of collaterals of the normal innervation (Naus et al., 1986). In contrast, Murakami and colleagues (Murakami and Higashi, 1988) found in the cat that individual corticorubral neurons project bilaterally in normal development and following unilateral cortical lesions. In the present study midline crossing axons with branches and terminal arbors on both sides of the midline were frequently observed. The suggestion that most of these crossing fibers are collaterals from preexisting,

ipsilaterally projecting corticorubral axons is supported by the unchanged fiber number in the cerebral peduncle in all adult lesioned groups. This is in contrast to the situation after P2 lesions, where the number of corticofugal axons was increased and, in parallel and at almost the same proportion, the number of crossing corticorubral axons.

In the pons the ratio of the fiber density in the termination area to the number of midline crossing axons (not increased in mAb IN-1 treated rats; greatly increased in P2-lesioned animals) indicates that a large relative increase in terminal arborization mainly occurs in the newborn animals after a lesion. In contrast, in the animals lesioned as adults, the contralateral pontine innervation is mainly due to an increased number of midline crossing corticopontine axons or axon collaterals.

The myelin-associated neurite growth inhibitors (IN-1 antigens) probably influence plasticity after adult CNS lesions

After unilateral cortical ablation and treatment with mAb IN-1 we could demonstrate structural plasticity that is normally restricted to a short postnatal period. In earlier studies corticorubral sprouting occurred only after lesions up to 17 days of age and corticopontine sprouting was never observed when lesions were made at 20 days of age (Leong, 1976). However, lesion-induced sprouting can take place well after this developmental period if a growth permissive environment is created by applying the neutralizing antibody IN-1. Whether the mAb IN-1 treatment has functional benefits in this lesion paradigm is currently under investigation.

Clinical considerations and conclusion

Human CNS myelin was shown to contain inhibitory proteins with similar biochemical properties and comparable high molecular weight (200-300 kD) as bovine (bNI-220; Spillmann et al., 1998) and rat material (NI-35/250; Spillmann et al., 1997). The rat monoclonal antibody IN-1 was able to neutralize the human CNS myelin inhibitory property in vitro (Spillmann et al., 1997). Taking into account that mAb IN-1 directed against rat NI-250 could also neutralize the inhibitory activity of frog, opossum and bovine CNS myelin (Lang et al., 1995;

Varga et al., 1995b; Spillmann et al., 1998) it is very likely that the IN-1 antigen is highly conserved among different species. A well-established observation is that the outcome of a CNS lesion in humans also depends on the age at which the lesion occurred (Kennard, 1936; Kennard, 1938). A similar approach might therefore be useful to improve the functional outcome after brain lesions in humans. New tools like humanized IN-1 Fab fragments are currently becoming available (Bandtlow et al., 1996; Brösamle et al., 1996).

In conclusion, our results indicated that new, crossed corticofugal projections form and terminate somatotopically following unilateral motor cortex lesion and mAb IN-1 treatment. Neutralization of the myelin-associated neurite growth inhibitors might therefore be a promising tool to improve the functional recovery of patients after CNS lesions.

Acknowledgments

The authors acknowledge Werner Z'Graggen and Joachim Tönnes for valuable discussions, Roland Schöb for photographic support and Eva Hochreutener for help with the graphics. We thank Regula Schneider for help with the histology, Barbara Niederöst for supporting us with hybridoma cells and Johanna Höhn and Denise Nierentz for their skilful care of the rats.

Chapter 6

Conclusion and Outlook

In the eighties a new concept concerning the failure of growth in the mature CNS was born: It was shown that a sufficient supply of the appropriate neurotrophic factors to cultured neurons was not able to induce fiber growth into explants of CNS tissue (Schwab and Thoenen, 1985). In the following, a series of elegant in vitro experiments gave evidence for factors within the CNS that are inhibitory for neurite outgrowth (Caroni and Schwab, 1988a; Schwab and Caroni, 1988). This inhibitory property of the CNS tissue was especially strong in the white matter, whereas various grades of inhibition were found in gray matter. When neurites contacted oligodendrocytes in culture their growth cones collapsed immediately and stayed paralyzed for a long time (Bandtlow et al., 1990). The inhibitory activity in rat myelin was biochemically purified and called NI-35/250. A monoclonal antibody (mAb IN-1) was raised against a gel-purified fraction of NI-250 (Caroni and Schwab, 1988b). This antibody was able to bind NI-35/250 and to neutralize its inhibitory activity. Application of mAb IN-1 in vitro allowed the outgrowth of neurites on CNS tissue or on purified myelin (Caroni and Schwab, 1988b). In vivo studies in adult rats with spinal cord lesions showed that treatment with mAb IN-1 promoted the regeneration of CST fibers over several millimeters; such kind of long-distance regrowth had never been observed until then (Schnell and Schwab, 1990; Schnell and Schwab, 1993). The local injection of NT-3 at the lesion site increased the regenerative sprouting, but without evoking regeneration (Schnell et al., 1994). The anatomical changes after mAb IN-1 application were paralleled by a functional recovery in a number of locomotor functions (Bregman et al., 1995). The cloning of the cDNA of the myelin-associated neurite growth inhibitors was recently finished and showed that these inhibitors, now called Nogo, are novel proteins (Chen et al., 1998). The expression pattern of the *nogo* splice forms suggests that the Nogo proteins may have several roles within the CNS and other tissues (Huber et al., 1998).

In the adult CNS an important role of the myelin-associated neurite growth inhibitors may be the stabilization of neuronal connections. These inhibitors may play a role in terminating the postnatal growth-permissive period by rendering the microenvironment inhibitory for growing neurites. A lot of evidence supports this hypothesis; for example, a negative regional and temporal distribution is

found for the growth-associated protein GAP-43, a marker for growth and plasticity, and myelin (Kapfhammer and Schwab, 1994). In addition, experimental manipulations that suppress myelination were shown to extend the growth-permissive period, thus, allowing sprouting and regeneration in the adolescent and adult CNS (Keirstead et al., 1995; Keirstead et al., 1992; Savio and Schwab, 1990; Schwegler et al., 1995).

After a unilateral lesion of the CST at the level of the medulla oblongata rostral to the pyramidal decussation, the lesioned CST degenerates and the contralateral spinal half looses most of its CST input. If such a lesion is performed in neonatal rats or hamsters many anatomical changes occur: The stump of the lesioned tract in the caudal medulla oblongata gives rise to sprouts rostral to the lesion site; some of these sprouts elongate, cross to the contralateral half of the brainstem and travel down to the cervical spinal cord in an abnormal position (Kalil and Reh, 1982). In addition, the contralateral, unlesioned CST forms new collaterals in the spinal cord that cross the midline to re-innervate the denervated spinal half resulting in a bilateral innervation originating from one hemisphere (Kuang and Kalil, 1990b). Interestingly, a similar sprouting mechanism is suggested for human patients with hemiplegic palsy and mirror movements (Carr et al., 1993; Farmer et al., 1991; Woods and Teuber, 1978). If their lesion occurred early in life, their mirror movements were more pronounced than in patients with lesions acquired later in life (Carr et al., 1993; Woods and Teuber, 1978). On the other hand, lesions acquired at early age can often be compensated functionally to a very high degree. This is in contrast to cortical or internal capsule lesions in adults which lead to unilateral and persistent loss of motor control (hemiplegia). Thus, it was suggested by several clinicians that the persistence of mirror movements might be an indicator of more extensive compensatory motor system reorganization following early lesions (Cao et al., 1994; Woods and Teuber, 1978). Studies using EMG recordings, focal magnetic stimulation, cross-correlation analysis and reflex testing indeed indicated that the CST originating in the intact hemisphere established abnormal branches and projected bilaterally to homologous motoneuron pools on both sides of the spinal cord (Cao et al., 1994; Carr et al., 1993; Farmer et al., 1991). Obviously, a method to enhance

plastic remodeling in the adult CNS might be a desirable clinical tool to improve the functional outcome after traumatic brain lesions or vascular insults.

In the experiments presented in this thesis we showed that neutralization of the myelin-associated neurite growth inhibitors is able to increase structural plasticity in the mature mammalian CNS. The growth-permissive period can be extended into adult life if we either suppress myelination (and thus the expression of the myelin-associated neurite growth inhibitors) or neutralize NI-35/250 by the monoclonal antibody IN-1. Fibers of the unlesioned CST formed collaterals that crossed the spinal cord midline and terminated in the denervated half. The fibers either crossed through the dorsal commissure or directly through the area of the lesioned, degenerated CST. This sprouting was significantly increased after mAb IN-1 treatment and showed a tendency to be enhanced after hrIN-1 Fab treatment (chapter 3 and 4). In non-myelinated segments of experimentally treated spinal cords we observed an even larger increase in the number of sprouting fibers (chapter 2). In addition, we observed that mAb IN-1 application induced sprouting of the lesioned CST to the contralateral red nucleus and pons resulting in a bilateral corticofugal projection to these important brainstem nuclei. The sprouting was topographically organized and stable over time (chapter 3). These results showed that the corticospinal neurons retain their ability to induce fiber growth in the adult CNS. These growing fibers apparently respond to specific guidance factors that are maintained or re-expressed in the brainstem and spinal cord after lesions. In parallel to the structural rearrangements in the adult rat brainstem and spinal cord, we showed an almost complete behavioral recovery (chapter 3). After a unilateral motor cortex lesion we observed a similar structural remodeling in the pons and red nucleus (chapter 5): The intact hemisphere established bilateral projections to these brainstem nuclei in a topographically organized manner.

All these results showed that lesion-induced structural plasticity can take place in the mature CNS if the tissue microenvironment is altered to a more growth-favorable substrate.

Outlook

The next important step in this project will be to determine if the newly sprouted fibers do establish connections and if these synapses are functional. We tested several approaches to address this question and plan to use some of them. Electron microscopy may allow the identification of BDA-labeled synapses in the denervated spinal half and would provide anatomical evidence for the presence of synaptic connections. As a quantification of the synapses in the electron microscope might be very labor intensive, another, maybe easier way to get anatomical evidence for terminal boutons and to quantify them is the co-localization of BDA-labeled fibers and a synaptic marker protein (synaptophysin or synapsin) using confocal microscopy. To determine if the new synaptic connections are really functional one can use a marker protein that is upregulated after excitation of the neuron. If the connections are functional, a strong stimulation of corticofugal fibers would upregulate such a marker resulting - in our case - in a bilateral upregulation after sprouting in the spinal cord. In normal animals, the induction of the marker would be mainly unilateral. The immediate early genes *c-fos* or *zif268* are such "cellular markers" – they are expressed in response to many stimuli and it was shown that their expression can be used to map functional pathways (Chaudhuri, 1997; Dragunow and Faull, 1989; Herrera and Robertson, 1996; Kaczmarek and Chaudhuri, 1997; Sagar et al., 1988). To perform such neural activity mapping the fiber pathways needs to be strongly stimulated, for corticofugal pathways either electrical stimulation (e.g. Sgambato et al., 1997; Wan et al., 1992) or injection of kainate, a potent glutamate agonist, (Curfs et al., 1995; Curfs et al., 1996) is often used. At the moment we are establishing a protocol for c-fos immunohistochemistry that allows us to detect also this IEG in the spinal cord after kainic acid injections into the forelimb motor cortex.

Another important question is how the newly sprouted fibers find their way to the new target area. A challenging project is to identify some of the guidance molecules and chemoattractors that are involved in target finding and synapse formation of the sprouted collaterals. Several experimental approaches might be useful to elucidate the nature of these factors. Immunohistochemistry and in situ hybridization could give a first idea about molecular candidates. A targeted

differential display using reverse transcriptase polymerase chain reaction (RT-PCR) has been used to show the changes in mRNA levels of several neurotrophic factors in the hippocampus after denervation (Fagan et al., 1997). We plan to compare the expression of potential guidance factors following a unilateral pyramidal lesion.

Combinations of mAb IN-1 and one or more trophic factors should be done. Indeed, NT-3 and hrIN-1 Fab fragment increased lesion-induced sprouting in the spinal cord, but we need to confirm these data by additional experiments.

Finally, a very exciting question is whether a different motor tract system, e.g. the rubrospinal tract (RST), could sprout and functionally compensate in response to a CST lesion. These studies are in progress (in collaboration with Olivier Raineteau).

Based on the anatomical and functional results after lesions and neutralization of the myelin-associated neurite growth inhibitors, we suggest that structural plasticity is an important mechanism for functional recovery after lesions in the brain and spinal cord. The presence of specific neurite growth inhibitors in the CNS myelin allows only a very limited amount of lesion-induced sprouting in the mature CNS. Tools like the mAb IN-1 and the hrIN-1 Fab fragment should be further studied and developed to allow their application in clinical trials in spinal cord and brain injured patients some day in the future.

Abbreviations

3.V	third ventricle
ABC	avidin-biotin-complex
BDA	biotinylated dextran amine
BSA	bovine serum albumin
Cc	corpus callosum
CCD	charge coupled device
cer.ped.	cerebral peduncle
CNS	central nervous system
Cp	cerebral peduncle
CST	corticospinal tract
DAB	3,3' diaminobenzidine tetrahydrochloride
ELISA	enzyme linked immunosorbent assay
FITC	fluorescein isothiocyanate
GAP-43	growth associated protein 43
Hi	hippocampus
HRP	horseradish peroxidase
ICMS	intracortical microstimulation
IN-1	inhibitor neutralizing protein 1
iOD	integrated optical density
i.m.	intramuscular
i.p.	intraperitoneal
l.V	lateral ventricle
mAb	monoclonal antibody
MBP	myelin basic protein
MCID	microcomputer imaging device
NI	neurite inhibitor
OD	optical density
P	postnatal day
P3U	mouse myeloma cell line (P3X63Ag8U.1)
R	regression coefficient

SEM	standard error of the mean
T	tumor
TBST-X	Tris buffered saline solution with Triton X-100
vs.	versus
WGA	wheat germ agglutinin

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List of publications

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Acknowledgments

In general, my thanks go to the whole Schwab group for creating a nice atmosphere and for the helpfulness within the group. In particular I want to thank:

Prof. Martin Schwab – for his scientific support and for giving me the opportunity to meet many interesting scientists – in Zürich and abroad. He had always some time if I wanted to talk to him, he found the right words even when all experiments failed and he never lost his trust in me.

Prof. Ueli Suter who volunteered to be my co-examiner.

My thanks go to Josef Kapfhammer and Patrik Vanek who introduced me into the field of plasticity and many techniques - Josef for his critical mind and for fruitful discussions and Pädu for many many things ...

Regula Schneider for many many hours at the cryostat and at the bench and for her friendship ...

Lisa Schnell for teaching me the Morf-lab "rules" and other stories ...

Barbara Niederöst for her patience in providing me with the cells.

"My" students Werner Z'Graggen, Christian Wenk and Toni Iania.
Werner, we had a "tough" start but finally we became good colleagues. Thanks!
Mäx, thanks for your collaboration and for introducing me to the fun of triathlon.
Toni, I want to thank you for your enthusiasm, critical remarks and your company (e.g. early Tuesday morning around the Greifensee or midnight-talks and -surgeries in the lab).

Isabel Klusman for talks, discussions and help in the lab.

Olivier Raineteau and Karim Fouad for good collaborations and scientific discussions (as well as for Glühwein, Chääsfondue and nice evenings) and copies of papers that I would maybe miss otherwise. Olivier, merci!

Gerlinde Metz for her patience with our rats and for our collaboration.

Roland Schöb and Eva Hochreutener for support with the photos and graphics.

Marcus Franc, Marjan van der Haar, Christine Bandtlow and the "rest of the gang" for being good colleagues.

Silvia Kaufmann for sending out reprints and help when I fighted against the bureaucracy.

Ruedi Kägi, Hansjörg Kasper and Hanspeter Rothenbühler for technical support.

The Morf-WG (incl. the old one with Christian Brösamle) – for being a "home" and for support in difficult periods.

Tammi for everything and especially for taking me to the mountains for climbing and hiking (last august was just perfect).

Thanks to all my friends that didn't give up their friendship with me although I had not much time for them.

Thousand thanks go to my mother and Peter who always supported me and helped me to find my way. Mama, Du weißt, daß alle Worte der Welt nicht reichen, mich für das, was Du für mich tust, zu bedanken.

Last but not least I want to thank Klaus who is the best friend I ever had.
Klosi, Danke für die vielen Kilometer, die Tausenden von Telefoneinheiten und all die anderen "Strapazen", die durch unsere getrennten Wohnsitze so auf Dich zukamen. (...) Ohne Dich hätte ich Vieles nicht geschafft! Und auf Dich möchte ich auch im nächsten "Arbeitsabschnitt" nicht verzichten.