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Scientific Review

Axon regeneration of spinal motoneurons following a lesion at the cord-ventral root interface†

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Those insults to the spinal cord which occur when ventral or dorsal roots are avulsed from the surface of the cord have been considered unfavourable with regard to both survival and axon regeneration of lesioned neurons. In this review, we describe the development of a surgical procedure aiming at a restoration of motor function after ventral root avulsion lesions. This development includes a series of investigations in animals, where an unexpected capacity for cell survival and axon regeneration of motoneurons after a cut lesion in the spinal cord was demonstrated and analyzed in great detail. Based on these findings, a surgical technique was tested, where avulsed ventral roots were replanted into the cord. After confirmation that such implanted roots could serve as a conduit for outgrowing motor axons in animals, the technique has been evaluated in a limited number of human cases of root avulsion lesions. We conclude that surgical intervention may indeed lead to return of motor function also in human cases of ventral root avulsion lesions. Interestingly, the procedure also seems to have an attenuating effect on the pain that develops in cases with a combined dorsal root avulsion. Lastly, we conclude that the cut lesion in the ventral part of the spinal cord, followed by axon regeneration in motoneurons may serve as a model for axon regeneration in the central nervous system.

Keywords: spinal cord; spinal cord injury; muscle reinnervation

Introduction

The type of nerve lesion that occurs when spinal nerve roots are torn off, avulsed, from the spinal cord surface, has in many respects been alikened traumatic lesions of the spinal cord itself. Thus, avulsion of ventral (mediating motor functions) or dorsal roots (meditating sensory functions) leads to the interruption of axons at the border between the central (CNS) and peripheral (PNS) nervous systems and in both cases a repair process requires axon growth within CNS tissue. From this reason, the prognosis for avulsion lesion victims has been regarded very unfavourable and considered not amenable to surgery.[1]

Root avulsion lesions occur most frequently in spinal cord segments supplying nerve fibres to the arm, particularly in conjunction with road traffic accidents or as a result of severe arm traction during complicated births.[2] It may also be seen in segments supplying the leg and in cases of injury to the cauda equina. Patients with these types of lesion suffer from paralysis and sensory dysfunctions, sometimes including an extreme, almost unbearable pain.[1,3] The conventional approach to treat these injuries has been to apply non-curative, palliative methods, or to transfer intact neighbouring nerves to the distal stump of the avulsed root in attempts to compensate for the loss of sensimotor function.[4] Recently, however, a new surgical technique was introduced for treatment of avulsion lesions to ventral roots.[5] This method is based on results from a long series of animal experiments, where the regenerative capacity of motoneurons after lesions near the transition zone between the CNS and PNS, which is located at the ventral root exit from the spinal cord. In this review, we summarise the results of these basic studies and also present clinical data on the outcome for patients after surgical treatment of ventral root avulsion lesions.

The spinal motoneurons have their cell bodies and the initial part of their axons located in the CNS, while the longest part of the axons are found in the

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PNS, ie in the ventral roots, the spinal nerves and the peripheral nerves. In line with the concept that axon regeneration is possible in the PNS, but not in the CNS, motoneurons have been shown to accomplish axon regeneration and reinnervation of muscle fibres after peripheral nerve lesions, especially after surgery that aims at connecting cut nerve ends, if necessary with nerve implant.[6,7] In case of ventral root avulsion lesions, however, the proximity of the lesion and its location at the CNS-PNS border have led to the view that motoneurons probably die as a result of the lesion or in any case are incapable of axon regeneration. This notion has later been supported in animal experiments, where ventral root avulsion has been shown to induce death in up to 90% of the lesioned motoneurons.[8–11]

Motoneurons may regenerate after an axon lesion in the ventral funiculus of the spinal cord

Against this background, it came as a surprise for us to see that motoneurons do indeed have a strong ability to survive and produce new axons, even after an axon lesion with a site even more proximal than at the CNS-PNS border zone. Thus, in cats that had been subjected to a cut lesion in the ventral funiculus of the spinal cord, which accomplishes an axotomy of motor axons within the CNS, we could unequivocally show that lesioned motoneurons could produce new axons bridging over the lesion area, by use of intracellular labelling with horseradish peroxidase (HRP) of motoneurons.[12] Such axons could subsequently regrow into adjacent denervated ventral roots. We have estimated the frequency of surviving motoneurons after this type of lesion to about 50%, of which probably the majority are able to produce new axons through the lesion.[13]

Which circumstances allow or promote the regeneration?

Properties of the scar

With this initial finding as a basis, we have tried to identify properties of severed motoneurons and the lesion area that allow or promote the axon regeneration in our lesion model. A promoting factor of obvious potential is the denervated peripheral nerve environment in the ventral root just outside the lesion area. Still, the new axons from the motoneurons must pass through a scar that is built up by CNS elements. Thus, the scar contains both astrocytes and oligodendrocytes, which myelinate the new axons[12] (Figure 1a,b). Since CNS glial scars are regarded as impermissive for axon growth, it was of interest to see, whether the lesion area in our model differed from other types of scar, where no axon regrowth has been reported. By use of intracellular labelling with HRP and electron microscopy axons from surviving motoneurons could be followed from the ventral horn into a scar tissue composed of a trabecular framework of astrocytic processes and invading leptomeningeal cells surrounded by an expanded extracellular space containing numerous collagen fibres.[12] It should also be pointed out that no examples of regenerating axons entering more intact areas of the white matter from the scar tissue could be observed. However, several examples of axons leaving the scar tissue and entering

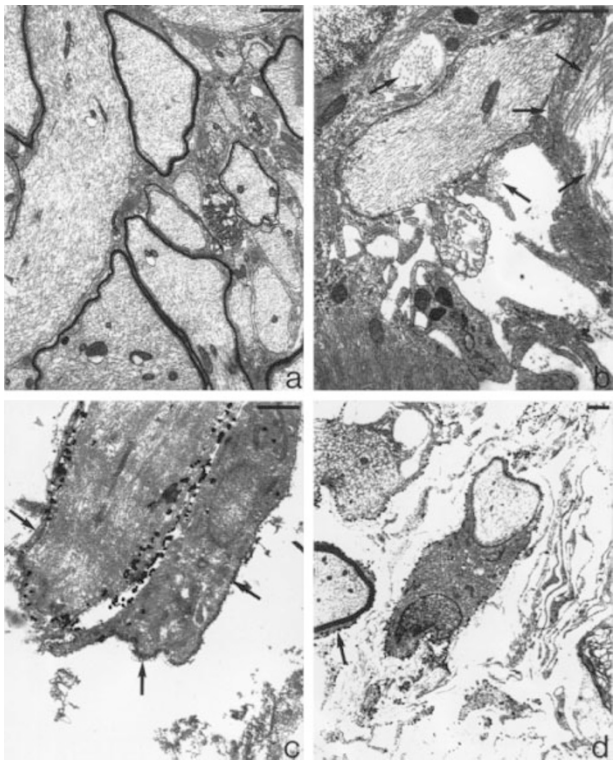

Figure 1 (**a**) Electron micrograph showing an ultrathin section from the ventral funiculus of an adult cat 41 days after a cut lesion in the funiculus. Several large calibre axonal profiles are surrounded by glial processes. Scale bar = 1 μm. (**b**) Electron micrograph showing an unmyelinated axon profile surrounded by astrocytic processes, 41 days after a ventral funiculus lesion. The arrows indicate collagen fibres in an expanded extracellular space. Note the vesicle-filled process which is emitted into the extracellular space by this axon. Scale bar = 1 μm. (**c**) Ultrastructural localization of p75- and laminin-like immunoreactivity in the spinal cord of an adult cat, 3 weeks after a cut lesion in the ventral funiculus. An axon and a glial cell are surrounded by an expanded extracellular space. p75-like immunoreactivity is visualised at the axonal membrane with silver-enhanced immunogold technique (black particles). Note that the glial cell is not p75 positive. Laminin-like immunoreactivity was visualised with ABC methodology (dark peroxidase reaction product) and was primarily observed in the surrounding basal membrane (arrows). Scale bar = 1 μm. (**d**) Ultrastructural localisation of laminin-like immunoreactivity at the spinal cord ventral root junction, 3 weeks after a cut lesion in the ventral funiculus lesion of an adult cat. Laminin-like immunoreactivity was in this specimen visualised with silver-enhanced immunogold technique (black particles) and is detected on the surface of axons, glial processes and scattered Schwann cells (arrow). Scale bar = 1 μm

a neighbouring ventral root were found. The CNS/PNS border was located roughly at the spinal cord surface. Some axons were found to be unmyelinated in the CNS scar tissue but became myelinated by Schwann cells in the ventral root. We could however not find any signs of Schwann cells invading the spinal cord scar tissue, whereas numerous glial processes were found in the proximal part of the ventral root. Substantial changes in the relative proportion of glial cells were observed during the first weeks after the operation. Less than 5% of the glial cells in the scar could be identified as oligodendrocytes 1 week after the lesion to be compared with around 60% in the ventral funiculus of normal adult cats. A similar but much slower elimination of oligodendrocytes deprived of axonal contact has been reported during Wallerian degeneration in the spinal cord.[14] In the light of numerous reports on the nonpermissive properties of CNS myelin,[15] it seems like an unavoidable conclusion that this rapid death of oligodendrocytes after the ventral funiculus incision may be of relevance for the observed regenerative response. An increase in the number of astrocytes was evident and several examples of mitotic cleavage of astrocytes were observed in the cicatrix. In addition, recent experiments have revealed that new astrocytes can be generated from ependymal stem cells after spinal cord injury.[16] Substantial numbers of phagocytotic cells and inflammatory cells were observed in the trabecular scar tissue during the first 3 weeks after the injury.

One of the properties that characterise the scar in our lesion model turned out to be a very long-standing defect in the blood–brain barrier.[17] Thus, such a defect could be demonstrated still at 2 years after the induction of the lesion, while substantially shorter defects in blood–brain barrier function (4–6 weeks) have been reported in other types of CNS lesions.[18,19] The implication of this finding for the regenerative capacity is unknown, but it is of interest that in scars with short-lasting barrier dysfunction, there is a transient presence of numerous axon sprouts in the scar for a time period that is equivalent with that of the barrier defect. One possible reason for the lively regenerative activity in our lesion model could then be that blood-borne substances that stimulate nerve growth and that are normally excluded from the nervous tissue, are in fact flooding into the scar area. The possibility that such substances could be of importance is supported by the fact that large amounts of receptors for members of the neurotrophin family are expressed by cells in the lesion area. Thus, reactive astrocytes express a truncated species of the trkB receptor,[20,21] whereas invading leptomeningeal cells contain both the low affinity p75 receptor[22] (Figure 2a) and functional trkA receptors.[23] These studies also indicate that the invading leptomeningeal cells may respond with increased process formation upon NGF that is released from reactive astrocytes[23] or from the blood stream via a defect blood–brain barrier (Figure 2b). Axon growth is also dependent on the presence of extracellular matrix molecules with adhesive properties. The lesion area in our model contains a number of such molecules, such as laminin, tenascin and various types of collagen, often arranged in tubular formations, in which new outgrowing axons can be seen[24,25] (Figures 1c,d and 2 and 3).

As a logical result of the vascular injury and the blood–brain barrier defect, the lesion area is invaded by blood-borne macrophages[26] and T-cells[27] within a few days after the injury. Interferon-gamma receptors and relevant JAK kinases are distributed in the lesion area in endothelial cells and reactive astrocytes as well as on the invading macrophages. T-cell derived interferon-gamma may then activate these different cell types. As a result, an upregulation of MHCII proteins and iNOS can be observed within a week after the lesion.[27] It seems possible that prostaglandins may act as mediators for this cytokine reaction and it has been shown that receptors for prostaglandin E2 and F2alfa are induced or upregulated in reactive astrocytes after 4–5 days after the spinal cord injury.[28] Hence, an inflammatory reaction seems to play a prominent role in the early response to the spinal cord lesion. It is also possible that the deposition of matrix molecules may be of importance for the survival and function of the inflammatory cells in the developing scar tissue. In order to test this hypothesis we have injected small volumes of prostaglandins or interferon gamma into the spinal cord and observed that this chemical lesion results in a similar scar formation as observed after the mechanical penetration of the ventral funiculus.

Properties of the motoneurons

Thus, it seems as if the scar formed after a mechanical lesion in the ventral white matter of the spinal cord promotes outgrowth of axons from lesioned motoneurons. The motoneurons themselves also seem to be very potent in producing new axons. Thus, during the early response to the lesion, the motoneurons may produce more than one myelinating axonlike process through the lesion area, and in some cases such axons may derive from distal dendrites ('dendraxons').[29] Remaining dendrites are reduced in size, and the number of synaptic connections with the lesioned cells is diminished, especially on the cell body and proximal dendrites.[30] There also seems to be a preferential loss of excitatory inputs to the motoneuron in this situation, probably leaving the cells under an inhibitory influence during the repair process.[30] These events may mirror a shift in the metabolism of the severed motoneurons from subserving the role for the motoneuron as a commander of muscle activity to a state where the primary goal is to survive and produce new axons. This shift is indeed reflected in an increase in mRNA expression of proteins linked with cell survival, such as brain-derived nerve growth factor (BDNF) and its high-affinity receptor trkB, or axon growth, such as GAP-43.[11,31] After a proximal lesion,

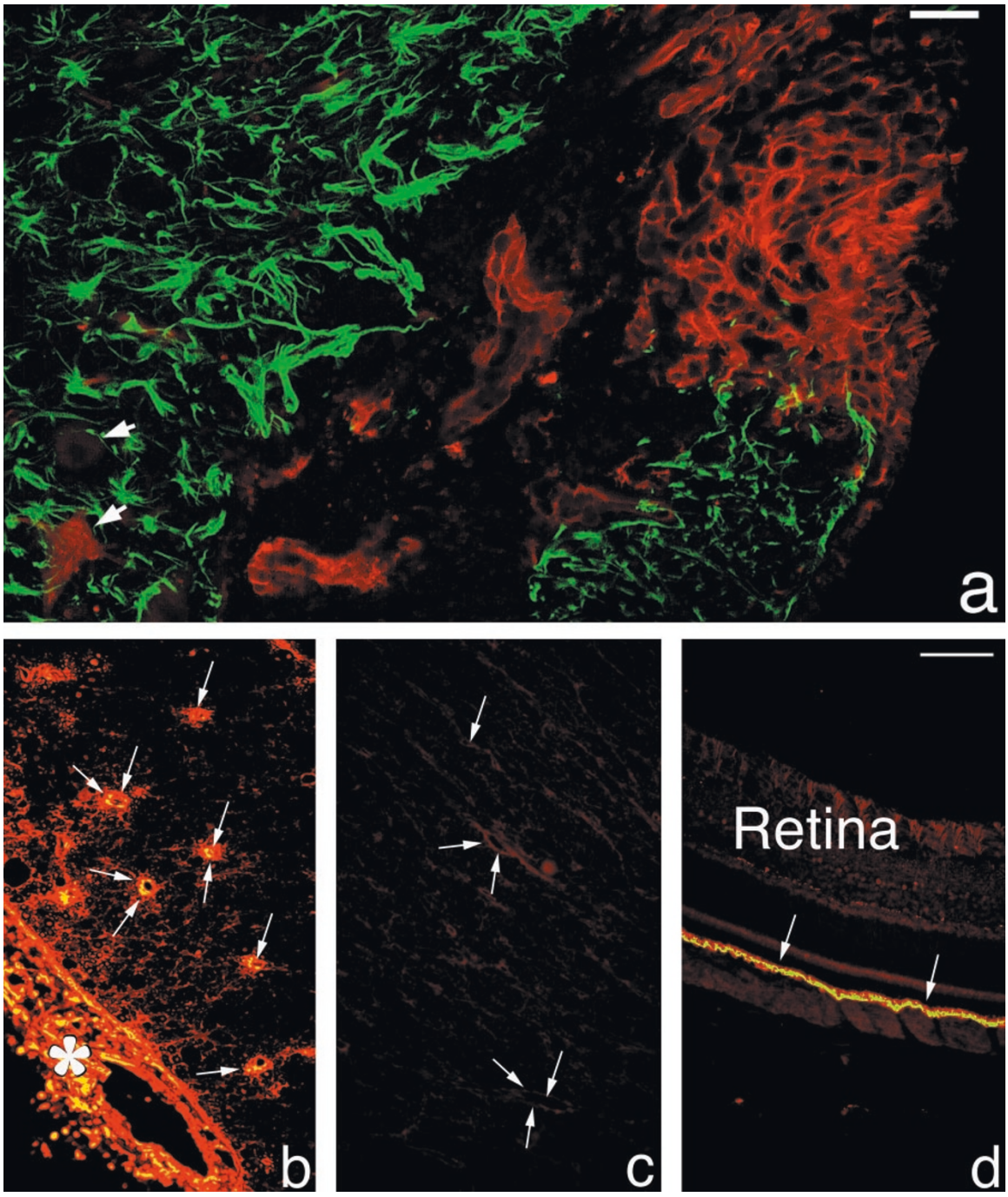

Figure 2 (**a**) Micrograph showing a transverse section from an adult rat 7 days after a ventral funiculus lesion. GFAP-(Cy2 = green) and p75-like (Cy3 = red) immunoreactivity in the injured rat spinal cord. p75 positive leptomeningeal cells invade the lesion area. The arrows indicate p75-positive motoneurons adjacent to the lesion. Scale bar = 50 μm. (**b**–**d**) Micrographs showing sections from an adult cat at 3 days after a lesion in the ventral funiculus and 30 min after an intravenous injection with 5 ml of the supernatant from a nerve growth factor (NGF) producing cell line. Sections were obtained from the spinal lesion area (**b**), the contralateral side of the injured spinal cord (**c**) and the eye (**d**). Examination of these sections revealed that NGF-like immunoreactivity (Cy 3) could be detected surrounding blood vessels in the lesion area (indicated by arrows in **b**), in the hypertrophic pia mater adjacent to the lesion (arrowheads in **b**) and in the choroidea of the eye (arrows in **d**). No immunoreactivity could be observed in the intact spinal cord (**c**) (arrows in **c** indicate blood vessels) or the retina (**d**), indicating that the injected NGF, as expected, did not cross the blood–brain barrier nor the blood–retina barrier. However, in the lesion area and the reactive pia mater the presence of NGF indicated an increased vascular permeability. Scale bar = 250 μm (**b** = **c** = **d**)

there is also an increase in immunoreactivity for the low affinity neurotrophin receptor ($p75^{NGFR}$), as well as immunoreactivity and mRNA for calcitonin gene related peptide (CGRP).[11] Of substances related to the insulin-like growth factor family, the mRNA for the binding protein IGFBP-6 shows a dramatic increase after lesion.[32] On the other hand, the NMDA type of glutamate receptor, as well as trkC, which is a high-

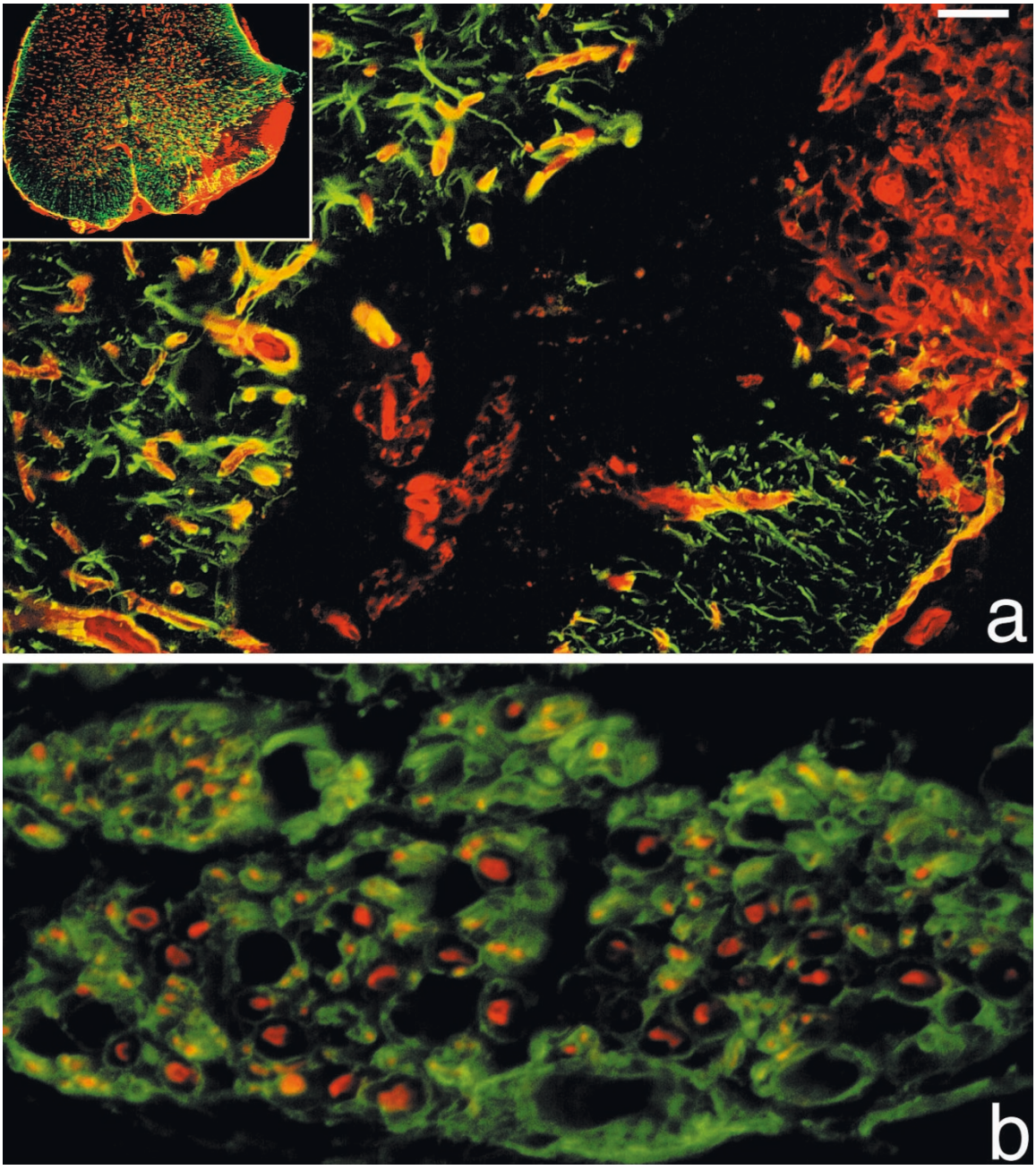

Figure 3 (**a**) GFAP- (Cy2 = green) and laminin-like (Cy3 = red) immunoreactivity in the injured rat spinal cord after a cut lesion at the ventral gray – ventral funiculus border. Deposition of laminin is observed in the lesion area 7 days after the lesion. At this stage the lesion area is essentially devoid of GFAP positive astrocytes. Note the laminin positive covering of blood vessels in the areas adjacent to the lesion. The inset shows a section from a rat spinal cord 3 weeks after the same type of lesion. Note a further increase in the content of laminin-like immunoreactivity in the scar tissue. (**b**) Micrograph showing a transverse section from an adult rat 3 weeks after a ventral funiculus lesion. Tube-shaped laminin-like immunoreactivity (Cy2 = green) surrounds axon-associated neurofilament-like (Cy3 = red) immunoreactivity. Note that the yellow color in the picture is the result of an optical overlap of the two immunoreactivities at different levels within the 14 μm thick section. Scale bar = 50 μm (a = b)

affinity receptor for neurotrophin (NT)-3, are down-regulated after a proximal axon injury in motoneurons.[33]

Axon regeneration of primary afferent neurons in the dorsal funiculus of the spinal cord

One explanation to the vivid axonal regeneration of the motoneurons after intramedullary axotomy could be that motoneurons constitute a very special population of nerve cells. In order to examine the possibility that also other types of neurons may accomplish axon growth under similar circumstances, we performed experiments in the adult rat, where a transverse cut lesion was made in the dorsal funiculus of the spinal cord. With tracer substances and immunohistochemistry we could show that ascending primary afferent fibres generated new axons within the scar tissue, but that these nerve fibres stopped at the border towards mechanically intact tissue on the other side of the scar.[34] If lengthening the scar by combining the transverse lesion with a rostrally oriented, longitudinal lesion along the dorsal column, newly formed axons grew for considerably longer distances. This growth occurred only within the lesion area.

Thus, it seems as if the traumatic glial scar in our lesion models offers a favourable environment for axon growth, while the degenerative zone that is induced distal to the lesion as a consequence of the death of the distal axon stump, ie the Wallerian degeneration zone, has a negative influence on axon growth. From this perspective, it seems tempting to try to find ways to affect the Wallerian zone towards the more growth-promoting type of CNS tissue found within the mechanical scars. A first step in such a process is to identify in greatest possible detail the different types of growth-related substances and signalling molecules acting in the mechanical lesion areas.

Experiments in animal models for ventral root avulsion lesions

As a direct consequence of the demonstrated regenerative ability of the motoneurons after a lesion in the spinal cord, we initiated a series of animal experiments with the aim to imitate the lesion in man described in the introduction, ie avulsion of ventral roots from the spinal cord surface. These lesions occur after strong traction violence on the upper extremity – in adults this is mostly seen after motor-bicycle accidents, and in newborn as a birth complication.[1,2,35] We performed such avulsions of lumbar ventral roots supplying the hindleg muscles in cat and rat. The avulsed roots were thereafter replanted into the lateral part of the spinal cord through a small opening in the pia mater and were fixed in position by sutures in surrounding pia. After 3–12 months the reinnervation of implanted roots was examined morphologically after axonal uptake and retrograde transport of a tracer substance (HRP), or after direct intracellular labelling of single motoneurons. With these techniques, we could show that a large number of neurons, presumably motoneurons, are able to let new axons grow out into the implanted roots.[36] By electrical stimulation of the operated roots it was also possible to show that the new axons could take functional contact with denervated muscle and induce muscle contractions (Figure 4).

A prerequisite for a meaningful recovery of motor function after ventral root replantation is that the motoneurons that reinnervate paralysed muscles are connected adequately to other neurons. By intracellular recordings from single nerve cells it became possible to demonstrate that regenerating motoneurons may be positioned in reflex arcs with excitatory and/or inhibitory effects on the motoneurons (Figure 4b–d). With regard to the ability of the motoneurons to find the same muscle that they initially innervated, the results from rat and cat suggested that there occurred a substantial degree of cross innervation, with probable negative functional consequences[36,37] (Figure 4e).

The next step was to perform the same type of operation in monkey,[38] and in this case the location of the ventral root avulsion lesion was changed to involve the lower part of cervical spinal cord with projections to the upper limb. The avulsion and replantation of avulsed roots were otherwise done in the same way as in rat and cat. Morphological identification of motoneurons by an HRP retrograde labelling technique showed that regenerating axons from a large number of motoneurons could be found in a small muscle nerve. In fact, the number of labelled motoneurons was larger on the lesion side than on the intact side. This could be interpreted as if motoneurons after replantation of a ventral root produce several axons in different nerve branches. The most important result from these studies, however, was that the monkeys, after an initial reinnervation phase with synkinesis of antagonistic musculature, with time were able to perform adequate voluntary movements in primarily proximal muscle groups in the arm.[38,39]

The first patients

The results from the animal experiments were so encouraging that we decided to test the method on cases of ventral root avulsion lesions in man. The first patient that underwent surgical treatment was a 25 year-old man, who was subjected to a strong, caudally oriented trauma against his left shoulder after falling off a motor-bicycle.[5] All ventral and dorsal roots from C6 to T1 on the left side were thereby avulsed. At the time for operation, which was performed within a month from the accident, the C6 roots were found subdurally near the spinal cord, while the other roots had been torn out from the spinal canal. It was possible to establish a direct replantation of the C6

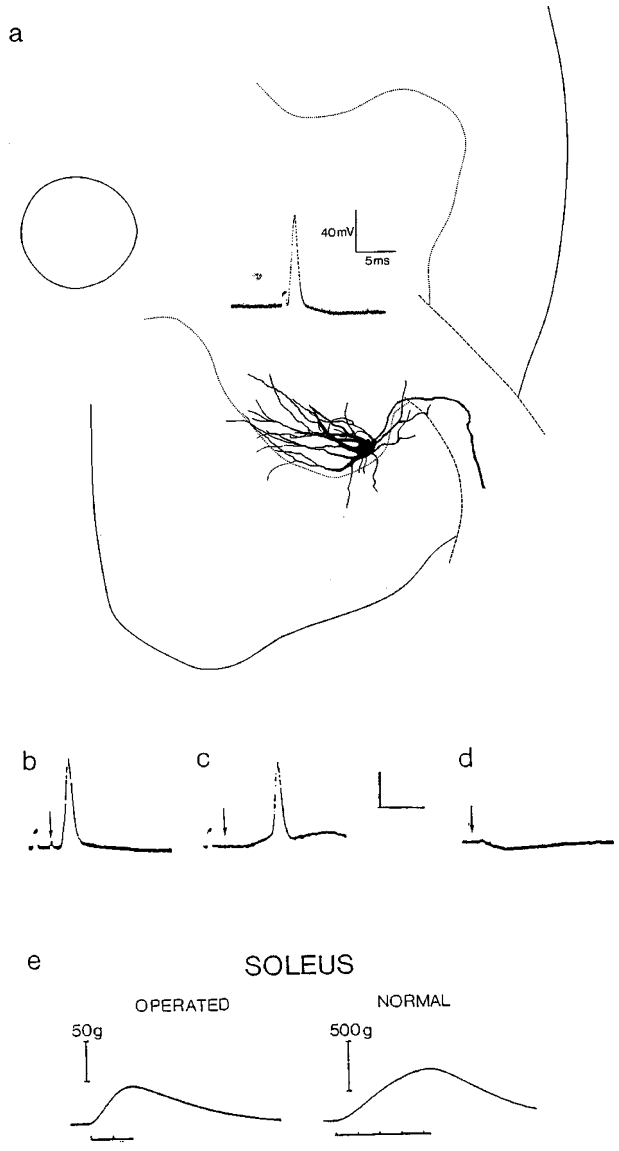

Figure 4 (**a**) A cat spinal lumbar motoneuron visualised by intracellular staining with horseradish peroxidase. After avulsion and reimplantation of a ventral root laterally has a new axon grown out into the implant. An action potential can be elicited by antidromic electrical stimulation of the ventral root. (**b**,**c**,**d**) Intracellular recordings from different motoneurons with axon outgrowth via implanted ventral roots. In (**b**) is shown an antidromically evoked action potential after stimulation of the implanted root. In (**c**) the same motoneuron has been activated orthodromically by stimulation of the tibial nerve in the popliteal fossa. In this way an excitatory postsynaptic potential (EPSP) is produced via primary afferent fibres. This EPSP in turn elicits an action potential. In yet another motoneuron (**d**) is an inhibitory postsynaptic potential (IPSP) elicited after stimulation of the tibial nerve. Scale bars: vertical = 20 mV, horizontal = 2 ms. (**e**) Electrical stimulation of an implanted ventral root gives rise to a contraction in the soleus muscle. The much faster contraction time in the operated animal than in the normal one strongly suggests that reinnervating motoneurons normally support fast-twitch muscle fibres. Reproduced by permission of Läkartidningen

ventral root in the cord. In order to create a continuity between the spinal cord an the C7 root, a sural nerve implant was used. No attempts were made to replant the C8 and T1 roots. The position of the replanted nerves was secured with the tissue glue Tisseal.

The first signs of reinnervation within the normal innervation area of the C6 and C7 segments were reinnervation potentials in the biceps brachii muscle. These potentials could be demonstrated at around 9 months postoperatively. The first clinical signs of return of function were found at about 15 months postoperatively. The muscle strength was thereafter slowly gradually recovering, especially in the biceps muscle, but also in the deltoid, triceps and brachioradial muscles. In order to establish to what degree the non-avulsed C5 root had contributed to the return of function in the biceps muscle, a selective blockade of the C5 root was performed. As a result of this blockade, the power in the deltoid muscle disappeared almost totally, while only negligable changes were recorded in the biceps muscle. The strength in elbow flexion was estimated to M4 (according to MRC 0-5) The activity in the different muscles that were

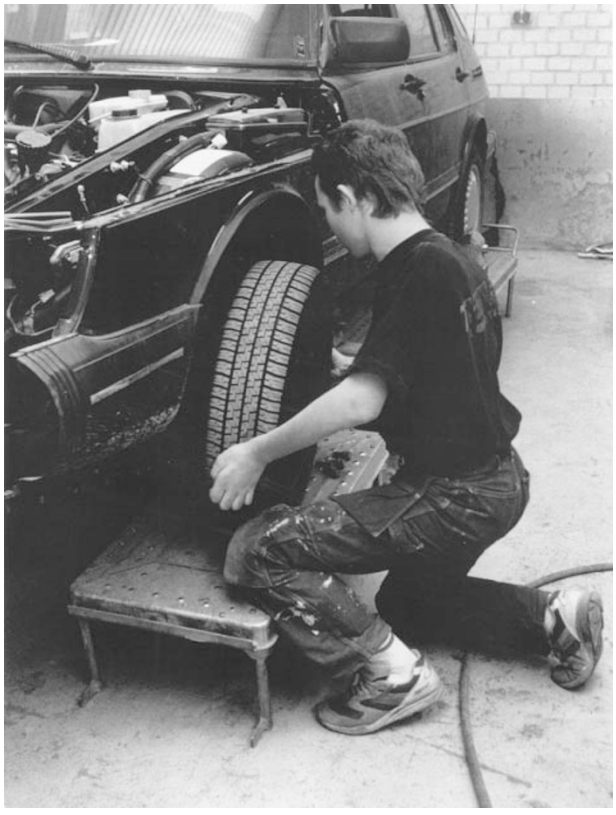

Figure 5 The first operated human case of ventral root avulsion lesion 2 years after the operation. The lesion engaged all ventral roots from C6 to T1 on the left side and there was initially a total paralysis of elbow and hand muscles. After recovery of especially elbow flexion, the patient could return to his work in a garage. Reproduced by permission of Läkartidningen

Table 1 Outcome in the first eight patients operated for intraspinal brachial plexus lesions

Patient	*Surgical procedure*	*Observation time (Years)*	*Motor function (MRC scale 0–5)*	*Sensory status*
AR	Repair of C8–Th1	3	Flexor carpi ulnaris = 4 Flexor dig. prof (Dig 2-5) = 4 Intrinsics = 0	No sensory return No pain
RP	Implantation C5, C6	3	Supraspin = 2 Biceps = 1	Moderate pain
TW	Implantation C6, C7	3	Biceps = 4 Supraspin, deltoid, triceps, brachiorad = 2 Infraspin = 0 Co-contractions	Mild pain No sensory return Severe pain
TS	Implantation C5, C6, C7	2	Triceps, supinator = 3 Biceps = 2 Supraspin, infraspin, deltoid = 1 Co-contractions	Mild pain Sensory return
KE	Implantation C5, C6	2	0	Severe pain
SO	Implantation C5, C6	1	Biceps = 1	No pain
SE	Implantation C5, C6	1	Pectoralis major = 1	Moderate pain
LS	Implantation C5, C6	1	Biceps = 1	Severe pain

reinnervated was always coupled so that co-contractions were common. Still, the patient could obviously benefit from the regained power, in that he could subsequently return to his work in a garage (Figure 5).

In a total of eight patients (Table 1), surgery was performed within 10 days to 10 months after the trauma. Restitution of connectivity with muscles regularly innervated by the implanted spinal cord segments was noted with electromyography (EMG) in all but one case. In three cases there was useful functional restitution similar to that described above. Activity in reinnervated muscles was always linked. As was found also in animal experiments, there was synkinesis, in that antagonistic muscles contracted simultaneously at volition. The power in the strongest muscles was estimated to be M4 (MRC 0-5) 2–3 years postoperatively. This corresponds to only a 20% reduction of normal power. In conjunction with the return of muscle activity, it was also noted by the patients that the initial severe pain was alleviated. A successful outcome of the surgery occurred only in patients operated within a couple of weeks after the injury.

In summary, then, functional return from implantation of avulsed ventral roots into the spinal cord is possible. The clinical application of a surgical strategy to repair this type of spinal cord injury demonstrates that motor recovery, leading to gross movements is possible if surgery is performed early after the trauma. However, future efforts are necessary in order to refine the surgical technique, to find ways to arrest or prevent the process leading to post-injury neuronal death, and to direct neurite outgrowth to the appropriate, specific target. Restoration of sensory connections with the spinal cord is also essential for the recurrence of volitional movements. Lastly, the axon regenerative capacity of motoneurons after a cut lesion in the spinal cord will be used as a model for the study of axon regeneration in the cord.

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