Motoneurons of the injured spinal cord of the adult dog can grow lengthy axons into an autologous peripheral nerve graft. A retrograde axonal tracing study

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To our knowledge, the capacity of injured spinal neurons to regenerate axons into peripheral nerve autografts has not yet been documented with axonal tracing methods in large adult mammals such as dogs. In the present study, one end of an autologous peripheral nerve graft (PNG), 10–15 cm long, was introduced dorsally into the lumbar (L4) spinal cord of six adult beagle dogs, thus producing a small focal lesion. The other end of the PNG was driven outside the spinal cord, then crushed and tied to nearby peripheral tissue with non-absorbable suture. Clinical examination of the operated animals was performed throughout the postoperative period. In five animals (out of six), the neurological deficit induced by the grafting procedure disappeared within five days. Four months after surgery, application of horse radish peroxidase (HRP) to the transected peripheral tip of the PNG led to the retrograde axonal labelling of about 30 lumbar neurons. The labelled cells, which had extended lengthy (up to 10 cm) axons into the PNGs, were mainly located in the vicinity of the intraspinal tip of the grafted nerve. Upon specific criteria, most of them were characterized as motoneurons. As the surgical procedure probably left the original stem axon of these neurons uninjured, it is suggested that axonal production from the labelled motoneurons might have arisen either from collateral axonal sprouts or even directly from the neuronal soma.

Keywords: dog; spinal cord injury; peripheral nerve grafting; axonal regeneration; retrograde axonal tracing

Introduction

In adult mammals, spontaneous regrowth of cut axons is known to occur in the peripheral nervous system whereas regeneration of injured axons in the central nervous system (CNS) is generally abortive and functional connections fail to be established. However, this failure appears to be related to factors present in the micro-environment of the CNS milieu rather than to an intrinsic inability of CNS neurons for axonal regrowth and reestablishment of functional connectivity. Using modern neuroanatomical tracing techniques, it has been demonstrated that most injured CNS neurons of the adult rat can grow axons along peripheral nerve grafts (PNGs) that are either blind-ended[1–4] or connected to central[5] or peripheral[6,7] targets. This effect is thought to be related to the specific structure of the peripheral nerves[1,8] as well as to the regrowth promoting properties of their non-neuronal elements, Schwann cells and extracellular matrix.[9–12]

In small mammals such as rats, the length of axonal regrowth from CNS neurons is obviously limited by the size of available peripheral nerve autografts (4 cm). In addition, neurological deficit following PNG implantation is rarely observed. Thus, the rat model does not appear to be directly transposable to man. Axonal regrowth into PNGs that were transplanted to the dog spinal cord has been documented[13] but studies with axonal tracers have so far not been reported in such large animals.

Thus, the aim of the present study was to evaluate: (1) the surgical feasibility of peripheral nerve grafting into the spinal cord of the adult dog; (2) the consequences of such a procedure on gait; (3) the capability of injured canine spinal neurons to extend axons all the way through long segments of peripheral nerve grafts by using horse radish peroxidase (HRP) as a retrograde axonal tracer.

Materials and methods

Six adult male (two) and female (four) beagle dogs (body weight 8–12 kg) were used in the present study.

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Grafting procedure

Prior to surgery, the dogs were given cephalexine (Rilexine, Reading, 30 mg/kg intravenously) and methyl-prednisolone (Solumedrol, Upjohn, 30 mg/kg intravenously) and were prepared for an aseptic procedure. General anaesthesia was induced with an intravenous injection of diazepam (Valium, Roche, 0.2 mg/kg) and thiobarbital (Nesdonal, Rhône Mérieux, 15 mg/kg), and maintained with oxygen (100%) isoflurane (2%) (Forane, Abbott) using a Bain inhalator.

For graft removal, the dogs were secured on the table in lateral recumbancy. A medical approach to the femoral region was performed, the saphenous nerve (made up of one, two or three fascicles) was identified, removed (10–15 cm long) and briefly stored in saline solution at 4°C before grafting. For PNG implantation, the dogs were secured to the table in ventral recumbancy. Approach to the dorsal thoraco-lumbar vertebrae was made through a dorsal midline incision[14] centered on L3 and extending from L1 to L5. Subcutaneous fat and fascia were cut until the lumbosacral fascia and the supraspinous ligament were reached. Then an incision was made around each spinous process. The *Multifidus lumborum* muscle was elevated from each spinous process and freed from the mamillary processes. Lateral retraction of the muscle exposed the dorsal laminae of the vertebrae. A hemilaminectomy was then performed.

Hemostatis was obtained with electric coagulation at the beginning of the procedure and by clamps when approaching the spine. After exposure of the spinal cord and durotomy, implantation of the nerve graft was carried out using a stylet. A slit was made in the meningeal membranes and spinal cord tissue on the left side.

A complete lesion was made from the dorsal to the ventral horn of the grey matter. One, two or three fascicles of the PNG were then introduced into the grey matter. The transplant was secured to the dura mater with two 0.3/10 (9/0) non-absorbable sutures (Prolene Ethnor SA, France). The other end of the PNG was driven through the paraspinal muscles and a non-absorbable suture (Prolene 3.5/10, Ethnor SA, France) was tied around its distal extremity in order to recover the transplant in the subcutaneous tissue for application of HRP (Figure 1A).

Closure of the surgical wound was performed in layers. Prednisolone (30 mg/kg) was administered *per os* (1 mg/kg) for 4 days following surgery.

Tracer application

Four months after grafting, the dogs were anaesthetized again, using the same anaesthetic procedure as described above. After surgical preparation of the lumbar region, a skin incision was performed and the subcutaneous tissue was dissected. The PNG was recovered, cleared from surrounding tissues and transected. HRP was applied to the extremity of the

A

B
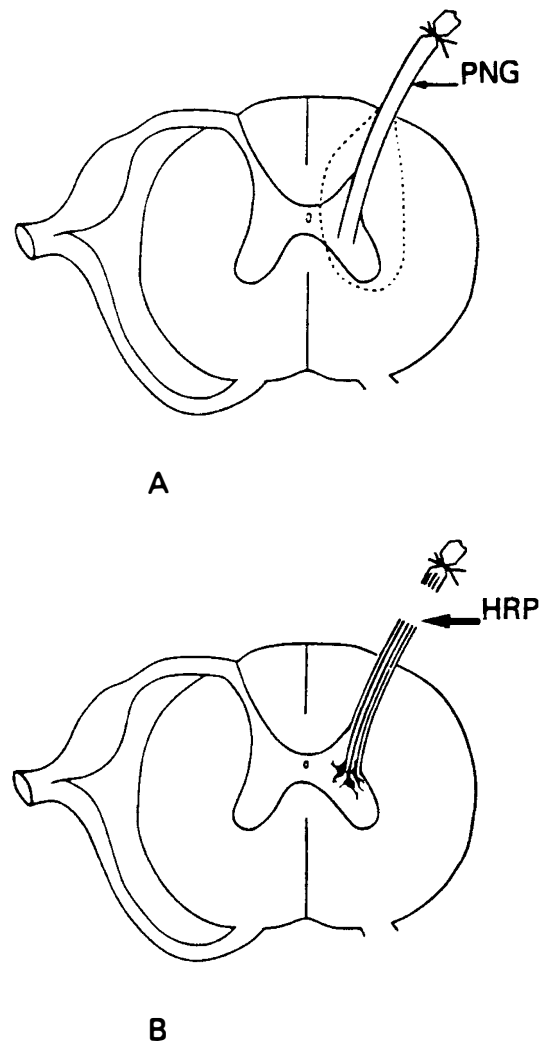
Figure 1 Schematic representation of the experimental procedures. (A) Intraspinal implantation of a peripheral nerve autograft (PNG). The outlined zone (dotted line) corresponds to the area into which the PNGs were recovered after histological processing of the different experimental animals. (B) Retrograde axonal tracing with horse radish peroxidase (HRP). The labelled neurons are those neurons that have grown an axon into the PNG at least up to the site of tracer application

proximal nerve stump (Figure 1B). In order to avoid tracer contamination of the neighbouring tissues, the cut end of the nerve was placed on a parafilm sheet and surrounded with petroleum jelly. Then, a small Gelfoam (Upjohn Comp.) pellet, soaked in 30% HRP (Sigma type VI) solution, was left in contact with the tip of the PNG for 1 h.[15] After that, the exposed area was washed several times with saline.

Forty-eight hours later, the dogs were sacrificed by barbiturate overdosing after sedation with ketamine. A lateral thoractomy was performed and the aorta was canulated. The animals were first perfused with an

isotonic heparinized saline solution, then with 10–12 liters of 3% glutaraldehyde in 0.1 M phosphate buffer at 4°C.

Histology of the PNG and spinal cord

The spinal cord was removed after dissection and kept in the same fixative. Each spinal cord segment was sectioned longitudinally in a cryostat. The sections, 40 μm thick, were processed for HRP histochemistry according to Mesulam,[16] then counterstained with neutral red. In every section, only labelled neurons displaying a nucleolus were counted.
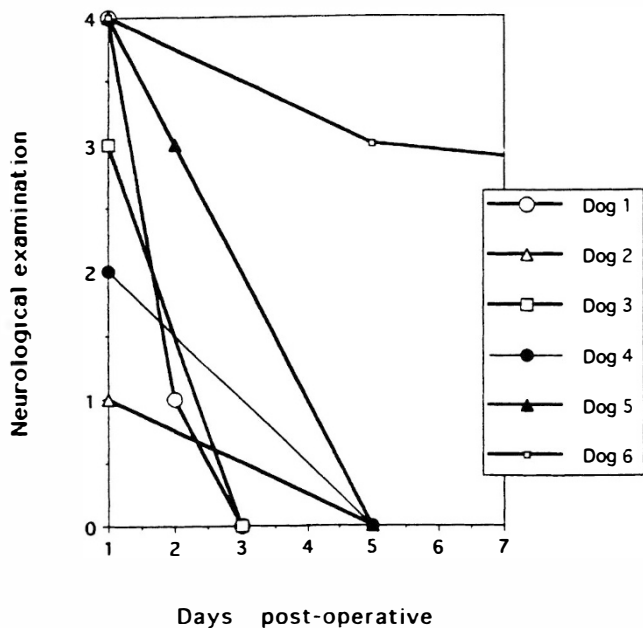
Results

Clinical assessment

All dogs developed neurological deficits (including paraplegia, paraparesia, left pelvic limb monoplegia or left pelvic limb monoparesia). Total recovery was generally obvserved within 5 days following surgery with the exception of dog #6 which displayed paraparesia until p.o. day 45 and left monoparesia until p.o. day 60, but recovered a normal gait after that period (Figure 2).

Gross examination and histological survey

Post-mortem gross examination of the operated spinal cord revealed that the grafted nerves had a healthy appearance and were seen to have remained firmly attached to the dorsal aspect of the cord. However, at the lesion and grafting site, the dura adhered to the underlying neural tissue, thus making its removal rather delicate.

The light microscope survey of longitudinal sections of the lumbar spinal cord indicated that fibrous scar tissue had stuck together the dura, the grafted nerve and the dorsal aspect of the spinal cord. The PNG penetrated the spinal tissue 1 to 1.5 mm deep (Figure 3).

Figures 3–5 Implantation of one end of a peripheral nerve graft (PNG) into the lumbar spinal cord of an adult beagle dog. Sagittal sections. HRP histochemistry and counter-staining with neutral red. Four and a half months post grafting. In Figures 4 and 5, all neurons appear dark on black and white photographs. However, HRP labelled neurons can be distinguished from unlabelled neurons as they appear to be filled with fine dark granules of the reaction product

Figure 3 Low magnification of the lesion and grafting site. G = intraspinal end of the PNG; F = extraspinal part of the PNG, embedded in fibrous scar tissue; asterisks = cysts; arrows point to strands of Schwann cells and associated elements, interwoven with the lesioned spinal tissue. Scale bar: 300 μm

Figure 2 Evolution of the neurological status in the grafted dogs. Full recovery (0) of dog six was observed after 60 days. Deficits are scored using the following scale: 0, no deficit; 1, monoparesia; 2, monoplegia; 3, paraparesia; 4, paraplegia

Local cavitation of the spinal tissue (micro and macrocyst formation) was a constant finding (Figures 3, 4A, B and C). The cysts were located inside the grey matter, either close to the intraspinal extremity of the grafted nerve or at some distance (1 to 10 mm) from it. They were lined with an acellular, amorphous membrane. The intraspinal end of the PNG generally appeared to be dissociated into small strands of Schwann cells and associated elements that were interwoven with the spinal tissue (Figures 3, 4C and D).

Five nerve grafts (out of six) were recovered. One graft was embedded into scar tissue and penetrated the white matter. Four grafts were in contact with the gray matter (Table 1). Retrograde axonal tracing with HRP

resulted in the labelling of neuronal somata of spinal neurons which were seen to be close to the intraspinal tip of the PNG (Figures 4 and 5). With regard to their location, size and morphology, most (77%) of these labelled cells were assumed to be motoneurons. In dogs (#3 to 6) with a positive neuronal labelling, the labelled neurons were always observed in the vicinity of the PNG. In dog number six, the 67 labelled neurons appeared to be located up to 3 mm from the implantation site, both cranially and caudally. Full results of PNG implantation experiments are summarized in Table 1).

Discussion

The main result of the present experimentation indicates that intrinsic neurons of the damaged spinal cord of the adult dog have the capacity to form and grow axonal extensions into peripheral nerve segments that have been deeply implanted into the spinal tissue. The grafted nerves act by providing a new microenvironment which appears to be much more permissive to axonal growth and elongation than the surrounding injured CNS tissue of the adult animal. According to their location, morphology and size, most of the neurons which could be retrogradely labelled from the grafted nerve were assumed to be motoneurons.

These findings are in full agreement with previous experiments which were carried out in adult rodents.[2,6-7] The longer axonal extension obtained in the adult dog (10–15 cm), *vs* the adult rat (3–4 cm), must probably be attributed to the available length of the grafted nerves rather than to different intrinsic growth capacities from canine and rodent spinal neurons. The limit of such axonal elongation from
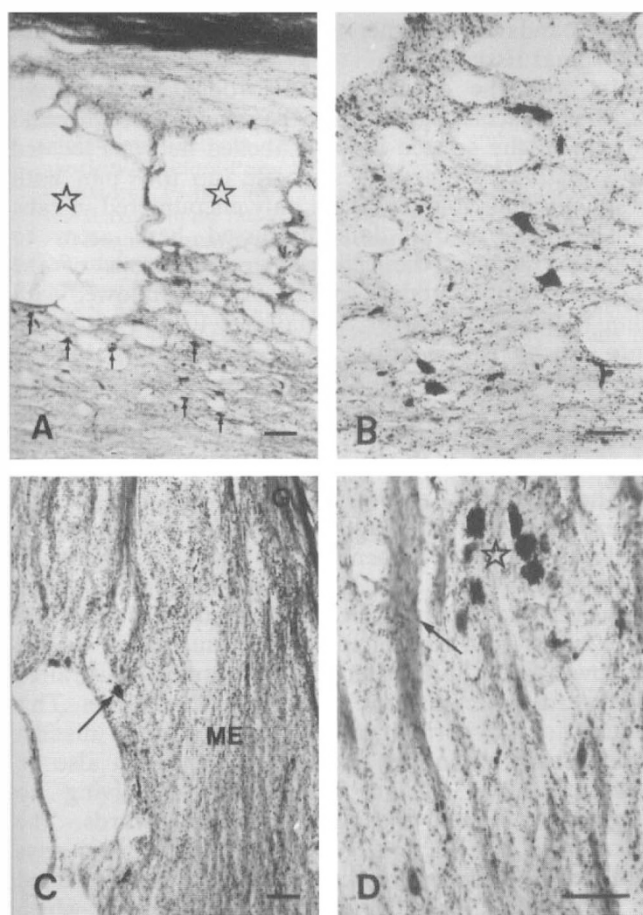
Figure 4 (A) The nerve graft (dark area, above) appears to be separated from the unlabelled neurons (arrows) (only stained with neutral red) by large lesion cavities of the spinal tissue (stars). (B) Enlargement of a section adjacent to that shown in A, displaying spinal neurons stained with neutral red but not labelled with HRP reaction product. (C) An HRP labelled neuron (arrow), filled with fine dark granules, is seen in close contact with the intraspinal tip of the grafted nerve (G). ME = spinal cord. (D) A cluster of HRP labelled neurons (stars) is located in between dissociated fascicules (arrow) of the PNG which are interwoven with the spinal tissue. Scale bars: A, 200 μm; B, C and D: 100 μm

Table 1 Results of PNG implantation

Dog number	Number of grafted fascicles	Gross examination of the grafting site	Number of HRP labelled neurons
1	2	1 fascicle found included in fibrous scar and implanted into the white matter	0
2	2	1 fascicle found into the grey matter	0
3	1	1 fascicle into the grey matter	5
4	3	2 fascicles implanted into the white matter. 1 fascicle implanted into the grey matter.	10
5	2	1 fascicle implanted into the grey matter	40
6	2	2 fascicles deeply implanted into the grey matter	67
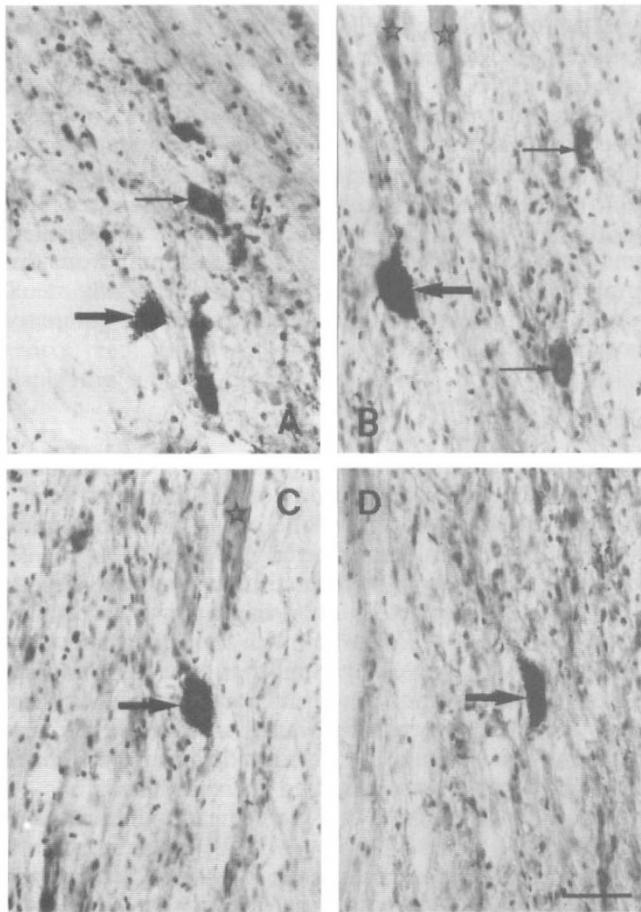
Figure 5 Large neurons, probably motoneurons, appear to be filled with HRP reaction product (large arrows) (and thus are assumed to have grown an axon into the PNG at least up to the site of tracer application). Neurons that are only stained with neutral red are generally smaller and do not contain granular material (thin arrows). Stars indicate nearby small PNG fascicles. Scale bar in D, for A, B, C, D: 50 μm

mammalian CNS neurons into peripheral conduits is unknown. However, at least in some instances, the length of axonal extension was proved to exceed that of the corresponding axons of the intact adult rat.[1]

In the present study, as well as in previous studies carried out in the adult rat,[6,7] the original stem axon was probably uninjured, since the corresponding ventral root was left intact and the PNG was inserted dorsally. This suggests that axonal production might have originated from damaged intraspinal collateral branches or, alternatively, directly from the neuronal soma, as was observed in the adult cat.[17,18]

The retrogradely HRP-labelled neurons are those nerve cells which have grown an axon into the PNG at least up to the site of tracer application. In PNG implantation studies to the cervical spinal cord of adult rats,[6,7] the average number of retrogradely labelled neurons was more than 200. In the present experiment, the average number of such labelled cells is about 20 when all experimental dogs are considered

and close to 30 if negative labellings are discarded. In addition, the highest value in a single animal did not exceed 67. These low values can be accounted for on the basis of technical considerations, since the number of labelled neurons is apparently correlated with the technical improvement of the surgical procedures with time, from dog one to dog six; secondly, to obvious differences between dogs and rats in terms of reactivity to the trauma induced by the implantation of the PNG. Unlike what is generally observed in rats under similar experimental conditions, cysts and cavitations develop at the lesion and grafting site in dogs. Labelled neurons were very rarely seen in areas where the tip of the grafted nerve appears to be more or less separated from the spinal tissue by such cavities. Conversely, labelled spinal neurons can be frequently observed in the continuation of bands of Schwann cells (bands of Büngner) which are interwoven with the spinal tissue.

In both the canine and the rodent spinal cord, labelled neurons appeared to be concentrated around the tip of the grafted nerve. Labelled neurons located at a distance from the lesion site (up to 8 mm both crainially and caudally) were only encountered in rats.

The comparative data discussed here seem to indicate that, at the cellular level, neurons of the injured canine or rodent spinal cord are endowed with similar potentialities for axonal growth and extension into nearby PNGs. What is different is probably the quality (more or less inhibitory) and the length of the spinal tissue that the regrowing axons have to run across prior to reaching the 'permissive' non-neuronal components of the grafted nerves.

The transient neurological deficit, consistently observed in the dog and only occasionally in the rat, appears to be dependent on the development of cysts and cavities at the lesion and grafting site. However, the quick return to a normal motor function is obviously not correlated with the success of axonal regrowth into PNGs since in this study the grafted nerves are not connected to the peripheral target. This restoration of normal gait depends on quite distinct (and greatly unknown) mechanisms and can also be observed after similar focal lesions involving no peripheral nerve grafting. In other words, the restoration of function, observed within a few days with only one exception, can in no way be considered as a functional test of the axonal regrowth which originates from the damaged spinal cord.

A major conclusion of the present study is that it raises once more the question of the validity of animal models in general, as far as they are considered as useful references for possible clinical applications. For many evident reasons, rats are irreplaceable experimental animals for fundamental and pre-clinical neuroscience studies. However, their brain and spinal cord are much smaller, more simply organized and probably more plastic than those of man. Let us recall that, in the eighties, the skipping of an intermediate animal model led to a considerable disappointment in

the therapy of Parkinson's disease using neural transplantation methodology. Thus, the use of bigger and/or higher (on the evolution scale) animals than rodents appears to be a necessary, or at least desirable, step in studies aimed at proposing therapeutic approaches in mankind.

Acknowledgements

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