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Axonal regeneration after crush injury of rat central nervous system fibres innervating peripheral nerve grafts

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Summary

Recent experimental studies in adult rodents indicate that neurons in many regions of the brain and spinal cord are capable of extensive axonal growth along peripheral nerve grafts inserted into the C.N.S. To explore further the capacity of damaged intrinsic C.N.S. neurons to initiate and sustain fibre growth we have studied the regenerative response of brain stem and spinal neurons to the crushing of their axons after such axons had already grown across peripheral nerve 'bridges' linking both these levels of the neuraxis. In adult rats, an autologous segment of sciatic nerve approximately 4 cm long was used to connect the medulla oblongata and the lower cervical spinal cord. After 6–42 weeks, when C.N.S. axons are known to have regenerated across these 'bridges', the nerve grafts were crushed near both their rostral and caudal insertions into the C.N.S. Axonal regeneration beyond the sites of injury was investigated 4–11 weeks after crush by retrogradely labelling C.N.S. neurons with horseradish peroxidase (HRP) applied 1 cm away from the injured site, along the assumed course of the C.N.S. fibres regrowing across the graft. The number and distribution of HRP-labelled neurons was found to be similar to that in rats with uncrushed grafts. To prove that such axonal regrowth from spinal and brain stem nerve cells did originate from injury of central nerve fibres innervating the graft and not by sprouting from undamaged C.N.S. neurons at both ends of the 'bridge', we first labelled with the fluorescent dye Fast Blue (FB) the cells whose axons were interrupted by the crush and, after two weeks, applied a second dye, Nuclear Yellow (NY) 1 cm beyond the site of injury. The presence of FB and NY double-labelled C.N.S. neurons in these animals, together with the results of the HRP-labelling experiments, suggest that central neurons whose axons innervate peripheral nerve grafts are capable of renewed growth after axonal injury. Under such experimental conditions these intrinsic C.N.S. neurons respond to axonal interruption in a manner that resembles the responses of cells that normally project along peripheral nerves. We believe this to be an additional indication of the powerful role in regeneration of interactions between neurons and the axonal environment.

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Introduction

The inability of axons injured in the C.N.S. to regrow long distances may be responsible for many of the functional deficits that follow injury to the adult mammalian C.N.S. On the other hand, injured axons in the P.N.S. can successfully regrow when they become associated with Schwann cells and other non-neuronal P.N.S. components (reviewed by Aguayo *et al.*, 1982). Experiments in which C.N.S. glia have been transplanted into peripheral nerve (Aguayo *et al.*, 1978; Weinberg & Spencer, 1979), or those in which peripheral nerve segments have been grafted into the C.N.S. (Aguayo *et al.*, 1983), suggest that differences in the long range growth of certain axons damaged in the P.N.S. or C.N.S. may depend more on the non-neuronal environment of these axons, than upon the central or peripheral location of their cell bodies.

We have previously demonstrated that adult rat spinal and medullary neurons elongate axons for distances of several centimetres through peripheral nerve grafts used as 'bridges' to connect the medulla oblongata and the lower cervical spinal cord (David & Aguayo, 1981). The neurons giving rise to these axons include cells that normally project to peripheral nerves (e.g. motor neurons) but also intrinsic neurons whose somata and projections are normally confined to the C.N.S.

Successful elongation of axons from intrinsic C.N.S. neurons has also been documented after the insertion of peripheral nerve segments into several other regions of the adult rat brain and spinal cord (Aguayo *et al.*, 1983; Richardson *et al.*, 1984). Although some of the regrown central fibres may originate by sprouting of undamaged neurons, many of the axons entering these grafts arise from nerve cells damaged at the time of grafting (Richardson *et al.*, 1984; Friedman & Aguayo, 1985; So & Aguayo, 1985). To explore further the capacity of damaged intrinsic C.N.S. neurons to sustain renewed fibre growth we have now studied the response of brainstem and spinal cord neurons to the crushing of their axons after they had already grown across peripheral nerve 'bridges' linking both these levels of the neuraxis. Using several retrogradely transported tracers, we demonstrate anatomically that newly extended axons arising from C.N.S. neurons are capable of regrowth when damaged within the environment provided by these P.N.S. grafts. Under these experimental conditions the axonal responses to injury by these intrinsic central neurons resemble the responses of peripheral nerve fibres known to be capable of regrowth after repeated injury. These findings provide further support to the hypothesis that the type of environment that surrounds the axon tip is an important determinant of its regenerative response.

Materials and methods

In adult Sprague–Dawley rats (250–300 g), an autologous segment of the sciatic nerve (nearly 4 cm long) was used to connect the medulla oblongata to the lower cervical spinal cord. The two ends of the grafts were introduced into the C.N.S. using a fine glass rod (tip diameter 150 μm). The mid-portion of the grafts was placed extraspinally in the soft tissues of the neck (David & Aguayo, 1981). Six to 42 weeks later, the grafts were crushed with a pair of jewellers forceps cooled in liquid

nitrogen. Such crush injuries were made approximately 10 mm from the rostral and caudal ends of the nerve graft (Fig. 1). We have previously reported that such crush results in the interruption of virtually all axons within the P.N.S. grafts (David & Aguayo, 1981). The sites of crush were marked with size 10.0 epineurial sutures for later identification. Axonal regrowth following crush injury was examined with two different retrograde tracer techniques: (1) horseradish peroxidase (HRP), and (2) fluorescent dyes, Fast Blue and Nuclear Yellow.

Studies using HRP

In eight rats the grafts were re-exposed 4–11 weeks after crush injury and transected at the mid-point of the graft, a distance of about 10 mm from the two sites of crush. A small Gelfoam pad (Upjohn) soaked in a 20% solution of HRP (Sigma type VI) was applied to the cut ends of the graft for approximately 1 h (Fig. 1). Grafts were placed on a small piece of Parafilm and the region of the crush and the surrounding soft tissues were sealed with petroleum jelly to prevent HRP diffusion. Twenty-four hours later, animals were perfused with 0.1 M phosphate buffer pH 7.4 followed by fixation with 3% glutaraldehyde. Cryostat sections (40 μm) of the medulla and spinal cord were processed for HRP histochemistry using tetramethylbenzidine as the chromagen (Mesulam, 1978).

Studies using fluorescent dyes

In two rats, the grafts were labelled with Fast Blue (FB) at the time of crush (Fig. 1), by applying a small Gelfoam pad soaked in 3% FB to the crush sites. The Gelfoam pads were left in place for 30–45 min and the surrounding areas sealed with petroleum jelly. After two weeks the regrowth of axons beyond the site of crush injury was assessed by retrogradely labelling this population of neurons with a second fluorescent marker Nuclear Yellow (NY) (Hoechst S 769121). This was accomplished by transecting the grafts at the mid-point between the two sites of crush and applying a small pad of Gelfoam soaked in 2% NY to the cut ends for 30–45 min (Fig. 1). After a 40–50 h survival period the animals were perfused as follows: firstly, with chilled physiological saline, followed by chilled 10% formalin in 0.1 M phosphate buffer, pH 7.4, and finally, with a cold solution containing 10% phosphate buffered formalin and 10% sucrose (Sawchenko & Swanson, 1981). The medulla and spinal cord were then removed and placed overnight in 10% phosphate-buffered formalin containing 30% sucrose. After rinsing the tissues for 5 min with cold saline (4° C), frozen sections 20 μm thick were cut on a cryostat, picked up on glass slides and air-dried. Sections were examined with a Leitz fluorescence microscope equipped with a 360 nm excitation wavelength filter.

For routine histological examination, the grafts were dissected after perfusion of the rats and placed in fixative (2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4) for several hours, postfixed in osmium tetroxide, dehydrated and embedded in Epon. Sections of 0.5 μm were stained with toluidine blue for light microscopy.

For analysis, the HRP labelling in rats with crushed grafts was compared to the labelling obtained in eight rats that we previously reported, in which the grafts were not crushed (David & Aguayo, 1981). The grafting procedure used in these animals is similar to that described above, and their postgrafting survival times ranged from 12–30 weeks.

Results

Four to 11 weeks after crushing peripheral nerve grafts that are known to be innervated by axons from spinal and medullary neurons (David & Aguayo, 1981), cross-sections of the graft distal to the sites of crush showed many axons ensheathed and myelinated by Schwann cells (Fig. 2A, B).

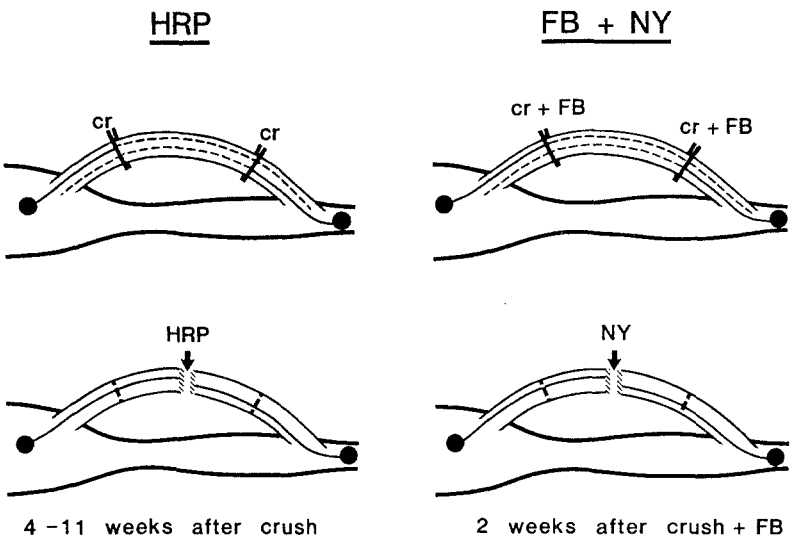
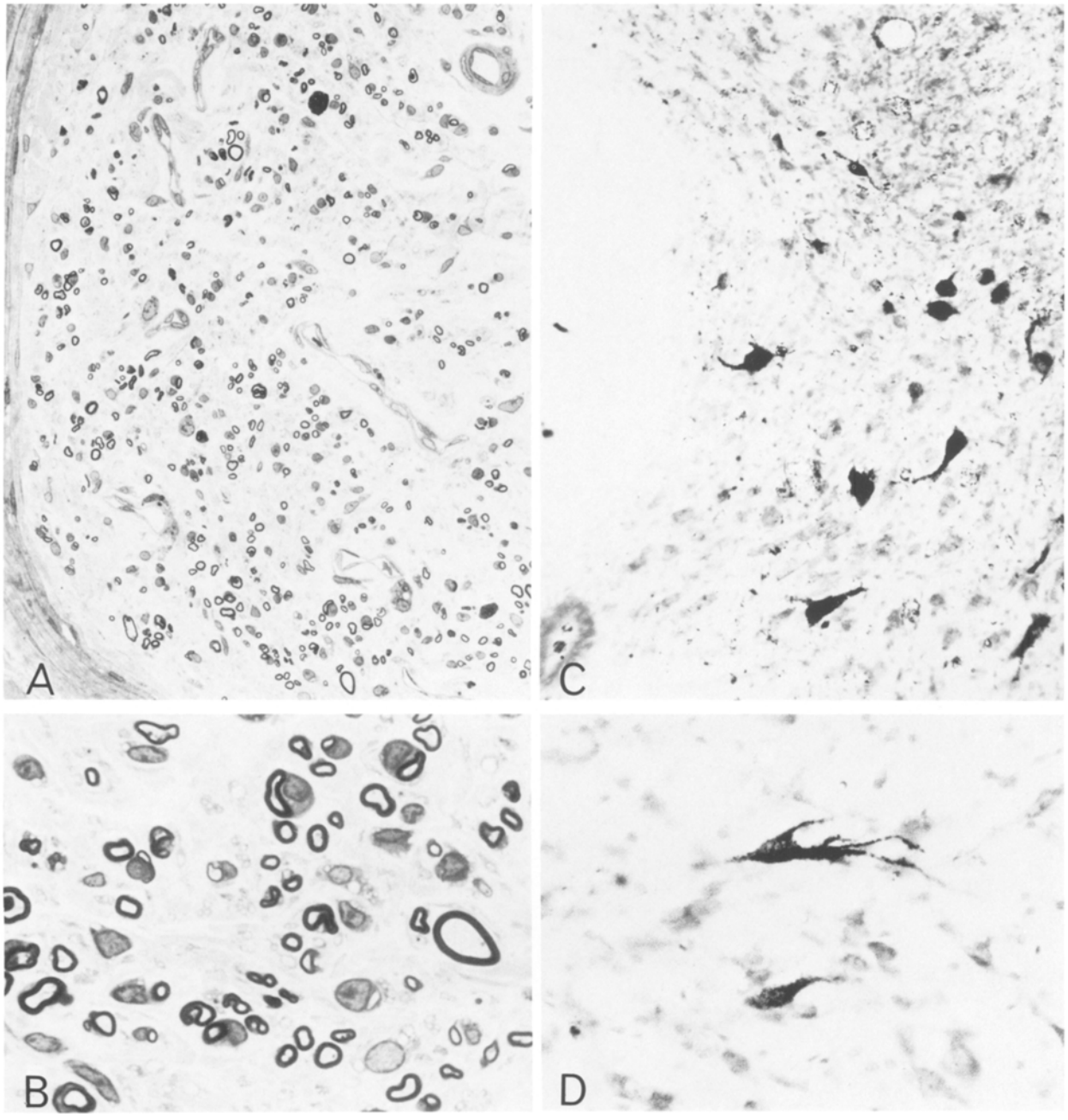


Fig. 1. Schematic diagram illustrating the grafting technique, the sites of crush injury (cr) and the application of the retrograde tracers.

HRP studies

Neurons in the medulla oblongata and spinal cord were retrogradely labelled with HRP applied distal to the site of injury 4–11 weeks after the crush (Fig. 2C, D). The labelled central neurons were located within a rostro-caudal distance of 4–6 mm near the site of insertion of the graft, and their distribution within the medulla and spinal cord (Fig. 5A, B) is similar to that in grafted animals in which the grafts were not crushed. In the medulla oblongata the labelled neurons were located ipsilaterally in the nucleus gracilis and cuneatus, nucleus intercalatus, nucleus tractus spinalis nervi trigemini, nucleus tractus solitarius, nucleus originis dorsalis vagi, nucleus reticularis lateralis, nucleus reticularis paramedianus, nucleus reticularis medullae oblongatae pars dorsalis and ventralis, nucleus olivaris inferior, nucleus accessorius olivaris inferior and nucleus raphae pallidus (Palkovits & Jacobowitz, 1974). In the spinal cord, labelled cells were found in all regions of the lower cervical spinal grey matter largely on the ipsilateral side near the site of insertion of the graft. Many of these neurons were in regions of the spinal cord and brain stem that contain only intrinsic neurons whose axons do not normally

Fig. 2. (A) Cross-section of a graft distal to the site of crush from an animal sacrificed eight weeks after crush injury. × 390. (B) Higher magnification of a portion of the graft seen in (A) showing both myelinated and bundles of unmyelinated fibres. × 1200. (C) HRP-labelled neurons in the ipsilateral dorsal horn of the spinal cord. The top of the figure represents lamina II, while the central canal can be seen at the lower left hand side. HRP was applied to the graft distal to the site of crush eight weeks after the injury. × 140. (D) Neurons in the region of the ipsilateral nucleus olivaris inferior labelled with HRP eight weeks after crush. × 276.


A
C
B
D

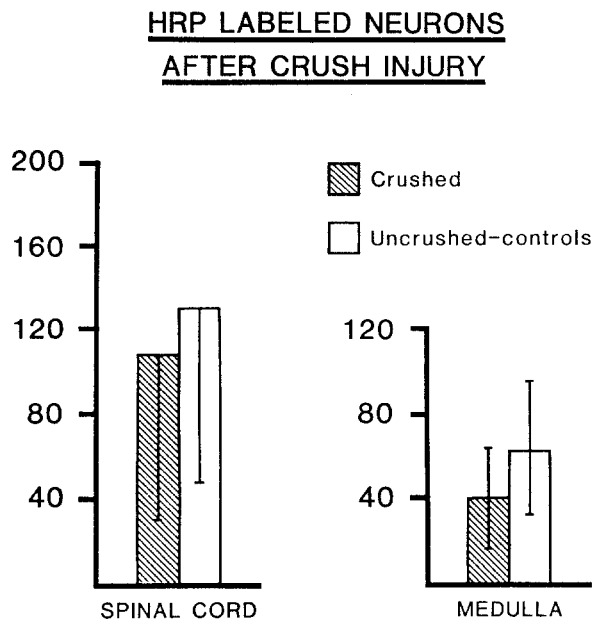


Fig. 3. Histogram of the mean number of HRP-labelled neurons in the spinal cord and medulla oblongata of rats with crushed (hatched lines) and uncrushed grafts. There are no significant differences between the two groups ($P \geq 0.1$ Student's t-test); $n = 8$ in both groups.

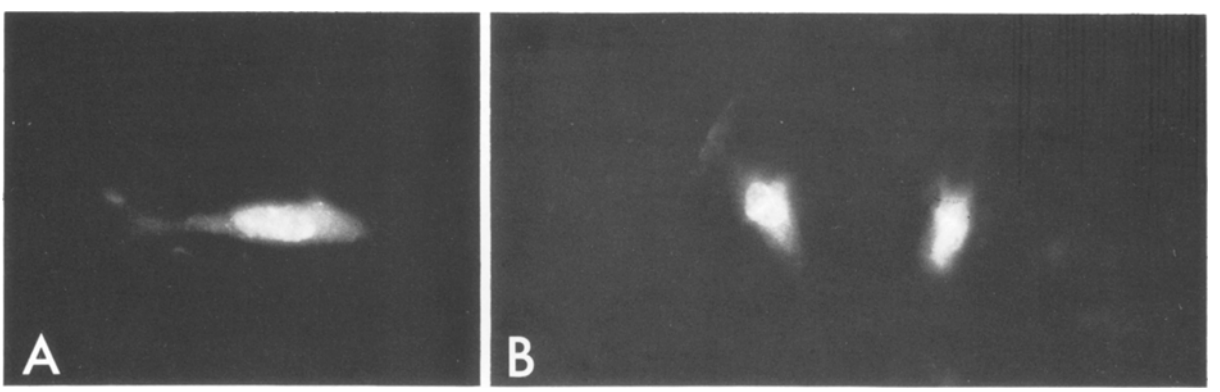


Fig. 4. Fluorescence photomicrograph of neurons double labelled with FB and NY, located in lamina X of the spinal cord (A) × 437, and in the nucleus reticularis paramedianus of the medulla oblongata (cell on the left in B). The neuron on the right in B is singly labelled with FB. × 500.

Fig. 5. Distribution of HRP (A and B) and FB and NY double labelled (C and D) neurons in the medulla oblongata and spinal cord that were retrogradely labelled after crush injury.

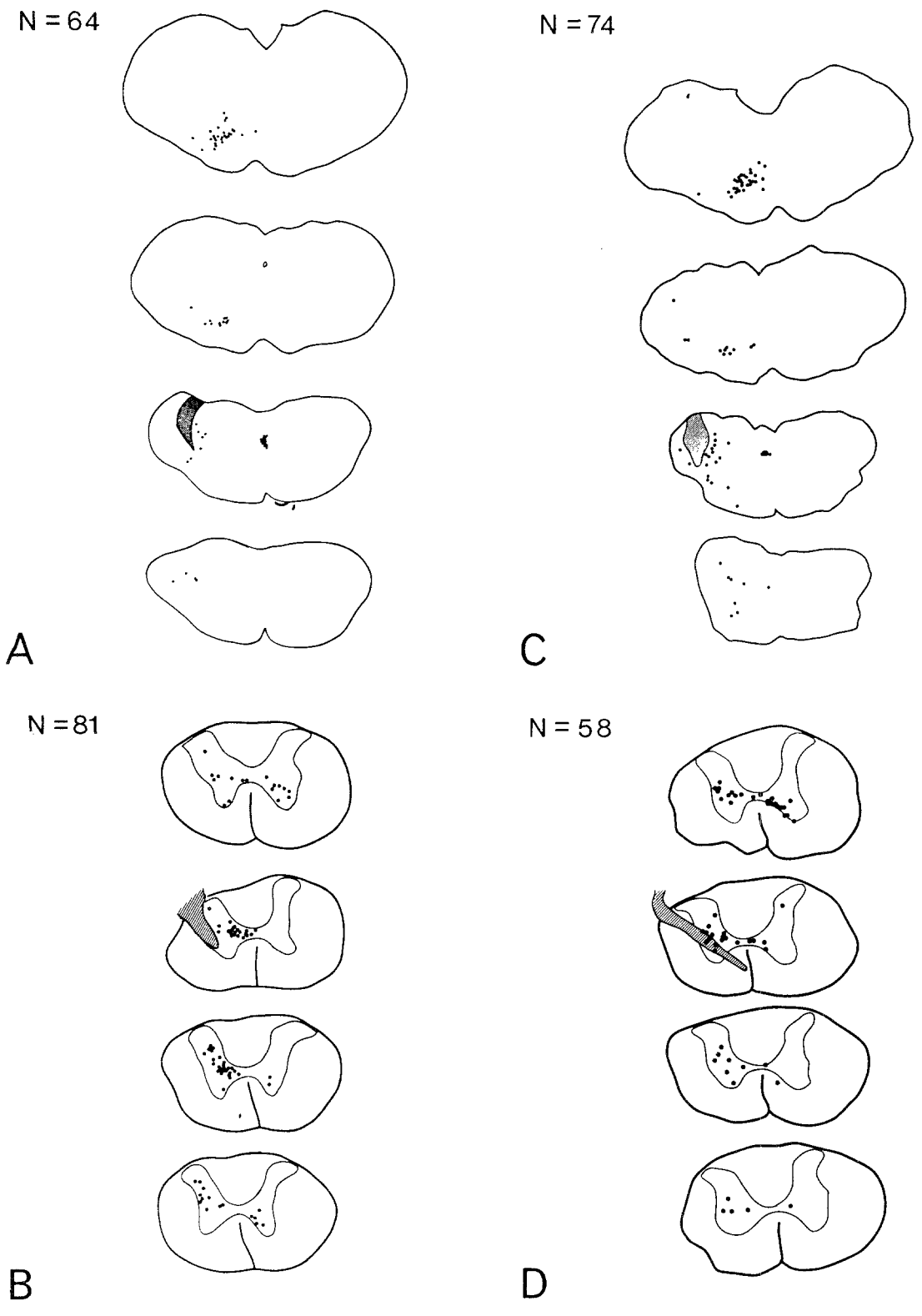

HRP
FB + NY
N = 64
N = 74
A
C
N = 81
N = 58
B
D

Table 1. The total numbers and percentages of neurons that were singly labelled with FB or NY and double labelled with both FB and NY (FB + NY) in the spinal cord (SC) and medulla oblongata (MED). FB was applied at the time of crush and NY was applied two weeks later. Approximately 14% of the neurons in the spinal cord and medulla oblongata are double labelled with FB and NY.

	FB		*FB + NY*		*NY*	
	SC	*MED*	*SC*	*MED*	*SC*	*MED*
No. 112	210	170	72	6	1	0
No. 114	568	341	58	74	0	3
Total	778	511	130	80	1	3
%	85.6	86	14.3	13.5	0.1	0.5

project into peripheral nerves. The distribution of HRP-labelled neurons in rats with uncrushed grafts has been reported previously (David & Aguayo, 1981). The total number of HRP-labelled neurons obtained after crush injury is also similar to that in rats with uncrushed nerve grafts (Fig. 3).

Fluorescent dye studies

In the experiments with fluorescent dyes we used short survival times of two weeks after crush injury, to minimize the diffusion of the fluorescent dyes from labelled neurons into neighbouring cells.

Two weeks after crushing of the graft, approximately 14% of all labelled C.N.S. neurons were double labelled with FB and NY (Fig. 4, Table 1), while less than 0.5% were labelled with NY alone. However, the total number of neurons labelled with the fluorescent dyes was 4–6-fold greater than that obtained with HRP.

The distribution of the double-labelled neurons (FB and NY) in the medulla and spinal cord was similar to that seen in the HRP studies (Fig. 5C, D).

Since NY diffuses out of cells more readily than FB (Bentivoglio *et al.*, 1980; O'Leary *et al.*, 1981), a short survival time of 40–50 h following NY application was used. We found that a 30 hour survival period was insufficient to label neurons (data not shown). These survival times following NY application are similar to those used by other investigators (Huisman *et al.*, 1981).

Discussion

When axons in the adult mammalian C.N.S. are injured they undergo regenerative sprouting, but these sprouts fail to grow for more than a few millimetres through C.N.S.

tissue (Ramón y Cajal, 1928). In contrast, injured peripheral nerve axons can regrow for considerable distances. There is increasing experimental evidence to suggest that differences in the regrowth of many damaged P.N.S. and C.N.S. axons is determined by interactions between neurons and non-neuronal components of the environment that surrounds the damaged axons (reviewed by Aguayo *et al.*, 1981, 1983).

Neurons in several regions of the adult rat forebrain, midbrain, pons, medulla, spinal cord and cerebellum are capable of extensive axonal growth through peripheral nerve grafts (Aguayo *et al.*, 1983). It is possible that the central axons growing into such peripheral nerve grafts may arise from either injured cells, or from collaterals of uninjured neurons. However, in recent studies in which peripheral nerve grafts were introduced into the rat olfactory bulb (Friedman & Aguayo, 1985), and retina (So & Aguayo, 1985), the majority of the axons innervating the grafts were derived from axotomized cells. Therefore, injured C.N.S. axons appear capable of regrowth and may contribute significantly to the innervation of peripheral nerve grafts inserted into the C.N.S. (Aguayo *et al.*, 1983).

In this study, we have examined the response of C.N.S. neurons to a crush injury of their axons, after they have already grown along peripheral nerve grafts. When peripheral nerve grafts containing central axons coursing between the medulla oblongata and the lower cervical spinal cord were crushed 6–42 weeks after grafting, both spinal and medullary neurons were retrogradely labelled with HRP several weeks later. Even though the neuronal population innervating the grafts prior to the crush injury is not known in these experiments, the distribution of the HRP-labelled cells and their total numbers after crush injury are similar to that in rats with uncrushed grafts. These findings suggest that the majority of the axons of C.N.S. origin found within the grafts may have regrown following the crush injury. However, it is possible that the interruption of the axons within the graft could have triggered collateral sprouting and regrowth from neighbouring uninjured C.N.S. neurons other than those which had initially grown along these grafts. Thus, the population of HRP-labelled neurons observed in these experiments might be similar in distribution but not identical to the neurons innervating the grafts prior to injury. To provide direct evidence for axonal regrowth after injury, the initial population of neurons innervating the grafts was labelled at the time of the crush with the fluorescent dye Fast Blue. Two weeks later, cells whose axons had regrown were again traced anatomically by applying Nuclear Yellow approximately 1 cm distal to the site of crush along the course they would follow during regrowth. In these experiments approximately 14% of the labelled neurons were double labelled with FB and NY, suggesting that they had initially innervated the graft, been subsequently interrupted by the crush and eventually regrown to reach the site of application of the second dye (NY).

The small percentage of double-labelled neurons might reflect: (1) the short postcrush survival period of two weeks, labelled cells representing only the source of the fastest growing axons; (2) an artifactual increase in the numbers of FB-labelled cells due to diffusion of the dye from labelled neurons into neighbouring nerve cells that do not

project into the graft; (3) that NY might be less effective than FB in labelling neurons; and (4) that only a small proportion of injured C.N.S. neurons is capable of axonal regrowth.

Survival times of 3–8 days are generally used following True Blue or Fast Blue application because the intensity of the fluorescence increases over the first eight days (Bentivoglio *et al.*, 1979). With longer survival times there is danger of diffusion of the dye (Bentivoglio *et al.*, 1979; O'Leary *et al.*, 1981). However, O'Leary *et al.* (1981) found little diffusion of True Blue with survival periods of up to 19 days. To minimize the possible inclusion of neurons spuriously labelled through diffusion we have killed the rats 16 days after FB application, and have not counted lightly labelled cells. Thus, diffusion of FB is unlikely to have contributed substantially to the small percentage of double-labelled neurons. Secondly, although exact estimates of the efficiency of labelling with FB and NY are not known, no marked differences between these dyes have been reported. It therefore seems likely that the small percentage of double-labelled neurons is due to the short postcrush injury survival period, and may represent mostly neurons with fast-growing axons. With longer survival periods of 4–11 weeks that were used for the HRP-labelling experiments, the majority of the neurons appear to have regrown as indicated by the lack of any significant difference in the number of HRP-labelled cells between rats with crushed and uncrushed grafts.

Another finding was that less than 0.5% of the fluorescent dye labelled neurons were singly labelled with NY. The presence of these neurons might be due to: (1) diffusion of NY into neighbouring cells with no projections into the graft; (2) a loss of FB labelling in double-labelled neurons; or (3) new growth into P.N.S. grafts following injury. Their small numbers (four out of a total of 1503 neurons) indicates that new C.N.S. neurons do not contribute significantly to the axonal population growing after crush injury of these grafts.

The 4–6-fold greater labelling obtained with the fluorescent dye Fast Blue as compared to HRP labelling in rats with uncrushed grafts probably reflects the greater sensitivity of the fluorescent dyes as retrograde tracers (Sawchenko & Swanson, 1981).

Conclusive evidence of *axonal regeneration* requires documentation of: (1) the interruption of axons; (2) axonal regrowth from an injured neuron; and (3) the distance of axonal regrowth. Evidence for all three have been presented in the present report. (1) The technique used to crush the graft has been shown previously to result in the interruption of virtually all axons innervating the P.N.S. grafts (David & Aguayo, 1981; Benfey & Aguayo, 1982). (2) In the experiments using fluorescent dyes, the neurons whose axons were crushed were tagged with FB. Neurons which had regrown their axons after the crush were then retrogradely labelled with NY. The presence of FB and NY in the same neuron provides strong evidence for regrowth from injured C.N.S. axons. The lack of any significant differences between the number and distribution of the HRP-labelled neurons in animals in which the grafts were either crushed or uncrushed further suggests regrowth of the majority of C.N.S. axons injured within the graft. (3) Finally, in these experiments central axons were demonstrated to have regrown a distance of approximately 10 mm from the site of crush injury.

These data suggest that axons from intrinsic nerve cells in the brain and spinal cord of adult rats can regrow when severed in an environment where they are able to interact with non-neuronal components of the P.N.S. The nature of these interactions is unknown but could involve changes in the neuronal perikaryon (Grafstein & McQuarrie, 1978; Skene & Willard, 1981; Richardson & Issa, 1984) perhaps triggered by signals received through growth cones in the damaged tip of axons (Bray & Bunge, 1973; Gundersen & Barrett, 1980) from molecular cues secreted by the ensheathing cells (Varon *et al.*, 1981; Richardson & Ebendal, 1982; Skene & Shooter, 1983) or present on cell surfaces and/or the extracellular matrix (Carbonetto *et al.*, 1982).

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