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# Extensive elongation of axons from rat brain into peripheral nerve grafts

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**The failure of axons to elongate in the injured central nervous system (CNS) of adult mammals restricts drastically the establishment of connections with target tissues situated more than a few millimetres away. Mechanisms that include a primary inability of some nerve cells to support renewed axonal growth, a premature formation of synapses on nearby neurones[1], an obstruction caused by the formation of a glial scar[2,3] and other influences of the microenvironment[4–7] are presumed to contribute to the failure of nerve fibres to regenerate as effectively in the CNS as in the peripheral nervous system (PNS). Support for the hypothesis that conditions in the glial environment of injured fibres have a decisive role in successful axonal elongation has recently come from studies using transplants containing either central glia or peripheral nerve segments as conduits of axon growth[7,8]. While CNS glial grafts have been shown to prevent growth of PNS fibres[7–9], experiments which used labelling techniques to trace the source of axons growing into PNS grafts provided evidence that processes from nerve cells in the spinal cord and medulla oblongata of adult rats may increase in length by 1 or more centimetres when the CNS glial environment is replaced by that of peripheral nerves[10,11]. Here we report for the first time the extensive elongation of axons from neurones in the brain of adult rats through PNS grafts introduced into the cerebral hemispheres.**

In 20 Sprague–Dawley rats weighing ~300 g the end of a 15-mm segment of an autologous sciatic nerve was inserted close to the basal ganglia or to the cerebral cortex (Fig. 1*A*). The animals had previously been anaesthetized with sodium pentobarbital administered intraperitoneally (5 mg per 100 g body weight). A glass rod held in a micromanipulator was inserted through an opening in the frontal bone and dura to a depth calculated to produce lesions extending to the sensory cortex or basal ganglia. The sciatic nerve graft was then introduced into each of these lesions and its epineurium anchored to the dura mater with 10-0 nylon sutures. The space between the nerve and the surrounding skull was sealed with bone wax and the free outer end of the nerve sutured to the temporalis muscle. The animals survived without any apparent neurological deficit other than limb weakness resulting from the removal of one sciatic nerve. The rats were examined 5–23 weeks after grafting to determine whether axons from neurones within the brain had elongated along the graft. For this purpose the graft was dissected along its extracranial course, transected 2 mm from its insertion into the temporalis muscle, and the free end placed for 50 min in a pad of Gelfoam soaked in a 20% solution of horseradish peroxidase (HRP; type VI, Sigma). The remaining portion of the graft was placed over a plastic sheath and covered with vaseline to avoid contamination of the surrounding tissue by HRP (Fig. 1*A*). In five additional animals, used as controls, the mid-portion of the regenerated graft was crushed three times with fine-tip forceps cooled in liquid nitrogen, 9–16 weeks after grafting and half an hour before the application of HRP to the distal end of the crushed nerve. About 48 h after applying HRP, the animals were perfused through the heart with a 0.1 M phosphate buffer solution followed by 3% glutaraldehyde. The brain was removed and rinsed in 10% sucrose buffer overnight. Then, 40 μm-thick coronal sections were cut in a cryostat, reacted with tetramethylbenzidine and hydrogen peroxide[12], counterstained with neutral red and mounted in Permount.

Cross-sections of the outermost portion of each graft were examined using a light microscope and found to contain regenerated fibres surrounded by Schwann cells and myelin. The overall appearance of these fibres resembled that of any regenerated peripheral nerve. HRP-labelled neurones were sought by dark- and brightfield light microscopy in sections rostral to and including the midbrain. An average of 145 sections of the brain were examined per rat. In 10 experimental animals the graft terminated close to the cortex; in 8 it was found within the caudate putamen and in 1 rat each it reached the hippocampus or the ventral nucleus of the thalamus. Of a total of 444 HRP-labelled neurones (Fig. 1*B*, *C*), most were found in the caudate putamen and sensory cortex (Table 1). The labelled nerve cells in the sensory cortex were scattered through layers II–VI. Both large and small cortical neurones were labelled but the HRP-containing cells tended to be of larger size than the unlabelled ones in the same regions (Fig. 2). Most (79%) of the labelled cells found in the brain of these animals were within 1.5 mm of the intracerebral tip of the graft; the rest were located between 1.5 and 6.5 mm from the graft. The greatest distance between the graft and the soma of the labelled cells was found for neurones in the substantia nigra (SN). Because all labelled neurones in the experimental rats were confined to a well circumscribed area close to the graft and as only two neurones were labelled in the five controls in which the graft was crushed before HRP application, we believe that most of the cells in the experimental animals used in this study were not falsely labelled by haematogenous spread or by diffusion along the grafted nerve. Thus, on the basis of these HRP tracing studies, we conclude that axons of cells in different regions of the rat brains examined have become elongated by at least 13 mm, the length of nerve graft used in the labelling experiments.

The fact that the largest number of labelled neurones was found in the caudate putamen may reflect a greater ability of

**Table 1** Distribution of HRP-labelled cortical and subcortical neurones in the brain of rats having PNS grafts

| Site | No. of HRP-labelled neurones |
|---|---|
| Caudate putamen | 218 |
| Globus pallidus | 42 |
| Thalamus | 10 |
| Substantia nigra | 22 |
| Ventral tegmental area | 20 |
| Zona incerta | 4 |
| Amygdala | 1 |
| Claustrum | 1 |
| Cortex | 126 |
| Total | 444 |

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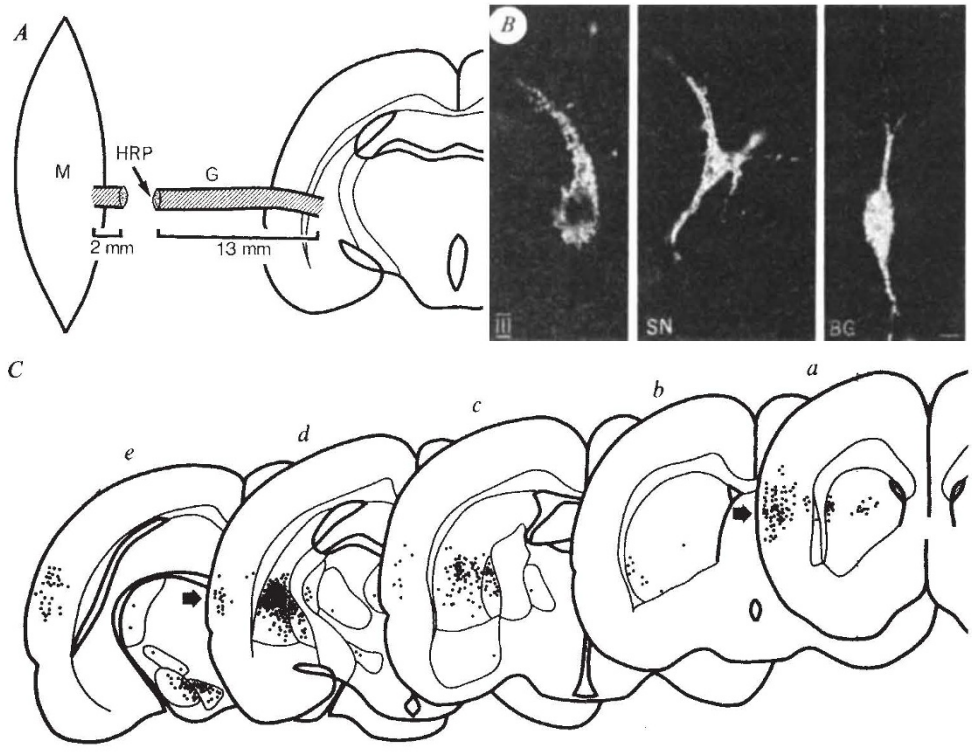


**Fig. 1** *A*, Schematic representation of the experimental design: a PNS graft (G) has been introduced into the rat brain. The intracranial tip of the graft can be positioned close to the cortex or subcortical nuclei within the hemisphere. The outer end of the graft, initially attached to the temporalis muscle (M), is cut to apply HRP. The advantages of this design are: (1) as most of the graft is extracranial, the cells from which the axons growing into the graft have originated can be determined by retrograde labelling with little risk of contamination from spread of HRP into the brain; (2) the length of axons regenerating within the graft can be measured by determining the distance between the site of HRP application and the soma of the labelled cells; (3) the grafts may be positioned in several regions of the brain to test the regenerative capacity of different neuronal populations. *B*, dark-field light micrographs of HRP-labelled neurones in layer III of the sensory cortex, from the pars compacta of the substantia nigra (SN) and from basal ganglia (BG). Calibration bar, 10 μm. *C*, transverse sections of the brain, obtained at 1.5-mm intervals, are arranged in a rostral (*a*) to caudal (*e*) direction to illustrate the approximate position of the 444 labelled neurones (●) observed in 20 grafted animals. The arrow in *d* indicates the site of 15 graft insertions made stereotactically 4.4 mm rostral to the inter-aural line in the horizontal plane[19]. In five other animals the graft was inserted 9 mm rostral to and 2 mm above these two reference lines (arrow in *a*).

these cells to regenerate in the experimental conditions used, or may simply be the consequence of their proximity to the transplanted nerve; different graft locations may elucidate this question. Most of the labelled cerebral neurones were situated near the tip of the graft, which suggests that the remarkable elongation of their axons results from local sprouting triggered by injury[13–15] resulting from the grafting procedure and facilitated by growth-favouring conditions in the PNS transplant. It is also possible that growth factors released by the segment of nerve[16,17] may influence only the soma of nearby cells. The demonstration that the cerebral neurones that give rise to the re-growing axons are confined to an area within a few millimetres of the end of the graft is consistent with similar observations in the rat spinal cord and brain stem[10,11]. These findings suggest that it may be possible to use similar grafting methods to investigate further neuronal plasticity in other specific areas of the CNS of mature and immature animals and in conditions associated with ageing and disease.

Although the axons of neurones labelled in these experiments would, in the intact animal, reside and project exclusively within the CNS, they have successfully elongated along the implanted grafts in a manner similar to that of fibres injured in peripheral nerves. This indicates that such elongation may depend on properties shared by populations of intrinsically and extrinsically projecting nerve cells, the potential for the growth of intrinsic CNS neurones being expressed when the neuroglial environment in the brain is substituted by that in peripheral nerves. It is not known, however, whether the cells labelled in these experiments indicate a more general potential for regeneration by CNS neurones when interacting with Schwann cells and other PNS components.

These results also suggest that in conditions created by nerve grafting, the axons of some CNS neurones can grow to lengths greater than those normally reached in the intact animal. Axons from the striatal neurones, which are thought normally to project only to the nearby globus pallidus and SN[18], appear to have grown along the entire length of the grafts. Furthermore, because the intracerebral tip of the 13-mm-long graft was as far as 6.5 mm from the labelled SN cells in some animals, we calculate that the axons from nigral neurones must have attained a total length of almost 20 mm. The extent of this axonal growth can be placed in perspective by comparing it with the maximum direct distance between the SN and the cerebral cortex: 14–15 mm in 300 g rats[19].

The hypothesis that the microenvironment at the tip of the

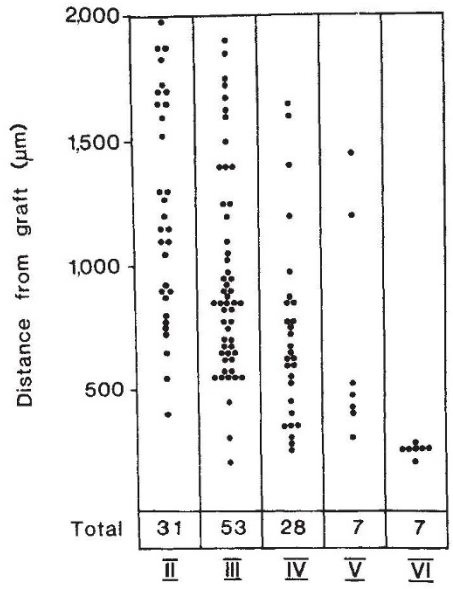


**Fig. 2** Distribution of 126 HRP-labelled neurones in layers II–VI of the somatosensory cortex of grafted rats. The vertical axis indicates the distance between the labelled soma and the intracerebral tip of the graft. The cells in the more superficial layers are nearly 2 mm from the graft. The diameter of the labelled cortical neurones averaged 24.9 ± 4.05 μm (mean ± s.d.) (range 16.8–38.4 μm) while that of 250 unlabelled neighbouring cells was 17.9 ± 3.63 μm (range 12.0–28.8 μm). The difference in size between the labelled and unlabelled cells may result from a neuronal reaction to axon interruption at the time of HRP application to the graft.

re-growing axon has a decisive role in the success or failure of regeneration implies that non-neuronal PNS components influence neuronal mechanisms that regulate elongation[20,21] whereas the CNS milieu of adult mammals either lacks these growth-promoting factors or exerts an inhibitory influence. The critical differences between the CNS and PNS microenvironment are unknown but may include specific substances released by cells[6,22], influences arising from extracellular components[23], surface properties of sheath cells[24] and, possibly the different spatial arrangement of glia in the CNS and PNS. Further questions which require investigation are whether central axons elongate into PNS grafts by the regrowth of the damaged fibres, by collateral sprouting or by both. Will long projecting axons such as those of the corticospinal tract, also grow along PNS grafts after they are interrupted in the brain? What connections, if any, can be established between the growing CNS axons and the target tissues to which the nerve graft is attached? Our findings suggest that the elongation of axons from these central neurones is governed by interactions between the growing nerve fibres and their surrounding tissues, and that abortive regeneration is not the result of an intrinsic lack of neuronal potential to support renewed growth.

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# Functional synapses are established between ciliary ganglion neurones in dissociated cell culture

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**The formation of specific synaptic connections is a central part of development in the nervous system. The parasympathetic ciliary ganglion of the chick is a useful population of neurones for studying synaptogenesis both because its development has been well characterized *in vivo*[1] and because its neurones can be maintained and examined in long-term dissociated cell culture[2,3]. The ciliary ganglion contains two classes of neurones: choroid neurones that innervate smooth muscle in the choroid layer of the eye, and ciliary neurones that innervate striated muscle in the iris and ciliary body. Both classes of neurone are cholinergic and both receive excitatory cholinergic input from preganglionic neurones in the accessory oculomotor nucleus. Ciliary ganglion neurones do not seem to innervate each other *in vivo*[4,5], even though they have matching neurotransmitter and receptor types. In cell culture, the neurones acquire high levels of choline acetyltransferase activity, form cholinergic synapses on skeletal myotubes when present in the cultures, and have significant levels of acetylcholine (ACh) sensitivity[2,6], as they do *in vivo*. We report here that cholinergic synaptic transmission does occur between the neurones in cell culture. These results indicate that ciliary ganglion neurones can innervate each other, and suggest that additional constraints exist *in vivo* to prevent them from doing so.**

Dissociated ciliary ganglion neurones were obtained from 7½-day-old chick embryos and, unless otherwise indicated, were grown in 35-mm dishes with skeletal myotubes as previously described[2]. In some cases the neurones were grown alone, using a collagen substratum coated with fibroblast material[2]. Culture medium was as previously described for nerve–muscle cultures[2] except that some cultures of neurones alone received medium with 3% (v/v) embryonic chick eye extract instead of 5% (v/v) whole embryo extract[2]. After 5–14 days the neurones were examined using intracellular recording techniques[2,6]; microelectrodes were filled with 1 M potassium acetate and had tip resistances of 80–200 MΩ. Recordings were accepted only if impaled neurones had resting potentials exceeding −45 mV and fired impulses in response to intracellular stimulation.

Intracellular recordings from the neurones revealed discrete spontaneous depolarizations that ranged in amplitude over 0.5–15 mV and in half rise time over 0.6–1.2 ms, and appeared to decay exponentially (Fig. 1). The frequencies of such depolarizations among neurones varied from 2 to 300 per min and persisted throughout recordings that lasted up to 45 min. At high frequencies the depolarizations often summed and occasionally triggered action potentials in the neurones (Fig. 1). Over 90% of the neurones grown with myotubes displayed such depolarizations whereas only 42% of the neurones grown alone did so (Table 1).

Pharmacological experiments indicated that the spontaneous depolarizations were cholinergic in origin. Perfusion of the cultures with 25 μM(+)tubocurarine (TC) completely abolished the depolarizations in all neurones within 5 min; partial recovery of the spontaneous activity was obtained 4 min after washout of the drug was initiated (Fig. 2). After 30 min of washout, the occurrence of depolarizing potentials among randomly selected, treated neurones was indistinguishable from that obtained in control cultures (Table 1).

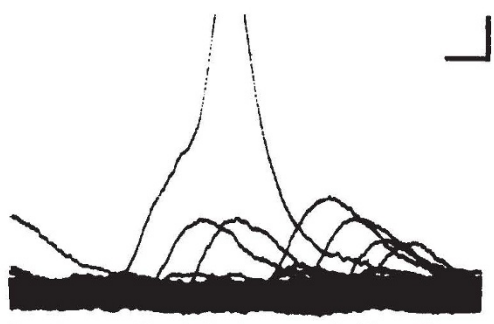

**Fig. 1** Spontaneous depolarizations recorded in a ciliary ganglion neurone grown with myotubes. A number of oscilloscope traces have been superimposed showing about 15 depolarizations. In one case a depolarization has triggered an action potential. Resting potential: −61 mV. Calibration bars: vertical, 5 mV; horizontal, 2 ms.

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