AXONAL GROWTH AND ITS INHIBITION

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Regeneration of axons in the central nervous system (CNS) is defined as a growth response to transection in which newly formed sprouts traverse the lesion site and extend for a substantial distance into distally located neuropil which may be either white or grey matter. Functional regeneration is a corollary defined as the invasion of regenerating fibres into the original or new targets, where synaptic connections are established which restore lost function. Regeneration of axons in the mammalian and avian CNS is, however, largely impossible, but the reasons for this are unknown.

The classical accounts of the injury response of CNS axons describe an initial abortive regenerative phase lasting up to 14 days, after which growth stops and most fibres die back into the lesion area.[1] The idea that the scar tissue deposited in the wound acts as a physical barrier, obstructing the growth trajectory to the target,[2–5] is contraindicated by an absence of neuromata on the proximal side of the scar, except in very rare instances.[6] The once strongly held view that mature CNS axons are inherently incapable of regrowth beyond the abortive post-injury response[1] has been refuted by Richardson *et al.*[7] who showed that neurons from most, if not all, CNS areas regenerate their axons into peripheral nervous system (PNS) grafts implanted into the brain, the spinal cord, and also onto the cut retinal stump of the optic nerve[8] (Fig. 1).

These findings represent a milestone in the history of regenerative research, not only by showing that CNS axons can regrow, but also in defining a feasible strategy for future research, with a new priority directed towards discovering what it is about the environment of the CNS, on the one hand, which impedes regrowth, and of the PNS, on the other, which promotes regeneration. Subsequently, at least two plausible explanations (which may not be mutually exclusive) were proposed for the growth arrest which underlies the failure of CNS axons to regenerate.

First, it was once thought that neurotrophic factors, essential for sustaining fibre growth during development and also mandatory for regeneration, were absent from the mature CNS. Growth-promoting substrates may also be important for regrowth since substrate maps are also present during development for redirecting growth into targets.[9] We do not know whether substrate maps either persist or are redrawn in the adult CNS after injury, but some neurotrophins are found in the mature CNS[10–12] which might be available to support a regenerative response. There is no evidence, however, that regenerating neurons recapitulate their developmental growth status. By contrast, in a mature peripheral nerve, trophic molecules and a guidance substrate are both provided by Schwann cells after damage,[13] which probably explains not only why regeneration is possible in this system but also why CNS axons readily regrow into peripheral nerves implanted into the brain[8,14] and into areas of the CNS in which Schwann cells are seeded.[15,16]

A second possible explanation for the failure of CNS regeneration is contact inhibition[17] – a concept originally introduced by Abercrombie and Heaysman[18] after observing fibroblast behaviour in monolayer cultures. In the CNS, inhibition of growth might be brought about by interaction between receptors on axons and glial surface membrane ligands which prevents growth cone adhesion to the substrate or causes growth cone collapse.[19] It is possible that in the normal developing CNS such axon/ligand interactions might function to confine growing axons within permissive pathways, limit the overgrowth of tracts, and prevent the mixing of functionally different fibre systems.[20,21]

It is assumed that growth cone collapsing and anti-adhesive ligands are absent from peripheral nerves, which would explain why motor axons readily regenerate across the CNS/PNS interface into ventral roots and cranial motor nerves.[22,23] However, the latter are one-way conduits, presumably because the ligand-bearing astrocytes at the CNS/PNS junction prevent peripheral sensory axons from penetrating the root/cord junction.[24–26] At an early stage of development the axons of neural crest derived dorsal root ganglia (DRG) are able to invade the cord to establish the definitive dorsal roots,[27] possibly because either receptor and/or ligand expressing glia cells are absent.

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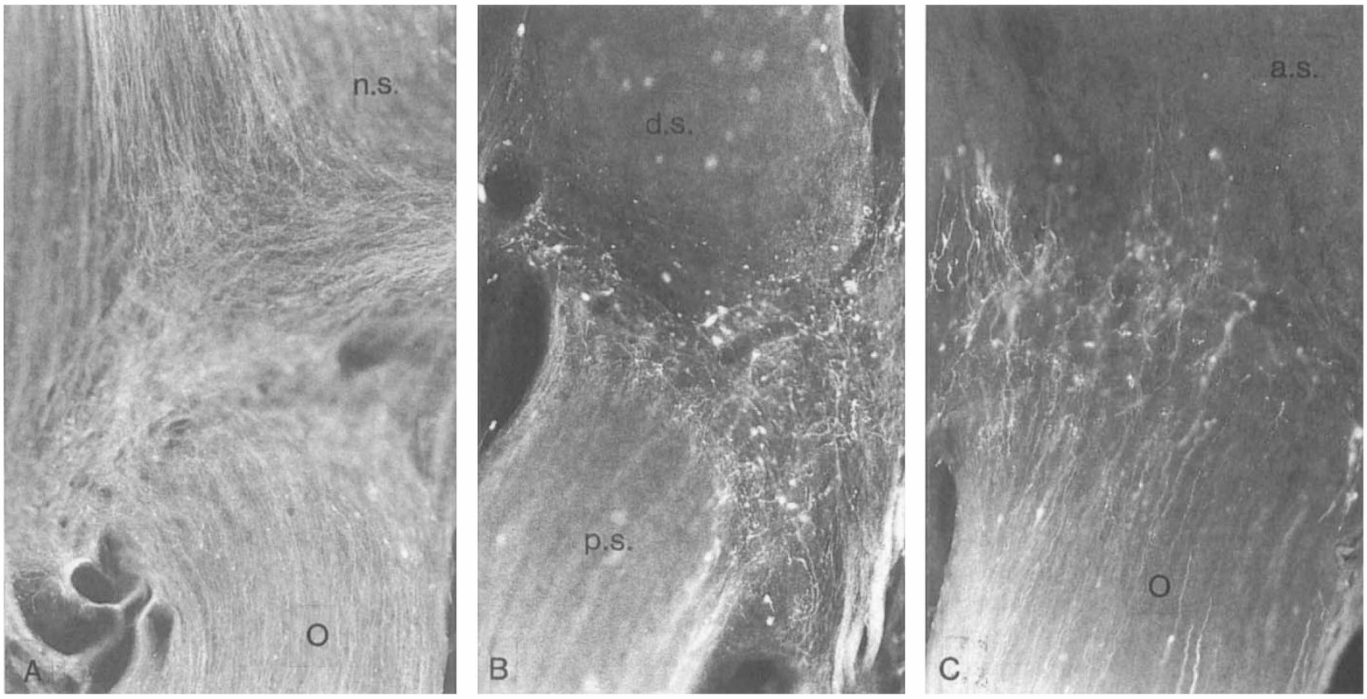

Fig. 1. *(A) Anastomotic site between optic nerve (O) and normal sciatic nerve (n.s.) showing rhodamine isothiocyanate-B (RITC-B) anterograde labelling of retinal glial cell (RGC) axons passing into the peripheral nerve (PN) segment from the optic nerve. (B) Anastomotic site between the cut ends of the optic nerve (d.s., distal segment; p.s., proximal segment) showing RITC-B anterograde labelling of RGC axons. No axons pass from the proximal segment of the optic into the distal segment, but fibres do course freely within the connective tissue scar and the dural sheath of the optic nerve. (C) Anastomotic site between optic nerve (O) and acellular sciatic nerve (a.s.) showing RITC-B anterograde labelling of RGC axons. Note that the axons cross the scar tissue but only small numbers ramify for short distances within the acellular PN segments. All labelled fibres are of RGC origin. Apparent lateral entry of fibres into the PN in (A) on the distal side of the scar is caused by fibre clustering in the basal lamina tubes in the proximal part of the PN before entry into the less disorganised distal part of the PN. Thirty days post-injury; magnification: (A) and (B) ×110; (C) ×220. (Reproduced with permission from Berry* et al.[8]*)*

CELLS EXPRESSING CONTACT INHIBITORY LIGANDS

The cells that mediate the contact inhibition which might prevent regeneration in the CNS are thought to be the macroglia, but the relative contribution of each is unknown. A major inhibitory role[28–33] has been attributed to oligodendrocytes, which are thought to express unique molecules on their surface and in the myelin sheaths. Two proteins of 35 kDa and 250 kDa have been isolated and partially purified from CNS myelin which inhibit axon growth[34] by inducing growth cone collapse.[35] When a hybridoma producing monoclonal antibodies against the 35 kDa protein is implanted into the subarachnoid space of experimental animals with partial spinal lesions, high titres of the antibody appear in the cerebrospinal fluid, and some regeneration appears to be promoted in the cord.[36,37] It is assumed that neutralising antibody penetrates the CNS parenchyma and reacts with the oligodendrocyte proteins, thereby blocking axonal receptor engagement. Regrowth is as incomplete in grey as it is in white matter, and it is therefore unlikely that inhibition by contact with oligodendrocytes/CNS myelin provides a complete explanation for the failure of CNS regeneration.

In vitro, neonatal CNS neurons are unable to grow neurites over cryosections of both pre- and post-myelination optic nerves.[38] Furthermore, *in vivo* experiments have shown that adult rat CNS fibres will not regenerate into embryonic/neonatal rat optic nerve grafts in which both oligodendrocytes and CNS myelin are absent.[39] Replication of these experiments in the periphery has confirmed the findings of Giftochristos and David[40] that regenerating peripheral axons do not penetrate either myelin/oligodendrocyte-free adult[41] or fetal/neonatal[39] optic nerve grafts unless Schwann cells co-migrate with the growing axons. Perhaps a more direct test for the hypothesis that oligodendrocyte/CNS myelin inhibits regeneration is provided by studying the injury response of axons in unmyelinated CNS *in vivo*. Although some workers have reported axonal sprouting proximal to a lesion in hypomyelinated CNS,[42,43] regeneration is not seen after complete transections of the Browman/Wyse (BW) rat mutant optic nerve[15] in which both oligodendrocytes and CNS myelin are entirely absent[44,45] (Fig. 2). Thus, if the explanation for non-growth of axons into myelin/oligodendrocyte-free mammalian CNS is inhibition, astrocytes must be active in this process, at least as early as the late fetal stage of development.

Although a correlation is lacking between regenerative failure and the onset of myelination in the developing mammalian CNS, in the chick there is a strong relationship between myelinogenesis and functional and anatomical restitution of the lesioned spinal cord.[46–49] Myelination of fibre tracts in the avian spinal cord begins on embryonic day (E) 13. Transection at the thoracic level

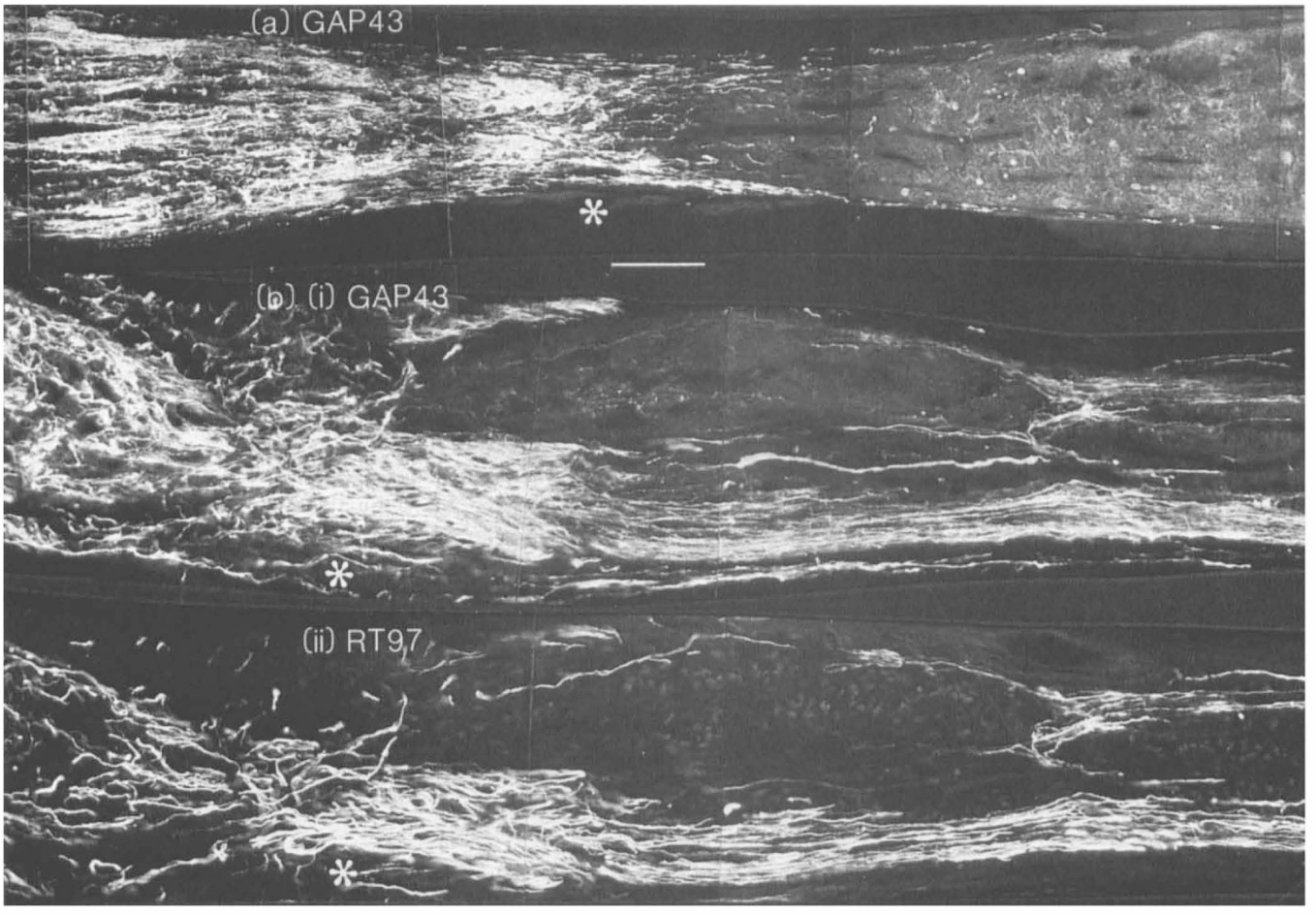

Fig. 2. *(A) Growth-associated protein 43 (GAP43) staining of axons at the crush site in a BW optic nerve in which oligodendrocytes are absent, and (B) GAP43 positive processes (i) and RT 97 (neurofilament) positive regenerated axons (ii) in the same section of a BW optic nerve in which Schwann cells are resident. An asterisk marks the centre of the lesion. The faintly stained cells seen in the unmyelinated segment of the BW optic nerve, distal to the crush site, are probably oligodendrocyte precursors. Note the co-localisation of GAP43 and neurofilament protein in many of the axons in (B) (i) and (ii). Regenerating fibres are largely confined to parellel arrays in one sector of the nerve containing similarly orientated Schwann cell basal lamina tubes; large areas of the unmyelinated proximal segment not occupied by Schwann cells are free of axons. Schwann cell basal lamina tubes were laminin-positive in an adjacent section. Scale bar represents 0.08 mm. (Reproduced with permission from Berry* et al.[15]*)*

before E13 is associated with complete anatomical repair and functional recovery, but both diminish over the period E13–E14, and are unachievable beyond E15. Delaying the onset of myelination by complement-mediated killing of oligodendrocytes with a mouse galactocerebroside monoclonal antibody extends the repair/recovery period at least until E15,[48] when double retrograde tract-tracing unequivocally demonstrates regeneration of axotomised cord axons.[46]

Reactive astrocytes in a CNS lesion have long been implicated in the failure of CNS axons to regenerate.[50–54] As already mentioned, it is unlikely that the astrocytes in the scar act as a physical barrier to growing axons but they could provide a 'physiological stop signal' for axonal growth,[55] possibly mediated by the anti-adhesive properties of either secreted or cell surface membrane bound molecules such as sulphated proteoglycans[56,57] or tenascin.[58]

Tenascin exhibits paradoxical enhancing and inhibitory effects on neurite outgrowth which have been mapped to different domains of the molecule.[59] Tenascin is present in very low titres in normal adult brain but is markedly upregulated in injured adult mouse cerebellum and cerebrum[60] but not in mouse optic nerve.[61] By contrast, in regenerating peripheral nerve tenascin expression is also enhanced.[62] An extracellular matrix protein J1-160/180 with about 40% sequence homology with mouse tenascin is secreted by oligodendrocytes and also has growth cone substrate anti-adhesive properties.[30,63,64] Chondroitin-6-sulphate proteoglycan inhibits neurite outgrowth *in vitro*[65] and its expression is upregulated in astrocytic scar tissue *in vivo*.[66] The ability of astrocytes to support neurite growth *in vitro*[67] and *in vivo*[68,69] is age related and could be correlated with the synthesis of cell surface inhibitory proteoglycans,[67] or a progressive downregulation in the production of neurotrophic/tropic molecules.[70]

AXONS EXPRESSING THE RECEPTOR FOR THE INHIBITOR LIGAND

The results of transplantation studies have provided evidence that the receptor for putative glial inhibitory ligands may not be expressed on axons early in development

before target engagement. For example, transplanted post-mitotic neuroblasts exhibit a florid regenerative response equally well in adult CNS white and grey matter,[71,72] despite the evidence for the presence of uncompromising growth inhibitory molecules in both sites (Fig. 3). This remarkable regenerative capacity of transplanted neuroblasts might be explained if all axons growing *de novo* do not elaborate the receptors for the putative inhibitory molecules. Thus, during normal development the presumed absence of receptor expression in post-mitotic neuroblasts would allow pioneering axons to find their way into targets even if the ligand is elaborated by immature astrocytes. Moreover, it is possible that a signalling molecule secreted by the target, and taken up by the 'homed' axon terminals, is retrogradely transported to the perikarya to initiate receptor protein production. It is possible that the inhibitor ligand is present from an early age in astroglia (see above) and, if so, both growth *de novo* and regeneration of axons are a function of receptor expression.

GROWTH INHIBITION ASSAYS

Several tissue culture techniques have become available recently to detect axonal growth inhibition including cryoculture and growth cone collapse assays. The latter technique is also a powerful tool in the investigation of the second messenger systems that subserve receptor/ligand interaction leading to the disassembly of cytoskeletal elements that presumably underlies growth arrest.[19,73]

The Cryoculture Technique

The cryoculture method measures the neurite outgrowth response of isolated neurons seeded onto cryosections of tissue (Fig. 4). Cryoculture was first introduced by Carbonetto *et al*,[74] Sandrock and Matthew[75] and Covault *et al.*[76] in 1987. Since then, the technique has been used widely in axon growth studies, the results of which are summarised in Table I. The technique allows controlled experiments to be designed investigating the fundamental mechanisms underlying the inhibition of axon growth. Thus, the overall growth response of test neurons and the behaviour of growth cones on substrates can be directly observed and quantified. Moreover, the growth inhibitory properties of the substrate may also be investigated by, for example, enzymatic or neutralising antibody methods.

Table I. Results with cryoculture technique

Reference	Test neurons	Growth/adhesive response	Substratum
Sandrock and Matthew[75]	Neonatal rat superior cervical ganglion explants	Regeneration on: No regeneration on:	adult rat intact sciatic nerve adult rat CNS tissue
Carbonetto *et al.*[74]	Embryonic chick DRG explants or dissociated DRG neurons	No regeneration on: Regeneration on:	adult rat optic nerve and spinal cord adult rat sciatic nerve, goldfish optic nerve, embryonic rat spinal cord, lesioned rat sciatic nerve
Savio and Schwab[79]	Neuroblastoma cells, sympathetic and neonatal rat DRG neurons	Poor regeneration and cell adhesion on: Regeneration and cell adhesion on:	white matter of adult rat brain, spinal cord and optic nerve grey matter of adult rat brain and spinal cord, optic nerve, sciatic nerve, mature trout CNS, and demyelinated rat spinal cord
Crutcher[78]	Embryonic chick sympathetic ganglion explants	Poor regeneration and cell adhesion on: Regeneration and cell adhesion on:	white matter of adult rat brain and spinal cord grey matter of adult rat brain and spinal cord
Watanabe and Murakami[80]	Dissociated embryonic chick neocortical cells	Cells adhere to grey matter on: Extensive cell adhesion on: Cell adhesion on white matter near lesion site on:	adult rat brain developing rat brain lesioned rat brain
	Embryonic chick DRG explants	Extensive cell adhesion on: Regeneration preferentially on grey matter of:	adult frog brain adult spinal cord
David *et al.*[81]	Embryonic chick DRG explants	Regeneration on or near lesioned site on: No regeneration on:	lesioned adult rat optic nerve normal adult rat optic nerve
Bedi *et al.*[77]	Adult rat DRG	No regeneration on: Regeneration on:	adult intact rat sciatic nerve adult lesioned rat sciatic nerve
	Embryonic rat DRG (E16–E20)	Regeneration on:	both intact and lesioned rat sciatic nerve
Shewan *et al.*[38]	Perinatal rat DRG (E18–P3)	Regeneration but poor cell adhesion on: Regeneration and cell adhesion on: No regeneration and poor cell adhesion on:	adult intact rat sciatic nerve adult lesioned rat sciatic nerve both adult and perinatal (E18–P1) rat optic nerve
	Adult rat DRG	No regeneration and poor adhesion on: Regeneration but poor adhesion on:	adult intact rat sciatic nerve, and both adult and perinatal optic nerve adult lesioned rat sciatic nerve
	Neonatal rat retinal ganglion cells	No regeneration and poor adhesion on: No regeneration but cell adhesion on:	adult intact rat sciatic nerve, both adult and perinatal optic nerve adult lesioned rat sciatic nerve

DRG, dorsal root ganglia.

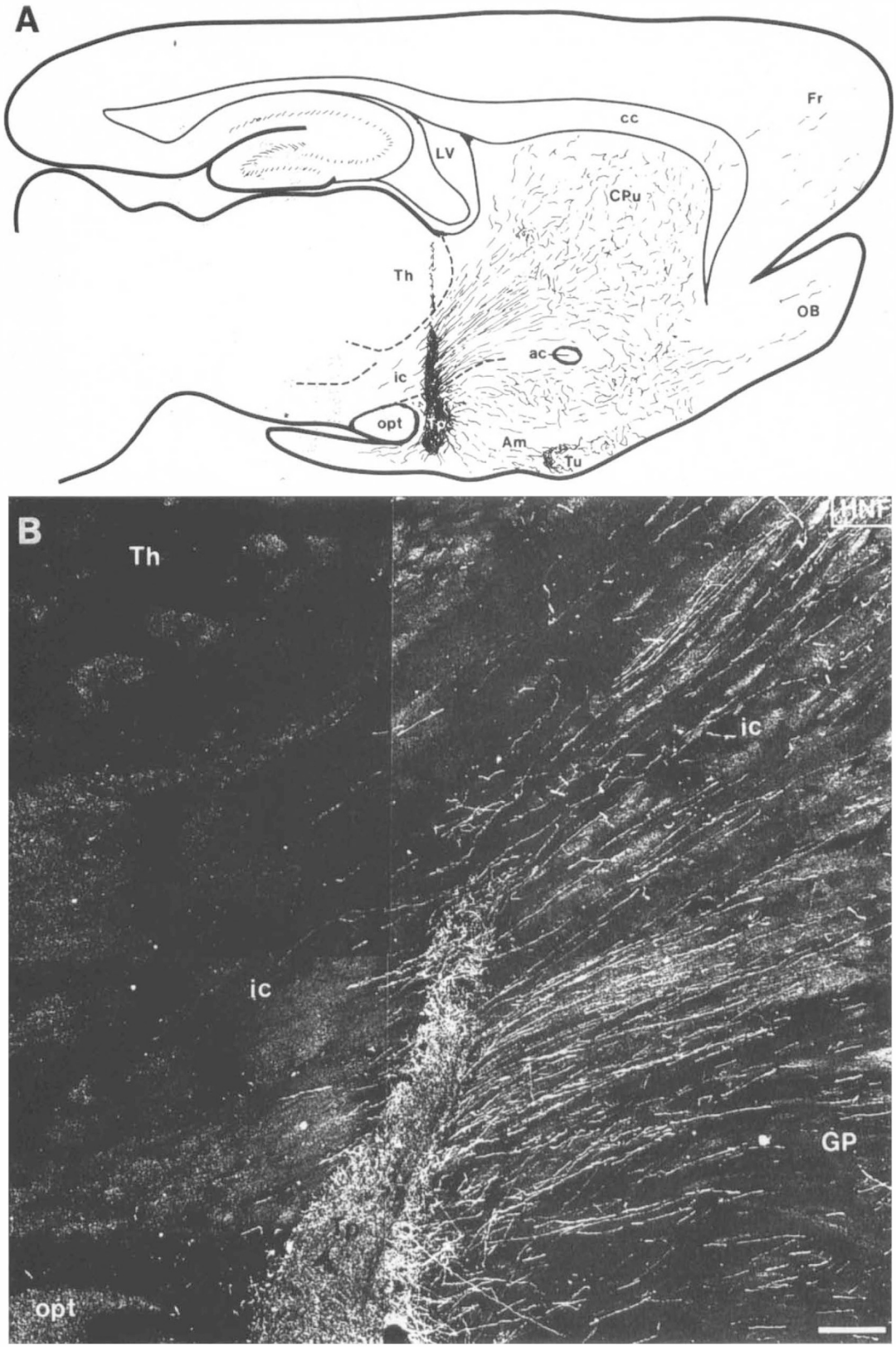

Fig. 3. *(A) Semi-schematic camera lucida drawing of a sagittal section from one of the human ventral mesencephalic (VM) tissue transplants placed in the internal capsule (ic), illustrating the highly polarised projection of graft-derived human neurofilament (HNF)-positive fibres rostrally along the internal capsule into the caudate putamen (CPu), the amygdala (Am) and the ventral striatum. ic, internal capsule; ac, anterior commissure; cc, corpus callosum; Th, thalamus; opt, optic tract; Fr, frontal cortex; OB, olfactory bulb; LV, lateral ventricle, Tu, olfactory tubercle. (B) Dark-field micrographs of a human VM transplant placed in the internal capsule (ic) stained for HNF. Note the marked polarity of the graft-derived fibre growth rostrally within the ic. Am, amygdala; GP, globus pallidus; opt, optic tract; Th, thalamus. Scale bar represents 0.1 mm. (Reproduced with permission from Wictorin* et al.[72]*)*

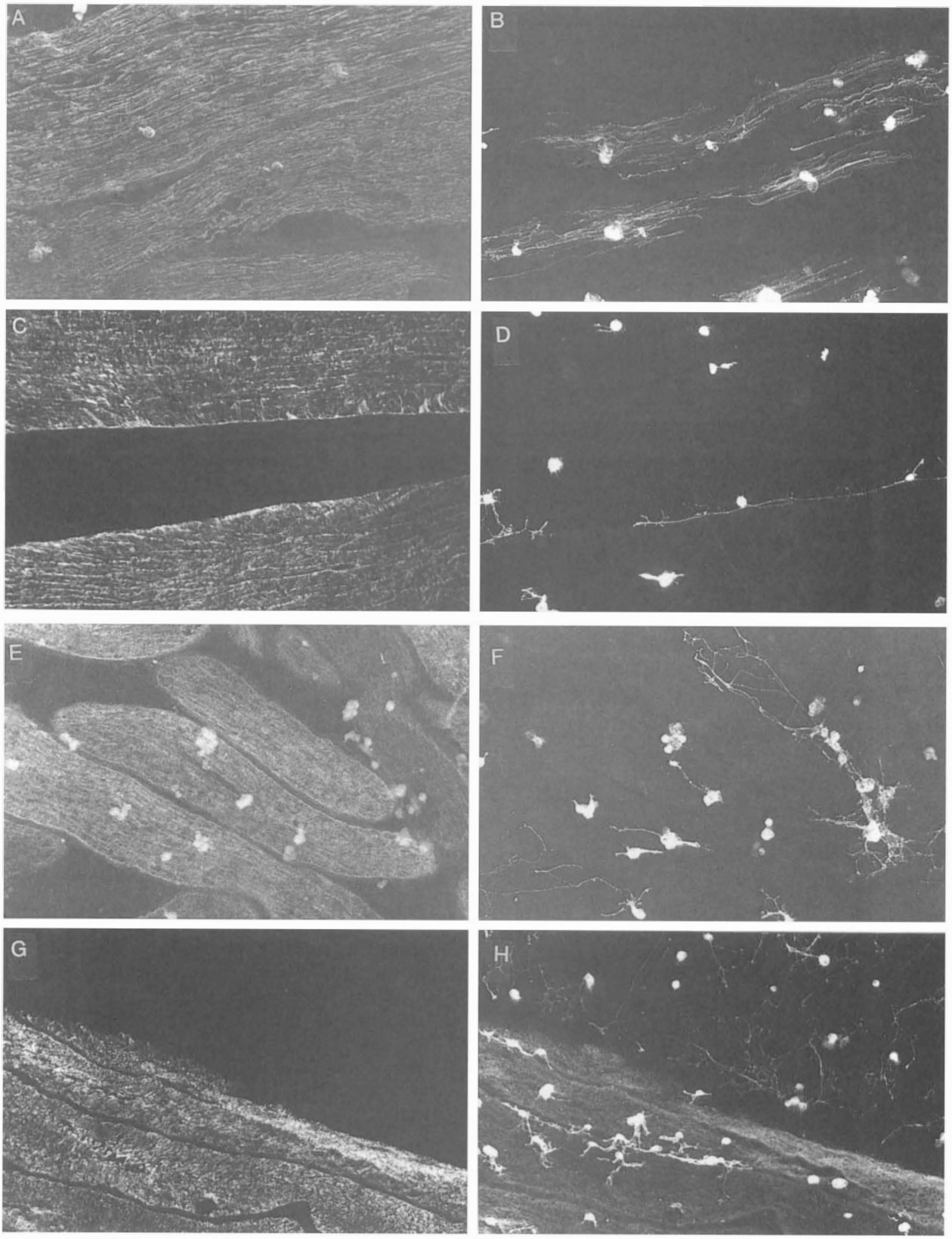

Fig. 4. *Growth of adult (A–D, G, H) and neonatal (E, F) dorsal root ganglion (DRG) on cryosections of sciatic nerve 10 days post-lesion (A, B) and on adult (C), E20 (E) and P1 (G) optic nerves. Fluorescence photographs of growth-associated protein (GAP43) (B, D, F, H) stained neurites, glial fibrillar acidic protein (GFAP) (C, E, G) and nerve growth factor receptor (NGFR) (A) immunostained cryosections. Adult and neonatal DRG (not shown) extend neurites only on 10-day lesioned sciatic nerve (A, B). Adult and neonatal DRG do not grow on cryosections of either adult optic nerve (C, D), E 20 optic nerve (E, F), or P1 optic nerve (G, H) but readily grow on the surrounding polylysine-coated glass. No putative myelin-associated inhibitory molecules are present in E20 and P1 optic nerves (×10).*

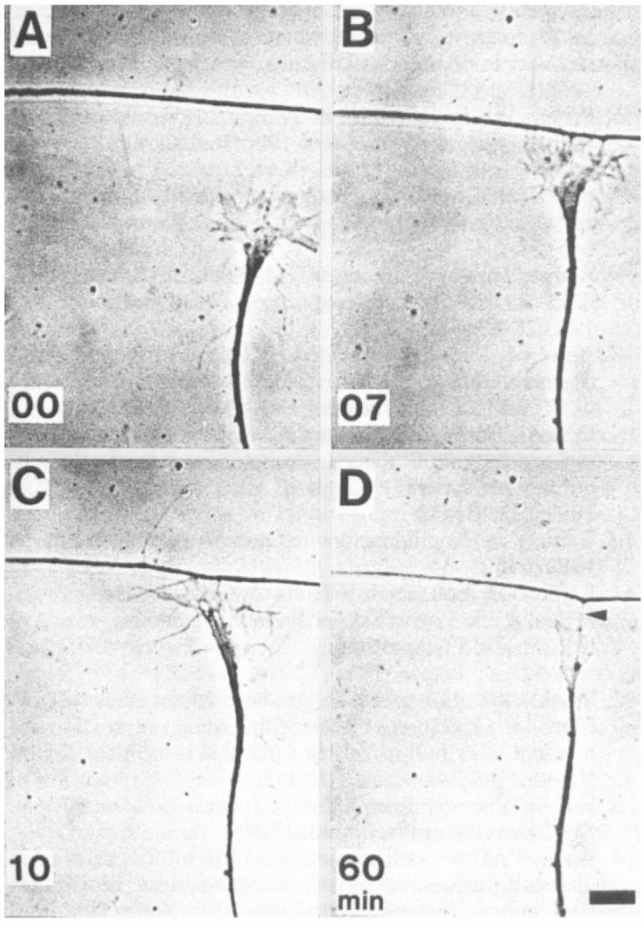

Fig. 5. *Retinal growth cone collapse on meeting a sympathetic neurite. In this example, retraction and collapse is incomplete, and the growth cone never recovers normal motility. Time in minutes is indicated at the lower left of each frame. Calibration bar represents 10 μm. (Reproduced with permission from Kapfhammer and Raper.[84])*

Interpretation of the results of such experiments does, however, require caution. For example, since growth inhibition is mediated by receptor/ligand interaction, the method tests not only the potency of the cryosection substrate to inhibit neurite outgrowth of the test neurons but also the receptor expression of the neurites. The differential growth response of neurites over cryosections seen is related to age and source of test neurons in the assay – two properties which probably correlate with receptor expression. For example, neonatal rat DRG neurites grow over cryosections of unlesioned adult sciatic nerve[38,74,77,79] but the neurites of adult rat DRG do not,[38,77] and neonatal rat retinal ganglion cells (RGC) neurites fail to grow over normal and lesioned adult rat sciatic nerve.[38] Adhesivity of the cultured test cells to the substrate is, in most cases, positively correlated with the regenerative response, but for neonatal RGC, despite good attachment to the surface of cryosections of lesioned rat sciatic nerve, no neurite extension occurs.[38] During normal development inhibitory ligands appear to be expressed in the rat CNS at least by E20, since the neurites of neither adult nor neonatal neurons will grow over cryosections of E20 optic nerve (Fig. 4).[38] Evidence from cryoculture experiments also suggests that receptors for inhibitory ligands are expressed on growth cones at least by birth in the rat, since the neurites of 1-day-old RGC and DRG are unable to grow over either neonatal unmyelinated or adult myelinated CNS neuropil.[38] On the other hand, Ard *et al.*[82] have shown that E15 rat RGC will extend neurites in culture among oligodendrocytes synthesising myelin basic protein.

In order for the results of cryosection experiments to be relevant to CNS regeneration, mature rather than immature neurons should form the test system and the growth characteristics of their neurites carefully documented on a wide range of substrates and correlated with *in vivo* behaviour.

Growth Cone Collapse

The growth of axons is defined as the forward extension of the tip of the neurite. The latter is expanded into a specialised structure called a growth cone, the integrity of which is essential for axon extension. From the cone emanate filopodia and lamellipodia which sample the immediate local microenvironment. The cone contains the organelles and metabolic machinery for activities such as membrane expansion, cytoskeletal actin and tubulin assembly and surface membrane adhesion, all of which contribute to axonal elongation.[83]

Growth cone collapse (Fig. 5) occurs in response to particular environmental signals and is defined as a dramatic change in both shape (from a spread to a collapsed state) and behaviour (with the temporary cessation of motility).[84,85] Collapse is probably associated with actin and tubulin depolymerisation.[73] The phenomenon can be observed *in vitro* and quantified to assay the potency of growth inhibitory molecules and substrates.[86] Multiple glycoproteins, including myelin derived proteins[33,87] and peanut lectin binding proteins,[88,89] cause growth cone collapse. *In vivo*, growth cone collapsing molecules may act transiently in development as pathway boundary defining molecules (e.g. preventing the invasion of (1) spinal nerves into the caudal half of each somite[90] and (2) temporal retinal fibres into the posterior tectum[91]) and in discouraging interfasciculation of heterogenous groups of axons (e.g. peripheral nerve axons with CNS axons.[84,92] Permanent growth cone collapse, which presumably accounts for the failure of axonal regeneration in the CNS, has not been observed *in vivo* but might occur when growth cones enter an environment where the inhibitory substrate is ubiquitous. There is, however, evidence that growth cones can adapt to prolonged exposure and become desensitised to growth cone collapsing molecules.[93,94] Neurite growth cone collapse is induced *in vitro* on contact with both oligodendrocytes[28–33] and liposomes coated with CNS myelin proteins, but blocked in the presence of neutralising anti-CNS myelin protein antibodies.[36,37,87]

LIGAND–RECEPTOR INTERACTIONS

Growth cone collapse assays have demonstrated that

ligand–transmembrane receptor interactions in some cases activate second messenger transduction systems within the growth cones by the release of Ca^{2+} from intracellular stores after activation of G protein linked receptors.[95–97] The large increases in levels of growth cone Ca^{2+} concentrations which occur in the presence of growth inhibitory myelin proteins are abolished after treatment with neutralising antibodies.[35] Collapse is also prevented by treatment with blockers of Ca^{2+} released from intracellular stores[33] and enhanced by G protein binding agonists.[97] However, some ligand–receptor interactions effect growth cone collapse without mobilising intracellular Ca^{2+}.[98]

The best-characterised growth cone collapsing molecule is collapsin, a 100 kDa non-membrane-spanning secreted glycoprotein.[99] Originally isolated from chick embryo brain, its recent cloning and sequencing reveal sequence homology with both fasciclin IV, an insect growth cone guidance molecule,[100] and a single C2-type immunoglobulin-like domain. It is expressed at high levels also in adult brain, and in muscle and lung tissue.

CONCLUSIONS

Permanent inhibition of axon growth could account for the abortive regeneration that occurs after CNS injury. This growth arrest could result from either collapse of the growth cone or its detachment from the substrate. A variety of glial-derived ligands have been identified which are thought to mediate contact inhibition through receptors on the surface of axon growth cones linked to an intracellular second messenger system. For example, avian and mammalian oligodendrocytes and CNS myelin express surface membrane bound growth cone collapsing proteins which inhibit axon growth on contact, and mammalian astrocytes appear to elaborate growth inhibitory anti-adhesive molecules. Receptors for the ligands mediating growth cone collapse and anti-adhesive activity have not been identified, but their expression on axons appear to be developmentally regulated.

Understanding more about receptor expression and the receptor–ligand interactions leading to arrest of axon growth, together with the characterisation of the molecules responsible, could suggest ways of preventing inhibition and thereby provide clues as to whether regeneration is possible after injury of the adult CNS.

Key words: Axon growth inhibition, Growth cone collapse, Growth inhibition assays, Growth inhibitory molecules, Ligand/receptor interaction.

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