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REVIEW

Factors secreted by Schwann cells stimulate the regeneration of neonatal retinal ganglion cells

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Abstract

The adult mammalian central nervous system (CNS) does not repair after injury. However, we and others have shown in earlier work that the neonatal CNS is capable of repair and importantly of allowing regenerating axons to re-navigate through the same pathways as they did during development. This phase of neonatal repair is restricted by the fragility of neurons after injury and a lack of trophic factors that enable their survival. Our aim is to define better the factors that sustain neurons after injury and allow regeneration to occur. We describe some of our work using Schwann cells to promote the regeneration of neurons from young postnatal rodents. We have established rapid methods for purifying Schwann cells without the use of either anti-mitotic agents to suppress contaminating fibroblasts or mitotic stimulation to generate large numbers of Schwann cells. The rapidly purified Schwann cells have been used to generate conditioned medium that we have shown stimulates axon regeneration in cultured retinal ganglion cell neurons. We also show that the positive effects of Schwann cells are still present after pharmacological blockade of the neurotrophin receptors, suggesting that novel factors mediate these effects.
Key words glial cell; neurotrophin; peripheral nerve; regeneration.

The failure of the adult CNS to support regeneration

The adult mammalian CNS does not support axonal regeneration. This means that damage to the CNS through disease or injury results in permanent loss of function. A major goal for neuroscience is to understand the constraints that exist in the CNS which prevent repair, and to devise strategies which allow us to overcome these constraints without damaging side-effects (Horner & Gage, 2000).

It was long thought that the adult CNS showed no signs of regenerative response after injury. However, it is now clear that those neurons that survive injury do attempt to regenerate (Richardson et al. 1980; Schwab, 2002). The regeneration of these axons is prevented by the environment of the CNS, which is inhibitory, and by the lack of positive stimulating conditions for axon growth. The glial cell-mediated inhibition is via astrocytes that form inhibitory scars (Fawcett & Asher, 1999; Logan & Berry, 2002) and oligodendrocytes that express inhibitory myelin proteins (Filbin, 2003; Woolf, 2003). One suggestion concerning the inhibitory nature of the CNS environment is that it is designed to maintain stability. The astrocytes respond to injury by sealing the damaged area from the rest of the unaffected CNS and therefore allow the maintenance of whatever function is left (Morgenstern et al. 2002; Rhodes et al. 2003). The normal function of myelin-associated inhibition may be a mechanism that is used to prevent sprouting of neurons after the period when connections have been made (Colello & Schwab, 1994; Liu et al. 2002).

It is also clear that after an injury to the CNS, uninjured neurons are able to sprout and grow new processes, which clearly demonstrates the remarkable plasticity of the CNS, even in adult life (Raineteau & Schwab, 2001; Raineteau et al. 2002). On an optimistic level, this plasticity provides hope for functional recovery, both

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through regeneration of damaged neurons and also through sprouting and re-wiring of spared or associated axon tracts. However, it also raises a major problem of control of the regenerative process, as aberrant sprouting and establishment of abnormal connections may have consequences that are worse than the loss of function associated with the original injury (Edgerton et al. 2001).

In contrast to the CNS, the peripheral nervous system (PNS) does regenerate and function can be restored after peripheral nerve injury (Fawcett & Keynes, 1990). The major factor enabling the successful regeneration of peripheral nerves is the presence of Schwann cells. Schwann cells are the myelinating glial cells of the PNS, but following peripheral nerve damage they de-differentiate and secrete survival- and growth-promoting factors (Fawcett & Keynes, 1990). It is the precise nature of these factors and their effects on CNS neurons that we have been interested in exploring. On one level of investigation, we can consider Schwann cells as transplants that could provide a stimulus for CNS regeneration. This has been explored extensively in the adult lesioned CNS, where there is considerable debate and controversy about how positive the effects of such transplants might be (Li & Raisman, 1994; Dezawa & Adachi-Usami, 2000; Iwashita et al. 2000). Secondly, if we can understand more clearly the crucial factors made by Schwann cells that promote CNS regeneration, we may be able to manipulate other cells to provide these effects. It is to this end that we have tried to define the nature of Schwann cell factors and their effects on regenerating neurons.

Regeneration in the neonatal system

Whereas the adult CNS shows little signs of repair, the neonatal CNS offers some hope for successful repair. In the neonate, the CNS is still in the developmental phase, so the conditions promoting the growth of axons and the formation of connections are working in favour of regeneration. It is our belief that if we can repair the nervous system at this stage, we may take advantage of the relative plasticity to optimize functional recovery. There are obvious advantages of trying to stimulate CNS regeneration at this early stage as the growth-promoting environment that exists in the developing nervous system may still be present during the neonatal period. Indeed in certain systems, such as the corticospinal tract where axon growth continues postnatally, the necessary substrates and guidance cues are still expressed in an appropriate fashion to be used by regenerating axons (Schreyer & Jones, 1988; Cohen et al. 1998).

Investigating CNS repair at this stage also has a logical advantage in allowing the factors known to impede regeneration in the adult CNS to be addressed independently of the repair process. In the neonate, the inhibitory environment of the adult CNS is not yet developed so this major limitation does not have to be overcome. The astrocytes are positive stimulators of axon growth, secreting permissive extracellular matrix. Although there are limited data available, it is not thought that astrocytes at this stage form the same inhibitory scars as are found in the adult system. As myelination proceeds, the effects of the neurite growth inhibitors NOGO and myelin-associated glycoprotein (MAG) can be assessed and manipulated to optimize repair (Filbin, 2003).

Furthermore, the target cells of most systems are within the major phase of developmentally associated plasticity. This period is associated with large-scale neuronal death and the refinement of nerve connections (O'Leary et al. 1986). Although it is clear that the dated view of the hard-wired nervous system has been revised, because even the adult system shows plasticity, for most systems the early postnatal phase is when the CNS is most easily re-wired.

One useful animal model in which CNS regeneration during the early postnatal period has been explored is the marsupial (Nicholls & Sanders, 1996; Maclaren & Taylor, 1997b). Marsupial CNS development is largely postnatal, so experimental interference can be made both during and shortly after the major period of axon growth to study both normal development and neonatal regeneration.

We used the short grey tailed opossum to study both retinofugal pathway development and regeneration of this pathway during the period towards the end of normal visual system development (Maclaren & Taylor, 1997a,b). Retinal ganglion cells (RGCs) were axotomized and a double labelling protocol used to detect regenerating axons. In brief, RGCs were labelled at their terminal mitosis using tritiated thymidine and these cells were then allowed to initiate their axons. We showed that the axons of these marked RGCs had reached the optic chiasm in the ventral diencephalon after 2 days. We therefore chose to lesion the retina 3 days after thymidine labelling, severing the RGC axons. Two to 3 weeks were allowed for the axons

to regenerate and the cells in the retina were then labelled retrogradely with horse radish peroxidase (HRP) from the optic tract. By lesioning the temporo-ventral retina, the ability of the ipsilaterally projecting RGCs to re-navigate a correct path through the optic chiasm could also be assessed (Maclaren & Taylor, 1995). Our results showed that retinal axons could not only regenerate but also re-navigate the visual pathways and re-innervate the correct target nuclei within the CNS (Fig. 1). However, we found that there was a developmental time window after which axons failed to regenerate. The end of this neonatal regeneration period does not correlate with myelination (Maclaren, 1996a), nor with the expression of injury associated inhibitory molecules such as Chondroitin sulphate proteoglycans (CSPGs) (Maclaren, 1996b). The loss of regenerative ability does correlate with the time of innervation of the superior colliculus. In other studies this period of innervation has been shown to correspond to the peak period of susceptibility of neurons to cell death and hence an increased susceptibility to axotomy (Fagiolini et al. 1997; Ma et al. 1998).

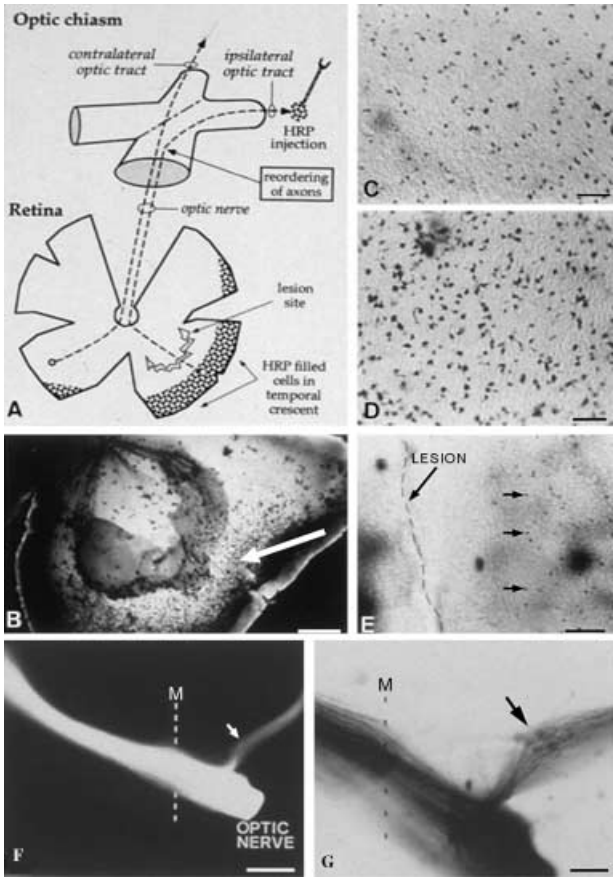

Fig. 1 Lesions of the marsupial (*Monodelphis domestica*) retina during the neonatal period result in regeneration with correct re-navigation of the visual pathways. (A) Schematic diagram showing the lesion of the temporo-ventral retina and position of HRP labelling in the ipsilateral optic tract, which was used to define the successful regeneration of RGCs with uncrossed projections. (B) The retina 10 days after lesion showing retrogradely labelled cells in the periphery behind the lesion (arrow). (C) Higher magnification view of regenerated retrogradely labelled retinal ganglion cells, which are fewer in number than those retrogradely labelled in un-lesioned control retinae (D). (E) The lesion and the regenerated RGCs. (F, G) Axons labelled with DiI (1, 1′ -dioctadecyl -3, 3, 3′, 3′ -tetramethylindocarbocyanide perchlorate) and photo-converted DiI, respectively, show the anterograde tracing of regenerating axons from behind a lesion in the temporo-ventral retina showing successful re-navigation of the optic chiasm. Scale bars in B and G, 200 μm; C and D, 25 μm; E, 50 μm; F, 500 μm.

Schwann cells as sources of regeneration stimulating factors

In the neonatal system it seemed that the major limitation on successful regeneration was not the inhibition generated by CNS glial cells or the inability of axons to grow and navigate along the correct CNS pathways, but that the cells did not survive the injury. We therefore switched our focus to trying to find factors that would allow retinal ganglion cells to both survive and then encourage the outgrowth of axons. As peripheral nervous system grafts or Schwann cells have been shown to encourage adult CNS regeneration (David & Aguayo, 1981; Li & Raisman, 1994; Cheng et al. 1996; Xu et al. 1997, 1999), including regeneration of adult retinal ganglion cells (So & Aguayo, 1985; Plant et al. 1995; Berry et al. 1996; Negishi et al. 2001), we focused on Schwann-cell-derived factors. We have isolated Schwann cells *in vitro* to examine the CNS regeneration-promoting factors that they produce. These effects have not been studied in neonatal neurons, in which the factors promoting regeneration may be different to those that are effective in the adult.

If the sciatic nerve is simply dissociated and grown in culture a mixture of Schwann cells and fibroblasts results. In such cultures the fibroblasts, with a greater mitotic index, rapidly dominate. To generate pure Schwann cells, fibroblasts must either be eliminated or suppressed. Many published methods use combinations of anti-mitotic agents to suppress early fibroblast division followed by mitogens to stimulate Schwann cell division once the fibroblasts have been eliminated (Raff et al. 1978; Brockes et al. 1979). We have used immunopanning to purify both rat and mouse

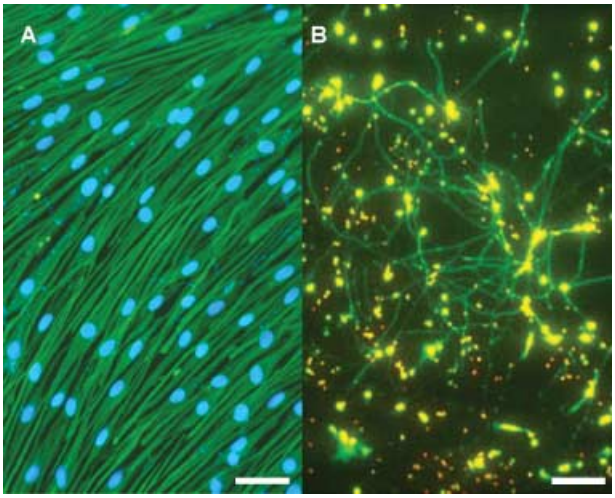

Fig. 2 Purified rat P4 Schwann cells 3 days after immunopanning stained with S100. Retinal ganglion cells isolated from P4 rat retina, stained with carboxyfluorescein diacetate, growing in the presence of Schwann cell conditioned medium. The extensive neurite outgrowth of the neurons is regenerative. Scale bars, 50 μm.

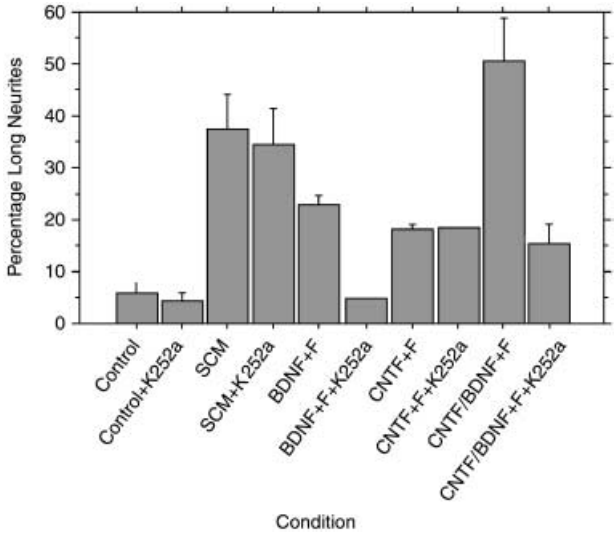

Fig. 3 Graphs showing the effects of different Schwann cell conditioned media (SCM) on RGC regeneration. Positive control medium containing BDNF or a combination of CNTF/BDNF and forskolin have slightly better neuritogenic effects than SCM. The effects of BDNF, but not those of CNTF (which does not act through a Trk receptor), are lost when the kinase inhibitor K252a is added. By contrast, SCM shows minimal loss of function when K252a is added. This suggests that the SCM positive regeneration-promoting factors are not acting through neurotrophin receptors.

Schwann cells taken from the sciatic nerves of neonatal animals (Barres et al. 1988; Bampton & Taylor, 2001). This technique allows pure Schwann cells to be isolated rapidly (Fig. 2A) without the use of mitotic inhibitors or stimulators commonly used in previous studies. These agents may affect the properties of the Schwann cells, particularly by changing the factors that they secrete (Yamamoto et al. 1993; Bermingham et al. 2001).

We used medium conditioned by these immunopurified Schwann cells to examine the effects on postnatal retinal ganglion cell survival and regeneration. We again used immunopanning (Barres et al. 1988) to isolate pure retinal ganglion cells. We have shown that Schwann cell conditioned medium has clear survival and neuritogenic properties (Fig. 2B). Because the axons of these RGCs would have been innervating their target cells at the time of their isolation, the promotion of neurite growth in these neurons is a clear measure of regeneration. The neuritogenic effects of Schwann cell conditioned medium were as good as a positive control medium containing a combination of brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and forskolin (Fig. 3). We also examined whether two Schwann cell lines, SCTM41 and PVGSV40T, had similar regeneration-promoting properties. We showed neither cell line has any significant effect in stimulating retinal ganglion cell regeneration at these stages.

An important question is what do Schwann cells make that underlies these effects? We know that Schwann cells produce a range of factors including neurotrophins (Funakoshi et al. 1993; Cai et al. 1999; Meier et al. 1999), but it is likely that, in addition, other factors are involved. For example, blocking neurotrophin effects by inhibiting tyrosine kinases (which include the major neurotrophin receptors of the Trk family), does not not significantly diminish the effects of Schwann cell medium on neuritogenesis. To identify these other factors we have used a radiolabelling assay, devised by Aviva Tolkovsky, in which the Schwann-cell-secreted proteins are labelled and fed to target cells (Fig. 4A; Bampton et al. 2003). By blocking protein synthesis in the target cells, any radioactive proteins must have been taken up from the conditioned medium. This assay revealed that only a very few proteins were taken up from the Schwann cell conditioned medium by the regenerating retinal ganglion cells (Fig. 4B; Bampton et al. 2003). Our efforts are now directed to isolating and purifying these proteins to test on purified RGC cultures both alone and in combination with other neurotrophic factors to try to stimulate optimal regeneration.

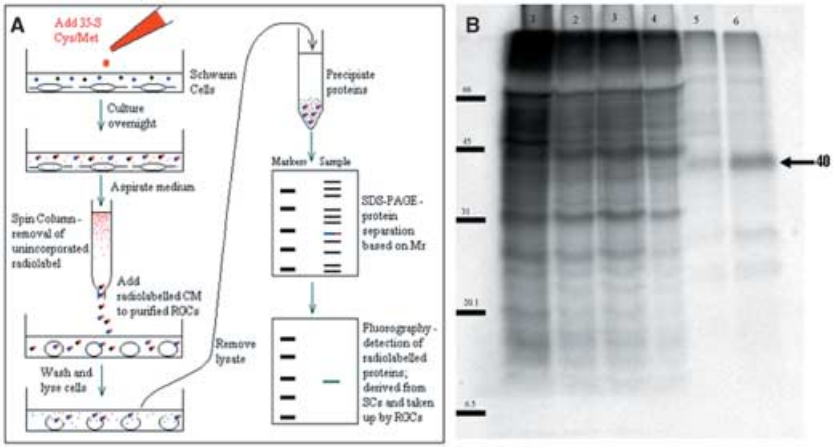

Fig. 4 (A,B) A method used to radiolabel proteins made by Schwann cells, which are then fed to RGCs. The Schwann-cell-derived factors are taken up by RGCs and then isolated by fluorography. (B) Radiolabelled proteins in Schwann cell conditioned medium (lane 1) and after passing through a spin column to remove unbound radiolabel (lane 2). Lanes 3 and 4 show the residual radiolabelled SCM after incubation with retinal ganglion cells (different preparations). Lanes 5 and 6 show two different exposures of RGC lysates after 24 h incubation, showing a number of radiolabelled bands, including a clear band at 40 kDa (indicated by arrow), which has been taken up by RGCs from the Schwann cell conditioned medium.

One criticism of this work is that RGCs never encounter Schwann cells, so factors that enhance survival and regrowth may simply be a part of a more generalized response, but one that could only be used therapeutically if Schwann cells were transplanted to the retina. For obvious reasons, using the natural glia of the retina, the Müller glia, to induce positive stimulation of regeneration would be advantageous. We can purify Müller glial cells and have shown that these again produce factors that stimulate sympathetic neuronal survival and outgrowth (Reis et al. 2002). The Müller glial cell conditioned medium has also been shown to promote retinal ganglion cell survival and regeneration, to a level that is better than the defined neurotrophin-containing medium (Reis et al. 2003).

In summary, we think that the neonatal visual system offers a very useful model for studies of CNS repair. First, the factors acting against regeneration are fewer in the neonatal CNS and the major obstacle is maintaining cells after axotomy. To prevent neuronal cell death after axotomy, neurotrophins can be used (Lui et al. 1998), but we have also found that other factors may be used as alternatives, or in combination with neurotrophins. Experiments using Schwann cells, and current work on Müller glial cells, show that there are novel regeneration-promoting factors that are taken up by axotomized retinal ganglion cells. Using the neonatal visual system, in which information on the normal pathfinding cues that are used by developing axons is perhaps best described (Mason & Sretavan, 1997), we can analyse the continued or re-expression of these cues during regeneration. We have shown that within this early postnatal phase the regenerating axons do have the ability to re-navigate that provides hope that long-distance projection neurons may be stimulated to restore functional connections.

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