CHAPTER 2

The Glial Response to Injury and Its Role in the Inhibition of CNS Repair

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The failure of axon regeneration after CNS injury is due to an inadequate or inappropriate regenerative response from damaged CNS axons and to a CNS environment that inhibits regeneration. This inhibitory environment contains many molecules that promote axon growth as well as molecules that inhibit it, but the balance of activities in the damaged CNS does not favour the regeneration of adult CNS axons. In principle, therefore, axon regeneration could be achieved in three ways: (1) Inhibitory molecules might be destroyed or blocked, (2) the amount of permissive molecules might be increased, or new permissive molecules introduced, (3) Axons might be altered so that they can grow in the inhibitory CNS environment. Some success has been achieved with all three of these approaches. This review addresses the inhibitory properties of the glial scar, a structure which forms wherever the CNS is damaged, and which is one source of axon growth inhibitory molecules in CNS injuries.

What Is the Glial Scar?

The end stage of glial scar formation, which is seen weeks after injury, is a largely astrocytic structure. The astrocytes are hypertrophic, with many tightly interweaving processes, many of them joined with junctional complexes.[1] The appearance is similar in many ways to the astrocytic glia limitans which lies around the surface of the CNS under the meninges. However the scar evolves over the weeks following injury, and other cell types play critical roles. Wherever the CNS is damaged a scarring process is initiated, which involves microglia, oligodendrocyte precursors, astrocytes, meningeal cells and vascular endothelial cells. The first change seen after injury is the appearance at the injury site of blood-borne cells, particularly monocyte/macrophages.[2] Within a few hours the endogenous macrophage lineage cells of the CNS, the microglia, begin to respond. They hypertrophy, begin to divide, and upregulate many molecules including complement receptors, which are the antigens often used to identify the cells and their state of reactivity.[3] Starting at about 24 hours oligodendrocyte precursors (OPCs) become reactive, with a burst of cell division; they hypertrophy and increase in cell surface levels of the chondroitin sulphate proteoglycan (CSPG) NG2 which is the most useful marker of these cells.[4,5] Astrocytes show reactivity after one to two days. They hypertrophy with an increase in size and and length and number of processes, a proportion of the cells divide, and they greatly upregulate the intermediate filament proteins GFAP, vimentin and nestin.[6-8] The GFAP upregulation is widespread, the vimentin and nestin upregulation much more restricted to the site of the lesion. Most lesions are invaded by meningeal cells, either from the meningeal

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layer surrounding the CNS, or from the meningeal-like cells that surround major blood vessels. Meningeal cell invasion is a particularly marked feature of spinal cord injuries, where there is often a large plug of these cells filling the lesion cavity.[1] Around CNS injuries capillaries become hypertrophic, with a considerable increase in the amounts of laminin and other matrix molecules, and endothelial cells can be seen invading lesions and forming part of the plug of cells that may fill them. Over time, most of the reactive microglia and oligdendrocyte precursors disappear, leaving behind the reactive astrocytes surrounding the injury and the meningeal cells and vascular endothelial cells filling it.

Control of Glial Scar Formation

The initiation and control of scar formation is an extremely complex subject that needs a review to itself. A recent review summarises current knowledge in this area.[3] The control of scar formation has been most extensively studied in the type of reactive gliosis that occurs when a peripheral nerve is injured. Many of the studies have been performed in the facial nerve nucleus, where there is recruitment and activation of microglia and reactive astrocytosis after nerve crush. However oligodendrocyte precursors are not activated in these lesions. The control of glial reactivity involves a complex interplay between microglia, neurones and astrocytes with signals probably passing in both directions between these three cell types. The molecules involved include IL-6, TGFbeta, FGF-2, MCSF and other cytokines.

The Glial Scar and Axon Regeneration

Numerous in vivo experiments have shown that the glial scar inhibits axon regeneration. There is obviously a correlation between the environment in which axon regeneration fails and scar formation, since a scar will form where ever axons are cut. Even very small lesions, which are insufficient to excite an visible disruption of glial architecture can cause changes in the CNS environment sufficient to block axon regrowth.[9] In order to show more than a correlation between glia scarring and inhibition of axon growth various transplant experiments have been performed. CNS tissue, even immature CNS tissue containing largely astrocytes and few oligodendrocytes blocks axon regeneration when it is transplanted to peripheral nerves, and tissue removed from scarred areas is very inhibitory.[10-13] Until recently the question of whether all CNS tissue is equally inhibitory to axon regeneration, or whether scar tissue is particularly inhibitory was not resolved. Two experiments from Davies and Silver show convincingly that the glial scar is much more inhibitory than the rest of the CNS tissue. The first experiment was to transplant adult sensory neurones into the corpus callosum of adult rats, using an atraumatic microtransplantation technique that does not usually initiate scar formation. In most animals there was extensive regeneration of axons from the transplanted neurones through the corpus callosum. However in a few cases regeneration failed, and it was in these cases that the transplant had been more traumatic, and had excited reactive gliosis around the transplant.[14] In the second experiment adult sensory neurones were transplanted into the spinal cord and a distance from a spinal cord injury. Again axons regenerated from the transplanted neurones through the spinal cord white matter, but growth was blocked when the axons approached the scar tissue surrounding the injury site.[15] The general conclusion is that all CNS tissue is inhospitable to axon regeneration, but glial scar tissue is much more inhibitory than undamaged tissue.

Inhibitory Glial Boundaries

In addition to the inhibition of axon regeneration by normal and damaged CNS tissue, there are situations where axon growth stops at places where the glial environment changes sharply from one type of glial cell to another; these are called glial boundaries. The most studied of these boundaries is that found between peripheral nerve tissue and CNS tissue, where

the Schwann cell environment of peripheral nerve changes to the astrocytic environment of the CNS, as is seen at the dorsal root entry zone (DREZ). Axons will regenerate within the dorsal root following a crush, particularly if the regenerative response is increased by a concomitant crush of the peripheral nerve attached to the same dorsal root ganglia. However the axons stop precisely at the DREZ, being unable to transit from a Schwann cell environment to an astrocyte/oligodendrocyte environment.[16-19] A similar situation occurs when a peripheral nerve or a Schwann cell transplant is placed in the CNS. These transplants attract axons successfully, so axons experience no difficulty in passing from astrocytes to Schwann cells. However when the axons reach the other end of the graft most are blocked at the Schwann cell/astrocyte boundary that exists there.[20] Meningeal cells also appear to have boundary-forming properties. Most CNS injuries become lined with invading meningeal cells within a few days. Some axons are able to regenerate to the interface between astrocytes and meningeal cells, but regeneration into the meningeal cell plug is very rarely seen. For instance in optic nerve crush lesions a largely astrocyte-free zone is seen at the crush site which is invaded by meningeal cells, microglia and oligodendrocyte precursors (OPCs).[21] Axons can be seen that have grown to the edge of the astrocyte zone, but they do not penetrate into the crush lesion unless they accompany astrocytic processes, some of which eventually invade these injuries.

Inhibitory Molecules in the Damaged CNS

There are many molecules that have inhibitory activity towards axon growth in the adult CNS. These can be divided into three categories: (1) Molecules present in myelin produced by oligodendrocytes which are present in the normal and damaged CNS, (2) Molecules upregulated in reactive glial cells around areas of injury, which are mostly chondroitin sulphate proteoglycans, (3) Axon guidance molecules which play a role in the develpment of the CNS, and which are also present in CNS injuries.

Inhibitory Molecules Produced by Oligodendrocytes

Oligodendrocytes produce several molecules which are extremely inhibitory to axon growth, and which play an important part in the inhibition of axon growth in the CNS. The subject of this review is the glial scar-related molecules, so only a brief description is given here. NogoA is a molecule of the reticulon family, expressed in oligodendrocytes and also some classes of neuron, which is inhibitory to all classes of axon except some embryonic axons.[22] Much work has been done with a blocking antibody, IN-1 and more recently with other antibodies with blocking activity. These antibodies have been shown to stimulate axon regeneration in a variety of lesion models in the spinal cord and elsewhere.[23] A receptor molecule has recently been identified.[24,25] MAG is expressed in myelinating oligodendrocytes, and is also released from the cell surface to diffuse more widely. It is inhibitory to many types of axon, although inhibition varies with axonal type and age.[26-28] A MAG knockout showed little regeneration in the CNS, demonstrating that other inhibitory mechanisms are able to block regeneration even in the absence of MAG, although the properties of MAG in vitro make it clear that it must be an important inhibitor of axon regeneration in vivo. Tenascin-R is a member of the tenascin family with axon growth inhibitory properties expressed at particularly high level in white matter, produced by oligodendrocytes and oligodendrocyte precursors. Also produced by oligodendrocytes is the CSPG versican, which is described below.

Inhibitory Molecules Produced in the Glial Scar

The first investigations into glial scar inhibitory molecules were performed in astrocytes, since they are the main component of mature scar tissue. The first in vitro experiments did not reveal inhibitory properties, since axons grow fairly well on monolayers of astrocytes. However

a glial scar is a three-dimensional tissue of tightly interwoven astrocytes, not a monolayer, and when astrocytes are grown as three-dimensional tissues axons grow through them very poorly.[29] In order to try and identify the types of inhibitory molecules produced by astrocytes a variety of astrocytic cell lines were produced, some of which were inhibitory to axon regeneration, some of which were permissive. Comparisons between these cells showed that the ability of the different cell lines to support axon regeneration was mirrored closely by the growth-promoting properties of their extracellular matrix. Yet the matrix of even the most inhibitory lines contained large amounts of laminin and other growth-promoting molecules. Clearly these cells were producing extracellular matrix molecules with the ability to block the growth-promoting effects of laminin. Various lines of evidence showed that these inhibitory molecules were CSPGs, and that much of the inhibition due to these CSPGs could be abrogated either by digesting away their glycosaminoglycan chains (GAGs) with chondroitinase ABC, or by preventing the sulfation of the GAGs with chlorate, or by preventing the GAGs from attaching to their protein cores by treatment with beta-D-xylosides.[30,31] For one particular inhibitory astrocyte line the main inhibitory molecule was the CSPG NG2.[32] That CSPGs are important inhibitory molecules for primary astrocyte cultures was shown by treating three-dimensional cultures with chlorate to block GAG sulfation, resulting in astrocytes that were much more permissive for axon regeneration than untreated cells.[30]

There were also indications from in vivo work that CSPGs might be important inhibitory molecules in glial scars. Using an antibody, CS56, that binds to sulfated forms of the chondroitin sulfate (CS) GAG chain, it was shown that in many types of CNS injury CS is upregulated within a few days of injury, and remains increased for a month or more afterwards.[33] Two types of experiment had also shown that these molecules are inhibitory. In the first, filter material was implanted into a CNS lesion, then removed several days later covered in scar astrocytes. These provided an inhibitory environment for axon growth, but when digested with chondroitinase the cultures were more permissive.[11,34] A second approach was to dissect scar tissue from around a CNS lesion and extract inhibitory material from it. This investigation showed that the inhibition produced by these extracts was sensitive to chondroitinase and heparitinase.[35,36]

More recently infusions of chondroitinase ABC to the damaged CNS have shown that digesting away GAG chains from CSPGs can make the glial scar less inhibitory to axon regeneration. In the first experiment chondroitinase was infused into a knife cut lesion of the nigro-striatal tract. Since the turnover of matrix in the CNS is fairly slow, these injections were given as bolus injections on alternate days, ensuring that the enzyme was present at a high enough concentration to be effective. In animals treated in this way around 2000 out of a complement of 45,000 axons were able to regenerate back to the striatum, compared with no regeneration in controls.[37] In a second set of experiments chondroitinase ABC has been infused into lesions of the dorsal columns of the rat spinal cord at level C4. These animals have shown regeneration of both sensory and corticospinal axons, with return of postsynaptic potentials resulting from cortical stimulation below the level of the lesion. The animals were assessed behaviourally, and showed a dramatic and rapid return of almost normal function in beam and grid walking tasks, but no improvement in a pure sensory task that would require axons to regrow back to the dorsal column nuclei.[38]

When astrocytes participate in the glial scar they greatly upregulate two cytoskeletal proteins, GFAP and vimentin. There has always, therefore, been an question as to whether this process is responsible for some of the inhibition seen in the glial scar. This has recently been tested in animals in which the GFAP and vimentin genes have been knocked out. Animals with a GFAP knockout showed increased axon regeneration after spinal cord injuries, but there was no effect after vimentin knockout.[39]

Individual CSPGs

Neurocan

Neurocan belongs to the family of lecticans, which also includes versican, brevican and aggecan. These molecules are all secreted molecules, which have a hyaluronate-binding motif. In the adult CNS much of the neurocan is in a processed form, in which the molecule is cleaved by proteolytic action into an N and C terminal fragment, known as neurocan N and neurocan C.[40] When the CNS is extracted into detergent-free saline much of the intact neurocan and neurocan C is removed, indicating that they are not attached to the cell surface. However a proportion of the intact neurocan and about half the neurocan N can only be extracted if the brain homogenate is treated with hyaluronidase, indicating that this neurocan is hyaluronate-bound.[41] When production of neurocan is investigated in purified glial cell populations in vitro it is produced by astrocytes and by OPCs, but not by mature oligodendrocytes. Neurocan is greatly upregulated following CNS damage. Immunostaining of normal brain showns neurocan to be present in white matter and at the glia limitans and also in perineuronal nets around many neurones. After cortical damage intense immunoreactivity is seen in grey and white matter surrounding the lesion. Western blots show that much of this upregulation is of the intact form of neurocan.[41,42] When proteoglycans are run on western blots they are generally present as a long diffuse smear rather than as a single discrete band. This is because of the variable quantity of GAG attached to the molecules, giving a range of molecular weights. However if the extract is treated with chondroitinase the GAGs are removed and the core protein runs as a single band. From this the amount of GAG (glycanation) can be estimated. Estimates of this type for neurocan show that glycantion is increased following CNS injury, each neurocan molecule carrying more GAG. Neurocan is inhibitory for axon growth: in a stripe assay in which stripes of L1 alternated with L1 plus neurocan axons from cerebellum chose to grow on the neurocan-free stripes.[41] Neurocan has been shown to interact with N-CAM, Ng-CAM/Ll, TAG-l/ axonin-l, and tenascin, and to inhibit axon growth mediated by Ng-CAM/ Ll.[43,44] The difference in inhibitory properties between monolayers and three-dimensonal cultures of astrocytes is probably due to the way in which neurocan associates with astrocytes. In monolayer culture neurocan does not associate with the astrocyte surface, although it sticks to the dish surrounding the cells. Thus in astrocyte monolayers most of the neurocan is secreted into the medium, is diluted away and therefore is unable to inhibit axon growth on the astrocyte surface. In three-dimensional cultures, however, the neurocan cannot diffuse away and is trapped in between the cells, It is therefore present at high concentration in the environment in which the axons are attempting to grow, and can therefore block axon regeneration. Neurocan production by astrocytes is upregulated by TGFalpha, TGFbeta and FGF-2.

Versican

Versican can exist in four splice variants. The major form in the normal CNS is the smallest form, V2.[45] In purified glial cultures versican is not produced by astrocytes, but the V2 form is produced in a differentiation-related fashion by cells of the OP lineage.[46,47] Undifferentiated bipolar OPCs do not make versican, but mulitpolar precusors and pre-oligodendrocytes do. There is less production by fully mature oligodendrocytes with myelin-like sheets. Versican is also made by meningeal cells in vitro, but these cells produce the larger V0 and V1 forms of the molecule. Extracting versican with saline, and following hyaluronidase digestion showns that about half the versican in the brain is bound to hyaluronate, about half is in a form which can be extracted by detergent-free saline. The versican from brain appears to be less glycanated than neurocan, since even without chondroitinase treatment it runs on western blots as a single band. However this band shifts slightly after chondroitinase digestion showing that there is

some glycanation. Versican produced in vitro, however, is much more glycanated, and much of it will not enter a gel unless chondroitinase is used. In the normal brain versican is present in the white matter, and after injury it is upregulated in and around the injury in white and grey matter.[46] This upregulation is presumably due to production by the OPCs that are recruited in large numbers to CNS injuries, although the secreted versican diffuses too readily to be identified around individual OPCs in immunostains. Versican has been shown to be inhibitory to axon growth in stripe and other assays.[46,48] In addition medium conditioned by oligodendrocyte cultures has large amounts of versican and brevican in it, and is inhibitory to axon growth. When the medium is depleted of versican it is less inhibitory.[47] Versican production by OPCs in culture is increased by TGFbeta, Il-1 and CNTF.[41]

Brevican

Brevican is the smallest of the lecticans found in the CNS. It can be produced by astrocytes, is upregulated following injury and has axon growth inhibitory properties.[49,50]

NG2

NG2 is produced as a membrane spanning molecule. It is a part time proteoglycan in that it can be produced both with and without GAG chains attached. NG2 can be cleaved from the cell surface by the action of an unidentified metalloproteinase, so that in the normal CNS around half of the NG2 is membrane associated, and half can be extracted without the use of detergents.[51] In the CNS NG2 is seen on three types of cell, oligodendrocyte precursors, vascular endothelial cells and meningeal cells, and these cell types also produce NG2 in vitro. NG2 positive OPCs are present throughout the CNS in both grey and white matter, where they may contact nodes of Ranvier and synapses.[4] After CNS injury there is rapid proliferation of OPCs within 2mm of the injury site, and the OPCs hypertrophy and greatly increase the around of NG2 on the cell surface. Western blot analysis shows a large increase in levels of NG2 after injury, starting at 24 hours and peaking around 7 days, after which levels decline over the following two or three weeks.[52] Comparisons between chondroitinase digested and undigested lanes show that the glycanation of NG2 is increased after injury. NG2 is strongly inhibitory to axon regeneration, and a blocking antibody to NG2 has been shown to promote axon growth on an inhibitory astrocyte cell line that produces large amounts of the proteoglycan (32). NG2 has other functions. It associates with the PDGFalpha receptor and acts as a necessary cofactor to its ability to transduce effects from this growth factor.[53,54] Since a large proportion of the NG2 in the CNS is in the released form, it is possible that there is some functional competition between released and cell surface NG2, but this issue has not yet been addressed experimentally.

Phosphacan

Phosphacan and its mouse homologue DSD-1 belong to a family of alternatively spliced molecules. Phosphacan, which is a secreted molecule, is the extracellular domain of the receptor tyrosine phosphatase RPTPbeta/zeta. RPTPbeta/zeta itself is found in two forms, a short receptor form and the full length form. In vitro phosphacan is expressed by astrocytes and by OPCs. Both cell types also make RPTPbeta/zeta, with OPCs making predominantly the short receptor form.[42,55,56] Phosphacan is inhibitory to some axons, but promotes growth in others.[43,57]

Glycanation of CSPGs

Various experiments described above demonstrate that the inhibitory properties of CSPGs are partly dependent on the sulphated GAGs attached to the protein cores. The biology of this form of inhibition is not established. To some extent the inhibitory properties of CSPGs can be

reproduced by GAGs by themselves, if presented at high concentration.[58] However in general the GAGs and proteoglycan core proteins must be attached to one another for them to show their normal inhibitory properties. Exactly how the inhibition works is not established. Many of the CSPG core proteins bind to other matrix molecules such as laminin and tenascin, or to cell surface adhesion molecules such as L1.[44] It seems probable that if the highly charged sulfated GAGs are localised to a region of a protein by the binding of the CSPG protein core, there might be masking of epitopes that promote axon growth, or a change in the tertiary structure of the protein sufficient to alter its function. Some experiments have examined whether the position in which the GAGs are sulfated affects their inhibitory function. These experiments suggest that the 6-sulfated GAGs are particularly inhibitory, while GAGs sulfated in the 6 position and the 2 position on the glucuronic acids are more permissive to axon growth.[59]

We have examined glycanation of NG2, neurocan and versican in the normal and injured brain. This can be done on western blots by comparing lanes in which the extract has or has not been digested with chondroitinase ABC. In undigested lanes GAGs of various lengths cause the CSPGs to run as a smear, which resolves to a single band after digestion. Densitomitry of the smear region gives an estimate of glycanation. These studies show that for NG2 and neurocan both core protein and the extent of glycanation are increased following cortical injury. Versican, however, is less glycanated in vivo than the other two CSPGs, and this does not change greatly after injury. When CSPGs from glia grown in vitro are examined in the same way, the degree of glycanation is greater than is seen in vivo, particularly for versican which is so highly glycanated when produced by oligodendrocyte lineage cells in vitro that it will not enter a gel at all unless digested with chondroitinase ABC.[41,46,51,60]

Chondroitin Sulphate Proteoglycans and Regeneration in the CNS

Since CSPGs are expressed in large amounts in CNS injuries, and since most of them are inhibitory to axon growth they must play some part in blocking axon regeneration after injury. Some of the evidence that injury-related CSPGs are a significant factor in CNS regeneration is discussed above. The most direct evidence are the experiments in which the enzyme chondroitinase has been infused into brain and spinal cord injuries, also described above.

If CSPGs are inhibitory to axon regeneration, what strategies might be used to counteract their effects and so promote regeneration after CNS injury? The first consideration in planning a strategy is to decide where CSPGs exert their effect. The molecules are expressed to some extent in the normal CNS, particularly in white matter, but are upregulated with an increase in their glycanation around injuries. If the molecules only have to be counteracted in the immediate vicinity of an injury, the task will be easier than trying to clear inhibition from the whole region where axon regeneration is needed. There is little evidence on this issue. Three experiments from Davies and Raisman and Davies and Silver, mentioned previously, suggest that inhibition due to CSPGs is much greater around injuries than in normal CNS.[9,14,15] The first experiment examined axon regeneration after very small lesions in the spinal cord which did not disturb glial architecture: following this cut axons sprouted away from the lesion through undamaged tissue but not through it, suggesting that only the lesion area is very inhibitory. The second and third experiments were to transplant adult sensory neurones into the rat CNS using an atraumatic technique. Axon growth from these transplants was only blocked where they encountered glial scar tissue, with high levels of CSPG. The conclusion is that normal CNS is probably moderately inhibitory to axon growth, but scar tissue is highly inhibitory. If axons can be enabled to pass through the scar area they therefore stand a chance of being able to regenerate on through the undamaged tissue. What treatment might be applied? The problem is that there are many CSPGs in the injured CNS, all of them inhibitory. It will be difficult to design treatments for each CSPG individually. However all CSPGs possess GAG chains, and much of their inhibitory activity relies on them, making them an attractive therapeutic

target. Moreover the experiments in which chondroitinase has been infused into the CNS to remove GAG chains have been successful in promoting regeneration. In principal inhibition of GAG synthesis would be a good way of preventing the buildup of inhibition in CNS injuries. However at present on sodium chlorate and beta-D-xylosides are able to do this, and both promote axon regeneration in in vitro models, but there are problems with both these agents.

Glial Boundaries in the CNS

The inhibitory mechanisms due to CSPGs and myelin-related molecules can be called surround inhibition, since the inhibitory molecules are diffusely expressed within the glial scar. There are places where axons meet boundaries between different glial cell types, and at some of these boundaries axon growth is stopped abruptly. For CNS repair two types of boundary are significant, Schwann cell/astrocyte and astrocyte/meningeal cell boundaries.

Schwann Cell/Astrocyte Boundaries

Boundaries between Schwann cells and astrocytes occur wherever peripheral nerves contact the CNS, for instance at the dorsal and ventral roots. Schwann cells and astrocytes show no ability to mix, leading to sharp boundaries between the cell types and dorsal root and ventral root entry zones.[20,61-63] The form of myelination changes at these entry zones, so that motor and sensory axons as they enter or leave the CNS have a node of Ranvier with an oligodendrocyte on one side, a Schwann cell on the other. If motor axons are damaged within the spinal cord, they can usually regenerate out of the CNS across the ventral root entry zone and into peripheral nerve, indicating that these axons can cross from astrocytes onto Schwann cells.[16] However if the dorsal root is crushed the axons will regenerate back towards the spinal cord, particularly if the peripheral nerve is crushed at the same time to increase the vigour of regeneration, but when the axons encounter astrocytes at the dorsal root entry zone their growth is blocked.[17,64] The stopped axon growth cones undergo morphological changes that indicate that the axon has received a stop signal as if it has reached an appropriate target.[65,66] These axons are unable, therefore to cross from Schwann cells to astrocytes. Similar boundary behaviours are seen when a peripheral nerve or Schwann cells are transplanted into the CNS in an attempt to bridge across an injury. Many types of CNS axons will grow into these grafts quite readily, indicating that they can cross from astrocytes to Schwann cells. However when axons attempt to leave the grafts back into an astrocytic environment their growth is blocked, as they are unable to cross from a Schwann cell environment back into an astrocytic one.[20,67,68]

Just as Schwann cells and astrocytes seldom mix in vivo, so they mix poorly in vitro. Therefore when Schwann cells and astrocytes are placed together in the same cultures they tend to separate out into separate patches.[61] This makes it possible to examine the ability of axons to grow across boundaries between Schwann cells and astrocytes in vitro. We have examined the growth of sensory axons in such cultures. Their behaviour is very similar to that seen in vivo. Where axons growing on astrocytes encounter a patch of Schwann cells almost all of them will cross onto the Schwann cells. However when axons growing on Schwann cells encounter a boundary with astrocytes only between 10% and 30% are able to cross.[69] One of the reasons why axons do not like to leave a Schwann cell environment for an astrocyte one may involve the adhesion molecule L1, a potent promoter of axon growth that is present on Schwann cells but not astrocytes. Thus when exogenous L1 in the form of L1-Fc is applied to the cultures, or when L1 is blocked by a functional blocking antibody the proportion of axons able to cross boundaries onto astrocytes is greatly increased. We were not able to find evidence of inhibition by astrocyte-produced CSPGs as a factor in preventing boundary crossing, probably because the main astrocyte CSPG, neurocan, is not attaced to the astrocyte cell surface but released into the culture medium. The recognition of Schwann cell astrocyte boundaries that leads to the blockage of growth appears to involve a signalling pathway that has been shown to

affect growth in other models. cAMP levels in the growth cone has been shown to have a strong effect on the ability of axons to grow on inhibitory molecules such as MAG. We find that raising cAMP levels in axons allows more of them to cross from Schwann cells onto astrocytes. Developmental changes are also significant. Axons growing from embryonic sensory neurones or embryonic retina can cross from Schwann cells to astrocytes in much larger numbers than axons from postnatal neurones (Adcock, Shewan, Czvitkovich, Fawcett unpublished results).

Boundary crossing behaviour has also been studied in a different in vitro model, using frozen sections of the dorsal root entry zones a the culture surface. The behaviour of axons in this model is similar to that seen in vivo, and to that seen in the culture model described above. Axons cross readily from the CNS to peripheral nerve, but not in the other direction. Developmental age has a critical influence. Thus when the substrate is taken from embryonic spinal cord the boundary is crossed by many more axons, and embryonic axons can cross adult boundaries. Calcium signalling plays a part in axonal behaviour at these boundaries, since blocking calcium release from internal stores with dantrolene increases the number of axons crossing dorsal root boundaries.[18,19]

Astrocyte/Meningeal Cell Boundaries

Meningeal cells are specialised fibroblast-like cells that surround the CNS and its major blood vessels, and are responsible for co-operating with astrocytes in the setting up of the glia limitans.[70-72] This is a layer of hypertrophic astrocyte processes running parallel to the surface of the brain and spinal cord, with a layer of basal lamina on top in between the astrocytes and meningeal cells. Following CNS injuries, particularly those that penetrate the meninges, the meningeal cells divide, and migrate into the injury cavity. Within a few days they line the entire injury cavity, and if the injury has reduced the density of astrocytes and created space within the tissue meningeal cells may invade more diffusely.[71,73] As the invading meningeal cells come into contact with astrocytes, they induce the same changes that are seen at the glia limitans. This leads eventually to the formation of a new glia limitans and often more general reactive astrocytic changes. Just as axons do not grow out of the brain or spinal cord through the normal glia limitans, the new glia limitans that forms after CNS injury appears to present a barrier to regenerating axons. In various regeneration studies axons can be seen to have approached the astrocyte-meningeal cell boundary and stopped at that point.[74,75] For instance Beattie et al[76] have reported that corticospinal axons retract from the injury site after spinal cord injury, but will then show some regenerative growth leading them to the meningeal boundary, where they stop. Davies and Silver performed transplants of adult sensory axons into the injured spinal cord, placed so that axons would grow through the dorsal columns until they encounter the injury. When the transplants were performed some time after the cord injury the CSPG reaction had abated sufficiently that a proportion of the axons were able to penetrate the glial scar tissue to reach the meningeal boundary, and there they stopped.[15] In all the experiments in which regeneration has been induced in the spinal cord, by blocking NogoA, chondroitinase and other treatments, the regenerating axons have gone around the meningeal boundary, not through it. However, by inhibiting the buildup of collagen and extracellular matrix in the injury it has been possible to induce axon regeneration across the injury site.[77,78]

We have been able to model axon behaviour at astrocyte/meningeal cell boundaries in vitro. As with Schwann cells and astrocytes, meningeal cells and astrocytes separate out in vitro into separate territories, the result being islands of meningeal cells in a lawn of astrocytes. We have plated sensory and other neuronal types onto these cultures to observe their behaviour at boundaries. Axons will grow on both astrocytes and meningeal cells, although axons growing on meningeal cells tend to be fasciculated and tortuous. When axons growing on meningeal cells reach a boundary of astrocytes around 90% will cross, with the remainder following around the interface between the cell types. However, when axons growing on astrocytes meet meningeal

cells only 15-30% will cross, many of the axons turning to follow the interface between the cell types.[79] Why are meningeal cells inhibitory? In principle this could be because they express inhibitory molecules, or because they lack the growth-promoting molecules present on astrocytes. Meningeal cells express at least three inhibitory molecules; the CSPGs NG2 and versican, and the axon guidance molecule Semaphorin 3A/ collapsin1. We have applied blocking antibodies to both NG2 and to the semaphorin receptors plexin 1 and plexin 2, and we find that the NG2 and plexin 2 antibodies both ncrease the number of axons that can cross onto meningeal cells from arond 20 to around 40%. There is no specific way of blocking inhibition due to versican. The main growth-promting molecule that is present on astrocytes but absent on meningeal cells is N-Cadherin, but we find that blocking this molecule with HAV peptides does not enhance boundary crossing onto meningeal cells. As with Schwann cell/astrocyte boundaries, it is possible to promote boundary crossing by manipulating growth cone signalling. Thus increasing cAMP levels and blocking the GTPase Rho both increase the number of axons crossing boundaries.[79,80]

Glial Boundaries and Repairing the CNS

To date the only treatment designed specifically to deal with glial boundaries is the inhibition of collagen synthesis in the lesion, which has succeeded in promoting some regeneration.[77,78] In addition two of the treatments that affect axonal boundary crossing as well as growth on inhibitory substrates, namely increasing cAMP levels and blocking Rho have been applied. The Rho ribosylating toxin, C3 from clostridium botulinum, has been applied to optic nerve and spinal cord injuries. In both cases axon regeneration was promoted.[81,82] In the optic nerve experiments it is likely that the axons encountered a meningeal boundary, since these cells invade optic nerve crushes within 24 hours, and the crushed axons retracted from the injury and probably did not grow through it for at least 24 hours after crush. There has only been one experiment to manipulate cAMP levels in vivo, by injecting it directly into dorsal root ganglia. This promoted some regeneration in the spinal cord, although it is not clear that any glial boundaries were involved. However the actions of neurotrophins are at least in part though cAMP, and these molecules do promote boundary crossing. Many experiments have attempted to promote axon growth across the Schwann cell/astrocyte boundary of the dorsal root entry zone. The most successful techniques have used neurotrophns. NT3 infused into the region of the entry zone allowed many axons to cross the boundary into the CNS.[83,84] Neurotrophins have also been expressed in the cord by adenoviral infection, and NGF and FGF-2 have attracted axons across the boundary.[85,86] The technique has been used with a degree of success to attract axons out of Schwann cell grafts to the spinal cord back into cord tissue. An infusion of BDNF and NT-3 allowed a modest number of axons to grow across the boundary.[67]

Strategies for Repairing the CNS

The events that remodel the CNS glial environment after injury are complex, leading to a terrain with several types of inhibitory obstacle. It would be daunting to devise a treatment that modifies all these inhibitory mechanisms simultaneously. However, it may not be necessary to deal with every inhibitory molecule. Axon growth cones integrate together all the various signals that they receive both growth promoting and growth inhibitory. In principle, therefore, axon regeneration could be achieved by increasing the amount of growth promoting activity or by reducing the amount of inhibition. Various different treatments to block inhibition in the damaged CNS have been applied, particularly anti NogoA antibodies, chondroitinase and demyelination all promote growth to a comparable degree. It will be important to find out whether these treatments have additive effects, or whether axon growth is the same once a certain level of permissiveness in the environment has been achieved. The issue of whether

treatments are additive also applies to the other ways in which axon growth can be promoted, by manipulating signalling pathways and with neurotrophins. If all these treatments converge on the same growth cone mechanisms it is unlikely that they will be additive. All the treatments that have been identified to date are able to promote growth of a fairly modest number of axons over distances of 1-2 cm. This is just at the margin of what would be useful to a spinal injury patient, where bringing the effective level of an injury down by even one spinal level would be of great benefit to patients with cervical level injuries. However, achieving robust growth of large numbers of axons over long distances is still an elusive aim. It is likely that there are axon growth control mechanisms that are yet to be discovered which we will need to manipulate to achieve this aim.

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