SCHWANN CELL PHENOTYPE

DURING PERIPHERAL NERVE DEVELOPMENT

AND DURING MYELINATION IN VITRO

by

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ABSTRACT

SCHWANN CELL PHENOTYPE DURING PERIPHERAL NERVE DEVELOPMENT AND DURING MYELINATION IN VITRO

The appearance of a variety of Schwann cell associated molecules has been followed during development of rat sciatic nerve. Cell surface antigens laminin, N-CAM, low affinity NGF-R, sulphatide, GAL-C, A5E3, L1 and intracellular antigens vimentin, S100, GFAP and P0 were examined in cells dissociated from sciatic nerves of rats aged between embryonic day 14 and adult. Changes in phenotype as early Schwann cells develop into myelin forming and non-myelin forming Schwann cells are described.

Axonal control of Schwann cell phenotype was explored using an in vitro system. It is shown that the axon dependent expression of myelin proteins can be mimicked in vitro by drugs that elevate Schwann cell intracellular cAMP levels. In defined medium, the myelin specific protein P0 is expressed by Schwann cells in the presence of cAMP elevating agents. Using in situ hybridisation and northern blots, the changes in levels of P0 protein are shown to be the result of increased mRNA levels. The full myelin phenotype is displayed by some of these cells, with new expression of surface lipids sulphatide and GAL-C and loss of the low affinity NGF-R accompanying P0 induction. Expression of the myelin phenotype is shown to occur only in the absence of cell division.

Growth factors modulate the response of Schwann cells to cAMP elevation. When acidic FGF, basic FGF, GGF, PDGF or serum are present in the culture medium, cAMP elevation is followed by Schwann cell division and little or no P0 elevation. On the other hand, elevation of sulphatide is less affected by the presence of growth factors since the rapid division is accompanied by a large induction in the levels of sulphatide.

One of the effects of axonal signals could be to elevate Schwann cell cAMP levels, causing sulphatide expression and rapid division during embryonic development and, later, induction of myelin in non dividing cells.
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ABBREVIATIONS

cAMP; Cyclic adenosine monophosphate.
8b-cAMP; 8-bromoadenosine 3'5' cyclic monophosphate.
db-cAMP; N6,2'-0-dibutyladenosine 3'5' cyclic monophosphate.
ATP; Adenosine triphosphate.
BrdU; Bromodeoxyuridine.
BSA; Bovine serum albumin.
CNS; Central nervous system.
CNTF; Ciliary neuronotrophic factor.
CRE; cAMP response element.
CREB; cAMP response element binding protein.
CS; Calf serum.
CTx; Cholera toxin.
DAG; Diacylglycerol.
DMEM; Dulbecco's modified Eagle's medium.
DRG; Dorsal root ganglia.
E; Embryonic day (e.g. E15 = embryonic day 15).
ECM; Extracellular matrix.
EDTA; Ethylenediaminetetraacetic acid.
FCS; Foetal calf serum.
aFGF; acidic fibroblast growth factor (FGF-1).
bFGF; basic fibroblast growth factor (FGF-2).
kFGF; Kaposi's sarcoma derived fibroblast growth factor (HST/FGF-4).
GDP; Guanosine diphosphate.
GDN; Glial derived nexin.
GFAP; Glial fibrillary acidic protein.
GGF; Glial growth factor.
GTP; Guanosine triphosphate.
HEPES; N[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid].
HLH; Helix-loop-helix.
IF; Intermediate filament.
IFNy; Interferon y.
Ig; Immunoglobulin.
IL-1; Interleukin-1.
InsP3; Inositol trisphosphate.
MAG; Myelin associated glycoprotein.
MEM; Minimal essential medium.
MEM-H; Hepes buffered minimal essential medium.
N-CAM; Neural cell adhesion molecule.
NDF; neu differentiation factor, (neu ligand).
NDGF; Neuron-derived growth factor.
NF1; Neurofibromatosis type I.
OAG; 1-oleoyl-2-acylglycerol.
P; Postnatal day (eg. P2 = 2 day old)
PDGF; Platelet derived growth factor.
PIP₂; Phosphatidylinositol bisphosphate.
PKA; Protein kinase A.
PKC; Protein kinase C.
PLC; Phospholipase C.
PLL; Poly-L-lysine.
PMP22; peripheral myelin protein, 22 kD.
PMSF; Phenylmethylsulphonylfluoride.
PNS; Peripheral nervous system.
SCG; Superior cervical ganglion.
SCIP; Schwann cell cAMP-inducible POU.
SDS; Sodium dodecyl sulphate.
SEM; Standard error of mean.
SSC; Sodium chloride/sodium citrate buffer at pH 7.
TGFα; Transforming growth factor α.
TGFβ; Transforming growth factor β.
TNF; Tumour necrosis factor.
VIP; Vasoactive intestinal peptide.
CHAPTER 1
GENERAL INTRODUCTION:

Over the past 150 years there has been a constant interest in the Schwann cell. As new techniques have become available, much information has been gathered about the role of these cells in nerve development, in maintaining nerve function and during repair after nerve damage. The wealth of information available about the molecular phenotype of Schwann cells and their production of, and response to, growth and trophic factors provides plenty of tools for those interested in exploring the interactions between cells of different lineages. In spite of the enormous interest in this area, the interdependence between the axons of peripheral nerves and their glial cells is still a long way from being understood. This study concentrates on axonal control of Schwann cell differentiation.

Thesis aims and objectives

1. To extend the characterization of the Schwann cell phenotype in development to the earliest stage at which the cells can be obtained. There is an enormous literature on postnatal Schwann cells, but few studies before birth.
2. To investigate axonal control of Schwann cell phenotype by examining the effect of removing axons both in vivo and in vitro.
3. To develop an in vitro model for Schwann cell myelination, and to compare this model with the situation in vivo during development and regeneration.
4. To use this model to investigate control of Schwann cell myelination with a view to understanding the mechanism(s) leading to myelination.

Theodor Schwann and Early Studies of the Schwann Cell

In 1839 Theodor Schwann (1810–1882) gave the first clear description of the cells that were later given his name in a book that proposed the cellular nature of plants and animals. This theory extended the work of the botanist Schleiden, who was also at the University of Louvain and who first saw the importance of the nucleus of vegetable cells. The similarity between these plant cells and organs Schwann had observed in the dorsal cord was the trigger for his theory that all parts of all living things were of cellular origin. In his examination of the elementary parts of animal tissues, he described a fifth class of tissues, (grouping nerves with skeletal muscle and capillary
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blood vessels) where the cells fused together in development. He described the development of nerves from "primary nerve cells" that coalesce and form a syncytium of secondary cells from which the nerve fibre was then differentiated. This theory was the result of an analogy being drawn between development of nerve and skeletal muscle.

Schwann showed the uninterrupted course of nerve fibres from peripheral organs to the central nervous system. He also described the production of a white material (myelin) formed by these secondary cells around their central core (the axon), which he considered as part of the same cell. 40 years later Ranvier (1878) showed that this central core within Schwann cells was an outgrowth from neurons located in the CNS or in ganglia, and not a part of the Schwann cell. Using osmium tetroxide to stain the lipids of myelin, Ranvier was able to show that the outer sheath was divided into internodes, each associated with only one nucleus and separated from each other by nodes, or breaks in the myelin. He was not able to determine whether the "Schwann membrane", the sheath surrounding the axon was continuous with this outer nucleus and its associated cytoplasm. A full description of the morphology of the myelinating Schwann cell, essentially as it is understood today was given by Cajal in 1909, illustrating that Schwann cell cytoplasm in the perinuclear space is continuous with that over the inner and outer surfaces of myelin, in the Schmidt and Lanterman incisures and in the nodal projections, and that myelin is an integral part of the cell.

Remak fibres seen in visceral nerves by Remak in 1838 were described as naked fibres with nuclei along their length. Schwann interpreted these fibres as secondary nerve cells in which no white matter had been deposited, and he considered them to have halted at an earlier stage of development. In 1909, Cajal saw that these fibres were present in the majority of nerves, mixed in with the myelin fibres. They were first visualised clearly with their axon cores by Tuckett in 1896 (Reviewed by Causey 1960)

The advent of electron microscopy in the late 1940s allowed the fine structure of the myelinating and also the more elusive non myelinating cells to be determined. The non–myelin forming Schwann cell.
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These are the cells that ensheath small unmyelinated axons in peripheral nerves. Other peripheral glial cells that do not synthesise myelin (and which are probably from the same lineage) including the satellite cells which surround PNS neuronal cell bodies, and glial cells at the motor end plate, the terminal Schwann cells, are not included in this description.

Unmyelinated axons are wrapped in a flap of Schwann cell cytoplasm so that they lie in a trough along the Schwann cell surface. The extension from the Schwann cell varies in the amount to which it encloses each axon, occasionally the trough is open on one side and the axon surface is only separated from the endoneurium by the basal lamina, more usually however the Schwann cell wraps all the way around the axon and back over itself to form a complete seal. Thus, in the main body of the nerve axons are prevented from touching each other by a Schwann cell process. The number of axons wrapped by one Schwann cell can be over 20 \((\text{ranges between 7 and 21 in rat trigeminal nerve, Dixon 1963})\). At the junctions between adjacent non–myelin forming Schwann cells the axons are not exposed and one Schwann cell overlaps the next \((\text{Eames and Gamble 1970})\). The axons do not travel the nerve as family groups, passing together from one non–myelin forming Schwann cell to the next, rather they separate and regroup continuously along the nerve so that a Schwann cell can ensheath one set of axons at its proximal end, and a different set at its distal end \((\text{Aguayo et al. 1976a})\). Recently dye injection experiments have revealed gap junctions between adjacent non–myelin forming Schwann cells \((\text{Konishi 1990})\). Occasionally non–myelin forming Schwann cells will wrap collagen bundles as if they were axons \((\text{Gamble and Eames 1964})\).

Axonal diameter is not the same along the axon's length but irregular, with organelle–rich varicosities where the diameter is greatest \((\text{Greenberg et al. 1990})\), the living axon is considered to change diameter continuously, presumably as these organelles are transported along its length.

Non–myelin forming Schwann cells are surrounded by basal lamina which is continuous between one cell and the next.

The myelin forming Schwann cell.

Unlike non–myelin forming Schwann cells, myelin forming Schwann cells never
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associate with more than one axon. Myelin is synthesised around the axon by the
Schwann cell to form a collar separating the axon from the endoneurium, this length
of myelin is called an internode, and the gap between two Schwann cells is called a
node. Myelin is composed of many layers of specialised Schwann cell plasma
membrane compacted together. The extracellular surface of one wrap of membrane is
pushed against the extracellular surface of the next and the intracellular surface of that
wrap is likewise against the next intracellular layer. The compacted layers of
membrane are visible in EM preparations as regular bands with a periodicity of 12–19
nm. Within each band is a major dense line 2.5 nm thick, separated by a pale
intrapерiod line, within the intraperiod line are two faint bands 2nm apart. The major
dense line is composed of two adjacent intracellular lipid bilayers so close together
that at EM they present one profile. The intraperiod line is composed of two
extracellular faces of Schwann cell plasma membrane, the faint bands are made up of
extracellular proteins (Napolitano and Scallen 1969). Myelin thickness depends on
the surface area of axolemma, this in turn depends on axonal diameter and internodal
length, larger axons are, therefore, generally surrounded by thicker myelin, but in
special cases where the internode is unusually short for the axonal diameter, myelin is
less thick (Spencer et al. 1973, Smith et al. 1982).

Cytoplasm is restricted to discrete areas, there is a cylinder of cytoplasm in the
adaxonal or inner part of the cell and more in the abaxonal or outer part, especially
near the nucleus which is located centrally in the abaxonal space. Myelin contains no
cytoplasm, but is broken up by the Schmidt-Lanterman incisures where the major
dense line opens out leaving a thin, spiral ribbon of cytoplasm connected to the
abaxonal cytoplasm, often connecting right through to the adaxonal cytoplasm. Longitudinal
incisures connect Schmidt-Lanterman incisures with cytoplasm contained in the
paranodal projections and these also connect with the abaxonal and adaxonal spaces
(Mugnaini et al. 1977). The extracellular space contained within the spiralling folds
of the Schwann cell is separated from the endoneurium and the peri-axonal space by
tight junctions located where the outer flap seals over itself (outer mesaxon), and at
the corresponding fold adaxonally, the inner mesaxon. At the paranodes, the terminal
loops touch the axon surface and adhere to the axolemma (Robertson 1957, Ochoa et
al. 1972).
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The sides of the loops are joined to each other and contain both tight junctions (Mugnaini and Schnapp 1974) and desmosome–like structures (Harkin 1964). Thus the periaxonal and endoneurial spaces only connect through the spiral path left between the paranodal loops and the axonal surface, a tortuous route between myelin wraps. At the node, where there is a gap between the myelin of one cell and its neighbour, adjacent Schwann cells form a loose cytoplasmic collar and send irregular processes (nodal fingers) into the space between the axolemma and the basal lamina. The basal lamina forms an unbroken sheath around each Schwann cell that is continuous along the fibres length, with no gaps between adjacent cells.

There is no evidence of dye coupling between adjacent myelin forming Schwann cells, so it is not likely that they contain gap junctions (Konishi 1990).

The rat sciatic nerve.

The sciatic nerve of the rat originates from spinal segments L4–L6; it is a mixed nerve containing axons of motor, sensory and sympathetic neurons. In the main body of the nerve (distal to the sciatic notch, and before any main branches) there are about 27,000 axons, 29% myelinated and 71% unmyelinated. The myelinated axons range in diameter from 1.5\(\mu\)m to 12.5\(\mu\)m and unmyelinated axons have a mean diameter of between 0.7 and 0.8\(\mu\)m (Schmalbruch 1986).

The relative numbers of myelin and nonmyelin forming Schwann cells are hard to measure directly when examining cells dissociated from an adult nerve, this is due to preferential adhesion of the nonmyelin Schwann cells to the culture dish in a short term culture. This problem can be partially overcome if cell suspensions are dried onto slides, but extensive damage to myelin cells during dissociation makes total cell counts inaccurate. Calculations can be made from published information taken from EM and light measurements as to the relative numbers of myelinated and unmyelinated axons, the average length of myelin internodes, the average number of axons to each non–myelin forming cell and the average length of a non–myelin forming Schwann cell. Such a calculation predicts that sciatic nerve would contain 65% non myelin Schwann cells and 35% myelin Schwann cells (see Appendix 1 at the end of this chapter for an example calculation).

Development of peripheral nerve.

The final adult relationship of axons with their associated glia, the myelin forming
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and non-myelin forming Schwann cells, is not complete in the rat until several weeks after birth when the last non-myelin forming Schwann cells develop a mature relationship with their axons (Diner 1965, Jessen et al. 1985 and Eccleston et al. 1987). After this date, although myelin forming Schwann cells continue to grow in length and thickness as the rat grows, there is little change in the number of Schwann cells or axons. A description of neural crest cell migration into the nerve, axon ingrowth and the morphology changes during nerve development is given in Chapter 2.

The role of Schwann cells.

The Schwann cell is famous for its ability to make the myelin sheath. Whilst this function is important, since demyelinating diseases cause paralysis, the Schwann cell has many other roles and some of these are described below.

1. Schwann cells in the mature, intact nerve

The advantages of myelin: Schwann cells and oligodendrocytes of vertebrates make the myelin of the PNS and CNS respectively. Myelin acts as an insulating collar forming internodes along the axon's length, separating the nodes of Ranvier, specialised areas of the axon surface that are electrically excitable. Because of the myelin insulation, an electrical impulse can travel passively a long way down the non-excitable internode. In this way the action potential leaps from one node to the next, a method of conduction called saltatory conduction (from the Latin saltatio: a dance). Saltatory conduction is faster than conduction along a continuously active unmyelinated axon. By making myelin, Schwann cells increase the speed of conduction without an undue increase in axon diameter. Invertebrates do not make myelin, the squid manages to coordinate fast movements by fusing many axons together into one giant axon that can be up to 1mm in diameter. There would not be room for many of the 7830 myelinated axons of the rat sciatic nerve if they had to be that big. Thus, myelination "enables us to carry a much greater number of separate high-speed communication channels packed into a small bundle." (B. Katz., Nerve muscle and synapse. McGraw-Hill 1966).

Prevention of axonal cross talk: Schwann cell processes separate axons from their neighbours to prevent inappropriate excitation of adjacent axons.
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Ion homeostasis: The presence of ion channels on the surface of Schwann cells has led to speculation of a role for Schwann cells in controlling the ionic environment of axons, and in regulating the volume of the periaxonal space. Voltage gated potassium channels are present on non-myelin forming Schwann cells and in the paranodal region of myelin forming Schwann cells (Chiu 1991). It is not clear whether these channels are also on the peri-axonal Schwann cell surface. Extracellular K⁺ in a confined space can be reduced by either removing K⁺ through K channels, or by actively pumping K⁺ into and Na⁺ out of the cell through the electrogenic Na⁺–K⁺–ATPase.

It is not known to what extent Schwann cells really do participate in regulating the immediate surroundings of their axons. The ion channels present on Schwann cells, and their likely activity during the conditions in the endoneurium during and after nerve activity have been reviewed recently (Bevan 1990, Barres et al. 1990, Chiu 1991, Black and Waxman 1990).

Gap junctions, present only between non-myelin forming Schwann cells (Konishi 1991) might also help non-myelin forming Schwann cells prevent a local build up of ions.

Transfer of macromolecules between Schwann cells and axons: In squid, macromolecular transport has been shown to occur bilaterally between Schwann cells and axons (Grossfeld et al. 1988, Tytell and Lasek, 1984). The hypothesis that Schwann cell-derived molecules can be passed to the axon is an attractive one as it would reduce the metabolic burden of transport from the neuronal cell body which can be a great distance away. In addition, Schwann cells could deliver informational molecules (neurotrophic factors) or factors to protect the axon from stress. The 70 kD heat-shock protein (HSP70), a protein with known cytoprotective properties, is one of the molecules demonstrated to be transferred from squid Schwann cells to their axon. The ability of Schwann cells to transfer this molecule to the axon without waiting for transfer of neuron-derived molecules to travel down the axon, a process that moves molecules at less than 4mm each day, may well be important in maintaining axons after injury or metabolic stress (reviewed in Buchheit and Tytell 1992).

The transfer of molecules from the squid Schwann cells to their axon is one that requires metabolic energy. The molecules transferred include, in addition to
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HSP70, actin, glycoproteins, phospholipids, and some of the transferred matter is particulate (reviewed by Buchheit and Tytell 1992). The molecules are transferred as vesicles that leave the Schwann cell and appear in the axonal cytoplasm. The method of this transfer has not been defined, but two models are proposed. Firstly, thin projections from the Schwann cell extend into the axon interior, portions of these are possibly pinched off and internalized by the axon. The alternative mechanism is that the Schwann cell externalizes the vesicles, using a mechanism that has been described for maturing reticulocytes and cultured rat embryonic cells and that is distinct from classic exocytosis (reviewed by Buchheit and Tytell 1992).

Schwann cells synthesise and express Na channels, the voltage activated channels that are responsible for the action potential in excitable cells. These are on the cell body of non-myelin forming Schwann cells (Chiu 1987) and also the channels and/or their precursors are seen in the cytoplasm of paranodal loops of myelin forming Schwann cells (Ritchie et al. 1990). Gray and Ritchie (1985) suggested that Na channels could be manufactured by Schwann cells for transfer to the axon and not for use by the Schwann cell. However, as the Na channels of Schwann cells do not have the same kinetics as those in axons, this hypothesis would require a change in channel properties once in the axolemma (Barres et al. 1990). It has not been shown that such assistance is necessary and in fish, demyelinated axons express increased Na channels (England et al. 1990).

There is also evidence for neuron-to-glia transfer, glia remove damaged materials from drug-intoxicated neurons (Cavanagh et al. 1990). A recent preliminary report has also indicated that Schwann cells are capable of removing retrogradely transported foreign substances taken up by crush injured axons (Gatzinsky and Persson 1992). This indicates a novel aspect of the protective role of Schwann cells after nerve injury.

Axolemmal ion channel distribution: Myelin forming Schwann cells organise axonal ion channel distribution. In a myelinated axon, Na channels are primarily located at the node, whilst fast K channels are located at the internode (Black and Waxman 1990). In fish, demyelination is followed by redistribution of Na channels to the internodal axonal surface (England et al. 1990).
Neurotrophic effects: Two cytoplasmic molecules present in Schwann cells of the intact adult nerve and known to have neurotrophic effects are ciliary neurotrophic factor (CNTF), predominantly expressed by myelin forming Schwann cells (Stöckli 1991, Rende et al. 1992, Friedman et al. 1992) and S-100β, present in both myelin forming and non-myelin forming Schwann cells (Mata et al. 1990). CNTF is a survival and/or differentiation factor for many neurons, including sympathetic neurons (Saadat et al. 1989), primary sensory neurons, (Manthorpe et al. 1986) and motoneurons both in vivo and in vitro (Arakawa et al. 1990, Lin et al. 1990a, Sendtner et al. 1990, Oppenheim et al. 1991). S-100 enhances CNS neuronal survival and neurite outgrowth and DRG neurite outgrowth in vitro (Winningham-Major et al. 1989, Van Eldik et al. 1991) and S100 rescues motor neurons, but not sensory neurons from naturally occurring cell death in vivo (Bhattacharyya et al. 1992). These actions could indicate that Schwann cell synthesis of CNTF and S-100 is required to maintain neuronal function and survival in some systems. If this is so, then a novel method for secretion of both S-100 and CNTF must be used as neither protein has a N-terminal hydrophobic domain (Kligman and Hilt 1988, Stöckli et al. 1989). If the delivery of vesicles from the Schwann cell to its axon described in the invertebrate nervous system (see above) also occurs between mammalian Schwann cells and their axons, then vesicles containing Schwann cell cytoplasm would deliver these neurotrophins into the axonal cytoplasm. The possible role of both S100 and CNTF after nerve injury is described later.

Schwann cells influence axon calibre, neurofilament phosphorylation, and slow axonal transport: The presence of a myelin sheath is essential for axon growth above 1μm in diameter. Axons of animals suffering from demyelinating neuropathies, axons of the hypomyelinating mutant mouse trembler and the naked axons in the roots of dystrophic mice, all have a reduced diameter and if remyelination occurs, or if normal Schwann cells are grafted in, then the axon diameter returns to normal (reviewed in Dyck 1984, Chapter 16 by Aguayo and Bray). In tissue culture, when myelination is prevented, rat DRG neurons never grow axons larger than 1.25 μm in diameter (Windebank et al. 1985).

Neurofilament phosphorylation, cytoskeletal organisation and axonal transport
rates depend on close intercellular contacts between myelinating Schwann cells and axons (de Waegh et al. 1992).

**Schwann cell may have a role during axon firing:** During action potentials, frog Schwann cells at the motor end plate show a transient increase in intracellular calcium, the release is from intracellular stores and can be mimicked by local application of the cotransmitters acetylcholine and ATP (Jahromi et al. 1992). In this preparation, the adjacent myelin-forming Schwann cells did not respond to axon stimulation.

Recently, in three preliminary reports, Schwann cell responses to axonal activation and applied neurotransmitters have been examined. Frog myelin forming Schwann cells in single fibre preparations, unlike those described above adjacent to the intact neuromuscular junction, do increase intracellular Ca^{++} in response to axon activation, and release is from intracellular stores by a ryanodine-sensitive mechanism (Lev-Ram et al. 1992). Cultured rat Schwann cells also show acetylcholine stimulated calcium increase, the action is via nicotinic receptors, release is from intracellular stores, but, unlike in the frog, release may be mediated by inositol triphosphate receptors rather than ryanodine receptors (Yoder et al. 1992). ATP, bradykinin, histamine and glutamine also stimulate Ca^{++} release from a subpopulation of cultured neonatal rat Schwann cells (Lyons et al. 1992).

### 2. Schwann cells after axotomy and in disease

If a peripheral nerve is damaged so that the axons are severed *in vivo* the axon distal to the break degenerates; the resulting changes in the distal nerve are called Wallerian degeneration. During Wallerian degeneration the Schwann cells divide until they fill the spaces inside the basement membranes that previously contained the Schwann cell-axon units. After Wallerian degeneration the distal nerve contains tubes of basement membrane full of Schwann cells, called the bands of Bugner. The degenerated distal nerve provides a favourable environment for axonal regrowth (for reviews see Perry and Brown 1992, Hall 1989).

**As source of neurotrophic factors:** Schwann cells provide survival factors for neurons after nerve lesion. After axotomy, Schwann cells in both the distal and proximal parts of the nerve gain nerve growth factor (NGF) mRNA and secrete NGF protein
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(Heumann et al. 1987). Schwann cells in the distal part of a transected nerve also express brain-derived neurotrophic factor (BDNF) mRNA and secrete BDNF-like activity (Acheson 1991, Meyer et al. 1992). Both these molecules are neurotrophic for neural crest derived sensory neurons in the periphery, BDNF also supports placode-derived sensory neurons (Lindsay et al. 1985).

The developmental expression of CNTF in Schwann cells coincides with the ability of motoneurons to survive after axotomy (Lowrie et al. 1987, 1982, Stöckli et al. 1989), and this has led to speculation that Schwann cell-derived CNTF is a neuron survival molecule at this time. This has recently been questioned however, because nerve lesion causes a reduction in Schwann cell CNTF protein and mRNA levels in the distal stump (Sendtner et al. 1992, Rabinovsky et al. 1992). The CNTF loss after lesion is slow, and the levels that remain in the distal stump, although they are 3–4 times less than the levels seen in the unlesioned nerve, still represent a considerable neurotrophic activity (Sendtner et al. 1992). Schwann cells in the proximal stump, those between the neuronal cell body and the lesion, maintain normal levels of CNTF after lesion (Sendtner et al. 1992). These cells are therefore another possible source of CNTF available to promote motoneuron survival after sciatic nerve lesion, but only if a mechanism exists to release CNTF from the intact cells (see above). Another possibility is that CNTF could leak from the myelin rejected by Schwann cells during Wallerian degeneration. The high CNTF levels in myelin forming Schwann cells might provide a reservoir of trophic factor, available for release during the disruption of myelin forming Schwann cells after injury without the need for de novo protein synthesis.

As described above, S-100 is expressed in Schwann cells, and S-100 is known to have neurotrophic effects, Schwann cell–released S-100 could be active in supporting neuronal survival and axon regeneration after nerve lesion. Like CNTF however, S-100 is expressed in greatly reduced amounts after nerve lesion (Kato and Satoh 1983, Perez and Moore 1968, Spreca et al. 1989).

Two other molecules synthesised by Schwann cells that have neurite-promoting activities are glial maturation factor beta (GMFβ) (Lim and Miller 1988) and the glia-derived nexin (GDN or protease nexin I) (Meier et al. 1989, Bleuel and
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Cell-attached and extracellular matrix (ECM) molecules that affect axon regrowth: Adhesion molecules expressed on the Schwann cell surface, and adhesive ECM proteins that were laid down by the Schwann cell before lesion are important in providing a permissive environment for axonal regrowth. After nerve lesion, regeneration depends on cell-cell and cell-matrix interactions, as well as on the soluble factors described above. Frozen sections of adult rat sciatic nerve are a permissive substrate for neurite outgrowth from embryonic but not adult DRG neurons. When sections are taken from lesioned nerve, then both adult and embryonic neurons are able to extend an extensive network of neurites over the tissue. This indicates that nerve lesion enhances Schwann cell expression of insoluble or surface bound neurite-promoting molecules (Bedi et al. 1992).

The adhesion molecules L1 and N-CAM are both expressed on the surface of Schwann cells after nerve section (Jessen et al. 1987a, Martini and Schachner 1988, Daniloff 1986, Nieke and Schachner 1985). These adhesion molecules are important for Schwann cell-axon interactions (Seilheimer et al. 1989, Wood et al. 1990), and for functional recovery after nerve lesion (Remsen et al. 1990). There is some evidence that selective expression of adhesion molecules by Schwann cells in motor nerves is instrumental in leading regrowing axons of motor neurons into motor, rather than sensory nerves during regeneration. The L2/HNK-1 carbohydrate epitope is expressed at higher levels by the Schwann cells of ventral roots than in dorsal roots. Motor neurons extend more and longer neurites over sections of ventral roots than they do over dorsal root sections. This preference for ventral roots as a substrate is not seen in sensory neurons and is blocked by antibodies to L2/HNK-1 (Martini et al. 1992).

Schwann cell associated N-cadherin is a permissive substrate for neurite outgrowth (Letourneau et al. 1990, 1991) and is present on the surface of Schwann cells in culture.

p30, a molecule recently identified in the chicken PNS (Daston and Ratner
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1991), is also expressed on the extracellular surface of Schwann cell membranes after nerve transection. This molecule is known to promote neurite outgrowth by cultured embryonic CNS neurons (Rauvala and Philaskari 1987) and so p30, like N-cadherin, has been proposed as a factor that enhances axon regrowth.

Schwann cells synthesize a basement membrane, some of the ECM molecules synthesized by Schwann cells and secreted into the surrounding tissues will also associate directly with the Schwann cell surface. These ECM molecules include laminin (Rogers et al. 1986) and tenascin (cytotactin/J1) (Danillof et al. 1989), both of which have neurite-promoting properties (reviewed by Reichardt and Tomaselli 1991).

Schwann cell-secreted ECM molecules laminin, fibronectin, collagens, proteoglycans and tenascin all interact with neurites and to varying degrees all permit neurite outgrowth in culture (reviewed in Sanes 1989 and Tomaselli and Neugebauer 1991, Reichardt and Tomaselli 1991). ECM remains around the bands of Büngner in cut nerve after Wallerian degeneration. The presence of these ECM molecules alone is not, however, sufficient for axon penetration into peripheral nerve stumps. This is shown by experiments where acellular nerve grafts, in which the Schwann cells have been killed but which still contain ECM components, are placed in the path of cut axons. No axon ingrowth occurs in the PNS if Schwann cells are prevented from entering the graft with the ingrowing axons (Hall 1986) and in the CNS, (where there are no Schwann cells to enter with the ingrowing axons) no axons will enter acellular PNS nerve grafts (Smith and Stevenson 1988). If Schwann cells are available, then growth cones and Schwann cells will travel into an acellular graft together, the growth cones using the basement membrane as a scaffold (Osawa et al. 1990).

Phagocytosis of myelin debris and axon debris: Although Schwann cells are capable of myelin phagocytosis and degradation of myelin debris in vitro (Bigbee et al. 1987), they will not remove myelin in vivo if macrophages are prevented from entering the endoneurium (reviewed in Hall 1989, Perry and Brown 1992). It is clear that whilst macrophages are the main cells involved in myelin degradation, and that Schwann cells will not initiate phagocytosis in vivo in their absence, Schwann cells are responsible for some of the myelin clearing (Stoll et al. 1989). In addition, after nerve
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Injury, Schwann cells take on some of the macrophage-related phenotype as they express the galactose specific lectin MAC-2, a molecule that is characteristically expressed by mature inflammatory macrophages (Rotshenker et al. 1992). Schwann cells are capable of synthesising IL1 in response to cytokines and bacterial antigens (Bergsteinsdóttir et al. 1991, Bergsteinsdóttir 1992). IL1 is chemotactic for both macrophages and leukocytes. It has not yet been demonstrated directly that Schwann cells do secrete IL1 after nerve damage in vivo, if they do then Schwann cells might be responsible for the chemotactic signal that calls macrophages to the injured nerve.

Schwann cell division after axotomy: After nerve lesion Schwann cells divide distal to the disruption (Abercrombie and Johnson 1946, Bradley and Asbury 1967, Clemence et al. 1989, Griffin et al. 1990). The resulting increase in cell numbers is critical if regeneration is across a gap (Scaravilli et al. 1986).

When Schwann cells are dissociated from nerves undergoing Wallerian degeneration the yield is higher than when normal nerves are dissociated (Ansselin et al. 1992). This is likely to be partly the result of increased Schwann cell numbers, but higher levels of adhesion molecules on the Schwann cell surface enhancing attachment to the culture substrate may also play a part.

Schwann cell migration: Although mature Schwann cells are generally stationary in vivo and in vitro, they are capable of migration after a demyelinating signal (Griffin et al. 1987), presumably this allows them to collect at the site where axons may need to be re-ensheathed.

Acetyl choline release by denervated Schwann cells: Schwann cells at the frog motor end plate secrete acetyl choline after axotomy (Bevan et al. 1973, Dennis and Miledi 1974). It is suggested that this release of neurotransmitter maintains the motor end plate until axonal regrowth to the endplate.

Schwann cells as antigen presenting cells: Schwann cells in culture are capable of expressing MHC class I and II molecules (Wekerle et al. 1986, Armati et al. 1990, Samuels et al. 1987). MHC class II expression by adult Schwann cells, both in vivo and in vitro is stimulated by interferon γ and soluble or whole M. leprae, (Bergsteinsdóttir 1992, Bergsteinsdóttir et al. 1992) and these activated Schwann cells can induce proliferation in antigen-specific T cells (Kingston et al. 1989).
Presentation of antigens by Schwann cells implies that they may participate in initiating T cell activation after mycobacterial and other infections, functioning in the same way that dendritic cells outside the nervous system act to maintain immune surveillance.

3. Schwann cells during development

Axon guidance: The evidence for Schwann cells, or their neural crest-derived progenitor cells, leading axons to their target was reviewed by Keynes (Keynes 1987). Neural crest ablation, whilst not totally preventing motor innervation, does cause some deficiencies of innervation, suggesting that Schwann cells are necessary for axon growth over long distances, but also suggesting that they are more likely to give trophic support than axon guidance. More recently, work using chick/quail chimeras has shown that the Schwann cells and axons enter the mesenchyme together. Schwann cell progenitor cells do not grow in advance of the growth cone (Carpenter and Holliday 1992a) and seem to be guided by axons to their final destination (Carpenter and Holliday 1992b). Invading growth cones contact both mesenchymal cells and migrating neural crest cells, but neural crest cells are not seen in advance of the axons (Haninec and Dubovy 1992). In the Splotch mutant mouse, the lumbo-sacral nerves are devoid of Schwann cells, but the lumbo-sacral plexus develops, and its branches grow into the hindlimb, and although outgrowth is slower than in normal littermates, they reach the topographically correct position (Grim et al. 1992). These new observations strengthen the conclusion that Schwann cells are not guiding axons to their destination.

Schwann cell growth control

Second messenger systems implicated in Schwann cell growth control:

cAMP elevating agents: When the actions of a hormone are mediated by raising cAMP, a classic series of events follows the binding of the hormone to its receptor. Receptor binding activates a stimulatory GTP-binding regulatory protein (G_s), by phosphorylation of the inactive GDP-bound protein. The G_s protein is made up of three subunits, the α, β and γ subunits, and the GTP binding site is on the α subunit. Activation dissociates the α subunit from the β and γ subunits, in which state it is able to activate adenylyl cyclase. Adenylyl cyclase is a membrane bound enzyme, that
controls synthesis of cAMP from ATP. The holoenzyme protein kinase A (PKA) is an inactive tetramer composed of a regulatory subunit dimer and two catalytic subunits. In the presence of cAMP, two cAMP molecules will bind to each part of the regulatory subunit, releasing the active catalytic subunits. The active catalytic subunits phosphorylate the proteins that mediate the cells' response (e.g., division, differentiation). The levels of cAMP are controlled by a balance in the activity of adenylyl cyclase and the cAMP phosphodiesterases which hydrolyse cAMP to the inactive 5'-AMP. The duration of the effect of cAMP is dependent on the activity of protein phosphatases, these act to dephosphorylate proteins and so terminate the effect of cAMP elevation (Reviewed in Alberts et al. Chapter 12 1989). Thus events that depend on cAMP elevation can be modulated by controlling the level of agonist, amount or activity of receptor, availability or activity of G proteins, adenylyl cyclase, PKA, phosphodiesterases, proteases and protease inhibiting proteins.

**Phospholipase C (PLC):** Signal transduction by many ligands is through the inositol phospholipid pathway. Activation of a receptor, again through a GTP-binding regulatory protein indirectly activates an enzyme, in this case PLC. PLC cleaves PIP$_2$ into inositol 1,4,5-triphosphate (InsP$_3$) and diacylglycerol. It is the action of these two molecules in releasing intracellular Ca$^{2+}$ and activating PKC respectively that mediates the measureable actions of the ligand, i.e., increasing intracellular calcium and phosphorylating proteins (reviewed in Alberts et al. Chapter 12 1989).

**Other calcium:** PLC induced calcium release mediated by InsP$_3$ is only one of the ways in which cells can raise intracellular calcium. Some cells, notably cardiac muscle cells, have calcium channels that can be opened by cAMP dependent phosphorylation (reviewed by Fischmeister and Hartzel 1991). Calcium-evoked calcium release from intracellular stores, through ryanodine-sensitive channels, is induced when intracellular calcium levels become greater than 1$\mu$M in smooth muscle cells (reviewed by Inio 1990). How much these or similar mechanisms are involved in mediating the response of Schwann cells to growth factors and axonal signals is not known.

**Tyrosine kinases:** Surface receptors with intrinsic tyrosine kinase activity mediate the effects of a large group of growth and differentiation factors. Factors in this group

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include bFGF, aFGF, PDGF, IGF-1, insulin, EGF and NGF. The biological effects of these factors interacting with their receptors include cell growth and proliferation and also cell survival, growth arrest and differentiation. One factor can inhibit or promote cell proliferation, depending on the cellular environment in which the receptor is expressed. For example, aFGF, bFGF and NGF have mitogenic effects on fibroblasts that either naturally (aFGF, bFGF) or after transfection (NGF) express the appropriate receptor. When the same factors interact with the same receptors in neuronal cells, receptor activation inhibits cell proliferation and enhances differentiation (reviewed in Schlessinger and Ullrich 1992).

Ligand binding causes dimerization of the receptor, tyrosine autophosphorylation and substrate tyrosine phosphorylation. Intracellular signalling proteins bind the tyrosine phosphorylated region of the receptor via a conserved region (called the SH2 domain for src homology 2 domain), and become tyrosine phosphorylated themselves. Proteins that contain the SH2 domain and act to mediate the effects of growth factor binding, include PLC-γ, the GTPase activating protein (GAP) of ras and a large number of small proteins that act as adaptors or regulatory components of catalytic subunits (reviewed in Schlessinger and Ullrich 1992).

Not every receptor-associated autophosphorylation site binds to the SH2 domains of every SH2-containing protein. Thus, the possible effect of activation of a particular receptor is defined by the number and the nature of tyrosine autophosphorylation sites on the intracellular portion of that receptor, and the availability of correct substrates. It is proposed that receptor specificity is controlled by the combination of proteins expressing the SH2 domain available in the cell (reviewed in Schlessinger and Ullrich 1992).

Growth factors that use tyrosine kinase receptors with possible effects on Schwann cell biology include aFGF, bFGF, PDGF, insulin and the ligand for p185^neu (NDF).

Receptors with serine/threonine kinase activity: TFGβ receptor. TGFβ binds to cells through a number of receptors, some of these are ECM proteins, others are integral membrane proteins (Lin and Lodish 1993). The type II TGFβ receptor has serine/threonine kinase activity (Lin et al. 1992).
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Interactions between second messenger pathways: The multiple steps in the cAMP and phospholipase C pathways lend themselves to interactions where the product of one pathway activates or suppresses a step in another, for example, the Ca\(^{++}\) and cAMP pathways are known to interact. The Ca\(^{++}\)-calmodulin complex will bind to and regulate the enzymes that synthesise cAMP synthesis and breakdown. Calmodulin and cAMP act together to control the activity of the enzyme phosphorylase kinase in mammalian muscle where the catalytic subunit activation requires both adenylyl cyclase phosphorylation of the β and α subunits, and calcium binding to calmodulin, the δ subunit (reviewed by Alberts et al. Chapter 12 1989).

One of the effects of cAMP elevation is to modulate expression of genes that contain a promoter element termed CRE (cAMP response element). PKA controls transcription of these genes through the cAMP response element binding protein (CREB). CREB is a nuclear protein that binds to the CRE element as a dimer, and PKA controls the ability of CREB to enhance transcription from CRE. The CREB protein isolated from PC12 cells has been shown to contain, in addition to PKA recognition sites, PKC and casein kinase II recognition sites, suggesting an interaction between several second messenger pathways in the control of the expression of one gene (Gonzalez et al. 1989).

As discussed later, in order to stimulate Schwann cell proliferation in vitro, it is often necessary to use a combination of drugs that will together activate more than one second messenger system. It is known that activation of one path can act to change the sensitivity to another in an indirect way, as shown by the increased expression of PDGF receptors by Schwann cells in response to cAMP elevation, explaining the dependence on raised cAMP levels before PDGF has mitogenic effects (Weinmaster and Lemke 1990). However, it is also possible that direct interactions between the second messenger systems occur, in a similar way to the interactions described above.

Multiple pathways can be activated in response to activation of one receptor: It was discussed above how activation of tyrosine kinase receptors can activate PLC, and will also phosphorylate other SH2 containing proteins. In an other example, the hormone erythropoietin activates a serine/threonine-specific phosphatase-dependent
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pathway, and also activates PKC. Erythropoietin regulates the growth and differentiation of red blood progenitor cells and activation of these two enzymes that may have opposite effects on the same substrate implies that the receptor activated kinase and phosphatase are kept separate, possibly through subcellular compartmentalization (Patel et al. 1992).

Mitogens for cultured Schwann cells

**cAMP elevation:** When applied to Schwann cells in tissue culture, both forskolin and cholera toxin, drugs which activate adenylyl cyclase and elevate cAMP, cause Schwann cell mitosis if added with serum or (to a lesser extent) with plasma (Raff et al. 1978b, Davis and Stroobant 1990). Dibutyryl cAMP (db-cAMP), a cell soluble analogue of cAMP that mimics the actions of naturally occuring cAMP, also causes Schwann cell mitosis in the presence of serum (Eccleston et al. 1989b). In defined medium neither forskolin nor db-cAMP cause Schwann cell mitosis (Stewart et al. 1991). As described later (see below), PDGF, aFGF, bFGF and GGF only show their maximal mitotic effects on Schwann cells if added together with a cAMP elevating drug. cAMP increases the number of PDGF receptors expressed by Schwann cells (Weinmaster and Lemke 1990) and it is possible that it also acts to elevate other growth factor receptors. No defined growth factors elevate Schwann cell cAMP levels, and this has led to a search for novel, axon-derived molecules that may elevate Schwann cell cAMP. Such a molecule could then, together with a growth factor, cause the axon-related Schwann cell division described below.

The neuropeptides vasoactive intestinal peptide (VIP) and secretin both increase Schwann cell cAMP levels in vitro. The adrenergic agonists isoproterenol, noradrenaline and adrenaline can also raise Schwann cell cAMP levels, and their activity is via β1 adrenergic receptors. Synergistic effects are seen when VIP or secretin are added together with noradrenalin (Yasuda et al. 1988). VIP, noradrenalin and adrenaline, however, are not Schwann cell mitogens (Davis and Stroobant 1990).

Increased expression of Schwann cell extracellular matrix molecules including laminin is seen after cAMP elevation (Baron–Van Evercooren et al. 1986). As described later, matrix molecules affect the response of Schwann cells to mitogens. This raises the possibility that an autocrine loop exists, in which the Schwann cell
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response to a mitogen is modulated by changed expression of matrix molecules. 

1-oleoyl-2-acetylglycerol (OAG) and calcium ionophore A23187: It is possible to mimic the effect of growth factors whose receptors cause activation of PLC by adding the calcium ionophore A21387 to elevate intracellular Ca\(^{++}\) and simultaneously activating PKC by adding OAG, an active analogue of diacylglycerol. Saunders and DeVries have used the calcium ionophore A21387 and OAG together to stimulate Schwann cell division, mitosis requires both drugs. The maximal response achieved, however, is only 30% of the response to axolemma- or myelin-fractions (see below) (Saunders and DeVries 1988).

Axon contact in vitro induces Schwann cell mitosis. Sensory neurons (Salzer et al. 1980), granule cells (Mason et al. 1989), PC12 cells (Cochran 1985), ciliary neurons (Muir et al. 1990) and growth cones (Dent et al. 1992) all induce Schwann cell division in vitro. This stimulation has been shown to require direct contact, as enhanced division by Schwann cells co-cultured with neurons is restricted to the cells touching neurites (Salzer et al. 1980).

Axolemma and myelin–enriched fractions: Fractions of neuronal membranes have been purified from CNS and PNS tissues of rat and cow and all of these fractions induce Schwann cell mitosis (reviewed in Pleasure et al. 1985). An axolemma–induced increase in Schwann cell cAMP levels has been predicted and searched for, but two groups did not find that CNS–derived axolemma increased Schwann cell cAMP levels (Meador-Woodruff et al. 1984, Pleasure et al. 1985). Another group, however, found that axolemma from cultured rat sensory ganglia and from PC12 cells did increase Schwann cell cAMP (Ratner et al. 1984).

Myelin–enriched fractions extracted from the CNS of both rat and cow are capable of inducing Schwann cell mitosis (Meador-Woodruff et al. 1984), but purified rat PNS myelin is not mitogenic (Sobue et al. 1983). Treatment with CNS–derived myelin does not elevate Schwann cell cAMP levels (Meador-Woodruff et al. 1984). Both axolemma and myelin cause diacylglycerol production, implying that they may both activate the inositol phospholipid pathway (Saunders and DeVries 1988).

Enzymes that affect Schwann cell division: The plasminogen activator urokinase is
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secreted by neurons, and if extrinsic urokonase is added to cultured Schwann cells both the basal and cholera toxin-induced rates of division are increased in serum free medium (Baron-Van Evercooren et al. 1987). Another type of plasminogen-activator activity is secreted by Schwann cells, this is increased when Schwann cells are induced to divide in the presence of GGF or cholera toxin in serum (Kalderon 1984, Baron-Van Evercooren et al. 1987).

Neuron-derived growth factor (NDGF): A heparin-binding protein with Schwann cell mitogenic activity is associated with axonal membranes (Ratner et al. 1988). This activity has been isolated from developing brain. The partially purified protein, which has a major component of 50kD and a minor component of 30kD, stimulates Schwann cell mitosis in serum free medium (Nordlund et al. 1992). This factor is possibly the active factor expressed on the axon surface and in axolemma fractions.

Schwann cell growth factors from macrophages and lymphocytes: Macrophages that have digested myelin membrane fragments and rat spleen-derived lymphocytes and monocytes that have been activated with concanavalin-A both secrete factors that are mitotic for Schwann cells (Baichwal et al. 1988, Lisak et al. 1985). The active factors in these conditioned media have not been identified, but activated macrophages secrete PDGF, TNF, TGFβ, TGFα, prostaglandin E2, IL-1α, IL-1β as well as enzymes associated with phagocytosis (discussed in Lindholm et al. 1987, Besner et al. 1990). aFGF and bFGF are contained in the cytoplasm of macrophages, but are not secreted (Besner et al. 1990), of these factors, PDGF, TGFβ, aFGF, bFGF, as described later, all enhance Schwann cell mitosis.

Basic fibroblast growth factor (bFGF/FGF–2) and Acidic fibroblast growth factor (aFGF/FGF–1): bFGF has been reported to be inactive (Stewart et al. 1991), or weakly mitotic (Schubert 1992) in defined medium. bFGF is described as variously mitogenic (Chen et al. 1991) or not mitogenic (Davis and Stroobant 1990) in the presence of serum. In the presence of cAMP elevating agents bFGF becomes a potent Schwann cell mitogen both with (Davis and Stroobant 1990, Chen et al. 1991) and without (Stewart et al. 1991) serum. The matrix molecules laminin, fibronectin and collagen type I enhance the mitogenic effects of bFGF (Chen et al. 1991).

aFGF is either non-mitogenic (Davis and Stroobant 1990) or weakly
mitogenic for cultured Schwann cells (Chen et al. 1991) in the presence of serum. 
aFGF becomes a strong mitogen when added with cAMP elevating agents and plasma 
or serum, (Davis and Stroobant 1990, Chen et al. 1991). Unpublished results from 
this laboratory indicate that aFGF, like bFGF, is not a mitogen in defined medium 
alone, and is strongly mitogenic in the presence of cAMP elevating agents, even in the 
absence of serum (H. J. S. Stewart, A. Sinanan personal communication).

Activated macrophages are capable of making aFGF and bFGF. Both factors 
are present in neurons, aFGF is present in DRG neurons, primary sensory neurons and 
retinal ganglion neurons (Schnürrch and Risau 1991, Elde et al. 1991). bFGF is 
located in the cytoplasm, aFGF is membrane bound, on the cytoplasmic face of the 
plasma membrane of the cell body, dendrites and axons (Elde et al. 1991). The 
absence of a hydrophobic signal sequence for translocation across the endoplasmic 
reticulum, characteristic of secreted proteins, in both bFGF and aFGF has led to the 
speculation that these factors are sequestered in the synthesising cell and only released 
after cell injury or death (Elde et al. 1991). The level of FGF receptor mRNA 
decreases over the first 3 postnatal days in rat sciatic nerve, but FGF itself is present in 
both neonatal and adult rat sciatic nerve (Davis 1992).

The actions of bFGF and aFGF are modulated by, and to some extent 
dependent on cell-surface associated heparan sulphate proteoglycans (HSPGs). At 
the cell surface, HSPGs bind bFGF with low affinity and present bFGF to the high 
affinity tyrosine kinase receptors. HSPG is present on the cell surface of Schwann 
cells (Baron–Van Evercooren et al. 1986), this is likely to mediate their response to 
both factors. HSPGs in the ECM bind bFGF and ECM bound bFGF forms a reservoir 
for soluble bFGF, that can be released after injury by heparin-like molecules and 
heparanase (Vlodavsky et al. 1991). In addition, both growth factors are protected 
from degradation by heparin binding, and aFGF is only active in promoting Schwann 
cell division in culture when soluble heparin is present, possibly due to this protective 
effect of heparin (reviewed in Basillico and Moscatelli 1992). 

Kaposi sarcoma FGF (kFGF, FGF–4): Unpublished results by Andrea Sinanan (UCL 
Department of Anatomy and Developmental Biology UCL, BSc. project 1992) 
indicate that this member of the FGF family is also mitogenic for Schwann cells, it is
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slightly less potent than bFGF, and active under the same conditions as those described for bFGF above.

Platelet-derived growth factor (PDGF) (BB, AB forms only) will not stimulate Schwann cell division in tissue culture in defined medium (Eccleston et al. 1990, Stewart et al. 1991). In the presence of serum PDGF is either mitogenic (Eccleston et al. 1990) or not mitogenic (Davis and Stroobant 1990). However, PDGF becomes a potent mitogen in the presence of cAMP elevating agents (Davis and Stroobant 1990, Stewart et al. 1991), this increase in mitogenicity after cAMP elevation has been shown to be due to increased expression of receptors for PDGF after cAMP treatment (Weinmaster and Lemke, 1990). PDGF-AA is not mitogenic for Schwann cells (Davis and Stroobant, 1990), this is because Schwann cells predominantly express PDGF-β receptors (Eccleston et al. 1990, Weinmaster and Lemke, 1990). Activation of PDGF receptors causes immediate elevation in Schwann cell expression of the proto-oncogene *c-fos*, an immediate–early transcription factor that has been widely implicated as an early mediator of mitogenic and differentiation stimuli (Weinmaster and Lemke, 1990).

PDGF is present in megakaryocytes and platelets, and platelet release is responsible for the PDGF in serum (Davis and Stroobant 1990). Activated macrophages release PDGF (Lindholm et al. 1987). Neurons contain PDGF–A and –B chains (Yeh et al. 1991, Sasahara et al. 1992), both these factors are secreted from cells via the exocytotic pathway and so PDGF–BB need not be restricted to acting only after cell damage. Schwann cells are capable of secreting PDGF–BB isoform in culture which could, therefore, act in an autocrine manner to stimulate proliferation (Eccleston et al. 1990). The level of PDGFβ receptor mRNA in rat sciatic nerve declines over the first 3 postnatal days to be maintained at low levels in the adult (Davis 1992, Hardy et al. 1992). Hardy and coworkers did, and Davis did not, find PDGF–B chain mRNA in sciatic nerve (Hardy et al. 1992, Davis 1992). Therefore, if PDGF–B chain is present in the sciatic nerve, is not clear whether the source is Schwann cells or neurons.

Transforming growth factor beta 1 (TGFβ1) is a mitogen for Schwann cells in the presence of serum (Eccleston et al. 1989b, Ridley et al. 1989), but is not mitotic, or is
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only weakly mitotic in defined medium (Stewart et al. 1991, Schubert 1992). cAMP elevating agents at low concentrations enhance mitosis in response to TGFβ1 in serum. Unlike PDGF, aFGF and bFGF above, cAMP is not a co-mitogen for TGFβ1 in the absence of serum (Stewart et al. 1991). Synergistic effects on Schwann cell division are, however, seen when TGFβ1 is added with bFGF or PDGF–BB in defined medium (Schubert 1992). TGFβ can therefore replace cAMP elevation to enhance the mitogenic effects of growth factors or serum on Schwann cells. There is no evidence that TGFβ elevates Schwann cell cAMP.

The richest source of TGFβ1 is in blood platelets, however it is expressed in many embryonic and adult tissues (Massagué 1990). In the embryonic mouse, TGFβ1 is only present in small amounts in the embryonic and adult nervous system (Flanders et al. 1991, Massagué 1990). TGFβ1 contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum, characteristic of secreted proteins (Massagué 1990) and activated macrophages release TGFβ (Lindholm et al. 1992). TGFβ1 is synthesised as a latent complex of high molecular weight, whose activity is controlled by a specific binding protein (Miyazono et al. 1991), the bioactive molecule is cleaved from the large pro–form of TGFβ (Massagué 1990). The biological actions of TGFβ are varied, and include upregulation of ECM synthesis, inhibition of ECM degradation and enhancement of both cell–cell and cell–matrix adhesion; TGFβ stimulates or inhibits cell proliferation, depending on the system examined. PDGF–B expression is induced by TGFβ in some cells, and this can have autocrine growth stimulatory effects. The growth–inhibitory effects of TGFβ1 are proposed to be via control of the activity of the (tumour suppressor) retinoblastoma gene product, a protein that inhibits c–myc transcription, preventing c–myc induced cell proliferation (reviewed by Massagué 1990, Moses et al. 1990).

Schwann cell proliferation is affected by both ECM molecules and PDGF. TGFβ1–induced changes in Schwann cell secretion of ECM molecules or PDGF secretion have not been examined, but it is likely that the observed effects of TGFβ1 are in part secondary to effects on Schwann cell ECM synthesis or growth factor production.

Transforming growth factor beta 2 (TGFβ2), like TGFβ1, is a mitogen for Schwann
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cells in the presence of serum, and cAMP enhances this mitogenic effect (Ridley et al. 1989). TGFβ2 is released by platelets, and is present in motorneurons, DRG neurons, autonomic neurons and in peripheral nerve fibres, and during early development TGFβ2 expression is detectable in the fibres before the cell bodies of developing PNS neurons (Massagué 1990, Flanders et al. 1991).

Glial growth factor (GGF) is mitogenic for Schwann cells in the presence of serum (Davis and Stroobant 1990) and it is not generally considered to be mitogenic in defined medium (Stewart et al. 1991), however Schubert (1991) has described GGF-induced Schwann cell mitosis in defined medium. The addition of cAMP elevating agents enhances the mitogenic effect of GGF on Schwann cells both with (Raff et al. 1978a) and without (Stewart et al. 1991) serum. GGF activity is extracted from pituitary glands, and has recently been purified from bovine pituitary, GGF consists of two peptides, one of 33 kD, the other 59 kD, named GGF–I and GGF–II respectively. The two proteins are highly homologous to each other (Goodearl et al. 1992), and it has been suggested that they are also homologous to NDF, the neu ligand (A. D. J. Goodearl, personal communication to R. Mirsky; see below for discussion of the neu ligand). The distribution of GGF in the PNS is unknown, but with oligonucleotide probes for GGF mRNA now available (Marchionni et al. 1992), the location of GGF synthesis in vivo will soon be determined, and it will be possible to determine whether GGF mediates Schwann cell mitosis in vivo.

p185<sup>neu</sup> (c-erb B–2, HER–2) and Neu differentiation factor (NDF or neu ligand): p185<sup>neu</sup> is a tyrosine kinase with homology to the EGF receptor family, it is expressed on the surface of Schwann cells during development and after nerve lesion, but not in the adult nerve (Cohen et al. 1992). This distribution has led to speculation that p185<sup>neu</sup> stimulates Schwann cell division, as it is expressed in vivo at times when Schwann cells are dividing. There is evidence for p185<sup>neu</sup> involvement in regulation of Schwann cell division; amplification or over-expression of p185<sup>neu</sup>, as seen after chemically induced mutations, causes increased tyrosine kinase activity and is oncogenic (Bargmann and Weinberg, 1988, Perantoni et al. 1987, Nikitin et al. 1991). The levels of p185<sup>neu</sup> can, however, be high in non-dividing Schwann cells, this is seen 3 weeks after permanent nerve lesion, when Schwann cell division has peaked
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and returned to near zero, but p185<sup>neu</sup> levels remain high (Cohen et al. 1992). In culture, Schwann cells express neu mRNA and p185<sup>neu</sup>, the level of neu mRNA is increased after adding GGF and forskolin, or forskolin alone in defined medium (Cohen et al. 1992).

The ligand for p185<sup>neu</sup>, NDF, has recently been described; NDF is a transmembrane glycoprotein that has extracellular EGF-homology and one immunoglobulin homology unit. The secreted protein is presumed to be cut from this membrane associated molecule, and the EGF-like domain is probably the receptor recognition site (Wen et al. 1992). NDF is expressed by ras oncogene-transformed fibroblasts (Wen et al. 1992). In mammary carcinoma cells, NDF inhibits DNA synthesis and promotes differentiation, and it is proposed that p185<sup>neu</sup> mutations lower NDF binding to the receptor, leading to loss of growth control (Peles et al. 1992, Wen et al. 1992).

These observations are clearly contradictory, if constitutive activation of neu receptor causes increased tyrosine kinase activity and oncogenesis, why does normal activation by the ligand of the receptor have differentiating and growth-inhibiting effects, apparently opposite results from activation of one receptor? Possibly there is more than one ligand for neu, one with inhibitory and the other excitatory effects. It is not yet clear how neu and NDF are involved in regulation of Schwann cell differentiation and division.

**Autocrine Schwann cell growth–promoting factors:** Some of the factors that affect Schwann cell division possibly act through changed expression of one or all of the Schwann cell autocrine factors described below.

1. Primary Schwann cell cultures secrete a factor that enhances their own division, this factor requires laminin as a co-mitogen (Porter et al. 1987). As described later (see below), laminin enhances the effect of some mitogens on Schwann cells. There is no evidence to show whether this factor is the same as one of the autocrine Schwann cell growth–promoting factors described below.

2. Schwannoma–derived growth factor (SDGF): The schwannoma line JS1 secretes a heparin–binding factor that is possibly mitogenic for Schwann cells. The factor has a molecular weight of between 31 and 35kD. Low amounts of mRNA for this factor
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are present in foetal sciatic nerve. SDGF applied to Schwann cells in defined medium for 7 days will double Schwann cell numbers, this could be a very weak mitogenic effect, but may indicate enhanced Schwann cell survival or attachment to the substrate (Kimura et al. 1990).

3. Glial maturation factor-beta (GMF): GMF is an acidic protein isolated from adult brain, it is mitotic for Schwann cells in culture, the mitotic effects of GMF are not enhanced by cholera toxin or pituitary extract (Bosch et al. 1984). GMFβ is a 17kD protein purified and sequenced from this extract. GMFβ is expressed by Schwann cells in culture and after axotomy but not in intact adult nerve (Bosch et al. 1989). It is therefore possible that GMFβ is an autocrine factor, important in the Schwann cell mitotic response to axotomy, in addition to having possible neurotrophic effects after axotomy.

4. PDGF-BB is synthesised by long-term cultures of Schwann cells in which normal growth control is lost, and antibodies to PDGF reduce DNA synthesis in these cells (Eccleston et al. 1990, 1991). Normal, short term cultured Schwann cells also secrete PDGF-like activity (Eccleston et al. 1990).

5. Laminin, or the laminin isoform merosin, is synthesised by and expressed on the surface of Schwann cells both in vivo and in culture (see Chapter 2). As described later, extrinsic laminin on the tissue culture substrate enhances the response of Schwann cells to mitogens, so it is likely that this intrinsic laminin also modulates the response of Schwann cells to mitogens.

Foetal calf serum, neonatal calf serum and foetal calf plasma are all co-mitogens for various Schwann cell growth factors (see individual factors described above). Different batches of serum give different basal and stimulated levels of Schwann cell division (Davis and Stroobant 1990), batches of serum are usually screened for low basal activity before use. Plasma (Davis and Stroobant 1990) or defined medium (Stewart et al. 1991) can be used as a substitute for serum to reduce platelet-derived and other factors when screening for mitogens.

Effect of tissue culture substrate on Schwann cell division: The tissue culture substrate can have a profound effect on the basal rate of Schwann cell division, and on their response to mitogens. When Schwann cells are grown on the complicated
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mixtures of matrix components from corneal endothelial cells or basement membrane
gel from EHS sarcoma, basal rates of division are raised whether the cells are grown
in serum or defined medium (Baron-Van Evercooren et al. 1986). These matrix
mixtures are not pure, and reconstituted basement membrane (Matrigel) is known to
contain several growth factors including TGF\(\beta\), EGF, bFGF, IGF-1 and PDGF
(Vucicevic et al. 1992), so mitotic effects could be due to growth factors bound to, or
released from, these substrates. HSPG on the cell surface and in ECM binds bFGF,
cell surface HSPG acts as a low-affinity FGF receptor, concentrating bFGF near to
the high affinity receptor, and possibly also changing the interaction between bFGF
and the high affinity receptor (reviewed by Klagsbrun and Baird 1991).

Serum contains many growth factors, and batches of serum vary in the amount
of factors they contain. If cells are plated on to substrates in a serum-containing
medium, then differing amounts of these growth factors are likely to bind to the
substrate and some will remain after the serum is removed. These serum-derived
growth factors are probably the reason for inconsistent Schwann cell responses to
mitogens occasionally seen by different groups.

Fibronectin has been shown to increase Schwann cell division, both with and
without added serum (Baron-Van Evercooren et al. 1986, 1987). Laminin has been
described as reducing (Baron-Van Evercooren et al. 1986) or raising basal rates of
Schwann cell division (McGarvey 1984) in defined medium. However, Chen and
coworkers (1991) found that neither fibronectin, laminin nor collagen type 1 had any
effect on basal rates of Schwann cell division in serum. Matrix proteins have a
promoting effect on Schwann cell response to growth factors; laminin, fibronectin and
collagen type 1 all increase the mitotic response of Schwann cells to bFGF in serum
(Chen et al. 1991).

Changes in Schwann cell response to mitogens with age: Between postnatal day (P)2
and P6, Schwann cells increase in their ability to respond to myelin-enriched
membrane fractions. Yoshino and coworkers found that this increased ability to
respond to myelin was due to more Schwann cells becoming sensitive, and not an
increased rate of division in the whole Schwann cell population (Yoshino et al. 1987).

Neonatal Schwann cells show no increase in Schwann cell mitosis after nerve
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transection, rather, in postnatal day 3 or 10 rats, nerve transection is followed by a decline in Schwann cell mitosis (Komiyama et al. 1992).

Schwann cell division in vivo:

In the adult animal, under normal conditions, Schwann cells do not divide (Terry et al. 1974, Pellegrino and Spencer 1985).

Schwann cells divide in vivo during development, in response to cut or crush injury of their axons (Abercrombie and Johnson 1946, Romine et al. 1976), and after demyelination even when the axon is undamaged (Griffin et al. 1990, Toews et al. 1990). This damage-induced Schwann cell division is not maintained, either ending when axons are re-ensheathed, or being greatly reduced 1–2 weeks after permanent axotomy (Pellegrino and Spencer 1985). There is a later burst of Schwann cell division if axons are allowed to grow into a long-term transected nerve (Pellegrino and Spencer 1985).

What are the signals for mitosis in vivo? Despite the many factors described above that induce Schwann cell mitosis in vitro, the causes of Schwann cell division in vivo are not clear. In the following section some of the possible factors that might act in vivo to stimulate Schwann cell division are discussed.

Axonal contact. During Schwann cell division during development, regeneration and repair after demyelination the cells are in contact with the axonal membrane, a known mitogen source in vitro.

Macrophages and Schwann cell division in vivo: Wallerian degeneration after nerve transection is accompanied by invasion by blood borne monocytes, which become active macrophages in the nerve. After nerve transection, the time course of cell division in the distal stump is consistent with division being driven by factors released by activated macrophages as they invade the nerve (Clemence et al. 1989).

Macrophage entry from the blood can be prevented after a nerve is cut if a piece of the cut nerve is placed in a Millipore chamber designed to exclude cell entry, but allowing nutrients to pass through into the nerve, and the chamber is implanted in the abdominal cavity. Without macrophage invasion, the Schwann cells reject their myelin, but they do not divide, and they do not degrade myelin, axon degradation, however, continues as normal. If the chamber is designed to allow macrophage entry
in the same system, then Wallerian degeneration continues as normal (Beuche and Friede 1984).

C57BL/01a mice have a dominant mutation which is characterised by the absence of Wallerian degeneration after nerve injury. The cut axon does not degenerate and there is no macrophage invasion, the Schwann cells remain attached to the axon, they reject their myelin very slowly, and they do not divide (reviewed by Perry and Brown 1992). From the above, it seems that degenerating axons produce an insufficient stimulus for Schwann cell mitosis (although as axon degeneration is present in nerve explants in Millipore chambers, this may stimulate myelin rejection), and that macrophage-derived signals are necessary to stimulate Schwann cell division during Wallerian degeneration (reviewed by Perry and Brown 1992).

When a nerve containing predominantly unmyelinated axons is cut, the mitosis seen in the Schwann cells during Wallerian degeneration is less than after transection of a nerve containing myelinated axons. This has been shown to be due to fewer mitotic stimuli in the cut unmyelinated nerve, rather than due to an inherent lack of responsiveness in the non–myelin forming Schwann cells as in a mixed nerve they divide nearly as much as the myelin forming Schwann cells (Clemence et al. 1989). The reason for this difference is not clear, the presence of degrading myelin could itself be mitogenic, or the mixed nerve could attract and activate more macrophages, which then release more mitogens.

Schwann cell sensitivity to mitogens in vivo: Mature Schwann cells contact the axon surface and they do not divide. There are other situations in vivo when Schwann cells are in the presence of known mitogens, but they do not divide. The first is seen in post-ganglionic sympathetic neurons; the axons of these neurons occasionally have a double myelin sheath, where an internode of myelin has formed underneath an established myelin sheath leaving the outer cell without axonal contact (Kidd and Heath 1988a, 1988b). After axon destruction, as demyelination proceeds the axon degenerates, macrophages invade and the inner Schwann cell divides, however many of the outer sheaths remain intact, showing no division and no myelin degradation (Kidd et al. 1992, Kidd and Heath, 1991). The second example is after IDPN-induced paranodal demyelination. The paranodes are loosened, but full
demyelination does not occur, the myelin forming cell does not divide, but nearby Schwann cells of unmyelinated axons do divide. In this case it is less certain that the mitogen is from macrophages, as few phagocytic cells are seen (Griffin et al. 1987). Thirdly, in a partially damaged nerve, the undamaged axons and myelin Schwann cells are not attacked by the macrophages collected around the cut axons nearby, and the myelin forming Schwann cells do not divide even though the macrophages are activated, and the Schwann cells of the cut axons are dividing (see Perry and Brown 1992 for review).

After lysophosphatidyl choline (LPC) or tellurium induced demyelination, macrophages invade the nerve, the myelin forming Schwann cells do divide even in the absence of a degenerating axon to render the cells sensitive to mitogens secreted by macrophages. As these toxins act directly on the Schwann cell, it may be that Schwann cell damage can induce mitogen sensitivity directly (Toews et al. 1990, 1992, Griffin 1990).

From these examples it is likely that mature myelin forming Schwann cells are not sensitive to mitogens, and that a disruption (axon degeneration, toxic insult) is necessary to render them sensitive to mitogens. The response to paranodal demyelination would suggest that non-myelin forming Schwann cells are more responsive to mitogens than the myelin forming Schwann cells, they may require less of a stimulus to render them sensitive to mitogens.

Schwann cell growth–inhibition in vitro:
As discussed above, there are conditions in vivo when Schwann cells do not divide, even in the presence of known mitogenic factors. In vitro, some factors have been described that suppress basal levels of Schwann cell mitosis or reduce the effects of mitogens on Schwann cells.

Autocrine growth inhibitory activity: Schwann cells in short term cultures secrete a factor that inhibits their own division (Muir et al. 1990, Eccleston et al. 1991). This factor inhibits unstimulated Schwann cell division, and division stimulated by cholera toxin, laminin, ciliary neurons, (Muir et al. 1990), GGF and 8-bromo cAMP (8b-cAMP) (Eccleston et al. 1991) in the presence of 10% serum. This factor has been identified as a 55-kD protein with metalloprotease activity and stromelysin
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immunoreactivity (Muir et al. 1990, Muir and Manthorpe 1992). It probably works by cleaving a fragment from the fibronectin molecule (present in serum) that is itself antiproliferative when added to Schwann cells (Muir and Manthorpe 1992).

Contact inhibition: Densely plated Schwann cells respond less to combinations of growth factors known to be highly mitogenic for more sparse cultures. It is possible that this is in part due to contact inhibition, a direct inhibition of division due to a physical contact between adjacent cells.

In other cell types cell–cell contact has been shown to inhibit cell division, and this effect has been separated from the effects of secreted growth inhibitory factors, or limiting amounts of growth factors (Galkina et al. 1992). In some cells intercellular coupling through gap junctions is proposed to mediate contact inhibition of proliferation (Naus et al. 1992).

Enteric neurons express an axon–associated growth inhibition molecule: When long term cultured Schwann cells are co–cultured with purified neurons from enteric ganglia their basal rate of division is reduced (Eccleston et al. 1989a). This is unlike other neuron–associated molecules which are are mitogens for Schwann cells.

Interferon–γ inhibits the mitotic response of Schwann cell to cAMP analogues in serum, but not to axolemma or GGF (Eccleston et al. 1989b). Peripheral and central neurons have been reported to express a molecule related to γ interferon (Ljungdahl et al. 1989).

Collagen type 1: Some preparations of collagen type 1 inhibit Schwann cell division (Eccleston et al. 1989c). However this is not always the case, and Chen and co–workers (Chen et al. 1991) found that type 1 collagen did not affect basal rates of Schwann cell division and that it enhanced the mitotic response of Schwann cells to forskolin and bFGF in serum.

Gangliosides: The gangliosides GM1 and GM3 are present in both the CNS and PNS. Addition of either GM1 or GM2 inhibits both the basal level of Schwann cell division and axolemma and cAMP stimulated division in vitro. The presence of gangliosides does not prevent cAMP elevation in response to forskolin (Yasuda et al. 1988). Gangliosides inhibit autophosphorylation of EGF–Rs and possibly PDGF–Rs in other cell types (Bremer et al. 1986). It is possible that gangliosides make Schwann cells
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unresponsive to mitotic stimuli by inactivating growth factor receptors.

**Neurofibromin, p21\textsuperscript{ras} and p120\textsuperscript{GAP}**: Neurofibromatosis type 1 (NF1, von Recklinghausen neurofibromatosis) is a genetic disease that is characterized by café-au-lait spots and neurofibromas, benign, but often disfiguring Schwann cell growths that emerge from peripheral nerves. It has been shown that the Schwann cells in neurofibromas have changed angiogenic and invasive properties, making them unlike normal Schwann cells (Sheela et al. 1990). There is an increased incidence in malignant tumours in some NF1 patients. NF1 is the result of disruption of one gene, called the NF1 gene, and a point mutation is sufficient to cause the disease. The neurofibromin gene encodes a 220 kD protein called neurofibromin (Daston et al. 1992) that is related to p120\textsuperscript{GAP}, the ubiquitous GTPase activating protein (Xu 1990). Neurofibromin antibody recognises non-myelin forming, but not myelin forming Schwann cells (Daston et al. 1992).

\(p21\textsuperscript{ras}\) is a regulatory membrane protein that exists in GTP- and GDP-bound forms, and \(p21\textsuperscript{ras}\) is part of the signal transduction pathway, linking external signals to cellular effects. \(p120\textsuperscript{GAP}\) converts the active GTP-bound \(p21\textsuperscript{ras}\) to the inactive GDP-bound form and acts to control the levels of active \(p21\textsuperscript{ras}\). Oncogenic transformation via \(p21\textsuperscript{ras}\) uncouples the transduction pathway from the requirement for an external signal, and since the oncogenic form of \(p21\textsuperscript{ras}\) lacks intrinsic GTPase activity, it is permanently active (Leevers and Marshall 1992). In normal cells GAP is also associated with EGF and PDGF receptors (Kaplan et al. 1990, Ellis et al. 1990) and scrape loading of \(p21\textsuperscript{ras}\) oncoprotein into Swiss 3T3 cells activates PKC in 5 minutes, \(c\text{-myc}\) in 1–2 hours and transforms the cells (discussed in Leevers and Marshall 1992).

In Schwann cells, GTP–\(p21\textsuperscript{ras}\) binds to both neurofibromin and its homologue \(p120\textsuperscript{GAP}\). GTP–\(p21\textsuperscript{ras}\) is growth–inhibitory for Schwann cells when bound to neurofibromin, and growth–stimulatory when alone, or if bound to \(p120\textsuperscript{GAP}\) suggesting that in Schwann cells, neurofibromin rather than \(p120\textsuperscript{GAP}\) acts to curb the effects of GTP–bound \(p21\textsuperscript{ras}\) (Basu et al. 1992, DeClue et al. 1992). Added \(p21\textsuperscript{ras}\) is normally growth inhibitory for primary Schwann cells in culture, unlike fibroblasts where \(p21\textsuperscript{ras}\) stimulates division. In combination with nuclear
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oncogenes, however, p21\textsuperscript{ras} proteins can stimulate Schwann cell proliferation (Ridley et al. 1988).

In the benign tumours of NF1, the cells will have a normal allele for neurofibromin and a defective allele. With intermediate levels of neurofibromin, these cells are able to partially control the level GTP–bound p21\textsuperscript{ras} and show only a partial loss of growth control (discussed in Bollag and McCormick 1992).

NF1 malignant tumours contain normal p21\textsuperscript{ras} and p120\textsuperscript{GAP} (Basu et al. 1992). Neurofibromin is low or lost in malignant NF1 tumours, where a "second hit" destroys the single normal allele for the NF1 gene that is present in the benign tumour (Basu et al. 1992). It is suggested that neurofibromin maintains growth–arrest of mature non–myelin forming Schwann cells and is therefore a tumour–suppressor protein.

Growth–arrest–specific gene 3 (\textit{gas3}, PMP22, SR13, CD25, PASII). Growth–arrest–specific genes are only expressed in the G0 phase of the cell cycle and their expression is negatively correlated with cell division. They may have a functional role in maintaining growth arrest (Schneider et al. 1988). In theory, there are two types of genes that are selectively expressed during growth arrest. The first type of gene in this category is a tumour–suppressor gene. Tumour suppressor genes act as a break to mitosis, the cell being prevented from entering mitosis as a result of this gene being expressed, and loss of one of these genes would be expected to cause uncurbed division. The second set of growth arrest specific genes are only expressed during growth arrest because they are part of the differentiated phenotype, expression of which in some cells is only possible during growth arrest, but which is not directly related to growth arrest. Loss of one of these genes would be expected to cause some loss of function to the mature cell, but no loss of growth control.

\textit{gas3} is one of these genes; in NIH 3T3 cells, growth arrest by serum and growth factor deprivation, or by density dependent growth inhibition, induces expression of the \textit{gas3} protein (Manfioletti et al. 1990). \textit{gas3} encodes a membrane protein that is 98% homologous to the myelin protein PASII (Kitamura et al. 1976, Welcher et al. 1991, Spreyer et al. 1991). This protein is now variously referred to as PMP22 (Snipes et al. 1992) and CD25 protein (Spreyer et al. 1991) and the gene is
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called SR13 (Snipes et al. 1992) and CD25 (Spreyer et al. 1991). PMP22 is expressed in compact myelin of adult rat sciatic nerve, and during development the pattern of PMP22 expression is the same as other myelin proteins (Snipes et al. 1992). The protein and mRNA are lost after nerve transection, remain low if axon-loss is permanent but return if nerve regeneration is permitted (Spreyer et al. 1991, Snipes et al. 1992). Cultured neonatal rat Schwann cells express PMP22 mRNA, and the levels are increased by forskolin treatment (Spreyer et al. 1991).

Whilst PMP22 is only expressed in situations in where at least some cells are in growth-arrest, expression is not entirely consistent with that of a growth arrest-specific gene as some non-dividing Schwann cells fail to express PMP22 protein or mRNA. Nerve section produces a burst of Schwann cell division that lasts for about 12 days, after which the cells stop dividing and remain quiescent (Pellegrino and Spencer 1985). Levels of PMP22 mRNA drop during the burst of Schwann cell division, after 2 weeks however, as the Schwann cells drop out of division, they fail to re-express PMP22, so at this time growth arrest is achieved in the absence of PMP22 (Spreyer et al. 1991).

In the hypomyelinating mouse mutant trembler, the defect is thought to be associated with a defect in the PMP22 gene that alters a putative membrane-associated domain of the PMP22 protein (Suter et al. 1992). This disorder only affects peripheral myelin forming cells, since both non-myelin forming Schwann cells and oligodendrocytes appear normal. The myelin cells are hypomyelinated and show poor compaction of existing myelin, and there is constant myelin degradation and remyelination. Schwann cells of myelinated nerves divide abnormally fast, to reach a steady Schwann cell count 10x more dense than in the normal nerve. This implies constant Schwann cell death, as the level of division does not decrease when this number is reached (reviewed in Dyck, 1984 Chapter 16, Aguayo and Bray). Although these cells show abnormal division associated with PMP22 miss-expression, it does not necessarily follow that the PMP22 is acting as a growth suppressor. If PMP22 is necessary for normal myelin synthesis or compaction, its absence would cause myelin disruption, this in itself could be mitogenic. There is therefore no direct evidence for PMP22 acting as a growth-arrest specific gene in
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Schwann cells. It behaves more like a myelin protein, or a differentiation-specific gene. In neither fibroblasts nor myelin forming Schwann cells has loss of PMP22 or gas3 been shown to be the direct cause of lost growth control, so it is has not yet been shown that PMP22 is involved in growth arrest.

Schwann cell differentiation

The phenotype of mature non-myelin forming and myelin forming Schwann cells changes when cells are taken into culture or after axon lesion in vivo. Both non-myelin forming and myelin forming cells lose galactocerebroside and the lipid recognised by the O4 antibody, myelin forming cells lose their myelin-specific proteins. Various proteins, generally only expressed by developing Schwann cells and non-myelin forming cells, are re-expressed by the myelin forming cells, these phenotypic changes are discussed further in Chapter 2 and illustrated in Figure 2.1.

What signals cause differentiation?: Mature Schwann cells express one of two phenotypes and it is clear that axons determine which Schwann cell phenotype is expressed. This has been demonstrated by two classic experiments. Firstly, Aguayo placed pieces of a small, unmyelinated nerve in the path of a cut, regenerating nerve containing many large axons. He was able to show that when cells that had previously ensheathed small axons without making myelin ensheath the larger ingrowing axons they started to make myelin (Aguayo et al. 1976b). In the second experiment, small sympathetic neurons grew in size after their target fields were artificially increased. As the neurons grew, the axons increased in diameter and the associated Schwann cells that would otherwise have become non-myelin cells, synthesised myelin sheaths (Voyvodic 1989). The opposite experiment, myelin forming cells being induced to ensheath small axons and express the non-myelin forming phenotype has not been done.

There are similarities between the two differentiated cells, since axons induce the lipids sulphatide and galactocerebroside and increase synthesis of basement membrane constituents in both cell types. The difference between the non-myelin forming and myelin forming cells is more than just the expression of myelin proteins, however, since myelin forming cells also suppress a group of non-myelin cell specific proteins (see Chapter 2 for a full description of Schwann cell phenotypes).
The possible mechanisms by which axons can induce two different Schwann cell phenotypes include:

1. **There is one factor, or combination of factors that are common to all axons.** Low amounts of these factors will induce expression of surface lipids and upregulate expression of ECM components, causing cells to become mature non-myelin forming Schwann cells. Higher amounts are only present in large axons, and high concentrations in addition to induction of lipids and ECM molecules, will induce the myelin and suppress the non-myelin proteins.

2. **There is one factor, or combination of factors that are common to all axons, these are responsible for the induction of lipids and ECM molecules in all differentiated Schwann cells.** Another set of factors, uniquely expressed by larger axons will induce myelin proteins and suppress non-myelin proteins.

3. Small and large axons do not express common Schwann cell differentiation factors, even though some of the effects of these factors are the same.

4. Small and large axons give the same signals to their Schwann cells, but only big axons are singly ensheathed, this allows the cells to polarise as the axonal and abaxonal sides of the cell become clearly defined. This polarity is essential before the cell can begin to make myelin (discussed by Bunge et al. 1986).

There is no evidence that any one of these mechanisms describes the real situation. It is unclear whether many differentiating factors, or only one is present, and if this is separate from axon derived mitogens.

One candidate molecule for mediating messages from axon to Schwann cell is NDGF, the axon-associated Schwann cell mitogen, extracted, and partially purified from brain (Nordlund et al. 1992). The addition of NDGF to cultured Schwann cells, however, induces division rather than differentiation.

**How do Schwann cells stop dividing during development?:** In development and during repair after nerve damage, Schwann cells divide, and in both these situations they are in contact with axons. Although the evidence that axonal contact causes this division is only indirect, axon contact is mitogenic *in vitro* (reviewed above) and is probably also mitogenic *in vivo*. It is also axonal contact that directs Schwann cell differentiation after division ends. How can the Schwann cell stop division and
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differentiate in the developing or regenerating nerve whilst in contact with axon-associated mitogens? Does the axon suddenly stop expressing mitogens? Schwann cells could initiate a negative feedback loop in which cover by confluent Schwann cells down regulates expression of mitogens, and induces a factor in the axon, or an autocrine factor in the Schwann cell that is mitosis-inhibiting for the Schwann cell (gangliosides and interferon-γ are both growth-inhibiting factors contained in axons). Another possibility is that Schwann cells may become insensitive to axonal mitogens due to loss or inactivation of growth factor receptors.

These questions have not been answered but, as discussed above, there is growing evidence that in the mature nerve, myelin forming Schwann cells are insensitive to mitogenic signals until they are sensitized by some disruption. This disruption could either release the cells from autocrine inhibition, or stimulate expression of growth factor receptors. The real situation in development could be a combination of the above, with autocrine factors allowing the Schwann cells to stop dividing and so facilitating differentiation, and then differentiation could be accompanied by loss of growth factor receptors.

The mature phenotype is never seen in dividing Schwann cells: In the developing nerve, the myelin and non-myelin cells do not mature until division has ceased (Diner 1965, Martin and Webster 1973). In damaged nerve, Schwann cell division is accompanied by loss of the mature phenotype. How mutually exclusive are Schwann cell division and Schwann cell differentiation? Is it essential for division to stop before the mature genes can be expressed? Conversely, is it obligatory for the genes responsible for the mature phenotype to be turned off before the cell can divide?

There is some evidence that Schwann cells in division actively suppress the myelin phenotype, since the transcription factor SCIP (for suppressed cAMP-inducible POU) is expressed only in dividing cells, and represses expression of myelin genes (see below).

POU transcription factor SCIP (tst-1, mouse homologue of oct-6): In co-transfection assays SCIP acts as a repressor of myelin-specific genes (Monuki et al. 1990). SCIP is expressed by Schwann cells as they develop, but lost in the mature nerve (Monuki et al. 1990, Collarini et al. 1992, Scherer et al. 1992b). Peripheral nerve transection
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causes a temporary return of SCIP mRNA (Monuki et al. 1990, Scherer et al. 1992b),
whilst after crush injury the increase in SCIP is maintained (Scherer et al. 1992b).
The pattern of SCIP expression in vivo is consistent with SCIP acting to prevent
myelin–specific gene expression during Schwann cell mitosis. In vitro, Schwann
cells express low levels of SCIP mRNA, however, SCIP expression is up–regulated
by treatment with forskolin in the presence of serum (Monuki et al. 1989). This is not
consistent with a myelin gene suppressing role for SCIP as myelin genes are also
expressed in these conditions, possibly this is an artefact produced by using forskolin
instead of the natural agonist.

In this thesis I will describe evidence indicating that under different
conditions, the axonal signals that induce Schwann cell division and those that cause
differentiation can both be mimicked in vitro by cAMP elevation. Some of the effects
of cAMP elevation on Schwann cell differentiation are only seen if mitosis is
prevented.
cAMP and Schwann cell differentiation: I have described above the mitogenic effects
of cAMP–elevating drugs on Schwann cells, how Schwann cells tend to stop division
before differentiation, and how they lose their mature phenotype as they divide.
Paradoxically cAMP is also implicated in Schwann cell differentiation.

Surface galactocerebroside is re–expressed on cultured Schwann cells exposed
to db–cAMP or forskolin (Sobue and Pleasure 1984, Sobue et al. 1986a, 1986b).
When the induction of galactocerebroside was related to Schwann cell division, it was
clear that the dose of drug that induced galactocerebroside was higher than the dose
needed for peak Schwann cell division (Sobue et al. 1986a).

Some other effects of axons on Schwann cell differentiation are also mimicked
by raising intracellular cAMP levels; P_{170k}, a protein expressed on all Schwann cells
in vivo, but lost in vitro (Shuman et al. 1988), is induced by cAMP, but not P_{0}
(Shuman et al. 1988, Kreider et al. 1988). In Schwann cell lines, db–cAMP decreases
the levels of P_{0} message and protein (Shuman et al. 1988, Gandelman et al. 1989).
More recently, however, other groups have shown that cAMP does elevate P_{0}, P_{2} and
MBP mRNA and protein (Lemke and Chao 1988, Kamholz et al. 1992, Monuki et al.
1989, 1990). cAMP will also reduce NGF–R levels, and after 8 days no NGF
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receptor protein is detectable (Mokuno et al. 1988) indicating that cAMP can mimic down-regulation of the non-myelin forming Schwann cell phenotype as well as inducing myelin proteins.

The effects of cAMP on the expression of P_q mRNA levels are delayed, and 24 hours of continuous cAMP expression is needed before an increase is seen (Monuki et al. 1989). This is consistent with a model in which cAMP activates regulatory proteins and these control expression of immediate early genes, the products of which control expression of the myelin genes (Monuki et al. 1989).

A rapid response to cAMP elevation has been seen in situations where myelin genes are already being expressed at relatively high levels. When endoneurial tissue from normal or crush injured nerve (5 weeks after crush) is treated with a very high dose of forskolin (100µM), levels of P_q mRNA are greatly increased within 90 minutes. No increase in myelin gene expression is seen if endoneurial tissue taken from permanently transected nerve is treated with forskolin or db-cAMP in the same way (LeBlanc et al. 1992). As the P_q genes are already activated in the normal and crush injured nerve, cAMP elevation in the Schwann cells, or a signal secondary to raised cAMP in the axons that are still present in the excised tissue can, possibly, modulate the level of myelin gene mRNAs more quickly. The P_q gene, however, does not contain any known cAMP response element (CRE) (Lemke et al. 1988) so the short term effects are also unlikely to be through direct action of cAMP on the P_q gene. These experiments indicate that although a prolonged cAMP elevation is necessary to promote myelination, once the myelin genes are active, then acute changes in this activity can be induced by short term changes in cAMP levels.

Maintaining Schwann cell differentiation: The surprising ability of myelin forming Schwann cells to remain differentiated without axonal contact described by Kidd and Heath (1988a, 1988b) has caused a re-evaluation of the Schwann cell-axon interaction. As described above, the axons of post-ganglionic sympathetic neurons occasionally have a double myelin sheath, where an internode of myelin has formed underneath an established myelin sheath. The outer myelin sheath is stable, and remains indefinitely without any contact with the axon, and even remains undisturbed after degeneration of the inner Schwann cell in response to axon degeneration (Kidd
1. General Introduction


In no other situation has a Schwann cell been demonstrated to be able to express the mature myelin forming phenotype without axonal contact. In all other cases Schwann cell dedifferentiation is seen either in response destruction of the axon that they are touching, or after interference with Schwann cell metabolism directly by toxins. The formation of the outer myelin sheath is the result of a new internode being formed underneath the original myelin. The new myelin gradually separates the original cell from the axon until the outer cell no longer contacts any axolemma, and faces only the basal lamina of the new cell on its abaxonal surface (Kidd et al. 1988a, 1988b). If, as discussed above, a signal from a degenerating axon, or toxin is necessary to render the Schwann cell sensitive to mitogens and to trigger destruction of myelin, then the outer Schwann cell is never in contact with such a signal. The novelty is in the idea that it takes an active signal to destroy the myelin phenotype, and not, as generally presumed, that in order to maintain the myelin phenotype a constant signal from the axon is required.

Species comparisons

Glial cells are present in early invertebrates, surrounding neuronal cell bodies and ensheathing axons, but myelin is only made by vertebrates, and, as described above, invertebrates can only increase conduction velocity through increasing axonal diameter.

\(P_o\) is the major component of both PNS and CNS myelin in fish. The first species to express PLP were lungfish, and amphibia and all higher vertebrates have no \(P_o\) in their CNS myelin. The more complicated chemical composition of CNS myelin has possibly evolved to meet the requirements of the more complicated topography of oligodendrocytes (see review by Hudson 1990 and references therein).

Regeneration in the CNS is far greater in lower vertebrates than in mammals. Fish and amphibia have a similar capacity to regenerate damaged axons in the CNS to that seen in the PNS of mammals (reviewed by Bunge and Hopkins 1990).

CNS and PNS myelin

CNS myelin is made by oligodendrocytes. How different are oligodendrocytes from Schwann cells? Is the expression of myelin in the CNS subject to the same controls as
1. General Introduction

in the PNS? In the following discussion, the two cells are compared.

**Morphology.** At the electron microscopy level, CNS and PNS myelin differences include:

1. The periodicity of the bands is smaller in the CNS, although the major dense lines, and the intraperiod lines show the same arrangement (reviewed by Hudson 1990).
2. There is less cytoplasm in CNS myelin, both at the internal mesaxon and at the abaxonal space outside the sheath.
3. The myelin in the CNS tends to be thinner than that in the PNS for axons of the same diameter.
4. There is no basement membrane surrounding oligodendrocytes.
5. Oligodendrocytes are not restricted to associating with only one axon, they can ensheath up to 60 axons.
6. The oligodendrocyte cell body is distant from the myelin sheath, and there are cytoplasmic processes connecting the sheaths with the cell body (2–6 reviewed by Peters et al. 1976).
7. In the PNS, the threshold for myelination is 1 \( \mu m \) in diameter, however in the CNS axons as small as 0.2 \( \mu m \) are myelinated (Franson and Hildebrand 1975).

**Chemical differences between CNS and PNS myelin:**

1. Peripheral myelin is richer in phospholipid and has less glycolipid (Peters et al. 1976) than CNS myelin.
2. Sphingolipids are 2% of PNS, and 5–20% of CNS total fatty acids (Peters et al. 1976), although the lipids GAL–C and sulphatide are expressed by both cell types (Mirsky et al. 1980).
3. The myelin proteins are different in the CNS and PNS. 60–70% of PNS, but only 5% of CNS total myelin protein is glycoprotein (Smith et al. 1990).

See Table 1.1 for a summary of these differences.

**Development:** Oligodendrocytes and Schwann cells derive from different lineages. Schwann cells are neural crest derived, whereas oligodendrocytes develop from a precursor cell, the highly migratory O2A progenitor that itself develops from the neural tube. As oligodendrocytes mature, they go through 4 identifiable stages, O2A progenitor (A2B5\(^+\), O4\(^-\)), committed proligodendrocyte (O4\(^+\), GAL–C\(^-\)), early
1. General Introduction

oligodendrocyte (GAL-C⁺, MBP⁻) and mature oligodendrocyte (MBP⁺, O4⁺, GAL-C⁺) (Gard and Pfeiffer 1990, Bansal et al. 1992). In culture, the O2A progenitor cell also has the ability to differentiate into an astrocyte–like cell, the type 2 astrocyte. In the optic nerve, all the oligodendrocytes develop from the O2A cells that migrate into the nerve from germinal zones near the optic chiasma (Small et al. 1987). However stem cells that are precursors for O2A cells, described as pre–progenitor cells are present in other regions of the brain. 6 day old rat cerebral white matter contains these pre–progenitor cells, that is cells that are A2B5⁻, that differentiate into A2B5⁺ O2A cells in culture (Grinspan et al. 1990). These pre–progenitor cells are immature neuroectodermal cells of the subventricular zone, the major germinal zone of the cerebral hemispheres (Altman 1966, Paterson et al. 1973, Pringle et al. 1992) and the ventral half of the spinal cord (Warf et al. 1991).

Ability to divide: growth factors and oligodendrocytes: In normal adult animals, oligodendrocytes, like Schwann cells, proliferate very little however new oligodendrocytes are produced at low levels in the adult (McCarthy and Leblond 1988). In vivo, all cells that express GAL–C, have dropped out of division, but the progenitor, and the proligodendrocyte both divide (Gard and Pfeiffer 1990).

In vitro, oligodendrocytes from adult rats are stimulated to divide by bFGF, aFGF and neuronal surface mitogens, but they do not respond to PDGF (Vick and DeVries 1992). The membrane–associated mitogen (NDGF) extracted from developing brain is a mitogen for the developing oligodendrocyte in vitro (Nordlund et al. 1992). The O2A progenitor cell divides in response to both PDGF and bFGF, but the division in response to PDGF is not sustained (McKinnon et al. 1990). In a mixture of PDGF and bFGF together the cells will divide indefinitely and not differentiate (Bögl er et al. 1990). O2A cells express predominantly PDGFα receptors, the level of these receptors declines as the cells mature (Hart et al. 1989, McKinnon et al. 1990).

There is some evidence that mature oligodendrocytes are more sensitive than their adult stem cells to surface mitogens on cultured DRG neurons, and that they are more capable of generating new myelinating oligodendrocytes (Wood and Bunge 1991).
1. General Introduction

In summary, there are differences and similarities in the way Schwann cells and oligodendrocytes respond to mitogens. aFGF, bFGF, PDGF and neuronal surface molecules are mitogenic for both Schwann cells and oligodendrocytes or oligodendrocyte precursor cells, but the response to PDGF is mediated by activation of a different class of receptor. Schwann cells require a co-mitogen to raise intracellular cAMP before aFGF, bFGF or PDGF cause proliferation, while oligodendrocytes respond to these growth factors alone. Both cells tend to down-regulate their growth factor receptors as they mature.

Oligodendrocyte phenotype is less dependent than the Schwann cell on axonal signals: In development, myelin proteins are expressed by oligodendrocytes before myelin formation (Sternberger et al. 1978). This is unlike Schwann cells, which do not express myelin proteins until they begin to make myelin (Trapp et al. 1981).

When oligodendrocytes are taken into tissue culture and deprived of axonal contact, the de-differentiation that follows is not as dramatic as that seen in the Schwann cell. Sulphatide and galactocerebroside and the myelin proteins MBP, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), MAG and PLP are retained (Mirsky et al. 1980, Dubois-Dalcq et al. 1986). This implies that, unlike Schwann cells, oligodendrocytes are independent of axon-derived signals for expression of the myelin phenotype. There is evidence, however that in oligodendrocytes, the level of myelin gene expression is regulated by axonal contact as MAG, PLP and MBP mRNAs are reduced in optic nerve after enucleation (Scherer et al. 1992a, McPhilemy et al. 1990).

cAMP effects: In tissue culture, oligodendrocyte differentiation from a CNPase− to a CNPase+ cell is enhanced by cAMP elevating agents (Raible and McMorris 1990). cAMP analogues also increase oligodendrocyte synthesis of galactocerebroside (Pleasure et al. 1986). Nuclei isolated from myelinating oligodendrocytes show a cAMP-stimulated increase in protein phosphorylation (Sato-Bigbee and Yu 1991).

The different level of independence of axonal signals and cAMP levels between Schwann cells and oligodendrocytes might be expected if the different morphology of the two cells is considered. The oligodendrocyte forms internodes that are distant from the cell body, around axons that are often very small and so may not
give a large myelin inducing signal. Once a process from a developing oligodendrocyte locates an axon to be myelinated, it will not need to wait for axonal signals to initiate myelin protein gene expression in a distant cell body. In the Schwann cell, the cell body is not distant, and so no delay would follow axon triggering of the myelin genes.

**Ability of oligodendrocytes to re-myelinate:** Mature oligodendrocytes isolated from adult rats are capable of myelinating DRG neurons *in vitro* (Rosen et al. 1989, Wood and Bunge 1986, 1991). *In vivo*, after demyelination, CNS remyelination can be the result both of division by surviving oligodendrocytes, and of stem cell division (Raine et al. 1988).

Thus the ability to divide and to remyelinate axons after damage is shared by Schwann cells and oligodendrocytes (Griffin et al. 1990, Aguayo et al. 1976b), however, there has been no description of a stem cell for Schwann cells in the adult mammalian peripheral nerve.

**Oligodendrocytes do not provide a favourable environment for axon regrowth:** This is the main area in which these two cell types differ. As discussed above, Schwann cells secrete and express on their surface factors that enhance neuronal survival and promote neurite outgrowth. Oligodendrocytes express on their surface factors that repel neurite outgrowth (reviewed by Schwab 1990). This neurite repelling surface, lack of ECM and the formation of astroglial scar tissue (reviewed by Bunge and Hopkins 1990) is likely to be the reason that CNS tissue is unable to support neuronal regeneration after a CNS lesion, when regeneration is possible from the same neurons into a PNS graft.

In general, it would appear that these two myelin producing cells show sufficient diversity in behaviour and morphology to make it unsafe to assume that the mechanisms controlling myelin expression are the same.

**Experimental approaches used to study Schwann cell–axon interactions**

Schwann cell dysfunction *in vivo*. One way to understand the normal interactions between axons and their glia is to examine the effect of diseases where this interaction has broken down. Some of these diseases and their animal models are listed in the tables below.
1. General Introduction

Genetic defects of Schwann cell or oligodendrocyte function:
are listed in Table 1.2.

Human diseases showing Schwann cell dysfunction are listed in Table 1.3.

In vivo models of demyelination are listed in Table 1.4. Another animal model that has recently been developed is the transgenic mouse where a gene is selectively added to the normal genome. Messing and coworkers recently used this technique to add the gene encoding bacterial diptheria toxin under the control of the \( P_q \) gene promoter. When Schwann cells are stimulated to myelinate in this mouse, they are also stimulated to synthesise diptheria toxin, selectively killing only those cells that synthesise diptheria toxin. The gene is specifically expressed by the myelin forming Schwann cells; non-myelin forming Schwann cells and oligodendrocytes do not express it. Oligodendrocytes are normal, but secondary changes are seen in the non-myelin forming Schwann cells of mixed nerves, but not of unmyelinated nerves (Messing et al. 1992).

This model is particularly useful as aspects of both Schwann cell-axon and Schwann cell-Schwann cell interactions are affected by loss of the cells induced to myelinate their axons. First, the expected specificity of expression of the \( P_q \) gene was confirmed. Second, the sensitivity of the non-myelin forming Schwann cells to disruption of adjacent exposed axons of large diameter, previously demonstrated by their division in response to paranodal demyelination (Griffin et al. 1987, 1990), was confirmed. Thirdly, a new system is set up for further study of the effects of myelin on axon development and function.

In vitro models: The other approach to examine axon-Schwann cell interactions is to culture the Schwann cells, and induce the myelin phenotype either by adding appropriate neurons, or attempting to mimic the effect of axons with drugs.

Neuron-Schwann cell co-cultures have been useful in determining the requirements of Schwann cells for myelination, and to screen neurons from different sources for their ability to induce Schwann cell proliferation or myelin synthesis. The in vitro neuron-Schwann cell coculture is also used to screen for agents that disrupt myelin formation (antibodies, toxins).

 Cultures of non glial cells transfected with glia-specific proteins have also
been used to separate the contribution of the various components of myelin to the adhesive and neurite promoting properties of the Schwann cell surface. $P_0$ (d'Urso et al. 1990, Schneider-Schaulies et al. 1990, Filbin et al. 1990), L- and S- forms of MAG (Afar et al. 1991, Johnson et al. 1989), and the various MBP isoforms (Staugaitis et al. 1990) have all been expressed in cell lines.

The approach chosen for the work described in this thesis is to induce expression of the myelin phenotype by Schwann cells in culture using cAMP elevating drugs, and to modulate their response to those drugs by adding growth factors. In this way it has been possible to demonstrate that the ability of Schwann cells to differentiate into mature myelin forming Schwann cells in response to myelin-inducing signals is closely linked to their ability to cease division.
Table 1.1

MYELIN PROTEINS

<table>
<thead>
<tr>
<th>Protein Zero (P₀)</th>
<th>PNS myelin</th>
<th>CNS myelin</th>
<th>Alt. sp. forms</th>
<th>location in cell</th>
<th>posttransl. modification</th>
<th>location mouse c'some</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;50%</td>
<td>0%</td>
<td>24.9 kDa&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CM</td>
<td>Glycosylated, Phosphorylated</td>
<td>1</td>
</tr>
</tbody>
</table>

| Proteolipid (PLP) protein (DM20) | trace | 40% | 30.0 kDa, 26.5 kDa | CM (CNS), N–CM (PNS) | Acylated | X (jimpy) |

| Myelin Basic Protein (MBP) | 5–15% | 30–40% | 21.4 kDa, 20.1 kDa, 18.4 kDa, 17.1 kDa (2 forms), 14.1 kDa | CM | Phosphotyalted, Methylated | 18 (shiverer) |

| Myelin Associated Glycoprotein (MAG) | <1% | 1% | 68.9 kDa<sup>a</sup>, 64.0 kDa<sup>a</sup> | N–CM, S–L inc. mesaxon, paranodes | Glycosylated (HNK–1), Phosphorylated | 7 |

CM, in compact myelin, N–CM, not in compact myelin. S–L inc., Schmidt–Lanterman incisures.
<sup>a</sup> MWt of the glycosylated form. <sup>b</sup> MWt based on SDS electrophoretic mobility.
<table>
<thead>
<tr>
<th></th>
<th>PNS myelin</th>
<th>CNS myelin</th>
<th>Alt. sp. forms</th>
<th>location in cell</th>
<th>posttransl. modification</th>
<th>location mouse c'some</th>
</tr>
</thead>
<tbody>
<tr>
<td>2',3'-Cyclic nucleotide 3'-Phosphohydrolase (CNPase)</td>
<td>&lt;1%</td>
<td>4%</td>
<td>48.0 kD(^b)</td>
<td>N-CM, Plasma membrane, cytoplasmic face.</td>
<td>Phosphorylated</td>
<td></td>
</tr>
<tr>
<td>Peripheral Myelin Protein 22 (PMP22)</td>
<td>minor myelin component</td>
<td>0%</td>
<td>22 kD(^a) CM</td>
<td>Glycosylated</td>
<td>11 (trembler)</td>
<td></td>
</tr>
<tr>
<td>P2 Basic Protein (P2)</td>
<td>0.05-1%</td>
<td>&lt;10% of fibres +</td>
<td>14.4 kD</td>
<td>CM, only in large axons</td>
<td>Amidated</td>
<td></td>
</tr>
<tr>
<td>Myelin/Oligodendrocyte Glycoprotein (MOG)</td>
<td>0</td>
<td>minor component</td>
<td>26-28 kD(^a)</td>
<td>N-CM, outer surface of cell</td>
<td>Glycosylated</td>
<td></td>
</tr>
</tbody>
</table>

CM, in compact myelin, N-CM, not in compact myelin. S–L inc., Schmidt–Lanterman incisures.
\(^a\) MWt of the glycosylated form. \(^b\) MWt based on SDS electrophoretic mobility.

## Table 1.2

MYELIN MUTANTS  
(Adapted from G. Lemke 1988 Unwrapping the genes of myelin. Neuron 1 535–543.)

<table>
<thead>
<tr>
<th>Mouse Mutant</th>
<th>Genetic Symbol</th>
<th>C'some Number</th>
<th>Mutated Gene</th>
<th>Gene Defect</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>shiverer mouse</td>
<td><em>shi</em></td>
<td>18</td>
<td>MBP</td>
<td>Deletion</td>
<td>Hypomyelination of CNS, lack of MDL; ataxia &amp; tremor; recessive</td>
</tr>
<tr>
<td>shiverer mouse</td>
<td><em>shi</em>&lt;sup&gt;mld&lt;/sup&gt;</td>
<td>18</td>
<td>MBP</td>
<td>Duplication</td>
<td>Like <em>shi</em>, but less severe; recessive</td>
</tr>
<tr>
<td>jimpy mouse</td>
<td><em>jp</em></td>
<td>X</td>
<td>PLP</td>
<td>Point mutation and splice defect</td>
<td>Hypomyelination of CNS, abnormal IPL; tremor and seizures; oligodendrocytes die; recessive</td>
</tr>
<tr>
<td>jimpy msd mouse</td>
<td><em>jp</em>&lt;sup&gt;msd&lt;/sup&gt;</td>
<td>X</td>
<td>PLP</td>
<td>point mutation</td>
<td>Like <em>jp</em>, but less severe; recessive</td>
</tr>
<tr>
<td>Mouse Mutant</td>
<td>Genetic Symbol</td>
<td>C'some Number</td>
<td>Mutated Gene</td>
<td>Gene Defect</td>
<td>Phenotype</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
<td>---------------</td>
<td>--------------</td>
<td>-------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>quaking mouse</td>
<td>qk</td>
<td>17</td>
<td>ND</td>
<td>-</td>
<td>Hypomyelination of CNS and PNS; sperm defect; non lethal; recessive. Possible inability to remove MAG to form compact myelin.</td>
</tr>
<tr>
<td>Trembler mouse</td>
<td>Tr</td>
<td>11</td>
<td>PMP22</td>
<td>Point mutation</td>
<td>Hypomyelination of PNS; tremor and seizures; semidominant</td>
</tr>
<tr>
<td>OLA mouse</td>
<td></td>
<td></td>
<td>single autosomal dominant gene</td>
<td>Normal until nerve lesion; abnormal Wallerian degeneration</td>
<td></td>
</tr>
<tr>
<td>Twitcher mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal CNS and PNS myelin</td>
</tr>
<tr>
<td>Dystrophic mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PNS demyelination; impaired basal lamina formation</td>
</tr>
<tr>
<td>myelin deficient rat (mld)</td>
<td>PLP</td>
<td></td>
<td></td>
<td></td>
<td>CNS demyelination, PNS normal</td>
</tr>
<tr>
<td>shaking pup rat</td>
<td>PLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human diseases showing Schwann cell dysfunction</th>
<th>symptom</th>
<th>cause</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Guillain-Barré syndrome (GBS), and Chronic inflammatory demyelinating neuropathy (CIDP)</strong></td>
<td>1° PNS demyelination</td>
<td>unknown</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td><strong>Multiple sclerosis (MS)</strong></td>
<td>1° CNS demyelination</td>
<td>unknown</td>
<td>Martin et al. 1992</td>
</tr>
<tr>
<td><strong>Neurofibromatosis (NF-1)</strong></td>
<td>benign Schwann cell tumour</td>
<td>defect in NFI gene</td>
<td>Xu et al. 1990</td>
</tr>
<tr>
<td><strong>Charcot-Marie-Tooth, Djerine-Sottas (hereditary motor and sensory neuropathies I and III)</strong></td>
<td>hypertrophic; 2° PNS demyelination</td>
<td>inherited</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td><strong>Leprosy</strong></td>
<td>Schwann cell proliferation; demyelination</td>
<td>M. Leprae invasion of nerve</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td><strong>Pelizaeus-Merzbacher disease</strong></td>
<td></td>
<td>PLP gene mutation, X-linked</td>
<td>Hudson 1990</td>
</tr>
<tr>
<td>Model Description</td>
<td>How Formed</td>
<td>Model for</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-----------------------------</td>
<td>-----------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Experimental allergic neuritis (EAN)</td>
<td>Peripheral myelin protein injection</td>
<td>MS</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td>Experimental allergic encephalomyelitis (EAE)</td>
<td>CNS myelin protein injection</td>
<td>GBS</td>
<td>Martin et al. 1992</td>
</tr>
<tr>
<td>Dietary toxin</td>
<td>Tellurium in diet</td>
<td>1° PNS demyelination</td>
<td>Toews et al. 1990</td>
</tr>
<tr>
<td>IP injected toxin</td>
<td>Iminodipropionitrile (IDPN)</td>
<td>1° PNS paranodal demyelination</td>
<td>Hall &amp; Gregson 1971</td>
</tr>
<tr>
<td>Intra-neural injection of toxin</td>
<td>Lysophosphatidyl choline (lysolecithin or LPC)</td>
<td>Local 1° PNS demyelination</td>
<td>Griffin et al. 1987, 1990</td>
</tr>
<tr>
<td>Intracranial injection of toxin</td>
<td>Coronavirus injection</td>
<td>CNS demyelination</td>
<td>Armstrong et al. 1990</td>
</tr>
<tr>
<td>Permanent peripheral nerve transection</td>
<td>Nerve cut</td>
<td>Nerve damage/ permanent 2° demyelination</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td>Temporary peripheral nerve transection</td>
<td>Nerve crush</td>
<td>Nerve damage/ temporary 2° demyelination</td>
<td>Dyck et al. 1984</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>Guanethidine injection</td>
<td>Death of sympathetic neurons</td>
<td>Kidd et al. 1986, 1992</td>
</tr>
</tbody>
</table>
1. General Introduction

Appendix 1

Schmalbruch (1986) described the rat sciatic nerve as containing 27,000 axons, 29% myelinated and 71% unmyelinated (7,830 and 19,170 respectively).

Peters et al. 1976 (page 186) in their review of the peripheral nervous system, state that non-myelin Schwann cell length in the rat sural nerve and sympathetic trunk averages 100\mu m and that in rat trigeminal nerve 7–21 (average 14) nonmyelin axons share one Schwann cell.

The internodal length of a myelin-forming Schwann cell is roughly equivalent to 100–200x the axonal diameter (also reviewed in Peters et al., 1976 page 190), thus as rat sciatic nerve contains myelinated axons of between 2 and 12\mu m diameter (Schmalbruch, 1986) if the average diameter is taken as 7\mu m, then the internodal length is between 700 and 1,400\mu m (with an average of 1.050mm).

Using these estimates, in the rat sciatic nerve, 19,170 unmyelinated axons would contain \( \frac{14 \times 19,170}{1,369} \) Schwann cell/axon units in cross section, and in 1cm, \( 100 \times \frac{1,369}{1,369} = 136,900 \) unmyelinated Schwann cells.

For myelinated axons, if each Schwann cell projects 1,050\mu m, then 9.52 myelin-forming Schwann cells per cm will be associated with each axon, giving a total of \( 9.52 \times 7,830 = 74,571 \) myelin-forming Schwann cells per cm.

This predicts a total of 211,471 Schwann cells/cm of adult rat sciatic nerve, with 35% myelin and 65% non-myelin forming Schwann cells.
CHAPTER 2
INTRODUCTION

In this chapter the phenotype of Schwann cells in adult peripheral nerve will be reviewed. Experiments to determine the pre-natal Schwann cell phenotype using selected antibodies will be described and these cells will be compared to mature Schwann cells and to the cells of the neural crest. A scheme of Schwann cell development will be proposed. The role of axons in inducing and maintaining Schwann cell phenotype will be discussed.

The two types of glia associated with axons in peripheral nerves, the myelin and the non-myelin forming Schwann cells, have been extensively classified. Markers in common and markers specific to one or other cell type have been described so that it is possible to identify one from the other in many preparations, for example, in sections of nerve or in cells dissociated from a nerve.

The phenotypes of myelin forming and non-myelin forming Schwann cells are summarised in the tables below.

Table 2.1: Schwann cell phenotype; alphabetical list
Table 2.2: Commonly used markers for adult Schwann cells. In three groups:
(1) on both types of Schwann cell,
(2) on non-myelin only,
(3) on myelin only.

Schwann cell phenotype. The first table is a list of antigens found in Schwann cells. Many of these, whilst essential for understanding Schwann cell function, are not useful for identifying Schwann cells as they are also expressed in the axons, the surrounding tissue or both and so are generally used only in combination with other Schwann cell markers, an example is seen in the L1 and N-CAM proteins, which are also expressed by many axons and fibroblasts.

The most commonly used Schwann cell markers, shown in Table 2:2, are described in more detail below.

Group 1: Markers common to myelin and non-myelin forming Schwann cells.
S100. S100 proteins are a group of 10–12kDa acidic, Ca++ binding proteins of the "E–F hand" group of molecules shown to regulate several cell activities, including
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regulation of microtubule assembly and disassembly. They have potential \textit{in vivo} roles in the regulation of glial cell morphology, cytoskeletal organisation and cell proliferation. S100β, in the form of the homodimer S100b, is present in the cytoplasm of all Schwann cells and in CNS glia. S100 is differentially expressed during the cell cycle in C6 glioma cells, greater levels being expressed during the G1 phase (reviewed in Kligman and Hilt 1988). As discussed in Chapter 1, S100 may also have extracellular roles; exogenous S100 enhances CNS neuronal survival and neurite outgrowth (Winningham-Major et al. 1989).

Myelin forming Schwann cells express higher levels of S100 protein than the non myelin forming cells, and the amount of S100 is directly related to the thickness of the myelin sheath (Mata et al. 1990).

\textbf{Galactocerebroside (GAL-C):} The glycosphingolipid GAL-C is expressed on the cell surface of myelin-forming and non-myelin-forming Schwann cells (Jessen et al. 1985). GAL-C is detectable just before the myelin proteins of the myelin forming cells (Jessen et al. 1987b, Kelly et al. 1992) and in the non-myelin forming Schwann cells, GAL-C expression appears as the cells achieve their mature morphology (Diner 1965, Jessen et al. 1985).

Galactocerebroside is necessary for myelin formation, since if antibodies are bound to GAL-C, myelination cannot proceed (Owens and Bunge 1990, Ranscht et al. 1987).

\textbf{Sulphatide:} Sulphatide is the sulphated form of GAL-C. Like GAL-C, it is expressed on the surface of both myelin-forming and non-myelin-forming Schwann cells \textit{in vivo} (Mirsky et al. 1980, 1990).

\textbf{Basement Membrane Components:} As described in Chapter 1, all mature Schwann cells are surrounded by a basement membrane, this is synthesised by the Schwann cell. Basement membrane is essential for myelin formation (reviewed by Bunge et al. 1989) and the basement membrane tubes that remain after Wallerian degeneration are important in regeneration (reviewed by Fawcett and Keynes 1990).

Schwann cell basement membrane contains laminin (merosin-B1-B2 isoform), nidogen, collagen type IV (α1 and α2 isoforms), fibronectin, tenascin (cytotactin/J1), entactin (Baron-Van Evercooren et al. 1986) and heparin sulphate
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Laminin: Laminin is a major component of basement membranes. Laminin is a large glycoprotein made up of 3 protein chains, two light chains of 200 kD, and one heavy chain of 400 kD, that interact together to form a cruciform structure (Nissinen et al. 1991). Laminin isoforms are formed by combinations of the different isoforms of these chains. There are three isoforms of the light chains (B1, B2 and s-laminin) (Timpl et al. 1979, Timpl 1989, Hunter et al. 1989) and two related heavy chains (A chain and merosin) (Timpl et al. 1979, Timpl 1989, Leivo and Engvall 1988, Ehrig et al. 1990). The laminin isoform containing the merosin chain with the the B1 and B2 short chains has recently been shown to be the major form of laminin in both normal and cut peripheral nerve (Leivo and Engvall 1988, Sanes et al. 1990, also Reichardt and Tomaselli 1991 for review).

Both myelin-forming and non-myelin-forming Schwann cells synthesize a basal lamina (Bignami et al. 1984a, Billings-Gagliardi et al. 1974) and make large amounts of laminin (Cornbrooks et al. 1983). The laminin in adult nerve is not restricted to the basement membrane, being also associated with the Schwann cell surface membrane, possibly even on its adaxonal face, so that axons may contact laminin in the adult nerve (Kuecherer-Ehret et al. 1990).

Laminin receptors: Laminin interacts with cells through a variety of cell receptors. The integrins are a family of high affinity receptors that mediate many of the effects of laminin, the integrins are integral membrane proteins, that form heterodimers containing $\alpha$ and $\beta$ subunits. 6 $\beta$-subunit homologues, and 12 $\alpha$-subunit homologues have been described so far. The integrins $\beta_1\alpha_1$, $\beta_1\alpha_2$, $\beta_1\alpha_3$, $\beta_1\alpha_6$, and $\beta_1\alpha_7$ have been shown to bind laminin, of these, $\beta_1\alpha_1$, $\beta_1\alpha_3$ and $\beta_1\alpha_6$ are present in the nervous system (reviewed by Reichardt and Tomaselli 1991). Integrins interact directly with the cytoskeleton, but can also activate second messenger systems.

Laminin also interacts with cells using non-integrin laminin receptors. These include integrin-related proteins, gangliosides, sulphatides, and a cell surface enzyme called galactosyltransferase that interacts with carbohydrate substrates on laminin. Gangliosides also modulate the functions of integrins (reviewed by Reichardt and
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Tomaselli 1991). It is unclear which of these laminin binding molecules are present on Schwann cells, so how laminin mediates its effects on Schwann cells remains to be determined.

Other integrin receptors. Recently the mRNA of another integrin isoform, β4, has been found to be expressed by Schwann cells. β4 integrin mRNA is expressed by Schwann cells at low levels at birth, and increases from P15. β4 integrin mRNA is lost after crush injury and returns as the nerve regenerates, in tissue culture, β4 mRNA expression is increased by forskolin. This distribution, and the dependence of myelin formation on the presence of an intact basal lamina has led to the suggestion that β4 integrin functions in axon–Schwann cell interactions during myelination (Feltri et al. 1992). The ligand for β4 is not known (Reichardt and Tomaselli 1991).

Intermediate Filaments (IFs): Intermediate filaments are 10 nm diameter, with heterogeneity in polypeptide subunits (discussed in Kelly et al. 1992).

6 classes of intermediate filament (I–VI) have been described:

I. & II. keratins in epithelial cells

III. vimentin, desmin, GFAP, peripherin

IV. NF–L, NF–M, NF–H, α–internexin

V. nuclear lamins

VI. nestin

IFs are linked to both the nuclear and plasma membranes, indicating a role for IFs in the spatial organisation of cytoplasm, in transport of macromolecules between nucleoplasmic and cytoplasmic compartments, or the transfer of information from the periphery to the nucleus. The function of one IF has recently been determined, the keratin cytoskeleton in skin epidermal cells has been demonstrated to confer strength and mechanical resistance. Co-expression of the type II keratin, K5 and the type I keratin, K14 is essential for the assembly of epidermal keratin intermediate filaments. Mutation of the gene encoding either K5 or K14 will prevent filament assembly, the keratin molecule clumps in the cytoplasm, leaving the cell without a cytoskeleton. The result is a skin with no resistance to compaction stresses, the cells are fragile and rupture on trauma and patients with this defect show hereditary skin blistering (Lane et al. 1992).
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The forms of IF expressed by cells often change during differentiation. A dramatic example of this is seen in neuronal development when nestin expression is replaced by first α-internexin which in turn is replaced by NFs L, M and H in the mature neuron (discussed in Kelly et al. 1992). Similar changes in the type of intermediate filament expressed are seen during muscle development where vimentin is changed for desmin. Oligodendrocytes lose vimentin as they mature and do not express any IFs. This remodelling of IFs during cell maturation might indicate that IFs also play a part in cell differentiation (discussed in Kelly et al. 1992). It may, however, only demonstrate the changing cytoskeletal requirements of cells at different stages of development.

**Nestin**: Nestin is a type VI intermediate, recognised by the rat 401 monoclonal antibody (Lendhal et al. 1990). Species of 400 and 175 kD are seen in the rat sciatic nerve (Friedman et al. 1990). Nestin is expressed by both myelin-forming and non-myelin-forming Schwann cells although the levels are higher in the myelin-forming cells (Friedman et al. 1990).

**Vimentin**: Vimentin is a type III intermediate filament. Vimentin is expressed by both myelin-forming and non-myelin-forming Schwann cells (Yen and Fields 1981). The presence of vimentin in fibroblasts reduces the usefulness of vimentin as a marker to define Schwann cells in culture.

**Group 2: Markers restricted to non-myelin-forming Schwann cells**

**Low Affinity Nerve Growth Factor Receptor (NGF–R, p75NGFR)**: There are two types of NGF–R. The low affinity receptor, a 75 kD glycoprotein (Hosang and Shooter 1985, Chao 1986, Radeke et al. 1987) is a cell surface associated protein with an extracellular domain rich in cysteine and acidic amino acids, with a single transmembrane domain and a short cytoplasmic domain that is required for biological activity (Hempstead et al. 1990, Yan et al. 1991). The high affinity receptor, trk (tyrosine protein kinase), is not present on Schwann cells (Zimmerman and Sutter 1983). It is the trk receptor that mediates most of the known effects of NGF (for review see Chao 1992).

The surface marker recognised by the mouse polyclonal antibody Ran–1 raised against 33B cells (Fields et al. 1975, Brockes et al. 1977) was the first surface
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marker for Schwann cells in tissue culture. The monoclonal antibody 217c (Peng et al. 1982), raised against the C6 rat glioma cell line, has been shown to recognise the same epitope as Ran−1 (Fields and Dammerman 1985) Ran−1, however, also binds another antigen, as it binds myelin-forming Schwann cells as well as non-myelin-forming Schwann cells, whilst 217c only binds to non-myelin-forming Schwann cells (Jessen et al. 1990). The monoclonal antibody 192-IgG recognises rat NGF−R (Chandler et al. 1984). It has since been demonstrated that 217c recognises the same molecule as 192-IgG, but not the same epitope (Kumar et al. 1990, Ferrari et al. 1991, Stemple and Anderson 1991), so now both 217c and Ran−1 are known to recognise the p75NGF−R.

The levels of NGF−R protein in rat sciatic nerve decrease during development. It is not easy to detect 125I NGF binding in adult rat sciatic nerve (Taniuchi et al. 1986). Binding of 217c to teased or dissociated adult sciatic nerve and sympathetic trunk (a largely unmyelinated nerve) reveals that the low affinity NGF−R remains on the surface of non-myelin-forming Schwann cells in the adult rat, although the levels are clearly lower than in developing nerve or on cultured Schwann cells (Jessen et al. 1990).

Schwann cell expression of both L1 and glia-derived nexin (GDN) mRNA are increased by NGF in culture. This indicates that some of the increased Schwann cell adhesiveness and increased production of neurite-promoting factors that act to promote neurite outgrowth after nerve damage is an autocrine response to the increased NGF secreted by Schwann cells after nerve injury (Seilheimer and Schachner 1987, Heumann et al. 1987, Bleuel and Monard 1992). Recently, a novel function for p75NGF−R has been proposed; Schwann cells migrate faster over the surface of cryostat sections of denervated sciatic nerve than over sections of normal nerve, and this migration is further increased if the sections are pretreated with NGF, implying that Schwann cells use p75NGF−R to migrate in the NGF-rich environment after nerve damage (Anton and Matthew 1992). Other functions for the p75NGF−R have been suggested (reviewed by Chao 1992). First, Schwann cells might store a reservoir of NGF ready to present to neurons. Second, p75NGF−R may transmit intracellular signals through G−protein mediated mechanisms (possibly this mediates
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the NGF induced elevation of GDN and L1 discussed above). Both of these functions, if evidence is found that they exist, would be applicable to the Schwann cells. The other suggested functions would only be applicable to neurons, or cells co-expressing the trk receptor with p75NGF-R. These include a role for p75NGF-R in retrograde transport of NGF, discriminating among neurotrophic factors and signalling or providing substrates for trk. It has also been suggested that expression of both p75NGF-R and trk is necessary before high affinity binding of NGF is possible (reviewed by Chao 1992).

Ran-2: The antigen recognised by the Ran-2 antibody is present on astrocytes in vitro and non-myelin forming, but not on myelin forming Schwann cells in vivo (Bartlett et al. 1981, Jessen and Mirsky 1984). Expression of Ran-2 is not maintained in culture (Mirsky and Jessen 1984).

Neural Cell Adhesion Molecule (N-CAM): The N-CAM family includes three major forms of N-CAM. The N-CAM 180 kD and N-CAM 140 kD are integral membrane proteins, whilst N-CAM 120 kD is inserted into the plasma membrane by a phosphatidylinositol anchor. There is one N-CAM gene, and alternative RNA splicing gives rise to the three main types of chain and other less common N-CAM molecules (for review see Edelman and Crossin 1991). Soluble and ECM associated forms of N-CAM have also been found (discussed in Probstmeier et al. 1992). The form of N-CAM predominant in embryonic tissue is highly sialylated and the level of α-2,8-linked polysialic acid modulates cell–cell interactions. The more α-2,8-linked polysialic acid bound, the lower the binding rate (reviewed by Rutishauser and Jessell 1988, Edelman and Crossin 1991). The N-CAM molecule also contains other carbohydrate modifications (the HNK-1 epitope is on some N-CAM molecules) and is phosphorylated (for review see Edelman and Crossin 1991). The N-CAM molecule contains Ig-like and fibronectin III-like domains in its extracellular portion. The actions of N-CAM tend to be mediated by homophilic binding (reviewed by Schachner 1990).

Developing Schwann cells express N-CAM on their cell surface, but as myelination proceeds N-CAM is lost selectively from the myelin–forming Schwann cells. In the mouse, some peri-axonal N-CAM remains (Martini and Schachner
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1986), but in the rat this down regulation is complete and no N–CAM is detectable immunocytochemically in the mature myelin–forming Schwann cells (Jessen et al. 1987a, Mirsky et al. 1986).

The contribution of N–CAM to enhanced neurite outgrowth over Schwann cells is less than that of L1, but N–CAM molecules, both on the Schwann cell surface and ECM–bound are likely to contribute to axonal regrowth through bands of Bugner after Wallerian degeneration in vivo, interacting with N–CAM molecules on the axonal surface (reviewed by Schachner 1990).

L1 / NGF-inducible large external glycoprotein (NILE). L1 is a cell surface adhesion molecule of 200 kD (Rathjen and Schachner 1984), with extracellular, transmembrane and intracellular domains. L1 belongs to the Ig superfamily with 6 Ig–like domains, L1 also shares homology with the type III repeating units of the ECM molecule fibronectin (reviewed by Schachner 1990).

L1 is expressed on non–myelin–forming Schwann cells in the adult sciatic nerve and on their axons (Martini and Schachner 1986). In development, L1 is expressed on all fasciculating axons (before they are separated from each other by their Schwann cells) and on Schwann cells before myelin is expressed (Faissner et al. 1984). After 1.5 loops of myelin are formed, L1 is lost from the Schwann cell, its associated axon, and from the nodal area (Martini and Schachner 1986).

L1 antibodies prevent myelination in culture (Seilheimer et al. 1989, Wood et al. 1990) suggesting a role for L1 in Schwann cell–neuron interactions. L1 is a major mediator of axonal growth over cultured Schwann cells and as the levels of L1 on Schwann cells increase after nerve transection, L1, like N–CAM, could be an important promoter of axonal regrowth after nerve damage (reviewed by Schachner 1990).

L1 mediated neuron–glial adhesion is via a heterophilic mechanism, whereas neuron–neuron interactions are mediated by homophilic, L1:L1 binding (Grumet and Edelman 1988).

There is evidence that functional co-operation between L1 and N–CAM enhances cell–cell interactions (Kadmon et al. 1990a & b).

Neuron–glia adhesion molecule (Ng–CAM). Originally identified in chick, Ng–CAM
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was thought to be the avian homologue for mammalian L1 and human NILE glycoproteins, but recently it has become clear that they are separate molecules, however, Ng-CAM and L1 do bind to each other (for review see Grumet 1992).

Glial Fibrillary Acidic Protein (GFAP). GFAP is a 49 kD type III intermediate filament. GFAP is expressed by non-myelin-forming Schwann cells but not by myelin forming Schwann cells (Jessen and Mirsky 1980 & 1985, Feinstein et al. 1992, Mokuno et al. 1989). The GFAP gene (Feinstein et al. 1992) and protein (Jessen and Mirsky 1980, 1985, Mokuno et al. 1989) in Schwann cells are not the same as those found in astrocytes.

Group 3: Markers restricted to myelin-forming Schwann cells: The proteins of PNS myelin. Non-myelin forming Schwann cells express very little, if any myelin protein (Inuzuka et al. 1988) or mRNA (Griffiths et al. 1989).

Myelin Protein Zero ($P_q$): $P_q$ accounts for more than 50% of the protein present in purified peripheral myelin (Greenfield et al. 1973). $P_q$ is a 28–30 kD transmembrane protein with a single extracellular Ig-like domain (Lemke and Axel 1985, Lemke et al. 1988). The $P_q$ molecule undergoes many post-translational modifications, including glycosylation (Everly et al. 1973, Kitamura et al. 1976, Matthieu et al. 1975, Roomi et al. 1978), phosphorylation (Brunden and Poduslo 1987b, Singh and Spritz 1976, Wiggins and Morell 1980), sulphation (Matthieu et al. 1975), and acylation (Agrawal et al. 1983). The $P_q$ molecule changes during development, between 5 and 9 days postnatally in the rat the nature of the oligosaccharide moiety changes from uniformly endo-H-insensitive, to a mixture of endo H-sensitive and insensitive in the adult. This indicates a change from complex to high mannose carbohydrate and possibly altered homophilic ($P_q$-$P_q$ intercellular, or intracellular binding, see below) or heterophilic ($P_q$-$X$, Schwann cell-axon) binding with age (Brunden 1992).

In the rat, $P_q$ is expressed exclusively by myelin forming Schwann cells. $P_q$ protein (Trapp et al. 1981) and mRNA (Lamperth et al. 1990) are seen developmentally after Schwann cells have formed the 1:1 relationship with their axons that precedes myelin formation. In the adult nerve, $P_q$ is present in all compact myelin and in small amounts in the perinuclear cytoplasm, $P_q$ is absent from the...
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As expected from the presence of an Ig–like domain, $P_0$ has adhesive properties. Transfected cells expressing $P_0$ protein on their surface show increased cell–cell attachment (d'Urso et al. 1990, Schneider–Schaulies et al. 1990, Filbin et al. 1990) and increased neurite outgrowth–promoting properties (Schneider–Schaulies et al. 1990). Cell–cell and cell–neurite interaction can be reduced by adding the $P_0$ extracellular domain as a soluble protein, confirming the ability of the extracellular portion of the $P_0$ molecule to bind to itself, as well as to unidentified molecules on the neurite surface (Schneider–Schaulies et al. 1990). Homophilic $P_0$ binding has been shown to depend on expression of the complex sugar residues added to the molecule by glycosylation as they are synthesised into the rough ER lumen. Glycosylation mutant Chinese hamster ovary (CHO) cells transfected with $P_0$ express $P_0$ molecules with high mannose sugar residues rather than complex sugars, and these cells show decreased adhesion compared to wild–type cells transfected in the same manner (Filbin and Tennekoon 1991).

It is predicted that in peripheral myelin, $P_0$ mediates compaction of myelin, acting between both the extracellular and intracellular myelin layers to promote adhesion (Lemke and Axel 1985). The demonstration of homophilic binding between $P_0$ molecules strengthens this hypothesis. The cytoplasmic domain of $P_0$ is very basic and it could act in the PNS in the same way that the myelin basic protein family acts in the CNS to maintain the major dense band of myelin (reviewed in Lemke 1988, Hudson 1990). The importance of $P_0$ in maintaining the myelin sheath has been demonstrated by preventing normal expression of $P_0$. Mutation of the $P_0$ gene results in hypomyelination, small areas of myelin compact, but the myelin sheath is never complete around the axon (Giese et al. 1992). In coculture with neurons, Schwann cells are prevented from synthesising normal myelin if they are infected with antisense $P_0$ mRNA (Owens and Boyd 1991).

Avian $P_0$ expression has recently been shown to be less stringently restricted to myelin forming cells, and $P_0$ is seen developmentally a long time before myelin wraps are present (Barbu 1990, Bhattacharyya et al. 1991), therefore, in avies, $P_0$ may have additional roles during nerve development, before myelin formation, possibly
mediating intercellular interactions.

**Myelin Basic Protein (MBP, P<sub>1</sub>):** MBP accounts for between 2 and 16% of peripheral nerve myelin protein (Greenfield et al. 1973). The myelin basic proteins are a family at least six related proteins of 14–21.5 kD that are derived from one gene that, through a complicated system of alternative splicing, produces seven different mRNA species. The MBP molecule is located on the intracellular face of myelin, at the major dense line. There is strong evidence from the MBP-deficient mutant mice *shiverer* and *myelin-deficient* that in the CNS, MBP maintains myelin compaction on the cytoplasmic side (reviewed in Lemke 1988). The role of MBP in the PNS is not known, as, although MBP is located in compact myelin, MBP deficient mutants have apparently normal peripheral myelin (reviewed in Lemke 1988).

**Myelin Protein 2 (P<sub>2</sub>, P<sub>2</sub> basic protein):** P<sub>2</sub> is a minor component of peripheral myelin, accounting for only 0.05–1% of total myelin protein (Milek et al. 1981, Hahn et al. 1987). P<sub>2</sub> is a 14.8 kD basic protein present in compact myelin and excluded from the Schmidt–Lanterman incisures (Hahn et al. 1987). As P<sub>2</sub> protein is only a minor component of myelin, in thinly myelinated axons the levels are very low and hard to detect, but P<sub>2</sub> is probably present in all compact myelin, and not restricted to myelin surrounding the larger fibres (Trapp et al. 1979, 1983, Winter et al. 1982, Hahn et al. 1987). P<sub>2</sub> is detectable by immunolabelling later in development than P<sub>0</sub> and MBP (Hahn et al. 1987).

P<sub>2</sub> has been speculated to be involved in the synthesis of long chain fatty acids, in their transport to myelin and in myelin assembly (reviewed by Hudson 1990).

**Myelin Associated Glycoprotein (MAG):** MAG is a transmembrane protein that constitutes less than 1% of PNS myelin protein. MAG is present in two isoforms, 67 kD and 72 kD, the 67 kD form predominates in the Schwann cell, which only expresses trace amounts of the 72 kD isoform. The extracellular domain of MAG contains 5 Ig-like domains, the intracellular domain is the site of differences between the two MAG isoforms. MAG is restricted to myelin forming Schwann cells in the PNS and is located in the mesaxons, the Schmidt–Lanterman incisures and the paranodal loops. No MAG is present in compact myelin (reviewed by Hudson 1990).
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There are two postulated functions for MAG. Firstly, the MAG molecule contains Ig-like domains making it likely to have adhesive properties. The long and short forms of MAG both increase the adhesiveness of MAG-transfected fibroblasts (Afar et al. 1991, Johnson et al. 1989), whilst the short form of MAG also increases the neurite-promoting properties of the same cells (Johnson et al. 1989). The MAG molecule is thought to mediate Schwann cell-axon interactions, and to maintain the periaxonal space and preventing compaction at the Schmidt-Lanterman incisures and the paranodal loops (reviewed by Hudson 1990). Removal of MAG is essential for myelin compaction and in the quaking mouse, poor compaction, hypomyelination, demyelination, remyelination and increased Schwann cell division are the result of Schwann cells inability to remove MAG (Trapp 1988). The second function for MAG is mainly in the CNS, where the 72 kD form of MAG is present on the large multivesicular bodies that may be associated with retrograde transport of membrane components from the periaxonal space to the oligodendrocyte cell body. MAG could be the receptor initiating endocytosis and thus could be involved in delivering messages from the axon to the oligodendrocyte cell body (reviewed by Hudson 1990).

Peripheral myelin protein 22 (PMP22, gas-3, PAS-II): PMP22 is a 22 kD glycoprotein that is selectively expressed in PNS myelin. PMP22 is expressed in compact myelin of adult rat sciatic nerve, and during development the pattern of PMP22 expression is broadly the same as other myelin proteins (Snipes et al. 1992). PMP22 dysfunction is probably responsible for the mouse mutation trembler in which axons are hypomyelinated and there is poor myelin compaction, constant myelin degradation and remyelination and the Schwann cells of myelinated nerves divide abnormally fast (Suter et al. 1992). As discussed in Chapter 1, PMP22 is homologous with a putative growth arrest specific protein, and it has been suggested that PMP22 expression is a signal for growth arrest (Welcher et al. 1991, Spreyer et al. 1991). However, any factor that interferes with myelin formation or maintenance will cause myelin degradation and Schwann cell division (reviewed in Chapter 1). If PMP22 is an essential component of PNS myelin, its loss would cause these effects without PMP22 acting as a growth arrest specific gene.

2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase): There are two forms of
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CNPase, 46 kD and 48 kD. CNPase is a cytoplasmic protein associated with myelin forming cells, Schwann cells and oligodendrocytes, but which is also expressed in non-neuronal tissue (thymus). The levels of CNPase are much lower in Schwann cells than in oligodendrocytes, and unlike oligodendrocytes, Schwann cells do not incorporate CNPase into compact myelin (Matthieu et al. 1979, reviewed by Lemke 1988 and Hudson 1990). Developmental expression of the CNPase gene during peripheral nerve development, unlike in the CNS, does not correlate with myelin formation (Stahl et al. 1990, Edwards and Braun 1988).

Several functions for CNPase have been suggested. CNPase in the CNS may participate in intracellular trafficking of myelin membrane components (RNA or protein), in GTP binding, and in organizing cytoskeletal networks. The presence of CNPase outside the nervous system suggests that it may have general functions, not restricted to myelin formation or maintenance (reviewed by Hudson 1990).

Proteolipid Protein (PLP and DM20): There are two isoforms of PLP, PLP at 24 kD, and DM20 at 20 kD, formed by alternative splicing from one gene. The PLP proteins in the CNS are transmembrane proteins, with 3 transmembrane domains, and a large extracellular domain, and PLP comprises 50% of CNS myelin protein (reviewed Hudson 1990, Lemke 1988). During early development, DM20 is the predominant species in oligodendrocytes, but as myelination peaks, PLP 24 kD becomes, and thereafter remains, the major species (reviewed by Hudson 1990).

There is good evidence for a structural role for PLP in CNS compact myelin, acting at the intraperiod line to maintain normal spacing between the bilayers (reviewed by Hudson 1990). In the CNS, an additional role for PLP is proposed, as during development, PLP mRNA has been detected in the mouse, long before myelin is first seen. PLP-deficient mutants show excessive proliferation of oligodendrocyte precursor cells, in spite of this they are profoundly short of mature oligodendrocytes. The mutant PLP has been demonstrated to have no toxic effects, rather, the normal PLP is proposed to be necessary for oligodendrocyte differentiation (reviewed by Hudson 1990). PLP/DM20 acts as an ion channel when incorporated into lipid bilayers and it has been suggested that PLP may be involved in receiving or signalling messages between oligodendrocytes and surrounding astrocytes or neurons (Hudson...
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DM20 is present in murine heart tissue, this may also indicate that PLP/DM20 is involved in more biological processes and is not only a structural component of compact myelin (Campagnoni et al. 1992).

In the PNS, PLP and DM20 are present in Schwann cell cytoplasm, but not in compact myelin (Puckett et al. 1987). Developmentally, and after nerve transection, PLP gene expression in the PNS does not correlate with myelination or parallel expression of the major myelin gene P0 suggesting that PLP is not acting as a myelin-specific protein in the PNS (Kamholz et al. 1992, Stahl et al. 1990).

"Neuron-specific" proteins: tubulin II and neurofilaments L and M (NF-L, NF-M): NF-L and NF-M are respectively 68 kD and 150 kD type IV intermediate filament proteins. In rat, NF-M is expressed by early myelin-forming Schwann cells in the first postnatal week (Kelly et al. 1992). The mRNAs for NF-L and NF-M are not detectable in adult rat sciatic nerve, but are induced after nerve transection and during tellurium induced demyelination (Roberson et al. 1992). Thus it is unlikely that neurofilaments are myelin-specific proteins. The mRNA for neuron-specific microtubule protein tubulin II is not present in mature Schwann cells, but is induced to detectable levels after nerve transection or tellurium induced demyelination (Roberson et al. 1992).

The expression of neuron-specific genes in the Schwann cells of developing and lesioned nerve has not been explained, although it must be pointed out that many other molecules are shared between neurons and glia (examples include N-CAM, L1, NGF-R, S100 and A5E3).

Changes in Schwann cell phenotype after axon loss. These changes are summarized in Figure 2.1.

Markers expressed by cultured Schwann cells and by Schwann cells after Wallerian degeneration: As discussed in Chapter 1, when myelin-forming Schwann cells are disrupted by nerve transection, toxic insult, or when sciatic nerves are excised and either dissociated with enzymes or explanted and placed in culture, myelin rejection and myelin destruction follow. What happens to Schwann cell expression of myelin genes and proteins in these cells, and are there other changes in the Schwann cell phenotype? How much do the non-myelin forming Schwann cells change their
phenotype in similar conditions?

The change in Schwann cell phenotype at these times are summarized below.

Markers that show no change, or only variations in level of expression. The intracellular proteins S100 (Brockes et al. 1979, Holton and Weston 1982a, 1982b, Mirsky and Jessen 1984), vimentin (Autilio-Gambetti et al. 1982) and nestin (Hockfield and McKay 1985) and the surface glycoprotein laminin (McGarvey et al. 1984) all continue to be expressed by Schwann cells in tissue culture and after axotomy in vivo.

Markers lost by both non-myelin forming and myelin forming Schwann cells. GAL-C is lost from both non-myelin and myelin forming Schwann cells when they are taken into tissue culture (Mirsky et al. 1980) and after axons are severed in vivo (Jessen et al. 1987b). Sulphatide is lost from myelin forming Schwann cells when they are taken into culture (Mirsky et al. 1980). The levels of both these lipids, whilst undetectable by routine immunocytochemistry, remain biochemically detectable and above those of non-glial cells (Fryxell 1980, Rutkowski et al. 1990). Purified Schwann cells in culture do not generally form a basal lamina, although they may continue to express some of the individual components of the basal lamina at lower levels when they are taken into culture and the distribution of laminin is altered after axon lesion in vivo (McGarvey et al. 1984, Bignami 1984b).

Myelin proteins are no longer expressed in large amounts by Schwann cells after they are placed in culture or after axotomy. Immunodetectable levels of $\alpha_0$, MBP, and $\beta_2$ disappear from rat Schwann cells in culture (Mirsky et al. 1980, Brockes et al. 1980, Winter et al. 1982, Lemke and Chao 1988), and this is accompanied by loss of $\alpha_0$ and MBP mRNA (Lemke and Chao 1988). $\alpha_0$, MBP, $\beta_2$ and MAG protein, and $\alpha_0$, MBP and MAG gene expression (LeBlanc and Poduslo 1990) are also greatly reduced after axon lesion in vivo. The $\alpha_0$ protein remaining in the membranes of cultured Schwann cells is 1000 times less than that seen in the myelin membrane of the intact nerve (Brockes et al. 1981). The loss is not absolute, and detectable amounts of $\alpha_0$ and MBP gene and protein are left in both cultured Schwann cells and in Schwann cells remaining after Wallerian degeneration (LeBlanc and Poduslo 1990, Brunden et al. 1990a, 1990b, Rutkowski et al. 1990). The $\alpha_0$ expressed by Schwann cells in
2. Schwann cell Phenotype

permanently transected nerves is not incorporated into the cell membrane, rather it is transported to the lysosomes and degraded (Poduslo 1984, Brunden and Poduslo 1987a, Brunden et al. 1985, 1990b). PMP22 mRNA is also lost on nerve transection (Spreyer et al. 1991, Welcher et al. 1991).

The levels of PLP and DM20 mRNAs during development and after crush injury do not parallel those of the major myelin proteins, suggesting that these transcripts are not under stringent axonal control (Gupta et al. 1991).

Non-myelin forming Schwann cell markers gained by myelin forming Schwann cells in culture and in transected nerve. The surface proteins L1, N-CAM and NGF-R and the cytoplasmic protein GFAP are expressed only by non-myelin forming Schwann cells in vivo in the adult mammal. In culture and after nerve transection, N-CAM is also synthesised by the previously myelin forming Schwann cells (Jessen et al. 1987a, Nieke and Schachner 1985, Daniloff et al. 1986). The levels of GFAP mRNA are reduced after nerve transection, however, indicating that the levels of GFAP expressed by non-myelin forming Schwann cells is maintained at higher levels by contact with small axons (Mokuno et al. 1989). After nerve transection, the type of N-CAM molecule expressed is also changed, more of the E form, or highly sialylated N-CAM is expressed after nerve damage (Remsen et al. 1990). L1 expression is also increased after nerve transection (Nieke and Schachner 1985). NGF-R protein and mRNA expression is upregulated after nerve transection (Taniuchi et al. 1986) and when Schwann cells are taken into culture (Lemke and Chao 1988).

The changes in Schwann cell phenotype seen during Wallerian degeneration are reversible. After crush injury, axon regrowth reverses all the changes described above and all the Schwann cells become either non-myelin forming or myelin forming cells once more.

Schwann cells in culture are very similar to those seen in the long-term transected nerve. Schwann cells in tissue culture, and those in the long-term transected nerve express very similar phenotypes. The non-myelin forming and myelin forming cells de-differentiate into a homogenous population. In this chapter I will examine the expression of O4 and A5E3 antigens and GFAP in transected nerves and on cultured Schwann cells in more detail.
Development of the rat sciatic nerve

Axon penetration of the limb-bud, axon maturation: The sciatic nerve of the rat originates from spinal segments L4–L6. At E11, motor axons leave the ventral spinal cord, and sensory axons begin to leave the dorsal root ganglia (DRG) at E12. By E12, the motor and sensory axons begin to join together into a nerve, and they grow towards the hindlimb. The growing tips and the axons form a disorganised plexus at the base of the limb, but do not enter the limb, and this plexus remains at the base of the limb until E14–15. At E13 some of the axons leave the plexus and penetrate the limb where they grow in a tight, unbranched bundle down the centre of the tissue. At E14 the first branches leave the main bundle and project towards the skin, and skin innervation begins between E14 and E15. By E19 there is extensive branching of the nerve, branches are seen throughout the limb, and skin innervation has begun at the tip of the toes. However, maturation of the peripheral nerve terminals is still not complete by P29. Branches from the nerve grow towards the hindlimb muscles at E15, and sprays or clusters of immature terminals are formed between E17 and E21 within the muscles. More mature, but still polyinnervated, nerve terminals are seen in muscles at P2, and the mature, singly innervated terminals develop over the next two weeks (Reynolds et al. 1991).

From E15, there are many degenerating axons present as some of the motoneurones die (Ziskind-Conhaim 1988).

Axon activity: Sensory axons are capable of transmitting messages to the spinal cord from electrical stimuli at E16, response to muscle stretch is seen from E17, and from natural skin stimuli at E19 (Fitzgerald 1987, 1991). Spontaneous movement in foetal rats peaks at E18–19 (Fitzgerald 1987).

Neural crest cells enter with the axons: Neural crest cells migrate from the dorsal margin of the neural tube. These highly migratory cells move along well characterised pathways away from the spinal cord and differentiate into a diverse number of tissues (for review see Le Douarin and Smith 1988).

In lumbar regions the spinal ganglia as well as Schwann cells are generally considered to both derive from neural crest cells (discussed in Altman and Bayer 1984). In the rat, segmentation of somites is complete by E10, and myotome growth
2. Schwann cell Phenotype

is in progress at lumbar levels by E12. At E11 neural crest cells are visible leaving the crest, but DRG are not identifiable in the lumbar region until E12 (Lawson et al. 1974, Altman and Bayer 1984). Rat lumbar DRG neurons undergo their final mitosis over the period E11–E15 (Lawson et al. 1974, Altman and Bayer 1984). Neurons in rostral ganglia tend to develop up to 2 days in advance of those in caudal ganglia and large neurons develop before small ones \(^1\) (reviewed by Altman and Bayer 1984). The first axonal projections leave the ganglia at E11–12 (Reynolds et al. 1991). At an equivalent stage in the development of brachial nerves in chick, from the time these axons and those leaving the ventral root emerge, they are sparsely populated with cells of neural crest origin (Carpenter and Holliday 1992a). As development continues, the axons are never without these neural crest cells, but as the nerve projects into the limb, the neural crest cells do not travel in advance of the invading growth cones (Carpenter and Holliday 1992a, 1992b). The evidence for Schwann cells, or their neural crest-derived progenitor cells, leading axons to their target was discussed in Chapter 1. It is unlikely that they are active in pathfinding.

There is some evidence that a few Schwann cells do not develop from the neural crest, but rather are formed from the neural tube around the ventral root axons as they leave the spinal cord (Lunn et al. 1987). Loring and Erickson saw neural crest cells migrate ventrally over the neural tube surface to the site where ventral root axons would later emerge, these cells, and the neural crest cells that migrate under the dermatome/myotome would be in an appropriate location to adhere to ventral root fibres as they emerged. They were nevertheless unable to exclude the possibility that some of the ventral root-associated cells had migrated from the neural tube with the roots (Loring and Erickson 1987). There is no evidence of a difference in phenotype between these cells and the neural crest derived cells, and as described above, Carpenter and Holliday also saw neural crest cells at the site of exit of ventral root axons from the time the first growth cones left the spinal cord (Carpenter and Holliday 1992a).

\(^1\) The data from Altman and Bayer 1984 quoted in this section date the pregnancy by counting the day of vaginal plug as day 1. We take the day of vaginal plug to be day 0. To avoid confusion I have taken one day off their published data.
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Schwann cell–axon interactions that precede development of the mature Schwann cells: At E14, the rat sciatic nerve contains many small axons packed together with no glial processes to separate them. The bundles of axons are separated into defined territories within the nerve by processes from the neural crest–derived cells, the presumed precursor cells of Schwann cells (Hashimoto, Jessen and Mirsky 1992, submitted). These cells are distributed throughout the body of the nerve, and not restricted to the periphery of the nerve bundle as has been described for smaller nerves at the same stage of development (Ziskind-Conhaim 1988). As development proceeds, the Schwann cells divide the axons into smaller bundles and by E20 a few of the larger axons are singly wrapped (Hashimoto, Jessen and Mirsky 1992, submitted). By birth, the first myelin wraps are seen around the larger axons that are now no longer sharing their Schwann cells and new myelin wraps are still being formed during the first three weeks after birth (Ziskind-Conhaim 1988). The final separation of the last small, unmyelinated axons from each other by Schwann cell processes is complete two to three weeks after birth (Diner 1965).

At E14 no basal lamina or collagen fibrils are present within the nerve, and adjacent Schwann cells contact each other although no clearly defined cell junctions are seen in electron micrographs. There is however some amorphous extracellular material. By E17, the main body of the nerve shows no increase in extracellular material, but at the edges of the nerve, where cells contact the surrounding mesenchyme, the beginnings of basal lamina are visible, and some collagen fibrils are forming. The Schwann cells at E17 show distended rough ER, consistent with increased secretion of ECM molecules. At E18, collagen fibrils are forming throughout the larger fascicles, and by E19 an incomplete basal lamina is present around many Schwann cells (Hashimoto, Jessen and Mirsky 1992, submitted).

There is a proximal–distal gradient in the level of development, with the distal portions of the nerve behind the proximal parts (Reynolds et al. 1991, Hashimoto, Jessen and Mirsky 1992, submitted).

Development of nerve ultrastructure: blood vessel invasion, perineurium and epineurium development: The first blood vessels invade the nerve at E18, endoneurial fibroblasts are first seen at E22. An incomplete perineurium is first seen at E17.
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(Hashimoto, Jessen and Mirsky 1992, submitted). At birth, there are a large number of activated macrophages in the sciatic nerve (Stoll and Muller 1986), and possibly these are involved in removing the debris from the degenerating motor nerve axons (Rootman et al. 1981).

Division profile. During peripheral nerve development, as the nerve increases in size, and as many axons acquire a one-to-one relationship with their axons, there is a need for more Schwann cells. This need is met by Schwann cell proliferation, which reaches very high levels at birth in the mouse, and then declines over the first 12 postnatal days (Asbury 1967, Brown and Asbury 1981). The degree of proliferation by Schwann cells and their precursor cells has recently been defined in the embryonic and neonatal rat sciatic nerve. The early cells are dividing rapidly before birth, division reaches a peak E19–E20 and then drops abruptly after birth (Stewart, Morgan, Mirsky and Jessen 1992, submitted). These changes are illustrated in Figure 2.2.

When the phenotype of these Schwann cells is examined, cells expressing \( P_0 \), the first of which are seen at birth when many sister Schwann cells are still dividing, are never synthesising DNA (Stewart, Morgan, Mirsky and Jessen, 1992 submitted).

NEURAL CREST PHENOTYPE, see Table 2.3

Descriptions of mammalian neural crest cells are fewer than those of avian crest, this is, presumably, due to the greater accessibility of avian embryos.

Extracellular matrix molecules

The migrating neural crest cell does not have a basal lamina (Billings-Gagliardi et al. 1974, Rogers and Edson 1986). However, in chick, the basal lamina component laminin is seen on migrating neural crest cells before basal lamina is present (Rogers et al. 1986, Rogers and Edson 1986). The mRNA for tenascin (also called J1 or cytotactin) is made by neural crest cells (Tucker and McKay 1991). Fibronectin is not present on migrating neural crest cells, but cultured neural crest cells are induced to express fibronectin by TGF\( \beta \) treatment (Rogers et al. 1992).

Other molecules expressed by neural crest cells

HNK-1 antibody recognises a glycolipid epitope expressed on the surface of many avian neural crest cells before, during and after migration. It is not seen on the non
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crest derived cells in the path of migrating neural crest cells. This marker is used extensively to determine neural crest migration paths, and for lineage studies of neural crest derived cells in avian systems (Loring and Erickson 1987). Unfortunately, it is not possible to use this antibody in mammalian studies since the epitope is not present on many neural crest cells (Smith-Thomas and Fawcett 1989).

The type VI intermediate filament nestin is recognised by rat 401 antibody. It is present on all neural tube cells, and is on the crest cells as they emerge and migrate away. Nestin is retained in both adult myelin-forming, and non-myelin-forming Schwann cells (Hockfield and McKay 1985).

S100 (in the β homodimer isoform) is a soluble cytoplasmic protein with calcium binding properties, which is not present on migrating neural crest cells, but is induced on cells destined to become Schwann cells after migration is complete (Holton and Weston 1982a).

Schwann cell myelin protein (SMP) is a surface glycoprotein that is present on all quail and chick Schwann cells, and not, as the name would suggest, restricted to Schwann cells synthesising myelin. SMP appears on neural crest cells as they differentiate and is not on migrating cells (Dupin 1990).

P0, the major protein of peripheral myelin, is expressed late in developing mammalian nerve and is not seen until, or just before, the onset of myelination (Trapp et al. 1981, Uyemura et al. 1979, Martini et al. 1988, Baron et al. 1981). In birds, however, IE8 a monoclonal antibody to P0, labels a subset of neural crest cells in the ventral migratory pathways (Bhattacharyya et al. 1992). These cells are the precursors of Schwann cells. IE8 binds both non-myelin-forming and myelin-forming Schwann cells in the adult chicken. Also in chick, another monoclonal antibody to P0 (112-2) detects P0 at least 2 days before myelination, however as this group do not look earlier than this, and it is not clear whether this antibody is the same as 1E8 (Barbu et al. 1990). If the 1E8 antibody is really only recognising the P0 molecule, these experiments imply that control of P0 expression is not the same in avian and mammalian species and predict extra functions for the P0 molecule in avian species, possibly as an adhesion molecule. The 1E8 antibody will be very useful in avian neural crest lineage studies as a marker for neural crest cells destined to form
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Schwann cells.

Environment of migrating Neural crest cells

The extracellular environment encountered by migrating avian neural crest cells has been reviewed by Perris and Bronner-Fraser (1989). Both basement membranes and interstitial matrices are present in the paths taken by neural crest cells. Outlining the adjacent epithelia is a basal lamina, an amorphous material separates this from a parallel array of collagenous fibrils. Immunocytochemically, these molecules have been identified in the neural crest pathways: laminin, collagen type IV, HSPG and fibronectin. Perris and co-workers (1991) have shown more recently that collagens I, III and IV (avian) are present in the path of migrating neural crest cells, but not collagens II, V and IX. The ability of these collagens to support or inhibit neural crest migration has been related to their location in pathways chosen by neural crest cells.

In the following experiments, glial cells taken from the sciatic nerve of developing rats are compared to neural crest cells and to Schwann cells. At E15, the cells are clearly very different to Schwann cells, but the amount that they have diverged from the cells that left the neural crest has not been easy to determine.
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METHODS

ANIMALS
Sprague Dawley albino rats bred by the Joint Animal House at University College London were used throughout. The day of the maternal vaginal plug was taken to be day 0, and the age of the pups was confirmed by comparison against a developmental chart compiled by Christie (1964).

CELL CULTURE
Sciatic nerves were taken from rats aged from embryonic day 14 (E14) to adult. Animals were killed by decapitation up to postnatal day 9, older rats were killed with excess CO₂ followed by exsanguination. The sciatic nerves were dissected out, pooled in MEM containing 15mM HEPES (no bicarbonate) buffered to pH 7.4 (MEM–H) and stored on ice. The epineural sheath was removed (if rats were older than E19), and the nerves moved to enzymes in Ca²⁺ and Mg²⁺ free DMEM and chopped finely with scissors. The tissue and enzymes were then placed in an incubator at 37°C in 5/95% CO₂/air for the times indicated below.

A. Trypsin containing enzyme mixture (used when total dissociation is required, or if surface antigens to be examined are insensitive to trypsin)
   2mg.ml⁻¹ collagenase (Lorne Diagnostics)
   1.25mg.ml⁻¹ Trypsin (trypsin 1:300 Gibco)

Times in trypsin containing enzymes:
- embryo–10 days 40 minutes
- 10 days–15 days 1h
- 15 days–adult 3 x 30 minutes
  (enzyme replaced at 30 minute intervals)

B. Enzyme Cocktail (used to preserve trypsin sensitive surface antigens)
   2mg.ml⁻¹ collagenase (Lorne Diagnostics)
   1.2mg.ml⁻¹ hyaluronidase (Sigma)
   0.3mg.ml⁻¹ trypsin inhibitor from chicken egg white (Sigma)
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Times in enzyme cocktail:

<table>
<thead>
<tr>
<th>Age</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>embryo</td>
<td>45 min</td>
</tr>
<tr>
<td>0–10 days</td>
<td>1h</td>
</tr>
<tr>
<td>10 days – adult</td>
<td>2.75h</td>
</tr>
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</table>

After incubation in enzyme as A or B above, an equal volume of MEM–H containing 10% calf serum (CS) was added and the tissue gently triturated with a plastic tip attached to a Gilson automatic pipette. The resulting cell suspension was spun at 500g for 10 minutes, the pellet of cells resuspended in DMEM with 10% CS and the cells were plated onto poly L lysine (PLL) coated glass coverslips. If the cells were to be dried on to slides, then the pellet was resuspended in phosphate buffered saline (PBS), and the cell suspension dried on to gelatin coated glass slides and stored dry at 4°C overnight before labelling.

Dorsal root ganglion conditioned medium. DRG were dissected from neonatal rats and dissociated in trypsin and collagenase as described for adult rat sciatic nerve above. The cells were cultured on laminin coated tissue culture plastic, in DMEM with 10% foetal calf serum (FCS) and NGF, cytosine arabinoside $10^{-5}$M was added for 1 week to reduce the number of Schwann cells and fibroblasts. The cells were washed and the medium was changed to DMEM with 10% CS and left for 2 days, this medium was collected, filtered through a 0.22μM Millipore filter and used immediately.

Denervation. Rats weighing between 90 and 100g were anaesthetized and the left sciatic nerve cut 2–4 mm below the sciatic notch and a 2–3mm segment of the nerve was removed. The proximal stump was ligated and rerouted into an adjacent muscle. After surgery, rats were kept up to 2 months before nerves were removed for dissociation and analysis. All these operations were performed by K. R. Jessen.

LABELLING WITH ANTIBODIES

Cells start attaching to PLL coated glass cover slips within 20 minutes. After a minimum of 2 hours they were washed in MEM–H and incubated directly with primary antibodies where the antigens were expressed on the cell surface or after fixing and permeablising where antigens were intracellular.

Surface antigens (GAL–C, O4, Ran–1(217c), Ran–2, NGF–R, A5E3 Laminin, N–
CAM, L1, LB1)
The cells were incubated in primary antibody diluted in MEM–H with 10% FCS for 30 minutes at room temperature, then fluorescein or rhodamine conjugated anti rabbit or mouse secondary antibodies diluted in MEM–H with 10% FCS for 30 minutes. The cells were then fixed in 4% paraformaldehyde for 15 minutes, mounted in Citifluor antifade mountant (Citifluor UK) on glass slides and sealed with clear nail varnish.

Intracellular antigens (GFAP, Vimentin, S100, P0)
The cells were fixed in 4% paraformaldehyde and then acid/alcohol (95% ethanol, 5% acetic acid at −20°C) for 15 minutes (S100 only) or acid/alcohol alone (all other internal antigens) for 15 minutes. After washing in PBS they were incubated in primary antibody diluted in PBS containing 10% FCS, 0.1M lysine and 0.02% sodium azide for 30 minutes. The cells were then washed and incubated in fluorescein or rhodamine conjugated anti mouse or rabbit secondary antibodies as appropriate for 30 minutes, mounted in Citifluor and sealed as above.

Where two antibodies were used to label the same cells, the antibodies were added sequentially e.g. first primary antibody then first secondary antibody followed by the second primary antibody then the second secondary antibody. Only when both antigens were on the cell surface were the two primary antibodies were mixed together to avoid long incubations of living cells.

ANTIBODIES
Primary antibodies
A5E3 (monoclonal IgG2a) hybridoma supernatant and ascites fluid (Mirsky et al. 1985) were used at dilutions of 1:2 and 1:500 respectively.
Anti bromodeoxyuridine (BrdU) Bu20a (monoclonal IgG1) supernatant (Gratzner 1982) was a gift from D.Y. Mason and was used at a dilution of 1:20.
Anti galactocerebroside (GAL–C) (monoclonal IgG3) (Ranscht et al. 1982) ascites fluid prepared by E. Abney or hybridoma supernatant from the same clone were used at dilutions of 1:200 and 1:2 respectively.
GFAP (rabbit polyclonal against cow GFAP protein) was from Dako Immunoglobulins A/s; Dakopatts, Copenhagen, Denmark and used at a dilution of
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LI Rabbit polyclonal (Faissner et al. 1984). Used at 1:100.

Anti laminin (rabbit polyclonal against mouse laminin) a gift from R. Timpl, used at a dilution of 1:800.

Neural cell adhesion molecule (N–CAM) (rabbit polyclonal) This antibody recognises all N–CAM species (Genarini et al. 1984). A gift from C. Goridis, used at a dilution of 1:500.

Q4 (monoclonal IgM) hybridoma supernatant (Sommer and Schachner, 1981) was used at a dilution of 1:2.

P0 (rabbit polyclonal against rat protein zero) three batches of P0 antibodies were used, first a gift from J. P. Brockes, second a gift from B. D. Trapp and third made in this laboratory essentially as described in Brockes 1980, all were used diluted 1:200.

P04 (mouse monoclonal) a gift from C. Linington. Used at a dilution of 1:100.

Ran–1(217c) (mouse monoclonal IgG2a) (Peng et al. 1982), a gift from K. L. Fields, was used at a dilution of 1:500.


S100 (rabbit polyclonal against cow S100 protein) was from Dako Immunoglobulins A/s; Dakopatts, Copenhagen, Denmark and used at a dilution of 1:1,000, or at 1:400 on dried cell preparations.

Anti vimentin (monoclonal) antibodies were from Boehringer and used at a dilution of 1:100.

Secondary antibodies

- Goat anti mouse Ig conjugated to tetramethyl rhodamine.
- Goat anti rabbit Ig conjugated to tetramethyl rhodamine.
- Goat anti mouse Ig conjugated to fluorescein.
- Goat anti rabbit Ig conjugated to fluorescein.

All above were from Cappel Organon Teknika Corp., West Chester P.A., and used at dilutions of 1:200–1:300 depending the strength of each batch. Cross reactivity to rabbit/mouse as appropriate was removed by adsorption against mouse/rabbit Igs.

Donkey anti rabbit conjugated to biotin
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Sheep anti mouse conjugated to biotin
Streptavidin conjugated to fluorescein
Streptavidin conjugated to texas red

All above were from Amersham International, Amersham, UK, and used diluted 1:100.

Non-specific binding to rat nerve by anti rabbit biotin was removed by incubation overnight at 4°C with chopped adult rat nerve.

MICROSCOPE

Immunolabelled slides were viewed and photographed on a Zeiss Axioskop or Zeiss Student microscope with epi-illumination and phase optics. A dark field condenser was attached to view the silver grains of autoradiographs.

ANALYSIS OF RESULTS

A minimum of 200 cells were counted on each coverslip, each experiment included at least two coverslips and each experiment was repeated three times. The cells were viewed with a 63x oil immersion or 40x dry objective as appropriate. Photographs were taken using Ilford HP5 (400 asa) black and white film.
2. Schwann cell Phenotype

RESULTS

Cells in the embryonic sciatic nerve

Morphology of cells from the prenatal sciatic nerve.

The cells from E15-16 sciatic nerve are very different to postnatal Schwann cells. They are flattened, polygonal cells that form pavements over the tissue culture substrate (Figure 2.3a). These cells are reminiscent of the rat neural crest cells described by Smith-Thomas and coworkers (1990). Postnatal Schwann cells in culture are spindle-shaped, bipolar cells that do not adhere closely to the culture substrate, rather they string out, tending to make swirl patterns over the dish, with little contact between adjacent cells (Figure 2.2c).

When cells from E15 nerve are maintained in culture overnight in medium that supports neonatal or adult Schwann cells, very few cells survive. This death can be prevented or reduced if the cells are kept in medium conditioned by DRG neurons.

Phenotype of prenatal Schwann cells.

Cell suspensions were made from nerves taken from rats aged between E14 and birth. The cells were attached to coverslips and then labelled with antibodies after cell attachment, and before 3 hours of culture. The results are summarized in Table 2.4 and Figures 2.4, 2.5 and 2.6.

It was important to ascertain that the cells studied were derived from the developing nerve, and not from some other part of the developing limb bud. This is certain to be the case for the following reasons; firstly, from the studies of the intact nerve by Hashimoto and coworkers (Hashimoto, Mirsky and Jessen 1992, submitted), there are no other cells present at E15 in the nerve to contaminate the cultures. The dissection, although it takes some time to master does, with practice, produce a clean piece of nerve without pieces of the surrounding tissues attached. In the first cultures, the nerves were always dissected back towards the spinal cord to be sure that they emerged from the DRGs and the spinal cord, however this was not necessary each time as the nerve tissue has a characteristic texture and the surrounding tissue is amorphous. In addition, when the surrounding mesenchymal tissues were dissociated and plated separately, it became clear that the nerve cultures were very different as the surrounding mesenchymal tissue, unlike nervous tissue, did not spread over the...
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coverslip, and the cells remained rounded after 3h of culture.

The cells present in the sciatic nerve at E15 share some markers with mature Schwann cells. Laminin and vimentin are present, and also the non-myelin Schwann cell–specific markers L1, N–CAM, A5E3 and NGF–R. Over the time examined, all the cells became positive for O4 and S100, and many of them started to express GAL–C and GFAP.

By birth (P0, E22) a few of the cells have begun to express the myelin phenotype and express detectable levels of the myelin protein P0.

Non-myelin Schwann cell marker expression is lost as myelin forming cells develop. Non-myelin forming Schwann cells express proteins that are absent from myelin forming cells; these include N–CAM, L1, A5E3, NGF–R and GFAP. All of these except GFAP are present in high amounts on the precursor cells and embryonic Schwann cells. It is evident then, that cells destined to become myelin forming Schwann cells will express these markers early in development, have to down-regulate their expression as they mature.

Monitoring the expression of the myelin protein P0 is the most certain way to determine that a cell has been induced to become a myelin forming cell. Before the myelin proteins are easily detected by immunohistochemistry, however, GAL–C is induced on the cell surface (Jessen et al. 1987a, Kelly et al. 1992). Using these two markers, it has been possible to relate the loss of non-myelin proteins to the acquisition of myelin.

The presence of GAL–C on 40% of the cells at birth, when over 90% of the cells express NGF–R, A5E3, N–CAM and L1 indicates that these markers are co-expressed. If the GAL–C labelled cells from neonatal rats are double labelled with GFAP, it is seen that 43 ± 8.0% (n = 3, ± S.E.M.) of the GAL–C+ cells also co-express GFAP. Evidently GAL–C expression is compatible with continued expression of the non-myelin markers.

P0 expression reaches 48 ± 3.9% (n = 3, ± S.E.M.) of the cells by the second postnatal day (P2), if these cells are double labelled with GFAP antibodies and dried on to glass slides to prevent GFAP induction during the first hours of culture (see below), 10% of the P0 labelled cells co-express GFAP. This co-expression of the
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non–myelin and myelin forming Schwann cell markers is temporary, and by P10 the
P₀ labelled cells never co-express any of the non–myelin forming cell markers.
Non–myelin Schwann cell marker expression is induced on myelin cells in culture
and after transection. It has already been demonstrated that N–CAM expression by
myelin forming Schwann cells is due to suppression of N–CAM synthesis on
myelination. Disruption of the axon–Schwann cell relationship, seen when Schwann
cells are taken into tissue culture, causes a relaxation of this suppression, and N–CAM
is re–expressed, within hours in culture, or after weeks in vivo (Jessen et al. 1987).

To determine whether this suppression is a general phenomenon, or a feature
unique to the control of N–CAM expression, similar experiments were performed
with the other non–myelin forming Schwann cell markers.

Sciatic nerves of 4 week old (90–100g) rats were permanently transected, and
4–8 weeks later, the nerves were dissociated, dried and immunolabelled with NGF–R,
A5E3 and GFAP antibodies. In each case, a large increase in immunolabelling was
seen compared to control nerves, with over 90%, 98 ± 0.8%, 90 ± 5.5% (n = 3 ±
S.E.M.) of S100⁺ Schwann cells expressing NGF–R, A5E3 and GFAP respectively.
Counts of control cells are not possible due to the nature of the dried preperations,
extensive myelin debris makes identification of individual cells difficult, and counting
unreliable, however it has already been well documented from experiments where
nerves were teased on to slides that the mature myelin cells do not express A5E3,
NGF–R or GFAP (Jessen and Mirsky 1984, Jessen et al. 1990).

In in vitro experiments, a rapid and dramatic increase in the level of non–
myelin cell markers is seen when myelin forming cells are taken into culture, shown
in Figure 2.7. GFAP and A5E3 are detectable in some P₀⁺ cells within 3 hours.
Further increase is suppressed by the protein synthesis inhibitor cycloheximide (50
µgml⁻¹), indicating that de–novo protein synthesis is involved. A similar induction
was seen for NGF–R (Jessen et al. 1990).

It is clear then, that myelin forming Schwann cells are capable of re–
expressing the non–myelin forming markers that they lost during maturation. These
return of N–CAM is not the only non–myelin forming Schwann cell marker that
returns to myelin forming Schwann cells when they break down their myelin sheath.
DISCUSSION

How like migrating neural crest cells are the cells taken from E15 nerve?

In spite of the morphological similarity between the cells at E15 and those of the neural crest, there is too little published data on the neural crest phenotype for a full comparison (compare Tables 2.3 and 2.4).

Neural crest cells are generally cultured in the presence of chicken or mouse embryo extracts (Bernd 1985, Rogers et al. 1992, Dupin et al. 1990, Sieber-Blum 1991, Cohen and Konigsberg 1975, Morrison-Graham et al. 1990). Smith-Thomas and coworkers (1990) and Smith-Thomas and Fawcett (1989) start their neural crest cultures in embryo extract, and then maintain them in neural tube conditioned medium. Neural crest cells, therefore, are always cultured in the presence of growth factors. This dependence on extrinsic factors is likely to be one aspect of the cells in the E15 nerve that makes them like neural crest cells. Another similarity is the presence of laminin and the absence of basal lamina on chick neural crest cells.

The presence of NGF-R on all the cells does, however, seem to indicate that they have changed since leaving the neural tube as quail neural crest cells do not bind NGF as they leave the neural tube (Bernd 1985). Recently, however NGF-R antibodies have been used to purify cells that have migrated from neural tube explants (Stemple and Anderson 1992), by 24h in vitro most of these cells express NGF-R. From these experiments, it has become clear that the cells in the rat sciatic nerve might have not changed much from the cells that migrate from the neural tube.

Figure 2.8. There are 4 stages in Schwann cell development. These experiments and published work have been used to compile the scheme of Schwann cell development shown in Figure 2.8. The cells in the sciatic nerve go through three identifiable stages before birth.

1: The cells taken from E15 nerve are morphologically very like neural crest cells, but, as discussed earlier, the lack of data on neural crest phenotype in rat prevents a close comparison of how much these cells have changed since they migrated away from the neural tube. At this stage they are the precursors of Schwann cells, rather than Schwann cells. There is one big difference between these cells and Schwann cells (in addition to the morphological differences seen) namely, when they are taken
2. Schwann cell Phenotype

into culture they do not survive unless neuron-derived factors are present.

2: It is clear that between E15 and E16 a big change happens. The cells become like Schwann cells morphologically, they start to express the Schwann cell-specific markers O4 and S100 and they become independent of neuronal factors for survival (the details of the change into independent cells is described in more detail by Jessen, Gavrilovic, Brennan, Morgan, Mirsky, in preparation). These cells are still distinct from mature Schwann cells, but they share enough of the Schwann cell phenotype to be called embryonic Schwann cells.

3: The third stage of Schwann cell development in the prenatal sciatic nerve is seen in the cells that have begun to diverge towards the myelin phenotype. Most of the cells that express GAL-C before birth and during the first postnatal week are those that have been induced towards the myelin phenotype (Jessen et al. 1985).

The fourth stage in Schwann cell development is the maturation of the non-myelin forming Schwann cells and this, as discussed earlier, is a postnatal event (Diner 1965, Jessen et al. 1985).

The division profile of the cells during the prenatal period is illustrated in Figure 2.2 (taken from Stewart, Morgan, Mirsky and Jessen 1992, submitted). The dividing cells are evenly distributed between O4+ and O4− cells at E16–17, and between GAL-C+ and GAL-C− cells at E20. Differentiation from precursor to embryonic Schwann cell, and from embryonic to early myelin forming Schwann cell is possible in the presence of a high level of cell division. However, as myelination proceeds, and the cells begin to express P0, they drop out of division, at birth, P1 and P2 the cells that have begun to express P0 are never seen in division (Stewart, Morgan, Mirsky and Jessen 1992, submitted).

The scheme in Figure 2.8 describes the progression through these stages during development. This figure was taken from Jessen and Mirsky: Current Opinion in Neurobiology 2 575–581 (1992).

Some of the data in Chapter 2 has been published. See Jessen et al. 1990 and Mirsky et al. 1990. These papers also include experiments that were done by others, these have not been included.
2. Schwann cell Phenotype

The experiments defining the co-expression of GFAP with GAL-C and P_0 were done in collaboration with H. J. S. Stewart. The experiments to determine expression of GFAP and NGF–R in transected sciatic nerve done in collaboration with K. R. Jessen and R. Mirsky.
Table 2.1

ADULT SCHWANN CELL PHENOTYPE
(ALPHABETICAL LIST)

Phenotype of normal Schwann cells in the intact adult nerve

M: Myelin-forming Schwann cells
NM: Non-myelin-forming Schwann cells

<table>
<thead>
<tr>
<th>M</th>
<th>NM</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>(in BL)</td>
</tr>
<tr>
<td>+</td>
<td>(in BL)</td>
</tr>
<tr>
<td>+</td>
<td>(in BL)</td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>(in BL)</td>
</tr>
<tr>
<td>+</td>
<td>− (in node BL)</td>
</tr>
<tr>
<td>+(n)</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

continued on next page
### ADULT SCHWANN CELL PHENOTYPE (alphabetical list) (continued from previous page)

<table>
<thead>
<tr>
<th>MY</th>
<th>NM</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>−</td>
<td>Myelin basic protein (MBP) (Martini and Schachner 1986)</td>
</tr>
<tr>
<td>+(n)</td>
<td>−</td>
<td>Na⁺ Channels (Konishi 1990)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Nestin (401 antigen)(Hockfield and McKay 1985, Lendhal et al. 1990)</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>Nerve growth factor (NGF–R) (Ran–1(217c) epitope) (Jessen et al. 1990)</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>Neurofibronin (NF–1) (Daston et al. 1992)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>Neurofilament M (NF–M) (Kelly et al. 1992)</td>
</tr>
<tr>
<td>+/−</td>
<td>+</td>
<td>Neuronal cell adhesion molecule (N–CAM)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>O7, O8, O9 (Eccleston et al. 1987)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>O11 (Eccleston et al. 1987)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>P₀ (Brockes et al. 1980)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>P₁70K (Shuman et al. 1983, 1988)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>P₂ (Trapp et al. 1979, Winter et al. 1982, Hahn et al. 1987)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>Peripheral myelin protein 22 (PMP22) (Snipes et al. 1992)</td>
</tr>
<tr>
<td>+</td>
<td>ND</td>
<td>Proteolipid protein (PLP) (Puckett et al. 1987)</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>Ran–2 (Jessen and Mirsky 1984)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>S100 (Brockes et al. 1979, Mata et al. 1990))</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Schwann cell membrane glycoprotein (SAG) (Diepernik et al 1992)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Schwann cell myelin protein (SMP) (Dupin et al. 1990) (avian)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Seminolipid (224–58 monoclonal antibody) (Guerci et al. 1986)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Sulphatide (O4 and O07 monoclonal antibody epitopes) (Mirsky et al. 1990)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>Transferrin (Lin et al. 1990b)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Vimentin (VN) (Yen and Fields 1981)</td>
</tr>
</tbody>
</table>

**MY** = myelin Schwann cells, **NM** = non myelin Schwann cells. *(n)* = only at node. **ND** = not determined. **BL** = Basal Lamina. */−* = regional variations.
Table 2.2

COMMONLY USED SCHWANN CELL MARKERS

| Group 1, markers common to both myelin forming and non-myelin forming Schwann cells |
|------------------------------------|----------------------------------|
| S100                               | In cytoplasm of some axons       |
| GAL-C                              |                                  |
| O4 epitope (sulphatide)            |                                  |
| Laminin                            | On perineural cells              |
| Vimentin                           | On fibroblasts                   |
| Nestin                             |                                  |
| SMP (avian only)                   |                                  |

<table>
<thead>
<tr>
<th>Group 2, Markers restricted to the nonmyelin forming Schwann cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-CAM</td>
</tr>
<tr>
<td>GFAP</td>
</tr>
<tr>
<td>NGF–R</td>
</tr>
<tr>
<td>L1</td>
</tr>
<tr>
<td>A5E3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3, markers restricted to myelin forming Schwann cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀</td>
</tr>
<tr>
<td>P₁ (MBP)</td>
</tr>
<tr>
<td>P₂</td>
</tr>
<tr>
<td>MAG</td>
</tr>
<tr>
<td>PMP22</td>
</tr>
</tbody>
</table>
### TABLE 2.3

<table>
<thead>
<tr>
<th>MARKER (in alphabetical order)</th>
<th>LOCATION AND WHEN SEEN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Lamina (EM)(frog)</td>
<td>none associated with NC cells until migration stops</td>
<td>Billings-Gagliardi et al. 1974</td>
</tr>
<tr>
<td>FN (#) (avian)</td>
<td>none on migrating NC cells</td>
<td>Rogers et al. 1992</td>
</tr>
<tr>
<td>HNK-1 (*) (avian)</td>
<td>on all migrating NC cells</td>
<td>Vincent 1984, Tucker 1984</td>
</tr>
<tr>
<td>LN (#)(rat)</td>
<td>none at E13 in nerves</td>
<td>Bignami et al. 1984a</td>
</tr>
<tr>
<td>LN (#)(chick)</td>
<td>present on migrating NC cells</td>
<td>Rogers et al. 1986</td>
</tr>
<tr>
<td>Nestin (*) (rat)</td>
<td>in pre-migrating and migrating NC cells</td>
<td>Hockfield and McKay 1985</td>
</tr>
<tr>
<td>NGF-R (*) (rat)</td>
<td>on some NC cells after 1 day in culture, 50% NGF–R+ with longer in culture</td>
<td>Smith-Thomas and Fawcett 1989, Lo et al. 1991, Bernd 1985</td>
</tr>
<tr>
<td>NGF-R (£) (quail)</td>
<td>none as leave neural tube reaches max of 35% with time in culture</td>
<td></td>
</tr>
<tr>
<td>NGF-R ($) (quail)</td>
<td>none as leave neural tube, becomes 28% with time in culture</td>
<td>Greiner et al. 1986</td>
</tr>
</tbody>
</table>
### NEURAL CREST PHENOTYPE (continued)

<table>
<thead>
<tr>
<th>MARKER</th>
<th>LOCATION AND WHEN SEEN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀ (*) (chick)</td>
<td>on Schwann cell progenitor cells as NC cells migrate</td>
<td>Bhattacharyya et al. 1991</td>
</tr>
<tr>
<td>P₀ (chick)</td>
<td>present 2 days before myelin</td>
<td>Barbu 1990</td>
</tr>
<tr>
<td>P₀ (#) (mouse)</td>
<td>only present 2 days before myelin</td>
<td>Martini et al. 1988</td>
</tr>
<tr>
<td>P₀ (#) (Rat)</td>
<td>just in advance of myelin</td>
<td>Trapp et al. 1981</td>
</tr>
<tr>
<td>S100 (@) (quail)</td>
<td>not seen until after migration</td>
<td>Holton and Weston 1982a</td>
</tr>
<tr>
<td>SMP (*) (quail)</td>
<td>not on migrating NC cells</td>
<td>Dupin et al. 1990</td>
</tr>
<tr>
<td>Tenacin (* and =) (chick)</td>
<td>mRNA present in migrating cells</td>
<td>Tucker and McKay 1991</td>
</tr>
</tbody>
</table>

Key to detection methods used:

(*) monoclonal antibody, (#) rabbit polyclonal antibody, (@) radioimmunone assay,
(=) in situ hybridization, (EM) electronmicroscopy, (£) binding of ¹²⁵I labelled NGF,
($) monoclonal antibody binding to bound extrinsic NGF.

NC = Neural crest
Table 2.4. Schwann cell markers in prenatal Schwann cells

<table>
<thead>
<tr>
<th>age</th>
<th>LN</th>
<th>VIM</th>
<th>S100</th>
<th>O4</th>
<th>N–CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15</td>
<td>#100 ± 0.4</td>
<td>0 ± 0.0(6)</td>
<td>0 ± 0.03</td>
<td></td>
<td>100 ± 0</td>
</tr>
<tr>
<td>E16</td>
<td>#100 ± 0.0</td>
<td>17 ± 8.0(5)</td>
<td>19 ± 11.0(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E17</td>
<td>97 ± 2.6</td>
<td></td>
<td></td>
<td></td>
<td>96 ± 2.5</td>
</tr>
<tr>
<td>E18</td>
<td>98 ± 0.4</td>
<td>#100 ± 0.0</td>
<td>81 ± 5.0(7)</td>
<td>43 ± 15.7(4)</td>
<td>90 ± 2.7</td>
</tr>
<tr>
<td>E19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>64 ± 7.2</td>
</tr>
<tr>
<td>E20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>97 ± 3.0</td>
<td></td>
<td>#94 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>#92 ± 0.6</td>
<td>100 ± 0.0</td>
<td></td>
<td>98 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

Adult cells:
myelin  +  +  +  +  -
non myelin +  +  +  +  +
Table 2.4. continued:

<table>
<thead>
<tr>
<th>age</th>
<th>Ran-2</th>
<th>L1</th>
<th>NGF-R</th>
<th>ASE3</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15</td>
<td></td>
<td>81 ± 13.2</td>
<td>#81 ± 3.5(6)</td>
<td>#79 ± 5.8</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>E16</td>
<td>71 ± 5.5</td>
<td>90 ± 11.6</td>
<td>#91 ± 1.8(5)</td>
<td>#73 ± 18.9</td>
<td>1 ± 1.5</td>
</tr>
<tr>
<td>E17</td>
<td></td>
<td>98 ± 1.4</td>
<td>#98 ± 1.5(5)</td>
<td>#98 ± 1.3(4)</td>
<td>0 ± 0.1(7)</td>
</tr>
<tr>
<td>E18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 ± 1.1</td>
</tr>
<tr>
<td>E19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 ± 1.2</td>
</tr>
<tr>
<td>E20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 ± 0.7</td>
</tr>
<tr>
<td>P0</td>
<td>97 ± 0.9</td>
<td>96 ± 2.9</td>
<td>#99 ± 0.2(5)</td>
<td>#99 ± 0.7</td>
<td>46 ± 9.8</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td>#100 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Adult cells:
- myelin: –
- non myelin: +

Numbers shown are % ± standard error of mean (S.E.M.) of NGF-R⁺ cells except where marked #, when % ± S.E.M. of total cells is given. n = 3 (n shown in brackets when >3). NGF-R labelled with 217c antibody. VIM; vimentin. LN; laminin.

Embryonic Schwann cells were dissociated from rat sciatic nerves at the ages indicated, and attached to glass coverslips (as described in Methods) for 1.5–3 hours before staining.
This chart summarises the adult Schwann cell phenotypes, and that shared by Schwann cells in culture and after Wallerian degeneration.
Figure 2.1 Plasticity of the adult Schwann cell phenotype

Schwann cells before Wallerian degeneration

Key to Schwann cell markers
- All Schwann cells
- Schwann cells not making myelin
- Schwann cells on axons
- Myelin Schwann cells

Schwann cells without axonal contact

NGF-R
GFAP
N-CAM
L1
A5E3
S100 laminin nestin
GAL-C
O4
P0
MBP
P2
MAG
PMP22

Injury
Repair

Non-myelin Schwann cells
Myelin Schwann cells
DNA synthesis during peripheral nerve development.

Taken from Stewart, Morgan, Jessen and Mirsky: Schwann cell division during rat sciatic nerve development: relationship between division and final differentiation of the Schwann cells. (1992) submitted.

Pregnant rats were injected with BrdU and left for 1h, the embryos were then removed and the sciatic nerves dissociated and dried on to glass slides. The dried cells were immunolabelled with BrdU antibodies and either $P_0$, O4 or GAL–C antibodies. The percent of BduR antibodies is indicated by the unbroken line, and the percent of $P_0$ positive cells is indicated by the broken line. The increase in O4 and GAL–C during development is shown in the bottom two graphs. BrdU labelled cells never labelled with $P_0$ antibodies, however, O4 and GAL–C labelled cells were equally BrdU positive and BrdU negative.
Figure 2.3

Morphology of cells dissociated from rat sciatic nerve 3 hours in culture.

a and b: E16 rat, phase and NGF–R (fluorescein).
c: Schwann cells from neonatal rat, phase.
Cells were plated on PLL coated glass coverslips for 3 hours after dissociating in enzyme cocktail, as described in Methods. The cells from E16 rat are flattened and tend to adhere to each other in pavements. The Schwann cells from neonatal sciatic nerve show the bipolar morphology seen in long term Schwann cell cultures, even within 3 hours of plating, the neonatal cells tend not to adhere to each other. The change from flattened to bipolar morphology occurs between E17 and E18.

Figure 2.3d was reproduced from Morrison–Graham, et al. 1990 for comparison. Neural crest cells from mouse, 48 hours in culture.


a, b, bar = 20µ.
c, bar = 50µ.
d, bar = 150µ.
Figures 2.4, 2.5 and 2.6

These graphs illustrate the data given in Table 2.5, together with published data on the developmental appearance of 
\( P_0 \) and GAL-C.

Figure 2.4
NGF-R
A5E3
N-CAM
L1

Figure 2.5
O4
S100
GFAP
GAL-C
\( P_0 \)

Figure 2.6
Vimentin
Laminin

\( P_0 \) taken from Stewart, Morgan, Jessen and Mirsky: Schwann cell division during rat sciatic nerve development: relationship between division and final differentiation of the Schwann cells. submitted (1992).
Figure 2.7

Cycloheximide prevents upregulation of A5E3 and GFAP on myelin forming Schwann cells in culture.

a: A5E3 induction on P^+ cells in culture.
b: GFAP induction on P^0+ cells in culture.

The level of both GFAP and A5E3 expression after 6h cycloheximide treatment is significantly less than that seen in control cultures 9h after plating (P>0.001 for A5E3 and P <0.02, >0.01 for GFAP, Students t-test). The inhibition of induction of non-myelin forming Schwann cell markers by cycloheximide indicates that de-novo protein synthesis is required for this expression.

Cells were dissociated from the sciatic nerves of P10 rats using enzyme cocktail as described in Methods and plated on PLL coated glass coverslips. After 3h, cycloheximide, 50μg.ml^-1 was added to some of the cultures. 9h after plating, cycloheximide treated and control cultures were immunolabelled with A5E3 and P^0 polyclonal, or GFAP and P^0,4 monoclonal antibodies. 200 cells on each of at least 2 coverslips were counted for each experiment, and each experiment was repeated 3 times.

The arrows at 3h indicate that cycloheximide was added to half of the cultures at this time.
A5E3 induction on myelin cells

- **Cycloheximide**
- **Control**

GFAP induction on myelin cells

- + **Cycloheximide**
- **Control**
Figure 2.8
Development of mature myelin-forming and non-myelin forming Schwann cells from neural crest cells.

Adapted from Jessen and Mirsky 1992.
3. cAMP on Schwann cells

CHAPTER 3
INTRODUCTION

cAMP effects on cultured Schwann cells. As described in Chapter 1, Schwann cell differentiation can, to a certain extent, be induced by cAMP elevating drugs. Surface GAL-C and the intracellular proteins P₀ and MBP are induced in cultured Schwann cells exposed to cAMP inducing agents, or cAMP analogues (Sobue and Pleasure 1984, Sobue et al. 1986a, 1986b, Lemke and Chao 1988, Kamholz et al. 1992). However, in some experimental systems GAL-C is induced without P₀ (Shuman et al. 1988, Kreider et al. 1988).

In this chapter, the response of Schwann cells to cAMP elevation in the absence of growth factors is further examined. Changes in the level of expression of the myelin protein P₀ and its mRNA are monitored with immunolabelling, immunoblot, in situ hybridisation and northern blot. The induction of the lipids GAL-C and the O4 antigen, and the loss of non–myelin markers GFAP, A5E3, N-CAM, L1 and NGF–R that accompany the induction of myelin proteins are monitored by immunolabelling.

Growth factors modulate the effect of cAMP on Schwann cells

When serum or various growth factors are present whilst Schwann cells are treated with cAMP elevating agents, I have found that the induction of P₀ protein, as detected by immunocytochemical labelling, is greatly reduced. Schwann cells in these conditions do not remain quiescent however, since they divide rapidly and many of them are still induced to express the surface antigen O4. When cells are prevented from dividing by contact inhibition, cAMP elevation will induce the myelin phenotype, even in the presence of growth factors.

The possibility that differentiation is prevented by cell division and not by repressors of differentiation in serum and growth factors is explored.
Schwann Cell Culture

Sciatic nerves were dissected from early postnatal rats (5d to 8d) and Schwann cell suspensions made as described in the previous chapter. This method is essentially the same as described by Brockes and coworkers (1979), but the need to kill contaminating fibroblasts with Thy-1 antibodies and complement was avoided by removal of the epineurial sheath. The number of fibroblasts at the start of the culture is greatly reduced by this method, and so cytosine arabinoside treatment alone is sufficient to control the fibroblast population. The cells were maintained in DMEM with added glutamine, insulin and 10% CS on PLL treated tissue culture plastic. Cytosine arabinoside, $10^{-5}$M, was added after 24h for 48h to give cultures which were at least 95% pure. On the fourth day cells were removed from the tissue culture dish with 125$\mu$g.ml$^{-1}$ trypsin in PBS containing 2mM EDTA, resuspended in culture medium and plated onto glass coverslips. The cells were then left for 24h before further treatment.

**Defined medium** Contains a 1:1 mixture of DMEM and Ham's F12 medium supplemented with BSA (Pentex) 30mg.ml$^{-1}$, dexamethasone (38ng.ml$^{-1}$), insulin (5$\mu$g.ml$^{-1}$), progesterone (60ng.ml$^{-1}$), putrescine (16$\mu$g.ml$^{-1}$), thyroxine (0.4$\mu$g.ml$^{-1}$), transferrin (100$\mu$g.ml$^{-1}$), triiodothyronine (10ng.ml$^{-1}$) and selenium (160ng.ml$^{-1}$).

**Cell culture in the presence of growth factors**

GGF (A generous gift from A. D. J. Goodearl. Semi-pure fraction (GGF--CM) prepared as in Raff et al. 1978) used at 1.8$\mu$g.ml$^{-1}$.

Recombinant bFGF 10ng.ml$^{-1}$ (Prepro Biotechnology).

aFGF 10ng.ml$^{-1}$ (A generous gift from M. Jaye of Rorer Biotechnology) (always used with heparin 20$\mu$g.ml$^{-1}$).

PDGF-BB (porcine) 5ng.ml$^{-1}$ (British Biotechnology Oxford UK).

TGF$\beta$ 1ng.ml$^{-1}$ (British Biotechnology Oxford UK).

**Cell culture in presence of other drugs**

A21387 (Sigma Chemical Co.)
Phorbol dibutyrate (PDB) (Sigma Chemical Co.)

4α-Phorbol (Sigma Chemical Co.)

PDB and 4α-phorbol were dissolved in DMSO, 2 x 10^{-2}M stock solutions were made and the drugs diluted in aqueous solutions from these.

Dimethylsulphoxide (DMSO) (Sigma Chemical Co.)

Sphingosine (Sigma Chemical Co.)

Amiloride (Sigma Chemical Co.)

Cycloheximide (Sigma Chemical Co.)

Cell culture in the presence of cholera toxin (CTx). CTx (Sigma Chemical Co.) (150ng.ml^{-1}) was added to Schwann cell cultures for 1h at the start of experiments, and 24h later for an additional h. In experiments lasting more than 4d, CTx was added for 1h at 96h and 168h. As a control for the specificity of the CTx, the β subunit of CTx (Sigma Chemical Co.) was used in some experiments at the same concentration and using a similar regime to that used for CTx.

Cell culture in the presence of forskolin. In some experiments forskolin (Calbiochem), a reversible activator of adenyl cyclase, was added to cells cultured in media as described above. A 10mM stock in alcohol was diluted in medium, and a control containing alcohol alone was included in the experiments. The forskolin was replaced in new medium every 24h.

Cell culture in the presence of cyclic AMP analogues. In these experiments, Schwann cells were plated on coverslips coated with PLL, laminin or extracellular matrix. Cyclic AMP analogues, N^6,2'-0-dibutyryladenosine 3':5'-cyclic monophosphate (db-cAMP) plus 8-bromoadenosine 3':5'-cyclic monophosphate (8b-cAMP) both at concentrations of 5 x 10^{-4}M were added at the start of the experiment. At 24h and 48h, the medium was replaced with fresh medium containing the two analogues, each at a reduced concentration of 5 x 10^{-5}M. If experiments lasted more than 3d, this dose was repeated every day until the end of the experiment. Similar concentrations of sodium butyrate were used in control experiments.

Cell culture in 3-isobutyl-1-methyl-xanthine (IBMX). IBMX (Sigma Chemical Co.), an inhibitor of cAMP hydrolysis, was added at 100μM to Schwann cell cultures at the start of the experiment. IBMX was replaced in new medium every 24h.
3. *cAMP on Schwann cells*

Control cultures contained 0.1% ethanol.

**Substratum coating.** Thirteen mm diameter, round glass coverslips were placed in 95% ethanol/1% HCl for 1h, washed in many changes of distilled water over 1h and stored in Analar ethanol. To coat in PLL, coverslips were rinsed in sterile distilled water, incubated with 1 mg ml$^{-1}$ PLL (mwt 500,000) (Sigma Chemical Co) at room temperature for 24h, washed with 6 changes of sterile distilled water over a period of 4d, and allowed to dry. Laminin coated coverslips were prepared by incubating PLL coated coverslips with 50µl of laminin solution (Gibco) (20µg.ml$^{-1}$ in DMEM) for 2h prior to plating with Schwann cells. Extracellular matrix coated coverslips were prepared from bovine endothelial cells as described previously (Eccleston et al. 1987) and stored in sterile PBS at 4°C until required for use.

**DNA replication.** DNA synthesis by Schwann cells was detected by addition of bromodeoxyuridine (BrdU) at a concentration of 2.5x10$^{-5}$M for the final 24h of culture (Gratzner 1982). In double labelling experiments with $\text{P}_\text{O}$ antibodies cells were washed, fixed with 95% ethanol/5% acetic acid at -20°C for 10 min. They were then treated with 2M HCl for 20 min to denature DNA and 10 min with 0.1M sodium borate (pH 8.5). Coverslips were incubated sequentially with anti-BrdU in PBS containing 0.1% Triton X-100 for 40 min, then with G-anti-Mlg-Rd for 30 min., the cells were then blocked with 3% gelatin (bovine, Sigma Chemical Co.) in PBS for 30 min., and incubated in $\text{P}_\text{O}$ antibodies then G-anti-Rlg-F1 for 30 min. each. In double labelling experiments with 04 antibodies cells were labelled with 04 antibodies followed by G-anti-Mlg-Rd, fixed in 2% paraformaldehyde for 10min, 95% ethanol/5% acetic acid, 2N HCl and sodium borate as above, followed by anti-BrdU and G-anti-Mlg-Rd. There was no difficulty in distinguishing 04 surface labelling from the nuclear BrdU labelling despite the fact that both antibodies were raised in mouse as the fixation used after use of the first two antibody layers reduced cross-reactivity.

All coverslips were mounted on microscope slides and viewed as described in Chapter 2.

**Immunoblotting (Western blot).**

This was carried out using Schwann cells cultured in 25cm$^2$ tissue culture
3. cAMP on Schwann cells

flasks coated with polylsine and laminin. Schwann cells were seeded at 5x10^5 - 10^6 per flask and treated with doses of cAMP analogues, forskolin or CTx for 3d, in defined medium as described above. Control cultures were maintained without addition of analogues. Proteins were extracted from the cells or sciatic nerve using 0.1ml of 2% SDS in 5mM Tris–Cl, pH 6.8, containing 2mM EGTA, 2mM EDTA and 2mM phenyl methylsulphonyl fluoride. The sample was boiled for 5 min, spun at 14,000g for 5 min and 10μl of supernatant withdrawn for protein determination using a Bio–Rad protein estimation kit. 2–mercaptoethanol (2%) was then added to the supernatant and the extracts subjected to SDS–polyacrylamide gel electrophoresis using a 10–15% gradient acrylamide slab gel. The separated proteins were then transferred to nitrocellulose and immunoblotted essentially as described previously (Jessen et al. 1984) using the Pq antiserum at a concentration of 1:5000, and 125I labelled donkey anti–rabbit Ig (ICN Biomedicals Ltd), 15 x 10^6 cpm/blot diluted in 20ml of PBS containing 3% haemoglobin.

Later immunoblots were run using a Bio–Rad mini–gel apparatus, in this case the acrylamide gel was 12% non–gradient. After transfer on to nitrocellulose paper (transfer for 1h at 4°C, using 100V with 0.2% SDS added to the transfer buffer to improve transfer) the nitrocellulose sheet was blocked with Tween–20 0.2%, Blotto (fat–free dried milk) 5% in PBS and then exposed to anti Pq diluted 1:4000 in the same solution overnight at 4°C. Anti rabbit biotin 1:1000 was applied for 1h followed by Vector Stain Elite ABC substrate for 30 minutes, made up according to the manufacturers instructions. The signal was visualised with diaminobenzidine 1mg.ml^-1, 1% nickel chloride in a solution containing imidazole.

**In situ hybridisation.** Cells cultured on cover slips were fixed for 20 minutes in 4% paraformaldehyde and dehydrated in increasing ethanol concentrations up to 70% ethanol, cells were then rehydrated and rinsed in hybridization buffer containing 4x SSC, 50% deionised formamide, 100μg.ml^-1 poly–adenosine, 120μg.ml^-1 heparin sulphate, 100μg.ml^-1 acid/base cleaved salmon sperm DNA, 50% dextran sulphate and 20mM di–thiothreitol (DTT) in 1x Denharts buffer. Preparations were then exposed to 35S ATP labelled 50 base pair oligonucleotide probe (see below for labelling of probe) diluted in hybridization buffer so that each coverslip was exposed
3. cAMP on Schwann cells
to 83,300 dpm in 10μl. The coverslips were incubated at 42°C overnight. After washing, the cells were dried and dipped in Kodak K2 emulsion, dried again and stored at 4°C for 2–4 weeks before developing and mounting in either glycerol based mountant (Citifluor U.K.) or stained with toluidine blue, dehydrated and mounted in DPX mountant (BDH).

The oligonucleotide used is complementary to base pairs 488–538 of the P_q mRNA that codes for part of the P_q molecule that spans the cell membrane, amino acids 124–140 (Lemke and Axel 1985, Kreider et al. 1988). The probe was a gift from L. D. Hudson (NIH, Bethesda, USA).

RNA extraction and Northern Blots. Whole RNA was extracted from desheathed sciatic nerves of P8 rat pups and Schwann cell cultures by the method of Chomczynski and Sacchi (1987). Whole nerve, cultured Schwann cells or cultured 3T3 cells were homogenised in a mixture of guanidine thiocyanate (Fluka) (4M), 0.75M sodium citrate pH 7 (25mM), 1% sarcosyl and 2-mercaptoethanol (0.1M). The solution was made to pH 4 with sodium acetate, an equal volume of phenol and 1/10 volume of chloroform/isoamyl alcohol (49/1) were added sequentially with mixing between additions. The RNA was in the aqueous phase, the protein and DNA being in the phenol or at the interphase after centrifugation for 1h at 10,000g. The RNA was precipitated from solution by incubating at −20°C with an equal volume of isopropanol, collected by centrifugation and washed by dissolving in guanidine thiosulphate solution as above and re-precipitating with isopropanol at −20°C. The RNA was collected by centrifugation for 30 minutes at approximately 13,000 rpm (fast setting in microfuge) and the pellet stored under 900μl of 75% ethanol at −70°C.

The RNA was recovered by adding 100μl 3M sodium acetate pH 5 to the 75% ethanol and centrifuged for 30 minutes at 4°C in microfuge as above. 50μl of 0.5% SDS at 65°C was added to the pellet to re-dissolve. Optical density readings at 260nm and 280nm were performed on a spectrophotometer to determine the amount and purity of the RNA. At 260nm, an optical density reading of 1 is equivalent to a RNA concentration of 40μg.ml⁻¹. A 260/280 ratio of >1.8 confirms that there is no appreciable protein contamination.

The RNA in sample buffer containing ethidium bromide was run on agarose
3. cAMP on Schwann cells

gels, examined under ultra violet light, photographed and the distance travelled by the molecular weight markers and ribosomal RNA measured. The RNA was then transferred to nylon membranes (Genescreen) and hybridized in a sealed bag with $^{32}$P labelled cDNA (1–4 x 10$^5$ dpm.ml$^{-1}$ for optimal signal:background ratio) at 60°C for 16h with shaking, then the nylon membrane was rinsed, washed for 1h in 2x SSC with 1% SDS at 60°C, followed by 1h in 0.1x SSC before exposing to X–ray film (Sigma Chemical Co.).

The cDNA used for Northern blots was from the plasmid pSN63c (Lemke and Axel 1985, Griffiths et al. 1989). This contains a 1.85 kb insert of cDNA (λ SN63) isolated from a cDNA library made from P8–10 rat sciatic nerve mRNA, screened for selective expression in the PNS, and shown to encode the P$^0$ protein (Lemke and Axel 1985). The cDNA was inserted into the plasmid vector pUC8 using an ECO R1 linker, grown up in bacteria, and then cut out from the plasmid using R1 enzyme. The pSN63c plasmid was a gift from G. Lemke to L. D. Hudson, NIH, Bethesda, USA. The cDNA was grown up, cut from the plasmid DNA and isolated by R. Mirsky and K. R. Jessen.

Labelling of probes for in situ hybridisation and for Northern blots

In Situ. 10ng of oligonucleotide probe was added to tailing buffer and 20 units of terminal transferase (Tdt) enzyme (both from Amersham UK) and 1.5μl $^{35}$S dATP (1200 Ci/mmol New England Nuclear) and the volume made to 25μl with water. The mixture was incubated at 37°C for 1–1.5h and the volume made up to 50μl with water. Unbound radioactive nucleotide was removed by running through a Sephadex G–25 mini spin column and the bound activity estimated by reading the activity of 1μl on a scintillation counter. The degree of labelling was calculated from the amount of radioactivity incorporated.

For example:

15.9pmol of ATP was incubated with 0.625pmol of oligonucleotide probe, a ratio of 26.5 : 1. This amount of ATP contains 41.25x10$^6$ dpm of $^{35}$S.

At the end of the incubation, after unbound radioactivity was removed, 1μl of the probe solution was counted in a scintillation counter and it was found that 9.15x10$^6$ dpm had been incorporated into the probe, or 22.2% of the total radioactivity present.
3. cAMP on Schwann cells

at the start.

So \(22.2 \times 26.5 \div 100 = 5.9\) molecules of \(^{35}\)S have bound to each molecule of probe.

Generally, 6 radioactive tails bound to each oligonucleotide, and this was found to give clear labelling of myelin forming cells, with low background on the slide, or on fibroblastic cells.

Northern blotting. The cDNA was labelled using a Boehringer Mannheim random primed DNA labelling kit, following the manufacturers instructions. 25ng of DNA was denatured by heating to 95°C for 2 min. prior to labelling. Equal amounts of dATP, dGTP and dTTP (0.5pmol of each) were mixed together and added, together with the reaction mixture and then 50μCi\(^{32}\)P-dCTP, in aqueous solution. The volume was made up to 19μl with dH\(_2\)O. Finally 1μl Klenow enzyme was added and the mixture incubated 30 min. to 1h at 37°C. The reaction was stopped by adding EDTA to 0.2M (pH 8.0) and the total volume made up to 150μl with water. Non incorporated deoxyribonucleoside triphosphates were removed by chromatography through a Bio–Spin column (Biorad). The incorporated radioactivity in 1μl was counted in a scintillation counter measuring the Cerenkov emission, a method that is only 44% efficient.

For example:

25ng cDNA was used as a template, and at the end of the incubation (after the non incorporated deoxyribonucleoside triphosphates were removed) 1μl contained a measured radioactivity of 26,503cpm. After accounting for the fact that only 44% of the radioactivity is detected in this method of measuring there are 60,234dpm.μl\(^{-1}\), and as the total volume was 150μl, 9x10^6dpm had incorporated into the newly synthesised cDNA.

As 2.2 x10^6dpm ≡ 1μCi; 9x10^6dpm ≡ 4.1μCi.

The added \(^{32}\)PdCTP contained 50μCi, as 4.1μCi of this was incorporated into the new probe, the efficiency was 8.2%.

The dCTP used contained 3Ci.mol\(^{-1}\), so the 50μCi used contained 16.7pmol, and 8.2% of this, or 1.37pmol of dCTP had incorporated into the newly synthesised cDNA.
RESULTS

**Schwann cell yield:** When the cells from a 6d rat sciatic nerve are dissociated, and the resulting cell suspension dried on to slides, 87 ± 1.7% of the total cells are S100+ Schwann cells. After 24h in culture, 94 ± 0.8% of the total cells are S100+ Schwann cells, indicating that many of the contaminating S100- cells are either killed in the dissociation, do not survive in regular culture medium, or do not attach to the tissue culture substrate. When the cells are cultured as described in Methods on tissue culture plastic in the presence of cytosine arabinoside $10^{-5}$M from 24–72h, then moved to laminin coated glass coverslips for 24h (the cells are now 5d in vitro), 99 ± 0.4% of the total cells are S100+ Schwann cells.

In cultures from 5–7d rats, 42 ± 3.3% of the Schwann cells that have attached to the coverslip express $P_0$ after 24h in culture. In other experiments (taken from Stewart, Morgan, Mirsky, and Jessen 1992, submitted), when cells were not cultured but dried onto glass slides after the nerve was dissociated, 45 ± 1.1% (n = 3) of the Schwann cells were $P_0^+$.  

**cAMP elevation induces expression of the myelin protein $P_0$ in short term cultures in defined medium.** To avoid the complicating effects of serum, which has been included in most previous studies on cAMP and Schwann cell differentiation, initial experiments studied the effect of cAMP elevation on cultured short term Schwann cells from 5d rat sciatic nerve in serum-free, defined medium. Experiments were started after 5d in vitro. By this time the cells had stopped expressing immunocytochemically detectable levels of myelin proteins and glycolipids, such as $P_0$, GAL-C or 04 (see Chapter 2).

After elevation of cAMP by use of CTx, forskolin or cAMP analogues for three days, expression of the major myelin protein $P_0$ was clearly seen in the majority of Schwann cells when they were examined by immunocytochemical methods using $P_0$ antibodies. $P_0$ was also induced, albeit to a lesser extent, by IBMX, an inhibitor of phosphodiesterase, as expected if expression was dependent on cAMP elevation.
3. cAMP on Schwann cells

Table 3.1

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>% P_0^+ Schwann cells (± SEM)</th>
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</thead>
<tbody>
<tr>
<td>CTx (150ng.ml^{-1})</td>
<td>62 ± 6.5 (n = 14)</td>
</tr>
<tr>
<td>Forskolin (2-4μM)</td>
<td>37 ± 10.7 (n = 11)</td>
</tr>
<tr>
<td>cAMP analogues</td>
<td>51 ± 5.7 (n = 22)</td>
</tr>
<tr>
<td>IBMX (100μM)</td>
<td>9 ± 3.5 (n = 8)</td>
</tr>
</tbody>
</table>

Control Experiments

| β subunit of CTx (150ng.ml^{-1}) | 0 ± 0 (n = 4) |
| Ethanol (0.5%) | 0 ± 0 (n = 3) |
| Butyrate | 0.3 ± 0.40 (n = 3) |
| GGF (1.8μg.ml^{-1}) | 0.2 ± 0.10 (n = 4) |
| bFGF (10ng.ml^{-1}) | 0.8 ± 1.0 (n = 3) |
| aFGF (10ng.ml^{-1} + 10μg.ml^{-1} heparin) | 0 (n = 2) |
| PDGF (5ng.ml^{-1}) | 0 (n = 2) |
| TGFβ (1ng.ml^{-1}) | 0 (n = 2) |
| Calf serum (10%) | 0 ± 0 (n = 4) |
| Calcium ionophore A21387 (5-7x10^{-8}M) | 0 ± 0.3 (n = 5) |
| Calcium ionophore A21387 (5-7x10^{-8}M) with PDB (pulsed) (10^{-8}M) | 0 ± 0 (n = 3) |
| PDB (pulsed) (10^{-8}M) | 0 ± 0 (n = 3) |
| PDB (constantly applied) (10^{-8}M) | 0 ± 0 (n = 3) |
| DMSO 2% | 0 (n = 2) |

0-1 d in vitro (no additions) 42 ± 3.0 (n = 3)
5 d in vitro (no additions) 0 (n = 1)
8 d in vitro (no additions) 0.1 ± 0.10 (n = 10)

Schwann cells were removed from 5-7d rats and cultured for 5d before addition of drugs that elevate cAMP or other agents. The cultures were examined with P_0 antibodies 3d after the addition of the drugs, except in experiments where the cells were examined at 24h, 5d or 8d in vitro. cAMP analogues, dbcAMP and 8bcAMP were used together each at a concentration of 5 x 10^{-4}M during d1 of treatment and at 5 x 10^{-5}M during d2 and d3. CTx and the β subunit of CTx were added to the cells for only 1h at 0h and again at 24h. In each experiment (n), a minimum of 200 cells was counted on each of at least two coverslips. The number of P_0 positive cells is expressed as a percentage of the total Schwann cells identified by phase-contrast illumination.
These results, and results from several control experiments are summarized in Table 3.1, and the typical appearance of cells induced to express P_0 is illustrated in Figure 3.1c. As previously reported (Sobue et al. 1986a), a characteristic morphological change in many of the Schwann cells from a bipolar shape to cells of a more flattened morphology which is less phase bright, is induced by cAMP analogues (compare Figure 3.1a and Figure 3.1c). The induced cells are notably larger in size and more flattened than control cells. The same morphological change accompanies P_0 induction in response to CTx and forskolin.

P_0 induction could be detected using either polyclonal or monoclonal P_0 antibodies, although the monoclonal antibodies labelled only a few cells and generated a more speckled and less intense labelling pattern. As the cells do not have an axon to ensheath, they can not form compact myelin and it is likely therefore, that they do not complete all the post-translational modifications of P_0 that occur in the intact nerve (discussed in Chapter 2). The difference in staining with the monoclonal antibody would then be explained if the monoclonal anti P_0 recognises an epitope that is only present after these post-translational changes have occurred.

The percentage of cells induced to express P_0 varied quite widely from experiment to experiment. The highest percentage seen in individual experiments was 84% and 85% following exposure to forskolin and CTx respectively, while the average percentage was considerably lower (Table 3.1). The cells in each culture that do not express P_0 in response to elevation of cAMP remains unexplained, however this possibly reflects the different stages of development of the cells when they were taken from the rat. By 6d after birth, some of the cells had been synthesising myelin for at least 6 days, whilst others were still immature GAL-C^- cells, not destined to become mature non-myelin forming Schwann cells for another 1-2 weeks in the animal. It may be that only as the cells mature do they become able to respond to cAMP elevation by synthesising myelin, or that they respond more quickly to changes in intracellular cAMP than the less mature cells.

No P_0 was seen in control experiments with the β subunit of CTx, ethanol, DMSO, butyrate, GGF, bFGF, aFGF, PDGF, TGFβ or CS or in experiments with serum-free media alone (Table 3.1).
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**Time course of protein induction** The time course of P_q induction in response to cAMP analogues in defined medium is indicated in the table below.

<table>
<thead>
<tr>
<th>Time</th>
<th>P_q^+ cells as % of total Schwann cells</th>
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</thead>
<tbody>
<tr>
<td>24h</td>
<td>0 ± 0% (n = 3)</td>
</tr>
<tr>
<td>48h</td>
<td>27 ± 17.1% (n = 4)</td>
</tr>
<tr>
<td>72h</td>
<td>51 ± 5.7% (n = 22)</td>
</tr>
<tr>
<td>4d</td>
<td>70 ± 9.0% (n = 3)</td>
</tr>
<tr>
<td>5d</td>
<td>36 ± 7.2% (n = 4)</td>
</tr>
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</table>

The delay before P_q can be detected after cAMP elevation confirms the observations of Monuki et al. 1989. When the cells are left longer than 5 days, there is a marked deterioration in their morphology. The cells retract their processes, become granular and many peel off their coverslips. This deterioration is more marked with cAMP analogues than with forskolin or CTx.

**P_q protein induced by elevation of cAMP can be detected in an immunoblot.** When cells were exposed to agents that elevate cAMP or mimic its effect, a clear band of P_q protein was detectable by immunoblotting (Figure 3.2). The extract of freshly excised sciatic nerve from a 85 rat used as a positive control showed a greater intensity of P_q protein than the cultured induced cells, even though less total protein was loaded on the gel track (25μg, compared to 31μg for cultured cells). On prolonged exposure of the immunoblot to X-ray film, a very faint band was also visible in the extract of cells cultured for 8d in the absence of agents which elevate cAMP or mimic its effects, even though no label was visible in immunofluorescence experiments see Figure 3.1b. This probably indicates that cells from 85 rats, many of which have already begun to make myelin, continue to synthesize very low residual levels of P_q in the absence of axonal contact (Poduslo et al. 1985).

**The level of P_q mRNA is also induced by cAMP elevation.** In situ hybridization experiments using an oligonucleotide probe show that the level of mRNA for P_q in the cells is elevated by agents that elevate Schwann cell cAMP. Figure 3.3 shows an autoradiograph of cells exposed for 48h to cAMP analogues (g and h), forskolin (c and f), CTx (c and d). In a and b, untreated cells are shown for comparison. It is clear that cAMP elevation is raising the levels of P_q mRNA.
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The time course of this induction is shown in Figure 3.4. The levels of $P_0$ mRNA are clearly higher than in control cultures after 24h of treatment with forskolin, a time when no $P_0$ protein is detectable on the cells immunocytochemically.

**Northern Blot.** Northern blots of whole RNA extracted from Schwann cell cultures also show increased levels of $P_0$ message after addition of forskolin (see Figure 3.5, lanes 3 and 4). Small, but readily detectable amounts of $P_0$ message are present in cells cultured in defined medium alone, and are maintained up to at least 13 days in vitro (not shown). This confirms the observations of Lemke and Chao (1988) who saw a 40 fold drop in $P_0$ mRNA expression over the first 5d in vitro when Schwann cells from P2–3 sciatic nerve were maintained in culture. This reduced level of $P_0$ mRNA expression was readily detectable, and maintained by the Schwann cells indefinitely (>4 months). Kreider and coworkers (Kreider et al. 1988) saw only trace levels of $P_0$ mRNA in neonatal rat Schwann cells after 7d in culture, and Morrison and coworkers (Morrison et al. 1991) saw no $P_0$ message in cultures from P5 rats after 10d in culture. The amount of time that a blot is exposed to X-ray film will determine the density of the band produced, it is possible that by exposing the X-ray film to the blot for a long time I routinely detected basal levels of $P_0$ mRNA in cultured Schwann cells, whilst others adjusted exposure to avoid this. Lemke and Axel (1985) demonstrated that the $P_0$ mRNA is transcribed as a single RNA species at 1.9 kb. When the distance travelled by the $P_0$ mRNA was compared to the distance travelled by the molecular weight markers, the cDNA was found to have migrated to a position between the 1.4 and 2.4 kb RNA markers, as expected.

In agreement with the levels of $P_0$ protein induced by cAMP in culture, the levels of $P_0$ mRNA never reach the levels seen in RNA taken from developing nerve at the time of myelin synthesis, compare Figure 3.5a, lanes 7 (2μg RNA from whole nerve) and 4 (10μg RNA from cAMP treated cells in defined medium). Using very high levels of forskolin (100μM) Lemke and Chao (1988) found that $P_0$ mRNA levels were only ever induced to 37% of the levels seen in neonatal (2–3 day old) rat sciatic nerve.

When total RNA from 3T3 cells is probed with cDNA complementary to $P_0$
3. cAMP on Schwann cells

mRNA, no hybridization at all is seen (see Figure 3, lane 2), and even after prolonged exposure of the blot to X-ray film no \( P_0 \) mRNA is detected (not shown).

Elevation of cAMP induces GAL-C and the O4 antigen on cultured Schwann cells. As reported previously for Schwann cells cultured in serum (Sobue and Pleasure 1984), cAMP elevated GAL-C expression on the surface of some of the Schwann cells cultured in defined medium. The levels of antigen recognised by the monoclonal antibody O4 were also raised. 19 ± 4.3% (n = 4) and 54 ± 8.0% (n = 8) of the cells treated with a mixture of db-cAMP and 8b-cAMP for 72 h, as described in Methods expressed GAL-C and O4 respectively, see Figure 3.6. When the cells are left longer in cAMP-elevating agents, the number of cells expressing O4 approaches 100%. After 6 days, 71%, 89%, 89% and 99% of the cells in one experiment expressed O4 after 6d treatment with IBMX, forskolin, CTx and cAMP analogues respectively. In these cultures, however the cells were no longer phase bright, and a few were peeling from the coverslip.

Division profile of Schwann cells treated with cAMP elevating agents in defined medium. DNA synthesis, assessed by incorporation of BrdU into the nucleus of Schwann cells as described in Methods, was not elevated above control levels. After addition of forskolin, CTx or cAMP analogues, BrdU was incorporated into the nucleus of 3 ± 2.5%, 3 ± 2.8% and 2 ± 1.5% of the cells in the final 24h of culture respectively. In control cells, BrdU incorporation was 1 ± 1.4%.

Withdrawal of cAMP: When cAMP elevating drugs were withdrawn, \( P_0 \) expression declined to undetectable levels in most of the cells. However, a few cells in each culture remained \( P_0^+ \) and retained their flattened morphology. In one experiment, 70% of the cells became \( P_0^+ \) after 72h of treatment with cAMP analogues. When cells in sister cultures, treated in the same way, were then washed and maintained in defined medium without drugs for a further 72h, most of the cells returned to their bipolar morphology, and lost \( P_0 \) immunoreactivity and mRNA, see Figure 3.7f. 5% of the cells, however, remained flattened and \( P_0^+ \), see Figure 3.7a and b.

Other drugs. In Chapter 1 the ability of one second messenger pathway to enhance or control the activity of another pathway was discussed. In the experiments described below, the effect of raising Schwann cell cAMP levels in the presence of drugs that
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Elevate intracellular calcium and drugs that activate or suppress the enzyme PKC was examined.

Calcium ionophore A21387: Addition of the calcium ionophore A21387 enhances the effect of cAMP elevation on GAL-C expression. If $5 \times 10^{-8}$M calcium ionophore is present when cAMP analogues are used to induce the myelin phenotype, the number of cells expressing GAL-C after 72h is $45 \pm 5.3\%$ ($n = 4$), compared to $19 \pm 4.3\%$ ($n = 4$) when cells are treated with cAMP analogues alone ($p < 0.05$, Students t-test, indicating a significant change). The level of $P_q$ expression was not increased in these cultures, $77 \pm 2.5\%$ ($n = 4$) of the cells expressed $P_q$ when A21387 was present compared to $77 \pm 8.3\%$ ($n = 4$) when the cells were treated with cAMP analogues alone. In these experiments, however, the $P_q$ induction was already high, nevertheless it was clear when looking at the cells that they were more strongly labelled with anti $P_q$ than those without calcium ionophore. Calcium ionophore alone at $5-7.5 \times 10^{-6}$M did not induce $P_q$ protein ($0 \pm 0.3\%$, $n = 5$) or GAL-C ($0 \pm 0$, $n = 3$).

It will be interesting to quantify this increased $P_q$ expression in individual cells, this could be done by photometric analysis of individual cells. Alternatively, using larger numbers of cells, cell extracts of treated cells could be analysed on a Western blot, an increase in total $P_q$ levels would be indicated by a heavier band of $P_q$ immunoreactivity produced by cells treated with A21387.

Pilot studies monitoring levels of $P_q$ mRNA by in situ hybridization indicate that the level of $P_q$ mRNA is enormously increased when cAMP is raised in the presence of with A21387 compared to the effect of cAMP elevation alone, see Figure 3.8.

Agents that enhance or inhibit the action of PKC: In some systems, agents that activate PKC will enhance the amount of cAMP released in response to other drugs, whilst having no cAMP elevating effects themselves (Shenolikar et al. 1986). If such a cooperative mechanism were to exist in Schwann cells, then it is possible that agents that activate PKC may enhance the effects of cAMP elevating drugs. Phorbol esters are tumour promoters which mimic DAG and activate PKC (see Harper 1988 for review).
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The phorbol ester phorbol dibutyrate (PDB) caused no elevation in \( \text{P}_o \) levels when the drug was applied constantly at \( 10^{-8}\text{M} \) (\( \text{P}_o = 0 \pm 0\% \), \( n = 3 \)) or pulsed on to the cells for 1h each day (\( \text{P}_o = 0 \pm 0\% \), \( n = 3 \)). The cells showed an immediate change in morphology with cell processes becoming distorted and wavy. When PDB was added at \( 10^{-8}\text{M} \), pulsed on to the cells for 1h each day as above, in combination with the calcium ionophore A21387, again no \( \text{P}_o \) was induced (\( \text{P}_o = 0 \pm 0\% \), \( n = 3 \)).

When cells were treated with the cAMP analogues 8b-cAMP with db-cAMP in the presence of PDB \( 10^{-8}\text{M} \) for 72h, elevation of immunodetectable \( \text{P}_o \) protein was completely abolished (reduced from \( 40 \pm 9.7 \) to \( 0 \pm 0.3\% \), \( n = 7 \)) (\( p <0.05 \), Students t-test, indicating a significant change). The inactive phorbol ester 4\( \alpha \) phorbol had no effect on cAMP analogue-induced \( \text{P}_o \) expression. \( \text{P}_o \) expression was \( 67 \pm 16.1\% \) when cAMP analogues were added alone, and \( 61 \pm 12.1\% \) in the presence of 4\( \alpha \) phorbol \( 10^{-8}\text{M} \) (\( n = 3 \)) (\( p >0.5 \), Students t-test, indicating no significant change).

When PDB was applied for only 1h each day, then the induction of \( \text{P}_o \) by cAMP analogues was not blocked, occasionally more labelled cells were seen, but this was not reproducible. Only 3 times out of 10 was the number of cells expressing \( \text{P}_o \) doubled when compared to cells induced without PDB treatment.

Phorbol esters initially activate PKC, but the enzyme can be inactivated after longer exposure to these drugs (Shenolikar et al. 1986, or Harper 1988 for review). It was hoped that long term activation of PKC might have been achieved by carefully limiting the time for which the cells are exposed to PDB. However, it was not possible to use this screening method to detect an enhancement of \( \text{P}_o \) induction in response to cAMP analogues in the presence of activated PKC. If such an effect exists, then it might be demonstrated by running extracts of the treated cells on Western blots, to show an increased level of \( \text{P}_o \) in each cell, rather than looking for increased numbers of cells expressing \( \text{P}_o \), as discussed above for calcium ionophore.

Although it is clear that long term exposure to PDB prevents induction of the myelin phenotype, the reason for this is not clear. Whether this is the result of reduced or increased PKC activity could only be determined by measuring the activity of this enzyme. When another PKC-inhibiting drug was used in an attempt to block
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PKC activity, no reduction in $P_0$ induction was seen. The lysosphingolipid sphingosine is competitive with DAG, and inhibits PKC (for review see Harper 1988). When $10^{-7}M$ sphingosine was present as cAMP analogues were added to cultured Schwann cells, no inhibition in $P_0$ protein induction was seen. $69 \pm 11.0\%$ of the cells became $P_0^+$ when cAMP analogues added alone, compared to $59 \pm 11.0\% P_0^+$ when sphingosine was present ($n = 4$) ($p > 0.5$, Students t-test, indicating no significant change).

DMSO induces differentiation in several transformed cell lines, but suppresses differentiation of glial cells from embryonal tetracarcinoma cells (see Sutrina et al. 1987 for discussion). DMSO inhibits the synthesis of glactoceramide and sulphatide in transformed rat Schwann cells (Sutrina et al. 1987). As DMSO was used to dissolve the phorbol esters in my experiments, it was important to determine that the levels used did not affect differentiation. The addition of 0.5% DMSO induced half maximal suppression of sulphatide synthesis (Sutrina et al. 1987), when DMSO was used to dissolve PDB at $10^{-2}M$, the final concentration of DMSO when PDB was used at $10^{-8}M$ was 0.0001% and thus well below the levels expected to suppress glial differentiation.

**Cycloheximide.** The protein synthesis inhibitor cycloheximide blocks the elevation of $P_0$ mRNA seen when cAMP mimicking drugs are added to cultured Schwann cells. Pilot studies (repeated twice) indicate a complete block of $P_0$ mRNA induction when 25–100$\mu$g.ml$^{-1}$ cycloheximide was added for 24h with 8b-cAMP and db-cAMP each at $5 \times 10^{-4}M$, see Figure 3.9d. Cycloheximide reduces the level of $P_0$ mRNA in Schwann cells to below that seen in control cells that have not been exposed to cAMP, even in the presence of cAMP elevating agents (compare Figures 3.9b and 3.9d).

**The role of the tissue culture substrate.** Laminin–coated substrates were not obligatory for induction of the myelin phenotype, since cells cultured on PLL coated coverslips or tissue culture plastic could be induced to make $P_0$ in response to elevation of cAMP. In a representative experiment, 63% of cells plated on laminin and 42% of cells plated on PLL expressed $P_0$ after a 3d exposure to cAMP analogues. Extracellular matrix from bovine corneal endothelial cells was also effective although
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In this case the cells assumed a process-bearing morphology rather than the flattened sheet-like morphology more typical of cells on laminin.

Growth factors and serum prevent $P_0$ induction


When cAMP elevating or mimicking drugs were added to cultured Schwann cells the induction of $P_0$ detectable by immunocytochemical labelling was greatly reduced in the presence of serum or growth factors, see Table 3.3. When the level of DNA synthesis was measured at the same time then, as expected, it was seen that the cells were dividing. In cultures where division was highest, $P_0$ induction was most suppressed. Serum and GGF both induce division and suppress differentiation in response to cAMP analogues, CTx, forskolin and IBMX. bFGF suppresses $P_0$ induction in response to forskolin, and early experiments with aFGF, and PDGF indicate that this is a general property of growth factors that are Schwann cell comitogens with cAMP, see Table 3.3. The morphology of these proliferating cells, and the relative amount of $P_0$ immunolabelling is shown in Figure 3.1. The Western blot shown in Figure 3.10 indicates that $P_0$ induction in response to cAMP analogues is greatly reduced by serum and only a faint band is seen that was not present in control cultures (see Figure 3.10).

Serum. In Figure 3.11 the effect of increasing concentrations of serum on the number of cells induced to express $P_0$ is demonstrated. The induction over 4 days is shown and it is clear that the levels of $P_0$ continue to rise after 72h. The effect of serum is, therefore, to delay rather than to prevent differentiation, and when cells are left 7 days in 10% serum with cAMP analogues, many more cells differentiate. Unlike the cells treated with cAMP in defined medium these cells show no deterioration in morphology, even after exposure to cAMP analogues for times of up to at least 3 weeks. When other sera were tested, foetal calf serum and horse serum had similar effects, both supported proliferation and reduced $P_0$ elevation after 3d treatment with cAMP elevating agents.

Cell density. The common action of serum and growth factors on Schwann cells is to

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generate conditions under which cAMP elevation increases DNA synthesis and the most likely interpretation of these results is that a mitogenic response involving cAMP is incompatible with myelin–related differentiation. Alternatively it is possible that serum and growth factors block induction of the myelin phenotype directly, and that the mitogenic effects of serum and growth factors are unrelated to the inhibition of myelin–related differentiation.

To test for a block of differentiation–specific genes in Schwann cells by growth factors in serum, advantage was taken of the observation that Schwann cells show a strong density–dependent inhibition of division as described in Chapter 1. Cells were exposed to agents which elevate cAMP in the presence of 10% CS at low plating densities, allowing a strong proliferative response, and at a series of higher densities when proliferation was increasingly suppressed. Cells were plated at densities ranging from 2,500 – 20,000 cells per coverslip and exposed to cAMP analogues for 5d in the presence of 10% CS. A labelling index of 82 ± 4% was observed at the lowest plating density, and no \( P^q \) expression was seen. At the highest plating density the labelling index observed was 26 ± 6.7% and 21 ± 8.5% of the cells expressed \( P^q \). Thus even in the presence of serum an inverse relationship between proliferation and myelin–related differentiation was revealed. These observations explain the delayed differentiation described above where little differentiation was seen after 72h, but the cells went on to differentiate after 7 days. The cells in these cultures become more dense as they divide, and the increased density will curb division and permit differentiation. These results indicate that either a block on differentiation produced by cells in division is acting, or that growth factors do prevent differentiation, but this block is ineffective when the cultures become dense.

Individual cells expressing \( P_0 \) do not incorporate BrdU and are therefore not synthesising DNA. During development, all of the \( P_0 \) expressing cells have been demonstrated to be non dividing (Stewart, Morgan, Mirsky and Jessen 1992, submitted, see Chapter 2, Figure 2.2). The results above show that Schwann cell populations responded to elevation of cAMP by a high degree of myelin–related differentiation and low proliferative activity, or by a low level of differentiation and rapid DNA synthesis depending on other conditions in the culture. This suggests that
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In individual Schwann cells cAMP elevation will either stimulate DNA synthesis or induce myelin-related differentiation, but not both. To test this, DNA synthesis in the P₀ negative and P₀ positive Schwann cells within the same culture were compared. In these experiments the plating density was adjusted to obtain substantial proportions of both BrdU positive cells and of P₀ positive cells in the presence of cAMP analogues and 10% CS as described above.

In four experiments the number of P₀ positive cells averaged 23 ± 3.7% (± SEM) and the number of cells synthesizing DNA within the P₀⁺ population was only 1 ± 0.3% (± SEM), while the number of cells synthesizing DNA within the P₀⁻ population was 31 ± 3.2% (± SEM). This shows that cells acquiring the myelin-related phenotype and dividing cells are largely non-overlapping populations, suggesting that DNA synthesis and myelin-related differentiation are essentially incompatible within individual Schwann cells. In pilot studies, in which the cells were only exposed to BrdU for the final 2h of culture, none of the P₀⁺ cells incorporated BrdU. This result indicates that the few P₀⁺ cells that did incorporate BrdU above had incorporated BrdU during their final S-phase, and had had time to differentiate since then (data not shown). This parallels the situation in development where all of the cells that have begun to express the myelin phenotype (as determined by P₀ expression) have dropped out of division.

Other agents that inhibit cell division. The Na⁺-H⁺ exchanger is activated by many protein growth factors in the course of stimulating cell proliferation, and in many systems this has been shown to be through PKC-mediated phosphorylation of the exchanger (reviewed by Alberts et al. Chapter 6 and Chapter 12, 1989). The resulting increase in intracellular pH is thought to play an important part in initiating cell proliferation. The drug amiloride inhibits the exchanger and prevents the cells from dividing in response to these growth factors (reviewed by Alberts et al. Chapter 6 1989). In order to determine whether, by simply preventing cells from responding to mitogens, a mitogenic signal could be converted into a differentiation signal, amiloride was used to prevent mitosis in response to cAMP analogues and serum. When Schwann cells were treated with cAMP analogues in defined medium in the presence of amiloride, P₀ induction was little changed, 84% of the cells expressed P₀.
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without amiloride, and 65% expressed \( P_0 \) when amiloride was present (\( n = 1 \)), indicating that amiloride did not prevent \( P_0 \) synthesis or expression. Amiloride at concentrations between 200 and 500\( \mu \)M reduced the proliferative response of the Schwann cells to cAMP and serum, from 66 ± 5% to 1 ± 0.7% (\( n = 3 \)) of the cells synthesising DNA. No increase in \( P_0 \) induction was seen, however, since 5 ± 1.4% of the cells expressed \( P_0 \) after 72h in response to cAMP with 10% CS alone, and in the presence of amiloride 1 ± 0.8% (\( n = 3 \)) of the cells expressed \( P_0 \).

Whilst an increase in intracellular pH is necessary to permit cell proliferation in response to mitogens, it is not in itself a sufficient signal to induce cell proliferation (discussed in Fine et al. 1985 and Galinka et al. 1992). There must be other cellular events triggered by the interaction between cAMP elevation and growth factor binding that, together with the rise in intracellular pH, induce cell proliferation. When amiloride prevents Schwann cells from responding to the signal to proliferate, enough of the other intracellular changes associated with cell division may still occur, and it is these that act to inhibit expression of \( P_0 \) protein. In the presence of amiloride then, Schwann cells are still receiving a message to proliferate, but they are prevented from responding to it, so they do not differentiate. When high density plating prevents induction of DNA synthesis in response to growth factors and cAMP elevation, presumably the Schwann cell intracellular environment has changed, so that it now perceives the increased cAMP levels as a differentiation-inducing signal rather than as a proliferation signal. More experiments will be necessary to understand the mechanism underlying the block of differentiation seen in the presence of serum.

In situ hybridization. When in situ hybridization experiments were performed on Schwann cells induced to proliferate in response to cAMP elevating drugs and growth factors, the change in of \( P_0 \) mRNA levels were not easy to determine. The plating density required for high proliferation in response to growth factors and cAMP elevation is low and although at low power the cultures did not appear to be heavily labelled, individual cells clearly had more silver grains than control cultures. The elevation was seen more clearly in Northern blots.

Northern blot When total mRNA was extracted from cultures of cells grown in serum and treated with forskolin, a large induction of \( P_0 \) mRNA is seen see Figure 3.5,
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compare lanes 5 and 6. Schwann cells are evidently using post-translational steps to control the levels of P\textsubscript{0} protein in these conditions as there was very little P\textsubscript{0} protein in identical cultures (see Figure 3.1h, and Figure 3.10 lane labelled CS + cAMP). Altering the level of P\textsubscript{0} mRNA is evidently not the only method the cell has to control P\textsubscript{0} protein levels.

cAMP induced O4 expression is not blocked by proliferation. In vivo it is clear that expression of O4, unlike that of P\textsubscript{0}, is compatible with proliferation during development as 100% of the Schwann cells at E21 are O4\textsuperscript{+}, and many of these cells are proliferating (Stewart, Morgan, Mirsky and Jessen 1992, submitted). To test whether O4 expression and DNA synthesis were compatible in cultured Schwann cells these parameters were monitored in cells exposed for 3d to cAMP elevating agents in defined medium and in medium containing growth factors or serum. The results are displayed in Table 3.4. It is clear that O4 induction in response to cAMP elevation is much less affected by the presence of growth factors than P\textsubscript{0} induction. It is also evident that whilst serum or GGF together with cAMP elevation induce similar amounts of cell division, serum reduces O4 induction in response to cAMP elevation to a far greater extent.

In the presence of GGF, O4 induction in response to forskolin was reduced from 59 ± 7.2% (n = 9) (BrdU positive cells 4 ± 2.5%) in defined medium alone, to 40 ± 8.1% (n = 5) (BrdU positive cells 85 ± 3.3%). This reduction in O4 expression is not statistically significant (p > 0.1, Students t-test). Many of the cells synthesising DNA must also be expressing O4, because only 15% of the cells are not synthesising DNA, and with 40% of the total cells expressing O4, the O4\textsuperscript{+} and DNA synthesising populations must overlap. When cells are plated more densely, enough to reduce, but not block, the proliferative response the effect of serum was less, and many cells expressing O4 and BrdU are induced even in serum, see Figure 3.12.

Elevation of cAMP in cultured Schwann cells mimics the inversion in protein expression characteristic of myelination in vivo. When a Schwann cell is induced to form myelin in vivo, the induction of myelin specific proteins is followed by down-regulation of a set of proteins that in the normal adult nerve are only seen on the non-myelin forming Schwann cells. These include GFAP, N-CAM, A5E3, L1 and NGFR.
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(Jessen et al. 1984, 1987a, Martini and Schachner 1986 and 1988). This inversion of protein expression is reversed when myelin-forming cells are taken into culture and in transected nerve, as described in Chapter 2. Therefore, in the absence of drug treatment, essentially all the cells in culture expressed GFAP, L1, N-CAM, A5E3 and NGFR in addition to being P\textsubscript{0} negative. Elevation of cAMP was found to induce the myelination-specific combination of protein up- and down-regulation in many cells, since the expression of GFAP, N-CAM, L1, A5E3 and NGFR was strikingly suppressed in a subset of the P\textsubscript{0} positive cells, see Figure 3.13.

Of all the conditions tested, cAMP elevation in high density cultures with 10% CS gave the best induction of the myelin phenotype in individual cells, i.e. highest levels of P\textsubscript{0} expression as judged by the intensity of immunolabelling, and also down-regulation of GFAP, N-CAM, L1, A5E3 and NGFR in the largest proportion of the P\textsubscript{0} positive cells. These observations raise the possibility that serum not only contains factors which block cAMP induction of the myelin phenotype, either directly, or through stimulating division, but also might contain factors that act together with cAMP to promote maximal expression of the myelin phenotype, once DNA synthesis is suppressed. Bologa and coworkers (Bologa et al. 1988) have demonstrated that serum enhances GAL-C expression in cultured oligodendrocytes. When serum derived factors were added to the cultures individually, they found that 2 fractions of serum enhanced GAL-C expression, whilst a third fraction repressed GAL-C expression. In my cultures, however, Schwann cells plated at high density in the presence a single growth factor, GGF, together with cAMP analogues also showed enhanced immunolabelling. This might indicate that one factor can act in two opposing ways, enhancing differentiation or enhancing division depending on other conditions in the culture. GGF is, however, only a semi pure fraction, and the possibility that more than one factor is present, one acting to promote differentiation, and the other acting to induce division has not be ruled out. The experiment will have to be repeated with a pure growth factor before it can be determined for certain whether single growth factors act both to promote differentiation and division in Schwann cells.
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DISCUSSION

The experiments in Chapter 3 demonstrate that elevation of intracellular cAMP in short term Schwann cell cultures induces expression of the major protein of peripheral myelin, \( \text{P}_0 \), and that this is accompanied by an increase in the levels of the mRNA for \( \text{P}_0 \). GAL–C and the lipid antigen O4 are both induced by cAMP elevation. The same cells also suppress a number of proteins that are also suppressed by myelin forming Schwann cells during development, and that are only seen on the non–myelin forming Schwann cells in the adult nerve. Thus cAMP elevation produces Schwann cells that very closely resemble myelin forming cells in mature peripheral nerve. The level of myelin protein and gene expression is, however, never as high as the levels seen in the developing nerve during myelin synthesis.

In another *in vitro* model of myelination when Schwann cells are cocultured with DRG neurons, myelin synthesis is prevented in the absence of basal lamina assembly. Basal lamina assembly is prevented when the synthesis of one of the components of the basal lamina is blocked by growing the cells (i) in the presence of the collagen secretion inhibitor, cishydroxyproline, (ii) without the collagen synthesis cofactor ascorbate or (iii) in the presence of the proteoglycan synthesis inhibitor \( \beta \)-xyloside (for review see Bunge et al. 1989). These Schwann cells are in the presence of myelin–inducing factors from the DRG axons, but no myelin is formed. The cells express MAG, O4, MBP and GAL–C, but are \( \text{P}_0 \) negative when stained with antibodies and synthesise \( \text{P}_0 \) at a level that can only be detected using immunoblotting (Morrison et al. 1991, Owens and Bunge 1989, Brunden et al. 1990a, 1992). Similar disruption to myelin assembly is seen when antibodies to GAL–C are added to cocultures of DRG neurons and Schwann cells in the presence of medium that would normally allow myelin synthesis. In these cultures basal lamina and MAG levels are normal, but \( \text{P}_0 \) and MBP are not detectable by immunocytochemistry (Ranscht et al. 1987, Owens and Bunge 1990). These experiments demonstrate that when Schwann cells receive a signal to initiate myelin assembly, if they are prevented from assembling a myelin sheath (either because they have no basal lamina, or because of the presence of GAL–C antibody), then a feedback mechanism will
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prevent maximal synthesis of the myelin components. There may be a similar negative feedback when Schwann cells are stimulated to express the myelin phenotype by cAMP elevation in culture. In these conditions, the cell is unable to compact the plasma membrane into myelin as there is no axon to spread around, and so maximal synthesis of the myelin proteins is not achieved.

The block of mRNA induction by cycloheximide is consistent with the hypothesis, discussed by Monuki and coworkers, that $P_0$ induction by cAMP is mediated by changed expression of regulating proteins, and not by a direct effect on the $P_0$ gene by cAMP. This hypothesis is based on the observation that the $P_0$ gene does not contain any known cAMP responsive elements and on the delayed upregulation of myelin protein expression in response to cAMP elevation (Lemke et al. 1988, Monuki et al. 1989). The reduction of $P_0$ mRNA to levels below those seen in untreated cells shows that (1) the $P_0$ mRNA remaining in the control cells is constantly turned over and (2) that even these basal levels also require protein synthesis for maintenance.

cAMP treated Schwann cells differentiate more completely in the presence of calcium ionophore A21387. In another system, the differentiation of the mastocytoma cell line P-815, cAMP arrests cell growth and induces differentiation as measured by the increased activity of the enzyme L-histidine decarboxylase. The calcium ionophore A21387 has no significant effect on differentiation of these cells, but cAMP and A21387 together synergistically increase cell differentiation (Miyazaki et al. 1992). If the axonal agent (or agents) normally activate cAMP, but also activate another pathway that elevates intracellular calcium, then when we elevate cAMP levels, we are only mimicking a part of the axonal signal. As discussed in Chapter 1, activation of one receptor can alter the activity of more than one second messenger pathway. If both cAMP and Ca++ levels are elevated in vivo when a Schwann cell is induced to produce myelin, then the enhanced Schwann cell differentiation produced when both intracellular cAMP and calcium are elevated could indicate one of two possibilities. The axon derived factor may activate more than one pathway to induce Schwann cell differentiation, or there might be two factors, one of which raises cAMP levels, and the other elevating Schwann cell intracellular Ca++ levels.
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The few cells that do not lose or even reduce their myelin phenotype after cAMP removal are intriguing, and it is tempting to speculate that they are similar to the outer cells of the double myelinated axons in the sympathetic nervous system (Kidd and Heath 1988a, 1988b). Once those cells are fully separated from their axon, they do not dedifferentiate when that axon degenerates after injury (Kidd et al. 1992, Kidd and Heath, 1991). Possibly in this tissue culture system, a few of the cells differentiate fully, and become independent of external signals and are able to maintain their differentiated phenotype. These cells will be interesting to examine further, to see if they last longer in culture, and if they do, what signals will cause them to de-differentiate.

How do growth factors curb Schwann cell differentiation; do they inhibit myelin gene expression directly, or is the inhibition secondary to proliferation? Growth factors and serum modulate the response of Schwann cells to elevated intracellular cAMP levels. Increases in intracellular cAMP to levels that would induce expression of P0 in defined medium will, in the presence of growth factors, induce proliferation and prevent induction of the myelin phenotype. As discussed in Chapter 1, cAMP unmasks the proliferative effect of growth factors, most of which are poor mitogens for Schwann cells when added alone. In the case of PDGF, it has been shown that cAMP-mediated induction of PDGF receptors is responsible for this cooperation (Weinmaster and Lemke 1990).

When cAMP promotes the proliferative effects of growth factors, there are at least two possibilities as to how the inhibition of cAMP induced differentiation is mediated. Firstly, activation of growth factor receptors could block the expression of differentiation-specific genes, so that when the cells differentiate in dense cultures it is because they have become unresponsive to growth factors. This could be due to down-regulating growth factor receptors, growth factor receptors becoming non-functional, or a change in the Schwann cell intracellular environment so that activation of growth factor receptors no longer induces proliferation. Recently it has been demonstrated that the levels of mRNA for PDGF-β and FGF receptors declines rapidly during the first three postnatal days in rat sciatic nerve (Davis 1992). This loss coincides with the developmental decrease in Schwann cell proliferation. The
The second possible mechanism by which growth factors might prevent differentiation in response to cAMP elevation is through a suppression of differentiation–specific gene expression that is secondary to proliferation. In contact inhibited cells, with a proliferation–mediated block on differentiation removed, the cells would be able to activate differentiation–specific genes, even if growth factor receptors are still activated. Possible experiments to separate these two mechanisms would include measuring the levels of growth factor receptors expressed by Schwann cells in dense culture. The autophosphorylation of growth factor receptors in response to the addition of growth factors could be measured to determine whether the receptors are functional. It will be interesting to determine whether the cAMP mediated increase in PDGF receptors is reduced in dense, non proliferating cultures.

An interesting parallel is seen in the differentiation of skeletal muscle. A suppression of differentiation during proliferation similar to that described for Schwann cells is seen in muscle cell lines (including the cell lines C2 (Franco and Lansman 1990), F3 (Song et al. 1992), G8, C2C12 and 23A2 (Tweedie et al. 1991)), where proliferation is high, and differentiation is suppressed when the cells are grown in high levels of serum. Growth arrest and differentiation can be induced in all of these cell lines by reducing the level of serum in the culture medium. Differentiated quail myocytes down regulate expression of differentiation–specific muscle proteins in the presence of mitogens (Latham and Konigsberg 1989). Normal myoblasts and satellite cells from adult muscle in tissue culture will at first proliferate, and then some will withdraw from the cell cycle, fuse and differentiate in the continued presence of myoblast mitogens serum and embryo extracts (Bischoff 1990, Curtis and Zalin 1985). Terminal differentiation in muscle cell lines is associated with permanent loss of FGF and EGF receptors (Olwin and Hauschka 1988).

A family of myogenic regulatory genes, the MyoD family, has been described, these genes share homology with helix–loop–helix (HLH) gene transcription regulatory proteins. These molecules share the ability to convert non–muscle cell lines to stable myogenic cells with the potential to undergo myogenesis. Members of the MyoD family include MyoD, myd, myf–5, MRF–4 (herculin), myf–6 and
myogenin, which all bind DNA at muscle-specific control regions (for review, see Olson 1990, 1992). High expression of MyoD suppresses cell growth and leads to differentiation, even in the presence of high levels of serum, which as described above, induces proliferation and inhibits expression of muscle-specific genes. This antagonism between the actions of growth factors and MyoD has been discussed in several reviews (see, for example Olson 1990, 1992). It is not clear whether members of the MyoD family independently suppress proliferation, or if this is a result of differentiation.

During muscle development in vivo, myoblast proliferation is maintained by factors secreted from the apical ectodermal ridge, and these factors maintain the cells in a proliferative and dedifferentiated state. Myoblast differentiation and growth arrest in vivo are accompanied by loss of Hox-7.1, a homeobox containing gene (discussed in Song et al. 1992). Forced expression of Hox-7.1 in F3 myoblast cells prevents terminal differentiation when serum levels are reduced, blocks MyoD expression, and transforms the cells (Song et al. 1992). Thus there are two genes identified, Hox-7.1 and MyoD which have opposite effects on myoblast proliferation and differentiation.

Growth factor mediated negative effects on myogenesis are likely to be due to interactions between MyoD and other HLH-containing regulatory proteins. MyoD and the ubiquitous HLH-containing protein E12 form heterodimers that turn on the muscle differentiation-specific genes, whilst MyoD-family homodimers only to bind control regions of these genes with a low affinity, and do not turn the genes on. Another HLH-containing protein Id (inhibitor of DNA binding) also binds E12, but Id heterodimers lack the ability to bind DNA and so can not initiate expression of the myogenic genes. By binding to E12, growth factor-induced Id reduces the amount of E12 available to bind to MyoD and so prevents differentiation. It is proposed that Id is lost on differentiation, releasing E12 from the inactive Id–E12 heterodimer, and allowing formation of the active E12–MyoD heterodimer (reviewed by Olson 1990). Myoblasts express high levels of MyoD prior to differentiation, and it is only after serum deprivation that the MyoD is bound to the muscle gene enhancer regions (reviewed by Olson 1990). As F3 cells constitutively expressing Hox-7.1 do not
express MyoD, it is likely that MyoD is not maintained at high levels if differentiation is prevented by division. Recently, the FGF-mediated block on myogenesis has been shown to be due to inactivation of myogenic HLH proteins. FGF activates PLC and induces phosphorylation of a conserved site in the DNA binding domain of myogenin, preventing DNA binding and thus preventing activation of muscle-specific genes (Li et al. 1992). PLC activation directly (without activating the FGF receptors) will substitute for FGF to produce this block.

It is possible that some similar mechanism is working during Schwann cell proliferation when cAMP is elevated in the presence of growth factors, acting to prevent expression of myelin genes. The transcription factor SCIP (also known as tst-1 and oct-6), a POU domain protein that has myelin gene-repressing actions has been identified (He et al. 1991, Monuki et al. 1989). SCIP is the predominant octamer binding protein in cultured proliferating Schwann cells (Monuki et al. 1989, 1990, Kuhn et al. 1991). The POU proteins are HLH containing proteins, the HLH region is contained in the POU homeodomain, close to the POU-specific domain and both of these regions are necessary for interactions with the octamer binding sites on the DNA (see reviews by Ruvkun and Finney 1991 and Busch and Sassone-Corsi 1990). SCIP binds to the Pₒ promoter and suppresses transcription (Monuki et al. 1990). SCIP is expressed by Schwann cells at birth, but is lost in the mature nerve (Monuki et al. 1990, Collarini et al. 1992, Scherer et al. 1992b). Peripheral nerve transection causes a temporary return of SCIP mRNA (Monuki et al. 1990, Scherer et al. 1992b), whilst after crush injury the increase in SCIP is maintained for at least 58 days (Scherer et al. 1992b). The pattern of SCIP expression in vivo is generally consistent with SCIP acting to prevent myelin-specific gene expression during Schwann cell mitosis during development and after transection. The failure of SCIP to downregulate after recovery from a crush injury, however, is inconsistent with this proposed role for SCIP. After crush injury, once the new axonal sprouts have grown through the distal part of the nerve, proliferation ceases and Schwann cells differentiate into the quiescent non-myelin forming and myelin forming cells, unexpectedly, these cells do not down regulate SCIP. In vitro, Schwann cells express very low levels of SCIP mRNA, and SCIP expression is up-regulated by treatment
3. cAMP on Schwann cells

with forskolin in the presence of serum (Monuki et al. 1989). Again, this does not appear to be consistent with a myelin gene suppressing role for SCIP, as myelin genes are expressed in these experiments. The levels of myelin genes induced in response to cAMP elevating drugs never reach the levels seen in intact nerve during myelinogenesis, however, and so the possibility that SCIP–mediated repression is acting to curb Schwann cell differentiation in response to cAMP in vitro has not been excluded. The ability of SCIP to suppress expression of myelin genes and the fact that SCIP is lost from Schwann cells at the same time that myelin commences during normal development makes it a candidate molecule for preventing myelin gene expression during early nerve development.

The observation by Li and coworkers that PLC activation prevents activation of muscle-specific genes (Li et al. 1992) confirms the need for more experiments to determine the activity of PKC when Schwann cells are treated with cAMP elevating agents in the presence of phorbol esters, discussed in Results (page 134). Phorbol dibutyrate prevented cAMP induction of myelin-specific genes, this could be due to PKC activation by the phorbol ester that is mimicking the effects of growth factor mediated PLC activation.

The parallel between Schwann cell and muscle differentiation is not exact as whilst both myoblast fusion and differentiation are enhanced by activation of adenylyl cyclase (Curtis and Zalin 1985), the absence of growth factors alone is sufficient to induce myoblast differentiation. In contrast, Schwann cells require, in addition to the removal of mitogenic signals, a positive inducing signal, a signal that is mimicked by cAMP elevation in vitro to initiate differentiation. The general principle that growth factors will act to inhibit differentiation by inducing proliferation, and that this inhibition is not absolute, but can be overcome if division is prevented is, however, the same.

Although the myelin phenotype is not induced by the combination of cAMP elevation and growth factors if the Schwann cells divide, a change in the Schwann cell phenotype away from that of control cells is induced by this combination of drugs, as O4 is induced on many of these cells. In these conditions, the phenotype produced is the same as that of Schwann cells in the developing nerve before the
mature myelin forming or non-myelin forming Schwann cells are seen.

The induction of O4 in response to cAMP elevation is prevented or reduced by serum, but not by GGF. It is possible that serum not only contains growth factors, but in addition contains inhibitors of differentiation, possibly the same serum-derived factor isolated by Bologa and coworkers that suppresses GAL-C expression in oligodendrocytes (Bologa et al. 1988) acting to inhibit O4 expression by Schwann cells.

These observations beg the question: how closely does cAMP elevation mimic the axonal signal? Two axon-induced events have been mimicked by cAMP elevation in these experiments. Firstly the O4+ proliferating cell first seen at E16 in vivo can be produced in vitro by cAMP elevation in the presence of growth factors. Additionally, the quiescent, myelin-expressing cell first seen 5 days later during normal development of the rat sciatic nerve is produced in vitro by cAMP elevation. It is possible that this axonal signal is acquired the ability to elevate Schwann cell cAMP levels. Thus these Schwann cells in the developing nerve resemble Schwann cells in vitro treated with cAMP elevating agents in defined medium or in dense culture as division is prevented. One hypothesis is that the ojmininduced events decrease in a cAMP elevation when the myelin forming cells differentiate. Schwann cells in the developing nerve behave very like Schwann cells in vitro that are expressing O4 and they proliferate. Later, as intracellular cAMP is elevated, that is express O4 and they proliferate. Later, as intracellular cAMP is elevated, that is express O4 and they proliferate. Later, as intracellular cAMP is elevated, that is express O4 and they proliferate.

If axonal contact elevates Schwann cell intracellular cAMP, then the Schwann cells could determine whether this signal induces (1) division, partial differentiation, or myelin expression or (2) full myelin differentiation. The Schwann cell intracellular environment will determine how the cell responds to this cAMP elevation. If growth factors are present in addition to cAMP, the Schwann cells could determine whether this signal induces division, partial differentiation, or myelin expression or (2) full myelin differentiation.
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available, and no autocrine (or other) mechanisms are active to inhibit the proliferative response, then course (1) will be taken. However, a signal that prevents the Schwann cells from proliferating (either the autocrine growth inhibition described in my experiments, or a signal with similar effects from the axon) could modulate this and then course (2) would be chosen. Course (2) would also be chosen if growth factors were present in limited amounts during development (probably an unlikely event), or if the Schwann cells no longer responded to the presence of growth factors by increasing DNA synthesis.

These experiments support the hypothesis that axonal contact elevates cAMP levels in Schwann cells at all stages in development of the nerve. This cAMP elevation could induce the burst of proliferation seen between E15 and birth, the expression of O4 in all Schwann cells after B21 and then later it could be instrumental in inducing the myelin phenotype.

These experiments do not touch on the differentiation of the non-myelin forming Schwann cell. The differentiation of these cells offers a third option to course (1) and (2) described above. An examination of the conditions that allow induction of the non-myelin forming Schwann cells can help us to understand the results of experiments where Schwann cells are cultured in the presence of myelin inducing drugs. Non-myelin forming Schwann cells are in contact with the axons of several neurons and so each Schwann cell could well be exposed to a similar total surface area of axolemma to that contacted by myelin forming Schwann cells, even though the axons themselves are smaller. It might be supposed that these cells are receiving a substantial amount of axon-derived cAMP elevating signal, and indeed they do they express O4 and GAL-C, surface lipids that are induced by cAMP in culture. In spite of this, these cells do not make myelin (Aguayo et al. 1976a) they do not express either the proteins of myelin or their genes (Jessen et al. 1985, Brunden et al. 1990a, Morrison et al. 1991), neither do they proliferate (Asbury 1967, Terry et al. 1974). How do the cells maintain this phenotype? Evidently the proposal that during nerve development an axonal signal elevates Schwann cell cAMP and that this elevation will produce either a proliferating, O4+ cell similar to embryonic Schwann cells, or a mature myelin forming Schwann cell, depending on the Schwann cells
Three possibilities can be suggested that would explain the ability of small and large axons to induce non-myelin forming and myelin forming phenotypes includes:

1. Larger axons induce a bigger cAMP elevation in their associated Schwann cells, this overcomes growth factor mediated inhibition of myelin expression. In non-myelin forming Schwann cells the level of cAMP elevation is never high enough to overcome this inhibition.

2. Larger axons express factors that are absent in smaller axons and these act to enhance the response to elevated cAMP. The factor could down regulate Schwann cell growth factor receptors or act in the same way that calcium ionophore acted in my \textit{in vitro} experiments to enhance the differentiation in response to cAMP elevation.

3. Larger axons induce myelin because the larger diameter induces cell polarity, when a big difference between adaxonal side and abaxonal side develops this is a signal in itself. This polarity might act by preventing proliferation, or by allowing compaction in to myelin of the two surfaces of the Schwann cell where they meet at the mesaxon.

Several laboratories have tried to demonstrate a difference in the chemical nature of the signal given to Schwann cells by large and small axons, the results of these experiments, however, have not been conclusive. Schwann cells co-cultured with PC12 cells (Cochran 1985) do not synthesise myelin and when SCG neurons were cocultured with Schwann cells, no myelin was reported (Estridge and Bunge 1979, Johnson et al. 1980, Roufa et al. 1986). The Schwann cells in some cultures containing neurons from sympathetic ganglia express easily detectable amounts \( P_{\text{O}} \) mRNA, but the levels are lower than those induced by DRG derived neurons (Morrison et al. 1991), and SCG from 4 day old rat does contain \( P_{\text{O}} \) mRNA (Morrison et al. 1991). It has been suggested that this indicates that small axons are capable of inducing myelin gene expression (Morrison et al. 1991). It must be pointed out, however, that SCG neurons do have some post ganglionic axons that are myelinated (Kidd and Heath 1988a, 1988b, 1991) although the majority are unmyelinated (Voyvodic 1989), so the expression of myelin genes in SCG cultures does not necessarily indicate a myelin-inducing ability in the smaller axons. It has also been demonstrated that the amount of myelin that SCG neurons induce can be
3. cAMP on Schwann cells

altered by the amount of target tissue-derived growth factors they are exposed to at critical developmental times (Voyvodic 1989). The ability to induce myelin is secondary to alterations in neuronal size, and this depends on the availability of growth factors, thus it follows that the ability of SCG neurons to induce Schwann cell myelin is likely to depend on culture conditions that may, in different laboratories, be more or less able to maintain the neurons at the critical size needed to induce myelin. Indeed Brunden and coworkers, unlike Morrison above, found that SCG neurons in culture failed to induce \( P_0 \) mRNA, and that \( P_0 \) and MBP proteins were undetectable (Brunden et al. 1992). They concluded that smaller axons are incapable of triggering Schwann cells to produce myelin proteins and suggest that screening purified SCG and DRG neuron populations for molecules expressed predominantly in the DRG could produce candidate molecules for axonal regulation of myelin protein expression.

A model of how axons control Schwann cell phenotype can be proposed. All neuronal surfaces, whether growth cone, small immature axons small mature axons or large mature axons, may elevate Schwann cell cAMP levels. This cAMP elevation is part of the neuronal signal that at early stages in nerve development induces Schwann cell division, and O4 expression but later acts to induce both of the mature Schwann cell phenotypes. Before cAMP elevation can induce either mature Schwann cell phenotype the cells must drop out of division. In order for the cells to differentiate into myelin forming Schwann cells, they must, in addition, neutralize, or down regulate an inhibitory factor that prevents myelin gene activation in response to cAMP induced transcription factors. This factor might act in a similar way to the HLH-protein Id in proliferating myoblasts described above that prevents differentiation in response to MyoD related molecules.

The small, mature axons that induce expression of the non–myelin Schwann cell phenotype may also induce GAL–C and O4 on the Schwann cell surface, through a cAMP-dependent mechanism. These cells may, like the myelin forming cells need to drop out of division before they achieve the mature morphological interaction with their axons. Although induction of these cells requires elevated cAMP, upregulation of myelin gene expression and down regulation of the non–myelin specific genes does
not follow. If the myelin-suppressing factor proposed above is only removed by contact with large, myelin-inducing axons, these cells will continue to contain myelin-suppressing activity so they will not make myelin and will retain non-myelin forming Schwann cell-specific markers. A possible mechanism would be that mature non-myelin forming Schwann cells represent an intermediate stage between the O4+, dividing and P₀⁻ cells induced in the presence of growth factors and myelin forming Schwann cells, in that they maintain just enough growth factor receptors active to mediate the block on myelin genes, but not enough to induce division.

When my cultures are treated with cAMP elevating drugs in the presence of serum, P₀ mRNA is induced but the levels of P₀ protein remain low. This is not the first demonstration that the levels of P₀ can be controlled post-transcriptionally, downstream from controls on the level of P₀ mRNA. When DRG neurons and Schwann cells are co-cultured in defined medium, myelin formation is prevented, but normal axon-induced Schwann cell proliferation and the isolation of larger axons by Schwann cells occurs as normal. In these cultures, the mRNAs for P₀ and MBP are present in high levels, and immunoblot analysis showed clear bands for both proteins, in spite of the absence of myelin-formation in these cultures (Brunden et al. 1992). In apparently identical cultures, Morrison and coworkers could not detect P₀ protein using immunocytochemistry, however, in situ hybridization demonstrated P₀ mRNA was present in the majority of the cells (Morrison et al. 1991). In these cultures the major myelin proteins are synthesised in response to axonal signals as normal, but in these special culture conditions, where the inability to synthesise a basal lamina prevents assembly of the myelin sheath, the synthesised myelin proteins are not concentrated into myelin lamellae. When Schwann cells are grown in the absence of axons they redirect newly synthesised P₀ protein to the lysosomes (Brunden and Poduslo 1987a). This pathway may also be used when Schwann cells are prevented from forming a myelin sheath, since if the myelin proteins can not inserted into myelin, they may be re-routed into lysosomes, and this may be the reason that the P₀ can not be detected by immunocytochemistry.

In my experiments, however, the levels of P₀ protein induced by cAMP elevation in the presence of serum as detected by immunocytochemistry and
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immunoblot is greatly reduced. This indicates that in neuron–free cultures the difference between immunocytochemically detectable P₀ protein and mRNA levels can not be explained in terms of different processing of newly synthesised protein. A possible explanation is that the rate of P₀ mRNA translation rather than transcription is altered when growth factors are present, so that although the level of P₀ mRNA is high, this is not being translated into protein.

The amount of myelin gene and protein induced when Schwann cells are treated with cAMP elevating agents in the presence of serum varies in different laboratories. At one extreme, Kreider and coworkers (1988) and Shuman and coworkers (1988) saw no increase in P₀ mRNA or protein when Schwann cells were treated with cAMP analogues in serum using Western blot, Northern blot and immunocytochemistry. Both groups published their plating densities and they are both using densities above that necessary for contact mediated inhibition of Schwann cell division in response to cAMP and serum, so it is not possible to explain the lack of differentiation in their cultures in terms of a strong proliferation response preventing differentiation. One explanation for the absence of differentiation in these experiments could be reduced detection of P₀ due to use of a different antibody. When the monoclonal antibody P₀4 is used to label sections of nerve or freshly dissociated myelin forming Schwann cells, the pattern and intensity of labelling is indistinguishable from that produced with polyclonal antibodies, during development, however the cells become labelled with polyclonal antibodies about one day before they become labelled with the P₀ 4 antibody. The induction of P₀ by cAMP elevating agents is, however, hard to detect with this antibody. If the polyclonal antibody used by Kreider and Shuman and their coworkers (who work in the same laboratory and so are likely to use the same antibody) only recognises the epitope that is detected by P₀4, an induction in P₀ could be missed. This does not explain the lack of induction in P₀ mRNA levels seen by Kreider and coworkers. As they are unusual in using an oligonucleotide probe, rather than cDNA for detecting P₀ mRNA in Northern blots, it is possible that in Northern blots this method may be less sensitive. They do not show the levels of P₀ in whole sciatic nerve, however, so it is not possible to determine the sensitivity of their detection system and if the detection method was less sensitive
then the cAMP-induced increase might be missed. The inability to detect MBP mRNA is less unexpected as MBP induction requires a greater cAMP elevation stimulus than induction of P₀ mRNA (Lemke and Chao 1988).

In most laboratories, however, it has generally been found that cAMP analogues induce expression of myelin proteins and their genes. P₀ protein and mRNA (Lemke and Chao, 1988), MBP mRNA (Lemke and Chao 1988), P₂ mRNA (Monuki et al. 1989), and PMP–22 mRNA and protein (Spreyer et al. 1991, Pareek et al. 1992), are all induced when cAMP levels are elevated. In all the above experiments, this elevation has been detected in the presence of serum, conditions where a substantial reduction in the amount of P₀ protein induced was seen in my cultures. Whilst none of these reports indicate either the cell density or the rate of DNA synthesis induced by cAMP, and as Schwann cell proliferation was inhibited and P₀ protein strongly induced in my cultures at plating densities of 2.6x10⁴ cells/cm² and above, it is likely that only a subpopulation of the Schwann cells were dividing. The induction of the myelin phenotype is likely to be in a population of cells within each culture where division has ceased.

The down regulation of non myelin specific markers that accompanies myelin upregulation in my experiments confirms the results of Mokuno and coworkers who have demonstrated loss of NGF–receptors and NGF–R mRNA from Schwann cells when cAMP levels are elevated for prolonged times in culture (Mokuno et al. 1988). The reduction was only detectable after 4 days of continuous exposure to 10⁻³M cAMP analogues, and after adding forskolin for 36h, Lemke and Chao (1988) were only able to detect a minimal reduction in the levels of NGF–R mRNA. The incomplete differentiation in the cultures of Lemke and Chao and the delayed downregulation in the cultures of Mokuno and coworkers could be due to serum-mediated inhibition of Schwann cell differentiation that is only overcome when, after a few cycles of division, proliferation is inhibited by contact inhibition.

One other Schwann cell gene that is down regulated by cAMP elevation is the proto–oncogene c–jun. Within 30 minutes of adding forskolin there is a dramatic reduction in c–jun mRNA levels (Monuki et al. 1989). Like the NGF–R, c–jun mRNA and protein expression is low in intact nerves, elevated during Wallerian
3. cAMP on Schwann cells
degeneration, and returns to low levels after recovery from a crush injury of the nerve
(Shy et al. 1992). It would seem unlikely that c–jun downregulation depends on
reduced division as the effect is immediate and dramatic (Monuki et al. 1989).

In summary, both the axonal signal that induces Schwann cell proliferation
and O4 expression prenatally, and the signal that induces myelin gene expression, first
seen at birth, can be mimicked by cAMP elevation. The Schwann cell response to
cAMP elevation in vitro can be altered by adding or withholding growth factors.

The Northern blots were done in collaboration with R. Mirsky. The Western blot
shown in Figure 3.10 was done in collaboration with I. Ahmed.
Some of the results in Chapter 3 have been published, see Mirsky et al. 1990, Morgan
et al. 1991.
### Table 3.3

**THE INVERSE RELATIONSHIP BETWEEN AMP MEDIATED P_o INDUCTION AND DNA SYNTHESIS**

<table>
<thead>
<tr>
<th></th>
<th>cAMP analogues</th>
<th>Forskolin 2-4μM</th>
<th>CTx</th>
<th>IBMX 100μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_o % BrdU%</td>
<td>P_o % BrdU%</td>
<td>P_o % BrdU%</td>
<td>P_o % BrdU%</td>
</tr>
<tr>
<td>Defined medium</td>
<td>51 ± 5.7</td>
<td>37 ± 10.7</td>
<td>62 ± 6.5</td>
<td>9 ± 3.5</td>
</tr>
<tr>
<td>Calf serum (10%)</td>
<td>1 ± 2.1</td>
<td>0 ± 0</td>
<td>0 ± 0.1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>GGF 1.8ng.ml⁻¹</td>
<td>15 ± 4.0</td>
<td>5 ± 4.0</td>
<td>4 ± 1.5</td>
<td>-</td>
</tr>
<tr>
<td>bFGF 10ng.ml⁻¹</td>
<td>-</td>
<td>1 ± 1.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>aFGF 10ng.ml⁻¹</td>
<td>-</td>
<td>2 (n = 2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGF 5ng.ml⁻¹</td>
<td>-</td>
<td>10 (n = 2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TGFβ 1ng.ml⁻¹</td>
<td>-</td>
<td>7 (n = 1)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Numbers given are % of total Schwann cells ± S.E.M. n > 2, except where indicated.
**TABLE 3.4**

RELATIONSHIP BETWEEN O4 EXPRESSION AND DNA SYNTHESIS

<table>
<thead>
<tr>
<th>cAMP analogues</th>
<th>Forskolin 2-4μM</th>
<th>CTx</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>O4% BrdU%</td>
<td>O4% BrdU%</td>
<td>O4% BrdU%</td>
<td>O4% BrdU%</td>
</tr>
</tbody>
</table>

| defined medium | 76 ± 9.4 (9) | 59 ± 7.2 (9) | 77 ± 3.9 (6) | 4 ± 2.0 (4) |
| 10% serum      | 2 ± 1.0 (6)  | 4 ± 2.5 (4)  | 3 ± 2.8 (4)  | 1 ± 0.9 (6) |
| GGF            | 14 ± 96.6 (5) | 0 ± 0.3 (3)  | 9 ± 6.6 (3)  | 1 ± 1.0 (3) |
|                | 86 ± 4.4 (5)  | 90 ± 3.5 (3) | 86 ± 3.4 (3) | 50 ± 12.0 (3) |

Numbers given are % of total Schwann cells ± S.E.M. (n).
Schwann cells were dissociated from P6 rat sciatic nerve and purified as described in Methods. After 4d the cells were replated onto laminin coated glass coverslips and 24h later the cells were treated as described below:

a, b, defined medium 72h.
c, d, defined medium with cAMP analogues 72h.
e, f, 10% calf serum 72h.
g, h, 10% calf serum with cAMP analogues 72h.

Phase and fluorescein optics.

cAMP analogues were added as described in Methods. At the end of the experiment the cells were fixed and labelled with polyclonal anti \( P_0 \).

The photographs and prints of fluorescently labelled cells were made under identical conditions. The time taken for an automatic camera to photograph the image in 3.1d was measured, all the other photographs of fluorescent images were taken on manual settings to ensure all the exposures were the same. Similarly, the prints of the negatives were all exposed for the same time taken to produce the image in 3.1d.

Bar = 20\( \mu \).
Figure 3.2

P₀ immunoblot.

Lanes
SN:  25μg protein extracted from whole sciatic nerve from a P5 rat.
1:  31μg protein extracted from cAMP treated Schwann cells.
2:  31μg protein extracted from CTx treated Schwann cells.
3:  31μg protein extracted from forskolin treated Schwann cells.
4:  31μg protein extracted from control Schwann cells.

Cultured cells were treated for 72h in defined medium as described above. The bound P₀ antibody was detected using ¹²⁵I labelled donkey anti rabbit Ig as described in Methods. In the sciatic nerve extract a smaller breakdown product of P₀ is also visible.
Figure 3.3

P₀ mRNA, 48h after drug treatment.

a, b: Control culture.
c, d: CTx treated culture.
e, f: Forskolin treated culture.
g, h: cAMP analogue treated culture.

Bright field and dark field images. Toluidine blue stained cells. Schwann cells were treated with cAMP elevating drugs in defined medium as indicated. After 48h the cells were fixed in paraformaldehyde and hybridized with ³⁵S labelled oligonucleotide as described in Methods.

Each field was chosen to contain a fibroblast (indicated with arrows and arrowheads). None of the fibroblasts and few of the untreated Schwann cells contain detectable levels of P₀ mRNA. Treatment with cAMP elevating drugs produces a large increase in the levels of P₀ mRNA. When cultures were left longer before developing the emulsion, basal levels of P₀ mRNA were detected in untreated Schwann cells, but not in fibroblasts.

Bar = 20µ.
Figure 3.4

Time course of $P_0$ mRNA induction after treatment of Schwann cells with cAMP analogues.

a, b: Untreated Schwann cells.
c, d: Schwann cells treated with cAMP analogues for 24h.
e, f: Schwann cells treated with cAMP analogues for 36h.

Bright field and dark field images. Toluidine blue stained cells.

Schwann cells were treated with cAMP analogues in defined medium for the times indicated. The cells were then fixed in paraformaldehyde and hybridized with $^{35}$S labelled oligonucleotide as described in Methods.

The levels of $P_0$ mRNA are greatly induced within 24h of cAMP elevation, and virtually all of the cells are labelled after 36h. Compare with Table 3.2 where no $P_0$ protein is detectable by immunolabelling with $P_0$ polyclonal antibodies at 24h, and only 27% of the cells are $P_0^+$ after 48h.

Bar = 50μ.
Figure 3.5
Northern Blot.

Total mRNA probed with cDNA complementary to $P_0$ mRNA.

Lanes:
1: 3μg mRNA molecular weight markers at 9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kb.
   This lane is not present in (a).
2: 10μg mRNA from 3T3 cells.
3: 10μg mRNA from Schwann cells in defined medium, 48h.
4: 10μg mRNA from Schwann cells treated with 4μM forskolin
   in defined medium, 48h.
5: 10μg mRNA from Schwann cells in 10% calf serum, 48h.
6: 10μg mRNA from Schwann cells treated with 4μM forskolin
   in 10% calf serum, 48h.
7: 2μg mRNA extracted from P8 rat sciatic nerve.

(a): Hybridized $P_0$ cDNA.
(b): Photograph of ethidium bromide bound to RNA within the agarose gel before
transfer to the nylon membrane. Excited with u.v. light. Bands are seen from
the molecular weight markers and the ribosomal RNA (18S and 28S) extracted from
the cells. This image indicates firstly, that there is little mRNA degradation, and
secondly, confirms that the same amount of RNA was added to each lane in lanes 2–6
and less in lane 7. Only lane 4 shows some degradation, indicating that the levels of
$P_0$ mRNA induced in the forskolin treated cells in defined medium may be even
greater than indicated by the band seen in lane 4 in (a).
Figure 3.6

GAL−C and O4 induction in Schwann cells by cAMP elevating drugs.

a: \( P_0 \)
b: phase
c: GAL−C

a, b and c show the same field of cells viewed with fluorescein, phase and rhodamine optics.

d: \( P_0 \)
e: phase
f: O4
d, e and f show the same field of cells viewed with fluorescein, phase and rhodamine optics.

Schwann cells treated with cAMP analogues for 72h in defined medium and immunolabelled as described in Methods. Untreated cultures contained no GAL−C\(^+\) cells, and <1% O4\(^+\) cells.

Bar = 20\( \mu \).
Figure 3.7
Schwann cells 72h after removal of cAMP analogues.

a, b: Phase and P_0 (fluorescein) optics. P_0 antibodies bind to 3 of the cells shown in this field 72h after cAMP analogues were removed. These cells have retained the flattened morphology associated with Schwann cells induced to differentiate by cAMP elevation in culture. These cells are 5% of the total Schwann cells in this culture. The other cells in the culture, 70% of which were flattened, P_0^+, cells after 72h cAMP analogue treatment, have lost P_0 immunoreactivity and returned to the classic bipolar morphology generally maintained by untreated Schwann cells in culture.

Bar = 20 μ.

c-f: In situ hybridization with oligonucleotide complementary to P_0 mRNA. c, d: Cells treated with cAMP elevating agent for 72h.

e, f: Cells treated with cAMP analogues for 72h, then maintained in culture for a further 72h without drugs. Bright field and dark field optics.

Bar = 100 μ.

Most of the cells are induced to express P_0 mRNA in response to cAMP elevation, the majority of these cells do not maintain high levels of P_0 mRNA after the drugs are removed.

cAMP analogues were applied as described in Methods.
Figure 3.8
Calcium Ionophore A21387 enhances $P_Q$ mRNA elevation in response to cAMP analogues.

a, b: A21387 alone.
c, d: untreated Schwann cells.
e, f: cAMP analogues alone.
g, h: cAMP analogues with A21387.

Phase and dark field optics. Bar = 100μ.

Schwann cells were treated for 48h as indicated above in defined medium. cAMP analogues were added as described in Methods, A21387 was used at a concentration of $5 \times 10^{-8}$M. At the end of the 48h incubation, the cells were fixed with paraformaldehyde and hybridized with oligonucleotide complementary to part of the $P_Q$ mRNA molecule as described in Methods.

A21387 does not elevate $P_Q$ mRNA levels alone (compare b and d), but does enhance $P_Q$ mRNA elevation in response to cAMP analogues (compare f and h).
Figure 3.9

Cycloheximide blocks cAMP analogue-mediated induction of $P_0$ mRNA.

a, b: Untreated Schwann cells in defined medium.
c, d: Schwann cells treated with cAMP analogues in defined medium, in the presence of $25\mu g.ml^{-1}$ cycloheximide.
e, f: Schwann cells treated with cAMP analogues in defined medium alone.

Schwann cells were treated as indicated above for 24h. cAMP analogues 8b–cAMP and db–cAMP were used together, each at a concentration of $5\times10^{-4}M$. After 24h, the cells were fixed in paraformaldehyde and hybridized with oligonucleotide complementary to part of the $P_0$ mRNA molecule as described in Methods.

Bar = 100$\mu$. 
Figure 3.10
Western blot comparing the induction of $P_q$ protein in the presence and absence of serum.

Lanes:

CS + cAMP: 50$\mu$g protein extracted from Schwann cells cultured in defined medium with 10% calf serum in the presence of cAMP analogues for 72h.

CS: 50$\mu$g protein extracted from Schwann cells cultured in defined medium with 10% calf serum for 72h.

SF: 50$\mu$g protein extracted from Schwann cells cultured in defined medium alone for 72h.

SF + SF: 50$\mu$g protein extracted from Schwann cells cultured in defined medium in the presence of cAMP analogues for 72h.

SN: 50$\mu$g protein extracted from adult rat sciatic nerve.

cAMP analogues 8b-cAMP and db-cAMP were added together, each at a concentration of 5x10$^{-4}$M for the first 24h, then at a reduced concentration of 5x10$^{-5}$M for the final 48h. Care was taken to plate the cells to be treated with calf serum and cAMP analogues at a density where the cells were still proliferating, and not contact inhibited, at the end of the incubation.
The inverse relationship between $P_o$ induction and increase in calf serum. This graph shows that $P_o$ is suppressed in the presence of increasing concentrations of calf serum. Schwann cells were cultured in the presence of cAMP analogues for up to 4d and the percentage of $P_o$ positive Schwann cells at each time point assessed by counting a minimum of 200 cells from each of duplicate coverslips. The results from a single representative experiment are shown.
Figure 3.12

O4 and P₀ induction in Schwann cells treated with cAMP elevating drugs in the presence of serum.

a: O4 (rhodamine optics).
b: phase.
c: BrdU (fluorescein optics).
The 3 cells indicated by arrows are synthesising DNA, and all 3 also express O4.

d: P₀ (rhodamine optics).
e: phase.
f: BrdU (fluorescein optics).

Schwann cells were plated at densities where some contact inhibition will occur. In these cultures, whilst a few of the cells differentiate, many cells are synthesising DNA.

Bar = 20μ.

The 3 cells indicated in (d) and (e) are expressing P₀, none of these cells are synthesising DNA (f).
Down regulation of non-myelin forming Schwann cell markers in Schwann cells induced to express P_0 by treatment with cAMP analogues.

a, P_0, polyclonal antibody (fluorescein optics).
b, NGF-R (rhodamine optics).
c, phase.
d, P_0, polyclonal antibody (fluorescein optics).
e, A5E3 (rhodamine optics).
f, phase.
g, P_0, monoclonal antibody (fluorescein optics).
h, N-CAM (rhodamine optics).
i, phase.
j, P_0, monoclonal antibody (fluorescein optics).
k, GFAP (rhodamine optics).
l, phase.
m, P_0, polyclonal antibody (fluorescein optics).
n, LI (rhodamine optics).
o, phase.
p, NGF-R (rhodamine optics).
q, GFAP (fluorescein optics).
r, phase.

Bar = 20μ.

Schwann cells from P5 rats were treated with cAMP analogues for 3d in 10% calf serum. The density was adjusted to produce reasonable numbers of induced and uninduced cells in the same culture. The P_0^+ cells have reduced expression of all the non-myelin forming Schwann cell related markers NGF-R, A5E3, N-CAM, GFAP and LI. In (p)–(r), cells are labelled with NGF-R and GFAP to demonstrate that in individual cells, all the non-myelin forming Schwann cell markers are downregulated together.
CHAPTER 4
GENERAL DISCUSSION

In the first part of this thesis, I have described the changes seen in the phenotype of cells dissociated from rat sciatic nerve at ages between E15 and birth. These cells undergo a profound change over this time, at E15 the cells assume a flat morphology in culture and tend to adhere to each other, whilst by birth the cells are bipolar and show no tendency to stay in groups. The embryonic cells depend on factors contained in the developing limb for survival and most die in the first 24h of culture without medium conditioned by neurons, whereas neonatal cells survive in ordinary culture medium without any additions. Over the 7d examined, the cells begin to express 2 of the markers associated with all Schwann cells in mature nerves, O4 antigen and S100. The cells present in the nerves of younger rats are so different that I have described them as precursors of Schwann cells, rather than Schwann cells. By E18, however, the cells express a phenotype similar enough to cultured Schwann cells to be called Schwann cells, even though most of them will not achieve their mature phenotype for some time in vivo. The development of the myelin phenotype from this early Schwann cell is described. A scheme is proposed that describes the phenotypic changes undergone by Schwann cells as they develop from a precursor cell into a cell that is recognisably a Schwann cell and this cell then matures into the myelin forming and non–myelin forming Schwann cells present in adult nerve.

The amount that this precursor Schwann cell has changed from the neural crest cell that it develops from is hard to determine due to the small amount of published data on rat neural crest cell phenotype and survival requirements. It would be interesting to repeat some of these experiments using neural crest cells to determine the extent of this difference. If the cells are very similar to neural crest cells, then it would be interesting to determine whether they are committed to becoming Schwann cells. For example, it would be interesting to see if they could be made to differentiate into another neural crest–derived cell (neurons or melanocytes) by varying the culture conditions.

The dependence of Schwann cells on axons for expression of the mature non–
4. General discussion

myelin forming and myelin forming phenotypes is discussed. When myelin forming Schwann cells are taken into culture they can not maintain their mature phenotype, and the upregulation of non-myelin forming markers is seen within hours, long before the disrupted myelin has been broken down. The mechanism driving this elevation is unknown, however this change in protein expression does depend on new protein synthesis. These proteins could be continually suppressed by mature myelin forming Schwann cells, and this suppression is removed or blocked by myelin disruption. As discussed in Chapter 1, the changes in Schwann cell phenotype after axon loss provide an environment that is favourable for axonal regrowth, and that promotes neuronal survival. Clearly, the faster these changes occur, the more complete recovery will be as fewer neurons are likely to die if survival factors reach them soon after injury, and a quick return of regrowing axons to their target tissue is likely to result in faster repair. The proteins measured, GFAP and A5E3 are not known to be involved in this repair, but their fast upregulation parallels that of N-CAM (Jessen et al. 1987a), a protein known to promote neurite outgrowth. The A5E3 antigen is unknown and a cytoskeletal protein like GFAP is unlikely to be involved in neurite adhesion, or secretion of neurotrophic factors. The general change in phenotype is likely to be reflected by these two proteins, and they help to demonstrate the speed with which the Schwann cell phenotype can change.

In the second section of this work, the effects of cAMP elevation on Schwann cell phenotype are examined. The response of Schwann cells to elevated cAMP levels depends on whether growth factors are present, and if cell proliferation is possible. When growth factors are present, the cells will proliferate in response to cAMP elevation. In the absence of growth factors, or in the presence of growth factors when proliferation is prevented by contact inhibition, cAMP elevation produces cells that express the myelin phenotype and do not proliferate. Schwann cells proliferating in response to growth factors and cAMP elevation do not express the myelin phenotype, however some of them are induced to express the phenotype of cells in the developing nerve and expresss the O4 antigen.

Future work will examine the intracellular responses of Schwann cells to cAMP elevation in different culture conditions to determine how differentiation is
4. General discussion

suppressed by growth factors. At what level in the response is there a difference; are the changes in immediate early genes different when cAMP is elevated in Schwann cells in defined medium or in the presence of growth factors or serum? Do the cells in serum treated with cAMP elevating agents respond with a different set of immediate early genes differently when they are in dense cultures from when they are sparsely plated?

The surface mitogen on axons NDGF, isolated by Nordlund and coworkers (Nordlund et al. 1992) is likely to be described in greater detail soon. If this factor mediates a part of axonal message to Schwann cells then does it elevate Schwann cell cAMP levels? It will be exciting to see to what extent the experiments described here can be repeated using NDGF instead of cAMP elevation. Another question that might be answered by NDGF is to what extent the small and large axons differ, in other words, do all axons express the same Schwann cell mitogens, and if they do, are the levels of expression different?

One other line of investigation that will be followed in future research will be to re-examine the effects of TGFβ on Schwann cells. The combination of TGFβ and FGF is mitogenic for Schwann cells (Schubert 1992), TGFβ acts as a Schwann cell comitogen mimicking the action of cAMP elevation. Other effects of TGFβ are to downregulate NGF-receptor expression by Schwann cells in culture (Chandross et al. 1992) and to enhance matrix formation (reviewed by Massagué 1990, Lin and Lodish 1993), both of these effects can also be achieved by cAMP elevation (Baron-Van Evercooren et al. 1986, Mokuno et al. 1988). There are many molecules that act as TGFβ receptors, recently reviewed by Lin and Lodish (1993), and one of these receptors is a serine/threonine kinase. The intracellular substrates for serine/threonine kinase receptors are not known, but activation of these receptors does not elevate intracellular cAMP levels. No effect of TGFβ on Schwann cell P0 expression has been described, and it is unlikely that TGFβ can mimic this action of cAMP (pilot studies in this laboratory have failed to detect an effect of TGFβ on either P0 mRNA or protein levels). It is possible that some of the intracellular changes seen in response to TGFβ are the same as those produced by cAMP elevation, but insufficient alone to produce differentiation. If TGFβ were used in combination with other
4. **General discussion**

agents, a combination might be found that does reproduce all of the changes produced by cAMP elevation that are necessary for Schwann cell differentiation. It will be exciting to examine TGFβ with other drugs to see if in combination with other factors Schwann cell differentiation can be induced. Calcium ionophore and NDGF would be obvious candidates.


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