

GROWTH FACTORS AND GROWTH ASSOCIATED PROTEIN  
(GAP-43) IN THE PERIPHERAL NERVOUS SYSTEM.

BY

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## ABSTRACT

Growth factors and growth associated protein (GAP-43) in the peripheral nervous system – H.J.S.Stewart

The factors required to stimulate short-term Schwann cell DNA synthesis in serum-free medium (containing insulin) are thoroughly analysed and compared with the growth factor requirements of long-term "autocrine" Schwann cells. Evidence is presented to demonstrate the importance of cAMP in Schwann cell DNA synthesis. Measurements of intracellular cAMP levels by radioimmunoassay in both Schwann cell types is related to the differing mitogen requirements of these cells.

Further tissue culture studies show that insulin-like growth factor-I (IGF-I) plays a role in Schwann cell DNA synthesis, and that cultured Schwann cells are IGF immunoreactive. Binding studies and Scatchard analysis using  $^{125}\text{I}$ -IGF-I provide evidence for a type 1 IGF receptor on cultured Schwann cells. An immunohistochemical analysis on the distribution of IGF *in vivo* in the rat sciatic nerve is reported. Dried cell preparations and 2hr cultures are used to document the presence of IGF in the Schwann cells from embryonic to adult rats. Teased nerve preparations from postnatal rats are used to study the distribution of IGF in neurones. Results obtained suggest that IGF-I may be acting as an autocrine/paracrine Schwann cell mitogen *in vivo*

An immunohistochemical investigation reveals that the growth associated protein GAP-43, hitherto considered to be associated with neurones and certain CNS glia, is present in non-myelin-forming Schwann cells. Nerve sections and dried cell preparations are used to document the developmental distribution of GAP-43 in Schwann cells *in vivo*. Further analyses on the developmental regulation of GAP-43 expression in Schwann cells has been carried out in tissue culture. Gel electrophoresis and western blotting techniques are used to confirm the identity of Schwann cell GAP-43.



Evidence that GAP-43 is widely distributed in the neurones of the adult peripheral nervous system (PNS) is provided. Immunohistochemical studies and western blotting demonstrate that GAP-43 is present in high amounts in all three sub divisions of the autonomic nervous system (ANS) of the adult rat. Taken together with known distribution of GAP-43 in areas of the CNS associated with plasticity, these findings suggest a role for GAP-43 in the plasticity of the ANS.

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## ABBREVIATIONS

BDNF – Brain derived neurotrophic factor  
CS – Calf serum  
DBcAMP – N<sup>6</sup>, 2'-O-dibutyryl adenosine 3':5' cyclic monophosphate  
DME – Dulbecco's modified eagles medium  
DRG – Dorsal root ganglion  
EGF – Epidermal growth factor  
FGF – Fibroblast growth factor  
Gal-C – Galactocerebroside  
GAP – Growth associated protein  
GFAP – Glial fibrillary acidic protein  
GGF – Glial growth factor  
IGF – Insulin-like growth factor  
IL1 – Interleukin 1  
IL2 – Interleukin 2  
MEMH – Minimal essential eagles medium  
M-F Schwann cell – Myelin-forming Schwann cell  
N-CAM – Neuronal cell adhesion molecule  
N-M-F Schwann cell – Non-myelin-forming Schwann cell  
NGF – Nerve growth factor  
PBS – Phosphate buffered saline  
PDGF – Platelet derived growth factor  
PGP 9.5 – Protein gene product 9.5kDa  
PLL – Poly-L-lysine  
PMSF – Phenylmethylsulphonylfluoride  
SCG – Superior cervical ganglia  
SDS – Sodium dodecyl sulphate  
TGF – Transforming growth factor  
VIP – Vasointestinal peptide



## CHAPTER 1

### GENERAL INTRODUCTION

## GROWTH FACTORS AND GROWTH ASSOCIATED PROTEIN (GAP-43) IN THE PERIPHERAL NERVOUS SYSTEM.

Development of the peripheral nervous system proceeds as complex series of well defined ontogenic processes. These processes include events such as cell migration, proliferation, differentiation, axonal elongation and synapse formation. In the past decade considerable advances have been made in determining the factors involved in the coordination, control and successful outcome of these events. These include not only agents that act on the outside of the cell, such as growth factors, but also molecules confined to the cell, such as receptors. Many of these factors are also important in regeneration, a process that is in many ways similar to development. The aims of this study have been :-

- a) To identify the growth factors involved in the control of Schwann cell mitosis *in vitro* and to demonstrate their prevalence *in vivo* at times of Schwann cell proliferation.
- b) To study the distribution of the growth associated protein GAP-43 in Schwann cells throughout development and in the mature peripheral nervous system.

To study the actions of a growth factor, or indeed to understand the function of a molecule, the biological context in which it acts must be considered. Thus detailed below are the aspects of PNS biology considered pertinent to this study.

### Early nerve development

The neurones and Schwann cells of the peripheral nervous system arise from the neural crest. Throughout development these neurones express high levels of GAP-43, a trait that has been exploited, using immunocytochemical techniques, to study the timing and pattern of innervation of the developing PNS (Reynolds et al., 1991; Fitzgerald et al., 1991). Cells from the neural crest migrate out to form the ganglia of the PNS from which emanate the neurones and accompanying Schwann cells (Le Dourain and Smith, 1988). Schwann cells are seen accompanying groups of axons from the earliest time that they project

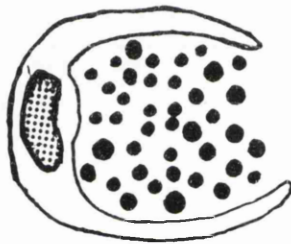
towards their targets. Even though single axons may sometimes be seen projecting towards their target without accompanying Schwann cells, in nerve trunks containing large numbers of axons there is always a close association between axons and supporting cells (eg Mirsky and Jessen, 1990). During early nerve development, at about embryonic days 14–15 in the rat, many precursor Schwann cells are seen lying at the edges of axon bundles, although some have already penetrated the nerve. At this stage these cells lack a basement membrane. Continuing proliferation increases Schwann cell numbers resulting in division of the nerve into separate axon–Schwann cell units or "families" (Fig 1.0). These families consist of a large number of axons communally enveloped by one or two Schwann cells (Gamble and Breathnach, 1965; Ziskind–Conhaim, 1988; Webster and Favilla, 1984). The ratio of axons to Schwann cells falls because of neuronal death and continuing Schwann cell proliferation. At this stage Schwann cells start to make the basement membrane (Webster and Favilla, 1984). Basement membrane formation is promoted by axonal contact (Bunge et al., 1982) and is an obligatory event in Schwann cell development. Experimental disruption of this process will prevent further Schwann cell maturation, particularly myelination (Bunge et al., 1986; Eldridge et al., 1987). By embryo day 17–18 the first premyelinated axons are isolated from the rest of the bundle by a spiral of Schwann cell cytoplasm (Ziskind–Conhaim, 1988). As development proceeds further the Schwann cell will eventually establish either one of two mature axon–Schwann cell relationships. The Schwann cell will either ensheath many small diameter axons to form a unmyelinated fibre, or when the axon diameter exceeds a critical value (Friede, 1972) the Schwann cell will wrap concentrically around it to form the myelin sheath (Fig 1.0). Myelination starts at around birth and is largely completed by the third postnatal week (Ziskind–Conhaim, 1988). The first mature unmyelinated fibres are formed about 10–14 days after birth, their development being completed 2–3 weeks later (Ziskind–Conhaim, 1988).

#### Schwann cell function and phenotype.

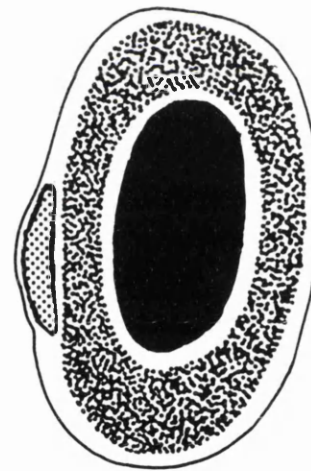
The Schwann cell fulfils several important functions *in vivo* such as provision of the myelin sheath, regulation of the extracellular environment and provision of adhesion molecules and trophic factors during nerve

FIGURE 1.0. Schematic diagram of the morphological differentiation of mature myelin-forming and non-myelin-forming Schwann cells from a precursor Schwann cell. In early development, Schwann cell precursors enclose large groups of axons. As development proceeds the Schwann cells proliferate and gradually segregate groups of axons until they finally assume the relationship with axons seen in mature fibres, a one-to-one relationship in the case of myelinated fibres, and several axons in the case of unmyelinated fibres. Reproduced with the kind permission of R.Mirsky and K.R.Jessen (Mirsky and Jessen, 1990).

NONMYELIN-FORMING  
SCHWANN CELL



PRECURSOR



MYELIN-FORMING  
SCHWANN CELL

development, maintenance and regeneration (Mirsky and Jessen, 1990). The function of the myelin sheath is to facilitate impulse conduction in large diameter fibres. In the mature nerve the two Schwann cell variants can be differentiated not only by their relationships with axons but also by their molecular phenotype. Phenotypic changes occur as these two Schwann cell types develop from early Schwann cells and their precursors (Fig 1.1) (Mirsky and Jessen, 1990).

The two distinct molecular phenotypes of the mature Schwann cells could, in theory, develop by induction of a specific set of antigens in both cell types, or by a mixture of induction and suppression in one cell type only. Current opinion suggests that the latter alternative is the more likely. Many of the developmental changes in Schwann cell phenotype, particularly in the myelin forming pathway, involve axonal signals (Jessen et al., 1987; Lemke and Chao, 1988; Mirsky and Jessen, 1990). These changes can, in many instances, be mimicked in tissue culture by agents known to elevate intracellular cAMP (Sobue and Pleasure, 1986; Morgan et al., 1991). The induction of the myelin protein P<sub>0</sub> occurs in response to cAMP when DNA synthesis is inhibited. Other characteristic changes which happen in myelin-forming Schwann cells such as down-regulation of expression of N-CAM, A5E3, NGF receptor and GFAP are also seen in response to cAMP elevation, particularly in those cells in which P<sub>0</sub> induction is strongest. The induction of the antigen 04 is not however affected by the proliferative state of the Schwann cell (Morgan et al., 1991).

When isolated Schwann cells are placed in tissue culture considerable phenotypic changes occur (Table 1.0). In myelin-forming cells, the myelin proteins are down regulated and N-CAM, GFAP, A5E3 and NGF receptors are all re-expressed (Jessen et al., 1990). Similarly, both myelin-forming and non-myelin-forming cells lose surface expression of 04 and galactocerebroside (Gal-C) (Mirsky et al., 1990). These phenotypic changes also occur in Schwann cells deprived of axonal contact as a consequence of nerve injury.

FIGURE 1.1. Development of the molecular phenotype of myelin-forming and non-myelin-forming Schwann cells from precursor cells in the rat sciatic nerve. Sch, Schwann cell; N-M Sch in stipple frame, cells with the molecular phenotype similar to mature non-myelin-forming cells, that will progress to myelination; M Sch, Myelin-forming Schwann cell; Gal-C, galactocerebroside. Arrow indicates molecules that are down-regulated during differentiation of the Schwann cell along the myelin pathway. All other Schwann cell molecules or properties are first seen at the time points indicate. Reproduced with the kind permission of R.Mirsky and K.R.Jessen (Jessen and Mirsky, 1991).

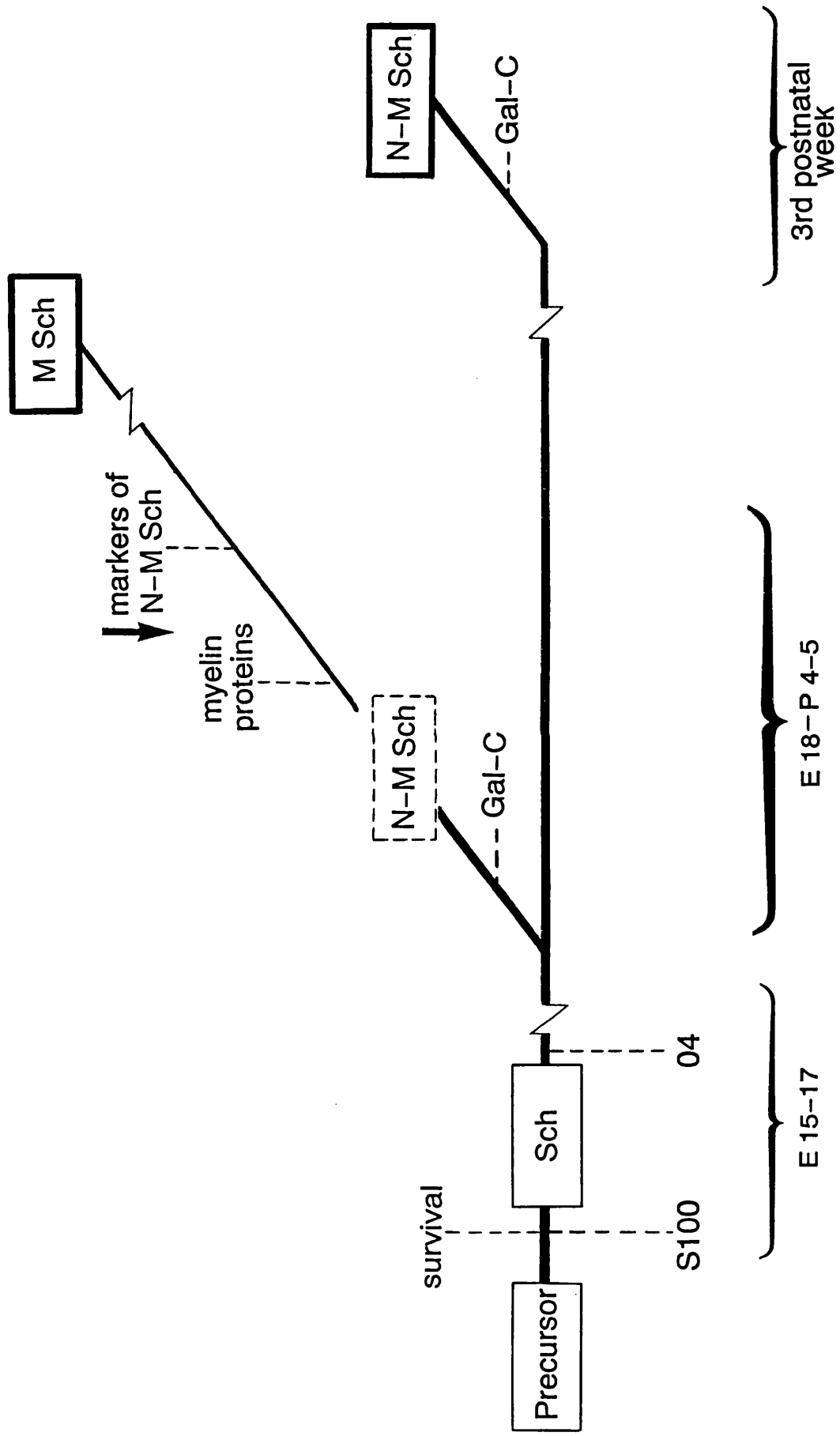




TABLE 1.0. The molecular phenotype of Schwann cells. Antigenic markers of non-myelin-forming, myelin-forming, short-term cultured and precursor Schwann cells are listed opposite. (Mirsky and Jessen, 1990). |

Table 1.0  
The Molecular Phenotype of Schwann cells.

ANTIGEN	Short-term			
	M-F Sch cells	N-M-F Sch cells	Cultured cells	Precursors
CNP'ase	+	n.d.	+	n.d.
P <sub>O</sub>	+	-	-	-
MBP	+	-	-	-
MAG	+	-	-	-
P <sub>170k</sub>	+	n.d.	-	n.d.
PLP	+	n.d.	+	n.d.
P <sub>2</sub>	+ a	-	-	n.d.
OII	+	-	-	n.d.
GFAP	-	+	+	-
NGF receptor	-	+	+	+
N-CAM	+	+	+	+
L-1	± b	+	+	+
A5E3	-	+	+	+
Ran-2	-	+	+	+
C4	-	+	n.d.	n.d.
S100	+	+	+	-
Vimentin	+	+	+	+
Laminin	+	+	+	+
O4, O8, O9	+	+	-	-
Gal-C	+	+	-	-
Seminolipid	+	+	+	-

a - Present on some m-f Schwann cells only

b - Present mainly at Nodes of Ranvier, very little elsewhere

n.d. - Not determined

### Peripheral nerve regeneration

Following nerve injury considerable changes occur in the resident Schwann cell population. Many of these regenerative events are reminiscent of events that occur in Schwann cell development. After axotomy, nerve fibres distal to the site of injury degenerate by a process known as Wallerian degeneration. Both the axon and the myelin sheath degenerate leaving behind Schwann cells in the basement membrane tubes that surrounded the original nerve fibre. These tubes are known as bands of Bünger or endoneurial tubes. Disruption of the axon-Schwann cell contact leads to de-differentiation of the Schwann cell as judged by phenotypic changes (as detailed above). In terms of protein expression the de-differentiated cell resembles that of the non-myelin-forming and cultured Schwann cell in many respects. After nerve injury rapid Schwann cell proliferation occurs (Brown and Asbury, 1981; Pelligrino et al., 1986; Clemence et al., 1989) reaching maximal rates 2-3 days post-operatively (Clemence et al., 1989). The molecular mechanisms responsible for triggering Schwann cell division after nerve injury are unclear. *In vitro* studies have shown that axonal and myelin debris stimulate Schwann cell mitosis (Salzer and Bunge, 1980; Salzer et al., 1980a) and that macrophages that have phagocytosed myelin produce a conditioned medium that is mitogenic for Schwann cells (Baichwal et al., 1988). Furthermore the accumulation of macrophages in the distal stump at around the time of Schwann cell proliferation has been documented (Clemence et al., 1989; Perry et al., 1987; Thomas 1966; Williams and Hall, 1971).

After axotomy most surviving neuronal cell bodies undergo changes in gene expression and metabolism. The onset of regeneration is associated with the induction of many genes and proteins many of which are the same as those encountered during neurite outgrowth in development. In particular synthesis of GAP-43 is significantly enhanced (Benowitz and Rottenberg, 1987) as are tubulin and actin synthesis (Hoffman and Cleveland, 1988). Synthesis of neurofilament proteins are not however elevated but are decreased during regeneration. This is associated with a decrease in quantity of neurofilaments and a decrease in diameter of the regenerating nerve fibres (Hoffman et al., 1987; Oblinger

and Lasek, 1988).

Several hours after axotomy axons begin to regenerate. Sprouts arising from the proximal stump grow across the site of injury into the distal stump (Fried and Bischhausen, 1980; Meller, 1987). Here the regenerating axons enter the bands of Bungner through which they grow towards the periphery to hopefully reinnervate the appropriate target (review Fawcett and Keynes, 1990).

### The autonomic nervous system

The autonomic nervous system (ANS) can be subdivided into the sympathetic, parasympathetic and enteric subdivisions (Langley, 1921). The main features of each system will be discussed below.

#### A) Sympathetic nervous system

The sympathetic division of the ANS consists of segmentally arranged ganglia that lie along the length of the vertebral column in the sympathetic chain (paravertebral ganglia). Sympathetic ganglia are also found in prevertebral masses of neurones around the abdominal aorta. In general the sympathetic nervous system is characterized by short preganglionic neurones and long postganglionic neurones. The latter generally synthesize the transmitter noradrenaline. The preganglionic fibres to the sympathetic chain leave the spinal cord via the ventral roots of the thoracic and lumbar vertebrae. The axons of postganglionic neurones very rarely have myelin sheaths.

#### B) Parasympathetic nervous system.

The parasympathetic ganglia, in contrast to the sympathetic ganglia, are usually found in association with, or very close to, their peripheral targets. In this system the preganglionic fibres are relatively long and the postganglionic fibres short. These fibres generally synthesize the transmitter acetylcholine as do the preganglionic fibres of both the parasympathetic and sympathetic nervous system. The parasympathetic division has a cranio-sacral outflow of preganglionic nerve fibres.

#### C) Enteric nervous system.

The enteric nervous system is an immense network of more than 10 million neurones in man (for reviews on the enteric nervous system see Furness and Costa, 1980; Llewellyn-Smith et al., 1983). The neurones are organized as two interconnected plexuses present along the entire

length of the gut. The myenteric plexus is situated between the longitudinal and circular muscles of the gut wall, whereas the submucosal plexus is in the submucosa. The enteric nervous system receives both sympathetic and parasympathetic extrinsic innervation (Gershon, 1981). The major feature that distinguishes the enteric nervous system from the other subdivisions of the ANS is its relative independence from the CNS. The supporting cells of the enteric plexus are also unusual in that they resemble CNS astrocytes more than they resemble other peripheral glia (Schwann or satellite cells) (Jessen and Burnstock, 1982).

Transmitters are released from autonomic nerve fibres from axonal swellings along the length of axons known as varicosities. This arrangement clearly differs from the classical neuromuscular presynaptic nerve ending seen in skeletal muscle, where transmitter is released from a single structure at the end of the axon.

#### Growth factors and the nervous system

It is becoming increasingly apparent that growth factors are important participants in the processes of PNS development, maintenance and regeneration. Nowadays it is widely accepted that development of the PNS of vertebrates is dependent in part on the interaction of immature sensory and autonomic neurones with specific survival factors derived from peripheral targets (Barde, 1988, 1989; Davis and Lumsden, 1990). Competition for limiting amounts of these factors results in significant neuronal losses during embryogenesis. In addition to the role of supporting particular neurones early in development, neuronotrophic factors are also required for the normal function of certain neurones in adult animals (Snider and Johnson, 1989). In addition, tissue culture studies have implicated growth factors in many other aspects of PNS development. Detailed below are the effects of growth factors relevant to this study, on both PNS and CNS derived cells. Where possible these effects have been related to the reported distribution of these factors and their receptors *in vivo*.

#### Nerve growth factor family

The first identified neuronotrophic factor was NGF discovered

more than 30 years ago by Levi-Montalcini and coworkers. In the PNS, NGF synthesized by target organs, supports the development and maintenance of sympathetic and sensory neurones derived from the neural crest (Levi-Montalcini and Angelletti, 1968; Thoenen and Barde, 1980; Levi-Montalcini, 1978). Both the levels of mRNA and protein correlate with the density of sympathetic innervation in adult rat tissues (Shelton and Reichart, 1984; Heumann et al., 1984). More recently NGF mRNA has been detected in the brain with the highest levels being in the hippocampus and cerebral cortex (Maisonpierre et al., 1990; Korshing et al., 1985) areas receiving major cholinergic projections from their basal forebrain. In much the same way that embryonic neurones die after interruption of contact with their targets, the basal forebrain cholinergic neurones die after axotomy (Korshing, 1986). These axotomized neurones can be rescued by the intraventricular injection of NGF (Williams et al., 1986; Hefti, 1986; Kromer, 1987; Montero and Hefti, 1988). In all NGF responsive neurones NGF has been shown to be retrogradely transported to the neuronal cell body. In the case of central cholinergic neurones the site of NGF synthesis is the pyramidal cells of the hippocampal neurones of the dentate gyrus (Ayer-le Lievre et al., 1988; Whittmore et al., 1988).

Recently factors related to NGF such as brain derived neurotrophic factor (BDNF)(Barde et al, 1982) and neurotrophin-3 (NT-3)(Hohn et al., 1990) have been discovered. BDNF is a protein of extremely low abundance that has so far only been isolated from pig brain (Barde et al., 1982; Hofer and Barde, 1988). It has been shown to induce sprouting of embryonic peripheral sensory ganglia *in vitro* in a manner indistinguishable from that of NGF (Davis et al., 1986). In addition neurones derived from the ectodermal placodes that are not responsive to NGF are responsive to BDNF and sympathetic neurones responsive to NGF are not responsive to BDNF (Lindsay et al., 1985; Davis et al., 1986). The third member of the NGF family, NT-3, has been shown to support the survival of 30% of neurones derived from the nodose ganglion the effect being additive to that of BDNF. Motoneurones, ciliary neurones and sympathetic neurones from embryonic chick did not survive in the presence of NT-3 (Hohn et al., 1990). The onset of gene expression for all three neurotrophins occurs between the 11th and 12th

day of rat embryogenesis, a time coinciding with the onset of neurogenesis (eg Altman and Bayer, 1984). The levels these factors achieve in early embryos however differ greatly. NT-3 mRNA is the most highly expressed in embryos whereas BDNF is expressed at the lowest levels and NGF expression varies locally during development (Maissonpierre et al., 1990).

#### Fibroblast growth factors

In addition to the NGF family many other growth factors have been shown to play varied roles in the nervous system. In particular the actions of the acidic and basic fibroblast growth factors (aFGF and bFGF) are becoming increasingly well documented. FGFs are 16kDa heparin binding growth factors thought to play a central role in many cellular processes such as development, wound healing and angiogenesis (Folkman et al., 1987; Gospodarowicz et al., 1987). In the nervous system, both factors are found in embryonic and adult brain tissues in relatively high amounts (Logan and Logan, 1986; Gospodarowicz, 1987; Risau et al., 1988; Gonzales et al., 1990) when compared with NGF or BDNF. Both the studies of Logan and Logan, (1986) and Caday et al., (1990) have shown that levels of FGF increase from embryonic to postnatal stages of brain development. In the CNS immunohistochemical studies have shown that bFGF is found primarily in neurones (Pettman et al., 1986; Finklestein et al., 1988). bFGF has been shown to support survival and/or neurite outgrowth of granule cell neurones (Hatten et al., 1988), hippocampal neurones (Walicke et al., 1986), mesencephalic neurones (Ferrari et al., 1989), cerebral cortical neurones (Morrison et al., 1986; Walicke 1988) chick spinal cord neurones (Unsiker et al., 1987), PC12 cells (Togari et al., 1983, 1985; Schubert et al., 1987; Rydel and Greene, 1987) and postnatal rat retinal ganglion cells (Lipton et al., 1988). Both oligodendrocytes (Eccleston and Silberberg, 1985; Saneto and de Vellis, 1985) astrocytes (Hatten et al., 1988), and neural precursor cells (Murphy et al., 1990) have been shown to respond mitotically to bFGF and astrocytes have been shown to synthesize bFGF (Hatten et al., 1988). bFGF, in combination with PDGF, has also been shown to cause prolonged proliferation of oligodendrocyte-type 2 astrocyte (O-2A) progenitors thus preventing their maturation into astrocytes (Bogler et al.,

1990).

The effects of FGF are presumably mediated by a cell surface receptor. Indeed two forms of basic FGF receptor have been detected in the developing mouse brain (Reid et al., 1990) and the adult rat brain (Imamura et al., 1988). The sequences of the mouse receptors reveal that they are both tyrosine kinases, the smaller receptor appears to be expressed at higher level during development and probably also binds aFGF. Studies of Wanaka et al., (1990) using *in situ* hybridization on CNS sections detected widely distributed populations of cells that express bFGF receptor mRNA. The distribution of receptor mRNA often correlated closely with populations of neurones responsive to FGF *in vitro*. Interestingly, neurones with bFGF receptors were largely devoid of NGF receptors. In the peripheral nervous system FGF receptor mRNA has been detected only in DRG during late development and early postnatal life. No mRNA was detected in sympathetic or parasympathetic ganglia at any developmental stage studied (Wanaka et al., 1991).

In the peripheral nervous system FGF has been isolated from the cat sciatic nerve (Logan and Logan, 1986) and bFGF has been detected immunohistochemically in the embryonic rat peripheral nerve where intense staining is found associated with the nerve fibres, fibroblasts, endothelial cells, and extracellular matrix (Gonzales et al., 1990). Both acidic (Claude et al., 1988) and basic FGF (Stemple et al., 1988) have been shown to promote neurite outgrowth and proliferation of chromaffin cells. The studies of Unsicker et al., (1987) on a wide range of peripheral neurones revealed neuronotrophic effects of FGF only on chick ciliary ganglion neurones, bFGF being more efficacious in this regard. In these studies FGF was found to support survival even when bound to polyornithine or polylysine substrates. Moreover FGF bound to nitrocellulose has been shown to enhance regeneration in the transected sciatic nerve (Danielson et al., 1988). Thus the neurotrophic effects of FGF are probably being receptor mediated by activation of an intracellular second messenger pathway (as apposed to retrograde transport of the ligand as is the case with NGF) or are mediated by non neuronal cells in the assay system. Indeed, studies using iodinated bFGF have failed to detect retrograde FGF transport in any neuronal type of the sciatic nerve (Ferguson et al., 1990). Contrastingly, receptor mediated internalization



and anterograde transport of bFGF has been reported in retinal ganglion cells (Ferguson et al., 1990).

Certain non-neuronal cells such as Schwann cells are known to respond to FGFs. Studies have shown that FGF can stimulate Schwann cell mitosis in the mouse (Krikorian et al., 1982) and that bFGF is weakly mitogenic for rat Schwann cells whereas aFGF is ineffective (Ratner et al., 1988). Both acidic and basic FGF can clearly be seen to be rat Schwann cell mitogens however provided forskolin is present in the culture medium to elevate intracellular cAMP levels (Davis and Stroobant, 1990). With regard to the above mentioned role of FGF as a Schwann cell mitogen, it has been reported that the neuronal cell surface molecule mitogenic for Schwann cells is a heparin binding growth factor (Ratner et al., 1988). Although this protein is not thought to be bFGF or aFGF it may be a member of the family of FGF related molecules.

Any consideration of FGFs as neurotrophic factors must take into account the fact that FGFs are not secretory proteins as they lack the appropriate signal sequence (Prats et al., 1989). While it is conceivable that FGFs are released from damaged cells and may thus play a role in nerve repair it is difficult to envisage a role as target derived neurotrophic agent regulating neuronal survival during development. It is possible however that an as yet undiscovered mechanism may enable FGF to be released from cells.

#### Ciliary neuronotrophic factor

Ciliary neuronotrophic factor (CNTF) is a 23kDa protein that is present in high concentrations in the sciatic nerve of adult rats (Manthorpe et al., 1986). At birth the levels of CNTF and its mRNA are just at the detection limit they rapidly rise during the first postnatal week reaching adult levels by the end of the second postnatal week (Stockli et al., 1989). *In vitro* it has been demonstrated that CNTF has a potent survival effect on various populations of neurones in particular neurones isolated from chick ciliary, dorsal root sensory and sympathetic ganglia (Barbin et al., 1984). More recently Sendtner et al., (1990) have demonstrated that CNTF prevents degeneration of motoneurones caused by nerve lesion in newborn rats. Furthermore Arakawa et al., (1990) have shown that CNTF exhibits a marked survival effect on chick motoneurones isolated from

embryos at the beginning of the period of naturally occurring motoneurone cell death. However it remains to be established whether CNTF is present at this early developmental stage. The suggestion has therefore been made that CNTF may function as a "lesion factor" preventing motoneurone cell death after lesion in the adult rat (Stockli et al., 1989). In the early postnatal period when CNTF levels in the sciatic nerve are very low (Sendtner et al., 1990) motor neurones are very sensitive to nerve injury. Moreover as CNTF, like FGF, lacks a secretory signal sequence (Stockli et al., 1989) thus supporting the hypothesis that CNTF plays an important role after cell injury.

The precise cellular location of CNTF in the PNS is at present unknown. Northern blot analysis of several adult rat tissues show detectable levels of mRNA only in the sciatic nerve (Stockli et al., 1989). Whether sciatic nerve CNTF has a neuronal or nonneuronal origin is not known. Support for a nonneuronal origin of CNTF comes from studies in the CNS where type 1 astrocytes have been reported to synthesize CNTF (Lillian et al., 1988; Hughes et al., 1988).

Tissue culture studies on the differentiation of CNS glia from O-2A progenitor cells show that CNTF in combination with the extracellular matrix cooperates in inducing type-2 astrocyte development (Lillien et al., 1990). Other roles for CNTF in differentiation have been provided by studies on PNS neurones where CNTF induces the expression of vasointestinal peptide immunoreactivity in embryonic chick sympathetic neurones (Ernsberger et al., 1989) and choline acetyltransferase activity in neonatal rat sympathetic neurones (Saadat et al., 1989). In both these studies a reduction in tyrosine hydroxylase immunoreactivity occurs concomitantly. CNTF also inhibits the proliferation of sympathetic neuroblasts (Ernsberger et al., 1989). Thus it may be that the physiological role of CNTF is as a differentiation factor rather than as a neuronotrophic agent during embryonic development, any neuronotrophic role being exerted only under pathophysiological conditions.

#### Transforming growth factor- $\alpha$ and Epidermal growth factor

Transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and epidermal growth factor (EGF) are related growth factors that both bind to the EGF receptor (Massague, 1983). The previously described activities of TGF- $\alpha$  have

been attributed to this binding (Deuel, 1987). EGF stimulates DNA synthesis (Leutz and Schachner, 1981; Raff et al., 1983) and proliferation of astrocytes (Simpson et al., 1982) and supports the survival of neonatal striatal, cortical, and cerebellar neurones *in vitro* (Morrison et al., 1987, 1988). EGF also induces enzymatic activities in glial cells (Honeggar and Guentert-Laubert, 1983; Guentert-Laubert and Honeggar, 1983; Almazan et al., 1985) and inhibits acetylcholine release from cholinergic nerves (Takayanagi, 1980). The presence of EGF in the mammalian CNS is however controversial. Immunoreactive EGF has been detected in the CNS of rats older than 2 weeks but not in younger rats (Fallon et al., 1984) and a prepro EGF mRNA has been detected, albeit at very low levels, in the adult mouse CNS (Rall et al., 1985). Conversely studies of Probstmeier and Schachner, (1986); and Poulsen et al., (1986) have failed to detect any EGF in the adult rat brain. Nevertheless receptors for EGF have been detected in the embryonic mouse brain (Adamson and Meek, 1984) and in the rat forebrain the numbers of binding sites declining with age (Quirion et al., 1988). EGF binding sites have also been detected in cultured astrocytes (Leutz and Schachner, 1981; Simpson et al., 1982) and oligodendrocytes but not neurones (Simpson et al., 1982). EGF receptor expression has also been detected in human glial tumours (Libermann et al., 1984).

The presence of TGF- $\alpha$  in the brain is less controversial. Immunoreactive TGF- $\alpha$  and mRNA have been detected in neurones of adult human, rat and mouse brain (Wilson and Derynck, 1988; Kudlow et al., 1989). In the fetal rat brain TGF- $\alpha$  mRNA was found to be 2-fold more abundant than in adult brain (Kudlow et al., 1989). Thus TGF- $\alpha$  is present in the brain during development (Kudlow et al., 1989) at a time when EGF receptor expression is high but EGF protein is not detectable (Adamson and Meek, 1984; Quirion et al., 1988). Therefore it is possible that *in vivo* TGF- $\alpha$  mediates many of the effects that are *in vitro* ascribed to EGF. In this regard TGF- $\alpha$  has been shown to stimulate neurite outgrowth from PC12 cells and support the survival of embryonic brain neurones (Zhang et al., 1990). TGF- $\alpha$  has also been shown to have similar activity to EGF in a number of other assay systems (for review see Derynck, 1989).

### Transforming growth factor- $\beta$

The transforming growth factors- $\beta$ 's (TGF- $\beta$ ) are a large family of growth factors involved in growth, differentiation and morphogenesis (review Massague, 1990). In the nervous system TGF- $\beta_1$  and TGF- $\beta_2$  stimulate Schwann cell proliferation (Eccleston et al., 1989; Ridley et al., 1989) the mitogenic response being modulated by forskolin mediated increases in intracellular cAMP (Ridley et al., 1989). Interestingly, the proliferation of long-term cultured "autocrine" Schwann cells is inhibited by TGF- $\beta_1$  (Eccleston et al., 1989). Thus in Schwann cells, as has been reported in other cells, TGF- $\beta$  may be involved as a switch where when some process is in an inactivated state TGF- $\beta$  will facilitate the activation of this process; conversely in the same cells once the process has been activated TGF- $\beta$  will function as a stop signal (review Sporn and Roberts, 1990). No role for TGF- $\beta$  in the CNS has yet been documented.

Little is known about the site of synthesis and distribution of TGF- $\beta$  in nervous tissues. Wilcox and Derynck, (1989) could detect no TGF- $\beta_1$  mRNA in adult mouse brain, however TGF- $\beta_3$  mRNA has been detected in the CNS of the developing mouse (Pelton et al., 1990), and TGF- $\beta_1$  mRNA has been reported mouse neural crest cells (Schmid et al., 1990).

### Platelet derived growth factor

The platelet derived growth factors (PDGF) are disulphide linked hetero- or homodimers of two distinct but related chains (PDGF A and PDGF B). In the CNS PDGF has been shown to have an important role in the proliferation and timing of oligodendrocyte development (Richardson et al., 1990). PDGF secreted by type-1 astrocytes *in vitro* keeps O-2A progenitors dividing and prevents their premature differentiation into oligodendrocytes (Richardson et al., 1988; Noble et al., 1988). mRNA for PDGF A has been detected in cultures of type-1 astrocytes and is present in the brain and optic nerve from late embryonic development throughout adulthood (Richardson et al., 1988; Pringle et al., 1988). PDGF A chain has also been shown to be produced by tumours of nerve tissue (Betsholtz et al., 1989). Recent studies, however have shown that high levels of PDGF A immunoreactivity and mRNA are found in the CNS and PNS neurones of both embryonic and developing mice. Some astrocytes and

oligodendrocytes were also seen to contain mRNA transcripts for PDGF A but at a much lower intensity than in neurones (Yeh et al., 1991). Thus it is possible that the source of the PDGF A required for oligodendrocyte development *in vivo* may be neuronal. O-2A progenitor cells are known to express  $\alpha$  receptors (Hart et al., 1989) which mediate the effects of all three forms of PDGF.

The role of PDGF B in the CNS is less well documented. B chain mRNA has been found in low but constant amounts throughout development in the rat CNS (Richardson et al., 1988) and B chain immunoreactivity has been detected at variable intensity in monkey CNS neurones (Sasahara et al., 1991). Most regions of the adult monkey brain were also found to express mRNA for PDGF A chain as well as  $\alpha$  and  $\beta$  receptors. Using transgenic mice the same authors that PDGF B chain transgene activity was greatest around birth and was expressed in higher levels in the CNS than in other tissues. Glial cells and ependymal cells were negative for immunohistochemically detectable levels of transgene expression and  $\beta$  receptor (Sasahara et al., 1991). PDGF B, possibly released from neurones, may also play a role in oligodendrocyte development as part of an AB heterodimer and influence O-2A progenitor and astrocyte chemotaxis (Bressler et al., 1985; Armstrong et al., 1990).

Recently several studies have implicated PDGF in the development of the PNS. Eccleston et al., (1990) and Yong et al., (1988) have shown that PDGF is able to stimulate Schwann cell mitosis in serum containing medium. In other studies no effect of PDGF was observed under these conditions however significant effects of PDGF BB (not PDGF AA) on DNA synthesis and proliferation were observed in the presence of the adenylate cyclase activator forskolin (Davis and Stroobant, 1990). The effect of forskolin in these experiments may be to upregulate PDGF receptor gene expression (Weinmaster and Lemke, 1990). In vitro Schwann cell PDGF receptors have been shown to be predominantly of the  $\beta$  type (Eccleston et al., 1990). The  $\beta$  type receptor is able to respond to PDGF BB and PDGF AB but not PDGF AA a fact that is consistent with the observed mitogenic effects of the PDGF types (Davis and Stroobant, 1990). Both short- and long-term cultured Schwann cells have been shown to secrete a PDGF-like molecule that may be involved in an autocrine proliferation loop in long-term cultured cells (Eccleston et

al., 1990). *In vivo* PDGF A chain transcripts and protein are detectable at high levels from late embryogenesis to adulthood in dorsal root ganglion neurones but are not detectable in the accompanying Schwann/satellite cells (Yeh et al., 1991). Using transgenic mice B chain gene expression has been demonstrated in the large sensory neurones of the dorsal root ganglia (Sasahara et al., 1991).

Thus in both the CNS and PNS, PDGF has been shown to have a variety of effect on glial cells. The source of PDGF mediating these effects *in vivo* is uncertain but it seems likely that much of it may be of a neuronal origin. Further studies will be required to determine if PDGF is transported in the axon and whether PDGF is released solely from the axon terminal or from the axon shaft.

#### Other growth factors

Aside from the growth factors mentioned above several others have been shown to elicit effects on neuronal and glial cells. Glial growth factor (GGF) a partially purified protein from bovine brain (Brockes et al., 1980) is of particular interest in this study. This molecule is a 31kDa basic protein that has been shown to be mitogenic for Schwann cells (Raff et al., 1978; Brockes et al., 1980; Davis and Stroobant, 1990) and astrocytes in dissociated cultures of the rat corpus callosum (Brockes et al., 1980). The GGF mediated stimulation of Schwann cell proliferation is not accompanied by a rise in intracellular cAMP (Brockes et al., 1980) and is dependent upon the presence of one or more components of fetal calf serum (Raff et al., 1978; Lemke and Brockes, 1984). GGF has subsequently been implicated in limb regeneration of Urodele amphibians (Brockes and Kintner, 1986) and GGF like polypeptides have been detected in Schwann cell tumours (Brockes et al., 1986). Further clarification of the role of GGF in the nervous system awaits the purification of this protein.

Interestingly several factors normally associated with the immune system are reported to have effects in the nervous system. Interleukin 2 has been shown to stimulate the proliferation of oligodendrocytes (Benviste et al., 1986) and to enhance neurite outgrowth from sympathetic but not sensory neurones (Keely Haughen and Letourneau, 1990).

Interleukin 1 (IL-1) has been shown to stimulate astrocyte proliferation (Guilian and Latchman, 1985) and to regulate NGF synthesis in astrocytes (Carman-Krzan et al., 1991) and in the non neuronal cells of the sciatic nerve (Lindholm et al., 1987) suggesting a possible role for IL-1 in the glial response to injury.

Many of the studies detailed above were carried out in serum containing medium. Thus a complete analysis of the actions of individual growth factors is impossible as serum is known to contain a multitude of hormones and growth factors. It is also becoming apparent that many of the cellular processes that occur during development and growth depend upon the complex interaction of a wide spectrum of growth factors. One of the major classes of serum growth factors that are known to have profound effects on animal growth and development are the insulin-like growth factors.

#### Insulin-like growth factors

The insulin-like growth factors (IGFs), or somatomedins, are polypeptide mitogens that play a fundamental role in mammalian growth processes. Their levels in serum are affected by nutritional status, adequate insulin secretion and in the case of IGF-I in postnatal life, growth hormone (Froesch et al., 1985). The structures of IGF-I and II have been elucidated by Rinderknecht and Humbel, (1978a, b). They consist of an A-domain homologous to the A-chain of insulin and a B-domain homologous to the B-chain of insulin. In contrast to insulin, the C domains are retained in both IGF-I and II. Although clearly separate entities, these two peptides have about 70% sequence homology and about 50% homology with proinsulin. IGF-I is a slightly acidic and IGF-II a basic peptide. Both have a molecular weight of about 7.5kDa. The complete sequences of rat IGF-I and II have been deduced (Shimatsu and Rotwein, 1987; Marquardt et al., 1981). Only three amino acid changes were noted between rat and human IGF-I and five amino acid differences between rat and human IGF-II.

## Other forms of IGF

### A)IGF-I.

The cDNA sequence for IGF-I predicts the existence of two different precursor molecules (Rotwein, 1986). The nucleotide sequence of the cDNA predicts an IGF-I molecule with a C terminal extension of 35 amino acids (IGF-IA) or 77 amino acids (IGF-IB). The extended carboxy terminus is known as the E peptide region. A larger form of IGF-I has been isolated from media conditioned by human fibroblast cell lines (Clemmons and Shaw, 1986). A biologically active peptide of 21.5 kDa was isolated that had an amino acid sequence that appeared to be identical to human IGF-IB. Cultured human fibroblasts have also been shown to secrete a peptide of 19kDa as estimated by SDS polyacrylamide gel electrophoresis (Conover et al., 1989). Using an antibody to the E peptide region of IGF-I this peptide was identified as IGF-IA. IGF-IA has also been detected in serum from children and adults with chronic renal failure (Powell et al., 1987). In addition Blum et al., (1987) have isolated at least six peptides from human plasma all with IGF action that were reactive with antisera against IGF-I or IGF-II. In the CNS a variant form of IGF-I has been characterized with a truncated N-terminal region (Sara and Carlsson-Skwirut, 1988).

### B)IGF-II

A single IGF-II precursor has been predicted to exist that has an E peptide carboxy terminal extension of 89 amino acids (Jansen et al., 1985). A number of variant forms of IGF-II have been reported. 25% of the IGF-II isolated from human plasma was found to lack the N-terminal alanine residue (Rinderknecht and Humbel, 1978b). Several other forms of IGF-II have been purified from the conditioned media of a rat epithelial-like cell line. These peptides were all in the molecular weight range 5-8kDa as determined by SDS polyacrylamide gel electrophoresis (Tanaka et al., 1989). Similarly a large molecular weight form (8.7-10kDa) of IGF-II has been isolated from pheochromocytomas, neural crest tumors (Gelato and Vassalotti, 1990). A large (9kDa) IGF-II form has also been isolated from human cerebrospinal fluid and detected in the brain (Hasselbacher and Humbel, 1982; Hasselbacher et al., 1985). Larger forms of IGF-II have been detected in several systems.



Gowan et al., (1987) have isolated a 15kDa form of IGF-II from human plasma that had equal affinity for plasma IGF binding proteins and for the IGF-II receptor of rat placenta when compared to 7.5kDa IGF-II. The amino acid composition of this peptide is however significantly different from that predicted from the cDNA sequence of an IGF-II of this mass. Further IGF-II peptides of molecular weights 8, 10 and 19kDa have been isolated from the conditioned medium of buffalo rat liver cells (Yang et al., 1985). They are postulated to be intermediates from 20k pro-IGF-II to the processed form.

### Binding proteins

The IGFs do not exist in serum (or other body fluids) in the free form but are bound to specific carrier proteins that extend the circulating half life of IGFs by 60-90 fold (review Holly, 1990). At present several distinct IGF binding proteins exist which appear to be independently regulated and almost certainly have separate roles. Only three of these binding proteins have been purified to homogeneity and their structures determined. These binding proteins may modulate the activities of the IGFs.

#### IGFBP-1

IGFBP-1 is a 25kDa protein that binds both IGF-I and IGF-II (Clemmons and Gardener, 1990). The mRNA for IGFBP-1 has been detected in rapidly growing/ differentiating tissues and in the liver. Immunohistochemical studies show however IGFBP-1 that the peptide is widely distributed throughout most tissues. The IGFBP-1 is unlikely to have biological action itself but has been shown both to inhibit the actions of IGF (reviewed by Holly, 1990) and to enhance the mitogenic effects of IGF-I (Elgin et al., 1987). The potentiation of the IGF-I response appears to be dependant on an integrin-like cell surface receptor. Indeed IGFBP-1 contains an RGD (Arg-Gly-Asp) sequence a feature common to several matrix proteins known to bind to receptors of the integrin family (Hynes, 1987).

#### IGFBP-2 and 3.

The IGFBP-2 a 32kDa protein that binds IGF-II with a higher

affinity than IGF-I (Brown et al., 1989). IGFBP-3 is a 53kDa growth hormone dependant glycoprotein that binds IGF-I and II with high affinity and can acts as an inhibitor or potentiator of IGF action (Blum et al., 1989; DeMellow and Baxter, 1988).

### IGF receptors

The IGFs can exert their effects via the insulin receptor and the type 1 and type 2 IGF receptors. The insulin receptor recognises insulin with 20–100 x higher affinity than IGF-II and I respectively (Gammeltoft et al., 1988).

#### A) Type 1 receptor.

The type 1 IGF receptor is a heterodimeric tyrosine specific protein kinase comprised of 2  $\alpha$  and 2  $\beta$  subunits joined by disulphide bridges . This receptor shows considerable homology with the insulin receptor notably in the  $\beta$  subunit (95kDa) tyrosine kinase domain (Massague and Czeck, 1982). Ligand binding occurs on the  $\alpha$  subunit (130kDa). IGF-I binds with a somewhat higher affinity than IGF-II and insulin binds weakly. A neuronal subtype of the type 1 receptor with an  $\alpha$  subunit of 115kDa has been isolated from fetal rat brain neurones (Burgess et al., 1987). The type 1 IGF receptor is believed to mediate the biological effects of IGF-I and IGF-II and is thought to mediate many of the response caused by supraphysiological concentrations of insulin.

#### B) Type 2 receptor

The type 2 IGF receptor is a 250kDa glycoprotein that binds IGF-II with high affinity, cross reacts weakly with IGF-I and does not bind insulin (Rechler and Nissley, 1985). Analysis of the cDNA of the human IGF-II receptor reveals that it is identical to the cation-dependant mannose-6-phosphate receptor (Morgan et al., 1987). Majority of type-2 receptors are found in intracellular organelles where they are involved in the sorting and transport of lysosomal enzymes from the Golgi apparatus to a prelysosomal compartment (Morgan et al., 1987; MacDonald et al., 1988). Recent studies of Senior et al., (1990) have shown that IGF-II receptor mRNA expression is high in the developing rat being strongly down regulated postnatally. Given that IGF-II expression is also regulated

in the same manner in most tissues (Beck et al., 1987, 1988; Stylianopolou et al., 1988) these authors suggest that high levels of IGF-II receptor present in embryonic and fetal tissue serve to stabilize local concentrations of IGF-II at required values by endocytosing excessive amounts of locally synthesized growth factor. A role for the IGF-II receptor in the metabolism of IGF-II has also been suggested by Czeck, (1989).

### IGFs and development

Both IGF-I and IGF-II are thought to play a role in embryonic and fetal development. IGF-II mRNA has been detected during embryogenesis in most mesodermally derived rat tissues, expression being switched off in adulthood in all tissue except the brain (Beck et al., 1987; Stylianopoulou et al., 1988). Intriguingly the pattern of IGF-II gene expression during embryogenesis overlaps significantly with the reported distribution of immunohistochemically detected TGF- $\beta_1$  (Stylianopoulou et al., 1988; Heine et al., 1987). Serum levels of IGF-II are also known to drop dramatically after birth (Moses et al., 1980) thus supporting the hypothesis that IGF-II is predominantly a fetal growth factor. Although most IGF-II gene expression is localized to tissues of mesodermal origin, IGF type 1 receptor (which can mediate the effects of IGF-I and IGF-II) expression is reputedly highest in tissues of ectodermal origin (Girbau et al., 1989). Thus in development IGF-II may be of prime importance in the development of ectodermal tissues. This interpretation must be treated with caution however as these studies were carried out in different species (rat vs chick).

IGF-I and IGF-I mRNA on the other hand are expressed by a variety of mesodermal and neuroectodermal derived tissues during embryonic and fetal development (for review see Hansson, 1990). For example vascular cell walls, endothelial cells, smooth muscle cells and fibroblasts show transient expression of IGF-I immunoreactivity, limited to early stages of their formation and maturation (Hansson et al., 1987b, 1989). The vascular walls of the mature animal are however able to re-express IGF-I immunoreactivity if exposed to increased blood flow or blood pressure (Hansson et al., 1989; Hansson et al., 1987). Similarly skeletal muscle cells show IGF-I immunoreactivity for only a short time

period during their postnatal development and differentiation (Jennische and Olivecrona, 1987).

Whether the IGFs exert their effects by autocrine/paracrine mechanisms, as well as, or as opposed to, endocrine actions unclear. In the fetus IGF-I and IGF-II genes are expressed by many tissues, and the serum levels of the IGFs are relatively low. This suggests a predominant role of autocrine/paracrine actions over endocrine actions. Consistent with this hypothesis is the observation that classical hormones (endocrine gland products) that are involved in postnatal growth do not appear to play key roles during embryonic development. In the postnatal animal however there is a progressive rise in serum IGF-I levels during the prepubertal years (Daughaday and Rotwein, 1989). Thus postnatally endocrine actions may interplay with autocrine/paracrine actions of IGF-I. Which mode of action predominates probably varies amongst tissues and with developmental stage. Autocrine/paracrine actions are likely to predominate in tissues where the entry of IGFs from the circulation is limited such as the nervous system, and in tissues where there are high local concentration of IGFs.

#### IGFs and nervous system development

IGF-I mRNA is expressed at high levels in the fetal brain declining to adult levels postnatally (Sandberg et al., 1988). *In situ* hybridization studies of Ayer-le Lievre et al., (1991) have noted high level of mRNA in the olfactory bulb and in discrete areas of the cranial sensory ganglia of the rat fetus. In the adult animal IGF-I mRNA is concentrated mainly in the olfactory bulb and cervical-thoracic spinal cord and to a lesser degree in the midbrain and cerebellum (Rotwein et al., 1988). IGF-I has also been detected by radioimmunoassay of adult brain tissue (D'Ercole et al., 1984). Short-term culture studies suggest that both the neurones and glia synthesize the IGF-I message in vivo (Rotwein et al., 1988). It must be noted however that reports have been published that fail to detect IGF-I mRNA in the adult brain (Lund et al., 1986) and fetal brain (Beck et al., 1987). IGF-I immunoreactivity has been detected in the neurones and glia of the developing cerebellum, the levels of protein declining with the onset of differentiation (Andersson et al., 1986).

In contrast IGF-II mRNA has been detected only in the choroid plexus, the leptomeninges (Stylianopoulou et al., 1988; Beck et al., 1987; Ayer-le Lievre et al., 1991) and the hypothalamus (Ayer-le Lievre et al., 1991). The expression of IGF-II in these cells continues throughout adult life (Stylianopoulou et al., 1987; Rotwein et al., 1988). The presence of IGF-II protein in the brain has been demonstrated by Hasselbacher et al., (1985). The persistence of relatively high levels of IGF-II in the CNS into adulthood contrasts with its more developmentally restricted expression in other rodent tissues (see above) and suggests that IGF-II is of importance in the brain.

Few studies have documented the distribution of the IGFs in the PNS. In the embryonic rat two groups have failed to detect any IGF-II mRNA (Beck et al., 1987; Stylianopoulou et al., 1988). A third group looking at the distribution of IGF-II in the craniofacial region of the rat fetus by *in situ* hybridization detected IGF-II mRNA only in association with the vascular endothelia of the ganglia (Ayer-le Lievre et al., 1991). In the same study IGF-I mRNA was detected in the iris primordium and ciliary body. Immunohistochemical studies that could not distinguish between IGF-I and 2 have found IGFs in the peripheral nerves of the mouse and chick embryo (Ralphs et al., 1990). Furthermore studies by other authors have shown that IGF-I immunoreactivity is present in the myenteric plexus, and at low levels in the axons and Schwann cells of the adult peripheral nerve (Hansson et al., 1988b; Andersson et al., 1988). It must be emphasized however that areas of IGF immunoreactivity do not necessarily reflect sites of IGF synthesis but may represent sites of IGF sequestration and utilization.

### In vitro actions

As noted above, two of the major growth promoting ingredients of serum, and those replaced by high concentrations of insulin in artificial media, are the IGFs. These growth factors mediate a plethora of effects on neuronal and glial cells via interaction with IGF receptors. Table 1.1 summarizes the effects of IGFs on the nervous system. In other systems IGFs have been implicated in many cellular processes such as matrix formation (eg Goldstein et al., 1989), migration (Stracke et al., 1989) and proliferation (eg Froesch et al., 1985).

Table 1.1. The actions of the IGFs in the nervous system *in vitro*.

TABLE 1.1  
Actions of IGFs in the nervous system

Peptide	Target	Effect	Reference
IGF-I	Fetal rat brain cells	DNA synthesis	Sara et al., 1979.
IGF-I	Fetal rat neurones	RNA synthesis	Burgess et al., 1987
IGF-I	O-2A progenitors	DNA synthesis, oligodendrocyte commitment	McMorris et al., 1986,1988.
IGF-I	Neonatal rat astroglial cells	DNA synthesis	Han et al., 1987
IGF-I	O-2A progenitors	MBP expression	Saneto et al., 1988.

IGF-I	Rat glial cell cultures	Expression of oligodendrocyte specific enzymes	van der Pal et al., 1988
IGF-I	Quail embryo DRG	Adrenergic differentiation	Xue et al., 1988
IGF-I	Chick embryo motoneurone	Neurite outgrowth	Caroni and Grandes, 1990
IGF-I	Chromaffin cells	Catecholamine secretion	Dahmer et al., 1990
IGF-I	Chick embryo brain cultures	Neuronal survival	Svic and Schubert, 1990
IGF-I	Rat fetus hypothalamus	Neuronal survival	Torres-Aleman et al., 1990*
IGF-I	Parasympathetic neurones	Survival	Crouch and Hendry, 1991



IGF-II	Chick embryo sensory neurones	Neurite outgrowth	Bothwell, 1982
IGF-II	Neuroblastoma SH-SY5Y cells	Neurite outgrowth	Recio-Pinto and Ishii, 1984
IGF-II	Neuroblastoma SH-SY5Y cells	Increased tubulin mRNA and neurite formation	Mill et al., 1985.
IGF-II	Chick embryo sensory and sympathetic neurones cultures	Neurite outgrowth and survival	Recio-Pinto et al., 1986
IGF-I/II	Neuroblastoma SH-SY5Y cells	DNA synthesis	Mattson et al., 1986.
IGF-I/II	Astrocytes	DNA synthesis 1987.	Bartoli et al.,

IGF-I/II	Rat sympathetic neuroblasts	DNA synthesis	DiCiccio-Bloom and Black 1988
IGF-I/II	Neuroblastoma SK-N-AS cells	DNA synthesis	El-Badry et al. 1989
IGF-I/II	Pontine and septal neurones	Transmitter specific differentiation.	Knusel et al., 1990 *

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\* In these studies an enhanced effect of IGF was observed in the presence of bFGF.

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### Actions of IGFs in vivo

Few studies have addressed the role of IGFs in the nervous system *in vivo*. Nevertheless Caroni and Grandes, (1990) demonstrated that injection of low quantites of IGF-I or IGF-II into the gluteus muscle of adult mice led to intramuscular nerve sprouting. Hence it is plausible that nerve sprouting after denervation may be enhanced by increased muscle synthesis of IGF. This hypothesis is supported by two observations of Ishii, (1989; 1990). Firstly, sciatic nerve transection produced a significant increase in muscle IGF-II mRNA, and secondly, treatment of rats pups with botulinum toxin prevented the developmental decline of IGF-II mRNA. Botulinum toxin is known to produce intramuscular nerve sprouting and paralysis by blockade of acetylcholine release at the neuromuscular junction. Furthermore correlative evidence of Ishii, (1989) suggests that IGF-II may be involved synaptogenesis. This study demonstrates that IGF-II mRNA levels in muscle are high during embryogenesis at a time when polyneuronal innervation is occuring. The developmental time course for the elimination of polyneuronal innervation is seen to closely follow that for decline in muscle IGF-II content. Thus factors regulating IGF-II gene expression may indirectly regulate synaptogenesis.

A role for IGF in peripheral nerve regeneration has been proposed by several authors (Hansson et al., 1986, 1987a, 1988a; Sjöberg and Kanje, 1989; Kanje et al., 1990). The latter authors showed that local administration of IGF-I to a crush lesion of the rat sciatic nerve stimulated nerve regeneration. Moreover this enhanced regeneration could be blocked by antibodies to IGF-I. A role for IGF-I during peripheral nerve regeneration is supported by immunohistochemical data which shows that IGF-I accumulates, primarily in Schwann cells, after nerve injury (Hansson et al., 1986, 1987a, 1988a). As regeneration is in many respects analogous to development it is conceivable that IGF may also play a role in development of the peripheral nerve.

### Growth associated proteins

In addition to the extracellular regulation of growth and development by distinct growth factors and hormones, the growth process

is also modulated by various intracellular and membrane associated molecules. These molecules include proteins as diverse as cytoskeletal elements, receptor molecules, ion channel proteins and components of intracellular second messenger systems. A molecule presumed to have a role primarily associated with growth should be expressed at high levels in situations in which growth occurs ie during development and regeneration.

In the nervous system, for example, a small set of genes have been identified that are selectively expressed at high levels during periods of axon growth and repressed upon differentiation to a stable mature state. One of these is an acidic protein designated GAP-43 (B50, pp46, F1, GAP-48, or p57) first described in 1981 by Skene and Willard.

### The GROWTH ASSOCIATED PROTEIN GAP-43

#### A) Association with axon growth

The protein GAP-43 is synthesized at high levels during periods of axonal outgrowth during development and regeneration. Synthesis declines somewhat during synaptogenesis, but remains well above normal levels throughout the period in which synaptic organization is being refined (Benowitz and Routtenberg, 1987). In the regenerating fish and amphibian optic nerves GAP-43 synthesis begins to increase approximately 4-5 days after axotomy coinciding with, or slightly preceding initiation of axon outgrowth (Skene and Willard, 1981a, b; Benowitz et al., 1981; Benowitz and Schmidt, 1987). A similar correlation of GAP-43 expression with axon outgrowth is seen in the rat sciatic nerve after nerve injury (Skene, 1989). Striking increases in GAP-43 have also been reported in the regenerating hypoglossal nerve of the rabbit (Skene and Willard, 1981c). In the central nervous system of mammals, where regeneration is abortive, axotomy does not lead to enhanced expression of GAP-43 (Reh et al., 1987; Kalil and Skene, 1986; Skene and Willard, 1981 a,b).

Consistent with a role in axon elongation GAP-43 has been demonstrated to be a major component of neuronal growth cone membranes *in vivo* (Skene et al., 1986; Meiri et al., 1986). In tissue culture studies Meiri et al., (1988) have confirmed this observation using immunohistochemical techniques. GAP-43 immunoreactivity was detected throughout the growth cone and in the filopodial extensions.

## B) Association with synaptic plasticity

Although levels of GAP-43 in the brain diminish greatly after birth, the protein persists in certain presynaptic membranes throughout life. Several studies have demonstrated that high levels of GAP-43 protein and mRNA remain in certain areas of the brain such as the hippocampus and many areas of the limbic system (Neve et al., 1987; Benowitz et al., 1988; Nelson et al., 1987; McGuire et al., 1988). These areas are believed to exhibit high levels of synaptic plasticity in adulthood. Further evidence for a role of GAP-43 in synaptic plasticity have been provided by Lovinger et al., (1986). These workers found that phosphorylation of a protein designated F1 (GAP-43), was associated with synaptic long-term potentiation (LTP) in the rat hippocampus. LTP is a form of synaptic plasticity whereby an increased post synaptic response to afferent stimulation is seen. The extent of protein kinase C mediated phosphorylation of GAP-43 was found to correlate highly with the persistence of LTP over several days (Lovinger et al., 1986). Thus GAP-43 in a phosphorylated state may play a role in synaptic plasticity in the adult brain.

It must be noted however that certain brain areas which are remarkably plastic in adult life (Kaas et al., 1983) have a relatively low concentration of GAP-43 (Benowitz et al., 1988). Furthermore, the increased phosphorylation of GAP-43 observed during LTP may be only be coincidental (Skene, 1989).

## Biochemical properties of GAP-43

GAP-43 is an extremely hydrophilic molecule of MW 24kDa (Basi et al., 1987). It is synthesized as a soluble protein that becomes attached to the membrane posttranslationally via acetylated residues near the N terminus (Skene and Virag, 1989). These features of GAP-43 are consistent with models that visualize the protein extending away from the cytoplasmic side of the plasma membrane into the cytosol where interactions with the membrane skeleton may take place. Indeed GAP-43 has been found in association with membrane skeletal components in the growth cone (Meiri and Gordon-Weeks, 1990; Moss et al., 1990).

Furthermore the carboxyterminus of GAP-43 has a limited sequence homology with neurofilament protein (La Bate and Skene, 1989).

### Biochemical actions of GAP-43

#### A) Phosphoinositide metabolism

Activation of phospholipase coupled receptors results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> stimulates the release of calcium from intracellular stores and DAG activates protein kinase C (PKC). Replenishment of PIP<sub>2</sub> is achieved by phosphorylation of phosphatidyl inositol 4-phosphate (PIP) by PIP kinase (eg Berridge, 1987). The activity of PIP kinase can be inhibited *in vitro* by GAP-43, the phosphorylated form of GAP-43 being more effective in this respect (van Dongen et al., 1985). The extent of GAP-43 phosphorylation has been shown to be correlated with the activity of PIP kinase (Jolles et al., 1980). Furthermore antibodies to GAP-43 which prevent its phosphorylation, also increase the production of PIP<sub>2</sub> (Oestreicher et al., 1983). *In vitro* the phosphorylation of GAP-43 is catalysed by PKC (Aloyo et al., 1983), an enzyme that is activated by DAG a product of PIP<sub>2</sub>. Thus *in vivo* GAP-43 may play a role in feedback inhibition of receptor mediated PIP<sub>2</sub> hydrolysis.

#### B) Calmodulin binding

GAP-43 is unusual amongst calmodulin binding proteins in that it binds calmodulin selectively in the absence of calcium (Andreason et al., 1983), a feature that is so rare that it has been exploited in the purification of GAP-43 (Andreason et al., 1983). In addition the association of GAP-43 with calmodulin can be regulated by high ionic strength and phosphorylation by PKC (Alexander et al., 1987). Cellular events resulting in the activation of PKC, and the subsequent phosphorylation of GAP-43, would result in the liberation of calmodulin which would then be able to interact with its target enzymes.

#### C) G protein interactions.

G proteins (GTP binding proteins) are a family of proteins involved in signal transduction mechanisms. They are activated by binding GTP

and then self inactivate due to intrinsic GTPase activity (Alberts et al., 1983). Strittmatter et al., (1990) have shown that GAP-43 stimulates GTP analogue binding to the G protein  $G_0$ . Furthermore these authors have demonstrated that  $G_0$  is a major component of the growth cone membrane. Thus *in vivo* GAP-43 may function as an intracellular regulator of  $G_0$  function.

It remains to be shown whether the actions of GAP-43 documented *in vitro* are also apparent *in vivo*. It seems likely however that GAP-43 plays a role in some aspect of signal transduction inside the cell.

#### Factors influencing GAP-43 expression

Several lines of evidence suggest that GAP-43 expression may be regulated by NGF. Firstly studies using PC12 cells have demonstrated that GAP-43 mRNA is induced by NGF (Basi et al., 1987; Federoff et al., 1988; Costello et al., 1990). Secondly, studies of Verge et al., (1990) provide evidence that GAP-43 mRNA expression in the mature PNS is related to expression of high affinity NGF receptors. Tissue culture studies using dissociated adult DRG neurones however, detected no effect of NGF on the expression of GAP-43 protein (Woolf et al., 1990). FGF, DBcAMP, phorbol ester, and EGF, listed in order of efficacy, are also potent in stimulating GAP-43 gene expression in PC12 cells (Costello et al., 1990 ). Expression of GAP-43 transcript by all these factors (including NGF) is subject to repression by dexamethasone, the degree of repression being roughly proportional to the potency of the inducer. The idea of a GAP-43 repressor molecule *in vivo* has been suggested by Woolf et al., (1990). Blockade of axonal transport whether by chemical or physical means was sufficient to induce GAP-43 expression in adult DRG neurones. This suggested that GAP-43 expression is controlled by a target derived fast axonally transported repressor molecule.

A single *in vivo* study has suggested that GAP-43 protein expression may be induced by IGFs. This correlative evidence is based on the observation that GAP-43 levels are higher in motornerves induced to sprout with IGF than in those induced to sprout with botulinum toxin (Caroni and Grandes, 1990).

### Concluding remarks

Increasingly developmental processes are seen to be orchestrated by a complex interplay of growth factors and growth associated molecules. In this study defined culture conditions have been employed to identify factors involved in Schwann cell proliferation, a process critical to successful nerve development. In addition the distribution of the growth associated molecule GAP-43 in the PNS has been examined with a view to unravelling the role of this enigmatic protein.



## **CHAPTER 2**

### **MATERIALS AND GENERAL METHODS.**

## MATERIALS

### Chemicals and growth factors:

The following compounds were obtained from Sigma Chemical Company (Poole, UK); transferrin, selenium, putrescine, triiodothyronine, thyroxine, carbachol, noradrenalin, histamine, 5-hydroxytryptamine (5HT),  $\alpha$ ,  $\beta$  methylene ATP, 2 methylthio ATP, adenosine, dopamine, neuromedin, bombesin, somatostatin, glyoxylic acid, dibutyryl cAMP (DBcAMP)), bovine insulin, gamma-aminobutyric acid (GABA), isoprenaline, phenylephrine, phenylmethanesulphonylfluoride (PMSF), glycine, cytosine arabinoside, 5-fluoro-2'-deoxyuridine (Fudr), lamimin and poly-L-lysine (PLL MW 300,000). Bovine serum albumin (Path-O-Cyte) was obtained from ICN Immunobiologicals (Bucks, UK). Forskolin was from Calbiochem-Behring Corporation (San Diego C.A.). The cAMP assay kit was obtained from Amersham International plc, (Berks, UK). The protein assay kit was from Bio-Rad (Munich, West Germany). Bromodeoxyuridine (BrdU) was from Boehringer Mannheim (Lewes, UK). Citifluor was obtained from City University (London). Porcine (BB) platelet-derived growth factor (PDGF) and TGF- $\beta_1$  were obtained from British Biotechnology (Oxford,UK). Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were obtained from Collaborative Research (Bedford, M.A). Atrial natriuretic peptide (ANP) and calcitonin gene related peptide (CGRP) were obtained from Cambridge Research Biochemicals (Cheshire, UK). Tumour Necrosis Factor  $\alpha$  (TNF) was a gift from Dr J Taverne (Department of Immunology, University College and Middlesex School of Medicine (London). Interleukin -  $1\beta$  (IL1) was from Genzyme (San Diego, C.A.), Interleukin-2 (IL2) was a gift from Dr A Kingston prepared according to the method of Schreier and Tess (1981), and Interleukin-4 (IL4) was obtained from DNAX Inc (Palo Alto C.A.). Nerve growth factor (NGF) was a gift from Dr J Winter (Winter et al., 1988). Glial growth Factor (GGF) was a gift from Dr A.D.J Goodearl prepared according to the method of Brockes, (1987). Insulin like growth factor-I (IGF-I) was prepared according to the method of Morrell et al., (1986). Iodinated IGF-I was a kind gift from Dr D. Morrell. Iodinated IGF-I was prepared using chloramine T to a specific activity of approximately  $100\mu\text{Ci}/\mu\text{g}$  and

purified by immunoaffinity chromatography on a column of antiserum R557A IgG covalently attached to cyanogen bromide activated Sepharose 4B (Pharmacia-LKB Bucks, UK). Purified GAP-43 was a gift from R Curtis prepared from 7-14 day rat pups using a modification of the method of Chan et al., (1986).

#### Tissue culture reagents:

Calf serum was from Imperial laboratories (Andover, Hants), Dulbecco's Modified Eagles Medium (DME) and trypsin were from Gibco Laboratories (Paisley, Scotland). Minimal essential medium containing 0.2M hepes (MEMH) and calcium-, magnesium-free MEMH were from the Imperial Cancer Research fund. Collagenase was obtained from Worthington Biochemical Corporation (Freehold N.J.). Tissue culture plastic was from Falcon (Plymouth, UK).

#### Antibodies:

Anti-BrdU was a gift from Dr D Mason (Gratzner, 1982). Polyclonal anti-rabbit S100 was from Dakopatts a/s (Denmark). Monoclonal anti-S100 was a gift from Dr G Gillespie. Monoclonal anti-IGF-I (sm1.2) was from the developmental hybridoma bank (North Carolina, USA), mouse anti-nerve growth factor receptor 192-IgG (1.3mg/ml) (NGF receptor) was a gift from Dr E Johnson Jr (Taniuchi and Johnson, 1985). Monoclonal anti glial fibrillary acidic protein (GFAP) and monoclonal anti-vimentin were from Boehringer Mannheim (Lewes, UK). Monoclonal and polyclonal anti-GAP-43 were a gift from R.Curtis. Polyclonal PGP 9.5 was from Ultracclone (Cambridge, UK), Anti IGF-II receptor (C2) was a gift from Dr C Scott (Scott and Baxter, 1987). Monoclonal anti-neurofilament (RT97) was a gift from Dr J Wood (Anderton et al., 1982) Anti substance P was from Cambridge Research Biochemicals (Cheshire, UK), Anti vasointestinal peptide (VIP) was from Biogenesis (Bournemouth, UK), Anti-rabbit biotin, anti-mouse biotin, fluorescein-streptavidin and texas red-streptavidin were obtained from Amersham International (Amersham, Bucks). Anti-rabbit and anti-mouse peroxidase were from Sigma Chemical Company (Poole, UK).

### Electrophoresis:

All electrophoresis apparatus and reagents were from Bio Rad (Munich, West Germany) unless otherwise stated. Nitrocellulose paper was from Schleider and Schvell (Dassel, West Germany).

## METHODS

### Animals

Sprague Dawley rats were used throughout this work. Neonates were killed by decapitation and adults (200–250g) were killed by a asphyxiation in CO<sub>2</sub>

### Preparation of Schwann cell cultures

Schwann cells were prepared using the method of Brockes et al., (1979). Sciatic nerves were dissected from Sprague Dawley rats of varying ages, from embryonic day 19 to adult. The epineurial sheath was removed where possible then the tissue was chopped and dissociated in 0.25% Trypsin, 0.4% collagenase in DME at 37°C and 5% CO<sub>2</sub>/95% air. Tissue from adult rats was digested for 3 x 0.5hr, from embryonic rats (E18–21) for 20min and from early postnatal rats 35min. After incubation an equal volume of MEMH containing 10% fetal calf serum was added and the tissue gently dissociated through a plastic pipette tip. The cells were centrifuged at 500xg for 10min, resuspend in DME containing 10% calf serum, then cultured in 25cm<sup>2</sup> flasks.

### Preparation of early embryonic Schwann Cells

The sciatic nerves from E14 or E15 rats were digested as stated above for 45min. Cells were plated on poly-L-lysine (PLL) or laminin coated coverslips in BALB-C 3T3 conditioned medium to ensure cell survival (Jessen et al., 1990). These cells were prepared by J. Gavrilovic and K.R. Jessen.

### Preparation of BALB-C 3T3 conditioned Medium

Confluent 25cm<sup>3</sup> flasks of Balb-C 3T3 fibroblasts were used to condition 3cm<sup>3</sup> of DME containing 10% calf serum for 24hr at 37°C.

### Long-term Schwann cell cultures

When cultures prepared as described above were not used within two weeks they were maintained by changing half of the culture medium once a week. This procedure was continued for approximately 3 months until a flattened population of cells appeared. Cultures were then passaged approximately once a month with 0.05% trypsin in 0.02% EDTA. Long-term Schwann cells were plated at a density of 5000 cells/cover slip.

### Cell culture

Schwann cells prepared from P1 sciatic nerves as described above were cultured in a 25cm<sup>2</sup> flask for 3 days with DME containing 10% calf serum supplemented with penicillin (100 IU/ml) streptomycin (100µg/ml) and glutamine (2mM) with 10<sup>-5</sup>M cytosine arabinoside to remove fibroblasts before passaging onto PLL or laminin (1µg/ml) coated 13mm glass coverslips, and culturing in serum-free medium. In some experiments cells were immediately plated onto PLL coverslips or laminin coated coverslips and cultured in either serum-free medium or DME containing 10% calf serum at 37°C 5%CO<sub>2</sub>, 95% air.

### Passaging cell cultures

Cells were removed from the culture dish using 50µl of 0.25% trypsin in 2ml of calcium- and magnesium-free MEMH for 10 min at 37°C in 5% CO<sub>2</sub>/95% air. Detached cells were pelleted by centrifugation at 500xg for 10 min. The pellets were resuspended in DME containing 10% CS then plated at between 10000 and 20000 cells/well on PLL or laminin coverslips.

### Dried cell preparations

Schwann cells prepared as described above were resuspended in a minimal volume of medium (50–100µl). 5–20µl were spread on gelatin-coated glass microscope slides with a plastic pipette tip and left to dry for a minimum of 2 hr at room temperature. Slides were then rehydrated in 4% paraformaldehyde and immunolabelled immediately or stored dessicated overnight at 4°C.

### Substratum coating of coverslips

Sterile 13mm diameter glass coverslips were incubated with PLL (1mg/ml) for 24hr at room temperature, washed with 12 changes of sterile distilled water over a 4 day period then allowed to dry. Laminin coated coverslips were prepared by incubating PLL coated coverslips in 50 $\mu$ l of laminin solution (20ng/ml in DME) for 1 hr at room temperature. Laminin was removed immediately prior to plating of Schwann cells.

### Serum-free medium

Serum-free medium used in this study was a modification of the medium of Bottenstein and Sato, (1979). DME was supplemented with transferrin (50  $\mu$ g/ml), progesterone (60 $\mu$ g/ml), putrescine (1.6 $\mu$ g/ml), insulin (5 $\mu$ g/ml), thyroxine (0.4 $\mu$ g/ml), selenium (100 $\mu$ g/ml), triiodothyronine (0.1  $\mu$ g/ml), BSA (0.3mg/ml), penicillin 100 IU/ml, glutamine 2mM and streptomycin 100  $\mu$ g/ml. In chapter 4 serum-free medium lacking insulin was used.

### Teased nerve preparations

Sympathetic trunk, sciatic or vagus nerves from varying age rats were excised and desheathed. Sections of nerve were then teased gently onto gelatin-coated microscope slides in a drop of PBS using 23 gauge needles as previously described (Jessen and Mirsky, 1984). The teased nerves were allowed to air dry for several hours, or stored dessicated at 4<sup>0</sup>C overnight before immunofluorescent labelling.

### Wax sections

Tissues for wax sectioning were removed from the animal and immediately fixed in 4% paraformaldehyde for 4hr at 4<sup>0</sup>C. After fixation tissue were processed through 80,90 and 100% ethanol for 30 min at each strength then embedded in polyester wax at 40<sup>0</sup>C. Sections of 1–7 $\mu$  were cut at 4<sup>0</sup>C and mounted onto PLL coated slides by Rory Curtis.

### Dilution of antibodies

Antibodies used on live cell cultures were diluted in MEMH containing 10% calf serum. Antibodies used on sections and fixed cells were diluted in PBS containing 0.1M lysine, 0.2% sodium azide and 10%

calf serum.

### BrdU Incorporation

Schwann cells seeded in DME containing 10% calf serum were washed 3 times in serum-free medium then cultured in serum-free medium with candidate mitogens. BrdU ( $10^{-5}$ M) was introduced into the culture medium for the final 24hr of 48hr experiments. Cultures were fixed for 10 min in 2% paraformaldehyde, permeabilized for 10min at  $-20^{\circ}\text{C}$  with methanol then treated with 2N HCl for 10 min to followed by a 10 min incubation with 0.1M sodium borate pH 8.5 to neutralize the acid, then coverslips were incubated with anti-BrdU (1:30) and anti-S100 (1:800), to identify Schwann cells, together in PBS containing 0.1% Triton X 100 for 40 min. After washing the coverslips were incubated with rhodamine conjugated goat anti-mouse immunoglobulins (pre absorbed with rabbit immunoglobulins) (1:100) and fluorescein conjugated goat anti-rabbit immunoglobulins (pre absorbed with mouse immunoglobulins) (1:100) together for a further 30 min, washed and mounted in Citifluor anti-fade mounting medium.

### Protein estimation

Cell and tissue samples used for electrophoresis and cAMP measurements were assayed for protein using the method of Bradford, (1976) and a Bio Rad protein assay kit with immunoglobulin as standard.

### Microscopy

Cells, teased nerves, whole mount preparations and wax sections were viewed for immunofluorescence with a Zeiss microscope using x 10, x 20, x 25, x 40 dry or x 63 oil immersion phase-contrast lenses, epi-illumination and rhodamine or fluorescein optics.

### Quantification

All results were based on a minimum of 3 separate experiments using duplicate, triplicate or quadruplicate coverslips. At least 200 cells were counted per coverslip to determine the proportion of BrdU labelled S100 positive cells.

## CHAPTER 3

THE INTERACTION BETWEEN cAMP ELEVATION, IDENTIFIED  
GROWTH FACTORS AND SERUM COMPONENTS IN  
REGULATING SCHWANN CELL GROWTH.



## INTRODUCTION

The Schwann cell is the major glial cell of peripheral nerve trunks, surrounding all nerve fibres and in many cases wrapping concentrically around the axon to form the myelin sheath. Understanding the factors controlling proliferation of this cell is of utmost importance when considering the maintenance of integrity and development of the peripheral nervous system. During early development Schwann cells migrate and proliferate along newly formed axons. High rates of division occur until a few days after birth when rates fall gradually coinciding with the onset of myelination (Brown and Asbury, 1981; Friede and Samorajski, 1968). Adult Schwann cells however are normally quiescent, proliferating only when nerve injury occurs or a Schwann cell tumour arises. *In vitro* Schwann cells normally divide very slowly in serum-containing medium (Raff et al., 1978; Brockes et al., 1980; Porter et al., 1986). However, if maintained in culture for a period of 2 – 3 months without passaging they will immortalize, being able to proliferate in the absence of exogenous growth factors (Eccleston et al., in press). Continual stimulation of Schwann cells with forskolin and glial growth factor for several months also results in an immortalized cell (Porter et al., 1987) which will, when injected into a syngeneic rat, form a tumour resembling a Schwannoma (Langford et al., 1988).

The factors required to stimulate mitosis in the Schwann cell have been analysed in many studies most of which have been complicated by the presence of serum and plasma in the culture medium eg. Eccleston et al., 1987; Davis and Stroobant, 1990, Raff et al., 1978; Ridley et al., 1989. The complex nature of serum and its multiplicity of actions renders difficult a complete analysis of factors required for Schwann cell DNA synthesis to occur. Therefore in this study serum-free medium has been used to analyse the mitogen requirements of short-term Schwann cells. Those mitogens found to be effective were then tested on long-term cultured Schwann cells to help us understand the mechanisms underlying Schwann cell growth control.

Results show that a combination of elevated cAMP and a growth factor are required to stimulate mitosis in short-term cultured Schwann cells whereas a single growth factor, but not exogenously added cAMP,

will stimulate division in long-term cultured Schwann cells. This can be directly related to the fact that intracellular levels of cAMP are elevated in long-term cultured Schwann cell which can therefore undergo mitosis in response to various growth factors known to stimulate mitosis in short-term cultured Schwann cells.

## METHODS

### Measurement of cAMP

Purified Schwann cells were placed onto PLL coated 4 well plates at 100,000 cells per well in DME containing 10% calf serum. After 7hr in culture medium, the medium was aspirated from each well and the cells frozen onto the dish with liquid nitrogen. 200 $\mu$ l of ice cold 5% trichloroacetic acid (TCA) were added and the cells frozen and thawed four times. The cells were scraped from the wells and centrifuged for 2 min at 14,000 x g. Supernatants were lyophilized then assayed for cAMP using a detection kit from Amersham International plc employing the acetylation procedure to increase sensitivity. TCA precipitated pellets were used for protein estimation.

### Mitogen screening

All the factors listed in table 1 were tested alone in serum free medium with GGF (0.9 $\mu$ g/ml) or with forskolin (0.1–20 $\mu$ M). In all cases where a dose range is given at least three concentrations of factor were tested and at least two different concentrations of forskolin were used.

## RESULTS

### Mitogens for Short-term cultured Schwann cell in serum-free medium.

Of the extensive list of potential mitogens screened in serum-free medium (Table 3.0) none of the factors alone were found to act as Schwann cell mitogens. Factors were screened with either GGF or forskolin. Only combinations of the adenylate cyclase activator forskolin with PDGF, GGF or bFGF resulted in DNA synthesis ( Fig.3.0). Similar results were obtained when DBcAMP ( $10^{-5}$  M ) was used to elevate intracellular cAMP levels instead of forskolin. GGF and forskolin were also able to cause dose dependant DNA synthesis when the Schwann cells were prepared and seeded in serum-free medium (data not shown). This indicates that any traces of serum remaining attached to coverslips after washing are not crucial for Schwann cell DNA synthesis. PDGF and bFGF were not tested in this manner. No single agent was able to initiate DNA synthesis in 48hrs. Agents that elevate cAMP were able to stimulate low levels of DNA synthesis (labelling index of 3-4%) in serum-free medium when used for longer time periods (> 48hr). TGF- $\beta_1$  (1-10 ng/ml) has previously shown to be a Schwann cell mitogen in serum containing medium (Eccleston et al., 1989; Ridley et al., 1989). In this study however TGF- $\beta_1$  did not stimulate DNA synthesis in serum-free medium when used alone or in combination with forskolin (0.1-10 $\mu$ M).

### Mitogens for Long-term cultured Schwann cells in serum free medium.

Only those combinations of mitogens that caused DNA synthesis in short-term Schwann cells were tested on long-term Schwann cells. These factors were applied singly to the the long-term cultures. The results show that long-term Schwann cells, unlike short-term Schwann cells, are able to proliferate in serum-free medium. This division rate can be enhanced by the polypeptide growth factors PDGF (5 ng/ml), GGF (0.9 $\mu$ g/ml) and FGF (100 ng/ml) but was not effected by DBcAMP ( $10^{-5}$  M ) or forskolin 2 $\mu$ M (Fig.3.0). As reported for short-term Schwann cells TGF- $\beta_1$  (2.5ng/ml) was unable to stimulate DNA synthesis of long-term Schwann cells.

#### cAMP levels in short- and long-term cultured Schwann cells

cAMP levels were measured in short- and long-term Schwann cell cultures. Long-term cultured Schwann cells had a 2–3 fold increase in intracellular cAMP compared to short-term cultures (Table 3.1).

TABLE 3.0

**FACTORS SCREENED AS POTENTIAL SCHWANN CELL MITOGENS**

<u>FACTOR</u>	<u>CONCENTRATION</u>	<u>ACTIVE WITH FORSKOLIN</u>	<u>ACTIVE WITH GLIAL GROWTH FACTOR</u>
IL1	1-200ng/ml		
IL2	1-500U/ml		
IL4	20-200U/ml		
EGF	1ng/ml		
PDGF	1-5ng/ml	*	
bFGF	5-100ng/ml	*	
GGF	0.1-4μg/ml	*	
NGF	0.2-1μg/ml		
TGF-β <sub>1</sub>	1-10ng/ml		
TNF	300 U		
5HT	10 <sup>-6</sup> -10 <sup>-8</sup> M		
α, β METHYLENE ATP	10 <sup>-6</sup> -10 <sup>-9</sup> M		
ADENOSINE	10 <sup>-6</sup> -10 <sup>-9</sup> M		
2 METHYLTHIO ATP	10 <sup>-7</sup> -10 <sup>-9</sup> M		
GABA	10 <sup>-5</sup> -10 <sup>-8</sup> M		

<u>FACTOR</u>	<u>CONCENTRATION</u>	<u>ACTIVE WITH FORSKOLIN</u>	<u>ACTIVE WITH GLIAL GROWTH FACTOR</u>
GLYCINE	$10^{-5}$ – $10^{-8}$ M		
PHENYLEPHERINE	$10^{-4}$ – $10^{-6}$ M		
CARBACHOL	$10^{-4}$ – $10^{-9}$ M		
ISOPRENALIN	$10^{-3}$ – $10^{-7}$ M		
DOPAMINE	$10^{-5}$ – $10^{-9}$ M		
HISTAMINE	$10^{-5}$ – $10^{-9}$ M		
CGRP	$10^{-6}$ M		
BOMBESIN	$10^{-8}$ – $10^{-9}$ M		
NEUROMEDIN	$10^{-8}$ – $10^{-9}$ M		
SOMATOSTATIN	$10^{-5}$ – $10^{-7}$ M		
FORSKOLIN	$10^{-5}$ – $10^{-8}$ M		*
DBcAMP	$10^{-3}$ – $10^{-5}$ M		*
ANP	$10^{-6}$ M		

TABLE 3.0. Each of the agents listed above was tested on short-term Schwann cell cultures in serum-free medium with either GGF (0.9 $\mu$ g/ml) or forskolin (0.1–20 $\mu$ M) as described in the text.

**FIGURE 3.0.** FGF,GGF and PDGF each synergize with forskolin to cause DNA synthesis in Schwann cells. Short-term Schwann cell cultures were incubated in serum-free medium for 48 hr with various concentrations of forskolin together with (A) FGF either 100 ng/ml (◻) or 50 ng/ml (◆); (B) GGF either 1.8 $\mu$ g/ml (◻) or 0.9 $\mu$ g/ml (◆); (C) PDGF either 5ng/ml (◆) or 2.5ng/ml (◻). DNA synthesis was measured by incorporation of BrdU over the last 24 hr . Each experimental value represents the mean of at least three separate experiments  $\pm$  SEM.



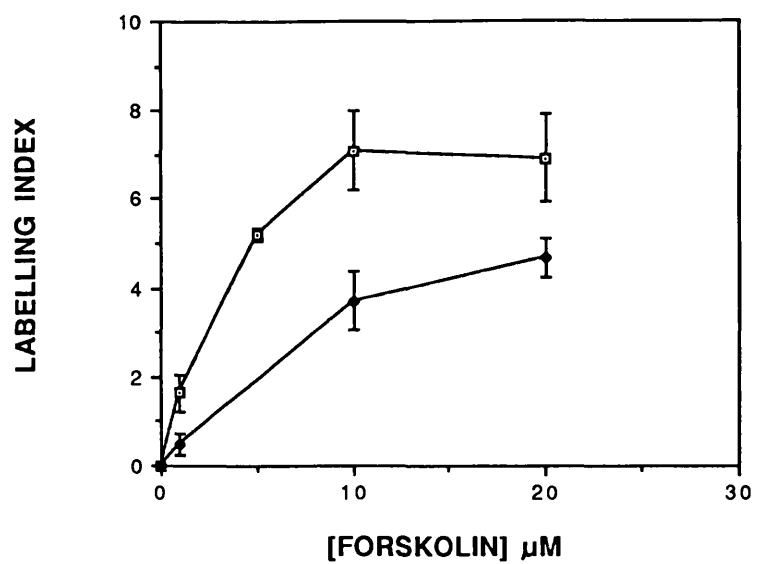
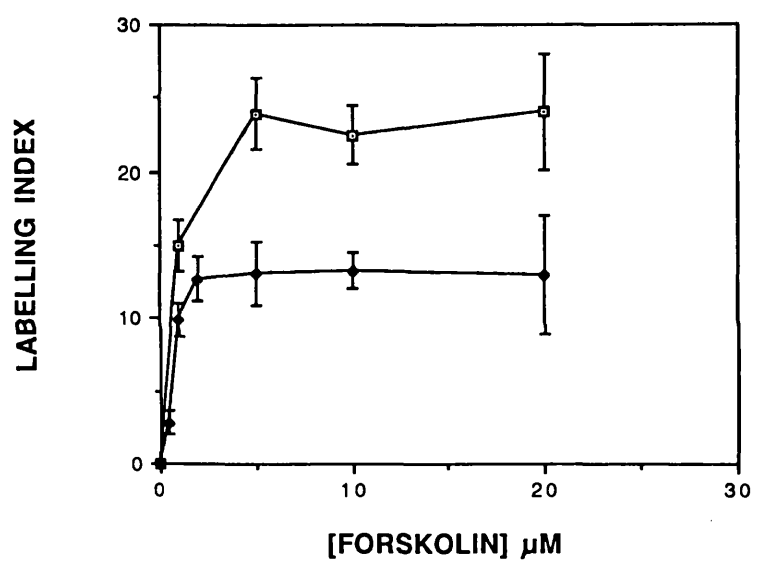
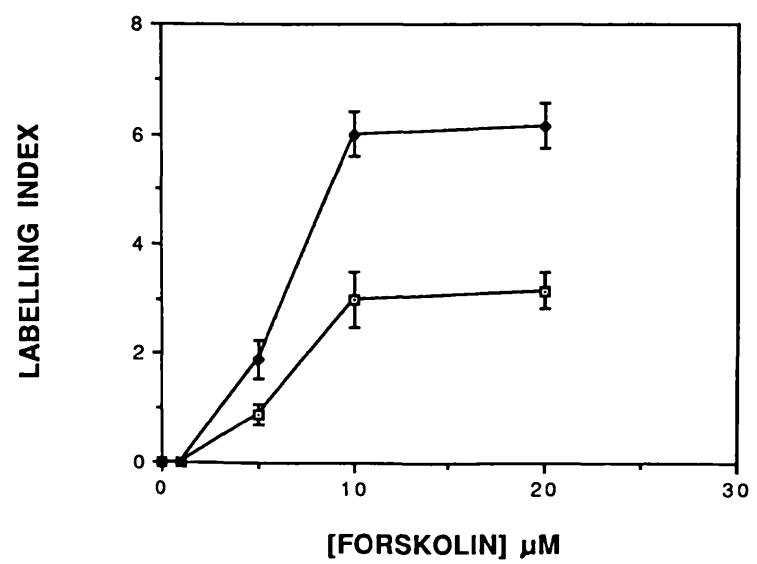
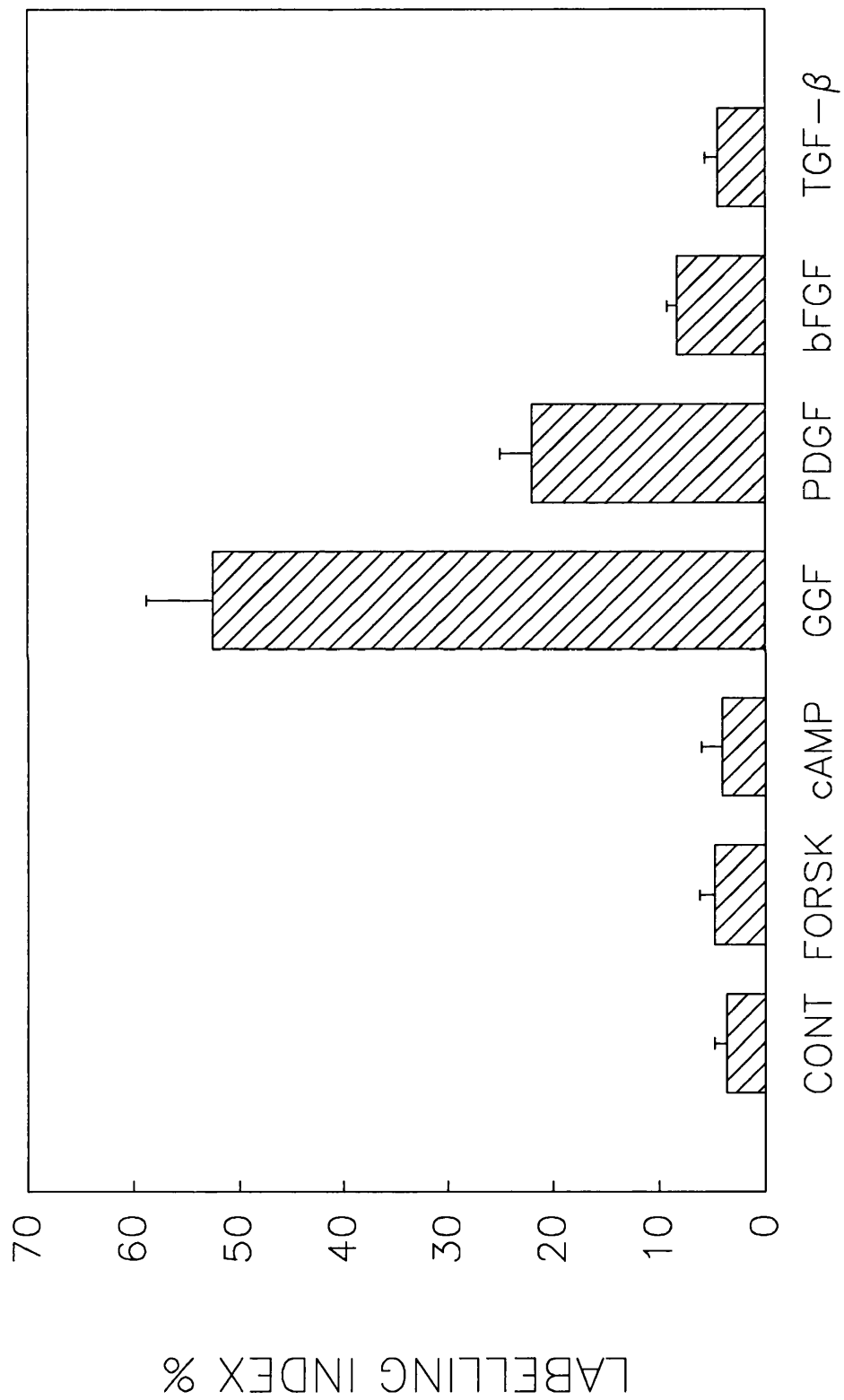
**A****B****C**

FIGURE 3.1. FGF (100ng/ml), GGF (0.9 $\mu$ g/ml) and PDGF (5 ng/ml) but neither cAMP (1 x 10<sup>-5</sup>M), forskolin (2 $\mu$ M) nor TGF- $\beta_1$  (2.5ng/ml) stimulate DNA synthesis in long-term Schwann cell cultures. BrdU was added for the last 24hr of culture. Each point represents the mean of at least three separate experiments. In all cases  $p < 0.01$ .



**TABLE 3.1**

**Intracellular cAMP is elevated in long-term cultured Schwann cells.**

=====		
Schwann cells	fmol/100,000 cells	fmol/ $\mu$ g protein
=====		
Short-term	196 $\pm$ 15.9 (n=5)	6.9 $\pm$ 0.8
Long-term	429 $\pm$ 45 (n=4)	19.0 $\pm$ 1.3
=====		

Short- or long-term cultured Schwann cells were trypsinised and seeded on PLL coverslips at a density 100,000 cells/well for 7 hr. Samples were then prepared as described in the text. Results show mean  $\pm$  SEM; n, number of samples from a representative experiment.  $p < 0.02$ .

## DISCUSSION

It is clear that short-term cultured Schwann cell in serum-free medium are quiescent and that they do not divide in response to a single growth factor. Rather the combination of a growth factor (bFGF, PDGF or GGF) with an agent that elevates intracellular cAMP is required. Interestingly, TGF- $\beta_1$  was unable to initiate DNA synthesis in serum-free medium, even when coupled with varying amounts of forskolin. Studies of Eccleston et al., 1989 and Ridley et al., 1989 have demonstrated that in serum-containing medium TGF- $\beta_1$  is a mitogen for Schwann cells. This effect could be enhanced when low levels of forskolin were used as a co-mitogen (Ridley et al., 1989). This suggests that in order for TGF- $\beta_1$  to act as a Schwann cell mitogen the presence of an as yet unidentified serum factor is required, although serum factors are not necessary for the mitogenic effect of bFGF or PDGF. Previous studies carried out in serum-containing medium have not clearly established the requirement of a cAMP elevating agent for Schwann cell mitosis to occur. Studies of Ratner et al., (1988), Eccleston et al., (1987,1990, in press) and Yong et al., (1988) have demonstrated that FGF and PDGF are mitogens for Schwann cells when used in isolation in serum-containing medium, whereas in other studies these growth factors did not cause DNA synthesis, (Salzer and Bunge, 1980 ; Davis and Stroobant, 1990; Weinmaster and Lemke, 1990). The use of the adenylate cyclase activator forskolin (Seamon and Daly, 1986) as a co-mitogen has however potentiated the response to these growth factors in cultures where the exogenously added growth factors alone had no effect (Weinmaster and Lemke, 1990; Davis and Stroobant, 1990). The apparent discrepancies between these studies are probably due to the use of different experimental regimes and sera. Some sera, for instance may contain agents that raise cAMP above basal levels. The presence of forskolin is not required to unveil the mitogenic effect of GGF in serum-containing medium but has been shown to potentiate effects observed (Ridley et al., 1989; Raff et al., 1978; Davis and Stroobant, 1990).

These observations in serum-free medium suggest that the short-term Schwann cell, like the Swiss 3T3 cell, requires the activation of at least 2 intracellular messenger pathways in order for DNA synthesis to occur

(Rozengurt, 1986). One of these pathways appears to act via intracellular cAMP. Evidence of Meador-Woodruff et al., (1984), Saunders and DeVries (1988a, 1988b), and Saunders et al., (1989), using axolemma and myelin fractions show that Schwann cell mitosis in response to these factors involves phosphoinositide metabolism, diacylglycerol production, protein kinase C activation and an increase in intracellular pH. These data indicate that in addition to cAMP elevation phosphoinositide metabolism and protein kinase C activation are required for Schwann cell mitosis to occur. Earlier studies carried out in serum-free medium have shown that both neurites (Salzer and Bunge, 1980) and axolemmal fractions (Sobue et al., 1983) can cause Schwann cell mitosis, in both these cases however, it is likely that more than one growth factor is involved. Another study has reported that cholera toxin, urokinase and fibronectin are all Schwann cell mitogens when applied singly in serum-free medium for 72hrs (Baron van Evercooren et al., 1987). Inconsistencies noted between studies may also variance due to the longer observation period used or be due to differences in the extent of washing of cell cultures to remove traces of serum before application of the mitogen in question. In this respect we have shown that it is possible to stimulate Schwann cell mitosis in serum-free medium by GGF and forskolin even when cells are prepared and seeded without serum.

The long-term cultured cell Schwann clearly differs from its short-term counterpart in that it is able to proliferate in serum-free medium without the addition of exogenous growth factors. It is also able to undergo enhanced proliferation rates in response to the polypeptide growth factors PDGF, FGF or GGF when used alone and is not stimulated to divide by agents known to elevate intracellular cAMP. Comparisons of the intracellular cAMP levels of the two cell types revealed that the long-term cultured Schwann cell has a constitutively higher basal cAMP level than the short-term cell. This observation may offer an explanation for the reduced growth factor requirements of long-term cultures. If, as has been demonstrated here activation of two intracellular messenger pathways are required for DNA synthesis, and the long-term Schwann cell has elevated intracellular cAMP levels, then application of a single mitogen that acts via a different second messenger pathway would be sufficient for DNA synthesis to occur. Conversely, as shown, further

stimulation of the adenylate cyclase pathway alone may not enhance division rates. An explanation is still required, however, for the proliferation of the long-term cultured Schwann cell observed in serum-free medium. This could be attributed to production of an autocrine growth factor(s) by this cell. This factor could stimulate a second messenger pathway that would couple with the already elevated cAMP resulting in DNA synthesis. Indeed Porter et al., (1987) reported that both mitogen treated Schwann cells and immortalized Schwann cells release autocrine growth factors and I have shown that both short- and long-term Schwann cells secrete a PDGF like molecule in similar amounts. Antibody blocking studies have demonstrated that this endogenous PDGF can act in an autocrine manner on long-term cells (Eccleston et al., 1990). The lack of response of the short-term cultured Schwann cell to PDGF alone in serum-free medium could be solely attributed to its lower intracellular cAMP levels. If this were the case one might expect application of a cAMP elevating agent alone to short-term Schwann cells in serum-free medium to result in DNA synthesis. These results show that in a 48 hr time period no Schwann cell division is seen in response to these agents. However, when studies were carried out over longer time period a small amount of cell division was observed. Since elevation of intracellular cAMP upregulates Schwann cell PDGF receptor mRNA and protein (Weinmaster and Lemke, 1990) it has been suggested that the observed mitogenic cooperativity between polypeptide growth factors and cAMP might result from increased expression of the corresponding growth factor receptor. In the short-term Schwann cell the observed lag in PDGF receptor gene expression induced by cAMP elevating agents may account for the delayed initiation of DNA synthesis seen in response to these agents. PDGF secreted by the cells themselves would only be able to initiate DNA synthesis when receptor levels were high enough. In other cells such as the Swiss 3T3 cell however, the synergistic effects of cAMP and polypeptide growth factors are not dependant on upregulation of growth factor receptors (Rozengurt, 1986).

At present the mechanisms responsible for producing elevated cAMP levels in long-term Schwann cells are unknown. Long-term Schwann cells may secrete an agent that acts in an autocrine manner to elevate intracellular cAMP. A factor that can elevate intracellular cAMP and

couple with growth factors to invoke DNA synthesis in the Schwann cell has, however, not yet been found. Recent studies (Yasuda et al., 1988) have shown that vasointestinal peptide (VIP), secretin and  $\beta$  adrenergic agonists markedly elevate Schwann cell cAMP levels but my studies and those of others (Davis and Stroobant, 1990; Raff et al., 1978) in which these substances, and many others known to raise intracellular cAMP levels in other cell types were screened, have not revealed any mitogenic effect. Elevation of cAMP levels *per se* may not be sufficient to stimulate DNA synthesis. The kinetics of the accumulation and differences in the intracellular localization of cAMP may need to be considered. Indeed Barsony and Marx, (1990) have shown using immunohistochemical techniques that the site of accumulation of cAMP varies depending upon the agonist used to stimulate cAMP production. Forskolin, for example, can cause nuclear accumulation of cAMP whereas calcitonin causes mainly cytoplasmic and perinuclear accumulation. Alternatively, the increased levels of cAMP detected in long-term cells could be due to an oncogenic mutation. Such a mutation has been described (Vallar et al., 1988; Lundis et al., 1989) in a subset of pituitary tumours, the mutation residing in the adenylate cyclase control protein Gs. A third possibility is that elevated cAMP levels found in the long-term cultured Schwann cell compared to the short-term cultured cell is not due to active stimulation of adenylate cyclase in the long-term cell but due to inhibition of adenylate cyclase in the short-term cell. The possibility that the Schwann cell may produce growth inhibitors has recently been addressed by Eccleston et al., (in press) and Muir et al., (1990). In these studies a factor has been described that inhibits short-term but not long-term Schwann cell mitosis. The mechanism of action of this inhibitor is at present unknown but it is tempting to speculate that it acts by inhibiting adenylate cyclase.

Changes in the molecular phenotype of the long-term Schwann cell, when compared to the short-term cell (Eccleston et al., in press), may also be related to cAMP levels. Differences such as low level P<sub>0</sub> expression, fibronectin expression and reduced NGF receptor levels (Eccleston et al., in press) have all been reported to be induced by elevating cAMP levels in short-term Schwann cells (Baron van Evercooren et al., 1986; Morgan et al., 1991; Monuko et al., 1988). The differential effects of TGF- $\beta$ , on short- and long-term cells may also be related to differences in



intracellular cAMP levels. The mitotic response of short-term Schwann cells to TGF- $\beta$  is regulated by forskolin levels. Maximal synergistic effects are seen at 0.5 $\mu$ M forskolin being completely abolished by 5 $\mu$ M forskolin where basal rates of DNA synthesis are inhibited (Ridley et al., 1989). In long-term Schwann cells where cAMP is constitutively elevated, TGF- $\beta$  has an inhibitory effect (Eccleston et al., 1989).

The sustained elevation of cAMP levels in the long-term Schwann cell may enable it to proliferate in serum-free medium in response to growth factors in an autocrine manner. This cell however is not fully transformed as judged by other criteria such as growth in soft agar and contact inhibition (Eccleston et al., 1990) and may thus represent an intermediate in the multi-step process of tumourigenesis. Two other Schwann cells types, the immortalized cell of Porter et al., (1987) and the SV40 Large T transfected cell of Ridley et al., (1988) are also defective in growth control and may also represent a intermediate tumour stage. In the latter case full transformation has been achieved by co-transfection with v-Ha ras. Whether these cells also have elevated cAMP levels is not known.

In summary this study shows that in serum-free medium it is necessary to elevate intracellular cAMP levels in order for DNA synthesis to occur in response to PDGF, FGF or GGF. The long-term Schwann cell has elevated basal levels of cAMP and therefore is able to undergo DNA synthesis in response to these growth factors without further cAMP elevation.

## CHAPTER 4

### ROLE OF IGF-I AS A SCHWANN CELL MITOGEN *IN VITRO* AND DISTRIBUTION OF IGF *IN VIVO*

## INTRODUCTION

The control of Schwann cell proliferation is a process crucial to the development and maintenance of the peripheral nerve. When these proliferative control mechanisms break down a Schwann cell tumour, such as a neurofibroma or Schwannoma, may arise.

It is now well established that the growth factors FGF, PDGF, GGF and TGF- $\beta$  are Schwann cell mitogens *in vitro* (Ratner et al., 1988; Raff et al., 1978; Krikorian et al., 1982; Ridley et al., 1989; Eccleston et al., 1989, 1990; Davis and Stroobant, 1990; Weinmaster and Lemke, 1990; and chapter 3). The mitogenic effects of these factors are potentiated by elevation of intracellular cAMP (Davis and Stroobant, 1990; Chapter 3) which may act by upregulating growth factor receptors (Weinmaster and Lemke, 1990). In addition Schwann cell mitosis is stimulated by sympathetic and sensory neurones (Wood and Bunge, 1975) and axonal membranes isolated from these nerves, PC12 cells (Ratner et al., 1984) and brain (Ratner et al., 1988; DeCosta and DeVries, 1989). In all these studies however, medium was used containing serum, plasma or high concentrations of insulin. One of the factors responsible for the mitogenic properties of serum on other cell types, and the factor which is mimicked by high insulin concentrations is IGF-I (Froesch et al., 1985; Rechler and Nissley, 1985). Thus it is plausible that any mitogenic effects of IGF-I on Schwann cells were masked in previous studies by the presence of serum.

Correlative evidence suggests that IGF-I may play a role in Schwann cell proliferation during nerve regeneration. Studies of Hansson et al., (1986, 1987) have shown that IGF-I immunoreactivity in Schwann cells rapidly increases after nerve injury, slightly preceding the period of maximal Schwann cell mitosis (Clemence et al., 1989; Pelligrino et al., 1986). Intense IGF-I immunoreactivity has also been detected in rapidly expanding, but not latent, neurofibromas (Hansson et al., 1988c). Moreover IGF-I and IGF-II have been described as mitogens for astrocytes (Han et al., 1987; Balloti et al., 1987), CNS glial cells that in certain respects resemble non-myelin-forming Schwann cells.

Using serum-free, insulin-free culture conditions this study demonstrates that IGF-I is a Schwann cell mitogen when used in

combination with forskolin and either FGF or PDGF. Furthermore this study shows that Schwann cell possess IGF receptors and that IGF immunoreactivity is present *in vivo* at a time of rapid Schwann cell proliferation ie during embryogenesis.

## METHODS

### IGF-type 2 receptor immunohistochemistry

Purified P1 schwann cells were plated at 10000 cells per well on PLL coverslips in DME containing 10% CS. After 24 hr in culture the medium was removed, the wells washed three times in MEMH and replaced with insulin-free serum-free medium. 24hr later cells were fixed for 20min in 4% paraformaldehyde, permeabilized with acetone at  $-20^{\circ}\text{C}$  for 4 min then labelled with rabbit polyclonal anti-IGF type 2 receptor C2 (1:2000) (Scott and Baxter, 1987) for 30 min. After washing in PBS coverslips were incubated in anti-rabbit biotin for 30min followed by fluorescein-streptavidin for 20 min. Control experiments were performed by substituting rabbit serum (1:2000) for anti-IGF type 2 receptor antibodies.

### Binding studies

Binding studies were carried out on short-term cultured Schwann cells using a modification of the method of Han et al., (1987). Cells were plated at a density of 100,000 cells per well in DME and 10% CS onto tissue culture plastic. Forty-eight hr before the start of an experiment the culture medium was removed, the wells washed 3 times with DME and the medium replaced with serum-free medium. Cells were incubated at  $4^{\circ}\text{C}$  in MEMH containing 1% BSA with radiolabelled IGF-I (100000 cpm) with or without varying amounts of cold IGF-I, or  $10^{-6}\text{M}$  insulin or  $10^{-9}\text{M}$  insulin for 6hr. At the end of the incubation time each well was washed five times with ice cold PBS then the cells were solubilized in 1M NaOH for 10-15 min. The solubilized material was collected. Each well was washed with 200 $\mu\text{l}$  of ice cold PBS which was combined with the solubilized material and counted in a gamma counter.

### Dried dissociated cells

Suspensions of Schwann cells from E14-16, P1 and adult rats prepared as described (General Methods) were spread on gelatin coated slides with a plastic pipette tip. Slides were left to dry then dessicated at  $4^{\circ}\text{C}$  overnight before immunolabelling. Before addition of antibody dried cell preparations and teased nerves were blocked with 5% fat free milk for 30 min .

### Preparation of dorsal root ganglion neurones

Dorsal root ganglia were removed from embryonic (E14), early postnatal (P1) and adult rats. Ganglia were dissociated in 0.25% trypsin, 0.4% collagenase for 90min at 37°C in 5% CO<sub>2</sub>/95% Air. The tissue was gently dissociated by triturating then centrifuged at 500xg for 10min with an equal volume of MEMH containing 10% CS. Cells were plated on laminin coated 13mm coverslips and cultured in DME containing 10% horse serum supplemented with nerve growth factor (0.2µg/ml), 10<sup>-5</sup>M cytosine arabinoside and 10<sup>-5</sup>M Fudr.

### IGF immunohistochemistry

All cells were fixed for 20min in 4% paraformaldehyde then permeabilized for 2min with 0.2% Triton X100 in PBS. Mouse monoclonal anti-IGF-I (Sm1.2) was used at a dilution of 1:200 for 1hr followed by biotin labelled goat anti-mouse immunoglobulin (1:200) (in PBS plus 10% calf serum, 0.01% sodium azide and 0.1M lysine) for 30min then fluorescein/streptavidin for 20min. Double labelling was performed with anti-S100 (1:1000) and rhodamine labelled goat anti-rabbit immunoglobulin (1:100). For early embryonic Schwann cells, sister cultures were labelled with anti-nerve growth factor receptor and anti-mouse rhodamine to identify Schwann cells.

### Controls for immunohistochemistry

Controls for immunohistochemistry consisted of omission of the primary antibody or substitution of mouse immunoglobulins (IgG1) for the primary antibody. In other experiments the diluted anti-IGF-I antiserum was incubated with a semi-pure preparation of IGF-I peptide (human) for 30 min at 37°C followed by overnight incubation at 4°C before immunolabelling was carried out.

### Electrophoresis and Western blotting

Schwann cells from postnatal day 1 rats were purified for 3 days as described above. The pure cultures were washed three times in MEMH then cultured for 2 days in serum-free medium. Cells were passaged in vercine containing 0.25% trypsin at 37°C for 10 min, then centrifuged for

10 min at 500 x g. The cell pellets formed were homogenized and solubilized in 0.1M Tris pH 8.8, 2% sodium dodecyl sulphate (SDS), 2mM EDTA, 2mM EGTA, containing 1mM PMSF. DRG samples were homogenized in the same way. Protein content was measured using a Bio Rad Kit (see General Methods) All samples were boiled for 2 min, centrifuged at 14000xg for 5 min then diluted 1:1 in 2x sample buffer (Laemmli, 1970) and re-boiled for 5 min. Approximately 35 $\mu$ g of samples were applied to 7.5–22.5% polyacrylamide gradient gels and subjected to electrophoresis as described by Laemmli (1970). Proteins were then electrophoretically transferred onto nitrocellulose overnight at 19 mA constant current. Lanes containing standards were excised and stained by amido black and the remainder of the nitrocellulose sheet was immunolabelled as follows. The nitrocellulose was blocked with 2.5% gelatin in Tris buffered saline (10mM Tris pH 7.3, 500mM NaCl) for 2 hr at room temperature and then incubated for 20hr at 4<sup>0</sup>C with mouse monoclonal anti-IGF-I (sm1.2) diluted 1:10000 or rabbit polyclonal anti-IGF-I (R557A) diluted 1:200,000 in PBS containing 1% BSA. The nitrocellulose was then washed in five changes of PBS for a total of 2hr before incubating with horse radish peroxidase linked anti-mouse, or anti-rabbit immunoglobulins diluted 1:1000 in PBS containing 1% BSA for 1 hr at room temperature. The blot was washed 5 times in PBS over a 1hr period then developed using diaminobenzidine as a chromagen in the presence of hydrogen peroxide.

## RESULTS

### IGF-I is Schwann cell mitogen.

Studies carried out in serum-free medium containing insulin revealed that bFGF, PDGF and GGF are Schwann cell mitogens provided intracellular cAMP levels are elevated (Chapter 3). As insulin at high concentrations can mimic the effects of IGF-I the mitogenic effects of IGF-I on Schwann cells were studied. As expected from observations in Chapter 3, IGF-I had no effect on Schwann cell DNA synthesis when used alone or when used in combination with forskolin (10 $\mu$ M). IGF-I was however able to induce DNA synthesis when combined with PDGF and forskolin. This effect was dose dependent and could be mimicked by high concentrations of insulin (1 $\mu$ M) (Fig 4.0). PDGF and forskolin were unable to cause Schwann cell mitosis in the absence of IGF-I or insulin. FGF and forskolin, on the other hand could stimulate DNA synthesis slightly (4%) in the absence of IGF-I (or insulin). This effect was nevertheless, potentiated by IGF-I in a dose dependent manner (Fig 4.0) or by 1 $\mu$ M insulin. Half maximal stimulation of DNA synthesis with both these growth factors was achieved by 6–8nM IGF-I. In contrast, DNA synthesis induced by GGF and forskolin was unaffected by IGF-I at all concentrations tested.

### Schwann cells express IGF receptors

Preliminary competitive binding studies (Table 4.0) showed that the Schwann cell IGF-I binding site has a higher affinity for IGF-I than for insulin. This indicated that the Schwann cell receptor was a type 1 IGF receptor. The Scatchard plot of the binding data for IGF-I (Scatchard, 1949) was curvilinear however suggesting two (or more) classes of receptor. More than two classes of receptor can rarely be resolved when only one unlabelled and labelled ligand are used (Glieman et al., 1985) Assuming that only two classes of receptor are present, the association constant of IGF-I for these receptors was about  $1.9 \times 10^9 \text{ M}^{-1}$  and  $0.06 \times 10^9 \text{ M}^{-1}$ . There were approximately  $1 \times 10^6$  high affinity IGF-I receptors per cell and approximately  $5 \times 10^5$  low affinity receptors per cell (Fig 4.1). Half maximal displacement of tracer binding could be achieved by 5nM IGF-I.



As the type 2 IGF receptor is primarily localized to the Golgi apparatus and secondarily to the cell surface (Valentino et al., 1988) Schwann cell cultures were permeabilized before immunolabelling. These studies showed that Schwann cells expressed low levels of type 2 IGF receptor (Fig 4.2). These receptors could only be detected on Schwann cells when biotinylated second antibodies were used. In contrast contaminating fibroblast were highly IGF type 2 receptor immunoreactive, majority of the label apparently being associated with the Golgi apparatus (Fig 4.2).

#### Schwann cell IGF expression is developmentally regulated

To establish whether IGF is expressed by Schwann cells *in vivo* when Schwann cells are proliferating, and is absent when Schwann cells are quiescent, 2–3hr cultures and dried dissociated cell preparations from varying age rats were used. In 2–3hr cultures IGF immunoreactivity was detected in Schwann cells from embryonic rats but not in Schwann cells from postnatal or adult rats (Fig 4.3). Identical results were obtained from dried dissociated cell preparations. Schwann cells from postnatal and adult rats were identified by morphology (short-term cultures only) or by S100 coexpression. Embryonic Schwann cells could not be unequivocally identified as a marker restricted to these cells *in vivo* does not exist. As all the cells in these preparations were immunoreactive however, it follows that all the embryonic Schwann cells must express IGF.

#### IGF expression is upregulated in tissue culture

Schwann cells, from P1 rats, were plated on PLL or laminin coated coverslips in serum-free or serum-containing medium, and labelled at various culture times up to 1 week. Schwann cells were identified by expression of S100 protein and/or by morphology. After 2–3 days in culture IGF immunoreactivity appeared in previously negative Schwann cells. All of the Schwann cells were IGF immunoreactive in some experiments whereas in others only a subpopulation were labelled (Fig 4.4). Immunoreactivity was detected on most or all cells at all later culture times (Fig 4.5). The immunoreactive IGF in these cultures was diffusely present throughout the cytoplasm. Neither the substratum nor the presence or absence of serum were shown to have any effect on IGF

expression.

IGF immunoreactivity was detected in all embryonic Schwann cells at all time points tested (up to 72hr). In both 2hr cultures and longer term cultures of embryonic Schwann cells two forms of IGF staining were seen. Many cells contained very bright granules of IGF immunoreactive material (Fig 4.3) whereas in a few cells faint IGF immunoreactivity was present in the cytoplasm. Neither the intensity of nor the appearance of the immunoreactivity varied with time in culture. Identification of embryonic Schwann cells, by comparing the morphology of the cells with NGF receptor labelled sister cultures, showed that cultures were over 90% pure.

#### IGF immunoreactivity is present in neurones

To determine whether neurones expressed immunoreactive IGF *in vivo* teased preparations were made from adult sciatic nerve. Axonal IGF immunoreactivity was found in unmyelinated axons (Fig 4.6) and on myelinated axons only at the node of Ranvier and at places where the myelin sheath was damaged (Fig 4.7). This latter observation suggests that the lack of immunoreactivity in myelinated axons was due to antibody penetration problems. No significantly immunoreactive Schwann cells were seen in these preparations. The presence of immunoreactivity in the unmyelinated axons and not the Schwann cells was determined by comparing the diameter of the IGF immunoreactive material with the diameter of the unmyelinated fibre and by examining the cytoplasm surrounding the Schwann cell nucleus.

To further establish the capability of neurones to express IGF cultures of sensory neurones were made from P1 DRG. After 2–4 weeks partially purified cultures were labelled with monoclonal anti-IGF-I. In these experiments all neuronal cell bodies were moderately IGF immunoreactive whereas axons and Schwann/ satellite cells were faintly labelled (Fig 4.8). Similar results were obtained from DRG cultures prepared from E16 and adult rats.

#### Controls

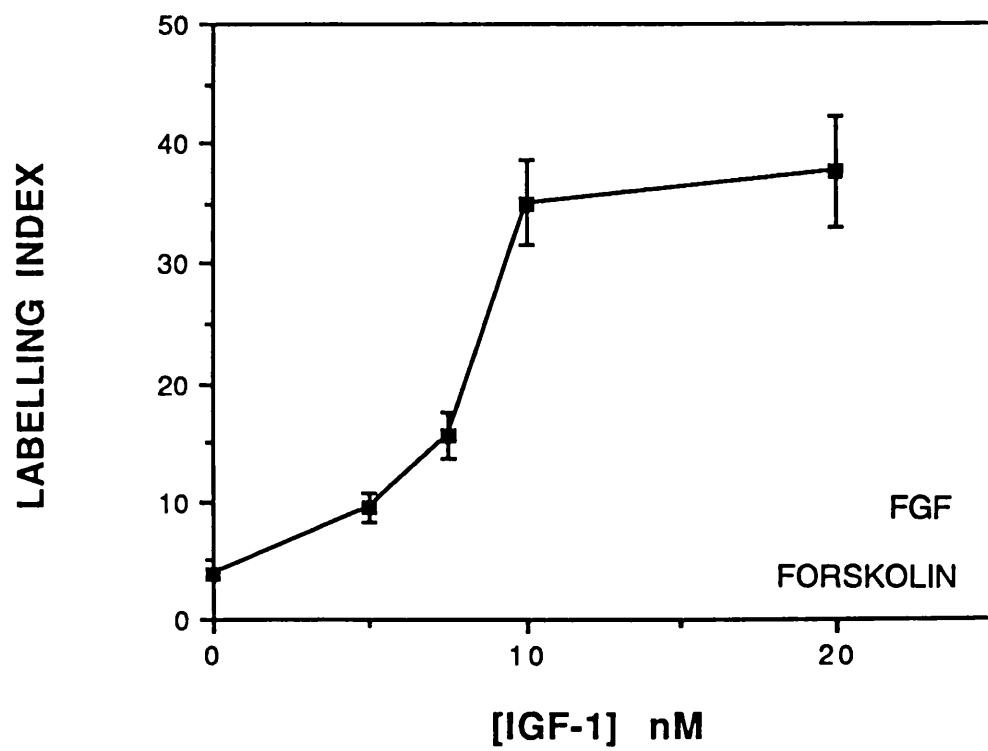
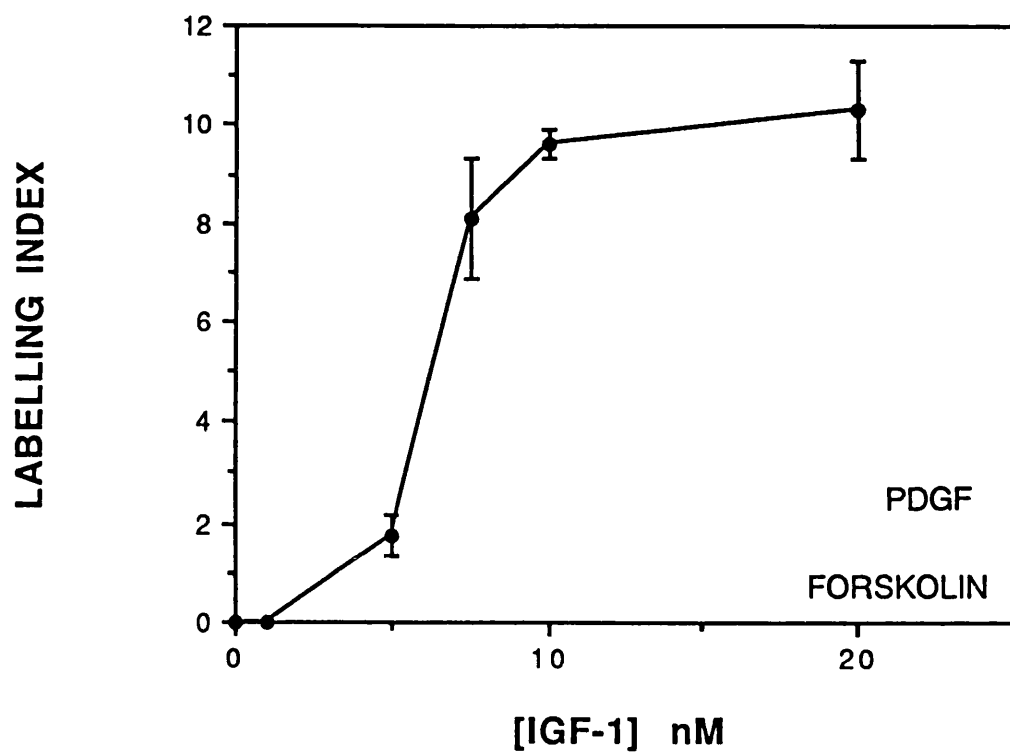
Several control experiments were carried out in immunohistochemical studies. In blocking studies 20 $\mu$ g /ml of semi-pure

human IGF completely blocked IGF staining. No IGF-I labelling was seen when primary antibody was omitted or when the primary antibody was replaced with control IgGs. The monoclonal anti-IGF-I (sm1.2) has a 1% cross reactivity with rat IGF-II (Clemmons and Van Wyk, 1983). Therefore although IGF immunoreactivity seen is probably due to IGF-I the possibility that the antibody is cross reacting with IGF-II cannot be eliminated.

#### High molecular weight forms of IGF immunoreactivity in the peripheral nervous system.?

Samples of P1 rat DRG and purified Schwann cells (4 days in culture) were electrophoresed on 7.5–22% gradient gels, blotted onto nitrocellulose and immunolabelled with either monoclonal anti-IGF-I (sm1.2) or polyclonal anti-IGF-I (R557A). No bands with the same relative mobility as IGF-I were seen. In all samples, 3 immunoreactive bands were seen with molecular weights of 11, 15 and 19kDa (Fig 4.9). Occasionally IGF immunoreactive bands with a molecular weight between 24–29kDa were detected. Identical bands were detected with both antibodies and were not seen in control experiments where the primary antibody was substituted with mouse or rabbit serum. 20 $\mu$ g of semi-pure human IGF-I showed a single band of molecular weight 7.5kDa.

FIGURE 4.0. IGF-I is a Schwann cell mitogen. FGF and PDGF synergize with forskolin and IGF-I to cause synthesis in Schwann cells. Schwann cell cultures from new born rats were incubated in serum-free medium for 48 hr with various concentrations of IGF-I together with PDGF 2.5 ng/ml and 10 $\mu$ M forskolin (●) (upper panel), or bFGF 100ng/ml and 10  $\mu$ M forskolin (⊗) (lower panel). DNA synthesis was measured by incorporation of BrdU over the last 24hr. Each experimental value represents the mean of at least three experiments.

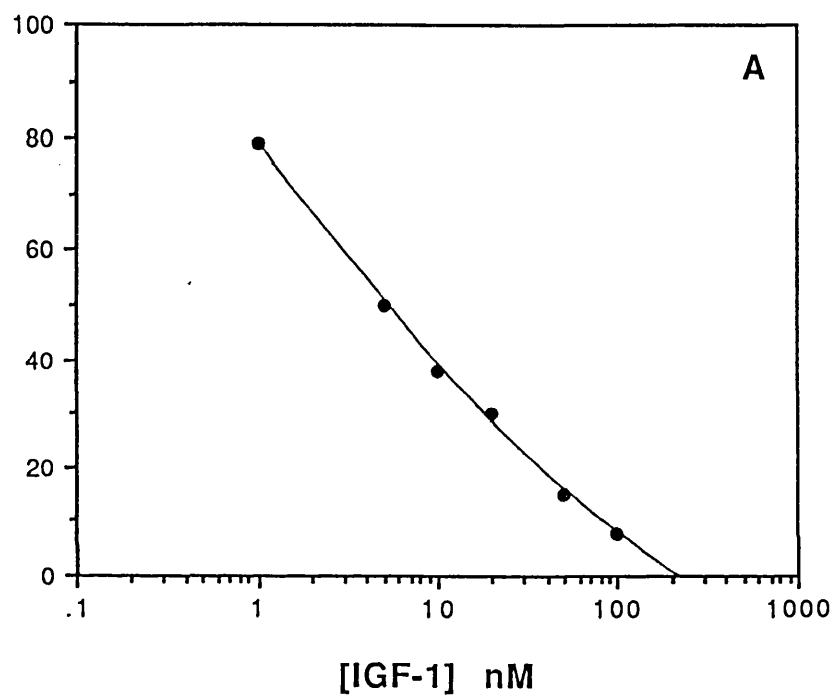


=====	
LIGAND	% BINDING
=====	
$^{125}\text{I}$ -IGF-I only	100.00
+ IGF-I $10^{-7}\text{M}$	$8.1 \pm 1.9$
+ INS $10^{-6}\text{M}$	$55.8 \pm 5$
+ INS $10^{-9}\text{M}$	$92.0 \pm 2$
=====	

Table 4.0. Binding of  $^{125}\text{I}$ -IGF-I to Schwann cells in 1 week-old cultures of new born rat sciatic nerve. Cells were incubated with  $^{125}\text{I}$ -IGF-I (100,000 cpm) with or without a 100 fold excess of unlabelled IGF-I, or with insulin for 6hr at  $4^{\circ}\text{C}$ . Binding was determined as described in Methods. Each result is the mean  $\pm$  S.E.M of 3 separate experiments.

FIGURE 4.1. Scatchard analysis of the binding of  $^{125}\text{I}$ -IGF-I to new born Schwann cells cultures, as determined by gamma counting. (A) The concentration dependence of  $^{125}\text{I}$ -IGF-I binding. Non specific binding (3%) was determined by adding  $1\ \mu\text{M}$  unlabelled IGF-I. (B). Scatchard analysis of data shown in (A). Each point is the mean of 3 replicates. The S.E.M of each data point was less than 5%. These data suggest that there is a high affinity binding site with a  $K_a$  of approximately  $2 \times 10^8\ \text{M}^{-1}$  and approximately  $1 \times 10^6$  receptors per cell. The low affinity binding site has a  $K_a$  of approximately  $0.6 \times 10^9\ \text{M}^{-1}$  and approximately  $6 \times 10^5$  receptors/cell.

% OF MAXIMUM 125-I-IGF-I BOUND



BOUND/FREE

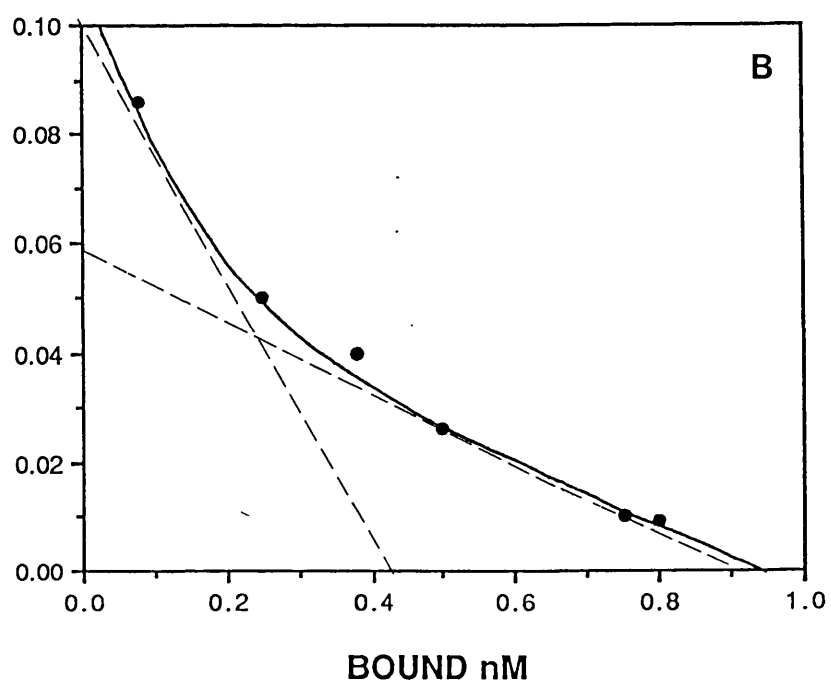




FIGURE 4.2 Schwann cells express IGF type 2 receptors. These photographs show cultured Schwann cells from P1 rat sciatic nerve. (A) phase contrast; (B) Fluorescein optics to visualize IGF type 2 receptor. Note the intense IGF-2 receptor immunoreactivity associated with the Golgi apparatus in fibroblasts (arrow) (C) Rhodamine optics to visualize NGF receptor. Bar = 10 $\mu$

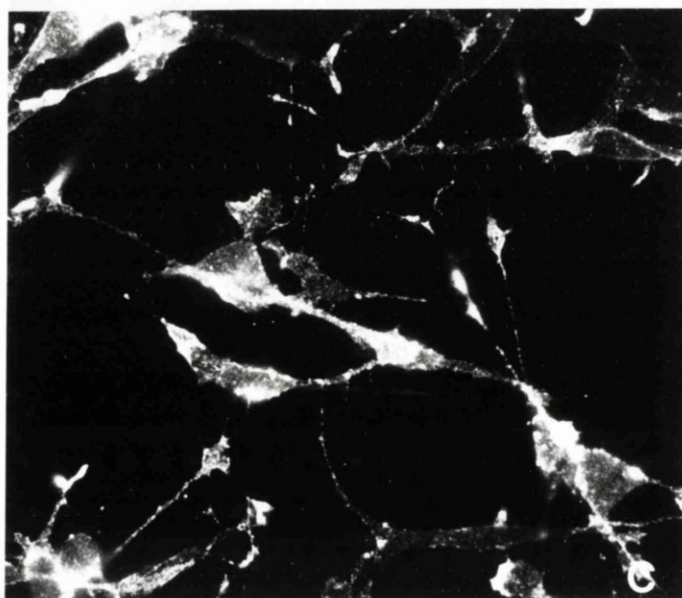
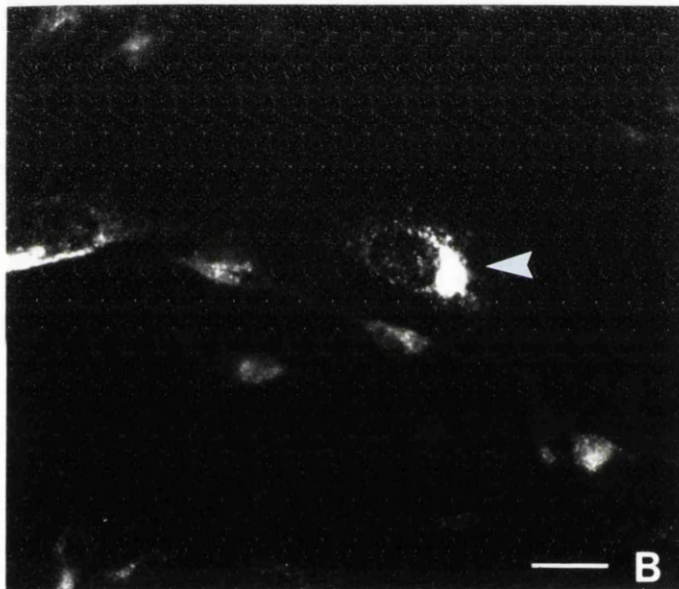
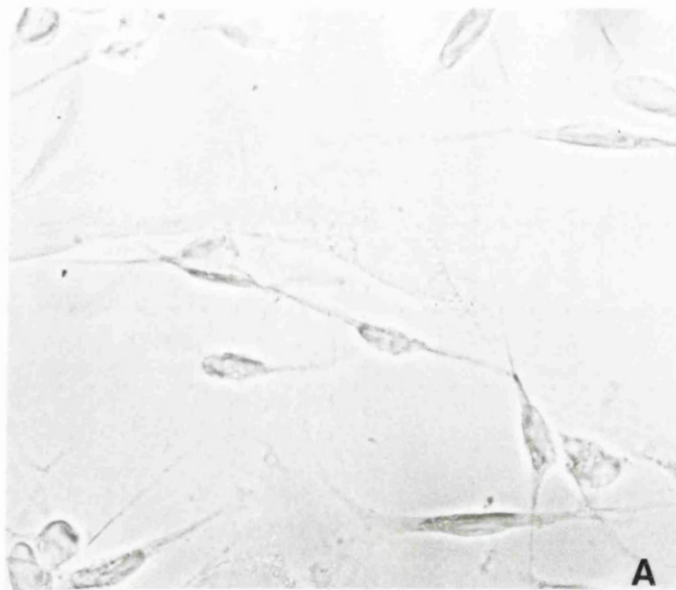


FIGURE 4.3. IGF immunoreactivity is downregulated in Schwann cells *in vivo*. These photographs show cells from E15 and P1 sciatic nerves after 2–3 hr in culture. Fluorescein optics were used to visualize IGF immunoreactivity in (A) E15 Schwann cells and (B) P1 Schwann cells . Bar = 10 $\mu$ .

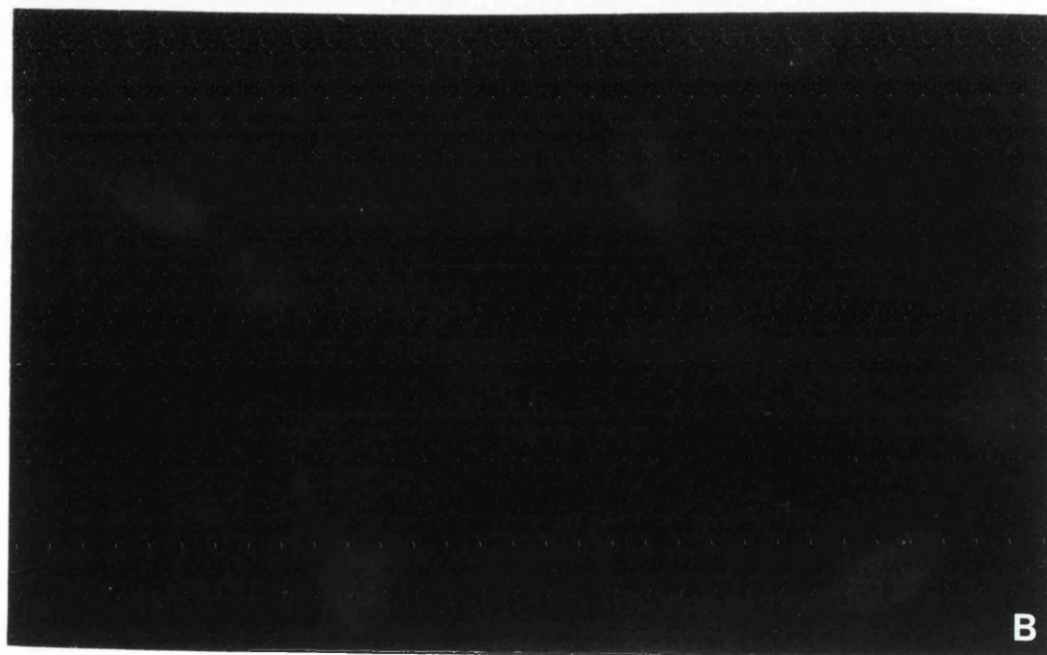
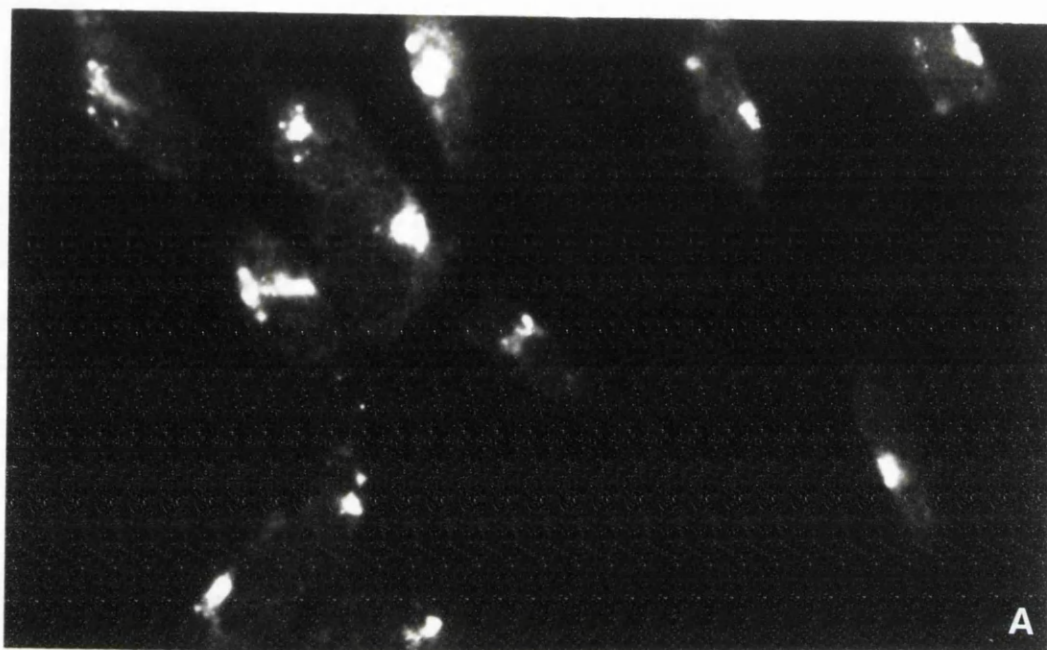


FIGURE 4.4. A subpopulation of Schwann cells express IGF immunoreactivity in after 2–3 days in culture. These photographs show P1 Schwann cells after 2 days in culture. (A) Fluorescein optics to visualize IGF; (B) phase contrast. Bar=10 $\mu$ .

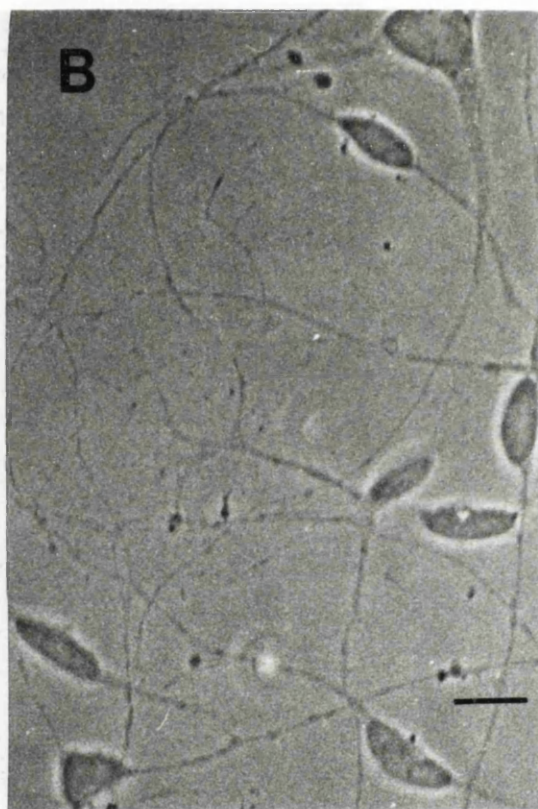
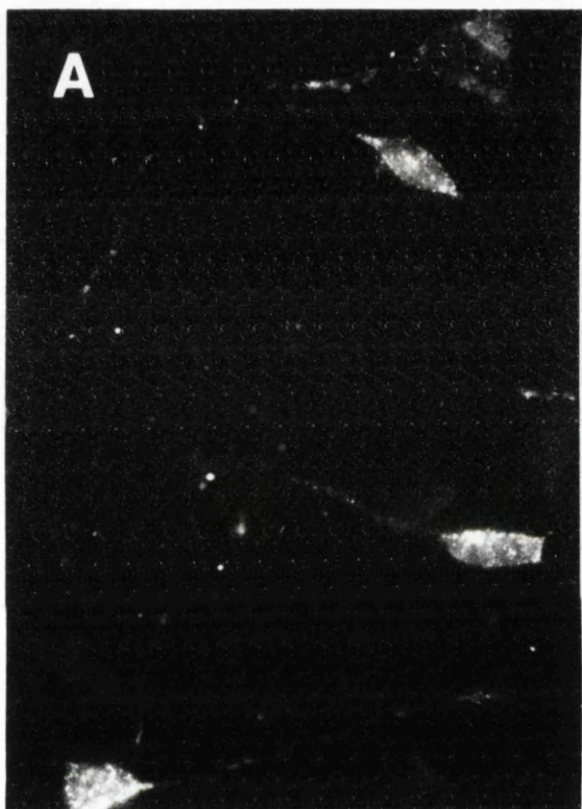


FIGURE 4.5. IGF immunoreactivity is expressed by all Schwann cells after 1 week in culture. These photographs show Schwann cells from P1 rats after 1 week in tissue culture. (A) Fluorescein optics to visualize IGF; (B) phase contrast . Bar= $10\mu$ .

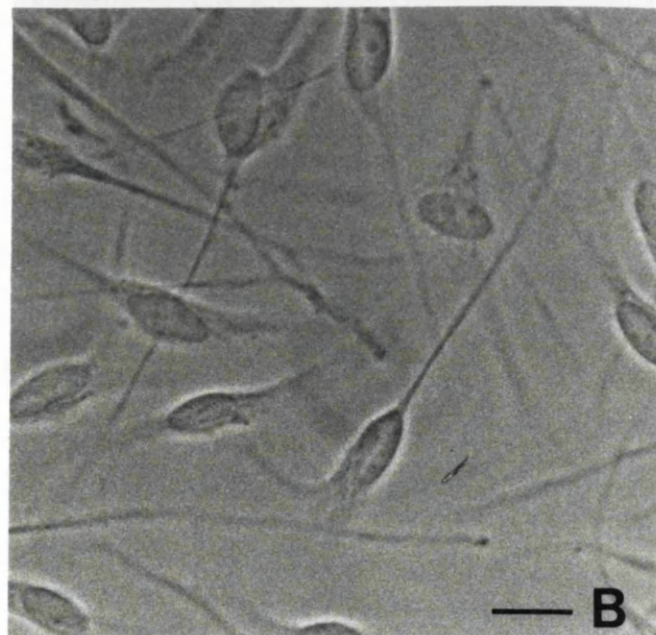
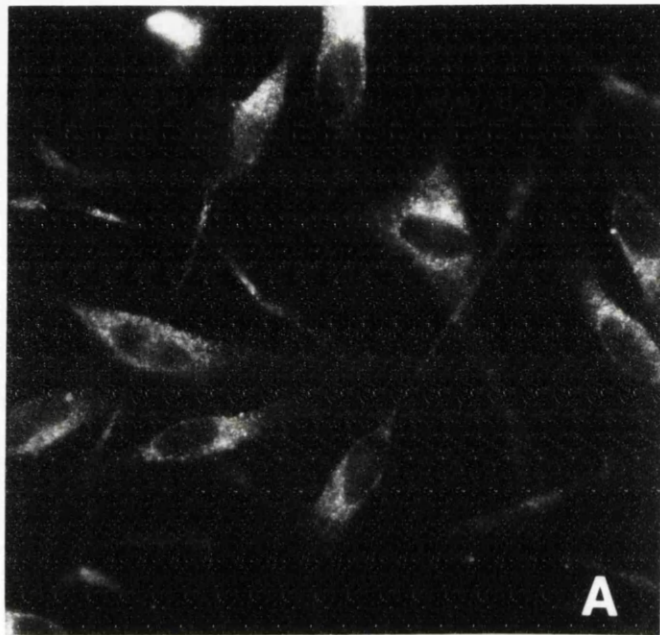
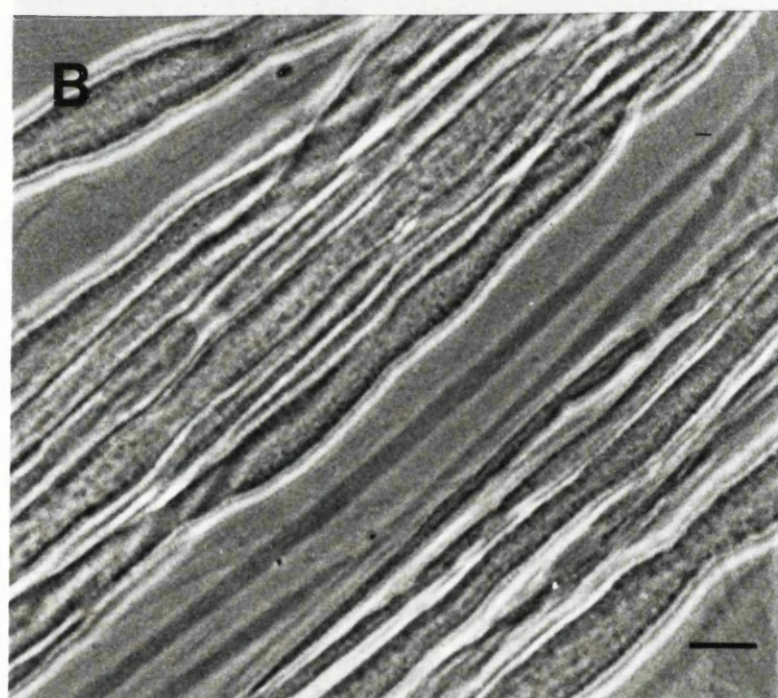
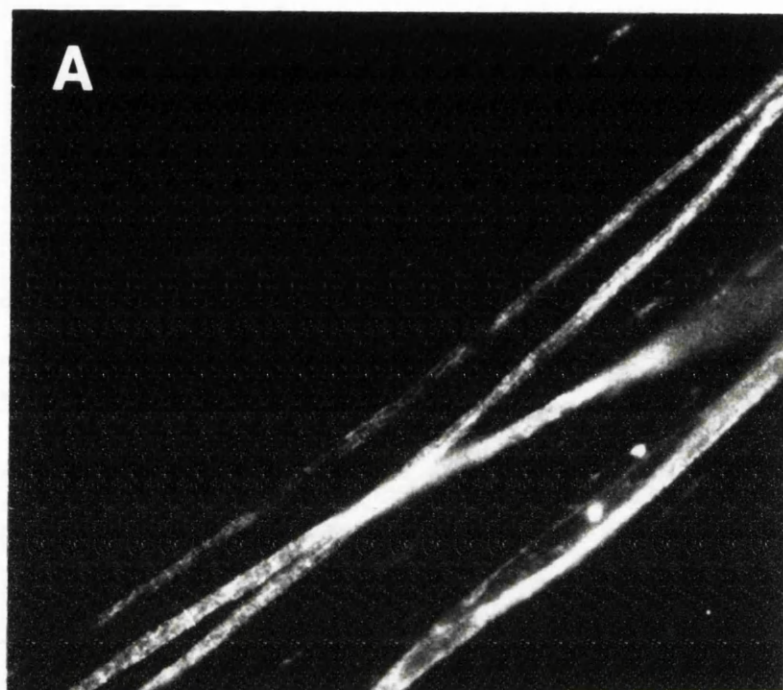




FIGURE 4.6. Unmyelinated axons in adult sciatic nerve express IGF *in vivo*. These photographs show IGF immunoreactivity in unmyelinated axons of teased sciatic nerve preparations from adult rat. (A) Fluorescein optics to visualize IGF in unmyelinated fibres; (B) Phase contrast. Bar = 10 $\mu$ .



**FIGURE 4.7. Myelinated axons are IGF immunoreactive in areas where the myelin sheath is torn. These photographs shows IGF immunoreactivity in a part of a myelinated axon where the myelin sheath is absent. (A) Fluorescein optics to visualize IGF; (B) phase contrast. Bar = 10  $\mu$ .**

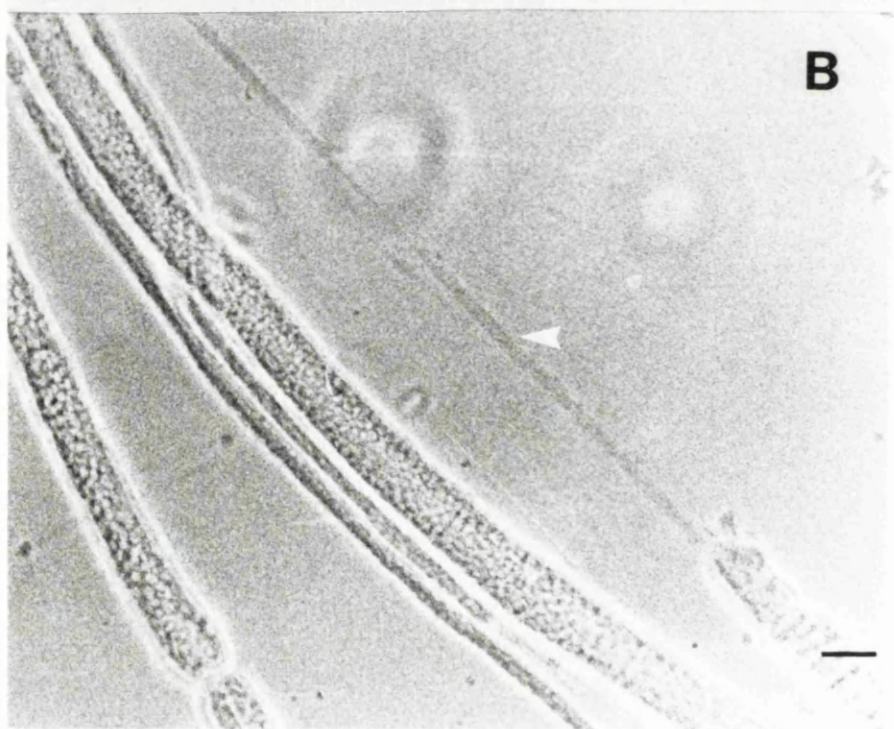
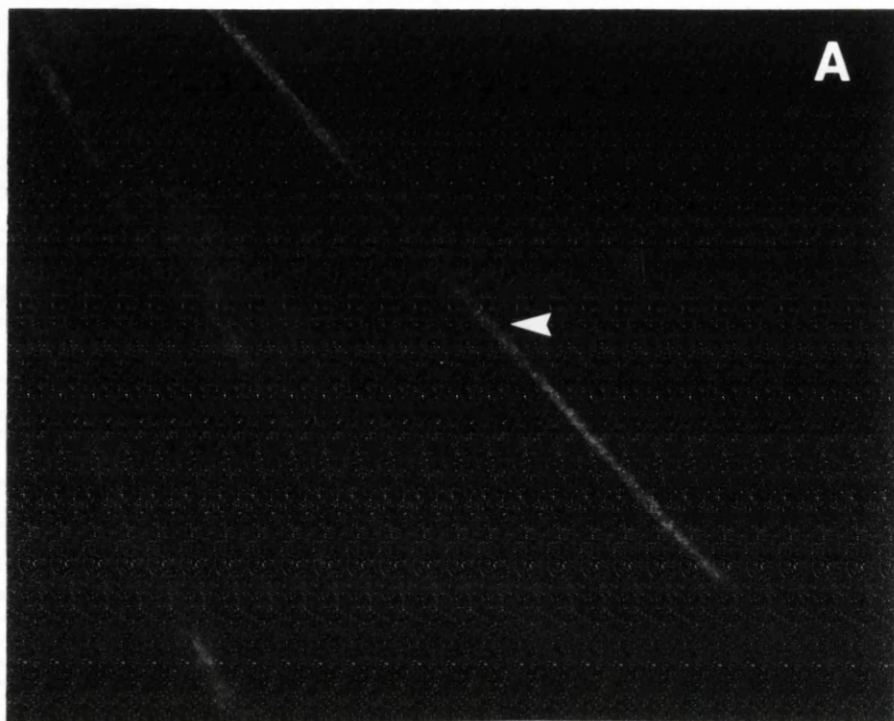


FIGURE 4.8. Sensory neurones are IGF immunoreactive *in vitro*. These photographs show DRG neurones prepared from P1 rats after 1 week in culture. (A) Fluorescein optics to visualize IGF; (B) phase contrast. Bar = 10 $\mu$ .



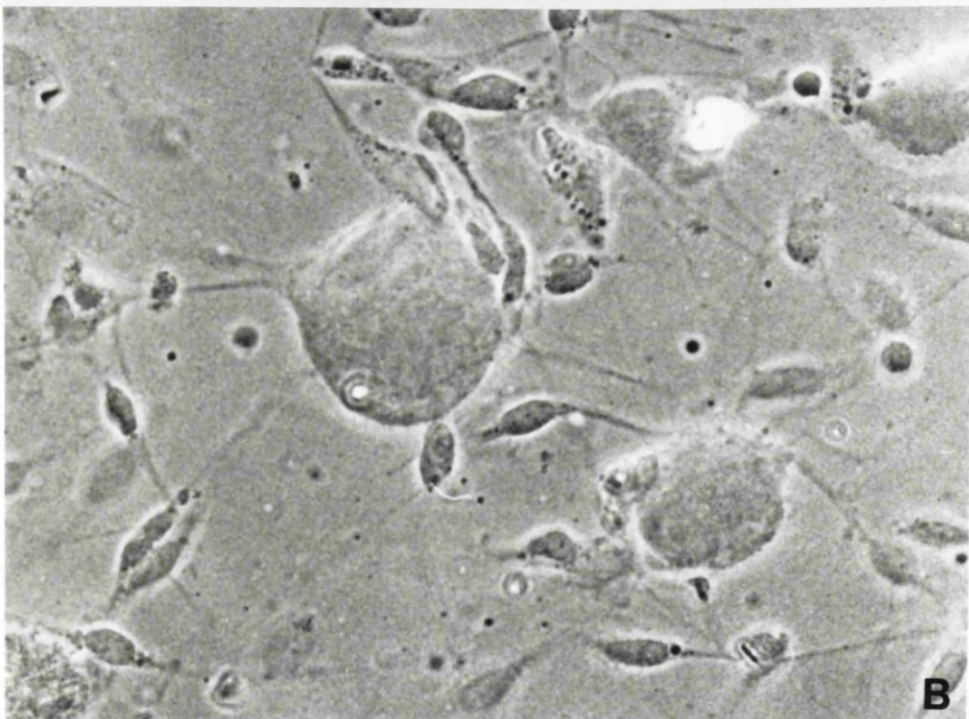
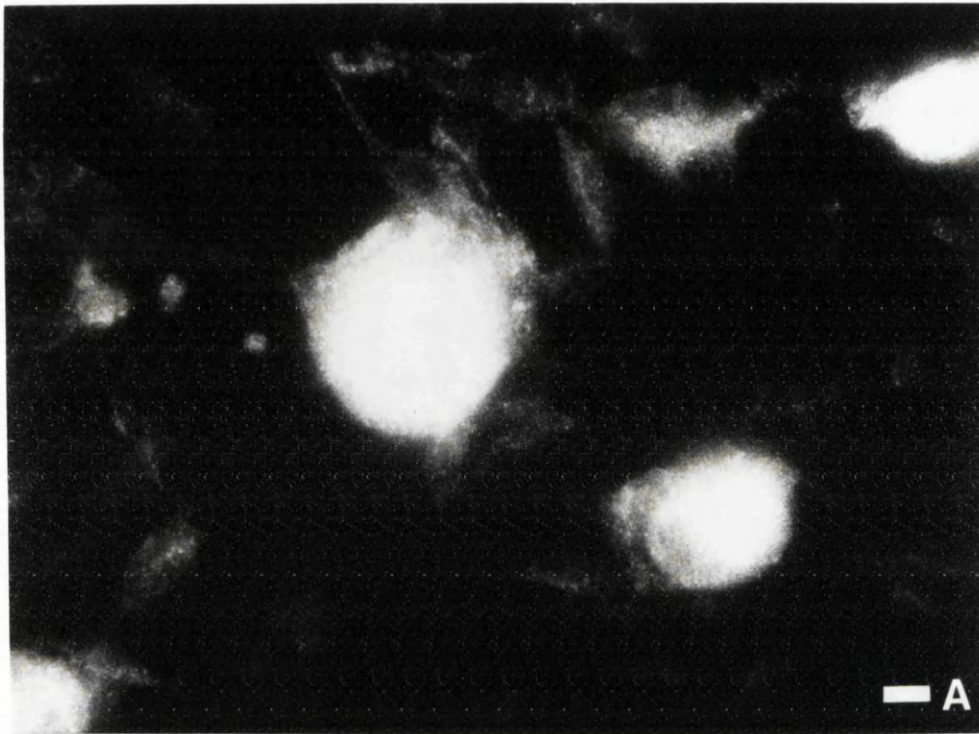
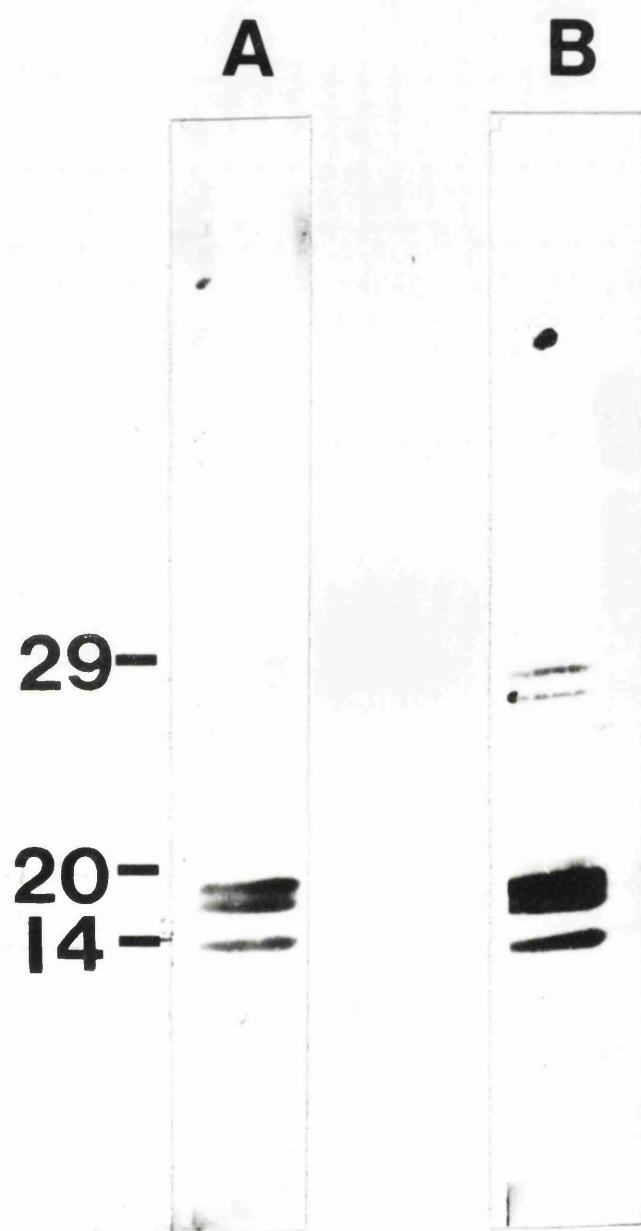


FIGURE 4.9. IGF immunoblotting. Preparations of P1 rat DRG and purified Schwann cells were electrophoresed on 7.5–22% gradient SDS gels, transferred to nitrocellulose and immunolabelled with mouse monoclonal anti-IGF-I. Blots were developed using diaminobenzidine as a chromophore in the peroxidase colorimetric reaction. Lane A = purified Schwann cells; Lane B = DRG.





## Discussion

This study demonstrates that IGF-I is a Schwann cell mitogen in tissue culture and may play a role in Schwann cell proliferation *in vivo*.

### Role of IGF-I in Schwann cell proliferation

Using serum-free medium lacking insulin we have shown that IGF-I stimulated Schwann cell DNA synthesis in a dose dependent manner in the presence of forskolin and either FGF or PDGF. IGF-I was essential for the mitogenic response of PDGF and forskolin and potentiated the mitogenic response of FGF and forskolin. In both cases half maximal response was achieved by 6–7nM IGF-I and could be mimicked by high concentrations of insulin. Both PDGF and FGF have previously been reported to be Schwann cell mitogens in medium containing serum (Krikorian et al., 1982; Ratner et al., 1988; Eccleston et al., 1990; Weinmaster and Lemke, 1990 ), plasma (Davis and Stroobant, 1990) or high concentrations of insulin (Chapter 3). Thus in all these studies the mitogenic effects of IGF-I would have been masked. The mitogenic effects of GGF and forskolin were not however effected by IGF-I. As the GGF in this study was impure no definite conclusions about the role of IGF-I in DNA synthesis caused by this agent can be made. In this study IGF-I alone did not cause DNA synthesis. Interestingly the astrocyte, a CNS glial cell that in many ways resembles the non-myelin-forming Schwann cell (Mirsky and Jessen, 1990), has been shown to respond mitogenically to IGF-I (Balloti et al., 1987; Han et al., 1987)

Binding studies using radiolabelled IGF-I and unlabelled IGF-I and insulin as competitors show that the Schwann cell receptor has a higher affinity for IGF-I than insulin. This observation coupled with the fact that IGF type 2 receptor does not bind insulin (Rechler and Nissley, 1985) suggest that Schwann cells possess type 1 IGF receptors. Scatchard analysis revealed however that IGF-I binds to a high affinity and low affinity receptor on Schwann cells. IGF-I can bind to the type 1 and type 2 IGF receptor and the insulin receptor (eg Rosenfeld, 1989). Based on the relative affinities of IGF-I for these receptors (and see above) it seems likely that the high affinity receptor is the type 1 IGF receptor. The low affinity receptor, on the other hand may be the IGF type 2 receptor (see

below) or the insulin receptor. Whether Schwann cells express all three receptor type cannot be determined from these studies. Further experiments using radiolabelled and unlabelled IGF-II and insulin should answer this question. The association constant of the high affinity receptor ( $1.9 \times 10^9 \text{ M}^{-1}$ ) and the amount of IGF-I required to cause half maximal displacement of tracer (5nM) are in close agreement with values reported for IGF-I receptors on other glial cells (Sheman et al., 1986, Balloti et al., 1987). Similarities between the dose response curves for IGF-I induced DNA synthesis and competition of tracer IGF-I suggest that these receptors probably mediate the mitogenic effect of IGF-I and high concentrations of insulin on Schwann cells.

Low levels of IGF type 2 receptor were detected on cultured Schwann cells. This receptor is identical to the mannose-6-phosphate receptor (MacDonald et al., 1988) and may thus be involved in lysosomal processing. In addition a potential role in modulation of IGF levels has been suggested (Senior et al., 1990). However this receptor is not believed to mediate the mitogenic effects of either IGF-I or IGF-II.

#### Is IGF-I a Schwann cell mitogen in vivo?

*mitogen*  
In order to determine whether IGF might have a role as a Schwann cell *in vivo*, this study examined the expression of immunoreactive IGF in cell suspensions dried on microscope slides immediately after dissociation of the nerve and in 2-3hr cultures. High levels of IGF immunoreactivity were seen in Schwann cells in early nerve development (E14-16). By birth and throughout adult life however, Schwann cells were not IGF-I immunoreactive. Thus the expression of high level IGF immunoreactivity correlates with the rapid Schwann cell proliferation occurring during embryogenesis (Jessen and Mirsky, personal communication, Webster and Favilla, 1984). While in the adult rat where Schwann cells are quiescent IGF immunoreactivity is undetectable. Other studies have detected very low levels of IGF-I immunoreactivity in some or all Schwann cells of the adult rat sciatic nerve (Hansson et al., 1986, 1987, 1988a, 1988b). These results taken together with the results of this study suggest that IGF-I is expressed in adult rat Schwann cells at very low levels that are barely detectable by immunohistochemical techniques.

In the chick peripheral axons Ralphs et al., (1990) detected dense

IGF labelling but failed to detect any Schwann cell associated IGF. The discrepancy between this study and ours may reflect species differences and/ or the stage of embryonic development. In addition, using *in situ* hybridization, no mRNA for IGF-I or IGF-II has been detected in the developing rat PNS (Beck et al., 1987, 1988; Stylianopoulou et al., 1988). However, in contrast to other studies (Sandberg et al., 1988; Rotwein et al., 1988; Brown et al., 1986; Lund et al., 1986), Beck et al., (1987, 1988) also failed to detect any IGF-I in the developing or mature brain. This discrepancy may reflect differences in sensitivity between the techniques used in these studies. Thus it is possible that low levels of IGF-I mRNA expressed in the peripheral nerve may not have been detected by these authors. Indeed the results of this study suggest that both Schwann cells and neurones are capable of synthesizing IGF at least in tissue culture.

Aside from embryogenesis, Schwann cells also proliferate during nerve regeneration and tumourgenesis. Evidence for a role of IGF-I in regeneration has been provided by several studies (Hansson et al., 1986, 1987a; Kanje et al., 1990; Sjöberg and Kanje, 1989). Cut or crush of the sciatic nerve resulted in increased Schwann cell IGF-I immunoreactivity within 1 day of injury, the levels remaining high for several weeks (Hansson et al., 1986, 1987a). The upregulation of IGF-I expression coincides with the increased Schwann cell proliferation rates seen after nerve injury (Clemence et al., 1989; Pelligrino et al., 1986). The levels of Schwann cell IGF-I remained elevated however, after the peak period of mitosis suggesting that IGF-I may also play other roles in regeneration. In this regard IGF-I has been shown to play a role in neurite outgrowth, and neuronal survival *in vitro* (Caroni and Grandes, 1990; Torres-Aleman et al., 1990; Svrzic and Schubert, 1990). Further evidence for an *in vivo* role of IGF-I in stimulating Schwann cell proliferation is provided by Sjöberg and Kanje, (1989) and Kanje et al., (1990). In these studies IGF-I perfusion was shown to enhance the regeneration rates of freeze injured sciatic nerves. Rates of non-neuronal cell proliferation were also elevated under these conditions. If neurite outgrowth was prevented however, perfused IGF-I had no effect on Schwann cell proliferation. Thus it appears that *in vivo* the axon is essential for Schwann cell mitosis in response to IGF-I. The role of the axon may be to elevate Schwann cell intracellular cAMP levels (Ratner et al., 1984) an effect which is

mimicked by forskolin in tissue culture. The results of this study predict that the presence of either FGF or PDGF may also be required for the observed mitogenic effects of IGF-I *in vivo*. Both of these factors are likely to be present in peripheral nerves (Yeh et al., 1991; Sasahara et al., 1991; Eccleston et al., 1990; Logan and Logan, 1986; Gonzales et al., 1990)

The presence of IGF-I has also been correlated with Schwann cell proliferation in von Recklinghausen's disease (Hansson et al., 1988c). Intense IGF-I immunoreactivity is seen in rapidly expanding lesions while quiescent neurofibromas are non IGF-I immunoreactive. In addition basic FGF has been detected in these tumours (Ratner et al., 1990). Thus the combined effects of FGF and IGF-I may be responsible for cell proliferation in neurofibromas. In the CNS enhanced levels of IGF-I and IGF-II mRNA have been detected in human gliomas (Sandberg et al., 1988) again suggesting a role for IGFs in proliferation of glial cells.

#### IGF immunoreactivity is expressed by neurones both *in vivo* and *in vitro*

Previous studies have demonstrated that peripheral nerves of the adult rat express low levels of IGF-I immunoreactivity *in vivo* (Hansson et al., 1986, 1987, 1988a, 1988b; Andersson et al., 1986). This study confirms the *in vivo* localization of IGF and shows that sensory neurones from varying developmental stages, express IGF in tissue culture. IGF immunoreactivity was detected in all neuronal cell bodies and at low levels in axons suggesting that neurones synthesize IGF *in vitro*. IGF expression was also seen in unmyelinated axons of teased adult sciatic nerves and in myelinated axons at the nodes of Ranvier. The presence of immunoreactive IGF in areas of myelinated axon where the myelin sheath was torn suggest that antibody penetration problems prevented the visualization of IGF expressing myelinated axons. *In vivo* the rapid axonal transport of IGF mainly in the anterograde direction has been reported (Hansson et al., 1988) confirming that neurones synthesize IGF. The rapid axonal transport of IGF suggests that it may play a role along the axon or at synapses. Indeed IGF-I has been suggested to play a role in stimulating neurotransmitter release in the sympathetic nervous system (Dahmer et al., 1990).

### Do Schwann cells synthesize IGF precursors?

Three bands of IGF immunoreactivity (MW 19,15 and 11kDa) were detected in Western blots of cultured Schwann cells, and postnatal rat DRG and sciatic nerve. The IGF detected in the nerve and DRG sample probably represents neuronal IGF as Schwann cells are not IGF-I immunoreactive at this developmental time. High molecular weight forms of IGF have been detected in several studies (Gelato and Vassolotti, 1990; Conover et al., 1989; Clemmons and Shaw, 1986; Tanaka et al., 1989; Powell et al., 1987; Hasselbacher and Humbel 1982; Hasselbacher et al., 1985, Gowen et al., 1987; Yang et al., 1985; Atkinson and Bala, 1981). The 19kDa IGF detected in this study may correspond to the IGF-I precursor (IGF-IA) described by Conover et al., (1989) and Powell et al., (1987) or the IGF-II described by Yang et al., (1985). A 15kDa form of IGF-II has previously been reported by Gowan et al., (1987). A 11 kDa form of IGF has not been reported before, however this band may be equivalent to the 10kDa form of IGF-II discovered in other studies (Yang et al., 1985; Tanaka et al., 1989). The identities of the higher molecular weight forms (24–29kDa) of IGF occasionally seen are not known. In this study no 7.5kDa IGF was detected suggesting that either this form is not synthesized in the nervous system or is present at levels too low to detect. Immunoprecipitation and pulse chase experiments using  $^{35}\text{S}$  labelled cysteine may help to address this point

In conclusion, this study shows that IGF-I can act as a Schwann cell mitogen *in vitro* when used in combination with forskolin and either PDGF or FGF. These effects are probably mediated by the type 1 IGF receptor. In addition this study shows that IGF immunoreactivity is prominent in early embryonic nerves, which is a period of particularly rapid Schwann cell proliferation.

## **CHAPTER 5**

### **GAP-43 EXPRESSION IN THE PERIPHERAL NERVOUS SYSTEM**

## INTRODUCTION

Early studies on growth associated protein showed that GAP-43 is one of a small set of neurone specific membrane bound proteins involved in neural development and repair. In the developing nervous system GAP-43 expression is seen all along the axon but is particularly enriched at the growth cone (Meiri et al., 1986) where it is presumed to play a role on neurite extension and synapse formation (Benowitz and Routtenburg, 1987; Skene, 1989; Snipes et al., 1987;). The synthesis and transport of GAP-43 is dramatically enhanced during regeneration of the mammalian PNS (Skene and Willard, 1981c; Tetzlaff et al., 1989) but levels do not increase or do so only transiently in the adult CNS where regeneration is abortive (Skene and Willard, 1981c; Kalil and Skene, 1986; Reh et al., 1987). In the CNS of lower vertebrates however, where regeneration does occur levels of GAP-43 increase significantly in response to injury (Skene and Willard, 1981a,b).

More recently however, it has been shown that GAP-43 expression is not confined to areas of axonal outgrowth or indeed restricted to neurones. Several studies have demonstrated that GAP-43 expression persists in several areas of the adult nervous system, such as the hippocampus, where a role in synaptic plasticity and remodelling is suggested (Benowitz et al., 1988; Benowitz et al., 1989; De La Monte et al., 1989; McGuire et al., 1988; Neve et al., 1987; Neve et al., 1988). Biochemically GAP-43 has been shown to be a substrate for protein kinase C (Aloyo et al., 1983), regulate phosphatidylinositol turnover (van Dongen et al., 1985; Oestreicher et al., 1983) and bind calmodulin (Andreason et al., 1983), suggesting a role in signal transduction. GAP-43 immunoreactivity has also been detected in glial cells of the CNS (Vitkovic et al., 1988; da Cunha and Vitkovic, 1989; Deloulme et al., 1990).

The presence of GAP-43 in the glial cells of the peripheral nerve, the myelin-forming and non-myelin-forming Schwann cells, is at present controversial. The experiments of Tetzlaff et al., (1989) have demonstrated the presence of GAP-43 immunoreactivity in reactive Schwann cells of the regenerating facial and sciatic nerves (Tetzlaff et al., 1989). The conclusion drawn from this study however was that the GAP-43 must be taken up from the regenerating axons by an unknown

mechanism. Likewise Woolf et al., (1990), using cultures of dorsal root ganglia, showed that some Schwann/satellite cells expressed GAP-43 albeit at low levels. Again the possibility of GAP-43 uptake from the neurones in the culture could not be eliminated. In contrast, no GAP-43 immunoreactivity was detected in pure Schwann cells or in sympathetic neurone / Schwann cell mixed cultures (Meiri et al., 1988). The studies of Verhaggen et al., (1986) also failed to detect any immunoreactive GAP-43 in intact rat sciatic nerve or in the distal stump following nerve transection and Fitzgerald et al., (1991) detected no neuroglial associated GAP-43 in sections of developing peripheral nerves. The presence of GAP-43 in glial cells suggests that this protein may play a more general role in the nervous system than previously supposed.

This study shows that the expression of GAP-43 in the rat peripheral nervous system is far from being restricted to developmental and regenerative situations. Using western blotting and immunohistochemical techniques significant levels of GAP-43 immunoreactivity have been detected in all areas of the adult autonomic nervous system examined and in mature non-myelin-forming Schwann cells and precursor Schwann cells.



## METHODS

### Denervation

Adult Sprague Dawley rats weighing 100g were anaesthetised with 1ml of chloral hydrate (45mg/ml in PBS). The left sciatic nerve was transected at mid-thigh level and the proximal portion deflected into a nearby muscle to prevent regenerating axons reaching the distal stump. The rats were then left for 4–13 days, after which they were killed by asphyxiation with carbon dioxide. These operations were carried out by Dr K.R.Jessen.

### Sympathectomy

Adult male Sprague Dawley rats (150g) were bilaterally sympathectomized by removal of the SCG under halothane anaesthesia. Before sacrifice the success of the operation was confirmed by the presence of Horner's syndrome in the eye. These operations were carried out by Dr T.Cowen.

### Stretch preparations of myenteric plexus

Segments of ileum, and colon were dissected out from adult and P7 Sprague Dawley rats, cut open lengthwise and stretched, mucosal side down, onto pieces of Sylgard silicon rubber. All tissues were fixed in 4% paraformaldehyde in PBS for 4 hr at 4°C, dehydrated and rehydrated in ethanol then treated with 0.1% Triton X100 in PBS for two periods of 15 min. The longitudinal and circular muscle layers, together with the myenteric plexus were peeled away from the rest of the intestine (Costa et al., 1979). This preparation was pinned down onto Sylgard longitudinal muscle surface upwards for immunohistochemistry.

### Whole mount preparations

Rats sympathectomized for 6 days and control rats were killed with a lethal dose of sodium pentobarbitone. Exsanguination was carried out by injecting 10ml of Tyrodes solution into the heart. The cerebral vessels (circle of Willis and the basilar artery) were removed and pinned out directly onto Sylgard. The tail vein and mesenteric veins were removed and opened out by cutting along the long axis of the vessel then pinned,

outer surface upwards, onto Sylgard. Connective tissue was gently cut away from the vessel walls. Iris preparations were made by pinning the eye out onto Sylgard, cutting and peeling back the cornea, then removing the iris by cutting it away from the muscle layer at the base of the eye. All preparations were fixed for 4 hr at 4°C in 4% paraformaldehyde then processed as described for gut whole mounts. After paraformaldehyde fixation any dura matter present on the cerebral vessels was removed.

#### Wax sections

Colon, ileum and stomach were prepared and fixed as described in General Methods. Fixed tissues were embedded in polyester wax then sectioned at 4°C and mounted onto PLL coated slides. Samples were dewaxed and rehydrated through 100, 90 and 70% ethanol then immunolabelled as described in General Methods.

#### Immunohistochemistry

##### Whole mounts and sections

Stretch preparations, whole mounts and wax sections were incubated in a humid chamber for 18hr with rabbit antiserum to GAP-43 diluted 1:5000. On some wax sections either monoclonal anti-S100 antibodies (1:500) or monoclonal anti-GFAP antibodies (1:10) were applied together with anti GAP-43 antibodies overnight. The preparations were washed in PBS then incubated for 1hr with fluorescein or rhodamine conjugated goat anti-rabbit immunoglobulin (1:100). When double labelling experiments were performed preparations were incubated in anti-mouse biotin antibodies (1:100) for 1 hr washed in PBS, incubated with fluorescein-streptavidin (1:100) for 20min then washed and incubated with anti-rabbit rhodamine for 1 hr.

Blood vessel and iris preparations were sequentially double labelled with monoclonal anti-GAP-43 antibodies (1:300) for 18hr at room temperature followed by anti-mouse fluorescein for 1hr. Preparations were washed in PBS then labelled overnight with anti-PGP 9.5 antibodies (Thompson et al., 1983) (1:400) followed by anti-rabbit biotin antibodies for 1hr and streptavidin-texas red for 20 min. Parasymathetic fibres and sensory fibres were labelled with rabbit polyclonal anti-vasointestinal

peptide antibodies (VIP 1:5000) and rat monoclonal anti-substance P antibodies (1:200) respectively, overnight at room temperature. Second antibodies were anti-rabbit and anti-rat fluorescein. Before mounting, blood vessel preparations were placed in 1% pontamine sky blue for 10 min to quench autofluorescence (Cowen et al., 1985).

All preparations were mounted in Citiflour anti-fade mounting medium and viewed for immunofluorescence with a Zeiss microscope using x63 oil immersion or x40, x25 or x10 dry phase contrast lenses, epi illumination and fluorescein and rhodamine optics.

#### Cultured Schwann cells

Long- and Short-term cell cultures (see General Methods) were stained with monoclonal anti-NGF receptor antibody used at a dilution of 1:5000 or by monoclonal anti-04 (1:2) followed by goat anti-mouse rhodamine. The cells were fixed in 4% paraformaldehyde in PBS for 20min then incubated in methanol for 10 min at  $-20^{\circ}\text{C}$  before applying rabbit anti-GAP-43 at a dilution of 1:1000 followed by fluorescein conjugated to goat anti-rabbit immunoglobulins. All incubations were carried out for 30min at room temperature.

#### Dried dissociated cell preparations

Dried dissociated cell preparations were rehydrated in 4% paraformaldehyde in PBS and permeabilised as described above then stained with monoclonal anti-GFAP antibodies (1:10) together with polyclonal anti-GAP-43 (1:5000) in a humid chamber for 18 hr at room temperature. In some experiments monoclonal anti-GAP-43 antibodies (1:300) and polyclonal anti-GFAP antibodies (1:100) were used. Cells were incubated in secondary antibodies and mounted as described for cell cultures. In some instances dried cells were incubated in goat anti-rabbit biotin second antibody for 30 min followed by fluorescein-streptavidin for 20 min.

#### Teased nerve preparations

Teased sciatic and vagus nerves and sympathetic trunk were fixed and permeabilized as described above. Preparations were labelled overnight at room temperature with polyclonal anti-GAP-43 (1:5000)

together with either monoclonal anti-GFAP (1:10) or monoclonal anti-vimentin (1:100). Second antibodies were used as described for wax sections and whole mounts (see above).

Teased preparations of the distal stump of cut sciatic nerve were prepared 4, 7 and 13 post lesion. Preparations were labelled with monoclonal anti-NGF receptor (1:5000) for 1 hr, rinsed in PBS then incubated with anti-mouse rhodamine (1:200) for 1hr. Preparations were fixed, permeabilized and labelled for GAP-43 as described above. To ensure that no axonal regeneration had taken place some preparations were labelled with RT97 (1:1000), which recognizes the phosphorylated form of the 210kDa neurofilament protein (Anderton et al., 1982). Preparations were fixed and permeabilized as described for GAP-43 immunohistochemistry. Anti-mouse rhodamine second antibodies (1:200) were used.

#### PGP 9.5 immunohistochemistry

Schwann cells and teased intact sciatic nerve and distal stump preparations (as described above) were fixed for 20min in 4% paraformaldehyde, washed in PBS, then permeablized with 0.1% triton for 10 min. Cells were incubated with polyclonal anti-PGP 9.5 (1:400) for 30 min followed by anti-rabbit fluorescien for 30 min. Coverslips were mounted and visualized as described in General Methods. Schwann cells were identified by morphology under phase contrast.

#### Controls

Controls were omission of the primary antibody or substitution of the primary antibody with pre-immune serum or application of the inappropriate second layer. As embryonic Schwann cells have to be cultured in BALB-C 3T3 conditioned medium to ensure cell survival (Mirsky and Jessen, 1990; Jessen personal communication) control experiments were carried out to ascertain the effect of this medium on GAP-43 expression by postnatal Schwann cells. P1 sciatic nerve Schwann cells were cultured in this medium or DME containing 10% calf serum for periods up to 1 week and immunolabelled for GAP-43.

### Catecholamine histochemistry

Catecholamine histochemistry was carried out according to the method of De le Torre and Surgeon, (1976). 2.55g sucrose, 1.2g  $\text{KH}_2\text{PO}_4$  and 0.375g of glyoxylic acid were dissolved in 25ml of distilled water. The pH of the solution was then adjusted to 7.4 with 1M NaOH. Tissue whole mounts dried onto glass microscope slides were dipped into the glyoxylic acid solution for three, 2 second periods. The tissues were dried with a hair drier then baked at  $100^\circ\text{C}$  for 4 min. Slides were mounted in liquid paraffin and viewed for fluorescence with an Olympus microscope using x 10 and x 20 dry lenses and 420 nm light.

### Forskolin treatment

Purified cultured Schwann cells were plated at a density of 10000 cells/well in DME containing 10% CS on PLL coated coverslips. Cells were treated with  $5\mu\text{M}$  or  $1\mu\text{M}$  forskolin or 1mM DBcAMP for 48hr at  $37^\circ\text{C}$  5% air/95%  $\text{CO}_2$ . Control cultures had no additions. All coverslips were labelled for GAP-43 and NGF receptor.

### Electrophoresis and immunoblotting

Preparations of longitudinal and circular muscle layers containing the myenteric plexus, adult rat sciatic nerve and sympathetic trunk were homogenized in 2% SDS, 2mM EDTA, 2mM EGTA, 5mM Tris pH 6.8, containing 1mM PMSF, and boiled for 2 min. Homogenized tissues were centrifuged for 2 min at 14000xg, supernatants removed and assayed for protein content using a Bio Rad kit (see General Methods). The remaining samples were diluted 1:1 with 2 x sample buffer containing 2% mercaptoethanol and boiled for 5 min. Preparations were applied to 10% polyacrylamide gels and subjected to electrophoresis as described by Laemmli, (1970). Mini gels were run at 15mA at constant current while stacking and then at 20mA. Proteins were then electrophoretically transferred onto nitrocellulose (pore size  $2\mu$ ) for 2.5hr at 150mA (constant current). Lanes containing standards were excised and stained with amido black for 3 min then destained with water. The remainder of the nitrocellulose was blocked with 2% gelatin in Tris buffered saline (pH 7.3) for 2 hr at room temperature, then incubated with pre-immune serum or rabbit anti-GAP-43 dil 1:10000 in 0.1% BSA for 18hr at room

temperature. The blots were then incubated with peroxidase linked goat anti-rabbit immunoglobulin (1:2000) for 1 hr at room temperature. After washing the blots were developed using 1mg/ml diaminobenzidine as a chromophore and hydrogen peroxide in the peroxidase colorimetric reaction. A sample of pure GAP-43 was used as a control.

Schwann cells from postnatal day 1 rats were purified with cytosine arabinoside for 3 days, cultured for a further 2 days and then passaged with vercine containing 0.25% trypsin. Cells were pelleted by centrifugation at 1000xg for 10 min, then homogenized in 2% SDS, 2mM EGTA, 2mM EDTA, and 1mM PMSF and subjected to electrophoresis and immunoblotted as described above. Schwann cell membranes prepared from P1 rats in conjunction with R Curtis were also blotted.

## RESULTS

### GAP-43 is expressed in the enteric nervous system

GAP-43 immunoreactivity was seen in sections from all areas of the enteric nervous system of the adult rat. Intense immunoreactivity could be seen in the myenteric, submucosal and mucosal plexuses (Fig 5.0, Fig 5.1). Immunoreactivity could also be seen in the nerves innervating the circular and longitudinal muscle layers. Neuronal cell bodies were generally unlabelled (Fig 5.2) as were glial cell bodies. No definitive proof of GAP-43 immunoreactivity in the enteric glia could be seen. Stretch preparations of the myenteric plexus from the colon and ileum revealed that in addition to the myenteric ganglia and interconnecting strands, the secondary and tertiary plexuses were also GAP-43 immunoreactive (Fig 5.3). Both varicose and non-varicose autonomic fibres were GAP-43 labelled (Fig 5.3). GAP-43 immunoreactivity was compared with the immunolabelling pattern of PGP 9.5 antibodies since this antigen is reported to be in all PNS neurones (Thompson et al., 1983; Gulbenkian et al., 1987). Comparison of the distribution of GAP-43 immunoreactivity with that of PGP 9.5 in similar preparations showed an essentially indistinguishable pattern of immunolabelling, suggesting that the entire myenteric plexus is GAP-43 immunoreactive (Fig 5.3). Stretch preparations prepared from the myenteric plexus of 7 day old rat pups showed a similar labelling pattern to that seen in the adult rat (Fig 5.4). The intensity of immunoreactivity was also comparable. Experiments carried out using monoclonal anti-GAP-43 gave essentially the same labelling pattern as described above. Control experiments using pre-immune serum and omission of the primary antibody showed no immunoreactive material.

To confirm the presence of GAP-43 in the enteric nervous system preparations of rat ileum containing the circular and longitudinal muscles and the myenteric plexus were immunoblotted. The bands detected in these samples had an identical electrophoretic mobility to pure GAP-43 protein on 10% SDS-polyacrylamide gels. Immunoreactive bands were seen in preparations of plexus from adult, 7 day, and 1 day rats (Fig 5.5). No immunoreactive bands were detected when gels were incubated with pre-immune serum.

### GAP-43 immunoreactivity is widespread in the sympathetic and parasympathetic nervous system

To establish whether GAP-43 was present in other areas of the mature autonomic nervous system preparations of various tissues (cerebral blood vessels, mesenteric vein, tail vein, iris) were immunolabelled with anti-GAP-43. The results showed that GAP-43 immunoreactivity was extensively and uniformly distributed in both varicose and non varicose fibres of all the tissues examined (Fig 5.6). Immunoreactivity was located exclusively to neuronal tissues. Double labelling of iris preparations with the neuronal marker PGP 9.5 and monoclonal anti-GAP-43 (91E12 Goslin et al., 1988) showed an apparently complete overlap in labelling patterns (Fig 5.7). Immunolabelling of the tail vein, mesenteric vein, and cerebral vessels (circle of Willis and basilar artery) with monoclonal anti-GAP-43 gave similar results to labelling experiments with polyclonal anti-GAP-43.

### The cellular localization of GAP-43 immunoreactivity in autonomic neurones.

In order to confirm the presence of GAP-43 immunoreactivity in sympathetic fibres, sympathectomy experiments were performed. Removal of the superior sympathetic ganglion resulted in a total loss of sympathetic fibres from both the iris and the circle of Willis. Partial loss of the sympathetic innervation of the basilar artery was seen. This was expected as the basilar artery also receives some sympathetic innervation from another source, probably the stellate ganglion (Tim Cowen personal communication). The loss of catecholamine fluorescence was not however accompanied by any obvious depletion of GAP-43 immunoreactive fibres suggesting that GAP-43 might be expressed by Schwann cells in the bands of Büngner. Surprisingly labelling of preparations with anti-PGP 9.5 antibodies also failed to reveal any sympathectomy induced changes in density of innervation (Fig 5.8). As expected VIP (marker of parasympathetic fibres in these preparations) and substance P (indicative of sensory fibres) immunoreactivity were unaffected by sympathectomy (Fig 5.7).



PGP 9.5 is expressed by Schwann cells in tissue culture and by reactive Schwann cells

As PGP 9.5 immunoreactivity was apparently unaffected by surgical sympathectomy the neuronal specificity of this marker (Thompson et al., 1983) was questioned. P1 Schwann cells cultured for 3 and 24 hr were immunolabelled for PGP 9.5. Fig 5.9 shows that all Schwann cells in these culture were PGP 9.5 immunoreactive. Teased preparations of 4 and 7 day denervated distal stumps also showed PGP 9.5 immunoreactivity associated with Schwann cells (Fig 5.8).

GAP-43 is expressed by some Schwann cells in the bands of Büngner

The experiments above suggested that reactive Schwann cells of the bands of Büngner express GAP-43. In order to confirm these observations teased preparations of the distal stump of transected sciatic nerve were made and labelled with GAP-43 antibodies. The absence of regenerating axons was determined by neurofilament immunohistochemistry. At 4, 7 and 13 days post cut GAP-43 immunoreactivity was associated with a sub-population of Schwann cells the numbers of which did not increase over the experimental period. In contrast, NGF receptor (a marker of non-myelin-forming Schwann cells) expression was upregulated being expressed by all Schwann cells at all time points examined. Morphological examination of the GAP-43 immunoreactive Schwann cells suggested that prior to denervation they were non-myelin-forming cells (Fig 5.9).

GAP-43 is expressed by non-myelin-forming Schwann cells *in vivo*

To establish whether Schwann cells in the intact nerve express GAP-43 *in vivo* teased nerve preparations and dried dissociated cell preparations were used. Teased nerve preparations from the mixed fibre nerves, the vagus and sciatic, showed that GAP-43 immunoreactivity was associated with unmyelinated fibres probably in non-myelin-forming Schwann cells. In some instances the axons of myelinating fibres were GAP-43 immunoreactive, whereas myelin-forming Schwann cells were never labelled. Non-myelin-forming Schwann cells were identified by morphology (5.10) and/or by GFAP co-expression.

To confirm the presence of GAP-43 immunoreactivity in non-myelin-forming Schwann cells teased preparations of the sympathetic trunk, which is 99% unmyelinated (Aguayo et al., 1976a) were examined. As shown in Fig 5.11 GAP-43 immunoreactivity was present in all nerves fibres that co-expressed GFAP. It was however impossible to determine unequivocally whether the GAP-43 immunoreactivity was associated with all the Schwann cells. Carefull examination of the teased nerves showed that GAP-43 was not exclusive to the non-myelin-forming Schwann cells. In certain areas it was possible to see GAP-43 immunoreactivity in the bead-like varicosities of autonomic axons (Fig 5.11).

In an attempt to unambiguously identify GAP-43 in mature non-myelin-forming Schwann cells dried dissociated preparations from various adult rat nerves and ganglia were made. The results are summarized in Table 5.0. In preparations made from myelinated nerves, non-myelin-forming Schwann cells, identified by phase contrast or GFAP co-expression, were only occasionally labelled with antibodies to GAP-43 (<5%) even when biotinylated second antibodies were used. Very rarely GAP-43 immunoreactivity could be detected in segments of myelinated axon but never in myelin-forming Schwann cells, confirming the results obtained from teased nerves. In sympathetic trunk preparations about 33% of cells expressed GAP-43. These results do not corroborate the data obtained from teased nerves, where the impression was obtained that a vast majority of Schwann cell were GAP-43 immunoreactive. This suggest that a technical problem may exist, possibly related to GAP-43 solubility, an issue that will be discussed below.

In preparations of autonomic ganglia a population of cells were labelled that had a more "compact" appearance than typical non-myelin-forming Schwann cells. These cells co-expressed GFAP, were devoid of myelin, and were thought to be satellite cells (Fig 5.12). In some instances cells known to be satellite cells from their association with neurones were GAP-43 immunoreactive (Fig 5.13) These cells were often intensely GAP-43 immunoreactive. In contrast only 25% of GAP-43 labelled cells from the sympathetic trunk were highly immunoreactive. In mixed nerves , the few GAP-43 immunoreactive cells seen were never intensely labelled. In addition approximately 70% of neurones present in

these cultures were GAP-43 immunoreactive to varying intensities.

In order to establish the localization of GAP-43 in satellite cells, and to determine whether all mature non-myelin-forming Schwann cells are GAP-43 immunoreactive, sections of adult ganglia were used. Sections of the DRG and associated nerve from adult rats showed that GAP-43 immunoreactivity was present in non-myelin-forming Schwann cells (Fig 5.14). The vast majority, if not all, of these Schwann cells were labelled. In the case of satellite cells it could be clearly seen that only a very small percentage of these cells were GAP-43 immunoreactive (Fig 5.14). In contrast many of the satellite cells in sections of the SCG were labelled (Fig 5.15). Again GAP-43 immunoreactivity could occasionally be seen in axonal varicosities in the associated nerve fibres of DRG sections (Fig 5.16). GAP-43 immunoreactivity could also be detected in some (10–20%) of the neuronal cell bodies in the DRG. Labelling was observed in many of the small and medium diameter cell bodies and never in the large diameter cell bodies. GAP-43 immunoreactivity in neurones was often concentrated in the perinuclear area. In sections of the SCG however neuronal cell bodies were only occasionally labelled.

#### Developmental regulation of GAP-43 in Schwann cells

The developmental regulation of GAP-43 was examined in short-term Schwann cell cultures and in dried dissociated cell preparations from embryonic and postnatal sciatic nerves. Dried dissociated cell preparations from E15–16 nerve (Fig 5.17) yielded approximately 87% GAP-43 immunoreactive cells (Table 5.0). A similar number ( $86\% \pm 1.2$ ) of labelled cells were seen after 2 hr culture. These cells could not be unequivocally identified as Schwann cells as a marker exclusive to Schwann cells *in vivo* does not exist at this developmental stage. As a vast majority of cells were labelled however, a large proportion of Schwann cells must be labelled. The main contaminating cells in these preparations are fibroblasts. Identical experiments carried out on P1 Schwann cells detected  $28.2\% \pm 6.2$  GAP-43 immunoreactive Schwann cells in dried cell preparations and  $39\% \pm 5$  in short-term cultures. Schwann cells were identified by co-labelling with S100. Notably the intensity of GAP-43 immunoreactivity in embryonic Schwann cells was also higher than in P1 cells.

In tissue culture GAP-43 expression is downregulated in embryonic Schwann cells, but not postnatal Schwann cells.

Schwann cells from E15-16 rats were cultured for periods up to 6 days. To ensure cell survival these cells were cultured in BALB C 3T3 conditioned medium. Schwann cells could be identified in these experiments by co labelling with NGF receptor antibodies. NGF receptor is present on fibroblasts *in vivo* but is down regulated in culture enabling unambiguous identification of Schwann cells. After 24hr in culture  $86.2 \pm 1.3\%$  (n=4) of NGF receptor positive Schwann cells were GAP-43 immunoreactive. The distribution and intensity of GAP-43 labelling varied somewhat from cell to cell. The majority of embryonic Schwann cells were moderately or intensely GAP-43 immunoreactive. In nearly all Schwann cells rings of immunoreactive material surrounding unlabelled areas could be seen (Fig 5.18) as well as areas of more diffuse labelling. Often a small number of NGF receptor negative cells with the morphology of fibroblasts, were found to be GAP-43 immunoreactive (<5% of the total number of cells identified by phase contrast). Schwann cells from E15 rats cultured for 6 days showed very low level GAP-43 immunoreactivity in approximately 30% of cells. The GAP-43 immunoreactivity present in these cultures was often present in groups of cells accumulated to one side of the nucleus, probably in the Golgi apparatus (Fig 5.19).

In contrast to embryonic Schwann cells expression of GAP-43 by P1 Schwann cells was not downregulated. Fig 5.20 shows that GAP-43 levels were relatively constant over a period of 3 weeks. The "ring like" structures apparent in embryonic Schwann cell cultures were also seen in these cells (Fig 5.21). In P1 Schwann cell cultures however majority of cells were weakly or moderately GAP-43 immunoreactive, (Fig 5.22) less than 5% of cells being intensely labelled. In these cultures, as in embryonic cultures, a few GAP-43 immunoreactive fibroblast-like cells were occasionally seen. Cultures prepared from adult rat sciatic nerve and sympathetic trunk gave essentially the same results as obtained from P1 Schwann cells. Between 25-30% of cells were consistently, although weakly, GAP-43 immunoreactive for a period of up to 4 weeks. Interestingly no GAP-43 immunoreactivity was detected in long-term

cultured "autocrine" Schwann cells.

Control experiments using pre-immune serum always gave negative results. Experiments carried out using BALB/c 3T3 fibroblast conditioned medium on P1 Schwann cells showed this medium had no effect on GAP-43 expression in these cells. This suggests that either 3T3 conditioned medium has no effect on embryonic Schwann cell GAP-43 expression or that postnatal Schwann cells are unable to respond to 3T3 conditioned medium possibly by lacking receptors to any active factors. Nevertheless, this experiment supports the hypothesis that GAP-43 expression is differentially controlled in embryonic and postnatal Schwann cells.

#### Postnatal Schwann cell GAP-43 expression is down-regulated by cAMP

In Chapter 3 it was shown that long-term cultured Schwann cells have constitutively high basal cAMP levels. The observation that these cells do not contain immunohistochemically detectable amounts of GAP-43 (see above) lead to the suggestion that cAMP may control Schwann cell GAP-43 levels. To test this hypothesis Schwann cells were incubated in 5 $\mu$ M or 1 $\mu$ M forskolin or 1mM DBcAMP for 48hr. In both cases down-regulation of GAP-43 expression was seen (Fig 5.23)

#### Schwann cell GAP-43 co-migrates with pure GAP-43

Samples of cultured Schwann cells (P1 rats), sympathetic trunk and sciatic nerve (adult rats) were run on 10% SDS polyacrylamide gels (Fig 5.5). In all cases a single immunoreactive band was detected of apparent molecular weight 45 kDa which co-migrated with a pure sample of rat brain GAP-43. Membrane preparations of cultured P1 rats Schwann cells, prepared in collaboration with, and immunoblotted by R.Curtis, were also seen to contain GAP-43. No immunoreactive bands were seen in control blots incubated in pre-immune serum.

TABLE 5.0.

Expression of GAP-43 in dried dissociated Schwann cell preparations.

NERVE	% of GAP-43 immunoreactive non-myelin-forming Schwann cells/satellite cells
Vagus	variable 0-5%
Sciatic	variable 0-5%
Sural	variable 0-5%
Brachial plexus	variable 0-5%
SCG	67.0 $\pm$ 1.7
MSG	72.1 $\pm$ 6.3
Sympathetic chain	48.5 $\pm$ 5.3
DRG	27.1 $\pm$ 5.9
Sympathetic trunk	33.0 $\pm$ 4.75
P1 Sciatic	28.2 $\pm$ 6.2
E15-16 Sciatic	87.3 $\pm$ 4.6

All nerves were from adult rats except where otherwise stated.

FIGURE 5.0. GAP-43 immunoreactivity is present in the mucosal plexus of the enteric nervous system. This photograph shows a wax section of the adult rat colon. (A) Fluorescein optics to visualize GAP-43; (B) phase contrast. Bar = 10 $\mu$ .

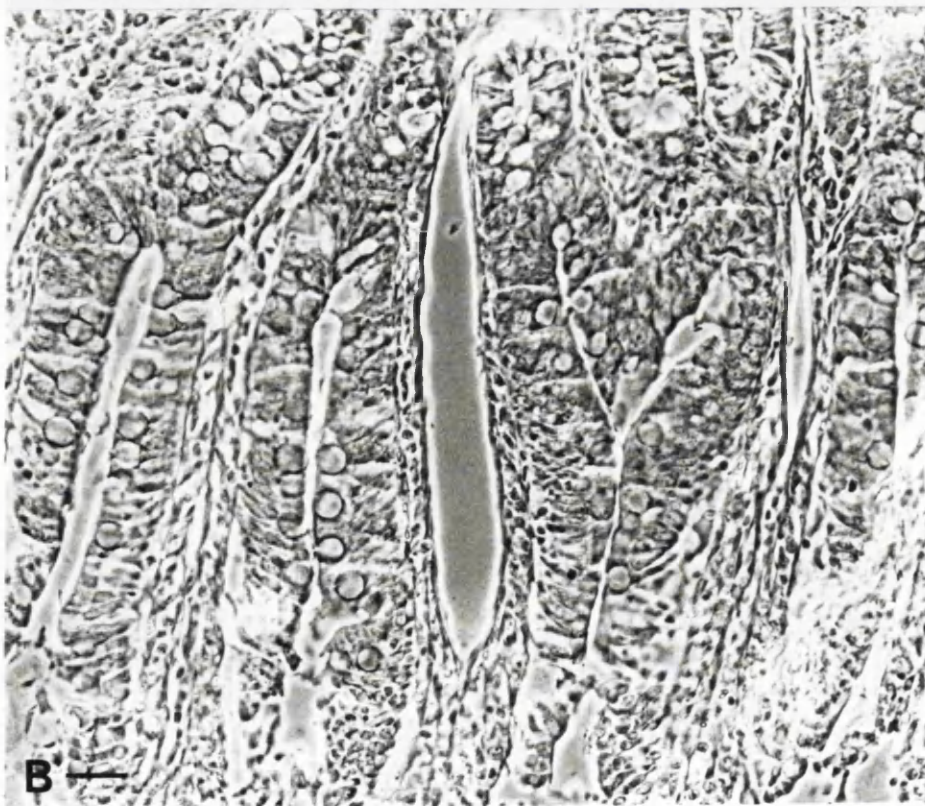
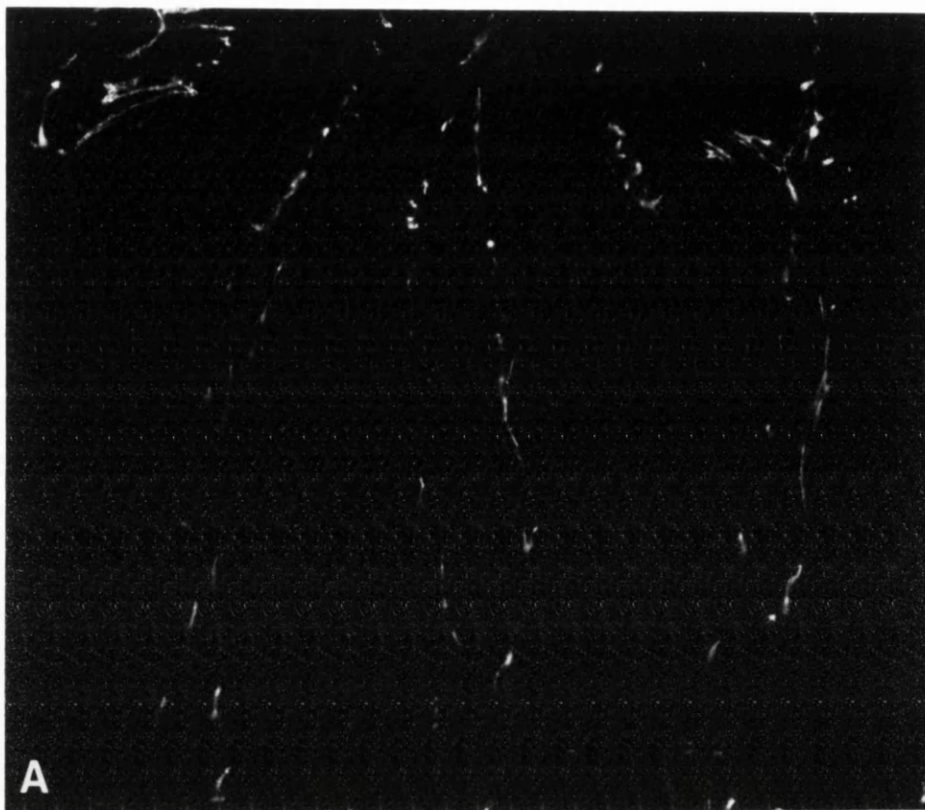




FIGURE 5.1. GAP-43 immunoreactivity in the submucosal (\*) and myenteric plexus (★) of the enteric nervous system. (A) phase contrast; (B) Fluorescein optics to visualize GAP-43; (C) Rhodamine optics to visualize S100. Note the presence of GAP-43 immunoreactivity in nerve fibres of the circular (CM) and longitudinal muscle (LM) layers. Bar = 10 $\mu$ .

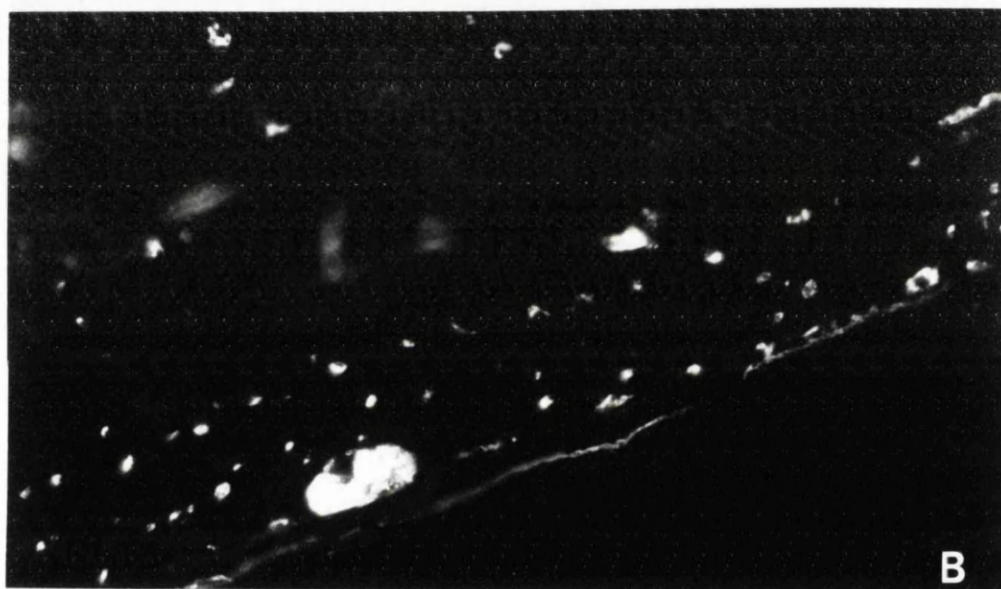
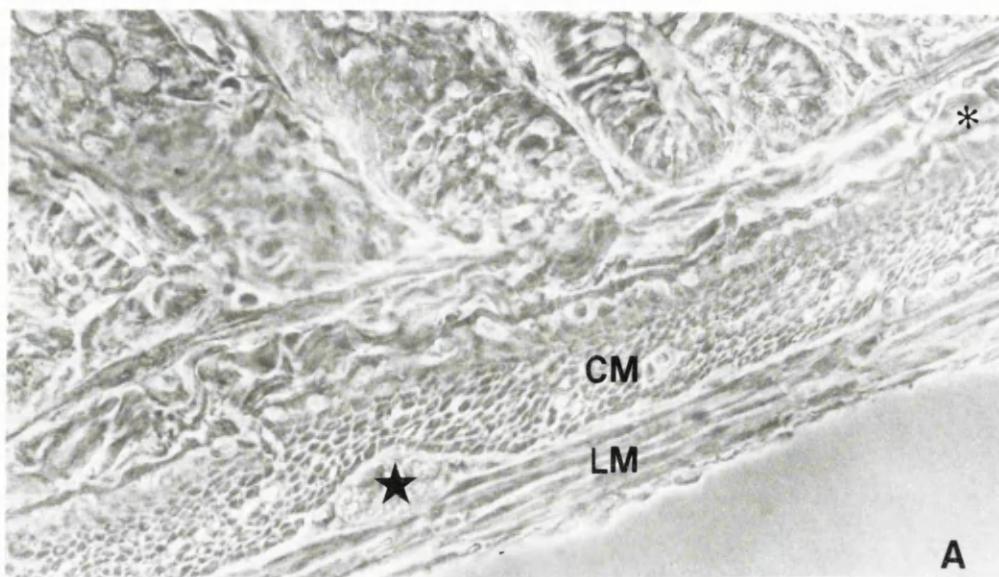


FIGURE 5.2. GAP-43 is expressed in the myenteric plexus. This photograph shows a wax section of the ileum. Neuronal cell bodies are not GAP-43 immunoreactive. Arrowhead in A indicates one of several GAP-43 negative neuronal cell bodies (A) Fluorescein optics to visualize GAP-43; (B) Glial cells labelled with anti-GFAP antibodies. Bar = 10 $\mu$ .

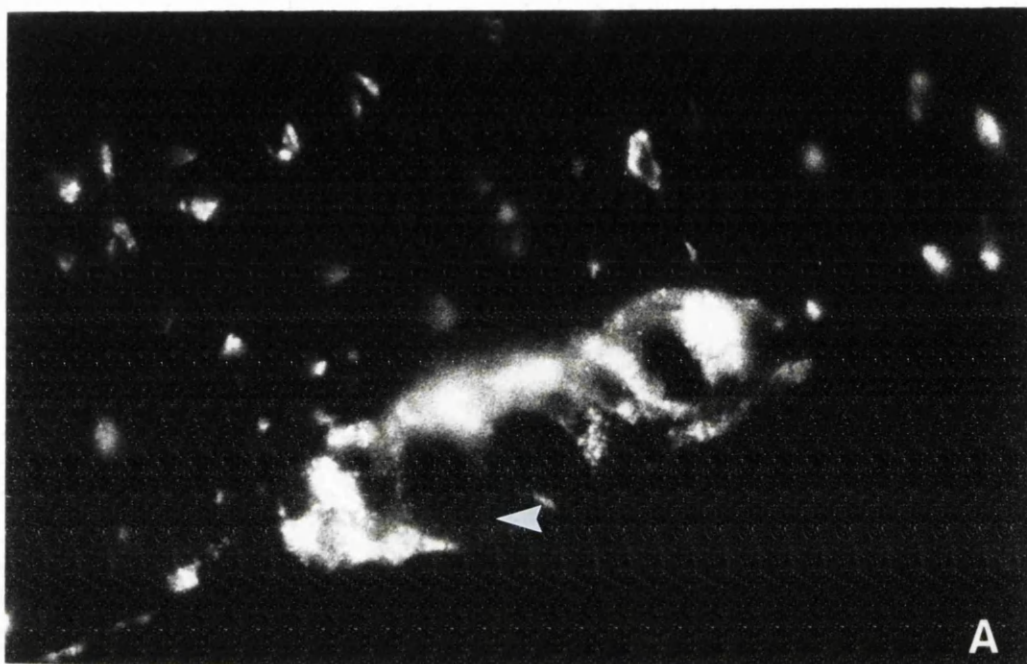


FIGURE 5.3. GAP-43 is present in all neurones of the myenteric plexus. This photograph shows a stretch preparation of the myenteric plexus of the adult rat. (A), (B) and (E) Fluorescein optics to visualize GAP-43. Note the presence of GAP-43 in axonal varicosities (Panel E arrow); (C) and (D) Fluorescein optics to visualize PGP 9.5 in sister preparations. (C) and (D) are the same magnification as are (B) and (E). Bar = 20 $\mu$ .



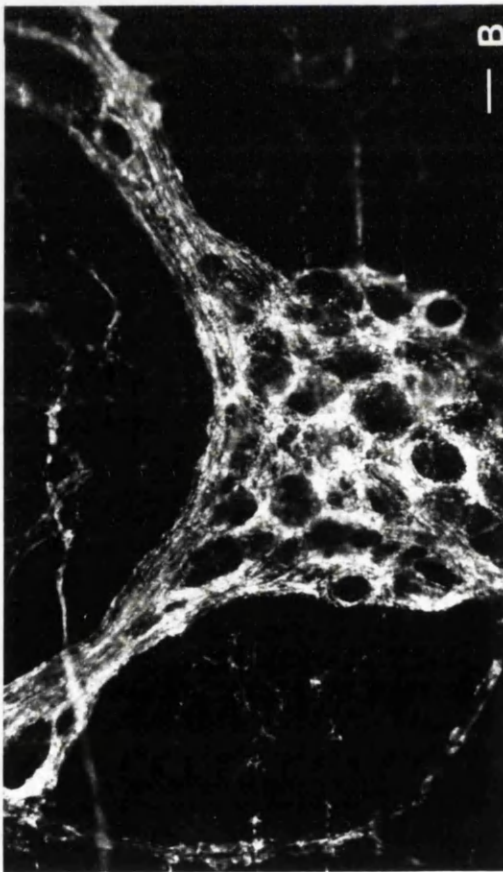
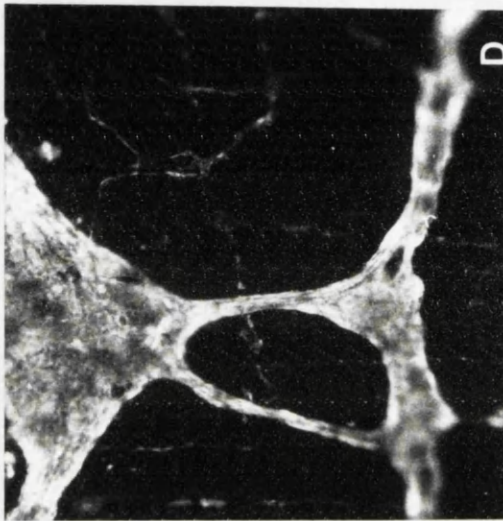
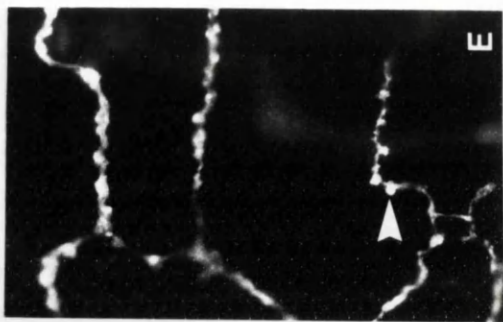
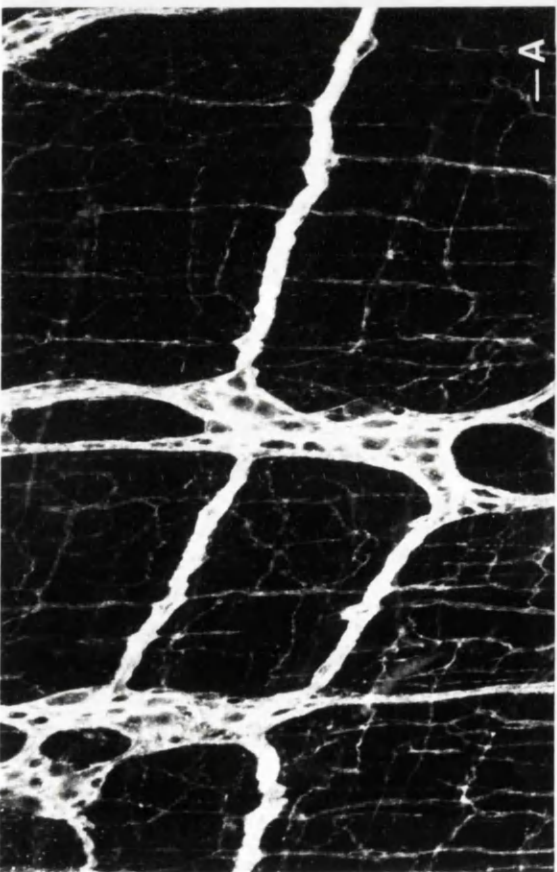
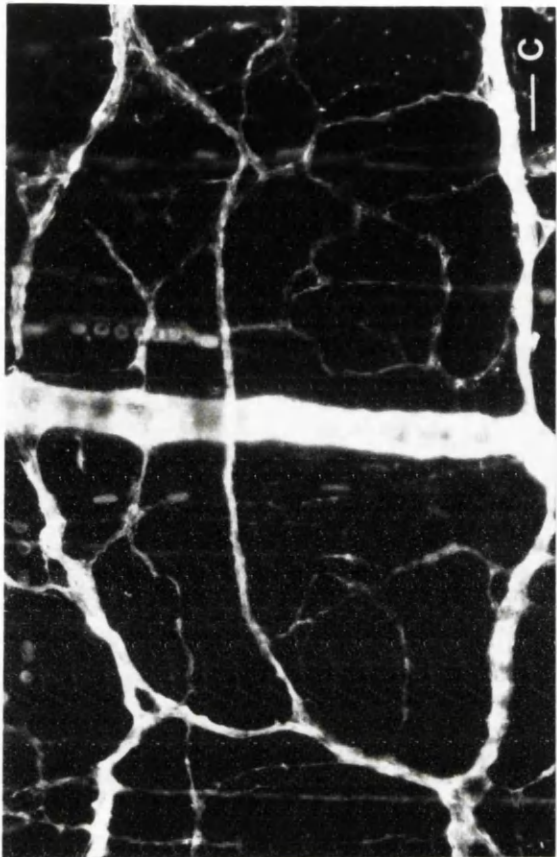


FIGURE 5.4. GAP-43 immunoreactivity is detected in the myenteric plexus of the developing rat. This photograph shows stretch preparation of the ileum of a P7 rat. (A) and (B) Fluorescein optics to visualize GAP-43. Bar = 20 $\mu$ .

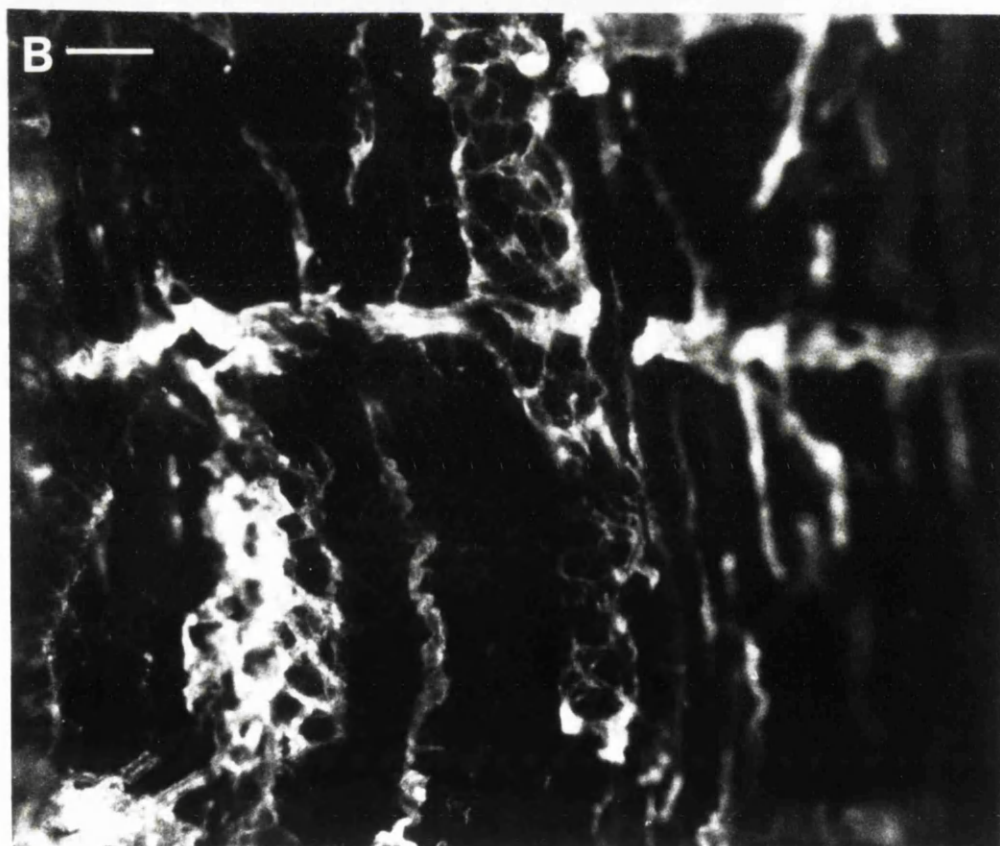
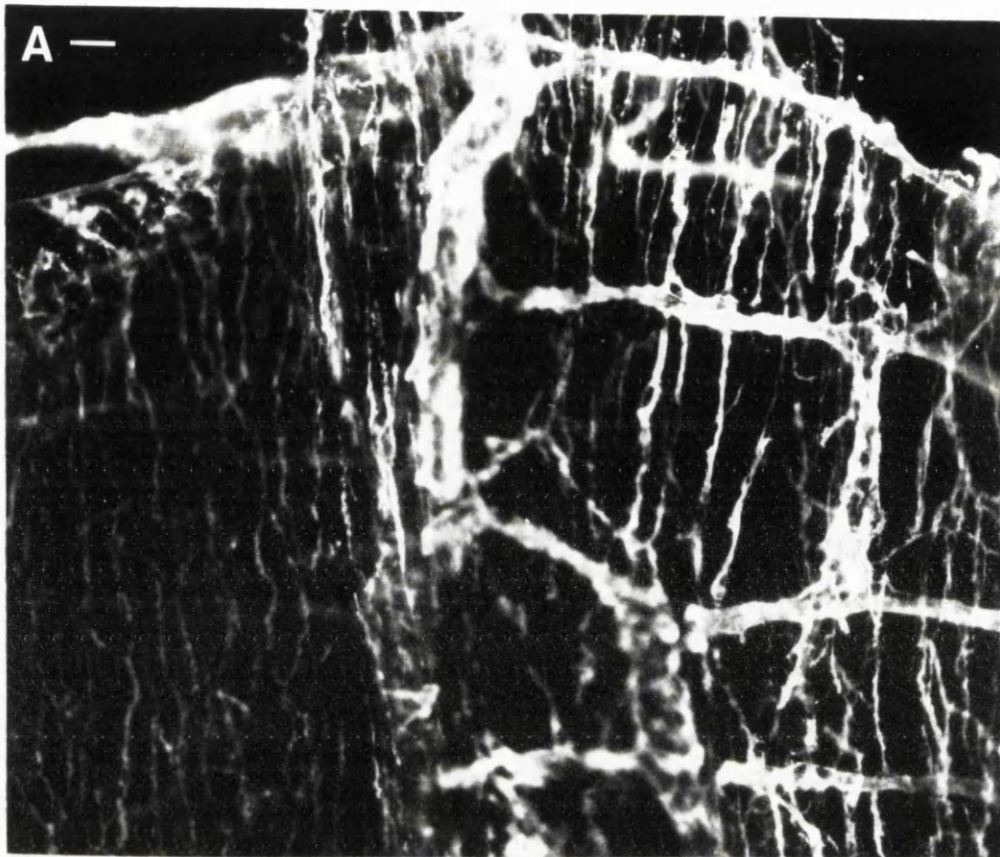


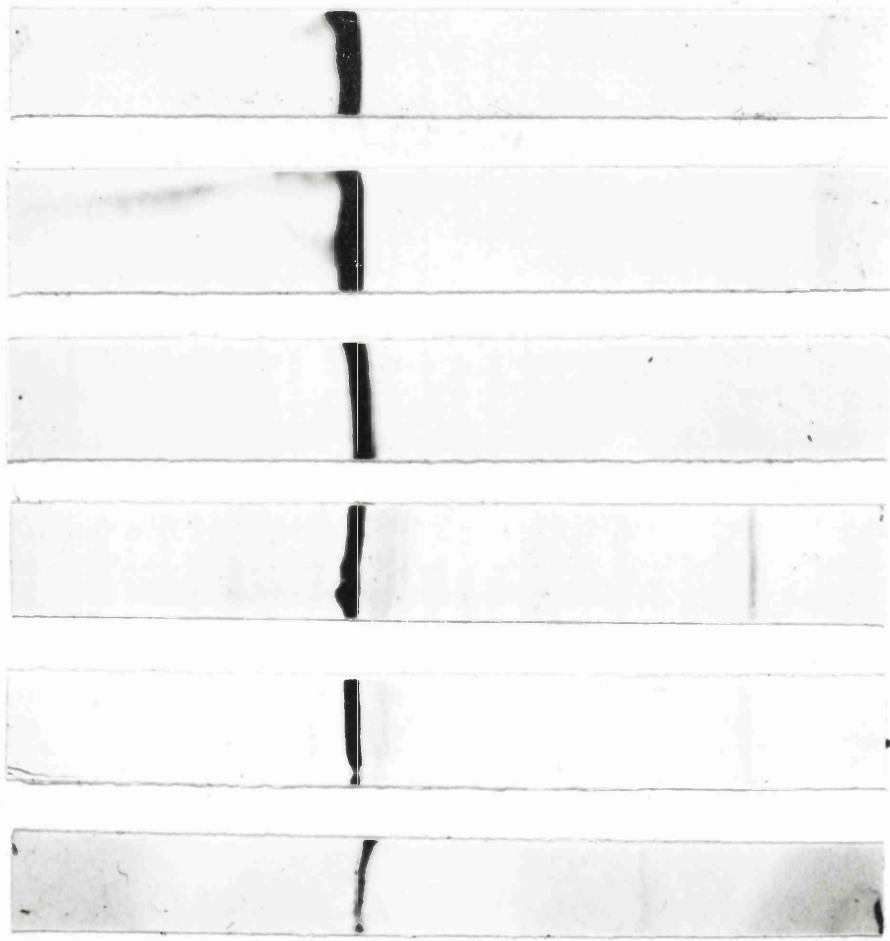


FIGURE 5.5. Western blot of various tissues from adult and developing rats. Preparations (10 $\mu$ g) were subjected to electrophoresis on a 10% SDS polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and incubated with anti GAP-43 antiserum. Blots were developed using diaminobenzidine as a chromophore in the peroxidase colormetric reaction.

enteric  
plexus

SC SN ST

P1 P7 AD AD AD



— 66 kDa

— GAP-43

— 29 kDa

FIGURE 5.6. GAP-43 immunoreactivity is widespread in the adult autonomic nervous system. These photographs show GAP-43 visualized by fluorescein optics in whole mount preparations of (A) Circle of Willis (cerebral vessel); (B) Mesenteric Vein; (C) Iris; (D) Basilar artery (cerebral vessel); (E) Tail vein vessel). A, C, D and E the same magnification. Bar = 20 $\mu$ .

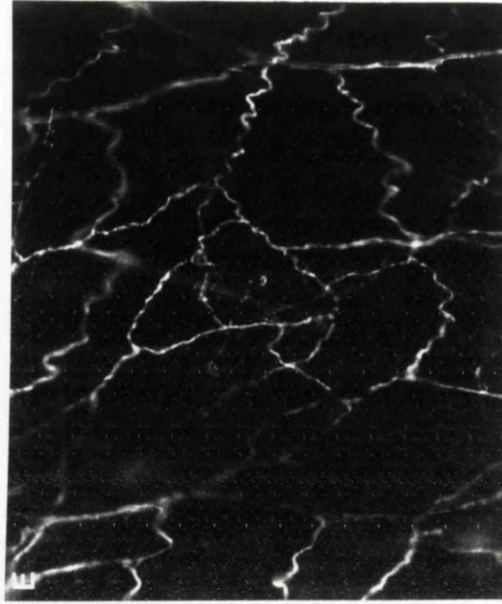
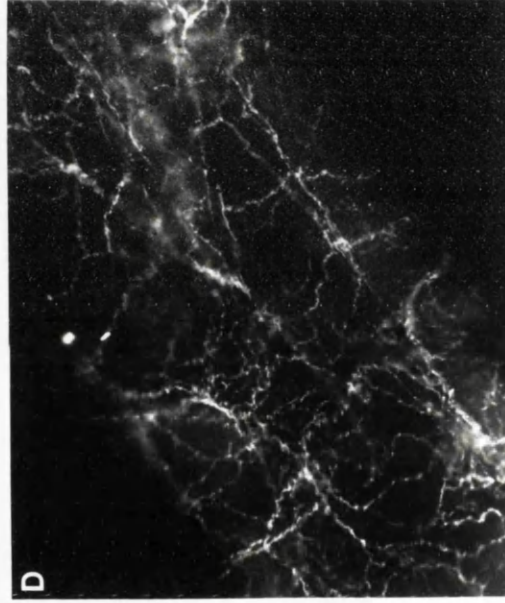
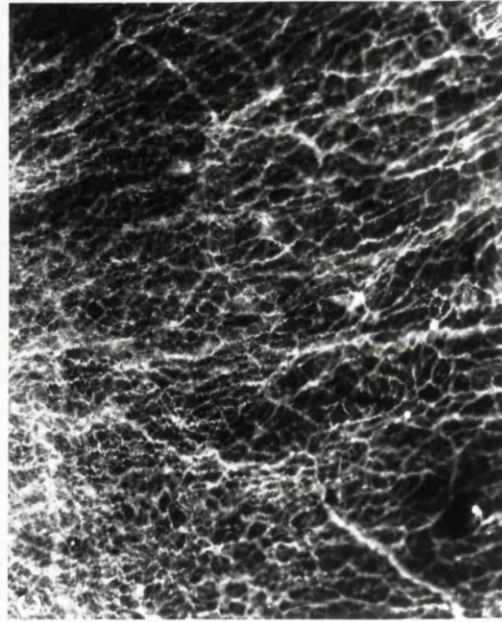
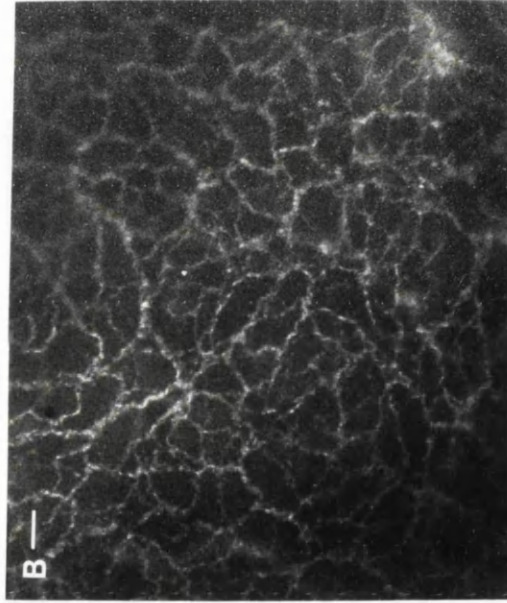
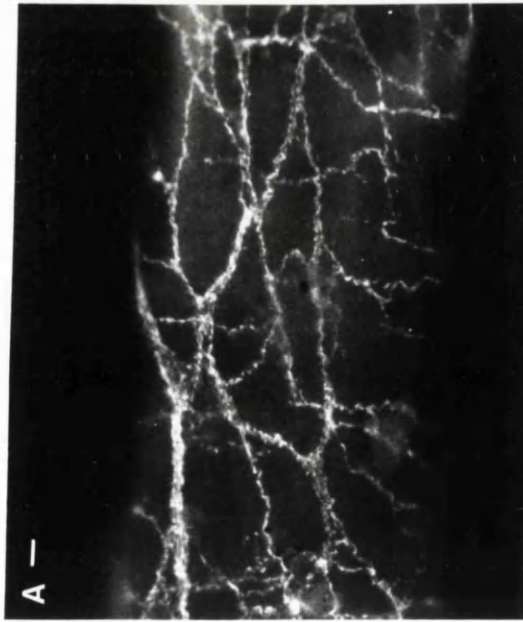
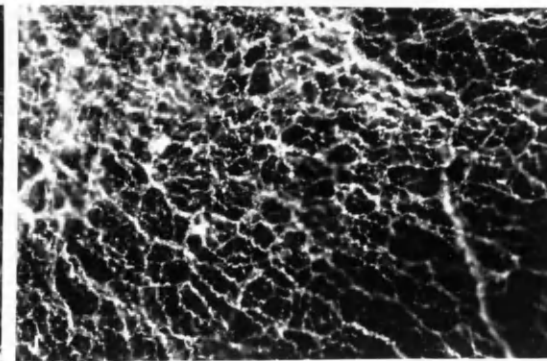
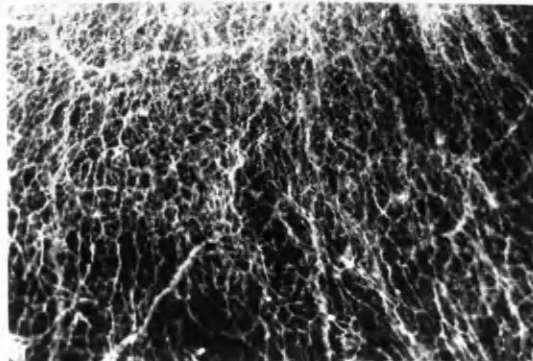
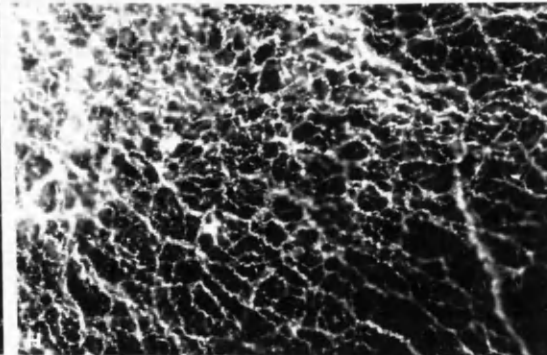
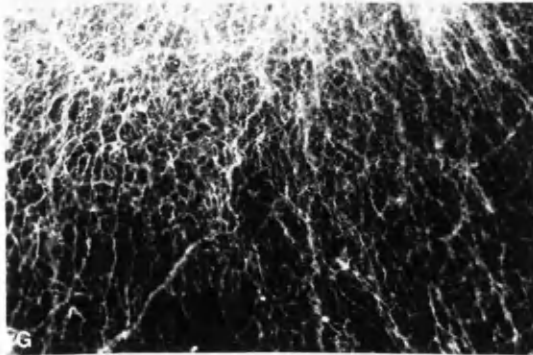
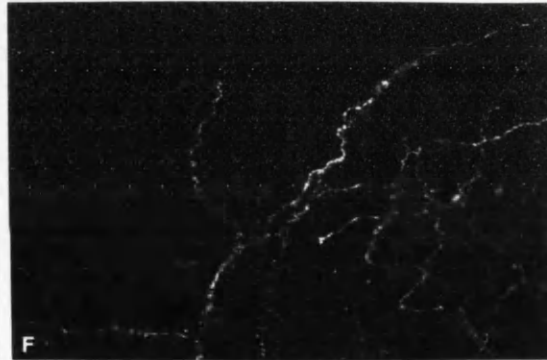
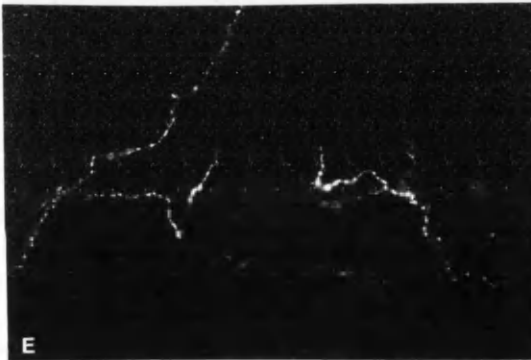
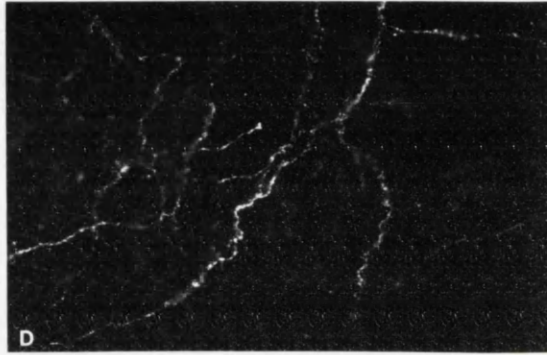
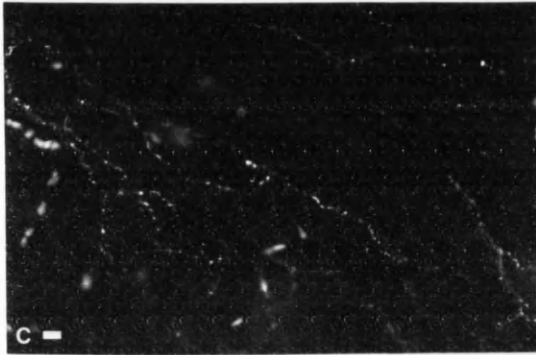
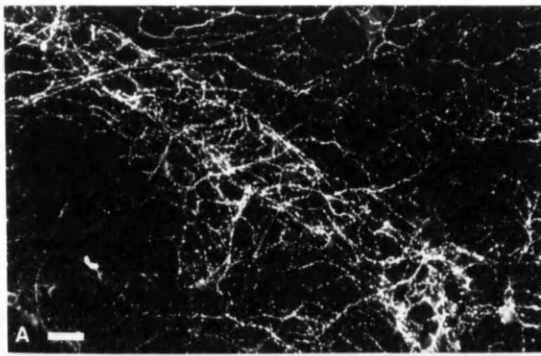


FIGURE 5.7. GAP-43 expression in the iris before and after sympathectomy. This photograph compares the distribution of (A) and (B) Catecholamines; (C) and (D) VIP; (E) and (F) substance P; (G) and (H) GAP-43; (I) and (J) PGP 9.5; before and after removal of the SCG. Fluorescein optics were used to visualize B-F and I and J. Rhodamine optics to visualize G and H, and 420nm excitatory light to visualize A. Photographs from the left hand side of the diagram are from unoperated rats and on the right hand side from operated rats. All photographs are the same magnification except C. Bar =20 $\mu$ .



**FIGURE 5.8.** PGP 9.5 is expressed by both cultured and reactive Schwann cells. This photograph shows PGP 9.5 immunoreactivity in Schwann cells from P1 rats after 24 hr in culture and PGP 9.5 immunoreactivity in the distal stump of the sciatic nerve 7 days post cut. (A) and (D) Fluorescein optics to visualize PGP 9.5; (B) and (C) phase contrast. Bar = 10 $\mu$ .



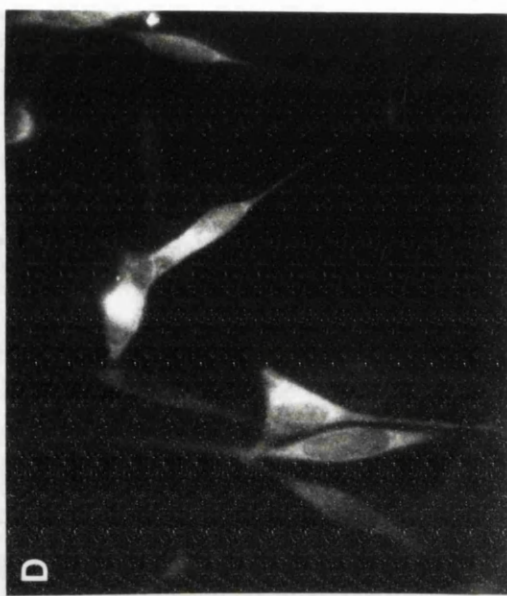
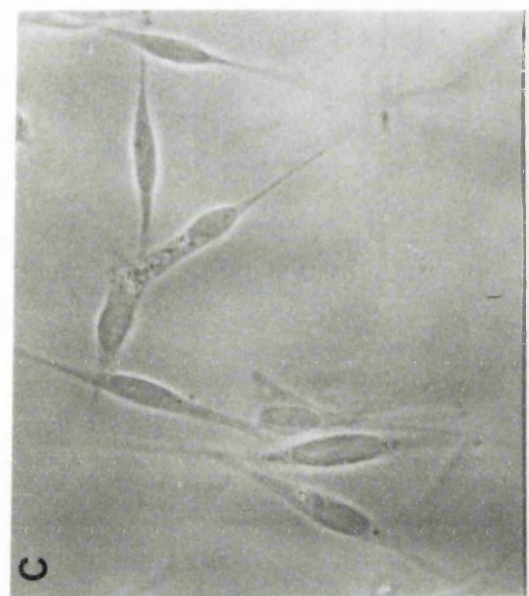
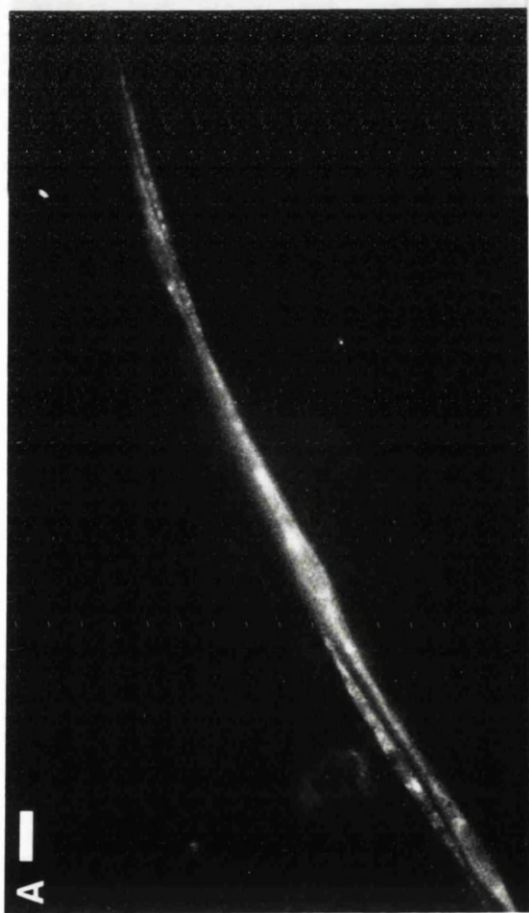




FIGURE 5.9. GAP-43 expression in Schwann cells is not rapidly upregulated after sciatic nerve transection. This photograph shows GAP-43 expression in Schwann cells 7 days after sciatic nerve transection. (A) phase contrast; (B) Fluorescein optics to visualize GAP-43; (C) Rhodamine optics to visualize NGF receptor. Bar = 10 $\mu$ .

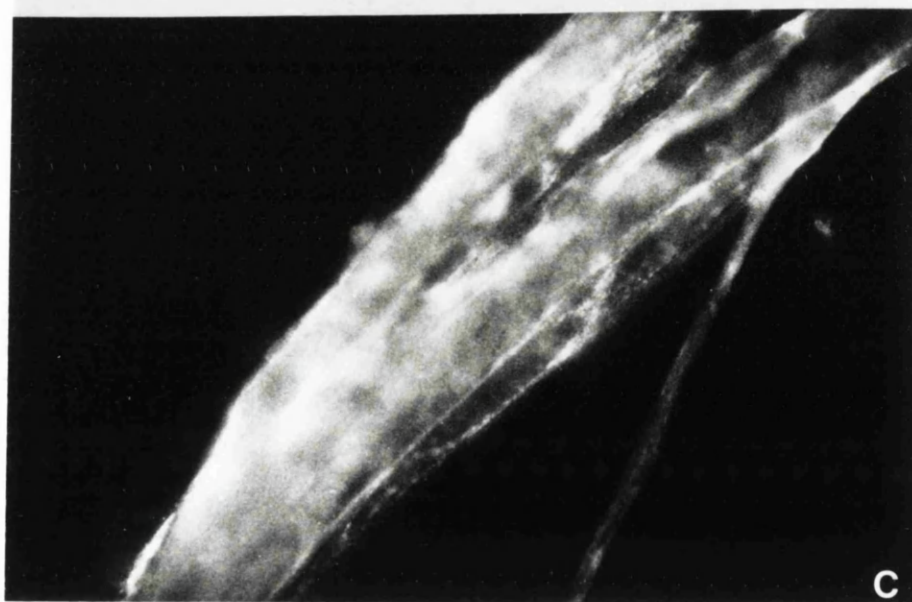
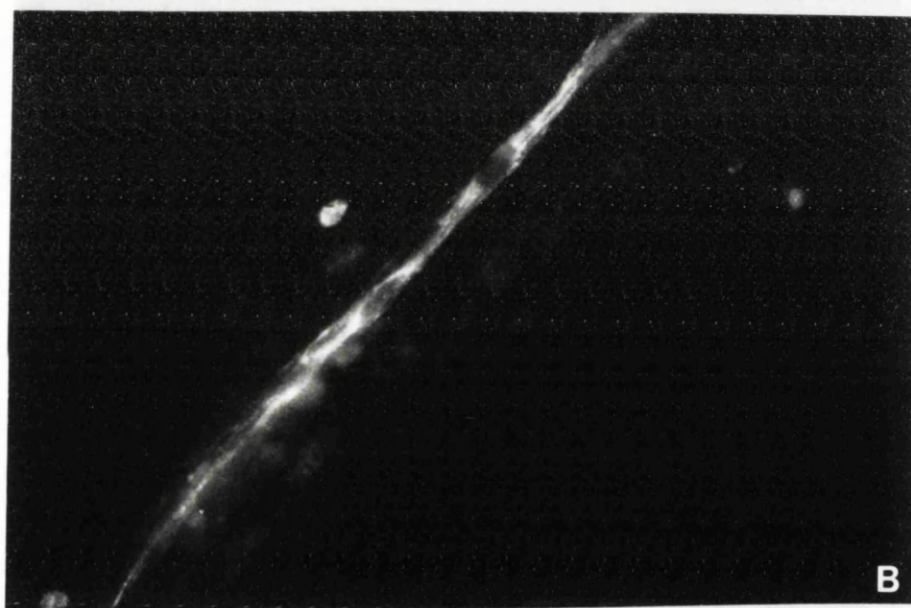
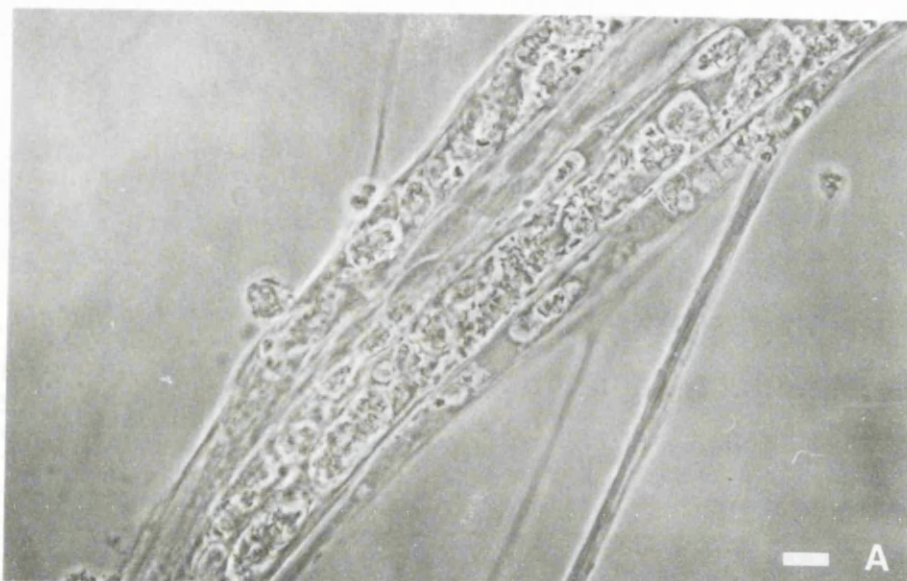


FIGURE 5.10. GAP-43 expression is restricted to non-myelin-forming Schwann cells *in vivo*. This photograph shows a unmyelinated and a myelinated nerve fibre from an adult sciatic nerve. (A) Fluorescein optics to visualize GAP-43; (B) Rhodamine optics to visualize vimentin; (C) phase contrast. Bar = 10 $\mu$ .

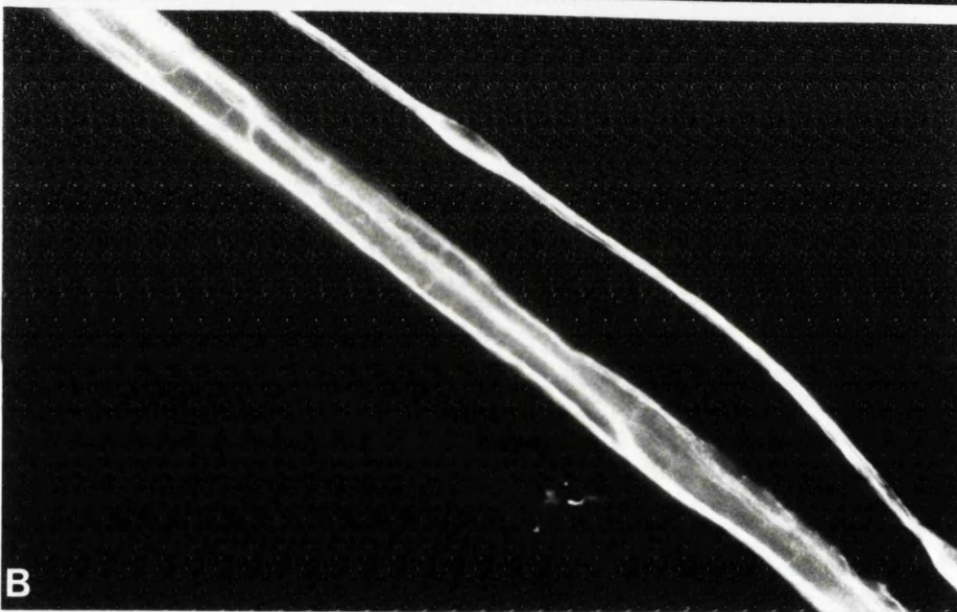
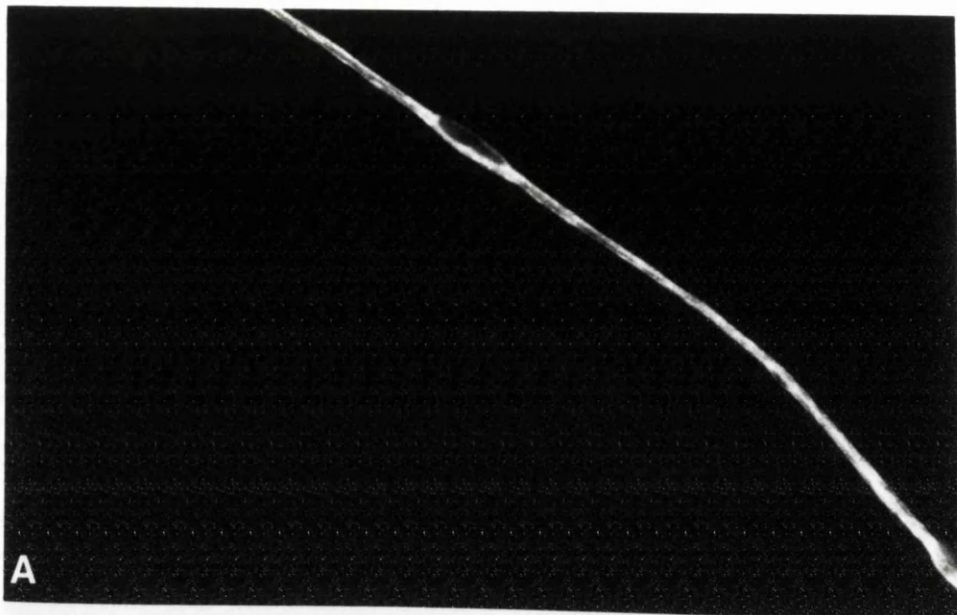


FIGURE 5.11. Non-myelin-forming Schwann cells express GAP-43. This photograph shows a bundle of unmyelinated fibres teased from the sympathetic trunk. (A) Fluorescein optics to visualize GAP-43; (B) Rhodamine optics to visualize GFAP; (C) phase contrast. Bar = 10 $\mu$ .



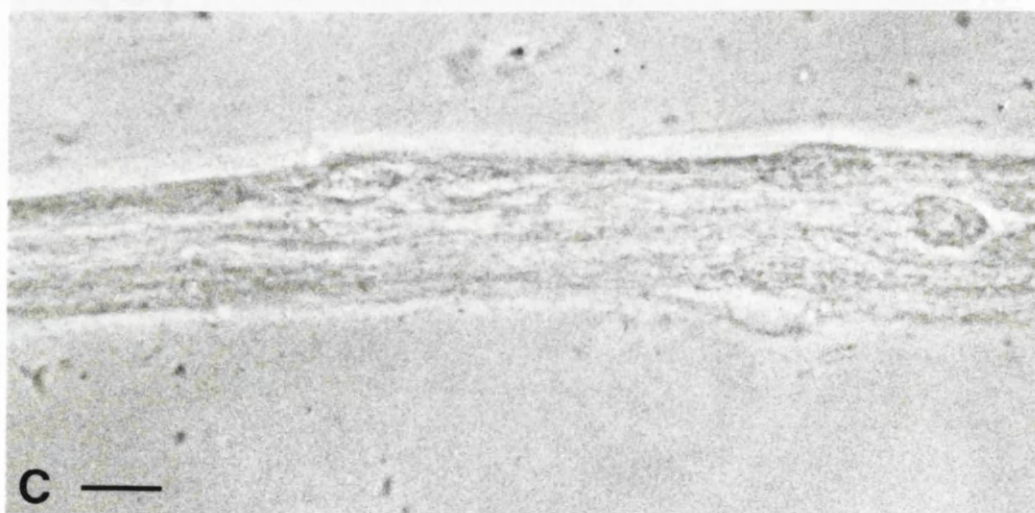
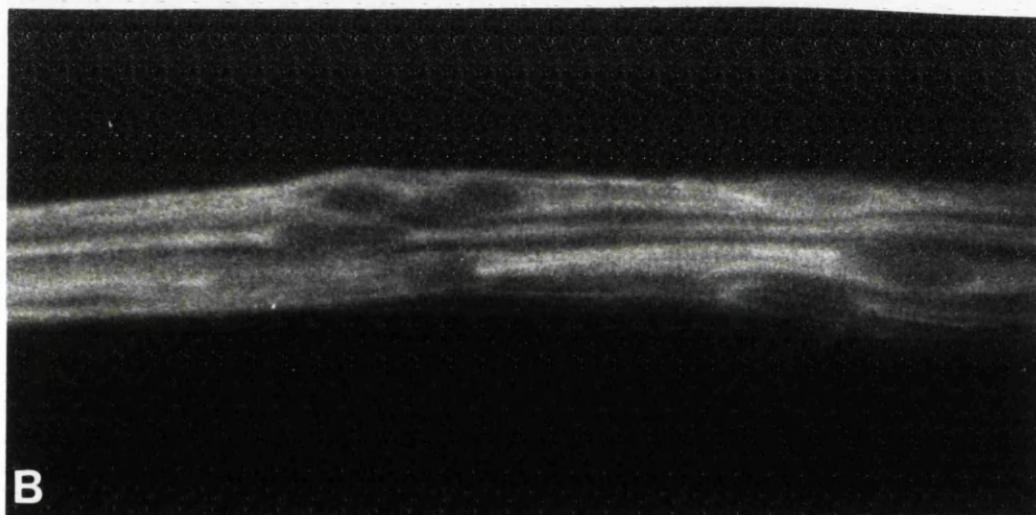
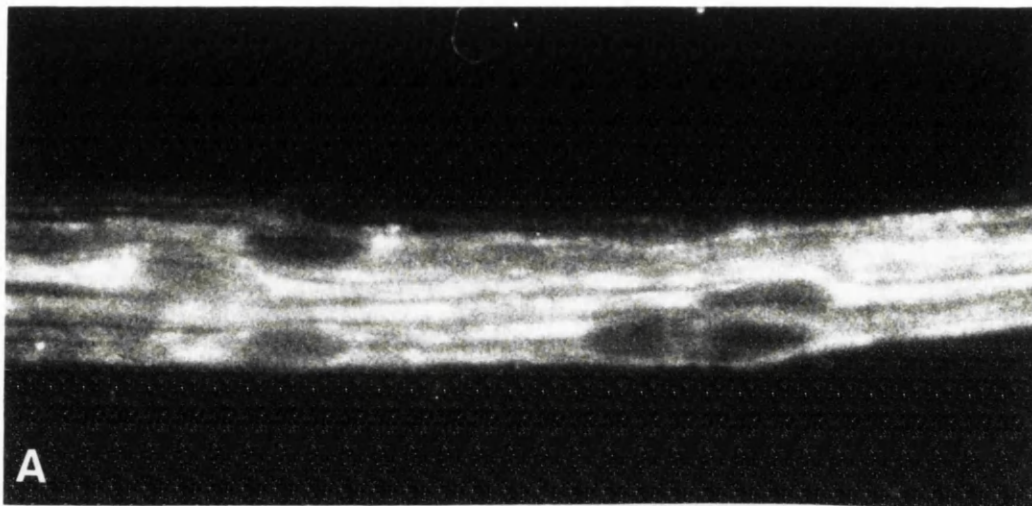


FIGURE 5.12. GAP-43 expression in dissociated cells from the adult SCG. This photograph shows that GAP-43 immunoreactivity is associated with a population of cells thought to be satellite cells. (A) fluorescein optics to visualize GAP-43 ; (B) Phase contrast. Bar = 10 $\mu$ .

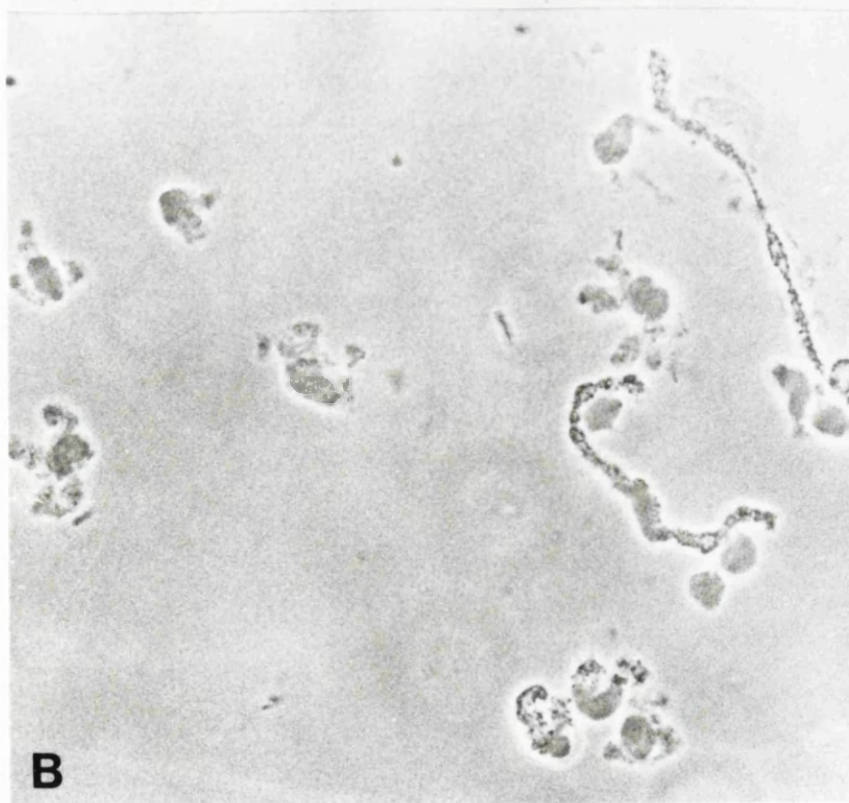
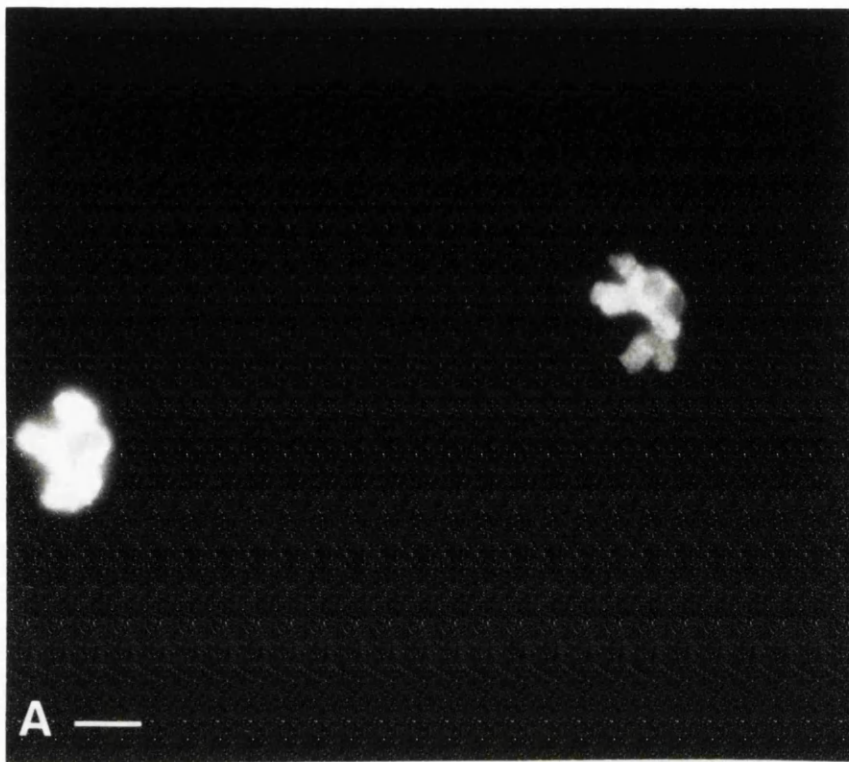




FIGURE 5.13. GAP-43 is expressed in by satellite cells in the SCG. This photograph shows a dried dissociated preparation of the adult SCG. GAP-43 immunoreactive satellite cells are identified by their association with SCG neurones. (A) Fluorescein optics to visualize GAP-43 ; (B) phase contrast. Bar = 10 $\mu$ .

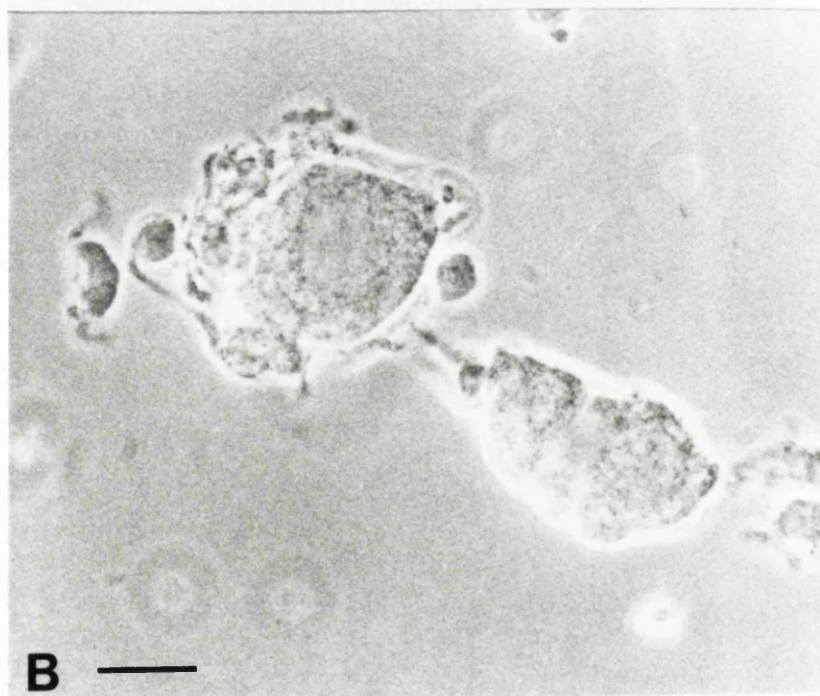
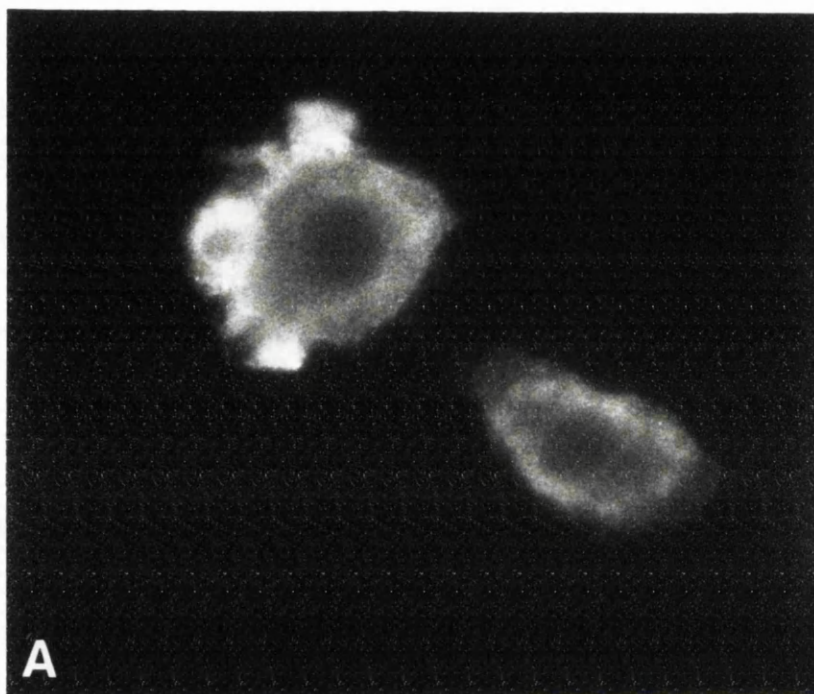


FIGURE 5.14. GAP-43 expression in the dorsal root ganglion. These photographs shows wax sections of an adult rat DRG. GAP-43 is expressed by some DRG neurones (A) and (D); a few satellite cells (D arrow). (B) and (C) phase contrast; (A) and (D) fluorescein optics to visualize GAP-43. Bar = 10 $\mu$ .

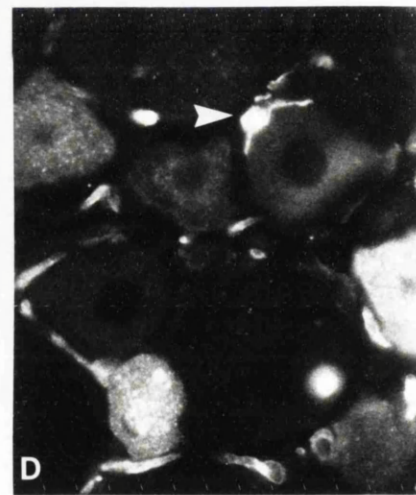
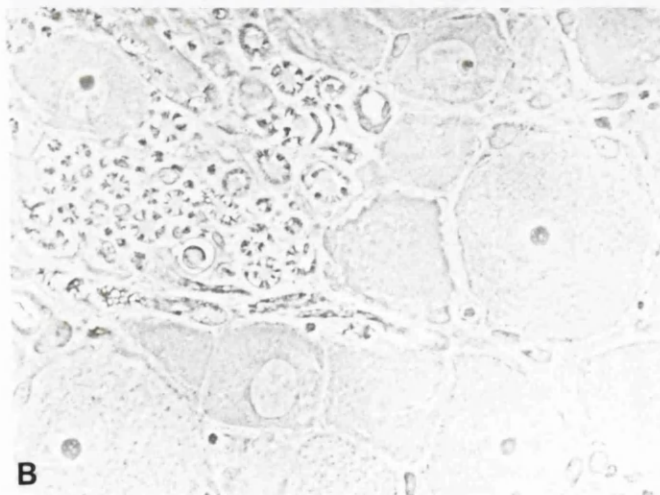
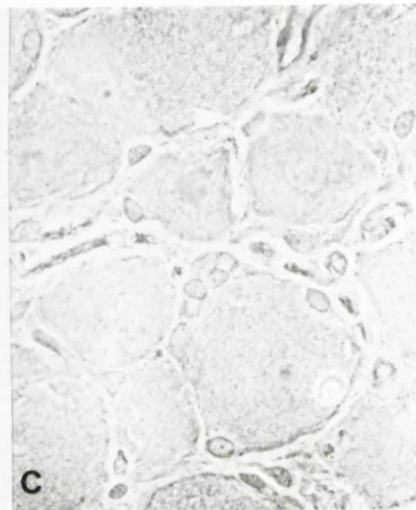
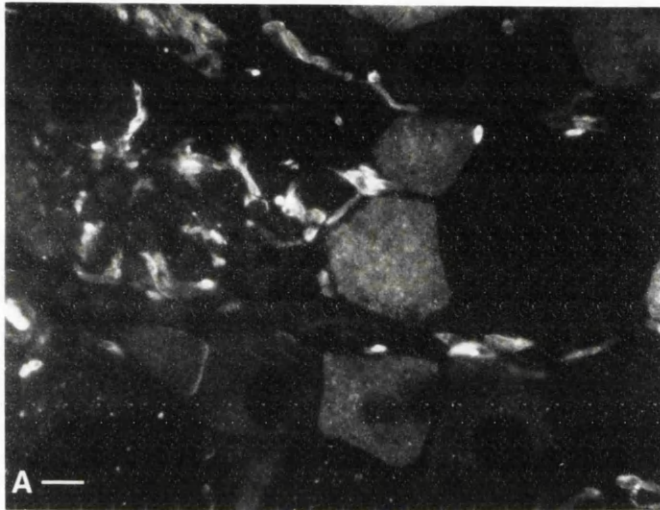


FIGURE 5.15. GAP-43 expression in the SCG. These photographs shows that GAP-43 is expressed by many satellite cells of the adult SCG. (A) Fluorescein optics to visualize GAP-43; (B) phase contrast. Bar = 10 $\mu$ .

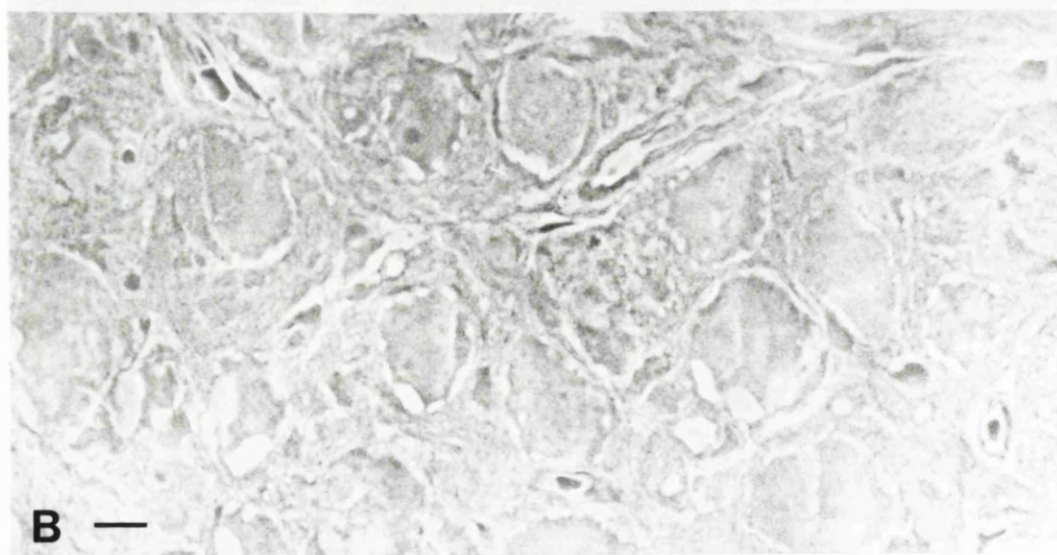
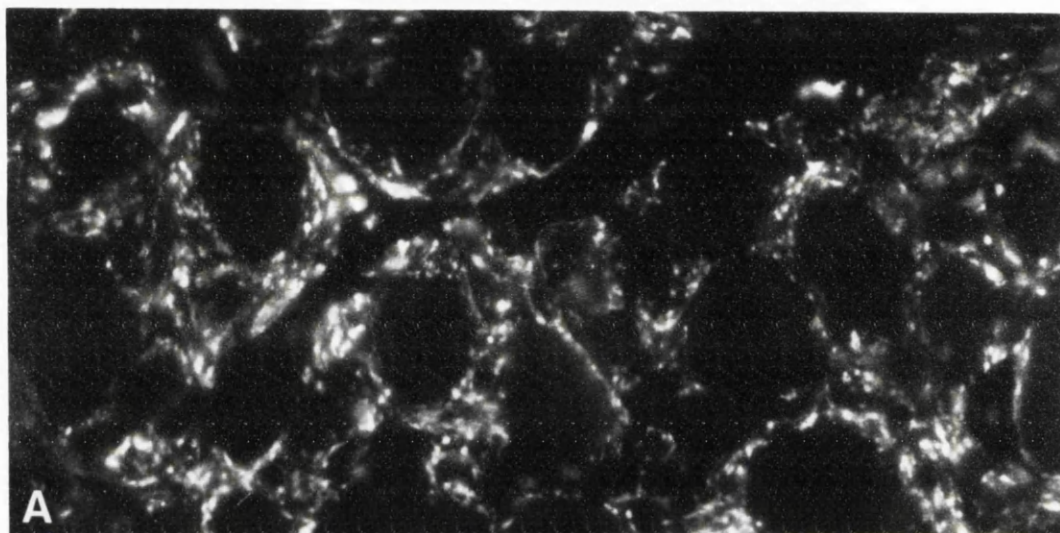


FIGURE 5.16. GAP-43 immunoreactivity in axonal varicosities. These photographs show a longitudinal section of the dorsal root of the DRG. (A) Fluorescein optics to visualize GAP-43; (B) Phase contrast. Bar = 10 $\mu$ .



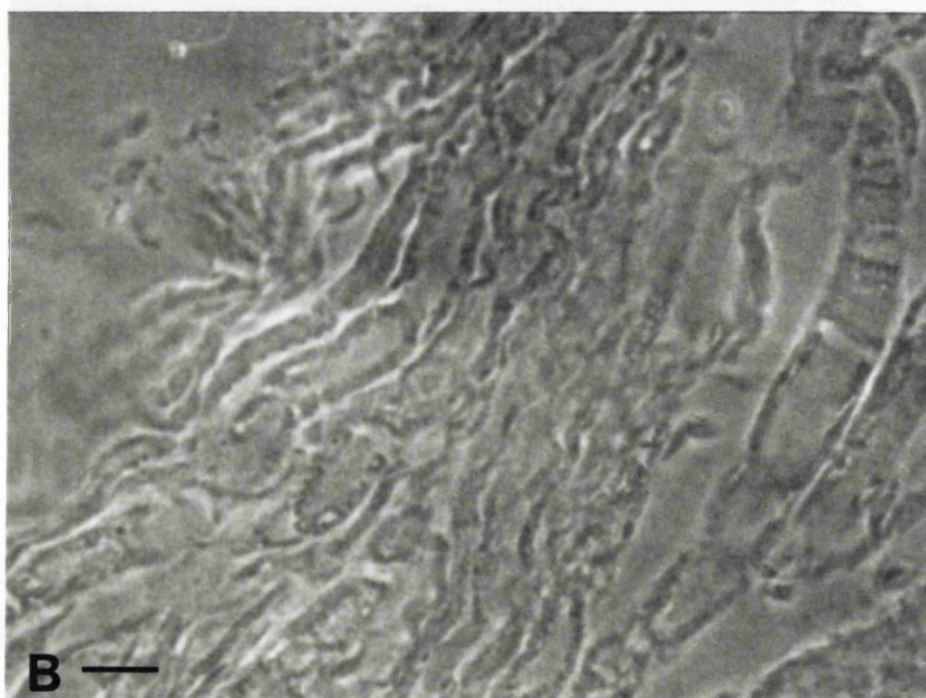
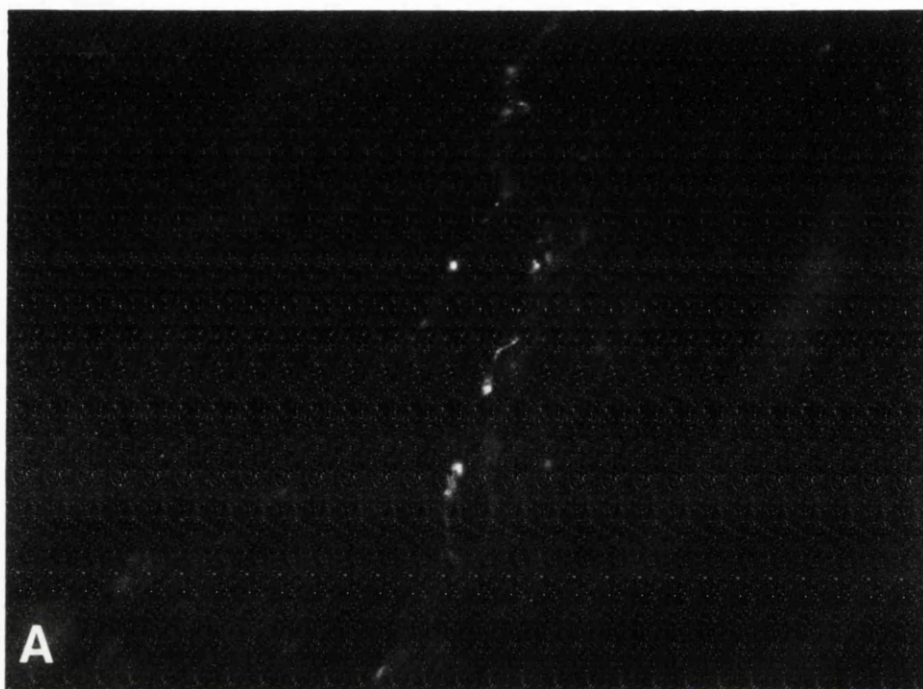




FIGURE 5.17. GAP-43 expression in dried dissociated embryonic Schwann cells. These photographs show dissociated cells prepared from an E15 sciatic nerve. (A) Fluorescein optics to visualize GAP-43; (B) phase contrast. Bar = 10 $\mu$ .

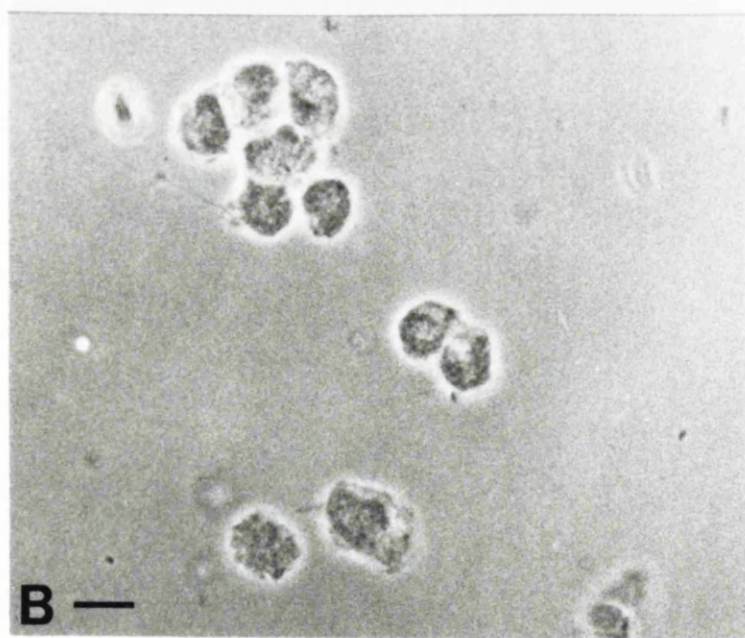
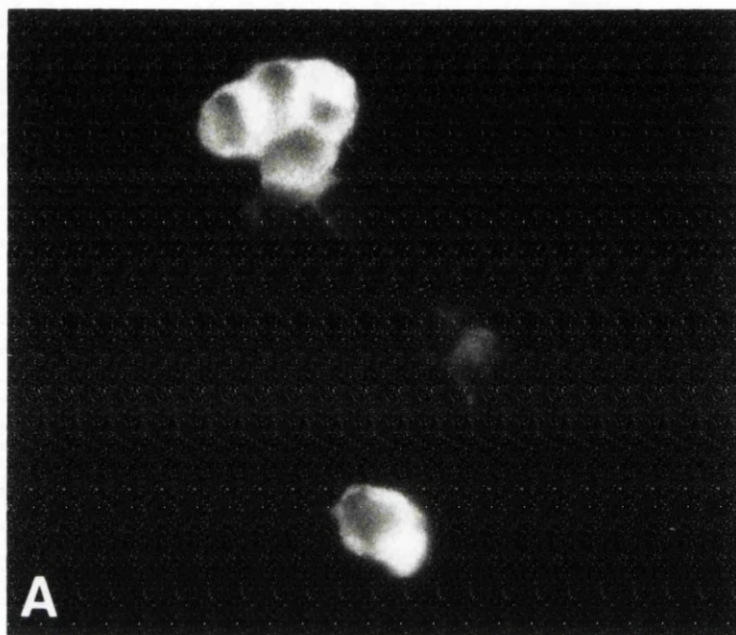
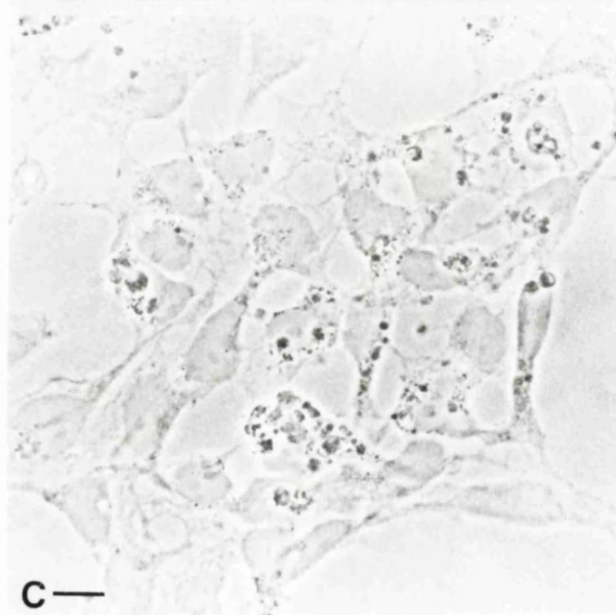
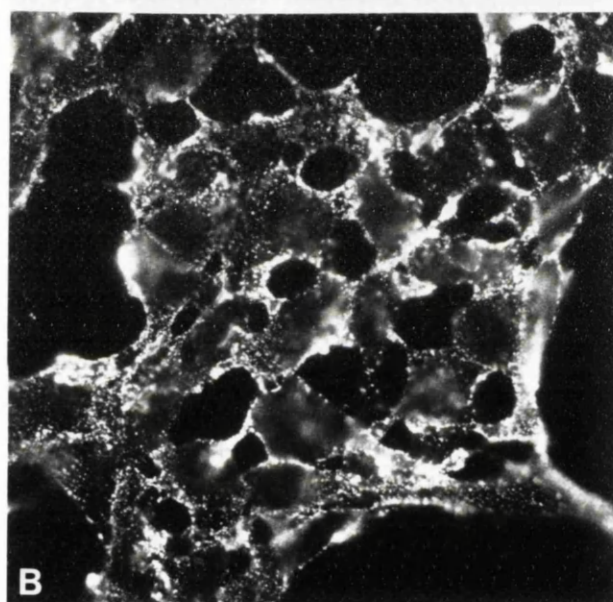
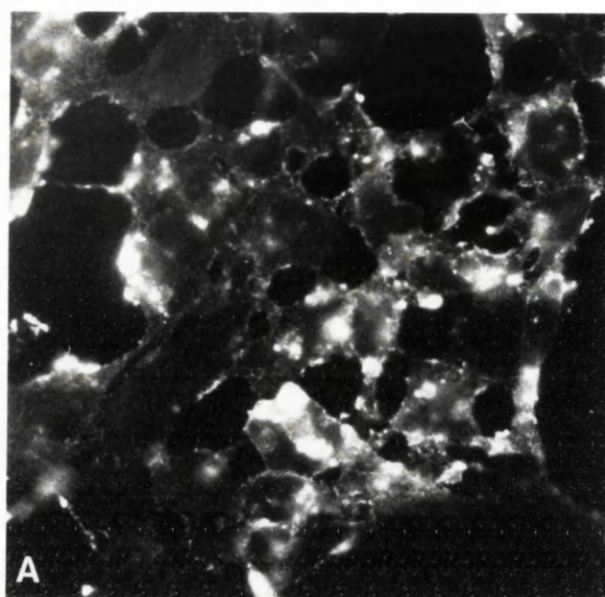
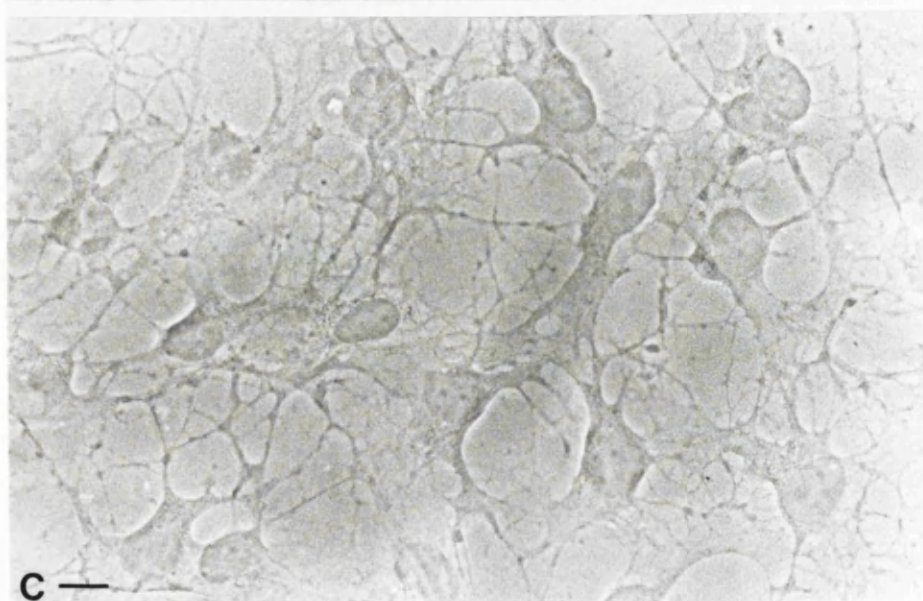
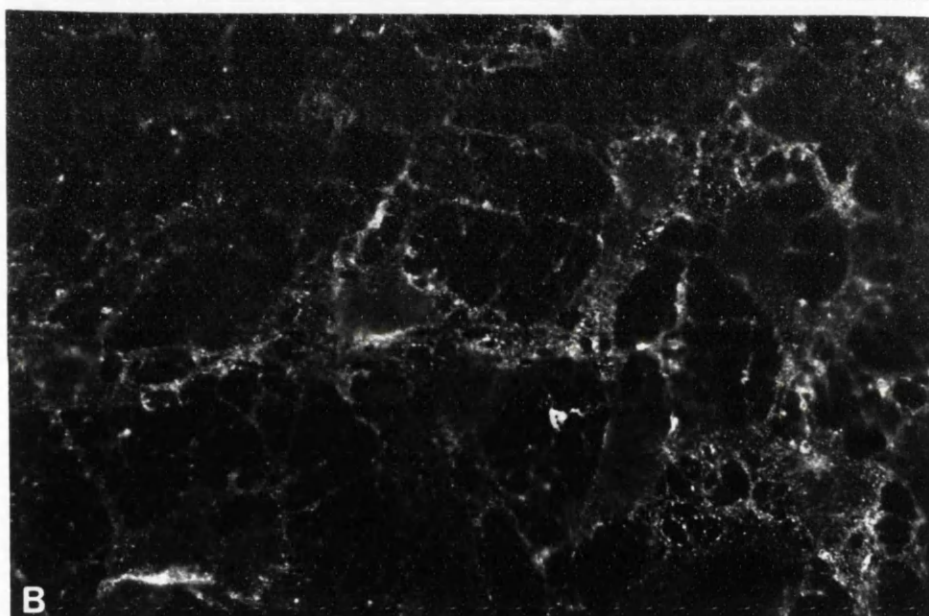
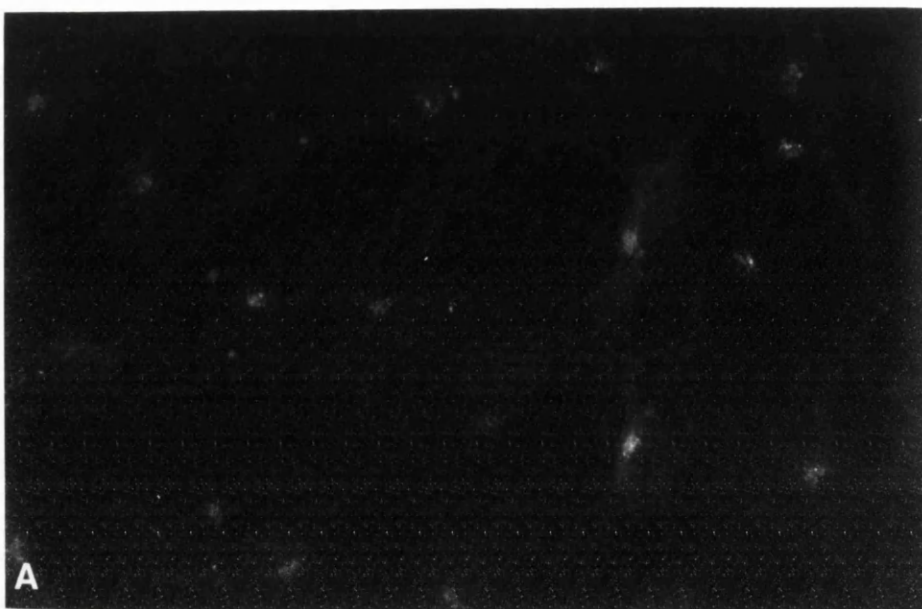


FIGURE 5.18. GAP-43 is expressed by embryonic Schwann cells. These photographs show that GAP-43 is expressed by the majority of E15 Schwann cells after 24hr in culture. (A) Fluorescein optics to visualize GAP-43; (B) Rhodamine optics to visualize NGF receptor; (C) Phase contrast. Bar =  $10\mu$ .



**FIGURE 5.19.** GAP-43 expression is downregulated by embryonic Schwann cells in culture. These photographs show GAP-43 expression by E15 Schwann cells after 6 days in culture. Note the distribution of GAP-43 in the perinuclear area. (A) Fluorescein optics to visualize GAP-43; (B) Rhodamine optics to visualize NGF receptor; (C) phase contrast. Bar = 10 $\mu$ .



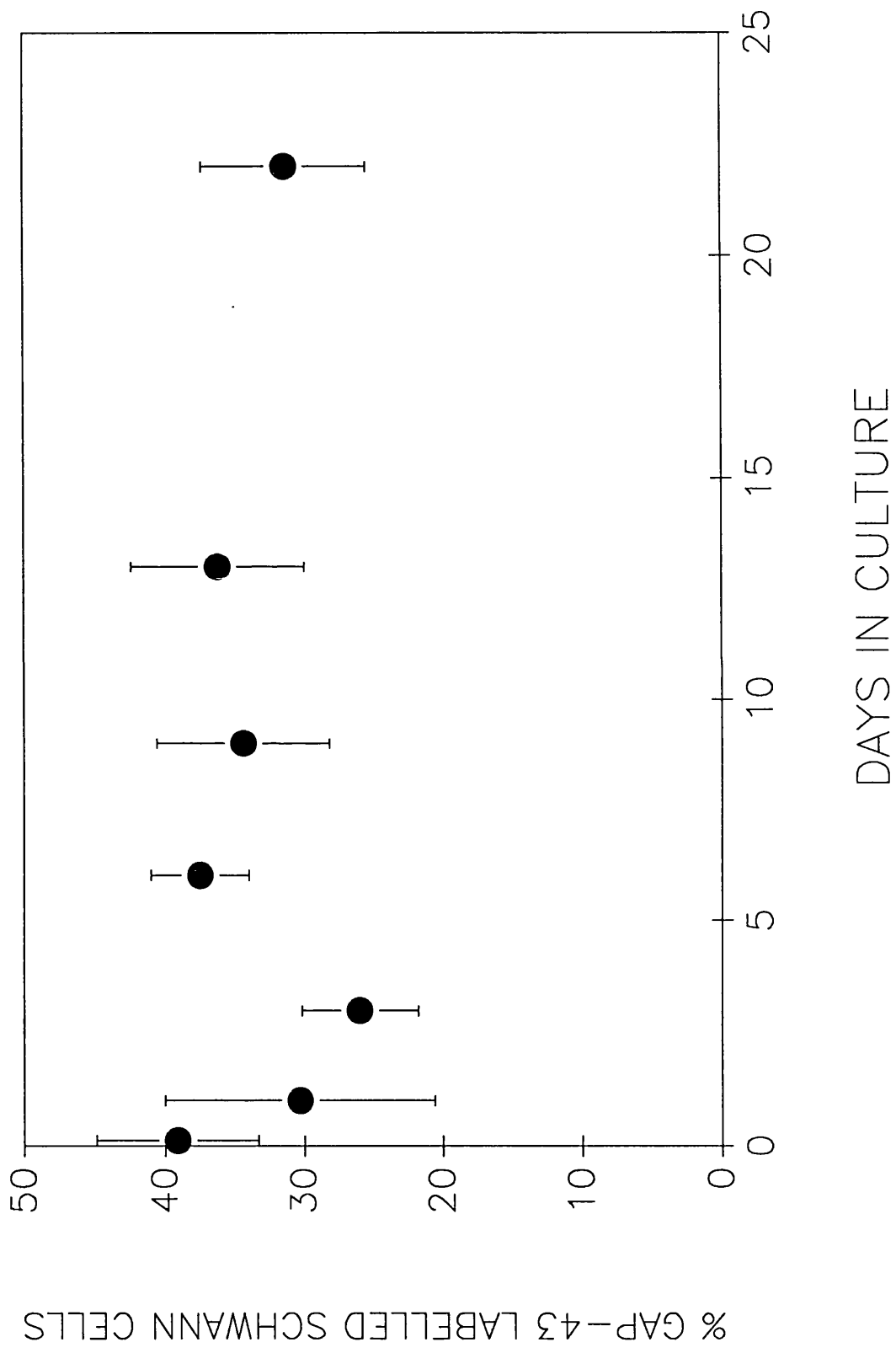
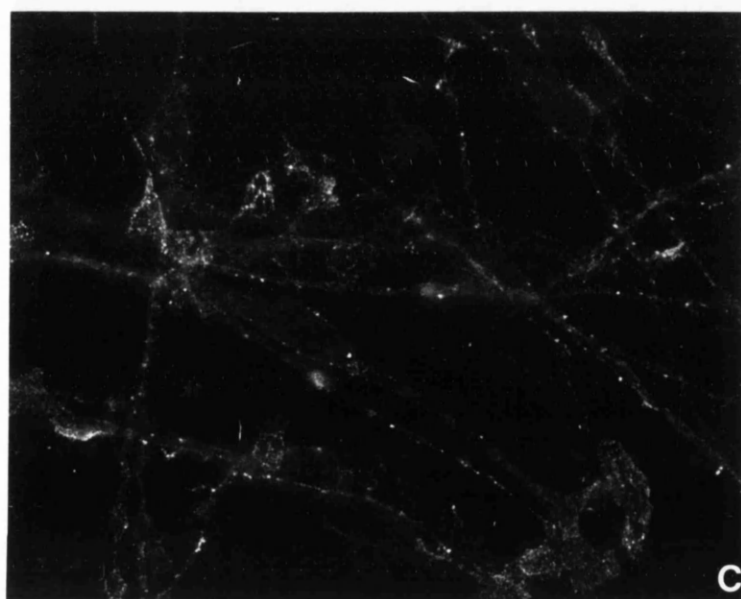
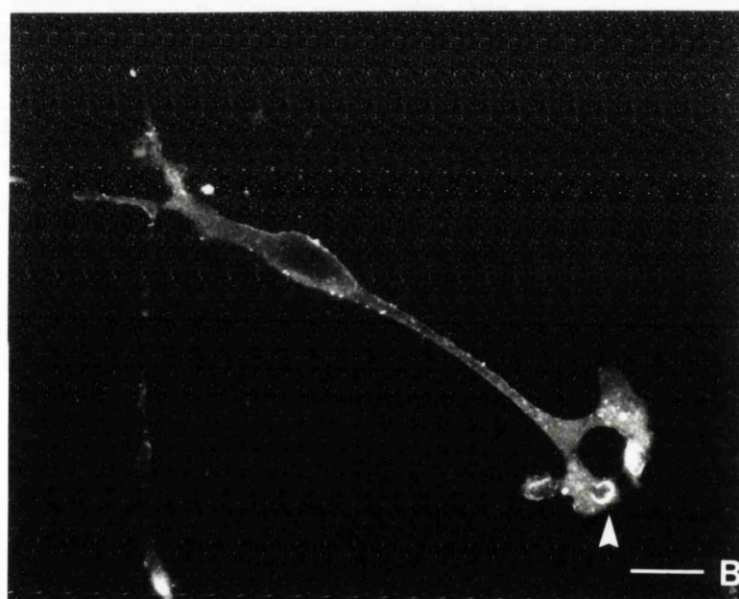
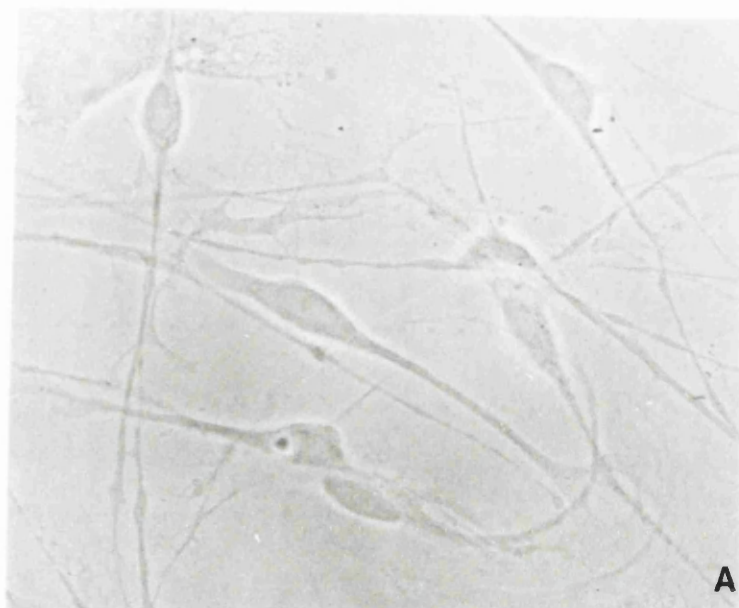


FIGURE 5.20. GAP-43 expression in postnatal Schwann cells is not downregulated in culture. Schwann cells from P1 rats were cultured for varying times up to 23 days. Schwann cells were identified by morphology and NGF receptor coexpression.



FIGURE 5.21. GAP-43 in Schwann cells is present in "ring like" structures. These photographs show several Schwann cells after 3 days in culture. Note the presence of GAP-43 in a structure at the end of the Schwann cell process (Arrowhead). (A) phase contrast; (B) Fluorescein optics to visualize GAP-43; (C) Rhodamine optics to visualize NGF receptor. Bar = 10 $\mu$ .



**FIGURE 5.22.** GAP-43 is expressed by a subpopulation of postnatal Schwann cells. These photographs show Schwann cells from a P1 rat after 3 days in culture. (A) Fluorescein optics to visualize GAP-43; (B) phase contrast. Bar = 10 $\mu$ .

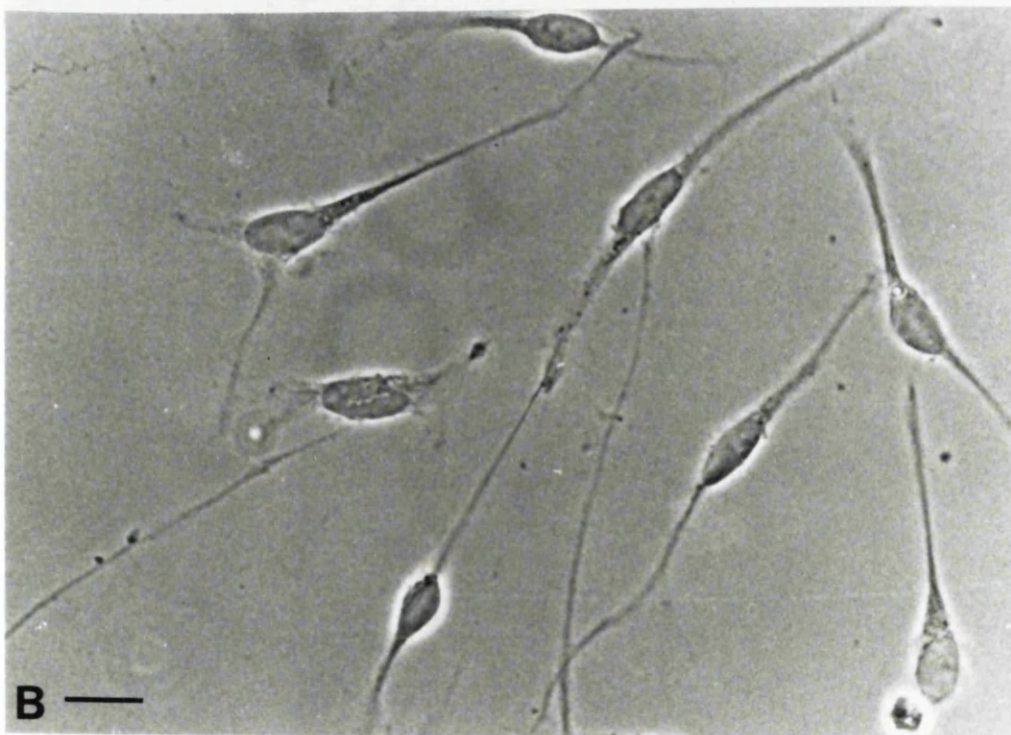
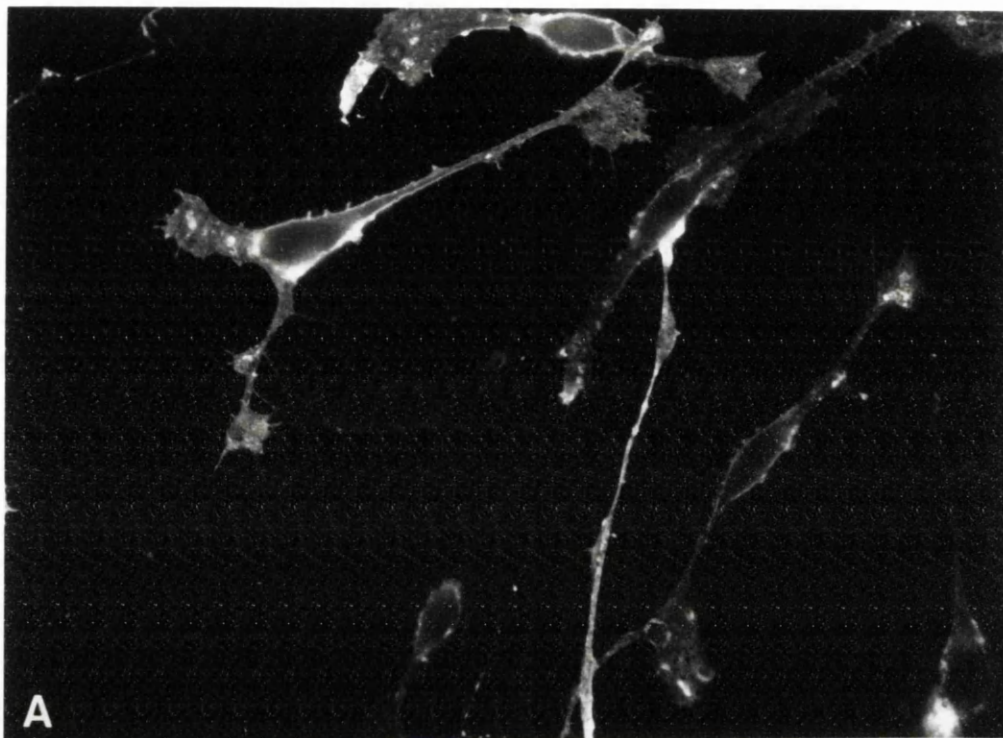
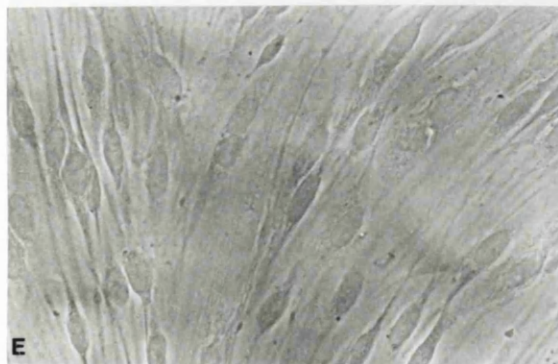
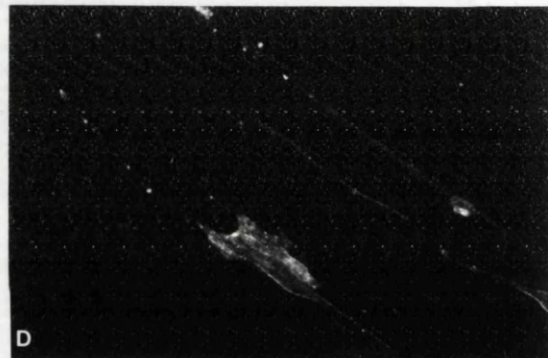
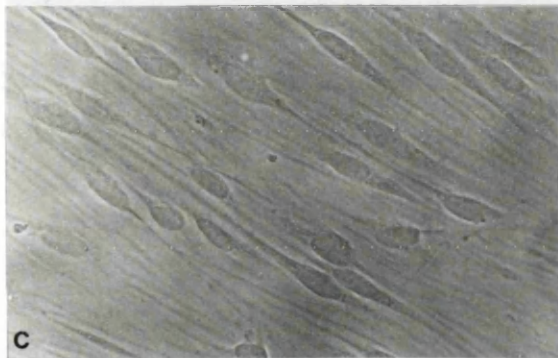
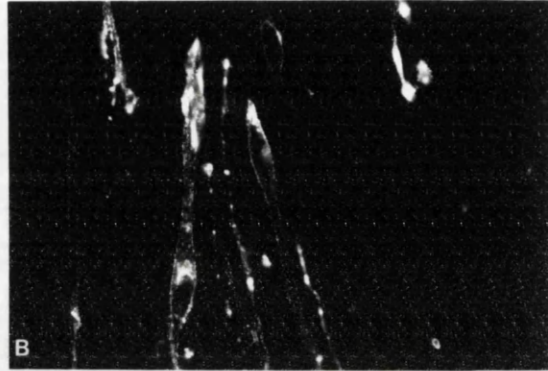
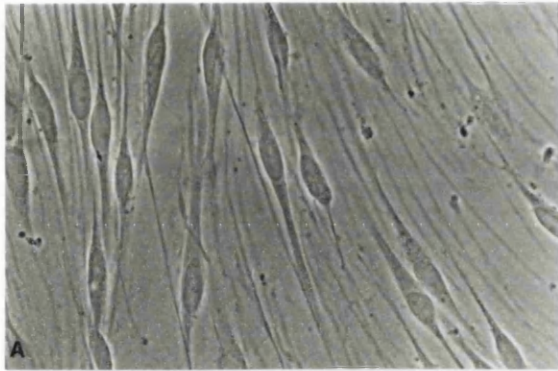


FIGURE 5.23. Postnatal Schwann cell GAP-43 expression is downregulated by cAMP. These photographs show P1 Schwann cells after treatment with forskolin for 48hr. (A) and (B) no treatment; (C) and (D) 1 $\mu$ M Forskolin; (E) and (F) 5 $\mu$ M forskolin. A,C and E, phase contrast; B,D, and F, Fluorescein optics to visualize GAP-43. Bar = 10 $\mu$ .



## DISCUSSION

This study shows that, suprisingly, GAP-43 is expressed extensively in the adult rat autonomic nervous system. Previous work has in general indicated that levels of the growth associated protein GAP-43 are high during development and regeneration of the nervous system and that GAP-43 levels decline precipitously on the establishment of mature synaptic terminals. Certain areas of the adult brain associated with synaptic plasticity and limbic function, such as the hippocampus, have been considered to be the major exception to this pattern since these areas continue to express high levels of GAP-43 throught adulthood.

In addition this study demonstrates that GAP-43 is expressed by developing Schwann cells and mature non-myelin-forming Schwann cells both *in vivo* and *in vitro*.

### Localization of GAP-43 in the autonomic nervous system

The presence of immunoreactive GAP-43 in nerve fibre varicosities of all the tissue studied strongly suggests that GAP-43 is expressed by the autonomic neurones. Indeed the studies of Sharkey et al., 1990 have demonstrated that the GAP-43 mRNA is present in the neurones of the myenteric plexus. The sympathetic neurones of the superior cervical ganglion have also been demonstrated to express GAP-43 mRNA during adulthood (Verge et al., 1990; Yamamoto and Kondo, 1990).

Sympathectomy experiments, designed to demonstrate the presence of GAP-43 in sympathetic neurones, had no discernible effect on GAP-43 expression. This suggested that GAP-43 was also expressed by the reactive Schwann cells of the bands of Büngner. Attempts to demonstrate the presence of GAP-43 in Schwann cells devoid of neurones, using the neurone specific marker PGP 9.5 (Thompson et al., 1983), also failed to reveal any sympathectomy induced changes in neuronal innervation. This lead to the suggestion that reactive Schwann cells may also express PGP 9.5. The presence of both GAP-43 and PGP 9.5 immunoreactivity in reactive Schwann cells was demonstrated using teased preparations of transected sciatic nerve distal stump.

PGP 9.5 was expressed by the entire Schwann



cell population whereas GAP-43 immunoreactivity was detected only in non-myelin-forming cells. The presence of PGP 9.5 in Schwann cells was confirmed by tissue culture studies.

Whether the enteric glial cells also express immunoreactive GAP-43 is at present unknown. At the level of the light microscope it is impossible to ascertain whether the GAP-43 immunoreactivity present in the neuropil of the enteric ganglia is associated solely with the neurones or with both neurones and the glial cell processes.

#### GAP-43 is expressed by non-myelin-forming Schwann cells *in vivo*

The expression of GAP-43 by Schwann cells in normal uninjured nerve was demonstrated using teased nerves and sections. This data shows that GAP-43 is expressed by many, if not all non-myelin-forming Schwann cells in the adult rat *in vivo*. Dried dissociated cell data however did not corroborate these results. The diminished GAP-43 immunoreactivity seen in these preparations from adult animals may be due to a technical problem. GAP-43 is initially synthesized as a soluble protein (Skene and Virag, 1989) and has been reported to exist in soluble form in the axons of mature hippocampus (Van Lookeren Campagne et al., 1990). Soluble GAP-43 was not however detected by these authors, in the hippocampal axons of the developing rat. By analogy it is possible that a similar situation exists in the Schwann cell. Mature Schwann cells may contain cytosolic GAP-43 whereas in the developing Schwann cell GAP-43 may exist in the membrane bound form. As GAP-43 is a highly hydrophilic protein (Basi et al., 1987) leaching of GAP-43 may have occurred during the preparative procedure. In dried cell preparations from embryonic nerves a vast majority of Schwann cells are highly GAP-43 immunoreactive, and there is no reason to believe that significant leaching has occurred from these cells.

Assuming that GAP-43 is universally present on non-myelin-forming Schwann cells it is not a typical marker of these cells. The expression of other non-myelin-forming Schwann cell markers such as GFAP, L1, N-CAM, A5E3, and NGF receptor, is rapidly upregulated when Schwann cells are removed from axonal contact and placed in tissue culture and after nerve transection (Nieke and Schachner, 1985; Taniuchi et al., 1986; Jessen et al., 1987; Jessen et al., 1990). Expression of GAP-



43, in contrast, continues to be restricted to a subpopulation of cells in tissue culture and is not upregulated in the transected sciatic nerve by 13 days post cut. GAP-43 is expressed however, by the entire Schwann cell population by 4 weeks post denervation (R. Curtis personal communication).

#### Developmental down regulation of Schwann cell GAP-43 expression

GAP-43 expression in Schwann cells is dramatically downregulated by birth. During the early periods of Schwann cell development (E15-16) majority of Schwann cells are highly GAP-43 immunoreactive. These levels drop until by birth only a third of the Schwann cells express GAP-43. The expression of GAP-43 can be correlated with Schwann cell motility. At E15 the Schwann cells are migrating and proliferating along the newly formed axons and express GAP-43. By birth many of these Schwann cells will have been committed to myelin formation and thus may downregulate GAP-43 expression. However, the present study does not address the issue of whether those cells that downregulate GAP-43 also upregulate markers of the myelin phenotype. This point will be addressed in future studies. If GAP-43 downregulation is related to acquisition of the myelin phenotype it occurs at an early developmental time relative to other markers of the non-myelin phenotype (Mirsky and Jessen, 1990).

In cultures of P1 Schwann cells expression of GAP-43 does not change during several days *in vitro*. Likewise the proportion of GAP-43 positive Schwann cells in adult Schwann cell cultures remains stable. Embryonic Schwann cells on the other hand, down regulate GAP-43 expression over a period of 5 days. The GAP-43 remaining in these cultures appears to reside in the Golgi apparatus. The differential regulation of GAP-43 expression by these cells may reflect differences in form of GAP-43 present in embryonic and adult Schwann cells, as postulated above. Variations between embryonic and adult GAP-43 may reflect differences in function of GAP-43 in embryonic and adult Schwann cells. GAP-43 in embryonic Schwann cells may be involved in cell migration, whereas in adult Schwann cells GAP-43 may play a role in process movement and shape remodelling (see below).

### Schwann cell GAP-43 expression is down regulated *in vitro* by cAMP

Both DBcAMP and the adenylate cyclase activator forskolin, downregulate postnatal Schwann cell GAP-43 expression within 48hr. This is a feature that is typical of all the markers of non-myelin forming Schwann cells (Morgan et al., 1991). In the latter case however, significant protein downregulation was not seen until after 72hr of forskolin or cAMP analogue treatment. The effect of cAMP on GAP-43 expression is further substantiated by the absence of GAP-43 immunoreactivity in long-term cultured Schwann cells, shown in Chapter 3 to have elevated basal levels of cAMP. In contrast GAP-43 mRNA expression in PC12 cells is induced by DBcAMP (Costello et al., 1990). Thus it seems as if neuronal and Schwann cell GAP-43 expression are controlled to some extent by different mechanisms. Whether the cAMP induced decrease in GAP-43 expression in Schwann cells is due to decreased synthesis or increased turnover of GAP-43 is at present unknown. The involvement of cAMP in GAP-43 expression may reflect the general importance of this second messenger molecule in Schwann cell biology, an issue that will be addressed in the General Discussion.

### Is GAP-43 expressed by mature sensory neurones?

The essentially identical labelling patterns seen with antibodies to PGP 9.5 and GAP-43 indicate that GAP-43 is present in all nerve fibres of the tissues examined. Thus in addition to being expressed by autonomic fibres GAP-43 may also be expressed by fibres of the sensory nervous system (shown to innervate the tissues examined by labelling with substance P). These observations however do not distinguish between an axonal and/or Schwann cell location of GAP-43. Nevertheless, in this study GAP-43 immunoreactivity was detected in some mature sensory neurones in sections of adult DRG. In addition studies of Verge et al., (1990) have detected GAP-43 mRNA in a subpopulation of mainly small sensory neurones. Expression of the GAP-43 gene was strongly correlated with expression of the high affinity NGF receptor. GAP-43 immunoreactivity was not detected however, in adult DRG in the studies of Woolf et al., (1990). The discrepancy between this study and the result reported here probably reflect differences in the sensitivity of GAP-43 antibodies used. The results of this study show that GAP-43 is expressed

by a subpopulation of mature sensory neurones.

#### Role of GAP-43 in adult autonomic neurones and non-myelin-forming Schwann cells

The widespread distribution of GAP-43 in developing, regenerating and adult autonomic neurones as well as in both central and peripheral glial cells suggests that GAP-43 may play a general role in the maintenance and integrity of the autonomic nervous system. Indeed the flanking sequence of the GAP-43 gene has several features common to "housekeeping genes" (Grabczyk et al., 1990). The persistent expression of GAP-43 by certain brain areas has been proposed to be related to synaptic plasticity, a phenomenon that encompasses a variety of cellular events ranging from changes in the coupling of depolarisation to synaptic and axonal sprouting to actual fibre outgrowth. Evidence for synaptic plasticity in the autonomic nervous system has been demonstrated by direct observation on single neurones of the living adult mouse (Purves et al., 1987; Purves and Voyvodyc, 1987). These studies showed firstly, that the pattern of presynaptic nerve endings on parasympathetic ganglia gradually changed throughout life and secondly, that the dendritic arbors of the SCG are continually remodelling. In contrast the mature neuromuscular junction does not show significant remodelling (Litchman et al., 1987). The enteric nervous system is also known to undergo remodelling, the neurones of the myenteric plexus experiencing considerable shape changes during physiological levels of contraction and relaxation (Gabella and Trigg, 1984). In view of the contractile and dilation properties of the blood vessels and the iris, autonomic innervation of these tissue may also be expected to undergo stress related remodelling. Additionally, recent studies have shown that unmyelinated axons are not of uniform calibre but are fundamentally varicose structures. These varicosities are believed to be dynamic structures created by the movement of membranous organelles (Greenburg et al., 1990). Hence the calibre of the unmyelinated axon may be constantly changing in both space and time. The expression of GAP-43 in the adult ANS may therefore reflect ongoing synaptic plasticity and remodelling.

Biochemical support for the involvement of GAP-43 in cell shape changes has been provided by (Moss et al., 1990; Meiri and Gordon-

Weeks, 1990). In these studies GAP-43 in the growth cone has been localized in areas of membrane adhesion and in association with the membrane skeleton, a structure that stabilizes the membrane and maintains cell shape while allowing deformations to occur (Bennett, 1985; Fox et al., 1988).

Shape changes and remodelling of the autonomic neurones may also be expected to effect the ensheathing Schwann cells. GAP-43 expression in non-myelin-forming Schwann cells may thus be related to dynamic changes in cell shape generated to maintain the normal axon/Schwann cell relationship. As mentioned above there are indeed reasons to expect that non-myelin-forming Schwann cells undergo shape changes that are not experienced by myelin-forming Schwann cells. In addition, junctions between adjacent non-myelin-forming Schwann cell processes or between these cells and the axons have not been seen (Aguayo et al., 1976b, Bray et al., 1981). Furthermore it has been noted that the position of the non-myelin-forming Schwann cell process and the axon is not fixed. As a result of anoxia Schwann cell tongues may retract exposing more of the axonal surface than is normal (Thomas and Ochoa, 1984). The axolemma-adaxonal Schwann cell plasmalemma association in myelinating fibres however is remarkably resilient to disruption in pathological conditions (Thomas and Ochoa, 1984). In contrast to the non-myelin-forming Schwann cell, the myelin-forming Schwann cell is secured to itself by tight junctions (Mugnaini and Schnapp, 1974; Shinowara et al., 1980) and is firmly adherent to the axolemma at the node of Ranvier (Bunge et al., 1967; Livingstone et al., 1973; Shinowara et al., 1980).

Interestingly GAP-43 immunoreactivity was also seen in satellite cells of the SCG, another population of cells that may well be undergoing shape changes in adulthood. As mentioned above the dendritic arborizations of the adult SCG have been shown to undergo dynamic rearrangements throughout adult life (Purves and Hadley, 1985; Purves and Voyvodic, 1987). Additionally it is likely that the synapses that impinge on these dendrites are constantly changing. Studies on the arrangement of preganglionic synapses on adult mice parasympathetic ganglia have revealed such changes do occur *in vivo* (Purves et al., 1987). Thus the satellite cells that surround these dendrites may be undergoing

GAP-43 mediated cell shape changes to accomodate these processes. Furthermore this study has shown that satellite cells in the DRG do not generally express GAP-43. The sensory neurones in the DRG are adendritic and do not have synapses impinging upon them. Hence the surrounding satellite cells may be relatively stationary abrogating the need for GAP-43.

The high level GAP-43 expression detected in embryonic Schwann cells may be related to motility. In the CNS GAP-43 may have a similar role. GAP-43 immunoreactivity has been detected in cultures of embryonic glia (0-2A progenitor cells) (Deloulme et al., 1990), which are thought to be motile *in vitro* (Small et al., 1987).

In summary this study shows that GAP-43 is not a neurone-specific marker *in vivo* or *in vitro*. GAP-43 has been detected in CNS glia in tissue culture (Vitkovic et al., 1988; daCuhna and Vitkovic, 1990, Deloulme et al., 1990) but its presence *in vivo* has not previously been documented. In addition GAP-43 has been detected in a mature population of sensory neurones. Taken together these results show that GAP-43 expression is not confined to situations of axonal outgrowth, but suggests that GAP-43 may play a more general role in the modelling of the nervous system. Indeed a recent study (Baetge and Hammang, 1991) has demonstrated that PC12 cells deficient in GAP-43 will initiate neurite outgrowth in a manner identical to that exhibited by GAP-43 expressing PC12 cells adding further support to this hypothesis.

## CHAPTER 6

### GENERAL DISCUSSION

The aims of this study were firstly, to identify factors capable of stimulating Schwann cell DNA synthesis *in vitro* and analyse their mode of action, secondly, to demonstrate the presence of active factors *in vivo* and thirdly, to examine the distribution of GAP-43 in the developing and adult PNS.

This study has extended previous studies by using serum-free medium to analyse the role of serum factors in Schwann cell mitosis by identified growth factors (PDGF, FGF and TGF- $\beta$ ). The results obtained reveal that IGF-I, a factor present in high concentrations in serum, is necessary for the mitogenic response of Schwann cells to PDGF and forskolin and potentiates the mitogenic effects of FGF and forskolin. In contrast Schwann cells in serum-free medium were unable to respond to TGF- $\beta$  and forskolin even in the presence of IGF-I. Whether these factors play a role in Schwann cell mitosis *in vivo* is currently unknown. This study shows however, that IGF immunoreactivity is present in the nerve when Schwann cells are rapidly proliferating during development.

The *in vitro* model for Schwann cell proliferation predicts that another growth factor other than IGF-I, such as PDGF or FGF, and a cAMP elevating agent are needed for *in vivo* Schwann cell proliferation. What the nature of the physiological cAMP elevating agent is, and whether Schwann cell cAMP is elevated *in vivo* is unclear. Several lines of evidence however, suggest that the axon is involved. Studies of Ratner et al., (1985) have shown that membranes from both PC12 cells and sensory neurones stimulate cAMP elevation in Schwann cells 2–4 fold. Furthermore Davis and Goodearl, (1990) have shown that brain axolemma synergizes with bFGF and PDGF to promote Schwann cell DNA synthesis in a manner similar to forskolin. Thus suggesting that the axon may act by elevating Schwann cell intracellular cAMP.

Although Schwann cell cAMP elevation induced by neurones is only 2–4 fold and long-term cultured Schwann cells have only 2–3 fold higher basal levels of cAMP than short-term cultured Schwann cells (Chapter 3), these values are similar to increases shown to be efficacious in other cell types. An example is that natural oscillations of cAMP in chick pineal cells result in a 2–4 fold increase in intracellular cAMP levels. These rises are sufficient to increase melatonin levels 10 fold (Nikaido and Takahashi, 1989).

The demonstration that bFGF immunoreactivity is present in the embryonic rat nerve (Gonzales et al., 1990) suggests that IGF in combination with FGF may play a role in Schwann cell proliferation during development. Using tissue culture techniques to model *in vivo* phenomena several studies have demonstrated the importance of interplay of growth factors in developmental processes. For example studies of Torres-Aleman et al, (1990) have demonstrated that bFGF and IGF-I act in an additive manner to support the survival of brain neurones. In addition studies carried out on chick parasympathetic neurones in serum-free medium showed both aFGF and phorbol esters when combined with IGF-I had a pronounced effect on neuronal survival (Crouch and Hendry, 1991). These agents were ineffective when used alone.

The results of the second part of this study show that GAP-43 is present in the mature autonomic nervous system. This observation is consistent with the hypothesis that the ANS is constantly remodelling throughout life as suggested by studies of Purves and Hadley, (1985), Purves et al., (1986). In addition GAP-43 is present in immature Schwann cells and mature non-myelin-forming cells.

An intriguing finding in this study was that Schwann cell GAP-43 levels are downregulated by agents that elevate intracellular cAMP. The downregulation of phenotypic markers associated with non-myelin-forming Schwann cells has previously been reported (Monuko et al., 1988; Morgan et al., 1991). In contrast, induction of the myelin phenotype in cultured Schwann cells is associated with cAMP elevation (Shuman et al., 1988; Lemke and Chao, 1988; Morgan et al., 1991). A simple interpretation of this data suggests that mature non-myelin-forming Schwann cells have lower basal cAMP levels than myelin-forming Schwann cells. cAMP has however been shown to trigger the expression of the lipids 04 (Morgan et al., 1991) and Gal-C (Sobue et al., 1986; Mirsky et al., 1990), which are both expressed by all mature Schwann cells (see Mirsky and Jessen, 1990). Upregulation of basement membrane components (Baron van Evercooren et al., 1986) and Schwann cell proliferation are other cAMP inducible effects.

*In vivo* the developmental events mimicked by cAMP elevation occur over a wide time scale (Chapter 1, Fig 1.0). To reconcile the effects of cAMP in tissue culture with Schwann cell development *in vivo*, a



mechanism whereby different levels of cAMP induce different phenotypic changes in Schwann cells may exist. Thus intracellular levels of cAMP in Schwann cells rise gradually until they reach a threshold value that stimulates the synthesis of basement membrane components and 04. The cAMP level in Schwann cells associated with larger axons may then rise rapidly inducing the expression of Gal-C. As levels rise higher still the myelin proteins will be induced and the non-myelin markers down-regulated. In the non-myelin-forming Schwann cell cAMP levels may continue to rise gradually reaching peak levels upon maturation. This level is hypothesized to be sufficient to induce Gal-C expression but inadequate to stimulate the down-regulation of the non-myelin phenotype. In tissue culture the levels of Schwann cell cAMP are proposed to be equivalent to very early developmental levels prior to axon induced elevation. As suggested above, the axon is thought to be responsible for stimulating Schwann cell cAMP elevation.

In order to test this hypothesis it will be necessary to examine cAMP levels in the single cell. Indeed immunohistochemical (Barsony and Marx, 1990) and fluorescent imaging techniques (Adams et al., 1991) have recently been developed that may be useful in this regard.

#### Future studies

The use of serum-free medium in this study has allowed a precise analysis of the factors controlling Schwann cell proliferation *in vitro* to be made. In addition this work has localized IGF in the nerve during development when Schwann cell proliferation is rapidly occurring. Future studies mapping the distribution of Schwann cell mitogens and their receptors *in situ* will enable the *in vivo* role of these factors to be assessed. *In situ* hybridization, autoradiography and immunohistochemical techniques may be employed in this regard.

These studies have suggested a role for GAP-43 in Schwann cell motility. Further studies on Schwann cell motility using time-lapse videomicroscopy and Boyden chambers would enable the effects of growth factors on Schwann cell motility to be observed. The effect of active factors on Schwann cell GAP-43 expression may clarify the role of GAP-43 in Schwann cell motility. In addition further studies on the effect of cAMP on GAP-43 mRNA and protein expression may provide

insights into the mechanism of cAMP induced Schwann cell differentiation.

In summary this study demonstrates the importance of the interplay of growth factors in Schwann cell proliferation. In addition the presence of GAP-43 immunoreactivity in the mature nervous system and in Schwann cells suggests that dynamic changes and remodelling of the nervous system continue throughout life.☺

## CHAPTER 7

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