GROWTH-ASSOCIATED PROTEIN-43
IN THE RAT NERVOUS SYSTEM

by

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Abstract

Growth-associated protein-43 (GAP-43) is a developmentally-regulated growth cone protein involved in axonal extension, which is up-regulated during neuronal regeneration (Skene, 1989). Polyclonal antibodies were raised against a GAP-43/ß-galactosidase fusion protein in order to study regeneration after traumatic injury to the central nervous system (CNS).

The antiserum was characterised on Western blots of purified GAP-43 and homogenates of rat CNS. Staining of tissue sections revealed an extensive distribution of GAP-43 in the adult CNS. The high titre of the antiserum allowed regions of high GAP-43 concentration to be demonstrated by increased antiserum dilution.

Immunohistochemistry showed that retinal ganglion cells up-regulated GAP-43 after optic nerve transection. The antiserum was then used to assess the extent of axonal regeneration after spinal cord compression (Guth *et al.*, 1985). Although both GAP-43+ perikarya and axons could be seen around the lesion, the extensive regeneration suggested by these authors could not be confirmed and most axons appeared to be spared from axotomy.

In CNS glial cultures, GAP-43 was demonstrated in O-2A progenitors and type-2 astrocytes by immunocytochemistry and Western blotting but was not found in oligodendrocytes. In tissue sections, GAP-43 could be seen in O-2A progenitors and was down-regulated as these cells differentiated into oligodendrocytes but GAP-43+ astrocytes were not found.

In the peripheral nervous system, GAP-43 was found in non-myelin-forming Schwann cells but not in myelinating Schwann cells, and enteric glia were also GAP-43+. Sciatic denervation studies showed that GAP-43 in Schwann cells is regulated by axonal contact.

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Dedicated to the memory of my grandparents.

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LIST OF ABBREVIATIONS

Apo E	apolipoprotein E
AMP	adenosine monophosphate
BDNF	brain-derived neurotrophic factor
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase
CNTF	ciliary neurotrophic factor
CNS	central nervous system
CST	corticospinal tract
DABCO	1,4-diazabicyclo(2,2,2)octane
DAI	days after isolation
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle's Medium
DNase	deoxyribonuclease I
DNA	deoxyribonucleic acid
DREZ	dorsal root entry zone
DSCT	dorsal spinocerebellar tract
DTT	dithiothreitol
E. coli	Escherichia coli
EBS	Earle's balanced salts
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
g	units of gravity

GAP-43	growth-associated protein-43
GC	galactocerebroside
G_{D3}	disialo-galactoglucosyl ceramide
GFAP	glial fibrillary acidic protein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Ig	immunoglobulin
kbp	kilobase pair
kD	kilo Dalton
KPi	potassium phosphate buffer
LTP	long-term potentiation
MARCKS	myristoylated alanine-rich protein kinase C substrate
MBP	myelin basic protein
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NGFr	nerve growth factor receptor
NT-3	neurotrophin 3
N-CAM	neural cell adhesion molecule
Ng-CAM	neuron-glia cell adhesion molecule
O-2A	oligodendrocyte-type-2 astrocyte
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PIP	phosphatidylinositol 4-phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
RGC	retinal ganglion cell
RMM	relative molecular mass
RNA	ribonucleic acid
rpm	revolutions per minute

SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Tris/HCl	Tris adjusted with HCl to the pH stated
TRITC	tetramethylrhodamine B isothiocyanate
UV	ultraviolet

Chapter One

Introduction

1.1. Growth-Associated Protein-43

1.1.1. Historical Perspective

Growth-associated protein-43 (GAP-43) is also known by several other names (pp46, B-50, F1, p57, neuromodulin). This designation was first applied in 1981 in a series of papers by Skene and Willard (1981a, b, c) in which axonally transported proteins were metabolically labelled during axonal outgrowth in development and regeneration and compared to those labelled in the undamaged adult nervous system. Prominent amongst the 'growth-associated proteins' in the fast component of axonal transport during axonogenesis was one of relative molecular mass (RMM) 43 kD, estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and hence the epithet GAP-43. An association with growth was further suggested by studies which found GAP-43 to be identical to the phosphoprotein pp46, present in the tips of extending axons known as growth cones (Meiri *et al.*, 1986; Skene *et al.*, 1986).

Prior to these studies, Zwiers *et al.* (1976) had identified a protein, which came to be known as B-50, that was phosphorylated in rat synaptic plasma membranes. Furthermore, phosphorylation was effected by protein kinase C (PKC) and regulated the ability of B-50 to inhibit the activity of phosphatidylinositol 4-phosphate (PIP) kinase (Van Dongen *et al.*, 1985), a regulatory enzyme in the phosphatidylinositol intracellular second messenger system (Berridge and Irvine, 1984). B-50 has been shown to be identical to GAP-43 in terms of mobility in polyacrylamide and isoelectric focusing gels and immunological cross-reactivity (Jacobson *et al.*, 1986).

Phosphorylation of a protein designated F1 was shown to be directly related to the persistence of hippocampal long-term potentiation (LTP, a model of mammalian neuronal plasticity and memory) (Nelson and Routtenberg, 1985; Lovinger *et al.*, 1985, 1986). F1 has also been shown to be identical to GAP-43 by comigration in 2-dimensional gels, comigration of proteolytic

fragments (peptide mapping) and immunological cross-reactivity (Snipes *et al.*, 1987). Moreover, cDNA cloning and sequencing have revealed both F1 and GAP-43 to be the same gene product (Basi *et al.*, 1987; Karns *et al.*, 1987; Rosenthal *et al.*, 1987).

Gene cloning also revealed the unexpected identity of GAP-43 with the neural-specific calmodulin-binding protein p57, which is now referred to as neuromodulin (Cimler *et al.*, 1987).

The literature is somewhat confused as a result of several groups investigating the protein under different terminologies and no consensus has yet been reached on nomenclature. Rather, each section of the 'GAP-43 community' continues to use its own title for this protein. Throughout this thesis it will be referred to as GAP-43. Despite this confusion, a large body of evidence has accumulated concerning the molecular properties, biochemical interactions, and distribution of GAP-43 but, as yet, no clear role has emerged for GAP-43 in the development or maintenance of neuronal connections (Benowitz and Routtenberg, 1987; Skene, 1989; Liu and Storm, 1990).

1.1.2. Distribution Of GAP-43

1.1.2.1. GAP-43 is Neural-Specific

GAP-43 has been repeatedly identified in studies of neural tissue (as described above) and investigations in several species have shown that GAP-43 is restricted to the nervous system using antibody and DNA probes (Basi *et al.*, 1987; Karns *et al.*, 1987; Neve *et al.*, 1987; Rosenthal *et al.*, 1987; McGuire *et al.*, 1988).

Axonal transport experiments (Skene and Willard, 1981a, b; Benowitz and Lewis, 1983) and studies of synaptosomal subcellular fractions (Zwiers *et al.*, 1976; Nelson and Routtenberg, 1985) clearly demonstrated that GAP-43 is synthesised by neurons and accumulates at the axon terminal during growth and maturity. This led to speculation that GAP-43 may be restricted

to neurons, and studies with cell-specific markers appeared to substantiate this *in vivo* and *in vitro* (Basi *et al.*, 1987; Meiri *et al.*, 1988; Oestreicher *et al.*, 1988). Other evidence suggests that non-neuronal (glial) cells of both the central and peripheral nervous systems can also express GAP-43 (Vitković *et al.*, 1988; Tetzlaff *et al.*, 1989; Woolf *et al.*, 1990; see Chapters 6 and 7 for a full discussion of glial GAP-43). Despite this apparent contradiction, GAP-43 is still referred to as a 'neuron-specific' protein (Van Lookeren Campagne *et al.*, 1990; Strittmatter *et al.*, 1990; Coggins and Zwiers, 1991).

1.1.2.2. GAP-43 Is Developmentally Regulated

The initial experiments of Skene and Willard (1981a, b) demonstrated that greater amounts of GAP-43 were transported along the axon, away from the soma, during development than in the adult state, and that axonal transport of GAP-43 was elevated in neurons undergoing regeneration after damage. Synthesis was shown to decline 10-20 fold during the first few weeks of postnatal rat brain development, although overall levels of GAP-43 remained high for several weeks indicating a half-life in the order of weeks (Jacobson *et al.*, 1986). Regeneration of goldfish retinal ganglion cell (RGC) axons induced 100-200 fold increase in GAP-43 synthesis over undamaged control optic nerves (Benowitz and Lewis, 1983; Perry *et al.*, 1987).

Total GAP-43 mRNA levels and translatable GAP-43 mRNA declined in the rat brain in the postnatal period, indicating that GAP-43 synthesis is controlled at the transcriptional level (Jacobson *et al.*, 1986; Basi *et al.*, 1987; Karns *et al.*, 1987; Rosenthal *et al.*, 1987). GAP-43 mRNA remained detectable in the adult but levels were elevated at least 10-fold in the cell bodies of neurons undergoing axonal regeneration (Basi *et al.*, 1987; Hoffman, 1989). *In situ* hybridisation experiments have suggested that while all neurons synthesise GAP-43 during development, gene expression persists into adulthood only in some cells (Rosenthal *et al.*,

1987; Neve *et al.*, 1988; De la Monte *et al.*, 1989).

Similarly, immunoreactive GAP-43 remained detectable in the adult rat nervous system both by radioimmunoassay and immunohistochemistry, although the protein is more abundant in immature tissue (Oestreicher *et al.*, 1986; Oestreicher and Gispen, 1986; Gorgels *et al.*, 1987; McGuire *et al.*, 1988). In spinal cord, GAP-43 declined 10-fold relative to total protein between 7 and 90 days (Gorgels *et al.*, 1987). The distribution of GAP-43 in adulthood is reviewed in detail in Chapter 4.

1.1.2.3. GAP-43 Is Axon-Specific

Hippocampal neurons in culture develop distinct axons and dendrites in a highly polarised manner, and immunostaining has shown that GAP-43 is restricted to axonal processes in these cells (Goslin *et al.*, 1988). Before axonal outgrowth, when all the minor processes were of approximately equal length, GAP-43 was distributed equally in all growth cones (Goslin *et al.*, 1990). GAP-43 became preferentially localised in the axon as it developed, while the dendritic growth cones gradually lost their residual GAP-43. If the axon was transected close to the soma so that polarity was lost, GAP-43 accumulated in the remaining processes before extension of a new axon (Goslin and Banker, 1990).

The suggestion that GAP-43 is excluded from mature dendrites is supported by electron microscopy which revealed an exclusively presynaptic location of GAP-43 in synaptosomes and synaptic regions (Gispen *et al.*, 1985; Van Lookeren Campagne *et al.*, 1989, 1990). Dendrites were also not immunoreactive for GAP-43 in preparations examined by light microscopy (Oestreicher and Gispen, 1986; McGuire *et al.*, 1988). Destruction of axonal projections to the dentate gyrus resulted in the transient loss of GAP-43 from synaptic regions prior to collateral sprouting (Benowitz *et al.*, 1990), suggesting that the remaining dendritic structures did not contain GAP-43.

1.1.2.4. GAP-43 Is Present In Axonal Membranes

Many studies have shown that GAP-43 is present in membranes isolated from adult and neonatal rat brains. GAP-43 is initially synthesised as a soluble protein and then becomes attached to membrane vesicles in the soma (Skene and Virág, 1989) which are anterogradely transported in the fast component of axonal transport (Skene and Willard, 1981a, b; Benowitz and Lewis, 1983). During development GAP-43 is incorporated into the growing tips of axons, known as growth cones, which can be isolated by subcellular fractionation (Meiri *et al.*, 1986; Skene *et al.*, 1986) and synaptosomes similarly isolated from adult brain also contained GAP-43 (Chan *et al.*, 1986).

Immunogold electron microscopy has confirmed these results in isolated nerve terminals and ultrathin tissue sections (Gispen *et al.*, 1985; Van Lookeren Campagne *et al.*, 1989, 1990). Furthermore, these studies have shown that GAP-43 in the synapse is confined to the presynaptic membrane, although GAP-43 has been localised to axon shafts in developing and adult tissue (Gorgels *et al.*, 1989; Van Lookeren Campagne *et al.*, 1990). It is uncertain whether GAP-43 is inserted directly into the axolemma or arises by diffusion away from the growth cone or presynaptic terminal where it is initially incorporated.

1.1.3. Molecular Properties Of GAP-43

1.1.3.1. Relative Molecular Mass

Early estimates of the RMM of GAP-43 (and its synonymous proteins), by comparison to the electrophoretic mobility in SDS-PAGE of standard proteins of known RMM, ranged from 43 kD (Skene and Willard, 1981b) to 57 kD (Andreasen *et al.*, 1983). In an elegant experiment, Benowitz *et al.* (1987) showed that the mobility of GAP-43 varied inversely with the concentration of acrylamide monomer in the gel mixture and, using the method of Ferguson (1964) to calculate the retardation coefficient of GAP-

43, these authors determined the RMM to be 32.8 kD. Furthermore, in the absence of detergent, GAP-43 eluted from a size-exclusion chromatography column at an estimated RMM of 124+/-10 kD. This peak resolved as a single GAP-43 band in SDS-PAGE, suggesting that the native protein may be a multimer of the GAP-43 polypeptide (Benowitz *et al.*, 1987).

From calculations of the Stoke's radius and sedimentation coefficient, Masure *et al.* (1986) estimated the RMM of GAP-43 to be 25.7 kD. These authors also performed amino acid analysis of GAP-43, revealing a paucity of hydrophobic residues. This may explain the aberrant migration of GAP-43 during SDS-PAGE as interactions with hydrophobic amino acids are responsible for the binding of SDS (Reynolds and Tanford, 1970).

The amino acid sequence of GAP-43, as deduced from cloned cDNA sequences (Basi *et al.*, 1987; Cimler *et al.*, 1987; Karns *et al.*, 1987; Rosenthal *et al.*, 1987), confirmed the amino acid analysis of Masure *et al.* (1986) and definitively showed that GAP-43 consists of 226 amino acids with a total RMM of 23.6 kD.

1.1.3.2. Post-Translational Modifications Of GAP-43

1.1.3.2.1. Membrane Attachment

Examination of the deduced amino acid sequence of GAP-43 indicated a highly hydrophilic protein, which is not compatible with its known cellular localisation in plasma membranes (Chan *et al.*, 1986; Skene *et al.*, 1986) or the behaviour of GAP-43 in Triton X-114 phase partitioning, which indicated considerable hydrophobicity (Dosemeci and Rodnight, 1987). In addition, sequence analysis did not reveal any membrane-spanning domains or potential sites for N-linked glycosylation (Basi *et al.*, 1987). However, Skene and Virág (1989) have recently shown that GAP-43 undergoes addition of palmitic acid moieties via thioester linkages to cysteine residues near the amino terminus and that this modification underlies membrane attachment.

The enzymes responsible for the attachment of these fatty acids to

GAP-43 and its subsequent deacylation have been identified (S. Patterson and P. Skene, personal communication) suggesting that membrane attachment is regulated by dynamic acylation of cytosolic GAP-43 under cellular control and indeed a 'soluble' pool of GAP-43 has been noted in the supernatants from high speed centrifugation steps designed to pellet membranes (Skene and Virág, 1989; Meiri and Gordon-Weeks, 1990; Moss *et al.*, 1990). Immunogold electron microscopy has also revealed the presence of cytosolic GAP-43 (Gorgels *et al.*, 1989; Van Lookeren Campagne *et al.*, 1989, 1990).

1.1.3.2.2. Phosphorylation

GAP-43 is an endogenous substrate for PKC (Chan *et al.*, 1986) and is known to be phosphorylated in a number of experimental paradigms (Nelson and Routtenberg, 1985; Van Dongen *et al.*, 1985; Nelson *et al.*, 1989). PKC-stimulated phosphorylation has been shown to occur at a single serine residue at position 41 (Coggins and Zwiers, 1989; Apel *et al.*, 1990). GAP-43 has additionally been proposed as a substrate for casein kinase II (Pisano *et al.*, 1988) although phosphorylation occurs at a different serine residue located near the carboxyl terminus. The PKC-phosphorylated serine 41 is a substrate for the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Liu and Storm, 1989; Schrama *et al.*, 1989).

Phosphorylation has been shown to modulate the function of GAP-43. The persistence of long-term changes in synaptic efficacy in LTP was directly related to the degree of phosphorylation of GAP-43 in hippocampal synaptosomal membranes, regulated by the translocation of PKC to the membrane and its subsequent activation (Benowitz and Routtenberg, 1987; Linden and Routtenberg, 1989). This may be linked to the correlation between PKC-stimulated GAP-43 phosphorylation and neurotransmitter release (Dekker *et al.*, 1989a; Heemskerk *et al.*, 1990) and the inhibition of neurotransmitter release by antibodies to GAP-43 (Dekker *et al.*, 1989b). Phosphorylation by PKC also enhanced the ability of GAP-43 to inhibit the

enzyme PIP kinase (Van Dongen *et al.*, 1985), and thus to control phosphatidylinositol turnover. Binding of GAP-43 to calmodulin is also regulated by phosphorylation at serine 41 (Alexander *et al.*, 1987). These biochemical interactions are discussed further below.

1.1.4. Biochemical Interactions Of GAP-43

1.1.4.1. Structural Cellular Components

As described in Section 1.1.3.2.1. GAP-43 is associated with the plasma membrane via palmitic acid moieties at the amino terminus (Skene and Virág, 1989). GAP-43 is also associated with a sub-membrane fraction of the cytoskeleton known as the cortical cytoskeleton (Allsopp and Moss, 1989) or the membrane skeleton (Meiri and Gordon-Weeks, 1990; Moss *et al.*, 1990). This fraction contains actin, tubulin, fodrin, talin and a-actinin but it is uncertain with which of these proteins GAP-43 interacts directly (Meiri and Gordon-Weeks, 1990). Analyses of GAP-43 amino acid sequence and its evolutionary conservation have indicated that GAP-43 has an elongated rod-like structure which extends away from the membrane attachment site at the amino terminus and it is believed that the carboxy-terminal domain, showing limited homology with the low RMM neurofilament protein, may bind to one or more of these cytoskeletal components (LaBate and Skene, 1989).

GAP-43 may thus serve as a link between the membrane and the cytoskeleton, underlying events such as adhesion and motility. In support of this concept, GAP-43 has been shown to be associated with areas of membrane which are tightly adherent to the substrate (Meiri and Gordon-Weeks, 1990). Isolated growth cones and growing neurites mechanically dislodged from their substrate in culture left behind patches of membrane containing GAP-43. GAP-43 showed a punctate distribution at the cytoplasmic face of the plasmalemma of neurites growing in culture, which also suggests that it may be localised at sites of substrate adhesion (Meiri *et al.*, 1988).

1.1.4.2. Calmodulin

The Ca^{2+}-binding protein calmodulin is an important intermediate in the regulation of several proteins and enzymes (reviewed in Liu and Storm, 1990). In contrast to other calmodulin-binding proteins, GAP-43 binds to calmodulin with greatest affinity in the absence of Ca^{2+} ions (Andreasen *et al.*, 1983). The dissociation constant for GAP-43 and calmodulin has been shown to be 0.23 μM in the absence of Ca^{2+} ions and 1 μM in the presence of 500 μM Ca^{2+}. High levels of K^+ ions also reduced the affinity of binding (dissociation constant 3.4 μM at 150 mM potassium chloride) and binding was abolished by PKC phosphorylation of GAP-43 (Alexander *et al.*, 1987). The calmodulin binding site has been identified as residues 43-51 (Alexander *et al.*, 1988) and the proximity of serine 41 may underlie the regulation of calmodulin binding by PKC phosphorylation of GAP-43. Dephosphorylation of GAP-43 by the calmodulin-stimulated phosphatase calcineurin (Liu and Storm, 1989; Schrama *et al.*, 1989) would serve as a negative feedback loop to promote reassociation of calmodulin with GAP-43.

GAP-43 has been suggested to control the local free calmodulin concentration by sequestering calmodulin at the plasma membrane (Liu and Storm, 1990) since it has been calculated from the concentration of these two proteins in brain tissue and their affinity that most or all calmodulin would be bound to GAP-43 in unstimulated cells (Alexander *et al.*, 1987). If the GAP-43-bound calmodulin is inactive (an as yet unanswered question) this would serve to reduce the activity of calcium-calmodulin dependent enzyme systems. Increased Ca^{2+} ion concentration or K^+ depolarisation would reduce the affinity of GAP-43 for calmodulin leading to focal liberation of calmodulin and thus activation of calcium-calmodulin mediated processes and increased Ca^{2+} buffering. PKC is stimulated by Ca^{2+} ions, and therefore phosphorylation of GAP-43 (leading to dissociation of calmodulin) may act to attenuate the activity of PKC.

1.1.4.3. Second Messenger Systems

Phosphorylated GAP-43 (but not the dephosphorylated form) is an inhibitor of the enzyme PIP kinase which catalyses the phosphorylation of PIP to phosphatidylinositol 4,5-bisphosphate (PIP_2) (Van Dongen *et al.*, 1985). Antibodies to GAP-43 interfered with this function and enhanced the activity of PIP kinase in synaptic plasma membranes (Oestreicher *et al.*, 1983). Since PKC is activated by the products of receptor-mediated PIP_2 breakdown (Berridge and Irvine, 1984), the phosphorylation of GAP-43 would appear to regulate transmembrane signalling by negative feedback and thus further attenuate the activity of PKC.

In addition, GAP-43 has been suggested to interact with G_o (Strittmatter *et al.*, 1990), a guanidine triphosphate-binding protein that transduces information from transmembrane receptors to intracellular enzyme systems (Bourne *et al.*, 1990). G_o is most abundant in the brain and is a component of growth cone membranes (Strittmatter *et al.*, 1990). Furthermore, GAP-43 was proposed to stimulate guanidine triphosphate binding to G_o, implying that G_o can be activated by GAP-43 in the absence of receptor-ligand activation. However this data has not been universally accepted, especially due to the inability to isolate the GAP-43/G_o complex necessary for guanidine triphosphate binding and thus activation of G_o (Bourne *et al.*, 1990).

1.1.4.4. Neurotransmitter Release Mechanisms

Phosphorylation of GAP-43 has been shown to be correlated with the release of the neurotransmitters noradrenaline and aspartate from rat hippocampal slices (Dekker *et al.*, 1989a; Heemskerk *et al.*, 1990) and isolated synaptosomes (Dekker *et al.*, 1990). Release of noradrenaline could be partially inhibited by antibodies to GAP-43 which totally prevented GAP-43 phosphorylation (Dekker *et al.*, 1989b). This strongly suggests that phosphorylated GAP-43 plays some part in the events leading to vesicular neurotransmitter release. It remains, however, to be shown

how GAP-43 is involved in these events (and how release is effected in the absence of phosphorylated GAP-43) and whether other neurotransmitter systems are similarly influenced by GAP-43 phosphorylation.

This ability to influence neurotransmitter release may underlie the observed correlation of GAP-43 phosphorylation with LTP (Lovinger *et al.*, 1985, 1986). Increased presynaptic release of the excitatory neurotransmitter glutamate in LTP (Lynch *et al.*, 1985) is consistent with the proposed role of GAP-43 in synaptic potentiation (Linden and Routtenberg, 1989).

1.1.5. Role Of GAP-43

The preceding overview of the molecular properties and biochemical interactions of GAP-43 leads into a consideration of possible functions in the developing and adult nervous system. Despite the wealth of information concerning GAP-43, no causal role has yet been established for GAP-43 in any physiological event. Consequently, these considerations are highly speculative.

1.1.5.1. Function Of GAP-43 In Adult Presynaptic Terminals

The association of GAP-43 with calmodulin, its phosphorylation by PKC and ability to inhibit PIP kinase (and the reciprocal interactions between these molecular events) strongly suggest that GAP-43 modulates many of the cellular responses to PIP_2 breakdown stimulated by receptor-ligand interaction (Berridge and Irvine, 1984). The overall effect of GAP-43 would seem to be sharpening of the peak cellular responses, in terms of Ca^{2+} ion concentration and PKC activity (Skene, 1989). This may explain the influence of GAP-43 on neurotransmitter release, which is regulated by both Ca^{2+} ions and PKC (see Dekker *et al.*, 1989a), or alternatively GAP-43 may be directly involved in vesicular membrane fusion (Dekker *et al.*, 1989b).

The interaction of GAP-43 with cytoskeletal elements and its presence

at sites of substrate adhesion suggest that GAP-43 may form a functional linkage between the cytoskeleton and the plasmalemma (Meiri and Gordon-Weeks, 1990), contributing to the structural integrity of the axonal terminal and its adhesion to surrounding elements.

1.1.5.2. Function Of GAP-43 In Growth Cones

Several lines of evidence suggest that GAP-43 has a role in process extension in developing neurons. First, GAP-43 is transported to the growth cone, where it is inserted into the membrane (Skene and Willard, 1981a, b; Meiri *et al.*, 1986; Skene *et al.*, 1986). Secondly, GAP-43 is synthesised at high levels during periods of axonal outgrowth and is selectively reinduced by axotomy in neurons capable of regeneration (Skene and Willard, 1981a, b; Hoffman, 1989). Thirdly, the most direct evidence comes from studies of non-neuronal cells which were induced to extend filopodial processes when transfected with the GAP-43 gene, and this was dependent upon a 'membrane-targeting signal' in the amino terminus including the sites for palmitic acid addition (Zuber *et al.*, 1989a, b). Additionally, the presence of GAP-43 may determine neuronal polarity (Goslin *et al.*, 1990; Goslin and Banker, 1990).

As the leading edge of growing neurites, growth cones serve a unique and pivotal role in neural development. They must not only extend neuronal projections by being the locus of membrane addition, but they must also be able to respond to environmental clues to guide the processes to their correct destinations (Bray, 1987; Van Hooff *et al.*, 1989a). GAP-43 may be involved in either of these functions.

If GAP-43 is active in membrane fusion (as has been proposed for neurotransmitter release) then neurite extension would depend upon an adequate supply of GAP-43. The pheochromocytoma tumour cell line PC12 is a model system for the study of neurite extension in culture (Greene and Tischler, 1976) and GAP-43 has been shown to be present in the processes of these cells (Van Hooff *et al.*, 1989c). However, a PC12 subclone which

contains only trace amounts of GAP-43 is still able to extend neurites in response to NGF (Baetge and Hammang, 1991).

Guidance may be effected through receptors for substrate-bound adhesion molecules, soluble neurite guidance factors or neurotransmitters, which may be enriched in the membranes of growth cones (see Van Hooff *et al.*, 1989a). In addition to underlying adhesion (Meiri and Gordon-Weeks, 1990), GAP-43 may promote motility by transmitting force from the cytoskeleton to sites of membrane-substrate adhesion according to the 'Pull Hypothesis' (Bray, 1987; Turner and Flier, 1989). GAP-43 would act to link membrane receptors for extracellular adhesion molecules to the cortical cytoskeleton, as has previously been suggested by Bray (1987), and would enable activation of these receptors by substrate-bound ligands to modify the action of GAP-43 through second messenger systems (see Turner and Flier, 1989). In support of this concept, adhesion was substantially reduced in the PC12 subclone deficient in GAP-43 (Baetge and Hammang, 1991), although it is not known whether growth cone guidance was affected.

Growth cones also possess receptors for nerve growth factor (NGF), which is known to direct the growth of neurites (Gundersen and Barrett, 1979; Van Hooff *et al.*, 1989a). NGF stimulates morphological differentiation in the PC12 cell model of neurite outgrowth, concomitant with translocation of GAP-43 to the cell membrane (Van Hooff *et al.*, 1989c). NGF also activates PKC by translocation to the plasma membrane in these cells (Heasley and Johnson, 1989; Kondratyev *et al.*, 1990) and studies with inhibitors and activators of PKC have suggested that PKC may have a role in the biological action of NGF (Hashimoto and Hagino, 1989; Glowacka and Wagner, 1990). NGF induces PKC-dependent phosphorylation of GAP-43 in PC12 cells (Dr. K. Meiri, personal communication) and furthermore, Yankner *et al.* (1990) have shown that NGF-induced neurite outgrowth is correlated with GAP-43 levels in PC12 cells transfected with the GAP-43 gene, suggesting that GAP-43 may act in transduction of the intracellular response to NGF. Activation of PKC has been shown to promote

neuritogenesis in primary cultures, although this has not yet been correlated with GAP-43 phosphorylation (Cambray-Deakin *et al.*, 1990).

Alternatively, GAP-43 may mediate the response of growth cones to neurotransmitters. Muscarinic acetylcholine receptor activation has been shown to stimulate GAP-43 phosphorylation in isolated growth cones (Van Hooff *et al.*, 1989b), which are known to bear receptors for several other neurotransmitters (Lockerbie *et al.*, 1988). Neurotransmitters are known to influence growth cone motility by influx of Ca^{2+} ions (Mattson and Kater, 1987) through clusters of Ca^{2+} ion channels in the growth cone (Silver *et al.*, 1990).

Phosphorylation of GAP-43 or increased Ca^{2+} ion concentration would cause dissociation of GAP-43/calmodulin complexes, liberating calmodulin and leading to changes in the cohesion of cytoskeletal elements affected by calmodulin-dependent phosphorylation (Liu and Storm, 1990; Van Hooff *et al.*, 1989a). A local 'softening' of the membrane is envisaged leading to filopodial extension. Thus GAP-43 may regulate cytoskeletal proteins during neurite outgrowth by direct interaction (possibly via the neurofilament-like domain at the carboxy-terminus as proposed by LaBate and Skene, 1989) and through control of free calmodulin levels.

1.1.5.3. The 'GAP Hypothesis'

While the exact function of GAP-43 in the growth cone remains unknown, it appears evident from the high levels present during development and the re-expression in regeneration that GAP-43 has a role in the elongation or guidance of outgrowing axons. Furthermore, initial studies showed that GAP-43 synthesis was not enhanced by lesions to the mammalian central nervous system (CNS), in which there is a failure of the axons to regenerate (Skene and Willard, 1981b). These observations led to the 'GAP Hypothesis' that axonogenesis depends critically upon certain proteins (including GAP-43) that are synthesised during axonal outgrowth and (most likely) conveyed to the growing tip. Lack of these proteins in sufficient

quantities in the adult mammalian CNS was proposed to underlie the failure of regeneration (Skene and Willard 1981b; Skene, 1984).

The fact that GAP-43 persists in adult neurons requires a revision of this hypothesis. Whatever function(s) GAP-43 may serve in the adult synapse, the growth cones require a greater supply of GAP-43 to perform either the same or a different set of functions. The failure of mammalian CNS regeneration is suggested to depend therefore upon the inability to recapitulate a 'developmental' gene programme after injury, including *enhanced* expression of GAP-43. This failure could be due to an intrinsic inability of CNS neurons to respond to axotomy. Alternatively, the cell body may not receive the correct signals from the severed axon or the extraneuronal environment. These factors are discussed fully in the following section.

1.2. Neural Regeneration

Once neurons have elaborated processes and made the appropriate connections they are incapable of further migration. Although synaptic plasticity can take place (in the form of changes in synaptic efficacy or the generation of new synapses), the nervous system in the adult can be considered essentially 'hardwired'. Consequently, when the neuronal circuitry is interrupted by damage or disease, connectivity can only be re-established by the generation of new processes or regeneration of those destroyed. For the purposes of this discussion and the remainder of the text, *regeneration* refers strictly to the regrowth of preexisting but damaged neuronal processes, and *collateral sprouting* refers to the generation of new processes from undamaged axons to compensate for lost connections. Although synaptic plasticity can be a consequence of neuronal damage, it can occur independently of either damage or growth of processes, in normal events such as learning (Black *et al.*, 1990).

The 'GAP Hypothesis' predicts that the failure of neurons to regenerate axonal processes after injury lies in the regulation of GAP-43

(and/or other proteins essential for axonal growth). Specifically, this seeks to explain the lack of regeneration seen in the mammalian CNS in comparison to the CNS of lower vertebrates and the peripheral nervous system (PNS) of mammals which are capable of substantial axonal regeneration and functional reconnection (Guth *et al.*, 1983; Fawcett and Keynes, 1990).

The 'GAP Hypothesis' can be viewed as part of two broader hypotheses to explain the failure of mammalian CNS regeneration. One proposes that mammalian CNS neurons have an inherent incapacity for regeneration and are incapable of up-regulating GAP-43 in response to axotomy. An opposite school of thinking holds that the neural environment regulates the ability of neurons to survive and regenerate after axotomy and proposes that the mammalian CNS is an unconducive environment. In this case, up-regulation of GAP-43 might require extra-neuronal signals present in limiting quantities in the mammalian CNS. This section will examine the response to injury of regenerating and non-regenerating neural tissue and review the evidence for the 'Inherent Incapacity' and 'Neural Environment' hypotheses. This will include a consideration of the non-neuronal (glial) elements of the CNS and PNS and the glial response to neural injury. For further information on central and peripheral glial cells, the reader is referred to Chapters 6 and 7 respectively.

1.2.1. Peripheral Nervous System Response To Injury

The PNS is derived from ectodermal placodes in the head region and from the neural crest, which is a transient aggregation of cells that forms by separation from the edge of the neural plate during neurulation (Jacobson, 1978; Le Douarin, 1982). These cells proliferate and then migrate along defined routes to give rise to a large number of different tissues including all neurons of the PNS and their accompanying glia, the Schwann cells (Le Douarin, 1982; Purves and Lichtman, 1985).

The cell bodies of peripheral neurons are located in sensory and

autonomic ganglia throughout the body. Their axons extend from these ganglia to the CNS and to target organs in the periphery through nerve trunks bounded by epineurial sheaths (Ide *et al.*, 1990). Within the trunks are funiculi, each consisting of a bundle of axons surrounded by a common perineurial sheath. The bundles of axons are subdivided and surrounded by the Schwann cells. Large diameter axons are myelinated by these cells so that each Schwann cell enwraps one axon. Smaller axons are not myelinated and several of these are enclosed by a common Schwann cell. Around each Schwann cell/axon unit lies a basal lamina tube, secreted by the Schwann cells, which is continuous from the cell body to the target organ. In addition, some CNS neurons project axons to the periphery (ie motor neurons) and these are similarly contained within Schwann cells in the nerve trunks.

If the continuity of the axons is disrupted by transection or focal injury of the nerve fibres, then the portion distal to the cell body degenerates, as first described by Waller (1850). Both axonal debris and the myelin sheath are phagocytosed in Wallerian degeneration, although the Schwann cells themselves survive and the basal laminae remain intact. Phagocytosis is accomplished by macrophages (Beuche and Friede, 1984; Lunn *et al.*, 1989) which migrate from the blood vessels in the nerve trunk to the damaged fibres and penetrate the basal lamina tubes (Stoll *et al.*, 1989). Mitogens are liberated from axonal and myelin membranes which promote the division of Schwann cells (Baichwal and DeVries, 1989; DeCoster and DeVries, 1989). Schwann cells thus fill the basal lamina tubes, becoming elongated and interdigitating fine processes, to form the Bands of Büngner.

Within a few hours after injury, the segments of the axons proximal to the lesion (ie still connected to the cell body) start to regenerate axonal sprouts towards the distal portion of the nerve trunk (Fawcett and Keynes, 1990). If the structural integrity of the trunk has been maintained, as in crush injury, or if the distance separating the stumps of

a transected nerve is not too great then the axons regenerate along the Bands of Büngner inside the basal lamina tubes (Hall, 1989; Fawcett and Keynes, 1990). Sprouting is abortive, however, if the interstump distance is in excess of 5 mm, although distances of up to 15 mm can be negotiated if both stumps are contained within a tube (Lundborg *et al.*, 1982; Dahlin *et al.*, 1988). Placement of only the proximal stump in a tube does not enhance axonal outgrowth, suggesting that the distal stump exerts a positive influence on regeneration (Longo *et al.*, 1983).

1.2.2. Mammalian Central Nervous System Response To Injury

The neural plate closes during neurulation to become the neural tube and give rise to the CNS. Neuroblasts and glioblasts are generated from (possibly separate) precursors in the neural tube and the germinal layers derived from it (Purves and Lichtman, 1985). While neurons are post-mitotic, it has been shown that myelin-forming oligodendrocytes (Sturrock and McRae, 1980; Ludwin and Bakker, 1988) and mature astrocytes (Latov *et al.*,1979; reviewed by Lindsay, 1986) are capable of mitosis in response to traumatic brain injury. Microglia, which have a mesodermal origin and invade the CNS around birth, proliferate and become phagocytic after direct brain injury or transection of the peripheral axons of motor neurons leading to the death of their centrally-located somata (Streit *et al.*, 1988).

The CNS lies within the cranium and the vertebral column and is structurally much more complex than the PNS. The cell bodies of central neurons are located in clusters called nuclei throughout the brain and spinal cord. Axons extend through a heterogeneous mass of cell bodies, neuronal and glial processes and synaptic regions. Individual oligodendrocytes contribute segments of myelin sheath to many axons while astrocytes enwrap the unmyelinated axons. There is no basal lamina within the adult CNS parenchyma, being present only around blood vessels and at the external limiting membrane (*glia limitans*) (McLoon, 1986; Sosale *et*

al., 1988).

After injury to the CNS, severed axons undergo Wallerian degeneration and there is proliferation of microglia and invasion of circulating macrophages in the vicinity of breached blood vessels (Lindsay, 1986). Activated microglia and blood-borne macrophages phagocytose neuronal and myelin debris and release peptides and cytokines which promote the mitosis of astrocytes (Giulian and Baker, 1986; Giulian *et al.*, 1988, 1989). The increase in astroglial number is accompanied by hypertrophy of these cells, consisting of cytoplasmic swelling with increases in nuclear size, microtubule content and intracellular membrane associated with the Golgi complex and rough endoplasmic reticulum (Reier, 1986). This suggests greater synthesis of proteins, which is reflected in up-regulation of the glial-specific intermediate filament protein, glial fibrillary acidic protein (GFAP), demonstrated by immunohistochemical methods (Lindsay, 1986; McLoon, 1986). These 'reactive' astrocytes also increase the number and size of their processes, eventually filling the extracellular spaces left by phagocytosis of the degenerating axons and myelin. Reactive gliosis is characterised in the light and electron microscopes by a meshwork of interdigitating astrocytic processes which are highly immunoreactive for GFAP (Reier, 1986), and which may extend considerable distances away from the site of injury (Mathewson and Berry, 1985).

As well as maintaining the structure of the CNS in the face of progressive neuronal loss, reactive gliosis also serves to seal surfaces of the neural parenchyma exposed to a non-neural environment by invasive trauma. In this event the reactive astrocytes additionally secrete a basal lamina to form a new *glia limitans* which can be detected by immunocytochemistry and electron microscopy 3-5 days after the insult; complete encapsulation occurs between 10-20 days depending on the severity of the lesion (Feringa *et al.*, 1980; Bernstein *et al.*, 1985). Formation of a basal lamina seems to be associated with astrocytic surfaces apposing connective tissue, especially collagen matrices. Although the exact

stimulus is unknown, it appears that the presence of fibroblasts may cause synthesis of basal lamina, concomitant with collagen deposition (Berry *et al.*, 1983; Reier *et al.*, 1983a).

Infiltration of fibroblasts and other meningeal elements is associated with penetrating injury to the CNS, leading to a complex glial-mesodermal scar with deposition of collagen matrix and astrocytic basal lamina (Krikorian *et al.*, 1981; Reier, 1986). Although reactive gliosis both in the presence and absence of mesodermal connective tissue elements have been described as 'glial scarring' (Reier, 1986), it seems pertinent to draw a distinction between the two events, at least with respect to the involvement of the additional cell types in penetrant injuries. In the absence of connective tissue and subsequent formation of a *glia limitans*, hyperplasia and hypertrophy of astrocytes will be referred to as *reactive gliosis*, while the more complex interaction with non-neural elements during penetrant lesions will be defined as *glial scarring*.

Despite the apparently reparative nature of reactive gliosis in reforming an external limiting membrane and maintaining structural integrity in the CNS, central neurons only show abortive regenerative sprouting in response to axotomy, often terminating in contact with densely-packed reactive astrocytes (Cajal, 1928; Hall and Berry, 1989). This seems surprising in view of the growth of neuronal processes over astrocyte surfaces *in vitro* (Noble *et al.*, 1984; Neugebauer *et al.*, 1988) and their secretion of neurotrophic factors (reviewed by Lindsay, 1986). The molecular mechanisms underlying the regenerative failure of the mammalian CNS, reviewed below, fall into 4 categories; a) lack of adequate neuronal response, b) lack of permissive factors, c) inhibitory factors, and d) physical barriers.

Beforehand, it is pertinent to examine CNS systems in which regeneration does occur. The regenerative capacities of the CNS of neonatal mammals and of inframammalian adults, which are more akin to the response mounted by the PNS, may highlight some of the factors affecting

regeneration in the mammalian CNS. Also the capacity of the mammalian olfactory bulb to receive incoming axons, even after induction of reactive gliosis, will be discussed.

1.2.2.1. Olfactory Bulb

The primary sensory neurons located in the olfactory neuroepithelium of the nose are unique in two ways. First, they are the only mammalian neurons that undergo continuous generation from stem cells followed by maturation and cell death, and secondly, they are the only neurons to extend newly-formed axons into the mature CNS and form functional synaptic connections therein (Graziadei and Monti Graziadei, 1978). This cycle of neurogenesis is necessary to replace neurons lost by mechanical wear of the olfactory epithelium and by degeneration due to noxious chemicals.

The unmyelinated axons of the sensory neurons project through peripheral nerve bundles into a laminated telencephalic extension of the cerebral cortex called the olfactory bulb. Lesions to the peripheral section of the olfactory receptor neuron fibres and damage to the olfactory bulb itself result in a hypertrophic glial scar at the superficial surface of the bulb, although connective tissue infiltration is minimal (Graziadei and Monti Graziadei, 1978; Doucette *et al.*, 1983). After a period in which axons and cell bodies of the damaged receptor neurons degenerate, newly-generated neurons project axons to the olfactory bulb, which penetrate the gliosed tissue and make apparently normal synapses in the appropriate area (Doucette *et al.*, 1983). Although this does not represent genuine axonal *regeneration*, the terrain of the reactive astrocytes is not only conducive to axonal growth but also appears able to guide the incoming fibres to their correct laminar destinations. This is accompanied by GAP-43 synthesis by the newly generated olfactory receptor neurons and GAP-43 can be visualised in their axons arriving in the olfactory bulb after lesioning (Verhaagen *et al.*, 1990).

1.2.2.2. Neonatal Central Nervous System

Unlike the situation in the adult CNS, injury to neonatal brain and spinal cord often does not lead to permanent functional deficits (Goldberger, 1986; Stelzner and Cullen, 1988) and may result in axonal elongation around or through the lesion site (Berry *et al.*, 1983; Bregman *et al.*, 1989; Merline and Kalil, 1990). The 'critical' period for axonal outgrowth appeared to correlate with differences in astrocytic reaction to injury and collagenous scar deposition, after which normal gliosis, scarring and lack of regeneration were seen (Berry *et al.*, 1983; Barrett *et al.*, 1984; Smith *et al.*, 1986; Bregman *et al.*, 1989). Fewer astrocytes were present in the tissue adjacent to penetrant lesions in neonates up to 8 days of age and no fibroblasts infiltrated the lesion to form collagenous deposits during this period (Berry *et al.*, 1983).

In an elegant series of experiments, Silver and colleagues showed (Smith *et al.*, 1986; Rudge *et al.*, 1989) that reactive astrocytes migrating onto implanted Millipore filters after brain lesioning during the 'critical' period (less than 8 days postnatal) were able to support axonal outgrowth and were different from those attaching to filters at later stages. These astrocytes, which were not accompanied by fibroblasts, proliferated and migrated rapidly and extended long, aligned processes. A mixed population of astrocytes and fibroblasts were found on filters implanted in older animals and these did not promote axonal regeneration. Moreover, implants removed from animals during the 'critical' period were able to support regeneration in older animals. This was confirmed by Bregman *et al.* (1989), who showed that foetal spinal cord transplants extended the 'critical' period for outgrowth of lesioned corticospinal tract (CST) axons. Kalderon (1988b) used peripheral nerve regeneration into synthetic tubes filled with cultured astrocytes to show that astrocyte maturation was accompanied by 'down-regulation of axonal growth'.

Alternatively, the regenerative capacity of the neonatal CNS may correlate with the functional status of the oligodendrocytes rather than

the astrocytes. Myelination is incomplete in animals less than 8 days old (see, for example, Schwab and Schnell, 1989).

1.2.3. Inframammalian Central Nervous System Response To Injury

The lower vertebrate CNS shares the same structural complexity and cell types as its mammalian counterpart, although ependymal cells are more prevalent (Simpson, 1983). It is however capable of considerable regeneration, even to the extent of repairing the spinal cord following complete transection and removal of a 1 mm segment (reviewed in Guth *et al.*, 1983). Astrocytes proliferate in response to injury and penetrant lesions are invaded by mesenchymal cells rather than fibroblasts. Additionally, ependymal cells (arising in the spinal cord from the central canal) proliferate and migrate into the glial/mesenchymal plug, fasciculating to form a bridge over which axons subsequently regenerate (Simpson, 1983). Interestingly, regeneration in lower vertebrates is accompanied by elevated synthesis of GAP-43 and its transport to growth cones (Skene and Willard, 1981a; Benowitz and Lewis, 1983).

1.2.4. Lack Of Mammalian Central Nervous System Regeneration

1.2.4.1. Lack Of Neuronal Response To Axotomy

In order to regenerate, the neuronal soma must receive a signal that axotomy has occurred and then mount a response that leads to axonal sprouting and elongation. This entails a transition from a *resting* state, characteristic of a normally-functioning mature adult neuron, to a *growth* state reminiscent of development. At the extreme, this transition would entail 'switching on' of developmental gene programmes not used in the *resting* state, perhaps to meet the increased demand for protein and lipid components of the membrane and cytoskeleton at the growth cone. The 'Inherent Incapacity Hypothesis' suggests that mammalian CNS neurons are incapable of such a transition, although whether this fault lies in the

transmission of the axotomy signal to the soma or the somatic reaction has not been addressed (Willard and Skene, 1982).

The initial signal received by the cell body of an axotomised neuron is the interruption of retrograde flow from the axon terminals, including target-derived neurotrophic factors (Greene and Shooter, 1980; Forman, 1983). This is believed to put the cell body in a state of readiness to initiate regeneration but secondary signals may be required to promote the outgrowth of the axon (Schwartz, 1987; Fawcett and Keynes, 1990). Nevertheless, the loss of target-derived neurotrophic factors may pre-empt any regenerative response by killing neurons which depend on a continual supply of these factors for their survival (Kromer, 1987; Otto *et al.*, 1987, 1989).

It is not within the scope of this thesis to review the highly variable responses of cell bodies in different neuronal systems to axotomy. For the purposes of this discussion, it is sufficient to note that there is no direct relationship between the initial morphological changes and regenerative capacity (Schwartz, 1987; Fawcett and Keynes, 1990), although Barron (1983) has suggested that accumulation of ribosomal RNA is deficient in non-regenerating CNS systems, which would be expected to lead to reduced capacity for protein synthesis. However, it has been shown that fast axonal transport of newly-synthesised proteins is increased in both regenerating and non-regenerating systems while differences have been noted in the spectra of proteins synthesised (Skene and Willard, 1981b). These results are inconsistent with the view that regeneration is limited by protein synthesis and it seems unlikely that production of GAPs would be specifically hampered by a lack of ribosomal RNA. Alternatively, the lack of RNA synthesis may be related to loss of trophic support for the neuron after axotomy (ie the early stages of cell death).

The 'Inherent Incapacity Hypothesis' has recently been shown to be largely incorrect by the transplantation experiments of Aguayo and colleagues, which demonstrated that many neurons from different regions of

the mammalian CNS can regenerate axons through grafts of peripheral nerve trunk (Richardson *et al.*, 1980; Aguayo, 1985; So and Aguayo, 1985). In some instances a few of the regenerating fibres re-entered the CNS at the termination of the grafted segment and formed well-differentiated, electrophysiologically active synapses within their original target region (Carter *et al.*, 1989; Keirstead *et al.*, 1989). This suggests that the cell body response of central neurons is sufficient to mount a regenerative response leading to functional restoration, given the correct factors a) to promote survival of the neuron, and b) to direct outgrowth of the axon.

Furthermore, these experiments demonstrated that the PNS is capable of supplying the necessary permissive factors to the neuron which are not available in the CNS even after trauma. Alternatively, the PNS may lack inhibitory factors or cells which prevent axonal regeneration. These observations are compatible with the 'Neural Environment Hypothesis'. Interestingly, grafts of PNS segments devoid of living cells were not as effective in promoting axonal regeneration from the CNS (Berry *et al.*, 1988a; Smith and Stevenson, 1988). This suggests that at least some of the putative permissive factors are produced by the non-neuronal cells of the peripheral nerve trunk (Berry *et al.*, 1987, 1988a). These possibilities are discussed in the following sections.

1.2.4.2. Lack Of Permissive Factors

Permissive factors perform 2 functions; they may promote a) the survival of neurons (*trophic* factors), and b) sprouting or elongation of neurites. In the second category are those factors that provide chemotactic guidance along a chemical gradient (*tropic* factors). These factors may be soluble or attached to the extracellular matrix (ECM) or the surface of other cells.

Since the necessary complement of permissive factors are, by definition, present during development, it seems appropriate first to review their expression during the developmental period and then ask which

factors are not active in non-regenerating adult CNS lesions. Examples will also be drawn from regenerating systems to illustrate the potential role of specific factors.

1.2.4.2.1. Diffusible Factors

Diffusible factors may be produced within the nervous system or by non-neuronal target organs. The best characterised soluble factor is NGF which has a neurotrophic effect upon certain classes of neurons (neural crest-derived peripheral sympathetic and sensory neurons and some central cholinergic macroneurons, see Lander, 1987; Vantini *et al.*, 1989). NGF is synthesised in limiting quantities by the target fields of these neurons and appears to determine the final density of innervation (Korsching and Thoenen, 1983; Large *et al.*, 1986; Davies *et al.*, 1987). NGF binds to specific receptors on neurons which mediate its uptake into the cell. Retrograde transport of NGF to the soma, or some other intracellular signal acting at the cell body (Greene and Shooter, 1980; Johnson and Taniuchi, 1987), promotes survival of neurons innervating the target field, thus regulating the degree of naturally-occuring cell death (Cowan *et al.*, 1984). NGF appears to be continuously required throughout adulthood (Ruit *et al.*, 1990), as demonstrated by the NGF-dependency of neurons after both peripheral (Bjerre *et al.*, 1974; Otto *et al.*, 1987) and central lesions (Kromer, 1987). This is reflected in the up-regulation of NGF synthesis in adulthood, when naturally occurring cell death has finished and the density of innervation has been established (Maisonpierre *et al.*, 1990b).

In addition, NGF can act directly on neurites to induce sprouting (Campenot, 1987) and direct the growth of neurites in a neurotropic manner (Gundersen and Barrett, 1979), but it is doubtful whether NGF is expressed early enough to influence the direction of outgrowth, at least during innervation of the skin (Davies *et al.*, 1987). However, target-derived neurotropic factors have been described (Lumsden and Davies, 1986; Tessier-Lavigne *et al.*, 1988; Heffner *et al.*, 1990).

A family of NGF-related neurotrophic factors has been recently described, including brain derived neurotrophic factor (BDNF, Leibrock *et al.*, 1989) and neurotrophin-3 (NT-3, Maisonpierre *et al.*, 1990a). BDNF is similar to NGF in promoting the survival of distinct populations of peripheral and central neurons during development, followed by up-regulation in adulthood (Johnson *et al.*, 1986; Barde, 1989; Leibrock *et al.*, 1989; Thanos *et al.*, 1989; Maisonpierre *et al.*, 1990a, b). NT-3, in contrast, shows down-regulation during maturation, suggesting that NT-3 acts to preserve cells only through the period of naturally occurring cell death (Maisonpierre *et al.*, 1990a, b). NGF, BDNF and NT-3 act on distinct but overlapping populations of neurons and this is reflected in their sites of synthesis (Maisonpierre *et al.*, 1990a, b; Hofer *et al.*, 1990).

Other factors reported to promote the survival of central neurons during development and/or adulthood include ciliary neurotrophic factor (CNTF, Blottner *et al.*, 1989a), basic fibroblast growth factor (FGF, Blottner *et al.*, 1989b; Otto *et al.*, 1989), epidermal growth factor (Morrison *et al.*, 1987, 1988) and insulin-like growth factors (Recio-Pinto and Ishii, 1988).

Trophic factors accumulated over time in the fluid secreted from damaged CNS tissue in a number of paradigms and this seemed to correlate with the survival of implants into cavity lesions (Manthorpe *et al.*, 1983; Politis, 1985). Specific neurotrophic factors that were elevated in the damaged CNS included NGF and both the acidic and basic forms of FGF (Nieto-Sampedro *et al.*, 1988; Finklestein *et al.*, 1988; Lorez *et al.*, 1988, 1989).

Astrocytes have been shown to produce several neurotrophic factors *in vitro* including NGF (Lindsay, 1979; Yamakuni *et al.*, 1987), and proliferating astrocytes are believed to be a source of neurotrophic activity after central neural injury (Lindsay, 1986). Release of NGF from astrocytes is stimulated by interleukin-1, which may be produced by infiltrating macrophages and activated microglia in brain lesions, and basic FGF (Giulian and Baker, 1986; Spranger *et al.*, 1990). Alternatively,

trophic factors may be released from cells which are damaged by trauma. This is the theory behind the action of the 'lesion factors', which support neuronal survival but are not secreted and are thus unlikely to act as target-derived neurotrophic factors, such as CNTF (Stöckli *et al.*, 1989; Sendtner *et al.*, 1990) and basic FGF (Janet *et al.*, 1987; Unsicker *et al.*, 1987). Although neurotrophic factors are released upon CNS damage there is still a considerable loss of neurons, due to the absence of the correct neurotrophic factors or accumulation that is too slow. Consequently, exogenously added neurotrophic factors have been shown to be active in promoting the survival of lesioned adult central neurons.

NGF rescues basal forebrain cholinergic neurons after axotomy by transection of the fimbria fornix commissural tract (Kromer, 1987; Rosenberg *et al.*, 1988) and prevents degenerative changes in these cells (Gage *et al.*, 1988a). Death of these neurons does not occur after chemical ablation of their target structures, suggesting that axotomy is crucial to their NGF dependence rather than simply loss of their endogenous supply of trophic factors (Sofroniew *et al.*, 1990). Alternatively, the cells deprived of their target cells by chemical ablation may get NGF from another source, such as reactive astrocytes (Lindsay, 1979). NGF also supports the survival of RGCs after optic nerve transection (Carmignoto *et al.*, 1989). Basic FGF supports NGF-sensitive populations of cholinergic medial septum neurons after fimbria fornix lesions (Anderson *et al.*, 1988; Otto *et al.*, 1989; Groethe 1989) and RGCs after optic nerve transection in adult rats (Sievers *et al.*, 1987). The effects of basic FGF may be indirect, however, as basic FGF causes NGF synthesis *in vivo* and in astrocyte cultures (Spranger *et al.*, 1990). BDNF also promotes the survival of RGCs and regrowth of their axons in adult retinal explants *in vitro* (Thanos *et al.*, 1989).

It would seem, then, that the CNS is deficient in the production of neurotrophic factors immediately after injury, although neurotrophic activity does accumulate in the lesioned CNS with time (Manthorpe *et al.*,

1983; Politis, 1985) and this may correlate with the time course of reactive gliosis (Lindsay, 1986). This contrasts with lesions to the PNS where trophic factors are rapidly elaborated by the damaged tissue and may accumulate in synthetic tubes linking the proximal and distal stumps of a severed peripheral nerve trunk (Longo *et al.*, 1983; Windebank and Poduslo, 1986). Schwann cells in the denervated distal segment of transected peripheral nerves were found to increase the synthesis of NGF in a biphasic pattern, the second phase of which was dependent on interleukin-1 from macrophages (Heumann *et al.*, 1989). Increased NGF has been shown to have a positive influence on the survival and regeneration of neurons axotomised by such transections (Otto *et al.*, 1987; Rich *et al.*, 1987, 1989), whereas injection of anti-NGF antiserum retarded regeneration after chemical axotomy (Bjerre *et al.*, 1974). Basic FGF also increased the survival of transected dorsal root ganglia neurons (Otto *et al.*, 1987) and promoted axonal regeneration (Danielsen *et al.*, 1988; Aebischer *et al.*, 1989). Activated macrophages, which are abundant in the distal segment of transected peripheral nerves, are directly capable of basic FGF synthesis (Baird *et al.*, 1985).

Although many tropic factors and neurite promoting factors have been identified *in vitro*, their effects are hard to monitor *in vivo*, unlike neuronal death which is used as an index of neurotrophic activity. Therefore information on the levels of soluble neuritogenic agents in the damaged CNS is as yet unavailable. Circumstantial evidence for a deficiency of such factors comes, however, from the PNS grafting experiments mentioned earlier (Vidal-Sanz *et al.*, 1987; Carter *et al.*, 1989) in which very few of the regenerating fibres present in the PNS graft actually re-entered the CNS and showed maximum penetration of 500 μm. Although no data have been reported on what proportion achieved re-entry, these authors stated that "the number of axons seen to extend beyond the graft and into the midbrain was only a small fraction of the fibre population (in the graft)" (Vidal-Sanz *et al.*, 1987). Alternatively these

results could be interpreted in terms of lack of substrate-bound elongation and/or guidance factors or the presence of non-permissive factors in the CNS.

Other factors, while not having a direct trophic or tropic effect on neurons, may aid the regenerative effort by providing the molecules required for axonal elongation. Apolipoprotein E (Apo E) is involved in the recycling of cholesterol from degenerating axons to growth cones (Ignatius *et al.*, 1987; Boyles *et al.*, 1989). Apo E synthesis is equivalent in both PNS and CNS development and declines in both adult systems. Within days of peripheral nerve injury, invading macrophages increase 3-fold the secretion of Apo E, which accumulates within the nerve sheath and represents 2-5% of the total extracellular protein until reinnervation (Skene and Shooter, 1983; Stoll and Müller, 1986; Müller *et al.*, 1985). In normal adult CNS Apo E is located in astrocytes, but after injury synthesis is up-regulated predominantly by invading macrophages (Stoll and Müller, 1986), although Apo E does not appear to accumulate within the neural tissue as it does in the PNS (Müller *et al.*, 1985). Shortage of Apo E in the CNS after injury, due either to insufficient secretion or retention, may constrain the ability of neurons to elongate axons by limiting recycling of membrane components.

1.2.4.2.2. Extracellular Matrix Molecules

Adhesion molecules in the ECM influence the direction of growth of neuronal processes during development (Purves and Lichtman, 1985; Dodd and Jessell, 1988). The ECM contains laminin, fibronectin and collagen which promote neurite outgrowth through interactions with a class of cell surface receptors known as integrins (Carbonetto *et al.*, 1988; Sanes, 1989). It is uncertain from the location and timing of appearance of these molecules during development what role they play in neurite guidance *in vivo* (Sanes, 1983, 1989; McLoon *et al.*, 1988; Sosale *et al.*, 1988; Rogers *et al.*, 1989).

Continual expression of laminin by glial cells appears to correlate

with the regenerative capacity of neural tissue. Laminin persists in the Schwann cell basal lamina tubes of peripheral nerves, and in astrocytes of the mammalian olfactory system and the entire CNS of lower vertebrates (Liesi, 1985; Hall, 1989). Moreover, lesions to the inframammalian CNS caused massive expression of astrocytic laminin (Liesi, 1985). The mammalian CNS down-regulates the adhesive ECM proteins laminin and fibronectin in adulthood and shows only limited and transient re-expression of these molecules after lesioning. Reactive astrocytes synthesised laminin after injury to some CNS regions (Liesi *et al.*, 1984; Liesi, 1985), but the distribution of laminin did not correlate with the regenerative sprouting of axons in lesions examined (Giftochristos and David, 1988a; Sosale *et al.*, 1988). Fibronectin reappeared after CNS lesions in the cytoplasm of glia and neuronal processes, although it is unclear whether it was synthesised by these cells or taken up from plasma leaking into the tissue after injury (Mizutani and Kimura, 1987). Although the expression of adhesion molecules does occur in the damaged CNS, it would appear that these adhesion molecules are inaccessible to axonal growth cones. Alternatively, it may be that adult mammalian CNS neurons down-regulate the integrin receptors necessary to respond to these adhesion molecules, as has been demonstrated in the development of chicken peripheral and central neurons (Cohen *et al.*, 1986; Tomaselli and Reichardt, 1988). Indeed, central neurons regenerating through cellular and acellular segments of sciatic nerve have been shown not to associate with Schwann cell basal lamina tubes which contain laminin (Berry *et al.*, 1988a; Smith and Stevenson, 1988; Hall and Berry, 1989).

Alternatively, the balance between proteolytic enzymes and proteases in the CNS may be inappropriate for axonal extension (Monard, 1988). Plasminogen activator (PA), which indirectly generates the protease plasmin and promotes neurite outgrowth in culture (Pittman and Buettner, 1989), was expressed by proliferating Schwann cells after injury to the PNS (Bignami *et al.*, 1982) and by astrocytes after injury to the goldfish visual pathway

(Sallés *et al.*, 1990). Developing CNS contained more PA than adult tissue, but lesions to adult optic nerve did not lead to detectable changes in PA activity (Bignami *et al.*, 1982). Using an *in vitro* model of astrocyte maturation, Kalderon *et al.* (1988a) showed that adult astrocytes synthesised less PA than immature astrocytes and that PA was not re-induced by passaging of mature astrocytes (as a model for astrocyte 'reactivity'). The serine protease nexin 1, which is derived from astrocytes and promotes neurite growth (Monard, 1988), was elevated during regeneration in the PNS (Meier *et al.*, 1989) and was found to be present in the adult olfactory bulb (Reinhard *et al.*, 1988). Levels in the normal and lesioned adult CNS have not yet been reported. The inability of the central glia to regulate the proteolytic activity in the ECM may prevent regeneration of axons.

1.2.4.2.3. Cell Surface Adhesion Molecules

The growth of axons is influenced by adhesion molecules on the surface of glial processes (Silver and Rutishauser, 1984) and other axons (Chang *et al.*, 1987). These may provide either a permissive substrate for outgrowth or demarcate specific pathways for axonal guidance, acting as haptotactic factors (providing chemotactic guidance over a surface). Neural cell adhesion molecule (N-CAM) and neuron-glia cell adhesion molecule (Ng-CAM) are expressed throughout development (for a recent review see Dodd and Jessell, 1988) but are down-regulated in the mature nervous system (Linnemann and Bock, 1989). Furthermore, the adhesiveness of N-CAM is modified by changes in sialic acid content during development. Specifically, the embryonic form is modified post-translationally by the addition of long polysialic acid chains which reduce the affinity of homophilic binding by charge repulsion, steric hindrance, or induction of conformational change (Edelman, 1983). The polysialic acid chains are reduced in number or length in the adult form of N-CAM, increasing the rate of binding (free-energy of binding) and hence the stability of neuronal interaction (Edelman, 1983; Chuong and Edelman, 1984). The embryonic form

can be distinguished immunohistochemically by a monoclonal antibody that recognises polysialic acid chains with at least three residues (Rougon *et al.*, 1986).

Immunohistochemical studies have shown that, in contrast to the rest of the mammalian CNS, N-CAM and Ng-CAM continued to be expressed in the olfactory system throughout adulthood and N-CAM was present in the embryonic form (Miragall *et al.*, 1989).

Denervation in the PNS produced changes in the expression of these molecules. N-CAM and Ng-CAM were up-regulated on the surface of Schwann cells (Nieke and Schachner, 1985) and N-CAM sialylation increased after sciatic nerve transection, suggesting that Schwann cells produce the less adhesive embryonic form of N-CAM (Daniloff *et al.*, 1986).

Unfortunately, no information is available regarding the expression of these (or other) cell adhesion molecules during the CNS response to damage. It is possible that lack of these developmentally-regulated molecules on surviving neuronal processes or the surface of reactive astrocytes may limit the capacity of damaged neurons to regenerate axons. It is useful, however, to examine the expression of cell surface molecules by glia in culture and their contribution to neurite outgrowth over glial monolayers.

Schwann cells in culture supported the outgrowth of neurites and this was inhibited by antibodies to Ng-CAM, integrin receptors and the Ca^{2+} dependent cell adhesion molecule N-cadherin (Bixby *et al.*, 1988). N-CAM did not appear to contribute to process outgrowth. Neonatal astrocytes in culture promoted neurite outgrowth by mechanisms involving N-cadherin, integrins and, notably, N-CAM (Neugebauer *et al.*, 1988). However, mature astrocytes showed loss of N-CAM and Ng-CAM activity and ligands for integrin receptors (such as laminin), associated with decreased ability to support neurite outgrowth (Smith *et al.*, 1990). This is consistent with the 'critical' period theory, whereby maturation of astrocytes *in vivo* leads to functional changes in the ability of these cells to support

development or regeneration of axons (Smith *et al.*, 1986).

In addition to the presence of adhesion molecules, neurotrophic factors bound to receptors on the surface of glial cells may act as haptotactic factors. Schwann cells distal to axotomy transiently up-regulated the receptor for NGF in response to loss of axonal contact (Taniuchi *et al.*, 1986, 1988; Raivich and Kreutzberg, 1987). This appeared to be the low affinity form of the receptor (Taniuchi *et al.*, 1988), which is not internalised upon binding of the NGF molecule (Johnson and Taniuchi, 1987), leading to the proposition that the receptors may act to trap NGF on the surface of the Schwann cells where it would be available to receptors on the advancing axons. In contrast, lesions to the CNS did not result in increased expression of NGF receptors (Taniuchi *et al.*, 1988).

In vitro experiments have shown that NGF bound to cryostat sections of denervated sciatic nerve provides a favourable substrate for neurite outgrowth (Sandrock and Matthew, 1987) and the haptotaxis hypothesis has found recent support from the synthesis of NGF receptors by spinal cord motor neurons after peripheral damage to their axons (Ernfors *et al.*, 1989). These cells are not dependent upon NGF for their survival and have not been shown to internalise NGF, suggesting that the receptor is the low affinity form acting to promote regeneration across substrate-bound NGF. Alternatively, the surface-bound NGF may be available for uptake by high affinity receptors on the axons of NGF-responsive sensory neurons, thus providing trophic support.

1.2.4.3. Chemical Barrier

The lack of regeneration in the adult mammalian CNS may be due to the presence of non-permissive factors preventing axonal elongation, which are absent from regenerating neural systems (PNS, olfactory bulb, immature and inframammalian CNS). Much tissue culture evidence points to the mammalian oligodendrocyte as being the source of such factors. Neurons grew readily on astrocytes in mixed glial cultures, but avoided oligodendrocytes which

caused growth cone collapse (Schwab and Caroni, 1988; Fawcett *et al.*, 1989a). Neurons cultured on tissue sections of adult CNS avoided white matter tracts while growing readily on gray matter, unmyelinated immature CNS, myelinated PNS and myelinated fish CNS (Savio and Schwab, 1989; Watanabe and Murakami, 1990). Cultured neurons also grew through explants of peripheral nerves, but not optic nerves which contained oligodendrocytes (Schwab and Thoenen, 1985). Myelin isolated from the CNS had a similar repulsive effect on neurite outgrowth whereas PNS myelin did not (Schwab and Caroni, 1988).

Two non-permissive factors have been identified by Schwab and colleagues in the myelin membranes of oligodendrocytes. Two proteins were isolated from CNS myelin which were non-permissive for neurite growth (RMM 35 kD and 250 kD respectively) and this activity could be blocked with monoclonal antibodies against these proteins (Caroni and Schwab, 1988a, b). These antibodies also partially blocked the non-permissive nature of mammalian white matter in optic nerve explants (Caroni and Schwab, 1988b), in tissue sections (Savio and Schwab, 1989) and *in vivo* (Schnell and Schwab, 1990). Appearance of the non-permissive proteins was specific for each region of the neonatal CNS but was correlated with oligodendrocyte differentiation after the period of axonal outgrowth (Caroni and Schwab, 1989). This agrees broadly with the end of the 'critical' period of neonatal regeneration (Berry *et al.*, 1983).

Oligodendrocytes also expressed the 160 kD and 180 kD isoforms of the J1 ECM glycoprotein which have been shown to be non-permissive for neuronal growth (Pesheva *et al.*, 1989). These molecules were present in CNS myelin (but not in the PNS) and appeared in developing white matter during the period of myelination.

Glial hyaluronate-binding protein has been implicated as a non-permissive factor for neurite extension synthesised by white matter astrocytes of the CNS, which appears late in development after axonal growth has been completed (Bignami *et al.*, 1988). It was not produced by

reactive astrocytes after injury, but it persisted for several months in white matter tracts undergoing Wallerian degeneration (Mansour *et al.*, 1990), suggesting that inhibition of regeneration may be an effect of a slowly-cleared mature CNS white matter component rather than a newly-synthesised product of reactive astrocytes. The effects of reactive astrocytes on regenerating axons are reviewed below.

1.2.4.4. Physical Barrier

Until recently, it was widely held that injury to the CNS resulted in the formation of a reactive glial barrier that was somehow impenetrable to growing axons (Cajal, 1928; Reier *et al.*, 1983b). However, this view has changed in recent years with our increasing understanding of astrocyte biology (Lindsay, 1986) and the appreciation that reactive gliosis is affected by interactions with fibroblasts in penetrant injuries, leading to the formation of a fibrous glial-mesodermal scar and deposition of a *glia limitans* which may instead be the impenetrable elements (Reier, 1986; Reier and Houle, 1988). There is still considerable evidence to suggest that astrocytes are non-permissive for axonal growth. This will be briefly reviewed before consideration of the evidence in favour of a positive role for astrocytes in CNS regeneration.

Transplantation experiments have shown the dichotomous nature of CNS and PNS tissue on axonal regeneration. PNS grafts to the CNS encouraged prolific outgrowth of severed axons through the grafted tissue (Aguayo, 1985). In contrast, segments of optic nerve prevented regeneration of peripheral neurons in the absence of either oligodendrocytes or a *glia limitans* (Reier *et al.*, 1983b). Surprisingly, grafts of immature optic nerve produced the same result (Giftochristos and David, 1988b). These effects may be due, however, to the relative levels of permissive and non-permissive factors discussed earlier.

Direct evidence for an astrocytic effect on regenerating peripheral axons comes from studies of the dorsal root entry zone (DREZ) where the

axons of primary sensory neurons enter the CNS. These axons can be lesioned peripherally without any direct injury to the CNS and without the formation of a glial scar or *glia limitans* (Reier, 1986). In lower vertebrates and neonatal mammals these axons regenerated successfully into the CNS, but in adult mammals they were arrested at the astrocytic interface at the DREZ (Liuzzi and Lasek, 1985; Carlstedt, 1988). Electron microscopical studies of arrested axons at the DREZ have suggested that growth cones contact astrocytes and are prevented from elongating by a process that is dependent upon activation of proteases within the growth cone leading to a transition to a non-growing state (Liuzzi and Lasek, 1987; Liuzzi, 1990). Glial elements of the CNS would therefore appear to regulate the regenerative capacity of neurons, including those with cell bodies extrinsic to the CNS.

Further evidence for a difference between astrocytes of regenerating and non-regenerating systems comes from electron microscope studies of astrocytic surfaces (reviewed by Reier, 1986). Orthogonal arrays of intramembranous particles, which are characteristic of mammalian astrocyte membranes, were absent from the membranes of astrocytes of several inframammalian species. These arrays were also lacking in the developing mammalian CNS and in the astrocytic membranes of the adult olfactory bulb. The effect of these orthogonal arrays on axonal growth is unknown and it is unclear whether their density increases during reactive gliosis, but differences in the surface properties of astrocytes correlate with the ability of CNS tissue to regenerate. Interestingly, astrocytic surfaces which provide a good substrate for neurite growth in culture possessed relatively few orthogonal arrays (Landis and Weinstein, 1983)

On the other hand, evidence exists to suggest that reactive astrocytes may exert a positive influence on axonal sprouting *in vivo*. After lesions to afferent pathways to the adult hippocampus, increased immunoreactive GFAP characteristic of reactive astrocytes was associated with collateral sprouting from undamaged fibres (Gage *et al.*, 1988b).

Although this does not represent *regenerative* axonal outgrowth, it is interesting that astrocytes not only appeared to support the elongation of these axons but may have also initiated sprouting through NGF secretion. Synthesis of NGF by astrocytes may be stimulated by interleukin-1, released from activated microglia in response to the lesion (Giulian and Baker, 1986; Spranger, 1990).

The realisation that introduction of fibroblasts in penetrant lesions altered the nature of reactive gliosis (to produce a glial-mesodermal scar) encouraged Guth and co-workers to examine lesions from which these cells were excluded (Guth *et al.*, 1986). Initially the model used was transection of the spinal cord of the hibernating ground squirrel, in which body temperature is reduced to 8^{o}C and metabolic rate is depressed. This resulted in reduced astrocyte reaction and infiltration of fibroblasts, with concomitant reduction in collagen deposition and glial scarring. Regeneration of fibres up to the edge of the lesion was extensive. This was extended to a more common laboratory animal with the development of a compression model of spinal cord injury in the rat. The lesion was produced with blunt forceps and this procedure prevented infiltration of fibroblasts by leaving the meninges intact (Guth *et al.*, 1985). Astrocytes and ependymal cells proliferated to produce longitudinal bridges and axons were observed to regenerate along these cellular paths, in a manner reminiscent of the response of inframammalian CNS to transection (Guth *et al.*, 1983). Although it remained unclear whether astrocytes were the direct substrate for axonal elongation, it appeared that regeneration could occur in the presence of a robust gliosis.

1.2.5. Summary-The Role Of Glia In Axonal Regeneration

It is clear from experimental grafting of PNS segments that neurons of the CNS are capable of regeneration and that the neural environment regulates the success of the regenerative response to axotomy (Aguayo, 1985). Much evidence has accumulated to suggest that the critical

difference between the PNS and CNS lies in the glial cells and, more specifically, that Schwann cells provide a favourable environment for regeneration whereas oligodendrocytes and astrocytes do not.

First, the induction of regeneration from axotomised PNS and CNS neurons correlates with the presence of Schwann cells in peripheral nerve segments. If the Schwann cells were killed by freezing of a *distal* nerve segment, the regeneration rate of axotomised peripheral neurons was reduced and axons only elongated through the graft in the company of Schwann cells migrating from the *proximal* segment (Hall, 1986a; Gulati, 1988; Sjöberg *et al.*, 1988). Likewise, peripheral nerve fibres regenerated into muscle-derived ECM, but only in the presence of Schwann cells originating from the peripheral nerve tissue (Fawcett and Keynes, 1990). However when mitosis was prevented (Hall and Gregson, 1977) in the proximal stump of a transected nerve anastomosed to an acellular (freeze/thawed) distal segment, both Schwann cell migration and axonal elongation into the graft were delayed until cell division restarted (Hall, 1986b). Similar results have been obtained with acellular peripheral grafts to transected optic nerve, utilising the RGCs to show that an endogenous population of Schwann cells is required to support axonal elongation from regenerating central neurons (Berry *et al.*, 1987, 1988a, b; Smith and Stevenson, 1988; Hall and Berry, 1989).

Secondly, purified populations of Schwann cells have been shown to promote neuronal survival and induce regeneration when transplanted into CNS lesions (Kromer and Cornbrooks, 1985; Maffei *et al.*, 1990). It is noteworthy that these lesions were also ameliorated by infusions of NGF in other studies by the same authors (Kromer 1987; Carmignoto *et al.*, 1989). In contrast, astrocytes have the capacity to prevent the normally robust regeneration of peripheral neurons *in vivo* or *in vitro* (Kalderon, 1988b; Fawcett *et al.*, 1989b). This inhibition of growth was only seen with mature astrocytes, supporting the notion that astrocytes have a 'critical' period, early in postnatal development, when they are able to support

axonal elongation (Smith *et al.*, 1986; Rudge *et al.*, 1989). This may be linked to changes in the surface properties or neurotrophic secretions of astrocytes as they mature (Reier, 1986; Smith *et al.*, 1990).

Thirdly, myelin derived from Schwann cells in the PNS did not contain the repulsive proteins responsible for the non-permissive effects of central myelin and oligodendrocytes (Caroni and Schwab, 1988b).

1.3. The Present Studies

A polyclonal antibody was raised against GAP-43, using recombinant DNA technology. The specificity of the antiserum was tested on Western blots of purified GAP-43 and extracts of CNS tissue and with immunohistochemistry of CNS tissue sections. The highly sensitive antiserum obtained revealed an unexpected distribution of GAP-43 in the CNS and the presence of an immunoreactive protein band in Western blots which may represent an aggregated form of GAP-43. These results are presented in Chapters 3 and 4.

Experimental manipulations which selectively prevent or allow CNS regeneration were used to examine the capability of central axons to express GAP-43, as an index of the cell body response to axotomy. In this way, a correct transduction of the 'axotomy signal' into a regenerative response was demonstrated in rat RGCs. The knowledge that central axons can synthesise GAP-43 during the course of regeneration enabled a study to be undertaken to confirm the assertions of Guth *et al.* (1985) that some spinal cord axons can regenerate after non-penetrant lesions, although most axons were spared from axotomy by the surgical procedure. These results are presented in Chapter 5.

In the course of these studies it became apparent that both CNS and PNS glia displayed immunoreactivity to GAP-43. Western blotting was used to confirm the presence of GAP-43 in glial cells and the specific populations of glia expressing GAP-43 were delineated. These results are presented in Chapters 6 and 7.

Chapter Two

Materials and Methods

2.1. Materials

2.1.1. Chemicals

Fine chemicals and enzymes were bought from Sigma. All other chemicals and solvents were of analytical grade or better and obtained from BDH, Fisons or Sigma, except where noted in the text. Electrophoresis grade materials were purchased from BDH Electran.

2.1.2. Sterile Tissue Culture Plastics

Hypodermic needles and syringes were bought from Sabre. Petri dishes and centrifuge tubes were obtained from Falcon. Pipettes, flasks and multiwell plates were supplied by Flow laboratories.

2.2. Animals

Sprague-Dawley rats were used throughout. These were obtained from the Departmental breeding colony and maintained in the animal house. Half Lop rabbits were bought in from Olac and kept in the Departmental animal facility.

2.2.1. Surgery

All surgical procedures were performed on adult male rats weighing 200-220 g under aseptic conditions using sterile gloves and scalpel blades and heat sterilised surgical instruments throughout. Anaesthesia was produced with Halothane (May and Baker) in medical oxygen:nitrous oxide (2:1, v:v) delivered at 1.5 l/minute. 3% Halothane was used for induction and this was reduced to 2% for the duration of the surgery.

2.2.1.1. Spinal Compression

5A watchmakers forceps were used to compress the exposed spinal cord at the fifth thoracic vertebra (T5), as described by Guth *et al.* (1985). The points of these forceps had previously been blunted on fine

grade glass paper and polished to remove any sharp edges (in order to avoid puncturing the meninges during compression) and a hole had been drilled through one blade. The forceps were mounted in a metal block as shown in Figure 2.1. This block was attached to a sterotaxic frame, which allowed precise positioning and stability of the forceps during insertion into the vertebral column. The block contained two pins which could be advanced towards the forceps by means of calibrated screws and thus controlled the distance between the blades at rest and at maximum closure to within 0.1 mm (see legend Figure 2.1.).

Animals were placed in a sterotaxic frame and their backs were shaved and then scrubbed with iodine solution (Povidone). An incision was made along the midline starting between the shoulder blades and extending 2 cm caudally. *m. trapezius* were separated from the fourth to sixth thoracic vertebrae (T4-T6) and retracted, and the paravertebral muscles were then cleared from the dorsal surface of T4 and T5. The dorsal lamina of T5 was then cut on either side with angled iridectomy scissors and removed to expose the spinal cord (see Figure 2.2.a).

After lowering the forceps into the laminectomy site (see Figure 2.2.b), the blades were carefully closed until they just contacted the spinal cord on both sides using a binocular dissecting microscope. The blade distance at rest was read from the calibrated screw of the *closing pin*, giving the diameter of the cord. Initial experiments indicated that a 60% compression for one second produced a lesion as described by Guth *et al.* (1985). The *limiting pin* was introduced through the drilled blade to a distance allowing closure of the blades to 40% of the cord diameter. Activation of the electromagnet by a one second 12 volt direct current closed the blades of the forceps (transformer with timer provided by Dr. R.M. Lindsay of Sandoz, London), producing a 60% compression of the spinal cord. The reader is referred to Chapter 5 for a full discussion of the spinal compression. Control animals received laminectomy but the forceps were not inserted.

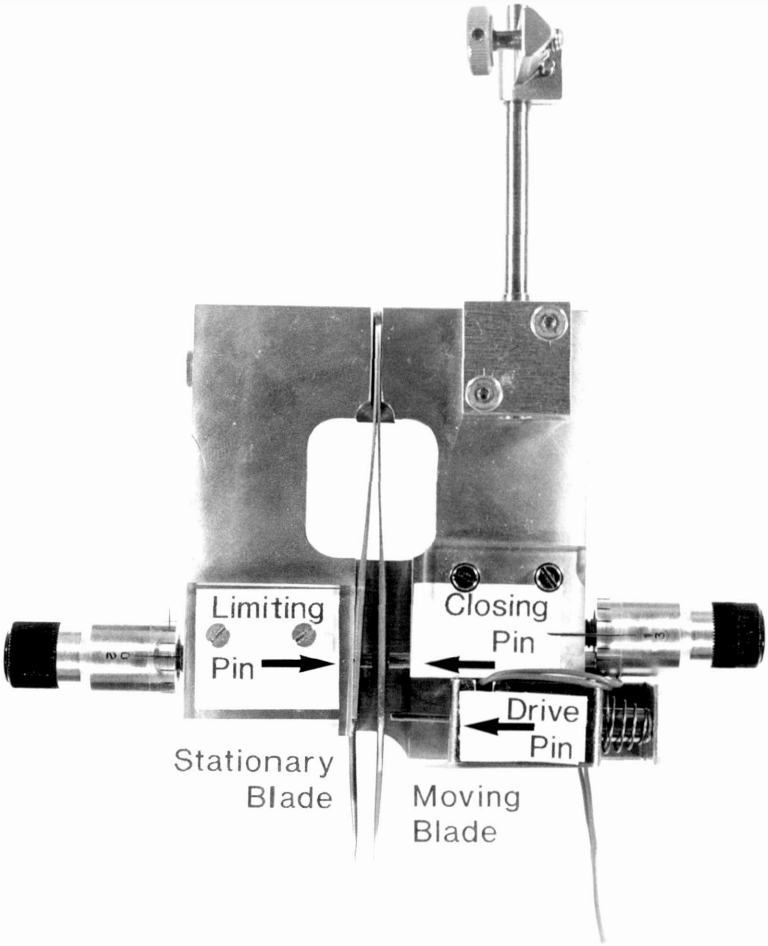


Figure 2.1.

Activation of the electromagnet by 12 volt direct current forces the *drive pin* against the *moving blade* of the forceps. Total closure is prevented by the *limiting pin*, which passes through the drilled hole in the *stationary blade*. The *moving blade* can also be advanced by the *closing pin* to position the blades in contact with the spinal cord prior to compression.

Calibrated screws control the position of both the *limiting pin* and the *closing pin* to within 0.1 mm. This allows the diameter of the cord to be measured from the position of the *closing pin* (set here at 3 mm). The maximum closure of the blades is controlled by the position of the *limiting pin* (set here at 2 mm). Under these conditions a 33% compression would be applied by activation of the *drive pin*.

Figure 2.2.

A and B. Surgical exposure and compression of the spinal cord.

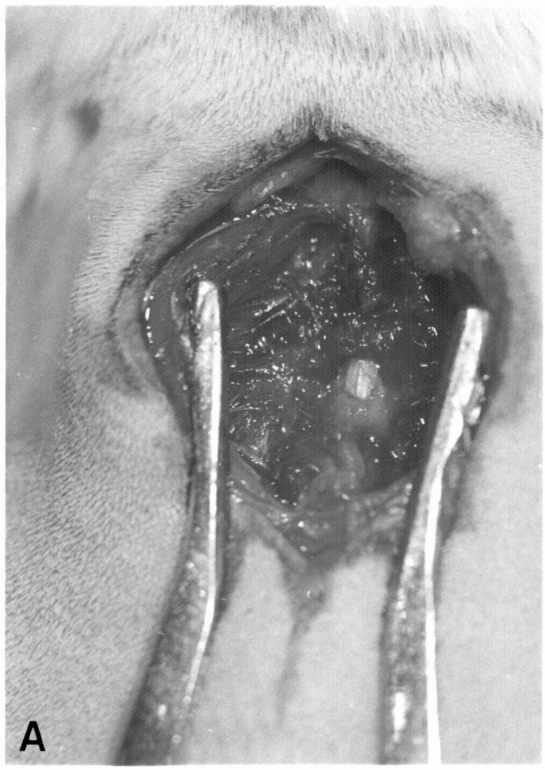
A. After laminectomy at T5, the dorsal surface of the spinal cord is exposed. The incision is longer than usual to increase the visibility of the laminectomy site.

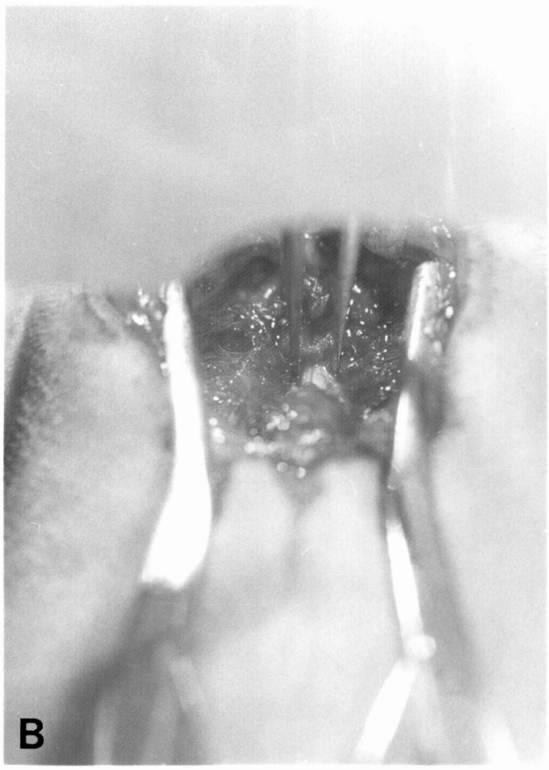
B. The forceps have been lowered so that the blades are inside the vertebra and are just in contact with the spinal cord. Closure of the forceps will now compress the cord, as determined by the position of the *limiting pin*. The metal block holding the forceps can be seen at the top of the photograph.

C.D. Exposure and lesioning of the sciatic nerve.

C. Exposure of the sciatic nerve in the thigh. The site of transection or compression is marked with an arrowhead. Once again, the incision is longer than normal to increase visibility, and *m. biceps femoris* has been retracted.

D. After transection, the proximal stump is inserted between *m. vastus lateralis* and *m. rectus femoris* (marked with a star).


A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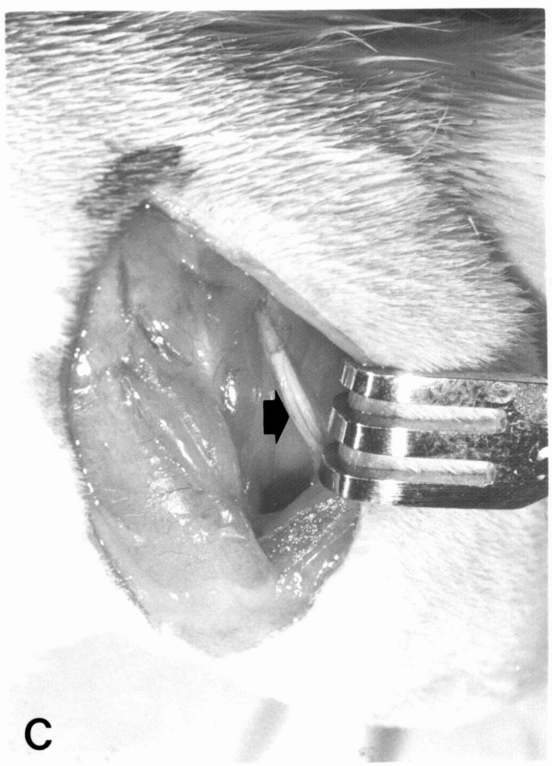
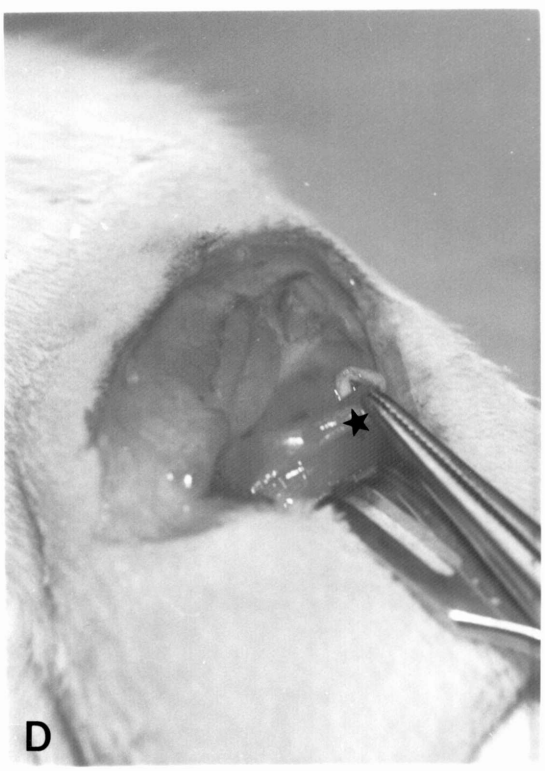

C


D

The forceps and retractor were then removed and the muscles and overlying fat pads were sutured with 1 gauge metric silk. The skin was closed with 2 gauge monofilament and anaesthesia was withdrawn. 2 ml 5% glucose, 0.9% sodium chloride (w:v, glucose saline) were injected subcutaneously for post-operative shock and 250 mg carbenicillin (Pyopen, Beecham) was injected intramuscularly. Animals were housed in cages (maximum 6/cage) lined with absorbent paper and with glucose saline *ad libitum* for one day and then moved to cages with sawdust and water as normal. Antibiotic treatment continued for 2 days (250 mg carbenicillin/day). Bladder massage was applied twice daily in those animals showing impaired renal function. All animals showed return of unassisted urination within 3 days and there were no instances of infection at the surgical site. Indeed, no animals were lost as a result of the surgical intervention, either through respiratory dysfunction under anaesthesia or post-operative shock or infection.

2.2.1.2. Sciatic Denervation.

The sciatic nerve was exposed at mid-thigh level by incision of the overlying skin and separation of *m. gluteus superficialis* and *m. biceps femoris* (see Figure 2.2.c). The nerve was then crushed or transected (see below) and the muscles were sutured with 1 gauge metric silk and the wound closed with 2 gauge monofilament. Animals were housed in the same way as the spinally damaged animals but no antibiotic or glucose saline injections were given.

2.2.1.2.1. Sciatic Nerve Transection

Permanent denervation was achieved by complete transection of the sciatic nerve in the thigh. To prevent regeneration of axons into the distal segment, the proximal stump was inserted between *m. rectus femoris* and *m. vastus lateralis* (see Figure 2.2.d) and held in place with a suture of 1 gauge metric silk. The absence of axons in the

distal stump was confirmed at the time of sacrifice by immunohistochemistry for neurofilament protein (see Section 2.9.1.2.).

2.2.1.2.2. Sciatic Nerve Compression

Temporary denervation followed by reinnervation of the distal portion was achieved by crushing a 2 mm wide segment for 2 seconds with blunt-edged 5A watchmakers forceps. An epineurial suture was placed around the nerve distal to the lesion to mark the site of axotomy.

2.2.1.3. Kainic Acid Injection

The excitatory neurotoxin kainic acid (2 μg in 2 μl filter-sterilised PBS) was injected sterotaxically into the cerebellar vermis at a depth of 1.2 mm.

2.2.1.4. Optic Nerve Transection and Sciatic Nerve Grafting

Surgery was performed by Professor M. Berry at the Department of Anatomy, Guy's Hospital, London, as described previously (Berry *et al.*, 1988b). Briefly, the optic nerve was exposed intraorbitally and transected 2 mm from the eyeball, ensuring that the vascular supply to the retina was not disrupted. A 20 mm segment of sciatic nerve was sutured onto the severed optic nerve and the free end was led out of the orbit and positioned subcutaneously on *m. temporalis*. In some animals the severed segments of the optic nerve were reapposed without a sciatic nerve graft and held in place with a single suture.

2.2.2. Antisera

Rabbits were first tested for endogenous circulating antibodies cross reacting with rat CNS tissue. Pre-immune serum was obtained (see below) and used to stain sections of adult rat cerebellum by immunofluorescence histochemistry (see Section 2.9.). Rabbits showing no cross reacting antibodies were selected and the pre-immune sera were

stored frozen for later use.

GAP-43/ß-galactosidase fusion protein (see Section 2.4.) was used to raise antibodies against GAP-43. Rabbits were inoculated with approximately 100 μg fusion protein in Freund's complete adjuvant and boosted one month later with 100 μg in incomplete adjuvant. Injections were made subcutaneously and 15 ml blood were collected from the marginal ear vein at weekly intervals. Blood was left to clot at room temperature and the serum was transferred to centrifuge tubes to precipitate erythrocytes at 1000*g*. Cleared serum was collected and stored at -70^{o}C.

2.3. GAP-43 Purification

GAP-43 was purified according to the method of Chan *et al.* (1986) with modifications, including the addition of calmodulin affinity chromatography (Andreasen *et al.*, 1983). To minimise enzymatic degradation of GAP-43, all procedures were carried out on ice with pre-cooled instruments and solutions. Centrifugation steps were performed in a refrigerated Beckman L8-55 ultracentrifuge at 4^{o}C and column chromatography was performed in a cold room at the same temperature.

2.3.1. Membrane Preparation

This procedure was modified from Chan *et al.* (1986) who prepared a synaptosomal fraction from adult cortex as a source of GAP-43. In order to increase the yield and reduce the cost of the purification, membranes were prepared from whole neonatal brain tissue in this study, in accordance with the greater synthesis of GAP-43 during axon outgrowth in brain development (Jacobson *et al.*, 1986).

Rat pups aged 7-14 days were decapitated and the brains were removed and pooled in hypotonic lysing buffer (2 mM DTT, 10 mM EDTA, 10 mM Tris/HCl, pH 7.5) using 2 ml per pup. After coarse chopping with a scalpel blade and scissors, the tissue was transferred to a glass

homogeniser and disrupted with 10 strokes of a motor-driven teflon pestle (950 rpm). An equal volume of lysing buffer was then added (total 4 ml lysing buffer per brain, average weight 0.8 g) and the crude homogenate was transferred to centrifuge tubes and spun at 1000*g* (60Ti rotor) for 10 minutes to remove nuclei and any undisrupted tissue. The supernatant was retained and the pellet was re-homogenised in an equal volume of lysing buffer and centrifuged as above. The pooled supernatants were centrifuged at 17,500*g* (60Ti rotor) for 20 minutes to pellet the membranes, which were resuspended in a minimal volume (2 ml per tube) of 1 mM magnesium acetate.

2.3.2. Alkali and Acid Extraction

GAP-43 was extracted from the membranes by addition of enough 5 M sodium hydroxide to adjust the pH to 11.5, according to Chan *et al.* (1986). Skene and Virág (1989) have subsequently shown that GAP-43 is inserted into the plasma membrane via thioester-linked palmitic acid residues, which are known to be alkali-labile (Kaufman *et al.*, 1984). Lipid membranes were removed by centrifugation at 130,000*g* (SW60Ti rotor) for 20 minutes and supernatants were pooled and acidified to pH 5.5 with 1 M sodium acetate (buffered to pH 5.0 with acetic acid). Insoluble proteins were pelleted by centrifugation at 58,000*g* (SW60Ti rotor) for 20 minutes.

2.3.3. Ammonium Sulphate Precipitation

Supernatant containing soluble GAP-43 was subjected to 40-80% ammonium sulphate precipitation. First, ammonium sulphate was added to 40% saturation (ie 0.229 g/ml supernatant) and the resulting solution was subjected to a further 58,000 *g* centrifugation for 20 minutes. Insoluble proteins were discarded and the supernatant containing soluble GAP-43 was supplemented with a further 0.262 g ammonium sulphate/ml (to give a final concentration of 80% saturation) and centrifuged again at

58,000*g*. The pellets were resuspended in a minimal volume (1 ml/tube) of 5 mM potassium phosphate buffer (KPi, pH 7.5) containing 1 mM EDTA and dialysed extensively against the same at 4^{o}C.

2.3.4. Hydroxylapatite Column Chromatography

A 5 x 1.5 cm glass column (bed volume 10 ml) was packed with hydroxylapatite (Bio-Rad) and equilibrated with 5 mM KPi at 4^{o}C at a free-drip flow rate of 1-2 ml/minute. The dialysed sample (5 ml) was loaded onto the column, collected and reloaded. GAP-43 has been shown to bind to hydroxylapatite at low salt concentrations and to be eluted between 30-75 mM KPi (Chan *et al.*, 1986). Accordingly, the column was washed with 40 ml 30 mM KPi and then GAP-43 was eluted with 100 ml 75 mM KPi, followed by 50 ml 400 mM KPi to remove remaining proteins and regenerate the column. The 75 mM KPi fraction was extensively dialysed against 20 mM Tris/HCl, 5 mM magnesium chloride and 1 mM EDTA (pH 7.5).

2.3.5. Calmodulin-Agarose Column Chromatography

Chan *et al.* (1986) utilised phenyl sepharose column chromatography to further purify GAP-43, although some contaminating proteins remained. Subsequently, Storm and colleagues have shown that GAP-43 is identical to the calmodulin-binding protein P57 (Cimler *et al.*, 1987). Calmodulin, which binds P57/GAP-43 only in the absence of free Ca^{2+} ions, can be used to purify GAP-43 to apparent homogeneity (Andreasen *et al.*, 1983). Accordingly, 2 ml calmodulin-agarose were packed into a 3 x 1 cm glass column and equilibrated with 20 mM Tris/HCl, 5 mM magnesium chloride, 1 mM EDTA, 5 mM EGTA (pH 7.5) at 4^{o}C. The dialysed 75 mM KPi fraction from the hydroxylapatite column was made up to 5 mM EGTA (pH was readjusted to 7.5) and applied to the column at a free-drip flow rate of 5-10 ml/minute, then collected and reloaded. The column was washed with 10 ml equilibration buffer and then GAP-43 was eluted with the same buffer containing 3 mM calcium chloride and no EGTA (15 ml

aliquots), followed by regeneration of the column with 100 ml 500 mM sodium chloride. The first three aliquots of eluted GAP-43 were combined and freeze-dried. Protein assay was performed on the resulting white powder using a Bio-Rad kit based upon the absorbance shift of Coomassie blue G250 (as described by the manufacturer) to establish the yield of GAP-43.

2.4. GAP-43/ß-galactosidase Fusion Protein

Full-length GAP-43 cDNA (Rosenthal *et al.*, 1987) in the pGEM3 circular plasmid vector was obtained from Dr. D.V. Goeddel (Genentech, California).

2.4.1. Amplification Of GAP-43 cDNA

The plasmid was amplified by transformation into *E. coli* (Glover, 1984). Colonies were selected for ampicillin-resistance (overnight at 37^{o}C on L-agar containing 50 μg/ml ampicillin) to indicate incorporation of the plasmid, and grown overnight at 37^{o}C in 200 ml Luria Bertani medium (L-broth) containing 100 μg/ml ampicillin (for formulations see Maniatis *et al.*, 1982). Cells were collected by centrifugation at 600*g* for 10 minutes, then resuspended in 10 ml 25 mM Tris/HCl (pH 8.0) containing 50 mM glucose, 10 mM EDTA and 5 mg/ml lysozyme, and subjected to SDS-alkaline lysis by addition of 20 ml 0.2 M sodium hydroxide, 1% SDS (w:v). Cell debris and bacterial DNA (chromosomal) were precipitated by addition of 10 ml 2.5 M potassium acetate (buffered to pH 4.8 with acetic acid), then the plasmid DNA was collected by isopropanol precipitation and equilibrium density centrifugation through 1 g/ml caesium chloride containing 1 mg/ml ethidium bromide (Glover, 1984). Plasmid DNA was identified under UV light.

2.4.2. Expression Of GAP-43 As A Fusion Protein With ß-galactosidase

GAP-43 was cloned into the pUR 292 circular plasmid expression

vector for synthesis as a fusion protein with ß-galactosidase (Rüther and Müller-Hill, 1983). First, a 610 bp fragment encoding residues 17-219 of GAP-43 (total length 226 residues, 678 bp, Rosenthal *et al.*, 1987) was excised from the pGEM3 plasmid with the *Ava II* and *Ava I* restriction endonucleases, both of which cut double-stranded DNA unevenly to leave cohesive ends (Maniatis *et al.*, 1982). The excised fragment was separated from the plasmid DNA by electrophoresis through 1% agarose (ICN Biochemicals) in 40 mM Tris/HCl, 1 mM EDTA, 5 mM sodium acetate (pH 7.8) containing 2 μg/ml ethidium bromide. The 610 bp GAP-43 fragment was identified by its position relative to a 1 kbp ladder of DNA standards (Gibco-BRL) under UV light, then cut out and purified using the Geneclean kit according to the manufacturer's instructions (Stratech Scientific). Secondly, as *Ava II* and *Ava I* recognition sequences do not exist in the pUR vectors (Rüther and Müller-Hill, 1983), the cohesive ends of the GAP-43 fragment were filled in by base pairing using the Klenow fragment of *E. coli* DNA polymerase (1 unit/25 μl) and a mixture of deoxyribonucleotidetriphosphates (80 μM) (Maniatis *et al.*, 1982) to produce blunt-ended DNA. Thirdly, the pUR 292 plasmid was cut with the *Bam H1* restriction endonuclease and end-filled with the Klenow fragment as described above (Maniatis *et al.*, 1982). Finally, the 610 bp GAP-43 fragment was mixed in 6 molar excess with the pUR 292 plasmid and DNA ligase was added (1 unit/10 μl) to achieve blunt-end ligation and re-circularisation of the plasmid.

The ligated plasmid was transformed into *E. coli* and plasmid DNA was prepared by SDS-alkaline lysis (except that equilibrium density centrifugation was not performed) as described in Section 2.4.1. Plasmids which had re-circularised without incorporating the GAP-43 fragment were identified by restriction analysis using the *Eco R1* endonuclease enzyme, recognition sites for which exist either side of the *Bam H1* cloning site. The DNA was separated by electrophoresis through 1% agarose as described and the bands were identified under UV

light. Colonies showing the presence of the GAP-43 cassette were grown up overnight in 200 ml L-broth containing 100 μg/ml ampicillin and 0.5 mM isopropyl ß-D-thiogalactoside to stimulate production of ß-galactosidase (Glover, 1984). Cellular proteins were analysed by SDS-PAGE in 7.5% acrylamide gels followed by Coomassie blue staining, as described in Section 2.6. Plasmids which had incorporated the 610 bp GAP-43 fragment in the wrong orientation only showed synthesis of ß-galactosidase, whereas the presence of a higher RMM protein indicated synthesis of the GAP-43/ß-galactosidase fusion protein.

2.4.3. Purification Of GAP-43/ß-galactosidase Fusion Protein

3 ml PAGE sample of one colony expressing the fusion protein were loaded on a preparative 6% acrylamide gel and proteins were separated as described in Section 2.6.2.2. The fusion protein band was identified by soaking the gel in 250 mM potassium chloride overnight at 4^{O}C then it was cut out and placed in dialysis tubing with 10 ml electroelution buffer (25 mM Tris, 192 mM glycine, 0.1% (w:v) SDS, pH 8.3). Electroelution was performed in a horizontal gel apparatus at 150 volts for 4 hours in sufficient buffer to just cover the tubing. Current was reversed for the last 2 minutes to release protein bound to the tubing and the eluted protein was recovered from the dialysis bag.

Protein concentration was estimated by spotting the eluate and BSA standard concentrations onto filter paper (Whatman No. 1) and staining with 0.1% Coomassie blue (G250 in (v:v) 10% acetic acid, 25% isopropanol) followed by washing in tap water. The fusion protein was stored in 1 ml aliquots at -20^{O}C prior to inoculation of rabbits (Section 2.2.2.).

2.5. Tissue Culture

2.5.1. Tissue Culture Media

Powdered media were purchased from Imperial Laboratories. The media used were Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12, according to the manufacturer's formulation, and a modified DMEM containing 187.2 mg/l D-valine instead of L-valine which is present in regular DMEM at a concentration of 93.6 mg/l. This modification significantly reduces fibroblast contamination of cultures (Estin and Vernadakis, 1986; Cholewinski *et al.*, 1989). The media were dissolved in an appropriate volume of double distilled deionised water and supplemented with sodium bicarbonate according to the manufacturer's specification, then filter sterilised. Foetal calf serum (FCS) was purchased from Flow Laboratories and dialysed against 3 changes of 50 volumes 0.9% (w:v) sodium chloride at 4^{o}C to remove valine (Estin and Vernadakis, 1986), followed by filter sterilisation. All procedures were carried out under aseptic conditions and, unless stated otherwise, media were supplemented with (v:v) 0.5% gentamicin (Flow Laboratories) to prevent microbial contamination.

Cells were maintained in an incubator with a water saturated atmosphere of 95% air/5% carbon dioxide at 37^{o}C. The bicarbonate-buffered media used equilibrate at pH 7.4 under such conditions.

Earle's balanced salts solution (EBS, pH 7.4,) consisted of 116 mM sodium chloride, 26.2 mM sodium bicarbonate, 5 mM glucose, 5mM potassium chloride, 1 mM calcium chloride, 1 mM sodium dihydrogen orthophosphate, 0.8 mM magnesium sulphate and 10 μg/ml phenol red.

2.5.2. Astroglial Cultures: Cerebellum And Cerebral Cortex

These cultures were established from 2 day old rats according to the procedure of Cholewinski *et al.* (1988), with the modification of Cholewinski *et al.* (1989) to reduce the proliferation of fibroblasts.

Cerebral cortices and cerebella from 4 animals were stripped of meninges, pooled separately and then chopped twice at right angles on a McIlwain Tissue Chopper (0.4 mm blade advance). Tissue was then suspended in 5 ml EBS containing (w:v) 0.025% trypsin (type III from bovine pancreas), 0.3% bovine serum albumin (BSA, fraction V) and 0.002% deoxyribonuclease I (DNase) by 5 strokes through a sterile Pasteur pipette and digested at 37^{o}C for 15 minutes with shaking. The enzymatic activity of the trypsin was quenched by addition of an equal volume of DMEM/10% FCS and the tissue was collected by centrifugation at 600*g* for 5 minutes. The cells were dissociated in 10 ml EBS containing (w:v) 0.3% BSA and 0.002% DNase by trituration through a Pasteur pipette (20 strokes). Tissue clumps were allowed to settle for 10 minutes and the cell suspension was collected and centrifuged at 600*g* for 5 minutes. Cells were then gently resuspended in a known volume of DMEM/10% FCS and counted using a haemocytometer.

For immunofluorescence studies, cells were plated at a concentration of $2x10^5$ onto 13 mm diameter glass coverslips (previously autoclaved and coated with 5 μg/ml poly-L-lysine) in sterile 24-well multiwell plates. After the medium was replaced with fresh DMEM/10% FCS on day 3, it was changed at day 5 to modified DMEM (as described above) containing 10% FCS to reduce fibroblast contamination (Cholewinski *et al.*, 1989).

2.5.3. Astroglial Cultures: Optic Nerve

Cultures were established from 2 day old rat optic nerves by a modification of the procedure of Raff *et al.* (1983a). Aseptic conditions were used and media were supplemented with 0.5% gentamicin. Optic nerves from 40 animals were finely chopped with scissors, then suspended in 5 ml of EBS containing (w:v) 0.025% trypsin, 0.3% BSA, 0.002% DNase and shaken at 37^{o}C for 20 minutes. An equal volume of DMEM/10% FCS was then added and the tissue was triturated by 30 strokes

through a Pasteur pipette. After centrifugation at 600*g* for 5 minutes the cells were resuspended in DMEM/10% FCS, then counted in a haemocytometer and plated in multiwell plates at a density of $5x10^5$ per well in aliquots of 50 μl directly onto the middle of poly-L-lysine coated coverslips. After allowing the cells to adhere for 1 hour in the incubator the wells were each flooded with a further 1 ml of medium. Medium was replaced with fresh DMEM/10% FCS on day 3 and changed at day 5 to modified DMEM/10% FCS to reduce fibroblast contamination.

2.5.4. Schwann Cell Cultures

Tissue culture media were supplemented with 0.5% gentamicin. Schwann cells were purified by a modification of the method of Brockes *et al.* (1979). Briefly, sciatic nerves were removed from one day old rat pups and the epineurial sheaths were removed. The nerves were finely chopped with scissors in a minimal volume of calcium- and magnesium-free DMEM containing (w:v) 0.2% collagenase and 0.125% trypsin, then digested at 37^oC for 30 minutes and dissociated by trituration through a 0.2 ml Gilson pipette tip. Cells were recovered by centrifugation at 600*g* for 5 minutes and gently resuspended in DMEM/10% FCS. Cells from eight nerves were seeded in 25 cm^2 tissue culture flasks and cytosine arabinoside (10^{-5}M) was added to kill rapidly dividing fibroblasts. After three days, purified Schwann cells were passaged with 0.05% (w:v) trypsin in EBS then resuspended in DMEM/10% FCS and counted in a haemocytometer. 10^5 cells were plated in 50 μl aliquots on coverslips which were flooded with a further 1 ml of medium after 1 hour as described.

2.5.5. Purification of O-2A Progenitor Cells

Culture media were supplemented with 200 units/ml penicillin and 200 μg/ml streptomycin. Primary mixed glial cultures were established from cerebral cortices of newborn pups (< 12 hours old) by

coarse chopping with a razor blade followed by mechanical dissociation in 5 ml DMEM/10% FCS with 30 strokes through a 10 ml pipette. Clumps were allowed to settle out and the cell suspension was recovered. Another 5 ml of medium was added and the clumps were triturated again. Cell suspensions were pooled and centrifuged at 600*g* for 5 minutes. The pelleted cells were gently resuspended in a known volume of DMEM/10% FCS, counted in a haemocytometer and then seeded at $5x10^4/cm^2$ in 150 cm^2 tissue culture flasks coated with 5 μg/ml poly-L-lysine. Medium was replaced every 3-4 days.

After 12 days O-2A progenitors were separated from the underlying monolayer of type-1 astrocytes and fibroblasts by a modification of the shaking procedure of McCarthy and DeVellis (1980). Flasks were subjected to preliminary agitation at 37^{o}C on an orbital shaker (2.5 cm throw) for 30 minutes at 150 rpm, to remove dead cells, then the medium was replaced with DMEM/15% FCS and the flasks were returned to the incubator to equilibrate for 1 hour. The flasks were then shaken at 250 rpm for 18 hours and the cell suspension was collected and filtered through 70 μm and 25 μm sterile nylon gauze. Microglia were allowed to adhere to untreated 150 cm^2 tissue culture flasks for 30 minutes at 37^{o}C. Floating cells were recovered by centrifugation at 600*g* for 5 minutes and oligodendrocytes were removed by incubation for 1 hour in EBS containing 0.3% BSA, 0.004% DNase (w:v), 10% (v:v) normal rabbit serum as a source of complement and monoclonal antibody against galactocerebroside (GC hybridoma supernatant diluted 1/20, Ranscht *et al.*, 1982) at 37^{o}C with agitation. Following centrifugation at 600*g* for 5 minutes, purified O-2A progenitors were resuspended in a known volume of DMEM/10% FCS, counted in a haemocytometer and replated on coverslips at $2.5x10^4$/well for immunocytochemistry (as described in Section 2.5.2.) or at 10^6/35 mm diameter Petri dish for preparation of membranes.

2.5.5.1. Enriched Type-1 Astrocyte Cultures

After removal of O-2A progenitors by overnight shaking, flasks containing monolayers of type-1 astrocytes and fibroblasts were incubated at 37^{o}C with 10 ml 0.05% trypsin in EBS (w:v) for 5 minutes. 10 ml DMEM/10% FCS were added and sheets of cells which had become dislodged were dissociated by trituration through a 19 gauge hypodermic needle. Following centrifugation at 600*g* for 5 minutes, cells were resuspended in a known volume of DMEM/10% FCS, counted and then replated at 5x10^{4}/well for immunocytochemistry as described in Section 2.5.2. Medium was replaced with fresh DMEM/10% FCS every 3-4 days.

2.5.5.2. Enriched Type-2 Astrocyte Cultures

O-2A progenitor cells which were maintained in DMEM/10% FCS differentiated into enriched type-2 astrocytes. Medium was changed every 5 days.

2.5.5.3. Enriched Oligodendrocyte Cultures

In serum-free medium O-2A progenitors differentiate into oligodendrocytes (Raff *et al.*, 1983b, 1984a). 24 hours after replating the enriched populations of O-2A progenitors, the medium was changed to a defined serum-free medium consisting of equal volumes of DMEM and Ham's F12 supplemented with insulin (10 μg/ml), transferrin (50 μg/ml), triiodothyronine (15 nM) and sodium selenite (30 nM). The medium was not changed thereafter.

2.6. Electrophoretic Protein Analysis

Proteins were separated according to RMM by SDS-PAGE essentially as described by Laemmli (1970) and transferred to nitrocellulose paper (Towbin *et al.*, 1979) for immunostaining.

2.6.1. Sample Preparation

PAGE sample buffer consisted of 62.5 mM Tris/HCl (pH 6.8) with 2% SDS (w:v), 5% ß-mercaptoethanol (v:v), 10% glycerol (v:v), 0.003% bromophenol blue (w:v). Samples for electrophoresis were boiled in this for 10 minutes (to promote the denaturation of proteins to their component polypeptide chains and the solubilisation of proteins by SDS binding) followed by centrifugation for 10 minutes in a microfuge to precipitate insoluble material.

2.6.1.1. Pure GAP-43

Pure GAP-43 (prepared as described in Section 2.3.) was dissolved directly by boiling in sample buffer at a concentration of 1 mg GAP-43/ml.

2.6.1.2. Tissue Homogenates

Dissected tissues from central and peripheral nervous system (obtained as described in Section 2.7.1.) were homogenised in 5 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 2% SDS (w:v) (pH 6.8) with 2 μg/ml leupeptin and 1 mM PMSF to inhibit proteases. Hand-held glass homogenisers were used, which were periodically cooled on ice or cardice to further limit protein degradation. The homogenate was boiled for 10 minutes to aid protein solubilisation and centrifuged to pellet insoluble debris and then the supernatant was collected. 1 μl was removed and diluted to 1 ml (to prevent interference from SDS) before being subjected to protein assay using a Bio-Rad kit (as described). The remainder was made up with an equal volume of double strength sample buffer and reboiled for 10 minutes.

2.6.1.3. Purified Cell Membranes

Cells maintained in tissue culture (Section 2.5.) were removed from the substrate by shaking or trypsinisation (0.05% in EBS) at 37^{o}C. Proteolytic activity was quenched with DMEM/10% FCS and the cells were washed 3 times in this medium to remove all traces of trypsin prior to cell lysis. All cells were resuspended in chilled isotonic sucrose (0.32 M) buffered with 10 mM HEPES (pH 7.5) and containing 2 μg/ml leupeptin and 1 mM PMSF.

Cells were sheared through a pre-cooled ball-bearing cell disrupter with a clearance of 12 μm (Balch *et al.*, 1984) and centrifuged at 5000*g* for 5 minutes. The post-nuclear supernatant was then centrifuged in a Beckman L8-55 ultracentrifuge at 100,000*g* (SW60Ti rotor) at 4^{o}C for 1 hour to pellet the membranes.

Membranes were either solubilised directly in sample buffer or solubilised in 5 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 2% SDS (w:v) (pH 6.8) with protease inhibitors as above followed by addition of an equal volume of double strength sample buffer. The second method allowed a 1 μl sample to be taken and diluted to 1 ml for protein assay (Bio-Rad) before addition of bromophenol blue and mercaptoethanol, which interfere with the protein assay (W. Whitfield, personal communication).

a) O-2A Progenitor Membranes

Freshly purified cells were resuspended directly in isotonic sucrose as stated and sheared.

b) Enriched Type-1 Astrocyte Membranes

Monolayers remaining after overnight shaking to remove O-2A progenitors, as described, were trypsinised and washed before resuspension in isotonic sucrose and shearing.

c) Enriched Type-2 Astrocyte Membranes

5 days after isolation (DAI), type-2 astrocytes maintained in DMEM/10% FCS were trypsinised and washed before shearing in isotonic sucrose.

d) Schwann Cell Membranes

Schwann cells were grown in flasks with 10^{-5} M cytosine arabinoside as described (Section 2.5.4.) for 3 days. After trypsinisation, the cells were washed and resuspended in isotonic sucrose and then sheared, as above.

2.6.1.4. *E. coli* Lysate

Transformed cells grown in L-broth were collected by centrifugation at 600*g* for 10 minutes. Cells were resuspended in PBS, centrifuged again and resuspended in distilled water, followed by addition of an equal volume of double strength sample buffer. Sonication (150 watts, probe tip 9.5 mm diameter) for 20 seconds at $4^{o}C$ was used to disrupt the cells and fragment the bacterial DNA prior to boiling.

2.6.2. SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated according to RMM by discontinuous buffer electrophoresis through 10% acrylamide resolving gels (Laemmli, 1970; Hames and Rickwood, 1981). 375 mM Tris/HCl (pH 8.8) containing (w:v) 10% acrylamide, 0.267% bisacrylamide and 0.1% SDS was polymerised with 0.075% ammonium persulphate (catalyst) and 0.05% TEMED (initiator). Stacking gels consisted of 3% acrylamide, 0.08% bisacrylamide and 0.1% SDS buffered with 125 mM Tris/HCl (pH 6.8), polymerised as above.

Gels for the separation of *E. coli* lysates were identical except that the composition of the resolving gel was reduced to 7.5% acrylamide and 0.2% bisacrylamide.

2.6.2.1. Analytical Gels

The electrophoresis system used was a minigel apparatus purchased form Hoeffer. 6 x 8 cm resolving gels were cast at a thickness of 0.75 mm and overlaid with 0.1% SDS. After 30 minutes polymerisation the overlay was poured off and stacking gels were cast using the 10-well gel combs supplied. Each well held up to 20 μl sample, loaded using a Hamilton syringe. Samples were stacked at 150 volts and resolved at 250 volts in the water-cooled electrophoretic cell (Hoeffer). Upper and lower chambers were filled with reservoir buffer (50 mM Tris, 384 mM glycine, 0.1% SDS, pH 8.3) according to Chan *et al.* (1986).

Prestained and unstained protein markers (RMM 180-26 kD, Sigma) were used on 10% resolving gels. For the 7.5% gels used to separate *E. coli* lysates, unstained protein markers (RMM 200-45 kD, Bio-Rad) were used.

2.6.2.2. Preparative Gels

A maxigel system was used for the isolation of the GAP-43 fusion protein (Protean I, Bio-Rad). 12 x 14 cm resolving gels containing 6% acrylamide and 0.16% bisacrylamide were poured at a thickness of 3 mm as described. Stacking gels were cast without a comb, leaving a 1 cm space at the top, and the gels were transferred to the water-cooled electrophoretic cell. 3 ml *E. coli* lysate in PAGE sample buffer was quickly loaded with a Gilson pipette and 150 volt current was applied immediately. Samples were stacked and resolved at this voltage for 4 hours.

2.6.3. Gel Staining

Proteins in the matrix of the gel after electrophoresis retain the ability to bind silver ions and some dyes and this property forms the basis of protocols for the localisation of total proteins after electrophoresis.

2.6.3.1. Coomassie Blue Staining

Gels were soaked in 0.1% Coomassie blue G250 in distilled water:methanol:acetic acid (2:2:1, v:v) for 1 hour. Excess dye was removed in (v:v) 12.5% isopropanol, 10% acetic acid to the desired intensity.

2.6.3.2. Silver Staining

This technique, which is over 1000 times more sensitive than dye-based stains, is essentially the method of Merril *et al.* (1981) with the modifications of Thompson (1987). Briefly, gels were fixed in (v:v) 50% methanol, 12% acetic acid for 30 minutes and soaked in 3 changes of (v:v) 10% ethanol, 5% acetic acid (10 minutes each) to remove bound SDS from the proteins which were then oxidised in 3 mM potassium dichromate, 3 mM nitric acid for 5 minutes. After rinsing in 4 changes of distilled water (1 minute each), gels were incubated with 12 mM silver nitrate for 30 minutes on an illuminated light box as a source of uniform intensity light. All the above procedures were performed on an orbital shaker. Proteins were revealed by developing the bound silver ions in 280 mM sodium carbonate, 0.02% (w:v) formalin and the reaction was stopped at the desired intensity of staining with 1% acetic acid (v:v).

2.6.4. Western Blotting

GAP-43 was identified in separated gel samples by electrophoretic transfer of proteins to nitrocellulose (Towbin *et al.*, 1979) followed by staining with antibodies against GAP-43.

2.6.4.1. Electrophoretic Transfer

Proteins were transferred onto nitrocellulose paper (0.2μm pore, Sartorius) in 25 mM Tris, 192 mM glycine, 20% (v:v) methanol (pH 8.3) using a horizontal semi-dry blotting apparatus according to the manufacturers instructions (Bio-Rad) and the modifications of Jacobson

and Kårsnäs,(1990). Current was applied at a constant rate of 3 amp/cm^2 of gel for 20 minutes.

2.6.4.2. Immunostaining

The nitrocellulose blot was incubated in blocking buffer (10% FCS (v:v), 0.9% sodium chloride (w:v) in 25 mM Tris/HCl, pH 7.5) for 1 hour to prevent non-specific binding of immunoglobulins to unoccupied protein binding sites. Anti-GAP-43 fusion protein antiserum was diluted 1/1000 in blocking buffer and the blot incubated overnight with shaking at 4^oC. The blot was then washed in 10 mM Tris/HCl (pH 7.5), 0.9% (w:v) sodium chloride for 1 hour (at least 6 changes) and incubated with horseradish peroxidase-conjugated swine anti-rabbit IgG (DAKO) diluted 1/200 in the same buffer for 3-5 hours at 4^oC with shaking. After washing as above, immunoreactive bands were visualised by addition of 0.0015% (v:v) hydrogen peroxide as substrate and 0.03% (w:v) chloronaphthol as chromogen. This reaction was terminated by washing the blot in tap water. Preimmune serum at the same concentration served as a negative control and ß-galactosidase in the RMM standards served as a positive control for the transfer and visualisation of immunoreactive protein.

2.6.4.3. Elution of Bound Immunoglobulins

Antisera can be affinity-purified by removing bound primary immunoglobulins from individual protein bands on blots (Talian *et al.*, 1983) instead of visualisation with secondary antibodies. After overnight incubation with the anti-GAP-43 antiserum and washing, horizontal strips of blots containing the proteins of interest were incubated with 3 ml 0.2 M glycine (buffered to pH 2.8 with HCl) for 5 minutes with agitation. The blot strips were then removed and the eluted antibodies were neutralised to pH 7.4 with 1 M sodium hydroxide. Eluted antibodies were then used to stain other blots (as above).

2.7. Tissue Harvesting

All animals were killed under pentabarbitone sodium anaesthesia (Sagatal, May and Baker, 60 mg/kg body weight injected intraperitoneally).

2.7.1. Fresh Tissue

The animals were exsanguinated by cardiac puncture and the central and peripheral nervous tissue was quickly dissected.

2.7.1.1. Frozen Sections

Tissue was placed on cork blocks and covered with mounting medium (Tissue Tek, Miles), then snap frozen by immersion in isopentane cooled on cardice. 5 μm sections were cut at -18^{O}C on a Cryocut E cryostat (Reichert Jung) and thaw-mounted onto slides previously coated with (w:v) 0.5% gelatin, 0.05% chrome alum. After air drying for 2 hours the sections were processed further by fixation on the slide with 4% (w:v) paraformaldehyde in PBS or 5% (v:v) acetic acid in ethanol. Alternatively sections could be stored desiccated at -20^{O}C once dried.

2.7.1.2. Teased Nerve Preparations

After dissection tissue was stored briefly in EBS with 25 mM HEPES (ph 7.4). Normal and lesioned peripheral nerves were desheathed and split along the long axis into manageable strands. The nerve fibres were then gently teased apart in a pool of PBS with 23 gauge hypodermic needles on gelatin-coated slides as previously described (Jessen and Mirsky, 1984). The teased nerves were allowed to air dry for 2 hours before immunostaining or overnight storage desiccated at 4^{O}C.

2.7.2. Perfusion Fixed Tissue

Animals were perfused transcardially with PBS at 37^{O}C, to remove blood from the vasculature, followed by 4% paraformaldehyde in PBS

(w:v). Tissue was carefully dissected out and postfixed in the same fixative for 4 hours or overnight.

2.7.2.1. Frozen Sections

Tissue was trimmed into blocks 5 mm thick and equilibrated in 30% (w:v) sucrose in PBS. Subsequent processing and sectioning was as for fresh tissue (see Section 2.7.1.1.) except that the temperature of the cryostat was -25^{o}C. Sections were either allowed to air dry or kept in a humidified atmosphere during slide adhesion (2 hours). This latter procedure was found to be necessary for preservation of GC and ganglioside G_{D3} in tissue sections (Curtis *et al.*, 1988).

2.7.2.2. Wax Embedded Sections

After overnight fixation above, tissue samples were dehydrated through a graded ethanol series (50%, 70%, 90% ethanol (v:v) in distilled water and 3 changes of absolute ethanol, 45 minutes each). Tissue was then infiltrated at 40^{o}C with polyester wax by immersion in a graded series (50%, 75% wax (v:v) in absolute ethanol and 3 changes of neat wax, 1 hour each) and allowed to set at room temperature. The block was trimmed and 5 μm sections were cut at 4^{o}C on a rotary microtome.

Sections were mounted at 4^{o}C on slides coated with 1 mg/ml poly-L-lysine (Huang *et al.*, 1983). After air drying at room temperature for 30 minutes the sections were baked at 30^{o}C to aid adherence to the slides, and could then be stored at room temperature. Prior to further processing the sections were dewaxed (2 changes of absolute ethanol, 10 minutes each) and rehydrated in a graded ethanol series (90%, 70%, 50% (v:v) ethanol in distilled water, 2 minutes each) and rinsed in several changes of distilled water.

2.8. Histochemical Stains

2.8.1. Toluidine Blue Staining

Sections were incubated in (w:v) 0.1% toluidine blue (R.A. Lamb), 1% sodium tetraborate for 5 minutes and rinsed in tap water.

2.8.2. Silver Staining For Axons

Many histological procedures are based on the affinity of neurons for silver ions ('argentaffinity' stains), the chemical basis for which is not yet fully understood. It is thought that a proteinaceous component of the axoplasm causes the reduction of silver ions to 'nuclei' of metallic silver (Kiernan, 1981). The method used here is that described by Kiernan (1981) including gold enhancement. Slides were washed 3 times in distilled water between changes of chemicals.

Sections were immersed in 20% (w:v) silver nitrate for 30 minutes in darkness to allow reduction of silver ions at axoplasmic sites of argentaffinity. Chemically bound but unreduced silver ions ('argyrophilic' binding) were removed by 5 minutes in 3% (w:v) sodium sulphite. The nuclei of metallic silver were expanded by autocatalytic deposition of silver metal in a 'physical developer' solution (w:v, 2% silver nitrate, 0.8% citric acid, 0.8% gelatin, 0.8% hydroquinone) until black reaction product could be seen (2-3 minutes).

For gold toning sections were immersed sequentially for 5 minutes each in (w:v) 0.2% sodium chloroaurate, 1% oxalic acid and finally 5% sodium thiosulphate. These procedures deposit gold atoms on the silver metal, so increasing the staining contrast by changing the colour of the metal particles and decreasing the background argyrophilic stain (Kiernan, 1981). Slides were then rinsed in tap water prior to dehydration through graded ethanols, clearing in xylene and mounting in Ralmount (R.A. Lamb).

2.9. Immunofluorescence

Indirect immunofluorescence (Cuello, 1983) was used to identify and localise antigens at the light microscope level by the binding of monospecific mouse monoclonal or rabbit polyclonal primary antibodies. These were subsequently visualised with secondary antibodies specific for immunoglobulins of these two species which were conjugated to fluorescent molecules. Two fluorochromes were used which emitted light at different wavelengths (FITC 525 nm, TRITC 570 nm). When conjugated to secondary antibodies with different specificities, the use of these two fluorochromes together allowed localisation of two different antigens in cultured cells or tissue sections and comparison of their distributions (Vandesande, 1983).

2.9.1. Antibodies

Many different antigens were studied in the course of these investigations, often in double labelling experiments with other antigens. For the sake of clarity, each antigen is listed here with information on the source and dilution of primary and secondary antibodies. These antigens are categorised by the nature of the primary antibodies as most double labelling experiments involved the use of one polyclonal rabbit primary and one mouse monoclonal antibody.

2.9.1.1. Rabbit Polyclonal Primary Antisera

All the following antisera were visualised with TRITC-conjugated goat anti-rabbit IgG (Cappel) diluted 1/500.

a) Rabbit Anti-GAP-43

The antiserum raised against the GAP-43/ß-galactosidase fusion protein described earlier (Section 2.2.2.) was used at dilutions from 1/5,000 to 1/50,000, except for use on cultured cells (see Section 2.9.4.).

b) Rabbit Anti-GFAP

This antiserum was obtained from DAKO and was diluted 1/500.

c) Rabbit Anti-Von Willebrand's Factor

Antiserum was obtained from DAKO and used at 1/200. Paraformaldehyde fixation resulted in loss of antigenicity but this was restored by proteolysis, as described by the manufacturers. 25 mM Tris/HCl, 5 mM EDTA, 0.9% sodium chloride (pH 7.4) containing 0.05% (w:v) trypsin was applied for 5 minutes (at room temperature to reduce enzymatic damage to the tissue sections) before incubation in the primary antiserum.

d) Rabbit Anti-CNP

Antiserum was raised by Raible and McMorris (1989) and was a kind gift from these authors. It was diluted 1/300.

e) Rabbit Anti-MBP

Antiserum was donated by Dr. R. Reynolds (Reynolds *et al.*, 1989) and was used at 1/500.

f) Rabbit Anti-S100

Antiserum was obtained from DAKO and used at a dilution of 1/200.

2.9.1.2. Mouse Monoclonal Primary Antibodies

FITC-conjugated secondary antisera specific for individual subclasses of mouse immunoglobulins were used to label the following monoclonal antibodies.

a) Mouse Anti-Neurofilament

RT97 monoclonal antibody, which is specific for the phosphorylated form of the high RMM neurofilament protein (210 kD, Anderton *et al.*, 1982), was a gift from Dr. J. Wood. Ascites fluid was diluted 1/2000 and visualised with goat anti-mouse IgG1 (Nordic) diluted 1/100.

b) Mouse Anti-MBP

Monoclonal antibody against human MBP was a gift from Dr. R. Thompson (Elfman *et al.*, 1986) as ascites fluid, which was diluted 1/10,000 and visualised with anti-mouse IgG1, as above.

c) Mouse Anti-Vimentin

This antibody was purchased as tissue culture supernatant from DAKO and diluted 1/15 for use. It was also of the IgG1 subclass and was visualised as described above.

d) Mouse Anti-GFAP

This antibody was obtained from Boehringer Mannheim as lyophilised IgG1 immunoglobulins. It was used at a concentration of 0.8 μg/ml and visualised with goat anti-mouse IgG1, described above, or with biotin-conjugated goat anti-mouse immunoglobulins followed by FITC-streptavidin. The biotin and streptavidin reagents were purchased from Serotec and applied at a dilution of 1/1000 for 1 hour each, separated by 3 washes in PBS (5 minutes).

e) Mouse Anti-GAP-43

91E12 monoclonal antibody was a kind gift from Dr. D. Schreyer (Goslin *et al.*, 1988), supplied as ascites fluid. Final working dilution was 1/3000 and staining was visualised with the biotin and streptavidin reagents described above.

f) Mouse Anti-G_{D3} Ganglioside

The LB_1 hybridoma cell line was donated to our laboratory by Dr. J. Cohen and shown to be specific for the ganglioside G_{D3} by thin layer chromatography (Curtis *et al.*, 1988). Ascites fluid was diluted 1/500 before application to perfusion-fixed frozen tissue sections which had been kept moist during slide adhesion, as described above. LB_1 was visualised with goat anti-mouse IgM (Cappel) diluted 1/200.

g) Mouse Anti-GC

Hybridoma cell line was a gift from Dr. B. Ranscht (Ranscht *et al.*, 1982). Cells were grown to confluence and supernatant was diluted 1/10 for application to perfusion-fixed frozen tissue sections which had been kept moist during slide adhesion. Bound antibody was visualised with goat anti-mouse IgG3 (Nordic) diluted 1/150.

h) ED1 Mouse Monoclonal Antibody

This antibody, which recognises an uncharacterised antigen on macrophages (Dijkstra *et al.*, 1985), was purchased as ascites fluid from Serotec and used at 1/500. It was also of the IgG1 subclass and was visualised with the biotin and streptavidin reagents described above.

i) Mouse Anti-NGFr

192-IgG monoclonal antibody (Taniuchi *et al.*, 1986) was obtained as ascites fluid from Prof. R. Mirsky and was diluted 1/100 for use and visualised with polyvalent anti-mouse immunoglobulins (Sigma) diluted 1/200. This 'polyvalent' preparation consists of equal amounts of affinity purified antibodies against mouse IgA, IgG and IgM

2.9.2. Immunohistochemistry Of Tissue Sections

Before application of primary antibodies, sections were treated with 0.5 mg/ml sodium borohydride for 5 minutes to reduce background fluorescence, as described by Osborn and Weber (1982) and then rinsed twice with PBS. Both primary antibodies (diluted in PBS) were applied simultaneously overnight followed by PBS washes (3 x 5 minutes) and then both fluorescent secondary antibodies (diluted in PBS) were applied for 1 hour. This did not lead to cross-species interactions, as staining patterns were confirmed in sections labelled for one antigen only. Controls were performed by omission of the primary antibodies or substitution with pre-immune serum.

2.9.3. Immunohistochemistry Of Teased Nerve Preparations

Dried teased nerve preparations were rehydrated and fixed in 4% paraformaldehyde in PBS for 20 minutes and washed in PBS and then permeabilised in methanol at -20^{o}C for 2 minutes. Sodium borohydride treatment was not required after brief fixation. After PBS washes the teased nerves were stained as described for tissue sections.

2.9.4. Immunocytochemistry Of Cultured Cells

Indirect fluorescent immunocytochemistry (Schachner, 1983) was used to identify antigens in cells maintained in tissue culture on 13 mm diameter glass coverslips and to localise these antigens to specific cellular compartments. Many of the antibodies listed above were applied to cultured cells, although the methodology differed from the staining of tissue sections. Unless otherwise noted the primary and secondary antibodies were used as described in Sections 2.9.1.1. and 2.9.1.2., but in some instances dilutions of antibodies were altered.

2.9.4.1. Cell Surface Antigens

GC and ganglioside G_{D3} were immunolabelled on living cells, therefore primary and secondary antibodies were diluted in EBS containing 5% (v:v) normal goat serum and 0.3% (w:v) BSA. All primary and secondary antibody incubations were of 20 minutes duration, followed by 3 washes (2 minutes each) in EBS/0.3% BSA. Cells were then fixed with 4% paraformaldehyde (w:v, in PBS) for 20 minutes.

To preserve NGFr antigenicity, cells were lightly fixed (with 2% paraformaldehyde (w:v, in PBS) for 5 minutes), washed in PBS and then incubated with primary antibodies diluted in PBS for 30 minutes. Following PBS washing, secondary antibodies were applied for 30 minutes diluted in PBS. Cells were then fixed again with 4% paraformaldehyde (w:v, in PBS) for 20 minutes.

2.9.4.2. Intracellular Antigens

Permeabilisation of cell membranes is necessary to visualise antigens having no extracellular domains, including GAP-43. Permeabilisation was achieved with methanol at -20^{o}C for 2 minutes, after fixation with 4% paraformaldehyde. Cells were subsequently washed in PBS and immunostained as follows. Primary antibodies were applied for 30 minutes followed by washing in PBS (3 x 2 minutes) and then

secondary antibodies were applied for 30 minutes.

2.9.4.2.1. Double Labelling With A Surface Antigen

Following surface staining, the cells were fixed and permeabilised. Primary antibodies against a single intracellular antigen were then applied followed by the appropriate secondary antibodies, diluted in PBS. Anti-GAP-43 antiserum was diluted 1/1000 for use on cells.

2.9.4.2.2. Simultaneous Labelling Of Two Intracellular Antigens

Cells were immediately fixed and permeabilised as described. Primary antibodies against two intracellular antigens (one mouse monoclonal and one rabbit polyclonal antiserum) were applied together diluted in PBS. Anti-GAP-43 antiserum was diluted 1/1000. After washing in PBS both secondary antibodies were applied together. Biotin and streptavidin reagents were not necessary to visualise mouse monoclonal antibodies against either GFAP or GAP-43, so goat anti-mouse IgG1 was used diluted 1/100.

2.9.5. Fluorescence Microscopy

On completion of staining cells and tissue sections were mounted in a drop of glycerol:PBS (9:1, v:v), containing 2.5% DABCO (w:v) to prevent fading of the fluorochromes, and sealed with clear nail varnish. Slides were viewed on a Reichert Jung Polyvar microscope fitted with phase contrast and epifluorescence optics. Filter sets were appropriate for the visualisation of FITC and TRITC with minimal fluorescent overlap or breakthrough.

Phase contrast and fluorescent images were photographed on Ilford HP5 Plus black and white film (400 ASA). Negatives were printed on Kentmere Multigrade paper and developed using Ilfospeed 2000 chemicals (Ilford).

Chapter Three

Characterisation of GAP-43
and Anti-GAP-43 Fusion Protein Antiserum

3.1. Introduction

GAP-43 was selected as a marker to identify regenerating axons in the CNS for the following reasons;

a) GAP-43 is expressed by neurons extending axons in development, during the regeneration of PNS and inframammalian CNS, and in tissue culture (Skene and Willard, 1981a, b; Verhaagen *et al.*, 1986; Goslin *et al.*, 1988; Meiri *et al.*, 1988). It seemed reasonable to assume that neurons of the mammalian CNS would also express GAP-43 if they were capable of regeneration. In recent studies by Benowitz *et al.* (1990), collateral sprouting in the CNS has been shown to be accompanied by increased expression of GAP-43.

b) GAP-43 is downregulated in the adult CNS (Jacobson *et al.*, 1986) and previous reports in the literature had suggested that immunoreactive GAP-43 has only a limited distribution in the adult brain and spinal cord (see Chapter 4 for a detailed description and references). GAP-43 is upregulated again in adult neurons undergoing regeneration (Skene and Willard, 1981a, b) and axons regenerating through the PNS can be visualised by immunohistochemistry (Verhaagen *et al.*, 1986). It also seemed reasonable to assume that regenerating CNS neurons expressing elevated levels of GAP-43 would be distinguishable against the low background levels expected in the adult.

c) GAP-43 is present in growth cones (Meiri *et al.*, 1986; Skene *et al.*, 1986) and becomes incorporated in the membrane of the axon shaft and thus marks regenerated axons along their entire length (Verhaagen *et al.*, 1986; Meiri *et al.*, 1988).

As no antibodies to GAP-43 were commercially available it was decided to raise an antiserum against purified GAP-43. Unfortunately GAP-43 yields were very small and the purified protein did not prove sufficiently immunogenic in small quantities to generate usable antiserum. In 1987, several groups reported the cloning and sequencing of the GAP-43 gene and Dr. D.V. Goeddel of Genentech was kind enough to

provide an aliquot of cloned GAP-43 cDNA in the pGEM3 plasmid. With the help of Dr. B.A. Spruce in our Department, this was used to synthesise a chimeric protein of GAP-43 and ß-galactosidase (described in Materials and Methods) as an immunogen to raise antisera in rabbits.

This Chapter describes the characterisation of the antiserum against the GAP-43/ß-galactosidase fusion protein by Western blotting of purified GAP-43 and crude brain extracts. In the course of these studies it became apparent that GAP-43, purified as described in Section 2.3., showed an unexpectedly irregular banding pattern when analysed by SDS-PAGE. Consequently, this Chapter also describes the characterisation of purified GAP-43.

3.2. Results

3.2.1. GAP-43/ß-Galactosidase Fusion Protein

Figure 3.1. illustrates stages in the production of the fusion protein. Single recognition sequences for the *Ava II* and *Ava I* restriction endonuclease enzymes exist at opposite ends of the GAP-43 cDNA sequence and these enzymes were selected to liberate a 610 bp GAP-43 fragment from the pGEM3 plasmid vector. This was confirmed by electrophoresis through 1% agarose (Figure 3.1.a). This band was subsequently cut out and the DNA was purified and ligated into the pUR 292 expression vector. To show incorporation of the GAP-43 cassette, the vector DNA was amplified by transformation into *E. coli* followed by restriction analysis using the *Eco R1* endonuclease enzyme, recognition sites for which exist either side of the 'polylinker' cloning site (Rüther and Müller-Hill, 1983), and 1% agarose electrophoresis. Of six colonies tested, four had been transformed with plasmids containing the GAP-43 cassette (Figure 3.1.b).

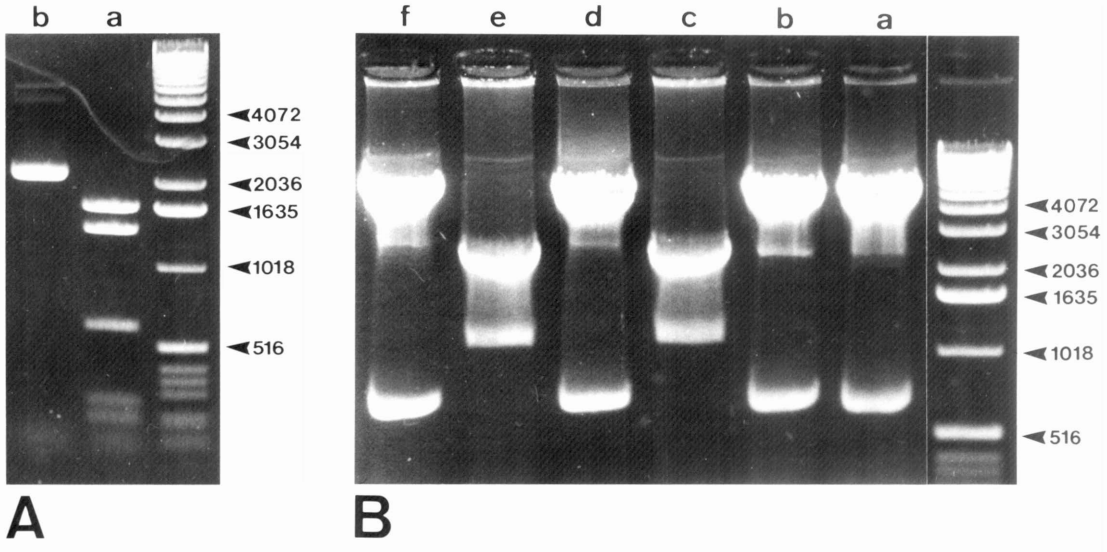
Cellular proteins from these colonies were analysed by SDS-PAGE (Figure 3.1.c). Two colonies had synthesised only ß-galactosidase, indicating that the GAP-43 cassette had been incorporated in the wrong orientation, while the other two colonies showed the presence of a chimeric fusion protein. A small amount of unfused ß-galactosidase was also present in these colonies, which is normal in cells synthesising fusion proteins (Maniatis *et al.*, 1982). Fusion protein from one of these colonies was purified and used to inoculate rabbits, as described in Materials and Methods.

Figure 3.1.

A. pGEM3 plasmid containing the GAP-43 cDNA was cut with the restriction endonucleases *Ava I* and *Ava II* (lane a) to liberate a 610 bp GAP-43 gene fragment, which was identified relative to a 1 kbp DNA ladder (Gibco-BRL). Lengths of DNA standards in bp are marked to the right. Lane b) shows uncut pGEM3 plasmid which had been incubated in the absence of restriction enzymes.

B. *Eco R1* restriction endonuclease analysis of colonies of *E. coli* transformed with the pUR 292 expression plasmid to demonstrate the presence of the GAP-43 cassette. Incorporation of the GAP-43 cassette in four colonies (lanes a, b, d, and f) is indicated by the presence of a DNA band at approximately 610 bp relative to a 1 kbp DNA ladder. Lengths of DNA standards in bp are marked to the right.

C. 7.5% acrylamide gel of cell lysates from these four colonies stained with Coomassie blue. The RMM in kD of protein standards is shown to the left and the position of ß-galactosidase is indicated. Lanes a and b show the presence of the GAP-43/ß-galactosidase fusion protein (major band above ß-galactosidase), indicating incorporation of the GAP-43 cassette in the correct orientation. Lanes c and d show synthesis only of ß-galactosidase, indicating reversed orientation of the GAP-43 cassette.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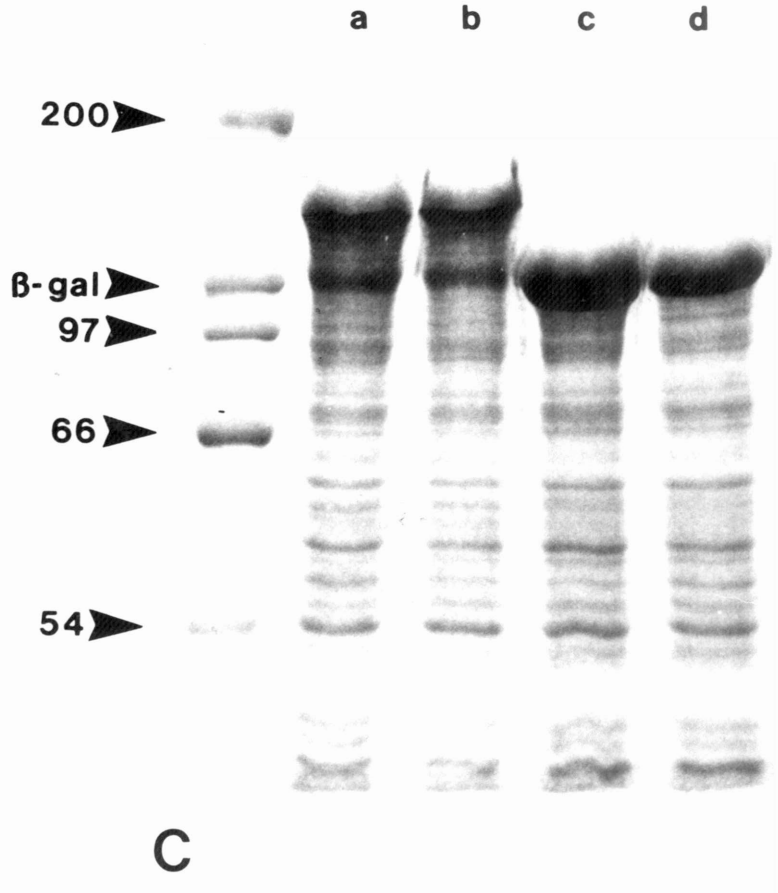
a
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200
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C

3.2.2. Characterisation Of Purified GAP-43

GAP-43 was purified from neonatal CNS as described in Materials and Methods. Analysis by SDS-PAGE of the purified GAP-43 eluted from the calmodulin-agarose column showed the presence of one or more protein bands in addition to GAP-43 (Figure 3.2.a). These bands migrated in 10% acrylamide gels in the region of ß-galactosidase, indicating a RMM in excess of 100 kD. However, the exact position of these bands varied between different gels as did the number of bands visible and their intensity. Most commonly there were two bands in this region, but occasionally only one additional band was visible and sometimes only GAP-43 was present.

It should be noted that these additional bands were only observed in early experiments. The same samples of purified GAP-43 reproducibly resolved as a single band at approximately 43 kD in later experiments (see Figure 3.2.c). This suggests that the inconsistent appearance of the additional bands was due to differences in the composition of the acrylamide gels rather than variability between different batches of GAP-43.

3.2.3. Characterisation Of Anti-GAP-43 Fusion Protein Antiserum

Serum from the inoculated rabbits were tested for recognition of purified GAP-43 in Western blots. All sera stained GAP-43 and ß-galactosidase (included in the RMM standard proteins) as expected and, more surprisingly, the additional high RMM bands were also stained in early experiments (Figure 3.2.b).

To confirm the specificity of the antiserum, Western blots prepared from homogenates of neonatal and adult CNS were stained. GAP-43 and the additional high RMM bands were both recognised in early experiments (Figure 3.2.b) but later, when GAP-43 routinely resolved as a single band, GAP-43 was the only immunoreactive protein stained in purified preparations and crude brain extracts (Figure 3.2.c).

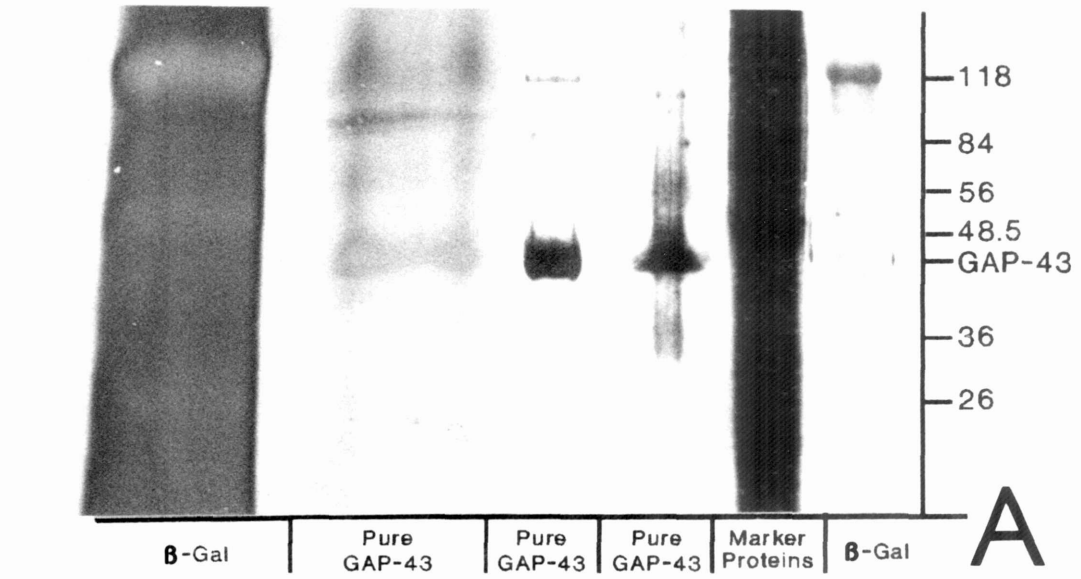
Figure 3.2.

A and B. In early experiments, GAP-43 resolved in 10% acrylamide gels as multiple bands.

A. Silver stained gels of purified GAP-43 show the presence of GAP-43 and additional bands, which are of variable RMM relative to standard proteins (RMM in kD indicated to the right). In two instances ß-galactosidase in adjacent lanes has been included as a reference for RMM. The position of ß-galactosidase in the lane to the far left is indicated by the lighter area at the top of the lane, caused by reflection of light during photography by the heavy deposition of silver due to prolonged developing of this gel. The very dark lane of marker proteins has not been removed in order to preserve the continuity of the area of gel to the right and is not intended as a guide to RMM.

B. Western blots of purified GAP-43 and tissue extracts of adult and neonatal CNS stained with anti-GAP-43 fusion protein serum and pre-immune serum diluted 1/1000. Anti-GAP-43 serum stained GAP-43 and the higher RMM bands in these preparations, and in addition stained ß-galactosidase, which was also included in the unstained protein markers in the far right lane. Pre-immune serum did not recognise GAP-43 or the higher RMM bands, nor did it stain ß-galactosidase (not shown).

C. In later experiments, purified GAP-43 resolved as a single band in silver stained gels and a single immunoreactive band was present in Western blots of purified GAP-43 and tissue extracts of neonatal and adult CNS stained with anti-GAP-43 fusion protein antiserum diluted 1/1000. Pre-stained fumarase (48.5 kD) is included in the Western blot as a reference for RMM.


118
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GAP-43
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26
β-Gal
Pure GAP-43
Pure GAP-43
Pure GAP-43
Marker Proteins
β-Gal
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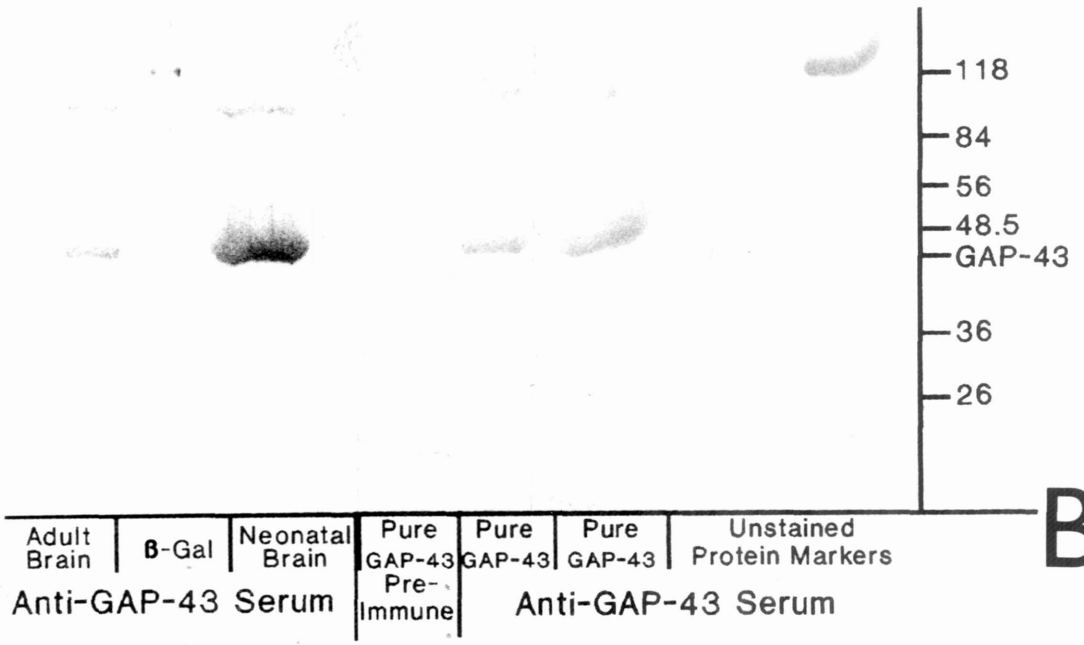
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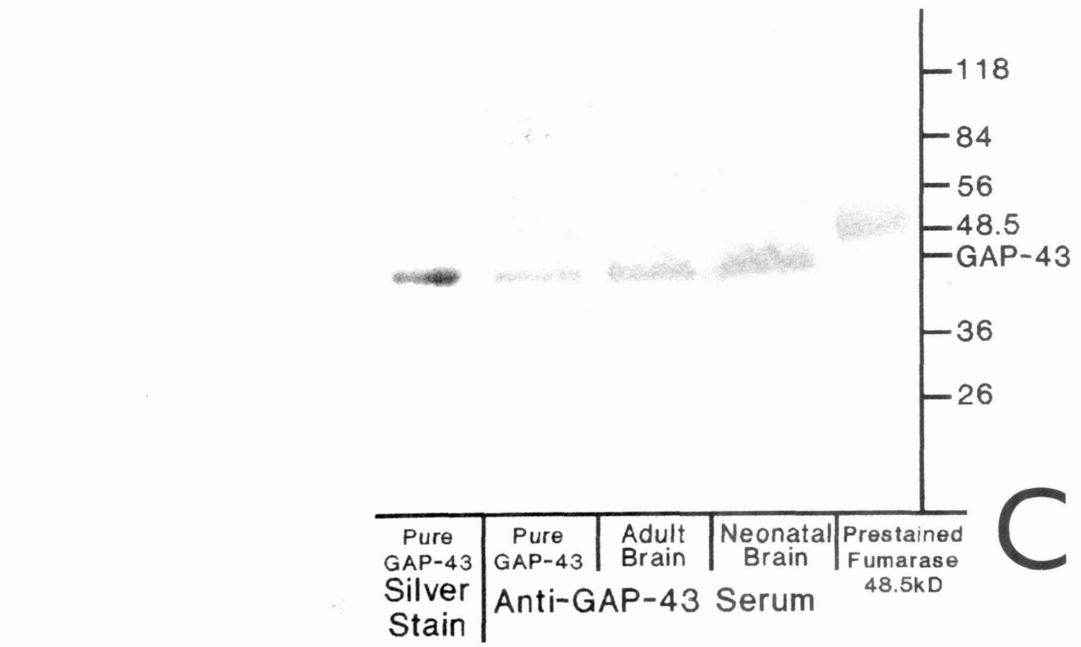
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Pure GAP-43
Adult Brain
Neonatal Brain
Prestained Fumarase 48.5kD
C
Silver Stain
Anti-GAP-43 Serum

3.2.4. Characterisation Of The High RMM Bands

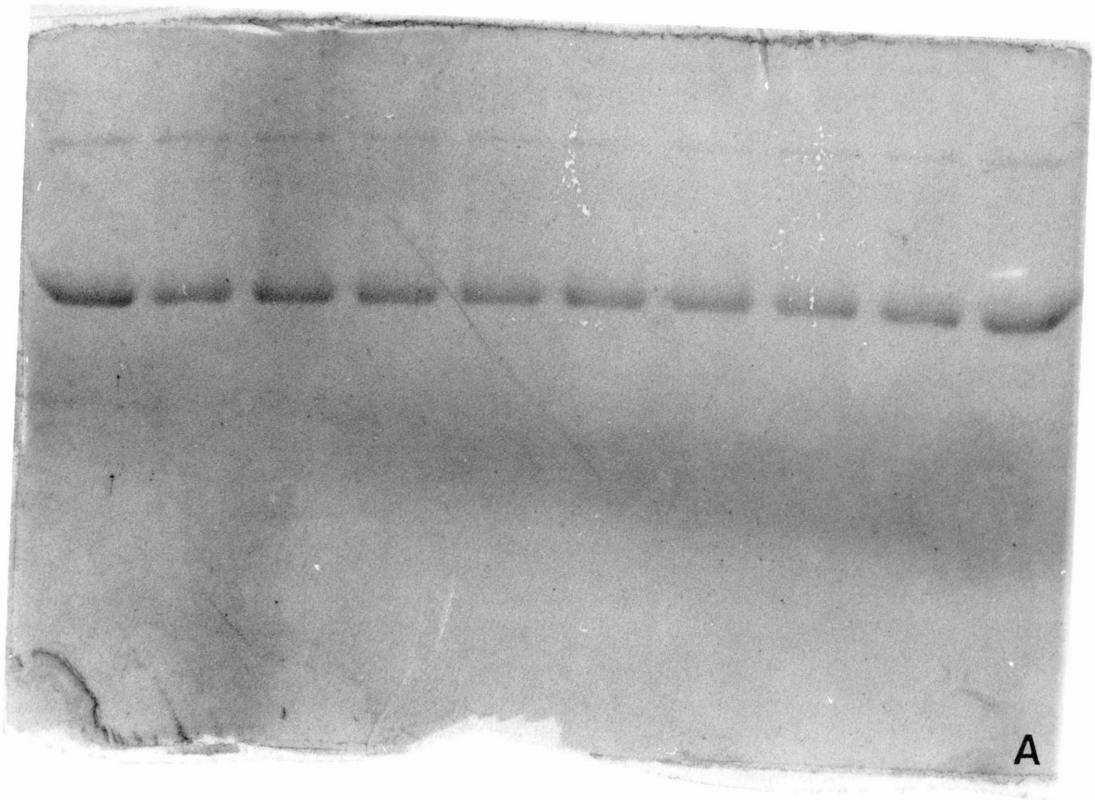
To address the nature of the additional bands, antibodies were affinity-purified separately from blot strips containing GAP-43, on the one hand, and the higher RMM proteins, on the other. Purified GAP-43 was resolved in all the wells of a gel and, after electrophoretic transfer to nitrocellulose, the residual proteins were visualised by Coomassie blue staining (see Figure 3.3.a). Strips corresponding to the two bands visualised were cut from the nitrocellulose blot and antibodies were eluted as described in Materials and Methods.

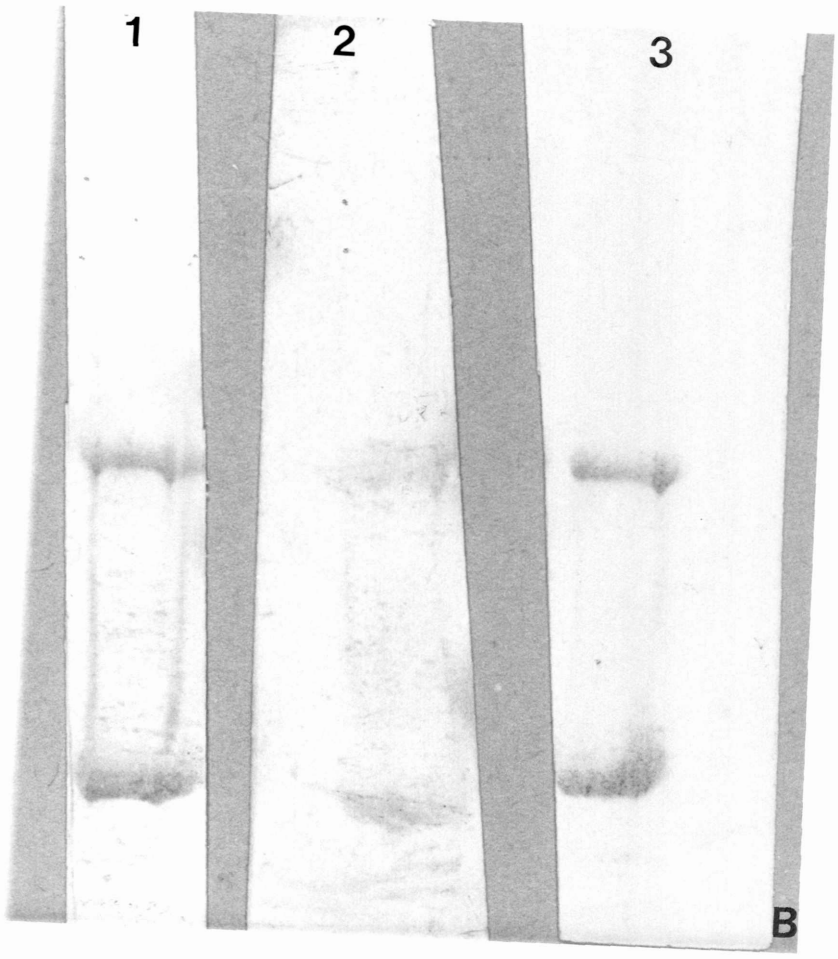
These affinity-purified antibodies were then used to probe nitrocellulose blots containing both GAP-43 and the higher RMM band. Figure 3.3.b shows that antibodies eluted from GAP-43 also cross-reacted with the higher RMM band and that the staining intensity was similar to that achieved with 'whole anti-GAP-43 fusion protein antiserum'. Antibodies eluted from the high RMM band stained both GAP-43 and the additional band but at a lesser intensity. This was probably due to the relative amounts of the two bands in the original gel (see Figure 3.3.a) rather than the affinity of the eluted antibodies for GAP-43. These results suggest that the proteins present in the additional band(s) are very similar to GAP-43, or at least share some antigenic determinants.

Figure 3.3.

A. Residual protein in a 10% acrylamide gel of purified GAP-43 after electrophoretic transfer to nitrocellulose, stained with Coomassie blue. The major band is GAP-43 and a faint higher RMM band can also be seen.

B. Western blot strips of purified GAP-43. Strip 1 was stained with antibodies eluted from GAP-43. Strip 2 was stained with antibodies eluted from the higher RMM band. Strip 3 was stained with 'whole anti-GAP-43 fusion protein antiserum'. GAP-43 (bottom band) and the higher RMM band are stained in all three strips.


A


1
2
3
B

3.3. Discussion

3.3.1. Identity Of GAP-43/ß-galactosidase Fusion Protein

The presence of GAP-43 cDNA was followed through the production of the GAP-43/ß-galactosidase fusion protein by restriction endonuclease analysis of plasmid DNA. The *Ava I* and *Ava II* restriction endonucleases were used to cut the pGEM3 plasmid containing the full-length GAP-43 cDNA, and this liberated a 610 bp fragment of the GAP-43 gene which could be identified by its size through the subsequent manipulations (see Figure 3.1.a, b).

The synthesis of a fusion protein by *E. coli* (transformed with the pUR 292 expression vector) was demonstrated by SDS-PAGE of cellular proteins which revealed a major protein band migrating more slowly than ß-galactosidase (see Figure 3.1.c), indicating a protein of higher RMM. Although it was to be expected that this would be a chimeric protein of GAP-43 and ß-galactosidase, this could only be shown directly by the reactivity of the antiserum raised against the fusion protein.

3.3.2. Specificity Of Anti-GAP-43 Antiserum

The antiserum against the fusion protein recognised both GAP-43 and ß-galactosidase, indicating that a GAP-43/ß-galactosidase fusion protein had been successfully synthesised. In addition, the antiserum recognised the high RMM bands which resolved in early polyacrylamide gels of purified GAP-43.

3.3.3. Nature Of The Higher RMM Bands

The ability of the antiserum to recognise both GAP-43 and the additional bands in Western blots enabled several questions to be answered about the high RMM bands. First, these additional bands were present in crude homogenates of neonatal brain showing that their appearance was not an artefact of the purification procedure and

secondly, homogenates of adult brain also showed the presence of these bands. Thirdly, cross-reactivity of eluted antibodies showed the higher RMM bands to be immunologically related to GAP-43. The possibilities that these bands either represent contaminating, but cross-reacting, proteins or aggregates of GAP-43 are discussed below.

3.3.3.1. Cross-Reacting Contaminating Protein

The appearance of additional bands in a preparation of purified GAP-43 was surprising. GAP-43 is the only protein known to bind calmodulin in the absence of Ca^{2+} ions (but not in their presence) and calmodulin column chromatography, which was the final purification step, has previously been shown to yield *pure* GAP-43 with no contaminating proteins from bovine brain (Andreasen *et al.*, 1983).

Even more surprising was the fact that the antiserum raised against the GAP-43/ß-galactosidase fusion protein recognised the higher RMM bands in addition to GAP-43. This was shown to be due to a population of antibodies recognising determinants on GAP-43 as antibodies eluted from GAP-43 cross-reacted with the additional bands and *vice versa*. This clearly shows that the higher RMM bands shared some determinants with GAP-43, and one might therefore expect some similarities between the primary amino acid sequences of GAP-43 and the additional bands. However, restriction endonuclease analyses of genomic DNA from rat and several other species (Southern blots) hybridised with GAP-43 cDNA probes have demonstrated that GAP-43 is the product of a single gene, and searches of the GenBank and EMBL sequence databases have shown no significant homology with any previously reported DNA sequences (Basi *et al.*, 1987; Cimler *et al.*, 1987; LaBate and Skene, 1989).

This does not exclude the possibility that a protein bearing little or no homology to GAP-43, in terms of nucleotide or amino acid sequences, might show sufficient similarities at the level of tertiary

structure to cause immunological cross-reactivity. Such a protein would also have to show certain biochemical similarities in order to copurify with GAP-43. A candidate would be the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) protein, which shares considerable biochemical and structural features with GAP-43 and is widespread in rat brain and other tissues (Albert *et al.*, 1986; Ouimet *et al.*, 1990). As its name implies, MARCKS protein is myristoylated at the amino terminus which promotes association with plasma membranes (as opposed to the palmitoylation of GAP-43), and like GAP-43, it is a substrate for PKC (Stumpo *et al.*, 1989). Also like GAP-43, MARCKS protein contains a high proportion of alanine (>20%) and other hydrophilic amino acid residues and thus shows aberrant migration under SDS-PAGE (RMM of 32 kD, estimated at 60-87 kD in polyacrylamide gels). MARCKS protein binds to calmodulin and this is prevented by phosphorylation of serine residues by PKC (Graff *et al.*, 1989). The gene encoding bovine MARCKS protein has recently been sequenced and shows no significant homology with any previously reported nucleotide sequences (including the GAP-43 gene), and the deduced amino acid sequence bears little resemblance to GAP-43 (Stumpo *et al.*, 1989).

Several lines of evidence argue against MARCKS protein being a cross-reacting contaminant in purified GAP-43. First, MARCKS protein would not be expected to copurify with GAP-43, as it does not bind calmodulin in the absence of Ca^{2+} ions (Graff *et al.*, 1989), and it would therefore be unlikely to be retained on the calmodulin-agarose column in the presence of excess EGTA. If a small amount had been retained, it would show *increased* binding to the column during *elution* of GAP-43 with 3 mM calcium chloride (Andreasen *et al.*, 1983; Graff *et al.*, 1989). Secondly, MARCKS protein showed migration consistent with a RMM of 60-87 kD in SDS polyacrylamide gels whereas the additional high RMM bands in the GAP-43 preparation appeared to migrate in excess of 100 kD. Thirdly, tissues such as lung and spleen which are rich in MARCKS

protein (Albert *et al.*, 1986) but devoid of GAP-43 showed no immunoreactivity to the anti-GAP-43 fusion protein antiserum described here (data not shown).

3.3.3.2. Aggregation Of GAP-43

Alternatively GAP-43 may form molecular complexes (either with itself or with other proteins) which are not disrupted during sample preparation and electrophoresis. This would provide an explanation for the presence of the higher RMM bands in crude tissue extracts and purified GAP-43 as additional proteins might copurify in a physical complex with GAP-43. The presence of GAP-43 in the higher RMM bands would provide a basis for the observed immunological cross-reactivity with GAP-43.

GAP-43 can be isolated in molecular complexes with PKC and PIP kinase (Jolles *et al.*, 1980), calmodulin (Andreasen *et al.*, 1983) and certain cytoskeletal proteins (Meiri and Gordon-Weeks, 1990), and it is thought to bind to G_o although this has not been shown directly (Strittmatter *et al.*, 1990). It is unlikely that weak non-covalent hydrogen bonds and electrostatic interactions between proteins (Alberts *et al.*, 1989) would resist the conditions of reduction and denaturation employed in SDS-PAGE (see Section 2.6.1.). Indeed, this was demonstrated for the interaction of GAP-43 and calmodulin (data not shown). GAP-43 was bound to a calmodulin-agarose column, as described in Section 2.3.5., and the column was washed to remove unbound proteins. Prior to eluting GAP-43, a sample of the agarose matrix was removed and then boiled in sample buffer and subjected to SDS-PAGE. The GAP-43/calmodulin complex resolved as two bands of approximately 43 kD (GAP-43) and 18 kD (calmodulin).

Furthermore, additional proteins copurifying with GAP-43 as a molecular complex would still be visible on silver stained gels, even after dissociation from GAP-43. Yet individual samples of GAP-43 were

inconsistent with respect to the presence of additional bands and, in later experiments, the same samples of GAP-43 routinely resolved as a single band. It therefore seems unlikely that the higher RMM bands represent complexes of GAP-43 with other proteins.

The ability of individual samples to resolve as either multiple bands or a single GAP-43 band supports the possibility that GAP-43 may form a multimeric aggregate with other GAP-43 molecules, thus giving rise to a variable number of higher RMM bands. GAP-43 has been suggested to exist as a multimeric complex in excess of 100 kD in the absence of detergent but this complex resolved as a single band under SDS-PAGE (Chan *et al.*, 1986; Benowitz *et al.*, 1987). The native structure of GAP-43 in intact cells has not yet been addressed nor has the nature of the potential interaction between GAP-43 molecules, but it is possible that multimeric GAP-43 complexes might be resistant to the conditions of SDS-PAGE. For example, hydrophobic interactions between the covalently-attached palmitic acid residues on GAP-43 might lead to the formation of 'micellar' structures in the presence of detergents (Helenius and Simons, 1975; Alberts *et al.*, 1989). However, thioester-linked palmitic acid moieties would not be expected to remain attached in the purified GAP-43 preparation as these linkages are not resistant to the alkaline conditions used to liberate GAP-43 from axonal membranes in Section 2.3.2. (Kaufman *et al.*, 1984; Skene and Virág, 1989).

In an attempt to demonstrate complex formation by GAP-43, samples were run on polyacrylamide gels under a number of different conditions to eliminate, or increase the intensity of, the higher RMM bands. This would be seen as dissociation or association of GAP-43 complexes, respectively. Gels run under non-reducing conditions in the absence of ß-mercaptoethanol (Hames and Rickwood, 1981) did not promote association of high RMM complexes, while causing control immunoglobulin protein to migrate in its native form. In contrast, acetylation of reduced cysteine thiol groups with iodoacetamide (Skene and Virág, 1989) failed

to eliminate the high RMM bands, yet the same treatment disrupted immunoglobulin proteins into their component polypeptide chains (data not shown). These results suggests that the high RMM bands are not due to formation of covalent disulphide bonds between cysteine residues in the amino terminus of GAP-43. Cleavage of thioester bonds linking palmitic acid moieties to GAP-43 with either high (alkaline) pH or hydroxylamine (Kaufman *et al.*, 1984; Skene and Virág, 1989) similarly failed to eliminate the high RMM bands (data not shown), arguing against hydrophobic interactions of GAP-43 through covalently-attached fatty acids. Chelation of Ca^{2+} ions with EGTA or excess Ca^{2+} ions in the gel samples did not affect the electrophoretic pattern, suggesting that GAP-43 does not aggregate in a calcium-dependent manner (data not shown).

Despite these efforts, the problem of identifying the additional immunoreactive bands proved insurmountable. Direct examination of these bands by isoelectric focusing or by elution from gels and subsequent analysis by peptide mapping or protein sequencing was pre-empted by the routine resolution of GAP-43 as a single band, both in purified preparations and in crude tissue extracts.

3.3.4. Concluding Remarks

While it was not a major goal of these studies to examine the biochemistry of GAP-43, the appearance of additional immunoreactive bands initially cast some doubt on the specificity of the antiserum raised against the GAP-43/ß-galactosidase fusion protein and the experiments described in this Chapter were performed in order to understand the nature of these bands. It is acknowledged with regret that these efforts did not yield a definitive identification, however the evidence points to an undefined aggregation of GAP-43, dependent upon uncharacterised factors in the matrix of the polyacrylamide gels. The greatest support for this explanation comes from the ability of a *single* aliquot of GAP-43 in sample buffer to resolve as either one band

or multiple bands in *different* gels. Moreover, GAP-43 has been reported to aggregate at high concentrations (Alexander *et al.*, 1987) and to form multimeric complexes (Chan *et al.*, 1986; Benowitz *et al.*, 1987).

It should be noted that additional high RMM bands have been observed by other investigators using well-characterised monoclonal and polyclonal antibodies against GAP-43 (K. Meiri, D. Schreyer and P. Skene, personal communications) and can be seen on gels and blots in some published papers (McIntosh *et al.*, 1989; Zuber *et al.*, 1989a; Moss *et al.*, 1990), although these authors have often chosen not to comment on their presence.

Throughout this thesis it will be assumed that immunostaining with antibodies against the GAP-43/ß-galactosidase fusion protein represents the distribution of genuine GAP-43 in tissue sections and cultured cells. Furthermore, Western blots are presented throughout that show recognition of a single GAP-43 band to support the specificity of this staining. However, it should be noted that some samples resolved as several bands in early experiments and other samples, which were prepared when additional high RMM bands were no longer apparent, might have the potential to resolve as several bands under certain electrophoretic conditions.

In the absence of any direct proof that the high RMM bands were aggregates of GAP-43 (either alone or in complexes with other proteins) one might suspect the specificity of the antiserum described here and refer to any staining as 'GAP-43-like immunoreactivity'. However it seems likely that these sera recognise GAP-43 specifically in the mammalian nervous system because, first, the appearance of these high RMM bands was highly variable and GAP-43 routinely resolved as a single band in later experiments and, secondly, other well-characterised antibodies specific for GAP-43 also recognised these bands.

Chapter Four

GAP-43 In The Adult Central Nervous System

4.1. Introduction

The wide distribution of GAP-43 in the nervous system of several species and the developmental regulation of its synthesis were quickly recognised by many investigators and led to speculation that GAP-43 may be a key molecule in the regulation of neuronal growth (Jacobson *et al.*, 1986; Benowitz and Routtenberg, 1987). Consequently, the levels of GAP-43 and its sites of synthesis have been extensively investigated throughout development and adulthood using a range of immunological and molecular techniques. Unless specified, the examples quoted in the following overview of the literature refer to evidence obtained from the rat.

4.1.1. Distribution Of GAP-43 In Adult Central Nervous System

4.1.1.1. Immunohistochemistry

Immunohistochemical examinations have revealed that GAP-43 is widespread throughout the fibre pathways and neuropil of the developing rat brain and spinal cord (Oestreicher and Gispen, 1986; Gorgels *et al.*, 1987; McGuire *et al.*, 1988; Erzurumlu *et al.*, 1990) and the developing visual system of cats and hamsters (Moya *et al.*, 1989; McIntosh *et al.*, 1989) and also that expression of GAP-43 is co-ordinate with the growth of axons and expansion of terminal arbors (Skene and Willard, 1981b; Goslin *et al.*, 1988; Moya *et al.*, 1989; Erzurumlu *et al.*, 1990). Adult CNS shows greatly reduced staining with only discrete regions immunoreactive for GAP-43, although there is some disagreement as to which regions. In a survey of all brain regions, Benowitz *et al.* (1988) found staining in layer 1 of the cortex, in the hippocampus and dentate gyrus and in a cerebral 'subcortical continuum', but not in the cerebellum as reported by Oestreicher and Gispen (1986) and McGuire *et al.* (1988).

Gorgels *et al.* (1987) showed that faint GAP-43 immunoreactivity

remains in the superficial laminae of the gray matter and the corticospinal tract (CST) in the adult spinal cord. Verhaagen *et al.* (1989) utilised the olfactory system to show that neurons which extend axons during adulthood express GAP-43. Immunoreactivity was only found in the cell bodies of immature olfactory receptor neurons in the olfactory epithelium, adjacent to the basal cell layer, but not in more mature neurons located in the superficial layers and expressing the olfactory marker protein. Discrete groups of fibres in the olfactory nerve bundles showed immunoreactivity but, unexpectedly, the terminals of these fibres in the glomeruli of the olfactory epithelium were only patchily stained.

4.1.1.2. Radio-Immunoassay

Verhaagen *et al.* (1986) could not demonstrate staining of GAP-43 in normal adult peripheral nerve, but reported 5.1 +/- 0.2 ng GAP-43/mg protein in sciatic nerve using radioimmunoassay. Using the same technique Oestreicher *et al.* (1986) mapped different brain regions and found GAP-43 levels varied from 84.4 +/- 7.3 μg/g wet weight of tissue (in the septum) to 17.6 +/- 0.5 μg/g tissue (in the cerebellum), and Gorgels *et al.* (1987) reported a value of 4.7 +/- 0.56 μg/g tissue for cervical spinal cord. It should be noted that detectable levels of GAP-43 in the spinal cord and sciatic nerve (respectively 50 ng/mg and 5 ng/mg total protein) were associated with faint or absent GAP-43 immunoreactivity. This suggests that some cells in the adult transport GAP-43 in amounts below the detection limit of these immunohistochemical studies. Consequently, molecular biological techniques were employed to demonstrate the presence and regulation of mRNA encoding GAP-43.

4.1.1.3. Northern Blotting

Northern blots from various regions of the adult rat brain revealed the highest levels of GAP-43 mRNA to be in the posterolateral

('association') cortex, striatum and 'midbrain', with modest levels in the hippocampus, septum, anterior cortex and medulla, while the cerebellum contained very low levels (Neve *et al.*, 1987). Adult human brain showed significant levels in the associative cortical areas, but not in any subcortical structures, and low levels in the cerebellum (Neve *et al.*, 1987, 1988).

4.1.1.4. *In Situ* Hybridisation

The location of GAP-43 mRNA can be determined at the single cell level by *in situ* hybridisation. Using human tissue, Neve *et al.* (1988) showed that most GAP-43 mRNA in the cortex is located in pyramidal neurons in layer 2 (especially in associative areas such as the inferior temporal cortex), while a small number of cells in deeper layers were also positive. Pyramidal cells and granule cells of the hippocampus and dentate gyrus also showed hybridisation, while in the cerebellum mRNA was predominantly located in the molecular layer, suggesting that the inhibitory interneurons of the molecular layer (basket and stellate cells) synthesise GAP-43 rather than the granule cells.

These results were partially confirmed in the rat (Rosenthal *et al.*, 1987; De la Monte *et al.*, 1989). Pyramidal cells throughout the dentate gyrus and hippocampus were positive for GAP-43 mRNA, but the granule cells of the dentate gyrus were not. The number of cells and intensity of hybridisation varied in the cerebral cortex, but no detailed study was undertaken in relation to different areas or layers. While De la Monte *et al.* (1989) reported no hybridisation in the cerebellar cortex, Rosenthal *et al.* (1987) showed intense hybridisation to the granule cell layer, suggesting that granule cells may synthesise GAP-43 in the rat. In addition, some cells in the molecular layer showed hybridisation.

In the olfactory bulbs the mitral cells showed intense hybridisation (Rosenthal *et al.*, 1987; De la Monte *et al.*, 1989). The

distribution of GAP-43 mRNA in the olfactory epithelium was determined by Verhaagen *et al.* (1990). Most hybridisation was to immature olfactory receptor neurons adjacent to the basal cell layer. Some mature neurons in the superficial layers were also positive (see Figure 4a of Verhaagen *et al.*, 1990), although it was not determined whether these were the cells which had matured most recently.

4.1.2. GAP-43 May Have A Role In Learning

The distribution of GAP-43 and its mRNA in development and adulthood, and the presence of GAP-43 in the growth cone during axonal elongation and synaptogenesis (Meiri *et al.*, 1986, 1988; Skene *et al.*, 1986; Baizer and Fishman, 1987), have led Benowitz to suggest that GAP-43 may be involved in the processes of synaptic plasticity underlying memory formation in the adult brain (Benowitz and Routtenberg, 1987; Neve *et al.*, 1987, 1988; Benowitz *et al.*, 1988). The hippocampus is known to be involved in short-term memory storage, both experimentally and clinically (Carpenter, 1978; Linden and Routtenberg, 1989). The so-called 'associative' areas of the cortex that contain most GAP-43 receive no direct sensory input: instead projections from the association nuclei of the thalamus and the primary sensory areas of the cortex converge to form the basis for multisensory perception and conscious memory (Carpenter, 1978). Lesions to these areas result in a related group of disorders known as agnosias, whereby specific stimuli fail to evoke the associated memory.

This hypothesis is supported by biochemical evidence that GAP-43 influences the presynaptic release of neurotransmitter or the postsynaptic responses to receptor activation (Oestreicher *et al.*, 1983; Van Dongen *et al.*, 1985; Dekker *et al.*, 1989b), and that GAP-43 is selectively and proportionally phosphorylated in the paradigm of memory formation known as hippocampal LTP (Lovinger *et al.*, 1985, 1986; Linden and Routtenberg, 1989).

4.1.3. The Present Study

As a means of determining the specificity and titre of serum obtained from rabbits inoculated with the GAP-43/ß-galactosidase fusion protein, polyester wax embedded sections of adult rat CNS were stained and compared with the reported distribution of GAP-43 detailed in Section 4.1.1. Furthermore, regions of adult central nervous system were homogenised and samples subjected to SDS-PAGE to separate proteins according to RMM. Western blots prepared from these gels were stained with the anti-GAP-43 fusion protein antiserum to reveal the nature of any immunoreactive proteins.

These studies showed that anti-GAP-43 fusion protein antiserum recognised a single protein in the rat CNS, which co-migrated with purified GAP-43. Furthermore, the antiserum was of very high titre and revealed GAP-43 in a more extensive distribution than previously documented. These results are discussed in terms of the properties of GAP-43 immunoreactive neurons and their possible role in memory formation. The use of different dilutions of anti-GAP-43 fusion protein antiserum in revealing varying amounts of GAP-43 in tissue sections is also discussed.

4.2. Results

4.2.1. Western Blotting

Figure 4.1. (upper panel) shows Western blots of crude extracts of adult olfactory bulb, cerebellum, spinal cord and whole brain stained with anti-GAP-43 fusion protein antiserum at a dilution of 1/1000. This concentration was used to show any and all immunoreactive protein bands, although identical results were achieved with dilutions as high as 1/10,000.

Immunoreactivity appeared as a single protein band of approximately 45 kD which co-migrated with GAP-43 purified from neonatal rat brain as described in Materials and Methods.

4.2.2. Immunohistochemistry

4.2.2.1. Olfactory Bulb

GAP-43 levels have not been directly quantified previously in the olfactory bulbs of the rat and staining of cryostat sections with avidin-biotin-HRP Vector-staining kit has shown that only small patches of immunoreactivity remained in the nerve fibre layer and synaptic glomeruli of adult rats (Verhaagen *et al.*, 1989). Immunohistochemistry of wax embedded sections appeared more sensitive (Figure 4.1. lower panel). Figure 4.2. shows the laminar structure of the olfactory bulb in diagrammatic form as a reference for the fluorescence micrograph. The incoming olfactory nerve fibres and the glomeruli of the olfactory bulbs were intensely stained even at high dilutions of the antiserum (up to 1/50,000, see Figure 4.3.). The mitral cell bodies and their processes in the external plexiform layer were not stained while the neuropil of the granule cell layer showed less intense staining which was not detected at dilutions higher than 1/20,000.

Figure 4.1.

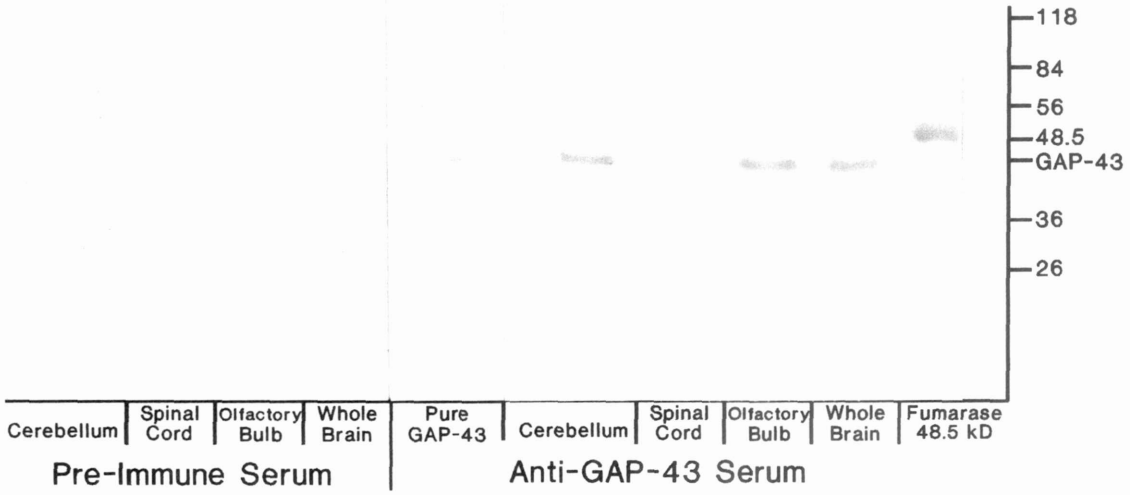
Upper Panel

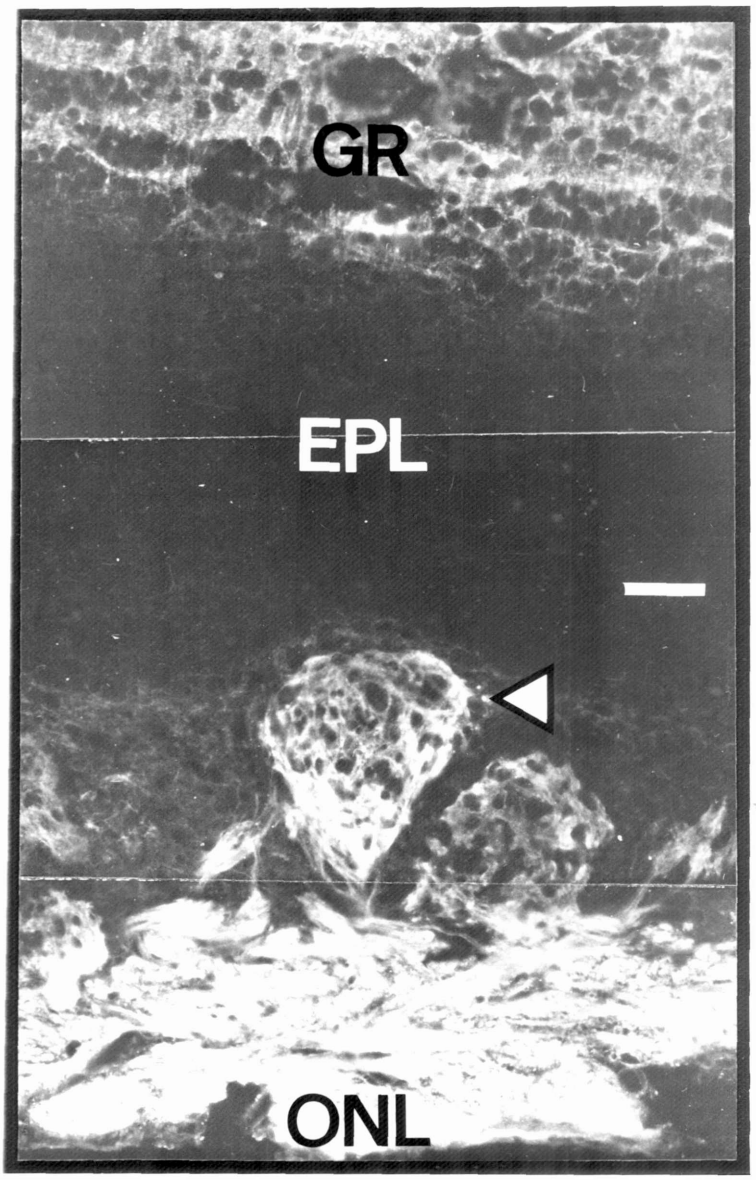
Immunoblots of crude tissue extracts from CNS regions. Cerebellum, spinal cord and olfactory bulb were removed and separately homogenised. The remainder of the brain was also homogenised ('whole brain'). 25 μg protein from each tissue were loaded on 10% acrylamide gels. Pre-immune serum showed no immunoreactivity. Anti-GAP-43 fusion protein antiserum revealed a single immunoreactive band which was identified by its position relative to protein standards (at right, RMM in kD) and purified GAP-43. Pre-stained fumarase (48.5 kD) is included as a reference for RMM.

Lower Panel

Olfactory bulb stained with anti-GAP-43 fusion protein antiserum at a dilution of 1/10,000. Olfactory nerve layer (ONL) and synaptic glomerulus (arrowhead) are more intensely stained than the granule cell layer (GR) while the external plexiform layer (EPL) shows no staining at all.

Scale bar, 50 μm.


118
84
56
48.5
GAP-43
36
26
Cerebellum
Spinal Cord
Olfactory Bulb
Whole Brain
Pure GAP-43
Cerebellum
Spinal Cord
Olfactory Bulb
Whole Brain
Fumarase 48.5 kD
Pre-Immune Serum
Anti-GAP-43 Serum


GR
EPL
ONL

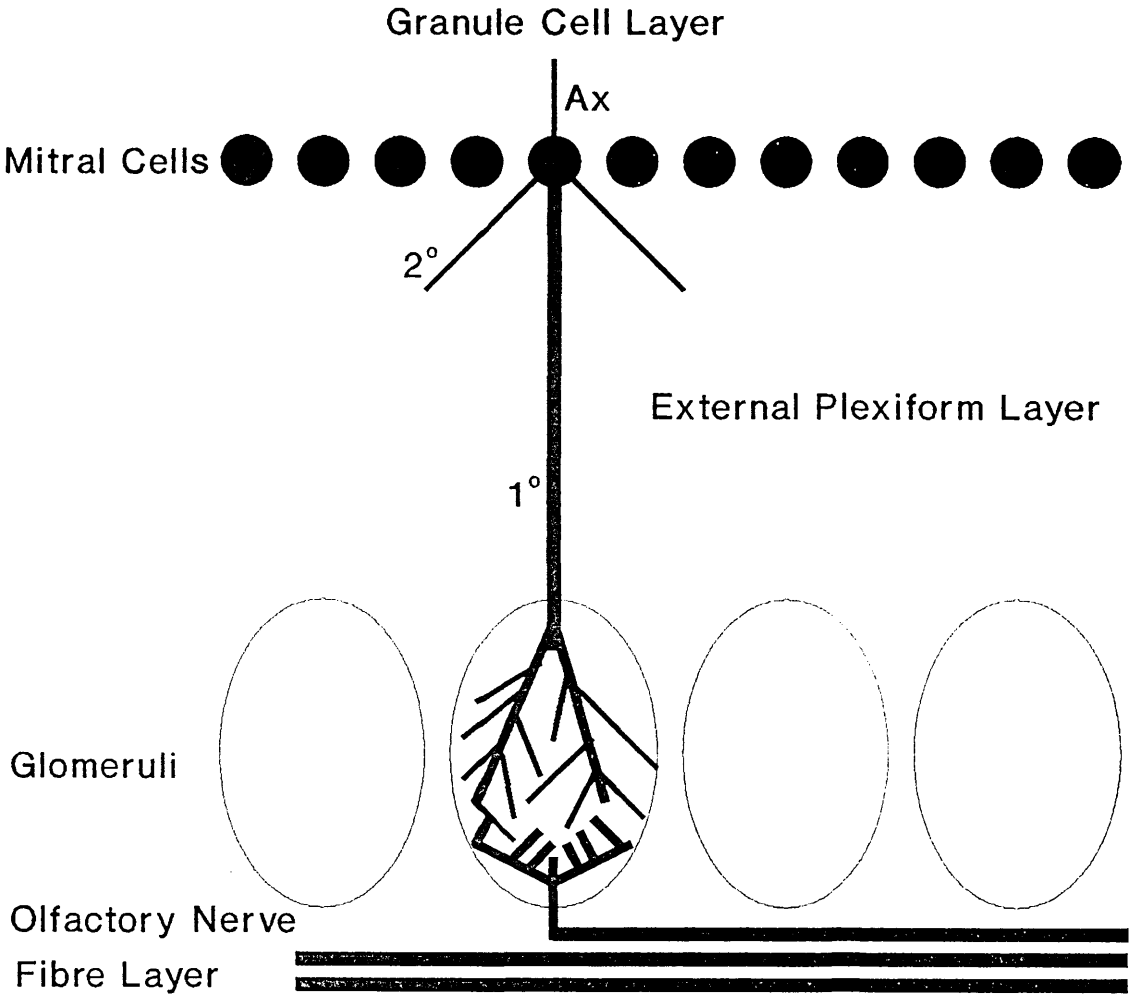


FIGURE 4.2.

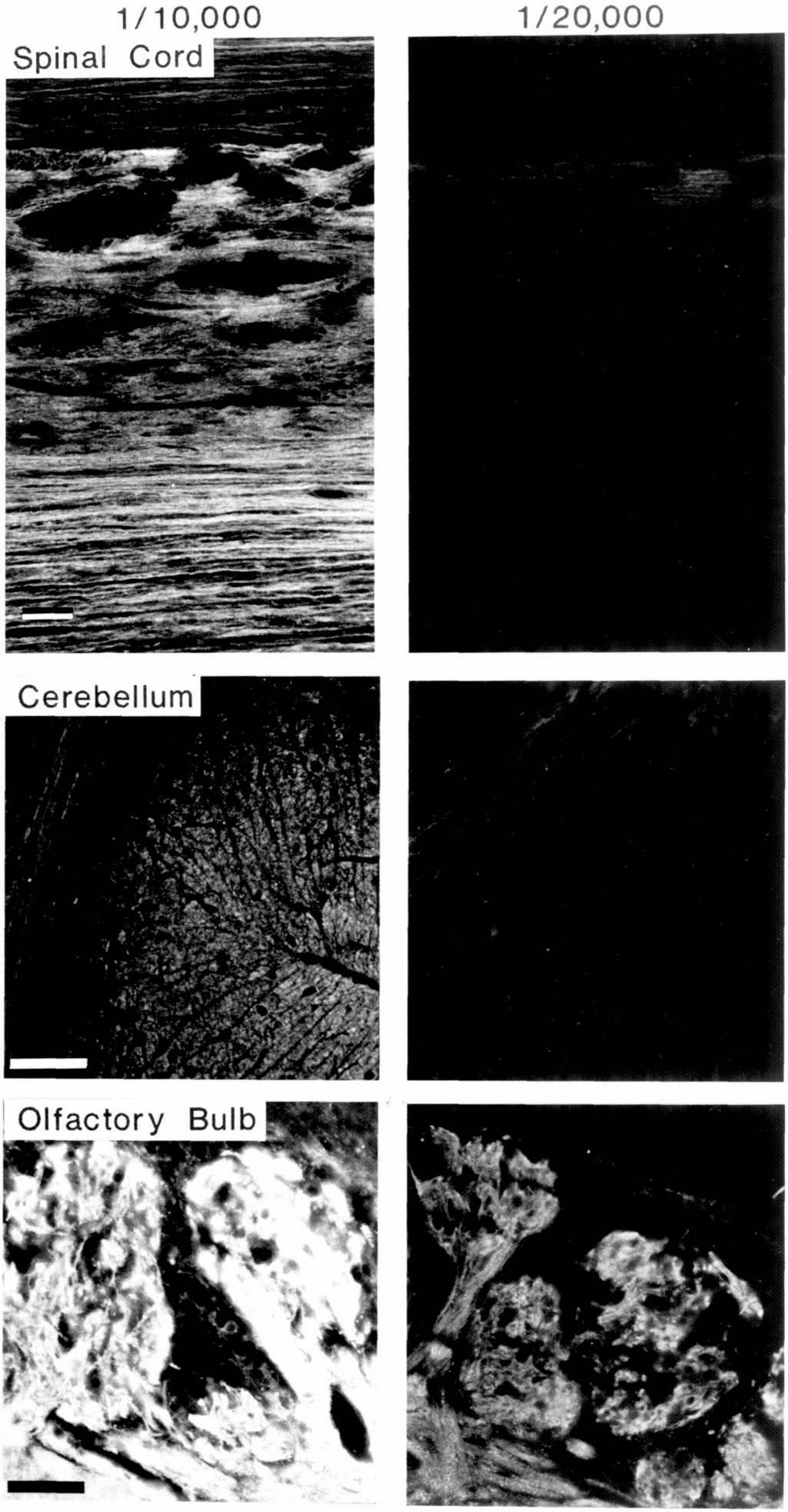
Diagrammatic representation of the laminar structure of the olfactory bulb. The surface of the bulb is covered by the olfactory nerve fibre layer which contains afferent fibres from the primary receptor neurons. These axons terminate on the dendritic arbors of the mitral cells in the synaptic glomeruli. The mitral cell bodies are located in a single layer and extend primary (1^{o}) and secondary (2^{o}) dendrites into the external plexiform layer towards the glomeruli. Mitral cell axons (Ax) extend through the granule cell layer to the anterior olfactory nucleus.

Figure 4.3.

Sections of spinal cord, cerebellum and olfactory bulb stained with different concentrations of anti-GAP-43 fusion protein antiserum. GAP-43 can be demonstrated in spinal cord and cerebellum at a dilution of 1/10,000 but not 1/20,000, whereas olfactory bulb shows robust staining at 1/20,000.

For each region, immunostaining at 1/10,000 and 1/20,000 were performed in parallel and sections were photographed and printed with identical exposure times. Indeed, the sections from cerebellum and olfactory bulb were all identically exposed and are therefore directly comparable. A lower power lens was used for photographing the spinal cord sections.

Scale bars, 50 μm.


1/10,000
1/20,000
Spinal Cord
Cerebellum
Olfactory Bulb

4.2.2.2. Cerebellum

The cerebellum contains one of the lowest concentrations of GAP-43 in the adult rat brain as measured by radioimmunoassay (17.6 +/- 0.5 μg/g wet weight of tissue, Oestreicher *et al.*, 1986) but GAP-43 has been localised in the cerebellum using antibodies by several groups (Oestreicher and Gispen, 1986; Benowitz *et al.*, 1988; McGuire *et al.*, 1988). The results are not consistent but labelling has been variously reported in the molecular layer and adjacent to the Purkinje cell bodies, and 'immunoreactive spots' were noted in the fibre tracts of the white matter.

The staining pattern revealed with the antiserum described here (Figure 4.4.b, c) confirmed the presence of GAP-43 in all these regions and highlighted the immunoreactive nature of fibres in the white matter of the cerebellum. The neuropil of the molecular layer is brightly stained, silhouetting the dendritic trees of the Purkinje cells, the cell bodies of the inhibitory interneurons and the radially-oriented fibres of the Bergman glia. Immunoreactivity can be seen adjacent to the basal surfaces of Purkinje cells (Figure 4.4.b, c). In some instances this surrounds the basal half of the Purkinje cell body but most commonly a single patch of staining can be seen, corresponding to the initial segment of the axon. Numerous fibres are visible in the white matter and radiating out through the granule cell layer, although it is clear from examination of sections double labelled for GAP-43 (Figure 4.4.c) and neurofilament protein (Figure 4.4.d) that not all axons are GAP-43+.

In addition to the previously reported staining, bright immunolabelling was noted in the climbing fibres of the olivocerebellar tract in the cerebellar commissure (Figure 4.4.a).

All staining in the cerebellum was lost by dilution of the antiserum beyond 1/20,000 (see Figure 4.3.).

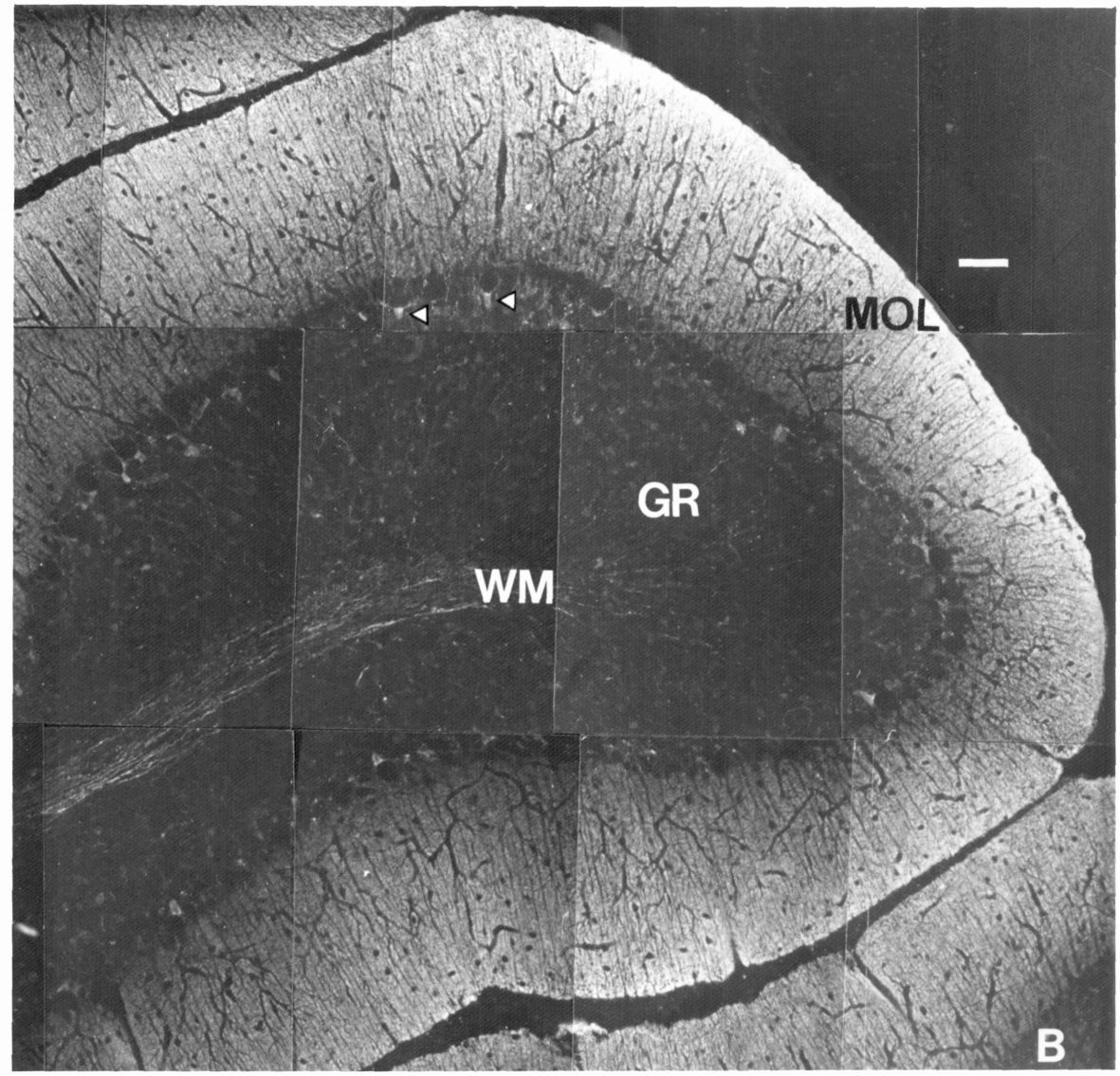
Figure 4.4.

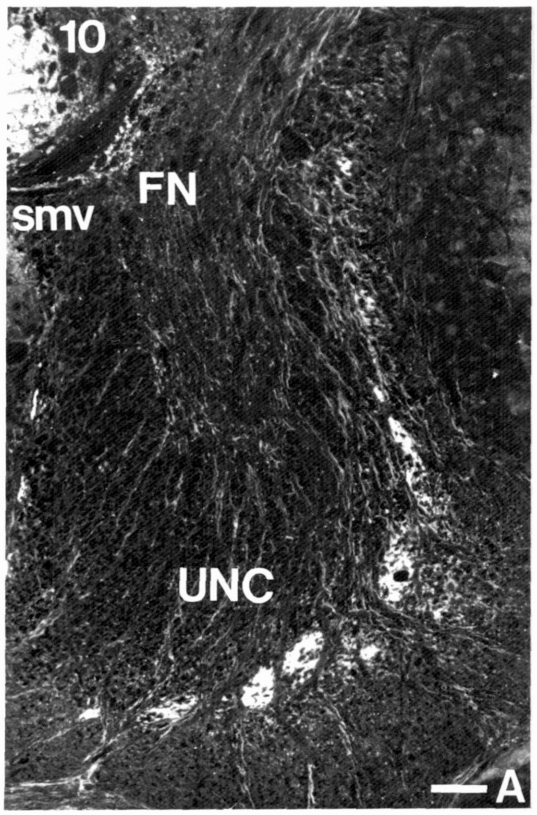
A. The cerebellar commissure in the base of the cerebellum stained with anti-GAP-43 fusion protein antiserum at a dilution of 1/10,000. The position of the molecular layer of lobule 10 and the junction with the superior medullary velum (smv) are indicated for reference. Bundles of climbing fibre axons in the olivocerebellar tract are stainedat the perimeter of the uncinate tract (UNC) ventral to the fastigial nucleus (FN).

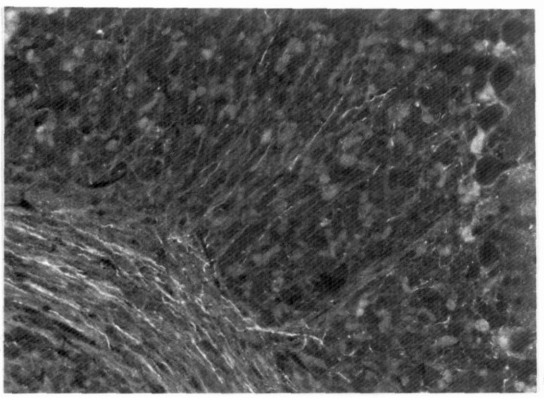
B. Photomontage of one folium of the cerebellum stained with anti-GAP-43 fusion protein antiserum at a dilution of 1/10,000. The neuropil of the molecular layer (MOL) is brightly stained, silhouetting the branched processes of the Purkinje cells and the radial processes of the Bergman glia. Cell bodies of inhibitory interneurons in the molecular layer are also unstained. The basal surfaces of the Purkinje cells and the initial segment of their axons are stained (arrowheads). Some axons in the white matter tract (WM) also show immunofluorescence.

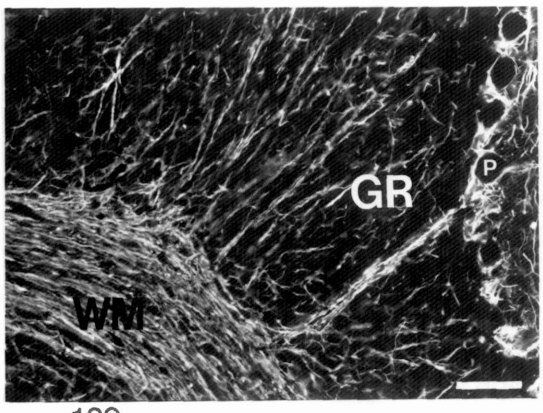
C,D. Double labelling of axons in the white matter of one folium and adjacent Purkinje cells with anti-GAP-43 fusion protein antiserum (c) and monoclonal antibody RT97 against neurofilament protein (d). Only a small proportion of the RT97+ axons in the white matter (WM) and granule cell layer (GR) are positive for GAP-43. Purkinje cells (P) show GAP-43 staining around their cell bodies and initial axon segments.

Scale bars, 50μm.


MOL
GR
WM
B


10
FN
smv
UNC
A


C


GR
P
WM
D

4.2.2.3. Spinal Cord

GAP-43 has previously been measured by radioimmunoassay in the adult rat spinal cord, and is reported to be present at 50 ng/mg protein (representing 4.7 μg/g wet weight of tissue) at cervical segment three (Gorgels *et al.*, 1987). Immunohistochemistry of Epon embedded tissue using the peroxidase-antiperoxidase technique (Gorgels *et al.*, 1987) revealed faint staining in the CST and the superficial laminae of the dorsal horn of the gray matter (corresponding to Rexed's laminae 1 and 2) and a more intense band in the dorsal gray commissure (midline continuation of Rexed's lamina 4). Rexed's cytoarchitectural division of the spinal gray matter in the cat into ten layers (Rexed, 1952) has also been shown to apply to the rat spinal cord (Brichta and Grant, 1985; Molander *et al.*, 1989).

A more extensive distribution was revealed by fluorescence immunohistochemistry of polyester wax embedded sections. Figure 4.5. shows the thoracic region of the rat spinal cord in transverse and longitudinal section. The gray matter neuropil showed staining throughout but with a pronounced decreasing dorsoventral gradient, while cell bodies remained unlabelled. Rexed's laminae 1 and 2 were most intensely stained and laminae 3 and 4 less so, while lamina 5 and the ventral horn showed uniformly lower staining. Lamina 10, which surrounds the central canal, showed brighter staining than the adjacent gray matter, approximating to the intensity of lamina 3. At thoracic levels the dorsal nucleus (Clarke's column) extends dorsally almost to occlude the dorsal gray commissure. While the dorsal nucleus was unstained, a thin strip of lamina 4 was brightly labelled above it. The tract of Lissauer at the dorsal surface of the gray matter was also brightly stained, as was the *fasciculus interfascicularis* in the dorsal funiculus. Both of these tracts contain dorsal root afferent fibres or their collaterals (Carpenter, 1978; Grant, 1985).

Staining in the white matter was generally less than in the gray

matter. The CST was brightly stained both in the crossed CST at the base of the dorsal funiculus and in the uncrossed ventral CST lying in the ventral funiculus at the midline (Armand, 1982; Tracey, 1985). The dorsolateral funiculus was also brightly labelled. The remaining white matter was predominantly unlabelled in transverse section but GAP-43+ fibres can be discerned radiating from the gray matter. In particular, the axons of the dorsal spinocerebellar tract can be visualised as they emerge laterally from the dorsal nucleus in the gray matter (Clarke's column) before entering the dorsolateral funiculus (Tracey, 1985). Staining of ascending and descending white matter axons was more apparent in longitudinal sections of the thoracic spinal cord, although the dorsolateral funiculus and the dorsal and ventral paths of the CST still showed the greatest immunoreactivity. All staining in the spinal cord was lost by dilution of the antiserum beyond 1/20,000 (see Figure 4.3.).

Figure 4.5.

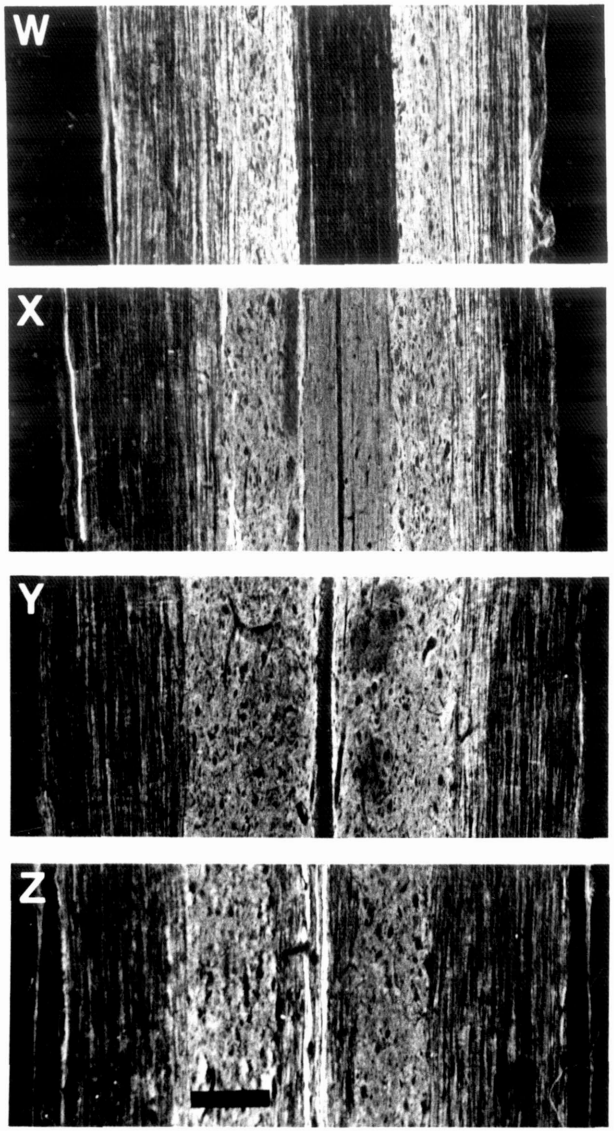
Transverse and horizontal sections of thoracic spinal cord, stained with anti-GAP-43 fusion protein antiserum at a dilution of 1/10,000.

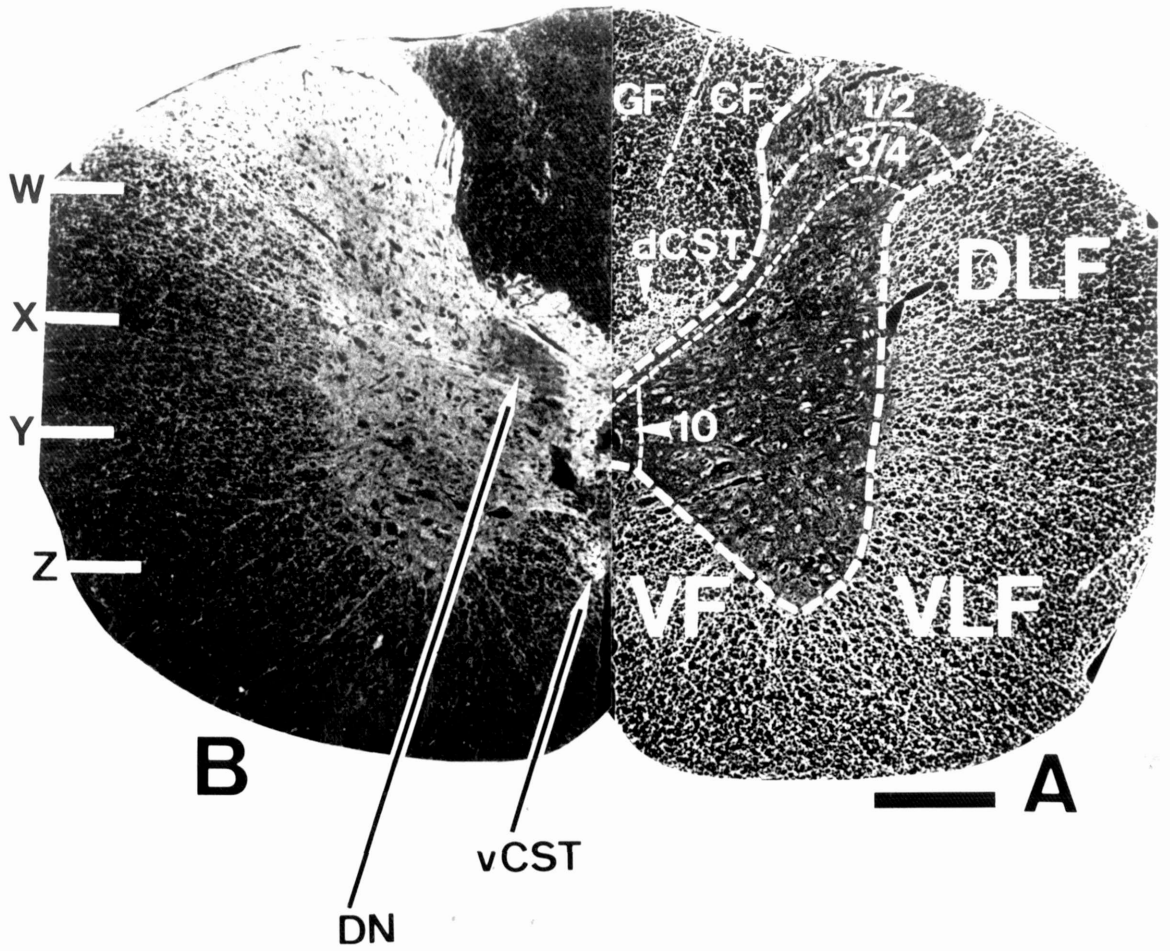
A. The major divisions of the spinal cord white matter are identified in a toluidine blue-stained hemi-section, printed as a negative image on reversal paper for greater contrast; VF, ventral funiculus; VLF ventrolateral funiculus; DLF dorsolateral funiculus. The individual components of the dorsal funiculus are identified; GF, gracile fascicle; CF, cuneate fascicle; dCST, dorsal corticospinal tract. The position of the *fasciculus interfascicularis* is indicated by the dashed line between GF and CF. Divisions of the gray matter are identified by laminar number, according to Rexed (1952).

B. Hemi-section showing GAP-43 immunoreactivity mainly in the superficial laminae of the gray matter (1/2) and lamina 10, with a decreasing dorsoventral gradient throughout the other laminae. The dorsal nucleus (DN, Clarke's column) and other cell bodies in the gray matter remain unstained. The dorsolateral funiculus and both dorsal and ventral corticospinal tracts (dCST, vCST) are also intensely stained.

W,X,Y,Z. Full width views of horizontal sections (photographed and printed at identical exposures) emphasise the staining of the regions described and also show that some axons are positive for GAP-43 throughout the white matter tracts. The planes of section are indicated at left of B.

Scale bars, 0.25 mm.


W
X
Y
Z


GF
CF
1/2
3/4
aCST
DLF
10
W
X
Y
Z
VF
VLF
B
A
vCST
DN

4.3. Discussion

4.3.1. Western Blots

Although GAP-43 immunoreactivity resolved as several protein bands in early blots, the appearance of the high RMM bands was highly variable. The nature of the high RMM bands is unknown, as discussed in Chapter 3, but their appearance has been noted by several investigators (K. Meiri, P. Skene, D. Schreyer, personal communications). Moreover, the antiserum reproducibly stained a single protein band which co-migrated with purified GAP-43 in later blots. It seems reasonable to conclude that the antiserum is specific for GAP-43 in crude extracts of CNS tissue and that the staining pattern revealed by immunohistochemistry represents the distribution of GAP-43, rather than any cross-reacting proteins.

4.3.2. Immunohistochemistry

4.3.2.1. Olfactory Bulb

The olfactory bulbs are the only part of the CNS which continue to receive ingrowth of new axons throughout adulthood. New olfactory receptor neurons, generated from stem cells in the olfactory epithelium, extend axons to the glomeruli in the olfactory bulbs (Graziadei and Monti Graziadei, 1978). Verhaagen *et al.* (1989) reported that immature receptor neurons expressed GAP-43 but mature cells (characterised by expression of the olfactory marker protein) and their projections to the glomeruli showed very little immunoreactivity.

In contrast, the distribution reported here shows that GAP-43 is present in large quantities in the axons of olfactory receptor neurons in the nerve fibre layer and their terminations in the glomeruli. This is consistent with transport of GAP-43 to the growth cones of axons arriving in the olfactory bulb and undergoing synaptogenesis in the

glomeruli. Synthesis and transport of GAP-43 by newly generated olfactory receptor neurons has been shown after lesions to the olfactory neuroepithelium (Verhaagen *et al.*, 1990). Similarly, *in situ* hybridisation has shown that some mature receptor neurons synthesise GAP-43 in the normal olfactory neuroepithelium (Verhaagen *et al.*, 1990).

It is not clear whether GAP-43 is down-regulated in these neurons after synaptogenesis is complete, as has been shown for other areas (Skene and Willard, 1981b; Oestreicher and Gispen, 1986; Erzurumlu *et al.*, 1990) and suggested by the study of Verhaagen *et al.* (1989). However, collaborative work with Dr. J. Schwob and Dr. K. Meiri (SUNY Syracuse, New York) using the antiserum described here has shown that GAP-43 can be detected in mature receptor neurons in the olfactory neuroepithelium, co-expressed with olfactory marker protein (unpublished observations), suggesting that mature cells continue to synthesise GAP-43. It should be noted that the relative ages of the mature cells expressing GAP-43 (identified by *in situ* hybridisation) was not addressed in the study of Verhaagen *et al.* (1990).

In addition, GAP-43 immunoreactivity was detected in the granule cell layer although at a lower level than in the glomeruli. The neuropil was stained while the granule cell bodies were not. These cells receive projections from the contralateral anterior olfactory nucleus and recurrent collateral fibres from the ipsilateral anterior olfactory nucleus (Carpenter, 1978). The distribution of GAP-43 is consistent with a presynaptic location in these afferent fibres. Alternatively, mitral cell bodies showed high levels of GAP-43 mRNA by *in situ* hybridisation (De la Monte *et al.*, 1989) and axons from these cells pass through the granule cell layer (Carpenter, 1978).

It is noteworthy that the external plexiform layer contained no GAP-43. The mitral cell dendrites which project through this layer to the glomeruli contain microtubule-associated proteins characteristic of immature neurons, suggesting that these dendrites are maintained in a

high state of plasticity to receive new synapses from the incoming olfactory nerve fibres (Viereck *et al.*, 1989). This confirms that GAP-43 protein is a strictly axonal protein (Goslin *et al.*, 1988; Goslin and Banker, 1990), rather than simply being associated with sites of ongoing synaptic plasticity.

4.3.2.2. Cerebellum

The bright labelling in fibre tracts of the cerebellar commissure is localised to the olivocerebellar tract, which contains the incoming climbing fibres. These small diameter myelinated axons arise in the inferior olivary nucleus and terminate in the cerebellar cortex, constituting one of only two afferent projections to the cerebellum, the other being the mossy fibre system (Palay and Chan-Palay, 1974). Climbing fibres exert an excitatory action on the Purkinje cells, possibly by release of the neurotransmitter aspartate (Ito, 1984). It is believed that all neurons in the inferior olivary nucleus send axons to the cerebellum (Flumerfelt and Hrycyshyn, 1985), and that this is the only source of climbing fibres (Ito, 1984). This would apparently make the olivocerebellar tract a suitable system for the study of GAP-43 synthesis and axonal transport in the adult CNS.

The predominant neuronal elements in the molecular layer of the cerebellum are the parallel fibre axons arising from the granule cells of the granule cell layer, which form *en passant* synapses on the dendritic spines of the Purkinje cell dendritic trees. Evidence has accumulated that the excitatory neurotransmitter glutamate is released at these sites (Ito, 1984). In rats, synaptic varicosities occur at 2.5 μm intervals along the parallel fibres, occupying nearly 40% of their length (Palay and Chan-Palay, 1974). The GAP-43 staining pattern in the molecular layer is consistent with a presynaptic location in these varicosities, but an extrasynaptic location in the axon shaft of the parallel fibres cannot be ruled out. Immuno-electron microscope studies

are currently underway in collaboration with Dr. S. Standring and Dr. A. Kent (Guy's Hospital, London) to determine if GAP-43 is present in the axolemma of the axon shaft, as has been shown for axons of the adult CST (Gorgels *et al.*, 1989). The synthesis of GAP-43 by granule cells is not universally accepted. *In situ* hybridisation has shown GAP-43 mRNA to be located in granule cells in one study (Rosenthal *et al.*, 1987) while others have failed to demonstrate GAP-43 mRNA in granule cell bodies (De la Monte *et al.*, 1989) and one study revealed diffuse hybridisation over the molecular layer of human cerebellum (Neve *et al.*, 1988), suggesting that inhibitory interneurons may synthesise GAP-43, as discussed below, or that GAP-43 may be locally synthesised in the parallel fibres.

The GAP-43 visualised around the basal surface of the Purkinje cell soma and the initial segments of their axons is consistent with a location in the presynaptic terminals of basket cells. These are inhibitory interneurons located in the deeper half of the molecular layer, which are stimulated by the parallel fibres and release gamma-aminobutyric acid at axo-somatic synapses on the Purkinje cell bodies and at axo-axonic plexuses, called pinceau, on the initial segment of the Purkinje cell axons (Palay and Chan-Palay, 1974; Ito, 1984). *In situ* hybridisation has shown that cells located in the molecular layer adjacent to the Purkinje cells contained GAP-43 mRNA, consistent with synthesis in the basket cells (Rosenthal *et al.*, 1987).

The staining in the white matter tracts could be due to the presence of GAP-43 in Purkinje cell axons or afferent fibres (mossy fibres or climbing fibres). The bright labelling in the olivocerebellar tract suggests that a substantial proportion of the climbing fibres are GAP-43+. Staining of DSCT axons in the spinal cord (see Section 4.3.2.3. below) suggests that at least one population of mossy fibre afferents contains GAP-43. However, detailed mapping of the origin of the GAP-43 immunoreactive fibres in the cerebellar white matter was beyond the scope of this study.

4.3.2.3. Spinal Cord

These data show a more extensive distribution of GAP-43 in the adult rat spinal cord than has previously been reported (Gorgels *et al.*, 1987). The differences are consistent with a greater sensitivity of the antiserum used as the regions stained by these authors (CST, superficial laminae of the dorsal horn, and the dorsal gray commissure) are also those most brightly stained in this study. Staining of the ventral tract of the CST was not apparent in their published micrograph, despite showing bright staining in both transverse and longitudinal sections (Figure 4.5.). However, GAP-43 staining of this tract has been noted in a recent study of neonatal spinal cord (Fitzgerald *et al.*, 1991).

In the gray matter, staining was predominant in the superficial laminae with a diminishing dorsoventral gradient. Lamina 10, surrounding the central canal, showed greater immunoreactivity than adjacent gray matter. This distribution is consistent with a location in fine calibre primary afferent fibres. The terminals of primary sensory neurons show a laminar distribution in the gray matter with fine calibre fibres (both unmyelinated and myelinated) distributing preferentially in the superficial laminae and coarse calibre myelinated fibres terminating more ventrally, although this is not an absolute division (Grant, 1985). In addition the tract of Lissauer and the *fasciculus interfascicularis*, which contain fine calibre primary afferent fibres (Chung *et al.*, 1979; Grant, 1985; Alvarez *et al.*, 1989), also showed GAP-43 immunoreactivity.

It is possible that axonal calibre is a determinant of GAP-43 expression as Gorgels *et al.* (1989) found that only small diameter unmyelinated and myelinated axons in the CST contained GAP-43. Interestingly, the small diameter myelinated axons of the olivocerebellar tract (climbing fibres) also contained GAP-43 (see previous Section).

GAP-43 expression may be restricted to specific populations of small diameter axons. Afferent fibres expressing the neuropeptides

calcitonin gene-related peptide or substance P, terminating principally in the superficial laminae (Aronin *et al.*, 1983; Tashiro and Ruda, 1988; Traub *et al.*, 1989), originate from cell bodies in the DRG which have been shown by *in situ* hybridisation to contain GAP-43 mRNA in normal adult rats (Verge *et al.*, 1989, 1990). However, cell bodies in the DRG containing somatostatin did not show significant GAP-43 mRNA (Verge *et al.*, 1990) and these also send projections to the superficial laminae (Mizukawa *et al.*, 1988). Primary sensory neurons containing these neuroactive molecules also innervate lamina 10 and may account for the GAP-43 staining around the central canal (Miller and Seybold, 1987). The dorsal CST also contributes fibres directly to the medial portions of the superficial laminae of the dorsal horns (Casale *et al.*, 1988) in a distribution that could account for some of the GAP-43 immunoreactivity in laminae 3 and 4. Intense GAP-43 staining was noted in the dorsal CST and it is likely these gray matter projections might also contain detectable amounts of GAP-43.

Alternatively, the presence of GAP-43 in the spinal gray matter might indicate ongoing synaptic reorganisation particularly in the superficial laminae. Injury-induced reactive synaptogenesis in the central nervous system is accompanied by increased levels of GAP-43 (Benowitz *et al.*, 1990) and GAP-43 staining in the central terminals of primary sensory neurons in the superficial laminae of the dorsal horn has been shown to increase after damage to the peripheral branch of these neurons (Woolf *et al.*, 1990). Although synapto-neogenesis occurs in these laminae after peripheral injury to primary sensory neurons in primates (Knyihár-Csillik *et al.*, 1985), there is no evidence of degenerative or regenerative changes in the dorsal horn of the normal spinal cord (Knyihár-Csillik *et al.*, 1982).

Neuronal soma in the laminae of the gray matter were not stained for GAP-43. The dorsal nucleus (or Clarke's column) was also unstained but axonal projections to the dorsal spinocerebellar tract (DSCT) from

the dorsal nucleus were GAP-43+. The DSCT consists of the afferent projections from the dorsal nucleus to the cerebellum and ascends mainly in the dorsolateral funiculus, although retrograde tracing and degeneration studies have demonstrated axons throughout the lateral columns (Zemlan *et al.*, 1978; Tracey, 1985). In addition, the dorsolateral funiculus contains other ascending and descending fibre tracts (Lundberg and Oscarsson, 1961; Clark, 1983), primary afferent fibres and propriospinal fibres (Chung *et al.*, 1987), and in some instances a branch of the crossed dorsal CST can be demonstrated in the dorsolateral funiculus by anterograde tracing (Casale *et al.*, 1988). The dorsolateral funiculus showed intense GAP-43 immunoreactivity and this might be due to GAP-43 in axons of the DSCT or CST or in calcitonin gene-related peptide-containing primary afferent fibres (as discussed above) (McNeill *et al.*, 1988).

The CST was the only identified population of axons in the white matter stained for GAP-43, due to the dorsolateral funiculus containing a mixed population of ascending and descending axons as described. The CST originates in the primary somatosensory cortex in the rat and descends the full length of the cord in the ventral aspect of the dorsal funiculus after decussation in the caudal medulla or in an uncrossed branch in the ventral funiculus adjacent to the midline (Armand, 1982; Tracey, 1985). The axons terminate in all laminae (Casale *et al.*, 1988) and may account for the GAP-43 staining in the ventral horns of the gray matter. Both the dorsal and ventral CST showed GAP-43 immunoreactivity, although the ventral tract was brighter. At the electron microscope level GAP-43 has been detected in the adult dorsal CST, located at the internal face of the axolemma (Gorgels *et al.*, 1989). Interestingly, large diameter axons ($>1\mu m$) were not stained, suggesting that GAP-43 has a role in the mature axon shaft of unmyelinated and smaller myelinated axons. Similar electron microscope data would be of interest with respect to the location of GAP-43 in the ventral CST, but this was not

examined in this study and no other ultrastructural studies have been reported in the literature, so it is not possible to correlate GAP-43 expression with axonal calibre in this region.

4.3.3. GAP-43 And Memory

GAP-43 has been implicated in the changes of synaptic structure and function underlying memory formation (Benowitz and Routtenberg, 1987; Neve *et al.*, 1987, 1988). GAP-43 has been shown to be involved in regulating the efficiency of synaptic transmission (Oestreicher *et al.*, 1983; Dekker *et al.*, 1989b) and GAP-43 is also associated with phases of synaptogenesis in the mammalian nervous system during development (Moya *et al.*, 1989; Verhaagen *et al.*, 1989; Erzurumlu *et al.*, 1990) and after denervation (Bisby, 1988; Verhaagen *et al.*, 1988; Van der Zee *et al.*, 1989; Benowitz *et al.*, 1990). Although the degree of GAP-43 phosphorylation has been shown to be correlated with the persistence of enhanced postsynaptic responses in the dorsal hippocampus (LTP) (Lovinger *et al.*, 1985, 1986), no causal role has yet been established for GAP-43 in memory formation. Indeed the evidence for such a role is circumstantial, being based mainly upon the distribution of GAP-43 in 'associative' cortical areas and the hippocampus (Benowitz and Routtenberg, 1987; Neve *et al.*, 1987, 1988; Benowitz *et al.*, 1988). The evidence presented here both strengthens and weakens this argument by showing that GAP-43 is expressed in areas involved in association and learning and in axons for which no such function has been proposed.

4.3.3.1. Olfactory Bulb

While the GAP-43 visualised in the glomeruli of the olfactory bulb is probably located in newly arrived axons, the staining in the granule cell layer may be associated with synapses on the granule cells. Granule cells have been suggested to serve an associative function by relaying information from both ipsilateral and contralateral anterior

olfactory nuclei to the mitral cells (Carpenter, 1978).

4.3.3.2. Cerebellum

Synaptic transmission at parallel fibre synapses with the Purkinje cell dendritic spines is known to undergo long-term modulation. Repetitive parallel fibre activation leads to potentiation of transmission, probably through increased neurotransmitter release (Hirano, 1990a, b; Crepel and Jaillard, 1991), whereas convergence of climbing fibre and parallel fibre impulses upon the same Purkinje cell results in persistently reduced transmission at the parallel fibre synapses in a process known as long-term depression (Ito, 1989). Stimulation from climbing fibres (which are axons from inferior olive neurons) leads to entry of Ca^{2+} ions into the Purkinje cell dendrites through voltage sensitive Ca^{2+} channels and desensitisation of glutamate receptors in the dendritic spines, at the postsynaptic side of parallel fibre-Purkinje cell synapses (Ito, 1989). Glutamate is believed to be the neurotransmitter released from parallel fibre varicosities (Ito, 1984) and synaptic transmission is thus reduced at the parallel fibre synapse and remains depressed for at least 1 hour.

The results presented here show that GAP-43 is present in both the climbing fibres and parallel fibres, suggesting that GAP-43 may have a role in memory formation in the cerebellum. A presynaptic role for GAP-43 in increased glutamate release from the parallel fibres (Hirano, 1990a, b; Crepel and Jaillard, 1991) is compatible with the proposed presynaptic role of GAP-43 in hippocampal LTP (Linden and Routtenberg, 1989). It should be noted that increased presynaptic release of glutamate is also associated with hippocampal LTP (Lynch *et al.*, 1985). The role of GAP-43 at the climbing fibre synapse is currently unknown.

Furthermore, the cerebellum has been implicated in 'motor learning' (the formation and execution of the motor 'programmes' necessary to perform learned, skilled tasks and movements) (Ito, 1984).

Motor learning of skilled acrobatic movements resulted in formation of new synapses (synapto-neogenesis) in the molecular layer, whereas repetitive exercising (non-learned locomotor activity on a treadmill) led to angiogenesis instead (Black *et al.*, 1990). It is possible that the presence of GAP-43 in parallel fibres may predispose these structures toward synapto-neogenesis. Some examples of classical conditioning (induction of a physiological response to a nonphysiological stimulus) also involve the cerebellum (Ito, 1984), although this is believed to be mediated by long-term depression (Ito, 1989).

4.3.3.3. Spinal Cord

The CST is involved in the generation of skilled movements (Carpenter, 1978), although learning is believed to occur at the level of the cerebellum (Ito, 1984). Furthermore, the cells of origin of the CST (in the rat, cortical layer 5B of the primary somatosensory cortex, see Tracey, 1985) are not those which showed high levels of GAP-43 mRNA by Northern blotting or *in situ* hybridisation (Neve *et al.*, 1987, 1988), although these studies were conducted on human tissue. This suggests that CST cells do not synthesise and transport large amounts of GAP-43, and therefore the greater part of the GAP-43 present in the axons of the CST is probably associated with the axolemma, as suggested by Gorgels *et al.* (1989). This, in turn, suggests that GAP-43 has an extrasynaptic role in the functioning of some axons, unrelated to memory storage or any other synaptic events. Likewise, the DSCT has no known role in learning.

4.3.4. Sensitivity Of Anti-GAP-43 Fusion Protein Antiserum

The distribution of GAP-43 revealed in these studies was compatible with the reported radioimmunoassay, Northern blot and *in situ* hybridisation data (see Section 4.1.). In contrast, previous

immunohistochemical studies have not revealed such an extensive distribution. This could be due to a) the fixation and processing of the tissue; b) the sensitivity of the antiserum; or c) the method of primary antibody detection.

The paraformaldehyde-fixed polyester wax methodology used in these studies has the advantages that tissue morphology is well preserved and lipids are extracted from the tissue during treatment in graded ethanols. This may well allow greater access of antibodies to GAP-43 which is located just inside the lipid-rich plasma membrane. For the combined reasons of morphological and antigen preservation, this was the method of choice, but staining of paraformaldehyde-fixed frozen sections revealed the same distributions at similar concentrations (data not shown). Moreover, McGuire *et al.*, (1988) stained paraffin wax sections of cerebellum with antibodies against GAP-43, but did not detect GAP-43+ axons in the white matter tracts. Tissue processing would appear not to determine the distribution of GAP-43 immunoreactivity, although *in situ* fixation of tissue was found to be essential.

Previous studies have mostly utilised GAP-43 purified by PAGE or column chromatography which is not highly immunogenic in rabbits. Indeed, we failed to obtain usable antiserum from rabbits inoculated with purified protein. Only Dr. K. Meiri (SUNY, Syracuse) was able to provide any polyclonal antiserum for use in comparative studies but this had to be used at much lower dilution to reveal similar staining patterns (1/100), whereas the mouse monoclonal antibody provided by Dr. D. Schreyer (Stanford University) readily reproduced the staining pattern detected with the anti-GAP-43 fusion protein antiserum. No attempt was made to compare indirect immunofluorescence with other primary antibody detection methods. However, it is unlikely that the use of indirect immunofluorescence underlies the low threshold of GAP-43 detection as both the peroxidase-antiperoxidase and avidin-biotin methods used by other investigators (Gorgels *et al.*, 1987; Verhaagen *et*

al., 1989) are more sensitive due to extra amplification steps and the affinity of avidin-biotin binding.

This would suggest that the high titre of anti-GAP-43 antibodies resulting from inoculation of GAP-43/ß-galactosidase fusion protein in rabbits accounts for the sensitivity of GAP-43 detection.

Furthermore, it was possible to distinguish between areas of high and low GAP-43 concentration within tissue sections. Although some areas appeared brighter than others at low dilutions (ie 1/10,000) this is a highly subjective and qualitative analysis, especially when coupled with the variability inherent in photographic processing. A degree of quantification can be introduced by using the antiserum at different dilutions (see Figure 4.3.). Locally higher concentrations of GAP-43, such as occur in the newly-arriving olfactory receptor afferents to the olfactory bulb, can be detected at higher dilutions (ie 1/20,000) than are required to visualise GAP-43 in mature axons and neuropil in the cerebellum and the spinal cord (ie 1/10,000).

Chapter Five

GAP-43 Expression After Central Nervous System Injury

5.1. Introduction

S. Ramón y Cajal noted in his book 'Degeneration and Regeneration of the Nervous System' (English translation by R.M. May, 1928):

"Pathologists consider it an unimpeachable dogma that there is no regeneration of the central paths, and therefore that there is no restoration of the normal physiology of the interrupted conductors in the spinal cord. A vast series of anatomico-pathological experiments in animals, and an enormous number of clinical cases that have been methodically followed by autopsy, serve as a foundation for this doctrine, which is universally accepted today. Nevertheless, some neurologists, setting to one side the incontestable (sic) disturbing fact that functional damage is irreparable, have made known histological observations of the partial regeneration of neurones and nerve fibres ... (These investigations) have also confirmed the old concept of the essential impossibility of regeneration, showing that, after a more or less considerable period of progress, the restoration is paralyzed, giving place to a process of atrophy and definitive breakdown of the nerve sprouts ... The generative acts occur only in a small number of fibres which are usually of moderate and small diameter. The immense majority of the conductors of the white matter, and especially those of large calibre, undergo degeneration and atrophy exclusively ... We may add that the sprouting of which we speak is more general and vigorous in young animals such as cats and dogs a few days old than in adult animals ...

As is well known, the optic nerve is a central path, organised anatomically like the white matter of the cord ... (and) will react to traumatic violence, not like peripheral nerves, but like the spinal cord, that is, by small and frustrated regenerative acts ..."

Over half a century has passed since Cajal and his contemporaries

made these observations, but our understanding of the failure of adult mammalian CNS regeneration has advanced little and there is still no clinical treatment to promote restoration of function after traumatic CNS injury. The Introduction to this thesis (Chapter 1) details the current state of CNS regeneration research and the reader is referred to this for a fuller discussion of the literature. However, some concepts and definitions bear repetition here. Firstly, *regeneration* refers specifically to regrowth of interrupted axons and is distinct from reparative processes such as elongation of collateral branches from either damaged or uninjured neurons.

Secondly, regenerative failure could result from the inability of central neurons to respond to axotomy (the 'Inherent Incapacity Hypothesis') or the effects of environmental factors which limit any regenerative response (the 'Neural Environment Hypothesis'). The 'GAP Hypothesis' suggests that certain proteins are absolutely required for axonal growth and that such proteins may be incorrectly regulated after CNS injury, due either to 'inherent' or 'environmental' factors.

Thirdly, transplantation studies using PNS segments grafted to the CNS have shown that central neurons can regenerate when provided with the correct environment (initiated by Tello, Leoz, Arcuate and Cajal, see Cajal, 1928, and conclusively recapitulated with modern tracing techniques by Aguayo and colleagues, see Bray *et al.*, 1987). These results prove the 'Inherent Incapacity Hypothesis' to be incorrect and demonstrate that the PNS provides a favourable environment for axonal growth from central neurons. It is unclear whether the CNS does not provide the necessary signals to initiate a regenerative response from the neural soma, or does not sustain directed outgrowth of axons.

Fourthly, although our understanding of the chemical factors that limit regeneration in the CNS is far from complete, the cellular elements constituting a physical barrier to elongating axons have been partially delineated. Reactive astrocytes themselves may not prevent

regeneration after CNS injury and may indeed supply necessary neurotrophic factors to surviving neurons (Lindsay, 1986). Invasion of fibroblasts on the other hand leads to the formation of a fibrous glial-mesodermal scar and deposition of a *glia limitans*, either of which may be impenetrable (Reier, 1986). Recent studies by Guth *et al.* (1985, 1986) have suggested that CNS regeneration may occur after spinal compression lesions from which fibroblasts are excluded. However, these experiments did not exclude the possibility that some axons may have been spared by the compression procedure, nor that the axons visualised may have arisen from collateral sprouting.

5.1.1. The Present Study

In collaborative experiments with Prof. M. Berry, Dr. L. Rees and Dr. S. Standring (UMDS, Guy's Hospital, London), expression of GAP-43 by retinal ganglion cells (RGCs) was examined by immunocytochemistry after transection of the optic nerve. Despite the failure of regeneration, axotomy induced expression of GAP-43 in RGCs, suggesting that these neurons are capable of initiating a regenerative response.

In a second set of experiments, with the expert assistance of the departmental animal surgeon D. Green, the model of spinal compression developed by Guth *et al.* (1985) to exclude fibroblasts was examined for elevated GAP-43 expression in neurons. Results presented in Chapter 4 showed that the spinal cord contains significant amounts of GAP-43 under normal circumstances, but that titration of the anti-GAP-43 fusion protein antiserum allows high local concentrations of GAP-43 to be visualised in actively elongating axons. Antiserum was therefore used at a concentration which allowed elevated levels of GAP-43 to be discerned in axons penetrating the lesion, showing that these axons are newly sprouted and not merely spared from destruction during trauma. It was not possible to determine whether these are regenerating axons or collateral sprouts.

5.2. Results

5.2.1. Optic Nerve Studies

5.2.1.1. GAP-43 In Non-Regenerating Retinal Ganglion Cells

To examine the response of RGCs to axotomy, sections of retina and optic nerve were examined by double immunofluorescence with anti-GAP-43 fusion protein antiserum (diluted 1/10000) and monoclonal antibodies against myelin basic protein (MBP) at several time points up to 15 days after intraorbital transection of the optic nerve and re-anastomosis of the severed ends. In control animals and in operated animals at 2 days after transection, GAP-43 was restricted to the plexiform layers of the retina and was absent from the axons of the RGCs in the nerve fibre layer and the head of the optic nerve, including the unmyelinated portion behind the globe (*lamina cribrosa*) (Figure 5.1.). No GAP-43 was detected in RGC axons of unoperated optic nerve, whereas a few GAP-43+ axons were present at the retinal side of the anastomosis 2 days after transection. By 5 days after transection the entire retinal stump was packed with GAP-43+ RGC axons which extended to the site of anastomosis and in some instances sprouts appeared to penetrate into the distal stump. These GAP-43+ fibres persisted to 15 days after transection, but gradually became restricted to the retinal side of the anastomosis, as if the penetrant sprouts had degenerated (Figure 5.2.). The nerve fibre layer of the retina and the *lamina cribrosa* were both packed with GAP-43+ axons at 15 days after transection, confirming the RGCs as the source of the GAP-43 immunoreactivity (Figure 5.1.).

The nature of the GAP-43 immunoreactivity was investigated by Western blotting of optic nerve 10 days after transection without re-anastomosis. A single immunoreactive band was found, which co-migrated with purified neonatal GAP-43 (Figure 5.3.).

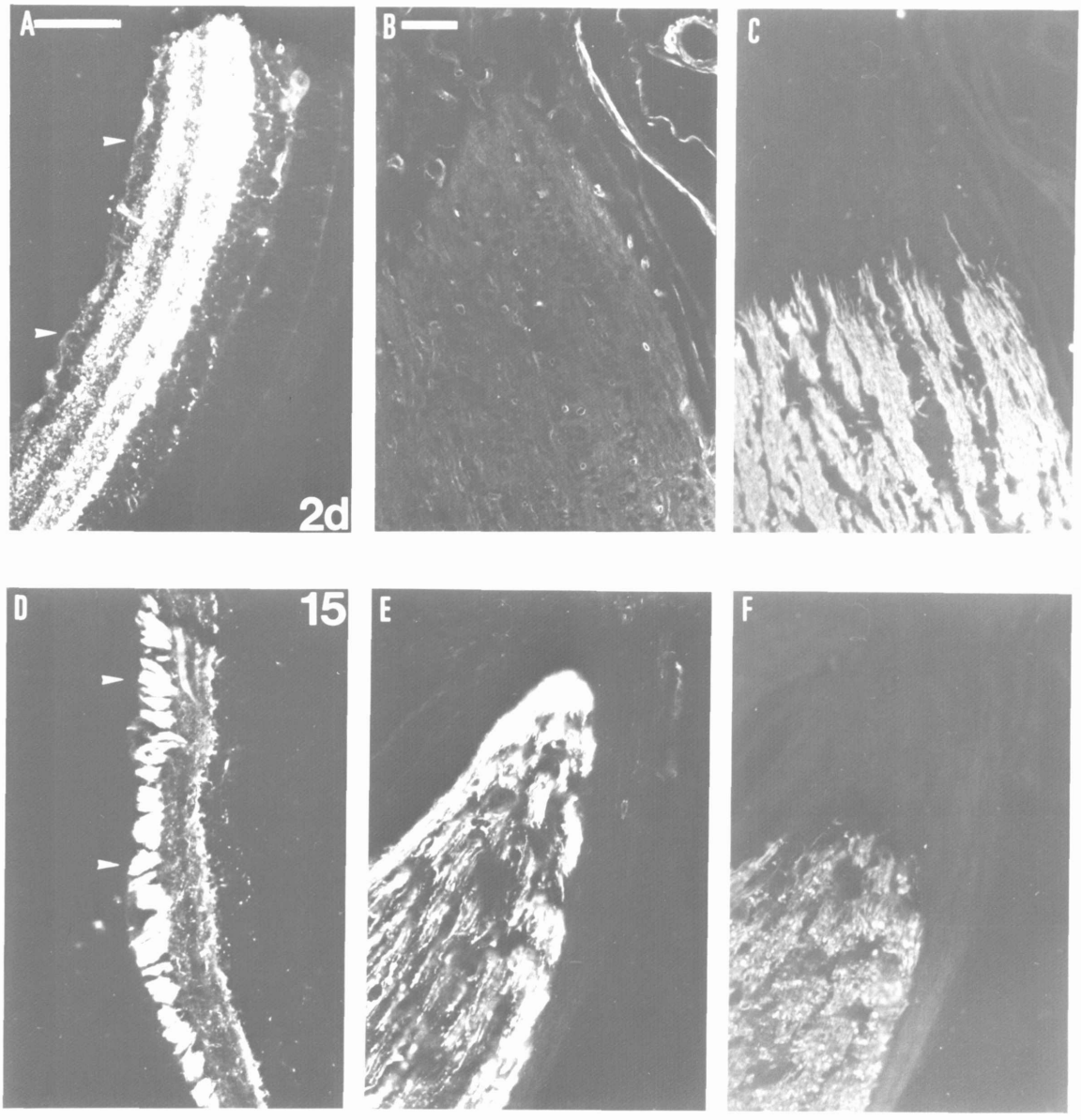


Figure 5.1.

Staining of retina and the head of the optic nerve with anti-GAP-43 antiserum (a,b,d and e). Double labelling of the optic nerve head with a monoclonal antibody against MBP is also presented (c and f).

A,B and C. 2 days after transection, GAP-43 is restricted to the plexiform layers of the retina (a) and is absent from RGC axons in the nerve fibre layer (arrowheads) and the head of the optic nerve (b). MBP is absent from the *lamina cribrosa* at the retinal tip of the optic nerve (c).

D,E, and F. 15 days after transection, GAP-43 can be seen in RGC axons in the nerve fibre layer of the retina (arrowheads) and the *lamina cribrosa* (e), which still contains no MBP (f).

Scale bars in a and b (refers also to c,d,e and f), 50 μm.

Figure 5.2.

Double labelling of the site of optic nerve transection with anti-GAP-43 fusion protein antiserum diluted 1/10,000 (a,c,e,g and i), and monoclonal antibodies against MBP (b,d,f,h and j). The retinal stump is to the left in all instances.

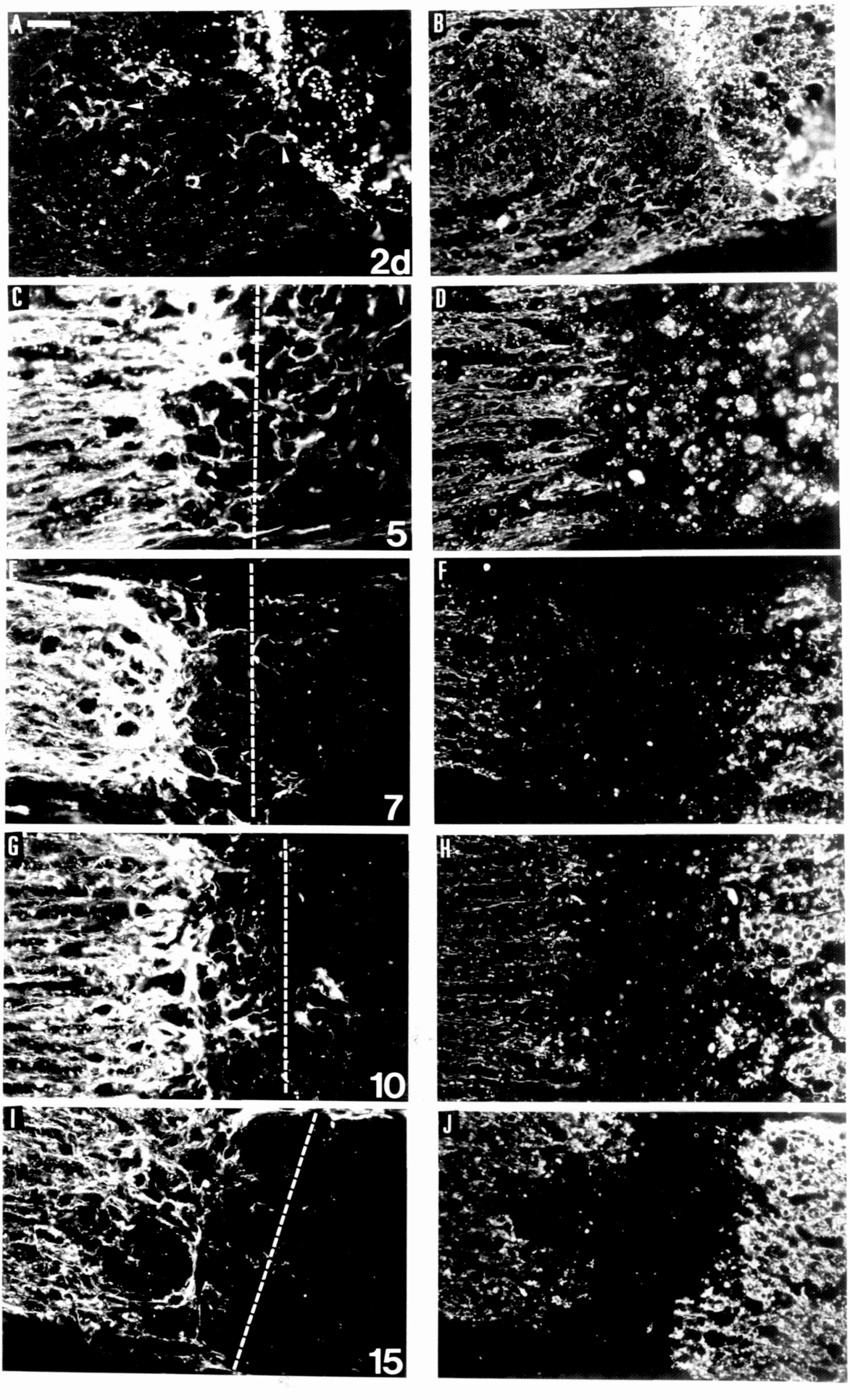
A and B. At 2 days after transection, brightly fluorescent erythrocytes mark the site of transection. A few severed axons are GAP-43+ on the retinal side of the anastomosis (arrowheads in a).

C and D. At 5 days after transection, GAP-43+ axons (c) accumulate in the retinal stump and a few appear to penetrate the degenerating myelin in the junctional zone. Site of transection is marked with a dashed line.

E,F,G and H. Although the myelin debris has been removed from the junctional zone (f and h), most of the GAP-43+ axons at 7 and 10 days after transection appear to be restricted to the retinal stump of the optic nerve (e and g), although a few still penetrate beyond the site of transection (marked with a dashed line).

I and J. At 15 days after transection, GAP-43+ axons are restricted to the retinal stump of the optic nerve and do not penetrate beyond the site of transection (marked with a dashed line).

Scale bar in a, 50 μm.


A
2d
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H
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J

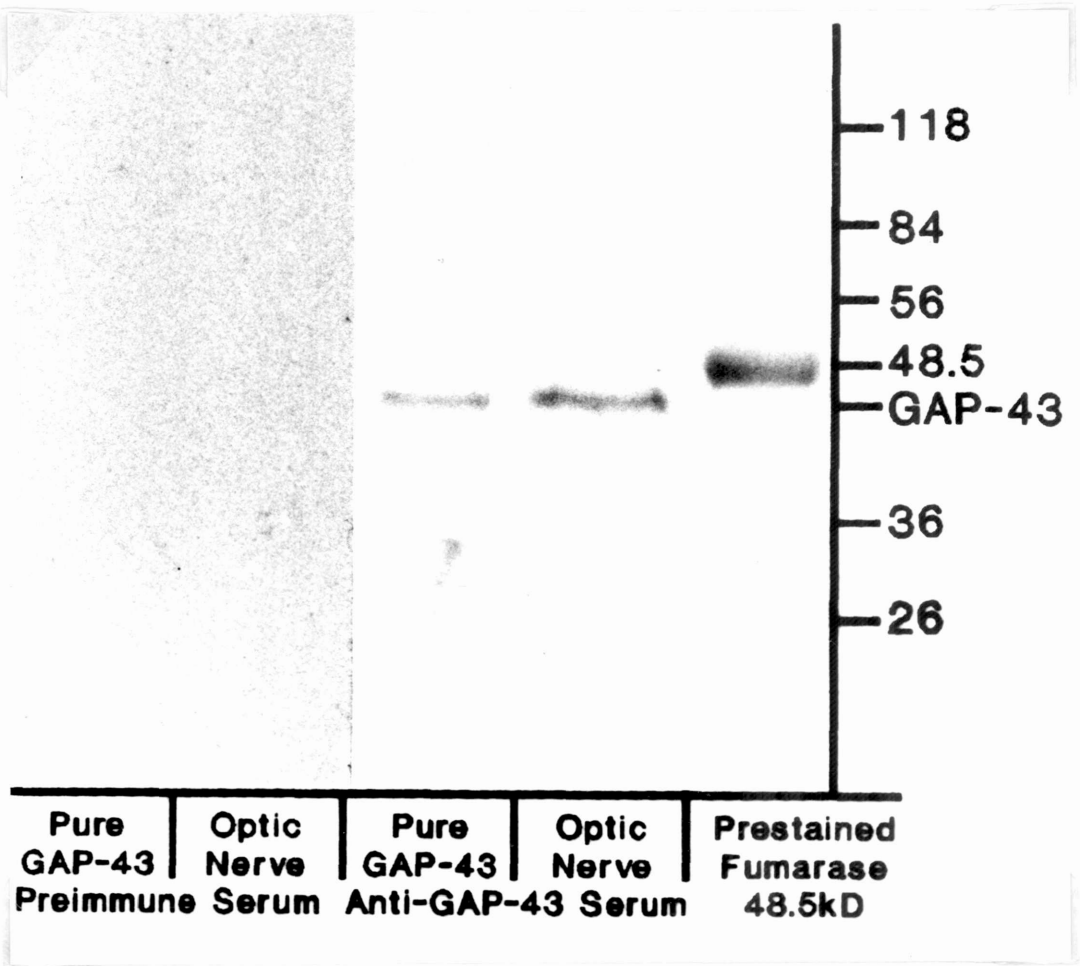


Figure 5.3.

Western blots of homogenised retinal stump of optic nerve 10 days after transection (without reanastomosis) and purified CNS GAP-43 stained with anti-GAP-43 fusion protein antiserum or pre-immune serum diluted 1/1000. The RMM of standard proteins are marked to the right and prestained fumarase (RMM 48.5 kD) is included as a reference for these.

Pre-immune serum did not recognise any proteins in these preparations. Anti-GAP-43 fusion protein antiserum only recognised GAP-43 and there were no cross-reacting proteins in the optic nerve stump.

5.2.2. Spinal Cord Studies

Initial studies were undertaken to replicate the spinal cord compression model of Guth *et al.* (1985) to produce a lesion which interrupted white matter axon tracts, but did not allow infiltration of connective tissue elements, eventually leading to spinal cord reconstruction and axonal growth. Using a 1 second compression time, it was found that complete closure of the forceps produced a total and irreparable interruption of the spinal cord (gray and white matter), which did not show any signs of reconstruction although the meninges remained intact. However, limiting the compression to a set distance (1.5 mm) for 1 second yielded highly variable results, from negligible tissue damage to almost total interruption. These studies also showed our initial assumption, that cord diameter would be constant amongst animals of the same body weight, to be incorrect.

Consequently, the compression device described in Materials and Methods was designed and constructed with the aid of Dr. R. Lindsay (Sandoz Institute, London). This enabled the cord diameter to be measured to within 0.1 mm, prior to compression by a predetermined percentage of this distance. Histological damage was found to be related to the severity of the lesion, in accordance with other models of spinal cord injury (Wrathall *et al.*, 1985). 50% compression gave minimal signs of tissue disruption, while compression by 70% of the cord diameter produced results similar to total occlusion (data not shown). However, compression by 60% of the cord width yielded an intermediate lesion essentially as described by Guth *et al.* (1985). (These authors claimed that the initial phase of axonal loss was followed by regeneration of the white matter tracts but, while we observed loss of axons in white matter tracts adjacent to the lesion, no regeneration of these pathways was evident.) Accordingly, a 60% compression for 1 second was adopted as a standard surgical trauma and the subsequent studies were all performed on animals receiving this treatment. It

should be noted that the lesion is unilateral, in that there is movement of only one blade of the forceps. Control animals received laminectomy at T5 only, without insertion of the forceps.

Post-operative degenerative changes and reconstruction of the cord were followed in animals sacrificed 1 hour, 24 hours, 48 hours, 4 days, 1 week, 2 weeks and 3 weeks after surgery, with at least 3 animals at each time. Long-term changes were also observed in animals maintained up to 12 weeks post-operative. Cords were embedded in polyester wax and 5 μm serial horizontal sections were cut. Areas of interest were identified by toluidine blue staining, and consecutive sections from these regions were subjected to silver staining (for axons) and immunostaining for GFAP (as a marker for astrocytes), MBP (as a marker for oligodendrocytes), neurofilament (as a marker for axons), Von Willebrand's Factor (as a marker for endothelial cells) and GAP-43. Anti-GAP-43 fusion protein antiserum was used at dilution of 1/30,000, which is below the limit for detecting GAP-43 in the normal adult rat spinal cord.

5.2.2.1. Development Of The Lesion

The development of the lesion was followed by silver staining for axons and by GFAP, MBP and neurofilament immunohistochemistry. All these procedures allowed clear distinction between white and gray matter and identification of damaged tissue. In addition, the site of compression could often be identified by restriction in the width of the cord which persisted for several weeks. At 1 hour post-operative, the lesion was characterised by extensive haemorrhage and accumulation of tightly-packed erythrocytes, which appeared dark in silver stained material and fluoresced brightly in immunostained sections (Figure 5.4.). The lesion was restricted to the core of the spinal cord, affecting the gray matter predominantly. The white matter was largely undamaged, although the tissue had a 'spongy' appearance (due to the

separation of axon fascicles) and reduction of neurofilament and silver staining was evident in tracts adjacent to the lesion. There were no signs of reactive gliosis and myelin was intact in adjacent white and gray matter, including the separated axon fascicles mentioned above.

At 24 hours post-operative, the lesion had become pale in silver stained material, suggesting dispersion of erythrocytes and these cells were found to be less densely packed in immunostained sections (Figure 5.5.). This revealed the presence of cavities, which were filled with the dispersed erythrocytes and a few axon fascicles of the white matter, which appeared to be myelinated. Disruption of the tissue was more apparent in the gray matter, while the white matter showed considerable structural continuity. Numerous retraction buds were visible in the white matter around the lesion in silver and neurofilament stained sections, in agreement with Cajal (1928), and the lateral funiculi were bilaterally affected by the loss of silver and neurofilament staining, suggesting axotomy of some long fibre tracts. However, other axons in the white matter remained intact. Extensive astrocytic gliosis was evident at the margins of the lesion, especially in the lateral funiculi where large reactive astrocytes could be seen extending processes diagonally towards the *glia limitans*. Reactive astrocytes could be seen in 'spongy' regions of the lateral funiculi where axonal damage had occurred. However, the distribution of myelin around the cavities appeared normal, including the axons in the 'spongy' white matter.

At 48 hours post-operative, the lesion appeared generally similar to that seen at 24 hours. Reactive astrocytes had withdrawn from the 'spongy' regions of the lateral funiculi but the myelin remained intact. Gray matter had completely degenerated within the cavities, but remaining white matter could be identified by the presence of myelinated axons. There was, however, considerable loss of axons in the white matter and many retraction buds were visible.

Figure 5.4.

1 hour after 60% spinal cord compression.

A. Full width view of a section impregnated with silver to reveal axons (x10). Scale bar, 250 μm.

B. High power view of the same section (x25, asterisk marks equivalent position in both micrographs). Scale bar, 100 μm.

Darkly stained erythrocytes reveal extensive haemorrhage predominantly in the gray matter (GM) but also in the white matter (WM). The axons of the white matter appear to be mostly intact.

C and D. Double label with monoclonal antibody against MBP (c) and polyclonal antiserum to GFAP (d). Scale bar in c, 250 μm.

Although brightly fluorescent erythrocytes can be seen, the glial response to trauma is minimal at 1 hour. Beyond the area of haemorrhage myelin distribution appears to be normal in white and gray matter and there is no evidence of astrocytic reaction.

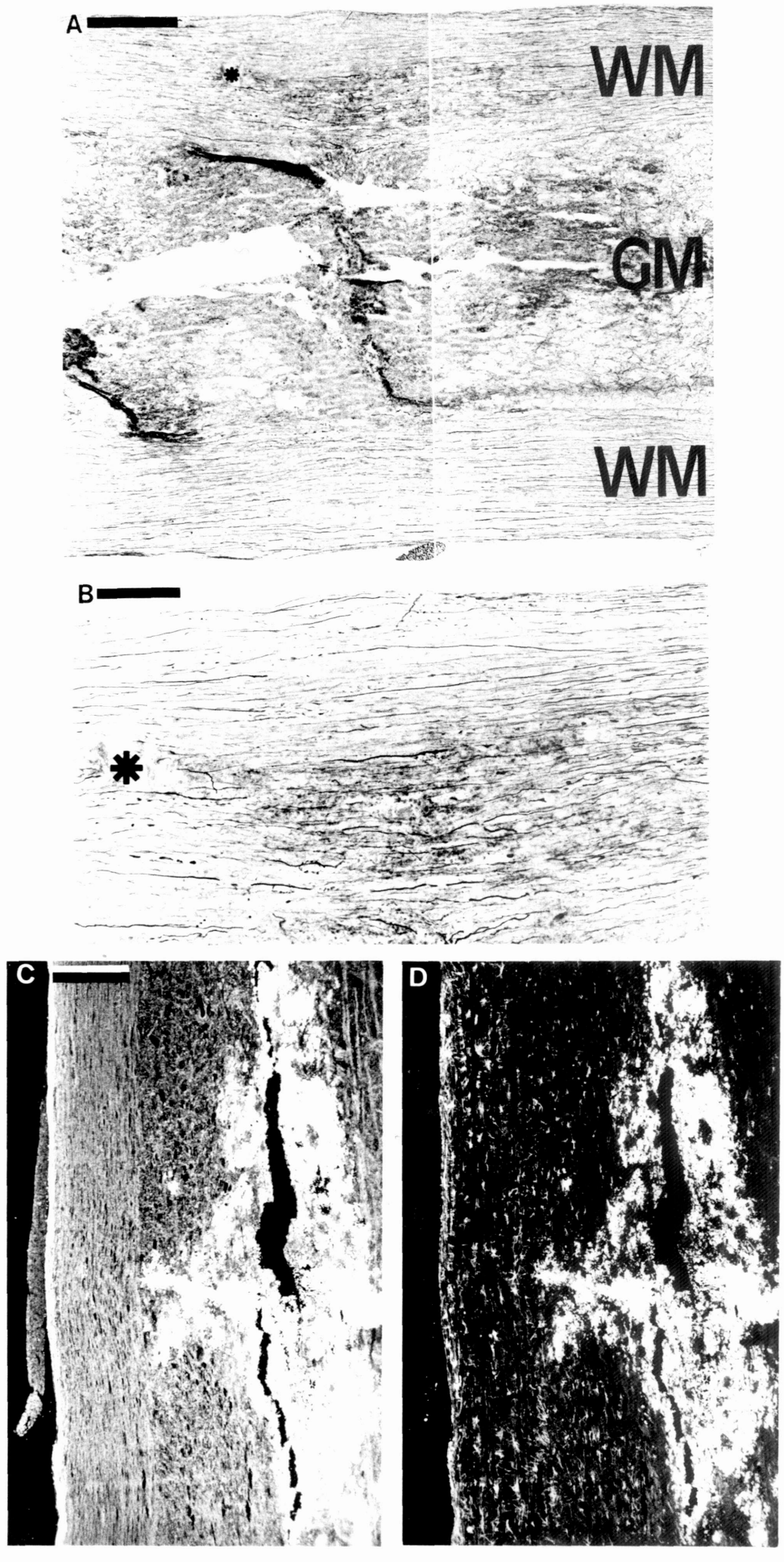

A
WM
GM
WM
B
C
D

Figure 5.5.

24 and 48 hours after 60% spinal cord compression.

A. Full width view of a 24 hours post-operative section impregnated with silver to reveal axons (x10). Scale bar, 250 μm.

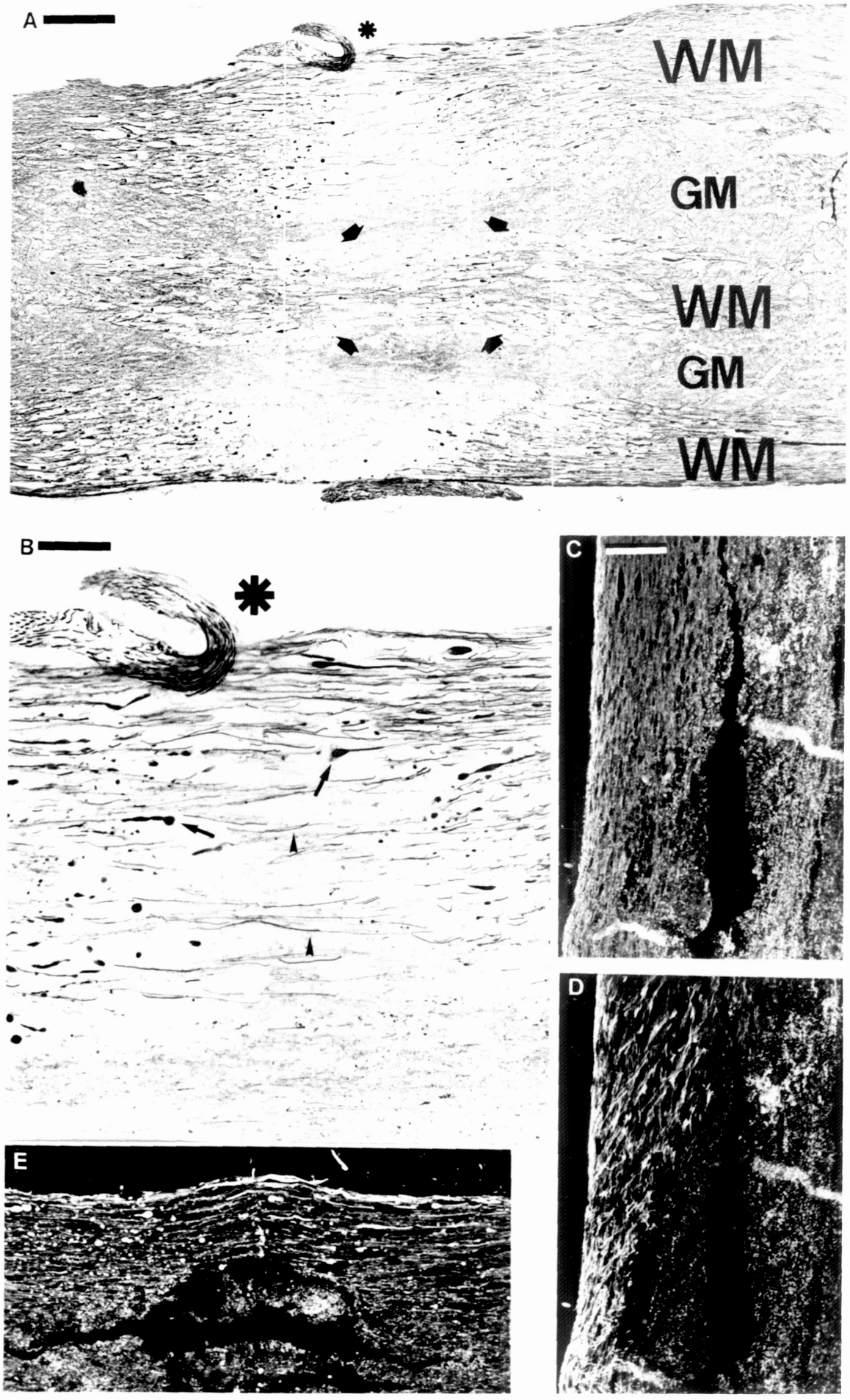
B. High power view of the same section (x25, asterisk marks equivalent position in both micrographs). Scale bar, 100 μm.

The structure of the cord is well preserved either side of the lesion site. Within the lesion, the gray matter (GM) has been destroyed and replaced by erythrocytes. The white matter (WM) is characterised by a reduction in axonal staining (silver impregnation) and the presence of numerous retraction buds (arrows in B). Many axons remain undamaged however, and pass through the lesion (arrowheads in B). The dorsal funiculus is particularly well preserved (area marked by large arrows in A).

C and D. Double label at 48 hours post-operative with monoclonal antibody against MBP (c) and polyclonal antiserum to GFAP (d). Scale bar in c, 250 μm.

An intense gliosis is evident around the lesion with reactive astrocytes extending diagonally oriented processes towards the *glia limitans* at the meningeal surface of the cord. Adjacent white matter tracts remain myelinated, including the axons in the 'spongy' tissue within the ring of reactive astrocytes.

E. Adjacent section to C and D stained with RT97 monoclonal antibodies against neurofilament protein (48 hours post-operative). Although retraction buds can be seen containing the phosphorylated RT97 epitope, some white matter axons are preserved intact adjacent to the lesion cavity. Scale as in C.


A
WM
GM
WM
GM
WM
B
C
D
E

Degenerative changes continued at 4 and 7 days post-operative, with the cavities and 'spongy' white matter becoming more defined (Figure 5.6.). Retraction buds were apparent at the margins of the cavities, particularly in the white matter. Most of the erythrocytes had disappeared and the cavities were now filled with larger round cells, which were translucent in silver stained material and non-fluorescent in immunostained sections. These most likely correspond to the mononuclear cells observed by Guth *et al.* (1985) in cavities resulting from spinal cord compression. Reactive astrocytes were occasionally present within the cavities which were bounded by an intense astrocytic gliosis, although the adjacent 'spongy' white matter remained free of astrocytes. Axons and myelin were still intact in these regions, existing inside the ring of reactive gliosis. Interestingly, the processes of the reactive astrocytes had become more longitudinally aligned by 7 days.

The extent of the lesion was highly variable, comprising one or more cavities. Single cavities were in the order of 1 mm long and wide, but multiple cavity lesions extended for as much as 10 mm. Notably, white and gray matter were not disrupted away from the vicinity of the cavities, except where white matter tracts were interrupted by two cavities and the intervening segment contained many retraction buds and much axonal debris.

Figure 5.6.

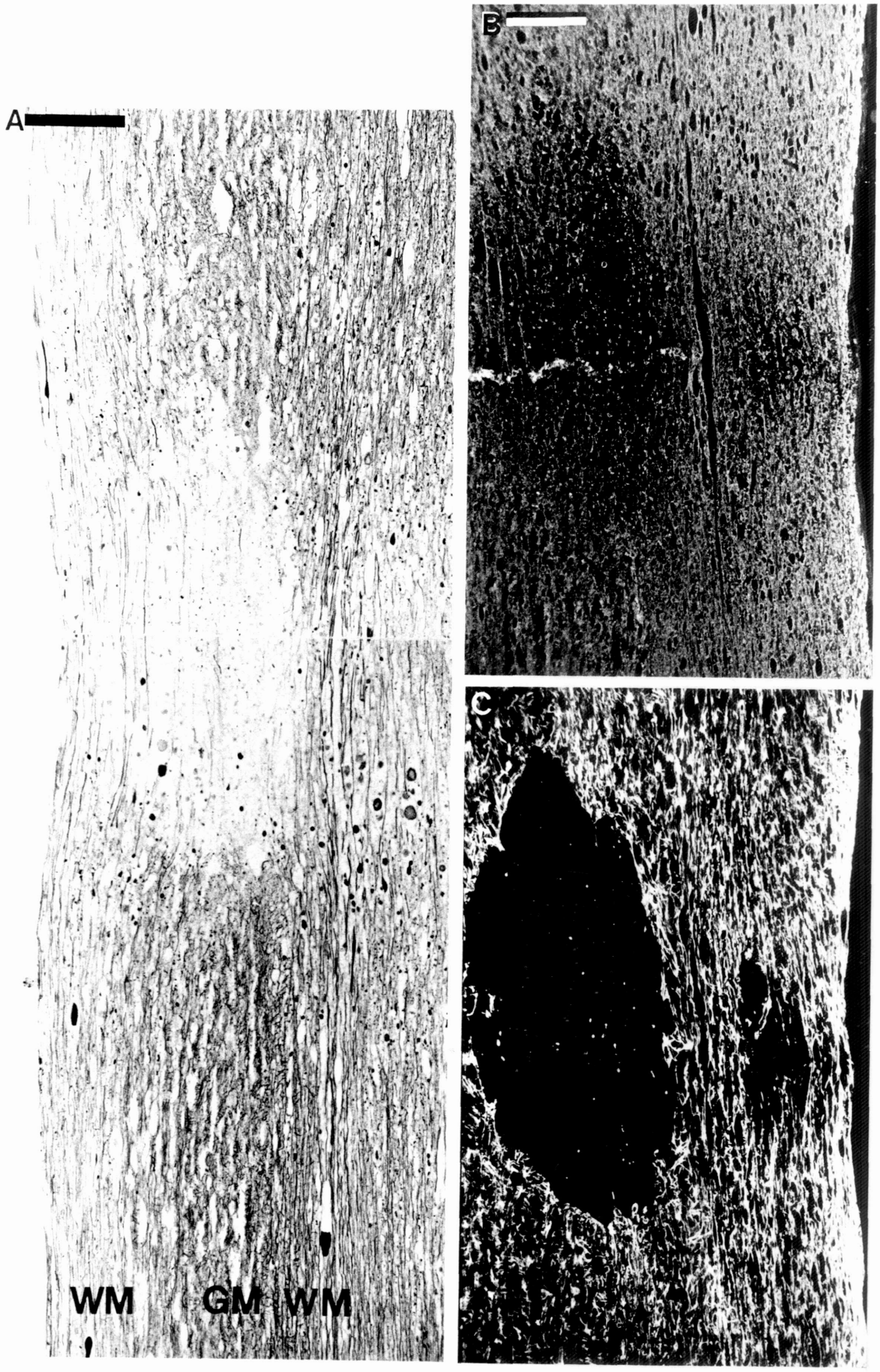
7 days after 60% spinal cord compression.

A. 3/4 width view of a section impregnated with silver to reveal axons (10x lens). Scale bar, 250 μm.

Gray matter (GM) cavities have become well defined and white matter (WM) contains axonal debris and retraction buds, although some axons appear to remain intact.

B and C. An adjacent section to A (from the same animal) double labelled with monoclonal antibody against MBP (b) and polyclonal antiserum to GFAP (c). Scale bar in b, 250 μm.

The cavity is bordered by normally-distributed myelin and densely packed reactive astrocytes in white and gray matter. Some myelinated axons persist inside the border of astrocytes. Note that the astrocytic processes have become predominantly longitudinal in orientation.


A
B
C
WM
GM
WM

At 2 and 3 weeks post-operative, degenerative changes appeared to have ceased and there was some evidence of reconstruction, although less than suggested by Guth *et al.* (1985). Astrocytes began to repopulate the areas of 'spongy' white matter, so that by 3 weeks the gray matter cavities were bounded by intact myelinated white matter tracts of the lateral funiculi containing large numbers of reactive astrocytes (Figure 5.7.). The dorsal and ventral funiculi appeared to consist largely of uninterrupted axons, leaving parallel cavities in the gray matter separated by strips of white matter. Some axons penetrated the ring of gliosis and entered the cavity, which contained mononuclear cells engorged with axonal and myelin debris. Structures resembling growth cones were seen adjacent to, and in some instances inside, the cavities. However, it was not possible to distinguish unambiguously these structures from retraction buds, which show similar morphology to growth cones in the light microscope.

The lesion remained essentially static at later stages with rings of intense gliosis persisting around one or more cavities up to 12 weeks post-operative.

Control animals, which received only T5 laminectomy, mostly showed no signs of histological damage and appeared quite normal in silver stained material and immunostained sections at all time points. Both white and gray matter remained intact and there was no astrocytic reaction. In a few cases, however, morphological damaged was noticed which was associated with loss of axons and reactive gliosis. This was probably due to contact of the scissors with the cord during the laminectomy.

Figure 5.7.

3 weeks after 60% spinal cord compression.

A. Full width view of a section impregnated with silver to reveal axons (x10). Scale bar, 250 μm.

B. High power view of the same section (x40 oil immersion phase contrast, asterisk marks equivalent position in both micrographs). Scale bar, 50 μm.

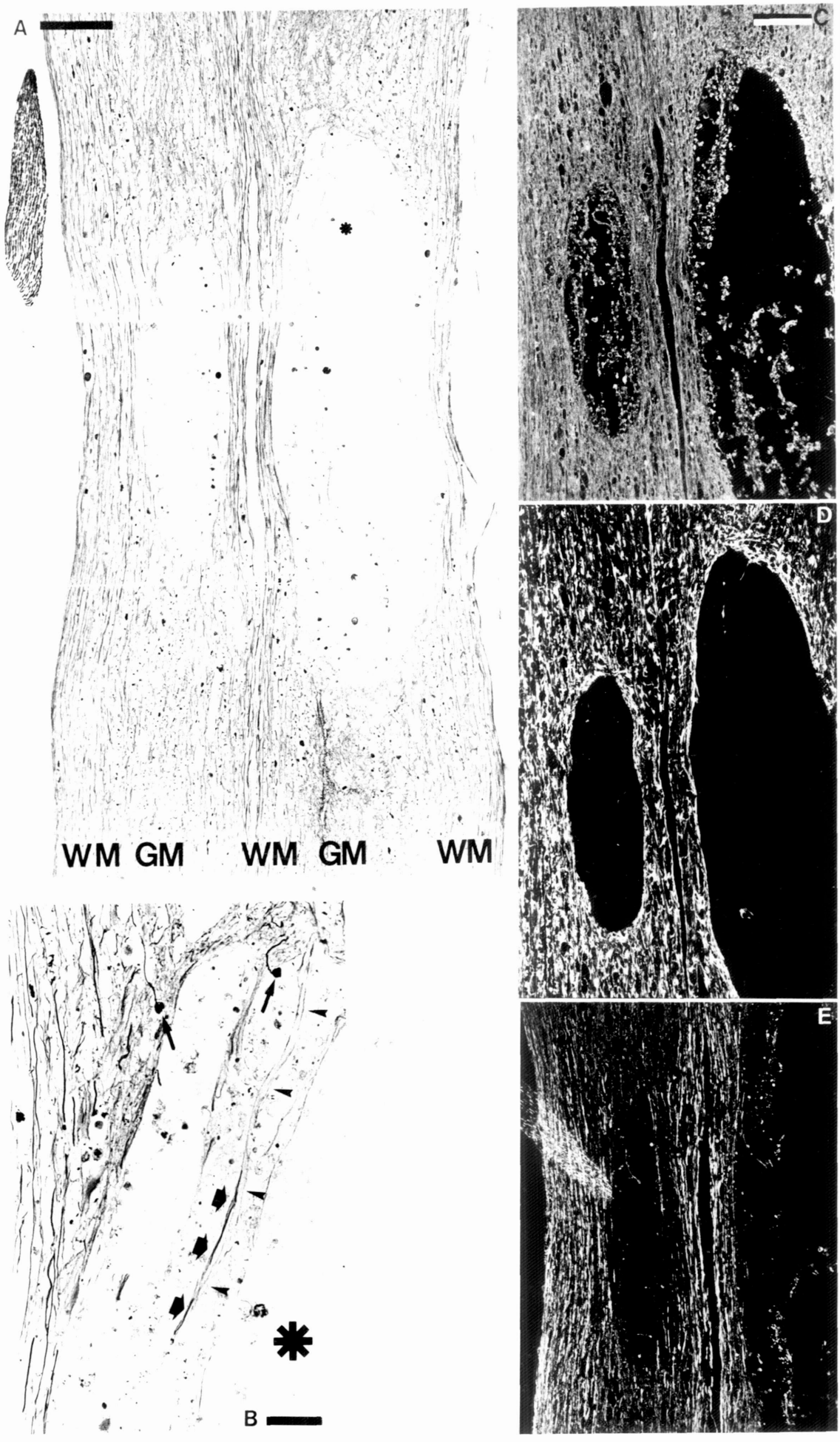
Gray matter (GM) cavities are bordered by normal-looking gray and white matter. Axons remaining in white matter (WM) tracts appear to be uninterrupted and span the lesion site. Two potential growth cones are visible at the gray matter boundary of one cavity (small arrows in b). Axons inside the cavity (large arrows) can be seen running along a tubular structure (arrowheads, possibly a capillary) made visible with phase contrast optics.

C,D and E. Adjacent sections to A (from the same animal).

C and D. Section double labelled with monoclonal antibody against MBP (c) and polyclonal antiserum to GFAP (d). Scale bar in c, 250 μm.

Myelin appears to be distributed normally around the cavities, which are bordered by a dense ring of reactive astrocytes.

E. Stain for axonal neurofilament protein (RT97 monoclonal antibody) showing that intact axons traverse the lesion site in the white matter, same scale as C.


A
WM GM WM GM WM
B
C
D
E

5.3.2.2. Expression Of GAP-43 After 60% Spinal Cord Compression

Previous studies have shown that the levels of GAP-43 in the normal adult spinal cord can be visualised with the anti-GAP-43 fusion protein antiserum at a concentration of 1/10,000 and that staining is lost by further dilution to 1/20,000 (see Chapter 4). Therefore, elevated expression of GAP-43 by regenerating neurons was monitored by the use of the antiserum at a dilution of 1/30,000. Two changes were noted in the distribution of GAP-43 after spinal cord compression. First, neuronal perikarya in the gray matter adjacent to the cavities became GAP-43 immunoreactive (and some also expressed RT97 staining), and secondly, GAP-43+ axons were visible around, and sometimes within, the lesion (Figure 5.8.).

GAP-43 was first apparent at 2 days after surgery in neuronal soma in the gray matter adjacent to the lesion and by 4 days post-operative a regenerative response was evident by the expression of GAP-43 in axons around the cavities. GAP-43+ cell bodies were restricted to the gray matter within 1 mm of cavities and in many instances these also contained RT97 immunoreactive neurofilament protein, and therefore probably represented neurons axotomised by the physical trauma of the surgery (Goldstein *et al.*, 1987; Shaw *et al.*, 1988; Mansour *et al.*, 1989). These GAP-43+ perikarya became more numerous by 7 days post-operative, but then began to decrease in number and staining intensity so that they were not detected after 3 weeks.

GAP-43+ axons (containing RT97+ immunoreactivity) were present in the white and gray matter at the margins of the cavities, and could be seen in white matter tracts several millimetres from the lesion. By 7 days post-operative, GAP-43+ axons could be seen entering the cavities and these increased in number up to 3 weeks after surgery (Figure 5.9.a, b). However, most axons in the white matter tracts at the edges of the cavities did not contain elevated levels of GAP-43 and were therefore probably spared from axotomy by the physical trauma of the surgery

(Figure 5.9.c, d). This is consistent with the neurofilament and silver staining data presented earlier, which demonstrated that white matter remained largely intact. These GAP-43+ axons persisted inside the cavities and at their edges up to 12 weeks post-operative, showing that these regenerative sprouts survive for considerable periods of time. However, bundles of GAP-43+ axons could not be traced around or through the lesions, suggesting that successful regeneration had not occurred and that the axons observed by Guth *et al.* (1985) to be bridging the cavities had probably been spared from axotomy by the surgical trauma (see Discussion).

In the majority of control animals, elevated GAP-43 was not detected in neuronal cell bodies or axons. However, elevated GAP-43 levels were associated with those animals showing signs of morphological damage. It is likely that these animals had received uncontrolled spinal cord trauma during laminectomy, resulting in some axotomy and subsequent GAP-43 up-regulation.

Figure 5.8.

GAP-43 expression (revealed with anti-GAP-43 fusion protein antiserum diluted 1/30,000) in neuronal perikarya and axons 1-2 weeks after 60% spinal cord compression.

A and B. Double labelling of two cell bodies in gray matter adjacent to a cavity 1 week post-operative with anti-GAP-43 antiserum (a) and RT97 monoclonal antibody (b) against neurofilament protein. Scale bar in a, 50 μm.

C and D. An axon in 'spongy' white matter (arrowheads) adjacent to a cavity at 1 week after surgery contains both GAP-43 (c) and RT97 (d) immunoreactivities. Note the presence of GAP43+ club-like ending with filopodia (arrow), which may represent a growth cone. Scale bar in c, 50 μm.

E and F. Axons and a cell body (arrowhead) expressing GAP-43 (e) in the gray matter 2 weeks post-operative. Note that the cell body also contains RT97+ neurofilaments. Scale bar in e, 50 μm.

G. Two GAP-43+ axons terminate at the boundary of a large cavity at 2 weeks after surgery. Scale bar, 50 μm.

A
B
C
D
E
F
G
CAVITY

5.2.2.3. Characterisation Of Axonal Substrates

Using the presence of elevated GAP-43 to identify elongating axons, an attempt was made to establish the substrates for axonal outgrowth in the adult CNS. Guth *et al.* (1985) proposed that, in the absence of glial-mesodermal scar formation, longitudinally oriented ependymal cells, astrocytic processes or capillary endothelia formed the 'specific cellular terrain for growth of axons into a spinal cord lesion'. To address the role of astrocytes in axonal elongation, double labelling was performed with anti-GAP-43 fusion protein antiserum (diluted 1/30,000) and monoclonal antibodies against GFAP. Due to the preservation of numerous axons and the extensive gliosis around the lesion, analysis of GAP-43 and GFAP staining patterns was only feasible within the cavities. Here, most GAP-43+ axons could be clearly seen in the absence of astrocytic processes (see Figure 5.9.e, f), although sometimes GFAP+ processes were associated with these growing axons. This does not, therefore, exclude a positive role for astrocytes in the guidance of axon sprouts, but strongly suggests that other cellular elements can provide appropriate substrates.

In some instances, axons within the lesion cavities were seen to be in apposition to elongated tubular structures (see Figures 5.7.b and 5.9.f), probably corresponding to the newly-formed capillaries which infiltrate the lesion site (Guth *et al.*, 1985). Capillary endothelia are known to proliferate and migrate in response to CNS trauma (Orita *et al.*, 1989) and also to possess several adhesion molecules on their surface (Pákáski *et al.*, 1990; Unemori *et al.*, 1990). To investigate this further, double labelling was attempted with the 91E12 monoclonal antibody against GAP-43 (Goslin *et al.*, 1988) and polyclonal antiserum against Von Willebrand's factor (factor VIII-related antigen) which is a marker for endothelial cells (Sehested *et al.*, 1981). Unfortunately, the protease treatment required to visualise Von Willebrand's factor in paraformaldehyde-fixed polyester wax-embedded tissue was not compatible

with maintenance of the 91E12 epitope, so double labelling was not feasible and it could not be excluded that the tubular structures in association with GAP-43+ axons were composed of ependymal cells rather that epithelia. However, the distribution of Von Willebrand's factor revealed the nature of vascular changes in the compressed cord to be consistent with a role in directional axonal guidance through the lesion (data not shown).

In normal unoperated animals, there was a distinctive pattern of staining associated with the capillary endothelia. Gray matter contained a large number of apparently randomly-oriented blood vessels, evidenced by rings of immunoreactivity. There were fewer capillaries in the white matter but these were more longitudinally oriented, although it was difficult to follow these vessels for any distance indicating a somewhat erratic course through the tissue. By 24 hours post-operative, substantial changes were evident indicating neovascularisation towards and into the lesioned area. In the gray matter, capillaries had become more longitudinally oriented while the white matter contained an increased number of longitudinal vessels. In addition, large branching vessels were seen obliquely penetrating the white matter. This trend continued at 2, 4 and 7 days post-operative when increasing numbers of large longitudinal capillaries could be seen traversing the lesion site in the 'spongy' white matter tracts and entering the cavities.

Figure 5.9.

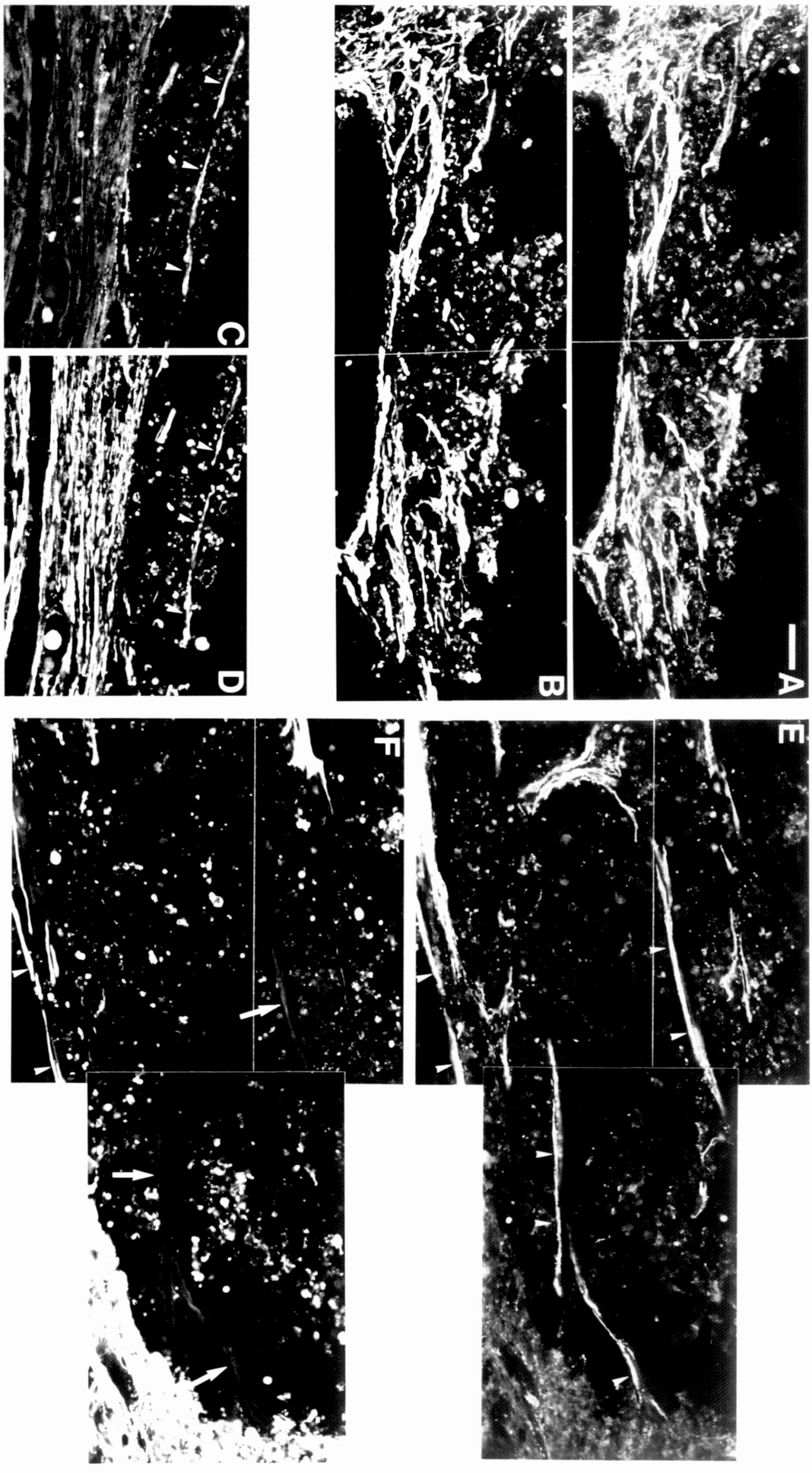
Expression of GAP-43 (revealed with anti-GAP-43 fusion protein antiserum diluted 1/30,000) by axons inside lesion cavities at 2 and 3 weeks after 60% spinal cord compression.

A and B. Double labelling of a bundle of axons with anti-GAP-43 antiserum (a) and RT97 monoclonal antibodies against neurofilament protein (b). All RT97+ axons in the cavity at 3 weeks after surgery are also GAP-43+.

C and D. Axons in a 3 weeks post-operative cavity (arrowheads) contain GAP-43 (c) and RT97 (d) immunoreactivities, while undamaged RT97+ axons in the 'spongy' white matter at the edge of the cavity (bottom half of field) do not contain GAP-43.

E and F. Double labelling of axons in a 2 weeks post-operative cavity with anti-GAP-43 antiserum (e) and monoclonal antibodies against GFAP (f). GAP-43+ axons (arrowheads in e) are mostly associated with GFAP- tubular structures (possibly capillaries, arrows in f), although a few astrocytic processes (arrowheads in f) appear to be co-localised with some axons.

Scale bar in a, 50 μm.


A
B
C
D
E
F

5.3. Discussion

5.3.1. Optic Nerve Studies

5.3.1.1. GAP-43 Expression In Non-Regenerating Retinal Ganglion Cells

Immunoreactive GAP-43 is essentially absent from RGC axons in the normal retina and optic nerve. At 2 days after transection, no GAP-43 could be visualised in the nerve fibre layer of the retina and the *lamina cribrosa* suggesting that axonal transport of GAP-43 by RGCs had not increased at this stage. However, GAP-43 could be detected at the distal tips of these transected axons in the retinal stump of the optic nerve. This could be due to accumulation in the severed axons of GAP-43 which is known to be synthesised and anterogradely transported in small amounts in the RGCs of normal animals (Skene and Willard, 1981b). The dilution of anti-GAP-43 fusion protein antiserum for immunohistochemical staining of these small amounts was not determined. It is sufficient to note that the dilution used in this study (1/10,000) was able to discriminate between the levels of GAP-43 in the normal adult optic nerve and higher levels induced by RGC axotomy. From 5 days after transection, GAP-43 could be visualised throughout the entire extent of the RGC axons, from the nerve fibre layer in the retina to the anastomosis site. In the absence of a peripheral nerve graft, GAP-43+ regenerative sprouts, which at 5 days after transection extended across the anastomosis and into the distal stump, subsequently disappeared and by 15 days after transection GAP-43 became restricted to the retinal stump. This is consistent with the degeneration of initially regenerating axons after optic nerve section, as noted by Cajal (1928). Anastomosis of a peripheral nerve segment, however, enabled these GAP-43+ regenerative sprouts to persist and elongate beyond the site of transection (data not shown).

The nature of the GAP-43 immunoreactivity expressed by adult RGCs

was investigated in Western blots of proteins from optic nerve after transection without anastomosis. Only GAP-43 was detected in these blots and there were no cross-reacting protein bands.

It is unclear from the limited time course of these studies how long GAP-43 would remain elevated in the transected RGC axons. The regulation of GAP-43 by intrinsic and environmental factors is incompletely understood, but studies of peripheral nerve (Bisby, 1988) have shown that GAP-43 remains elevated for over 100 days if regeneration is impeded whereas levels return to control values over this period if regeneration is allowed to proceed. This suggests that GAP-43 synthesis may be regulated by a target-derived factor(s), although this does not appear to be NGF (Verge *et al.*, 1990).

It has been proposed that neurons of the CNS do not respond to axotomy by increased synthesis and axonal transport of GAP-43 and that this underlies the failure of these cells to regenerate successfully (Skene and Willard, 1981b; Skene, 1984). The results presented here show that GAP-43 becomes detectable by immunocytochemical means within 5 days of optic nerve transection. The failure of Skene and Willard (1981b) to detect changes in axonally-transported GAP-43 after optic nerve lesion may be due to the nature (crush) or site of the injury (near the optic chiasm) being inadequate to promote a somatic reaction from the RGCs. Aguayo and colleagues have suggested that the proximity of the axotomy site to the cell body determines the ability of central neurons to mount a regenerative response (Richardson *et al.*, 1984; Bray *et al.*, 1987).

The elevation of GAP-43 in axotomised RGCs shows that at least some CNS neurons are capable of responding to traumatic injury by recapitulating developmental events necessary for axon extension. This suggests that both the 'GAP Hypothesis' and the 'Inherent Incapacity Hypothesis' are inadequate to explain the failure of regeneration in these neurons, and further supports the 'Neural Environment Hypothesis'.

It seems, therefore, that certain elements of the adult CNS are unfavourable for axonal elongation, leading to the degeneration of GAP-43+ axon sprouts after optic nerve transection, whereas elements in the PNS graft allow the RGCs to regenerate axons beyond the transection site (So and Aguayo, 1985; Berry *et al.*, 1988b). Up-regulation of GAP-43 after RGC axotomy seems to be an intrinsic response, but the success of regeneration is determined by extrinsic factors.

The intrinsic ability of different populations of neurons to up-regulate synthesis and transport of GAP-43 may vary, and this may explain the inconsistent capacity of axotomised central neurons to regenerate into peripheral nerve grafts (Friedman and Aguayo, 1985; Bray *et al.*, 1987). Alternatively, populations with higher 'basal' levels of GAP-43 may show greater regenerative propensity, such as the mitral cells of the olfactory bulb (Friedman and Aguayo, 1985; Rosenthal *et al.*, 1987) and neurons of Clarke's column in the spinal gray matter (Richardson *et al.*, 1984; this Thesis, Chapter 4). It is interesting in this regard that GAP-43 staining is increased in the central terminals of primary sensory neurons in the superficial laminae of the spinal gray matter after damage to the peripheral branches of these neurons (Woolf *et al.*, 1990), a manipulation which also increases the regenerative propensity of these central axons (Richardson and Verge, 1986).

5.3.2. Spinal Cord Studies

5.3.2.1. Re-expression Of GAP-43 After 60% Spinal Cord Compression

As discussed in Chapter 4, the anti-GAP-43 fusion protein antiserum can be used to distinguish areas of locally high GAP-43 concentration by dilution beyond the detection limits of the levels which exist in the normal adult CNS. The dilution used here (1/30,000) did not yield any GAP-43 immunostaining in normal spinal cord sections (unoperated animals) or in the majority of laminectomy-only control

sections. However, supranormal levels of GAP-43 were detected 2-4 days after 60% spinal cord compression and this was assumed to represent up-regulation of GAP-43 synthesis and axonal transport after axotomy, as has been reported for axotomised neurons in the mammalian PNS and inframammalian CNS (Skene and Willard, 1981a, b; Verhaagen *et al.*, 1986). This is in agreement with the up-regulation of GAP-43 in axotomised RGCs, which occurred between 2 and 5 days after optic nerve transection.

Furthermore, GAP-43 was observed both in neuronal perikarya and axons. Although GAP-43 mRNA is increased in neuronal cell bodies after axotomy in the PNS (Basi *et al.*, 1987; Verge *et al.*, 1990), this is the first report of GAP-43 *protein* accumulating in the soma of either central or peripheral neurons. This accumulation may be due to axotomy close to the cell body, which is followed by up-regulation of GAP-43 synthesis in the absence of a target for axonal transport, and may persist until a 'critical length' of axon is available for transport away from the soma. GAP-43+ perikarya were only found immediately adjacent to lesion cavities, consistent with this notion of proximal axotomy. In addition, many of these soma contained the phosphorylated neurofilament epitope recognised by the RT97 monoclonal antibody, confirming the neuronal identity of these cell bodies and indicating recent axotomy. Neurofilament phosphorylation is usually restricted to axons, but axotomy leads to the transient detection of phosphorylated epitopes (including that recognised by RT97) in perikarya of peripheral (Shaw *et al.*, 1988) and spinal cord neurons (Goldstein *et al.*, 1987; Mansour *et al.*, 1989). It has also been suggested that accumulation of phosphorylated neurofilament epitopes is proportional to the proximity of axotomy to the cell body (Goldstein *et al.*, 1987). Both GAP-43 and RT97 immunoreactivity diminished in cell bodies by 3 weeks after surgery, suggesting that these neurons had either died or successfully regenerated axons, consistent with previous studies of neurofilament

phosphorylation (see above references).

GAP-43+ axon sprouts were frequently seen in the vicinity of the lesion and were occasionally observed to circumnavigate the cavities in the relatively undamaged 'spongy' white matter tracts. However, most of the axons in these tracts did not contain elevated levels of GAP-43 and were probably spared from axotomy. This is in accordance with the anatomical data derived from silver and neurofilament stained sections which showed that much of the white matter remained intact, although there was some loss of axons. GAP-43+ axons were also found *within* the cavities, and these increased in number up to 3 weeks, suggesting that some axons were able to regenerate through the surrounding gliotic tissue. These axons persisted inside and around the cavities up to 12 weeks after surgery, but did not increase further in number or appear to elongate past or through the cavities. These observations are discussed below in terms of spinal cord regeneration in the compression model.

5.3.2.2. Spinal Cord Regeneration

The report of Guth *et al.* (1985) was the first to indicate significant growth of central axons after spinal cord injury, without pharmacological intervention or peripheral nerve grafting. This was based on morphological observations using conventional histological stains to identify various tissue elements. The axons visualised in the lesion at 2 and 3 weeks post-operative were assumed to represent new growth, possibly by regeneration of severed axons. Our study of this model was undertaken initially to determine by immunohistochemical means the cellular elements with which these sprouting axons were associated. It was found that the severity of the lesion was a critical factor determining the tissue damage and any subsequent reconstruction of the spinal cord. Specifically, compression in excess of 70% produced total and irreversible destruction of white and gray matter. 60% compression was found to produce cavity formation in the gray matter and loss of

some white matter axons. Although this seemed to replicate the model of Guth *et al.* (1985), an obvious problem was that such a 'sub-total' lesion spared large numbers of white matter axons from axotomy, which could not be distinguished from potentially regenerating axons by the histological and immunochemical stains available. Consequently, GAP-43 was selected as a suitable marker for newly sprouting axons for the reasons discussed in Chapter 3 (see Section 3.1. for references), leading to the studies reported here.

It is clear from the silver and neurofilament staining of sections at various times after surgery, and from the distribution of GAP-43+ axons, that regenerative sprouting in the spinal cord compression model is less substantial than previously thought (Guth *et al.*, 1985) and is probably abortive in nature (see Cajal, 1928). It seems likely that the longitudinal nerve fibres observed by Guth *et al.* (1985) to be crossing the lesion had in fact been spared from axotomy by the surgical trauma. Data presented here show that white matter tracts persisted from 1 hour post-operative until 3 weeks, and that the dorsal and ventral funiculi separated gray matter cavities, forming the apparent 'bridges' described by Guth *et al.* (1985). Most axons in these white matter tracts did not show elevated GAP-43 levels, suggesting that they were not sprouting and had indeed been spared from axotomy.

Spinal cord neurons were shown, however, to be capable of mounting a regenerative response to axotomy by the appearance of GAP-43 in both neuronal soma and axons. This response seems to have been abortive by the apparent failure of axons to elongate around or through the cavities that formed. Although it is possible that GAP-43 may have been down-regulated in those axons which crossed the lesion site, considerable experimental evidence suggests that GAP-43 persists in the shafts of elongating axon until regeneration is complete and contact has been made with target cells (Verhaagen *et al.*, 1986; Baizer and Fishman, 1987; Bisby, 1988; Meiri *et al.*, 1988; Savage *et al.*, 1990). The inability to

trace GAP-43+ axons through the lesion site at any time point suggests that regeneration had not occurred. This further suggests that despite the regenerative reaction to axotomy, the environment of the CNS is not conducive to axonal elongation even in the absence of glial-mesodermal *scarring*.

Although it was not possible to reach a definitive conclusion, some progress was made towards identifying the cellular substrates for axonal elongation after spinal cord compression. Morphological observations suggested that, in many cases, GAP-43+ axons in the cavities were associated with capillary endothelia involved in the neovascularisation of the lesion site. Neovascularisation is known to occur in models of CNS injury including spinal cord compression (Guth *et al.*, 1985; Orita *et al.*, 1989; Shigematsu *et al.*, 1989; Gelderd, 1990) and regenerating axons have been shown to associate with capillaries in collagen implants placed in spinal cord lesions (Gelderd, 1990). Immunohistochemical identification of endothelial cells partially confirmed this suggestion by showing that capillaries in white and gray matter around the lesion become more longitudinally oriented and penetrate the cavities, although double labelling with GAP-43 was not possible for technical reasons.

Moreover, successful double labelling experiments with GAP-43 and GFAP showed that elongating axons were often not in association with astrocytic processes. As the role of astrocytes in the failure of recovery from CNS injury is currently unknown (see Section 1.2.4.4.), it is significant that astrocyte surfaces did not appear to promote axonal outgrowth *in vivo*, in contrast to their neuritogenic properties *in vitro* (Lindsay, 1979, 1986; Noble *et al.*, 1984; Neugebauer *et al.*, 1988). However, reactive astrocytes do not seem to form a totally impenetrable barrier to axons as the GAP-43+ sprouts inside the cavities must have grown through the border of gliotic tissue.

5.3.2.3. Criticisms Of The Compression Model

The lack of agreement between this study and that of Guth *et al.* (1985) invites criticism of the compression model of spinal cord trauma. This model was chosen for the property of excluding connective tissue elements from the damaged CNS and for the reported ability to sustain axonal regeneration. One major drawback of the model is the large variability in the lesion produced by compressive injury, even with the precision-timed 'percentage' compression employed here. Although 60% compression inevitably yielded cavity formation within the cord, the number and size of the cavities both varied so that lesions ranged from 1 to 10 mm in length. One can only assume that the manually-timed compression inflicted by Guth *et al.* (1985) with hand held forceps was inherently even more variable. Their observation of axonal growth through the lesion was based on comparison of histologically stained sections from animals sacrificed at increasing times after surgery. In view of the variable nature of the lesion and in the absence of corroborating immunohistochemical data, this conclusion no longer seems tenable.

Secondly, any model of axonal regeneration should ideally guarantee that a defined population of neurons are axotomised and offer a means of tracing the regrowth of their axons. Unfortunately, the compression model satisfies neither of these criteria. Transection models are more commonly used, but these permit invasion of fibroblasts and axotomised neurons show limited regeneration in the absence of PNS transplants. GAP-43 may be useful in evaluating the regenerative response of central neurons after transection.

Thirdly, no good surgical control exists for this lesion. Laminectomy only, without insertion of the forceps, was chosen to differentiate between the effects of physical injury (compression) and reduced blood flow (which has been shown to decline along the entire length of the spinal cord after laminectomy, Anderson *et al.*, 1978). In

most instances this resulted in no histological damage to the spinal cord and no detectable elevation of GAP-43, but in a few cases both tissue damage and elevated GAP-43 were noted. This is most likely due to accidental compression of the cord during the laminectomy procedure. This may partially explain the variability in the morphology of the compressed cords.

5.3.3. Concluding Remarks

The studies reported in this Chapter utilised the anti-GAP-43 fusion protein antiserum to show the response of CNS neurons to traumatic lesions. The optic nerve provided a discrete population of neurons which could be totally axotomised. GAP-43 was found to be up-regulated by the RGCs and could be visualised throughout the RGC axons regardless of the success of regeneration. This suggests the 'axotomy signal' is correctly transduced into a regenerative response in these cells, at least with respect to GAP-43 synthesis and transport. It would seem that the environment in the damaged CNS restricts the outgrowth of axons leading to abortive regeneration, although it cannot be excluded that some other element of the regenerative 'machinery' is regulated by Schwann cells allowing regeneration into grafted peripheral nerves.

GAP-43 expression was also investigated in a model of spinal cord compression. While it is of interest that elevated GAP-43 was noted both in neuronal soma and axons, these results must be interpreted with caution. First, up-regulation of GAP-43 synthesis only demonstrates a regenerative response by axotomised neurons, rather than the success of axonal regeneration, as shown in the optic nerve studies. Also GAP-43+ sprouts might arise by regeneration following axotomy or by collateral sprouting from undamaged neurons. Secondly, it should be noted that the source of these GAP-43+ sprouts was not determined and those could have originated from fibre tracts of the white matter or from intrinsic

interneurons located in the gray matter. It is tempting to speculate that the GAP-43+ perikarya in the gray matter adjacent to the cavities may have been the origin of some of these sprouts.

Chapter Six

GAP-43 In Macroglial Cells Of The Central Nervous System

6.1. Introduction

Oligodendrocytes and astrocytes, which comprise the macroglial cells of the CNS, are both derived from the neural tube (Purves & Lichtman, 1985). The identification of intermediate stages has proved difficult and controversial (reviewed by Miller *et al.*, 1989) and the precise lineage relationship between these two cell types is not yet fully understood. The development of *in vitro* techniques for culturing and purifying macroglial cells, and the production of antibodies to molecules which are expressed by one or both of the macroglial classes have provided opportunities to study this relationship.

6.1.1. Studies *In Vitro*

GFAP is a cell-specific marker for astrocytes in the CNS (Bignami *et al.*, 1972), although glial cells in the PNS also express an immunologically related protein (Jessen *et al.*, 1984). In culture, two distinct morphological forms can be identified amongst astrocytes binding anti-GFAP antibodies (Wilkin *et al.*, 1983). These two types of astrocyte can also be distinguished immunologically by the expression of surface gangliosides and have been named according to the designation of Raff *et al.* (1983a). Flat epithelioid astrocytes are termed type-1 and do not bind the monoclonal antibodies A2B5 and LB_1, while process-bearing stellate astrocytes express the surface gangliosides binding A2B5 and LB_1 and are designated type-2 (Raff *et al.*, 1983a; Curtis *et al.*, 1988).

Oligodendrocytes are the myelinating cells of the CNS and have been shown to synthesise myelin lipids and proteins in culture, even in the absence of axons (Sarlième *et al.*, 1983; Dubois-Dalcq *et al.*, 1986). Furthermore, maturing oligodendrocytes in culture express GC, CNP and MBP sequentially in a defined developmental progression (Hardy and Reynolds, 1991).

Of particular note are the observations of Raff and colleagues who

have shown in culture that oligodendrocytes and stellate type-2 astrocytes arise from a common progenitor cell (Raff *et al.*, 1983b, 1984). Epithelioid type-1 astrocytes appear to be derived from another, as yet unidentified, precursor cell. Bipotential oligodendrocyte/type-2 astrocyte (O-2A) progenitors differentiate into oligodendrocytes in serum-free medium but develop into type-2 astrocytes in the presence of 10% FCS (Raff *et al.*, 1983b; Temple and Raff, 1985). O-2A progenitors express surface gangliosides binding A2B5 and LB_1, which are maintained as they develop into type-2 astrocytes. These gangliosides are lost however as the O-2A progenitors differentiate into oligodendrocytes and acquire GC (Raff *et al.*, 1983b; Levi *et al.*, 1987).

The isolation of bipotential O-2A progenitors in dissociated cell cultures (McCarthy and DeVellis, 1980) and single-cell microcultures (Temple and Raff, 1985) has allowed dissection of the factors that regulate their *in vitro* differentiation into oligodendrocytes or type-2 astrocytes. Single O-2A progenitors maintained in serum-free medium develop into oligodendrocytes, suggesting that this is the default pathway for differentiation (Temple and Raff, 1985). However, O-2A progenitors are mitotic and their cell cycle is driven by platelet-derived growth factor (PDGF) which delays differentiation into oligodendrocytes (Raff *et al.*, 1988; Noble *et al.*, 1988). Raff proposes that PDGF-responsiveness is regulated by an internal 'clock' which 'counts' the number of divisions prior to oligodendrocyte differentiation, possibly by limiting mitotic dilution of an unidentified molecule (Raff *et al.*, 1985; Hart *et al.*, 1989b). Differentiation into type-2 astrocytes requires extracellular signals either produced by other cell types in mixed glial cultures, or present in FCS (Temple and Raff, 1985). These signals would appear to be ciliary neurotrophic factor (Hughes *et al.*, 1988) and a nondiffusible component associated with the extracellular matrix (reviewed by Lillien and Raff, 1990). The relevance of these *in vitro* observations depends

critically on the identification of the O-2A progenitors and their descendants *in vivo*.

6.1.2. Studies *In Vivo*

Many of the antibodies used to define glia in culture can also be used to identify these cells in tissue sections from developing and adult brain.

It has been shown in our laboratory that O-2A progenitors can differentiate into oligodendrocytes *in vivo*. O-2A progenitors can be visualised in frozen tissue sections with the LB_1 monoclonal antibody (Curtis *et al.*, 1988), which can be used to show the migration of these cells from the germinal layers followed by the sequential expression of the oligodendrocyte-specific markers GC, CNP and MBP (Curtis *et al.*, 1988; Reynolds and Wilkin, 1988; Hardy and Reynolds, 1991; Reynolds and Wilkin, 1991). LB_1 binding is lost as the immature oligodendrocytes acquire GC, although some cells can be double labelled.

Astrocytes can be readily detected by the presence of GFAP (Bignami *et al.*, 1972) but the demonstration of two distinct astrocytic types has proved more difficult. In mixed glial cultures, O-2A progenitors and type-2 astrocytes can be distinguished on the basis of morphology and their expression of surface ganglioside(s) binding the monoclonal antibodies A2B5 and LB_1 (Raff *et al.*, 1983b; Levi *et al.*, 1987) but the identification of type-2 astrocytes in tissue sections has proved problematic. A2B5 binds an unspecified epitope on several ganglioside species which is also present on neuronal surfaces (Eisenbarth *et al.*, 1979; Fredman *et al.*, 1984) but, on the basis of colabelling with GFAP, Miller and Raff (1984) have suggested that type-2 astrocytes correspond to the fibrous astrocytes found in white matter, while type-1 astrocytes represent the counterpart of the protoplasmic astrocytes located mainly in grey matter. These authors have recently questioned the reliability of A2B5 as a marker of type-2 astrocytes in

tissue sections (see Miller *et al.*, 1989) and therefore the existence of type-2 astrocytes has been called into doubt.

The possibility that type-2 astrocytes do not exist *in vivo* is supported by results from our laboratory using LB_1. The binding of this monoclonal antibody is specific for ganglioside G_{D3} and can be detected on O-2A progenitors in cryostat sections of neonatal brain but cannot be colocalised to cells expressing GFAP in such sections (Curtis *et al.*, 1988; Hardy and Reynolds, 1991). While it is possible that astrocytes express G_{D3} below the level of detection of fluorescent light immunohistochemistry, or that they cease to express G_{D3} before they acquire GFAP, these data suggest that signals specifying the astrocytic pathway for O-2A differentiation *in vitro* may be missing from the developing rat brain.

If this is correct, differentiation of O-2A progenitors into oligodendrocytes is the default pathway *in vivo* as well as *in vitro*, and therefore type-2 astrocytes *in vitro* may be misdirected oligodendrocytes resulting from the presence of FCS in the tissue culture medium. Oligodendrocytes have indeed been shown to express GFAP transiently during development (Choi and Kim, 1984). It had been hoped that protein markers of type-2 astrocytes would help resolve this issue.

6.1.3. The Present Study

GAP-43 was initially considered to be restricted to neurons (see Section 1.1.2.1.) but examination of astrocytes in mixed glial cultures from rat cortex by 2-D gel electrophoresis and immunoblotting of PKC-phosphorylated membrane proteins and by immunofluorescence revealed the presence of GAP-43 (Vitković *et al.*, 1988). Immunocytochemical and Western blotting studies with anti-GAP-43 fusion protein antisera were undertaken to confirm that GAP-43 is indeed present in astrocytes and to determine if it is restricted to particular types of astrocyte or astrocytes from different regions.

Mixed glial cultures from cortex, cerebellum and optic nerve all contained GAP-43+ astrocytes, suggesting that GAP-43 expression is not determined by the area of the CNS from which glia are cultured. However, GAP-43 was predominantly associated with stellate astrocytes and could be colocalised with LB_1, suggesting that GAP-43 might be a marker for the type-2 astrocyte lineage. To verify this, O-2A progenitors (purified by R. Hardy in our laboratory) were maintained in medium containing 10% FCS. Immunocytochemistry and Western blotting showed that O-2A progenitors expressed GAP-43 and that this was retained as these cells differentiated into type-2 astrocytes. In contrast, GAP-43 was lost from O-2A progenitors maintained in the absence of serum as they began to acquire GC. Thus GAP-43 has the same distribution as LB_1 in CNS macroglial cells and their O-2A progenitors *in vitro*.

Immunohistochemical studies were then performed in an attempt to localise GAP-43 in O-2A progenitors and their descendants *in vivo*. O-2A progenitors and immature oligodendrocytes contained GAP-43, but mature oligodendrocytes expressing CNP or MBP did not. However, GAP-43 did not colocalise with GFAP+ astrocytes in tissue sections of developing or adult brain. Furthermore, induction of reactive gliosis in the adult CNS did not induce GAP-43 in astrocytes. These results support the contention that O-2A progenitors differentiate into oligodendrocytes constitutively *in vivo* and that type-2 astrocytes *in vitro* are tissue culture artefacts, or at least differ phenotypically *in vivo*.

These results formed the basis of a paper which has been accepted for publication (Curtis *et al.*, 1991).

During the course of these studies two reports were published which described GAP-43 in cultured oligodendrocytes (da Cunha and Vitković, 1990; Deloulme *et al.*, 1990), although this was not confirmed by characterisation of these cells using conventional markers of oligodendrocyte maturation (CNP or MBP) and no staining of tissue sections was performed. This disagrees with my results and the causes

of this disagreement are discussed.

6.2. Results

6.2.1. GAP-43 In Mixed Glial Cultures

The expression of GAP-43 by CNS macroglial cells was initially examined by immunocytochemistry of mixed glial cultures from neonatal cortical hemispheres, cerebellum and optic nerve. GAP-43 was found mainly in stellate astrocytes and bipolar cells, and occasionally in flat astrocytes. Double labelling of these cells with the LB_1 monoclonal antibody to identify bipolar O-2A progenitors and stellate type-2 astrocytes (Curtis *et al.*, 1988) showed that both cell types expressed GAP-43 (see Figure 6.1.). Some flat astrocytes which were GAP-43+ did not bind the LB_1 monoclonal antibody (which is specific for ganglioside G_{D3}), suggesting that these were type-1 astrocytes.

To investigate further the cell specificity of GAP-43 expression, a modification (Hardy and Reynolds, 1991) of the shaking procedure of McCarthy and DeVellis (1980) was utilised to separate enriched populations of type-1 astrocytes, type-2 astrocytes, oligodendrocytes and O-2A progenitors. These were subjected to immunocytochemistry and Western blotting with the anti-GAP-43 antiserum.

6.2.2. GAP-43 Is Expressed By O-2A Progenitors *In Vitro*

Figure 6.2. shows that O-2A progenitors, isolated by orbital shaking and purified by complement-mediated cytolysis, were immunoreactive for GAP-43 and ganglioside G_{D3}. In addition, some small process-bearing cells were immunoreactive for GAP-43 but not ganglioside G_{D3}. These cells may represent the 'pre-progenitor' cells which are isolated with the O-2A progenitors in these cultures (Hardy and Reynolds, 1991). As yet there is no specific molecular marker to identify these 'pre-progenitor' cells.

Figure 6.1.

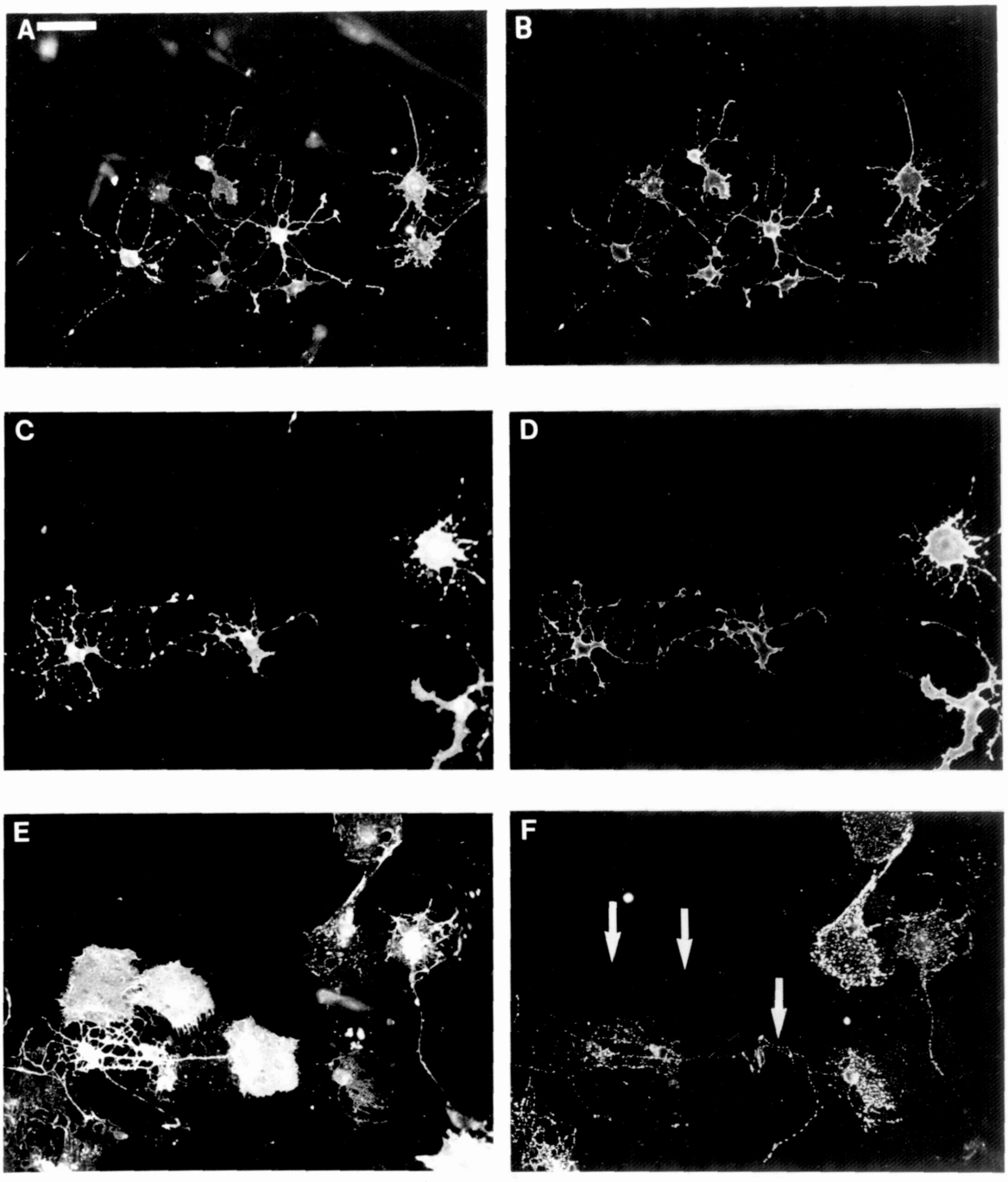
Double labelling of mixed glial cultures from different CNS regions with anti-GAP-43 fusion protein antiserum diluted 1/1000 (a,c and e) and LB_1 monoclonal antibodies against ganglioside G_{D3} (b,d and f).

A and B. Optic nerve culture 2 days *in vitro*. GAP-43 is restricted to G_{D3}+ immature type-2 astrocytes.

C and D. Cerebellar culture 4 days *in vitro*. GAP-43 is restricted to G_{D3}+ type-2 astrocytes. Note the variety of morphologies displayed by these cells.

E and F. Cortical culture 7 days *in vitro*. GAP-43 is expressed by G_{D3}+ type-2 astrocytes (some of which have assumed a more flattened morphology) and by G_{D3}- epithelioid cells which may be type-1 astrocytes (arrows).

Scale bar in a, 50 μm.


A
B
C
D
E
F

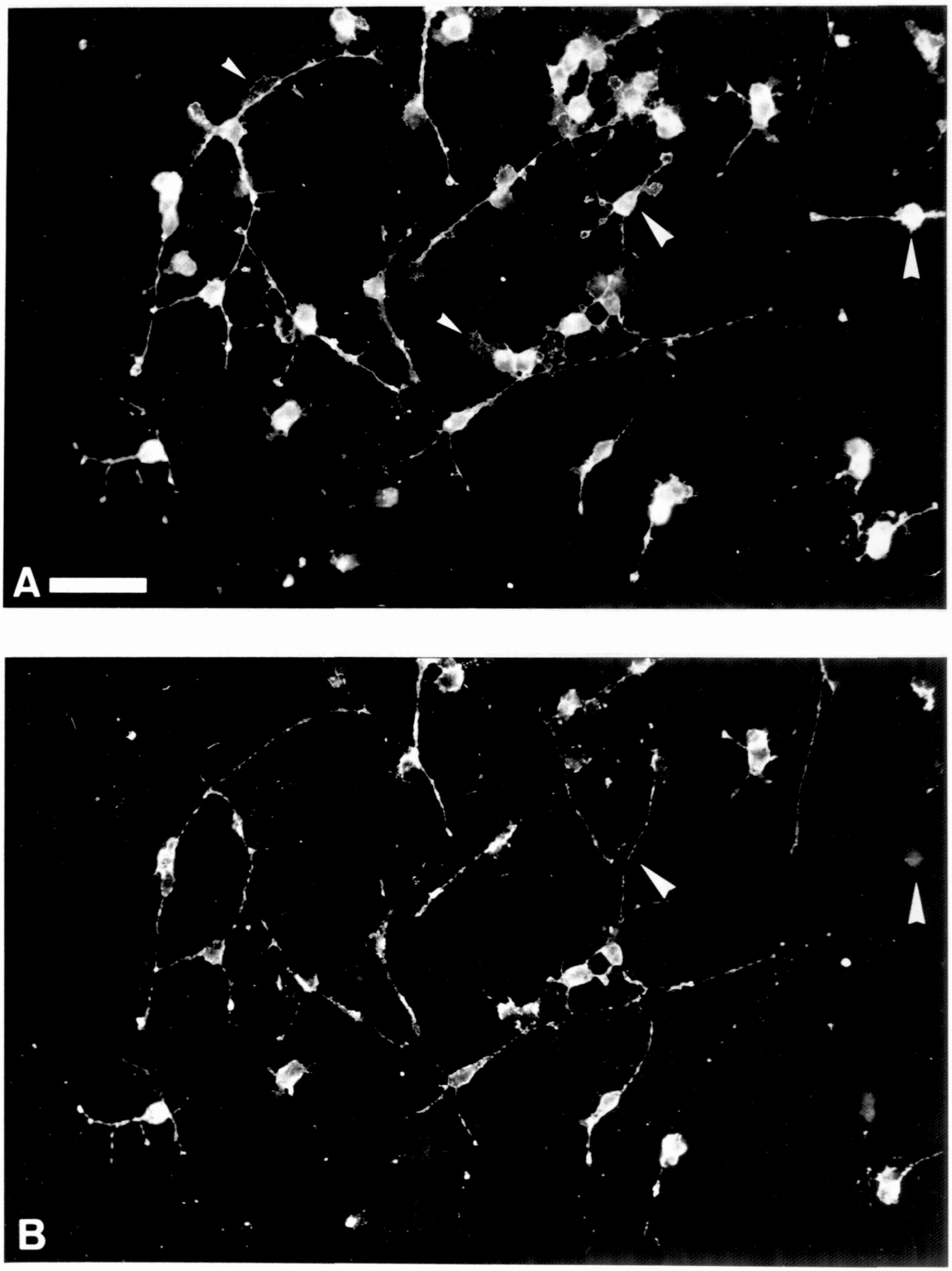


Figure 6.2.

O-2A progenitor cells 1 DAI, stained with anti-GAP-43 antiserum (a) and LB_1 monoclonal antibody (b). GAP-43 stains many membranous extensions (small arrowheads). Note small process-bearing cells, which contain GAP-43 but not ganglioside G_{D3} (large arrowheads).

Scale bar in a, 50 μm.

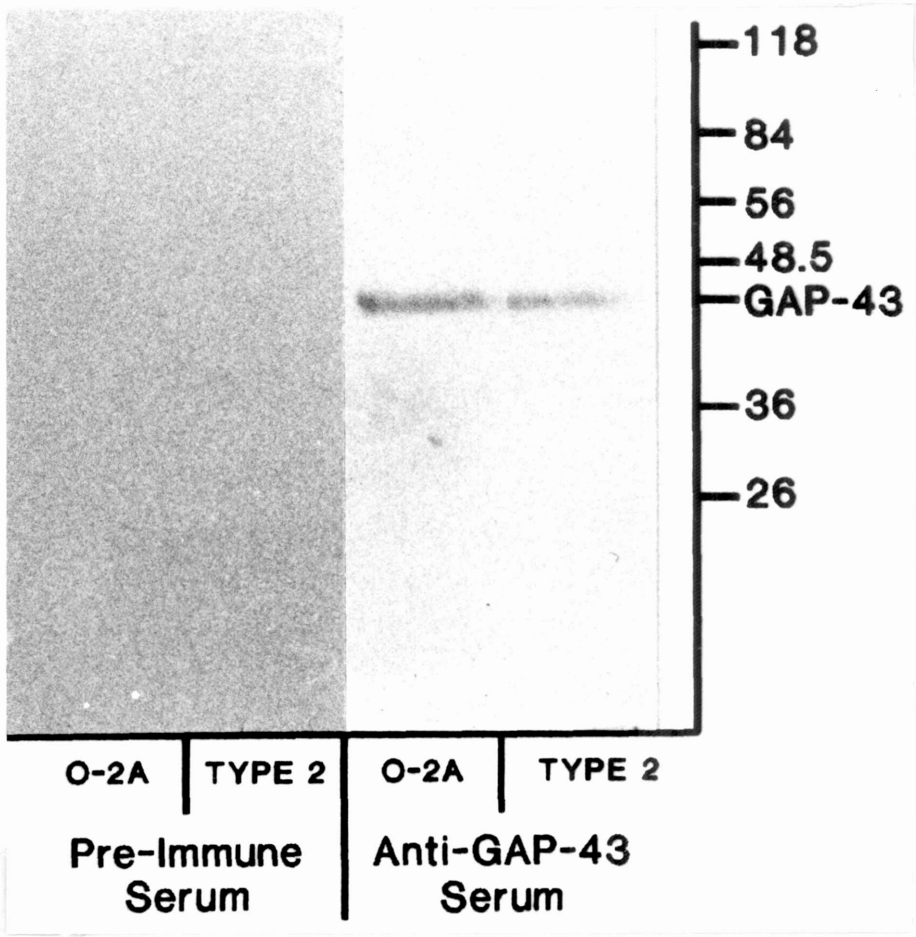


Figure 6.3.

Western blots of membranes prepared from enriched cultures of O-2A progenitors and type-2 astrocytes (O-2A progenitors 5DAI, maintained in medium containing 10% FCS) stained with anti-GAP-43 fusion protein antiserum or pre-immune serum diluted 1/1000. The RMM of standard marker proteins and the position of GAP-43 are marked to the right.

Pre-immune serum did not bind any membrane proteins from these cells. Only GAP-43 was recognised by the anti-GAP-43 fusion protein antiserum and there were no cross-reacting proteins.

Western blotting of proteins from membranes of freshly isolated O-2A progenitors confirmed the presence of membrane-bound GAP-43 (Figure 6.3.).

6.2.3. GAP-43 Is Down-Regulated During Oligodendrocyte Differentiation *In Vitro*

O-2A progenitors transferred to serum-free medium (after 1 day in medium containing 10% FCS) have been shown in parallel studies (Hardy and Reynolds, 1991) to differentiate into oligodendrocytes over a period of 5-6 days, sequentially expressing the oligodendrocyte-specific markers GC, CNP, MBP. Double labelling with antibodies against GAP-43 and GC or MBP showed that oligodendrocyte maturation was accompanied by down-regulation of GAP-43 (Figure 6.4.a, b, c, d).

GAP-43 was lost as these cells became increasingly multipolar and began to synthesise GC, the earliest oligodendrocyte-specific marker. The less differentiated cells possessed fewer processes and were still strongly immunoreactive for GAP-43 whereas the GC+ cells had a more more mature morphology and contained much less GAP-43. The cells which were strongly stained for GAP-43 at this stage were also positive for ganglioside G_{D3} (data not shown). It has previously been shown that G_{D3} is associated with an early stage of oligodendrocyte differentiation (Levi *et al.*, 1987) and in this study it was found that this was lost along with GAP-43 as the cells acquired GC.

At a later stage in culture the more mature oligodendrocytes synthesised MBP but none of these cells contained GAP-43. Western blot analysis of the membranes of maturing oligodendrocytes was not possible due to the continued presence of GAP-43+ O-2A progenitors at later stages in these serum-free cultures.

Figure 6.4.

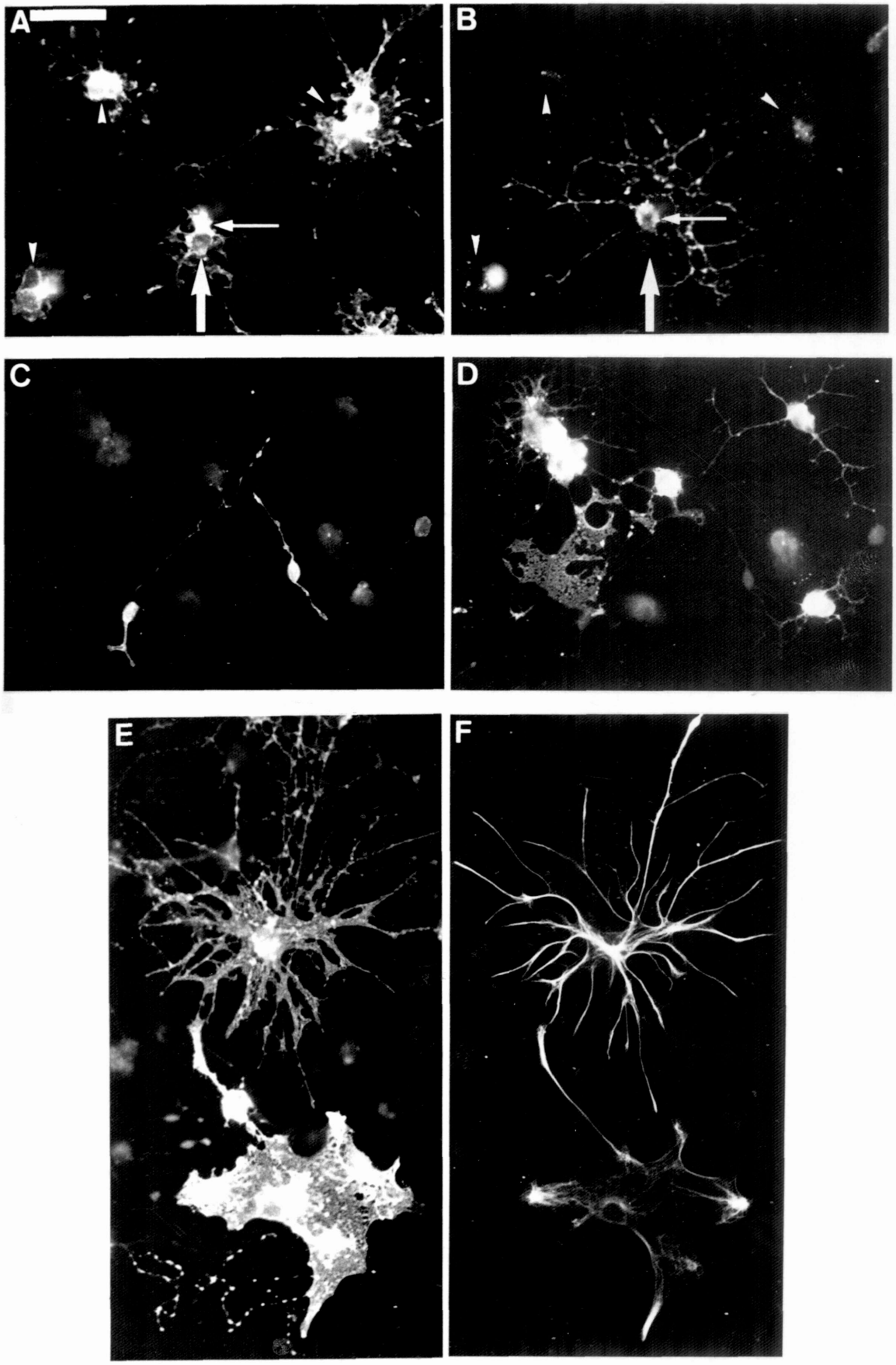
A,B,C and D. O-2A progenitors were maintained in serum-free medium and double labelled with anti-GAP-43 fusion protein antiserum diluted 1/1000 (a and c) and monoclonal antibodies against the oligodendrocyte developmental markers GC (b) or MBP (d).

A and B. At 4 DAI, an immature oligodendrocyte expresses GC (b) but not GAP-43 (small arrow) while an adjacent cell is GAP-43+ (a) but contains no GC (large arrow). 3 cells can be seen which are strongly GAP-43+ and are beginning to express GC (arrowheads).

C and D. At 5 DAI, some mature oligodendrocytes can be seen expressing MBP (d) and in one instance elaborating an area of velate membrane. These cells contain no GAP-43 (c), but two GAP-43+ O-2A progenitors can be seen in this field.

E and F. Double labelling of type-2 astrocytes (O-2A progenitors 14 DAI, maintained in medium containing 10% FCS) with anti-GAP-43 fusion protein antiserum diluted 1/1000 (e) and monoclonal antibodies against GFAP (f). These cells displayed a variety of morphologies ranging from a highly process-bearing form to a flat, polygonal shape, as illustrated in this field.

Scale bar in a, 50 μm.


A
B
C
D
E
F

6.2.4. GAP-43 Is Expressed By Type-2 Astrocytes *In Vitro*

O-2A progenitors maintained in medium containing 10% FCS differentiated into type-2 astrocytes, expressing GFAP and ganglioside G_{D3}. These cells also expressed GAP-43 at all time points examined (up to 14 DAI, Figure 6.4.e, f). Typical stellate morphologies predominated but some type-2 astrocytes were more polygonal, consistent with the contact-mediated shape change suggested by previous studies from our laboratory (Wilkin *et al.*, 1983; Johnstone *et al.*, 1986).

Western blotting of proteins from membranes isolated from cultures enriched in type-2 astrocytes confirmed the presence of membrane-bound GAP-43 (Figure 6.3.).

6.2.5. GAP-43 Is Down-Regulated During Oligodendrocyte Differentiation *In Vivo*

The loss of GAP-43 during oligodendrocyte differentiation was also observed *in vivo* at a similar point in maturation (Figure 6.5.). At 1 day of age GAP-43 immunoreactivity was widespread throughout the cortex (Benowitz *et al.*, 1988; McGuire *et al.*, 1988) but, in cryostat sections from corpus callosum, GAP-43 could be seen in the cell bodies of O-2A progenitors stained for G_{D3} ganglioside lying amongst the strongly GAP-43+ developing neuropil. At 5 days of age GAP-43 was present in the numerous GC-stained oligodendrocytes which had begun to differentiate in rows between fascicles of elongating axons. Previous electron microscopic studies have shown that rows of interfascicular oligodendrocytes are joined by tight junctions and are not interposed by neuronal or astrocytic processes (Peters *et al.*, 1976; Massa and Mugnaini, 1982). Therefore the GAP-43 located between the nuclei is most likely in the membranes of the immature oligodendrocytes.

In older animals, GAP-43 was not apparent in mature oligodendrocytes (identified by their expression of the myelin proteins CNP or MBP) in the granule cell layer of the dentate gyrus and the

Figure 6.5.

5 μm sections from developing rat brain double labelled with antibodies against GAP-43 (a,c,e and g) and the oligodendrocyte developmental markers ganglioside G_{D3} (b), GC (d), CNP (f) or MBP (h).

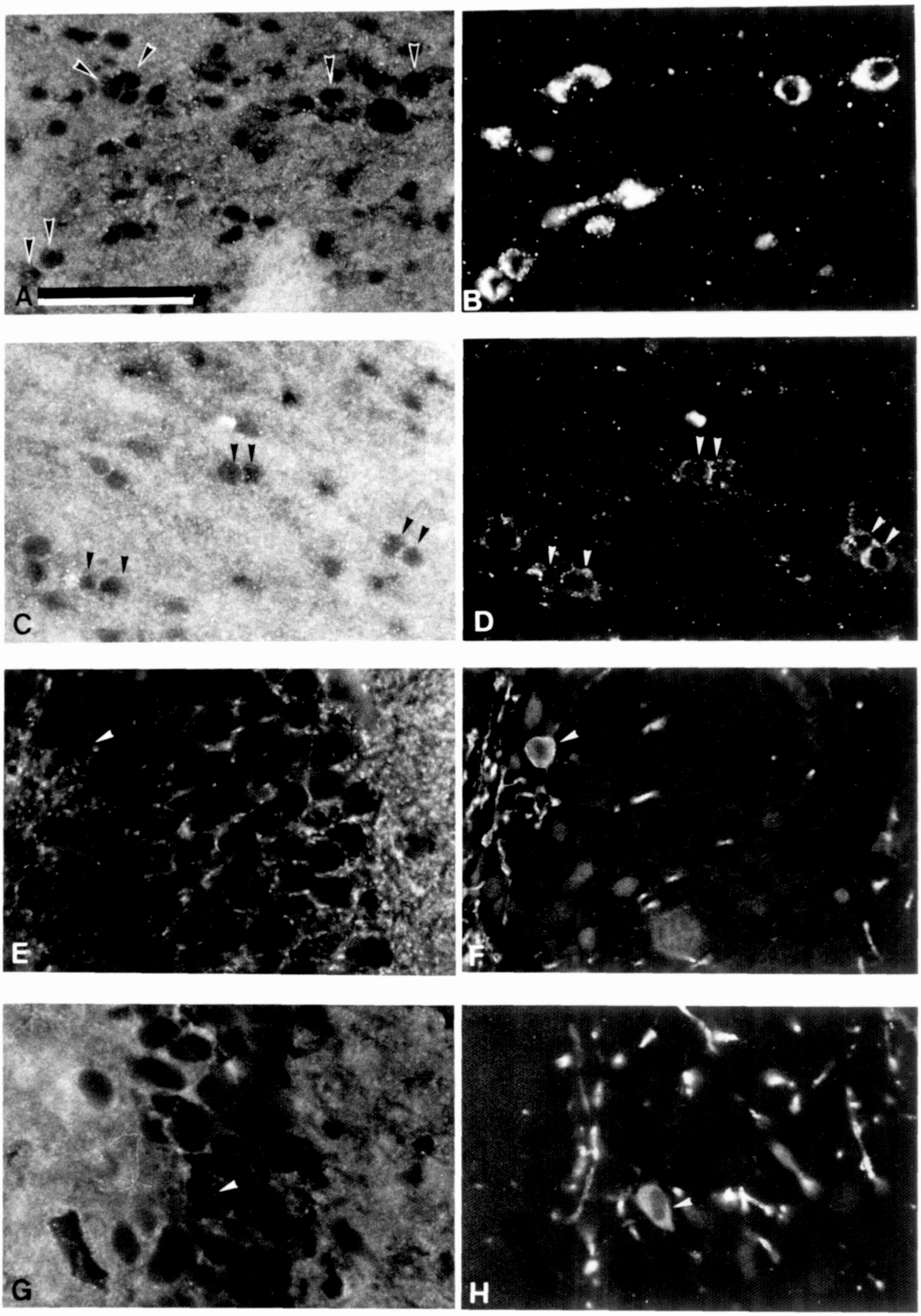
A and B. The cell bodies of O-2A progenitors in the cingulum of 1 day rat (identified by the presence of ganglioside G_{D3}) contain less GAP-43 than the surrounding neuropil, but are clearly above the background nuclear staining. Frozen section, anti-GAP-43 fusion protein antiserum diluted 1/5000, ganglioside G_{D3} monoclonal antibody.

C and D. The membranes of interfascicular oligodendrocytes (labelled with GC) contain GAP-43 in the 4 day rat corpus callosum. Frozen section, anti-GAP-43 fusion protein antiserum diluted 1/5000, GC monoclonal antibody.

E and F. At 20 days the molecular layer (MOL) of the dentate gyrus is stained for GAP-43 while a CNP+ oligodendrocyte (arrowhead) at the inner edge of the granule cell layer (GR) contains no GAP-43. Polyester wax-embedded section, 91E12 monoclonal antibody against GAP-43, polyclonal antiserum against CNP.

G and H. At the same age (20 days) the pyramidal cell layer of the hippocampus contains an MBP+ oligodendrocyte which does not contain GAP-43. Polyester wax-embedded section, monoclonal antibody against MBP, anti-GAP-43 fusion protein antiserum diluted 1/10,000.

Scale bar in a, 50 μm.


A
B
C
D
E
F
G
H

pyramidal cell layer of the hippocampus, which are known to display minimal GAP-43 immunoreactivity (Oestreicher and Gispen, 1986; Benowitz *et al.*, 1988; McGuire *et al.*, 1988). In adult white matter tracts the only GAP-43 staining was restricted to subsets of axons, while astrocytes and oligodendrocytes remained unlabelled (see Figure 6.6.).

6.2.6. Astrocytes *In Vivo* Do Not Express GAP-43

As there are currently no specific markers to identify type-2 astrocytes *in vivo*, the anti-GAP-43 antiserum was used on sections of both normal and lesioned adult rat cerebellum to see if GAP-43 immunoreactivity could be colocalised with GFAP+ astrocytes. In common with previous immunohistochemical studies of adult rat central nervous system at the light and electron microscope levels (Oestreicher and Gispen, 1986; Oestreicher *et al.*, 1988; Benowitz *et al.*, 1988; McGuire *et al.*, 1988; Gorgels *et al.*, 1989) there was no evidence for an astrocytic localization of GAP-43.

Figure 6.6. shows white and gray matter astrocytes and Bergman glia in formaldehyde-fixed cerebellar sections, identified by the presence of GFAP. None of these astrocytes showed immunoreactivity for GAP-43 in the normal cerebellum, despite staining of neuronal elements in the molecular layer and the white matter tracts. Similarly, reactive astrocytes in lesions caused by kainic acid injection to the cerebellum did not show GAP-43 immunoreactivity despite extensive damage to the granule cell layer, although GAP-43+ axons in the white matter and terminals in the molecular layer remained intact. Freshly frozen tissue, sectioned and fixed on the slide with acid:alcohol has been reported to show selectively increased staining of GFAP in white matter astrocytes (Shehab *et al.*, 1990). GAP-43 could not be visualised in astrocytes revealed by this method (data not shown).

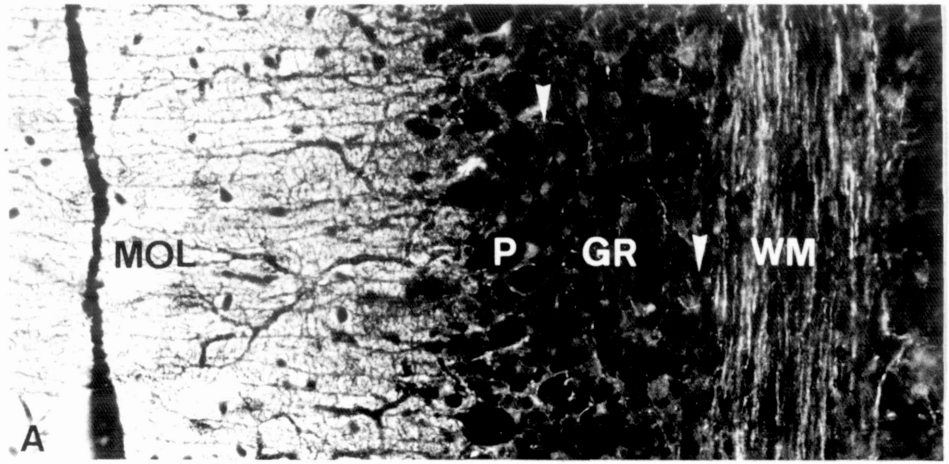
Figure 6.6.

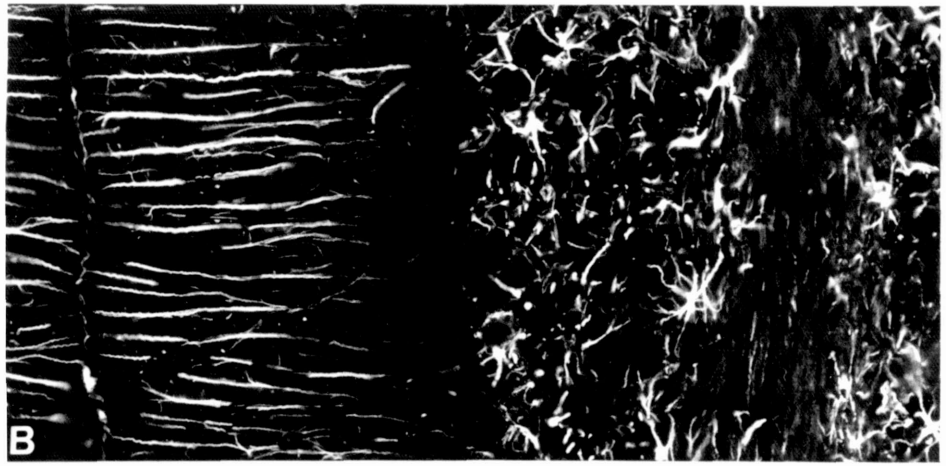
5μm polyester wax sections of adult rat cerebellum double-labelled with anti-GAP-43 fusion protein antiserum diluted 1/10,000 (a,c) and monoclonal antibodies against GFAP (b,d).

A and B. In unlesioned tissue, GAP-43 (a) stains axons in the white matter (WM), some neuronal processes in the granule cell layer (GR) and parallel fibre terminals in the molecular layer (MOL). Bergman glia and Purkinje cell dendrites in the molecular layer remain unstained, while some labelling can be seen around the Purkinje cell bodies (P). GFAP+ astrocyte cell bodies and processes (b) contain no GAP-43 immunoreactivity (arrowheads).

C and D. 21 days after injection of kainic acid, the granule cell layer (GR) at the top of the section has been destroyed, leaving a rim of highly GFAP immunoreactive astrocytes (d). Reactive astrocytes can also be seen in the white matter. GAP-43 staining (c) persists in white matter axons and the molecular layer, but neither the rim of astrocytes nor those in the white matter (arrowheads) show GAP-43 immunoreactivity. Note also the absence of Purkinje cell bodies, indicating the death of these cells.

Scale bar in d, 50 μm.


MOL
P
GR
WM
A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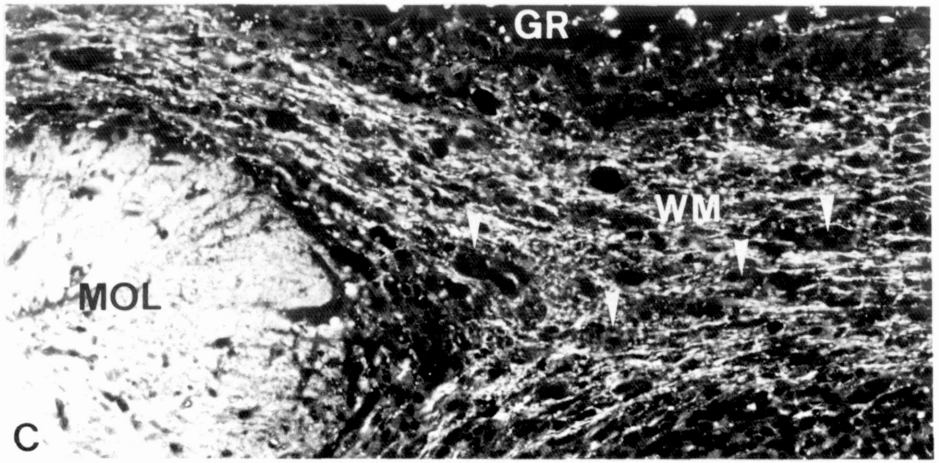
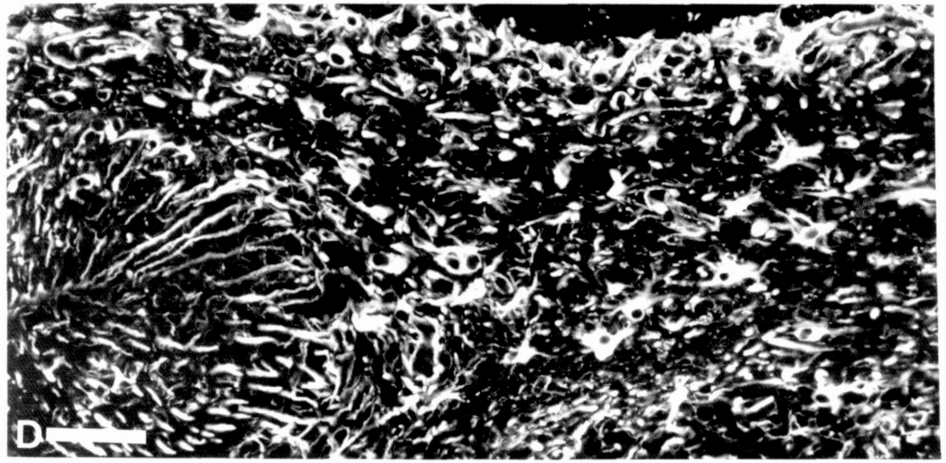

GR
WM
MOL
C


D

6.3. Discussion

These data show that O-2A progenitors express GAP-43 both *in vivo* and *in vitro* and that this is lost as the cells differentiate into oligodendrocytes. Type-2 astrocytes *in vitro* continue to express GAP-43 up to 14 DAI, the longest period studied, but GFAP+ astrocytes could not be detected in sections of developing, adult or damaged brain. This is reminiscent of the ganglioside G_{D3} which is also a marker of O-2A progenitors that is lost by oligodendrocytes as they differentiate but retained by type-2 astrocyte *in vitro*. Ganglioside G_{D3} is not detectable on astrocytes *in vivo* (Curtis *et al.*, 1988; LeVine and Goldman, 1988).

6.3.1. Oligodendrocyte Differentiation

O-2A progenitors can differentiate in culture into type-2 astrocytes or oligodendrocytes respectively in the presence or absence of FCS (Raff *et al.*,1983b, 1984; Temple and Raff 1985). In defined, serum-free medium the O-2A progenitors undergo a series of maturation steps as they differentiate into oligodendrocytes, sequentially expressing the markers G_{D3}, GC, CNP, MBP (Dubois-Dalcq *et al.*, 1986; Hardy and Reynolds, 1991). GAP-43 was lost by these cells as they began to acquire GC, the earliest marker of immature oligodendrocytes. None of the mature oligodendrocytes, as assessed by the expression of MBP, co-expressed GAP-43. This is in contrast to the findings of da Cunha and Vitković (1990) and Deloulme *et al.* (1990) who proposed that GAP-43 is expressed constitutively by oligodendrocytes *in vitro*.

Deloulme *et al.* (1990) performed biochemical studies on GAP-43 from secondary cultures derived from mixed glial primaries by differential adhesion. However, it is the experience in our laboratory that differential adhesion yields a mixed population of O-2A progenitors, type-2 astrocytes, oligodendrocytes and microglia (Hardy and Reynolds, 1991, R. Reynolds personal communication) and that the

proportion of type-2 astrocytes increases with the duration of the primary culture, due to factor(s) present in FCS promoting differentiation of O-2A progenitors into type-2 astrocytes (Raff *et al.*, 1983b, 1984). Deloulme *et al.* (1990) maintained their mixed glial cultures for 18-20 days before subculturing, during which time a substantial proportion of the O-2A progenitors are likely to have differentiated into type-2 astrocytes not oligodendrocytes. The isolated cells were assumed to be purified oligodendrocytes as they were maintained in supplemented Waymouth's MD 705/1 medium to select against astrocytes, however no evidence was presented to show that the secondary cultures had been depleted of astrocytes, for instance by the use of GFAP immunocytochemistry. Indeed, their published photograph of a 'purified oligodendrocyte' looks remarkably like a type-2 astrocyte (compare Figure 3d of Deloulme *et al.*, 1990, with Figure 6.4.e this Chapter) and no double labeling with any oligodendrocyte marker was used to confirm the proposed identity of their isolated cells. The authors note that 'morphological differentiation' of oligodendrocytes was accompanied by loss of GAP-43, in accordance with the immunocytochemical data presented here. Furthermore, as Figure 6.4.c demonstrates, GAP-43+ O-2A progenitors persist in these serum-free secondary cultures (possibly by proliferation of 'pre-progenitor' cells, which are isolated along with the O-2A progenitors, Hardy and Reynolds, 1991). It is possible that the GAP-43 protein and mRNA isolated from these secondary cultures by Deloulme *et al.* (1990) is derived from contaminating O-2A progenitors and type-2 astrocytes rather than the oligodendrocyte population.

The conclusions of da Cunha and Vitković (1990) were based on GAP-43 immunostaining of O-2A progenitors maintained in culture medium containing 0.5% FCS and costained only with GC to demonstrate progression along the oligodendrocyte lineage. Oligodendrocytes cultured in up to 1% FCS can initiate differentiation with the extension

of processes and the synthesis of oligodendrocyte markers including GC and MBP (Dubois-Dalcq *et al.*, 1986), however they do not appear to be fully differentiated with long MBP+ processes or elaborate MBP+ sheets between these processes (see Figure 1h in Dubois-Dalcq *et al.*, 1986). With specific regard to the GAP-43+ oligodendrocytes documented by da Cunha and Vitković (1990), the cells were not shown to be mature by the use of MBP staining and appeared from their published photographs to be of an immature shape, unlike the mature oligodendrocytes in our serum-free cultures which contained MBP and demonstrated a more differentiated form, with the presence of velate MBP+ structures resembling flattened membranes.

In vivo staining with the same markers of oligodendrocyte maturation revealed a similar decline in GAP-43 expression to that demonstrated by the studies of O-2A precursors maintained in defined medium. This lends support to the contention that culturing O-2A progenitors in medium containing 0.5% FCS prevents their normal maturation into oligodendrocytes. In contrast to defined medium in which all the components are catalogued, FCS contains a wide spectrum of unknown factors which may vary between manufacturers and indeed batches of serum. These molecules may not be endogenous to the developing rodent central nervous system due either to the bovine origin of the serum or the action of the Blood Brain Barrier, which prevents free diffusion of molecules from the circulation into central neural tissue and maintains the differences between plasma and the cerebrospinal fluid (Fenstermacher, 1985). Any one of these 'foreign' factors could affect O-2A progenitors and prevent differentiation into the *in vitro* counterparts of mature oligodendrocytes.

6.3.2. Role of GAP-43 in Oligodendrocyte Motility

O-2A progenitors cultured from optic nerve have been shown to be motile *in vitro* and have been suggested to populate the developing optic

nerve by migration from their germinal zone, probably in the base of the preoptic recess above the optic chiasm (Small *et al.*, 1987). This is supported by the absence in the developing optic nerve of the 'pre-progenitor' precursor cells of O-2A progenitors, which have been isolated from rat cerebral white matter during development (Grinspan *et al.*, 1990). Migration of O-2A progenitors has also been suggested by the apparent movement of these cells from the area around the fourth ventricle into the developing folia during cerebellar maturation (Curtis *et al.*, 1988) and from the region of the third ventricle through the corpus callosum (Hardy and Reynolds, 1991). In contrast, oligodendrocytes are not motile *in vitro* (Small *et al.*, 1987) and *in vivo* are firmly anchored to axons by their myelin sheaths.

In neurons, GAP-43 is associated with the plasma membrane and is particularly enriched in the growth cone (Meiri *et al.*, 1986; Skene *et al.*, 1986) where it interacts with both the plasmalemma and a sub-membrane cytoskeleton fraction containing fodrin, a-actinin, talin and actin (Moss *et al.*, 1990; Meiri and Gordon-Weeks, 1990). Isolated growth cones dislodged from their substrate in culture leave behind patches of membrane and associated sub-membrane cytoskeleton, which represent adhesion plaques, and also contain immunoreactive GAP-43, leading to the suggestion that GAP-43 is localised at sites of substrate adhesion (Meiri *et al.*, 1988; Meiri and Gordon-Weeks, 1990). Activation of receptors by substrate-bound ligands may modify the function of GAP-43 to link the plasma membrane to the cytoskeleton, thus stabilising the adhesion plaques and generating an anchor for the subsequent motility of the cell or growth cone (Bray, 1987; Turner and Flier, 1989). In O-2A progenitors GAP-43 is also associated with the plasma membrane where it decorates a large variety of membrane extensions. GAP-43 may regulate membrane-substrate interactions in these cells and contribute to their motility. The down-regulation of GAP-43 in maturing oligodendrocytes may underlie the cessation of motility, which is coincident with the

final mitotic division and the start of oligodendrocyte differentiation.

6.3.3. Role of GAP-43 in Oligodendrocyte Differentiation

One factor known to have a significant role in O-2A progenitor differentiation is PDGF, which is synthesised by type-1 astrocytes during the period of gliogenesis in the optic nerve and drives the division of the O-2A progenitors (Noble *et al.*, 1988; Raff *et al.*, 1988; Richardson *et al.*, 1988). PDGF also promotes the *in vitro* motility of the O-2A progenitors (Noble *et al.*, 1988). A 'developmental clock' in the O-2A progenitors counts the number of divisions and causes the cells to drop out of the mitotic cycle and differentiate into oligodendrocytes in the absence of FCS (Raff *et al.*, 1985). This is coordinated with the loss of motility in the newly-formed oligodendrocytes and the disappearance of GAP-43 from these cells. The timing of the 'developmental clock' has been postulated to be due to the limiting dilution by successive cell divisions of a molecule critical for PDGF-responsiveness (Raff *et al.*, 1988, Hart *et al.*, 1989b).

Although mature oligodendrocytes lose receptors for PDGF, recently differentiated oligodendrocytes still express these receptors (Hart *et al.*, 1989a). The PDGF-receptor activates a range of intracellular signals, including intrinsic tyrosine kinase activity and elevations in both cyclic AMP and intracellular calcium ions (Ca^{2+}). Newly-formed oligodendrocytes show a robust elevation in cytosolic Ca^{2+}, consistent with activation of the inositol phospholipid pathway in response to PDGF-receptor activation (Berridge and Irvine, 1984; Hart *et al.*, 1989b). Neither the PDGF-receptor nor one of the early intracellular signals would therefore appear to be the regulatory element in the responsiveness of O-2A progenitors to PDGF.

Although speculative, the loss of GAP-43 coincident with PDGF-dependent cessation of motility and cell division prior to oligodendrocyte differentiation suggests some interesting possibilities.

First, the GAP-43 gene may be regulated by the same PDGF-activated second messenger system which drives mitosis and retards differentiation, in which case loss of motility would be incidental to the differentiation process. Secondly, GAP-43 may be the missing regulatory element controlling PDGF-responsiveness, either by limiting dilution or by acute down-regulation of the gene. GAP-43 is distributed inside the membrane, in the vicinity of receptor-activated events, and is biochemically well-suited to the regulation of intracellular signals, at least those generated by the inositol phospholipid pathway. GAP-43 is an endogenous substrate for protein kinase C and shows Ca^{2+}-stimulated dissociation from calmodulin (Chan *et al.*, 1986; Skene, 1989). GAP-43 could be the 'switch' regulating the loss of cellular responses to PDGF-generated second messenger systems. Finally, of course, there may be no causal link between GAP-43 and the PDGF response.

6.3.4. Type-2 Astrocyte Differentiation

Differentiation into oligodendrocytes appears to be the default pathway for O-2A progenitors, as shown in single cell microculture in the absence of serum (Temple and Raff, 1985). PDGF released from type-1 astrocytes functions to maintain a mitotic population of O-2A progenitors which can migrate and give rise to oligodendrocytes where they are required, as discussed above. The other branch of the O-2A lineage gives rise to type-2 astrocytes *in vitro* but the differentiation of type-2 astrocytes is much more complex and has been recently reviewed (Lillien and Raff, 1990). Soluble CNTF released by type-1 astrocytes causes transient expression of the astrocyte marker GFAP in developing O-2A progenitors, followed by differentiation into oligodendrocytes (Hughes *et al.*, 1988). Further matrix-bound factors are required a) to prevent oligodendrocyte differentiation and b) to induce permanent type-2 astrocyte differentiation. One of these factors appears to be basic

FGF (either substrate-bound or in solution) which can inhibit differentiation along the oligodendrocyte pathway. Cultures containing endothelial cells produce an extracellular matrix suitable for type-2 astrocyte differentiation, and this cell type may be the source of the basic FGF-like activity. Remaining factors have yet to be identified, but they appear to be strictly matrix-bound molecules which are not produced by neurons, oligodendrocytes or type-1 astrocytes.

The identification of type-2 astrocytes *in vivo* has proved highly controversial in recent years. Initially the monoclonal antibody A2B5 (Eisenbarth *et al.*, 1979) was used as a marker *in vivo* as it binds selectively to O-2A progenitors and type-2 astrocytes in mixed glial cultures (Miller and Raff, 1984; Miller *et al.*, 1985). Unfortunately, the use of A2B5 in studies of neural tissue sections is limited by the fact that it recognises several ganglioside species with at least one sialic acid residue (Fredman *et al.*, 1984) some of which are present on neurons as well as cells of the O-2A lineage. Consequently, the results obtained with this antibody *in vivo* have been questioned by the original authors (Miller *et al.*, 1989). Another monoclonal antibody, which is monospecific for the ganglioside G_{D3}, has been used to identify O-2A progenitors and type-2 astrocytes *in vitro* and also labels migrating O-2A progenitors in tissue sections (Curtis *et al.*, 1988). This antibody has not revealed the presence of any type-2 astrocytes in sections from optic nerve, cerebellum, forebrain or any other central nervous system region studied (Curtis, Reynolds and Wilkin, unpublished observations), confirming the unreliability of the A2B5 antibody. In addition cerebellar type-2 astrocytes *in vitro* showed a neuron-like uptake of the neurotransmitter gamma-aminobutyric acid which can be inhibited by *cis*-1,3-aminocyclohexane (Johnstone *et al.*, 1986) but this could not be detected in slices of developing rat cerebellum of the same age. Instead astrocytes in slices and freshly dissociated preparations from 8 day cerebellum showed ß-alanine-sensitive gamma-aminobutyric acid uptake

which is characteristic of type-1 astrocytes *in vitro* (Cohen *et al.*, 1980; G.P. Wilkin unpublished observations). These results indicate the possibility that type-2 astrocytes *in vitro* are different from the astrocytes that are found in the developing and adult brain. Viewed conservatively, this can be seen just as the aberrant expression by type-2 astrocytes in culture of a few molecular properties, such as ganglioside G_{D3} and GAP-43 expression and *cis*-1,3-aminocyclohexane-sensitive gamma-aminobutyric acid uptake. The more extreme view is that type-2 astrocytes are an artefact of tissue culture that do not exist *in vivo*.

Recently, a study, using the classical techniques of ^{3}H-thymidine and electron microscopy to identify newly-differentiated cells (Skoff, 1990), demonstrated that there is no astrogliogenesis in the optic nerve after the appearance of oligodendrocytes in the first and second postnatal weeks. This is in conflict with the studies of Raff and colleagues using the A2B5 antibody which suggest that type-2 astrocytes are generated after oligodendrocytes, starting in the second postnatal week (reviewed by Miller *et al.*, 1989). It is agreed that type-1 astrocytes first appear at late embryonic stages (E16 in the rat) and develop prenatally and in the first postnatal week (Miller *et al.*, 1989; Skoff, 1990). In the light of these findings it seems unlikely that the type-2 astrocytes that we recognise in tissue culture actually exist *in vivo*. Undoubtedly two types of astrocyte can be discerned *in vivo* by various light and electron microscope techniques (reviewed in Miller *et al.*, 1989) but these do not conform to the tissue culture designation of type-1 and type-2 astrocytes.

The failure of a sensitive antibody against GAP-43 to identify astrocytes in tissue sections whilst readily labelling type-2 astrocytes in culture lends support to the foregoing argument that these cells are artefacts arising from misdirected differentiation of O-2A progenitors, which constitutively become oligodendrocytes *in vitro* (Temple and Raff,

1985; Miller *et al.*, 1989) and probably also *in vivo* (Hardy and Reynolds, 1991; Reynolds and Wilkin 1991). Previous studies have indicated that oligodendrocytes may transiently express GFAP during development both *in vivo* (Choi and Kim, 1984) and *in vitro* (Ogawa *et al.*, 1985) and it is shown here that oligodendrocytes express GAP-43 at an early stage in their development. Therefore the GFAP+, GAP-43+, ganglioside G_{D3}+ phenotype seen in neonatal mixed glial cultures may represent an immature oligodendrocyte prevented from undergoing terminal differentiation by factor(s) present in FCS or produced by other cell types *in vitro*.

6.3.5. GAP-43 In Type-1 Astrocytes

Previous investigations have failed to agree on the expression of GAP-43 by type-1 astrocytes *in vitro*. Initial studies by Vitković suggested that type-1 astrocytes show GAP-43 immunoreactivity but that this gradually disappears from the cells by 10 days *in vitro* (Vitković *et al.*, 1988; da Cunha and Vitković, 1990). However, my interpretation of the evidence presented in these publications was that GAP-43 appeared to be associated with debris (possibly axonal) on the surface of the type-1 astrocytes rather than being in the plasma membranes of these cells. Phagocytosis of this debris could account for the disappearance of the GAP-43. Indeed, Deloulme *et al.* (1990) showed that GAP-43 protein and mRNA were absent from type-1 astrocytes using Western and Northern blotting techniques, although it was unclear how these astrocytes were purified in this study.

In experiments with the anti-GAP-43 fusion protein antiserum, some epithelioid astrocytes in mixed glial cultures showed GAP-43 immunoreactivity at all time points studied and, furthermore, this was associated with both the astrocyte plasma membrane and surface-bound debris. Live staining of mixed glial cultures, which did not allow access of anti-GAP-43 antibodies to the interior of the cells, revealed

the presence immunoreactive debris which could also be visualised on the surface of the cells by phase contrast microscopy and this GAP-43+ debris was found to disappear within 10 days *in vitro* (data not shown). However, permeabilisation of parallel cultures did reveal the presence of GAP-43 in epithelioid cells which were ganglioside G_{D3}-, suggesting they were type-1 astrocytes (see Figure 6.1.e, f).

Consequently, enriched type-1 astrocyte/fibroblast cultures were prepared from the monolayer remaining after removal of O-2A progenitors by overnight shaking and used for immunostaining and Western blotting (data not shown). While most of the flat cells were GAP-43-, some were found to be GAP-43 immunoreactive to greater or lesser degrees of intensity, and this was associated with a single band in Western blots that co-migrated with purified CNS GAP-43. However, GAP-43+ O-2A progenitors and type-2 astrocytes were also found in these cultures (identified by the presence of ganglioside G_{D3}), probably due to the persistence of 'pre-progenitor' cells in these monolayers (R. Reynolds, personal communication).

While these studies suggest that a small population of type-1 astrocytes express GAP-43 to varying degrees, several experimental and theoretical factors stand in the way of a definitive conclusion. Foremost is the absence of a specific marker for type-1 astrocytes. These cells are operationally defined as flat astrocytes, expressing GFAP but not ganglioside G_{D3}. Triple labelling techniques would be required to show the presence of GAP-43+/ganglioside G_{D3}+/GFAP+ astrocytes in either mixed glial or type-1 astrocyte enriched cultures, although such techniques have been recently utilised in the study of glial cells (Armstrong *et al.*, 1990; Vaysse and Goldman, 1990). Secondly, 'pre-progenitor' cells continue to be present in type-1 astrocyte/fibroblast cultures after overnight shaking, although experiments are currently in progress to isolate these cells by a further differential adhesion step (R. Reynolds, personal

communication). These cells are capable of giving rise to the type-2 astrocytes contaminating these type-1 astrocyte/fibroblast cultures. Thirdly, type-2 astrocytes *in vitro* are known to undergo a shape change from the familiar stellate morphology to a flattened form, possibly induced by contact with other cells (Wilkin *et al.*, 1983; Johnstone *et al.*, 1986), and this renders morphology an unreliable indicator of 'astrocyte type'.

Consequently, in the double labelling studies performed, GFAP+/GAP-43+ flat astrocytes could represent type-2 astrocytes that have changed shape while ganglioside G_{D3}-/GAP-43+ flat cells might be fibroblasts rather than type-1 astrocytes. Isolation of monolayers devoid of 'pre-progenitor' cells, or the use of triple labelling techniques, would allow further delineation of the cell specificity of GAP-43 expression.

Chapter Seven

GAP-43 In Glial Cells Of The Peripheral Nervous System

7.1. Introduction

There are several types of glial cells in the PNS which all derive from the neural crest. The most numerous and widely studied class of peripheral glia, and the main subject of this Chapter, is the Schwann cells associated with axons in peripheral nerve trunks. In addition, satellite cells surround neuronal cell bodies in autonomic and sensory ganglia, and enteric glia perform the same role in the ganglia of the intestinal nervous system, while specialised glial cells are present at nerve terminals in the periphery.

7.1.1. Molecular Phenotypes Of Schwann Cells

Schwann cells can either provide myelin membrane insulation for axons (corresponding functionally to oligodendrocytes in the CNS) or ensheath unmyelinated axons in membrane-bound troughs within their cytoplasm. These phenotypes are regulated by axonal contact (Lemke and Chao, 1988; Brunden *et al.*, 1990; Jessen *et al.*, 1987a, 1990; Mirsky *et al.*, 1990). Immature Schwann cells at late embryonic stages (E16 to birth) express the protein markers S100, GFAP, vimentin, Ng-CAM, N-CAM, laminin, the cell surface protein A5E3 and the receptor for nerve growth factor (NGFr) (Jessen *et al.*, 1984; Martini and Schachner, 1986; Mirsky and Jessen, 1991).

Some Schwann cells in contact with axons are induced to express the major myelin proteins P_0, myelin basic protein (MBP), myelin-associated glycoprotein and become mature myelin-forming Schwann cells (Lemke and Chao, 1988; Brunden *et al.*, 1990; Mirsky and Jessen, 1991). It appears that axon diameter is the main determinant of differentiation along the myelination pathway and primarily axons of greater than 1 μm diameter are capable of such induction, but the nature of the axon-associated signal is unknown (Friede, 1972; Griffin *et al.*, 1988). Subsequent to the induction of myelin proteins, the early Schwann cell markers N-CAM, Ng-CAM, GFAP, A5E3 and NGFr are down-regulated by the

myelin-forming Schwann cells (Martini and Schachner, 1986; Jessen *et al.*, 1987b, 1990), which continue to express S100, laminin and vimentin.

Those cells not induced to form myelin continue to express all the early Schwann cell markers but do not synthesise significant amounts of myelin proteins. Thus it would appear that the non-myelin-forming Schwann cell phenotype is expressed by default in the absence of axons and that up-regulation of myelin proteins and down-regulation of N-CAM, GFAP, A5E3 and NGFr are coordinately regulated by axonal contact and that this process is reversible (see below).

7.1.2. Schwann Cell Phenotypes Are Acutely Regulated By Axons

Removal of axonal contact by either surgical denervation of nerves *in vivo* or by dissociation of nerves *in vitro* leads to loss of the major myelin proteins and up-regulation of Ng-CAM, N-CAM, A5E3 and NGFr by previously myelin-forming Schwann cells (Nieke and Schachner, 1985; Taniuchi *et al.*, 1986, 1988; Lemke and Chao, 1988; Trapp *et al.*, 1988; Jessen *et al.*, 1990). Another consequence is the production of NGF by Schwann cells, although this is regulated in part by macrophages (Heumann *et al.*, 1989). In the case of GFAP there is some disagreement over the effects of axonal deprivation (Mokuno *et al.*, 1989; Neuberger and Cornbrooks, 1989; Condorelli *et al.*, 1990; Jessen *et al.*, 1990; see Discussion for details).

The dedifferentiation of myelin-forming Schwann cells in the absence of axons is fully reversible. If axons are allowed to repopulate denervated nerves, some Schwann cells are induced to become myelin-forming and undergo the same change in phenotype as during development (Nieke and Schachner, 1985, Jessen *et al.*, 1987a, Gupta *et al.*, 1988, Martini and Schachner, 1988, Neuberger and Cornbrooks, 1989). Similarly, Schwann cells co-cultured with axons *in vitro* are capable of myelination, and down-regulate proteins characteristic of non-myelin-forming Schwann cells (Eldridge *et al.*, 1987; Seilheimer *et al.*, 1989).

Intracellular cyclic AMP has been suggested to mediate the axonal signals specifying differentiation along the myelination pathway (Sobue *et al.*, 1986). Elevation of cyclic AMP by addition of cholera toxin or forskolin, or addition of cyclic AMP analogues, to Schwann cells in culture induces expression of myelin proteins P_0 and MBP and suppresses the expression of GFAP, A5E3 N-CAM and NGFr (Lemke and Chao, 1988; Mokuno *et al.*, 1988; Morgan *et al.*, 1991).

7.1.3. GAP-43 In Schwann Cells

GAP-43 was first detected in Schwann cells by Tetzlaff *et al.* (1989) in immunostained sections of regenerating facial and sciatic nerves. It was concluded that the Schwann cells had taken up GAP-43 from elongating axons by an unknown mechanism. GAP-43 was also demonstrated in Schwann cells of dissociated dorsal root ganglia by Woolf *et al.* (1990), although the immunostaining was of very low intensity and there existed the formal possibility of uptake from axons of the primary sensory neurons present in the cultures.

In contrast, some studies by other groups have failed to demonstrate the presence of GAP-43 in Schwann cells. Meiri *et al.* (1988) failed to detect immunoreactive GAP-43 in purified Schwann cell cultures or in the glial component of sympathetic ganglion cultures. Immunohistochemistry of crushed peripheral nerve only revealed GAP-43 in regenerating axons, identified by the presence of neurofilament protein, while control nerves showed no immunoreactivity (Verhaagen *et al.*, 1986). To my knowledge, there have been no published reports showing GAP-43 expression in Schwann cells by metabolic labelling, Northern blotting or *in situ* hybridisation. Although no evidence or methodology was presented, Basi *et al.* (1987) reported that no mRNA for GAP-43 could be detected in distal stumps of transected peripheral nerve, however GAP-43 mRNA has been faintly detected in some Northern blots of such tissue (M. Bisby, personal communication).

7.1.4. The Present Study

In the studies on sciatic nerve grafts to transected optic nerve (Chapter 5) it was observed that cells in the peripheral nerve stained with antibodies against GAP-43 prior to the arrival of regenerating retinal ganglion cell axons and that these cells increased in number with time. These cells were spindle shaped and existed in columns along the axis of the grafted segment, and some appeared to migrate out into the junctional zone between the optic and sciatic nerves. Therefore it was concluded on the grounds of morphology and location that there were Schwann cells in the grafted material that expressed GAP-43.

In conjunction with markers of both myelinating and non-myelin-forming Schwann cells, the anti-GAP-43 fusion protein antiserum was used to demonstrate the expression of GAP-43 in the normal sciatic nerve and after either crush or transection at mid-thigh level. While transection denervates the nerve permanently, the crushed nerve is able to support regeneration of sensory and motor axons into the distal portion (Verhaagen *et al.*, 1986, Hall, 1989; Fawcett and Keynes, 1990). This allowed Schwann cells to be studied in the absence of an axonal source of GAP-43 and to evaluate the temporal changes in GAP-43 expression during denervation and reinnervation in order to establish the phenotypic segregation of the protein. The cervical sympathetic trunk which is 99% unmyelinated (Aguayo *et al.*, 1976) was also examined to address the nature of the Schwann cells expressing GAP-43. In addition, Schwann cells cultured in the absence of axons were studied to further exclude the possibility of uptake of neuronal GAP-43.

7.2. Results

7.2.1. GAP-43 In Normal Sciatic Nerve

The expression of GAP-43 in normal peripheral nerve tissue was initially examined in the sciatic nerve. This was chosen because of its

large size, ease of dissection, and the presence of both myelinated and unmyelinated fibres. In transverse frozen sections, GAP-43 could be visualised in sparsely scattered cells (see Figure 7.1.). Neurofilament protein had a different staining pattern, as visualised with the RT97 monoclonal antibody, being evenly distributed throughout the nerve as discrete spots representing large diameter axons. Close examination of sections double labelled for GAP-43 and neurofilament revealed that the GAP-43+ cells did not surround these large calibre axons.

Non-myelin-forming Schwann cells, labelled with GFAP, were also sparsely distributed in transverse sections and double staining with GAP-43 revealed extensive co-labelling in these structures. However, a few GFAP+ cells did not stain for GAP-43. The co-distribution of GAP-43 and GFAP suggests that GAP-43 is present in most non-myelin-forming Schwann cells. S100 and vimentin both showed a wider distribution than GAP-43, labelling the entire population of Schwann cells including those myelinating the RT97+ large diameter axons.

GAP-43 was also seen in GFAP+ non-myelin-forming Schwann cells in longitudinal sections (Figure 7.2.). However the ubiquitous distribution of MBP did not allow the presence of GAP-43 in myelin-forming Schwann cells to be assessed. Consequently, teased nerves were prepared in order to examine the expression of GAP-43 in individual Schwann cell cables (Figure 7.3.). The nature of the GAP-43-immunoreactive Schwann cells was determined by phase contrast microscopy, to reveal the presence of myelin, and by double labelling with antibodies against GFAP or MBP. GAP-43 was clearly present in GFAP+ non-myelin-forming Schwann cells, but was excluded from myelin-forming Schwann cells.

Figure 7.1.

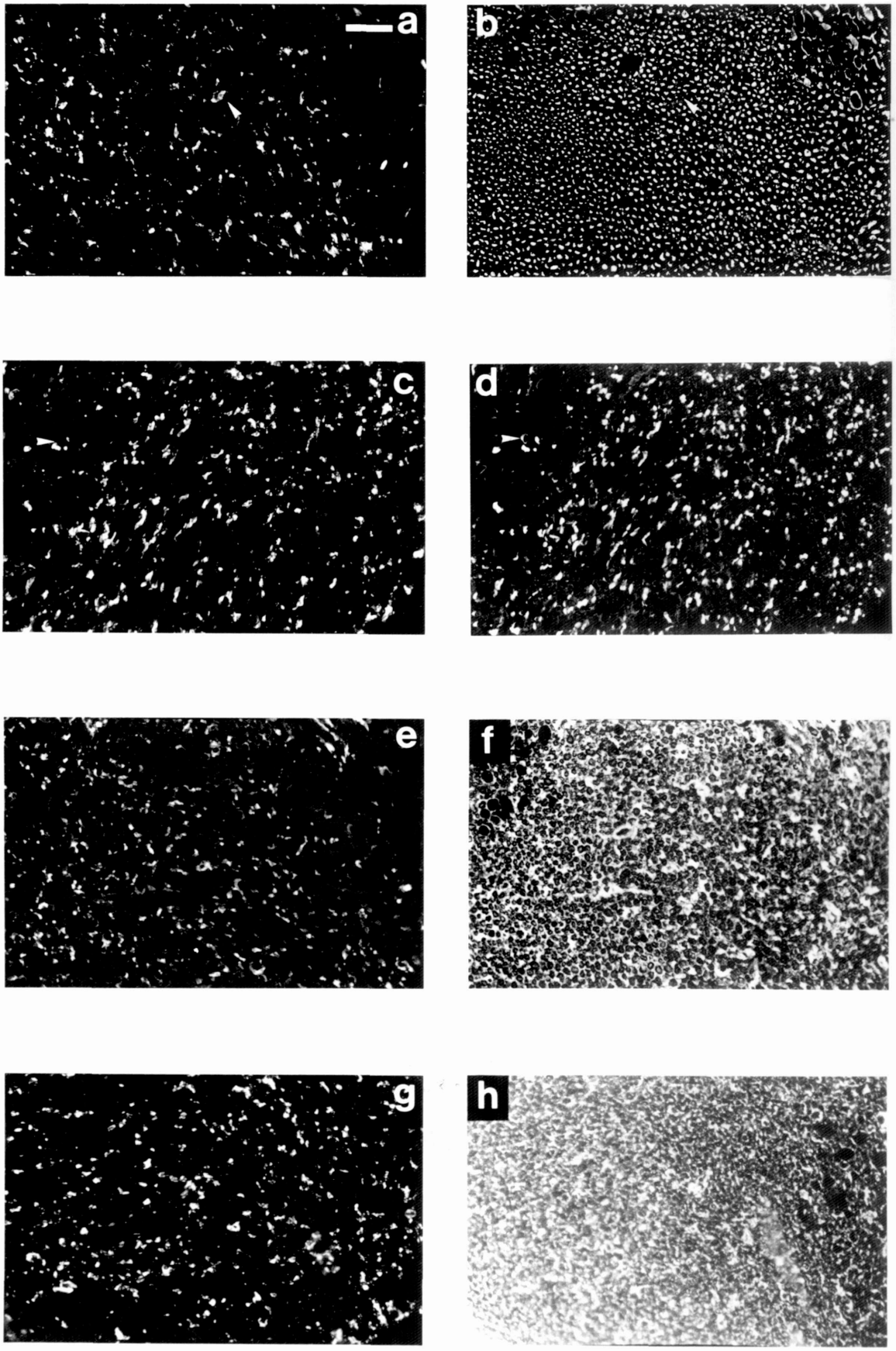
Transverse sections of normal sciatic nerve double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,c,e,g) and monoclonal antibodies against neurofilament (b), GFAP (d), S100 (f) or vimentin (h).

A and B. RT97+ axons show a more extensive distribution than GAP-43. While neurofilament appears as evenly distributed dots (corresponding to large diameter axons) GAP-43 is more sparse and sometimes form rings (arrowhead in a). These rings do not surround the neurofilament+ axons (arrowhead in b).

C and D. GAP-43 and GFAP have an almost identical distribution, but a few GFAP+ cells do not stain for GAP-43 (arrowheads).

E,F,G and H. S100 (f) and vimentin (h) are more extensively distributed than GAP-43 (e,g), labelling the entire population of Schwann cells, including those surrounding the large diameter axons.

Scale bar in a, 50 μm.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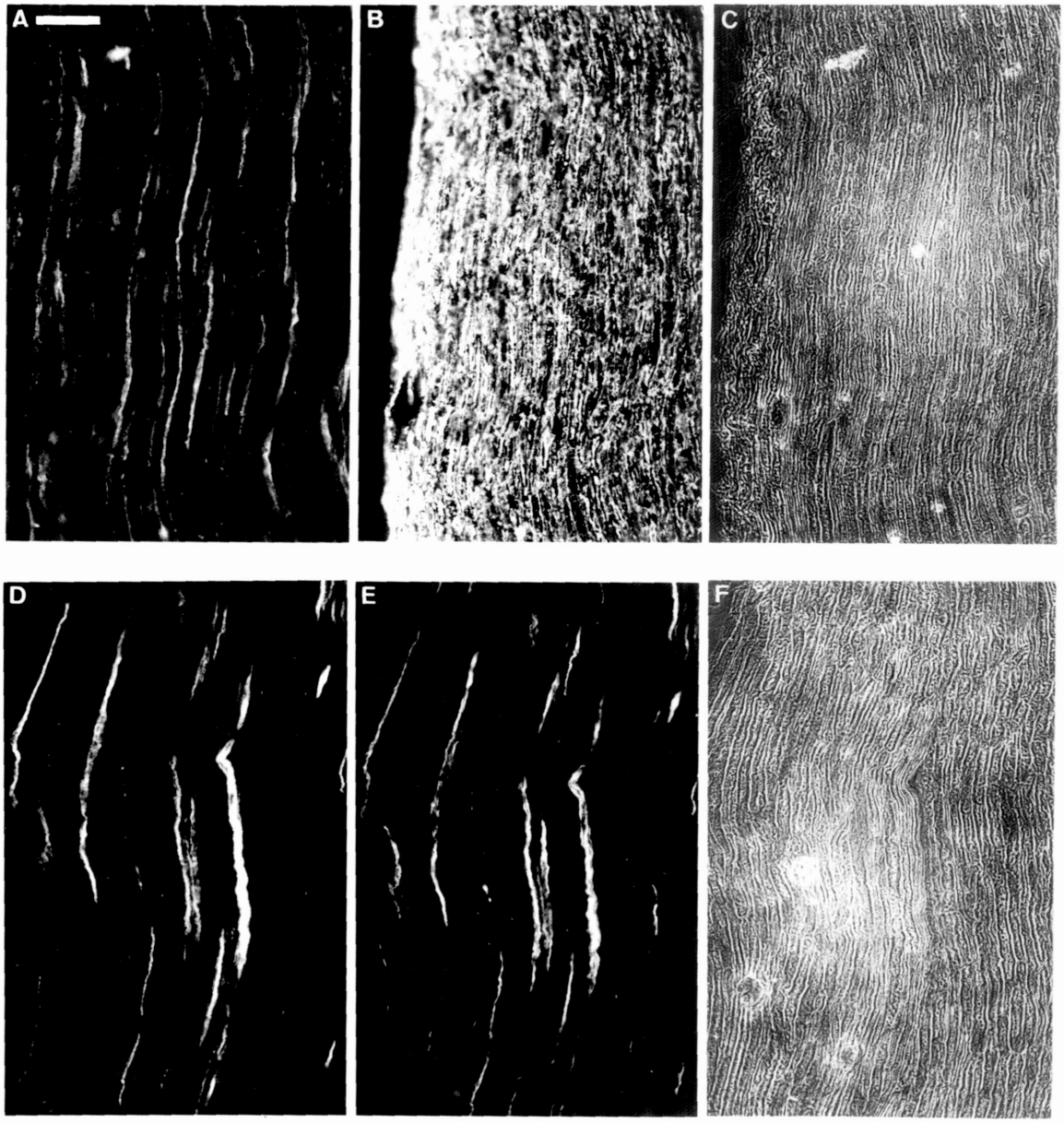


Figure 7.2.

Longitudinal sections of normal sciatic nerve double labelled with antibodies against GAP-43 (a and d) and MBP (b) or GFAP (e). The corresponding phase contrast images are also presented (c and f).

GAP-43 (d) co-localises with GFAP (e) in non-myelin-forming Schwann cells, but the extensive distribution of myelin renders the identification of these cells impossible in the phase contrast and MBP-stained images (b,c and f).

Scale bar in a, 50 μm.

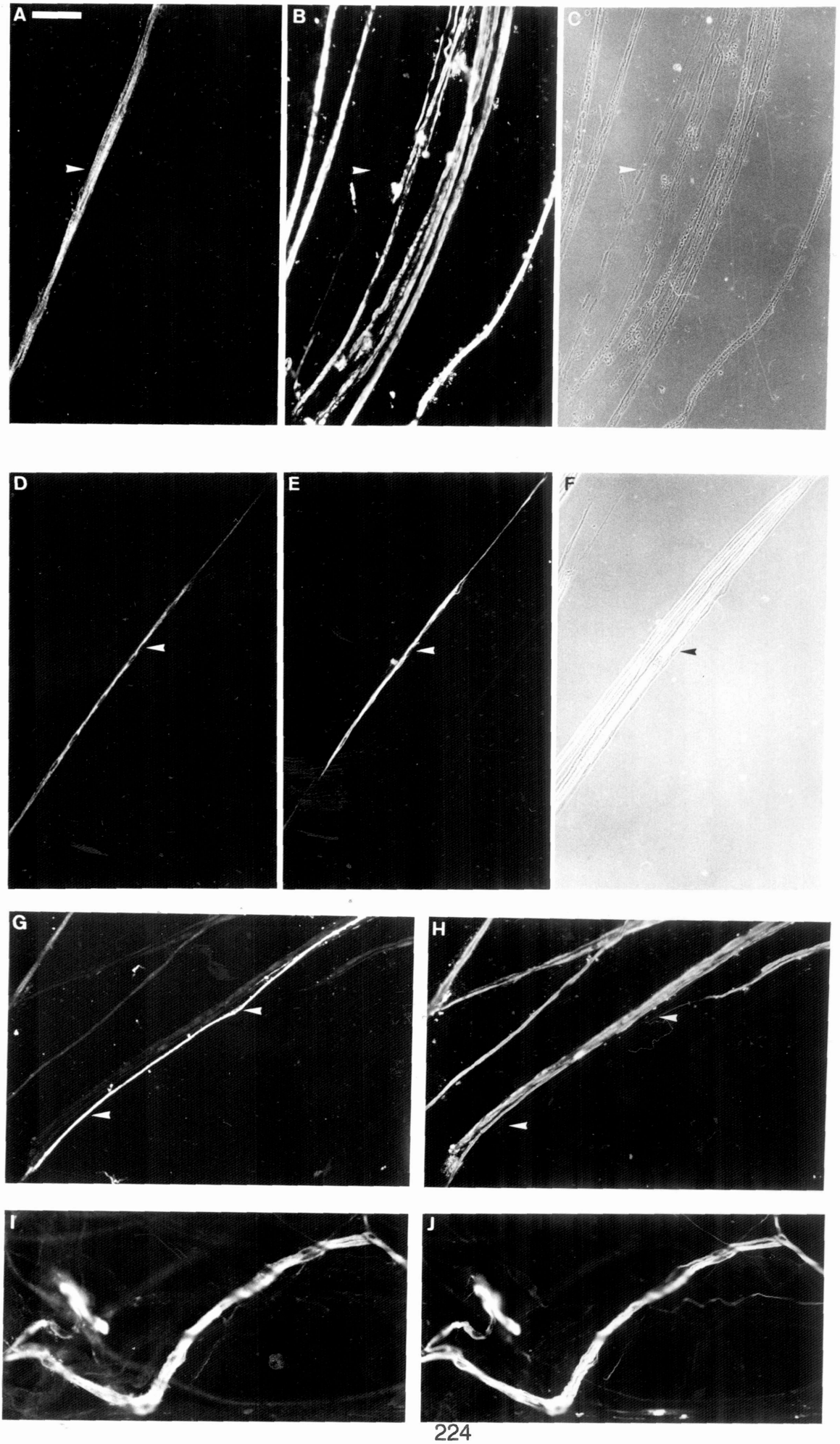
Figure 7.3.

Teased preparations of normal sciatic nerve double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,d,g and i) and monoclonal antibodies against MBP (b and h) or GFAP (e and j). Phase contrast images are also presented in some instances (c and f).

A,B and C. G and H. Non-myelin-forming Schwann cells (arrowheads) contain GAP-43 (a and g) but do not stain for MBP (b and h). In the absence of a corresponding phase contrast image, micrograph of the GAP-43 staining (g) has been overexposed to show the position of MBP+ myelin-forming Schwann cells. Only the cell marked with arrowheads is GAP-43+.

D,E and F. I and J. Non-myelin-forming Schwann cells stained for both GAP-43 (d and i) and GFAP (e and j). Arrowheads (in d,e and f) mark the position of the cable of non-myelin-forming Schwann cells adjacent to several myelin-forming Schwann cells visible under phase contrast (f). Myelin-forming Schwann cells which are not stained for GAP-43 or GFAP can also be seen in micrograph of GAP-43 (i) which has been slightly overexposed in the absence of a corresponding phase contrast image.

Scale bar in a, 50 μm.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7.2.2. GAP-43 In Cervical Sympathetic Trunk

To verify the expression of GAP-43 by non-myelin-forming Schwann cells, teased preparations were made from the sympathetic trunk of the superior cervical ganglion which is 99% unmyelinated (Aguayo *et al.*, 1976). Figure 7.4. shows that nearly all Schwann cells in these preparations were double labelled for both GAP-43 and GFAP, but that GAP-43 was not present in those few cells expressing MBP. This confirms that GAP-43 is restricted to non-myelin-forming Schwann cells in the normal PNS. Western blots of homogenised cervical sympathetic trunk confirmed the presence of GAP-43 (Figure 7.5.).

However, these experiments did not exclude the possibility that GAP-43 is present in unmyelinated axons rather than the non-myelin-forming Schwann cells themselves. Therefore, Schwann cells were examined in the absence of axons, both in dissociated cell culture and in chronically denervated nerve segments *in vivo*.

7.2.3. GAP-43 In Cultured Schwann Cells

To examine individual Schwann cells deprived of axons, dissociated cell cultures (prepared from sciatic nerves of newborn rats by H.J.S. Stewart, University College, London) were double labelled with anti-GAP-43 fusion protein antiserum and the Schwann cell markers S100 and NGFr. Some Schwann cells were intensely GAP-43+ while others did not stain at all. Fibroblasts remaining in these cultures were not stained. GAP-43 had the same cellular distribution as NGFr, being present in sheet-like membranous extensions. These structures did not contain S100, which appeared restricted to the cytoplasm of the Schwann cell bodies.

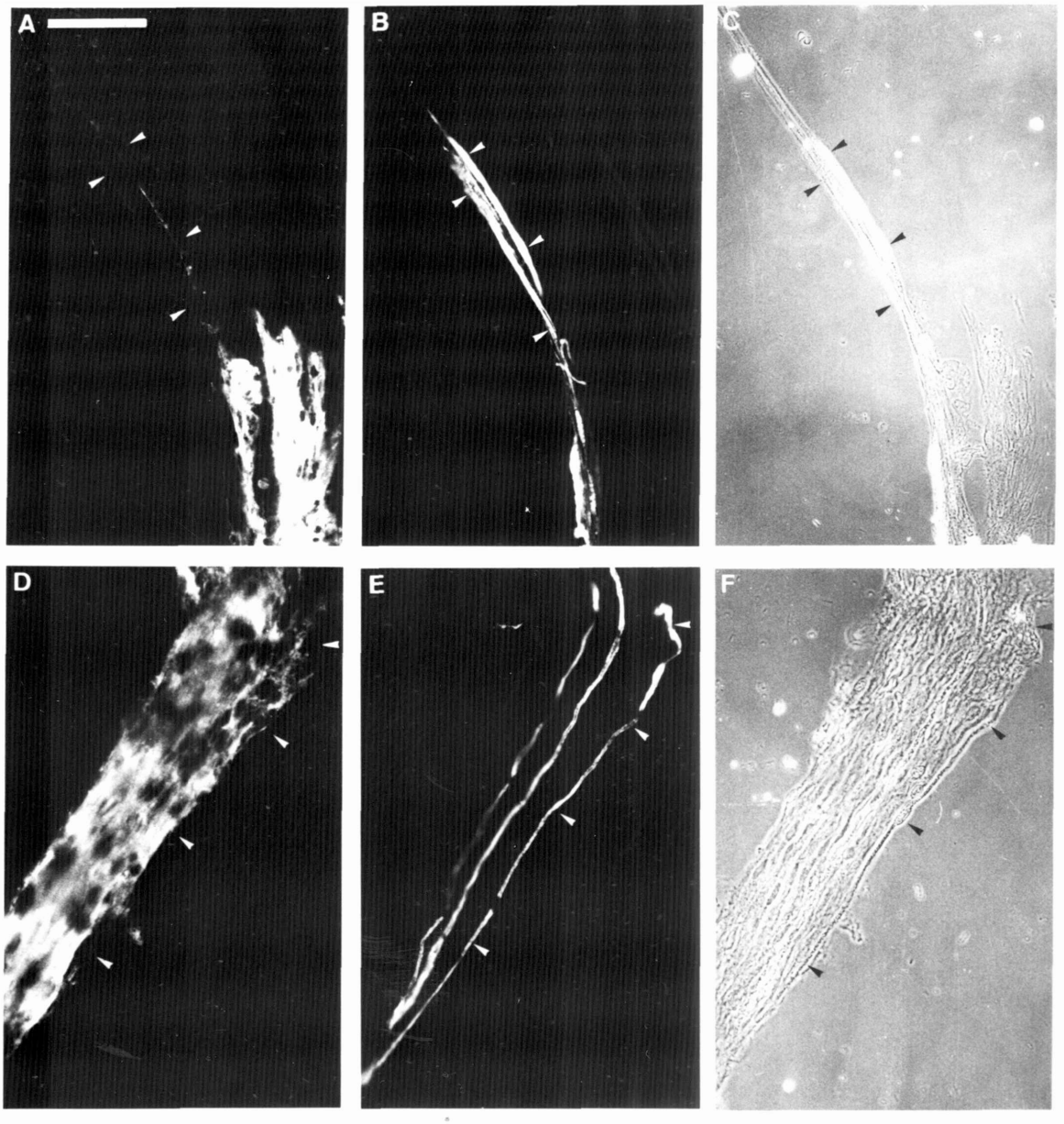
Western blotting of proteins from membranes of cultured Schwann cells confirmed the presence of membrane-bound GAP-43 (Figure 7.5.).

Figure 7.4.

Teased preparations of cervical sympathetic trunk double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,d,g and i) and monoclonal antibodies against MBP (b and e) or GFAP (h and j). Phase contrast images are also presented in some instances (c and f).

A,B and C. D,E and F. Sympathetic trunk consists almost entirely of non-myelin-forming Schwann cells which contain GAP-43 (a and d) but do not stain for MBP (b and e). The few myelin-forming Schwann cells in this nerve do not contain GAP-43 (arrowheads). Scale bar in a, 50 μm.

G and H. I and J. Non-myelin-forming Schwann cells contain both GAP-43 (g and i) and GFAP (h and j). Scale bar in g, 50 μm.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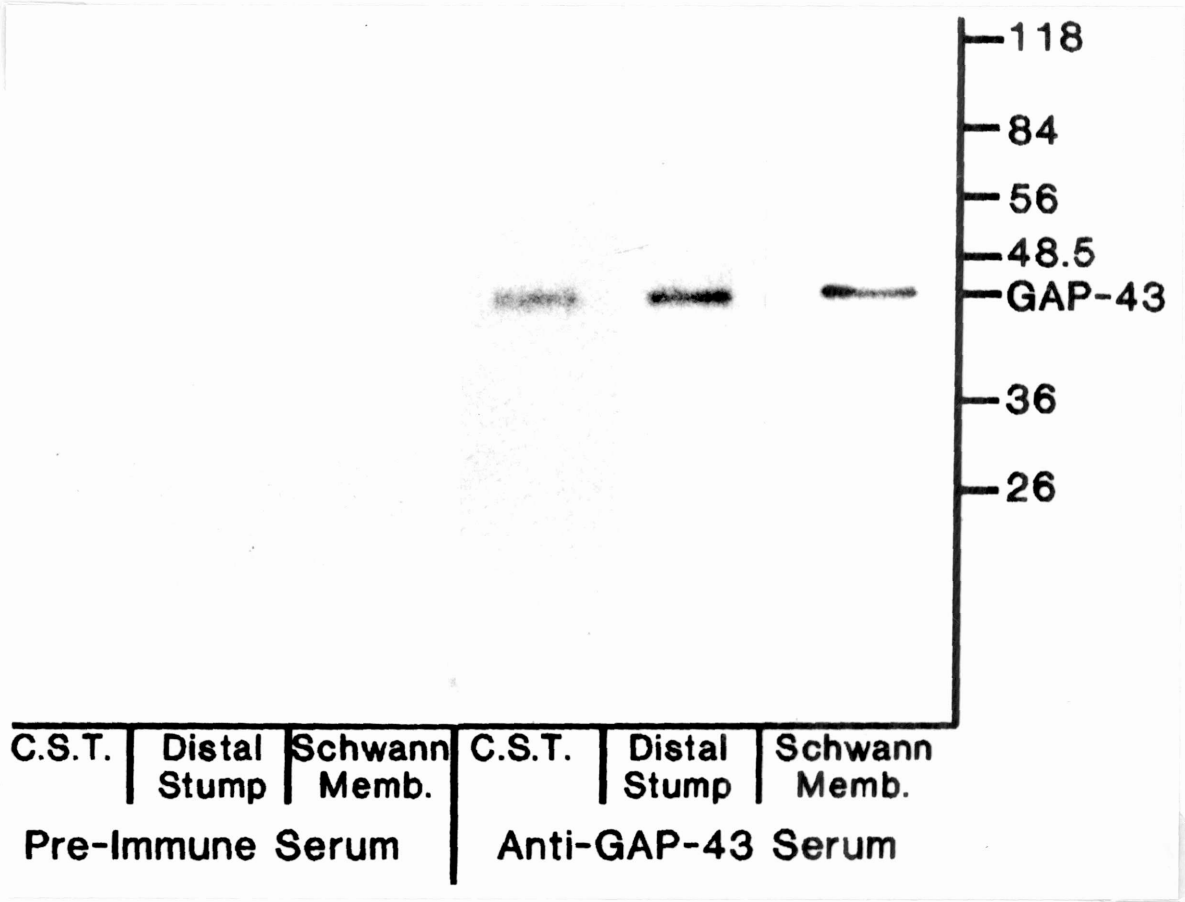


Figure 7.5.

Western blots of purified membranes from cultured Schwann cells (SCHWANN MEMB.) and whole tissue homogenates of cervical sympathetic trunk (CST) or denervated distal stump of sciatic nerve 4 weeks after transection (DISTAL STUMP) stained with anti-GAP-43 fusion protein antiserum or pre-immune serum diluted 1/1000. The RMM of standard marker proteins and the position of GAP-43 are marked to the right.

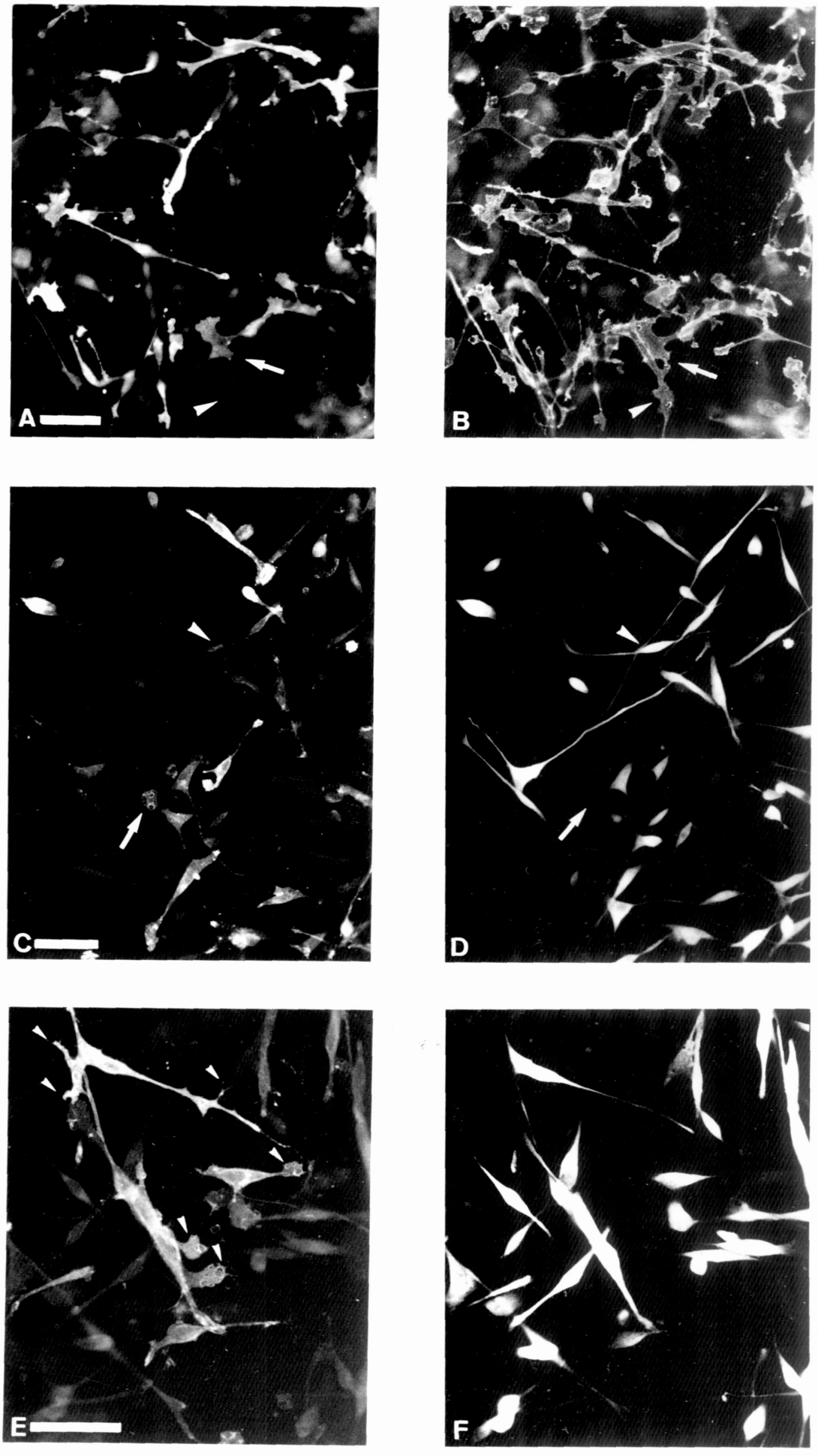
Only GAP-43 was recognised by anti-GAP-43 fusion protein antiserum and there were no cross-reacting proteins. Pre-immune serum did not recognise any proteins in these preparations.

Figure 7.6.

Cultured Schwann cells double labelled with either anti-GAP-43 fusion protein antiserum diluted 1/1000 and monoclonal antibodies against NGFr (a and b) or 91E12 monoclonal antibody against GAP-43 and polyclonal antiserum against S100 (c,d,e and f).

A and B. C and D. GAP-43 (a and c) is variable in the membranes of Schwann cells containing NGFr (b) and S100 (d). Some cells do not contain GAP-43 immunoreactivity (arrowheads) while others are brightly stained. Both GAP-43 and NGFr label velate membrane extensions which are not labelled by S100 (arrows). Scale bars in a and c, 50 μm.

E and F. Higher magnification micrograph showing a large number of S100+ Schwann cells (f) which show variable staining for GAP-43. Note the presence of several GAP-43+ membrane extensions (arrowheads in e). Scale bar in e, 50 μm.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7.2.4. GAP-43 In Chronically Denervated Sciatic Nerve

Sciatic nerves were surgically denervated by transection, as described in Materials and Methods, and reinnervation was prevented by deflecting the proximal stumps into adjacent muscle blocks. The absence of axons in distal segments was confirmed by RT97 immunohistochemistry up to 8 weeks after denervation (Figure 7.7.) and tissue from these animals was used to assess the expression of GAP-43 by Schwann cells in double labelling experiments.

GAP-43 was present in a minority of Schwann cells up to 2 weeks after transection, as shown in teased preparations of distal segments stained for GAP-43 and vimentin (Figure 7.8.). By 4 weeks however, nearly all Schwann cells appeared GAP-43+ in teased nerves and essentially the entire population were immunoreactive at 8 weeks. This was confirmed in frozen sections (Figure 7.9.) which showed that GAP-43 was restricted to a small population of cells which were also GFAP+ up to 2 weeks after transection, while at 4 and 8 weeks, GAP-43 was widespread throughout the denervated segments. At these later time points the distribution of GAP-43 exceeded that of GFAP and many GAP-43+ Schwann cells were not immunoreactive for GFAP.

Western blotting cofirmed the presence of GAP-43 in distal segment homogenised 4 weeks after denervation (Figure 7.5.). The absence of axons in this tissue was confirmed by prior removal of a 3 mm portion of the *proximal* end of the *distal* segment, which was teased apart and stained with the RT97 monoclonal antibody against neurofilament protein (data not shown).

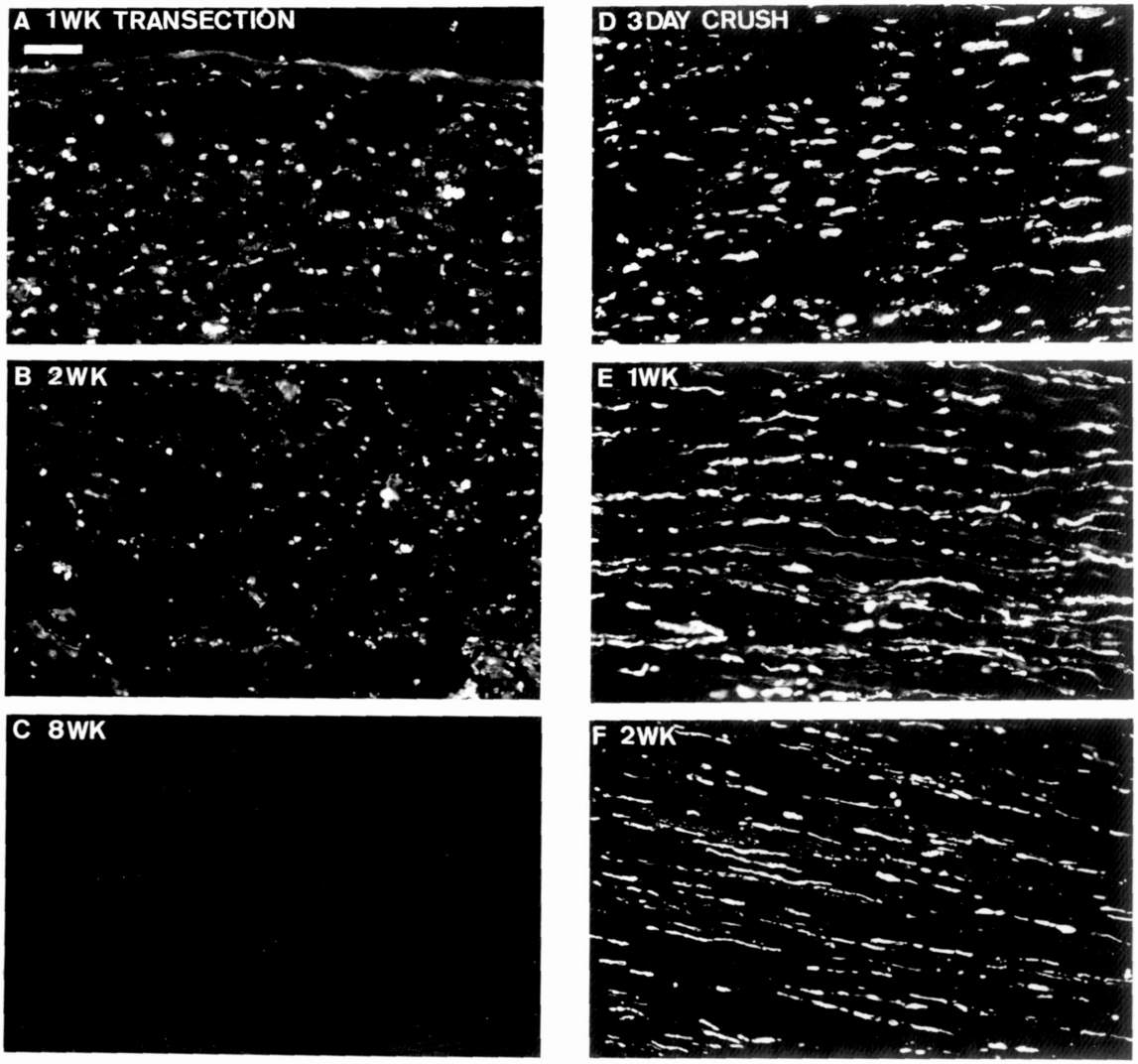


Figure 7.7.

Sections of transected (a,b and c) and crushed (d,e and f) sciatic nerve stained with the monoclonal antibody RT97 to demonstrate the presence of axons (see Materials and Methods).

A,B and C. Axons are absent from the distal stump of transected sciatic nerves up to 8 weeks after axotomy.

D,E and F. After sciatic crush, only axon debris is present at 3 days (d), but regenerating axons are visible at 1 and 2 weeks (e and f).

Scale bar in a, 50 μm.

Figure 7.8.

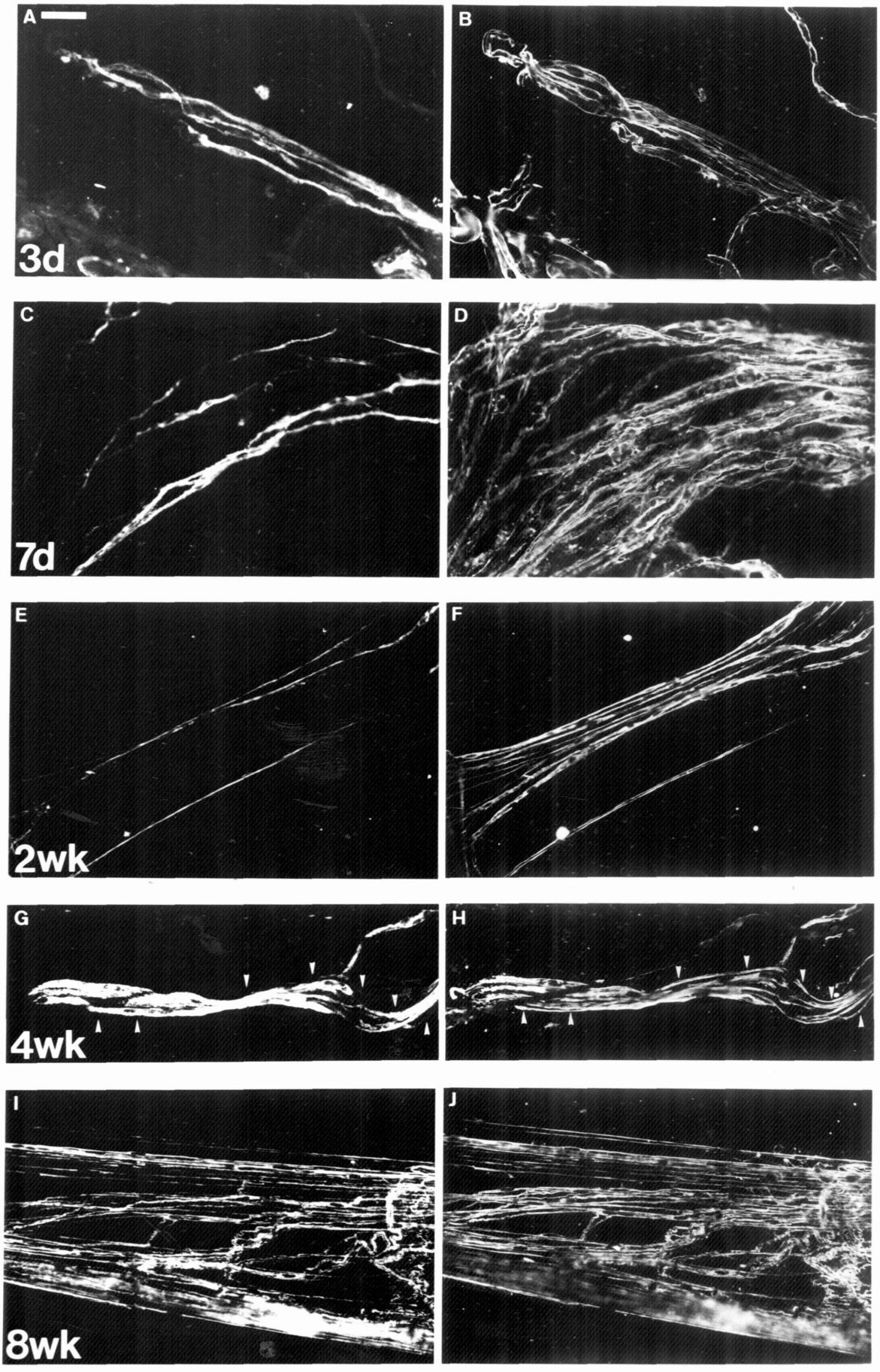
Teased preparations from denervated distal segments of sciatic nerve double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,c,e,g and i) and monoclonal antibodies against vimentin (b,d,f,h and j).

A,B. C,D. E,F. Up to 2 weeks, only a minority of Schwann cells visualised with vimentin (b,d and f) also contain GAP-43 (a,c and e).

G,H. At 4 weeks, nearly all Schwann cells contain GAP-43 (g). However, a few cells visualised with vimentin (h) do not stain for GAP-43 (arrowheads). A blood vessel, which is not labelled by antibodies against either GAP-43 or vimentin, can be seen running diagonally across the Schwann cell bundle.

I,J. At 8 weeks, essentially all the Schwann cells visualised with vimentin (j) also contain GAP-43 (i).

Scale bar in a, 50 μm.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8wk

Figure 7.9.

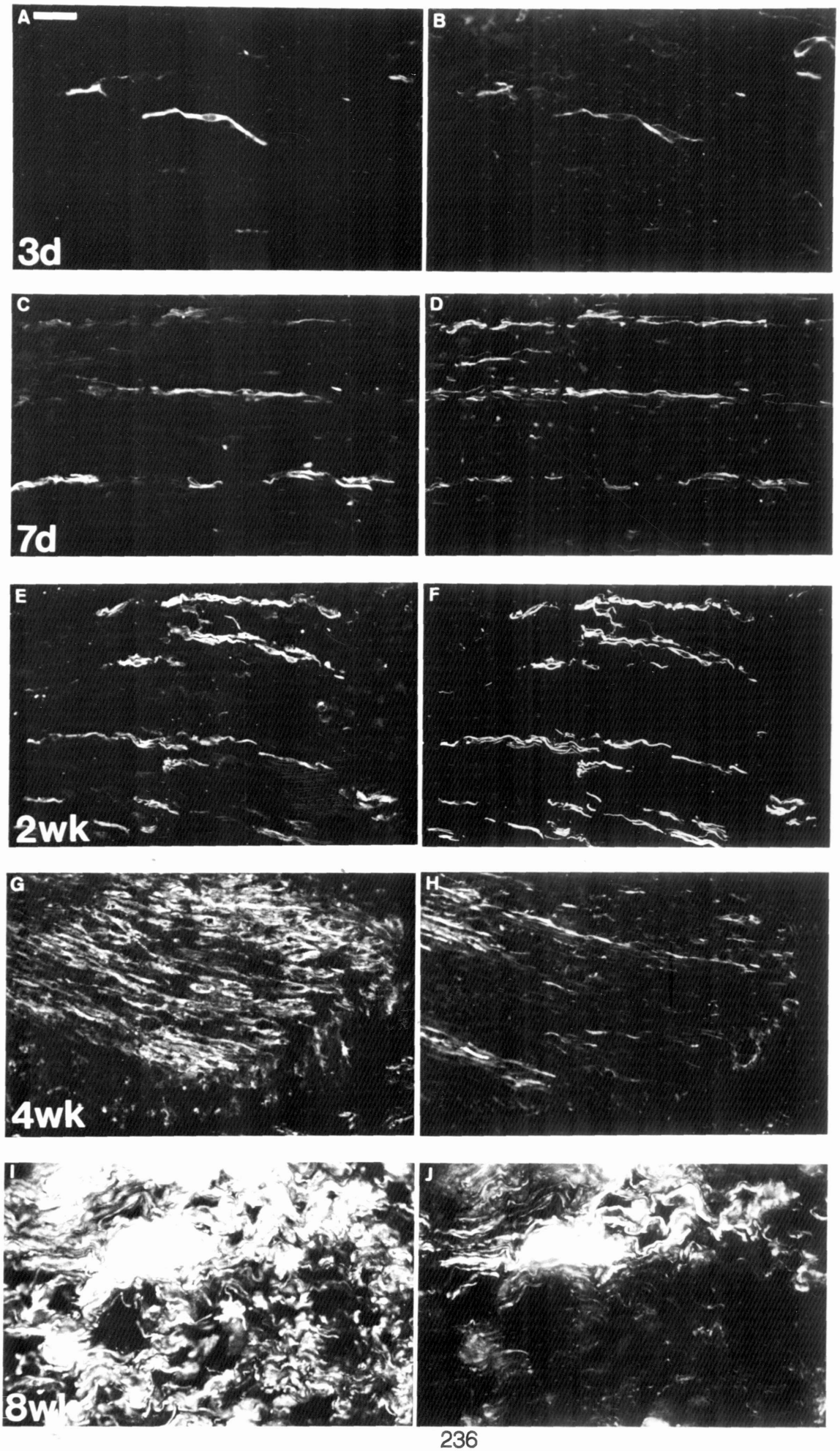
Longitudinal sections of denervated distal segments of sciatic nerve double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,c,e and i) and monoclonal antibodies against GFAP (b,d,f and j). Adjacent sections are shown at the 4 week time point, stained with anti-GAP-43 antiserum (g) and polyclonal antiserum against GFAP (h).

A,B. C,D. E,F. Up to 2 weeks after transection, GAP-43 (a,c and e) is expressed by a minority of Schwann cells, which also express GFAP (b,d and f).

G,H. At 4 weeks, the majority of Schwann cells in the denervated stump contain GAP-43 (g), while GFAP labels very few cells in an adjacent section (h).

I,J. At 8 weeks, most or all Schwann cells are GAP-43+ (i) but only a few also stain for GFAP (j).

Scale bar in a, 50 μm.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7.2.5. GAP-43 Expression During Reinnervation Of Crushed Sciatic Nerve

The regeneration of axons after crushing of the sciatic nerve was followed by RT97 immunohistochemistry. Although RT97+ regenerating axons could be seen by 1 week (Figure 7.7.), GAP-43 immunoreactivity was unexpectedly restricted to GFAP+ non-myelin-forming Schwann cells in sections (Figure 7.10.) and could not be seen in the regenerating axons.

Staining of teased tissue distal to the lesion site (Figure 7.11.) revealed that the number of GAP-43+ Schwann cells did not increase as had been found in the transected nerves. GAP-43 was restricted to a minority of the vimentin+ Schwann cells at all survival times (up to 30 days).

7.2.6. GAP-43 Is Not Expressed By Macrophages

The intermediate filament protein vimentin is a molecular marker for several cell types in addition to Schwann cells, including the macrophages which invade peripheral nerve tissue after damage. To exclude the possibility that GAP-43 in lesioned sciatic nerve is due to invading macrophages, these cells were visualised with the ED_1 monoclonal antibody (Dijkstra *et al.*, 1985) in frozen sections double labelled with anti-GAP-43 antiserum. Macrophages did not contain immunoreactive GAP-43 and the ED_1 staining pattern was complementary to that of GAP-43 (Figure 7.12.).

7.2.7. GAP-43 Is Expressed By Enteric Glial Cells

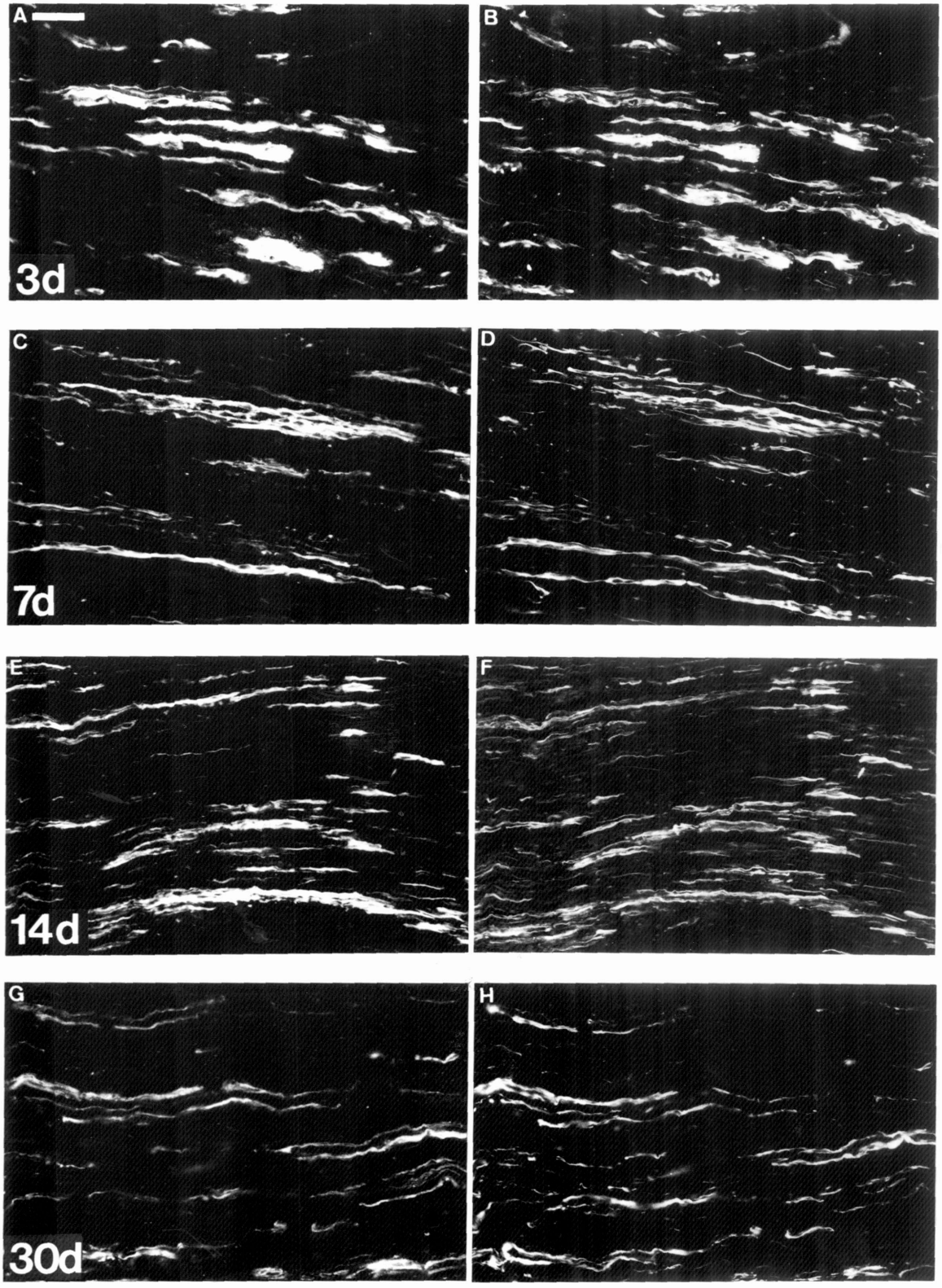
Glial cells of the enteric nervous system, which bear some antigenic similarities to non-myelin-forming Schwann cells (Jessen and Mirsky, 1984), were examined in polyester wax embedded sections of stomach, small intestine (ileum) and large intestine (colon). Glial cells of the ganglia of the myenteric and submucosal plexuses were immunoreactive for GAP-43 in sections double labelled for GAP-43 and S100 or GFAP (Figure 7.14.).

Figure 7.10.

Longitudinal sections of sciatic nerve distal to crush injury double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,c,e and g) and monoclonal antibodies against GFAP (b,d,f and h).

At all times up to 30 days, GAP-43 and GFAP show the same distribution. GAP-43+ regenerating axons are not visible despite the ingrowth of RT97+ axons as early as 1 week after axotomy (see Figure 7.7.).

Scale bar in a, 50 μm.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30d

Figure 7.11.

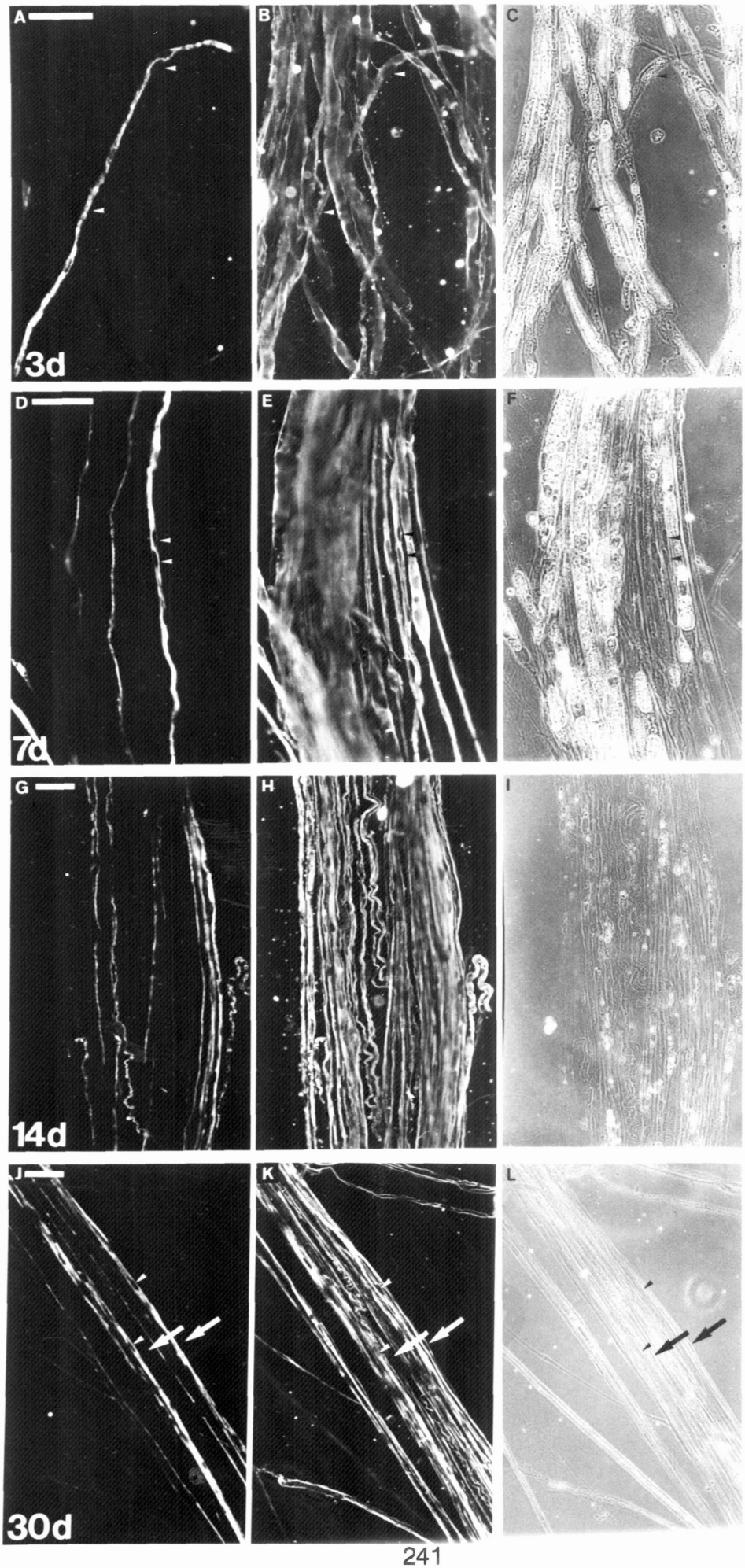
Teased preparations from distal segments of crushed sciatic nerve double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a,d,g and j) and monoclonal antibodies against vimentin (b,e,h, and k). Corresponding phase contrast images are also presented (c,f,i and l).

A,B and C. D,E and F. At 3 and 7 days after crushing, single GAP-43+ (a and d) non-myelin-forming Schwann cell cables are visible (arrowheads) in the bundles of phase bright myelin-forming Schwann cells (c and f). However, phase dark non-myelin-forming Schwann cells can be seen which do not stain for GAP-43.

G,H and I. At 14 days, myelin debris has been removed (i) but only a few Schwann cell cables contain GAP-43 (g).

J,K and L. At 30 days, regenerating axons have initiated myelin formation in some Schwann cells (arrows) which are thus phase bright (l) but do not contain GAP-43 (j). At this stage, it is still a minority of Schwann cells that are GAP-43+ (arrowheads)

Scale bars, 50 μm.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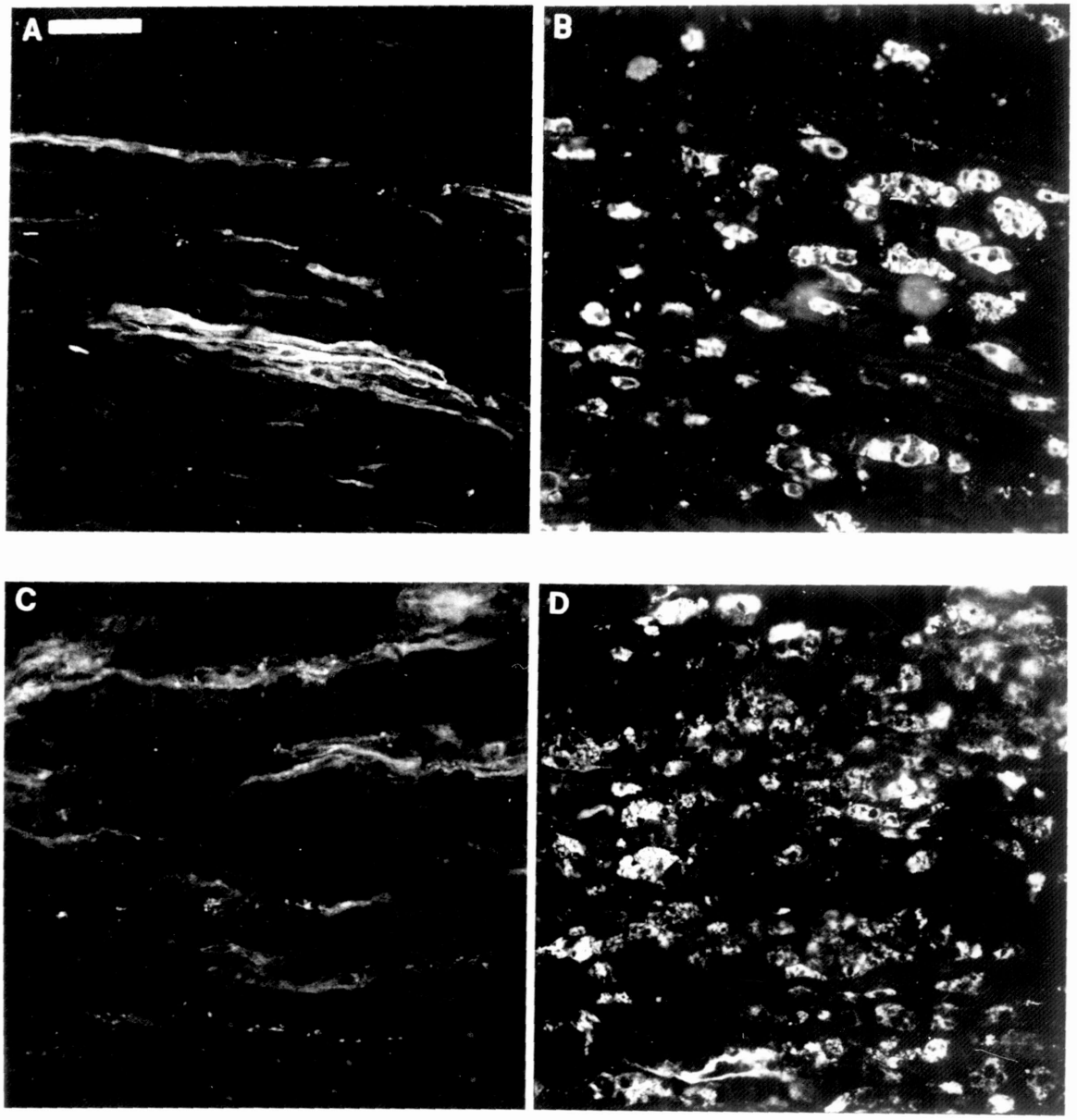


Figure 7.12.

Longitudinal sections of sciatic nerve 7 days after transection (a and b) and 3 days after crushing (c and d) double labelled with antibodies against GAP-43 (a and c) and the macrophage marker ED_1 (b and d).

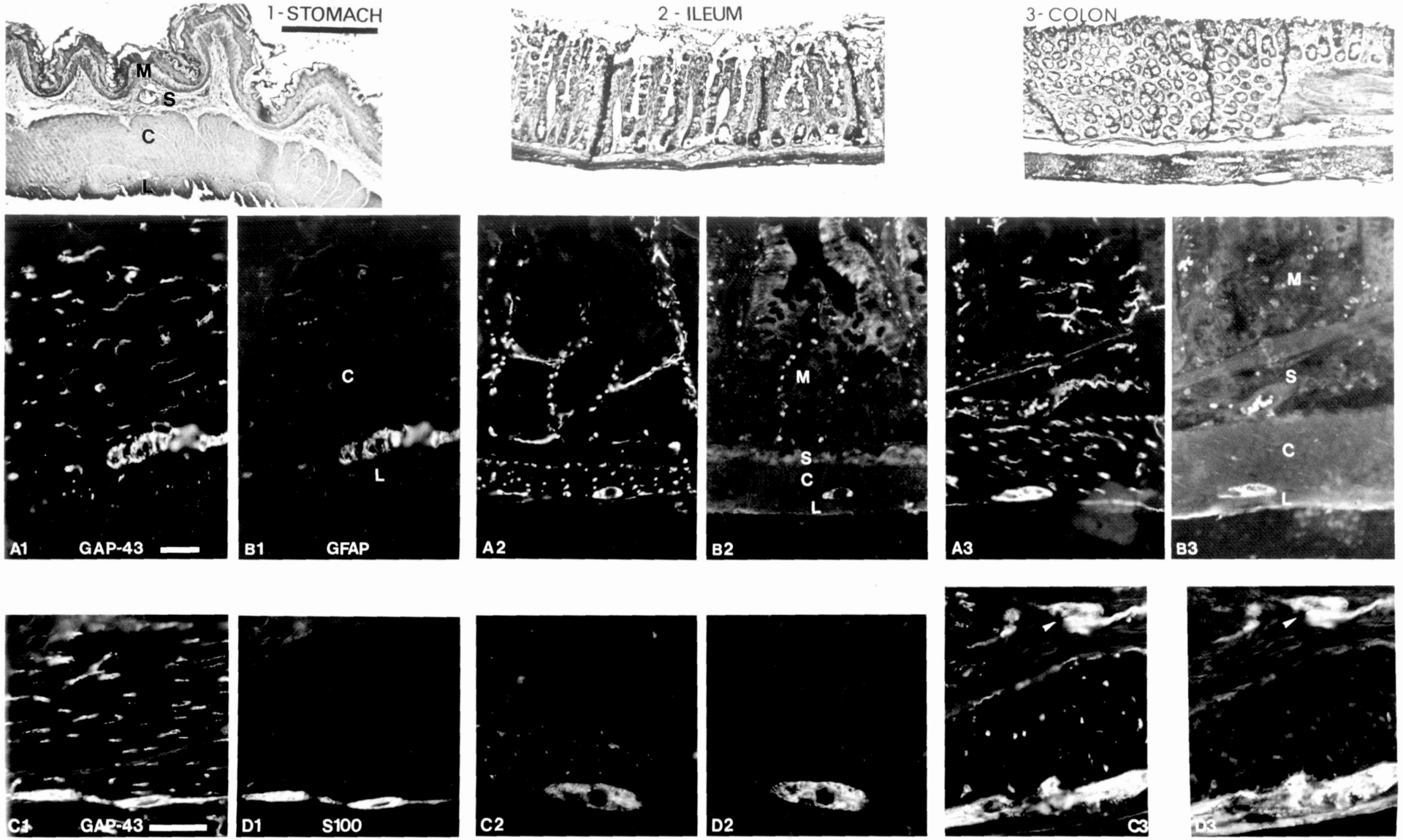
Scale bar in a, 50 μm.

Figure 7.13.

This figure is composed of 3 panels, referring to 1) stomach, 2) ileum and 3) colon. Each panel contains a reference section stained with toluidine blue (scale bar in 1, 5 mm), a section double labelled with anti-GAP-43 fusion protein antiserum diluted 1/5000 (a) and monoclonal antibodies against GFAP (b) (scale bar in a1, 50 μm) and a high power view of a section double labelled with monoclonal antibodies against GAP-43 (c) and polyclonal antiserum against S100 (d) (scale bar in c1, 50 μm). The different layers of the intestinal wall are labelled in the reference section of stomach (M, mucosa; S, submucosa; C, inner circular muscle layer of *m. externa*; L, outer longitudinal muscle layer of *m. externa*) and these layers are also marked in micrograph b of each panel.

Neuronal cell bodies in ganglia of the myenteric plexus (located between the circular and longitudinal muscle layers) are unlabelled by antibodies against GAP-43, while the glial cells surrounding them are positive for GAP-43 (a and c), GFAP (b) and S100 (d) in all three regions. Likewise, enteric glia of the submucosal ganglia are also S100+ and GAP-43+ (arrowheads in c3 and d3).

GAP-43 also stains axons and their accompanying Schwann cells ramifying through the muscle layers and mucosa.


1 - STOMACH
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2 - ILEUM
3 - COLON
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C
L
A3
B3
M
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C
L
C1 GAP-43
D1 S100
C2
D2
C3
D3

7.3. Discussion

7.3.1. GAP-43 Expression By Non-Myelin-Forming Schwann Cells

In the normal sciatic nerve both non-myelin-forming and myelin-forming Schwann cells are present, which can be distinguished immunocytochemically by the expression of distinct sets of proteins (Mirsky and Jessen, 1991). Using GFAP to mark the non-myelin-forming Schwann cells (Jessen *et al.*, 1984) or MBP to demonstrate the presence of myelin, double labelling experiments revealed GAP-43 to be expressed exclusively by non-myelin-forming Schwann cells. In teased nerve preparations, GAP-43 can be clearly seen to label only those cells expressing GFAP and to be excluded from cables of MBP+ Schwann cells. Consequently, the cervical sympathetic trunk was examined. This nerve has been shown to consist mainly of unmyelinated fibres (approximately 99%, Aguayo *et al.*, 1976). GAP-43 was widespread in the non-myelin-forming Schwann cells in these preparations but was not present in the few Schwann cells expressing MBP.

Thus GAP-43 appears to join the growing list of protein markers for non-myelin-forming Schwann cells, including GFAP, N-CAM, Ng-CAM and NGFr. These proteins are expressed early in development by all Schwann cells and are subsequently down-regulated by those cells induced to myelinate large diameter axons (Nieke and Schachner, 1985; Jessen *et al.*, 1987b, 1990; Mirsky and Jessen, 1991). Collaborative studies with K.R. Jessen, R. Mirsky and H.J.S. Stewart (University College, London) have shown that GAP-43 is present in Schwann cells derived from sciatic nerve at embryonic days 15 and 16, prior to the induction of myelination (manuscript in preparation). This suggests that GAP-43 is expressed constitutively by Schwann cells and is down-regulated in myelin-forming Schwann cells by axonal contact.

7.3.2. GAP-43 Expression By Schwann Cells In The Absence Of Axons

To exclude the possibility that GAP-43 in the membranes of unmyelinated axons was responsible for the staining in the normal sciatic nerve and to test the contention that GAP-43 expression by Schwann cells is regulated by axonal contact, immunocytochemical studies were performed on Schwann cells deprived of axons either by denervation *in vivo* or by dissociation of nerves *in vitro*. Previous studies have shown that lipids and proteins characteristic of myelinating Schwann cells are regulated by axon contact and that Schwann cells rapidly revert to a non-myelin-forming phenotype when contact is lost (Mirsky *et al.*, 1980; Lemke and Chao, 1988; reviewed by Mirsky and Jessen, 1991).

Some cultured Schwann cells, identified by the expression of either S100 or NGFr, were positive for GAP-43. This suggests that non-myelin-forming Schwann cells contain GAP-43 in the absence of axons, but does not exclude the possibility of GAP-43 uptake from axons prior to dissociation *in vitro*. However after sciatic nerve denervation *in vivo*, GAP-43 was present in a minority of Schwann cells up to 2 weeks but labelled nearly all Schwann cells in the distal segment at 4 and 8 weeks. This shows that synthesis of GAP-43 can occur in the absence of neurons and that immunoreactivity in non-myelin-forming Schwann cells is not due to GAP-43 present in (or taken up from) axons.

These studies also showed that GAP-43 is regulated differently from other non-myelin-forming Schwann cell protein markers. While NGFr was expressed by all S100+ Schwann cells after 4 days in culture (data not shown), GAP-43 was only expressed by a minority of these cells. Indeed, collaborative studies with K.R. Jessen, R. Mirsky and H.J.S. Stewart (University College, London) have shown that GAP-43 is only present in 25-40% of Schwann cells maintained in culture at all time points studied up to 3 weeks (manuscript in preparation). Furthermore, GAP-43 was up-regulated between 2 and 4 weeks after denervation *in vivo*

while N-CAM, Ng-CAM and NGFr are rapidly synthesised by previously myelinating Schwann cells deprived of axonal contact by Wallerian degeneration (Nieke and Schachner, 1985; Taniuchi *et al.*, 1986; Jessen *et al.*, 1987b).

Cyclic AMP has been implicated as the intracellular second messenger mediating some effects of axonal contact in Schwann cells (Lemke and Chao, 1988; Mirsky *et al.*, 1990; Morgan *et al.*, 1991). Preliminary experiments with K.R. Jessen, R. Mirsky and H.J.S. Stewart (University College, London) have shown that GAP-43 is down-regulated in cultured Schwann cells by addition of forskolin, which elevates intracellular cyclic AMP, in common with other protein markers of the non-myelin-forming Schwann cell phenotype (Morgan *et al.*, 1991).

7.3.3. GAP-43 Expression In Regenerating Sciatic Nerve

Crush injury to the sciatic nerve did not cause an increase in the number of Schwann cells expressing GAP-43. Thus the delayed expression of GAP-43 by Schwann cells 4 weeks after transection did not occur in the presence of regenerating axons. This suggests that reestablishment of axonal contact perpetuates the suppression of GAP-43 synthesis.

Surprisingly, GAP-43 was not visualised in the axons regenerating through the sciatic nerve after crush injury. Previous studies have shown that GAP-43 is localised in axons stained with neurofilament in regenerating peripheral nerves (Verhaagen *et al.*, 1986) and that axonal transport of GAP-43 increases 9-fold after crush injury (Skene and Willard, 1981b). Northern blotting and *in situ* hybridisation studies have shown that GAP-43 mRNA is increased in dorsal root ganglia after sciatic nerve injury (Basi *et al.*, 1987; Verge *et al.*, 1990). This suggests that GAP-43 in regenerating peripheral axons is below the limit of detection of the highly sensitive antiserum used in this study, which

readily labelled Schwann cells. Similar results have been reported by Tetzlaff *et al.* (1989).

Therefore the immunocytochemical data of Verhaagen *et al.* (1986) must be questioned. Examination of their micrographs reveals GAP-43 in structures closely resembling the Schwann cells visualised in this study. However these structures were also immunoreactive for neurofilament protein using a monoclonal antibody against the low molecular weight neurofilament protein, with which GAP-43 shares a region of limited sequence homology (52% conservation at the amino acid level, LaBate and Skene, 1989). It is therefore possible that this antibody may have cross-reacted with GAP-43 leading to the apparent co-distribution of low molecular weight neurofilament protein and GAP-43. Indeed, the radioimmunoassay data presented by these authors supports the concept of expression by Schwann cells. GAP-43 levels did not change significantly 6 days after sciatic nerve transection, consistent with expression by a stable population of Schwann cells and contrary to a location in axons which are phagocytosed within a week of transection (see for instance, Beuche and Friede, 1984; Lunn *et al.*, 1989).

7.3.4. Regulation Of GFAP After Sciatic Nerve Transection

There are conflicting reports concerning the regulation of GFAP after peripheral nerve transection. Neuberger and Cornbrooks (1989) have suggested that the number of Schwann cells expressing GFAP in frozen sections decreases while Jessen *et al.* (1990) have shown an increase in GFAP+ Schwann cells 4 to 8 weeks after transection by staining of cell suspensions prepared from denervated tissue. At the mRNA level, reduction of GFAP expression has been reported distal to transection (Mokuno *et al.*, 1989), but other studies have been unable to confirm this (Condorelli *et al.*, 1990). In this study, GFAP immunocytochemistry of frozen sections (see Figure 7.9.) and teased nerves (not shown) suggests that the proportion of Schwann cells

expressing GFAP is not significantly altered by peripheral nerve transection. In common with previous studies (Neuberger and Cornbrooks, 1989; Jessen *et al.*, 1990), strongly immunoreactive cells remained in the denervated segment, most likely representing the original population of non-myelin-forming Schwann cells. It is interesting that these GFAP+ cells were also GAP-43+ up to 2 weeks after transection, suggesting that non-myelin-forming and myelin-forming Schwann cells may remain immunocytochemically distinguishable even after denervation and loss of myelin proteins and lipids. However, corroboration of these findings required further investigation that was beyond the scope of this study.

7.3.5. Role Of GAP-43 In Non-Myelin-Forming Schwann Cells

It has been suggested previously that astrocytes of the CNS, non-myelin-forming Schwann cells of the PNS and glial cells of the enteric nervous system may represent a functionally-related group of cells on the basis of their common expression of protein markers, such as GFAP and the A5E3 cell surface antigen, and their lack of myelin proteins and lipids (Jessen and Mirsky, 1984). The findings presented here and in the previous Chapter are consistent with this view. GAP-43 is expressed by some astrocytes (Curtis *et al.*, 1991), non-myelin-forming Schwann cells and enteric glia but not by mature oligodendrocytes or myelin-forming Schwann cells.

It is unclear what role GAP-43 may play in non-myelin-forming Schwann cells and enteric glia. In neurons, GAP-43 associates both with the plasma membrane and elements of the cytoskeleton (Meiri and Gordon-Weeks, 1990) and GAP-43 may have a role in the adhesion and/or motility of O-2A progenitors and type-2 astrocytes (see previous Chapter). The presence of GAP-43 in the membranes of Schwann cells suggests that it may play a similar role in peripheral glia, stabilising the membranes of the non-myelin-forming Schwann cells.

Unmyelinated axons are enclosed in membrane-bound troughs within

non-myelin-forming Schwann cells. Adhesion molecules on the axonal and Schwann cell surfaces 'anchor' the axons in these troughs (Martini and Schachner, 1986; Bixby *et al.*, 1988; Seilheimer and Schachner, 1988), and GAP-43 may stabilise the Schwann cell membrane relative to the cytoskeleton and so prevent movement during mechanical distortion of the nerve trunk through injury or exertion. Myelin-forming Schwann cells on the other hand enwrap larger diameter fibres in several layers of myelin membrane, which form specialised junctions with the axolemma at the paranodal regions (Livingston *et al.*, 1973; Peters *et al.*, 1976). There appears little danger of myelinated axons moving relative to their associated Schwann cells and thus GAP-43 may not be required for membrane stabilisation in these cells. Furthermore, recent data has shown that unmyelinated axons are not constant in diameter and that varicosities are present along the axon shaft, consistent with axonal transport of organelles (Greenberg *et al.*, 1990). This suggests that axons may undergo periodic swelling as these varicosities move along the axon. GAP-43 may serve to regulate the fluidity/stability of Schwann cell membranes under these circumstances.

In addition, Schwann cells are known to be motile in culture (Crang and Blakemore, 1987) and have been shown to divide and migrate from distal segments of peripheral nerve after denervation (Weinberg and Spencer, 1976). GAP-43 may underlie the motility of Schwann cells and therefore play an important role in the glial response to peripheral nerve injury.

Chapter Eight

General Summary

8.1. Summary

The studies reported in this thesis have utilised a specific antiserum of high titre (raised against a GAP-43/ß-galactosidase fusion protein) to document the distribution of GAP-43 in the peripheral and central nervous systems of the rat during development, in adulthood and after traumatic injury.

8.1.1. GAP-43 In The Normal Adult Central Nervous System

GAP-43 was found to be more widespread in the adult CNS than previously reported in the literature. Synaptic glomeruli in the olfactory bulb contained immunoreactive GAP-43, consistent with a location in growing axons of the primary olfactory receptor neurons. Climbing fibre axons and parallel fibres terminals were stained in the cerebellum, indicating a possible role for GAP-43 in cerebellar learning. GAP-43 was widely distributed in the spinal cord. Titration of the anti-GAP-43 fusion protein antiserum allowed different levels of GAP-43 to be discerned in these tissues, with olfactory synaptic glomeruli showing the greatest concentration of GAP-43.

8.1.2. GAP-43 Expression After Central Nervous System Injury

Elevation of GAP-43 was demonstrated in optic nerve and spinal cord axons after traumatic injury by using anti-GAP-43 fusion protein antiserum at dilutions below the limits of detection of GAP-43 levels existing in the normal adult.

Transection of optic nerve led to expression of GAP-43 by RGCs within 5 days. Axons of these cells in the retina and optic nerve remained GAP-43 immunoreactive up to 15 days after transection in the absence of successful regeneration. This contradicts the findings of Skene and Willard (1981b) and shows that the failure of regeneration by central neurons is not due to the inability of these cells to synthesise GAP-43.

Compression of the spinal cord led to up-regulation of GAP-43 in some spinal cord axons and neuronal perikarya adjacent to lesion cavities. The presence of phosphorylated neurofilament epitopes in these soma suggests

axotomy close to the cell body. Although GAP-43+ axons were found within cavities in the lesions which appeared to have regenerated from the adjacent tissue, most axons were spared from axotomy in the region of the lesion in contrast to the morphological observations of Guth *et al.* (1985). GAP-43+ axons in the cavities were mostly not associated with astrocytic processes and could sometimes be seen adjacent to tubular structures (possibly capillaries).

These studies demonstrate that GAP-43 can be up-regulated by central neurons in response to injury but that this does not correlate with the success of regeneration. Thus caution should be exercised when using GAP-43 as a marker for regenerating axons.

8.1.3. GAP-43 Expression By Macroglial Cells Of The Central Nervous System

O-2A progenitor cells and type-2 astrocytes were shown to contain GAP-43 *in vitro*. O-2A progenitors maintained in serum-free medium lost GAP-43 as they differentiated into oligodendrocytes. A similar progression was observed *in vivo* in that O-2A progenitor cells contained GAP-43, but this was lost during oligodendrocyte differentiation. This is the first *in vivo* description of a GAP-43+ non-neuronal cell type in the CNS.

GAP-43 could not be detected in astrocytes in the normal adult CNS, or after the induction of 'reactive gliosis'. This suggests that O-2A progenitors do not give rise to type-2 astrocytes in adult rats.

A paper based on these results has been accepted for publication (Curtis *et al.*, 1991).

8.1.4. GAP-43 Expression By Schwann Cells Of The Peripheral Nervous System

Non-myelin-forming Schwann cells *in vivo* were shown to express GAP-43, but myelin-forming Schwann cells did not contain GAP-43 immunoreactivity. Permanent denervation of sciatic nerve led to expression of GAP-43 by previously myelinating Schwann cells, suggesting that GAP-43 may

be suppressed in myelin-forming Schwann cells by axonal contact.

This emphasises that GAP-43 can no longer be considered as a neuron-specific protein, and that care must be taken when using GAP-43 as a marker of axonal growth in the PNS.

8.2. Future Directions

These results raise several interesting questions which might be addressed in future studies. The following considerations are speculative, although the techniques described have previously been applied to the study of GAP-43.

8.2.1. GAP-43 In The Normal Adult Central Nervous System

Further neuroanatomical mapping with anti-GAP-43 immunohistochemistry might be used to reveal other populations of axons expressing high levels of GAP-43 in adulthood. With respect to the climbing fibres of the cerebellum, injection of radiolabelled amino acids into the inferior olive followed by analysis of axonally transported proteins arriving in the cerebellum could be used as a direct means to show GAP-43 synthesis by these cells. Alternatively, immuno-electron microscopy could be used to examine these axons for GAP-43 associated with the mature axon shaft, as has been shown for the CST (Gorgels *et al.*, 1989). Indeed, collaborative experiments with Dr. A. Kent and Dr. S. Standring (UMDS, Guy's Hospital, London) are underway to demonstrate the location of GAP-43 in the molecular layer of the cerebellum.

8.2.2. GAP-43 Expression After Central Nervous System Injury

The expression of immunoreactive of GAP-43 could be investigated after injury to different CNS regions, as an index of neuronal cell body response to axotomy. In this way it might be possible to determine if populations of neurons differ in their response and if this correlates with the ability to regenerate when provided with an appropriate environment (ie a PNS

graft). Alternatively, GAP-43 up-regulation might be used as a probe to determine the factors influencing the cell body response to axotomy. For instance, Bray *et al.* (1987) have suggested that the proximity of the axotomy site to the perikaryon is an important determinant of the regenerative response. This could be assessed directly by GAP-43 immunohistochemistry after severance of the optic nerve at intraorbital or intracranial sites.

In the compression model of spinal cord injury (documented in Chapter 5) the nature of factors regulating axonal growth could be investigated using the anti-GAP-43 fusion protein antiserum at a dilution of 1/30,000 to mark elongating axons. Infusion of growth factors such as NGF or antibodies to putative non-permissive CNS factors (Caroni and Schwab, 1988a, b) could be used to stimulate neuronal growth through the lesion. Alternatively, the nature of axonal substrates could be addressed using monoclonal antibodies to ECM and cell surface adhesion molecules in double labelling experiments with anti-GAP-43 fusion protein antiserum.

8.2.3. GAP-43 Expression By Macroglial Cells Of The Central Nervous System

In situ hybridisation would be necessary to confirm the expression of GAP-43 by O-2A progenitor cells *in vivo*.

The putative regulation of PDGF-responsiveness by GAP-43 is an intriguing possibility. O-2A progenitor cells isolated from adult tissue have recently been shown to be unresponsive to PDGF (Chan *et al.*, 1990). Immunocytochemistry and Western blotting might show that this is correlated with the absence of GAP-43 from these cells.

GAP-43 may also prove useful as a marker for type-2 astrocytes, as these cells have not yet been conclusively identified *in vivo*. One possibility is that *neonatal* O-2A progenitors may give rise to type-2 astrocytes after traumatic injury in young animals but a similar process does not occur in older animals. Alternatively, *adult* O-2A progenitors may generate astrocytes which do not express GAP-43. This is currently under investigation in collaborative

experiments with Prof. M. Berry (UMDS, Guy's Hospital, London), using a penetrating injury to induce gliosis at different times after birth. The glial response to such injury has previously been shown to depend on the age of the animals at surgery (Berry *et al.*, 1983).

8.2.4. GAP-43 Expression By Schwann Cells Of The Peripheral Nervous System

Synthesis of GAP-43 by non-neuronal cells *in vivo* is still a contentious issue but this could be demonstrated directly by two methods. First, *in situ* hybridisation is currently being used in collaborative studies with Prof. R. Mirsky, Dr. K. Jessen and Dr. H. Stewart (University College, London) to show the presence of GAP-43 mRNA in non-myelin-forming Schwann cells of the cervical sympathetic trunk. Secondly, either Northern blotting or metabolic labelling could be used to show GAP-43 up-regulation in the denervated sciatic nerve 2-4 weeks after transection.

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