

**Zinc-Finger Transcription Factors in the Schwann Cell
Lineage**

By

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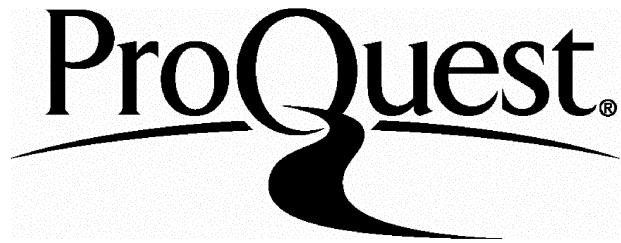
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ABSTRACT

Myelin forming and non-myelin forming Schwann cells are the major glia within peripheral nerves. Recent studies have revealed the importance of the transcription factors Sox10, Pax3, Krox-20 and Oct-6 in Schwann cell development. This work describes one novel Schwann cell zinc-finger transcription factor, Zfp-57, and investigates the phenotype of Schwann cells deficient in another, Krox-24, with a view to discovering a function for these genes in Schwann cells.

Zfp-57 is expressed in nerves from embryo day 12 to adult and is present in Schwann cell nuclei. To investigate whether Zfp-57 plays a role in myelination, Zfp-57 cDNA was overexpressed in Schwann cells. No effects were detected on Schwann cell differentiation towards a myelin phenotype, suggesting that Zfp-57 may not be involved in this process

I undertook a detailed investigation of nerves of Krox-24 null mutant and heterozygous mice in which expression of the LacZ gene is controlled by the Krox-24 promoter. Krox-24 activation occurs in late embryonic/ early postnatal peripheral nerve development. In nerves, mRNA levels of typical Schwann cell molecules are normal, proliferation and the ultrastructural morphology is not affected.

In regenerating nerves Krox-24 deficient Schwann cells down-regulate myelin genes and up-regulate molecules required for regeneration as efficiently as wildtype cells except for p75NTR mRNA, which is increased in Krox-24 deficient mice. Furthermore axonal regeneration occurs normally.

Schwann cell death occurs developmentally and the effect of Krox-24 deficiency on this phenomenon has been investigated using TUNEL. 1 day after transection of newborn sciatic nerves cell death is elevated threefold in Krox-24 $^{-/-}$ mice compared to wildtype littermates. In a low density culture assay where cell death is measured in the absence of autocrine Schwann cell survival factors, there is no difference between Krox-24 null cells and normal Schwann cells. Additionally cell death induced by TGF β is unaltered.

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ABBREVIATIONS

ADS; antibody diluting solution
APS; Ammonium persulphate
BSA; Bovine serum albumin
BrdU; bromodeoxyuridine
Bluogal; 5-Bromo-3-Indolyl-B-D-Galctopyranoside
CNS; Central nervous system
Dhh; Desert hedgehog
dbcAMP; dibutryl cAMP
DMEM; Dulbecco's modified Eagle's medium
DRG; Dorsal root ganglion
EDTA; ethylenediaminetetraaceticacid
ECM; extracellular matrix
EGR; Early growth response
FCS; Foetal calf serum
bFGF; basic FGF, FGF-2
GFAP; Glial fibrillary acidic protein
HBS; HEPES buffered saline
HEPES; N[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP; horse radish peroxidase
IEG; Immediate early gene
IPTG; Isopropyl B-D-thiogalactoside
Krox; Kruppel like hox protein
MAG; Myelin associated glycoprotein
Mitf; microphthalmia gene
N-CAM; Neural cell adhesion molecule
NDF; *neu* differentiation factor
p75NTR; Low affinity neurotrophin receptor
P₀; major peripheral myelin protein zero

Pax; Paired box like protein
PBS; Phosphate buffered saline
PLL; Poly-L-lysine
PLO; Poly-DL-ornithine
PMP22; peripheral myelin protein, 22kD
PMSF; phenylmethylsulphonylfluoride
PNS; Peripheral nervous system
RT-PCR; Reverse transcription polymerase chain reaction
SCIP; Schwann cell cAMP inducible POU
SDS; Sodium dodecyl sulphate
Sox; SRY like box protein
Sp; Splotch mutant
Spd; Splotch delayed mutant
TAE; Tris acetate EDTA buffer
TE; Tris EDTA buffer
TBE; Tris borate EDTA
TGF β ; Transforming growth factor β
TUNEL; Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
VEGF; Vasal epithelial growth factor
WS4; Waardenburg-Shah syndrome
Xgal; 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside

CHAPTER 1

GENERAL INTRODUCTION

The major glial cells of the peripheral nerve are the myelinating and non-myelinating Schwann cells. Glial cells are supporting cells surrounding neurones and their processes throughout the central nervous system (CNS) and peripheral nervous system (PNS) and take their name from the Greek word for glue, “glia”. Theodor Schwann, pioneer of the cell theory, gave the first description of the cells that would later bear his name in 1839 (Shepherd, 1991). He described the developing nerve fibres, from primary nerve cells and the course from the CNS to peripheral organs. In addition he described the secondary cells (Schwann cells) producing a white material (myelin) surrounding a central core (the axon). A more current description of the Schwann cell was given some 70 years later by the brilliant Spanish neuroscientist Ramon y Cajal. Using the silver impregnation technique, developed by Camillo Golgi in 1873, he was able to illustrate the Schwann cell, the node of Ranvier, Schmidt and Lanterman incisures and that the cytoplasm was continuous within myelin. This showed that myelin, rather than being a separate entity, was integral to the cell (Cajal, 1928). Cajal also described the presence of fibres without myelin found in most nerves, first seen by Robert Remak as naked, organic fibres reported in 1837. These were also noted by Schwann as fibres without white matter although he incorrectly considered these to be in an earlier state of development rather than a sub-type of axon. The advent of the electron microscope, as in so many instances, allowed the ultrastructure and morphology of both Schwann cell forms to be revealed (Geren and Raskind, 1953).

Mature myelinating Schwann cells form 1:1 relationships with larger diameter nerve axons (such as large A fibre afferents and motor neurones) and facilitate rapid saltatory conduction, while non-myelinating Schwann cells support a number of smaller diameter axons (e.g. Small C-fibre afferents) in membrane invaginations.

Development of the Schwann cell lineage

Schwann cells develop in characteristic fashion through a series of stages beginning with the formation of a migratory population of cells known as the neural crest. These cells migrate away from the neural tube and a sub-population form a transient cell group, the Schwann cell precursors, which associate closely with outgrowing axons from the DRG (sensory in nature and also of crest origin) and ventral neural tube (motor neurons). These cells develop in contact with axons into immature Schwann cells and subsequently into the mature myelin and non-myelin forming Schwann cells (Mirsky and Jessen, 1999).

Neural Crest

These two Schwann cell types are derived from a group of cells that dorsally delaminates from the newly formed neural tube upon completion of neurulation. This transient, migratory, group of cells, known as the neural crest, is destined to develop into a diverse array of cell types, including the sensory and autonomic components of the peripheral nervous system as well as a range of non-neuronal tissues (Le Douarin and Smith, 1988). A major part of the newly formed neural crest cells will form the myelinating and non-myelinating Schwann cells (Le Douarin and Smith, 1988, Le Douarin *et al.*, 1991). Ablation of the dorsal neural tube in frogs has been shown to

result in DRG absence and a lack of Schwann cells to associate with the motor neurons, derived from the remaining ventral neural tube (Harrison, 1924). This study clearly demonstrated the dorsal origin of cells that were destined to form Schwann cells. Other studies in the chick show that a small but significant population of Schwann cells that ensheathe proximal ventral routes may be of ventral origin (Weston, 1963, Loring and Erickson, 1987, Lunn *et al.*, 1987). In contrast more recent investigations suggest that glial cells populating ventral roots are of neural crest origin (Carpenter and Hollyday, 1992a, Bhattacharyya *et al.*, 1994). Taken together these data argue strongly that the majority of Schwann cells are derived from neural crest but that a small minority may be ventrally derived.

The emergence of neural crest cells from the neural tube and their migration is dependent on the differential expression of cell adhesion molecules. During the closure and formation of the chick embryo neural tube, stages 8 – 9, cadherin 6 (cad6) is expressed in the dorsal lip and roof plate while neural crest cells express cadherin 7 (cad7) which is persistent throughout their migration (Nakagawa and Takeichi, 1995). Subsequent migration paths and terminal destinations of these cells have been elucidated by a number of different techniques such as tritiated thymidine injections to radiolabel nuclei and avian chimeras (Weston, 1963, LeDouarin, 1973).

Pathways of migration

The first insight into the migratory routes of trunk neural crest was gained by injections of tritiated thymidine which is incorporated into DNA, labels all subsequent progeny and becomes progressively diluted with cell division. Using this method two main pathways

of neural crest migration were discovered: one dorsolateral, beneath the ectoderm and the other ventrally through the somite, between sclerotome and dermamyotome (Weston, 1963). Ventrally migrating cells give rise to cells in the DRG, sympathetic ganglia, adrenomedullary cells, aortic plexuses and Schwann cells and follow a segmental pattern and move through the rostral half of the somite only (Rickman *et al.*, 1985, Weston, 1963, Erickson *et al.*, 1992), while the dorsolateral cell group do not show such a pattern (Serbedzija *et al.*, 1989, 1990, Erickson *et al.*, 1992). The dorsolateral cell population migrates out later than the ventral group (Erickson and Goins, 1995) and gives rise to melanocytes (Dusahne, 1935, Dorris 1939, Rawles, 1947, Mayer 1973).

Extending this work to fate map neural crest cells in yet more detail comes from an elegant chimeric avian model, based on the differences in cell size and or staining properties of the cells found in two species, chick and quail (Le Douarin, 1973). This method has led to the discovery of three additional pathways of migration; an intersomitic population, a longitudinally migrating group passing along the length of the neural tube and a group that travel along the dorsal aorta (Le Douarin, 1982, Teillet *et al.*, 1987, Erickson *et al.*, 1989).

Neural crest cell potential

The question of how divergent types of differentiated cells evolve from a multipotent homogenous cell population, such as the neural crest, is interesting to developmental biologists. Lineage studies have been conducted *in vivo* and *in vitro* demonstrating that neural crest contains a mixture of both committed progenitor cells as well as multipotent

precursors (Bronner-Fraser and Fraser, 1988, 1989, Barrofio *et al.*, 1991, Fraser and Bronner-Fraser, 1991, Frank and Sanes, 1991, Serbedzija *et al.*, 1994, Sieber-Blum, 1989, Dupin *et al.*, 1990). Similar studies suggest that the divergence of glial cell precursors and neuronal precursors occurs shortly after gangliogenesis (Duff *et al.*, 1991, Hall and Landis, 1991, 1992). These studies are indicative that glial cell differentiation may be induced by local interactions, such as cues from the extracellular environment. However, new evidence shows that distinct fate restricted glial and neuronal precursors can be detected earlier, one day after the crest cells begin to emigrate from the dorsal midline and this supports the theory that at least in some instances glial cell fate is intrinsically predetermined (Henion and Weston, 1997). Studies of both chick and rat have shown that low levels of P0 protein, a marker of myelin forming Schwann cells, is detectable in the late migrating crest (Bhattacharya *et al.*, 1994, Zhang *et al.*, 1995, Lee *et al.*, 1997).

Molecules with potential to bias glial cell formation from neural crest

As well as lineage strategies designed to investigate the relationship of the divergent crest cell derivative populations at different developmental stages, other studies have attempted to discover the molecular cues that may underlie this diversity. Members of the neuregulin family of growth factors (discussed in more detail below) have been shown to promote the formation of glial cells from neural crest cells *in vitro* (Shah *et al.*, 1994). More recently, transient Notch signaling has been shown to suppress neuronal differentiation and induce glial cell differentiation *in vitro* (Morrison *et al.*, 2000,

Wakamatsu *et al.*, 2000). These data demonstrate that glial cell differentiation can be regulated by protein signals derived from the neurons that they accompany and that ultimately a number of factors may, in combination, be responsible for terminal differentiation.

Neuronal differentiation and axon sprouting predates the Schwann cell precursor

The sciatic nerve is composed of a mixed population of sensory and motor neuron derived axons and originates from the lumbar spinal segments L4-6. In the rat, neural crest cells that form the DRG begin to aggregate in the anterior somite adjacent to the neural tube between embryonic day 11 (E11) and E13 (Angulo, 1951, Sobkowicz *et al.*, 1973, Lawson *et al.*, 1974). The neurons in lumbar ganglia begin to differentiate at E11, the peak for large ventrolateral neurons is E12 and for small dorsomedial cells is E13 and differentiation is complete by E15 (Lawson *et al.*, 1974).

Motor neurons of the ventral neural tube begin to project axons at E11, and these are loosely bundled with those from the DRG in early nerve routes at E12 (Reynolds *et al.*, 1991). These growing nerves enter the proximal part of the developing hindlimb at E14, and most remain tightly bundled with a few branches beginning to project to superficial tissues. By E15 the nerves extend into two thirds of the hindlimb and the process of branching into muscle tissue is well underway, the most distal aspects of the hindlimb are innervated by E19 (Reynolds *et al.*, 1991).

The close association of glial cells to early projecting axons has been shown in frogs (Harrison, 1924), tadpole tail (Billings-Gagliardi *et al.*, 1974), and chick limb (Weston, 1963, Loring and Erickson *et al.*, 1987, Dahm and Landmesser, 1988, Carpenter and

Hollyday, 1992a, 1992b). In chick and mouse Schwann cell precursors are closely associated and migrate with outgrowing axons, but are absent from the most distal leading tip of the axon (Dahm and Landmesser, 1988, Carpenter and Hollyday, 1992b, Bogusch, 1992). Some data suggest that Schwann cell precursors migrate ahead of the axon, pioneering a growth path (Noakes and Bennett, 1987, Serbedzija *et al.*, 1990, Haninec and Dubovy, 1992). However analysis of splotch mutant mice and ErbB2 null mutant mice show that for most of their trajectory motor neurons are able to project normally in the absence of Schwann cells (Grim *et al.*, 1992, Woldeyesus *et al.*, 1999).

Ultrastructural analysis of developing rat sciatic nerves at E14 has revealed that glial cells are located around the perimeter of the tightly packed axon bundle, separating the axons from surrounding mesenchyme (Peters and Muir, 1959, Jessen *et al.*, 1994). Larger branches contain inside nuclei of presumptive glial cells and these can be seen to extend processes that contact many axons within the bundle (Jessen *et al.*, 1994).

Schwann Cell Precursors

By E 14 in the rat (E 12 mouse) developing glial cells associated with outgrowing axons display specific characteristics and these cells have been termed Schwann cell precursors (Jessen *et al.*, 1994). These cells represent an intermediate state of differentiation in the Schwann cell lineage and can be identified by the expression of growth-associated protein-43 (GAP-43), their survival responses *in vitro* and the expression of brain fatty acid binding protein (B-FABP) (Jessen *et al.*, 1994, Kurtz *et al.*, 1994). B-FABP is not detectable in neural crest cells at E10.5 in mouse but by E11.5 is present in DRGs and spinal nerves and labels various peripheral glial cell populations (Kurtz *et al.*, 1994). In

culture, Schwann cell precursors, unlike mature Schwann cells, undergo rapid apoptotic death when removed from associated axons, an effect that may be countered by the incubation with 100pM of β -neuregulin (Dong *et al.*, 1995). The morphology of the Schwann cell precursor in culture is markedly different from that of mature Schwann cells; unlike the bi- or tripolar shape of typical neonatal Schwann cells, precursors are more flattened with a less distinct shape. Cultured precursors tend to be found in clustered groups in contrast to later neonatal Schwann cells that grow more evenly distributed.

In the rat, the brief period of E 16 to 17 (E 15 to E16 in mouse) sees the transition of the Schwann cell precursors into early Schwann cells, a process that has been shown not to require DNA synthesis (Dong *et al.*, 1995) (Fig. 1.). From this point on Schwann cells are distinguishable from other cell types, such as fibroblasts and perineurial cells, by their continual expression of the calcium binding protein S100. Shortly after the appearance of S100 the Schwann cells begin to express the surface lipid 04. These immature Schwann cells proliferate extensively, reaching a DNA synthesis peak at around E 19 or 20 in the rat sciatic nerve (Stewart *et al.*, 1993). In vitro these cells now show the classical bi- or tripolar morphology seen in mature Schwann cells and when cultured at moderate density do not die by apoptosis, having established autocrine survival loops (Jessen *et al.*, 1994, Dong *et al.*, 1999). The components of this intrinsic survival capability have recently been investigated by attempting to reproduce the positive effects of Schwann cell conditioned medium on cultures of Schwann cells. In such assays medium conditioned by Schwann cells, cultured at high density, rescue others cultured at a lower density and the combined action of three factors NT-3, IGF

and PDGF can mimic this effect suggesting that together they form the basis of a Schwann cell autocrine loop (Meier *et al.*, 1999).

Schwann cells may then proceed along one of two developmental paths, culminating in the formation of either myelinating or non-myelinating Schwann cells. Differentiation into both lineages has been shown to require the initial formation of a basal lamina upon contact with axons (Bunge *et al.*, 1986). Those immature cells destined to become myelinating Schwann cells begin to express the glycolipid galactocerebroside at E 19, shortly before forming 1:1 relationships with axons along the entire length of each axon. Schwann cells at this stage have stopped dividing and are found in the sciatic nerve at birth (P0) in both rat and mouse. The first wrappings of the myelin sheath are seen at this time together with high level expression of the major myelin proteins; the glycoprotein protein zero (P_0) and myelin basic protein (MBP), myelin associated-glycoprotein (MAG), proteolipid protein (PLP), peripheral myelin protein of 22kDa (PMP22) and P2, CNPase and periaxin (Mirsky and Jessen, 1999).

The development of the non-myelinating Schwann cell is delayed compared to the myelinating cells. They do not express galactocerebroside until shortly before elaborating their distinctive pattern of multi-axonal support, which does not begin until the third postnatal week (Diner *et al.*, 1965, Jessen *et al.*, 1985; Jessen *et al.*, 1987).

Neuregulins and their receptors mediate axon Schwann cell signaling

The neuregulin (NRG) family of proteins and the ErbB subfamily of receptor protein tyrosine kinase molecules (PTKs) together perform key roles in both neural and cardiac development (reviewed in Adlkofer and Lai, 2000, Garratt *et al.*, 2000). Currently four

genes are known that encode the distinct neuregulin proteins, NRG1, NRG2, NRG3 and NRG4 (Adlkofer and Lai, 2000, Garratt *et al.*, 2000). Neuregulin-1 (NRG1) is the consensus name given to a group of molecules isolated from different systems and named independently: Neu-differentiation factor (NDF) being cloned from rat (Wen *et al.*, 1992), heregulin from human (Holmes *et al.*, 1992), acetylcholine receptor inducing activity (ARIA) from chick (Falls *et al.*, 1993) and glial growth factor (GGF) from bovine brain (Marchionni *et al.*, 1993). The pursuit of a ligand for the ErbB2 receptor resulted in the naming of NDF (Wen *et al.*, 1992) and heregulin (Holmes *et al.*, 1992) from which the name, neuregulin was later derived.

The structure of neuregulins is modular and they may be divided into four main parts; an N-terminal domain which is either Ig like or cysteine rich (CRD), an EGF (epidermal growth factor) like domain, a transmembrane domain and finally an intracellular C-terminal domain (Adlkofer and Lai, 2000). The EGF like domain found in neuregulins typifies ErbB ligands. The C-terminal region of this domain may be of α or β forms, arising from alternative splicing, which modulates ErbB heterodimer binding affinities (Jones *et al.*, 1999, Pinkas-Kramarski *et al.*, 1998). NRG1 splice variants number greater than 15 and proteins have been characterised differing in both N-terminal and C-terminal domains (reviewed in Lemke 1996).

Neuregulins bind to and activate members of EGF receptor subfamily of PTKs known as “ErbBs” taking their name from the viral oncogene, v-Erb-B, present in avians and encoding mutant epidermal growth factor receptor (EGFR) (Adlkofer and Lai, 2000). Four receptors of this class have been isolated to date; EGFR (ErbB1/ HER1), ErbB2

(neu/ HER2), ErbB3/ HER3 and ErbB4/ HER4 (Lemke 1996). ErbB receptors are large, 170-185kDa molecules, consisting of a cysteine rich extracellular domain, a transmembrane domain and a long, tyrosine rich, intracellular domain. The ErbB receptors signal as heterodimers and many combinations occur normally (Riese *et al.*, 1995, Sliwkowski *et al.*, 1994).

NRG-1 activates ErbB2 indirectly through its heterodimeric association with ErbB3 and ErbB4 and the heterodimers formed by ErbB2/ ErbB3 and ErbB2/ ErbB4 are currently considered to be the high affinity receptors for NRG1 (Pinkas-Kramarski *et al.*, 1996, Pinkas-Kramarski *et al.*, 1998, Jones *et al.*, 1999).

NRG was initially studied as GGF where a mitogenic effect was elicited on Schwann cells *in vitro* (Raff *et al.*, 1978, Marchionni *et al.*, 1993). Additionally, as mentioned earlier, NRG1 has been shown to promote the differentiation of glial cells from neural crest (Shah *et al.*, 1994). Further detailed studies *in vitro* have shown that β forms of NRG both promote the survival of Schwann cell precursors and induce differentiation to form the mature phenotype (Dong *et al.*, 1995). Conversely, by the addition of the extracellular domain (EGF like region) of ErbB4 molecules the survival promoting activity of neuron conditioned medium on Schwann cell precursors can be blocked (Dong *et al.*, 1995). These data strongly suggest that NRG/ ErbB ligand receptor partners are the mechanism whereby neurons and glia signal and that NRG may be required for Schwann cell differentiation, survival, proliferation and maturation.

Studies of null mutant transgenic mice demonstrate the requirement of NRG1, ErbB2 and 3 receptor function in Schwann cell development (Lee *et al.*, 1995, Meyer and Birchmeier 1995, Riethmacher *et al.*, 1997). NRG and ErbBs are also expressed in the heart and NRG1, ErbB2 and ErbB4 null mutant mice die midgestation (E10.5) from a

failure to develop ventricular trabeculae within the heart (Gassmann *et al.*, 1995, Lee *et al.*, 1995, Meyer and Birchmeier, 1995). Consequently the study of the development of the PNS is limited to early stages and NRG1 and ErbB2 deficient mice have been shown to have a loss of Schwann cell precursors (Lee *et al.*, 1995, Meyer and Birchmeier 1995). Mice lacking ErbB3 are able to survive until birth in limited numbers and show severely reduced numbers of both Schwann cell precursors and mature Schwann cells (Riethmacher *et al.*, 1997). In elegant transgenic mouse lines Woldeyesus and colleagues and also Lee and colleagues have genetically rescued the ErbB2 mutant by expression of ErbB2 cDNA under the control of the heart specific promoters Nkx2.5 and heart muscle actin promoters, respectively. These mice lack mature Schwann cells as well as other neural crest derivatives (Woldeyesus *et al.*, 1999, Morris *et al.*, 1999). These findings are indicative that axonally derived neuregulin function is essential for Schwann cell development.

Schwann cells respond to neuronal activity

Spontaneous neuronal activity is a feature of the developing nervous system and helps to shape the pattern of mature connectivity (reviewed by Goodman and Shatz, 1993). Neurones of the PNS are not an exception and have been shown to exhibit depolarizing electrical activity early in development, in advance of reaching target tissues (Del Castillo and Vizoso, 1953, Fitzgerald *et al.*, 1987). Recently an exciting finding demonstrates that *in vitro*, Schwann cells respond to extracellular ATP release derived from electrically stimulated DRG neurons, *in vitro*, by increasing levels of intracellular calcium (Stevens and Fields, 2000). Further to this the authors demonstrate that this leads to increases of phosphorylated CREB accompanied with elevated c-fos and Krox-

24 expression in Schwann cells. Finally, they show that such stimulation causes a decrease in Schwann cell proliferation and they suggest that Schwann cells respond to neuronal activity by dropping out of the cell cycle and that this may in turn modulate Schwann cell response to other axonally derived signals that cause differentiation. Other studies have shown that activity inhibits myelination, promotes myelination or has no effect (Stevens *et al.*, 1998, Demerens *et al.*, 1996, Colello *et al.*, 1995). These data are hard to reconcile but the majority of evidence suggests that Schwann cell differentiation is modulated by axonal electrical activity, and from the Stevens and Fields study it seems likely that Schwann cells respond to such cues by reduced mitosis and a greater tendency to differentiate.

The plausibility of such a mechanism comes from the finding that Schwann cells express P2Y receptors and respond to ATP application by elevation of intracellular calcium levels (Ansselin *et al.*, 1997, Green *et al.*, 1997, Lyons *et al.*, 1995, Robitaille, 1995, Mayer *et al.*, 1998).

Myelin forming Schwann cells

Invertebrates with simple nervous systems, such as the squid, which have only unmyelinated axons, but still require fast action potential propagation for survival have developed a single large composite “Giant axon”. Vertebrates do not possess giant axons but have the equivalent, myelin produced by myelin forming Schwann cells which represent an evolutionary more advanced cell. The myelin sheath that these cells form around single axons is a multilayered myelin membrane that acts as an insulative conduit. The sheath is discontinuous along the length of the axon. At the junction of adjacent Schwann cells there are specialised areas of electrically excitable axon surface,

known as the node of Ranvier. Action potentials are propagated from one node to the next, effectively leaping along the axon in a process known as saltatory conduction (Ranvier, 1878, Ritchie, 1983). For unmyelinated axons the conduction velocity is proportional to the root of the diameter of the axon. With saltatory conduction the velocity of a given action potential is significantly increased, approximately ten times, compared to a similar sized unmyelinated axon (Jacobson, 1993, Ritchie, 1983). Thus myelin enhances conduction efficiency of axons. Axon calibers as small as $0.2\mu\text{m}$ in diameter are myelinated in the CNS, while in the PNS, $1\mu\text{m}$ is on average the smallest myelinated fibre present (Waxman and Bennett, 1972, Ritchie, 1983).

The myelin sheath when viewed by high power Transmission Electron Microscopy (TEM) shows a pattern of band periodicity, 12-19 nm in size. Each band consists of a dark major dense line, 2.5 nm thick separated by paler interperiod lines. The major dense line is formed by the close approximation of two intracellular lipid bilayers, that are indistinguishable by TEM, while the interperiod line is formed by the two extracellular surfaces of the Schwann cell membrane (Napolitano and Scallen, 1969). In the myelin forming Schwann cell, cytoplasm is excluded from myelin and restricted to certain areas such as the adaxonal space (the area immediately surrounding the axon) and the perinuclear abaxonal space (farthest from the axon). At either end of the cell, approaching the node of Ranvier, terminal loops rich in cytoplasm form the paranodal region. These loops adhere to the axolemma and form a series of septate-like junctions that serve to anchor the myelin membrane, providing a diffusible barrier and preventing lateral diffusion of membrane proteins (reviewed in Peles and Salzer, 2000). On the axon side these junctions contain the protein Caspr/ Paranodin. The juxtaparanodal

region is a zone just under the edges of the compact myelin sheath beyond the paranodal region and is rich in Caspr2 and a number of different delayed-rectifier potassium channels that may promote repolarisation or maintain resting potential (Peles and Salzer, 2000). One myelin forming Schwann cell is separated from the next at the node of Ranvier, but the cells send irregular processes, known as nodal fingers, into the nodal space. The node of Ranvier is rich in sodium channels including $\text{Na}_v1.6$ and these channels allow the depolarization events in saltatory conduction (Peles and Salzer, 2000). Other proteins found in this zone include ankyrin G, that interacts with sodium channels, and also CAMs. Myelin sheaths are regularly interspersed by Schmidt-Lanterman incisures, crevasse like fissures traversing the entire thickness of the sheath, and also longitudinal incisures, connecting paranodal cytoplasm to adaxonal and abaxonal spaces (Mugnaini *et al.*, 1977).

Myelin composition

Myelin consists of a number of different glycoproteins; P_0 , MBP, MAG, PLP, PMP22 and P2, CNPase. Of these the most abundant is P_0 accounting for more than 50% of the total peripheral protein (Greenfield *et al.*, 1973).

P₀

P₀ is a 28-30 kD transmembrane protein with a single extracellular Ig-like domain (Lemke and Axel, 1985, Lemke *et al.*, 1988). The presence of this Ig-like domain, found in cell adhesion molecules such as N-CAM (for review see Edelman, 1983), is indicative that P₀ is somehow involved in adhesion. It is predicted that P₀ mediates myelin compaction by adhering the extracellular and intracellular myelin layers together (Lemke and Axel, 1985). In support of this, the homophilic adhesive nature of P₀ has been demonstrated by transfection assays where increased cell-cell attachment occurs (d'Urso *et al.*, 1990, Schneider-Schaulies *et al.*, 1990, Filbin *et al.*, 1990). The crystal structure of P₀ has been resolved and recombinant extracellular domains have been shown to form dimers and tetramers *in vitro* leading to the proposal that P₀ acts by forming a lattice network securing opposing Schwann cell membranes (Shapiro *et al.*, 1996).

In maturing cells high level P₀ expression is associated with myelin forming Schwann cells (Trapp *et al.*, 1981, Webster and Favilla, 1984, Hahn *et al.*, 1987). Both P₀ mRNA levels and protein synthesis rise about 30 – 40 fold from birth to a peak during the second post-natal week before dropping off in the adult (Lemke and Axel, 1985, Trapp *et al.*, 1988, Stahl *et al.*, 1990, Baron *et al.*, 1994). Schwann cells require continuous axonal contact to maintain high level P₀ expression (Weinberg and Spencer, 1976, Trapp *et al.*, 1988, Mirsky *et al.*, 1980, Lemke and Chao, 1988, Jessen and Mirsky, 1991, Fernandez-Valle *et al.*, 1993, Scherer *et al.*, 1994, Gupta *et al.*, 1993). Recently, the gene encoding the transcription factor Sox10 has been suggested to regulate P₀

expression (Peirano *et al.*, 2000). Here the authors show that Sox10 expression is sufficient to induce P₀ in a neuroblastoma cell line with *in vitro* co-transfection assays, while this effect is absent when known Sox10 mutants are used in the same assays. Also they show that the P₀ promoter contains two Sox10 binding sites. Additionally developing sciatic nerves of Dom mutants, that are deficient in Sox10, were analysed at E12 and were found not to express P₀.

The creation of a P₀ deficient animal has shown that not only is P₀ involved in myelin compaction during development but is required for the long term maintenance of the sheath via protein turnover (Giese *et al.*, 1992). Human peripheral neuropathies have been identified that are linked to mutations in the P₀ gene (reviewed by Keller and Chance, 1999, see below). Recently, a study of P₀ gene overexpression highlights the importance of dosage and stoichiometry of the different myelin genes in the production of a stable myelin sheath (Wrabetz *et al.*, 2000). A series of transgenic mice overexpressing P₀ have been generated by introducing increasing copies of the P₀ gene, and it was found that increasing dysmyelination occurred with increased P₀ gene dosage. Increased P₀ gene dosage resulted in reduced P₀ protein expression and interestingly, also caused a reduction of normal levels of MBP, which is suggestive that myelin assembly employs a process linking the various constituents in a mechanism that closely controls the final stoichiometry.

MBP

MBP is the next most abundant protein accounting for between 2% and 16% of peripheral myelin (Greenfield *et al.*, 1973). MBPs consist of a family of six proteins,

14-21.5kD, that are located on the intracellular face of compact myelin in the major dense line (reviewed by Lemke and Barde, 1998). Both oligodendrocytes and Schwann cell target MBP proteins to the sheath via free ribosomes located close to the site of myelin formation (Colman *et al.*, 1982, Trapp *et al.*, 1987, Ainger *et al.*, 1993). The naturally occurring mouse mutation *shiverer* (*shi*) has been shown to be deficient in MBP. Within the CNS the major dense line fails to form (Privat *et al.*, 1979, Popko *et al.*, 1988) while in the PNS only a slight thinning of myelin could be seen (Kirschner and Ganser, 1980, Rosenbluth *et al.*, 1980). In accordance with the higher density of MBP found in the CNS (30%) these data demonstrate that MBP has a more important role in the CNS compared to the PNS. *Shi* phenotype includes tremors, convulsions and early death can be rescued by the introduction of the wild-type MBP gene (Readhead *et al.*, 1987, Kimura *et al.*, 1989). *Shi* mice also exhibit double the number of Schmidt-Lanterman incisures in myelin forming Schwann cells (Gould *et al.*, 1995). Double mutants have been generated deficient for both P_0 and MBP which show a severe hypomyelination devoid of major dense lines, indicating that in the PNS both MBP and P_0 proteins are required for normal myelination (Martini *et al.*, 1995).

MAG

MAG is a transmembrane protein constituting less than 1% of peripheral myelin. It exists in two isoforms, 67 kD and 72 kD forms, with the former predominantly in Schwann cells and the latter localised to oligodendrocytes of the CNS as well as Schwann cells. MAG is found in areas of Schwann cell cytoplasm where P_0 is absent, such as the mesaxon, Schmidt-Lanterman incisures and paranodal loops but not in compact myelin and is expressed earlier than all other myelin genes (Hudson *et al.*,

1990). This restricted expression pattern is related to MAG function as a preventor of compaction at paranodal loops and Schmidt-Lanterman incisures (Trapp *et al.*, 1984, Trapp and Quarles, 1984). The extracellular aspect of MAG contains 5 Ig like domains, usually associated with adhesive properties. Upon transfection of MAG into fibroblasts, not usually expressing MAG, an increase in adhesiveness was noted together with a neurite outgrowth promoting activity, suggesting that MAG may have dual roles (Afar *et al.*, 1991, Johnson *et al.*, 1989). A naturally occurring *Quaking* mouse mutant shows a disrupted MAG expression pattern although the mutation is not in the MAG locus, suggesting that MAG may be, at least in part responsible for the phenotype (Trapp *et al.*, 1988). MAG deficient mice have an obvious phenotype within the PNS although enhanced regeneration following sciatic nerve injury has been reported (Li *et al.*, 1994, Montag *et al.*, 1994, Filbin, 1995). However, the onset of maturity is accompanied with progressive axonopathy and axonal degeneration suggesting that MAG may also stabilize myelin (Yin *et al.*, 1998). Other studies showing that MAG inhibits extention of neurites of older ganglia *in vitro*, although such an effect is absent from DRGs of young, newborn, animals suggesting that MAG is inhibitory for mature neurons (DeBellard *et al.*, 1996, Tang *et al.*, 1997).

PLP

PLP exists in two isoforms, PLP at 24 kD and DM20 at 20 kD formed by alternative splicing of one gene (reviewed in Suter and Snipes, 1995). The majority of PLP is seen in the CNS where it contributes 50% of total central myelin whereas in the PNS low amounts are expressed. During development of oligodendrocytes DM20 is initially expressed at higher levels of the two genes but is later superceded by PLP at the peak of

myelination (Schindler *et al.*, 1990, Timsit *et al.*, 1992, 1995). In mature myelin PLP is proposed to have a structural role being localised in the interperiod line and PLP null mutants show CNS myelinopathies (Hodes *et al.*, 1993, Griffiths *et al.*, 1995). As well as this function PLP deficient mice show increased oligodendrocyte proliferation and a lack of mature oligodendrocytes suggesting that PLP is required for the maturation of this cell type (Suter and Snipes, 1995). As for the P₀ gene, gene dosage appears to be critical for normal PLP function. Overexpressing PLP transgenic mice display CNS phenotypes similar to that of the null mutant (Redhead *et al.*, 1994, Kagawa *et al.*, 1994). The importance of PLP protein in the normal function of PNS myelin is controversial. One study shows PLP and DM20 are excluded from compact myelin but are found in Schwann cell cytoplasm (Puckett *et al.*, 1987) and the idea that developmental expression is not coordinated with myelination suggests that both isoforms are not required for myelination (Kamholz *et al.*, 1992, Stahl *et al.*, 1990). Another, more recent study demonstrates the presence of low amounts of PLP protein within compact myelin and show that PLP deficiency leads to a degree of demyelination showing that as well as in the CNS the PNS requires normal PLP function (Garbern *et al.*, 1997).

PMP22

PMP22 is a 22 kD glycoprotein expressed in mammalian sciatic nerve in a pattern similar to that of other myelin gene products and is localised to compact myelin (Snipes *et al.*, 1992, reviewed in Werner *et al.*, 1998). PMP22 protein expression is detectable after MAG but before MBP onset in rodent sciatic nerve, both *in vivo* and *in vitro* cocultures of DRG neurones and Schwann cells (Notterpek *et al.*, 1999). In the mature

nerve PMP22 is found in the myelin sheath but also in the membranes that surround unmyelinated axons (Snipes et al., 1992, Haney et al., 1996). The highest levels of PMP22 were associated with late stages of myelination, but an early presence of PMP22 in both myelin forming and non-myelin forming Schwann cells suggest that PMP22 has dual functions in the PNS (Notterpek *et al.*, 1999). Support for these data may be found from studies that show that PMP22 gene expression is under the control of two differentially expressed tissue specific promoters. Promoter 1 is expressed in myelinating Schwann cells while promoter 2 is found in tissues that do not form myelin (Bosse *et al.*, 1994, Suter *et al.*, 1994).

The mouse mutants, *trembler* (Tr) and *trembler-J* (Tr^J) display poor peripheral myelin compaction and hypomyelination together with increased Schwann cell proliferation and associated with inappropriate myelin degradation and remyelination (Suter *et al.*, 1992a, b). Missense mutations in the transmembrane domains of the PMP22 gene are the cause of this phenotype showing how it may be important for myelin compaction and maintenance of mature myelin (Suter *et al.*, 1992b, Suh *et al.*, 1997). Tr mutants have a G150D mutation in the last transmembrane domain that causes a transport arrest of PMP22 in the endoplasmic reticulum (ER) (Naef *et al.*, 1997, D'Urso *et al.*, 1998). Tr^J mice have a Leu16Pro mutation and PMP22 proteins are also transported abnormally (Notterpek *et al.*, 1997, Tobbler *et al.*, 1999). PMP22 mutations are associated with three different phenotypes in humans; Charcot-Marie-Tooth (CMT) disease, Dejerine-Sottas syndrome (DSS), hereditary neuropathy with liability to pressure palsies (HNPP) (reviewed in Keller and Chance 1999). The majority of patients diagnosed as CMT1A have a duplication including the complete PMP22 gene

although point mutations also exist (Lupski *et al.*, 1991, Raeymaekers *et al.*, 1991). One Dutch family has the same mutation as in Tr^J mutants, Leu16Pro, and shows a severe DSS phenotype (Valentijn *et al.*, 1992). Interestingly this mutation results in an 80% reduction in myelinated fibre density in humans compared to 20% in the Tr^J mouse (Gabreels-Festen *et al.*, 1995, Robertson *et al.*, 1997). In addition to the Tr and Tr^J mutants identified, PMP22 deficient mice have been generated by the complete disruption of the PMP22 gene and these animals display hypermyelination and demyelinating peripheral neuropathies in the homozygous and heterozygous state (Adlkofer *et al.*, 1995, Adlkofer *et al.*, 1997). The phenotype of PMP22 null mutants and the natural Tr mutants vary in severity and a toxic gain-of-function effect has been proposed to be the basis of this difference, caused by aberrant intracellular protein trafficking (Naef *et al.*, 1999, Tobler *et al.*, 1999, Robertson *et al.*, 1999). Robertson and colleagues have shown that Tr^J mice and transgenic mice overexpressing the PMP22 gene (7copies) differ with respect to axon/Schwann cell interactions (Robertson *et al.*, 1997, Robertson *et al.*, 1999). They show that Tr^J homozygote Schwann cells fail to make a full turn around axons and the mesaxon is therefore incomplete, which was not seen in PMP22 overexpressing transgenic mice.

Recently transgenic mice have been created overexpressing increasing copies of the PMP22 gene which show that peripheral neuropathies develop when 4 copies or more of the transgene are present and that with 7 copies hypomyelination is severe (Huxley *et al.*, 1996, 1998). More significantly it has been found that Tr^J mutants and mice with 7 copies of the PMP22 gene are able to form 1:1 associations with axons but that many fibres could be visualized as being incompletely surrounded by Schwann cell cytoplasm (Robertson *et al.*, 1999). Delays in the onset of myelination have also been observed in

mouse and rats overexpressing the mouse PMP22 protein (Magyar *et al.*, 1996, Serada *et al.*, 1996). Further analyses of these PMP22 overexpressing rats shows that myelination is blocked at the promyelin stage, not delayed, and Schwann cell differentiation at the mRNA level is not perturbed (Niemann *et al.*, 2000). Thus it is likely that PMP22 is involved early on in the process of myelination, following the establishment of 1:1 relationships with axons.

To summarise these findings, the localization of PMP22, the discovery of tissue specific promoters and the data obtained from analysis of natural and man made mutants (human, rat and mouse) suggest that PMP22 may have 2 roles; an earlier role in the establishment of a complete Schwann cell to axon association and a later role as a component of mature compacted myelin. PMP22 mutations can lead to toxic gain-of-function effects caused by aberrant intracellular protein trafficking (Naef *et al.*, 1999, Tobler *et al.*, 1999) and this may also explain the large variation in severity in neuropathies seen both in patients with mutations in the PMP22 gene and animal models.

P2, CNPase

The products of both P2 and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) genes are believed to play a role in myelin assembly (reviewed by Hudson, 1990). P2 is a small, 14.8 kDa protein, comprising less than 1% of total myelin protein and is only found within compact myelin (Hahn *et al.*, 1987). This protein is proposed to function by regulating the synthesis and transport of long chain fatty acids (Hudson 1990).

CNPase is a cytoplasmic protein, found as two isoforms (46 and 48 kDa) within glial cells as well as other non-neuronal tissues. Within the CNS it is likely that CNPase is involved in intracellular transportation of mRNA, effectively guiding specific proteins to their site of myelin assembly (Hudson, 1990). In contrast to the CNS the developmental expression of CNPase within the peripheral nerve does not correlate with myelination, making its role less clear within the PNS (Stahl *et al.*, 1990).

Periaxin

Periaxin is a member of the PDZ domain protein family and is expressed at high levels by myelinating Schwann cells, where it is associated with the cytoskeleton. The murine periaxin gene encodes two splice variant mRNAs, differing in the 3' untranslated region, which translate into two periaxin forms: L-periaxin and S-periaxin (Gillespie *et al.*, 1994, Dytrych *et al.*, 1998). The two periaxin forms are targeted to different subcellular compartments within mature myelinating Schwann cells, L-periaxin is localized to the plasma membrane while S-periaxin is expressed within the cytoplasm (Dytrych *et al.*, 1998).

The onset of periaxin expression in rat sciatic nerve occurs early in development and peaks during the first two weeks after birth, the period of active myelination (Gillespie *et al.*, 1994). Expression is initially nuclear, but just prior to myelination it is seen adaxonally and progressive myelination sees a shift in this localisation to the abaxonal Schwann cell membrane (Scherer *et al.*, 1995). Within compact myelin, periaxin is localized to the periaxonal sheath, similar to MAG, leading to the theory that it may be involved early on in the process of axonal ensheathment (Gillespie *et al.*, 1994). Cytoplasmic periaxin expression is regulated by axonal contact in a similar fashion to

myelin genes and decreases following nerve injury, with the exception that periaxin expression predates major myelin gene expression as it is detectable in developing nerves and in regenerating nerves in advance of P₀ (Scherer *et al.*, 1995). It has recently been shown that periaxin may be detected as a nuclear product in the Schwann cell precursors of embryonic mouse nerve at E14, prior to the appearance of plasma membrane localized periaxin seen at E17 (Dytrych *et al.*, 1998). The significance of this observation to the development of Schwann cells has yet to be further investigated. A periaxin null mutant mouse has been generated, which shows a normal pattern of myelination during development but goes on to present severe demyelination and associated reduced nerve conduction velocities in adults (Gillespie *et al.*, 2000).

Recently, mutations in the periaxin gene have been reported in peripheral neuropathies including DSS (Boerkel *et al.*, 2001) and an autosomal recessive form of CMT (ARCMT) termed CMT4F (Guilbot *et al.*, 2001). Taken together these data strongly suggest that periaxin plays a role in either myelin stability and / or protein turnover in mature myelin forming Schwann cells.

Connexin 32

Gap junctions are intercellular channels allowing the passage of small molecules such as ions, second messengers and metabolites (<1000 Da) (reviewed by Bruzzone and Ressot, 1997). They consist of oligomeric connexin proteins, that are four-pass-transmembrane proteins and differential expression is seen in many different tissues. Connexin 32 (Cx32) is highly expressed in the myelin-forming cells of the CNS and PNS (Scherer *et al.*, 1995). In the mature PNS, Cx32 is localized to the paranodal region and Schmidt-Lanterman incisures of myelin and may form a pathway for

diffusion across areas of non-compacted myelin (Scherer *et al.*, 1995, Paul, 1995). Cx32 deficient mice display late-onset peripheral neuropathy with the progressive appearance of many onion bulb structures, thinly myelinated axons and increased periaxonal collars in the sciatic nerve, showing that Cx32 is important for myelin maintenance (Anzini *et al.*, 1997). Similarly, in humans more than 80 mutations in the Cx32 gene are associated with peripheral nerve degeneration as seen in X-linked forms of Charcot-Marie-Tooth neuropathies (Bergoffen *et al.*, 1993, Bruzzone *et al.*, 1994, Ionasescu *et al.*, 1994, Bone *et al.*, 1995, Fairweather *et al.*, 1994).

A study of the PNS of Cx32 deficient and P₀ haplo-deficient mice demonstrates an accelerated peripheral neuropathy compared to either individual mutant suggesting that both of these proteins, implicated in myelin maintenance, may have a combined effect on myelin stability despite having different sub-cellular localizations (Neuberg *et al.*, 1998).

Non-myelin forming Schwann cells

Non-myelin forming Schwann cells support a number of smaller diameter axons, up to 20, in membranous channels that may or may not completely enclose each axon, allowing the axons to be isolated from one another. Adjacent non-myelin forming Schwann cells, unlike myelin forming Schwann cells, overlap one another and no part of the axonal surface is exposed (Eames and Gamble 1970). Non-myelin forming Schwann cells express gap junctions, located at the interphase of two cells, allowing these small ionic pores to facilitate intercellular communication. This suggests that non-myelin forming Schwann cells act as a continuous chain of support cells (Konishi, 1990).

As the name suggests this Schwann cell type does not make myelin or express high levels of the myelin genes, rather they express markers associated with developing Schwann cells. The low affinity neurotrophin receptor, p75 (p75NTR) is a 75 kD cell surface associated glycoprotein that is able to bind the neurotrophins, including NGF (Hosang and Shooter, 1985, Chao *et al.*, 1986, Radeke *et al.*, 1987). This molecule is expressed in all Schwann cells during development, down regulated in myelin forming Schwann cells but maintained on non-myelin forming Schwann cells in the adult sciatic nerve (Jessen *et al.*, 1990). Binding studies show that p75NTR protein in the adult sciatic nerve does not bind NGF in large quantities (Taniuchi *et al.*, 1986).

Non-myelin forming Schwann cells express two different adhesion molecules, the neural cell adhesion molecule (N-CAM) and L1. Both are members of the large Ig superfamily, with Ig-like domains in combination with fibronectin III-like domains, facilitating homophilic binding (reviewed Edelman and Crossin, 1991, Schachner, 1990).

Both L1 and N-CAM have been shown to promote neurite outgrowth *in vitro*, L1 being the most potent, suggesting an important function in axon growth both in development and following injury (reviewed Schachner, 1990).

N-CAM

N-CAM can exist in three moieties generated by alternative splicing of one gene, 180 kD, 140 kD and 120 kD, the two larger proteins are integral membrane proteins while the smaller is tethered by a phosphatidylinositol anchor (reviewed by Edelman and Crossin, 1991). Developing Schwann cells express N-CAM on their cell surface but as myelination begins this molecule is down-regulated on myelin forming cells. In the mouse a small amount of N-CAM expression remains in the periaxonal space (Martini

and Schachner, 1986) while in the rat N-CAM is not detectable on myelin forming Schwann cells (Jessen *et al.*, 1987a, Mirsky *et al.*, 1986).

L1

L1 is a large, 200 kD, cell surface adhesion molecule expressed on mature non-myelin forming Schwann cells and their axons (Rathjen and Schachner, 1984, Martini and Schachner, 1986). During Schwann cell development L1 is expressed on all Schwann cells prior to the onset of myelination as well as on axons within the developing nerve (Faissner *et al.*, 1984). Subsequently L1 expression is down-regulated from all myelin forming Schwann cells together with the axon which is being myelinated, just as the myelin wrapping process is underway (Martini and Schachner, 1986). *In vitro* assays have demonstrated that addition of L1 antibodies prevent myelination suggesting that L1 binding is required for the process of myelination and more specifically the establishment of adhesion between axon and Schwann cell (Selheimer *et al.*, 1989, Wood *et al.*, 1990).

Recently L1 deficient mice have been generated and these display normal development of non-myelin forming Schwann cells and associated unmyelinated axons within the sciatic nerve (Haney *et al.*, 1999). In contrast, in the mature adult sciatic nerve (P60) many unmyelinated axons were either partially surrounded or completely bereft of Schwann cell processes and these Schwann cells showed a discontinuous basal lamina. A degree of degeneration was also seen in the mutant nerves and von Frey pressure tests revealed that this was due to a sensory deficit caused by a sensory axon loss in L1 deficient mice (Haney *et al.*, 1999). Further to this the authors went on to show by sciatic nerve grafting, wildtype into L1 mutants and vice versa, that this deficit

was due to a lack of axonally derived L1 and not Schwann cell derived L1 and that axon-Schwann cell interactions in unmyelinated fibres mediated by heterophilic binding of axonal L1 with a Schwann cell ligand other than L1.

Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is a 49 kD type III intermediate filament, expressed by astrocytes within the CNS and in the non-myelin forming Schwann cells of the PNS (Jessen and Mirsky 1980, 1985, Feinstein *et al.*, 1992, Mokuno *et al.*, 1989).

Markers shared by both Schwann cell types

S100 proteins.

These small proteins, 10-12 kD in size, are acidic calcium binding proteins found mainly in the cytoplasm and at the cell surface of all Schwann cells. They have potential roles in proliferation and regulation of glial cell morphology via cytoskeletal organisation (reviewed by Kligman and Hilt, 1988). S100 may have a neurotrophic role as excess S100 has been shown to enhance CNS neuronal survival and neurite outgrowth (Winningham-Major *et al.*, 1989). The onset of S100 expression by Schwann cells marks the transition of the Schwann cell precursor cell to the immature Schwann cell, found at E15-E17 in the rat sciatic nerve (equivalent to the period E14-E16 in the mouse) (Jessen *et al.*, 1994, Dong *et al.*, 1999). The expression of S100 by only the Schwann cells within mature nerves (connective tissue cells such as fibroblasts are S100 negative) and the continued expression following axotomy or when taken into culture make S100 a good experimental marker of Schwann cells (reviewed by Jessen and Mirsky 1990). Myelin forming Schwann cells express higher levels of S100 compared to

non-myelin forming cells, which is directly related to the quantity of myelin associated with a given cell (Mata *et al.*, 1990).

One interesting aspect of S100 is that once expression is induced it remains a feature of Schwann cells and unlike many other markers it is not reversible on denervation.

Other Intermediate Filaments.

Nestin is a type VI intermediate filament and two forms can be found in the rat sciatic nerve, 175 kD and 400 kD (Friedman *et al.*, 1990). Although nestin is expressed in both myelin forming and non-myelin forming Schwann cells, higher levels are seen in myelin forming Schwann cells (Friedman *et al.*, 1990).

Vimentin is a type III intermediate filament expressed in myelin forming and non-myelin forming Schwann cells (Yen and Fields 1981).

Galactocerebroside (GAL-C).

GAL-C is a glycosphingolipid expressed on the cell surface of both myelin forming and non-myelin forming Schwann cells of the PNS and oligodendrocytes of the CNS (Jessen *et al.*, 1985, Mirsky *et al.*, 1980). GAL-C expression is associated with maturing Schwann cells, appearing first in myelin forming Schwann cells just before myelination (at birth in mouse sciatic nerve) (Jessen *et al.*, 1987a, Kelly *et al.*, 1992) and two to three weeks later in non-myelin forming cells (Jessen *et al.*, 1985, Jessen *et al.*, 1987). Antibody blocking experiments prevent myelination in co-cultures of DRG neurons and Schwann cells demonstrating that GAL-C may be necessary for myelination (Owens and Bunge, 1990, Ranscht *et al.*, 1987). Gal-C is normally synthesized in cells by UDP-galactose ceramide galactosyltransferase (CGT) and this enzyme has been targeted to

create CGT null mutant mice that are therefore unable to produce Gal-C (Bosio *et al.*, 1996, Coetzee *et al.*, 1996). In one of these mice, growth is retarded and premature death occurs within the fourth post-natal week, and a pronounced whole body tremor develops on or about P12 (Bosio *et al.*, 1996). In both strains, all of the myelin gene proteins are expressed typically and normal myelin ultrastructure is exhibited by mutant sciatic nerve. Nerve conduction in CGT null mutants is however reduced to levels normally associated with non-myelin fibres alone, suggesting that saltatory conduction is dramatically impaired (Bosio *et al.*, 1996, Coetzee *et al.*, 1996). CGT deficient myelin-forming cells do not express Gal-C or sulphatide but instead nonhydroxy and hydroxy fatty acid substituted glucosylceramides (Glc-C) are substituted in their place (Bosio *et al.*, 1998). The result of this substitution is that, although the myelin sheath appears normal, there is increased fluidity and permeability of the sheath together with abnormal nodal membrane organization. This disables saltatory conduction demonstrating that Gal-C is required for normal myelin function. In the sciatic nerves of mice deficient in galactocerebroside and sulphatide axonal retraction was noted at Postnatal day 10 suggesting that Schwann cell axon signaling may be impaired (Dupree *et al.*, 1998). As in CGT deficient mice normal myelin is present in the adult, but in this case the authors have not tested conduction velocities and so it remains to be seen if nerve impulses are also impaired.

Interestingly a single human case has been reported, diagnosed as pharyngeal-cervical-brachial Guillain-Barre syndrome, associated with auto-antibodies to Gal-C (Anti Gal-C IgM) (Kasuya *et al.*, 1999). Anti Gal-C IgM induced bulbar paralysis which is most likely to be due to disrupted saltatory conduction in a hypomyelination associated neuropathy.

This protein takes its name from the 04 monoclonal antibody which has been shown to bind to the sulphated form of Gal-C, as well as some unidentified lipids (Bansal and Pfeiffer, 1987). 04 is expressed two days earlier than Gal-C in the development of both Schwann cells and oligodendrocytes (Sommer and Schachner, 1981, Wolswijk and Noble, 1989, Mirsky *et al.*, 1990). Expression of 04 is first detectable in the rat sciatic nerve at E16-17 and by E18 the majority of cells are 04 positive (Mirsky *et al.*, 1990). 04 is regulated by axon contact *in vivo*, in a similar fashion to Gal-C, which is reproducible by cAMP elevation *in vitro* (Mirsky *et al.*, 1990). This study demonstrates the possibility that 04 may recognize other epitopes other than sulphated Gal-C.

Laminin.

Both myelin forming and non-myelin forming Schwann cells synthesise basal lamina (Bignami *et al.*, 1984, Billings-Gagliardi *et al.*, 1974). Schwann cells make large amounts of laminin, the major component of basal lamina (Cornbrooks *et al.*, 1983). Laminins comprise a large, growing family of molecules and are a 3 sub-unit glycoprotein made up of two light chains, 200 kD, and one heavy chain, 400 kD, that form a cruciform structure (Nissinen *et al.*, 1991). The major form of laminin expressed by Schwann cells consists of $\beta 1$ and $\beta 2$ light chains and the $\alpha 2$ heavy chain (merosin) (reviewed in Reichardt and Tomaselli, 1991). A naturally occurring mutant mouse known as dy/dy has been shown to have reduced levels of the laminin $\alpha 2$ chain (Arahata *et al.*, 1993). Peripheral nerves in these mice have reduced numbers of myelinated axons (Harris *et al.*, 1972) and atypical patterns of Schwann cell ensheathment

suggesting that axon Schwann cell interactions may be, in part, dependent on laminins (Bradley and Jenkinson, 1973, Stirling, 1975, Madrid *et al.*, 1975, Uziyel *et al.*, 2000).

Integrins.

Integrins are cell surface glycoproteins that mediate local interactions between cells and the ECM and facilitate bi-directional signaling and such interactions may mediate cell proliferation, migration and differentiation (Hynes, 1992). They comprise a large family of non-covalently bound α and β subunit heterodimers; each subunit comprises a large extracellular domain involved in ligand binding and a short intracellular that can interact with elements of the cytoskeleton and adaptor molecules. 20 subunits have been identified that have been shown to form 22 differing α/β heterodimers (reviewed in Previtali *et al.*, 2001). Each integrin heterodimer may bind ligands that include members of the ECM and transmembrane proteins of the Ig superfamily, e.g. L1 (termed in-trans binding) with signaling being further modulated by interactions with cell-surface/cytoskeletal proteins such as tetraspan molecules, e.g. CD9 (termed in-cis binding) (Previtali *et al.*, 2001).

Integrins have been identified throughout Schwann cell development but the heterodimeric combinations seen vary according to the specific stages of Schwann cell differentiation. Neural crest cells express a number of different integrins and their migratory routes are lined with ECM integrin ligands such as the laminins. RGD peptide and integrin antibody blocking experiments cause disruption of neural crest cell migration, similarly peptides competing for ECM ligands and growth factor receptor mutants show disruption of migration (Previtali *et al.*, 2001). Seven integrin

heterodimers have been described in mature Schwann cells of chick, rodent and human: $\alpha 1\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 8$, $\alpha 2\beta 1$, $\alpha 6\beta 4$, $\alpha 5\beta 1$ and $\alpha v\beta 3$ (Previtali *et al.*, 2001).

The differentiation of neural crest into Schwann cell precursors is accompanied, in the chick, by expression of $\alpha 6\beta 1$ integrin (Lallier *et al.*, 1992). Similarly the rodent cell transition to Schwann cell precursors is accompanied by altered integrin expression; $\alpha 1\beta 1$ integrin expressed in the neural crest is down-regulated to very low levels in forming Schwann cell precursors but is high in mature non-myelin forming Schwann cells (see below) (Stewart *et al.*, 1997, Perris *et al.*, 1997). These data suggest that the specific stages of Schwann cells development are accompanied by differential integrin expression.

$\alpha 6\beta 4$ integrin

The integrin $\alpha 6\beta 4$ is the most highly regulated heterodimer during postnatal development of Schwann cells and in maturity is localized to myelin forming Schwann cells (Previtali *et al.*, 2001). In both rats and humans the expression of $\beta 4$ integrin subunit dramatically increases in myelin forming Schwann cells, is localised abaxonally and is axon dependent (Einheber *et al.*, 1993, Feltri *et al.*, 1993, Niessen *et al.*, 1994). $\beta 4$ sub-unit null mutants have been generated which die shortly after birth allowing only a glimpse at the onset of myelination, but show that myelination can occur and that $\beta 4$ is not required for Schwann cell differentiation (Frei *et al.*, 1999). However, the possibility that $\beta 4$ may have a role in the longer term in myelin maintenance cannot be ruled out.

$\alpha 6\beta 1$ integrin

In contrast to $\alpha 6\beta 4$ the integrin $\alpha 6\beta 1$ is not regulated but is continually expressed throughout Schwann cell development (Einheber et al., 1993, Feltri et al., 1993). The dynamics of integrin heterodimeric subunit association are beginning to be understood and it has been shown that the $\alpha 6$ subunit preferentially dimerizes with $\beta 4$ over $\beta 1$ (Giancotti et al., 1992). Thus an increase in $\beta 4$ subunit expression could lead to the preferential formation of $\alpha 6\beta 4$ integrins over $\alpha 6\beta 1$ in myelinating Schwann cells. Apart from such hypotheses the explicit role of $\alpha 6\beta 1$ integrin remains unknown.

$\alpha 1\beta 1$ integrin

The integrin $\alpha 1\beta 1$ is associated with non-myelin forming Schwann cells. Expression has been visualized at low levels in developing rat Schwann cells, very low levels in myelinating Schwann cells and high levels within non-myelin forming Schwann cells from two weeks postnatally into adulthood (Stewart et al., 1997). Experiments conducted *in vitro* and *in vivo* suggest that the expression of $\alpha 1\beta 1$ integrin is regulated at least in part by axon contact (Fernandez-Valle et al., 1994, Stewart et al., 1997).

$\beta 1$ integrin subunit

The interactions of $\beta 1$ integrin subunit with adaptor and cytoskeletal proteins suggest that it may have a role in the process of Schwann cell myelination. Merlin (Schwannomin) undergoes cytoskeletal translocation during Schwann cell differentiation and is associated with the $\beta 1$ subunit suggesting a link between $\beta 1$ and the cytoskeleton during myelination (Obremski et al., 1998). The $\beta 1$ subunit is also associated with focal adhesion kinase (FAK) and paxillin in differentiating Schwann cells in glial-neuronal

cocultures, again linking $\beta 1$ to the cytoskeleton and the onset of myelination (Chen *et al.*, 2000).

In addition to these findings suggestive of a role for $\beta 1$ subunit in myelination, an *in vitro* study shows that $\beta 1$ facilitates Schwann cell migration. Using the Varani migration assay it was found that laminin-1 and -2 increase Schwann cell migration and that antibodies to the $\beta 1$ subunit can block this migration (Milner *et al.*, 1997). It is therefore likely that $\beta 1$ integrins mediate Schwann cell migration on laminin substrates.

$\alpha v \beta 3, \alpha v \beta 8$ integrins

Both $\alpha v \beta 3$ and $\alpha v \beta 8$ integrin expression has been demonstrated in mature Schwann cells by immuoprecipitation (Milner *et al.*, 1997). To date, a systematic study of these integrins in Schwann cell development is absent and no direct immunolocalisation has been shown. However, functional data suggests, at least *in vitro*, that αv integrins mediate Schwann cell migration on fibronectin (Milner *et al.*, 1997).

Studies of integrin function in the PNS using null mutants have been of limited success, many resulting in embryonic lethality such as integrin $\beta 1$ or without phenotype as in $\alpha 1$ integrin knockouts (reviewed in Previtali *et al.*, 2001). Further studies employing Cre-loxP system and P₀-Cre transgenic mice may consolidate our knowledge of integrin function in Schwann cell differentiation.

Apoptosis in Schwann cell development

It is known that cell death is a feature of mammalian development and the nervous system is no exception with about 50% of neurons of the peripheral nervous system

dying during normal development (Jacobson *et al.* 1997). This phenomenon has also been shown to be present in the development of glial cells. Data suggests that about 50% of oligodendrocytes formed during optic nerve development are lost due to cell death (Barres *et al.*, 1992). Recently cell death in the Schwann cell lineage has been discovered (Ciutat *et al.*, 1996, Trachtenberg and Thompson, 1996, Syroid *et al.*, 1996, Grinspan *et al.*, 1996, Nakao *et al.*, 1997). These findings are discussed in detail in chapter 6.

Transcription Factors in Schwann cell development

Multicellular eukaryotic organisms are composed of a large number of different tissue types. Cells from these phenotypically dissimilar tissues, such as brain and heart, have identical genotypes but they possess a different spectrum of cellular mRNA. It is now accepted that tissues forming such diverse phenotypes are different as a result of gene regulation at the level of transcription. The identification of mechanisms by which gene transcription is regulated in a precise temporal and spatial pattern is fundamental to our understanding of the development of the multicellular eukaryote. Patterns of gene expression are particularly dynamic during development, a function of the complexity of the processes involved in the generation of a complex organism in a relatively short time. These processes encompass proliferation and differentiation, the generation of many cells by cell division and the reversible commitment of a cell to a given phenotype determined by both genetic and epigenetic factors as well as programmed cell death.

Transcription factors are responsible both for the modulation of cell-type specific gene expression and the gene expression seen during development that regulates and

coordinates this expression. Transcription factors effect a response by binding to specific regions of DNA and interacting with other proteins to upregulate and/or repress gene expression. To date a number different types of transcription factor have been identified in mammalian development and these are grouped into classes based upon the structure of the DNA binding domain. Schwann cells have been shown to express a number of representatives from the different classes of transcription factors (Topilko and Meier, in press and see also Fig 1.1).

The development of Schwann cells involve two distinct, but overlapping patterns of transcription factor expression; those present during neural crest formation, migration and subsequent Schwann cell precursor development, which I shall term early transcription factors and those involved in the differentiation of the two, mature Schwann cell forms, here termed late transcription factors.

Early Transcription Factors

Sox10

The prototypical gene, the sex determining factor, SRY lends its name to this class of transcription factors, SRY box, which are characterised by the possession of a high mobility group (HMG) DNA binding domain (reviewed by Wegner, 1999). The HMG domain is not highly conserved (>50% amino acid homology), binds the heptameric sequence ^{5'} (A/T)(A/T)CAA(A/T)G _{3'} and unusually for a transcription factor binds the minor groove of DNA. Using degenerate PCR, Sox10 was isolated from primary Schwann cell cultures revealing a protein of 466 amino acid residues and 56 kD in size (Kuhlbrodt *et al.*, 1998) with greater than 90% homology to murine Sox8 and 9 (Wright *et al.*, 1993). At the mRNA level Sox10 expression is seen mainly in the nervous

system, and in the CNS is found in the brain where it is localised to the oligodendrocytes of white matter tracts such as the corpus callosum and the internal and external capsules (Kuhlbrodt *et al.*, 1998). In the PNS the expression of Sox10 is seen in the dorsal aspect of the newly closed mouse neural tube, albeit weakly at E8.5 and then, more crucially in a proportion of all the emerging neural crest (Kuhlbrodt *et al.*, 1998, Southard-Smith *et al.*, 1998, Britsch *et al.*, 2001). The majority of PNS ganglia express Sox10 and nerve fibres emanating from them, first seen at E10.5, are also positive for Sox10 mRNA; this latter expression, localised to Schwann cells continues into early adulthood (Kuhlbrodt *et al.*, 1998, Southard-Smith *et al.*, 1998, Britsch *et al.*, 2001). This data suggests that Sox10 is expressed throughout Schwann cell development at the mRNA level in at least a proportion of cells, however it remains unclear if the relative levels of Sox10 fluctuate temporally. The importance of Sox10 in the PNS is demonstrated in isolated cases presenting lesions together with hypomyelination attributable to heterozygous mutations in the Sox10 gene (Inoue *et al.*, 1999, Pingault *et al.*, 2000, Touraine *et al.*, 2000). One possible cause of this neuropathy is a reduction in P_0 expression. Sox10 expression is sufficient to induce P_0 in a neuroblastoma cell line, and as this effect is absent in known Sox10 mutants, it may well form the basis of this neuropathy (Peirano *et al.*, 2000). Additionally, the authors have shown that Sox10 can bind the P_0 promoter at two sites suggesting a direct interaction.

Waardenburg-Hirschprung's disease or Waardenburg-Shah syndrome (WS4) are human hereditary disorders resulting in deafness, pigmentary loss and aganglionic megacolon (Herbath *et al.*, 1998, Kuhlbrodt *et al.*, 1998b). These disorders arise due to neural crest abnormalities and in some cases WS4 is linked to a mutation in the Sox10 gene (Herbath

et al., 1998, Kuhlbrodt *et al.*, 1998b, Pingault *et al.*, 1998). A model for WS4, *Dom* Hirschprung's mouse, a naturally occurring mutant, shows homozygote embryonic lethality and a failure to produce melanoblasts, while heterozygous animals show intestinal aganglionosis and spotted pigmentation (Lane and Liu, 1984). As in WS4 the *Dom* mutation has been localized to a mutation in the *Sox10* gene (Southard-Smith *et al.*, 1998, 1999).

Sox10 regulates diverse genes

Sox10 expression continues into the lineage diversification of both glial cells and melanocytes, but is down-regulated in other neural crest cell derivatives (Herbarth *et al.*, 1998, Kuhlbrodt *et al.*, 1998, Pusch *et al.*, 1998). *Sox10* has now been implicated in the regulation of two genes found within glial cell lineages of the PNS, the *P₀* gene (see above) (Peirano *et al.*, 2000) and more recently *ErbB3* (Britsch *et al.*, 2001). In this most recent study, a *Sox10* null mutant has been generated by LacZ gene insertion and in the heterozygous state a phenotype including pigmentation and megacolon defects, similar to the WS4 and *Dom* mutants, was obtained. Even more significantly, a reduction of the neuregulin receptor, *ErbB3*, has been identified at E10.5 *in vivo*, which has severe consequences for future glia development, and *in vitro* *Sox10* overexpression can induce *ErbB3* expression (Britsch *et al.*, 2001). The *Sox10* null mutant does not show any expression of B-FABP, a marker of Schwann cell precursors, in DRGs and spinal nerves at E11.5 or E12.5, indicating that these cells are completely absent while TUJ-1 labels a neuronal population that begins to develop normally.

In another derivative of the neural crest, the melanocyte, a number of possible downstream target genes have been identified that Sox10 may regulate (Lee *et al.*, 2000, Britsch *et al.*, 2001). One study noted that similar pigmentation deficiencies were present in mice with mutations in the microphthalmia gene (Mitf). Mitf encodes a basic helix-loop-helix-leucine zipper transcription factor essential for the development of the melanocyte lineage (Lee *et al.*, 2000). Sox10 can directly bind and activate the mitf gene; whereas a WS4 mutant acts as a dominant negative repressor of mitf expression (Lee *et al.*, 2000, Bondurand *et al.*, 2000). Another investigation examined three markers expressed in melanocytes, with the finding that *c-kit* positive cells are reduced to 25% in Sox10 null mutants while *mi* and *trp-2* positive cells are completely absent (Britsch *et al.*, 2001). Further to this the authors show that Sox10 can directly induce expression of the *trp-2* gene *in vitro*.

Pax3

Pax3 is an example of a murine Paired box DNA binding domain transcription factor first found in Drosophila segmentation genes (Bopp *et al.*, 1986), encoding a 479 amino acid protein containing both paired domain and paired type homeodomain (Goulding *et al.*, 1991). Pax3 mRNA is expressed in the dorsal part of the neural tube (roof plate), in neural crest and in somitic mesoderm (Goulding *et al.*, 1991). Two naturally occurring Pax3 mouse mutants, *splotch* (Sp) and *splotch delayed* (Spd) have been identified, the former having a deletion in the homeodomain (Epstein *et al.*, 1991) and the latter a point mutation in the paired domain (Moase and Trasler, 1990). Homozygous Sp mutants die at E13 showing central nervous system deficits such as exencephalus and spina bifida (Epstein *et al.*, 1991). Spd mutants survive until E18 (Moase and Trasler, 1990) and fail

to develop early Schwann cells, determined by the absence of S-100 immunoreactivity in the embryonic nerve (Franz, 1993). Other neural crest derivatives disrupted in these mutants include the spinal ganglia, heart tissues and pigment cells (Gruss and Walther, 1992). It is clear from these data that Pax3 has a role in the early emergence and development of neural crest cell derivatives and is required for normal Schwann cell development. However, Pax3 is not restricted to these early developmental stages; Pax3 mRNA is detectable in the sciatic nerve into early post-natal life with a peak of expression around E17 (Blanchard *et al.*, 1996). Further evidence comes from the finding that micro-injection of exogenous Pax3 into Schwann cells *in vitro* causes down regulation of myelination markers such as MBP, and upregulation of those associated with non-myelin forming Schwann cells such as L1, GFAP, p75NTR and N-CAM (Kioussi *et al.*, 1995). This, together with the finding that Pax3 can repress the MBP promoter in co-transfection assays, has lead the authors to suggest that Pax3 has a role in Schwann cell terminal differentiation. Taken together these data suggest that Pax3 is important throughout the development of the Schwann cell lineage with diverse specific functions at different time points that remain to be further investigated.

Late Transcription Factors

Oct-6

Of the few late transcription factors identified within the developing Schwann cell lineage, Oct-6/ SCIP/ Tst-1 is evident first. Oct-6 is a member of the POU domain transcription factor family. This protein family is characterised by a POU binding domain which can be subdivided into a POU-specific and a POU homeodomain which are joined by a short linker. The name POU is derived from the discovery of this protein

type in three different systems, Pit-1, Oct-6 and Unc (reviewed by Latchman, 1999). POU proteins bind the consensus sequence ^{5'} ATGCAAAT ^{3'} with the POU-specific domain contacting the initial 4 bases and the POU homeodomain contacting the last four bases (Klemm *et al.*, 1994). Oct-6 was isolated from three different systems concurrently; sciatic nerve, testes and embryonic stem cells of the central nervous system (Monuki *et al.*, 1989, He *et al.*, 1989, Meijer *et al.*, 1990 and Suzuki *et al.*, 1990). Oct-6 mRNA and protein have been detected in Schwann cell precursors at E12 and E14, mouse and rat respectively, and persist until postnatal day 12 (P12) (Blanchard *et al.*, 1996). Oct-6 expression is regulated; reaching a peak around birth, prior to maximal myelin gene expression (Monuki *et al.*, 1990, Scherer *et al.*, 1994b, Arroyo *et al.*, 1998).

Oct-6 is inducible in Schwann cells *in vitro* by the elevation of cytoplasmic cAMP (Monuki *et al.*, 1989) and *in vivo* Schwann cells require axonal contact for Oct-6 to be expressed (Scherer *et al.*, 1994). Two null mutant lines have been created independently and demonstrate that Schwann cells form typical 1:1 ensheathment relationships with axons but the majority fail to subsequently myelinate or show delayed myelination (Bermingham *et al.*, 1996, Jaegle *et al.*, 1996). Together these data are consistent with a theory that Oct-6 is required for accurate temporal Schwann cell myelination.

In one null mutant line a reduced level of P_0 and MAG at the mRNA level occurs (Jaegle *et al.*, 1996). Further evidence suggesting that Oct-6 is involved in the transcription of the myelination genes comes from the findings that the P_0 gene promoter includes multiple Oct-6 binding sites and that Oct-6 is able to repress P_0 expression *in vitro*

(Monuki *et al.*, 1993). In addition, a supposed dominant negative truncated Oct-6 transgene driven by the P0 promoter, known as delta SCIP, results in premature Schwann cell differentiation, overexpression of the myelin genes and hypermyelination (Weinstein *et al.*, 1995). It has since been shown that delta SCIP can rescue the Oct-6 null mutant phenotype suggesting that delta SCIP does not act as a dominant negative, rather an over-expressor of Oct-6. Given the conflicting nature of the data derived from experiments conducted *in vivo* and *in vitro*, the effect of Oct-6 on myelination is unclear. It will be interesting to see what genes are direct targets of Oct-6.

Brn-5

Recently, a second member of the POU domain family of transcription factors, Brn-5, has been described in Schwann cells (Wu *et al.*, 2001). This gene was originally cloned from rat brain and is localised within a subpopulation of cortical neurons of the CNS (Anderson *et al.*, 1993). Brn-5 in the PNS is localised to the myelin-forming Schwann cells of the mature nerve and developmental expression is reciprocal to that of Oct-6 (Wu *et al.*, 2001). In addition the authors show that the inverse is also true following sciatic nerve axotomy, with the loss of Brn-5 expression and transient up-regulation of Oct-6. This data together with the finding that Brn-5 expression is inducible by β NRGs lead to the suggestion that this gene may be the hitherto elusive transcriptional regulator of myelin maintenance in mature myelin forming Schwann cells and may be responsible for the ability to generate myelin with a delayed schedule in Oct-6 *-/-* mice.

Krox-20

The gene Krox-20 (Egr-2/ NGF-IB), taking its name from *krüppel box*, encodes for a zinc finger transcription factor containing three zinc finger motifs and was isolated from both pheochromocytoma (PC12) and 3T3 fibroblast cell lines following induction by nerve growth factor (NGF) (Hazel *et al.*, 1988, Ryseck *et al.*, 1989, Milbrandt, 1988). The rapid onset of Krox-20 expression, following noxious stimulation, is analogous to that of c-Fos and c-Jun and has therefore been termed an immediate early gene (IEG). Krox-20 is expressed in a number of different tissues, including lung, heart, muscle, thymus, spleen, testes and both CNS and PNS (Chavrier *et al.*, 1988, Watson and Millbrandt, 1990). Expression has been described in the CNS, e.g. in the neocortex, striatum, hypothalamus, amygdala and olfactory bulb (Bhat *et al.*, 1992, Mack *et al.*, 1992, Herdegen *et al.*, 1993). The localisation of Krox-20 is predominantly nuclear and a moderate increase may be elicited following bicuculline-induced seizures, in cortical and subcortical areas (Herdegen *et al.*, 1993).

During the development of the central nervous system Krox-20 is an essential component of hindbrain segmentation (Wilkinson *et al.*, 1989). It is expressed in rhombomeres 3 and 5 and in Krox-20 null mutant embryos these rhombomeres are either partially formed or ablated entirely (Schneider-Manoury *et al.*, 1993). Furthermore Krox-20 has been shown to directly regulate two homeobox genes, Hoxb-2 and Hoxa-2 in rhombomeres 3 and 5 via cis-activation (Sham *et al.*, 1993, Nonchev *et al.*, 1996a, Seitanidou *et al.*, 1997). Transfection experiments demonstrate that Krox-20 can induce ectopic expression of Hox genes acting via conserved Krox-20 binding sites found in the

enhancer elements of these genes (Sham *et al.*, 1993, Nonchev *et al.*, 1996b, Seitanidou *et al.*, 1997).

In the PNS Krox-20 has a fundamental role in the development of the sub-population of Schwann cells destined to myelinate (Topilko *et al.*, 1994). In mice the onset of Krox-20 expression has been demonstrated to occur in the transitory period where precursor cells become immature Schwann cells, at E13-14, approximately 1 day after SCIP expression begins (Blanchard *et al.*, 1996, Topilko *et al.*, 1994, Topilko *et al.*, 1997). This pattern of expression co-ordinates with the appearance of the molecule S100, a marker of differentiating glia, and is dependant on continuous, direct, axonal contact (Murphy *et al.*, 1996). Krox-20 expression is regulated reaching a peak around birth, the point at which the process of myelination is beginning (Watson and Milbrandt, 1990, Blanchard *et al.*, 1996). In Krox-20 null mutant mice myelinating Schwann cells pass the promyelinating phase, where they form 1:1 relationships with axons, and continue to wrap the axon about 1-2 turns before becoming stalled at this point (Topilko *et al.*, 1994). The fact that late markers of myelination, P₀ and MBP, are present at very low levels together with the finding above suggest that Krox-20 is required for and controls transcription of the genes required for myelination (Topilko *et al.*, 1994). *In vitro* Krox-20 can be induced, in Schwann cells by differentiation conditions using molecules that elevate cytoplasmic cAMP and by the addition of NRG β (Murphy *et al.*, 1996, D. Parkinson unpublished data). Such conditions also result in the upregulation of periaxin (D. Parkinson unpublished data). The synchrony of the Krox-20 and periaxin gene expression in combination with the physical abnormality found in both the Krox-20 and periaxin null mutant mice suggest that Krox-20 may interact with genes involved in the

regulation of the cytoskeleton. (Topilko *et al.*, 1994, Gillespie *et al.*, 2000). Further studies of Krox-20 null mutant mice have demonstrated that the cessation of mitotic activity and apoptosis normally seen within the first two postnatal weeks does not occur (Zorick *et al.*, 1999). In addition SCIP expression is altered from a transient pattern to one that is sustained significantly past that seen in the wildtype animal (Zorick *et al.*, 1999). This data supports the theory that Schwann cells are stalled at a particular stage in development, most likely to be the promyelinating phase, and continue to cycle as they would in the late embryonic/ newborn animal well into the second postnatal week. Schwann cell maturation is accompanied by Krox-20 expression in 55% of all Schwann cells, those destined to become myelinating Schwann cells, and is sustained in this sub-cellular population in the adult (K.R. Jessen unpublished data, Herdegen *et al.*, 1993, Topilko *et al.*, 1997). The adult pattern contrasts with that for another, closely related, transcription factor, Krox-24 which is expressed highly in non-myelinating Schwann cells of the adult and at high levels early in development (Topilko *et al.*, 1997). This data together with the finding that during Wallerian degeneration Krox-20 is gradually downregulated while Krox-24 is dramatically upregulated in all Schwann cells has lead to the suggestion that the two genes have antagonistic roles in Schwann cells (Topilko *et al.*, 1997). However, both genes demonstrate a peak of expression around birth, and with Krox-24 being present in all cells at this time point, mutually exclusive expression patterns can be ruled out. This point is addressed in this thesis (see chapter 4). Human Egr-2 mutants have been identified that give rise to debilitating CMT1 neuropathies, discussed below.

Krox-24

Krox-24 (Lemaire *et al.*, 1988) also known as Egr-1 (Sukhatme *et al.*, 1987), NGFI-A (Milbrandt, 1987), zif268 (Lau and Nathans, 1987) and tis8 (Lim *et al.*, 1987) is another member of the murine zinc finger transcription factor family with homology to the prototypical example of this gene type, *Xenopus* TFIII-A. As the names suggest this gene was independently and simultaneously cloned from different systems by similar differential screening strategies designed to identify novel genes with a role in cellular growth control. Screens were performed searching for genes with low expression in cDNA transcripts isolated from quiescent cells but that were rapidly upregulated following mitogenic stimulation or conditions favouring differentiation. Egr-1 was isolated from cDNA derived from serum and cycloheximide stimulated Balb/c 3T3 cells, NGFI-A was isolated as a NGF induced transcript from rat pheochromocytoma cells (PC12) and zif268 was isolated from serum treated Balb/c 3T3 cells. Tis8 was cloned as a phorbol-inducible gene in 3T3 cells and Krox-24 was isolated through hybridisation to a highly conserved domain of the drosophila *krüppel* gene from serum stimulated 3T3 cells. In addition, a chicken homolog exists, isolated as v-src-inducible gene from chicken embryo fibroblasts (Simmons *et al.*, 1989) and the gene was cloned as a T-cell activated transcript and named gene 225 (Wright *et al.*, 1990).

Krox-24 differs from the Krox-20 gene as it is expressed in response to diverse stimuli which can be classified in four groups; induction by mitogens, induction during development and differentiation, induction due to injury and induction in neuronal signalling (reviewed in Gashler and Sukhatme 1995). High levels of Krox-24 mRNA

are expressed in a number of different tissues, including heart, lung, muscle, bone and both CNS and PNS with lower levels seen in the kidney, spleen and liver (Sukhatme *et al.*, 1988, Lemaire *et al.*, 1988, Christy *et al.*, 1988, McMahon *et al.*, 1990). In the CNS studies on the rat brain have revealed expression of Krox-24 throughout cortical development, with expression low in the neonate but reaching high levels in the mature cortex suggesting a requirement in cortical maturation (Watson and Millbrandt 1990). This rise in Krox-24 expression correlates with the process of differentiation and is not restricted to neuronal lineages, a similar pattern is seen in cardiac, osteoblast and monocyte differentiation (Gashler and Sukhatme, 1995). Two Krox-24 null mutants have been generated and mice homozygous for the mutation display a 25% reduction in body size and sterility (Lee *et al.*, 1996, Topilko *et al.*, 1998). These defects have been identified as being caused by anterior pituitary deficits in both sexes together with ovary abnormalities (Lee *et al.*, 1996, Topilko *et al.*, 1998). Analysis of the anterior pituitary revealed disrupted morphology associated with a complete loss of luteinising hormone (LH) producing cells, somatotropes and LH was not produced. Additionally gonadotrophin (GH) producing cells, gonadotropes, showed a substantial reduction in GH production and no reduction in cell numbers (Topilko *et al.*, 1998).

Expression of Krox-24 mRNA has been identified in the sciatic nerve where it was found to be developmentally regulated postnatally with the highest levels found at birth followed by a steady decline to the low levels seen in the adult (Watson and Millbrandt, 1990). Krox-24 protein expression has since been described in the development of Schwann cells, where as mentioned earlier, it is suggested to have an antagonistic role with Krox-20 (Topilko *et al.*, 1997). Additionally a small proportion of Schwann cells

were identified that continued to express Krox-24 into adulthood, and these were considered to be non-myelin forming Schwann cells although this has yet to be confirmed (Topilko *et al.*, 1997).

Zinc Finger Transcription Factors and Disease

Altered transcription factor expression is a common molecular mechanism in tumorigenesis and a number of different forms of cancer are caused by mutations in the DNA binding regions of known zinc finger transcription factors (Call *et al.*, 1990; Little *et al.*, 1992; Mesa *et al.*, 1996). The human pediatric nephroblastoma, Wilms tumor, is caused by mutations in the Wilm's tumor suppressor gene (WT1) encoding for a zinc finger transcription factor (Call *et al.*, 1990; Little *et al.*, 1992; Rauscher 1993, reviewed by Lee and Haber, 2001). Zinc finger transcription factors are also responsible for inherited disease. Greig cephalopolysyndactyly syndrome (GCPs) is caused by translocational interruption of the human Gli-3 gene (Kinzler *et al.*, 1987; Vortkamp *et al.*, 1991). Gli-3 is a mammalian homologue of the *Drosophila* segment polarity gene, *cubitus interruptus*, and the mouse mutant extra-toes (Xt) also displays polysyndactyly and is a model of GCPs (Schimmang *et al.*, 1992).

Krox-24 mRNA levels have been found to be increased in human and mouse adenocarcinomas, suggesting that it may have a role in prostate cancer (Thigpen *et al.*, 1996, Eid *et al.*, 1998, Svaren *et al.*, 2000). However, in other tumours, such as lung tumours, reduced Krox-24 expression has been observed (Levin *et al.*, 1994). In addition a number of different human cell lines, such as breast carcinoma and osteogenic sarcoma show reduced growth and tumorigenicity when overexpressing Krox-24 by transfection

(Huang *et al.*, 1995). These conflicting effects of Krox-24 observed in different cells could be indicative of the dual functions that Krox-24 possesses, both acting as an inducer or repressor of transcription depending on the cellular context. Recently increased Krox-24 expression has been identified in both mouse and human atherosclerosis lesions (Du *et al.*, 2000, McCaffrey *et al.*, 2000). As mentioned earlier novel Egr-2 mutants have been identified causing CMT1 neuropathies, discussed in the next section.

Peripheral neuropathies

Inherited peripheral neuropathies are chronic disorders of the peripheral nervous system that may cause progressive muscle weakness and sensory dysfunction. Different sub-forms of this disease are distinguished depending on severity and the mutated gene involved and include Charcot-Marie-Tooth (CMT) disease, Dejerine-Sottas syndrome (DSS), hereditary neuropathy with liability to pressure palsies (HNPP) and congenital hypomyelination (CH)(reviewed in Schenone and Mancardi 1999, Keller and Chance 1999, Warner 1999).

Charcot Marie Tooth neuropathies (CMT) are a heterogenous group of inherited peripheral neuropathies described by Parisian physicians Dr. Charcot and Dr. Marie and the London based Dr. Tooth in the late 19th century (reviewed in Schenone and Mancardi, 1999, Keller and Chance, 1999, Warner 1999). They represent one of the most common neurodegenerative disorders affecting humans with a prevalence of 1 in 2500 (Skre, 1974). CMT disorders are further sub-classified according to the cellular basis of the disease, the gene involved and chromosomal location. CMT1 is the most

common form and presents with PNS demyelination, associated with glial cell abnormalities and mutations in any one of a number of genes expressed in Schwann cells (reviewed in Kamholz *et al.*, 2000). CMT2 is believed to be caused by axonal dysfunction and one CMT family has been identified with a mutation in the neurofilament-light gene (NF-L) (reviewed in Gemignani and Marbini 2001). Recently, a second CMT family has been identified with NF-L mutations suggesting that intrinsic axonal dysfunction can lead to CMT (De Jonghe *et al.*, 2001). In addition an X-linked variant of CMT (CMTX) is caused by mutations in the connexin-32 gene as discussed earlier (Bergoffen *et al.*, 1993, reviewed in Nelis *et al.*, 1999).

The majority of patients with CMT1 have a duplication in a region of chromosome 17, designated CMT1A, a region containing the myelin gene PMP22, while the less common CMT1B is caused by a mutation in the major peripheral myelin protein, P₀ as discussed earlier (reviewed in Kamholz *et al.*, 2000).

Peripheral neuropathies associated with mutations in the Krox-20 and Periaxin genes

Krox-20

Recently, a rarer and severe form of CMT1 has been identified caused by a number of different mutations within the human Krox-20 gene (Warner *et al.*, 1998, Timmerman *et al.*, 1999, Latour *et al.*, 1999, Bellone *et al.*, 1999, Yoshihara *et al.*, 2001). A single case of a de novo missense mutation (Arg359Trp) in the region of the first zinc-finger domain has been identified (Timmerman *et al.*, 1999). The identification of four different mutations leading to amino-acid substitutions (Arg381His, Arg381Cys, Ser382Arg, Asp383Tyr) in the second zinc-finger domain is suggestive that this region

may be a mutational hotspot (Latour *et al.*, 1999, Warner *et al.*, 1999, Yoshihara *et al.*, 2001). Within the third zinc finger domain a missense mutation (Arg409Trp), again causing an amino-acid substitution has been reported (Warner *et al.*, 1998). A de novo mutation (D305V) in the region ahead of the first zinc-finger has also been identified (Bellone *et al.*, 1999). Finally, a patient diagnosed with recessive CH has a substitution (Ile286Asn) within the R1 repressor domain (Warner *et al.*, 1998).

Periaxin

Periaxin has recently become implicated in an autosomal recessive form of CMT (ARCMT) otherwise termed CMT4F and Dejerine-Sottas syndrome (Boerkoel *et al.*, 2001, Guilbot *et al.*, 2001). The CMT4F mutation found in a large consanguineous Lebanese family has been mapped to 19q13 and more specifically to the periaxin gene (Prx) where analysis of morphology revealed hypomyelination and onion bulb structures. Characterisation revealed a nonsense mutation (R196X) leading to a premature stop codon predicted to cause truncated L-periaxin, which was confirmed using sural nerve biopsies where L-periaxin protein was undetectable (Guilbot *et al.*, 2001). The more severe DSN phenotype, also present sural nerve hypomyelination and onion bulb structures are attributable to both nonsense and frameshift mutations leading once again to truncated L-periaxin protein (Boerkoel *et al.*, 2001).

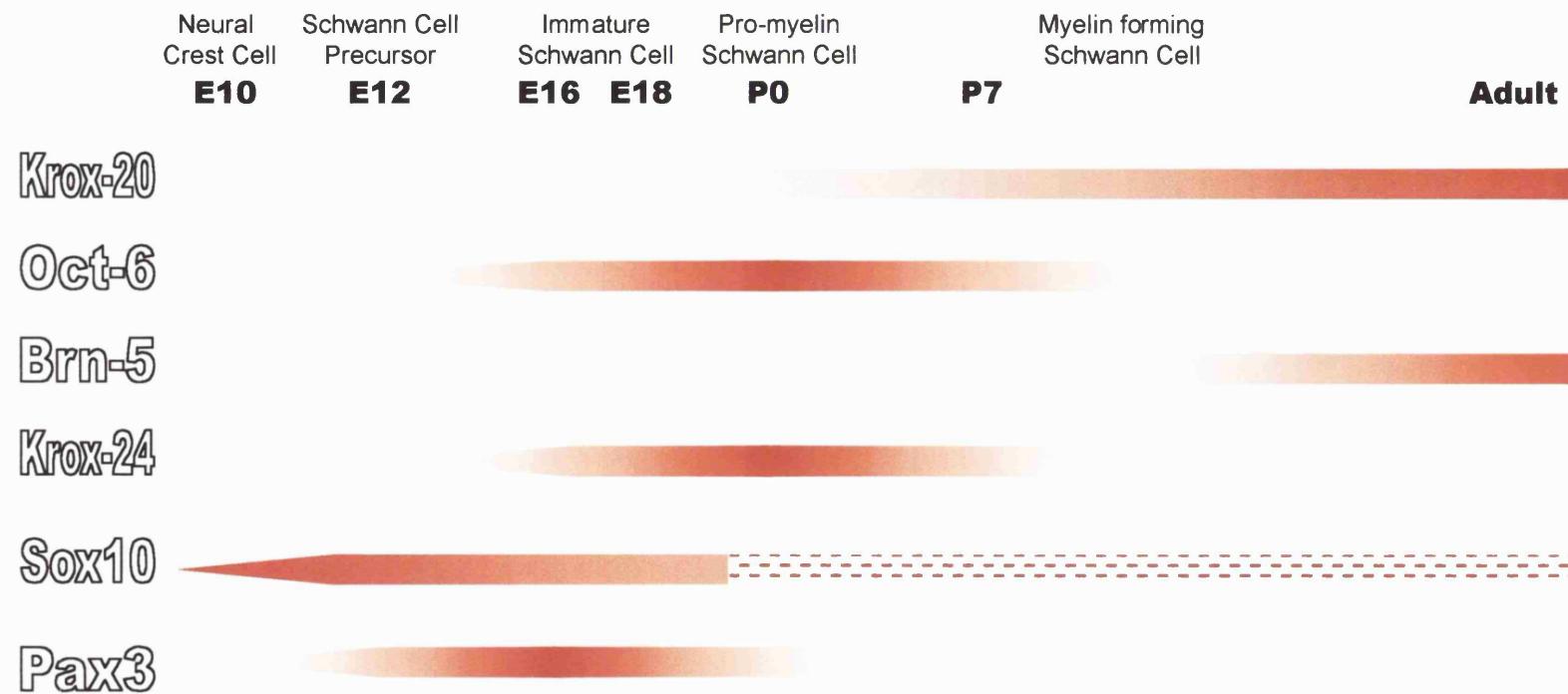
Schwann cell response to axotomy

One aspect of the mature peripheral nervous system which is of particular interest is the ability it has to regenerate following injury. Following axotomy the distal peripheral nerve undergoes the process of Wallerian degeneration. The degeneration of the distal

axon is accompanied by the dedifferentiation and subsequent proliferation of Schwann cells. Schwann cells stop myelinating and down-regulate the production of P_0 , MAG, MBP and PMP22 (Lemke and Chao, 1988; Lemke *et al.*, 1988; Mirsky and Jessen, 1990). Invading macrophages phagocytose and degrade the degenerating distal axon and myelin proteins, aided in part by Schwann cells (reviewed in Kiefer *et al.*, 2001, Fernandez-Valle *et al.*, 1995). Schwann cells upregulate the nerve growth factor receptor (P75NTR) and associated cells upregulate the secretion of the nerve growth factor (NGF) (Heumann *et al.*, 1987; Lemke and Chao, 1988, Mirsky and Jessen, 1990). GAP-43 immunoreactivity is restricted to non-myelinating Schwann cells in mature peripheral nerve glia (Curtis *et al.*, 1992; Hall *et al.*, 1992) following injury, previously myelinating Schwann cells begin to express GAP-43 mRNA and protein, increasing progressively for the following three weeks (Hall *et al.*, 1992; Scherer *et al.*, 1994a). The transcriptional regulation occurring during Schwann cell dedifferentiation following sciatic nerve transection is something a number of investigators have attempted to unravel. There is an associated down-regulation of genes involved in myelination, such as P_0 , and an increase of genes usually only expressed by non-myelin forming Schwann cells such as N-CAM, and these changes are addressed in more detail in chapter 5.

Figure 1.1 A schematic diagram showing the temporal expression pattern of known transcription factor mRNAs within the myelin-forming Schwann cell lineage

The approximate temporal expression pattern of Schwann cell transcription factors Krox-20, Oct-6, Brn-5, Krox-24, SOX10 and Pax-3 is shown. The stages of myelin-forming Schwann cell development are shown together with time points in mouse development. For simplicity only the myelin-forming Schwann cell expression is shown. The hatched region in the SOX10 band indicates uncharacterised expression.



CHAPTER 2

MATERIALS AND METHODS

Reagents for molecular biology

Taq DNA-polymerase, RNase H- Reverse Transcriptase (superscript II), Ampicillin, T4 DNA ligase, T4 buffer, Klenow (DNA polymerase), dNTPs and the 1KB DNA ladder were from GibcoBRL (GibcoBRL Life Technologies, Paisley, UK). Random hexamers were from Promega Corporation (Madison, USA). EDTA disodium salt, ethidium bromide, Tween20, deoxycholic acid, phenylmethylsulphonylfluoride (PMSF), bromophenol blue and NP40 (now superseded by IGEPAL CA-630) were obtained from Sigma (Poole, UK). ECL Plus Kit and Hybond-N nitrocellulose membrane were from Amersham Pharmacia Biotech (UK). Agarose was from Appligene ONCOR (France). Sodium dodecyl sulphate (SDS) and Glycerol were from BDH Lab. Supplies (Poole, UK). Ultraspec RNA total RNA isolation agent was from Biotecx Laboratories (TX, USA). Seeblue pre-stained standards and Multimark multi-colored standard were from Novex, (CA USA). Kaleidoscope pre-stained standards were from Biorad (CA, USA). Vectastain Elite Kit was from Vector Laboratories (CA, USA). Bromodeoxyuridine, terminal transferase and biotinylated-d-UTP were from Roche Diagnostics (Germany).

Reagents for tissue culture

Dulbecco's modified Eagles medium (DMEM), minimum essential medium (MEM) Ham's F-12 medium, L-15 medium, trypsin, glutamine, penicillin, streptomycin were

from GibcoBRL (GibcoBRL Life Technologies, Paisley, UK). Transferrin, selenium, putrescine, triiodothyronine (T3), thyroxine (T4), progesterone, insulin (10^{-3} M), bovine serum albumin (BSA), cytosine arabinoside (Ara C), poly-L-lysine (molecular weight 300,000), poly-L-ornithine, lysine, dibutyryl-cAMP (dbcAMP - 1 mM) and laminin were obtained from Sigma (Poole, UK). Collagenase was obtained from Worthington (Lorne Laboratories, Reading, UK). Foetal calf serum (FCS) was from Bioclear, UK and Fugene 6 transfection reagent was from Roche Diagnostics (Germany). Tissue culture petri dishes and 24-well plates were from Falcon (Becton-Dickinson, Cowley, UK), NDF- β was from R&D Systems (Oxford, UK) and forskolin was from Calbiochem (CA, USA).

Reagents for immuno-labelling

Triton X-100, Hoechst dye H33258, DAPI were from Sigma (Poole, UK). Citifluor was from Citifluor Ltd. (London, UK). Paraformaldehyde and Proteinase K were obtained from Fluka Chemicals Ltd. (Buchs, Switzerland).

Reagents for histology

Glutaraldehyde, OCT compound (Tissue Tek), araldite CY212, DDSA, BDMA, dibutyl pthalate and rubber coffin moulds were from Agar Scientific(UK). DPX mountant, sodium cacodylate and Superfrost Plus microscope slides were from Merck (Poole, UK).

Immunohistochemistry

Labeling with antibodies

Cryostat sections, 8-10 μ m, were collected on Superfrost Plus microscope slides and air dried for at least 45 minutes. Teased nerve preparations were made by dissecting out sciatic nerves into L15 medium on ice and desheathing followed by the teasing of small nerve pieces using fine 27 gauge needles. These were also left for 45 min to allow the nerve fibres to adhere well to the slide. In the case of fresh frozen tissue, teased nerve preparations and coverslips, fixing was 10 min in 4% paraformaldehyde at room temperature followed by 3, 5 min washes in PBS. In the case of pre-embedded fixed cryostat sections the time for fixing was reduced to 5 min only. Tissue was then blocked using PBS containing 10% calf serum, 0.1% lysine and 0.02 % sodium azide, known as antibody diluting solution (ADS), for one hour at room temperature. Antibodies were diluted in antibody diluting solution and applied overnight at 4 $^{\circ}$ C. The following day the slides were washed three times for 5 min each in PBS and incubated in secondary fluorescent antibodies for 25 min at room temperature. Samples were washed again, mounted using Citifluor antifade mountant and sealed with clear nail varnish. In the case of BrdU and P₀ antibodies, paraformaldehyde fixation was replaced by another three step staining protocol given below and double labelling with these two antibodies was performed by incubating both primary antibodies, at the same time, in parallel followed by both secondary antibodies in parallel. All other double labelling involved the sequential application of primary and secondary antibodies separated by a brief fixation step to preserve the first initial antibody staining.

Antibody controls

Controls were routinely carried out to validate antibody specificity. Primary antibody exclusion from a single sample in each experiment was employed to ensure that the second or third antibody layers used did not cause any non-specific background. In the case of the primary Zfp-57 antibody titration experiments were carried out to determine a sensitive and specific concentration at which to use the antibody in further work. Competition assays using the derivative peptides (where available) were carried out to ascertain the specificity of primary antibodies in use on components of the sciatic nerve.

Primary antibodies

Anti Protein zero (P₀) Rabbit polyclonal antibody against rat protein zero was generated and characterized in the laboratory by Louise Morgan essentially as described by Brockes et al., 1980 (Morgan *et al.*, 1994). The antiserum was purified by S. Namin by incubating with chloroform-extracted newborn rat skin for 48 hr, followed by precipitation with caprylic acid, then 40% ammonium sulphate.

Fresh frozen tissue and cells were prefixed in ice cold methanol for 10 min followed by fixation in 2N HCl for 15 min, washed in PBS then further neutralized with 0.1M sodium borate solution for 10 min. The cells were then blocked for 2 hr with antibody diluting solution, washed and incubated in anti-P₀. The antibody was used at a dilution of 1:500 overnight at 4°C and visualized using fluorescein-conjugated anti-rabbit Ig's.

Anti myelin basic protein (MBP) Mouse monoclonal against rat MBP from was from Roche Diagnostics (Germany) and used at a final dilution of 1:100.

Anti Zfp-57 Rabbit polyclonal against rat Zfp-57 fusion protein was made by Dr. G. Zoidl and used at a final dilution of 1:500.

Anti glial fibrillary acidic protein (GFAP) Rabbit polyclonal antibody against cow GFAP protein was from Dako Immunoglobulins (Dakopatts, Copenhagen, Denmark.) and used at a final dilution of 1:200.

Anti calcium binding protein S100 Rabbit polyclonal antibody against cow S100 protein was from Dako Immunoglobulins (Dakopatts, Copenhagen, Denmark.) and used at a final dilution of 1:1000.

Anti RT-97 mouse monoclonal antibody (IgG1) supernatant was a gift from J. N. Wood (Wood and Anderton, 1981) and used at a final dilution of 1:4000.

Anti L1 Rat monoclonal antibody (clone 324) was a gift from Dr. R. Martini (Martini *et al.*, 1994). This antibody was used in the form of hybridoma supernatant and diluted with MEM-H 10% calf serum (CS) 1:1 and placed on prefixed cells. Incubation was for 1 hr at room temperature, followed by Cy3-conjugated anti-rat IgGs for 30 min.

Anti p75NTR Rat polyclonal antibody against mouse p75NTR (MAD357) was from Chemicon (Harrow, UK) and used at a final dilution of 1:1000.

Anti p75NTR Mouse monoclonal antibody against rat p75NTR was a gift from Dr. E. Johnson Jr. (Taniuchi *et al.*, 1986) and used at a final dilution of 1:800.

Anti Krox-20, Egr-2, NGFIIA Rabbit polyclonal against mouse Krox-20 was from

Cambridge Bioscience (UK) and used at a final dilution of 1:600.

Anti Krox-24, Egr-1, NGFIA Rabbit polyclonal antibody against mouse Krox-24 (C-19) was from Santa Cruz (California, USA) and used at a final dilution of 1:600.

Anti-L-periaxin Rabbit polyclonal antibody against L-periaxin was a gift from Prof. P.J. Brophy (University of Edinburgh, Scotland, UK) and used at a final dilution of 1:500.

Anti bromodeoxyuridine (BrdU) Mouse monoclonal (IgG1) supernatant (Gratzner 1982) was a gift from Dr.D. Mason and used at 1:500.

Secondary antibodies

Goat anti-mouse Ig conjugated to tetramethyl rhodamine. Used at 1:200.

Goat anti-rabbit Ig conjugated to fluorescein. Used at 1:600.

The above antibodies were from Cappel (Cappel Organon Teknika Corp, PA, USA)

Donkey anti-rabbit Ig conjugated to biotin.

Sheep anti-mouse Ig conjugated to biotin.

Streptavidin conjugated to fluorescein.

All above were from Amersham (Amersham Pharmacia Biotech, UK) and used at a dilution of 1:100.

Goat anti-rat Ig Cy3. 1:200

Goat anti-rabbit Ig Cy2. 1:100

The above were from Jackson laboratories (Pennsylvania, USA.)

Removal of cross-reacting antibodies in second layers

Sepharose-linked immunoglobulins were packed into two columns of 0.5ml bed volume on nylon wool. The columns were washed in PBS and the relevant conjugated antibody was run through the columns in succession, using a small amount of PBS to elute the last of the unbound antibody. The columns were regenerated with 15ml 5M magnesium chloride followed by 7ml PBS. Sodium azide was added to the purified antibody at a final concentration of 0.02% prior to storage. The titre of each batch of antibody was tested before use and adjusted so that fluorescein-conjugated anti-mouse IgGs and rhodamine-conjugated anti-rabbit IgGs were used at dilutions of 1:100, and rhodamine-conjugated anti-mouse IgGs and fluorescein-conjugated anti-rabbit IgGs were used at dilutions of 1:200.

Nuclear counter staining

A stock solution of 1mg/ml Hoechst dye H33258 in water, was diluted 1:1000 in PBS and applied to fixed cell cultures for 10 min prior to mounting and viewing with Hoechst optics. Alternatively a stock solution of 10uM DAPI in water and diluted at 1:20,000 in PBS and incubated for 5 min on tissue sections.

Primer design

One pair of oligonucleotide primers per specific mRNA (20-35 nucleotides in length) was designed by eye using the sequence information available in EMBL databases (GibcoBRL Life Technologies, Paisley, UK). They were designed in such a way to minimise hairpin structures in individual primers and dimerisation between primer pairs and were checked against databases to ensure specificity. The sequence of the specific primers used in this study are detailed in Table 2.1.

Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from freshly dissected sciatic nerves and branchial plexus or cultured cells of mice of the appropriate ages using Ultraspec RNA total RNA isolation agent. RNA concentration was determined by spectroscopy and analysed for integrity of the RNA by agarose gel electrophoresis under denaturing conditions. Total RNA (500ng) was reversed-transcribed in a 50 μ l reaction mix containing 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM DTT, 0.5mM dNTPs, 20ng random hexamers primers and 200U RNase H- Reverse Transcriptase (superscript II) as recommended in the manufacturers protocol (GibcoBRL Life Technologies, Paisley, UK). After incubation for 90 min at 42⁰C followed by 15 min at 70⁰C, RNA was removed by digestion with both RNase H (2 units) and RNase A (0.1 μ g/ μ l) for 30 min at 37⁰C.

The relative amount of cDNA synthesized from each sample was determined by PCR amplification using specific primers for 18S RNA (Owens and Boyd, 1991; Zoidl *et al.*, 1995). Equal amounts of cDNA, equivalent to 500ng total RNA from the various tissues were used for PCR together with a water control with oligonucleotide primer pairs. PCR was performed in 50 μ l reaction volumes containing 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM MgCl₂, 0.2mM dNTPs, 25pmoles of each primer and 2.5 units Taq DNA-polymerase. The reaction conditions for each primer pair were optimised with respect to MgCl₂ concentration, annealing temperature, extension time and cycle number.

PCR reactions were performed in a Perkin Elmer Gene Amp 2400 thermocycler. Cycling conditions were one initial cycle of 3 min at 94⁰C followed by 26-40 cycles of 30 sec at 94⁰C, 45 sec at specified annealing temperatures and 45 sec at 72⁰C before a final extension period of 10 min at 72⁰C. Upon completion gel loading buffer (10x) was added to each sample and 10 μ l of each reaction was electrophoresed on 2% agarose gels including ethidium bromide in 1x Tris Acetate EDTA (TAE) buffer in a Horizon 58 gel apparatus (BRL-Life Technologies, Gaithersburg, MD). Loading buffer consisted of 0.5M EDTA, pH 7.5, 10% SDS, 50% Glycerol and 0.25% bromophenol blue. Electrophoresed bands were visualised on a UVP dual intensity transilluminator and documented using polaroid photography.

Genotyping of Transgenic mice

1. Maintenance of transgenic mice

All the mice used in this study are derived from a null mutant transgenic line, originally generated by Dr. Piotr Topilko in the laboratory of Dr. Patrick Charnay, Paris. This transgenic line was created using homologous recombination by the insertion of the LacZ gene proximal to the Krox-24 start codon and after the promoter of the gene. The transgenic has been characterised in some detail by the Charnay laboratory (Topilko *et al.*, 1997, 1998). The $-\text{-}$ mice are 25% smaller as adults when compared with their wild type litter mates and homozygotes of both sexes are sterile due to a pituitary defect. Therefore heterozygote matings only are possible with this line (Topilko *et al.* 1997). All the mice used in this study were generated in house from three litters of founder animals. Developing eggs at the morula post-implantation stage of development were dissected from donor females and kept at body temperature while in transit to London. Eight of each were then injected into the uterus of pseudo pregnant female C57/BL6 mice in London. Subsequent progeny were genotyped and crossed out with C57/BL6 females to expand the colony size. It was found that the mice maintained on this background had a small litter number (7 on average) and few if any homozygotes were generated. The colony was therefore transferred onto an CD1 background by crossing out one generation and backcrossing the resulting F1 progeny. This resulted in an increased litter size (>10 on average) and usually at least one homozygote mutant per litter was obtained. No less than 6 outbred lines were maintained at any given time to maintain colony diversity.

During the course of this study 716 transgenic mice have been sacrificed and the genotype determined. The resulting numbers of animals obtained were 121 wildtype (+/+), 471 heterozygous for the mutation (+/-) and 124 homozygous for the mutation (-/-). According to Mendelian genetic principles both the numbers of wildtype and Krox-24 -/- animals are under represented by around 50% when compared to the heterozygotes or the total number of animals analysed. The loss of knockout animals in utero in mouse lines that usually survive beyond birth, is not unusual and in this study occasional embryos being reabsorbed were genotyped and found to be Krox-24-/. It is not understood why the wildtype population is under represented in this study.

2. Genomic DNA Extraction.

Genomic DNA was isolated from tissue samples derived from each transgenic mouse used in this study. From adult and sub-adult (weaner) mice, 0.5cm of tail was collected into separate tubes on ice using sterile scalpel blades. From neo-natal and early post-natal animals, an equivalent weight of tissue was collected; either a hemisphere of brain or a lobe of liver was used in this case. To each tissue sample 500 μ l DNA extraction buffer (0.1M Tris pH8.5, 0.5mM EDTA, 0.2% SDS and 0.2M NaCl) and 1.5 μ l of a 20mg/ml Proteinase K stock was added to give a final concentration of 1.65 μ g/ml. The samples were incubated overnight at 45 $^{\circ}$ C in a waterbath. The next day the samples were inverted a few times and left for one further hour to ensure complete digestion. Samples were centrifuged for 15 min at 13,000rpm at 4 $^{\circ}$ C and the supernatant collected to a fresh

tube. An equal volume of cold isopropanol was added to each tube to precipitate the DNA, which was hooked from the solution and washed in 70% ethanol. After centrifugation for 8 min at 13,000rpm at 4⁰C the DNA pellet was air dried for 15 min and resuspended in Tris EDTA (TE) buffer, pH 8.0. Once dissolved the samples were analysed by PCR and stored at -20⁰C until genotyping could be confirmed.

3. Genotyping by PCR

PCR was performed in 50 μ l reaction volumes containing 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM MgCl₂, 0.2mM dNTPs, 25pmole of each primer and 2.5 units Taq DNA-polymerase. The reaction conditions for all three primers, in this triplicate reaction, were optimised with respect to MgCl₂ concentration and annealing temperature. The sequence of the three Krox-24 genotyping primers is detailed in Table 2.1.

PCR reactions were performed in a Perkin Elmer Gene Amp 2400 thermocycler. Cycling conditions were one initial cycle of 3 min at 94⁰C followed by 30 cycles of 30 sec at 94⁰C, 45 sec at 58⁰C and 45 sec at 72⁰C before a final extension period of 10 min at 72⁰C. At the end of the reaction 1/5 volume of a 5 X loading buffer was added to each sample at which point the samples could be stored at -20⁰C. 10 μ l of each reaction was separated on 2% agarose gels including ethidium bromide in 1x TAE buffer in a Horizon 58 gel apparatus. Electrophoresed bands were visualised on a UVP dual intensity transilluminator and documented using polaroid photography.

β-Galactosidase histochemical staining

Embryos and nerves were dissected out into L15 medium on ice, washed three times in PBS containing 0.02% NP40 (now superseded by IGEPAL CA-630, Sigma Chemical Co.) and fixed overnight with 1% paraformaldehyde, 0.2% glutaraldehyde, 2mM MgCl₂, 5mM EGTA and 0.02% NP40 at 4⁰C. The following day the tissue was washed three times for 30 min with Phosphate buffered Saline (PBS) containing 0.02% NP40 and placed in staining solution containing 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆.H₂O, 2mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP40 and 1mg/ml 5-Bromo-3-Indolyl-B-D-Galctopyranoside (Bluogal) or 5-Bromo-4-Chloro-3-Indolyl-B-D-Galctopyranoside (Xgal) (Sigma Chemical Co.). The staining reaction was carried out overnight, in the dark, at 30⁰C. The tissue was washed three times for 30 min with PBS containing 0.02% NP40 and then cryoprotected in PBS containing 15% sucrose overnight at 4⁰C. Whole mount photographs were taken on a Leica dissecting microscope using Kodak Ektachrome 64 Tungsten slide film. If sectioning was required the tissue was embedded in OCT compound in a liquid nitrogen cooled bath of isopentane and stored at -70⁰C.

Electron Microscopy

Krox-24 mutant mice of various ages were anaesthetised by intraperitoneal injection of hypnorm, hypnovel and distilled water (1:1:2) appropriate for the age of the animal. The mice were then intracardially perfused with freshly made 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M sodium cacodylate pH 7.4 using a perfusion pump (Watson Marlow, USA). Following perfusion of between 5 to 10 min the sciatic nerves were dissected out, with care to avoid any mechanical stress, and placed on a slow rotator in the same fixative at 4⁰C. The next day the tissue was washed three times for 15 min in

0.1M sodium cacodylate buffer, pH 7.4 and then stained with 1% osmium tetroxide for 45 minutes. The tissue was washed three more times and then dehydrated as follows; 20% acetone, 40% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 96% ethanol, 100% ethanol and 100% acetone (Greenfield *et al.* 1990). Each step involved a 5 min incubation and was carried out twice to 90% ethanol and three times thereafter. Tissue samples were then embedded in araldite in two stages; overnight incubation in 50% araldite and 50% acetone mixture followed the next day by an 8 hour infiltration in araldite alone, all at room temperature. Araldite consisted of equal parts araldite CY212 and DDSA with accelerator, BDMA and plasticizer, dibutyl phthalate. The tissue samples were then placed into a rubber coffin mould and thermo-cured at 65°C for 48 hours. Semi-thin sections, 0.5µm and ultra-thin sections, gold, silver/ gold or silver were taken using an Ultracut E ultramicrotome (Leica, Germany). Semi-thin sections were taken using fresh glass knives and collected on microscope slides and ultra-thin sections on copper grids (Agar Scientific, UK). Semi-thin sections were stained with 0.1% toluidine blue in ethanol, rinsed with distilled water, dried and mounted in DPX mountant. These sections were then analysed by light microscopy and photographs were taken using 35mm, Ilford Pan F (50 asa) high contrast black and white film. Thicker ultra-thin sections (Gold), for lower magnification work, were collected on plastic coated copper slot grids (Agar Scientific, UK) and thinner sections (silver), for higher power work, were collected on copper New 200 grids (Agar Scientific, UK). Grids were stained for 15 min in lead citrate solution, washed in distilled water and dried. Grids were viewed in a Jeol 1010 electron microscope (Jeol, Japan) and images captured on X-ray Film (Ilford, UK). Films were printed using a Devere enlarger on Ilford multigrade paper using Ilford multigrade gel filters (Ilford, UK). Low magnification montages were

captured by overlapping each individual image by 30%, the resulting films were printed, trimmed with a guillotine and joined with Magic Tape (3M, UK).

Peripheral Nerve Injury Experiments

Sciatic nerve transection

Adult normal and transgenic mice, between 2 and 4 months of age, were anaesthetised using halothane and the left sciatic nerve exposed at mid-thigh level. The nerve was tied with two sutures of 8/0 black polyamide monofilament, ethilon (Becton Dickinson, USA), the first, 2-3mm below the sciatic notch, and the second 2-3mm distal to the first. A segment 1-2mm in size was then excised from between the two sutures and the distal end of the sciatic nerve diverted and sutured into muscle to limit the possibility of re-ligation. Resulting wounds were closed with surgical clips (Becton Dickinson, USA). At a number of different time points following transection, the animals were culled and both the distal stump of the left, transected sciatic nerve and the contralateral control nerve were excised.

Sciatic nerve crush

In a similar operation, the left sciatic nerve was exposed at mid-thigh level and was then crushed 2-3mm below the sciatic notch, 3 times at the same position, using No.3 forceps that were sharpened to a fine point. Care was taken to prevent damage to the sheath surrounding the sciatic nerve and the same forceps were employed in all operations in this study. A single suture of 8/0 black polyamide monofilament, ethilon was then

applied to a fine branch of the sciatic nerve, at the same level as the crush site, to allow future identification of the wound. Injured and contralateral control sciatic nerves were excised at a number of different time points, from 5 days to 2 months following transection.

Embedding

The mice were then killed by cervical dislocation and sciatic nerves were dissected out and immediately embedded in OCT compound (fresh frozen) in a liquid nitrogen cooled bath of isopentane before storage at -70°C.

Western Blotting

1. Sample preparation, gel separation and blotting

Tissue was extracted as soon as possible following dissection in protein extraction buffer with phenylmethylsulphonylfluoride (PMSF) and proteinase inhibitors using a Dounce homogeniser. Protein extraction buffer: 5mM Tris pH 6.8, 2mM EDTA, 2mM EGTA and 2% SDS including 1:100 PMSF from 0.1M PMSF stock and 1:100 protein inhibitor cocktail. Protein inhibitor cocktail: antipain (0.5µg/ml), pepstatin (0.5µg/ml), amastatin (0.5µg/ml), apoprotein (3U/ml), leupeptin (0.5µg/ml), bestatin (0.5µg/ml) and trypsin inhibitor (0.5µg/ml) all from Roche diagnostics (Germany).

Normally four complete adult mouse sciatic nerves (desheathed) were extracted in 500µl of buffer. Samples were separated using SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) under denaturing conditions, with a mini Protean II gel electrophoresis apparatus (Biorad, CA USA). One of three different molecular weight standards were included to enable band size identification, either Kaleidoscope pre-stained standards, Seebblue pre-stained standards or Multimark multi-colored standard. Separated proteins were then transferred to a nitrocellulose membrane, Hybond-N, in a mini gel transfer tank (Biorad, CA USA).

2. Blot staining

Non-specific binding sites on the membrane were first blocked for at least 2 hr using 5% fat free milk powder in PBS at 4⁰C. Primary antibodies were incubated overnight at 4⁰C in 1% fat free milk powder in PBS, at concentrations demonstrated to give the best signal to noise ratios, on a slow rotator (Gallenkamp, UK). The following day the blots were washed in PBS containing 0.05% Tween 20, usually 5 x 15 min at room temperature. Primary antibody staining was further revealed using either a two or three layer system. The former involved incubation with a secondary antibody, directed against the primary immunoglobulin, conjugated to the enzyme horseradish peroxidase (HRP), 1:2000 in PBS + 0.05% Tween 20, for 50 min at room temperature. The three layer system employed a secondary antibody conjugated to biotin, used at a concentration of 1:2000 in PBS + 0.05% Tween 20, incubated for 50 min at room temperature. After washing in PBS containing 0.05% Tween 20, usually 5x 10 min at room temperature, blots were incubated with Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA) for 30 min as recommended in the manufacturers protocol.

Using either two or three layer systems blots were finally washed in PBS containing 0.05% Tween 20, usually 5x 10 min at room temperature, before being developed for 5 min using the chemi-luminescent substrate ECL Plus (Amersham Pharmacia Biotech, UK) as recommended in the manufacturers protocol. The blots were covered in cling film and placed in an autoradiography cassette with intensifying screens (Appligene, USA) and visualised by brief exposure to Kodak BioMax Mr-1 film which was developed from 45secs to 10 min later in an X-Ograph Compact X2 automatic developer.

Cell Proliferation (BrdU) and Cell Death assays

Cell Proliferation assay

To quantify Schwann cell proliferation, the relative proportion of Schwann cells undergoing DNA synthesis, during the S phase of the cell cycle, was determined by injections of bromodeoxyuridine (BrdU). Transgenic mice of various ages were injected intraperitoneally with 100 μ g BrdU per g of body weight in saline together with 0.007M NaOH and allowed to rest without disturbance for one and a half hours. The mice were then killed by means appropriate for the age of the animal and either whole embryos (early embryonic ages) or sciatic nerves (all other ages) were dissected out and fixed in 2% paraformaldehyde in PBS for 1-8 hr at 4 $^{\circ}$ C. The tissue was then cryoprotected in 15% sucrose in PBS overnight at 4 $^{\circ}$ C, before being embedded in OCT compound in a liquid nitrogen cooled bath of isopentane before storage at -70 $^{\circ}$ C.

Cryostat sections of both whole embryos and nerves of various ages, were cut at between 6 and 8 μ m and mounted on Superfrost Plus microscope slides. Tissue was allowed to dry for 45 min and then fixed by submersion in methanol for 10 min at room temperature. This was followed by a 20 min incubation in 2M HCl to denature the DNA, followed by 10 min with 0.1M sodium borate (pH8.5). The sections were washed, usually 3 times in PBS for 5 min and then incubated in anti-BrdU antibody overnight at 4°C. The sections were again washed 3 times in PBS for 5 min and the staining revealed by incubation with goat-anti-mouse Ig rhodamine (25 min at room temperature). Finally the sections were washed 3 times in PBS for 5 min and incubated with 1mg/ml DAPI for 5 min, to label all nuclei, before being mounted in Citifluor mounting medium. In double labelling experiments using GFAP antibodies sections were incubated sequentially with first BrdU antibodies (overnight at 4°C), followed by goat anti-mouse Ig rhodamine (25 min at room temperature) and then 5 min fixation with 4% paraformaldehyde. The sections were then exposed to GFAP antibodies (30 min at room temperature) followed by goat anti-rabbit Ig FITC (25 min at room temperature). Nuclei were then labelled with DAPI as detailed above and the sections mounted in Citifluor mounting medium

Cell Death Assay

Cells in the process of apoptotic cell death were analysed, using sections to estimate *in vivo* cell death and with cultured cells to measure *in vitro* cell death, by *in situ* labeling of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). Cryostat sections of fresh-frozen nerves, of various

ages, were cut at between 6 and 8 μ m and mounted on Superfrost Plus microscope slides. Tissue was allowed to dry for 45 min and then fixed by submersion in 4% paraformaldehyde for 10 min at room temperature. The sections were washed, usually 3 x 5 min in PBS and then pre-incubated in terminal transferase (TdT) buffer (30mM Tris buffer pH 7.2, 140mM sodium cacodylate and 1mM cobalt chloride) for 15 min at room temperature. Terminal transferase and biotinylated-d-UTP were added to the sections in a TUNEL reaction mixture, as recommended in the manufacturers' protocol and incubated at 37 $^{\circ}$ C for 60 min. The reaction was terminated by washing 2 x 5 min in ultrapure H₂O and once in PBS. Non-specific binding sites were blocked using PBS with 10% FCS for at least 60 min at room temperature. The sections were then incubated with streptavidin conjugated to FITC, at a concentration of 1:100 in ADS with 0.1% Triton X-100 for 25 min at room temperature. Nuclei were then labelled with DAPI as detailed above and the sections mounted in Citifluor mounting medium.

Microscopy and quantification

Immunolabelled slides and coverslips were viewed using either a Zeiss Axioskop fluorescence light microscope with epi-illumination and phase optics (Carl-Zeiss, Germany) or a TCS Laser Scanning Confocal microscope (Leica, Germany). TUNEL-positive and BrdU stained nuclei were counted as a percentage of all nuclei within a given field, using an ocular grid with a 40x objective lens or by importing images into NIH image, using Photoshop 5.0 on an Apple Macintosh G3 computer. At least 5 fields per section for three sections separated by 64 μ m were counted and each experiment

repeated at least three times. Confocal images were directly imported into Photoshop and light microscope photographs were taken using 35mm film, either Ilford HP5 (400 asa) black and white film or Kodak EPY 64 T (64 asa) colour reversal film.

Cell Culture Experiments

Isolation and Culture of Schwann Cells

Schwann cells were prepared using the method of Brockes *et al.*, (1979). Newborn Sprague-Dawley rats were killed by decapitation and the sciatic nerves dissected out using a Leica MZ6 dissection microscope. Nerves were placed in medium buffered for air, usually L15, and kept on ice. The epineurial sheath was removed and the cells dissociated by digestion in 0.25% trypsin, 0.4% collagenase in DMEM at 37°C and 5%CO₂ and 95% air for 50 min with single trituration at the half way stage. The cells were then washed in DMEM with 10% FCS and centrifuged for 10 min at 500xg at 4°C to pellet, resuspended in the same medium and cultured in polylysine and laminin coated 25cm³ flasks. For rat Schwann cell cultures used for transient transfection assays, impure Schwann cells were cultured in DMEM containing 10% FCS and 10⁻⁵ M AraC to remove dividing fibroblasts from the culture (Brockes *et al* 1979). Cells were expanded in DMEM containing 10% FCS and 4μM forskolin. Transgenic mouse cell cultures were not subject to such purification as mouse Schwann cells do not quiesce, instead purity was analysed postexperiment by immunocytochemistry using a range of specific Schwann cell antibodies.

Three types of culture media were used in this study: DMEM; supplemented serum free defined medium referred to as defined medium (DM) (Jessen *et al* 1994); and non-supplemented serum free defined medium known as simple defined medium (s-DM) (Meier *et al* 1999). Each medium was used with additions (eg. FCS or forskolin) as stated.

Defined Medium (DM) contained 1:1 Hams F12/ DMEM supplemented with 100 μ g/ml glutamine, 0.03% bovine serum albumin (BSA), 100 μ g/ml transferrin, 16 μ g/ml putrescine, 38ng/ml dexamethasone, 60ng/ml progesterone, 400ng/ml thyroxine (T4), 5ng/ml insulin (low insulin) or 5 μ g/ml (high insulin), 10.1ng/ml triiodothyronine (T3), 160ng/ml selenium and 100U/ml each of penicillin/ streptomycin.

Simple Defined Medium (s-DM) contained 1:1 Hams F12/ DMEM supplemented with 0.03% bovine serum albumin (BSA) and 100U/ml each of penicillin/ streptomycin.

Growth medium (GM) contained DMEM + 10%FCS including 4 μ M forskolin, 3 μ g/ml basic fibroblast growth factor (bFGF) and 100U/ml each of penicillin/ streptomycin.

Schwann Cell survival assay

The sciatic nerve and branchial plexus were dissected and dissociated from a range of early postnatal ages of entire Krox-24 mutant mouse litters, as described above. Following centrifugation the cells were resuspended in s-DM (Meier *et al.* 1999) and counted with a haemocytometer and plated at 300 cells per 30 μ l drop onto poly-L-ornithine coated 13mm round coverslips in triplicate. Following incubation for three hours at 37°C and 5%CO₂ and 95% air, one set of coverslips, representing a sample of

cells from each animal of the litter were fixed for immunocytochemistry. The remaining two sets of cells were topped up with s-DM and culture for 24 hr in total. These cells were then also fixed, typically the cells were washed twice with PBS and then placed into 4% paraformaldehyde for 10 min after which they were washed in PBS three times for 5 min each and stored at 4°C in PBS prior to analysis.

Quantification of Schwann cell survival

The number of living Schwann cells in the above experiments is expressed as survival percent and was carried out using a method previously described (Meier *et al.*, 1999, Parkinson *et al.*, In press). Survival percent is the number of living cells present at 24 hr on the coverslip as a percentage of the number of cells that had attached to the substrate in sister cultures at 3 hr. Dead cells were identified by observations of nuclear staining (Hoechst) and morphology, typically nuclear condensation was visualized as intensely staining small nuclei. Occasionally pyknotic nuclei, a feature of apoptotic cell death, were visible as multiple punctate Hoechst positive bodies. Associated with these nuclear changes, processes were retracted and the cytoplasm appeared granulated and vacuolated. Also both surviving and dying Schwann cells populations were positively identified using S-100 immunocytochemistry.

To further validate that these cells were dead or dying TUNEL method was used.

Statistical analysis

Unless otherwise stated, the statistical significance of data was evaluated using the paired students t-test.

Zfp-57_EGFP Construct

The full length cDNA of Zfp-57 was cloned, in frame, into pEGFP-N3, a mammalian expression vector integrating EGFP to form a fusion protein and was prepared by Dr.G. Zoidl. The full length coding sequence was amplified by PCR using Pfu-DNA polymerase including synthetic SalI and XholI restriction sites. A schematic diagram shows how the Zfp-57 cDNA was inserted into the plasmid used in this study to form the carboxyterminus EGFP fusion protein (Fig 2.2). The construct was sequenced to check for mutations and transient transfections carried out into Hela cells to check EGFP expression.

Schwann cell transfection with Zfp-57_EGFP using Fugene 6

Schwann cells plated onto coverslips DM +0.5% FCS after Ara C purification were placed in an incubator for 2 hours (at 37°C and 5% CO₂) - allowing them to adhere to the substrate. They were then topped up carefully with 500 µl of the same medium used to plate the cells and incubated overnight. The following day, the medium was changed to 400 µl of DM +0.5% FCS (+/-dbcAMP [+/- NDF-β, 20ng/ml]). Coverslips were transfected with the appropriate plasmid(s) as follows: for each coverslip one tube (A) with 97µl of DMEM was supplemented with 3µl of Fugene 6 transfection reagent. They

were left for 15 min while the appropriate plasmid(s) for each coverslip were pipetted into a separate tube (B). Tube A was then added to B and incubated for a further 15 min at room temperature. The resulting ~100 μ l was added to the corresponding 400 μ l of medium on the coverslips, which were incubated as above. The coverslips were incubated for 48 hr, after which they were fixed and stained.

Substrate preparation

Coverslips

Round 13 mm coverslips (Merck) were baked at 140°C for 4 hr under dessicated conditions and coated with 1mg/ml poly-L-lysine in dH₂O overnight at room temperature. Coverslips were then washed in distilled water 3 times overnight at room temperature on a shaker, before being air dried in a flow cabinet and stored dessicated. For cell survival assay coverslips were coated with 1.5ug/ml poly-L-ornithine in dH₂O for 15 min at room temperature followed by 1 hr at 37°C. These were then washed three times in dH₂O and dried as above.

Dishes and flasks

Plastic tissue-culture dishes and flasks were coated with PLL by incubating them with 20 μ g/ml PLL for 2 hours at room temperature. These were then drained and dried in preparation for use. Prior to adding cells, a dish or flask would be coated with laminin (10 μ g/ml in DMEM) for 1 hour.

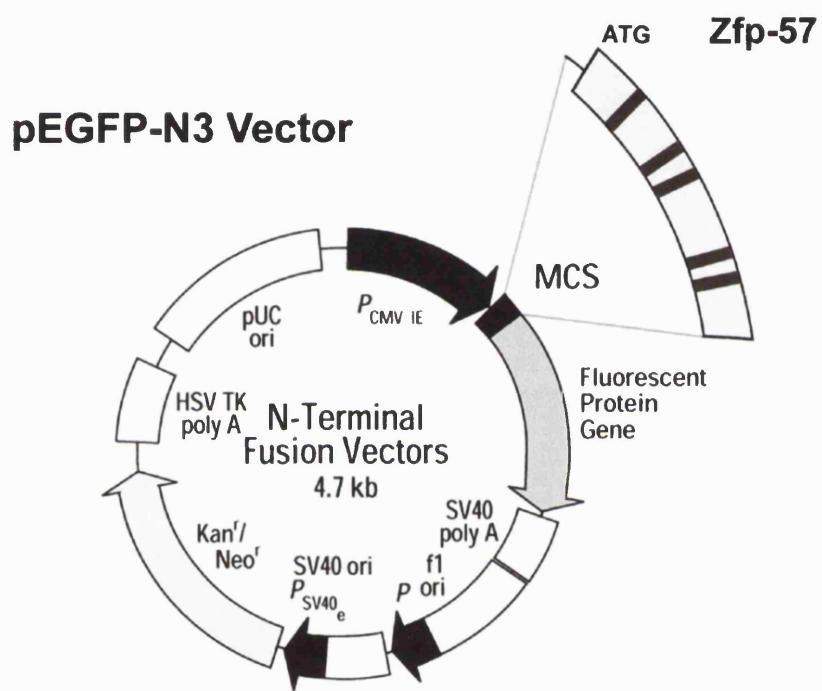
Table 2.1 Sequence of RT-PCR oligonucleotide primers

Shown are the primer sequences for the ten target molecules used in this study. The sense (forward) primer, upper line, and anti-sense (reverse) primer, lower line, is shown for each pair. The specific primer pair for Krox-24 is shown on one line and the specific LacZ primer, used in genotyping, is shown in brackets below. The third column details the temperature used during the annealing phase of the PCR reaction, and the 5th column shows the total number of cycles used in the reaction. The exact size of the amplified fragment (in base pairs) for each primer pair is shown in the 4th column.

Target molecule	Primer sequences	Annealing Temp.	Expected size (bp)	PCR cycles
Zfp-57	5' GGA C T G A G C C G T C A C C G A C G T G C 3' 5' C A G A A G T G C T G G T T G G G T T G A C G 3'	52°C	427	36
Krox-24/ Egr-1 (+ LacZ)	5' G A G T G T G C C C T C A G T A G C T T 3' 5' G G T G C T C A T A G G T T G T T C G C T 3' (5' G T T G C A C C A C A G A T G A A A C G 3')	58°C	412 (500)	34 (30)
Krox-20	5' A C A T G A C T G G A G A G A A G A G A C C C T 3' 5' A C C T C C T A T C A C A A C C T T C T G C T G G 3'	60°C	854	34
Egr-3	5' A C A G A G A A T G T G A T G G A C A T C G G T C 3' 5' G C A C T C A T G A G G C T A A T G A T G T T G 3'	58°C	172	34
Oct-6/ SCIP/ Tst-1	5' A T T C C C C G G G A G A A T G G A C G A A G A G A G G A G A G T C C 3' 5' C T G C G A G C G C C G C G G A G A G G C C C G C T A A A G T G A C 3'	56°C	534	35
P0	5' T T G G T G C T T C G G C T G T G G T C 3' 5' T G T T G C T G C T G T T G C T C T T C 3'	52°C	181	27
p75NTR	5' C C G A T A C A G T G A C C A C T G T G A T G 3' 5' A G C A G C C A A G A T G G A G C A A T A G A C 3'	56°C	103	30
NCAM	5' G G T G A A C A T C A C C T G T G A G G T C 3' 5' C T T G T G C T G T G C T G G A G T A T G G 3'	56°C	280	36
Dhh	5' C A T G T G G C C C G G A G T A C G C C 3' 5' C G C T G C A T C A G C G G C C A G T A 3'	65°C	346	33
18SrRNA	5' C C T C G A A A G A G T C C T G T A 3' 5' G G G A A C G C G T G C A T T T A T 3'	52°C	341	22

Figure 2.2 A schematic diagram of the Zfp-57_EGFP construct

The 1.3 kbp cDNA encoding Zfp-57 is shown together with the pEGFP vector. Zfp-57 was inserted, in frame, in the orientation shown into the multiple cloning site (MCS) of pEGFP, resulting in a fusion protein of EGFP fused to the carboxyterminus of Zfp-57. The fusion protein was driven by the powerful CMV promoter as shown. The five zinc-finger motifs are shown as dark bands within the Zfp-57 cDNA.



CHAPTER 3

Zfp-57 in the Schwann cell lineage

INTRODUCTION

Zinc Finger Transcription Factors

The discovery of the prototypical zinc-finger transcription factor TFIIIA (the *Xenopus laevis* RNA polymerase III transcription factor) was significant as it represented the first example of a motif capable of binding DNA other than the known helix-turn-helix DNA binding motif (Miller *et al.*, 1985). It has since been demonstrated that zinc finger transcription factors are the largest family of transcription factors and are a common form of DNA binding protein (Berg, 1986). The large variations in the structure of motifs found in different species have lead some to speculate that these transcription factors are evolutionarily the oldest form of binding protein (Evans and Hollenberg, 1988).

The first insight into the functional significance of zinc finger transcription factors has come from work on the fruit fly, *Drosophila melanogaster*. The molecular cues that define the temporal and spatial organisation within the egg, forming the genetic basis for subsequent body patterning have been characterized (reviewed by Rivera-Pomar and Jackle, 1996). These cues precede any morphogenetic phenotype and involve four different gene families in sequential pattern, each being dependent on the preceding one. The first, the product of the maternal coordinate gene bicoid, forms a concentration gradient along the length of the egg; this is followed by the expression of the second family, the gap gene family, members of which are expressed in large discrete blocks spanning the length of the egg. These genes are followed by the expression of two

families of genes that are expressed in smaller, discrete stripes; namely the pair rule and segment polarity genes (Pankratz and Jäckle, 1990).

Pertinent to this discussion, the gap gene family is the earliest to be detected in *Drosophila* and has four members; *hunchback*, *krüppel*, *knirps* and *giant*. All of these genes, identified by analysis of mutations, encode proteins containing DNA binding motifs of the zinc finger type. It is suggested that these genes act by regulating the expression of other genes further downstream such as the pair rule genes. Mutations in any of these genes result in abnormal body patterning (Rivera-Pomar and Jackle, 1996).

The existence of vertebrate zinc finger transcription factors has since been demonstrated (Chowdhury *et al.*, 1987; Bellefroid *et al.*, 1989), where they have roles in the regulation of cell type specific gene expression (El-Baradi *et al.*, 1991), cell proliferation (Chavrier *et al.*, 1988) and differentiation (Chowdhury *et al.*, 1987; Okazaki *et al.*, 1993; Bellefroid *et al.*, 1996). To date, only two zinc-finger transcription factors have been described in the mammalian peripheral nervous system: Krox-20 and Krox-24 (Topilko *et al.*, 1994, Topilko *et al.*, 1997). Also a third member of this transcription family, Egr-3, has been shown to be present at the mRNA level in Schwann cells (D. Parkinson, personal communication). Krox-20 has a well defined role in the development of myelinating Schwann cells (Topilko *et al.*, 1994) and the onset of expression occurs in the transitory period where Schwann cell precursors become immature Schwann cells (Blanchard *et al.*, 1996, Topilko *et al.*, 1994). In Krox-20 null mutant mice myelinating Schwann cells pass the promyelinating phase, where they form 1:1 relationships with axons, and continue to wrap the axon about 1-2 turns before becoming stalled at this point (Topilko *et al.*, 1994). This data together with the finding that late markers of myelination, such as P₀ and MBP, are largely absent suggest that

Krox-20 is required for and controls transcription of myelination genes (Topilko *et al.*, 1994). In vitro Krox-20 can be induced in Schwann cells by differentiation conditions using molecules that elevate cytoplasmic cAMP. This expression is enhanced in the presence of Neuregulin (NDF β) (Murphy *et al.*, 1996, Parkinson *et al.*, unpublished data). The role that Krox-24 plays in peripheral glia is less clear. Krox-24 was first identified in the PNS following injury to the sciatic nerve, where the gene, at the mRNA level, was found to be rapidly upregulated (Herdegen *et al.*, 1991). Krox-24 expression has since been described in the development of Schwann cells, where it is suggested to have an antagonistic role with Krox-20 (Topilko *et al.*, 1997).

The Structure of the Zinc Finger Motif DNA binding Domain

The large zinc finger group of motifs incorporate one or more zinc molecules into their structure. Proteins in this family usually contain tandem repeats of the zinc finger motif, e.g. the *Drosophila krüppel* gene contains 4 C2H2 motifs (Rosenberg *et al.*, 1986). This well conserved motif occurs in the yeast proteins SW15 and ADR1 and in mammalian regulatory proteins such as the testis determining factor ZFY, the enhancer Sp1 and the Wilms tumour suppressor, WT1. A single zinc finger motif has been demonstrated to be ineffective in binding to specific DNA sequences in isolation (Parraga *et al.*, 1988) A single motif consists of about thirty amino acids and has the conserved formula-



The structure of this motif has been investigated using the extended X-ray absorption fine structure (EXAFS) technique on the prototypical TFIIIA zinc finger transcription factor (Diakun *et al.*, 1986). This demonstrated the coordination sphere of the zinc atom sites to consist of two cysteine and two histidine residues. This

knowledge, in conjunction with known metalloprotein structures, has allowed the structure of zinc finger motifs to be predicted (Berg, 1988) and subsequently to be confirmed by 2 dimensional nuclear magnetic resonance (2D NMR) (Parraga *et al.*, 1988 and Lee *et al.*, 1989) and also by analysis of the crystal structure (Pavletich and Pabo, 1991).

Isolating Zinc Finger Transcription Factors

As indicated above any given zinc finger protein usually has at least two, tandemly repeated, zinc finger motifs. The amplification and isolation of novel transcription factors with tandemly repeated zinc finger motifs has been demonstrated by using PCR (Pellegrino *et al.*, 1991). This technique employs degenerate oligonucleotide primers, designed against different regions within the zinc finger motif.

Dr. G. Zoidl, in this laboratory, has applied a similar strategy to screen for regulated zinc finger transcription factors in Schwann cells and has been described (Zoidl *et al.*, 1997). Degenerate primers were designed according to the structure of the zinc finger motif of the *Drosophila* gene transcript, *kruppel*. The sense primer is based on the Cys-Xaa2-Cys region (tgcccnngagtgyggnaar) and the antisense on the H/C link region (nggcttctcnccngtrtg) of the zinc finger motif. PCR using these primers was performed on cDNA synthesised from total RNA from E 12 and E 13 sciatic nerves. The amplified fragments were then eluted from display gels and directly subcloned. Amplified cDNA was fixed on nylon membranes and differential hybridisation performed using radiolabelled total cDNA synthesised from total E 12 and E 13 RNA. Positive clones, representing fragments of novel zinc finger transcription factors, were then used to search Genbank and EMBL databases using BLASTn for nucleotide sequences and BLASTx for amino acid sequences, which lead to the identification and isolation of Zfp-

57. Zfp-57 is a previously described gene thought to be down-regulated during early mammalian differentiation (Okazaki *et al.*, 1994). The authors employed a promoter trap technique to isolate Zfp-57 from undifferentiated cells of a teratocarcinoma stem cell line (F9 cells). Two Zfp-57 transcripts were identified, 1.8 and 3.2 kb in size, but only the smaller of the two could be identified in mouse testis and the authors show by immunocytochemistry that Zfp-57 protein is localized in nuclei, consistent with transcription factor function.

AIMS

Zfp-57 has been cloned in this laboratory in a screen of zinc-finger transcription factors regulated during Schwann cell development. Experiments in this chapter were designed to explore the temporal expression pattern of Zfp-57 at the mRNA and protein level during development and investigate the localisation in the two adult Schwann cell populations. Preliminary luciferase reporter assays suggest that Zfp-57 may repress myelin gene promoters *in vitro* and we hypothesise that Zfp-57 may be involved in the repression of myelination. Additional experiments are shown to test this hypothesis.

RESULTS

Zfp-57 mRNA can be detected in Schwann cells throughout development

A developmental time series of mouse sciatic nerve was prepared and probed by RT-PCR using specific oligonucleotide primers for Zfp-57 (Fig 3.1). Zfp-57 mRNA could be detected at all the time points assayed and is therefore present throughout development (Fig 3.2). This experiment was repeated at least three times and carried out on two different sets of cDNA samples. The levels of Zfp-57 mRNA show a distinct pattern of regulation; being detectable at the Schwann cell precursor stage at E12, expressed maximally at the immature Schwann cell stage at E17 and then decaying steadily through postnatal development into the low levels of mature Schwann cells found in the adult. This pattern of expression differs markedly from that of Krox-20mRNA shown for comparison (Fig 3.2). Krox-20 has been shown to be required for the process of myelination and Krox-20 mRNA expression is detectable at E17, in immature Schwann cells, in advance of when myelination is most vigorous in the early postnatal animal (Topilko *et al.*, 1994). Krox-20 mRNA expression continues in the postnatal nerve and is maintained in the adult population of myelinating Schwann cells.

Zfp-57 protein is expressed in mouse Schwann cells *in vivo*.

In order to investigate the protein expression pattern of Zfp-57 in Schwann cells during development and in maturity, teased nerve samples were prepared and probed by immunohistochemistry, using a polyclonal antibody derived from a fusion protein (produced in the laboratory by Dr. G. Zoidl).

Zfp-57 protein could be detected at all time points assayed; in embryonic immature Schwann cells at E16 (Fig 3.3 A, B), in new born Schwann cells, at the onset of myelination (C, D), Zfp-57 protein expression continues in maturing Schwann cells at P7 (E, F) and is maintained into the adult state (G, H). Zfp-57 protein is localised to both subpopulations of Schwann cells present in adult sciatic nerve: myelin forming and non-myelin forming Schwann cells. Schwann cells that are MBP positive and therefore myelin forming clearly express Zfp-57 protein (Fig 3.3 I & J arrows) which is also seen in presumptive non-myelin forming Schwann cells, negative for MBP (arrowheads).

Zfp-57 protein is absent from the adult sympathetic trunk

The sciatic nerve is an example of a mixed nerve being composed of axons that are myelinated by myelin forming Schwann cells and fibres that are not myelin insulated but are supported by non-myelin forming Schwann cells. The sympathetic trunk in contrast is a nerve fibre bundle consisting almost entirely of axons that are not myelinated and are supported by non-myelin forming Schwann cells. Double immunohistochemistry was used to investigate the localisation of Zfp-57 protein in cryo-sections of the sympathetic trunk. An antibody recognising the cell adhesion molecule L1, which is localised to the cytoplasm was used to specifically label all Schwann cells in the trunk (Fig 3.4 B & D). Zfp-57 antibodies did not stain any of the Schwann cell nuclei within the sympathetic trunk (Fig 3.4 A & D) suggesting that Zfp-57 expression is restricted to glial cell populations within the sciatic nerve and brachial plexus. Alternatively Zfp-57 may be expressed exclusively within nerves having mixed fibre composition.

Zfp-57 protein is expressed in mouse Schwann cells *in vitro*.

In order to investigate the protein expression pattern of Zfp-57 in Schwann cells *in vitro* rat Schwann cells were isolated from the sciatic nerves and brachial plexuses of P3 rats and cultured overnight in DMEM supplemented with 10% FCS on PLL Laminin coverslips.

Double immunocytochemistry using antibodies recognising a Zfp-57 fusion protein and p75 low affinity neurotrophin receptor that specifically labels Schwann cells in culture was carried out. All Schwann cells cultured in this fashion express p75 and the cytoplasmic staining is shown in Fig 3.5 B. Zfp-57 protein is localised to the nuclei of all p75 positive cells and therefore all of the Schwann cells in this culture.

Zfp-57 overexpression does not induce differentiation of Schwann cells *in vitro*.

In order to investigate the possible function of Zfp-57 in Schwann cells purified primary Schwann cell cultures were prepared and transfected with an EGFP plasmid containing the full length Zfp-57 protein. This fusion protein construct was prepared by Dr. G. Zoidl in the laboratory.

When in a relatively poor medium, defined medium supplemented with 0.5% serum, Schwann cells quiesce, and no longer express markers of differentiated Schwann cells such as the transcription factor Krox-20 or the myelin gene P₀. Instead the cells adopt an immature phenotype up-regulating markers such as p75. Schwann cells maintained under these conditions for three days are shown in Fig 3.6 A-G. Periaxin antibody was used to label cells that are differentiating and at no time was periaxin staining seen in Schwann cells under these conditions (Fig 3.6 A). When parallel cultures were

transfected with EGFP vector expressing Zfp-57 and subsequently stained for periaxin there were no cells that stained positively for periaxin (Fig 3.6B).

These experiments were quantified by comparing Schwann cells transfected with EGFP expressing Zfp-57 and those transfected with an empty vector control, with 0% periaxin positive cells in each case (Fig 3.9). The transfection efficiency averaged 18% for Schwann cells transfected in these conditions.

It has been shown by this laboratory and others that elevators of Schwann cell intracellular cAMP can mimic Schwann cell differentiation *in vitro* (Sobue and Pleasure, 1984, Sobue, *et al.*, 1986, Morgan *et al.*, 1991). The addition of dibutyl cAMP (dbcAMP) to Schwann cells cultured as above results in striking differentiation along the myelination pathway, with cells up-regulating markers such as Gal-C, P₀ and periaxin. (Sobue, *et al.*, 1986, Scherer *et al.*, 1995). To investigate whether over-expression of Zfp-57 could modulate the process of differentiation, along the myelination pathway, under these conditions, cells were transfected with EGFP vector expressing Zfp-57 and subsequently stained for periaxin. Figure 3.8 B shows that differentiating Schwann cells transfected with Zfp-57 express periaxin to a similar degree compared to controls (Fig 3.7 A). Within this group of cells some are clearly positive for GFP in both periaxin positive and periaxin negative cells (Fig 3.8 D).

These experiments were quantified by comparing Schwann cells transfected with EGFP expressing Zfp-57 and those transfected with an empty vector control, with 41% and 39% periaxin positive cells respectively (Fig 3.9). Counts were also made of the number of periaxin positive cells also positive for GFP (Table 3.1). Those Schwann cells transfected with Zfp-57 showed 13% +/- 2.1 periaxin-GFP positive cells compared to

14% +/- 1.6 for empty vector control. The transfection efficiency averaged 30% for Schwann cells transfected in these conditions.

NRG is a known Schwann cell mitogen (Raff *et al.*, 1978, Marchionni *et al.*, 1993) and promotes Schwann cell precursor survival and differentiation (Dong *et al.*, 1995). Subsequent studies of null mutants for NRG1, and the receptors ErbB2, ErbB3 and ErbB4 show that these receptor ligand interactions are required for Schwann cell development (Gassmann *et al.*, 1995, Lee *et al.*, 1995, Meyer and Birchmeier 1995, Riethmacher *et al.*, 1997). To investigate if Schwann cells cultured in NRG were influenced by Zfp-57 over-expression, parallel experiments to those described above were carried out with a single change in that the medium was supplemented with 20ng/ml NRG + 10uM forskolin. Under these conditions NRG acts as a mitogen and few periaxin positive cells were seen in empty vector controls and Zfp-57 vector (Fig 3.8 A and B respectively)

Quantification of these experiments revealed that Schwann cells transfected with EGFP expressing Zfp-57 and those transfected with an empty vector control both showed 1% positive staining periaxin cells (Fig 3.9). Those Schwann cells transfected with Zfp-57 showed 30% +/- 4.6 periaxin-GFP positive cells compared to 28% +/- 3.5 for empty vector control. These data suggest that Zfp-57 cannot modulate Schwann cell differentiation in the presence of the known Schwann cell mitogen NRG at least under the single set of conditions used here. The transfection efficiency averaged 35% for Schwann cells tranfected in these conditions.

DISCUSSION

We have characterised the expression of a novel zinc-finger transcription family member, Zfp-57 at both the mRNA and protein levels in the development of the sciatic nerve and in the principal glial cells within this system, Schwann cells.

The description of this gene in the Schwann cell lineage represents the third transcription factor of the zinc-finger class to be found in these cells, after Krox-20 and Krox-24 (Topilko *et al.*, 1994, Topilko *et al.*, 1997). Our knowledge of this class of transcription factors is distinctly under-represented given the suggestion that this may be the most common mammalian transcription factor (Berg, 1986). The importance of these transcription factors in mammalian organisms has been further highlighted with the publication of the first drafts of the human genome. Two parallel projects of unprecedented scale, one private (Celera) and one public (International Human Genome Sequencing consortium, I.H.G.S.C.) funded estimated the total number of human genes to between 30,000 and 40,000 a considerable drop from previous estimates of 100,000 (Venter *et al.*, 2001, I.H.G.S.C. 2001). Of perhaps greater interest than actual gene numbers themselves are the preliminary analyses of predicted human protein coding genes which have been carried out, based on alignments with known genes in databases such as RefSeq, Pfam and SMART. These analyses reveal a massive proportion of potential C2H2 zinc-finger transcription factor proteins in *H. sapiens* with estimates of actual numbers ranging from 600 (Celera) to 700 (I.H.G.S.C) (Venter *et al.*, 2001, I.H.G.S.C. 2001). Further study of these proteins is likely to reveal many homologues in rodents probably culminating in the discovery of diverse functions in different cell types. In fact Zfp-57 is a member of an ever increasing class of zinc-finger transcription factors

(Zfps). Many molecules of this class have been identified in different mammalian systems including: Zfp-1, Zfp-35, Zfp-37, Zfp-38, Zfp-51, Zfp-60, Zfp-64 (Chowdhury *et al.*, 1989, Cunliffe *et al.*, 1990, Chowdhury *et al.*, 1992, Burke *et al.*, 1994, Marakis *et al.*, 1996, Peres *et al.*, 1996, Mack *et al.*, 1997).

Zfp-57 is a novel zinc-finger transcription factor of the PNS and is localised to Schwann cells. This gene is developmentally regulated at the mRNA level with a transient peak of expression on or about embryonic day 17, the later stage of embryonic development, equivalent to the immature Schwann cell stage (Fig 3.2). This time point precedes the major onset of Schwann cell differentiation towards the myelinating pathway, as determined by the onset of high level myelination gene expression (e.g. P₀), and by morphology by two days. The subsequent down-regulation of Zfp-57 expression as Schwann cell differentiation proceeds suggests that it may play a role in myelination, although the transfection experiments described in this chapter do not support this idea. Significantly the temporal expression of Krox-20 mRNA expression, a transcription factor required for myelination (Topilko *et al* 1994.), is reversed compared to Zfp-57; Krox-20 expression onset begins with the appearance of the immature Schwann cell and steadily increases as myelination progresses (Blanchard *et al.*, 1996).

Another major difference between the expression patterns of these two genes is that Zfp-57 can be detected at the mRNA level throughout the development of PNS, from the Schwann cell precursor (E12), continuing throughout development in Schwann cells and (E17, P2, P7.) and is present in the mature Schwann cells of the adult sciatic nerve. Krox-20 differs in that expression is absent in both neural crest and Schwann cell precursors (Topilko *et al.*, 1994, Blanchard *et al.*, 1996).

Zfp-57 protein is detectable in Schwann cells *in vitro* (Fig 3.5) and at all stages of development *in vivo*, a result consistent with the mRNA expression finding (Fig 3.2). The localistion of Zfp-57 is nuclear, consistent with the identification of a possible nuclear localisation signal in the carboxy terminus (Fig 3.1) and its putative function as a transcription factor (Fig 3.3). These findings are consistent with other work using a different antibody directed against Zfp-57 protein also showing nuclear localisation (Okazaki *et al.*, 1994). It was found that 100% of Schwann cells at all stages of development, assayed express the Zfp-57 protein (data not shown). Clear nuclear expression can be seen in immature Schwann cells (E16), promyelinating Schwann cells (NB), maturing Schwann cells (P7) and adult Schwann cells. In addition the expression of Zfp-57 in the mature adult sciatic nerve is not restricted to one or other sub-population of Schwann cells. The developmental regulation of Zfp-57 seen at the mRNA level was not discernable by immunocytochemistry with the antibody in the conditions used. This technique, although qualitative, can at times allow the visualisation of different protein expression levels but does not seem to be possible in this case. The turnover of cellular proteins is highly variable and is mediated by ubiquitination which targets proteins to degradation proteases (reviewed in Bohley 1996). This mechanism of protein turn over has been suggested to be one of the ways that transcripton factors such as c-Fos and c-Jun are regulated (Hermida-Matsumoto *et al.*, 1996). It is possible that Zfp-57 could be a very stable protein with a long half life, leading to the maximal nuclear staining seen at all stages. Attempts were made to quantify Zfp-57 protein expression in Schwann cell development by Western blotting but were inconclusive (data not shown). The experiments resulted in a number of faint

bands of different sizes and it was not possible to determine which corresponded to Zfp-57.

Zfp-57 positive nuclei can be seen in myelin forming Schwann cells expressing MBP while it is also expressed in presumptive non-myelin forming Schwann cells, that do not express MBP (Fig 3.3). Taken together these data suggest that Zfp-57 is not directly involved in myelination and Schwann cell autonomous function is more likely. Intriguingly Zfp-57 expression is absent from the sympathetic trunk, a peripheral nerve fibre consisting entirely of non-myelin forming Schwann cells (Fig 3.4). One possibility is that Zfp-57 is somehow required for the development and maintenance of Schwann cells within complex mixed nerve fibre bundles such as the sciatic nerve and brachial plexus.

The results of a functional analysis of Zfp-57 over-expression in primary rat Schwann cells show that Zfp-57 does not induce or modulate the process of Schwann cell differentiation *in vitro* as determined by the expression of the PDZ domain protein periaxin. Periaxin is required for myelin stability and mutations have been identified that cause human CMT neuropathies (Boerkoel *et al.*, 2001, Guilbot *et al.*, 2001). This data suggests that Zfp-57 does not play a role in the process of Schwann cell differentiation. This lack of apparent function in the experiments performed here could be related to low level expression of the exogenous Zfp-57 gene. In the experiments described above the expression of GFP was visible but was relatively low, and as such it is possible that the experiments failed to over express the Zfp-57 gene significantly above the normal endogenous levels. Also it remains possible that the construct employed here has interfered with Zfp-57 function. The Zfp-57 gene is fused to the GFP reporter and it is possible that the resulting fusion protein function is altered or reduced. It would be

gene of interest are run independently from different promoters separated by an IRES sequence.

What could the function of Zfp-57 be? The peak of mRNA expression coordinates with the peak of Schwann cell proliferation (Stewart *et al.*, 1993, U. Lange personal communication) and preliminary results of Zfp-57 over-expression studies, in conditions that favour Schwann cell proliferation, showed that cells transfected with Zfp-57 may have reduced DNA synthesis as determined by BrDU incorporation (preliminary data not shown). Zfp-57 could therefore be involved in the control of Schwann cell proliferation

In summary Zfp-57 is a novel gene expressed in Schwann cells and Zfp-57 mRNA is detected throughout Schwann cell development and is maximal in the late embryonic stage at which time immature Schwann cells are prevalent. Zfp-57 protein is localised to the nuclei of Schwann cells from early in development into maturity where it can be identified in both myelin forming and non-myelin forming Schwann cells. We have found that over expression of Zfp-57 in primary Schwann cells *in vitro* is not able to modulate the process of differentiation in four different paradigms, suggesting that it is not directly involved in Schwann cell maturation. Further investigations could look into how Zfp-57 functions may be targeted to earlier events such as Schwann cell proliferation, migration or Schwann cell axon interactions and the transduction of signals involved such as β NRG/ ErbB ligand receptor mechanisms.

CONCLUSIONS

I conclude that Zfp-57 is expressed throughout Schwann cell development at both the mRNA and protein levels. The localisation of Zfp-57 to both adult Schwann cell sub-types and over-expression analyses indicate that Zfp-57 is probably not involved in myelination.

Figure 3.1 The Zfp-57 protein contains three complete, presumptive functional zinc finger motifs

The complete coding region of Zfp-57 is given, the cDNA sequence is shown on the upper lines with the amino acid (One-letter symbol code) translation beneath. Zfp-57 is 421 amino acids long. The amino acids boxed in green show the five potential zinc-finger, DNA binding, domains that typify zinc finger transcription factors. Zfp-57 is a member of the C₂H₂ class of zinc finger transcription factors and the conserved C₂H₂ residues are highlighted blue and yellow respectively. Open boxes within the domains 1 and 5 denote exchanged residues, indicative of incomplete non-functional zinc-finger motifs. Conversely, motifs 2-4 are completely conserved and are likely to be responsible for the DNA binding capability of this protein.

Figure 3.2 Detection of Zfp-57 mRNA in a developmental time series of mouse sciatic nerve, using Reverse transcription polymerase chain reaction (RT-PCR); comparison with Krox-20

Zfp-57 mRNA is detectable by RT-PCR throughout Schwann development at all time points assayed. Expression is predominantly embryonic beginning as early as E12 and peaking at the late embryonic stage, E17. Conversely Krox-20 is not expressed at E12 but is up-regulated at late embryonic stages and highly expressed postnatally, a process associated with and critical for the onset and continuation of myelination in the developing nerve (Topilko *et al.*, 1994). Krox-20 mRNA expression is maintained in adult sciatic nerve contrast while by contrast Zfp-57 mRNA expression is significantly reduced after birth.

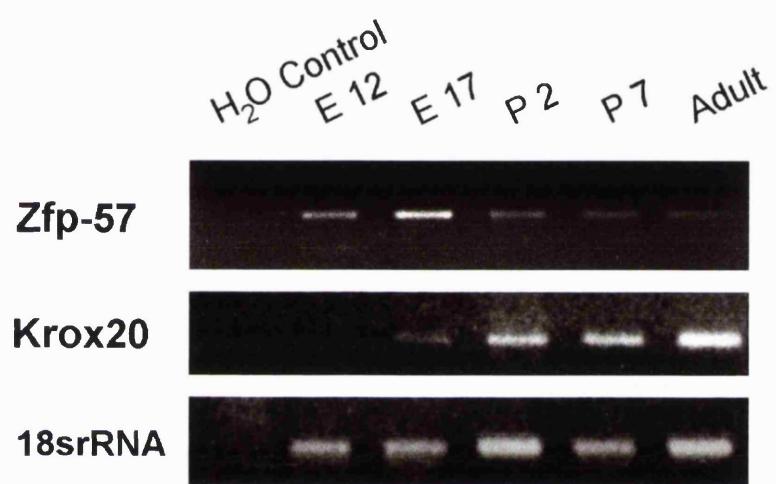


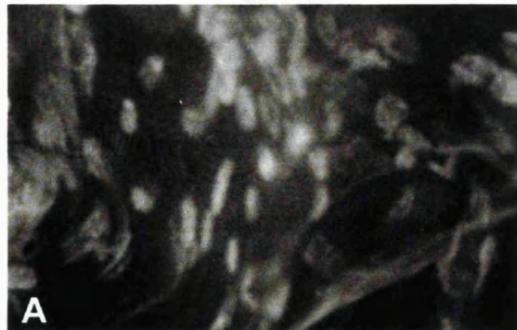
Figure 3.3 Zfp-57 protein is expressed in mouse Schwann cells *in vivo*

Immunohistochemistry using an antibody recognising a Zfp-57 fusion protein was carried out on teased sciatic nerve preparations and viewed using FITC optics. Fluorescence (A, C, E, G) and corresponding phase contrast images (B, D, F, H) show the nuclear localisation of the Zfp-57 protein in Schwann cells *in vivo*. Zfp-57 protein expression is clearly present in immature Schwann cells at E16.5 (A, B) and is also prevalent in new born Schwann cells, at the onset of myelination (C, D). Zfp-57 protein expression continues in maturing Schwann cells at P7 (E, F) and is maintained into the adult state (G, H).

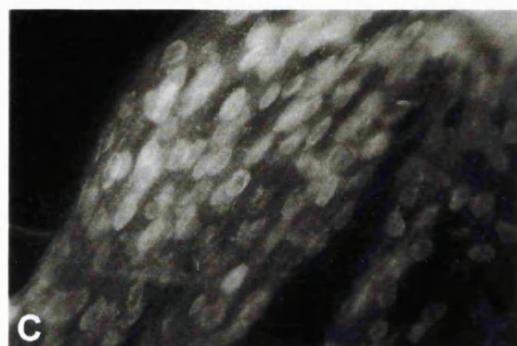
Double immunohistochemistry was used to label an adult tease nerve preparation with Zfp-57 (J) and MBP (K). Zfp-57 staining is localised to MBP positive myelin forming Schwann cells (arrows) and is also seen in presumptive non-myelin forming Schwann cells, negative for MBP (arrowheads).

Scale bars = 50 μ m.

Zfp-57



A



C

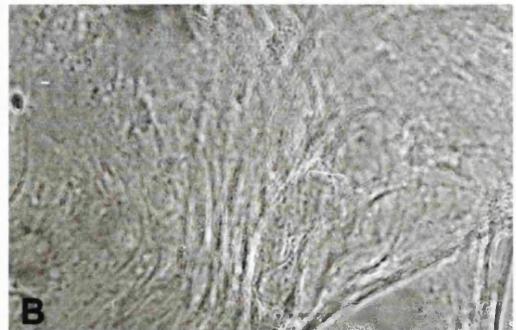


E

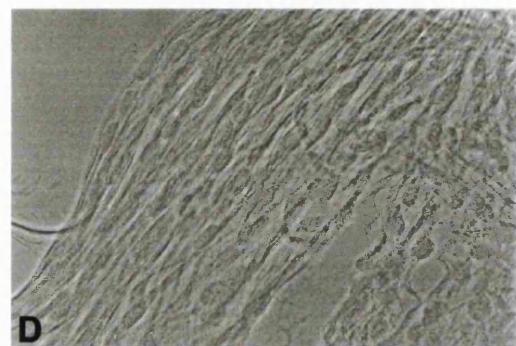


G

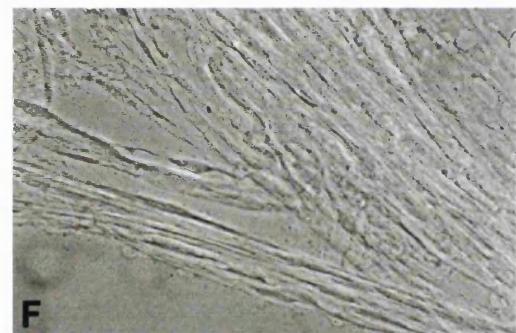
Phase



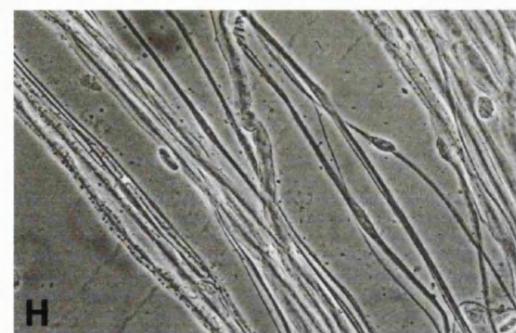
B



D



F



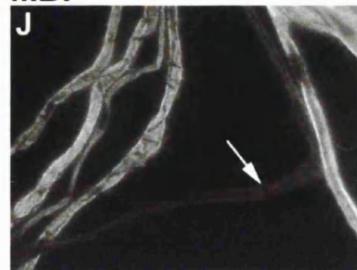
H

Zfp-57



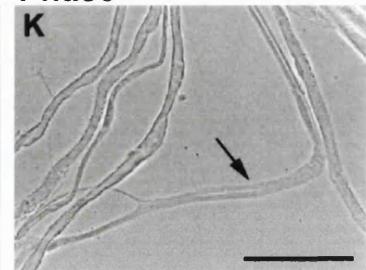
I

MBP



J

Phase



K

Figure 3.4 Zfp-57 protein is absent from the adult sympathetic trunk

Double immunohistochemistry using antibodies recognising a Zfp-57 fusion protein and mouse L1 cell adhesion molecule was carried out on adult mouse sympathetic trunk cryosections. Fluorescence images show the localisation of L1 to the cytoplasm of all the Schwann cells with the sympathetic trunk (B and overlay D). Zfp-57 is absent from all the nuclei of the sympathetic trunk (A and overlay D). Nuclei are stained with DAPI (C and overlay D). This experiment was performed twice.

Scale bar = 50 μ m.

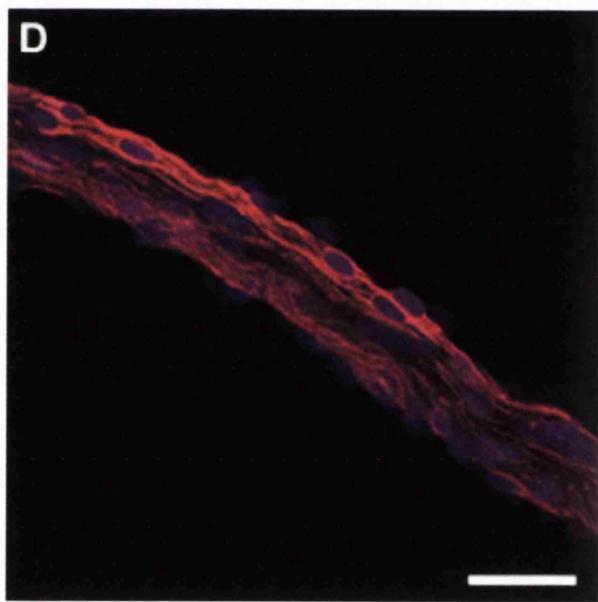
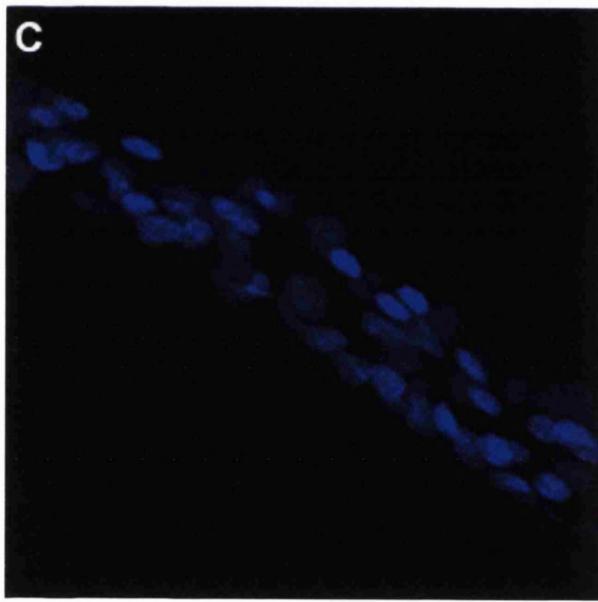
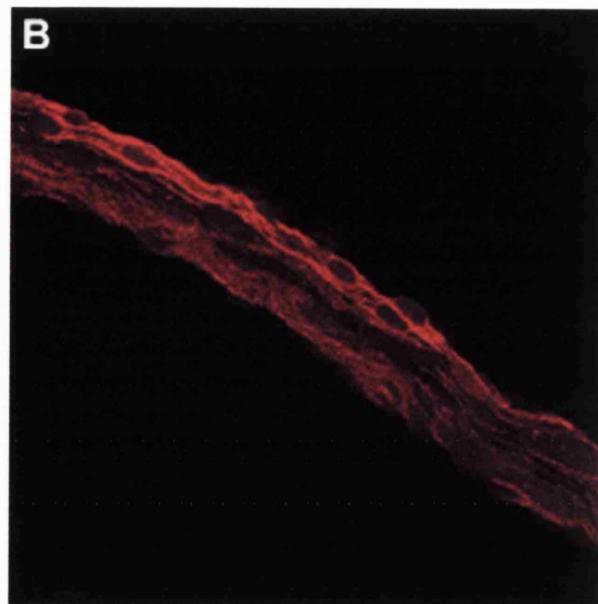
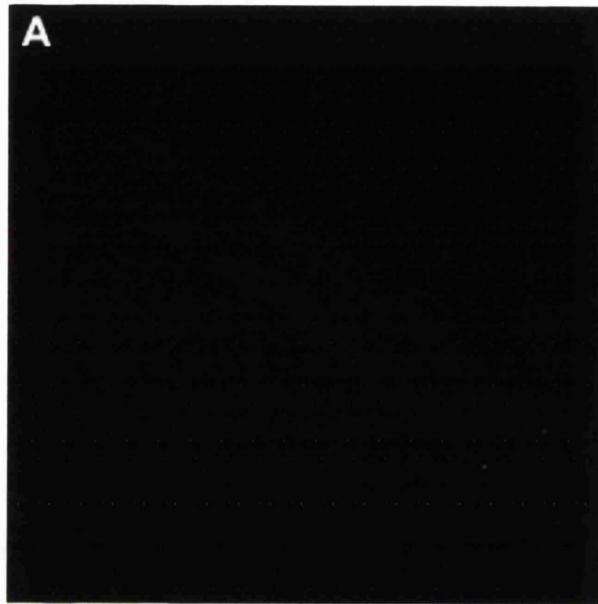
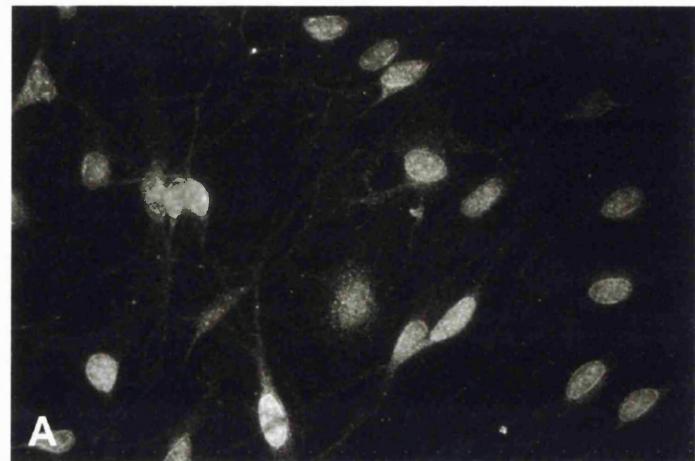


Figure 3.5 *In vitro* expression of Zfp-57 protein in Schwann cells

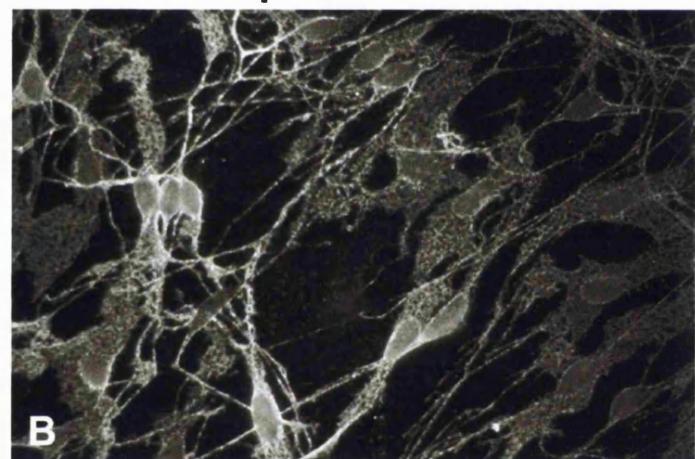
Double immunocytochemistry using antibodies recognising a Zfp-57 fusion protein and p75NTR on P3 rat Schwann cells cultured for one day. These fluorescence images show that cultured Schwann cells display a strong nuclear signal of Zfp-57 as viewed with FITC optics (A) and these cells stain positively for P75NTR (B), a known marker for Schwann cells (viewed with rhodamine optics). The corresponding field viewed by phase contrast is shown in C.

Scale bar = 50 μ m.

Zfp-57



p75NTR



Phase

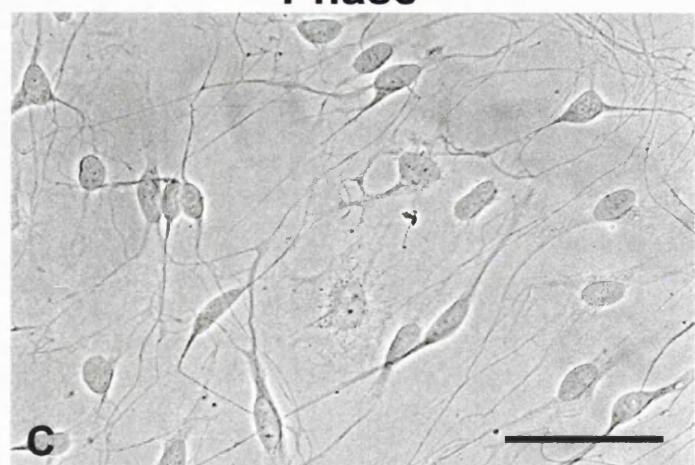


Figure 3.6 Over-expression of Zfp-57, A; periaxin, a marker of differentiating Schwann cells, is not induced in quiescent Schwann cells

Fluorescent and light micrographs of pure primary rat Schwann cells transiently transfected with Zfp-57 in EGFP expression vector (B, D, F, H.) or empty vector controls (A, C, E, G.). Schwann cells were cultured in defined medium supplemented with 0.5% serum for three days. An antibody against periaxin was used to immunolabel any differentiating Schwann cells and was viewed with rhodamine optics (A, B.). Positively transfected Schwann cells were identified with fluorescein optics detecting GFP fluorescence, which was always localised to the nucleus (C, D.). DAPI was employed to label the nuclei of all Schwann cells within the cultures (E, F.). The typical bi- or tripolar morphology of quiescent Schwann cells in vitro is clearly visible by phase contrast (G,H.). This experiment was performed 5 times on triplicate coverslips.

Scale bars = 50 μ m.

EMPTY VECTOR



A

Zfp-57_GFP



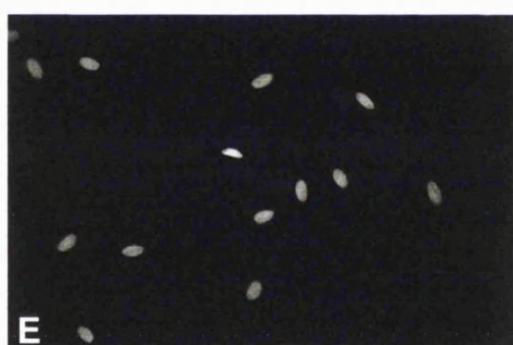
B



C



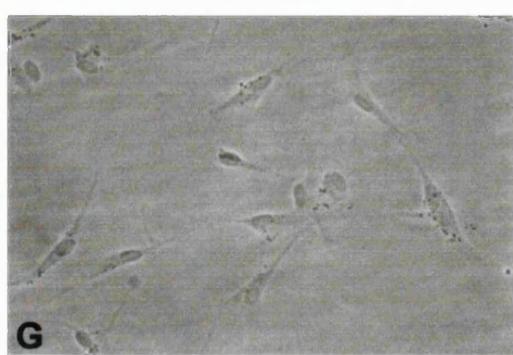
D



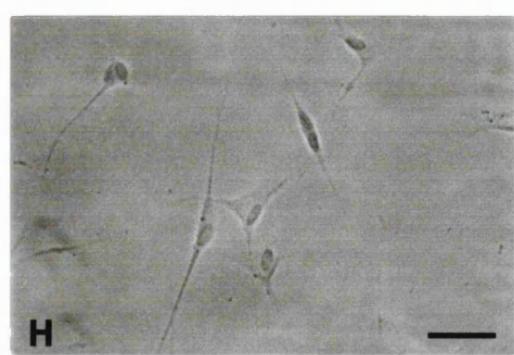
E



F



G



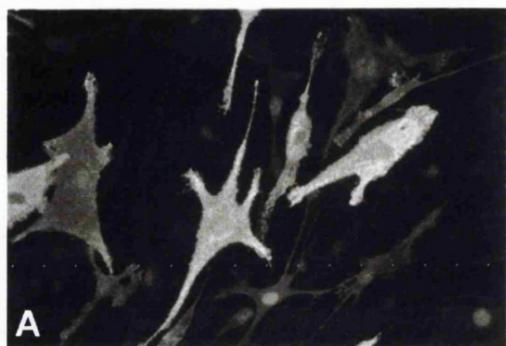
H

Figure 3.7 Over-expression of Zfp-57, B; periaxin expression is not modulated in Schwann cells differentiating in response to dbcAMP

Fluorescent and light micrographs of pure primary rat Schwann cells transiently transfected with Zfp-57 in EGFP expression vector (B, D, F, H.) or empty vector controls (A, C, E, G.). Schwann cells were cultured for three days in defined medium supplemented with 10uM dibutryl cAMP. Differentiating Schwann cells extended processes and lamellae as they differentiate and a proportion stain positively with an antibody against periaxin (A, B.). Positively transfected Schwann cells were identified with fluorescein optics detecting GFP fluorescence (C, D.). DAPI was employed to label the nuclei of all Schwann cells within the cultures (E, F.). By phase contrast only the nucleus of these Schwann cells is obvious as many of the processes are very fine and the lamellae very flat (G,H.). Both periaxin positive cells are seen within the Zfp-57_EGFP and EGFP alone populations. This experiment was performed 5 times on triplicate coverslips.

Scale bars = 50 μ m.

EMPTY VECTOR



Zfp-57_GFP

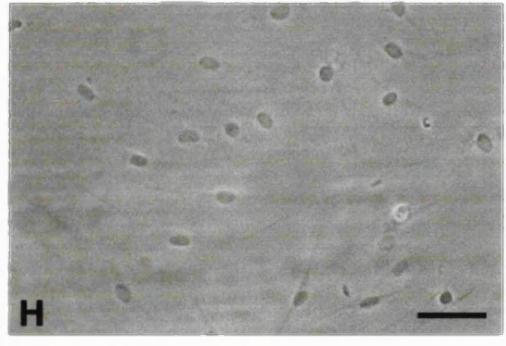
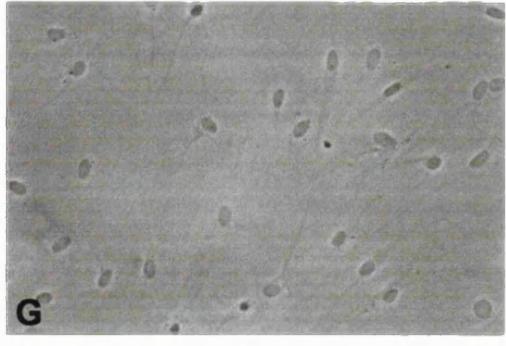
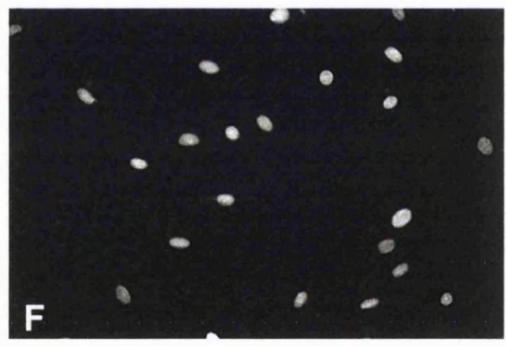
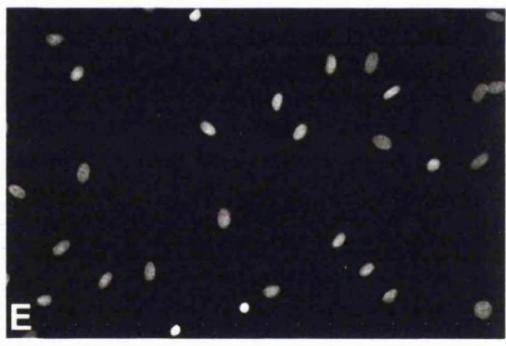
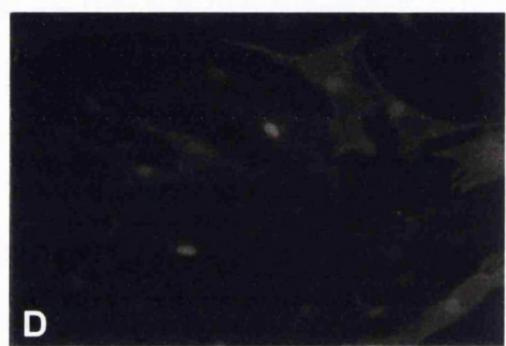
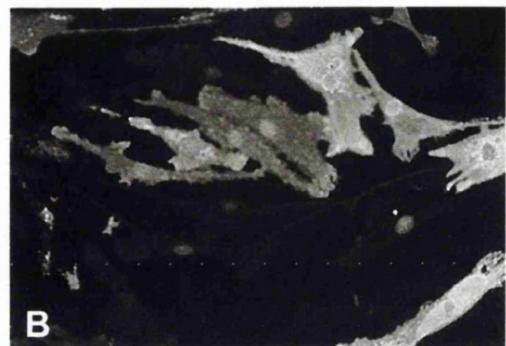
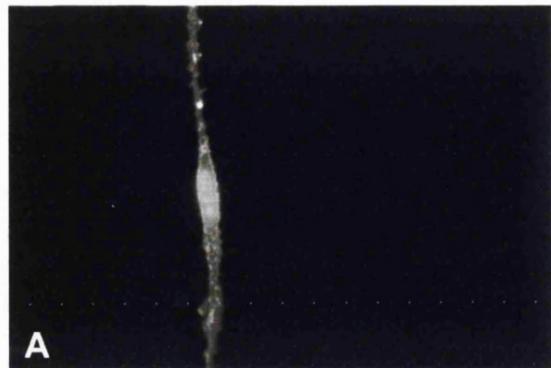


Figure 3.8 Over-expression of Zfp-57, C; periaxin expression is not modulated in Schwann cells differentiating in response to β NRG

Fluorescent and light micrographs of three day cultures of pure primary rat Schwann cells transiently transfected with Zfp-57 in EGFP expression vector (B, D, F.) or empty vector controls (A, C, E.). An antibody against periaxin was used to immunolabel any differentiating Schwann cells and was viewed with rhodamine optics (A, B.). Positively transfected Schwann cells were identified with fluorescein optics detecting GFP fluorescence which was always localised to the nucleus (C, D.). The morphology of Schwann cells in these conditions is shown by phase contrast (E, F.). This experiment was performed 3 times on triplicate coverslips.

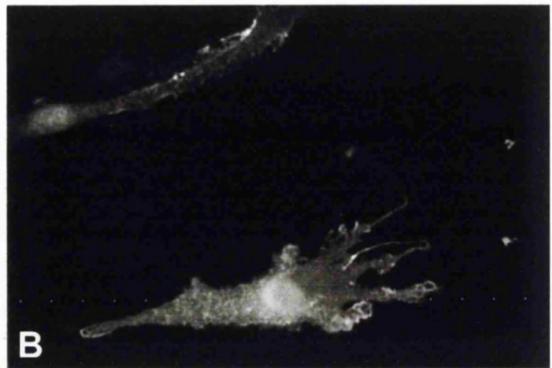
Scale bars = 50 μ m.

EMPTY VECTOR



A

Zfp-57_GFP



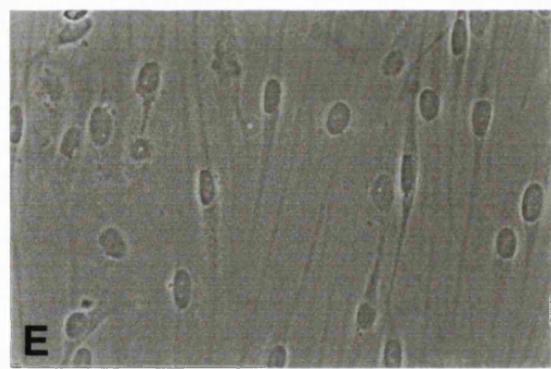
B



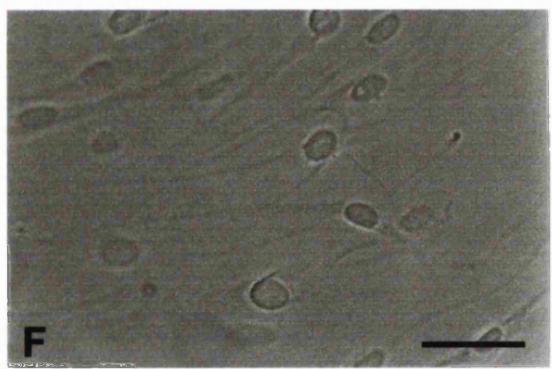
C



D



E



F

Figure 3.9 Graph to show percentage of Schwann cells induced to express periaxin when transfected with Zfp-57.

Shown are the percentage of Schwann cells immuno-positive for periaxin in three conditions: defined medium (Def. Med.), defined medium plus dibutyryl cAMP (+db-cAMP) and defined medium plus forskolin and NRG-1 (NDF β). Light grey bars represent empty EGFP vector (EV) and dark grey bars represent Zfp-57_EGFP (Zfp-57).

Zfp-57 over-expression; percentage periaxin positive cells

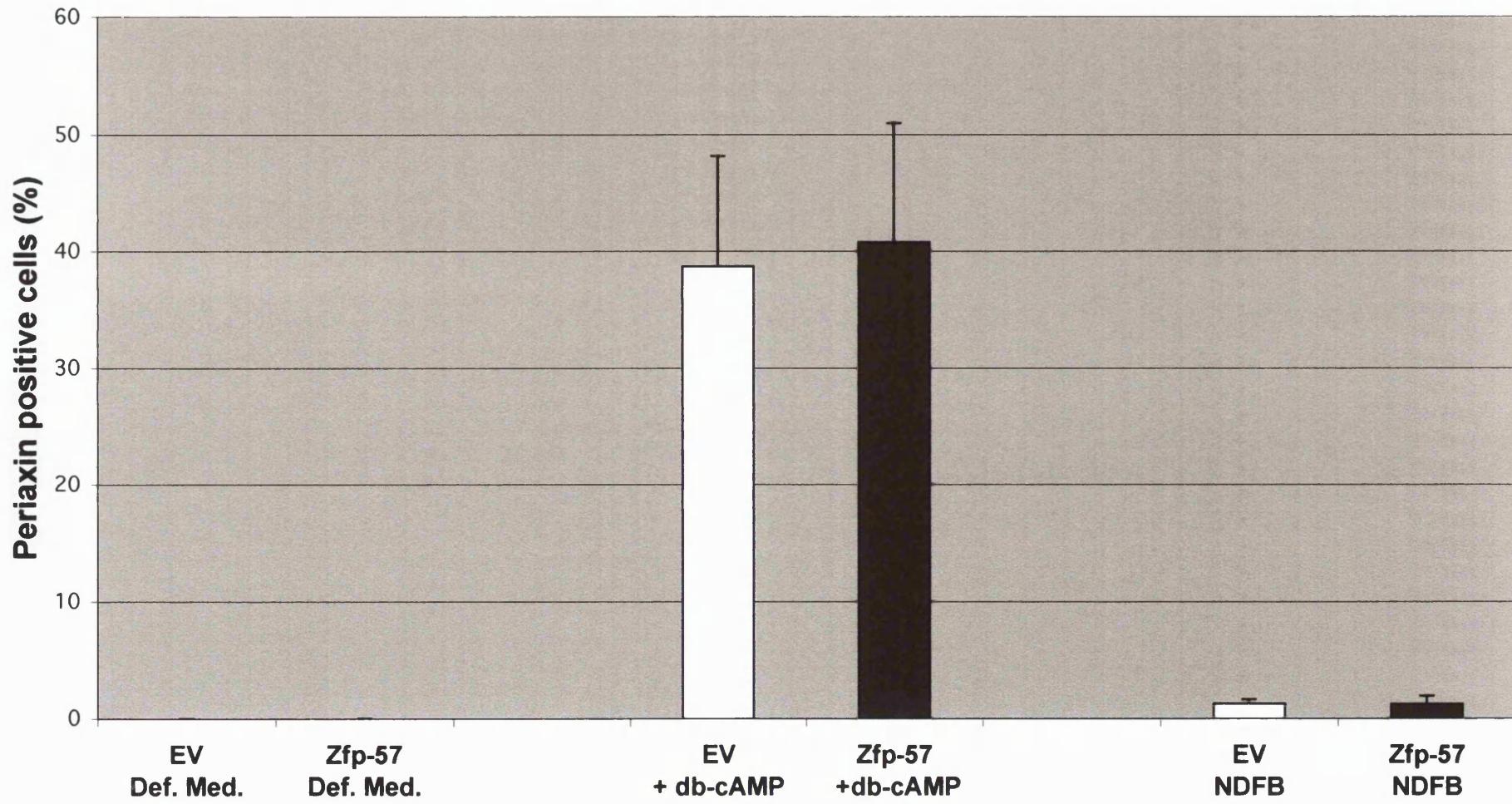


Table 3.1 Table to show percentage of GFP positive Schwann cells that also express periaxin.

Counts were also made of the number of periaxin positive cells also positive for GFP in conditions with either 10uM dbcAMP or 20ng/ml NRG + 10uM forskolin (Table 3.1). With dbcAMP, Schwann cells transfected with Zfp-57 showed 13% +/- 2.1 periaxin-GFP positive cells compared to 14% +/- 1.6 for empty vector control. With NRG and forskolin, Schwann cells transfected with Zfp-57 showed 30% +/- 4.6 periaxin-GFP positive cells compared to 28% +/- 3.5 for empty vector controls.

	<i>Zfp57_EGFP</i>	<i>Empty Vector</i>
dbcAMP	13% +/- 2.1	14% +/- 1.6
NRG	30% +/- 4.6	28% +/- 3.5

CHAPTER 4

Krox-24 in the sciatic nerve and phenotype of Krox-24 null mutant nerves

INTRODUCTION

Krox-24 belongs to the Egr family of transcription factors, which is comprised of four members: Krox-24 (Egr-1, NGF1-A, zif268, tis8), Krox-20 (Egr-2, NGF1-B), Egr-3 (Pilot), Egr-4 (NGF1-C) (reviewed by O'Donovan *et al.* 1999). All the members of this family possess a highly conserved DNA binding domain composed of three zinc fingers that recognise a nine base pair sequence GCG(T/G)GGGCG.

Null mutant transgenic mice have been generated for all four of these transcription factors (O'Donovan *et al.* 1999). Krox-20 null mutants display defects in hindbrain development and Schwann cell myelination (discussed in general introduction). Egr-3 deficient mice suffer from sensory ataxia, scoliosis and resting tremor which are attributable to a complete loss of muscle spindles (Tourtelotte *et al.* 1998). Egr-4 deficient mice have an autonomous germ cell defect that leads to male infertility (O'Donovan *et al.* 1999).

Krox-24 deficient mice

Two transgenic mouse lines with a targeted disruption of the Krox-24 gene have been generated by two independent laboratories (Lee *et al.* 1996, Topilko *et al.* 1997, 1998). As discussed in the general introduction, these mice exhibit sterility due to a specific loss of somatotropes from the anterior pituitary (Lee *et al.* 1996, Topilko *et al.* 1998). Behavioural analysis of these mice has revealed that while short-term memory is normal, performance in tests requiring long-term memory is impaired compared to wildtype animals (Jones *et al.* 2001). This deficit is attributable to a

loss of late long-term potentiation (LTP) in the dentate gyrus within the hippocampus in null mutants and demonstrates a requirement for Krox-24 in synaptic plasticity of late LTP (Jones *et al.* 2001). This data endorses the work of others that demonstrated Krox-24 up-regulation in the hippocampus following stimulation is capable of inducing LTP (Cole *et al.* 1989, Wisden *et al.* 1990, Richardson *et al.* 1992).

Krox-24 expression in the CNS mediates synaptic plasticity

Accumulating data demonstrating up-regulation of Krox-24 expression in other CNS regions in response to diverse stimuli suggest that this gene may have a general role in synaptic plasticity (O'Donovan *et al.* 1999). Regions of visual cortex of monkeys undergoing visual learning tasks have increased Krox-24 immunostaining, showing that Krox-24 expression in neurons is involved in visual learning processes (Okuno and Miyashita, 1996). In the zebrafinch a Krox-24 ortholog, ZENK, has been described in the CNS (the name is an acronym of Zif268, Egr-1, NGF1-A and Krox-24) (Reviewed by Long and Salbaum, 1998). Playing a species specific song recording has been shown to induce Krox-24 in central auditory nuclei in zebrafinch and the act of singing itself induced even higher levels of the gene in associated song motor nuclei (Mello *et al.* 1995, Jarvis and Nottebohm, 1997). Thus it seems likely that Krox-24 expression is associated with auditory and visual learning processes. Circadian- rhythm entrainment occurs by an unknown mechanism but involves the suprachiasmatic nucleus of the hypothalamus, and exposure to light in the dark cycle can induce a phase shift which is accompanied by altered immediate early gene expression including Krox-24 (O'Donovan *et al.* 1999).

In addition to the importance of Krox-24 in the mechanism of late LTP within the hippocampus and the participation in learning processes, up-regulation has also been identified in association with the induction of epileptic seizures (Gass *et al.* 1992, Lanaud *et al.* 1993, Liang and Jones, 1997).

Targets of Krox-24 regulation

Given the diverse nature of Krox-24 expression, it is perhaps not surprising that it has been implicated in the induction of a range of different genes. Of particular interest to the above studies within the CNS is the finding that Krox-24 may regulate three genes that encode protein components of the synapse, synapsin I and II and SNAP 45 (Thiel *et al.* 1994, Chin *et al.* 1994, Svaren *et al.* 2000). The possibility that Krox-24 controls synaptic plasticity by regulating genes encoding proteins involved in synapse formation is a very real one.

Krox-24 has been shown to induce several members of the growth factor family *in vitro*, including platelet derived growth factor A chain (PDGF-A), PDGF-BB, basic fibroblast factor (bFGF; FGF2) and also transforming growth factor β (TGF β) and its receptor in different cell types (Liu *et al.* 1996, Khachigian *et al.* 1995, Silverman *et al.* 1997, Rafty and Khachigian 1998, Biesiada *et al.* 1996, Wang *et al.* 1997, Liu *et al.* 1999, Du *et al.*, 2000). Components of the cell cycle modulation mechanism such as p53 can also be induced (Nair *et al.* 1997, Svaren *et al.*, 2000). Cytokines such as human tumor necrosis factor (TNF-alpha), and adhesion molecules such as intercellular adhesion molecule (ICAM-1) and CD44 can be regulated (Kramer *et al.* 1994, Malzman *et al.* 1996a, Malzman *et al.* 1996b, McCaffrey *et al.*, 2000). Finally a number of components of the coagulation system as well as metalloproteinases appear to be influenced by Krox-24 (Cui *et al.* 1996, Verde *et al.* 1988, Haas *et al.*

1999). Recently two independent laboratories have investigated the possible targets of Krox-24 in prostate cells and those isolated from atherosclerotic lesions by screening cDNA microarrays (McCaffrey *et al.*, 2000, Svaren *et al.*, 2000). These studies have isolated previously identified Krox-24 targets such as ICAM-1, CD44, p53, PDGF-A and TGF β s further implicating Krox-24 in the modulation of these molecules. These screens have also identified a number of diverse targets, not previously described, which include copper-zinc superoxide dismutase (SOD1), crystatin M protease, the signalling molecule Rad (related to Ras) and connexin 26. In primary Schwann cell cultures transfection of antisense Krox-24 has been shown to reduce the expression of p75NTR suggesting that Krox-24 might regulate the expression of this receptor (Nikam *et al.* 1995).

The Structure of Krox-24

The Egr-1 protein product is conserved across species with the cDNAs for rat, mouse, chicken, zebrafish and human being highly homologous. The amino acid sequence of murine Krox-24 is 533 residues in length and can be subdivided into three regions; a serine threonine-rich N-terminal domain, a central basic region containing the three zinc finger binding motifs that recognise the consensus sequence GCG(T/G)GGGCG and a proline/ serine/ threonine-rich C-terminus (Sukhatme *et al.* 1988). Deletion analysis has demonstrated the N-terminal domain to be a strong trans-activation domain (Gashler and Sukhatme 1995). In addition these analyses revealed a compact domain including residues 281- 304, just ahead of the zinc finger region, with powerful repressor like activity, the removal of which causes increased Egr protein activity. This novel repressor domain, known as R1, is serine, threonine rich, conserved in vertebrates and has since been shown to be a target of a novel

protein family known as NAB proteins (for NGFI-A-Binding protein) (reviewed by O'Donovan *et al.*, 1999).

NAB protein corepressors

The NAB proteins are among a growing group of transcription factor repressors known as corepressors. These proteins are able to repress the activity of the promoter to which they are recruited when bound to their transcription factor even if it is normally a transcriptional activator (Svaren *et al.*, 1996). Two NAB proteins, NAB1 and NAB2 were originally isolated from a yeast two hybrid screen of proteins capable of interacting with an Egr "bait" that encompassed the inhibitory R1 domain (Russo *et al.*, 1995; Svaren *et al.*, 1996). NAB1 is able to repress the activity of Krox-24, Krox-20 and Egr-3 whilst being unable to influence Egr-4 transcriptional activation (Russo *et al.*, 1995). Accordingly only Egr-4 is lacking in the R1 domain and is therefore not influenced by NABs.

Within both of the NAB proteins there are two conserved domains termed NAB Conserved Domains 1 and 2 (NCD1 and NCD2), NCD1 is located near the aminoterminus and NCD2 is found towards the carboxyterminus. The NCD1 region of the NAB proteins mediates the binding with the R1 domain of Egr proteins (Svaren *et al.*, 1996). The second region, NCD2 has been shown to contain both a nuclear localisation signal (NLS) as well as the source of transcriptional repressor activity (Swirnoff *et al.* 1998). Further investigations into NAB function have revealed dominant negative mutants unable to bind Egrs that enhance their activation (Svaren *et al.* 1998). The authors show that these mutants exert such an effect by sequestering wildtype NABs and propose that NAB protein multimerization may be important in NAB function.

In vivo evidence for a negative feedback loop involving NAB2 and Krox-20 in rhombomeres 3 and 5 of the developing hindbrain has recently been presented (Mechta-Grigoriou *et al.*, 2000). Disruption of Krox-20 function, by its targeted inactivation, leads to aberrant hindbrain segmentation (Schneider-Maunoury *et al.*, 1993, 1997), and experiments where NAB is misexpressed produced effects similar to those of Krox-20 inactivation (Mechta-Grigoriou *et al.*, 2000). Furthermore, Krox-20 with a mutation in its NAB-binding domain R1 is insensitive to NAB repression (Svaren *et al.*, 1996) and a recessive human myelinopathy (CHN) has been associated with such a mutation (Warner *et al.*, 1998). This presumably results from uncontrolled Krox-20 transcriptional activation of myelin genes due to a lack of NAB-mediated negative regulation. Evidently NAB proteins are important in controlling Krox-20 target gene activation in the hindbrain and in Schwann cells.

Krox-24 expression in the PNS

As outlined briefly in the general introduction, Krox-24 expression has been described in Schwann cells at both the mRNA and protein level in developing Schwann cells (Watson and Milbrandt, 1990, Topilko *et al.*, 1997). Also Krox-24 expression in adult non-myelin forming Schwann cells has been identified (Topilko *et al.*, 1997). Finally the nerve sheath, that encapsulates peripheral nerves, shows a degree of Krox-24 expression. In this chapter experiments are described to investigate further the expression pattern of Krox-24 in Schwann cells and also the ultrastructure of sciatic nerves deficient in Krox-24.

AIMS

Krox-24 is reported to have an antagonistic role to Krox-20, a gene required for the normal development of myelin forming Schwann cells. I hypothesised that Krox-24 is therefore involved in Schwann cell differentiation towards the non-myelin phenotype. Additionally, high level Krox-24 expression has been shown at birth the time when Schwann cells are engaged in proliferation suggesting a role for Krox-24 in this process. To test these hypotheses I explored the phenotype of Schwann cells deficient in the Krox-24 gene at two developmental stages: new born when Krox-24 is maximal and adult when expression is low.

RESULTS

Krox-24 expression is a feature of late embryonic and early postnatal sciatic nerve development.

As mentioned previously (see methods) the null mutant mouse used in this study was created by homologous recombination employing a transgenic construct engineered to incorporate the *E. coli* gene, Lac Z (Topilko *et al.* 1997, 1998). The incorporation of the Lac Z gene, which encodes β -galactosidase, enables the expression of Krox-24 to be investigated *in vivo*. When tissue is processed, β -galactosidase activity results in the cleavage of the chromogenic substrate, X-Gal, resulting in the formation of a blue product. Previous work using this transgenic mouse combined with immunohistochemistry has shown that the β -galactosidase expression pattern observed faithfully recapitulates that of Krox-24 (Topilko *et al.* 1997, 1998).

This technique has been used to investigate the expression of Krox-24 throughout the development of the sciatic nerve within the hindlimb. At the earliest time point assayed here, E12, no blue product could be visualised in the developing nerve routes (arrows in Fig 4.1 A). This data suggests that Krox-24 is not expressed in Schwann cell precursors which predominate at this stage in the rat and mouse (Jessen *et al.* 1994, Dong *et al.* 1999). In fact blue product was not detected anywhere in this section, notably the neural tube and the developing DRG (arrowhead). At E15 the picture remained unchanged, and blue product could not be seen in the developing nerve routes, arrows, or associated with the DRG, arrowhead (Fig 4.1 B). A population of cells that are positively staining for β -galactosidase can be seen in the developing epithelial flanking the spinal cord. These cells are located in the deepest

epithelium layer, which is the region where the pigment cells of the epithelium, the melanocytes, are found.

Three days later, at E18, a dramatic change has occurred and the nerve can be seen to be completely β -galactosidase positive, full of a diffuse blue product (Fig 4.1 C). By this stage Schwann cells are maturing, express S100, and begin to form close relationships with axons. This pattern of expression is also found in the newborn sciatic nerve (Fig 4.1 D) and the intensity of the blue product may be even more intense. The most intense staining was seen at this stage and suggests that Krox-24 expression is maximal in the newborn sciatic nerve. Less than 1 week after birth, at P5, the level of blue product was observed to have decreased significantly (Fig 4.1 E) and was completely absent in the adult nerve (Fig 4.1 F). In fact by P10 no blue product was observed (data not shown) and these results suggest that Krox-24 expression is restricted to approximately 1 week of sciatic nerve development, beginning on or possibly just before E18 and continuing to approximately P7.

Krox-24 null mutant sciatic nerves do not have morphological abnormalities

Krox-24 deficient mice develop into adulthood successfully but have been observed to be up to 25% smaller in body weight, attributable to pituitary abnormalities (Topilko *et al.* 1998). The gross morphology of sciatic nerves from these animals appears normal (Topilko *et al.* 1997) but observations of the ultrastructure remained to be undertaken. Transmission electron microscopy was employed to analyse the sciatic nerves of three month old adult wildtype and Krox-24 null mutant mice. Observations at high power (6,000 times) showed the presence of myelin forming Schwann cells and compact myelin sheaths. A single myelin-forming Schwann cell

ensheathing a small axon is shown in wildtype (Fig 4.2 A) and similarly from a Krox-24 null mutant (Fig 4.2 B). Even higher power observations suggested that the organisation of compact myelin sheath was not perturbed, with the major dense line clearly present (data not shown). These findings suggested that the myelin sheaths are normal in Krox-24 deficient nerves and further investigations were not considered to be necessary. Therefore calculations of the G-ratio, a measure of the myelin thickness to axon diameter, were not carried out.

Non-myelin forming Schwann cells were also present in the mutant nerve (Fig 4.2 D) and these compared favourably with those seen in the wildtype control nerve (Fig 4.2 C). Finally the perineurium, the sheath that surrounds the nerve, was inspected. This is noteworthy as recently this laboratory has demonstrated the importance of the hedgehog family of signalling proteins in sheath development (Parmantier *et al.* 1999). This data demonstrated the importance of desert hedgehog (Dhh) showing that expression in early peripheral nerve development is required for the correct formation of the perineurium. The perineurium is composed of a number of concentric layers of flattened fibroblastic cells that surround nerve fibre bundles (Thomas, 1963). In normal adult nerve the perineurium consists of five layers of fibroblast derived connective tissue (Fig 4.2 E). In nerves derived from Krox-24 null mutant mice the five layers making up the sheath display comparable morphology as compared to the control (Fig 4.2 F). Thus it was concluded that the ultrastructural morphology of Krox-24 deficient sciatic nerve appeared normal.

Schwann cells form the major cellular component of peripheral nerves and closely associate with the many axons that project from the CNS to the periphery. As

previously mentioned, the sciatic nerve is mixed in nature and contains axons derived from four sources. These include myelinated motor axons (of ventral origin), myelinated afferents (sensory), sympathetic unmyelinated axons and unmyelinated afferents (sensory) (Jenq and Coggeshall, 1985, Smalbruch, 1986). Aguayo and colleagues (Aguayo *et al.*, 1976) have demonstrated that axon derived signals can determine Schwann cell phenotype; thus it was interesting to count myelinated and unmyelinated axons in the Krox-24 deficient sciatic nerve to quantify any differences.

Counts of axons were made of the two large branches of the sciatic nerve, the tibial and peroneal nerves. Unmyelinated axons were counted in ultra-thin transverse sections directly while viewing in the TEM, and myelinated axons were counted on low-magnification micrograph montages of the same sections. All of the fibres within these two nerve branches were counted from three pairs of animals. The sum of the total number of myelinated axons within these two branches was found to be 4064 +/-48 (n=3) in the wildtype nerve and 4036 +/-112 (n=3) in the Krox-24 deficient nerve (Fig 4.3 A). The total number of unmyelinated axons was found to be 6855 +/-45 (n=3) in the wildtype nerve and 6922 +/-78 (n=3) in the Krox-24 deficient nerve (Fig 4.3 A). These data are not statistically significant (P>0.05) and demonstrate that the number of myelinated and unmyelinated axons are not abnormal in Krox-24 deficient sciatic nerves. To further analyse the relationship of unmyelinated axons and associated Schwann cells counts were made of the number of axons supported by individual non-myelin forming Schwann cells. The highest frequency was represented by Schwann cells supporting groups of axons ranging in number between 1 and 10 (Fig 4.3 B). The lowest frequencies were those cells

supporting in excess of 40 axons. Wildtype non-myelin cells supporting 1 to 10 axons had a frequency of 169+/-9 and a similar frequency of 173+/-11 was found for null mutant cells. As the axon group size increases, the frequency decreases and there was little difference between control and null mutant nerves in all group sizes tested. These data are indicative that non-myelin forming Schwann cells of Krox-24 null mutant sciatic nerves support unmyelinated axon numbers similar to those found in wildtype nerves.

mRNA analysis of known transcription factors and markers of Schwann cells

Investigation into a possible phenotype in Krox-24 null mutant mice was initiated by an RT-PCR screen. This technique is relatively fast, requires small amounts of starting material (mRNA) and is very sensitive, being able to detect rare transcripts. Specific oligonucleotide primers were designed to recognise four transcription factors: Krox-24, Krox-20, Egr-3 and Oct-6. Also primers were created to distinguish the two subpopulations of Schwann cells; P_0 to detect myelin and myelin forming Schwann cells and p75NTR and NCAM to detect non-myelin forming Schwann cells. Finally Dhh primers were used to evaluate if the expression of Dhh was altered which could be indicative of perineurium abnormalities (Parmantier *et al.*, 1999). Two stages of Schwann cell development were chosen for this analysis; newborn when Krox-24 expression is high and adult when expression is low or absent.

Newborn sciatic nerve contains high levels of all four transcription factors assayed here (Fig 4.4). Krox-24 deficient sciatic nerve contains similar mRNA levels, compared to wildtype controls, in the case of Krox-20, Egr-3 and Oct-6 but note that

mRNA isolated from Krox-24 mutant nerve does not show the presence of Krox-24 transcripts. Expression of P_0 , p75NTR, NCAM and Dhh are also readily detectable at this stage and similarly no difference could be detected in Krox-24 deficient samples.

In adult wildtype samples, levels of the transcription factors Krox-24, Egr-3 and Oct-6 are much lower than in newborn samples (Fig 4.2). In contrast the expression of the transcription factor Krox-20 and myelin protein P_0 is maintained in the adult nerve consistent with the association of these genes with the process of myelination. The level of NCAM remains constant in the samples, despite being down-regulated in myelin forming Schwann cells, which is due to axonally derived N-CAM. Additionally p75NTR and Dhh transcripts were also dramatically reduced in the adult nerve. As was discovered at the newborn stage, no differences in transcript levels could be detected for those analysed from Krox-24 deficient adult nerves as compared to those derived from wildtype animals. These results are consistent with the finding that adult Krox-24 deficient sciatic nerves display normal ultrastructural morphology (see above).

The onset and progression of myelination

The normal mRNA expression patterns described above in newborn Krox-24 deficient nerves provided strong evidence that development of Schwann cells occurs normally. Given that Krox-24 expression is maximal at birth which corresponds to the time when the process of myelination is beginning, further investigations seemed pertinent. Having established that the expression of P_0 mRNA at birth in Krox-24 deficient cells was comparable to wildtype controls and that compact myelin was

visualised in the adult, it was interesting to investigate if the temporal expression of P_0 protein remained intact.

Immunohistochemistry revealed little or no P_0 staining in transverse sections of wildtype and Krox-24 null mutant nerve at E18 (Fig 4.5 A, B). By contrast, at two days after birth, P_0 immunoactivity was clearly visible in nerves from both animals (Fig 4.5 C, D). Expression of P_0 varies over the nerve sections, some areas are positive (green) while others are negative (black) consistent with the process of myelination beginning at this stage. P_0 is a good marker of myelin formation as it is the major component of the myelin sheath (Greenfield *et al.* 1973), however it still only accounts for 48% of compact myelin. Therefore, to confirm that the onset of myelination was not affected in Krox-24 null mutants, the ultrastructure of sciatic nerves from newborn mice was analysed.

Careful observations of transverse ultra-thin sections of wildtype newborn nerves revealed that myelin forming Schwann cells could be seen at three different stages in the process of myelination. A proportion of Schwann cells could be seen in the earliest stage in this process having assumed 1: 1 relationships with axons; a number of cells had made a few turns around an axon while others already display, thin, compacted myelin sheaths seen as intense black rings (Fig 4.6 A). Similarly observations of nerves from Krox-24 null mutant mice revealed myelinating Schwann cells at varying stages in the process of myelination (Fig 4.6 C).

Non-myelin forming Schwann cells were also investigated in newborn nerves. At this stage in wildtype controls Schwann cells were typically found to have

surrounded a group of axons but as yet have not assumed the adult configuration where axons are segregated to isolate them from each other (Fig 4.6 B). In the Krox-24 null mutant, non-myelin forming cells were identified that had reached a similar stage to that of wildtype controls (Fig 4.6 D). These data show that the process of myelination is initiated on schedule in nerves of Krox-24 deficient mice as compared to wildtype littermates. Additionally the development of non-myelin forming Schwann cells also appears to progress in a normal fashion.

Rate of DNA synthesis in Krox-24 null mutant nerves is unaltered

Schwann cells have been shown to proliferate extensively during development and the peak of proliferation occurs a day or two before birth in the rat (Stewart *et al.* 1993). If Schwann cells lose axon signals through an injury response, resulting in axon degeneration, they are stimulated to enter the cell cycle and will proliferate extensively. Interestingly Krox-24 is expressed at high levels in both scenarios and suggests that it is somehow involved in proliferation.

BrdU injections were carried out at three different developmental time points and the number of BrdU positive cells within sciatic nerves was evaluated by immunohistochemistry. The highest level of BrdU incorporation was found at E18 the earliest time point assayed (Fig 4.7 A, B). By postnatal day 5, the number of nuclei within the nerve showing BrdU incorporation is greatly reduced (Fig 4.7 C, D, E, F) and in adult nerve no BrdU positive cells were observed (Fig 4.7 G, H). To quantify the BrdU incorporation within the sciatic nerve counts were made of BrdU positive nuclei expressed as a percentage of total nuclei present. At E18, 28.7 %+/- 3.5 (n=3) were found to be BrdU positive for wildtype nerves and 28 %+/- 1.5 (n=3)

for Krox-24 null mutants (Fig 4.8). At P5 less cells were BrdU positive with 3.8 % \pm 2.5 (n=3) for wildtype nerves and 4.2 % \pm 2.2 (n=3) for Krox-24 null mutant nerves. In the adult nerves isolated from either animal, BrdU positive nuclei were not detected. Comparison of percentages from wildtype and Krox-24 null mutant nerves at each stage were not statistically significant (P>0.05). These data demonstrate that proliferation, as assayed by DNA synthesis, is unaltered in sciatic nerves at the time points tested in Krox-24 deficient mice as compared to wildtype controls. Attempts were made to double immuno-stain to specifically label Schwann cells within the nerve that were BrdU positive. Technically this is a challenge because the protocol to reveal BrdU positive cells requires acid fixation (see methods). Two antibodies were found to give good labelling under these conditions, anti-GFAP (Fig 4.7 C, D) and anti-P₀ (Fig 4.7 E, F). These markers are of limited use as GFAP protein can be expressed by neuronal processes while P₀ is only expressed by approximately 60% of Schwann cells, namely those that are myelin forming. Although both of these proteins are cytoplasmic, the very close association of GFAP or P₀ positive cell cytoplasm (revealed by FITC) to BrdU positive nuclei leads to a yellow signal (arrows Fig 4.7, C, D, E, F). Although inconclusive, these findings suggest quite strongly that the nuclei taking up BrdU in these experiments were Schwann cells.

DISCUSSION

In this chapter data has been presented to explore the temporal expression of Krox-24, within the sciatic nerve of mice heterozygous for a Krox-24 “knock in” mutation utilizing the LacZ gene. Also the phenotype of nerves of Krox-24 null mutant mice has been investigated at the mRNA, protein and ultrastructural level in development and maturity. Finally the rate of proliferation, as assayed by DNA synthesis, within Krox-24 null mutant mice has been analysed.

Using the LacZ gene as a marker of Krox-24 gene transcription, I have shown that expression is predominantly a feature of late embryonic and early postnatal development. High levels of expression are detected at E18 and the highest at the newborn stage where the nerves appear diffusely blue. It is possible that all of the Schwann cells are positive, but the diffuse signal generated with this mouse line means one cannot be certain that this is the case. The transgenic construct used here utilised a version of the LacZ gene without a nuclear localisation signal (NLS), so β -galactosidase expression is cytoplasmic. The incorporation of an NLS would have localised expression to individual nuclei and allowed estimation of the total number of Schwann cells expressing Krox-24 at any given stage. By P5 the level of Krox-24 expression is markedly reduced, about 50%, compared to the newborn stage and in the adult is not detectable. I was unable to detect β -galactosidase expression in the nerve roots at E12 or E15, the Schwann cell precursor and immature Schwann cell stages, respectively. This contrasts with another study that found β -galactosidase expression in the nerve roots using this same mouse line at E11 (Topilko *et al.* 1997). This expression was detected at the thoracic level in spinal nerves, while here I have

investigated the lumbar hind limb region only and this difference may represent a rostral caudal difference in Krox-24 expression within embryonic spinal nerves. Also the authors did not assay the Schwann cell precursor stage, E12, as documented in this study. A more probable explanation for this difference could be the relative sensitivity of this technique in different hands, since Topilko and colleagues were able to detect low expression in adult nerves (possibly associated with non-myelin forming Schwann cells) which was not the case here (and see next chapter). Taking these anomalies into account, Krox-24 expression is clearly maximal over a period of about one week focused around birth. This data is consistent with the demonstration that Krox-24 mRNA is present in the sciatic nerves at high levels at birth and that levels decay as development proceeds, reaching low levels present in the adult (Watson and Millbrandt, 1990).

Does this expression pattern give any clues as to the putative function of Krox-24 in Schwann cells? The expression of Krox-24 would categorise it as a late transcription factor such as Krox-20 and Oct-6, rather than early transcription factors Sox10 or Pax3. The fact that the majority of Schwann cells are probably expressing Krox-24 at the newborn stage contrasts that of another Egr family member, Krox-20. Krox-20 is expressed by a minority of cells at the immature Schwann cell stage, in advance of myelination, and is then expressed by and localised to myelin forming Schwann cells as development ensues (Topilko *et al.* 1994, Murphy *et al.* 1996, Blanchard *et al.* 1996). Krox-20 is maintained in the adult myelin forming Schwann cells while Krox-24 expression is down-regulated within mature nerve. Similarly expression of the myelin gene, P_0 , is up-regulated and maintained only in myelin forming Schwann

cells (Lee *et al.* 1997). Thus a role for the Krox-24 gene in myelination seems unlikely.

The expression pattern of Krox-24 has similarities with that of a transcription factor from a different family, Oct-6. Oct-6 is expressed at highest levels at birth and is down-regulated in the mature nerve (Bermingham *et al.* 1996, Jaegle *et al.* 1996, Blanchard *et al.* 1996). Like Krox-24, but unlike Krox-20, Oct-6 expression can also be detected in all Schwann cells at the protein level (Blanchard *et al.* 1996). Two Oct-6 null mutant lines demonstrate that Schwann cells form typical 1:1 ensheathment relationships with axons but then myelination is somehow delayed by about two weeks (Bermingham *et al.* 1996, Jaegle *et al.* 1996). This is clearly different from Krox-24 null mice, where as I have shown myelination appears normal and on schedule.

The developmental expression pattern of Krox-24 and likewise Oct-6 bears a striking similarity to the rate of DNA synthesis in developing Schwann cells *in vivo*. Proliferation occurs during early Schwann cell development, with a peak on or about birth which decreases to zero by the second postnatal week (Stewart *et al.* 1993, U. Lange personal communication). Experiments designed to test directly if Krox-24 is required for Schwann cell proliferation have been presented here. No difference could be detected between wildtype and Krox-24 null mutant DNA synthesis rates within the sciatic nerve at three stages: E18, the peak of Schwann cell proliferation, at P5 a stage when proliferation is decreasing, and in adult nerve, when proliferation has ceased. An involvement of Krox-24 in Schwann cell proliferation is not indicated by these data. This data is consistent with that found in the developing

telencephalon of Krox-24 deficient mice where no loss of mitotic activity was detected (Ghorbel *et al.*, 1999).

Analysis of the ultrastructure of the components of mature sciatic nerve has revealed that Krox-24 deficiency does not result in any phenotypic abnormalities of myelin forming or non-myelin forming Schwann cells. This data is supported by the finding that the transcription factors Krox-20 and Oct-6 and proteins P_0 , p75NTR and NCAM, are expressed at normal levels, at the mRNA level, in Krox-24 null mutant nerves. Further to this, I have shown that the ultrastructure of sciatic nerves at the newborn stage is also normal with Schwann cells visible in normal stages of development whether myelin forming or non-myelin forming. This suggests that myelination occurs on schedule and this has been confirmed by the result that normal P_0 levels were detectable at the mRNA and protein level in the early postnatal animal. No differences were detected for all transcription factors and markers at the mRNA level at the newborn stage. I have also demonstrated that the perineurial sheath is normally formed in the nerves of Krox-24 deficient mice. The developmental expression of the signalling molecule Dhh is required for the formation of perineurium (Parmantier *et al.* 1999) and I have shown that Krox-24 deficient sciatic nerves do indeed express Dhh at the mRNA level at birth.

Taken together, these data suggest that mice lacking a functional Krox-24 gene do not experience any deficiency in the development and maturation of both myelin forming and non-myelin Schwann cells in the sciatic nerve. A number of studies have identified up-regulation of another Egr protein family member, Egr-3, in addition to Krox-24 upon stimulation of the suprachiasmatic nucleus and in the

hippocampus following seizure activity (Yamagata *et al.* 1994, Morris *et al.* 1998, O'Donovan *et al.* 1998). This raises the possibility of genetic redundancy between some Egr protein family members (Swirnoff and Millbrandt, 1995). To address this point the expression of Egr-3 mRNA was assessed at the newborn and adult stages where little difference could be detected between Krox-24 deficient and wildtype control samples. Egr-3 has not yet been described in Schwann cell development although D. B. Parkinson in the laboratory has identified mRNA in Schwann cells during development, with a peak at birth. The presence of Egr-3 at characteristic levels in Krox-24 deficient Schwann cells raises the possibility that Egr-3 can compensate for a lack of Krox-24 protein. In the hippocampus the profiles of Egr-3 and Krox-24 mRNA expression in response to a stimulus have been shown to be identical, however the protein profiles are very different with Krox-24 being transient and Egr-3 being sustained for considerably longer (O'Donovan *et al.* 1998). It may be interesting to explore if any differences exist in absolute Egr-3 protein quantities and if any modulation of Egr-3 protein longevity occurs in Schwann cells deficient in Krox-24 that may compensate for this deficiency. Recently estimates of the number of zinc-finger transcription factors proteins in *H. sapiens* of 600 to 700 raises the likelihood of genetic redundancy being a common feature of mammalian development and it remains to be seen how many more of these proteins are present in Schwann cells (Venter *et al.* 2001, I.H.G.S.C. 2001).

CONCLUSIONS

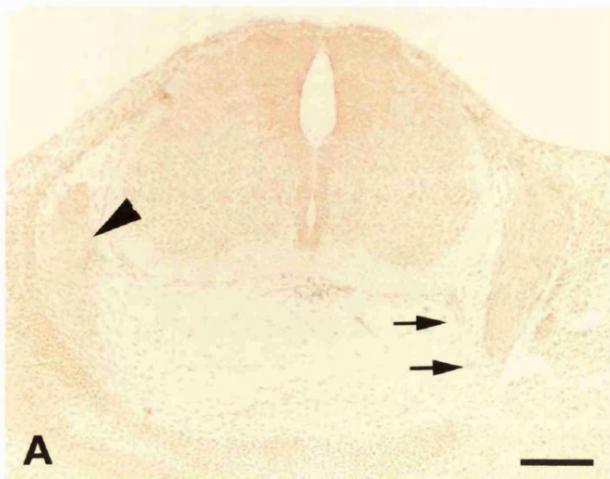
I conclude that Krox-24 is highly expressed in all Schwann cells at birth suggesting non-reciprocal expression patterns with Krox-20. I have shown that the phenotype and numbers of the two Schwann cell forms are normal in the adult Krox-24 null mutant mouse and therefore Krox-24 is not required for the normal development of non-myelin forming Schwann cells. Additionally an absence of the gene *in vivo* does not affect proliferation during development or alter the timing of the onset of myelination.

Figure 4.1 X-Gal staining reveals Krox-24 driven β -galactosidase expression

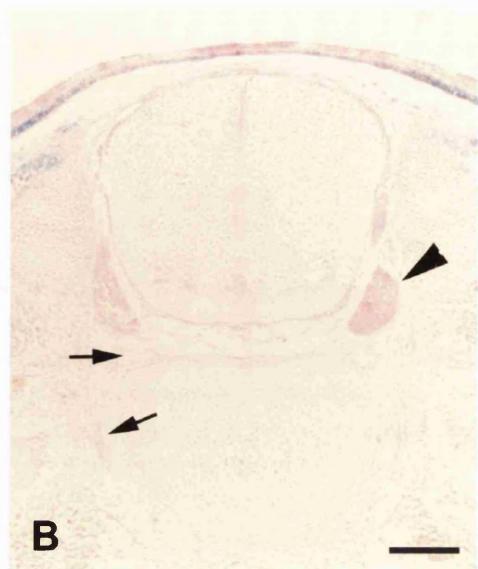
Light micrographs of sections through tissue derived from mice heterozygous for the Krox-24 mutation, assayed for β -galactosidase activity and counter stained with haematoxylin and eosin. Shown are sections through whole embryos at E12 (A), E15 (B) and longitudinal sections through sciatic nerves at E18 (C), P0 (D), P5 (E) and adult (F). Krox-24/ LacZ expression, visualised as a blue product, is absent from the developing nerve roots at E12 and E15 (arrows) as well as DRGs (arrowheads). Some expression can be seen in the deep epithelial cell layer flanking the neural tube at E15. High level expression can be seen at E18 and P0 with lower levels at P5. In the adult no blue product was detected.

Scale bars = 100 μ m.

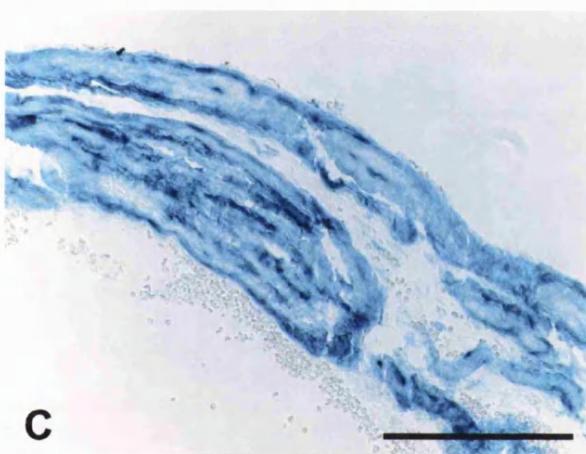
E12



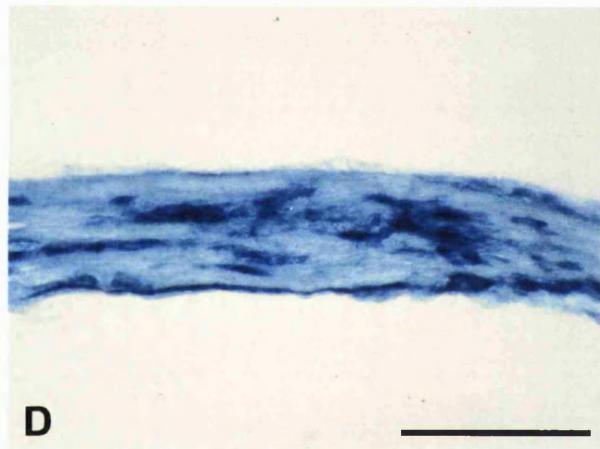
E15



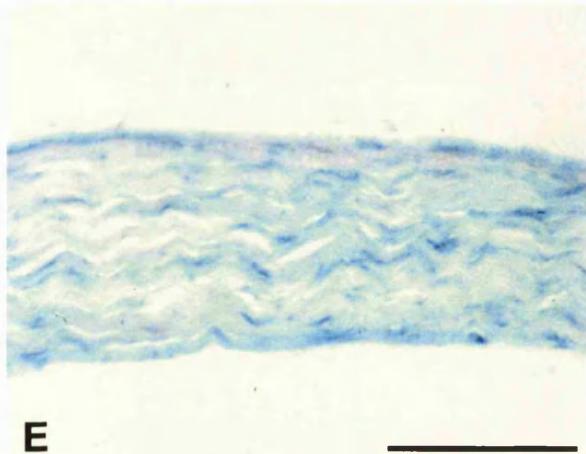
E18



P0



P5



Adult

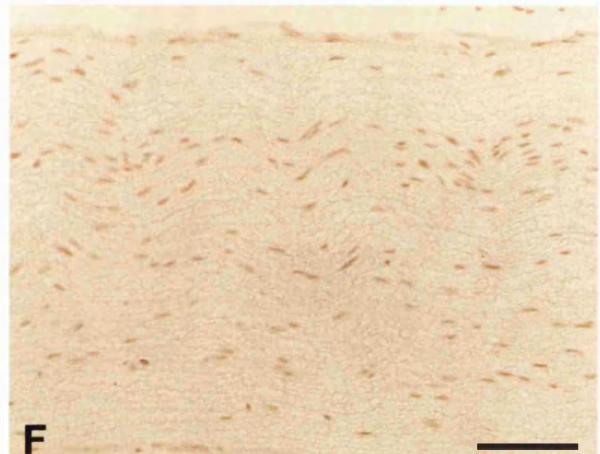


Figure 4.2 The ultrastructural morphology of adult Krox-24 deficient sciatic nerve

Transmission electron micrographs of cross sections through sciatic nerves, at the mid-thigh level, from wildtype (A, C, E $+/+$) and Krox-24 null mutant (B, D, F $-/-$). A single myelin forming Schwann cell surrounding a single small axon is shown (A, B). Note the nucleus of the Schwann cell and myelin sheath appearing as a dark band around the axon. Non-myelin forming Schwann cells characteristically support a number of smaller diameter axons and do not make myelin (C, D). The perineurium consists of 5, closely apposed, endothelial like cell layers with collagen fibrils in between that work in combination to protect and isolate nerve fibres bundles (E, F). No striking differences are apparent between wild-type and mutant perineurium.

Scale bars = 1 μ m

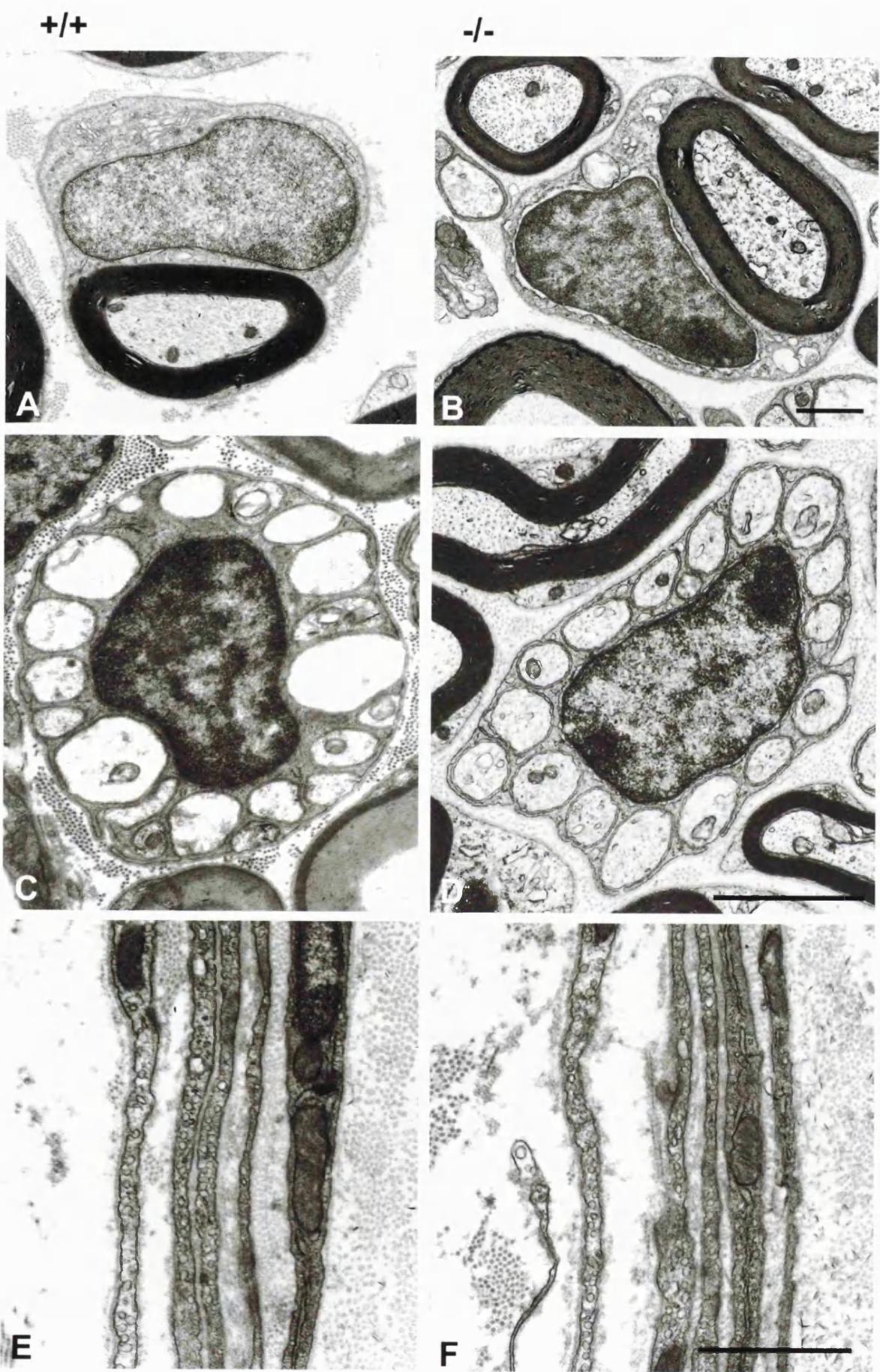
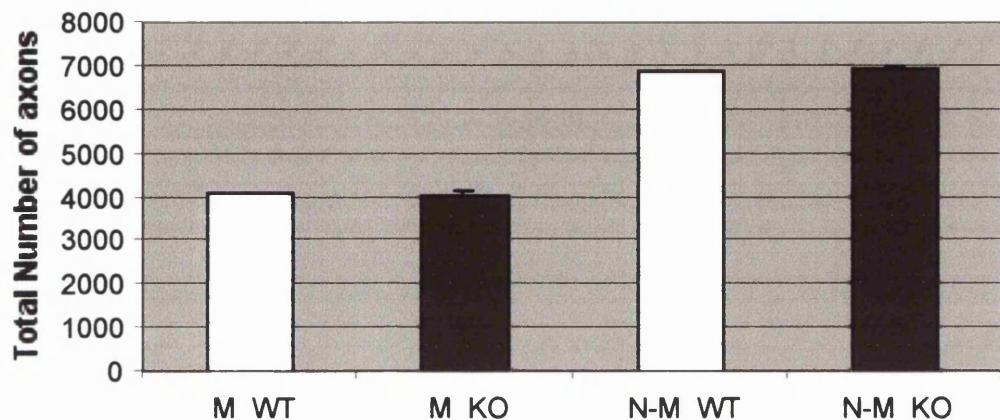


Figure 4.3 Quantification reveals normal numbers of myelinated and unmyelinated axons in Krox-24 null mutant sciatic nerves and the number of unmyelinated axons supported by individual non-myelin forming Schwann cells is also normal

Graphs to show relative numbers of myelinated (M) and unmyelinated (N-M) axons in wildtype (WT, white bars) and Krox-24 null mutant nerves (KO, dark grey bars) (A) and number of axons supported by individual non-myelin forming Schwann cells (B). Number of axons supported is represented in groups, 1-10, etc., which is plotted against frequency.

A

Number of Myelinated (M) and non-myelinated (N-M) axons in wildtype (WT) and Krox-24 deficient (K/O) sciatic nerves

**B**

Number of axons supported by single non-myelin forming Schwann cells

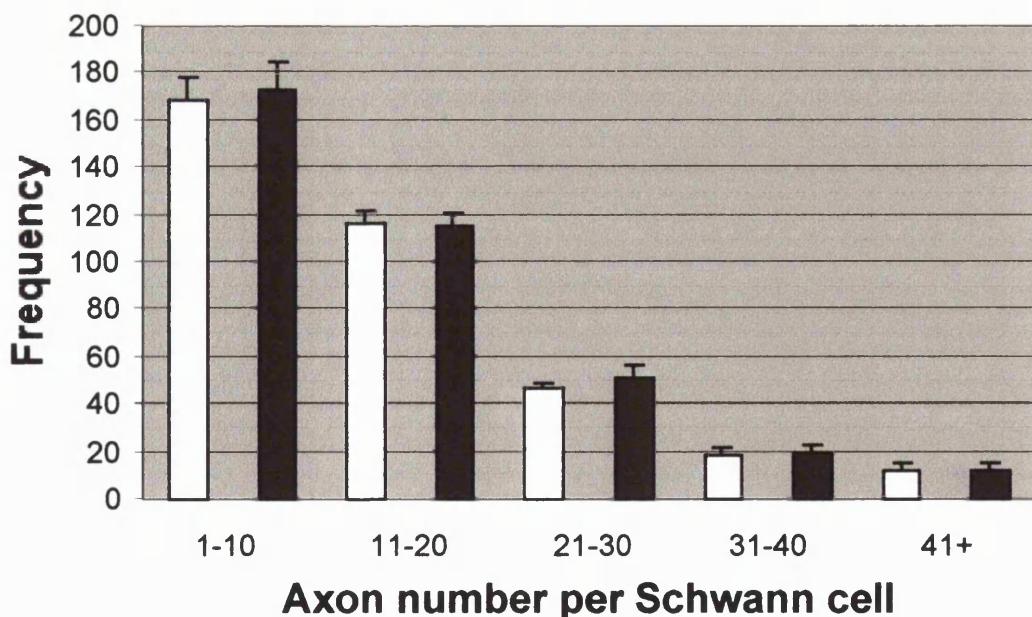


Figure 4.4 Detection of mRNA by RT-PCR of transcription factors and markers of Schwann cells in mouse sciatic nerve at birth and in the adult; comparison of wildtype and Krox-24 null mutant cDNAs.

Pooled cDNAs from sciatic nerves of newborn (left hand panels) and adult (right hand panels) were prepared from wildtype (+/+) and Krox-24 null mutants (-/-). Specific oligonucleotide primers were designed to compare the mRNA levels of markers by RT-PCR. The transcription factors Krox-24, Krox-20, Egr-3 and Oct-6 were analysed. Also a marker of myelin forming Schwann cells, P_0 , and non-myelin forming Schwann cells, p75NTR and NCAM were tested. Finally the signalling molecule, Dhh was tested at both stages.

Three of the transcription factors, Krox-24, Egr-3 and Oct-6 are highly expressed at birth but are reduced in the adult. In contrast Krox-20 mRNA expression is maintained at a similar level at both stages. Note that Krox-24 -/- cDNAs do not contain detectable levels of Krox-24 mRNA. P_0 is abundant at both stages, NCAM is reduced slightly in the adult and p75NTR is dramatically reduced in the adult from high levels at birth. For all of the primers tested (other than Krox-24), no differences were discernable between cDNAs isolated from wildtype or Krox-24 null mutant nerves.

Equal loading of template cDNAs was controlled using primers designed against 18S rRNA (Owens and Boyd, 1991). In all PCR reactions a water control (H_2O) was included to ensure against contamination.

RT-PCR

new born

+/- -/- H₂O



Krox-24
(34 cycles)



Krox-20
(34 cycles)



Egr-3
(34 cycles)



Oct-6
(35 cycles)



P0
(27cycles)



p75NTR
(30 cycles)



NCAM
(30 cycles)



18s
(24 cycles)



Dhh
(30 cycles)

adult

+/- -/- H₂O



Krox-24
(34 cycles)



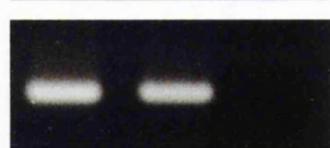
Krox-20
(34 cycles)



Egr-3
(34 cycles)



Oct-6
(35 cycles)



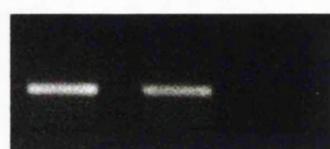
P0
(27cycles)



p75NTR
(30 cycles)



NCAM
(30 cycles)



18s
(24 cycles)



Dhh
(30 cycles)

Figure 4.5 Expression of P₀ protein occurs on schedule in Krox-24 deficient sciatic nerves.

Fluorescence images of transverse sections through wildtype (A, C +/+) and Krox-24 mutant (B, D -/-) sciatic nerves at E18 (A, B) and P2.5 (C, D). Sections have been stained with an antibody against P₀ and revealed with FITC (fluorescing green). Note that P₀ staining is absent from both nerves at E18 (A, B). Three days later at P2.5 P₀ positive staining is visualised as circular, doughnut, shaped structures, dispersed across the nerve consistent with this being an initial stage of myelination.

Nuclei within the nerves are labelled with DAPI and fluoresce blue.

Scale bars = 100μm.

+/+

-/-

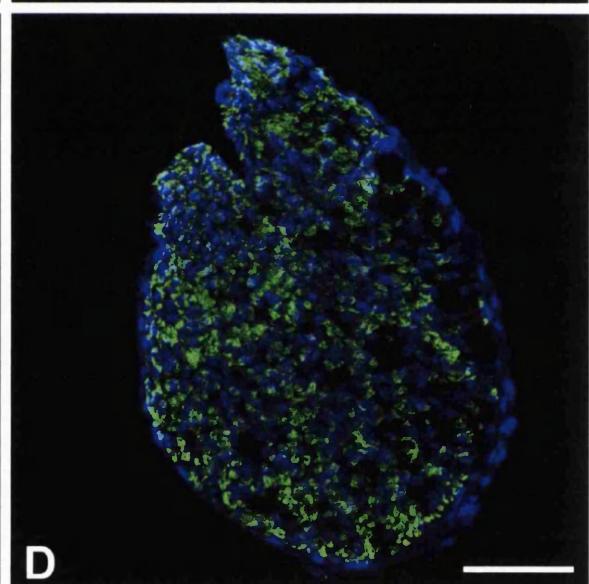
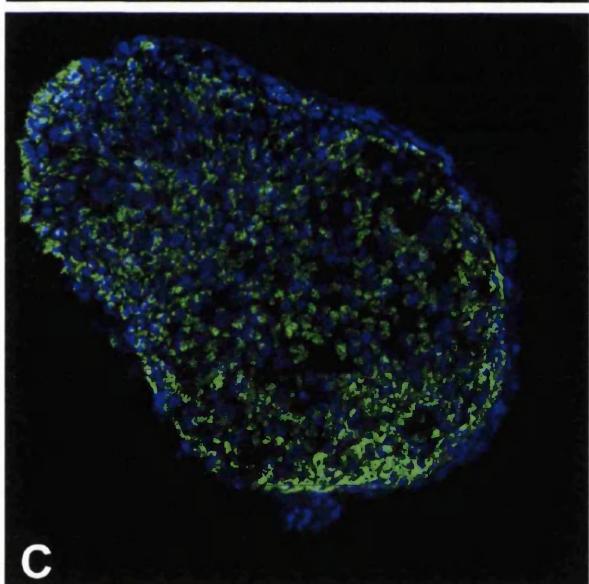
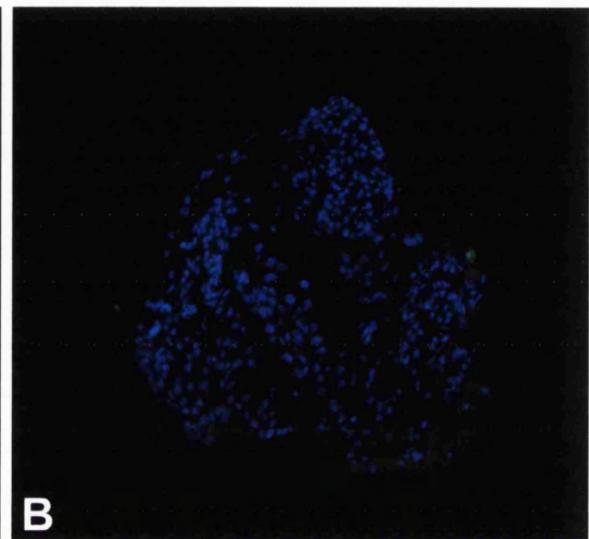
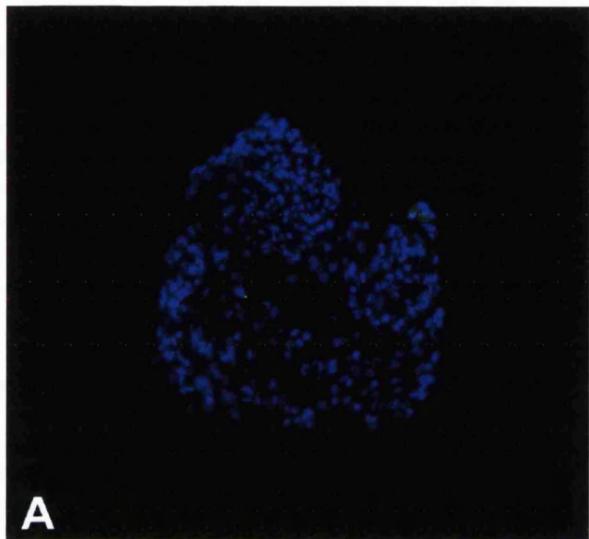
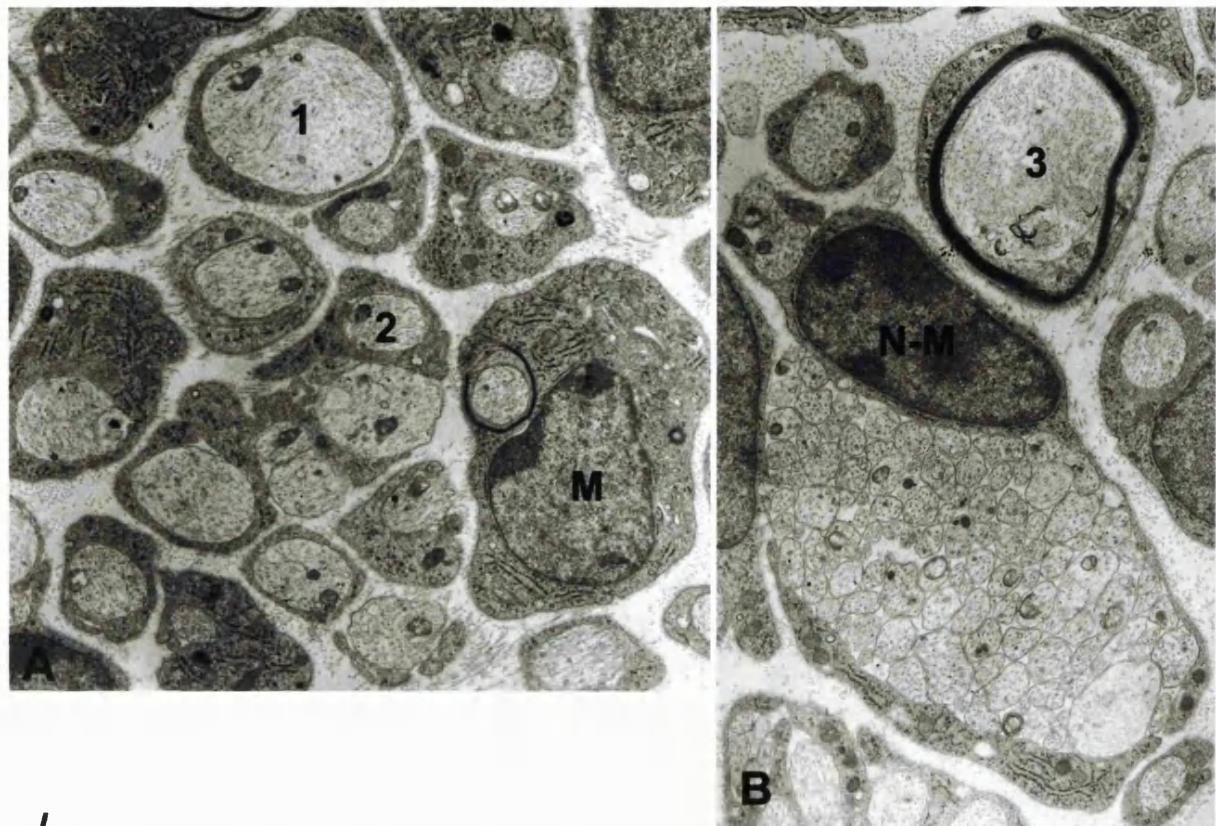


Figure 4.6 The ultrastructural morphology of newborn Krox-24 deficient sciatic nerve at the newborn stage

Transmission electron micrographs of cross sections through sciatic nerves, at the mid-thigh level, from wildtype (A, B) and Krox-24 null mutants (C, D). The nucleus of a myelin forming Schwann cell (M) can be seen in both +/+ and -/- nerves. Myelin forming Schwann cells can be identified that have formed 1:1 relationships with axons (1), that have initiated myelin wrapping (2) and that possess compacted myelin (3). Non-myelin forming Schwann cells (N-M) characteristically support a number of smaller diameter axons and can be seen to have begun this process as they surround families of axons (B, D).

Scale bars = 1 μ m

+/+



-/-

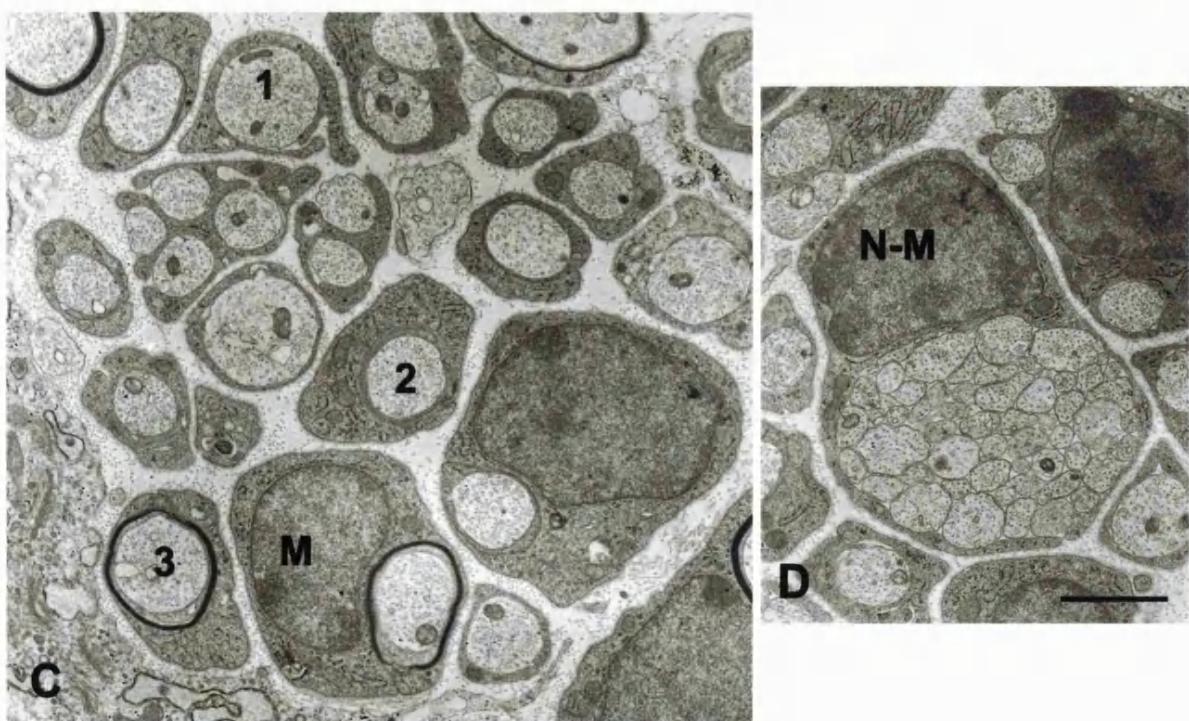


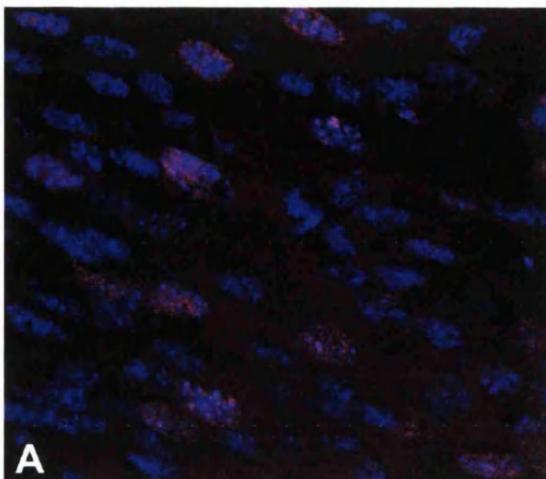
Figure 4.7 BrdU positive nuclei are detected in wildtype and Krox-24 deficient sciatic nerves.

Fluorescent images of sections through wildtype (A, C, E, G $+/+$) and Krox-24 mutant (B, D, F, H $-/-$) sciatic nerves. Longitudinal sections were taken of E18 (A, B) and P5 (C, D, E, F) and transverse sections of adult nerve (G, H). Sections have been stained with an antibody against BrdU and revealed with rhodamine (fluorescing red). Nuclei within the nerves are labelled with DAPI and fluoresce blue, except E, F. Nuclei that are BrdU positive and that are counterstained with DAPI appear magenta. Double immunohistochemistry was carried out with GFAP (C, D) and P₀ (E, F, G, H). Many BrdU positive nuclei can be seen at E18 (A, B), less are visible at P5 (C, D, E, F) and are absent in the adult

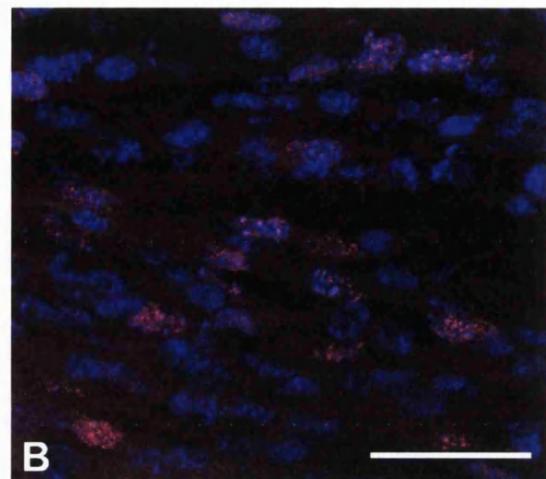
Scale bars = 50 μ m.

+/+

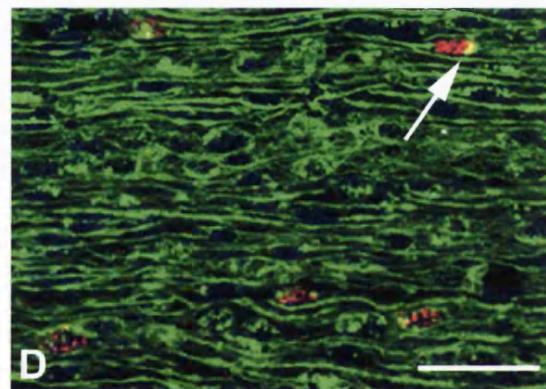
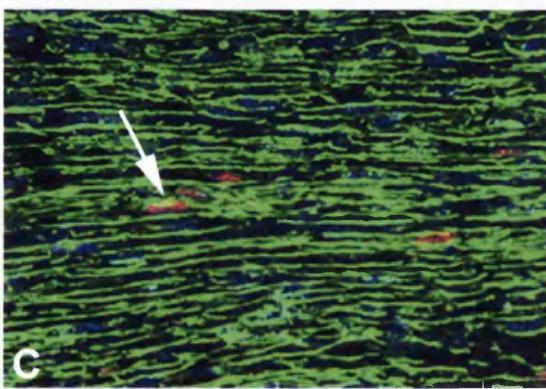
E18



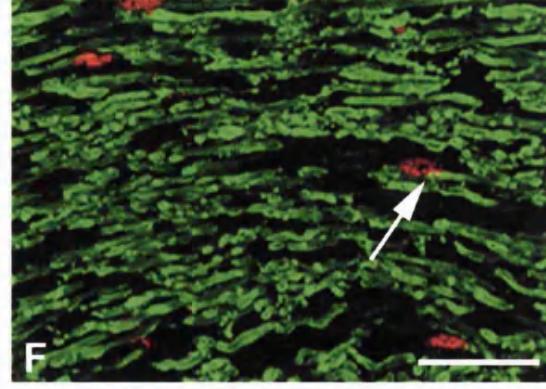
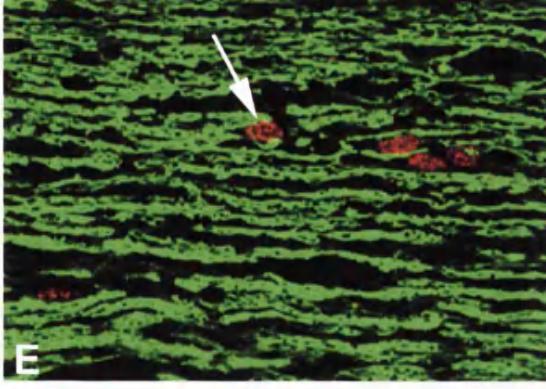
-/-



P5



P5



Ad

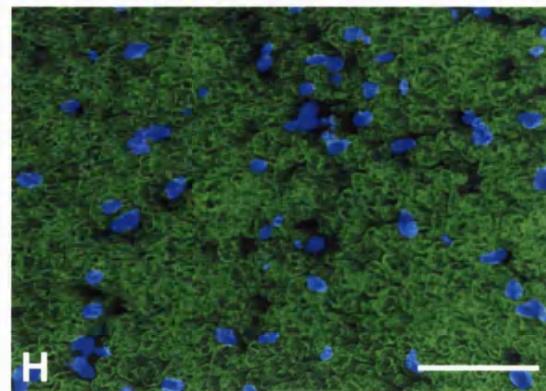
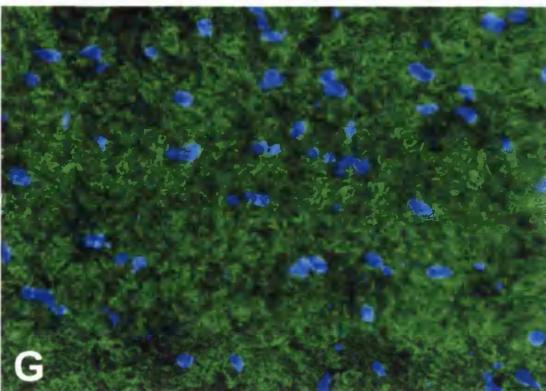
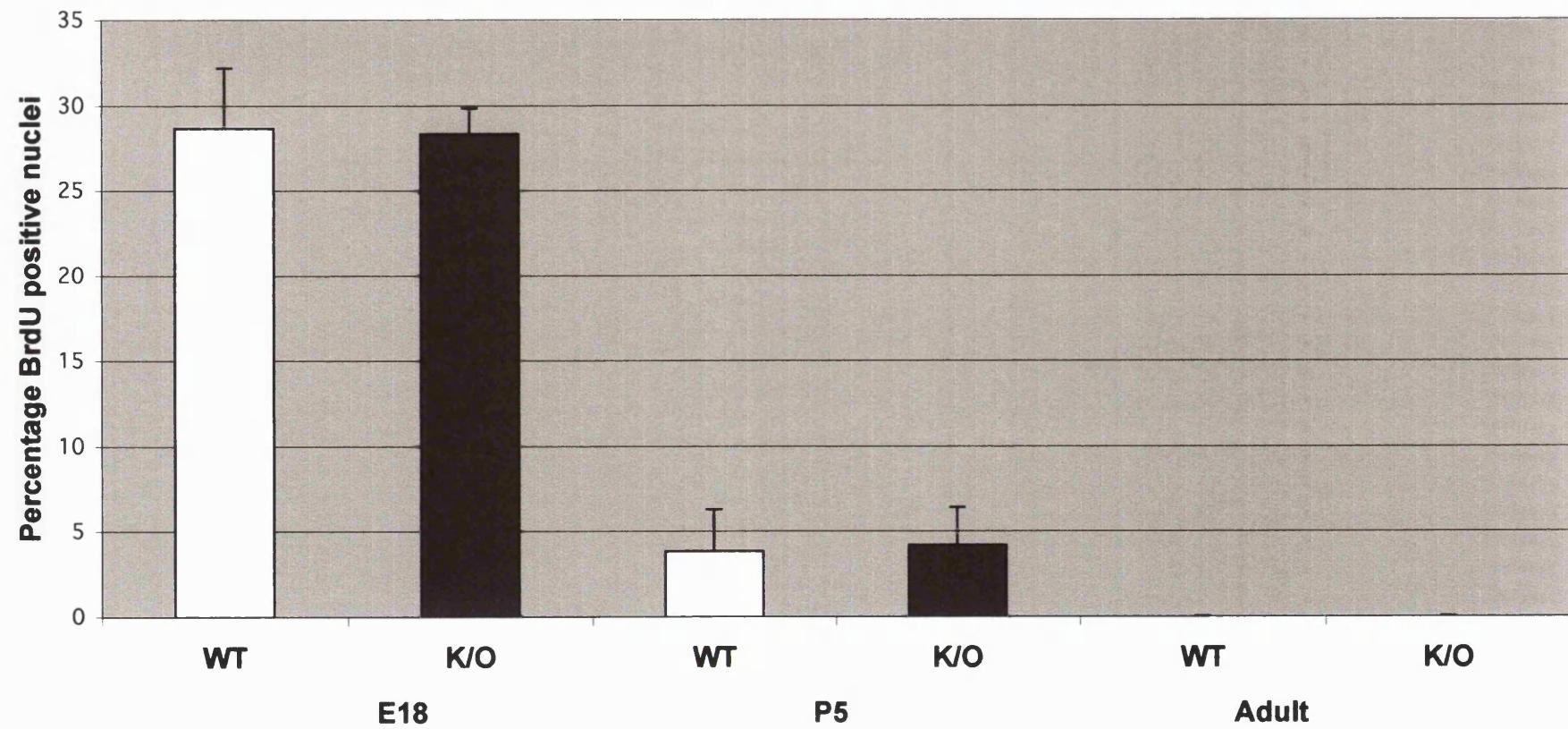


Figure 4.8 Quantification of DNA synthesis at three time points reveals little difference between wildtype and Krox-24 null mutant sciatic nerves.

Graph to show relative numbers of BrdU positive nuclei in wild type (WT) and Krox-24 null mutant nerves (KO) (A) at E18, P5 and adult.

Rate of DNA synthesis at E18, P5 and adult



CHAPTER 5

Regeneration of the PNS in mice deficient in Krox-24

INTRODUCTION

The plasticity of Schwann cells is perhaps nowhere more evident than during the processes of degeneration and regeneration. An injury resulting in the severing of an axon stops the distal portion of the axon from functioning as the connection to the neuronal cell body is interrupted. Such damage results in the initiation of a characteristic pattern of changes termed Wallerian degeneration after Augustus Waller, 1850. These changes include the breakdown of the axon, macrophage invasion, myelin clearance and Schwann cell reorganisation (reviewed by Fu and Gordon 1997, Scherer and Salzer, 1996).

Axon degeneration

After transection distal axons degenerate rapidly, and a process that begins within the first 24 hours is usually completed within 3 days (Ramon y Cajal 1928). This rapid degeneration can be delayed by the introduction of the protease inhibitor, leupeptin (Hurst *et al.*, 1984). In addition, breakdown of axonal cytoskeletal elements such as neurofilament has been shown to be dependent on the protease calpain together with Ca^{2+} ions (reviewed by Lundborg, 1988, George *et al.*, 1995). The mechanism of axon degeneration does not involve the caspase family of cysteine proteases, which mediate apoptotic cell death present in many systems during this development (Finn *et al.*, 2000). The slow Wallerian degeneration (Wld^{S}) mouse (formerly known as C57BL/Ola or *Ola* for short) has a dominant mutation that delays the processes of Wallerian degeneration substantially. Axons remain physiologically active 14 days after transection, associated

myelin remains intact and there is a delay in the down-regulation of the P_0 gene. (Lunn *et al.*, 1987, Thomson *et al.*, 1991). In wildtype mice axons are dysfunctional within 2 days and completely degenerate by the fourth day after axotomy. Mice with chimeric bone marrow were generated to demonstrate that the *Ola* mouse phenotype was not caused by a defect in a hematogenous element (Perry *et al.*, 1991). Sciatic nerve chimeras demonstrate that *Ola* derived Schwann cells are not responsible for the phenotype rather it is an intrinsic property of the axon itself (Glass *et al.*, 1993). The genomic region responsible for *Wld^S* has been mapped to the distal part of chromosome 4 and to a 85 kb tandem triplication mapping therein (Lyon *et al.*, 1993, Coleman *et al.*, 1998). Three genes within this repeat unit have been sequenced and identified; the ubiquitination factor *Ufd2*, a previously undescribed gene *D4Cole1e* and a novel retinoid-binding protein *Rbp7* (Conforti *et al.*, 2000). *Ufd2* and *D4Cole1e* are fused in frame producing a chimeric protein that is expressed in the nervous system, while *Rbp7* is intact and is overexpressed in tissues other than the NS eg. adipose tissue. *Ufd2* is involved in multiubiquitination proteolytic pathways in yeast and may underlie the *Wld^S* phenotype by competing with normal ubiquitination factors.

These studies clearly show the requirement of proteases for axon degeneration and the mechanism of ubiquitination that is involved. However the molecular executors in this process remain to be discovered.

Macrophages in the injured nerve

Macrophages mediate the demyelination in nerve injuries and influence Schwann cell proliferation and subsequent axon regeneration and remyelination (reviewed by Kiefer *et al.*, 2001). The injured nerve is quickly invaded by myelomonocytic cells, and in

particular macrophages, which are recruited to the injury site as well as the distal nerve portion (O'Daly and Imaeda, 1967, Olsson and Sjostrand, 1969, Clemence *et al.*, 1989, Stoll *et al.*, 1989, Monaco *et al.*, 1992). The principle role of these hematogenous macrophages is to phagocytose degenerating myelin (O'Daly and Imaeda, 1967, Perry *et al.*, 1987, Stoll *et al.*, 1989). There is strong evidence to suggest that myelin phagocytosis is complement-mediated both from *in vitro* (Beuche and Friede, 1984, 1986, DeJong and Smith, 1977) and *in vivo* studies (Hirata *et al.*, 1999). Other roles have been attributed to these cells including the secretion of Schwann cell mitogens (Baichwal *et al.*, 1988) and the release of interleukin-1 which in turn induces endoneurial neurotrophin expression (Heumann *et al.*, 1987, Lindholm *et al.*, 1987). Schwann cells themselves are able to degrade myelin *in vitro* (Bigbee *et al.*, 1987, Fernandez-Valle *et al.*, 1995) and, to a limited extent, are responsible for myelin clearance *in vivo* (Stoll *et al.*, 1989). The transition from myelinating Schwann cells to those capable of phagocytosis correlates with the expression of heme oxygenase 1 (HO-1), a 32 kDa heat shock protein (HSP-32) (Hirata *et al.*, 2000). In order to realise this function it is suggested that Schwann cells require the presence of macrophages (Perry and Brown 1992, reviewed in Fu and Gordon 1997).

Schwann cell responses to axotomy

Dennervated Schwann cells assume a phenotype more in line with those of non-myelin forming Schwann cells, characterised by the expression of p75NTR, GAP43, L1 and N-CAM (Scherer and Salzer, 1996). This process begins with the separation of myelin sheaths at incisures to form so-called ovoids. As myelin forming Schwann cells lose axonal contact they begin to down-regulate the expression of the specific myelin genes;

in transected nerves an almost complete loss of P₀, MBP, P2 and MAG proteins is seen after 14 days (Poduslo *et al.*, 1985, Gupta *et al.*, 1988, LeBlanc and Poduslo 1990). The de-differentiation of myelin forming Schwann cells is accompanied by a dramatic increase in proliferation (Abercrombie and Johnson 1946, Thomas 1948, Bradley and Ashbury 1970, Pelligrino *et al.*, 1986, Griffin *et al.*, 1990) which, at early stages, is attributable equally to both Schwann cell forms (Clemence *et al.*, 1989). Proliferating Schwann cells stain positively for antibodies to activated ErbB2 suggesting that NRG may be the mitogen involved, but the source of this factor remains unknown (Kwon *et al.*, 1997). Subsequent reinnervation induces Schwann cells to remyelinate and they stop expressing p75NTR, GAP43, L1 and N-CAM and begin to express genes associated with myelination such as MAG (Martini and Schachner, 1988, Taniuchi *et al.*, 1988, Hall *et al.*, 1992).

Schwann cells create a favourable environment for axon regeneration

The many Schwann cell basal laminae, formed during development, remain intact during degeneration and form Schwann cell packed conduits, known as bands of Büngner through which re-growing axons may travel (Ramon y Cajal 1928, Ide *et al.*, 1983, Bunge *et al.*, 1987, Hall 1989, Anderson *et al.*, 1983, Zhang *et al.*, 1995, reviewed Fu and Gordon 1997). Nerve cryoculture assays demonstrate that the neurite out-growth promoting ability of degenerated nerve segments is significantly enhanced compared to normal nerve and that nerve regeneration in basal lamina tubes is poor in the absence of living Schwann cells (Bedi *et al.*, 1992, Danielsen *et al.*, 1994, Agius and Cochard, 1998). Normal nerve forms a poor substrate for neurite outgrowth in such assays (Bedi

et al., 1992, Zuo *et al* 1998a) and this differential growth effect is due largely to the altered gene expression of Schwann cells.

Molecules upregulated by Schwann cells after lesion

Extracellular matrix molecules

In addition to the persistent basal laminae tubes, Schwann cells synthesise and secrete the ECM molecules laminin, fibronectin, entactin/nidogen, P200, F-spondin, collagens, proteoglycans and tenascin-C which are permissive for neurite outgrowth *in vitro* (Scherer and Salzer, 1996, Patton *et al.*, 1997, Burstyn-Cohen *et al.*, 1998, Chernousov *et al.*, 1999). These molecules may enhance regeneration but are not sufficient alone to allow axon regrowth, similarly the basement membrane is not sufficient for regeneration in an acellular graft where Schwann cells are prevented from entering with growing axons (Hall 1986). If Schwann cells are allowed to enter an acellular graft with invading axons, the axons can use the existing basement membrane as a substrate to efficiently reinervate the distal nerve stump (Ide *et al.*, 1983, Osawa *et al.*, 1990). Antibodies to F-spondin and laminin-2 can partially block neurite outgrowth on Schwann cells (Burstyn-Cohen *et al.*, 1998, Agius and Cochard, 1998). Integrins are receptors for ECM molecules (see general introduction) and antibodies to the $\beta 1$ subunit can inhibit outgrowth *in vivo* and *in vitro* (Toyota *et al.*, 1990, Agius and Cochard, 1998)

Laminin is a potent stimulus of axon out-growth (Bixby and Harris, 1991, Wang *et al.*, 1992) and Schwann cell migration (Bailey *et al.*, 1993, Milner *et al.*, 1997). In the normal nerve Laminin is masked by inhibitory chondroitin sulphate proteoglycan (CSPG), which can be disinhibited by treatment with matrix metalloproteinases (MMPs) (Zou *et al.*, 1998). Further, MMP-2 and MMP-9 expression are up-regulated after

sciatic nerve transection (Ferguson *et al.*, 2000), suggesting that MMPs may have a role in the creation of a more favourable axon growth environment. Recently, repeated freeze-thaw treatment to generate acellular nerve grafts has shown this process to remove laminin-1 and -2 which may explain the lack of axonal outgrowth within such a graft (Dubovy *et al.*, 2001). In addition the authors demonstrate that expression of laminin-1 and -2 and the integrin receptor $\alpha 6\beta 1$ are associated with Schwann cells that enter the nerve graft and goes some way in explaining the requirement for Schwann cell presence in nerve grafts for axon out growth.

Cell Adhesion molecules

Denervated Schwann cells express cell adhesion molecules (CAMs) including L1, CHL1, N-CAM, N-Cadherin and ninjurin (Scherer and Salzer, 1996, Araki and Milbrandt, 1996, Zhang *et al.*, 2000a). The expression of the adhesion molecules L1, CHL1 and N-CAM is up-regulated in the distal stump following nerve injury (Nieke and Schachner 1985, Daniloff *et al.*, 1986, Zhang *et al.*, 2000a). More specifically L1 and N-CAM have been shown to be dramatically increased on the surface of dedifferentiating myelin forming Schwann cells, while being sustained by the non-myelin forming Schwann cells (Jessen *et al.*, 1987a, Martini and Schachner, 1988). These molecules mediate Schwann cell interactions and are required for functional nerve recovery (Seilheimer *et al.*, 1989, Wood *et al.*, 1990, Remsen *et al.*, 1990, reviewed by Fu and Gordon 1997).

Neurotrophins

There are four members of the neurotrophin family (NT) in mammals: NGF, BDNF, NT-3 and NT-4 (reviewed in Ip and Yancopolous, 1996). NTs signal by high affinity interactions with specific tyrosine kinase receptors (Trks) and p75NTR. Schwann cells of the distal sciatic nerve secrete these factors and have been shown to promote neuronal survival and neurite outgrowth (Scherer and Salzer, 1996, Fu and Gordon 1997).

NGF

Expression of nerve growth factor (NGF) is increased at both the mRNA and protein level in transected nerves (Heumann *et al.*, 1987, Rush *et al.*, 1995). The NGF response to injury *in vivo* is biphasic with an initial peak within 24 hours followed by a slower increase after 2 days (Heumann *et al.*, 1987). This effect can be reproduced *in vitro* by the addition of activated macrophages, leading to the suggestion that macrophages modulate NGF expression in the response to injury (Heumann *et al.*, 1987). The expression of p75NTR, able to bind NGF, is also up-regulated in dedifferentiated Schwann cells of the distal stump (Taniuchi *et al.*, 1986, Heumann *et al.*, 1987, Robertson *et al.*, 1995). P75NTR up-regulation is first seen 24 hours after nerve transection, reaches a peak after 1 week and is maintained some 10 weeks after injury (Heumann *et al.*, 1987, Taniuchi *et al.*, 1988, Robertson *et al.*, 1995). In a crush injury, where regeneration occurs, the levels of both p75NTR mRNA and protein are up-regulated to a lesser extent and the effect is transient, with expression being suppressed by axonal contact (Taniuchi *et al.*, 1988, Robertson *et al.*, 1995).

In oligodendrocytes, the CNS equivalent of Schwann cells, NGF has been shown to bind to p75NTR and cause apoptosis *in vitro* (Casaccia-Bonelli *et al.*, 1996). There has been a similar demonstration in Schwann cells *in vitro*, an effect shown to be independent of Bcl-2 and absent in p75NTR mutant Schwann cells (Soilu-Hanninen *et al.*, 1999). In support of this, reduced apoptosis in the distal stump of mice lacking p75NTR 3 weeks after crush injury has also been seen (Ferri and Bisby 1999).

As well as a pro-apoptotic signal mediated via jun kinase (JNK) phosphorylation ligand binding to p75NTR also activates nuclear factor kB (NF-kB) in another intracellular pathway (Gentry *et al.*, 2000). Here NF-kB promotes survival in the RN22 Schwannoma cell line and blocking with NF-kB antibodies resulted in the cells becoming more susceptible to NGF induced cell death (Gentry *et al.*, 2000). The effect of increased p75NTR on the Schwann cell surface and extracellular NGF on Schwann cells themselves is unclear. One possibility is that Schwann cells present NGF to axons, and that availability of NGF controls axon number which in turn regulates the required number of Schwann cells via apoptosis (Taniuchi *et al.*, 1988).

BDNF, NT-3 and NT-4

BDNF and NT-4 have been identified as molecules upregulated following injury, but the temporal pattern of expression is less rapid than for NGF (Acheson 1991, Meyer *et al.*, 1992, Funakoshi *et al.*, 1993). The majority of BDNF mRNA expression is derived from Schwann cells (Friedman *et al.*, 1996). NT-3 expression is also increased after axotomy but the level is lower than for BDNF and NT-4 (Funakoshi *et al.*, 1993). NT-3 and BDNF promote axon regeneration (Utley *et al.*, 1996, Sterne *et al.*, 1997a), and

exogenous NT-3 promotes motor neuron regeneration and reinnervation of skeletal muscle (Sterne *et al.*, 1997b). When BDNF antibodies are applied locally to a site of lesion,

axon regeneration is retarded and myelination reduced by 83%, suggesting that Schwann cell derived BDNF is required for regeneration (Zhang *et al.*, 2000b). A chimeric protein that can activate all trks called, pan-neurotrophin-1, when overexpressed in Schwann cells (driven by the BDNF promoter) has been found to improve regeneration of all neuron classes in transgenic mice (Funakoshi *et al.*, 1998).

Cytokines

Three cytokines have been described in Schwann cells ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) (Scherer and Salzer, 1996). Motor, sensory and autonomic neurons retrogradely transport LIF and CNTF (Curtis *et al.*, 1993, 1994). CNTF is expressed by myelinating Schwann cells and expression is down-regulated following axotomy. CNTF and LIF may normally supply trophic support for motor neurons and adult null mutants develop motor neuron disease (Sendtner *et al.*, 1994, 1996). CNTF release occurs after axotomy by an unknown mechanism and is believed to encourage motor neurone regrowth (Sendtner *et al.*, 1997). LIF increases in the distal sciatic nerve following axotomy and is derived from Schwann cells (Banner and Patterson, 1994, Curtis *et al.*, 1994, Ito *et al.*, 1998). IL-6 and the IL-6 receptor (IL-6R α) are rapidly induced distal to nerve injury but the cellular source is disputed (Bolin *et al.*, 1995, Hirota *et al.*, 1996, Reichert *et al.*, 1996, Ito *et al.*, 1998).

The importance of these molecules in the process of peripheral regeneration is demonstrated by investigations of null mutants (Yao *et al.*, 1999, Zhong *et al.*, 1999). CNTF null mutant mice have been shown not to fully recover after undergoing sciatic nerve crush operations, in contrast to wildtype mice (Yao *et al.*, 1999). In mice lacking the cytokine IL-6 there is delayed sensory axon regeneration as determined by compound action potential measurement and the sciatic functional index (SFI) (Zhong *et al.*, 1999). Conversely, transgenic mice overexpressing IL-6 display enhanced motor neuron regeneration (Hirota *et al.*, 1996).

GAP-43

GAP-43 immunoreactivity is restricted to non-myelinating Schwann cells in mature peripheral nerve glia (Curtis *et al.*, 1992; Hall *et al.*, 1992). Following injury, previously myelinating Schwann cells begin to express GAP-43 mRNA and protein, increasing progressively for the following three weeks (Hall *et al.*, 1992; Scherer *et al.*, 1994a). Terminal Schwann cells located at the neuromuscular junction rapidly up-regulate GAP-43 following sciatic nerve denervation (Woolf *et al.*, 1992).

Other factors expressed by Schwann cells

Netrin-1

During development of the nervous system, axonal guidance is under trophic as well as attractive and repulsive chemotropic cues that are mediated by a diverse group of molecules (Song and Poo, 2001). Netrin-1 is just one example and new evidence suggests that in addition to developmental functions a role in regeneration is likely

(Madison *et al.*, 2000). Adult rat sciatic nerve shows low level expression of netrin-1 mRNA and following axotomy this is dramatically up-regulated.

Immunohistochemistry shows that netrin protein expression is associated with Schwann cells which may be the cellular source (Madison *et al.*, 2000).

Transforming growth factor- β (TGF β)

The TGF β family comprises 3 isoforms: TGF β 1, TGF β 2 and TGF β 3, and is closely related to activin, bone morphogenetic protein (BMP) and glial derived neurotrophic factor (GDNF) families (Scherer and Salzer, 1996). Their receptors are heterodimeric serine/threonine kinases, referred to as type I and type II receptors (Wrana *et al.* 1994). They also bind a third, type III receptor which is an ECM molecule β -glycan. Schwann cells synthesize all three TGF β s and expression is regulated by axonal contact (Mews and Meyer, 1993, Scherer *et al.* 1993, Einheber *et al.* 1995). During degeneration TGF β 1 mRNA increases while TGF β 3 decreases and regeneration results in a reversal of this reciprocal pattern. In culture TGF β 1 increases Schwann cell expression of L1 and N-CAM (Einheber *et al.* 1995, Stewart *et al.* 1995a) and inhibits forskolin induced differentiation to the myelin forming Schwann cell (Mews and Meyer, 1993, Einheber *et al.*, 1995, Stewart *et al.*, 1996). Also TGF β 1 can inhibit myelination in neuron / Schwann cell cocultures (Einheber *et al.*, 1995, Guenard *et al.*, 1995). These data suggest that TGF β 1 may act to maintain the undifferentiated state in immature Schwann cells or those undergoing Wallerian degeneration. Recently it has been shown that TGF β s can mediate the negative regulation of Schwann cell survival acting through caspase dependent mechanisms (Parkinson in press).

Transcription Factors

The proto-oncogenes c-fos, and c-jun, are immediate early genes, that have been shown to be highly expressed by Schwann cells some 12 hours after axotomy at the mRNA level and protein level (Liu *et al.*, 1995, Stewart *et al.*, 1995b, Shy *et al* 1996, Soares *et al.*, 2001). C-fos and c-jun belong to the basic-zipper (bZip) superfamily of transcription factors that form homo- and heterodimeric complexes (Kerppola and Curran, 1993). Thus, the concurrent expression of these two factors within denervated nerve probably forms the basis of a signaling mechanism involved in regeneration that has yet to be fully understood (Soares *et al.*, 2001).

Oct-6 expression is transiently upregulated, from low levels, 2 days after transection of adult rat sciatic nerves (Monuki *et al.*, 1990; Scherer *et al.*, 1994b). Krox-20 is down regulated following sciatic nerve transection, relatively slowly, some 3 to 10 days later (Herdegen *et al.*, 1993, Topliko *et al.*, 1997). Krox-24 is upregulated following sciatic nerve transection but in contrast to Krox-20 the onset is rapid correlating with the onset of p75NTR expression (Topliko *et al.*, 1997, Nikam *et al.*, 1995). Increased Krox-24 expression after injury is a response of peripheral glia alone as axotomised neurons do not up-regulate Krox-24 gene expression (Herdegen *et al.*, 1992). Interestingly Krox-24 is able to transactivate the p75NTR promoter and it is suggested that Krox-24 is required for the up-regulation of p75NTR in Schwann cells following nerve injury (Nikam *et al.*, 1995).

In this chapter a series of experiments are outlined to investigate the role of Krox-24 in axonal degeneration and regeneration, macrophage recruitment and Schwann cell dedifferentiation as well as subsequent re-differentiation in the mouse sciatic nerve.

AIMS

The Krox-24 gene is dramatically up-regulated in the sciatic nerve following denervation. I wanted to test the hypothesis that increased Krox-24 expression in Schwann cells may be involved in the processes of Wallerian degeneration and regeneration that occur with characteristic efficiency within the PNS.

RESULTS

Krox-24 expression is upregulated in both transection and crush paradigms

To check our experimental paradigms and assess at first hand the resulting expression pattern of Krox-24 in the sciatic nerve following injury, transection and crush operations were carried out on three month old, adult mice, heterozygous for the Krox-24 mutation. These animals, carrying a single allele for the inserted β -galactosidase construct, were allowed to survive for 1 week and were then sacrificed, the nerves dissected out and processed for β -galactosidase histochemistry using the chromogenic substrate, Bluogal. Any Krox-24 driven expression of β -galactosidase appears as blue staining, is visible by the naked eye and allows observations of the gross localisation of Krox-24 expression.

Krox-24 expression, visualised as a blue product, can be clearly seen in the region of sciatic nerve distal to the crush site together with a small amount of staining upstream of the wound site, marked with an arrowhead (Fig 5.1 A, B). The crush site is marked by an arrow, and does not display any blue staining; note that during removal of the nerve from the mouse the suture has drifted distally. Care was taken in future experiments to avoid this physical disruption of the suture marker. In the transected nerve, a similar pattern is seen (Fig 5.1 C, D) but the intensity of the blue product is increased. This suggests that Krox-24 expression is higher in this model of permanent injury (nerve transection), as compared to an injury allowing regeneration (crush injury). In particular the staining seen upstream of the wound site in the proximal stump is much more significant than in the crushed nerve.

At no time was blue staining seen in any of the contralateral control nerves. The blue product is localised to the nerve interior and is not seen in the perineurium, nerve sheath (Fig 5.1 D, arrow). Within one week of permanent transection all axons in the distal nerve have degenerated, and Schwann cells form by far the largest cellular component within the nerve, as they proliferate extensively. Thus it seems likely that the Krox-24 driven β -galactosidase expression seen is Schwann cell derived, but a contribution derived from endoneurial fibroblasts or invading macrophages in these experiments cannot be ruled out.

The RT-97 antibody labels axons within peripheral nerves

To address the potential of the peripheral nerves of mice deficient in Krox-24 to degenerate and regenerate in cut and crush paradigms, a good axon marker was required. The monoclonal antibody, RT-97, recognizing neurofilaments and generated by Prof. J. N. Wood was chosen (Wood and Anderton, 1981). To check the validity of using this antibody, immunohistochemistry was carried out on sections of adult sciatic nerve, taken just below the sciatic notch. RT-97 stains axons throughout a transverse section of sciatic nerve (Fig 5.2 A). This confirms other findings that suggest that RT-97 labels axons irrespective of whether they are afferent or efferent in nature (P. Anderson personal communication). Thus RT-97 was judged to be a good marker of axons for the following experiments. Shown for comparison is a consecutive section immunolabeled with an antibody to the major myelin protein P_0 (Fig 5.2 B). The numerous P_0 immunopositive myelin sheaths within this sciatic nerve section appear as concentric FITC positive rings surrounding hollow tubes that are unstained, the axons.

Axon degeneration is unaffected in Krox-24 mutants

When the sciatic nerve is injured by crushing or cutting, axon transection results and the distal portions of these axons are rapidly degraded. To test whether this rapid degradation is related to the upregulation of Krox-24 expression, the sciatic nerves of Krox-24 null mutant mice and wildtype litter mates were permanently transected and assayed four days later. Nerves were sectioned and immunolabelled for RT-97.

In the transected wildtype nerve RT-97 immunostaining is completely absent indicating that little or no intact neurofilaments remain and that the process of axonal degeneration is complete (Fig 5.3 A). For comparison a normal, un-operated, wildtype segment of nerve is shown (Fig 5.3 D). Axons within this section are clearly visible, stained by RT-97 and appear as longitudinal tracts within the nerve. The null mutant nerve closely resembles that of the wildtype litter mate. No RT-97 positive staining is seen indicating that the axons have degenerated with a similar time course (Fig 5.3 E). These results indicate that the initiation of the normal pattern of Wallerian degeneration, marked by the rapid digestion of axons in the distal peripheral nerve stump is not affected in Krox-24 deficient mice.

Krox-24 null mutants show efficient axon regeneration

When the sciatic nerve is subjected to a crush injury in which axons become completely transected but the perineurial sheath remains intact, axons proximal to the wound site regenerate while axons within the distal segment degenerate. Proximal to the wound site axons degenerate a short distance retrogradely and the corresponding nerve cell bodies

undergo characteristic changes associated with the process of regeneration (reviewed by Lieberman, 1971, Lundborg, 1988). Subsequently these proximal axons begin to regrow by sending out many terminal and collateral sprouts, which progress distally.

To ask whether Krox-24 is in any way required to create a permissive growth environment for peripheral axons, sciatic nerve crush operations were performed on three month old Krox-24 null mutant mice and their wildtype litter mates. Experiments were repeated 5 times and each group consisted of 6 animals, usually 3 wildtype and 3 Krox-24 null animals, with the occasional substitution of a heterozygous null mutant in place of a wildtype. Animals were sacrificed after 1 week and the nerves were sectioned and stained with RT-97 to label the newly regenerating axons. In both animals the degree of regeneration, as determined by positive immunostaining for RT-97, was found to be comparable (Fig 5.4). Axons can be clearly seen tracing a path from the more proximal aspect (left hand side in Fig 5.4) into the denervated region located more distally (the right hand side in Fig 5.4). These composite images represent half of the distance to the proximal injury site and the amount of axon regeneration equates to a distance grown for the majority of axons of 14mm.

To more accurately measure the extent of regeneration a modified experimental approach was adopted. A single group of 6 mice consisting of three wildtype and three Krox-24 null mutant mice were operated, crushing the nerves. After one week the sciatic nerves were removed as outlined in Figure 5.5. They were placed onto a grid and a 0.5cm nerve segment the most distal part of the tibial nerve was excised and then embedded for transverse sections (Fig 5.5 C). Sections were stained with RT-97 and the

few fast growing axons which had regenerated through the nerve could be observed (Fig 5.6). The number of axons that had reached this point was low and there was no obvious difference between Krox-24 null mutant nerves (Fig 5.6 B) and wildtype controls (Fig 5.6 A).

To confirm this, counts were made of the total number of axons that had regenerated to this point, 2 cm distal to the original crush site in three pairs of animals. It was found that in wildtype nerves $48 +/- 2$ RT-97 positive axons were present and in the Krox-24 null mutant nerves $44 +/- 4$ were found (Fig 5.7). These data were not significantly different ($P > 0.05$) and show that the population of rapidly regrowing axons is not altered in Krox-24 deficient mice. Thus it was concluded that the regeneration of both the bulk of axons together with the faster regenerating group occurs normally in the peripheral nerves of mice deficient in Krox-24.

Krox-24 null mutant Schwann cells down-regulate the myelin gene, P_0 , normally and myelin clearance within the mutant nerves occurs typically

Having established that the processes of axonal degeneration and regeneration occurred normally in Krox-24 null mutant animals, attention was turned to the cells that express Krox-24 in the sciatic nerve, the Schwann cells. As Schwann cells lose contact with axons through degeneration, they down-regulate the expression of the specific set of genes associated with myelination and as mentioned previously, existing derelict myelin proteins are phagocytosed (Poduslo et al., 1985, Gupta et al., 1988, LeBlanc and Poduslo 1990).

To investigate whether myelin genes are down-regulated following nerve transection the mRNA was isolated from the nerves of adult wildtype and Krox-24 null mice that underwent permanent sciatic nerve transection 1 week earlier as well as the contralateral controls. RT-PCR using primers directed against P₀ shows high level expression of the gene at the mRNA level in both control wildtype nerves (Fig 5.8 +/+) and control Krox-24 null mutant nerves (Fig 5.8 -/-). These results are consistent with the ongoing process of myelin protein turnover associated with normal myelin present in wildtype nerves and Krox-24 null mutant nerves (see also Chapter 4). Schwann cells stop expressing myelin genes when they lose contact with axons and this is reflected in the lack of P₀ mRNA expression seen here (Fig 5.8 +/+T). Schwann cells that are deficient in Krox-24 also down-regulate the expression of P₀ mRNA following transection (Fig 5.8 -/-T) suggesting that Schwann cells with this null mutation respond as do wildtype cells to denervation at the mRNA level.

To investigate whether myelin genes are down-regulated at the protein level and that myelin is cleared in the nerves of Krox-24 mutant mice, whole protein extracts were collected from 1 week post transected nerves and contralateral control nerves from Krox-24 mutant mice together with wildtype litter mates and immunoblotted for P₀. The single large band, 32kD in size, seen in lane 1 reflects the large quantities of P₀ protein that are found in the mature sciatic nerve and this can be observed to be reduced 1 week after transection (Fig 5.9, lane 2). This difference can just be seen in the upper panel with 1 μ g total loading but becomes more obvious with the lower total protein loading of 250ng. An almost identical pattern of bands is revealed for the null mutant animal also,

lanes 3 and 4 (Fig 5.9), suggesting that not only is P_0 present in normal quantities in mature nerves but that it is removed during myelin phagocytosis with equal efficiency.

p75NTR expression is up-regulated by denervated wildtype Schwann cells and in Schwann cells of Krox-24 null mutants expression is enhanced at both mRNA and protein levels

Expression of p75NTR mRNA in the normal adult mouse sciatic nerve is present at relatively low levels and is associated with non-myelin forming Schwann cells (Taniuchi *et al.*, 1986, 1988, Heumann *et al.*, 1987 and see also Chapter 4). This was confirmed for both wildtype and Krox-24 deficient sciatic nerves by RT-PCR (Fig 5.8 +/- and -/-). Following transection a modest up-regulation of p75NTR mRNA was seen in wildtype nerve which is just visible (Fig 5.8 +/T). This is consistent with data that shows that p75NTR is up-regulated following injury (Taniuchi *et al.*, 1986, Heumann *et al.*, 1987, Robertson *et al.*, 1995). Interestingly, up-regulation of p75NTR mRNA in the sample derived from Krox-24 null mutants was increased compared to the wildtype (Fig 5.8 -/T). This difference could represent an increase of between 2 and 3 fold as compared to the wildtype, but estimates of fold differences are very approximate using RT-PCR, as this technique is at best semi-quantitative.

To investigate if this mRNA difference was accompanied by a difference at the protein level, immunohistochemistry using an antibody against p75NTR was carried out. Schwann cell expression of p75NTR protein is difficult to detect in adult mouse sciatic nerves as it is present at relatively low levels (Fig 5.10 A.). Following transection p75NTR protein is present at increased levels in the nerves of wildtype mice (Fig 5.10

C.). This pattern of up-regulation was also seen in the Krox-24 null mutant, but the level of p75NTR protein appeared to be greater than for the wildtype, closely matching the pattern of mRNA expression found above (Fig 5.10 E.). Together these results showing increases in both p75NTR mRNA and protein strongly suggest that p75NTR up-regulation following axotomy is enhanced in Schwann cells deficient in Krox-24 in comparison to wildtype controls.

Cell adhesion molecule expression is up-regulated by Schwann cells of Krox-24 null mutants

Dedifferentiating Schwann cells have been shown to up-regulate the expression of the cell adhesion molecules L1 and N-CAM (Jessen *et al.*, 1987a, Martini and Schachner 1988).

L1 mRNA was analysed by RT-PCR and similar levels were found in both wildtype (+/+) and Krox-24 null sciatic nerves (-/-) (Fig 5.8). Interestingly the levels within both nerve samples following transection were lower (+/+T, -/-T) than in unoperated samples. L1 immunohistochemistry shows that L1 expression is up-regulated after injury in dedifferentiating Schwann cells (Fig 5.11 D.) from an existing pattern where expression is exclusively localised to the non-myelin forming Schwann cells (Fig 5.11 A.). In the sciatic nerve lacking Krox-24 L1 expression due to injury is also up-regulated (Fig 5.11 D.). There appears to be little difference in L1 expression, or up-regulation after injury in the sciatic nerves of Krox-24 null mice compared to wildtypes. It was interesting to note that the expression of L1 mRNA 1 week after transection was lower than in normal controls and that the protein expression did appear to be increased. Possibly L1 protein

stability is enhanced, or turnover increased following injury or the peak of L1 mRNA expression occurs earlier than was tested here.

N-CAM is normally expressed in three different isoforms running at three different sizes, 180 kD, 140 kD and 120 kD as shown in lane 1 (Fig 5.12) in an immunoblot of N-CAM on wildtype sciatic nerve (reviewed in Edelman and Crossin, 1991). One week after sciatic nerve transection the expression pattern of these three N-CAM isoforms changes; the largest 180 kD isoform is down-regulated while the 140 kD is up-regulated (Fig 5.12 lane 2) (Remsen *et al.*, 1990). This differential regulation of the different isoforms is a result of different cellular localisation, with the large 180 kD isoform being present within axons and the 140 kD isoform associated with Schwann cells. Thus axon degeneration leads to a loss of the large isoform and Schwann cell de-differentiation leads to an increase in the 140 kD N-CAM isoform.

Krox-24 mutant sciatic nerves show a normal level of expression of the three different N-CAM isoforms (Fig 5.12 lane 3) and the trend in changes of these isoforms following sciatic nerve transection closely parallels that seen in the wildtype (Fig 5.12 lane 4).

Krox-24 null mutants show normal remyelination following injury

Having demonstrated that axons can regenerate with equal efficiency in Krox-24 null mutant mice the question whether the Schwann cells of these animals were competent to remyelinate the new axons remained. It is possible to visualise the morphology of nerve fibres and associated myelin using toluidine blue staining and high magnification light microscopy. Semi-thin transverse sections of sciatic nerve two weeks post crush injury exactly 5mm distal to the original crush site show the presence of myelin, visible as concentric black rings (Fig 5.13). These are clearly visible in both the wildtype nerve

(Fig 5.13 A) and the Krox-24 null mutant nerve (Fig 5.13 B). Counts were made at that same level from 2 wildtype and 2 Krox-24 null mutant sciatic nerves two weeks post crush. 1235 +/- 60 myelinated fibres were found in the wildtype nerves while the Krox-24 null mutant nerves showed 1268 +/- 86, with no significant difference between the two ($P>0.05$). These numbers are comparable with those found in previous studies of regeneration in mouse sciatic nerve (Tanaka and Webster, 1991, Uziyel *et al.*, 2000). Finally to quantify the progression of myelination at the molecular level immuoblotting for P_0 was employed. Four pairs of 3 month old mice were operated, crushing one nerve per animal, and these nerves together with the contralateral controls were excised after two weeks and the proteins extracted. Similar, high, levels of P_0 protein are present in the control non-transected samples of wildtype and Krox-24 null mutant nerves (Fig 5.15 lane 1 and 3). Lower levels of the protein are detected in both wildtype and Krox-24 null mutant lesion samples, consistent with the ongoing process of remyelination that may take months to complete (Fig 5.15 lane 2 and 4). No difference could be seen between the wildtype and Krox-24 deficient regenerating samples, suggesting that P_0 protein is re-expressed at normal levels by Krox-24 deficient Schwann cells. These data provide strong evidence that Krox-24 deficient Schwann cells have the capacity to myelinate after a period when contact with axons is lost, and that the temporal progress of myelination is unaltered compared with wildtype controls.

DISCUSSION

Krox-24 gene expression has been shown to increase in the PNS, specifically in the distal sciatic nerve, after injury at the protein level (Topilko *et al.*, 1997). The experiments presented in this chapter represent an attempt to elucidate the possible function for this gene in the response to peripheral injury. They demonstrate that Krox-24 gene up-regulation is a feature of the entire distal sciatic nerve following injury and to a lesser extent the proximal nerve portion, local to the wound site. Also they show that a mutation in the Krox-24 gene, rendering it dysfunctional, does not affect the processes of degeneration and regeneration in peripheral neuronal axons. The response of the major glial cell of the peripheral nerve, the Schwann cell, to axonal loss remains unmodified, at least with respect to the parameters examined in this chapter. Myelin gene expression is down-regulated as assayed by P_0 , N-CAM gene expression increases and L1 expression alters in the normal fashion. Interestingly the up-regulation of p75NTR associated with Schwann cells after denervation is enhanced in the Krox-24 deficient sciatic nerve. In addition, the ability of mutant Schwann cells to respond to axonal contact and to subsequently remyelinate regenerating axons is not compromised.

In this study the expression of Krox-24 within the sciatic nerve has been shown, one week following crush or transection. Krox-24 is found throughout the distal nerve portion and, to a lesser extent, in the proximal stump close to the site of injury (Fig 5.1) and this pattern is consistent with known data (Topilko *et al.* 1997). The expression of Krox-24 is most likely to be Schwann cell derived and associated with a loss of axonal contact, although this data does not prove this unequivocally. The intensity of staining

was typically more intense in the model of permanent transection (Fig 5.1 C, D), when compared to the crush nerve (Fig 5.1 A, B). This difference probably reflects the degree of axon loss in these two paradigms: the former results in complete axon loss and prevents regrowth while the latter allows axon regeneration. Thus one week after crush considerable numbers of axons have regenerated and a proportion of Schwann cells are now in contact with axons again. Similar crush experiments were carried out except that the recovery period was extended to two months, and in this case no β -galactosidase expression was seen indicative that Krox-24 expression was absent. Thus it is likely that Krox-24 expression is down-regulated upon reinervation (data not shown). These experiments reinforce existing data showing that Krox-24 is up-regulated in nerve injury paradigms and show additionally that the degree of Krox-24 expression is probably proportional to total axon loss.

Both of the processes of degeneration and regeneration occur in a temporally correct sequence and with normal efficiency in Krox-24 null mutants when compared with wildtype litter mates (Fig 5.3, 5.4, 5.6, 5.7). Degeneration of distal axons is a rapidly occurring process and is completed early after an injury (Ramon y Cajal 1928, Hurst *et al.*, 1984, reviewed by Lundborg, 1988, George *et al.*, 1995). In the slow Wallerian degeneration (Wld^S) mouse axons remain physiologically active 14 days after transection, and associated myelin remains intact which is probably due to the production, by duplication, of a chimeric protein of the ubiquitination factor, Ufd2 (Lunn *et al.*, 1987, Thomson *et al.*, 1991, Conforti *et al.*, 2000). The results obtained here show that this process is not delayed in the nerves of Krox-24 deficient mice (Fig 5.3 E) when compared to wildtype controls (Fig 5.3 A) and axons are completely

degraded within four days. The Krox-24 gene is unlikely, therefore, to be involved in the process of Ca^{2+} and protease dependant axonal degradation mechanisms.

The sciatic nerve is a composite structure consisting of an efferent population of myelinated motor axons and unmyelinated sympathetic axons, together with an afferent population of both myelinated and unmyelinated sensory axons (Schmalbruch, 1986). Recently the speed of regeneration of these different populations, in the injured rat peroneal nerve, has been shown to vary. Myelinated sensory afferents are the first to regenerate, within the first two weeks, followed by myelinated motor axon regeneration some 10 weeks later (Kawasaki *et al.*, 2000). In mice lacking the multifunctional cytokine IL-6 there is delayed sensory axon regeneration as determined by compound action potential measurement and the sciatic functional index (SFI) (Zhong *et al.*, 1999). The experiments shown in this chapter do not directly address the question of the differential rate of regeneration of the various sub-populations of neuronal projections within the sciatic nerve. However, counts of axons that had regenerated 2cm or more in only one week showed no difference between Krox-24 deficient and wildtype nerves (Fig 5.7). These axons are fast growing at a rate in excess of 2.86 mm per day and it is possible that they are sensory in nature as suggested by Kawasaki and colleagues (2000). Also, it was noted that animals allowed to recover completely following sciatic nerve crush, after three months, did not display any visible impairment suggesting a degree of functional recovery (data not shown). In support for this finding efficient myelination was evident two weeks post sciatic nerve crush in Krox-24 mutant animals (Fig 5.13, 5.14, 5.15).

The rate of axon outgrowth of at least 2.86 mm arrived at in this study, is comparable to studies of both rat and rabbit, which have shown a range of rates varying

between 3.0 – 4.4 mm per day (reviewed by Lundborg, 1988). In the mouse such a rate, allowing for a 1 – 2 day initial delay period would mean axons could reach target tissue within 1 week of injury.

The regeneration seen in the null mutant animal used in this study is comparable to that of the wildtype litter mates and this finding is reflected in the changes in the expression pattern of two Schwann cell proteins, L1 and N-CAM that are implicated in nerve regeneration. These molecules are expressed at low levels in adult sciatic nerve and are restricted to the non-myelin forming Schwann cells (with the exception that a small amount of N-CAM is detectable on murine myelin-forming Schwann cells) (Jessen *et al.*, 1990, Martini and Schachner 1986, Jessen *et al.*, 1987, Mirsky *et al.*, 1986). L1 and N-CAM expression is up-regulated by dedifferentiating myelin-forming Schwann cells (Jessen *et al.*, 1987a, Martini and Schachner 1988). N-CAM encourages axon outgrowth *in vitro* while antibodies to L1 prevent such outgrowth suggesting that they may be required for regeneration *in vivo* (Schachner 1990, Seilheimer *et al.*, 1989, Wood *et al.*, 1990, Remsen *et al.*, 1990, reviewed by Fu and Gordon 1997). No differences were detected for both of these molecules in sciatic nerves of mice deficient in Krox-24. This shows that the Schwann cells within Krox-24 deficient nerves are competent to produce favourable environments for regeneration, with respect to these two molecules assayed in these experiments.

P75NTR gene expression is up-regulated after injury (Taniuchi *et al.*, 1986, Heumann *et al.*, 1987, Lemke and Chao, 1988, Mirsky and Jessen, 1990, Robertson *et al.*, 1995). Experiments above show that the relative expression of p75NTR is enhanced at both mRNA and protein levels in Krox-24 deficient sciatic nerves when compared to wildtype controls. Many attempts were made to quantify the difference at the protein

level by western blotting; however these were not successful, since a large number of positive staining bands running at different sizes were seen. The feeling was that this was due to background staining caused by the secondary antibody and this meant that identification of the p75NTR protein was not possible with any degree of confidence. Krox-24 interactions with p75NTR have been previously reported where it was shown that overexpression of Krox-24 antisense cDNA in cultured Schwann cells resulted in ablation of p75NTR expression (Nikam *et al.* 1995). This has led the authors to suggest that Krox-24 is required for the expression of p75NTR *in vitro*. The results arrived at in this study are conflicting, suggesting that Krox-24 deficient Schwann cells respond to axonal loss with increased p75NTR expression. This difference is hard to reconcile but it should be noted that normal adult levels of p75NTR protein were detected in Krox-24 deficient sciatic nerves (data not shown) and that p75NTR mRNA is detectable in neonates (see chapter 4). These findings are indicative that Krox-24 is not required for p75NTR expression *in vivo* in the transgenic mutant animal studied here.

The significance of the p75NTR up-regulation on Schwann cells and its relevance to the process of regeneration remains unknown. Taniuchi and colleagues (1988) postulate that p75NTR expressed in large quantities on the Schwann cell surface binds NGF and other neurotrophins and presents a substrate loaded with trophic support to growing axons. The assumption to be drawn from this hypothesis would be that regeneration in p75NTR knockout mice would be impaired, but this experiment has yet to be carried out. In the CNS it has been shown that transgenic mice overexpressing NGF, but lacking p75NTR, have enhanced sympathetic fibre growth which suggests that the regenerative action of neurotrophins does not require p75NTR, at least in the CNS (Walsh *et al.*, 1999). Presumably interactions of excess NGF with the high affinity trk A

receptor are responsible for this result, which really throws doubt on p75NTR up-regulation as part of a regenerative mechanism. It will be interesting see what occurs during regeneration in p75NTR mutant mice within the PNS. Alternatively, there is now a body of evidence to suggest that the p75NTR mediates apoptosis in glial cells (Cassacia-Bonnel *et al.*, 1996, Soiliu-Hanninen *et al.*, 1999, Ferri and Bisby 1999), and this point is addressed in greater detail in the following chapter.

In the Krox-24 null mutant at least one element of the inflammatory response, phagocytic macrophage invasion, remains unaltered (Fig 5.13 and data not shown), a feature that is, perhaps, indicative that Krox-24 does not play a role in this response. Further support for this finding comes from the demonstration that myelin clearance is normal in Krox-24 mutants (Fig 5.9) suggesting that the macrophages are actively engaged in their main function, namely that of myelin phagocytosis (O'Daly and Imaeda, 1967, Perry *et al.*, 1987, Stoll *et al.*, 1989).

Growth factors may also warrant investigation as Krox-24 is implicated in the transcriptional regulation of PDGF-A, PDGF-B, bFGF and also TGF β (Khachigian *et al.*, 1995, Silverman *et al.*, 1997, Rafty and Khachigian 1998, Biesiada *et al.*, 1996). Of these PDGF-B has been shown to be one of three factors contributing to the Schwann cell survival autocrine loop (Meier *et al.*, 1999), while TGF β is made by Schwann cells and is implicated in the active killing of Schwann cells (Morgan *et al.*, 1991, Parkinson in press).

Thus despite thorough investigation, none of the parameters tested here, except p75NTR, were altered by lack of Krox-24 expression.

CONCLUSIONS

I have shown that degeneration and regeneration occur normally in the adult Krox-24 null mutant mouse indicative that Krox-24 is not required for these processes. However one parameter tested in this study, expression of p75NTR, was found to be increased in Krox-24 null mutant mice as compared to the wildtype controls, suggesting that Krox-24 may be involved in the regulation of p75NTR in Schwann cells.

Figure 5.1 Krox-24 is induced in response to injury in the murine sciatic nerve

Whole mount light micrographs of sciatic nerves from mice heterozygous for the Krox-24 mutation. Nerves were assayed for β -galactosidase activity 1 week after both crush (A, B) and transection (C, D) operations. Contralateral control nerves are shown for comparison. Crush site in A is labelled with a single arrow and a single suture, placed on a small fibre, marks the most distal part of the crush site.

Krox-24/ LacZ expression, visualised as a blue product, is up-regulated in the distal aspect of both crushed and transected sciatic nerves and is more intense in the latter. Expression can also be seen, to a lesser degree, in the region proximal to the wound site, extending for 2-3mm.

Scale bars = 2mm.

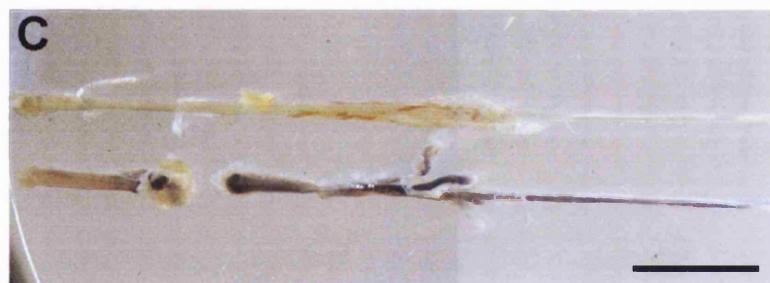
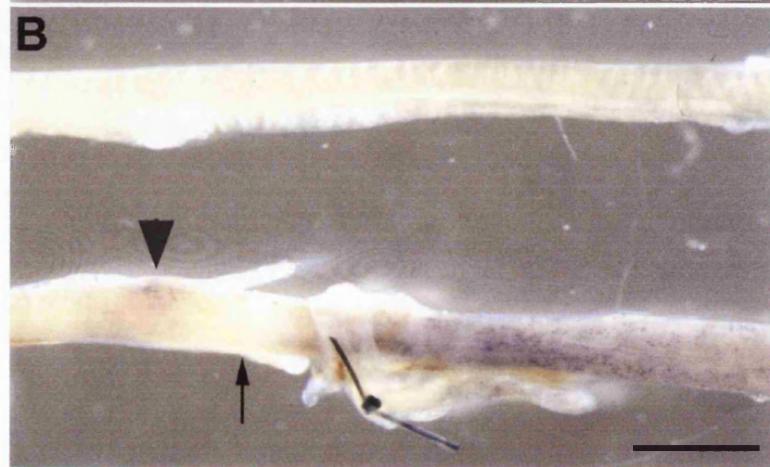


Figure 5.2 Anti-RT-97 antibodies label axons within the sciatic nerve

Fluorescence micrographs showing sections through a single Krox-24 null mutant sciatic nerve from an adult (3 month old) mouse. These transverse sections are consecutive and were taken at the level of the sciatic notch. The upper section has been immunolabelled with an antibody against RT-97 recognising neurofilament (A) and the lower with anti- P_0 (B). In addition the nuclei contained within each section have been labelled with DAPI (fluorescing blue). Rhodamine has been used to reveal RT-97 (red) and many brightly stained fibres, axons, can be seen throughout this section (A). RT-97 stains axons irrespective of calibre suggesting that axons derived from different neuron subtypes are labelled. P_0 has been visualised with FITC (green) and can be seen as many concentric rings, consistent with its presence in compacted myelin sheaths that surround myelinated axons (B). The nuclei of cells contained within the perineurium (nerve sheath) can be seen as a halo around the entire nerve and are P_0 negative.

Scale bar = 100 μ m

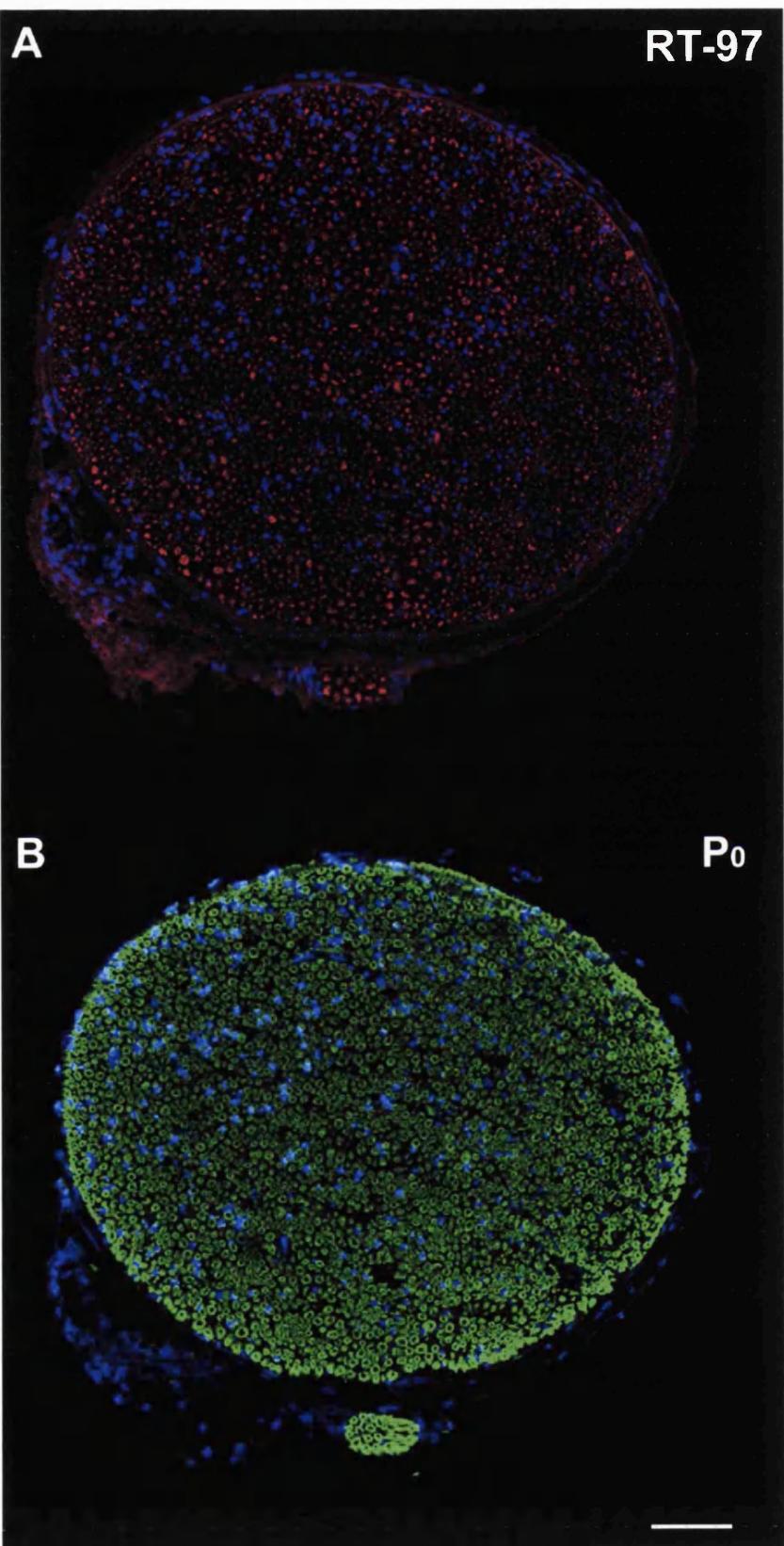


Figure 5.3 Axon degeneration 4 days after sciatic nerve transection

Fluorescence and light micrographs of longitudinal sections through sciatic nerves of adult wildtype (A, B, C, D $+/+$) and Krox-24 null mutants (E, F, G $-/-$) 4 days after sciatic nerve transection. Nerve samples were taken at the same level, 5mm distal to the site of transection. An antibody against RT-97 was used to label axons and was viewed with rhodamine optics (A, D, E.). Hoechst 33258 was employed to label the nuclei of presumptive Schwann cells within the nerve sections (B, F.). The discontinuous appearance of the degenerating nerve can be seen with phase contrast in both animals (C, G.). The normal appearance of axons as assayed with RT-97 is shown in (D), many brightly stained conduits, axons, can be seen traversing the section from left to right. 4 days after transection the axons have become completely degraded in wildtype and Krox-24 mutant nerves.

Scale bars = 50 μ m

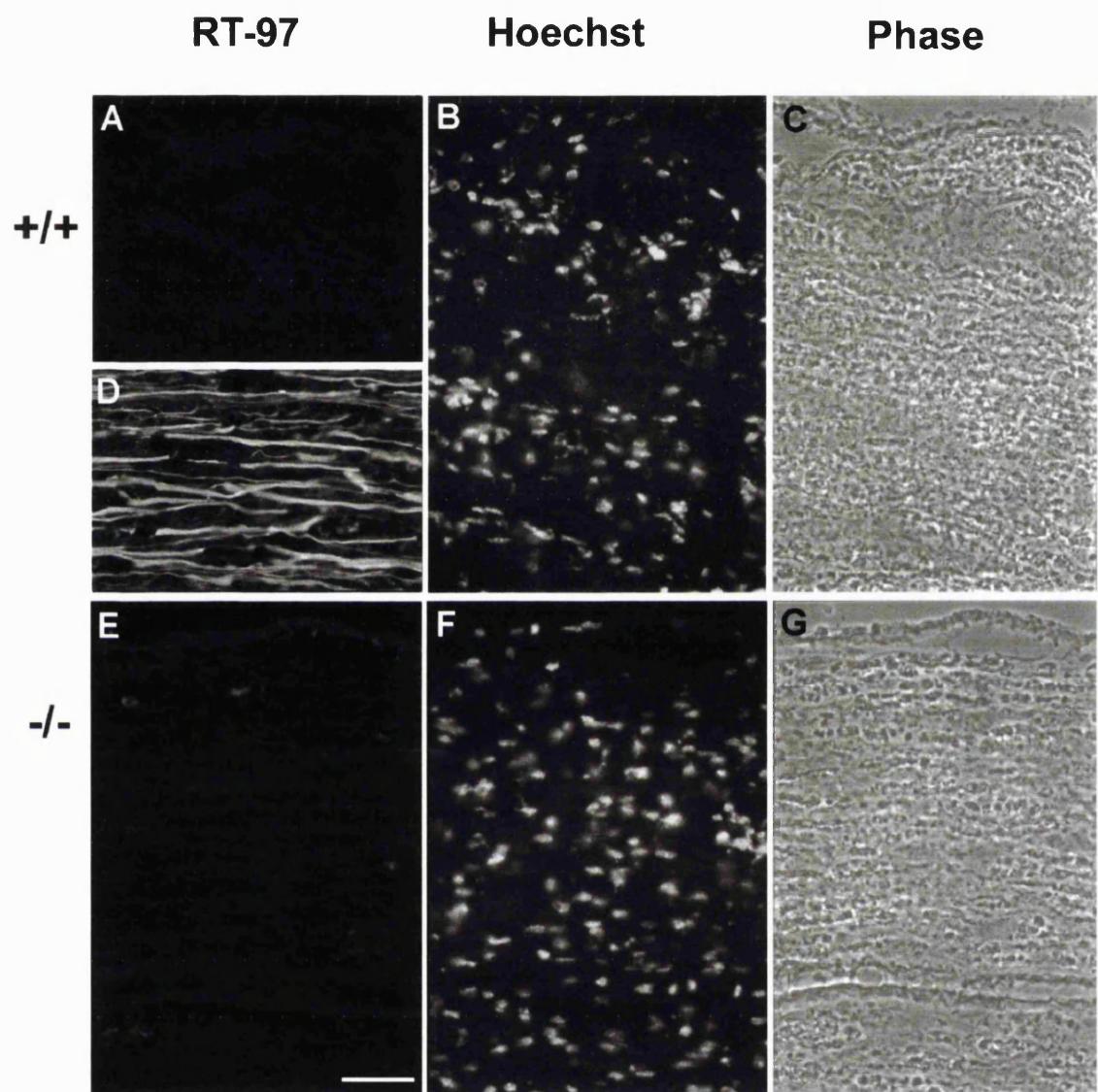


Figure 5.4 Axon regeneration 1 week after sciatic nerve crush

Fluorescence and light micrograph montages of longitudinal sections through wildtype (A, B +/+) and Krox-24 null mutant (C, D -/-) sciatic nerves 1 week after crush. A 10mm nerve sample was taken 10mm distal to the crush site, sectioned, stained with an antibody against RT-97 to label regenerating axons and viewed with rhodamine optics (A, C.). Corresponding phase contrast images show the complete length of the sciatic nerve samples (B, D.).

Axons appear as bright white lines (growing from left, proximal to right, distal) and the majority appear to have grown only half way through these nerve segments in both wildtype and the Krox-24 null mutant, arrows (A, C).

Scale bars = 100 μ m.

+/+



Figure 5.5 Low magnification images of sciatic nerve dissection and measurement

Photomicrographs showing the exposed right hand sciatic nerve of a three month old, adult, mouse 1 week following a crush injury (A, B). By removing dorsal thigh muscle tissue the sciatic nerve is clearly visible as a white conduit running caudal to and parallel with the femur (not visible).

The crush site was marked during the operation with a small suture of black ethilon, arrow (B). The nerve can be seen to divide upon reaching the knee into four branches, three of which are visible here: the peroneal (P), the sural (S), the tibial (T), blue arrows (B). Note that the smallest branch, known as the cutaneous nerve, is not visible here. To allow quantification the nerves were removed to a grid with 0.5cm graduations covered with wax (to prevent tissue dessication), the suture was aligned to zero and the distal nerve accurately divided into segments that were embedded ready for sectioning (C).

Scale bars = 500 μ m.

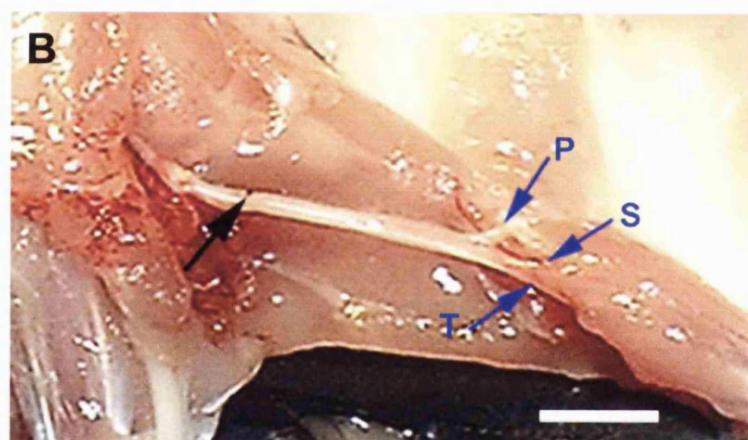
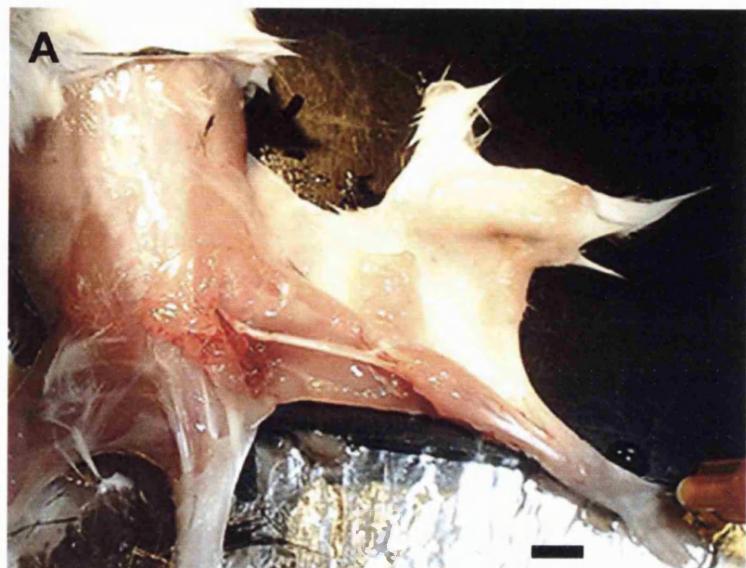


Figure 5.6 A small proportion of axons are able to regenerate a distance exceeding 2cm within 1 week.

Fluorescence micrographs of transverse sections through wildtype (A, C $+/+$) and Krox-24 null mutant (B, D $-/-$) sciatic nerves 1 week after crush. Sections of tibial nerve were taken at the most distal region, 2cm from the crush site (see fig 5.5 C). Anti-RT-97 antibody was used to label regenerating axons and was revealed with rhodamine (red) (A, B). Corresponding reflected light, false colour images show the morphology of these sciatic nerve samples (C, D). A small number of axons have grown this far, appearing as bright red spots and are representative of fast growing axons (A, B).

Scale bars = 100 μ m.

+/+

-/-

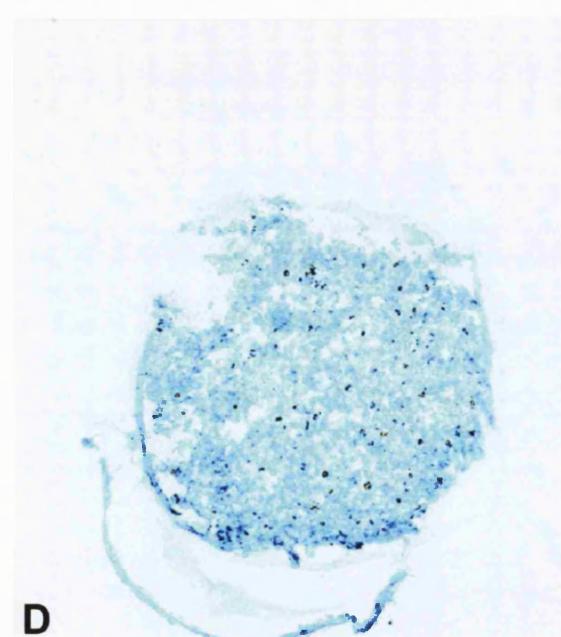
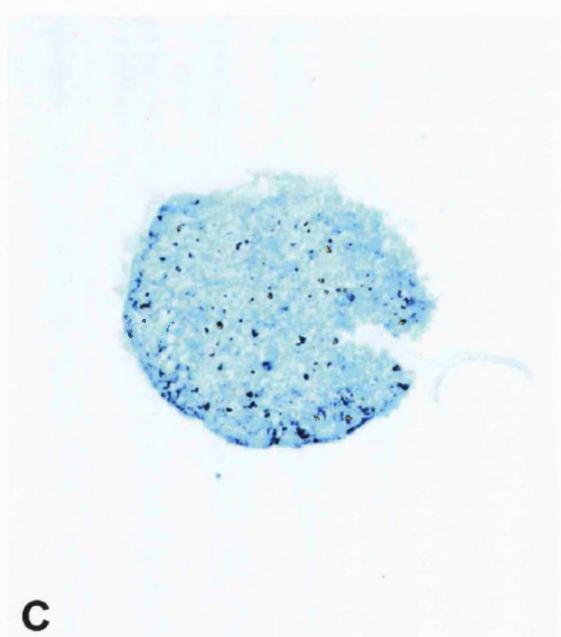
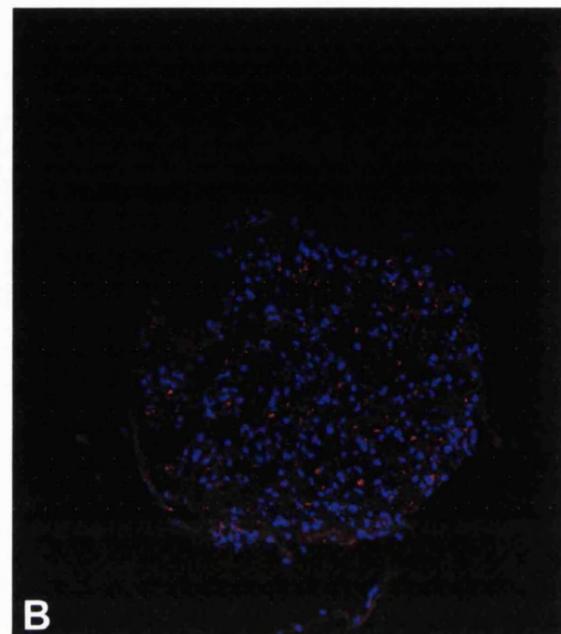
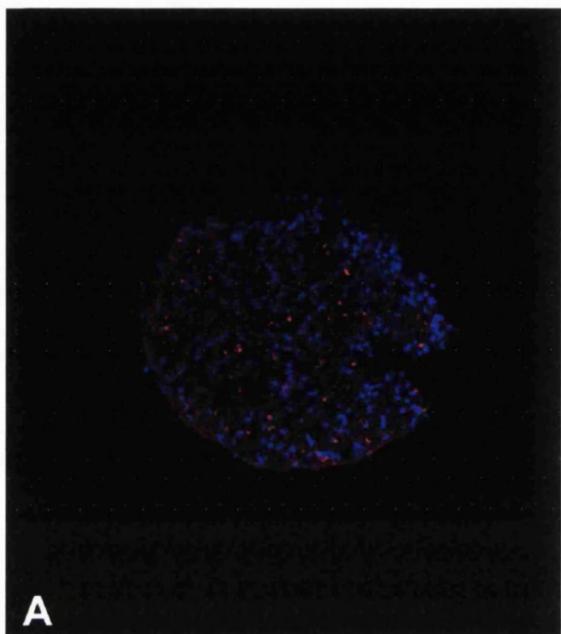


Figure 5.7 A comparison of the numbers of fast growing axons shows that equal numbers are present in the tibial nerves of wildtype and Krox-24 null mutant mice.

Graph to show the number of fastest growing axons that have reached a point 2cm distal to the original crush injury site in 1 week. Counts of axons from 3 wildtype tibial nerves (WT)+/- SEM are shown together with counts from 3 Krox-24 null mutant nerves (KO)+/- SEM.

**Number of axons found 2cm distal to a
crush site, after 1 week of regeneration**

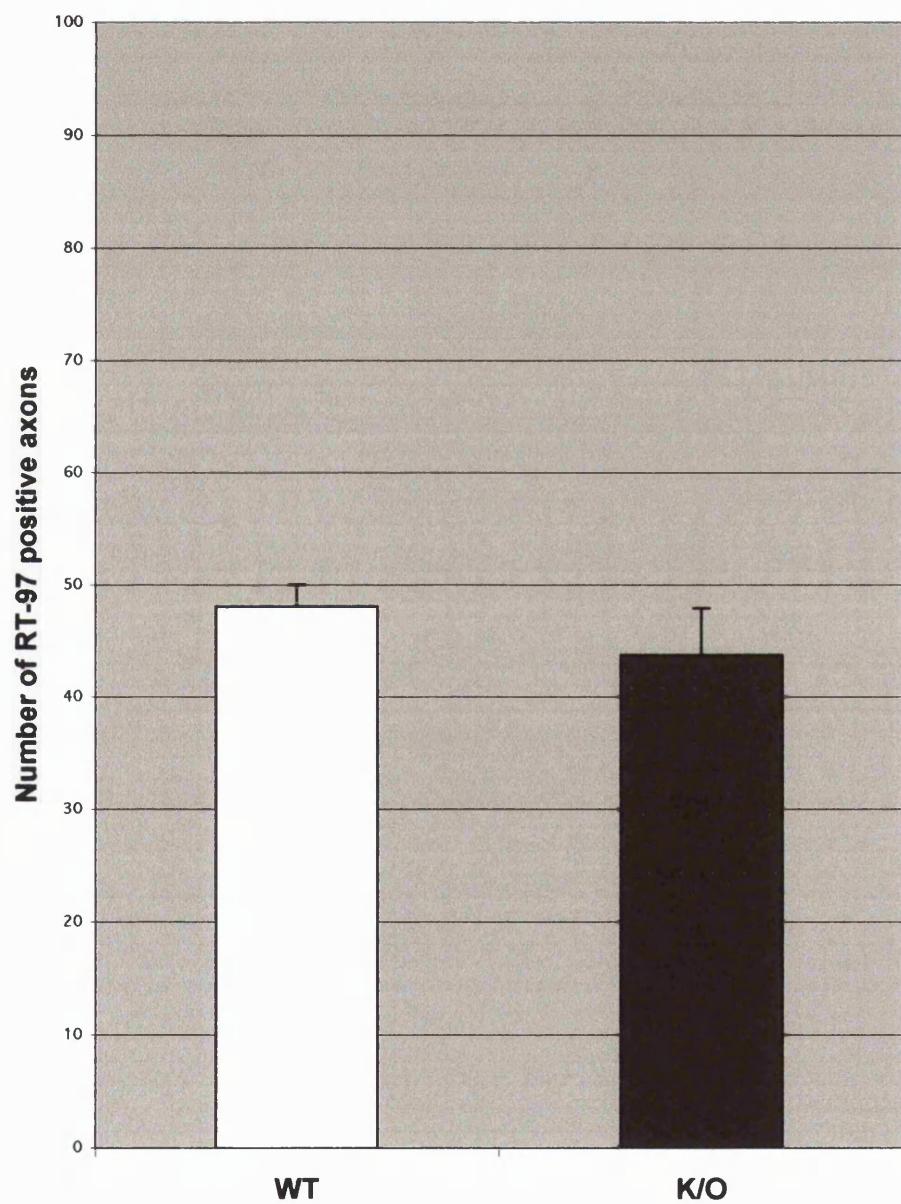


Figure 5.8 Detection of mRNA by RT-PCR of Schwann cell markers down-regulated (P₀) and up-regulated (p75) in the distal sciatic nerve in response to transection

Pooled cDNAs from sciatic nerves of adult mice were prepared from control wildtype (+/+) and Krox-24 null mutants (-/-) as well as 1 week after transection in wildtype (+/T) and Krox-24 null mutants (-/-T). Specific oligonucleotide primers were designed to check the mRNA levels of markers by RT-PCR. A single marker of myelin forming Schwann cells, P₀, which is down-regulated after injury was tested. P₀ mRNA is abundant in control wildtype (+/+) and Krox-24 null mutant (-/-) samples but after transection is not detectable in either wildtype (+/T) or Krox-24 null mutant (-/-T) samples. Levels of p75 mRNA in control wildtype (+/+) and Krox-24 null mutant (-/-) samples are very low with these PCR conditions. The level increases modestly in the wildtype transected sample (+/T). However the level of p75 mRNA in the Krox-24 null mutant (-/-T) sample is higher than for wildtype (+/T), which may represent a two fold increase. L1 mRNA levels appear similar in control wildtype (+/+) and Krox-24 null mutant (-/-) samples and after transection these levels drop but are once again similar in wildtype (+/T) or Krox-24 null mutant (-/-T) samples.

Equal loading of template cDNAs was controlled using primers designed against 18S rRNA (Owens and Boyd, 1991). In all PCR reactions a water control (H₂O) was included to ensure against contamination. The PCRs were repeated three times on a single set of cDNAs.

RT-PCR
Adult Sciatic Nerve and 1 week post-transection

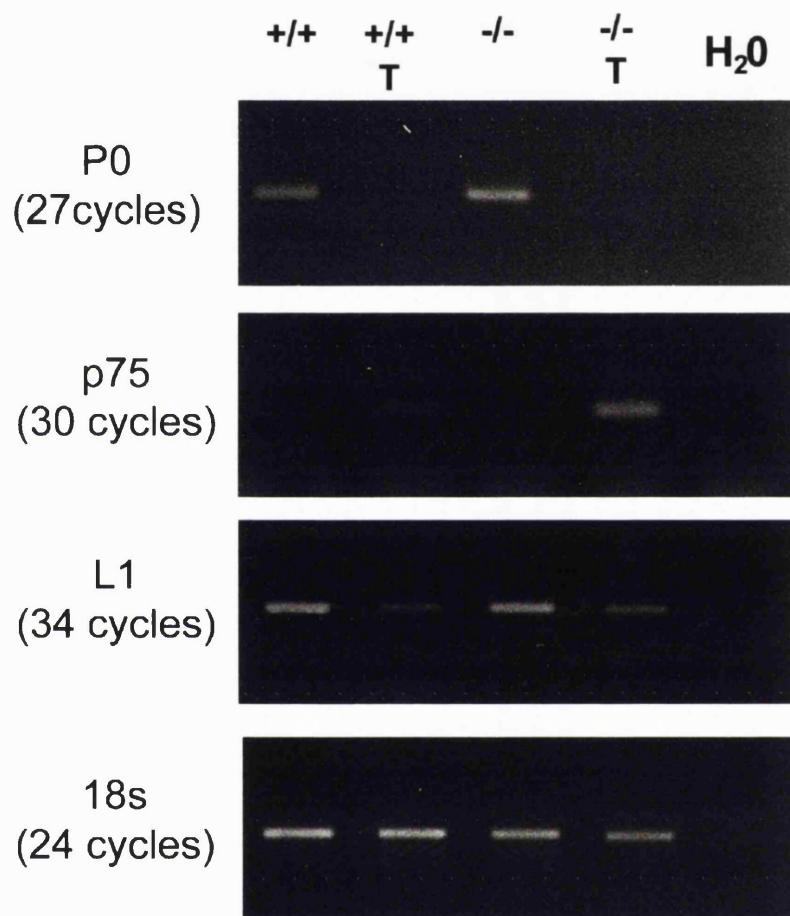
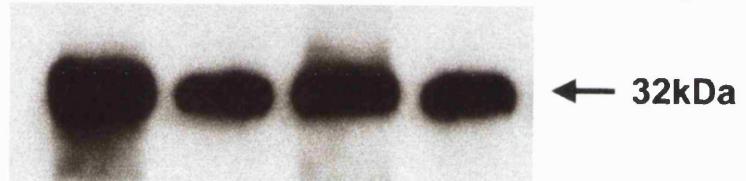


Figure 5.9 Western blot analysis of P₀ protein expression following sciatic nerve injury

The presence of P₀ protein in 1 μ g (upper panel) and 250ng (lower panel) total protein extracts of distal sciatic nerve 1 week after permanent transection was assayed by immunoblotting. The 32kDa P0 protein can be seen in all lanes: Wildtype control nerve extract (Lane 1 +/+) wildtype transected nerve extract (Lane 2 +/+T), Krox-24 null mutant control nerve extract (Lane 3 -/-) and Krox-24 transected nerve extract (Lane 4 -/-T). The level of P₀ protein is similarly down-regulated in transected nerves of wildtype and Krox-24 mice (Lanes 2 and 4, respectively) when compared to control nerves (Lanes 1 and 3), and the validity of this observation is most obvious with the lower total protein loading

	1	2	3	4	
+/-	+/-	+/-	-/-	-/-	Mol. Wgt.
T	T			T	

α P0
1ug



α P0
250ng

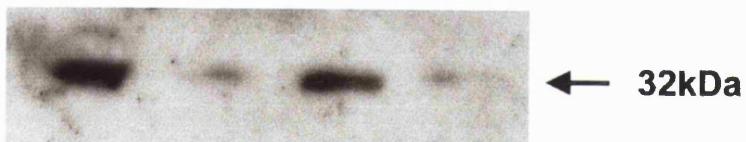


Figure 5.10 p75NTR expression 1 week after sciatic nerve transection

Fluorescence and light micrographs of transverse sections through adult sciatic nerves of wildtype (A, B, C, D $+/+$) and Krox-24 mutants (E, F $-/-$) 1 week after sciatic nerve transection. Nerve samples were taken at the same level, 5mm distal to the wound site. Sections were incubated with an antibody against p75NTR and revealed with rhodamine optics (A, C, E.). corresponding phase contrast images are also shown (B, D, F.). p75NTR expression is low in the normal wildtype nerve shown as weak immunoreactivity in (A), and this increases 1 week after transection (C). Krox-24 mutant tissue also shows increased p75NTR immunoreactivity after transection and the intensity of staining appears increased when compared to wildtype (E).

Scale bars = 50 μ m.

p75NTR

Phase

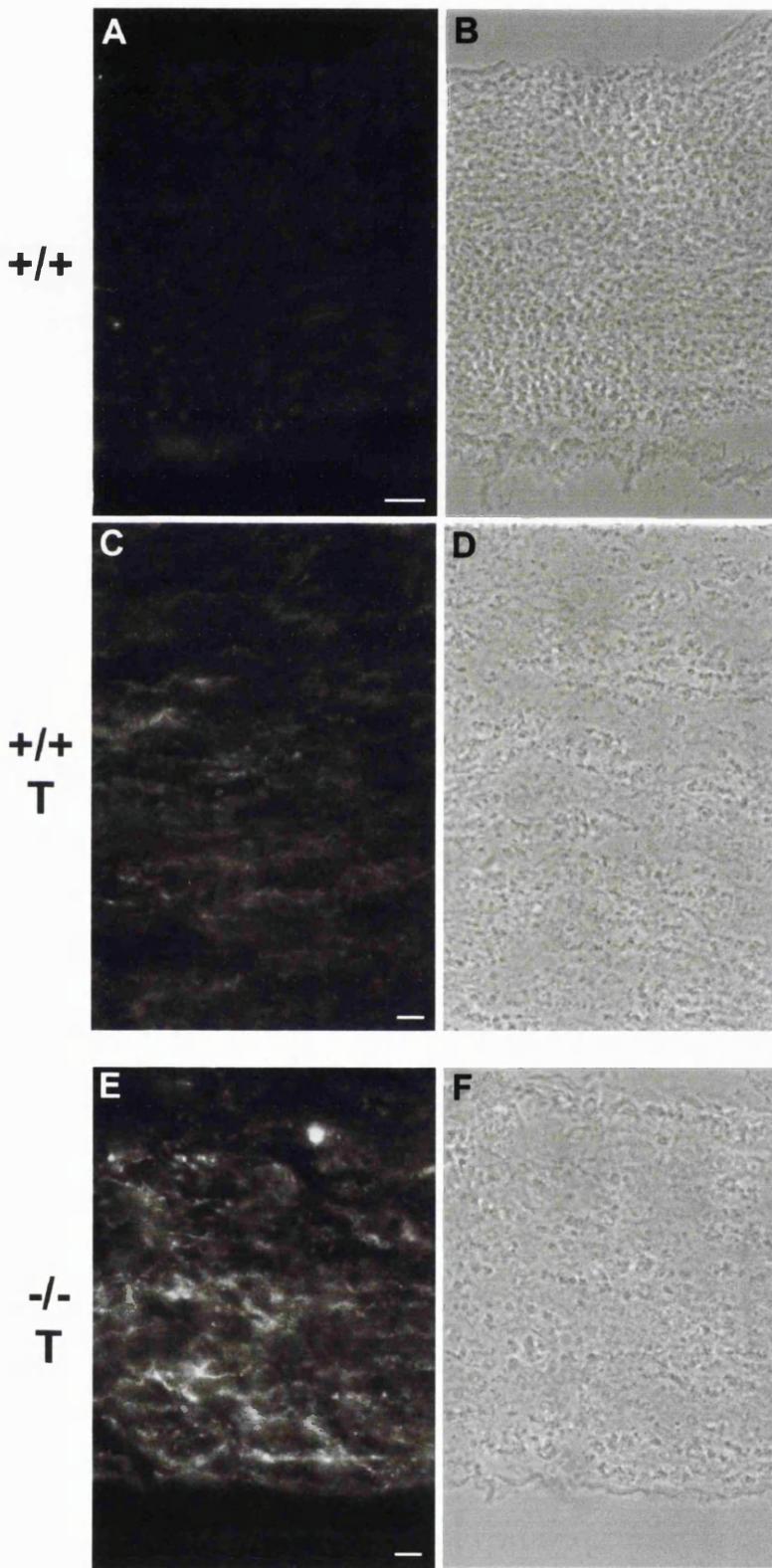
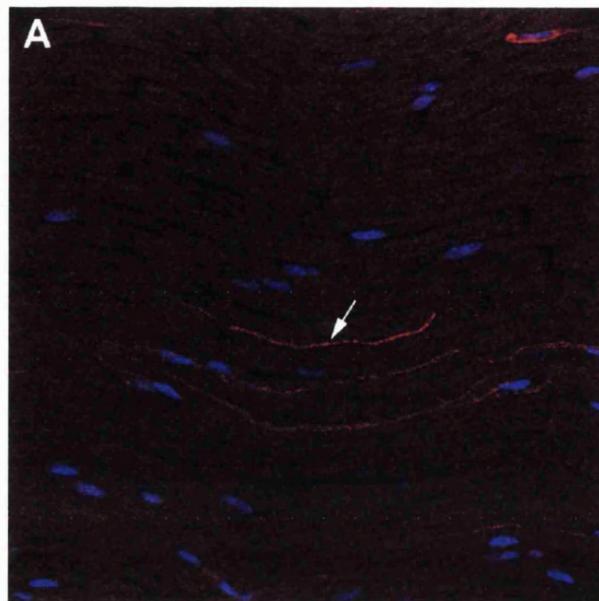


Figure 5.11 L1 expression within adult sciatic nerve and 1 week post transection

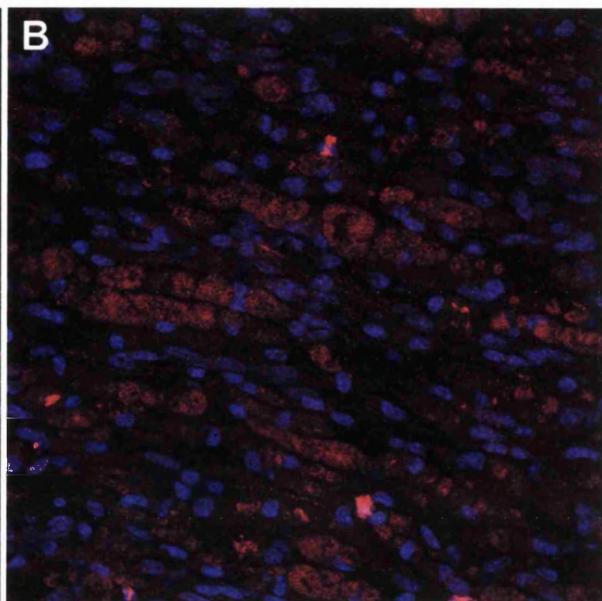
Fluorescence micrographs of longitudinal sections through adult sciatic nerves, of wildtype (A, B $+/+$) and Krox-24 null mutants (C, D $-/-$). Nerve samples were taken at the same level, 5mm distal to the wound site in controls (A, C) or 1 week after sciatic nerve transection (B, D). Sections were incubated with an antibody against L1 and revealed with a rhodamine secondary antibody layer (A-D). L1 expression is localised to the non-myelin forming Schwann cells and associated unmyelinated axons in the wildtype and Krox-24 null mutant nerve (A, C). The nuclei in the intact nerve, shown with Hoechst, are aligned in the plane of the nerve fibres (A, C). L1 expression pattern alters 1 week after transection, with a general increase in expression in dedifferentiating Schwann cells, although overall the levels appear lower than in intact unmyelinated fibres, where part of the labelling may be axonally derived (B, D). Krox-24 null mutant tissue (D) shows similar increased L1 immunoreactivity after transection compared to wildtype (C).

Scale bars = 50 μ m.

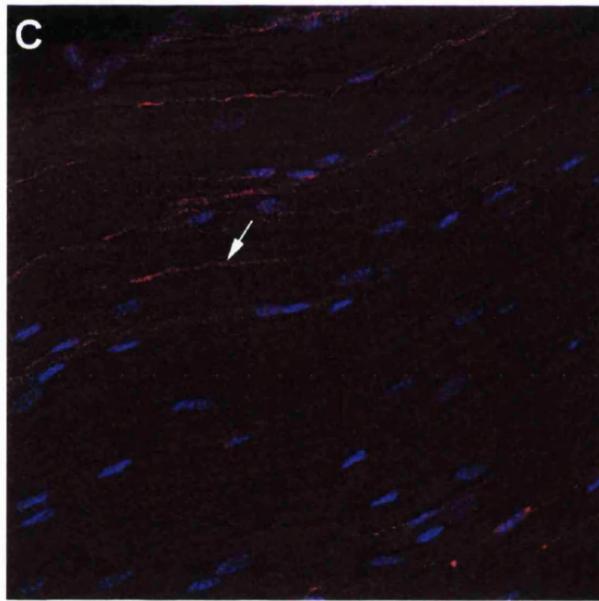
+/+



+/+ T



-/-



-/- T

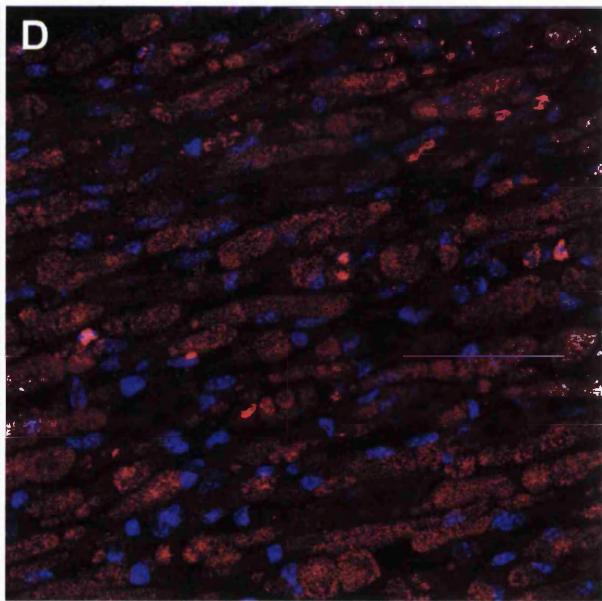


Figure 5.12 Western blot analysis of N-CAM protein expression following sciatic nerve injury

The presence of N-CAM protein in 5 μ g total protein extracts of distal sciatic nerve 1 week after permanent transection was analysed by immunoblotting. The three N-CAM protein isoforms, 180 kDa, 140 kDa and 120 kDa can be seen in wildtype control nerve extract (Lane 1. arrows) as well as in the Krox-24 null mutant control nerve extract (Lane 3.). Complete loss of the largest 180kDa isoform, found in axons, is found in both lanes 2 and 4. Increased expression of the 140kDa isoform is visible in both wildtype transected nerve extract (Lane 2.), and Krox-24 transected nerve extract (Lane 4.).

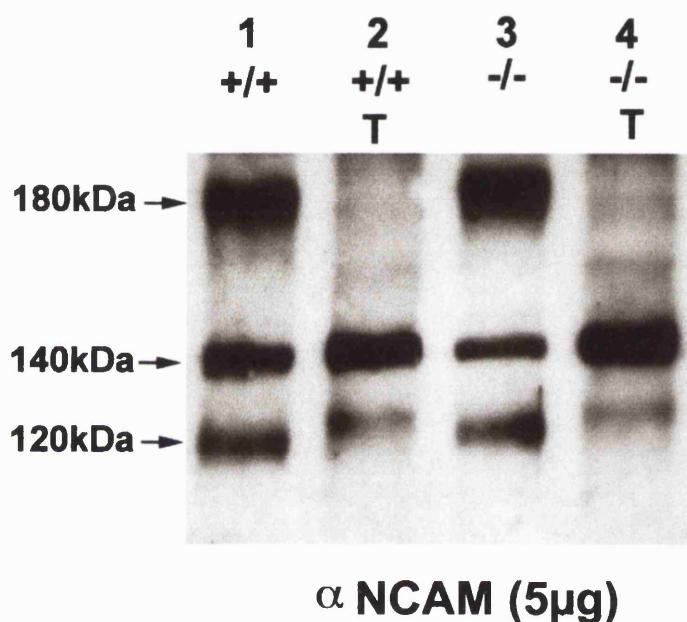


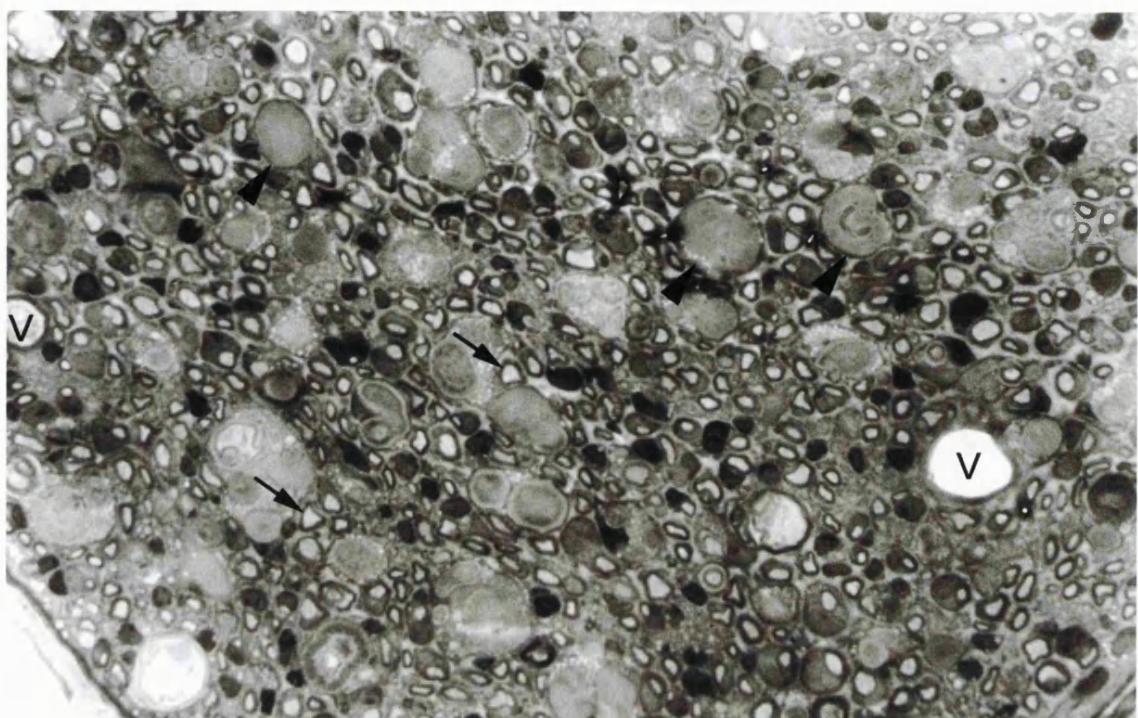
Figure 5.13 Myelination of regenerating axons two weeks after sciatic nerve crush

Light micrographs of araldite embedded, osmicated semi-thin cross sections through sciatic nerve 2 weeks after injury. Semi-thin sections were taken 5mm distal to the site of injury from wildtype mice (A^{+/+}) and Krox-24 mutant animals (B^{-/-}) and counterstained with toluidine blue. In both samples regenerating axons have associated myelin, visualised as dark concentric rings surrounding a paler core (arrows).

Many macrophages remain in the nerve at this time point following crush injury, seen as large polymorphic spheres (arrow heads). Capillaries in the regenerating nerve are also visible (V).

Scale bars = 5 μ m

A +/+



B -/-

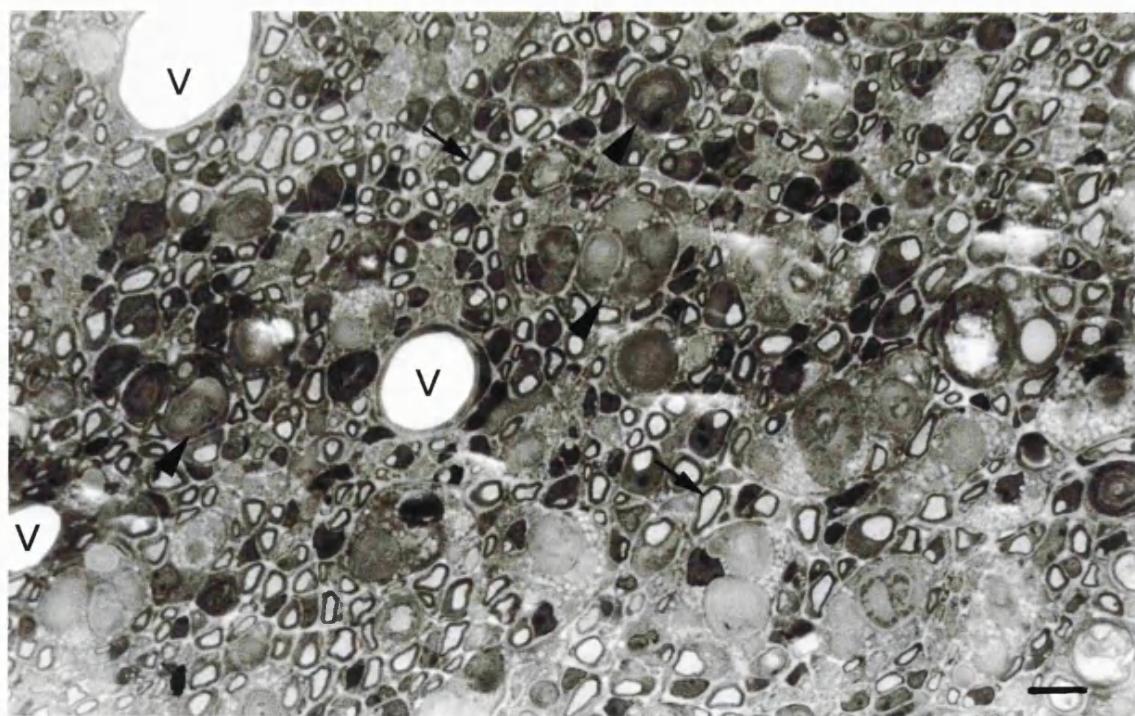


Figure 5.14 A comparison of the total number of myelinated axons with the sciatic nerve two weeks after crush injury

Graph to show the total number of axons that are myelinated after 2 weeks. Counts of axons from 2 wildtype sciatic nerves (WT) +/- SD are shown together with counts from 2 Krox-24 null mutant nerves (K/O) +/- SD.

**Total number of myelinated axons,
after 2 weeks of regeneration**

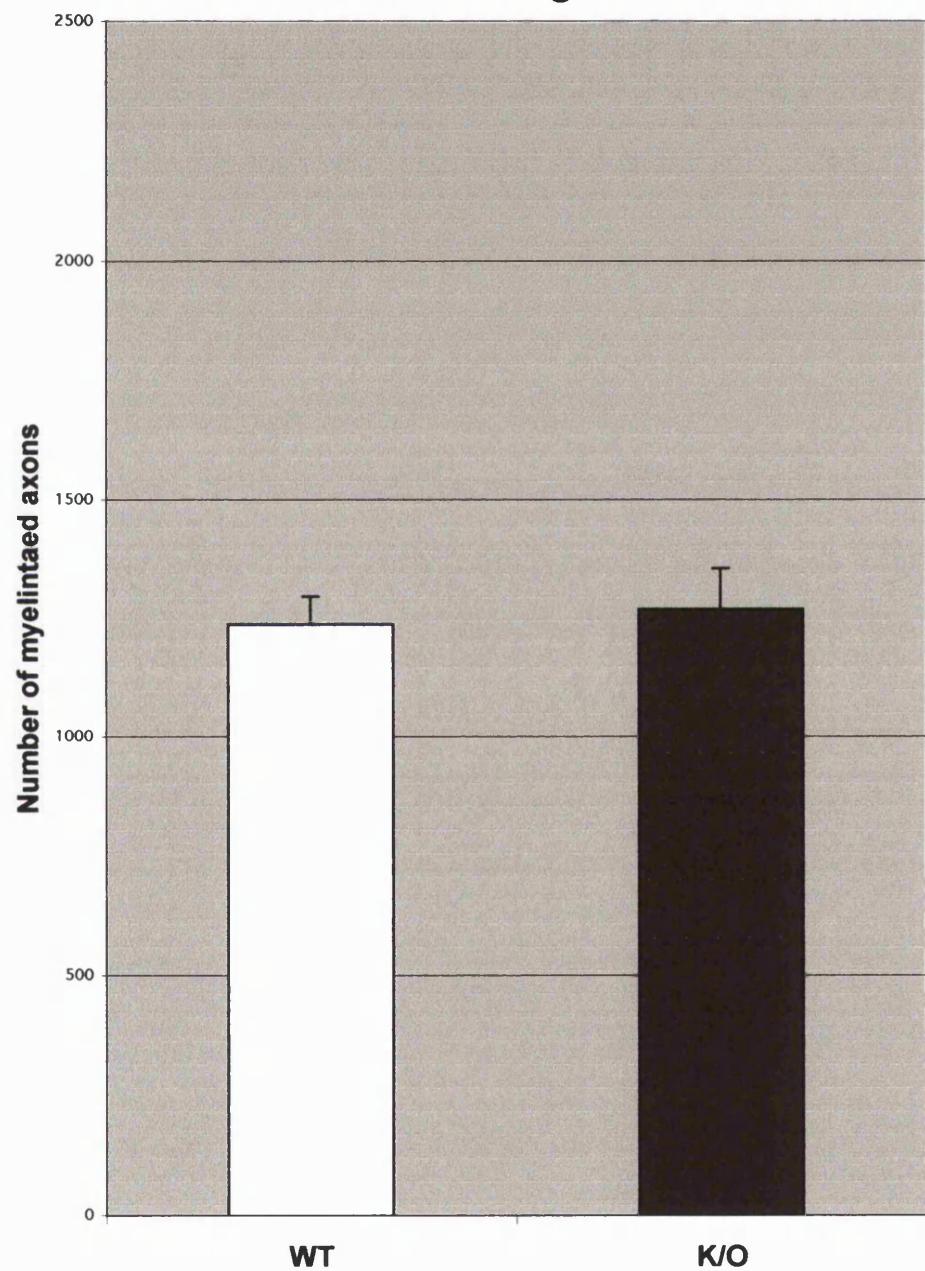
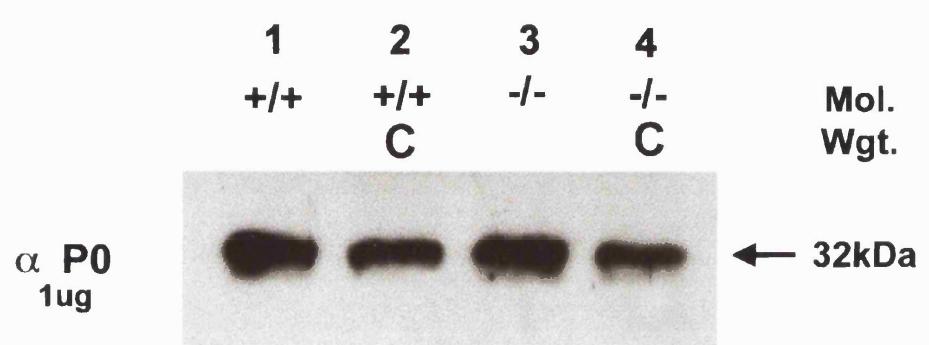


Figure 5.15 Western blot analysis of P₀ protein within regenerating sciatic nerve

The presence of P₀ protein in 1 μ g total protein extracts of distal sciatic nerve 2 weeks after crush injury was assayed by immunoblotting. The 32kDa P0 protein can be seen in all lanes: Wildtype control nerve extract (Lane 1 $^{+/+}$), wildtype crush nerve extract (Lane 2 $^{+/+}$ C), Krox-24 null mutant control nerve extract (Lane 3 $^{-/-}$) and Krox-24 crush nerve extract (Lane 4 $^{-/-}$ C). The level of P₀ protein is similarly up-regulated in regenerating nerves of wildtype and Krox-24 mice (Lanes 2 and 4, respectively) when compared to control nerves (Lanes 1 and 3.).



CHAPTER 6

Schwann cell death *in vivo* and *in vitro* in the Krox-24 null mouse

INTRODUCTION

Cell death is a normal event in the development of the majority of tissues in living organisms (reviewed in Jacobson *et al.*, 1997). Homeostatic mechanisms require continuous cellular turnover, which may occur at a rapid rate. For example mammalian epithelial tissue is often exposed to mechanical stress and damage and as a result has one of the fastest turnovers. From this perspective the nervous system is extraordinary, in that during maturity most neurons do not turn over at all. In contrast to this stability, it is now accepted that about 50% of neurons of the peripheral nervous system die during normal development (Jacobson *et al.*, 1997). Histogenetic cell death (the diffuse loss of cells from an organ during the course of normal development) was alluded to by a number of eminent scientists such as Collins, (1906) and Cajal (1928) in the early part of the last century. Due to the transient nature of cell elimination, cell death was not appreciated as an important mechanism in development until researchers including Glücksman, (Glücksman, 1951) and Saunders and Fallon (1966) made counts of cells in a particular sub-population before and after the complete development of that population.

Cell death during normal development is believed to occur via different developmental strategies. Programmed cell death (PCD) has been well documented in invertebrates, such as *Caenorhabditis elegans* (*C.elegans*), where the development of

the nervous system relies on strict lineage programs to generate small and precise numbers of nerve cells in appropriate distributions (reviewed in Ellis *et al.*, 1991). Not all cell death in invertebrates appears to be programmed and there is evidence to demonstrate that cell death in higher organisms does not occur in such a predictable manner but rather is a function of extrinsic signals or the lack of them. Therefore the definition of PCD in mammalian terms does not imply such a strict delineation as applied to invertebrates such as *C.elegans*.

At present there is strong evidence to suggest that the mechanism of cell death, involved in the development of an efficient nervous system, is genetically regulated. There are currently three major roles postulated for programmed cell death in neurons; adjustment of target innervation to its size, the adjustment of neuron numbers to match a presynaptic neuron pool and the elimination of incorrect connections.

Morphology of apoptosis

The description of a form of cell death distinct from necrosis and the introduction of the name “apoptosis” to distinguish this alternative mechanism represented the first attempt at identifying discrete cell death pathways and was initially met with scepticism (Kerr *et al.*, 1972). Features of the original description of apoptosis are morphological and include cell shrinkage, membrane blebbing, preservation of organelles such as mitochondria and nuclear condensation (pyknosis) and margination of chromatin and finally cellular fragmentation to form a number of apoptotic bodies.

In contrast within cells undergoing necrosis, the organelles become disrupted and the cytoplasm expands causing the cells to swell, leading to rupturing of the cell membrane releasing the cellular contents (reviewed in Studzinski, 1999). Generally necrosis involves local groups of cells and is associated with inflammation while single cells may die by apoptosis and inflammation is absent.

During apoptosis DNA is characteristically fragmented into 300kb to 50kb oligonucleosomes that may be visualised as DNA “ladders” when separated on agarose gels. However DNA degradation is associated with all forms of cell death including necrosis which is typically visualised as a smear but occasionally appears as a ladder (reviewed in Studzinski, 1999). Apoptotic bodies are removed by phagocytosis, a rapid process that may be completed within a single hour, which explains the difficulty earlier researchers had in identifying and quantifying such a large scale loss of cells during development (reviewed in Platt *et al.*, 1998).

Mechanisms in cell death

It is now generally thought that cells dying during normal mammalian development, as a direct result of trophic factor deprivation, referred to as apoptosis, or normal cell death or programmed cell death (PCD) have activated an intrinsic death programme with the intention of killing themselves. The first evidence for the intrinsic cell death programme came from experiments where inhibitors of RNA or protein synthesis caused the suppression or postponing of apoptosis, suggesting that dying cells need to synthesise new proteins to die in this fashion. Staurosporine, a protein kinase

inhibitor, and corticosteroids are able to induce many different cell types to undergo apoptosis (reviewed in Jacobson *et al.*, 1997).

Clues to the molecular components of cell death mechanisms began with the identification of “Ced” genes in the hermaphroditic worm *C.elegans*, where 131 PCDs occur during the course of normal development (reviewed in Ellis *et al.*, 1991). Two genes have been identified from *C.elegans*, Ced-3 and Ced-4, that have been shown to be required for PCD to occur and inactivation causes abnormally large size worms to develop (Ellis and Horvitz 1986, Yuan and Horvitz 1990, reviewed in Nicholson and Thornberry 1997). A third gene, Ced-9 has been identified that is capable of preventing cells undergoing PCD, and inactivation leads to the death of many cells usually destined to survive, and consequently worms die during early development (Hengartner *et al.*, 1992). The fate of any given cell in *C.elegans* is therefore determined by the regulation of expression of these opposing genes, Ced-3 and Ced-4 being pro-apoptotic and Ced-9 favouring survival. Mammalian homologues of Ced-3, Ced-4 and Ced-9 have been identified: Apoptotic protease activating factor 1 (Apaf1) is homologous to Ced-4 (Zou *et al.*, 1997), members of the Bcl2 family are homologous to Ced-9 (reviewed in Newton and Strasser 1998) and a family of mammalian cysteine proteases are homologous to Ced-3 (reviewed in Nicholson and Thornberry 1997).

Caspases mediate proteolytic cleavage in apoptosis

The gene Ced-3 encodes a cysteine protease homologous to the mammalian gene Interleukin-1 β -converting enzyme (ICE). ICE is responsible for the cleavage of the inactive cytokine precursor pro-interleukin 1 β (proIL-1 β) to its active form and has since been named caspase 1 for cysteinyl aspartate specific proteinase (Nicholson and Thornberry, 1997). Over expression of ICE induces PCD in rat fibroblasts and is suppressed by Bcl-2. Caspases exist in dormant proenzyme forms that are catalytically cleaved into competent proteases at Asp-x bonds and at least 11 Caspases have now been identified including Caspase 3 (CPP32/apopain/Yama) and Caspase 2 (Ich-1/Nedd2). (Nicholson and Thornberry, 1997).

Knockouts have been generated for a number of caspases with a striking apoptotic phenotype being found in the Caspase 3 mutant (Nicholson and Thornberry, 1997). A large increase in brain volume occurs together with ectopic cell masses and area duplications in the cortex and optic stalk associated with a specific blockage of cell death within the CNS (Kuida *et al.*, 1996). The selective phenotype of Caspase 3 mutants suggests that this gene has a dominant role in neural apoptosis and it is likely that differential caspase expression is tissue specific.

Caspase 9 deficient mice have also been generated and these animals display a severe phenotype and only 8% of homozygous mutants survive to birth (Haken *et al.*, 1998). Similar to Caspase 3 null mutants, Caspase 9 deficient animals also have a severely disrupted CNS, with defects in cortex associated with a lack of apoptosis. In this comprehensive study the authors identify that ES cells are not susceptible to

PCD induced by UV irradiation in both caspase-9 and -3 null mutants. They suggest that a caspase-9 and -3 dependent pathway is selectively triggered in UV irradiated ES cells and that this pathway may be important for normal CNS development (Haken *et al.*, 1998). In addition they identify 3 further possible pathways: one that is caspase 9 and caspase 3 independent (UV irradiated thymocytes and splenocytes), a second that is caspase-9 independent and caspase-3 dependent (T cell induced PCD) and a third that is caspase-9 dependent and caspase-3 independent (dexamethasone induced thymocyte PCD).

These studies are indicative that the mechanisms underlying apoptosis are tissue specific and include multiple pathways of caspase mediated cell death as well as at least one pathway independent of caspases.

Caspase substrates during apoptosis

In light of the complex morphological events that occur in cells during apoptosis it is not surprising to learn that the proteolytic substrates of caspases are many and varied. Activated caspases cleave other caspases and Bcl2 family members causing irreversible cell death (Cheng *et al.*, 1997, Cohen 1997, Clem *et al.*, 1998). Other targets include DNA repair proteins (poly (ADP-ribose) polymerase, PARP), regulators of cell adhesion (focal adhesion kinase, FAK) and cytoskeleton components (Actin) (reviewed in Nicholson and Thornberry 1997). One of the first detectable events in an apoptotic cell is the cleavage of the DNA repair protein PARP, which is a substrate of Caspase 3 (Lazebnik *et al.*, 1994).

Recently the mechanism which is responsible for membrane blebbing has been elucidated, with the discovery of a novel Rho-associated kinase ROCK I, an effector of the small GTPase Rho. ROCK I has been found to phosphorylate myosin light chain (MLC) which leads to membrane blebbing and ROCK I is a substrate of Caspase 3 (Sebbagh *et al.*, 2001). The authors show that to obtain active ROCK I requires Caspase 3 cleavage and that the *in vitro* inactivation of ROCK I prevents membrane blebbing but does not hinder chromatin condensation.

The cytochrome c/ Apaf1/ caspase-9 apoptosome

The cytochrome c/ Apaf1/ caspase-9 mechanism of apoptosis is a major pathway underlying cell death (Zou *et al.*, 1999). In response to death stimuli, mitochondrial membranes become permeable and cytochrome c is released (Newmeyer *et al.*, 1994, Kroemer and Reed, 2000). Cytochrome c associates with Apaf1 and procaspase-9 triggering a caspase activation cascade that may include caspase-3 activation and culminates in apoptosis (Zou *et al.*, 1997, Liu *et al.*, 1996, Li *et al.*, 1997, Zou *et al.*, 1999).

Support for the suggestion that CNS development relies on a caspase-9 and caspase-3 dependent mechanism of PCD comes from the finding that Apaf1 null mutants, similar to caspase -9 and -3 null mutants, display CNS abnormalities (Yoshida *et al.*, 1998). This study also demonstrated that Apaf1 was required for apoptosis dependent on mitochondrial pathways of activation. Recently cytochrome

c null mutants have been created that show embryonic lethality due to dramatically reduced levels of PCD (Li *et al.*, 2000).

Apoptosis inducing factor (AIF) in cell death

The studies of different cell populations, derived from caspase deficient mice, and their responses to death inducing stimuli discussed above were suggestive that another pathway of cell death involving caspase independent mechanisms exist. The cloning of apoptosis inducing factor (AIF), localised to mitochondria, represents the first identification of a component of just such a mechanism (Susin *et al.*, 1999). AIF is normally present in the mitochondrial inter-membrane space, similar to cytochrome c, and is released in response to stimuli inducing cell death (Daugas *et al.*, 2000). The creation of embryonic stem cells (ES) null for AIF by homologous recombination, one of the initial stages of transgenesis, was carried out but subsequent attempts to generate mice deficient for AIF failed (Joza *et al.*, 2001). Study of the mutant ES cells revealed that they were resistant to growth factor deprivation and the process of cavitation associated with the development of the embryoid body (EB) was absent. The cell death induced in wildtype EBs by AIF has the characteristics of apoptosis and these data suggest that AIF is essential for the first wave of cell death associated with early embryogenesis (Joza *et al.*, 2001).

Mammalian cell death suppression by Bcl-2

As mentioned earlier Ced-9 has a mammalian homologue, the proto-oncogene Bcl-2, first isolated from human follicular B cell lymphomas, where it is overexpressed. Bcl-2 can also suppress PCD in *C.elegans* suggesting evolutionary conservation of PCD mechanisms (Vaux *et al.*, 1992, Hengartner and Horvitz, 1994). Bcl-2 is a membrane bound protein thought to be associated with the cytoplasmic surface of the nuclear envelope, endoplasmic reticulum and the outer mitochondrial membrane. As well as Bcl-2 other members of this gene family have been identified and can be divided into 2 groups depending on whether they promote apoptosis (Bax, Bcl-x_S, Bak, Bad, Bik, Bid and Harakari) or suppress it (Bcl-2, Bcl-x_L, Bcl-w, A1 and MCL-1) (reviewed by Newton and Strasser, 1998). Bax, Bak and Bik have been found to heterodimerize with and inhibit the binding of Bcl-x_L to Ced-4 suggesting that pro-apoptotic members of this family regulate cell death by controlling caspase mediated apoptosis (Chinnaiyan *et al.*, 1997). When Bcl-2 is absent in null mutants Bax can promote apoptosis, again suggesting that intrafamily interactions between these two subpopulations of proteins regulates cell death (Knudson and Korsmeyer 1997).

The precise mechanism by which the suppressors of apoptosis function is poorly understood but the crystal structure of Bcl-x_L.has revealed a similarity to membrane channel forming protein of bacterial toxins such as diphteria toxin, so it may regulate membrane trafficking (Muchmore *et al.*, 1996). Both Bcl-2 and Bcl-x_L are able to block cytochrome c release from mitochondrial membranes which in turn prevents apoptosis (Kluck *et al.*, 1997, Kharbanda *et al.*, 1997). Thus the

cytochrome c/ Apaf1/ caspase-9 apoptosome would not be able to form and caspase mediated cell death would be prevented. The Bcl-2 function is not restricted to controlling the release of cytochrome c alone, but it has also been shown to prevent AIF release suggesting that Bcl-2 also regulates caspase independent cell death mechanisms (Susin *et al.*, 1996).

The IAP protein family are inhibitors of cell death

This family of proteins was discovered in baculoviruses and functions in such a way as to suppress host cell death (Crook *et al.*, 1993, Birnbaum *et al.*, 1994). They are characterised by a novel ~70 amino acid domain termed the baculoviral IAP repeat (BIR) which occurs tandemly repeated up to three times (reviewed in Deveraux and Reed 1999). At least 16 IAP family members have been discovered in organisms from viruses to yeast, invertebrates and mammals. Overexpression of members of the six strong group of human IAPs, XIAP, cIAP1, cIAP2, NAIP and survivin have been shown to suppress apoptosis induced by diverse stimuli (Deveraux and Reed 1999).

The basis of the inhibitory function has recently been revealed by a number of investigators by X-ray crystallography. XIAP binds both Caspase 3 and Caspase 7 across the caspase substrate groove representing a steric blockade thereby preventing the binding of these caspase proteins to possible substrates (Reidl *et al.*, 2001, Chai *et al.*, 2001). Surprisingly the BIR domains are not directly involved, but rather the

linker regions between these domains interact with the substrate grooves of the caspase protein suggesting that BIR domains may be superfluous to IAP function (Chai *et al.*, 2001, Huang *et al.*, 2001).

Apoptosis in *Drosophila*

The fruit fly, *Drosophila melanogaster*, has proven a useful tool for developmental biologists and the study of mutants has allowed the cloning of many novel proteins. Homologues of mammalian components of the cell death pathways have been discovered in *Drosophila*. Five caspases have been identified so far: DREDD, DRONC, drICE, Dcp-1 and DECAcY, a single homologue of ced-4/ Apaf1 known as Apaf1 related killer (Ark) has been isolated, one member of the Bcl-2 family, known as DROB-1 and two IAP homologues, dIAP1 and dIAP2 (reviewed by Bangs and White, 2000).

Intriguingly three genes involved in PCD in *Drosophila* development have been identified, and as yet, no mammalian homologues have been found. These are *reaper* (*rpr*), *grm*, and *head involution defect* (*hid*) and the proteins of these genes are involved in the initiation of all PCD in *Drosophila* (White *et al.*, 1994, Grether *et al.*, 1995, Chen *et al.*, 1996). In mutant embryos devoid of these genes, PCD can be induced by X-irradiation, indicating that cell death mechanisms are still present but suggesting that these genes are responsible for the signal activation of intrinsic PCD programmes which may be found in most cells. Over expression studies reveal that

each of these three genes cause extensive, abnormal cell death and that this induction is caspase dependent (Bangs and White, 2000).

Cell death in the mammalian nervous system

Although more complex nervous systems may employ programmed cell death at an early stage in development, it is suggested that a second more flexible strategy is more prevalent, namely target dependent cell death. In 1909 M.L.Shorey demonstrated that target ablation (removal of limb buds) in chick and amphibian embryos resulted in the depletion of motor neurons and neurones in the related sensory ganglia. In the period of 1920 to 1936 S. Detweiler confirmed these results and expanded on them showing that the inverse was also true. Implantation of an extra limb bud produced an increase in both sensory and motor neurons. In 1934 Viktor Hamburger suggested that there was a proportional relationship between the amount of the target removed and the number of neurons in the innervating population (Jacobson, 1993). Each of these experiments indicates that the target is instrumental in defining the final population of innervating cells, a developmental mechanism ensuring that all target tissue become innervated. Confirmation of this theory is found in an elegant experiment involving chick/ quail chimeras, exploiting the large difference in size between the two species. Chick lumbosacral cords were transplanted into quails and *vice versa* prior to limb innervation and it was found that more quail motor neurons survived in the chick than the quail and fewer chick motor neurons survived in the quail than the chick (Tanaka and Landmesser, 1986). These and other data provide a

body of evidence documenting cell death in both the developing CNS and PNS (reviewed in Raff *et al.*, 1993).

Subsequently the discovery and isolation of molecules that are indirectly responsible for target dependent cell death, namely NGF and later BDNF, NT3 and CNTF have lead to the development of a neurotrophic theory (reviewed in Levi-Montalcini, 1987, Mudge, 1993). In the PNS, it is known that developing neurons undergo a period of cell death related to target derived neurotrophin availability (Davies, 1988, Barde, 1989). The trophic nature of NGF was initially demonstrated in chick embryo explants where sensory and sympathetic ganglia were incubated for 24 hours in a semi-solid medium and developed a halo of neurites, which were of maximal density on the side facing an NGF source (Levi-Montalcini, 1987). Further to this work it has been discovered that neurones cultured *in vitro* undergo apoptosis if deprived of appropriate neurotropic factors (Davies, 1988, Barde, 1989).

Increases in the transcription of the immediate early genes of the fos family eg. c-fos have been found during the earliest stages of PCD in the DRG as well as sympathetic neurons. These transcription factors are encoded by genes induced during PCD.

The trophic theory no longer applies exclusively to neurons

It has been known since the early 1960s from studies by Smart and Leblond, Pannese and Ferannini that glial cells of the CNS undergo cell death during development, although the exact nature of the dying cells and the volume involved have only recently begun to be elucidated (Jacobson, 1993).

The proliferation and differentiation of the CNS glial cell precursors, O-2A progenitor cells, have been studied *in vitro* extensively. These cells are particularly accessible in the rat optic nerve, where they support retinal ganglion cells and may develop to form either oligodendrocytes or type-2 astrocytes *in vitro*. *In vivo*, type-1 astrocytes differentiate early (E14-P21) and are known to produce platelet-derived growth factor (PDGF) A chains, believed to be the source of PDGF that stimulates the proliferation of O-2A progenitor cells. Oligodendrocytes therefore appear later (P0-P21) (reviewed in Raff *et al.*, 1993). PDGF, Insulin-like growth factor (IGF) or insulin have been shown to be survival factors for purified 0-2A progenitor cells as well as oligodendrocytes (Barres *et al.*, 1992). *In vivo* counts enabling an estimation of the number of dying cells in developing optic nerve showed that between 300 and 500 cells undergo suspected PCD in the developmental period of P4-P10, and that 91% of the dyeing cells were GC⁺oligodendrocytes (Barres *et al.*, 1992). To investigate whether PDGF rescues oligodendrocytes *in vivo* PDGF A chain transfected COS cells were transplanted into the subarachnoid space of P8 rats to deliver the PDGF to the CNS and in particular the optic nerve. The average number of dying cells per section decreased by 85% and the total number of cells per nerve increased by 50%, an increase almost exclusively due to an increase in the number of oligodendrocytes. This data suggests that about 50% of oligodendrocytes formed during development are lost due to PCD, and that PDGF is acting as a trophic factor for oligodendrocytes paralleling the effect NGF has on sensory neurons in the periphery. It would appear that cell death due to competition for trophic factors

adjusts the number of oligodendrocytes required for complete myelination of the retinal ganglion cell axons in the nerve. Transgenic mice overexpressing PDGF have been generated and these animals show hypoproliferation of oligodendrocytes during development resulting in a gross excess of oligodendrocytes (Calver *et al.*, 1998). Interestingly these excess cells are removed in a mechanism leading to a normal level of myelination found in maturity suggesting that rather than acting as a survival signal, PDGF is mitogenic for oligodendrocytes

Cell death in Schwann cells of developing nerves

Many parallels may be drawn between neuronal survival and target innervation and the recruitment of Schwann cells by axons; both processes are dependent on trophic factors and involve matching the numbers of one cell population to that of another. This together with the data above showing that 50% of oligodendrocytes, die during optic nerve development makes it surprising to learn that until recently Schwann cell apoptosis had not been discovered.

Cell death in Schwann cells is predominantly associated with development, is attenuated following injury and absent in the mature nerve (Ciutat *et al.*, 1996, Trachtenberg and Thompson 1996, Syroid *et al.*, 1996, Grinspan *et al.*, 1996, Nakao *et al.*, 1997). The first observation that Schwann cells undergo apoptosis in development was made in the ventral roots of the embryonic chick where two waves were observed: the first at E5-6 and the second at E8.5 which are thought to be linked to motor neurone cell death (Ciutat *et al.*, 1996). Observations of mammalian

Schwann cell apoptosis began at neuromuscular junctions where the denervation of P4 rats results in a loss of Schwann cells at nerve terminals, with up to 27% being positive for TUNEL 1 day after injury (Trachtenberg and Thompson 1996). Schwann cell apoptosis is also found in the developing rat sciatic nerve albeit at very low levels, with estimates varying from 0.5-1% at the newborn stage which decreases to 0.1% or less within the first postnatal week (Grinspan *et al.*, 1996, Syroid *et al.*, 1996, Nakao *et al.*, 1997). The methods used to arrive at these figures are not a direct measure of the total number of Schwann cells that die during development, and given the rapidity with which apoptotic cells are removed the absolute number of Schwann cells that die by apoptosis is likely to be significantly higher. A systematic study of cell death throughout development of Schwann cells has not yet been carried out.

Interestingly, Schwann cell apoptosis within newborn nerves is increased 10 fold when assayed for TUNEL, 1 day after axotomy, demonstrating that Schwann cells at the pro-myelin stage of development are partially dependent on axonal contact for survival (Grinspan *et al.*, 1996).

Possible mechanisms of Schwann cell death

TGF β

The transforming growth factor- β (TGF β) superfamily encompasses 3 isoforms of TGF β as well as homologous proteins belonging to the bone morphogenetic protein (BMP) family and glial derived neurotrophic factor (GDNF) (reviewed Scherer and Salzer, 2001). Their receptors are heterodimeric serine/ threonine kinases. Schwann

cells synthesise all three TGF β isoforms and possess receptors type I, II and III. The effects of TGF β s on Schwann cells are complex and depend to a degree on axon contact. Early studies from this laboratory were suggestive that TGF β applied to cultures of rat Schwann cells caused a change in morphology (receding processes) together with a loss of cells and up-regulation of the cell adhesion molecules N-CAM and L1 (Stewart *et al.*, 1995, 1996). A more in depth study of this affect shows that TGF β induces Schwann cell apoptosis *in vitro* in a dose-dependent manner and that this effect can be mimicked *in vivo* by the introduction of exogenous TGF β to transected newborn nerves leading to two fold increase in apoptosis in the distal stump (Parkinson *et al.*, in press). Investigations into the intracellular mechanisms involved in this effect demonstrate that phosphorylation of c-jun and induction of AP1 dependent transcription occur and that caspase activity is required (possibly caspase 3).

NGF

The Schwann cells found to die by apoptosis in newborn rat sciatic nerves are premyelinating cells determined by the continued expression of p75NTR and lack of periaxin expression (Grinspan *et al.*, 1996). This in itself is interesting as p75NTR has been implicated in the induction of apoptosis in Schwann cells (Taniuchi *et al.*, 1988) and expression is up-regulated in the mature nerve following injury together with the ligand NGF (Taniuchi *et al.*, 1986). Schwann cell death can be induced by NGF *in vitro* and is completely blocked in cells lacking the p75NTR receptor in

similar experiments (Soilu-Hanninen *et al.*, 1999). It was also found that overexpression of Bcl-2 did not protect cells from apoptosis induced by NGF. In P1 sciatic nerves of both wildtype and p75NTR deficient mice less than 0.15% apoptosis of Schwann cells is seen suggesting no significant difference for cells lacking the p75NTR receptor (Syroid *et al.*, 2000). In contrast, if the sciatic nerves are transected and the distal aspects assayed for TUNEL 1 day later there is a 13 fold increase in Schwann cell apoptosis in the wildtype nerve but little or no increase in apoptosis in Schwann cells deficient for p75NTR (Syroid *et al.*, 2000). These data suggest that NGF may mediate Schwann cell death in certain conditions signalling via p75NTR receptor. For instance, the loss of axon contact during early development may result in lost positive survival cues such as integrin interactions or NRG β , and so previously silenced negative survival mechanisms such as NGF/ p75NTR binding become dominant and induce cell death.

IGF

Recently an autocrine survival loop has been identified in Schwann cells that develops as Schwann cells mature, and is absent from Schwann cell precursors. This explains the amazing lack of death that mature Schwann cells undergo even after denervation (Syroid *et al.*, 1999, Meier *et al.*, 1999). The study from this laboratory demonstrates that the synergistic action of three factors; Insulin-like growth factor (IGF), neurotrophin-3 (NT-3) and platelet-derived growth factor-BB (PDGF-BB),

can mimic the rescue effects of Schwann cell conditioned medium on Schwann cells cultured at very low densities, namely to rescue all cells (Meier *et al.*, 1999). The second investigation highlights IGF1, and IGF1 receptor as an autocrine trophic factor mechanism responsible for mature Schwann cell longevity (Syroid *et al.*, 1999).

Studies into the intracellular pathways underlying IGF1 mediated Schwann cell survival have revealed that the PI3K inhibitor, LY294002, blocks IGF1 rescue and that the caspase inhibitor bok-asp-fmk (BAF) has a similar effect suggesting that IGF1 rescues Schwann cells from caspase dependent apoptosis signalled via PI3K (Delaney *et al.*, 1999). In addition it has been shown that IGF1 can inhibit c-jun-N-terminal protein kinase (JNK) activation suggesting that IGF1 acts at least in part by inhibiting activation of JNK in surviving Schwann cells (Cheng *et al.*, 2001).

NRG β

The normal, low, levels of apoptosis seen *in vivo* and the inflated levels resulting from axotomy of newborn nerves can be suppressed by the presence of NRG β (Grinspan *et al.*, 1996). Similarly the Schwann cell death resulting from denervation seen at nerve terminals can be significantly reduced with the introduction of exogenous NRG β (Trachtenberg and Thompson 1996). These rescue effects can be reproduced *in vitro* if Schwann cells from early postnatal animals are plated in serum free medium at sub-confluent, low densities (Grinspan *et al.*, 1996, Nakao *et al.*, 1997). It is clear from these studies that Schwann cell death in response to loss of axonal contact through denervation can be reduced by exogenous NRG β , suggesting

that NRG β is a positive regulator of Schwann cell survival in early post-natal stages of development. Recently a possible intracellular mechanism of NRG β induced Schwann cell survival via binding to ErbB2/ ErbB3 receptor complex has been revealed. ErbB2/ ErbB3 ligand binding has been shown to cause phosphoinositide 3-kinase (PI3K) activation in rescued Schwann cells *in vitro* and conversely that a PI3K blocker increases the levels of apoptosis (Li *et al.*, 2001). Further to this the authors show that NRG β activates Akt kinase, downstream of PI3K, and this leads to Bad phosphorylation and ultimately to Schwann cell survival.

The majority of these studies implicate PI3K as an important step in the translation of a cell death signal in Schwann cells and indicate that a caspase dependent mechanism affects apoptosis. Support for this latter suggestion comes from overexpression studies where it was found that overexpression of the Bcl-X_L gene in Schwann cells subjected to hyperglycemic conditions protects them from apoptosis (Delaney *et al.*, 2001). JNK activation has also been investigated and is found in Schwann cells that are destined to undergo cell death an hour after serum starvation (Cheng *et al.*, 2001). Over expression of the Bcl-X_L gene in Schwann cells inhibits JNK activation suggesting that Schwann cell survival mechanisms may include Bcl family members (Cheng *et al.*, 2001). Another ced-9 homologue, Bcl-2 has also been found to suppress growth factor induced cell death *in vitro* when overexpressed in Schwann cells (Soilu-Hanninen *et al.*, 1999). Similarly caspase activation is the mechanism underlying oligodendrocyte cell death in different contexts. Caspase-3,

has been identified as a mechanism of cell death occurring in oligodendrocytes derived from the myelin deficient rat *in vivo* (Beesley *et al.*, 2001), while *in vitro*, the treatment of oligodendrocytes with NGF results in caspase-1, -2 and -3 activation (Gu *et al.*, 1999).

AIMS

One other feature of sciatic nerve development that is coincident with maximal Krox-24 gene expression is normal Schwann cell death. This in combination with a previous report that Krox-24 may regulate p75NTR, itself implicated in growth factor induced cell death, lead me to hypothesise that Krox-24 may be involved in Schwann cell death.

RESULTS

Apoptosis is a rare event in newborn sciatic nerve of both wildtype and Krox-24 deficient mice

The expression of Krox-24 in the Schwann cell lineage is predominantly early in normal development (Topilko *et al.*, 1997 and Chapter 4) and is maximal on or close to the day of birth. We hypothesised that an involvement of Krox-24 in Schwann cell apoptosis may be likely as apoptosis is a feature of early Schwann cell development (Ciutat *et al.*, 1996, Trachtenberg and Thompson 1996, Syroid *et al.*, 1996, Grinspan *et al.*, 1996, Nakao *et al.*, 1997, Syroid *et al.*, 2000).

In order to address this question cryostat sections of fresh frozen sciatic nerve from newborn Krox-24 mutants and wildtype litter mates were assayed for TUNEL an indicator of apoptosis. This technique, as detailed in methods, revealed very few TUNEL positive cells in the normal newborn nerves irrespective of whether the nerves were from Krox-24 mutants or not (Fig 6.1 and Fig 6.2 B, D.). Quantification of TUNEL positive cells expressed as a percentage of all nuclei (as revealed by DAPI staining) showed about 0.08% +/- (n=5) of cells were undergoing apoptosis in null mutant and 0.075 %+/- (n=5) in wildtype nerves (Table 6.1.). This result compares quite favourably with the value of 0.15% found in control nerves at this stage by

Syroid and colleagues (Syroid *et al.*, 2000): This suggests that developmental Schwann cell death is not perturbed by Krox-24 deficiency at the newborn stage.

Apoptosis is significantly increased one day after transection and Krox-24 null mutant nerves exhibit a 3 fold increase in apoptosis compared with wildtype mice.

Two studies have demonstrated that the number of Schwann cells dying by apoptosis one day after transection injury, is increased 10 fold in the rat and 13 fold in the mouse sciatic nerve (Grinspan *et al.*, 1996, Syroid *et al.*, 2000). In the case of the p75NTR deficient mouse, this increase is not seen and given the suggestion that Krox-24 is required for p75NTR expression I wished to test whether the level of apoptosis in Krox-24 null mutant nerves could also be attenuated following injury.

Experiments that were conducted in parallel to those above showed that apoptosis is significantly increased 1 day after transection (Fig 6.2 A, C., Fig 6.3 A, B.). TUNEL positive cells can be seen as bright FITC positive (green) nuclei that appear condensed as visualised by DAPI staining (blue). The amount of apoptosis had risen in the wildtype animal from 0.075% to 1.8% after transection, a 24 fold increase (Table 6.1, figure 6.1). Surprisingly, the sciatic nerve derived from Krox-24 deficient mice showed an even larger increase in apoptosis, increasing from 0.08% to 5.2%, a 65 fold increase (Table 6.1, figure 6.1). This difference in TUNEL positive nuclei is readily visible by eye at low magnification (Fig 6.3 A, B.) but also by higher

magnification (Fig 6.2 A, C.). This represents a dramatic three fold increase in apoptosis as compared to wildtype litter mates following sciatic nerve transection.

Apoptosis is absent from normal adult sciatic nerve and 1 day after transection

Schwann cell apoptosis is not a major feature of adult sciatic nerve and the cells are remarkably resistant to cell death following transection (Nakao *et al.*, 1997, Grinspan *et al.*, 1996, Ferri and Bisby 1999). Having revealed that Krox-24 deficient Schwann cells in sciatic nerves of newborn mice are more susceptible to cell death following axotomy we hypothesised that cell death in the adult nerve might also be modulated in a similar fashion.

Sections of adult sciatic nerve from wildtype and Krox-24 null mutant taken from the same, mid thigh level were assayed for TUNEL. The number of TUNEL positive nuclei within normal adult wildtype nerve and Krox-24 deficient nerve are practically zero (Fig 6.5). Many Schwann cell nuclei are visible as stained by DAPI (Fig 6.5 A, G.) and these do not show TUNEL positive FITC staining (Fig 6.5 B, H). Similarly nerves assayed for TUNEL one day after transection, also do not display prominent apoptotic nuclei, irrespective of whether the tissue is derived from wildtype (Fig 6.5 E). or Krox-24 null mutant mice (Fig 6.5 K).

Apoptosis is also absent from longer term denervated nerves.

Schwann cell apoptosis in adult sciatic nerve can be elicited in long term denervated preparations and is noticeable 5 days after transection and maximal at 21 days (Ferri

and Bisby 1999). We therefore wanted to determine if Krox-24 deficient Schwann cells in long term denervated adult sciatic nerves were more susceptible to cell death compared to wildtype litter mates.

To address this question the sciatic nerves of adult mice were transected and assayed for TUNEL after two weeks. Sections of adult sciatic nerve from wildtype and Krox-24 null mutant were taken from the same, mid thigh level. The number of TUNEL positive nuclei within normal adult wildtype nerve and Krox-24 deficient nerve were again found to be practically zero (Fig 6.6 B, H). Many Schwann cell nuclei are visible as stained by DAPI (Fig 6.6 A, G.) and these are not showing TUNEL positive FITC staining (Fig 6.6 B, H). Similarly nerves assayed for TUNEL 14 days after transection, showed a degree of apoptotic nuclei, irrespective of whether the tissue is derived from wildtype (Fig 6.6 E) or Krox-24 null mutant mice (Fig 6.6 K). Quantification showed a similar increase in apoptosis in wildtype 1.2% +/-SD (n=3) and Krox-24 null mutant mice 1.1% +/-SD (n=3).

Krox-24 deficient newborn Schwann cells survive at least as well as wildtype cells in low density survival assays

Given that cells in the newborn sciatic nerve are more susceptible to apoptosis following axotomy compared to wildtype we wanted to evaluate if this was a Schwann cell autonomous feature or if the effect was due to an extrinsic factor that actively killed the cells. In order to address this question Schwann cells were isolated from P1 sciatic nerves and plated at low density (300 cells) on polyornithine

(PORN) coverslips and maintained in a simple defined medium as described in Meier *et al.*, 1999 and in methods above. S100 staining was employed to label cells and counts were made of cells at the time of plating and 1 day later. Viable cells were counted as surviving when showing normal nuclear morphology and by S100 staining (Fig 6.7 A, D).

After one day in culture surviving Schwann cells of both Krox-24 null mutant and wildtype litter mates displayed similar bi or tripolar morphology typical of cultured Schwann cells (Fig 6.7 G, J, I, L.). On average 67% +/- 14 (n=13) of wildtype Schwann cells survived in this one day assay compared to 69% +/- 9 (n=8) for the Krox-24 deficient Schwann cells (Fig 6.8). Using the Mann-Whitney non-parametric two sample test, we were able to determine that there was little or no significance between these two sets of data (P>0.05). This data suggests that Schwann cell death occurs at a normal rate in cells deficient in Krox-24 when compared with wildtype cells.

These cultures were impure but nerves were desheathed to remove the perineurium and any fibroblasts contained therein. Counts to assess purity revealed that, on average, the cultures were 95% pure. Any contaminating fibroblasts present were not counted as they do not express S100, a single fibroblast can be seen by its large nucleus in Figure 6.5 C, which is S100 negative (Fig 6.10 A).

Schwann cells that are considered not to have survived in these assays did not become detached from the substrate but remained *in situ* as rounded up cells. These could be readily visualised by phase contrast (Fig 6.7 I, L.) even though they

continued to stain positively for S100 (Fig 6.7 G, J). These cells were confirmed as Schwann cells that were dying or indeed dead by the TUNEL assay (Fig 6.9 C, D.). Schwann cell derived from wildtype nerves showed 27.5% +/- 3 (n=3) TUNEL positive cells and similar percentages were found in cells deficient in Krox-24, 29% +/- 1.5 (n=3). Statistically there was no difference in the number of cells that were TUNEL positive between the two groups (P>0.05).

TGF β induces Schwann cell death

The data above suggested that Krox-24 deficient Schwann cells survive at least as well as in low density cultures compared to wildtype controls. To determine if these cells were more susceptible to known Schwann cell death inducing factors, the prime candidate being TGF β , cultures were set up as above with the introduction of 10 μ M TGF β for 1 day.

TGF β was found to increase the Schwann cell death seen in these low density cultures (Fig 6.7 M-R). The survival in the presence of TGF β was calculated to be 24.5% +/- 2.5 (n=3) for wildtype cells and 30.5% +/- 5.5 for Krox-24 null mutant cells (Fig 6.8). These results were not statistically significant (P>0.05) indicating that induction of cell death by TGF β was not altered in Schwann cells deficient in Krox-24 compared with wildtype cells.

DISCUSSION

In this series of experiments I have demonstrated that transection of sciatic nerves at the newborn, promyelin stage, results in an increase in Schwann cell death when compared with wildtype litter mates. Krox-24 deficient Schwann cells are somehow more sensitive to cell death at this critical stage of development. As well as differentiating along either the myelin forming or non-myelin forming phenotypes Schwann cells, at this time, are acquiring an autocrine capability (Syroid *et al.*, 1999, Mejier *et al.*, 1999), and are becoming increasingly less susceptible to death by apoptosis induced by denervation (Grinspan *et al.*, 1996, Nakao *et al.*, 1997). Using short term, low density, cultures designed to limit the survival effects of autocrine factors, I have shown that Krox-24 deficient Schwann cells survive in comparable numbers to wildtype cells. Also it is clear from the experiments outlined above that active killing by TGF β is not the basis for the *in vivo* effect.

Krox-24 has been shown to be expressed during the pro-myelin period which suggests it may transactivate genes associated in this transitory phase of Schwann cell development (Topilko *et al.*, 1997 and see chapter 4). One such gene is that encoding p75NTR which is thought to be directly regulated by Krox-24 in Schwann cells *in vitro* as overexpression of antisense Krox-24 inhibits p75NTR expression (Nikam *et al.*, 1995). From this data the prediction for Schwann cells null for Krox-24 would be that p75NTR might be completely lacking and the cells would show

responses similar to those derived from p75NTR deficient mice. As detailed above p75NTR null Schwann cell apoptosis is reduced following denervation (Syroid *et al.*, 2000), while the result obtained for Krox-24 deficient cells is quite the opposite suggesting that p75NTR could be expressed at levels above the normal. It would be interesting to quantify p75NTR expression of newborn Schwann cells before and after sciatic nerve transection by western blot protein analysis to determine whether Krox-24 regulates p75NTR in vivo. The data presented in the preceding chapter suggests that p75NTR expression, at both mRNA and protein levels is increased, following axotomy of adult nerves deficient in Krox-24 when compared to wildtype controls. However, no difference of p75NTR mRNA expression was found at the newborn stage in Krox-24 deficient nerves compared with wildtype litter mates. Time limitations prevented further experiments to be carried out to determine p75NTR expression following axotomy of newborn sciatic nerves. One other possibility is that another member of the EGR protein family, such as EGR-3 replaces the lost function of Krox-24 and enables p75NTR expression. Therefore the result described above could be due to an altered function of an as yet to be identified gene.

Quantification of this result showed that there is a 24 fold increase in Schwann cell death in wildtype nerves following axotomy compared to a 65 fold increase seen in Krox-24 null mutant cells. This first value appears quite high compared to the literature and in particular the study of Syroid and colleagues (Syroid *et al.*, 2000). This study also carried out on mouse, and the only published

article to date on this system gives a value of 13 fold induction following transection, a 50% less increase than calculated here. Comparing the data, the reason for this difference may stem from control untransected apoptosis values arrived at in the different studies. Here I have found 0.075% apoptosis while Syroid and colleagues find 0.15%, which is 1.8 times larger. This difference alone could account for the seemingly exaggerated increase in cell death following axotomy arrived at in this study. If a variation in sensitivity of TUNEL protocols is the reason for this difference it is surprising that the apoptotic counts of control nerves after axotomy is similar at 1.8%, and a more likely explanation could be as a result of mouse strain differences in Schwann cell death. The mice in this study are on a C57/bl6 CD1 background while those in the other study are of balb-C origin (Syroid *et al.*, 2000).

Schwann cell death is not a feature of normal adult sciatic nerve (Nakao *et al.*, 1997, Grinspan *et al.*, 1996, Ferri and Bisby 1999). The data presented here is in line with these studies and values of zero were obtained for both Krox-24 deficient and normal nerves. Long term denervation results in an increase in Schwann cell death to detectable levels (Ferri and Bisby 1999). Similarly I find that after 14 days of chronic axotomy about 1% of Schwann cells die via apoptosis. An absolute comparison between the data presented here and that cited is not possible due to the different time points assayed and the expression of data as a function of area as opposed to percentage of nuclei. Clearly though there is little difference in Schwann cell death numbers comparing wildtype controls to Krox-24 deficient sciatic nerves 14 days after denervation.

There would appear to be a difference in response of Schwann cells deficient in Krox-24 to axotomy at the newborn and adult stages; newborn cells are more susceptible to death while adult cells remain as stable as their wildtype counterparts. As described above, clear differences between newborn and adult cells exists; newborn cells in the process of acquiring autocrine function (Syroid *et al.*, 1999, Meier *et al.*, 1999), can be considered to be mid differentiation, (Jessen and Mirsky, 1999) and are sensitive to neuregulin signalling derived from the axon (Trachtenberg and Thompson 1996, Syroid *et al.*, 1996, Grinspan *et al.*, 1996, Nakao *et al.*, 1997). The available data and that documented in chapter 4 define one further difference; newborn Schwann cells normally express Krox-24 maximally while in adult cells very little krox-24 is detectable (Topilko *et al.*, 1997). Although Krox-24 is rapidly up-regulated in Schwann cells following axotomy of the adult sciatic nerve, the main difference in axotomy of newborn and adult sciatic nerves is that in newborn nerves pre-existing, high, levels of Krox-24 are present, which are absent in adult cells (Topilko *et al.*, 1997 and chapter 5). The possibility that Krox-24 regulates a glioprotective, anti-apoptotic gene such as a member of the Bcl or AIP families is an attractive one. These data would support a hypothesis that this protective function is focused in early development and that the stability of Schwann cells seen in the adult sciatic nerve is attributable to mature autocrine function and/or alternative regulation of genes that suppress cell death. It would be interesting to investigate what effect Krox-24 may have in earlier Schwann cell development given that expression is first seen at the precursor stage.

Low density survival assays have been employed to investigate if Schwann cells are more susceptible to cell death *in vitro* when null for Krox-24 in conditions that do not favour autocrine survival. The results suggest that Schwann cell survival is not perturbed in Krox-24 null mutants and that the cells survive at least as well as wildtype controls. Having established that at low densities the cells survived as well as controls I wanted to ascertain if the cells were more susceptible to active killing by a known factor or if the cells did not respond in the correct fashion to elements of the autocrine loop. I decided to investigate if TGF β could be responsible for the increased cell death seen in the newborn axotomised nerves *in vivo*. It was found that in the same low density cultures TGF β killed Schwann cells and approximately 25% survived after a 1 day exposure, this compares favourably for similar assays conducted on rat Schwann cells (Parkinson *et al.*, In press). This effect was similar for Schwann cells deficient for Krox-24 and suggests that increased TGF β sensitivity is not present on these cells. The possibility that another factor such as NGF is responsible remains to be investigated. Alternatively the cells may not be surviving in response to positive cues such as IGF, NT3 and PDG-BB, members of the autocrine loop, and it would be interesting to see if the responses of Krox-24 null mutant cells were attenuated to one or other of these factors.

To summarise: Schwann cell death in response to axotomy of newborn sciatic nerves is increased 3 fold in cells deficient in the transcription factor krox-24. This increase of Schwann cell death does not seem to be paralleled in the mature Schwann cell

population found in the adult sciatic nerve. Together these data suggest that the absence of Krox-24 expression confers a negative survival signal to developing Schwann cells in the presence of axons, which is not significant in mature adult Schwann cells. The survival of Schwann cells isolated from Krox-24 null mutants does not appear to be modified as they display similar low density survival patterns as compared to wildtype controls. Further work would be required to investigate if all components of the autocrine loop; IGF, NT3 and PDGF-BB, were able to rescue these cells as they can wildtype cells (Meier *et al.*, 1999). We have investigated the possibility that Krox-24 null-mutant Schwann cells are perhaps more susceptible to TGF β induced death and show that clearly this is not the case.

What could be causing increased Schwann cell death seen *in vivo*? A number of different factors could be causative of Schwann cell death in such a model of axonal loss including NGF and future work could include a study of the *in vitro* effect of exogenous NGF on Schwann cells deficient in Krox-24.

CONCLUSIONS

I have shown a three fold increase in cell death in the new born sciatic nerves of Krox-24 null mutant mouse as compared to the wildtype controls. I show that the cells are probably more susceptible to an *in vivo* death signal and that this signal is not TGF β . I conclude that Krox-24 may be either directly or indirectly involved in Schwann cell survival *in vivo*. My results from the previous chapter implicate Krox-24 in the regulation of p75NTR and this may form the basis for future work.

Figure 6.1 TUNEL positive nuclei in normal P1 sciatic nerve

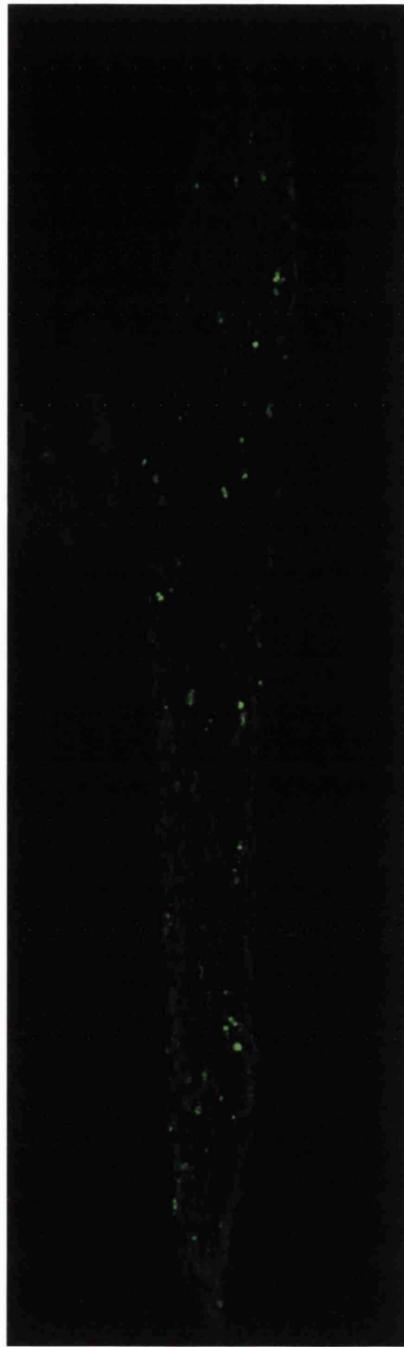
Low magnification fluorescence montage of a longitudinal section through a wildtype sciatic nerve. This single 1cm length of nerve encompasses a region from the sciatic notch through to the distal tip of the tibial branch that extends towards the foot.

Figure 6.2 P1 Schwann cell death increases upon sciatic nerve transection

Low magnification fluorescence image of sections through sciatic nerves, at the mid-thigh level, one day following transection. Longitudinal sections through a wildtype (+/+) and Krox-24 null mutant (-/-) sciatic nerves stained for TUNEL are shown. TUNEL positive nuclei revealed with FITC, again appearing green, are dramatically increased compared to control nerves (not shown). The Krox-24 null mutant has increased TUNEL positive nuclei (-/-) compared to the wildtype (+/+).

Scale bar = 200 μ m

+/+



-/-

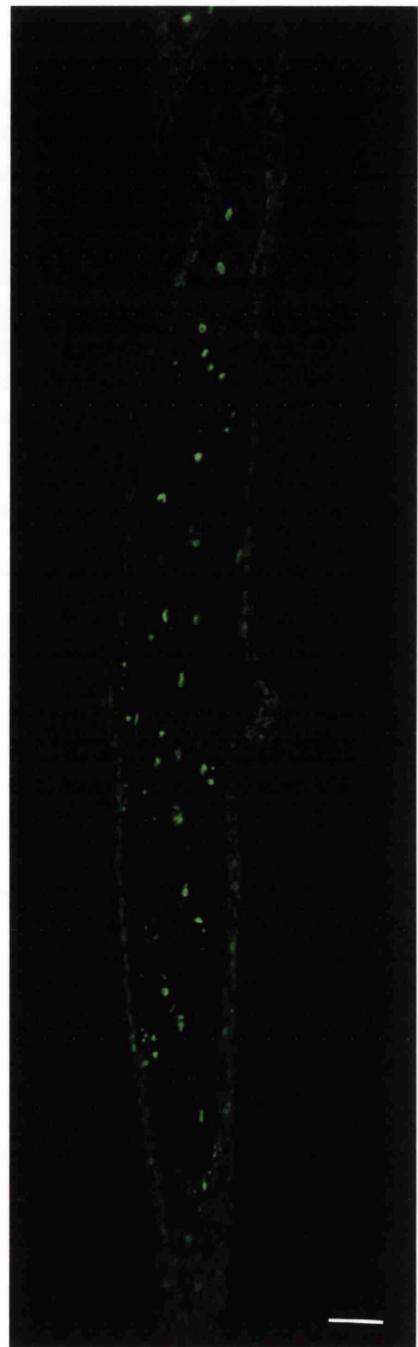
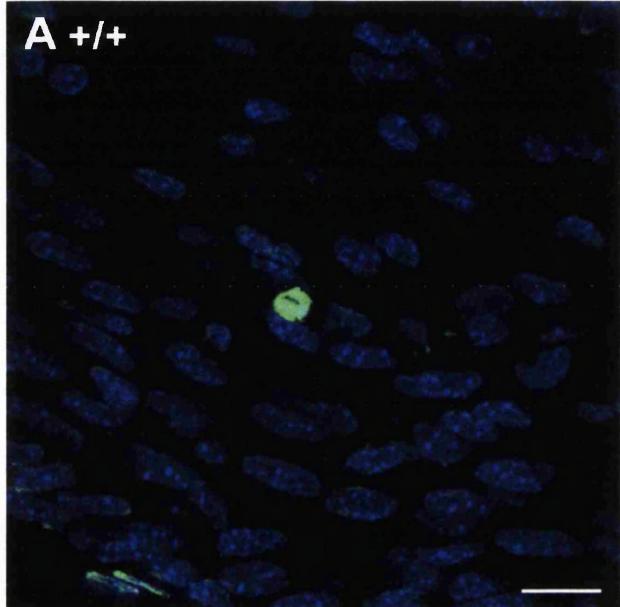


Figure 6.3 Krox-24 deficient Schwann cells are more susceptible to cell death in vivo

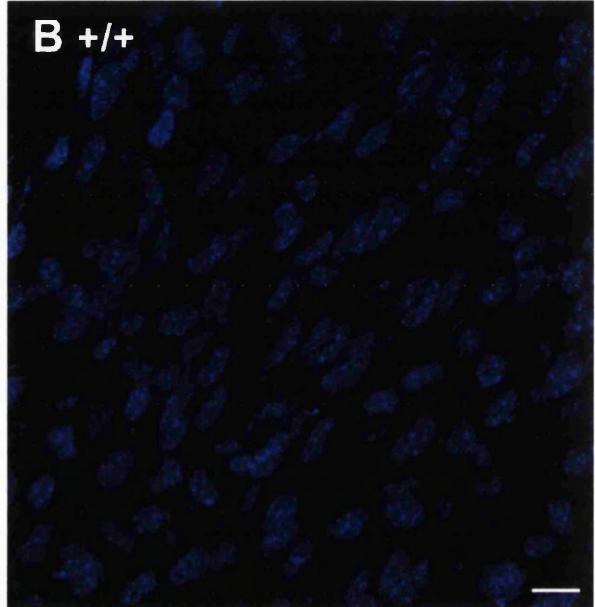
High magnification fluorescence image of sections through P1 sciatic nerves, at the mid-thigh level, one day following transection (A, C) or contralateral controls (B, D). Longitudinal sections through a wildtype (A, B) and Krox-24 null mutant (C, D) sciatic nerves stained for TUNEL. TUNEL positive nuclei revealed with FITC, again appearing green, are increased in mutant nerves (C) compared to wildtype (A). Nuclei are labelled with DAPI and fluoresce blue

Scale bars = 50 μ m

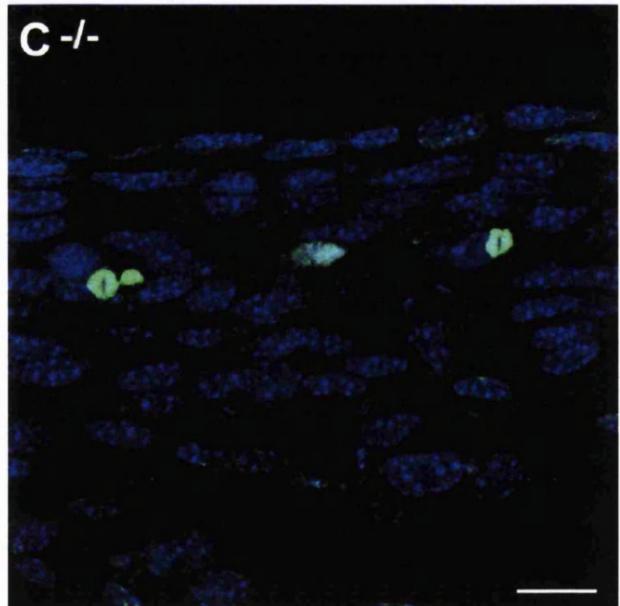
Transected



Control



C $-/-$



D $-/-$

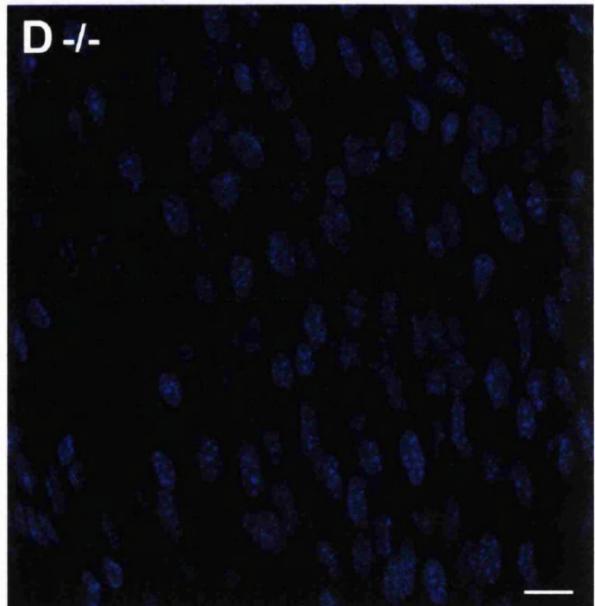


Figure 6.4 Quantification reveals a three fold increase in cell death of Krox-24 null mutant Schwann cells in vivo

Graph of percentage of apoptotic cells as measured by TUNEL for control nerves at P1 together with those of transected nerves. Krox-24 $-/-$ (K/O) untransected nerve showed 0.08 % $+$ / $-$ 0.012 (n=5) compared to 0.075 % $+$ / $-$ 0.15 (n=5) in wildtype nerves (WT). Following transection wildtype nerves (WT trans) showed 1.85 % $+$ / $-$ 0.064% TUNEL positive cells compared to 5.4 % $+$ / $-$ 1.184 for Krox-24 $-/-$ nerves (K/O trans).

**Cell death in normal P1 sciatic nerves and 1 day after denervation
(*in vivo*)**

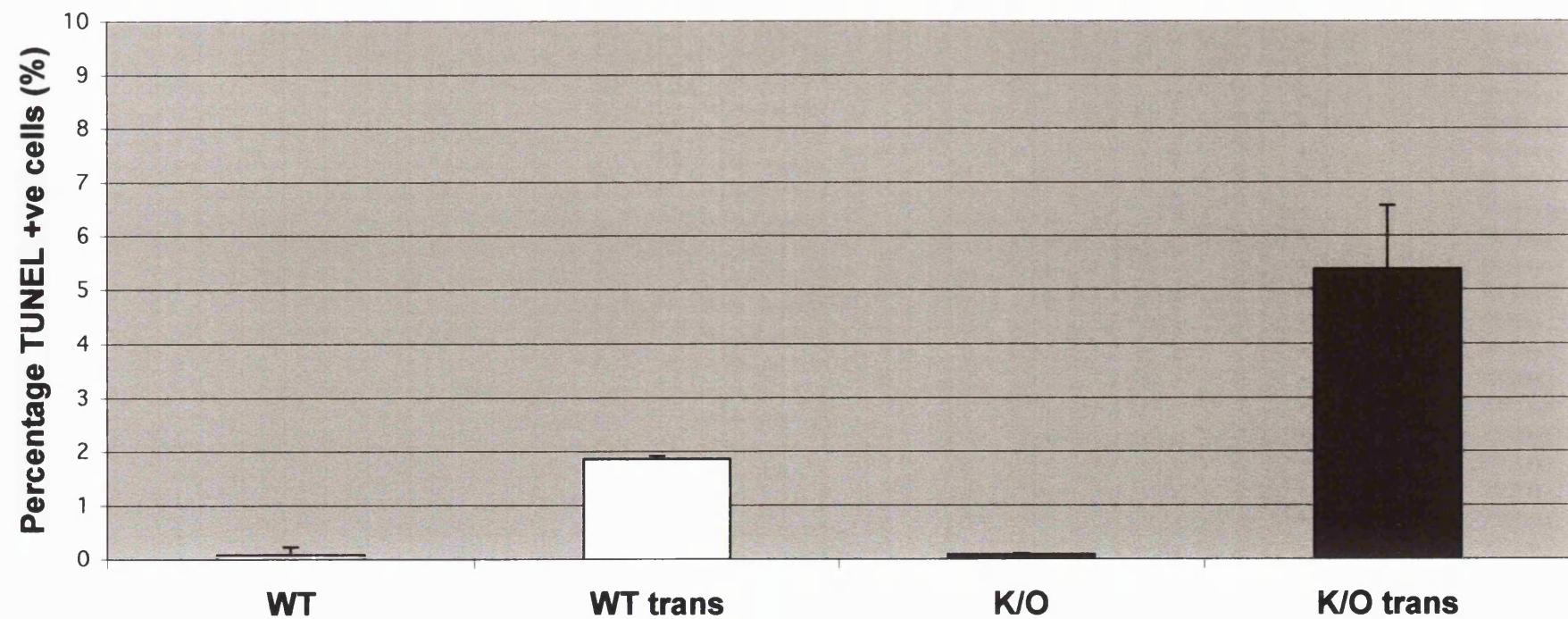


Figure 6.5 Schwann cell death is not a feature of adult sciatic nerve

Fluorescence images of longitudinal sections through adult sciatic nerves, at the mid-thigh level, of wildtype (A-F) and Krox-24 null mutants (G-L). Control nerves (A-C, G-I), and those one day following transection (D-F, J-L) are shown. TUNEL positive nuclei revealed with FITC are not present in all nerve samples assayed (B, E, H, K). Nuclei are labelled with DAPI and fluoresce blue (A, D, G, J). The continuous, tract like, morphology of these nerve samples can be seen with phase contrast (C, F, I, L).

Scale bars = 50 μ m

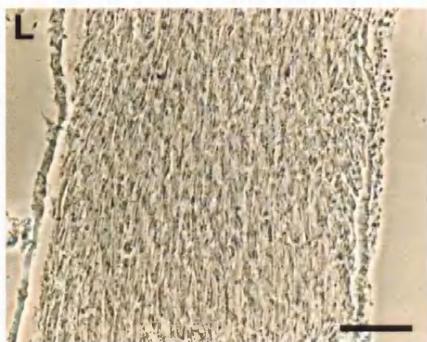
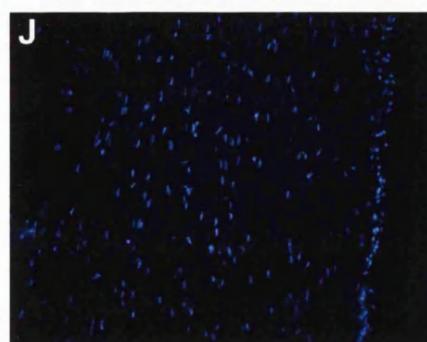
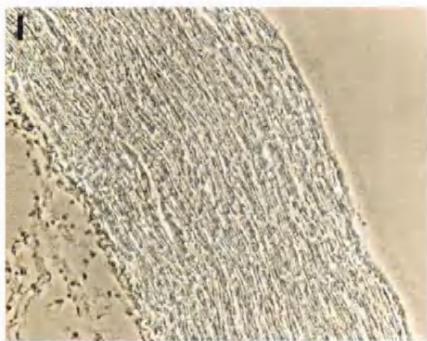
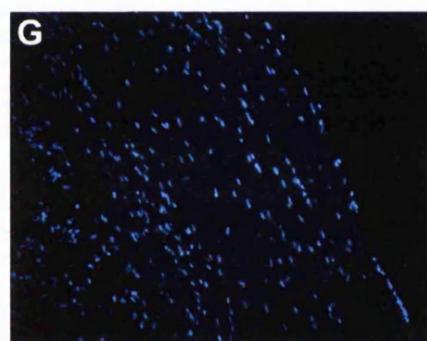
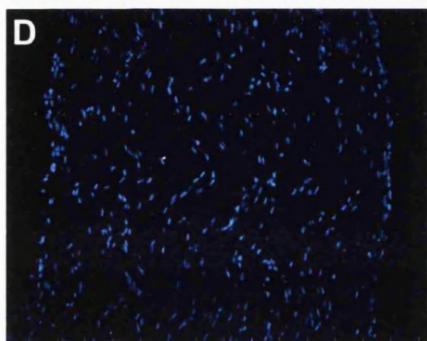
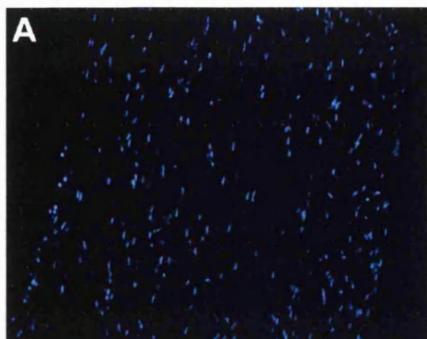
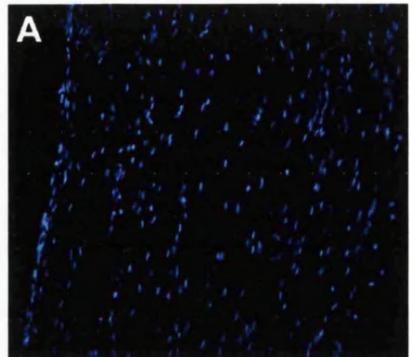
Dapi**Tunel****Phase**

Figure 6.6 Schwann cell death is present 14 days after transection

Fluorescence images of longitudinal sections through adult sciatic nerves, at the mid-thigh level, of wildtype (A-F) and Krox-24 null mutants (G-L). Control nerves (A-C, G-I), and those 14 days following transection (D-F, J-L) are shown. TUNEL positive nuclei are present in similar proportions in both wildtype (arrows, E) and Krox-24 deficient nerve samples (arrows, K). Nuclei are labelled with DAPI and fluoresce blue (A, D, G, J), Schwann cells proliferate within the distal stump and an increase in DAPI labelled nuclei can be seen in both wildtype (D) and null mutant (J) as compared to controls (A and G respectively). Phase contrast images (C, F, I, L) reveal the amorphous nature of degenerating nerves (F and L) compared to controls (C and I).

Scale bars = 50 μ m

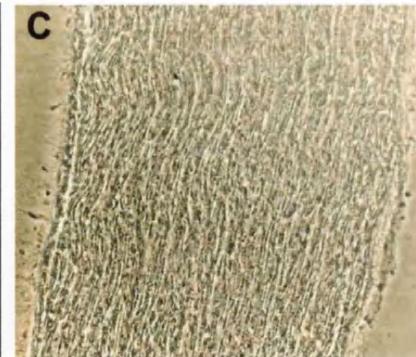
Dapi



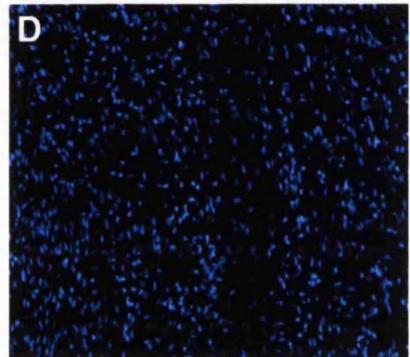
Tunel



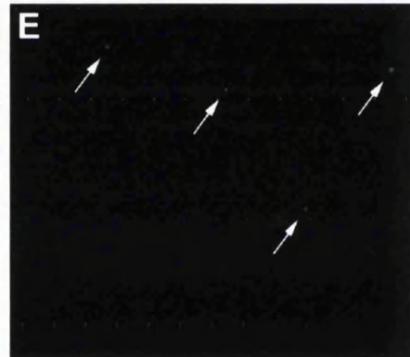
Phase



D



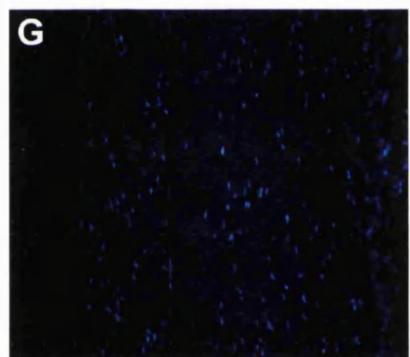
E



F



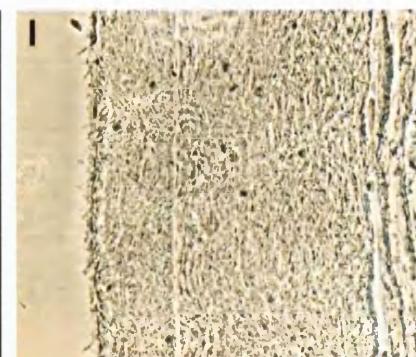
G



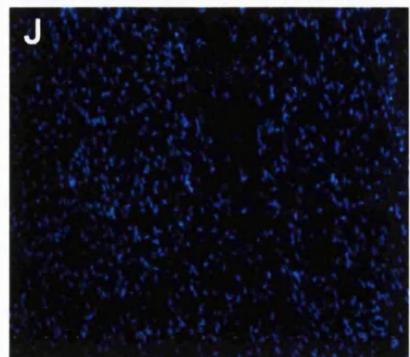
H



I



J



K



L

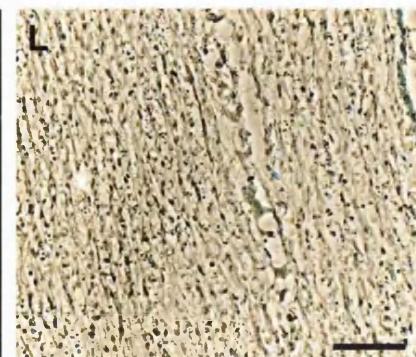


Figure 6.7 In vitro assays reveal similar survival at low density and in the presence of TGF β in Krox-24 deficient Schwann cells

Fluorescent and light micrographs of Schwann cells plated at low density and assayed 10 hour after plating (Control), one day later (1 Day after plating) and one day in the presence of TGF β 1 (1 Day + TGF). Schwann cells were identified by S100 staining and revealed with FITC (left-hand column). Nuclei are labelled with DAPI and fluoresce blue (central column). Cell morphology can be seen with phase contrast (right-hand column). Surviving Schwann cells were counted stained positively for S100, without nuclear condensation and showing clear processes of bi or tripolar nature.

Scale bar = 50 μ m

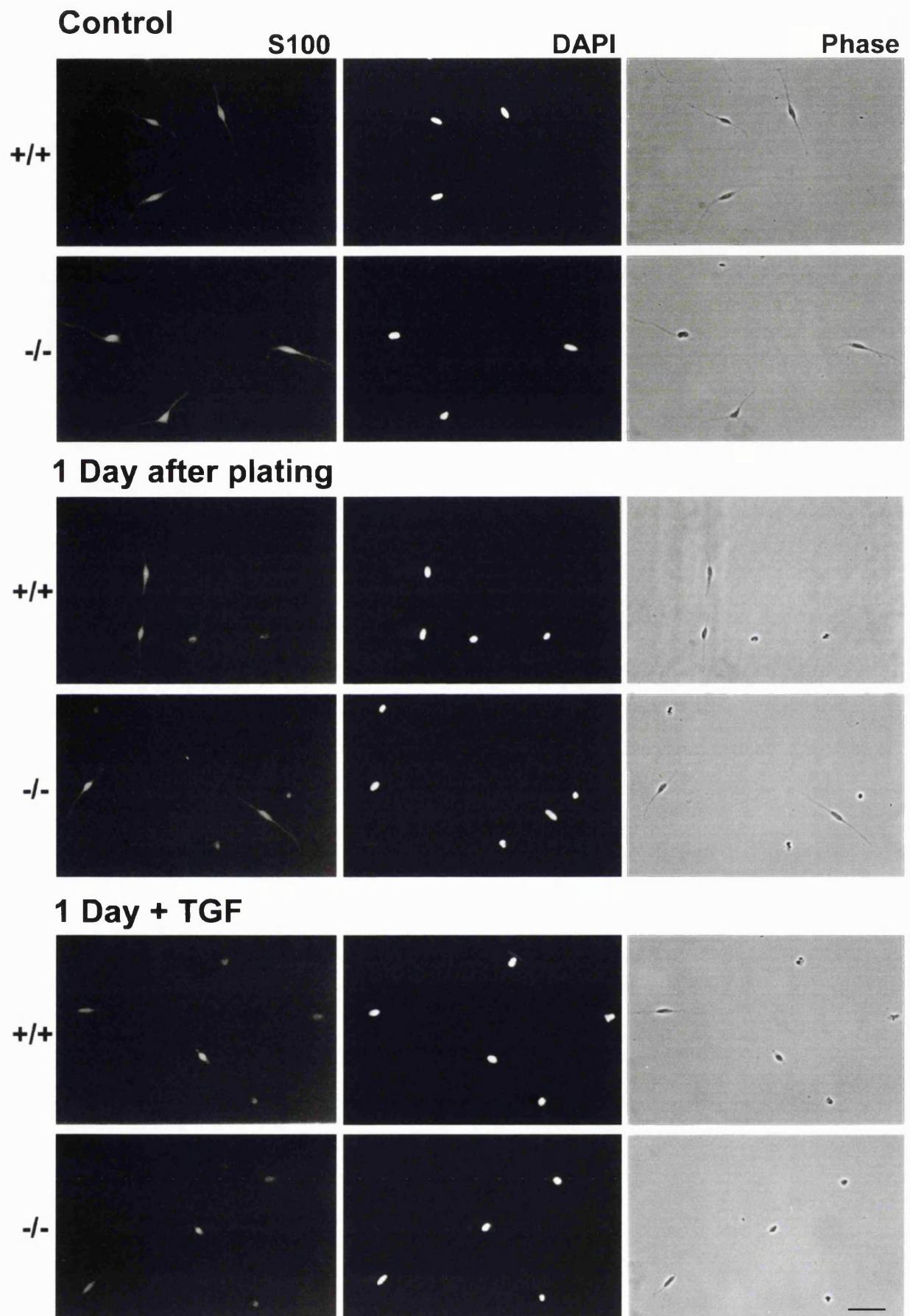


Figure 6.8 Quantification reveals normal survival at low density and in the presence of TGF β in Krox-24 deficient Schwann cells

Graph of percentage Schwann cell survival as a function of sister control cultures cells. One day after plating on average 67 % +/- 14 (n = 13) wildtype SCs survival was seen compared to 69 % +/- 9 (n = 8) in the null mutant and in the presence of TGF β 27.5% +/- 3 (n=3) wildtype survived compared to 29% +/- 1.5 (n=3) in the null mutant.

Schwann cell survival; 1 day low density assay

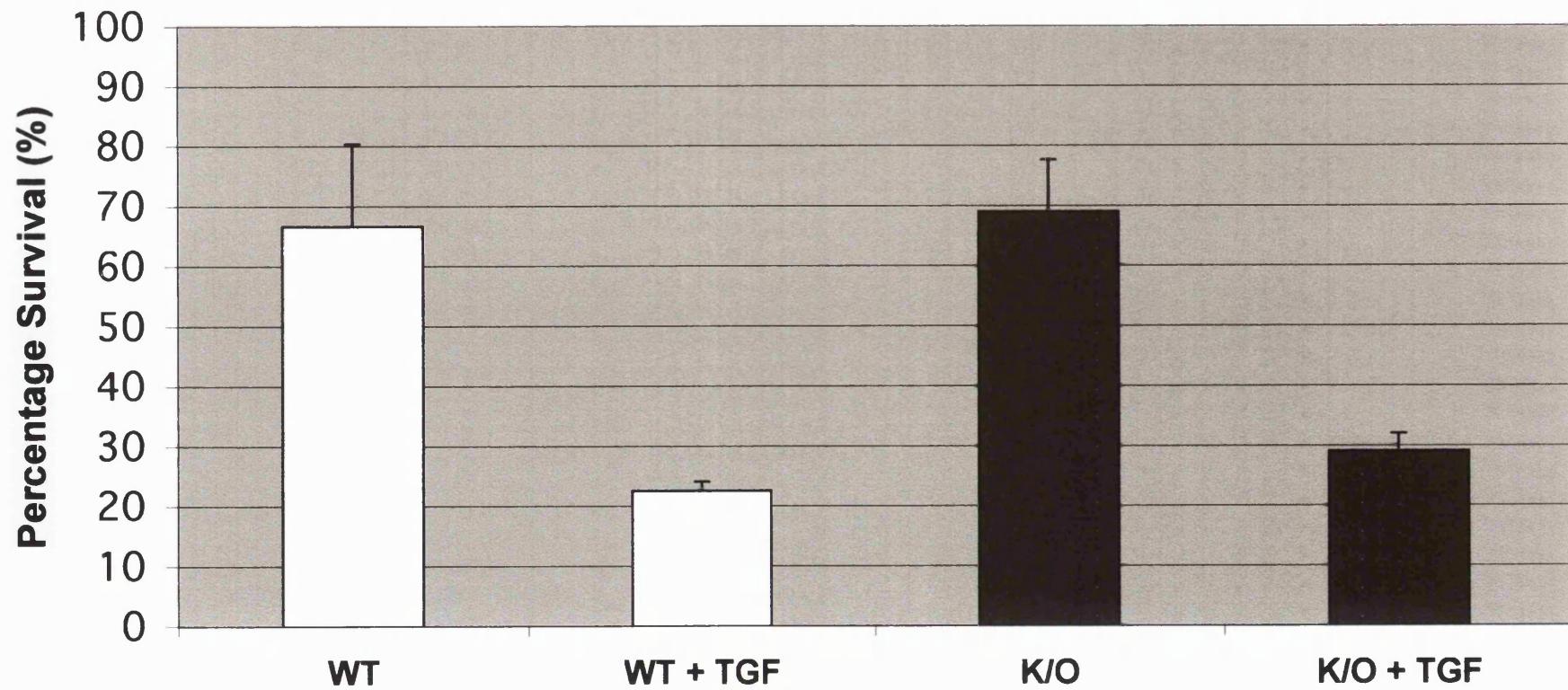
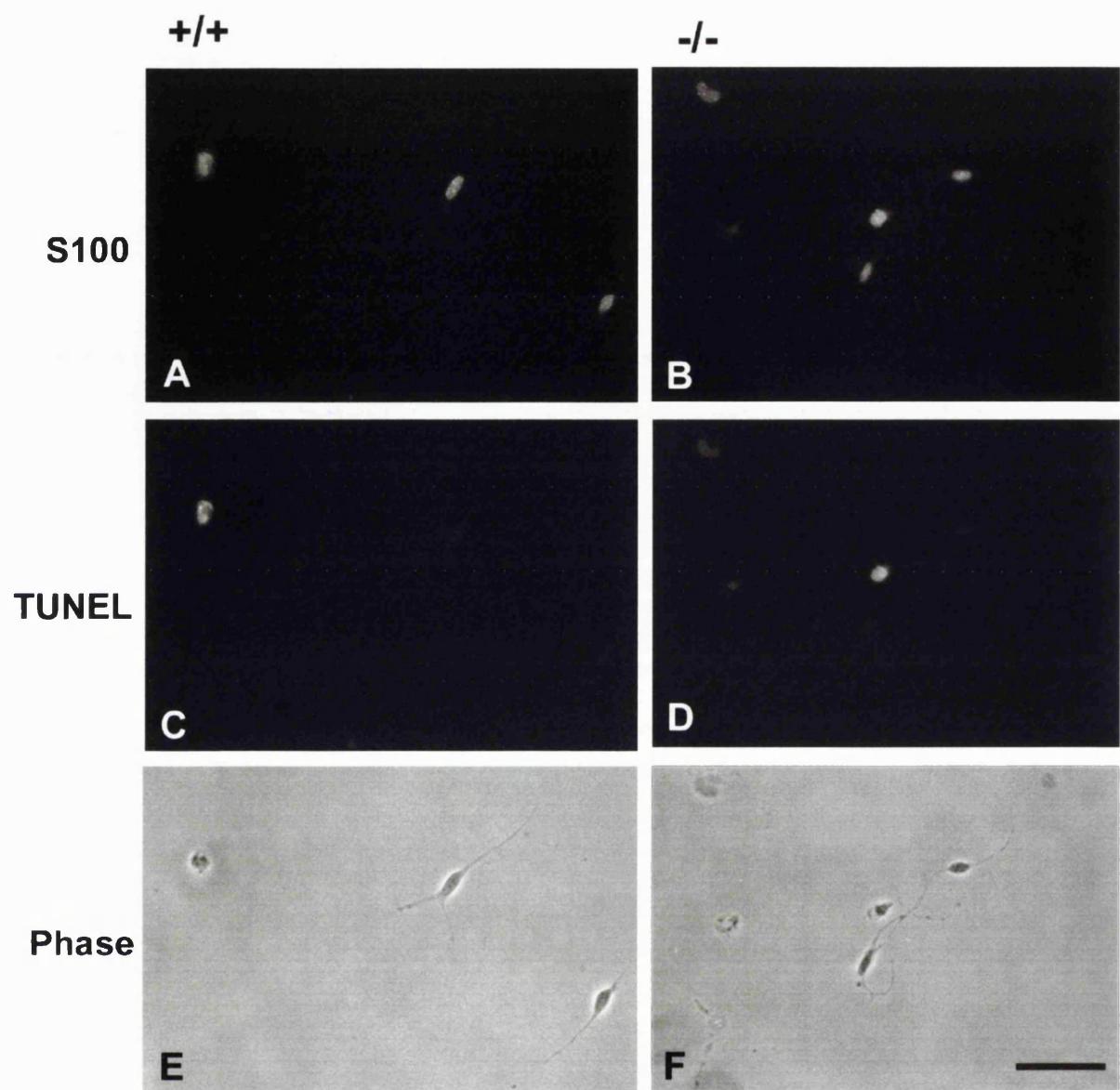


Figure 6.9 Schwann cells die by apoptosis in low density survival assays

Fluorescent and light micrographs of Schwann cells plated at low density from wildtype (A, C, E) and Krox-24 null mutant mice (B, D, F) and assayed for TUNEL one day after plating. Schwann cells were identified by S100 staining and revealed with rhodamine (A, B). Dying cells can be seen as TUNEL positive spheres, showing atypical nuclear morphology (C, D). These TUNEL positive cells continue to be S100 positive identifying them as Schwann cells (A, B), but have retracted processes and rounded up morphology by phase contrast (E, F).

Scale bar = 50 μ m

Quantification revealed similar levels of Schwann cells undergoing apoptotic cell death from wildtype, 27.5% +/- 3.3% (n=3), and Krox-24 deficient animals 28.8% +/- 1.5% (n=3).



Number of TUNEL positive Schwann cells

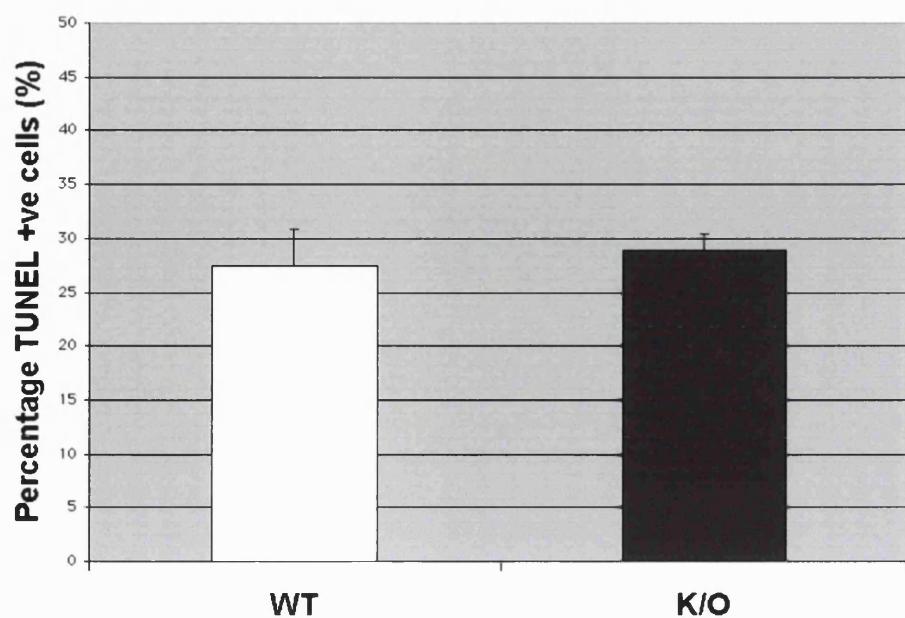


Figure 6.10 Low density cultures of mouse sciatic nerve are 95% pure Schwann cells

Fluorescent and light micrographs of Schwann cells plated at low density from wildtype (A, C) and Krox-24 null mutant mice (B, D). A single contaminating fibroblast nucleus visible as a large DAPI stained ovoid is present(C) and this cell is S100 negative (A).

Scale bar = 50 μ m

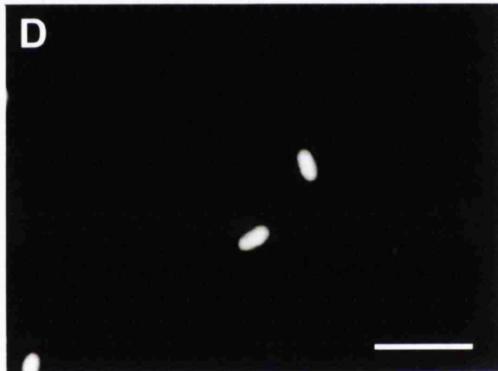
+/+

-/-

S100



DAPI



CHAPTER 7

GENERAL DISCUSSION

The results presented in this thesis expand our knowledge of the molecular events involved in Schwann cell development, and in particular of the role of the zinc-finger transcription factor population contained therein. I have described the expression pattern of a recently cloned zinc-finger transcription factor, Zfp-57, in Schwann cell development. Zfp-57 mRNA and protein are found in Schwann cells from early in development into maturity where they can be identified in both myelin forming and non-myelin forming Schwann cells (Fig 7.1). Zfp-57 is the third transcription factor of this class to be described in the Schwann cell lineage, after Krox-20 and Krox-24 (Topilko *et al.*, 1994, Topilko *et al.*, 1997). The number of genes encoding this class of proteins has recently been shown to be large and it is likely that even more will be discovered in the nervous system (Venter *et al.*, 2001, I.H.G.S.C. 2001). Work is continuing in the laboratory to further elucidate Zfp-57 expression by *in situ* hybridisation and a construct has been generated for the subsequent generation of a Zfp-57 knockout transgenic mouse line.

I have also investigated the expression of Krox-24 in the development of the sciatic nerve. Using the LacZ gene as a marker of Krox-24 gene transcription I have shown that high levels of expression are detected at E18 while the highest level was found at

the newborn stage which is consistent with other data (Watson and Millbrandt, 1990, Topilko *et al.*, 1997). It is possible that all of the Schwann cells are positive at this stage but further investigations would be necessary to confirm this. I have also explored the phenotype of Schwann cells of sciatic nerves of Krox-24 deficient mice. No difference in proliferation could be detected between wildtype and Krox-24 null mutant Schwann cells within the sciatic nerve at the three stages assayed. Analysis of the ultrastructure of the developing and mature sciatic nerve has revealed that Krox-24 deficiency does not result in any obvious phenotypic abnormalities of either the myelin forming or non-myelin forming Schwann cell populations. This data is supported by the finding that known Schwann cell markers such as Krox-20, Oct-6, P₀, p75NTR and NCAM, are expressed at normal (mRNA) levels in Krox-24 null mutant nerves. Taken together these data suggest that mice lacking a functional Krox-24 gene do not experience any deficiency in the development and maturation of either myelin forming or non-myelin Schwann cells in the sciatic nerve.

In this study the expression of Krox-24 following crush or transection within the sciatic nerve has also been investigated. I show that Krox-24 is found throughout the distal nerve portion and to a lesser extent, in the proximal stump close to the site of injury and that the intensity of this expression is higher in the model of permanent transection. The expression of Krox-24 is most likely to be Schwann cell derived and associated with a loss of axonal contact, although my data does not prove this

unequivocally. In the nerves of Krox-24 deficient mice I show that the processes of degeneration and regeneration are not perturbed. I show that the bulk of re-growing axons together with those that re-grow at the fastest rate are present in similar quantities in Krox-24 null mutants compared with wildtype animals. I have also shown that, with the exception of p75NTR, the Schwann cell response to axotomy and regeneration is normal in Krox-24 deficient mice.

Finally I have shown that cell death in the sciatic nerve occurs normally in development while the transection of sciatic nerves at the newborn, promyelin stage, results in an increase in Schwann cell death when compared with wildtype litter mates. Quantification of this result showed that there is a 24 fold increase in Schwann cell death in wildtype nerves following axotomy compared to a 65 fold increase seen in Krox-24 null mutant cells. This cell death is probably due to the specific loss of Schwann cells but this remains to be confirmed. Using short term, low density, cultures designed to limit the survival effects of Schwann cell autocrine factors, I have shown that Krox-24 deficient Schwann cells survive in comparable numbers to wildtype cells. This suggests that an extrinsic factor present within the sciatic nerve is responsible for the increase in cell death seen *in vivo*.

The most obvious extrinsic candidate was TGF β , which has been shown to actively kill Schwann cells *in vitro* and *in vivo* in this laboratory (Parkinson *et al.*, in press). I investigated whether TGF β could be responsible for the increased cell death seen in the newborn axotomised nerves *in vivo*. It was found that in low density Schwann cell cultures TGF β killed Schwann cells. Approximately 25% survived after a 1 day

exposure, in both Krox-24 deficient and wild type Schwann cell populations. This data suggests that TGF β is not responsible for the increased sensitivity to cell death seen in these Krox-24 deficient cells. The possibility that another factor is responsible remains to be investigated (see below).

The p75NTR molecule is normally up-regulated in the distal part of the sciatic nerve in Schwann cells after injury (Taniuchi *et al.*, 1986, Heumann *et al.*, 1987, Lemke and Chao, 1988, Mirsky and Jessen, 1990, Robertson *et al.*, 1995). My experiments show that the relative expression of p75NTR is enhanced at the mRNA level in Krox-24 deficient sciatic nerves when compared to wildtype controls. It will be interesting to see in future whether the actual quantity of p75NTR protein is increased in Krox-24 deficient Schwann cells following axotomy. Krox-24 interactions with p75NTR have been previously reported (Nikam *et al.* 1995). It was shown that overexpression of Krox-24 antisense cDNA in cultured Schwann cells resulted in ablation of p75NTR expression. The results arrived at in this study conflict with these results and the two studies are difficult to reconcile. It should be noted however, that normal adult levels of p75NTR protein were detected in uninjured Krox-24 deficient sciatic nerves (data not shown) and that p75NTR mRNA is detectable in neonates (see chapter 4). These findings suggest that Krox-24 is not required for the normal p75NTR expression *in vivo* in the transgenic mutant animal studied here. As previously discussed, Taniuchi and colleagues proposed that Schwann cell expression of p75NTR serves to enhance regeneration by the binding and presentation of NGF to growth cones (Taniuchi *et al.*, 1988). In this study, if

p75NTR is indeed increased over and above the levels that would normally be expressed by Schwann cells following injury, it is significant that no effect on nerve regeneration could be seen. This would argue against the proposed model assuming that other factors such as neurotrophins are not altered in this Krox-24 null mice (not tested here).

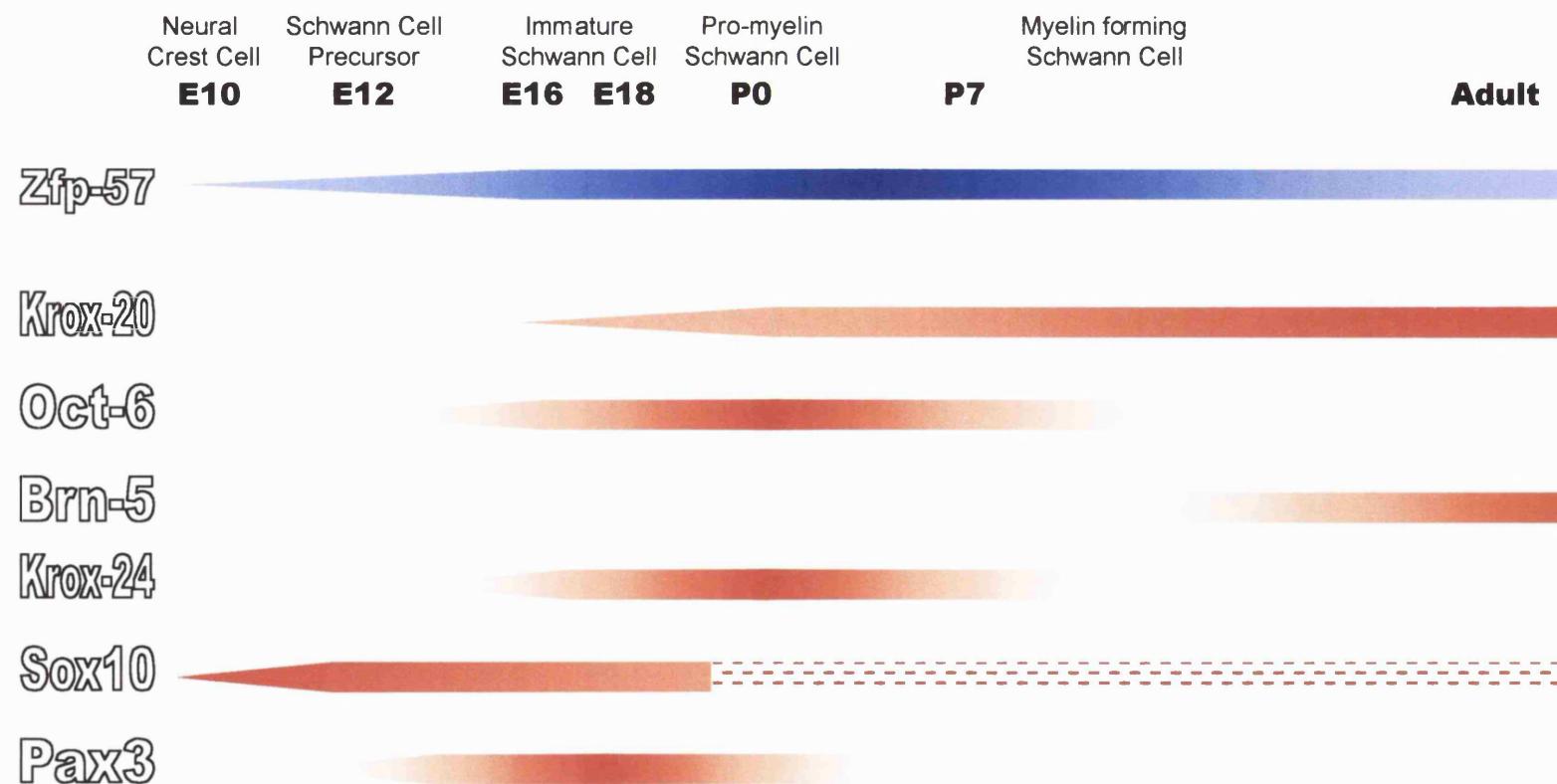
The classical view of the function of p75NTR is, however, beginning to change. Data suggest that p75NTR can augment the trophic response of a given cell to NGF by interacting with TrkA forms (reviewed in Carter and Lewin, 1997, Frade and Barde, 1998). In addition to this trophic function, which depends on heterogenous dimerization of TrkA and p75NTR, p75NTR may produce an alternative response in cell populations that express p75NTR together with low levels of trk receptors or truncated forms. Schwann cells provide an example of such a response since Schwann cells isolated from p75NTR knockout mice show impaired migratory function in response to neurotrophins compared with wildtypw cells (Anton *et al.*, 1994, Bentley and Lee, 2000). Additionally, there is a now a body of evidence to suggest an additional function of p75NTR in mediating apoptosis not only in Schwann cells but also in glial cells of the CNS (Casaccia-Bonelli *et al.*, 1996, Soilu-Hanninen *et al.*, 1999, Ferri and Bisby 1999). NGF acting via the p75NTR could therefore be responsible for the increased apoptosis seen after nerve transection in newborn Krox-24 *-/-* mice. Further experiments will be required to test this hypothesis. Experiments would include the quantification of p75NTR expression of newborn Schwann cells before and after sciatic nerve transection by western blot

protein analysis and a measurement of the effects of NGF on cell death *in vivo* and *in vitro* in Schwann cells from wildtype and mutant animals. Time limitations prevented such experiments from being carried out.

The lack of an obvious phenotype in Krox-24 deficient mice is, perhaps, surprising given the high Krox-24 expression levels observed during development and following axotomy. The possibility of genetic redundancy among Egr protein family members is likely. As well as Krox-20 I have shown that Egr-3 is also expressed, at the mRNA level, in Schwann cells during development. It may therefore be interesting to generate double knockouts of both Krox-24 and Egr-3 and also Krox-24 and Krox-20 and to analyse the phenotype presented in these new mutants.

Figure 7.1 A schematic diagram showing the temporal expression pattern of Zfp-57 in relation to known transcription factors within the myelin-forming Schwann cell lineage

In blue is the expression pattern of Zfp-57 mRNA predicted by the results in chapter 3. The approximate temporal expression pattern of Schwann cell transcription factors Krox-20, Oct-6, Brn-5, Krox-24, SOX10 and Pax-3 mRNA is shown for comparison in red. The stages of myelin-forming Schwann cell development are shown together with time points in mouse development. For simplicity only the myelin-forming Schwann cell expression is shown. Hatched region in SOX10 band indicates uncharacterised expression.



CHAPTER 8

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