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Signals that control embryonic Schwann cell development and myelination

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A Thesis Submitted for the Degree of Doctor of Philosophy

University of London

2006

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ABSTRACT

The generation of mature Schwann cells from their cells of origin, neural crest cells, proceeds through two transitional steps; first neural crest cells are specified to form Schwann cell precursors, which then mature into immature Schwann cells. These then generate the myelinating and non-myelinating Schwann cells found in mature nerves. Each of these steps has been well characterised phenotypically in terms of antigenic profile, survival mechanisms and morphological changes.

First of all, using adapted survival assays I found that there is a range of factors that supports the survival of crest-derived glial precursor populations that generate satellite cells and Schwann cells but not of crest cells themselves. I found also that satellite cells develop earlier than Schwann cells in a number of characteristics including survival mechanisms and antigenic profile.

I then examined the role of the Notch signalling pathway in the Schwann cell lineage using different *in vitro* and *in vivo* studies. I found that only Schwann cell precursor maturation is regulated by Notch signalling during early Schwann cell development. Notch signalling is also important in regulating cell division in immature Schwann cells and it acts as a negative regulator of myelination. During normal development, Notch signalling is attenuated, by a mechanism likely to involve the transcription factor Krox-20, to allow myelination to occur.

Finally, in collaboration with Professors W.F. Blakemore and R.J. Franklin (University of Cambridge), I made a comparative analysis of the transplant characteristics of Schwann cell precursors and Schwann cells in two animal models of Multiple Sclerosis. We found that Schwann cell precursors outperform Schwann cells in myelination potential, survival and migratory properties in these animal models.

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ABBREVIATIONS

ADAM	A Disintegrin And Metalloproteinase domain
AraC	Cytosine arabinoside
BDNF	Brain-Derived Neurotrophic Factor
BFABP	Brain Fatty Acid Binding Protein
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stem Cells
BrDU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CM	Crest medium
CNS	Central Nervous System
db cAMP	Dibutryl cAMP
DHH	Desert Hedgehog
DM	Defined supplemented medium
DMEM	Dulbecco's modified Eagles medium
DRG	Dorsal Root Ganglion
EGF	Epidermal Growth Factor
ERK	Extracellular signal-Related Kinase
FACS	Fluorescent-Activated Cell Sorting
FCS	Foetal Calf Serum
FDU	5-Fluoro-2'-Deoxyuridine
FGF	Fibroblast Growth Factor
GalC	Glycolipid Galactocerebroside
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
IB	Immunoblot
ICC	Immunocytochemistry
IGF	Interstitial Growth Factor
IHC	Immunohistochemistry
IPL	Interperiod Line
JNK	c-jun NH_2 – terminal kinase
LPA	Lysophosphatidic Acid
MAG	Myelin-Associated Glycoprotein

MAL	Myelin and Lymphocyte Protein
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MDL	Major Dense Line
MEM	Minimal Essential Medium
MS	Multiple Sclerosis
NCSC	Neural Crest Stem Cells
NGF	Nerve Growth Factor
NICD	Notch Intracellular Domain
NT-3	Neurotrophin-3
P0	Protein zero
PDGF	Platelet-Dervided Growth Factor
PDL	Poly-D-Lysine
PI3	Phospho-inositol 3
PLP	Proteolipid Protein
PMP-22	Peripheral Myelin Protein-22
PNS	Peripheral Nervous System
SM	Simple non-supplemented medium
SREBP	Sterol Regulatory Element Binding Protein
TGF	Transforming Growth Factor
WB	Western Blot

Units

bp	base pair
^{0}C	degrees Celcius
kb	kilo base
kDa	kilo Dalton
hr	hour
min	minute
sec	second
g	gram
mg	milligram
μg	microgram
L	litre
ml	millilitre
μl	microlitre
mm	millimetre
μm	micrometer
nm	nanometer

A note on conventions used in the text

When referring to a gene/mRNA, its name or abbreviations appears in italics in the text, as opposed to normal text for the product/protein.

ACKNOWLEGEMENTS

First and foremost, I would like to thank Prof. K.R. Jessen and Prof R. Mirsky for their help, advice and support during my PhD. I hope to enjoy the next few years in their lab as much as I have enjoyed these past years.

I would like also to thank a number of people who have helped me generally and with my work. These include Dr. D. Parkinson, Dr. M. Duran, Dr. A. Bhaskaran, Dr. N. Kazakova, Dr. S. Namini, Dr. M. D'Antonio, Ms A. Droggiti, Mr M. Turmaine, and everyone in the Biological Services Unit at UCL. I would also like to thank Ms J. Gilson, Dr. A. Setzu, Prof. W.F. Blakemore, Prof. R.J. Franklin and Rachel (University of Cambridge), Dr. M. Agudo and Prof. S. McMahon (King's College London). My deepest thanks also to everyone who have provided reagents and animals for all my experiments.

My time in London would never have been as nice as it has been without the help and love from my friends and family over here. In particular, I would like to thank Anna, Marta, Meena, Mewa and Minou.

Finally and more importantly I would like to thank my family back in Mauritius for their love, teachings and support all my life. I am especially indebted to my father and mother who have taught me the value of honest hard-work and resilience.

This thesis is dedicated to my nephews and niece; Keshil, Rea Devi, Souvir and Dhanveer, the beautiful angels in my life.

CHAPTER 1: General Introduction

1.1 Schwann cells and their lineage: an overview.

Schwann cells constitute the majority of glial cells in the peripheral nerves and they exist in two forms: myelinating Schwann cells, which form myelin around large diameter axons (> 1μM) and non-myelinating Schwann cells, which accommodate smaller diameter axons along troughs on their surface. The other distinct glial cell-types in the peripheral nervous system (PNS) include olfactory ensheathing cells (Chuah and West, 2002), the teloglia (terminal glia) of somatic motor nerve terminals (Robitaille, 1998), satellite glia that envelop the neuronal cell bodies in sympathetic, parasympathetic and sensory ganglia (Pannese, 1981), enteric glial cells in the autonomic ganglia in the gut wall (Gabella, 1981; Jessen and Mirky, 1983), and specialized glia found in association with sensory nerve endings such as Pacinian corpuscles (Spencer and Schaumburg, 1973).

Most of the Schwann cells found in mature nerves are derived from migrating neural crest cells (Anderson, 1997; Le Douarin *et al.*, 1991) and their development occurs through two transitional steps; first is the process of gliogenesis, whereby the neural crest cells are specified to form Schwann cell precursors, followed by a maturation of these cells into immature Schwann cells (Dong *et al.,* 1995, 1999; Jessen *et al.,* 1995). These cells then differentiate to form mature myelinating and non-myelinating Schwann cells. Each of these steps has been well characterized phenotypically in terms of their antigenic profile and survival mechanisms as well as in terms of morphological changes (reviewed in Jessen and Mirsky, 2005 *a, b*; Sherman and Brophy, 2005).

1.2 The origin of Schwann cells.

The neural crest is a transient migratory population of cells that delaminates from the dorsal part of the neural tube during embryonic development (Le Douarin and Smith, 1988; Le Douarin *et al.*, 1991). The neural crest also gives rise to a variety of other cell types and in the trunk, in addition to glia, crest cells give rise to sensory, sympathetic and parasympathetic neurons, chromaffin cells, and melanocytes. The cardiac crest, found in the more anterior part of the trunk, gives rise to smooth muscle cells and connective tissue, whilst the cephalic crest in the head region, additionally gives rise to cartilage and bone cells (Le Douarin and Kalcheim, 1999).

1.2.1 Neural crest induction, delamination and migration.

The neural crest is formed during the process of neurulation, when the neural plate folds over itself to give rise to the neural tube. Induction of the neural crest occurs at the border between the neural plate and the non-neural ectoderm. As the folding of the neural plate occurs, the neural folds (border regions of the ectoderm) come together and later fuse. The neural crest progenitors, thus present in and/ or immediately adjacent to the dorsal neural tube, leave the neural tube and migrate along defined paths in the embryo, where they differentiate into their different derivatives (Le Douarin and Kalcheim, 1999).

Induction of the neural crest occurs in multi-step manner and a number of factors, which are important in this process, have been identified. Firstly, a gradient of bone morphogenic proteins (BMPs) segregates the ectoderm into neural and non-neural domains, with the formation of the anterior neural folds occurring at a precise threshold level of BMP. Posteriorizing signals, including Fibroblast Growth Factors

(FGFs), Wnts and retinoic acid secreted from the epidermis and/or the paraxial mesoderm, transform these anterior neural folds into the prospective neural crest. BMPs are then thought to maintain the potential of the neural crest, and these precursor cells undergo proliferation in response to Wnt1 and Wnt3a and are prevented from differentiating prematurely by Pax3 (Goulding *et al.,* 1991; Sato *et al.,* 2005). Eventually, in response to Notch/Delta signalling and Foxd3, these precursors differentiate into either neural crest or dorsal root ganglion neurons (Aybar and Mayor, 2002; Basch *et al.*, 2004; Knecht and Bronner-Fraser, 2002).

Once induced, the neural crest cells undergo an epithelial to mesenchymal transition (EMT), involving a number of changes in cell morphology and cell surface adhesion and recognition molecules. More specifically, in the chick, the crest cells downregulate N-cadherin, molecules expressed generally by cells of the neural tube, as well as losing expression of cadherin-6b, which is present on cells of the dorsal-most region of the neural tube. These crest cells also upregulate cadherin-7 (Nakagawa and Takeichi, 1995, 1998). This delamination process is controlled by BMPs, more specifically BMP-4, through the upregulation of rhoB (Graham, 2003; Kalcheim and Burstyn-Cohen, 2005; Moore and Larue, 2004).

The delaminated cells then migrate to different parts of the body and differentiate into their derivatives. In vertebrates, trunk neural crest cells use two main pathways of migration: the first one is ventral migration through the anterior sclerotome and the neural crest cells give rise to cells in the dorsal root ganglia (DRG), sympathetic ganglia, adrenomedullary cells and Schwann cells (Erickson *et al.*, 1992; Le Douarin and Teillet, 1974; Weston, 1963). The second pathway of migration is a dorsolateral migration over the entire surface of the somite and the cells mainly give rise to melanocytes, the pigment cells of the skin and of fur in

mammals or feathers in avians (Erikson *et al.*, 1992; Erikson and Goins, 1995). Other secondary pathways of migration have also been identified; an intersomitic migration and longitudinal migration along the neural tube or the dorsal aorta (Le Douarin *et al.*, 1982; Teillet *et al*, 1987).

1.2.2 Neural crest cells and gliogenesis.

The multipotentiality of neural crest cells, as discussed above, has been thoroughly demonstrated in numerous *in vitro* studies (Cohen and Konigsberg, 1975; Dupin *et al.*, 1998; Le Douarin and Kalcheim, 1999, Le Douarin and Dupin, 2003). *In vivo* as well, it has been demonstrated that thoracic neural crest cells populate the gut and form enteric neurons when transplanted in the vagal axial level, whereas their normal fate is the formation of sensory and sympathetic neurons (Newgreen *et al.,* 1980). More dramatically, cultured trunk neural crest cells have been shown to contribute to cranial skeletal elements when they are grafted into the developing head (McGonnell and Graham, 2002). Given the multipotentiality of these cells, the formation of the vast array of derivatives most probably lies in the extrinsic cues they encounter on their path of migration or at the specific areas where they differentiate (Graham, 2003).

The development of glial cells seems to occur in 2 phases: some neural crest cells appear to have already entered the glial lineage at the onset of their migration, whereas other cells start glial differentiation later on, as they are migrating ventrally along the neural tube (Henion and Weston, 1997). Many *in vitro* studies have been performed and a role of a number of factors implicated in this process of gliogenesis.

Neuregulin-1 has received considerable attention since it was shown to be able to suppress neurogenesis in clonal cultures of rat neural crest cells as well as

significantly increase the percentage of $GFAP^{+ve}$ (Glial Fibrillary Acidic Protein) glial clones (Shah *et al.,* 1994). Whilst these data are consistent for an essential role of neuregulin-1 in promoting gliogenesis, specification of glial phenotype from neural crest cells is not dependent on neuregulin-1 since in the same studies, they show that glial clones are readily formed even in the absence of neuregulin-1. In chick neural crest cells as well, neuregulin-1 has been shown to significantly upregulate levels of *seraf*, which is a gene present in the newly formed Schwann cell precursors (Wakamatsu *et al.*, 2004). However, in similar cultures without neuregulin-1, *seraf* expression does still increase with time. These results suggest that neuregulin-1 might be instead expanding the population of Schwann cell precursors rather than specifying the crest cells into adopting a glial fate, hence increasing the expression of *seraf.*

More recent data has implicated Notch signalling in PNS gliogenesis. Notch activation leads to a suppression of neurogenesis in avian and rat neural crest cells, both *in vitro* and *in vivo* (Kubu *et al.*, 2002; Morrison *et al.,* 2000; Wakamatsu *et al.*, 2000) as well as significantly increasing the percentage of glial cells in clonal analysis experiments (Kubu *et al.*, 2002; Morrison *et al.,* 2000). Again, a similar parallel to the role of neuregulin-1 can be drawn in that, an absence of Notch activation, still leads to an appearance of glial clones.

1.2.3 The role of Sox10 in gliogenesis.

Sox family members have been implicated in the developmental regulation of a number of systems (Wegner, 1999), and in the PNS, Sox10 has been implicated in the development of glial cells from neural crest cells (Wegner, 2000; Wegner and Stolt, 2005).

Sox10 mRNA is expressed in migrating crest cells (Britsch *et al.*, 2001; Pusch *et al.*, 1998; Southard-Smith *et al.*, 1998) and dissociated crest cells express Sox10 protein (Paratore *et al.*, 2001). Sox10 expression remains high in developing glia in ganglia and in nerve trunks, but is downregulated in developing neurons (Kuhlbrodt *et al.*, 1998). In Sox10 mutants, peripheral glia are essentially absent, both in nerve trunks and in ganglia, whereas DRG sensory neurons are initially present, although they die later (Britsch *et al.*, 2001; Sonnenberg-Riethmacher *et al.*, 2001). In these mice, the nerve trunks and ganglia contain, in addition to neurons, a residual population of cells that seem to retain a neural crest phenotype. These data argue for an essential role of Sox10 in glial cell specification, as an absence of it appears to prevent gliogenesis in the PNS. *In vitro* studies support this notion as enforced expression of Sox10 in the neuroblastoma cell line N2A, causes upregulation of the *P0* gene, which is a marker of glial cell differentiation (Peirano *et al.*, 2000).

1.2.4 Novel origin of Schwann cells in spinal roots.

Glial differentiation markers, including S100β, appear relatively earlier in spinal roots than in limb nerves (Murphy *et al.*, 1996) and indicate differences in the formation of these structures. Whilst the origin of Schwann cells in spinal nerves from migrating neural crest cells has been well documented (see above), the development of the glia in the spinal roots has only recently been documented (Maro *et al.*, 2004); using elegant fate-mapping studies, the authors show that the glial cells populating these roots originate from boundary cap cells. These cells, of neural crest origin (Niederlander and Lumsden, 1996), appear transiently in small clusters at motor exit points and dorsal root entry zones (Altman and Bayer, 1982, 1984). They are identified by the zinc finger transcription factor, Egr2 (Krox-20) (Schneider-

Maunoury *et al.,* 1993; Wilkinson *et al.*, 1989). Using, *Egr2* driven Cre recombinase, Maro and colleagues (2004) traced the fate of these cells *in vivo*. They found that during embryogenesis the boundary cap cells left their point of origin and migrated along peripheral axons to colonize the spinal nerve roots and DRG, where they differentiated into both neurons and glial cells.

1.3 Schwann cell precursors.

Neural crest cells contribute to most of the glial cell types in the PNS, including satellite cells in the DRG and Schwann cells in nerve trunks, as mentioned above. Schwann cell development, however, proceeds through a distinct intermediate stage, the Schwann cell precursor. In the rat, Schwann cell precursors are present in the spinal nerves at around embryonic day 14/15 (E14/15) and they mature to the Schwann cells, present in the nerves at around E17/18 (Jessen *et al.*, 1994). In the mouse, however, this stage of Schwann cell development proceeds two days earlier; Schwann cell precursors are present in the nerves at around E12/13, and they give rise to Schwann cells, present at around E15/16 (Dong *et al.,* 1999).

In addition to generating Schwann cells, Schwann cell precursors have recently been shown to also generate endoneurial fibroblasts (Joseph *et al.*, 2004). Using Cre recombinase-mediated fate mapping, the authors show that in P11 sciatic nerves, the majority of endoneurial fibroblasts, as well as Schwann cells were derived from Schwann cell precursors. Since endoneurial fibroblasts represent only about 5-10% of the number of Schwann cells produced at birth (Wanner *et al.*, 2006), this implies that Schwann cell precursors are strongly glial-biased progenitors.

In vitro culture manipulation of Schwann cell precursors show that they can retain the intrinsic properties of multipotentiality, as seen in neural crest cells. They can transdifferentiate to form a number of neural crest derivatives in response to

different growth factors. In avians, melanocytes can be generated from developing Schwann cells either by exposure to FGF (Sherman *et al.*, 1993) or endothelin (Dupin *et al.*, 2003). In rats also, clonal analysis of a sub-population of E14.5 sciatic nerve glial cells, referred to as Neural Crest Stem Cells (NCSCs), have shown their multipotentiality by differentiating into neurons, Schwann cells or myofibroblasts with exposure to complex culture medium containing chick embryo extract and retinoic acid (Morrison *et al.*, 1999).

1.3.1 Neural Crest Stem Cells (NCSCs).

E14.5 rat sciatic nerve glial cells can be sub-divided into distinct sub-populations by Fluorescent Activated Cell Sorting (FACS), using antibodies to P0 and $p75^{NTR}$. One such population, cells sorted as $P0^{-ve}p75^{+ve}$, was shown to be able to self-renew and, in clonal analysis experiments, can generate neural crest derivatives, as mentioned above. When engrafted into chick embryos, these cells gave rise to neurons as well as glial cells (Morrison *et al.*, 1999). The authors argue that these cells represent a resident population of stem cells within the developing nerves and are qualitatively similar to migrating neural crest cells in terms of their developmental potential. However, subsequent studies have shown that these NCSCs are considerably less neurogenic and more gliogenic than migrating neural crest cells. E14.5 sciatic nerve NCSCs were found to generate significantly more glia and less neurons than migrating neural crest cells, when grafted in chick embryos (White and Anderson, 1999; White *et al.*, 2001). This could be explained by the fact that these cells are about 10 fold less sensitive to the neurogenic signal, BMP2 than migrating crest cells, which is correlated to a decrease in mRNA levels of the type Ia BMP receptor (White *et al.*, 2001). Similarly, in response to pro-gliogenic Delta-Notch signals (Morrison *et al.*, 2000), E14.5 NCSCs have been shown to generate

significantly more glial clones and fewer neuronal clones than migrating neural crest cells in clonal analysis experiments. Again, these differences in sensitivity can be explained by the fact that E14.5 NCSCs express higher levels of the Notch receptor, and lower levels of the Notch antagonist, Numb (Kubu *et al.*, 2002).

These studies suggest that glial cells present in E14.5 rat nerves are functionally different from migrating neural crest cells; whereas neural crest cells can generate a number of derivatives, E14.5 sciatic nerve glial cells are more restricted in potential, and are strongly glial biased. One possibility is that the population of cells examined in the experiments above are actually Schwann cell precursors, given their strong gliogenic potential and this is supported by the *in vivo* fate mapping studies, which showed that E14.5 glial cells generate predominantly Schwann cells and a small population of endoneurial fibroblasts. Expression of different antigenic markers as well shows that neural crest cells and Schwann cell precursors are phenotypically distinct (see below and figure 1).

1.3.2 Developmental profile of early PNS glia.

Schwann cell precursors are initially found at the edges of nerves as well as within mature nerve trunks. They have extensive sheet-like processes connected to each other and surrounding large groups of axons. The early nerves are a tightly packed structure with no extracellular matrix and no apparent connective tissue and blood vessels (Jessen *et al.*, 1994; Jessen and Mirsky, 2005b). In contrast, older nerves consist of numerous axon-Schwann cell units, surrounded by connective tissue and blood vessels.

Morphologically as well, differences are seen between Schwann cells and their precursors when they are dissociated and plated onto coverslips. Whereas

Schwann cell precursors are flattened and exhibit numerous cell-cell contacts between themselves, Schwann cells are bipolar or tri-polar in shape and show very little cell-cell contact. Furthermore, Schwann cell precursors show greater motility than Schwann cells, when cultured *in vitro* (Jessen *et al.*, 1994).

The study of the Schwann cell lineage has necessitated the development of molecular markers to distinguish the different stages. Nowadays, a rather complete set of antigens is available to distinguish their stages and they are classified into 5 major groups (Figure 1): antigens present in the whole of the early Schwann cell lineage (yellow box), antigens present only in crest cells and Schwann cell precursors (blue box), antigens present only in Schwann cell precursors (green box), antigens present in Schwann cell precursors and Schwann cells (orange box) and antigens present only in Schwann cells (red box) (Jessen *et al.*, 1994; Dong *et al.*, 1999; Jessen and Mirsky, 2005a).

Survival mechanisms also differ remarkably between Schwann cell precursors and Schwann cells. Schwann cells can support their own survival, when plated at high densities, by secreting factors including Insulin Growth Factor-2 (IGF-2), Neurotrophin-3 (NT-3) and Platelet-Derived Growth Factor-BB (PDGF–BB) into the culture medium, whereas Schwann cell precursors do not possess these autocrine survival circuits (Meier *et al.*, 1999). Schwann cell precursors require paracrine signals for their survival, including neuregulin-1 and a combination of IGF-2 and FGF-2 amongst others (Jessen *et al.*, 1994, Dong *et al.*, 1999).

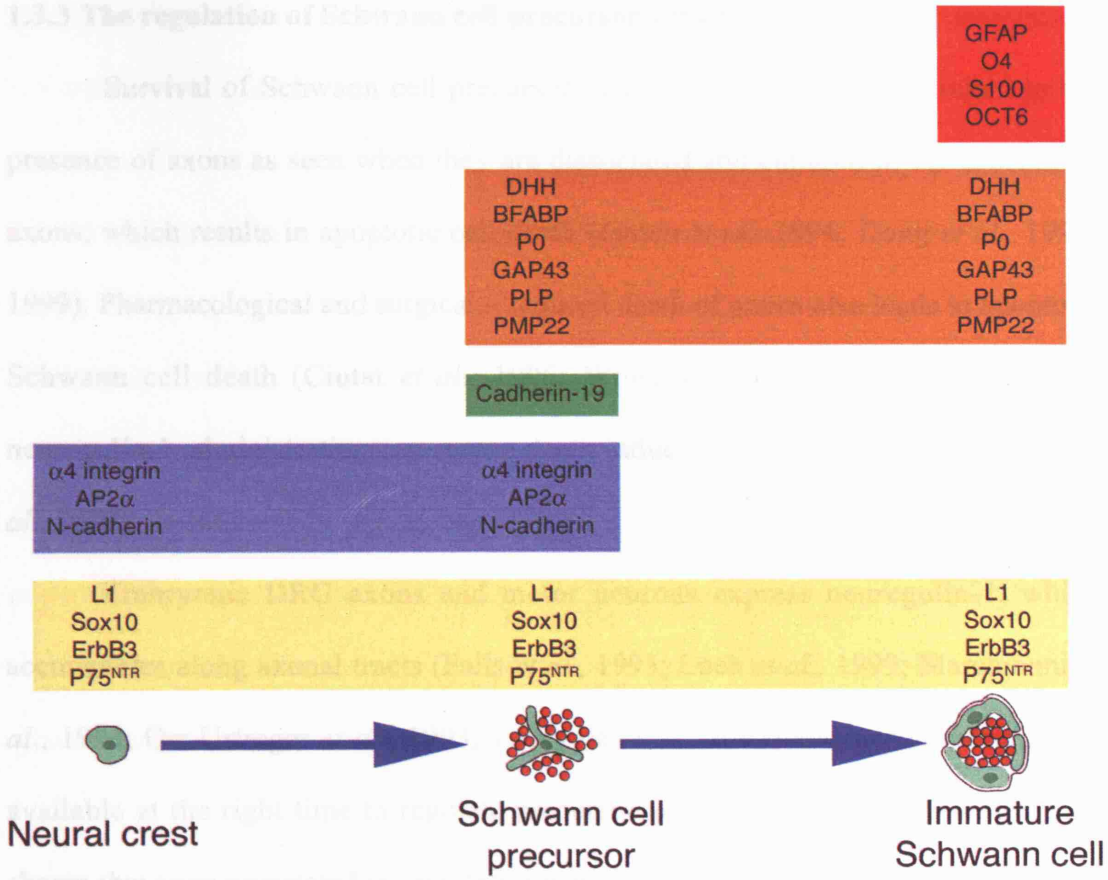


Figure 1.1: Antigenic profile of cells in the Schwann cell lineage.

Yellow box – antigens present in the whole of the early Schwann cell lineage; *Blue box* – antigens present only in crest cells and Schwann cell precursors; *Green box* – antigens present only in Schwann cell precursors; *Orange box* – antigens present in Schwann cell precursors and Schwann cells; *Red box* – antigens present only in Schwann cells (Jessen *et al.*, 1994; Dong *et al.*, 1999; Jessen and Mirsky, 2005a).

1.3.3 The regulation of Schwann cell precursor survival.

Survival of Schwann cell precursors seems to be intrinsically linked to the presence of axons as seen when they are dissociated and cultured in the absence of axons, which results in apoptotic cell death (Jessen *et al.,* 1994; Dong *et al.,* 1995, 1999). Pharmacological and surgical – induced death of axons also leads to apoptotic Schwann cell death (Ciutat *et al.*, 1996; Winseck *et al.,* 2002) and exogenous neuregulin-1 administration can rescue death induced by loss of axons (Winseck *et al.*, 2002).

Embryonic DRG axons and motor neurons express neuregulin-1, which accumulates along axonal tracts (Falls *et al.,* 1993; Loeb *et al.,* 1999; Marchionni *et al.*, 1993; Orr-Urtreger *et al.*, 1993, Taveggia *et al.*, 2005) and would therefore be available at the right time to regulate precursor survival. Cell culture studies have shown that axon associated or secreted signals from DRG neurons are able to support rat precursor survival and in both cases, the signal identified as neuregulin-1 (Dong *et al.*, 1995).

In mice, in which neuregulin signalling has been disrupted by genetic inactivation of either the neuregulin-1 gene or its receptors ErbB2 or ErbB3, Schwann cell precursor numbers are severely reduced, whereas development of satellite cells and DRG neurons appears relatively normal (Garratt *et al.,* 2000a; Meyer and Birchmeier, 1995; Morris *et al.*, 1999; Riethmacher *et al.*, 1997; Woldeyesus *et al.*, 1999). Absence of Schwann cell precursors in these mutant mice supports the idea of crucial role of neuregulin-1 signalling in their survival. Further support is provided by the phenotype seen in the genetic disruption of 3 of the neuregulin-1 isoforms. Whereas mice lacking isoforms I and II show normal peripheral glia development (Meyer *et al.*, 1997), mice in which isoform III has been

inactivated, show a severe depletion of Schwann cell precursors and consequently Schwann cells in peripheral nerves (Wolpowitz *et al.*, 2000).

1.3.4 The regulation of precursor – Schwann cell transition.

The precursor – Schwann cell transition involves a coordinated change in phenotypic variables including antigenic profile, survival mechanisms, motility and cellular interactions occurring in peripheral nerves. Many of these changes are faithfully reproduced in simple cell culture experiments, following the timing of normal development. When E14 Schwann cell precursors are cultured in medium containing neuregulin-1, at the end of 4 days (E14+4) they exhibit most of the characteristic phenotypes of Schwann cells that they would possess if they had been freshly dissociated at E18. Thus, the cultured precursors develop autocrine survival mechanisms and have the antigenic profile and mitogenic responses of Schwann cells at the end of the 4 days in culture (Dong *et al.,* 1995, 1999; Leimeroth *et al.*, 2002). These experiments show that neuregulin-1 as well as supporting the survival of Schwann cell precursors is sufficient for their maturation to Schwann cells. *In vitro* FGF-2 can accelerate neuregulin-mediated Schwann cell generation (Dong *et al.*, 1995), although this has not been demonstrated *in vivo.*

Another likely signal that could be involved in this transition is endothelin, acting through the endothelin B receptor. Both endothelin and its receptor are present in developing nerves (Brennan *et al.*, 2000). Although exposure to endothelin promotes the survival of Schwann cell precursors, their maturation to Schwann cells occurs very slowly. Furthermore, the maturation of the precursors in the presence of neuregulin-1 and endothelin, occurs at a rate intermediate to that seen in endothelin or neuregulin-1 alone (Brennan *et al.*, 2000). These data argue for antagonistic roles

of neuregulin-1 and endothelin on Schwann cell precursor maturation; whereas neuregulin-1 would promote Schwann cell generation, endothelin would retard it. In the spotting lethal rat, in which the endothelin B receptors are non-functional, Schwann cells are generated ahead of schedule (Brennan *et al.*, 2000), providing further evidence for the negative role of endothelin in Schwann cell maturation.

The only transcription factor shown to have a role in this transition is AP2α, which delays this transition when it is over-expressed in precursors *in vitro* (Stewart *et al.*, 2001). Expression of AP2α is sharply down-regulated, both in rats and in mice, at the transition point, suggesting its importance *in vivo* in the regulation of Schwann cell generation (Stewart *et al.*, 2001).

1.4 Immature Schwann cells.

As mentioned above, maturation of Schwann cell precursors leads to the formation of immature Schwann cells, which are present in the rat nerves at around E17/E18 and in mouse nerves, at around E15/16. The cytoarchitecture of the nerve changes considerably and the developing perineurium defines the endoneurial space, which contains 'axon – Schwann cell' families, endoneurial fibroblasts, blood vessels and extracellular matrix. These axon-Schwann cell units are similar to the ones described in newborn nerves (Webster and Favilla, 1984). Essentially, they consist of axons grouped together in small bundles by Schwann cells surrounding them.

As discussed above, the development of the Schwann cells from their precursors occurs through a complex series of phenotypic changes. At this stage there is a strict control of cell numbers, through a variety of signals, in preparation for the process of myelination, which, typically begins at around birth in rodents.

Also large diameter axons become selectively ensheathed by the immature Schwann cells, priming them for myelination. This process is called radial sorting and occurs until myelination is finished (see Section 1.4.2).

1.4.1. The control of Schwann cell numbers.

Developmental neuronal death has largely ceased in peripheral nerves during these late embryonic stages (Davies, 1996) when axon and Schwann cell numbers need to be matched. The major control of this process relies on the control of Schwann cell numbers. This is achieved in a number of ways, including proliferation, survival and death of these cells.

1.4.1.1 Schwann cell proliferation.

Cell division is a characteristic feature of the precursor and immature Schwann cell stages. Whilst proliferation, as measured by *in vivo* BrdU labeling, is high in Schwann cell precursors, the maximum rate of division occurs in the immature Schwann cells, at E18/19 in the rat (Stewart *et al.,* 1993). Similarly, in mice, proliferation is high at the Schwann cell precursor stage (E13), but significantly increases and reaches a peak at the immature Schwann cell stage (E15 onwards) (Yu *et al.*, 2005)

Schwann cell proliferation has been shown to be axonally-induced in *in vitro* studies, where direct contact with neurites and neurite fractions were shown to induce cell division in Schwann cells (Salzer *et al.*, 1980). After nerve transection of newborn animals, Schwann cell proliferation is also reduced when they lose contact with the axons (Komiyama and Suzuki, 1992). One of the axonal mitogenic factors

has been shown to be neuregulin-1, in *in vitro* Schwann cell-DRG co-culture studies (Morrissey *et al.*, 1995), although there is no direct *in vivo* evidence for this.

Another mitogen that could be important in controlling Schwann cell division is Transforming Growth Factor-β (TGF-β). TGF-β can induce proliferation in Schwann cell cultures (Einheber *et al.*, 1995; Guenard *et al.*, 1995; Ridley *et al.*, 1989) and in mice, in which the type II TGF-β receptor has been genetically inactivated in Schwann cells, proliferation as measured by *in vivo* BrdU labeling and presence of phospho-histone 3 (a marker of cell division) is reduced (D'Antonio M *et al.*, in press). This shows an *in vivo* role of these growth factors in controlling cell division, either directly or indirectly.

Laminins are also closely related to *in vivo* Schwann cell division. Genetic ablation of either the *laminin γ1* chain leads to reduced proliferation in the Schwann cells (Yu *et al.*, 2005). Also, laminin 2 and 8 have been shown to induce Schwann cell proliferation and in mice, in which both of these laminin chains are absent, Schwann cell mitosis is greatly reduced (Yang *et al.*, 2005).

1.4.1.2 Schwann cell survival and death.

The survival of immature Schwann cells in late embryonic and perinatal nerves is probably regulated by an interplay between survival and death signals.

Apoptosis, in the same way as proliferation, is a feature of immature Schwann cells during their normal development, as revealed by TUNEL labeling of dying cells in nerve sections (Grinspan *et al.*, 1996). Nerve transection experiments indicate that there is an increase in Schwann cell apoptosis suggesting a role for an axonal factor in regulating survival, since the axons degenerate following this procedure. This axonal factor appears to be neuregulin-1, as exogenously applied

neuregulin-1 is able to rescue some of the Schwann cells (Grinspan *et al.,* 1996). Nerve transections also induce apoptotic death of all teloglia at neuromuscular junctions and again, exogenous neuregulin-1 is able to rescue them from cell death (Trachtenberg and Thompson, 1996).

Two major death signals in Schwann cells have been identified *in vivo* and *in vitro.* Nerve Growth Factor (NGF), acting through its receptor $p75^{NTR}$, induces cell death in cultured Schwann cells and Schwann cells isolated from $p75^{NTR}$ knockout mice survive better than normal mice when subjected to serum or growth factor deprivation (Soilu-Hanninen *et al.,* 1999; Syroid *et al.*, 2000). Furthermore, Schwann cell apoptosis is markedly reduced in the $p75^{NTR}$ knockout mice following neonatal sciatic nerve transection, although the levels of apoptotic death in control and mutant mice during normal development are similar (Syroid *et al.*, 2000). These results suggest that $p75^{NTR}$-mediated Schwann cell death is not important for natural cell death seen *in vivo* during early development (Grinspan *et al.,* 1996), although it might be important during nerve injury.

The other death signal identified in Schwann cells during early development is TGF-β. Cultured Schwann cells die by apoptotic cell death with exposure to TGF-β (Parkinson *et al.*, 2001, Skoff *et al.*, 1998) and in mice, in which type II TGF-β has been genetically inactivated, there are reduced levels of cell death in E18 sciatic nerves, when compared to normal mice (D'Antonio *et al.*, in press).

As mentioned above, neuregulin-1 has been identified as a Schwann cell survival signal. Others factors that contribute to Schwann cell survival include autocrine survival circuits and laminin. It has been demonstrated *in vitro* that Schwann cells, when plated at high densities, are able to secrete factors including NT-3, PDGF-BB and IGF-2 that promote their survival (Meier *et al.*, 1999), although

at present there is no *in vivo* evidence for this. Laminins are major constituents of the basal lamina, which surrounds Schwann cells. In experiments, in which *laminin γ1* had been genetically ablated, survival of Schwann cells was considerably impaired and the increased apoptosis was mediated through a reduction in phosphatidyl-inosital-3 (PI3) kinase activity (Yu *et al.*, 2005). The PI3 kinase pathway is also involved in mediating lysophosphatidic acid (LPA)-induced Schwann cell survival following serum withdrawal (Weiner and Chun, 1999; Weiner *et al.*, 2001).

1.4.2 Radial sorting.

Further development of Schwann cells leads to the formation of myelinating and non-myelinating Schwann cells. Large diameter axons become surrounded by Schwann cell myelin whereas small diameter axons are accommodated in troughs along the surface of non-myelinating Schwann cells. Before birth, Schwann cells send cytoplasmic processes into groups of axons, progressively defasciculating them until there are individual axons surrounded by Schwann cells, destined to form the myelinating Schwann cells or groups of axons, destined to remain unmyelinated, which are surrounded by non-myelinating Schwann cells (Webster, 1984). The process, by which the axons are defasciculated until they are in a 1:1 relationship with a Schwann cell is called radial sorting, a process which is tightly related to Schwann cell proliferation (Webster, 1971; Martin and Webster, 1973; Webster *et al.*, 1973).

1.5 Myelination.

The process of myelination has evolved as a means to provide rapid saltatory conduction of nerve impulses along large axons. The physiological rationale for this

development of myelination in axons of a minimum calibre is that saltatory conduction in small diameter axons would probably not lead to more rapid impulse transmission (Sherman and Brophy, 2005). However, the mechanisms underlying this selective ensheathment of large diameter axons by Schwann cells are still unclear.

Immature Schwann cells destined to form myelin, start to express the glycolipid galactocerebroside (GalC) at around E19 (Jessen *et al.,* 1985), after which they ensheath the larger axons. This process of ensheathment starts at around birth and continues until all the appropriate axons are myelinated (Scherer and Slazer, 2004). Non-myelinating Schwann cells start their differentiation only until after the third postnatal week, expressing GalC just shortly before (Diner *et al.,* 1965, Jessen *et al.,* 1985; Jessen *et al.,* 1987). Like GalC, there are a number of molecular changes, which accompany the fate of immature Schwann cells into either, forming myelinating Schwann cells or non-myelinating Schwann cells (Figure 2). The formation of non-myelinating Schwann cells involve few molecular changes as they express most of the antigens expressed by immature Schwann cells. GalC is expressed in mature myelinating and non-myelinating Schwann cells but not in immature Schwann cells, whilst $\alpha_1\beta_1$ and $\alpha_7\beta_1$ integrins are expressed selectively in non-myelinating Schwann cells (Previtali *et al.*, 2003; Stewart *et al.*, 1997). Generation of myelinating Schwann cells, however, proceeds with more profound molecular changes as most of the antigens associated with immature Schwann cells are downregulated and there is an upregulation of a number of genes associated with control of myelination and formation of the myelin sheath.

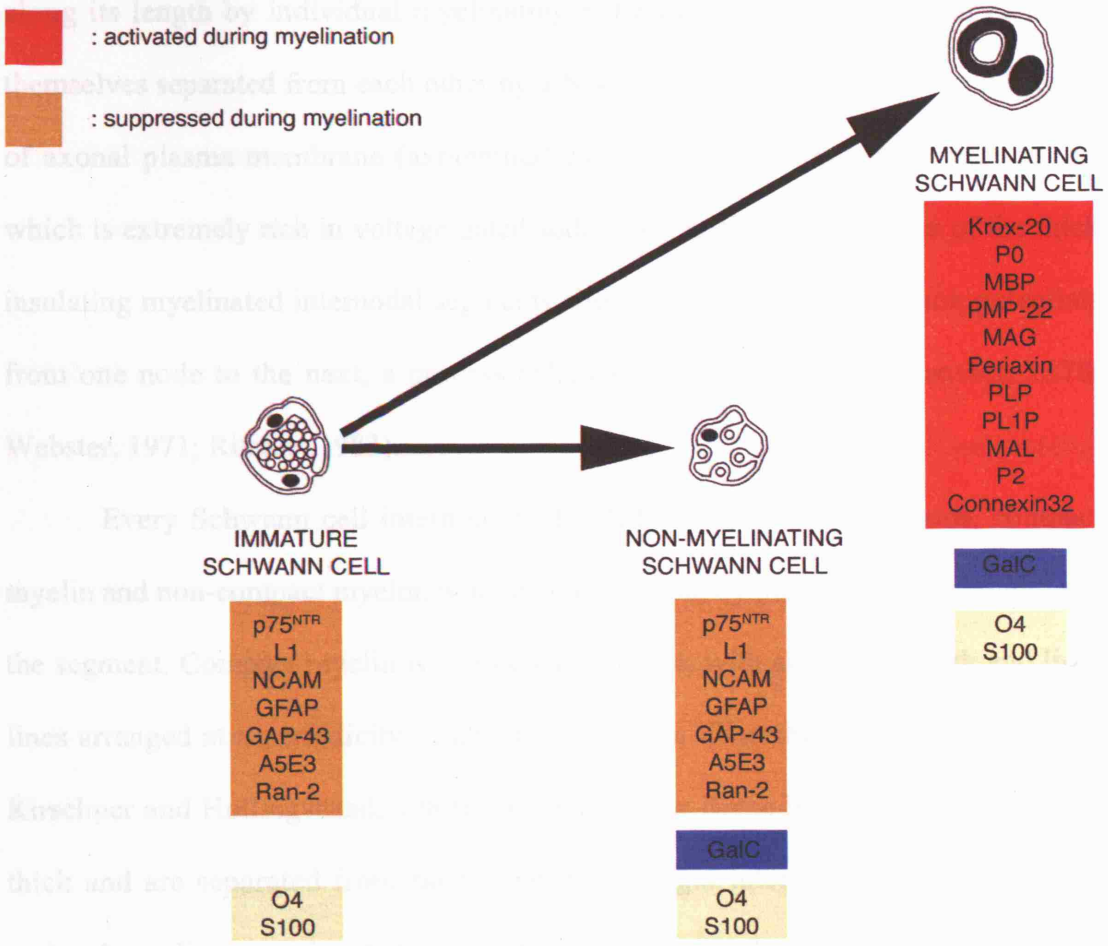


Figure 1.2: Antigenic profile of immature Schwann cells and mature myelinating and non-myelinating Schwann cells.

Yellow box – antigens present in immature Schwann cells and mature myelinating and non-myelinating Schwann cells; *Blue box* – antigens present in only myelinating and non-myelinating Schwann cells; *Orange box* – antigens present in immature and non-myelinating Schwann cells and downregulated in myelinating Schwann cells; *Red box* – antigens upregulated only in mature myelinating Schwann cells during myelination (Jessen and Mirsky, 2005b)

1.5.1 The structure of a myelinated fibre.

A myelinated fibre typically consists of a large diameter axon surrounded along its length by individual myelinating Schwann cells. The Schwann cells are themselves separated from each other by a Node of Ranvier, which is a small stretch of axonal plasma membrane (axolemma) exposed to the extracellular milieu, and which is extremely rich in voltage-gated sodium channels. The presence of the thick insulating myelinated internodal segments allows the propagation of axon potentials from one node to the next, a process called saltatory conduction (Ranvier, 1878; Webster, 1971; Ritchie, 1983).

Every Schwann cell internode is divided in two distinct domains, compact myelin and non-compact myelin, with the nucleus usually positioned in the middle of the segment. Compact myelin is a lamellar structure, with alternating dark and light lines arranged at a periodicity of about 13 – 19 nm (Kirschner and Sidman, 1976; Kirschner and Hollingshead, 1980). The dark major dense lines (MDL) are 2.5 nm thick and are separated from each other by the light interperiod lines (IPL). The major dense lines consist of the two intracellular lipid bilayers fused together, with the extrusion of the cytoplasm. The interperiod lines are formed by two extracellular surfaces of the Schwann cell membrane (Alberts *et al.*, 1994). The major function of myelin is thought to insulate the axon allowing rapid propagation of the impulses. Non-compact myelin includes the cytoplasmic channels present at the abaxonal and adaxonal surfaces of the cell, the Schmidt-Lanterman incisures, that radially transverse the compact myelin and the paranodal loops.

The paranodal loops are present at the end of the cell, when the major dense lines open up to accommodate cytoplasm. The paranodal junctions have been proposed to anchor the myelin loops to the axon through the septate-like junctions

that they form around the axons. They also help to form a diffusion barrier into the periaxonal spaces (reviewed by Peles and Salzer, 2000). The juxtaparanodes are found just beyond the innermost paranodal junction, under the compact myelin sheath and contain voltage-sensitive potassium channels. This region of the axon is thought to promote repolarization and maintain resting potential as well as preventing ectopic impulses (Peles and Salzer, 2000).

1.5.2 The composition of myelin.

Myelin membrane is composed of about 80 % of lipids and 20% of proteins. A number of studies (spontaneous mutations or targeted inactivation of genes) have revealed a structural role of the proteins in the stability of myelin. Protein zero (P0) is the major cell adhesion molecule of PNS myelin and forms the IPL and contributes to the MDL (Giese *et al.,* 1992; Martini *et al.*, 1995). Myelin Basic Protein (MBP) is another equally important structural protein involved in myelin compaction and is a major component of the MDL (Martini *et al.*, 1995; Privat *et al.*, 1979). Other proteins expressed in peripheral myelin include proteolipid protein (PLP), peripheral myelin protein-22 (PMP22), myelin and lymphocyte protein (MAL/MVP17) and myelin-associated glycoprotein (MAG) (Nave, 2001). GalC and galactosulfatide are two of the myelin glycolipids that have been identified as essential for proper node and paranode formation and for ensuring myelin stability (Nave, 2001).

1.5.3 The regulation of myelination.

As mentioned above, the molecular mechanisms controlling the selective ensheathment of large diameter axons are largely unknown as are the putative axonal signal(s) that initiate myelination in these cells. The basal lamina and more

specifically laminins, a major constituent of the basal lamina, and their receptors, integrins, have been shown to have integral functions in radial sorting and myelination. Other important factors that have identified in the regulation of myelination comprise Oct-6, Krox 20, Nab 1/2, Brn-2 and Sox10. Finally, recent evidence has shown that negative regulators of myelination exist as well (Jessen and Mirsky, 2005a).

1.5.3.1 The role of basal lamina and laminins in myelination.

Myelinating Schwann cells form a continuous basal lamina surrounding all the internodes of a myelinated fibre and *in vitro* studies first showed the importance of the basal lamina in myelination (Bunge, 1993). Further work showed that laminin, a major component of the basal lamina, rather than the basal lamina itself, was the factor required for myelination to occur. In cell culture experiments, myelin formation occurred in the absence of basal lamina, although laminin was present around the Schwann cells (Podratz *et al.*, 2001). In mutant mice, in which a continuous basal lamina is absent, myelination still occurred (Madrid *et al.*, 1975; Nagakawa *et al.*, 2001).

Laminins belong to a family of extracellular matrix proteins and exist as trimers made up of an α-,β- andγ-subunit. There are five α–subunits, four β–subunits and three γ–subunits and, in the basal lamina of Schwann cells, 2 laminins have been identified so far, laminin 2 (α2β1γ1) and laminin 8 (α4β1γ1). Laminin 2 and 8 are expressed during development, with laminin 8 expressed at higher levels during adulthood. The role of laminins has been elucidated with a number of genetic inactivation of some of the laminin genes (Feltri and Wrabetz, 2005).

The spontaneous mouse mutant *dystrophic* has a disruption in the laminin α2 chain and a number of abnormalities in the PNS occur, including varying levels of defects in radial sorting along the length of the nerve (Bradley and Jenkinson, 1973; Stirling, 1975). Some fibres in the proximal PNS and most fibers in the distal PNS are sorted out properly and achieve myelination, although the myelin is abnormal. Myelin thickness is altered, internodal length is decreased by half and nodes of Ranvier are abnormally wide (Bradley and Jenkinson, 1973; Bradley *et al.,* 1977; Jaros and Bradley, 1979). In mice with targeted inactivation of the laminin α2, similar phenotypes are obtained, and in addition the spinal roots are very abnormal (Yang *et al.*, 2005).

Mild sorting defects, as well as defects in myelin formed, are also seen in mice lacking laminin 8, obtained by targeted disruption of the laminin α4 chain, mostly in spinal roots and distal nerves (Wallquist *et al.*, 2005; Yang *et al.,* 2005). However, in the absence of both laminin 2 and 8, in mice in which both laminin α2 andα4 chains are absent, radial sorting is completely impaired and all axons remain unsorted (Yang *et al.*, 1995), suggesting a complementary role of laminin 2 and 8 in this process. In mice deficient in the laminin γ1 chain also, almost no axonal sorting occurs along the entire length of the nerve (Chen and Strickland, 2003; Yu *et al.,* 2005). These mice also lack both laminin 2 and 8 since the laminin γ1 subunit is present in both of these laminins.

Sorting and myelination defects are also seen in mice in which the laminin receptors have been inactivated. Schwann cells express 2 types of laminin receptors: dystroglycan, which appears in promyelinating Schwann cells (Schwann cells in a 1:1 relationship with axons just prior to myelination) (Masaki *et al.,* 2002; Previtali *et al.*, 2003b*)* and integrins, which are usually expressed continuously from

embryonic development onwards. Thus, Schwann cell precursors and embryonic Schwann cells express α6β1-, α2β1- and α3β1-integrins (Hsiao *et al.,* 1991; Previtali *et al.*, 2003b) whereas α6β4-integrin appears at the promyelin stage (Masaki *et al.,* 2002; Previtali *et al.*, 2003b*)* and α7β1-integrin is expressed in mature Schwann cells (Previtali *et al.*, 2003a; Feltri and Wrabetz, 2005). Disruption of the β1 integrin subunit leads to profound defects in radial sorting, although some Schwann cells are still able to myelinate, probably due to compensatory mechanisms by α6β4 integrin and dystroglycan (Feltri *et al.*, 2002). Inactivation of dystroglycan in Schwann cells did not however produce any major defects in cell sorting, although there was a late onset of neuropathy caused by hypomyelination and abnormally folded myelin sheaths (Saito *et al.*, 2003).

ADAM 22, a member of the ADAM (a disintegrin and metalloproteinase domain) family of proteins, is expressed highly in the nervous system (Sagane *et al.*, 1999) and ablation of the gene results in severe hypomyelination and ataxia (Sagane *et al.*, 2005). It has been suggested that these defects are due to impairment in integrin signalling, since it has been shown that several ADAMs interact with integrins via their disintegrin domains (White, 2003).

1.5.3.2 The role of Lgi4 in myelination.

The spontaneously occurring mutant *claw paw (clp)* mice also have defects in radial sorting in the PNS as well as peripheral hypomyelination and limb posture abnormalities (Darbas *et al.*, 2004; Henry *et al.*, 1991; Koszowski *et al.*, 1998). Recently the mutation in *clp* was mapped to *Lgi4*, a member of the leucine-rich glioma-inactivated (*Lgi)* family of genes encoding putative secreted proteins. This mutation leads to a protein that lacks exon 4, $Lgi4^{clp}$, which, instead of being

secreted, is retained in the endoplasmic reticulum. The loss of this Lgi4 protein was shown to be responsible for this claw paw phenotype (Bermingham *et al.*, 2005).

1.5.3.3 The role of Oct-6 in Schwann cell myelination.

Oct-6 (SCIP/ Tst-1) is a member of the POU domain transcription factor family, isolated from ES cells of the Central Nervous System (CNS), sciatic nerve, and testes (Suzuki *et al.*, 1990; Monuki *et al.*, 1989; He *et al.*, 1989; Meijer *et al.*, 1990). In the PNS, Oct-6 is present in Schwann cell precursors and persists at least until postnatal day 12 (P12) (Blanchard *et al.*, 1996). The peak of expression occurs at birth after which it is progressively downregulated (Monuki *et al.*, 1990, Scherer *et al.*, 1994, Arroyo *et al.*, 1998). Oct-6 inactivation leads to severe disruption of myelination, as the Schwann cells arrest at the promyelin stage for about 7-10 days, after which myelination proceeds normally (Bermingham *et al.*, 1996; Jaegle *et al.*, 1996). The normal upregulation of the myelin genes and proteins is blocked for the first 2 weeks, although they return to normal levels once myelination has started (Jaegle *et al.*, 1996).

The *Oct-6* gene contains Schwann cell specific elements within its promoter, which have been shown to control gene expression spatially and temporally during development (Mandemakers *et al.*, 2000). Genetic ablation of this promoter element leads to a phenotype similar to that seen in the Oct-6-deficient mice (Ghazvini *et al.*, 2002). The delay in myelination in the Oct-6 mice can be partly explained by the presence of a related transcription factor Brn-2. This factor shows similar patterns of expression during development and Brn-2 has been suggested to provide compensatory mechanisms to the loss of Oct-6, since loss of both Brn-2 and Oct-6 results in further delays in myelination (Jaegle *et al.*, 2003). Brn-2 inactivation alone

does not lead to any defects in myelination although Brn-2 expression, under the control of the Oct-6 promoter rescues the defects in myelination of Oct-6 mice (Jaegle *et al.*, 2003).

1.5.3.4 The role of Krox 20 in Schwann cell myelination.

Krox-20 (Egr 2) is a zinc finger transcription factor characterized by three zinc finger motifs, originally isolated from PC12 and 3T3 fibroblast cell lines (Hazel *et al.*, 1988, Ryseck *et al.*, 1989, Milbrandt, 1988). In the PNS, Krox-20 is selectively expressed in promyelinating Schwann cells, seen from E16 onwards in the mouse (Parkinson *et al.*, 2002; Zorrick *et al.*, 1996, 1999).

Loss of Krox-20 in mice leads to severe myelination defects (Topilko *et al.*, 1994). Axonal sorting occurs normally in the Krox-20 knockout mice but the Schwann cells arrest at the promyelin stage. They form the usual 1:1 relationship with the axon but myelination does not proceed further. Myelin proteins such as P0 and MAG, normally upregulated during myelination, remain at very low levels (Topilko *et al.*, 1994). Further evidence of the role of Krox-20 in regulating myelin gene expression were obtained by gene profiling studies of Krox-20 overexpression in cultured Schwann cells (Nagarajan *et al.*, 2001). In this study, the expression of a number of critical myelin genes such as P0, MAG, PMP22 and periaxin was induced by Krox-20, together with a series of other genes. More recently, direct regulation of the P0 gene expression by Krox-20 has been demonstrated and Krox-20 binding sites on the first intron of the P0 gene were found (LeBlanc *et al.*, 2005). Also, Krox-20 is able to upregulate myelin proteins P0 and periaxin in an unrelated cell type, NIH 3T3 cell (Parkinson *et al.*, 2004).

Lipid and cholesterol biosynthesis is also an essential step in myelin formation (Garbay *et al.*, 2000). Krox-20 has been shown to induce expression of a number of lipid and cholesterol biosynthetic enzymes including 3-hydroxyl-3-methylglutaryl coenzyme A reductase, 7-dehydrocholesterol reductase, stearoyl CoA desaturase, and lanosterol 14-demethylase (Nagarajan *et al.*, 2001). In the PNS, the SREBP1 (sterol regulatory element binding protein 1) gene was shown to be upregulated during myelination (Nagarajan *et al.* 2002; Verheijen *et al.* 2003). This gene is involved in the regulation of expression of cholesterol/lipid biosynthetic genes (Horton *et al.*, 2002) and recently, Krox-20 and SREBP were shown to synergistically activate the promoters of several SREBP target genes, providing a direct role of Krox-20 in controlling lipid and cholesterol biosynthesis (LeBlanc *et al.*, 2006).

In addition its role in regulating myelin gene and lipid expression, Krox-20 has been shown to influence a variety of other changes associated with a myelinating phenotype. In Schwann cell cultures, Krox-20 has been shown to downregulate L1, prevent TGFβ-induced cell death and block neuregulin-induced proliferation, changes associated with the development of both an *in vitro* and *in vivo* myelinating phenotype (Parkinson *et al.*, 2004). In Krox-20 null mice, as well, Schwann cell proliferation and death are increased supporting the view that Krox-20 is involved in these processes (Ghazvini *et al.*, 2002; Zorik *et al.*, 1999; reviewed in Topilko and Meijer, 2001).

Krox-20 also contains Schwann cell specific enhancers, which are transcriptionally regulated by axonal factors. The *Krox-20* gene contains the immature Schwann cell element (ISE), which is active in immature Schwann cells from E15.5, and the myelinating Schwann cell element (MSE), active in myelinating

Schwann cells, from E18.5 (Ghislain *et al.*, 2002). The MSE contains multiple Oct-6 binding sites and requires Oct-6 for activation (Ghislain *et al.*, 2002).

Recently, NAB proteins have been identified as essential transcriptional regulators of Krox-20 in PNS myelination (Le *et al.*, 2005). Inactivation of NAB 1 and NAB 2 result in myelination defects similar to that seen in Krox-20 deficient mice; Schwann cells arrest at the promyelin stage and myelination does not proceed, myelin genes like P0 and periaxin are not upregulated and Schwann cells do not exit from the cell cycle. Thus, both Krox-20 and NAB are required, forming protein complexes that regulate the myelination programme (Le *et al.*, 2005).

1.5.3.5 The role of Sox10 and NFkB in Schwann cell myelination.

Sox10 has been implicated in the early development of Schwann cells (discussed above) and it also seems to be involved in the regulation of myelination. Sox10 and Oct-6 function synergistically to modulate Krox-20 activity *in vitro* (Kuhlbrodt *et al.*, 1998) and Sox10 has been shown to regulate the P0 promoter either by itself (Peirano *et al.*, 2000) or in synergy with Krox-20 (LeBlanc *et al.*, 2005). Sox10 and Krox-20 also act together to regulate the connexin32 promoter and mutations in the Krox-20/Sox10 binding site of the human connexin32 promoter results in X-linked Charcot-Marie-Tooth neuropathy (Bondurand *et al.*, 2001).

Another transcription factor recently implicated in myelination is NFkB, which is highly expressed in premyelinating Schwann cells. Blocking the activity of NFkB or using cells that had non-functional NFkB in DRG-Schwann cell co-cultures resulted in reduced levels of myelination as well as the prevention of *Oct-6* gene upregulation (Nichols *et al.*, 2003).

1.5.3.6 The role of neuregulin-1 in myelination.

The role of neuregulin-1 in the early development of Schwann cells has been discussed above. Neuregulin-1 also serves important functions in Schwann cell myelination, particularly in the control of myelin thickness. In mice, in which the neuregulin-1 receptor, ErbB2 had been conditionally ablated in Schwann cells, abnormally thin myelin sheaths are formed (Garratt *et al.*, 2000) and more recent studies have shown that a reduced level of axonal neuregulin-1 leads to decreased myelin thickness and an increased level of neuregulin-1 leads to thicker myelin sheaths as well as myelination of smaller diameter axons (Michailov *et al.*, 2004).

Recently, it has been suggested that the neuregulin-1 itself, specifically the membrane-associated type III isoform, is the elusive axonal signal that triggers myelination, in addition to controlling the extent of myelin formation (Lemke, 2006). This suggestion stems from the work by Taveggia and colleagues (2005), who show that in DRG co-culture experiments, myelination does not occur in axons lacking the neuregulin-1 type III isoform although it is rescued when these axons are lentivirally infected with neuregulin-1 type III. More importantly they show that superior cervical ganglia (SCG) neurons, which are normally not myelinated due the small diameter size of their axons, are readily myelinated if they are lentivirally infected with neuregulin-1 type III. However, given that normal axon-Schwann cell relationships, including radial sorting, have to be established prior to myelination, it is hard to be sure that these experiments show the importance of neuregulin-1 for myelination or for the establishment of these axon-Schwann cell relationships.

1.6 Wallerian Degeneration.

PNS axons, as opposed to axons within the CNS, have the capacity for regeneration and re-establishing functional connections with target organs following injury. This unique feature of the PNS arises from the ability of Schwann cells, unlike oligodendrocytes, to support axonal regeneration.

After nerve injury in the PNS and CNS, a set of events, referred to as Wallerian degeneration, occurs in the distal part of the site of axotomy, which is essential for axonal regeneration (Fu and Gordon, 1997; Scherer and Salzer, 2001). Thus, the proximal portion of an injured nerve degenerates up to the first node of Ranvier, whereas the in the distal stump, all axons fragment and disappear. The myelin sheaths break down and are phagocytosed first by Schwann cells and then by macrophages. The basal lamina of the Schwann cells remains intact and they undergo extensive cell division between 3 and 5 days (Oaklander and Spencer, 1988). 'Denervated' Schwann cells undergo extensive changes in their phenotype and both myelinating and non-myelinating Schwann cells revert to an immature Schwann cell phenotype (Figure 2). Myelinating Schwann cells decrease synthesis of myelin proteins and lipids and upregulate cell adhesion molecules, including L1 and NCAM. Non-myelinating Schwann cells, on the other hand, reduce expression of GalC and sulfatide. At the transcriptional level as well, factors expressed in myelinating Schwann cells, including Krox-20 and Oct-6 are downregulated and denervated Schwann cells upregulate factors, such as Pax3 and c-jun, which are expressed in immature Schwann cells (Scherer and Salzer, 2001).

Crush injury leads to Wallerian degeneration followed by axonal regeneration. Initially, during the Wallerian degeneration, the Schwann cells de-differentiate and revert to their immature Schwann cell phenotype, but subsequent

reinnervation leads to remyelination and development of the myelinating phenotype (Fu and Gordon, 1997; Scherer and Salzer, 2001). Initially, the denervated Schwann cells surround the bundles of remyelinating axons and then establish the 1:1 relationships before myelinating them. This process is fundamentally similar to what happens during development (Webster, 1993), although the myelin thickness is less and internodal length of the remyelinated Schwann cells is usually shorter than in normal nerves (Beuche and Friede, 1985).

Remyelination in the PNS is possible for two main reasons. First, the rapid clearance of myelin debris by macrophages following injury enhances axonal regeneration (Dahlin, 1995) and the presence of the denervated Schwann cells themselves is as equally important (Sketelj *et al.,* 1989). Denervated Schwann cells secrete extracellular matrix molecules (F-spondin, collagen p200 and fibronectin isoforms) as well as cell adhesion molecules on their surface (N-cadherin, tenascin-C, laminin, L1 and NCAM), which promote neurite outgrowth. They are also a potent source of trophic and tropic factors including NGF and Brain-Derived Neurotrophic Factor (BDNF), which promote axonal regeneration (Fu and Gordon, 1997; Scherer and Salzer, 2001).

1.7 Neuregulin-1 signalling pathway.

Neuregulin-1 has a wide variety of functions in the Schwann cell lineage, as described above. Neuregulin-1 signals via receptor tyrosine kinases of the ErbB family, and two receptors, ErbB3 and ErbB4 bind to it with high affinity. However, functional neuregulin-1 signalling seems to occur when heterodimers are formed between ErbB2 and either of the two receptors. ErbB3-ErbB2 complexes are the primary receptors in peripheral glia development whereas ErbB4-ErbB2 complexes

signal in the myocardium (Garratt *et al.*, 2000). The *neuregulin-1* gene is about 1.4 megabases long, but less than 0.3% of it encodes proteins (Stefansson *et al*, 2002). More than 15 different neuregulin-1 isoforms are produced as a result of rich alternative splicing and multiple promoters. The different isoforms are broadly categorised in three major groups, type I, II and III based on their N-terminal sequence (Falls, 2003). The structure of the isoforms is shown in Figure 4. Common to all the isoforms is the epidermal growth factor (EGF)-like domain, which is sufficient for activation of the receptors (Holmes *et al.*, 1992).

The neuregulin-1 receptors are structurally similar and consist of an extracellular domain with two cysteine-rich boxes, a transmembrane domain and a cytoplasmic domain homologous to tyrosine kinases. The tyrosine kinase domains of ErbB2 and ErbB4 are catalytically active, whereas the corresponding domain in ErbB3 is not. ErbB2, unlike ErbB3 and ErbB4, cannot bind to a ligand and therefore can only signal in the context of a receptor heterodimer (Falls, 2003). The preferred heterodimerisation partner of ErbB2 is ErbB3 (Tzahar *et al.*, 1996) and this receptor complex is the most potent signalling module of the ErbB receptor family (Pinkas-Kramarski *et al.*, 1996; Wallasch *et al.*, 1995). Activation of the ErbB2-ErbB3 receptor complex results in the stimulation of several major pathways, including the mitogen-activated protein kinase (MAPK) (Ben-Levy *et al.*, 1994), PI3 Kinase pathway (Peles *et al.*, 1992; Prigent and Gullick, 1994), phospholipase-Cγ (Fazioli *et al.*, 1991; Peles *et al.*, 1991), protein kinase C, and the Janus kinase pathway (Liu and Kern, 2002).

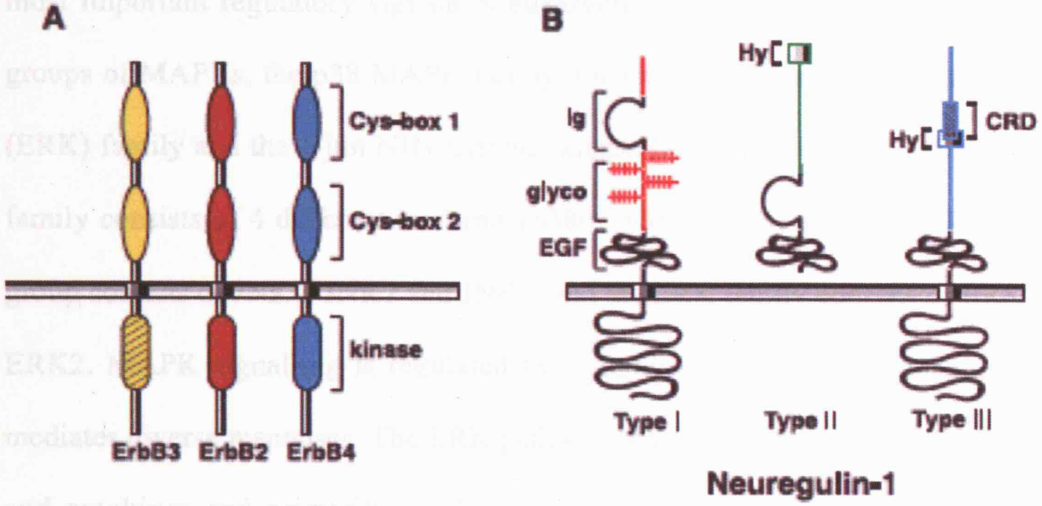


Figure 1.3: Schematic representation of the major neuregulin-1 isoforms and neuregulin-1 receptors ErbB2, ErbB3 and ErbB4.

A – The overall structures of the three neuregulin-1 receptors are similar and consist of two extracellular cysteine-rich domains, a transmembrane domain and a cytoplasmic domain homologous to tyrosine kinases.

B – The three major neuregulin-1 isoforms (Type I, II and III) have a common EGF-like domain. Other domains present are an immunoglobulin (Ig)-like domain, a domain rich in potential glycosylation sites (glyco), and a cysteine-rich domain (CRD). Grey shaded boxes indicate signal peptides or internal hydrophobic sequences (Hy in types II and III) or putative transmembrane domains. Sequences present in one of the three major isoforms only are shown in colour.

(Figure 4 and this legend have been adapted from Garratt *et al.*, 2000).

1.8 MAPK signalling pathway.

MAPKs are widely expressed serine-threonine kinases that are among the most important regulatory signals in eukaryotes. There are 4 distinctly regluated groups of MAPKs, the p38 MAPK family, the extracellular signal-regulated kinase (ERK) family and the c-jun NH_2-terminal kinase (JNK) family and ERK5. The p38 family consists of 4 different isoforms (p38α, p38β, p38γ, p38δ), the JNK kinase group consists of JNK1, JNK2 and JNK3, and the ERK family consists of ERK1 and ERK2. MAPK signalling is regulated by a number of upstream mechanisms and mediates diverse responses. The ERK pathway is activated by several growth factors and cytokines and primarily mediates cell survival and proliferation (Yoon and Seger, 2006). The p38 family is activated mostly by stress stimuli and cytokines and this pathway is important for cell differentiation, apoptosis, cell-cycle arrest, cytokine production and inflammation (Zarubin and Han, 2005). The JNK family is also activated by stress stimuli and growth factors and shares broadly similar functions to the p38 MAPK family (Weston and Davis, 2002). ERK5 is structurally similar to the classical ERK kinase group but seems to have distinct upstream and downstream effectors (Wang and Tournier, 2006).

MAPK activity is regulated by a three-tiered cascade of protein kinases. Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs, which are serine/threonine kinases, are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and

tyrosine residues (Figure 5)(reviewed in Roux and Blenis, 2004).

Figure 1.4: Schematic representation of the MAPK signalling pathway cascade.

1.9 Notch signalling.

The Notch signalling pathway is one of the most important cell signalling pathways essential for developmental patterning throughout the animal kingdom, from nematodes to man (Artavanis-Tsakonas *et al.*, 1999). It has been implicated in a wide variety of developmental mechanisms, from cell survival to proliferation, fate and morphogenesis (Artavanis-Tsakonas *et al.*, 1999, Harper *et al.*, 2003) as well as in various pathologies in humans (Joutel and Tournier-Lasserve, 1998).

Notch signalling is initiated when Notch receptors expressed in a cell interact with ligands present in an adjacent cell. In the classical pathway of canonical Notch signalling, proteolytic cleavage of the Notch receptor, upon ligand-binding, leads to the cytoplasmic release of an intracellular domain, NICD, which translocates to the nucleus and interacts with the CSL (named for CBF1, also known as RBP-Jk) transcriptional regulator and induces transcription of its effector genes Hes (Hairy/Enhancer-of-Split), Hey (hairy/Enhancer-of-split related with YRPW motif) and Herp (Hes-related repressor protein) (Harper *et al.*, 2003; Lai *et al.*, 2003). A CSL-independent mechanism of Notch signalling has been proposed, which acts through deltex to modulate Notch function (Martinez Arias *et al.*, 2002).

1.9.1 Notch receptors and ligands and their interaction.

Both the Notch receptors and ligands are type 1 transmembrane proteins. In mammals, there are 4 Notch receptors (Notch 1-4) and 2 structurally related groups of ligands, Delta (Delta-like 1-4) and Serrate (Jagged 1-2) in mammals (reviewed in Lai *et al., 2003;* Luo *et al.,* 2005; Mumm and Kopan, 2002). The ligands have a number of EGF-like repeats in the extracellular domain and a unique Delta or Serrate N-terminal domain (DSL domain) (reviewed in Allman *et al.,* 2002a,b). Recently, two novel tissue-specific ligands have been identified, Delta/Notch-like EGF-related receptor (DNER) (Eiraku *et al.,* 2002, 2005) and F3/contactin (Hu *et al.,* 2003; Hu *et al.,* 2004)

The Notch receptor is a heterodimeric protein and it contains an extracellular portion, which consists of tandem EGF-like repeats (29-36) that bind ligand, and three LIN-12/Notch repeats (LNR) that maintain the receptor in an inactivated state. The intracellular domain of Notch, NICD contains a RAM domain and ankyrin repeats, which interact with CSL, a transcriptional activator domain (TAD), which possibly amplifies target gene transcription, and a PEST (proline, glutamate, serine, threonine-rich) domain, which regulates protein stability (reviewed in Allman *et al.,* 2002a,b). Ligand – receptor binding occurs at specific sequences along the extracellular EGF-like repeats (Rebay *et al.,* 1991; Lieber *et al.,* 1992) and results in 2 proteolytic cleavages and subsequent release of the NICD from the membrane. The first cleavage occurs immediately external to the transmembrane domain, and is mediated by TACE (tumor necrosis factor-α converting enzyme) and Kuzbanian, which are members of the ADAM family of metalloproteases (Baron, 2003; Brou *et al.,* 2000; Lieber *et al.,* 2002; Pan and Rubin, 1997). The second cleavage event

requires presenilins and nicastrin and occurs at the inner half of the transmembrane domain (reviewed in Baron, 2003; Fortini, 2001; Lai, 2002).

1.9.1.1 CSL-dependent Notch signalling.

After the cleavage, NICD enters the nucleus and binds to the DNA binding domain of CSL (Jarriault *et al.*, 1995) and converts it from a transcriptional repressor complex to a transcriptional activator complex. In its basal state, CSL interacts with a co-repressor complex, containing a histone deacetylase and represses transcription (Kao *et al.,* 1998; Hsieh *et al.*, 1999; Zhou *et al.*, 2000). NICD replaces this repressor complex from CSL with a transcriptional activator complex that includes NICD, Mastermind, and histone acetyltransferase p300 (reviewed in Kadesh 2004). This leads to the activation of primary target genes including the Hes (1, 3, 5), Hey (1, 2 and L) and Herp (1, 2, 3) families, which are basic helix-loop-helix (bHLH) type of transcriptional repressor. More recently, CSL binding sites have been found in the promoter region of a number of other genes, including erbB2 and cyclin D1, although evidence of a regulation of these genes *in vivo* by the NICD-CSL complex is yet to be found (Iso *et al.*, 2003).

1.9.1.2 CSL-independent Notch signalling.

A novel pathway of Notch signalling has been proposed recently, one that does not require CSL-dependent transcription (Brennan and Gardner, 2002; Martinez Arias *et al.*, 2002). Several lines of evidence point to the idea that Notch functions in a number of cell types *in vivo* and *in vitro*, independently of CSL (Martinez Arias *et al.*, 2002). It has been postulated that NICD can interact with transcription factors other than CSL, such as Mef2 and LEF1 (Ross and Kadesh, 2001; Wilson-Rawls *et*

al., 1999). Another likely mechanism would be interaction with Deltex, an adaptor protein that binds to the ankyrin repeats of NICD (Diederich *et al.*, 1994). It has been shown that Notch signalling can inhibit the function of the transcription factors E47 in NIH 3T3 cells (Ordentlich *et al.*, 1998) and Mash1 in neural progenitor cells (Yamamoto *et al.*, 2001), in a CSL-independent, Deltex-dependent manner. Further studies have shown additional roles for deltex in mediating Notch function in Bergmann glia (Eiraku *et al.*, 2005; Patten *et al.*, 2006), oligodendrocyte (Hu *et al.*, 2003) and neural progenitor development (Cui *et al.*, 2004).

1.9.2 Regulation of Notch signalling.

Notch signalling is a vitally important in both invertebrates and vertebrates but signalling is regulated at several levels in the pathway, depending on the cell types and organisms, including the regulation of receptor activation and level and activity of NICD.

1.9.2.1 Regulation of receptor activation.

The *fringe* genes (*lunatic fringe, manic fringe* and *radical fringe*) encode glycosyltransferases that modify receptor response to their ligands (Moloney *et al.*, 2000). Fringe interacts directly with Notch, at either the EGF repeats 22 – 36 or at the LNR repeats (Ju *et al.*., 2000; Panin *et al.*, 1997). This leads to an increased sensitivity of the Notch receptor to Delta ligands and a decreased sensitivity to Serrate/ Jagged ligands through addition of two sugars to the EGF regions (Blair 2000; Hicks *et al.*, 2000; Munro and Freeman, 2000). However, ligand-induced signalling requires an initial modification of Notch by fucose, which is important for Fringe activity (Okajima *et al.*, 2003; Sasamura *et al.*, 2003; Wang *et al.*, 2001).

1.9.2.2 Regulation of NICD levels.

The half-life of nuclear NICD is relatively short (1-1.5 hr) and a number of molecules have been shown to be involved in the switching off of the Notch signal. Sel-10 is an E3 ubiquitin ligase that can stimulate phosphorylation-dependent ubiquitination of NICD and its subsequent proteosomal degradation (Gupta-Rossi *et al.*, 2001; Oberg *et al.,* 2001). Another E3 ubiquitin ligase, Itch, has been shown to promote ubiquitination of Notch by binding to the intracellular domain of membrane-tethered Notch (Qiu *et al.,* 2000). Mastermind also can stimulate phosphorylation and degradation of NICD (Fryer *et al.*, 2002), making activity of Notch self-regulating since mastermind is initially recruited as a transcriptional activator in the CSL complex.

Numb is another adaptor protein that has been shown to antagonize Notch signalling (Guo and Jan, 1996; Spana *et al.,* 1996). One possible mode of action of Numb is by binding to Itch and promoting ubiquitination and degradation of Notch (McGill and McGlade, 2003) or it might be interacting with components of the endocytic machinery such as a-Adaptin and Eps15, which promote endocytosis and degradation of Notch (Jafar-Nejad *et al.*, 2002).

Figure 1.5: The Notch signaling pathway.

Notch receptors are synthesized as single precursor proteins that are cleaved in the Golgi by a Furin-like convertase during their transport to the cell surface where they are expressed as heterodimers. Fringe glycosyltransferases modify EGF-like repeats by adding N-acetylglucosamine within the Golgi. Notch signalling is initiated after ligand-receptor interaction, which induces two sequential proteolytic cleavages. The first cleavage within the extracellular domain is mediated by the metalloprotease TACE (tumor necrosis factor α-converting enzyme). The cleaved extracellular subunit of the receptor is 'trans-endocytosed' by the neighbouring ligand-expressing cell. This process seems to be controlled by Neuralized and/or Mindbomb E3 ubiqutin ligases. The second cleavage occurs within the transmembrane domain and is mediated by the γ-secretase activity of the multi-protein complex of presenilins (PS), which includes Nicastrin, APH-1 and PEN-2. The liberated intracellular domain of Notch (NICD) translocates into the nucleus and binds to the transcription factor CSL (CBF1 in humans). This interaction leads to transcriptional activation by displacement of corepressors (CoR) and simultaneous recruitment of coactivators (CoA), including mastermind-like proteins (MAML1). Receptors modified by Fringe glycosyltransferases cannot mediate signalling via Jagged ligands, whereas Delta-mediated Notch signalling is still possible. (Adapted from Radtke *et al.*, 2005)

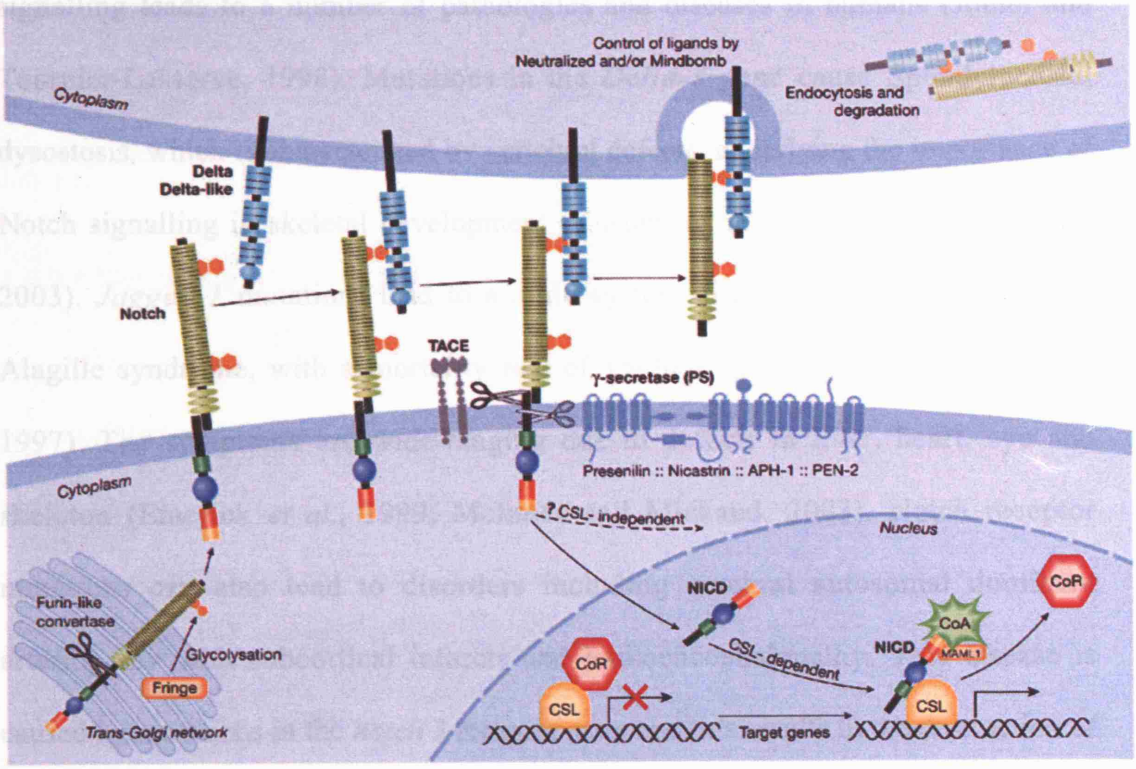


Figure 1.5: The Notch signalling pathway (taken from Radtke *et al.*, 2005)

1.9.3. The role of Notch signalling in development and disease.

Over the past few years, the role of Notch signalling in patterning vertebrate development has become increasingly important and nowadays it is difficult to find a tissue or organ that is not dependent on Notch function during some stage of its development. Like other developmental signalling pathways, dysregulation of Notch signalling leads to a number of pathologies and diseases in humans (Joutel and Tournier-Lasserve, 1998). Mutations in the *Delta-3* gene cause Spondylocostal dysostosis, which is characterized by vertebral defects, underlying the importance of Notch signalling in skeletal development (Kusumi *et al.*, 1998; Turnpenny *et al.*, 2003). *Jagged-1* mutations lead to a multi-system disease in humans, known as Alagille syndrome, with a mortality rate of 15-20% (Li *et al.*, 1997; Oda *et al.*, 1997). The symptoms are wide-ranging due to defects in liver, heart, eye and skeleton (Emerick *et al.*, 1999; McInnes and Michaud, 2003). Notch receptor mutations can also lead to disorders including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. This disease is caused by mutations in the *notch 3* receptor gene and this results in the destruction of arteriolar vascular smooth muscle cells, which causes malformation of arteries in the brain and subsequent subcortical white matter tissue death (Hungerford and Little, 1999; Joutel *et al.*, 1996; McInnes and Michaud, 2003).

It is not surprising that defects in Notch signalling result in so many wide-ranging disorders in humans, given that the receptors and ligands are widely expressed during embryogenesis and that this pathway has been shown to regulate tissue development from all three primary germ layers, the endoderm (pancreas development), mesoderm (skeleton, vasculature, haematopoietic cells) and ectoderm (neuronal and glial cells). Patterning of the nervous system, whether the CNS or

PNS, relies on signalling from a number of pathways, including Wnt signalling and Hedgehog signalling. Since its discovery, Notch signalling has become one of the most important pathways involved in proper development of the nervous system (Louvi and Artavanis-Tsakonas, 2006). It is involved in a number of processes, including cell fate specification, progenitor maintenance, survival and morphogenesis in at least one stage of the development of most of the cells that make the nervous system. Some specific roles of Notch signalling in nervous system development are discussed below.

1.9.3.1 Notch signalling and CNS patterning.

In the vertebrate CNS, neurons and glia are generated from the neuroepithelium; whereas neurons are generated during embryonic life from ventricular progenitors, most glial cells are generated at late embryonic and early postnatal stages in the proliferating subventricular zone with the exception of radial glia, which are generated in the ventricular zone (Goetz and Barde, 2005; Louvi and Artavanis-Tsakonas, 2006; Sauvageot and Stiles, 2002). It is thought that Notch signalling is required to maintain the population of neuronal progenitors, and selectively allow a few cells to enter the neuronal lineage (Lewis, 1996). Nascent neurons express Notch ligands and inhibit Notch-expressing progenitor cells from undergoing neuronal differentiation (de la Pompa *et al.*, 1997; Lewis, 1998) by repressing expression of MASH-1, which is a transcription factor required for neurogenesis (Lewis, 1998; Sasai *et al.*, 1992). It was originally thought that Notch signalling is involved in maintaining the population of neuronal progenitor cells and promoting glial differentiation only by the inhibition of neuronal differentiation. However, recent studies have suggested that Notch signalling might rather play an instructive role in promoting glial differentiation (Gaiano and Fischell, 2002),

including astrocytes, radial glia in the forebrain and cerebellum and Muller glia in the retina.

1.9.3.1.1 Muller glia.

Neurons and glia arise from a common progenitor in the retina (Cepko, 1999) and numerous studies have shown the involvement of Notch signalling in the differentiation of Muller glia. Notch activation in retinal progenitors inhibits neuronal differentiation, and promotes formation of Muller glia (Bao and Cepko, 1997; Furukawa *et al.*, 2000, Scheer *et al.,* 2001). Similarly, misexpression of Notch downstream effectors, including Hes1, Hes 5 and Hey2 leads to gliogenesis at the expense of neurogenesis in retinal progenitors (Furukawa *et al.*, 2000; Hojo *et al.*, 2000; Satow *et al.*, 2001). Ablation of Hes 5 and disruption of Hes 1 activity by a dominant-negative construct results in a decrease in the population of Muller glia in the retina (Furukawa *et al.*, 2000; Hojo *et al.*, 2000).

1.9.3.1.2 Radial glia and Astrocytes.

Radial glia have a critical role in the generation of the cytoarchitecture of the brain; they can contribute to the formation of neurons, astrocytes and oligodendrocyte progenitors (Alvarez-Buylla *et al,* 1990; Chanas-Sacre *et al*, 2000; Malatesta *et al.*, 2000; Noctor *et al.*, 2001) and also serve as a scaffold for the migration of newly born neurons (Rakic, 1971a, b). They are generated from the undifferentiated neuroepithelium before neurogenesis occurs and after neurogenesis and neuronal migration decrease, they are transformed into type I astrocytes (Chanas-Sacre *et al.*, 2000).

In vivo studies have shown that the formation of radial glia is promoted by ectopic expression of NICD in the progenitors (Gaiano *et al.*, 2000). Co-culture with cerebellar granule neurons leads to the transformation of astrocytes into radial glia and this *in vitro* system has been used to study Notch function in radial glia formation (Patten *et al.*, 2003; 2006). These studies have shown that Notch ligands expressed in the neurons activate Notch receptors in the astrocytes, enabling their transformation into radial glia.

1.9.3.1.3 Oligodendrocytes .

In contrast to the other glial subtypes in the CNS, Notch signalling has been shown to be inhibitory to oligodendrocyte differentiation. Oligodendrocytes are the myelinating glia in the CNS and are derived from oligodendrocyte progenitor cells (OPCs), via an intermediate stage, the immature oligodendrocyte (Richardson, 2001). *In vitro* studies have shown that Notch signalling inhibits the differentiation of OPCs into mature myelinating oligodendrocytes (Tanigaki *et al.*, 2001; Wang *et al.*, 1998). Further *in vivo* studies of conditional Notch1 ablation have shown premature differentiation of OPCs into immature oligodendrocytes (Genoud *et al.*, 2002).

In the rat optic nerve, OPCs express Notch 1 and retinal ganglion cells express Jagged 1. Jagged 1 expression, however, goes down after birth and this is coincidental with the differentiation of the OPCs into mature myelinating oligodendrocytes (Wang *et al.*, 1998). These results suggest that Notch signalling might be inhibitory to the process of myelination. Subsequent studies have supported this view and it was shown that oligodendrocyte myelination occurs prematurely in Notch 1 heterozygous mice, which presumably have lower Notch signalling levels (Givogri *et al.*, 2002).

1.9.3.2. Notch signalling and PNS development.

Nascent DRGs consist of a core of differentiating neurons that are surrounded by undifferentiated proliferative cells (Wakamatsu *et al.*, 2000). The undifferentiated cells initially give rise to additional neurons, after which they generate satellite glial cells (Wakamatsu *et al.*, 2000). Notch signalling seems to be involved in the initial patterning of the DRG, as the nascent neurons express Delta 1, whereas the undifferentiated progenitors express Notch 1. Notch activation inhibits neurogenesis, in a Sox2–dependent manner, allowing the maintenance of the progenitor cells, which later give rise to satellite cells (Wakamatsu *et al.*, 2000, 2004a). Also, it was shown that division of undifferentiated cells leads to asymmetrical localization of Numb in the daughter cells. This would allow the two daughter cells to assume different fates, since Numb, being a Notch antagonist, makes the cells differentially responsive to Notch activity (Wakamatsu *et al.*, 1999, 2000). However, Notch activation in cultured chick neural crest cells, by transfection with NICD, did not lead to increase in glial differentiation, as seen by expression of P0, which is a marker of glial differentiation (Wakamatsu *et al.*, 2000).

Similarly, the role of Notch signalling in Schwann cell differentiation is controversial. $p75^{+ve}P0^{-ve}$ NCSCs (see Section 1.3.1), isolated from explants of E10.5 rat neural tube, were clonally plated and subjected to Delta-IgG_{Fc} treatment, a soluble Delta protein that activates Notch signalling (Morrison *et al.*, 2000). These cells give rise to clones consisting of neurons, Schwann cells and melanocytes (Shah *et al.*, 1996; Stemple and Anderson, 1992) after long-term culture (typically 14 days after plating). In the presence of the Delta chimeric protein, the number of clones containing neurons decreased and the number of clones containing Schwann cells increased (Morrison *et al.*, 2000). The authors argue for an anti-neurogenic and pro-

gliogenic role of Notch signalling in Schwann cell gliogenesis. However, glial differentiation is not accelerated nor increased when DRGs are transfected *in ovo* with NICD (Endo *et al.*, 2002; Morrison *et al.*, 2000).

1.10 Myelin disorders.

Glial cells and their associated myelin are integral components of the nervous system and are required for its proper functioning. Invariably, glial cell dysfunction in both the PNS and CNS lead to a number of debilitating diseases. In the PNS, Type 1 Charcot-Marie-Tooth disease, which is a collection of inherited diseases causing malfunction of the peripheral nerves, is mostly due to mutations to some of the five Schwann cell genes coding for myelin proteins. Clinical disability occurs as a result of axonal damage induced by the instability and breakdown of myelin (Berger *et al.*, 2002). Schwann cells also form tumors, caused by mutations in 2 genes, neurofibromin that leads to neurofibromatosis (NF) type 1 and merlin, which gives rise to NF type 2 (Ratner and Daston, 2001). In the CNS, the majority of brain tumors are derived from glial cells and their progenitors, most notably from astrocytes (Nister *et al.*, 2001). However, the most common myelin disease is multiple sclerosis, which an inflammatory demyelinating disease predominantly affecting oligodendrocytes (Lucchinetti and Lassmann, 2001).

1.11 Multiple Sclerosis (MS).

This disease is relapsing or progressive disorder, characterized by focal areas of demyelination throughout the CNS with a predilection for brainstem, optic nerves, spinal cord and periventricular white matter (Lucchinetti and Lassmann, 2001). This disease more commonly affects young adults (20-40 years), particularly females,

leading to severe neurological disability within the next 10-15 years (Weinshenker, 1998).

MS is considered to be an autoimmune disease with activated T cells initiating a propagating destructive process (Wekerle *et al.*, 1986). It is thought that activated T cells migrate through the blood-brain barrier (Wekerle, 1998) and stimulate local and haematogenous macrophages that destroy myelin and result in further release of CNS autoantigens (Kornek and Lassmann, 2003). T-cell mediated inflammation alone, however, is not sufficient to produce the typical large demyelinating plaques. Other demyelinating amplification factors are required, which differ between patients (Kornek and Lassmann, 2003). Amongst the factors that are thought to make MS such a heterogeneous disease are genetic and environmental factors (Kornek and Lassmann, 2003; Kornek and Lassmann, 2003).

Eighty percent of patients typically have the 'relapsing-remitting' type of MS, which is characterized by a gradual progression of symptoms and signs over a period of days that stabilize, and often improve spontaneously. The disease often progresses between relapses and persistent signs of CNS dysfunction develop after relapses (secondary progressive MS). The remaining twenty percent of affected patients have the primary progressive type, which is characterized by a steady and gradual development of signs and symptoms (Noseworthy *et al.*, 2000).

The hallmarks of MS include loss of myelin and axons, which lead to the neurological symptoms. One striking observation of this disease is that oligodendrocytes are predominantly affected, whereas Schwann cells are unaffected. Another feature of the disease is the occurrence of spontaneous myelin repair in CNS lesions (Bunger *et al.*, 1961), particularly in MS (Perier and Gregoire, 1965).

A number of therapeutic strategies have been devised in order to alleviate the symptoms in MS or to cure the disease. Given the heterogeneity of the disease and the number of apparently different causative agents, no effective means of cure has been devised (Halfpenny *et al.*, 2002). A more detailed description of the different experimental strategies considered to promote neurophysiological functions is provided below.

1.11.1 Therapeutical strategies in MS.

As mentioned above, MS is an inflammatory disease of the CNS, which results in disseminated plaques of demyelination as well as axonal damage, which correlate with clinical disability. The only currently available treatments are targeted at the inflammatory phase of the disease and drugs including interferon-β, glatiramer acetate, mitoxantrone and natalizumab help to reduce frequency of relapses as well as appearance of new lesions (Frohman *et al.*, 2006; Lubetzki *et al.*, 2005; Noseworthy *et al.*, 2000). However, no net repair-promoting activity of these drugs has been found.

Remyelination, either occurring spontaneously or induced by exogenous cell transplants, has been shown to restore saltatory axonal conduction and hence, leads to functional recovery in animal models of MS (Jeffrey and Blakemore, 1997; Smith *et al.*, 1979). Additionally, remyelination could minimize axonal loss since it has been shown that intact myelin sheaths have a profound beneficial effect on axonal integrity (Edgar *et al.*, 2004; Griffiths *et al.*, 1998; Kornek *et al.*, 2000; Lappe-Siefke *et al.*, 2003; Xin *et al.*, 2005). Thus, most experimental strategies considered for repair in this disease have centered on the remyelination of the demyelinated axons, either by transplantation of exogenous myelinogenic cells or by enhancing the

process of spontaneous endogenous remyelination (Stangel and Hartung, 2002; Halfpenny *et al.*, 2002, Zhao *et al.*, 2005).

1.11.2 Spontaneous myelin repair.

Endogenous remyelination is likely to be mediated by adult OPCs rather than mature oligodendrocytes (Gensert and Goldman, 1997; Keirstead and Blakemore, 1997; Watanabe *et al.*, 2002). The OPCs are a ubiquitous population of cells, present in the developing and adult CNS, that have been shown to generate mature myelinating oligodendrocytes both *in vivo* and *in vitro* (Chari and Blakemore, 2002; Stangel and Hartung, 2002). In response to demyelination, these cells proliferate and migrate into the lesions, where they interact with the demyelinated axons and differentiate into mature myelinating oligodendrocytes (Chari and Blakemore, 2002; Zhao *et al.*, 2005).

Endogenous remyelination seems to occur only during the early stages of disease development in MS; about 40% of demyelination plaques are remyelinated, with at least 10% of the plaque volume remyelinated, during the initial inflammatory phase of the disease (Prineas *et al.*, 1993). The majority of chronic plaques, however, contain either unmyelinated axons or thinly-remyelinated axons (Ozawa *et al.*, 1994). A number of reasons have been proposed to explain the ultimate failure of endogenous remyelination in MS. Firstly, the OPCs themselves might be themselves directly targeted and recently, antibodies against OPC antigens were found in lesions (Cid *et al.*, 2004; Niehaus *et al.*, 2000), probably leading to a depletion of the OPCs. The limited migratory capacity of the OPCs could also be another contributory factor (Franklin *et al.*, 1997). Inhibitors of myelination, namely PSA-NCAM, are also re-expressed on chronically demyelinated axons, potentially making them unamenable to remyelination (Charles *et al.*, 2002). Finally, the astrocytic glial scar formed

around lesions could form a physical barrier to the migration of the OPCs into the lesions (Fawcett and Ascher, 1999).

One strategy for myelin repair in MS that has received considerable attention for the past years is the enhancement of the inherent endogenous remyelination that occurs during the course of the disease. Given that this remyelination is rather incomplete, different approaches to promote this remyelination have been considered. Administration of growth factors that protect against demyelination and promote myelination in experimental models of MS have been carried out with varying degrees of success; NGF, LIF or neuregulin-1 have been shown to decrease oligodendrocyte injury in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Butzkueven *et al.*, 2002; Cannella *et al.*, 1998; Villoslada *et al.*, 2000), FGF-2 improves the clinical course in EAE (Ruffini *et al.*, 2001) and PDGF has been shown to accelerate remyelination in chemically-induced demyelinating lesions (Allamargot *et al.*, 2001). IGF-1, as well, has been shown to enhance remyelination in EAE (Yao *et al.*, 1995), but this effect was shown to be transient in the acute phases of demyelination, and absent during the chronic phases (Cannella *et al.*, 2000). Given that there are a number of growth factors sequentially expressed during remyelination (Copelman *et al.*, 2000; Hinks and Franklin, 1999), generation of stable and long-lasting remyelination by the administration of a single factor is rather unlikely. Growth factor therapy has many potential drawbacks in that the sequence and route of administration are not known and the short half-life of growth factors and potential side effects might make them ineffective or even harmful (Lubetzki *et al.*, 2005; Stangel and Hartung, 2002). Indeed the first clinical trial of administration of a growth factor, namely IGF-1 in MS patients, was stopped early due to adverse effects (Halfpenny *et al.*, 2002).

1.11.3 Exogenous myelin repair.

Since the early 1970s, a number of different myelinogenic cells have been considered as a remyelination source in a number of different animal models of MS (Franklin *et al.*, 2002; Pluchino *et al.*, 2004). Numerous studies have been carried out to identify the cell type that would be the ideal choice for a cell-replacement therapy in MS. A number of properties that a cell type should possess, have been set for it to be considered as ideal choice (Blakemore and Franklin, 2000; Franklin, 2002).

1.11.3.1 Ideal cell choice for transplant (Franklin, 2002).

The first and most self-evident property a cell should possess would be the ability to remyelinate the demyelinated axons in MS lesions and restore saltatory conduction. Given that chronic demyelination plaques are characterized by a dense astrocytic glial scar surrounding the lesions, the cells should be able to integrate well with these astrocytes, such that there is no hindrance to remyelination. Also, since MS is a multi-focal lesion, ideally the cells should be able to migrate through the normal CNS and myelinate the scattered areas of demyelination present through the CNS. This would avoid the need for several potentially risky injection points along the CNS during the surgical procedure. The myelin formed by the transplanted cells should be stable in that it would be able to withstand the long-lasting disease process in MS. Since in MS, only myelin in the CNS is affected, remyelination by a peripheral myelin type would be less susceptible to repeated attacks during the disease process. Finally, the cells should be readily available in sufficient quantities and if possible, obtained from the patient such that immune rejection is circumvented and immunosuppression is not required.

1.11.4 Cell replacement therapy in MS.

A number of cell types have been considered for transplant and their properties have been extensively studied in a number of different animal models of MS. These include cells of the oligodendrocyte lineage, Schwann cells, olfactory ensheathing cells and stem cells.

1.11.4.1 Cells of the oligodendrocyte lineage.

Cells of the oligodendrocyte lineage, obtained by *in vitro* culture or freshly dissociated have been extensively used in transplant studies with varying levels of success in mediating myelination (Franklin, 2002). The early progenitors fare much better than more mature cells in myelinating wider areas (Rosenbluth *et al.*, 1990; Warrington *et al.*, 1993), reflecting the high proliferative and mobile nature of oligodendrocyte progenitors and the quiescent, non-mobile nature of the mature oligodendrocytes (Pfeiffer *et al.*, 1993). However, when transplanted in normal CNS white matter, OPCs survive and migrate very poorly (Franklin *et al.,* 1996; O'Leary and Blakemore, 1997).

1.11.4.2 Schwann cells.

Schwann cells have long been considered as an ideal choice for cell transplant given their numerous favorable characteristics. Although they are present in the PNS, they are able to remyelinate CNS axons (Blakemore, 1976, 1977) and can restore saltatory conduction (Felts and Smith, 1992; Honmou et al., 1996; Kohama et al., 2001). They can also be obtained readily from human biopsy material and expanded in vitro, making them suitable for autologous transplantation (Kohama et al., 2001; Rutkowski et al., 1995). The myelin sheath of the Schwann cells have

also the additional advantage of being likely to withstand the autoimmune inflammatory injury in MS.

Schwann cells, however, do not interact very well with astrocytes; when Schwann cells and astrocytes are co-cultured together, they separate into distinct and non-overlapping domains (Ghirnikar and Eng, 1994; Lakatos *et al.,* 2000), and when transplanted into demyelinating lesions, they are present only in regions devoid of astrocytes (Blakemore, 1976; Shields *et al.*, 2000). Neither Schwann cells nor OPCs can survive and migrate when transplanted into normal white matter making them unsuitable for remyelinating the scattered lesions present in MS (Iwashita and Blakemore, 2000; Iwashita *et al.*, 2000).

1.11.4.3 Olfactory ensheathing cells.

Olfactory ensheathing cells (OECs) are a unique group of glial cells present in the olfactory nerve and nerve fibre layer of the olfactory bulb. They ensheath the small diameter axons and have characteristics of both Schwann cells and astrocytes (Barnett *et al.,* 1993; Doucette, 1990; Ramon-Cueto and Nieto-Sampedro, 1992). These cells can remyelinate demyelinating lesions and restore saltatory conduction (Franklin *et al.*, 1996; Imaizumi *et al.*, 1998). The myelin formed by these cells shares the same ultra-structural and antigenic profile, namely the expression of P0, as Schwann cell myelin (Franklin *et al.*, 1996; Smith *et al.*, 2001).

Although it has been shown that OECs can migrate through normal CNS and integrate into areas of astocytosis in CNS trauma models (Boruch *et al.*, 2001; Ramon-Cueto *et al.,* 1998), the issue remains controversial in that the markers used for labeling these transplanted cells have proved unreliable (Iwashita *et al.*, 2000). More studies need to be performed for these cells to be met with universal approval, but the fact that they share the favorable characteristics of Schwann cells and at the

same time seem to fare better than Schwann cells in terms of their migration and survival properties suggest that they may offer distinct advantages.

1.11.4.4 Stem cells.

Over the past few years, stem cells, either embryonic or adult-derived, have received considerable attention as a cell-replacement therapy for MS. They are highly proliferative and are pluripotent, making them an unlimited cell source for transplants (Weissmann *et al.*, 2001). Embryonic stem cells, when transplanted into lesions in rodents can differentiate into glial cells and myelinate the demyelinated axons (Brustle *et al.,* 1999; Liu *et al.*, 2000; McDonald *et al.*, 1999) but the formation of teratomas and heterologous tissues make them unsuitable for repair (Yanai *et al.*, 1995; Brustle *et al.,* 1997; Deacon *et al,* 1998). This problem has been circumvented by the transplant of OPCs, derived from embryonic stem cells, which can myelinate demyelinating lesions (Glaser *et al.,* 2005).

Adult neural stem cells have been also used for transplant studies and they have been shown to differentiate into oligodendrocytes and myelinate the demyelinated axons and promote functional recovery. Importantly these cells do not form tumors (Ben-Hur *et al.*, 2003; Einstein *et al.*, 2003; Pluchino *et al.*, 2003). Adult bone-marrow derived stem cells also have neural potential (Brazelton *et al.*, 2000; Kopen *et al.*, 1999; Mezey *et al.*, 2000; Woodbury *et al.*, 2000), and bone marrow cells have been shown to remyelinate demyelinated axons in rodents (Sasaki *et al.*, 2001). More studies need to be performed to identify the stem cell source that could be used to promote repair in MS, one that is readily and easily available and which would prove safe when injected into humans.

CHAPTER 2: Materials and Methods

2.1 List of reagents

2.1.1 Reagents for tissue culture

Transferrin, selenium, putrescine, triiodothyronine, thyroxine, progesterone, insulin (10^{-3}M), bovine serum albumin (BSA), cytosine arabinoside (Ara C), poly-D-lysine, poly-L-ornithine, lysine, dibutyryl-cAMP (dbcAMP), 5-Fluoro-2'-Deoxyuridine (FDU), ascorbic acid, trypsin inhibitor, hyaluronidase, N-acetyl cysteine, fibronectin and laminin were obtained from Sigma (Poole, UK). Collagenase was obtained from Worthington (Lorne Laboratories, Reading, UK). 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). Foetal calf serum (FCS) was from Bioclear, UK. Tissue culture Petri dishes and 24-well plates were from Falcon (Becton-Dickinson, Cowley, UK), and forskolin was from Calbiochem (CA, USA). Round 13 mm coverslips were from BDH (Lutterworth, UK). Growth factors including PDGF-BB, IGF-1 and FGF-2 were from Peprotech Ltd (UK) and neuregulin-1 was obtained from R&D systems (UK). NGF was obtained from Invitrogen (UK).

2.1.2 Reagents for molecular biology

Random hexamers, Oligo-dT, RNAsin and AMV-Reverse Transcriptase were from Promega Corporation (Promega, Madison, USA). Taq DNA-polymerase, RNase H, Reverse Transcriptase (superscript II), T4 DNA ligase, T4 buffer, Klenow (DNA polymerase), DNA Polymerase I, dNTPs , 1KB plus DNA ladder, TRIzol reagent

and Agarose were from Invitrogen (Invitrogen Ltd, Paisley, UK). EDTA disodium salt, ethidium bromide, Sodium Citrate, Tween20, Phenol:Chloroform:Isoamyl Alchool 25:24:1, Chloroform:Isoamyl Alchool 24:1, phenylmethylsulphonylfluoride (PMSF) and bromophenol blue were from Sigma (Poole, UK). ECL Plus Kit, Western Blot Stripping Kit and Hybond-N nitrocellulose membrane were from Amersham Pharmacia Biotech (UK). Absolute Ethanol, Isopropanol, Methanol, Sodium dodecyl sulphate (SDS), Sodium Chloride (NaCl) and Glycerol were from BDH Lab. Supplies (Poole, UK). Kaleidoscope pre-stained standards were from Biorad (CA, USA). Bromodeoxyuridine, Terminal Transferase and biotinylated-d-UTP were from Roche Diagnostics (Germany). Diethyl pyrocarbonate (DEPC) and Phenol-chloroform were from Fluka Chemicals Ltd. (Buchs, Switzerland).

2.1.3 Reagents for immuno-labelling

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

2.1.4 Reagents for histology and Electron Microscopy

Glutaraldehyde, Agar 100 Resin, Methyl Nadic Anhydride (MNA), Dodecenyl Succinic Anhydride (DDSA), N-Benzyldimethylamine (BDMA), Tissue Tek OCT compound and rubber coffin moulds were from Agar Scientific (UK). DPX mountant and 13mm coverslips were from Merck (Poole, UK), Superfrost Plus microscope slides were from BDH (UK).

2.2 List of Antibodies

2.2.1 List of Primary antibodies

Active Caspase-3 (CM1): Rabbit polyclonal antibody specific for active cleaved caspase-3 was from BD Pharmingen (Oxford, UK) and used at a concentration of 1: 250 for ICC.

BFABP: Rabbit polyclonal antibody was a gift from Dr T. Muller (Centre National de la Recherche Scientifique, France) (Kurtz *et al.*, 1994) and used at a concentration of 1:2000 for IHC.

Bromodeoxyuridine (BrdU): Mouse monoclonal antibody was from Roche Diagnostics (UK) and used at a final dilution of 1:100 for ICC or IHC.

Cdk 2: Rabbit polyclonal antibody obtained from Santa Cruz Biolotechnology (USA) and used at a concentration of 1: 500 for WB.

Cyclin D1: Mouse monoclonal antibody obtained from Santa Cruz Biolotechnology (USA) and used at a concentration of 1: 500 for WB.

ErbB2 (Neu): Rabbit polyclonal antibody obtained from Santa Cruz Biolotechnology (USA) and used at a concentration of 1: 500 for WB.

ErbB3: Rabbit polyclonal antibody obtained from Santa Cruz Biolotechnology (USA) and used at a concentration of 1: 500 for WB.

Fibronectin: Rabbit polyclonal antibody obtained from Sigma (UK) and used at a concentration of 1:200 for ICC.

GalC: The GalC hybridoma cell line was a generous gift from Dr B. Ranscht (Max-Plank Institute, Germany) (Ranscht *et al*, 1982), and the mouse monoclonal supernatant was used at a 1:2 dilution for our FACS analysis.

GAPDH: mouse monoclonal antibody against GAPDH (Abcam Ltd, Cambridge, UK) was used at a dilution of 1:5000 for WB.

GFAP: Rabbit polyclonal antibody, obtained from Dako Immunoglobulins (Dakopatts, Copenhagen, Denmark) and used at a concentration of 1:500 for ICC and IHC.

Krox-20: Rabbit polyclonal antibody obtained from Covance (USA) and used at a concentration of 1:250 for ICC or 1:1000 for WB.

L1 (324): 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 1:1 with MEM-H 10% calf serum (CS)

L1 (ASCS4): Mouse monoclonal antibody (clone ASCS4) was a gift from Dr. P. Patterson (Caltech, USA). This antibody was used in the form of hybridoma supernatant and diluted 1:1 with MEM-H 10% calf serum (CS).

Laminin: Rabbit polyclonal antibody to mouse laminin α2 401 was a gift by Dr L. Sorokin (University of Erlangen- Nurnburg, Germany) (Ringelmann et al., 1999) and used at a concentration of 1:50 for ICC.

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 1:8000 for ICC.

Nestin (Rat 401): Mouse monoclonal antibody obtained from the Developmental Studies Hybridoma Bank (USA) and used at a concentration of 1:2000 for ICC or IHC.

NICD: Mouse monoclonal antibody obtained from Chemicon (USA) and used at a concentration of 1: 1000 for WB.

O4: The O4 hybridoma cell line was a generous gift from Dr. I. Sommer (University of Glasgow, Glasgow, UK) (Sommer and Schachner, 1981), and the mouse monoclonal supernatant was used at a 1:2 dilution for ICC.

OX7: OX7 hybridoma cell line secreting IgM recognizing Thy 1.1 was from the European Collection of Animal Cell Cultures (DERA, Wiltshire, UK) and used at a concentration of 1:1 for ICC.

p-AKT: Rabbit polyclonal antibody specific to the phosphorylated form (ser 473) of Akt were from Cell Signalling Tech (Hertfordshire, UK) and used at a concentration of 1:50 for ICC.

p-ERK 1/2: Mouse monoclonal antibody obtained from Sigma (UK) and used at a concentration of 1:2000 for WB.

p-JNK 1/2: Rabbit polyclonal antibody obtained from Cell signaling technology (USA) and used at a concentration of 1:1000 for WB.

p-p38: Rabbit polyclonal antibody obtained from Cell signaling technology (USA) and used at a concentration of 1:1000 for WB.

Protein zero (P0): Mouse monoclonal antibody, used at a concentration of 1:400 overnight at 4°C (for immunohistichenistry – IHC) or 1:2000 (for Western Blots – WB). It was obtained from Astexx (Austria).

Protein zero (P0): Rabbit polyclonal antibody against rat protein zero was generated and characterized in the laboratory by Louise Morgan (Morgan *et al.,* 1994). The antibody was used at a dilution of 1:500 overnight at 4°C for immunocytochemistry (ICC).

S100β: Rabbit polyclonal antibody against cow S100β protein was from Dako Immunoglobulins (Dakopatts, Copenhagen, Denmark.) and used at a final dilution of 1:1000 for ICC or IHC.

TuJ1: mouse monoclonal antibody against b Tubulin III was from Covance (USA) and used at a final dilution of 1:10,000 for IHC.

TuJ1: rabbit polyclonal antibody against b Tubulin III was from Covance (USA and used at a final dilution of 1:4000 for and ICC and IHC.

2.2.2 List of Secondary antibodies

Goat anti-mouse immunoglobulins (Ig) conjugated to tetramethyl rhodamine (used at a dilution of 1:100) and goat anti-rabbit Ig conjugated to fluorescein (used at 1:600) were from Cappel (Cappel Organon Teknika Corp, PA, USA). Donkey anti-rabbit Ig conjugated to biotin (used at 1:100), sheep anti-mouse Ig conjugated to biotin (used at 1:100), streptavidin conjugated to fluorescein (used at 1:100) and streptavidin conjugated to Cy3 (used at 1:50) were from Amersham (Amersham Pharmacia Biotech, UK). Goat anti-rat Ig Cy3 (used at 1:200) was from Jackson laboratories (Pennsylvania, USA). Goat anti-mouse Ig conjugated to horseradish peroxidase (HRP) (used at a dilution of 1:2500) and goat anti-rabbit Ig conjugated to horseradish peroxidase (HRP) (used at a dilution of 1:2500) were from Promega Ltd (UK).

2.3 Tissue culture techniques

2.3.1 Substratum coating of coverslips and dishes

Poly-D-lysine (PDL):

Round 13 mm coverslips were baked at 140^{0}C for 8 hr and then coated with 1mg/ml poly-D-lysine in dH_2O for 4 hours at room temperature. Coverslips were then washed in distilled water 3 times at room temperature on a shaker, before being air dried in a flow cabinet and stored dessicated.

Tissue culture dishes (35 mm, 60 mm and 90 mm) were coated with PDL (1 mg/ml) for 2 h at room temperature. The solution was then removed and the dishes were air-dried without washing.

Poly-L-ornithine:

Baked 13 mm glass coverslips were coated with 20 µl drops of poly-L-ornithine solution (0.1 mg/ml in deionized water) for 2 hr, then rinsed in deionized water for 15 min prior to drying.

Laminin:

20 µl drops of laminin (20 µg/ml) in Dulbecco's modified Eagles' medium (DMEM) were plated on PDL-coated coverslips and left for at least 1 h before removal prior to plating the cells. Alternatively, laminin was added to dishes, left for at least 1 h and removed just before plating cells or tissues.

Fibronectin:

20 µl drops of fibronectin (25 µg/ml in DMEM) were plated on PDL-coated coverslips for 30 min and removed prior to plating the cells. PDL coated dishes were coated with fibronectin, which was removed prior to plating the tissue.

2.3.2. Medium Components

Simple non-supplemented medium (SM):

A 1:1 mixture of DMEM and Ham's F12 medium was used and supplemented only with BSA (0.3 mg/ml). This is referred to as SM in the text.

Defined supplemented medium (DM):

This medium is a modification of the medium of Bottenstein and Sato (1979). A 1:1 mixture of DMEM and Ham's F12 was supplemented with transferrin (100 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), thyroxine (0.4 μg/ml), triiodothyronine (10.1 ng/ml), dexamethasone (38 ng/ml), selenium (160 ng/ml), BSA (0.3 mg/ml), penicillin (100 IU/ml), streptomycin (100 IU/ml) and glutamine (2 mM). This medium is referred to as DM in the text. When used for crest cells this medium contained thyroxine at 0.8 μg/ml, triiodothyronine at 20.2 ng/ml and glucose at 0.5 mg/ml.

Dorsal root ganglia (DRG) culture medium:

This medium consists of MEM containing 10% FCS, Insulin (10^{-3} M), glucose (32 ng/ml) and NGF (50 ng/ml).

Crest medium (CM):

This medium consists of DM (see above) supplemented with neuregulin-1 (10 ng/ml), IGF-1 (100 ng/ml), N-acetyl cysteine (1 mM), FGF-2 (3 ng/ml) and insulin (10^{-9} M). This id referred to as CM in the text.

2.3.3 Cell cultures

2.3.3.1 DRG satellite cells

DRGs were dissected out from embryos of different ages (as specified in the text) and plated on a small scratch on a PDL-laminin coated tissue culture dishes containing DM supplemented with neuregulin-1 (20 ng/ml) and insulin (10^{-6} M). They were cultured at 37°C and 5% CO_2 for 24 h to allow cell migration, after which the DRG and all the attached neurites were removed with fine needles. The remaining flattened satellite cells were briefly washed with versene (0.2 mg/ml EDTA, 0.01% PBS, 0.005% Phenol Red in ultra pure deionized water) and detached by incubating for 3-5 min at 37°C in 200 µl versene containing 3 drops of enzyme cocktail [collagenase (2 mg/ml), hyaluronidase (1.2 mg/ml) and trypsin inhibitor (0.3 mg/ml) in DMEM]. The cell suspension obtained was collected, centrifuged at 1,000 rpm for 10 min, resuspended in the relevant medium and plated.

2.3.3.2 Schwann cell and Schwann cell precursor replated cultures.

For replated cultures of Schwann cells and Schwann cell precursors, sciatic nerves from embryos at different ages were dissected and segments were plated on a small scratch, as described for the DRG. After culture for 24 h, the segments were lifted off and the glial cells that had migrated extensively from the nerve were replated in the same way as the DRG glia replates.

2.3.3.3 Neural crest cells

Neural tubes from E11 rat embryos were dissected out and plated on a PDL-fibronectin coated 35 mm Petri-dish containing 2 ml of CM, essentially as described by Smith-Thomas and Fawcett (1989). They were cultured for 24 h at 37°C and 5 % CO_2 after which the neural tubes were excised with a needle using an inverted

microscope, leaving the neural crest cells. These were then dissociated by incubating for 3 min in 200 µl versene containing 3 drops of enzyme cocktail as described above. After centrifugation the cells were resuspended in the relevant medium and plated.

2.3.3.4 Schwann cell precursors

For acute preparations of Schwann cell precursors, the sciatic nerves from E14.5 rat embryos were dissected and incubated for 1 h in enzyme cocktail at 37°C. The cell suspension obtained was collected, centrifuged and resuspended in the relevant medium and plated (Jessen *et al.,* 1994).

2.3.3.5 Serum-purified Schwann cells

Schwann cells were prepared using the method of Brockes et al., (1979). Newborn or post-natal day 3 (P3) Sprague-Dawley rats were killed by decapitation and the sciatic nerves dissected out. Nerves were placed in L15 medium, 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 37^0C and 5%CO2 and 95% air for 35 min with trituration at the end. The cells were then washed in DMEM with 10% FCS and centrifuged for 10 min at 1000 rpm at 4^0C to pellet, resuspended in the same medium with AraC (10^{-3} M) and cultured on PDL-laminin coated 35 mm tissue culture dishes for 3 days. FCS causes the contaminating fibroblasts present in these cultures to proliferate and these are killed by the anti-mitotic compound, AraC. After 3 days in culture, mostly Schwann cells are left in the culture dish.

2.3.3.6 Immunopanning of Schwann cells

Purification of Schwann cells was performed by reverse immunopanning using an antibody specific to fibroblasts, Thy 1.1. Schwann cells, cultured for 3 days in AraC (above), were detached from the dishes by 5 min treatment with 0.025% trysin-versene solution, centrifuged and resuspended in 10 ml DM containing 10% FCS. This cell suspension was placed onto a 90 mm Petri dish, coated with mouse IgG and Thy 1.1, and cultured at37^{0}C and 5%CO2 for 20 min, with a gentle shaking of the dish midway through. In this procedure, the remaining fibroblasts, found in the Schwann cell cultures after AraC purification, were removed as they bind to the Thy1.1-coated Petri dish and the cell suspension obtained was devoid of any fibroblasts. The cell suspension was then recovered, pelleted and resuspended in expanding medium [DM containing neuregulin-1 (10 ng/ml), FGF-2 (3 ng/ml), insulin (10^{-3} M), 3% FCS and Forskolin (4 μM)} and cultured onto PDL-Laminin coated dishes. The medium was changed every 3 days until the cells were confluent on the dish.

2.3.4 Glia conditioned medium

Glial conditioned medium was prepared essentially as described previously (Meier *et al.*, 1999). 2.5 x 10^6 P5 Schwann cells or E (15+1) satellite cells were plated onto a 35 mm PDL-Laminin coated dish in 1.2 ml of SM. After 24 h of culture, the conditioned medium was collected and stored in BSA-coated vials at -80°C until required. To prevent degradation of active factors in the medium, the cells were cultured in the presence of leupeptin (20 μM).

2.3.5 Adenoviral infections

Confluent expanded Schwann cells grown on PDL-Laminin dishes or serum-purified cells plated onto PDL-Laminin coverslips (5,000 cells/coverslip) were cultured in DM containing 0.5% FCS and Insulin (10^{-3}M) for 24 hrs. Adenoviral preparations were added to the cultures and left for 24 hrs and then the medium changed. The cultures were then fixed at different times and ICC performed or proteins were extracted for WB analysis.

Schwann cell precursors were dissected out and plated on either 35 mm PDL-Laminin dishes or onto PDL-Laminin coverslips (2,000 cells/coverslip). DM containing neuregulin-1 (20 ng/ml) and Insulin (10^{-3}M) was added to the cultures and 3 hrs later, adenoviral preparations added. 24 hrs later, the medium was changed and thereafter the cultures fixed at appropriate times for ICC or proteins extracted for WB analysis.

2.3.5.1 Adenoviral constructs and preparation

The adenovirus used for Notch activation was obtained from Dr GP Dotto (Harvard Medical School, USA). Briefly, the recombinant adenoviruses expressing the constitutively active form of Notch1 and the ANK domain alone (control) were obtained by inserting the corresponding cDNAs [amino acids 1758–2556 and 1852–2125, respectively, of human Notch1 (Capobianco *et al.*, 1997)] into the *Bam*HI–*Xho*I sites of pAdTrack-CMV, and then recombined into the adenoviral backbone plasmid pAdEasy-1 in bacteria (He *et al.*, 1998). The adenovirus expressing constitutively active Notch1 is referred to as Ad-NIC in the text and its matched control adenovirus is referred to as Ad-GFP. Both adenovirus express GFP protein as well after infections.

Adenovirus expressing either full-length Krox-20 or the appropriate empty vector was obtained as a gift from Dr J. Milbrant (Nagarajan *et al.*, 2001), and these were made in the laboratory of Dr M. Ehrengruber (Ehrengruber *et al.*, 2000). Briefly, wild-type EGR2 (Krox-20) (Chavrier *et al.*, 1998) were inserted into the E1 regions of Ad5PacIsGFP to yield AdGFP EGR2. Ad5PacIsGFP was prepared by ligating an expression cassette, CMV–sGFP–SV40pA, into the *Pac*I site (in the E3 deletion) of Ad5PacI as described (Ehrengruber *et al.*, 1998). The adenovirus expressing full-length Krox-20 is referred to as Ad-K20 in the text and its matched control adenovirus expresseing the empty vector plasmid, is referred to as Ad-GFP in the text. Both adenovirus express GFP protein as well after infections.

Adenoviral supernatants were prepared and titered as described previously (Parkinson *et al.*, 2001). Briefly, confluent HEK 293 cells were infected with adenovirus, and after 2 days in culture following cytopathic shock, the cell supernatant was collected and purified with the vivapure AdenoPACK 500 (Sartorius AG, UK) according to manufacturer's protocol. The virus were then titered by infection of serum-purified Schwann cells with different volumes of the virus and the volume, which resulted in infection rate of >95% (as seen by expression of GFP) and no cytopathic death was chosen. Please note that after each viral preparation, the amount of virus used was always titered.

2.3.6 Retroviral infections

The cDNAs for NICD, obtained by PCR from the full-length human Notch1 cDNA (amino acids 1760-2566)(Ross and Kadesch, 2001) was cloned into the retroviral plasmid vector pBABEpuro, and the GP+E ectotropic packaging cell line (Morgenstern and Land, 1990) was then stably transfected with the plasmid DNA. Plasmids, without the transgene, were used as control vector. Retroviral supernatant

obtained from the infected GP+E cells were then used to infect confluent Schwann cells and then the infected cells were puromycin-selected and then expanded. In the text, cells infected with the NICD-containing retrovirus are referred to as BP-NICD-Schwann cells and cells infected with the control retrovirus are referred to as BP2-Schwann cells.

The cDNAs for mouse full length Delta 1 (amino acids 14-2190) (Lowell *et al.*, 2000) was cloned into the retroviral plasmid vector pBABEpuro, and the GP+E ectotropic packaging cell line (Morgenstern and Land, 1990) was then stably transfected with the plasmid DNA. Plasmids, without the transgene, were used as control vector. Retroviral supernatant obtained from the infected GP+E cells were then used to infect confluent NIH 3T3 cells and then the infected cells were puromycin-selected and then expanded. In the text, cells infected with the Delta 1-containing retrovirus are referred to as BP-Delta 3T3 cells and cells infected with the control retrovirus are referred to as BP2 3T3 cells.

2.3.7 Myelination assays

2.3.7.1 cAMP myelination assay

A cAMP analogue, dibutryl cAMP (10^{-3}M), was added to cultures, as mentioned in the text. The cells were then fixed after 1 day, for Krox 20 ICC, 2 days, for periaxin ICC or 3 days for P0 ICC.

2.3.7.2 DRG-Schwann cell co-cultures myelination assay

DRGs (about 200 per experiment) were dissected from E15.5 rat embryos, dissociated in 1ml of 0.25% trypsin for 45 minutes and triturated at the end. The cells were washed in DRG medium and centrifuged for 10 minutes. The pellet was then resuspended in 10 ml of medium and then cultured onto PDL-coated dishes for 1 hr

to remove most of the fibroblasts and Schwann cells, which attach to the surface of the dish. The cell suspension was then pelleted again, reuspended in medium and then plated onto PDL-Laminin coverslips at a density of 75,000 cells per coverslip (50 ul drops). The cells were left in the incubator overnight after which they were topped up with 450 ul of DRG medium containing AraC (10^{-3}M). 3 days later, the medium was changed to fresh DRG medium and cultured for the next 3 days. The cells were further purified by adding DRG medium containing FDU (10^{-3}M) for 3 days, followed by 3 days in DRG medium alone, and then 3 more days with DRG medium and FDU. Following the last round of purification, the cells were cultured for at least 1 week in DRG medium alone, with fresh medium added every 3 days. Then, Schwann cells, infected with Ad-NIC or Ad-GFP adenovirus, were added to the cultures (50,000 cells per coverslip). The cultures were left for 2 weeks to allow the Schwann cells to associate with the DRG axons. Again, fresh DRG medium was added every 3 days. Then myelination was triggered by adding fresh DRG medium supplemented with ascorbic acid (50 ng/ml) to the cultures. Ascorbic acid triggers the formation of a basal lamina in the Schwann cells and then subsequent myelination (Eldridge *et al.*, 1989). Every 3 days, for up to 14 days, DRG medium containing freshly prepared ascorbic acid was added to the cultures. The cells were then fixed and immunolabelled for the myelin protein P0 and counter-stained with Hoescht nuclear dye.

2.3.8 Proliferation Assay

2.3.8.1 *In vitro* BrdU proliferation assay

BrDU solution (10^{-5}M) was added to cells in culture for 24 hrs and then fixed and immunolabelled using a monoclonal anti-BrdU antibody. Bromodeoxyuridine (BrdU), an analog of thymidine (derivative of uridine), is a uridine derivative that can be incorporated specifically into DNA in place of thymidine. Cells that are synthesizing DNA (in S-phase of the cell cycle) will incorporate BrdU into the DNA after a period of exposure to BrDU. Anti-BrdU immunolabelling can then be used to identify cells that undergo DNA synthesis.

2.3.8.2 *In vivo* BrdU proliferation assay

E17.5 Pregnant mice were injected intraperitoneally with 100μg BrdU solution per g of body weight and allowed to rest without disturbance for one hour. The mice were then killed and the embryos dissected out. The sciatic nerves were dissected out and the tissues embedded in OCT compound and stored at -70^{0}C. Frozen sections (10 μm thickness) were cut using a cryostat (Reichert-Jung, Germany) and BrdU ICC performed.

2.3.9 Cell Death Assay

Apoptotic cell death was analysed by *in situ* labeling of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed 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

cultures in a TUNEL reaction mixture, as recommended in the manufacturers' protocol and incubated at 37^0C for 60 min. The reaction was terminated by washing in ultrapure H_2O and then in PBS. Non–specific binding sites was blocked using PBS with 10% FCS for at least 60 min at room temperature. The cells 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 Hoechst as detailed above and the coverslips mounted in Citifluor mounting medium.

Alternatively, apoptotic cells were immunolabelled for active caspase-3 by the CM1 antibody. Caspase-3 activation is associated with apoptotic cell death (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998).

2.3.10 Inhibitor experiments

Different inhibitors were used to block specific pathways in our culture system, as mentioned in the text:

UO126 (Erk 1/2 phosphorylation blocker): obtained from Calbiochem (Nottingham, UK) and used at 10mM.

SB202190 (p38 phosphorylation blocker): obtained from Calbiochem (Nottingham, UK) and used at 10mM.

JNK peptide (JNK 1/2 phosphorylation blocker): obtained as a gift from Dr. H. Mehmet (Imperial College London, UK) (Borsello *et al.,* 2003) and used at 10mM.

2.4 Molecular Biology Techniques

2.4.1 RNA isolation

Total RNA was isolated from rat and mouse sciatic nerves or from cells using TRIzol reagent (Invitrogen Ltd, Paisley, UK), following the manufacturers' instruction. The RNA was then quantified using a spectrophotometer (Camspec, UK) and analysed for integrity on a 1% agarose gel under denaturing conditions.

2.4.2 Semi-quantitative Reverse Transcription Polymerase Chain Reaction.

Total RNA (200ng-1μg) was reversed-transcribed in a 20μl reaction mix containing 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 1mM dNTPs, xU RNAsin, 2U RNase H, 20ng oligo-dT and 200U AMV Reverse Transcriptase as recommended in the manufacturers' protocol (Promega Ltd, UK). After incubation for 5 min at 70^0C followed by 10 min at room temperature and 1 hour at 42^0C, 30μl TE pH 8.0 were added, and the cDNA aliquoted.

The relative amount of cDNA synthesized from each sample was determined by PCR amplification using specific primers for GAPDH mRNA. Equal amounts of cDNA, from the various samples, were used for PCR together with a water control with oligonucleotide primer pairs. PCR was performed in 25μl reaction volumes containing 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, 50pmoles of each primer and 2.5 units Taq DNA-polymerase. The reaction conditions for each primer pair were optimised with respect to $MgCl_2$ concentration, annealing temperature, extension time and cycle number. The primers used and the different reaction conditions for each primer sets are given in Appendix I.

PCR reactions were performed in a MWG Biothec Primus96 Thermal Cycler (Germany). Upon completion, loading buffer (10x) was added to each sample and

10µl of each reaction was electrophoresed on 1 % 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 (UK) and documented using polaroid photography.

2.4.3 Western Blotting

Frozen nerve samples or cells were transferred into lysis buffer (25 mM Tris-HCl, pH 7.4, 95 mM NaCl, 10 mM EDTA, 2%SDS, 1mM PMSF and protease inhibitors), and homogenized with a Dounce homogenizer. 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). Subsequently the lysates were boiled and centrifuged, and protein concentration in the supernatant was determined with the Biorad protein assay kit (Biorad, CA, USA), according to the manufacturers' instructions.

Samples (usually 5-50 µg of protein) were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, with a mini Protean II gel electrophoresis apparatus (Biorad, CA, USA). Kaleidoscope pre-stained standards molecular weight (Biorad) was included to enable band size identification. Separated proteins were then transferred to a nitrocellulose membrane, Hybond-N, in a mini gel transfer tank (Biorad).

Non-specific binding sites on the membrane were blocked for 2 hrs using 5% fat free milk powder in TBS. Then, primary antibodies were incubated in 1% fat free milk powder in TBS overnight at 4^0C on a slow rotator (Gallenkamp, UK). The blots were

then washed in TBS 3 times, for 5 minutes each, at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in TBS for 1 hr at room temperature, washed in TBS (3 X 5 min) and developed with ECL chemioluminescent reagent for 5 minutes in the dark (Amersham Biosciences Ltd, UK). The blots were 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 5 secs to 10 min later in an X-Ograph Compact X2 automatic developer (UK).

2.4.4 Genotyping

DNA extraction: 5 mm of tail samples were digested at 55^0 C overnight in lysis buffer (0.01M Tris pH 8.0, 0.05M EDTA pH8.0, 0.04M Nacl and 1% SDS in UP H_2O) containing 20 ng/ml Proteinase K (Sigma, UK). DNA was obtained from this mix using phenol-chloroform and ethanol using the maufacturer's instruction.

Genotyping: PCR reactions were set up for each sample using 100-200 ng of DNA and run at specific conditions for each primer set. Typically 50µl reaction volumes containing 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, 50pmoles of each primer and 2.5 units Taq DNA-polymerase were used. The reaction conditions and sequence of the primers used are found in the appendix II.

2.4.5 *In situ* Hybridisation

A digoxigenin-labelled cDNA probe was used to detect P0 mRNA in our cultures (Lee *et al.*, 1997; Morgan *et al.*, 1994). The cells were fixed in 4% paraformaldeyde and washed 3X in RNAse-free PBS (1X). They were then incubated with hybridization buffer containing 2.5 ng/ml of the P0 probe (Lee *et al.*, 1997) and left overnight at 65^0C in a humified box containing 2X SSC. The next day, the coverslips

were rinsed with 1X SSC at RT, after which they were washed with 1X SSC at 65^0C for 1 hr. They were then again washed with 1X SSC for 5 min at RT, after which they were incubated with buffer B1 containing 10% normal goat serum (Sigma, UK) for at least 1 hr. They were then incubated overnight with anti-DIG antibody (Roche diagnostics, USA) overnight at 4^0C. The next day, the coverslips were washed with buffer B1 3X for 5 min each, after which they were treated with buffer B3 for 5 min. Then buffer B4 was added to the coverslip and the plates wrapped in aluminium foil and developed according to manufacturer's protocol. The cells were then mounted in aquamount (VWR, UK) and allowed to dry. The composition of all the reagents used is found in appendix II.

2.5 Animals used

2.5.1 Animals used at University College London

CALSL-NICD mice: Transgenic line in which the *NICD* transgene is expressed under the control of the relatively strong (but mosaic) chicken β-actin promoter and a floxed "stop cassette". Recombination by Cre recombinase leads to the excision of the "stop" cassette and subsequent activation of the *NICD* transgene under the chicken β-actin promoter (Yang *et al.,* 2004). A schematic representation of the *CALSL-NICD* transgene is shown in Appendix III. These animals were kindly provided by Dr J. Shen (Harvard Medical School, USA)

RBP-J "floxed" mice: Transgenic line in which loxP sites were inserted on both sides of the RBP-J exons (6 and 7) encoding its DNA binding domain. Mating with Cre transgenic mice leads to deletion of the *RBP-J* allele (Han *et al.*, 2002). A schematic representation of the floxed *RBP-J* allele and deleted *RBP-J* allele, after Cre-mediated excision, is shown in Appendix IV. These animals were kindly provided by Professor M.J. Dallman (Imperial College London, UK)

Dhh-cre mice: Transgenic mice expressing Cre recombinase under a transgene driven by the Desert Hedgehog (Dhh) promoter. *Dhh Cre* activity is observed in the Schwann cell lineage from E12, in the testis and in the skin of the snout, but not in the brain and more specifically the hypothalamic region (Jaegle *et al.*, 2003). These animals were kindly provided by Dr D. Meijer (Erasmus University Medical Center, Netherlands)

Krox-20 mice: Knockout mice in which a null allele is created in the *Krox-20* gene, by an in-frame insertion of the *E. coli lacZ* gene (Schneider-Maunoury *et al.*, 1993). These animals were kindly provided by Dr P. Charnay (Ecole Normale Superieur, France)

Hes 1 mice: Knockout mice in which a null allele was created by targeted disruption of the *Hes 1* locus (Ishibashi *et al.*, 1995).

Hes 5 mice: Knockout mice in which a null allele was created in the *Hes 5* gene by targeted disruption. The first 2 exons of *Hes 5*, which encode the basic helix-loop-helix domain and part of the third exon were deleted and replaced by a PGK-neo^r cassette, and this results in loss of *Hes 5* expression and function (Cau *et al.*, 2000).

E11.5 embryos from Hes 1 and Hes 5 mutant mice were kindly provided by Dr F. Guillemot (NIMR, UK).

Sprague-Dawley rats: Time-mated pregnant females and post-natal pups were obtained from the Biological Services Unit at University College London.

2.5.2 Animals used at Cambridge University

GFP-transgenic rats: Transgenic rats that ubiquitously express the enhanced green fluorescent protein (GFP) under a chicken β-actin promoter and cytomegalovirus enhancer (CMV) were purchased from the National Bio Resource Project at Kyoto University (Japan).

Sprague-Dawley rats: Time-mated pregnant females and post-natal pups, obtained from crosses with GFP-transgenic rats as well as recipient females for transplant studies were obtained from the Biological Services Unit at University of Cambridge Department of Clinical Veterinary Medicine (UK).

2.6 Immunohistochemistry (IHC).

Embryos, nerve segments and spinal cord segments were embedded in 30% sucrose solution overnight at room temperature. The next day, excess sucrose solution was removed and the samples embedded in OCT compound in rubber coffin moulds and stored at -80^0C. Sections of various thickness were cut using a cryostat (Reichert-Jung, Germany), collected on Superfrost Plus microscope slides and then air dried for at least 1 hour. For immunolabelling of the sections, different fixatives and blocking solution (BS) were used. For GFAP, nestin, S100β and MBP IHC, the sections were fixed in 4% paraformaldehyde (PF), permeablised with ice-cold methanol, blocked with 0.2% tritonX-100 in ADS (PBS containing 10% calf serum, 0.1% lysine and 0.02 % sodium azide) and primary and secondary antibodies applied in BS. For TuJ1 and BFABP IHC, the sections were fixed in 4% PF for 10 min, blocked with 0.2% tritonX-100 in ADS for 1 hr and primary and secondary antibodies applied in BS. For monoclonal P0 IHC, sections were fixed in ice-cold methanol for 20 min, blocked with 0.1% tritonX-100 in ADS for 1 hr and primary and secondary antibodies applied in BS. For BrDU IHC, sections were fixed in 2M HCL for 10 min, neutralized for 7 min with 0.1M Sodium borate, and primary and secondary antibodies applied in 0.1% tritonX-100 in ADS. For the dilutions of different primary and secondary antibodies and time of incubation, please see Section 2.2. Between each step, the slides were washed 3 times with 1X PBS for 5 minutes each and finally, mounted using Citifluor antifade mountant and sealed with nail varnish. As control, 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.

2.7 Immunocytochemistry (ICC)

For ICC, cells were either unfixed or fixed in different fixatives, and immunolabelling performed in different conditions. For polyclonal TuJ1, Delta 1, Krox-20 and periaxin ICC, cells were fixed for 10 min with 4% PF for 10 min, blocked with 0.2% tritonX-100 in ADS for 1 hr and primary and secondary antibodies applied in blocking solution. For GFAP, nestin and S100β ICC, the sections were fixed in 4% PF, permeablised with ice-cold methanol, blocked with 0.2% tritonX-100 in ADS and primary and secondary antibodies applied in BS. For L1 (ASCS4), laminin α2 and fibronectin ICC, cells were fixed for 5 min with 4% paraformaldehyde, blocked with ADS for 30 min and primary and secondary antibodies applied in BS. For BrDU and P0 ICC, sections were fixed in 2M HCL for 10 min, neutralized for 7 min with 0.1M Sodium borate, and primary and secondary antibodies applied in 0.1% tritonX-100 in ADS. For O4 and L1 (324) double-immunolabelling, primary antibodies were to unfixed cells for 1 hr and then incubated with relevant secondary antibodies, after which they were post-fixed with PF. For p-AKT, active caspase-3 and p-c-jun, cells were fixed with 4% PF for 10 min, blocked with 50% goat serum and 1% BSA in TBS for 1 hr and primary and relevant secondary antibodies applied in BS. For the dilutions of different primary and secondary antibodies and time of incubation, please see Section 2.2. Between each step, the cells were briefly washed with 1X PBS and finally, mounted using Citifluor antifade mountant and sealed with nail varnish. As control, 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.

2.8 Microscopy

2.8.1 Electron Microscopy

The sciatic nerves of mutant mice at different ages were dissected out and placed in freshly prepared 2% glutaraldehdye in 0.1M sodium phosphate buffer pH 7.4, overnight at 4^0C. The next day, the tissue was washed three times for 15 min in 0.1M phosphate buffer, pH 7.4 and then stained with 1% osmium tetroxide for 1 hour and then washed three more times and dehydrated as follows; 25% ethanol (5 min), 50% ethanol (5 min), 70% ethanol (5 min), 90% ethanol (10 min), 100% ethanol (4 X 10 min) followed by propylene oxide (3 X 10 min).

The samples were then embedded in agar resin in two stages; a 3hr incubation in a 1:1 mixture of propylene oxide and resin, followed by two 12 hrs incubations in resin alone, all at room temperature. Resin consists of agar resin (12 g), DDSA (8 g), MNA (5 g) and BDMA (400 ml), stirred well. The tissue samples were then placed, in fresh resin, into a rubber coffin mould and thermo-cured at 65^0C for 12 hours. Semi-thin and ultra-thin sections were cut using an Ultracut E ultramicrotome (Leica, Germany). Semi-thin sections were cut using fresh glass knives and collected on microscope slides and ultra-thin sections were cut with a diamond knife (Diatome, USA) on copper grids (Agar Scientific, UK). Sectioning of semi-thin and ultra-thin sections was done by Mr M. Turmaine (Anatomy department, University College London)

Semi-thin sections were stained with 0.1% toluidine blue in ethanol, rinsed with distilled water, dried and mounted in DPX mountant and pictures taken with a

light microscope (Leica, UK). The ultra-thin sections were stained for 15 min in lead citrate solution, washed in distilled water, dried and then viewed in a Jeol 1010 electron microscope (Jeol, Japan) and images captured on X-ray Film (Ilford, UK). Films were printed using a Deverre enlarger on Ilford multigrade paper using Ilford multigrade gel filters (Ilford, UK).

2.8.2 Fluorescent microscopy

A fluroscent microscope (Nikon Eclipse E800, Nikon, UK) or a confocal microscope (Multi-photon UV, Leica, UK) was routinely used to acquire pictures after immunolabelling.

2.9 Quantification of myelination

Sciatic nerves from wild-type and mutant mice, were dissected out and processed for resin sections. Ultra-thin sections, at the level of the sciatic notch, were cut and viewed in an electron microscope. Pictures were then taken, with a good resolution to see the myelin profile of the individual Schwann cells, in the whole nerve and the number of myelinating Schwann cells per nerve counted. Myelin count was then expressed as the number of myelinated Schwann cells per 10,000 μm^2 of nerve area. Each experiment was based on 2 sets of samples, consisting of at least 2 mutant mice and several wild-type mice. Statistical difference was measured using the *student T-test.*

2.10 Fluorescent-Activated cell sorting (FACS)

FACS was used to separate a population of Schwann cells into myelinating and non-myelinated Schwann cells. 10 P5 rats were culled and the sciatic nerves and

brachial plexus dissected out. A cell suspension was then obtained and the cells purified by negative immunopanning and the fibroblasts removed. The cells were then lived stained with anti-GalC, present only on myelinating Schwann cells, using an anti-mouse FITC as a secondary antibody. The cells were then sorted in a Becton-Dickinson FACSCalibur machine (UK), which, first sorted the live cells from the dead cells and then separated the fluorescent ($GalC^{+ve}$) from the non-fluorescent cells ($GalC^{-ve}$). The two populations were collected and then the mRNA extracted and then processed for RT-PCR. The FACS procedure, after isolation and immunolabelling, was carried out by Ms Lola Martinez (Wolfson Institute of Biomedical Research, University College London, UK).

2.11 Animal transplant experiments

All animal surgeries for transplant experiments were carried out at the University of Cambridge Department of Clinical veterinary medicine. All EB lesions, transplants and dissections of the spinal cords were carried out by Ms Jenny Gilson, according to protocol published elsewhere (Blakemore and Crang, 1985; Hinks *et al.*, 2001). All retinal transplants were carried out by Dr Anna Setzu, according to protocol elsewhere (Setzu *et al.*, 2004).

2.12 Statistical analysis

All data are presented as arithmetic mean ± standard error of mean (SEM). Statistical significance was estimated by the Student's *t* test using Microsoft Excel program (Microsoft UK, UK).

CHAPTER 3: The trunk neural crest and its early glial derivatives: a study of survival responses, developmental schedules and autocrine mechanisms.

3.1 INTRODUCTION

Regulation of cell survival is a key control mechanism in developing organs. Therefore the availability of survival factors, and the competence to respond them are two important features of developing cells and tissues. The laboratory has shown previously that in the Schwann cell lineage, the transition between precursors and Schwann cells is characterized by a change in the way cell survival is regulated. Schwann cells possess autocrine survival circuits, which enable the survival of the Schwann cell population in the absence of axon-derived signals (Meier *et al.*, 1999). Schwann cell precursors on the other hand are unable to generate signals that support their own survival and appear to be entirely dependent on axonal survival signals. Strong evidence indicates that a major component of this signal is neuregulin-1 (Jessen *et al.*, 1994; Dong *et al.*, 1995; Meyer and Birchmeier, 1995). This comparison of precursors and Schwann cells provides a clear example of a developmental change in survival control brought about by changing the source or availability of essential survival factors.

Other observations indicate that the competence of early glial cells to respond to survival factors is also developmentally regulated. This is suggested by the finding that although Schwann cell survival is supported by Schwann cell conditioned medium, and by a minimal combination of NT-3/PDGF-BB/IGF-2 that mimics its effect, precursors show no response to the medium or to the growth factor cocktail despite the presence of the relevant growth factor receptors (Meier *et al.*, 1999).

To examine this issue further, I have asked whether the three main cell types involved in early PNS glial development (neural crest cells, Schwann cell precursors and developing satellite cells) differ significantly in their ability to respond to potential survival factors. For direct comparison, survival assays that allow the measurement of their response to applied factors under identical conditions were established. The competence of these cells to respond to a range of potential survival signals, all of which have previously been implicated in some way in survival control in this system were determined. I have also used these assays to identify autocrine survival mechanisms in developing satellite cells and studied the expression of differentiation markers in crest cells, Schwann cell precursors and satellite cells.

I find a number of differences in survival regulation between the cells. In particular, most of the factors tested fail to support survival of neural crest cells, although most of them support the survival of early glia, namely Schwann cell precursors and developing satellite cells from same age embryos. When taken together with previous comparisons of survival responses between precursors and Schwann cells, it appears that each developmental transition in the PNS glial lineage is characterized by an expansion in the number of factors that can block cell death. This may reflect a progressive reduction in the need for co-operative growth factor signalling to ensure survival. Thus instead of showing increasing selectivity and specificity in survival support as they differentiate, these cells become less stringent at least with respect to the factors tested here. The spectrum of potential survival factors for developing satellite cells, together with the timing of the appearance of autocrine survival signalling and antigenic differentiation markers, all indicate that the development of these cells is significantly ahead of that of Schwann cell precursors. The broad spectrum of survival factors and early autocrine support for

satellite cells provide a plausible explanation for the intriguing observation that in mice in which the glial survival factor neuregulin-1 or its receptors has been inactivated satellite cells persist while Schwann cell precursors are absent (Riethmacher *et al.*, 1997; Morris *et al.*, 1999; Woldeyesus *et al.*, 1999; Garratt *et al.*, 2000).

3.2 RESULTS

3.2.1 Assaying the survival of crest cells, Schwann cell precursors and satellite cells under identical conditions

To compare the survival properties of neural crest cells, Schwann cell precursors and developing satellite cells, under strictly similar conditions, I adapted methods for culturing the three cell types. In particular I devised a new method for the culture of highly pure populations of developing satellite cells in the absence of neurons and adapted our previous method for the preparation of Schwann cell precursors (Materials and Methods). Neural crest cell cultures were established using an adaptation of previously described methods (Smith-Thomas and Fawcett, 1989; Shah et al., 1994).

In this way the three cell types are initially obtained by migration from the parent tissue explant over a period of 24 h. In all cases, the explant is then removed, the cells enzymatically detached from the coverslips, replated on the substrate of choice and survival quantified over a period of 1 or more days as described elsewhere (Jessen *et al.*, 1994; Dong *et al.*, 1995; Parkinson *et al.*, 2001). The competence of the cells to respond to potential survival signals can be assessed by adding factors at the replating stage, and autocrine mechanisms can be examined by plating cells at different densities or in cell-conditioned medium. I have designated the developmental age of cells tested in survival assays as the embryonic age at the time of dissection +1 (see Section 3.2.4 below). Thus satellite cells, detached from coverslips 24 h after an E13 DRG was explanted and used for survival tests, are referred to as E13+1 cells.

To test the purity of these cultures, E14 +1 satellite cell and Schwann cell precursor replates were immunolabelled with antibodies to L1 and TuJ-1 to label neuronal cells (L1 positive/TuJ-1 positive) and glial cells (L1 positive only). A few

TuJ-1+ cells were seen (1.1 ± 0.9%, n=4) in both precursor and satellite cell replates. In four independent experiments an average of 95.2 ± 0.6% of the cells were L1 positive indicating that the majority of the cells were glia rather than fibroblasts. The L1 negative cells were confirmed as fibroblastic by their morphology and double-immunolabelling with L1 and Thy1.1 (Dong *et al.*, 1995). Neural Crest cells were immunolabelled with antibodies to L1 and Thy1.1, and an average of 97.6 ± 2.3% (n=4) were L1 positive with only a small percentage of Thy1.1 positive cells present (2.5 ± 1.7%, n=4).

3.2.2 The transition from crest cells involves a marked increase in responsiveness to potential survival factors.

To determine whether the competence to respond to potential survival signals was developmentally regulated at the transition between undifferentiated crest cells and Schwann cell precursors, cell survival was tested using a number of factors that have previously been implicated in survival regulation in crest cells and developing Schwann cells. Similar experiments were carried out on developing satellite cells to examine whether their survival requirements differed from those of Schwann cell precursors in same age embryos. All these experiments were carried out over a period of 20 h using DM (without insulin or IGF), unless otherwise stated. Although experiments of this kind can reveal developmentally regulated differences between different cell types, and show the competence of a cell to respond to a factor under the present conditions, it is clear that survival responses are context-dependent. Therefore other results with particular factors may be obtained using different media and culture conditions.

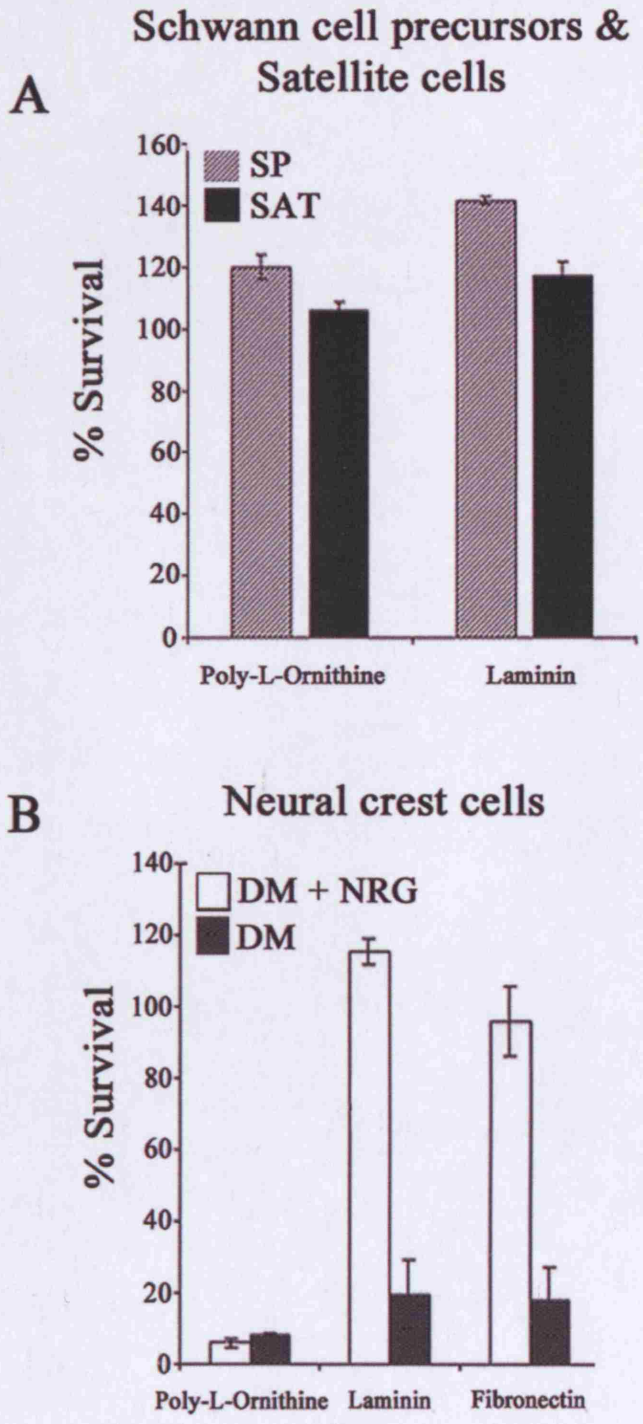
3.2.2.1 Crest cells need contact with extracellular matrix to respond to neuregulin-1 but early glia do not.

Freshly isolated Schwann cell precursors survive in the presence of neuregulin-1 when plated on either poly-L-lysine or laminin (Jessen *et al.*, 1994). Neuregulin-1supports neural crest cell survival in neural crest cells cultured on fibronectin (Bannerman *et al.*, 2000), although other experiments suggest that in a richer culture medium it does not enhance survival (Shah et al., 1994). I therefore tested whether neuregulin-1 would support neural crest cell survival in this replate assay. In the presence of neuregulin-1 (20 ng/ml), E14+1 satellite cells and Schwann cell precursors both survived equally well on laminin and poly-L-ornithine (Fig. 3.1A). In contrast, replate cultures of neural crest cells cultured on fibronectin, laminin or poly-L-ornithine showed a different pattern (Fig. 3.1B). Neuregulin-1 completely rescued neural crest cells plated on laminin or fibronectin substrate, but failed to support survival on poly-L-ornithine. These results represent a novel *in vitro* difference between rat Schwann cell precursors and developing satellite cells on the one hand, and neural crest cells on the other.

Figure 3.1: In crest cells, but not glia, the survival response to neuregulin-1 is extracellular matrix dependent.

A – In Defined Medium supplemented with neuregulin-1 (20 ng/ml) E14+1 Schwann cell precursors and satellite cells survive equally well on poly-L-ornithine. *B* – In the same medium, neural crest cells die unless they are plated on laminin or fibronectin. Survival above 100% is due to cell division (not shown). DM, Defined Medium; NRG, Neuregulin-1.

Figure 3.1


A
Schwann cell precursors &
Satellite cells
% Survival
160
140
120
100
80
60
40
20
0
SP
SAT
Poly-L-Ornithine
Laminin
B
Neural crest cells
% Survival
140
120
100
80
60
40
20
0
DM + NRG
DM
Poly-L-Ornithine
Laminin
Fibronectin

3.2.2.2 FGF-2 plus IGF-1 rescue early glia but not neural crest cells

The result above suggested that crest cells might have more stringent survival requirements than cells that have already entered the glial lineage. To explore this further I first tested FGF-2 and IGF-1. FGF-2 promotes crest cell survival in the presence of the neural tube in the rat (Bannerman and Pleasure, 1993) and supports crest cell survival in the chick (Kalcheim, 1989). FGF-2 plus IGF-1, but neither factor alone, supports the survival of rat Schwann cell precursors in a 20 h assay although survival is poorer at longer time points (Gavrilovic *et al.*, 1995). We found that on a laminin substrate neither FGF-2 (3 ng/ml) nor IGF-1 (100 ng/ml) applied separately supported crest cells or E14+1 Schwann cell precursor survival. These cells differed, however, in their response to FGF-2 (3 ng/ml) plus IGF-1 (100 ng/ml). This combination rescued Schwann cell precursors as reported previously but did not support crest cell survival either on laminin or fibronectin (Fig. 3.2A and not shown). This combination also supported the survival of E14+1 satellite cells (not shown).

It seems likely that the survival response of rat crest cells to FGF-2 plus IGF-1 in the presence of the neural tube seen in other studies on rat crest (Bannerman and Pleasure, 1993) reflects a need for co-operation with tube-derived factors. This is not required by Schwann cell precursors or satellite cells, supporting the notion that the survival requirements of crest cells are more demanding than those of early glia.

3.2.2.3 Endothelin-3 rescues early glia but not neural crest cells

Endothelins are required for the survival and differentiation of other crest cell lineages and a role in enteric neural crest stem cell migration/survival has recently been demonstrated (Baynash *et al.*, 1994; Hosoda *et al.*, 1994; Kurihara *et al.*, 1994; Clouthier *et al.*, 1998; Shin *et al.*, 1999; Sidebotham *et al.*, 2002). Endothelins also promote the survival and differentiation of Schwann cell precursors (Brennan *et al.*,

2000). Using laminin coated coverslips I compared the survival responses of crest cells and E14+1 Schwann cell precursors and satellite cells to endothelin-3 (10 ng/ml). Endothelin-3 alone rescued a significant proportion of E14+1 Schwann cell precursors and satellite cells from death as expected, an effect that was potentiated by IGF-1 (100 ng/ml). However, neither endothelin-3 nor endothelin-3 plus IGF-1 rescued crest cells (Fig. 3.2B).

3.2.2.4 NT-3 and PDGF-BB do not support the survival of neural crest cells, partially support Schwann cell precursors but rescue developing satellite cells more effectively

NT-3 and PDGF-BB have, together with IGF-2, been identified as components of autocrine survival signals for Schwann cells. When applied together they rescue Schwann cells at very low concentrations, even under very parsimonious conditions (Meier *et al.*, 1999). When applied singly they provide a significant survival support for Schwann cells as long as relatively high concentrations are used (5-10 ng/ml) (Meier *et al.*, 1999). At these higher concentrations NT-3 and PDGF-BB, singly and in combination, also partially rescue Schwann cell precursors (Lobsiger *et al.*, 2000). NT-3 has also been implicated in proliferation and differentiation of avian crest cells (Kalcheim *et al.*, 1992; Pinco *et al.*, 1993; Henion *et al.*, 1995).

I therefore tested the effects of NT-3 (5 ng/ml) and PDGF-BB (10 ng/ml) applied either singly or together. In all cases, the DM in these experiments contained 10^{-6} M insulin, a high concentration sufficient to activate type 1 IGF receptors. Crest cells showed no survival response to these factors, applied alone or together (Fig. 3.2C). At these relatively high concentrations, NT-3 or PDGF-BB alone supported some survival of Schwann cell precursors and in combination they appeared to have

additive effects, as reported previously (Lobsiger *et al.*, 2000). The response of satellite cells to NT-3 alone and to PDGF-BB alone was significantly greater ($P < 0.01$) and comparable to that shown by neonatal Schwann cells (Meier *et al.*, 1999).

3.2.3 IGF rescues developing satellite cells but not Schwann cell precursors

The experiments above raised the possibility that developing satellite cells might in general acquire mature survival responses ahead of developing Schwann cell precursors in the same age embryo. To test this, I examined the response to type 1 IGF receptor activation, and to FGF-2 alone. IGF-1 or -2 or 10^{-6} M insulin have no effect on the survival of Schwann cell precursors when applied alone in DM (Gavrilovic *et al.*, 1995), although they are potent survival signals for immature Schwann cells from E18 onwards (E16 in the mouse) (Campana *et al.*, 1999; Delaney *et al.*, 1999; Dong *et al.*, 1999; Meier *et al.*, 1999; Syroid *et al.*, 1999; Cheng *et al.*, 2000). Furthermore, the survival response to IGFs or high insulin is the earliest known change in phenotype in the transition between precursors and Schwann cells, appearing before other Schwann cell features such as high levels of cytoplasmic S100β expression (Dong *et al.*, 1999).

Using DM containing high insulin (10^{-6} M) and a laminin substrate I found a striking difference between satellite cells and Schwann cell precursors at E14+1. The precursors died as expected, while the satellite cells survived and proliferated, a behaviour not shown by cells in the Schwann cell lineage until about 2 days later in development (Fig. 3.3A). This response was density independent (Fig. 3.5B), independent of laminin substrate, and present even in SM (Fig. 3.3C). It was elicited under a number of conditions that stimulate type 1 IGF-1 receptors while low insulin (10^{-9} M) produced only a weak effect, suggesting that the survival effect is likely to be due to type 1 IGF receptor stimulation (Fig. 3.3C).

Figure 3.2: A number of signals rescues Schwann cell precursors and satellite cells from same age embryos, but has no effect on crest cell survival.

A – In combination, FGF-2 (3 ng/ml) and IGF-1 (100 ng/ml) rescue E14+1 Schwann cell precursors but not crest cells. DM, laminin substrate. *B* – ET-3 (10 ng/ml) has no effect on crest survival but rescues a significant proportion of Schwann cell precursors, an effect that is potentiated by IGF-1 (100 ng/ml). DM, laminin substrate. *C* – NT-3 (5 ng/ml) and PDGF-BB (10 ng/ml) alone support some E14+1 Schwann cell precursors but rescue a significantly ($P < 0.01$) larger number of satellite cells from the same age embryos. These factors, alone or together, fail to rescue crest cells in this assay. DM containing 10^{-6} M insulin, laminin substrate. NCC, Neural Crest Cells; SP, Schwann cell precursors; SAT, Satellite cells.

Figure 3.3: IGF-1 or 10^{-6} M insulin rescue developing satellite cells but not Schwann cell precursors or neural crest cells.

In *A* and *B*, the assays are on laminin substrate and in DM containing 10^{-6} M insulin using E14+1 cells. Most of the precursors die but the satellite cells survive and proliferate, their number at 20 h exceeding that initially plated. *A* – 10,000 cells/coverslip. *B* – 125 cells/coverslip. *C* – The assays are on poly-L-ornithine substrate using E14+1 cells in SM. Low (10^{-9} M) or high (10^{-6} M) insulin or IGF-1 (100 ng/ml) are added as indicated. Note that treatments that activate type 1 IGF receptors selectively rescue developing satellite cells. NCC, Neural Crest Cells; SP, Schwann cell precursors; SAT, Satellite cells

Figure 3.2

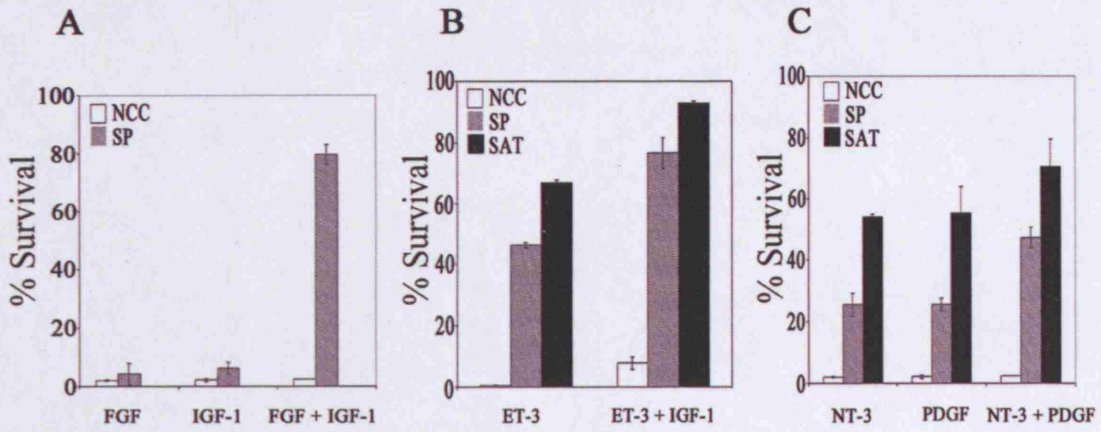
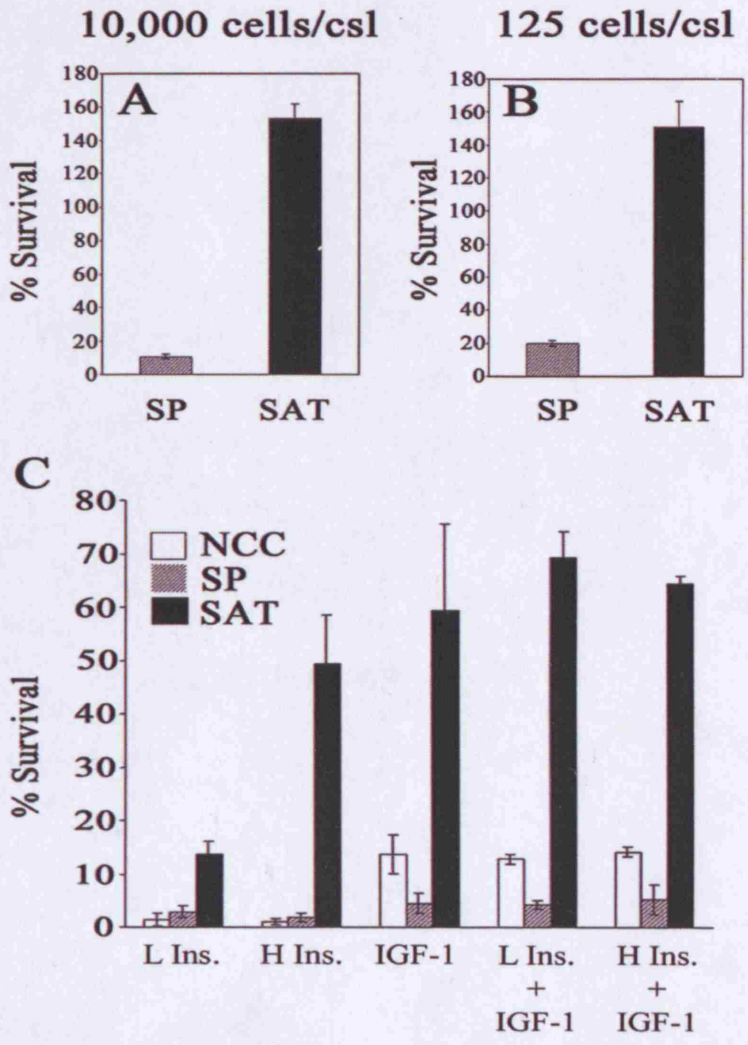


Figure 3.3



To confirm that IGF was rescuing cells from death, I counted the number of apoptotic dying cells visualized by TUNEL and caspase-3 immunolabelling under the conditions used in Fig. 3C (Parkinson *et al.*, 2001). The number of apoptotic cells was significantly reduced in media that activate type 1 IGF receptors, whereas in medium containing only 10^{-9} M insulin, a minimal reduction in cell death occurred compared to that seen in SM without insulin (Fig. 3.4A).

Using the same conditions, the survival effects of IGF-1 on developing satellite cells were dose-dependent with highest rescue at 100 ng/ml IGF-1 (Fig. 3.4B). These assays were independently confirmed by a death assay using active caspase-3 labelling of sister cultures, again showing that 100 ng/ml IGF-1 most effectively decreased the number of dead cells (Fig. 3.4B).

Lastly, I examined some of the molecular mechanisms underlying the survival effect of IGF-1 on developing satellite cells. IGF-1 can rescue Schwann cells from caspase-mediated apoptosis induced by survival factor withdrawal, via activation of the phosphatidylinositol-3 kinase (PI-3K) pathway and Akt, a downstream effector of PI-3K (Campana *et al.*, 1999; Delaney *et al.*, 1999; Cheng *et al.*, 2000). To test whether the survival response was mediated via activation of the Akt pathway, I plated E14+1 satellite cells at a density of 125 cells/20 µl drop on poly-L-ornithine and cultured them in SM for 6 h. At this time the medium was changed to SM, plus or minus 100 ng/ml IGF-1, and the cells were cultured for a further 1.5 h. The cells were then fixed and stained for the Serine-473 phosphorylated form of Akt. Clear cytoplasmic immunolabelling for phospho-Akt was seen in the cultures containing IGF-1, whereas no labelling was seen in cells grown in SM alone (Fig. 3.5A).

IGF-1 rescue of Schwann cells from apoptosis also involves the inhibition of c-jun N-terminal kinase (JNK) activation (Cheng *et al.*, 2001). Conversely, TGFβ induced cell death in neonatal Schwann cells involves phosphorylation of c-jun and activation of the JNK/c-jun pathway (Parkinson *et al.*, 2001). To test whether survival factor withdrawal in satellite cells would induce phosphorylation of c-jun and whether IGF-1 could affect this process, E14+1 satellite cells were plated at a density of 125 cells/20 µl drop on poly-L-ornithine and cultured in either SM or SM containing IGF-1 (100 ng/ml) for 3 h. The cells were labelled with antibodies to Serine-63 phospho-c-jun. In the presence of IGF-1, the number of positive cells was sharply reduced (Fig. 3.5B).

I also examined the response to FGF-2, since FGF-2 alone in DM does not rescue Schwann cell precursors (above) although it rescues Schwann cells. Exposure of E14+1 satellite cells to FGF-2 (3 ng/ml) alone, under conditions identical to those in Fig. 3.2A, rescued 75-80% of the cells survived while the survival of Schwann cell precursors was less than 5% in parallel experiments (not shown).

Together, these experiments indicate that in E14+1 satellite cells, as in neonatal Schwann cells, IGF-1 supports survival via activation of Akt and suppression of the JNK/c-jun pathway. In responding to IGF-1 alone and FGF-2 alone, satellite cells differ from Schwann cell precursors from the same age embryos, and show survival responses that in Schwann cell development appear at the precursor/Schwann cell transition.

Figure 3.4: IGF-1 or 10^{-6} M insulin block apoptosis in developing satellite cells. The assays are on poly-L-ornithine in SM using E14+1 satellite cells.

A – Low (10^{-9} M) or high (10^{-6} M) insulin or IGF-1 (100 ng/ml) is added as indicated. Note that signals that activate type-I IGF receptors reduce the number of cells with caspase positive, or TUNEL positive nuclei. The % decrease in caspase positive and TUNEL positive nuclei in low insulin is significantly different from each of the other treatments at the $P < 0.05$ level. *B* – IGF-1 is added at the concentrations indicated. Note that the effects of IGF-1 are dose-dependent and that maximal effects, both in terms of cell numbers and caspase positive nuclei, are seen in the same concentration.

Figure 3.5: IGF-1 inhibits apoptotic death in E14+1 satellite cells.

A – Phospho-Akt immunolabelling is seen in SM supplemented with IGF-1 (100 ng/ml) but not in SM alone (Hoechst staining show nuclei in blue). *B* – In SM alone most E14+1 satellite cells (also labelled with Hoechst nuclear stain) show phospho-c-jun immunoreactivity, but addition of IGF-1 (100 ng/ml) abolishes this in most cells.

Figure 3.4

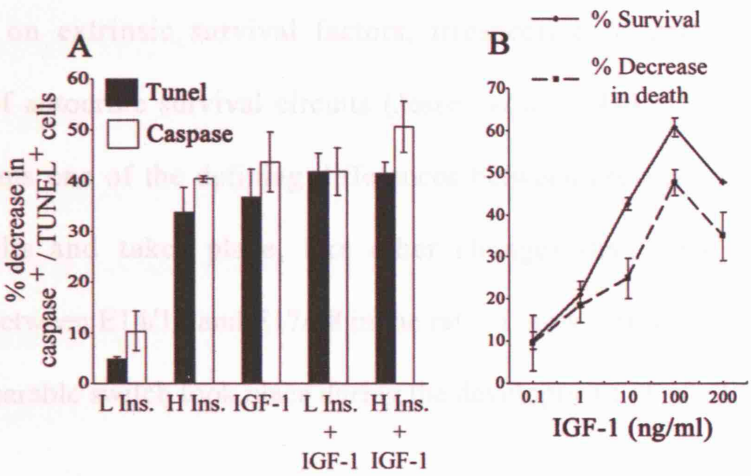
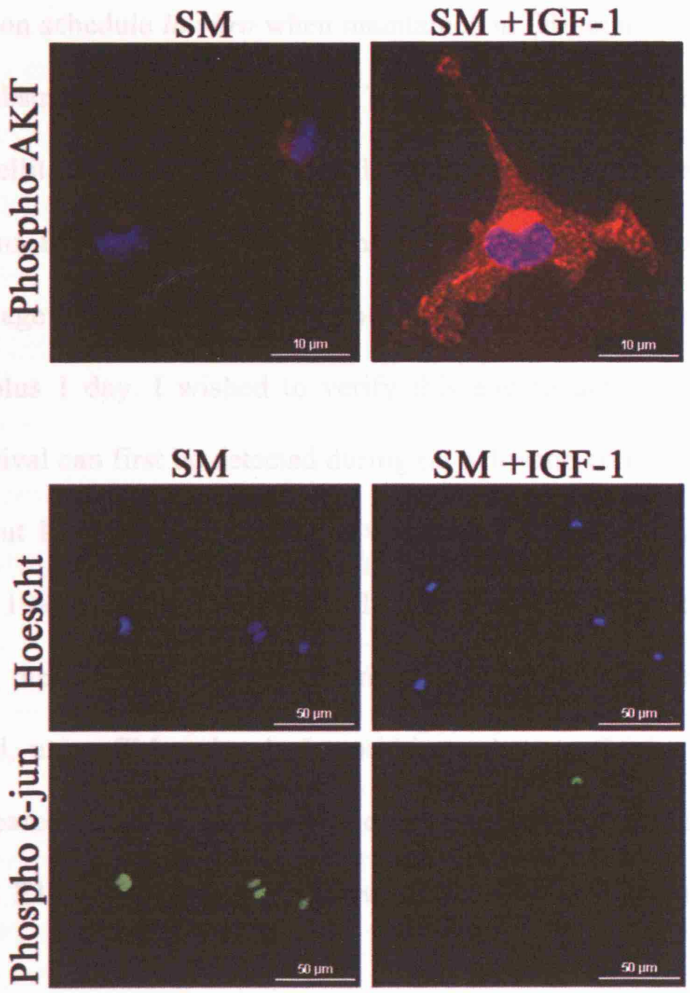


Figure 3.5



3.2.4. Developing satellite cells show autocrine survival early in development

Early Schwann cell development involves an unambiguous switch from the dependence on extrinsic survival factors, irrespective of cell density, to the expression of autocrine survival circuits (Jessen *et al.*, 1994; Meier *et al.*, 1999). This represents one of the defining differences between precursors and immature Schwann cells and takes place, like other changes that mark Schwann cell generation, between E14/15 and E17/18 in the rat. I now determined whether and when a comparable switch took place during the development of satellite cells.

3.2.4.1 A validation of the survival assays described above for studying the timing of glial development

This laboratory showed previously that Schwann cell precursors mature approximately on schedule *in vitro* when maintained in DM containing neuregulin-1 on a laminin substrate (Dong *et al.*, 1995). The methods described here for obtaining developing satellite cells and Schwann cell precursors involve growing cells under these conditions for 1 day prior to testing. This can be expected to make the developmental age of the cells, at the time of testing, equivalent to the embryonic age of the tissue plus 1 day. I wished to verify this and to determine when density dependent survival can first be detected during rat Schwann cell development, using medium without IGF or high insulin, as we have previously done in the mouse (Dong *et al.*, 1999). The ability of cells to survive at high density (10,000 cells/coverslip; therefore exposed to autocrine factors) was measured using a 1 day survival period, using SM and poly-L-ornithine substrate (Meier *et al.*, 1999). I therefore compared the survival of cells acutely dissociated and plated from E14, E15, E16 and E18 nerves, with the survival of cells replated at the expected

equivalent age, namely cells from 1 day outgrowth from E13 (+1=E14), E14 (+1=E15), E15 (+1=E16) and E17 (+1=E18) nerves.

The survival characteristics of the replate cultures were the same as those of cells acutely derived from embryos that are 1 day older (Fig. 3.6A). In accordance with previous reports, Schwann cell precursors from nerves at E14 did not have autocrine survival mechanisms and this was true for both the acute and equivalent replate preparations (E13+1). Cells from E16 and replated E15+1 cells also died, in agreement with previous observations on equivalent cells from mouse nerves tested, as here, without IGFs or high insulin (Dong *et al.*, 1999). The autocrine survival mechanisms were present only in the E18 acute Schwann cell cultures and the E17+1 replate ones.

3.2.4.2. Density-dependent survival appears early in satellite cell development

These experiments were repeated using replated satellite cells (Fig. 3.6B). Density dependent survival could be demonstrated from E15 (E14+1) onwards. The survival of E14+1 cells was above 30% (Fig. 3.6B), while the survival of these cells was less than 10% when they were plated sparsely to avoid the effects of autocrine support (not shown).

If the density-dependent survival seen in these experiments was due to autocrine signalling then medium conditioned by dense satellite cell cultures should rescue sparsely plated satellite cells (Meier *et al.*, 1999). To test this, conditioned medium from densely plated E15+1 satellite cells was added to E15+1 satellite cells plated sparsely (125 cells/coverslip) to see whether it would enhance survival. Conditioned medium was also prepared from immunopanned Schwann cells from 7 day old rats for comparison since this medium supports the survival of sparsely plated Schwann cells (Meier *et al.*, 1999). Both DRG glia and Schwann cell

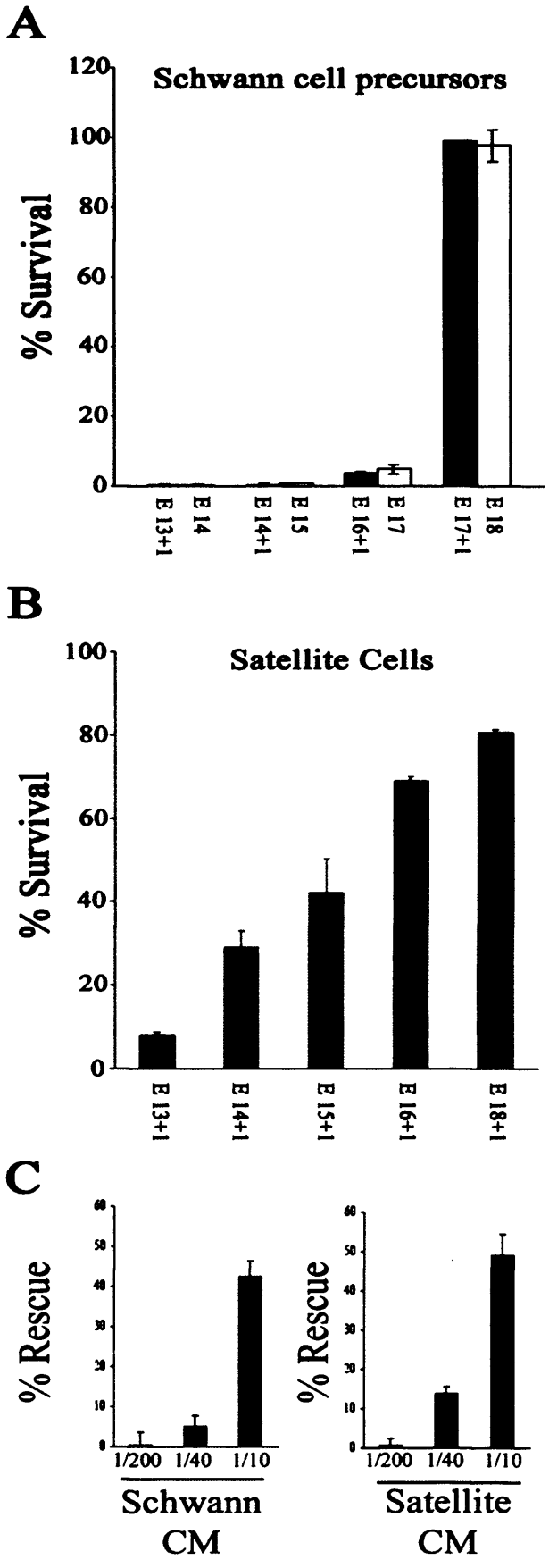
conditioned media rescued a large proportion of cells that would have died if grown only in SM (Fig. 3.6C,D). They acted in a dose-dependent manner with a ten-fold dilution being most effective in promoting survival as seen previously for Schwann cells (Meier *et al.*, 1999). Although I have not tried to establish the molecular identity of the survival activity in the satellite cell conditioned medium, it is likely to be similar to that of Schwann cell conditioned medium. This is suggested by the close relationship between the two systems, by the observation that Schwann and satellite cell conditioned medium rescued developing satellite cells with comparable effectiveness (Fig. 3.6C) and by the fact that Schwann cell conditioned medium contains IGF-2, NT-3 and PDGF-BB, factors that promote the survival of early developing satellite cells (above).

These experiments are consistent with the idea that during development most satellite cells within DRGs acquire the ability to survive in a density dependent way. This change in survival regulation parallels that shown previously for developing Schwann cells as they transit from Schwann cell precursors to immature Schwann cells. The switch can be detected in satellite cells about 2 days before a comparable change is seen in developing Schwann cells. This suggests that in the development of DRG satellite glia, autocrine survival circuits arise significantly earlier than they do in Schwann cell development.

Figure 3.6: Autocrine survival circuits in developing satellite cells.

The assays are on poly-L-ornithine substrate in SM using replated or acutely dissociated cells at the embryonic ages indicated. *A* – The appearance of density dependent survival during rat Schwann cell development using SM (without insulin or IGF) and 10,000 cells/coverslip. The cells were acutely dissociated (E14, etc) or replated at the equivalent age (E13+1, etc). Density dependent survival appears during E17. *B* – In satellite cells, density dependent survival appears early. The cells were replated in SM at the ages indicated using 10,000 cells/coverslip. *C* – Conditioned medium (CM) from dense cultures of satellite cells or Schwann cells rescues E14+1 satellite cells in sparse cultures (125 cells/coverslip). Maximum rescue is seen at a dilution of 1:10.

Figure 3.6


A
Schwann cell precursors
% Survival
120
100
80
60
40
20
0
E 13+1
E 14
E 14+1
E 15
E 16+1
E 17
E 17+1
E 18
B
Satellite Cells
% Survival
100
80
60
40
20
0
E 13+1
E 14+1
E 15+1
E 16+1
E 18+1
C
% Rescue
1/200
1/40
1/10
Schwann CM
% Rescue
1/200
1/40
1/10
Satellite CM

3.2.5. Early appearance of differentiation markers supports the notion that glia within DRGs mature ahead of glia within peripheral nerve trunks

I now examined the expression of glial differentiation markers to see if they appeared earlier in satellite cells than in developing Schwann cells. Expression of laminin α2 and fibronectin was compared in replated neural crest cells, and E13+1 Schwann cell precursors and satellite cells. Laminin α2 was present on all three cell types and Schwann cells (not shown), whereas fibronectin was expressed only by satellite cells (Fig. 3.7A) and Schwann cells (not shown) but not by neural crest cells or Schwann cell precursors.

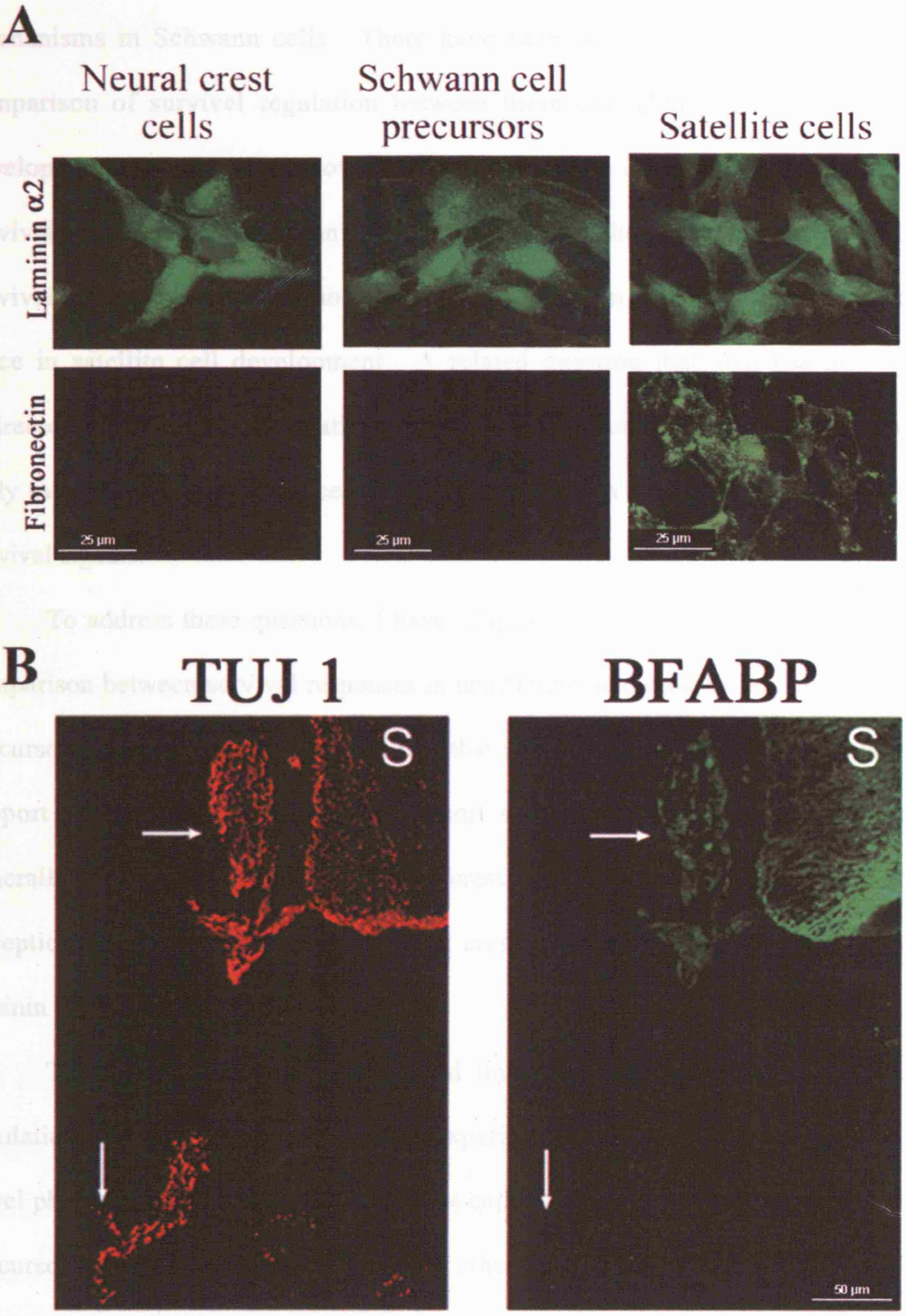
Since the calcium-binding protein S100β marks the transition between precursors and Schwann cells, I investigated its presence in satellite cells. Freshly dissociated cells from DRGs from E14-E16 embryos were immunolabelled with S100β antibodies 3 h after plating on laminin-coated coverslips. In cultures from E14 ganglia S100β was already present in about 28% of flat, non-neuronal cells on the coverslips, whereas less than 4% of Schwann cell precursors are S100β positive at this stage (Jessen *et al.*, 1994; Dong *et al.*, 1995). In cultures from E15 and E16 DRGs, this figure rose to 40% and 53%, respectively.

Brain fatty acid binding protein (BFABP) is an early glial differentiation marker in the mouse. It is present in DRG glia and Schwann cell precursors by E11.5 but absent from both DRG and nerve glia at lumbar levels at E10.5 (Kurtz *et al.*, 1994; Britsch *et al.*, 2001). To examine the early appearance of BFABP I immunolabelled sections from thoracic levels of E10.5 mouse embryos. I found numerous $BFABP^{+ve}$ cells in DRGs while glia in the distal nerves projecting towards the limbs containing Schwann cell precursors were $BFABP^{-ve}$ (Fig. 3.7B). In sum, three of the four differentiation markers tested here appear earlier in developing satellite cells than they do in Schwann cell development within limb nerves.

Figure 3.7: The appearance of differentiation markers in early glia.

A – Immunolabelling of the cell types indicated with antibodies to fibronectin and α2 laminin. Note that E13+1 satellite cells have already acquired fibronectin immunoreactivity while Schwann cell precursors from same age embryos are negative. Fibronectin immunoreactivity appears at later stages of Schwann cell development (not shown). *B* – Immunolabelling of sections of mouse embryos at the thoracic level with antibodies against BFABP (to identify early glia) and against the neuronal marker TuJ-1 (to identify nerves and ganglia). Note that satellite cells in DRGs are BFABP positive (horizontal arrow), while cells in distal nerves (vertical arrow) are still negative. S: spinal cord

Figure 3.7


A
Neural crest cells
Schwann cell precursors
Satellite cells
Laminin α2
Fibronectin
25 µm
25 µm
25 µm
B
TUJ 1
BFABP
S
S
50 µm

3.3 DISCUSSION

Little has been known about signals that might determine the survival of satellite glia. This contrasts with our relatively extensive information about survival mechanisms in Schwann cells. There have been no studies that allow a clear comparison of survival regulation between these two glial populations during development and it is not known whether the major developmental change in survival control in the Schwann cell lineage, namely, the appearance of autocrine survival circuits at the Schwann cell precursor/Schwann cell transition, also takes place in satellite cell development. A related question that also has not been addressed is whether the formation of early glia, i.e. Schwann cell precursors and early satellite glia, from crest cells involves a change in competence to respond to survival signals.

To address these questions, I have adapted survival assays to allow a direct comparison between survival responses in undifferentiated crest cells, Schwann cell precursors and early satellite cells. I find that while the majority of the signals that support Schwann cell survival also support survival of early glia, these signals generally do not support the survival of crest cells in identical assays. The sole exception is neuregulin-1, which rescues crest cells provided they are plated on laminin or fibronectin.

This shows that entry to the glial lineage is an important point for the regulation of survival responses. These experiments therefore define a number of novel phenotypic differences between crest cells on the one hand and Schwann cell precursors and early satellite cells on the other, and provide additional criteria by which crest cells and early glia can be distinguished experimentally.

Together with previous studies, the present observations show that each of the three main stages in embryonic Schwann cell development is characterized by a distinct and expanding profile of signals that block death. It is likely that the increase in the number of single survival factors at each developmental stage reflects a progressive reduction in the need for co-operative signalling to ensure survival, since some of the factors that fail to block death in these experiments do so using the same cells under more complex conditions.

The matrix-dependence of the neuregulin-1 response in crest cells represents another difference between these cells and early glia. Integrins that interact with fibronectin or laminin may be required for successful survival signalling by neuregulin-1 in rat neural crest cells. In oligodendrocyte progenitor cells α6 integrins, probably interacting with laminin α2, potentiate neuregulin-1 signalling through the ERK signalling pathway and promote survival (Colognato *et al.*, 2002), and a similar mechanism may be operating here. Previous studies on the neural crest have suggested important roles for extracellular matrix molecules and integrin receptors, principally in motility, migration and adhesion (reviewed in Perris and Perrisinotto, 2000; Lallier *et al.*, 1992; Previtali *et al.*, 2001). At least eight different integrin combinations have been reported to be expressed by avian and murine neural crest cells (Previtali *et al.*, 2001). Experiments from α4 integrin null mice suggest that this integrin, which binds to fibronectin, promotes survival of early glial progenitor/late migrating neural crest cells, and a role for α4β1 integrin in avian neural crest cell survival *in vitro* has recently been shown (Haack and Hynes, 2001; Testaz *et al.*, 2001).

I find no evidence for autocrine survival loops in crest cells, and confirm their absence in Schwann cell precursors. The earliest satellite cells I have tested, at

E13+1, also do not show significant density dependent survival. One day later, however, satellite cells already show significant survival in dense cultures, which is not seen in sparse cultures. Autocrine survival circuits can therefore be detected in developing satellite cells 2-3 days earlier than in cells in the Schwann cell lineage. This, together with the response of satellite cells to growth factors, in particular IGF-1 and FGF-2, and the early expression of differentiation markers, all argue that satellite cells develop ahead of cells in the Schwann cell lineage.

Two suggestions can be made as to why this might be. Firstly, the rate of progression from precursors to Schwann cells involves a balance between endothelin, which acts as a brake, and neuregulin-1, which promotes the transition (Brennan *et al.*, 1999). By analogy, it is possible that the neuronal soma may secrete or express factors not present in axons that accelerate the rate at which DRG glia mature. Secondly, isoform II, a soluble form of neuregulin-1, and neuregulin-2 are produced by DRG neurones and the spinal cord (Garratt *et al.*, 2000). Satellite cells, being close to these sources, may therefore be exposed to higher of neuregulin than Schwann cell precursors, that are likely to be chiefly regulated by the membrane associated isoform III (Wolpowitz *et al.*, 2000). Since neuregulin accelerates the precursor/Schwann cell transition (Brennan *et al.*, 2000) in addition to promoting survival and proliferation, this may result in advanced maturation of satellite cells.

Schwann and satellite cells are clearly closely related. Both cell types express S100β, BFABP, P75 NTR, L1, NCAM, and GFAP and basal levels of P_0 mRNA (although, as with Schwann cells, not all satellite cells express all of these markers at all times) (Jessen *et al.*, 1984; Mirsky *et al.*, 1986; Lamperth *et al.*, 1989; Britsch *et al.*, 2001; unpublished observations). In acoustic and vestibular ganglia, satellite cells commonly form non-compacted myelin-like sheaths around nerve cell bodies,

indicating an ability to adopt a differentiated Schwann cell phenotype (Pannese, 1981, 1994). *In vitro* observations also suggest that satellite cells can convert to Schwann cells, since the most plausible origin of the Schwann cells that ensheath axons radiating from ganglion explants is the satellite cells within the ganglia, although this has not been tested rigorously. Nevertheless differences exist. In avians, Schwann cells in developing nerve trunks *in vivo* express Schwann cell myelin protein (SMP) while satellite cells in DRG only do so when they are removed from the DRG microenvironment (Cameron-Curry *et al.*, 1993) while mouse and rat satellite cells express the Ets transcription factor Erm, which is also expressed by neural crest cells and peripheral neurons but not by Schwann cell precursors nor Schwann cells (Hagedorn *et al.*, 2000; Paratore *et al.*, 2002).

Although I find that neuregulin-1 has the potential to support crest cell survival, provided the cells are attached to extracellular matrix, neuregulin-1 is unlikely to control crest cell survival *in vivo* since crest cells are present in neuregulin mutants, and no increase in the death of these cells has been noted (Britsch *et al.*, 1998). Interestingly, in these mutants satellite cells are present and normal, but Schwann cell precursors are essentially absent, indicating that cells in the PNS glial lineage can be generated from crest cells without neuregulin-1 (Meyer and Birchmeier, 1995; Garratt *et al.*, 2000). Nevertheless, it seems surprising that cells that are as similar and closely related as developing satellite cells and Schwann cell precursors should be quite differently affected by the absence of neuregulin-1 signalling. The absence of Schwann cell precursors in mice lacking neuregulin-1 is likely to be due to the acute dependence of these cells on axonal- neuregulin-1 (Jessen and Mirsky, 1998; Mirsky and Jessen, 1999, 2001). The present observations may explain the persistence of satellite cells. We find that even the earliest satellite

cells (E13+1, equivalent to E12 in mouse) show a strong survival response to IGF-1 or high (10^{-6} M) insulin, not seen in Schwann cell precursors. Furthermore, E14+1 satellite cells are effectively rescued by NT-3 and PDGF-BB at concentrations that have little effect on Schwann cell precursor survival and unlike precursors, also respond to FGF-2 alone. These factors are widely expressed in embryos including the tissues near condensing DRG and are likely to be available to satellite cells (Hebert *et al.*, 1990; Kalcheim and Neufeld, 1990; Maisonpierre *et al.*, 1990; Bondy *et al.*, 1992; Pinco *et al.*, 1993). Therefore we suggest that in the absence of neuregulin-1, Schwann cell precursors die since they are not able to take effective advantage of other environmental signals such as IGFs, FGFs, NT-3 and probably other factors, while satellite cells survive due to their potent survival response to these signals, helped by the early appearance of autocrine survival signalling.

CHAPTER 4: The role of the Notch signalling pathway in Schwann cell development and myelination.

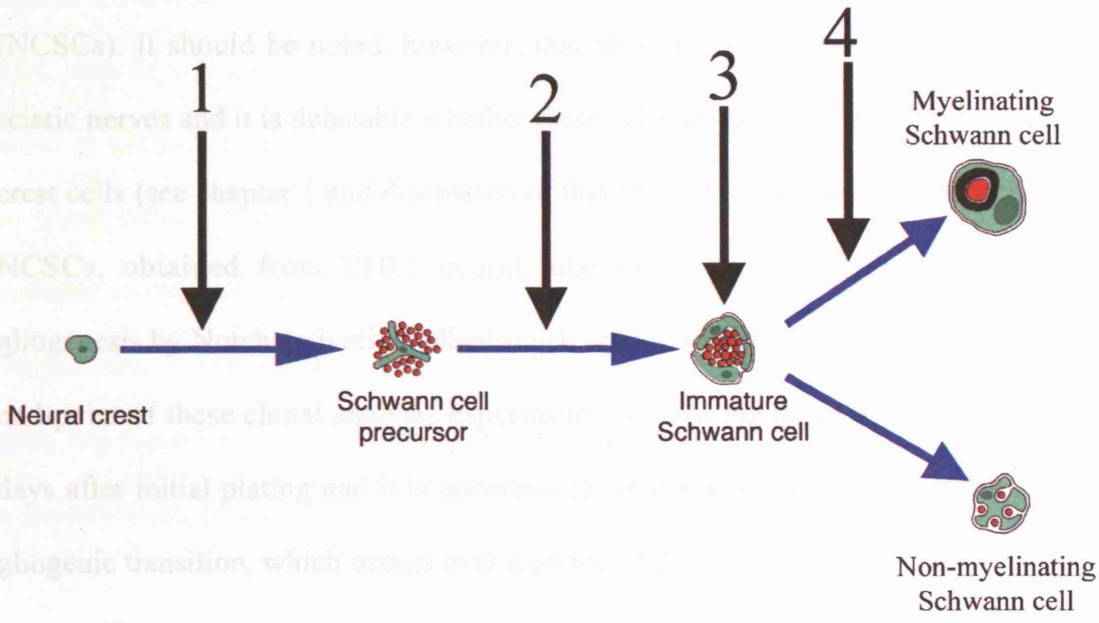
4.1 INTRODUCTION

The Schwann cell lineage has been well characterised and the antigenic profile and signalling pathways involved in the differentiation of the cells involved is discussed more in detail in Chapter 1. Briefly, neural crest cells undergo gliogenesis and form Schwann cell precursors, present in sciatic nerves at E14/15 in the rat. These cells then differentiate to give rise to immature Schwann cells, present at E17/18, which then further differentiate to either myelinating or non-myelinating Schwann cells present in adult nerves.

Notch signalling is involved in the patterning of the CNS, although some of its roles remain controversial. It has been shown to instruct gliogenesis, at least for Muller glia, radial glia and astrocytes, and maintain the pool of progenitor cells. It has also been shown to inhibit myelination by oligodendrocytes. In the PNS, however, its role in promoting gliogenesis from the neural crest is still controversial. Also, no study has been done to investigate the potential role of Notch signalling in the Schwann cell lineage. Given the numerous parallels that exist between glia from the CNS and PNS, I wanted to study the roles of Notch signalling at each of the stages of the Schwann cell lineage using well-characterised *in vitro* and *in vivo* models.

I first looked at the process of gliogenesis (arrow 1, figure below), and examined the effect of Notch signalling on glial differentiation in an *in vitro* model of neural crest differentiation and in Hes knockout mice. I next examined the effect of Notch activation, both *in vitro* and *in vivo*, on Schwann cell precursors and found

that Notch activation promotes their differentiation into immature Schwann cells (arrow 2, figure below). Using *in vitro* model of Schwann cell differentiation, I then investigated the possible mechanisms involved in this effect. Given that immature Schwann cells are highly proliferative cells and that Notch signalling promotes cell division in a number of cell types, I also wanted to test if Notch signalling had any role in this process (arrow 3, figure below). Finally, I studied the role of Notch signalling in Schwann cell myelination (arrow 4, figure below).



4.2 RESULTS

4.2.1 Notch signalling and PNS gliogenesis.

4.2.1.1 Notch activation does not induce expression of glial differentiation markers in neural crest cells.

There are conflicting reports, at least *in vitro*, of the putative role of Notch signalling in instructing gliogenesis from neural crest cells. Wakamatsu *et al.*, (2000) show that enforced activation of the Notch signalling pathway in avian cultured crest cells does not lead to an increase in expression of the glial membrane marker and major myelin protein P0, whereas Morrison *et al.*, (2000) suggest a robust instructive role of Notch signalling in promoting gliogenesis from rat Neural Crest Stem Cells (NCSCs). It should be noted, however, that these cells were obtained from E14.5 sciatic nerves and it is debatable whether these cells are the same as migratory neural crest cells (see chapter 1 and discussion of this chapter). Subsequent experiments on NCSCs, obtained from E10.5 neural tube explants, still show promotion of gliogenesis by Notch activation, albeit much less efficiently (Kubu *et al.*, 2002). The end-point of these clonal analysis experiments is culturing these cells for at least 14 days after initial plating and it is uncertain to what extent they represent the *in vivo* gliogenic transition, which occurs over a period of 2-3 days.

I have determined cell culture conditions that enable us to induce gliogenesis in neural crest cells after only 2-3 days in culture. *In vivo* neural crest cells do not express the glial membrane marker P0 whereas E14.5 Schwann cell precursors do (Lee *et al.*, 1997). *In vitro* as well, I show here that neural crest cells do not express P0, whilst Schwann cell precursors do (Figure 1A). Cultures of neural crest cells were established by an adaptation of the method of Smith-Thomas and Fawcett (1989). Briefly, neural tube from E11 rat embryos were dissected out and plated overnight in crest medium (CM) (see Chapter 2). The next day, the neural tubes were

removed from the dishes and the neural crest cells that had migrated out on the dish were replated onto coverslips and are referred to as 'explanted neural crest cells' in the text.

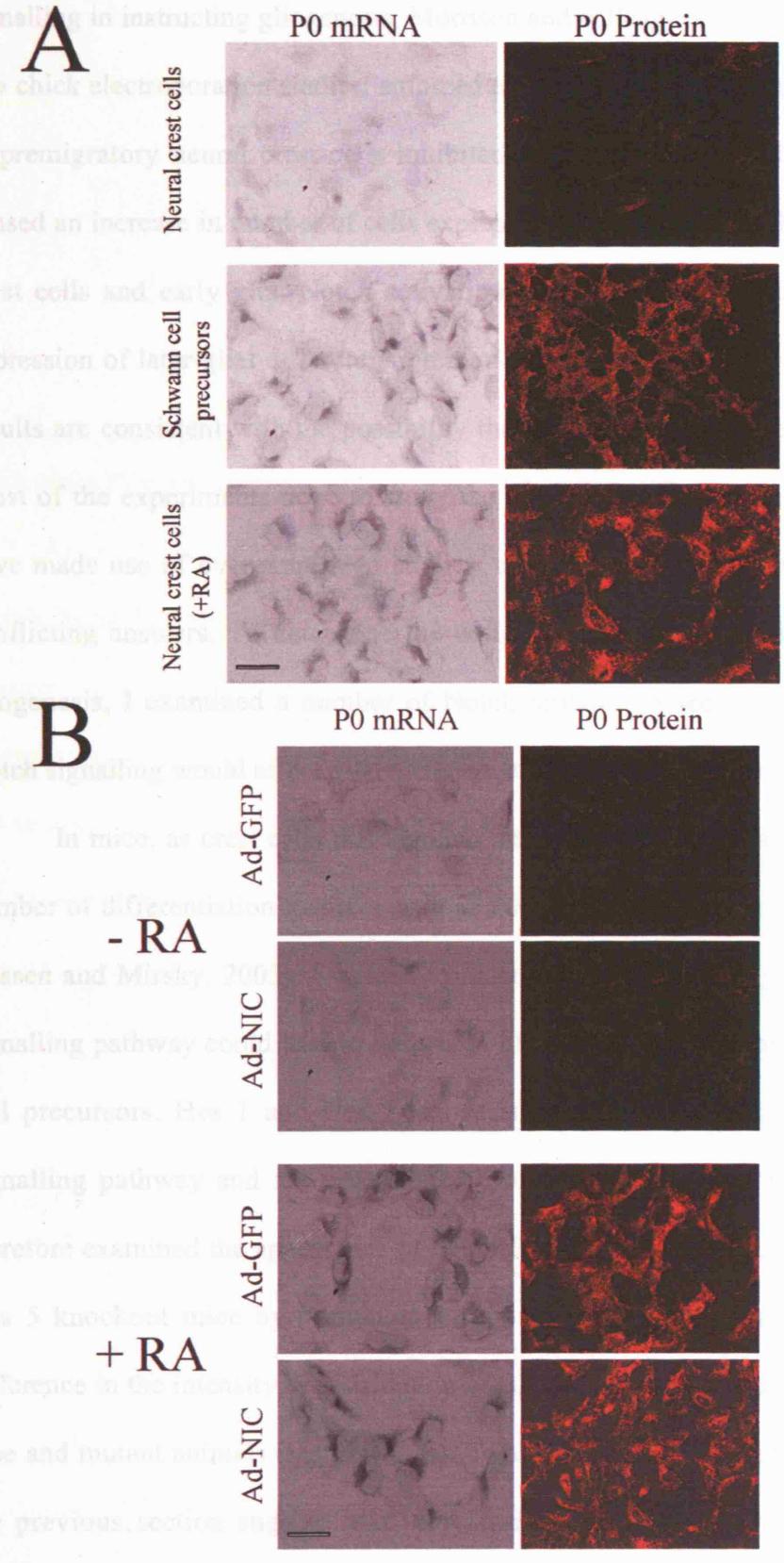
Explanted neural crest cells were cultured in CM supplemented with retinoic acid (100 ng/ml) and after 3 days in culture, they express P0 mRNA and protein (Figure 4.1A), reflecting the transitional stage of gliogenesis. I have adapted these culture conditions to see if Notch activation can induce expression of this glial differentiation marker and hence promote gliogenesis, similarly to retinoic acid. Thus, explanted neural crest cells were infected with an adenovirus encoding the Notch intracellular domain (Ad-NIC) and 24 hrs later, the medium was changed to CM, with or without retinoic acid and cultured for a further 3 days. Notch activation alone is not sufficient to induce either mRNA or protein expression of the P0 marker in these culture conditions, whereas in both control- or Ad-NIC-infected cells, cultured in retinoic acid, strong expression was seen (Figure 4.1B). This experiment suggest that Notch signalling is not involved in the directing neural crest cells to a glial fate since it is not sufficient in promoting expression of P0 expression, which is a characteristic feature of Schwann cell lineage specification.

Figure 4.1: Activation of Notch signalling in neural crest cells does not lead to upregulation of the Schwann cell precursor marker P0.

(A) Neural crest cells do not express P0 mRNA or protein, whereas freshly isolated Schwann cell precursors, fixed 3 hrs after plating, express both. However neural crest cells, cultured over a period of 3 days in retinoic acid (100 ng/ml)(+RA), express P0 mRNA and protein, reflecting *in vivo* gliogenesis. Scale bar, 25 μm.

(B) Neural crest cells, infected with control (Ad-GFP) or activated Notch (Ad-NIC) virus, cannot upregulate P0 mRNA or protein in the absence of retinoic acid, after 3 days in culture (-RA) (*top panel*), whereas in presence of retinoic acid (100 ng/ml)(+RA), the expected increase in P0 mRNA and protein levels is obtained, with or without Notch activation (*bottom panel).* Scale bar, 25 μm.

Figure 4.1


A
P0 mRNA
P0 Protein
Neural crest cells
Schwann cell precursors
Neural crest cells (+RA)
B
P0 mRNA
P0 Protein
- RA
Ad-GFP
Ad-NIC
+ RA
Ad-GFP
Ad-NIC

4.2.1.2 Notch signalling is not essential for gliogenesis.

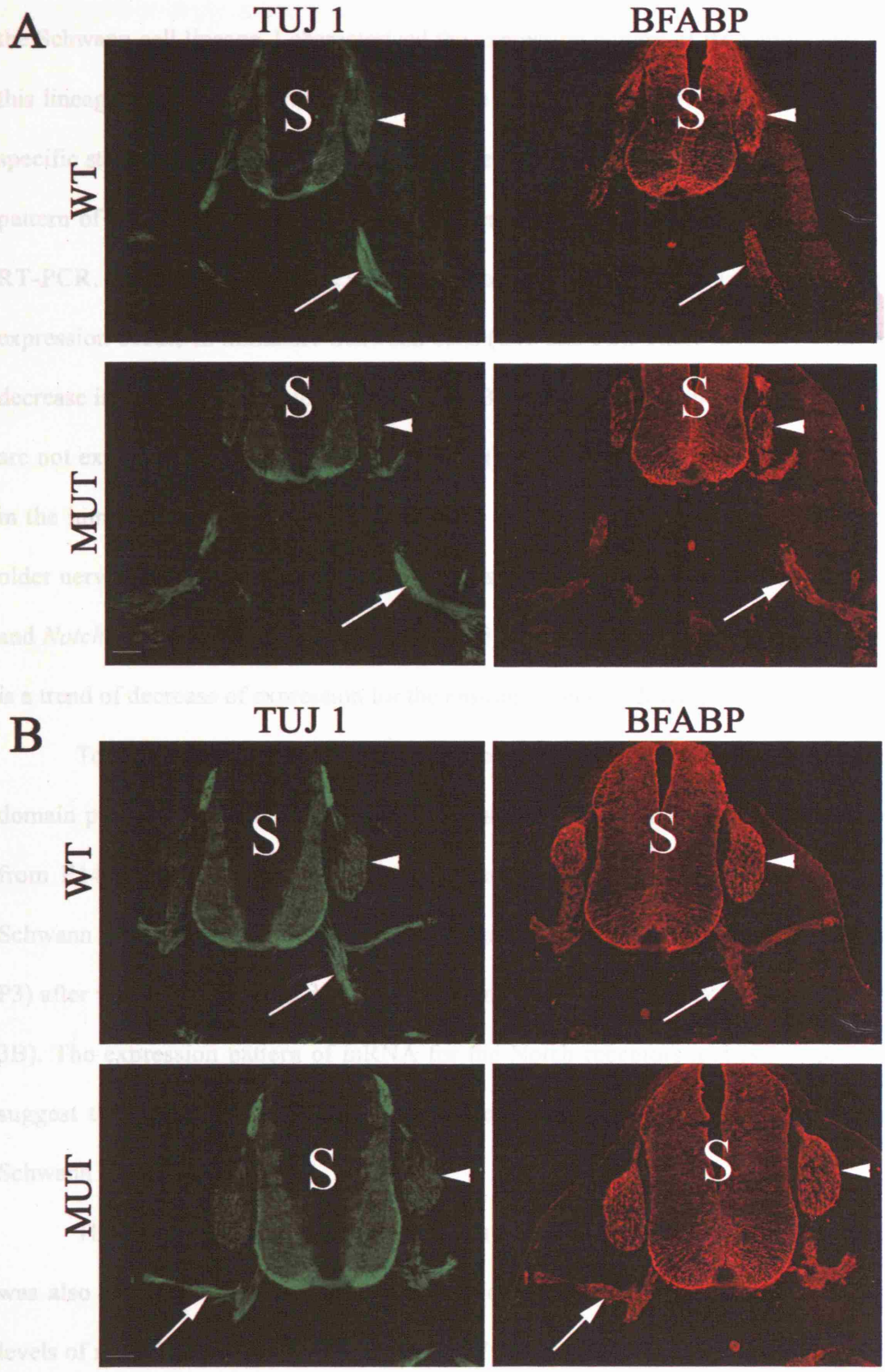
In vivo experiments also have been attempted to reconcile a role for Notch signalling in instructing gliogenesis; Morrison and colleagues (2000) found that in *in ovo* chick electroporation studies, enforced expression of an activated form of Notch in premigratory neural crest cells inhibited the generation of DRG neurones and caused an increase in number of cells expressing Sox10 cells, which is expressed by crest cells and early glia. Notch activation did not however induce an increased expression of later glial differentiation markers such as PLP. They argue that these results are consistent with the possibility that Notch promoted glial differentiation. Most of the experiments done to study the role of Notch signalling in gliogenesis have made use of overexpression studies, either *in vitro* or *in ovo* and have given conflicting answers. To determine the definitive role of Notch signalling in PNS gliogenesis, I examined a number of Notch mutants to see if loss-of-function of Notch signalling would affect glial differentiation from neural crest cells.

In mice, as crest cells differentiate into glia and peripheral nerves appear, a number of differentiation markers such as P0 and BFABP appear as early as E10.5 (Jessen and Mirsky, 2005). I wanted to determine whether disruption in the Notch signalling pathway could lead to defects in gliogenesis, i.e. appearance of Schwann cell precursors. Hes 1 and Hes 5 are essential effectors of the classical Notch signalling pathway and are upregulated through a CSL-dependent mechanism. I therefore examined the appearance of BFABP in early nerves at E11.5 in Hes 1 and Hes 5 knockout mice by immunolabelling on fixed/frozen sections. I found no difference in the intensity or distribution of BFABP immunolabelling between wild-type and mutant animals (Fig. 4.2A, B). Together these results and the results from the previous section suggest that the classical Notch signalling pathway is not involved in the of Schwann cell precursors from undifferentiated neural crest cells.

Figure 4.2: Disruption of the classical Notch signalling pathway does not lead to impairment in gliogenesis.

Hes 1 (A) and Hes 5 (B) knockout mice do not have any overt defects in gliogenesis judging from the appearance of BFABP. Both wild-type (WT) and mutant (MUT) embryos express normal levels of the Schwann cell precursor marker BFABP *(red)* in embryonic nerves. The sections were double immunolabelled with TUJ1 antibodies that bind to class III β-tubulin in axons *(green)*. DRGs (arrowheads) and nerve segments (arrows) are all BFABP positive. S: spinal cord. Scale bar, 50 μm.

Figure 4.2



4.2.2 Notch signalling pathway components are expressed in the Schwann cell lineage.

As a baseline for investigating the role of Notch signalling at later stages of the Schwann cell lineage, I characterized the expression pattern of its components in this lineage. Nerves, from rat embryos at time points that broadly correspond to the specific stages of the Schwann cell lineage, were isolated and the mRNA expression pattern of the Notch receptors and effector proteins examined by semi-quantitative RT-PCR. *Notch 1* mRNA is expressed throughout the lineage but its peak of expression occurs in immature Schwann cells (E18 and P3). There is also a trend of decrease in expression in older nerves (from P3 to P15). *Notch 2* and *Notch 3* mRNA are not expressed in Schwann cell precursors (E14) and show maximum expression in the immature Schwann cells, and similarly to *Notch 1*, expression goes down in older nerves. However, at all stages *Notch 1* is more highly expressed than *Notch 2* and *Notch 3*. *Hes 1* mRNA is highly expressed in Schwann cell precursors and there is a trend of decrease of expression for the ensuing stages of development (Fig. 3A).

To confirm these results, the expression of the activated Notch intracellular domain protein, NICD was studied using Western blotting on extracts from nerves from E14 to P15. This shows a gradual increase of the protein levels from the Schwann cell precursor stage (E14) to the immature Schwann cell stage (E18, P0 and P3) after which the levels go down, with the onset of myelination (P3 onwards) (Fig. 3B). The expression pattern of mRNA for the Notch receptors and NICD protein suggest that Notch signalling would have important regulatory functions in the Schwann cell lineage.

The expression of Notch ligands in peripheral neurones during development was also examined. DRGs at different developmental ages were isolated and the levels of mRNA for *Delta 1* and *3* and *Jagged 1* and *2* assessed by semi-quantitative

RT-PCR. At E15, all 4 ligands are highly expressed while at birth (P0), *Delta 1* and *Delta 3* expression disappear. *Jagged 1* and *Jagged 2* are still expressed at birth but expression is strongly downregulated thereafter (P7 onwards) (Fig. 3C).

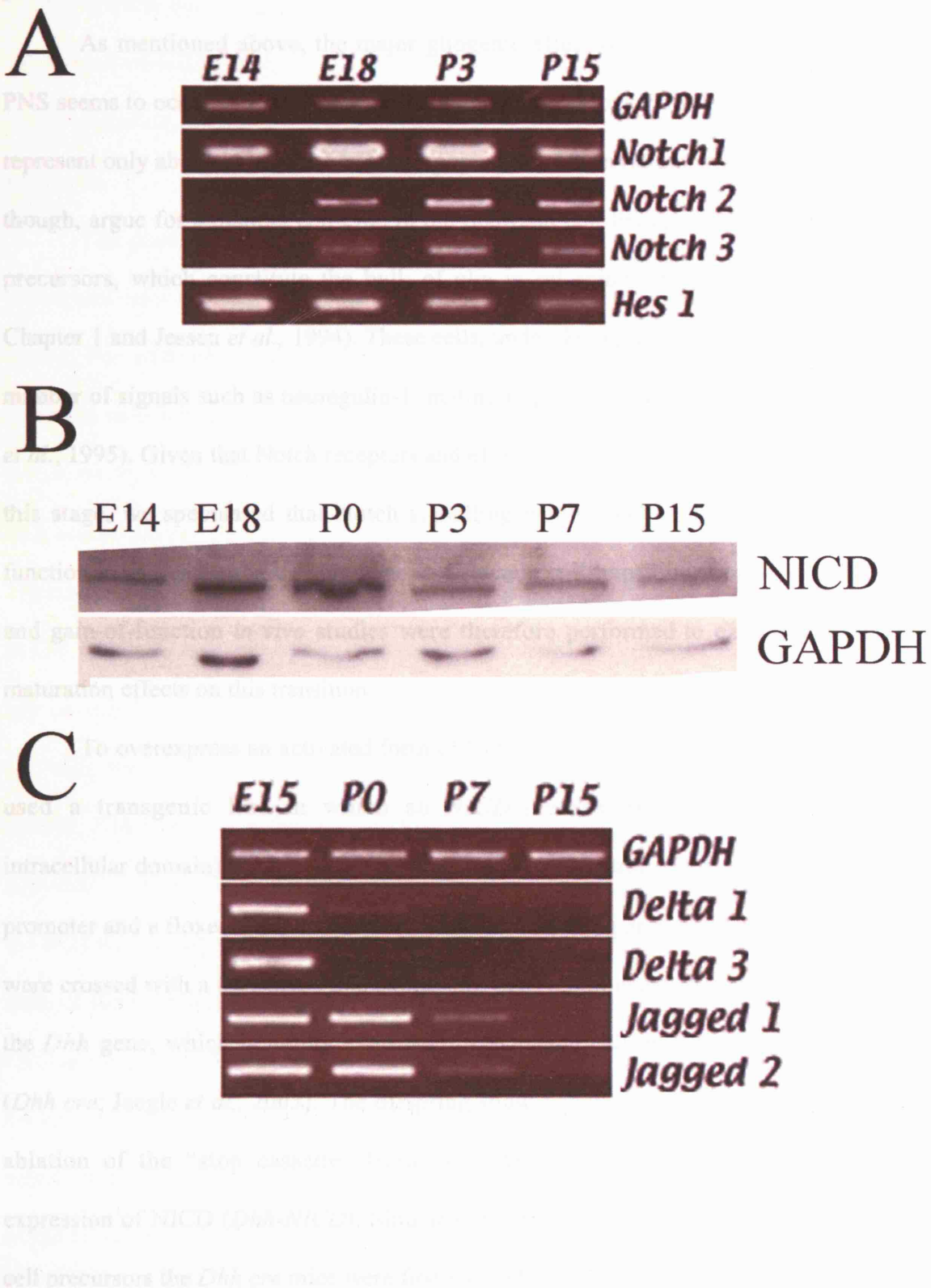
Figure 4.3: Expression pattern of Notch signalling pathway molecules in the PNS.

(A) RT-PCR. Semi-quantitative mRNA expression of *Notch* receptors (1-3) and Notch effector, *Hes 1* in freshly isolated nerves at E14, E18, P3 and P15. *Notch 1* mRNA levels increase from Schwann cell precursors (E14) to immature Schwann cells (E18 and P3) and go down as cells differentiate into myelinating and non-myelinating Schwann cells (P15). A similar pattern of expression seen for *Notch 2* and *Notch 3*, but their expression is lower than *Notch 1* and they are not expressed in Schwann cell precursors. *Hes 1* is mostly expressed in Schwann cell precursors and with development, expression goes down. *GAPDH* is used as a loading control.

(B) Western blot analysis of protein samples obtained from freshly isolated nerve extracts. NICD levels increase from Schwann cell precursors (E14) to immature Schwann cells (E18, P0) and then decrease as development proceeds (P3 onwards). GAPDH immunoblot (IB) is used as a loading control.

(C) RT-PCR analysis of Notch ligands in freshly isolated DRGs obtained at different ages. *Delta 1* and *3* are expressed only at E15, whereas *Jagged 1* and *2* are expressed at E15 and P0, after which the levels go down dramatically. *GAPDH* is used as a loading control

Figure 4.3


A
E14
E18
P3
P15
GAPDH
Notch1
Notch 2
Notch 3
Hes 1
B
E14
E18
P0
P3
P7
P15
NICD
GAPDH
C
E15
P0
P7
P15
GAPDH
Delta 1
Delta 3
Jagged 1
Jagged 2

4.2.3 Notch signaling and Schwann cell precursor maturation.

4.2.3.1 Notch activation promotes the *in vivo* maturation of Schwann cell precursors into Schwann cells.

As mentioned above, the major gliogenic effect of Notch signalling in the PNS seems to occur in E14.5 rat sciatic nerve NCSCs (Morrison *et al.*, 2000), which represent only about 15% of the total cell population. Observations in this laboratory, though, argue for a distinct cell-type in the Schwann cell lineage, the Schwann cell precursors, which constitute the bulk of glia in rat sciatic nerves at E14.5 (see Chapter 1 and Jessen *et al.,* 1994). These cells, under the regulatory mechanisms of a number of signals such as neuregulin-1, mature to give rise to Schwann cells (Dong *et al.*, 1995). Given that Notch receptors and effector proteins are highly expressed at this stage, we speculated that Notch signalling would have important regulatory function in this Schwann cell precursor – Schwann cell transition. Loss-of-function and gain-of-function *in vivo* studies were therefore performed to examine at any maturation effects on this transition.

To overexpress an activated form of Notch in the Schwann cell precursors, I used a transgenic line in which an *NICD* (constitutively activated Notch1 intracellular domain) transgene is expressed under the control of the chicken β-actin promoter and a floxed "stop cassette" (*CALSL-NICD*; Yang *et al.,* 2004). These mice were crossed with a transgenic line expressing Cre recombinase under the control of the *Dhh* gene, which is highly expressed in Schwann cell precursors from E12.5 (*Dhh cre*; Jaegle *et al.,* 2003). The offspring show Schwann cell precursor-specific ablation of the "stop cassette" from the CALSL-NICD transgene leading to expression of NICD (*Dhh-NICD).* Similarly, to remove Notch function in Schwann cell precursors the *Dhh cre* mice were first crossed with *RBP-J* floxed mice, to give a first generation of mice expressing the *Dhh cre* transgene and one floxed allele of the

RBP-J gene (N1 generation). This N1 generation was backcrossed with *RBP-J* floxed animals leading to the generation of mice in which *RBP-J* was selectively deleted from Schwann cell precursors (*Dhh-RBP-J*). Thus, mice, which had constitutive expression of NICD in Schwann cell precursors and mice, in which the classical Notch signalling pathway, had been disrupted in the Schwann cell precursors were generated.

The phenotypic changes that characterize the development of Schwann cell precursors into Schwann cells in mice have been well documented (Dong *et al.*, 1999). For example, the O4 antigen is expressed by about 90% of immature Schwann cells but no expression was seen in Schwann cell precursors obtained from mouse embryos. These cells were isolated from E12 (for Schwann cell precursors) or E16 sciatic nerves (immature Schwann cells) and plated onto coverslips for 3 hrs. They were then fixed and immunolabelled with the O4 antibody and the percentage of $O4^{+ve}$ cells determined in each culture determined. I adapted this method to study any potential role of Notch signalling in the maturation of Schwann cell precursors and thus examined the expression of the O4 antigen in wild-type and mutant mice. Briefly, E14.5 nerves, in the case of *Dhh/NICD* mice or E15.5 nerves, in the case of *Dhh/RBP-J,* mice were dissected out and the cells plated onto coverslips. 3 hrs later, the cells were fixed and immunolabelled for O4 and L1, which is a general marker of cells of the Schwann cell lineage and the percentage of O4-$L1^{+ve}$ cells determined. Overexpression of NICD resulted in a higher proportion of $O4^{+ve}$ cells in mutant nerves compared to wild-type animals suggesting that Notch activation leads to an enhanced maturation of the Schwann cell precursors (Fig. 4A). However, disruption of the classical Notch pathway at E15.5 did not have any effect on the appearance of

O4 from Schwann cell precursors suggesting that Schwann cells were generated on schedule in these animals (Fig. 4B).

4.2.3.2 Mechanisms involved in the Notch-mediated promotion of Schwann cell precursors maturation

The results in the previous section indicate that Notch activation might be involved in the Schwann cell precursor – Schwann cell transition. Although no difference is seen in the maturation schedule of Schwann cell precursors when the classical Notch pathway is disrupted, there is increased differentiation when NICD is overexpressed. This difference might be due to the involvement of the CSL-independent Notch pathway in this transition, thus explaining the apparent lack of effect when *RBP-J* is ablated (explained fully in the discussion). It appears likely that Notch signalling is required for this transition to occur, possibly by acting in a rate-limiting manner. I therefore decided to study the possible molecular mechanisms involved and set up *in vitro* culture conditions to study them. E14.5 rat Schwann cell precursors, cultured in defined medium (DM) supplemented with neuregulin-1, generate Schwann cells over a period of 4 days, based on appearance of Schwann cell antigenic markers such as GFAP, S100β and O4, and autocrine survival mechanims (reviewed in Jessen and Mirsky, 2005a,b).

I wanted to see if under similar culture conditions, activation of Notch would speed up this transition, reflecting what is observed in the *in vivo* experiments of NICD overexpression. Notch was activated in rat Schwann cell precursors in two ways. First, Schwann cell precursors were co-cultured with Delta1-expressing NIH 3T3 cells. A Delta 1 retrovirus and its matched control retrovirus were used to stably infect NIH 3T3 cells. Immunolabelling with an antibody against Delta 1 shows that it is expressed on the surface of the 3T3 cells infected with the Delta 1 retrovirus (BP-

Delta), whereas in the control-infected cells (BP2 3T3), no expression is seen (Figure 4C). Thus, when Schwann cell precursors are co-cultured with these Delta1-expressing cells, binding of Notch receptors, expressed by the Schwann cell precursors, to the Delta1 ligand, expressed on the surface of 3T3 cells would result in activation of the Notch signalling pathway in the Schwann cell precursors. Schwann cell precursors were co-cultured with either BP2 or BP-Delta 3T3 cells and the expression of the Schwann cell marker GFAP was examined over a period of 6 days. The cells were co-immunolabelled with nestin, which is a general marker of the cells of the Schwann cell lineage (Friedman *et al.*, 1990) to allow their identification from the 3T3 cells and the percentage of nestin^{+ve}GFAP^{+ve} cells determined. Co-culture with BP-Delta 3T3 cells results in an earlier maturation of Schwann cell precursors into GFAP-expressing Schwann cells. At 1, 2, 3, 4 and 6 days, there is respectively 84%, 73%, 74%, 46% and 28% more GFAP^{+ve} cells in the Schwann cell precursors/BP-Delta co-cultures than the Schwann cell precursors/BP2 co-cultures (Fig. 4D).

Figure 4.4: *In vivo* and *in vitro* activation of Notch signalling in Schwann cell precursors accelerate their differentiation into Schwann cells.

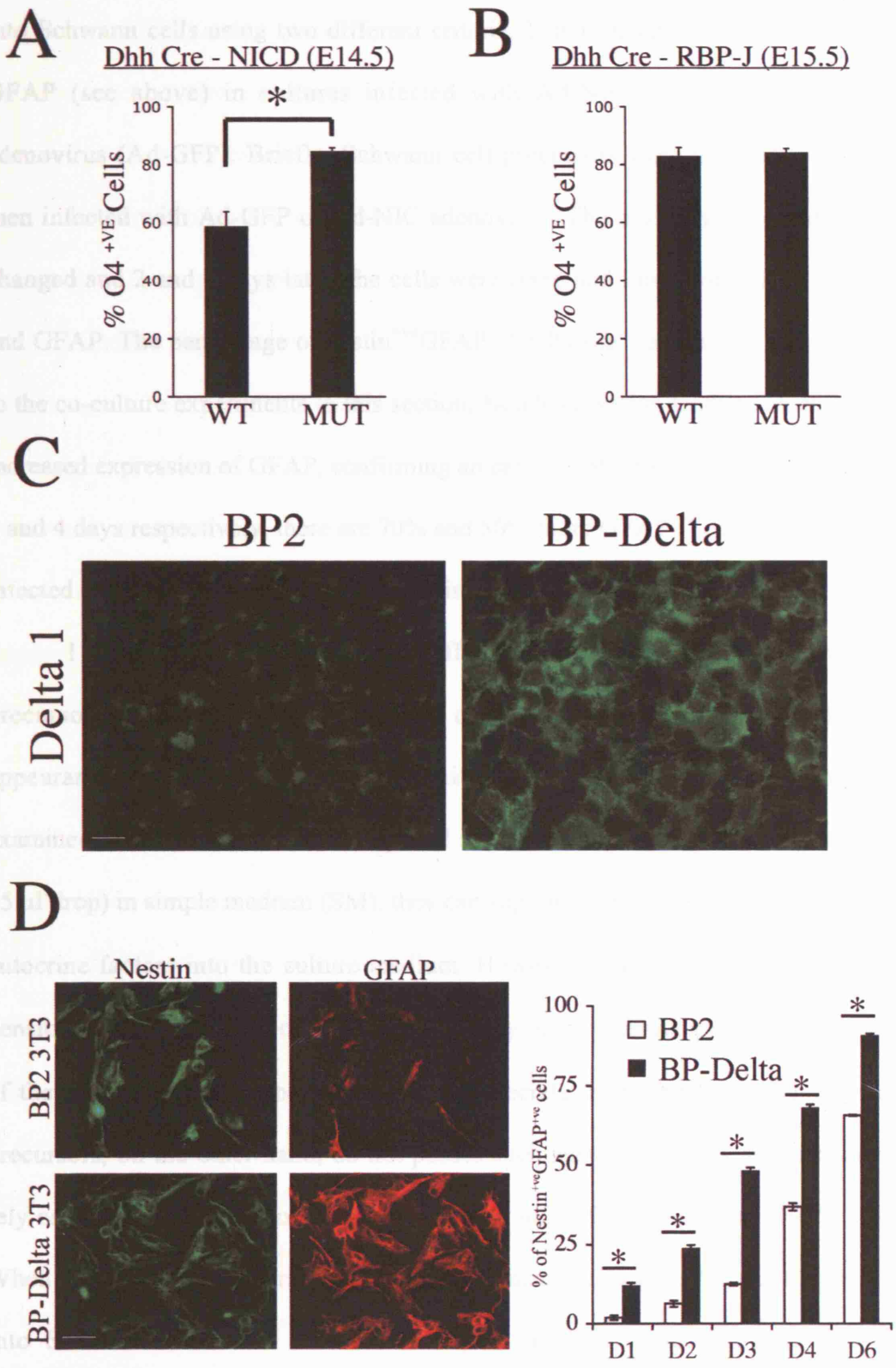
(A) E14.5 nerves from mice in which NICD is conditionally overexpressed (MUT), have higher percentage of $O4^{+ve}$ Schwann cells (84.6 ± 1.3%) than nerves from wild-type (WT) mice (63.5± 5.1%) (n=6). * denotes significant level of difference ($p<0.001$).

(B) E15.5 nerves from wild-type (WT) and mutant (MUT) mice, in which RBP-J has been selectively inactivated, show comparable expression of $O4^{+ve}$ Schwann cells (84.1± 1.5% compared to 82.9 ± 3.0%) (n=6).

(C) Delta 1 immunocytochemistry of NIH 3T3 cells, stably transfected with either Delta 1 (BP-Delta) retrovirus or control retrovirus (BP2). BP-Delta cells express high levels of Delta 1 on their surface *(right panel)* whereas BP2 cells express low levels of it *(left panel)*. Scale bar, 25 μm

(D) *Left panel* – After 4 days in culture, a greater proportion of Schwann cell precursors, plated on BP-Delta expressing 3T3 cells, differentiate into $GFAP^{+ve}$ Schwann cells *(red)*, compared to Schwann cell precursors plated on control BP2 3T3 cells. Cells were double immunolabelled using antibodies to nestin *(green)*, which is expressed in both Schwann cell precursors and Schwann cells. Scale bar, 20 μm. *Right panel* – Quantification of the percentage of $nestin^{+ve}GFAP^{+ve}$ cells, cultured on control BP2 and BP-Delta 3T3 over a 6-day period (n=6, $p<0.001$). * denotes significant level of difference. Scale bar, 20 μm.

Figure 4.4


A
Dhh Cre - NICD (E14.5)
% O4 +VE Cells
100
80
60
40
20
0
*
WT
MUT
B
Dhh Cre - RBP-J (E15.5)
% O4 +VE Cells
100
80
60
40
20
0
WT
MUT
C
BP2
BP-Delta
Delta 1
D
Nestin
GFAP
BP2 3T3
BP-Delta 3T3
% of Nestin+veGFAP+ve cells
100
75
50
25
0
BP2
BP-Delta
*
D1
D2
D3
D4
D6

Notch signalling was also activated in Schwann cell precursors by infection with the Ad-NIC adenovirus and this system was used to study their differentiation into Schwann cells using two different criteria. I first examined the appearance of GFAP (see above) in cultures infected with Ad-NIC or its matched control adenovirus (Ad-GFP). Briefly, Schwann cell precursors were isolated, plated and then infected with Ad-GFP or Ad-NIC adenovirus. The next day, the medium was changed and 2 and 4 days later, the cells were fixed and immunolabelled for nestin and GFAP. The percentage of nestin^{+ve}GFAP^{+ve} cells was determined and, similarly to the co-culture experiments in this section, Notch activation leads to an earlier and increased expression of GFAP, confirming an earlier maturation of the precursors. At 2 and 4 days respectively, there are 70% and 50% more GFAP^{+ve} cells in the cultures infected with Ad-NIC than in the cultures infected with Ad-GFP (Figure 5A).

I next wanted to investigate the effect of Notch activation on Schwann cell precursor maturation using a criterion different to appearance of GFAP. The appearance of autocrine survival circuits (Meier *et al.*, 1999) was therefore examined. When Schwann cells are plated at high densities (10,000 cells plated on a 15 μl drop) in simple medium (SM), they can support their own survival by secreting autocrine factors into the culture medium. However, when they are plated at low densities (125 cells plated on a 15 μl drop), they do not survive presumably because of the low amount of autocrine factors they secrete in the medium. Schwann cell precursors, on the other hand, do not posses such survival circuits and exclusively rely on paracrine factors for survival (Jessen *et al.*, 1994, Dong *et al.*, 1995, 1999). When Schwann cell precursors are cultured in neuregulin-1 for 4 days, they mature into Schwann cells and are most likely to acquire these autocrine survival mechanisms. We wanted to investigate whether Notch activation in Schwann cell

precursors could speed up their maturation into Schwann cells and result in an earlier appearance of these survival mechanisms. Schwann cell precursors were isolated and plated onto PDL-laminin coated 35mm tissue culture dishes. 3 hrs later, they were infected with Ad-NIC or control Ad-GFP adenovirus, and the next day replated at high or low densities onto PDL-laminin coated coverslips and cultured in SM. 24 hrs later, the surviving cells were immunolabelled for the L1 antigen (present in Schwann cell precursors and Schwann cells) and then the number of surviving cells, as detected by Hoechst immunolabelling, were counted and expressed as a percentage of the original number of cells present (sister cultures fixed and immunolabelled after 3 hrs following replating). Ad-NIC-infected Schwann cell precursors survived much better than control-infected cells, when cultured at high density in SM (88.4 ± 2.8 % compared to 24.8 ± 2.0 % respectively). This result suggests that Notch activation causes an earlier appearance of autocrine survival mechanisms in Schwann cell precursors in our culture system, most probably by accelerating their differentiation into Schwann cells. This effect on survival is unlikely to be a paracrine effect of Notch activation, since similar cultures plated at low densities were not able to survive in SM. Only 8.9 ± 2.1 % and 7.9 ± 0.8% of cells were able to survive when plated at low densities for Ad-GFP- and Ad-NIC-infected cultures respectively.

4.2.3.3 Notch activation in Schwann cell precursors increases their responsiveness to neuregulin-1.

The effects induced by Notch activation parallel, to a large extent, the effects of neuregulin-1 on Schwann cell precursors. As discussed in Sections 1.3.2 and 1.3.3,

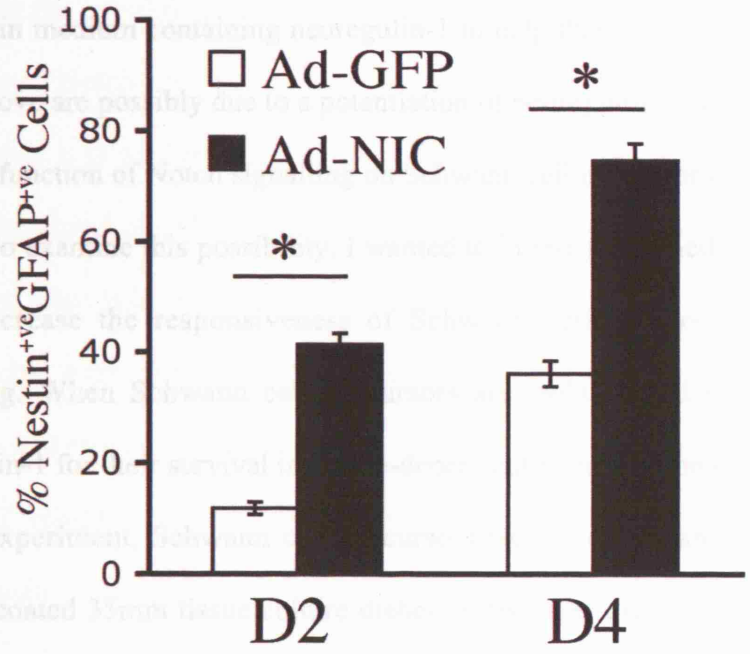
Figure 4.5: Adenoviral-mediated Notch activation in Schwann cell precursors accelerates their differentiation to Schwann cells.

(A) A greater proportion of Schwann cell precursors infected with activated Notch adenovirus (Ad-NIC) differentiate into $GFAP^{+ve}$ Schwann cells compared to Schwann cell precursors infected with control virus (Ad-GFP), after 2 days (D2) and 4 days (D4) post-infection. Cells were double immunolabelled using antibodies to nestin *(green),* which is expressed in both Schwann cell precursors and Schwann cells. The percentage of $nestin^{+ve}GFAP^{+ve}$ cells was quantified and at D2 and D4 respectively, there is approximately 70% and 50% more $GFAP^{+ve}$ Schwann cells in Ad-NIC-infected cultures than in Ad-GFP cultures (n=6). * denotes significant level of difference ($P<0.001$)..

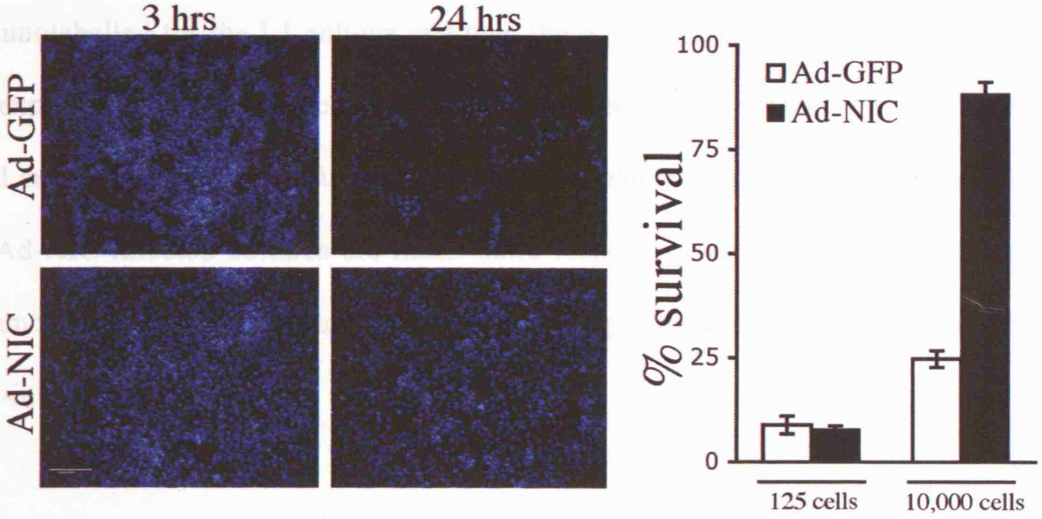
(B) Notch activation in Schwann cell precursors accelerates their differentiation into Schwann cells and leads to an earlier appearance of autocrine survival mechanisms. *Left panel* – Hoechst labelling shows that Ad-NIC-infected Schwann cell precursors are able to survive much better than control Ad-GFP–infected Schwann cell precursors. After 24 hrs in culture (24 hrs), there are very few live cells present in Ad-GFP-infected cultures when compared to sister cultures that had been fixed and labelled at 3 hrs post-plating (3hrs). In contrast, the majority of the cells in Ad-NIC-infected cultures are alive after 24 hrs, when compared to sister cultures after 3 hrs in culture. Scale bar, 100 μm. *Right panel* – Percentage survival of Ad-GFP- or Ad-NIC-infected Schwann cells after 24 hr culture in SM. At high densities (10,000 cells), Ad-NIC-infected cells are able to survive better than control Ad-GFP-infected cells (88.4 ± 2.8 % compared to 24.8 ± 2.0 % respectively). At low densities (125 cells) however, neither Ad-GFP- nor Ad-NIC-infected cells can survive suggesting that the effect seen on survival at high densities is not due to an intrinsic effect of Notch activation on blocking apoptosis.

Figure 4.5

A


100
80
60
40
20
0
% Nestin+veGFAP+ve Cells
Ad-GFP
Ad-NIC
*
*
D2
D4

B


3 hrs
24 hrs
Ad-GFP
Ad-NIC
100
75
50
25
0
% survival
Ad-GFP
Ad-NIC
125 cells
10,000 cells

neuregulin-1 is essential for Schwann cell precursors survival and their differentiation into Schwann cells (Jessen *et al.*, 1994; Dong *et al.*, 1995). Two independent groups have shown that radial glia maturation is regulated by neuregulin signalling, an effect which is potentiated by Notch signalling activity (Patten *et al.*, 2003; Schmid *et al.*, 2003). In the experiments described above on the effect of Notch activation on Schwann cell precursor maturation, the cells were always cultured in medium containing neuregulin-1 to help their survival. Thus the effects I found above are possibly due to a potentiation of neuregulin-1 function rather than an intrinsic function of Notch signalling on Schwann cell precursor maturation.

To examine this possibility, I wanted to investigate whether Notch activation could increase the responsiveness of Schwann cell precursors to neuregulin-1 signalling. When Schwann cell precursors are isolated and plated, they require neuregulin-1 for their survival in a dose-dependent manner (Dong *et al.*, 1995, 1999). In this experiment, Schwann cell precursors were isolated and plated onto PDL-laminin coated 35mm tissue culture dishes. 3 hrs later, they were infected with Ad-NIC or control Ad-GFP adenovirus, and the next day replated at low densities (125 cells plated on a 15 μl drop) onto PDL-laminin coated coverslips and cultured in increasing doses of neuregulin-1. 24 hrs later, the surviving cells were immunolabelled for the L1 antigen and then the number of surviving cells counted and expressed as a percentage of the original number of cells present (sister cultures fixed and immunolabelled after 3 hrs following replating). As shown in figure 6A, the Ad-NIC-infected cultures are much more responsive to neuregulin-1 and less amount of it is required to achieve 50% survival or proliferation, than in control Ad-GFP-infected cultures.

4.2.3.4 Notch signalling acts through erbB2 receptor upregulation to induce neuregulin-1 responsiveness in Schwann cell precursors.

In radial glia maturation, the potentiation of neuregulin-1 signalling by Notch activation occurs through the upregulation of the neuregulin-1 receptor, erbB2 (Patten *et al.,* 2003; Schmid *et al.*, 2003). Notch activation also leads to an increased responsiveness of Schwann cell precursors to neuregulin-1 (Section 4.2.6). To investigate the possibility that this effect is mediated by upregulation of erbB2 levels, the effect of Notch activation on the expression of neuregulin-1 signalling pathway components was analysed by Western blotting. Briefly, Schwann cell precursors were isolated and plated onto PDL-laminin coated 35mm tissue culture dishes. 3 hrs later, they were infected with Ad-NIC or control Ad-GFP adenovirus, and the next day proteins were extracted and analysed by Western blotting. Notch activation in Schwann cell precursors resulted in an increase in erbB2 receptor levels although the levels of erbB3 were relatively unaffected (Fig. 6B). The expression of the downstream pathways regulated by neuregulin-1 signalling, namely the MAPK pathways, was also examined. No effect on phosphorylation of ERK 1/2 were seen in these culture conditions, probably because the cells were cultured in saturating levels of neuregulin-1. However, a slight increase in JNK 1/2 phosphorylation was observed but the most dramatic effect was seen with a large upregulation of p38 phosphorylation.

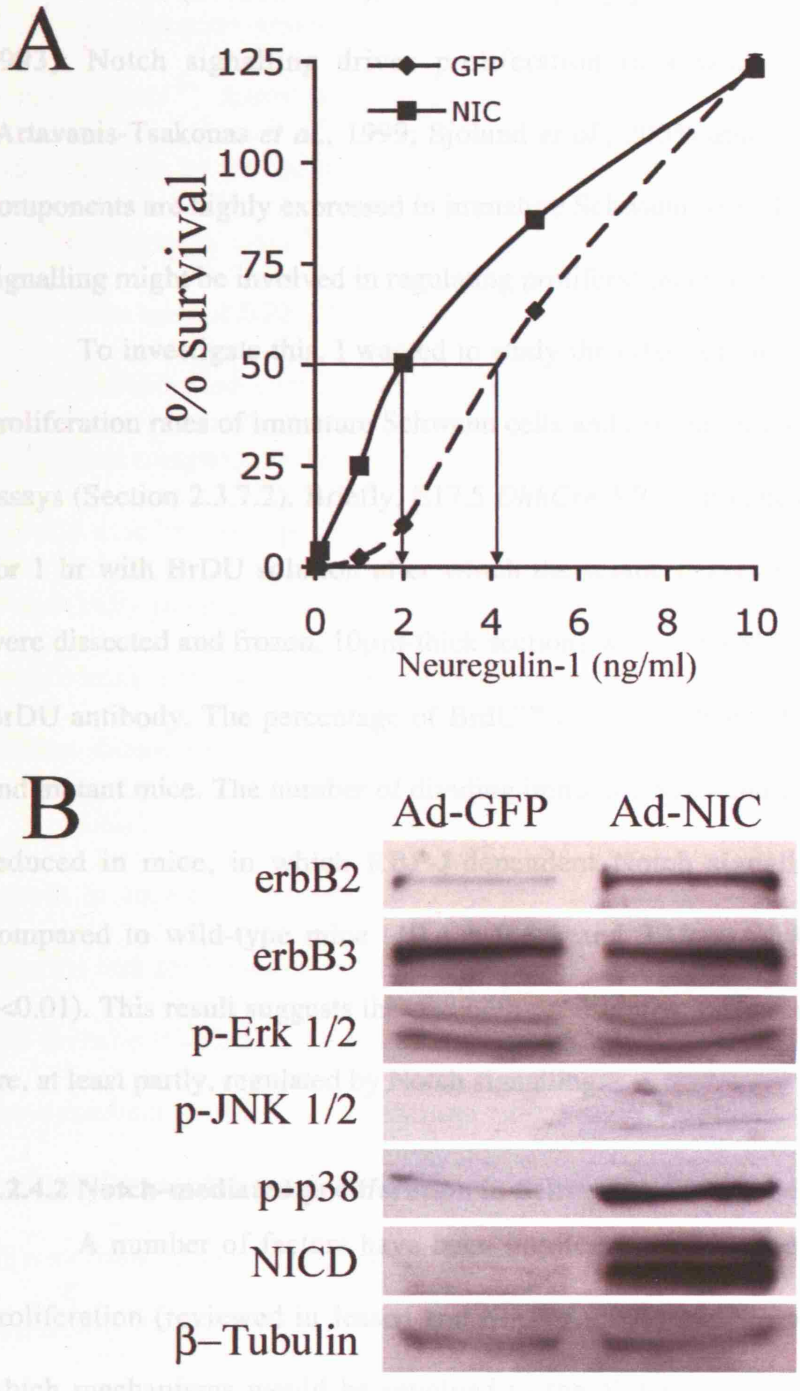
The results in the previous sections consistently show a strong effect of Notch signalling on maturation of Schwann cell precursors, which is most likely to be mediated through an enhanced responsiveness to neuregulin-1 signalling, via regulation of the erbB2 receptor.

Figure 4.6: Notch activation in Schwann cell precursors increase their responsiveness to neuregulin-1, a process possibly mediated by transactivation its receptor erbB2.

(A) Percentage survival of Ad-GFP- and Ad-NIC-infected Schwann cell precursors in increasing concentrations of neurgulin-1. Ad-NIC-infected Schwann cell precursors survive better then Ad-GFP-infected Schwann cell precursors in lower doses of neuregulin-1; 50% of the cells survive in response to only 1.96 ± 0.23 ng/ml of neuregulin-1 for Ad-NIC-infected Schwann cell precursors compared to 4.22 ± 0.19 ng/ml required for Ad-GFP-infected Schwann cell precursors, a difference of 2 fold ($n=4$, $P<0.01$).

(B) Western Blot analysis of components of the neuregulin-1 signalling pathway in Ad-GFP- and Ad-NIC-infected Schwann cell precursor cultures. Notch activation leads to an increase in ErbB2 levels, although ErB3 levels remain the same. Phosphorylation of Erk 1/2 is unaffected in these culture conditions, but a slight increase in JNK 1/2 phosphorylation is seen. P38 phosphorylation, however, is highly increased in Ad-NIC-infected Schwann cell precursors. β-tubulin IB is used as a loading control and NICD IB is used as a control for NICD expression.

Figure 4.6


A
125
100
75
50
25
0
% survival
GFP
NIC
0
2
4
6
8
10
Neuregulin-1 (ng/ml)
B
Ad-GFP
Ad-NIC
erbB2
erbB3
p-Erk 1/2
p-JNK 1/2
p-p38
NICD
β–Tubulin

4.2.4 Notch signalling and immature Schwann cell proliferation.

4.2.4.1 Notch is required for *in vivo* proliferation of immature Schwann cells.

Notch mRNA and NICD protein are most highly expressed in immature Schwann cells (Section 4.2.3), which are rapidly proliferating cells (Stewart *et al.*, 1993). Notch signalling drives proliferation in a wide variety of cell-types (Artavanis-Tsakonas *et al.*, 1999; Sjolund *et al.*, 2005) and, given that its pathway components are highly expressed in immature Schwann cells, I speculated that Notch signalling might be involved in regulating proliferation in immature Schwann cells.

To investigate this, I wanted to study the effect of Notch inactivation on the proliferation rates of immature Schwann cells and I set up *in vivo* BrDU proliferation assays (Section 2.3.7.2). Briefly, E17.5 *DhhCre-RBP-J* pregnant mice were injected for 1 hr with BrDU solution after which the sciatic nerves of individual embryos were dissected and frozen. 10μm-thick sections were cut and immunolabelled with a BrDU antibody. The percentage of $BrdU^{+ve}$ cells was then determined in wild-type and mutant mice. The number of dividing immature Schwann cells was considerably reduced in mice, in which RBP-J-dependent Notch signalling was inactivated compared to wild-type mice (19.4 ± 0.6% and 39.9 ± 5.8% respectively)(n=3, $p<0.01$). This result suggests that the high proliferative rates seen in nerves at E17.5 are, at least partly, regulated by Notch signalling.

4.2.4.2 Notch-mediated proliferation in Schwann cells can be assayed *in vitro.*

A number of factors have been implicated in the process of Schwann cell proliferation (reviewed in Jessen and Mirsky, 2005) and we wanted to investigate which mechanisms would be involved in the Notch-mediated process, described above. In order to study these mechanisms, we first had to establish culture conditions that would essentially reflect the mitogenic role of Notch activation on Schwann cells.

Notch was activated in purified Schwann cell cultures (Section 2.3.3.5) either by adenoviral infections with Ad-NIC or co-culture with BP-Delta 3T3. Briefly, Schwann cells were co-cultured with control BP2 or BP-Delta 3T3 and after 2 days, BrDU proliferation assays were performed (Section 2.3.7.1). The percentage of $nestin^{+ve}BrDU^{+ve}$ determined. Activation of the endogenous Notch receptors in Schwann cells by the Delta 1 ligand expressed on the BP-Delta 3T3 cells increased their proliferation rates by approximately 75% when compared to Schwann cells cultured on control BP2 3T3 (n=6) (Figure 4.7C). Activation of Notch signalling by adenoviral infection with Ad-NIC also increased the proliferation rates of Schwann cells when compared to Schwann cells infected with control Ad-GFP (68.8 ± 1.9% and 3.1 ± 0.7% respectively). In this experiment, Schwann cells were plated and adenovirally infected and 24 hrs later, the medium was changed. After 2 days BrDU proliferation assays were performed and the percentage of $BrDU^{+ve}$ cells in the cultures determined.

Cell division is tightly controlled by many regulatory mechanisms that either permit or inhibit its progression. Cyclin D1 and cdk2 are important regulatory proteins that are involved in early cell cycle progression and are upregulated during cell division (Gloias *et al.,* 2004). Notch activation leads to upregulation of both of these markers in cultures of Schwann cells suggesting that Notch signalling might intrinsically regulate Schwann cell proliferation. Purified Schwann cells, cultured on 60 mm tissue culture dishes, were adenovirally infected with Ad-GFP and Ad-NIC adenovirus. 24 hrs later, the medium was changed and proteins extracted after 2 days in culture. The proteins were analysed by Western blotting and immunoblotted using cyclin D1 and cdk2 specific antibodies. In the Ad-NIC-infected cells, there was a large increase in levels of cyclin D1 and cdk2 (Figure 4.7D)

Figure 4.7: Notch signalling is involved in the proliferation of immature Schwann cells.

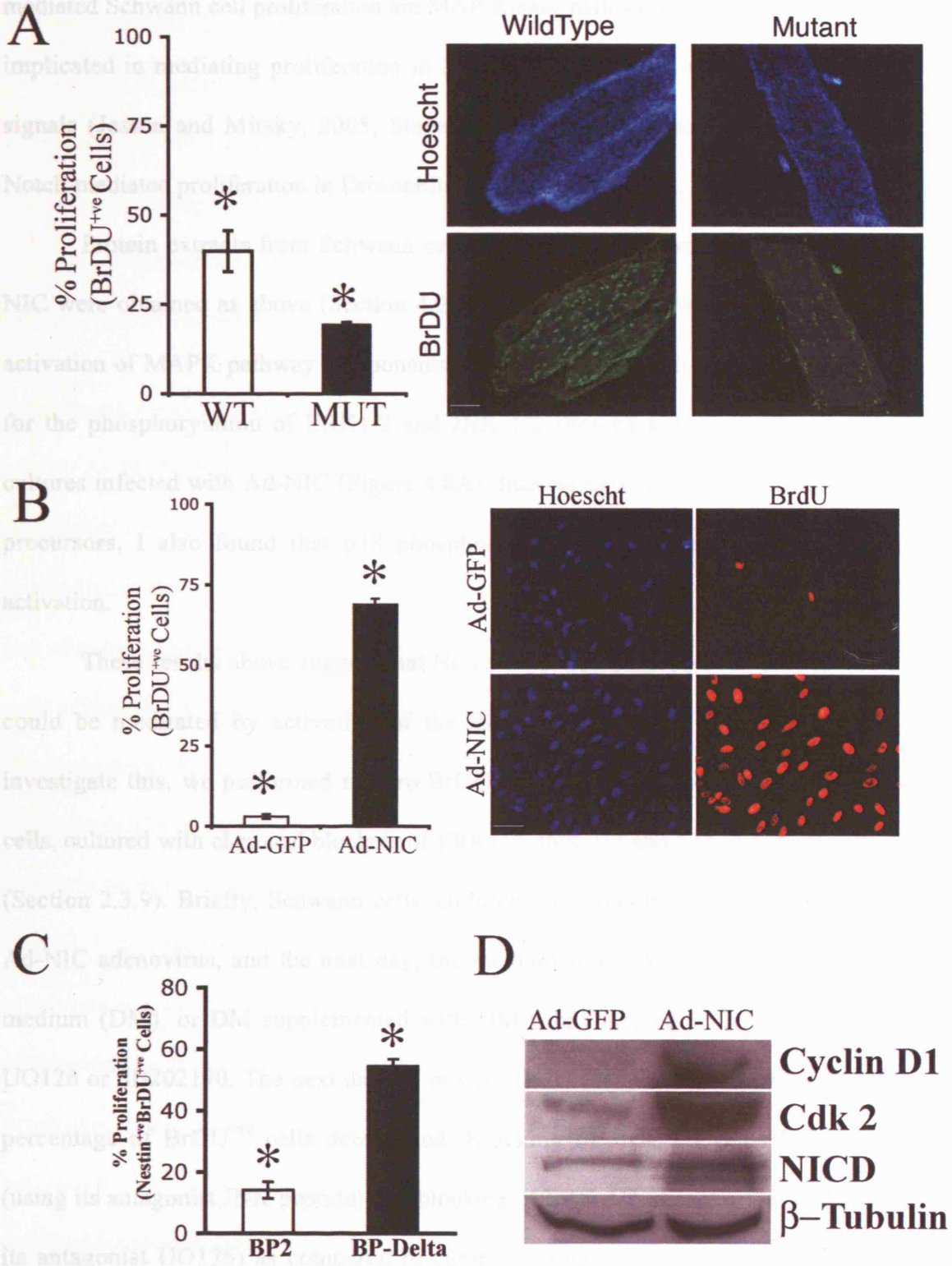
(A) Conditional ablation of *RBP-J* in immature Schwann cells (*Dhhcre/RBP-J)* leads to a reduction in proliferation as seen by *in vivo* BrDU immunolabelling. *Left panel* – The number of $BrDU^{+ve}$ cells (BrDU immunolabelling) were counted and expressed as a percentage of the total number of cells present per section (Hoechst immunolabelling). Mutant mice (MUT) have fewer proliferating immature Schwann cells in their sciatic nerves than wild-type mice (WT) (19.4 ± 0.6 % and 39.9 ± 5.8 % respectively) (n=3). * denotes significant level of difference ($p<0.01$). *Right panel* – cryosections of nerves from wild-type and mutant embryos show fewer $BrDU^{+ve}$ cells (*green)* in nerves counterstained with Hoechst nuclear stain (*blue).* NB. The pictures were taken at the same exposure time but in WT mice, a lot of non-specific staining is seen surrounding the nerve because of the presence of the epineurial sheath in this particular section. In the mutant sciatic nerves, the sections do not have this epineurial sheath and hence no non-specific staining is seen. Scale bar, 50 μm.

(B) Adenoviral-mediated Notch activation induces proliferation in Schwann cell cultures, as seen by *in vitro* BrDU immunolabelling *Left panel* – The number of $BrDU^{+ve}$ cells (BrDU immunolabelling) were counted and expressed as a percentage of the total number of cells present (Hoechst immunolabelling). Schwann cells proliferate more when infected with the Ad-NIC adenovirus than when infected with the control Ad-GFP adenovirus (68.8 ± 1.9% and 3.0 ± 0.7% respectively)(n=6). * denotes significant level of difference ($P<0.001$). *Right panel* – BrDU immunolabelling *(red)* shows a greater number of proliferating cells in Ad-NIC-infected cultures than in control Ad-GFP cultures. Hoechst staining *(blue)* labels the Schwann cell nuclei. Scale bar, 25 μm.

(C) Activation of the endogenous Notch receptors in Schwann cells, by co-culturing with Delta-expressing 3T3 (BP-Delta) induces proliferation in Schwann cell cultures, as seen by *in vitro* BrDU immunolabelling. The number of $BrDU^{+ve}$ cells (BrDU immunolabelling) were counted and expressed as a percentage of the total number of cells present (nestin immunolabelling). 54.5 ± 3.3% of Schwann cells divide in BP-Delta 3T3 co-cultures compared to only 14.1 ± 3.8% in BP2 co-cultures (n=4). * denotes significant level of difference ($P<0.01$).

(D) Western blot analysis of cell cycle markers cyclin D1 and cdk2 in Ad-GFP- and Ad-NIC-infected Schwann cells. Notch activation leads to upregulation of these markers confirming its role in cell cycle progression. β-tubulin IB is used as a loading control and NICD IB is used as a control for NICD expression

Figure 4.7


A
100
75
50
25
0
% Proliferation (BrDU+ve Cells)
*
*
WT
MUT
WildType
Mutant
Hoescht
BrDU
B
100
75
50
25
0
% Proliferation (BrDU+ve Cells)
*
*
Ad-GFP
Ad-NIC
Hoescht
BrdU
Ad-GFP
Ad-NIC
C
80
60
40
20
0
% Proliferation (Nestin+veBrDU+ve Cells)
*
*
BP2
BP-Delta
D
Ad-GFP
Ad-NIC
Cyclin D1
Cdk 2
NICD
β–Tubulin

4.2.4.3 Notch-mediated proliferation in Schwann cells is mediated by MAPK pathways.

Among the most interesting candidates that could be involved in the Notch-mediated Schwann cell proliferation are MAP Kinase pathways since they have been implicated in mediating proliferation in Schwann cells from a range of mitogenic signals (Jessen and Mirsky, 2005; Stevens, 2006) and are also implicated in the Notch-mediated proliferation in Drosophila (Giraldez and Cohen, 2003).

Protein extracts from Schwann cell cultures, infected with Ad-GFP or Ad-NIC were obtained as above (Section 4.2.9) and analysed for Western blotting for activation of MAPK pathway components. Immunoblotting with antibodies specific for the phosphorylation of ERK1/2 and JNK 1/2 showed a dramatic increase in cultures infected with Ad-NIC (Figure 4.8A). Interestingly, unlike in Schwann cell precursors, I also found that p38 phosphorylation was not affected by Notch activation.

These results above suggest that Notch-mediated Schwann cell proliferation could be meditated by activation of the ERK 1/2 and JNK 1/2 pathways. To investigate this, we performed *in vitro* BrDU assays in Ad-NIC-infected Schwann cells, cultured with chemical blockers of ERK1/2, JNK 1/2 and p38 phosphorylation (Section 2.3.9). Briefly, Schwann cells, cultured on coverslips, were infected with Ad-NIC adenovirus, and the next day, the medium was changed to either control medium (DM), or DM supplemented with DM supplemented with JNK peptide, UO126 or SB202190. The next day, an *in vitro* BrdU assay was performed and the percentage of $BrDU^{+ve}$ cells determined. Blocking of JNK 1/2 phosphorylation (using its antagonist JNK peptide) and blocking of ERK 1/2 phosphorylation (using its antagonist UO126) as compared to control-treated Ad-NIC-infected Schwann cells resulted in a reduction of number of $BrDU^{+ve}$ cells of about 82.8% and 98.5%

respectively. Predictably, blocking p38 phosphorylation, using the antagonist SB202190, did not result into any significant reduction in proliferation (Figure 4.8C).

To further illustrate the role of MAPK pathways in Notch-mediated Schwann cell proliferation, I performed Western blotting analysis of protein extracts from Schwann cells, infected with Ad-NIC adenovirus and cultured in either DM or DM supplemented with UO126. Protein extracts were obtained as described before (4.2.9) and immunoblotting was performed with antibodies specific for cyclin D1 and cdk2. Notch-mediated upregulation of cyclin D1 and cdk2 was abolished by this blocker, further confirming the role of ERK 1/2 in Notch-mediated proliferation.

4.2.4 Notch signalling and Schwann cell myelination.

At birth, immature Schwann cells exit the cell cycle and begin their differentiation into myelinating Schwann cells (Jessen and Mirsky, 2005). We have shown that Notch receptor mRNA levels and NICD protein levels are downregulated just after birth (Section 4.2.3). This might be just coincident with immature Schwann cells exiting the cell cycle and beginning their differentiation and we have shown that Notch mediates Schwann cell proliferation. The other interesting possibility is that a downregulation of Notch signalling is required for their differentiation to take place, similarly to that seen in oligodendrocytes. During development, downregulation of Jagged 1 in neurons seems to be essential for oligodendrocyte maturation and myelination (Wang *et* al., 1998). Furthermore, *in vivo* studies of oligodendrocyte differentiation have shown that a reduction in the levels of Notch 1 leads to premature myelination (Givogri *et al.,* 2002). This work suggests that Notch activation acts to block CNS myelination and that a reduction in signalling is required for myelination to occur. In Schwann cells too, a similar mechanism might

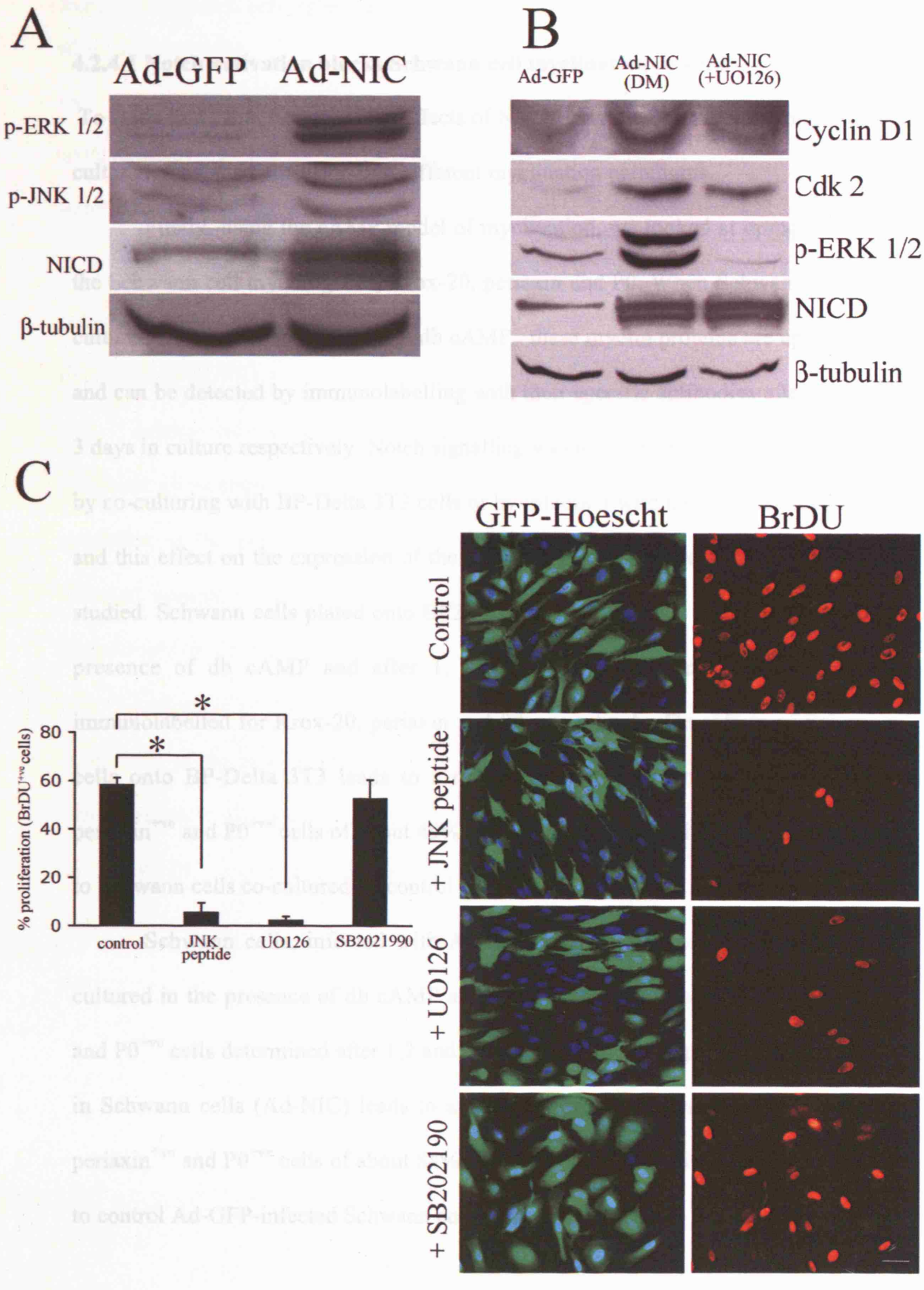
Figure 4.8: The MAPK pathway is involved in Notch-mediated Schwann cell proliferation.

(A) Western blot analysis of components of the MAPK signalling pathway from protein extracts in Ad-NIC-infected or Ad-GFP-infected Schwann cell cultures. Ad-NIC-infected cells express more p-ERK 1/2 and p-JNK 1/2 than control Ad-GFP-infected cells. p-P38 was not detected in these cultures, indicating an absence of regulation by Notch activation. β-tubulin is used as a loading control.

(B) Western blot analysis of cell cycle markers in Ad-GFP-infected Schwann cells and Ad-NIC-infected cells cultured in control medium (DM) or treated with UO126 (+UO126). Notch-mediated upregulation of the cell cycle progression markers, cyclin D1 and cdk 2 proteins is reduced by UO126, a chemical blocker of ERK 1/2 phosphorylation. This inhibitor is very potent in inhibiting ERK 1/2 phosphorylation, as shown here by the decrease in p-ERK 1/2 levels in Ad-NIC-infected Schwann cell cultures, treated with UO126. β-tubulin IB is used as a loading control and NICD IB is used as a control for NICD expression.

(C) Block of some of the components of the MAPK signalling pathway leads to a reduction in Notch-mediated Schwann cell proliferation. *Left panel* – Inhibition of JNK 1/2 and ERK 1/2 phosphorylation by treatment with JNK peptide and UO126 respectively reduces the Notch-mediated proliferation in Schwann cells, by approximately 90% and 95% respectively. Inhibition of P38 phosphorylation, by treatment with SB202190 however, has no effect. (n=6) * denotes significant level of difference ($P<0.01$). *Right panel* – There are fewer $BrDU^{+ve}$ cells *(red)* seen in Ad-NIC cultures treated with JNK peptide and UO126 than in control cultures. Cultures treated with SB202190 show a high percentage of $BrDU^{+ve}$ cells. Infected cells express GFP *(green)* and are counterstained with Hoechst nuclear staining *(blue)*. Scale bar, 25 μm.

Figure 4.8


A
Ad-GFP
Ad-NIC
p-ERK 1/2
p-JNK 1/2
NICD
β-tubulin
B
Ad-GFP
Ad-NIC (DM)
Ad-NIC (+UO126)
Cyclin D1
Cdk 2
p-ERK 1/2
NICD
β-tubulin
C
GFP-Hoescht
BrDU
Control
+ JNK peptide
+ UO126
+ SB202190
% proliferation (BrDU+ve cells)
0
20
40
60
80
*
*
control
JNK peptide
UO126
SB2021990

exist, which would correlate with our observations on the expression pattern of Notch signalling pathway components.

4.2.4.1 Notch activation blocks Schwann cell myelination *in vitro.*

To study this I first examined the effects of Notch activation on myelination in cultured Schwann cells using two different myelination paradigms.

Firstly, using the cAMP model of myelination, we looked at upregulation of the Schwann cell myelin genes, Krox-20, periaxin and P0. When Schwann cells are cultured with cAMP analogues e.g. db cAMP, these myelin proteins are upregulated and can be detected by immunolabelling with their specific antibodies after 1, 2 and 3 days in culture respectively. Notch signalling was activated in Schwann cells either by co-culturing with BP-Delta 3T3 cells or by infection with the Ad-NIC adenovirus and this effect on the expression of these myelin proteins, in response to db cAMP studied. Schwann cells plated onto BP2 or BP-Delta 3T3 cells were cultured in the presence of db cAMP and after 1, 2 and 3 days, the cells were fixed and immunolabelled for Krox-20, periaxin and P0 respectively. Co-culture of Schwann cells onto BP-Delta 3T3 leads to a reduction in the percentage of Krox-20^{+ve}, periaxin^{+ve} and P0^{+ve} cells of about 49%, 66% and 93% respectively when compared to Schwann cells co-cultured on control BP2 3T3 (Figure 4.9A).

Schwann cells, infected with Ad-GFP and Ad-NIC adenovirus were also cultured in the presence of db cAMP and the percentage of Krox-20^{+ve}, periaxin^{+ve} and P0^{+ve} cells determined after 1,2 and 3 days respectively. Again, Notch activation in Schwann cells (Ad-NIC) leads to a reduction in the percentage of Krox-20^{+ve}, periaxin^{+ve} and P0^{+ve} cells of about 84%, 77% and 91% respectively when compared to control Ad-GFP-infected Schwann cells (Figure 4.9B & C).

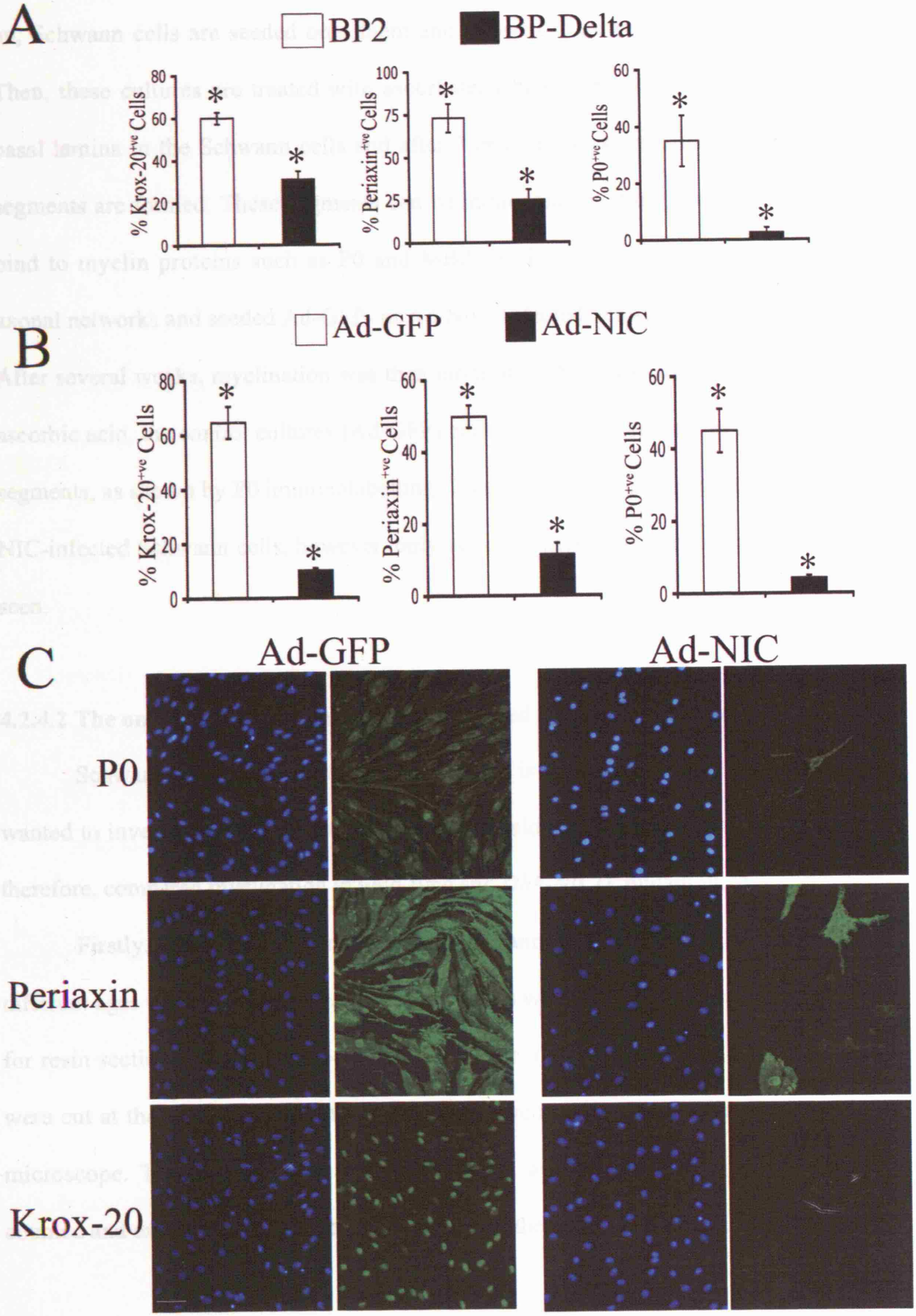
Figure 4.9: Notch activation in Schwann cells inhibits cAMP-induced upregulation of myelin proteins.

(A) Activation of the endogenous Notch receptors in Schwann cells, by co-culturing with Delta-expressing 3T3 (BP-Delta), inhibits cAMP-mediated upregulation of the myelin proteins Krox-20, periaxin and P0. There is a reduction of the percentage of Krox-20^{+ve}, periaxin^{+ve} and $P0^{+ve}$ cells in BP-Delta co-cultures compared to BP2 co-cultures by 49%, 66% and 93% respectively (n=6). * denotes significant level of difference ($P<0.001$).

(B) Adenoviral-mediated Notch activation in Schwann cells also inhibits cAMP-mediated upregulation of the myelin proteins Krox-20, periaxin and P0. There is a reduction of the percentage of Krox-20^{+ve}, periaxin^{+ve} and $P0^{+ve}$ cells in Ad-NIC-infected compared to control cultures (Ad-GFP) by about 84%, 77% and 91% respectively (n=6). * denotes significant level of difference ($P<0.001$).

(C) *Left panel* – cAMP treatment of control Schwann cultures (Ad-GFP) induces expression of the myelin proteins Krox-20, periaxin and P0 after 1, 2 and 3 days in culture respectively. *Right panel* – Adenoviral-mediated Notch activation in Schwann cells (Ad-NIC) however, prevents this cAMP-mediated upregulation of these myelin proteins. Hoechst staining *(blue)* is used to label the Schwann cell nuclei. Scale bar, 50 μm.

Figure 4.9


A
BP2
BP-Delta
% Krox-20+ve Cells
% Periaxin+ve Cells
% P0+ve Cells
B
Ad-GFP
Ad-NIC
% Krox-20+ve Cells
% Periaxin+ve Cells
% P0+ve Cells
C
Ad-GFP
Ad-NIC
P0
Periaxin
Krox-20

To validate these results, a more physiological model of myelination, the DRG-Schwann cell co-cultures (Section 2.3.6.2) was used. Briefly, in this model, a network of purified DRG neurons is first set up after several weeks in culture. Later on, Schwann cells are seeded onto them and allowed to associate with the axons. Then, these cultures are treated with ascorbate, which triggers the formation of a basal lamina in the Schwann cells and after 7 or more days treatment, myelinated segments are formed. These segments can be immunolabelled using antibodies that bind to myelin proteins such as P0 and MBP. In this study, I set up these DRG axonal networks and seeded Ad-GFP- or Ad-NIC- infected Schwann cells onto them. After several weeks, myelination was then monitored. After 14 days treatment with ascorbic acid, the control cultures (Ad-GFP) contained a good amount of myelinated segments, as shown by P0 immunolabelling (Figure 4.10). In cultures containing Ad-NIC-infected Schwann cells, however, only very few myelinated segments could be seen.

4.2.4.2 The onset of *in vivo* myelination is retarded by Notch activation.

Schwann cell myelination, at least *in vitro*, is blocked by Notch activation. I wanted to investigate whether Notch activation could block myelination *in vivo* and therefore, compared myelination in wild-type and *Dhh-NICD* mutant mice.

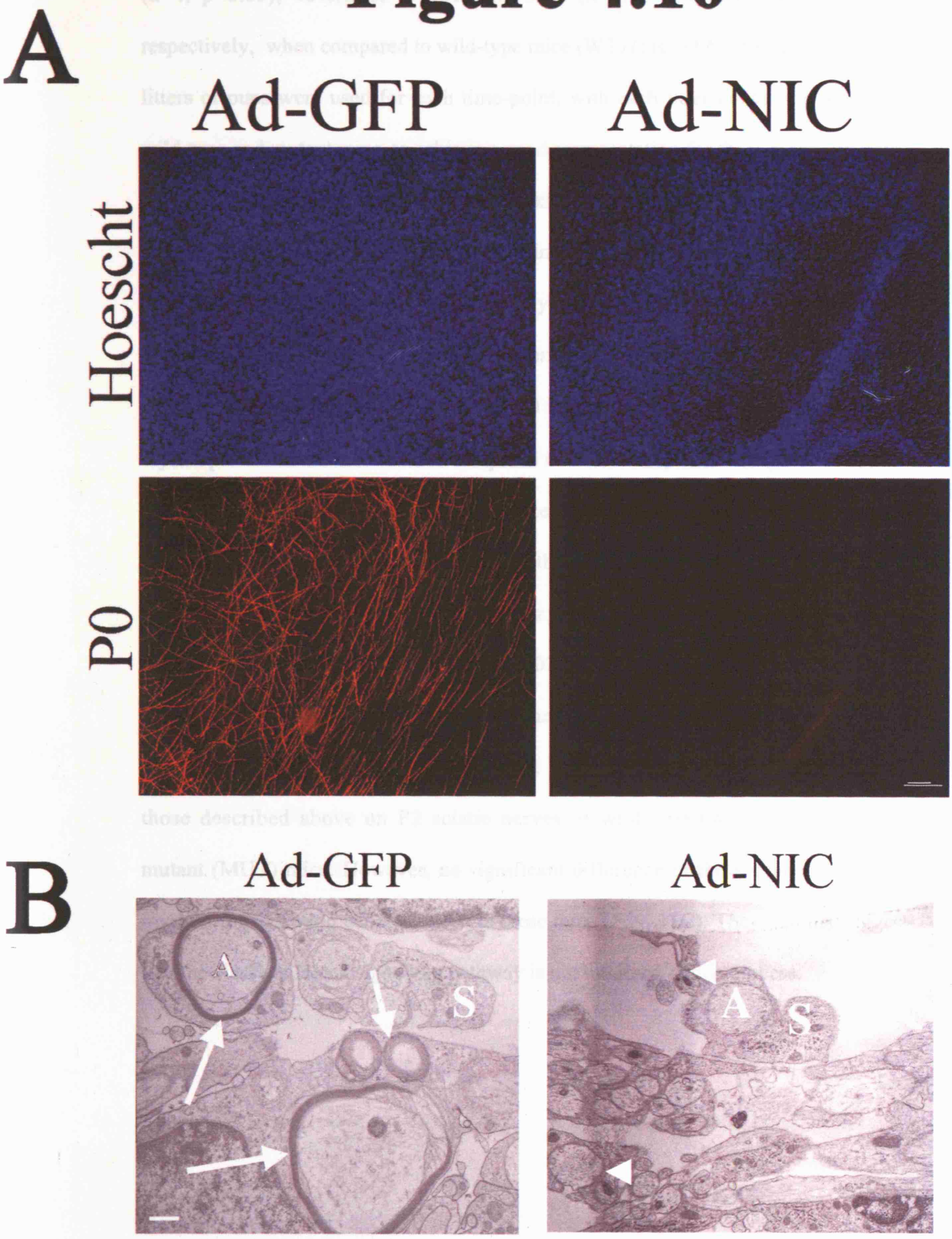
Firstly, the number of myelinating Schwann cells in the sciatic nerves at different ages was examined. Briefly, sciatic nerves were dissected out and processed for resin sections. Ultra-thin sections of new-born (NB), P2 and P5 sciatic nerves were cut at the level of the sciatic notch and viewed under a transmission electron microscope. The total number of Schwann cells with a myelinating profile was counted and expressed as percentage of the area of the nerve. In mutant mice (MUT),

Figure 4.10: Notch activation prevents myelination in Schwann cell-DRG co-cultures.

(A) Control Ad-GFP-infected Schwann cells are able to myelinate DRG axons, in response to ascorbic acid, and numerous $P0^{+ve}$ myelin segments *(red)* are seen. In contrast, Ad-NIC-infected Schwann cells are not able to myelinate similar DRG axonal networks and $P0^{+ve}$ myelin segments are rarely seen. Hoechst staining labels the Schwann cell and DRG nuclei. Scale bar, 100 μm.

(B) EM photomicrographs of DRG neurones co-cultured with Ad-GFP-infected *(left panel)* and Ad-NIC-infected *(right panel)* Schwann cells. In response to ascorbic acid, numerous myelinated axons are present in the control cultures (arrows) whereas Ad-NIC-infected Schwann cells only exist in 1:1 relationship with axons (arrowheads). A – Axon; S – Schwann cell. Scale bar 500 nm.

Figure 4.10



the average number of myelinating Schwann cells was drastically reduced by 85% ($n=4$, $p<0.05$), 40%($n=6$, $p<0.01$) and 35% ($n=4$, $p<0.05$) at NB, P2 and P5 respectively, when compared to wild-type mice (WT) (Fig. 11A). At least 4 different litters of pups were used for each time-point, with each litter containing at least 2 wild-type and mutant pups, to achieve a good representation for these measurements.

To validate the effect of this Notch-mediated block of myelination, we analysed the expression of the myelin protein P0 in sciatic nerve extracts of P2 wild-type (WT) and mutant (MUT) mice. Briefly, the sciatic nerves from P2 mice were dissected out and proteins extracted. Western blotting analysis was then performed using 5 μg of total protein per lane. The P0 immunoblot showed a reduction of this myelin protein in the mutant mice compared to the wild-type mice (Figure 4.11B).

Based on these results above, it is likely that overexpression of NICD leads to at least an initial block of myelination in the PNS. In the CNS, oligodendrocytes myelinate prematurely in Notch1 heterozygous mice, which presumably have reduced Notch signalling (Givogri *et al.*, 2002). Thus, we would predict that if Notch signalling was disrupted in Schwann cells, myelin differentiation could occur ahead of schedule similarly to oligodendrocytes. We performed experiments similar to those described above on P2 sciatic nerves of wild-type (WT) and *Dhh/RBP-J* mutant (MUT) mice. However, no significant difference in the average number of myelinating Schwann cells was seen in these mice (Fig., 11C). These results suggest that the classical Notch signalling pathway is not involved in this process.

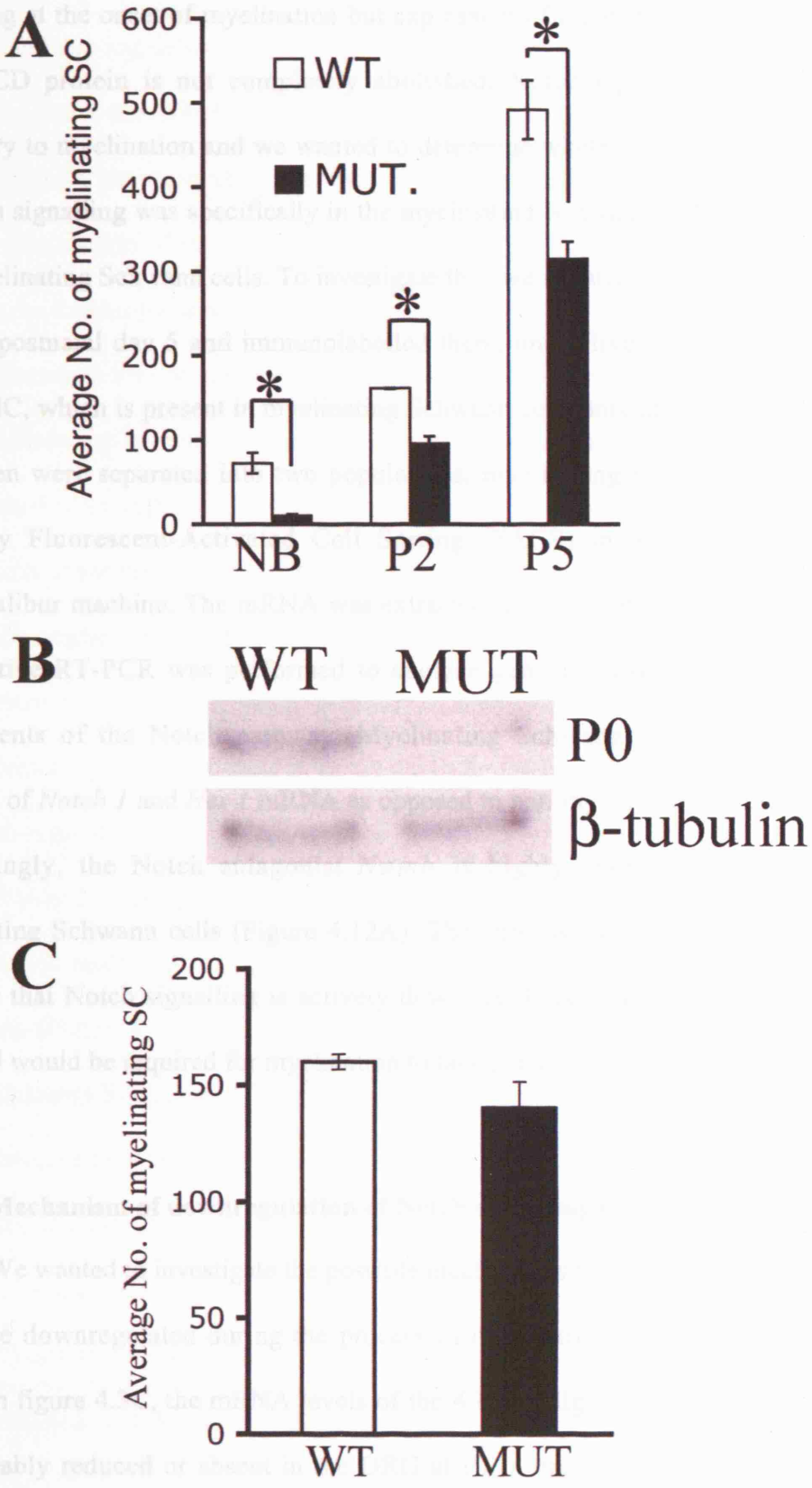
Figure 4.11: *In vivo* Notch activation in Schwann cells blocks myelination.

(A) The average number of myelinating Schwann cells in new-born (NB), P2 and P5 sciatic nerves were compared in wild-type mice (WT) and in *DhhCre/NICD* mice (MUT). In mutant mice, there was a reduction in the number of Schwann cells with a myelinating profile of 85%, 40% and 35% compared to wild-type animals in nerves at NB, P2 and P5 ages respectively. * denotes significant level of difference ($P<0.05$). The average number of myelinating Schwann cells in each nerve is expressed as the total count of myelinating Schwann cells per 10,000 μm^2 area of nerve.

(B) Western blot analysis of myelin protein P0 in P2 sciatic nerves from wild-type (WT) and *Dhh/NICD* (MUT). In mutant mice, there is a lower amount of the myelin protein P0 compared to normal mice. β-tubulin IB is used as a loading control.

(C) The average number of myelinating Schwann cells in P2 sciatic nerves were compared in wild-type mice (WT) and in *DhhCre/RBP-J* mice (MUT). The average number of myelinating Schwann cells in both wild-type and mutant mice shows no significant difference (160 ± 3.3 and $140.2 \pm 11.3\%$ respectively) (n=4). The average number of myelinating Schwann cell in each nerve is expressed as the total count of myelinating Schwann cells per 10,000 μm^2 area of nerve.

Figure 4.11


A
Average No. of myelinating SC
600
500
400
300
200
100
0
WT
MUT.
*
*
*
NB
P2
P5
B
WT
MUT
P0
β-tubulin
C
Average No. of myelinating SC
200
150
100
50
0
WT
MUT

4.2.4.3 Notch signalling is specifically downregulated in myelinating Schwann cells.

In peripheral nerves, there is a general trend in the downregulation of Notch signalling at the onset of myelination but expression of the mRNA of the receptors and NICD protein is not completely abolished. Notch signalling seems to be inhibitory to myelination and we wanted to determine whether the down-regulation of Notch signalling was specifically in the myelinating Schwann cells as opposed to non-myelinating Schwann cells. To investigate this, we isolated purified rat Schwann cells at postnatal day 5 and immunolabelled them, under live labeling conditions, with GalC, which is present in myelinating Schwann cells only at this age. The cells were then were separated into two populations, myelinating and non-myelinating cells, by Fluorescent-Activated Cell Sorting (FACS) in a Becton-Dickinson FACSCalibur machine. The mRNA was extracted from both populations and semi-quantitative RT-PCR was performed to analyze gene expression of some of the components of the Notch pathway. Myelinating Schwann cells express lower amounts of *Notch 1* and *Hes 1* mRNA as opposed to non-myelinating Schwann cells. Interestingly, the Notch antagonist *Numb* is highly expressed only in non-myelinating Schwann cells (Figure 4.12A). The expression pattern of these genes suggests that Notch signalling is actively down-regulated in myelinating Schwann cells and would be required for myelination to take place.

4.2.4.4 Mechanism of downregulation of Notch signalling during myelination

We wanted to investigate the possible mechanisms by which Notch signalling would be downregulated during the process of myelination in Schwann cells. As shown in figure 4.3C, the mRNA levels of the 4 Notch ligands examined are either considerably reduced or absent in the DRG at P7, a time at which myelination is

occurring rapidly. Absence of ligands would prevent activation of Notch receptors in Schwann cells and hence Notch signalling could be prevented.

The expression of *Numb* in myelinating Schwann cells, however, suggests that an intrinsic regulation of Notch signalling in Schwann cells could be taking place. One factor that could be involved in this process is Krox-20. This transcription factor is among the most important regulators of Schwann cell myelination and a detailed description of its role is given in Section 1.5.3.4. Recently, new roles of Krox-20, in downregulating negative regulators of myelination, have emerged. The c-Jun-N-terminal kinase pathway has been shown to inhibit myelination, and Krox-20 suppresses the activity of this pathway (Parkinson *et al.*, 2004; Parkinson DB, unpublished observations). We hence wanted to investigate whether Krox-20 could be the factor responsible for downregulating Notch signalling in Schwann cells.

We studied the expression of NICD protein in sciatic nerves of P7 Krox-20 mutant mice by Western blotting. In Krox-20 knockout mice, NICD protein levels are abnormally elevated compared to the normal wild-type mice. This suggests that the down-regulation of Notch signalling occurring at birth might be mediated by Krox-20. Also, NICD proteins show intermediate levels of expression in Krox-20 heterozygous mice, indicating an allele-dependent regulation by Krox-20 of NICD expression (Figure 4.12C).

Cultured Schwann cells express endogenous levels of NICD protein, which can be detected by Western blotting at long film exposures. We wanted to determine the effects of Krox-20 overexpression on these endogenous levels of NICD protein. Thus, Schwann cells were infected with an adenovirus encoding Krox-20 protein (Ad-K20) or its matched control virus (Ad-GFP) and after 2 days in culture, proteins were extracted and Western blotting performed. The films were exposed for 5

minutes to detect endogenous NICD levels. Krox-20 is able to greatly reduce the levels of endogenous NICD protein in Schwann cells (Figure 4.12B).

To further characterize this role of Krox-20 in reducing levels of NICD, we studied the effects of Krox-20 overexpression on artificially upregulated NICD protein in Schwann cells. Schwann cells were stably infected with a retrovirus, encoding NICD (NICD-Schwann cells). These cultures express high levels of NICD, which can be detected by Western blotting after only 3 seconds of film exposure. NICD-Schwann cells were infected with either control (Ad-GFP) or Krox-20 adenovirus (Ad-K20) and Western blotting of the protein extracts obtained after 1, 2 and 3 days following infection, carried out. Krox-20 is able to significantly reduce the expression of even these artificially elevated NICD levels in these cultures (Figure 4.12D). These results suggest that one of the functions of Krox-20 relates to the degradation of NICD protein, either directly or indirectly.

Figure 4.12: Notch signalling is downregulated in myelinating Schwann cells, a process likely mediated by Krox-20.

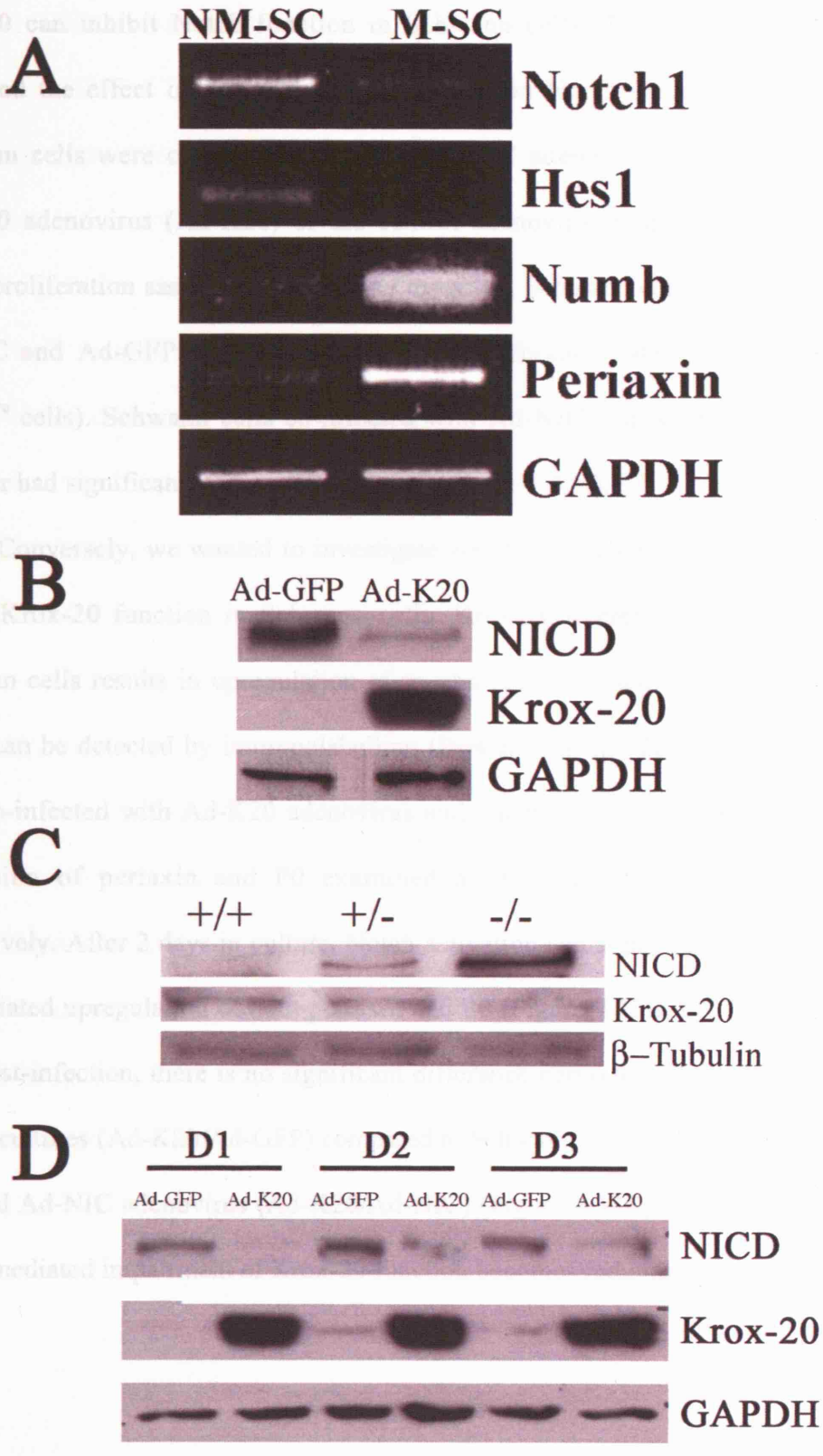
(A) Semi-quantitative RT-PCR analysis of different components of the Notch signalling pathway in myelinating (M-SC) and non-myelinating Schwann cells (NM-SC). *Notch 1* and *Hes 1* are present only in non-myelinating Schwann cells, whereas the Notch antagonist, *Numb* is highly expressed in myelinating Schwann cells. *GAPDH* is used as a loading control for both cell types. *Periaxin* is present only in myelinating Schwann cells and in this experiment, its expression in the myelinating Schwann cells population only demonstrates that the FACS procedure is highly efficient in separating the two populations of Schwann cells.

(B) Western blot analysis of NICD levels in Ad-GFP- or Ad-K20-infected Schwann cells. Krox-20 overexpression is able to reduce the endogenous levels of NICD present in Schwann cells. GAPDH IB is used as a loading control for both cultures and Krox-20 is present only in Krox-20-infected Schwann cells. (NB. Film exposure for NICD detection was 5 min.).

(C) Western blot analysis of NICD protein levels in P7 sciatic nerves of Krox-20 mutant mice. NICD protein levels are higher in Krox-20 knockout mice (-/-), than in wild-type mice (+/+). In Krox-20 heterozygotes (+/-), NICD protein shows intermediate levels of expression to wild-type and knockout mice. GAPDH IB is used as a loading control and Krox-20 IB is used as a control for Krox-20 expression.

(D) Western blot analysis of the effect of Krox-20 overexpression on NICD levels in NICD-infected Schwann cells. In Krox-20-infected cultures, NICD protein levels are greatly reduced when compared to control-infected cultures after 1 (D1), 2 (D2) and 3 days (D3) in culture. GAPDH IB is used as a loading control and Krox-20 IB is used as a control for Krox-20 expression. NB. Film exposure for NICD detection was about 2 sec.

Figure 4.12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NM-SC
M-SC
Notch1
Hes1
Numb
Periaxin
GAPDH
B
Ad-GFP
Ad-K20
NICD
Krox-20
GAPDH
C
+/+
+/-
-/-
NICD
Krox-20
β–Tubulin
D
D1
D2
D3
Ad-GFP
Ad-K20
Ad-GFP
Ad-K20
Ad-GFP
Ad-K20
NICD
Krox-20
GAPDH

4.2.4.5 Krox 20 inhibits Notch function in Schwann cells *in vitro.*

The results in the previous section suggest that Krox-20 is involved in the degradation of NICD protein, either directly or indirectly but it is not known whether Krox-20 can inhibit Notch function in Schwann cells. To investigate this, we examined the effect of Krox-20 overexpression on Notch-induced proliferation. Schwann cells were co-infected with the Ad-NIC adenovirus (Ad-NIC) and the Krox-20 adenovirus (Ad-K20) or the control adenovirus (Ad-GFP) and *in vitro* BrDU proliferation assays carried out. As expected, Schwann cells co-infected with Ad-NIC and Ad-GFP adenovirus had high proliferation rates (76.3 ± 0.8% of $BrDU^{+ve}$ cells). Schwann cells co-infected with Ad-NIC and Ad-K20 adenovirus, however had significantly fewer $BrDU^{+ve}$ cells (6.1 ± 1.4 %).

Conversely, we wanted to investigate whether Notch activation was able to impair Krox-20 function in Schwann cells. Krox-20 overexpression in cultured Schwann cells results in upregulation of myelin proteins such as periaxin and P0, which can be detected by immunolabelling (Parkinson *et al.,* 2003). Schwann cells were co-infected with Ad-K20 adenovirus and either Ad-GFP or Ad-NIC and the expression of periaxin and P0 examined after 2 and 3 days post-infection respectively. After 2 days in culture, Notch activation can significantly block Krox-20-mediated upregulation of both periaxin and P0 (Figure 4.13B, C). However, at 3 days post-infection, there is no significant difference periaxin and P0 expression in control cultures (Ad-K20/Ad-GFP) compared to Schwann cells, co-infected with Ad-K20 and Ad-NIC adenovirus (Ad-K20/Ad-NIC) (Figure 4.13B, C). Thus, with time Notch-mediated impairment of Krox-20 function becomes redundant.

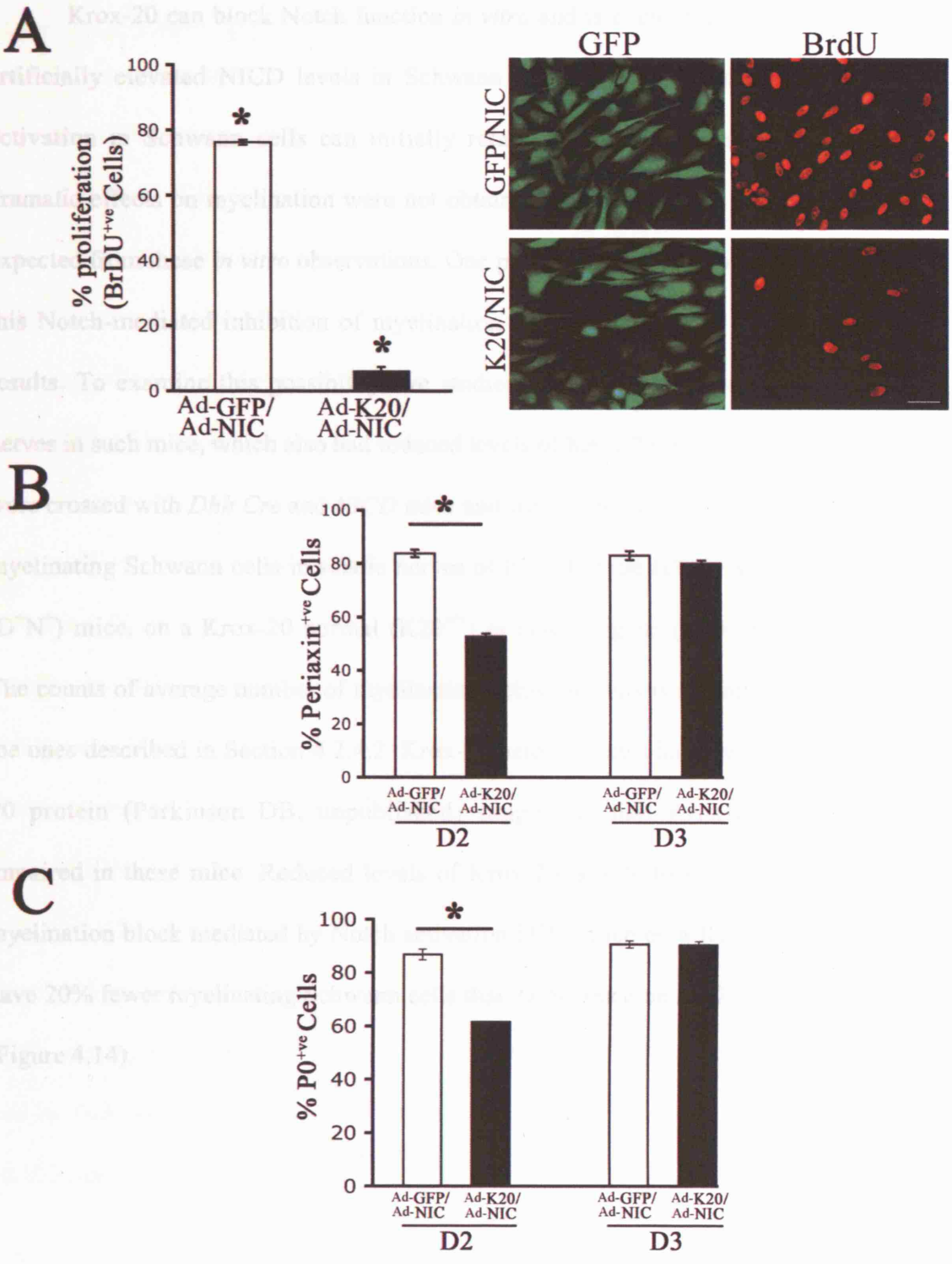
Figure 4.13: Krox-20 is able to block Notch function in Schwann cells.

(A) Krox-20 overexpression leads to a reduction in Notch-mediated proliferation in Schwann cells as shown by *in vitro* BrDU immunolabelling. *Left panel* –The percentage of $BrDU^{+ve}$ cells is lower in Ad-NIC cultures co-infected with the Krox-20 adenovirus (Ad-K20/Ad-NIC) than in Ad-NIC cultures co-infected with the control GFP adenovirus (Ad-GFP/Ad-NIC)(6.1 ± 1.4 % and 76.3 ± 0.8% respectively)(n=6). * denotes significant level of difference ($P<0.001$). *Right panel* – There are fewer $BrDU^{+ve}$ cells *(red)* seen in Ad-NIC cultures co-infected with the Krox-20 adenovirus (Ad-K20/Ad-NIC) than in Ad-NIC cultures co-infected with the control GFP adenovirus (Ad-GFP/Ad-NIC). Infected cells express GFP *(green)*. Scale bar, 25 μm.

(B) Notch activation can initially reduce the Krox-20-mediated upregulation of Periaxin. At 2 days post-infection, there are 83.8 ± 1.4% $periaxin^{ve}$ cells in control cultures (Ad-K20/Ad-GFP) and 52.7 ± 1.2% $periaxin^{ve}$ cells in Notch activated cells (Ad-K20/Ad-NIC). However no difference is seen in control cultures (Ad-K20/Ad-GFP) and Notch activated cells (Ad-K20/Ad-NIC) (83.1 ± 1.7% and 80.0 ± 1.0% respectively) (n=6) * denotes significant level of difference ($P<0.001$).

(C) Notch activation can initially reduce the Krox-20-mediated upregulation of P0. At 2 days post-infection, there are 86.8 ± 2.1% $P0^{+ve}$ cells in control cultures (Ad-K20/Ad-GFP) and 61.6 ± 0.03% $P0^{+ve}$ cells in Notch activated cells (Ad-20/Ad-NIC). However no difference is seen in control cultures (Ad-K20/Ad-GFP) and Notch activated cells (Ad-K20/Ad-NIC) (90.6 ± 1.4% and 90.3 ± 1.4% respectively) (n=6) * denotes significant level of difference ($P<0.001$).

Figure 4.13


A
GFP
BrdU
GFP/NIC
K20/NIC
% proliferation (BrDU+ve Cells)
100
80
60
40
20
0
*
*
Ad-GFP/ Ad-NIC
Ad-K20/ Ad-NIC
B
% Periaxin+ve Cells
100
80
60
40
20
0
*
Ad-GFP/ Ad-NIC
Ad-K20/ Ad-NIC
Ad-GFP/ Ad-NIC
Ad-K20/ Ad-NIC
D2
D3
C
% P0+ve Cells
100
80
60
40
20
0
*
Ad-GFP/ Ad-NIC
Ad-K20/ Ad-NIC
Ad-GFP/ Ad-NIC
Ad-K20/ Ad-NIC
D2
D3

4.2.4.6 *In vivo* block of myelination by Notch activation is further impaired by lack of Krox 20.

Krox-20 can block Notch function *in vitro* and is even able to downregulate artificially elevated NICD levels in Schwann cells. We have shown that Notch activation in Schwann cells can initially retard myelination *in vivo*, but more dramatic effects on myelination were not obtained in these mice as we would have expected from these *in vitro* observations. One possibility is that Krox-20 can inhibit this Notch-mediated inhibition of myelination in these mice, thus giving us such results. To examine this possibility, we studied the myelination profile of sciatic nerves in such mice, which also had reduced levels of Krox-20. Krox-20 mutant mice were crossed with *Dhh Cre* and *NICD* mice and we compared the average number of myelinating Schwann cells in sciatic nerves of P3 wild-type (D^+N^-) and *Dhh-NICD* (D^+N^+) mice, on a Krox-20 normal ($K20^{+/+}$) or heterozygous ($K20^{+/-}$) background. The counts of average number of myelinating Schwann cells is essentially similar to the ones described in Section 4.2.4.2. Krox-20 heterozygotes have reduced levels of P0 protein (Parkinson DB, unpublished) suggesting that Krox-20 function is impaired in these mice. Reduced levels of Krox-20 is able to further enhance the myelination block mediated by Notch activation D^+N^+ mice on a $K20^{+/-}$ background have 20% fewer myelinating Schwann cells than D^+N^+ mice on a $K20^{+/+}$ background (Figure 4.14).

Figure 4.14: Lower levels of Krox-20 are able to reduce further the myelination retardation defect in mice with Schwann cells that overexpress NICD.

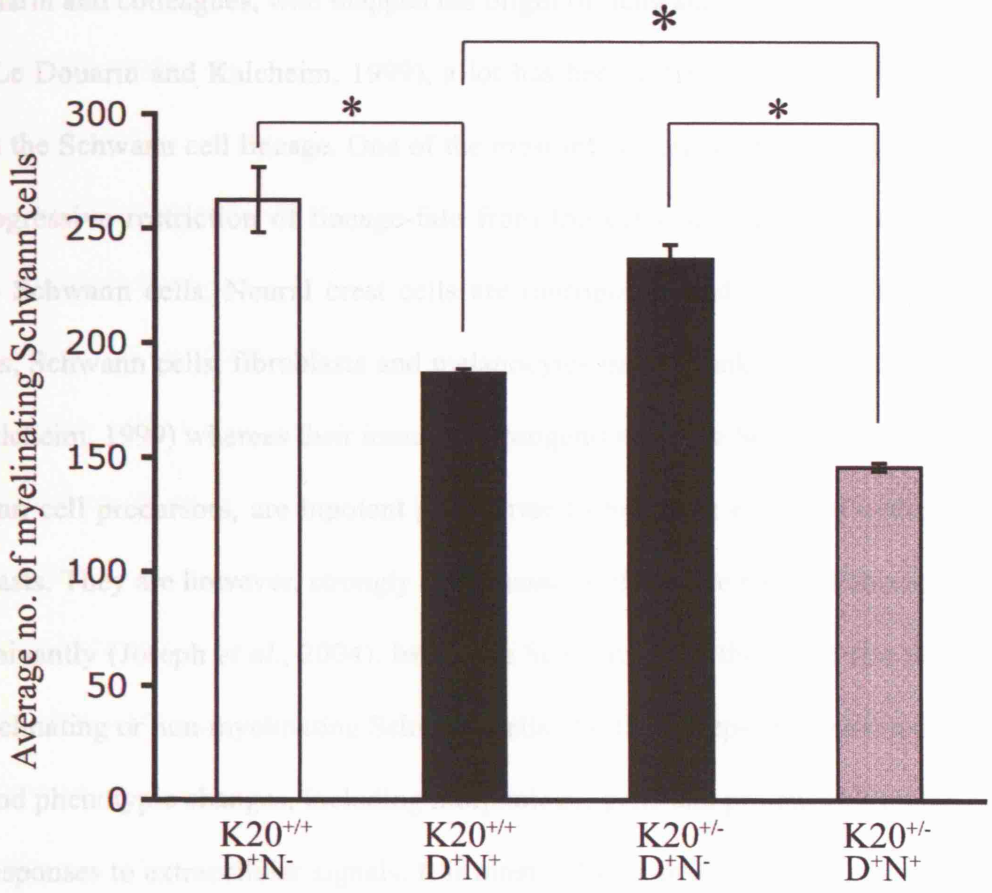
Average number of myelinating Schwann cells in wild-type mice (D^+N^-) and in mutant mice (*DhhCre/NICD;* D^+N^+) with either normal levels of Krox-20 (Krox20 wild-type; $K20^{+/+}$) or reduced levels of Krox-20 (Krox20 heterozygotes; $K20^{+/-}$).

Conditional activation of Notch in Schwann cells leads to a reduction in number of Schwann cells with a myelinating profile in mice with equivalent levels of Krox-20. In normal Krox-20 levels, forced Notch activation in Schwann cells ($K20^{+/+}$ D^+N^+) leads to a reduction of 30% in the average number of myelinating Schwann cells when compared to wild-type mice ($K20^{+/+}$ D^+N^-). At reduced Krox-20 levels, Notch activation ($K20^{+/-}$ D^+N^+) leads to a reduction of 35% in the average number of myelinating Schwann cells when compared to wild-type mice ($K20^{+/-}$ D^+N^-)(n=4). * denotes significant level of difference ($P<0.05$).

However, reduced levels of Krox-20 further enhance this block in myelination induced by forced Notch activation. $K20^{+/-}$ D^+N^+ mice have 20% fewer myelinating Schwann cells than $K20^{+/+}$ D^+N^+ mice (n=4). * denotes significant level of difference ($P<0.05$).

In wild-type mice (D^+N^-), the number of myelinating Schwann cells in $K20^{+/+}$ or $K20^{+/-}$ situations are not statistically different (n=6). The average number of Schwann cell in each nerve is expressed as the total count of myelinating Schwann cells per 10,000 μm^2 area of nerve.

Figure 4.14


*
*
*
300
250
200
150
100
50
0
Average no. of myelinating Schwann cells
K20+/+ D+N-
K20+/+ D+N+
K20+/- D+N-
K20+/- D+N+

4.3 DISCUSSION

Over the past thirty years, starting with the pioneering experiments by Nicole Le Douarin and colleagues, who mapped the origin of Schwann cells from neural crest cells (Le Douarin and Kalcheim, 1999), a lot has been learned about the biology of cells in the Schwann cell lineage. One of the most interesting aspects in the lineage is the progressive restriction of lineage-fate from the cells of origin, neural crest to mature Schwann cells. Neural crest cells are multipotent and give rise to sensory neurons, Schwann cells, fibroblasts and melanocytes in the trunk region (Le Douarin and Kalcheim, 1999) whereas their immediate progenitors in the Schwann cell lineage, Schwann cell precursors, are bipotent giving rise to Schwann cells and endoneurial fibroblasts. They are however, strongly glial-biased as they give rise to Schwann cells predominantly (Joseph *et al.*, 2004). Immature Schwann cells then give rise to either the myelinating or non-myelinating Schwann cells. All these steps are characterised by profound phenotypic changes, including morphology, gene and protein expression and their responses to extracellular signals. It is most unlikely that this lineage or even the individual transition steps are governed by one universal signalling pathway. Most of the processes occurring in the lineage are probably governed by a number of signalling pathways, many of which could be either redundant or acting co-operatively with one another. The one signalling pathway that has been shown to be vital in the Schwann cell lineage is the neuregulin pathway, in experiments done either *in vitro* and/or *in vivo*. It may be involved in the generation of Schwann cell precursors from neural crest cells, by directing them away from a neuronal fate (Shah *et al.*, 1994). It promotes the Schwann cell precursor maturation into immature Schwann cells (Dong *et al.*, 1995; Jessen *et al.*, 1994) and is involved in the control of myelination (Lemke, 2006). Nowadays, each step in this lineage has been extensively characterised in terms of its

phenotypic attributes and hence the mechanisms involved in the control of individual processes, including differentiation, proliferation or myelination can be extensively studied both *in vitro* and *in vivo*.

In this study, we wanted to examine the role of Notch signalling in the Schwann cell lineage, based on observations of its role in other systems, in particular in the differentiation of CNS glia. The expression pattern of Notch receptor mRNA and, more importantly, NICD protein, suggests that Notch signalling might have differential roles in the Schwann cell lineage. *In vitro* clonal analysis of the differentiation of E11.5 rat neural crest cells suggests that Notch signalling might be instructing them to enter the glial lineage (Kubu *et al.*, 2002). This work shows that the number of clones containing Schwann cells and other cell-types or clones containing Schwann cells only is increased with Notch activation. However, other work has shown than enforced activation of Notch *in ovo* does not lead to an increase in gliogenesis (Endo *et al.*, 2002; Morrison *et al.*, 2000), and other *in vitro* work shows that Notch activation in quail neural crest cells does not lead to differentiation into Schwann cells (Wakamatsu *et al.*, 2000). Our experiments with rat neural crest cells show similar results, as we do not see any increase in expression of Schwann cell precursor-associated markers when Notch is activated. *In vivo*, as well, our experiments show that a disruption in Notch signalling does not lead to any defect in gliogenesis, at least as observed in $Hes1^{-/-}$ and $Hes5^{-/-}$ embryos. If Notch activation was indeed required to instruct neural crest cells to enter the glial lineage, as proposed by Morrison and colleagues, we would anticipate that gliogenesis should have been considerably impaired in the Hes1 and Hes5 knockout mice, at least if Notch were signalling through the classical CSL-dependent pathway. Further work still needs to be done however, to provide a definitive answer to this still controversial matter. Glial

differentiation in Hes1 and Hes5 double-knockouts has to be characterised, as they both might have mutually redundant roles. Alternatively, RBP-J knockout mice can be studied and the role of CSL-dependent Notch signalling in gliogenesis determined. More importantly, glial differentiation in Notch receptor knockouts has to be considered as that would involve the disruption of both CSL-dependent and independent pathways. CSL-independent Notch signalling, mainly through Deltex, is increasingly becoming more prominent as a Notch signalling pathway of a number of cell-types, including glial cells in the CNS (see Section 1.9.1.2).

The experiments carried out by Kubu and colleagues (2002) involve the culture of a sub-population of E10.5 NCSCs, and show that Notch promotes glial differentiation over a period of 14 days. Neural crest cells, however, give rise to Schwann cell precursors, which then generate Schwann cells (Jessen *et al.*, 1994) and they can be easily distinguished by differential expression of antigenic markers (Dong *et al.*, 1999; Jessen *et al.*, 1994). The one possibility that might arise from this clonal analysis is that the long-term and complex culture conditions might initially cause the differentiation of the neural crest cells into Schwann cell precursors, which then mature to give rise to the Schwann cells. Thus, Notch function could possibly be important in the second step rather than the first step, explaining its pro-gliogenic effects. Importantly, the same studies show that cells isolated from E14.5 sciatic nerves, which correspond to the Schwann cell precursor stage, are much more sensitive to Notch activation and they have 10-fold higher levels of Notch receptors. Our initial experiments suggest that Notch activation is not involved in the differentiation of neural crest cells into Schwann cell precursors. We, however, show that Notch activation is highly potent in mediating the Schwann cell precursor – Schwann cell transition, both *in vitro* and *in vivo,* a mechanism that is most likely to be

mediated by enhanced responsiveness to neuregulin-1 signalling and involving the p38 MAPK pathway. Similar paradigms of Notch activation mediating function by enhancing neuregulin-1 signalling have been shown to be present in radial glia differentiation (Patten *et al.*, 2003; Schmid *et al.*, 2003).

As well as enhancing the generation of immature Schwann cells, Notch signalling seems to be important in their maintenance. Both *in vivo* and *in vitro*, Notch signalling is shown to stimulate cell division in Schwann cells, probably acting through the ERK 1/2 and JNK 1/2 MAPK pathways. Moreover, Notch signalling retards the further differentiation of immature Schwann cells into the mature myelinating phenotype. During normal development, Notch levels go down specifically in myelinating Schwann cells, and when Notch signalling is activated, a block in myelination is seen, both *in vivo* and *in vitro*. Myelination involves first an arrest in cell division and then subsequent upregulation of myelin genes, both processes that are likely to be mediated by the transcription factor Krox-20 (Parkinson *et al.*, 2004; Topilko *et al.*, 1998). In our experiments, we show that Krox-20 can decrease Notch levels and inhibit Notch function, at least *in vitro*. Furthermore, *in vivo* Notch activation seems to be regulated by the levels of Krox-20. In essence, during normal development, one likely role of the Notch signalling pathway seems to be the expansion of the immature Schwann cell population, which is required for matching the number of Schwann cells to the number of axons in the nerves. Notch signalling might then be specifically inactivated in the myelinating Schwann cells, through a mechanism involving Krox-20, to allow myelination to proceed normally.

Similar paradigms of Notch function exist in other systems, most notably during postnatal myogenesis. Following muscle injury, satellite cells, the primary constituents of regenerating muscles, begin to proliferate and then commit to a

myoblast fate. It has recently been shown that the initial wave of proliferation is mediated by Notch signalling as the satellite cells show increased expression of activated Notch protein (Conboy and Rando, 2002). Interestingly, the authors also show that Numb becomes then asymmetrically localised in daughter cells and this leads to an attenuation of Notch-mediated proliferation and promotion of myogenic differentiation. Our system shows similar parallels in that Notch signalling promotes the expansion of the immature Schwann cell pool, and subsequent downregulation is required for differentiation into myelinating Schwann cells. The upregulation of Numb protein could possibly explain this attenuation of Notch signalling in myelinating Schwann cells, as seen in our FACS analysis. Some unknown signal during the process of radial sorting could upregulate Numb in the Schwann cells destined to form myelinating Schwann cells, and this would inhibit Notch-mediated cell division, which is essential for subsequent myelination. Further characterisation of Numb function *in vivo*, could give us important clues in this process of radial sorting and myelination in peripheral nerves. In normal development, Schwann cells are likely to divide asymmetrically during radial sorting, as they separate a single large diameter axon from a group of axons and form a 1:1 relationship with it. Conditional loss of function of Numb in Schwann cells could possibly perturb this process, shedding more light on the mechanisms involved in radial sorting.

This study mostly involved overexpression of NICD in cells at different stages in the lineage and importantly, observations seen *in vitro* were also seen *in vivo*. However, the predicted outcome of the effects of Notch loss-of-function for some of these observations was not obtained. In view of the role of activation of Notch signalling in promoting the Schwann cell precursor – Schwann cell transition, loss of Notch signalling should have delayed this transition. Similarly, loss of Notch function

in the immature Schwann cells should have resulted in premature myelination, since Notch inhibits myelination. However, no effect was seen when RBP-J was conditionally inactivated in those cells. One possible explanation is that some of the effects of Notch signalling in these cells could be mediated through the CSL-independent pathway, and more specifically through Deltex. It is only recently that Notch signalling though CSL-independent mechanisms have been recognised (Martinez Arias *et al.*, 2002) and by now, the roles of Notch signalling through Deltex have been identified in a number of systems (Ciu *et al.*, 2004; Hu *et al.*, 2003; Kishi *et al.*, 2001; Luo and Rando, 2004; Yamamoto *et al.*, 2001). For example, it has been shown recently that both CSL-dependent and Deltex-dependent mechanisms are important in mediating the differentiation of radial glial cells from astrocytes (Patten *et al.*, 2006). Deltex-dependent Notch signalling could still take place in mice in which RBP-J was conditionally inactivated, explaining the lack of effect seen. To circumvent this shortcoming in the study of Notch disruption in our system, Notch receptors should be conditionally inactivated. Lack of Notch receptors would prevent both CSL-dependent and Deltex-dependent downstream signalling and would test whether a direct effect on Schwann cell generation and myelination could be seen.

Overall our study shows that Notch signalling is important for the generation and maintenance of immature Schwann cells. Block of Notch activation is then required for subsequent myelination and this effect is likely to be due to Krox-20 inhibition of Notch function. Interestingly, the evidence suggests that both CSL-dependent and CSL-independent downstream Notch signalling may be required for Notch function *in vivo*.

CHAPTER 5: A comparative analysis of the transplant properties of Schwann cells and Schwann cell precursors in animal models of Multiple Sclerosis (MS).

5.1 INTRODUCTION

As discussed in Chapter 1, MS is characterised by multiple focal demyelinating plaques within the CNS and axonal degeneration, leading to neurological impairment. One strategy considered for repair in MS is the transplant of myelinogenic cells that would remyelinate the demyelinated axons, and restore saltatory conduction as well as prevent further axonal degeneration. Ideally the cells to be transplanted should be able to survive and migrate in the CNS and populate and myelinate the multiple lesions in MS (Blakemore and Franklin, 2000; Franklin, 2002). A number of cell types have been considered, including cells of the oligodendrocyte lineage, Schwann cells, olfactory ensheathing cells and stem cells and the merits of each of them, in terms of the ideal properties they should possess, are described in more detail in Section 1.11.3.1 (Franklin, 2002, Pluchino *et al.*, 2004).

Schwann cells are the only cell types that have so far been considered for a clinical trial on patients with MS, but unfortunately no success was obtained. Brain biopsies performed 5 months after transplant revealed no surviving Schwann cells in the lesions and the clinical trial was abandoned in 2003 (Pluchino *et al.*, 2003; http://www.myelin.org/06232003.htm). These cells were considered because of the considerable advantages that they provide; they can easily remyelinate demyelinated CNS axons, the myelin they form should be able to withstand the autoimmune injury

in MS and they can easily be obtained from human biopsy material for autologous transplantation, avoiding the risk of immune rejection (Blakemore and Franklin, 2000; Franklin, 2002). Nevertheless, these cells have many drawbacks when investigated in animal models of MS, and these are described in Section 1.11.4.2, with all the references therein. Briefly, they do not interact very well with astrocytes, and when transplanted in demyelinating lesions, they are usually found in regions where astrocytes are absent. They are also unable to survive and migrate after transplant in normal CNS white matter.

In order to circumvent these drawbacks of Schwann cells, we wanted to investigate the transplant characteristics of an earlier stage of the Schwann cell lineage, Schwann cell precursors. These cells are an intermediate stage in the Schwann cell lineage, being derived from neural crest cells and giving rise to Schwann cells (Jessen *et al.,* 1994). They are present in rats at around E14/15 and factors including neuregulin-1 and endothelins have been shown to regulate their transition to Schwann cells. Schwann cell precursors and Schwann cells differ significantly in their antigenic profile and survival characteristics (Chapter 1; Jessen and Mirsky, 2004). Also, unlike Schwann cells, Schwann cell precursors are highly motile cells that accompany migrate growing axons in peripheral nerves, migrating with them as they extend. *In vitro*, they have also been shown to migrate at least 10 times faster than Schwann cells (Jessen et al., 1994).

We therefore proceeded to make a comparative analysis of the myelinogenic potential, survival and migratory properties and interactions with host CNS tissue, of Schwann cells and Schwann cell precursors transplanted in two animal models of MS. This work has been done in collaboration with Professors WF Blakemore and

RJM Franklin at the University of Cambridge (see Chapter 2 for details of their contribution to this study).

5.1.1 Animal Models of MS used in present study

5.1.1.1 Ethidium bromide (EB) model of focal demyelination

This model involves creating a focal area of demyelination in adult white matter by injection of a 0.01% solution of ethidium bromide, which is a glio-toxic agent (Blakemore and Crang, 1989; Shields *et al,* 2000). These lesions can provide a good model for characterising the remyelination potential of cells transplanted into them. An additional advantage of such lesions is that they undergo spontaneous remyelination, which is useful for studying interactions with host-derived cells. A variant of this model is the suppression of endogenous repair after lesions, by exposure to 40 Grays of irradiation (Blakemore and Crang, 1985; Hinks *et al.*, 2001).

5.1.1.2 Retinal Model of Myelination

The retina consists of a layer of unmyelinated large axons along its whole surface (nerve fibre layer). Throughout life, these axons remain unmyelinated due to the inability of OPCs to migrate out of the optic nerve into the retina (ffrench-Constant *et al.*, 1988; Perry and Lund, 1990). Previous studies have shown that the nerve fibre layer can be myelinated by transplant of myelinogenic cells in the retina (Ader *et al.*, 2000; Huang *et al.,* 1991; Laeng *et al.*, 1996), and recently techniques to quantify myelination in the retina after cell transplant have been described (Setzu *et al.*, 2004). This model provides an excellent model for a quantitative comparison of the myelinogenic potential between different cell types. An additional advantage of this model is that the unmyelinated axons are embedded in a dense matrix of astrocytes,

cells that are present in MS lesions and, which have adverse interactions with Schwann cells in most experiments (Blakemore, 1976; Ghirnikar and Eng, 1994; Lakatos *et al.,* 2000, Shields *et al.*, 2000). The retina is therefore particularly useful for the interactions of transplanted cells with these potential inhibitors of myelin repair.

5.2 RESULTS

5.2.1 Assaying the transplantation properties of Schwann cell precursors and Schwann cells under strictly comparable conditions.

To have a strict comparison of the transplantation properties of Schwann cells and Schwann cell precursors, we needed to standardize the method of obtaining pure populations of both cell-types using similar conditions.

Because Schwann cell precursors cannot be kept in culture for more than 4 days, without maturing irreversibly to Schwann cells (Jessen *et al.*, 1994; Dong *et al.*, 1995), we decided to use freshly dissociated cells for the transplants. Sciatic nerves and brachial plexus from E14.5 rat embryos were dissected and digested in enzyme cocktail (see Chapter 2) for 1 hr. The digested nerves were washed in L15 medium and the cells obtained were then resuspended in DMEM containing 10ng/ml neuregulin-1. 1μl of this cell suspension, at a density of 10,000 cells/μl, was then injected in all of our transplant experiments. We examined the purity of these cells suspensions to make sure that they did not contain any contaminating fibroblasts. Fibroblasts have deleterious effects in Schwann cell-mediated remyelination, when Schwann cells suspensions contaminated with fibroblasts are transplanted in rodent demyelinating lesions. Only Schwann cell suspensions containing less than 10% fibroblasts were associated with remyelination (Brierley *et al.*, 2001). In five independent tests, we calculated the purity of the cells obtained, by immunolabelling them with the antibodies to L1 to identify the glial cells. Some of the cells that were isolated for transplant studies were plated onto PDL-laminin coated coverslips at a density of 1000 cells/15μl. 3 hrs later, the cultures were immunolabelled for L1 and the percentage of $L1^{+ve}$ cells determined (average of

number of $L1^{+ve}$ cells per total number of cells). We found, on average, that 99.1±5.6% of the cells in these cultures were $L1^{+ve}$.

In most previous studies performed involving Schwann cells, they were transplanted only after long-term culture (Blakemore and Crang, 1985; Brierley *et al.*, 2001; Iwashita *et al.*, 2000, Shields *et al.*, 2000). In our studies, however, we wanted to use freshly dissociated Schwann cells to have a strict comparison with Schwann cell precursors that were freshly isolated. Schwann cells were obtained from sciatic nerves and brachial plexus of newborn or P1 rats. The cell suspensions obtained usually have a significant number of contaminating fibroblasts and we therefore decided to purify them by negative immunopanning. Briefly, after digestion, the cells obtained were resuspended in DMEM containing 10% foetal calf serum and then cultured for 20 minutes Petri dish coated with Thy1.1 antibodies. The fibroblasts present bound to the Thy1.1 antibody and remained attached to the dish, whereas the Schwann cells did not attach. The cell suspension was then recovered and washed in L15 medium. The cells were resuspended in DMEM containing 10ng/ml neuregulin-1 and 1µl of this cell suspension, at a density of 10,000 cells/µl, was used for all of our transplant experiments. The purity of the cells was determined by immunolabelling with L1 antibody (see above, as described for Schwann cell precursors) and in five independent observations, we found that 99.8±1.3% of the cells were $L1^{+ve}$ glial cells.

In order to fully characterise the properties of these cells, we had to be able to identify them after transplant. Since we used freshly isolated cells, we could not label them with a marker such as LacZ, which requires infection with a retrovirus after long-term culture. We therefore decided to use cells obtained from transgenic rats that ubiquitously express the enhanced green fluorescent protein (GFP) under a

chicken β-actin promoter and cytomegalovirus enhancer (CMV) (see Section 2.5.2). Transgenic males were crossed with normal Sprague-Dawley females and usually half the offspring obtained carried the transgene. Schwann cell precursors were obtained from such offspring at E14, and are hence referred to as GFP-Schwann cell precursors, and Schwann cells were obtained from newborn or P1 pups and are referred to as GFP-Schwann cells in this chapter. All of our transplant studies were repeated at least twice and usually involved 4 or more animals per group, unless stated otherwise.

5.2.2 Schwann cell precursors survive and myelinate at least as efficiently as Schwann cells in EB lesions.

Schwann cells show good remyelination when transplanted into the EB model of focal demyelination of the spinal cord and their myelinogenic properties have been extensively characterised. The cells used in the transplants, however, were always kept in culture for at least two weeks (Blakemore, 1977; Duncan *et al.*, 1981; Blakemore and Crang, 1985). Since we were using freshly dissociated Schwann cells for our experiments, we decided to investigate whether they had similar myelination and survival transplant properties to Schwann cells kept under longer-term culture conditions. GFP-Schwann cells were injected into an EB lesion and 1 month later, the spinal cord segment dissected out and processed for resin or frozen sections. The GFP-Schwann cells were well distributed within the EB lesions in the dorsal funiculus of the spinal cord (Figure 5.1A). Even after 1 month following transplant, the cells showed intense green fluorescence when viewed under a fluorescent

Figure 5.1: Freshly dissociated GFP-Schwann cells survive and myelinate demyelinated axons in an EB lesion.

GFP-Schwann cells were transplanted into an EB lesion at T13 and 1 month later, the spinal cord segment dissected out and processed for resin or frozen sections.

(A) *Left Panel* – Semi-thin section of a spinal cord 4 weeks after an EB lesion, stained with toluidine blue. Arrow and white line demarcation show part of dorsal funiculus devoid of oligodendrocyte myelin after EB injection. *Right Panel* - Frozen transverse section of a spinal cord segment showing the presence of the transplanted Schwann cells, as seen by the expression of GFP *(green),* within the EB lesion in the dorsal funiculus. The structure of the spinal cord is seen as red from a brightfield picture taken and the outline of the white matter is demarcated from the grey matter by the white line in the picture.

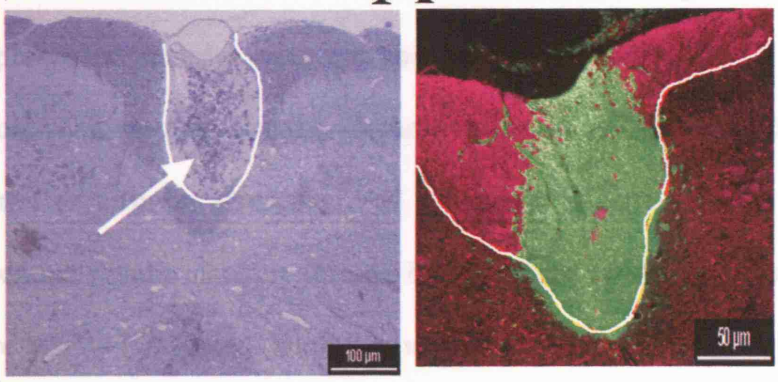
(B) Frozen sections were immunolabelled with P0 antibody. P0 immunoreactivity *(middle panel)* colocalises with GFP, expressed by the transplanted cells *(top panel)*. These pictures show that the transplanted Schwann cells can form $P0^{+ve}$ peripheral-like myelin in the CNS.

(C) *Left panel* – Semi-thin sections of a spinal cord segment showing part of an EB lesion containing transplanted Schwann cells. The sections were stained with toluidine blue. Dark circular structures represent myelin formed by the oligodendrocytes (arrows). Light circular structures represent myelin formed by transplanted Schwann cells (arrowheads).

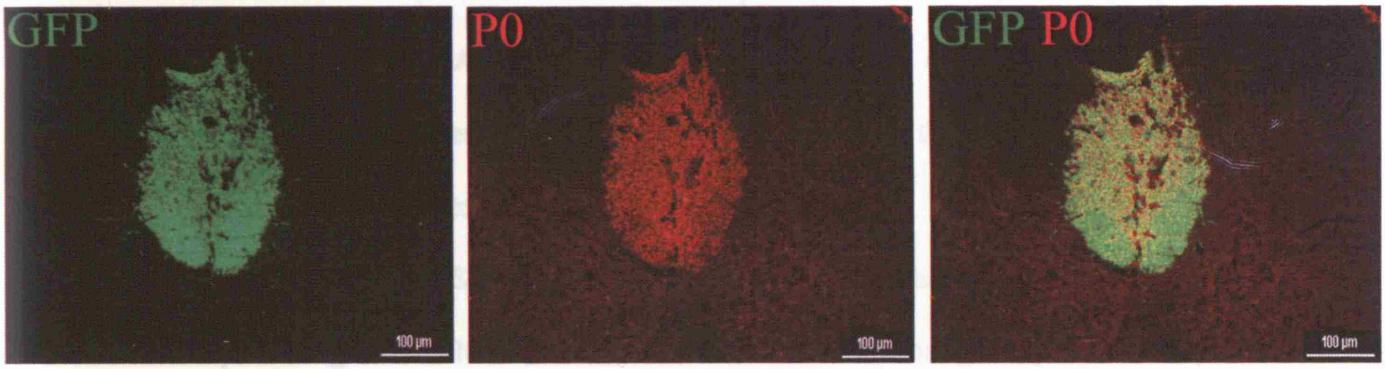
Right panel – Electron micrograph illustrating the myelinating profile of transplanted GFP-Schwann cells in EB lesions. They form the typical 1:1 relationship with the CNS axons and form myelin wraps around them. A, CNS axons; M, myelin.

Figure 5.1

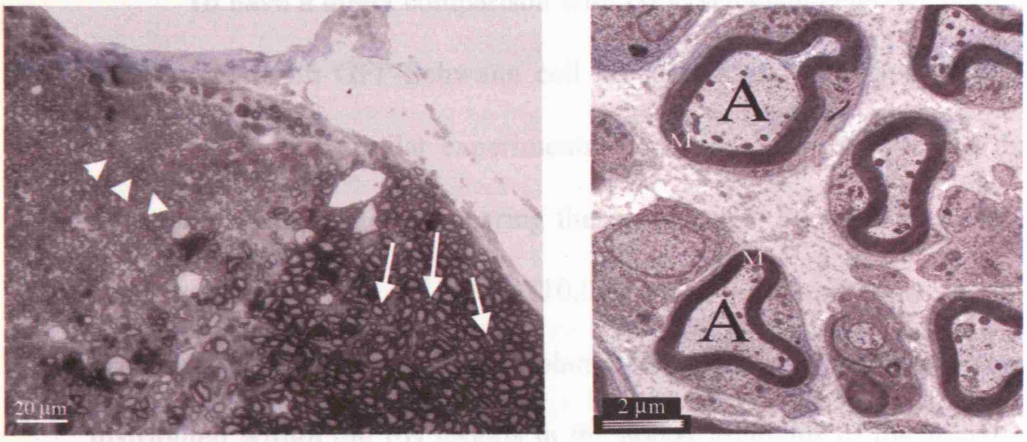
A



B



C



microscope, and no immunolabelling was required to detect the GFP protein. Frozen sections were immunolabelled with P0 antibody, which binds to myelin formed by Schwann cells. GFP-Schwann cells were able to remyelinate the demyelinated axons in the EB lesions and form $P0^{+ve}$ myelin sheaths (Figure 5.1B). Semi-thin sections of resin-processed samples were cut and stained for myelin with 1% toluidine blue solution. The myelin sheaths formed by the transplanted GFP-Schwann cells appeared as circular structures and were usually less heavily stained than the endogenous myelin sheaths formed by oligodendrocytes surrounding the lesions (Figure 5.1C – *left panel*). Ultra-thin sections of these EB lesions were also cut and viewed under a transmission electron microscope (TEM). The transplanted GFP-Schwann cells associated in 1:1 relationship with axons and individually myelinated only a single axon. Another characteristic feature these cells exhibited, which was similar to the features observed in peripheral nerves, is the significant extracellular space present between them (Figure 5.1C – *right panel*). These experiments suggest that freshly isolated Schwann cells are not significantly different from Schwann cells kept as long-term cultures, at least in terms of their myelinogenic properties (Shields *et al.*, 2000).

To have a direct comparison with GFP-Schwann cells, we performed similar experiments with GFP-Schwann cell precursors and analysed these features. As mentioned before, similar experimental conditions were maintained during all the transplant studies when comparing these cell-types. For example, same number of cells was injected at all times (10,000 cells) and lesions were reproduced as accurately as possible. These transplanted Schwann cell precursors were also nicely distributed within the EB lesions in the dorsal funiculus of the spinal cord (Figure 5.2A) and were able to remyelinate the demyelinated axons, with peripheral-like

properties. Thus, the myelin formed showed P0 immunoreactivity (Figure 5.2B) and had the characteristic periodicity of peripheral myelin (Figure 2C) (Shields *et al.*, 2000). These results suggest that GFP-Schwann cell precursors progress to form essentially normal Schwann cells in these transplants, although this is achieved in a CNS environment. For ease of description, we will generally refer to GFP^{+ve} cells observed in the CNS after transplant of GFP-Schwann cell precursors, as 'CNS-differentiated' Schwann cells and for GFP^{+ve} cells observed in the CNS after transplant of GFP-Schwann cells as 'post-natal' Schwann cells.

5.2.3 'CNS-differentiated' Schwann cells' survive better than 'post-natal' Schwann cells within the normal CNS.

Cultured Schwann cells show poor long-term survival, when transplanted in normal CNS, such that after about one month, very few surviving cells are present (Iwashita *et al.,* 2000). We wanted to compare the survival properties of GFP-Schwann cell precursors and GFP-Schwann cells under similar conditions. Thus, GFP-Schwann cells and GFP-Schwann cell precursors were transplanted into normal white matter (dorsal funiculus at thoracic level T13 of the spinal cord) and the survival of the cells was assessed after one and two months. The spinal cord segments were dissected out and processed for frozen sectioning. The sections were immunolabelled for MBP, which is present in both CNS and PNS myelin. However, in these sections, MBP was not detected in peripheral myelin since the intensity of immunolabelling is much brighter in CNS myelin. This is because MBP is much more abundant in CNS myelin than in PNS myelin. MBP immunolabelling was done to define the structure of the spinal cord and the transplanted cells were detected by the presence of GFP fluorescence.

Figure 5.2: 'CNS-differentiated' Schwann cells can survive and remyelinate CNS axons, at least as efficiently as freshly isolated GFP-Schwann cells.

GFP-Schwann cell precursors were transplanted into an EB lesion at T13 and 1 month later, the spinal cord segment dissected out and processed for resin or frozen sections.

(A) Frozen transverse section of a spinal cord segment showing the presence of 'CNS-differentiated' Schwann cells, as seen by the expression of GFP *(green),* within the EB lesion in the dorsal funiculus. The structure of the spinal cord is seen as red from a brightfield picture taken and the outline of the white matter is demarcated from the grey matter by the white line in the picture.

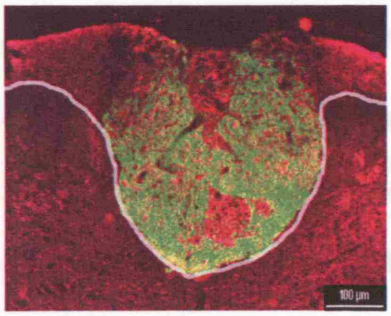
(B) Frozen sections were immunolabelled with P0 antibody. P0 immunoreactivity colocalises with GFP, expressed by the transplanted cells *(bottom left panel).* High magnification picture taken by confocal microscopy showing myelin *(red)* formed by the GFP^{+ve} transplanted cells *(green).* These pictures show that 'CNS-differentiated' Schwann cells can form $P0^{+ve}$ peripheral-like myelin in the CNS.

(C) *Left panel* – Electron micrograph illustrating the myelinating profile of 'CNS-differentiated' Schwann cells in EB lesions. They form the typical 1:1 relationship with the CNS axons and form myelin wraps around them. A, CNS axons; M, myelin.

Right panel – High magnification of an ultra-thin section showing part of the myelin sheath of a remyelinating cell with the characteristic periodicity of peripheral myelin (approximately 15 nm).

Figure 5.2

A



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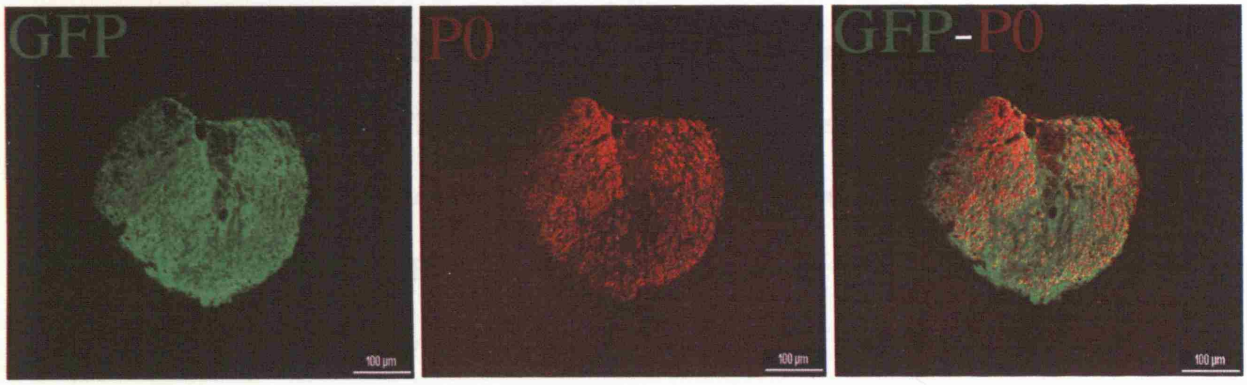
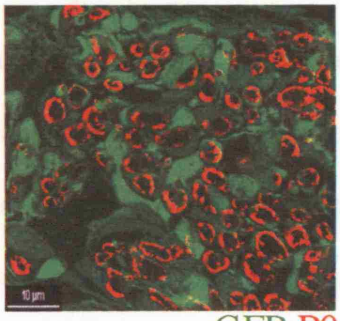
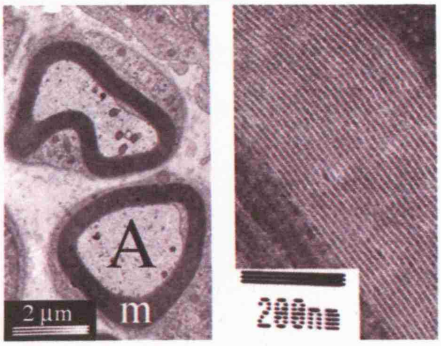




GFP P0

C



Not surprisingly, 'post-natal' Schwann cells showed very poor long-term survival and very few GFP^{+ve} cells were present in the white matter at 1 and 2 months (Figure 5.3A). In contrast, the 'CNS-differentiated' Schwann cells showed good long-term survival, as a substantial number of GFP^{+ve} cells were present in the white matter at 1 and 2 months (Figure 5.3B). We quantified the areas containing GFP^{+ve} cells and a substantial and statistically significant difference was obtained for the amount of surviving cells present after transplant of these two cell-types (Figure 5.3C).

The presence of a small number of GFP^{+ve} 'post-natal' Schwann cells in the white matter even after 2 months following transplant was strange in that we had expected all of them to have died. During the transplant procedure, some of the axons at the injection sites are damaged and become demyelinated. We wanted to examine whether the GFP^{+ve} cells could have associated with these axons and form stable myelin, which would explain their survival. We therefore immunolabelled frozen sections of the spinal cords, dissected out 2 months after transplant, with P0 antibody and found that most of the surviving GFP^{+ve} cells had indeed formed $P0^{+ve}$ myelin (Figure 5.3D). P0 immunolabelling was also done on frozen sections of the spinal cords containing 2-month old 'CNS-differentiated' Schwann cells, and we found a similar proportion of $P0^{+ve}$ cells scattered within the GFP^{+ve} cell mass (Figure 5.3D). These results are particularly important in that they show that 'CNS-differentiated' Schwann cells can survive normally within the normal CNS unlike 'post-natal' Schwann cells.

Figure 5.3: 'CNS-differentiated' Schwann cells survive much better than 'postnatal' Schwann cells in normal CNS tissue.

GFP-Schwann cells and GFP-Schwann cell precursors were transplanted in the normal white matter at spinal cord segment T13 and 1 and 2 months later, the spinal cords were dissected out and processed for frozen sections. The sections were immunolabelled for oligodendrocyte myelin using MBP antibody.

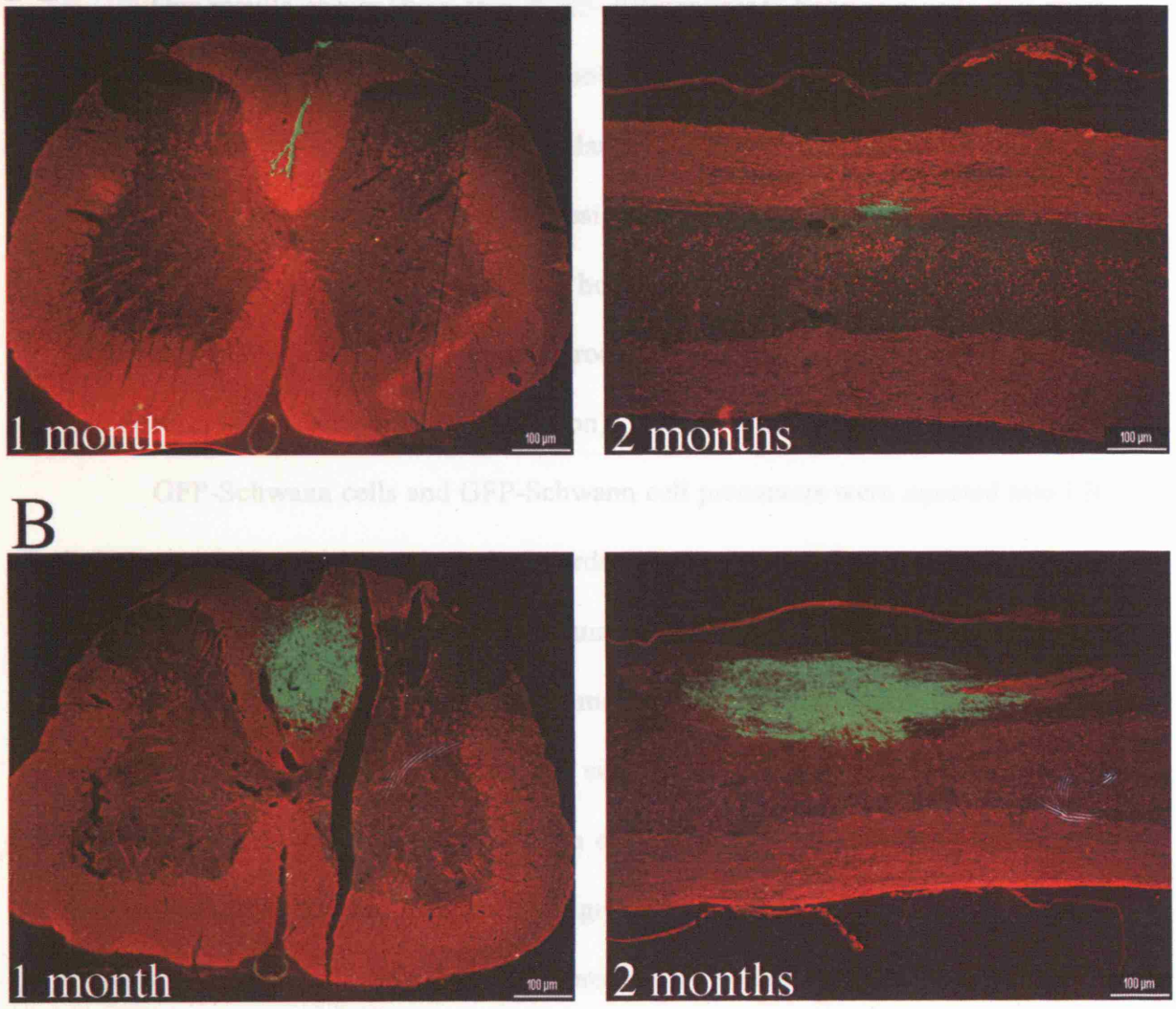
(A) 'Post-natal' Schwann cells cannot survive in the normal white matter at 1 month (*left panel* – transverse section of the spinal cord) and 2 months (*right panel* – parasagittal section of the spinal cord) and only very few GFP^{+ve} cells (*green*) are seen in the white matter. MBP immunolabelling (*red*) is present in oligodendrocyte myelin.

(B) 'CNS-differentiated' Schwann cells can survive much better than GFP-Schwann cells in normal white matter and at 1 month (*left panel* – transverse section of the spinal cord) and at 2 months (*right panel* – parasagittal section of the spinal cord), a large number of surviving GFP^{+ve} cells (*green*) are present in the white matter. MBP immunolabelling (*red*) is present in oligodendrocyte myelin.

(C) Quantification of the number of surviving cells 'post-natal' and 'CNS-differentiated' Schwann cells in normal white matter after 1 and 2 months following transplant. All the areas containing GFP^{+ve} cells were quantified using an image analysis software (Image J, USA) and this was compared in animals transplanted with GFP-Schwann cells and GFP-Schwann cell precursors. At 1 and 2 months, 'post-natal' Schwann cells occupy an area of 72.5±32.5 and 108.9±60.7 pixels respectively and 'CNS-differentiated' Schwann cells I areas occupy an area of 3297.6±226.7 and 2945±767.1 pixels respectively ($n=6$, $p<0.001$).

(D) Frozen sections of spinal cords after 2 month-transplant were immunolabelled with P0 antibody. Most of the surviving 'post-natal' Schwann cells (*green*) are associated with $P0^{+ve}$ myelin (*red*) (*top panel*), whereas very few of the surviving 'CNS-differentiated' Schwann cells (*green*) are associated with $P0^{+ve}$ myelin (*red*) (*bottom panel*).

Figure 5.3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1 month
100 µm
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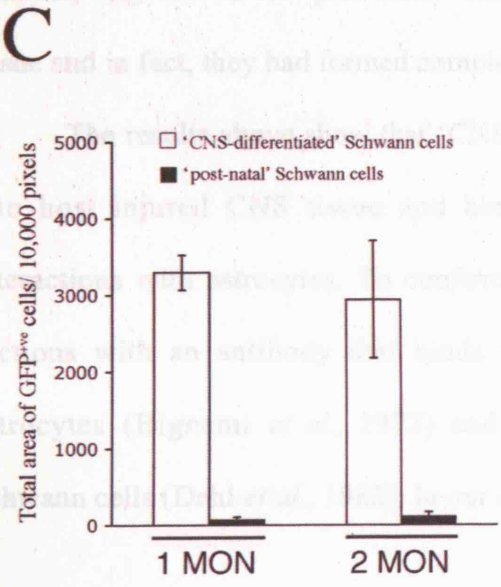
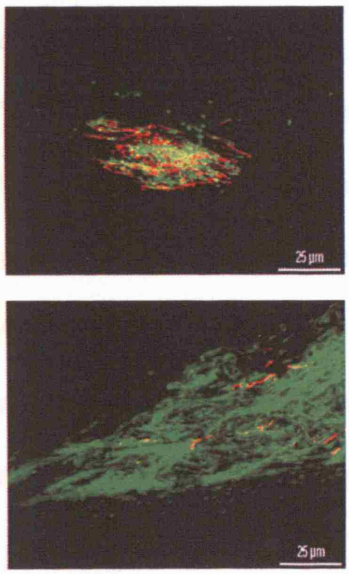

C
'CNS-differentiated' Schwann cells
'post-natal' Schwann cells
Total area of GFP+ve cells/ 10,000 pixels
5000
4000
3000
2000
1000
0
1 MON
2 MON


D
25 µm
25 µm

5.2.4 'CNS-differentiated' Schwann cells', unlike 'post-natal' Schwann cells, are able to integrate smoothly within injured CNS tissue.

Our results above show that 'CNS-differentiated' Schwann cells can exist within the normal CNS even after 2 months following transplant. We wanted to investigate whether they would have similar properties in injured CNS tissue. When Schwann cells are transplanted into EB lesions in the CNS, they are unable to leave the site of lesion and integrate into the host tissue (Shields *et al.*, 2000). This is largely due to the presence of a dense astrocytic glial scar surrounding these lesions (see Section 1.11.4.2 for detailed discussion).

GFP-Schwann cells and GFP-Schwann cell precursors were injected into EB lesions and 2 months later, the spinal cords dissected out and processed for frozen sections. Some of the sections were immunolabelled with MBP antibody to define the regions of the EB lesion. As mentioned above, MBP labels intact oligodendrocyte myelin and the lesion site can be identified by an absence of reactivity. 'CNS-differentiated' Schwann cells were present within the lesion and more importantly they had extensively migrated into the normal CNS tissue. A large number of GFP^{+ve} cells were found scattered within MBP^{+ve} areas (Figure 5.4A). In contrast, very few of the 'post-natal' Schwann cells were present in the intact CNS tissue and in fact, they had formed completely separated domains (Figure 5.4B).

The results above show that 'CNS-differentiated' Schwann cells can migrate into host injured CNS tissue and hence, suggest that they have no adverse interactions with astrocytes. To confirm this, we immunolabelled the spinal cord sections with an antibody that binds to GFAP. GFAP is highly expressed in astrocytes (Bignami *et al.*, 1972) and in comparatively much lower levels in Schwann cells (Dahl *et al.*, 1982). In our experiments, we found that GFAP could not

be detected in the transplanted cells, presumably because immunoreactivity with astrocytes was stronger than in Schwann cells. We found that most of 'post-natal' Schwann cells were confined almost exclusively to regions within the lesion devoid of astrocytes (figure 5.5B). These observations are in agreement with previous studies involving transplant of cultured Schwann cells (Blakemore, 1976; Shields *et al.*, 2000). 'CNS-differentiated' Schwann cells, on the other hand, had migrated into regions of high GFAP immunoreactivity and were intermingled with host astrocytes (Figure 5.5A).

Figure 5.4: 'CNS-differentiated' Schwann cells integrate much better in normal CNS tissue than 'postnatal' Schwann cells, after transplant in an EB lesion.

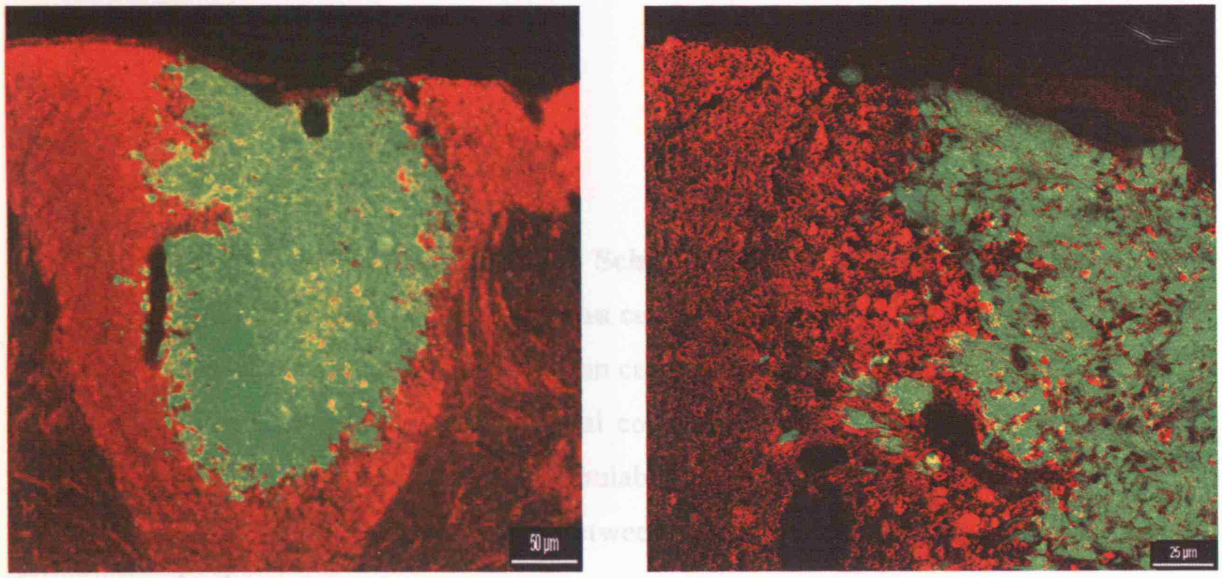
GFP-Schwann cells and GFP-Schwann cell precursors were transplanted into an EB lesion and 2 months later, the spinal cords were dissected out and processed for frozen sections. These were immunolabelled with MBP antibody, which labels oligodendrocyte myelin and hence shows the interface between the lesion and normal CNS white matter, uninjured by the gliotoxin.

(A) Low power *(left hand panel)* and high power *(right hand panel)* confocal picture illustrating the presence of 'CNS-differentiated' Schwann cells *(green)* within the lesion in the dorsal funiculus. MBP immunolabelling *(red)* shows the presence of intact oligodendrocyte myelin. Note that many of the transplanted cells are able to integrate deep into the normal CNS tissue and there is no clear demarcation between the lesion area and the normal CNS tissue.

(B) Low power *(left hand panel)* and high power *(right hand panel)* confocal picture illustrating the presence of 'post-natal' Schwann cells *(green)* within the lesion in the dorsal funiculus. MBP immunolabelling *(red)* shows the presence of intact oligodendrocyte myelin. Here there is a clear demarcation between the lesion area and the normal CNS tissue and the transplanted cells are very rarely seen in the normal CNS tissue.

Figure 5.4

A



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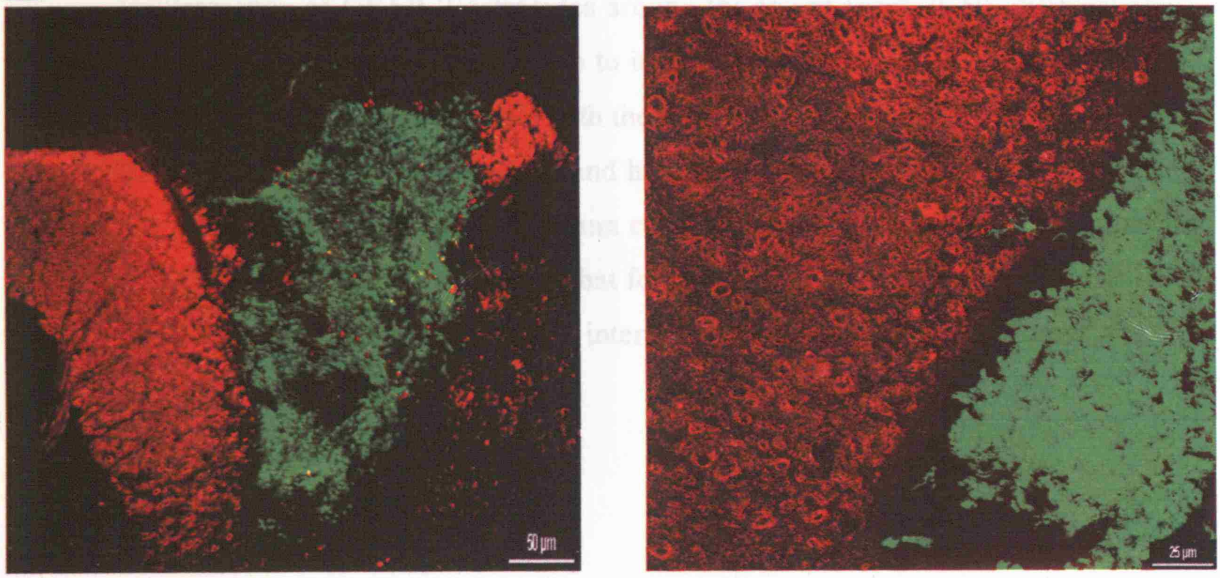


Figure 5.5: 'CNS-differentiated' Schwann cells interact much better with astrocytes than 'post-natal' Schwann cells.

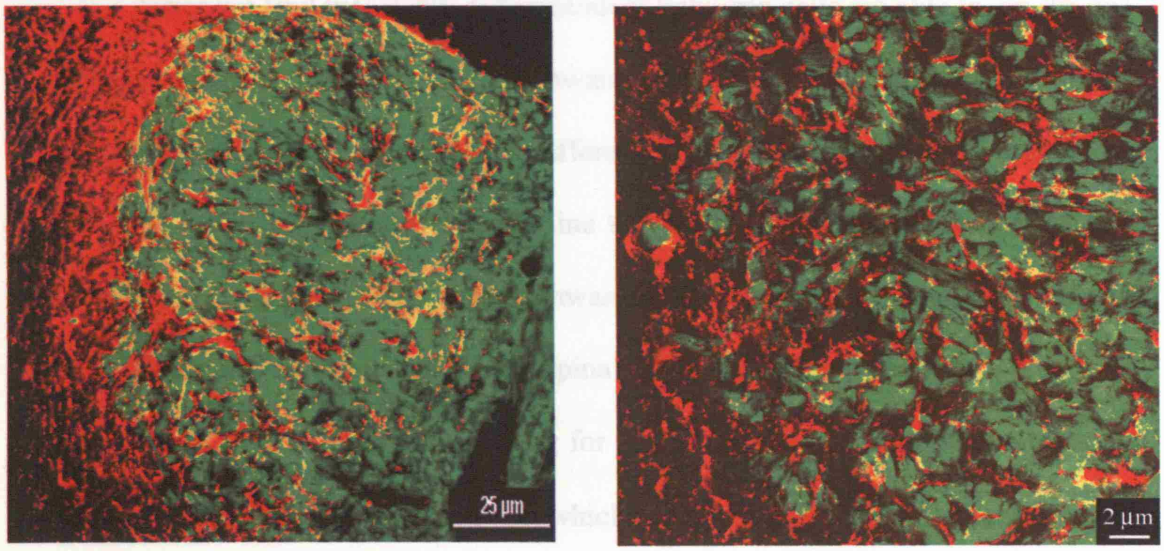
GFP-Schwann cells and GFP-Schwann cells precursors were transplanted into an EB lesion and 2 months later, the spinal cords were dissected out and processed for frozen sections. These were immunolabelled with GFAP antibody, which labels astrocytes and the interactions between the transplanted cells and astrocytes examined.

(A) Low power *(left hand panel)* and high power confocal picture illustrating the presence of $GFAP^{+ve}$ astrocytes around the lesion *(red)*. 'CNS-differentiated' Schwann cells *(green)* are able to intermingle with these astrocytes and many of them are in direct contact with the astrocytic processes.

(B) Low power *(left hand panel)* and high power confocal picture illustrating the presence of 'post-natal' Schwann cells *(green)*, which are separated from the $GFAP^{+ve}$ astrocytic scar *(red)* that form around the lesion. Both cell types exist as separate domains with little intermingling between the GFP^{+ve} cells and the astrocytes.

Figure 5.5

A



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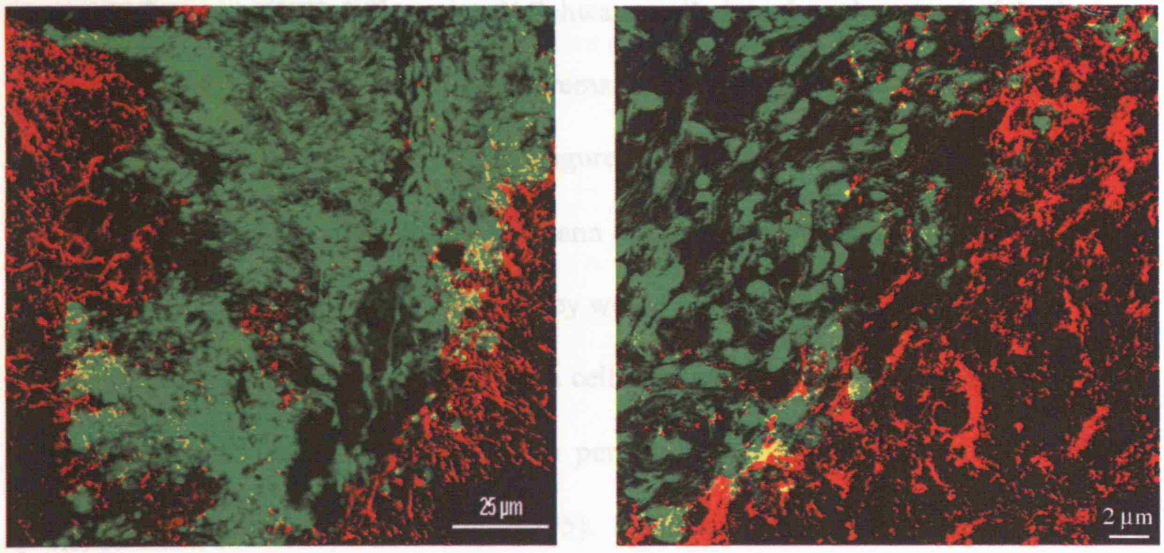


5.2.5 'CNS-differentiated' Schwann cells express markers of mature Schwann cells one month after transplant into CNS tissue.

Since we find that 'CNS-differentiated' Schwann cells are able to, on the one hand, remyelinate CNS axons with Schwann cell-like myelin characteristics but on the other hand, exhibit considerable differences from Schwann cells in interaction with host tissue, we wanted to determine the phenotypic characteristics of 'CNS-differentiated' Schwann cells. GFP-Schwann cell precursors were transplanted into an EB lesion and one month later, the spinal cords were dissected out and processed for frozen sections. Immmunolabelling for S100β, which is a Schwann cell marker (Jessen and Mirsky, 2004), and nestin, which is a marker of cells in the Schwann cell lineage and is not found in oligodendrocytes and astrocytes (Friedman *et al.*, 1990), was performed. 'CNS-differentiated' Schwann cells found on the edges of the lesion or inside the host tissue, which are so remarkably different from transplanted GFP-Schwann cells, still expressed S100β (Figure 5.6A) as well as nestin (Figure 5.6B).

These results suggest that Schwann cell precursors, when injected into CNS tissue, develop into Schwann cells as they would during normal development, at least in terms of S100β expression. Schwann cell precursors are essentially $S100\beta^{-ve}$, and as they mature into Schwann cells in peripheral nerves, they become $S100\beta^{+ve}$ (Jessen *et al.*, 1994; Dong *et al.*, 1995). Furthermore, the 'CNS-differentiated' Schwann cell precursors can myelinate CNS axons with Schwann cell-like characteristics (Figure 5.2). However, the interactions of these 'CNS-differentiated' Schwann cells with astrocytes are significantly different from the interactions of 'postnatal' Schwann cells with host CNS cells. Taken together, these results suggest that 'CNS-differentiated' Schwann cells are different from 'post-natal' Schwann cells although they do acquire some of phenotypic characteristics of Schwann cells.

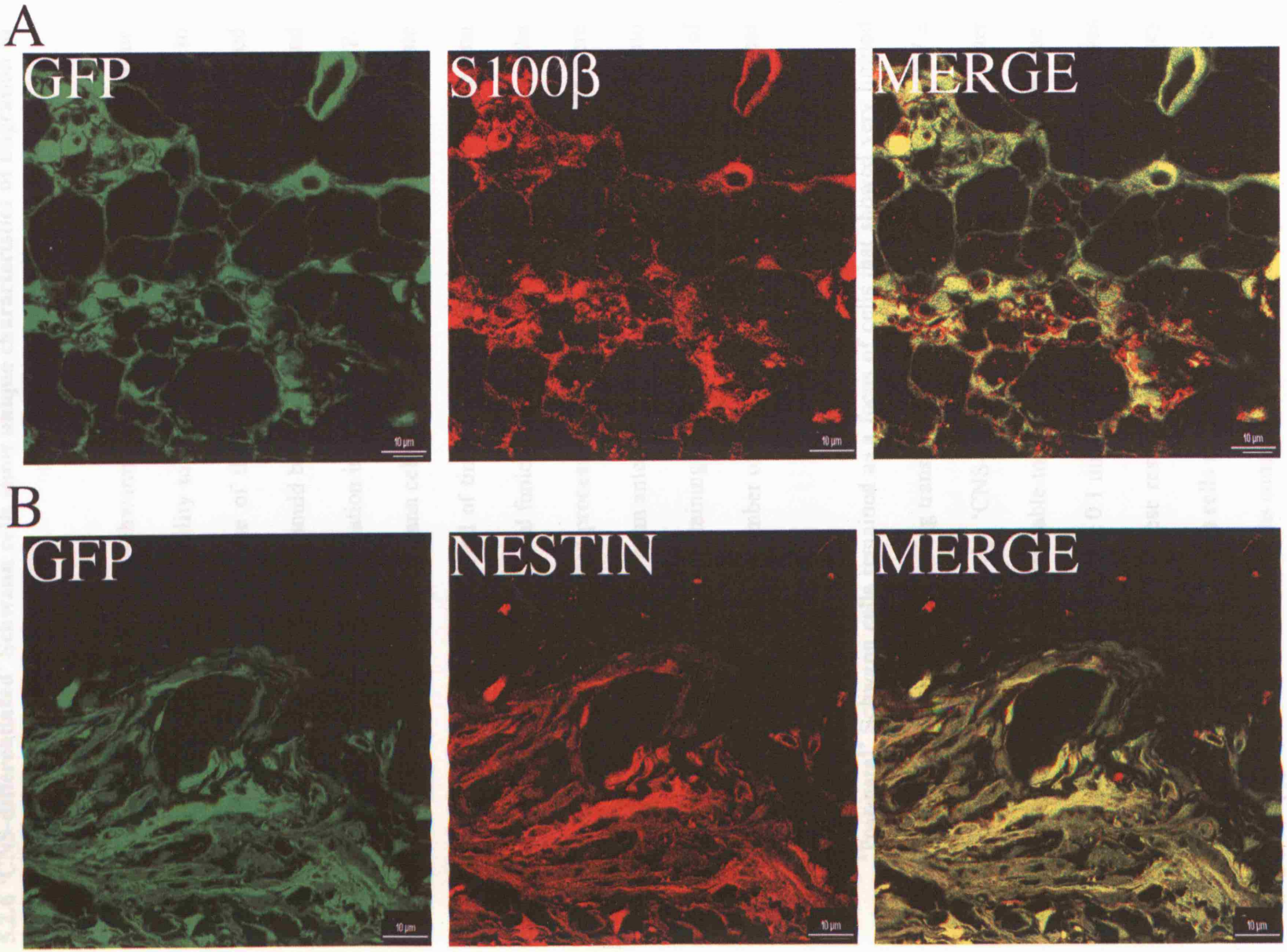
Figure 5. 6: Antigenic profile of 'CNS-differentiated' Schwann cells present in an EB lesion, one month after transplant.

GFP-Schwann cell precursors were transplanted into an EB lesion and 1 month later, the spinal cords were dissected out and processed for frozen sections. These were immunolabelled antibodies to S100β, which is a marker of mature Schwann cells, and to nestin, which is a marker of cells of the Schwann cell lineage.

(A) S100β expression *(red)* co-localises with GFP expression *(green)* showing that most of the 'CNS-differentiated' Schwann cells that are present outside of the lesion and within the normal CNS tissue express this marker of mature Schwann cells.

(B) The 'CNS-differentiated' Schwann cells also express nestin *(red)*, which co-localises with GFP expression *(green)* showing that they are likely to be still in the Schwann cell lineage and have not transdifferentiated into other cell-types.

Figure 5.6



5.2.6 'CNS-differentiated' Schwann cells show unique characteristics of migration in the CNS.

Our experiments show that Schwann cell precursors seem to have a unique combination of features, namely an ability to remyelinate CNS axons and an ability to integrate well in host CNS tissue. One of the criteria of an ideal cell for cell-based remyelination therapy is that the cells should be able to migrate within the host tissue and populate the numerous foci of demyelination in MS. We wanted to investigate how GFP-Schwann cell precursors and GFP-Schwann cells would compare in their ability to migrate within normal CNS tissue over a period of time. GFP-Schwann cells and GFP-Schwann cell precursors were injected in the dorsal funiculus at spinal level T13 and 1 and 2 months later, the spinal cords dissected out and processed for frozen sections. Serial sections were then cut for the spinal cord segments in an anterior-posterior orientation and collected onto slides. The total number of sections containing GFP^{+ve} cells was counted and the length of spread calculated by multiplying the number of sections with a factor of 20 μm (thickness of sections).

'Post-natal' Schwann cells remained as a focus of cells that showed very limited migration after 1 and 2 months following transplant. The length of spread was about 0.7 ± 0.1 mm and 0.9 ± 0.2 mm respectively. 'CNS-differentiated' Schwann cells, on the other hand, performed much better and were able to migrate within the normal CNS over time. At 1 month, they had spread about 3.7 ± 0.1 mm and at 2 months, the length of spread was about 7.73 ± 0.3 mm (Figure 5.7). These results are particularly important in that they show that 'CNS-differentiated' Schwann cells retain their migratory potential over time, as shown by the length of spread at 2 months compared to 1 month.

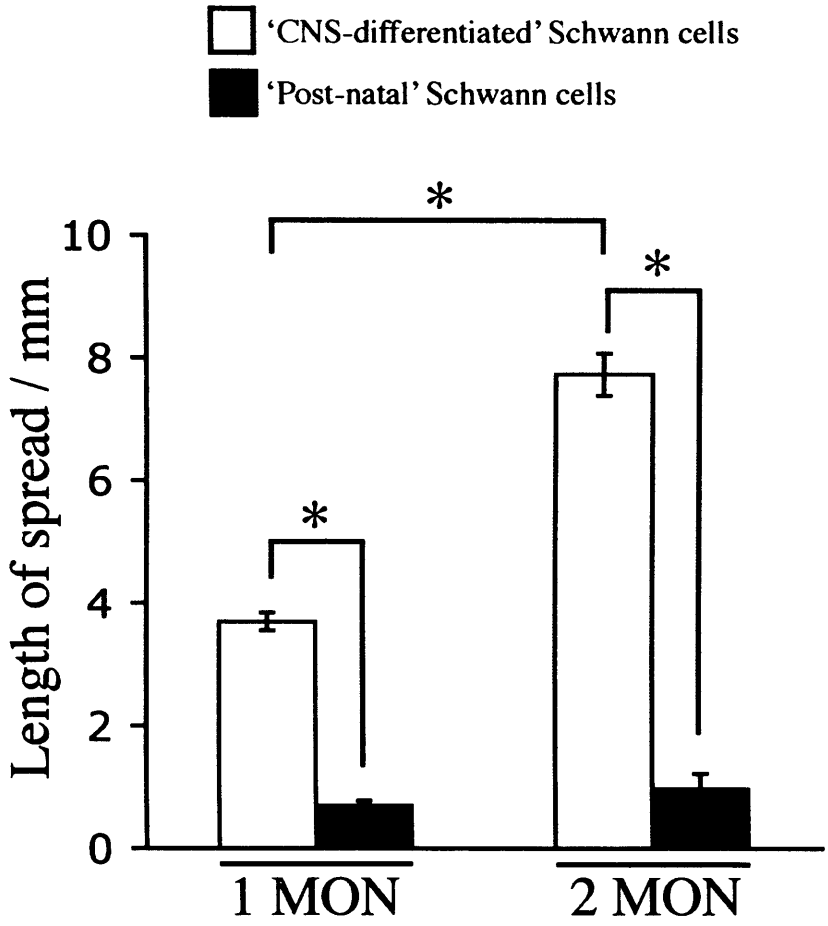


Figure 5.7

Figure 5.7: 'CNS-differentiated' Schwann cells are able to migrate further distances than 'post-natal' Schwann cells in normal white matter.

Length of spread of the 'CNS-differentiated' Schwann cells and 'post-natal' Schwann cells after 1 and 2 months following transplant was measured and is illustrated in this graph. 'CNS-differentiated' Schwann cells are able to migrate 3.70±0.14 mm and 7.73±0.34 mm in the white matter respectively, whereas the length of spread of post-natal Schwann cells is only about 0.71±0.07 mm and 0.98±0.24 mm after 1 and 2 months transplant respectively. (n=3, $p<0.005$, * denotes level of significance).

5.2.7 'CNS-differentiated' Schwann cells perform substantially better than postnatal Schwann cells in myelinating retinal axons and migrating in the astrocyte-rich environment of the retina.

Both Schwann cells and Schwann cell precursors are able to remyelinate axons when transplanted into an EB lesion, which is relatively devoid of any endogenous cells with the exception of macrophages. We now wanted to compare the remyelinating potential of these cell-types in a different environment, the retina, which consists of a large network of unmyelinated axons embedded in an astrocyte-rich matrix. The additional advantage of this model is that myelination can be quantified and a strict comparison of the quantity of myelin made by both cell-types in this environment can be made.

GFP-Schwann cells and GFP-Schwann cell precursors were injected in the retina of adult Sprague-Dawley rats and after 4 and 6 weeks, the retinae were dissected out and flat-mounted. They were than immunolabelled for P0 to reveal the extent of myelination. All the areas showing P0 immunoreactivity were acquired by confocal microscopy and quantified with image analysis. We found that at the 4-week point, 'CNS-differentiated' Schwann cells were 2 fold better than 'post-natal' Schwann cells in myelinating these axons. More remarkably, we found that there was a 17 fold increase in amount of myelin for the 'CNS-differentiated' Schwann cells at the 6 week-point compared to the 4-week point. In contrast there was only about a 2 fold increase in the case of the 'post-natal' Schwann cells (Figure 5.8A).

We wanted to investigate the reason for such startling differences and we postulated that even though an equal number of cells was injected in each case, the migration of the Schwann cells population in the astrocyte-rich retina could be the limiting factor in the formation of P0 myelin. This might not be the case for Schwann cell precursors, both because they are more migratory than Schwann cells *in vitro* (Jessen *et al.*,

1994) and because we found that they migrate out of the CNS lesions and with the CNS environment (above). We therefore decided to inject GFP-Schwann cells and GFP-Schwann cell precursors and monitor their distribution in the retina. The retinae were flat-mounted and montage of the distribution GFP^{+ve} cells made using confocal microscopy. We found here, at least qualitatively, that the 'CNS-differentiated' Schwann cells had migrated longer distances than 'post-natal' Schwann cells in the retina (Figure 5.8B).

These experiments suggest that the environment of the retina, probably as a result of the presence of astrocytes, considerably hinders the migration of 'post-natal' Schwann cells and that these are present within limited areas of the retina. Consequently, they are able to myelinate only these small areas of the axonal layer in the retina and with time, no big increase in the amount of myelin they make is seen. 'CNS-differentiated' Schwann cells, on the other hand, are not hindered in their migration and spread throughout most of the surface of the retina. Consequently, they are able to myelinate many more axons and this is the probable reason why we get such a massive increase in amount of myelin at the 6-week point compared to the 4-week point.

Figure 5.8: 'CNS-differentiated' Schwann cells perform substantially better than 'postnatal' Schwann cells in (A) myelinating retinal axons and (B) migrating in the astrocyte-rich environment of the retina.

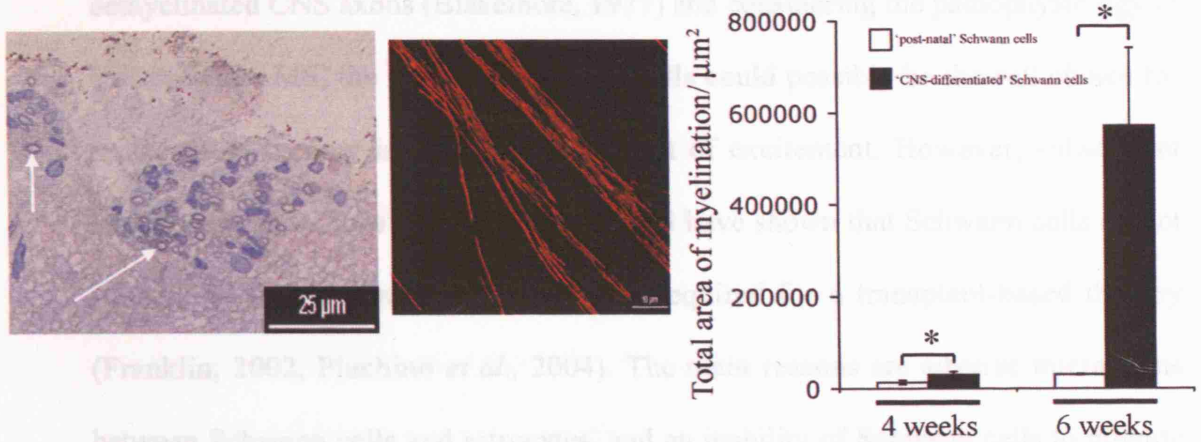
(A) GFP-Schwann cells and GFP-Schwann cell precursors were injected in the retina and 4 and 6 weeks later, the retinae were dissected out, flat-mounted and immunolabelled with the Schwann cell myelin marker P0 or processed for semi-thin resin sections . All areas containing $P0^{+ve}$ myelinating cells were acquired using a confocal microscope and the total area of myelination quantified. *Left Panel* – Toluidine blue stained semithin cross-section of the the retina. Myelin formed by transplanted cells are seen as circular black rings (arrows). *Middle panel* – Picture depicting the $P0^{+ve}$ myelinated segments *(red)* from a retina transplanted with GFP-Schwann cell precursors.

Right panel – Graph depicting the total amount of $P0^{+ve}$ myelin formed by 'post-natal' Schwann cells and 'CNS-differentiated' Schwann cells after 4 and 6 weeks following transplant. The area of myelination formed by 'CNS-differentiated' Schwann cells is 31,240 ± 5,997 μm^2 and 573, 278 ± 168,538 μm^2 whereas the area of myelination formed by 'post-natal' Schwann cells is only 13,837 ± 4,208 μm^2 and 30,815 ± 1,040 μm^2 respectively at 4 and 6 weeks following transplant (n=5, $P<0.05$, * denotes significant level of difference).

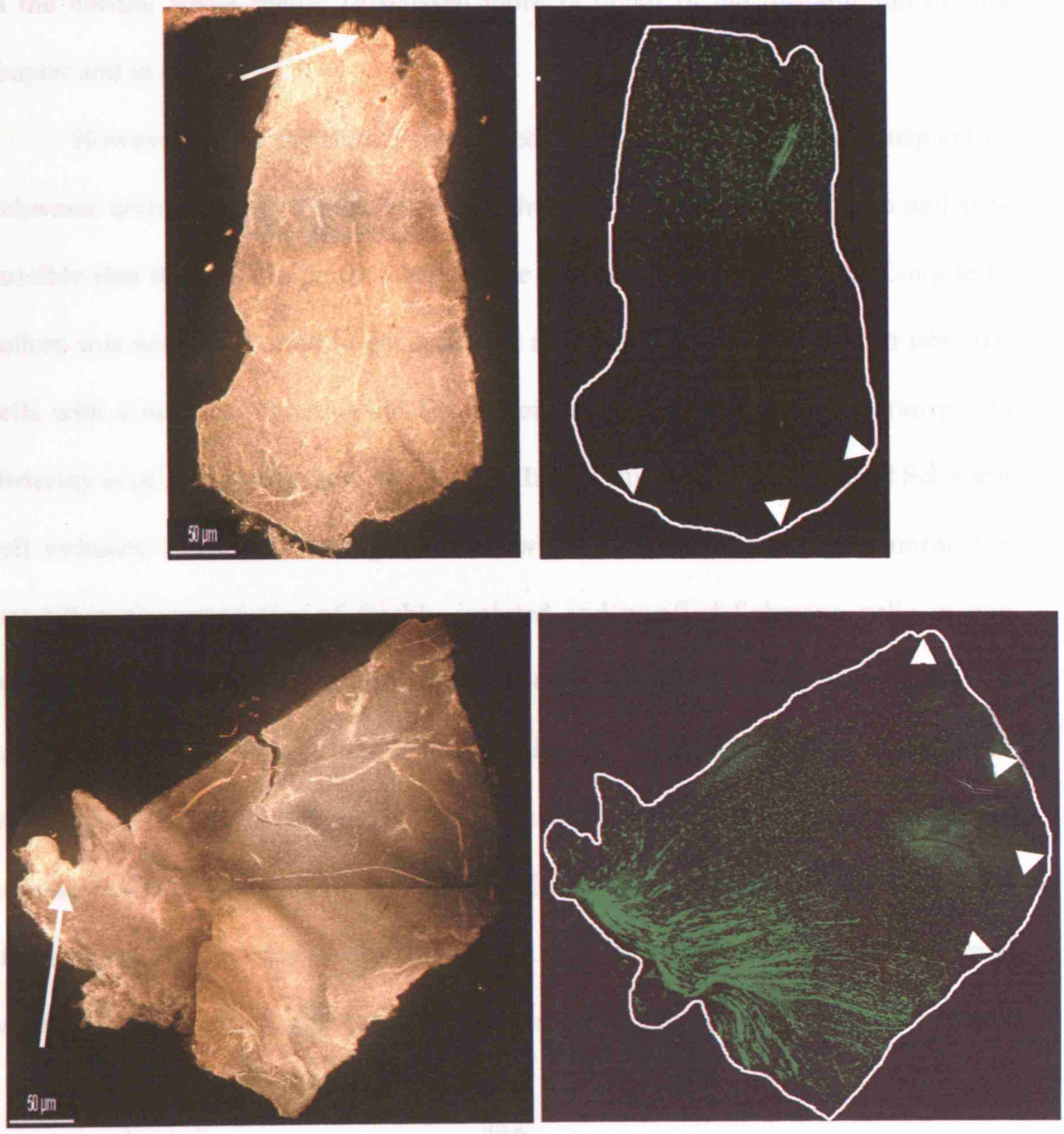
(B) Picture montage depicting the distribution of GFP^{+ve} cells in a flat-mount of t part of the retina, dissected out 6 weeks after transplant with either GFP-Schwann cells *(top panel)* or GFP-Schwann cell precursors *(bottom panel)*. The brightfield picture of the retinal flat-mounts is on the left and corresponding confocal pictures showing the distribution of the GFP+ve cells is on the right. 'CNS-differentiated' Schwann cells are able to migrate throughout most of the surface of the retina and present up to edges of the retinal flat-mounted (shown by the arrowheads), whereas most of the 'post-natal' Schwann cells are found near the point of injection (arrows) and are not able to migrate great distances over the retinal surface.

Figure 5.8

A


25 µm
800000
600000
400000
200000
0
Total area of myelination/ µm²
'post-natal' Schwann cells
'CNS-differentiated' Schwann cells
*
*
4 weeks
6 weeks

B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5.3 DISCUSSION

Schwann cells have been shown for some time to be able to myelinate demyelinated CNS axons (Blakemore, 1977) and considering the pathophysiology of a disease like MS, the idea that Schwann cells could possibly be the cell choice for replacement therapy in MS generated a lot of excitement. However, subsequent experiments in various animal models of MS have shown that Schwann cells do not possess all the favourable characteristics required for a transplant-based therapy (Franklin, 2002, Pluchino *et al.*, 2004). The main reasons are adverse interactions between Schwann cells and astrocytes, and an inability of Schwann cells to migrate in the normal white matter (discussed more in detail in the introduction of this chapter and in chapter 1).

However, all of the studies mentioned above have involved the transplant of Schwann cells that have been expanded *in vitro* over a period of time and it is possible that this culture period changes the characteristics of the cells. Long-term culture was needed in order to get sufficient numbers of purified cells or to label the cells with a marker, which would allow them to be identified after the transplants (Brierley *et al.*, 2001; Iwashita *et al.*, 2000). In this study, we have obtained Schwann cell cultures from GFP-transgenic rats, which has allowed us to examine the transplant characteristics of freshly isolated and purified Schwann cells in two models of MS. In fact, our results show that expanded and freshly isolated Schwann cells do not differ much in their characteristics. Freshly isolated Schwann cells can myelinate demyelinated axons, but do not interact well with astrocytes and cannot migrate from a lesion into the uninjured CNS. When examined one and two months after transplant, they also do not survive and migrate when transplanted into normal white matter. The few surviving cells seem to be present only because of their

association with demyelinated axons. The transplant process probably damages some of the axons and the Schwann cells myelinate them and hence do not die. The large majority of Schwann cells, however, die in the intact CNS, as shown before with expanded Schwann cells (Iwashita and Blakemore, 2000; Iwashita *et al.*, 2000).

Cells of the oligodendrocyte lineage have also been used in transplant studies (Franklin, 2002; Pluchino *et al.*, 2004). Mature oligodendrocytes seem to have a limited myelination potential, but earlier progenitors, like OPCs, show a far greater myelinating, migratory and proliferative potential and can generate more myelin over a wider area of demyelination (Rosenbluth *et al.*, 1990; Warrington *et al.*, 1993). This variability in the transplant characteristics of cells from different stages of the cell lineage probably reflects the biological differences between these cells; OPCs are motile and proliferative cells, whereas mature oligodendrocytes are non-motile and quiescent (Pfeiffer *et al.*, 1993). Cells of the Schwann cell lineage differ also in these characteristics and it has been shown, at least *in vitro*, that Schwann cell precursors, the progenitors of Schwann cells, are about ten fold more motile than Schwann cells (Jessen *et al.*, 1994).

Given that the transplant characteristics of cells in the oligodendrocyte lineage reflect their biological properties, we wanted to investigate whether the same would apply for cells of the Schwann cell lineage. A number of comparative studies were hence performed to investigate the properties of Schwann cells and Schwann cell precursors, freshly obtained from GFP-transgenic rats. First of all, the myelinating characteristics of the transplanted GFP-Schwann cell precursors were determined, since these cells were used for the first time in a transplant study. We show here that they make a Schwann cell-like myelin, as seen ultrastructurally and by the expression of the peripheral myelin marker, P0. Since the GFP-Schwann cell

precursors transplanted into the lesions had differentiated into cells with these Schwann cell characteristics, we decided to refer them to 'CNS-differentiated' Schwann cells and the cells present in the lesions after transplant of GFP-Schwann cells were referred to as 'post-natal' Schwann cells.

The other properties examined however, differed remarkably between the Schwann cells and the Schwann cell precursors, Whereas, 'post-natal' Schwann cells and endogenous astrocytes were clearly separated from each other, 'CNS-differentiated' Schwann cells were found intermingled with astrocytes, deep into the astrocytic scar domain surrounding the lesion. Also, 'post-natal' Schwann cells rarely migrated into the uninjured CNS. 'CNS-differentiated' Schwann cells, on the other hand, were able to integrate smoothly into CNS tissue. Survival and migration in normal CNS white matter, as well differed greatly; 'CNS-differentiated' Schwann cells were able to survive and spread along the neuraxis for at least 2 months following transplant, whereas 'post-natal' Schwann cells mostly died and did not spread. The myelinogenic potential of both cells was significantly different and when, quantified over a period of time, 'CNS-differentiated' Schwann cells are able to generate more myelin than 'post-natal' Schwann cells. Our retinal transplants provided a good model to study myelination and migration potential of cells in an astrocyte-rich matrix. We showed using this model that 'post-natal' Schwann cells cannot myelinate and migrate as well as 'CNS-differentiated' Schwann cells, probably as a result of the presence of astrocytes. These results support the view that Schwann cells do not interact very well with astrocytes, making this a limiting factor in MS cell transplant therapy.

The nature of such differences is more surprising because the expected differentiation of the Schwann cell precursors within only 4 days, as seen *in vitro* and

in normal development, would lead them to acquire the characteristic properties of Schwann cells, and hence behave like Schwann cells relatively rapidly after transplant. Indeed, most of the transplanted Schwann cell precursors acquire the antigenic profile of Schwann cells and express S100β, as seen 1 month after transplant. Also, they are not likely to have transdifferentiated into other glial cell types as they express nestin, which is present only in cells of the Schwann cell lineage and not in mature astrocytes and oligodendrocytes (Friedman *et al.,* 1990). These results imply that the differentiation of Schwann cells from Schwann cell precursors, seen in normal development in the PNS, differs significantly from the differentiation seen after transplant in the CNS. These "CNS-differentiated" Schwann cells appear to acquire only some of the characteristics of "normal" Schwann cells and perhaps keep the motility and survival properties of Schwann cell precursors.

When experimentally compared for the properties ideally needed for a cell to be considered as a cell replacement therapy, Schwann cell precursors fare much better than Schwann cells in the aspects that we considered. 'CNS-differentiated' Schwann cells have a better myelinogenic potential than 'post-natal' Schwann cells, are able to interact with astrocytes and can migrate outside of a lesion in the normal white matter. Also, they survive and migrate much better than 'post-natal' Schwann cells in normal CNS white matter. The Schwann cell-like myelin formed from Schwann cell precursors has this additional advantage of probably being able to withstand the long-term course of MS and be unaffected by the autoimmune injury that affects oligodendrocytes. Further experimentation is still needed to characterise the phenotype of the "CNS-differentiated" Schwann cells together with a more rigorous examination of the migratory and survival potential of these cells. One

experiment that could be done is to investigate the ability of 'CNS-differentiated' Schwann cells to migrate towards a focal area of demyelination and myelinate the axons, after they had been transplanted some distance away from the site of lesion. It would also be of interest to know whether Schwann cell precursors can be administered into the blood stream (intravenously) or into the cerebrospinal fluid circulation (CSF) (intracerebroventricularly) and myelinate axons present in a demyelinating lesion. Previous work has shown that when adult neural stem cells are injected in the blood stream or CSF circulation, they myelinate demyelinating plaques in an animal model of MS and promote functional recovery (Pluchino *et al.*, 2003). Cells injected this way seem to "home" specifically towards the sites of CNS trauma and it is believed that "chemoattraction" signals would guide the cells towards the injury sites (Pluchino *et al,* 2004).

Our results in this study show that Schwann cell precursors clearly have a good potential for repairing demyelination in MS lesions. Further studies would provide a more definitive case for Schwann cell precursors as a cell therapy for MS. However, for consideration, the cells should be readily available in sufficient quantities. Since these cells are present only during embryonic development, they could be obtained from aborted foetal tissue, but this could be ethically difficult and pose a problem of cell numbers. Alternatively, these cells could be obtained by differentiation from stem cells, with bone marrow stem cells (BMSCs) perhaps being the most promising. Previous work has shown that BMSCs can differentiate into Schwann cells *in vitro* (Dezawa *et al.*, 2001), and BMSCs-derived Schwann cells have been shown to remyelinate CNS axons (Akiyama *et al.*, 2002; Keilhoff *et al.*, 2006). Skin stem cells have recently been identified (Toma *et al.*, 2001) and Schwann cells have been derived from them (Toma *et al.*, 2005). The Schwann cell

lineage has been extensively characterised and Schwann cell precursors and Schwann cells can be easily differentiated by their antigenic profile (Jessen and Mirsky, 2005a). With further characterisation of the BMSCs differentiation, it would be possible to isolate Schwann cell precursors, which could potentially provide a good source of these cells.

CHAPTER 6: General Discussion

The generation of mature Schwann cells in adult nerves from their cells of origin, neural crest cells, occurs through different intermediate cell derivatives and involves complex phenotypic changes along the way. A lot has been learned over the past thirty years about some of the phenotypic changes; the different intermediates have distinct antigenic expression profiles and survival mechanisms and relationships to axons and connective tissue in peripheral nerves (see Jessen and Mirsky, 2005 a, b). Also, some of the signalling pathways involved in their development have been characterised. However, much still needs to be learned. In the work presented in this thesis, I have observed the changes in survival signalling that take place as the Schwann cell lineage emerges from the neural crest (Chapter 3), examined the role of Notch signalling in subsequent glial differentiation and myelination (Chapter 4) and lastly, studied how cells from different stages of the lineage compare as myelin repair cells in the CNS (Chapter 5).

One common theme that has emerged from these apparently distinct studies is the regulation of differentiation of cells of the Schwann cell lineage by different microenvironments. In Chapter 1, the survival characteristics of neural crest cells and their glial derivatives, Schwann cell precursors and satellite cells are described. As this differentiation takes place, these glial derivatives become less stringent in their survival requirements than neural crest cells. Together, with the results from the work by Meier and colleagues (1999), and the results described in Chapter 1, there is now a more complete characterisation of the factors required to promote survival of the cells in the Schwann cell lineage. In summary, it appears that there is a progressive expansion of factors that can block cell death at each transition point in

the lineage. In addition, satellite cells are likely to differentiate ahead of cells in the Schwann cell lineage as they acquire a more mature survival profile earlier. Although they share many common features including their antigenic profile and as shown in this study, survival characteristics, they are intrinsically different, perhaps because they develop in distinct microenvironments.

Satellite cells develop in close apposition to neuronal soma found in the DRG, whereas Schwann cell precursors contact axonal membranes in the peripheral nerves. It is possible that the neuronal soma express factors that are more conducive to satellite cell differentiation than axonal membranes. Schwann cell precursors mature into Schwann cells in response to neuregulin-1 (Jessen *et al.*, 1994; Dong *et al.*, 1995), an effect mediated principally by the axonal membrane-associated isoform III (Wolpowitz *et al.*, 2000). Satellite cells, on the other hand, are additionally exposed to isoform II, a soluble form of neuregulin-1 and neuregulin-2, produced by DRG neurones and the spinal cord. Exposure to higher levels of neuregulin might accelerate the maturation of satellite cells, at higher rates than Schwann cell precursors. Another signal that might explain such differences is Notch-Delta signalling. Whilst it has been shown, both *in vivo* and *in vitro*, that Notch signalling promotes the differentiation of satellite cells from crest-derived precursors in DRGs (Wakamatsu *et al.*, 2000; Wakamatsu, 2004), it appears unlikely that this is the case in the differentiation of Schwann cell precursors from neural crest cells (Chapter 4). Again, this difference in signals involved in differentiation might lead to more rapid maturation of satellite cells compared to Schwann cells.

Axonal contact is also vitally important for Schwann cells, regulating their survival, proliferation and differentiation (see Section 1.4 and 1.5). Schwann cell precursors also rely exclusively on axonal contact for their survival and

differentiation (see Section 1.3). Among the most important axonally-derived factors is neuregulin-1, which has been shown to regulate the survival of Schwann cell precursors as well as their maturation into Schwann cells. In Chapter 4, I describe the Notch signalling pathway as a novel factor in promoting this transition. In summary, it appears that Notch signalling potentiates the neuregulin-1 signal that is required for this transition to occur, by upregulating erbB2 receptors and thus increasing the responsiveness of Schwann cell precursors to neuregulin-1.

Schwann cell precursors, however, are bipotent and also rise to endoneurial fibroblasts, although they are very strongly glial-biased (Joseph *et al.*, 2004). Differentiation of Schwann cell precursors into their derivatives is accompanied by profound changes in the cytoarchitecture of the developing nerve. At E14 in the rat, the tightly packed nerves contain no extracellular matrix or apparent connective tissue and blood vessels. The Schwann cell precursors display extensive sheath-like processes surrounding large groups of axons. As the nerves mature, dramatic changes in the cytoarchitecture occur. At birth in the rat, the nerves are surrounded by a developing perineurium, which encloses the endoneurial space. Numerous axon-Schwann cell families, endoneurial fibroblasts, blood vessels are embedded in an extracellular matrix (Webster and Favilla, 1984). It is possible that the differentiation of Schwann cell precursors into their derivatives is influenced by such changes in the cytoarchitecture. In adult normal nerves, Schwann cells are found only in close apposition to axons and, as mentioned above, both Schwann cell precursors and Schwann cells require axonal membrane factors for survival and differentiation. Thus, as development proceeds, most of the Schwann cell precursors are likely to remain in contact with axons and consequently differentiate into Schwann cells. However, as the nerves develop and the axons become segregated into families and

the endoneurial space is filled with extracellular matrix, some of Schwann cell precursors might lose contact with axons and it is an attractive possibility that this triggers their differentiation into endoneurial fibroblasts. Contact with axons allows the Schwann cell precursors to receive the positive signals, including neuregulin-1 and Notch, which direct their differentiation into Schwann cells. It is also likely that these signals and/or other axonal signals might also inhibit the differentiation of the Schwann cell precursors into endoneurial fibroblasts. Thus, Schwann cell precursors would no longer receive such signals upon loss of axonal contact and, possibly in response to other factors present in the extracellular matrix, they would differentiate into endoneurial fibroblasts. This model of Schwann cell precursor differentiation has only partially been demonstrated but were more evidence to be found in the future, it would show how the different microenvironment present within the same structure (in this case, the developing nerve) can profoundly influence development.

The results described in Chapter 5 show an even more dramatic influence of different microenvironments on the development of Schwann cell precursors. In this study, the transplant properties of Schwann cells and Schwann cell precursors were examined in two animal models of MS. We found that Schwann cell precursors, transplanted into normal or injured CNS, differentiated into Schwann cells after a period of time. These 'CNS-differentiated' Schwann cells were apparently similar to post-natal Schwann cells in antigenic profile and they remyelinated demyelinated CNS axons with Schwann cell-like characteristics. However, they were remarkably different in other aspects, as examined in this study. We have shown that these 'CNS-differentiated' Schwann cells can integrate into the normal CNS white matter and form close contacts with astrocytes surrounding demyelinating lesions. Another feature that is markedly different from post-natal Schwann cells is their ability to

survive within the normal CNS. They also outperform post-natal Schwann cells in their ability to migrate in the spinal cord and retina.

These observations show that the Schwann cells formed by differentiation of Schwann cell precursors in the CNS are phenotypically partially different from the Schwann cells formed during normal development. In normal development, as mentioned above, axonal signals such as neuregulin-1 and Notch promote the maturation of Schwann cell precursors into Schwann cells. In the CNS also, neuregulin-1 is expressed by axons (Falls, 2003) and the Notch ligand, Jagged 1 is expressed by axons and astrocytes (John *et al.*, 2002; Stidworthy *et al.*, 2004) on their surfaces. The presence of these positive signals is likely to promote the natural progression of Schwann cell precursors into Schwann cells, explaining the Schwann cell-like myelin formed and their antigenic profile. However, the microenvironment in the CNS is likely to be very different from that in the PNS. The presence of astrocytes and oligodendrocytes as well as CNS axons themselves, which most probably express different molecules on their surfaces, are amongst the factors that might influence the differentiation of the Schwann cell precursors. The fact that the 'CNS-differentiated' Schwann cells survive, integrate and migrate in the CNS suggest that they are closer in their phenotype to CNS glia such as astrocytes than Schwann cells.

In this thesis, although I have worked on many different aspects of the developmental biology of the Schwann cell lineage as well as on a cell replacement therapy for MS, one common feature has emerged; differentiation of embryonic cells, at least in the Schwann cell lineage, is fundamentally dependent on the *in vivo* microenvironment in which they are present. I have learned that progenitor cells can follow completely different routes in their differentiation, if their microenvironments,

which are spatially separated by very small distances, are different. This shows how beautiful the study of developmental biology is and, at the same time shows how little we know and most possibly how little we will possibly ever understand.

REFERENCES

Ader, M., Meng, J., Schachner, M. and Bartsch, U. (2000). Formation of myelin after transplantation of neural precursor cells into the retina of young postnatal mice. *Glia* **30**, 301-10.

Akiyama, Y., Radtke, C., Honmou, O. and Kocsis, J. D. (2002). Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* **39**, 229-36.

Allamargot, C., Pouplard-Barthelaix, A. and Fressinaud, C. (2001). A single intracerebral microinjection of platelet-derived growth factor (PDGF) accelerates the rate of remyelination in vivo. *Brain Res* **918**, 28-39.

Allman, D., Aster, J. C. and Pear, W. S. (2002a). Notch signaling in hematopoiesis and early lymphocyte development. *Immunol Rev* **187**, 75-86.

Allman, D., Punt, J. A., Izon, D. J., Aster, J. C. and Pear, W. S. (2002b). An invitation to T and more: notch signaling in lymphopoiesis. *Cell* **109 Suppl**, S1-11.

Altman, J. and Bayer, S. A. (1982). Development of the cranial nerve ganglia and related nuclei in the rat. *Adv Anat Embryol Cell Biol* **74**, 1-90.

Altman, J. and Bayer, S. A. (1984). The development of the rat spinal cord. *Adv Anat Embryol Cell Biol* **85**, 1-164.

Alvarez-Buylla, A., Theelen, M. and Nottebohm, F. (1990). Proliferation "hot spots" in adult avian ventricular zone reveal radial cell division. *Neuron* **5**, 101-9.

Anderson, D. J. (1997). Cellular and molecular biology of neural crest cell lineage determination. *Trends Genet* **13**, 276-80.

Arroyo, E. J., Bermingham, J. R., Jr., Rosenfeld, M. G. and Scherer, S. S. (1998). Promyelinating Schwann cells express Tst-1/SCIP/Oct-6. *J Neurosci* **18**, 7891-902.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.

Aybar, M. J. and Mayor, R. (2002). Early induction of neural crest cells: lessons learned from frog, fish and chick. *Curr Opin Genet Dev* **12**, 452-8.

Bannerman, P. G. and Pleasure, D. (1993). Protein growth factor requirements of rat neural crest cells. *Journal Of Neuroscience Research* **36**, 46.

Bannerman, P. G., Puhalla, S., Sahai, A., Shieh, A., Berman, M. and Pleasure, D. (2000). Glial growth factor-2 promotes the survival, migration and bromodeoxyuridine incorporation of mammalian neural crest cells in caudal neural tube explant cultures. *Developmental Brain Research* **124**, 93.

Bao, Z. Z. and Cepko, C. L. (1997). The expression and function of Notch pathway genes in the developing rat eye. *J Neurosci* **17**, 1425-34.

Barnett, S. C., Hutchins, A. M. and Noble, M. (1993). Purification of olfactory nerve ensheathing cells from the olfactory bulb. *Dev Biol* **155**, 337-50.

Baron, M. (2003). An overview of the Notch signalling pathway. *Semin Cell Dev Biol* **14**, 113-9.

Basch, M. L., Garcia-Castro, M. I. and Bronner-Fraser, M. (2004). Molecular mechanisms of neural crest induction. *Birth Defects Res C Embryo Today* **72**, 109-23.

Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79**, 1277.

Ben-Hur, T., Einstein, O., Mizrachi-Kol, R., Ben-Menachem, O., Reinhartz, E., Karussis, D. and Abramsky, O. (2003). Transplanted multipotential neural precursor cells migrate into the inflamed white matter in response to experimental autoimmune encephalomyelitis. *Glia* **41**, 73-80.

Ben-Levy, R., Paterson, H. F., Marshall, C. J. and Yarden, Y. (1994). A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *Embo J* **13**, 3302-11.

Berger, P., Young, P. and Suter, U. (2002). Molecular cell biology of Charcot-Marie-Tooth disease. *Neurogenetics* **4**, 1-15.

Bermingham, J. R., Jr., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L. and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev* **10**, 1751-62.

Bermingham, J. R., Jr., Shearin, H., Pennington, J., O'Moore, J., Jaegle, M., Driegen, S., van Zon, A., Darbas, A., Ozkaynak, E., Ryu, E. J. et al. (2006). The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. *Nat Neurosci* **9**, 76-84.

Beuche, W. and Friede, R. L. (1985). A new approach toward analyzing peripheral nerve fiber populations. II. Foreshortening of regenerated internodes corresponds to reduced sheath thickness. *J Neuropathol Exp Neurol* **44**, 73-84.

Bignami, A., Eng, L. F., Dahl, D. and Uyeda, C. T. (1972). Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res* **43**, 429-35.

Blair, S. S. (2000). Notch signaling: Fringe really is a glycosyltransferase. *Curr Biol* **10**, R608-12.

Blakemore, W. F. (1976). Remyelination by Schwann cells of axons demyelinated by intraspinal injections of lysolecithin. *Neuropathology Applied Neurobiology* **2**, 21-39.

Blakemore, W. F. (1977). Remyelination of CNS axons by Schwann cells transplanted from the sciatic nerve. *Nature* **266**, 68-9.

Blakemore, W. F. and Crang, A. J. (1985). The use of cultured autologous Schwann cells to remyelinate areas of persistent demyelination in the central nervous system. *J Neurol Sci* **70**, 207-23.

Blakemore, W. F. and Crang, A. J. (1989). The relationship between type-1 astrocytes, Schwann cells and oligodendrocytes following transplantation of glial cell cultures into demyelinating lesions in the adult rat spinal cord. *J Neurocytol* **18**, 519-28.

Blakemore, W. F. and Franklin, R. J. (2000). Transplantation options for therapeutic central nervous system remyelination. *Cell Transplant* **9**, 289-94.

Blanchard, A. D., Sinanan, A., Parmantier, E., Zwart, R., Broos, L., Meijer, D., Meier, C., Jessen, K. R. and Mirsky, R. (1996). Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct-1, Krox-20, and Pax-3. *J Neurosci Res* **46**, 630-40.

Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O. and Goossens, M. (2001). Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Hum Mol Genet* **10**, 2783-95.

Bondy, C., Werner, H., Roberts, J. C. T. and LeRoith, D. (1992). Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: Comparison with insulin-like growth factors I and II. *Neuroscience* **46**, 909.

Borsello, T., Clarke, P. G., Hirt, L., Vercelli, A., Repici, M., Schorderet, D. F., Bogousslavsky, J. and Bonny, C. (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* **9**, 1180-6.

Boruch, A. V., Conners, J. J., Pipitone, M., Deadwyler, G., Storer, P. D., Devries, G. H. and Jones, K. J. (2001). Neurotrophic and migratory properties of an olfactory ensheathing cell line. *Glia* **33**, 225-9.

Bottenstein, J. E. and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci U S A* **76**, 514-7.

Bradley, W. G., Jaros, E. and Jenkison, M. (1977). The nodes of Ranvier in the nerves of mice with muscular dystrophy. *J Neuropathol Exp Neurol* **36**, 797-806.

Bradley, W. G. and Jenkison, M. (1973). Abnormalities of peripheral nerves in murine muscular dystrophy. *Journal of the Neurological Sciences* **18**, 227.

Brazelton, T. R., Rossi, F. M., Keshet, G. I. and Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1775-9.

Brennan, A., Dean, C. H., Zhang, A. L., Cass, D. T., Mirsky, R. and Jessen, K. R. (2000). Endothelins control the timing of Schwann cell generation in vitro and in vivo. *Dev Biol* **227**, 545-57.

Brennan, K. and Gardner, P. (2002). Notching up another pathway. *Bioessays* **24**, 405-10.

Brierley, C. M., Crang, A. J., Iwashita, Y., Gilson, J. M., Scolding, N. J., Compston, D. A. and Blakemore, W. F. (2001). Remyelination of demyelinated CNS axons by transplanted human schwann cells: the deleterious effect of contaminating fibroblasts. *Cell Transplant* **10**, 305-15.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev* **15**, 66-78.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes and Development* **12**, 1825.

Brockes, J. P., Fields, K. L. and Raff, M. C. (1979). Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res* **165**, 105-18.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5**, 207-16.

Brustle, O., Jones, K. N., Learish, R. D., Karram, K., Choudhary, K., Wiestler, O. D., Duncan, I. D. and McKay, R. D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* **285**, 754-6.

Brustle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S. and McKay, R. D. (1997). In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* **94**, 14809-14.

Bunge, R. P. (1993). Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. *Curr Opin Neurobiol* **3**, 805-9.

Butzkueven, H., Zhang, J. G., Soilu-Hanninen, M., Hochrein, H., Chionh, F., Shipham, K. A., Emery, B., Turnley, A. M., Petratos, S., Ernst, M. et al. (2002). LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. *Nat Med* **8**, 613-9.

Cameron-Curry, P., Dulac, C. and Le Douarin, N. M. (1993). Negative regulation of Schwann cell myelin protein gene expression by the dorsal root ganglionic microenvironment. *The European Journal Of Neuroscience* **5**, 594.

Campana, W. M., Darin, S. J. and O'Brien, J. S. (1999). Phosphatidylinositol 3-kinase and Akt protein kinase mediate IGF-I- and prosaptide-induced survival in Schwann cells. *Journal Of Neuroscience Research* **57**, 332.

Cannella, B., Hoban, C. J., Gao, Y. L., Garcia-Arenas, R., Lawson, D., Marchionni, M., Gwynne, D. and Raine, C. S. (1998). The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. *Proc Natl Acad Sci U S A* **95**, 10100-5.

Cannella, B., Pitt, D., Capello, E. and Raine, C. S. (2000). Insulin-like growth factor-1 fails to enhance central nervous system myelin repair during autoimmune demyelination. *Am J Pathol* **157**, 933-43.

Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. and Bishop, J. M. (1997). Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. *Mol Cell Biol* **17**, 6265-73.

Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R. and Guillemot, F. (2000). Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. *Development* **127**, 2323-32.

Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Curr Opin Neurobiol* **9**, 37-46.

Chanas-Sacre, G., Rogister, B., Moonen, G. and Leprince, P. (2000). Radial glia phenotype: origin, regulation, and transdifferentiation. *J Neurosci Res* **61**, 357-63.

Chari, D. M. and Blakemore, W. F. (2002). Efficient recolonisation of progenitor-depleted areas of the CNS by adult oligodendrocyte progenitor cells. *Glia* **37**, 307-13.

Charles, P., Reynolds, R., Seilhean, D., Rougon, G., Aigrot, M. S., Niezgoda, A., Zalc, B. and Lubetzki, C. (2002). Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? *Brain* **125**, 1972-9.

Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R. and Charnay, P. (1988). A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. *Embo J* **7**, 29-35.

Chen, Y., Fischer, W. H. and Gill, G. N. (1997). Regulation of the ERBB-2 promoter by RBPJkappa and NOTCH. *J Biol Chem* **272**, 14110-4.

Chen, Z. L. and Strickland, S. (2003). Laminin gamma1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve. *J Cell Biol* **163**, 889-99.

Cheng, H.-L., Steinway, M., Delaney, C. L., Franke, T. F. and Feldman, E. L. (2000). IGF-I promotes Schwann cell motility and survival via activation of Akt. *Molecular and Cellular Endocrinology* **170**, 211.

Cheng, H. L., Steinway, M. L., Xin, X. and Feldman, E. L. (2001). Insulin-like growth factor-I and Bcl-X(L) inhibit c-jun N-terminal kinase activation and rescue Schwann cells from apoptosis. *Journal Of Neurochemistry* **76**, 935.

Chuah, M. I. and West, A. K. (2002). Cellular and molecular biology of ensheathing cells. *Microsc Res Tech* **58**, 216-27.

Cid, C., Alvarez-Cermeno, J. C., Camafeita, E., Salinas, M. and Alcazar, A. (2004). Antibodies reactive to heat shock protein 90 induce oligodendrocyte precursor cell death in culture. Implications for demyelination in multiple sclerosis. *Faseb J* **18**, 409-11.

Ciutat, D., Caldero, J., Oppenheim, R. W. and Esquerda, J. E. (1996). Schwann cell apoptosis during normal development and after axonal degeneration induced by neurotoxins in the chick embryo. *J Neurosci* **16**, 3979-90.

Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E. and Yanagisawa, M. (1998). Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development (Cambridge, England)* **125**, 813.

Cohen, A. M. and Konigsberg, I. R. (1975). A clonal approach to the problem of neural crest determination. *Dev Biol* **46**, 262-80.

Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J. B., Baron-Van Evercooren, A., Georges-Labouesse, E. and ffrench-Constant, C. (2002). CNS integrins switch growth factor signalling to promote target-dependent survival. *Nature Cell Biology* **4**, 833.

Conboy, I. M. and Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell* **3**, 397-409.

Copelman, C. A., Cuzner, M. L., Groome, N. and Diemel, L. T. (2000). Temporal analysis of growth factor mRNA expression in myelinating rat brain aggregate cultures: increments in CNTF, FGF-2, IGF-I, and PDGF-AA mRNA are induced by antibody-mediated demyelination. *Glia* **30**, 342-51.

Crang, A. J., Franklin, R. J., Blakemore, W. F., Trotter, J., Schachner, M., Barnett, S. C. and Noble, M. (1991). Transplantation of normal and genetically engineered glia into areas of demyelination. *Ann N Y Acad Sci* **633**, 563-5.

Cryns, V. and Yuan, J. (1998). Proteases to die for. *Genes Dev* **12**, 1551-70.

Cui, X. Y., Hu, Q. D., Tekaya, M., Shimoda, Y., Ang, B. T., Nie, D. Y., Sun, L., Hu, W. P., Karsak, M., Duka, T. et al. (2004). NB-3/Notch1 pathway via Deltex1 promotes neural progenitor cell differentiation into oligodendrocytes. *J Biol Chem* **279**, 25858-65.

D'Antonio, M., Droggiti, A., Feltri, M.L., Roes, J., Wrabetz, L., Mirsky, R. and Jessen, K. R. (2006). TGFb Type II receptor signaling controls Schwann cell death and proliferation in developing nerves. *Journal of Neuroscience* **in press.**

Dahl, D., Chi, N. H., Miles, L. E., Nguyen, B. T. and Bignami, A. (1982). Glial fibrillary acidic (GFA) protein in Schwann cells: fact or artifact? *J Histochem Cytochem* **30**, 912-8.

Dahlin, L. B. (1995). Prevention of macrophage invasion impairs regeneration in nerve grafts. *Brain Res* **679**, 274-80.

Darbas, A., Jaegle, M., Walbeehm, E., van den Burg, H., Driegen, S., Broos, L., Uyl, M., Visser, P., Grosveld, F. and Meijer, D. (2004). Cell autonomy of the mouse claw paw mutation. *Dev Biol* **272**, 470-82.

Davies, A. M. (1996). Paracrine and autocrine actions of neurotrophic factors. *Neurochem Res* **21**, 749-53.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-48.

Deacon, T., Dinsmore, J., Costantini, L. C., Ratliff, J. and Isacson, O. (1998). Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. *Exp Neurol* **149**, 28-41.

Delaney, C. L., Cheng, H. L. and Feldman, E. L. (1999). Insulin-like growth factor-I prevents caspase-mediated apoptosis in Schwann cells. *Journal Of Neurobiology* **41**, 540.

Dezawa, M., Takahashi, I., Esaki, M., Takano, M. and Sawada, H. (2001). Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. *Eur J Neurosci* **14**, 1771-6.

Diederich, R. J., Matsuno, K., Hing, H. and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120**, 473-81.

Diner, O. (1965). [The Schwann cells during mitosis and their relation to the axons during the development of the sciatic nerve in the rat]. *C R Acad Sci Hebd Seances Acad Sci D* **261**, 1731-4.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-96.

Dong, Z., Sinanan, A., Parkinson, D., Parmantier, E., Mirsky, R. and Jessen, K. R. (1999). Schwann cell development in embryonic mouse nerves. *J Neurosci Res* **56**, 334-48.

Doucette, R. (1990). Glial influences on axonal growth in the primary olfactory system. *Glia* **3**, 433-49.

Duncan, I. D., Aguayo, A. J., Bunge, R. P. and Wood, P. M. (1981). Transplantation of rat Schwann cells grown in tissue culture into the mouse spinal cord. *J Neurol Sci* **49**, 241-52.

Dupin, E. and Le Douarin, N. M. (2003). Development of melanocyte precursors from the vertebrate neural crest. *Oncogene* **22**, 3016-23.

Dupin, E., Ziller, C. and Le Douarin, N. M. (1998). The avian embryo as a model in developmental studies: chimeras and in vitro clonal analysis. *Curr Top Dev Biol* **36**, 1-35.

Edgar, J. M. and Garbern, J. (2004). The myelinated axon is dependent on the myelinating cell for support and maintenance: molecules involved. *J Neurosci Res* **76**, 593-8.

Ehrengruber, M. U., Lanzrein, M., Xu, Y., Jasek, M. C., Kantor, D. B., Schuman, E. M., Lester, H. A. and Davidson, N. (1998). Recombinant adenovirus-mediated expression in nervous system of genes coding for ion channels and other molecules involved in synaptic function. *Methods Enzymol* **293**, 483-503.

Ehrengruber, M. U., Muhlebach, S. G., Sohrman, S., Leutenegger, C. M., Lester, H. A. and Davidson, N. (2000). Modulation of early growth response (EGR) transcription factor-dependent gene expression by using recombinant adenovirus. *Gene* **258**, 63-9.

Einheber, S., Hannocks, M. J., Metz, C. N., Rifkin, D. B. and Salzer, J. L. (1995). Transforming growth factor-beta 1 regulates axon/Schwann cell interactions. *J Cell Biol* **129**, 443-58.

Einstein, O., Karussis, D., Grigoriadis, N., Mizrachi-Kol, R., Reinhartz, E., Abramsky, O. and Ben-Hur, T. (2003). Intraventricular transplantation of neural precursor cell spheres attenuates acute experimental allergic encephalomyelitis. *Mol Cell Neurosci* **24**, 1074-82.

Eiraku, M., Hirata, Y., Takeshima, H., Hirano, T. and Kengaku, M. (2002). Delta/notch-like epidermal growth factor (EGF)-related receptor, a novel EGF-like repeat-containing protein targeted to dendrites of developing and adult central nervous system neurons. *J Biol Chem* **277**, 25400-7.

Eiraku, M., Tohgo, A., Ono, K., Kaneko, M., Fujishima, K., Hirano, T. and Kengaku, M. (2005). DNER acts as a neuron-specific Notch ligand during Bergmann glial development. *Nat Neurosci* **8**, 873-80.

Eldridge, C. F., Bunge, M. B. and Bunge, R. P. (1989). Differentiation of axon-related Schwann cells in vitro: II. Control of myelin formation by basal lamina. *J Neurosci* **9**, 625-38.

Emerick, K. M., Rand, E. B., Goldmuntz, E., Krantz, I. D., Spinner, N. B. and Piccoli, D. A. (1999). Features of Alagille syndrome in 92 patients: frequency and relation to prognosis. *Hepatology* **29**, 822-9.

Endo, Y., Osumi, N. and Wakamatsu, Y. (2002). Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development. *Development* **129**, 863-73.

Erickson, C. A., Duong, T. D. and Tosney, K. W. (1992). Descriptive and experimental analysis of the dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the chick embryo. *Dev Biol* **151**, 251-72.

Falls, D. L. (2003). Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* **284**, 14-30.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72**, 801-15.

Fawcett, J. W. and Asher, R. A. (1999). The glial scar and central nervous system repair. *Brain Res Bull* **49**, 377-91.

Fazioli, F., Kim, U. H., Rhee, S. G., Molloy, C. J., Segatto, O. and Di Fiore, P. P. (1991). The erbB-2 mitogenic signaling pathway: tyrosine phosphorylation of phospholipase C-gamma and GTPase-activating protein does not correlate with erbB-2 mitogenic potency. *Mol Cell Biol* **11**, 2040-8.

Feltri, M. L., Graus Porta, D., Previtali, S. C., Nodari, A., Migliavacca, B., Cassetti, A., Littlewood-Evans, A., Reichardt, L. F., Messing, A., Quattrini, A. et al. (2002). Conditional disruption of beta 1 integrin in Schwann cells impedes interactions with axons. *J Cell Biol* **156**, 199-209.

Feltri, M. L. and Wrabetz, L. (2005). Laminins and their receptors in Schwann cells and hereditary neuropathies. *J Peripher Nerv Syst* **10**, 128-43.

Felts, P. A. and Smith, K. J. (1992). Conduction properties of central nerve fibers remyelinated by Schwann cells. *Brain Res* **574**, 178-92.

Ffrench-Constant, C., Miller, R. H., Burne, J. F. and Raff, M. C. (1988). Evidence that migratory oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells are kept out of the rat retina by a barrier at the eye-end of the optic nerve. *J Neurocytol* **17**, 13-25.

Fortini, M. E. (2001). Notch and presenilin: a proteolytic mechanism emerges. *Curr Opin Cell Biol* **13**, 627-34.

Franklin, R. J. (2002). Remyelination of the demyelinated CNS: the case for and against transplantation of central, peripheral and olfactory glia. *Brain Res Bull* **57**, 827-32.

Franklin, R. J., Bayley, S. A. and Blakemore, W. F. (1996a). Transplanted CG4 cells (an oligodendrocyte progenitor cell line) survive, migrate, and contribute to repair of areas of demyelination in X-irradiated and damaged spinal cord but not in normal spinal cord. *Exp Neurol* **137**, 263-76.

Franklin, R. J. and Blakemore, W. F. (1997). To what extent is oligodendrocyte progenitor migration a limiting factor in the remyelination of multiple sclerosis lesions? *Mult Scler* **3**, 84-7.

Franklin, R. J., Gilson, J. M., Franceschini, I. A. and Barnett, S. C. (1996b). Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult CNS. *Glia* **17**, 217-24.

Friedman, B., Zaremba, S. and Hockfield, S. (1990). Monoclonal antibody rat 401 recognizes Schwann cells in mature and developing peripheral nerve. *J Comp Neurol* **295**, 43-51.

Frohman, E. M., Racke, M. K. and Raine, C. S. (2006). Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* **354**, 942-55.

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C. and Jones, K. A. (2002). Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes Dev* **16**, 1397-411.

Fu, S. Y. and Gordon, T. (1997). The cellular and molecular basis of peripheral nerve regeneration. *Mol Neurobiol* **14**, 67-116.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* **26**, 383-94.

Gabella, G. (1981). Ultrastructure of the nerve plexuses of the mammalian intestine: the enteric glial cells. *Neuroscience* **6**, 425-36.

Gaiano, N. and Fishell, G. (2002). The role of notch in promoting glial and neural stem cell fates. *Annu Rev Neurosci* **25**, 471-90.

Gaiano, N., Nye, J. S. and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26**, 395-404.

Garbay, B., Heape, A. M., Sargueil, F. and Cassagne, C. (2000). Myelin synthesis in the peripheral nervous system. *Prog Neurobiol* **61**, 267-304.

Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P. and Birchmeier, C. (2000). A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J Cell Biol* **148**, 1035-46.

Gavrilovic, J., Brennan, A., Mirsky, R. and Jessen, K. R. (1995). Fibroblast growth factors and insulin growth factors combine to promote survival of rat Schwann cell precursors without induction of DNA synthesis. *The European Journal Of Neuroscience* **7**, 77.

Genoud, S., Lappe-Siefke, C., Goebbels, S., Radtke, F., Aguet, M., Scherer, S. S., Suter, U., Nave, K. A. and Mantei, N. (2002). Notch1 control of oligodendrocyte differentiation in the spinal cord. *J Cell Biol* **158**, 709-18.

Gensert, J. M. and Goldman, J. E. (1997). Endogenous progenitors remyelinate demyelinated axons in the adult CNS. *Neuron* **19**, 197-203.

Ghazvini, M., Mandemakers, W., Jaegle, M., Piirsoo, M., Driegen, S., Koutsourakis, M., Smit, X., Grosveld, F. and Meijer, D. (2002). A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. *Embo J* **21**, 4612-20.

Ghirnikar, R. S. and Eng, L. F. (1994). Astrocyte-Schwann cell interactions in culture. *Glia* **11**, 367-77.

Ghislain, J., Desmarquet-Trin-Dinh, C., Jaegle, M., Meijer, D., Charnay, P. and Frain, M. (2002). Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. *Development* **129**, 155-66.

Giese, K. P., Martini, R., Lemke, G., Soriano, P. and Schachner, M. (1992). Mouse P0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. *Cell* **71**, 565-76.

Giraldez, A. J. and Cohen, S. M. (2003). Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**, 6533-43.

Givogri, M. I., Costa, R. M., Schonmann, V., Silva, A. J., Campagnoni, A. T. and Bongarzone, E. R. (2002). Central nervous system myelination in mice with deficient expression of Notch1 receptor. *J Neurosci Res* **67**, 309-20.

Glaser, T., Perez-Bouza, A., Klein, K. and Brustle, O. (2005). Generation of purified oligodendrocyte progenitors from embryonic stem cells. *Faseb J* **19**, 112-4.

Golias, C. H., Charalabopoulos, A. and Charalabopoulos, K. (2004). Cell proliferation and cell cycle control: a mini review. *Int J Clin Pract* **58**, 1134-41.

Gotz, M. and Barde, Y. A. (2005). Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. *Neuron* **46**, 369-72.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *Embo J* **10**, 1135-47.

Graham, A. (2003). The neural crest. *Curr Biol* **13**, R381-4.

Griffiths, I., Klugmann, M., Anderson, T., Yool, D., Thomson, C., Schwab, M. H., Schneider, A., Zimmermann, F., McCulloch, M., Nadon, N. et al. (1998). Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science* **280**, 1610-3.

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M. and Scherer, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J Neurosci* **16**, 6107-18.

Guenard, V., Rosenbaum, T., Gwynn, L. A., Doetschman, T., Ratner, N. and Wood, P. M. (1995). Effect of transforming growth factor-beta 1 and -beta 2 on Schwann cell proliferation on neurites. *Glia* **13**, 309-18.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., Ciechanover, A. and Israel, A. (2001). Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. *J Biol Chem* **276**, 34371-8.

Haack, H. and Hynes, R. O. (2001). Integrin Receptors Are Required for Cell Survival and Proliferation during Development of the Peripheral Glial Lineage. *Developmental Biology* **233**, 38.

Hagedorn, L., Paratore, C., Brugnoli, G., Baert, J.-L., Mercader, N., Suter, U. and Sommer, L. (2000). The Ets Domain Transcription Factor Erm Distinguishes Rat Satellite Glia from Schwann Cells and Is Regulated in Satellite Cells by Neuregulin Signaling. *Developmental Biology* **219**, 44.

Halfpenny, C., Benn, T. and Scolding, N. (2002). Cell transplantation, myelin repair, and multiple sclerosis. *Lancet Neurol* **1**, 31-40.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* **14**, 637-45.

Harper, J. A., Yuan, J. S., Tan, J. B., Visan, I. and Guidos, C. J. (2003). Notch signaling in development and disease. *Clin Genet* **64**, 461-72.

Hazel, T. G., Nathans, D. and Lau, L. F. (1988). A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc Natl Acad Sci U S A* **85**, 8444-8.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* **95**, 2509-14.

He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W. and Rosenfeld, M. G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340**, 35-41.

Hebert, J. M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G. R. (1990). Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Developmental Biology* **138**, 454.

Henion, P. D., Garner, A. S., Large, T. H. and Weston, J. A. (1995). trkC-Mediated NT-3 Signaling Is Required for the Early Development of a Subpopulation of Neurogenic Neural Crest Cells. *Developmental Biology* **172**, 602.

Henion, P. D. and Weston, J. A. (1997). Timing and pattern of cell fate restrictions in the neural crest lineage. *Development* **124**, 4351-9.

Henry, E. W., Eicher, E. M. and Sidman, R. L. (1991). The mouse mutation claw paw: forelimb deformity and delayed myelination throughout the peripheral nervous system. *J Hered* **82**, 287-94.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat Cell Biol* **2**, 515-20.

Hinks, G. L., Chari, D. M., O'Leary, M. T., Zhao, C., Keirstead, H. S., Blakemore, W. F. and Franklin, R. J. (2001). Depletion of endogenous oligodendrocyte progenitors rather than increased availability of survival factors is a likely explanation for enhanced survival of transplanted oligodendrocyte progenitors in X-irradiated compared to normal CNS. *Neuropathol Appl Neurobiol* **27**, 59-67.

Hinks, G. L. and Franklin, R. J. (1999). Distinctive patterns of PDGF-A, FGF-2, IGF-I, and TGF-beta1 gene expression during remyelination of experimentally-induced spinal cord demyelination. *Mol Cell Neurosci* **14**, 153-68.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F. and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. *Development* **127**, 2515-22.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-10.

Honmou, O., Felts, P. A., Waxman, S. G. and Kocsis, J. D. (1996). Restoration of normal conduction properties in demyelinated spinal cord axons in the adult rat by transplantation of exogenous Schwann cells. *J Neurosci* **16**, 3199-208.

Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* **109**, 1125-31.

Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A. and Yanagisawa, M. (1994). Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79**, 1267.

Hsiao, L. L., Peltonen, J., Jaakkola, S., Gralnick, H. and Uitto, J. (1991). Plasticity of integrin expression by nerve-derived connective tissue cells. Human Schwann cells, perineurial cells, and fibroblasts express markedly different patterns of beta 1 integrins during nerve development, neoplasia, and in vitro. *J Clin Invest* **87**, 811-20.

Hsieh, J. J., Zhou, S., Chen, L., Young, D. B. and Hayward, S. D. (1999). CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proc Natl Acad Sci U S A* **96**, 23-8.

Hu, Q. D., Ang, B. T., Karsak, M., Hu, W. P., Cui, X. Y., Duka, T., Takeda, Y., Chia, W., Sankar, N., Ng, Y. K. et al. (2003). F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. *Cell* **115**, 163-75.

Hu, Q. D., Cui, X. Y., Ng, Y. K. and Xiao, Z. C. (2004). Axoglial interaction via the notch receptor in oligodendrocyte differentiation. *Ann Acad Med Singapore* **33**, 581-8.

Huang, P. P., Alliquant, B., Carmel, P. W. and Friedman, E. D. (1991). Myelination of the rat retina by transplantation of oligodendrocytes into 4-day-old hosts. *Exp Neurol* **113**, 291-300.

Hungerford, J. E. and Little, C. D. (1999). Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. *J Vasc Res* **36**, 2-27.

Imaizumi, T., Lankford, K. L., Waxman, S. G., Greer, C. A. and Kocsis, J. D. (1998). Transplanted olfactory ensheathing cells remyelinate and enhance axonal conduction in the demyelinated dorsal columns of the rat spinal cord. *J Neurosci* **18**, 6176-85.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev* **9**, 3136-48.

Iwashita, Y., Crang, A. J. and Blakemore, W. F. (2000a). Redistribution of bisbenzimide Hoechst 33342 from transplanted cells to host cells. *Neuroreport* **11**, 1013-6.

Iwashita, Y., Fawcett, J. W., Crang, A. J., Franklin, R. J. and Blakemore, W. F. (2000b). Schwann cells transplanted into normal and X-irradiated adult white matter do not migrate extensively and show poor long-term survival. *Exp Neurol* **164**, 292-302.

Jaegle, M., Ghazvini, M., Mandemakers, W., Piirsoo, M., Driegen, S., Levavasseur, F., Raghoenath, S., Grosveld, F. and Meijer, D. (2003). The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev* **17**, 1380-91.

Jaegle, M., Mandemakers, W., Broos, L., Zwart, R., Karis, A., Visser, P., Grosveld, F. and Meijer, D. (1996). The POU factor Oct-6 and Schwann cell differentiation. *Science* **273**, 507-10.

Jafar-Nejad, H., Norga, K. and Bellen, H. (2002). Numb: "Adapting" notch for endocytosis. *Dev Cell* **3**, 155-6.

Jaros, E. and Bradley, W. G. (1979). Atypical axon-Schwann cell relationships in the common peroneal nerve of the dystrophic mouse: an ultrastructural study. *Neuropathol Appl Neurobiol* **5**, 133-47.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-8.

Jeffery, N. D. and Blakemore, W. F. (1997). Locomotor deficits induced by experimental spinal cord demyelination are abolished by spontaneous remyelination. *Brain* **120 (Pt 1)**, 27-37.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y. and Gavrilovic, J. (1994). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron* **12**, 509-27.

Jessen, K. R. and Mirsky, R. (1983). Astrocyte-like glia in the peripheral nervous system: an immunohistochemical study of enteric glia. *J Neurosci* **3**, 2206-18.

Jessen, K. R. and Mirsky, R. (1998). Origin and early development of Schwann cells. *Microscopy Research And Technique* **41**, 393.

Jessen, K. R. and Mirsky, R. (2002). Signals that determine Schwann cell identity. *J Anat* **200**, 367-76.

Jessen, K. R. and Mirsky, R. (2004). Schwann cell development. In *Myelin biology and disorders*, vol. 1 (ed. R. A. Lazzarini), pp. 329-370. Amsterdam; London: Elsevier Academic Press.

Jessen, K. R. and Mirsky, R. (2005a). The origin and development of glial cells in peripheral nerves. *Nat Rev Neurosci* **6**, 671-82.

Jessen, K. R. and Mirsky, R. (2005b). The Schwann cell lineage. In *Neuroglia*, (ed. H. Kettenmann and B. R. Ransom), pp. 85-100. Oxford: Oxford University Press.

Jessen, K. R., Mirsky, R. and Morgan, L. (1987). Axonal signals regulate the differentiation of non-myelin-forming Schwann cells: an immunohistochemical study of galactocerebroside in transected and regenerating nerves. *J Neurosci* **7**, 3362-9.

Jessen, K. R., Morgan, L., Brammer, M. and Mirsky, R. (1985). Galactocerebroside is expressed by non-myelin-forming Schwann cells in situ. *J Cell Biol* **101**, 1135-43.

Jessen, K. R. and Richardson, W. D. (2001). Glial cell development: basic principles and clinical relevance. Oxford; New York: Oxford University Press.

Jessen, K. R., Thorpe, R. and Mirsky, R. (1984). Molecular identity, distribution and heterogeneity of glial fibrillary acidic protein: an immunoblotting and immunohistochemical study of Schwann cells, satellite cells, enteric glia and astrocytes. *Journal Of Neurocytology* **13**, 187.

John, G. R., Shankar, S. L., Shafit-Zagardo, B., Massimi, A., Lee, S. C., Raine, C. S. and Brosnan, C. F. (2002). Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat Med* **8**, 1115-21.

Joseph, N. M., Mukouyama, Y. S., Mosher, J. T., Jaegle, M., Crone, S. A., Dormand, E. L., Lee, K. F., Meijer, D., Anderson, D. J. and Morrison, S. J. (2004). Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. *Development* **131**, 5599-612.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E. et al. (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383**, 707-10.

Joutel, A. and Tournier-Lasserve, E. (1998). Notch signalling pathway and human diseases. *Semin Cell Dev Biol* **9**, 619-25.

Ju, B. G., Jeong, S., Bae, E., Hyun, S., Carroll, S. B., Yim, J. and Kim, J. (2000). Fringe forms a complex with Notch. *Nature* **405**, 191-5.

Kadesch, T. (2004). Notch signaling: the demise of elegant simplicity. *Curr Opin Genet Dev* **14**, 506-12.

Kalcheim, C. (1989). Basic fibroblast growth factor stimulates survival of nonneuronal cells developing from trunk neural crest. *Developmental Biology* **134**, 1.

Kalcheim, C. and Burstyn-Cohen, T. (2005). Early stages of neural crest ontogeny: formation and regulation of cell delamination. *Int J Dev Biol* **49**, 105-16.

Kalcheim, C., Carmeli, C. and Rosenthal, A. (1992). Neurotrophin 3 is a mitogen for cultured neural crest cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 1661.

Kalcheim, C. and Neufeld, G. (1990). Expression of basic fibroblast growth factor in the nervous system of early avian embryos. *Development* **109**, 203.

Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M. and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* **12**, 2269-77.

Keilhoff, G., Goihl, A., Langnase, K., Fansa, H. and Wolf, G. (2006). Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. *Eur J Cell Biol* **85**, 11-24.

Keirstead, H. S. and Blakemore, W. F. (1997). Identification of post-mitotic oligodendrocytes incapable of remyelination within the demyelinated adult spinal cord. *J Neuropathol Exp Neurol* **56**, 1191-201.

Kirschner, D. A. and Hollingshead, C. J. (1980). Processing for electron microscopy alters membrane structure and packing in myelin. *J Ultrastruct Res* **73**, 211-32.

Kirschner, D. A. and Sidman, R. L. (1976). X-ray diffraction study of myelin structure in immature and mutant mice. *Biochim Biophys Acta* **448**, 73-87.

Kishi, N., Tang, Z., Maeda, Y., Hirai, A., Mo, R., Ito, M., Suzuki, S., Nakao, K., Kinoshita, T., Kadesch, T. et al. (2001). Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. *Int J Dev Neurosci* **19**, 21-35.

Knecht, A. K. and Bronner-Fraser, M. (2002). Induction of the neural crest: a multigene process. *Nat Rev Genet* **3**, 453-61.

Kohama, I., Lankford, K. L., Preiningerova, J., White, F. A., Vollmer, T. L. and Kocsis, J. D. (2001). Transplantation of cryopreserved adult human Schwann cells enhances axonal conduction in demyelinated spinal cord. *J Neurosci* **21**, 944-50.

Komiyama, A. and Suzuki, K. (1992). Age-related differences in proliferative responses of Schwann cells during Wallerian degeneration. *Brain Res* **573**, 267-75.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* **96**, 10711-6.

Kornek, B. and Lassmann, H. (2003). Neuropathology of multiple sclerosis-new concepts. *Brain Res Bull* **61**, 321-6.

Kornek, B., Storch, M. K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M. and Lassmann, H. (2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol* **157**, 267-76.

Koszowski, A. G., Owens, G. C. and Levinson, S. R. (1998). The effect of the mouse mutation claw paw on myelination and nodal frequency in sciatic nerves. *J Neurosci* **18**, 5859-68.

Kubu, C. J., Orimoto, K., Morrison, S. J., Weinmaster, G., Anderson, D. J. and Verdi, J. M. (2002). Developmental changes in Notch1 and numb expression mediated by local cell-cell interactions underlie progressively increasing delta sensitivity in neural crest stem cells. *Dev Biol* **244**, 199-214.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998). Sox10, a novel transcriptional modulator in glial cells. *J Neurosci* **18**, 237-50.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H. and Kamada et, a. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368**, 703.

Kurtz, A., Zimmer, A., Schnutgen, F., Bruning, G., Spener, F. and Muller, T. (1994). The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. *Development* **120**, 2637-49.

Kusumi, K., Sun, E. S., Kerrebrock, A. W., Bronson, R. T., Chi, D. C., Bulotsky, M. S., Spencer, J. B., Birren, B. W., Frankel, W. N. and Lander, E. S. (1998). The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat Genet* **19**, 274-8.

Laeng, P., Molthagen, M., Yu, E. G. and Bartsch, U. (1996). Transplantation of oligodendrocyte progenitor cells into the rat retina: extensive myelination of retinal ganglion cell axons. *Glia* **18**, 200-10.

Lai, E. C. (2002). Notch cleavage: Nicastrin helps Presenilin make the final cut. *Curr Biol* **12**, R200-2.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73.

Lakatos, A., Franklin, R. J. and Barnett, S. C. (2000). Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. *Glia* **32**, 214-25.

Lallier, T., Leblanc, G., Artinger, K. B. and Bronner-Fraser, M. (1992). Cranial and trunk neural crest cells use different mechanisms for attachment to extracellular matrices. *Development* **116**, 531.

Lamperth, L., Manuelidis, L. and Webster, H. d. (1989). Non myelin-forming perineuronal Schwann cells in rat trigeminal ganglia express P0 myelin glycoprotein mRNA during postnatal development. *Molecular Brain Research* **5**, 177.

Lappe-Siefke, C., Goebbels, S., Gravel, M., Nicksch, E., Lee, J., Braun, P. E., Griffiths, I. R. and Nave, K. A. (2003). Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. *Nat Genet* **33**, 366-74.

Le Douarin, N., Dulac, C., Dupin, E. and Cameron-Curry, P. (1991). Glial cell lineages in the neural crest. *Glia* **4**, 175-84.

Le Douarin, N. and Kalcheim, C. (1999). The neural crest. Cambridge: Cambridge University Press.

Le Douarin, N. M. and Dupin, E. (2003). Multipotentiality of the neural crest. *Curr Opin Genet Dev* **13**, 529-36.

Le Douarin, N. M. and Smith, J. (1988). Development of the peripheral nervous system from the neural crest. *Annu Rev Cell Biol* **4**, 375-404.

Le Douarin, N. M. and Teillet, M. A. (1974). Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev Biol* **41**, 162-84.

Le, N., Nagarajan, R., Wang, J. Y., Svaren, J., LaPash, C., Araki, T., Schmidt, R. E. and Milbrandt, J. (2005). Nab proteins are essential for peripheral nervous system myelination. *Nat Neurosci* **8**, 932-40.

LeBlanc, S. E., Jang, S. W., Ward, R. M., Wrabetz, L. and Svaren, J. (2006). Direct regulation of myelin protein zero expression by the Egr2 transactivator. *J Biol Chem* **281**, 5453-60.

Lee, M., Brennan, A., Blanchard, A., Zoidl, G., Dong, Z., Tabernero, A., Zoidl, C., Dent, M. A., Jessen, K. R. and Mirsky, R. (1997). P0 is constitutively expressed in the rat neural crest and embryonic nerves and is negatively and positively regulated by axons to generate non-myelin-forming and myelin-forming Schwann cells, respectively. *Mol Cell Neurosci* **8**, 336-50.

Lee, M. J., Calle, E., Brennan, A., Ahmed, S., Sviderskaya, E., Jessen, K. R. and Mirsky, R. (2001). In early development of the rat mRNA for the major myelin protein P(0) is expressed in nonsensory areas of the embryonic inner ear, notochord, enteric nervous system, and olfactory ensheathing cells. *Dev Dyn* **222**, 40-51.

Leimeroth, R., Lobsiger, C., Lussi, A., Taylor, V., Suter, U. and Sommer, L. (2002). Membrane-bound neuregulin1 type III actively promotes Schwann cell differentiation of multipotent Progenitor cells. *Dev Biol* **246**, 245-58.

Lemke, G. (2006). Neuregulin-1 and myelination. *Sci STKE* **2006**, pe11.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr Opin Neurobiol* **6**, 3-10.

Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol* **9**, 583-9.

Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J. et al. (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**, 243-51.

Lieber, T., Wesley, C. S., Alcamo, E., Hassel, B., Krane, J. F., Campos-Ortega, J. A. and Young, M. W. (1992). Single amino acid substitutions in EGF-like elements of Notch and Delta modify Drosophila development and affect cell adhesion in vitro. *Neuron* **9**, 847-59.

Liu, J. and Kern, J. A. (2002). Neuregulin-1 activates the JAK-STAT pathway and regulates lung epithelial cell proliferation. *Am J Respir Cell Mol Biol* **27**, 306-13.

Liu, S., Qu, Y., Stewart, T. J., Howard, M. J., Chakrabortty, S., Holekamp, T. F. and McDonald, J. W. (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci U S A* **97**, 6126-31.

Lobsiger, C. S., Schweitzer, B., Taylor, V. and Suter, U. (2000). Platelet-derived growth factor-BB supports the survival of cultured rat Schwann cell precursors in synergy with neurotrophin-3. *Glia* **30**, 290.

Loeb, J. A., Khurana, T. S., Robbins, J. T., Yee, A. G. and Fischbach, G. D. (1999). Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. *Development* **126**, 781-91.

Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat Rev Neurosci* **7**, 93-102.

Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000). Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. *Curr Biol* **10**, 491-500.

Lubetzki, C., Williams, A. and Stankoff, B. (2005). Promoting repair in multiple sclerosis: problems and prospects. *Curr Opin Neurol* **18**, 237-44.

Lucchinetti, C. and Lassmann, H. (2001). The neuropathology of multiple sclerosis. In *Glial cell development: basic principles and clinical relevance*, vol. 2001(ed. K. R. Jessen and W. D. Richardson), pp. 379-400. Oxford; New York.

Luo, D., Renault, V. M. and Rando, T. A. (2005). The regulation of Notch signaling in muscle stem cell activation and postnatal myogenesis. *Semin Cell Dev Biol* **16**, 612-22.

Madrid, R. E., Jaros, E., Cullen, M. J. and Bradley, W. G. (1975). Genetically determined defect of Schwann cell basement membrane in dystrophic mouse. *Nature* **257**, 319-21.

Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990). NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. *Neuron* **5**, 501.

Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-63.

Mandemakers, W., Zwart, R., Jaegle, M., Walbeehm, E., Visser, P., Grosveld, F. and Meijer, D. (2000). A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration. *Embo J* **19**, 2992-3003.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-8.

Maro, G. S., Vermeren, M., Voiculescu, O., Melton, L., Cohen, J., Charnay, P. and Topilko, P. (2004). Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. *Nat Neurosci* **7**, 930-8.

Martin, J. R. and Webster, H. D. (1973). Mitotic Schwann cells in developing nerve: their changes in shape, fine structure, and axon relationships. *Dev Biol* **32**, 417-31.

Martinez Arias, A., Zecchini, V. and Brennan, K. (2002). CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? *Curr Opin Genet Dev* **12**, 524-33.

Martini, R., Mohajeri, M. H., Kasper, S., Giese, K. P. and Schachner, M. (1995). Mice doubly deficient in the genes for P0 and myelin basic protein show that both proteins contribute to the formation of the major dense line in peripheral nerve myelin. *J Neurosci* **15**, 4488-95.

Martini, R., Xin, Y. and Schachner, M. (1994). Restricted localization of L1 and N-CAM at sites of contact between Schwann cells and neurites in culture. *Glia* **10**, 70-4.

Masaki, T., Matsumura, K., Hirata, A., Yamada, H., Hase, A., Arai, K., Shimizu, T., Yorifuji, H., Motoyoshi, K. and Kamakura, K. (2002). Expression of dystroglycan and the laminin-alpha 2 chain in the rat peripheral nerve during development. *Exp Neurol* **174**, 109-17.

McDonald, J. W., Liu, X. Z., Qu, Y., Liu, S., Mickey, S. K., Turetsky, D., Gottlieb, D. I. and Choi, D. W. (1999). Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* **5**, 1410-2.

McGill, M. A. and McGlade, C. J. (2003). Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem* **278**, 23196-203.

McGonnell, I. M. and Graham, A. (2002). Trunk neural crest has skeletogenic potential. *Curr Biol* **12**, 767-71.

Meier, C., Parmantier, E., Brennan, A., Mirsky, R. and Jessen, K. R. (1999). Developing Schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. *J Neurosci* **19**, 3847-59.

Meijer, D., Graus, A., Kraay, R., Langeveld, A., Mulder, M. P. and Grosveld, G. (1990). The octamer binding factor Oct6: cDNA cloning and expression in early embryonic cells. *Nucleic Acids Res* **18**, 7357-65.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-90.

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E. and Birchmeier, C. (1997). Isoform-specific expression and function of neuregulin. *Development* **124**, 3575-86.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. and McKercher, S. R. (2000). Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290**, 1779-82.

Michailov, G. V., Sereda, M. W., Brinkmann, B. G., Fischer, T. M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M. H. and Nave, K. A. (2004). Axonal neuregulin-1 regulates myelin sheath thickness. *Science* **304**, 700-3.

Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* **1**, 183-8.

Mirsky, R. and Jessen, K. R. (1999). The neurobiology of Schwann cells. *Brain Pathology* **9**, 293.

Mirsky, R. and Jessen, K. R. (2005). Molecular signalling in Schwann cell development. In *Peripheral neuropathy*, (ed. P. J. Dyck and P. K. Thomas), pp. 2v., various paging. Philadelphia, Pa.; [London]: Elsevier Saunders.

Mirsky, R., Jessen, K. R., Brennan, A., Parkinson, D., Dong, Z., Meier, C., Parmantier, E. and Lawson, D. (2002). Schwann cells as regulators of nerve development. *J Physiol Paris* **96**, 17-24.

Mirsky, R., Jessen, K. R., Schachner, M. and Goridis, C. (1986). Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats. *J. NEUROCYTOL.* **15**, 799.

Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-75.

Monuki, E. S., Kuhn, R., Weinmaster, G., Trapp, B. D. and Lemke, G. (1990). Expression and activity of the POU transcription factor SCIP. *Science* **249**, 1300-3.

Monuki, E. S., Weinmaster, G., Kuhn, R. and Lemke, G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* **3**, 783-93.

Moore, R. and Larue, L. (2004). Cell surface molecules and truncal neural crest ontogeny: a perspective. *Birth Defects Res C Embryo Today* **72**, 140-50.

Morgan, L., Jessen, K. R. and Mirsky, R. (1994). Negative regulation of the P0 gene in Schwann cells: suppression of P0 mRNA and protein induction in cultured Schwann cells by FGF2 and TGF beta 1, TGF beta 2 and TGF beta 3. *Development* **120**, 1399-409.

Morgenstern, J. P. and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**, 3587-96.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K.-F. (1999a). Rescue of the Cardiac Defect in ErbB2 Mutant Mice Reveals Essential Roles of ErbB2 in Peripheral Nervous System Development. *Neuron* **23**, 273.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F. (1999b). Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273-83.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Morrison, S. J., White, P. M., Zock, C. and Anderson, D. J. (1999). Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**, 737-49.

Morrissey, T. K., Levi, A. D., Nuijens, A., Sliwkowski, M. X. and Bunge, R. P. (1995). Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. *Proc Natl Acad Sci U S A* **92**, 1431-5.

Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev Biol* **228**, 151-65.

Munro, S. and Freeman, M. (2000). The notch signalling regulator fringe acts in the Golgi apparatus and requires the glycosyltransferase signature motif DXD. *Curr Biol* **10**, 813-20.

Murphy, P., Topilko, P., Schneider-Maunoury, S., Seitanidou, T., Baron-Van Evercooren, A. and Charnay, P. (1996). The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. *Development* **122**, 2847-57.

Nagarajan, R., Le, N., Mahoney, H., Araki, T. and Milbrandt, J. (2002). Deciphering peripheral nerve myelination by using Schwann cell expression profiling. *Proc Natl Acad Sci U S A* **99**, 8998-9003.

Nagarajan, R., Svaren, J., Le, N., Araki, T., Watson, M. and Milbrandt, J. (2001). EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. *Neuron* **30**, 355-68.

Nakagawa, M., Miyagoe-Suzuki, Y., Ikezoe, K., Miyata, Y., Nonaka, I., Harii, K. and Takeda, S. (2001). Schwann cell myelination occurred without basal lamina formation in laminin alpha2 chain-null mutant (dy3K/dy3K) mice. *Glia* **35**, 101-10.

Nakagawa, S. and Takeichi, M. (1995). Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **121**, 1321-32.

Nakagawa, S. and Takeichi, M. (1998). Neural crest emigration from the neural tube depends on regulated cadherin expression. *Development* **125**, 2963-71.

Nave, K. A. (2001). Myelin-specific genes and their mutations in the mouse. In *Glial cell development: basic principles and clinical relevance*, vol. 2001. (ed. K. R. Jessen and W. D. Richardson), pp. 177-208. Oxford; New York.

Nicholson, D. W. and Thornberry, N. A. (1997). Caspases: killer proteases. *Trends Biochem Sci* **22**, 299-306.

Nickols, J. C., Valentine, W., Kanwal, S. and Carter, B. D. (2003). Activation of the transcription factor NF-kappaB in Schwann cells is required for peripheral myelin formation. *Nat Neurosci* **6**, 161-7.

Niederlander, C. and Lumsden, A. (1996). Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves. *Development* **122**, 2367-74.

Niehaus, A., Shi, J., Grzenkowski, M., Diers-Fenger, M., Archelos, J., Hartung, H. P., Toyka, K., Bruck, W. and Trotter, J. (2000). Patients with active relapsing-remitting multiple sclerosis synthesize antibodies recognizing oligodendrocyte progenitor cell surface protein: implications for remyelination. *Ann Neurol* **48**, 362-71.

Nister, M., Urhbon, L., Hesselager, G. and Westermark, B. (2001). Glial tumours of the CNS. In *Glial cell development: basic principles and clinical relevance*, vol. 2001.(ed. K. R. Jessen and W. D. Richardson), pp. 437-456. Oxford; New York.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-20.

Noseworthy, J. H., Lucchinetti, C., Rodriguez, M. and Weinshenker, B. G. (2000). Multiple sclerosis. *N Engl J Med* **343**, 938-52.

O'Leary, M. T. and Blakemore, W. F. (1997). Oligodendrocyte precursors survive poorly and do not migrate following transplantation into the normal adult central nervous system. *J Neurosci Res* **48**, 159-67.

Oaklander, A. L. and Spencer, P. S. (1988). Cold blockade of axonal transport activates premitotic activity of Schwann cells and wallerian degeneration. *J Neurochem* **50**, 490-6.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem* **276**, 35847-53.

Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. et al. (1997). Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* **16**, 235-42.

Okajima, T., Xu, A. and Irvine, K. D. (2003). Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. *J Biol Chem* **278**, 42340-5.

Orr-Urtreger, A., Trakhtenbrot, L., Ben-Levy, R., Wen, D., Rechavi, G., Lonai, P. and Yarden, Y. (1993). Neural expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. *Proc Natl Acad Sci U S A* **90**, 1867-71.

Ozawa, K., Suchanek, G., Breitschopf, H., Bruck, W., Budka, H., Jellinger, K. and Lassmann, H. (1994). Patterns of oligodendroglia pathology in multiple sclerosis. *Brain* **117 (Pt 6)**, 1311-22.

Pan, D. and Rubin, G. M. (1997). Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. *Cell* **90**, 271-80.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-12.

Pannese, E. (1981). The satellite cells of the sensory ganglia. *Adv Anat Embryol Cell Biol* **65**, 1-111.

Paratore, C., Brugnoli, G., Lee, H.-Y., Suter, U. and Sommer, L. (2002). The Role of the Ets Domain Transcription Factor Erm in Modulating Differentiation of Neural Crest Stem Cells. *Developmental Biology* **250**, 168.

Paratore, C., Goerich, D. E., Suter, U., Wegner, M. and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128**, 3949-61.

Parkinson, D. B., Bhaskaran, A., Droggiti, A., Dickinson, S., D'Antonio, M., Mirsky, R. and Jessen, K. R. (2004). Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell proliferation and death. *J Cell Biol* **164**, 385-94.

Parkinson, D. B., Dickinson, S., Bhaskaran, A., Kinsella, M. T., Brophy, P. J., Sherman, D. L., Sharghi-Namini, S., Duran Alonso, M. B., Mirsky, R. and Jessen, K. R. (2003). Regulation of the myelin gene periaxin provides evidence for Krox-20-independent myelin-related signalling in Schwann cells. *Mol Cell Neurosci* **23**, 13-27.

Parkinson, D. B., Dong, Z., Bunting, H., Whitfield, J., Meier, C., Marie, H., Mirsky, R. and Jessen, K. R. (2001). Transforming growth factor B (TGFB) mediates Schwann cell death in vitro and in vivo: Examination of c-Jun activation, interactions with survival signals, and the relationship of TGFB-mediated death to Schwann cell differentiation. *Journal of Neuroscience* **21**, 8572.

Patten, B. A., Peyrin, J. M., Weinmaster, G. and Corfas, G. (2003). Sequential signaling through Notch1 and erbB receptors mediates radial glia differentiation. *J Neurosci* **23**, 6132-40.

Patten, B. A., Sardi, S. P., Koirala, S., Nakafuku, M. and Corfas, G. (2006). Notch1 signaling regulates radial glia differentiation through multiple transcriptional mechanisms. *J Neurosci* **26**, 3102-8.

Peirano, R. I., Goerich, D. E., Riethmacher, D. and Wegner, M. (2000). Protein zero gene expression is regulated by the glial transcription factor Sox10. *Mol Cell Biol* **20**, 3198-209.

Peles, E., Lamprecht, R., Ben-Levy, R., Tzahar, E. and Yarden, Y. (1992). Regulated coupling of the Neu receptor to phosphatidylinositol 3'-kinase and its release by oncogenic activation. *J Biol Chem* **267**, 12266-74.

Peles, E., Levy, R. B., Or, E., Ullrich, A. and Yarden, Y. (1991). Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase C gamma. *Embo J* **10**, 2077-86.

Peles, E. and Salzer, J. L. (2000). Molecular domains of myelinated axons. *Curr Opin Neurobiol* **10**, 558-65.

Perier, O. and Gregoire, A. (1965). Electron microscopic features of multiple sclerosis lesions. *Brain* **88**, 937-52.

Perris, R. and Perissinotto, D. (2000). Role of the extracellular matrix during neural crest cell migration. *Mechanisms of Development* **95**, 3.

Perry, V. H. and Lund, R. D. (1990). Evidence that the lamina cribrosa prevents intraretinal myelination of retinal ganglion cell axons. *J Neurocytol* **19**, 265-72.

Pfeiffer, S. E., Warrington, A. E. and Bansal, R. (1993). The oligodendrocyte and its many cellular processes. *Trends Cell Biol* **3**, 191-7.

Pinco, O., Carmeli, C., Rosenthal, A. and Kalcheim, C. (1993). Neurotrophin-3 affects proliferation and differentiation of distinct neural crest cells and is present in the early neural tube of avian embryos. *Journal Of Neurobiology* **24**, 1626.

Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J. and Yarden, Y. (1996). Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J Biol Chem* **271**, 19029-32.

Pluchino, S., Furlan, R. and Martino, G. (2004). Cell-based remyelinating therapies in multiple sclerosis: evidence from experimental studies. *Curr Opin Neurol* **17**, 247-55.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A. et al. (2003). Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* **422**, 688-94.

Podratz, J. L., Rodriguez, E. and Windebank, A. J. (2001). Role of the extracellular matrix in myelination of peripheral nerve. *Glia* **35**, 35-40.

Previtali, S. C., Dina, G., Nodari, A., Fasolini, M., Wrabetz, L., Mayer, U., Feltri, M. L. and Quattrini, A. (2003a). Schwann cells synthesize alpha7beta1 integrin which is dispensable for peripheral nerve development and myelination. *Mol Cell Neurosci* **23**, 210-8.

Previtali, S. C., Feltri, M. L., Archelos, J. J., Quattrini, A., Wrabetz, L. and Hartung, H.-P. (2001). Role of integrins in the peripheral nervous system. *Progress in Neurobiology* **64**, 35.

Previtali, S. C., Nodari, A., Taveggia, C., Pardini, C., Dina, G., Villa, A., Wrabetz, L., Quattrini, A. and Feltri, M. L. (2003b). Expression of laminin receptors in schwann cell differentiation: evidence for distinct roles. *J Neurosci* **23**, 5520-30.

Prigent, S. A. and Gullick, W. J. (1994). Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *Embo J* **13**, 2831-41.

Prineas, J. W., Barnard, R. O., Kwon, E. E., Sharer, L. R. and Cho, E. S. (1993). Multiple sclerosis: remyelination of nascent lesions. *Ann Neurol* **33**, 137-51.

Privat, A., Jacque, C., Bourre, J. M., Dupouey, P. and Baumann, N. (1979). Absence of the major dense line in myelin of the mutant mouse "shiverer". *Neurosci Lett* **12**, 107-12.

Pusch, C., Hustert, E., Pfeifer, D., Sudbeck, P., Kist, R., Roe, B., Wang, Z., Balling, R., Blin, N. and Scherer, G. (1998). The SOX10/Sox10 gene from human and mouse: sequence, expression, and transactivation by the encoded HMG domain transcription factor. *Hum Genet* **103**, 115-23.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T. and Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J Biol Chem* **275**, 35734-7.

Radtke, F., Schweisguth, F. and Pear, W. (2005). The Notch 'gospel'. *EMBO Rep* **6**, 1120-5.

Rakic, P. (1971a). Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res* **33**, 471-6.

Rakic, P. (1971b). Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. *J Comp Neurol* **141**, 283-312.

Ramon-Cueto, A. and Nieto-Sampedro, M. (1992). Glial cells from adult rat olfactory bulb: immunocytochemical properties of pure cultures of ensheathing cells. *Neuroscience* **47**, 213-20.

Ramon-Cueto, A., Plant, G. W., Avila, J. and Bunge, M. B. (1998). Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. *J Neurosci* **18**, 3803-15.

Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc Natl Acad Sci U S A* **79**, 2709-13.

Ranvier, L. and Weber, E. (1878). Leçons sur l'histologie du système nerveux. Paris: Librairie F. Savy.

Ratner, N. and Daston, M. M. (2001). Genetic and cellular mechanisms of Schwann cell tumour formation: neurofibromatosis type 1 and neurofibromatosis type 2. In *Glial cell development: basic principles and clinical relevance*, vol. 2001.(ed. K. R. Jessen and W. D. Richardson), pp. 355-378. Oxford; New York.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-99.

Ridley, A. J., Davis, J. B., Stroobant, P. and Land, H. (1989). Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. *J Cell Biol* **109**, 3419-24.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-30.

Ringelmann, B., Roder, C., Hallmann, R., Maley, M., Davies, M., Grounds, M. and Sorokin, L. (1999). Expression of laminin alpha1, alpha2, alpha4, and alpha5 chains, fibronectin, and tenascin-C in skeletal muscle of dystrophic 129ReJ dy/dy mice. *Exp Cell Res* **246**, 165-82.

Ritchie, J. M. and Rang, H. P. (1983). Extraneuronal saxitoxin binding sites in rabbit myelinated nerve. *Proc Natl Acad Sci U S A* **80**, 2803-7.

Robitaille, R. (1998). Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. *Neuron* **21**, 847-55.

Rosenbluth, J., Hasegawa, M., Shirasaki, N., Rosen, C. L. and Liu, Z. (1990). Myelin formation following transplantation of normal fetal glia into myelin-deficient rat spinal cord. *J Neurocytol* **19**, 718-30.

Ross, D. A. and Kadesch, T. (2001). The notch intracellular domain can function as a coactivator for LEF-1. *Mol Cell Biol* **21**, 7537-44.

Roux, P. P. and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* **68**, 320-44.

Ruffini, F., Furlan, R., Poliani, P. L., Brambilla, E., Marconi, P. C., Bergami, A., Desina, G., Glorioso, J. C., Comi, G. and Martino, G. (2001). Fibroblast growth factor-II gene therapy reverts the clinical course and the pathological signs of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice. *Gene Ther* **8**, 1207-13.

Rutkowski, J. L., Kirk, C. J., Lerner, M. A. and Tennekoon, G. I. (1995). Purification and expansion of human Schwann cells in vitro. *Nat Med* **1**, 80-3.

Ryseck, R. P., MacDonald-Bravo, H., Mattei, M. G. and Bravo, R. (1989). Cloning and sequence of a secretory protein induced by growth factors in mouse fibroblasts. *Exp Cell Res* **180**, 266-75.

Sagane, K., Hayakawa, K., Kai, J., Hirohashi, T., Takahashi, E., Miyamoto, N., Ino, M., Oki, T., Yamazaki, K. and Nagasu, T. (2005). Ataxia and peripheral nerve hypomyelination in ADAM22-deficient mice. *BMC Neurosci* **6**, 33.

Sagane, K., Yamazaki, K., Mizui, Y. and Tanaka, I. (1999). Cloning and chromosomal mapping of mouse ADAM11, ADAM22 and ADAM23. *Gene* **236**, 79-86.

Saito, F., Moore, S. A., Barresi, R., Henry, M. D., Messing, A., Ross-Barta, S. E., Cohn, R. D., Williamson, R. A., Sluka, K. A., Sherman, D. L. et al. (2003). Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron* **38**, 747-58.

Salzer, J. L. and Bunge, R. P. (1980). Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *J Cell Biol* **84**, 739-52.

Salzer, J. L., Bunge, R. P. and Glaser, L. (1980a). Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J Cell Biol* **84**, 767-78.

Salzer, J. L., Williams, A. K., Glaser, L. and Bunge, R. P. (1980b). Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *J Cell Biol* **84**, 753-66.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* **6**, 2620-34.

Sasaki, M., Honmou, O., Akiyama, Y., Uede, T., Hashi, K. and Kocsis, J. D. (2001). Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. *Glia* **35**, 26-34.

Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D. et al. (2003). neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development* **130**, 4785-95.

Sato, T., Sasai, N. and Sasai, Y. (2005). Neural crest determination by co-activation of Pax3 and Zic1 genes in Xenopus ectoderm. *Development* **132**, 2355-63.

Satow, T., Bae, S. K., Inoue, T., Inoue, C., Miyoshi, G., Tomita, K., Bessho, Y., Hashimoto, N. and Kageyama, R. (2001). The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina. *J Neurosci* **21**, 1265-73.

Sauvageot, C. M. and Stiles, C. D. (2002). Molecular mechanisms controlling cortical gliogenesis. *Curr Opin Neurobiol* **12**, 244-9.

Scheer, N., Groth, A., Hans, S. and Campos-Ortega, J. A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* **128**, 1099-107.

Scherer, S. S. and Salzer, J. L. (2001). Axonal-Schwann cell interactions during peripheral nerve degeneration and regeneration. In *Glial cell development: basic principles and clinical relevance*, vol. 2001.(ed. K. R. Jessen and W. D. Richardson), pp. 299-330. Oxford; New York.

Scherer, S. S., Wang, D. Y., Kuhn, R., Lemke, G., Wrabetz, L. and Kamholz, J. (1994). Axons regulate Schwann cell expression of the POU transcription factor SCIP. *J Neurosci* **14**, 1930-42.

Schmid, R. S., McGrath, B., Berechid, B. E., Boyles, B., Marchionni, M., Sestan, N. and Anton, E. S. (2003). Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. *Proc Natl Acad Sci U S A* **100**, 4251-6.

Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-214.

Setzu, A., Ffrench-Constant, C. and Franklin, R. J. (2004). CNS axons retain their competence for myelination throughout life. *Glia* **45**, 307-11.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P. and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* 77, 349-60.

Sherman, D. L. and Brophy, P. J. (2005). Mechanisms of axon ensheathment and myelin growth. *Nat Rev Neurosci* **6**, 683-90.

Sherman, L., Stocker, K. M., Morrison, R. and Ciment, G. (1993). Basic fibroblast growth factor (bFGF) acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes. *Development* **118**, 1313-26.

Shields, S. A., Blakemore, W. F. and Franklin, R. J. (2000). Schwann cell remyelination is restricted to astrocyte-deficient areas after transplantation into demyelinated adult rat brain. *J Neurosci Res* **60**, 571-8.

Shin, M. K., Levorse, J. M., Ingram, R. S. and Tilghman, S. M. (1999). The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* **402**, 496.

Sidebotham, E. L., Woodward, M. N., Kenny, S. E., Lloyd, D. A., Vaillant, C. R. and Edgar, D. H. (2002). Localization and endothelin-3 dependence of stem cells of the enteric nervous system in the embryonic colon. *Journal of Pediatric Surgery* **37**, 145.

Sjolund, J., Manetopoulos, C., Stockhausen, M. T. and Axelson, H. (2005). The Notch pathway in cancer: differentiation gone awry. *Eur J Cancer* **41**, 2620-9.

Sketelj, J., Bresjanac, M. and Popovic, M. (1989). Rapid growth of regenerating axons across the segments of sciatic nerve devoid of Schwann cells. *J Neurosci Res* **24**, 153-62.

Skoff, A. M., Lisak, R. P., Bealmear, B. and Benjamins, J. A. (1998). TNF-alpha and TGF-beta act synergistically to kill Schwann cells. *J Neurosci Res* **53**, 747-56.

Smith, E. J., Blakemore, W. F. and McDonald, W. I. (1979). Central remyelination restores secure conduction. *Nature* **280**, 395-6.

Smith, P. M., Sim, F. J., Barnett, S. C. and Franklin, R. J. (2001). SCIP/Oct-6, Krox-20, and desert hedgehog mRNA expression during CNS remyelination by transplanted olfactory ensheathing cells. *Glia* **36**, 342-53.

Smith-Thomas, L. C. and Fawcett, J. W. (1989). Expression of Schwann cell markers by mammalian neural crest cells in vitro. *Development* **105**, 251-62.

Soilu-Hanninen, M., Ekert, P., Bucci, T., Syroid, D., Bartlett, P. F. and Kilpatrick, T. J. (1999). Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. *J Neurosci* **19**, 4828-38.

Sommer, I. and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol* **83**, 311-27.

Sonnenberg-Riethmacher, E., Miehe, M., Stolt, C. C., Goerich, D. E., Wegner, M. and Riethmacher, D. (2001). Development and degeneration of dorsal root ganglia in the absence of the HMG-domain transcription factor Sox10. *Mech Dev* **109**, 253-65.

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat Genet* **18**, 60-4.

Spana, E. P. and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17**, 21-6.

Spencer, P. S. and Schaumburg, H. H. (1973). An ultrastructural study of the inner core of the Pacinian corpuscle. *J Neurocytol* **2**, 217-35.

Stangel, M. and Hartung, H. P. (2002). Remyelinating strategies for the treatment of multiple sclerosis. *Prog Neurobiol* **68**, 361-76.

Stemple, D. L. and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-85.

Stevens, B. (2006). Cross-talk between growth factor and purinergic signalling regulates Schwann cell proliferation. *Novartis Found Symp* **276**, 162-75; discussion 175-80, 233-7, 275-81.

Stewart, H. J., Brennan, A., Rahman, M., Zoidl, G., Mitchell, P. J., Jessen, K. R. and Mirsky, R. (2001). Developmental regulation and overexpression of the transcription factor AP-2, a potential regulator of the timing of Schwann cell generation. *Eur J Neurosci* **14**, 363-72.

Stewart, H. J., Morgan, L., Jessen, K. R. and Mirsky, R. (1993). Changes in DNA synthesis rate in the Schwann cell lineage in vivo are correlated with the precursor--Schwann cell transition and myelination. *Eur J Neurosci* **5**, 1136-44.

Stewart, H. J., Turner, D., Jessen, K. R. and Mirsky, R. (1997). Expression and regulation of alpha1beta1 integrin in Schwann cells. *J Neurobiol* **33**, 914-28.

Stidworthy, M. F., Genoud, S., Li, W. W., Leone, D. P., Mantei, N., Suter, U. and Franklin, R. J. (2004). Notch1 and Jagged1 are expressed after CNS demyelination, but are not a major rate-determining factor during remyelination. *Brain* **127**, 1928-41.

Stirling, C. A. (1975). Abnormalities in Schwann cell sheaths in spinal nerve roots of dystrophic mice. *J Anat* **119**, 169-80.

Suzuki, N., Rohdewohld, H., Neuman, T., Gruss, P. and Scholer, H. R. (1990). Oct-6: a POU transcription factor expressed in embryonal stem cells and in the developing brain. *Embo J* **9**, 3723-32.

Syroid, D. E., Maycox, P. J., Soilu-Hanninen, M., Petratos, S., Bucci, T., Burrola, P., Murray, S., Cheema, S., Lee, K. F., Lemke, G. et al. (2000). Induction of postnatal schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy. *J Neurosci* **20**, 5741-7.

Syroid, D. E., Zorick, T. S., Arbet-Engels, C., Kilpatrick, T. J., Eckhart, W. and Lemke, G. (1999). A role for insulin-like growth factor-I in the regulation of Schwann cell survival. *Journal of Neuroscience* **19**, 2059.

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H. and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. *Neuron* **29**, 45-55.

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shrager, P. et al. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* **47**, 681-94.

Teillet, M. A., Kalcheim, C. and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. *Dev Biol* **120**, 329-47.

Testaz, S. and Duband, J. L. (2001). Central role of the alpha4beta1 integrin in the coordination of avian truncal neural crest cell adhesion, migration, and survival. *Developmental Dynamics: An Official Publication Of The American Association Of Anatomists* **222**, 127.

Toma, J. G., Akhavan, M., Fernandes, K. J., Barnabe-Heider, F., Sadikot, A., Kaplan, D. R. and Miller, F. D. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* **3**, 778-84.

Toma, J. G., McKenzie, I. A., Bagli, D. and Miller, F. D. (2005). Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* **23**, 727-37.

Topilko, P. and Meijer, D. (2001). Transcription factors that control Schwann cell development and myelination. In *Glial cell development: basic principles and clinical relevance*, vol. 2001. (ed. K. R. Jessen and W. D. Richardson), pp. 223-244. Oxford; New York.

Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B., Seitanidou, T., Babinet, C. and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**, 796-9.

Trachtenberg, J. T. and Thompson, W. J. (1996). Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379**, 174-7.

Trapp, B. D., Pfeiffer, S. E., Anitei, M. and Kidd, G. J. (2004). Cell biology of myelin assembly. In *Myelin biology and disorders*, (ed. R. A. Lazzarini). Amsterdam; London: Elsevier Academic Press.

Turnpenny, P. D., Whittock, N., Duncan, J., Dunwoodie, S., Kusumi, K. and Ellard, S. (2003). Novel mutations in DLL3, a somitogenesis gene encoding a ligand for the Notch signalling pathway, cause a consistent pattern of abnormal vertebral segmentation in spondylocostal dysostosis. *J Med Genet* **40**, 333-9.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* **16**, 5276-87.

Verheijen, M. H., Chrast, R., Burrola, P. and Lemke, G. (2003). Local regulation of fat metabolism in peripheral nerves. *Genes Dev* **17**, 2450-64.

Villoslada, P., Hauser, S. L., Bartke, I., Unger, J., Heald, N., Rosenberg, D., Cheung, S. W., Mobley, W. C., Fisher, S. and Genain, C. P. (2000). Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. *J Exp Med* **191**, 1799-806.

Wakamatsu, Y. (2004). Understanding glial differentiation in vertebrate nervous system development. *Tohoku J Exp Med* **203**, 233-40.

Wakamatsu, Y., Endo, Y., Osumi, N. and Weston, J. A. (2004a). Multiple roles of Sox2, an HMG-box transcription factor in avian neural crest development. *Dev Dyn* **229**, 74-86.

Wakamatsu, Y., Maynard, T. M., Jones, S. U. and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* **23**, 71-81.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-21.

Wakamatsu, Y., Osumi, N. and Weston, J. A. (2004b). Expression of a novel secreted factor, Seraf indicates an early segregation of Schwann cell precursors from neural crest during avian development. *Dev Biol* **268**, 162-73.

Wallasch, C., Weiss, F. U., Niederfellner, G., Jallal, B., Issing, W. and Ullrich, A. (1995). Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *Embo J* **14**, 4267-75.

Wallquist, W., Plantman, S., Thams, S., Thyboll, J., Kortesmaa, J., Lannergren, J., Domogatskaya, A., Ogren, S. O., Risling, M., Hammarberg, H. et al. (2005). Impeded interaction between Schwann cells and axons in the absence of laminin alpha4. *J Neurosci* **25**, 3692-700.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63-75.

Wang, X. and Tournier, C. (2006). Regulation of cellular functions by the ERK5 signalling pathway. *Cell Signal* **18**, 753-60.

Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P. and Haltiwanger, R. S. (2001). Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *J Biol Chem* **276**, 40338-45.

Wanner, I. B., Guerra, N.K., Mahoney, J., Kumar, A., Wood, P., Mirsky, R. and Jessen, K.R. (2006). Role of N-cadherin in Schwann cell precursors of growing nerves. *Glia* **in press**.

Warrington, A. E., Barbarese, E. and Pfeiffer, S. E. (1993). Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. *J Neurosci Res* **34**, 1-13.

Watanabe, M., Toyama, Y. and Nishiyama, A. (2002). Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. *J Neurosci Res* **69**, 826-36.

Webster, H. and Favilla, J. T. (1984). Development of peripheral nerves. In *Peripheral neuropathy*, (ed. P. J. Dyck), pp. 329-359. Philadelphia; London: W.B. Saunders Co.

Webster, H. D. (1971). The geometry of peripheral myelin sheaths during their formation and growth in rat sciatic nerves. *J Cell Biol* **48**, 348-67.

Webster, H. D. (1993). Myelin injury and repair. *Advances in Neurology* **59**, 67-73.

Webster, H. D., Martin, R. and O'Connell, M. F. (1973). The relationships between interphase Schwann cells and axons before myelination: a quantitative electron microscopic study. *Dev Biol* **32**, 401-16.

Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* **27**, 1409-20.

Wegner, M. (2000). Transcriptional control in myelinating glia: flavors and spices. *Glia* **31**, 1-14.

Wegner, M. and Stolt, C. C. (2005). From stem cells to neurons and glia: a Soxist's view of neural development. *Trends Neurosci* **28**, 583-8.

Weiner, J. A. and Chun, J. (1999). Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. *Proc Natl Acad Sci U S A* **96**, 5233-8.

Weiner, J. A., Fukushima, N., Contos, J. J., Scherer, S. S. and Chun, J. (2001). Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. *J Neurosci* **21**, 7069-78.

Weinshenker, B. G. (1998). The natural history of multiple sclerosis: update 1998. *Semin Neurol* **18**, 301-7.

Weissman, I. L., Anderson, D. J. and Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol* **17**, 387-403.

Wekerle, H. (1993). T-cell autoimmunity in the central nervous system. *Intervirology* **35**, 95-100.

Wekerle, H. (1998). Immune pathogenesis of multiple sclerosis. Brain autoimmune reactivity and its control by neuronal function. *Mult Scler* **4**, 136-7.

Weston, C. R. and Davis, R. J. (2002). The JNK signal transduction pathway. *Curr Opin Genet Dev* **12**, 14-21.

Weston, J. A. (1963). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. *Dev Biol* **6**, 279-310.

White, J. M. (2003). ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr Opin Cell Biol* **15**, 598-606.

White, P. M. and Anderson, D. J. (1999). In vivo transplantation of mammalian neural crest cells into chick hosts reveals a new autonomic sublineage restriction. *Development* **126**, 4351-63.

White, P. M., Morrison, S. J., Orimoto, K., Kubu, C. J., Verdi, J. M. and Anderson, D. J. (2001). Neural crest stem cells undergo cell-intrinsic developmental changes in sensitivity to instructive differentiation signals. *Neuron* **29**, 57-71.

Wilson-Rawls, J., Molkentin, J. D., Black, B. L. and Olson, E. N. (1999). Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. *Mol Cell Biol* **19**, 2853-62.

Winseck, A. K., Caldero, J., Ciutat, D., Prevette, D., Scott, S. A., Wang, G., Esquerda, J. E. and Oppenheim, R. W. (2002). In vivo analysis of Schwann cell programmed cell death in the embryonic chick: regulation by axons and glial growth factor. *J Neurosci* **22**, 4509-21.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P. and Birchmeier, C. (1999). Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* **13**, 2538-48.

Wolpowitz, D., Mason, T. B., Dietrich, P., Mendelsohn, M., Talmage, D. A. and Role, L. W. (2000). Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron* **25**, 79-91.

Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* **61**, 364-70.

Xin, M., Yue, T., Ma, Z., Wu, F. F., Gow, A. and Lu, Q. R. (2005). Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. *J Neurosci* **25**, 1354-65.

Yamamoto, S., Nagao, M., Sugimori, M., Kosako, H., Nakatomi, H., Yamamoto, N., Takebayashi, H., Nabeshima, Y., Kitamura, T., Weinmaster, G. et al. (2001). Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. *J Neurosci* **21**, 9814-23.

Yanai, J., Doetchman, T., Laufer, N., Maslaton, J., Mor-Yosef, S., Safran, A., Shani, M. and Sofer, D. (1995). Embryonic cultures but not embryos transplanted to the mouse's brain grow rapidly without immunosuppression. *Int J Neurosci* **81**, 21-6.

Yang, D., Bierman, J., Tarumi, Y. S., Zhong, Y. P., Rangwala, R., Proctor, T. M., Miyagoe-Suzuki, Y., Takeda, S., Miner, J. H., Sherman, L. S. et al. (2005). Coordinate control of axon defasciculation and myelination by laminin-2 and -8. *J Cell Biol* **168**, 655-66.

Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R. and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol* **269**, 81-94.

Yao, D. L., West, N. R., Bondy, C. A., Brenner, M., Hudson, L. D., Zhou, J., Collins, G. H. and Webster, H. D. (1995). Cryogenic spinal cord injury induces astrocytic gene expression of insulin-like growth factor I and insulin-like growth factor binding protein 2 during myelin regeneration. *J Neurosci Res* **40**, 647-59.

Yoon, S. and Seger, R. (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* **24**, 21-44.

Yu, W. M., Feltri, M. L., Wrabetz, L., Strickland, S. and Chen, Z. L. (2005). Schwann cell-specific ablation of laminin gamma1 causes apoptosis and prevents proliferation. *J Neurosci* **25**, 4463-72.

Zarubin, T. and Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Res* **15**, 11-8.

Zhao, C., Fancy, S. P., Magy, L., Urwin, J. E. and Franklin, R. J. (2005). Stem cells, progenitors and myelin repair. *J Anat* **207**, 251-8.

Zhou, S., Fujimuro, M., Hsieh, J. J., Chen, L., Miyamoto, A., Weinmaster, G. and Hayward, S. D. (2000). SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Mol Cell Biol* **20**, 2400-10.

Zorick, T. S., Syroid, D. E., Arroyo, E., Scherer, S. S. and Lemke, G. (1996). The Transcription Factors SCIP and Krox-20 Mark Distinct Stages and Cell Fates in Schwann Cell Differentiation. *Mol Cell Neurosci* **8**, 129-45.

Zorick, T. S., Syroid, D. E., Brown, A., Gridley, T. and Lemke, G. (1999). Krox-20 controls SCIP expression, cell cycle exit and susceptibility to apoptosis in developing myelinating Schwann cells. *Development* **126**, 1397-406.

APPENDIX I

Primers and PCR conditions for RT-PCR

Notch 1 receptor

Notch 1F: GGC AAG CTT GAA TGG CCA G

Notch 1R: TTG GGA ACG GAA GCT GGG

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 59^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

Notch 2 receptor

Notch 2F: GAG GAA TAG CAA AAC CTG C

Notch 2R: TAG AGG AGC GGA GTG TTC C

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 55^{0}C, and 30 sec at 72^{0}C, before a final extension period of 5 min at 72^{0}C.

Notch 3 receptor

Notch 3F: CCA TCC TTG GAC TCA GGC

Notch 3R: AGC TGG TGT TAG TAG CTC C

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 55^{0}C, and 30 sec at 72^{0}C, before a final extension period of 5 min at 72^{0}C

Hes 1

Hes 1F: AGC CAA CTG AAA ACA CCT GAT T

Hes 1R: GGA CTT TAT GAT TAG CAG TGG

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 35 cycles of 30 sec at 94^{0}C, 30 sec at 58^{0}C, and 30 sec at 72^{0}C, before a final extension period of 5 min at 72^{0}C.

Numb

Numb F: CAA ACC AGT GAC ATT AGT GG

Numb R: GAT ACT TCT TCT AAC CAA CGG

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 56^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

Jagged 1

Jagged 1F: GTT CTC CAA ATA ACT GTT CCC

Jagged 1R: ATT TCA TTC TGA CAG TGA CCC

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 37 cycles of 30 sec at 94^{0}C, 30 sec at 58^{0}C, and 30 sec at 72^{0}C, before a final extension period of 10 min at 72^{0}C.

Jagged 2

Jagged 2F: TGC TGT CTG GCT TTG AAT GCC

Jagged *2R:* AGC ATT AAG GCA CGG TTT CCC

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 63^{0}C, and 20 sec at 72^{0}C, before a final extension period of 10 min at 72^{0}C.

Delta 1

Delta 1F: TTG TTC TTT CTC AGT GCC TCG

Delta 1R: CCC TTC TTG TTG ACG AAC TCC

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 61^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

Delta 2

Delta 2F: ATT CCC AGA CGA GTG TGA AGC

Delta 2R: CAG GTA AAA GGC ATC CAG TGC

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 61^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

Periaxin

Periaxin F: CCT GAA TTC ACC TTC TTC CCG GTC

Periaxin R: ACG TCA CCA GTG AGT AGC CAC GC

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 33 cycles of 30 sec at 94^{0}C, 30 sec at 56^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

GAPDH

GAPDH F: ACC ACA GTC CAT GCC ATC AC

GAPDH R: TCC ACC ACC CTG TTG CTG TA

PCR reaction conditions: 1 initial cycle of 2 min at 95^{0}C followed by 26 cycles of 30 sec at 94^{0}C, 30 sec at 63^{0}C, and 30 sec at 68^{0}C, before a final extension period of 5 min at 68^{0}C.

Primers and PCR conditions for genotyping

Dhh cre

Dhh creF: ACC CTG TTA CGT ATA GCC GA

Dhh creR: CTC CGG TAT TGA AAC TCC AG

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 35 cycles of 30 sec at 94^{0}C, 30 sec at 56^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

RBP-J floxed

RBP-F: TTG CCA AGC CAA AGC CC

RBP-R: GAA GGC GAT TGA ACA GTG CG

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 35 cycles of 30 sec at 94^{0}C, 45 sec at 60^{0}C, and 1 min at 72^{0}C, before a final extension period of 10 min at 72^{0}C.

CALSD-NICD

NICD F: GGA CGA CAA CCA GAA TGA G

NICD R: TGA GCG GGG TGA AGC CAT C

PCR reaction conditions: 1 initial cycle of 4 min at 94^{0}C followed by 35 cycles of 1 min at 94^{0}C, 1 min at 63^{0}C, and 1 min at 72^{0}C, before a final extension period of 7 min at 72^{0}C.

Krox-20 knockouts

K20 430C: ACA TGA CTG GAG AGA AGA GAC CCC T

K20 1233 NC: GCG TTT TGC TGG GCC TGT TAG GGT A

LacZ 1591NC: TTG GTG TAG ATG GGC GCA TCG TAA C

PCR reaction conditions: 1 initial cycle of 3 min at 94^{0}C followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 59^{0}C, and 30 sec at 72^{0}C, before a final extension period of 3 min at 72^{0}C.

Hes 1 knockouts

Hes 1D730: TGG GAT GTG GGA CAT GAG GG

Hes 1R1581: TCA CCT CGT TCA TGC ACT CG

neo4: GCA GCG CAT CGC CTT CTA TC

Wildtype allele: Hes 1 D730 + Hes 1R1581

Mutant allele: Hes 1R1581 + neo4

PCR reaction conditions: 1 initial cycle of 10 min at 95^{0}C followed by 35 cycles of 1 min at 94^{0}C, 1 min at 60^{0}C, and 1 min at 72^{0}C, before a final extension period of 10 min at 72^{0}C.

Hes 5 knockouts

Hes 5L: GCT GGG GGC CGC TGG AAG TGG

Hes 5U: CCG CTC CGC TCC GCT CGC TAA

neo4: GCA GCG CAT CGC CTT CTA TC

Wildtype allele: Hes 5L + Hes 5U

Mutant allele: Hes 5L + neo4

PCR reaction conditions: 1 initial cycle of 10 min at 95^0C followed by 35 cycles of 1 min at 94^0C, 1 min at 62^0C, and 1 min at 72^0C, before a final extension period of 10 min at 72^0C.

APPENDIX II

Buffer B1:

0.1M Tris-HCl

0.15M NaCl

in UP H_20.

Buffer B2:

Buffer B1 + 1% heat inactivated goat serum

Buffer B3:

0.1M tris (pH 9.5)

0.1M NaCl and 50 mM $MgCl_2$

in UP H_20.

Buffer B4:

Buffer B3 containing

3.5 ml/ml NBT (Roche Diagnostics, Germany)

3.5 ml/ml BCIP (Roche Diagnostics, Germany)

0.24 mg/ml levamisole (Sigma, UK)

Hybridisation Buffer:

1X SALTS

50% formamide (Sigma, UK)

0.1 mg/ml yeast total RNA (Invitrogen, UK)

10% w/v dextran sulphate (Fluka Chemicals Ltd, Switzerland)

1X Denhart's (Sigma, UK)

in autoclaved UP H_20.

10X Salts

2M NaCl

50mM EDTA

100mM Tris-Cl (pH 7.5)

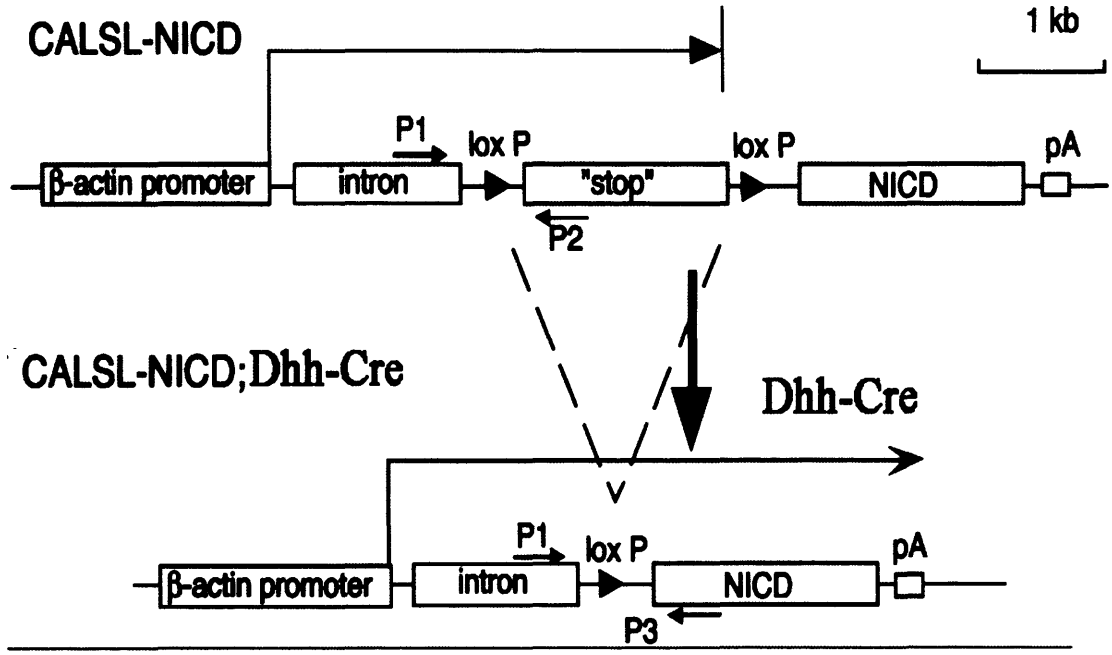
50 mM $NaH_2PO_4.2H_2O$

50 mM Na_2HPO_4

in autoclaved UP H_2O

APPENDIX III:

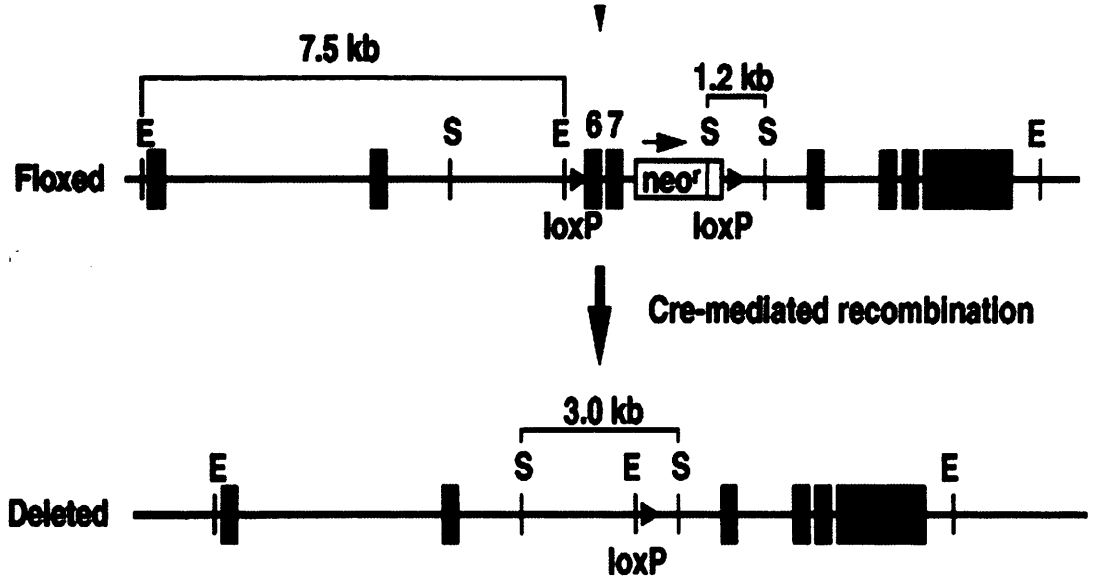
Schematic representation of the CALSL-NICD transgene



The *CALSL-NICD* transgene contains the chicken *β-actin* promoter, a hybrid 5' intron, a transcriptional and translational stop cassette flanked by two loxP sites, cDNA encoding human NICD (amino acid residues 1762–2304), and the SV40 polyadenylation signal. In *CALSL-NICD* transgenic mice, transcription and translation of the *NICD* cDNA are blocked by multiple polyadenylation sites and the stop codon present in the stop cassette. In the presence of Cre recombinase, the stop cassette is excised, thus allowing the transcription and translation of NICD (see arrow) (adapted from Yang *et al.*, 2004).

APPENDIX IV:

Schematic representation of RBP-J 'floxed' and 'deleted'allele.



A loxP site was inserted upstream of the sixth exon and the other downstream of the seventh exon together with a neomycin phosphotransferase gene-expressing cassette (PGK-neo). The DNA binding domain of RBP-J is encoded by the sixth exon (Kawaichi *et al.*, 1992) and this domain is essential for gene function (Chung *et al.*, 1994; Oka *et al.*, 1995). Cre-mediated recombination results in formation of deleted allele, with loss of RBP-J function (Han *et al,* 2002; Moriyama *et al.*, 2006).