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A Molecular Study of the Signals that control Schwann Cell Development and Myelination \textit{in vivo}

By

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ABSTRACT

To identify novel genes that may be involved in Schwann cell lineage development and in myelination, we combined microarray based gene expression profiling with a refined bioinformatic analysis. We compared mRNA from rat sciatic nerves at embryonic day 14 (E14), E18, postnatal day 7 (P7), P12 and in the distal stump of P12 sciatic nerves cut five days previously and in which, therefore, all the myelin related genes should be strongly down-regulated. 1609 genes and expressed sequence tags (ESTs) were found to have at least a two-fold change in their level of expression either during development or after nerve injury. On this set of genes/ESTs a statistical analysis was performed to identify those that were significantly different between two time points. This was then followed by a sequence mapping and a protein structural and functional annotation carried out with Biopendium™ software, which enabled us to substantially minimise the number of unknown sequences. We identified agrin and two members of the collapsin response mediated protein family (CRMP) as possibly involved in the transition between Schwann cell precursors (E14) and immature Schwann cells (E18). Our data also suggest that the cartilage related proteins type II collagen and chondromodulin-1, and the cytoskeletal protein synuclein-1 may have important functions during peripheral nerve myelination. In addition, to clarify the role of transforming growth factor beta (TGFβ) during Schwann cell development in vivo, mice carrying a floxed segment of the TGFβ type II receptor have been crossed with mice in which the CRE recombinase is expressed under the control of the P0 myelin protein promoter. Although myelination appears to occur normally, we found that Schwann cells death and proliferation rate are impaired in the perinatal period, both during normal development and after nerve injury, indicating a key role for TGFβ in controlling these events.
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CHAPTER 1

GENERAL INTRODUCTION

Myelin is a multilamellar membrane that surrounds and supports axons in both the central nervous system (CNS) and the peripheral nervous system (PNS). Its main function is to isolate axons and facilitate rapid saltatory conduction. In myelinated vertebrates, two types of myelinating cells exist: oligodendrocytes in the CNS and Schwann cells in the PNS. Both synthesize similar sets of protein, but each has unique proteins that may account, at least in part, for the differences in myelin structure and formation that characterise each nervous system. In the mature CNS a single oligodendrocyte can ensheath many axons via multiple processes, while in the PNS a single Schwann cell myelinates a single axonal segment.

Myelinating and non-myelinating Schwann cells are undoubtedly the major glial cells in the peripheral nerve. They were first described in 1839 by Theodor Schwann, the pioneer of the cell theory, whose name they would later bear (Shepherd, 1991). Ramon y Cajal was the first to appreciate and to describe in incredible detail the intricate relationship between the various components of the nervous system (Ramon y Cajal, 1928; Ramon y Cajal, 1999-2002, edited translation). However, due to the limitations of light microscopy, he failed to realize that myelin was an extension of glial cells, and not foreign to them. The advent of the electron microscope allowed the ultrastructure and morphology of both Schwann cell forms to be revealed (Geren and Raskind, 1953; Geren 1954). It was thus elucidated that mature myelinating Schwann cells form 1:1 relationships with larger diameter nerve axons, while non-myelinating Schwann cells support a number of smaller diameter axons in membrane invaginations.
Development of the Schwann cell lineage

Most of the Schwann cells within the developing nerve derive, through a series of characteristic steps, from the neural crest, a population of migratory cells (Le Douarin et al., 1991; Anderson, 1997). The formation of mature myelinating and non-myelinating Schwann cells from the neural crest involves the generation of two main intermediates, the Schwann cell precursor and the immature Schwann cell (Dong et al., 1995, 1999; Jessen and Mirsky, 1999), and therefore, the overall process involves three main transitions: from crest cells to precursors, from precursors to immature Schwann cells and finally the formation of the two mature Schwann cell types (Mirsky and Jessen, 1996; Jessen and Mirsky, 1999, 2002).

Neural crest

The neural crest is a transitory, migrating group of cells that segregate from the tips of the neural folds just before or just after they fuse to give rise to the neural tube. Neural crest cells will develop into a complex array of cell types, including the sensory and autonomic components of the peripheral nervous system as well as a range of non-neuronal tissues (Le Douarin and Smith, 1988: Le Douarin and Kalcheim, 1999). A major part of the newly formed neural crest cells will form the myelinating and non-myelinating Schwann cells, as well as the other glial cell types in the PNS, such as the satellite cells, the teloglia and the enteric glial cells (Le Douarin and Smith, 1988, Le Douarin et al., 1991, Anderson, 1997). Yet, some studies conducted in the chick seem to suggest that the ventral neural tube itself contributes some Schwann cells that localize in the proximal part of the peripheral nerve in vivo (Rickmann et al., 1985; Lunn et al., 1987) or migrate away from ventral neural tube explants in vitro (Loring and Erickson, 1987). In contrast more recent investigations suggest that glial cells
populating ventral roots are of neural crest origin (Carpenter and Hollyday, 1992a, Bhattacharyya et al., 1994; Golding and Cohen, 1997). Finally, a recent paper indicates that boundary cap (BC) cells, a population of cells that forms clusters at the entry and exit points of peripheral nerve roots, could represent a source of components of the PNS (Maro et al., 2004). Taken all together these data suggest that the vast majority of Schwann cells are derived from neural crest but that a small minority may be ventrally derived.

The induction of the neural crest

Prior to their migration, neural crest cells are an integral part of the neuroepithelium. Thanks to a morphogenetic conversion that causes these epithelial progenitors to become mesenchymal, the neural crest cells become motile and engage in migration (LeDouarin and Teillet, 1974; Bronner-Fraser, 1995; LaBonne and Bronner-Fraser, 1999). The formation of neural crest precursors at the neural plate border involves many signalling events. Wnt proteins, Bone Morphogenetic Proteins (BMPs) and Fibroblast Growth Factors (FGFs) have all been shown in various assays to mimic the tissue interactions that induce neural crest (reviewed in Gammil and Bronner-Fraser, 2003). Another factor, called Noelin-1, appears to be of crucial importance in the timing of neural crest formation (Knecht and Bronner-Fraser, 2002). A Wnt protein, Wnt6, seems to be the inducing signal from non-neural ectodermal tissue (Garcia-Castro et al, 2002), while the BMP signal is required only after the initial induction step, indicating that BMPs might serve for maintenance of the induction process (Selleck et al, 1998) or for initiation of migration (Sela-Donenfeld and Kalcheim, 1999). In amphibians FGF signalling can induce neural crest in neuralized ectoderm (LaBonne and Bronner-Fraser, 1998; Mayor et al, 1995).
Neural crest extrinsic markers

Extrinsic markers have also been applied to neural crest precursor cells. Initial studies used donor tissue labelled with tritiated thymidine (Weston, 1963). However this technique has a main limitation in the fact that the label rapidly becomes diluted as a result of cell proliferation. The modern versions of this approach are more efficient and involve the use of vital dye such as DiI or DiO for staining the premigratory crest cells (Stern, 1990; Serbedzija et al., 1989,1990; Hatada and Stern, 1994). The dye, incorporated in the cell membrane, is transferred to the daughter cells through a certain number of generation and provides a means for marking for 2-4 days.

LeDouarin developed an elegant method based on the observation that quail cells have a nuclear marker that distinguishes them from chick cells (LeDouarin, 1973, 1982). When quail cells are transplanted into a chick embryo the cells from each species retain their nuclear characteristics and can be identified in the chimera. The technique is so powerful that a single quail cell can be identified in a chick tissue.

Neural crest intrinsic markers

Different methods have been used to shed light on the pathways of neural crest cell migration: extirpation or in situ destruction of the neural crest, often used in lower and higher vertebrates, have yielded significant information, but the results obtained are relatively crude (reviewed by Hörstadius, 1950; Weston, 1970). More precise information on the migration process and on the fate of neural crest cells have been obtained by means of cell marking experiments. In pioneering experiments cytoplasmic inclusions or differences in cell size have been used (Raven, 1937; Triplett, 1958). More recently other experiments have followed neural crest cells by labelling them with
monoclonal antibodies HNK-1 and NC1. These antibodies recognise a glucuronic acid-containing carbohydrate carried by several surface glycoprotein and glycolipids (Chou et al, 1986). However, the epitope is only transiently expressed on some neural crest cells (Dupin et al, 1990), is present only in a subset of rat neural crest cells and no reactivity could ever be detected with mouse neural crest cells (Erickson et al, 1989, Pomeranz et al, 1993). Because of this most of the work using HNK-1 has been done in avian embryos. The low-affinity neurotrophin receptor p75 has in some cases been used to study migration of murine and rat neural crest cells (Stemple and Anderson, 1992; Chalazonitis et al, 1997).

The transcription factor Slug, a member of the zinc finger family isolated in chick embryos (Nieto et al, 1994) is expressed at high level in neural crest precursor cells prior to the onset of motility. Its close relative Snail is expressed in mouse and fish (Locascio et al, 2002). Both seem to be target of neural crest induction and, interestingly, Slug promoter contains a binding site shown to mediate transcription in response to Wnt6 (Wu et al., 2003). Inhibition of Slug/Snails inhibits the migration of the neural crest cells (LaBonne and Bronner-Fraser, 2000). Their targets are not known yet, but recently they have been shown to regulate the epithelial-mesenchymal transition (EMT) in vitro via repression of E-cadherin and Claudins/Occludins, thus triggering the breaking of adherens and tight-junctions (Cano et al, 2000; Ikenouchi et al, 2003).

The transcription factor Sox-9 is a marker for murine premigratory and migratory neural crest in fish, mouse and chick (Mori-Akiyama et al, 2003; Cheung and Briscoe, 2003), while Sox-10 in these species marks only migratory neural crest cells (Cheng et al, 2000; Britsch et al, 2001). Other markers such as Pax-3 and Notch are expressed in the neural plate but elevated in the neural crest (Monsouri et al, 2001; Coffman et al, 1993; Williams et al, 1995). The transcription factor Pax-3 is mutated in the Splotch mouse, in
which the development of the neural tube is severely affected (Epstein et al., 1991). Notch is a membrane-anchored protein that is activated through binding of ligands expressed by neighboring cells. Upon binding to one of the ligands such as Delta, the intracellular domain of Notch is cleaved and translocated to the nucleus (Kopan, 2002). Although Delta/Notch signalling is required for neural crest formation (Endo et al., 2002), overexpression of Notch results in a loss of neural crest derivatives (Coffman et al., 1993) stressing the importance of having finely tuned levels of Notch/Delta in the neural crest. The transcription factor AP-2, initially expressed at the neural plate border is later enhanced in neural crest migrating from the cranial neural folds (Mitchell et al., 1991; Luo et al., 2002). Null mutation of this gene revealed its importance for the development of many cranial facial structures, including the cranial ganglia (Schorle et al., 1996). During peripheral glia development, AP-2 appears to play a role in the timing of Schwann cell formation, since its down-regulation coincides with the precursor-immature Schwann cell transition (Stewart et al., 2001). Finally RhoB, a target of BMP signalling, that is expressed in the dorsal neural tube and in migrating neural crest is necessary for the delamination of neural crest cells, although it is not required for neural crest specification, (Liu and Jessel, 1998; Henderson et al., 2000).

Pathways of migration in the trunk

In the trunk of vertebrate neural tube two main directions of migration have been recognized: a first one, ventral, between neural tube and somites that gives rise to cells in the dorsal root ganglia (DRG), sympathetic ganglia, adrenomedullary cells, aortic plexuses and Schwann cells (Weston, 1963; Le Douarin and Teillet, 1974; Erickson et al., 1992) and a second, lateral, over the entire dorso-lateral surface of the somite, that
will 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). Moreover, the work of Le Douarin and colleagues with the chick-quail chimeras has revealed the existence of three additional migration pathways: an intersomitic group of cells, a longitudinal population migrating along the neural tube and another longitudinal group that migrates along the dorsal aorta (Le Douarin et al, 1982; Teillet et al, 1987).

The pattern of migration of neural crest cells is largely mediated by inhibitory signals that actively exclude cells from the caudal halves of the somites. In fact, in elegant experiments in which the somites were rotated 180°, so that the anterior-posterior axis is reversed, neural crest cells will migrate only through what was originally the anterior portion of the somite (Bronner-Fraser and Stern, 1991). Several molecules in the posterior half of the somites are inferred as inhibitory, among them extracellular matrix molecules (ECM) such as collagen IX, F-spondin, versican, chondroitin 6-sulfate-rich proteoglycans and ephrin B2 (Perris et al, 1991; Ring et al, 1996; Landolt et al, 1995; Debby-Brafman et al, 1999; Smith et al., 1997; Wilkinson, 2001). Some permissive ECM-associated signals have also been identified; fibronectin for example has been shown to play an important role in the migration of the neural crest in vivo and enhances its motility in vitro (Thiery et al, 1982; reviewed in Duband et al, 1986). Diverse type of collagen, including collagen I, IV and VI are also able to mediate neural crest cell motility in vitro (Perris et al, 1993).

The multipotentiality of the neural crest

The question of how crest cells are able to generate such a diversity of cell types is of great interest for developmental biologists (reviewed in LaBonne and Bronner-Fraser
2000; Kalcheim, 2000; LeDouarin and Dupin, 2003). Many experiments conducted in vivo and in vitro suggest that neural crest is composed of an heterogeneous population of cells endowed with different proliferation and differentiation potentials. For example, the fate of individual cells can be changed by transplanting them to different locations along the neural tube or exposing them to different factors in vitro (Dupin et al., 1990; LeDouarin et al., 1993). Rare totipotent progenitors, able to generate all the different derivatives (neurons, glial cells, mesenchymal cells, melanocytes) were identified (Baroffio et al., 1988, 1991). Yet, if post-migratory crest cells are transplanted back into the neural crest of younger hosts they can no longer give rise to the full array of derivatives (LeDouarin, 1996). Evaluations in vitro indicated that more of 70% of the progenitors were fate restricted by 30-36 hours after leaving the neural tube (Henion and Weston, 1997). This suggests that as migration proceeds, neural crest cells gradually undergo a restriction of their potentiality. Neural crest cells have the capability of self-renewal in culture (Stemple and Anderson, 1992) and recently the presence of a population of neural crest stem cells in the peripheral nerve has been suggested on the basis of expression of p75 low affinity NGF receptor and myelin proteins (Morrison et al., 1999). These cells, accounting around 10% of the total cells in E14 peripheral nerves, appear to be both multipotent and capable of self-renewal (Morrison et al., 1999, 2000).

A large amount of work in vivo, through the analysis on mouse mutants, as well as in vitro, has led to the identification of several growth factors/receptors implicated in the development of specific neural crest derivatives (reviewed in Anderson, 1997; Sieber-Blum, 2000). Melanocyte lineage differentiation is stimulated by endothelin-3 (ET3) and stem cell factor (SCF) (Opdecamp et al., 1998: Yoshida et al., 2001). The
ET3/endothelin receptor B (ETRB) pathway plays a crucial role also in enteric gangliogenesis, a process in which glial derived neurotrophic factor (GNDF) is also involved (Kruger et al, 2003; Airaksinen and Saarma, 2002). In humans Hirschsprung disease is linked with a defect in these signal pathways, and the colonization of the gut by neural crest cells is abnormal (McCallion and Chakravarti, 2001; Iwashita et al, 2003). In the PNS, gliogenesis is regulated by the neuregulin family of growth factors (discussed in more details below), that signals through the ErbB-receptors family (Shah et al, 1994; reviewed in Jessen and Mirsky, 2002, 2004). Since in the PNS neurons and glia differentiate in the same environment the question of how they choose to adopt either neuronal or glial fate has been puzzling. In vivo BMPs (in particular BMP2), regulatory genes and cell-cell interaction interplay to generate neurons first and glial cells later (Morrison, 2001) and in vitro studies showed that BMP neurogenic activity is dominant over the gliogenic effect of neuregulin (Shah and Anderson, 1997). Recent work has suggested that the activation of the Notch signalling pathway initiates the irreversible switch from neurogenesis to gliogenesis (Wakamatsu et al, 2000; Morrison et al, 2000; Kubu et al., 2002). The soluble form of the Delta-1 ligand promotes Schwann cell differentiation in cultured neural crest stem cells even in the presence of BMP2 (Morrison et al, 2000). This effect seems to be regulated by the relative expression of Notch and its antagonist Numb: cells that express Numb will go through a neuronal fate while those that don't express it will become glial cells. Accordingly, over-expression of Numb in crest cells culture promotes neuronal differentiation (Wakamatsu et al, 2000; Kubu et al, 2002). Neural crest stem cells (NCSC) from the peripheral nervous system show also some intrinsic differences. In fact E14 NCSC isolated from gut are more responsive to neurogenic factors than NCSC isolated from sciatic nerves, which are more responsive to gliogenic factors (Bixby et al, 2002).
However, one possible explanation for this difference may reside in the method used to isolate these cells. They have in fact been FACS sorted as P₀⁻ and p75⁺ with an anti-P₀ antibody that is probably not suitable for this purpose, leading many to believe that this population of cells represent Schwann cell precursors (Jessen and Mirsky, 2004). This is also supported by the observation that the population that sorts as P₀⁺ and p75⁻ does not form Schwann cells in the presence of neuregulin-1 (Morrison et al., 1999), raising many concerns about the technique used in these experiments.

Neural crest derivatives maintain partial plasticity and a recent paper demonstrated that ET3 is capable of reprogramming the melanocyte phenotype in vitro inducing de-differentiation and subsequent activation of glial markers (Dupin et al., 2000). Recently the converse transition from Schwann cell to glial-melanocyte precursors has been obtained after exposure to ET3 (Dupin et al, 2003), underlying the relative instability of neural crest derived pigment and glial cells in vitro.

Axon/Schwann cell relationships during peripheral nerve development

Several groups have carried on experiments aimed at a better understanding of the close interaction between axons and Schwann cells in the early stages of nerve development, and many different animal models have been used, such as frog (Harrison, 1924) chick (Weston, 1963; Carpenter and Holliday, 1992a, b) and rat (Jessen et al, 1994). Development seems to follow a rostro-caudal pattern and also a proximo-distal gradient along the nerve outgrowth from the spinal cord. Some authors have suggested that Schwann cell may act as pioneer cells, migrating ahead of the axon and guiding peripheral nerve outgrowth (Noakes and Bennet, 1987). This theory appears to be ruled out by the findings in transgenic mice lacking neuregulin or its receptor ErbB3. In fact in these mice, although Schwann cells are basically absent in the nerve, axons grow and
reach their target normally (Mayer and Birchmeier, 1995; Riethmacher, 1997). Similarly, in the *splotch* mouse, the absence of Schwann cells does not impede motor neurons from projecting correctly, although it results in motor neurons migration from the spinal cord in to the periphery (Grim *et al*, 1992; Vermeren *et al*., 2003). Rather, it seems that the presence of the neurite outgrowth from the ventral motor axon is necessary for guiding Schwann cells into their final position in the peripheral nerve (Bhattacharyya *et al*, 1994).

**The Schwann cell precursor**

During rat development, axons project into the hind limb between embryonic day 13 (E13) and E14 (E11-E12 in the mouse). Ultrastructural analysis reveals bundles of small diameter axons tightly packed with apparently no extracellular matrix within them, and surrounded along their perimeter by glial cells separating the axons from the mesenchymal tissue (Jessen *et al*, 1994). By this stage the glial cells associated with the outgrowing axon display specific characteristics that differentiate them from both migrating crest cells and immature Schwann cells, and are therefore named Schwann cell precursors (Jessen *et al*, 1994). Unlike migrating crest cells, Schwann precursors express the growth-associated protein 43 (GAP-43), the brain fatty acid binding protein (B-FABP), and the myelin proteins P0, PLP and PMP-22 (Jessen *et al*, 1994; Kurtz *et al*, 1994; Lee *et al*, 1997; Hagedorn *et al*, 1999; Britsch *et al*, 2001). If dissociated from the axon and plated, precursor cells undergo rapid apoptotic death (Jessen *et al*, 1994; Dong *et al*, 1995), unlike mature Schwann cell that survive *in vitro* owing to autocrine loops (Meier *et al*, 1999). Schwann cell precursor death *in vitro* can be rescued by incubation with 100pM of β-neuregulin-1 (Jessen *et al*, 1994; Dong *et al*, 1995), a growth factor that is likely to be the axonal signal responsible for precursor cell survival.
(reviewed in Mirsky and Jessen, 1999). When plated, Schwann cell precursors exhibit a morphology markedly different from that of neonatal and adult Schwann cells; instead of a bi- or tri-polar shape, they are more flattened and tend to cluster together in a pavement-like array, while Schwann cells from later stages grow more evenly distributed. Moreover, in vitro studies showed that precursor cells are much more motile than mature Schwann cell, with a migration rate similar to that of crest cells (Jessen et al, 1994).

The brief period spanning between E16 and E17 (E14/E15 in the mouse) sees the transition of Schwann cell precursors into immature Schwann cells (also called pre-myelinating Schwann cells). These cells are clearly distinguishable from precursors thanks to their cytoplasmic immunoreactivity for the calcium binding protein S100 (Jessen et al, 1994). Shortly after S100, immature Schwann cell start expressing the surface lipid O4 (Dong et al, 1999). When in vitro, they tend to assume a bi- tri-polar shape, have low motility and proliferate in the presence of fibroblast growth factor (FGF) (Jessen et al, 1994; Dong et al, 1999). Probably the most striking characteristic of immature Schwann cells is that when removed from the axon they do not die by apoptosis, having established an autocrine survival loop (Dong et al, 1999; Meier et al, 1999). This means that during development there is a change in survival regulation: the survival of Schwann cell precursors depends on axonal signals, whereas Schwann cell survival is axon independent. The nature of this loop has been studied in detail, and different experiments aimed at mimicking the positive effect of Schwann cell conditioned medium on Schwann cell cultures, suggested that the main component of the autocrine survival process is a cocktail of three growth factors, NT-3, IGF-2 and PDGFBB (Meier et al, 1999). Other potential autocrine Schwann cell survival factors
include leukaemia inhibitory factor (LIF) and lysophosphatidic acid (LPA) (Dowsing et al., 1999; Weiner and Chun, 1999).

**Neuron-Schwann cell signals**

As already mentioned, Schwann cells from E18 onwards have the capability to survive when dissociated from the axon, while Schwann cell precursors undergo rapid death by apoptosis in this condition, but can be rescued by the growth factor β-neuregulin-1 (Dong et al., 1995). At present, the Neuregulin family accounts for four different genes, NRG1, NRG2, NRG3 and NRG4, whose signals, implicated in many different systems, are transduced via the ErbB-receptor family (reviewed in Adlkofer and Lai, 2000; Garrat et al., 2000; Falls, 2003). Neuregulin-1 is the best known, and encodes for many different isoforms, isolated in various systems, and named Neu differentiation factor (NDF; Wen et al., 1992), heregulin (Holmes et al., 1992), glial growth factor (GGF; Marchionni et al., 1993), acetylcholine receptor inducing activity (ARIA; Falls et al., 1993) or sensory and motor neuron derived factor (SMDF; Ho et al., 1995). The term "neuregulin" stems from the contraction of Neu differentiation factor and heregulin, the first two published by groups seeking for the ligands of the ErbB2 receptor.

**Structure of NRG1**

The Neuregulin-1 gene has been recently sequenced and assembled (Stefansson et al., 2002). The gene is on the short arm of chromosome 8, it is about 1.3 megabases long, but only less than 0.3% of it encodes for protein. Thus the rich alternative splicing and use of multiple promoters, result in at least 15 different isoforms (reviewed in Lemke, 1996). With respect to the in vivo functions and cell biological properties, three
characteristics of the molecules play crucial roles: the epidermal growth factor-like (EGF-like) domain, the N-terminal sequence and whether the isoform is initially synthesized as a transmembrane (TM) or not TM protein. The EGF-like domain on its own seems sufficient for ErbB receptor activation (Holmes et al., 1992; Wu et al., 1994). Alternative splicing gives rise to variants in the C-terminal part of this domain, originating "α" or "β" forms, with different affinities for the ErbB receptors (Pinkas-Kramarski et al., 1998; Jones et al., 1999; reviewed in Adlkofer and Lai, 2000). The N-terminal region can contain either an immunoglobulin-like (Ig-like) domain, or a cysteine rich domain (CRD), the latter one mainly found in the neuregulins predominant in the nervous system (Ho et al., 1995).

Receptors for NRGs

Neuregulin-1 signalling involves binding to members of the EGF-receptor subfamily of phospho-tyrosine kinases (PTKs) known as the ErbBs. The name derives from the avian virus oncogene v-Erb-B, found in avian erythroblastosis, where it encodes for a mutated form of the EGF receptor (Adlkofer and Lai, 2000). The activation of these receptors in various cell types results in cellular responses that include differentiation, proliferation, apoptosis, migration and adhesion (reviewed in Yarden and Sliwkowski, 2001; Peles et al., 1992; Falls et al., 1993). Although NRG1 was first identified by groups seeking for ligands for the ErbB2 receptor, it is now known that NRG1 binds initially to the high affinity receptors ErbB3 and ErbB4, and that the activation of ErbB2 takes place indirectly after its heterodimerization with either Erb3 or ErbB4 (Riese et al., 1995, Sliwkowski et al., 1994; Carraway and Cantley, 1994). In the past years various experiments have indicated that while NRG1 signals mainly through ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers, NRG2 preferentially activates the ErbB1/ErbB3
heterodimer and the ErbB4 homodimer (Pinkas-Kramarski et al., 1998; Jones et al., 1999; Carraway et al., 1997; Crovello et al., 1998). These findings support the general idea that the different bioactivities of NRG1 and NRG2 are a direct consequence of the activation of different ErbB receptor combinations.

**Role of NRGs in Schwann cell development**

The first implication of the importance of NRG in Schwann cell biology comes from in vitro experiments that identified GGF as a Schwann cell mitogen (Raff et al., 1978; Marchionni et al., 1993). Moreover, as mentioned previously, work on crest cell clones showed that the addition of NRG1 to the cocktail in which the cells were grown, strongly pushed the crest cells towards a glial choice, selectively suppressing the generation of neurons (Shah et al., 1994; Shah and Anderson, 1997). Further in vitro studies on Schwann cell precursors have shown that β-neuregulin-1 is capable of blocking apoptosis and can stimulate precursor proliferation (Dong et al., 1995). Even more striking is that this work demonstrated that, in the presence of NRG-1, Schwann cell precursors generate Schwann cells with a time course similar to the one seen in vivo in peripheral nerve (Dong et al., 1995). Additionally, the effect of NRG on Schwann cell is abolished via the exposure to a soluble form of the EGF-like domain of ErbB4 (Dong et al., 1995). Taken together, these data strongly suggested that the interaction NRG/ErbBs was the axon-derived signal responsible for Schwann cell survival, proliferation and differentiation.

A powerful boost to these studies arrived from the analysis of null mutant mice for NRG1, ErbB2, ErbB3 and ErbB4 (Lee et al., 1995; Meyer and Birchmeier 1995; Riethmacher et al., 1997; Gassman et al., 1994). NRG -/- and ErbB2 -/- mice die at midgestation, due to the arrest of trabeculation of the heart ventricle. However, already
at E10.5 it is possible to notice in these mice a significant reduction in the number of Schwann cell precursors (Meyer and Birchmeier, 1995). This phenotype is shared by the ErbB3 -/- mice, which have proven to be particularly informative since around 20% of these mice survive until birth. Their analysis reveals a complete loss of Schwann cell precursors and around 80% reduction in motor and sensory neurons (Riethmacher et al., 1997). The loss of neurons could be due to a lack of all the Schwann cell derived factor such as CNTF, PDGF, LIF, and proves the importance of Schwann cells as trophic partners for neurons. The essential role of ErbB2 for peripheral nerve development has been underlined in elegant experiments where the cardiac defect has been rescued (Morris et al., 1999; Woldeyesus et al., 1999). These groups have created transgenic mouse lines in which the ErbB2 cDNA is expressed under the control of either the heart specific promoter Nkx2.5 or the heart muscle actin promoter. These mice lack mature Schwann cells as well as other crest derivatives, and although axons project relatively normally, motor and sensory axons are severely defasciculated and disorganized. Similar results are seen in mice in which only the CRD-NRG-1 isoform is missing, underlying that this is the predominant isoform in peripheral nerve (Wolpowitz et al, 2000).

**Neuregulin and myelination**

The role of NRG in later events of Schwann cell differentiation, including myelination, is less clear. Neurons, including motor and large sensory neurons, express neuregulin-1 in the adult nervous system (Chen et al., 1994; Bermingham-McDonogh et al., 1997). These findings are consistent with a possible role of neuregulin-1, in the adult, in the maintenance of the myelin sheath. According with this hypothesis, the conditional ablation of ErbB2 in developing nerve, via a Krox-20/Cre allele, results in a deficit in
Schwann cell number and in myelin thickness (Garratt et al., 2000b). In contrast, addition of the soluble isoform of neuregulin-1, GGF, in co-cultures of dorsal root ganglia neurons and Schwann cell, results in block of myelination in a dose dependent manner. If GGF is added to co-cultures where myelin had already formed, the result is a striking demyelination accompanied by Schwann cell dedifferentiation and proliferation (Zanazzi et al., 2001). These results are consistent with the findings that neuregulin and expression of ErbBs is substantially increased in Schwann cell during Wallerian degeneration (see below) and demyelination, suggesting a possible autocrine signalling mechanism (Carroll et al., 1997; Hall et al., 1997). Taken together, these data suggest a role for neuregulins and ErbB-receptors not only during Schwann cell generation and maturation but also in later events of peripheral nerve development and in the adult. Nevertheless, recent experiments with transgenic mice challenge the idea that Nrg is a demyelination signal.

Neuregulin-1 type III controls myelin thickness

Recent experiments with mutant and transgenic mice have shown that neuregulin (in particular neuregulin-1 type III) is the key axonal signal for the regulation myelin thickness (Michailov et al., 2004). In this paper the authors show that mice heterozygotes (+/-) for Nrg-1 produced a thinner myelin sheath than normal littermates and had reduced conduction velocity. In contrast when Nrg-1 type III is specifically overexpressed postnatally by motorneurons and DRG neurons in transgenic mice, thanks to a Thy 1.2 promoter, there is a dramatic increase in myelin thickness and in the number of wraps. Moreover many small caliber axons are myelinated (Michailov et al., 2004). However, the precise mechanisms that link neuregulin expression and axon diameter are still unclear.
The role of Endothelins in the timing of Schwann cell generation

The generation of Schwann cells from their precursors can be broadly reproduced *in vitro* under simple conditions. Thus, E14 precursors, plated in defined medium containing neuregulin-1, generate, after four days (E14+4=E18), cells that show the phenotype of immature Schwann cells rather than that of precursors (Dong *et al.*, 1995). However, a percentage of the cells, calculated to be around 15%, do not convert to Schwann cells. Therefore, it is probable that other factors contribute to the Schwann cell precursor to Schwann cell transition *in vivo*. Endothelins (ET), acting through the endothelin B receptor, are likely to be one of these factors (Brennan *et al.*, 2000). ETs 1, 2 and 3 are encoded by three different genes, and share strong sequence and structural homology (Yanagisawa *et al.*, 1988). Similarly to neuregulin, all of them can support the survival of Schwann cell precursors *in vitro* but, unlike neuregulin, they do not induce proliferation in precursors. Moreover, if cultured in the presence of ETs for four days, Schwann cell precursors tend to remain precursors instead of converting to Schwann cells. If E14 precursors are cultured in the presence of both neuregulin and ET, the Schwann cell generation rate is an intermediate of that seen with neuregulin alone or ET alone (Brennan *et al.*, 2000). These data indicate that ET act as a brake on Schwann cell generation, a finding confirmed by the accelerated precursor to Schwann cell transition in mice deficient for the endothelin B receptor (Ceccherini *et al.*, 1995; Brennan *et al.*, 2000). Interestingly, in the presence of ET, Schwann cell precursors convert to Schwann cells after 7 days, even in the absence of neuregulin, indicating that the action of ET is not to block, but rather to delay the generation of Schwann cells (Brennan *et al.*, 2000). On the other hand, this also means that neuregulin is not absolutely required for the generation of Schwann cells, although it promotes this process. Finally, it has been shown that *in vitro*, the addition of FGF2 to neuregulin,
accelerates Schwann cell maturation (Dong et al., 1999). Taken together these observations indicate that a fine regulation of these factors is necessary for a perfectly timed glial development in peripheral nerve.

In this respect of highly coordinated gene control, it is surprising that, to date, only one transcription factor, AP2α, has been implicated in this process. AP2α in fact, is strongly down-regualated in vivo during the precursor to Schwann cell transition, and its overexpression in vitro delays the generation of Schwann cells (Stewart et al., 2001).

**Signals that promote Schwann cell survival**

As discussed above, there is now strong evidence that the survival of Schwann cell precursors is acutely dependent on axonal signals produced by neuregulin-1. However, the survival of Schwann cells must rely on different signals, since the transection of adult nerves does not lead to Schwann cell death (Grinspan et al., 1996; Syroid et al., 1996; Trachtenburg and Thompson, 1996). In fact, in the distal stump of transected nerves, Schwann cell can survive for a considerable time, although they decline in number and become less responsive to extrinsic signals (Li et al., 1998; Sulaiman and Gordon, 2002). This ability of Schwann cells to survive in the absence of the axon is crucial for nerve regeneration and for remyelination after injury, since Schwann cell create a permissive environment that promotes axonal growth. The establishment of an autocrine loop is a major reason why Schwann cell survive (Cheng et al., 1998; Meier et al., 1999; Dowsing et al., 1999; Weiner and Chun, 1999).

**Schwann cell proliferation and death**

Co-culture of DRG neurons and Schwann cells have been extensively used to investigate Schwann cell proliferation, establishing that neuregulin-1 is the primary
axonal mitogen for Schwann cells (Morrissey et al., 1995; Bunge et al., 1996). Schwann cell DNA synthesis and proliferation can also be studied in purified culture. In this second case, the presence of cAMP, or of factors that mimic it, is necessary, since, with the exception of NRG-1 and HGF, in the absence of cAMP the mitogenic response of Schwann cell is minimal (reviewed in Mirsky and Jessen 2001; Jessen and Mirsky, 2004). Using purified cultures, in the presence of cAMP, it has been found that several growth factors, including TGFβs, PDGF-BB, FGF-1 and -2, and Reg-2 act as Schwann cell mitogens. The additional presence of IGFs potentiates the effect of most Schwann cell mitogens, including neuregulin-1 (Stewart et al., 1996; Cheng and Feldman, 1997; Cheng et al., 1999; Conlon et al., 2001). Proliferation in damaged nerves appears to be regulated by different mechanisms than those involved in nerve development, as indicated by the study of cyclin D1 and D2 null mice (Kim et al., 2000; Atanasoski et al., 2001). This issue, and the role of TGFβs in Schwann cell proliferation will be addressed extensively in chapter 5.

In addition to signals that promote Schwann cell survival and proliferation, there are factors that play a key role in Schwann cell death by apoptosis, during development an after nerve damage. (Trachtenberg and Thompson, 1996, Syroid et al., 1996, Grinspan et al., 1996, Nakao et al., 1997). Two factors, nerve growth factor (NGF) and TGFβ have been directly implicated in these processes (Soilu-Hanninen et al., 1999; Parkinson et al., 2001). These findings are discussed in detail in chapter 5.

Early transcription factors in Schwann cell lineage development

In eukaryotes, a great deal of biological regulation takes place at the transcriptional level. Cells from tissues phenotypically different, such as brain and muscle, share identical genotypes but possess a different spectrum of cellular mRNA. It is Nowadays
clear that gene regulation is the basis for forming such diverse phenotypes. Apart from containing coding regions, genes are sprinkled with “enhancer” and “silencer” sites, often located near the promoter, but sometimes thousand of base-pairs distant from it. Transcriptional activators and repressors act at these sites, recruiting the transcriptional initiation machinery to a gene’s promoter. The identification of the mechanisms that regulate gene transcription in a precise temporal and spatial pattern is fundamental to our understanding of the development of the multicellular eukaryote. Transcription factors are responsible for the modulation of tissue specific gene expression and for the control of gene expression seen during development. To date several different transcription factors have been identified in mammalian development and they are grouped into classes based upon the structure of the DNA binding domain. Schwann cells have been shown to express a number of representatives from the different classes of transcription factors, which modulate the expression of distinct set of genes that both mark and lead Schwann cell differentiation (reviewed in Topilko and Meier, 2001). To date, a first group of transcription factors, including Sox-10 and Pax-3, is known to be present during neural crest specification, migration and during the subsequent Schwann cell precursor development. These can be considered early transcription factors. A second group, comprising Oct-6 and Krox-20, is then involved in Schwann cell differentiation, and are here termed late transcription factors. However, it should be noted that these two groups often overlap and, as we will see, act coordinately to control Schwann cell development.

Sox-10

The sex determining factor, SRY, encodes a transcription factor characterised by a DNA-binding motif known as the high mobility group (HMG) domain. The Sox (SRY
box) gene family consists of genes related to SRY, with a sequence homology to the HMG box, which is involved in developmental regulation in a number of different systems (reviewed by Wegner, 1999). The HMG domain is not highly conserved (>50% amino acid homology), binds the heptameric sequence 5' (A/T)(A/T)CAA(A/T)G 3' and, unusually for a transcription factor, binds the minor groove of DNA. Binding of the Sox protein results in strong DNA bending that could facilitate the interaction of other transcription factors in either side of the Sox binding site (Pevny and Lovell-Badge, 1997). The transcription factor Sox-10 appears to be directly involved in glial development from the neural crest (Kuhlbrodt et al., 1998; Britsh et al., 2001; Paratore et al., 2001; Peirano et al., 2000). Sox-10 was isolated using degenerate PCR from primary Schwann cell cultures, revealing a protein of 466 amino acid residues and 56 kD in size (Pingault et al., 1998; Kuhlbrodt et al., 1998) with greater than 90% homology to murine Sox8 and 9 (Wright et al., 1993). Sox-10 expression, at least at the mRNA level, is seen mainly in the nervous system. In the CNS, it is found in the brain where it localises in the oligodendrocytes of white matter tracts, such as the internal and external capsules and the corpus callosum (Kuhlbrodt et al., 1998). In the PNS, expression of Sox-10 mRNA is seen in the dorsal aspect of the closing neural tube, and then, more crucially, in the migrating neural crest (Kuhlbrodt et al., 1998, Southard-Smith et al, 1998, Britsch et al., 2001; Paratore et al., 2001). Sox-10 is subsequently down-regulated in early neurons, but remains expressed in the developing glia, both in the ganglia and along the nerve trunks emanating from them. This latter expression, localised to Schwann cells, continues into early adulthood, although at lower levels (Kuhlbrodt et al., 1998, Southard-Smith et al, 1998, Britsch et al., 2001, Lange and Jessen, unpublished observation). This data suggests that Sox-10,
at least at the mRNA level, is expressed throughout Schwann cell development. However it remains unclear if the relative levels of Sox-10 fluctuate temporally.

Heterozygous mutations in the human SOX-10 gene are associated with isolated cases presenting growth retardation, impairment of both the central and autonomic nervous system, and, in some circumstances, peripheral neuropathy with hypomyelination and deafness (Touraine et al., 2000; Pingault et al., 2000). It is possible that this neuropathy associated with hypomyelination is due to reduced myelin protein zero (P₀) expression. Sox-10 expression is in fact sufficient to induce P₀ in a neuroblastoma cell line, and as this effect is absent in known Sox-10 mutants (Peirano et al., 2000).

Mutations in the Sox-10 gene have been associated with Waardenburg-Hirschprung’s disease or Waardenburg-Shah syndrome (WS4), which are human hereditary disorders arising due to neural crest abnormalities and resulting in deafness, pigmentary loss and aganglionic megacolon (Herbath et al., 1998, Kuhlbrodt et al., 1998b, Pingault et al., 1998). The Dominant Megacolon (Dom) Hirschprung’s mouse, a naturally occurring mutant, characterised by a nonsense or frameshift mutation in the Sox-10 gene (Southard-Smith et al., 1998, 1999), has been widely used to study the effects of Sox-10 inactivation. These mice show homozygote embryonic lethality and a failure to produce melanoblasts, while heterozygous animals show intestinal aganglionosis and spotted pigmentation (Lane and Liu, 1984).

Sox-10 expression continues during the diversification of both the melanocyte and glial lineages, while it is down-regulated in other neural crest cell derivatives (Herbarth et al., 1998, Kuhlbrodt et al., 1998, Pusch et al., 1998). The role of Sox-10 in peripheral glia development has been investigate further in Sox-10 null mice (Britsch et al., 2001). In this study, a Sox-10 null mutant was generated by LacZ gene insertion and in the heterozygous state a phenotype including pigmentation and megacolon defects, similar
to the WS4 and Dom mutants, was obtained. Even more significantly, in homozygous Sox10 null mice early peripheral glial cells, which can be identified by expression of the brain-specific fatty acid binding protein (B-FABP), are missing. This applies to both Schwann cell precursors in peripheral nerve and to satellite cells in the DRGs where, on the other end, sensory neurons are initially present in normal numbers, although they start dying later (Sonnenberg-Riethmacher et al., 2001). One reason for this could be the reduction of the neuregulin-1 receptor, ErbB3, which is observed in Sox10 null mice. (Britsch et al., 2001). Neuregulin-1 signalling does not appear to be essential for the survival of migrating crest cells in vivo (Britsch et al., 1998), but neuregulin-1 and its receptor ErbB3 are then important for the survival and proliferation of Schwann cell precursors (Britsch et al., 1998; Dong et al., 1995; 1999). It follows that one of the consequences of the reduced expression of ErbB3 receptor in Sox-10 null mice could be the death and decreased proliferation of those crest cells that have started glial differentiation, resulting in a severely reduced number of Schwann cell precursors in nerve trunks. This however does not account for the lack of glia in the DRGs in Sox-10 null mice. In fact, in mice in which neuregulin-1 signal has been inactivated DRG glia develop normally (Garrat et al., 2000; Woldeyesus et al., 1999; Meyer and Birchmeier, 1995). It is therefore possible that one of the major functions of Sox10 is in glial specification, the process by which crest cells change to become early glia (Britsch et al., 2001).

In another derivative of the neural crest, the melanocyte, a number of other possible downstream target genes of Sox-10 have been identified (Lee et al., 2000, Potterf et al., 2000). One study noted that pigmentation deficiencies similar to those observed in the Sox-10 heterozygous mice, were present in mice with mutations in the microphthalmia gene (Mitf) which encodes a transcription factor essential for the development of the
melanocyte lineage (Lee et al., 2000). Sox-10 can directly bind and activate the Mitf gene, and this activation is enhanced by Pax-3, whereas a mutant form of the Sox-10 genes acts as a dominant negative repressor of Mitf expression (Potterf et al., 2000; Lee et al., 2000, Bondurand et al, 2000).

Sox-10 remains expressed, although at lower levels, in postnatal nerves, and has been shown to synergise with Pax-3, Oct-6 and Krox-20, functioning as a transcriptional modulator (Kuhlbrodt et al., 1998). In vitro studies have shown that Sox-10 in synergy with Krox-20 strongly activates connexin-32 expression by directly binding to its promoter (Bundurand et al., 2001), suggesting a possible role for Sox-10 in the regulation of myelin genes. This is in agreement with experiments that have demonstrated that Sox-10 is able to bind the Po promoter and could have a role in the expression of this gene, which encodes for the major protein in peripheral myelin (Peirano et al., 2000).

**Pax-3**

Pax-3 belongs to the murine Paired box (Pax) DNA binding domain transcription factor family, and encodes a 479 amino acid protein containing both paired domain and paired type homeodomain (Goulding et al., 1991) and therefore binds DNA through its bipartite DNA-binding domains (Phelan and Loeken, 1998). Pax-3 mRNA is expressed in the roof plate of the neural tube, in the neural crest and in somitic mesoderm (Goulding et al., 1991). Mutations in a number of Pax genes have been shown to result in developmental defects in human and mice (Mansouri et al., 1996). In humans, disruption to the Pax-3 gene results in Waardenburg syndrome, while the fusion of Pax-3 with the forkhead gene is the cause of the childhood cancer rhabdomyosarcoma (Galili et al., 1993; Shapiro et al., 1993). Two naturally occurring
Pax-3 mouse mutants, *splotch* (Sp) and *splotch delayed* (Spd) have also been identified, the former having a deletion in the homeodomain (Epstein *et al.*, 1991) and the latter a point mutation in the paired domain (Moase and Trasler, 1990; Franz, 1993). Homozygous Sp mutants die at around E14 showing central nervous system deficits such as excencephalus and spina bifida, a lack of musculature and defects in the PNS and in pigmentation (Epstein *et al.*, 1991). Sp mice embryonic nerves have no Schwann cells in the lower trunk (Grim *et al.*, 1992), while Spd mice, which survive until E18 (Moase and Trasler, 1990) have severely reduced numbers of Schwann cells (Franz, 1993). Other neural crest derivatives are disrupted in these mutants, including neurons in the spinal ganglia and melanocytes (Gruss and Walther, 1992). These data indicate that Pax-3 has a role in the early emergence and development of neural crest cell derivatives and is required for normal Schwann cell development. However, Pax-3 is not restricted to these early developmental stages and its mRNA is detectable in the sciatic nerve throughout embryonic development and after birth (Blanchard *et al.*, 1996; Kioussi *et al.*, 1995). Interestingly, micro-injection of exogenous Pax-3 into Schwann cells *in vitro* causes down-regulation of myelin markers such as P0 and MBP, and up-regulation of those associated with non-myelin forming Schwann cells such as GFAP, L1, N-CAM and p75NTR (Kioussi *et al.*, 1995). These results are in agreement with the finding that in adult nerve Pax-3 expression is confined to non-myelin forming Schwann cells. Moreover, Pax-3 can repress the MBP and P0 promoters in co-transfection assays. Taken together these data suggest that Pax-3 has a role in Schwann cell terminal differentiation, favouring the generation of non-myelinating Schwann cells by suppressing the myelin-forming pathway.
Myelination

The process of myelination represents one of the most striking examples of cell-cell cooperation. The differentiation of myelin-forming Schwann cells is governed and dependent on the contact with another cell, the neuron, via specialised processes, the axons. All Schwann cells have the potential to form myelin, but they only do so after contact with certain axon, the large diameter ones. As we have seen, neuregulin-1 type III plays a crucial role in determining myelin thickness, but the participation of additional axonal signals and growth factors in the overall process cannot be ruled out (Michailov et al., 2004; reviewed in Mirsky and Jessen, 2004).

Myelinating Schwann cells polarize along the axon, with an abaxonal plasma membrane that is in contact with the extracellular matrix and interacts with it, and an adaxonal membrane which is in direct contact with the axon (Bunge et al., 1986). The abaxonal membrane directs the formation of a basal lamina, an essential prerequisite for myelination (Bunge et al., 1986), while the adaxonal membrane is enriched in myelin-associated glycoprotein (MAG) (Trapp et al., 1984; reviewed in Trapp et al., 2004). Immature cells destined to become myelinating Schwann cells begin to express the glycolipid galactocerebroside (GalC) at around E19 (Jessen et al., 1985). Shortly after, they start to segregate the bigger axons forming 1:1 relationships with them, while smaller caliber axons remain in polyaxonal pockets, surrounded by a single Schwann cell (Webster, 1971). The process of axonal segregation and Schwann cell myelination continues until all appropriate axons are myelinated. In the PNS, 1μm is on average the smallest myelinated fibre present (Ritchie, 1983). Schwann cells at the 1:1 stage have stopped dividing and are found in the sciatic nerve at birth in both rat and mouse (reviewed in Jessen and Mirsky 2002, 2004). The first wrappings of the myelin sheath are seen at this time together with high level expression of the major myelin proteins:
the glycoprotein protein zero (P₀), peripheral myelin protein 22kDa (PMP22), myelin basic protein (MBP), proteolipid protein (PLP), myelin associated-glycoprotein (MAG), plasmolipin, myelin and lymphocyte protein (MAL), P2, periaxin (Prx) and CNPase (Jessen and Mirsky, 1999; 2002).

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

Myelin structure

The myelin sheath that Schwann cells synthesise around a single axon is a multilamellar membrane that acts as an insulating conduit. The sheath is discontinuous along the length of the axon. At the junction of adjacent Schwann cells there are specialised areas of electrically excitable axon surface, known as the node of Ranvier. Action potentials are propagated from one node to the next in a process known as saltatory conduction (Ranvier, 1878, Webster, 1971; Ritchie, 1983). In unmyelinated axons the conduction velocity is directly proportional to the root of the diameter of the axon. For this reason, invertebrates such as the squid, that need rapid nerve conduction but have only unmyelinated axons, have developed "giant axons" with large diameter. Myelin, and thus saltatory conduction, increases the velocity of a given action potential up to ten times, compared to a similar sized unmyelinated axon (Jacobson, 1993, Ritchie, 1983). Every Schwann cell forms a single internode, which can be divided in two distinct domains, compact myelin and non-compact myelin. The Schwann cell nucleus is usually positioned in the middle of the internode. Compact myelin accounts for the majority of the myelin in the internode, and, if observed with a Transmission Electron
Microscope (TEM), appears as a lamellar structure, alternating dark and light lines that wraps a central axon. The spiral membranes of compact myelin have a periodicity of 13 to 19 nm, depending on the technique used to measure it (Kirschner and Sidman, 1976; Kirschner and Hollingshead, 1980). The periodicity derives from the presence of dark major dense lines, 2.5 nm thick, separated by paler interperiod lines. The major dense line is formed by the fusion of two intracellular lipid bilayers, with the squeezing of the cytoplasm, while the interperiod line is formed by the two extracellular surfaces of the Schwann cell membrane (Alberts et al., 1994; reviewed in Trapp et al., 2004). Non-compact myelin, on the other hand, provides cytoplasmic continuity over the length of the myelinating Schwann cell. It includes the cytoplasmic channels at the abaxonal and adaxonal surfaces and Schmidt-Lanterman incisures, that radially transverse the compact myelin. A major channel that extends the entire length of the internode contains the outer mesaxon. At either end of the myelinating Schwann cell, approaching the node of Ranvier, the major dense line opens up to accommodate cytoplasm, forming the paranodal loops. These loops are a major site of myelin axon adhesion via a series of septate-like junctions and provide a diffusible barrier and preventing lateral diffusion of membrane proteins (reviewed in Peles and Salzer, 2000; Scherer et al., 2004). The 10 -15 nm area of the myelin internode adjacent to the paranodal loops is known as juxtaparanodal region. In TEM it is basically indistinguishable from the internode, but it is enriched in delayed-rectifier potassium channels and in the protein Caspr 2, a homologue of Caspr (Peles and Salzer, 2000). The node of Ranvier is a small stretch of bare axolemma (axonal plasma membrane), directly exposed to the extracellular milieu, that separates myelin segments along individual nerve fibers. This area is extremely rich in voltage-gated Na channels, and is therefore directly responsible for saltatory conduction (Scherer et al., 2004). The nodal axolemma is surrounded by Schwann cell
microvilli, which extend from the outer border of the adjacent myelin internodes and contain F-actin, ezrin, radixin and moesin (reviewed in Scherer et al., 2004).

**Myelin composition**

Since the major function of compact myelin is to insulate the axon, it does not need to be a particularly complex and dynamic structure. Myelin membrane comprises ~75% lipids and ~25% proteins, which serve mainly, but not only, a structural function. Myelin proteins are encoded in the CNS and PNS by an overlapping but not identical set of genes. As we shall see, natural mutants and man-made transgenic mice have been widely used for the analysis of the function of these proteins, showing that they are involved in several stages of Schwann cell development.

**Po**

Myelin protein zero (P₀) is the main protein in the myelin of the PNS, accounting for more than 50% of the total proteins (Greenfield et al., 1973). The P₀ gene encodes a single RNA species of 1.9 Kb which results in a protein consisting of a 29-residue signal peptide, a 124-residue extracellular domain, a 26-residue transmembrane domain and a 69-residue intracellular domain (Sakamoto et al., 1987; reviewed in Kirschner et al., 2004). P₀ is therefore a 28-30 kDa, glycosilated, integral membrane protein, that belongs to the immunoglobulin gene superfamily, thanks to its extracellular immunoglobulin(Ig)-like domain (Lemke and Axel, 1985, Lemke et al., 1988). The presence of this Ig-like domain, found in cell adhesion molecules such as N-CAM (for review see Edelman, 1983), and the abundance of P₀ in PNS myelin, suggested that P₀ could be somehow involved in both the extracellular and intracellular membrane adhesion, which result in compact myelin (Lemke and Axel, 1985). The homophilic
The adhesive nature of Po has been demonstrated by transfection assays where increased cell-cell attachment occurs (D'Urso et al., 1990, Schneider-Schaulies et al., 1990, Filbin et al., 1990). To date, only the recombinant extracellular domain corresponding to the sequence for rat has been crystallized, showing that this domain has to assemble into a tetrameric complex in order to serve such an adhesive function (Shapiro et al., 1996).

Po is expressed almost exclusively in Schwann cells and at dramatically high levels during myelination (Trapp et al., 1981, Webster and Favilla, 1984, Kirschner et al., 2004). However, Po mRNA has been detected in the notochord, in otic placode and vesicle, in the enteric neural crest and in olfactory ensheathing cells (Lee et al., 2001). Prior to birth, Po mRNA first appears in a subset of neural crest cells and this probably represents the first sign of glial commitment. Subsequently it remains expressed, at basal levels, in Schwann cell precursors and immature Schwann cells (Bhattacharyya et al., 1991; Lee et al., 1997). After birth, both Po mRNA levels and protein synthesis rise about 30 – 40 fold in myelinating Schwann cells, and peak during the second postnatal week before dropping off in the adult (Lemke and Axel, 1985; Trapp et al., 1988; Stahl et al., 1990; Baron et al., 1994). Schwann cells require continuous axonal contact to maintain high level Po expression (Trapp et al., 1988; Mirsky et al., 1980; Lemke and Chao, 1988; Jessen and Mirsky, 1991; Fernandez-Valle et al., 1993; Scherer et al., 1994; Gupta et al., 1993). Po expression regulation is thought to occur mainly at the transcriptional level (Stahl et al., 1990; Lee et al., 1997). So far, the strongest evidence for a direct regulation of the Po gene is for the Sox-10 transcription factor. Sox10, in fact, up-regulates both endogenous Po expression as well as the Po promoter 10- to 20-fold when ectopically expressed in neuroblastoma cells, while this effect is absent when known Sox10 mutants are used in the same assays (Peirano et al., 2000). Moreover, the authors show that the Po promoter contains two probable Sox-10 binding sites.
The function of the $P_0$ protein has been extensively studied thanks to a series of transgenic mice. Homozygous and heterozygous null mice have shown that not only $P_0$ is involved in myelin compaction during development but it is also required for the long term maintenance of the myelin sheath via protein turnover (Giese et al., 1992). Moreover, a series of observations indicates a role for $P_0$ in axon maintenance. In fact, $P_0$ null mice develop axonal degeneration (Giese et al., 1992; Frei et al., 1999; Martini et al., 1995), and several point mutation in the $P_0$ gene cause the axonal form of Charcot-Marie-Tooth disease (CMT2) (reviewed in Wrabetz et al., 2004). Human peripheral neuropathies such as CMT and Dejerine-Sottas syndrome (DSS) have been identified that are linked to various mutations in the $P_0$ gene (reviewed by Keller and Chance, 1999; Wrabetz et al., 2004). Work on $P_0$ overexpressing mice has suggested that myelin assembly employs a process linking the various constituents in a mechanism that closely controls the final stoichiometry (Wrabetz et al., 2000). In this work, a series of transgenic mice overexpressing $P_0$ has been generated by introducing extra copies of the $P_0$ gene. It was found that increasing dysmyelination occurred with increased $P_0$ gene dosage. Surprisingly, increased $P_0$ mRNA expression resulted in reduced $P_0$ protein expression and interestingly, also caused a reduction of normal levels of MBP, highlighting the importance of dosage and stoichiometry of the different myelin genes in the production of a stable myelin sheath.

**MBP**

MBP is the second most abundant protein in peripheral myelin, accounting for up to 16% of the total proteins (Greenfield et al., 1973). MBP is encoded by a gene 105kb in length in the mouse and 180kb in humans, and consists of at least six different isoforms,
ranging from 14 to 21.5 kDa, generated through alternative splicing of the gene (reviewed by Lemke and Barde, 1998; Campagnoni and Campagnoni, 2004). A second family of MBP proteins, the *golli-MBPs*, is generated from a different transcription start of the gene. The golli proteins are present in the nervous system but at levels significantly lower than the MBPs, and are also expressed in the immune system, including spleen and thymus (Campagnoni *et al.*, 1993). MBP, in all its classic isoforms, is located on the intracellular face of compact myelin in the major dense line, and its major role appears to be that of structural component. Both oligodendrocytes and Schwann cells target MBP proteins to the sheath via free ribosomes located close to the site of myelin formation (Colman *et al.*, 1982, Trapp *et al.*, 1987, Ainger *et al.*, 1993).

The *shiverer* (*shi*) mouse is a naturally occurring mutant that has been shown to be deficient in MBP. In these mice the major dense line fails to form within CNS myelin, while in the PNS myelin is formed and functional, but it is slightly thinner than in a normal mouse (Privat *et al.*, 1979; Popko *et al.*, 1988; Kirschner and Ganser, 1980, Rosenbluth *et al.*, 1980). One reason for this difference probably lays in the fact that MBP is much more abundant in the CNS (30% of myelin proteins) and therefore has a more important function than in the PNS. Moreover, the other main protein in the CNS, PLP, does not have a cytoplasmic tail, whereas P₀ in the PNS does. Therefore MBP is probably needed in the CNS for myelin stability while it is not crucial in the PNS. *Shi* phenotype includes tremors, convulsions and early death that can be rescued by the introduction of the wild-type MBP gene (Readhead *et al.*, 1987, Kimura *et al.*, 1989). *Shi* mice also exhibit double the number of Schmidt-Lanterman incisures in myelin forming Schwann cells, although the meaning of this difference is not yet clear (Gould *et al.*, 1995). Double mutants have been generated deficient for both P₀ and MBP. These mice show a severe hypomyelination, and the thin myelin sheath formed is
devoid of major dense lines, indicating that in the PNS both MBP and Po proteins are required for normal myelination (Martini et al., 1995).

**PMP22**

PMP22 is a 22 kD glycoprotein that is a minor component of the myelin sheath of peripheral nerve. It belongs to a family of proteins characterised by four transmembrane domains and conserved amino acid motifs (Jetten and Suter, 2000). PMP22 expression pattern is similar to that of other myelin gene products, and it is localised to compact myelin (Snipes et al., 1992, reviewed in Werner et al., 1998; Suter, 2004). PMP22 is widely expressed in neural and non-neural tissues during embryonic development and also in the adult (Notterpek et al., 1999). PMP22 protein is most highly expressed in myelinating Schwann cell where it is detectable after MAG but before MBP onset in rodents (Notterpek et al., 1999). In the mature nerve PMP22 is found in the plasma membrane of non-myelinating and myelinating Schwann cells, and in the compact portion of myelin (Snipes et al., 1992, Haney et al., 1996). The PMP22 gene spans approximately 40kb in humans and consists of six exons (Patel et al., 1992). On the transcriptional level, two different promoters, P1 and P2, have been identified (Suter et al., 1994). While P1 appears to control myelinating Schwann cell-specific expression, P2 is more widely active (Suter et al., 1994). PMP22 and Po are regulated in a similar way during development, myelination and after nerve injury, and colocalise in compact myelin. This raises the hypothesis of an association between these two proteins, where PMP22 would interact with the Po tetrameric complex (D'Urso et al., 1999). This idea fits well with the finding that mutations affecting PMP22 and Po result in similar inherited demyelinating neuropathies (Scherer, 1997; Snipes and Suter, 1995; Wrabetz et al., 2004).
Most of the knowledge about the role of PMP22 in peripheral nerve comes form genetics, since PMP22 is the most common cause of neuropathies in humans and rodents. The mouse mutants trembler (Tr) and Tr-J display peripheral myelin uncompaction and hypomyelination together with increased Schwann cell proliferation, associated with abnormal myelin degradation and remyelination (Suter et al., 1992a, b; 1993). These mice carry a point mutation in hydrophobic regions of the PMP22 protein, indicating that the transmembrane domains may be important for myelin compaction and for maintenance of mature myelin (Suter et al., 1992b, Suh et al., 1997). These two mutations are now well characterised: Tr mutants have a G150D mutation in the last transmembrane domain that causes a transport arrest of PMP22 in the endoplasmic reticulum (ER) (Naef et al., 1997, D’Urso et al., 1998). Tr-J mice have a Leu16Pro mutation and PMP22 proteins are also transported abnormally (Notterpek et al., 1997, Tobbler et al., 1999). To better understand the function of PMP22, PMP22 deficient mice have been generated by the complete disruption of the PMP22 gene. These animals display walking difficulties due to progressive limb weakness. From a morphological point of view they display a mildly delayed onset of myelination followed by hypermyelination and demyelinating peripheral neuropathies in both the homozygous and heterozygous state, characterized by tomacula, onion bulbs and aberrant Schwann cell proliferation and death (Adlkofer et al., 1995, 1997; Sancho et al., 1999; 2001).

Recently, transgenic mice and rats overexpressing increasing numbers of copies of the PMP22 gene have been created (reviewed in Wrabetz et al., 2004). Overexpressing mice show that peripheral neuropathies develop when 4 copies or more of the transgene are present and that with 7 copies hypomyelination is severe (Huxley et al., 1996,
1998). Moreover, it has been found that TrJ mutants and mice with 7 copies of the PMP22 gene are able to form 1:1 associations with axons but that many fibers were not completely surrounded by Schwann cell cytoplasm (Robertson et al., 1999). Delays in the onset of myelination have also been observed in mouse and rats overexpressing the mouse PMP22 gene (Magyar et al., 1996, Sereda et al., 1996). Further analyses of these PMP22 overexpressing rats shows that myelination is blocked at the promyelinating stage, not simply delayed, and Schwann cell differentiation at the mRNA level is not perturbed (Niemann et al., 2000). Thus it is likely that PMP22 is involved early on in the process of myelination, immediately after the establishment of 1:1 relationships with axons.

In humans PMP22 mutations are associated with three different neuropathies: Charcot-Marie-Tooth (CMT) disease, Dejerine-Sottas syndrome (DSS) and hereditary neuropathy with liability to pressure palsies (HNPP) (reviewed in Keller and Chance 1999; Wrabetz et al., 2004). Duplications of the complete PMP22 gene account for most cases of CMT1A, although point mutations also exist (Lupsik et al., 1991, Raeymaekers et al., 1991). Intriguingly, a Dutch family with a Leu16Pro mutation, the same that occurs in Tr-J mutants, shows a severe DSS, with an 80% reduction in myelinated fibre density as compared to 20% in the Tr-J mouse (Valentijn et al., 1992; Gabreels-Festen et al., 1995, Robertson et al., 1997)

The large variation in severity in neuropathies seen both in patients with mutations in the PMP22 gene and animal models suggest that these mutations, as well as mutations in P0, could act via at least two different mechanisms, gain of function or loss of function. In the latter case, as a result of the mutations, the protein is either not produced at all or is quickly degraded. Loss of function is therefore the likely cause of the less severe phenotypes observed. In the gain of function mechanism, the mutated
proteins gains an abnormal or toxic function that perturbs the biology of the cell much more severely, and usually results in the more severe phenotypes (reviewed in Wrabetz, 2004).

To conclude, all these observations indicate that PMP22 may have two roles in the PNS; an earlier role in the establishment of a complete Schwann cell to axon association and a later role as a component of mature compacted myelin.

**PLP**

The PLP gene, located on the X chromosome, spans around 17kb, consists of seven exons and is controlled by a single promoter region (Stoffel et al., 1984; Diehl et al., 1986). PLP exists in two isoforms, PLP (24 kDa) and DM20 (20 kDa) as a result of an uncommon exonic alternative splicing (reviewed in Suter and Snipes, 1995). PLP is mainly expressed in the CNS where it contributes 50% of total myelin proteins whereas in the PNS much lower amounts are present. Two transcription initiation sites have been identified in the PLP gene (Milner et al., 1985). Both are employed in the CNS, while the downstream one is predominant in the PNS (Kamholz et al., 1992; Scherer et al., 1992). The significance of this seems to lie in the influence of promoters on splice site selection. The upstream promoter selectively drives the expression of PLP, and as a result, this is the form predominant in the CNS. The downstream promoter selectively expresses DM20, which is the major isoform expressed in the PNS, although myelinating Schwann cell also express PLP. Of the two genes, during development of oligodendrocytes DM20 is initially expressed at higher levels but is later superseded by PLP at the peak of myelination (Schindler et al., 1990, Timsit et al., 1992, 1995; Scherer et al., 1995; reviewed in Hudson, 2004). PLP is localised in the interperiod line in mature myelin and is therefore proposed to have a structural role. Gene targeting of
the PLP locus has generated PLP null mutant as well as mice lacking both PLP and DM20 or DM20 only (Boison and Stoffel, 1994; Boison et al., 1995; Klugmann et al., 1997; Stecca et al., 2000; Sporkel et al., 2002; Uschkureit et al., 2001). In each transgenic model, oligodendrocyte development occurs normally, and they assemble myelin. However, this myelin, in the PLP/DM20 double mutant, has defects in the intraperiod line, with reduced conduction velocity and impaired neuromotor coordination (Boison and Stoffel, 1994). An age-related degeneration of oligodendrocytes is also seen in PLP and PLP/DM20 mutants (Klugmann et al., 1997; Sporkel et al., 2002; Stecca et al., 2001; Yool et al., 2001). Interestingly, while PLP/DM20 double KO mice show axonal swelling and loss of compact myelin in small caliber axons (Yool et al., 2001), these defects are spared in mice that express only DM20 (Boison et al., 1995), suggesting a possible redundancy for these two proteins.

In humans, mutations in both PLP and DM20 cause Pelizaeus-Merzbacher disease (PMD), an X-linked a leukodystrophy and, in some cases, a dysmyelinating neuropathy. The range of mutations is extremely varied, from point mutations to deletions to duplication (reviewed in Yool et al., 2000). The relationship between genotype and phenotype is remarkably close in the animal models and the PMD cases, making them useful models for studying the mechanisms of PLP gene-related disease. As a result, it has become clear that the PLP gene plays a wider role in neural development in addition to its function as a structural component of myelin. It has also emerged that duplications of the PLP gene are the commonest mutation in PMD. Genetic disorders arising from a dosage effect may be more common than previously recognized.
MAG

MAG is a type I membrane glycoprotein consisting of an N-terminal extracellular domain, a single transmembrane domain and a small cytoplasmic domain (Lai et al., 1987; Scherer et al., 1987). The gene spans approximately 16kb and is composed of 13 exons. MAG protein is encoded in two isoforms, 67 kD (S-MAG) and 72 kD (L-MAG) forms, with the former predominantly in Schwann cells and the latter localised to oligodendrocytes of the CNS as well as Schwann cells. In the PNS MAG constitutes less than 1% of peripheral myelin, and is found mainly in non-compact myelin, such as the mesaxon, Schmidt-Lanterman incisures and paranodal loops but not in compact myelin and is expressed before the massive up-regulation of the other myelin genes (Hudson et al., 1990; reviewed in Georgiou et al., 2004). This observation has suggested a role for MAG in mediating axon-myelin sheath interactions, and supported a role for MAG in the initial stages of myelin formation (Georgiou et al., 2004). The extracellular portion of MAG contains 5 Ig-like domains, which as already seen for P0, are often associated with adhesive properties. The adhesive properties of MAG have been demonstrated experimentally with different approaches. The use of antibody against MAG prevents the binding of oligodendrocytes to neurons (Poltorak et al., 1987), and recombinant MAG confers adhesive properties to liposomes and to different cell types, such as fibroblasts (Johnson et al., 1989; Afar et al., 1991). One of the first insights into the role of MAG in mediating axon-myelin sheath interactions came from the naturally occurring Quaking mouse mutant. The mutation, which does not occur in the MAG gene but in the QKI gene, directly affects MAG mRNA and protein expression and MAG localisation (Trapp et al., 1988; Bartoszewics et al., 1995; Fujita et al., 1988). MAG deficient mice, on the other hand, reveal only a subtle phenotype within the PNS, although the onset of maturity is accompanied with progressive
axonopathy, axonal degeneration and onion bulbs formation, suggesting that MAG may also stabilize myelin (Yin et al., 1998; Weiss et al., 2001). Moreover, in MAG null mice enhanced regeneration following sciatic nerve injury has been reported (Li et al., 1994, Montag et al., 1994, Filbin, 1995). This latter finding is in agreement with other studies in the CNS where has been reported that, while during early development MAG promotes axonal growth, it becomes an inhibitory molecule for axonal regrowth in the adult (reviewed in Georgiou et al., 2004).

The fact that the neuropathology in MAG knockout is not severe together with the ability to detect it only later in life, suggest that MAG mutations in humans would be difficult to diagnose, and in fact, to date, no human mutation in the MAG gene has been identified.

Periaxin

Periaxin (Prx) is a member of the PDZ domain protein family and is primarily expressed by myelinating Schwann cells, where it associates with the cytoskeleton. The periaxin gene, located in mouse on chromosome 7, is composed of seven exons and encodes two mRNAs of 5.2 and 4.6kb, resulting from different splicing, which translate into two periaxin forms: S-periaxin (16kDa) and L-periaxin (147kDa) (Gillespie et al., 1994, Dytrych et al., 1998; Gillespie et al., 2000). It has been reported that, in human PNS, the smaller mRNA is predominant, suggesting that L-periaxin is the main isoform in human Schwann cells (Boerkoel et al., 2001). Both isoform contain a PDZ domain, which is believed to be involved in protein-protein interaction at the plasma membrane. In the mouse, although they have similar abundance, only L-periaxin is localized to the Schwann cell plasma membrane while S-periaxin is distributed within the cytoplasm (Dytrych et al., 1998).
In Schwann cells, periaxin expression is detectable early in development, at around the time when precursors start to differentiate in immature Schwann cells, and peaks during the first two weeks after birth, the period of active myelination (Gillespie et al., 1994; Sherman and Brophy, 2000). Initially, L-periaxin expression is nuclear, but just as myelination starts, it concentrates at the adaxonal membrane and progressive myelination sees a shift in this localisation to the abaxonal Schwann cell membrane (Gillespie et al., 1994; Scherer et al., 1995; Sherman and Brophy; 2000). The meaning of this early nuclear localisation is not clear yet: one explanation is that the nuclear targeting serves to sequestrate the protein from the cytoplasm, avoiding inappropriate interaction in the cytoplasm. Alternatively, it is possible that L-periaxin may have a role in co-transcriptional regulation in embryonic nerve (reviewed in Sherman and Brophy, 2004). Similarly to MAG periaxin is localized, in the cytoplasm-filled periaxonal regions of the sheath but is excluded from compact myelin, suggesting that it may be involved in the early process of axonal ensheathment (Gillespie et al., 1994). Periaxin expression is axonally regulated, in a similar fashion to myelin genes and is down-regulated following nerve injury. Interestingly, periaxin expression in developing and in regenerating nerves predates major myelin gene expression being, for example, detectable in advance of P0 (Scherer et al., 1995).

To study periaxin function further, null mutant mice (Prx<sup>−/−</sup>) have been generated. These mice are able to form and assemble compact myelin, but, already at 6 weeks, the sciatic nerves contain some tomacula (Gillespie et al., 2000). With ageing, Prx<sup>−/−</sup> mice show severe demyelination and associated reduced nerve conduction velocities (Gillespie et al., 2000). It appears then that L-periaxin in needed to stabilise myelin and the axon-Schwann cell unit. One way by which this could happen is through complexes that periaxin forms with the dystrophin-related protein 2 (DRP2), a member of the...
dystrophin-like protein family. This family of proteins, which comprises dystrophin, utrophin and dystrobrevin, binds with the plasma membrane protein dystroglycan. The study of DRP2-L-periaxin-dystroglycan complexes in Prx<sup>−/−</sup> mice has demonstrated that they are fundamental for stable axon-glia interaction (Sherman<sup>et al.</sup>, 2001).

Mutations in the periaxin gene have been reported in peripheral neuropathies in humans, including DSS (Boerkel<sup>et al.</sup>, 2001) and an autosomal recessive form of CMT termed CMT4F (Guilbot<sup>et al.</sup>, 2001; Takashimi<sup>et al.</sup>, 2002). It is likely that the identification of novel components of the DRP2-Prx-dystroglycan complex, will lead to possible candidates for other forms of CMT.

Recently, it has been demonstrated that in Prx<sup>−/−</sup> mice longitudinal bands of cytoplasm are missing, internodal lengths are reduced and, as a result, nerve conduction velocity is slower (Court<sup>et al.</sup>, 2004). Moreover, with brilliant experiments using chimaeric mice, the authors showed that the ability of Schwann cells to elongate in response to axonal growth is cell-autonomous (Court<sup>et al.</sup>, 2004). Taken together these data are the first experimental proof that internodal length directly influences nerve conduction velocity.

**Connexin 32 and Connexin 29**

The connexins family is composed of at least 21 different genes (Willeke<sup>et al.</sup>, 2002). Connexins encode a group of channel forming proteins that share a common four transmembrane domain topology (Bruzzone<sup>et al.</sup>, 1996; White and Paul, 1999; reviewed in Scherer and Paul, 2004). Gap junctions are intercellular channels that allow the passage of small molecules such as ions, second messengers and metabolites (<1000 Da), and control cell proliferation and differentiation (reviewed by Bruzzone and Ressot, 1997; Simon, 1997). They consist of two apposed hemichannels, each
composed of six connexins arranged to form a central pore. Interestingly, only some combinations of connexins are functional.

Connexin 32 (Cx32), the first connexin to be cloned (Paul, 1986), is expressed in the myelin-forming cells of the CNS and PNS, where it parallels the expression of myelin-related genes (Scherer et al., 1995; Sohl et al., 1996). Two candidates for Cx32 regulation in the PNS are the transcription factors Sox-10 and Krox-20. In fact, the Cx32 promoter contains putative binding sites for these two transcription factors (Bondurand et al., 2001; Musso et al., 2001). In the mature PNS myelin, Cx32 is localized to the paranodal region and Schmidt-Lanterman incisures and may therefore form a pathway for diffusion across areas of non-compacted myelin (Scherer et al., 1995, Paul, 1995). Cx32 deficient mice have been generated and they display a late-onset peripheral neuropathy (Balice-Gordon et al., 1998). This neuropathy is characterized by the progressive appearance of onion bulbs, thin myelin sheath and increased periaxonal collars, suggesting that Cx32 is important for myelin maintenance (Anzini et al., 1997). In mice that are Cx32 "+" and P0 haplo-deficient the resulting peripheral neuropathy is accelerated compared to either individual mutant. This suggests that both of these proteins, despite having different sub-cellular localizations, may have a combined effect on myelin stability (Neuberg et al., 1998). In humans more than 80 mutations in the Cx32 gene have been identified as associated with peripheral nerve degeneration, which is called X-linked form of Charcot-Marie-Tooth disease (CMTX). In some of these, the mutation is not in the open reading frame (ORF), but instead it can be found in non-coding regions (Bergoffen et al., 1993, Bruzzone et al., 1994, Ionasescu et al., 1994, Bondurand et al., 2001).

Connexin 29 (Cx29) has been recently identified (Altevogt et al., 2000). Its expression appears to be restricted to myelinating glial cells both in the CNS and PNS (Sohl et al.,
In myelinating Schwann cells Cx29 is expressed earlier than Cx32, but in the adult its levels are lower than those of Cx32. It localizes in particular in the juxtaparanodal region of myelinating Schwann cells, and in the inner mesaxon, incisures and paranodal loops (Altevogt et al., 2002).

**P2 and CNPase**

The product of the P2 gene is a small, 14.8 kDa protein, that accounts for less than 1% of total myelin proteins, and is only found within compact myelin where is believed to function by regulating the synthesis and transport of long chain fatty acids (Hahn et al., 1987; Hudson 1990). Similarly, the product of the 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) gene is thought to play a role in myelin assembly (reviewed by Hudson, 1990).

CNPase is a cytoplasmic protein, found as two isoforms (46 and 48 kDa) within glial cells as well as other non-neuronal tissues (reviewed in Braun et al., 2004). In the PNS it comprises only 0.4% of total myelin protein. While in the CNS the developmental expression of CNPase parallels that of other myelin-related proteins, in the peripheral nerve it peaks around birth but decreases gradually in older animals, making its role in myelination unclear (Stahl et al., 1990). The generation of CNPase null mice is very recent (Lappe-Siefke et al., 2003). These mice develop a normal myelin but adults manifest a severe neurodegenerative disorder, characterized by axonal swelling and degeneration, suggesting a role for CNPase in axonal survival, although the mechanisms involved are still unknown. Moreover, a role for CNPase in RNA metabolism, regulation and degradation has been proposed, based on structural studies that revealed a resemblance of CNP with some bacterial RNA ligases (Hudson, 1990; Mazumder et al., 2002).
Plasmolipin and MAL

Plasmolipin and myelin and lymphocyte protein (MAL/MVP17) are, like PLP and PMP22, members of the four transmembrane domain protein family.

The plasmolipin gene encodes a single 1.7kb mRNA which results in a 157aa, 18kDa protein (Fisher and Sapirstein, 1994). Plasmolipin was initially thought to be restricted to nervous system and kidney, but successive work demonstrated that it is in fact expressed in a variety of tissues, including thymus, lung and thyroid gland (Hamacher et al., 2001). In the PNS, plasmolipin mRNA expression is restricted to myelinating Schwann cells, and its regulation during neural development and sciatic nerve regeneration shows a tight correlation with myelination (Gillen et al., 1996; Bosse et al., 2003). Interestingly, plasmolipin expression after nerve cut is induced even when axonal regeneration in prevented by ligation of the nerve stump (Bosse et al., 2003), suggesting an additional role for plasmolipin different from myelin biogenesis and maintenance. Moreover, plasmolipin in mature myelin has a distinct lipid rafts (see below) association, indicating a possible role for plasmolipin in intracellular protein trafficking (Bosse et al., 2003).

Plasmolipin shares some structure and expression similarity with MAL/MVP17. The MAL gene encodes for a 153aa, 17kDa protein that is expressed in kidney, spleen, brain and peripheral nerve, with the highest mRNA levels during CNS and PNS myelination (Kim et al., 1995; Schaeren-Wiemers et al., 1995). In the PNS, MAL is strongly expressed in myelinating Schwann cells where it colocalizes with MBP and PLP in compact myelin (Frank et al., 1998). MAL immunoreactivity is also detectable in immature Schwann cells and persists in non-myelinating Schwann cells (Frank et al., 1999). In epithelial cells, it localizes in the apical plasma membrane, where specific glycosphingolipids such as sulfatide and galactosylceramide are enriched. These lipids
are also enriched in myelin, and MAL associates with them in myelinating Schwann cells and oligodendrocytes (Carduff \textit{et al.}, 2001; Kim and Pfeiffer, 2002). Therefore, one possible role for MAL is in the formation and stabilization of this specific lipids microdomains (reviewed in Frank, 2000).

\textbf{Integrins.}

Integrins are glial cell surface receptors that mediate local interactions between cells and the ECM and provide the cues necessary for myelination and that may also mediate cell migration, proliferation and differentiation (Hynes, 1992; 2002; ffrench-Constant, 2004). Each integrin is composed by heterodimers of $\alpha$ and $\beta$ subunits, coded by separate genes; each subunit comprises a short intracellular domain directly linked to cytoskeleton molecules such as vinculin and talin, a single transmembrane domain and a large extracellular domain. The combination of two extracellular domains creates a ligand binding site (Hynes, 1992). So far, 26 subunits have been identified (18 $\alpha$ and 8 $\beta$), that have been shown to form 24 differing $\alpha$/ $\beta$ heterodimers (Hynes and Zhao, 2000; reviewed in Previtali \textit{et al.}, 2001). Each integrin heterodimer has specificity for a single ligand or range of ligands, that include members of the ECM (Ruoslhti \textit{et al.}, 1996) and surface proteins of the Ig superfamily, such as L1 and Thy-1. The binding with these ligands is called \textit{in-trans} binding, while the binding with cell-surface/cytoskeletal proteins such as tetraspan molecules is termed \textit{in-cis} binding (Previtali \textit{et al.}, 2001).

Various integrins are expressed throughout Schwann cell development but the heterodimeric combinations seen vary according to the specific stages of Schwann cell differentiation. \textit{In vivo} studies of chick neural crest cells have shown that these cells express the laminin receptors $\alpha1\beta1$ and $\alpha6\beta1$. Integrin antibody blocking experiments
cause disruption of neural crest cell migration. Similarly, peptides competing for ECM ligands and growth factor receptor mutants show disruption of migration (Previtali et al., 2001). α1β1 integrin is expressed in the neural crest, is down-regulated to very low levels during the transition to Schwann cell precursors but is high again in mature non-myelin forming Schwann cells. It is probably regulated by axonal contact, being up-regulated in myelinating Schwann cells following nerve injury (Stewart et al., 1997, Perris et al., 1997). In mature Schwann cells of chick, rodent and human, at least seven integrin heterodimers have been identified: α1β1, α6β1, αvβ8, α2β1, α6β4, α5β1 and αvβ3 (Previtali et al., 2001). These data suggest that the specific stages of Schwann cells development are accompanied by differential integrin expression.

During embryonic development, α6 is associated with β1, but it switches partner, to β4, at the onset of myelination. In both rats and humans the expression of β4 integrin subunit dramatically increases in myelin forming Schwann cells, is localised abaxonally and is axon dependent (Einheber et al., 1993, Feltri et al., 1993, Niessen et al., 1994). β4 sub-unit null mutants have been generated but, unfortunately, die shortly after birth allowing only a glimpse at the onset of myelination, but show that myelination can occur normally and that β4 is not required for Schwann cell differentiation (Frei et al., 1999). However, the possibility that β4 may have a role in the longer term in myelin maintenance cannot be ruled out.

Studies of integrin function in the PNS using null mutants have been of limited success, many resulting in embryonic lethality such as integrin β1, or without phenotype as in α1 and α7 integrin knockouts (reviewed in Previtali et al., 2001; Previtali et al., 2003). Cre/loxP technology can be used to overcome the problem of embryonic lethality, and to ablate a given gene in a cell specific way (see chapter 5). This approach has been applied to ablate integrin β1 specifically in Schwann cells (Feltri et al., 2002). In this
work, mice carrying floxed β1 alleles have been crossed with mice in which the cre recombinase is expressed under the control of the P0 promoter. These mice show a clear phenotype, characterized by a peripheral neuropathy, due to the inability of Schwann cells to form and maintain proper contact with the axon. Migration, proliferation and survival of precursors cells occur normally, indicating that β1 integrin is not required in the embryonic stages of development. Interestingly, in some cases, the block of myelination can be overcome, and Schwann cells synthesise myelin, albeit with a delay. An explanation for this could be a compensatory mechanism via dystroglycan or α6β4 (Feltri et al., 2002). Taken together these data suggest that β1 integrin is required for Schwann cells to interact properly with axons. Further similar studies will be required to understand the role of other integrins in Schwann cell development.

Laminin

Laminins comprise a large family of glycoproteins made up of 3 sub-units: two light chains, 200 kDa, and one heavy chain, 400 kDa, that together form a cruciform structure (Nissinen et al., 1991). Schwann cells express large amounts of laminin, the major component of basal lamina (Cornbrooks et al., 1983; Podratz et al., 2001). Both myelin forming and non-myelin forming Schwann cells synthesise a basal lamina that, in myelinating ones, is necessary for axon ensheathment (Bignami et al., 1984, Bunge et al., 1986; reviewed in Bunge, 1993). Interestingly, laminin deposition on the Schwann cell surface is sufficient for the onset of myelination even in the absence of a basal lamina (Podratz et al., 2001). On the Schwann cell surface, integrins α6β1, α6β4 and dystroglycan are the major receptors for laminins (reviewed in Previtali et al., 2001). The main form of laminin expressed by Schwann cells consists of β1 and β2 light chains and the α2 heavy chain (merosin) (reviewed in Reichardt and Tomaselli,
Mutations in merosin have been identified in both a naturally occurring mutant mouse known as dy/dy, and in merosin-deficient congenital muscular dystrophy (CMD) patients (Xu et al., 1994; Sunada et al., 1995) Both human and mouse disorders result in a peripheral neuropathy, characterised by abnormal bundles of naked axons not surrounded by any Schwann cell process, particularly in the dorsal roots, and by a discontinuous basal lamina (Bradley and Jenkinson, 1973, Madrid et al., 1975, Uziyel et al., 2000). Taken together, these findings and the data from mutant in integrin and transgenic mice, point at the laminin-integrin complex as a crucial actor in the process of peripheral nerve myelination.

**Laminin and leprosy**

Leprosy is a disease caused by the bacterium *Micobacterium leprae* and is characterised by infiltration and infection of the Schwann cells of sensory nerves that often result in a rapid immune response that cause extensive inflammation. Laminin2 interacts with the phenolic glycolipid-1 (PGL-1) of the bacterium. This disrupts the interaction of laminin with dystroglycan, and in myelinating Schwann cells result in rapid demyelination and dedifferentiation, while in non-myelinating Schwann cells bacteria are taken up (Brophy, 2002; Sherman et al., 2001; Rambukkana et al., 1997; 1998; 2002). The binding of PGL-1 to myelinating Schwann cells is sufficient to induce their de-differentiation, probably following disruption of the dystroglycan-DRP-2 complex. The de-differentiation makes myelinating Schwann cells similar to non-myelinating ones and thus more susceptible to bacterial invasion (Rambukkana et al., 2002; Brophy et al., 2002).
The role of non-myelinating Schwann cells

As the name suggests this Schwann cell type does not make myelin and does not express high levels of the myelin genes. Rather non-myelin forming Schwann cells support bundles of up to 20 smaller diameter axons (less than 1μm), in membranous channels that may or may not completely enclose each axon, allowing the axons to be isolated from one another. Unlike myelin forming Schwann cells, adjacent non-myelin forming Schwann cells overlap one another so that no part of the axonal surface is exposed to the extracellular space (Eames and Gamble 1970). Intercellular communications between non-myelin forming Schwann cells are facilitated by gap junctions, small ionic pores located at the interphase of two cells. This suggests that non-myelin forming Schwann cells act as a continuous chain of support cells (Konishi, 1990), an idea supported by the fact that unmyelinated fibres are surrounded by a continuous basal lamina.

Non-myelinating Schwann cell express markers associated with developing Schwann cells such as the low affinity neurotrophin receptor, p75 (P75NTR), the adhesion molecules neural cell adhesion molecule (N-CAM) and L1 and a distinct set of cytoskeletal proteins, including glial fibrillary acid protein (GFAP). All of these molecules are expressed in all Schwann cell during development and subsequently down-regulated in myelin forming Schwann cells. P75 a 75 kD cell surface associated glycoprotein that is able to bind the neurotrophins, including NGF and in adult sciatic nerve is present only in non-myelinating cells (Hosang and Shooter, 1985, Chao et al., 1986; Jessen et al., 1980).

Both N-CAM and L1 are members of the large Ig superfamily, with Ig-like domains in combination with fibronectin III-like domains, facilitating homophilic binding
LI and N-CAM have been shown to promote neurite outgrowth in vitro, L1 being the most potent, suggesting an important function in axon growth both in development and following injury (reviewed Schachner, 1990).

In the mouse a small amount of N-CAM expression remains in the periaxonal space (Martini and Schachner, 1986) while in the rat N-CAM is not detectable on myelin forming Schwann cells (Jessen et al., 1987a, Mirsky et al., 1986).

L1 is a large, 200 kD, cell surface adhesion molecule expressed on mature non-myelin forming Schwann cells and their axons (Rathjen and Schachner, 1984, Martini and Schachner, 1986). Precisely, a shorter form of L1, L1cs, that has a four amminoacids deletion in the cytoplasmic domain, is expressed by Schwann cells, while the complete L1 is expressed only by neurons (Uyemura, 1993). In vitro assays have demonstrated that addition of L1 antibodies prevent myelination suggesting that L1 binding is required for the process of myelination and more specifically the establishment of adhesion between axon and Schwann cell (Selheimer et al., 1989, Wood et al., 1990).

L1 deficient mice have been generated and they display normal development of non-myelin forming Schwann cells and associated unmyelinated axons within the sciatic nerve (Haney et al., 1999). However, in the mature adult sciatic nerve many unmyelinated axons were either partially surrounded or completely bereft of Schwann cell processes and these Schwann cells showed a discontinuous basal lamina. A degree of degeneration was also seen, due to a sensory deficit caused by a sensory axon loss in L1 deficient mice (Haney et al., 1999).
Myelin lipids

Around 70.5% of myelin is composed of lipids. The ratios of sphingolipids:glycerolphospholipids:sterols are 28:44:28 in the myelin sheath (Norton and Cammer, 1984; Morell et al., 1994; Taylor et al., 2004). Although there are no myelin specific lipids, myelin is particularly enriched in certain lipids, such as galactocerebroside (GalC) and cholesterol. However, a mice deficient in UDP-galactose:ceramide galactosyltransferase, an enzyme necessary for GalC byosynthesis has surprisingly normal myelin in the PNS (Coetzee et al., 1996a; 1996b, 1998). However, during development a significant number of axons loose contact and pull away form their myelin sheath (Dupree et al., 1998). On the other hand, a mouse deficient in cholesterol has severe hypomyelination (Saher et al., 2002).

It is now clear that myelin can no longer be seen as a inert insulating material that simply assists fast nerve conduction. Rather, myelin is part of a complicated network between the environment, the Schwann cell and the axon. Lipids play a crucial role in this event. For example phospholipids, such as diacylglycerol (DAG) has a function of signal transduction and, in particular in the CNS, various gangliosides may have a role in the maintenance of myelin stability and in the control of nerve regeneration (reviewed in Taylor et al., 2004).

Recently it has been proposed that both in the CNS and the PNS glycosphingolipids and cholesterol can form specialised microdomains, called lipid rafts, with which proteins may or may not interact (Simons and Ikonen, 1997; Simons and Toomre, 2000). In the CNS, MBP and PLP have been shown to associate with these microdomains, while in the PNS, P0, PMP22 and plasmolipin interact with the rafts (Hasse et al., 2002). Although the raft hypothesis is still subjected to strong criticisms, possible functions for
these structure have been proposed, including protein trafficking and signal transduction (reviewed in Taylor et al., 2004).

Late transcription factors

Myelin formation represents a huge task for the Schwann cell. The transcription, synthesis and targeting to the right site of all the membrane components (proteins and lipids) requires a finely tuned cascade of events. Microtubules, consisting of heterodimers of α and β tubulin (Dustin et al., 1984), play a crucial role in this process, by organizing organelle distribution and by providing the substrate for the translocation of myelin constituents along the length of the internode (reviewed in Trapp et al., 2004). Recently, neuregulin-1 type III has been indicated as the axonal signal implicated in controlling myelin thickness and the wraps number (Michailov et al., 2004).

One field in which major progress has been made is the analysis of transcriptional control. In particular, two transcription factors, Oct-6 and Krox-20 play crucial roles in myelination (Monuki et al., 1990; Bermingham et al., 1996; Jaegle et al., 1996; Topilko et al., 1994; Murphy et al., 1996; Zorick et al., 1999; Parkinson et al., 2004; for a review see Topilko and Mejier, 2001).

Oct-6

Oct-6 (SCIP/ Tst-1) is a member of the POU domain transcription factor family. The name POU derives from the discovery of these proteins in three different systems, Pit-1, Oct-6 and Unc (reviewed by Latchman, 1999). This protein family is characterized by a POU binding domain that can be subdivided into a POU-specific and a POU
homeodomain. POU proteins bind the consensus sequence 5'-ATGCAAAT-3' with the POU-specific domain interacting with the initial 4 bases and the POU homeodomain interacting with the last four bases (Klemm et al., 1994). Oct-6 was isolated from three different systems simultaneously: embryonic stem cells of the central nervous system, sciatic nerve, and testes (Suzuki et al., 1990; Monuki et al., 1989; He et al., 1989; Meijer et al., 1990). Oct-6 mRNA and protein have been detected in Schwann cell precursors and persist at least until postnatal day 12 (P12) (Blanchard et al., 1996). Its expression reaches a peak around birth, prior to maximal myelin gene expression and is then progressively down-regulated (Monuki et al., 1990, Scherer et al., 1994, Arroyo et al., 1998). Oct-6 expression in vivo requires axon-Schwann cell contact, while, in vitro, elevation of intracellular cAMP induces Oct-6 (Scherer et al., 1994; Monuki et al., 1989). In Oct-6 deficient mice Schwann cells form typical 1:1 ensheathment relationships with axons but in the majority of them myelination is severely delayed (Bermingham et al., 1996, Jaegle et al., 1996). In this mutant line the mRNA levels of Krox-20 (see below) and of other myelin genes such as P0 and MAG fail to be elevated at the appropriate time (Jaegle et al., 1996). However, after about two weeks, Schwann cells of Oct-6 deficient mice start to myelinate and express high levels of Krox-20 and myelin genes. There is now evidence that this is due to the action of a second transcription factor, Brn-2, which is closely related to Oct-6 (Jaegle et al., 2003). The Brn-2 expression pattern is very similar to that of Oct-6, but Brn-2 deficient mice do not show any abnormality in myelination. However, in nerves lacking both Oct-6 and Brn-2 myelination is even more delayed than in Oct-6 null mice alone. Moreover, overexpression of Brn-2 can partially rescue the Oct-6 phenotype (Jaegle et al., 2003; Ghazvini et al., 2002; Sim et al., 2002). Intriguingly, even in Oct-6/Brn-2 double mutants myelination eventually occurs,
although whether this is due to the intervention of other transcription factors or to different mechanisms is not yet clear.

Analysis of the Oct-6 promoter has demonstrated the existence of Schwann cell specific enhancer (SCE) elements, which are probably regulated by axonally-induced transcription factors. One of the SCE, situated 12kb downstream of the transcription initiation site, is sufficient to correctly drive Oct-6 expression, both during development and regeneration (Mandemakers et al., 2000; Ghazvini et al., 2002).

**Krox-20**

The gene Krox-20 (Egr-2/NGF-IB) takes its name from *krüppel* box and encodes for a zinc finger transcription factor characterized by three zinc finger motifs. It was isolated from both PC12 and 3T3 fibroblast cell lines following induction by nerve growth factor (NGF) (Hazel et al., 1988; Ryseck et al., 1989; Milbrandt, 1988). Krox-20 is expressed in a number of different tissues, including heart, muscle, thymus, testes, spleen, lung and both CNS and PNS (Chavrier et al., 1988; Watson and Millbrandt, 1990). Expression has been described in the CNS, e.g. in the neocortex, striatum, hypothalamus, amygdala and olfactory bulb (Bhat et al., 1992; Mack et al., 1992; Herdegen et al., 1993).

In the CNS, during embryonic development, Krox-20 is a fundamental component of hindbrain segmentation (Wilkinson et al., 1989). It is expressed in rhombomeres 3 and 5 and in Krox-20 deficient embryos these rhombomeres are either ablated entirely or only partially formed (Schneider-Manoury et al., 1993). Furthermore, Krox-20 has been shown to directly regulate two homeobox genes, Hoxb-2 and Hoxa-2 in rhombomeres 3 and 5 via cis-activation. Transfection experiments demonstrate that Krox-20 can induce ectopic expression of Hox genes acting via conserved Krox-20
binding sites found in the enhancer elements of these genes (Sham et al., 1993; Nonchev et al., 1996b; Seitanidou et al., 1997).

In the PNS Krox-20 is crucial for the development of myelinating Schwann cells (Topilko et al., 1994; reviewed in Topilko and Meijer, 2001). In those cells that have been signaled to myelinate, Krox-20 starts being expressed around E16 (in mouse), approximately 1-2 days after Oct-6 (Blanchard et al., 1996; Topilko et al., 1994; Zorik et al., 1996, 1999). This pattern of expression co-ordinates with the appearance of Schwann cell markers, such as S100, and is dependent on continuous axonal contact. In fact, after nerve transection, Krox-20 levels fall sharply, and Krox-20 is re-expressed in regenerating nerve (Murphy et al., 1996; Zorik et al., 1999). In Krox-20 deficient mice myelination does not occur. In these mice Schwann achieve the 1:1 relationships with axons, and continue to wrap the axon about 1-2 turns before becoming stalled at this point (Topilko et al., 1994). In Krox-20 null mice, markers of myelination, such as the myelin proteins P0 and MBP, are present at very low levels, suggesting that Krox-20 is required for and controls transcription of the genes required for myelination (Topilko et al., 1994). Moreover, in these mice, Oct-6 is expressed on time and remains expressed at a higher level than normal after birth, suggesting that Krox-20 could negatively regulate Oct-6. Schwann cells in Krox-20 null mice also have a higher DNA synthesis and death rate, indicating a possible role for Krox-20 in controlling these events (Ghazvini et al., 2002; Zorik et al., 1999; reviewed in Topilko and Meijer, 2001). This data supports the theory that Schwann cells are stalled at a particular stage in development, most likely to be the promyelinating phase, and continue to cycle as they would in the late embryonic/newborn animal well into the second postnatal week.

The hypothesis that Krox-20 is directly involved in regulating myelin genes has been reinforced by recent gene expression profiling of cultured Schwann cells in which
Krox20 expression has been adenovirally enforced (Nagarajan et al., 2001). The authors showed that under these conditions, myelin-related genes and lipid genes, as well as a series of other genes, are up-regulated by Krox-20 (see chapter 3 for further comments). Further work carried on in our laboratory, has demonstrated that enforced expression of Krox-20 alone is sufficient to determine a range of phenotypic changes in cultured Schwann cells, associated with the transition from proliferating immature Schwann cells to quiescent myelinating ones (Parkinson et al., 2002a,b; 2003a,b). In fact, these experiments show that in addition to the up-regulation of myelin-related genes such as P0 and periaxin, and the down-regulation of L1, neuregulin-1 induced proliferation and TGFβ induced death are prevented, similarly to what happens at the beginning of myelination in vivo. There is now evidence that at the basis of these changes is the ability of Krox-20 to inhibit the JNK/c-Jun pathway. This pathway is necessary for blocking myelination and to promote Schwann cell proliferation and death, meaning that, by preventing the action of this pathway, Krox-20 exerts direct control on Schwann cell proliferation, death and myelination (Parkinson et al., 2003a). Most interesting, Krox-20 is also able to induce the expression of myelin specific genes in NIH 3T3 cells, which are completely unrelated to Schwann cells. This ability points to Krox-20 as a master regulatory gene (Parkinson et al., 2004; reviewed in Jessen and Mirsky, 2004).

The mechanisms by which Krox-20 is regulated by the axon are not yet clear. As for Oct-6 (above), specific Schwann cell enhancers (SCE) have been identified in the Krox-20 regulatory region. In particular, the Krox-20 promoter contains two elements: an immature Schwann cell element (ISE) and a myelinating Schwann cell element (MSE). The ISE is active only in pre-myelinating cells, while the MSE is active from E18 onwards, in myelinating cells. Both enhancers are up-regulated in regenerating nerves.
(Ghislain et al., 2002). Interestingly, the MSE appears to be dependent on Oct-6 for activation, and contains multiple Oct-6 binding sites (Ghislain et al., 2002).

As we have seen previously, Sox-10 can function synergistically with Oct-6 in modulating Pax-3 positively and Krox-20 negatively (Kuhlbrodt et al., 1998). In turn, once activated, Krox-20 probably down-regulates Oct-6.

**Other transcription factors identified in Schwann cells**

In adult PNS, Krox-20 is expressed only in myelinating Schwann cells. This pattern contrasts with that for another closely related transcription factor, Krox-24, which binds to some of the sequences recognised by Krox-20 too (Topilko et al., 1997). Krox-24 is highly expressed early in development, remains expressed in non-myelinating Schwann cells of the adult and is up-regulated during Wallerian degeneration (Topilko et al., 1997). In Krox-24 null mice myelination occurs normally, but increased death rates are observed after nerve transection of neonatal nerves (Topilko et al., 1998; Harris, PhD thesis, University of London, 2001).

The expression of Krox-24 is regulated in Schwann cells by the B class helix-loop-helix (HLH) factor Mash2 (Kury et al., 2002). Mash2 has been identified in adult Krox-20 positive Schwann cells, is down-regulated after nerve transection and inhibits Schwann cell proliferation *in vitro* (Kury et al., 2002). Moreover, all the four HLH Id genes are expressed during Schwann cell development. In particular, Id1 and Id3 are present in the embryo, then downregulated at P10 (around the peak of myelination) and up-regulated again in the adult. In addition, both are up-regulated after nerve transection and inhibit myelin gene expression (Stewart et al., 1997; Thatikunta et al., 1999).
Finally, in coculture of neurons and Schwann cells in vitro, the transcription factor NF-kB is expressed before Oct-6, and its inhibition prevents myelination, indicating that NF-kB may have a role in myelination (Nickols et al., 2003).

**Wallerian degeneration**

One of the most striking characteristics of Schwann cells is their plasticity. If axonal contact is lost, due to injury or cell dissociation, the Schwann cell in the distal stump of the nerve start to de-differentiate, proliferate and down-regulate all the myelin genes (see also chapter 3). At the same time adhesion molecules, neurotrophins, cytokines and their receptors are up-regulated to create an environment that allows axonal re-growth. All the events taking place distal to the site of nerve injury are known as Wallerian degeneration. (reviewed in Stoll and Muller, 1999; Scherer and Salzer, 2001). In the first week after axotomy, distal axons fragment and disappear, and myelin breaks down into so-called ovoids. Myelin debris is rapidly cleared by macrophages and Schwann cell themselves, probably to facilitate axonal re-growth, since it has been shown that myelin inhibits axonal regeneration (reviewed in Filbin et al., 2001). While myelin degenerates, the basal lamina is maintained by Schwann cells, which organise themselves in columns called bands of Bungner. It is still unknown whether Wallerian regeneration begins with a positive signal resulting form axonal degeneration or it is due to the interruption of the supply of neuronal factors that maintain the myelinating phenotype. The slow Wallerian degeneration mouse (Wld(S)) (formerly known as Ola) is a natural mutant in which myelinated axons remain phsiologically active for weeks after axotomy. Schwann cell proliferation, down-regulation of myelin genes, up-regulation of NGF, tenascin and p75NGFR, as well as macrophages infiltration are also
delayed (Lunn et al., 1989; Thomson et al., 1991; Fruttiger et al., 1995). The genetic mutation is a 85Kb tandem triplication on mouse chromosome 4. This results in the formation of a chimeric protein formed via the fusion of the N-terminus of the multi-ubiquitination factor Ube4b and a novel gene, D4Cole1e/Nmat, a NAD synthesising enzyme (Lyon et al., 1993; Conforti et al., 2000; Mack et al., 2001). A third gene lies in the area of the triplication, the retinoic binding protein Rbp7, and is therefore overexpressed in the Wld(S) mice. However, northern blot experiments failed to detect the mRNA for Rbp7 in the nervous system and is therefore not a likely candidate for Wld(S) (Conforti et al., 2000). Although it is known that ubiquitination plays an important role in axonal degeneration, the mechanism by which the fusion gene Ube4b/Nmat protects the Wld(S) gene from degeneration are still unclear.

**Axonal regeneration**

Degenerating peripheral nerve represents the best substrate to support axonal regeneration (Ide et al., 1990; Dahlin, 1995; reviewed in Scherer and Salzer, 2001). Axonal regeneration is not effective in normal untransected nerve stumps or in distal nerve stumps of Wld(S) mice (Brown et al., 1991). Two factors seem to play a key role in regeneration: the degradation of myelin in lesioned nerves and the presence of Schwann cells. Axon can regenerate partially even in the absence of Schwann cells, when only the extracellular matrix is present, but regenerates much better in nerves that contain Schwann cells (Sketely et al., 1989). Schwann cells in fact maintain their basal laminae, which form the bands of Bungner, through which re-growing axons can travel (Ramon y Cajal, 1928; Bunge et al., 1987; reviewed in Fu and Gordon, 1997). In vitro studies have been performed by culturing neurons on cryo-sections of nerve. In these assays, degenerated nerve promotes neurite out-growth much better than normal nerve,
and the presence of Schwanns cell is a crucial factor (Bedi et al., 1992; Agius and Chochard; 1998). The changes occurring to Schwann cells after nerve axotomy are discussed further in chapter 3.

**Schwann cells control neuronal survival and perineurium formation**

As we have seen, Schwann cells rely heavily on axonal signals for their survival, in particular during development. However, it is now becoming clear that they are not just passive targets of extrinsic signals, but that Schwann cell are a source of signals which are crucial for peripheral nerve development and neuron survival. In ErbB3 -/- and ErbB2-/- mice with a targeted rescue of the heart defect (above), Schwann cell are severely reduced in number (Riethmacher et al., 1997; Woldeyesus et al., 1999; reviewed in Mirsky and Jessen, 2001; Mirsky et al., 2002). Surprisingly, in these mice most of DRG neurons were missing by E14, while motor neurons died in the trunk limb region slightly later, being reduced by ~80% at E18. Since at E14 most axons have not yet reached their target, the most likely explanation for death of DRG neurons is the lack of survival signals from Schwann cell precursors. Motor neuron death is instead likely to depend on the lack of signals from immature Schwann cells. This idea as been strengthened by the observation that Sox-10-/- mice, where neither satellite cells nor Schwann cell precursors are generated, have severely reduced numbers of DRG neurons and motor neurons (Britsh et al., 2001). The nature of the glial-derived neuronal survival signals has not been identified yet, but neurotrophin-3 (NT-3) and glial-derived neurotrophic factor are possible candidates.

In addition to promoting neuronal survival, Schwann cells also control Na+ channels clustering at the node of Ranvier, and can influence axonal calibre and neurofilament
phosphorylation in myelinating axons (reviewed in Mirsky et al., 2002; Mirsky and Jessen, 2001).

Schwann cell and Schwann cell precursors, besides signalling to neurons, are also involved in the formation of the perineurial sheath that surrounds and protects peripheral nerve (Bunge et al., 1989; reviewed in Mirsky and Jessen; 2001). Work in our lab has shown that in desert hedgehog (Dhh) null mice, the perineurium in much thinner than in normal nerves and perineurial cells have a patchy basal lamina. Moreover, the epineurial collagen sheath is often disrupted and there is infiltration of perineurial cells in the endoneurium, indicating that the nerve-tissue barrier is compromised (Parmantier et al., 1999).

Taken together these observations indicate that in peripheral nerve Schwann cells are involved in the regulation of three main cell types: neurons, connective tissue cells and the Schwann cells themselves.

AIMS

As we have seen, there is much left to learn about the complex mechanisms taking place during the transition from Schwann precursors to immature Schwann cells and in the myelination of peripheral nerve. To try to add valuable information to the knowledge of these events, and to identify genes of potential functional importance that have not been previously implicated in Schwann cell development, we have performed, using Affymetrix GeneChip Technology, gene expression profiling on mRNA extracted from rat sciatic nerves at different embryonic stages, during myelination and after nerve injury. DNA microarrays and their possible applications are discussed in detail in chapter 3. In chapter 4 I will analyse the expression and localisation of a series of genes that have been identified through the gene profiling as possibly involved in Schwann
cell differentiation and myelination. Finally, in chapter 5 I will discuss the phenotype of transgenic mice in which the TGFβ receptor type II has been ablated specifically in Schwann cells thanks to a P0 CRE transgene and in which, therefore, TGFβ signalling is not active in Schwann cells.
FIGURE 1.1. Differentiation markers in embryonic Schwann cell development. In grey are highlighted those markers shared by neural crest cells, Schwann cell precursors and immature Schwann cells. In blue, those markers present on migrating neural crest, on Schwann cell precursors but not in immature Schwann cells. In green are highlighted markers present on Schwann cell precursors and immature Schwann cells, but absent from the migrating neural crest. (from Jessen and Mirsky, 2004).
AP-2α
N-cad*
S100
GFAP
Oct-6**
Q4**
B-FABP
Po
Dhh
CD9*
GAP-43*
PMP22*
PLP*
Sox10
ErbB3
P75
L1
AP-2α
N-cad*
Sox10
ErbB3
P75
L1
B-FABP
Po
Dhh
CD9*
GAP-43*
PMP22*
PLP*
Neural
crest
Associate with ECM
NRG survival is ECM dependent
Schwann-cell Precursors
Survival response to FGF+IGF, ET+IGF, PDGF+NT3+IGF
Survival response to FGF+IGF, ET+IGF, PDGF+NT3+IGF
Autocrine
Basal lamina
Immature
Schwann cells
Associate with axons
NRG survival is ECM independent
Survival response to FGF+IGF, ET+IGF, PDGF+NT3+IGF
Survival response to FGF+IGF, ET+IGF, PDGF+NT3+IGF
CHAPTER 2

MATERIALS AND METHODS

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, TRlzol reagent and agarose were from Invitrogen (Invitrogen Ltd, Paisley, UK). EDTA disodium salt, ethidium bromide, sodium chloride, sodium citrate, tween20, phenol:chloroform:isoamyl alchohol 25:24:1, chloroform:isoamyl alchohol 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) was from Fluka Chemicals Ltd. (Buchs, Switzerland).

Reagents for tissue culture

Transferrin, selenium, putrescine, triiodothyronine (T3), thyroxine (T4), progesterone, insulin (10^{-3}M), bovine serum albumin (BSA), cytosine arabinoside (Ara C), poly-D-lysine, poly-L-ornithine, lysine, dibutyryl-cAMP (dbcAMP - 1 mM) and laminin were obtained from Sigma (Poole, UK). Collagenase was obtained from Worthington (Lorne...
Laboratories, Reading, UK). 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), NDF-β and TGFβ-1 were from R&D Systems (Oxford, UK) and forskolin was from Calbiochem (CA, USA).

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).

Reagents for histology and Electron Microscopy

Glutaraldehyde, OCT compound (Tissue Tek), araldite CY212, DDSA, BDMA, dibutryl pthalate and rubber coffin moulds were from Agar Scientific(UK). DPX mountant and 13mm coverslips were from Merck (Poole, UK), and Superfrost Plus microscope slides were from BDH.

Animals

Rats

Sprague-Dawley rats, maintained in the Main Biological Unit at UCL, were used. Embryos from ages E11-18 were removed from pregnant females; the day of the vaginal plug was taken as day 0.
Transgenic mice

TGFβRIIf/f mice (129sv/BALB/c) were from Dr. Jurgen Roes (UCL, London, UK). P0 CRE mice (FVB) were from Dr. Lawrence Wrabetz (San Raffaele Scientific Institute, DIBIT, Milan, Italy). Synuclein null mice (C57BL/6J inbred strain from Harlan UK) were from Dr. Ralf Shoepfer (UCL, London, UK).

Generation of P0 CRE/ TGFβRIIf/f mice

TGFβRIIf/f mice were initially crossed with P0 CRE mice, to generate P0 CRE/ TGFβRIIf/+ mice (1/2 of the offspring). These TGFβRIIf/+ mice, called N1, were then back-crossed with TGFβRIIf/f mice to obtain the P0 CRE/ TGFβRIIf/f genotype (1/4 of the offspring). The littermates (TGFβRIIf/f, TGFβRIIf/+ P0 CRE/ TGFβRIIf/f) were used as controls. The genotypes were determined by PCR on genomic DNA extracted from tail samples.

Genomic DNA extraction and genotyping

Tail samples were placed in 500 µl of SNET (10 mM Tris/Hcl pH 8.0, 5 mM EDTA, 0.25M NaCl, 1% SDS) plus 5 µl of 20mg/ml proteinase K, and incubated overnight at 55°C in a waterbath. The day after the DNA was phenol:chloroform extracted, ethanol precipitated and resuspended in 100 µl TE, pH 8.0. 1µl of DNA solution was used for PCR amplification in a standard PCR mix (see below, in the paragraph entitled “semi-quantitative RT-PCR”). The conditions for the P0 CRE PCR where: 94°C for 4 minutes followed by 94°C 30 seconds, 50°C 1 minute, 72°C 1 minute, for 30 cycles. Primers for P0 CRE amplification were as follows: P0 CRE sense 5’-GCTGGGCCCAAAATGTGCTGGG-3’; P0 CRE AS2 5’-CCACCACTCTCCATTGCAC-3’. In the presence of the CRE transgene a single band of ~ 480bp resulted from the amplification
reaction. For the TGFβRII floxed PCR the conditions were: 94°C 2 minutes, followed by 94°C 30 seconds, 63°C 1 minute, 72°C 2 minutes, for 30 cycles. Primers for TGFβRII floxed PCR were as follows: TβRII sense 5’-CATGAAGTCTGCGTGGCCGTGTG-3’; TβRII AS 5’-TGTAATCGTTGCACACTCT-TCCATGT-3’. In case of TGFβRII f/f mice, a single band of 1.7kb resulted from the amplification reaction. In case of TGFβRII f/c mice, two bands of 1.7 (floxed) and 1.5kb (wt) were amplified.

**Southern blotting**

To establish the percentage of recombination we performed Southern blotting on DNA extracted from tails (control) and purified Schwann cells from P0CRE/TGFβRII f/f and P0CRE/TGFβRII f/+ mice. 10 μg of DNA were digested with the restriction enzyme NcoI, at 37°C, overnight. The day after the digested DNA was separated by 1% agarose gel electrophoresis. The DNA was then transferred to a nylon membrane (Hybond-N+) by upward capillary transfer (Southern, 1975; Sambrook and Russell, 2001). Briefly, after electrophoresis the gel was soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45min and then neutralized in re-naturing solution (1M Tris, 1.5M NaCl) for 30min. After neutralization the DNA was transferred to the membrane with capillary transfer for 16-18 hours; the transfer buffer was 20xSSC. After the transfer the DNA was crosslinked to the membrane with a Stratalinker (Stratagene).

**Probe preparation**

TGFβRII exon II probe (Cazac and Roes, 2000), was radiolabelled with high specific activity (> 10⁹ cpm/μg) α³²P-dCTP (Amersham Biosciences), using Nick Translation Kit (Amersham Biosciences) following the manufacturer’s instructions. The probe was
then run onto a TE pre-equilibrated Microspin S-200 HR column (Amersham Pharmacia Biotech, Inc.).

Hybridisation and washing

The membrane was inserted in a glass tube and equilibrated in Hybridisation Solution (Research Genetics, USA) for 2 hours at 42°C in a rotating oven. The exon II probe was added and the tube left to rotate for at least 18 hours. The membrane was then removed and submerged in a tray containing 500ml of 2x SSC, 0.1% SDS and left with gentle agitation in a water-bath at 42°C for 30min. The rinse solution was then substituted with 500ml of fresh 2x SSC, 0.1% SDS and the tray incubated at 50°C for 30min, with agitation. Finally, the membrane was rinsed in 0.2x SSC, 0.1% SDS for 30min at 65°C for 25min.

Autoradiography

After washing, the membrane was wrapped in Saran Wrap and placed in an autoradiography cassette and exposed to a Biomax X-ray film (Kodak), for 24 hours. The film was then developed with a X-Ograph Compact X2 automatic developer. The percentage of recombination was calculated with a program called MCD Basic.

Peripheral Nerve Injury Experiments

All the experiments where animals were involved were performed following the UK Home Office guidelines.

Sciatic nerve transection

Seven days old (P7) Wistar rats and adult transgenic mice were anaesthetised with halothane, and the right sciatic nerve was exposed at mid-thigh level. The sciatic nerve was then cut and the distal end diverted to limit the possibility of re-ligation. Resulting
wounds were sutured with 3/0 black polyamide monofilament, mersilk (Johnson&Johnson, USA). Two, five or seven days following transection, the animals were culled and both the distal stump of the right, transected sciatic nerve and the contralateral control nerve were excised, frozen and immediately processed for RNA extraction.

RNA isolation

Total RNA was isolated from rat sciatic nerves at different time points using TRIzol reagent (Invitrogen Ltd, Paisley, UK), following the manufacturer's instruction. The RNA was then quantified using a spectrophotometer and analysed for integrity on a 1% agarose gel under denaturing conditions. When the RNA was used for the preparation of targets for microarray analysis a supplemental clean-up step using the Quiagen RNeasy isolation kit (QUIAGEN Ltd, UK) was performed.

Target preparation for microarrays analysis

All the procedures in the following paragraphs were performed as indicated in the Affymetrix Expression Analysis Technical Manual (Affymetrix Inc., MA, USA). Note that "probes" are the oligonucleotides synthesised on the surface of the arrays and that "target" is the biotin labelled cRNA that is being interrogated.

First strand cDNA synthesis

Total RNA (5-40μg) was resuspended in a total of $x$ μl DEPC-treated water, where $x = 20 - (8+z^*)$ (i.e. final reaction volume 20 μl). 1μl of T7-(dT)24 primer (100pM/μl) was added and the mix is incubated for 10min at 70°C. 4μl of 5x first strand cDNA
buffer, 2μl of 0.1 M DTT, 1μl of 10mM dNTPs mix were added and the mix incubated at 42°C for 2min. z μl of SSII RT were added and the reaction incubated at 42° for 1 hour.

The volume z* depended on how much RNA there was in the initial step:

For 5-8μg RNA use 1μl RT
For 8.1-16μg RNA use 2μl RT
For 16.1-24μg RNA use 3μl RT
For 24.1-32μg RNA use 4μl RT
For 32.1-40μg RNA use 5μl RT

Second strand cDNA synthesis

The first strand reaction was placed on ice and 91 μl DEPC-treated water, 30μl 5x second strand buffer, 3μl of 10mM dNTPs mix, 1μl of 10 U/μl DNA ligase, 4μl of 10U/μl DNA Polymerase I, 1μl of 2U/μl Rnase H were added. After incubation at 16°C for 2 hours 2μl of 5U/μl T4 DNA Polymerase were added and the mix re-incubated at 16°C for 10min. The reaction was inactivated with 10μl 0.5M EDTA. The double stranded cDNA was then cleaned with a phenol:chloroform extraction, ethanol precipitated and resuspended in 12μl DEPC-treated water.

Synthesis of biotin-labelled cRNA (in vitro transcription, IVT)

This reaction was performed using the HighYield RNA Transcript Labeling Kit (Enzo BioArray, NY, USA) following the manufacturer's instructions. 6μl of the double stranded cDNA (corresponding to 12-15μg of total RNA) were reverse transcribed and
labelled in a reaction containing 16μl RNase free water, 4μl of 10x hybridisation reaction buffer, 4μl of 10x biotin labelled riboligos, 4μl of 0.1M DTT, 4μl of RNase inhibitor mix and 2μl of 20x T7 RNA polymerase. The reaction was incubated for 4 hours at 37°C and the resulting cRNA was cleaned with Quiagen RNeasy kit. The cleaned biotin labelled cRNA was then fragmented with 5x fragmentation buffer (200mM Tris-acetate pH 8.1, 500mM KOAc, 150mM MgOAc) for 35min at 94°C. The yield and size distribution of labelled transcript was estimated with a gel electrophoresis through a 1% agarose gel.

Target hybridisation

The fragmented cRNA (x μl, to have 15μg) is mixed with 5μl of 3nM control oligonucleotide B2, 15μl of 20x eukaryotic hybridisation controls, 3μl of 10mg/ml herring sperm DNA, 3μl of 50mg/ml acetylated BSA, 150μl of 2x hybridisation buffer and water to a final volume of 300μl. This hybridisation cocktail is heated for 5min at 99°C while the probe arrays is equilibrated with 200μl of 1x hybridisation buffer. The cocktail is then transferred at 45°C for 5min and, following the removal of the buffer from the array, is hybridised to the probes. The hybridisation is carried on in a rotisserie box in a 45°C oven for 16 hours.

Washing, Staining and Scanning the Probe Arrays

Washing and staining

The GeneChip Fluidics Station 400 (Affymetrix, MA, USA) was used to wash and stain
the probe arrays. It is operated using GeneChip software. The *Antibody Amplification Washing and Staining Protocol* (EukGE-SW2) was used.

After the 16 hours the hybridisation cocktail was removed from the probe array and replaced with the appropriate volume of Non-Stringent Wash Buffer.

**Table 2.1: fluidics protocol**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post Hyb Wash 1</td>
<td>10 cycles of 2 mixes/cycles with Wash Buffer A at 25°C</td>
</tr>
<tr>
<td>Post Hyb Wash 2</td>
<td>4 cycles of 15 mixes/cycles with Wash Buffer B at 50°C</td>
</tr>
<tr>
<td>Stain</td>
<td>Stain the probe array for 10min in SAPE solution at 25°C</td>
</tr>
<tr>
<td>Post Stain wash 1</td>
<td>10 cycles of 4 mixes/cycles with Wash Buffer A at 25°C</td>
</tr>
<tr>
<td>2nd Stain</td>
<td>Stain the probe array for 10 min in antibody solution at 25°C</td>
</tr>
<tr>
<td>3rd Stain</td>
<td>Stain the probe array for 10 min in SAPE solution at 25°C</td>
</tr>
<tr>
<td>Final Wash</td>
<td>15 cycles of 4 mixes/cycles with wash Buffer A at 30°C. The holding temp. is 25°C</td>
</tr>
</tbody>
</table>

- Wash Buffer A = non-stringent wash buffer
- Wash Buffer B = stringent wash buffer
- SAPE = Streptavidin Phycoerythrin

**Scanning**

After the completion of the hybridisation procedure, the chips were scanned with an Agilent scanner. This scanner uses an argon-ion laser, and is also controlled by the GeneChip software. It performs a 2x Image scan at a wavelength of 570nm.
Data Analysis

Analysis with Affymetrix Suite 5.0

The first data analysis was performed with GeneChip Expression Analysis Software, Affymetrix Suite 5.0. The software applies a series of computational techniques (algorithms) to analyse the data generated from the Expression Analysis probe arrays. The algorithms are used in two types of analysis: an *Absolute Analysis*, used to determine whether transcripts represented on the probe array are detected or not within one sample and a *Comparison Analysis*, used to determine the relative change in abundance for each transcript between a baseline and an experimental sample. This second analysis employs *Normalisation or Scaling* techniques to minimise differences in overall signal intensities between the two arrays allowing for more reliable detection of biologically relevant changes in the sample. A first Absolute Analysis is used as the source of Baseline data and a second probe array experiment as the source of experimental data to be compared to the Baseline. Some of the Comparison Analysis metrics are used in a decision matrix to derive a Signal Log ratio, which indicates whether a transcript has increased (I), decreased (D), marginally increased (MI), marginally decreased (MD) or exhibits no change (NC) in expression level.

Analysis with GeneSpring

After the experiments have been validated with the Affymetrix Suite 5.0, the data from all the 14 experiments were loaded onto Genespring software (Silicon Genetics, USA) for statistical analysis. Only chips with acceptable data distributions (assessed using Genespring), 5'3' ratios, % call, spike-in sensitivity, background and noise levels were included in the analysis. Data were normalised to the median expression level within,
and per gene across arrays. Genes that fell below the level of confident detection (p > 0.06 in MAS5) in two or more of the triplicate samples were excluded from the analysis. We next excluded genes with less than a two-fold change in expression level (signal log2 ratio > 1), either during development from E14 to P12 or after nerve injury. 1609 genes/ESTs that passed this first restriction were considered for downstream analysis. The following step was a restriction by statistical group comparison, using the ANOVA/Welch's approximate t-test (p < 0.05), not considering variances equal, between the time points E14 and E18, E18 and P12, P12 and P12cut. The probes that were significantly changed between any two conditions were subjected to cluster analysis to identify genes with similar expression profile across multiple experiments. Both a Hierarchical clustering and a K-means clustering were performed.

**Hierarchical clustering**

In hierarchical clustering, the genes are connected iteratively based on their similarity. The genes with similar expression patterns are grouped together and are connected by a series of "branches", which is called dendrogram (or clustering tree). To make the dendrogram, GeneSpring calculates the correlation for each gene with every other gene in the set. Then it takes the highest correlation and pairs those two genes, averaging their expression profiles. GeneSpring then compares this new composite gene with all of the other unpaired genes. This is repeated until all of the genes have been paired. In our case the similarity of gene expression patterns has been calculated with GeneSpring using Pearson correlation. The Pearson correlation coefficient is often used in microarray data analysis because it is sensitive not only to the direction of change (increasing or decreasing), but also to magnitude of change.
K-means clustering

K-means clustering divides genes into groups based on their expression patterns. The goal is to produce groups of genes with a high degree of similarity within each group and a low degree of similarity between groups. K-means clusters are constructed so that the average behavior in each group is distinct from any of the other groups. For example, in a time series experiment k-means clustering can be used to identify unique classes of genes that are up-regulated or down-regulated in a time dependent manner. GeneSpring's k-means clustering algorithm divides genes into a user-defined number (k) of equal-sized groups, based on the order in the selected gene list. It then creates centroids (in expression space) at the average location of each group of genes. With each iteration genes are reassigned to the group with the closest centroid. After all of the genes have been reassigned, the location of the centroids is recalculated and the process is repeated until the maximum number of iterations has been reached. It is also possible to perform a "Test Additional Random Starting Clusters", a feature that makes clustering as tight as possible by performing clustering several times, each time starting from a different random grouping of genes, and choosing the best result. We have used this test when generating our clusters in all the comparisons, so that the number of clusters obtained is the one considered optimal by the GeneSpring software.

Data Availability

All data were saved in MAGE-ML files and combined with experimental information in MIAME compliant format using a locally designed tool (MEditor) for submission to the ArrayExpress database (EBI, Hinxton UK).
Bioinformatic Analysis

This part of my work was performed in collaboration with Dr David Michalovich and Dr Morris Paterson at Inpharmatica.

Mapping the Probes to Transcripts

Inpharmatica's Biopendium™ (http://www.inpharmatica.co.uk/biopendium.htm) was used to identify protein for each of the probes found to be differentially expressed. This probe-to-protein mapping was generated with an automated technology in which probes were first mapped to appropriate transcripts and then to the corresponding protein. In this process, the 'target' nucleotide sequence for each probe set was obtained from Affymetrix (http://www.affymetrix.com/index.affx), and aligned to the rat genome (version 3.1, http://www.hgsc.bcm.tmc.edu/projects/rat/) using a rapid alignment tool BINSEQ (unpublished results). Transcripts that overlapped with the target sequence, or that were 2kb upstream were identified using a database of genomic annotations. Assignments were made using in an order of descending confidence Refseq (http://www.ncbi.nih.gov/RefSeq/), Genbank (http://www.ncbi.nih.gov/Genbank/index.html), ENSEMBL (Birney et al., 2004) and then TWINSCAN (Korf et al., 2001). Exonic relationships were confirmed using a BLASTN search of the target nucleotide sequences against the Refseq, GENBANK, ENSEMBL, then TWINSCAN nucleotide coding sequences. Probe mappings were assigned confidence level "high", "medium" or "low" depending on how much each probe overlapped with each exon. The corresponding protein sequence was identified for each transcript by cross-referencing to protein databases. Probes that were not confidently mapped automatically were examined by hand. In cases where the rat gene had not been publicly identified an orthologous human or mouse protein was taken forward for annotation when appropriate.
Annotating the Proteins

Once an amino acid sequence had been identified for each probe set, protein functional annotation was generated using Biopendium™ and publicly available data sources. In this application, family alignments were derived from secondary databases (for example, PFAM, CDD, PRINTS) and converted to a standardised PSI-BLAST (Altschul et al., 1997) profile format. For structural classification of proteins (SCOP), a domain-delineated profile is generated by running PSI-BLAST across a database of non-redundant protein sequences. These profiles were then added into the pre-calculated Domain Professor™. Structural annotation was made using the GenomeThreader™ application within Biopendium™. Matches of only 100% confidence to a PDB (protein database) structure were displayed. In addition, to describe each protein sequence, the HUGO Gene Nomenclature Committee (HGNC) curated gene title and gene symbol was used by preference. Failing this, the description was taken from a Refseq or SWISSPROT or Genbank. Keywords were extracted from SWISSPROT (or Genbank) protein sequences that were over 95% ID and 90% length of our sequences. Gene Ontology (GO) annotation was obtained for each protein sequence using gene to GO mappings from both LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/) and GOA. OMIM identifiers were also extracted from Locus Link.

To assess the chances of the protein being secreted, SignalP (Nielsen et al., 1997) was used. If both the NN and HMM method identified a signal peptide, the protein was described as ‘highly likely’. If one method found a signal peptide, signal peptide was assigned as ‘likely’, if neither prediction method was positive the signal annotation was assigned as, ‘unlikely’. TMHMM (Krogh et al., 2001) was used to predict the number of transmembrane regions.
Primer design

Oligonucleotide primers per specific mRNA (20-25 nucleotides in length) were designed by eye using the sequence information available in the NCBI databases. They were designed in such a way to minimise hairpin structures in individual primers and dimerisation between primer pairs and were checked against databases using the BLAST software to ensure specificity. The sequence of the specific primers used in this study are detailed in Table 2.2.

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

Total RNA (200ng-1μg) was reversed-transcribed in a 20μl reaction mix containing 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM DTT, 1mM dNTPs, 1U 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°C followed by 10 min at room temperature and 1 hour at 42°C, 30μl TE pH 8.0 were added, and the cDNA aliquoted. For the analysis of ESTs expression total RNA (500ng) was reversed-transcribed in a 50μl reaction mix containing 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM DTT, 0.5mM dNTPs, 20ng random hexamers primers and 200U RNase H- Reverse Transcriptase (superscript II) as recommended in the manufacturers’ protocol (GibcoBRL Life Technologies, Paisley, UK). After incubation for 90 min at 42°C followed by 15 min at 70°C, RNA was removed by digestion with both RNase H (2 units) and RNase A (0.1μg/μl) for 30 min at 37°C.

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 MgCl₂, 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 MgCl₂ concentration, annealing temperature, extension time and cycle number.

PCR reactions were performed in a MWG Biotech Primus96 Thermal Cycler or in a Perkin Elmer Gene Amp 2400 thermocycler (the latter for genotyping only). Cycling conditions were one initial cycle of 4 min at 94°C followed by 28-35 cycles of 30 sec at 94°C, 1 min at specified annealing temperatures (see Table 2.2) and 1 min at 72°C before a final extension period of 10 min at 72°C. 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.

**Western Blotting**

Frozen tissues were pulverized, 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 5-30 µgs of protein extract) 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 molecular weight standards (Biorad) were 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 overnight at 4°C using 5% fat free milk powder in PBS/0.05% Tween 20. The following day, primary antibodies were incubated in 1% fat free milk powder in PBS/0.05% Tween 20 for 1 hour at room temperature, on a slow rotator (Gallenkamp, UK). The blots were then washed in PBS containing 0.05% Tween 20, usually 1 x 15 min followed by 4 x 5 minutes at room temperature. Subsequently, the membranes were washed and incubated with horseradish conjugated secondary antibody diluted in the same solution as the primary antibody, and developed with ECL chemioluminescent reagent (Amersham Biosciences Ltd, UK). The blots were covered in Saran Wrap film and placed in an autoradiography cassette with intensifying screens (Appligene, USA) and visualised by brief exposure to Kodak BioMax Mr-1 film which was developed from 45secs to 10 min later in an X-Ograph Compact X2 automatic developer.

Immunohistochemistry

Cryostat sections, 6-8µm thick, were collected on Superfrost Plus microscope slides (BDH) and air dried for at least 1 hour. Teased nerve preparations were obtained by dissecting out sciatic nerves into L15 medium on ice and desheathing followed by the teasing of small nerve pieces using fine needles. These were also left for at least 1 hour at room temperature, to allow the nerve fibres to dry and adhere well to the slide. Unless
stated otherwise for fresh frozen tissue, teased nerve preparations and coverslips, fixing was 10 min in 4% paraformaldehyde at room temperature followed by 3, 5 minutes washes in 1xPBS. Tissue was then blocked using antibody diluting solution, known as ADS (PBS containing 10% calf serum, 0.1% lysine and 0.02 % sodium azide), for one hour at room temperature. Antibodies were diluted in ADS and applied overnight at 4°C. The following day the slides were washed three times for 5 min each in 1xPBS and incubated in secondary fluorescent antibodies for 25 min at room temperature. In those cases were a three layer system was used, biotin was applied as second layer for 25 min at room temperature. The slides or coverslips where then washed three times for 5min each in 1xPBS. The third layer was usually streptavidin conjugated FITC or Cy3 applied for 20 min at room temperature. Samples were washed again, 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.

**Primary antibodies**

Anti protein zero (P_0) Rabbit polyclonal antibody against rat protein zero was generated and characterized in the laboratory by Louise Morgan essentially as described by Brockes et al., 1980 (Morgan et al., 1994). The antiserum was purified by S. Namini.

Fresh frozen tissue and cells were prefixed in ice cold methanol for 10 min followed by fixation in 2N HCl for 15 min, washed in PBS then further neutralized with 0.1M sodium borate solution for 10 min. The cells were then blocked for 2 hr with antibody diluting solution, washed and incubated overnight with anti-P_0. The antibody was used
at a dilution of 1:500.

**Anti protein zero (P₀)** mouse monoclonal antibody against protein zero was a gift from J. Archelos, and used at a dilution of 1:1000.

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

**Anti synuclein-1** mouse monoclonal antibody (IgG1) against α-synuclein was from BD Transduction Laboratories (UT, USA), and used at a final dilution of 1:500.

**Anti collagen type II** mouse monoclonal antibody against collagen type II (clone CIIC1), in the form of ascites, was from Developmental Studies Hybridoma Bank (University of Iowa, USA), and used at a final dilution of 1:100.

**Anti collagen type II** Rabbit polyclonal antibody against collagen type II was a gift from Dr. L. Sandell, (Washington University School of Medicine, St. Louis, USA) and used at a dilution of 1:2000.

**Anti α-B-crystallin** mouse monoclonal antibody against B-crystallin (Stressgen Biotechnologies, CA, USA) was used at a final dilution of 1:2000.

**Anti agrin** mouse anti-agrin antibody was from Stressgen (Stressgen Biotechnologies, CA, USA), and used at a final dilution of 1:100.

**Anti Sox-9** rabbit polyclonal antibody against Sox-9 was a gift from Dr. M. Wegner (Institut für Biochemie, Erlangen, Germany) and used at a final dilution of 1:2000.

**Anti chondromodulin-I** Rabbit polyclonal antibody against chondromodulin-I was a gift from Dr. Haraki and Dr. Sukunami (University of Kyoto, Japan) and used at a final dilution of 1:600.

**Anti Jip-1** mouse monoclonal antibody against Jip-1 was a gift from A. Whitmarsh (University of Manchester, Manchester, UK), and used at a dilution of 1:200.
Anti L1 rat monoclonal antibody (clone 324) was a gift from Dr. R. Martini (Martini et al., 1994). This antibody was used in the form of hybridoma supernatant and diluted with MEM-H 10% calf serum (CS) 1:1 and placed on prefixed cells. Incubation was for 1 hr at room temperature, followed by Cy3-conjugated anti-rat Iggs for 30 min.

Anti-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.

Anti-neurofascin Rabbit polyclonal antibody against neurofascin was a gift from Prof. P.J. Brophy, and used at a dilution of 1:100.

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

Anti phospho-histone-H3 Rabbit polyclonal antibody against phospho-histone H3 (PH3) (Upstate, Inc., USA) was used at a final dilution of 1:5000.

Anti Smad4 mouse monoclonal antibody against Smad4 was purchased from Santa Cruz Biotechnologies Inc. (CA, USA), and used at a dilution of 1:400.

Anti Smad2 mouse monoclonal antibody against Smad2 was purchased from BD Bioscience, and used at a dilution of 1:500.

Anti-MUPP-1 rabbit polyclonal antibody against Mupp-1 was a gift from E. Peles (Weizmann Institute of Science, Rehovot, Israel), and used at a final dilution of 1:100.

Anti p75NTR rabbit polyclonal antibody against p75NTR (Advance Targeting Systems, CA, USA), was used at a final dilution of 1:1000.

Secondary antibodies

Goat anti-mouse 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).

**In situ hybridisation**

**Tissue preparation**

Sciatic nerves dissected from P3 rats were fixed in 4%PF for 4-6 hours and transferred to 30% sucrose (in 0.1M phosphate buffer) for 24 hours. The nerves were then embedded in OCT compound, and 15-20μm sections were cut and collected onto Superfrost slides. The sections were dried for 30min-1hour at room temperature, fixed in 4%PF for 10min and washed 3x in PBS for 3 min each time. Sections were then acetylated (29.5 ml dH2O, 0.4ml triethanolamine, 0.05 ml concentrate HCl, 0.075 ml acetic anhydride) for 10min with shaking, washed 3x in PBS, 5min each and dehydrated with 50%, 75%, 80%, 90%, 100% ethanol, 2 minutes each and then air-dried.

**Hybridisation**

200-300μl hybridisation buffer (1x salts, 50% formamide, 0.1 mg/ml yeast total RNA, 10% w/v dextran sulphate, 1x Denhardt’s) were placed on each slide, and incubated at room temperature at least 2h in a closed box containing 2xSSC, for pre-hybridisation. The pre-hybridisation solution was carefully removed and replaced with 200μl hybridisation buffer containing the DIG labelled probe at 200-400 ng/ml, previously heated at 80°C for 5min. Each slide was covered with a piece of plastic bag, and the hybridisation box (humidified) placed in an oven at 65°C overnight. “Total” collagen type II and “II A” probes were a generous gift from Dr. Linda Sandell (Washington 94
Washing and staining

Each slide was submerged in 0.2xSSC in Coplin jars at room temperature and subsequently transferred to preheated 0.2xSSC at 65°C for 1h, with rocking. The slides were then transferred to room temperature 0.2xSSC for 5min and then in buffer 1 (B1) (0.1M Tris pH 7.5, 0.15M NaCl) for 5min. The slides were then incubated for 1h with B2 (B1 plus 1% heat inactivated sheep or goat serum) at room temperature. 0.5ml of anti-DIG antibody (diluted 1:4000 in B2) were then placed on each slide, and incubated at 4°C overnight in a humidified box. The day after, the slides were washed 3x with B1, equilibrated for 5min with B3 (0.1M tris pH 9.5, 0.1M NaCl, 50mM MgCl₂), and incubated for 6h-3 days in solution B4 (2xB3 in 1:1 solution with polyvinyl alcohol, plus 3.5µl of NBT per ml of solution, 3.5µl BCIP, 0.24 mg/ml levamisole). The reaction was stopped placing the slides in PBS; the slides were mounted in aquamount and allowed to dry.

Cell Culture Experiments

Coverslips preparation

Round 13 mm coverslips (Merck) were baked at 140°C for 8 hr under dessicated conditions and coated with 1mg/ml poly-D-lysine (PDL) in dH₂O 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.

Isolation and Culture of Schwann Cells

Schwann cells were prepared using the method of Brockes et al., (1979). Newborn Sprague-Dawley rats were killed by decapitation and the sciatic nerves dissected out
using a dissection microscope. 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°C and 5% CO2 and 95% air for 50 min with single trituration at the half way stage. The cells were then washed in DMEM with 10% FCS and centrifuged for 10 min at 500xg at 4°C to pellet, resuspended in the same medium and cultured on PDL and laminin coated coverslips. For non-myelinating Schwann cells, 6 week old rats were killed in a CO2 chamber and the sympathetic trunk was dissected out. The epineurial sheath was removed and the cells dissociated with trypsin and collagenase, resuspended in defined medium (DM), and plated on PDL and laminin coated coverslips for 24 hours. After the 24 hours the cells were fixed for 10 minutes in 4% PF and immunolabelled with the antibody of interest.

For the db-cAMP stimulation assay, P3 sciatic nerve Schwann cells were plated in DM + 0.5% foetal calf serum (FCS) for 3 days, and subsequently stimulated with 1mM db-cAMP for 24 hours.

**Defined Medium (DM)** contained 1:1 Hams F12/DMEM supplemented with 100μg/ml glutamine, 0.03% bovine serum albumin (BSA), 100μg/ml transferrin, 16μg/ml putrescine, 38ng/ml dexamethasone, 60ng/ml progesterone, 400ng/ml thyroxine (T4), 5ng/ml insulin (low insulin) or 5μg/ml (high insulin), 10 ng/ml triiodothyronine (T3), 160ng/ml selenium and 100U/ml each of penicillin/ streptomycin.

**Immunopanning**

For immunopurification, Schwann cells from rat or mouse sciatic nerves were purified by negative immunopanning on dishes coated with Thy1.1 antibodies as described in Dong et al., (1997). Briefly, 2-3 90mm Petri dishes (Falcon) were coated with antimouse or anti-rat IgG (Thy 1.1, Dako, final concentration 50-60μg/ml in 7 ml 50mM
Tris pH 9.5), wrapped in nescofilm and left overnight at 4°C. The following day the IgG solution was removed from the plates and replaced with a solution containing 4ml Ox-7 supernatant, 2ml LI5 and 400µl 35% BSA. After 2 hours at room temperature the Ox-7 solution was removed and the dishes washed 3 times in PBS. The Schwann cells, dissociated from the sciatic nerves as described previously, were resuspended in 7-8 ml DM and transferred to the Thy 1.1 coated dish. The dishes were shaken vigorously and incubated at 37°C for 10min. The dishes were shaken once more and incubated for a further 10min. The cell suspension is transferred to another coated dish and the two previous steps are repeated. The cell suspension was collected, spun, and the cells resuspended in the relevant medium. The cell purity was determined with S100 staining.

**Schwann Cell Precursor Cultures**

Schwann cell precursor cultures were prepared according to the method form Jessen et al. (1994). Sciatic nerves were dissected from E14 rat embryos. The nerves were cut in small pieces and incubated with 600µl enzyme cocktail solution (2mg/ml collagenase, 1.2 mg/ml hyaluronidase, 0.3 mg/ml trypsin inhibitor) for 1 hour, before being triturated through a 1ml pipette and then through a 200µl pipette. They were then re-incubated for a further 15min before being triturated again. The cell suspension was then transferred to a centrifuge tube and the volume made up to 10ml with defined medium (DM), in order to dilute the enzymes. The tube was then centrifuged for 10min at 1000rpm and the cells were counted and resuspended at a concentration of 3000 cell in 15µl in the relevant growth factor (usually β-neuregulin). These cells were plated on PLL and laminin coated glass coverslips and after 2.5-3 hours topped up in DM containing β-neuregulin. After 6, 24 or 48 hours the cells were fixed and stained with the antibody of interest.
Neural crest cell cultures

Neural crest cell cultures were prepared essentially as described in Woodhoo et al., 2004. Neural tubes were dissected out from E11 rat embryos and plated on a PDL-fibronectin-coated 35-mm Petri dish containing 2 ml of DM supplemented with insulin (10-9 M), bFGF (3 ng/ml), Nrg-1 (10 ng/ml), IGF-1 (100 ng/ml) and N-acetyl cysteine (1 mM). After 24 hours at 37°C, 5% CO2, the tubes were removed from the dish, the cells fixed with 4% paraformadehyde (PF) and immunostained with the antibody of interest.

FACS sorting

P3 rat Schwann cells were resuspended in L15 medium + 10% FCS and immunolabelled with anti-mouse GalC supernatant (Ranscht et al., 1987), at a 1:1 dilution. Anti-mouse FITC was used as second layer. The cells were then centrifuged and resuspended in L15+10%FCS to a final concentration of 1-6 million cells/ml. GalC positive and negative cells were selected using a EPICS Elite ESP flow cytometer (Beckman Coulter).

Cell Proliferation and Cell Death assays

Cell Proliferation

Phospho-histone H3 (PH3) staining

The ratio of cells undergoing proliferation was measured with PH3 immunlabelling. Cryostat sections, 6µm thick, were cut from fresh frozen sciatic nerves at different ages, mounted on Superfrost slides and allowed to dry for 45min-1 hour at room temperature. Sections were fixed for 10 minutes with 4% PF, and, after washing, they were blocked
using ADS for 1 hour. PH3 antibody, diluted 1:5000 in ADS, was applied either for 1 hour at room temperature or overnight at 4°C. Sections were then washed and incubated with anti-rabbit FITC for 30 minutes. Nuclei were labelled with Hoechst (diluted 1:1000 in ADS) and the sections mounted in Citifluor mounting medium.

Cell Death Assay

Cells in the process of apoptotic cell death were analysed using sections to estimate in vivo cell death by in situ labeling of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). Cryostat sections of fresh-frozen nerves, of various ages, were cut at between 6 and 8μm and mounted on Superfrost Plus microscope slides. Tissue was allowed to dry for 45 min and then fixed by submersion in 4% paraformaldehyde for 10 min at room temperature. The sections were washed, usually 3 x 5 min in PBS and then pre-incubated in terminal transferase (TdT) buffer (30mM Tris buffer pH 7.2, 140mM sodium cacodylate and 1mM cobalt chloride) for 15 min at room temperature. Terminal transferase and biotinylated-d-UTP were added to the sections in a TUNEL reaction mixture, as recommended in the manufacturers’ protocol and incubated at 37°C for 60 min. The reaction was terminated by washing 2 x 5 min in ultrapure H2O and once in PBS. Non-specific binding sites were blocked using PBS with 10% FCS for at least 60 min at room temperature. The sections were then incubated with streptavidin conjugated to Cy3, at a concentration of 1:50 in ADS with 0.1% Triton X-100 for 30 min at room temperature. Nuclei were then labelled with Hoechst dye and the sections mounted in Citifluor anti-fade mounting medium.
**TGFβ survival assays**

The sciatic nerve was dissected and dissociated from newborn entire litters, of P0 CRE/TGFβRII mice. Following centrifugation the cells were resuspended in DM + 0.5% FCS, counted with a haemocytometer and plated at 3000 cells per coverslips onto Pdl and laminin coated coverslips in triplicate. Following incubation for three hours at 37°C and 5% CO2 and 95% air, one set of coverslips, representing a sample of cells from each animal of the litter were fixed for immunocytochemistry. The remaining two sets of cells were topped up with DM + 0.5% FCS and treated with 20ng/ml TGFβ-1 for 24 hr. These cells were then also fixed into 4% PF for 10 min, washed twice with PBS, and Hoechst dye was used for nuclear staining.

**Quantification of Schwann cell survival**

The number of living Schwann cells in the above experiment is expressed as survival percent and was carried out using a method previously described (Meier et al., 1999, Parkinson et al., 2001). Survival percent is the number of living cells present at 24 hr on the coverslip as a percentage of the number of cells that had attached to the substrate in sister cultures at 3 hr.

**Microscopy and quantification**

Slides were mounted in Citifluor (Citifluor, Ltd, UK) and examined with a fluorescence microscope (Eclipse E800, Nikon). Images were captured with a digital camera (DMX1200, Nikon), and ACT-1 acquisition software (Nikon). UMAX PowerLookII was used to digitalize the images.

TUNEL-positive and BrdU stained nuclei were counted as a percentage of all nuclei within a given field, using a 40x objective lens or by importing images into NIH image,
using Photoshop 5.0 on an Apple Macintosh G4 computer. At least 1000 cells were counted and each experiment repeated at least five times. All images were directly imported into Photoshop.

**Statistical analysis**

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

**Electron Microscopy (EM)**

Mutant and control mice of various ages were killed with a method appropriate for the age of the animal. The sciatic nerves were rapidly dissected out, with care to avoid any mechanical stress and placed in freshly prepared 2% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.4 at 4°C. 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 45 minutes. The tissue was washed three more times incubated with 2% uranyl acetate in H2O for 45 minutes, and then dehydrated as follows; 25% ethanol (5 min), 50% ethanol (5 min), 70% ethanol (5 min), 90% ethanol (10 min), 100% ethanol (10 min x 4) and propylene oxide (10 min x 3). Tissue samples were then embedded in araldite in two stages; overnight incubation in 50% araldite and 50% propylene oxide mixture followed the next day by an 8 hour infiltration in araldite alone, all at room temperature. Araldite consisted of equal parts araldite CY212 and DDSA with accelerator, BDMA and plasticizer, dibutryl pthalate. The tissue samples were then placed into a rubber coffin mould and thermo-cured at 65°C for 48 hours. Semi-thin sections, 1μm and ultra-thin sections, were taken using an Ultracut E ultramicrotome (Leica, Germany). Semi-thin sections were taken using fresh glass knives and collected on microscope slides and
ultra-thin sections on copper grids (Agar Scientific, UK). Semi-thin sections (STS) were stained with 0.1% toluidine blue in ethanol, rinsed with distilled water, dried and mounted in DPX mountant. These sections were then analysed by light microscopy. Ultra-thin sections (silver), for higher power work, were collected on copper New 200 grids (Agar Scientific, UK). Grids were stained for 15 min in lead citrate solution, washed in distilled water and dried. Grids were viewed in a Jeol 1010 electron microscope (Jeol, Japan) and images captured on X-ray Film (Ilford, UK). Films were printed using a Deverre enlarger on Ilford multigrade paper using Ilford multigrade gel filters (Ilford, UK).
Table 2.2, primers for RT-PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Annealing Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col2a1</td>
<td>5'-TCGGGGCTCCCCAGTCGCTGGT-3'</td>
<td>5'-TCCAGGGGTACCAGGTTTAC-3'</td>
<td>58°C</td>
<td>30</td>
</tr>
<tr>
<td>ChM-1</td>
<td>5'-GGAAAGGCAAAGATCATGCAGGGG-3'</td>
<td>5'-ACACCATGCCCCAAGATGCGG-3'</td>
<td>56°C</td>
<td>32</td>
</tr>
<tr>
<td>Snca</td>
<td>5'-TGC-TGTGGATATTGTTGTTG-3'</td>
<td>5'-AGGTGCTGTCGTCATGCT-3'</td>
<td>54°C</td>
<td>33</td>
</tr>
<tr>
<td>Jip-1</td>
<td>5'-CGACTGTCTGCTCACTCCAGG-3'</td>
<td>5'-CATAGACAGTGCAAGTTGCG-3'</td>
<td>57°C</td>
<td>32</td>
</tr>
<tr>
<td>Sox9</td>
<td>5'-GAATCTCCTGGAGCCCTCTCCA-3'</td>
<td>5'-CCCTCCAGCCTGCTGACC-3'</td>
<td>54°C</td>
<td>37</td>
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<tr>
<td>COUP-TFI</td>
<td>5'-AGCCATCGTGCTGTGACCACCACC-3'</td>
<td>5'-CCTGCCCTGATACGCAT-3'</td>
<td>55°C</td>
<td>33</td>
</tr>
<tr>
<td>Agrin</td>
<td>5'-CCGTAAGGAGTCGTGAAGAATG-3'</td>
<td>5'-TTTGTTGCGAGACTGTTTG-3'</td>
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<td>eMAF</td>
<td>5'-GTGATGGCTCCTTTTGAATGTTG-3'</td>
<td>5'-AGAGGCTCAGGAAACACAGGA-3'</td>
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<tr>
<td>CRMP-1</td>
<td>5'-ATAGACAGAGGCCAGCCTAGC-3'</td>
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<tr>
<td>CRMP-3</td>
<td>5'-CCCCCTCCCATAAAACCTCTCCTTTGG-3'</td>
<td>5'-CTGGAAGATCAGGCTGCGG-3'</td>
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<tr>
<td>Gapdh</td>
<td>5'-ACCACAGTCCATGCACCTCAC-3'</td>
<td>5'-TCCACCGCCTGCTGTA-3'</td>
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<td>H33426</td>
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<td>AA892486</td>
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<tr>
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<td>5'-CACAGAACATTTCAAAAGGCCG-3'</td>
<td>55°C</td>
<td>30</td>
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</table>
CHAPTER 3

Gene profiling and bioinformatic analysis of the Schwann cell lineage

INTRODUCTION

As already discussed in the general introduction, the great majority of Schwann cells in the peripheral nervous system derive from the neural crest (Le Dourain et al., 1991; Anderson, 1997). Two main intermediates, the Schwann cell precursor (E14-15 in the rat, E12-13 in the mouse), and the immature Schwann cell (from E17 in the rat, from E15 in the mouse), are then involved in the formation of mature myelinating and non-myelinating Schwann cells (Dong et al., 1995, 1999; Mirsky and Jessen, 1996; Jessen and Mirsky, 1999, 2002). The transition from Schwann cell precursor to immature Schwann cell is a process that takes place somewhat abruptly. Immature Schwann cells are clearly distinguishable form precursors thanks to their in vitro bi-polar shape, immunoreactivity to S100 and O4 (in vivo), and ability to survive in the absence of axonal contact, due to autocrine loops (Jessen et al., 1994; Dong et al., 1999; Meier et al., 1999). The precursor/Schwann cell transition is also accompanied by the down-regulation of the transcription factors Sox10 and AP-2, while the expression of the transcription factor Oct-6 increases during this period (Kuhlbrodt et al., 1998; Britsch et al.; 2001 Stewart et al., 2001; Blanchard et al., 1996). However, much remains to be learned about the molecular regulation of this event and the change in gene expression profile involved in this transition.

In Chapter 1 we have seen that the cells destined to myelinate carry out large amounts of membrane synthesis and wrapping to form the myelin sheath. Myelin proteins, such as P0, MBP and PLP, are strongly up regulated, and so are the genes involved in lipid synthesis. One of the most striking features of this process is its reversibility: if axonal contact is lost, the Schwann cells de-differentiate, proliferate and the myelin related
genes are down regulated, while adhesion molecules, neurotrophins, cytokines and their receptors are up-regulated to create an environment that allows axonal re-growth (reviewed in Stoll and Muller, 1999; Scherer and Salzer, 2001). To date, five transcription factors have been shown to be involved in Schwann cell development and in the myelination programme: Sox10 (Kuhlbrodt et al., 1998; Britsh et al., 2001), Oct-6 (SCIP/Tst-1; Monuki et al., 1990; Bermingham et al., 1996; Jaegle et al., 1996), Brn-2 (Jaegle et al., 2003), NF-kappaB (Nickols et al., 2003) and Krox-20 (Topilko et al., 1994; Murphy et al., 1996; Zorick et al., 1999). The latter has recently been suggested to act as master regulatory gene, due to its capability to drive the expression of myelin specific genes in cell types other than Schwann cells (Parkinson et al., 2004). Still, many of the molecular mechanisms involved in the myelination process remain poorly understood.

Schwann cell response to nerve injury

Axonal loss results in all the Schwann cells distal to the site of injury assuming the phenotype of denervated Schwann cells. This can be considered as a third phenotype of Schwann cells along with myelinating and non-myelinating Schwann cells. Denevated Schwann cells are broadly similar to non-myelinating ones, and are characterised by the expression of p75NTR, GAP-43, N-CAM and L1. This means that after nerve injury the bigger changes are seen in myelinating Schwann cells that dramatically decrease their synthesis of lipids and myelin-related proteins. However, even non-myelinating Schwann cells are affected by axonal loss, and reduce their expression of galactocerebroside and sulphatide (reviewed in Scherer and Salzer, 2001).

The switch to denervated Schwann cells is accompanied by a massive increase in proliferation, attributable to both Schwann cell forms (Abercrombie and Johnson, 1946;
Abercrombie et al., 1959; Bradley and Ashbury, 1970; Clemence et al., 1989). Subsequently, as reinnervation occurs, Schwann cell stop proliferating, and those Schwann cells destined to re-myelinate down-regulate L1, N-CAM, p75NTR and GAP-43 and start expressing MAG and other myelin-associated genes (Taniuchi et al., 1988; Martini and Schachner, 1988; Hall et al., 1992).

As already mentioned in Chapter 1, denervated Schwann cells create a favourable environment for axonal regeneration. Besides eliminating myelin debris and forming persistent basal lamina tubes, denervated Schwann cells also secrete a series of extracellular matrix (ECM) molecules, including F-spondin, collagen P200, laminin, tenascin-C and fibronectin, which appear to be permissive for axonal regrowth (Burstyn-Cohen et al., 1998; Chernousov et al., 1999; Patton et al., 1997; Scherer and Salzer; 2001). Moreover, denervated Schwann cells up-regulate the expression of cell adhesion molecules, including N-CAM, L1, N-cadherin and ninjurin (Martini, 1994; Araki and Milbrandt, 1996; Scherer and Salzer, 2001). In particular, L1 and N-CAM have been shown to increase dramatically on the surface of de-differentiating myelinating Schwann cells, and to remain highly expressed in non-myelinating ones (Martini and Schachner, 1998; Jessen et al., 1987).

The injured nerve is quickly invaded by macrophages, which mediate demyelination and influence Schwann cell proliferation and successive remyelination (reviewed in Kiefer et al., 2001). Macrophages are recruited to the injury site as well as the distal nerve portion and their principal function is to phagocytose degenerating myelin (Perry et al., 1987; Stoll et al., 1989; Monaco et al., 1992). The macrophage complement receptor type 3 is the main surface receptor involved in myelin recognition and uptake (Bruck, 1997). Schwann cell are also responsible for myelin clearance in vivo (Stoll et
al., 1989). Interestingly, while myelin phagocytosis by Schwann cells appears to be
lectin mediated, myelin phagocytosis by macrophages is opsonin-dependent (reviewed
in Hirata and Kawabuchi, 2002). Other roles have been attributed to macrophages,
including the secretion of Schwann cell mitogens and the release of interleukin-1
(Baichwal et al., 1988; Heumann et al., 1987).

Degenerating peripheral nerve, and in particular formerly myelinating Schwann cells, is
also the source of trophic factors. This idea was already suggested by Ramon y Cajal
(1928), who also suggested that these factors could also have guidance effects on the
regenerating axons. It is now widely accepted that Schwann cell are the main source of
factors such as neurotrophins, cytokines and TGFβs.

**Neurotrophins**

The neurotrophin (NTs) family consists of four members, brain derived neurotrophic
factor (BDNF), nerve growth factor (NGF), neuroptrophin-3 (NT-3) and -4 (NT-4)
(reviewed in Lewin and Barde, 1996; Ip and Yancopolous, 1996). They signal through
high-affinity tyrosine kinase receptors (Trks) and low-affinity p75NTR.

NGF expression at both the mRNA and protein level is sharply increased in the distal
stump of transected nerves (Rush et al., 1995; Heumann et al., 1987). NGF activation is
enhanced by the addition of macrophages, suggesting that they may modulate Schwann
cell synthesis of NGF (Heumann et al., 1987; Robertson et al., 1995). The expression of
the NGF receptor p75NTR is also increased in Schwann cells, and is not influenced by
macrophages (Heumann et al., 1987). P75NTR up-regulation is seen 24 hours after
transection, peaks at around 1 week and is maintained for up to 10 weeks, and axon
regeneration down-regulates p75NTR (Robertson et al., 1995; Taniuchi et al., 1988).
P75NTR activates the transcription factor NF-κB, which could be involved in re-myelination (Carter et al., 1996; Nickols et al., 2003).

BDNF, NT-3 and NT-4 are also up-regulated after nerve injury, but with a temporal pattern retarded compared to NGF (Acheson et al., 1991; Funakoshi et al., 1993). Formerly myelinating Schwann cell, rather than non-myelinating ones, appear to be the main source of BDNF (Friedman et al., 1996). BDNF and NT-3 have been shown to promote axonal regeneration, and exogenous NT-3 promotes the regeneration of motor neurons and their reinnervation of skeletal muscle (Utley et al., 1996; Sterne et al., 1997a, 1997b). The interaction of regenerating axons with the Schwann cell suppresses the expression of BDNF and NT-4. In recent years various experiments have shown that BDNF and NGF promote Schwann cells myelination, while NT-3 appears to inhibit it (Chan et al., 2001; Chan et al., 2004). On the other hand, NT-3 enhances Schwann cell migration while both BDNF and NGF inhibit it (Yamauchi et al., 2003; Yamauchi et al., 2004). These data indicate that neurotrophin have a key role not only during the degeneration/regeneration process but also during normal Schwann cell development.

**Cytokines**

Ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF), are a family of structural related cytokines the expression of which has been detected in Schwann cells (Scherer and Salzer, 2001). The membrane protein g130 is the signal transduction subunit common to these three factors. G130 interacts with LIFβ receptor, IL-6α receptor and with CNT-Fc receptor to transduce the signal of LIF, IL-6 and CNTF respectively (reviewed in Ip and Yancopoulos, 1996).

CNTF has a trophic effect on a variety of PNS and CNS neurons (Sendter et al., 1994). In the PNS, CNTF is expressed by myelinating Schwann cells, and its levels fall
dramatically after nerve injury, indicating that CNTF could be a trophic factor for uninjured neurons, an hypothesis sustained by the observation that CNTF null mice eventually develop motor neuron disease (Sendtner et al., 1996; 1997). During Wallerian degeneration CNTF is released in the extracellular space, but the mechanism is unknown (Sendtner et al., 1997).

LIF levels increase sharply after nerve injury, and Schwann cells appear to be the main source (Curtis et al., 1994; Ito et al., 1998). LIF enhances axon re-growth, and in CNTF/LIF double mutants motor neuron death is increased compared to CNTF alone null mice (Sendtner et al., 1996).

Similarly to LIF, IL-6 expression is induced by nerve axotomy, but the cellular source is not clear (Bolin et al., 1995; Ito et al., 1998; Hirota et al., 1996). Recent work indicates that Schwann cell derived IL-6 and LIF are involved in the recruitment of macrophages to the nerve after injury, suggesting that Schwann cells are also active regulators of the inflammatory response, rather than simply passive targets of extrinsic signals (Tofaris et al., 2002).

**TGFβs**

Schwann cells express all the three forms of transforming growth factor-β (TGFβ), TGFβ-1, -2, and -3. The role of the TGFβ family during Schwann cell development will be discussed in chapter 5.

**Transcription factors expressed after nerve injury**

The oncogene c-Jun and c-Fos have been shown to be highly expressed at both the mRNA and protein level some 12 hours after nerve injury (De Felipe and Hunt, 1994; Liu et al., 1995; Stewart et al., 1995; Shy et al., 1996; Parkinson et al., 2004). C-Jun
and c-Fos are able to form homo- and heterodimers, therefore their expression after axotomy forms the basis for a signal cascade involved in nerve regeneration that is not fully understood yet (Kerppola and Curran, 1993; Soares et al., 2001).

In adult nerves, Oct-6 expression is transiently up-regulated around 2 days after injury, while Krox-20 expression is strongly down-regulated (Monuki et al., 1990; Scherer et al., 1994; Topilko et al., 1997). Krox-24 is up-regulated in Schwann cell following sciatic nerve transection, and there is an indication that its up-regulation is necessary for p75NTR induction (Topilko et al., 1997; Nikam et al., 1995).

To try to elucidate further the events accompanying Schwann cell development and myelination I have performed a gene expression profiling study of embryonic, myelinating and de-differentiating Schwann cells, using Affymetrix Gene Chip Technology. In the following paragraphs a brief overview on DNA microarrays, their history and applications is provided.

**DNA microarrays: an overview**

The fundamental principle of DNA microarrays is the process of hybridisation, which has been used for decades by molecular biologists as the basis for techniques such as Southern and Northern blotting. In these methods a small sequence of DNA, an oligonucleotide, often radioactively labelled, is used to hybridise to complementary fragments of DNA or to a messenger RNA. If the oligonucleotide is radioactive, the hybridisation can be visualised as a band on a photographic film. In the case of Northern blotting the intensity of the resulting band depends, to some extent, on the amount of messenger RNA and therefore on its level of expression.
Southern and Northern blotting, although powerful techniques, allow the study of a single gene and messenger at a time. The first example of large-scale analysis was introduced in the late 1970s, and was named dot-blot (Kafatos et al., 1979). In this technique multiple hybridization targets, instead of being distributed in a gel containing DNA or RNA, are attached to a filter and can be analysed in parallel. Moreover, imaging methods allow parallel measurement of the signals as well. Hoheisel et al., (1994), took this approach a step further and used multiple libraries arrayed on filters at high density for cross-correlating cloned sequences. The high density spotting was achieved by replacing manual procedures with robotics, which increased the speed of the operation, improved the accuracy of placing the samples and removed human errors. This represented the first step towards microarrays.

What really distinguishes a DNA microarray from a dot blot is that while in the dot blot multiple targets are arrayed on the support (usually a nylon membrane) and the probe, normally a single sequence, is applied under hybridisation conditions, in a microarray multiple probes are attached to the support and the target to be analysed is labelled. It is therefore to be noticed that, from now on, the term *probe* will be used for the nucleic acid of known sequence, which will be attached to the surface in the case of the microarray, and *target* will describe the collection of sequences to be analysed (total RNA or purified polyA\(^+\) mRNA).

There are currently two main types of DNA microarrays (or chips): in the first one, probes are synthesised *in situ* directly on a rigid support (usually glass), while in the second pre-synthesised probes are attached to the platform (nylon or glass). This second technology offers more flexibility, in that it is possible to design any probe for spotting on the array. On the other hand, the *in situ* technology is easier to control and results in greater uniformity of hybridization between chips, which can facilitate data
analysis when comparing multiple experiments, and, although more expensive, is nowadays generally preferred (Schena, 1999; 2004; Knudsen, 2004; Baldi and Hatfield, 2003).

**Affymetrix GeneChip Technology**

Probably, the most widely used *in situ* technology at the moment is Affymetrix GeneChip (Fodor *et al.*, 1993; Pease *et al.*, 1994). Affymetrix uses high density oligonucleotides photochemically synthesised on the surface of a glass support. There are up to 40 oligonucleotides (each one up to 25 bases long) for each gene spotted on the chip. These oligonucleotides correspond to regions of the gene that are, presumably, the least similar to other genes, to guarantee the highest specificity for each gene (Chee *et al.*, 1996; Lockhart *et al.*, 1996; Lipshutz *et al.*, 1999). From this region 11 to 20 oligos are chosen as perfect matches (PM), meaning that they are perfectly complementary to the mRNA of that gene. In addition Affymetrix has generated 11 to 20 mismatch (MM) oligos, which differ from the PM only in the nucleotide positioned at the centre of the sequence. These MM oligos serve as internal control and are able to detect background and non-specific hybridisation, which is extremely important for the quantification of weakly expressed mRNAs. As described in detail in Chapter 2, the target mRNA to be interrogated, is then labelled and applied to the chip for hybridisation. After the hybridisation is completed the chips are washed and scanned. Computation of the relative expression level of all the probes is performed by the Affymetrix software.
Application of DNA microarrays

The development of DNA microarray technology has made possible the analysis of the expression profile of thousands of genes simultaneously. This can potentially provide a snapshot of the level of expression of all the genes in a cell under a given set of conditions. These expression profiles can be used, among other possibilities, to determine the function of new genes, for studying development and evolution, to evaluate the effect of drugs on gene expression or to analyse alternative splicing (Lockhart and Winzeler, 2000; Smith and Greenfield, 2003; Levy, 2003; Shaw and Morrow, 2003; Holloway et al., 2002). Another powerful application of microarrays is in diagnostic and prognostic: DNA polymorphisms, first proposed as a tool for mapping the human genome (Botstein et al., 1980), have allowed the isolation of a number of disease related genes. Analyzed on a large scale using microarrays, polymorphisms are expected to permit genetic studies aimed at finding the genes associated with common diseases and inherited diseases susceptibilities (Cargill et al., 1999).

The use of Bioinformatics in Arrays

The most common application of DNA microarrays is the comparison of gene expression levels under different conditions, and one of the most typical goals is to find genes that are statistically significantly up- or down-regulated. It is therefore important to make sure that the samples that we are comparing are really comparable. The chips have to be the same under the different conditions and also the amount of sample applied to each chip has to be comparable. It is then necessary to normalize the data, so that the quantified values would only represent true differences in gene expression. This process is automatically performed by Affymetrix Suite (See Material and Methods), as
well as by other commercially available packages. But how do we determine whether an observed variation in the expression level of a gene in two or more different conditions is significant? At present the best way is to repeat the experiments and to measure the variation. Of course the lower the number of replicates, the more difficult it is to estimate the variance. It is now widely accepted that three is the minimum number of replicates that allows a reliable statistical analysis. Only after that normalization and statistical analysis have been performed can the real analysis of the results start.

**DNA microarray in the study of peripheral nerve biology**

DNA microarray technology has been previously used to study Schwann cell embryonic development (Buchstaller et al., 2004), and to decipher Schwann cell expression profiling during myelination and after nerve injury (Nagarajan et al., 2001; Araki et al., 2001; Nagarajan et al., 2002; Kubo et al., 2002; Verheijen et al., 2003). The use of DNA microarrays to analyse the response of cultured Schwann cells to enforced expression of Krox-20, has shown that the mRNA levels for myelin proteins and lipids, as well as the mRNAs for a variety of unknown genes, are strongly up-regulated by Krox-20. This finding indicates that this transcription factor plays a crucial role in the transition from immature Schwann cells to myelinating ones (Nagarajan et al., 2001). Moreover, microarray analysis of the genes up-regulated after nerve injury in the adult sciatic nerve has given important indications about genes that could be important for nerve regeneration, such as the novel protein nin283 (Araki et al., 2001; Kubo et al., 2002). Finally, a study of gene expression profiling during Schwann cell development from pre-myelinating to post-myelinating Schwann cells has led to the identification of a group of genes, maximally expressed in the adult nerve, linked with the metabolism of
energy lipids, providing important clues about the association of peripheral neuropathy with diseases such as diabetes and lipodystrophy (Verheijen et al., 2003).

Noticeably, all of these peripheral nerve expression profiling studies have been performed in mouse. In our experiments we decided to use rat for several reasons. The main is a technical one: since we wanted to analyse mRNA expression in embryonic Schwann cells, we needed to collect a high number of sciatic nerves to obtain the ~30μg of total RNA that were necessary for each hybridisation at the time we started our study (2001). Dissecting sciatic nerves from E14 rat embryos is much easier than from E12 mouse embryos (the time of appearance of Schwann cell precursors), and the number of pregnant females that we needed to sacrifice was thus much smaller. Moreover, most of the knowledge about Schwann cell development comes from studies performed in rat, where the Schwann cell precursor was first identified (Jessen et al., 1994), making rat a more obvious choice for our goals.

There are other novelties in our study. Contrary to previous experiments that have looked at genes involved in regeneration after nerve injury in the adult (Araki et al., 2001; Kubo et al., 2002), in our transection studies we have used nerves at the peak of myelination. We have chosen to analyse in detail all those genes that are up-regulated in sciatic nerve after birth and sharply down-regulated following nerve injury, and which should be, therefore, involved in myelination.

Finally, in most expression profiling work, the number of sequences which are completely unknown or only weakly similar to an expressed sequence tag (EST) is very high, and this can result in a significant reduction of the information that can be extracted from an experiment. Therefore, the expression profiling that we performed was accompanied by a refined bioinformatics analysis using Biopendium™ software.
from Inpharmatica, an extensive database of sequence and structural relationships. This was aimed to minimise the number of unknown sequences among the differentially expressed genes. We found that with this approach nearly all the ESTs could be mapped either to rat genes or to the mouse or human orthologues, and that a large number of the encoded proteins could be subjected to a detailed structural and functional annotation.
RESULTS

General approach

Affymetrix Rat U34A Chips were hybridized using cRNA probes obtained from E14, E18, P7, P12 rat sciatic nerves and from P12 nerves that have been cut five days previously. In Fig 3.1 a schematic explanation of the procedure is shown.

Since Schwann cells account for the vast majority of cells in peripheral nerve, and considering that the mRNA levels in the axons of sensory and motor neurons are supposed to be very low (Mohr, 1999; Mohr and Richter, 2000), our analysis should provide an accurate overview of the expression profile of Schwann cells, during the transition from Schwann cell precursors (E14) to immature Schwann cells (E18), and during the active phases of myelination (E18-P7-P12). Moreover the comparison between P12 and P12cut (P12 nerves previously cut at P7) will provide us information on the initial stages of Wallerian degeneration, a process during which all myelin-related genes are strongly down-regulated.

1069 probes have at least a two-fold change during PNS development or after nerve injury

The data obtained from the chip hybridizations, performed in triplicate for each time point to minimize the number of false positives and to allow a correct statistical analysis, were first analysed with the Affymetrix Suite 5.0 and subsequently loaded onto Genespring software. A first restriction by expression percentage was performed, to exclude those probes that did not have at least a two fold change (log2>1) between at least two of the developmental time points or after nerve cut. 1069 probes, out of the
more than 8500 present on the U34A chip, passed this first restriction, and were considered for further analysis. Importantly, all the known myelin-related genes such as protein zero (P₀), myelin basic protein (MBP), peripheral myelin protein 22 (PMP22), myelin associated glycoprotein (MAG), proteolipid protein (PLP), periaxin, and the transcription factors Oct-6 and Krox-20 were detected as changing, as well as many genes involved in cholesterol synthesis and in lipid metabolism. This finding, in agreement with previous papers on expression profiling in mouse peripheral nerve (Nagarajan et al., 2001; Verheijen et al., 2003; Buchstaller et al., 2004), represents a valid internal control for our system, since genes encoding for myelin proteins or for members of the cholesterol synthesis chain are expected to increase during peripheral nerve development and to decrease dramatically after nerve injury (Stoll and Muller, 1999; Scherer and Salzer; 2001).

Clustering of the 1609 probes

Having performed multiple experiments over a series of time points, it made sense to try to group, or cluster, the genes that behaved in a similar way across the experiments. In fact, finding genes with similar expression patterns can lead to better understanding of the functions of genes. Gene clustering can be achieved using various procedures that apply different algorithms. One of the most widely used is hierarchical clustering (Eisen et al., 1998). It requires two main steps that are repeated in order to find the genes that are most similar. Basically, hierarchical clustering finds the pair of genes that are most similar, joins them together, and then identifies the next most similar pair of genes. This process continues until all of the genes are joined into one giant cluster (See Chapter 2 for details). The same process is applied to generate a tree for the
experiments. Using Genespring, we performed Hierarchical clustering of the 1609 probes and, in parallel, the clustering of the 14 experiments (triplicates for E14, E18, P12, P12cut, duplicate for P7). This generated the mock-phylogenetic tree (often referred to as "dendrogram") shown in Fig 3.2. This kind of clustering provides information on the behavior of the genes considered and also on how similar and therefore reliable the replicates are.

To investigate further the changes occurring in between two successive time points we performed a statistical analysis followed by hierarchical and K-means clustering comparing E14 with E18, E18 with P12, and P12 with P12cut. K-means clustering divides genes based on their expression patterns. The goal is to produce groups of genes with a high degree of similarity within each group and a low degree of similarity between groups. K-means clusters are constructed so that the average behavior in each group is distinct from any of the other groups. For example, in a time series experiment k-means clustering can be used to identify unique classes of genes that are up-regulated or down-regulated in a time dependent manner (See Chapter 2 for more details). Moreover, using the Biopendium™ software from Inpharmatica to complement publicly available databases, we carried out a rigorous mapping and protein annotation of all the probes found to be statistically changed.

130 probes are statistically different between E14 and E18

To try to elucidate the molecular mechanisms involved in the transition between Schwann cell precursors (E14) and immature Schwann cells (E18), the 1069 probes found to significantly change during nerve development were subjected to an ANOVA/Welch's approximate t-test (p<0.05) (not assuming variances are equal). 130
were found to be statistically changed. Of those, 53 were up-regulated and 77 down-regulated.

Hierarchical and K-means clustering of the 130 probes statistically different at E14 compared with E18

On this set of 130 probes both hierarchical and K-means clustering were performed, to group genes that behaved in a similar way in our experimental conditions. The first resulted in the dendrogram shown in Fig 3.3, while K-means clustering, performed with the "random starting clusters" option, to allow the software to identify the optimal number of different clusters, resulted in 10 different sets (Fig. 3.4). K-means clustering can be more effective in time series experiments, to identify unique classes of genes that are up-regulated or down-regulated in a time dependent manner. Therefore, we decided to refer to the sets deriving from this kind of clustering for our subsequent analysis.

Interestingly, for the vast majority of the 130 probes, the change in expression level was much more dramatic for those that were down-regulated when compared to those that were up-regulated. (See in particular set 3, 6 and 9 in Fig 3.4). The list of the 130 probes, including the p values scored in the ANOVA/Welch’s t-test and the raw and normalized level of expression at all the time points analyzed, is provided in Table 3.1.

Mapping of the probe sets

The analysis of the molecular mechanisms involved in the transition between precursors and immature Schwann cells was complicated by the fact that 50% (65 out of 130) of the probes detected as changing corresponded to ESTs. To try to bypass this obstacle and to gather as much information as possible on each gene, we performed a comprehensive bioinformatic analysis using the Biopendium™ software from
Inpharmatica (www.inpharmatica.com). First, an automated probe mapping technology was used to identify open reading frames (ORF) for each probe set identified. After alignment to the rat genome using the rapid tool BINSEQ, assignments were made using, in order of descending confidence, RefSeq, Genebank, ENSEMBL or TWINSCAN. The sequences that were not confidently mapped to an ORF yet, were examined by eye and hand mapped. In those cases where the rat gene had not been identified, the mouse or human orthologues were used for protein annotation, when appropriate. This analysis effectively allowed us to reduce the number of unknown sequence from the starting 65 (50%), to only 6 (~ 4%) of the total of the differentially expressed probes (Table 3.2).

**Protein structural and functional annotation**

Once the amino acid sequences had been identified, we generated a protein structural and functional annotation, using Biopendium™ complemented by publicly available databases. Initially, each protein was described using the HGNC gene symbol and, when available, Gene Ontology (GO) annotation was obtained from both LocusLink and GOA. We also assessed, using SignalP (Nielsen *et al.*, 1997) whether it was likely or not for each protein to be secreted and, with TMHMM (Krogh *et al.*, 2001), we predicted the number of transmembrane domains. Finally, Domain Professor™ and Genome Threader™, both part of the Biopendium™, were used for a complete functional and structural annotation of all the proteins identified. The resulting tables are added as Supplementary Material in the Appendix.
**Categorization of the changed genes**

The information obtained from this bioinformatic analysis was used to group the differentially expressed genes based on the probable biological function (examples are given in Tables 3.3.1 and 3.3.2). As shown in Table 3.3.1, among the up-regulated genes in the transition from E14 and E18, are adhesion molecules such as integrin alpha1 (Itgal) and decorin (Dcn), a chondroitin sulphate proteoglycan already known to be expressed by Schwann cells (Hanemann et al., 1993). Interestingly, a decorin-like molecule is up-regulated during mouse sciatic nerve regeneration (Braunewell et al., 1995a,b), suggesting that decorin may have similar functions during sciatic nerve development and regeneration. The transcription factors NF1-X and Cebpd (also known as C/EBP-Delta), the latter recently identified as expressed in embryonic Schwann cells and in adult sciatic nerves on myelination (Buchstaller et al., 2004; Verheijen et al., 2003), are also up-regulated. At E14 they are essentially absent, a pattern of expression shared by the cytoplasmic protein alpha-Synuclein (Snca, see Chapter 4) and by the actinin associated protein Pdlim3.

Table 3.3.2 shows the categorization of the down-regulated genes. We found that the transcription factors Sox-10, Ets-1 and Id3, all known to be expressed in embryonic peripheral nerve (Kuhlbrodt et al., 1998; Parkinson et al., 2002; Stewart et al., 1997; Thatikunta et al., 1999), were strongly down-regulated. Likewise, the neural adhesion molecule L1, crucial for the adhesion of Schwann cells to the axon and for the formation of the first Schwann cell loops around the axons that will be myelinated (Martini and Schachner, 1986; Wood et al., 1990; reviewed in Martini, 1994) appeared to be down-regulated in peripheral nerve during embryonic development. Interestingly, we also found that cyclinD1 (Ccnd1), a gene previously identified as crucially involved in Schwann cell proliferation after nerve injury (Kim et al., 2000), was strongly down-
regulated during the precursor to Schwann cell transition.

**CRMP-1 and CRMP-3 are down-regulated during embryonic nerve development**

Among the down-regulated genes, the cytoplasmic related proteins CRMPs and agrin intrigued us. Two members of the CRMP family, CRMP-1 and -3, thought to be expressed mainly by neurons, and involved in neuronal plasticity in response to semaphorin signals (Wang and Strittmatter, 1996; Rosslenbroich *et al.*, 2003), were strongly down-regulated. To confirm our finding, semi-quantitative RT-PCRs were performed on mRNA extracted from E14 and E18 nerves. For both CRMP-1 and CRMP-3 a band of the expected size is clearly present at E14 and is strongly decreased at E18 (Fig. 3.5), suggesting that CRMPs are expressed by Schwann cell precursors and are down-regulated during the transition to immature Schwann cells.

**Rat agrin is expressed in embryonic and perinatal peripheral nerve**

It was also surprising to find that agrin (Agrn), a molecule known to be of crucial importance for acetylcholine receptor clustering at the neuromuscular junction (NMJ) (reviewed in Bezakova and Ruegg, 2003), was strongly expressed by Schwann cell precursors. Again, to confirm the results of the microarray hybridizations, we performed a semi-quantitative RT-PCR, with primers specific for the neural form of agrin, which showed a clear band at E14 and a very reduced one by E18 (Fig. 3.5). To investigate agrin expression further, we immunolabelled a series of nerves/cultures with an antibody that recognises all the active forms of agrin. Figure 3.6 shows that agrin protein appears to be already expressed in the migrating neural crest, the cell population
from which Schwann cells originate, and it is subsequently highly expressed by Schwann cell precursors. In newborn rat Schwann cell cultures, agrin protein expression was substantially lower than in precursors, and was restricted to Schwann cells, with no staining detectable in fibroblasts or other cell types, as confirmed by similar staining performed with antibody to the Schwann cell specific protein S100β (Fig 3.7). Thus, agrin could be used as a Schwann cell marker in dissociated cultures from perinatal nerves. Little or no staining was detectable in teased nerve preparations from adult rats, in agreement with the array results.

378 probes are statistically different between E18 and P12

As myelination takes place, myelin proteins and genes involved in lipid synthesis are strongly up-regulated. When axonal contact is lost, the Schwann cells de-differentiate and the myelin related genes are down regulated. We took advantage of this characteristic, and focussed our attention on those genes that were up regulated between E18 and P12, and also down-regulated at P12 after nerve cut at P7.

To evaluate how many of the 1609 probes found to have at least a two fold change in our experimental conditions were statistically different between E18 and P12 sciatic nerves, we performed, with GeneSpring, an ANOVA/Welch's t-test (p<0.05, not assuming variances are equal). 378 probes resulted to be significantly changed. Of these, 361 were up-regulated, while only 17 were down-regulated.

Clustering of the 378 probes

As we have seen previously, clustering is a fast way to group genes based on similar expression profiles. Following a similar procedure to that used in the first comparison
(E14 compared with E18), we used GeneSpring to cluster the 378 probes that were statistically different between E18 and P12. Hierarchical clustering resulted in the dendrogram in Fig 4.8, while K-means clustering yielded 15 clusters as the optimal representation possible (Fig. 4.9).

237 probes are statistically different between P12 and P12cut

To determine how many of the 1609 probes were significantly changed between P12 sciatic nerves and P12 nerves cut five days previously (P12cut) the statistical test used in the previous conditions (Anova/Welch's t-test, p<0.05), was again applied. 237 probes were differentially expressed; 162 were down-regulated, while 75 were up-regulated. Interestingly, the majority of the down-regulated probes (110 out of 162, see Table 3.4) were among the 361 that showed a significant increase in expression levels between E18 and P12. This once more emphasises the importance of axon-Schwann cell contact during myelination in peripheral nerve, and strongly points to those 110 probes as myelin-related.

Clustering of the 237 probes

Hierarchical clustering of the 237 probes differentially expressed between P12 and P12cut sciatic nerves resulted in the dendrogram in Fig 3.10. K-means clustering yielded 13 different sets as optimal representation, according to the GeneSpring software (Fig. 3.11).

Mapping of the 237 probes

As we found when comparing E14 and E18 nerves, the number of ESTs present in this second comparison (96 out of 237 probes) was very high. Therefore, we carried out a
bioinformatic analysis identical to the one performed for the embryonic comparison in order to map and annotate to proteins as many differentially expressed probes as possible. In the following paragraph this analysis will be illustrated for a particular probe, the EST AI639533.

**Mapping and annotation of the rat ETS AI639533**

The EST AI639533 was included in set 14 (Fig 3.11), alongside the myelin-related genes PLP, periaxin, Krox-20 and 3-hydroxy-3-methylglutaryl-CoA-synthase-1 (HMGCS1), and was among the 110 probes up-regulated between E18 and P12 and down-regulated after nerve cut, strongly suggesting that it may play a fundamental role in myelination. Applying the bioinformatic analysis described previously, we were able to map this EST to the 3'UTR of a novel rat gene representing the orthologue of the mouse cDNA AK030342, which encodes the un-characterised protein BAC26192 (Fig 3.12A). Using the mouse orthologue as a seed for the GENEWISE algorithm (Birney et al., 2004), we were able to re-predict a rat open reading frame of 500 amino acids which shares 90% identity over the full length of the mouse orthologue and 79% identity over the full length of the human un-characterised protein AAH31099 (Fig 3.12B). The use of Biopendium™ GenomeThreader™ structural alignment tool allowed us to identify in BAC26192 a relationship with bacterial phospholipase D/nuclease structures (Fig 3.12C). The consensus sequence SxK(x)₄D(x)₆GSₓS, highly similar to the consensus HxK(x)₄D(x)₆GSₓN that defines phospholipase D/nuclease activity, is present, suggesting a similar function for the protein BAC26192 (Fig 3.12D). An identical result was found when threading the rat prediction. The EST AI639533 may therefore represent a phospholipase D with a role in myelination, although this awaits...
confirmation. A similar analysis was performed for all the other unmapped ESTs (See Supplementary Material, Appendix).

**Categorisation of the differentially expressed genes**

The bioinformatic analysis reduced the number of unknown sequences from the original 96 (40.5%) to only 4 (1.7%) of the total of differentially expressed probes (Table 3.5). This, of course, was of invaluable help in the categorisation of the genes based on the probable biological function. As expected, among the up-regulated genes (Table 3.6.1) we found molecules involved in the inflammatory response, such as members of the major histocompatibility complex (MHC) and the C4 complement protein, known to be activated after peripheral nerve injury (Bonnard et al., 1997). We also identified molecules that are known to be involved in Schwann cell de-differentiation and proliferation, like the transcription factor c-Jun (De Felipe and Hunt, 1994; Stewart, 1995; Shy et al., 1996; Parkinson et al., 2004), and others involved in nerve regeneration, such as the extracellular matrix protein tenascin (Tnn) (Martini et al., 1990; Kiernan et al., 1999), which is important in creating a permissive environment for axonal regrowth.

Among the genes that were down regulated after nerve cut (Table 3.6.2), and also up-regulated between E18 and P12, there were many implicated in myelin synthesis and maintenance, whether because they are responsible for the expression of myelin constituents or because implicated in lipid metabolism. Here it should be noted that \( P_0 \) and MBP, although detected among the 1609 differentially expressed probes, did not make it through the statistical t-test for the P12 versus P12cut comparison. This was due to the massive mRNA expression at P12 of these two genes, which caused a saturation of the mismatch probes in the first hybridisation performed. The problem, imputable to
scanner settings, was subsequently solved, but resulted in the two genes being too variable among the experiments to pass the t-test. Since these were the only two genes for which this occurred, we did not consider it necessary to repeat the experiment.

**Semi-quantitative RT-PCR experiments confirmed the hybridisation results**

In addition to genes that showed anticipated changes in expression levels, a considerable number of unexpected genes were found to be up-regulated during myelination and down-regulated in cut P12 sciatic nerves. A selection of these genes (chosen based on the high level of expression and on the massive fold-change), including known genes and annotated ESTs, was subjected to semi-quantitative RT-PCR analysis, to confirm the hybridisation results. Gapdh, a housekeeping gene that does not vary in the different experimental conditions, was used as internal control, and the myelin-related protein periaxin as positive control (Parkinson *et al.*, 2003) (Fig 3.13A and 3.13B). In every case considered the RT-PCR results obtained were in agreement with the array data. Further analysis of these genes is reported in the sections below.

**Snca, ChM-1 and PEA-15 are enriched in myelinating Schwann cells**

To verify whether any of the selected genes shown in Figure 3.13, was specific for myelinating Schwann cells, we performed semi-quantitative RT-PCR experiments on mRNA extracted from P3 rat Schwann cells that had been FACS sorted using antibodies against galactocerebroside (GalC), which, at this stage of development, is specific for myelinating Schwann cells (Mirsky *et al.*, 1980; Jessen *et al.*, 1985). The confinement of periaxin expression to the GalC⁺ cell population confirmed the validity
of this approach (Fig 3.14). Although all of the genes examined here were up-regulated between E18 and P12 and down-regulated after nerve cut and therefore behaved like myelin-related genes (above), these experiments showed that, at this early developmental stage, most of them were equally expressed in both cell types. Three of them however, were clearly enriched in myelinating Schwann cells, namely the cytoplasmic protein α-Synuclein (Snca), the cartilage related protein Chondromodulin-1 (ChM-1, Lect-1) and a phosphoprotein enriched in astrocytes, 15-kd (PEA15, annotated with Biopendium™ from the EST AA894345) (Fig 3.14).

Some of the genes down-regulated after nerve cut are induced by dbcAMP

It is well known that activation of cAMP pathways can, under certain conditions, partially mimic the initial steps of myelination, inducing the expression of genes like P0 and periaxin (Monuki et al., 1989; Morgan et al., 1991; reviewed in Jessen and Mirsky, 1991; 2004). We therefore tested the cAMP responsiveness of the genes examined in Fig. 13, including the three that were enriched in myelinating Schwann cells at P3. The periaxin gene was used as positive control (Parkinson et al., 2003). In spite of the apparent axonal dependence of all these genes in vivo (Fig 3.13), only 5 of them, namely Collagen type II, Snca, ChM-1, PEA15 and the EST H33246 (mapped with Biopendium to a farnesyl-diphosphate-farnesyl-transferase-1), were clearly elevated following exposure of cultured Schwann cells to dbcAMP (Fig 3.15) (see Chapter 4 for further comments).
DISCUSSION

Typically, expression profiling experiments reveal a high number of differentially expressed sequences that are either unknown or only weakly similar to ESTs. Often, this significantly limits the information that can be extracted from an experiment. Therefore, we combined the use of microarray based gene expression profiling with a refined bioinformatic analysis performed with the Biopendium™ software, to identify genes potentially involved in embryonic Schwann cell development and myelination. The Biopendium™ allowed us to considerably reduce the number of unknown sequences, and so to have a clearer view of the biological events taking place. Combining publicly available sources and the Biopendium™ it was possible to map the large majority of the ESTs identified in the screening studies and to provide a comprehensive biological annotation for the sequences identified.

In the present experiments in fact, 65 out of 130 differentially expressed probes in the first comparison (E14 compared with E18), and 96 out of 237 in the second (P12 compared with P12 cut) were ESTs. Our approach allowed us to reduce the number of unmapped sequences to only 6 and 4 respectively, enabling us to better categorise the genes based on the biological function.

Genes regulated in the embryonic transition from Schwann cell precursors to immature Schwann cells

The number of repressed and induced genes in the transitional phase from Schwann cell precursor to immature Schwann cell was comparable (77 down-regulated, 53 up-regulated). It was however intriguing that the number of genes that were strongly
suppressed in this transition was much larger than the number of those strongly induced. Thus, all of the 53 up-regulated genes showed only a moderate change in their expression levels, comparable to that shown by many of the down-regulated genes. A group of the down-regulated genes, however, showed a substantially greater degree of change in expression levels (set 3, 6 and 9 in Fig 3.4 and Supplementary Material). It is possible that some of the extensive gene suppression at the E14/E18 transition is related to the narrowing of developmental options that characterises the transition form Schwann cell precursors to immature Schwann cells. E14 Schwann cell precursors are highly motile cells that interact intimately with axons rather than with the extracellular matrix and many, perhaps most, of these cells retain some developmental plasticity, and can experimentally be diverted to other crest derived lineages. Immature Schwann cells, on the other hand, are less motile, anchored to the extracellular matrix and harder to divert to form other cell types (Sherman et al. 1993, Jessen and Mirsky, 1999; 2004; Morrison et al., 1999; Paratore et al., 2002).

Down-regulated genes

The categorisation of the down-regulated genes, based on their biological function, showed that at least three transcription factors, Sox-10, Ets-1 and Id3, already known to be expressed in embryonic nerve (Kuhlbrodt et al., 1998; Parkinson et al., 2002; Stewart et al., 1997; Thatikunta et al., 1999), were repressed during the transition to immature Schwann cells.

As already discussed in the general introduction, Sox-10 is crucial for peripheral glia specification and development (Britsch et al., 2001). Our results confirm the general idea that Sox-10, although present throughout the Schwann cell lineage is expressed at
much lower level after birth than in the embryo (Lange and Jessen, unpublished observation).

Previous observations had indicated that Id3 (and Id1) is expressed in embryonic nerve, down-regulated around the peak of myelination, and induced after nerve injury (Stewart et al., 1997). Moreover, Id3 can inhibit the expression of myelin genes (Stewart et al., 1997; Thatikunta et al., 1999). Our data, showing that Id3 is already strongly down-regulated in immature Schwann cells, represent the first indication of a regulation of Ids transcription factors in embryonic nerve, and suggest that their suppression could be involved in the precursor to Schwann cell transition.

Several Ets transcription factors are known to be expressed by Schwann cells (Parkinsin et al., 2002), and their activity modulates the expression of neuregulin. It is therefore possible that Ets-1 down-regulation reflects the decreased dependency of immature Schwann cell on neuregulin for survival.

A number of receptors/signalling molecules are also down-regulated during the transition towards immature Schwann cells. Among them is the EST AA800790 that we mapped with Biopendium to the endothelin receptor type B. Endothelins (ETs) play a crucial role in the timing of the transition from precursors to Schwann cell (Brennan et al., 2000). In rats with a non-functional type B receptor Schwann cell generation is ahead of schedule, and various in vitro experiments indicated that endothelins act as a brake on the embryonic transition from precursors to Schwann cells (Brennan et al., 2000). Consequently, it looks possible that endothelin receptor down-regulation is one of the mechanisms involved in the generation of immature Schwann cells. It is interesting to notice that recent work has suggested that, in the enteric nervous system, Sox-10 may directly regulate the spatio-temporal expression of the endothelin receptor B gene via binding to a specific enhancer (Zhu et al., 2004). Therefore there may be a
direct correlation between the parallel embryonic down-regulation of these two genes observed in our microarray analysis.

Among the down-regulated genes, the ones related to the cytoskeleton showed the strongest downregulation. While the neurofilament protein NF-M was known to be transiently expressed in Schwann cells (Kelly et al., 1992; Fabrizi et al., 1997), the identification of CRMP family members and agrin was rather surprising. The CRMP (ULIP/DRP) family consists of at least five phosphoproteins, homologous to the *C. elegans* protein Unc-33, which are essential for growth cone collapse in response to semaphorin 3A signals (Wang and Strittmatter, 1996). They have been linked to other cellular events such as differentiation, apoptosis/proliferation and migration (reviewed in Charrier et al., 2003). Recently CRMPs have also been involved in neurite extension in response to signals from neurotrophins in DRG sensory neurons (Quach et al., 2004). Interestingly, a member of the family, Ulipl/CRMP4, has been identified as suppressed by the cAMP activator forskolin in cultured Schwann cells (Bermingham et al., 2001), in agreement with our array results that indicate these genes as strongly down-regulated during myelination. CRMPs may be involved in Schwann precursor cytoskeletal reorganization and possibly in their migration along the outgrowing axon, being then downregulated once the cell has reached a more stationary phase.

Agrin is an heparan sulfate proteoglycan initially isolated from the basal lamina of the neuro-muscular junction (NMJ) in *Torpedo californica* (Nitkin et al., 1987), and it is the nerve derived factor responsible for the assembly of the post-synaptic apparatus *in vivo* (reviewed in Hoch, 1999). Agrin deficient mice die around E18 and show profoundly impaired post-synaptic differentiation (Gautam et al., 1996). More recently agrin roles in other contexts, such as the "immunological synapse" and cytoskeletal actin reorganisation have been elucidated (Khan et al., 2001; reviewed in Bezakova and
Ruegg., 2003; Finn et al., 2003). It is also interesting that agrin and neuregulin-1 colocalise and have a synergistic effect on acetylcholine receptor expression in muscle (Li et al., 2004). In muscle, and other tissues, agrin binds to α-dystroglycan and laminins (O'Toole et al., 1996; Sugiyama et al., 1994; Matsumura et al., 1997), and an agrin "minigene" has been used to rescue dystrophic mice, through the stabilisation of the complex formed by α-dystroglycan and laminin α5 (Moll et al., 2001). Agrin has been previously detected in developing and adult Schwann cells in chick and frog, where its role has been related to acetylcholine receptor clustering and synapse formation (Ma et al., 1994; Yang et al., 2001). Our results, showing that agrin is present in the rat Schwann cell lineage as early as E11, that its expression peaks in Schwann cell precursors while it is subsequently down-regulated, suggest an alternative function. Since agrin has been implicated in the control of the actin cytoskeleton and of a number of cytoskeletal proteins in skeletal muscle (Bezakova and Lomo, 2001), it is possible that agrin has a similar role in Schwann cell development. This is also supported by the high expression of agrin in Schwann cell precursors that are much more motile than immature Schwann cells (Jessen et al., 1994). Alternatively, agrin could interact with dystroglycan and laminin complexes on the Schwann cell surface and have a role in their stabilization of the initial interaction between axons and glia, although the strong down-regulation of agrin observed after birth and in adult mice seems to be against this hypothesis.

Up-regulated genes

Among the up-regulated genes we found that the extracellular matrix protein decorin (Dcn) was strongly induced (see table 3.3.1). Decorin expression has been already documented in Schwann cells (Hanemann et al., 1993), and decorin-like molecules are
up-regulated in regenerating peripheral nerve (Braunewell et al., 1995a,b). Taken together with our data, these findings suggest a role for decorin in both peripheral nerve development and regeneration.

Integrin α1 also appears to be up-regulated at the mRNA level in this transition. Integrin α1 is known to be expressed in the chick neural crest but is not expressed in Schwann cell precursors (Stewart et al., 1997). In adult nerve it is restricted to non-myelinating Schwann cells (reviewed in Previtali et al., 2001). It is possible that the up-regulation that we observed represents integrin α1 expression by cells that will be destined to become non-myelinating ones.

Two transcription factors, Cebpd (Cebp/Delta, CELF, CRP3) and NF1-X are switched on, although at low levels, in the transition from precursors to Schwann cells. Cebpd has been previously detected as increasing in embryonic Schwann cells compared with neural crest cells (Buchstaller et al., 2004) and in mouse peripheral nerve during myelination (Verheijen et al., 2003). Depending on the cell type and physiological conditions, Cebpd has been shown to act on cell proliferation, differentiation and apoptosis (Darlington et al., 1998; Menard et al., 2002). Recent in vitro work has demonstrated that, in a melanoma cell line and in dissociated DRG cells, Cebpd can induce myelin gene expression and suppress the expression of GFAP and Pax-3 (Kamaraju et al., 2004). Taken together these data suggest a role for Cebpd in the onset of myelination.

NF1-X belongs to the nuclear factor 1 (NF1) family of transcription factors, which are widely expressed in various tissues and regulate the transcription of many genes (Paonessa et al., 1988; Roulet et al., 1995). NF1-X binds with high affinity to the regulatory region of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Osada et
al., 1999), a gene involved in cholesterol metabolism, and could therefore be involved in cholesterol synthesis during myelination.

Overall, it was somehow surprising not to find any myelin-related gene as induced in immature Schwann cells. A more careful analysis of the raw data revealed that most of them (P0, MBP, periaxin, Krox-20 and Oct-6 among the others) were absent at E14 and present at E18 (based on the “call” with Affymetrix Suite 5.0; data available upon request in the MEditor database, ICH, London). However, the levels of expression at E18 were low for all of them, and this caused their exclusion after the statistical test (p<0.05).

Genes regulated during myelination and after nerve injury

The genes that are directly involved in myelination are massively up-regulated in Schwann cells during the first three weeks after birth (reviewed in Jessen and Mirsky, 2002, 2004; Garbay et al., 2000). Following nerve damage, in a process known as Wallerian degeneration, the Schwann cells in the distal stump de-differentiate, proliferate and strongly down-regulate all the myelin-related genes (reviewed in Muller and Stoll, 1998; Scherer and Salzer, 2001). In our experimental conditions we found that 378 genes were differentially regulated between E18 and P12. The vast majority of them (361) was up-regulated, and only 17 were down-regulated.

Genes down-regulated between E18 and P12

Among the down-regulated ones we found the neurofilament middle chain (NF-M), that we already found down-regulated between E14 and E18, and the calcium dependent adhesion molecule N-cadherin. The expression of N-cadherin has previously been
shown to be sharply down-regulated *in vivo* at the precursors to Schwann cell transition, and it appears to mediate adhesion between Schwann cells (K.R. Jessen, personal communication; Wanner and Wood, 2002).

The mRNA for cyclin E was also found as suppressed between E18 and P12. The down-regulation of genes involved in cell cycle is in agreement with the observation that while immature Schwann cells are actively proliferating, myelinating Schwann cells are quiescent (reviewed in Jessen and Mirsky, 2004). Various cell cycle related genes have been detected as down-regulated between E17 and P0 in expression profiling experiments in mouse (Verheijen *et al.*, 2003). Moreover, cyclin E has been indicated as the major down-stream target of cyclin D1 (Geng *et al.*, 1999), that we found down-regulated in the transition from E14 to E18, suggesting a possible cascade of event involved in blocking Schwann cell proliferation prior the onset of myelination. However it is important to notice that cyclin D1 is not required for Schwann cell proliferation during development (Kim *et al.*, 2000).

**Genes up-regulated between E18 and P12 and down-regulated after nerve cut**

237 genes were statistically changed after nerve cut. 162 of them were down-regulated and 175 were up-regulated. Importantly, 110 out of the 162 genes/ESTs down-regulated after nerve cut, were also induced during the active phases of myelination, a combination that strongly indicates that they are myelin related.

As expected, in this category we found numerous genes already known to be involved in myelin synthesis, including the transcription factors Oct6 and Krox20, the myelin proteins PLP, MAG, periaxin, plasmolipin and MAL, as well as many genes related to cholesterol and lipid metabolism.
Previous studies had indicated that the expression of the transcription factor Oct6 peaks around birth and is subsequently rapidly down-regulated (Monuki et al., 1990, Scherer et al., 1994, Arroyo et al., 1998). Moreover, nerve cut in adult nerve induces a transient up-regulation of Oct-6 around two days after injury (Monuki et al., 1990, Scherer et al., 1994). If the sciatic nerve is cut at P1, Oct-6 expression is instead sharply down-regulated (Scherer et al., 1994). Somehow surprisingly, we found that Oct-6 is strongly expressed at least until P12, and according to the hybridisation's result, its expression is actually stronger at P7 and P12 than not at E18 (see Table 1), in agreement with previous work showing that Oct-6 immunoreactivity is present in Schwann cells at P12 (Blanchard et al., 1996). In our conditions, Oct-6 is down-regulated even when the injury is performed at P7, indicating that Oct-6 expression may need to remain sustained during myelination.

Perhaps even more surprising was the finding that Sox-10 is down-regulated after cut of myelinating nerves. As expected we found that Sox-10 expression is down-regulated from E14 to E18 (above) and from E18 to P12, but our result also indicate that the mRNA levels for Sox-10 remain sustained during myelination and are suppressed by loss of axonal contact. These findings are in agreements with previous observation indicating that Sox-10 binds P₀ and Cx32 promoters and could therefore be implicated in the transcriptional control of at least these two proteins in peripheral myelin (Peirano et al., 2000; Bondurand et al., 2001).

The use of Biopendium allowed us to map and annotate to proteins most of the ESTs that we found statistically changed between P12 and P12 cut. Two of them were particularly interesting, the ESTs AA639533 and AA894345.
The EST AA639533 clustered, in the K-means clustering, with the myelin-related genes PLP, periaxin, Krox-20 and HMGCS1 (see Fig. 3.4 and Supplemental Material), indicating a possible role in myelination for this gene. Biopendium annotated this EST to an uncharacterized mouse protein that shares structural homology with bacterial phospholipase D. It is therefore possible that this novel protein has a phospholipase-like function during myelination. Phospholipase D catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline in response to various signals, including neurotransmitters, hormones and growth factors (Exton, 1997). Phosphatidic acid is then broken down into two second messengers, diacylglycerol and lysophosphatidic acid (LPA). LPA, a normal constituent of serum, has various effects on Schwann cells, including survival, actin cytoskeleton reorganisation and myelin genes synthesis (Li et al., 2003). It is therefore possible that this new phospholipase D is involved in some of these complex events.

The EST AA894345 clustered alongside numerous genes involved in lipid metabolism. Semiquantitative RT-PCR experiments confirmed its regulation after nerve cut and suggested that the gene product is enriched in myelinating Schwann cells and up-regulated after db-cAMP treatment of cultured Schwann cells with cAMP, a series of findings that strongly point to this gene as myelin-related. Biopendium mapped the EST AA894345 to the gene encoding for a phosphoprotein enriched in astrocytes of 15kDa (PEA15). PEA15 was initially identified as a target of protein kinase C in astrocytes (Araujo et al., 1993). It is also called PED, for phosphoprotein enriched in diabetes. In fact it has been found to be up-regulated at both the mRNA and protein levels in various tissues, including adipose tissue and skeletal muscle, in patients with type II diabetes mellitus (Condorelli et al., 1998). It should be noticed that more than 30% of patients with type II diabetes mellitus develop also a peripheral neuropathy. This observation,
taken together with our data that indicate that PEA15 is highly expressed in myelinating
Schwann cells make PEA15 an interesting candidate for further studies in this field. It
has also been shown that PEA15 could have a role in controlling cell proliferation by
preventing ERK localisation in the nucleus (Formstecher et al., 2001). In fact, genetic
deletion of PEA15 leads to increased ERK nuclear localisation and c-Fos induced
proliferation. Therefore PEA15 could be part of the mechanisms that control Schwann
cell proliferation. Finally, the generation of PEA15 null mice has demonstrated that this
protein can also protect astrocytes from tumor necrosis factor (TNF)α induced death in
vitro (Kitsberg et al., 1999). TNFα, in combination with TGFβ, can also induce
Schwann cell death in vitro. It would be interesting to investigate whether PEA15 has a
similar protective role in Schwann cells too.

The presence among the 110 genes of novel genes known to be related to cartilage/bone
synthesis and maintenance or to cytoskeletal reorganization suggests that they may also
have a role in peripheral nerve development and myelination. This group of genes will
be analysed further in chapter 4.

**Genes up-regulated after nerve cut**

Although the identification of genes up-regulated during nerve degeneration was not
one of our goal, a brief analysis of this group of genes was carried on, to validate, based
on the existing literature, our general approach.

The injured nerve is quickly invaded by myelomonocytic cells, in particular
macrophages, that are important in mediate demyelination and influence Schwann cell
proliferation and successive remyelination (reviewed in Kiefer et al., 2001). The
principal role of macrophages is to phagocytose and degrade myelin debris (Stoll et al.,
1989; Perry et al., 1987). It appears that Schwann cells themselves are able to degrade
myelin, but to do so they require the presence of macrophages (reviewed in Fu and Gordon, 1997). Therefore, it was not surprising to find, among the up-regulated genes, molecules involved in the inflammatory response, such as at least two members of the major histocompatibility complex (MHC), also detected as induced after nerve injury in adult mice (Araki et al., 2001). We also identified other molecules involved in the immune response such as the leukocyte common antigen and β2-microglobulin. Moreover, we identified the C4 complement protein, which is known to be activated after peripheral nerve injury (Bonnard et al., 1997).

In agreement with the literature, we also identified molecules that are known to be involved in Schwann cell de-differentiation and proliferation, like the growth associated protein 43 (Gap-43), NGFR, the IL-6R and the transcription factor c-Jun (Hall et al., 1992; Heumann et al., 1997; De Felipe and Hunt, 1994; Stewart, 1995; Shy et al., 1996; Parkinson et al., 2004; reviewed in Scherer and Salzer, 2001).

Similarly to previous work on gene profiling of injured nerve (Kubo et al., 2002; Araki et al., 2001), we detected various molecules involved in protein metabolism as up-regulated. Among them were the cathepsins L and S (Kubo et al., 2002), the matrix-metalloprotease Mmp12 and kallikrein, a trypsin-like serine protease.

We also identified the extracellular matrix protein tenascin (Tnn), which is important in creating a permissive environment for axonal regrowth (Martini et al., 1990; Kiernan et al., 1999). However, we did not identify any of the other adhesion molecules or extracellular matrix molecules known to be involved in the process of regeneration, such as L1, N-Cam, N-cadherins, laminin-2, spondin, P200, fibronectin or collagens (reviewed in Scherer and Salzer, 2001).

There are two possible explanations for this. The first is a technical one: not all of these genes are present on the rat U34 microarray (for example, P200 and laminin-2 are not
present) and therefore for some of them the detection is not possible. The second possible explanation is that since we have harvested the nerves only five days after nerve cut, many of the genes involved in regeneration are not yet expressed at sufficient high levels to be detected as increased.
Figure 3.1. Standard gene expression profiling assay. The basic concept behind the use of GeneChip arrays for gene expression is simple: labelled cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented. Although hybridization has been used for decades to detect and quantify nucleic acids, the combination of the miniaturization of the technology and the large and growing amounts of sequence information, have enormously expanded the scale at which gene expression can be studied. (Adapted from Affymetrix GeneChip Technical Manual).
Total RNA

Reverse Transcription

cDNA

In Vitro Transcription

Biotin-labeled cRNA

GeneChip Expression Array

Hybridization

Fragmentation

Fragmented, Biotin-labeled cRNA

Wash and Stain

Scan and Quantitate
Figure 3.2. Hierarchical clustering, performed with GeneSpring, of the 1609 probes shown to have at least a two fold change at some point in our experimental conditions. The genes are connected iteratively, based on their similarity. The genes with similar expression patterns are grouped together and are connected by a series of branches (clustering tree, on top). With the same method, experiments with similar expression profiles are also grouped together (horizontal bars). The length of each horizontal bar tells how similar the replicates are. Colors: red, high level of expression; yellow, moderate level of expression; blu, low level of expression.
Figure 3.3. Hierarchical clustering of the 130 probes statistically different between E14 and E18. Note the presence of two distinct groups of genes at E14, one with high level of expression (red group, on the upper left side) and one with low level of expression (blue group, upper right side). The genes with the highest level of expression are then massively down-regulated and remain very low in the following time points considered. One the other hand, the genes with low level of expression are only mildly up-regulated at E18 but then their expression gradually increases, reaching the peak at P12. Most of them are then down-regulated following nerve injury. The substantial absence of horizontal bars in between the replicates for E14 and E18 indicates that the results from the replicates are very consistent.
A

M  E14  E18  P7  P12  CUT -

Col2a1
Cry-ab
ChM-I
Coup-TFI
Jip-1b
Sox-9
Maf-2
Snca
Prx
Gapdh

B

P12 SN  P12 SN cut

24  26  28  30  32 cycles

1

2

3

4

M  +  P12 cut

Gapdh
### Table 3.1 LIST OF THE 130 PROBES DIFFERENTIALLY EXPRESSED BETWEEN E14 AND E18

<table>
<thead>
<tr>
<th>Gene Accession</th>
<th>Entrez ID</th>
<th>Description</th>
<th>E14 expression levels</th>
<th>E18 expression levels</th>
<th>Log2 Fold Change</th>
<th>p Value</th>
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<tbody>
<tr>
<td>AAI95569</td>
<td>1046000</td>
<td>Rat tissue factor X</td>
<td>1.26</td>
<td>1.34</td>
<td>0.70</td>
<td>0.08</td>
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<td>L014600</td>
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<td>1.34</td>
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Table 3.2 Mapping and annotation of the 130 genes/EST differentially expressed between E14 and E18

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<tr>
<th>Total number of probes</th>
<th>130 (of which 65 corresponded to ESTs)</th>
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<tr>
<td>probes mapped to proteins</td>
<td>124 (94 mapped with Biopendium, 30 mapped by hand, 6 unmapped)</td>
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<tr>
<td>Proteins with Gene Symbol</td>
<td>110 (99 unique symbols)</td>
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<td>Proteins for which Gene Ontology annotation was available</td>
<td>86</td>
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Table 3.3.1 Examples of genes up-regulated in the E14/E18 transition

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<th>P value</th>
<th>E14 exp</th>
<th>E18 exp</th>
<th>Nr</th>
<th>%</th>
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Set, set in which they cluster (Fig 3.4)
P value, value in the ANOVA/Welch t-test (p<0.05)
E14 exp., raw level of expression at E14
E18 exp., raw level of expression at E18
Nr, number of genes that fall in the category
%, percentage of genes that fall in the category relative to the total number of up-regulated genes
(A), absent.
Table 3.3.2 Examples of genes down-regulated in the E14/E18 transition.

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<th>Set</th>
<th>P value</th>
<th>E14 exp.</th>
<th>E18 exp.</th>
<th>Nr</th>
<th>%</th>
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<td>L10640 Acvrllb</td>
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<td>D83948 Rbm10</td>
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<td>0.03321</td>
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<td>J03969 Npp1</td>
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<td>AF020618 Myd116</td>
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<td>0.03856</td>
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<td>U52013 Crmp-3</td>
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<td>422</td>
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<td>U52102 Crmp-1</td>
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<td>0.02751</td>
<td>336</td>
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<td>0.02072</td>
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<td>X59149 L1</td>
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<td>IMMUNE RESPONSE</td>
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Set, set in which they cluster (Fig 3.4);
P value, value in the ANOVA/Welch t-test (p<0.05);
E14 exp., raw level of expression at E14;
E18 exp., raw level of expression at E18;
Nr, total number of genes that fall in the category;
%, percentage of genes that fall in the category relative to the total number of down-regulated genes.
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<td>M29249cds RAT3H3M Rat 3-hydroxy-3-methylglutaryl coenzyme A reductase gene</td>
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<td>U03416_at</td>
<td>U03416 Rattus norvegicus neuronal ofactomedin-related ER localized protein (D2Sut1e) mRNA</td>
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<tr>
<td>U67995_s_at</td>
<td>U67995 Rattus norvegicus stearyl-CoA desaturase 2 mRNA</td>
</tr>
<tr>
<td>X55286_g_at</td>
<td>X55286 R.norvegicus mRNA for HMG-CoA reductase</td>
</tr>
<tr>
<td>AF003835_at</td>
<td>AF003835 Rattus norvegicus isopentenyl diphosphate-dimethylallyl diphosphate isomerase mRNA</td>
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<tr>
<td>AF007758_g_at</td>
<td>AF007758 Rattus norvegicus synuclein 1 mRNA</td>
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<tr>
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<td>Z49855 R.norvegicus mRNA for plasmolipin</td>
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<td>AF005720mRNA#3 Rattus norvegicus chloride channel (CIC-2) gene</td>
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<tr>
<td>A224879_at</td>
<td>A224879 Rattus norvegicus mRNA for collagen alpha 1 type II</td>
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<tr>
<td>M72711_at</td>
<td>M72711 Rat transcriptional repressor of myelin-specific genes (SCIP) mRNA</td>
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<td>rc_AA892496 EST196299 Rattus norvegicus cDNA</td>
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<td>rc_AI172293 EST218294 Rattus norvegicus cDNA</td>
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<td>rc_AI180442 EST224188 Rattus norvegicus cDNA</td>
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<tr>
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<td>S79711 CD3 gamma-chain</td>
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<td>U02983 Rattus norvegicus secretogranin III (Sglll) mRNA</td>
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<td>AJ001029_at</td>
<td>AJ001029 Rattus norvegicus mRNA for Sox10 protein</td>
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<td>Z12152_s_at</td>
<td>Z12152 R.norvegicus mRNA for neurofilament protein middle (NF-M)</td>
</tr>
<tr>
<td>D30666_s_at</td>
<td>D30666 Rat mRNA for brain acyl-CoA synthetase II</td>
</tr>
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<td>D37920_s_at</td>
<td>D37920 Rat mRNA for squalene epoxidase</td>
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<td>L12016_s_at</td>
<td>L12016 Rat tricarboxylate transport protein mRNA</td>
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<tr>
<td>M17527_s_at</td>
<td>M17527 Rat GTP-binding protein (G-alpha-i1) mRNA</td>
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<td>M60322_s_at</td>
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<td>rc_AI638993_s_at</td>
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<td>U75916 Rattus norvegicus zonula occludens 2 protein (ZO-2) mRNA</td>
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<td>L01624 Rattus norvegicus serum and glucocorticoid-regulated kinase (sgk) mRNA</td>
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<td>L13619 RATC66A Rattus rattus insulin-induced growth-respons protein (CL-6) mRNA</td>
</tr>
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<td>M15883 Rat clathrin light chain (LClb2) mRNA</td>
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<td>MS5534mRNA Rat alpha-crystallin B chain mRNA</td>
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<td>M62763completemRNA</td>
<td>M62763completemRNA Rat 60 kDa protein and non-specific lipid transfer protein mRNA</td>
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<td>M73714_at</td>
<td>M73714 Rat microsom aldehyde dehydrogenase mRNA</td>
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<tr>
<td>M89945mRNA_g_at</td>
<td>M89945mRNA RATFARDIPH Rat farnesyl diphosphate synthase gene</td>
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<tr>
<td>M95591_at</td>
<td>M95591 RATSSST Rattus rattus hepatic squalene synthetase mRNA</td>
</tr>
<tr>
<td>M95591_g_at</td>
<td>M95591 RATSSST Rattus rattus hepatic squalene synthetase mRNA</td>
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<td>rc_AA91797_at</td>
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Table 3.5. Mapping and annotation of the 237 genes/ESTs differentially expressed between P12 and P12cut

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<td>Probes mapped to proteins</td>
<td>233 (194 mapped with Biopendium, 39 mapped by hand, 4 unmapped)</td>
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<td>Proteins for which Gene Ontology Annotation was available</td>
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Set, set in which they cluster (Fig.3.11);
P value, value in the ANOVA/Welch t-test (p<0.05);
P12 exp., raw level of expression at P12;
P12 cut exp., raw level of expression after nerve cut;
Nr, number of genes that fall in the category;
%, percentage of genes that fall in the category relative to the total number of up-regulated genes.
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CHAPTER 4

Novel cartilage/bone related and cytoskeletal genes identified in the peripheral nervous system

INTRODUCTION

In chapter 3 we have seen that, along with the expected myelin-related genes, a number of genes not previously characterised in Schwann cells were up-regulated during development and suppressed after nerve injury, a combination that points to them possibly being involved in myelination. Two groups were particularly interesting, due to the high level of expression and dramatic down-regulation. The first, including collagen alpha1 type II and chondromodulin-1, is composed of genes that encode for proteins known to localise in the extracellular matrix (ECM) in cartilage and/or bone, and that are involved in both the development and maintenance of these two tissues. The second is a small group of genes, such as synuclein-1 and alpha-B-crystallin, encoding for proteins that localise in the cytoplasm and could be related to cytoskeleton rearrangements and to signalling (see Table 3.6.2).

Both the ECM and cytoskeleton play crucial roles during nerve development and myelination, which makes these two groups of genes even more interesting.

The ECM in peripheral nerve

The ECM is a fundamental component of the PNS, where it is involved in cell migration, proliferation and maintenance of cell shape. The components of the ECM, collagens, glycoprotein and proteoglycans, are synthesised by the surrounding cells, and form a complex lattice that provides mechanical strength and acts as a guide for the developing neural crest (Chernousov and Carey, 2000). The ECM, and in particular
fibronectin and laminins, creates a permissive substrate for axon migration, facilitating nerve development (Krull and Koblar, 2000). Other molecules, such as tenascin-C and collagen types II, III and IX are non-permissive during neural crest migration and axon pathfinding (Krull and Koblar, 2000; Perris, 1997). Finally, molecules such as aggrecans have an inhibitory role in neural crest migration, meaning that they can either directly or indirectly interact with the neural crest and impede cell motility (Perris, 1997). Neural cells interact with the ECM via integrins (see chapter 1), which in turn interact with the cytoskeleton and trigger intracellular signalling pathways (Previtali et al., 2001).

Collagens

Collagens are a major constituent of the mammalian ECM. So far at least 19 different collagen molecules have been identified, and can be subdivided into fibrillar and non-fibrillar collagens. In fibrillar collagens, such as type I, II and III, a highly stable triple helix forms cable like molecules which confer tensile strength to structures like tendons and ligaments. Non-fibrillar collagens, like collagen type IV, act as adaptors between matrix components (Carey et al., 1983).

Many collagens are expressed throughout the nervous system. In mouse, endoneurial collagen type III appears around E15, and seems to be synthesised by Schwann cells (Osawa and Ide, 1986). Type I collagen is instead found mainly in the epineurium, and its marked reduction could be at the base of the disrupted integrity of the blood-nerve barrier in the desert hedgehog null mouse (Osawa and Ide, 1986; Parmantier et al., 1999).

Collagen type IV is highly expressed in peripheral nerve, where it forms stable sheets within basal laminae (Carey et al., 1983). Together with laminin, collagen type IV is
organised in a network like structure that provides physical support to the endoneurium and forms a potential basis for the selective barrier function of the perineurium (Williams et al., 2000).

Schwann cells also synthesise collagen alpha 4 type V (also known as p200), which binds with high affinity to heparin-like glycosaminoglycans (Chernousov et al., 2000). In culture, this collagen actively promotes the migration of pre-myelinating Schwann cells, but inhibits axonal outgrowth (Chernousov et al., 2001). This difference could facilitate Schwann cell ensheathment of axons and at the same time restrict the movement of axons promoting fasciculation (Chernousov et al., 2001).

Another collagen expressed by Schwann cells is collagen alpha 1 type VI (Col6α1) (Jaakkola et al., 1989). It interacts with many other components of the ECM, including collagen IV, fibronectin, biglycan and decorin (Vitale et al., 2001). Collagen VI is not expressed by the neural crest, but is actively synthesised by Schwann cells from around E15 (in the mouse) onwards and the activation of its transcription is stimulated by neuregulin, as part of the differentiation program from precursors to Schwann cells (Vitale et al., 2001). After the acquisition of the Schwann cell phenotype, the expression of col6α1 becomes independent of neuregulins, and is maximised when the cell withdraws from the cell cycle (Vitale et al., 2001).

More recently, collagen alpha 1 type XIII has been localised to CNS neurons and to developing PNS (Sund et al., 2001). Its function is not clear yet, but it has been suggested that it may facilitate cell-cell and cell-ECM interactions (Kvist et al., 2001).

**Fibronectin**

Schwann cell synthesise a fibrillar network consisting of laminin, fibronectin and collagen type IV, which is believed to be involved in Schwann cell proliferation
immediately before myelination (Chemousov et al., 1998). Fibronectin deposition depends on the presence of collagen IV in the ECM. Treatment with collagenase dramatically decreases the amount of fibronectin fibrils, and Schwann cells cultured on collagen IV coated coverslips deposit a rich network of fibronectin (Chemousov et al., 2001). Fibronectin null mice die during gestation, probably as a result of cardiovascular defects (George et al., 1993; 1997). Development of the nervous system is also disrupted, with absence of the notochord and incomplete closure of the neural tube (George et al., 1993).

**Tenascins**

The tenascin family consists of five members, tenascin-C, R, X, Y and W. They are involved in many events such as morphogenesis, tumor metastasis and neural migration (Mackie and Tucker, 1999). Tenascin-C and -R contain repetitive fibronectin III and EGF-like domains and, along with tenascin-Y are expressed in the nervous system (Joester and Faissner, 2001). Tenascin-R is expressed mainly in the CNS by oligodendrocytes during myelination (Xiao et al., 1997), while the expression of tenascin-Y has been detected in the developing PNS in chick, although its function remains obscure (Joester and Faissner, 2001). Tenascin-C is expressed in both CNS and PNS. It has the ability to both stimulate and inhibit growth cone extension and is actively involved in sciatic nerve regeneration (Joester and Faissner, 2001). Surprisingly, tenascin-C null mice did not present any obvious phenotypic abnormality (Forsberg et al., 1996; Kiernan et al., 1999), suggesting a possible redundancy of tenascin-C resulting in the ability of tenascin-R or others to take its place. However, a more careful analysis of the tenascin-C null mice revealed more subtle phenotypes, such as deficits in coordination and hyperlocomotion (Kiernan et al., 1999).
**Laminins**

As we have already seen in the general introduction, Schwann cells synthesise large amounts of laminins, which are one of the major components of the basal lamina (Cornbrooks et al., 1983; Podratz et al., 2001). In the ECM, laminins associate with collagen IV; integrins and dystroglycan are the major receptors for laminins on the Schwann cell surface (reviewed in Previtali et al., 2001). Laminin-2 is produced by Schwann cells and appears to be involved in nerve regeneration (Kamiguchi et al., 1998). Aberrant expression of laminins has been associated with various muscular dystrophies in which normal nerve development, regeneration and function are impaired (Gustafsson and Fassler, 2000; Uziyel et al., 2000).

**Proteoglycans**

In recent years the interest in proteoglycans in the nervous system has increased dramatically. This is mainly due to the idea that the environment that favours nerve regeneration may involve proteoglycans that have undergone enzymatic cleavage (Ferguson and Muir, 2000; reviewed in Hartmann and Maurer, 2001). Among the proteoglycans with emerging roles in the PNS are the testicans (Hartmann and Maurer, 2001), although the expression of most proteoglycans in sciatic nerve remains to be established. In contrast, in the CNS proteoglycan turnover is not present and this could be one of the reasons for the lack of neuronal regeneration.
MMP and TIMPs

The ECM in peripheral nerve is also rich in matrix metalloproteinases (MMP) and in tissue inhibitors of matrix metalloproteinase (TIMPs). The role of MMP is probably that of remodelling the ECM to allow processes such as migration, wound repair and cell death, necessary to maintain the health of the individual (reviewed in Murphy and Gavrilovic, 1999). MMP are endopeptidases that can degrade many constituents of the ECM. They are synthesised as inactive precursors and become active after removal of the propeptide (Sternlicht and Werb, 2001). Excessive activity of MMPs is limited by TIMPs (Baker et al., 2002). MMP-2, -3, -9 and -13 are expressed in the nervous system during normal development and after injury, as well as in some disease states, such as after ischaemia (reviewed in Platt et al., 2001).

The role of cytoskeleton in myelination

There is a strong correlation between the ECM and Schwann cell cytoskeleton. As we have already mentioned in Chapter 1, myelinating Schwann cells polarize along the axon and the abaxonal membrane directs the formation of the basal lamina, which is essential for myelination (Bunge et al., 1986; Bunge 1993). Laminin 2 associates on the Schwann cell surface with integrins, in particular with integrin α6β1, and it appears likely that integrin β1 is necessary to link the laminin-2 in the basal lamina to the cytoskeleton (Bunge 1993; Feltri et al., 2002). The severe phenotype observed in both laminin-2 and β1mutants (see chapter 1) suggests that the laminin-integrin complex is fundamental for the proper onset of myelination (Uziyel et al., 2000; Feltri et al., 2002; reviewed in Previtali et al., 2001; Jessen and Mirsky, 2004).
Neuron-Schwann cell co-cultures have demonstrated that integrin β1 interacts with the actin-linked protein paxillin and with focal adhesion kinase (Chen et al., 2000). Interestingly, in vitro experiments showed that the disruption of the actin cytoskeleton blocks myelination (Fernandez-Valle et al., 1997). In turn, paxillin binds merlin (also known as Schwannomin), the product of the neurofibromatosis type 2 tumor suppressor gene, a protein that could have a role in the control of Schwann cell proliferation. Merlin, which is related to the ezrin/moesin family of protein, localises in paranodes and Schmidt-Lanterman incisures, and interacts with spectrin, a cytoskeletal protein that can bind actin. This interaction results in an indirect link between merlin and actin, and treatment of Schwann cells with antisense nucleotide to merlin cause dramatic changes in the cytoskeleton (Scherer et al., 2001; Scoles et al., 1998). As mentioned in Chapter 3, the actin cytoskeleton can also be influenced by treatment of cultured Schwann cells with LPA and sphingosine 1-phosphate (SIP) (Weiner et al., 2001; Li et al., 2003; Barber et al., 2004). Both LPA and SIP determine rearrangements in actin cytoskeleton via the small GTPase Rac1 (Barber et al., 2004). LPA also induces focal adhesion of paxillin and vinculin, and induces N-cadherin/catenin mediated Schwann cell-Schwann cell interactions (Wanner and Wood, 2002). Rho and its downstream effector Rho kinase (ROCK) have been shown to be important regulators of the Schwann cell cytoskeleton: pharmacological inhibition of ROCK results in loss of stress fibres and microvilli in Schwann cells and in aberrant myelination in neuron-Schwann cell cocultures (Melendez-Vasquez et al., 2004). Actin also binds dystonin, and mice with mutations in this cytoskeletal protein have an abnormal myelination and disorganized cytoskeleton (Bernier et al., 1998).

Besides integrins, laminins also bind to dystroglycan. Mice in which dystroglycan has been specifically ablated in Schwann cells present severe neurological dysfunction,
have slow conduction velocity accompanied by disorganized microvilli, and reduced
sodium channel density at the Node of Ranvier (Saito et al., 2003). In skeletal muscle, α
and β-dystroglycans form complexes with the dystrophin-like family of proteins, which
comprises dystrophin, utrophin (DRP1) and dystrobrevin. These complexes link the
ECM to the cortical actin cytoskeleton (reviewed in Sherman and Brophy, 2004).
Schwann cells highly express another member of the dystrophin-like family, DRP2
(Sherman et al., 2001). DRP2-dystroglycan complexes bind the cytoskeletal protein L-
periaxin (see chapter 1), and this binding is necessary to stabilize axon-glia interaction
(Gillespie et al., 2000; Sherman et al., 2001). While periaxin is already present during
embryonic development, dystroglycan appears perinatally, and DRP2 soon after birth
(Sherman et al., 2001). It is possible that the formation of the dystroglycan-DRP2-
periaxin complex alters the cytoskeletal organisation at the moment when Schwann
cells exit the cell cycle and begin to synthesise myelin (Wrabetz and Feltri, 2001).
Moreover, recent studies have demonstrated the importance of the complex for the
formation of cytoplasmic channels and for internodal length (Court et al., 2004).

Taken altogether, these data show that ECM and cytoskeleton play a crucial role in
many aspects of Schwann cell biology, such as migration, proliferation, differentiation
and myelination. The identification of new ECM and cytoskeletal proteins could be
very helpful in the understanding of these events.
RESULTS

Cartilage gene expression during peripheral nerve development

Probably the most surprising finding in our array profiling was that a group of genes, relevant for cartilage and bone formation, was developmentally regulated in myelinating nerves. Collagen alpha1 type II (Col2a1), Chondromodulin-1 (ChM-1) Osteonectin (SPARC), Osteoadherin (Omd), and bone sialoprotein II (Ibsp) increased in expression levels during nerve development up to P12, and were strongly down-regulated after nerve cut (Table 4.1).

Col2a1 and ChM-1 RT-PCRs

Table 4.1 shows that Col2a1 and ChM-1 were, among the cartilage related ones, the two genes with the highest mRNA level of expression, and with the most dramatic fold change following nerve transection. For these reasons we chose to analyse them further. In Chapter 3 we have seen that RT-PCR experiments confirmed their regulation during development and after nerve cut (Fig 3.13A). While Col2a1 appeared to be evenly distributed between myelinating and non-myelinating cells, ChM-1 seemed to be enriched in myelinating Schwann cells (Fig 3.14), and both genes were up-regulated after stimulation of cultured Schwann cells with dbcAMP (Fig 3.15).

In situ hybridization and multiplex RT-PCR showed that type IIA is the Col2a1 isoform expressed in peripheral nerve

Col2a1 can be generated in two isoforms, due to alternative splicing of the precursor mRNA. Type IIA, the form containing a cysteine-rich domain in the NH2-propeptide (exon 2), is expressed by precartilage and also by noncartilage epithelial and

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mesenchymal cells, while type IIB, in which exon 2 is absent, is characteristic of chondrocytes only (Sandell, 1994; Zhu et al., 2001). *In situ* hybridisation on sections from P1 sciatic nerves, performed with a probe that recognises the fibrillar part of the collagen, and thus both isoforms, and with another probe specific for exon 2, showed a clear signal in both cases, suggesting that the IIA isoform is the main one in peripheral nerve (Fig 4.1A). To confirm this result we performed multiplex RT-PCR experiments (as in Urabe et al., 2003), which clearly showed that the expression of the IIB isoform is only marginal compared to that of the IIA form (Fig 4.1B).

**Type II collagen protein is down-regulated after nerve injury**

The down-regulation of Col2a1 after nerve cut was confirmed at the protein level too: Western blot experiments, performed with an antibody that recognises specifically the type IIA collagen, showed a band of the expected size in P12 sciatic nerve extracts, a band which is strongly reduced in extracts from nerves subjected to transection (Fig 4.2A). Often, two bands of smaller molecular weight were also visible, both in whole sciatic nerve extract and in protein samples from immunopurified Schwann cells (Fig 4.2B). These bands are likely to represent cleaved NH₂-propeptides of the type IIA collagen, as bands of similar molecular weight have been seen in serum (L. Sandell, personal communication) and after treatment of type IIA procollagen with matrix metalloproteinases (MMPs)(Fukui et al., 2002).

**Col2a1 expression during Schwann cell development**

The array results suggested that Col2a1, at the mRNA level, was expressed at least as early as E14. To assess when the protein was expressed, we immunolabeled a series of cell cultures. These immunolabellings showed that the protein was already detectable in
the migrating neural crest and its expression was maintained throughout development (Fig 4.3). In immature Schwann cells examined in teased E18 nerves, the protein was mainly found in the perinuclear area, while in mature myelinating fibres Col2a1 accumulated in the paranodal region. Prominent staining was also detectable between tightly apposed fibres in teased adult nerves (Fig 4.4).

**Col2a1 is evenly expressed in myelinating and non-myelinating Schwann cells**

It was interesting to notice that although Col2a1 seemed to be equally distributed in myelinating and non-myelinating Schwann cells (Fig 3.14), its expression was completely abolished by nerve cut at both mRNA and protein level (Fig 3.13A and 4.2), suggesting an axonal regulation in both cell types. In order to confirm that Col2a1 is expressed by both Schwann cell lineages, we immunolabelled a series of teased fibres and cultured Schwann cells from the sympathetic trunk, were most of the cells are of the non-myelinating type. These experiments confirmed that Col2a1 is widely expressed in non-myelinating Schwann cells *in vivo* and *in vitro* (Fig 4.5).

**The transcription factor Sox9 is expressed and regulated in peripheral nerve**

As already mentioned, to date, only three transcription factors, Sox10, Oct-6 and Krox-20 have been shown to be of crucial importance for Schwann cell development and myelination *in vivo*. It is therefore highly likely that other transcription factors not yet identified concur in the regulation of these complex events. The transcription factor Sox9 is required for neural crest development and glial fate choice, and controls Col2a1 expression during chondrocyte differentiation (Cheung and Briscoe, 2003; Stolt *et al.*, 2003; Bell *et al.*, 1997; Akiyama *et al.*, 2002). Therefore we asked if it could have a similar function in Schwann cells. Sox9 is not present on the Affymetrix U34A array,
therefore as a preliminary experiment we performed a series of RT-PCRs to determine whether it was expressed in peripheral nerve. Fig 3.13A (Chapter 3) shows that Sox9 mRNA is expressed as early as E14 and that it decreases, although not dramatically, after sciatic nerve injury. Intriguingly Maf-2, a transcription factor that collaborates with Sox9 in controlling Col2a1 (Huang et al., 2002), was also detected in our screening and decreased after nerve transection, a finding confirmed by RT-PCR (Fig 13.3A) that suggest a possible interaction of these transcription factors in controlling Col2a1 in peripheral nerve.

**Sox-9 protein expression in Schwann cells**

To verify whether Sox-9 mRNA expression in the peripheral nerve reflected a true protein expression, we performed immunolabelling on cell cultures and on teased nerve preparations. These experiments showed that the protein was expressed in Schwann cell precursors and in Schwann cell cultures from new-born sciatic nerves, with only marginal expression detectable in fibroblasts (Fig 4.6). In adult nerves Sox9 remained strongly expressed, although, contrary to earlier stages of development, it seemed to accumulate more in the perinuclear area than in the nucleus, possibly indicating an inactive state (Fig 4.7).

**Cytoskeletal genes in peripheral nerve: Snca and Cry-ab**

It was extremely interesting to notice that two of the genes that were strikingly up-regulated during myelination and down-regulated in cut sciatic nerves, αSynuclein (Snca) and alpha-B-crystallin (Cry-ab) (Table 4.2), colocalize in glial cytoplasmic inclusions in multiple system atrophy (MSA), and have both been implicated in neurological diseases, including Alzheimer's and Parkinson's diseases (Gai et al., 1999;
Kahle et al., 2002; reviewed in Eriksen et al., 2003). Immunoreactivity to Snca in Schwann cells has been previously reported (Mori et al., 2002), and Snca was detected in array profiling of mouse sciatic nerve (Nagarajan et al., 2002), while Cry-ab is constitutively expressed in the lens of the eye, in kidney epithelium, and has been identified as a candidate autoantigen in multiple sclerosis (van Noort et al., 1995). However, the expression of these proteins in peripheral nerve has not been investigated in detail and their roles remain unknown.

As described earlier, RT-PCR experiments showed a weak band corresponding to Snca at E14, while it was clearly expressed at E18 and found at much higher levels at P7 and P12, with sciatic nerve cut resulting in a large down-regulation of mRNA levels. Cry-ab was expressed later in development, with a clear band visible only at P7, and there was a significant down-regulation in the cut nerve (Fig. 3.13A). Snca appeared to be enriched in myelinating Schwann cells at P3, while Cry-ab was equally expressed in myelinating and non-myelinating Schwann cells at that stage. Snca, but not Cry-ab, was up-regulated after treatment of cultured Schwann cells with dbcAMP (Fig 3.14 and 3.15).

**Snca and Cry-ab proteins are down-regulated after nerve cut**

Western blot and immunolabelling experiments confirmed that Snca and Cry-ab were highly expressed in mature nerves, and that the down-regulation after nerve injury happens at the protein as well as mRNA level (Fig 4.8). These western blot experiments also suggested that Schwann cells are the main cell type to express the proteins in peripheral nerve, since the protein levels in immunopanned Schwann cell cultures are comparable to those in the whole sciatic nerve before injury.
Snca and Cry-ab expression in adult sciatic nerves

To localise the proteins expression in adult sciatic nerve, teased nerve preparation were immunolaballed with antibodies against Snca and Cry-ab. Cry-ab immunolabelling was detected in the perinuclear area and in the cytoplasmic collar but not in Schmidt-Lanterman incisures, while these structures showed Snca immunoreactivity (Fig 4.9). To confirm this finding, we carried out double immunolabelling with MUPP-1 and neurofascin (Nfasc), two proteins known to locate in incisures and in paranodal loops (Poliak et al., 2002; Tait et al., 2000). Figure 4.10 shows that Snca colocalised with MUPP-1 and Nfasc in incisures and at nodes of Ranvier, suggesting a possible role for synuclein in the cyto-architecture of these complex structures.

Snca protein expression is developmentally regulated and enriched in myelinating Schwann cells

The RT-PCR experiments previously performed (Fig 3.13 and 3.14) suggested that Snca expression is regulated during development and is higher in myelinating Schwann cells than in non-myelinating ones. To verify whether the developmental regulation was detectable at the protein level too, we cultured E14 Schwann cell precursors in the presence of β-neuregulin, a condition under which they convert to Schwann cells with a time course that is broadly similar to that with which Schwann cells appear in embryonic nerves in vivo (Dong et al., 1995). In line with RT-PCR data, we found only very low Snca immunoreactivity in E14 Schwann cell precursors. When E14 precursors were maintained in β-neuregulin for 4 days allowing them to convert to Schwann cells, Snca immunoreactivity reached the levels seen in immature Schwann cells from E18 nerves (Fig 4.11). Moreover, to confirm the enrichment of Snca in myelinating cells, we performed double immunolabelling of cultured Schwann cells from the sympathetic
trunk with antibodies against Snca and against the myelinating Schwann cell specific protein periaxin (Prx). These experiments showed that Snca in expressed mainly by myelinating Schwann cells, although it can also be occasionally found at lower levels in non-myelinating Schwann cells (Fig4.12). Similar staining performed with antibodies against Cry-ab and the Schwann cell marker S100, showed that Cry-ab is equally expressed by the two Schwann cell types (Fig4.13), in agreement with the RT-PCR results.

**Snca -/- mice do not show any gross abnormalities in peripheral nerve**

The mouse C57BL/6J inbred strain from Harlan UK has a ~2cM deletion in the synuclein-1 locus that results in the complete inactivation of the gene (Specht and Shoepfer, 2001). We therefore analysed the morphology of the sciatic nerve in these mice, to test whether Snca could play a role either during myelination or in the maintenance of the myelin sheath. Semi thin sections were obtained from adult (3-6 month old) sciatic nerves and stained with toluidine-blue (Fig.4.14). No gross abnormality in the nerve structure or in myelin thickness was identified in any of the animals analysed compared to control mice.

As we have seen previously, in adult rat sciatic nerve, Snca localises in Schmidt-Lantermann incisures and in nodes of Ranvier. To test whether the lack of Snca could result in an impairment in these structures, we immunolabelled teased nerve fibres from Snca -/- and control mice with antibody against Snca, Nfasc and Mupp-1. Fig. 4.15 shows that in the absence of Snca, Mupp-1 and Nfasc appear to localise correctly in Schmidt-Lantermann incisures and nodes, indicating that Snca is probably dispensable for the cyto-architecture of these structures.
DISCUSSION

As mentioned in Chapter 3, in our experimental conditions we found that 110 out of the 162 genes/ESTs down-regulated after nerve cut, were also induced during the active phases of myelination, a combination that strongly indicates that they are myelin related. As expected, we found numerous genes already known to be involved in myelin synthesis, including the transcription factors Oct6 and Krox20, the myelin proteins PLP, MAG and plasmolipin, as well as many genes related to cholesterol and lipid metabolism. The presence in the same group of novel genes known to be related to cartilage/bone synthesis and maintenance or to cytoskeletal reorganization suggests that they may also have a role in peripheral nerve development and myelination.

Cartilage/bone related genes in peripheral nerve

Collagen alpha1 type II (Col2a1)

Type II collagen is a major component of cartilage and provides structural integrity to the tissue. Type II procollagen can be expressed in two forms by differential splicing of the primary gene transcript. The two mRNAs either include (type IIA) or exclude (type IIB) exon 2, which encodes the major portion of the amino (NH2)-propeptide (Ryan and Sandell, 1990). The expression of type IIB is spatially correlated with the high level expression of the cartilage proteoglycan aggrecan, establishing type IIB procollagen and aggrecan as markers for the chondrocyte phenotype (Sandell et al., 1991). Transcripts of type II collagen, primarily type IIA, are also expressed in embryonic spinal ganglion and in other tissues, including the notochord, during development (Sandell et al., 1991; Ng et al., 1993; Sandell et al., 1994; Oganesian et al., 1997).

Mutations in collagen type II in humans cause a variety of diseases such as
achondrogenesis, hypochondrogenesis, Stickler syndrome, Kniest dysplasia, Wagner syndrome and spondyloepiphyseal dysplasia (SED) (Chan et al., 1995; Horton et al., 1992; Ahmad et al., 1993; Wilkin et al., 1999). In many cases the patients present also ocular abnormality, such as myopia and retinal detachment, probably due to the fact that type II collagen is also expressed in the vitreous (Ahmad et al., 1991; Korkko et al., 1993). Collagen type II seems to be also involved in the sensorineural deafness that accompanies SED and Stickler syndrome and maybe the target of an autoimmune process in cases of acquired bilateral sensorineural hearing loss (Helfgott et al., 1991). Mice lacking type II collagen produce abnormal cartilage, develop a skeleton without endochondral bone and are unable to dismantle the notochord. They are usually delivered vaginally but die either immediately before or soon after birth (Li et al., 1995; Aszodi et al., 1998).

Our findings show that type IIA collagen is expressed in peripheral nerve by the Schwann cell lineage during embryonic development and by both myelinating and non-myelinating Schwann cells and is regulated by axonal signals. The expression of col2a1 by the neural crest is surprising since type II collagen is supposed to be non-permissive for crest cell migration (Perris, 1997). However, it is possible that the deposition of type II collagen is needed to prevent improper migration of the cells, and that col2a1 is therefore involved, together with molecules such as tenascins and aggrecans in “migratory directionality” (Perris, 1997). The expression of col2a1 increases during peripheral nerve development. The most obvious role that we can infer is a structural one. However, we can also speculate that col2a1 may have other functions. Intriguingly in fact, the NH2-propeptide of type IIA procollagen binds to TGFβ-1 and to BMP-2, influencing their distribution and action in the extracellular matrix in chondrogenic tissue (Zhu et al., 1999). Both of these growth factors can, at least in vitro, divert early
glia to other lineages (Morrison et al., 2000). Therefore one function of type IIA collagen, in particular in embryonic nerves, could be to bind BMP and TGFβ, and prevent excessive exposure of differentiating glia to these factors, thus favouring Schwann cell development. Interestingly, in our analysis we have identified another gene, the uterine sensitization-associated gene-1 (Usag-1, annotated with Biopendium™ from the EST AA892798, see table 3.6.2), belonging to the cysteine knot-containing family, that is likely to accumulate in the extracellular matrix, where it can act as a BMP antagonist (Avsian-Kretchmer and Hsueh, 2004; Yanagita et al., 2004). During myelination col2a1 is highly expressed, and in common with many myelin-related genes it is up-regulated by treatment of cultured Schwann cells with cAMP. Surprisingly, although it appears to be equally expressed by myelinating and non-myelinating Schwann cells, nerve cut results in a complete down-regulation of both the mRNA and the protein for col2a1, meaning that even in non-myelinating Schwann cell its expression is axonally dependent. This unusual behaviour is shared by galactocerebroside and sulfatide, which are down-regulated in non-myelinating Schwann cells after peripheral nerve injury (Scherer and Salzer, 2001).

**Chondromodulin-1 (ChM-1)**

ChM-1 is cartilage matrix protein that stimulates the growth of chondrocytes and that inhibits angiogenesis (Hiraki et al., 1997; Hiraki and Shukunami, 2000). In the chick embryo ChM-1 is also expressed in developing heart and eye, and its mRNA has been detected in the notochord and in the neural tube (Dietz et al., 1999; Shukunami et al., 1999). ChM-1 mice do not show any abnormality in vascular invasion, cartilage development and endochondral bone formation (Brandau et al., 2002; Nakamichi et al.,
2003). However, adult mutant mice showed a significant increase in bone mineral
density and reduced bone resorption relative to bone formation suggesting that ChM-1
is a bone remodeling factor in mice (Nakamichi et al., 2003). We have shown that
ChM-1 mRNA is expressed mainly by myelinating Schwann cells and that it is
regulated by axonal contact. We were however unable to detect significant levels of
ChM-1 protein by Western blotting and immnunofluorescence. This could mean either
that the expression and regulation of ChM-1 mRNA in nerves is anomalous and without
function or that Schwann cells express a ChM-1 variant that is not recognised by the
ChM-1 antibody. In the latter case, it could be speculated that the function of ChM-1 in
peripheral nerve was negative control of angiogenesis, and that its down-regulation after
nerve damage could facilitate revascularization and macrophage recruitment during
regeneration.

Osteonectin/SPARC, Osteoadherin (Omd) and bone sialoprotein (Ibsp II)

Osteonectin/SPARC, Osteoadherin (Omd) and bone sialoprotein (Ibsp II) is a matrix-
associated protein that elicits changes in cell shape, inhibits cell-cycle progression, and
influences the synthesis of ECM (Termine et al., 1981; Bradshaw et al., 2003).
Osteonectin binds collagen fibrils and accounts for the unique property of bone collagen
to undergo calcification. Osteonectin deficient mice appear normal until around 6
months of age, when they develop severe eye pathology characterized by cataract
formation and rupture of the lens capsule (Gilmour et al., 1998). Moreover, the absence
of osteonectin in mice gives rise to aberrations in the structure and composition of the
extracellular matrix that result in generation of cataracts, development of severe
osteopenia, and accelerated closure of dermal wounds (Bradshaw et al., 2003).
Osteonectin mRNA had been previously detected in peripheral nerve (Holland et al.,
1987), and expression profiling experiments have shown that it increases after nerve injury (Gillen et al., 1995; Nagarajan et al., 2003).

Omd is a small cell-binding proteoglycan, isolated from rat and bovine bone that belongs to the family of leucine-rich repeat proteins (Wendel et al., 1998). It binds osteoblasts via the integrin α5β3 and it is located to the mineralized bone matrix, with highest concentration of marker at the border between bone and cartilage remnants, with a distribution that is highly similar to that of bone sialoprotein (Ramstad et al., 2003).

Ibsp II is an acidic glycoprotein that constitutes around 12% of non-collagenous proteins in human bone. It belongs to the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family of proteins, alongside osteopontin, a protein that, in agreement with previous experiments (Jander et al., 2002), we found up-regulated after nerve cut. Both Omd and Ibsp are suggested to have a role in bone mineralisation (reviewed in Qin et al., 2004).

We have not investigated in detail the distribution of SPARC, Omd, and Ibsp in peripheral nerve, and at present it is difficult to speculate on their possible function. However, one can argue, based on the existing literature, that they may have a role in ECM structure and could, for example, link the extracellular matrix to the Schwann cell via integrins. Their down-regulation after nerve injury may be involved in creating a favourable environment for axon regeneration.

The transcription factor Sox-9 is expressed in Schwann cells in the PNS

The observation that Col2a1 was so strikingly regulated during Schwann cell development raised the question of which transcription factor was responsible for its expression in peripheral nerve. During chondrogenesis Sox9 directly regulates the expression of Col2a1 (Bell et al., 1997). Sox-9 plays also an essential role in sex
determination, being up-regulated in male and downregulated in female genital ridges. It is possibly immediately downstream of SRY in mammals, and functions as a critical Sertoli cell differentiation factor, perhaps in all vertebrates (Morais da Silva et al., 1996). Interestingly, recent work has demonstrated a role for Sox9 in neural crest development (Mori-Akiyama et al., 2003; Cheung and Briscoe, 2003). In humans, mutation in Sox-9 cause Campomelic dysplasia, a disorder of the newborn characterized by congenital bowing of long bones, together with other skeletal and extraskeletal defects. Up to two-thirds of affected XY individuals have a gradation of genital defects or may develop as phenotypic females (Wagner et al., 1994). Heterozygous Sox-9 mutant mice reproduce most of the skeletal abnormalities of campomelic dysplasia. The heterozygous Sox9 mice dies perinatally with cleft palate, as well as hypoplasia and bending of many skeletal structures derived from cartilage precursors (Bi et al., 2001). The role of Sox-9 in collagen type II control and skeletal development, as well as its expression in the neural crest made this transcription factor particularly interesting for our studies. I have shown that Sox9 is expressed throughout the development of the Schwann cell lineage from the neural crest to adult mature myelinating and non-myelinating Schwann cells. While in the embryonic and early post-natal stages Sox9 is found in the nucleus, in adult Schwann cell we found a strong cytoplasmic immunoreactivity. It is possible that this represents an inactive state of the protein, since in gonads Sox9 activity is regulated by a nucleo-cytoplasmic shuttling (Gasca et al., 2002). Sox-9 mRNA is expressed by both myelinating and non-myelinating Schwann cells, and after nerve injury the mRNA levels for Sox-9 drop, although not dramatically, indicating that its expression depend on axonal signals. These results, combined with the recent findings that Sox9 is determinant in the glial fate choice in the spinal cord
(Stolt et al., 2003), strongly suggest that Sox9 is involved in Schwann cell differentiation.

**Expression and localisation of Synuclein-1 and αB-crystallin in peripheral nerve**

A central role for the Schwann cell cytoskeleton in the processes of differentiation and myelination has long been suggested, and several molecules that play an important part, such as actin, periaxin and DRP-2, have been identified (Gillespie et al., 1994; Fernandez-Valle et al., 1997; Sherman et al., 2001). Our analysis led us to the identification of many cytoskeletal proteins, including synuclein-1 (Snca) and αB-crystallin (Cry-ab). Snca is a small protein which forms aggregates in various neuropathologies such as Parkinson's disease (PD), Alzheimer's disease and multiple system atrophy (MSA) (reviewed in Eriksen et al., 2003; Kahle et al., 2002). Snca belong to the synuclein family that includes also β- and γ-synuclein (George, 2002).

The gene for Snca spans about 117 kb and encodes a 14kDa protein that presents 6 imperfect repeats of 11 aminoacids at the N-terminus. This region acquires alpha-helical secondary structure similar to that of A2-apolipoproteins upon lipid binding (Davidson et al., 1998; Eliezer et al., 2001). In neurons, Snca is concentrated in presynaptic nerve terminals and in cultured hyppocampal neurons it associates with synaptic vesicles (Jakes et al., 1994; Murphy et al., 2000), suggesting that Snca could be involved in synapse formation. Snca null mice exhibited intact brain architecture and possessed normal dopaminergic cell bodies, fibres, and synapses. However they displayed enhanced dopamine release at nigrostriatal terminals only in response to paired electrical stimuli, and attenuation in amphetamine induced locomotion. These findings suggested that Snca is not essential for synapse formation but plays an important role in the maintenance of synaptic function (Abeliovich et al., 2000). Snca as been also
proposed to act as a chaperone since, besides lipids, it interacts with a variety of cellular proteins, thus modifying their activity. In particular it shares sequence homology and interacts with the molecular chaperone 14-3-3 (reviewed in Recchia et al., 2004). The 14-3-3 protein plays critical roles in cell signaling events that control progress through the cell cycle, transcriptional alterations in response to environmental cues, and programmed cell death. Due to the structural similarity and interaction between Snca and 14-3-3, it is possible that Snca may be involved in some of these events.

Our data show that Snca expression in peripheral nerve parallels that of most myelin genes, being strongly induced from E16-E17 onwards, suppressed in cut nerves and up-regulated in Schwann cell cultures by treatment with dbcAMP. Moreover Snca localises in Schmidt-Lanterman incisures and in paranodal loops suggesting that it could have a role either in the architecture of these complex structures or in the signalling between axon and Schwann cell. However, the function of Schmidt-Lanterman incisures is not fully understood yet. It is possible that they are involved in myelin formation, but so far little evidence of protein or lipid transport through incusures exists (reviewed in Trapp and Kidd, 2004). One function of Schmidt-Lanterman incisures could be that of facilitating the communication between the outer and inner aspects of the myelin internode, as radial diffusion of small molecules through the gap-junctions present in the incisures membranes has been recently demonstrated (Balice-Gordon et al., 1998).

Recently we have proposed that the scaffold protein JIP-1 could be a component of the pathway by which Krox20 regulates the activity of JNK, controlling Schwann cell death and proliferation during development (Parkinson et al., 2004). In neuronal cells transfected with Snca under oxidative stress conditions, the levels of JIP-1 are highly increased and vice-versa, suggesting that they may interact in response to injury and stress (Hashimoto et al., 2002). During peripheral nerve development the expression
patterns of JIP-1 and Snca are similar, and both proteins accumulate in paranodal loops (Parkinson et al., 2004; this work). This raises the possibility of an interaction of these two proteins in Schwann cells too and therefore of an involvement of Snca in controlling Schwann cell development. We were however unable to detect any abnormality in the peripheral nerve of a Snca mutant strain of mice (Specht and Shoepfer, 2001). It is possible that Snca is redundant and that either β- or γ-synuclein take over its function, if any, during myelination. Another possibility is that a subtle phenotype is actually present that could not be detected with a simple morphological analysis.

In pathological circumstances, such as in MSA, Cry-ab colocalises with Snca. Cry-ab is a major component of the lens of the eye, but it is also widely distributed in different tissues. It is a member of the alpha-crystallin/small heat-shock protein (alpha/HSP) superfamily, characterised by a highly conserved C-terminal "α-crystallin domain" (reviewed in de Jong et al., 1998). Therefore, Cry-ab possesses some molecular chaperone-like functions (reviewed in MacRae, 2000; Horwitz, 2003). Cry-ab had been previously detected in Schwann cells (Iwaki et al., 1990), but its role in peripheral nerve has never been investigated. Null mice for Cry-ab have normal lens development, but reduced life span with a severe osteoarthritis (Brady et al., 2001). Interestingly, recent work has identified mutations of the highly conserved "HSP20/α-crystallin domain" of the small heat shock proteins 22 and 27 (HSP22 and HSP27) that cause Charcot-Marie-Tooth disease and distal hereditary motor neuropathy in humans (Irobi et al., 2004; Evgrafov et al., 2004). Transgenic mice, overexpressing alpha A-crystallin, the other alpha-crystallin gene, under the control of the vimentin promoter, develop demyelination and axonal dystrophy (De Rijk et al., 2000), while mutations in Cry-ab cause a desmin-related myopathy, a neuromuscular disorder (Vicart et al., 1998). This
findings, combined with our data that showed that Cry-ab is highly induced during peripheral nerve development and that it depends on axonal signals for its expression, raises the possibility that Cry-ab is involved myelination.
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CHAPTER 5

TGFβ controls Schwann cell death and proliferation during embryonic and perinatal development

INTRODUCTION

The TGFβ superfamily

The transforming growth factor β (TGFβ) superfamily of cytokines controls many cell processes, including cell proliferation, recognition, differentiation and apoptosis, both during embryonic development and in mature tissue in a variety of species, ranging from flies to mammals (Massague, 1998; Massague et al., 2000; Shi and Massague, 2003; ten Dijke and Hill, 2004). Disruption or mutations in TGFβ pathway component are associated with several human diseases, including cancer, fibrosis and auto-immune diseases (Derynck et al., 2001; ten Dijke and Hill, 2004).

The TGFβ superfamily contains two subfamilies, the TGFβ/Activin/Nodal subfamily and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) subfamily, defined by the specific signalling pathways that they activate (Izzi and Attisano, 2004). TGFβ signalling is initiated by binding and bringing together type I and type II receptor serine/threonine kinases on the cell surface. Usually, the active form of a TGFβ cytokine is a dimer, the arrangement of which suggests the formation of a complex with two type I and two type II receptors. TGFβ access to the receptors is regulated by a large family of proteins, collectively known as ligand traps (Shi and Massague, 2003). After TGFβ binding, type II receptor phosphorylates the type I receptor kinase domain, which then propagates the signal through phosphorylation of Smad proteins.
There are eight Smad proteins, constituting three functional classes: the receptor regulated Smad (R-Smad), the co-mediator Smad (Co-Smad) and the inhibitory Smad (I-Smad). The R-Smad class is comprised of Smad1, 2, 3, 5 and 8. These Smads are directly activated through phosphorylation by the type I receptor kinase and undergo homotrimerization and formation of heterodimeric complexes with the Co-Smad, Smad4. These activated complexes are translocated to the nucleus and in collaboration with other cofactors regulate the transcription of target genes. I-Smads, Smad6 and Smad7, negatively regulate TGFβ signalling by competing with R-Smads for Co-Smad and by targeting the receptors for degradation (Izzo and Attisano, 2004; Shi and Massague, 2003).

In humans, the receptor serine/threonine kinase family comprises 12 members, 7 type I and 5 type II receptors (Mannig et al., 2002). They are all organized sequentially into an N-terminal extracellular ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain. Immediately N-terminal of this kinase domain, the type I receptor has a characteristic sequence, termed the GS domain, the phosphorylation of which by the type II receptor is required for type I receptor activation (Shi and Massague, 2003). Type I receptor activation results in increased affinity for the R-Smads, which are directly phosphorylated by the type I receptor (Kretzschmar et al., 1997).

While BMP ligands exhibit a high affinity for the extracellular binding domain of the type I receptors, TGFβ/activin display high affinity for the type II receptors and do not interact with isolated type I receptors (Massague, 1998; Kirsch et al., 2000). So in the TGFβ/activin case, the binding to the type II receptor is necessary to allow the subsequent incorporation of the type I receptor, leading to a large complex comprising a ligand dimer and four receptor molecules (Shi and Massague', 2003). Interestingly,
each member of the TGFβ superfamily binds to a characteristic combination of type I and type II receptors (ten Dijke and Hill, 2004). However, significant variation exists for the binding between different type II receptors and their ligands (Hart et al., 2002). For example, the activin type II receptor has a broad specificity and can bind to both activin and BMP ligands, while the TGFβ receptor II has low affinity for the other members of the superfamily (Hart et al., 2002).

Ligand access to receptors is also controlled by membrane-anchored co-receptors that play an important role in signalling. For example, the membrane-anchored proteoglycan beta-glycan, also known as TGFβ type III receptor, mediates TGFβ binding to the type II receptor, a role that is particularly critical for TGFβ-2 (Brown et al., 1999).

As mentioned previously, phosphorylation via type I receptors of R-Smads permits their association with Co-Smad. Once formed, this complex translocates to the nucleus and interacts with the DNA to regulate transcriptional responses. Intriguingly, stimulation with TGFβs leads immediately to positive and negative changes in the expression of several hundred genes (Kang et al., 2003). Since the same set of Smad proteins is used for activation and repression of gene expression, the recruitment of co-activator or co-repressor is required, and probably depends on the cell type and other physiological conditions at the time of TGFβ stimulation (Massague, 2000). Smad partners in this process include the DNA binding factor FoxH1, c-Jun/Fos, CREBP and homeobox proteins (e.g. Mixer) (ten Dijke et al., 2000; Attisano and Wrana, 2000; Massague and Wotton, 2000; Shi and Massague, 2003). The positive regulation of gene expression is achieved by recruitment of positive regulators such as CBP/p300, while the negative regulation of gene expression includes direct recruitment by Smads of co-repressors such as c-ski and SnoN, two members of the Ski family of protooncoproteins (Liu et al., 2001; Derynck and Zhang, 2003; Shi and Massague, 2003).
Once activated, the TGFβ signalling needs to be tightly regulated and properly terminated. I-Smads (Smad6 and 7) negatively regulate the pathway, by competing for Co-Smad. Smad7 also binds to the activated receptors leading to the ubiquitination and degradation of the receptors with the help of the E3 ubiquitin ligases, the Smad ubiquitination regulatory factors (Smurfs) (Suzuki et al., 2002; Tajima et al., 2003; Izzi and Attisano, 2004).

Accumulating data suggest that TGFβ also acts through Smad independent pathways. For instance, TGFβ has been shown to activate mitogen-activated protein kinase (MAPKs) signalling pathways, including MKK4/c-Jun N-terminal kinases (JNK) and MKK3/p38 kinases pathways (Adachi-Yamada et al., 1999; Massague’ and Chen, 2000). The link between the receptors and these pathways is not clear, but may involve TAK1 (TGFβ-activated kinase 1), which acts directly on MKKs that activate either JNK or p38 (Zhou et al., 1999). Activation of TAK1 has also been linked to induction of apoptosis in eye development in Drosophila (Takatsu et al., 2000). Activation of JNK and p38 through TGFβ signalling can lead to rapid transcriptional responses, by activating AP-1 complexes via phosphorylation of the transcription factor c-Jun (Hocevar et al., 1999) or CRE (cAMP responsive element) regulatory complexes via phosphorylation of ATF-2 (Sano et al., 1999). C-Jun phosphorylation has been linked with cell cycle progression and apoptosis in various systems (Watson et al., 1998; Wisdom et al., 1999). Interestingly, it has been reported that activated Smad complexes can form physical links with Jun or ATF-2 complexes (Wong et al., 1999; Sano et al., 1999). Moreover, in certain circumstances Smad undergoes activating phosphorylation by JNK (Engel et al., 1999). Taken together these observations suggest an interplay between the Smad and JNK or p38 pathways indicating diverse forms of integration between TGFβ signalling and other pathways in the cell (reviewed in Massague, 2000).
TGFβ and Schwann cells

TGFβs, and in particular TGFβ-1, -2 and -3 are expressed by Schwann cells and exert a variety of effects on these cells (reviewed in Scherer and Salzer, 2001; Jessen and Mirsky, 2004). In cultured Schwann cells, in the presence of serum or of cAMP elevating agents or both, TGFβ promotes Schwann cell DNA synthesis and thus proliferation (Eccleston et al., 1989; Ridley et al., 1989). Interestingly, the mitogenic effect of TGFβ is enhanced by low (0.5 μM) concentrations of forskolin, which is a potent activator of cAMP, while at higher concentrations of forskolin (5 μM) the synergistic interaction of forskolin and TGFβ is abolished (Ridley et al., 1989). In the same situation, even if applied at concentrations too low to induce Schwann cell proliferation, TGFβ suppress cAMP-induced P0, galactocerebroside and O4 induction (Mews and Meyer, 1993; Morgan et al., 1994; Stewart et al., 1995). However, if TGFβ is applied to DRG neuron/Schwann cell co-cultures, it acts to inhibit Schwann cell proliferation induced by contact with neurites (Guenard et al., 1995b; Einheber et al., 1995). Moreover, in these co-cultures TGFβ suppresses P0 and galactocerebroside induction and blocks myelination (Guenard et al., 1995a; Einheber et al., 1995).

TGFβ and Schwann cell apoptosis

As we have seen in the general introduction, a variety of signals, either axonally derived (NRG-1) or of autocrine origin, exert a positive control on Schwann cell survival. In addition to these positive signals, there are at least two factors, TGFβ and NGF, which actively promote Schwann cell death by apoptosis after nerve injury. NGF, acting through the p75NTR, promotes Schwann cell death in vitro, and mice lacking p75NTR show reduced cell death both after neonatal nerve injury and three weeks after nerve crush in the adult (Khursigara et al., 2001; Ferri and Bisby, 1999; Syroid et al., 2000),
and cultured Schwann cell from p75 null mice survive better than normal ones when deprived of growth factors and serum (Soilu-Hanninen et al., 1999; Syroid et al., 2000). However, the NGF signalling pathway appears to be extremely complex, since under some conditions NGF can promote Schwann cell survival (Khursigara et al., 2001). Schwann cell response to NGF signalling strongly depends on the presence of the protein RIP-2 (receptor interacting protein-2), which interacts with p75. In the presence of RIP-2, Schwann cell death in response to NGF is prevented, while the absence of RIP-2, achieved in vitro by expression on dominant negative RIP-2 or by prolonged culturing, Schwann cells become sensitive to NGF-induced death (Khursigara et al., 2001).

Similarly to NGF, TGFβ can induce Schwann cell death in a variety of conditions in vitro (Skoff et al., 1998; Parkinson et al., 2001). Treatment with TGFβ kills freshly isolated neonatal Schwann cells, and the apoptotic effect is completely inhibited by the combined presence of neuregulin-1 and autocrine signals. The c-Jun phosphorylation pathway via JNK is directly involved in TGFβ-mediated Schwann cell death, since expression of a constitutively active v-Jun promotes Schwann cell death while dominant negative c-Jun blocks TGFβ induced apoptosis (Parkinson et al., 2001). These data are supported by the finding that TGFβ is unable to kill Schwann cell from post-natal day 4 (P4) nerves, due to its inability to phosphorylate c-Jun in myelinating Schwann cells (Parkinson et al., 2002, 2004). In vitro, enforced expression of the transcription factor Krox-20 is sufficient to block both serum deprivation and TGFβ-induced Schwann cell death via inactivation of the JNK-c-Jun pathway (Parkinson et al., 2004). The scaffold protein JIP-1, identified with the gene profiling described in Chapter 3 and 4, is likely to be a component of the mechanism by which Krox-20 regulates JNK activity in Schwann cells (Parkinson et al., 2004).
In vivo, TGFβ is expressed by Schwann cell precursors, immature Schwann cells and its expression remains sustained in the adult (Scherer et al., 1993; Parkinson et al., 2001). Moreover, TGFβ-1 mRNA and protein expression are elevated in the distal stump of injured nerves both in neonatal and in adult mice, while TGFβ-3 levels are reduced in the same circumstances, indicating that TGFβ-1 may have a role in the early events following nerve damage (Scherer et al., 1993; Parkinson et al., 2001). In agreement with this, injection of TGFβ increases Schwann cell death in injured, but not normal, neonatal nerves (Parkinson et al., 2001). Interestingly, if combined with TNFα, TGFβ can induce Schwann cell death even in the presence of serum, and the combination of these two factors in serum-free conditions kills Schwann cell more potently than TGFβ alone (Skoff et al., 1998; Parkinson et al., 2004).

Taken together all these data indicate that the effects of TGFβ on Schwann cells strongly depend on the context, and its role in nerve development is very difficult to predict.

Mice in which the gene for TGFβ-1 has been knocked out die 3-4 weeks after birth, due to a massive inflammatory response that results in lymphocyte and macrophage invasion in many organs but in particular in heart and lungs (Kulkarni et al., 1993; Geiser et al., 1993). Moreover, about 50% of the homozygous TGFβ-1 null mice die prenatally, due to defective haematopoiesis and endothelial differentiation (Dickson et al., 1995). TGFβ-2 and TGFβ-3 null mice have also been generated. TGFβ-3 deficient mice die perinatally, due to delayed pulmonary development and defective palatogenesis, implicating TGFβ-3 in epithelial-mesenchymal interactions (Kaartinen et al., 1995), while TGFβ-2 mutants die soon after birth and present a wide range of developmental defects that, interestingly, are not overlapping with the defects seen in
TGFβ-1 and -3 knockouts. They include lung, cardiac, craniofacial, eye and urogenital malformations (Sanford et al., 1997).

The early mortality and the number of developmental defects make these animals unsuitable to comprehensively study peripheral nerve development in the absence of TGFβ. Recently, TGFβ-1 null mice that can survive into adulthood have been generated by breeding TGFβ-1 +/− mice with nude mice, which lack T cells and thus inflammatory response, which is the major cause of death in TGFβ-1 null mice (Day et al., 2003). The adult TGFβ-1 +/−/nude mice thus produced presented hypermyelination and tomacula-like structures, with the major abnormalities in the node of Ranvier region, suggesting a possible role for TGFβ-1 in myelin maintenance (Day et al., 2003). However, axonal sorting and myelination in the PNS in those TGFβ-1 null mice that are able to survive up to 3-4 weeks appears to be normal (McLennan et al., 2000). One possible explanation for this is that TGFβ-1 has no role in controlling normal myelination. On the other hand, this is difficult to verify since until weaning null mice take-up TGFβ-1 through maternal milk (Letterio et al., 1994). Thus, the role of TGFβ-1 in nerve development remains unclear.

AIMS

To try to bypass these obstacles and to shed some light on the possible functions of TGFβ in nerve development and myelination we decided to generate a conditional knockout mouse in which the TGFβ receptor type II (TGFβRII) is specifically ablated only in Schwann cells. To do so, we crossed mice carrying a floxed type II receptor (Cazac and Roes, 2000) with P0 CRE mice in which the CRE recombinase protein is expressed under the control of the P0 promoter (Feltri et al., 1999a, 1999b).
Both these mice have been successfully used in recent years: the TGFβ type II receptor floxed mouse has been used to specifically inactivate TGFβ signalling in B cells (Cazac and Roes, 2000), while the P0 CRE mouse has been used to ablate β1 integrin and dystroglycan in Schwann cells (Feltri et al., 2002; Saito et al., 2003), suggesting that the combination of the two should allow us to inactivate TGFβ signalling in Schwann cells only.

In the following paragraph a brief overview of CRE/loxP technology is provided.

**CRE/loxP technology**

Gene targeted mice, derived from embryonic stem cells, are often useful tools to study gene function. However, if the gene inactivation results in embryonic lethality, the function of the gene in later stages cannot be further studied. Another pitfall of classical gene targeting is that the resulting phenotype can be extremely complex, and it can be difficult to distinguish cell-autonomous from more complex lesions. The CRE recombinase/loxP sites (CRE/loxP) system has been developed to overcome these limitations, confining the inactivation of a gene in a tissue- or cell-specific manner. This system allows the inactivation of a target gene in a single cell type, thereby allowing the analysis of the pathophysiological consequences of genetic alteration in mature animals (Ramirez-Solis et al., 1995; Smith et al., 1995; Raiewsky et al., 1996). In the CRE/loxP technology a mouse is generated, in which the targeted gene is flanked by two loxP sites. The loxP sequences consists of two 13 base pair (bp) inverted repeats and an 8-bp asymmetrical core spacer region. The CRE recombinase leads to the site-specific recombination between the two loxP sites with consequent inversion or excision of the region in between the two loxP sequences (Fig 5.2) (Ramirez-Solis et al., 1995; Li et al., 1996; Van Duyne, 2001; Branda and Dymecki, 2004). Indeed, CRE will remove the
sequence in between two directly repeated target sites, and invert the sequence in between two inverted sites. Therefore, only those cells where the CRE recombinase is expressed will bear the mutation. One of the first reports of conditional gene modification examined the effect on T cells of a null mutation in the essential DNA polymerase β gene (Gu et al., 1994). Since then, this technology has been used to generate a plethora of conditional mutants. Nowadays conditional targeting is commonly used not only for gene inactivation but also for the introduction of mutations and also for the activation of a gene (Torres et al., 1996; Lakso et al., 1992). In particular, ligand-regulated forms of CRE have been developed with the goal of adding temporal control to the recombinase activity, enabling the induction of the genetic changes late in embryogenesis and/or in adult tissues. One particular successful strategy has involved fusing a mutant estrogen receptor (ER) ligand-binding domain to the C-terminus of CRE (reviewed in Brenda and Dymecki, 2004). In these mice CRE activity is induced only after administration of tamoxifen, allowing temporal and spatial control of the recombinase activity. Thus, both cell-type-specific and inducible gene targeting are feasible using the CRE/loxP recombination system, and, importantly, the efficiency in the system can reach 100% (Kuhn et al., 1995). However, in many cases, gene inactivation is not complete, due to a failure of the recombinase to modify the target gene in all cells expressing the recombinase. The resulting mosaicism precludes the analysis of a null phenotype in the targeted lineage, due to the presence of wild-type cells that can mask a potential defect. Is therefore always necessary to carefully determine the percentage of recombination. Still, the recombinase tool remains a powerful technology that will help to determine the contribution of any given gene in development, disease and disability.
RESULTS

Generation of the P0 CRE/TGFβRII"f" mice

To disrupt the TGFβRII gene specifically in Schwann cells, TGFβRII"f" mice (Cazac and Roes, 2000) were crossed with P0 CRE mice (Feltri et al., 1999), which induce efficient Schwann cell lineage-specific deletion of loxP-flanked target sequences (Feltri et al., 2002; Saito et al., 2003) (Fig 5.3A and B). In the TGFβRII"f" mouse loxP sites flank exon 3 of the TGFβRII gene, which encodes for the membrane-proximal extracellular part of the protein (Cazac and Roes, 2000; Fig 5.3A). If exon 3 is ablated the resulting protein is truncated and translation terminates before the extracellular domain. This should result in a type II receptor complete inactivation.

To determine the recombination efficiency, Southern blot analysis of Schwann cell isolated from the sciatic nerve of P21 P0 CRE/TGFβRII"f" and P0 CRE/TGFβRII"f" mice was performed (Fig 5.3C). Unfortunately, the purification of the Schwann cells via negative immunopanning was not particularly efficient, and the number of fibroblasts in the culture, measured with S100β staining, was around 20-25% of the total cells. Densitometric analysis of the Southern blot indicated that the recombination efficiency was 65 and 67% for the heterozygous and the homozygous respectively. This result normalized for the Schwann cells purity (75-80%), means that at this stage the recombination is probably somewhere around 90%.

Therefore, to better determine the efficiency of deletion, we decided to use an indirect approach. TGFβ binding to the type II receptor results in type I receptor recruitment and phosphorylation, which in turn phosphorylates R-Smads. Phoshorylated R-Smads then form a heterodimeric complex with the Co-Smad Smad4. The activated complex is
translocated to the nucleus where, in collaboration with various cofactors, it regulates the transcription of various genes (see above). Loss of type II receptor should result in the absence of Smad nuclear localisation. We therefore cultured P5 Schwann cells from TGFβRII<sup>WT</sup> (from now-on called wild-type (wt)), P0 CRE/TGFβRII<sup>+/+</sup> and P0 CRE/TGFβRII<sup>+/−</sup> mice (from now often referred to as mutants). After 24 h the cells were stimulated with 10 ng/ml TGFβ-1, fixed and immunolabelled with antibodies against Smad2 and Smad4 (Fig 5.4A and B respectively). In the cells isolated from P0 CRE/TGFβRII<sup>+/−</sup> mice there is basically no nuclear localisation when compared to wt and P0 CRE/TGFβRII<sup>+/−</sup> cells, indicating that already at P5 the recombination is almost complete.

**P0 CRE/TGFβRII<sup>+/−</sup> mice myelinate correctly**

Previous studies had indicated that, in DRG neuron/Schwann cell co-cultures *in vitro*, TGFβ-1 acts to inhibit Schwann cell proliferation, suppresses P<sub>0</sub> and galactocerebroside induction and blocks myelination (Guenard *et al.*, 1995a,b; Einheber *et al.*, 1995). Therefore we asked whether TGFβ-1 could have a function in negatively controlling myelination *in vivo*. If so, we would expect to find either premature myelination or hypermyelination or both. To answer this question we analysed by electron microscopy (EM) transverse sections from wt and P0 CRE/TGFβRII<sup>+/−</sup> sciatic nerves. This analysis showed that at P1 Schwann cells have started segregating the big caliber axons in a 1:1 relationship and that in many cases myelination has started, with one or more loops of myelin already formed (Fig 5.5), but no significant differences between the wt and the mutant mice were detectable. By P5 basically all the big caliber axons are segregated and myelination is proceeding correctly (Fig 5.5 and 5.6). At P21, in both wt and mutant nerves, myelination is nearly completed and smaller caliber axons are
segregated in bundles surrounded by a single non-myelinating Schwann cell. Moreover, the myelin sheath appears to be morphologically normal and stable in the adult (Fig 5.7).

Interestingly, in a P21 P0 CRE/TGFβRII<sup>ΔT</sup> mouse we identified a bundle of smaller caliber axons that were surprisingly surrounded by a myelin sheath (Fig 5.8). However, only one of these structures was present in this animal, and we never came across this kind of abnormality in any of the other mutant mice analysed (more than 30 mutants were analysed at time points P1, P5, P15, P21, P30 and adult). This led us to consider this as an unusual but random event, probably not attributable to the lack of TGFβ signalling.

Myelin-related protein expression appears to be normal in the mutant mice

As mentioned previously, in vitro TGFβ-1 suppresses the expression of myelin-related proteins such as P<sub>0</sub> and galactocerebroside (Einheber et al., 1995). Lack of TGFβ-1 signalling could therefore lead to an over-expression of myelin-related proteins. However, the EM analysis described above suggests that an abnormal expression of myelin proteins is unlikely since we did not observe any difference in the onset of myelination and/or in myelin structure between wt and mutant mice. To confirm this observation we immunolabelled newborn sciatic nerves from wt and mutant mice with an antibody against the myelinating Schwann cell protein periaxin. No clear difference was detectable between the wt and the P0 CRE/TGFβRII<sup>ΔT</sup> mouse (Fig 5.9A). Similarly, no differences were seen when we immunolabelled new-born sciatic nerves with an antibody against the p75NTR (Fig 5.9A). Moreover, western blot experiments performed on sciatic nerve extracts during the active phases of myelination (P10, P21 and P30) and in the adult, showed that there were no gross abnormalities between wt
Schwann cell death is reduced in perinatal normal and injured sciatic nerve in P0 
CRE/TGFβRII^{fl/fl} mice

Previous experiments demonstrated that TGFβ-1 acts as a Schwann cell killer in freshly 
isolated cells from newborn rat nerves and that injection of TGFβ-1 increases Schwann 
cell death into injured, but not normal, neonatal nerves (Skoff et al., 1998; Parkinson et 
al., 2001). These findings suggest that TGFβ may have a role as negative regulator in 
the control of Schwann cell numbers during development. To test this hypothesis, we 
immunolabelled transverse sections of sciatic nerves from E18 wt and mutant mice with 
the TUNEL technique, to detect nuclear fragmentation. These experiments revealed that 
the percentage of TUNEL-positive nuclei was considerably lower in mutant nerves 
compared with the wt ones, decreasing from 0.41% to 0.16% (Fig 5.10 A and B; \( p< 
0.005 \)). Similarly, Schwann cell apoptosis in P2 nerves is significantly reduced from 
0.34% in the wt controls to only 0.09% in mutant mice (Fig 5.10 A and C; \( p< 0.001 \)).

In normal neonatal nerve Schwann cell survival depends on the combined action of 
axonally derived NRG-1 and of autocrine signals (Meier et al., 1999). If the neonatal 
nerve is transected the survival of Schwann cells is sustained only by autocrine signals, 
and, although most of the cells survive, death by apoptosis is highly increased 
(Grinspan et al., 1996). Interestingly, this increase in Schwann cell death is not 
observed in null mice for the p75NTR, suggesting that p75 plays a crucial role in 
mediating Schwann cell apoptosis after injury (Syroid et al., 2000). Moreover, if TGFβ 
is injected in the distal stump of a wt transected nerve the number of TUNEL-positive
nuclei is increased further, indicating that Schwann cells in the distal stump are sensitive to TGFβ killing (Parkinson et al., 2001).

We therefore asked whether the loss of TGFβ receptors could result in reduced Schwann cell death after axotomy in P1 nerves. We performed TUNEL staining on nerve sections from wt and P0 CRE/TGFβRII f/f mice 24 hours after nerve transection. This experiment showed that in the wt mice cell death increases 13.85 fold, in agreement with previous results (Grinspan et al., 1996; Syroid et al., 1996, 2000; Parkinson et al., 2001). Similarly, in the mutant nerves Schwann cell death is increased 13.22 fold, but remains nearly four fold lower than in the wt mouse (Fig 5.10 and 5.11), indicating a role for TGFβ in the induction of Schwann cell death after nerve injury in the neonatal nerve.

**Reduced cell death is accompanied by reduced proliferation in E18 P0 CRE/TGFβRII f/f nerves**

TGFβ, in the presence of serum or of cAMP elevating agents or both, promotes Schwann cell DNA synthesis and thus proliferation in vitro (Eccleston et al., 1989; Ridley et al., 1989). This, taken together with the finding that in the nerve of P0 CRE/TGFβRII f/f mice Schwann cell death by apoptosis was reduced by approximately 2.5 fold raised the possibility that TGFβ may have a key role in controlling Schwann cell numbers during peripheral nerve development. This hypothesis predicts that, in the absence of TGFβ signalling, the reduction of Schwann cell death should be accompanied by a similar reduction in Schwann cell proliferation. This assumption was tested by labelling transverse sections of E18 sciatic nerves from wt and mutant mice with an antibody against the phospho-histone-3 (PH3), that specifically labels those cells that are actively dividing. Figure 5.12 shows that in the mutant nerves the rate of PH3-
positive nuclei is 2.3 fold reduced when compared to the wt controls (p< 0.001). This reduction rate is very close to the one observed in Schwann cell death, suggesting that these two events could be related.

**TGFβ receptor type II recombination has already occurred at E18**

To verify whether the observed reductions in Schwann cell death and proliferation in E18 and in newborn sciatic nerves from mutant mice were attributable to the loss of type II receptor and therefore of TGFβ signalling, we had to make sure that by E18 the recombination of the floxed segment had already occurred. To do so we used the indirect approach already used at P5 and immunolabelled for Smad 2 and Smad 4 Schwann cells from wt and mutant mice after treatment with TGFβ. These experiments showed that in the mutant mice there was basically no nuclear localisation of Smad proteins (Fig 5.13 and 5.14), indicating that the recombination has already occurred at least as early as E18, pointing at the lack of TGFβ receptor as the likely candidate for the observed phenotype.

**Schwann cells from newborn P0 CRE/TGFβRII<sup>fr</sup> sciatic nerves are resistant to TGFβ killing**

As already mentioned, treatment with TGFβ actively kills freshly isolated Schwann cells from newborn rat nerves. Therefore, to further test the hypothesis that the reduction in Schwann cell death observed in perinatal nerves of P0 CRE/TGFβRII<sup>fr</sup> mice is due to the loss of sensitivity to TGFβ killing, we performed an *in vitro* survival assay in the presence of TGFβ. Freshly isolated Schwann cells from the sciatic nerves of newborn mutant and wt type mice were plated at a density of 3000 cells per coverslips on a laminin substrate. Cells were allowed to attach to the substrate for about
3 hours and then exposed to 10ng/ml TGFβ-1 for one day. At the end of the experiment the cultures were fixed and Hoechst stained. The number of living cells is expressed as survival percentage, that is the number of living cells present at the end of the experiment as a percentage of the number of cells present (i.e. that had attached) after 3 hours. We observed that, as expected, only 33 ± 5% of the Schwann cells from the wt sciatic nerves were still alive after 24 hours, while 55 ± 2% of the cells from the mutant mice survived (Fig 5.15), a percentage consistent with the number of cells that usually survive in this condition in untreated cultures (Parkinson et al., 2001), indicating that these cells are not sensitive to TGFβ killing.

**Schwann cell death and proliferation are not affected in adult nerves from P0 CRE/TGFβRII \( \text{\textsuperscript{\text{\textregistered}}} \) mice after axotomy**

In the normal adult nerve Schwann cells are present in a quiescent state. Following nerve injury they undergo a wave of proliferation that is part of the process of Wallerian degeneration. However, contrary to what happens in the perinatal nerve (see above), Schwann cells in the adult nerve do not undergo rapid apoptosis after axotomy (Scherer et al. 1993; Grinspan et al., 1996; Scherer and Salzer, 2001). However, after axotomy TGFβ-1 mRNA levels are up-regulated in the distal stump (Scherer et al., 1993), suggesting that TGFβ could have a role in the early events following nerve injury. Therefore we tested cell death and proliferation in normal and axotomised nerves from P0 CRE/TGFβRII \( \text{\textsuperscript{\text{\textregistered}}} \) and wt mice. No apoptosis or proliferation were detectable in the adult nerves of either wt or mutant mice (Fig 5.16). Moreover, 7 days after nerve cut there was basically no apoptosis in the wt or the mutant nerves (Fig 5.17), while there was clear proliferation, as indicated by PH3 staining (Fig 5.18). However, no significant difference in the proliferation rate was detectable between the wt and the mutant nerves
suggesting that TGFβ does probably not have a role in the control of these events in the adult peripheral nerve.

Myelin-related proteins are correctly down-regulated in mutant nerves after axotomy

Previous experiments indicated that TGFβ, at least in DRG neuron/Schwann cell cocultures can inhibit myelin protein expression (Guenard et al., 1995a; Einheber et al., 1995). Moreover, after nerve injury in the adult nerve, while TGFβ-1 mRNA is upregulated, myelin-related gene mRNAs and proteins are strongly down-regulated in the distal stump. To test whether TGFβ has a role in controlling the down regulation of myelin genes, we immunolabelled transverse sections of normal and transected (2 days and 7 days after axotomy) adult nerves from wt and mutant mice with antibodies against P₀ and periaxin. We could not detect any clear difference in the levels of expression of the two proteins between mutant and wt nerves in any of the conditions analysed (Fig 5.19 and 5.20).

However, we noticed an interesting difference between mutant and wt nerves in the expression of the p75NTR. In fact, in the mutant mouse immunoreactivity for p75 is already detectable two days after axotomy, while it is absent at this stage in the wt nerve. 7 days after transection the levels of p75 are comparable between wt and mutant nerves (Fig 5.21). The meaning of this difference is currently under investigation.
DISCUSSION

The TGFβ superfamily of cytokines is involved in many aspects of the development and maintenance of various tissues, and alterations in its signalling pathway are associated with a range of human diseases (Massague, 2000). In the peripheral nervous system Schwann cells express TGFβ-1, -2 and -3 (Stewart et al., 1995), and these factors have a variety of proliferative and phenotypic effects on these cells (Scherer and Salzer, 1996; Jessen and Mirsky, 2004). The first indication that TGFβ-1 and -2 could have a proliferative role for peripheral glia came from studies on purified rat Schwann cells (Eccleston et al., 1989; Ridley et al., 1989). However, in DRG/Schwann cell co-cultures TGFβ inhibits Schwann cell proliferation, blocks myelination and suppresses the expression of P0 and galactocerebroside (Guenard et al., 1995a,b; Einheber et al., 1995). Moreover, in a variety of conditions in vitro and in vivo TGFβ induces Schwann cell death (Parkinson et al., 2001, Skoff et al., 1998). So, what is the real function of TGFβ in vivo?

In our experiments we have tried to answer this question by generating a conditional knockout mouse that does not express functional TGFβ receptor type II specifically in Schwann cells. In conditional targeting experiments, one of the main pitfalls is that sometimes the percentage of recombination is far from being 100% (Rajewsky et al., 1996; Brenda and Dymecki, 2004). This of course could mask the phenotype, due to the presence of wild-type cells.

The first obstacle that we encountered in estimating the percentage of recombination in our mice was that the purification of mouse Schwann cells by negative immunopanning was not very efficient. In fact, we were never able to obtain Schwann cells at a purity greater than 80%. Still, the analysis by Southern blot of the DNA thus obtained indicated that the recombination was probably higher than 90%, a value that should
guarantee that the effects of the lack of TGFβ signalling are not masked by the presence of unrecombined cells. This was confirmed indirectly by experiments in which we tested for Smad nuclear localisation. As we have mentioned in the introduction, one of the main consequences of TGFβ binding to type II receptors is Smad protein phosphorylation and nuclear translocation (Derynck and Zhang, 2003; Shi and Massague, 2003). We demonstrated that as early as E18, and then at P5, in the P0 CRE/ TGFβRII^{flfl} mice there is basically no nuclear localisation of the Smad proteins Smad2 and Smad4. This strongly suggests that the recombination of exon3 of the type II receptor has already occurred at this stage. Moreover, in vitro experiments on Schwann cells dissociated from P1 mutant and wt mice showed that, at this developmental time-point, the cells from P0 CRE/ TGFβRII^{flfl} mice are not sensitive to TGFβ mediated apoptosis, which is another indirect indication that the recombination has successfully occurred. At present, experiments are in progress to try to better purify Schwann cells from wt and mutant P3 mice, with the aim of performing a Southern blot that should give a definitive answer on the precise percentage of recombination soon after birth.

One of the most interesting effects observed in vitro in DRG-neuron/Schwann cell co-cultures treated with TGFβ was the block of myelination (Guenard et al., 1995a). This finding suggests that, in the absence of TGFβ signalling, myelination may occur earlier than normal. In contrast with this idea, in those TGFβ \(-/-\) mice that survive until 3-4 weeks after birth the onset of myelination is normal, and myelin appears morphologically normal (McLennan et al., 2000). However, these mice can probably take-up TGFβ from maternal milk and this could mitigate the effects of the mutation. The analysis of our mice showed that the onset of myelination does not appear prematurely in mice lacking TGFβ signalling. Moreover, myelination proceeds correctly and myelin maintenance does not present any abnormality, even in 1-year-old
mice. This is in contrast with findings in adult (6-13 week old) TGFβ−/− nude mice, which present striking abnormalities in myelin compaction, with tomacula-like and "honeycomb" structures, particularly frequent in the node of Ranvier region (Day et al., 2003). However, the results of this study are debatable: in fact only 11 TGFβ−/− nude pups were found out of nearly 1000 pups genotyped, indicating that most of the double transgenic die during embryonic development, and suggesting that any phenotype observed may not be Schwann cell-autonomous. Moreover, 7 of these pups died at weaning, and the autopsies did not show any pathology. Of the remaining 4 mice, one was killed when 6 weeks old, and the other three were sacrificed when 13 weeks old. Surprisingly, although the mice looked healthy, EM analysis showed a grossly abnormal myelin. Unfortunately, no behavioural or elettrophysiological tests were performed on these mice, and therefore too many questions about the observed phenotype remain unanswered.

In addition to blocking myelination, in DRG/Schwann cell co-cultures, treatment with TGFβ suppresses the expression of myelin-specific genes, and in purified Schwann cell cultures TGFβ blocks cAMP induced expression of various myelin genes (Mews and Meyer, 1993; Morgan et al., 1994; Stewart et al., 1995; Guenard et al., 1995b; Einheber et al., 1995). Moreover, recent experiments have shown that in Schwann cell cultures TGFβ antagonises the effects of forskolin on the mRNA levels of many myelin-related genes as well as of Oct-6 and Krox-20. In contrast with this, TGFβ effects on the non-myelinating Schwann cell specific genes GAP-43, p75, NCAM and L1 are complex, varying from the induction of NCAM and L1 to suppression of p75 and GAP-43 (Awatramani et al., 2002). Our experiments however suggested that TGFβ does probably not have a direct effect on the expression level of myelin proteins. In fact periaxin and P0 expression, tested via immunohistochemistry and western blot did not
appear to be different between wild-type and mutant mice. Similarly, the developmental expression of p75 and L1 looked normal. These results, taken together the observation that myelin is morphologically normal in P0 CRE/TGFβRII^OT^ mice strongly suggest that TGFβ is not fundamentally involved in the process of myelination in vivo.

In recent years our lab has shown that TGFβ, in freshly purified neonatal Schwann cells in vitro and in perinatal injured nerve in vivo, actively kills Schwann cells, and that this effect can be blocked in vitro through enforced expression of the transcription factor Krox-20, that leads to inactivation of the JNK-c-Jun pathway (Parkinson et al., 2001, 2004). In agreement with these observations we found that in P0 CRE/TGFβRII^OT^ mice Schwann cell death is significantly decreased in embryonic (E18) and perinatal stages, both during normal development and after nerve injury. Interestingly, after nerve injury at P1, Schwann cell death is increased in the mutant mouse too, although it remains nearly 4 fold lower that in the wild-type. It is likely that the increase of Schwann cell death even in the absence of TGFβ signalling is due to the action of the NGF/p75 pathway, which has been shown to be crucial in mediating Schwann cell apoptosis after neonatal nerve injury (Syroid et al., 2000).

The observed reduction in Schwann cell death was also accompanied by a reduction in Schwann cell proliferation of similar magnitude. This is in agreement with previous studies that showed that TGFβ has a mitogenic effect on Schwann cells (see citations above). A control of TGFβ on both Schwann cell death and proliferation would also account for the normal appearance of the sciatic nerve in the mutant mouse. In fact, it can be speculated that if only Schwann cell death was affected we would have probably observed some morphological abnormalities. However, to definitely confirm that there
are no significant differences in the overall number of Schwann cells between wt and mutant mice, stereological experiments are currently in progress in the laboratory.

It must be reported that recent work has identified the protooncogene ski as probably involved in controlling Schwann cell exit from the cell cycle, differentiation and myelination (Atanasoski et al., 2004). Ski is a repressor of TGFβ signalling through direct interactions with Smad2, 3 and 4. Ski probably interacts with the co-repressor N-CoR, thereby negatively modulating gene transcription by Smad complexes, and probably interferes with Smad binding to the coactivator of gene transcription p300/CBP (Akiyoshi et al., 1999). The work from Atanasoski and colleagues shows that ski antagonises the mitogenic effect of TGFβ in cultured Schwann cells, and ski overexpression in Schwann cell cultures prevents the cells from re-entering the cell cycle. On the other hand, in DRG/Schwann cell cocultures ski expression (as well as myelination) is blocked by treatment with TGFβ. Finally, ski seems to be involved in myelination and myelin gene expression. In fact, although ski -/- mice are embryonic lethal, late embryonic stages could be analysed, and myelin proteins appeared to be reduced at the mRNA level (Atansoski et al., 2004). In this respect, analysis of myelination in ski conditional knockouts would be very helpful in determining the precise role of ski in this process. It would also be very interesting to evaluate the levels of ski in the P0 CRE/TGFβRII Cre mice, to test whether the lack of TGFβ signalling has a direct consequence in modulating ski expression.

After injury in the adult sciatic nerve there is a dramatic increase in Schwann cell proliferation, while cell death, at least in the first weeks, is not affected (Scherer and Salzer, 2001). However, the mechanism involved in controlling these events can be different from those modulating the same mechanisms during development. For example, while during embryonic development cyclin D1 null mice do not present any
impairment in Schwann cell proliferation, after nerve injury Schwann cell proliferation is basically blocked in these mice, suggesting a role for cyclin D1 in controlling Schwann cell de-differentiation and re-entry in the cell cycle (Kim et al., 2000; Atanasoski et al., 2001). We showed that TGFβ is probably not involved in controlling Schwann cell proliferation and death after nerve injury in the adult nerve. As in the wild-type, no cell death or proliferation could be detected in the uninjured adult nerve in mutant mice. 7 days after sciatic nerve transection the Schwann cells in the distal stump are actively proliferating, but no significant differences were visible in the mutant mouse when compared with the wild-type. Our findings, taken together the results in the cyclin D1 -/- mice, strongly indicate that the molecular mechanisms that regulate Schwann cell proliferation during development or after axonal damage are fundamentally different.

We also tested the adult mutant nerve for Schwann cell-related proteins expression before and after nerve injury. These immunofluorescence experiments showed that there was no significant difference in the expression levels of P0 or periaxin in the uninjured adult nerve between wt and P0 CRE/TGFβRII -/- mice. Similarly, 2 or 7 days after nerve cut, the levels of P0 and periaxin drop in a comparable fashion between mutant and wt mice.

However, during these experiments we made an interesting observation regarding the p75NTR. In fact, in P0 CRE/TGFβRII -/- mice p75 is already clearly detectable 2 days after nerve cut, while it is still undetectable at this time-point in the wild-type mice. P75 is known to be up-regulated after nerve injury (Heumann et al., 1987). Its up-regulation is seen at the mRNA level 24 hours after transection, peaks at around 1 week and is maintained for up to 10 weeks, and axon regeneration down-regulates p75 (Robertson et al., 1995; Taniuchi et al., 1988). Moreover, TGFβ treatment down-regulates p75 in
cultured Schwann cells (Mews and Meyer, 1993; Awatramani et al., 2002). It is therefore possible that the lack of TGFβ results in an early and massive up-regulation of p75. 1 week after nerve transection p75 levels have probably reached the peak of expression in the wild-type nerve too, and no difference is detectable at this stage. As we have discussed previously, the role of the NGF/p75 pathway in the PNS is not completely clear. P75 is crucial for modulating Schwann cell death after nerve injury (above), but no other role has been identified so far. The meaning and consequences of the up-regulation that we observed are therefore not clear. The fact that we did not detect any Schwann cell death in the transected nerves of the mutant mice indicates that in this situation p75 is probably not involved in modulating Schwann cell apoptosis. The regulation of other genes, such as L1 and NCAM is currently under investigation to elucidate if there is any similarity in their control after nerve injury to the one observed for p75.

It must be also noted that the pattern of labelling that we observed for p75 raises some doubts about the antibody we used. In fact in our experiments p75 appears to be localised as bright dots; however, p75 is a surface protein and therefore the labelling should be more membrane-associated. More experiments are therefore requested to test the antibody specificity.

In conclusion, with this study we provide the first in vivo evidence that TGFβ signalling is crucially involved in controlling Schwann cell death and proliferation during peripheral nerve development. The P0 CRE/TGFβRII f/f mouse that we have generated could also be a useful tool to study the role of TGFβ during the events following nerve injury, such as Wallerian degeneration and during nerve regeneration.
Figure 5.1
A  CRE recombinase target site (loxP)

\[
\begin{align*}
5' \quad &\text{ATAACTTC} &\text{GTATAATG} &\text{TATGCTA} &\text{ACGAAGTTAT} &3' \\
&\text{TTTTAAGC} &\text{ATATTACGAT} &\text{ATGCTTCAAT} &\text{AATA} &3'
\end{align*}
\]

B  Excision/Insertion

C  Inversion

Figure 5.2
A

PH3 positive nuclei at E18

<table>
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<tr>
<th></th>
<th>WT</th>
<th>P0CRE/TGFβRIIff</th>
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<tr>
<td>%</td>
<td>1.16± 0.0010</td>
<td>0.50± 0.0012</td>
</tr>
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</table>

B

![Bar chart showing PH3 positive nuclei comparison between WT and P0CRE/TGFβRIIff](chart.png)

C

![Images showing PH3 localization in WT and P0CRE/TGFβRIIff](images.png)
A

Control + TGFβ (24hrs)

WT

PO CRE

TGFβRII f/f

B

Survival

WT PO CRE/TGFβRII f/f
PO CRE
TGFβRII f/f

Ad C
2d cut
7d cut

WT
POCRE
TGFβRII f/f

WT
CONCLUSIONS AND GENERAL REMARKS

The interaction between axon and Schwann cell represents one of the most striking examples of cell:cell interaction and the process of myelination is an incredible example of cell specialization. During the course of my studies I have tried to add valuable information to the knowledge of the molecular mechanisms that control Schwann cell differentiation and myelination. To achieve this goal I used two technologies that in recent years have radically changed the way researchers address biological questions: DNA microarrays and the CRE/loxP system.

DNA microarrays allow the analysis of the expression of thousand of genes simultaneously. This means that is possible to examine, in a fairly straightforward way, the overall transcriptional response of a tissue or of a cell type in normal conditions or in disease state, after biological, genetic or chemical stimuli or during biological processes such as cell-cycle progression or development. This kind of analysis is commonly referred to as gene expression profiling. The large amount of data generated from microarray analysis represents a great strength of the system, but it can also be seen as a weakness. This happens because very often gene profiling experiments generate more questions than answers. In fact, commonly, hundreds if not thousands of genes greatly change their expression levels between two different conditions. How should a researcher proceed from here? At present many programs are available for the analysis of the data obtained from a comparison. These tools, such as the software GeneSpring that we used in our analysis, facilitate the processing of multiple data sets and assessment of data quality. Nevertheless, even using these tools, the amount of information generated by a microarray analysis remains difficult to handle. For this
reason it is important to make available to the scientific community the raw data obtained from each experiment. The data obtained from our experiments can be a clear example: we were interested in those genes up-regulated during myelination and down-regulated after nerve injury, since the co-existence of these two conditions indicates that a gene may be myelin-related. However, we found that tens of genes are strongly up-regulated after nerve injury. They could be of great interest for researchers studying the initial events leading to nerve degeneration and to inflammatory response. It is therefore important to store them in a database where they can be accessible to the scientific community. For this reason our data, combined with experimental information in MIAME (minimal information about microarray experiments) compliant format, are stored at the Institute of Child Health (ICH) in London, ready for submission to the ArrayExpress database (EBI, Hinxton UK).

Another strength/weakness of microarrays is the number of ESTs present on the chips. Very often ESTs can represent up to 50% of the sequences that are differentially expressed in two or more conditions. If from one point of view this can be exciting, since the study of an EST could lead to the discovery of a novel gene, on the other hand it greatly reduces the ability to interpret and wholly understand the results of a set of experiments. To try to bypass this problem, in collaboration with the biotech company Inpharmatica (www.inpharmatica.com), we have mapped and annotated nearly all the ESTs that we found differentially expressed in our experimental conditions. This allowed us to better analyse our data and to generate and make available a sort of database for each one of the probes that changed in the embryonic comparison (E14vsE18) or after peripheral nerve injury (P12vsP12cut) (see Appendix). In this database we indicated how the gene has been mapped, provide a series of keywords related to the gene function and, if available, the Gene Ontology annotation. Moreover
we indicate if the gene has a signal peptide that makes the protein likely to be secreted or if transmembrane (TM) domains are present. Finally we provide a sequence and a structural annotation. To our knowledge this is the first time that such a complete and detailed analysis and description of the data sets from a microarray analysis is provided.

Of course the great challenge now would be to verify whether the predictions made with the use of the Biopendium are totally reliable and therefore if the functions inferred for the novel sequences identified correspond to the real function of the proteins. Nevertheless we believe that this represents a powerful way to handle data obtained from microarray analysis and its potential could be investigated further to improve the outcome even more.

The gene profiling that we performed also brought to our attention a series of genes, already known to be important in other systems, that had not been previously identified in peripheral nerve. In particular, the group of genes related to cartilage/bone development could represent an intriguing source of new experiments. We have in fact shown that type II collagen and the transcription factor Sox9 are highly expressed by Schwann cells, and that their regulation probably depends on axonal signals. However at present their function in peripheral nerve remains unknown. Unfortunately both type II collagen and Sox9 null mutants are embryonic lethal, therefore we could not use these animals to study peripheral nerve development in the absence of these genes.

The second technology that we used, the CRE/loxP system, provides a way to overcome the problem of embryonic mortality. I believe that it would be extremely interesting to study Schwann cell specific knockouts for type II collagen and Sox9 (and of course many other genes identified with our screening) by crossing the P0 CRE mouse with mice in which col2a1 or Sox9 genes have been floxed. The analysis of mice thus
obtained should provide strong indications about the functions of these genes during Schwann cell development.

On the other hand the analysis of the P0CRE/TGFβRII mouse suggested that during peripheral nerve development TGFβ is probably involved in the control of Schwann cell death and proliferation. But what are the molecular mechanisms involved in these events? Recent work in our laboratory indicated that the c-Jun/JNK pathway is involved in TGFβ-mediated apoptosis (Parkinson et al., 2001). Is this the only pathway involved or there are other pathways that play a role in this process? And what about TGFβ-mediated proliferation?

The use of DNA microarrays could be a way to answer these questions. Comparing the gene profiling of Schwann cells from normal mice and from mice in which the TGFβ receptor type II has been specifically ablated, under various conditions, could provide important clues in this direction. For example it would give an indication of the downstream targets of Smad proteins in Schwann cells, or it could provide a general idea of the genes directly controlled by TGFβ during peripheral nerve development in vivo.

In conclusion, the experiments here described provide some novel information about genes that could play a part in the molecular mechanism involved in Schwann cell differentiation and myelination, and represent a broad platform for the plan of future experiments.
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APPENDIX

Mapping and structural annotation of the probes found to be differentially expressed between E14 and E18 and between P12 and P12cut.

In the following documents, the mapping and structural annotation of the 130 probes differentially expressed between E14 and E18 and of the 237 probes differentially expressed between P12 and P12cut is provided.

The probes are divided based on the set in which they clustered in the K-means clustering performed with GeneSpring and shown in Figures 3.4 and 3.11.

The 10 sets for the E14vsE18 comparison are provided first, followed by the 15 sets resulting from the P12vsP12cut comparison.

These documents have been produced by Dr David Michalovich and Dr Morris Paterson, at Inpharmatica.
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  - Symbol: ID3
  - OMIM: 600277
  - Keywords: GO:0016307 nucMut unlikely
  - Structure: Unlikely to have helix-loop-helix domain

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  - Myeloid differentiation primary response gene 118
  - Symbol: Myd116
  - OMIM: 00*00030154
  - Keywords: GO:0005622 Alternative splicing unlikely

- **P12464**: insulin-like growth factor binding protein 2, Igf2ip2, 304
  - Insulin-like growth factor binding protein 2
  - Symbol: Igf2ip2
  - OMIM: 00*00069146
  - Keywords: Unlikely to be an insulin-like growth factor binding protein homologue
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<td>IL8</td>
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<tr>
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**Gene Symbol**
- Vcam1: Vascular cell adhesion molecule 1
- IL8: interleukin 8
- PC: Pyruvate carboxylase

**GO Terms**
- GO:0016337: cell adhesion
- GO:0005615: high affinity
- GO:0005515: protein binding
- GO:0005515: cell adhesion molecule
- GO:0007155: cell adhesion
- GO:0016021: integral to membrane
- GO:0005515: protein binding
- GO:0005194: cell adhesion

**Structure Annotation**
- Interleukin 8-like chemokines
- Pyruvate carboxylase

**Function Annotation**
- Vascular cell adhesion molecule 1
- Interleukin 8-like chemokines
- Pyruvate carboxylase
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**Note:** This table contains data related to gene expression and function, including GO terms such as "signal transduction," "cellular component," and "molecular function."
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Notes:
- Dppal - Dihydroorotase and related cytosolic dihydroorotase, Bub3-Family: C. d. dom.
- E. coli 2.30.31.1.1 - E. coli 2.30.31.1.1
- X. laevis - Xenopus laevis
- DAP (UDP)-dihydronicotinamide deaminase (UDP-glucose deaminase) DNA binding, intermediate filament head (DNA binding), intermediate filament head (DNA binding)
- Intermediate filament head (DNA binding), intermediate filament head (DNA binding)
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**GO Terms:**
- GO:0000287 magnesium K+ binding
- GO:0004872 receptor activity likely
- GO:0004895 catenin adhesion receptor activity
- GO:00005194 integrin adhesion molecule activity
- GO:00007155 cell-matrix adhesion
- GO:0006305 integrin complex
- GO:0016021 integral to membrane
- GO:0030593 neutrophil chemotaxis
- GO:0045123 cellMar extravasation

**Super-Families:**
- Death domain
- Cytochrome P450 family
- Integrin family

**Probes:**
- Hand mapped to BAC 35150.1 1157 hypothetical protein
- Probe mapped to directly with high confidence to Gentamicin
- Probe mapped to directly with high confidence to Gentamicin
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- **Protein Name**: Proenkephalin
- **Accession**: S10565_6_at
- **Score**: 313
- **Function**: Synclen domain

**S13507**
- **Protein Name**: Microtubule-associated protein 2
- **Accession**: S13507
- **Score**: 1825
- **Function**: Synclen domain
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*Family: BSH-domain*<br>**Super-Family:** CIC chloride channel | 16 | 0.0005667 | transcription factor complex | 100% GT confidence |

*Family: BSH-domain*<br>**Super-Family:** FabDHe | 16 | 0.0005667 | transcription factor complex | 100% GT confidence |

*Family: POU-domain*<br>**Super-Family:** Thio-kinase | 20 | 0.0005667 | transcription factor complex | 100% GT confidence |

*Family: POU-domain*<br>**Super-Family:** FabDHe | 16 | 0.0005667 | transcription factor complex | 100% GT confidence |

*Family: POU-domain*<br>**Super-Family:** FabDHe | 16 | 0.0005667 | transcription factor complex | 100% GT confidence |

*Family: POU-domain*<br>**Super-Family:** FabOHe | 16 | 0.0005667 | transcription factor complex | 100% GT confidence |
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**Probeset Description**

- **Probe**: A probe is a segment of DNA or RNA that is complementary to a specific stretch of DNA or RNA.
- **Mapping**: The location of the probe on the genome.
- **Accession**: The accession number of the gene.
- **Lent.**: The length of the probe.
- **Description**: A description of the gene or protein.
- **Gene**: The gene symbol.
- **Family**: The family to which the gene belongs.
- **Databases**: The databases where the gene is found.
- **Keywords**: Keywords related to the gene.
- **GO**: Gene Ontology terms associated with the gene.
- **Supp**: Support information.
- **This**: The presence of the gene in the current study.
- **Sequence Annotation**: Annotation related to the gene's sequence.
- **Structure Annotation**: Annotation related to the gene's structure.
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</table>
rc AA863302_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005887 integral to plasma membrane
- GO:0005922 complex coherence
- GO:0005524 ATP binding
- GO:0040007 growth
- GO:0046668 transcription factor activity

rc AA864436_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005783 endoplasmic reticulum
- GO:0006695 cholesterol biosynthesis
- GO:0006610 lipid biosynthesis
- GO:0016323 apical plasma membrane
- GO:0016324 apical plasma membrane

rc R351997_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0045028 purinergic nucleotide receptor activity
- GO:0016323 basal plasma membrane
- GO:0007186 G-protein coupled receptor activity
- GO:0005887 integral to plasma membrane
- GO:0005922 complex coherence

rc R354298_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005783 endoplasmic reticulum
- GO:0006695 cholesterol biosynthesis
- GO:0006610 lipid biosynthesis
- GO:0016323 apical plasma membrane
- GO:0016324 apical plasma membrane

rc U58639_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005783 endoplasmic reticulum
- GO:0006695 cholesterol biosynthesis
- GO:0006610 lipid biosynthesis
- GO:0016323 apical plasma membrane
- GO:0016324 apical plasma membrane

rc X04970_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005783 endoplasmic reticulum
- GO:0006695 cholesterol biosynthesis
- GO:0006610 lipid biosynthesis
- GO:0016323 apical plasma membrane
- GO:0016324 apical plasma membrane

rc X144866_at probe mapped to Ensembl Gene Model. The probe mapped to AHAH17534.1 with high confidence to identified with the following GO terms:

- GO:0005783 endoplasmic reticulum
- GO:0006695 cholesterol biosynthesis
- GO:0006610 lipid biosynthesis
- GO:0016323 apical plasma membrane
- GO:0016324 apical plasma membrane
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<th>Strain/Genus Accession</th>
<th>Last Name</th>
<th>Description</th>
<th>Genome</th>
<th>OMIM</th>
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<th>GO</th>
<th>Split ID</th>
<th>Title</th>
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<th>Structure Annotation</th>
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**Probe 12**

- **Probe Name**: AA1401033_at
  - **Strain/Genus**: 902368mB64_1
  - **Mapping**: direct with high confidence
  - **Sequence**: 131370

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**Probe 50**

- **Probe Name**: AA1401033_at
  - **Strain/Genus**: 902368mB64_1
  - **Mapping**: direct with high confidence
  - **Sequence**: 434
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<td>U65217_i_at</td>
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**Note:** The table above lists genes with associated GO terms and confidence levels. The GO terms include GO:0032876 (protein binding) and GO:0016042 (lipid catabolism). The confidence levels are indicated as high, medium, and low. The references are not provided in the image.
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<th>Function</th>
<th>Expression</th>
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<td>GO:0003775</td>
<td>membrane spanning</td>
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<td>Fatty acid binding protein 4 polypeptide</td>
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