Diss. ETH No. 13321

Effects of the Absence of Schwann Cell Membrane Proteins on Myelin Formation and Survival of Axons

Electron microscopic investigations on myelin formation in β4 integrin-deficient mice and on survival of axons in aged P0-deficient mice

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZURICH

For the degree of
Doctor of Natural Sciences

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1999

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1 Kurzfassung

Die schnelle Fortleitung von elektrischen Impulsen im peripheren Nervensystem beruht auf der Isolation der Axone durch die Mark- (Myelin-) scheiden. Eine Voraussetzung für den Aufbau einer Markscheide ist die Wechselwirkung zwischen der Schwannschen Zelle und ihrer Basallamina. Unter mehreren Kandidaten-Molekülen, welche diese wichtige Interaktion vermitteln können gibt es die Integrine, die als spezialisierte Zelloberflächenmoleküle in der Lage sind, extrazelluläre Matrixkomponenten zu binden. Obwohl Schwannsche Zellen eine Auswahl von verschiedenen Integrin-Untereinheiten exprimieren, ist gerade das β4 Integrin ein vielversprechender Kandidat für die Vermittlung spezifischer Wechselwirkungen in myelinisierenden Schwannschen Zellen, weil es zeit- und gewebe-spezifisch während des Beginns der Myelinisierung hochreguliert wird. Um zu klären, ob Schwannsche Zellen auch zur Markscheidenbildung fähig sind, wenn das β4 Integrin fehlt, untersuchten wir β4 Integrin-defiziente Mäuse. Diese Tiere sterben wenige Stunden nach Geburt, weshalb wir nur femorale Quadricepsnerven von neugeborenen Tieren elektronenmikroskopisch untersuchen konnten. Die Quadricepsnerven von β4 Integrin-defizienten Mäusen zeigten dieselbe gemischte Population von frühen Myelinisierungsstadien wie die Kontrolltiere. Zusätzlich kultivierten wir Spinalganglien unter Bedingungen, welche eine Markscheidenbildung erlaubten. Die Quantifizierung von bemarkten Segmenten in den Kulturen, welche mit Antikörpern gegen etablierte Myelinmarker gefärbt wurden, ergab keine Unterschiede zwischen β4-defizienten und Kontrolltieren. Die Ultrastruktur dieser Myelinsegmente war normal. Aus diesen Ergebnissen zogen wir den Schluss, dass β4 Integrin für die Markscheidenbildung nicht notwendig ist.

Im zweiten Projekt fokussierten wir unser Interesse auf das Zelloberflächenmolekül P0, welches das häufigste Protein im peripheren Myelin ist. Das Fehlen von P0 führt zu schwerwiegender, kongenitaler Dysmyelinisierung und zur Bildung von nichtkompaktiertem Myelin, was eine erschwerte Fortleitung des Aktionspotentials in den peripheren Nerven zur Folge hat. P0 ist eines der ursächlichen Gene für das Déjérine-Sottas Syndrom, eine klinisch schwere Form der humanen

hereditären Neuropathien. Diese Krankheit ist gekennzeichnet durch ihren Beginn in der Kindheit, Symptome von distaler Muskelschwäche, reduzierte Nervenleitgeschwindigkeiten von weniger als 10m/s und morphologischen Merkmalen von De- und Remyelinisierung. Zusätzlich zur Demyelinisierung wurden in Patienten wie auch in P0-defizienten Mäusen degenerierende Axone gefunden. Wir bestimmten das Ausmaß der axonalen Schädigung im P0-defizienten Mausmodell mit dem Ziel, eine Grundlage für Untersuchungen zu therapeutischen Möglichkeiten in Myelinkrankheiten zu finden. In verschiedenen proximalen Nervenästen fanden wir reduzierte Durchmesser von bemarkten Axonen, jedoch war keine signifikante Reduktion der Anzahl der Axone festzustellen. Distale sensible Nervenäste zeigten eine deutliche Reduktion myelinisierter Axone und die Anzahl der epidermalen sensorischen Rezeptorzellen war auffallend gering. Muskelfasern von P0-defizienten Mäusen erschienen atrophisch und zeigten Merkmale von Denervierung und Reinnervierung durch Nervensprossung aus benachbarten motorischen Einheiten. Zusammenfassend kann festgestellt werden, dass Axone in P0-assoziierten Myelinerkrankungen von distal nach proximal fortschreitend degenerieren. Unsere Beobachtungen stützen die Annahme, dass die klinischen Symptome der Patienten, welche an einer schweren Form von hereditärer motorisch-sensibler Neuropathie erkrankt sind, möglicherweise durch die axonale Degeneration bedingt sind.

Bei anderen, nicht-hereditären, neurodegenerativen Erkrankungen, wie der Amyotrophen Lateralsklerose oder der diabetischen Neuropathie, wurden bereits neurotrophe Faktoren als vielversprechende Agenzien in das Behandlungsspektrum aufgenommen. Aufgrund unserer Resultate schlagen wir vor, dass die P0-defizienten Mäuse zum Austesten des therapeutischen Potentials von neurotrophen Faktoren in hereditären primär myelin-bedingten Neuropathien benutzt werden können.

2 Summary

The fast propagation of electric signals in the peripheral nervous system depends on the insulation of axons by myelin sheaths. A prerequisite for the formation of myelin is the interaction of the Schwann cell with its basal lamina. Among several candidate molecules that mediate this important interaction are the integrins - specialized cell surface receptors - which are able to bind to extracellular matrix components. Although Schwann cells express a set of different integrin subunits, the β4 integrin is a promising candidate for mediating specific interactions in myelinating Schwann cells being expressed in a temporal and tissue-specific manner at the onset of myelination. To elucidate the competence of Schwann cells to myelinate in the absence of β4 integrin, we investigated β4 integrin-deficient mice. β4 knockout mice die within a couple of hours after birth, therefore, we examined the femoral quadriceps nerves of newborn animals at the electron microscopic level. The femoral quadriceps nerves of β4-deficient mice showed the same diversity of early myelination stages as those of control animals. Furthermore, we cultured dorsal root ganglia under conditions allowing myelination. Quantification of cultured myelin-segments that were immunolabeled for established myelin proteins revealed no differences between control and knockout animals. The ultrastructure of these myelin-segments was normal. Based on these results, we concluded that the β4 integrin subunit is not required for myelin formation.

In the second project we focused on the cell adhesion molecule P0 which is the most abundant protein in peripheral myelin. A lack of functional P0 leads to severe and congenital dysmyelination and the formation of noncompacted myelin which results in impaired propagation of action potentials in peripheral nerves. P0 is the culprit gene for a clinically severe form of human hereditary motor and sensory neuropathies, the P0-related Déjérine-Sottas syndrome. Having its onset in infancy, the disease presents with symptoms of distal muscle weakness, slowed nerve conduction velocity below 10m/s and nerve fibers that show signs of de- and remyelination. In addition to demyelination, profiles of degenerating axons were observed in patients and in the P0-deficient mouse model. We assessed the extent of axonal involvement in the P0 mouse model with the aim to provide a basis for investigations on therapeutic possibilities in myelinopathies. In different proximal nerve branches we detected reduced calibers of myelinated axons,

but no significant reduction of the axon numbers. Distal sensory nerve branches displayed a marked loss of myelinated axons and the number of sensory epidermal target cells was significantly reduced. Muscle fibers of P0-deficient mice were atrophic and demonstrated signs of denervation and reinnervation by sprouts of neighboring motor units. In conclusion, we found that axons in P0-related myelin deficiencies degenerated in a dying-back mechanism. Our observations provide additional evidence for the assumption that the clinical symptoms of patients affected by severe forms of hereditary motor and sensory neuropathies may predominantly result from axonal degeneration.

Therapeutic approaches to treat other, non-hereditary, neurodegenerative disorders such as amyotrophic lateral sclerosis or diabetic neuropathy, included neurotrophic factors as promising beneficial agents. We propose that the P0-deficient mice may be used for testing the therapeutic potential of neurotrophic factors also in hereditary myelinopathies.

3 Introduction

3.1 Myelin Formation in the Peripheral Nervous System

For the correct function of the nervous system, rapid conduction of action potentials along the axons is needed. Rapid conduction of electrical signals can be achieved either by increasing the diameter of the conducting cable or by efficient electrical insulation of the axon. Lower invertebrates use the first strategy, whereas organisms with a more complex nervous system insulate their axons with a fatty coat named myelin. Thus they achieve conduction velocities of 40-60 m/s and more, and use less than 1% of the diameter that an axon without insulation needs to conduct an action potential at the same speed.

In the peripheral nervous system (PNS), myelin sheaths are formed by specialized glial cells, the Schwann cells. Schwann cells extend processes and wrap them around the axon. After several turns, the cytoplasm residing in the membrane layers is squeezed out and additional layers are tightly wrapped in subsequent turns. This compaction of the membrane layers results in a myelin sheath which is highly resistant to currents. In myelinated axons, the conduction of the action potential is restricted to the nodes of Ranvier that are specialized sites between the internodal myelin sheaths of adjacent glial cells. At the axonal plasmalemma, the nodes of Ranvier are characterized by an accumulation of ion channels that are needed for the propagation of the action potentials and which are very rare in the internodes (for review see Burden and Yarden, 1997).

The formation of a normal and efficient myelin sheath in peripheral nerves is critically dependent on the correct development of the myelinating Schwann cells. Subsequently, the initial development of these glial cells will be described and their final differentiation explained, focusing on the influences of Schwann cell-axon and Schwann cell-extracellular matrix interactions.

3.1.1 From the neural crest to myelinating Schwann cells

The neural crest is a transient population of migratory cells which develops at the dorsal side of the neural tube during neurulation. The cells migrate throughout the

embryo along well-defined pathways to their final destination. Neural crest cells (NCCs) are initially pluripotent (Stemple and Anderson, 1992; Shah et al., 1994). They can differentiate into a variety of cell types, such as sensory and sympathetic ganglia of the PNS, glial cells (Schwann cells), secreting cells of the adrenal gland or pigment cells of the skin. In the head, NCCs give rise to bone, cartilage and other connective tissue (Le Douarin and Ziller, 1993). The NCCs become restricted to specific fates at various time points during development. The mechanisms for cell fate restrictions are not clear yet, but Shah et al (1994) found evidence that glial growth factors (neuregulins) instruct NCCs to differentiate into glial cells (Shah et al., 1994). NCCs give rise to Schwann cell precursors. Those differentiate into immature Schwann cells which undergo final differentiation steps into either non-myelin- or myelin-forming Schwann cells (Jessen and Mirsky, 1998) (Fig. 1).

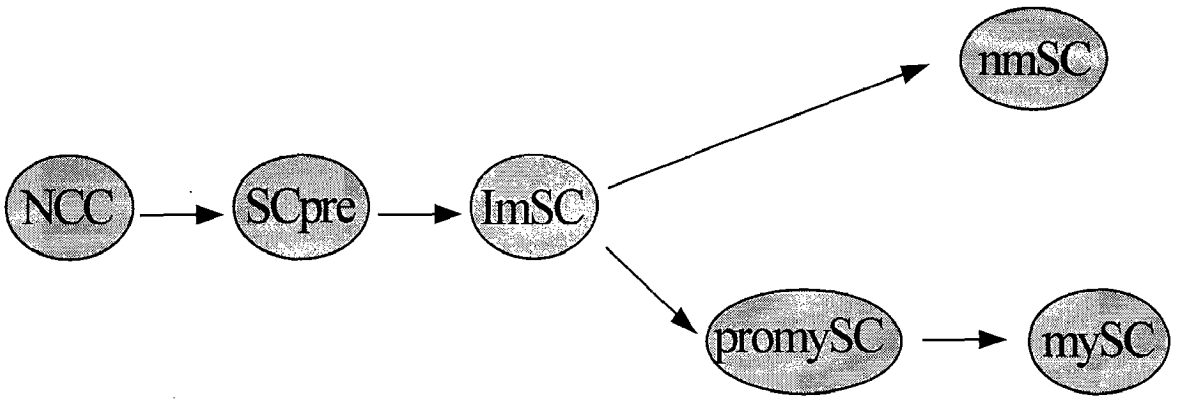
Molecular markers for different developmental stages of Schwann cells

NCC change their expression of cell surface molecules in a developmentally regulated pattern. One of the earliest markers for glial lineage found in NCC is the low affinity receptor for nerve growth factor (LNGFR, $p75^{NGF}$). When the transition of NCC into proliferative Schwann cell precursors is completed, around E12 in the mouse, these cells start to produce other glial cell markers such as the Neural Cell Adhesion Molecule (N-CAM), the cell adhesion molecule L1 and the Glial Fibrillary Acidic Protein (GFAP). It has recently been shown that Schwann cell precursors already express the myelin Protein Zero (P0) at a low basal level (Lee et al., 1997). Schwann cell precursors are critically dependent on axonal contact for their survival, because they need growth factors (neuregulins, NRGβ) which are provided by outgrowing neurons (Jessen and Mirsky, 1998) (Fig. 1).

Between E12/13 and E14/15 in the mouse, Schwann cell precursors undergo fundamental changes in order to become immature Schwann cells (Dong et al., 1999). When plated in culture at normal densities, immature Schwann cells survive without axons *in vitro*. Recent findings show that Schwann cells acquire independence from axons by establishing an autocrine loop involving different growth factors (Meier et al., 1999). Immature Schwann cells express N-CAM, L1, GFAP, the cytosolic Ca^{2+}-binding protein S100β (Kligman and Hilt, 1988), and galactocerebrosides (GalC), glycolipids

that are highly enriched in myelin (Coetzee et al., 1998). They also keep their basal P0 expression. Immature Schwann cells differentiate reversibly into non-myelinating or myelinating Schwann cells. Non-myelinating Schwann cells keep the early markers of glial cells (N-CAM, L1, GFAP, LNGFR), but they suppress the basal expression of P0, most probably due to their contact with small caliber axons. The final differentiation into this type of glial cell occurs postnatally. The transition from immature into myelinating Schwann cells is characterized by a downregulation of LNGFR, N-CAM, L1 and GFAP and a strong upregulation of myelin genes, including P0, Myelin Basic Protein (MBP), and Peripheral Myelin Protein 22 (PMP22) (Jessen and Mirsky, 1998) (Fig. 1). The expression of myelin genes peaks between two and four weeks postnatally and is later downregulated to intermediate levels when myelination is completed.

The different stages of Schwann cell development are also characterized by specific transcription factors: NCC, Schwann cell precursors, immature Schwann cells and promyelinating Schwann cells express Pax3. Immature Schwann cells begin to express SCIP (suppressed cAMP-inducible POU protein) and, at least 24 hours later, EGR2/Krox20 (early growth response 2 gene product; Krox20 is the mouse homologue) (Topilko et al., 1994). Schwann cells that further differentiate into non-myelinating cells maintain SCIP expression, but downregulate EGR2. Differentiation into myelinating Schwann cells leads to a prolonged expression of EGR2 and a downregulation of SCIP before myelination is completed (Jessen and Mirsky, 1998) (Fig. 1).



	Neural crest cells	Schwann cell precursors (SCpre)	Immature Schwann cells (ImSC)	Promyelinating Schwann cells (promySC)	Myelinating SC (mySC)	Non myelin-forming SC (nmSC)
Time schedule, mouse	E8.5	E12/13	E15/16	(E17/18)	(E18)	(postnatally)
Transcription factors	Pax3	Pax3 c-Jun	Pax3 SCIP EGR2	Pax3 SCIP EGR2	EGR2 (SCIP)	SCIP
Molecular markers, cellular properties	LNGFR	LNGFR NCAM, L1 GFAP GAP43 basal P0 Need NRGβ (provided by axonal contact)	S100 basal P0 LNGFR NCAM, L1 GFAP GalC Autocrine survival loop	S100 high P0 MAG GalC	S100 high P0 MAG PMP22 MBP Cx32	S100 no P0 LNGFR NCAM,L1 GFAP

Figure 1 *Different stages of Schwann cell development.*

Neural crest cells (*NCC*) undergo several maturation steps during differentiation into myelinating Schwann cells (*mySC*) and non-myelinating Schwann cells (*nmSC*). During these transitions, the cells change the expression pattern of transcription factors and molecular markers as is shown in the table. In the mouse, the most important transition from Schwann cell precursors to immature Schwann cells takes place between E13 and E15 and is characterized by the gain of independence of neuron-derived β-forms of neuregulins (*NRGβ*). Promyelinating Schwann cells (*promySC*) are defined as Schwann cells that form a typical one-to-one ratio with an axon, but that have not formed myelin yet. For abbreviations of molecular markers and transcription factors, see text (adapted from Jessen and Mirsky, 1998).

3.1.2 Axons provide instructive signals for the Schwann cells to initiate myelination

In the PNS development axons innervate the limbs in fascicles. At this stage, Schwann cell precursors migrate into developing limbs in conjunction with the axon fascicles. When the neurons have reached their targets, further differentiated Schwann cells start introducing processes among the fasciculated axons. Larger axons are segregated by prospective myelinating Schwann cells and one-to-one ratios between an

axon and a Schwann cell are accordingly formed (Martini et al., 1988; Martini, 1994). Non-myelinating Schwann cells keep contact with a bundle of smaller axons that will never be myelinated, thereby forming cytoplasmic processes around single axons to separate them from each other. The first cue for the Schwann cell to commit to a myelinating phenotype seems to come from the axon. These signals are presumably transduced by cell adhesion molecules that are expressed by axons and Schwann cells. The caliber of the axon appears to play an important role for myelin-competence (Voyvodic, 1989), but the mechanisms how the Schwann cells perceive the size of the axon are not clear.

Interactions of the Schwann cell with the axon are mediated by the cell adhesion molecules N-CAM, L1 and MAG, which interact in a homophilic or heterophilic binding. L1 and N-CAM expression at the adaxonal side has been shown for early Schwann cells surrounding fasciculating axons and for non-myelinating Schwann cells (Martini and Schachner, 1986; Martini, 1994). Myelinating Schwann cells downregulate the expression of L1 and N-CAM after approximately one and a half turn around the axon, while MAG expression is simultaneously increased. The myelin protein P0 is upregulated when the Schwann cells have established a 1:1 ratio with an axon (Martini et al., 1988). At this early stage of myelination, MAG and P0 are located in the adaxonal membrane of the first noncompacted turn. When myelination proceeds, MAG expression becomes restricted to the noncompacted parts of the myelin sheath, the Schmidt/Lanterman incisures, the paranodal and the adaxonal cytoplasmic loops, whereas P0 becomes confined to compacted areas of the myelin (Martini, 1994).

After myelination has begun, both axons and Schwann cells undergo further maturation steps, such as extension of axonal diameter and increase of myelin thickness. Normal adult nerve fibers acquire a specific correlation between axon caliber and thickness of the myelin sheath which usually is described by the g-ratio. The final length of the internodes is also related to the myelin thickness, i.e., larger caliber fibers present with long and small fibers with short internodes. The maturation of axons and Schwann cells most probably requires signaling from both partners. Changes in the mutual influence of Schwann cells and axons lead to severe dysfunctions of the nerve fibers.

3.1.3 Interaction of the Schwann cell with the basal lamina allows myelination

In the course of differentiation, Schwann cells assemble a specialized extracellular matrix at their abaxonal side. The Schwann cells that ensheathe outgrowing axon bundles start expressing and secreting extracellular matrix (ECM) components (for review see Martini, 1994). In the PNS, both non-myelinating and myelinating Schwann cells are surrounded by this basal lamina. The basal lamina consists of different matrix components like the glycoproteins laminin 1 and 2, fibronectin and entactin/nidogen, as well as type IV collagen and heparansulfate-proteoglycan (Martini, 1994). The role of basal lamina components in the formation of myelin has been extensively studied, resulting in the proposition that interaction of the Schwann cells with their basal lamina is important for myelination (for review see Bunge, 1993).

How is the Schwann cell induced to assemble its basal lamina? Schwann cells cultured in the absence of neurons are not able to form basal lamina. When Schwann cells are cultured with neuron-conditioned medium or when neuron-Schwann cell cocultures are used that do not allow direct contact between the two cell types, only patchy basal lamina is formed. From these results it has been concluded that neurons provide a diffusible factor that initiates the expression of basal lamina components in the Schwann cells (Clark and Bunge, 1989). However, such a factor is not yet known. Investigations of the basal lamina of Schwann cells cultured with neuron-conditioned medium revealed an abnormal ultrastructure, suggesting that a diffusible neuronal factor is not sufficient to initiate the correct assembly of basal lamina (for review see Bunge, 1993).

In neuron-Schwann cell cocultures, all Schwann cells in contact with neurites upregulate the expression of basal lamina components. The myelination of larger caliber axons *in vitro*, however, requires the presence of ascorbic acid. Ascorbic acid promotes the formation of triple-helical collagen IV by hydroxylation of the prolin-residues in the collagen subunits. Inhibition of this form of collagen strongly interferes with the assembly of basal lamina and with myelination (Eldridge et al., 1987; Eldridge et al., 1989). The requirement of ascorbic acid can be circumvented by the addition of exogenous ECM (Eldridge et al., 1989). From these studies it emerges that Schwann cells need extracellular matrix containing laminin and triple-helical collagen IV to

initiate proper myelination. Additional evidence for the importance of complete basal lamina for myelination in the PNS comes from studies in dystrophic mice. Two strains of dystrophic mice either producing a non-functional truncated form or lacking laminin 2 show dysmyelination in proximal and distal parts of peripheral nerves (Stirling, 1975; Weinberg et al., 1975; for review see Matsumura et al., 1997).

The mechanism by which basal lamina supports myelination in the PNS is not clear. It has been suggested that the assembly of the basal lamina helps to keep specific ECM components in close apposition to their corresponding Schwann cell surface receptors, such as the integrins. The interaction of Schwann cell surface receptors with the basal lamina may provide essential structural stability for the Schwann cell during the spiraling process in myelination. In addition, integrins and possibly other ECM receptors on the abaxonal membrane of the Schwann cell may convey important signals from the extracellular environment to the myelinating cell.

In the CNS, myelin is formed by oligodendrocytes. Oligodendrocytes do not form one-to-one ratios with axons but they are able to myelinate several different axons. They lack a basal lamina similar to that of Schwann cells. Therefore, it was suggested that oligodendrocytes do not need the structural stability provided by ECM. Investigations on the myelination in the CNS have been difficult because of the lack of an appropriate *in vitro* model. However, recent studies on slice cultures of brain tissue argue for an involvement of ECM-like molecules also in CNS myelination (Raval-Fernandes and Rome, 1998).

The basal lamina is a specialized kind of extracellular matrix

Extracellular matrices are composed of insoluble fibers like collagens and soluble polymers like glycoproteins and proteoglycans. They fulfill diverse functions: Glycoproteins have the ability to influence cell behavior by allowing attachment and migration of cells, thus are important players in developmental and pathological changes. The binding of ECM components to cell surface receptors may result in significant changes in the gene expression of the cells. Interaction between the ECM ligands and their receptors can be localized to specific domains within the matrix components, such as fibronectin type III repeats (FNIII), epidermal growth factor- (EGF-) like domains or Arg-Gly-Asp- (RGD-) sequences (Ayad et al., 1998).

A most important component of Schwann cell basal lamina is the glycoprotein laminin. The laminins are able to interact with cells via cell surface receptors, as well as with other basement membrane components, thereby organizing and stabilizing the ECM. Laminins are composed of three genetically distinct chains (5 α, 3 β, and 2 γ chain isoforms) which assemble into more than 7 different isoforms by interaction in triple helical coiled-coil structures. Isomeric forms of laminin differ in their specific interactions with cell surface receptors and exhibit developmentally regulated and tissue-specific patterns of expression (Ayad et al., 1998).

Integrins

Schwann cells express receptors for basal lamina components that belong to the large family of integrins. Integrins are heterodimeric, non-covalently associated cell-surface proteins. Currently there are 14 α and 9 β subunits known that can associate to form approximately 20 different integrin receptors (Fernandez-Valle et al., 1998). Additional diversity of the receptors is achieved by alternative splicing of the individual subunits. The integrin subunits comprise a large extracellular domain, a transmembrane segment and a short cytoplasmic sequence (approximately 50 amino acids) (Fig. 2*A*).

Integrins are the primary cellular receptors for many ECM constituents, including fibronectin, laminin, thrombospondin and several collagens. They are expressed by a wide variety of cells, such as cells of the immune system, endothelial and epithelial cells, neurons and glial cells. Some integrin subunits are almost ubiquitously expressed, while others have a very restricted expression pattern. The ligand specificities of different integrins overlap in the way that one integrin binds several ligands and one ligand may be bound by different integrins (Hynes, 1992).

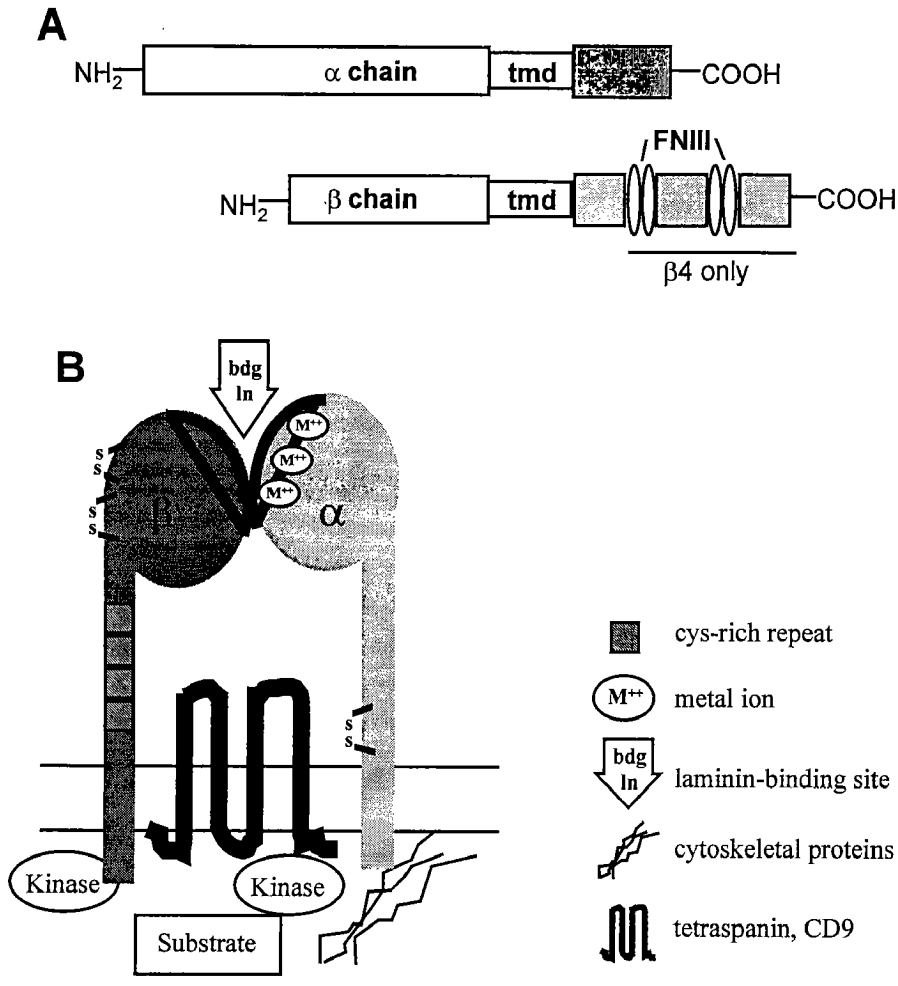


Figure 2 *Structural characteristics of integrins*

(*A*) All integrins are transmembrane heterodimers composed of an α subunit non-covalently associated with a β subunit. Whereas short β subunits have a cytoplasmic domain of approximately 50 amino acids, the β4 subunit has a long intracellular domain (1000 amino acids) that includes two pairs of fibronectin III (FNIII) repeats. *tmd*, transmembrane domain; (adapted from Archelos et al., 1999). (*B*) Hypothesized overall shape of an integrin receptor, showing the putative locations of the cystein-rich repeats of the β subunit (*crosshatched*) and metal-binding sites in the α subunit (M^{++}). In both, the α and the β subunit, there are several disulfide bonds. The *arrow* points to the area representing the ligand-binding region (*bdg ln*: binding of laminin), which is known to be made up from both subunits based on cross-linking and binding data. Integrin subunits have been shown to interact with cytoskeletal proteins, with kinases and with linker proteins of the tetraspanin family that are able to recruit kinases and their substrates (adapted from Hynes, 1992, and Hadjiargyrou et al., 1996).

The functions of integrins are diverse. In the development of the nervous system they mediate neuronal attachment and control growth cone movement (for review see Reichardt and Tomaselli, 1991). Experiments with integrin antisense oligonucleotides

resulted in dramatic inhibition of the migration of cranial neural crest cells (Kil et al., 1996). The importance of integrins in cell migration and attachment was first explained by their ability to bind cytoskeletal structures, thereby directly altering the cytoskeletal architecture. However, there was increasing evidence from studies on immune cells that integrins are able to mediate signals in a more complex way (Juliano and Haskill, 1993). First, integrins are able to transduce signals by regulating the levels of second messengers inside the cell; secondly, integrins recruit cytoplasmic kinases that are involved in phosphorylation cascades used to change gene expression; thirdly, the affinity of integrin receptors for their ligand and the ligand specificity can be altered significantly through other surface receptors on the same cell (Fernandez-Valle et al., 1998; for review see Reichardt and Tomaselli, 1991; Dedhar and Hannigan, 1996). Finally, integrins associate with transmembrane linker proteins, such as proteins of the tetraspanin family (Maecker et al., 1997), which are known to be involved in the clustering of membrane receptors and their intracellular signaling partners (Fig. 2*B*). In summary: Cells that express integrins have numerous possibilities to change the functional characteristics of integrin receptors. This enables the cells to alter the quality of signals that they get from the ECM according to their own differentiation state.

3.1.4 Does the α6β4 integrin have a pivotal role in PNS myelination?

Schwann cells change their integrin expression pattern at the onset of myelination.

Schwann cells express several integrins. The heterodimers α1β1, α2β1, α6β1, α6β4, αvβ8 and αvβ3 have been detected in human myelinating Schwann cells (Archelos et al., 1999) as well as in cultured rat Schwann cells (Milner et al., 1997). However, there is not much known about their function in these cells. Non-myelinating Schwann cells and Schwann cells in a promyelinating stage express α1β1 and α6β1 integrin (Einheber et al., 1993; Feltri et al., 1994; Stewart et al., 1996). Upon myelination both *in vivo* and *in vitro*, the β4 integrin subunit is upregulated while α6 and β1 mRNA levels are not increased (Feltri et al., 1994). The switch from α1β1 or α6β1 to α6β4 expression in myelinating Schwann cells is well documented (Sonnenberg et al., 1990; Einheber et al., 1993; Feltri et al., 1994; Fernandez-Valle et al., 1994). After nerve lesion, the expression of β4 is downregulated in Schwann cells,

very similar to the downregulation of myelin proteins (Einheber et al., 1993). Together, these studies demonstrate that in Schwann cells the expression of α6β4 integrin is regulated in a similar pattern as the typical myelin genes.

Expression pattern of α6β4 in various tissues

The expression of α6β4 integrin is restricted to a number of specialized cells and tissues. Proliferating basal keratinocytes of stratified squamous epithelia that form the skin express α6β4 in high amounts (Sonnenberg et al., 1990). In addition, α6β4 integrin is present in epithelial tissues of intestine, lung and kidney, in the perineurium and in Schwann cells, whereas no expression of α6β4 integrin was detected in the CNS (Sonnenberg et al., 1990). The α6β4 complex is essential for the assembly and maintenance of hemidesmosomes in epithelial keratinocytes, whereas Schwann cells do not form such structures. Hemidesmosomes are specialized sites for the attachment of the cell to the extracellular matrix and they are critically dependent on α6β4 for their adhesive function. A loss of either integrin subunit is detrimental, as has been shown in α6 and β4 integrin-deficient mice which die around birth due to extended skin detachment (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). In humans, mutations in the α6 or β4 integrin lead to various forms of a skin disease named epidermolysis bullosa (Pulkkinen et al., 1998a,b). The tissue-specific expression pattern of α6β4 integrin and the fact that the function of α6β4 integrin cannot be compensated for by other integrins in epithelial cells, pointed to a possibly essential role for this ECM receptor in the function of myelinating Schwann cells.

The β4 integrin subunit is unusual in its structure and in its intracellular interactions.

The primary explanation for the unique functions of the α6β4 integrin lies in the special amino acid sequence of the β4 integrin subunit. β4 is the only integrin subunit with a long cytoplasmic tail of 1000 amino acids. It comprises two pairs of FNIII repeats separated by a linker sequence and seems to be unique, since no homologous proteins have been found. The similarity of the extracellular domains of the β4 and other β integrin subunits is approximately 35%. Four different isoforms of the β4 integrin

subunit are known to date, all of which are differentially spliced in their intracellular domain (Hogervorst et al., 1990) (Fig. 2*A*).

Like other integrins, α6β4 binds to laminin isoforms with its extracellular domain. However, the intracellular interactions of the β4 integrin subunit differ significantly from those of other β integrin subunits. Short β subunits are known to interact with actin-linked cytoskeletal proteins. They also recruit the tyrosine kinases FAK (focal adhesion kinase) or ILK (integrin-linked kinase) to activate further intracellular signaling cascades (Dedhar and Hannigan, 1996). In the β4 integrin subunit, however, the linker sequence of the intracellular part contains a specific sequence that associates with intermediate filaments. This interaction is pivotal for the function of α6β4 integrin in hemidesmosomes. Besides the difference in interacting with cytoskeletal proteins, the β4 integrin subunit also engages different protein kinases for signal transduction. The Shc (Src homology collagen) adaptor protein, which links receptors to intracellular signaling pathways, is able to bind to the β4 integrin subunit and interactions of the β4 with other - yet unknown - tyrosine kinases have been proposed (Mainiero et al., 1995). These characteristics suggest an important role for the β4 integrins not only in skin, but also in Schwann cells.

Does the lack of β4 integrin compromise the formation of myelin?

In myelinating Schwann cells the α6β4 integrin shows an expression pattern similar to that of typical myelin proteins. Hence it was proposed that the interaction of the α6β4 integrin with the basal lamina is functionally important for myelin formation. Due to the lack of convenient experimental approaches like, for instance, function blocking antibodies, no extensive studies on the functional role of β4 integrin in myelination have been carried out. In our study, we investigated the progression of myelination in newborn β4 integrin-deficient mice. We also assessed the potential of β4 integrin-deficient Schwann cells to myelinate outgrowing neurites over a longer time period in DRG explant cultures.

3.2 Disorders of the Peripheral Nervous System

Disorders of the PNS are characterized by an inefficient propagation of action potentials, which results in clinical presentation of impaired muscle performance and sensation, in electrophysiological abnormalities and in morphological pathology of the nerve fibers. In the majority of peripheral neuropathies, the Schwann cells and the myelin are primarily affected, often leading to a secondary damage of the demyelinated axons. Common causes of demyelinating peripheral neuropathies comprise environmental toxins, therapeutic drugs, metabolic disorders such as diabetes, viral and bacterial infections, and immune-mediated events. In addition, a number of chronic hereditary forms of peripheral neuropathies exist. The latter have come into focus during the last decade because of extensive progress in genetic analyses.

3.2.1 Hereditary motor and sensory neuropathies

The first descriptions of patients with a hereditary motor and sensory neuropathy (HMSN) have been published in the late 19th Century by Charcot and Marie in France and by Tooth in England. Therefore, HMSNs have also been named Charcot-Marie-Tooth (CMT) disorders, and both terms are often used as synonyms. The clinical presentation of CMT includes distal muscle weakness and muscular atrophy, affecting feet and lower legs much earlier than hands and arms. Most CMT patients also have mild but significant sensory involvement. Major forms of CMT can be distinguished according to their electrophysiological and morphological pathologies (for review see Dyck et al., 1993; Harding, 1995; De Jonghe et al., 1997).

CMT1 is the most common of these three forms presenting with a variable onset from early childhood to adolescence. Electrophysiological abnormalities that can be measured early in all patients demonstrate low nerve conduction velocities (NCVs) (for review see Harding, 1995). The reduction of NCVs is variable but progressive in the first few years of life (for review see Ionasescu, 1995). Major morphological features described for CMT1 patients are demyelination, onion bulb formation, supernumerary Schwann cells and thinly remyelinated axons. De- and remyelination also results in an increased variability of internodal length that can be observed in single fiber preparations (for review see Dyck et al., 1993).

X-linked CMT (CMTX) is characterized by only slightly reduced NCVs and decreased amplitudes (Hahn, 1993). Nerve biopsies of patients frequently show thinly remyelinated axons and axonal sprouts in the form of regenerating clusters, whereas onion bulb formation is scarce (Hahn et al., 1990; Sander et al., 1998; for review see Martini, 1997; Martini et al., 1998). Other prominent pathological alterations include enlarged periaxonal collars (the innermost Schwann cell loop that remains filled with cytoplasm even when the myelin is compacted) (Hahn et al., 1990; for review see Martini, 1997; Martini et al., 1998). Patients show a mild to moderate clinical phenotype. On the average, male CMTX1 patients show a more severe neuropathy than CMT1A patients with the duplication of the *PMP22* gene (for review see De Jonghe et al., 1997). Male patients are usually also more affected than female carriers who often do not show any clinical symptoms (Hahn, 1993; for review see De Jonghe et al., 1997).

CMT2 encompasses axonal forms of peripheral neuropathies as is demonstrated by near normal NCVs and reduced amplitudes which are indicative of axonal degeneration. Peroneal muscular atrophy and distal muscle weakness are more pronounced in CMT2 than in CMT1, whereas hands are usually less affected. Other differences include that the onset of CMT2 is usually later and pathological alterations suggesting primary axonal impairment rather than myelin degeneration (for review see Dyck et al., 1993; De Jonghe et al., 1997).

CMT3 comprises the most severe disorders of the hereditary neuropathies, Déjérine-Sottas syndrome (DSS) and Congenital Hypomyelination (CH). Clinical, hallmarks include the early affection of fingers and hands and onset of the disease in infancy presenting with delayed motor development. The severity of the disease is also reflected in nerve biopsies frequently presenting with demyelination, onion bulb formation and reduced numbers of myelinated fibers (for review see Ionasescu, 1995). There is some confusion with regards to the clinical classification of DSS patients, because criteria for the diagnosis are rather ill-defined (for review see De Jonghe et al., 1997).

Approximately 76% of all CMT cases are inherited in an autosomal dominant way. However, X-linked (as mentioned above) and autosomal recessive forms have also been described (for review see Ionasescu, 1995; De Jonghe et al., 1997). CMT disorders are genetically heterogeneous (Harding and Thomas, 1980). Using molecular methods

the first culprit gene for CMT1 was mapped to chromosome 1 in linkage with the Duffy blood group marker (Bird et al., 1982). Subsequently approximately 18 different loci that are associated with the different types of CMT have been mapped, but only four genes have been identified so far (for review see De Jonghe et al., 1997; Nelis et al., 1999). They are the culprit genes for CMT1, CMTX and the myelin-related forms of CMT3, DSS and CH. In the PNS, the expression of these identified genes is confined to myelinating Schwann cells. A description of the encoded proteins and the corresponding CMT disorders follows below. In addition, natural or genetically engineered mouse models exist for these disorders, which will also be discussed.

3.2.2 Peripheral myelin protein 22-related neuropathies

The peripheral myelin protein of 22kDa molecular weight (PMP22) is highly expressed in myelinating Schwann cells of the PNS. Low expression of PMP22 has also been found in other tissues such as intestine, lung and heart (Spreyer et al., 1991; Welcher et al., 1991). The tissue-specific expression is regulated by alternative use of two different promoters (Suter et al., 1994). PMP22 is upregulated in Schwann cells upon axonal contact and at the onset of myelination (Spreyer et al., 1991). It is located in the compacted myelin and comprises 5-7% of the protein contents of peripheral myelin (Snipes et al., 1992; Haney et al., 1996). PMP22 is a hydrophobic protein of 160 amino acids containing four membrane-associated domains (Manfioletti et al., 1990; Suter et al., 1992b; D'Urso and Müller, 1997). On the first extracellular loop, a N-glycosylation site carries a L2/HNK-1 glycosylation in humans and in cats (Hammer et al., 1993; Pareek et al., 1993; Snipes et al., 1993). The function of PMP22 is still debated. Its structural similarity to pore forming proteins like connexins suggests a role as a channel protein (Suter et al., 1992b). Similarities also exist to the major structural protein of the CNS, the proteolipid protein PLP, which in turn suggests a function of PMP22 in the architecture of the myelin sheath (Suter et al., 1993). Recently, D'Urso and colleagues found that PMP22 interacts with the major structural protein of PNS myelin, P0. This interaction further supports a structural role for PMP22 (D'Urso et al., 1999). PMP22 has first been identified as a growth arrest-specific protein in fibroblasts suggesting a regulatory function for PMP22 in cell growth (Manfioletti et al., 1990; Zoidl et al., 1997).

PMP22 is the culprit gene for CMT1A. Approximately 70% of CMT1A cases are caused by the duplication of a region of 1.5 Mb on chromosome 17 (17p11.2-12) which includes the *PMP22* gene (Lupski et al., 1991; Raeymaekers et al., 1991; Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992). The duplication results from an unequal crossing-over event (Chance et al., 1994). Recombination hotspots lying on both sides of the duplicated DNA sequence facilitate this frequent misalignment (Reiter et al., 1996). Such CMT1A-REP sequences have been analyzed in different CMT1A pedigrees (Chance et al., 1993; Timmerman et al., 1997; Yamamoto et al., 1997). It has been suggested that a small homologous region of 700bp is sufficient to allow the recombination (Yamamoto et al., 1998). In sporadic CMT1A patients without a family history of CMT, *de novo* duplications of the 1.5 Mb region are often found (Hoogendijk et al., 1992). Interestingly, the duplications are usually of paternal origin (Palau et al., 1993; Lopez et al., 1997). As a consequence of the unequal crossing-over event a deletion of the *PMP22* gene occurs on the second chromatide (Chance et al., 1994). The deletion of the *PMP22* gene results in the hereditary neuropathy with liability to pressure palsies HNPP (Davies, 1954; Chance et al., 1993). HNPP is characterized by a moderate slowing of NCVs, focal myelin thickenings (tomacula) and a variable degree of segmental demyelination and axonal loss (Meier and Moll, 1982; Verhagen et al., 1993). With regards to the clinical presentation, HNPP is a rather mild form of peripheral neuropathy.

In a minority of CMT1A patients, the disorder is caused by point mutations in the *PMP22* gene (Valentijn et al., 1992b; Roa et al., 1993a,b; for review see De Jonghe et al., 1997; Nelis et al., 1999). The severity of the clinical phenotype in these cases depends on the kind of mutation. Furthermore, several mutations in the *PMP22* gene have been shown to cause DSS, the much more severe form of HMSN (for review see Nelis et al., 1999). Frameshift and nonsense mutations have also been found to cause HNPP. Presently, approximately 30 different mutations are known (for review see De Jonghe et al., 1997; Nelis et al., 1999).

PMP22-related neuropathies can also be observed in the genetically engineered animal models. *Pmp22*-deficient mice have been generated by the targeted disruption of the *pmp22* gene (Adlkofer et al., 1995). The homozygous animals present with a congenital dysmyelination including tomacula, followed by demyelination, onion bulb

formation and axonal degeneration (Adlkofer et al., 1995). Heterozygous *pmp22*-deficient mice show a slowly progressing neuropathy with observable electrophysiological alterations at approximately 12 months of age (Adlkofer et al., 1995; Adlkofer et al., 1997a). Tomacula and onion bulb formation is delayed in comparison to the homozygous knockout mice and clinically, the mice are normal (Adlkofer et al., 1995; Adlkofer et al., 1997a). Heterozygous *pmp22*-deficient mice serve as animal models for HNPP. In addition to the knockout mice, rats and mice with additional copies of the *pmp22* gene have been generated (Magyar et al., 1996; Sereda et al., 1996). These transgenic animals show an early demyelinating phenotype or a total congenic absence of myelin, depending on the number of *pmp22* genes expressed. The rather severe phenotype may be used as model for severe forms of CMT1A or for DSS (Magyar et al., 1996; Sereda et al., 1996).

In addition to the genetically engineered animals, two natural mouse mutants - *trembler* and *tremblerJ* - exist that carry point mutations in the *pmp22* gene (Suter et al., 1992a,b). Hypomyelination and Schwann cell overproduction in these animals is more pronounced in the homozygous than the heterozygous mice (Henry and Sidman, 1988; Ericson and Borg, 1999). The same mutations have also been identified in CMT1A (*tremblerJ* mutation; Valentijn et al., 1992a) and DSS (*trembler* mutation; Ionasescu et al., 1997) patients.

The identification of the *PMP22* gene as the culprit gene for hereditary neuropathies subsequently led to investigations on the possible disease mechanisms. The observations in CMT1A patients and corresponding animal models support the notion that *pmp22* is a dosage-sensitive gene. Dosage-sensitivity has also been found for other major myelin proteins of the central (for review see Shine et al., 1992; Griffiths et al., 1998) and peripheral nervous system (Martini et al., 1995b). Alterations in the amount of such myelin proteins are suggested to interfere with myelinogenesis and with the structural stability of the myelin sheath (Lupski, 1998), but alternative explanations such as influences on the Schwann cell differentiation or viability can not be excluded.

Heterozygous point mutations in the *pmp22* gene often lead to a more severe disease course than the lack of one total allele of the gene. This observation suggests a dominant-negative effect of most of the mutations on the function or the processing of PMP22. In vitro studies have revealed that mutated forms of PMP22 interfere with the

transport of wild-type PMP22 into the plasma membrane, thereby leading to an accumulation of PMP22 in the endoplasmic reticulum (Naef et al., 1997; Notterpek et al., 1997; D'Urso et al., 1998; Tobler et al., 1999). Endoplasmic accumulation and interrupted transport of PMP22 to the membrane compartment appear to be detrimental for normal function of the Schwann cells.

3.2.3 Connexin32-related neuropathies

Most cells establish a direct communication with their neighboring cells by the formation of gap junctions, channels that are composed of two hemichannels from opposing membranes. Two hemichannels interact with each other to form one single pore connecting the cytoplasms of both cells (Kumar and Gilula, 1996). The inner pore diameter is approximately 1.5 nm, which limits the passive diffusion of cell components to fluctuations of ions, small metabolites and second messengers of less than 1000Da weight (Kumar and Gilula, 1996). The hemichannels or connexons consist of six connexin subunits (Cascio et al., 1995). Connexins are the basic molecular elements of the gap junctions. They form a multigene family of at least 13 members (Nicholson and Bruzzone, 1997), which are named after the apparent molecular weight of the different mammalian proteins. In most cells, more than one connexin are present. The expression of connexins in different tissues and cell types is specific but overlapping. Some connexins are present in a wide variety of cells, whereas others show a very restricted distribution (Bruzzone et al., 1996a). Gap junctions in different tissues exhibit divers properties with respect to ion and metabolite permeability, which is due to the individual characteristics of the different connexins (Bruzzone et al., 1996b). Connexons from neighboring cells are able to form homotypic gap junctions consisting of two identical connexons or heterotypic gap junctions consisting of two different connexons. The great number of possible compositions of gap junctions reflects the importance of the availability of subtle differences in channel function for the cells (for review see Kumar and Gilula, 1992; Kumar and Gilula, 1996; Bruzzone and Ressot, 1997).

In contrast to gap junctions that link cytoplasms of two different cells, the junctions in myelinating Schwann cells form reflexive intracellular channels, thereby connecting different cytoplasmic compartments within the same cell. Myelinating Schwann cells predominantly express connexin32 (32 kDa, Cx32), whereas mRNA for

Connexin43 is present in very low amounts (Mambetisaeva et al., 1999). Cx32 is located in the membranes that form the paranodal loops and in Schmidt-Lanterman incisures, regions where the cytoplasm has not been squeezed out during the process of myelin compaction (Bergoffen et al., 1993; Scherer et al., 1995). The reflexive gap junctions provide a direct radial diffusion pathway between the inner and the outer cytoplasmic region of the Schwann cell (Bergoffen et al., 1993; Bone et al., 1997; for review see Paul, 1995). *Cx32* is expressed from the more proximal of two possible promoters of this gene in a pattern similar to that of other myelin proteins (Neuhaus et al., 1996; Sohl et al., 1996). This promoter holds a potential binding site for the myelin-specific transcription factor SCIP, which is expressed in Schwann cells at the initiation of myelination (Neuhaus et al., 1996; Sohl et al., 1996).

The gene for *Cx32* lies on chromosome X (Xq13.1). *Cx32* has been identified as the culprit gene for the dominant X-linked form of CMT, CMTX1 (Bergoffen et al., 1993). Approximately 200 different mutations in the *Cx32* gene are known to be associated with the disease. They include missense, nonsense and frameshift mutations, as well as deletions and insertions within the coding region (for review see Nelis et al., 1999). Studies on the relationship between the genotype and clinical phenotype of CMTX patients by Ionasescu and colleagues revealed that missense mutations tend to result in milder forms of the disorder, whereas nonsense mutations cause moderate to severe phenotypes (Ionasescu et al., 1996). To explain the differences in the severity of the phenotype of CMTX patients, functional studies investigated the ability of various mutant Cx32 proteins to form gap junction channels. Ectopic expression in Xenopus oocytes demonstrated that some mutations interfere with channel formation, whereas others allow functional coupling of adjacent cells (Bruzzone et al., 1994; Oh et al., 1997; Ressot et al., 1998). Similar results were found in investigations on channel formation in transfected mammalian cell lines (Omori et al., 1996; Deschênes et al., 1997; Yoshimura et al., 1998). Recently, more sophisticated experiments on channels formed by mutant Cx32 showed that electrophysiological properties are differentially affected by distinct mutations (Ressot et al., 1998). In addition, the ability of Cx32 to form functional connexons appears to depend on the length of the protein: Connexins longer than 215 amino acids form functional connexons, whereas shorter proteins tend to be retained within the cell (Castro et al., 1999). Interestingly, several mutations after

the 215th amino acid impede the formation of proper gap junctions from two functional connexons (Castro et al., 1999).

In 1996, Nelles and colleagues generated the *cx32*-deficient mice, which serve as an animal model for CMTX (Nelles et al., 1996). The mice are viable and fertile. As Cx32 is highly expressed in liver, Cx32 knockout mice show abnormalities in the function of the liver caused by inefficient cell coupling (Nelles et al., 1996). The lack of Cx32 in the myelinating Schwann cells leads to a late onset progressive neuropathy (Neuhaus et al., 1996; Scherer et al., 1998). 4-week-old *cx32*-deficient mice have normal nerve fibers; mice older than 4 months develop onion bulb structures and enlarged periaxonal collars (Anzini et al., 1997). Electrophysiological measurements indicate a mild axonal and demyelinating pathology (Anzini et al., 1997). Similar to CMTX patients, *Cx32*-deficient mice show axonal degeneration and regenerating clusters (Anzini et al., 1997; for review see Martini, 1997; Martini et al., 1998).

3.2.4 Early growth response 2 gene-related neuropathies

The early growth response 2 (EGR2) gene product is a member of the EGR family of transcription-regulatory factors. The members of the EGR family are important mediators of changes in gene expression in neuronal cells. They are involved in the upregulation of various proteins in response to environmental cues, such as light or growth factors (for review see O'Donovan et al., 1999). EGR2 and its mouse homologue Krox20 have been published in 1988 (Chavrier et al., 1988; Joseph et al., 1988) and display an overall amino acid identity of approximately 90% (Warner et al., 1999). Sequence analysis revealed that EGR2 contains an inhibitory domain and three zinc-finger domains in the C-terminal half of the protein. EGR2 binds to DNA in a sequence-specific manner and its activity is regulated by proteins binding to the inhibitory domain (Russo et al., 1995; Svaren and et al, 1996; for further references see Warner et al., 1999).

EGR2/Krox20 is present during development of the CNS in rhombomers 3 and 5 (Chavrier et al., 1990; Schneider-Maunoury et al., 1993) and in immature Schwann cells in the developing PNS (Topilko et al., 1994; Murphy et al., 1996). The importance of EGR2/Krox20 for the normal development and survival of mammals has been

demonstrated by the phenotype of homozygous *krox20*-deficient mice, which show a severe reduction or elimination of the rhombomers 3 and 5 and die within the first two weeks after birth (Schneider-Maunoury et al., 1993). Whereas the heterozygous *krox20*-deficient mice appear to be normal, the homozygous knockout mice display an interesting phenotype in the PNS (Topilko et al., 1994). In these animals, myelin is totally absent, presumably due to an early block in the differentiation of myelinating Schwann cells (Topilko et al., 1994). EGR2/Krox20 is suggested to be required for the transactivation of PNS myelination-specific genes. This hypothesis is supported by the finding that *krox20*-deficient Schwann cells express the early myelin marker MAG but lack late myelin markers such as P0, MBP and P2 (Topilko et al., 1994).

Because these results indicated an important role for EGR2/Krox20 in the myelination process in the PNS, patients with a peripheral neuropathy have been screened for mutations in this gene that - in humans - has been localized to chromosome 10 (10q21-22) (Joseph et al., 1988). Warner and colleagues (1998) described patients having mutations in the second or third zinc-finger domain or in the inhibitory domain of EGR2. A fourth mutation has lately been identified in the first zinc finger domain (Timmerman et al., 1999). Although only four different mutations have been reported so far, a considerable variability in the clinical phenotype and the mode of inheritance was found (Warner et al., 1998; Timmerman et al., 1999; Warner et al., 1999). The clinical phenotype of patients with mutations in the *EGR2* gene ranges from mild, CMT1-like, symptoms to DSS and severe CH. Three of the four pedigrees were diagnosed with autosomal dominant inheritance, whereas autosomal recessive inheritance has been suggested for one pedigree diagnosed with CH (Warner et al., 1999). Warner and colleagues investigated functional implications of the four known mutations that lead to a peripheral neuropathy (Warner et al., 1999): They were able to show that the severity of the resulting disorder correlates with the ability of the mutant protein to bind to specific DNA sequences (EGR2 binding sites) and the elicited transcriptional activity. The most severe phenotype is associated with the mutant which confers the greatest level of DNA binding (Warner et al., 1999). These observations suggest a dominant-negative mechanism for the *EGR2* mutations possibly resulting in significant alterations of expression levels of major myelin genes.

3.2.5 Protein zero-related neuropathies

Protein zero (P0, MPZ) is the major protein of the peripheral myelin. This protein comprises approximately 50% of the total protein contents of the myelin in the PNS (Greenfield et al., 1973). The cDNA of *P0* is 1.9 kb in length, with an open reading frame of 744 bp (Lemke and Axel, 1985; Hayasaka et al., 1991). The genomic location of the *P0* gene is on chromosome 1. The *P0* gene is 7 kb long and consists of 6 exons (Lemke et al., 1988; Hayasaka et al., 1993c). The segregation of the exons is consistent with the different suggested domains (Hayasaka et al., 1993c). From the deduced amino acid sequence it was inferred that P0 has a N-terminal signal sequence of 29 amino acids (aa), a large hydrophilic extracellular domain (124 aa), a short transmembrane sequence (26 aa) and a basic intracellular domain (69 aa). The amino acid sequence of the extracellular domain is similar to the variable region of immunoglobulins (Hayasaka et al., 1993c). Whereas Ig-like domains in immunoglobulins are encoded in one exon, the Ig-like domain of P0 is encoded in two exons (Lemke et al., 1988). Therefore it has been suggested that P0 is more closely related to the hypothetical ancestral half-domains of this structural unit. The apparent molecular weight of P0 is 29 kDa (D'Urso et al., 1990). The nascent P0 is cotranslationally inserted into the endoplasmic reticulum, where it is posttranslationally modified by glycosylation, phosphorylation and palmitoylation; The signal peptide is cleaved off (D'Urso et al., 1990; Suzuki et al., 1990; Mendelez and Bizzozero, 1996). The N-type glycosylation site in the extracellular domain of P0 can carry the L2/HNK-1 epitope (Bollensen and Schachner, 1987; Griffith et al., 1992; Hammer et al., 1993).

P0 is highly expressed by myelinating Schwann cells in the PNS, but not by glial cells of the CNS (Mirsky et al., 1980; Lemke and Axel, 1985). During development the levels of P0 mRNA and protein are increasing after E18 (rat), peaking at postnatal day 14 and decreasing thereafter to an intermediate level (Baron et al., 1994). This expression depends on axonal contact. When myelinating Schwann cells loose axonal contact, for example under culture conditions or after nerve injury, they downregulate the expression of P0 (Mirsky et al., 1980; Trapp et al., 1988; De Leon et al., 1991). A recent publication by Jessen's group showed that P0 is expressed at basal levels already in a population of migrating NCC and in Schwann cell precursors (Lee et al., 1997). Upon differentiation of Schwann cells into the myelinating type, they strongly

upregulate the P0 expression, while those cells differentiating into the non-myelinating type reduce this basal expression.

The time course of the upregulation of P0 in myelinating Schwann cells and the Ig-like structure of the extracellular domain lead to the proposition that P0 has an essential function as adhesion molecule during the formation and maintenance of peripheral myelin (Lemke and Axel, 1985). Immunolocalization of P0 to the compacted regions of myelin has strengthened this hypothesis (Martini et al., 1988; Martini, 1994). Several studies with transfected cells showed that P0 confers adhesion by homophilic binding of the extracellular domains (D'Urso et al., 1990; Filbin et al., 1990; Schneider-Schaulies et al., 1990; Filbin and Tennekoon, 1991; Filbin and Tennekoon, 1993). This interaction mediates the compaction of the intraperiod lines of the myelin sheath. The intraperiod line is formed by tight apposition of the extracellular leaflets of adjacent membrane turns in the myelin sheath, whereas the leaflets of the lipid bilayer facing the cytoplasm constitute the major dense line. Shapiro et al. have described the structure and the binding characteristics of the extracellular domain of P0 by X-ray crystallography (Shapiro et al., 1996). In their crystal model they propose that P0 forms cis-tetramers with P0 molecules in the same membrane. These tetramers interact with tetramers of the opposing membrane to convey homophilic binding. The tetrameric model explains dominant-negative effects of some mutated P0 forms, but it is not able to clarify the role of the carbohydrates in the adhesion (Kirschner et al., 1996). From studies in the *shiverer* (MBP-deficient) mice it was speculated that P0 must also have adhesive properties in its intracellular domain, since such animals lack a major dense line in the CNS. In the PNS, where P0 remains to confer adhesion in the major dense line, myelin is unaffected (Kirschner and Ganser, 1980; Lemke, 1988). The highly basic intracellular amino acid sequence of P0 electrostatically interacts with negatively charged phospholipids, which may mediate the compaction of the major dense line. This affinity is partially dependent on the phosphorylation state of P0 (Suzuki et al., 1990; Ding and Brunden, 1994).

The assumption that P0 plays an essential function in myelin formation and maintenance has been elegantly demonstrated by the generation of P0-deficient mice (Giese et al., 1992). The homozygous P0-deficient mice (P0-/-) show deficits in motor coordination, they exhibit tremors and occasional convulsions. Pathological alterations

in motor and sensory nerves include hypomyelination, myelin degeneration, onion bulb formation and occasional degeneration of axons (Giese et al., 1992). The ultrastructure of the myelin in P0-/- mice is abnormal. Compacted myelin having a regular spacing of the major dense lines is present in only 10-15% of the fibers. Most axons are surrounded by noncompacted myelin with split intraperiod lines and often undulated major dense lines. Other morphological features include axons that are totally devoid of myelin and axons in association with myelin debris. These findings, along with studies performed in MBP/P0 double mutants (Martini et al., 1995a), clearly demonstrate that P0 does indeed have an essential function in myelin compaction, both at the intraperiod and major dense line. The functional analysis of P0-/- nerves demonstrated that NCV are strongly reduced in P0-/- mice; amplitudes and duration of compound muscle action potentials show striking pathological changes (Zielasek et al., 1996). The electrophysiological results suggest a severe demyelinating neuropathy involving the axons. They provide evidence for the deleterious effects of the P0-deficiency on the function of the nerves.

The heterozygous P0-deficient mice (P0+/-) show a more subtle phenotype, with onset of morphological and electrophysiological pathology after 4 to 6 months (Martini et al., 1995b). Myelin degeneration and onion bulb formation is mainly confined to motor nerves, whereas sensory nerves remain unaffected. The morphological and electrophysiological alterations of P0+/- and P0-/- mice are reminiscent of the pathology described for CMT1B and DSS patients, respectively (Martini et al., 1995b; Zielasek et al., 1996; Martini, 1997; Martini et al., 1998).

After the mapping of the human *P0* gene, a number of different mutations in this gene have been described in CMT1B and DSS patients (Hayasaka et al., 1993a,b; Nelis et al., 1994; Rautenstrauss et al., 1994; Roa et al., 1996; Warner et al., 1996; Bort et al., 1997; Tachi et al., 1998; for review see De Jonghe et al., 1997; Nelis et al., 1999). Several of these mutations show a dominant-negative effect (Kirschner and Saavedra, 1994; Warner et al., 1996; Wong and Filbin, 1996; Zhang and Filbin, 1998; for review see Nelis et al., 1999). Dominant-negative effects are particularly frequent when multimers need to be formed for the proper function of the molecules as is suggested for P0. The increasing knowledge of the binding characteristics of the extracellular domain of P0 allows to propose a relation between the different CMT mutations and the severity of the phenotype of the disorder (Warner et al., 1996). Mutations may, for instance,

interfere with the stabilization of the P0 tetramers, whereas other mutations directly affect the binding of the opposite P0-molecules thereby impairing the adhesive function of these molecules.

3.2.6 Axonal degeneration in P0-deficient mice

Electrophysiological and morphological investigations revealed a considerable axonal impairment in P0-deficient mice (Giese et al., 1992). Reduced calibers of demyelinated axons and features of axonal degeneration implicated that the lack of the structural myelin protein P0 can influence axonal properties. However, there are still unanswered questions concerning the mechanisms how the lack of P0 can lead to an axonal involvement. It is assumed that abnormal myelin results in an impaired signaling from the Schwann cell to the axon which consequently leads to pathological alterations in the neuron. A number of studies support the notion that myelination alters axonal properties. In early studies, de Waegh and colleagues compared axonal calibers and neurofilament phosphorylation of *trembler* and wild-type mice (de Waegh and Brady, 1990; de Waegh et al., 1992). They were able to show that axonal diameters and neurofilament phosphorylation in *trembler* mice were reduced and that these alterations depend on the phenotype of the myelinating Schwann cells. A decrease in axonal diameter was also observed in optic nerve fibers that were demyelinated by irradiation (Colello et al., 1994). In the normal, non-myelinated stem processes of DRG neurons reduced axonal calibers were accompanied by a decrease in neurofilament number and phosphorylation (Hsieh et al., 1994). These observations demonstrated that the induction of axonal changes by the lack of myelination is a general mechanism that appears to be independent of specific mutations in myelin genes.

Axonal involvement is also a common observation in nerve biopsies of CMT and DSS patients (Hahn et al., 1990; Sander et al., 1998; for review see Gabreëls-Festen et al., 1993; De Jonghe et al., 1997). In addition to a reduction of axonal diameters, axonal degeneration is often observed in nerve biopsies and is reflected in electrophysiological measurements presenting with reduced amplitudes. As mentioned above, this pathology is particularly prominent in CMTX patients. A similar situation has been found in *cx32*-deficient mice which display morphological signs of axonal degeneration and regeneration (Anzini et al., 1997). Recent investigations in PMP22-deficient mice also

presented evidence for significant axonal loss in the femoral nerves of these mutants (Sancho et al., 1999).

Although substantial evidence exists for the axonal involvement in myelinopathies, studies on the extent of induced axonal degeneration and secondary effects on targets that are innervated by myelinated axons are scarce. We, therefore, quantified axonal degeneration in P0-deficient mice and investigated the pathology of target tissues such as muscle and skin.

4 Materials and Methods

This chapter provides further unpublished information regarding experimental procedures, materials used for experimental work, and abbreviations.

4.1 Genotyping of P0- and β4 Integrin-Deficient Mice: Amplification of Specific DNA Fragments by PCR

The presence of wild-type or mutant P0 alleles was detected using a set of three specific primers in the polymerase chain reaction (PCR). PCR-fragments of 500 bp resulted for the P0 wild-type allele and fragments of 334 bp for the P0 mutant allele. For the amplification of P0 gene fragments, the oligonucleotide primer S1295 (1 mM, 5'-TCAGTTCCTTGTCCCCCGCTCTC-3') was used for amplification of both wild-type and mutant alleles, while the oligonucleotide sequences AS1606 (0.5 mM, 5'-GGCTGCAGGGTCGCTCGGTGTTC-3') and AS1772 (1 mM, 5'-ACTTGTCTC-TTCTGGGTAATCAA-3') were specific for the mutant and wild-type allele, respectively. The reactions for detection of wild-type and mutant alleles were carried out separately using 2μM of each primer of the corresponding pairs. The PCRs were carried out in a final volume of 20μl (1.5 mM $MgCl_2$, 1 x PCR buffer, 0.2 mM dATP, dCTP, dGTP, dTTP, 0.5 - 1 U Taq DNA polymerase, 100-500 pg genomic DNA). The reaction product was denatured at 94°C for 1 minute, followed by 35 cycles (94°C, 15 s; 53°C, 30 s; 72°C, 30 s) and a final extension at 72°C for 7 minutes using a Perkin Elmer thermal cycler (Perkin Elmer Gene Amp 9700).

For the demonstration of the inserted *neo* gene in both β4 integrin- and P0-deficient mice a PCR was carried out with oligonucleotide sequences specific for the neo-resistance cassette: 5' (1 mM): 5' CTGCATACGTTGATCCGGCTACCTGCC-CATTCG 3', and 3' (1 mM): 5' ACTCGTCAAGAAGGCGATAGAAGGCGA-TGCG 3'). The resulting PCR-fragment had a size of 565 bp. The PCR was carried out in a final volume of 20 μl (using the same concentrations as described above). The reaction product was denatured at 94°C for 2 minutes, followed by 25 cycles (94°C, 30s; 65°C, 1 minute; 72°C, 1 minute) and a final extension at 72°C for 10 minutes using a Perkin Elmer PCR system (Perkin Elmer Gene Amp 2400).

4.2 Solutions

Anesthetics

Mix 1 ml Ketanest 50, 0.25 ml Rompun and 5ml 0.9M NaCl in a sterile tube. Use 10 µl/g bodyweight for surgery, or approximately 400µl per mouse for lethal injections.

Ascorbic acid

Dissolve 50 mg ascorbic acid in 10 ml distilled water. Make freshly for feeding of the cell cultures and add to the medium at a concentration of 1:100.

Collagen

Freeze 1-2 rat tails overnight
Wash tails with 70% ethanol
Pull long silvery tendons apart
Put tendons in sterile petridish
Wash twice with 70% ethanol
Wash twice with sterile distilled water
Put tendons in sterile bottle adding 150 ml 0.1% acetic acid per 1g tendons
Stir slowly at 4°C until dissolved (2-4 days)
Centrifuge at 3000 rpm for 1 hour.

NGF

Dissolve powder in 10 ml EMEM medium containing 10% FCS.

Formvar

Dissolve 0.5% Formvar powder in pure chloroform and stir gently overnight to get a clear solution. Keep the bottle wrapped in aluminum at room temperature.

Lead citrate (Reynolds)

Use freshly boiled and cooled distilled water for all solutions
Dissolve 1.33 g lead-(II)-nitrate in 15 ml water
Dissolve 1.76 g sodium citrate in 15 ml water
Mix the two solutions by shaking well
Add 8 ml 1M NaOH, made freshly by dissolving NaOH pellets
Gently mix until the solution is clear
Add 12 ml water (end volume: 50 ml)
Filter (0.22 µm) solution before use.

OsO_4

Carefully severe the ampoule containing OsO_4 by sawing until easily breakable. Add the whole ampoule to 0.1 M cacodylate buffer, close the bottle tightly and break the ampoule by vigorously shaking. Then stir gently overnight until the OsO_4 is totally dissolved. Make solutions of 2% OsO_4 w/v.

Phosphate buffer

Prepare 1 M Na_2HPO_4 * 2 H_2O (I) and 1M NaH_2PO_4 * 2 H_2O (II) solutions. For 1 liter phosphate buffer (0.1 M, pH 7.4), take 77.4 ml (I) and 22.6 ml (II) and add distilled water.

PBS

Dissolve 7.2 g NaCl in a solution of 80 ml 0.2 M Na_2HPO_4, 20 ml 0.2 M NaH_2PO_4 and distilled water. Add distilled water up to 1 liter.

Spurr's medium (Serva, Heidelberg, Germany)

Mix 10 g ERL, 6 g DER, 26 g NSA, and 0.4 g DMAE in a plastic beaker and stir well (1 hour).

Uranyl acetate

Dissolve 1g uranyl acetate in 50ml distilled water. Filter (0.22μm) the solution before use.

4.3 Primary Antibodies

Mouse monoclonal MAG (undiluted supernatant 513; Poltorak et al., 1987)
Mouse monoclonal P0-7 (1:100, gift from J. Archelos; Archelos et al., 1993)
Rabbit polyclonal N-CAM (1:100)
Rat monoclonal β4 integrin (1:50 in cell culture, 1:200 on cryosections, clone 346-11A, Pharmingen)
Rat monoclonal β1 integrin (1:50 in cell culture, clone 9EG7, Pharmingen)
Rat monoclonal α6 integrin (1:50 in cell culture, clone GoH3, Pharmingen)
Rabbit polyclonal β4 integrin (1:50, gift from E. Ruoslahti; Giancotti et al., 1992)

4.4 Secondary Antibodies

Goat-anti-mouse-rhodamine (1:100, Sigma)
Goat-anti-rat-fluorescein (1:200, Sigma)

Sheep-anti-rabbit-fluorescein (1:200, Sigma)
Goat-anti-rabbit-rhodamine (1:100, Sigma)
Goat-anti-rat-rhodamine (1:100, Sigma)

4.5 Substances

2-Amino-2-Methyl-1-Propanol buffer (2-AMP)	Sigma (Lot-nr. 107H6063, Cat. nr. 221)
5'-Fluoro-2'-DeoxyUridine (FDU)	Sigma (F-0503)
Ammonium sulfide solution (>20%)	Merck (5442)
Ascorbic Acid	ICN Biochemicals Inc.
Bovine Serum Albumin (BSA)	Roth (8076.2)
Cacodylic acid sodium salt trihydrate (Cacodylate)	Serva (15540)
Cytosine β-D-Arabino-furanoside (AraC)	SIGMA (C-1768)
DER	Serva (18247)
Di-methyl amino-ethanol (DMAE)	Serva (20130)
Dulbecco's Minimal Essential Medium (DMEM)	Gibco (22320-022)
Eagle's Minimal Essential Medium (EMEM)	Gibco (61100-053)
Fetal Calf Serum (FCS)	Hyclone (Lot-nr. AGD6387, Cat. nr. SH30070)
Formvar	Serva (21740)
Glutaraldehyde (GA)	Serva (23115)
Hydrofluoric acid	J.T. Baker (6054)
Ketanest 50 (5%; 50mg/ml)	Parker-Davis, Berlin
L15 medium	Gibco (11415-049)
Lead-(II)-nitrate	Merck (7398)
Na2HPO4*2H2O	Merck (6580)
NaH2PO4*H2O	Merck (6268)
NGF 7S	Boehringer Mannheim (1014331)
Nonenyl Succinic Anhydride (NSA)	Serva (30812)
Normal Goat Serum (NGS)	Dianova (Lot-nr. 005000121)
OsO4	Chempur (006051)
Paraformaldehyde (PFA)	Merck (4005)
poly-L-lysine	Sigma (P-1274)
Proteinase K	Boehringer-Mannheim (1373196)
Rompun (2%)	Bayer, Leverkusen
Sodium barbital	Merck (1.06316.01000)
Sodium citrate	Merck (6448)
Sucrose	Roth (4621.1)
Triton X100	Roth (6683.1)
Uranyl acetate	Merck (8473)
Vinyl cyclohexene dioxide (ERL)	Serva (38216)

4.6 Abbreviations

BDNF	Brain-derived neurotrophic factor
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EGR2	early growth response 2 (gene)
FNIII	Fibronectin III repeat
GalC	Galactocerebroside
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
Ig	Immunoglobulin
IGF	Insulin-like growth factor
LNGFR	Low affinity nerve growth factor receptor
m/s	Meter per second
MBP	Myelin basic protein
N-CAM	Neural cell adhesion molecule
NCC	Neural crest cells
NGF	Nerve Growth Factor
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
P0	Protein zero
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMP22	Peripheral myelin protein (22kDa)
PNS	Peripheral Nervous System
SCIP	suppressed cAMP-inducible POU protein

5 Results I

5.1 Myelin Formation by Schwann Cells in the Absence of β4-Integrin

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published in Glia 28 (1999), in press

5.1.1 Summary

The interaction of the Schwann cell with its basal lamina has been hypothesized to be an important prerequisite for the formation of a myelin sheath in the peripheral nervous system. One possible player in this interaction is β4 integrin; it is upregulated during myelin formation and, in association with α6 integrin, can interact with particular components of the Schwann cell basal lamina. In order to characterize the functional roles of β4 integrin during myelination, we investigated myelination in the absence of β4 integrin, i.e., in peripheral nerve tissue from β4 integrin-deficient mice. Because the mutants die within several hours after birth, we cultured dorsal root ganglia from neonatal mutants under conditions that promote myelination, quantified the myelin segments by immunofluorescence, and investigated the ultrastructure of the cultured myelin sheaths. In another approach, we quantified the few myelin sheaths that are detectable in femoral nerves of newborn animals. Based on both approaches, we conclude that myelination by Schwann cells can occur in the absence of β4 integrin demonstrating that this Schwann cell component is dispensable for myelin formation in peripheral nerves.

5.1.2 Introduction

The development of myelin sheaths that mediate the rapid propagation of action potentials along axons is one important prerequisite for the optimal function of the nervous system of vertebrates. In the peripheral nervous system (PNS), Schwann cell-related cell surface molecules such as MAG, Connexin32 (Cx32), E-Cadherin, P0, and PMP22, have been identified as components of the sophisticated insulating Schwann cell sheath (Fannon et al., 1995; for review see Martini and Schachner, 1997; Scherer, 1997). These components are strongly upregulated at early stages of myelination (Snipes et al., 1992; Fannon et al., 1995; Lee et al., 1997; Scherer et al., 1998; for review see Martini, 1994). In the mature state, these myelin components are differentially expressed at distinct sites of the myelin sheath, including compact myelin, (P0 and PMP22; Martini et al., 1988; Haney et al., 1996), noncompacted myelin (E-Cadherin, Cx32 and MAG; Martini and Schachner, 1986; Fannon et al., 1995; Scherer et al., 1998) and at periaxonal and abaxonal myelin compartments (MAG) where the molecules could fulfill distinct functions (Martini and Schachner, 1986). Studies in mice deficient in particular myelin components demonstrated that myelin molecules can play distinct, but also overlapping roles during myelin formation and maintenance (Martini and Schachner, 1997; Martini and Carenini, 1998).

A regulation pattern similar to typical myelin components has been described for β4 integrin (Einheber et al., 1993; Feltri et al., 1994). As a member of a large family of cell surface glycoproteins forming heterodimeric extracellular matrix (ECM) receptors (Hynes, 1992; Schwartz et al., 1995), this molecule is, in contrast to myelin molecules, confined in its expression to the abaxonal Schwann cell membrane (Einheber et al., 1993; Feltri et al., 1994). This localization suggests an interaction of β4 integrin with components of the Schwann cell basal lamina, an extracellular matrix structure that has been purported to be an important prerequisite for myelin formation (Eldridge et al., 1987; Bunge et al., 1989). Because β4 integrin is upregulated when myelination starts, it is tempting to speculate that the interaction of β4 integrin with the basal lamina is functionally important for myelin formation, possibly by the transduction of the relevant signals into the cell interior as has been shown in other systems (Hynes, 1992; Schwartz et al., 1995).

Although it was possible to characterize the functional roles of distinct myelin components by investigating the corresponding knockout mice (for review see Martini and Schachner, 1997), the role of β4 integrin in Schwann cell myelination could not be evaluated from mouse mutants deficient in β4 integrin, because the mutants die shortly after birth and prior to myelin formation as a consequence of severe dysfunction of epidermis and other stratified squamous epithelia (Dowling et al., 1996; van der Neut et al., 1996). To circumvent this, we cocultured dorsal root ganglion neurons and Schwann cells from newborn β4 knockout mice under conditions that allow myelination. In addition, we used electron microscopy to quantify the few myelin sheaths that are detectable in femoral nerves of newborn β4–null animals. Our analyses reveal that myelination by Schwann cells can occur in the absence of β4 integrin, demonstrating that this Schwann cell component is dispensable for myelin formation in the PNS.

5.1.3 Materials and methods

Animals and Determination of Genotype

Mice homozygously deficient for β4 integrin (β4-/-) were obtained by breeding heterozygous β4-deficient mice (β4+/-; Dowling et al., 1996). β4-/- and β4+/- mice were distinguished from wild-type (β4+/+) littermates by PCR with primers specific for the *neo*-gene. β4-/- mice were further distinguished from β4+/- mice by their severe skin blistering and by the absence of β4 integrin immunoreactivity in cryosections of forelimbs (see below).

Cell Culture

Dorsal root ganglia (DRGs) were dissected from E19.5 β4-/- and β4+/+ mouse embryos and explanted on glass coverslips as previously described (Carenini et al., 1998). The cultures were kept in myelin-promoting medium for up to 7 weeks. Semiquantitative analysis of the number of internodes per culture was performed using a Zeiss light microscope at a final magnification of X400. In each culture, four non-overlapping visual fields adjacent to the ganglion were selected and the mean number of myelinated internodes in all four fields was determined. A total number of five, seven, and nine cultures derived from β4+/+ (n = 2), β4+/- (n = 3), and β4-/- (n = 3) mice, respectively, were investigated.

Electron Microscopy

The femoral nerve branches of decapitated newborn mice were exposed and fixed *in situ* with 4% paraformaldehyde (PFA) and 2% glutaraldehyde (GA) in 0.1 M cacodylate buffer (pH 7.4) overnight, followed by excision and postfixation of the nerves in the same fixative. After osmification with 2% OsO_4 in the same buffer, the samples were dehydrated in acetone and embedded in Spurr's medium (Serva, Heidelberg, Germany). Ultrathin sections were counterstained with lead citrate (Reynolds) and examined in a Zeiss electron microscope (EM10B). DRG explants were processed for electron microscopic investigation as described (Carenini et al., 1998). In brief, cultures were fixed in 2% GA in 0.05 M phosphate buffer containing 100 mM sucrose and osmificated in 2% OsO_4 followed by dehydration in ethanol and embedding

in Spurr's medium. Glass coverslips were removed by treatment with hydrofluoric acid, and areas adjacent to the ganglion were cut out and reembedded in Spurr's medium for sectioning.

Immunofluorescence Microscopy

For the investigation of β4 integrin expression in forelimbs of neonatal mice, cryosections were permeabilized with absolute methanol for 10 min at -20°C, fixed with 4% PFA in 0.1 M phosphate buffered saline (PBS), and incubated overnight with a rat monoclonal antibody (mAb) against β4 integrin (1:100, clone 364-11A), followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibody (Sigma, Deisenhofen, Germany) for 1 h.

For immunostaining of cell cultures, the cultures were rinsed in PBS, fixed with 4% PFA and permeabilized with 100% methanol (-20°C) for 10 min. After washing with 0.2% TritonX-100 in PBS for 10 min, the cultures were blocked in 10% normal goat serum (NGS) in PBS, and incubated with an antibody directed against either P0 (mouse mAb, P0-7, 1:100, generously provided by Dr. Juan Archelos; Archelos et al., 1993) or myelin-associated glycoprotein (MAG, undiluted supernatant from mouse hybridoma clone 513; Poltorak et al., 1987) for 3-4 h on ice, followed by incubation with the corresponding FITC-conjugated secondary antibody for 40 min on ice. Nerve cryosections of adult wild-type mice that were used as positive controls were stained according to the same protocol.

Statistical Analysis

Statistical significance was determined by a Student's *t*-test using Excel software.

5.1.4 Results

Early stages of myelin formation in peripheral nerves of neonatal β4-deficient mice

Myelination in most peripheral nerves occurs postnatally, so mature myelin sheaths could not be studied in β4-deficient (β4-/-) mice, which die shortly after birth. However, even though peripheral nerves of neonatal mice do not yet contain mature myelin profiles, early developmental stages of myelin formation do occur in peripheral nerves of wild-type mice. Thus, to investigate whether β4 integrin is an essential component for myelin formation in peripheral nerves, we examined myelination in β4-/- mice.

We used quantitative electron microscopy to examine myelination in the quadriceps branch of the femoral nerve of β4-/- and wild-type littermates (β4+/+). In adult wild-type mice, this nerve contains approximately 500 myelinated axons, of which about 140 axons have a particularly large caliber of more than 8 μm (Carenini et al., 1997; Lindberg et al., 1999). For our present study in newborn mice, the presence of such large axons was of particular advantage because many are already myelinated at birth.

Low power electron microscopy revealed that the nerves of the different genotypes were very similarly organized, without detectable differences in nerve diameters (data not shown). With higher magnification of nerves from wild-type mice, approximately 300 axons were recognized as prospective myelinated axons due to their typical association with Schwann cells. The Schwann cells were always surrounded by a continuous basal lamina and showed a high degree of developmental variability, showing profiles without turning loops or with processes that have not yet formed a complete loop (promyelin stage), with one to four noncompacted loops or some compacted loops (Fig. 3). In neonatal β4-/- mice, the degree of segregation of prospective myelinated axons was similar to that seen in β4+/+ mice (Table 1). Moreover, the Schwann cells ensheathing the prospective myelinated axons were always surrounded by a continuous basal lamina and showed a comparable degree of developmental variability comparable to that of Schwann cells from β4+/+ mice (Table 1). Thus, at early stages of postnatal development, β4 integrin did not seem to be required for myelin formation.

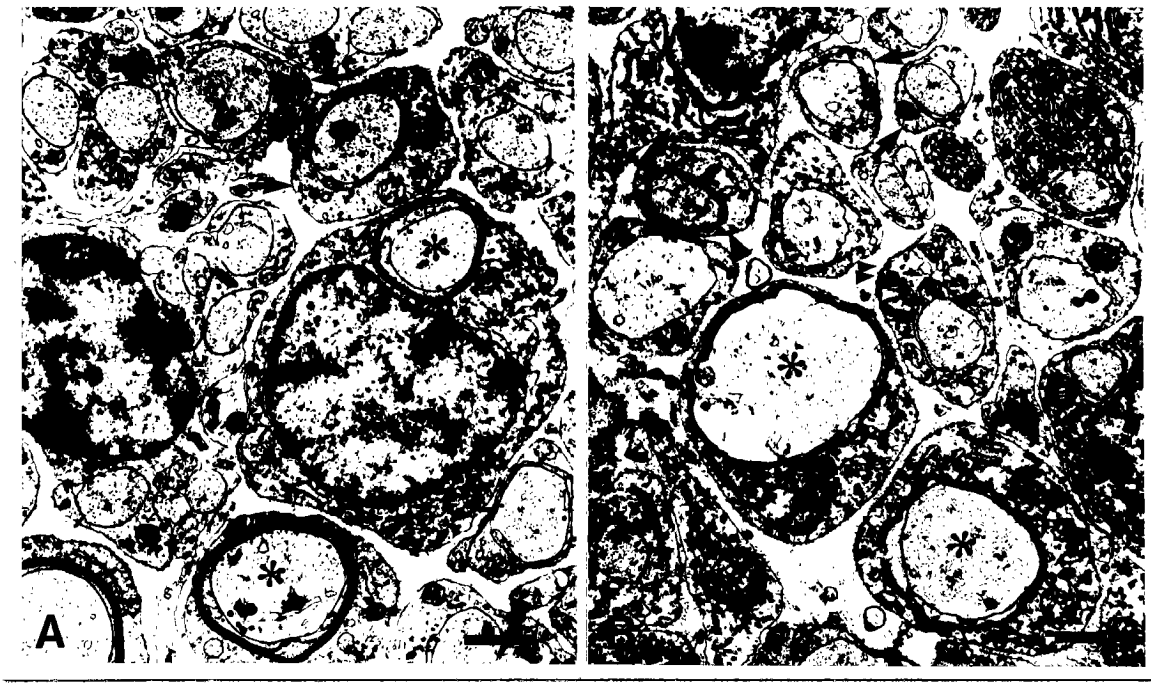

Figure 3

Electron micrographs of femoral quadriceps nerves of neonatal β4+/+ (*A*) and β4-/- mice (*B*). Note that, in both β4+/+ and β4-/- mice, the Schwann cells are surrounded by a continuous basal lamina (*B*, *double arrowhead*) and show a high degree of developmental variability. *Arrows* indicate profiles without turning loops or with processes that have not yet formed a complete loop. The single *arrowhead* in (*B*) points to a Schwann cell with more than one noncompacted loop and the *asterisks* indicate axons that have been surrounded by a few compacted turns. Scale bars = 1 μm.

Table 1 Quantitative analysis of segregated axons and of various developmental stages of Schwann cells in femoral quadriceps nerves of neonatal β4+/+ and β4-/- mice*

	Total number of segregated axons	Promyelin stage[a]	Fibers with more than 1 turn[b]	Fibers with compacted myelin
β4+/+ (n = 11)	272 ± 41	219 ± 43 (80% ± 11%)	18 ± 14 (7% ± 7%)	36 ± 19 (13% ± 6%)
β4-/- (n = 8)	259 ± 21	218 ± 22 (84% ± 6%)	17 ± 12 (7% ± 5%)	24 ± 10 (9% ± 4%)

*Values are given as mean ± standard deviation. n indicates number of animals included in the analysis. Differences between values from β4+/+ and β4-/- were not significant ($P = 0.8$; Student's *t*-test).

[a] Schwann cells without turning loops or with processes that have not yet formed a complete turn.

[b] Schwann cells with noncompacted processes that have formed at least one turn around an axon.

Schwann cells from β4-/- mice myelinate dorsal root ganglion (DRG) neurons in culture

Although the absence of β4 integrin did not interfere with initial steps of myelin formation in neonatal mice, β4 integrin might nevertheless be important for the formation and maintenance of more mature myelin sheaths. To explore this possibility, we cultured DRGs from newborn β4+/+, β4+/-, and β4-/- mice under conditions that allow myelination to occur *in vitro* (Eldridge et al., 1987; Carenini et al., 1998). Irrespective of genotype, Schwann cells from all explants increased in number and aligned along outgrowing neurites within the first two weeks in myelin-promoting medium containing ascorbic acid. The formation of myelin could easily be identified by its typical refractivity when investigated by bright field microscopy (not shown).

To quantify the myelin segments in such cultures, immunofluorescence microscopy was performed using two antibodies to well-established myelin proteins, P0

and MAG. We have used these markers previously with cocultures of Schwann cells and DRG neurons in order to visualize compacted (P0) and noncompacted (MAG) regions of myelin sheaths (Carenini et al., 1998). Myelin segments from β4-/- and wild-type mice showed similar staining patterns (Fig. 4A-D). Despite considerable variability among individual cultures, overall the numbers of the myelin sheaths were statistically comparable in the cultures from wild-type and knockout animals (Table 2).

We next investigated the possibility that absence of β4 integrin might result in subcellular abnormalities of myelin sheaths. Electron microscopy revealed that myelinating β4-null Schwann cells were uniformly surrounded by a continuous basal lamina, as were their wild-type counterparts. In addition, compact myelin sheaths showed normal periodicity of major dense lines in the β4-null cell cultures (Fig. 4E,F). Although myelin thickness within individual cultures varied considerably, no correlation was seen between this feature and the genotype of the cultures. These observations provided compelling evidence that myelin formation can occur correctly in the absence of β4 integrin.

Table 2 **Number of myelinated internodes in DRG explants from β4+/+, β4+/-, and β4-/- mice***

	Genotypes		
	β4+/+ (n = 5)	β4+/- (n = 7)	β4-/- (n = 9)
Number of myelinated internodes per visual field	10 ± 7	19 ± 16	13 ± 11

*Values are given as mean ± standard deviation. n indicates the number of cultures from three (β4+/-, β4-/-) or two (β4+/+) different animals.

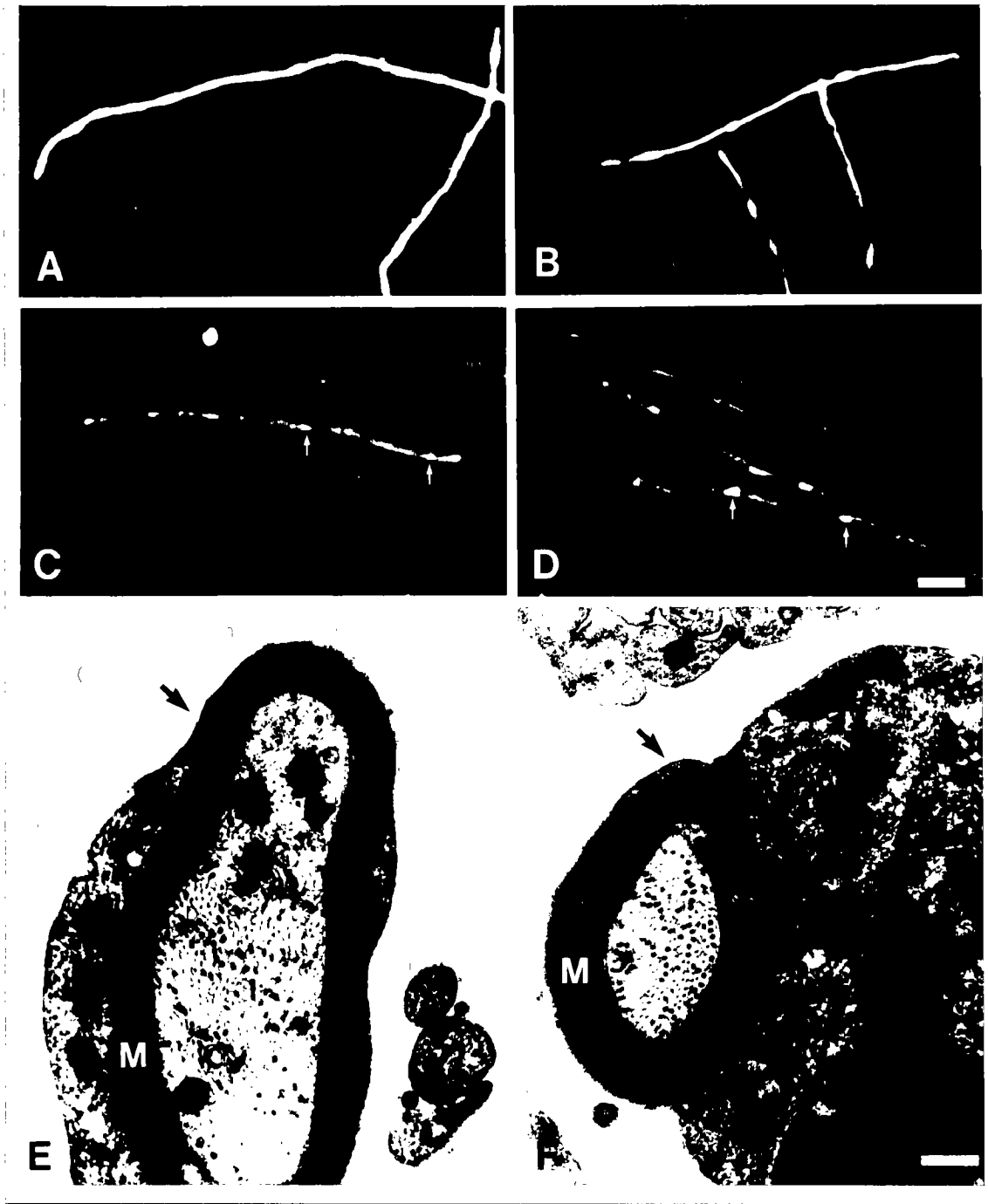

Figure 4

Immunofluorescence micrographs of myelinating DRG cultures from β4+/+ (*A*, *C*) and β4-/- mice (*B*, *D*) using antibodies against P0 (*A*, *B*) and MAG (*C*, *D*) and electron micrographs of DRG cultures from β4+/+ (*E*) and β4-/- mice (*F*).

A-D, Note that P0 antibodies label myelin internodes homogeneously (*A*, *B*), whereas MAG-antibodies preferentially label the noncompacted sites of the internodes (*C*, *D*, *arrows*). No principle differences are detectable between the different genotypes.

E, *F*, In cultures from both β4+/+ (*E*) and β4-/- mice (*F*), compacted myelin (*M*) is detectable. Arrows indicate the continuous basal laminae of the myelinating Schwann cells.

Scale bar: (shown in *D*) *A-D*, 50μm; (shown in *F*) *E*, *F*, 0.3μm.

5.1.5 Discussion

Based on the observation that α6β4 integrin is expressed concomitantly with the major components of the myelin sheath of the peripheral nervous system, it has been proposed that this integrin may play a central role in regulating myelin formation in the developing Schwann cell (Sonnenberg et al., 1990; Einheber et al., 1993; Feltri et al., 1994; Niessen et al., 1994). Despite this correlation in expression, we found no evidence of a myelination defect in either newborn β4-/- mice or in myelinating cultures from these mutants. Rather, our findings demonstrated clearly that myelin formation can occur in the absence of β4 integrin.

While β4 integrin-deficient Schwann cells can myelinate, we cannot rule out the possibility that the integrin might play some role in myelin formation *in vivo*. Because α6 can partner with b1 in the absence of β4 (Dowling et al., 1996; van der Neut et al., 1996), it is possible that in the Schwann cells of β4-null mice α6b1 assumes the functional roles normally provided by α6β4. Indeed, b1 integrin is detectable on cultured myelinating Schwann cells of both β4+/+ and β4-/- mice (Fernandez-Valle et al., 1994); R.F. and R.M., unpublished data) and α6b1 binds to the same basal lamina components as α6β4, namely laminins 1, 2 and 5 (Hynes, 1992). The fact that antibodies against b1 integrin interfere with early steps of myelination *in vitro* is in support of this view (Fernandez-Valle et al., 1994). However, in contrast to the putative compensatory role of b1 in myelin formation, the b1 integrin cannot rescue the organization of stratified epithelia in β4-null mutants, as reflected by strong b1 immunoreactivity in blistering skin of β4-deficient mice (Dowling et al., 1996). It is tempting to speculate that the inability of epithelial cells to compensate for the lack of β4 is due to the fact that this integrin is indispensable for the organization of hemidesmosomes by which epithelial, but not Schwann cells, interact with the basal lamina (Dowling et al., 1996; van der Neut et al., 1996).

Our observation that myelin formation can occur in the absence of β4 could also mean that integrins are not involved in myelination. This view would fit with the observation that Schwann cells devoid of an ultrastructurally defined basal lamina were able to form compact myelin *in vitro* (Podratz et al., 1998). These findings may call into question the previous model saying that the Schwann cell basal lamina serves as a

framework stabilizing the position of the Schwann cell body while the inner lip turns around the axon in a spiral-like manner (Bunge et al., 1989). That the interaction between the Schwann cell and its basal lamina is entirely dispensable *in vivo* seems unlikely; mice deficient in the basal lamina component laminin 2 (merosin), a ligand of α6β4, are impaired in myelination (Xu et al., 1994; for review see Matsumura et al., 1997). Thus further experimental studies are needed to characterize the significance of the Schwann cell-basal lamina interaction during myelin formation *in vivo*.

A possible functional role of β4 is the stabilization and maintenance of the unique architecture of the mature myelin sheath rather than its generation per se. Owing to the early death of β4-null mice, we could not address the role of β4 in myelin maintenance. Intriguingly, we have recently found that the development of the myelin sheath is dependent on only a few key molecules, whereas the maintenance of myelin depends on a much higher number of myelin components (Martini and Schachner, 1997). Thus, for example, the mature myelin sheath is much more vulnerable than the developing one in MAG- or Cx32-deficient mice, which show normal myelin formation in young mice, but myelin degeneration in the corresponding adult mutants (Martini and Carenini, 1998). Similarly, mice heterozygously deficient for PMP22 or P0 show myelin degeneration in adulthood but not at neonatal ages (Martini and Carenini, 1998). Interestingly, this reduced ability to maintain myelin is confined to motor nerves and ventral spinal roots, while myelin sheaths of sensory cutaneous nerves of knockout mice appear much more stable (Martini, 1997; Martini and Carenini, 1998). Given that our culture studies were confined to sensory neurons, and that we were unable to monitor long-term survival of myelination in either cultured β4-null Schwann cells or in β4-null mice, we have yet to test vigorously for a role for β4 in myelin maintenance. To test whether β4 integrin is a crucial component for myelin maintenance will require tissue-specific gene targeting directed by a myelin-specific promoter. Though beyond the scope of the present study, such investigations in the future will be instrumental in improving our understanding of the roles of integrins in the peripheral nervous system and the possible implications for integrin-mediated diseases.

5.1.6 Acknowledgments

The authors are grateful to Heinrich Blazyca and Kathrin Rohner for skillful technical assistance. This work was supported by Deutsche Forschungsgemeinschaft (MA 1053) and by research funds of the University of Würzburg.

6 Conclusions and Perspectives I

6.1 Myelination is not impaired in β4 integrin-deficient mice

β4-integrin-deficient mice die shortly after birth. Studying myelination in the absence of this integrin subunit is, therefore, only possible in cell culture and in the nerves of newborn animals. Our investigations in femoral nerves of newborn β4-deficient mice demonstrated an early formation of myelin similar to that of control animals. The number of axons segregated by Schwann cells into a one-to-one relationship and the progress of myelination on these axons did not differ between wild-type and knockout mice. In DRG explant cultures under myelin-promoting conditions, we detected similar numbers of myelin segments for both genotypes. Contrasting the usually very thin myelin (up to approximately five compacted turns) found in femoral nerves of newborn mice, the cultured myelin sheaths were up to 15 turns thick. They showed normal ultrastructural compaction of the membrane layers. Based on these observations, we concluded that β4 integrin is dispensable for the formation of myelin in the peripheral nervous system. However, the tightly regulated expression pattern of β4 integrin and its unique molecular structure suggest a specific function for this cell adhesion molecule in myelinating Schwann cells. With our approaches we could not address several questions that partly will be discussed below.

6.1.1 Hypothetical functions for β4 integrin in myelinating Schwann cells

In myelinating Schwann cells, β4 integrin was presumed to function as a mainly structural protein and to mediate attachment of the Schwann cell to the ECM during the process of myelination. This hypothesis is mainly based on the capability of the integrins to bind to ECM components on the extracellular and to cytoskeletal components on the intracellular side. β4 integrin possesses a variety of binding sites to interact with cytoskeletal and signaling proteins. The diverse functions of such interactions have mainly been described in immune cells, keratinocytes or cancer cells (for review see Juliano and Haskill, 1993; Clark and Brugge, 1995; Schwartz et al.,

1995; Burridge and Chrzanowska-Wodnicka, 1996; Nixon et al., 1998), therefore, most of them need yet to be proven in Schwann cells. Early myelinaton is not impaired in the absence of β4 integrin although β4 is specifically upregulated in normal Schwann cells at the onset of myelin formation. Therefore, β4 may be important for late effects in myelin formation and maintenance. Two ways of signal transduction are conceivable for β4 integrin to mediate such effects: In contradiction to earlier hypotheses, an interaction of β4 integrin with actin filament was reported and shown to be essential for the protrusion of filopodia and the anchoring of lamellae in carcinoma cells (Rabinowitz and Mercurio, 1997). The interaction of β4 integrin with laminin has been found to be particularly strong. The adhesion of these cells to the matrix is mediated, however, by β1 integrin (Rabinowitz and Mercurio, 1997). Therefore, it could be hypothesized in parallel that, while β1 is able to compensate for mechanical adhesion of the Schwann cell to the basal lamina in newborn animals and in cell culture, it may not provide strong enough interactions to prevent mechanical damage to thickly myelinated nerve fibers in adult animals.

The integrin α6β4 plays a major role in the stabilization of actin filaments in filopodia of migrating carcinoma cells (Rabinowitz and Mercurio, 1997). α6β4 integrin reduces the rearward flow of actin at these protruding sites. It is conceivable, albeit unlikely, that the cytoskeletal stabilization in Schwann cells, which possibly is mediated by β4 integrin, is only needed in later stages of myelination when the amount of myelin membranes that need to be wrapped around the axon reaches a certain mass. In our studies, we investigated only myelin sheath of approximately 15 to 20 membrane layers. These myelin sheaths are rather thin and the lack of β4 integrin at this stage may not have visible consequences on the mechanism of membrane wrapping.

Other most important functions of integrins are activation and modification of intracellular signaling cascades (for review see Juliano and Haskill, 1993; Clark and Brugge, 1995; Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Nixon et al., 1998). Different integrins activate specific signaling pathways that often converge to result in modified sensitivity of the cell to external stimuli or in altered gene expression. Because there is very little known about the signaling cascades elicited by

integrins in Schwann cells, the possibility exists that the lack of β4 integrin may lead to changes in the gene expression and even in the myelin composition.

Both ideas about a potential function of β4 integrin in later stages of axon myelination might be tested in experiments where β4 integrin-deficient Schwann cells are grafted into wild-type mice, or in tissue-specific β4 knockout mice.

6.1.2 Can β1 integrin compensate for the function of β4 integrin?

As mentioned in the discussion of the paper (on page 49), β1 integrin has been shown to be expressed on myelinating Schwann cells and inhibition of its function leads to disturbance of myelination *in vitro* (Fernandez-Valle et al., 1994). Therefore, it is possible that β1 compensates for a putative role of β4 integrin in myelination. However, β1 is not able to carry out the function of β4 integrin in the stratified epithelia of β4 knockout mice. A possible explanation for this discrepancy lies in the dual functional properties of the β4 integrin. The assembly of hemidesmosomes in keratinocytes critically depends on the binding of other hemidesmosomal proteins to the hinge-region between the fibronectin repeats in the intracellular domain of β4 (Mainiero et al., 1995). Another major function of β4 integrin is the activation of various intracellular signaling cascades. Whereas the β1 subunit lacks the hinge-region important for the assembly of HD and, therefore, is not able to provide the attachment of other hemidesmosomal proteins, it is perfectly capable of activating intracellular signaling cascades. Although the cascades activated by β4 and β1 integrin presumably differ at least slightly, the activation events elicited by the β1 integrin may be sufficient to compensate for the lack of β4-mediated signaling. However, the nature and functional consequences of these putative signaling events in myelinating Schwann cells are still unknown.

7 Results II

7.1 Loss of Distal Axons and Sensory Merkel Cells and Features Indicative of Muscle Denervation in Hindlimbs of P0-Deficient Mice

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published in The Journal of Neuroscience 19(14) (1999), 6058-6067

7.1.1 Abstract

Mice lacking the major Schwann cell myelin component P0 show a severe dysmyelination with pathological features reminiscent of the Déjérine-Sottas syndrome in humans. Previous morphological and electrophysiological studies on these mice did not only demonstrate a compromised myelination and myelin maintenance, but were suggestive of an impairment of axons as well. Here, we studied the axonal pathology in P0-deficient mice by quantitative electron microscopy. In addition, we investigated epidermal receptor end organs by immunocytochemistry and muscle pathology by histochemistry.

In proximal sections of facial and of femoral nerves, axon calibers were significantly reduced, whereas the number of myelin-competent axons was not diminished in 5- and 17-month-old P0-deficient mice. However, in distal branches of the femoral and sciatic nerve (digital nerves innervating the skin of the first toe) the numbers of myelin-competent axons were reduced by 70% in 6-month-old P0-deficient mice. Immunolabeling of foot pads revealed a corresponding loss of Merkel cells by 75% suggesting that survival of these cells is dependent on the presence or maintenance of their innervating myelinated axons. In addition, quadriceps and gastrocnemius muscles showed pathological features indicative of denervation and axonal sprouting. These findings demonstrate that loss of an important myelin component can initiate degenerative mechanisms not only in the Schwann cell but also in the distal portions of myelinated axons leading to the degeneration of specialized receptor end organs and impairment of muscle innervation.

7.1.2 Introduction

Inherited demyelinating neuropathies are chronic disorders of the peripheral nervous system that cause muscle weakness and sensory dysfunction. Particularly in lower limbs, irreversible degenerative processes such as muscle atrophy are typical, and the increasing loss of muscle strength results in malformation of the skeleton (Dyck et al., 1993). So far, four genes have been identified that are related to these disorders, including the peripheral myelin protein (PMP) 22, the myelin protein zero (MPZ, P0), the gap junction protein connexin 32 (Cx32), and the early growth responsive gene (EGR) 2 (Warner et al., 1998; for review, see De Jonghe et al., 1997; Martini et al., 1998). Depending on the mutated gene and on the severity of the resulting disorder, different subforms can be distinguished. These include various types of the Charcot-Marie-Tooth (CMT) disorder and particularly severe variants, such as the Déjérine-Sottas syndrome (DSS) and congenital hypomyelination (Warner et al., 1998; for review, see De Jonghe et al., 1997; Martini et al., 1998).

A common histopathological feature in nerve biopsies is the presence of abnormal myelin sheaths and reduced numbers of myelin profiles (Dyck et al., 1993), a finding that is in concord with the fact that the culprit genes are expressed by Schwann cells. Electrophysiologically, the disrupted myelin formation or myelin degeneration is reflected by lowered conduction velocities, increased muscle response latencies, and dispersed compound action potential profiles (Dyck et al., 1993). Paradoxically, the disorders are often associated with reduced amplitudes of compound action potentials, a feature that is indicative of compromised axon properties rather than of myelin disruption (Sghirlanzoni et al., 1992; Garcia et al., 1998; Marrosu et al., 1998). The surprising observation that mutations in Schwann cell-associated genes cause axonal abnormalities or damage is of particular interest, because dysfunction of axons may have robust functional consequences.

A tight link between the Schwann cell phenotype and axonal properties has been experimentally demonstrated in the Trembler (Tr) mouse (de Waegh et al., 1992), a spontaneous mouse mutant carrying a Asp to Gly substitution at codon 150 of the CMT-related gene PMP22 (Suter et al., 1992b). In mice deficient in the myelin-associated glycoprotein (MAG), reduced neurofilament spacing, altered phosphorylation of neurofilaments and axonal loss are leading features in the peripheral nerves of these

mutants (Fruttiger et al., 1995a; Carenini et al., 1997; Yin et al., 1998). Axonal impairment has also been described in sciatic and femoral nerves of mice homozygously deficient in P0 (Giese et al., 1992), an animal model for P0-related DSS (Martini et al., 1995b; Martini, 1997). To determine the extent of axonal changes in these mice, we measured the calibers of myelinated axons and quantified the numbers of myelinated axons in various peripheral nerves by electron microscopy. We found that in proximal parts of peripheral nerves, axons were reduced in their calibers but not in their numbers. In distal parts of the nerves, the number of axons was drastically reduced, accompanied by muscle denervation and a significant loss of sensory Merkel cells.

7.1.3 Materials and methods

Animals

Wild type mice (P0++) and P0-deficient mice (P0--) were taken from our own breeding colony. The genotypes were determined by their striking and typical phenotype (Giese et al., 1992). In some mice determined as P0-- mice, presence of the inserted *neo* gene was confirmed by PCR using appropriate primers.

Table 3 indicates the number of nerves of P0++ and P0-- mice investigated at different postnatal ages.

Table 3. Number of nerves of P0++ and P0-- mice analyzed at different postnatal ages

	P0++				P0--			
Age (months)	CF	FQ	FS	TN	CF	FQ	FS	TN
1.5				5				3
4				2				4
6	4	4	4	4	3	3	3	3
17	2	4			5	5		

CF, Cervical branch of facial nerve; FQ, femoral quadriceps nerve; FS, femoral saphenous nerve; TN, toe nerves.

Surgery

To confirm the sciatic and saphenous origin of the plantar and dorsal toe nerves, respectively, sciatic or saphenous nerves were transected as described (Fruttiger et al., 1995b), followed by an electron microscopic investigation of nerve-lesion-induced degeneration in the first toe of the lesioned side at postoperative day 14.

Tissue preservation for light and electron microscopy

Mice were transcardially perfused with 0.1 M cacodylate buffer containing 4% freshly depolymerized paraformaldehyde and 2% glutaraldehyde. Facial and femoral nerves comprising quadriceps and saphenous branches were dissected at the level of the

stylomastoid foramen and of the inguinal ligament, respectively. For analysis of the facial nerve, we selected the branch that bifurcates into the cervical branch, innervating the platysma, and the marginal mandibular branch, innervating the muscles of the lower lip. For simplicity, we called this common branch cervical branch. In addition, the first toes containing the terminal branches of the femoral saphenous and the sciatic nerves were dissected. Tissue specimens were postfixed for 12-24 hours in the perfusion fixative and processed for transmission electron microscopy as described (Carenini et al., 1997).

Immunolabeling of Merkel cells

Immunolabeling of Merkel cells was performed on 16 µm thick acetone-fixed serial cryosections of foot pads from 1.5- and 6-month-old P0++ and P0-- mice using antibodies to cytokeratin 20 as described previously (Airaksinen et al., 1996). Instead of fluorescent markers, biotinylated secondary anti-mouse antibodies and an avidin-biotin complex coupled to horse radish peroxidase were used (Sigma, St. Louis). Peroxidase activity was detected by a Tris-buffered solution containing diaminobenzidine-HCl and 0.03% H_2O_2.

Immunolabeled Merkel cells were counted on every second section. The total lengths of the dermis/epidermis interfaces of these sections were measured and the number of Merkel cells per mm dermis/epidermis interface was determined. Statistical analysis was performed using a Student's *t*-test.

Quinacrine fluorescence of Merkel cells

To quantify the number of Merkel cells in the back skin, we used the fluorescent vital dye quinacrine (quinacrine hydrochloride, Sigma-Aldrich, Deisenhofen, Germany) which is taken up by Merkel cells after it is injected systemically (Airaksinen et al., 1996). 6-month-old P0++ and P0-- (n = 4 in each group) were injected intraperitoneally with quinacrine/saline solution (1.5 mg quinacrine/100 g body weight) as described previously (Airaksinen et al., 1996). After 18-20 hrs, the animals were killed by CO_2 inhalation and the back skin was shaved and depiliated with depiliating cream. Approximately 1 x 1 cm of back skin was excised and embedded with the hairy side up in Aquatex (Merck, Darmstadt, Germany). Whole mounts of skin were viewed under an

Axiophot epifluorescence microscope with a filter for fluorescein isothiocyanate. The entire tissue was examined and the number of Merkel cells per cm^2 was determined. Statistical analysis was performed using a Student's *t*-test.

Type grouping of muscle fibers

Quadriceps and gastrocnemius muscles were dissected from 6-month-old P0++ and P0-- mice followed by immediate freezing in liquid nitrogen-cooled isopentane. Cryosections (12 µm thick) were mounted on poly-L-lysine-coated glass slides and air-dried. Myofibrillar actomyosin ATPase histochemistry has been carried out at pH 4.3, 4.6 and 9.4 as described (Hämäläinen and Pette, 1993).

Morphometry

For quantitative analysis, all myelinated axons (including axons without myelin but having achieved a 1:1 ratio with Schwann cells and abnormally myelinated axons in P0-- mice) of the facial cervical nerve and the femoral quadriceps and saphenous nerves were considered. In addition, the myelinated axons of the first toe were investigated.

The numbers and diameters of myelinated axons were determined on electron micrographs at a final magnification of 2,000-4,000. For each nerve, all axons were considered. The axons were categorized according to their diameters (<1 µm, 1-3 µm, 3-6 µm, >6 µm). Significance of differences between mean values was determined by a two-sided Student's *t*-test.

7.1.4 Results

To study the morphological appearance and number of axons in mice deficient in P0, we investigated various peripheral nerves. In particular, we selected two muscle nerves, the cervical branch of the facial nerve and the femoral quadriceps nerve. Because we have shown previously that the neuropathy in P0-deficient mice progresses with age, we investigated the mice at 6 and 17 months. In addition to the muscle nerves, we scored the saphenous nerve, a cutaneous and particularly long branch of the femoral nerve, at 6 months of age. The cervical facial nerve was investigated ~4 mm distal to the stylomastoid foramen, the femoral quadriceps and saphenous nerves at the level of the inguinal ligament.

Reduced axon diameters in peripheral nerves of P0-deficient mice

In peripheral nerves of wild-type mice (P0++), axons of larger caliber were always surrounded by normal myelin sheaths. In P0-deficient mice (P0--), various forms of abnormal axon-Schwann cell units were found in all nerves investigated. As shown previously, axons had either achieved a 1:1 relationship to Schwann cells without myelin or were surrounded by abnormally compacted myelin (Giese et al., 1992; Martini et al., 1995a; Carenini et al., 1999). For simplicity, axons that had achieved a 1:1 relationship with Schwann cells and axons surrounded by abnormal myelin were considered to be abnormally myelinated axons. A typical pathological feature in the nerves of the P0-- mice was the presence of supernumerary Schwann cells forming onion bulbs around abnormal axon-Schwann cell units. Such onion bulbs are indicative of myelin degeneration-induced Schwann cell proliferation (Martini, 1997).

Cervical branch of the facial nerve

In 6- and 17-month-old P0++ mice, the cervical branch of the facial nerve (CF) contained 1180 (±180) myelinated axons (Fig. 5*A*). Approximately 80% of the myelinated axons had diameters between 3 and 6 μm (Fig. 5*B*). Myelinated axons with diameters smaller than 1 μm were apparently absent and axons exceeding 6 μm were rarely found. In 6- and 17-month-old P0-- mice, a similar number of larger caliber axons was determined as in P0++ mice (Fig. 5*A*). However, a significant reduction in axon caliber was found at both ages (Fig. 5*B*). Whereas in P0++ mice most myelinated axons

were between 3 and 6 μm in diameter, in P0-- mice ~70% of the axons had a caliber of 1-3 μm. Furthermore, axons displaying a diameter <1 μm were present in the mutants.

Femoral quadriceps nerve

The femoral quadriceps nerve (FQ) contains 560 (±40) myelinated axons in both P0++ and P0-- mice (Fig. 1*C*). As shown in Fig. 5*D*, most of the myelinated axons of P0++ mice were larger than 3 μm in diameter and ~40% exhibited calibers of >6 μm. In P0-- mice, however, the situation was dramatically changed (Fig. 5*D*). Most axons were <3 μm in diameter, and this shift to smaller caliber axons was at least partially at the expense of the categories containing axons with a diameter >3 μm. Similar to the CF, a significant number of axons were <1 μm in P0-- mice (Fig. 5*D*).

Femoral saphenous nerve

In P0++ mice, the femoral saphenous nerve (FS) contains 780 (±80) myelinated axons (Fig. 5*E*). In P0-- mice, this number was reduced by ~25%, but this reduction was not statistically significant (Fig. 5*E*). A very significant change, however, was found when the axon calibers were compared (Fig. 5*F*). In P0++ mice most axons had a diameter between 3 and 6 μm; in the mutants 80% of the axons had a diameter of 1-3 μm only. Similar to the CF and FQ, a significant number of axons had a diameter of <1μm in the P0-- mice. In all nerves of P0-- mice examined, electron microscopy revealed a substantially elevated density of neurofilaments in myelin-competent axons (see Fig. 8*A*,*C*).

Reduced numbers of axons and features indicative of axonal degeneration in distal parts of FS and sciatic nerves of P0-deficient mice

We have shown that in proximal aspects of three different peripheral nerves of P0-- mice axon calibers are significantly altered, but the number of myelinated axons was not significantly reduced. Because it is known that in inherited peripheral neuropathies the clinical and pathological alterations are usually most severe in lower parts of the extremities with features reflecting axonal damage (Dyck et al., 1993), we investigated the myelinated axons of the first toe. In cross sections through the basis of the first toe, two plantar and two dorsal nerves can be found (Fig. 6*A*). All myelinated axons innervating the plantar side are derived from the sciatic nerve, whereas the axons

innervating the dorsal side are derived from the FS. These anatomical conditions were confirmed by transecting either the sciatic or the FS nerve of normal mice followed by studying the lesion-induced degeneration of axons in the respective location of the toe (Fig. 6*B-E*). When sciatic nerves had been transected, all axon-Schwann cell units from the plantar side degenerated (Fig. 6*D*), whereas transection of the FS resulted in the degeneration of all dorsal axon-Schwann cell units (Fig. 6*C*).

Each plantar nerve of a P0++ mouse contains ~40 myelinated axons, and each dorsal nerve contains ~25 myelinated axons. In addition, several small nerve branchlets derived from the FS can be detected in the dorsal aspect of the toe. When investigating the plantar and dorsal nerve branches in the toes of P0-- mice, we found a dramatic reduction of myelin-competent axons (Fig. 7). In general, the strongest reduction was found in 6-month-old P0-- mice, the oldest age investigated, with ~50% of reduction in the sciatic nerve derivatives (plantar) and ~75% of reduction in the FS branches (dorsal) (Fig. 7).

Whereas in the proximal parts of the peripheral nerves the abnormal axon-Schwann cell units were often associated with supernumerary Schwann cells reflecting myelin degeneration-induced Schwann cell proliferation, such features were not detectable in the distal nerve parts (Fig. 8). Instead, Schwann cell profiles reminiscent of bands of Büngner were occasionally found suggesting previous axonal loss (Fig. 8*B*). The number of bands of Büngner, however, was clearly smaller than the number of putatively degenerated axons, possibly reflecting Schwann cell degeneration.

Reduced numbers of Merkel cells in P0-deficient mice

It has been shown previously that sensory Merkel cells are dependent on the presence of their innervating myelinated axons (Mills et al., 1989; Airaksinen et al., 1996). We therefore determined the numbers of these specialized terminal cells in the foot pads by immunohistochemistry using antibodies to cytokeratin 20. Merkel cells were detected at the basal side of the epidermis close to the border of the dermis. In 1.5-month-old P0++ mice, approximately two (1.8 ± 0.4) Merkel cells per mm dermis/epidermis interface could be detected. A similar number of Merkel cells could be found in 1.5-month-old P0-- mice (1.5 ± 0.3). At 6 months of age, the number of Merkel cells was not significantly altered in P0++ mice (2.2 ± 0.3), whereas in 6-month-old P0-

- mice, the number of Merkel cells was reduced to 0.5 (± 0.1) per millimeter dermis/epidermis interface (Figs. 9*A*,*B*, 10). Thus, in P0-- mice, loss of sensory axons is accompanied by a profound reduction of Merkel cells.

We also investigated the numbers of Merkel cells in the hairy skin of the back of P0++ and P0-- mice. For this purpose, we injected mice with quinacrine, a fluorescent vital dye that labels Merkel cells (Airaksinen et al., 1996). Again, the number of Merkel cells was dramatically reduced in 6-months-old P0-- mice when compared with age-matched P0++ (2.2 ± 3.9 Merkel cells per cm^2 in P0-- mice versus 248.5 ± 86.8 cells per cm^2 in P0++ mice; p=0.003; Fig. 9*C*,*D*).

Type grouping of muscle fibers

Based on our observation that in cutaneous sensory nerves terminal axons degenerate and cause loss of Merkel cells, we considered the possibility that terminals of motor axons degenerate as well. As one possibility one might investigate distal aspects of muscle nerves close to their entrance into the corresponding muscle. However, since muscle nerves contain both motor and sensory axons, a loss of axonal profiles in muscle nerves does not stringently reflect a loss of motor axons. We, therefore, investigated quadriceps and gastrocnemius muscles with respect to established histopathological features that are indicative of loss of motor axons.

Although proximal aspects of motor nerves did not show significant axonal loss by electron microscopy, quadriceps muscles and, even more striking, gastrocnemius muscles of 6-month-old P0-- mice showed features indicative of denervation. For instance, in the lateral head of the gastrocnemius muscle of P0++ mice, ATPase staining at pH 4.3 revealed a checkerboard pattern of relatively few, darkly labeled type I myofibers (Fig. 11*A*). Labeling for type II myofibers at pH 9.4 resulted in a complementary staining pattern (data not shown). In the same muscle of P0-- mice, a grouped arrangement of type I myofibers was striking at pH 4.3 (Fig. 11*B*). Such groups usually contained 4 to 7 tightly apposed myofibers. It is well established that such a staining pattern of grouped myofibers results from denervation of muscle fibers followed by collateral reinnervation by sprouts from neighboring motor units (De Girolami et al., 1997). In addition to the type grouping of myofibers, small, angulated myofibers that were often associated with grouped myofibers of normal size were

detected (Fig. 11*C,D*). Such myofibers that were never detected in muscles from P0++ mice are indicative of denervation and represent neurogenic muscle atrophy (De Girolami et al., 1997).

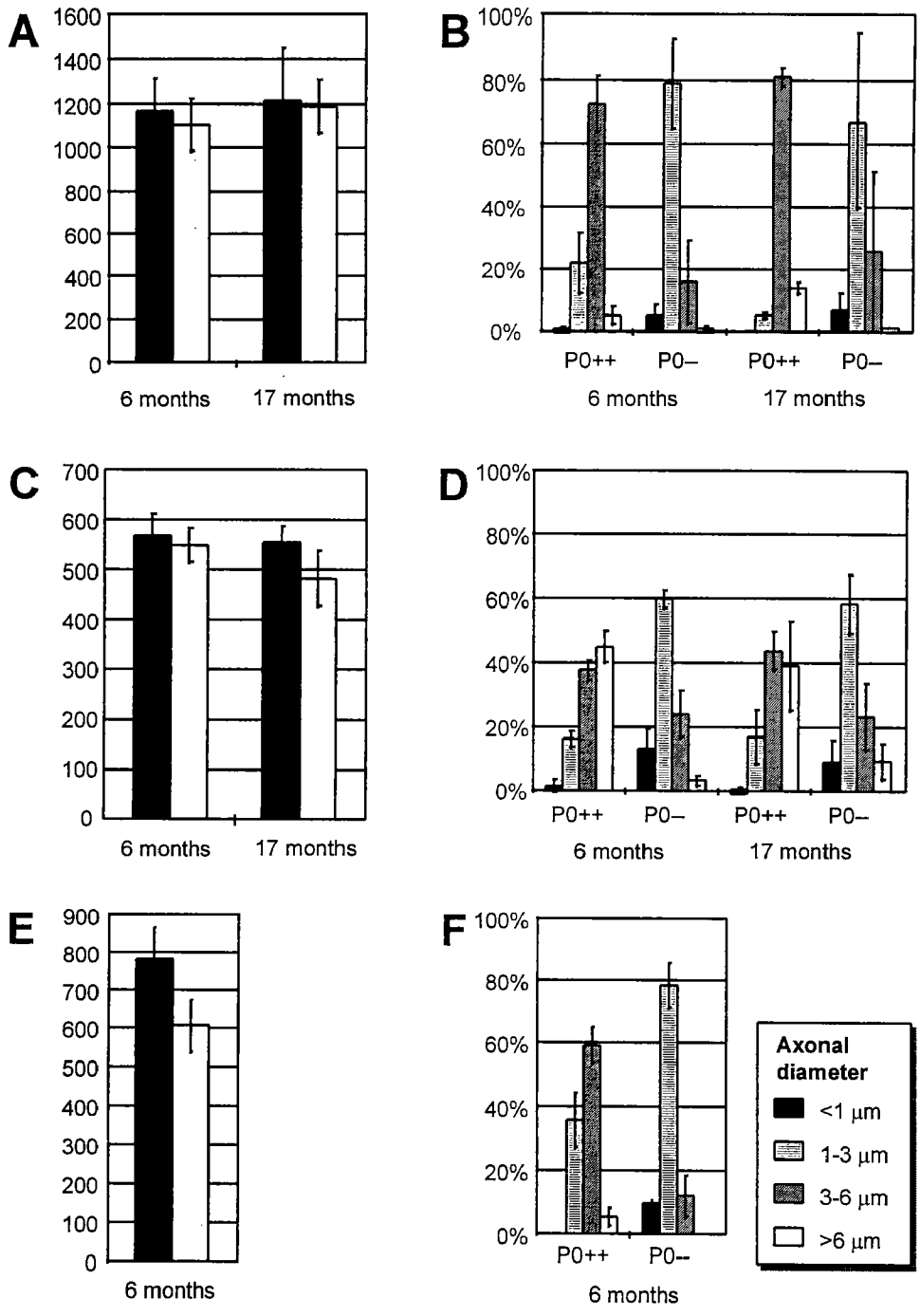

Figure 5

Representations of the number of myelin-competent axons (*A,C,E*) and size-frequency histograms (*B,D,F*) of the cervical branch of the facial nerve (*A,B*), of the femoral quadriceps nerve (*C,D*) and of the femoral saphenous nerve (*E,F*) of P0++ and P0-- mice. The total number of myelin-competent axons in the proximal nerves of P0-- mice is not significantly changed at all ages investigated (*A,C,E*; $p > 0.05$). Note the reductions of axonal diameters in P0-- animals. All differences between P0++ and P0-- mice in the axon number per size category were significant. Mean ± SD are shown. *Inset* in *E* applies to *A*, *C*, *E*. *Inset* in *F* applies to *B*, *D*, *F*.

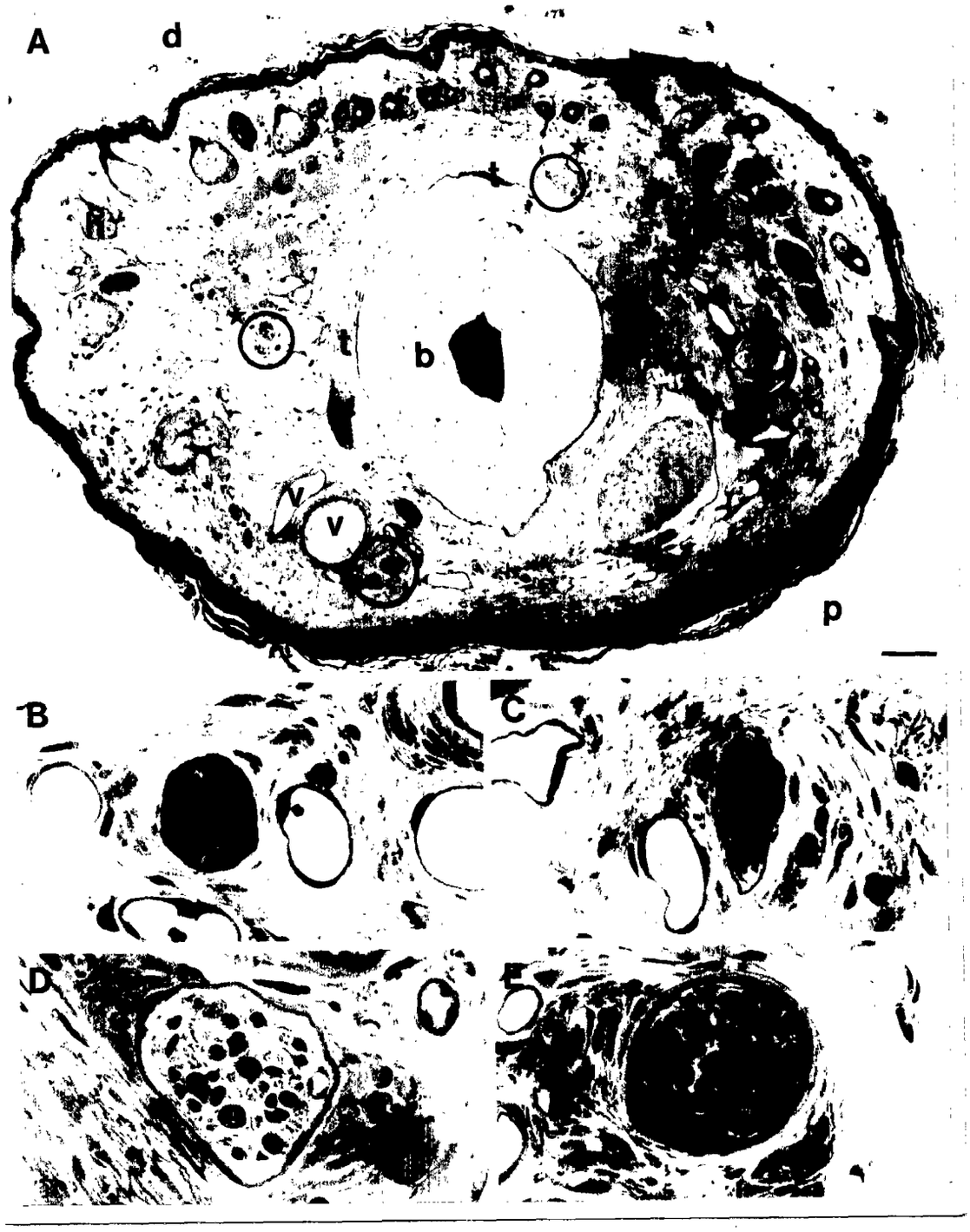

Figure 6

Micrographs of semithin cross sections of the base of the first toe of P0++ mice. *A*, Low power micrograph of the toe showing the position of dorsal branches of the femoral saphenous nerve (*circles with stars*) and plantar branches of the sciatic nerve (*circles*). Dorsal (*B*, *C*) and plantar (*D*, *E*) nerve branches of the toe after transection of the sciatic (*B*,*D*) and the femoral saphenous (*C*,*E*) nerves. Wallerian degeneration in *C* and *D* reflects the femoral saphenous and sciatic nerve origin of the dorsal and ventral nerves, respectively. *b*, bone, *d*, dorsal, *h*, hair follicles, *p*, plantar, *t*, tendons, *v*, vessels. Scale bar (shown in *A*): *A*, 300 μm; *B-E*, 15 μm.

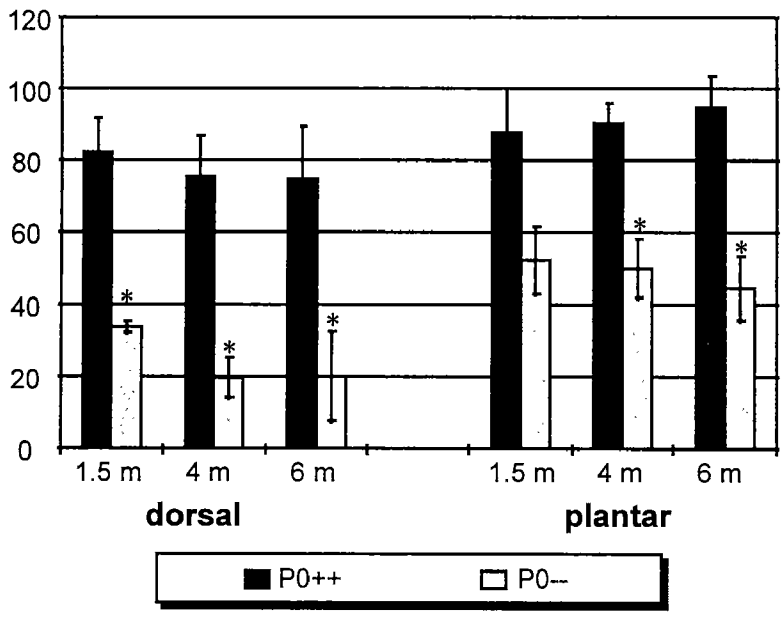

Figure 7

Representation of the number of myelin-competent axons in the nerve branches innervating the first toe of P0++ and P0-- mice at 1.5, 4, and 6 months (*m*) of age. Note the significant reduction of axons in the distal nerve branches of 4- and 6-month-old P0-- mice (* $p \leq 0.01$). Mean ± SD are shown.

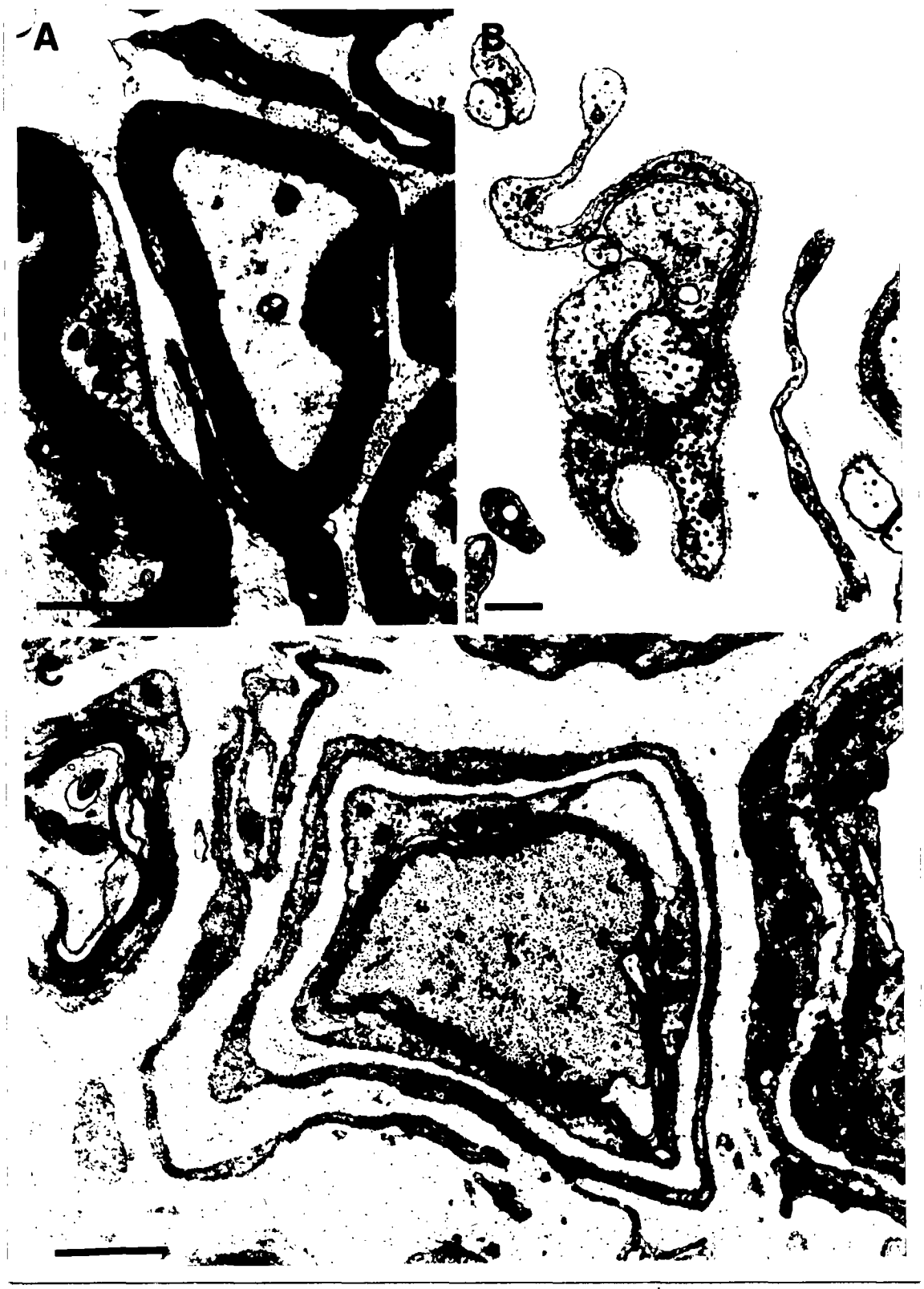

Figure 8

Electron micrographs of dorsal nerve branches of the first toe (*A*, *B*) and of femoral quadriceps nerves (*C*) of P0++ (*A*) and P0-- (*B*, *C*) mice. *A*, In the toes of P0++ mice, large axons with compact myelin sheaths are found. *B*, In the toes of P0-- mice, Schwann cell profiles reminiscent of bands of Büngner and representing Wallerian degeneration are pathological hallmarks. *C*, In proximal nerve parts of P0-- mice such as in femoral quadriceps nerves, abnormal myelin profiles, associated with onion bulb cells are typical. Scale Bars: *A*, *C*, 1 μm; *B*, 0.25 μm.

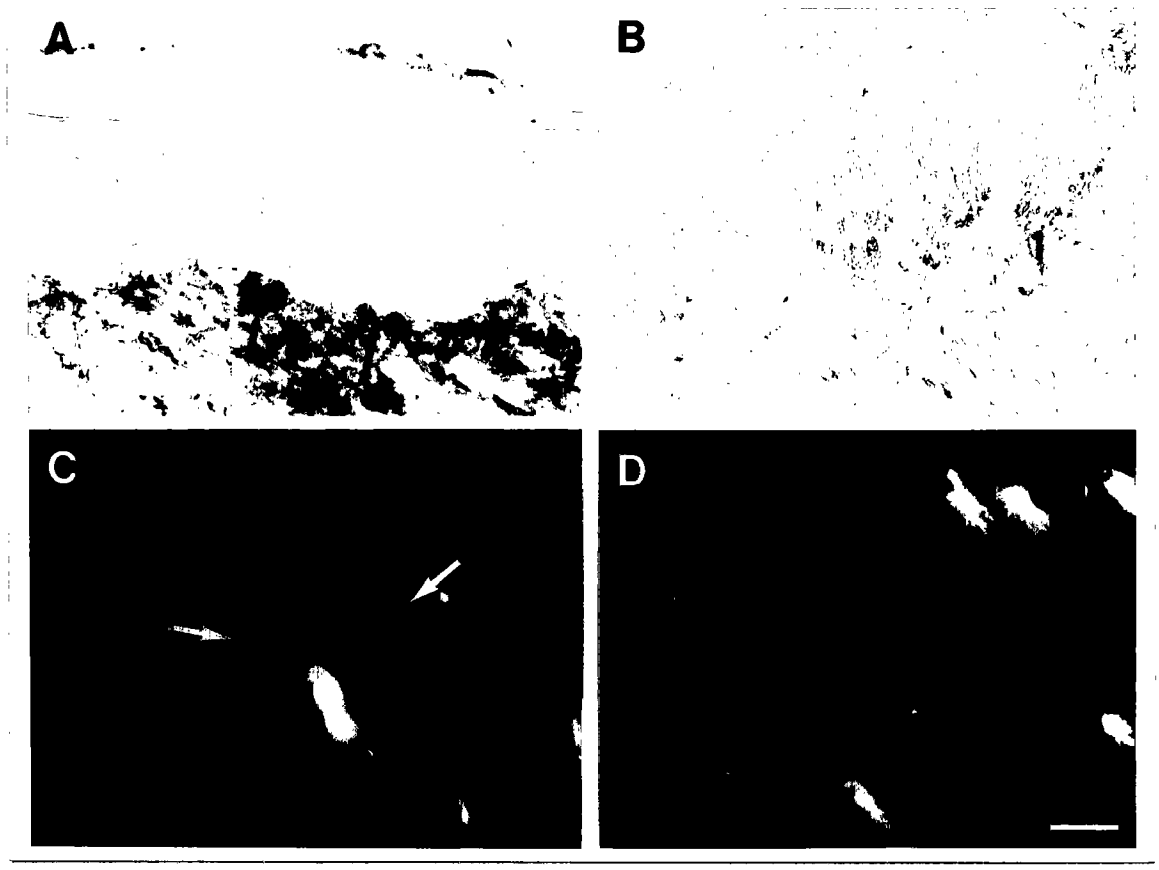

Figure 9

Light microscopy of Merkel cells in the glabrous (A, B) and hairy skin (C, D) of 6-month-old P0++ (A, C) and P0-- mice (B, D) using antibodies to cytokeratin 20 (A, B) and the fluorescent vital dye quinacrine (C, D). A and B show cryosections of the footpads, C and D whole mount preparations of the back skin. A, In P0++ mice, Merkel cells (arrows) are detectable at the dermis/epidermis interface. B, In P0-- mice, the number of Merkel cells is strongly reduced so that long stretches of dermis/epidermis interface are devoid of Merkel cells. C, In hairy skin of P0++ mice, groups of Merkel cells (between arrows) representing touch domes are frequently found. D, In hairy skin of P0-- mice, Merkel cells are only rarely found. Highly fluorescent conical structures visible in hairy skin of both P0++ and P0-- mice represent the hair shafts. Scale bar (shown in D): A, B, 25μm; C, D, 50μm.

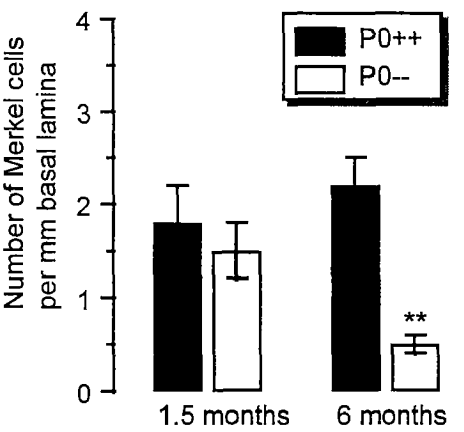

Figure 10

Quantification of Merkel cells in the glabrous skin of P0++ and P0-- mice. At the age of 6, but not at 1.5 months, the number of Merkel cells in the skin of P0-- mice is significantly reduced compared to age-matched P0++ mice. (**$p < 0.01$). Mean ± SD are shown.

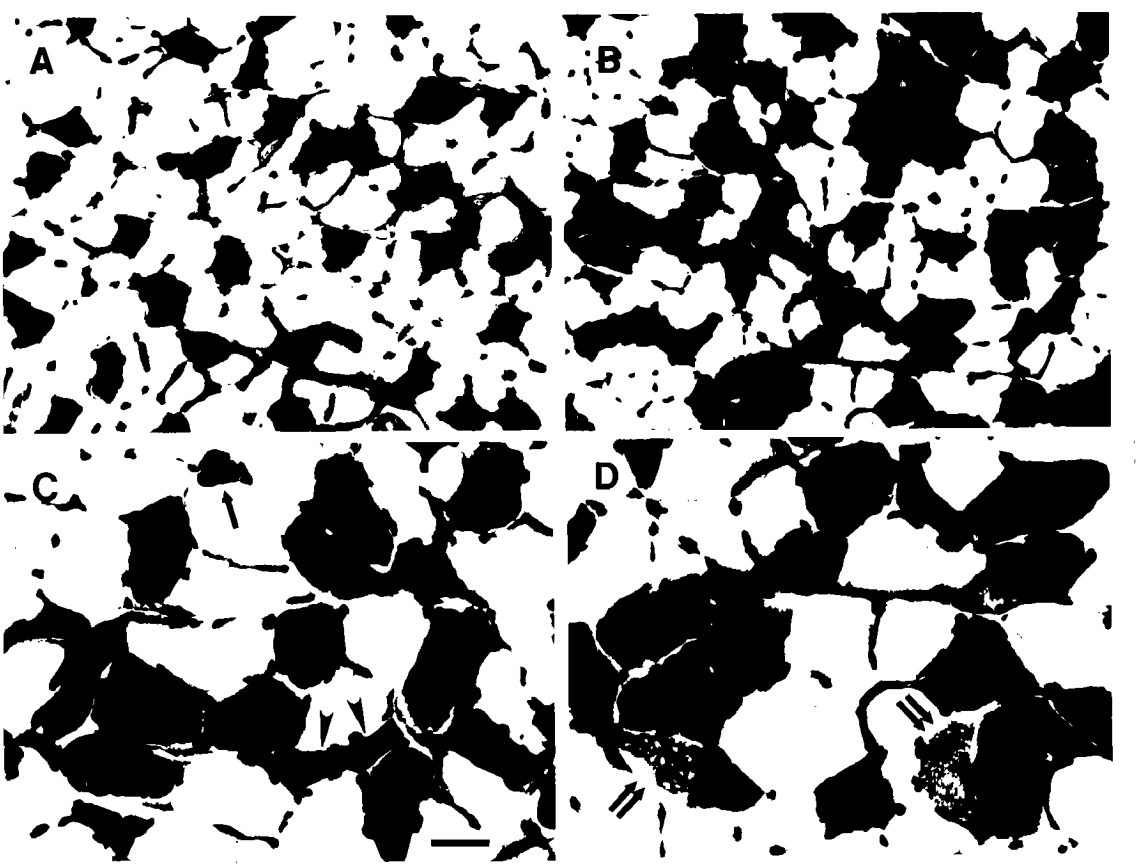

Figure 11

ATPase activity in the gastrocnemius muscle of 6-month-old P0++ (*A*) and P0-- mice (*B-D*) at pH 4.3. *A*, Labeling for ATPase activity in gastrocnemius muscle of P0++ mice reveals the typical checkerboard staining pattern. *B*, In the P0-- mice, type I muscle fibers are grouped. *C*, *D*, Larger magnification of gastrocnemius muscle of P0-- mice. Note small groups of atrophic myofibers (*arrow*), angulated fibers (*arrowheads*) and fibers of intermediate staining intensity (*double arrows*) probably reflecting a change of fiber type. Scale bar (shown in *C*):*A*, *B*, 25 μm; *C*, *D*, 12.5 μm.

7.1.5 Discussion

We have shown that in mice deficient in the Schwann cell component P0 axonal properties are significantly changed. In proximal parts of peripheral nerves, axons are reduced in their calibers, but there is no significant loss of axons. In distal parts of the nerves, a robust loss of axons could be found as reflected by reduced numbers of axons, hallmarks of Wallerian degeneration, and features indicative of denervation of peripheral target organs.

Myelinating glia cells determine axonal properties and axon survival

The finding that axonal properties, such as axon size, are strongly dependent on glial partners has been described in a variety of different systems. In MAG-deficient mice, myelination is initially normal, but in mice older than several months, axons and myelin degenerate (Fruttiger et al., 1995a; Carenini et al., 1997). A characteristic abnormality is the presence of paranodal myelin tomacula (Carenini et al., 1997; Yin et al., 1998). These tomacula have been suggested to form as a result of reduced axon calibers at the paranode caused by reduced neurofilament phosphorylation and spacing (Yin et al., 1998). However, it is also conceivable that myelin tomacula cause a physical constriction of axons attributable to a local overproduction of myelin turns, as has been proposed in other tomaculous peripheral neuropathies (Meier and Moll, 1982; Adlkofer et al., 1997b). This constriction could lead, in turn, to axonal strangulation. In a very elegant series of experiments, De Waegh and colleagues transplanted nerve stumps from Trembler (Tr) mice into transected nerves of wild-type mice, allowing regrowing axons from wild-type mice to become myelinated by Schwann cells from Tr mutants (de Waegh and Brady, 1990; de Waegh et al., 1992). Abnormal Schwann cells of Tr mice were associated with smaller diameters of the wild-type axons and with a reduced degree of phosphorylation and spacing of the neurofilaments. Investigations on dorsal root ganglion neurons *in vivo* confirm the view that the Schwann cell phenotype can influence axon properties. Each dorsal root ganglion neuron extends a non-myelinated stem process that bifurcates into two myelinated axons, one projecting into the spinal cord via the dorsal root and the other into the spinal nerve. In line with the view that glial cells can modify axonal properties, the non-myelinated stem process of the neuron has a smaller diameter and more dense neurofilament packing with lower

phosphorylation status than the myelinated central and distal axons that are myelinated (Hsieh et al., 1994). The influence of myelinating glial cells on the axonal phenotype is not confined to Schwann cells. In optic nerves that have been depleted of oligodendrocytes by X-ray irradiation, the retina ganglion cell axons do not reach the calibers of myelinated retina ganglion cell axons (Colello et al., 1994). In line with this observation is the finding that enwrapping (Sanchez et al., 1996) or formation of compact myelin (Windebank et al., 1985) by oligodendrocytes leads to an increase of axonal calibers.

The loss of terminal axons in P0-deficient mice could be viewed as an extreme form of modulatory effect of glial cells on axonal properties. This phenomenon is not unique to mice deficient in P0 and has also been described in other experimental models or disorders. Sahenk and Chen (1998) recently demonstrated the vulnerability of distal axons from mice that are associated with mutant Schwann cells from humans. Sural nerve biopsies from CMTX patients were grafted into transected nerves of immune-deficient nude mice. Axons were reduced in their calibers and displayed tightly spaced neurofilaments in the human xenografts. In distal, but not in proximal aspects of the grafts, features indicative of axon degeneration were found. It is interesting to mention in this context that CMTX patients often suffer from an axonopathy rather than from a myelinopathy (Timmerman et al., 1996; De Jonghe et al., 1997; Sander et al., 1998) and mice deficient in Cx32 display features indicative of axon degeneration and regrowth at later ages (Anzini et al., 1997; Scherer et al., 1998). A robust effect of oligodendrocytes on survival of axon terminals has recently been demonstrated in multiple sclerosis. In this kind of neurological disorders, oligodendrocytes suffer from autoimmune attacks against particular membrane components (Archelos et al., 1998). The chronic impairment of oligodendrocytes apparently leads to a robust damage of axons as reflected by the frequent occurrence of terminal axonal ovoids in multiple sclerosis lesions (Trapp et al., 1998).

How can glial cells influence axonal properties and impair the survival of terminal axons? It is conceivable that molecules at the axon interface, such as MAG and possibly others, might be important mediators for the modulation of axonal properties by myelinating glial cells (de Waegh et al., 1992; Yin et al., 1998). Intriguingly, MAG is only weakly expressed at the Schwann cell-axon interface in P0-deficient mice

(Carenini et al., 1999). The compromised axon-glia interactions could also alter slow axonal transport, as has been shown in trembler mutants (de Waegh and Brady, 1990; de Waegh et al., 1992). It is tempting to speculate that axonal receptors of glial molecules could locally modify axonal transport, possibly by signaling into the cell interior. It has recently been shown that particular mutations in the copper/zinc superoxide dismutase linked with amyotrophic lateral sclerosis in human lead to impaired slow axonal transport of neurofilaments in transgenic mice, long before motor neuron decline occurs (Williamson and Cleveland, 1999). In this model, it is suggested that the impaired transport mechanism leads to the typical hallmarks of the disease, such as neurofilament-containing axonal swellings that result in axonal strangulation and eventually death of motor neurons (Williamson and Cleveland, 1999). In the case of inherited neuropathies, we propose a different mechanism, because axonal swellings and death of motor neurons are not hallmarks of these diseases. It is conceivable that, in glia-mediated neuropathies, impaired slow axonal transport in all parts of the nerve leads to a deprivation of vital cell soma-derived molecules, particularly in the distal aspects of the axons. This in turn could induce local detrimental mechanisms that eventually lead to the decline of the axon terminal, whereas proximal aspects of the axons and the neuronal cell bodies (H. Lassmann, personal communication; M. Sendtner, personal communication) remain preserved. The implication of anterograde axonal transport in the degeneration of axons in various forms of hereditary neuropathies could explain the paradox observation that, although all myelinating Schwann cells are abnormal, degeneration of axons is most severe at the distal ends of long nerves (Dyck et al., 1993). On the other hand, it is conceivable that Schwann cells at the terminal regions of the nerves suffer particularly severe from the absence of P0 and cause distal axon loss. We consider this possibility as unlikely, however, because there is no evidence for such a mechanism, neither in young P0-deficient mice, nor in other myelin mutants (R.M. unpublished observations).

Clinical implications

The fact that axon-glia interactions results in robust axonal changes has important clinical implications. A particularly striking phenomenon in our mouse model is the loss of axons at distal aspects of longer nerves. Interestingly, recent electrophysiological

studies in young children diagnosed as CMT1A patients revealed that the first pathophysiological signs of the disease are a reduction of the amplitude of compound muscle action potentials, possibly reflecting axonal degeneration (Garcia et al., 1998). Similarly, several patients suffering from CMTX have previously been misdiagnosed as CMT2 because of a robust reduction of the amplitude of compound action potentials that is indicative of substantial axonal loss (Timmerman et al., 1996; De Jonghe et al., 1997). The increased vulnerability of relatively long nerves is most probably responsible for the well known phenomenon that the peroneal muscles and the intrinsic foot muscles of patients are first and most severely affected, followed by muscle atrophy in the hands (Dyck et al., 1993). This leads to irreversible skeletal deformities such as pes cavus and clawhand formation caused by unopposed action of long toe and finger muscles, respectively. In P0-- mice, muscle atrophy is not as severe as in human. This might be explained by the shorter live span of the animals and also by the reduced vulnerability of the nerves due to their shorter extension in a small-sized animal.

The robust loss of axons in at least some forms of inherited neuropathies has important implications for possible treatment strategies. One possibility might be to mimic correct Schwann cell-axon interactions, possibly by activating axonal receptors for glial cell surface molecules with the appropriate ligands. Furthermore, it might be promising to treat the distal aspects of the nerves with trophic factors in the hope to prevent or to reduce axonal loss. A promising approach has recently been presented by Haase et al. (1997) using adenoviral vectors for gene transfer of neurotrophin-3 into muscles of spontaneous mouse mutants suffering from progressive motor neuronopathy. Loss of motor axons could be attenuated and neuromuscular function was improved. Similar approaches could be of interest in hereditary neuropathies with the aim to rescue axon terminals of particularly severely affected nerves. Alternatively, it might be helpful to foster axonal regrowth or intramuscular sprouting before muscle atrophy will occur. Interestingly, insulin-like growth factor-I strongly fosters intramuscular axonal sprouting (Caroni and Grandes, 1990). In addition, this factor accelerates myelination by Schwann cells (Feldman et al., 1997) and therefore could preserve abnormal myelin sheaths that would otherwise be prone to degeneration. The availability of animal models will be instrumental in searching for the appropriate strategies to prevent degenerative processes that result in irreversible degenerative changes in hereditary neuropathies in humans.

7.1.6 Acknowledgments

The study was supported by the Swiss National Research Foundation (to R.M.), the Deutsche Forschungsgemeinschaft (to R.M.), by research funds of the University of Würzburg, and by the European Union (Clinical, Genetic, and Functional Analysis of Peripheral neuropathies: An Integrated Approach). We are grateful to Carsten Wessig, Stefano Carenini and K.V. Toyka for discussions, Kathrin Rohner and Heinrich Blazyca for skillful technical assistance, and Michael Sendtner and Hans Lassmann for sharing unpublished data.

8 Conclusions and Perspectives II

8.1 Distal Axons and Target Tissues are Affected in P0-Deficient Mice

Despite earlier observations that suggested axonal degeneration (Giese et al., 1992) we found no loss of axons in proximal motor nerves in our mouse model, the P0-- mutant. This finding was independent of the age of the animals investigated. In the proximal sensory nerves of our mice, we detected only a non-significant trend towards axonal degeneration. However, when we investigated the distal sites of sensory nerve branches, we detected a highly significant loss of myelinated axons already at a very young age. In addition, we could determine a concomitant loss of sensory target organs in the skin that are usually innervated by myelinated axons. In contrast to the sensory branches, the distal motor branches showed no significant axonal degeneration when analyzed close to the muscle (data not shown). However, the muscles of the P0-- mice displayed extensive pathological alterations indicative of fiber denervation. Histologically, we demonstrated atrophic myofibers and fiber type grouping resulting from de- and reinnervation of the muscle fibers. Our results clearly demonstrate an involvement of axons in the pathology of the P0-- mice. The axons generally suffer from a degeneration that begins in distal regions and manifests as a dying-back neuropathy. Because degeneration of axons is a feature described in patients suffering from myelin-related neuropathies, the P0-- mice are valuable models not only for further investigations on the possible pathomechanisms that operate in myelinopathies but also for testing putative therapeutic agents for the treatment of hereditary myelin-related neuropathies of the CMT-type.

8.2 Therapeutic approaches in peripheral neuropathies

Treatment strategies for different forms of HMSNs are scarce. In the past, the lack of adequate animal models and the fact that hereditary diseases are a clinically less important group of neuropathies retarded attempts to search for therapies. Investigations in therapeutic possibilities have now been reinforced, taking into account the rapid

progress in the discovery of CMT culprit genes and the availability of genetically engineered animal models (for review see De Jonghe et al., 1997; Martini, 1997). Intensive research on the trophic requirements of neurons provided a large number of neurotrophic factors capable of influencing neuronal growth behavior and survival (for review see Davies, 1994; Oppenheim, 1996; Abel et al., 1999).

Neurotrophic factors promote the survival of neurons

Neurotrophic factors are a heterogeneous group of proteins including members of different families of growth factors (see table 4). Their survival-promoting effect has first been demonstrated on cultured sensory and motor neurons. Further analyses of the *in vivo* function of these factors revealed a surprising complexity and diversity of their actions: They act directly on neurons to support their differentiation and growth (for review see Snider, 1994). Several factors promote survival of postmitotic developing neurons in the period of naturally occurring programmed cell death and of adult axotomized neurons (for review see Oppenheim, 1996; Price et al., 1996).

Table 4 **List of neurotrophic factors with effects on spinal motor neurons and dorsal root ganglion sensory neurons**

Neurotrophins	Neuropoietic cytokines (IL-6-family)	Insulin-like growth factors (IGFs)	Transforming growth factor beta family (TGF-beta)	Fibroblast growth factors FGFs	Other growth factors
NGF[1]	CNTF[1,2]	IGF-I[1,2]	TGFβ1[1,2], β2[1,2], β3[1,2]	FGFa[1,2]	TGFα[1,2]
BDNF[1,2]	LIF[1,2]	IGF-II[1,2]	GNDF[2]	FGFb[1,2]	PDGF[2]
NT-3[1,2]	CT-1[3]				Stem cell factor[1]
NT-4/5[1,2]					

[1] action of neurotrophic factor on dorsal root ganglion sensory neurons, [2] action on spinal motor neurons (adapted from Yuen and Mobley, 1996); [3] Reference: Pennica et al., 1996.

BDNF, brain-derived neurotrophic factor; *CNTF*, ciliary neurotrophic factor; *CT-1*, cardiotrophin; *GDNF*, glial-derived neurotrophic factor; *IL-6*, interleukin-6; *LIF*, leukemia inhibitory factor; *NGF*, nerve growth factor; *NT*, neurotrophin; *PDGF*, platelet-derived growth factor.

Neurotrophic factors are able to regulate axonal outgrowth and neuronal sprouting after nerve lesions. The diversity of protective actions renders the neurotrophic factors ideal candidates in the treatment of neuropathies. They may be successful as therapeutic agents, not only when the disease directly involves a lack of neurotrophic factors or their receptors but also in neuropathies of other underlying mechanisms (for review see Yuen and Mobley, 1996).

To investigate the extent of beneficial effects of neurotrophic factors, inducible rat and mouse models representing metabolic or diabetic neuropathies have been used (table 5) (Elias et al., 1995; Gao et al., 1995; Contreras et al., 1997). In addition, natural mouse mutants exist that display phenotypes resembling human motor neuronopathies. The progressive motor neuronopathy in *pmn* mice (Schmalbruch et al., 1991) is commonly accepted as a distant model for motor neurodegenerative diseases in humans like amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA). The gene locus of the *pmn* mutation lies on chromosome 13 and appears to be homologous to the human disease locus of SMA but not ALS (Jockusch et al., 1997). *pmn* mice show a progressive motor neuronopathy leading to paralysis of hindlimbs, which is reflected by signs of neurogenic atrophy of muscles and later involvement of forelimbs. Homozygous mice die within six to seven weeks, whereas the heterozygous animals are normal (Schmalbruch et al., 1991). The *wobbler* (*wr*) mouse presents with muscle atrophy in early postnatal development leading to general muscle weakness and wasting. Signs of de- and reinnervation are prominent. The *wr* mice are used as a model for lower motoneuron diseases (Hantaï et al., 1995). The autosomal-recessive inheritance pattern and the chromosomal location make the *wr* gene a potential homologue for a human muscular atrophy (AR-DMD) (Jockusch et al., 1997).

Another important issue in the development of treatment strategies are feasible delivery systems for therapeutic drugs. The administration of purified neurotrophic factors by intramuscular, subcutaneous or intravenous injections allowed statements on the beneficial effects of the agents regarding the disease course, but left several concerns without satisfying solutions: Problems encountered with systemic administration of neurotrophic factors included poor bioavailability due to short half life (CNTF) or specific binding proteins (IGF-I), toxic side effects (CNTF), and the mounting of immune responses against the foreign compounds (for review see Henderson, 1995;

Yuen and Mobley, 1996). An elegant solution to some of these problems has been found in the possibility of direct gene delivery to the target tissues via adenoviral gene transfer.

Several neurotrophic factors have a potentially beneficial effect on neurons affected in hereditary peripheral neuropathies

The therapeutic potential of several neurotrophic factors, like NT-3, IGF-I, CNTF or BDNF, has been assessed in animal models for human neurodegenerative disorders (see table 5) (for review see Yuen and Mobley, 1996). In contrast to hereditary peripheral neuropathies, these disorders often involve a loss of motoneurons. The survival-promoting neurotrophic factors are ideal compounds to counteract motoneuron death in motor neuronopathies. In myelin-related hereditary neuropathies, neuronal apoptosis has not been described and only lately increasing evidence supports the hypothesis that the axonal involvement is predominantly responsible for the more severe clinical symptoms of HMSN patients. Presuming their potent action on sprouting and regeneration of axons, neurotrophic factors promise to exert beneficial effects on the damaged and degenerating axons. In the following paragraphs, the most promising neurotrophic factors will be discussed with view to the present possibilities to test them in animal models for their therapeutic potential in HMSNs.

Table 5 Neurotrophic factors and their clinical potential.

Member	*In vitro and in vivo effects, neurotrophic action*	*Effects in animal models for neuropathies and in clinical trials.*
NT-3	NT-3 promotes the survival of neonatal and adult sensory (Maisonpierre et al., 1990) and motor (Hughes et al., 1993) neurons *in vitro*. NT-3 promotes differentiation of muscle spindle afferents (Oakley et al., 1997). NT-3 promotes profuse DRG neurite outgrowth (Maisonpierre et al., 1990) and induces branching *in vitro* (Lentz et al., 1999). NT-3 (-/-) show marked loss of proprioceptive neurons (70%), resulting in abnormal postures (Ernfors et al., 1994a; Farinas et al., 1994, 1996) and loss of mechanoreceptors in the skin (Airaksinen et al., 1996).	**Cisplatin-exposed rats**: NT-3 treatment reverts the induced demyelination and decreased NCV* of sensory neurons (Gao et al., 1995). **Diabetic rats**: NT-3 attenuates the reduction of myelinated nerve fibers (Elias et al., 1995). Adenoviral NT-3 gene transfer into ***pmn**** **mice** reduces axonal degeneration and enhances muscle (re-)innervation (Haase et al., 1997; Haase et al., 1998). **Phase I** clinical trial in **healthy** volunteers: doses similar to those used to produce beneficial effects in animals appear to be tolerated by humans. Phase II trials are planned (for review see Yuen and Mobley, 1996).
IGF-I	*In vitro*, IGF (II) induces neurite outgrowth of sympathetic and sensory neurons (Recio-Pinto et al., 1986); IGF-I enhances survival of embryonic spinal cord motoneurons (Neff et al., 1993). *In vivo*, IGF promotes survival of motoneurons during development and after axotomy (Lewis et al., 1993a; Pu et al., 1999a). Exogenous IGF induces sprouting of intact adult motor neurons (Caroni and Grandes, 1990) and fosters reinnervation of muscle after crush lesion (Contreras et al., 1993). IGF-I (-/-) mice have reduced sensory and motor NCV, and show a shift to smaller myelinated fibers (Gao et al., 1999).	IGF-I prevents **vincristine** neuropathy in mice (Contreras et al., 1997). IGFs protect against **diabetic** neuropathy in **rat** (Ishii and Lupien, 1995). Administration of IGF-I to **IGF-mutant** mice restores NCVs (Gao et al., 1999). In **ALS* patients** phase III trials: IGF-doses shown to be beneficial in mice are well tolerated by humans. A decrease of clinical progression was statistically significant, but biological benefits were modest. Serum of treated patients contains IGF-antibodies, but no neutralization of IGF-I activity has been shown (Lewis et al., 1993b; Ishii et al., 1994).

Member	*In vitro and in vivo effects, neurotrophic action*	*Effects in animal models for neuropathies and in clinical trials.*
CNTF	CNTF promotes survival of motoneurons *in vitro* (Arakawa et al., 1990). CNTF promotes survival of embryonic motoneurons (Oppenheim et al., 1991). CNTF promotes survival of motoneurons after axotomy (Sendtner et al., 1990). CNTF influences the differentiation of OL progenitor cells (O2A-progenitor cells) (Stöckli et al., 1991). CNTF protects OL against natural and TNF-induced death (Louis et al., 1993). Subcutaneous injection of CNTF in normal mice stimulates terminal sprouting (Gurney et al., 1992) and may enhance regeneration (Sahenk et al., 1994). CNTF (-/-) mice show extensive motor neuron loss after birth (Masu et al., 1993).	***wr**** **mice**: exogenous CNTF increases grip strength and running speed, improves gait pattern and reduces muscle atrophy (Mitsumoto et al., 1994a,b; Ikeda et al., 1995a). ***pmn* mice**: CNTF application slows axonal degeneration (Sagot et al., 1995) and prevents neuronal degeneration (Sendtner et al., 1992a). Adenoviral CNTF gene transfer and systemic availability of CNTF inhibits demyelination and axonal degeneration (Haase et al., 1999). Toxic side effects manifest as fever, extreme weight loss and induction of acute phase proteins in liver (Shapiro et al., 1993; Dittrich et al., 1994; Henderson et al., 1994). Clinical trials in **ALS** patients (Cedarbaum et al., 1995; Miller et al., 1996): in two trials there were no significant treatment benefits for patients, but predominant side effects - cough, asthenia, nausea, anorexia, weight loss, fever - resulted in a reduction of the dose. Circulating antibodies against CNTF were found (ALS CNTF Treatment Study (ACTS) Group, 1996).
BDNF	BDNF promotes survival of sensory neurons (for review see Lewin and Barde, 1996). BDNF promotes survival of motor neurons after axotomy (Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993) and in development (Oppenheim et al., 1992). Lack of BDNF (-/-) leads to extensive loss of cranial and DRG sensory neurons (Ernfors et al., 1994b; Jones et al., 1994).	***wr* mice**: subcutaneous injections improve grip strength, muscle twitch and increase the number of myelinated fibers in ventral roots (Mitsumoto et al., 1994b; Ikeda et al., 1995b) There is less denervation-induced muscle atrophy. In **ALS** phase I - II trials: patients tolerate BDNF doses shown to be effective in *wr* mice. No beneficial effects are reported except significant improvement of FVC* (Bradley and the BDNFTrialGroup, 1995).

* *ALS* = amyotrophic lateral sclerosis, *DRG* = dorsal root ganglion, *FVC* = forced vital capacity, *NCV* = nerve conduction velocity, *OL* = oligodendrocyte, *pmn* = progressive motor neuronopathy mouse mutant, *wr* = wobbler mouse mutant;

(Table adapted from Yuen and Mobley, 1996)

8.2.1 Neurotrophin-3

Neurotrophin-3 (NT-3) is a promising candidate for the treatment of HMSN patients

NT-3 is expressed during development and in adult tissue. The mRNA expression is highest in kidney and spleen and reaches intermediate levels in brain, muscle and skin (Maisonpierre et al., 1990). NT-3 is retrogradely transported in PNS and CNS neurons, which demonstrates that it is a target-derived neurotrophic factor (DiStefano et al., 1992). In addition, NT-3 is capable of improving the retrograde transport in axons of *pmn* mice (Sagot et al., 1998) which might also be an important issue in the treatment of HMSN patients.

Table 5 summarizes present knowledge on the neurotrophic actions of NT-3. Although the mechanisms responsible for the beneficial effects of NT-3 are presently unclear, an alleviation of pathological phenotypes has been shown repeatedly in different neuropathic models. The injection of a replication-deficient adenoviral vector into muscles of *pmn* mice resulted in an ameliorated motor performance and an increased lifespan of the treated animals (Haase et al., 1997; Haase et al., 1998). A histological analysis revealed that these benefits were caused by a reduced loss of myelinated fibers and an increase in intramuscular terminal nerve sprouting. Both effects would be highly desirable in the treatment of CMT-like disorders. In addition, NT-3 seems to be well tolerated by humans at concentration levels that were therapeutically effective in mice (Yuen and Mobley, 1996). The use of NT-3 alone, however, may not reach the most beneficial effect, as it has been shown that a simultaneous treatment of *pmn* mice with BDNF and CNTF adenoviral vectors synergistically increased the protective effects (Haase et al., 1997).

Approaches to test the therapeutic potential of NT-3 in HMSNs

At present, two very elegant ways are possible to study the therapeutic potential of NT-3 in mouse models for myelin-related neuropathies. NT-3 adenoviral vector may be injected into muscles of newborn P0-deficient mice. Transfected cells express NT-3 constitutively, because the gene is under the control of a viral promoter. The constitutive expression of NT-3 is of advantage for the assessment of the effects of long-term

application of NT-3. However, because NT-3-treated *pmn* mice die at the age of approximately two months, further investigations on the long-term expression of NT-3 in the transfected muscle cells are needed. In the P0-deficient mice, such long-term studies can easily be attempted because the mice have a near-normal lifespan. Disadvantages that need to be addressed are the mounting of an immune response against viral proteins in the host and the possibility that the localized expression of NT-3 in injected muscles may ask for multiple injections in different parts of the body. In mice, however, solutions to these problems are known: Injection of the adenoviral vector into the maturing thymus can induce immune tolerance toward the vector proteins. It has also been shown that NT-3 from muscle is systemically available in the treated mice leading to ameliorated muscle innervation not only in the injected muscles but in all muscles analyzed (Haase et al., 1997). The use of NT-3 adenoviral vector in P0-deficient mice, therefore, constitutes a straightforward experimental approach to address the question of the putative therapeutic potential of NT-3 in CMT-like neuropathies.

Alternatively, mice deficient in a myelin protein may be crossbred with transgenic mice expressing NT-3 under a muscle-specific promoter. Wright et al (1997) showed that in such mice high levels of NT-3 mRNA are present in muscles up to six weeks after birth, until the used myogenin promoter is downregulated. NT-3 overexpression in wild-type muscle results in a higher number of DRG neurons and an increase in muscle spindle density in some muscles (Wright et al., 1997). Crossbreeding of NT-3 transgenic with NT-3 knockout mice selectively rescued proprioceptive neurons innervating muscle spindles, whereas cutaneous sensory innervation could not be preserved (Wright et al., 1997). The great advantage of this approach would be the lack of immune reactions that might interfere with the trophic action of NT-3. In addition, NT-3 would be expressed very early which may be important to ameliorate congenital disorders of the CMT3-type. Although it would be possible in this approach to assess the principle potential of NT-3 to confer beneficial effects on hereditary myelinopathies, the short-time overexpression of NT-3 in muscle may not be sufficient to study long-term effects of NT-3 on affected demyelinated axons.

8.2.2 Insulin-like growth factor-I

Insulin-like growth factor-I (IGF-I) has pleiotropic effects on the neuromuscular system

Insulin-like growth factor-I (IGF-I) is expressed in a variety of tissues including fetal and adult brain, adult nerves, motor, sensory and autonomic neurons, and developing and adult muscle tissue. As a pleiotropic growth factor IGF-I acts on neurons, glial cells and a variety of other cells (Caroni and Grandes, 1990; Neff et al., 1993). Similar to other neurotrophic factors, IGF-I is able to induce intramuscular sprouting (Caroni and Grandes, 1990) and to foster reinnervation after nerve lesion (Contreras et al., 1993). In support of the indispensable role of IGF-I in muscle reinnervation, exogenous application of IGF-I binding proteins inhibits axonal regeneration (Caroni, 1993; Caroni et al., 1994). IGF-I and IGF-II are upregulated in muscle tissue after denervation, specifically at sites enriched in neuromuscular endplates (Pu et al., 1999b). IGF-I prevents Wallerian degeneration in neuropathic animal models when administered simultaneously with the toxic agent (see table 5).

In addition to the neurotrophic effects, IGF-I supports the survival of Schwann cells at different developmental stages. For instance, Schwann cells need IGFs to become independent from axonal contact *in vitro* (Gavrilovic et al., 1995; Meier et al., 1999; Syroid et al., 1999). A positive action on glial cells is also well documented in EAE (experimental autoimmune encephalomyelitis) animal models for multiple sclerosis, where IGF-I was shown to reduce lesions, foster remyelination and induce increased expression of myelin genes in oligodendrocytes (Liu et al., 1995; Yao et al., 1995). Administration of IGF-I in EAE rats also reduces the number of infiltrating immune cells (Liu et al., 1997; Li et al., 1998). Together, these findings support the assumption that IGF-I has a great therapeutic potential in CNS demyelinating disorders. It will be interesting to investigate whether IGF-I may produce similar effects in the PNS of animals suffering from a congenital impairment of myelination. Because IGF-I is able to exert beneficial effects on both, impaired glial cells and degenerating axons, this growth factor is thought to ameliorate the functional state of the neuromuscular system in general.

Experimental approaches to investigate the therapeutic potential of IGF in models for CMT-like disorders are limited to the systemic administration of the active compound, because alternative strategies like adenoviral gene transfer or transgenic animals are lacking at the moment. Although this delivery route has been proven effective without negative side effects in neuronopathies and lesion experiments, it may hold certain disadvantages for the use in peripheral myelin-related neuropathies. It has been hypothesized that in demyelinating neuropathies mostly the myelinated axons are prone to degenerate (R. Martini, personal communication). A possible induction of increased expression of myelin proteins in Schwann cells by systemically available IGF-I may, therefore, render the axons more susceptible to degeneration due to increased myelin production. Such a counteractive effect would neutralize a possible beneficial effect of IGF-I on the axonal regeneration. A restricted expression of IGF-I in muscle might be able to confine neurotrophic effects of IGF to intramuscular location.

8.2.3 Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) promotes survival of spinal motor neurons

CNTF is a 20 kDa protein initially purified from chick eye (Barbin et al., 1984) that is expressed postnatally in PNS and CNS glial cells. The regional distribution and developmental expression of CNTF exclude a role of this neurotrophic protein as a target-derived factor (Stöckli et al., 1991). Lacking a signaling sequence, CNTF is located in the cytosol of Schwann cells and astrocytes (Stöckli et al., 1989). Due to the late expression, CNTF is hypothesized to lack a physiological role in the development, but to be essential in the postnatal maintenance of responsive neurons. This view is supported by findings in CNTF knockout mice which loose approximately 25% of their spinal motor neurons during the first half year of life (Masu et al., 1993).

A number of neurotrophic effects of CNTF are shown in table 5. Because of its survival-promoting activity on motor neurons, the therapeutic potential of CNTF has been intensively studied in animal models for neuronopathies culminating in the clinical application of CNTF in the treatment of ALS patients (see table 5 for references). Beneficial effects of CNTF have been determined in *wr* and *pmn* mice, accepted models for axonal degeneration and motor neuronopathies. The analysis of motor tasks in

treated animals revealed increased grip strength and ameliorated gait patterns resulting from decreased muscle atrophy and increased maintenance of myelinated axons (Haase et al., 1997). The therapeutic effects of CNTF are increased when applied in synergy with other neurotrophic factors, like NT-3 or BDNF (Mitsumoto et al., 1994b; Haase et al., 1997). The potent ability of CNTF to reduce axonal degeneration is also of interest for the treatment of CMT diseases, whereas the potential to promote neuronal survival is of less importance, because neuronal degeneration appears to be minor or absent in myelin-related peripheral neuropathies. At least in aged P0-deficient mice, the number of facial motoneurons is normal (M. Sendtner, personal communication).

Poor bioavailability and toxic side effects complicate the use of CNTF as a therapeutic agent

The administration of CNTF by conventional delivery routes has revealed major disadvantages of this neurotrophic factor. Promising results regarding axonal preservation have been accompanied by serious side effects (see table 5). Further clinical use has been abandoned because of unacceptable negative complications (Cedarbaum et al., 1995; Miller et al., 1996; for review see Yuen and Mobley, 1996). This disadvantage is partly due to the inevitability to use rather high doses of CNTF to achieve therapeutic effects, despite the high potency of CNTF to promote neuronal survival *in vitro*. The bioavailability of CNTF is low because of rapid degradation and production of antibodies that together lead to a strong reduction of CNTF protein levels.

To circumvent the deleterious side effects, injections of a CNTF adenoviral vector aimed at a more localized expression of the protein. However, Haase et al. (1999) provided evidence that both, side effects and therapeutic benefits, appear to depend on elevated serum activities of CNTF (Haase et al., 1999). Therefore, other innovative delivery strategies are needed to keep CNTF in the repertoire of clinically usable therapeutic agents. To investigate the potentially beneficial effects of CNTF in hereditary demyelinating neuropathies, the possibility to localize the expression of CNTF specifically to Schwann cells might be essential. An expression of CNTF confined to Schwann cells might be able to circumvent the toxic side effects elicited by systemic presence of CNTF.

8.2.4 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) maintains muscle innervation

BDNF is expressed in embryonic and adult skeletal muscle and - as a target-derived neurotrophic factor - is retrogradely transported to the cell somata (DiStefano et al., 1992). As listed in table 5, BDNF exerts positive effects on both, sensory and motor neurons. However, BDNF-deficient mice display a loss of sensory neurons, whereas motor neurons develop normally (Ernfors et al., 1994b; Jones et al., 1994). Injection of BDNF into muscles of *wr* mice results in beneficial effects that include a reduction of denervation-induced muscle atrophy and an increase in myelinated nerve fibers (Mitsumoto et al., 1994b; Ikeda et al., 1995b). BDNF has similar neurotrophic effects as CNTF and antagonizes the dying-back of axons in *wr* mice. Application of BDNF in galactose-fed rats, a model for diabetic neuropathy presenting with reduced NCVs and splitting of myelin, resulted in the attenuation of these functional and structural disorders (Mizisin et al., 1997). However, beneficial effects of BDNF on myelin splitting and on NCVs in these animals were not consistently found in all nerves investigated. In addition, these findings suggest that BDNF might exert its effects via changing the metabolism of Schwann cells (Mizisin et al., 1997) which may be inefficient in CMT disorders, because of the known dominant-negative effect of some mutations leading to CMT. BDNF has been proposed as a putative therapeutic agent to treat traumatic injuries to spinal nerves and roots, because in rats BDNF promotes the survival of neurons that are axotomized closely to their somata (Novikov et al., 1997; Novikova et al., 1997). The therapeutic effects of BDNF in these lesions also include an enhancement of neuronal regeneration close to the CNS (Novikov et al., 1997). With view to the axonal impairment demonstrated in the P0-deficient mice, however, the enhancement of proximal axonal regeneration appears to be less important for the treatment of CMT-like disorders.

In summary, an increasing number of neurotrophic factors are analyzed for their therapeutic potential in human motor neuronopathies and in traumatic nerve lesions. Because of their regeneration-promoting capacities, particular factors appear to be well suited to exhibit beneficial effects also in inherited myelin-related peripheral

neuropathies. It will be important to test these putatively beneficial effects of neurotrophic factors rigorously in adequate animal models for myelinopathies. Our study, together with recent detailed analyses of other mouse mutants, provide an important basis for investigations in the clinical potential of neurotrophic factors. Considering its specific promotion of nerve regeneration and muscle reinnervation, NT-3 may indeed be the most promising candidate to be investigated for its therapeutic use in myelin-related neuropathies.

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11 Acknowledgments

- I would like to thank Prof. Dr. R. Martini for giving me the opportunity to thoroughly study morphological aspects of the nervous system. I greatly appreciated his continuous interest in the projects and his unconditional support in solving problems. He also gave me the opportunity to be part of the establishment of his new lab in Würzburg.
- I would also like to thank Prof. Dr. U. Suter, who accepted me as his external Ph.D.-student, for his encouragement and advice regarding the less scientific side of labwork and research. I am very grateful for his support and mentoring in all the situations when I felt uncertain about the presence and the future.
- My scientific work considerably profited from the help and advice of my colleagues. I therefore would like to thank:
  C. Schmid (Dr. in spe), for help with various tasks in the lab, including computer problems and molecular biology, and for interesting discussions on scientific problems;
  Dr. S. Carenini, for sharing his knowledge on cell cultures and on various morphological details;
  Dr. Med. C. Wessig and Dr. Med. M. Mäurer, for discussions on scientific and clinical aspects of neuropathies: I very much enjoyed our collaboration;
  Our technical assistants, K. Rohner and H. Blazyca, who always provided for a pleasant atmosphere in the lab.
- I especially would like to thank J. Dowling (by now possibly Dr. Med.), who spent a month in our lab. He introduced me to the fascinating fields of integrins and epithelial tissue and additionally became a very good friend in a hard time.
- My time in Würzburg would have been much harder without all my new friends outside the lab who encouraged me and made me keep in mind that there are other important things beside research work.
- Finally I would like to thank my family for keeping in touch and being ready to help when help was needed.