

# Open Research Online

---

The Open University's repository of research publications  
and other research outputs

## A transgenic mouse model of hereditary motor and sensory neuropathy

### Thesis

#### How to cite:

Dati, Gabriele (2009). A transgenic mouse model of hereditary motor and sensory neuropathy. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2009 The Author

Version: Version of Record

---

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

---

[oro.open.ac.uk](http://oro.open.ac.uk)

Gabriele Dati

A Transgenic mouse model of  
Hereditary Motor and Sensory  
Neuropathy

Doctor of Philosophy  
In  
Molecular and Cellular Biology

2009

## DECLARATION OF WORK

I declare that all the work for this thesis has been performed by myself, except for the semi thin sections of sciatic nerves (Fig. 2.4, page 84 and Fig. 2.9, page 98), for the electromicrographs of sciatic nerves (Fig. 2.5, page 85), all performed by Dr. A. Quattrini in the Neuropathology Unit at San Raffaele Hospital and for the P0 glycosylation assay (Fig2.11, page 102), performed by Dr. Pietro Fratta after I left Dr. Wrabetz 's lab.

In addition, some of the immunohistochemistry in Figure 2.10 has been published in: Wrabetz L, D'Antonio M, Pennuto M, Dati G, Tinelli E, Fratta P, Previtali S, Imperiale D, Zielasek J, Toyka K, Avila R, Kirschner DA, Messing A, Feltri ML and Quattrini A. Different intracellular pathomechanisms produce diverse *MPZ*-neuropathies in transgenic mice, *J. Neurosci.*, 26:2358-2368, 2006.

# **CONTENTS**

pages

<b>Contents</b>	<b>1</b>
<b>Abstract</b>	<b>4</b>
<b>Chapter 1: Introduction</b>	<b>6</b>
<b>1.1 The Nervous System</b>	<b>6</b>
<u>1.1.1 Nerve cells</u>	<u>6</u>
<u>1.1.2 Glial cells</u>	<u>7</u>
<b>1.2 The Peripheral Nervous System (PNS)</b>	<b>8</b>
<b>1.3 Myelin</b>	<b>10</b>
<b>1.4 Myelin Proteins</b>	<b>13</b>
<u>1.4.1 Proteolipid protein (PLP)</u>	<u>13</u>
<u>1.4.2 Myelin Basic Protein (MBP)</u>	<u>15</u>
<u>1.4.3 P2 protein</u>	<u>17</u>
<u>1.4.4 Peripheral myelin protein 22 (PMP22)</u>	<u>18</u>
<u>1.4.5 Myelin Associated Glycoprotein (MAG)</u>	<u>20</u>
<b>1.5 Myelin Protein Zero</b>	<b>21</b>
<u>1.5.1 P0 cDNA</u>	<u>23</u>
<u>1.5.2 Mpz gene</u>	<u>23</u>
<u>1.5.3 Mpz gene expression</u>	<u>25</u>
<u>1.5.4 Mpz gene expression regulation</u>	<u>26</u>
<u>1.5.5 P0 glycoprotein synthesis</u>	<u>27</u>
<u>1.5.6 P0 glycoprotein function</u>	<u>28</u>
<u>1.5.7 P0 cytoplasmic tail function</u>	<u>30</u>
<u>1.5.8 P0 prenatal function</u>	<u>33</u>
<b>1.6 PNS Development</b>	<b>34</b>
<u>1.6.1 Schwann Cell development</u>	<u>35</u>
<u>1.6.2 Neural crest – SC precursor transition</u>	<u>36</u>
<u>1.6.3 Precursor – immature SC transition</u>	<u>40</u>
<u>1.6.4 Immature to myelinating or non-myelinating SC transition</u>	<u>41</u>



<b>1.7</b>	<b>Transcriptional control during SC development</b>	<b>43</b>
<u>1.7.1</u>	<u>Oct-6/SCIP/Tst1</u>	45
<u>1.7.2</u>	<u>Krox-20</u>	46
<b>1.8</b>	<b>The Inherited Neuropathies</b>	<b>48</b>
<u>1.8.1</u>	<u>Charcot-Marie-Tooth disease (CMT)</u>	49
<u>1.8.1.1</u>	<u>CMT 1</u>	49
<u>1.8.1.2</u>	<u>CMT 2</u>	53
<u>1.8.1.3</u>	<u>CMT 4</u>	55
<u>1.8.2</u>	<u>Dejerine-Sottas Disease (DSD)</u>	57
<u>1.8.3</u>	<u>Congenital Hypomyelination Neuropathy (CHN)</u>	58
<u>1.8.4</u>	<u>Hereditary Neuropathy with Liability to Pressure Palsies (HNPP)</u>	58
<b>1.9</b>	<b>Animal models of CMT disease</b>	<b>59</b>
<u>1.9.1</u>	<u>PMP22 animal models</u>	60
<u>1.9.1.1</u>	<u>Trembler mouse</u>	60
<u>1.9.1.2</u>	<u>Trembler-J mouse</u>	63
<u>1.9.1.3</u>	<u>PMP22 transgenic mouse</u>	63
<u>1.9.1.4</u>	<u>PMP22 transgenic rat</u>	64
<u>1.9.1.5</u>	<u>Conditional PMP22 overexpressing mouse</u>	64
<u>1.9.1.6</u>	<u>Mice with decreased PMP22 gene dosage</u>	65
<u>1.9.2</u>	<u>P0 animal models</u>	65
<u>1.9.2.1</u>	<u>LoF Mouse models</u>	66
<u>1.9.2.2</u>	<u>GoF Mouse models</u>	68
<b>Chapter 2:</b>	<b>RESULTS</b>	<b>72</b>
<b>2.1</b>	<b>Patients carrying P0Q215X mutation</b>	<b>72</b>
<b>2.2</b>	<b>Generation of Mpz Q215X/+ mice</b>	<b>73</b>
<b>2.3</b>	<b>Expression and synthesis of Q215X MPZ</b>	<b>76</b>
<b>2.4</b>	<b>Effect of the LoxP site in intron 5 on MPZ expression</b>	<b>79</b>
<b>2.5</b>	<b>Q215X/+ mice develop transient Congenital Hypomyelination</b>	<b>81</b>
<b>2.6</b>	<b>Q215X/+ mice present a neuromuscular defect</b>	<b>88</b>
<b>2.7</b>	<b>Q215X MPZ is not trafficked correctly</b>	<b>90</b>

<b>Chapter 3: DISCUSSION</b>	<b>95</b>
<b>3.1 Generation of Mpz Q215X/+ mice, expression and synthesis of Q215X MPZ and effect of the LoxP site in intron 5 on MPZ expression</b>	<b>95</b>
<b>3.2 Q215X/+ mice develop transient Congenital Hypomyelination</b>	<b>101</b>
<b>3.3 Q215X/+ mice present a neuromuscular defect</b>	<b>105</b>
<b>3.4 Q215X MPZ is not trafficked correctly</b>	<b>106</b>
<b>3.5 Conclusions</b>	<b>107</b>
 <b>Chapter 4: Materials and Methods</b>	 <b>111</b>
<b>4.1 Production of Targeting Vector</b>	<b>111</b>
<b>4.2 Generation of recombinant embryonic stem (ES) cells</b>	<b>112</b>
<b>4.3 Generation of chimeric mice and germline transmission of the Mpz targeted allele</b>	<b>112</b>
<b>4.4 Western Blot Analysis</b>	<b>113</b>
<b>4.5 Semi-quantitative RT-PCR</b>	<b>114</b>
<b>4.6 Morphological Analysis</b>	<b>116</b>
<b>4.7 Behavioral Analysis</b>	<b>117</b>
<b>4.8 Immunohistochemistry</b>	<b>117</b>
<b>4.9 Image Analysis</b>	<b>118</b>
 <b>Reference list</b>	 <b>119</b>

## ABSTRACT

Congenital Hypomyelination (CH) is the most severe demyelinating form of Hereditary Motor and Sensory Neuropathies and manifests at birth in human. Some subtypes of CH are due to dominant mutations in the gene coding for P0 glycoprotein, which functions as a homophilic adhesion protein, responsible for compaction of opposing myelin lamellae. By homologous recombination in ES cells, we have generated a mouse containing a nonsense mutation in the intracellular portion of P0 (Q215X) that, in the heterozygous state, is associated with CH neuropathy in humans. This mutation is predicted to encode a truncated P0 protein, lacking part of the cytoplasmic domain. Expression analysis demonstrated that Q215X heterozygous mice produce both wildtype P0 and a smaller, truncated P0 glycoprotein; furthermore, the levels of mRNA and protein produced by the mutated allele are less abundant, relative to the wildtype. We demonstrated then that this reduction in mRNA and protein levels could be partially explained by inefficient transcription of the mutated allele, due to the presence of a LoxP site with intron 5. Behavioral analysis of the Q215X/+ mice revealed reduced motor performance at 11 days after birth. Morphological analysis performed on sciatic nerves of mice between P1 and P14 revealed defects in the process of axonal sorting by Schwann cells, with the presence of bundles of mixed large and small calibre axons surrounded, but not ensheathed, by single Schwann cells. These morphological defects are rescued after the second week of life: sciatic nerves from adult mice, in fact, show only mild hypomyelination, which is much less severe than the morphology reported in patients. From these preliminary data, we conclude that the Q215X mutation results in a truncated P0 protein; since the phenotype of Q215X/+ mice and P0 +/- mice differs, Q215X probably produces a gain of function. Finally, we studied *in vivo* the intracellular location of the truncated protein, in order to clarify aspects of the pathogenetic mechanism of the Q215X P0 mutation: we found that the mutated protein is not properly trafficked within Schwann cells, being partially retained in the ER compartment. The phenotype we have

observed in the mutant mice presents similarities to other hypomyelinating mice that carry mutations in different genes, that are all involved in the laminin pathway: this suggests that mutant P0 may interfere with laminin signaling, required for the correct timing of axonal sorting by Schwann cells. Thus, from these data we conclude that the Q215X mouse is a partial model of Congenital Hypomyelination, less severely affected, if compared to human patients. This difference in disease severity could be partially explained by the inefficient transcription of the Q215X allele. Thus, we believe that the mechanism of this hypomyelination is likely to be related to the mechanism of the more severe neuropathy in human. This mouse will be useful to reveal the pathogenesis of the mutation.

# 1. INTRODUCTION

## 1.1 The Nervous system

Neurons and glia, together with other supporting cells like blood vessels, meninges, immune cells and fibroblasts are components of the Nervous System

### ▪ 1.1.1 Nerve Cells

Within a neuron, four morphologically different regions can be distinguished: the cell body (or the perikaryon), the dendrites, the axon and the presynaptic terminal of the axon. What differentiates a nerve cell from other cell types is the ability to generate electrical signals. The metabolic center of the neuron, the *perikaryon*, usually gives rise to a series of extensions, the *dendrites*, that form the receptive apparatus of the neurons. The other characteristic neuronal structure, the *axon*, the conducting unit of the neuron, is a tubular process that can extend for a long distance in the body. The high speed conduction of the action potential is facilitated by the myelin sheath which is present in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS), except at the level of the Node of Ranvier. Close to its end the axon divides into many specialised extremities, called *presynaptic terminals*, which constitute the transmitting elements of the neuron. It is through them that a neuron contacts and transmits information to the receptive surfaces of any effector cells. The point of contact is called *synapse*. Three different components give rise to a synapse: the presynaptic terminal of a cell, the *receptive surface* of the other cell and the space between them, the *synaptic cleft*. The terminals of the presynaptic neuron sometimes contact the postsynaptic neuron directly on its cell body; more commonly they make contact with dendrites. In the case of the motor axons of the PNS, the receptive cell is a skeletal muscle cell and the synaptic unit is referred to as the neuromuscular junction. In each neuronal

cell information goes from the cell body and dendrites, to the region from where the impulse initiates and finally to the presynaptic site of the axon that communicates with other cells. Between nerve cells there is no cytoplasmic continuity and each cell has specific and precise connections only with some nerve cells and not with others.

On the basis of the number of processes that arise from the cell body, neurons are classified into three groups, unipolar, bipolar and multipolar. *Multipolar neurons* predominate in the vertebrate nervous system, they have one or more dendritic processes and a single axon. In a typical multipolar cell, dendrites emerge from all parts of the neuronal body. Even within the category of the multipolar neurons the size and the shape of different cells vary greatly. Different types of multipolar cells account for all of the distinguishable neuronal types. The morphological differences among multipolar cells are due largely to variations in the number and length of dendrites and length of the axon. The number and extent of dendritic processes in a given cell correlate with the number of synaptic contacts that other neurons make on that cell, while the length of the axon reflects the signalling function of a neuron.

#### ▪ 1.1.2 Glial Cells

Glial cells are found contacting nerve cell bodies and also contacting and sometimes ensheathing axons, where they serve different functions: they provide myelin, they support brain structure, they remove debris coming from neuronal death or injury, they drive the migration of neurons and guide the outgrowth of axons, they provide nourishment for nerve cells and finally they modulate synaptic transmission.

Glial cells are generally divided into *macroglia* and *microglia*. The macroglia include myelin forming cells: Oligodendrocytes (OL) in the CNS and Schwann cells (SC) in the PNS, astrocytes and ependymal cells. Microglia includes several phagocytic cells that can be mobilised after injuries, infections or other diseases.

The major function of OL and SC is to generate myelin respectively in the CNS and in the PNS, although in the PNS SC also surround small calibre axons without myelinating them, providing a support and protective function. They form this sheath by spirally wrapping their plasma membrane. Probably the major difference between these two cell types is that one OL can envelop several axons at the same time in the CNS and make myelin around them, on the contrary a single SC myelinates only one axon, with a diameter larger than 1 $\mu$ m (Friede and Samorajski, 1968).

## **1.2 The Peripheral Nervous System (PNS)**

The peripheral nervous system (PNS) includes the cranial nerves, the spinal nerves with their roots, the peripheral nerves and the peripheral components of the autonomic nervous system and the enteric nervous system. Spinal alpha-motoneurons, which extend axons into the PNS, have their soma located in the ventral horn of the spinal cord and their axons leave the CNS through the ventral roots. Primary sensory neurons are located in the dorsal root ganglia (DRG) of the PNS and project axons both centrally to the CNS and peripherally through the dorsal roots to their peripheral targets. The dorsal and ventral roots are attached to the spinal cord and the attachment site is considered as the border between the peripheral and the central nervous systems. Of note that, while axons penetrate the transition zone, the surrounding glia cells do not. As a consequence, Schwann cells (SCs), the glia cells of the PNS, are only associated with the parts of the axons that are located in the PNS. The outer connective tissue layer of the CNS, the dural sheath, becomes merged with the outer connective tissue layer of peripheral nerves, the epineurium, where the dorsal and ventral roots merge to form a spinal nerve. As the spinal nerves leave the spinal canal, they quickly divide into dorsal and ventral rami. The dorsal rami supply the back, while the ventral

rami send projections to the ventrolateral part of the body wall and the limbs. The ventral rami supplying the limbs form plexuses at the cervical and lumbosacral regions, from which the major peripheral nerves emerge to reach the limbs.

The epineurium, the perineurium and the endoneurium, three layers of compact connective tissue separate and protect single nerve fibers. The epineurium surrounds the fascicles of the peripheral nerve; it is constituted by fibroblasts lacking a basal lamina and by collagen type I and III. Of note that the epineurium is the only part of the PNS where a lymphatic capillary network is present and is connected to regional lymph nodes. Two other components are relevant for the epineurium histoarchitecture: first, the *vasa nervorum*, a network of arterioles and venules that provides blood support and that extends to the perineurium and the endoneurium; second, variable amounts of fat, working as pillows to protect the fascicles against damage by compression.

Six to eight layers of epithelial cells constitute the perineurium. All the layers consist of concentric flattened epithelial cells with a basal lamina connected to each other by tight junctions and gap junctions. Of these cell layers, both the inner and the outer produce an organized basal lamina, made of collagen type IV fibers, fibronectin and proteoglycan. Two main functions are accomplished by the perineurium: first, to allow a selective transport mechanism, able to restricts transfer of molecules into the endoneurium; second to protect nerve fibers from damage through an organized structure of collagen layers (Parmantier et al., 1999).

The endoneurium, the intrafascicular compartment of the nerve, is made of collagen fibrils running parallel along the nerves. It reaches 50% of the intrafascicular space, giving support and protection to the fascicles. Embedded in the endoneurium are axons ensheathed by SC. Endoneurial fibroblasts are the main producers of the collagen that is present in this structure.

Finally, within the peripheral nerves, SCs are the major cellular component of the intrafascicular space, together with a smaller number of endoneurial fibroblasts. Rows of SCs are arranged longitudinally along the axons, forming myelin sheaths around them. As a rule, large caliber axons



are surrounded by myelin, whereas small caliber axons are segregated by SCs, but not myelinated. Around the SCs, a carbohydrate rich basal lamina covers the surface.

### 1.3 Myelin

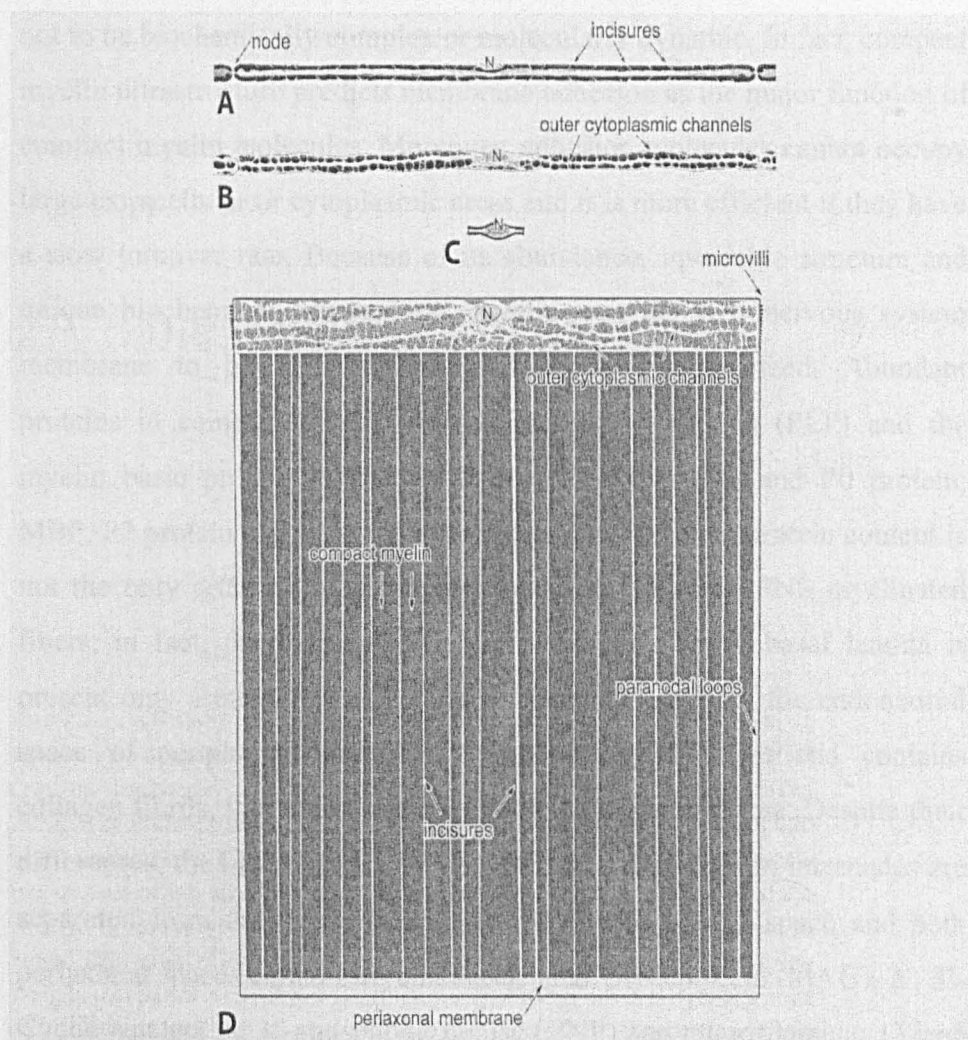
Myelin is one of the fundamental adaptations of vertebrates: it is a spiraled extension around an axon of, respectively, the Schwann cell plasma membrane in the PNS and the oligodendrocyte plasma membrane in the CNS. Myelin promotes rapid and repetitive communication between neurons and it modulates the maturation and survival of axons. Neurons communicate by depolarizing the electrical potential of their axons; this occurs through channel-mediated exchange of  $\text{Na}^+$  in an energy dependent manner. Rapid unmyelinated axonal communication would demand energy and space requirements that are not consistent with evolution of the complex mammalian brain. It was therefore advantageous to evolve a mechanism to propagate neuronal communication through thin axons in a more efficient manner. Myelin-forming cells serve this function by producing a series of discontinuous insulation units called internodes along single axons.

Each myelin internode can be divided into two structurally and functionally distinct domains: compact myelin and non-compact myelin (the paranodal and Schmidt-Lantermann regions). Compact myelin inhibits ion exchange during nerve conduction, while the paranodes, which demarcate the longitudinal ends of each internode, facilitate ion exchange at the Node of Ranvier, a part of the axon that separates each internode. The nerve impulse is transmitted from node to node by electrotonic spread and the action potentials regenerated at each Node of Ranvier; this process is commonly referred to as saltatory conduction.

In 1928, Ramon y Cayal provided the first description of a teased myelinated PNS axon (Fig 1.1). Compact myelin includes the majority of the internode. Non-compact myelin provides cytoplasmic continuity

between the myelin forming cell and its various regions of myelin internode. Since the majority of myelin components are synthesized in the cytoplasm of myelin-forming cells, cytoplasmic channels at the abaxonal surface (not in direct contact with the axon, but in contact with SC cytoplasm) are needed to form and maintain the myelin internode. These channels contain cytoskeletal components for transport and stability, mitochondria for energy and endoplasmic reticulum for local membrane biosynthesis. The cytoplasmic channels at the lateral ends of the internode (paranodal loops) are major sites of myelin-axon adhesion. The membranes of the adaxonal surface are in direct contact with the axons and their cytoplasmic channels may transmit axonal signals that regulate myelin formation, determining the length and thickness of the myelin internode. Finally, Schmidt-Lanterman incisures cross compact PNS myelin and connect outer and inner regions of the internode. These structures are more visible in the PNS than in the CNS, and constitute a continuous channel of cytoplasm, which extends through the sheath from the periaxonal process to the soma. The incisure membrane is characterized by the presence of tight junctions that separate paranodes and incisures from extracellular space, adherens junctions, that link together consecutive layers of the sheath, and gap junctions, that mediate diffusion across incisures by forming a radial pathway for ions and small molecules (Balice-Gordon et al., 1998).

By Transmission Electron Microscopy (TEM), compact myelin appears as a lamellar structure of alternating dark and light lines that spiral around the axon. During the process of myelin compaction, both in oligodendrocytes and Schwann cells, the cytoplasm is partially excluded. Cytoplasm membrane leaflets are closely apposed to form the major dense lines (MDL), while extracellular leaflets of adjacent lamellae become closely apposed to form the intraperiod lines (IPL), separated by 2.0 nm in the CNS and 2.5 nm in the PNS. The spiral membranes of compact myelin have a periodicity (distance from dense line to dense line) of approximately 13 to 14 nm when fixed with aldehydes and embedded in epoxy resins (Kirschner and Hollingshead, 1980) and is slightly greater in PNS myelin than in CNS myelin. *In situ*, however, the distances are larger



**Fig 1.1:** Myelin internodes in the peripheral nervous system (PNS). Drawings by Ramon Y Cajal of osmic acid fixed (A) and silver impregnated (B) myelinated fibers from the PNS. Compact myelin membranes surround the axon (A, in black) and comprise the majority of the myelin internode. To illustrate the membrane expansion that occurs during myelination, the size of a Schwann cell before myelination (C) is compared to a Schwann cell and its “unrolled” myelin internode (D). Cytoplasmic domains at the outer surface of the internode (B, D) are contiguous with the cytoplasmic channels that surrounds (paranodes and inner mesaxon) and traverse (Schmidt-Lanterman incisures) the compact myelin. (Adapted from “Myelin biology and disorders”, chapter 1, page 4).

(17 to 18 nm) when measured by X-ray diffraction (Kirschner and Sidman, 1976).

Since the major function of compact myelin is insulation, it needs not to be biochemically complex or molecularly dynamic. In fact, compact myelin ultrastructure predicts membrane adhesion as the major function of compact myelin molecules. Moreover, adhesion molecules cannot occupy large extracellular or cytoplasmic areas and it is more efficient if they have a slow turnover rate. Because of its abundance, invariable structure and unique biochemical composition, myelin was the first nervous system membrane to be isolated and molecularly characterized. Abundant proteins in compact myelin include proteolipid protein (PLP) and the myelin basic protein (MBP) in CNS compact myelin and P0 protein, MBP, P2 protein and PMP-22 in the PNS. The different protein content is not the only criterion to distinguish between CNS and PNS myelinated fibers; in fact, they have a different periodicity and a basal lamina is present only around PNS myelinated fibers. In addition, the endoneurial space of peripheral nerves is conspicuously abundant and contains collagen fibrils, that are not present in the CNS parenchyma. Despite their differences, the inner surface of both PNS and CNS myelin internodes are separated from the axon by a 12 to 14 nm periaxonal space and both periaxonal spaces contain Myelin Associated Glycoprotein (MAG), 2', 3'-Cyclic Nucleotide 3'-phosphodiesterase (CNP) and microfilaments (Trapp et al., 1988).

## 1.4 Myelin Proteins

### ▪ 1.4.1 Proteolipid protein (PLP)

PLP is the most abundant CNS myelin protein; it constitutes approximately 50% of the total. The gene gives rise to two alternatively spliced transcripts that encode the classical PLP protein (a hydrophobic

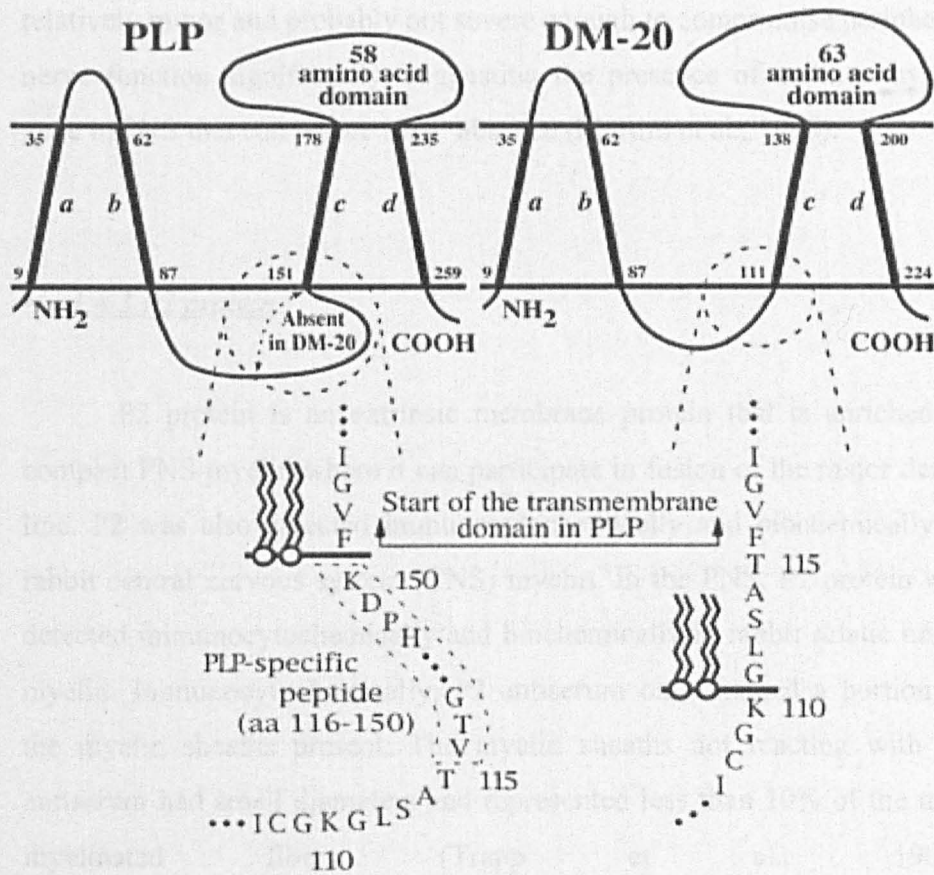
protein with four transmembrane domains and a MW of 26kD) and a smaller isoform, named DM20. The only difference between them is the presence of an additional 35 aminoacid residues in the intracellular region of PLP. The PLP/DM20 gene is expressed primarily by mature oligodendrocytes, although small amounts of the proteins have been detected in embryonic CNS, PNS, heart, spleen, thymus and lymphnodes (Bongarzone et al., 1999; Bronstein, 2000; Garbern et al., 1997). In humans, PLP duplication is a common cause of a dysmyelinating disorder, Pelizaeus-Merzbacher disease: affected patients present a range of phenotypes, whereby larger duplications or point mutations can result in more severe clinical manifestations (Inoue et al., 1999). Increase of the PLP gene dosage in nonmutant mice with only a 2-fold transcriptional overexpression results in a dysmyelinating phenotype characterized by severe hypomyelination and astrocytosis, seizures, and premature death. This demonstrates that precise control of the PLP gene is a critical determinant of terminal oligodendrocyte differentiation. Oligodendrocytes in the dysmyelinated CNS express a range of genes typical of mature cells, yet are unable to assemble sufficient myelin. Oligodendrocytes contain abnormal vacuoles and stain intensely for PLP and other proteins such as MAG. The findings suggest that with high gene dosage much of the PLP, and possibly other proteins, is missorted and degraded in the lysosomal system. Surprisingly, mutant mice that lack expression of a targeted PLP gene fail to exhibit the dysmyelinated phenotype (Boison and Stoffel, 1994). Oligodendrocytes are still competent to myelinate CNS axons of all calibers and to assemble compacted myelin sheaths. Ultrastructurally, however, the electron-dense 'intraparallel' lines in myelin remain condensed, correlating with its reduced physical stability. This suggests that after myelin compaction, PLP forms a stabilizing membrane junction, similar to a "zipper." Animal models of PMD, the *jimpy* and the *msd* spontaneous mutant mice, accumulate Plp gene products in the perinuclear region, are unable to transport them to the cell surface and undergo a two- to threefold increase in oligodendrocyte cell death (Gow et al., 1998). Recently, transgenic mice expressing only the DM-20 isoform were created. Although DM-20 is incorporated into functional compact myelin

sheaths in young animals, the analysis of these mice revealed that the 35 amino acid PLP-specific peptide is required to engender the normal myelin period and to confer long-term stability on this multilamellar membrane (Stecca et al., 2000). As already mentioned above, both PLP and DM20 are expressed in the PNS. The role of the two molecules within the PNS has not been clarified yet, but it is known that several PLP mutations (null- and point mutations) have been identified in different patients affected by peripheral neuropathies (Shy et al., 2003).

▪ 1.4.2 Myelin basic protein (MBP)

MBP accounts for approximately 35% of total protein in CNS myelin and 15% in PNS myelin. It has been found in all vertebrates that have myelin, both in the CNS and in the PNS. It is localized at the cytoplasmic surface of both central and peripheral compact myelin. It belongs to a family of alternatively spliced, highly positively charged extrinsic membrane proteins (Zeller et al., 1984), which bind negatively charged lipids. It is localized in the major dense line of myelin and in CNS it is required for normal myelin compaction. The MBP-deficient shiverer mice, natural mouse mutants carrying a deletion of the 3' end of the myelin basic protein gene, which completely prevents production of mature mRNA and protein, show a severe hypomyelination, a delamination in the CNS major dense line, tremors and tonic seizures (Molineaux et al., 1986). Although MBP is also expressed in the PNS, examination by electron microscopy showed that the peripheral nervous system in the shiverer mice, in contrast to the markedly abnormal central nervous system, is grossly normal. Myelin sheaths are of the usual thickness and exhibit normal periodic structure consisting of alternating major dense and intermediate lines. Subtle abnormalities do occur, however, consisting of increased numbers of Schmidt-Lanterman incisures, aberrant terminations of myelin lamellae in internodal regions, invagination of the axon by the inner tongue of the myelin sheath, myelin debris in both axon and Schwann cells, and disruption of outer myelin





**Fig 1.2** Topographical models of PLP and DM-20 in a membrane bilayer. Four transmembrane domains, *a-d*, are depicted for both proteins, in which numbers represent the amino acids in the vicinity of the membrane surfaces. In this model, the amino acids K150 of PLP and K110 of DM-20 define the beginning of the third transmembrane domain for each protein (Hartmann et al., 1989). Note that F151 in PLP is at the bilayer surface but that F116 in DM-20 is displaced toward the center of the membrane by the five amino acids, Gly-Leu-Ser-Ala-Thr. This displacement could increase the size of the second extracellular domain. (Adapted from Stecca et al., 2000).

lamellae (Rosenbluth, 1980; Gould et al., 1995). Despite the absence of myelin basic protein, the peripheral manifestations of this gene are relatively minor and probably not severe enough to compromise peripheral nerve function significantly, suggesting the presence of another myelin gene in PNS that can rescue MBP absence (Martini et al., 1995).

▪ 1.4.3 P2 protein

P2 protein is an extrinsic membrane protein that is enriched in compact PNS myelin where it can participate in fusion of the major dense line. P2 was also detected immunocytochemically and biochemically in rabbit central nervous system (CNS) myelin. In the PNS, P2 protein was detected immunocytochemically and biochemically in rabbit sciatic nerve myelin. Immunocytochemically, P2 antiserum only stained a portion of the myelin sheaths present. The myelin sheaths not reacting with P2 antiserum had small diameters and represented less than 10% of the total myelinated fibers (Trapp et al., 1983). The species differences in the expression of P2 are also confirmed by the differential potentials of the protein to induce an autoimmune demyelinating disease both in PNS and CNS. In fact, in inbred Lewis rats, P2 basic protein from bovine PNS myelin produced experimental allergic neuritis (EAN) without involvement of the brain or spinal cord. In guinea-pigs, bovine P2 did not produce EAN but large doses produced mild experimental allergic encephalomyelitis (EAE). In rabbits, bovine P2 produced both mild EAE and EAN. Finally, P2 protein shares significant homology with cellular retinoic acid binding protein (CRABP), and cellular retinol binding protein (CRBP), a family of proteins that have lipid binding activities and function in fatty acid transport (Uyemura et al., 1984).



▪ 1.4.4 Peripheral myelin protein 22 (PMP22)

It is a minor, but crucial component of the myelin sheath of peripheral nerves. It represents approximately 2-5% of total myelin in PNS. PMP22 belongs to a family of membrane proteins that is characterized by four hydrophobic domains and conserved amino acid motifs. The group includes epithelial membrane protein-1 (EMP-1), EMP-2, and EMP-3 (Jetten and Suter, 2000). PMP22 is widely expressed in neural and non-neural tissues, during embryonic life and in the adult (Baechner et al, 1995). PMP22 is most highly expressed by myelinating Schwann cells and its highest levels of expression were associated with late stages of myelination; however the presence of the protein in nonmyelinating SCs and in SCs commencing myelination supports multiple roles for PMP22 in peripheral nerve biology (Notterpek et al, 1999). Detailed immunohistochemical analyses have localized PMP22 to the plasma membrane of non-myelinating and myelinating Schwann cells as well as to the compact portion of myelin (Haney et al., 1996; Snipes et al., 1992). PMP22 is also found in the CNS, but at much lower levels than in peripheral nerves (Parmantier et al., 1995). Its transcripts have been found also during mouse CNS development (Parmantier et al., 1997). In the adult, PMP22 mRNA levels are approximately 10-fold higher in sciatic nerve, compared to the lung and intestine, and about 50 to 100-fold higher than in brain. Studies conducted in primary rat SC cultures showed that the PMP22 gene is regulated by two alternative promoters that are located immediately upstream of two alternative 5' noncoding exons (exons 1A and 1B). While both transcripts are coexpressed in tissues and cell lines, the transcripts containing exon 1A are preferentially expressed in myelinating Schwann cells, while transcripts containing exon 1B are preferentially expressed in tissues that do not form peripheral myelin (Suter et al., 1994). PMP22 encodes a hydrophobic integral membrane protein of 160 amino acids, with a predicted non-glycosylated molecular weight of approximately 18kD. Given the fact that the protein is not

purified yet, computational analysis of these hydrophobic domains suggested four potential transmembrane domains. These putative intracellular domains are small and unlikely to be involved in specific interactions with intracellular proteins. On the other hand, the extracellular loops of PMP22 may directly interact with other molecules, but so far no such extracellular interaction has been shown, although direct association of PMP22 with P0 in myelin membrane has been highlighted by co-immunoprecipitation experiments (D'Urso et al., 1999). Interestingly, PMP22 protein has been found to be glycosylated. Human and cat PMP22 carry the HNK-1 carbohydrate epitope, previously found on other cell surface glycoproteins, including P0 and many other proteins involved in cell-cell and cell-extracellular matrix adhesion (Hammer et al., 1993). While P0 is well characterized as a major adhesion molecule in PNS myelin (see chapter 1.5), the function of PMP22 is still unknown; it might be involved in regulating cell proliferation, death, differentiation and adhesive processes (Quarles 2002), although strong homophilic interactions have been excluded (Takeda et al., 2001). Most of what is known about the crucial function of PMP22 in proper development and maintenance of the nervous system has been learned from genetics, since PMP22 is the culprit gene in the most common form of hereditary motor and sensory neuropathies (HMSN) in human and rodents (Naef and Suter, 1998). This critical role of PMP22 in peripheral nerves became clear after the mouse PMP22 gene was mapped on chromosome 11, a chromosomal segment that is syntenic to human chromosome 17p11.2 (Suter et al, 1992a, 1992b). This human chromosomal region had been previously linked to the most common form of HMSN, called CMT1A (see chapter 1.8). Furthermore, the importance of PMP22 in PNS was also supported by the findings that the mouse mutants *Trembler* (*Tr*) and *Trembler-J* (*Tr-J*), natural mouse mutants recognized as potential animal models for severe forms of HMSN, Dejerine-Sottas Syndrome (DSS), carry point mutations in hydrophobic regions of the PMP22 protein (Suter et al., 1992a, 1992b, 1993). Finally, it has been recently showed, in a novel *pmp22* knock-out mouse line, generated by replacing the first two coding exons with the *lacZ* reporter, that PMP22 can bind the integrin/laminin

complex, mediating the interaction of SCs with the extracellular environment. These PMP22-deficient mice present peripheral nerves that display the characteristics of tomaculous neuropathy. In the absence of PMP22, myelination of peripheral nerves is delayed, and numerous axon–SC profiles show loose basal lamina. PMP22 and  $\beta 4$  integrin are coexpressed at the cell surface and can be coimmunoprecipitated together with laminin and  $\alpha 6$  integrin (Amici et al., 2006).

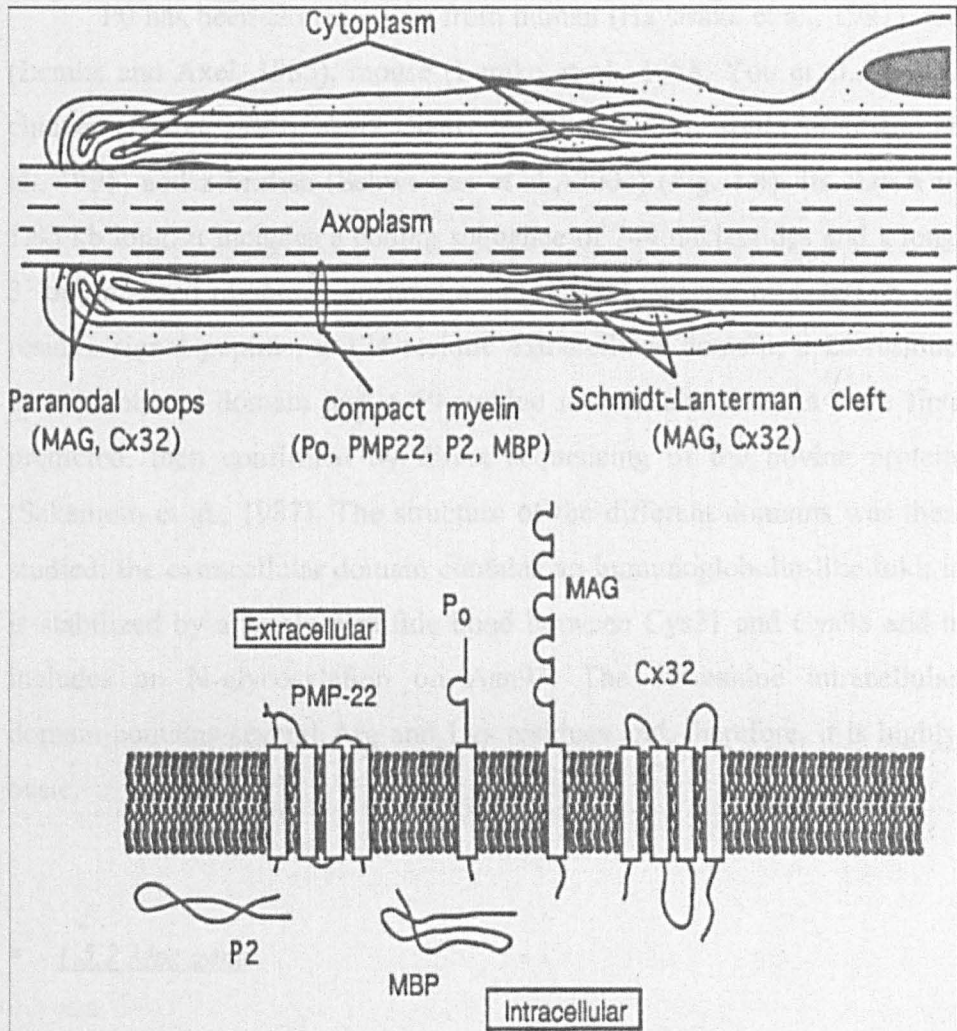
▪ 1.4.5 Myelin Associated Glycoprotein (MAG)

MAG is a minor myelin constituent, comprising approximately 1% and 0.1% of total myelin proteins in CNS and PNS respectively. It is a glycoprotein of the Ig-superfamily, with a high homology to N-CAM. Two distinct isoforms, large MAG (L-MAG) and small MAG (S-MAG), are produced through the alternative splicing of the primary MAG transcript and differ in their cytoplasmic domain. L-MAG is necessary for CNS myelin integrity, while S-MAG is sufficient to maintain PNS integrity (Fujita et al, 1998). To elucidate the role of MAG in the axon–Schwann cell interaction leading to myelination, neonatal rodent Schwann cells were infected in vitro with a recombinant retrovirus expressing MAG antisense RNA. A proportion of the Schwann cells infected with the MAG antisense virus did not myelinate axons and expressed lower levels of MAG than control myelinating Schwann cells, as measured by immunofluorescence. Electron microscopy revealed that the affected cells failed to segregate large axons and initiate a myelin spiral despite having formed a basal lamina, which normally triggers Schwann cell differentiation. Taken together these observations strongly suggested that MAG was the critical Schwann cell component induced by neuronal interaction that initiates peripheral myelination (Owens and Bunge, 1991). Conversely, experimentally increased levels of MAG in SCs accelerated initial ensheathment of dorsal root ganglia (DRG) neurites Owens et al., 1990). However, MAG-deficient mice have been produced; they appeared

normal in motor coordination and spatial learning tasks. Normal myelin structure and nerve conduction in the PNS was observed, with N-CAM overexpression at sites normally expressing MAG, indicating a possible compensatory effect. On the other hand, in the CNS the onset of myelination was delayed, and subtle morphological abnormalities were detected indicating that MAG participates in the formation of the periaxonal cytoplasmic collar of oligodendrocytes and in the recognition between oligodendrocyte processes and axons (Montag et al., 1994). The analysis of adult and aged MAG  $-/-$  mice revealed degenerative alterations of myelin in the PNS, with the formation of classical onion bulbs; the presence of such alterations suggest an *in vivo* role for MAG in the maintenance of peripheral myelin integrity. Mutant mice expressing only one of the two isoforms alternatively showed a differential role of the L-MAG isoform in CNS and PNS myelin.

### **1.5 Myelin Protein Zero**

Protein zero is the major protein in myelin of the peripheral nervous system (PNS), where it constitutes 50 to 60% of total protein content in peripheral myelin. It was discovered and characterized more than 30 years ago. The human protein is a member of the immunoglobulin gene superfamily; it is a ~30 kD integral membrane protein, constituted by a single disulfide-stabilized  $V_H$ -like domain, a single transmembrane domain, one glycosylation site and relatively few other post-translational modifications. It contributes to peripheral myelin structure across a wide phylogenetic range that includes mammals, reptiles, birds and amphibian; only in fish, it is abundant also in the CNS (Schweitzer et al., 2003).



**Fig. 1.3: Upper panel:** Schematic longitudinal section through a single myelinated axon showing the distribution of some of the peripheral nerve myelin proteins, MAG, Po, PMP22, P2, MBPs and Cx32 and their association with the major domains of compact and noncompact myelin. **Lower panel:** Schematic diagram of the putative topology and orientation of the major myelin proteins with respect to the Schwann cell plasmalemma (modified from Suter et al. 1993).

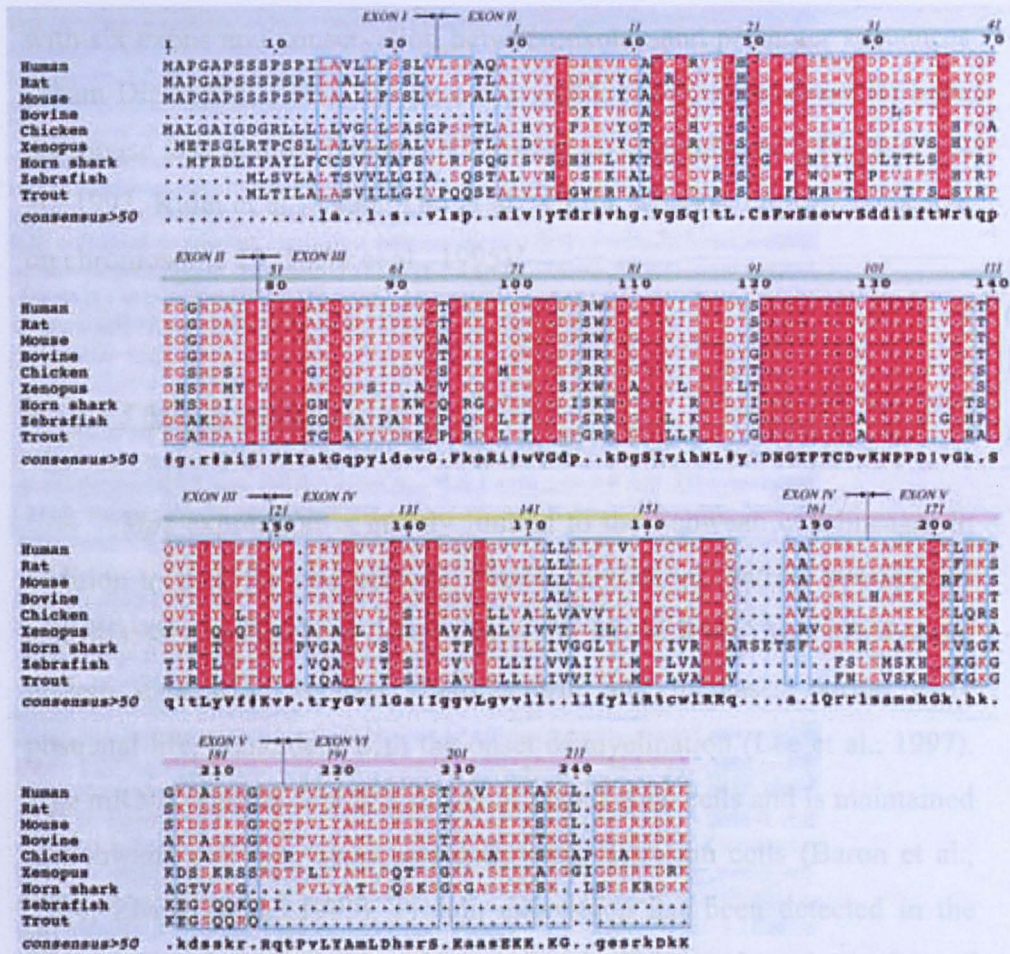
### ▪ 1.5.1 P0 cDNA

P0 has been cloned so far from human (Hayasaka et al., 1991), rat (Lemke and Axel, 1985), mouse (Lemke et al., 1988; You et al., 1991), chicken (Barbu, 1990), shark (Saavedra et al., 1989), trout (Stratmann et al., 1995) and zebrafish (Schweitzer et al., 2003) (Fig. 1.4). Its cDNA is 1.85 kb long: it includes a coding sequence of 744 nucleotides and a long 3' untranslated region. It encodes a single RNA species of 1.9kb. A 29-residue signal peptide, a 124-residue extracellular domain, a 26-residue transmembrane domain and a 69-residue intracellular domain were first predicted, then confirmed by direct sequencing of the bovine protein (Sakamoto et al., 1987). The structure of the different domains was then studied: the extracellular domain contains an immunoglobulin-like fold; it is stabilized by a single disulfide bond between Cys21 and Cys98 and it includes an N-glycosylation on Asn93. The 69-residue intracellular domain contains several Arg and Lys residues and, therefore, it is highly basic.

### ▪ 1.5.2 *Mpz* gene

The gene codifying for P0 glycoprotein was first isolated and characterized in rat and mouse. It is relatively small (7kb), consisting of 6 exons (Lemke et al., 1988; You et al., 1991). Careful analysis of gene and protein structure revealed that the separation of the different domains mirrors the exon segregation; in fact, exon 1 codifies for the 5' untranslated region of the mRNA and for the major part of the protein signal peptide (from residue -29 to residue -7); both exon 2 and 3 code for the remaining part of the signal peptide and for the extracellular domain (from residue -6 to residue 120); exon 4 includes the DNA region giving rise to the transmembrane domain (from residue 121 to residue 165) and, finally, exon 5 and 6 codify the P0 cytoplasmic domain and the 3' untranslated region (Lemke et al., 1988). Despite its difference in length with rodent *Mpz* gene, human *MPZ* preserves its genomic organization,





**Fig 1.4:** Structural homologies between P0 molecules of different vertebrate species. The deduced amino acid sequence of zebrafish P0 is aligned with trout IP1, as well as shark, human, rat, mouse, bovine, Xenopus and chicken P0. The sequence boundaries corresponding to exons I to VI of the human P0 gene are indicated. The extracellular, transmembrane and cytoplasmic domains are indicated by the horizontal bands of green, yellow and pink respectively. The multiple species alignment for the nine different species reveals the extent of homology: 100%, white letters on red field, >50%, red letters. (Adapted from Myelin Biology and Disorders, chapter 20, Kirschner, Wrabetz and Feltri, figure 20.1).

with six exons and conservation between exonic and promoter sequences (Pham-Dinh et al., 1993). The chromosomal location is conserved for both the mouse *Mpz* and the human *MPZ* (being on chromosome 1 Hayasaka et al., 1993; Kuhn et al., 1990; You et al., 1991), whereas rat *Mpz* is located on chromosome 13 (Liehr et al., 1995).

- 1.5.3 *Mpz* gene expression

*Mpz* expression is mainly limited to the Schwann cell lineage. In addition to that, the otic placode, vesicle, notochord, enteric neural crest and olfactory ensheathing cells express the *Mpz* mRNA. *Mpz* mRNA and protein expression is basal before birth and increases during early postnatal life, coincident with the onset of myelination (Lee et al., 1997). *Mpz* mRNA appears first in a subset of neural crest cells and is maintained in Schwann cell precursors and embryonic Schwann cells (Baron et al., 1994; Zhang et al., 1995). Protein expression has been detected in the neural crest of chicken (Bhattacharyya et al., 1991) and on the surface of freshly cultured cells from E14.5 rat nerve (Lee et al., 1997).

Postnatal expression of P0 mRNA increases and distinguishes the differentiation of non myelin-forming from myelin-forming Schwann cells: levels are undetectable in the former, whereas they are strongly induced in the latter. The fate choice between the two different cell types and the levels of *Mpz* expression are determined by their contact with axons. In fact, myelinating Schwann cells require contact with axons for both the induction and the maintenance of high-level *Mpz* expression (Lemke and Chao, 1988; Gupta et al., 1988; Trapp et al., 1988). The inductive axon signals include increased intracellular levels of cAMP and brain-derived neurotrophic factor (BDNF). On the other hand, various agents have been identified, that are able to repress *Mpz* expression in Schwann cells: SV40 T-antigen with c-Jun (Bharucha et al., 1994), c-jun on its own (Parkinson et al., 2004), serum, GGF, TGF betas, bFGF2 (Cheng and Mudge, 1996; Fernandez-Valle et al., 1993; Mews and Meyer, 1993; Morgan et al., 1991; 1994) and NT3 (Cosgaya et al., 2002). Some of



these may be responsible for maintenance of low levels of *Mpz* expression in premyelinating Schwann cells and during differentiation of non myelin-forming Schwann cells.

▪ 1.5.4 *Mpz* gene expression regulation

A quantitative analysis in the rat sciatic nerve of myelin protein gene expression during development (Stahl et al., 1990), revealed that *Mpz* expression is thought to be primarily transcriptionally regulated; in fact, *Mpz* mRNA appears immediately before P0 glycoprotein and is remarkably upregulated during myelination.

After the genomic structure of *Mpz* was revealed, a promoter region, without a canonical TATAA box was identified; a 1.1 kb region was in fact capable of driving expression of a reporter gene specifically only in transfected Schwann cells, and not in other cell types. Using the same 1.1 kb promoter region, activated expression of different proteins was specifically achieved in Schwann cells of transgenic mice (Messing et al., 1992). Nevertheless, levels of transgene expression were not consistent, indicating that the 1.1 kb of proximal *Mpz* promoter were sufficient to activate appropriate Schwann cell-specific expression, but not enough to achieve full amplitude of *Mpz* expression. Further studies conducted on cultured Schwann cells allowed the dissection of different *cis*-acting elements within the 1.1 kb *Mpz* promoter (Brown and Lemke 1997). Transcription factors like Sp1 and NF-Y are bound to different DNA sequences in the proximal 350 nucleotides, but the basis for cell-specific activation remains unclear. Moreover, several other transcription factors are upstream regulators of *Mpz* in developing nerve, including Oct6, Krox20 and Sox10. Oct6 strongly represses the *Mpz* promoter *in vitro* (Monuki et al., 1989), but functions as an activator *in vivo* (Bermingham et al., 1996; Jaegle et al., 1996). Krox20 is necessary *in vivo* for terminal differentiation of myelin-forming Schwann cells (Topilko et al., 1994), as *Krox20*-null mice produce none of the normal up-regulation of *Mpz* expression in post-natal nerve. Finally, the transcription factor Sox10,

which is required for formation of Schwann cells (Britsch et al., 2001), is expressed at all stages of Schwann cell life and is able to upregulate both endogenous *Mpz* expression and the *Mpz* promoter, when ectopically expressed in N2A neuroblastoma cell lines (Peirano et al., 2000). Whether this regulation is direct remains unclear for all three.

In order to produce levels of mRNA and protein expression of exogenous genes, comparable to endogenous *Mpz*, with a correct topographical and temporal pattern, insertions into exon 1 of an *Mpz*-based transgene, containing 6kb of 5' flanking region and 400 nucleotides of 3' region were generated (Feltri et al., 1999, Previtali et al., 2000; Wrabetz et al., 2000; Yin et al., 2000). Recently, a highly conserved element within the first intron of the *Mpz* gene has been identified, which contains binding sites for the early growth response 2 (Egr2/Krox20) transcription factor. Egr2 can act synergistically with Sox10 to activate this intron element and to induce *Mpz* expression to the high levels found in myelinating Schwann cells (LeBlanc et al., 2006).

#### ▪ 1.5.5 P0 glycoprotein synthesis

Like the proteins of other plasma membranes, P0 synthesis is localized to the endoplasmic reticulum, then processed through the Golgi network and transported intracellularly in membrane vesicles that finally fuse with the polarized plasma membranes of Schwann cells (Mellman and Warren, 2000; Eichberg et al, 2002). Microtubule disassembly experiments, associated with confocal microscopy and electron microscopic immunocytochemistry, showed that microtubules are necessary for specific myelin protein transport; in fact, following colchicine-mediated microtubule disassembly, P0, MAG and laminin accumulated in Schwann cell perinuclear cytoplasm (Trapp et al., 1995). However, the process through which P0 and MAG reach their final location within the SC plasma membrane seems to be independent of microtubules and most likely occurs by ligand receptor binding mechanisms, between carrier vesicles and target membrane. Like other

myelin proteins, P0 possesses targeting sequences that act in polarized cells; in fact, transfection of P0 glycoprotein in non-polarized HeLa cells produce surface membrane polarization, through P0 mediated homophilic adhesion. In addition, P0 glycoprotein has been stably transfected into other cell types, for instance, Madin-Darby canine kidney (MDCK) cells. MDCK cells are ideal for this purpose because they are an easily transfected cell line that polarizes into two membrane domains. In MDCK transfected cells, P0 is targeted to the basolateral surface. When the same cells are transfected with a deleted form of P0, lacking its cytoplasmic domain, the intracellular localization of P0 changes to the apical surface (Kidd et al., 2006).

Post-translational modifications of P0 occur both in the extracellular and intracellular domain; the extracellular region undergoes disulfide bond formation between Cys21 and Cys98 (Shapiro et al., 1996), glycosylation of Asn93 with a complex carbohydrate (Gallego et al., 2001; Poduslo, 1990) and sulfation of the N-acetylglucosamine residues of the carbohydrate. In the cytoplasmic domain, the post-translational modifications include acylation of Cys153 with palmitic acid (Bizzozero et al., 1994) and phosphorylation of Tyr191, Ser181 and Ser204 (Eichberg et al., 2002; Hilmi et al., 1995; Iyer et al., 1996). Of particular interest for the topic of this thesis is the fact that in physiological conditions, cleavage of P0 can occur at the level of its cytoplasmic domain, generating a truncated glycoprotein of approximately 25kD (Agrawal et al., 1990): this shorter protein has been isolated and characterized in bovine; it is truncated at the level of residue Q215 (numbering of residues comprehensive of 29-residue signal peptide) (Qualtieri et al., 2006).

#### ▪ 1.5.6 P0 glycoprotein function

The hypothesis that P0 glycoprotein was a transmembrane protein responsible for interactions at both the cytoplasmic and extracellular appositions in PNS myelin originated from application of x-ray diffraction techniques to study myelin membrane packing in sciatic nerves from

normal mice and the shiverer mouse mutant (Kirschner & Ganser, 1980). The shiverer mouse has an extensive deletion in the gene encoding MBP; as a result, the mutant is not able to express functional MBP mRNA or protein. In the CNS, this mutation results in a severe hypomyelinated phenotype, whereas in the PNS the myelin appears normal. This finding, together with the fact that P0 is the most abundant protein in PNS myelin, supported the conclusion that P0 protein specifically accounts for spacing and adhesion at both Schwann cell membrane surfaces. Several different functional studies, conducted in vitro on various transfected cell lines or myelinating cultures, and in vivo on transgenic mice supported this role of P0 in promoting peripheral myelin membrane adhesion and compaction through homophilic interactions. First of all, cells transfected with P0 acquire the ability to adhere to each other, while anti-P0 antibodies inhibit the adhesion (D'Urso et al., 1990; Filbin et al., 1990; Schneider-Schaulies et al., 1990). Then, the cytoplasmic domain of P0 is required for extracellular adhesion to occur: its truncation (at the level of the last 52 or 59 residues) inhibits adhesion of full-length P0 by a dominant negative mechanism (Wong and Filbin, 1994, 1996). Finally, the use of transfected cell lines helped also in identifying the possible role of post-translational modifications for P0 adhesive function. In fact, glycosylation at Asn93 with complex carbohydrate (Filbin and Tennekoon, 1991, 1993), disulfide bond formation (Zhang and Filbin, 1994) and possibly acylation (Gao et al., 2000) all apparently modulate P0 adhesive function. Moreover, antibodies against the HNK1 carbohydrate chain, expressed by P0 at its single N-glycosylation site, partially inhibit P0 homophilic interaction (Griffith et al., 1992). In vitro and in vivo myelination experiments further confirm the role of P0 in adhesive homophilic interaction and formation of the intraperiod line: first, Schwann cells infected with a retrovirus coding for P0 antisense RNA show diminished levels of P0 protein and are unable to myelinate, or form myelin with uncompacted lamellae when co-cultured with DRG neurons (Owens and Boyd, 1991). Second, mice lacking P0 glycoprotein (P0<sup>-/-</sup> mice) are able to form a multilamellar spiral structure around larger axons. This structure is uncompacted and therefore results in reduced nerve conduction velocities. In addition, in P0<sup>-/-</sup> mice intraperiod

lines are absent. Finally, some myelin sheaths show compaction, particularly at the major dense line, suggesting that other molecules can compensate for the loss of P0 (Giese et al., 1992). The mechanism through which P0-mediated homophilic interaction occurs was clarified by the X-ray resolution of P0 extracellular domain structure: in the myelin sheath, P0 forms tetramers in the plane of the membrane; these tetramers can interact with other tetramers on the opposing cell membrane. The tetrameric assembly of P0 proteins is supported by analytical ultracentrifugation data showing that oligomerization of the extracellular domains of the rat recombinant protein is energetically favorable in solution (Shapiro et al., 1996, Inouye et al., 1999 – Fig 1.5).

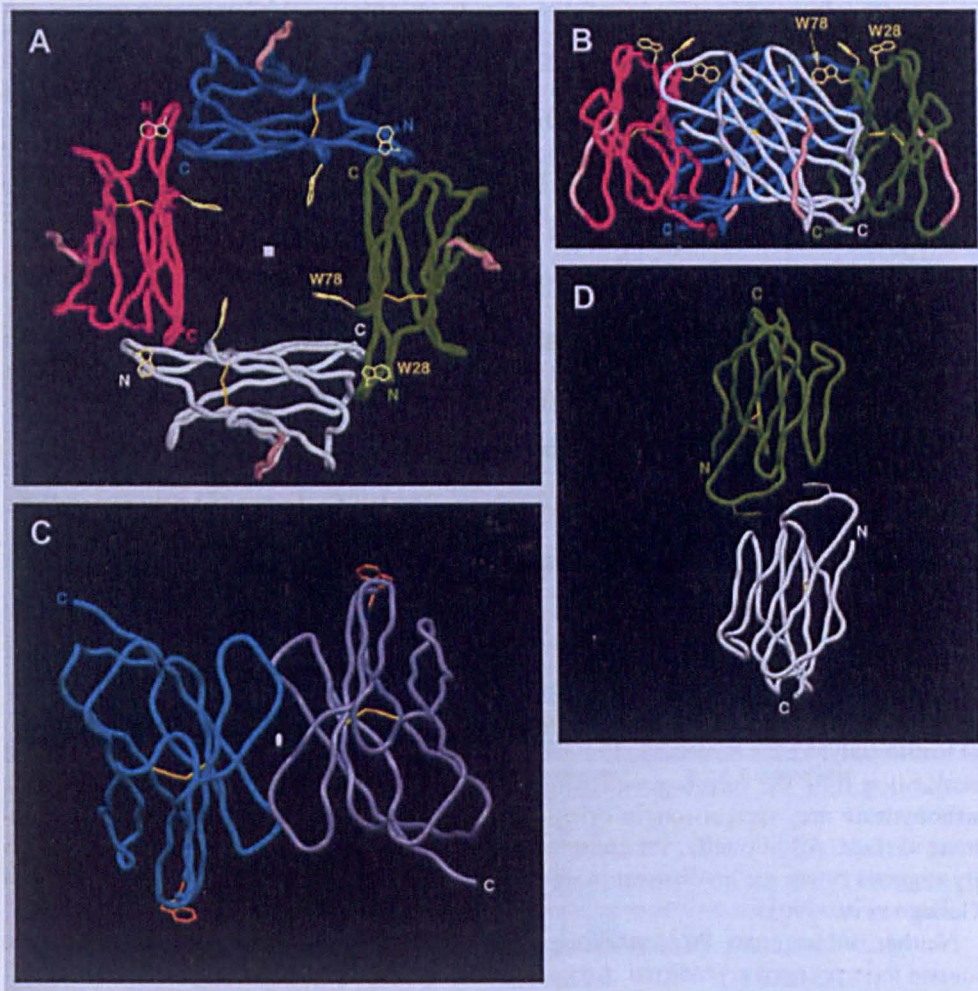
- 1.5.7 P0 cytoplasmic tail function

Electron microscopy and membrane diffraction studies helped to clarify the cytoplasmic apposition between Schwann cell plasma membranes in compact myelin. It is in fact commonly thought that the formation of the so-called Major Dense Line, is due to electrostatic interactions between the basic cytoplasmic domain of P0 and the acidic phospholipids present in SC plasma membrane; this hypothesis is supported by the fact that a peptide containing 65 of the 69 residues of the intracellular domain of P0 is able to bind to and to aggregate artificial phospholipid vesicles (Ding and Brunden, 1994). However, this hypothesis is strongly questioned by the evidence that even if pH and ionic strength vary, the Major Dense Line remains unchanged. As a consequence, it has been proposed that the cytoplasmic apposition is likely to result from lipid anchoring and hydrophobic interactions between P0 intracellular domains (Inouye et al., 1999). Furthermore, evidence from P0 and MBP knock-out animals seems to indicate that P0 is not the only molecule responsible for Major Dense Line compaction: in fact, in mice lacking both molecules axons are enwrapped by myelin-like processes devoid of the major dense line, while mice deficient in either protein showed partial and normal compaction (Martini et al., 1995).

In addition to that, P0, like other adhesion receptors, could also have a signal transduction role; this hypothesis is supported by the finding of phosphorylation of serine and tyrosine residues in the cytoplasmic domain (Brunden and Poduslo, 1987; Hilmi et al., 1995; Iyer et al., 1996). Other evidences for this role of P0 glycoprotein comes from transfection experiments in HeLa cells, which indicate that P0 can control aspects of Schwann cell differentiation, such as polarization and gene expression: in fact, HeLa cells expressing P0 ectopically can augment the adhesion program in the cells, by the placement of junctional proteins at cell-cell contacts, promoting the assembly of desmosomes and adherens junctions and suppressing the transformed phenotype to an epithelioid phenotype; this response is paralleled by an increase in expression levels for proteins that are normally associated with epithelial junctions (i.e. N-cadherin,  $\alpha$ -catenin, and vinculin; Doyle et al., 1995). Furthermore, when P0 is introduced in HeLa cells, the carcinoma cell line regains adhesion-mediated growth control, together with the acquisition of contact inhibition and loss of anchorage-independent growth. Finally, P0-expressing HeLa cells lose their tumorigenic and metastatic potency when injected into nude mice. Other findings supporting the potential signal transduction role for P0 arise from in vivo studies conducted on engineered mice: in P0 null mice myelin gene expression is altered and MAG, E-cadherin and beta-catenin are mislocalized.

Given the fact that P0 could signal to regulate Schwann cell polarization, junction formation and gene expression during myelination, recent disease-related evidence further suggests that the cytoplasmic domain of P0 may signal to the extracellular domain, regulating its own adhesiveness. Serine residues in the cytoplasmic domain of P0 are phosphorylated by Protein Kinase C (PKC). When the PKC target motif (RSTK motif) or an adjacent serine residue is mutated, P0 adhesive function is abolished and peripheral neuropathy can develop in humans.





**Fig. 1.5:** Molecular structure of P0 extracellular domain and its tetrameric arrangement. The molecule is represented by its backbone and the disulfide bonds are indicated in yellow. **A)** The tetramer (formed of 4 P0 molecules) is viewed looking down onto the membrane surface. **B)** The tetramer is viewed perpendicular to that in **A)**, parallel to the membrane surface. **C)** Lateral view, parallel to the membrane surfaces, of two P0 glycoproteins forming the adhesive interface. **D)** View of the head-to-head interface, early event in myelin formation (Adapted from Shapiro et al 1996).

Consistent with these data, PKC alpha together with the PKC binding protein RACK-1 and p65 adaptor protein are immuno-precipitated with P0; moreover, inhibition of PKC activities abolishes P0-mediated adhesion in an in vitro system (Xu et al., 2001, Gaboreanu et al., 2007). Finally, the role of tyrosine phosphorylation remains to be determined. Tyrosine phosphorylation occurs mainly at residue Tyr191 (Iyer et al., 2000; Xu et al., 2000); however, mutations at this site do not influence P0 adhesiveness (Xu et al., 2001).

In summary, the cytoplasmic tail of P0 has several hypothetical functions in trafficking, Major Dense Line compaction, extracellular domain adhesiveness and intracellular signaling. All these different functions may have important implication for the pathogenesis of CMT1b neuropathy (see chapter 1.8).

#### ▪ 1.5.8 P0 prenatal function

As already discussed in this chapter, P0 mRNA and protein are expressed at low levels expression during pre-natal life in a subpopulation of neural crest cells and in Schwann cell precursors (Baron, 1994; Bhattacharyya, 1991; Lee, 1997); the presence of P0 mRNA is also found in the otic placode, enteric nervous system and olfactory ensheathing cells (Lee et al., 2001). Such a widespread prenatal expression indicates possible additional functions of P0, outside the myelin sheath. One possibility is that P0 mediates heterophilic adhesion to neurites: in fact, cells expressing ectopic P0 are able to promote neurite outgrowth in dorsal root ganglion neurons with neurites of a mean length of about 150 microns. (Schyneider-Schaulies, 1990). In addition, several observations predict a possible role for P0 in the maintenance of axons. First, both heterozygous and homozygous P0 null mice develop axonal degeneration (Frei et al., 1999; Giese et al., 1992; Martini et al., 1995/2). Second, several P0 mutations in patients cause the axonal form of Charcot-Marie-



Tooth, with minimal myelin involvement, pupillary signs and deafness (De Jonghe et al., 1999; Marrosu et al., 1998).

## **1.6 PNS Development**

Different components of the PNS originate from different germ layers. The neuroectoderm gives rise to neurons and glial cells, while the nerve sheath and nerve vasculature derive from the mesoderm. The cells that differentiate into neurons and glial cells in the trunk region are the neural crest cells. During embryonic development, neural crest cells are formed at the border between the neural plate and the presumptive epidermis, overlaying the lateral plate mesoderm in a gastrulating embryo.

Two processes determine neural crest cell development: delamination and fate determination.

The process of neural crest delamination is important for differentiation and migration of these cells towards target organs. As a first step, the cells must switch from an epithelial to a mesenchymal morphology. The zinc-finger protein *Slug* is considered a good marker for neural crest cell induction and subsequent delamination in *Xenopus*, chicken and mouse embryos, even if its involvement in neural crest induction and delamination remain unclear; in fact its deletion does not cause any failure in this process (Jiang et al., 1998). Delamination of neural crest cells starts at the rostral end of the embryo in *Slug* positive migrating cells. It was also demonstrated that ectopic expression of *Noggin*, a BMP-4 inhibitor, can prevent migration and positively regulate the epithelial-mesenchymal transition (Sela-Donenfeld et al., 1999).

Neural crest, originating from rostral to caudal levels, can generate distinct but also overlapping sets of derivatives (Baker et al., 1997). In order to demonstrate this hypothesis, a series of homotypic graft experiments were performed; these experiments helped to demonstrate for example that only a small population of trunk neural crest generate chromaffin cells of the adrenal, while SC derive from neural crest cells of

the entire antero-posterior axis (Le Douarin et al., 1993). Furthermore, several tracing experiments were performed to understand if the fate decision of neural crest cells is due to an instructive action of the environment on a homogeneous population of cells or to a selective action on a collection of committed cells. The results of these studies show that while most pre-migratory cells, such as sensory neurons and glia, are multipotent, there are also precursors generating a single unique neural crest derivative (Frank and Sanes, 1991). Rat and mouse neural crest cells were grown at clonal density, in order to understand their entire repertoire: the results indicate that many cells, before leaving the neural crest, are multipotent self-renewing stem-like cells (Ito et al., 1993). Using these clonal cell culture techniques, several factors that are potentially involved in the lineage determination of the neural crest were identified. One of these factors is GGF-2. This is the product of the neuregulin gene and it can induce differentiation of neural crest cells into SCs (Shah et al., 1994). On the contrary, BMP-4 or BMP-2 promote neuronal differentiation, while smooth muscle cells are induced by TGF $\beta$  (Shah et al., 1996).

▪ 1.6.1 Schwann Cell development

As already introduced in the previous paragraph, during development, SCs originate from a multipotent migratory cell population that derives from the neural crest.

The process of SC development involves three transitions:

- 1- from neural crest cells to precursor SCs;
- 2- from precursor to immature SCs;
- 3- from immature to the myelinating and the non myelinating SCs (Fig 1.6).

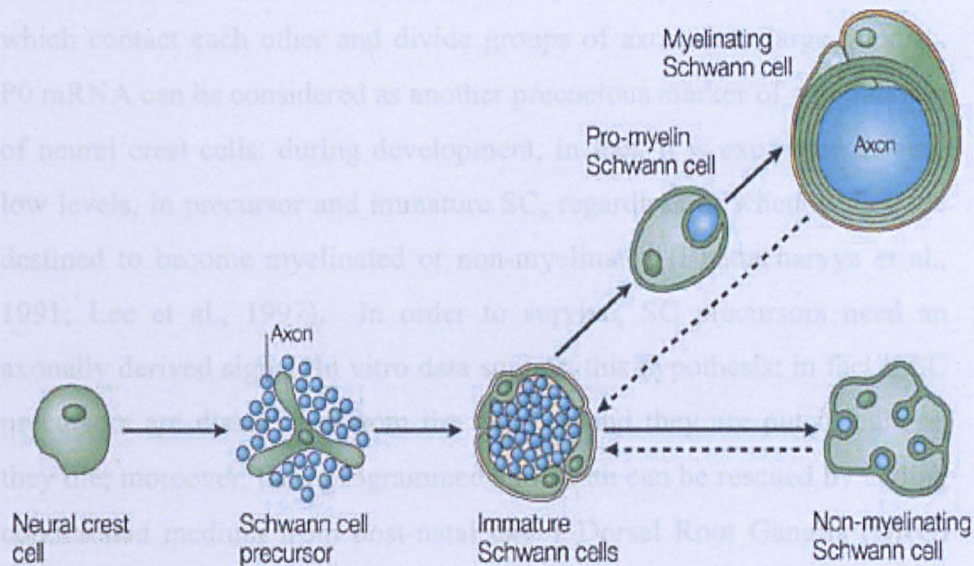
Only the first and the last of this transition points involve a fate choice decision. The regulation of gliogenesis from the neural crest is not clear yet; some future glial cells already enter the glial lineage at the onset of crest migration, while other cells start glial development later. The signals important for inducing glial development from both early and late entry

crest cells still need to be clarified. In vitro and in vivo experiments identified two growth factor signals that are involved in regulating early SC development in embryonic nerves:  $\beta$  neuregulin 1 and endothelin (Brennan et al., 2000; Garratt et al., 2000). Very likely these factors have a role in the establishment of the PNS glial lineage. Neuregulin in fact strongly suppresses neuronal differentiation of rat neural crest stem cells while promoting or allowing glial differentiation. (Shah et al., 1994). In addition,  $\beta$  neuregulin 1 is required for some aspects of neural crest migration: in fact the blockade of the neuregulin signalling pathway results in inefficient development of the sympathetic ganglia, due probably to the failure of sympathogenic neural crest cells to migrate to the appropriate site (Garratt et al., 2000).

One key regulator of PNS glial differentiation is the transcription factor Sox-10, which is initially expressed in the earliest migrating neural crest cells. Interestingly, mice that carry a spontaneous or a targeted mutation of Sox10 show neuronal cells in dorsal root ganglia, but no Schwann cells or satellite cells (Britsch et al., 2001). Therefore, it is very likely Sox 10 has a major function in undifferentiated crest cells, to drive their choice between neuronal and glial development. In this light, it is interesting to know that ErbB3 gene expression in neural crest cells is under Sox10 control. ErbB3 gene product is a neuregulin receptor: in fact, as observed in Sox10 mutant mice, the down-regulation of ErbB3 receptor is one of the causes of several changes in development of neural crest cells.

#### ▪ 1.6.2 Neural crest – SC precursor transition

As already mentioned above, one of the major problems in defining the development of SC from neural crest cells is the absence of early differentiation markers.



Copyright © 2005 Nature Publishing Group  
 Nature Reviews | Neuroscience

**Fig. 1.6:** Schematic illustration of the main cell types and developmental transitions involved in Schwann cell development. Dashed arrows indicate the reversibility of the final, largely postnatal transition during which mature myelinating and non-myelinating cells are generated. The embryonic phase of Schwann cell development involves three transient cell populations. First, migrating neural crest cells. Second, Schwann cell precursors (SCPs). These cells express various differentiation markers that are not found in migrating neural crest cells, including brain fatty acid-binding protein (BFABP), protein zero (P0) and desert hedgehog (DHH). At any one time, a rapidly developing population of cells — such as the glia of embryonic nerves — will contain some cells that are rather more advanced than others. Third, immature Schwann cells. All immature Schwann cells are considered to have the same developmental potential, and their fate is determined by the axons with which they associate. Myelination occurs only in Schwann cells that by chance envelop large diameter axons — Schwann cells that ensheath small diameter axons progress to become mature non-myelinating cells. (Adapted from Jessen and Mirsky, 2005)

In rat sciatic nerve at E14 precursor SC present sheath like processes, which contact each other and divide groups of axons into large bundles. P0 mRNA can be considered as another precocious marker of a population of neural crest cells: during development, in fact, it is expressed at very low levels, in precursor and immature SC, regardless of whether they are destined to become myelinated or non-myelinated (Bhattacharyya et al., 1991; Lee et al., 1997). In order to survive, SC precursors need an axonally derived signal. In vitro data support this hypothesis: in fact if SC precursors are dissociated from the neurons and they are put in culture, they die; moreover, their programmed cell death can be rescued by adding conditioned medium from post-natal day 1 Dorsal Root Ganglia (DRG) neurons to the cultures, or putting SC precursors in close proximity to neurites of DRG neurons or finally exposing the cultures to axonal membranes isolated from cultured DRG neurons (Jessen et al., 1994; Dong et al., 1995). These in vitro findings were confirmed in vivo in chick embryos, where programmed cell death of Schwann cells occurs both during normal development and after axonal degeneration induced by neurotoxin treatment. Interestingly, in those embryos Schwann cell apoptosis during development coincides with normally occurring motoneuron death. All together these in vitro and in vivo data indicate that axonal-derived trophic signals are involved in the regulation of Schwann cell survival in peripheral nerves during development. This axonal signal is  $\beta$  Neuregulin 1(NRG) (Mirsky and Jessen, 1999). Four different genes code for neuregulins: *NRG-1*, *NRG-2*, *NRG-3* and *NRG-4*. Little is known of the role of *NRG-2*, *NRG-3* and *NRG-4*, whereas *NRG-1* has been shown to be fundamental for SC and OL survival and differentiation. Neuregulins are a family of growth factors, characterised by the combination of different domains: an Epidermal Growth Factor (EGF)-like motif, a signal peptide, an immunoglobulin (Ig)-like domain, a cysteine-containing N-terminal domain, a glycosylation domain and a transmembrane domain. All NRG-1 isoforms can be proteolytically cleaved and released from the cell surface.

In E14 rats, the beta forms of NRG prevent apoptosis of Schwann cell precursor and stimulate DNA synthesis. When precursors are exposed to NRG in defined medium, they generate Schwann cells, with a time course that is similar to that occurring in embryonic nerves *in vivo*; moreover, if SC precursors are exposed to the extracellular domain of the ErbB4 NRG receptor, a protein that specifically blocks the action of NRG, the neuronal signal that mediates precursor survival and maturation can be blocked (Dong et al., 1995). These *in vitro* findings are supported by *in vivo* data generated in  $\beta$  neuregulin 1 null mice: neuregulin  $-/-$  embryos die during embryogenesis, due to cardiac malformations. Interestingly, before the embryos die, they are almost completely devoid of SC precursors (Meyer and Birchmeier, 1995).

NRG-1 is expressed at the right time and place to act as signal from neurons to precursors, but also to play a role at later stages of development. In fact, the level of NRG-1 protein in adult mice is strongly reduced as compared to rat embryos at E14 through to adult life. (Bermingham-McDonogh *et al.*, 1997; Marchionni *et al.*, 1993).

The four members of the EGF family of receptor tyrosine kinases, ErbB1 (EGF-receptor), ErbB2, ErbB3 and ErbB4, function as receptors for neuregulins. The different ErbB receptors contain a large extracellular ligand-binding domain, a single transmembrane domain and an intracellular part with a COOH-tail and a tyrosine kinase domains, with few exceptions: ErbB2 has no affinity for NRGs, while ErbB3 receptor lacks the tyrosine kinase activity. The ErbB receptors usually form heterodimers on the cell surface, with nearly all the possible combinations, in a process of ligand induced dimerization, in which one of the other ErbB members is recruited as co-receptor. Finally, ligand-binding mechanism leads to receptor phosphorylation and activation of downstream signalling pathways.

Many of these receptors are expressed in neurons and in glial cells during development (Burden and Yarden, 1997). The ErbB2/ErbB3 heterodimer is the primary NRG-1 receptor in SC (Canoll et al., 1996). *In vivo* data coming from engineered mice support this finding: first,



knockout mice for *ErbB3* die soon after birth, and their nerves are devoid of SC (Riethmacher et al., 1997). Second, like  $\beta$ -NRG null mice, mice lacking ErbB2 receptor die from cardiac defects, at an embryonic stage in which it is not possible to evaluate the effect on SC development. Third, some knock-out animals with cardiac rescue of erbB2 survive until birth and completely lack SCs (Lee et al., 1995; Garratt et al., 2000; Lin et al., 2000).

The survival and progression of Schwann cell precursors to Schwann cells is regulated in vitro and in vivo by another family of factors, endothelins (ETs). In vitro data confirm this role for ETs: in fact, when added in vitro to rat Schwann cell precursors, ETs promote survival without stimulation of DNA synthesis (Brennan et al., 2000). This action of ETs is mediated by the ET(B) receptors, that are expressed in developing peripheral nerves. Interestingly, within the complex growth factor interactions controlling the timing of Schwann cell development in embryonic nerves ETs seem to act as negative regulators of Schwann cell generation: in fact, in the combined presence of  $\beta$ -neuregulin and ETs Schwann cell generation is significantly slower than in  $\beta$ -neuregulin alone. A further in vivo confirmation of these in vitro data came from the characterization of spotting lethal rats, in which functional ET(B) receptors are absent: these animals presented accelerated expression of the Schwann cell marker S100 in developing nerves (Brennan et al., 2000).

#### ▪ 1.6.3 Precursor – immature SC transition

This transition occurs between E14 and E17 in rat and in vivo it is characterized by a series of morphological changes mainly related to regulation of survival and response to mitogens. As opposed to Schwann cell precursors, immature SC can survive when cultured at high density in a defined medium without external addition of NRG or DRG conditioned medium (Jessen et al., 1994). As SC mature in peripheral nerves, they move gradually from axon-dependent to axon-independent survival. This

capacity of SC to survive for an extended period of time in the absence of axons is crucial for nerve regeneration: for example, during Wallerian degeneration, near the lesioned point a retraction of proximal and distal stumps of the nerve occurs; as a consequence, SC de-differentiate to an immature phenotype and provide both trophic factors and adhesive substrates that promote axonal growth in the distal stump. The ability of Schwann cells to survive without axons is due to the establishment of an autocrine survival loop that is absent in precursors. Insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB are important components of this autocrine survival signal. Schwann cells have receptors for these factors: when they are applied at very low concentrations in Schwann cell conditioned medium, they promote and support survival; moreover, if the action of these factors is blocked (using specific blocking antibodies) the SC survival activity is blocked (Meier et al., 1999). Finally, another factor secreted by SC that can promote SC survival in the presence of other growth factors is Leukemia Inhibitory Factor (LIF) (Dowsing et al., 1999).

In addition to positive survival signals, other factors that actively promote apoptosis may also play an important role in SC death after injury: the Nerve Growth Factor (NGF) promotes cell death in SC, via the p75 neurotrophin receptor (Soiliu-Hanninen et al., 1999). Finally, TGF $\beta$  can have a similar effect on developing SC, both in vitro and in vivo (D'Antonio et al., 2006).

- 1.6.4 Immature to myelinating or non-myelinating SC transition

Once formed, immature SC invade bundles of axons and sort them into smaller groups. Depending on the size of axons, they associate with only one large axon or with multiple small caliber axons, in order to adopt a pro-myelinating or a non pro-myelinating phenotype, respectively. The decision to become a myelinating versus non-myelinating SC is determined by cell-extrinsic signals coming from the axons. The identity



of the axonal signals responsible for this choice is not known, although it is determined by the axonal diameter; in fact only axons with a diameter bigger than 1  $\mu\text{m}$  will become myelinated, whereas smaller axons will not. However, it has been recently demonstrated that NRG1 Type III is involved in this choice, in fact myelinated axons express it at higher levels as compared to ensheathed axons; moreover, the analysis of neurons from NRG1 Type III null animals revealed thinner myelin sheaths; lentiviral-mediated expression of NRG1 Type III in neurons is able to rescue these defects and drive Schwann cells to myelinate axons that would normally not be myelinated (Taveggia et al., 2005).

On the other hand, SC also determine multiple properties of axons and neurons. They directly control the number of neurofilaments of the axons and their phosphorylation state. This is particularly important because a change in neurofilament number is important in determining axon diameter (Martini, 2001). As it is for Wallerian degeneration, this is another process where SC exert a fundamental effect on neuronal final development. Further evidence of the reciprocal relationship of SC and axons comes from the analysis of the ErbB3 knock out mice: these mice die soon after birth, but their nerves are devoid of SC, presumably because they lose SC precursors even if axons can reach their normal target with normal innervation (Riethmacher et al., 1997). However analysis of DRG survival in these mice revealed that 80% of them die before reaching the final target between E13 and E18, indicating that sensory neuron death is due to lack of SC and their precursors.

In addition to that, SC mediate also the spacing of  $\text{Na}^+$  channel clusters along axonal membranes, during development of the Node of Ranvier (Salzer, 2002).  $\text{Na}^+$  channels are positioned in the middle of the Node of Ranvier and their distribution is particularly important for the saltatory conduction of impulses. Myelinating glia regulate the targeting of these channels at the Node of Ranvier, not only in the PNS, but also in the CNS (Boiko et al, 2001). Other soluble factors could be involved in the clustering of these channels at the Node of Ranvier (Kaplan et al., 1997; Martini, 2001).

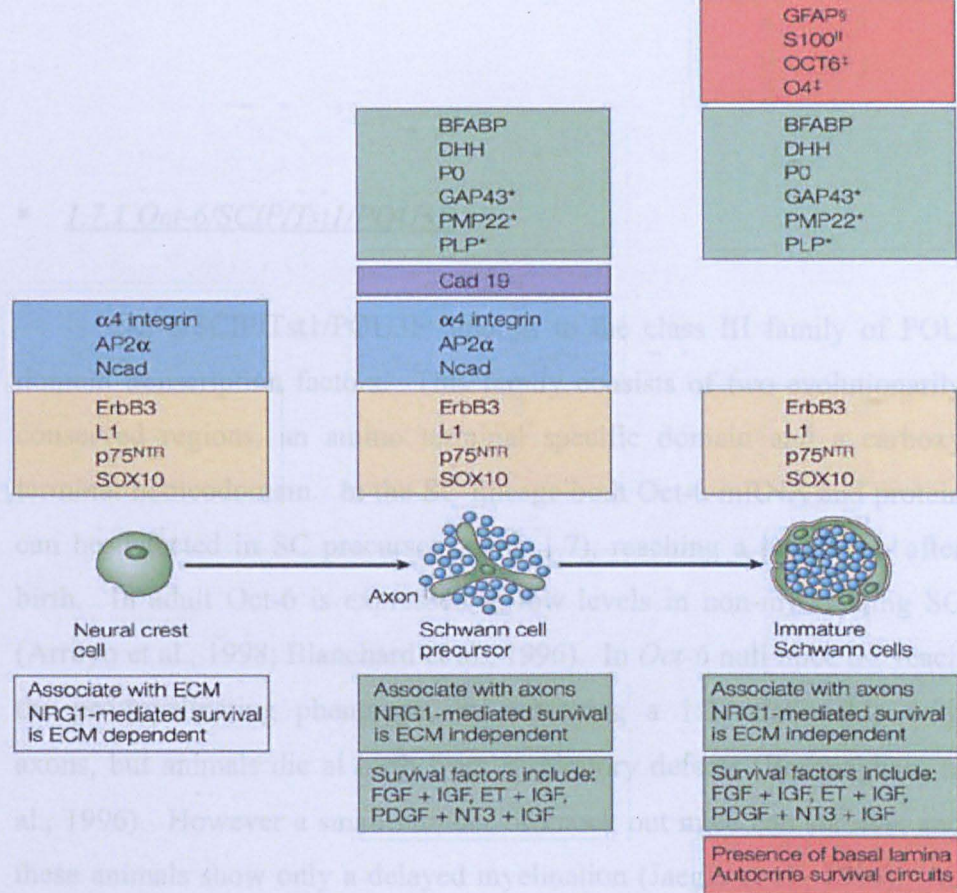
Finally, the effect of SC on the axons is proven by the fact that some of the mutations that cause human inherited neuropathies, such as Charcot Marie Tooth (CMT) disease, Dejerine Sottas Syndrome (DSS) and Congenital Hypomyelination (CH) disease, are due to a primary defect in SC. However, as a consequence of these mutations, some patients show also changes in the axons, such as reduced axonal caliber and alteration in the phosphorylation state of neurofilaments (Bjartmar et al., 1999). These secondary defects affecting the axons could lead to axonal degeneration with subsequent severe clinical consequences (Sahenk 1999).

All these observations strongly support the existence of a strict reciprocal relationship between SC and axons. Both axons and SC cooperate for development and maintenance of the PNS.

Finally, immature SC can generate also non-myelinating SC: these cells can be identified by the expression of several characteristic surface markers such as p75, Glial Fibrillary Acidic Protein (GFAP), N-CAM, L1, GAP-43. These markers are all suppressed in myelinating Schwann cells, when the myelination process takes place (Fig. 1.7).

## **1.7 Transcriptional control during SC development**

Although the molecular identity of the axonal signals that induce the myelinating or non-myelinating phenotype in SC is only partially known, relevant information on the transcription factors regulating myelination in SC is broader. Three transcription factors are known to be important in the development of the glial lineage: the first of these, Sox 10 has been discussed in a previous section, the other two, Oct 6 (also known as SCIP and Tst 1) and Krox 20 are both involved in the myelination program and are important for the topic of this thesis.



Copyright © 2005 Nature Publishing Group  
Nature Reviews | Neuroscience

**Fig. 1.7:** Changes in phenotypic profile as cells progress through the embryonic Schwann cell lineage. Shared profiles are indicated by distinct colours. The boxes above the lineage drawing indicate the changes in gene expression that take place during embryonic Schwann cell development. The gene expression shown here is based on observations of endogenous genes rather than on observations of reporter genes in transgenic animals. Each developmental stage also involves characteristic relationships with surrounding tissues, and distinctive cell signalling properties (boxes below lineage drawing). For instance, neural crest cells migrate through extracellular matrix. By contrast, SCPs and Schwann cells are embedded among neurons (axons) with minimal extracellular spaces separating them from nerve cell membranes, a characteristic feature of glial cells in the CNS and PNS. Basal lamina is absent from migrating crest cells and SCPs, but appears on Schwann cells. \*Proteins that also appear on neuroblasts/early neurones. ‡Markers that are acutely dependent on axons for expression. §Glial fibrillary acidic protein (GFAP) is a late marker of in vivo Schwann cell generation, as significant expression is not seen until about the time of birth. GFAP + SCs can be distinguished from astrocytes for their peculiar shape. The expression of GFAP is suppressed in cells that form myelin but retained in non-myelin-forming Schwann cells. The early expression of GFAP has not yet been carefully examined in mice. ||SCPs have been shown to be S100 calcium-binding protein (S100)-negative and Schwann cells S100-positive using routine immunohistochemical methods. (Adapted from Jessen and Mirsky 2005)

▪ 1.7.1 Oct-6/SCIP/Tst1/POU3F

Oct-6/SCIP/Tst1/POU3F belongs to the class III family of POU domain transcription factors. This family consists of two evolutionarily conserved regions, an amino terminal specific domain and a carboxy terminal homeodomain. In the SC lineage both Oct-6 mRNA and protein can be detected in SC precursors (Fig. 1.7), reaching a peak soon after birth. In adult Oct-6 is expressed at low levels in non-myelinating SC (Arroyo et al., 1998; Blanchard et al., 1996). In *Oct-6* null mice SC reach the pro-myelinating phenotype, by achieving a 1:1 relationship with axons, but animals die at birth from respiratory defects (Bermingham et al., 1996). However a small number of knock out mice can survive, and these animals show only a delayed myelination (Jaegle et al., 1996). An explanation for this rescue is that Oct-6 might be involved in determining the transition from pro-myelinating to myelinating SC. It is also possible that other POU genes compensate for its absence; this is the case for two different POU domain transcription factors, Brn-2, that is normally expressed by the SC lineage and Brn-1 that is not normally expressed in Schwann cells; both proteins are able to rescue the developmental delay phenotype, when ectopically expressed in cultured Oct-6 null Schwann cells or in Oct-6  $-/-$  animals (Jaegle et al., 2003; Friedrich et al., 2005). Other in vitro and in vivo studies, using a dominant negative form of Oct-6, postulated that it can act as a negative transcriptional regulator and a general repressor for myelin genes in immature SC in vitro (Monuki et al., 1993; Weinstein et al., 1995). However a recent paper showed that Oct-6 is a direct Krox-20 activator. In particular the authors showed that it acts as a positive regulator at the beginning of the myelination process (Ghislain et al., 2002). Thus, Oct-6 probably serves to regulate the timing of myelination by regulating the transition of SC from pre-myelinating to pro-myelinating and to the myelin-forming phenotype.

### ▪ 1.7.2 Krox-20

Egr-2 (Krox-20) is a zinc finger transcription factor belonging to the Early Growth Response (Egr) family; it is expressed in the SC lineage (Fig. 1.7). This family includes also *Egr-1*, *Egr-3* and *Egr-4* genes. Krox-20 contains different domains: a DNA binding domain, an activation domain and a domain that is able to interact with two co-factors NAB1 and NAB2 (NGF-1A-binding protein) (Russo et al., 1995; Svaren et al., 1996). The gene is activated around E10.5; it is not expressed in SC precursors while it is present in the dorsal and ventral roots close to the neural tube. In the peripheral nerve Krox-20 is activated around E15, very likely with the acquisition of a one-to-one relationship between SC and axons. During adulthood, Krox-20 expression marks myelinating SC (Topilko et al., 1997). This axonal regulation is evident after nerve damage. During Wallerian degeneration, SC de-differentiate to an immature SC phenotype, losing their myelin sheath, while Krox-20 is downregulated (Topilko et al., 1997).

The function of Krox-20 in myelination has been studied in engineered mice. Mice carrying a targeted deletion of *Krox-20* die soon after birth, and in addition to defects in hindbrain segmentation and in bone formation, they are defective in SC differentiation (Topilko et al., 1994; Schneider-Maunoury et al., 1993). All SC destined to myelinate in these mice acquire a one-to-one relationship with the axon, but they seem to be arrested at the pro-myelinating stage. This impairment is also paralleled by a strong reduction in the expression of P0, MBP and PMP22 myelin genes. The peripheral nerves of the few animals that survive after birth were analyzed: in these nerves, both SC proliferation and apoptosis are increased as compared to control littermates. Therefore, it is likely that Krox-20 is involved in regulating the transition into the pro-myelinating phenotype, not only by activating specific myelin gene expression, but also by inactivating different signaling pathways present in immature SC and responsible for the inhibition of their differentiation. One example is the c-Jun-amino-terminal kinase (JNK) pathway that is active in SC from E18 to birth; this pathway is inactivated as SC start to myelinate with a



mechanism that is Krox-20 dependent (Topilko et al., 1994; Jessen and Mirsky, 2005).

In humans, mutations in *Krox-20* are associated with peripheral neuropathies such as CMT, CH and DSS neuropathies (Warner et al., 1998; Warner et al., 1999). Co-transfection experiments show that Krox-20 can partially trans-activate the P0 promoter (Zorick et al., 1999). Another paper describes an induction of mRNAs for P0, MBP, MAG, PMP22 Cx32 and MAG by Krox-20 (Nagarajan et al., 2001). Some of the mutations described in humans have a dominant negative effect on the wild type Krox-20, affecting, at the end, the expression of myelin genes. Recently, a highly conserved element has been found in the first intron of the *Mpz* gene, which contains binding sites for Krox20 and Sox 10 transcription factors (LeBlanc et al., 2006; LeBlanc et al., 2007). *Egr2* mutants specifically affect this element in the *Mpz* first intron. Both *Egr2* and *Sox10* are able to bind this element, when myelination takes place in the sciatic nerve. A dominant *Egr2* mutant does not impede *Egr2* binding to *Mpz*, but reduces *Sox10* binding to the *Mpz* intron element, thereby resulting in the disruption of the genetic program that controls myelination (LeBlanc et al., 2007).

Another mutation that causes human neuropathy is recessive and is located in the Krox-20 domain interacting with the NAB transcriptional co-factors (Warner et al., 1999). The location of this mutation strongly suggests that NAB proteins, complexed with Krox-20, are key regulators of the Schwann cell myelination program. This hypothesis is supported by in vivo data coming from mice lacking both *Nab1* and *Nab2*; these mice show elevated *Egr2* expression, but a severe congenital hypomyelination of peripheral nerves, with a block of Schwann cell development at the pro-myelinating stage (Le et al., 2005).

## 1.8 The Inherited Neuropathies

The Inherited Neuropathies are a clinically and genetically heterogeneous group of diseases that affect the peripheral nervous system (PNS) and can result in a severe neuromuscular deficit.

These neuropathies were first identified more than one century ago. In fact, in 1886 J.M Charcot and P.Marie in Paris, and T.T.Tooth in London described a syndrome, which they defined respectively as “une forme peculi re d’atrophie musculaire progressive” and “the peroneal type of progressive muscular atrophy”. The syndrome, that carries their names, was characterized by a decrease in strength and distal muscular atrophy, a minimal sensory component, the presence of foot deformities, a rare, but present, infantile onset and a frequent familiar recurrence (Charcot, Marie, 1886; Tooth, 1886). Due to the vast clinical heterogeneity of these diseases, a chaotic classification followed the first description, where ambiguous and overlapping definitions were used (“Charcot-Marie-Tooth disease”, “peroneal muscular atrophy”, “hypertrophic neuropathy”, “Dejerine-Sottas disease or syndrome”, “Roussy-L vy syndrome” – Harding and Thomas, 1980). The coming of electromyographical (EMG) techniques produced a significant improvement in the nosography of inherited neuropathies. At present, the most used clinical classification (Dick et al. 1983) includes eight different types of Hereditary Motor and Sensory Neuropathies (HMSN), subdivided by means of clinical and electromyographical reports. Only three types of HMSN exclusively affect the PNS, while in the other forms the neuropathic status is associated with other neurological and non-neurological problems.

HMSN I (demyelinating or type 1 Charcot-Marie-Tooth disease) and HMSN III (Dejerine-Sottas disease) are characterized by a moderate to severe reduction in motor nerve conduction velocities (NCV – in normal individuals it reaches 40-50 m/s, while in affected patients it goes down to 10-30 m/s); the very early onset and the higher severity distinguish HMSN III from HMSN I.

On the other hand, HMSN II (axonal or type 2 Charcot-Marie-Tooth disease) is characterized by slight or absent reductions in NCV, accompanied with EMG signs of chronic axonal damage.

Recently, in the last decade, the identification of HMSN patients with mutations in specific myelin genes moved the principles of classification from the clinical-electrophysiological phenotype to the original genetic alteration. So far, myelin gene defects were identified in patients suffering from Charcot-Marie-Tooth type 1,2 and 4 diseases, Dejerine-Sottas disease, Hereditary Motor and Sensory Neuropathy with Liability to Pressure Palsies and Congenital Hypomyelination (Fig. 1.8).

- 1.8.1 Charcot-Marie-Tooth disease (CMT)

CMT is the most common inherited peripheral neuropathy in humans, with a prevalence of 40 in 100000. According to electrophysiological reports, two distinct forms of CMT can be identified, one demyelinating (CMT 1) and one axonal (CMT 2) (Lupski et al., 1991; Kaku et al., 1993; Dyck et al., 1983).

1.8.1.1 CMT 1, the most frequent form of CMT, is caused by abnormalities intrinsic to the Schwann cells, the myelin-producing cells of the peripheral nervous system; therefore it is associated with severe PNS demyelination, as demonstrated by slowed nerve conduction velocities; its onset usually occurs in the first two decades and is characterized by a progressive distal muscular strength deficit (Lupski et al., 1991): Patients may require foot care (*pes cavus* and *pes equinovarus*) or bracing to ambulate normally (*steppage gait*), and sometimes become unable to walk. Usually osteotendinous reflexes are absent or very weak and rarely, a sensory impairment is present. The typical electrophysiological reports are always present in CMT 1 patients several years before clinical disease onset (Kaku et al., 1993; Garcia et al., 1998). From the histopathological point of view, peripheral nerves of affected individuals present a reduction in the number of myelinated fibers, characterized by very thin sheaths and

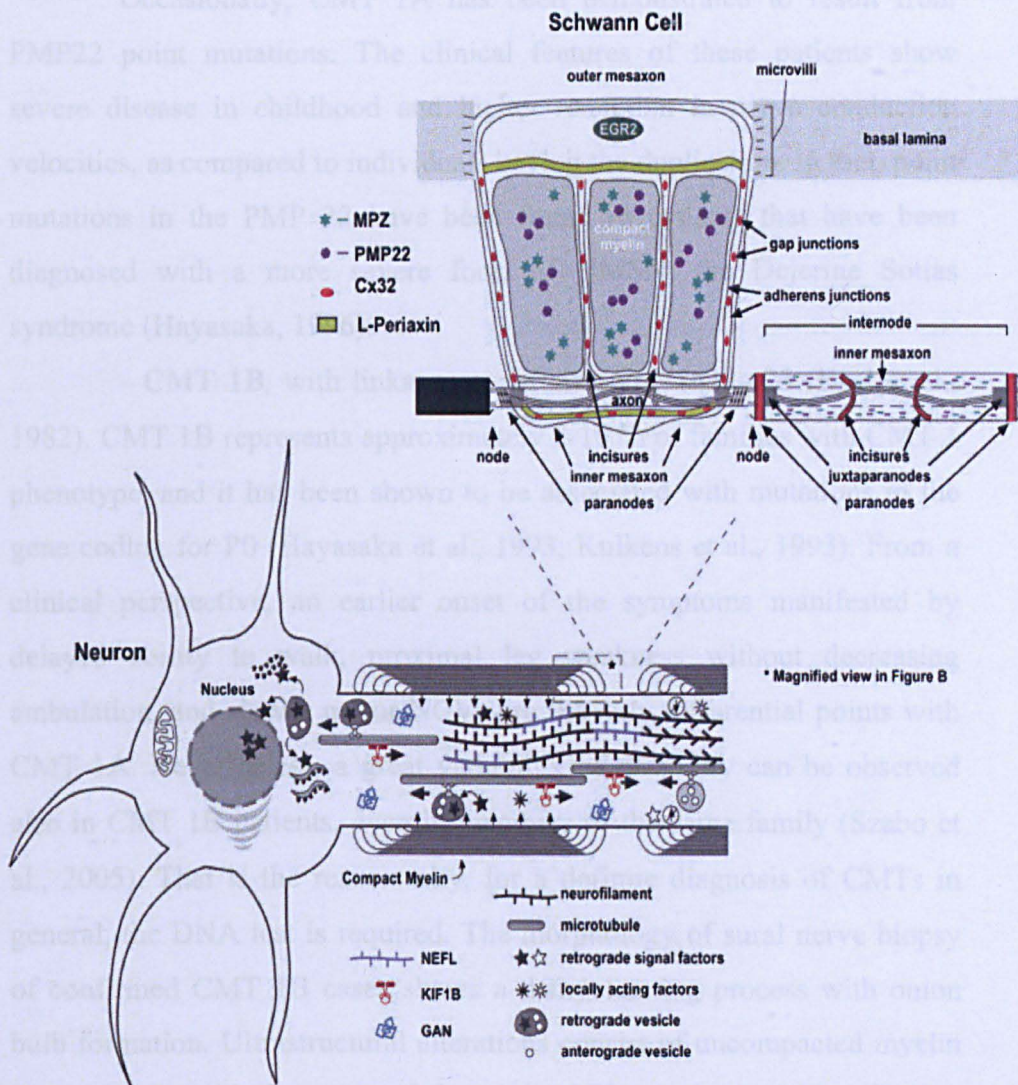


the emergence of hypertrophic “onion bulb changes”, that represent morphological features of a chronic process of demyelination/remyelination (Dyck et al., 1983). A trait of CMT 1 patients is the huge clinical variability, observed not only in unrelated individuals (Lupski et al., 1991), but also between members with a common pedigree (Kaku et al., 1993) and even between identical twins (Garcia et al., 1995).

Type I CMT is usually inherited as an autosomal dominant disorder, although recessively inherited, sporadic and X-linked forms do occur. According to genetic linkage analysis data, CMT 1 has been subdivided in:

- **CMT 1A**, with linkage on chromosome 17p11.2 (Vance et al. 1989). About 90% of CMT 1 patients are CMT 1A; the disease condition is most commonly due to segmental duplication of a region of chromosome 17 (71% of all CMT 1A cases), leading to the presence of an extra copy of the gene for peripheral myelin protein 22 (PMP22) (Lupski et al., 1991; Raeymaekers et al., 1991, 1992; Nelis et al., 1999).

Inheritance is autosomal dominant in pattern and analysis of nerve biopsies suggests that the disorder is caused by increased gene dosage (Hanemann et al., 1994; Yoshikawa et al., 1994; Vallat et al., 1996). As demonstrated by Pentao et al., in 1992, the duplication arises from an unequal crossing-over and recombination occurring between homologous sequences flanking the duplicated genomic region. The clinical symptoms in patients carrying the duplication may appear in the first decade or early in the second decade. Muscle weakness starts in the feet and legs. Infants and children manifest the disease by walking on their toes and inability to walk on their heels. Older patients consult a physician because of abnormality of the gait, foot deformities, or loss of balance. Steppage or equine gait, pes cavus deformity and claw toe are usually present in CMT1A patients. Hand tremors are a frequent complaint and are most likely related to hand weakness or to coexisting essential tremor. Enlargement of nerves can be seen or palpated, predominantly in male patients. Muscle stretch reflexes disappear early in the ankles and later in



**Fig. 1.8:** In the last decade, several studies contributed to the identification of HMSN patients with mutations in specific myelin genes. In this schematic view of myelinated axon and myelinating Schwann cell, the localization within the cell of some proteins mutated in patients with inherited neuropathies is illustrated. The region enclosed in the rectangle on the bottom panel is shown in detail on the top. MPZ is localized to compact myelin, whereas Cx 32 is localized to the paranodal loops, incisures and inner mesaxon composed of noncompact myelin. (The figure has been adapted from Shy et al., 2002)

the patella and upper limbs. The plantar responses are frequently absent. Finally, mild sensory loss may be seen in some patients.

Occasionally, CMT 1A has been demonstrated to result from PMP22 point mutations. The clinical features of these patients show severe disease in childhood and higher reduction in nerve conduction velocities, as compared to individuals having the duplication. In fact, point mutations in the PMP 22 have been found in patients that have been diagnosed with a more severe form of HMSN, the Dejerine Sottas syndrome (Hayasaka, 1996).

- **CMT 1B**, with linkage on chromosome 1q22-q23 (Bird et al., 1982). CMT 1B represents approximately 5-10 % of families with CMT 1 phenotype, and it has been shown to be associated with mutations in the gene coding for P0 (Hayasaka et al., 1993; Kulkens et al., 1993). From a clinical perspective, an earlier onset of the symptoms manifested by delayed ability to walk, proximal leg weakness without decreasing ambulation, and slower motor NCVs are the only differential points with CMT 1A. Nevertheless, a great variability of disability can be observed also in CMT 1B patients, even in members of the same family (Szabo et al., 2005). That is the reason why, for a definite diagnosis of CMTs in general, the DNA test is required. The morphology of sural nerve biopsy of confirmed CMT 1B cases shows a demyelinating process with onion bulb formation. Ultrastructural alterations consist of uncompacted myelin in agreement with the accepted function of P0 as a homophilic adhesion molecule (Gabreels-Festen et al., 1996): To date over 110 different mutations in MPZ have been identified, that result not only in the mild CMT 1B phenotype, but also in more severe phenotypes like Dejerine-Sottas syndrome and Congenital Hypomyelination.

- **CMT X**, the X-linked form of demyelinating CMT, accounts for 7-10% of CMT. It is caused by point mutations in the *connexin-32* gene, a gap junction protein expressed in myelinating Schwann cells in the paranodal loops and Schmidt-Lanterman incisures (=SLI), but not incorporated into the myelin sheath (Bergoffen et al., 1993). CMT X is mainly inherited as a dominant form (about 90% of the cases). The clinical

features and clinical variability are similar to the CMT 1A and 1B patients, but the family pedigree reveals lack of male-to-male transmission and, moreover, the affected males have more severe phenotypes than the affected females. The nerve conduction studies have shown intermediate motor slowing in most families; the velocities are usually between 30 and 40 m/s in males and can be faster or near-normal in females: these values are consistent with a demyelinating form of the disease (Bergoffen et al., 1993; Nicholson and Nash, 1993; Lewis and Shy, 1999). However, there are intriguing reports describing CMT X patients with primarily axonal features on electrophysiological and morphological analyses (Timmerman et al 1996; Birouk et al 1998; Gutierrez et al., 2000; Hattori et al., 2003).

- Finally, an additional, less common form of CMT1, sometimes indicated as **CMT 1C**, exists; it refers to a group of CMT 1 with no detectable mutations in *PMP 22* or *MPZ* genes. Two causative genes have been identified so far: the *egr 2* gene on chromosome 10q21-22A, coding for the zinc finger transcription factor EGR2, expressed in myelinating Schwann cells and discussed above (Warner et al., 1998), and a putative protein degradation gene, *LITAF/SIMPLE*, on chromosome 10p13.1-12.3, coding for a ubiquitous lysosome protein that may be involved in cell proliferation and apoptosis (Street et al., 2003; Bennett et al., 2004).

1.8.1.2 CMT 2 (or Type II) is the axonal or neuronal form of CMT; it has been previously suggested to represent about one-third of autosomal dominant CMT families. CMT 2 is mainly distinguishable from CMT 1 by the presence of normal or slightly slowed NCV; the classical clinical symptoms are similar to the ones of CMT 1 patients, but some differences are present: the onset usually occurs later, hypertrophic changes on biopsies are absent and foot muscle weakness is often more severe. Morphological studies on patient biopsies showed only occasional, small *onion bulbs*, but mainly a reduction in the number of myelinated fibers, more pronounced distally. Nevertheless, the clinical phenotype within the CMT 2 subgrouping can vary more than CMT 1. Due to the absence of the electrophysiologic changes, peculiar to CMT 1, CMT 2 is more difficult to diagnose.

Several unique genes have been found for CMT 2, mapping to at least seven distinct loci and originating the different forms of axonal CMT. Between them: a mitochondrial membrane protein *MFN2* (Mitofusin2), a protein involved in axonal transport *KIF1B* (Kinesin family member 1B), a protein involved in endosomal trafficking *RAB7* (Ras-associated protein), a protein involved in RNA processing *GARS* (Glycyl-tRNA synthetase), a neuronal structural protein *NEFL* (Neurofilament light), a protein involved in chromatin organization *LMNA* (Lamin A/C), a heat-shock 27-kD protein-1 (*HSPB1*) and finally *P0* (Zuchner and Vance, 2006). The classification of CMT 2 subgroups is actually based on the genomic linkage analyses; some of these forms present the classical CMT 2 phenotype, while others are characterized by specific clinical findings:

- **CMT 2A** was mapped on chromosome 1p35-36 (Ben Othmane et al., 1993), in an area that is believed to be gene rich; this, coupled with the instability of the region, has made progress on identifying this locus very difficult. Patients show the traditional CMT phenotype. Recently it has been linked to mutations in *MFN2* (Zuchner et al., 2004) and *KIF1B* (Bissar-Tadmouri, 2004)

- **CMT 2B** mapping on chromosome 3q13-22 (Auer-Grumbach et al., 2000). Families with CMT 2B are likely to be distinct in their clinical presentation, with sensory symptoms being unusually severe; patients; in fact, commonly had ulcerations leading to amputations of the feet. Recently it has been linked to mutations in *RAB7* (Houlden et al., 2004)

- **CMT 2C**, in 2003 Klein and colleagues found linkage to a region at 12q23-q24. Patients suffering from this subform of type II CMT have been noted to have diaphragm and vocal cord paresis that can lead, in most severe cases, to respiratory failure (Dyck et al., 1994). No gene has been yet associated to this subform.

- **CMT 2D**, mapped on chromosome 7p14 and sometimes associated with an upper extremities onset of symptoms (Ionanescu et al., 1996). Screening of different families diagnosed with CMT2D led to the identification of a mutation in the *GARS* gene (Antonellis et al., 2003).

- **CMT 2E**, related to a mutation in the NF-L gene on chromosome 8p21 (Mersiyanova et al., 2000).

- **CMT 2F**, linkage of the disorder has been found on chromosome 7q11-q21 (Ismailov et al. 2001). Affected members of two different families showed mutations in the HSPB1 gene (Evgrafov et al. 2004).

- **CMT 2 with MPZ mutations**, characterized by the classical axonal CMT phenotype (Marrosu et al., 1998; Senderek et al., 2000; Boerkoel et al., 2002).

- **Autosomal recessive CMT 2**, mapped on chromosome 1q21.2 q21.3 (Bouhouche et al., 1999). In 3 consanguineous Algerian families with autosomal recessive CMT2 linked to chromosome 1q21, a homozygous mutation in the LMNA gene was identified (De Sandre-Giovannoli et al. 2002).

1.8.1.3 CMT 4 is an autosomal recessive form of CMT, with 10 loci and five causative genes identified. According to the disrupted gene, 5 different forms of CMT 4 has been identified:

- **CMT 4A**, mapped on chromosome 8q21 and often classified as a recessive form of an axonal CMT. CMT 4A patients carry mutations in the *GDAP1* gene. This gene is predominantly expressed in neural tissue, including brain, spinal cord, dorsal root ganglia and sural nerve and it is probably involved in neural differentiation (Cuesta et al., 2002). CMT 4A is characterized by early age of onset, severe sensorimotor impairment and foot deformities. Nerve conduction studies and nerve biopsy findings are highly variable both between families and within families, some showing prominent demyelination while others showing primary axonal degeneration (Nelis et al., 2002).

- **CMT 4B**, mapped on chromosome 11q22 (Ben Othmane et al. 1999). It is a recessively inherited demyelinating neuropathy characterized by early onset (age 2-3 years), severe phenotype (loss of ambulation in adult), mild facial weakness and hearing loss in some patients, and demyelination with distinct redundant focally folded myelin shown on nerve biopsies (Quattrone et al., 1996; Gambardella et al., 1999). The



defective genes have been identified: one is myotubularin-related protein 2 (MTMR2) gene, encoding a ubiquitously expressed dual specificity phosphatase, whose main substrate are phosphorylated phosphoinositides (Bolino et al., 2000; Nelis et al., 2002; Berger et al., 2002); the second one is the myotubularin-related 13 (MTMR13) gene, coding for a protein in which key catalytic residues are missing ("pseudophosphatase", Azzedine et al., 2003).

- **CMT 4C**, linked to chromosome 5q23-33; the presenting problem of CMT 4C is disabling scoliosis, usually apparent by age 10 years. The severity of distal sensorimotor neuropathy varies and foot deformities are common. Sural nerve biopsies show demyelinating features with focal myelin thickenings (Gabreels-Festen et al., 2002). The disrupted gene has been identified with the SH3TC2 gene (or KIAA1985; Senderek et al., 2003).

- **CMT 4D**, mapped on chromosome 8q24.3 and also known as hereditary motor and sensory neuropathy-Lom type (HMSN-LOM); it is a demyelinating neuropathy that presents at age 5-6 years with gait difficulty from leg weakness and is associated with hearing loss. It was initially identified in the gypsy community. A homozygous nonsense mutation in the N-myc downstream-regulated gene 1 (*NDRG1*) has been found in all affected individuals. *NDRG1* is a ubiquitous gene with the highest levels of expression in Schwann cells and with no expression in axons, probably involved in the Schwann cell-axon interaction (Kalaydjieva et al., 2000).

- **CMT 4F**, mapped on chromosome 19q13, is a sensory-motor demyelinating neuropathy with neuropathic pain. It is caused by homozygous mutations in periaxin (Guilbot et al., 2001). Periaxin mutations can cause recessively inherited demyelinating neuropathies with a broad variety of phenotypes, ranging from Dejerine-Sottas disease to CMT 4F. Hearing loss is a distinctive associated feature of CMT 4F patients. Nerve biopsies show evidence of demyelination and remyelination with some onion bulb formation and segments of hypermyelination (or *tomacula*) that can contribute to unusual

susceptibility to fiber dysfunction or degeneration after modest compression (Takashima et al., 2002).

- 1.8.2 Dejerine-Sottas Disease (DSD)

With this name is indicated a third form of Hereditary Motor and Sensory Neuropathies, equivalent to HMSN III and sometimes also indicated as CMT type 3; it is defined as a severe demyelinating neuropathy, presenting in infancy with delayed motor development, very slow nerve conduction velocities (less than 10-12 m/s) and usually elevated Cerebro-Spinal Fluid (CSF) proteins. Progression is severe and walking is lost early. Inheritance is thought to be autosomal dominant (Dyck et al., 1975). Hypomyelination of the peripheral nerves is seen as a pathological hallmark and it is more severe than in CMT patients. Classic onion bulbs, made of concentric thin Schwann cell lamellae, and myelin breakdown products were considered evidence of an ongoing demyelinating process (Dyck et al., 1971; Ouvrier et al., 1987). Recent genetic investigations showed that several of the earlier published cases of DSD result from *de novo* heterogeneous dominant point mutations of the PMP 22 gene (Roa et al., 1993; Gabreels-Festen et al., 1995; Valentijn et al., 1995), or the MPZ gene (Hayasaka et al., 1993; Nakagawa et al., 1999). More recently it has been demonstrated that also autosomal dominant mutations of EGR2 might result in DSD (Warner et al., 1998). Furthermore, an autosomal recessive inherited form of DSD may result from mutations in the *periaxin* gene (PRX – Boerkoel et al., 2001). In addition, one mutation in the inhibitory domain of EGR2 and at least one PMP22 mutation in the C-terminal intracellular domain of the protein are inherited as an autosomal recessive trait (Warner et al., 1998; Parman et al., 1999). These mutations are silent in the heterozygous parents, but cause a DSD in the homozygous children. Two specific mutations of MPZ lead to a DSD phenotype in the homozygous state, but result in a mild CMT phenotype in the heterozygous state (Ikegami et al., 1996; Pareyson et al., 1999). Finally, the phenotypic expression of the few reported cases



of a homozygous PMP22 duplication may cause a DSD (LeGuern et al., 1997; Sturtz et al., 1997).

- 1.8.3 Congenital Hypomyelination Neuropathy (CHN)

Lyon in 1969, and Kennedy and colleagues in 1977 had discerned a variant of HMSN III, distinct from DSD; they called it Congenital Hypomyelination Neuropathy (CHN) and defined as a severe neuropathy with a congenital or early infantile onset; peripheral nerve biopsies from CHN patients present no or extremely thin myelin sheaths and atypical onion bulbs of mainly basal lamina (Bornemann et al., 1996). Several cases with a similar pathology have been described, with distal muscle weakness, hypotonia, areflexia, and severe slowing of nerve conduction velocities. The findings of CHN patients led CHN to be considered distinct from DSD, the first being a non-progressive disorder with a defect in myelin formation and the second a progressive demyelinating disorder (Harati and Butler, 1985). In most severe cases of CHN, articular contractures or *arthrogryposis multiplex congenital* are reported (Boylan et al., 1992). The majority of CHN cases occurred sporadically, but in some of them clear evidence of autosomal recessive inheritance was present. Although CHN is a distinct clinical entity, it may share similar genetic features with DSS; in fact molecular genetics analysis of CHN patients revealed mutations in genes coding for MPZ (Warner et al., 1996; Mandich et al., 1999), PMP 22 (Fabrizi et al., 2001) and EGR 2 (Warner et al., 1998).

- 1.8.4 Hereditary Neuropathy with Liability to Pressure Palsies (HNPP)

Hereditary neuropathy with liability to pressure palsies (HNPP) is a frequent demyelinating neuropathy (10 to 50 per 100.000), which is sometimes classified with the CMT 1 syndromes because of its genetic and functional relationship to the latter group of peripheral neuropathies.

HNPP is an autosomal dominant disorder, first described by De Jong in 1947 and often viewed as an entrapment or compressive neuropathy that may affect the peroneal, median or ulnar nerves at vulnerable sites. The onset of HNPP is usually in childhood or adolescence. When palsies occur, they may be debilitating in that they may last for days to weeks and may require installation of a lower limb brace in the cases of prolonged peroneal palsies. The abnormal neurophysiological features of HNPP are consistent with demyelination, showing mildly prolonged motor and sensory nerve conduction velocities in a symmetrical, generalized pattern. Mild electrophysiological signs of demyelination are present also in nerves not affected by palsy (Earl et al., 1964). Histological assessment of sural nerve biopsies reveals the presence of tomacula, the pathological signature of HNPP in which there is massive redundancy or overfolding of layers of the myelin sheath, with predominance in the paranodal region. Rare patients showing axonal regeneration and lacking tomacula have been observed (Sessa et al., 1997). The spectrum of clinical presentation in HNPP is broad and may range from clinically asymptomatic persons (obligate gene carriers), to those who more typically present with recurrent palsies and in some advanced cases may mimic smouldering forms of CMT 1.

The genetic locus for HNPP maps to chromosome 17p11.2-12, where it is often associated (90% of HNPP cases) with a large 1.5-Mb DNA deletion, involving the same DNA fragment that is duplicated in CMT 1A patients and that harbors the peripheral myelin protein-22 gene (Chance et al., 1993). Rarely, point mutations have been observed in HNPP patients (Nicholson et al., 1994; Lenssen et al., 1998; Sahenk et al., 1998).

### **1.9 Animal models of CMT disease**

As we have seen in the prior chapter, even if genetically heterogeneous, the CMT phenotype is similar among the majority of

patients and includes progressive distal weakness and wasting in the limbs with less evident sensory loss, deformities in the feet and reduced or absent tendon reflexes. Even if the majority of CMT disorders start as demyelinating diseases, disability in the patients mainly correlates with axonal damage; essentially, most of CMTs evolve to a disturbance of the SC/axon unit, rather than isolated damage to myelin-forming SC or axons. In order to clarify the pathogenetic mechanisms of the different CMT neuropathies, animal models have taken on a key role. In fact, it is there that the normal three-dimensional and reciprocal relationships between SC and axons can be highlighted and that pathologic changes can be monitored, with the aim of understanding first how mutations in different genes, some of which are expressed specifically by SC, can lead to similar disabling axonopathies, and second how the different CMT phenotypes (CMT1, DSS, CH and CMT2) can be generated from different mutations in the same gene.

Here below I include a short review of the main CMT animal models known so far, that can be somehow helpful for the purpose of this thesis.

#### ▪ 1.9.1 PMP22 animal models

As discussed in the dedicated chapter, mutations affecting the PMP22 gene are the most frequent causes of inherited peripheral neuropathies (Fig. 1.9). Various animal models for PMP22 point mutations are currently available. Some of them carry naturally occurring PMP22 mutations (*Tr* and *Tr-J* mice), while others were artificially generated through common transgenic techniques (PMP22 transgenic mice; PMP22 transgenic rats; conditional PMP22 overexpressing mice and mice with decreased PMP22 gene dosage).

1.9.1.1 Trembler mouse: this spontaneous mouse mutant was described more than 50 years ago (Falconer, 1951). It is characterized by

autosomal dominant inheritance, unsteady gait, weakness of the hindlimbs, axial tremor and stress-induced convulsions. The Trembler mutation is a Glycine to Aspartic Acid substitution at position 150, in the last hydrophobic domain of PMP22 protein (Suter et al., 1992). The same mutation has been found in a family diagnosed with a severe DSS phenotype (Ionasescu et al., 1997). Within the cell, the mutation leads to a trafficking defect of the PMP22 protein, associated with the high tendency of the *Tr* protein to form aggregates (Tobler et al., 2002). At the morphological level, adult *Tr* mutants show severe hypomyelination of peripheral nerves, with increased SC number and SC proliferation (Perkins et al., 1981; Sancho et al., 2001) and reduced NCV (< 10 m/s; Low and McLeod, 1975). During development, the onset of myelination is delayed: Schwann cells are late in progressing from the promyelination stage to myelination (Henry et al., 1983). Finally, hypomyelination in the *Trembler* mutant mice is associated with a general downregulation of myelin protein components, including PMP22; in fact quantitative and qualitative immunocytochemical analysis performed in the sciatic nerve of the *Tr*/+ mouse showed a significant decrease in PMP22, P0 and MBP (Vallat et al., 1999).



1.9.1.2 Trembler-J mouse: it is again a spontaneous mutant mouse, carrying a missense mutation exchanging a Proline residue for a Leucine at position 16 in the first hydrophobic domain of PMP22 (Suter et al., 1992b). The same mutation has also been described in a family with a severe CMT 1 (Valentijn et al., 1992). At the intracellular level, the Tr-J protein appears to reach the intermediate compartment between the endoplasmic reticulum and the Golgi, but cannot proceed further (D'Urso et al., 1998; Tobler et al., 1999). The pathology in adult heterozygous *Tr-J* mice is qualitatively similar (thinly myelinated axons, SC onion bulb formation, abnormalities in myelin compaction and perturbances of the axon-glia compartment), but less pronounced than in the *Tr* (Henry et al., 1983).

1.9.1.3 PMP22 transgenic mouse: transgenic mice carrying 16 to 30 additional copies of the PMP22 gene have been generated (Magyar et al., 1996). These mice display a severe congenital hypomyelinating neuropathy, characterized by an almost complete lack of myelin and marked slowing of nerve conductions. An increased number of amyelinating Schwann cells can be found in affected nerves. These amyelinating SC do not form onion bulbs, but associate with axons. The expression of embryonic Schwann cell markers (p75<sup>NTR</sup>, N-CAM and L1) indicates that the mutant Schwann cells are characterized by a premyelination-like phenotype. In addition, Schwann cells continue to proliferate during adulthood. Thus, these data show that mutant Schwann cells are unable to proceed in their differentiation into the myelinating phenotype. Further analysis of PMP22 transgenics revealed that the mutant mice develop also a distally accentuated axonopathy. Interestingly, degenerating axons seem to be preferentially associated with demyelinating or dysmyelinating SC (Sancho et al., 1999). In addition to these, another set of PMP22 transgenic lines, carrying fewer copies (about 8 copies) of the gene, have been generated. These mice show a peripheral



neuropathy, similar to the human pathology: the disorder is dominant, causes progressive weakness of the hind legs, and there is severe demyelination in the peripheral nervous system including the presence of onion bulb formations (Huxley et al, 1996).

1.9.1.4 PMP22 transgenic rat: a single line carrying three transgenic *Pmp22* copies was generated (Sereda et al., 1996). PMP22-transgenic rats develop gait abnormalities caused by a peripheral hypomyelination, Schwann cell hypertrophy (onion bulb formation, suggesting active demyelination), and muscle weakness. Myelin abnormalities are more pronounced in ventral than in dorsal roots. Reduced nerve conduction velocities closely resemble recordings in human patients with CMT1A. Furthermore, when bred to homozygosity, transgenic animals completely fail to elaborate myelin. Trafficking of the overexpressed PMP22 myelin protein through the endoplasmic reticulum is not significantly impaired in fact the molecule acquires complex glycosylation and is blocked in the Golgi compartment. Finally, in PMP22-transgenic rats the program of myelin gene expression, analyzed using semiquantitative reverse transcription-PCR and immunofluorescence techniques, is not affected (Niemann et al., 2000).

1.9.1.5 Conditional PMP22 overexpressing mouse: this is a transgenic mouse model in which mouse *pmp22* overexpression can be regulated. In this mouse model, Schwann cells specifically overexpress *pmp22* in of the peripheral nerves; when the mice are fed with tetracycline overexpression is turned off. When *pmp22* overexpression occurs, it causes demyelination. In contrast, when *pmp22* overexpression is off, myelination is nearly normal. When overexpression of *pmp22* is switched off in adult mice, correction begins within 1 week and lead to advanced myelination by 3 months. Nevertheless the myelin sheaths are still thinner than normal. Conversely, when the gene is upregulated in adult mice, active demyelination starts 1 week after and progresses for about 8 weeks.

All together these data indicate that Schwann cells with mature myelin are sensitive to increased amounts of pmp22 (Perea et al., 2001).

1.9.1.6 Mice with decreased PMP22 gene dosage: mice with genetic disruption of the PMP22 gene have been generated (Adlkofer et al., 1997); they present a delay in the onset of myelination and develop abundant tomacula at a young age. The presence in older animals of thinly myelinated axons and SC onion bulbs, together with very slow nerve conduction velocities indicate that the mice develop demyelination and remyelination. In homozygous *Pmp22* null mice distal axonopathy develops: it differs from the one described in PMP22-transgenic mice because of the presence of clear signs of active axonal degeneration, like accumulation of dense bodies and vesicles, vacuolization of axonal organelles, disruption of the axolemma and disorganization of cytoskeletal elements. Heterozygous mutant mice are less affected than homozygous null animals, but they also exhibit focal tomacula (Sancho et al., 1999). These sausage-like hypermyelination structures are comparable to the morphological features in hereditary neuropathy with liability to pressure palsies (HNPP). Therefore, analysis of knock-out animals indicates that *Pmp22* is required for the correct development of peripheral nerves, the maintenance of axons and the determination of myelin thickness and stability.

▪ 1.9.2 P0 animal models:

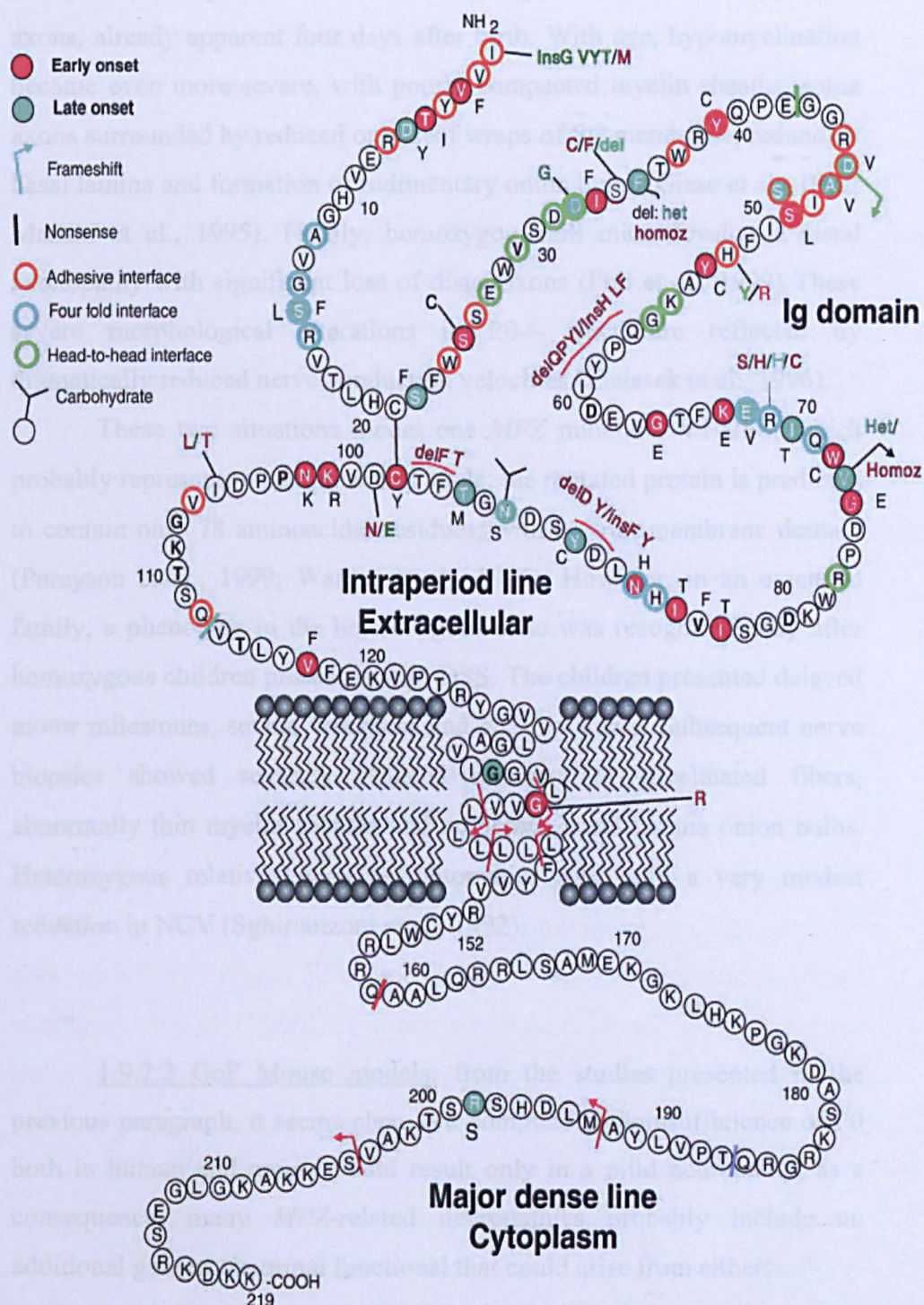
As previously discussed, more than 110 mutations *MPZ* have been identified, causing CMT 1B in humans. They reach 4 to 14% of CMT not due to chromosome 17 duplication. No deletion or duplication of the gene has been reported, but only missense, nonsense and frameshift mutations,. Most of the mutations are distributed in the extracellular domain, but also mutations within the transmembrane and intracellular domains have been described. Mutations in *MPZ* probably generate the widest variety of



CMT phenotypes, ranging from mild CMT 1B to more severe DSS, CH or even CMT 2 (Fig. 1.10). This variety in CMT phenotypes is paralleled by a great diversity in pathological traits, including demyelination, dysmyelination, tomacula, myelin outfolding or axonal loss with clusters of regenerating axons. These different phenotypes indicate different mutation-specific pathogenetic mechanisms that can be investigated in animal models.

In contrast to PMP22, there are no naturally occurring mouse mutants with *Mpz* point mutations. In the last decade, mice have been engineered in order to introduce specific *P0* mutations and produce both Loss-of-Function (LoF) and Gain-of-Function (GoF) models of *MPZ*-related neuropathies.

1.9.2.1 LoF Mouse models: knock-out mice for *P0* have been generated using homologous recombination in embryonic stem cells to replace the endogenous *P0* gene on mouse chromosome 1 with an inactivated *P0* gene (Giese et al., 1992). Heterozygous null mice develop a late-onset neuropathy. The myelin sheaths of *P0*<sup>+/-</sup> mice were indistinguishable from those of *P0*<sup>+/+</sup> mice until four months of age, when mild hypomyelinated profiles appeared and occasional onion bulbs were identified. These morphological abnormalities were more evident in one-year-old animals: segmental demyelination and formation of onion bulbs become predominant pathological features in peripheral nerves, resulting in moderate electrophysiological alterations (significant increase in compound muscle action potential latencies; Martini et al., 1995). In contrast, homozygous-null mice showed a very early-onset neuropathy: when the mice were two-weeks old they presented a phenotype characterized by weak vibrations of the animal body; four-week old mutants developed clasping of hindlimbs when lifted by the tail, slight tremors and jerky movements of the hindlimbs; with age, this behaviour became more pronounced and some of the animals developed convulsions.



**Fig. 1.10:** Mutations in the open reading frame of MPZ, which are associated with early onset or late onset symptoms of neuropathy. Early onset cases are defined as those with a delayed onset of walking or other early milestones. Late onset cases are defined as those with their first symptoms of neuropathy after age 21. (This figure is taken from Shy et al., 2004).

Their peripheral nerves showed severe morphological abnormalities such as myelin uncompaction and an unusually high number of non-myelinated axons, already apparent four days after birth. With age, hypomyelination became even more severe, with poorly compacted myelin sheaths, some axons surrounded by reduced or absent wraps of SC membrane, redundant basal lamina and formation of rudimentary onion bulbs (Giese et al., 1992; Martini et al., 1995). Finally, homozygous-null mice develop a distal axonopathy with significant loss of distal axons (Frei et al., 1999). These severe morphological alterations in P0<sup>-/-</sup> mice are reflected by dramatically reduced nerve conduction velocities (Zielasek et al., 1996).

These two situations model one *MPZ* mutation, V102FS, which probably represents a complete null allele; the mutated protein is predicted to contain only 78 aminoacidic residues, with no transmembrane domain (Pareyson et al., 1999; Warner et al., 1996). However, in an extended family, a phenotype in the heterozygous state was recognized only after homozygous children presented with DSS. The children presented delayed motor milestones, severe weakness and NCV < 4 m/s. Subsequent nerve biopsies showed severely reduced numbers of myelinated fibers, abnormally thin myelin sheaths and numerous basal lamina onion bulbs. Heterozygous relatives were asymptomatic, with only a very modest reduction in NCV (Sghirlanzoni et al., 1992).

1.9.2.2 GoF Mouse models: from the studies presented in the previous paragraph, it seems clear that complete haploinsufficiency of P0 both in human and mouse could result only in a mild neuropathy; as a consequence, many *MPZ*-related neuropathies probably include an additional gain of abnormal functional that could arise from either:

- 1) a dominant-negative effect that originates from the myelin sheath or,
- 2) a toxic effect of the mutant protein that initiates from an intracellular location during the synthesis and trafficking of P0.

Support for the notion of gain of function comes from different evidence, in vitro experiments and transgenic mice. In human samples,

high magnification electron microscopical analyses of CMT patient nerve biopsies showed that peripheral demyelinating neuropathies have distinguishable ultrastructural phenotypes that depend on the alteration in primary structure of P0; this suggests that patients with P0 mutations could express the mutated abnormal protein that is inserted into the myelin sheath where it could lead to specific impairments in inter-membrane appositioning (Kirshner et al., 1996). In addition, in vitro coexpression of full-length P0 and mutated P0 (missing either 52 or 59 aminoacids from the cytoplasmic domain) in CHO cells, prevents the full-length P0 protein from behaving as an adhesion molecule; this shows that the mutated forms of P0 can have a dominant-negative effect on the adhesiveness of the WT protein (Wong and Filbin, 1996).

Further evidence for gain of function of P0 mutations come from transgenic mice (Wrabetz et al., 2000; Previtali et al., 2000; Wrabetz et al., 2006). To test for GoF mechanisms, several *MPZ* mutations have been inserted into the *Mpz* gene, which was then inserted as a random transgene in the mouse genome, in addition to the two endogenous *Mpz* alleles. In this way loss of mutant P0 function in these mice should not be visible. As a preliminary control for this kind of approach, additional copies of wild-type *Mpz* have been inserted randomly in the mouse genome. The analysis of these transgenic mice showed that normal peripheral nerve myelination depends on strict dosage of *Mpz* (Wrabetz et al., 2000). The mice in fact manifested a dose-dependent, dysmyelinating neuropathy, ranging from transient perinatal hypomyelination to arrested myelination and impaired sorting of axons by Schwann cells. Such defects represent a gain of normal P0 function and could be associated with premature arrival of P0 in the membrane spiraling around the axon (= inner mesaxon; Yin et al., 2000). Myelination was restored by breeding the transgene into the *Mpz*-null background, demonstrating that dysmyelination does not result from a structural alteration or Schwann cell-extrinsic effect, but only to P0 glycoprotein overexpression. *Mpz* mRNA overexpression in the transgenic animals ranged from 30-700%. Breeding experiments placed the threshold for dysmyelination between 30 and 80% *Mpz* overexpression.

Following on these studies, we have investigated gain of abnormal function in authentic mouse models of CMT1B mutations. S63 (34th residue of MPZ, after cleavage of the signal peptide) is conserved from fish to human (Kirschner et al., 2004), and mutations of S63 in the P0ECD determine diverse neuropathy phenotypes. Lupski and colleagues (Warner et al., 1996) proposed that P0 deleted for S63 (P0S63del) and associated in humans with CMT1B phenotype (Kulkens et al., 1993) would be unstable, resulting in the loss of one-half of P0 function, whereas substitution of serine to cysteine (P0S63S), associated with Déjérine-Sottas syndrome in humans (Hayasaka et al., 1993) might produce aggregates and a toxic gain of function in the myelin sheath. To test this hypothesis, we produced transgenic mouse models expressing the mutated alleles, using the same approach as for *Mpz* overexpression (a random transgene inserted in the mouse genome, in addition to the two endogenous *Mpz* alleles, in a way that only phenotypes resulting from gain of function can be observed). Both S63del and S63C mice develop neuropathies that resemble the corresponding human neuropathy. Each P063 mutant acts via gain of abnormal function, but their pathogenetic mechanisms originate from different intracellular locations. S63C arrives to the myelin sheath where it produces a packing defect, whereas S63del is retained in the ER and induces an Unfolded Protein Response (UPR), probably toxic to the myelin-forming Schwann cell. Interestingly, this was the first evidence for UPR associated with peripheral neuropathies (Wrabetz et al., 2006).

In this thesis, I describe the generation and characterization of one authentic mouse model of an HMSN mutation, associated in humans with the most severe phenotype resulting from MPZ mutations, the Congenital Hypomyelination Neuropathy. Thus, to generate this authentic model, we decided to use homologous recombination in ES cells in order to replace one mouse endogenous *Mpz* allele, with one human MPZ allele, carrying the specific mutation in its open reading frame. This mutation is a C to T transition in exon 5 that, once translated, generates a premature stop codon in the cytoplasmic domain of P0. Our additional hope then is that the

characterization of this mouse model could give more indication of the role of the P0 cytoplasmic tail in peripheral nerve myelination.



## 2. RESULTS

The purpose of this thesis is to describe and discuss the creation and characterization of a new transgenic mouse model of P0Q215X mutation associated with Congenital Hypomyelination neuropathy in humans.

### 2.1 Patients carrying P0Q215X mutation

In 1996, Warner et al. described a patient that presented early in life (10 months of age) with delayed motor milestones and severely reduced nerve conduction velocity (= NCV, 6 m/s), indicative of a generalized demyelinating peripheral neuropathy. A sural nerve biopsy was performed 8 months later and revealed abnormally thin myelin sheaths with a mild reduction of the number of myelinated fibers; no axonal degeneration was visible and no macrophages could be observed. Rare rudimentary onion bulbs were seen. Occasionally, large axons were found that had no myelin and were surrounded by Schwann cells with abundant cytoplasm. All these morphological defects were more indicative of an abnormal development of myelin rather than the result of a failed remyelination process, following demyelination. For this reason the patient was diagnosed with a Congenital Hypomyelination. The patient was then examined at 6 and 8 years of age and nerve conduction velocity confirmed the previous findings.

Heteroduplex analysis of the patient highlighted the presence of a base change in exon 5 of the *MPZ* gene, a C to T transition at nucleotide 643 that creates a premature stop in the Gln 215 codon (numbering includes 29-residue leader sequence). Further analyses indicated that the mutation was a de novo mutation, not inherited from one of the parents, and that it was a disease-associated mutation.

Another patient, carrying the same de novo mutation in the *MPZ* gene was then described (Mandich et al., 1999); the patient was 12 months old when she was first evaluated by a neurologist because of delayed

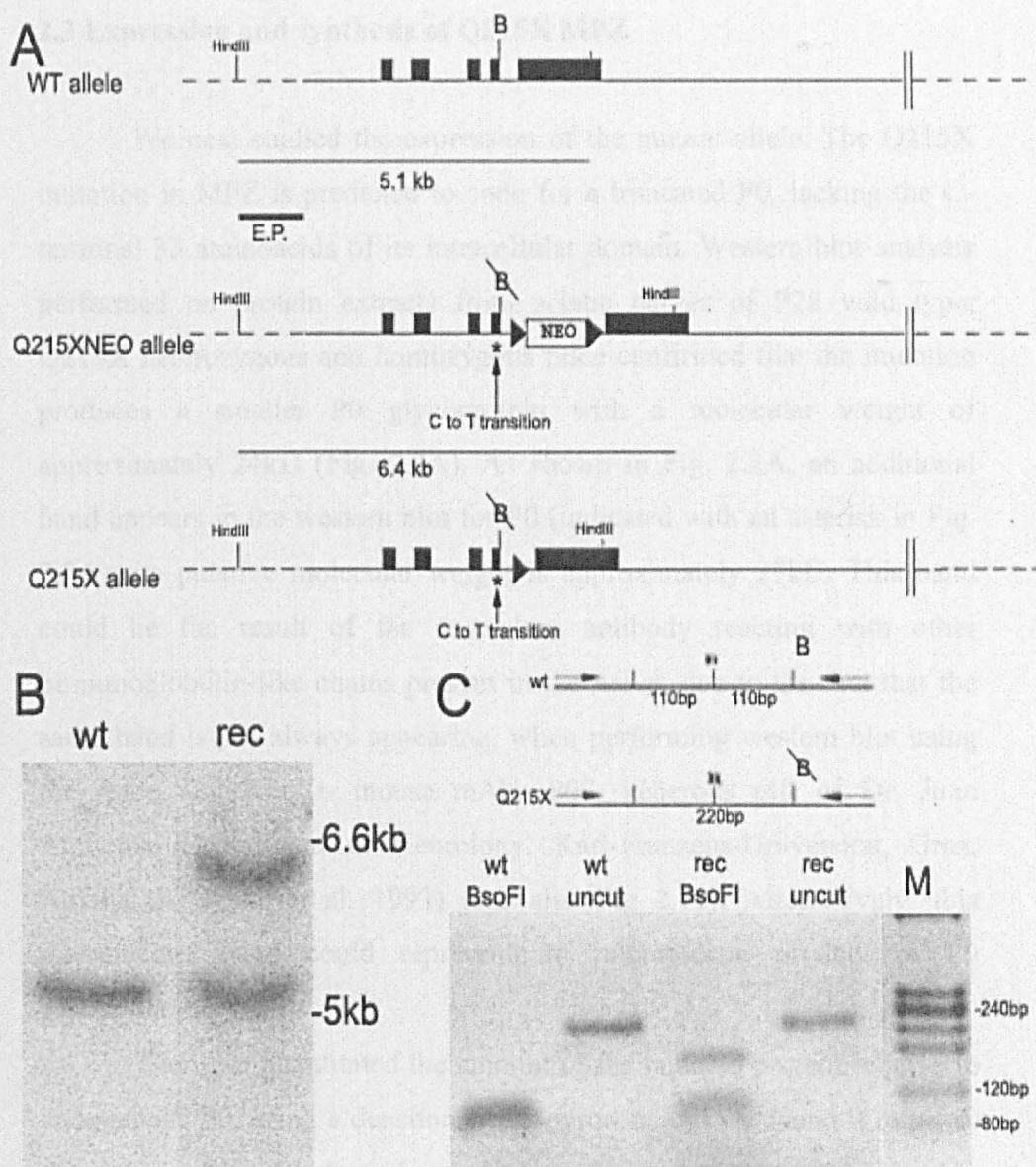
motor milestones; the neurological examination revealed diffuse muscle weakness, hypotonia, reduced deep tendon reflexes, scoliosis and foot deformities (pes planus). NCVs at that age were absent. At 7 years of age, the patient confirmed the reported symptoms and manifested in addition sensory ataxia and distal muscle wasting of the lower limbs. Nerve conduction velocities reached a value of approximately 4 m/s and sensory nerve action potentials were not recordable. A sural nerve biopsy confirmed the pathological findings described in the previous patient by Warner: loss of myelinated fibers, hypomyelination of all the remaining sheaths, several completely unmyelinated axons frequently surrounded by Schwann cells with copious cytoplasm and, finally, atypical onion bulbs.

## 2.2 Generation of *Mpz* Q215X/+ mice

To express the Q215X mutated MPZ in mice, we generated a targeting vector (Fig. 2.1A), which contains the whole *Mpz* gene, except for the 5' untranslated region, exon 1 and part of intron 2. This targeting vector carries the C to T mutation in exon 5 and a neomycin gene expression cassette, for selection, inserted in intron 5 and flanked by two LoxP sites. The linearized vector was electroporated into TBV2 *129SVPas* embryonic stem (ES) cells and one potential recombinant was identified among 168 G418 selected clones, using a Southern blot assay, with a hybridization probe 5' to the targeting construct. As expected for a homologous recombination event, the endogenous Hind III fragment was larger by 1.3kb, due to the presence of the neomycin insertion (Fig. 2.1B). We confirmed the absence of potential concatemers at the P0 locus using two different probes inside the targeting vector, in order to evaluate for concatamer junction fragments in Southern Blot analysis (data not shown). The C to T transition in exon 5 results in the ablation of a restriction site for BsoFI endonuclease. To confirm the presence of the Q215X mutation in the genomic DNA of the ES clone, we amplified by PCR the genomic region flanking the mutation and we digested it with BsoFI. Restriction enzyme digestion revealed the expected absence of the BsoFI site in the



PCR amplimer (Fig. 2.1C). Targeted ES cells were injected into C57Bl/6 blastocysts and one germline male chimera transmitted the mutation to the offspring. We obtained mice carrying the Q215X NEO allele (Fig. 2.1A); these mice were then crossed with mice expressing CRE recombinase ubiquitously, to excise the neomycin selection cassette and we obtained mice having the Q215X mutated allele, which still carries one Lox P site in intron 5 (Fig. 2.1A).



**Fig 2.1:** **A)** Schematic representation of the genomic organization of wild-type, Q215XNEO and Q215X *Mpz* alleles. The external probe used for Southern Blot analyses of ES cell clones is indicated (E.P.), together with the length of the DNA fragments, originating upon HindIII digestion. The C to T mutation in exon 5 is indicated by asterisks. The BsoFI site within exon 5 is indicated (B).

**B)** Southern Blot analysis of the genomic DNA of the ES cell clone where homologous recombination occurred.

**C)** BsoFI restriction enzyme digestion of the PCR-amplified genomic region flanking the C to T mutation in exon 5. The BsoFI site within exon 5 is indicated (B).

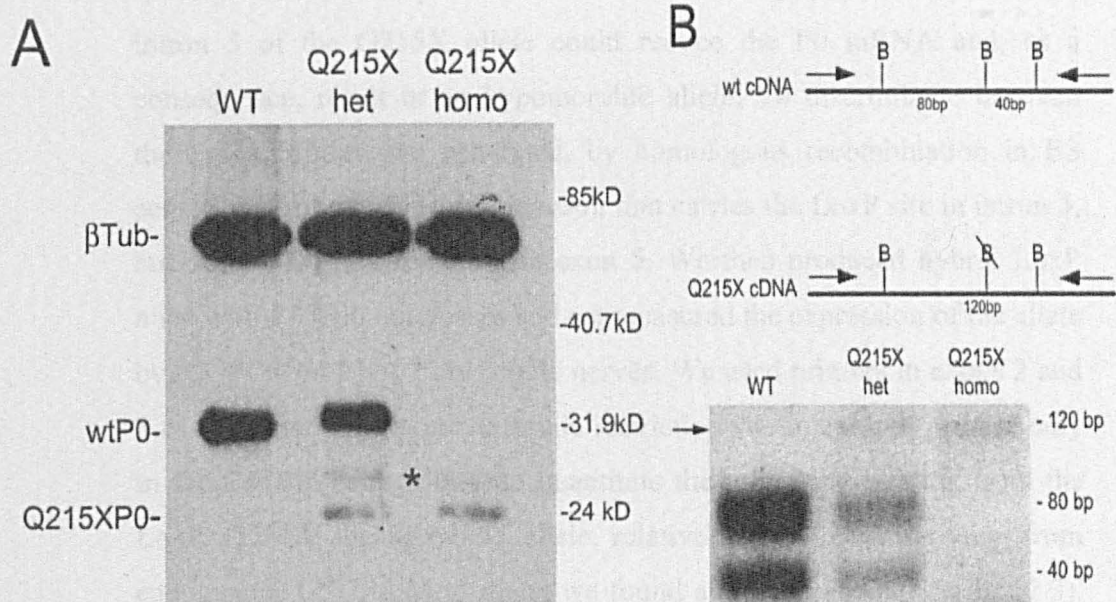
### 2.3 Expression and synthesis of Q215X MPZ

We next studied the expression of the mutant allele. The Q215X mutation in MPZ is predicted to code for a truncated P0, lacking the C-terminal 33 aminoacids of its intracellular domain. Western blot analysis performed on protein extracts from sciatic nerves of P28 wild type, Q215X heterozygous and homozygous mice confirmed that the mutation produces a smaller P0 glycoprotein with a molecular weight of approximately 24kD (Fig. 2.2A). As shown in Fig. 2.2A, an additional band appears in the western blot for P0 (indicated with an asterisk in Fig. 2.2A); its putative molecular weight is approximately 27kD. This band could be the result of the secondary antibody reacting with other immunoglobulin-like chains present in the nerve, due to the fact that the same band is not always appearing, when performing western blot using the same antibody (= mouse mAbs P07, generous gift of Dr. Juan Archelos, Department of Neurology, Karl-Franzens-Universitat, Graz, Austria; Archelos et al. 1993) (see also Fig 2.11). Alternatively, this intermediate band could represent an intermediate product of P0 glycosylation.

Then, we quantitated the amount of the mutated protein relative to endogenous P0, using a densitometric approach, and we found it to be 10 times less abundant than the endogenous one (ratio of approximately 1:10). Of note that the amount of protein extract loaded on each lane was normalized using  $\beta$ -Tubulin as a reference (Fig. 2.2A).

Thus, in order to understand if this reduction in protein amount was determined at the level of transcription or translation, we performed an RT-PCR analysis on RNA from P28 sciatic nerves of wild type, Q215X heterozygous and homozygous mice. We amplified the mutated region with primers that recognized exon 4 and 6 of Mpz, flanking the point mutation in exon 5 and we could distinguish and quantitate the Q215X mutated transcript relative to endogenous P0 mRNA, after digestion with BsoFI restriction enzyme. As shown in Fig 2.2B, this analysis indicated that the amount of transcript deriving from the mutated allele is

significantly lower (about 1:20) than the amount of transcript deriving from wild type *Mpz* allele, indicating that very likely the reduction in protein amount could be due to reduced steady state levels of mRNA. Notably, this reduction in the total amount of P0 mRNA is even more pronounced in the Q215X homozygous mice, where the amount of the mutated mRNA is less than double the levels of Q215X mutated transcript present in the heterozygous state.



**Fig 2.2: A)** P0 Western Blot analysis on protein extracts from sciatic nerves of wild type, Q215X/+ and Q215X/Q215X sacrificed at postnatal day 28. Normalization has been performed using  $\beta$  Tubulin as reference. The asterisk indicates an additional band appearing in the western blot for P0 with a putative molecular weight of approximately 27kD.

**B)** BsoFI restriction enzyme digestion of the RT-PCR-amplified cDNA, obtained RNA extracted from wild type, Q215X/+ and Q215X/Q215X P28 sciatic nerves. The BsoFI sites within the amplified cDNA are indicated (B).

## 2.4 Effect of the LoxP site in intron 5 on MPZ expression

This reduction in P0 mRNA steady state levels could be due to a specific effect of the point mutation. Alternatively, the LoxP site left in intron 5 of the Q215X allele could reduce the P0 mRNA and, as a consequence, result in an hypomorphic allele. To discriminate between these possibilities, we generated, by homologous recombination in ES cells, a control mouse (LoxP mouse), that carries the LoxP site in intron 5, but not the Q215X mutation in exon 5. We then produced hybrid LoxP mice with C57Bl6 outcrosses and we measured the expression of the allele by RT-PCR on RNA from sciatic nerves. We used primers in exons 2 and 3 of *Mpz* that flank a polymorphic restriction site in exon 3, present only in the C57Bl6 background, to quantitate the transcript deriving from the LoxP (129SV background) allele relative to the one deriving from endogenous C57Bl6 *Mpz* allele: we found a ratio of about 1:5 (Fig. 2.3). This indicated that the LoxP site has an effect on P0 mRNA stability, but this effect could not account completely for the severe reduction in Q215X MPZ amount (1/10 of endogenous P0).





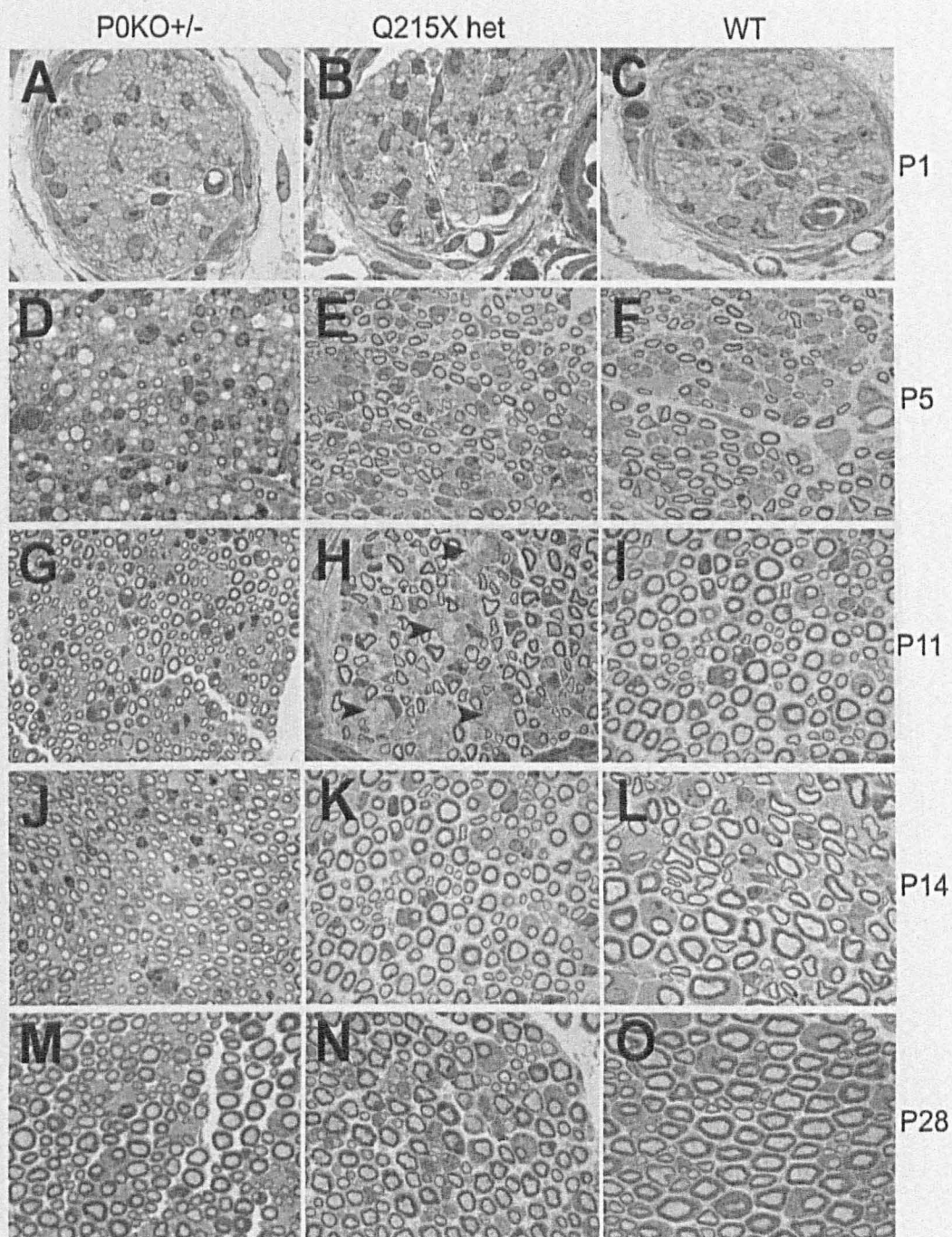
**Fig 2.3: A)** Schematic representation of the genomic organization of LoxP *Mpz* alleles.  
**B)** DpnII restriction enzyme digestion of the RT-PCR-amplified cDNA, obtained RNA extracted from wt (129SVPas/C57Bl6 - left lane) and LoxP(129SVPas/C57Bl6 - right lane) P28 sciatic nerves. The DpnII site within the amplified cDNA is indicated.

## 2.5 Q215X/+ mice develop transient Congenital Hypomyelination

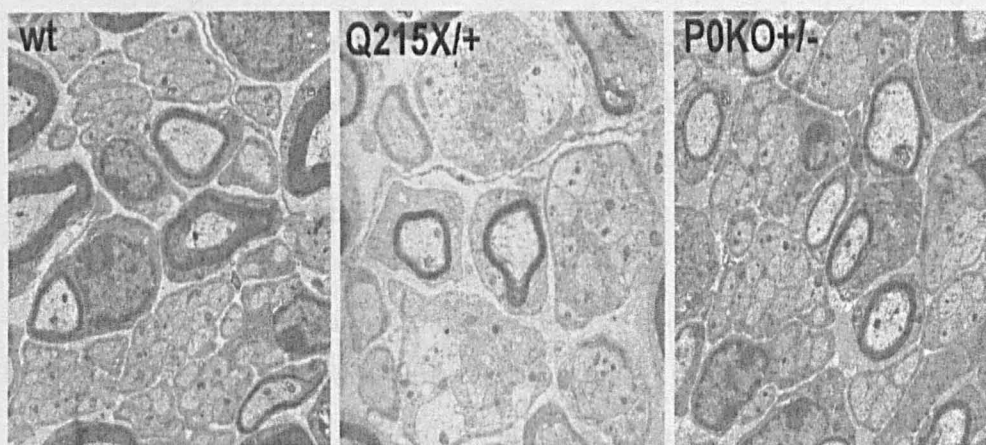
Since the Q215X mutation in human is autosomal dominant, we focused our phenotypical and morphological analyses on Q215X heterozygous mice. Surprisingly, although the phenotype in the patients has a very early onset and is remarkably severe, heterozygous mutant mice did not present any external sign of peripheral neuropathy (gait difficulty, reduced weight, tremor, atrophy of the paraspinal and hindlimb musculature). As shown in Fig. 2.4 (M-N-O), semi-thin section analysis of P28 sciatic nerves from Q215X heterozygous mice revealed only a mild hypomyelination, not consistent with the severe dysmyelinating neuropathy described in the patients. Thus, we analyzed the morphology of sciatic nerves in the first two weeks of postnatal life. During this period, when myelination takes place, P0 glycoprotein expression is induced at very high levels in myelin-forming Schwann cells. Sciatic nerves of P1 and P5 mutant mice did not show any obvious abnormality, when compared to wild type littermates (Fig. 2.4A to 2.4F). In contrast, at P11, some bundles of unsorted mixed calibre axons were present in Q215X heterozygous mice (Fig. 2.4H-arrowheads), and not in wild type littermates (Fig. 2.4I) or in P0 haploinsufficient mice (Fig. 2.4G). At P14, these radial sorting defects were not visible anymore indicating that the myelination deficit observed in P11 Q215X heterozygous mice is transient.

In order to confirm and characterize the radial sorting defects, we decided to study bundles of wt, P0KO +/- and Q215X/+ P11 nerves by electron microscopy (Fig. 2.5). In all the nerves we analyzed, the bundles are surrounded by Schwann cells, with normal basal lamina and include mixed calibre axons, with diameters ranging approximately from 0.2 to 2  $\mu\text{m}$ . In most of the bundles, surrounding SCs send their cytoplasmic processes within bundles, sorting single axons away from contact with their neighbours.





**Fig 2.4:** Semi-Thin Sections of sciatic nerves taken from wild type, Q215X/+ and P0KO +/- mice, at P1 (A-B-C), P5 (D-E-F), P11 (G-H-I), P14 (J-K-L) and P28 (M-N-O). In H, arrowheads indicate bundles of unsorted axons. Magnification 100X.

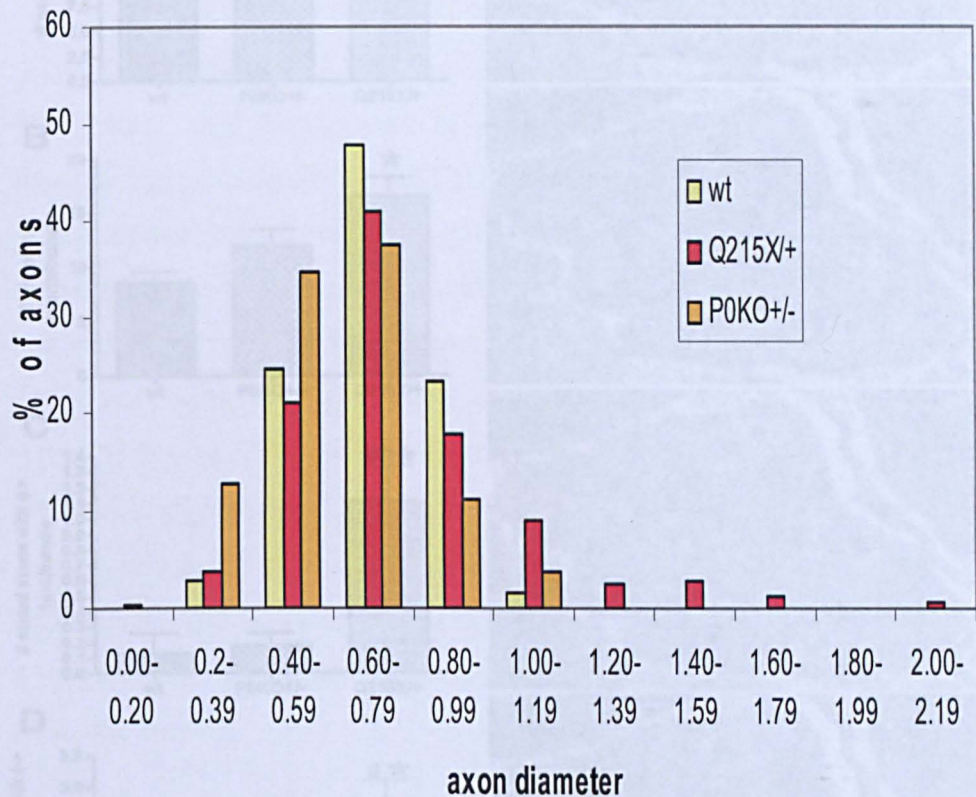


**Fig 2.5:** Electromicrographs of sciatic nerves taken from wild type, Q215X/+ and P0KO +/- mice at P11, showing details of bundles of unsorted axons. Magnification 20000X.

To quantitate these abnormalities, we used the NIH Image J software: first, we performed detailed morphometric analyses to look at the distribution of fiber diameters in unsorted bundles. As shown in Fig. 2.6, bundles from wt (in total 24 from n=3 mice) and P0KO +/- (in total 23 from n=3 mice) nerves at P11 contain none or few axons with diameter < 1 $\mu$ m (the maximum diameter they reach being < 1.2 $\mu$ m), whereas bundles from Q215X/+ animals (in total 34 from n=3 mice) include axons with diameters larger than 1.4 $\mu$ m (the maximum diameter they reach being approximately 2.2 $\mu$ m). In addition, we counted the total number of axons present in the bundles; as shown in Fig. 2.7A, our analysis revealed that the Q215X heterozygous mice present a higher, although not to a significant extent, number of axons in the bundles (indicated by asterisks in Fig 2.7A); secondly, we examined the number of unsorted axons (an unsorted axon being in direct contact with other axons and not surrounded only by SC processes; indicated by asterisks in Fig. 2.7B) and we found it significantly higher in Q215X/+, as compared to both wild type and P0KO heterozygous mice. Third, we also counted the number of sorted (not in close contact with other axons, but surrounded exclusively by SC processes, indicated by asterisks in Fig. 2.7C) and unsorted axons (indicated by asterisks in Fig. 2.7D) present within the bundles and having a diameter greater than 1  $\mu$ m; by this age (P11), in fact, every large calibre axon in a normal nerve has already been segregated and presents at least some turns of compact myelin. As shown by the graphs, both parameters are significantly higher in the Q215X/+ than in the other two genotypes examined; in fact, while in control nerves all the axons larger than 1 $\mu$ m in diameter had been already segregated and at least partially myelinated, in contrast, approximately 15% of the axons we measured in the bundles of mutant Q215X/+ mice had a diameter ranging from 1 to 2  $\mu$ m (Fig. 2.6). Taken together, these data showed that Q215X heterozygous mutant mice present a transient dysmyelinating neuropathy, due to a radial sorting defect that appears around P10 and that disappears around P14, leading to a delay in the myelination process. Since we have already demonstrated

that in Q215X heterozygous mutant mice the amount of the mutated protein relative to endogenous P0 was 10 times less abundant, one can argue that the transient phenotype we described could be the result of an overall reduction of the total amount of P0 glycoprotein in Q215X heterozygous mutant mice. Nevertheless, our morphological analysis demonstrated that the radial sorting defect observed in Q215X heterozygous mutants is specifically due to the presence of the Q215X mutated glycoprotein and not to the lower amount of MPZ in mutant mice. This is clearly demonstrated by the absence of similar unsorted bundles of large calibre axons in P0 null heterozygous mice; in P0KO +/- mice in fact the amount of total P0 mRNA is lower than in Q215X heterozygous mice. This absence demonstrates that the Q215X point mutation acts through a gain of function mechanism.





**Fig 2.6:** Axon diameter (in  $\mu\text{m}$ ) distribution within bundles of unsorted axons, in sciatic nerves taken from wild type, Q215X/+ and P0KO +/- mice at P11. This graph reports the percentage of axons/genotype, measured within the bundles, with a given diameter, over the total number of axons examined/genotype.

Fig 2.7: A) Total number of axons within the bundles (sorted + unsorted). Error bars represent SEM.

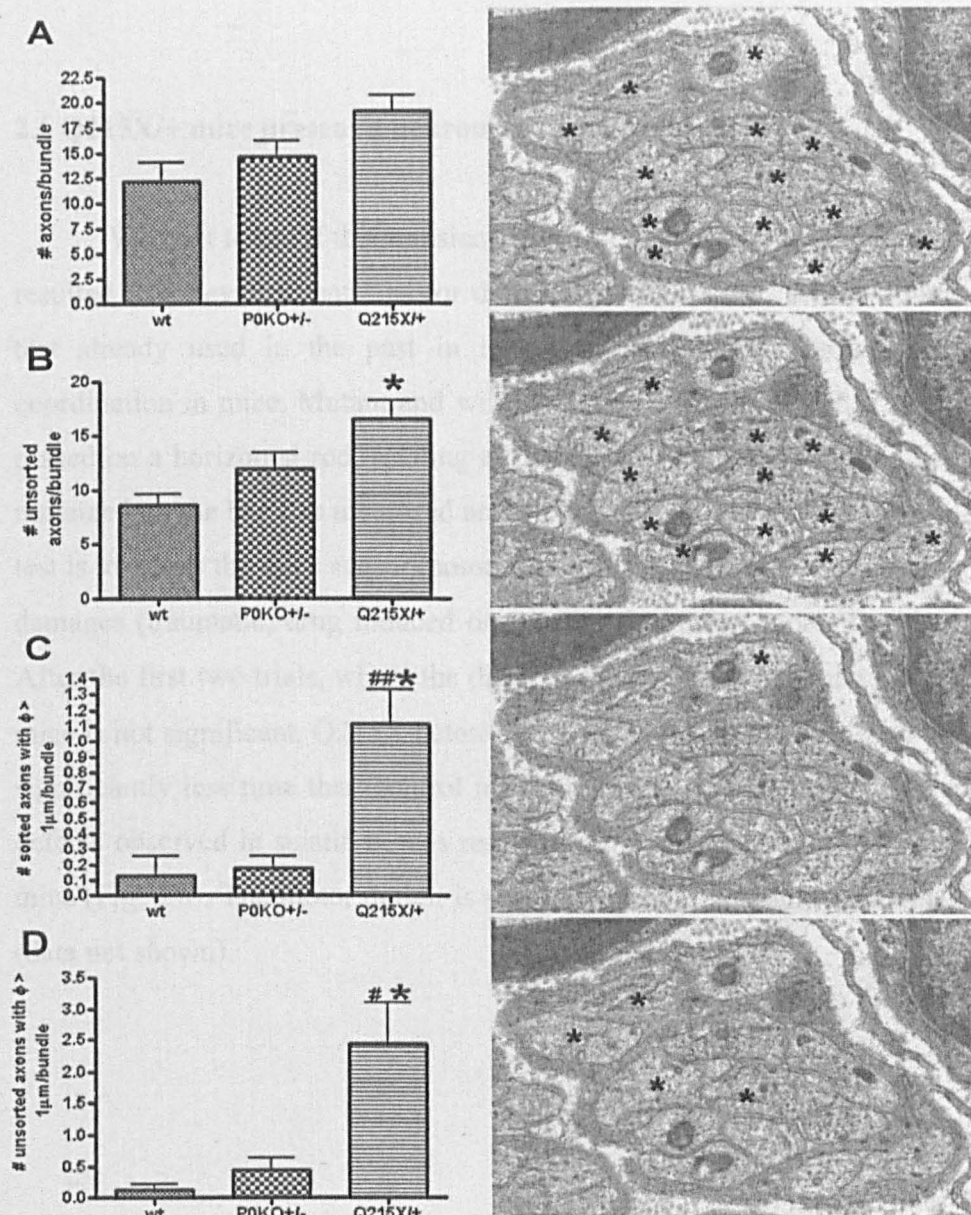
B) number of unsorted axons (in direct contact with other axons, indicated by asterisks in the right panel) within the bundles.

C) number of sorted axons (not in direct contact with other axons, but surrounded by P-0 processes, indicated by asterisks in the right panel) within the bundles, with a diameter  $> 1\mu\text{m}$ .

D) number of unsorted axons (in direct contact with other axons, indicated by asterisks in the right panel) within the bundles, with a diameter  $> 1\mu\text{m}$ .

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. wild type (one-way ANOVA followed by Tukey test).

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. P0KO +/- (one-way ANOVA followed by Fisher test).



**Fig 2.7: A)** Total number of axons within the bundles (sorted + unsorted). Error bars represent SEM.

**B)** number of unsorted axons (in direct contact with other axons, indicated by asterisks in the right panel) within the bundles.

**C)** number of sorted axons (not in close contact with other axons, but surrounded by SC processes, indicated by asterisks in the right panel) within the bundles, with a diameter > 1μm.

**D)** number of unsorted axons (in direct contact with other axons, indicated by asterisks in the right panel) within the bundles, with a diameter > 1μm.

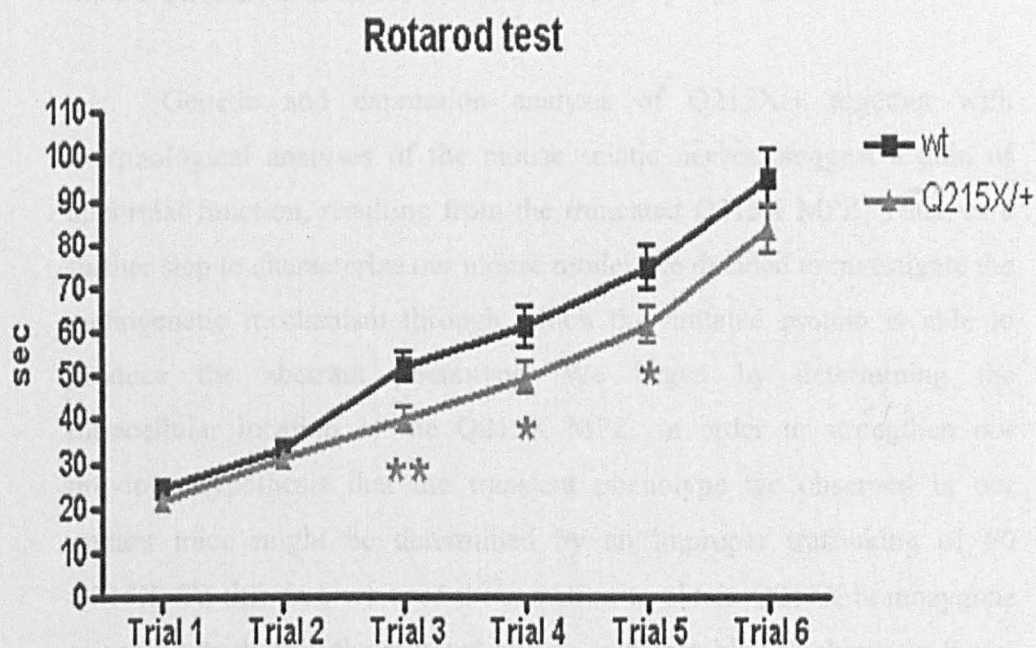
\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. wild type (one-way ANOVA followed by Tukey test)

# $p < 0.05$ ; ## $p < 0.01$ ; ### $p < 0.001$  vs. P0KO+/- (one-way ANOVA followed by Fisher test)

## **2.6 Q215X/+ mice present a neuromuscular defect**

We next tested if this transient defect in the process of myelination resulted in a developmental motor deficit, by rotarod test, a behavioural test already used in the past in Dr. Wrabetz's lab to assess motor coordination in mice. Mutant and wild type mice, from P10 to P12, were placed on a horizontal rod, rotating at increasing speed and the time they remained on the bar was measured and plotted. The purpose of the rotarod test is to assess the mice sensorimotor coordination. The test is sensitive to damages (traumatic, drug induced or genetic) that effect motor function. After the first two trials, where the difference between control and mutant mice is not significant, Q215X heterozygous mice remained on the bar for significantly less time than control mice, revealing that the radial sorting defects observed in sciatic nerves result in a motor deficit in P11 mutant mice (Fig. 2.8). The motor deficit is no longer present in adult (P28) mice (data not shown).



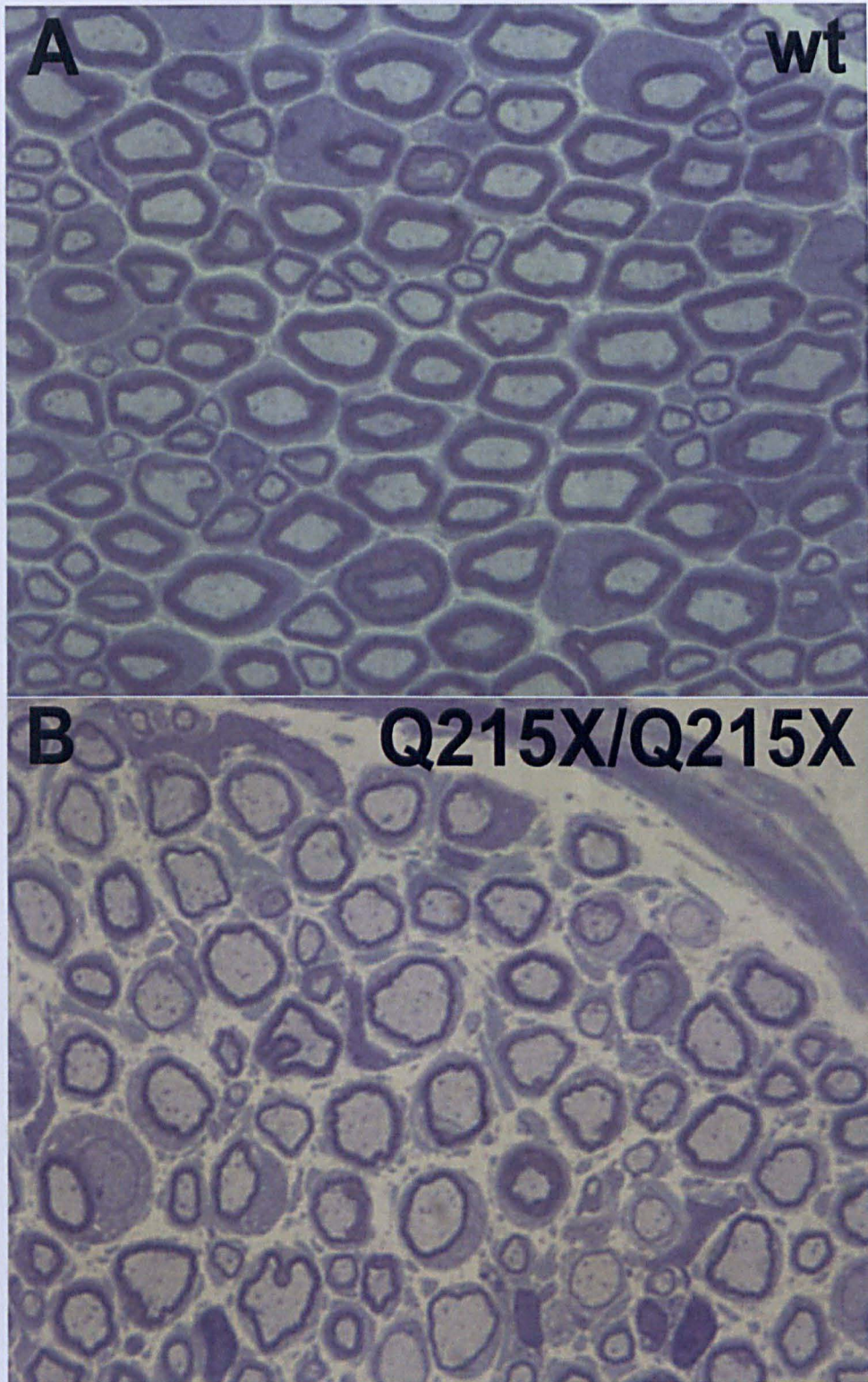


**Fig 2.8:** Rotarod analysis shows that Q215X/+ mice at P11 (n=72) remain on the accelerating cylinder less time than the wild type littermates (n=57). Error bars represent SEM. \*p<0.05; \*\*p<0.01 vs. wild type (t-test)



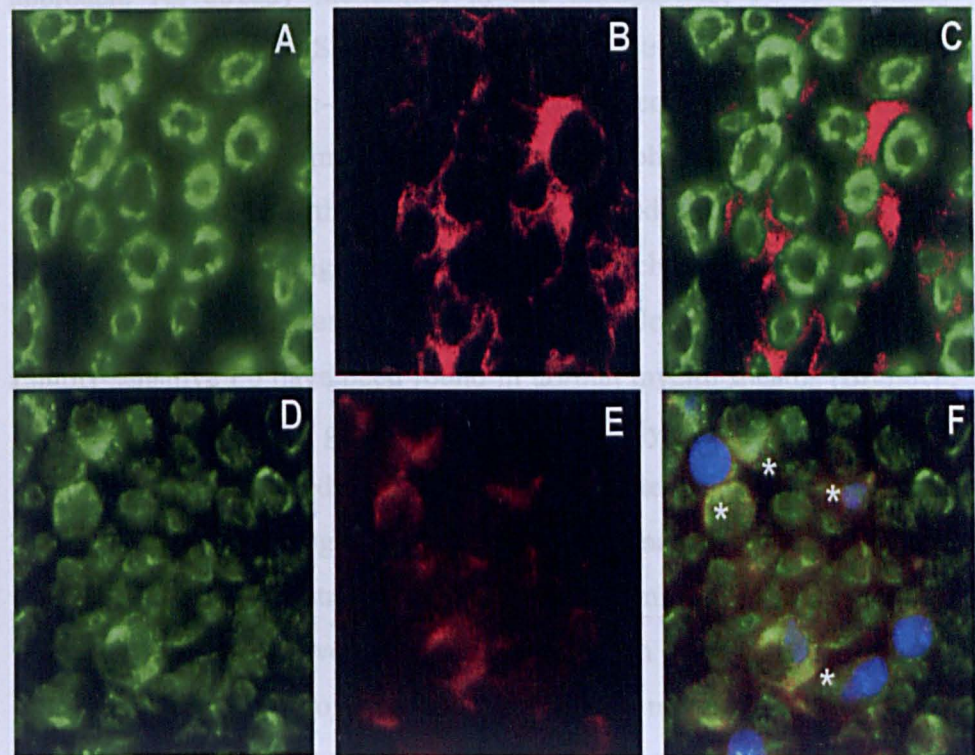
## 2.7 P0 Q215X MPZ is not trafficked correctly

Genetic and expression analysis of Q215X/+ together with morphological analyses of the mouse sciatic nerves, suggest a gain of abnormal function, resulting from the truncated Q215X MPZ. Thus, as a further step to characterize our mouse model, we decided to investigate the pathogenetic mechanism through which the mutated protein is able to produce the aberrant phenotype. We began by determining the intracellular location of the Q215X MPZ, in order to strengthen our previous hypothesis that the transient phenotype we observed in our mutant mice might be determined by an improper trafficking of P0 Q215X. To this end, we bred the mutation to obtain Q215X homozygous mice, in which only the mutated protein is detectable. As shown in figure 2.9A and B, semi-thin sections of these nerves, at P28, revealed a substantial hypomyelination, with the presence of many fibers with thin myelin sheaths for the diameter of axons, and few fibers in which single Schwann cells ensheathed axons, but were not able to form any myelin sheath around them. Then, we stained transverse sections of sciatic nerves from P28 homozygous mutant and control mice, using an antibody directed against the extracellular domain of P0. To localize intracellularly the mutated MPZ, we co-stained the sections with an antibody that recognizes the KDEL signal, a tetrapeptide located at the carboxy-terminal sequences of Endoplasmic Reticulum (= ER) luminal proteins, that perform essential functions related to protein folding as well as assembly. In control nerves (Fig. 2.10 A-B-C), almost all P0 glycoprotein was detected in compact myelin and its signal never co-localized with ER resident proteins. In contrast, in homozygous mutant mice (Fig. 2.10 D-E-F), Q215X truncated P0 partially co-localized with anti KDEL antibody, indicating that the mutated protein is, at least in part, sequestered in the ER.



**Fig 2.9:** Semi-Thin Sections of sciatic nerves taken from wild type (A) and Q215X/Q215X (B) mice at P28. Magnification 100X.





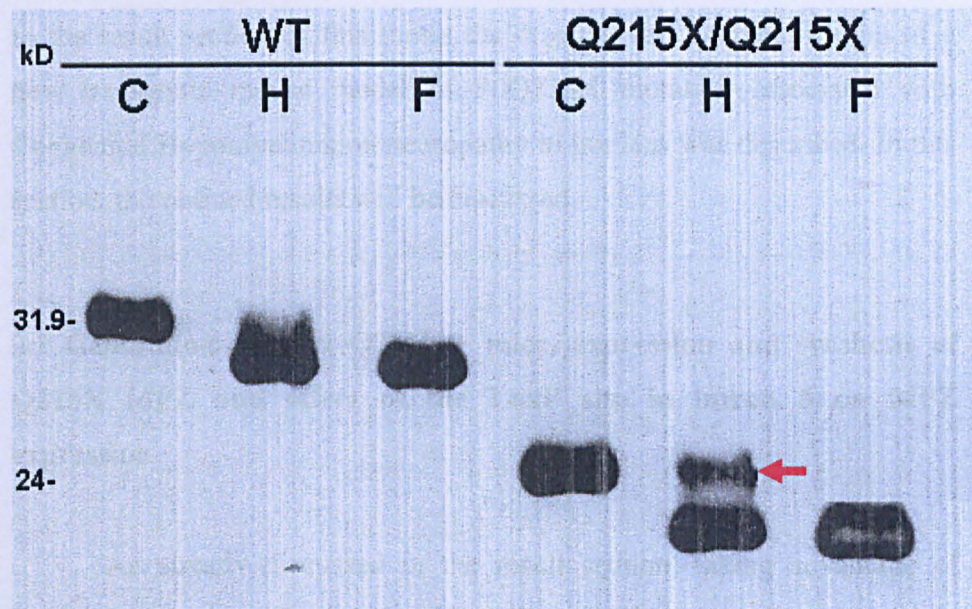
**Fig 2.10:** Immunofluorescence staining for P0 (A,D) or KDEL (B, E) or overlay of the two (C, F) in WT (A, B, C) and Q215X/Q215X (D, E, F) sciatic nerves at P28. P0 and KDEL stainings were almost mutually exclusive in wild-type nerves, with most P0 staining in circular myelin sheaths. In contrast, a part of P0 and KDEL staining coincided in Q215X/Q215X nerves (see asterisks). Magnification 100X.

In addition to the immunohistochemical analyses, to further characterize the intracellular location of the mutant protein, we analyzed the glycosylation of Q215X P0, using a specific glycosidase digestion approach. As already mentioned in the introduction, P0 is normally glycosylated within the Schwann cell ER, at the level of N122 (D'Urso et al., 1990). In the pre-Golgi and cis-Golgi compartments, N-linked glycoproteins contain immature high-mannose oligosaccharides that are sensitive to digestion with endoglycosidase H (EndoH). When the proteins arrive at the medial-Golgi compartment, oligosaccharides are processed to mature, becoming resistant to EndoH digestion. Both EndoH-resistant and EndoH-sensitive P0 have been found in normal myelin sheaths (Brunden, 1992). Another specific glycosidase, peptide *N*-glycosidase F (PNGaseF), is able to digest N-linked oligosaccharides, despite their maturation. After EndoH or PNGaseF digestion, Western Blot analysis of mutant nerve protein lysates demonstrated that our cytoplasmic tail truncated P0 is mostly glycosylated; nevertheless a small fraction of it is EndoH resistant (indicated by a red arrow in Fig. 2.11). Thus, most of P0 Q215X has immature glycosylation, which could be consistent with ER or Golgi localization, but some may arrive to the myelin sheath.

Of note that, in this western blot analysis no additional band for P0 with an approximate molecular weight of 27kD appears, even when endoglycosidase digestion occurred. This demonstrates that this intermediate band could not represent an intermediate product of P0 glycosylation, but it is very likely the result of the secondary antibody reacting with other immunoglobulin-like chains present in the nerve.



### 3. DISCUSSION



**Fig. 2.11:** Biochemical and expression analysis of Q215X mutants shows that the mutated P0 has altered post-translational modification.

Western analysis for P0 on sciatic nerve lysates from WT or Q215X homozygous mutants untreated (C) or after digestion with EndoH (H) or PNGaseF (F).

intron 5 (Fig. 2.1A). We then studied the expression of the mutant allele. We have shown that the Q215X mutation in MPZ produces a smaller P0 glycoprotein with a molecular weight of approximately 24kD (Fig. 2.2A). Since both Warner and colleagues and Mandich and her collaborators did not examine their patient biopsies for the presence of a truncated form of P0, lacking part of its intracellular domain, this is the first demonstration that the C to T transition in exon 3 of *MPZ* gene observed in the two different patients effectively results in a shorter form of P0 glycoprotein, with a lower molecular weight, as compared to wild type protein.

Then, we quantified the amount of the mutant protein relative to the the endogenous P0 and we found it to be 10 times less abundant than the endogenous one (ratio of approximately 1:10 - Fig. 2.2A). By RT-PCR analyses we then demonstrated that very likely the reduction in protein amount could be due to reduced steady state levels of mRNA in mutant mice. In addition, we generated and characterized a control *LoxP* mouse carrying the *LoxP* site in intron 3, but not the Q215X mutation in exon 3.

### 3. DISCUSSION

In the result section of this thesis the creation and characterization of a new transgenic mouse model of P0Q215X mutation associated with Congenital Hypomyelination neuropathy in humans was described. In this section, the obtained results will be discussed.

#### 3.1 Generation of *Mpz* Q215X/+ mice, expression and synthesis of Q215X MPZ and effect of the LoxP site in intron 5 on MPZ expression

As already discussed in the result section, taking advantage of homologous recombination in ES cells and electroporating them into mouse blastocysts, we obtained mice carrying the Q215X NEO allele (Fig. 2.1A) that were then crossed with mice expressing CRE recombinase ubiquitously, to excise the neomycin selection cassette in order to generate mice having the Q215X mutated allele and carrying one Lox P site in intron 5 (Fig. 2.1A). We then studied the expression of the mutant allele. We have shown that the Q215X mutation in MPZ produces a smaller P0 glycoprotein with a molecular weight of approximately 24kD (Fig. 2.2A). Since both Warner and colleagues and Mandich and her collaborators did not examine their patient biopsies for the presence of a truncated form of P0, lacking part of its intracellular domain, this is the first demonstration that the C to T transition in exon 5 of *Mpz* gene observed in the two different patients effectively results in a shorter form of P0 glycoprotein, with a lower molecular weight, as compared to wild type protein.

Then, we quantitated the amount of the mutated protein relative to the the endogenous P0 and we found it to be 10 times less abundant than the endogenous one (ratio of approximately 1:10 - Fig. 2.2A). By RT-PCR analyses we then demonstrated that very likely the reduction in protein amount could be due to reduced steady state levels of mRNA in mutant mice. In addition, we generated and characterized a control LoxP mouse carrying the LoxP site in intron 5, but not the Q215X mutation in exon 5.

We then measured the expression of the LoxP allele by RT-PCR on RNA from sciatic nerves, relative to the one deriving from endogenous C57Bl6 Mpz allele (Fig. 2.3). We demonstrated that the LoxP site has an effect on P0 mRNA stability, reducing it, but not to the same extent as for Q215X MPZ allele. Interestingly, such an effect of the LoxP insertion on mRNA steady state levels was never reported before. Therefore, the mechanism by which LoxP reduces mRNA levels is not clear. Of note that intron 5, the one bearing the LoxP site in the mutated Mpz allele, is very short, only 107bp in length. It is well known that the process of transcription is slow and, at least for highly expressed genes, transcription of long introns, particularly common in mammals, is expensive. As a consequence, it has been demonstrated that introns in highly expressed genes are shorter than those in genes that are expressed at low levels, and natural selection appears to favour short introns in highly expressed genes to minimize the cost of transcription and splicing (Castillo-Davis et al., 2002). In order to express the Q215X mutated MPZ in vivo, we generated mice having the Q215X mutated allele, carrying one Lox P site in intron 5; this means that an original intron of 107 bp has reached the “new” length of approximately 220bp. Such an increase in intron length could explain the less efficient transcription of the mutated Mpz allele in the LoxP mouse, but at the same time it may not be the only reason for the massive reduction of Q215X mutated transcript. One possible explanation for this further decrease in Q215X mRNA is Nonsense-Mediated Decay; it is known in fact that the strength of biological systems depends on the function of proofreading mechanisms preventing errors. Studies in yeast (Losson and Lacroute, 1979) and of human genetic disorders (Chang and Kan, 1979) allowed the identification of a conserved control mechanism that identifies faulty open reading frames and eliminates imperfect mRNAs that contain premature translation termination codons (PTCs) and code for nonfunctional or dangerous polypeptides; this mechanism has been termed nonsense-mediated mRNA decay (NMD) (Hentze and Kulozik, 1999). Anyhow, the hypothesis that NMD may be responsible for the reduction of Q215X mRNA is not supported by the position of the PTC in the Q215X mutated allele; it is well known in fact that the

distinction between a normal and a premature stop codon is made on the basis of its location with respect to the last exon-exon junction: in particular, the termination codon is considered premature when it is positioned > 50-55 nucleotides upstream of the last exon-exon junction (Nagy and Maquat, 1998). Here the Q215X mutation is only 3 nucleotides from the last exon-exon junction. In addition to that, recent data demonstrated that NMD could be one of the molecular mechanisms by which different mutations, in particular truncating mutations, in the same gene can result in different disease phenotypes; in particular, the more severe phenotypes occur only when the mutated mRNAs are able to escape NMD (Inoue et al., 2004). The authors focused their attention on SOX 10 and MPZ mRNA containing PTCs: mutant mRNAs that result in a less severe disease have decreased stability, while the more severe neurological diseases seem to result from a stable mRNA that is translated into a mutant protein with potent dominant-negative activity. More specifically the authors tested several MPZ mutated transcripts (produced in transfected human cell lines) and found that the PTCs associated with severe diseases are all located in the last exon (except for one, Q215X, that is located at the distal end of the penultimate exon) and resulted in an accumulation of mRNA at levels equivalent to the wild-type allele. Since no expression data are available from Q215X human patient samples, our mutant mouse could be useful to confirm in vivo these in vitro findings and could help in clarifying the mechanisms that are responsible for the massive reduction of Q215X mutated transcript. For instance, we can test Q215X sensitivity to NMD in mouse SN by treating excised and desheathed SNs with cycloheximide (CHX) in culture medium. CHX is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. As a positive control we can use SNs from a mouse line, generated in the lab and expressing an Mpz transgene containing lac-z and a premature stop codon, more than 50-55 nucleotides upstream of the last exon-exon junction and therefore predicted to undergo NMD. After CHX treatment, mRNA can be extracted and retro-transcribed and expression evaluated by RT-PCR. If no difference in Q215X and LoxP-WT expression is detected after treatment, while, as



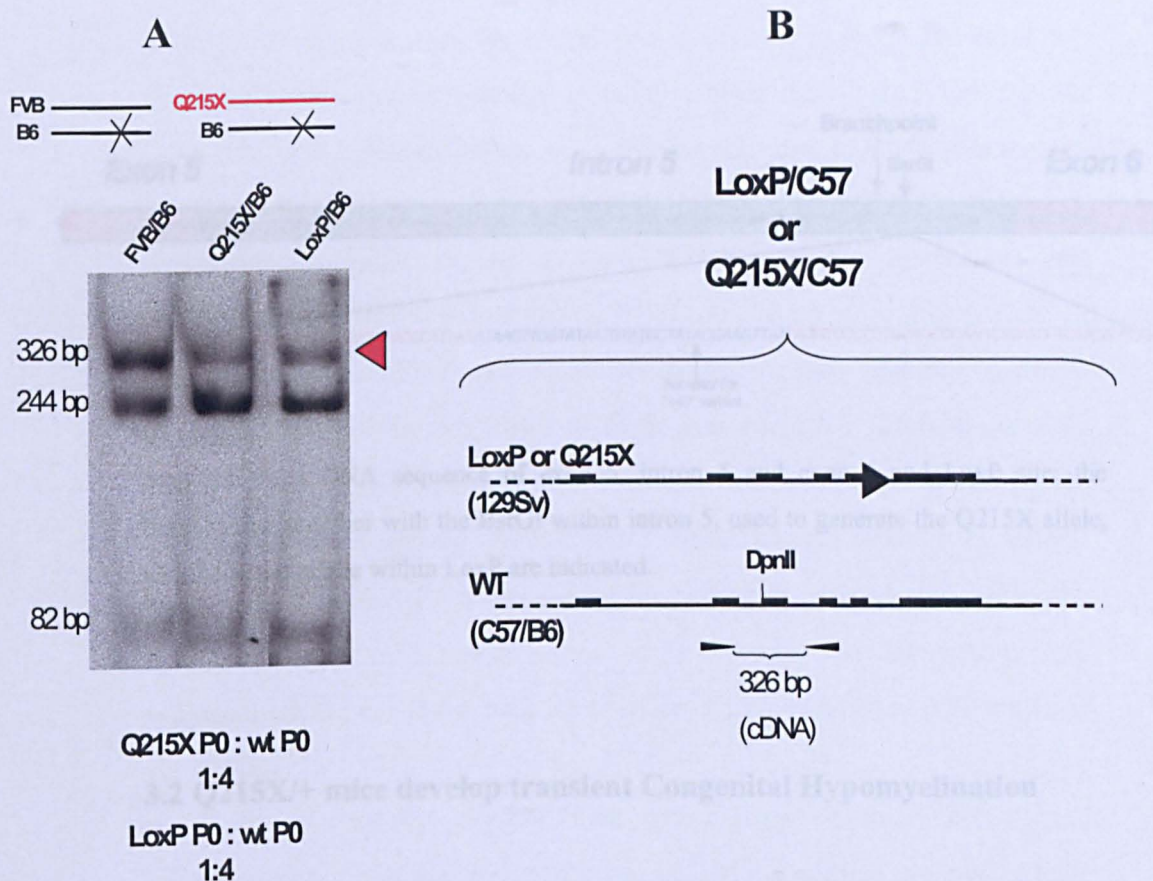
expected, there is a significant increase in the positive control, we could confirm that, also in vivo in a myelin context, Q215X does not undergo NMD.

Other potential mechanisms through which LoxP could negatively influence expression can act both at the transcriptional or post-transcriptional level. In fact, the LoxP-dependant expression reduction could be due to an effect of LoxP at the transcriptional level. Intronic sequences are known to be involved in transcriptional regulation and highly conserved sequences in *Mpz* intron 1 have previously been demonstrated to bind Sox10 and Egr2 transcription factors (TF) and that this binding plays a key role in *Mpz* expression (LeBlanc *et al.*, 2006). As already discussed, *Mpz* intron 5, where the LoxP site is inserted, is very short and highly conserved; there is the possibility to investigate potential TF binding sites, using the rVISTA software analysis and having intron 1 binding as positive control. Last year, a sequence in *mpz* intron 5 has been identified as a potential binding site for CTCF, the vertebrate insulator protein (Kim *et al.*, 2007). Interference with this binding could affect the euchromatin/heterochromatin status of the genomic region and thus alter the expression of *mpz* in our mice; we therefore can test by ChIP if CTCF does bind to *Mpz* intron 5 and if yes, ask in vivo in our LoxP control mouse if the presence of LoxP in intron 5 is able to abolish such binding.

Recently in Dr. Wrabetz's lab Q215X heterozygous mice have been brought into the C57 background; as a consequence, the *mpz* expression in Q215X heterozygous mice has been re-evaluated in relation to the WT allele, using semiquantitative radioactive RT-PCR and taking advantage of a DpnII restriction site present in the background strain allele (C57) and absent in the ES cell strain (Sv129), used for the generation of our transgene. Transgene expression in the line carrying the mutation (Q215X) and in the one carrying only the LoxP site was 1:4 compared to the WT allele, indicating that the mutation itself was not able to induce any reduction in P0 mRNA (Fig. 3.1). Interestingly, when examining *mpz* expression in the Q215X line through BsoFI digestion, the expression was quantified as 1:15 compared to WT. There is the possibility that such a difference in results, when analyzing two different segments of the same

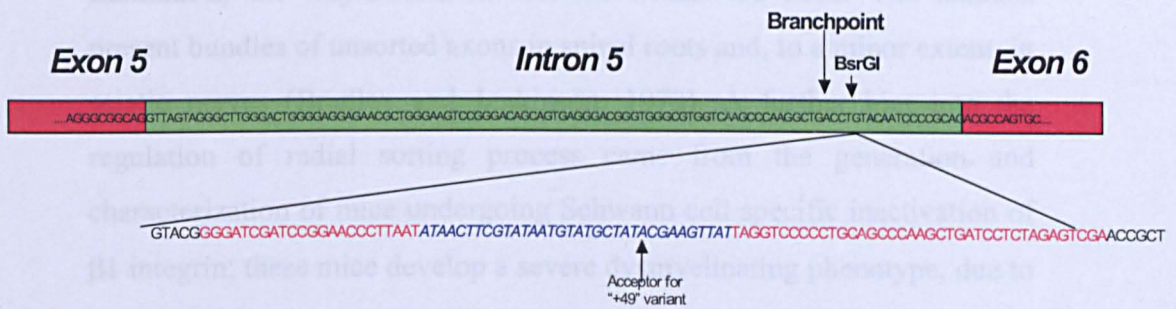
cDNA, could be due to the presence of alternative splicing products in the 3' region of the gene, where LoxP and the mutation are inserted. In order to test this hypothesis, different RT-PCRs from sciatic nerve cDNA from Q215X homozygous, LoxP homozygous and WT animals using primers extending from mpz exons 4 and 5 to exon 6 can be performed and all the resulting amplicon patterns can be carefully analyzed and compared. The possibility of alternative splicing occurring in all the lines carrying the LoxP in intron 5 is further supported by the finding of a detailed bioinformatic analysis of the exon 6 splice acceptor performed in Dr. Wrabetz's lab; this analysis contributed to identify a "weak" splicing acceptor site in exon 6. The presence of the LoxP site and its flanking sequence between the mpz intron 5 branchpoint and the exon 6 splice acceptor, along with the "weak" acceptor site in exon 6, may have contributed to the generation of these alternative splicing patterns. It's important to remark that the LoxP sequence, along with its flanking region, can function here as a cryptic splice acceptor (Fig. 3.2).

Alternative splicing at the exon 5-6 junction could explain the difference amongst the expression results using the polymorphic DpnII site in exon 3 or the BsoFI restriction site in exon 5, but doesn't justify the overall underexpression of the mpz-loxP alleles of 1:4 compared to WT mpz alleles. Increased instability of the alternatively spliced forms could explain this reduction. To test this hypothesis, LoxP homozygote sciatic nerves can be explanted, desheathed from the perinevrium to make them more accessible to treatment, and incubated at 37 C in culture medium with RNA polymerase II inhibitor actinomycin D (ActD). RNA at different time-points (1-2-4 hours) can be extracted and RT-PCRs for mpz performed, to analyze the stability of the different splice variants. This approach will give us the possibility to evaluate the half-life of the alternatively spliced variants, if any, and their contribution to the overall mpz expression reduction.



**Fig. 3.1:** A) DpnII restriction enzyme digestion of the RT-PCR-amplified cDNA, obtained from RNA extracted from wt (FVB/C57Bl6 - left lane), Q215X/+ heterozygous (129SVPas/C57Bl6 - mid lane) and LoxP/+ heterozygous (129SVPas/C57Bl6 - right lane) P28 sciatic nerves. The DpnII site within the amplified cDNA is indicated. The red arrow indicates the amplified DNA coming from the LoxP and Q215X Mpz alleles. B) Schematic representation of the genomic organization of LoxP and Q215X Mpz alleles. The DpnII site within exon 3 is indicated, together with the primer pair used to amplify the cDNA from sciatic nerve of Q215X heterozygous, LoxP heterozygous and WT animals using primers extending from mpz exons 2 to exon 4.





**Fig. 3.2:** Mpz DNA sequence of exon 5, intron 5 and exon 6 and LoxP site: the branchpoint, together with the BsrGI within intron 5, used to generate the Q215X allele, and the acceptor site within LoxP are indicated.

### 3.2 Q215X/+ mice develop transient Congenital Hypomyelination

As reported in the results, our phenotypical and morphological analyses on Q215X heterozygous mice revealed that heterozygous mutant mice present a transient dysmyelinating neuropathy, due to a radial sorting defect that appears around P10 and disappears around P14, leading to a delay in the myelination process. Furthermore, we demonstrated that the radial sorting defect observed in Q215X heterozygous mutants is specifically due to the presence of the Q215X mutated glycoprotein and not to the lower amount of MPZ in mutant mice. Finally, by comparing morphological features of our Q215X heterozygous with the ones of P0KO +/- mice, we clearly showed that the Q215X point mutation is acting through a gain of function mechanism.

What Gain of Function mechanism might explain the morphological phenotype we have observed in Q215X heterozygous animals? Analyses of mutant mice presenting similar phenotypes might be revealing. For example, one possible explanation for the transient dysmyelinating phenotype we observed in our mutant mice is that the protein, P0 Q215X, can somehow interfere with laminin signals, necessary for Schwann cells to segregate the axons. In fact, an impairment in the

process of radial sorting has been first described in mice deficient for Laminin-2, the major laminin isoform within the PNS. The animals present bundles of unsorted axons in spinal roots and, to a minor extent, in sciatic nerves (Bradley and Jenkinson, 1973). A further hint into the regulation of radial sorting process came from the generation and characterization of mice undergoing Schwann cell specific inactivation of  $\beta 1$  integrin; these mice develop a severe dysmyelinating phenotype, due to the inability of Schwann cells to form and maintain interactions with axons. In  $\beta 1$ -integrin null animals, Schwann cells can migrate, proliferate and survive as in normal mice. Careful ultrastructural analysis of mutant nerves revealed that  $\beta 1$  is necessary for Schwann cells to reorganize the cytoskeleton as they ensheath axons. In its absence, Schwann cells cannot segregate axons and leave bundles of naked axons in adult nerves (Feltri et al., 2002).

More recently, further data on engineered mice led to the identification of two genes downstream of laminin signals, necessary for a correct spatial and temporal axonal sorting within peripheral nerves. One of these genes is Rac1. It is known that the levels of the small GTPase Rac1 activity control extension of radial lamellae in different cell types (Pankov et al., 2005). Within the PNS, the segregation of axons by Schwann cells requires the activation of Rac1 by  $\beta 1$  integrin. It has been, in fact, demonstrated that cultured Schwann cells, devoid of  $\beta 1$ -integrin, can migrate and elongate correctly on axons, but are not able to extend radial cytoplasmic processes; in addition, in  $\beta 1$  null nerves the levels of active Rac-1 are decreased; on the other hand, ectopic expression of active Rac1 in  $\beta 1$  null nerves results in improvement of sorting defects. Finally, the generation and characterization of Rac1 null mice showed a delayed axonal sorting within peripheral nerves (Nodari et al., 2007; Benninger et al., 2007).

Despite the striking similarities, Rac1 null mice show milder radial sorting defects as compared to  $\beta 1$ -integrin knock-out animals. This indicates that  $\beta 1$  signalling in Schwann cells is not only acting via Rac1. This finding is supported by the empirical data showing that  $\beta 1$ -integrin is

able to physically associate to the nonreceptor tyrosine kinase FAK (Fernandez-Valle et al., 1998). Moreover, when the FAK gene is specifically inactivated in SC, mutant nerves are characterized by arrested sorting of large-calibre axons. Of note is that mutant SC can infiltrate processes between axons, but are not able to proliferate properly, indicating that the dysmyelinating phenotype can be the result of an insufficient number of SC (Grove et al., 2007). FAK is not the only gene whose ablation results in sorting defects, due to impaired SC proliferation; another example comes from *cdc42* knock-out mice, where the radial sorting defects could be related to a lowered SC proliferation rate. In contrast to FAK, Cdc42 activation is not dependent on  $\beta 1$  activity; in fact, the levels of active Cdc42 in  $\beta 1$  null nerves appear not to be substantially different to those of wildtype animals (Benninger et al., 2007; Nodari et al., 2007).

In this section, we presented a new transgenic animal with a heterozygous mutation in the intracellular domain of MPZ that developed a transient congenital hypomyelination phenotype. This hypomyelinating phenotype is highly similar, even if to a less severe extent, to other hypomyelinating mice that carry mutation in different genes, all involved in the laminin signalling events, responsible for controlling the myelination process and the cross talk between the Schwann cell compartment and the nerve environment. These similarities lead me to suggest that P0 Q215X could perturb events downstream of the laminin signalling pathways. This “laminin signalling pathway perturbation” hypothesis is further supported by *in vitro* findings, obtained in transfected MDCK cells (see paragraph 5.5). When these cells are stably transfected with P0 glycoprotein, they localize P0 within their basolateral surface; on the contrary, deletion of the P0 cytoplasmic domain results in apical targeting in MDCK cells. Using the same *in vitro* approach, the authors identified a novel aminoacidic sequence (=YAML motif) within the P0 cytoplasmic tail that includes active tyrosine-based and leucine-based signals and that is necessary for basolateral targeting of P0 (Kidd et al., 2006). Further support to this hypothesis came already from previous

studies performed on transgenic mice overexpressing wild type P0 glycoprotein. As we have seen in paragraph 1.9.2.2, these mice manifested a dysmyelinating neuropathy, that ranges from transient perinatal hypomyelination to arrested myelination and impaired sorting of axons by Schwann cells. Such defects have been associated with incorrect trafficking of P0 in the SC membrane (Yin et al., 2000). Thus, according to the data we collected, we can speculate that P0 Q215X is not properly trafficked within Schwann cells. Perhaps, it arrives at an inappropriate location (e.g. the basal surface near the basal lamina), where it perturbs laminin signalling, somehow altering the communication between axons and Schwann cells and generating the transient radial sorting phenotype we have described. This hypothesis could be tested using a strategy similar to the one used by Kidd et al. in 2006 and described later in section 2.7.

An alternative possibility to explain the phenotype of Q215X heterozygous mice is suggested by the similarity to claw paw mice. In 1991, the murine autosomal recessive mutation *claw paw* was described: homozygous *clp/clp* mice presented abnormalities of limb posture within the first one or two postnatal days; morphological analyses of *clp/clp* mutant sciatic nerves showed that affected animals present delayed and abnormal myelination in the peripheral nervous system; in addition, the nerves presented defects in radial sorting of the fibers, with blocked myelination of small calibre axons, that are myelinated in normal animals (Henry et al., 1991). The abnormalities highlighted in *clp/clp* mice represent Schwann cell difficulties in performing the transitional step from promyelin to compact myelin. A recent study, where reciprocal nerve grafting experiments between wild-type and *clp/clp* animals were performed, demonstrated that the *clp* mutation affects the Schwann cell compartment and possibly also the neuronal compartment, being likely involved in direct axon/Schwann cell interactions. Furthermore, within the Schwann cell compartment, *clp* affects a myelin-related signaling pathway that regulates the expression of one very important transcriptional regulator of myelin genes, Krox-20, but not Oct-6, another major transcriptional regulator of the myelination process (Darbas et al., 2004). More recently, the *clp* mutation has been identified as a 225bp insertion in



the *Lgi4* gene. This gene codes for a secreted and glycosylated leucine-rich repeat protein that is expressed in Schwann cells and in restricted populations of neurons. The *clp* mutation perturbs *Lgi4* mRNA splicing, precluding splicing of exon 4 of *Lgi4* and generating a mutated protein that is retained within the cell. Functional studies performed in sensory neuron-Schwann cell cocultures, where *Lgi4* was downregulated using siRNA, identified *Lgi4* as an important signalling molecule that controls axon sorting during peripheral nerve myelination: in fact, when siRNA for *Lgi4* is turned-off in cocultures, myelination is inhibited; in addition, administration of *Lgi4* to *clp/clp* neuron-Schwann cell cocultures is able to restore myelination (Bermingham et al., 2005). Given the phenotype similarities between the *clp/clp* and P0Q215X heterozygous mice, one might speculate that P0 Q215X alters normal *Lgi4* function.

### **3.3 Q215X/+ mice present a neuromuscular defect**

Using the Rotarod test we next demonstrated that this transient defect in the process of myelination we showed, resulted in a developmental motor deficit. These data, together with previous morphological observations in peripheral nerves, lead us to conclude that the Q215X heterozygous mice are a partial model of Congenital Hypomyelination neuropathy; partial in the sense that they present a dysmyelinating phenotype that appears very early in postnatal life and that results in a motor disability for the mice, as has been reported for the two patients presenting Q215X de novo mutations. Nevertheless, this dysmyelination is less severe if compared to the clinical and pathological picture of the patients. The possible reasons for this have been already discussed in the previous paragraphs and relate to dosage and efficient transcription of the mutated allele. However, it is also true that having no expression data from human patients, we can not be certain that the less severe phenotype we observed in our mouse model could be due to intrinsic differences in the control of the myelination process, or, more generally of the transcription machinery, between mice and men. In

support of the inefficient dosage hypothesis, we have demonstrated that the Q215X mutation is manifesting its phenotype through a Gain of Function mechanism. As shown in paragraph 1.9.2.2, we have investigated the mechanism of action of different P0 mutations and we have found it to be always dose-dependent. In addition, we have generated homozygous Q215X mutant mice: those mice express more mutant protein and present a more severe phenotype, as compared to heterozygous (Fig. 2.9 and data not shown). Thus, we can speculate that Q215X/+ mice are less severely affected than Q215X/+ humans, due to the LoxP-induced reduction of the expression of the mutated allele. Although the phenotype we have described is milder, it is Congenital Hypomyelination-like, suggesting that this could be useful to elucidate the cell biological pathophysiology underlying Q215X neuropathy.

### **3.4 Q215X MPZ is not trafficked correctly**

We then decided to investigate the pathogenetic mechanism through which the mutated protein is able to produce the aberrant phenotype. We determined the intracellular location of the Q215X MPZ, together with its glycosylation status. With both analysis, we found that most of P0 Q215X could be located in the ER or Golgi, but a portion of it may arrive to the myelin sheath. These data let us speculate that P0 Q215X may in part arrive to inappropriate locations, thereby generating the radial sorting defects we have described.

Recently (Wrabetz et al., 2006), we investigated gain of abnormal function also in mouse models of CMT1B and Déjérine-Sottas syndrome (DSS) mutations. We have chosen two different mutations in the same aminoacid S63, conserved from fish to human (Kirschner et al., 2004): P0S63del, associated with CMT1B (Kulkens et al., 1993) and P0S63C is associated with DSS (Hayasaka et al., 1993). We produced transgenic mouse models with these alleles and we showed that both alleles produce gain of function, but of different types, originating from diverse intracellular locations. In particular, our immunohistological analyses

revealed that P0S63C arrived to the myelin sheath, whereas P0S63del is mostly retained in the ER. In addition, to better characterize the intracellular location of the mutant proteins, we analyzed the glycosylation of both P0S63del and P0S63C, using the same approach reported for Q215X P0. After EndoH or PNGaseF digestion, Western Blot analysis of mutant nerve protein lysates demonstrated that P0S63C is glycosylated like WT, consistent with arrival to myelin sheath, whereas the majority of P0S63del is not glycosylated and completely resistant to any glycosidase treatment, consistent with ER retention.

Thus, the evidences of mislocalization of Q215XP0 that are reported in my thesis provide further support to the idea that wide-ranging MPZ-related neuropathy phenotypes result from different kinds of Gain of Abnormal Function, originating from diverse intracellular locations (Wrabetz et al., 2006).

### 3.5 Conclusions

In this manuscript we have described the generation and characterization of a mouse model of Congenital Hypomyelination neuropathy, due to a mutation, Q215X, in the cytoplasmic tail of P0 glycoprotein. By using homologous recombination into ES cells, we have inserted the C to T transition in exon 5 that generates the Q215X mutation into one endogenous allele of the mouse *mpz* gene. Then, we have generated a mouse carrying the mutated allele in heterozygosity. We analyzed the expression of P0 in Q215X/+ mice and we showed for the first time that the mutation was effectively translated into a shorter form of P0 glycoprotein, with a lower molecular weight, as compared to wild type protein. Morphological observations in sciatic nerves, together with the analysis of neuromuscular defects in the transgenic animals lead us to three main conclusions. First of all the Q215X heterozygous mice are a partial model of Congenital Hypomyelination neuropathy; in fact the animals present a dysmyelinating phenotype that appears very early in postnatal life and that results in a motor disability for the mice, as has been

reported for the two patients presenting Q215X de novo mutations. Nevertheless, this dysmyelination was less severe if compared to the clinical and pathological picture of the patients. The possible reasons for this mild phenotype have been already discussed in the previous paragraphs and relate to dosage and inefficient transcription of the mutated allele. Secondly, by comparing the phenotype of our P0 mutant mouse with the one of P0 +/- mice, we demonstrated that the Q215X mutation is acting through a Gain of Function mechanism. Finally, by analyzing the intracellular location of the mutated P0 glycoprotein and by comparing it with the ones of other P0 mutant proteins, we have provided further support to the hypothesis that the wide-range of neuropathy phenotypes due to mutations in MPZ come from different kinds of Gain of Abnormal Function, originating from diverse intracellular locations.

In addition to these findings on the molecular pathogenesis of Q215X MPZ Congenital Hypomyelination Neuropathy, the detailed morphological analysis of altered radial sorting in sciatic nerves of Q215X +/- mice suggests that Q215X may perturb the laminin signalling pathways and/or alter normal *Lgi4* function, that are necessary for the correct spatial and temporal occurrence of radial sorting of axons by SC, within peripheral nerves.

Finally, during the process of accomplishing a careful characterization of our Q215X mutant mice, we generated a control mouse (LoxP mouse), that carries the LoxP site in intron 5, but not the Q215X mutation in exon 5. We then found that the LoxP site by itself had an effect on P0 mRNA stability, reducing its amount as compared to a wildtype P0 allele. Interestingly, this is the first time that such an effect of the LoxP insertion on mRNA steady state has been reported. This effect of the LoxP site within intron 5 can explain why the Q215X/+ mice show a very mild phenotype compared to patients' clinical picture. In fact, recently in Dr. Wrabetz's lab, RNA obtained from skin biopsies taken from glabrous myelinated-fiber-containing surfaces of the finger and forearm of a patient carrying a Q215X de-novo mutation and from the mother as a control was extracted and retro-transcribed. By RT-PCR, a 149 bp fragment extending from exon 4 to exon 6 was amplified using the

same primer pair on patient and control cDNA and on Q215X/+ mouse and WT cDNA. Contribution from the mutated and normal allele was evaluated with BsoFI digestion, since in both the human and mouse sequence context, the C to T transition abolishes a BsoFI restriction site. This analysis confirmed the reduced expression of the mutated allele in the mouse model, previously shown in the thesis, while in the patient the two alleles showed similar levels of expression. These data taken together with the ones, discussed in paragraph 3.1, demonstrating that the mutation itself was not able to induce any reduction in P0 mRNA, strongly support our idea that the milder phenotype of our Q215X/+ mice, compared to patients' clinical picture, is due to the low expression of our transgene, carrying the LoxP in intron 5.

During the last year, new techniques have been validated to create transgenic, knock-in and knock-out mice (Copeland et al., 2001); in particular, the development of phage based homologous recombination systems made the generation of transgenic and knockout constructs simpler, enabling scientists to engineer large segments of genomic DNA, like those carried on bacterial artificial chromosomes (BACs) or P1 artificial chromosomes (PACs) that replicate at low-copy number in *Escherichia coli*. This technique, which takes advantage of phage recombination to perform genetic engineering, is called recombinogenic engineering or recombineering: it offers exciting opportunities to create mouse models of human disease and for gene therapy. The most important aspects of recombineering are that only short homology segments are required to direct the recombination, and recombination efficiency rates allow recombinants to be screened rather than selected. The fact that recombinants can be screened means that only one recombination step is required to create the desired modification. These plasmid and phage systems possess a high frequency of recombination, offering the possibility to manipulate the DNA without any drug selection. With this technique, virtually any kind of mutation can be engineered into a BAC in the absence of drug-selectable markers or of loxP or FRT sites, the presence of which, as we have clearly seen in our Q215X mutant mice, can affect the function of the region of the BAC being studied.



## 4. MATERIALS AND METHODS

### 4.1 Production of Targeting Vector

The complete *Mpz* was cloned from a  $\lambda$ -DASH II mouse (129S2 strain) genomic DNA library, a generous gift from Andras Nagy (Mount Sinai Hospital, Toronto, Canada), and subcloned into pBluescript SKII (Stratagene, La Jolla, USA), in order to obtain the *Mpz*blue plasmid. To generate the Q215X targeting vector, a 2.6-kb *Bsr*GI fragment, containing exons 2, 3, 4 and 5 and part of intron 5 was excised from *Mpz*blue, blunted and subcloned into blunt pBluescript (*Eco*RI digested and subsequently filled-in), to generate the plasmid SA<sub>wt</sub>blue. Polymerase chain reaction (PCR)-based, site-directed mutagenesis (Ho et al., 1989) was performed on an *Nco*I-*Eco*RV fragment of SA<sub>wt</sub>blue, in order to introduce the mutated cDNA sequence in exon 5. After sequence confirmation, the 2.6-kb *Sma*I-*Eco*RV mutated fragment was excised from the resulting plasmid (SA<sub>Q215X</sub>blue) and subcloned into a *Sma*I digested pLOX plasmid (kind gift of Dr. P Orban), 5' to a floxed neo selection minigene present in pLOX, to obtain the plasmid SA<sub>Q215X</sub>LOX. A 4-kb *Bsr*GI-*Sal*I fragment containing part of intron 5 exon 6 and flanking sequence was excised from *Mpz*blue and subcloned 3' to the floxed neo cassette, into *Sal*I-*Bsr*GI digested SA<sub>Q215X</sub>LOX, in order to generate a new plasmid termed SA+1/2LA<sub>Q215X</sub>LOX. Finally, to obtain the complete Targeting Vector, to be used for homologous recombination in ES cells, a 4-kb *Bsr*GI fragment was excised from *Mpz*blue and subcloned into *Bsr*GI digested SA+1/2LA<sub>Q215X</sub>LOX plasmid. The resulting TV<sub>Q215X</sub> plasmid was linearized using *Not*I endonuclease and electroporated into ES cells. In order to construct the TV<sub>wt</sub> for the generation of the control LoxP mouse, we used the same strategy, except that the 2.6 *Bsr*GI fragment was not mutated.



## **4.2 Generation of recombinant embryonic stem (ES) cells**

ES cells (TBV2 line - Corradi, 2003) were grown in Dulbecco's Modified Eagles Medium (DMEM) 15% fetal calf serum,  $10^{-4}$  M  $\beta$ -mercaptoethanol (all from GIBCO Industries, Langley, OK, US), 2 mM L-Glutamine and  $10^7$  U/ml LIF (CHEMICON Int.- Temecula, CA, US), on an embryonic fibroblast feeder layer previously inactivated with Mitomycin C. Electroporation and positive selection were performed as described (Joyner, 1993). Resistant colonies were picked after 8-10 days of selection. Genomic DNA was extracted from expanded clones, digested with HindIII and analyzed by Southern blotting at the 5' end of the recombinant locus (see Fig.1A for external probe). Homologous recombinant clones were analyzed at the 3' end by HindIII and BglII digestion and semi-quantitative Southern analysis, for copy number monitoring. Out of 168 ES clones screened, one scored positive for Q215XNEO homologous recombination and was propagated. 2 clones, out of 190, scored positive for LoxP homologous recombination and were propagated. The Q215XNEO positive clone was screened for the presence of the mutation by PCR analysis, using intron 4 and intron 5 specific primers (5'-CCCTAGACTGCTTCAGTGGTGG-3' and 5'-GGTCAGCCTTGGGCTTGAC-3' respectively), followed by restriction with BsoFI endonuclease (Fig.1C). PCR conditions were 95°C for 30s, 55°C for 60s and 72°C for 30s (30 cycles), followed by 10 min extension at 72°C, in a standard PCR reaction mix.

## **4.3 Generation of chimeric mice and germline transmission of the Mpz targeted allele**

All experiments performed on mice were conducted with appropriate anaesthesia, in accordance with experimental protocols approved by the Institutional Animal Care and Use Committee, San Raffaele Scientific Institute and the Italian ministry of Health. The targeted ES clones were injected into blastocysts derived from C57BL/6J females. The chimeric

embryos were then transferred into the uteri of 2.5 day pseudopregnant foster mothers. Chimeric males with 70-100% agouti color were bred by crossing with wild-type C57BL/6J females and germline transmission was identified by the presence of agouti offspring. Genotyping was carried out by multiplex PCR amplification with one primer in intron 4, another one in exon 6 and the third one on the neomycin minigene (respectively: 5'-CCCTAGACTGCTTCAGTGGTGG-3', 5'-GGTGCTTCGGCTGTGGTCC-3' and 5'-CAATGACGACGCTGGGCGGGG-3'). PCR conditions were 95°C for 60s, 68°C for 60s and 72°C for 120s (32 cycles), followed by 10 min extension at 72°C, in a standard PCR reaction mix. Heterozygous mice for the Q215XNEO allele were subsequently confirmed by Southern blotting using the 5' probe. In order to remove the neomycin minigene, we then crossed Q215XNEO heterozygous mice with transgenic mice expressing the Cre recombinase under control of the human cytomegalovirus minimal promoter (Schwenk et al., 1995). We obtained neo negative, Q215X heterozygous mice, that were then crossed to homozygosity. Genotyping of these mice was carried out by PCR using intron 4 and exon 6 specific primers that flanked the remaining LoxP site in intron 5. Both Southern blot and PCR analyses were performed on genomic DNA prepared from tail samples (Sambrook et al., 1985). Q215X heterozygous mice were then maintained by backcrosses to FVB/N mice (Charles River Lab.). Animals used for most experiments were congenic N5 to N11 in FVB/N background.

#### 4.4 Western Blot Analysis

Frozen sciatic nerves dissected from P28 Q215X heterozygous, homozygous and wild type mice were pulverized, sonicated in lysis buffer (95 mM NaCl, 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 2% SDS, and protease inhibitors), boiled for 5 min, and spun at 14,000 rpm in a microcentrifuge for 10 min at room temperature to eliminate insoluble material. The protein concentration in supernatants was determined by

BioRad protein assay according to the manufacturer's instructions. Equal amounts of homogenates (containing 2.5–10 µg of protein) were brought up to 5 µl with 8 M urea, to which was added 5 µl of 8 M urea, 0.05 M DTT, 1%SDS, followed by 10 µl of standard reducing sample buffer. The samples were denatured, resolved on a 14% SDS-polyacrylamide gel, and electroblotted onto PVDF membrane. To verify equal loading of protein, membranes were stained with amido black or ponceau red. Blots were then blocked with 0.05% Tween, 5% dry milk in PBS, and incubated with the appropriate antibody in 0.05% Tween and 1% dry milk in PBS. Mouse mAbs recognized P0 (P07, the generous gift of Dr. Juan Archelos, Department of Neurology, Karl-Franzens-Universitat, Graz, Austria; Archelos et al. 1993) and  $\beta$ -tubulin (Sigma Chemical Corporation, St. Louis, MO, US). Peroxidase-conjugated secondary antibodies (Sigma Chemical Corporation, St. Louis, MO, US) were visualized using the ECL method with autoradiography film (Amersham Biosciences AB, Uppsala, Sweden). The intensity of bands was quantified by densitometry, and the ratio of intensities for each myelin protein and  $\beta$ -tubulin was determined. For deglycosylation experiments with peptide N-glycosidase F (PNGaseF) and endoglycosidase H (EndoH), homogenates were digested per manufacturer's instructions (New England Biolabs, Beverly, MA) and visualized as described above.

#### **4.5 Semi-quantitative RT-PCR**

Sciatic nerves were dissected from mutant and control littermates at the ages indicated (n=3 mice/genotype/time point). Total RNA was isolated using the triazol reagents (Boehringer Mannheim, Mannheim, Germany) with minor modification. Nerves were homogenized in the presence of triazol, extracted with chloroform and precipitated in the presence of tRNA. A portion (500 ng) of total RNA was reverse transcribed using Moloney Murine Leukaemia Virus reverse transcriptase and oligo dT primers (Promega Corporation, Madison, WI, US). For analysis in sciatic

nerves, equal volumes of the reverse-transcribed product from nerves of mutant and control mice were amplified using GAPDH-specific primers (5'-GTATGACTCTACCCACGG-3' and 5'-GTTCAGCTCTGGGATGAC-3') in the presence of alpha <sup>32</sup>P-dCTP. PCR conditions were 95°C for 30s, 55°C for 60s and 72°C for 60s (30 cycles), followed by 10 min extension at 72°C, in a standard PCR reaction mix. Aliquots from the amplification were withdrawn at 22, 24 and 26 cycles, resolved on an acrylamide gel and visualized by autoradiography. The intensity of the bands was quantified by densitometry (Molecular Dynamics), to verify that amplification was logarithmic, and to determine the relative amount of starting cDNA from each sample. Equal amounts of RT product, as determined by the GAPDH signal, were amplified using P0 specific primers. Analysis of the products was conducted as for GAPDH. To analyse P0 expression in LoxP and wild type mice, we exploited the exon 3 polymorphism using the RT-PCR method of Fiering et al. (1995), as described in Wrabetz et al. (2000). Briefly, 200 ng of total RNA was reverse transcribed, PCR amplification was performed as described above in the presence of alpha <sup>32</sup>P-dCTP, using a single primer pair recognizing P<sub>0</sub> exon 2 (5'-GTCCAGTGAATGGGTCTCAG-3') and exon 4 (5'-GCTCCCAACACCACCCCATATA-3') that flank a polymorphic DpnII site present only in the C57BL/6J allele and not in 129S2Pas (the polymorphic BglII site spans the intron 2/exon 3 boundary, such that in the cDNA product, only its 4 nucleotide core, a DpnII site, remains). PCR conditions were 95°C for 30s, 63°C for 60s and 72°C for 60s (26 cycles), followed by 10 min extension at 72°C, in a standard PCR reaction mix. To avoid the formation of heteroduplexes between the DpnII-containing and non-DpnII-containing products, only cycles in the logarithmic range were chosen. Two microlitres of RT-PCR product were digested with DpnII for 60 min, phenol extracted, precipitated, resolved by acrylamide gel electrophoresis and visualized by autoradiography. The intensity of the bands was quantified by densitometry, and the ratio between DpnII-containing (from wild type mice) and non-DpnII-containing (from LoxP mice) products was calculated. The procedure described above was repeated three times to confirm reproducibility of the data presented. To

analyse P0 expression in Q215X mice, we took advantage of the BsoFI site that disappear when the C to T transition in exon 5 is present. We used the RT-PCR method of Fiering et al (1995) described above. We performed PCR amplification using a P0 specific primer pair that flanks the Q215X mutation in exon 5 (in exon 4: 5'-GGCAGGCTGCCCTGCAG-3' and in exon 6: 5'-CTTCTCACTGGCAGCTTTGGTGC-3'), in the presence of <sup>32</sup>P-dCTP. PCR conditions were 95°C for 30s, 63°C for 30s and 72°C for 30s (26 cycles), followed by 7 min extension at 72°C, in a standard PCR reaction mix. The RT-PCR products were digested with BsoFI for 60 min, phenol extracted, precipitated, resolved by acrylamide gel electrophoresis and visualized by autoradiography. The intensity of the bands was quantified by densitometry, and the ratio between BsoFI-containing (from wild type Mpz allele) and non-BsoFI-containing (from Q215X allele) products was calculated. The procedure described above was repeated three times to confirm reproducibility of the data presented.

#### **4.6 Morphological Analysis**

Mutant and control littermates were sacrificed at the ages indicated and sciatic nerves were dissected. In most cases, semi-thin section and electron microscope analyses of sciatic nerves was performed as described in Quattrini et al., 1996. For semi-thin analysis, a portion of the nerve was fixed in 2% buffered glutaraldehyde and postfixed in 1% osmium tetroxide. After alcohol dehydration, these samples were embedded in Epon. Transverse sections (0.5-1 µm thick) were stained with toluidine blue and examined by light microscopy. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy. Morphometric features in electromicrographs were determined using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

#### **4.7 Behavioral Analysis**

For Rotarod analysis, groups of P10 mutant and control littermates were placed on a round cylinder rotating first at 4 rotations per minute (rpm) and then accelerating at 7.2 rpm<sup>2</sup> (Ugo Basile, VA, Italy). The animals were allowed to stay on the rod for a maximum of 900 s and the time of hold on the rotating rod was measured in subsequent trials (2 trials per day from P10 to P12). Statistics was made using SigmaStat 3.0 software.

#### **4.8 Immunohistochemistry**

For cryosections, sciatic nerve segments from mutant and control animals were collected in PBS and either directly, or after overnight fixation in 4% paraformaldehyde in 0.1 M PBS at 4°C, cryoprotected in 20% sucrose, embedded in O.C.T. (Miles), and snap-frozen in liquid nitrogen. Indirect immunofluorescence was performed on 10- $\mu$ m thick cryosections fixed again in 4% paraformaldehyde in 0.1M PBS for 4 min and in cold acetone for 10 sec, rinsed twice in PBS, and blocked with 10% normal goat serum (Dako, Glostrup, Denmark), 1% bovine serum albumin, 0.1% Triton X-100 and 0.05% sodium azide. Double staining was performed with a rabbit polyclonal Ab recognizing P0 extracellular domain (a gift from Dr.D. Colman, Montreal Neurological Institute, Montreal, CA) and a monoclonal Ab recognizing the KDEL tetrapeptide at the carboxy-terminal sequences of ER-resident proteins (StressGen, Victoria, BC, Canada). Sections were then treated with FITC- or TRITC-conjugated secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, US), and viewed with a fluorescence microscope.



#### **4.9 Image Analysis**

Micrographs of morphological samples and radiographic films were digitalized using an AGFA Arcus 2 scanner and figures were prepared using Adobe ® Photoshop 7.0.

## 5. Reference List

- Adlkofer,K., Frei,R., Neuberg,D.H., Zielasek,J., Toyka,K.V., and Suter,U. (1997). Heterozygous peripheral myelin protein 22-deficient mice are affected by a progressive demyelinating tomaculous neuropathy. *J. Neurosci.* 17, 4662-4671.
- Agrawal,H.C., Agrawal,D., and Strauss,A.W. (1990). Cleavage of the P0 glycoprotein of the rat peripheral nerve myelin: tentative identification of cleavage site and evidence for the precursor-product relationship. *Neurochem. Res.* 15, 993-1001.
- Akagi,Y., Isaka,Y., Akagi,A., Ikawa,M., Takenaka,M., Moriyama,T., Yamauchi,A., Horio,M., Ueda,N., Okabe,M., and Imai,E. (1997). Transcriptional activation of a hybrid promoter composed of cytomegalovirus enhancer and beta-actin/beta-globin gene in glomerular epithelial cells in vivo. *Kidney Int.* 51, 1265-1269.
- Amici,S.A., Dunn,W.A., Jr., Murphy,A.J., Adams,N.C., Gale,N.W., Valenzuela,D.M., Yancopoulos,G.D., and Notterpek,L. (2006). Peripheral myelin protein 22 is in complex with alpha6beta4 integrin, and its absence alters the Schwann cell basal lamina. *J. Neurosci.* 26, 1179-1189.
- Antonellis,A., Ellsworth,R.E., Sambuughin,N., Puls,I., Abel,A., Lee-Lin,S.Q., Jordanova,A., Kremensky,I., Christodoulou,K., Middleton,L.T., Sivakumar,K., Ionasescu,V., Funalot,B., Vance,J.M., Goldfarb,L.G., Fischbeck,K.H., and Green,E.D. (2003). Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth disease type 2D and distal spinal muscular atrophy type V. *Am. J. Hum. Genet.* 72, 1293-1299.
- Arroyo,E.J., Bermingham,J.R., Jr., Rosenfeld,M.G., and Scherer,S.S. (1998). Promyelinating Schwann cells express Tst-1/SCIP/Oct-6. *J. Neurosci.* 18, 7891-7902.
- Azzedine,H., Bolino,A., Taieb,T., Birouk,N., Di,D.M., Bouhouche,A., Benamou,S., Mrabet,A., Hammadouche,T., Chkili,T., Gouider,R., Ravazzolo,R., Brice,A., Laporte,J., and LeGuern,E. (2003). Mutations in MTMR13, a new pseudophosphatase homologue of MTMR2 and Sbf1, in two families with an autosomal recessive demyelinating form of Charcot-Marie-Tooth disease associated with early-onset glaucoma. *Am. J. Hum. Genet.* 72, 1141-1153.
- Baechner,D., Liehr,T., Hameister,H., Altenberger,H., Grehl,H., Suter,U., and Rautenstrauss,B. (1995). Widespread expression of the peripheral myelin protein-22 gene (PMP22) in neural and non-neural tissues during murine development. *J. Neurosci. Res.* 42, 733-741.
- Baker,C.V., Bronner-Fraser,M., Le Douarin,N.M., and Teillet,M.A. (1997). Early- and late-migrating cranial neural crest cell populations have equivalent developmental potential in vivo. *Development* 124, 3077-3087.

- Balice-Gordon, R.J., Bone, L.J., and Scherer, S.S. (1998). Functional gap junctions in the schwann cell myelin sheath. *J. Cell Biol.* 142, 1095-1104.
- Barbu, M. (1990). Molecular cloning of cDNAs that encode the chicken P0 protein: evidence for early expression in avians. *J. Neurosci. Res.* 25, 143-151.
- Baron, P., Shy, M., Kamholz, J., Scarlato, G., and Pleasure, D. (1994). Expression of P0 protein mRNA along rat sciatic nerve during development. *Brain Res. Dev. Brain Res.* 83, 285-288.
- Ben, O.K., Middleton, L.T., Loprest, L.J., Wilkinson, K.M., Lennon, F., Rozear, M.P., Stajich, J.M., Gaskell, P.C., Roses, A.D., Pericak-Vance, M.A., and . (1993). Localization of a gene (CMT2A) for autosomal dominant Charcot-Marie-Tooth disease type 2 to chromosome 1p and evidence of genetic heterogeneity. *Genomics* 17, 370-375.
- Ben, O.K., Rochelle, J.M., Ben, H.M., Slotterbeck, B., Rao, N., Hentati, F., Pericak-Vance, M.A., and Vance, J.M. (1998). Fine localization of the CMT4A locus using a PAC contig and haplotype analysis. *Neurogenetics.* 2, 18-23.
- Bennett, C.L., Shirk, A.J., Huynh, H.M., Street, V.A., Nelis, E., Van, M.L., De, J.P., Jordanova, A., Guergueltcheva, V., Tournev, I., Van Den, B.P., Seeman, P., Mazanec, R., Prochazka, T., Kremensky, I., Haberlova, J., Weiss, M.D., Timmerman, V., Bird, T.D., and Chance, P.F. (2004). SIMPLE mutation in demyelinating neuropathy and distribution in sciatic nerve. *Ann. Neurol.* 55, 713-720.
- Benninger, Y., Thurnherr, T., Pereira, J.A., Krause, S., Wu, X., Chrostek-Grashoff, A., Herzog, D., Nave, K.A., Franklin, R.J., Meijer, D., Brakebusch, C., Suter, U., and Relvas, J.B. (2007). Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. *J. Cell Biol.* 177, 1051-1061.
- Berger, P., Young, P., and Suter, U. (2002). Molecular cell biology of Charcot-Marie-Tooth disease. *Neurogenetics.* 4, 1-15.
- Bergoffen, J., Scherer, S.S., Wang, S., Scott, M.O., Bone, L.J., Paul, D.L., Chen, K., Lensch, M.W., Chance, P.F., and Fischbeck, K.H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* 262, 2039-2042.
- Bermingham-McDonogh, O., Xu, Y.T., Marchionni, M.A., and Scherer, S.S. (1997). Neuregulin expression in PNS neurons: isoforms and regulation by target interactions. *Mol. Cell Neurosci.* 10, 184-195.
- Bermingham, J.R., Jr., Scherer, S.S., O'Connell, S., Arroyo, E., Kalla, K.A., Powell, F.L., and Rosenfeld, M.G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev.* 10, 1751-1762.

- Bermingham, J.R., Jr., Shearin, H., Pennington, J., O'Moore, J., Jaegle, M., Driegen, S., van, Z.A., Darbas, A., Ozkaynak, E., Ryu, E.J., Milbrandt, J., and Meijer, D. (2006). The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. *Nat. Neurosci.* 9, 76-84.
- Bharucha, V.A., Peden, K.W., and Tennekoon, G.I. (1994). SV40 large T antigen with c-Jun down-regulates myelin P0 gene expression: a mechanism for papovaviral T antigen-mediated demyelination. *Neuron* 12, 627-637.
- Bhattacharyya, A., Frank, E., Ratner, N., and Brackenbury, R. (1991). P0 is an early marker of the Schwann cell lineage in chickens. *Neuron* 7, 831-844.
- Birouk, N., LeGuern, E., Maissonobe, T., Rouger, H., Gouider, R., Tardieu, S., Gugenheim, M., Routon, M.C., Leger, J.M., Agid, Y., Brice, A., and Bouche, P. (1998). X-linked Charcot-Marie-Tooth disease with connexin 32 mutations: clinical and electrophysiologic study. *Neurology* 50, 1074-1082.
- Bissar-Tadmouri, N., Nelis, E., Zuchner, S., Parman, Y., Deymeer, F., Serdaroglu, P., De, J.P., Van, G., V, Timmerman, V., Schroder, J.M., and Battaloglu, E. (2004). Absence of KIF1B mutation in a large Turkish CMT2A family suggests involvement of a second gene. *Neurology* 62, 1522-1525.
- Bizzozero, O.A., Fridal, K., and Pastuszyn, A. (1994). Identification of the palmitoylation site in rat myelin P0 glycoprotein. *J. Neurochem.* 62, 1163-1171.
- Bjartmar, C., Yin, X., and Trapp, B.D. (1999). Axonal pathology in myelin disorders. *J. Neurocytol.* 28, 383-395.
- Blanchard, A.D., Sinanan, A., Parmantier, E., Zwart, R., Broos, L., Meijer, D., Meier, C., Jessen, K.R., and Mirsky, R. (1996). Oct-6 (SCIP/Tst-1) is expressed in Schwann cell precursors, embryonic Schwann cells, and postnatal myelinating Schwann cells: comparison with Oct-1, Krox-20, and Pax-3. *J. Neurosci. Res.* 46, 630-640.
- Boerkoel, C.F., Takashima, H., Stankiewicz, P., Garcia, C.A., Leber, S.M., Rhee-Morris, L., and Lupski, J.R. (2001). Periaxin mutations cause recessive Dejerine-Sottas neuropathy. *Am. J. Hum. Genet.* 68, 325-333.
- Boiko, M.I. and Nurimanov, K.R. (2001). [Modern concept of peripheral erectile mechanisms]. *Fiziol. Zh.* 47, 116-132.
- Boison, D. and Stoffel, W. (1994). Disruption of the compacted myelin sheath of axons of the central nervous system in proteolipid protein-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* 91, 11709-11713.
- Bolino, A., Muglia, M., Conforti, F.L., LeGuern, E., Salih, M.A., Georgiou, D.M., Christodoulou, K., Hausmanowa-Petrusewicz, I.,

- Mandich,P., Schenone,A., Gambardella,A., Bono,F., Quattrone,A., Devoto,M., and Monaco,A.P. (2000). Charcot-Marie-Tooth type 4B is caused by mutations in the gene encoding myotubularin-related protein-2. *Nat. Genet.* 25, 17-19.
- Bongarzone,E.R., Campagnoni,C.W., Kampf,K., Jacobs,E.C., Handley,V.W., Schonmann,V., and Campagnoni,A.T. (1999). Identification of a new exon in the myelin proteolipid protein gene encoding novel protein isoforms that are restricted to the somata of oligodendrocytes and neurons. *J. Neurosci.* 19, 8349-8357.
- Bornemann,A., Hansen,F.J., and Schmalbruch,H. (1996). Nerve and muscle biopsy in a case of hereditary motor and sensory neuropathy type III with basal lamina onion bulbs. *Neuropathol. Appl. Neurobiol.* 22, 77-81.
- Bouhouche,A., Benomar,A., Birouk,N., Mularoni,A., Meggouh,F., Tassin,J., Grid,D., Vandenberghe,A., Yahyaoui,M., Chkili,T., Brice,A., and LeGuern,E. (1999). A locus for an axonal form of autosomal recessive Charcot-Marie-Tooth disease maps to chromosome 1q21.2-q21.3. *Am. J. Hum. Genet.* 65, 722-727.
- Boylan,K.B., Ferriero,D.M., Greco,C.M., Sheldon,R.A., and Dew,M. (1992). Congenital hypomyelination neuropathy with arthrogryposis multiplex congenita. *Ann. Neurol.* 31, 337-340.
- Bradley,W.G. and Jenkinson,M. (1973). Abnormalities of peripheral nerves in murine muscular dystrophy. *J. Neurol. Sci.* 18, 227-247.
- Brennan,A., Dean,C.H., Zhang,A.L., Cass,D.T., Mirsky,R., and Jessen,K.R. (2000). Endothelins control the timing of Schwann cell generation in vitro and in vivo. *Dev. Biol.* 227, 545-557.
- Britsch,S., Goerich,D.E., Riethmacher,D., Peirano,R.I., Rossner,M., Nave,K.A., Birchmeier,C., and Wegner,M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* 15, 66-78.
- Bronstein,J.M. (2000). Function of tetraspan proteins in the myelin sheath. *Curr. Opin. Neurobiol.* 10, 552-557.
- Brown,A.M. and Lemke,G. (1997). Multiple regulatory elements control transcription of the peripheral myelin protein zero gene. *J. Biol. Chem.* 272, 28939-28947.
- Brunden,K.R. and Poduslo,J.F. (1987). A phorbol ester-sensitive kinase catalyzes the phosphorylation of P0 glycoprotein in myelin. *J. Neurochem.* 49, 1863-1872.
- Brunden,K.R. (1992). Age-dependent changes in the oligosaccharide structure of the major myelin glycoprotein, P0. *J. Neurochem.* 58, 1659-1666.

- Burden,S. and Yarden,Y. (1997). Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* 18, 847-855.
- Canoll,P.D., Musacchio,J.M., Hardy,R., Reynolds,R., Marchionni,M.A., and Salzer,J.L. (1996). GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. *Neuron* 17, 229-243.
- Castillo-Davis,C.I., Mekhedov,S.L., Hartl,D.L., Koonin,E.V., and Kondrashov,F.A. (2002). Selection for short introns in highly expressed genes. *Nat. Genet.* 31, 415-418.
- Chance,P.F., Alderson,M.K., Leppig,K.A., Lensch,M.W., Matsunami,N., Smith,B., Swanson,P.D., Odelberg,S.J., Distèche,C.M., and Bird,T.D. (1993). DNA deletion associated with hereditary neuropathy with liability to pressure palsies. *Cell* 72, 143-151.
- Chang,J.C. and Kan,Y.W. (1979). beta 0 thalassemia, a nonsense mutation in man. *Proc. Natl. Acad. Sci. U. S. A* 76, 2886-2889.
- Cheng,L. and Mudge,A.W. (1996). Cultured Schwann cells constitutively express the myelin protein P0. *Neuron* 16, 309-319.
- Cosgaya,J.M., Chan,J.R., and Shooter,E.M. (2002). The neurotrophin receptor p75NTR as a positive modulator of myelination. *Science* 298, 1245-1248.
- Cuesta,A., Pedrola,L., Sevilla,T., Garcia-Planells,J., Chumillas,M.J., Mayordomo,F., LeGuern,E., Marin,I., Vilchez,J.J., and Palau,F. (2002). The gene encoding ganglioside-induced differentiation-associated protein 1 is mutated in axonal Charcot-Marie-Tooth type 4A disease. *Nat. Genet.* 30, 22-25.
- D'Antonio,M., Droggiti,A., Feltri,M.L., Roes,J., Wrabetz,L., Mirsky,R., and Jessen,K.R. (2006). TGFbeta type II receptor signaling controls Schwann cell death and proliferation in developing nerves. *J. Neurosci.* 26, 8417-8427.
- D'Urso,D., Brophy,P.J., Staugaitis,S.M., Gillespie,C.S., Frey,A.B., Stempak,J.G., and Colman,D.R. (1990). Protein zero of peripheral nerve myelin: biosynthesis, membrane insertion, and evidence for homotypic interaction. *Neuron* 4, 449-460.
- D'Urso,D., Prior,R., Greiner-Petter,R., Gabreels-Festen,A.A., and Muller,H.W. (1998). Overloaded endoplasmic reticulum-Golgi compartments, a possible pathomechanism of peripheral neuropathies caused by mutations of the peripheral myelin protein PMP22. *J. Neurosci.* 18, 731-740.



- D'Urso,D., Ehrhardt,P., and Muller,H.W. (1999). Peripheral myelin protein 22 and protein zero: a novel association in peripheral nervous system myelin. *J. Neurosci.* 19, 3396-3403.
- Darbas,A., Jaegle,M., Walbeehm,E., van den,B.H., Driegen,S., Broos,L., Uyl,M., Visser,P., Grosveld,F., and Meijer,D. (2004). Cell autonomy of the mouse claw paw mutation. *Dev. Biol.* 272, 470-482.
- De Sandre-Giovannoli,A., Chaouch,M., Kozlov,S., Vallat,J.M., Tazir,M., Kassouri,N., Szepietowski,P., Hammadouche,T., Vandenberghe,A., Stewart,C.L., Grid,D., and Levy,N. (2002). Homozygous defects in LMNA, encoding lamin A/C nuclear-envelope proteins, cause autosomal recessive axonal neuropathy in human (Charcot-Marie-Tooth disorder type 2) and mouse. *Am. J. Hum. Genet.* 70, 726-736.
- De,J.P., Timmerman,V., Ceuterick,C., Nelis,E., De,V.E., Lofgren,A., Vercruyssen,A., Verellen,C., Van,M.L., Martin,J.J., and Van,B.C. (1999). The Thr124Met mutation in the peripheral myelin protein zero (MPZ) gene is associated with a clinically distinct Charcot-Marie-Tooth phenotype. *Brain* 122 ( Pt 2), 281-290.
- Ding,Y. and Brunden,K.R. (1994). The cytoplasmic domain of myelin glycoprotein P0 interacts with negatively charged phospholipid bilayers. *J. Biol. Chem.* 269, 10764-10770.
- Dong,Z., Brennan,A., Liu,N., Yarden,Y., Lefkowitz,G., Mirsky,R., and Jessen,K.R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* 15, 585-596.
- Dowsing,B.J., Morrison,W.A., Nicola,N.A., Starkey,G.P., Bucci,T., and Kilpatrick,T.J. (1999). Leukemia inhibitory factor is an autocrine survival factor for Schwann cells. *J. Neurochem.* 73, 96-104.
- Doyle,J.P., Stempak,J.G., Cowin,P., Colman,D.R., and D'Urso,D. (1995). Protein zero, a nervous system adhesion molecule, triggers epithelial reversion in host carcinoma cells. *J. Cell Biol.* 131, 465-482.
- Dyck,P.J., Lambert,E.H., Sanders,K., and O'Brien,P.C. (1971). Severe hypomyelination and marked abnormality of conduction in Dejerine-Sottas hypertrophic neuropathy: myelin thickness and compound action potential of sural nerve in vitro. *Mayo Clin. Proc.* 46, 432-436.
- Dyck,P.J., Lais,A.C., and Offord,K.P. (1974). The nature of myelinated nerve fiber degeneration in dominantly inherited hypertrophic neuropathy. *Mayo Clin. Proc.* 49, 34-39.
- Dyck,P.J., Ott,J., Moore,S.B., Swanson,C.J., and Lambert,E.H. (1983). Linkage evidence for genetic heterogeneity among kinships with hereditary motor and sensory neuropathy, type I. *Mayo Clin. Proc.* 58, 430-435.

Dyck,P.J., Litchy,W.J., Minnerath,S., Bird,T.D., Chance,P.F., Schaid,D.J., and Aronson,A.E. (1994). Hereditary motor and sensory neuropathy with diaphragm and vocal cord paresis. *Ann. Neurol.* 35, 608-615.

EARL,C.J., FULLERTON,P.M., WAKEFIELD,G.S., and SCHUTTA,H.S. (1964). HEREDITARY NEUROPATHY, WITH LIABILITY TO PRESSURE PALSIES; A CLINICAL AND ELECTROPHYSIOLOGICAL STUDY OF FOUR FAMILIES. *Q. J. Med.* 33, 481-498.

Eichberg,J. (2002). Protein kinase C changes in diabetes: is the concept relevant to neuropathy? *Int. Rev. Neurobiol.* 50, 61-82.

Evgrafov,O.V., Mersiyanova,I., Irobi,J., Van Den,B.L., Dierick,I., Leung,C.L., Schagina,O., Verpoorten,N., Van,I.K., Fedotov,V., Dadali,E., uer-Grumbach,M., Windpassinger,C., Wagner,K., Mitrovic,Z., Hilton-Jones,D., Talbot,K., Martin,J.J., Vasserman,N., Tverskaya,S., Polyakov,A., Liem,R.K., Gettemans,J., Robberecht,W., De,J.P., and Timmerman,V. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36, 602-606.

Fabrizi,G.M., Simonati,A., Taioli,F., Cavallaro,T., Ferrarini,M., Rigitelli,F., Pini,A., Mostacciuolo,M.L., and Rizzuto,N. (2001). PMP22 related congenital hypomyelination neuropathy. *J. Neurol. Neurosurg. Psychiatry* 70, 123-126.

FALCONER,D.S. (1951). Two congenital defects of the limb bones in the mouse. *Acta Anat. (Basel)* 13, 371-378.

Feltri,M.L., D'Antonio,M., Quattrini,A., Numerato,R., Arona,M., Previtali,S., Chiu,S.Y., Messing,A., and Wrabetz,L. (1999). A novel P0 glycoprotein transgene activates expression of lacZ in myelin-forming Schwann cells. *Eur. J. Neurosci.* 11, 1577-1586.

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

Fernandez-Valle,C., Fregien,N., Wood,P.M., and Bunge,M.B. (1993). Expression of the protein zero myelin gene in axon-related Schwann cells is linked to basal lamina formation. *Development* 119, 867-880.

Fernandez-Valle,C., Wood,P.M., and Bunge,M.B. (1998). Localization of focal adhesion kinase in differentiating Schwann cell/neuron cultures. *Microsc. Res. Tech.* 41, 416-430.

- Filbin,M.T., Walsh,F.S., Trapp,B.D., Pizzey,J.A., and Tennekoon,G.I. (1990). Role of myelin P0 protein as a homophilic adhesion molecule. *Nature* 344, 871-872.
- Filbin,M.T. and Tennekoon,G.I. (1991). The role of complex carbohydrates in adhesion of the myelin protein, P0. *Neuron* 7, 845-855.
- Filbin,M.T. and Tennekoon,G.I. (1993). Homophilic adhesion of the myelin P0 protein requires glycosylation of both molecules in the homophilic pair. *J. Cell Biol.* 122, 451-459.
- Frank,E. and Sanes,J.R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus. *Development* 111, 895-908.
- Frei,R., Motzing,S., Kinkel, I., Schachner,M., Koltzenburg,M., and Martini,R. (1999). Loss of distal axons and sensory Merkel cells and features indicative of muscle denervation in hindlimbs of P0-deficient mice. *J. Neurosci.* 19, 6058-6067.
- Friedrich,R.P., Schlierf,B., Tamm,E.R., Bosl,M.R., and Wegner,M. (2005). The class III POU domain protein Brn-1 can fully replace the related Oct-6 during schwann cell development and myelination. *Mol. Cell Biol.* 25, 1821-1829.
- Friede,R.L. and Samorajski,T. (1968). Myelin formation in the sciatic nerve of the rat. A quantitative electron microscopic, histochemical and radioautographic study. *J. Neuropathol. Exp. Neurol.* 27, 546-570.
- Fujita,N., Kemper,A., Dupree,J., Nakayasu,H., Bartsch,U., Schachner,M., Maeda,N., Suzuki,K., and Popko,B. (1998). The cytoplasmic domain of the large myelin-associated glycoprotein isoform is needed for proper CNS but not peripheral nervous system myelination. *J. Neurosci.* 18, 1970-1978.
- Gaboreanu,A.M., Hrstka,R., Xu,W., Shy,M., Kamholz,J., Lilien,J., and Balsamo,J. (2007). Myelin protein zero/P0 phosphorylation and function require an adaptor protein linking it to RACK1 and PKC alpha. *J. Cell Biol.* 177, 707-716.
- Gabreels-Festen,A. (2002). Dejerine-Sottas syndrome grown to maturity: overview of genetic and morphological heterogeneity and follow-up of 25 patients. *J. Anat.* 200, 341-356.
- Gabreels-Festen,A.A., Bolhuis,P.A., Hoogendijk,J.E., Valentijn,L.J., Eshuis,E.J., and Gabreels,F.J. (1995). Charcot-Marie-Tooth disease type 1A: morphological phenotype of the 17p duplication versus PMP22 point mutations. *Acta Neuropathol.* 90, 645-649.
- Gabreels-Festen,A.A., Hoogendijk,J.E., Meijerink,P.H., Gabreels,F.J., Bolhuis,P.A., van,B.S., Kulkens,T., Nelis,E., Jennekens,F.G., de,V.M., van Engelen,B.G., Van,B.C., and Mariman,E.C. (1996). Two divergent types

of nerve pathology in patients with different P0 mutations in Charcot-Marie-Tooth disease. *Neurology* 47, 761-765.

Gallego,R.G., Blanco,J.L., Thijssen-van Zuylen,C.W., Gotfredsen,C.H., Voshol,H., Duus,J.O., Schachner,M., and Vliegthart,J.F. (2001). Epitope diversity of N-glycans from bovine peripheral myelin glycoprotein P0 revealed by mass spectrometry and nano probe magic angle spinning 1H NMR spectroscopy. *J. Biol. Chem.* 276, 30834-30844.

Gambardella,A., Bono,F., Muglia,M., Valentino,P., and Quattrone,A. (1999). Autosomal recessive hereditary motor and sensory neuropathy with focally folded myelin sheaths (CMT4B). *Ann. N. Y. Acad. Sci.* 883, 47-55.

Gao,Y., Li,W., and Filbin,M.T. (2000). Acylation of myelin Po protein is required for adhesion. *J. Neurosci. Res.* 60, 704-713.

Garbern,J.Y., Cambi,F., Tang,X.M., Sima,A.A., Vallat,J.M., Bosch,E.P., Lewis,R., Shy,M., Sohi,J., Kraft,G., Chen,K.L., Joshi,I., Leonard,D.G., Johnson,W., Raskind,W., Dlouhy,S.R., Pratt,V., Hodes,M.E., Bird,T., and Kamholz,J. (1997). Proteolipid protein is necessary in peripheral as well as central myelin. *Neuron* 19, 205-218.

Garcia,A., Combarros,O., Calleja,J., and Berciano,J. (1998). Charcot-Marie-Tooth disease type 1A with 17p duplication in infancy and early childhood: a longitudinal clinical and electrophysiologic study. *Neurology* 50, 1061-1067.

Garcia,C.A., Malamut,R.E., England,J.D., Parry,G.S., Liu,P., and Lupski,J.R. (1995). Clinical variability in two pairs of identical twins with the Charcot-Marie-Tooth disease type 1A duplication. *Neurology* 45, 2090-2093.

Garratt,A.N., Britsch,S., and Birchmeier,C. (2000). Neuregulin, a factor with many functions in the life of a schwann cell. *Bioessays* 22, 987-996.

Ghislain,J., smarquet-Trin-Dinh,C., Jaegle,M., Meijer,D., Charnay,P., and Frain,M. (2002). Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. *Development* 129, 155-166.

Giese,K.P., Martini,R., Lemke,G., Soriano,P., and Schachner,M. (1992). Mouse P0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. *Cell* 71, 565-576.

Gould,R.M., Byrd,A.L., and Barbarese,E. (1995). The number of Schmidt-Lanterman incisures is more than doubled in shiverer PNS myelin sheaths. *J. Neurocytol.* 24, 85-98.

- Gow,A., Southwood,C.M., and Lazzarini,R.A. (1998). Disrupted proteolipid protein trafficking results in oligodendrocyte apoptosis in an animal model of Pelizaeus-Merzbacher disease. *J. Cell Biol.* 140, 925-934.
- Griffith,L.S., Schmitz,B., and Schachner,M. (1992). L2/HNK-1 carbohydrate and protein-protein interactions mediate the homophilic binding of the neural adhesion molecule P0. *J. Neurosci. Res.* 33, 639-648.
- Grove,M., Komiyama,N.H., Nave,K.A., Grant,S.G., Sherman,D.L., and Brophy,P.J. (2007). FAK is required for axonal sorting by Schwann cells. *J. Cell Biol.* 176, 277-282.
- Guilbot,A., Williams,A., Ravise,N., Verny,C., Brice,A., Sherman,D.L., Brophy,P.J., LeGuern,E., Delague,V., Bareil,C., Megarbane,A., and Claustres,M. (2001). A mutation in periaxin is responsible for CMT4F, an autosomal recessive form of Charcot-Marie-Tooth disease. *Hum. Mol. Genet.* 10, 415-421.
- Gupta,S.K., Poduslo,J.F., and Mezei,C. (1988). Temporal changes in PO and MBP gene expression after crush-injury of the adult peripheral nerve. *Brain Res.* 464, 133-141.
- Gutierrez,A., England,J.D., Sumner,A.J., Ferer,S., Warner,L.E., Lupski,J.R., and Garcia,C.A. (2000). Unusual electrophysiological findings in X-linked dominant Charcot-Marie-Tooth disease. *Muscle Nerve* 23 , 182-188.
- Hammer,J.A., O'Shannessy,D.J., De,L.M., Gould,R., Zand,D., Daune,G., and Quarles,R.H. (1993). Immunoreactivity of PMP-22, P0, and other 19 to 28 kDa glycoproteins in peripheral nerve myelin of mammals and fish with HNK1 and related antibodies. *J. Neurosci. Res.* 35, 546-558.
- Hanemann,C.O., Stoll,G., D'Urso,D., Fricke,W., Martin,J.J., Van,B.C., Mancardi,G.L., Bartke,I., and Muller,H.W. (1994). Peripheral myelin protein-22 expression in Charcot-Marie-Tooth disease type 1a sural nerve biopsies. *J. Neurosci. Res.* 37, 654-659.
- Haney,C., Snipes,G.J., Shooter,E.M., Suter,U., Garcia,C., Griffin,J.W., and Trapp,B.D. (1996). Ultrastructural distribution of PMP22 in Charcot-Marie-Tooth disease type 1A. *J. Neuropathol. Exp. Neurol.* 55 , 290-299.
- Harati,Y. and Butler,I.J. (1985). Congenital hypomyelinating neuropathy. *J. Neurol. Neurosurg. Psychiatry* 48, 1269-1276.
- Harding,A.E. and Thomas,P.K. (1980). Genetic aspects of hereditary motor and sensory neuropathy (types I and II). *J. Med. Genet.* 17, 329-336.
- Hattori,N., Yamamoto,M., Yoshihara,T., Koike,H., Nakagawa,M., Yoshikawa,H., Ohnishi,A., Hayasaka,K., Onodera,O., Baba,M., Yasuda,H., Saito,T., Nakashima,K., Kira,J., Kaji,R., Oka,N., and Sobue,G. (2003). Demyelinating and axonal features of Charcot-Marie-Tooth disease with mutations of myelin-related proteins (PMP22, MPZ and

Cx32): a clinicopathological study of 205 Japanese patients. *Brain* 126, 134-151.

Hayasaka,K., Nanao,K., Tahara,M., Sato,W., Takada,G., Miura,M., and Uyemura,K. (1991). Isolation and sequence determination of cDNA encoding the major structural protein of human peripheral myelin. *Biochem. Biophys. Res. Commun.* 180, 515-518.

Hayasaka,K., Himoro,M., Wang,Y., Takata,M., Minoshima,S., Shimizu,N., Miura,M., Uyemura,K., and Takada,G. (1993). Structure and chromosomal localization of the gene encoding the human myelin protein zero (MPZ). *Genomics* 17, 755-758.

Hayasaka,K. (1996). [Molecular genetics of Charcot-Marie-Tooth disease]. *Nippon Naika Gakkai Zasshi* 85, 1325-1330.

Henry,E.W., Cowen,J.S., and Sidman,R.L. (1983). Comparison of Trembler and Trembler-J mouse phenotypes: varying severity of peripheral hypomyelination. *J. Neuropathol. Exp. Neurol.* 42, 688-706.

Henry,E.W., Eicher,E.M., and Sidman,R.L. (1991). The mouse mutation claw paw: forelimb deformity and delayed myelination throughout the peripheral nervous system. *J. Hered.* 82, 287-294.

Hentze,M.W. and Kulozik,A.E. (1999). A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* 96, 307-310.

Hilmi,S., Fournier,M., Valeins,H., Gandar,J.C., and Bonnet,J. (1995). Myelin P0 glycoprotein: identification of the site phosphorylated in vitro and in vivo by endogenous protein kinases. *J. Neurochem.* 64, 902-907.

Houlden,H., Blake,J., and Reilly,M.M. (2004). Hereditary sensory neuropathies. *Curr. Opin. Neurol.* 17, 569-577.

Huxley,C., Passage,E., Manson,A., Putzu,G., Figarella-Branger,D., Pellissier,J.F., and Fontes,M. (1996). Construction of a mouse model of Charcot-Marie-Tooth disease type 1A by pronuclear injection of human YAC DNA. *Hum. Mol. Genet.* 5, 563-569.

Ikegami,T., Nicholson,G., Ikeda,H., Ishida,A., Johnston,H., Wise,G., Ouvrier,R., and Hayasaka,K. (1996). A novel homozygous mutation of the myelin Po gene producing Dejerine-Sottas disease (hereditary motor and sensory neuropathy type III). *Biochem. Biophys. Res. Commun.* 222, 107-110.

Inoue,K., Osaka,H., Imaizumi,K., Nezu,A., Takanashi,J., Arai,J., Murayama,K., Ono,J., Kikawa,Y., Mito,T., Shaffer,L.G., and Lupski,J.R. (1999). Proteolipid protein gene duplications causing Pelizaeus-Merzbacher disease: molecular mechanism and phenotypic manifestations. *Ann. Neurol.* 45, 624-632.

Inoue,K., Khajavi,M., Ohyama,T., Hirabayashi,S., Wilson,J., Reggin,J.D., Mancias,P., Butler,I.J., Wilkinson,M.F., Wegner,M., and Lupski,J.R. (2004). Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations. *Nat. Genet.* 36, 361-369.

Inouye,H., Tsuruta,H., Sedzik,J., Uyemura,K., and Kirschner,D.A. (1999). Tetrameric assembly of full-sequence protein zero myelin glycoprotein by synchrotron x-ray scattering. *Biophys. J.* 76, 423-437.

Ionasescu,V.V., Searby,C.C., Ionasescu,R., Chatkupt,S., Patel,N., and Koenigsberger,R. (1997). Dejerine-Sottas neuropathy in mother and son with same point mutation of PMP22 gene. *Muscle Nerve* 20, 97-99.

Ismailov,S.M., Fedotov,V.P., Dadali,E.L., Polyakov,A.V., Van,B.C., Ivanov,V.I., De,J.P., Timmerman,V., and Evgrafov,O.V. (2001). A new locus for autosomal dominant Charcot-Marie-Tooth disease type 2 (CMT2F) maps to chromosome 7q11-q21. *Eur. J. Hum. Genet.* 9, 646-650.

Ito,K., Morita,T., and Sieber-Blum,M. (1993). In vitro clonal analysis of mouse neural crest development. *Dev. Biol.* 157, 517-525.

Iyer,S., Rowe-Rendleman,C.L., Bianchi,R., and Eichberg,J. (1996). Tyrosine phosphorylation of myelin protein PO. *J. Neurosci. Res.* 46, 531-539.

Iyer,S., Bianchi,R., and Eichberg,J. (2000). Tyrosine phosphorylation of PNS myelin P(0) occurs in the cytoplasmic domain and is maximal during early development. *J. Neurochem.* 75, 347-354.

Jaegle,M., Mandemakers,W., Broos,L., Zwart,R., Karis,A., Visser,P., Grosveld,F., and Meijer,D. (1996). The POU factor Oct-6 and Schwann cell differentiation. *Science* 273, 507-510.

Jaegle,M., Ghazvini,M., Mandemakers,W., Piirsoo,M., Driegen,S., Levavasseur,F., Raghoenath,S., Grosveld,F., and Meijer,D. (2003). The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev.* 17, 1380-1391.

Jessen,K.R. and Mirsky,R. (1994). Neural development. Fate diverted. *Curr. Biol.* 4, 824-827.

Jessen,K.R. and Mirsky,R. (2005). The origin and development of glial cells in peripheral nerves. *Nat. Rev. Neurosci.* 6, 671-682.

Jetten,A.M. and Suter,U. (2000). The peripheral myelin protein 22 and epithelial membrane protein family. *Prog. Nucleic Acid Res. Mol. Biol.* 64, 97-129.

Jiang,R., Lan,Y., Norton,C.R., Sundberg,J.P., and Gridley,T. (1998). The Slug gene is not essential for mesoderm or neural crest development in mice. *Dev. Biol.* 198, 277-285.



Kaku,D.A., Parry,G.J., Malamut,R., Lupski,J.R., and Garcia,C.A. (1993). Uniform slowing of conduction velocities in Charcot-Marie-Tooth polyneuropathy type 1. *Neurology* 43, 2664-2667.

Kalaydjieva,L., Gresham,D., Gooding,R., Heather,L., Baas,F., de,J.R., Blechschmidt,K., Angelicheva,D., Chandler,D., Worsley,P., Rosenthal,A., King,R.H., and Thomas,P.K. (2000). N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. *Am. J. Hum. Genet.* 67, 47-58.

Kaplan,M.R., Meyer-Franke,A., Lambert,S., Bennett,V., Duncan,I.D., Levinson,S.R., and Barres,B.A. (1997). Induction of sodium channel clustering by oligodendrocytes. *Nature* 386, 724-728.

Kidd,G.J., Yadav,V.K., Huang,P., Brand,S.L., Low,S.H., Weimbs,T., and Trapp,B.D. (2006). A dual tyrosine-leucine motif mediates myelin protein P0 targeting in MDCK cells. *Glia* 54, 135-145.

Kirschner,D.A. and Sidman,R.L. (1976). X-ray diffraction study of myelin structure in immature and mutant mice. *Biochim. Biophys. Acta* 448, 73-87.

Kirschner,D.A. and Ganser,A.L. (1980). Compact myelin exists in the absence of basic protein in the shiverer mutant mouse. *Nature* 283, 207-210.

Kirschner,D.A. and Hollingshead,C.J. (1980). Processing for electron microscopy alters membrane structure and packing in myelin. *J. Ultrastruct. Res.* 73, 211-232.

Kirschner,D.A., Inouye,H., and Saavedra,R.A. (1996). Membrane adhesion in peripheral myelin: good and bad wraps with protein P0. *Structure.* 4, 1239-1244.

Kirschner,D.A., Szumowski,K., Gabreels-Festen,A.A., Hoogendijk,J.E., and Bolhuis,P.A. (1996). Inherited demyelinating peripheral neuropathies: relating myelin packing abnormalities to P0 molecular defects. *J. Neurosci. Res.* 46, 502-508.

Kleopa,K.A. and Scherer,S.S. (2002). Inherited neuropathies. *Neurologic Clinics* 20, 679-+.

Kuhn,R., Pravtcheva,D., Ruddle,F., and Lemke,G. (1990). The gene encoding peripheral myelin protein zero is located on mouse chromosome 1. *J. Neurosci.* 10, 205-209.

Kulkens,T., Bolhuis,P.A., Wolterman,R.A., Kemp,S., te,N.S., Valentijn,L.J., Hensels,G.W., Jennekens,F.G., de,V.M., Hoogendijk,J.E., and . (1993). Deletion of the serine 34 codon from the major peripheral myelin protein P0 gene in Charcot-Marie-Tooth disease type 1B. *Nat. Genet.* 5, 35-39.

Le Douarin, N.M. and Dupin, E. (1993). Cell lineage analysis in neural crest ontogeny. *J. Neurobiol.* 24, 146-161.

Le, N., Nagarajan, R., Wang, J.Y., Svaren, J., LaPash, C., Araki, T., Schmidt, R.E., and Milbrandt, J. (2005). Nab proteins are essential for peripheral nervous system myelination. *Nat. Neurosci.* 8, 932-940.

LeBlanc, S.E., Jang, S.W., Ward, R.M., Wrabetz, L., and Svaren, J. (2006). Direct regulation of myelin protein zero expression by the Egr2 transactivator. *J. Biol. Chem.* 281, 5453-5460.

LeBlanc, S.E., Jang, S.W., Ward, R.M., Wrabetz, L., and Svaren, J. (2006). Direct regulation of myelin protein zero expression by the Egr2 transactivator. *J. Biol. Chem.* 281, 5453-5460.

LeBlanc, S.E., Ward, R.M., and Svaren, J. (2007). Neuropathy-associated Egr2 mutants disrupt cooperative activation of myelin protein zero by Egr2 and Sox10. *Mol. Cell Biol.* 27, 3521-3529.

Lee, D.A., Zurawel, R.H., and Windebank, A.J. (1995). Ciliary neurotrophic factor expression in Schwann cells is induced by axonal contact. *J. Neurochem.* 65, 564-568.

Lee, M., Brennan, A., Blanchard, A., Zoidl, G., Dong, Z., Tabernero, A., Zoidl, C., Dent, M.A., Jessen, K.R., and Mirsky, R. (1997). P0 is constitutively expressed in the rat neural crest and embryonic nerves and is negatively and positively regulated by axons to generate non-myelin-forming and myelin-forming Schwann cells, respectively. *Mol. Cell Neurosci.* 8, 336-350.

Lee, M., Brennan, A., Blanchard, A., Zoidl, G., Dong, Z., Tabernero, A., Zoidl, C., Dent, M.A., Jessen, K.R., and Mirsky, R. (1997). P0 is constitutively expressed in the rat neural crest and embryonic nerves and is negatively and positively regulated by axons to generate non-myelin-forming and myelin-forming Schwann cells, respectively. *Mol. Cell Neurosci.* 8, 336-350.

Lee, M.J., Calle, E., Brennan, A., Ahmed, S., Sviderskaya, E., Jessen, K.R., and Mirsky, R. (2001). In early development of the rat mRNA for the major myelin protein P(0) is expressed in nonsensory areas of the embryonic inner ear, notochord, enteric nervous system, and olfactory ensheathing cells. *Dev. Dyn.* 222, 40-51.

LeGuern, E., Gouider, R., Mabin, D., Tardieu, S., Birouk, N., Parent, P., Bouche, P., and Brice, A. (1997). Patients homozygous for the 17p11.2 duplication in Charcot-Marie-Tooth type 1A disease. *Ann. Neurol.* 41, 104-108.

Lemke, G. and Axel, R. (1985). Isolation and sequence of a cDNA encoding the major structural protein of peripheral myelin. *Cell* 40, 501-508.

- Lemke, G. and Chao, M. (1988). Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* 102, 499-504.
- Lemke, G., Lamar, E., and Patterson, J. (1988). Isolation and analysis of the gene encoding peripheral myelin protein zero. *Neuron* 1, 73-83.
- Lenssen, P.P., Gabreels-Festen, A.A., Valentijn, L.J., Jongen, P.J., van Beersum, S.E., van Engelen, B.G., van Wensen, P.J., Bolhuis, P.A., Gabreels, F.J., and Mariman, E.C. (1998). Hereditary neuropathy with liability to pressure palsies. Phenotypic differences between patients with the common deletion and a PMP22 frame shift mutation. *Brain* 121 ( Pt 8), 1451-1458.
- Lewis, R.A. and Shy, M.E. (1999). Electrodiagnostic findings in CMTX: a disorder of the Schwann cell and peripheral nerve myelin. *Ann. N. Y. Acad. Sci.* 883, 504-507.
- Liehr, T., Ekici, A., Hillenbrand, R., and Rautenstrauss, B. (1995). Regional localization of rat myelin protein zero Mpz gene to chromosome 13q24-25 by means of FISH. *Mamm. Genome* 6, 824-825.
- Lin, W., Sanchez, H.B., Deerinck, T., Morris, J.K., Ellisman, M., and Lee, K.F. (2000). Aberrant development of motor axons and neuromuscular synapses in erbB2-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* 97, 1299-1304.
- Losson, R. and Lacroute, F. (1979). Interference of nonsense mutations with eukaryotic messenger RNA stability. *Proc. Natl. Acad. Sci. U. S. A* 76, 5134-5137.
- Lupski, J.R., de Oca-Luna, R.M., Slaugenhaupt, S., Pentao, L., Guzzetta, V., Trask, B.J., Saucedo-Cardenas, O., Barker, D.F., Killian, J.M., Garcia, C.A., Chakravarti, A., and Patel, P.I. (1991). DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 66, 219-232.
- Magyar, J.P., Martini, R., Ruelicke, T., Aguzzi, A., Adlkofer, K., Dembic, Z., Zielasek, J., Toyka, K.V., and Suter, U. (1996). Impaired differentiation of Schwann cells in transgenic mice with increased PMP22 gene dosage. *J. Neurosci.* 16, 5351-5360.
- Mandich, P., Mancardi, G.L., Varese, A., Soriani, S., Di, M.E., Bellone, E., Bado, M., Gross, L., Windebank, A.J., Ajmar, F., and Schenone, A. (1999). Congenital hypomyelination due to myelin protein zero Q215X mutation. *Ann. Neurol.* 45, 676-678.
- Marchionni, M.A., Goodearl, A.D., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., and . (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* 362, 312-318.

- Marrosu,M.G., Vaccargiu,S., Marrosu,G., Vannelli,A., Cianchetti,C., and Muntoni,F. (1998). Charcot-Marie-Tooth disease type 2 associated with mutation of the myelin protein zero gene. *Neurology* 50, 1397-1401.
- Martini,R., Mohajeri,M.H., Kasper,S., Giese,K.P., and Schachner,M. (1995). Mice doubly deficient in the genes for P0 and myelin basic protein show that both proteins contribute to the formation of the major dense line in peripheral nerve myelin. *J. Neurosci.* 15, 4488-4495.
- Martini,R., Zielasek,J., Toyka,K.V., Giese,K.P., and Schachner,M. (1995). Protein zero (P0)-deficient mice show myelin degeneration in peripheral nerves characteristic of inherited human neuropathies. *Nat. Genet.* 11, 281-286.
- Martini,R. (2001). The effect of myelinating Schwann cells on axons. *Muscle Nerve* 24, 456-466.
- Meier,C., Parmantier,E., Brennan,A., Mirsky,R., and Jessen,K.R. (1999). Developing Schwann cells acquire the ability to survive without axons by establishing an autocrine circuit involving insulin-like growth factor, neurotrophin-3, and platelet-derived growth factor-BB. *J. Neurosci.* 19, 3847-3859.
- Mellman,I. and Warren,G. (2000). The road taken: past and future foundations of membrane traffic. *Cell* 100, 99-112.
- Mersiyanova,I.V., Perepelov,A.V., Polyakov,A.V., Sitnikov,V.F., Dadali,E.L., Oparin,R.B., Petrin,A.N., and Evgrafov,O.V. (2000). A new variant of Charcot-Marie-Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene. *Am. J. Hum. Genet.* 67, 37-46.
- Messing,A., Behringer,R.R., Hammang,J.P., Palmiter,R.D., Brinster,R.L., and Lemke,G. (1992). P0 promoter directs expression of reporter and toxin genes to Schwann cells of transgenic mice. *Neuron* 8, 507-520.
- Mews,M. and Meyer,M. (1993). Modulation of Schwann cell phenotype by TGF-beta 1: inhibition of P0 mRNA expression and downregulation of the low affinity NGF receptor. *Glia* 8, 208-217.
- Meyer,D. and Birchmeier,C. (1995). Multiple essential functions of neuregulin in development. *Nature* 378, 386-390.
- Mirsky,R. and Jessen,K.R. (1999). The neurobiology of Schwann cells. *Brain Pathol.* 9, 293-311.
- Molineaux,S.M., Engh,H., de,F.F., Hudson,L., and Lazzarini,R.A. (1986). Recombination within the myelin basic protein gene created the dysmyelinating shiverer mouse mutation. *Proc. Natl. Acad. Sci. U. S. A* 83, 7542-7546.
- Montag,D., Giese,K.P., Bartsch,U., Martini,R., Lang,Y., Bluthmann,H., Karthigasan,J., Kirschner,D.A., Wintergerst,E.S., Nave,K.A., and . (1994).

- Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. *Neuron* 13, 229-246.
- Monuki,E.S., Weinmaster,G., Kuhn,R., and Lemke,G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* 3, 783-793.
- Monuki,E.S., Kuhn,R., and Lemke,G. (1993). Repression of the myelin P0 gene by the POU transcription factor SCIP. *Mech. Dev.* 42, 15-32.
- Morgan,L., Jessen,K.R., and Mirsky,R. (1991). The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. *J. Cell Biol.* 112, 457-467.
- Morgan,L., Jessen,K.R., and Mirsky,R. (1994). Negative regulation of the P0 gene in Schwann cells: suppression of P0 mRNA and protein induction in cultured Schwann cells by FGF2 and TGF beta 1, TGF beta 2 and TGF beta 3. *Development* 120, 1399-1409.
- Naef,R. and Suter,U. (1998). Many facets of the peripheral myelin protein PMP22 in myelination and disease. *Microsc. Res. Tech.* 41, 359-371.
- Nagarajan,R., Svaren,J., Le,N., Araki,T., Watson,M., and Milbrandt,J. (2001). EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. *Neuron* 30, 355-368.
- Nagy,E. and Maquat,L.E. (1998). A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. *Trends Biochem. Sci.* 23, 198-199.
- Nelis,E., Haites,N., and Van,B.C. (1999). Mutations in the peripheral myelin genes and associated genes in inherited peripheral neuropathies. *Hum. Mutat.* 13, 11-28.
- Nelis,E., Erdem,S., Van Den Bergh,P.Y., Belpaire-Dethiou,M.C., Ceuterick,C., Van,G., V, Cuesta,A., Pedrola,L., Palau,F., Gabreels-Festen,A.A., Verellen,C., Tan,E., Demirci,M., Van,B.C., De,J.P., Topaloglu,H., and Timmerman,V. (2002). Mutations in GDAP1: autosomal recessive CMT with demyelination and axonopathy. *Neurology* 59, 1865-1872.
- Nicholson,G. and Nash,J. (1993). Intermediate nerve conduction velocities define X-linked Charcot-Marie-Tooth neuropathy families. *Neurology* 43, 2558-2564.
- Nicholson,G.A., Valentijn,L.J., Cherryson,A.K., Kennerson,M.L., Bragg,T.L., DeKroon,R.M., Ross,D.A., Pollard,J.D., McLeod,J.G., Bolhuis,P.A., and . (1994). A frame shift mutation in the PMP22 gene in hereditary neuropathy with liability to pressure palsies. *Nat. Genet.* 6, 263-266.

- Niemann,S., Sereda,M.W., Suter,U., Griffiths,I.R., and Nave,K.A. (2000). Uncoupling of myelin assembly and schwann cell differentiation by transgenic overexpression of peripheral myelin protein 22. *J. Neurosci.* 20, 4120-4128.
- Nodari,A., Zambroni,D., Quattrini,A., Court FA, D'Urso,A., Recchia,A., Tybulewicz,V.L., Wrabetz,L., and Feltri,M.L. (2007). Beta1 integrin activates Rac1 in Schwann cells to generate radial lamellae during axonal sorting and myelination. *J. Cell Biol.* 177, 1063-1075.
- Notterpek,L., Snipes,G.J., and Shooter,E.M. (1999). Temporal expression pattern of peripheral myelin protein 22 during in vivo and in vitro myelination. *Glia* 25, 358-369.
- Ouvrier,R.A., McLeod,J.G., and Conchin,T.E. (1987). The hypertrophic forms of hereditary motor and sensory neuropathy. A study of hypertrophic Charcot-Marie-Tooth disease (HMSN type I) and Dejerine-Sottas disease (HMSN type III) in childhood. *Brain* 110 (*Pt 1*), 121-148.
- Owens,G.C. and Bunge,R.P. (1991). Schwann cells infected with a recombinant retrovirus expressing myelin-associated glycoprotein antisense RNA do not form myelin. *Neuron* 7, 565-575.
- Owens,G.C. and Boyd,C.J. (1991). Expressing antisense P0 RNA in Schwann cells perturbs myelination. *Development* 112, 639-649.
- Pankov,R., Endo,Y., Even-Ram,S., Araki,M., Clark,K., Cukierman,E., Matsumoto,K., and Yamada,K.M. (2005). A Rac switch regulates random versus directionally persistent cell migration. *J. Cell Biol.* 170, 793-802.
- Pareyson,D., Menichella,D., Botti,S., Sghirlanzoni,A., Fallica,E., Mora,M., Ciano,C., Shy,M.E., and Taroni,F. (1999). Heterozygous null mutation in the P0 gene associated with mild Charcot-Marie-Tooth disease. *Ann. N. Y. Acad. Sci.* 883, 477-480.
- Parkinson,D.B., Bhaskaran,A., Droggiti,A., Dickinson,S., D'Antonio,M., Mirsky,R., and Jessen,K.R. (2004). Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell proliferation and death. *J. Cell Biol.* 164, 385-394.
- Parman,Y., Plante-Bordeneuve,V., Guiochon-Mantel,A., Eraksoy,M., and Said,G. (1999). Recessive inheritance of a new point mutation of the PMP22 gene in Dejerine-Sottas disease. *Ann. Neurol.* 45, 518-522.
- Parmantier,E., Cabon,F., Braun,C., D'Urso,D., Muller,H.W., and Zalc,B. (1995). Peripheral myelin protein-22 is expressed in rat and mouse brain and spinal cord motoneurons. *Eur. J. Neurosci.* 7, 1080-1088.
- Parmantier,E., Braun,C., Thomas,J.L., Peyron,F., Martinez,S., and Zalc,B. (1997). PMP-22 expression in the central nervous system of the embryonic mouse defines potential transverse segments and longitudinal columns. *J. Comp Neurol.* 378, 159-172.

- Parmantier,E., Lynn,B., Lawson,D., Turmaine,M., Namini,S.S., Chakrabarti,L., McMahon,A.P., Jessen,K.R., and Mirsky,R. (1999). Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. *Neuron* 23, 713-724.
- Peirano,R.I., Goerich,D.E., Riethmacher,D., and Wegner,M. (2000). Protein zero gene expression is regulated by the glial transcription factor Sox10. *Mol. Cell Biol.* 20, 3198-3209.
- Pentao,L., Wise,C.A., Chinault,A.C., Patel,P.I., and Lupski,J.R. (1992). Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit. *Nat. Genet.* 2, 292-300.
- Perea,J., Robertson,A., Tolmachova,T., Muddle,J., King,R.H., Ponsford,S., Thomas,P.K., and Huxley,C. (2001). Induced myelination and demyelination in a conditional mouse model of Charcot-Marie-Tooth disease type 1A. *Hum. Mol. Genet.* 10, 1007-1018.
- Perkins,C.S., Aguayo,A.J., and Bray,G.M. (1981). Behavior of schwann cells from trembler mouse unmyelinated fibers transplanted into myelinated nerves. *Exp. Neurol.* 71, 515-526.
- Pham-Dinh,D., Fourbil,Y., Blanquet,F., Mattei,M.G., Roeckel,N., Latour,P., Chazot,G., Vandenberghe,A., and Dautigny,A. (1993). The major peripheral myelin protein zero gene: structure and localization in the cluster of Fc gamma receptor genes on human chromosome 1q21.3-q23. *Hum. Mol. Genet.* 2, 2051-2054.
- Poduslo,J.F. (1990). Golgi sulfation of the oligosaccharide chain of P0 occurs in the presence of myelin assembly but not in its absence. *J. Biol. Chem.* 265, 3719-3725.
- Previtali,S.C., Quattrini,A., Fasolini,M., Panzeri,M.C., Villa,A., Filbin,M.T., Li,W., Chiu,S.Y., Messing,A., Wrabetz,L., and Feltri,M.L. (2000). Epitope-tagged P(0) glycoprotein causes Charcot-Marie-Tooth-like neuropathy in transgenic mice. *J. Cell Biol.* 151, 1035-1046.
- Quattieri,A., Urso,E., Le,P.M., Scornaienchi,M., Quattrone,A., Di,D.L., Napoli,A., and Sindona,G. (2006). Proteomics of bovine myelin sheath: characterization of a truncated form of P0 by MALDI-TOF/TOF mass spectrometry. *J. Am. Soc. Mass Spectrom.* 17, 117-123.
- Quarles,R.H. (2002). Myelin sheaths: glycoproteins involved in their formation, maintenance and degeneration. *Cell Mol. Life Sci.* 59, 1851-1871.
- Quattrone,A., Gambardella,A., Bono,F., Aguglia,U., Bolino,A., Bruni,A.C., Montesi,M.P., Oliveri,R.L., Sabatelli,M., Tamburrini,O., Valentino,P., Van,B.C., and Zappia,M. (1996). Autosomal recessive hereditary motor and sensory neuropathy with focally folded myelin



- sheaths: clinical, electrophysiologic, and genetic aspects of a large family. *Neurology* 46, 1318-1324.
- Raeymaekers,P., Timmerman,V., Nelis,E., Van,H.W., De,J.P., Martin,J.J., and Van,B.C. (1992). Estimation of the size of the chromosome 17p11.2 duplication in Charcot-Marie-Tooth neuropathy type 1a (CMT1a). HMSN Collaborative Research Group. *J. Med. Genet.* 29, 5-11.
- Riethmacher,D., Sonnenberg-Riethmacher,E., Brinkmann,V., Yamaai,T., Lewin,G.R., and Birchmeier,C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389, 725-730.
- Riethmacher,D., Sonnenberg-Riethmacher,E., Brinkmann,V., Yamaai,T., Lewin,G.R., and Birchmeier,C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389, 725-730.
- Roa,B.B., Dyck,P.J., Marks,H.G., Chance,P.F., and Lupski,J.R. (1993). Dejerine-Sottas syndrome associated with point mutation in the peripheral myelin protein 22 (PMP22) gene. *Nat. Genet.* 5, 269-273.
- Rosenbluth,J. (1980). Peripheral myelin in the mouse mutant Shiverer. *J. Comp Neurol.* 193, 729-739.
- Russo,M.W., Sevetson,B.R., and Milbrandt,J. (1995). Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc. Natl. Acad. Sci. U. S. A* 92, 6873-6877.
- Saavedra,R.A., Fors,L., Aebersold,R.H., Arden,B., Horvath,S., Sanders,J., and Hood,L. (1989). The myelin proteins of the shark brain are similar to the myelin proteins of the mammalian peripheral nervous system. *J. Mol. Evol.* 29, 149-156.
- Sahenk,Z., Chen,L., and Freimer,M. (1998). A novel PMP22 point mutation causing HNPP phenotype: studies on nerve xenografts. *Neurology* 51, 702-707.
- Sahenk,Z. (1999). Abnormal Schwann cell-axon interactions in CMT neuropathies. The effects of mutant Schwann cells on the axonal cytoskeleton and regeneration-associated myelination. *Ann. N. Y. Acad. Sci.* 883, 415-426.
- Sakamoto,Y., Kitamura,K., Yoshimura,K., Nishijima,T., and Uyemura,K. (1987). Complete amino acid sequence of PO protein in bovine peripheral nerve myelin. *J. Biol. Chem.* 262, 4208-4214.
- Salzer,J.L. (2002). Nodes of Ranvier come of age. *Trends Neurosci.* 25, 2-5.
- Sancho,S., Magyar,J.P., Aguzzi,A., and Suter U (1999). Distal axonopathy in peripheral nerves of PMP22-mutant mice. *Brain* 122 ( Pt 8), 1563-1577.

- Sancho,S., Young,P., and Suter,U. (2001). Regulation of Schwann cell proliferation and apoptosis in PMP22-deficient mice and mouse models of Charcot-Marie-Tooth disease type 1A. *Brain* 124, 2177-2187.
- Schneider-Maunoury,S., Topilko,P., Seitandou,T., Levi,G., Cohen-Tannoudji,M., Pournin,S., Babinet,C., and Charnay,P. (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* 75, 1199-1214.
- Schneider-Schaulies,J., von,B.A., and Schachner,M. (1990). Recombinant peripheral myelin protein P0 confers both adhesion and neurite outgrowth-promoting properties. *J. Neurosci. Res.* 27, 286-297.
- Schweitzer,J., Becker,T., Becker,C.G., and Schachner,M. (2003). Expression of protein zero is increased in lesioned axon pathways in the central nervous system of adult zebrafish. *Glia* 41, 301-317.
- Schweitzer,J., Becker,T., Becker,C.G., and Schachner,M. (2003). Expression of protein zero is increased in lesioned axon pathways in the central nervous system of adult zebrafish. *Glia* 41, 301-317.
- Sela-Donenfeld,D. and Kalcheim,C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* 126, 4749-4762.
- Senderek,J., Hermanns,B., Lehmann,U., Bergmann,C., Marx,G., Kabus,C., Timmerman,V., Stoltenburg-Didinger,G., and Schroder,J.M. (2000). Charcot-Marie-Tooth neuropathy type 2 and P0 point mutations: two novel amino acid substitutions (Asp61Gly; Tyr119Cys) and a possible "hotspot" on Thr124Met. *Brain Pathol.* 10, 235-248.
- Senderek,J., Bergmann,C., Stendel,C., Kirfel,J., Verpoorten,N., De,J.P., Timmerman,V., Chrast,R., Verheijen,M.H., Lemke,G., Battaloglu,E., Parman,Y., Erdem,S., Tan,E., Topaloglu,H., Hahn,A., Muller-Felber,W., Rizzuto,N., Fabrizi,G.M., Stuhmann,M., Rudnik-Schoneborn,S., Zuchner,S., Michael,S.J., Buchheim,E., Straub,V., Klepper,J., Huehne,K., Rautenstrauss,B., Buttner,R., Nelis,E., and Zerres,K. (2003). Mutations in a gene encoding a novel SH3/TPR domain protein cause autosomal recessive Charcot-Marie-Tooth type 4C neuropathy. *Am. J. Hum. Genet.* 73, 1106-1119.
- Sereda,M., Griffiths,I., Puhlhofer,A., Stewart,H., Rossner,M.J., Zimmermann,F., Magyar,J.P., Schneider,A., Hund,E., Meinck,H.M., Suter,U., and Nave,K.A. (1996). A transgenic rat model of Charcot-Marie-Tooth disease. *Neuron* 16, 1049-1060.
- Sessa,M., Nemni,R., Quattrini,A., Del,C.U., Wrabetz,L., and Canal,N. (1997). Atypical hereditary neuropathy with liability to pressure palsies (HNPP): the value of direct DNA diagnosis. *J. Med. Genet.* 34, 889-892.

- Sghirlanzoni,A., Pareyson,D., Balestrini,M.R., Bellone,E., Berta,E., Ciano,C., Mandich,P., and Marazzi,R. (1992). HMSN III phenotype due to homozygous expression of a dominant HMSN II gene. *Neurology* 42, 2201-2204.
- Shah,N.M., Marchionni,M.A., Isaacs,I., Stroobant,P., and Anderson,D.J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* 77, 349-360.
- Shah,N.M., Groves,A.K., and Anderson,D.J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* 85, 331-343.
- Shapiro,L., Doyle,J.P., Hensley,P., Colman,D.R., and Hendrickson,W.A. (1996). Crystal structure of the extracellular domain from P0, the major structural protein of peripheral nerve myelin. *Neuron* 17, 435-449.
- Shy,M.E., Balsamo,J., Lilien,J., and Kamholz,J. (2001). A molecular basis for hereditary motor and sensory neuropathy disorders. *Curr. Neurol. Neurosci. Rep.* 1, 77-88.
- Shy,M.E., Hobson,G., Jain,M., Boespflug-Tanguy,O., Garbern,J., Sperle,K., Li,W., Gow,A., Rodriguez,D., Bertini,E., Mancias,P., Krajewski,K., Lewis,R., and Kamholz,J. (2003). Schwann cell expression of PLP1 but not DM20 is necessary to prevent neuropathy. *Ann. Neurol.* 53, 354-365.
- Snipes,G.J., Suter,U., Welcher,A.A., and Shooter,E.M. (1992). Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). *J. Cell Biol.* 117, 225-238.
- Soilu-Hanninen,M., Ekert,P., Bucci,T., Syroid,D., Bartlett,P.F., and Kilpatrick,T.J. (1999). Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. *J. Neurosci.* 19, 4828-4838.
- Stahl,N., Harry,J., and Popko,B. (1990). Quantitative analysis of myelin protein gene expression during development in the rat sciatic nerve. *Brain Res. Mol. Brain Res.* 8, 209-212.
- Stecca,B., Southwood,C.M., Gragerov,A., Kelley,K.A., Friedrich,V.L., Jr., and Gow,A. (2000). The evolution of lipophilin genes from invertebrates to tetrapods: DM-20 cannot replace proteolipid protein in CNS myelin. *J. Neurosci.* 20, 4002-4010.
- Stratmann,A. and Jeserich,G. (1995). Molecular cloning and tissue expression of a cDNA encoding IP1--a P0-like glycoprotein of trout CNS myelin. *J. Neurochem.* 64, 2427-2436.
- Street,V.A., Bennett,C.L., Goldy,J.D., Shirk,A.J., Kleopa,K.A., Tempel,B.L., Lipe,H.P., Scherer,S.S., Bird,T.D., and Chance,P.F. (2003).

Mutation of a putative protein degradation gene LITAF/SIMPLE in Charcot-Marie-Tooth disease 1C. *Neurology* 60, 22-26.

Sturtz,F.G., Latour,P., Mocquard,Y., Cruz,S., Fenoll,B., LeFur,J.M., Mabin,D., Chazot,G., and Vandenberghe,A. (1997). Clinical and electrophysiological phenotype of a homozygously duplicated Charcot-Marie-Tooth (type 1A) disease. *Eur. Neurol.* 38, 26-30.

Suter,U., Moskow,J.J., Welcher,A.A., Snipes,G.J., Kosaras,B., Sidman,R.L., Buchberg,A.M., and Shooter,E.M. (1992). A leucine-to-proline mutation in the putative first transmembrane domain of the 22-kDa peripheral myelin protein in the trembler-J mouse. *Proc. Natl. Acad. Sci. U. S. A* 89, 4382-4386.

Suter,U., Welcher,A.A., Ozcelik,T., Snipes,G.J., Kosaras,B., Francke,U., Billings-Gagliardi,S., Sidman,R.L., and Shooter,E.M. (1992). Trembler mouse carries a point mutation in a myelin gene. *Nature* 356, 241-244.

Suter,U., Welcher,A.A., and Snipes,G.J. (1993). Progress in the molecular understanding of hereditary peripheral neuropathies reveals new insights into the biology of the peripheral nervous system. *Trends Neurosci.* 16, 50-56.

Suter,U., Snipes,G.J., Schoener-Scott,R., Welcher,A.A., Pareek,S., Lupski,J.R., Murphy,R.A., Shooter,E.M., and Patel,P.I. (1994). Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP22) gene transcripts by two promoters. *J. Biol. Chem.* 269, 25795-25808.

Svaren,J., Sevetson,B.R., Apel,E.D., Zimonjic,D.B., Popescu,N.C., and Milbrandt,J. (1996). NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. *Mol. Cell Biol.* 16, 3545-3553.

Szabo,A., Zuchner,S., Siska,E., Mechler,F., and Molnar,M.J. (2005). Marked phenotypic variation in a family with a new myelin protein zero mutation. *Neuromuscul. Disord.* 15, 760-763.

Takashima,H., Nakagawa,M., Kanzaki,A., Yawata,Y., Horikiri,T., Matsuzaki,T., Suehara,M., Izumo,S., and Osame,M. (1999). Germline mosaicism of MPZ gene in Dejerine-Sottas syndrome (HMSN III) associated with hereditary stomatocytosis. *Neuromuscul. Disord.* 9, 232-238.

Takashima,H., Boerkoel,C.F., De,J.P., Ceuterick,C., Martin,J.J., Voit,T., Schroder,J.M., Williams,A., Brophy,P.J., Timmerman,V., and Lupski,J.R. (2002). Periaxin mutations cause a broad spectrum of demyelinating neuropathies. *Ann. Neurol.* 51, 709-715.

Takeda,Y., Notsu,T., Kitamura,K., and Uyemura,K. (2001). Functional analysis for peripheral myelin protein PASII/PMP22: is it a member of claudin superfamily? *Neurochem. Res.* 26, 599-607.

Taveggia,C., Zanazzi,G., Petrylak,A., Yano,H., Rosenbluth,J., Einheber,S., Xu,X., Esper,R.M., Loeb,J.A., Shrager,P., Chao,M.V., Falls,D.L., Role,L., and Salzer,J.L. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* 47, 681-694.

Timmerman,V., De,J.P., Spoelders,P., Simokovic,S., Lofgren,A., Nelis,E., Vance,J., Martin,J.J., and Van,B.C. (1996). Linkage and mutation analysis of Charcot-Marie-Tooth neuropathy type 2 families with chromosomes 1p35-p36 and Xq13. *Neurology* 46, 1311-1318.

Tobler,A.R., Notterpek,L., Naef,R., Taylor,V., Suter,U., and Shooter,E.M. (1999). Transport of Trembler-J mutant peripheral myelin protein 22 is blocked in the intermediate compartment and affects the transport of the wild-type protein by direct interaction. *J. Neurosci.* 19, 2027-2036.

Tobler,A.R., Liu,N., Mueller,L., and Shooter,E.M. (2002). Differential aggregation of the Trembler and Trembler J mutants of peripheral myelin protein 22. *Proceedings of the National Academy of Sciences of the United States of America* 99, 483-488.

Topilko,P., Schneider-Maunoury,S., Levi,G., Baron-Van,E.A., Chennoufi,A.B., Seitanidou,T., Babinet,C., and Charnay,P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* 371, 796-799.

Topilko,P., Schneider-Maunoury,S., Levi,G., Baron-Van,E.A., Chennoufi,A.B., Seitanidou,T., Babinet,C., and Charnay,P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* 371, 796-799.

Topilko,P., Levi,G., Merlo,G., Mantero,S., Desmarquet,C., Mancardi,G., and Charnay,P. (1997). Differential regulation of the zinc finger genes Krox-20 and Krox-24 (Egr-1) suggests antagonistic roles in Schwann cells. *J. Neurosci. Res.* 50, 702-712.

Trapp,B.D., Itoyama,Y., MacIntosh,T.D., and Quarles,R.H. (1983). P2 protein in oligodendrocytes and myelin of the rabbit central nervous system. *J. Neurochem.* 40, 47-54.

Trapp,B.D., Bernier,L., Andrews,S.B., and Colman,D.R. (1988). Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *J. Neurochem.* 51, 859-868.

Trapp,B.D., Hauer,P., and Lemke,G. (1988). Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. *J. Neurosci.* 8, 3515-3521.

- Trapp,B.D., Kidd,G.J., Hauer,P., Mulrenin,E., Haney,C.A., and Andrews,S.B. (1995). Polarization of myelinating Schwann cell surface membranes: role of microtubules and the trans-Golgi network. *J. Neurosci.* *15*, 1797-1807.
- uer-Grumbach,M., De,J.P., Wagner,K., Verhoeven,K., Hartung,H.P., and Timmerman,V. (2000). Phenotype-genotype correlations in a CMT2B family with refined 3q13-q22 locus. *Neurology* *55*, 1552-1557.
- Uyemura,K., Yoshimura,K., Suzuki,M., and Kitamura,K. (1984). Lipid binding activities of the P2 protein in peripheral nerve myelin. *Neurochem. Res.* *9*, 1509-1514.
- Valentijn,L.J., Baas,F., Wolterman,R.A., Hoogendijk,J.E., van den Bosch,N.H., Zorn,I., Gabreels-Festen,A.W., de,V.M., and Bolhuis,P.A. (1992). Identical point mutations of PMP-22 in Trembler-J mouse and Charcot-Marie-Tooth disease type 1A. *Nat. Genet.* *2*, 288-291.
- Valentijn,L.J., Ouvrier,R.A., van den Bosch,N.H., Bolhuis,P.A., Baas,F., and Nicholson,G.A. (1995). Dejerine-Sottas neuropathy is associated with a de novo PMP22 mutation. *Hum. Mutat.* *5*, 76-80.
- Vallat,J.M., Sindou,P., Preux,P.M., Tabaraud,F., Milor,A.M., Couratier,P., LeGuern,E., and Brice,A. (1996). Ultrastructural PMP22 expression in inherited demyelinating neuropathies. *Ann. Neurol.* *39*, 813-817.
- Vallat,J.M., Sindou,P., Garbay,B., Preux,P.M., Anani,T., Richard,L., and Diot,M. (1999). Expression of myelin proteins in the adult heterozygous Trembler mouse. *Acta Neuropathol.* *98*, 281-287.
- Vance,J.M., Nicholson,G.A., Yamaoka,L.H., Stajich,J., Stewart,C.S., Speer,M.C., Hung,W.Y., Roses,A.D., Barker,D., and Pericak-Vance,M.A. (1989). Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. *Exp. Neurol.* *104*, 186-189.
- Warner,L.E., Hilz,M.J., Appel,S.H., Killian,J.M., Kolodry,E.H., Karpati,G., Carpenter,S., Watters,G.V., Wheeler,C., Witt,D., Bodell,A., Nelis,E., Van,B.C., and Lupski,J.R. (1996). Clinical phenotypes of different MPZ (P0) mutations may include Charcot-Marie-Tooth type 1B, Dejerine-Sottas, and congenital hypomyelination. *Neuron* *17*, 451-460.
- Warner,L.E., Mancias,P., Butler,I.J., McDonald,C.M., Keppen,L., Koob,K.G., and Lupski,J.R. (1998). Mutations in the early growth response 2 (EGR2) gene are associated with hereditary myelinopathies. *Nat. Genet.* *18*, 382-384.
- Warner,L.E., Garcia,C.A., and Lupski,J.R. (1999). Hereditary peripheral neuropathies: clinical forms, genetics, and molecular mechanisms. *Annu. Rev. Med.* *50*, 263-275.
- Weinstein,D.E., Burrola,P.G., and Lemke,G. (1995). Premature Schwann cell differentiation and hypermyelination in mice expressing a targeted

- antagonist of the POU transcription factor SCIP. *Mol. Cell Neurosci.* 6, 212-229.
- Wong,M.H. and Filbin,M.T. (1994). The cytoplasmic domain of the myelin P0 protein influences the adhesive interactions of its extracellular domain. *J. Cell Biol.* 126, 1089-1097.
- Wong,M.H. and Filbin,M.T. (1996). Dominant-negative effect on adhesion by myelin Po protein truncated in its cytoplasmic domain. *J. Cell Biol.* 134, 1531-1541.
- Wrabetz,L., Feltri,M.L., Quattrini,A., Imperiale,D., Previtali,S., D'Antonio,M., Martini,R., Yin,X., Trapp,B.D., Zhou,L., Chiu,S.Y., and Messing,A. (2000). P(0) glycoprotein overexpression causes congenital hypomyelination of peripheral nerves. *J. Cell Biol.* 148, 1021-1034.
- Wrabetz,L., D'Antonio,M., Pennuto,M., Dati,G., Tinelli,E., Fratta,P., Previtali,S., Imperiale,D., Zielasek,J., Toyka,K., Avila,R.L., Kirschner,D.A., Messing,A., Feltri,M.L., and Quattrini,A. (2006). Different intracellular pathomechanisms produce diverse Myelin Protein Zero neuropathies in transgenic mice. *J. Neurosci.* 26, 2358-2368.
- Xu,M., Zhao,R., Sui,X., Xu,F., and Zhao,Z.J. (2000). Tyrosine phosphorylation of myelin P(0) and its implication in signal transduction. *Biochem. Biophys. Res. Commun.* 267, 820-825.
- Xu,W., Shy,M., Kamholz,J., Elferink,L., Xu,G., Lilien,J., and Balsamo,J. (2001). Mutations in the cytoplasmic domain of P0 reveal a role for PKC-mediated phosphorylation in adhesion and myelination. *J. Cell Biol.* 155, 439-446.
- Yin,X., Kidd,G.J., Wrabetz,L., Feltri,M.L., Messing,A., and Trapp,B.D. (2000). Schwann cell myelination requires timely and precise targeting of P(0) protein. *J. Cell Biol.* 148, 1009-1020.
- Yoshikawa,H., Nishimura,T., Nakatsuji,Y., Fujimura,H., Himoro,M., Hayasaka,K., Sakoda,S., and Yanagihara,T. (1994). Elevated expression of messenger RNA for peripheral myelin protein 22 in biopsied peripheral nerves of patients with Charcot-Marie-Tooth disease type 1A. *Ann. Neurol.* 35, 445-450.
- You,K.H., Hsieh,C.L., Hayes,C., Stahl,N., Francke,U., and Popko,B. (1991). DNA sequence, genomic organization, and chromosomal localization of the mouse peripheral myelin protein zero gene: identification of polymorphic alleles. *Genomics* 9, 751-757.
- Zeller,N.K., Hunkeler,M.J., Campagnoni,A.T., Sprague,J., and Lazzarini,R.A. (1984). Characterization of mouse myelin basic protein messenger RNAs with a myelin basic protein cDNA clone. *Proc. Natl. Acad. Sci. U. S. A* 81, 18-22.



Zhang,K. and Filbin,M.T. (1994). Formation of a disulfide bond in the immunoglobulin domain of the myelin P0 protein is essential for its adhesion. *J. Neurochem.* 63, 367-370.

Zhang,S.M., Marsh,R., Ratner,N., and Brackenbury,R. (1995). Myelin glycoprotein P0 is expressed at early stages of chicken and rat embryogenesis. *J. Neurosci. Res.* 40, 241-250.

Zielasek,J., Martini,R., and Toyka,K.V. (1996). Functional abnormalities in P0-deficient mice resemble human hereditary neuropathies linked to P0 gene mutations. *Muscle Nerve* 19, 946-952.

Zorick,T.S., Syroid,D.E., Brown,A., Gridley,T., and Lemke,G. (1999). Krox-20 controls SCIP expression, cell cycle exit and susceptibility to apoptosis in developing myelinating Schwann cells. *Development* 126, 1397-1406.

Zuchner,S., Mersiyanova,I.V., Muglia,M., Bissar-Tadmouri,N., Rochelle,J., Dadali,E.L., Zappia,M., Nelis,E., Patitucci,A., Senderek,J., Parman,Y., Evgrafov,O., Jonghe,P.D., Takahashi,Y., Tsuji,S., Pericak-Vance,M.A., Quattrone,A., Battaloglu,E., Polyakov,A.V., Timmerman,V., Schroder,J.M., and Vance,J.M. (2004). Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. *Nat. Genet.* 36, 449-451.

Zuchner,S. and Vance,J.M. (2006). Mechanisms of disease: a molecular genetic update on hereditary axonal neuropathies. *Nat. Clin. Pract. Neurol.* 2, 45-53.