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Review

Pelizaeus-Merzbacher disease: Genetic and cellular pathogenesis

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Abstract. Pelizaeus-Merzbacher disease (PMD) and the allelic spastic paraplegia type 2 (SPG2) arise from mutations in the X-linked gene encoding myelin proteolipid protein (PLP). Analysis of mutations affecting PLP, the major protein in central nervous system myelin, has revealed previously unsuspected roles for myelinating glia in maintaining the integrity of the nervous system. The disease spectrum for PMD and SPG2 is extraordinarily broad and can be best understood by accounting not only for the wide range of mutations that can occur but also for the effects of *PLP1* mutations on both cell autonomous and non-cell autonomous processes in myelinating cells. Appreciating the wide range of genetic and cellular effects of *PLP1* mutations is important for patient and family counseling, understanding disease pathogenesis, and, ultimately, for developing future disease-specific therapies.

Keywords. Pelizaeus-Merzbacher, proteolipid protein, myelin, leukodystrophy, unfolded protein response, gene duplication, axo-glial interactions

Clinical description and historical nosology of Pelizaeus-Merzbacher disease

Friedrich Pelizaeus described, well before the rediscovery of Mendel's rules of inheritance, a family with severe neurological impairment that began early in life and in which he observed the salient features that we now associate with X-linked recessive disorders, *i.e.* 'that the disease is passed on by the mother but does not hurt her,' [1]. Twenty-five years later, Ludwig Merzbacher re-investigated the same family, which by then had 12 affected individuals, and performed a detailed pathological analysis of the brain of one affected man. He described the widespread lack of myelin staining in the cerebral white matter [2]. Both Pelizaeus and Merzbacher noted the development in early infancy of involuntary eye movements, or nystagmus. Infants had poor head control, motor tone and control, and often had tremors or titubation of the head and neck when seated. The disease was slowly progressive, with additional signs including bradylalia, scanning speech, ataxia and intention tremor of the upper limbs, spasticity of the limbs, athetotic movements, and cognitive impairment [1, 2].

In 1954, Seitelberger described a disorder with more severe clinical signs and pathology than that described by Merzbacher [3]. In this condition, there was nearly complete absence of myelin sheaths, and a profound loss of oligodendrocytes, the myelin-forming cells in the central nervous system (CNS). Seitelberger recognized the similarities to the disorder described by Pelizaeus and Merzbacher and to distinguish them referred to their syndrome as 'classical,' while his more severe patients were described as having 'connatal' Pelizaeus-Merzbacher disease (PMD). Subsequently, clinical forms of severity intermediate between the connatal and classical syndromes were labeled as 'transitional' [4, 5]. The lack

of histochemical staining of the white matter in these patients was non-uniform and typically had areas of relatively preserved myelin staining, giving the white matter a patchy or 'tigroid' appearance, which Seitelberger maintained was pathognomonic and essential for making the diagnosis of PMD [5]. Zeman and colleagues stressed the importance of X-linked inheritance in PMD families and considered this to be relatively more important than the pattern of myelin staining [6], drawing strong criticism from Seitelberger [5]. Boulloche and Aicardi also stressed the importance of family history as well as cardinal neurological signs in identifying families with PMD, and these criteria were important for subsequent linkage studies that lead to the identification of the gene defects that cause PMD [7]. Zeman furthermore speculated that the fundamental defect in PMD affected a proteolipid [6], This prescient hypothesis was later strengthened by the mapping of the *proteolipid protein 1* (*PLP1;* formerly called *PLP*) gene to the X chromosome [8] and subsequently confirmed by the identification in 1989 of *PLP1* mutations in several families with the disease [9–11].

The phenotypic spectrum of *PLP1* mutations expanded when Boespflug-Tanguy and colleagues found *PLP1* mutations in patients who had the relatively mild spastic paraplegia syndrome (now specifically called spastic paraplegia 2 or SPG2), without the other major signs of PMD ([12], reviewed in [13]). Later studies further discriminated patients with mutations in the *PLP1* gene who had 'pure' spastic paraplegia, where neurological signs consisted of leg weakness and spasticity and associated autonomic signs, from those with 'complicated' spastic paraparesis, where additional CNS signs, such as ataxia, dysarthria and cognitive impairment, coexist. Clear discrimination between complicated SPG2 and mild PMD cannot be made in some cases, and is sometimes quite subjective. A clinically distinct null syndrome was described by Garbern and colleagues, and is characterized by complicated spastic paraparesis with mild to moderate demyelinating peripheral neuropathy, less distinct white matter changes on magnetic resonance imaging and reduced *N*-acetylaspartate, measured spectroscopically *in vivo*, in CNS white matter [14, 15].

Myelination and the role of myelin in CNS function

The efficient conduction of action potentials in the human nervous system is dependent upon the myelin sheath, a spirally wound band of oligodendrocyte cell membrane that segmentally envelops axons (Fig. 1a). Although the notion that the myelin sheath simply creates a region of high electrical resistance may not be totally correct, it does form a region of low capacitance that separates unmyelinated gaps [16], the nodes of Ranvier, where gated ion channels that mediate the ion fluxes that constitute the action potential are concentrated. Indeed, it appears that one important developmental role of myelination is to maintain the exquisite segregation of ion channels in the node and adjacent regions [17–19].

Oligodendrocytes are metabolically very active, especially during the time of peak myelination. A single oligodendrocyte can myelinate up to about 50 separate axonal segments, or internodes, requiring an area of cell membrane about 1000 times the surface area around its perikaryon [20]. It has been calculated that during the peak of myelination, each oligodendrocyte makes about 1.75×10^{-7} mg protein per day, which corresponds to three times the weight of the perikaryon. Since protein makes up about one third the dry mass of myelin, the oligodendrocyte must meet exceptionally high protein synthetic demands. Oligodendrocytes must not only synthesize large amounts of protein, but also synthesize, import, and sort lipids into the developing myelin membrane [21–25].

During myelination, the key proteins and lipids that comprise the myelin sheath undergo coordinate increases in expression. The expression of the major proteins is dependent on glial contact with axons [26, 27]. As the oligodendrocyte cell membrane spirals around the axon, most of its cytoplasmic contents between membrane leaflets are extruded to form so-called compact myelin. Within a single internodal myelin segment there are also areas of the myelin sheath where the cytoplasmic space remains relatively enlarged: (1) along the outer edges of the internodal sheath, at the paranodal loops, and (2) the clefts of Schmidt-Lanterman.

Proteolipid protein 1: The predominant protein in CNS myelin

Proteolipid protein is also known as lipophilin and Folch-Lees protein. PLP is the predominant protein in CNS myelin and constitutes about 50% of the protein mass of myelin (reviewed in [28]). PLP is believed to have a tetraspan topology, with both termini in the cytoplasm. PLP is so hydrophobic that is that requires organic solvents for efficient extraction. In addition to enrichment in hydrophobic amino acids, the protein also is covalently modified by fatty acid acylation. Six fatty acids are linked via cysteine residues to PLP molecule via an autocatalytic post-translational mechanism [29] (Fig. 1c). The fatty acids attached to the intracellular loop of PLP have been proposed to mediate the association of PLP with the adjacent lipid leaflet in compact myelin [30].

PLP is synthesized in the rough endoplasmic reticulum (RER) and subsequently transported through the Golgi complex, where the myelin lipid constituents, such as cholesterol, sulfatide and galactocerebroside, associate with PLP in membrane 'rafts' [31, 32]. Raft formation is one

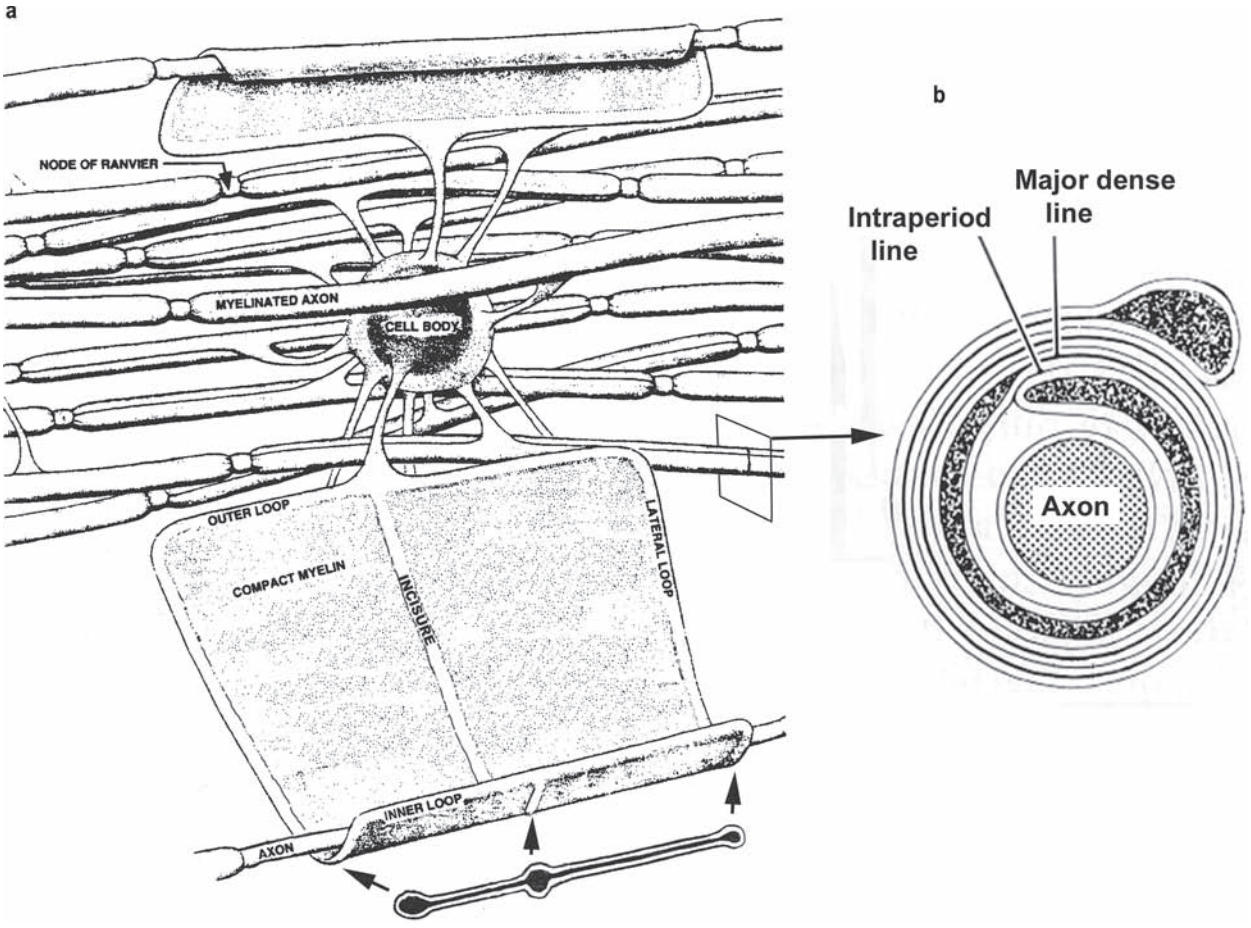

Figure 1. Central nervous system (CNS) myelin. A single oligodendrocyte can myelinate many axon segments. In (*a*), one internode is unraveled to show the widened regions that comprise the inner and outer loops or mesaxons, and the longitudinal incisures of Schmidt-Lanterman (modified from [170]). In (*b*) a cross-section shows the spiral lamellae that form compact myelin.

of the initial stages of myelin assembly, and is followed by the vesicular transport of PLP to the myelin membrane. In addition to its membrane anchoring role, N-terminal fatty acylation also appears to serve as a signal targeting PLP to newly synthesized myelin membrane and may be an important aspect of raft formation [21].

Processed PLP is a protein of 276 amino acids encoded by a single gene, composed of seven exons located on the X chromosome (Xq22.2) [33, 34]. The first exon encodes only the initiator methionine, which is cleaved off the nascent protein. The third exon contains an internal splice donor site that when used generates a transcript encoding a smaller (20 kDa) protein lacking 35 amino acids from the full-length protein and is designated DM20 [35, 36]. This exon is the last to be spliced, a process that is developmentally regulated [37]. While believed to share transmembrane topology similar to that of PLP, DM20 lacks part of the intracellular loop that contains two acylation sites. This difference may account for the altered conformation and physical properties observed for DM20 [31, 38, 39]. The two proteins can form heteromers [40]; however, their functional significance is not understood. DM20 and PLP are differentially expressed both during development and in different regions of the nervous system. DM20 is the predominant product at embryonic stages, but postnatally is overtaken by PLP, which accounts for the majority of the *PLP1* gene product in mature CNS myelin [41, 42]. In the peripheral nervous system, the level of DM20 is slightly greater than that of PLP; however, the total amount of PLP and DM20 in peripheral nervous system myelin is less than 0.01% of the total protein [43]. PLP is unusually well conserved among mammals. Sequence analysis indicates that a *GPM6/DM20*-like gene is the ancestral gene that arose in early bilateria, while *PLP1* probably arose in amphibians (reviewed in [44]). While PLP/DM20 is the predominant protein in reptilian, avian, and mammalian CNS myelin, in fish and amphibians, both P0 and DM20 coexist at high levels in myelin [45]. *PLP1* gene structure is preserved among tetrapods and readily discernible in the primordial gene of the *GPM6* family present in invertebrates [44]. Mammalian PLPs are virtually identical

at the protein level, with only one or two residues differing between most species. Human, mouse and rat PLP are identical. Moreover, no amino acid polymorphisms have been detected in the thousands of coding regions sequenced in the human *PLP1* gene. An additional exon with the potential of encoding an alternate N terminus was reported in mouse [46], but is not present in the human gene.

While distinct functions for PLP and DM20 have not been identified, the two proteins are not functionally equivalent, since isoform-specific knockout mice demonstrated that DM20 alone cannot fully compensate for lack of PLP [30, 47]. Proposed functions for PLP/DM20 include ion or other small molecule channel [48], precursor for secreted mitogen [49], as well as mediator of myelin compaction, perhaps functioning as an 'adhesive strut,' [50]. A role for PLP as a mediator of intercellular signaling [51] was substantiated by the discovery that PLP, but not DM20, interacts with α_v-integrin as part of a signaling complex that appears to play an important role in oligodendroglial development [52, 53]. In addition to oligodendrocytes, the *PLP1* gene is expressed elsewhere in the nervous system, such as in olfactory ensheathing cells [54], satellite cells [55], brainstem neurons [56] and Schwann cells [14, 57, 58], where the predominant isoform expressed is DM20 [43]. Schwann cell expression of PLP/DM20 is orders of magnitude lower than that observed in oligodendrocytes, and most of the protein produced is not normally incorporated into the myelin sheath [57, 59]. A low level of PLP/DM20 expression also occurs outside of the nervous system, in the heart [60], fetal thymus, spleen [61], thyroid, trophoblasts, spermatogonia, and skin [62]; however, no developmental or other physiological defects have been reported in these organs in either PMD patients or *Plp1* mutant animals, and therefore the significance of this expression is not established. In addition, some of these studies should be considered in light of recent observations that some antibodies to PLP epitopes may cross-react with other proteins, in particular other members of the proteolipid gene family, such as GPM6A and GPM6B [63]. In general, cells other than myelinating oligodendrocytes tend to favor the synthesis of DM20 over PLP.

Conformation-disrupting mutations and the unfolded protein response

A remarkable variety of *PLP1* mutations have been found that cause PMD or SPG2, and understanding them in both their genetic and cell biological contexts is important to fully appreciate disease pathogenesis. Somewhat ironically, there is better understanding of the biological effects of mutations affecting *PLP1* than there is of the essential biological functions of PLP itself. The most severe PMD syndromes usually are caused by missense mutations in the *PLP1* gene. Missense mutations cause a broad range of clinical phenotypes, from connatal PMD (*e.g.* [9–11]) to isolated spastic paraplegia [12, 64] and progressive ataxia with mild cognitive impairment [65]. After over 50 years of study, the precise functions of PLP are not understood. The high conservation of the entire PLP sequence, however, suggests that mutations anywhere in the gene would be deleterious. Indeed, mutations have been found throughout the gene. Mutations that affect only PLP, but not DM20, most often cause a relatively mild syndrome (see list at http://www.geneclinics.orgprofiles/pmd/pmd-table3.html). Mutations in the N-terminal third of the protein tend to cause severe PMD, while those affecting the second extracellular domain can cause the full range of phenotypes. Other than these, there are few correlations between mutation location and clinical severity.

Drawing from observations that intracellular trafficking of mutant Plp in transfected COS-7 cells is perturbed, Gow and Lazzarini [51] proposed that differences in clinical severity in patients with *PLP1* coding region mutations must take into account gain-of-toxic function of the mutant protein as well as the loss of function. Early (prenatal) development of oligodendrocytes is normal, but as the cells transform from premyelinating to myelinating cells, when high levels of myelin lipids and proteins, especially PLP, must be synthesized, mutant protein accumulates and may overwhelm the cell's ability to compensate [66]. In particular, the effects of the mutation on the folding and intracellular trafficking of the protein were hypothesized to play a significant role (reviewed in [67]). Mutations that affect the folding and transport to the cell surface of both PLP and DM20 are associated with the most severe PMD phenotypes and also increased oligodendrocyte cell death, while mutations that impair transport only of PLP, but not of DM20, produce a less severe phenotype with less or even no oligodendrocyte cell death [51, 66]. Since mutations that preclude expression of PLP (null mutations) cause milder disease, the predominant effect of severe *PLP1* coding region mutations was speculated to be caused by misfolded *PLP1* gene products, rather than just to loss of function. In addition, transgene-derived expression of normal Plp and Dm20 in *jimpy* (*jp*) mice, which have a severe C-terminal frame-shifting *Plp1* mutation, is not sufficient to rescue the mutant phenotype, and is consistent with mutant Plp1 having a dominant gain-of-toxic-function [68]. The cellular and molecular consequences of the accumulation of misfolded PLP and DM20 in the RER of oligodendrocytes, rather than the absence of these proteins in the myelin sheath, are thus a major cause of the clinical signs and symptoms of PMD.

There is good evidence that *PLP1* mutations induce the unfolded protein response (UPR), a group of intracellular signal transduction pathways that maintain ER homeosta-

sis (reviewed by [67, 69]). Broad functions of the UPR are to reduce transcription and translation rates and to induce ER-associated degradation (ERAD), (reviewed in [70]) to reduce synthetic demands on the ER, as well as to increase the folding and biosynthetic capacity of the ER. When these compensatory responses are overwhelmed, as in the case of virally transduced protein synthesis, the ultimate cellular reaction is to induce apoptosis.

Involvement of different components of the UPR has now been tested directly in PMD patients, and also examined experimentally in rodent PMD model systems [71]. Several well-characterized components of the UPR, including the heat shock protein HSPA5 (also known as BiP) and two bZip transcription factors, DNA damage induced transcript 3 (DDIT3; also known as CHOP, CEBPZ, CHOP10, GADD153 and MGC4154) and activating transcription factor 3 (ATF3), are induced in mice that carry missense mutations in *Plp1* and also in a patient who had severe PMD caused by a splice-site mutation that causes in-frame exon 6 skipping. Interestingly, this effect was observed with both a mild and a severe murine *Plp1* allele. Prior to this study, DDIT3 was considered to be a pro-apoptotic factor [72, 73]. *rumpshaker* (*rsh)* mice, which have a mild phenotype, when crossed into a *ddit3*-null background (*i.e. ddit3 null/rsh*mice) have a more severe phenotype than *rsh/y* mice, and therefore it was concluded that DDIT3 has an anti-apoptotic effect in this context [71]. It should be noted that *rsh* can be influenced by genetic background effects, where it has been shown that the phenotype is much more severe on the C57BL/6 than on the C3H background [74]. However, ATF3 can have opposite effects on cell survival [75, 76], depending on the mutational and cellular context in which it acts, providing precedence for a similar phenomenon with DDIT3.

Protein misfolding has been implicated as a pathogenic mechanism in several other neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases [77–80]. The morphological features associated with protein accumulation in these diseases include amorphous aggregates in the ER, cytoplasm or nucleus, and intermediate filament-containing aggresomes in the cytoplasm [81, 82]. Perinuclear inclusions are also observed in a variety of cell types, particularly in cultured cells treated with proteasome complex inhibitors, and are thought to form when the ER-to-cytoplasm delivery of unfolded proteins exceeds degradation by the proteasome complex. Aggresome-like inclusions are rarely found in PMD, however, probably because myelinating oligodendrocytes do not normally synthesize intermediate filaments. Proliferating oligodendrocyte precursor cells express vimentin and nestin in culture, but the expression of these genes is switched off as the cells differentiate [83]. Although protein misfolding has been implicated in all of these diseases, the molecular mechanisms of oligodendrocyte cell death in PMD may thus be different than those in the more classic neurodegenerative diseases.

Effects of *PLP1* mutations on axo-glial morphology and neuronal physiology

PLP1 mutations can have neurobiological effects in addition to dysmyelination. Scherer and collaborators [84] have shown that axo-glial junctions at the paranodal region are disrupted in *md* rats, an animal model with a *PLP1* point mutation, and that these changes are probably involved in disease pathogenesis. Effects on ion channel organization and localization and formation of axo-glial junctions (the contacts between the paranodal loops of myelinating cells with axons) have been observed in the dysmyelinating *shiverer* mouse that has a mutation in another major myelin gene, *myelin basic protein* (*mbp*) [18, 85, 86]. Thus, secondary effects of mutations are important to consider and may also have implications for designing symptomatic therapy.

Miller and colleagues have observed that *myelin deficient* (*md*) rats, which have a severe *Plp1* missense mutation, die at about 3 weeks of age due to respiratory drive failure [56]. Although the detailed mechanism is not fully understood, alterations in neurotransmitter receptors on brainstem neurons involved in ventilatory control were perturbed. Interestingly, these neurons were immunoreactive with Plp1 antibodies, suggesting a neuronal role for this protein. This and other studies based on immunohistochemical observations, however, should be interpreted cautiously with in view of a study by Greenfield et al. [63] that strongly suggests that cross-reactivity of Plp antibodies with homologous proteins, such as Gpm6a and Gpm6b, may give false-positive results.

The most common cause of PMD: Extra gene dosage and overexpression

Confusion arose after the initial discovery of *PLP1* mutations since many families with clinically apparent PMD showed linkage to the *PLP1* locus, but did not have intragenic *PLP1* mutations [87]. The paradox was resolved when Inoue and colleagues [88] recognized that submicroscopic interstitial duplications of the X chromosome that included the *PLP1* gene were in fact the most frequent cause of PMD, and not just a genetic curiosity [89]. Duplications of the entire *PLP1* gene account for about 60–70% of PMD cases [88, 90–92]. Duplication of the *PLP1* gene probably arises during meiosis in the maternal grandfather in those cases where a new mutation has been found [92, 93]. *PLP1* duplications are typically tandem in nature, involving a large genomic segment that includes neighboring genes [88, 93, 94]. Strik-

ing variation in the position of the breakpoints occurs in different PMD families [93–95], unlike the situation with other inherited duplications such as Charcot-Marie-Tooth disease type 1A (CMT1A), where the duplication is mediated by non-homologous recombination between flanking low copy repeats, and therefore is of constant size [96, 97]. In contrast, generation of *PLP1* duplications, inferred from analysis of duplication breakpoints, is thought to occur through a coupled homologous and nonhomologous recombination mechanism that involves repair of a double-stranded break (DSB) by one-sided homologous strand invasion of a sister chromatid, followed by DNA synthesis and nonhomologous end-joining with the other end of the break [95]. The duplicated segment can be as large as five megabases (Mb), which is over 150 times the size of the *PLP1* locus [93, 95]. Therefore, not only might PLP be overexpressed in these patients, but also a number of other X-linked genes may be inappropriately expressed. Segmental chromosomal duplications are probably a fairly frequent occurrence in the human genome, but because only a fraction of genes are sensitive to increased dosage effects, phenotypic effects are infrequently observed elsewhere in the genome [98, 99]. Families with duplications display a range of clinical severities [91, 93, 94] and personal observations. While it has been proposed that severity of the syndrome is proportional to the length of the duplication [93], this is not the case [95]. The natures of the other genes affected by the duplication as well as the positions of the breakpoints, which might disrupt other X-linked genes, could contribute to the overall phenotype. That *PLP1* is a dosage-sensitive gene is reinforced by the observation that three or more copies of the *PLP1* gene in patients with a more severe form of PMD [94, 100, 101]. The molecular mechanism for generation of *PLP1* triplications and quintuplication has not been identified. Additional mechanisms of genomic rearrangements operate at the *PLP1* locus, as indicated by several families in which the duplicated copy invades another spot on the X chromosome [102].

PLP1 duplications are presumed to cause overexpression of PLP. This has not been experimentally confirmed in human patients, but studies with transgenic mice and rats are consistent with this expectation. Since the smallest duplications observed are approximately 100 000 bp, the involvement of other dosage-sensitive genes at Xq22 cannot be fully excluded as contributing to the PMD phenotype. In rodents it has been possible to generate transgenic animals where only the *Plp1* gene is overexpressed [103–106]. In these animals, the severity of the neurological phenotype, which is comparable to that in PMD patients, is approximately proportional to the copy number of the transgene. Excessive amounts of normal PLP have been shown to accumulate in the late endosome and lysosomal compartments of rodent cells overexpressing PLP [22]. Since PLP typically associates with cholesterol and other lipids to form myelin 'rafts' as it traffics through the Golgi compartment [32], the shunting of excess PLP into the endosomal/lysosomal compartment depletes the Golgi of myelin lipids [22]. Presumably the transport and assembly of myelin constituents is altered in cells overexpressing PLP. Thus, while abnormal PLP proteins trigger a protein misfolding response in the RER, excessive PLP creates an imbalance in myelin constituents that adversely affects the subsequent stage of nascent myelin assembly in the Golgi network. Occasionally females with a duplication of the *PLP1* gene manifest an early-onset neurological phenotype [107]. Like some carriers of *PLP1* point mutations, these patients with mild PMD or spastic paraplegia show sustained clinical improvement. The recovery of these heterozygous females probably follows a mechanism similar to that thought to occur in heterozygotes with severe *PLP1* point mutations, namely compensatory myelin production by the normal oligodendrocytes that contain only a single copy of the *PLP1* gene [108, 109].

PLP1 null mutations and axonal damage

A third mechanism of molecular pathogenesis in PMD/SPG2 occurs through true loss-of-function, such as in patients with a deletion of the entire *PLP1* gene [110–112] or with point mutations at the beginning of the coding region that preclude translation or cause early termination [14, 113]. Null mutations are particularly important in deducing important functions of the native protein. Surprisingly, complete deficiency of PLP and DM20 causes a relatively mild neurological syndrome that can be classified either as complicated SPG2 or a mild form of PMD [15, 112–114]. In mice lacking Plp, oligodendrocytes develop normally and myelinate and animals have normal neurological function until late adulthood, when progressive deterioration occurs [115–117]. Pathologically the most striking abnormalities are not in myelin, where there are subtle abnormalities in compaction, but in axons [116]. These pathological changes suggest there is an absolute requirement for PLP, both to maintain the structure of compact myelin, and to maintain axonal integrity and function. Thus, the absence of PLP would neither trigger the UPR nor derail myelin assembly, but would instead negatively affect maintenance of the myelin sheath.

Axonal damage has been found in some PMD patients as well as in knockout mice, a finding that is important for future understanding of the pathogenesis of demyelinating disease and its treatment. Axonal pathology in *PLP1* mutations has been demonstrated in several rodent models, including those caused by *PLP1* point mutations [118], increased *PLP1* gene dosage [119] and *PLP1* null mutation [15, 116]. Consistent with this interpretation,

Garbern and coworkers have found evidence for axonal damage in both mice and patients with a *PLP1* null mutations by a combination of direct pathological examination of brain tissue and magnetic resonance spectroscopy ([15] and unpublished observations). The axonal injury is not due to demyelination since myelin is intact in both patients and experimental animals, or oligodendrocyte cell death since these cells appear healthy and ensheathe axons. The extent of axonal injury increases with age, and probably accounts for the progression of neurological signs and symptoms. Furthermore, the axonal degeneration is length dependent, suggesting that axonal transport is impaired. This has been shown to be the case in studies of axonal transport in Plp-deficient mice [120]. Interestingly, the disruption of axonal transport affects retrograde transport both earlier and to a more significant extent, suggesting that loss of signaling from the axon terminus is the critical event. These data suggest that progressive axonal damage is not only a common feature of the pathogenesis of PMD, it is also clinically relevant. Because axonal degeneration occurs without significant demyelination, it probably arises from the absence or perturbation of PLP-mediated oligodendrocyte-axonal interactions. Axonal degeneration also occurs as a result of mutations affecting at least one other myelin protein. Knockout of cyclic nucleotide phosphodiesterase 1 (*Cnp1*) also results in late onset progressive axonal degeneration [121]. Cnp1-deficient mice have abnormalities in the molecular organization of nodal and paranodal proteins, demonstrating the importance of myelin proteins in the formation and maintenance of the molecular and cellular architecture in the nodal region [122]. In an interesting experiment, Yin and colleagues [123] generated mice in which Plp was replaced by myelin protein zero (MPZ) in CNS myelin. Although myelin formed properly in these animals, by about 6 months of age, these mice developed neurological deterioration, reduced survival, and pathologically had axonal degeneration morphologically similar to that seen in *Plp1* null mice. Mice with equal proportions of MPZ and Plp had normal neurological function, longevity and axonal morphology. The authors speculate that during evolution, the switch from CNS myelin containing predominantly MPZ (as in cartilaginous fish) to that where PLP was the major protein was driven by the neuroprotective function of particular myelin proteins, such as PLP. Of note, not all myelin proteins have neuroprotective activity, since mice or rats with *mbp* mutations do not develop axonal swellings or transactions, although they do have severe dysmyelination and alteration of ion channels and axoglial junctions [116, 124–126]. The precise mechanism of the neuroprotective effects of myelin proteins remains to be defined.

The axonal abnormalities in PMD are very similar to those described by Trapp and coworkers [127] in multiple sclerosis (MS), suggesting that the axonal abnormalities in MS, like those in PMD, may likewise result from disruption of oligodendrocyte-axonal interactions. Axonal degeneration is clinically relevant in MS, since the *N*-acetyl aspartate/creatine ratio is decreased in the brains of patients with MS, even in regions outside of MS lesions, and correlates well with clinical disability [128–130]. Also, axonal damage in MS may underlie the secondary progressive phase of the disease, which does not respond significantly to immune modulation. Further understanding of the mechanisms of axonal degeneration in PMD will thus also be important in MS, and may lead to the development of new treatment strategies for both diseases.

Despite the large number of duplications that have been identified, the reciprocal deletion of the *PLP1* locus is rarely observed [110, 131]. Reported deletions of the *PLP1* gene encompass a much smaller segment of the X chromosome, with only two neighboring genes [131]. Presumably, the deletion of larger sections of the X chromosome, which would comprise the majority of reciprocal recombination events arising from duplications of PLP, would cause lethality, or infertility, or divergent syndromes. By examining the deletion breakpoints in the three identified families, Lupski and coworkers [112] discovered several different modes of genome rearrangement. This study reinforces the complexities of recombination involving the *PLP1* locus that were initially observed with the *PLP1* duplications, and suggests that non-homologous joining of ends, similar to the mechanism invoked with *PLP1* duplications [95], cause *PLP1* deletions [112]. In addition to the loss-of-function mutations arising from deletion events, two point mutations in the *PLP1* coding region at the initiation codon [113] or the second codon [14] are null for PLP expression. Unlike the *PLP1* deletions characterized to date, these null point mutations allow for a direct examination of *PLP1* loss without complicating considerations from deletion of those genes neighboring *PLP1*, *i.e.* the RAS superfamily member RAB9L and the thymosin β family member TMSNB [112]. Splice-site mutations that truncate *PLP1* often cause a mild syndrome [132–134] that closely resembles the PLP-null syndrome. Although needing direct verification, it is likely that these mutations result in loss of function through deletion of functional domains of the protein and/or nonsense-mediated decay of the mRNA [135].

Peripheral neuropathy in PMD

Some patients with PMD have a demyelinating peripheral neuropathy [14]. The neuropathy in these patients is mild, however, and not of major clinical significance. Electrophysiological studies demonstrate areas of modestly slowed nerve conduction velocities distributed non-

uniformly along the nerve [136]. Most of these patients have either null mutations or those that interrupt the PLP-specific region of the protein [14, 137]. A few other mutations that lie outside the PLP-specific domain and indicate other regions of the protein are necessary for proper peripheral nerve function as well ([138] and personal observations). How these mutations affect peripheral nerve function, while those associated with *PLP1* duplications or severe missense mutations do not, is not understood.

Additional genotype-phenotype correlations

Approximately 100 distinct mutations have been discovered to date (for an up-to-date accounting of the various point mutations, refer to http://www.geneclinics.org/img/table3-plp1.pdf). An extensive collection of missense mutations (those mutations that result in amino acid substitution) in the PLP/DM20 gene exist. Certain amino acid codons have a particularly rich array of changes that offer an opportunity to investigate the consequences of a specific protein alteration on myelination and the clinical manifestations of PMD/SPG2 [139, 140]. Three codons were mutated in two missense versions (V166E, V166G; L224I, L224P; Q234X, Q234P), and one codon was subjected to five different missense mutations: the aspartate at position 202 was changed to an asparagine, histidine, valine, glycine or glutamate residue in different PMD patients. Codon 202, located in the large external loop of PLP/DM20 (Fig. 2), represents a mutational 'hot spot'. Indeed, the entire external loop has an excessive number of mutations. While mutations are distributed throughout the PLP/DM20 coding sequence, appearing in both the transmembrane and extra-membrane domains, many of the missense mutations occur within the large external loop. The susceptibility of this region hints at conformational cues that may be important in maintaining the intraperiod line in compact myelin. A significant number of mutations are also available in the intracellular domain that is specific for PLP, including a nonsense mutation in exon3B, that enable a comparison of the roles of DM20 and PLP in the myelin sheath of man (Fig. 2).

A number of splice site mutations have been uncovered in PMD patients. Of most interest are the splicing mutations that are not located at the strictly conserved positions in the donor and acceptor splice sites, including a deletion of 19 bp within intron 3 and 26 bp in intron 5 [134, 140]. Although the spliced products have not been characterized in these families, splicing mutations would most likely result in exon skipping that would create an internally deleted and possibly a frame-shifted abnormal PLP protein. However, mutations within intron 3 that eliminate the donor splice site have the potential of leaving the DM20 transcript and protein unaffected, probably accounting for the mild syndrome that usually results. The atypical splicing mutations at the *PLP1* locus [132, 134, 140] suggest that even more splicing mutations may be found in PMD/SPG2 patients, mutations that have eluded detection because sequencing efforts usually concentrate on coding regions and intron/exon junctions.

Another category of point mutations is regulatory mutations that alter the expression of the *PLP1* gene without affecting the protein sequence. A putative promoter mutation has been reported in a PMD family at –34 of the *PLP1* gene [141]. Whether this mutation is the cause of the syndrome, or whether it alters PLP/DM20 expression in the reported family is not known, but its close proximity to the *PLP1* transcription initiation site and lack of identification of the mutation in normal control specimens (G. Hobson, personal communication) supports the possibility that it is pathogenic. Additional changes may occur within *PLP1* regulatory elements, which are not yet fully defined, or at splice sites that affect *PLP1* gene expression. Two patients with PMD or SPG2 phenotypes have been described where genomic rearrangement (inversion in the case of Muncke et al. [142], duplication in the case of Lee et al. [143]) occurs near, but not directly involving, the *PLP1* gene itself. In both cases, the authors speculated that position effects caused dysregulation of *PLP1* expression. Two families have been described with mutations that lie within the *PLP1*-specific region of the gene, yet do not change the coding sense [144, 145]. Although it is possible that these patients have additional undetected *PLP1* mutations or other disorders caused by mutations in heterologous genes, the possibility that these mutations affect gene regulation remains. Of interest, the PLP-specific region, including the 3rd base or 'wobble' positions, is the most highly conserved portion of *PLP1* among mammals, suggesting that there is important regulatory, as well as coding, information embedded there.

A second class of regulatory mutations that may cause a PMD-like disorder affects genes encoding transcription factors that are thought to regulate *PLP1*. The transcription factors that directly bind to the *PLP1* promoter are candidates, as are factors known to affect PLP expression, such as the homeodomain protein Nkx2.2 [146, 147] or the high-mobility-group regulator Sox10 [148]. One *SOX10* mutation has been described that combines features of PMD, Charcot-Marie-Tooth disease type 1 and Waardenburg-Hirschsprung syndrome [149]. The occurrence of such mutations is rare, as no additional *SOX10* mutations were found when screening 56 patients with uncharacterized hereditary peripheral neuropathy or 88 patients with uncharacterized leukodystrophies [150]. It is possible that some of these patients may be affected by other factors that potentially alter PLP expression, such as Nkx2.2 or MyT1 [151, 152]. Apart from the broader phenotypes expected from the mutation of a transcription factor that acts on multiple target genes, the absence of

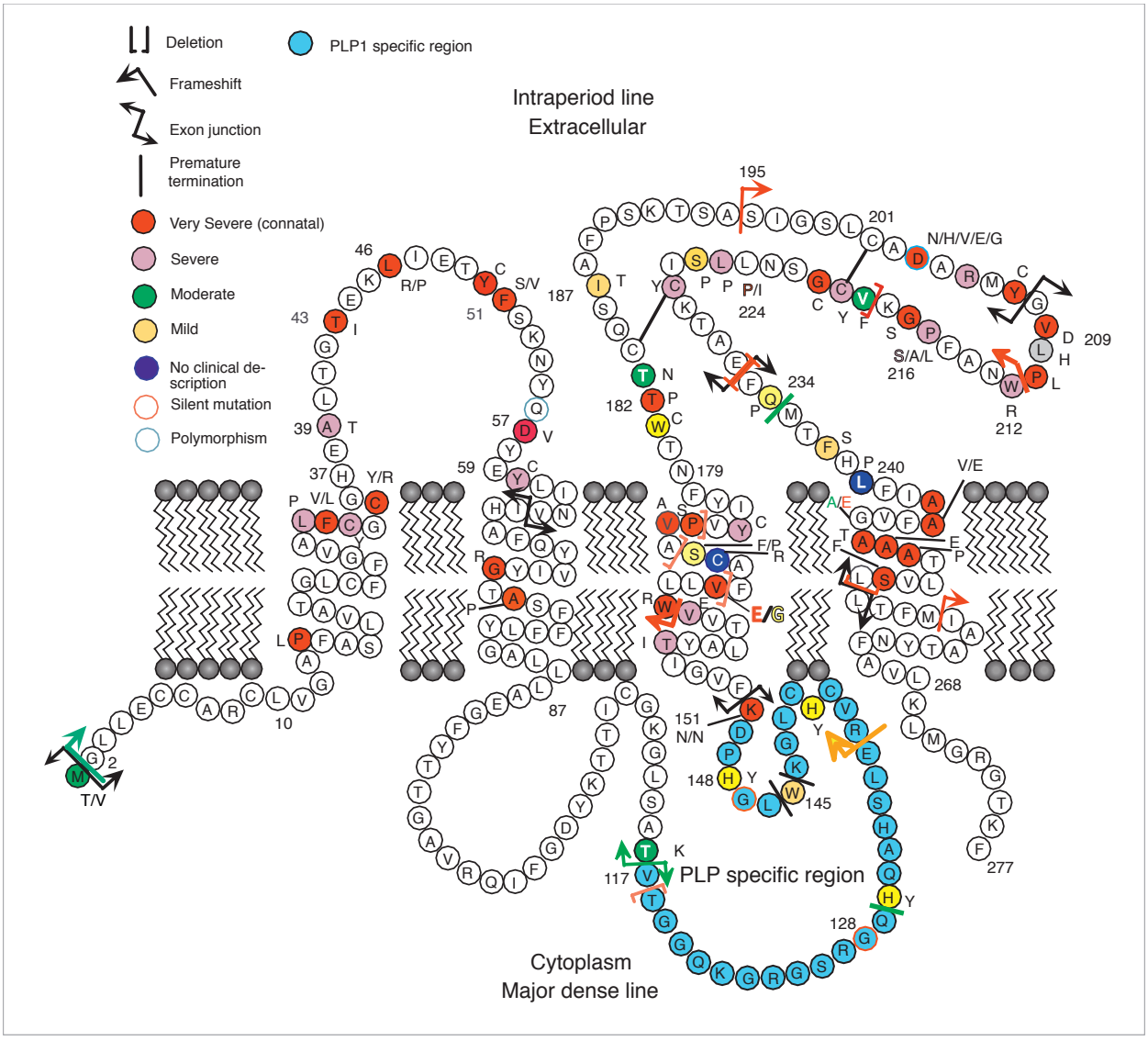

Figure 2. Proteolipid protein (PLP) alterations associated with Pelizaeus-Merzbacher disease/spastic paraplegia type 2 (PMD/SPG2). The figure shows a proposed model for the structure of PLP as it lies in the lipid bilayer of an oligodendrocyte membrane. The PLP-specific region that is missing in DM20 is shaded in blue. Cysteine residues (nos. 5, 6, 8, 108, 138 and 140) that are acylated are filled with black. Citations for the mutations demonstrated in the figure are available at http://www.geneclinics.org/img/table3-plp1.pdf. When more than one mutation occurs at a single position, the most severe phenotype is indicated. For completeness, some mutations are presented that have been described without clinical information. When limited clinical information was available, the disease severity was assigned as follows: very severe (equivalent to 'connatal') syndrome was ascribed if the patient was explicitly described as connatal, neurological signs were present at birth or death occurred before 20 years of age; severe: neurological signs were present in first few months of life, patient was described explicitly as intermediate or classical PMD or death occurred after 20 years of age; complicated SPG: patient was able to walk effectively for at least a few years, and had CNS signs in addition to spastic paraparesis; SPG2: 'pure' spastic paraparesis not associated with other CNS signs.

X-linkage in disorders caused by mutated transcription factors should distinguish them from PMD patients with mutations in the *PLP1* gene.

Approximately 80% of patients with a typical PMD syndrome have *PLP1* mutations [91, 92, 140]. Since clinical *PLP1* mutation testing only examines the exons, and the proximal portions of the 5′ and 3′ regions and of the introns, it is possible that some of the remaining patients have mutations in these non-coding regions that lie outside the currently analyzed regions. There is at least one well-characterized regulatory region deep within the large first intron of the murine *Plp1* gene [153]. Mutations in an equivalent region of the human gene would be expected to cause a PMD syndrome.

In addition to mutations affecting the SOX10 transcription factor, at least one other cause of locus heterogeneity can

account for a PMD-like syndrome. Uhlenberg et al. [154] identified mutations in the *GJA12* gene that encodes a gap junction protein as a cause of an autosomal recessive PMD-like disease. Interestingly, although knockout of the *Gja12* gene in mice does not cause an obvious phenotype, double knockouts deficient in both Gja12 and Gjb1 develop a equivalent dysmyelinating syndrome [155]. Although the mechanism is not elucidated, these observations strongly support the involvement of intercellular interactions in myelination or myelin maintenance.

Neurobiological effects in heterozygotes

Females heterozygous for *PLP1* gene mutations sometimes develop neurological signs and symptoms. In some PMD families, where female heterozygotes are clinically affected through adult life, as in the family described by Pelizaeus and Merzbacher [1], unfavorably skewed X-inactivation may be the mechanism. An alternative explanation is probably more common in most other families. Several investigators have observed an inverse correlation within PMD families: in families with severely affected males, the heterozygous women are unlikely to have clinical manifestations of PMD/SPG2, whereas in families with mildly affected males, heterozygous females are more likely to have symptoms [156–158]. In a family with a particularly mild syndrome characterized by ataxia and mild spastic paraplegia, all three heterozygous females had neurological signs [65]. Similar correlations have also been observed in animals with *Plp1* mutations where mild alleles in males are associated with persistent neurological signs in heterozygotes [159].

The resolution of this paradox must take into account the phenomena of X-inactivation and the cell-autonomous effects of *PLP1* mutations on oligodendrocytes. Because of random inactivation of the X chromosome, females who are heterozygous for *PLP1* mutations should express the abnormal protein in approximately 50% of their oligodendrocytes. Oligodendrocytes expressing a more severe *PLP1* mutation, however, in which both PLP and DM20 are affected, undergo increased cell death, as they do in males with these mutations, and are eliminated during myelination and replaced by normal oligodendrocytes. In contrast, oligodendrocytes expressing a less severe *PLP1* mutation that does not cause cell death are not eliminated, thus producing abnormal myelin and neurological dysfunction. Female PMD carriers are also usually clinically unaffected, although some may have transient neurological abnormalities as children [160]. Female dogs that are heterozygous for a severe mutation in the canine *Plp1 (shaking pup),* for example, have neurological abnormalities early in life, but by adulthood are clinically normal and have normal numbers of oligodendrocytes that express very little mutant *PLP1* messenger RNA [109]. Although the mechanism of oligodendrocyte elimination is not as well understood, rare females with *PLP1* duplications have been described that have transient neurological signs that resolve by adulthood [107]. For unknown reasons, most females heterozygous for *PLP1* duplications have favorably skewed X-inactivation [161]. Thus, the transmission of *PLP1* mutations can be either X-linked recessive or dominant, with reduced penetrance, depending on the nature of the mutation and its cell biological effects.

Future prospects

Although a rare disorder, systematic study of PMD has enhanced not only clinical understanding of disease pathogenesis, but also previously unrecognized important functions of glia in nervous system biology. A summary of the major genetic and cellular mechanisms that cause PMD/SPG2 is given in Figure 3. Much still remains to be learned, not least of which is identifying the essential biological functions of PLP and DM20. The mechanisms involved in PLP-dependent axo-glial interactions, such as those that modulate axonal transport and maintain axonal integrity also require further study. A major advantage in PMD studies is the existence of a large number of animals that model each of the major categories of pathogenesis underlying the human disease. These models are invaluable both for studying disease mechanisms as well as for evaluating potential therapies.

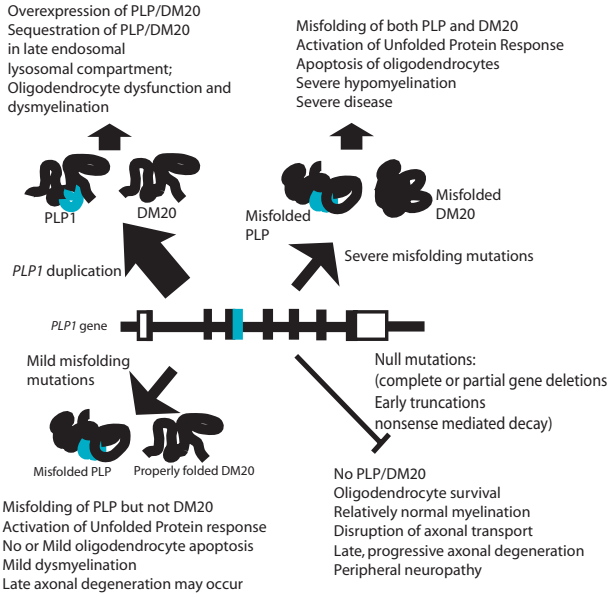

Figure 3. Summary of the major genetic and cellular mechanisms that cause PMD/SPG2.

Currently, there is no specific therapy for patients with PMD. Cellular therapy, such as transplantation of oligodendrocyte precursors into the CNS, has shown potential in animal models of PMD and is the most promising approach for true myelin repair [162–167]. Efficient delivery of sufficient cells, rather than just demonstration that transplanted cells survive and can myelinate, has not been achieved, however, and cellular therapy has not yet reversed the clinical deficits in animal models. In addition, for maximum effectiveness, this therapy may need to be initiated either *in utero* or shortly after birth.

The observation that most patients with PMD have a gene duplication, and thus overexpress PLP, or have a point mutation causing gain-of-function precludes simple replacement gene therapy, even if appropriate delivery vehicles were to become available. Instead, for most patients, the more appropriate goal might be to reduce PLP expression, such as through antisense or RNAi therapy, since absence of PLP results in a less severe syndrome than those caused by severe missense mutations or *PLP1* duplications. The potential for this strategy was demonstrated by Skoff et al. [168], who reported reduced oligodendrocyte apoptosis in *jp* mice after treatment with *Plp1* antisense RNA.

The finding that axonal degeneration is clinically relevant in the pathogenesis of PMD also raises the possibility that neuroprotective therapy directed at maintaining the integrity of axons might be effective in this disorder. Dysmyelination causes disorganization of the molecular architecture in the nodal region, such as unmasking of juxtaparanodal potassium channels. Therefore, ion channel blockers, such as potassium channel blockers, may help to compensate for the neurological abnormalities caused by the myelin defect [126, 169].

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