



Mechanisms and Treatments in Demyelinating CMT

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Abstract

Demyelinating forms of Charcot-Marie-Tooth disease (CMT) are genetically and phenotypically heterogeneous and result from highly diverse biological mechanisms including gain of function (including dominant negative effects) and loss of function. While no definitive treatment is currently available, rapid advances in defining the pathomechanisms of demyelinating CMT have led to promising pre-clinical studies, as well as emerging clinical trials. Especially promising are the recently completed pre-clinical genetic therapy studies in PMP-22, GJB1, and SH3TC2-associated neuropathies, particularly given the success of similar approaches in humans with spinal muscular atrophy and transthyretin familial polyneuropathy. This article focuses on neuropathies related to mutations in PMP-22, MPZ, and GJB1, which together comprise the most common forms of demyelinating CMT, as well as on select rarer forms for which promising treatment targets have been identified. Clinical characteristics and pathomechanisms are reviewed in detail, with emphasis on therapeutically targetable biological pathways. Also discussed are the challenges facing the CMT research community in its efforts to advance the rapidly evolving biological insights to effective clinical trials. These considerations include the limitations of currently available animal models, the need for personalized medicine approaches/allele-specific interventions for select forms of demyelinating CMT, and the increasing demand for optimal clinical outcome assessments and objective biomarkers.

Keywords Charcot-Marie-Tooth disease · Demyelinating neuropathy · Biological mechanisms · Treatment targets · Clinical trials · Therapeutic development

Introduction to Demyelinating CMT

Charcot-Marie-Tooth disease (CMT) refers to a heterogeneous set of genetic peripheral nerve disorders that collectively comprise the most common inherited neurological disease, with an estimated prevalence of 1:2500 individuals [1, 2]. The varied forms of CMT span the phenotypic spectrum from subclinical neuropathy to that resulting in early loss of ambulation in the setting of severe weakness and sensory loss. Most commonly, CMT presents as a sensory motor neuropathy, though primarily sensory and motor forms (hereditary sensory autonomic neuropathy and distal hereditary motor neuropathy, respectively) can also occur. Disease onset can

also vary widely, with some patients showing signs in infancy and others developing symptoms in their elderly years.

Historically, the classification of CMT has anchored on the mode of inheritance and the primary pathology observed in nerve, as reflected in nerve conduction studies. Demyelinating CMT, which is the focus of this review, is defined by upper extremity motor conduction velocities (CV) of less than 38 m/s, resulting from homogeneous demyelination of large, myelinated axons. Pathological evidence of demyelination can also be observed on nerve biopsy, though with the increasing yield of genetic testing, biopsy is generally reserved only for cases that pose a particular diagnostic challenge.

Demyelinating forms of CMT are characterized as CMT1 (autosomal dominant (AD) inheritance), CMT4-demyelinating (autosomal recessive (AR) inheritance), and X-linked forms (Table 1 and Fig. 1). In the era of increased genetic testing, the phenotypic spectrum associated with specific genes in CMT has broadened significantly, and the classification of CMT is gradually evolving to incorporate the specific gene that is mutated in individuals with CMT [3]. It should also be emphasized that while the term

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Table 1 Classification of Charcot-Marie-Tooth disease; demyelinating subtypes

Type	Gene/locus	Specific phenotype
<i>Autosomal dominant CMT1 (AD CMT)</i>		
CMT1A	Dup17p (PMP22)	Classic CMT1
CMT1B	MPZ	CMT1/DSN/CHN/intermediate/CMT2
CMT1C	LITAF	Classic CMT1
CMT1D	EGR2	Classic CMT1/DSN/CHN
CMT1E	PMP22 (point mutation)	Classic CMT1/DSN/CHN
CMT1F	NEFL	CMT2, can have slow MCVs in CMT1 range + 2 early-onset severe disease
CMT1G	PMP2	Classic CMT1
CMT1	FBLN5	CMT1/macular degeneration/hyperelastic skin
<i>Hereditary neuropathy with liability to pressure palsies (HNPP)</i>		
HNPP	Del 17p (PMP22)	Typical HNPP
	PMP-22 (point mutation)	Typical HNPP
<i>X-linked CMT</i>		
CMT1X	GJB1	Intermediate $\pm$ patchy MCVs/male MCVs, female MCVs
CMTX dominant—CMTX6	PDK3	Classic CMT1
CMTX recessive (Cowchock)—CMTX4	AIFM1	Axonal/infantile onset/learning difficulties
<i>Autosomal recessive demyelinating (CMT4)</i>		
CMT4A	GDAP1	CMT1 or 2, usually early-onset and severe/vocal cord and diaphragm paralysis Rare AD CMT2 families described
CMT4B1	MTMR2	Severe CMT1/facial/bulbar/focally folded myelin
CMT4B2	SBF2	Severe CMT1/glaucoma/focally folded myelin
CMT4B3	SBF1	Severe CMT/scoliosis/syndactyly/focally folded myelin
CMT4C	SH3TC2	Severe CMT1/scoliosis/cytoplasmic expansions
CMT4D (HMSNL)	NDRG1	Severe CMT1/gypsy/deafness/tongue atrophy
CMT4E	EGR2	Classic CMT1/DSN/CHN
CMT4F	PRX	CMT1/more sensory/focally folded myelin
CMT4G	HK1	Severe CMT1
CMT4H	FGD4	Severe CMT1
CMT4J	FIG4	Severe CMT1 $\pm$ ALS phenotype in adulthood
CCFDN	CTDP1	CMT1/gypsy/cataracts/dysmorphic features
CMT4	SURF	CMT1/nystagmus/ataxia
CMT1	PMP22 (point mutation)	Classic CMT1/DSN/CHN/HNPP
CMT1	MPZ	CMT1/DSN/CHN/intermediate/CMT2

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AD autosomal dominant, AR autosomal recessive, CMT Charcot-Marie-Tooth, DI dominant intermediate, RI recessive intermediate, CCFDN congenital cataracts, facial dysmorphism, and neuropathy, dup duplication, del deletion, PMP22 peripheral myelin protein 22, MPZ myelin protein zero, LITAF lipopolysaccharide-induced tumor necrosis factor alpha, EGR2 early growth response-2, DSN Déjérine-Sottas neuropathy, CHN congenital hypomyelinating neuropathy, NEFL neurofilament, FBLN5 Fibulin-5, GJ1 Gapjunction beta 1/connexin 32, PDK3 pyruvate dehydrogenase kinase isoenzyme 3, AIFM1 apoptosis-inducing factor mitochondrion associated 1, GDAP1 ganglioside-induced differentiation-association protein 1, MTMR2 myotubularin-related protein-2, SBF2 SET binding factor 2, SBF1 SET binding factor 1, SH3TC2 SH3 domain and tetratricopeptide repeat domain 2, NDRG1 N-myc downstream regulated 1, PRX periaxin, FDG4 frabin, FIG4 phosphoinositide phosphatase FIG4, CTDP1 C-terminal domain of RNA polymerase II subunit A, phosphatase of, subunit 1, HK1 hexokinase 1, SURF1 surfeit-1 protein, ALS amyotrophic lateral sclerosis

“demyelinating” CMT has historically been used, there is evidence to suggest that some forms of CMT actually involve a developmental abnormality of myelin formation

or “dysmyelination,” which may both cause abnormal nerve function and predispose nerves to demyelination throughout the course of disease [4–6].

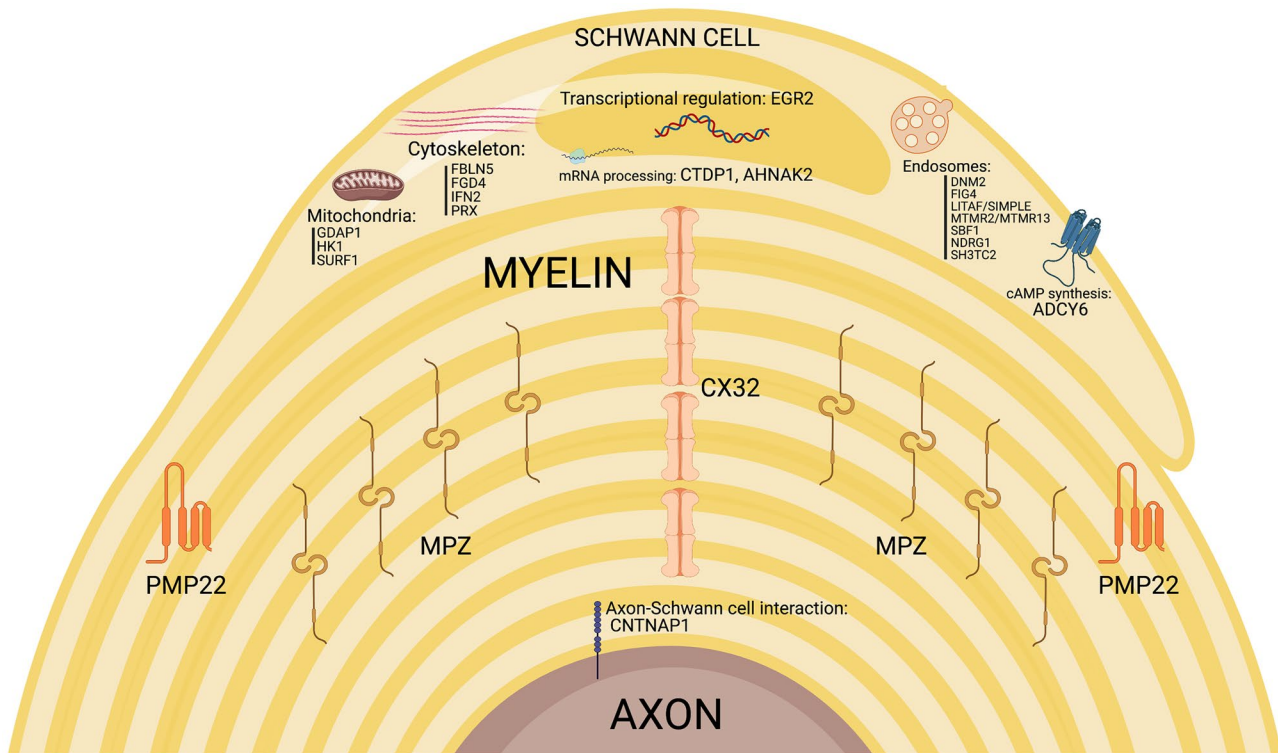


Fig. 1 Demyelinating CMT genes

Many of the forms of CMT result in similar clinical manifestations, which can pose a challenge in establishing a precise diagnosis at the bedside. While select subtypes do have distinguishing phenotypic features, the majority of patients with CMT present with foot deformities, including pes cavus, pes planus, or hammertoes, length dependent muscle weakness and atrophy, and length-dependent sensory loss. Deep tendon reflexes are typically diminished or absent however can also be preserved or rarely even heightened in select forms of CMT. While genetic testing in CMT continues to be guided by the clinical examination, family history, and electrophysiology, testing is increasingly being accomplished through the use of next-generation sequencing (NGS) panels containing multiple disease relevant genes, rather than through sequential gene testing [7, 8]. Given the high prevalence of CMT1A, however, it is still reasonable to exclude this possibility in patients presenting with the classic demyelinating CMT phenotype as a first step in the diagnostic evaluation [9].

With the increasing use of next-generation sequencing, gene discovery in CMT has rapidly accelerated over the past two decades, with over 120 causative genes identified to date [10]. New mutations in these genes are also continuously being discovered as captured in the inherited neuropathy variant browser (http://hihg.med.miami.edu/code/http/cmt/public_html/index.html#/) [11]. While this genetic diversity can be daunting in both the clinical and research settings, it should be emphasized

that of the ~65% of patients in whom a genetic diagnosis can be defined, ~90% have a mutation in one of the four most common genes known to underlie CMT, namely PMP-22, MPZ, GJB1, and MFN2 [9, 12–14]. For this reason, this review will primarily focus on PMP-22, MPZ, and GJB1, which together comprise the most common forms of demyelinating CMT.

Disease-causing mutations in CMT can result in clinical neuropathy through both loss and gain of function, with the latter being more common. Gain of function mutations can cause cellular derangements that are unrelated to the primary role of the encoded protein, whereas the loss of function mutations leads to either reduced levels or abnormal function of the protein. The majority of CMT1 is mediated by gain of function mechanisms, whereas the AR CMT-demyelinating forms are more commonly caused by loss of function [15]. In the heterozygous state, AD gain of function mutations also tend to cause a more severe neuropathy than those resulting in a loss of function [16]. These distinctions have important implications for therapeutic development in these disorders.

Despite rapid advances in understanding the biology of CMT, no definitive treatment is currently available, and a few pre-clinical studies have progressed to clinical trials. As reviewed by Juneja et al., there are several critical obstacles in identifying effective treatments in CMT [10]. These obstacles include the rarity of many of the forms of

CMT, the genetic and phenotypic heterogeneity of these disorders, and the challenge of developing optimal animal models and translating candidate treatments to humans. Many of the treatments investigated thus far in CMT also target pathways downstream from the initial biological insult that results from a given genetic mutation. Another important challenge therefore lies in defining ways to target upstream biological derangements as early as possible in the disease in order to minimize neurodegeneration.

As the CMT research community works to overcome these challenges, there is also increasing reason for optimism. Recent developments in the treatment of spinal muscular atrophy and transthyretin familial polyneuropathy have been transformative in paving the way for effective genetic therapy approaches for neuromuscular disorders; and the emergence of genetic therapies for select forms of CMT now offer tangible hope for people with these conditions [17–19].

Introduction to Myelin Biology

The myelin sheath plays a critical role in enabling rapid conduction of nerve impulses within the peripheral nervous system. Peripheral nerve myelin is a multi-layer structure

composed of Schwann cells, which surround single axons in a one-to-one ratio (Fig. 2). The myelin sheath includes compact regions of myelin (which are predominant) as well as non-compact regions, which include the paranode and juxtaparanode and are adjacent to the nodes of Ranvier (gaps between two myelin segments that enable the influx of ions) [20]. Outward potassium currents take place at the juxtaparanode and internode. This molecular architecture allows for preservation of the depolarization current by the high internodal capacitance and enables its efficient propagation until the next node of Ranvier, where a new action potential is triggered. This allows for a higher conduction velocity in myelinated nerve fibers than that observed in unmyelinated fibers. In addition to facilitating rapid conduction, myelin also protects axons, supports signaling and communication between axons and Schwann cells, and provides metabolic support to axons [21]. The compact and non-compact regions of myelin include distinct proteins, such as PMP-22, MPZ, and myelin basic protein within compact myelin [16]. The expression of these proteins is highly regulated within Schwann cells, with even slight changes resulting in abnormal development or maintenance of the myelin sheath [22, 23].

In humans, myelin formation occurs in the postnatal period; however, Schwann cells continue to maintain

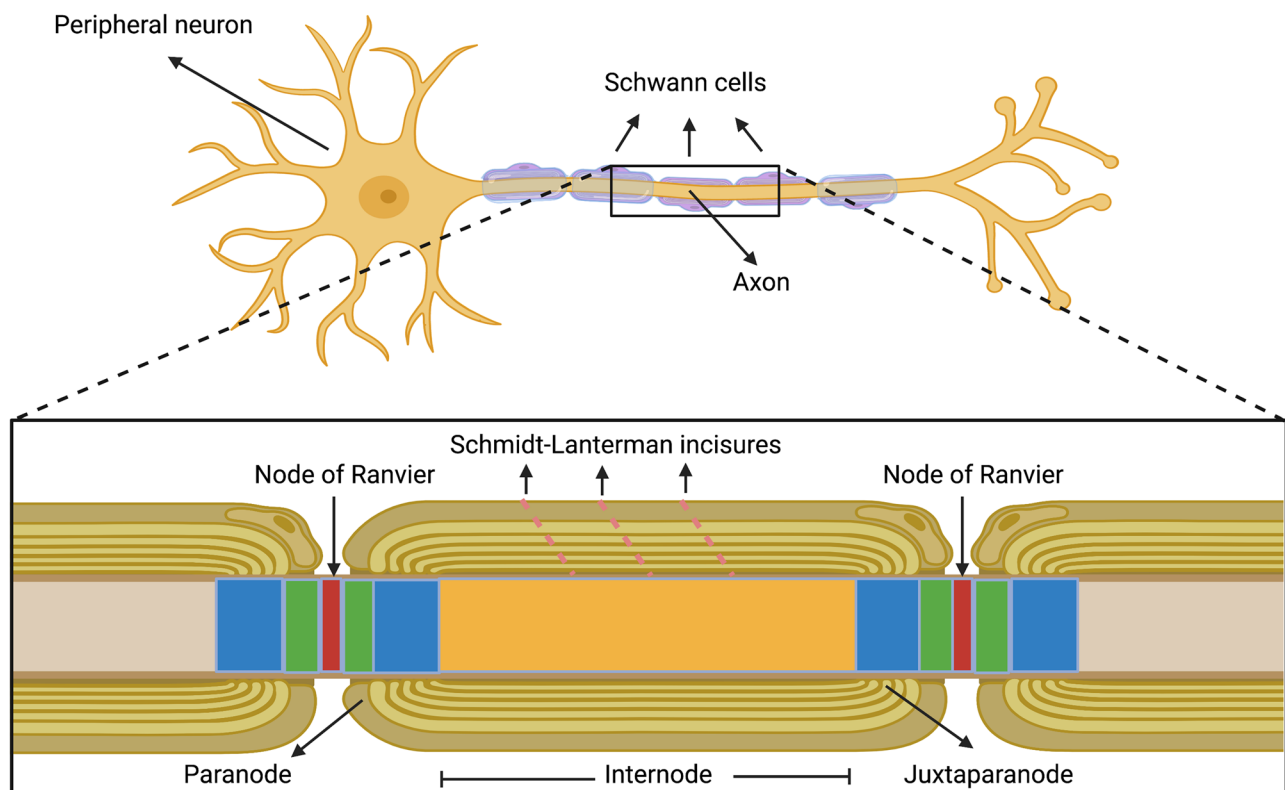


Fig. 2 Schematic of Schwann Cell

myelin integrity into adulthood. Importantly, in addition to causing demyelinating CMT, mutations in genes encoding myelin proteins can sometimes result in primarily axonal neuropathies [24–26]. As mentioned, it is also important to distinguish abnormal myelin development, or “dysmyelination,” from the disruption of normally formed myelin or “demyelination,” as the nature and timing of myelin injury are integral to the varied pathomechanisms of demyelinating CMT and have important implications for the development of treatments.

PMP-22- Associated Neuropathy: Epidemiology and Clinical Features

CMT1A is the most common hereditary neuropathy, accounting for ~50% of all CMT and 70–80% of CMT1 [9, 13, 14]. CMT1A has a *de novo* mutation rate of 10%, and patients therefore may report the absence of relevant family history [27]. The disease most commonly manifests with a “classic CMT phenotype,” namely pes cavus, length-dependent weakness and sensory loss resulting in gait difficulty, and hyporeflexia. The majority of patients present in the first decade of life, though later presentations are not uncommon. As CMT1A progresses, patients commonly require ankle foot orthotics but rarely lose ambulation. While CMT1A has historically been perceived as primarily impacting large, myelinated nerve fibers, several studies have confirmed injury to small unmyelinated fibers, which may explain the high prevalence of neuropathic pain observed in the disease (~20% of patients) [28, 29].

Nerve conduction studies in CMT1A reveal reduced sensory and motor compound amplitudes (SNAPs and CMAPs) and uniform slowing of motor conduction velocities in the demyelinating range. This uniformity of CV slowing (meaning that there is a similar degree of slowing in each nerve) contrasts with the patchy and heterogeneous slowing of CVs observed in acquired immune neuropathies. Regardless of the degree of conduction velocity slowing, it is the reduction in CMAPs that reflects the degeneration of motor axons and correlates with disability [30, 31]. Nerve biopsy, which is no longer routinely performed for diagnosis, demonstrates the presence of onion bulbs [32, 33]. While onion bulb formation in CMT1A has traditionally been attributed to recurrent demyelination and remyelination, this assumption has recently been put into question. A large study of electrophysiological data from patients with CMT1A revealed that both motor and sensory nerve conduction velocities increase with age and confirmed the absence of any acquired demyelinating features (i.e., partial conduction block) [4]. Given the striking uniformity of motor conduction velocities, the presence of slow conduction velocities in the first year of life, and the stability of the velocities over time, it has been

suggested that CMT1A is primarily dysmyelinating rather than demyelinating and that onion bulb formation actually reflects abnormal organization of Schwann cells around their axons [4–6, 34]. Examination of dermal myelinated nerve fibers in CMT1A has also revealed uniformly shortened internodes, potentially related to a developmental defect in internodal lengthening, which may account for the uniformity of conduction velocity slowing [35]. The possibility of a primary developmental defect in myelin formation in CMT1A underscores the need to treat early in the disease course in order to minimize disability.

It is important to emphasize that despite the relative genetic homogeneity of CMT1A as compared to other forms of CMT, the disease results in notable inter- and intra-familial variability, which may relate to the influence of genetic modifiers, as well as other epigenetic or environmental factors [36, 37].

PMP-22 Biology and Pathomechanisms

CMT1A most commonly results from a 1.4 Mb tandem duplication on chromosome 17 p11.2, following an unequal crossing over event in meiosis [38]. In contrast, depletion of the protein resulting from deletions in PMP-22 causes hereditary neuropathy with liability to pressure palsies (HNPP). HNPP manifests with a distinct clinical phenotype of recurrent compressive neuropathies superimposed on a slowly progressive polyneuropathy [39, 40]. CMT1, resulting from point mutations in PMP-22 (termed CMT1E), can also be phenotypically distinct, with an earlier disease onset, more notable disability, and more severe slowing of motor conduction velocities [41]. Rarely, CMT1E can also resemble HNPP [42]. Pathologically, CMT1E is associated with the aggregation of PMP22 in the cytoplasm of Schwann cells, a finding not observed in CMT1A [43].

PMP-22 encodes peripheral myelin protein 22, a 22 kDa hydrophobic transmembrane glycoprotein that accounts for approximately 2–5% of peripheral nerve myelin protein [44]. PMP-22 is expressed in Schwann cells during myelination and is thought to affect the organization of lipids in compact myelin [45–48]. The majority of PMP-22 is degraded immediately following translation due to inefficient folding, while the remaining ~10% of PMP-22 is folded in the endoplasmic reticulum (ER), glycosylated in the Golgi, and incorporated into the compacted regions of myelin [45, 49]. PMP-22 is critical for both the synthesis and maintenance of myelin and is believed to play a structural role in the myelin sheath, though the exact mechanisms by which the protein functions within myelin remain somewhat elusive [9, 50]. It has also been hypothesized that PMP-22 binds tetramers of myelin protein zero, thereby helping to compact and stabilize myelin [39, 51, 52].

The precise mechanisms by which PMP-22 duplications impact Schwann cell development and function have also not been determined; however, many studies have suggested the increased dose of PMP-22 resulting from the duplication underlies the development of neuropathy. Overexpression of PMP-22 in rodent models results in a demyelinating neuropathy, whereas reducing PMP-22 transcription in these models improves both myelination and neuropathy severity [19, 53–58]. Further underscoring that gene dosage of *PMP-22* is critical for nerve health are the observations that patients harboring a PMP-22 triplication have a more severe neuropathy phenotype and those with a PMP-22 duplication on one chromosome and a deletion on the other do not develop neuropathy [55, 59, 60].

While the dose of PMP-22 is clearly important to nerve health, pre-clinical work also suggests a straightforward dosage mechanism cannot fully explain the pathogenesis of CMT1A. Rodent models have shown elevated levels of PMP-22 mRNA; however, PMP-22 protein levels are highly variable and can even fall into the normal range [49, 55, 61]. Examination of the skin and sural nerve biopsies in humans with CMT1A also have not consistently demonstrated elevations in PMP-22 relative to controls [61, 62], and no clear correlation between the amount of PMP-22 expressed in intact myelin and disease severity has been established [39, 55]. This contrasts with the uniform reduction in dermal PMP-22 seen in patients with HNPP [63]. The levels of PMP-22 expression in CMT1A also fluctuate over time. It is increasingly recognized therefore that in addition to down-regulating the expression of the protein, effective treatments for CMT1A will also need to prevent the excessive fluctuations in PMP-22 [5, 39, 64].

PMP-22 expression is tightly regulated by two promoters (P1 and P2), which are tissue specific. The P1 promoter is expressed only in Schwann cells, and the P2 promoter is expressed in non-peripheral nervous system tissues [65]. In addition, a late myelinating Schwann cell enhancer (LMSE) has been identified upstream of the P1 promoter. LMSE is important in the later stages of myelination, as well as in remyelination following injury [66, 67], and Pantera et al. have shown that deleting the LMSE significantly reduces the expression of PMP-22 by disproportionately impacting the P1 promoter [68, 69]. Small duplications containing LMSE can also result in mild forms of CMT1A, suggesting that the additional copy of the super-enhancer region can be disease causing independently of PMP-22 [55].

PMP-22 is additionally regulated by multiple transcription factors, some of which have been evaluated as therapeutic targets in pre-clinical studies. Among these are EGR2/Krox20 (Early growth response protein 2) and SOX10 (SRY sex determining region Y-box 10), which bind PMP-22 within an intronic regulatory element and induce its expression by mechanisms that thus far are not well defined

[55, 70]. Other important regulators include YAP/TAZ and TEAD [71]. Makoukji et al. also demonstrated that oxysterols (molecules formed from the oxidation of cholesterol) inhibit the expression of both PMP-22 and MPZ in Schwann cells and that this inhibition is mediated by liver X receptors (LXRs) [72]. Furthermore, a selective LXR agonist (TO901317) successfully downregulated the expression of both PMP-22 and MPZ suggesting a potential therapeutic approach [72].

Post-transcriptionally, PMP-22 is regulated by select microRNAs (miRNAs), small regulatory molecules that target the 3'UTR of mRNA and inhibit its function [73–75]. Specifically, Verrier et al. demonstrated that miR-29a inhibits PMP22 reporter expression [74], and Lee et al. found that miR-381 is downregulated in the C22 mouse model of CMT1A (see section entitled “[Biological Models of PMP-22-Associated Neuropathy](#)” below for a detailed description of the C22 mouse model) (76). Furthermore, administration of an miR-381 expressing lentiviral vector into the sciatic nerves of C22 mice resulted in an improvement in both clinical and electrophysiological measures [76].

The notable intra-familial variability observed in CMT1A has led to a search for genetic modifier genes that can be targeted to ameliorate the CMT1A phenotype [77, 78]. Tao et al. performed a genome-wide analysis in 330 patients with CMT1A of European ancestry on the phenotypic extremes and identified four single nucleotide polymorphisms in the *signal-induced proliferation-associated 1 like 2* (*SIPA1L2*) gene, which was associated with foot dorsiflexion strength [78]. The authors further demonstrated that *SIPA1L2* is a part of a co-expressed network of key myelination genes under the regulation of SOX10 and that knockdown of *SIPA1L2* in Schwann cells results in reduced PMP-22 expression. Variants in other CMT-causing genes have also shown associations with the severity of CMT1A. Earlier and more severe manifestations of CMT1A were reported with a co-existing I92V variant in *LITAF/SIMPLE* [79, 80] and several SNP alleles in the *SH3TC2* gene associated with phenotypic differences in CMT1A [81]. As mentioned previously, miRNAs play an important role in the regulation of PMP-22 expression, and a variant in the miR-149 was closely associated with neuropathy severity in a Korean CMT1A cohort [82].

As is true of several forms of CMT, the aggregation of misfolded protein is believed to play a role in CMT1A [83–87]. Overexpression of PMP-22 (which is inefficiently folded even in a non-pathogenic state) can result in the accumulation of misfolded protein in the ER, with subsequent activation of the unfolded protein response (UPR), reduced protein translation, and potentially apoptosis of Schwann cells (see section titled “[Intracellular Changes in Response to MPZ Derangement](#)” and Fig. 4 for detailed discussion of the UPR). The aggregation of PMP-22 is observed more commonly in patients with point mutations in PMP-22 than

in those harboring the duplication [43] and is recapitulated in the C22 and trembler J mouse models, in which protein aggregates are seen in the cytoplasm of Schwann cells [86, 88]. Importantly, in vitro studies suggest that the retention of protein in the ER can affect the amount of PMP-22 present in the plasma membrane [86]. Furthermore, there is evidence to suggest that it is possible to reduce the impact of misfolded protein and prevent the formation of protein aggregates through modulation of chaperones that support effective protein trafficking [85, 89]. This approach has been explored in rodent models with promising results. Specifically, treatment of DRG explants from C22 mice with small-molecule inhibitors of heat shock protein 90 (HSP90) resulted in improved trafficking of PMP-22 and in myelination [90].

Schwann cells play an important role in axonal regeneration, and duplications in PMP-22 have been shown to impair the regeneration of large diameter axons [91]. Targeting of denervated Schwann cells to increase the efficiency of axonal regeneration has therefore been explored as a therapeutic avenue in CMT1A. Specifically, deficiency in NT-3 (a neurotrophic factor that plays a role in the Schwann cell autocrine loop and stimulates myelination) has been shown to impair nerve regeneration, and NT-3 knockout mice manifest a progressive motor neuropathy [92–94]. A small clinical trial assessing the efficacy of subcutaneously administered NT-3 did show clinical improvement; however, the short half-life of the drug limited further investigation [95]. As discussed later in this review, gene therapy studies using adeno-associated virus (AAV)-mediated neurotrophin 3 (NT-3) have since shown promise [96].

Multiple studies have suggested excess PMP-22 interferes with Schwann cell differentiation, and thereby with myelination, as evidenced by the abnormal expression of genes associated with immature Schwann cells, such as Sox2 and c-Jun [62]. This abnormal gene expression is particularly evident in vitro when Schwann cells are exposed to neurons, underscoring the role of Schwann cell axon interactions in the setting of abnormal PMP-22 dosing [97]. Fledrich et al. also found that in transgenic CMT1A rats, Schwann cells develop a persistent differentiation defect resulting from an imbalance of the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)-Akt and the mitogen-activated protein kinase 1 (Mek)-Erk intracellular signaling pathways, the latter of which is known to play a role in Schwann cell plasticity and nerve regeneration [62, 98]. The authors further demonstrated that enhancing PI3K-Akt signaling with epidermal growth factor (EGF)-like growth factor neuregulin-1 (NRG1) type I promoted Schwann cell differentiation. In contrast, Fornasari et al. found that different isoforms of NRG1 are actually strongly overexpressed in the nerves of transgenic CMT1A rats, suggesting that NRG1 may not be a viable treatment for CMT1A [99].

Another proposed mechanism for PMP-22 induced defects in Schwann cell differentiation is a rise in the influx of extracellular $[Ca^{2+}]$ into Schwann cells, which was shown to be related to an overexpression of the purinergic receptor P2X7 [100–102]. This was further explored by Vanoye and colleagues who demonstrated that PMP-22 specifically increases calcium influx through store-operated calcium channels, which help replenish $[Ca^{2+}]$ in the endoplasmic reticulum. The authors hypothesize that PMP-22 accumulation in the endoplasmic reticulum may therefore result in elevated intracellular $[Ca^{2+}]$ and subsequent demyelination [101, 103]. As discussed in more detail later in this review, inhibition of P2X7 was also found to be a viable therapeutic target in animal models of CMT1A [100, 104].

The importance of PMP-22 for effective intracellular lipid metabolism has recently been elucidated, presenting a new angle of the pathogenesis of CMT1A. Seventy percent of the myelin membrane is composed of lipids, including phospholipids, cholesterol, and glycosphingolipids [105]. Furthermore, cholesterol synthesis in Schwann cells is required for de novo synthesis of myelin. Fledrich et al. previously examined sciatic nerve and skin tissue mRNA extracts in CMT1A rats and demonstrated differential dysregulation of lipid metabolism-associated genes in mildly versus severely affected animals [106]. PMP-22 also interacts with cholesterol [107], and alterations in PMP-22 appear to impact cholesterol metabolism. Nerves from Trembler (Tr) mice were found to have reduced cholesterol synthesis [107–111], and both Schwann cells and nerves from *PMP22* knockout mice showed an abnormal cholesterol distribution [112]. Zhou et al. also recently found that PMP22, through interaction with the cholesterol efflux regulatory protein ABCA1, facilitates the efflux of cholesterol from Schwann cells [113]. In Schwann cells from homozygous Trembler J (TrJ) mice, cholesterol is retained in the Golgi along with PMP-22 and diminished in the plasma membrane [107], and it has been demonstrated that the cholesterol-binding motif known as CRAC of PMP-22 plays a particularly important role in PMP-22-mediated cholesterol localization within Schwann cells [107]. Taken together, these studies suggest that restoring cholesterol metabolism within Schwann cells could potentially offer therapeutic benefit in CMT1A.

Biological Models of PMP-22-Associated Neuropathy

The first animal models used to study myelin abnormalities associated with the *PMP22* gene were the naturally occurring mouse mutant Trembler (Tr) [114, 115] and Trembler J (Tr-J) [116]. The Trembler mice carry an

autosomal dominant missense mutation that substitutes an aspartic acid residue for a glycine in residue 150 (G150D) and present a severe phenotype characterized by spastic paresis, generalized tremor, and transient tonic–clonic seizures at an early age (after 10–14 days of age). Trembler-J (Tr-J) was produced by the Jackson Laboratory from the C57BL/6 J strain of Tr mice. Tr-J carries a missense mutation replacing a leucine with a proline residue at position 16 (L16P) in the first transmembrane domain of *PMP22* [116]. Tr-J mice present progressive limb weakness and tremor. It is important to note that despite their historical importance in understanding the connection between *PMP22* and demyelinating neuropathies, Tr and Tr-J mice are models of missense mutations in *PMP22* and therefore emulate the human disease CMT1E and not CMT1A, as they don't carry extra *PMP22* copies. Other Trembler lines include the Trembler-m1H, tr-m2H, and tr-m3H, all of which present a more severe phenotype than expected for a CMT1A model [117, 118].

To better recapitulate the biology of *PMP22* copy number variation characteristics of CMT1A, different *PMP22* overexpression models have been created since the late 1990s. These include transgenic mice lines C22 (carrying seven supplementary human *PMP22* transgene copies), C61 (carrying eight supplementary human *PMP22* transgene copies [119]), and C3 (carrying 3 to 4 *PMP22* transgene copies [119–121]). Despite the closer genotype and phenotype to CMT1A when compared to the Trembler models, several issues still remain regarding the reliability of these transgenic *PMP22* overexpressing mouse models. C22 and C61 models have to carry an excessive number of *PMP22* copies to demonstrate a phenotype and therefore do not adequately recapitulate the *PMP22* duplication characteristic of the human disease. Even when considering the more similar C3 model, which carries between 3 to 4 *PMP22* transgene copies, another issue common to all of these models is their inability to recapitulate genomic and epigenomic effects of the 1.4 Mb tandem duplication, including changes in microRNAs and long noncoding RNA, as well as possible modifier genes that may have important roles in disease biology and phenotypic expression. Other transgenic mice used in the study and modeling of CMT1A also include the JP18 and JP18/JY13 mouse models, which carry one and two extra copies of *PMP22*, respectively. It is important to note that the large duplicated 1.4 Mb DNA segment characteristic of CMT1A is beyond the limit of current cloning techniques. Therefore, none of the current animal models contains this large mutation and truly recapitulates the genetics of human CMT1A.

A *PMP22*-transgenic rat established in the late 1990s has become one of the most used rodent CMT1A models [122]. This transgenic rat carries three copies of a 43 kb

restriction fragment that contains the *PMP22* transcription unit, including 7 kb upstream of exon 1A and 4 kb downstream of exon 5, and presents behavioral, electrophysiological, and pathological features consistent with CMT1A in humans. This model has been used in several studies evaluating different treatments for CMT1A, including Onapristone [57], PXT3003 [123], AAV2/9 shRNA targeting *PMP22* mRNA [124], and antisense oligonucleotides against *PMP22* mRNA [19].

Cellular systems are also commonly used in pre-clinical studies for CMT1A, usually utilizing lines derived from the above-mentioned rodent models. A commonly used cell line is the S16 rat SC line, previously shown to sustain high levels of *PMP22* expression comparable to those in myelinating Schwann cells and demonstrating transcription factor binding patterns similar to rat sciatic nerve [70, 71, 125, 126]. These lines have been genetically engineered with different reporter systems and used in high-throughput screening assays to identify candidate compounds capable of reducing *PMP22* mRNA expression [15].

Unfortunately, due to the basic differences on a genetic level between rodent models of CMT1A and the human disease, the translation of pre-clinical studies into successful human trials have been challenging. Therefore, models that better recapitulate the genomic network involved in human CMT1A are urgently needed. As an alternative, human-induced pluripotent stem cells derived from patients with CMT1A could provide a superior model to study CMT1A in an authentic genetic background [10, 127]. However, challenges in fully differentiating these stem cells into mature myelinating Schwann cells capable of in vitro myelination still limit the use of this strategy in pre-clinical therapy development studies for demyelinating CMT.

Therapeutic Targets in PMP-22-Associated Neuropathy (Table 2 and Fig. 3)

Lowering PMP-22 in CMT1A

Since pre-clinical studies have underscored the importance of *PMP22* dosing in the pathogenesis of CMT1A, treatment efforts have focused on reducing the expression of *PMP22* and supporting effective myelination. One of the first candidate therapies examined was ascorbic acid (AA), an antioxidant with pro-myelinating effects that successfully reduced *PMP22* expression in Schwann cells via inhibition of adenylate cyclase and reduction of cyclic AMP levels [128, 129]. In vivo studies in C22 mice subsequently confirmed that AA suppresses *PMP22* expression and showed improved motor function in treated animals [56]. These promising results, together with the known safety profile of

Table 2 Treatment strategies in PMP-22-associated neuropathies

Treatments	Target	Model	Reference
<i>Lowering PMP22 in CMT1A</i>			
Ascorbic acid	Reduces PMP22 expression via adenylate cyclase inhibition	C22 mice	Passage (2004), Micallef (2009), Pareyson (2011)
Onapristone	Progesterone receptor 1 antagonist	CMT1A rats	Sereda (2003), Desarnaud (1998), Meyer (2007)
PXT3003		CMT1 rats	Chumakov (2014), Prukop (2019), Mandel (2015), Attarian (2014), NCT02579759
Baclofen	GABAB receptor agonist/reduces adenylate cyclase activity		
Naltrexone	Opioid receptor antagonist		
D-Sorbitol	Muscarinic receptor antagonist		
<i>PMP22 independent targets</i>			
A348079	P2X7 antagonist	CMT1A rats	Nobbio (2009), Sociali (2016)
Nano-Cur treatment (curcumin)	Reduces oxidative stress/SERCA inhibition	CMT1A rats	Caillaud (2020)
Heat shock protein inhibitors (HSP90 inhibitor)	Activate heat shock pathway/enhances protein trafficking	C22 mice	Evans (2010), Chittoor-Vinod (2019)
Histone deacetylase 6 (HDAC6) inhibitors -CKD-504	Acetylation of tubulin/inhibition of HSP90	CMT1A patients	Ha (2020)
<i>Emerging genetic therapies</i>			
AAV1.NT-3 gene therapy	NT-3 secretion	Trembler J mice	Sahenk (2014), Yalvac (2018) NCT03520751
ASO	PMP22 mRNA suppression	C22 mice and CMT1A rats	Zhao (2018)
siRNA	Reduces PMP22 expression	Trembler J mice	Lee (2017)
Squalenoyl siRNA PMP22 nano-particles	Reduces PMP22 mRNA	JY13 and JP18 mice	Lee (2017), Boutary (2021)
CRISPR/Cas9	Target the TATA-box of the P1 promoter/reduces PMP-22 expression	C22 mice	Lee (2020)

PMP22 peripheral myelin protein 22, *PXT3003* fixed-dose combination of baclofen, naltrexone, and sorbitol, *GABAB* gamma-aminobutyric acid B receptor, *SERCA* sarcoplasmic/endoplasmic reticulum Ca²⁺ -ATPase, *NT-3* neurotrophin-3, *ASO* antisense oligonucleotides

AA, motivated multiple clinical trials. Unfortunately, these trials failed to demonstrate clinical efficacy of AA in people with CMT1A [130–133].

The inhibition of progesterone, a neuroactive steroid involved in the myelination program of Schwann cells, similarly offered promise in pre-clinical studies of CMT1A [134]. In vitro studies in rat Schwann cells demonstrated that progesterone and its derivatives activate the P1 PMP-22 promoter and increase the expression of transcription factors SOX-10 and KROX-20, thereby further driving the expression of PMP-22 [134–136]. Sereda et al. targeted the action of progesterone in male transgenic CMT1A rats with onapristone, a progesterone/glucocorticoid receptor antagonist, and demonstrated reduced expression of PMP-22 and an improvement in the clinical phenotype [57, 137]. Due to concerns regarding their potential toxicity, progesterone inhibitors have not progressed to clinical trials.

A treatment that has more recently shown promise in CMT1A is PXT3003, a combination of compounds identified using a systems biology approach focused on pathways that promote myelination, while simultaneously

downregulating PMP-22. PXT3003 combines three repurposed drugs including (1) baclofen, a GABA receptor agonist that reduces PMP22 transcription in Schwann cells by reducing adenylate cyclase activity [138]; (2) naltrexone, an opioid receptor antagonist believed to potentiate Baclofen's mechanism of action; and (3) D-sorbitol, a natural metabolite involved in the polyol pathway postulated to stabilize misfolded proteins [139].

PXT3003 was demonstrated to successfully downregulate PMP-22 via the PI3K-AKT/MEK-ERK signaling pathway in transgenic CMT1A rats, with an improvement in myelination and in the clinical phenotype [123]. Combination therapy proved superior to treatment with the individual components of PXT3003 [139]. Prukop et al. also demonstrated that brief treatment of transgenic CMT1A rats with PXT3003 during early development delays disease onset in adulthood, with a dose-dependent improvement in limb strength [140]. Interestingly, despite the clinical improvement observed, the only electrophysiological measure that improved was distal motor latency. Furthermore, while treatment resulted in a shift towards large-caliber axons, there

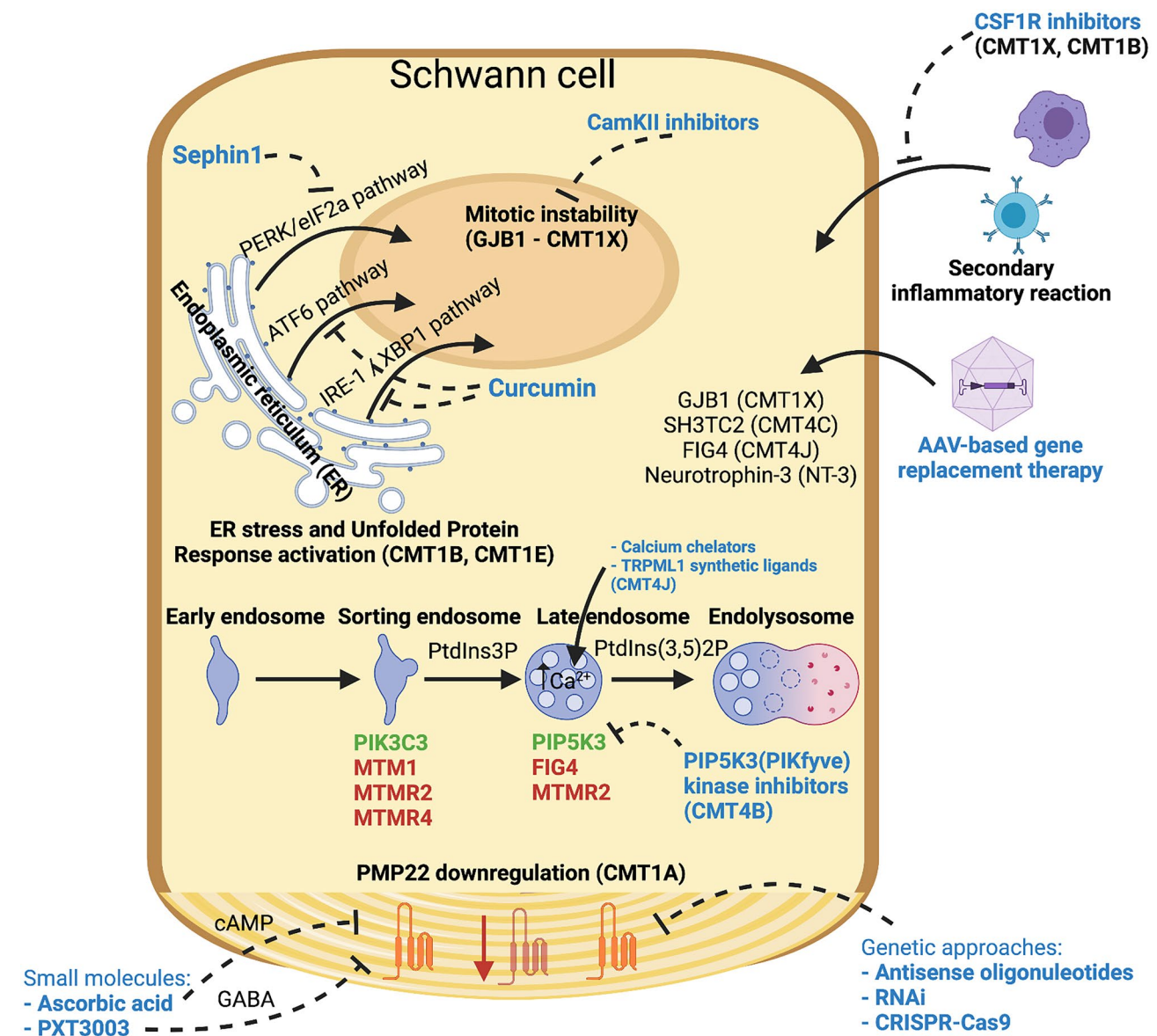


Fig. 3 CMT1 treatment targets and therapies

was no change in the total number of myelinated axons or in myelin thickness. The authors hypothesized the clinical benefit of PXT3003 may result in part from enhanced muscle innervation at the neuromuscular junction, uncoupled from the effects of demyelination. Recent *in vitro* and *in vivo* work has offered support for this hypothesis [139]. Specifically, transgenic CMT1A rats treated with PXT3003 showed an increased number of innervated neuromuscular junctions. Interestingly, PMP-22 expression did not decline in response to treatment, contrary to prior studies [139]. While the precise biological mechanisms underlying PXT3003-associated improvement in CMT1A will require further study, early clinical trials are promising. Notably, a randomized, double blind, placebo-controlled, phase 2 study including 80

CMT1A patients confirmed the safety and tolerability of PXT3003 and showed a significant improvement in the Charcot-Marie-Tooth Neuropathy Score (CMTNS) and the Overall Neuropathy Limitations Scale (ONLS) in those receiving the highest treatment dose for 12 months ($n = 19$) [141]. Results from a Phase III clinical trial (ClinicalTrials.gov identifier NCT02579759) have also shown a significant improvement in the 10-m walk test and ONLS in the higher dose group, with no serious adverse events [142].

PMP-22 Independent Targets in CMT1A

The identification of P2X7 channel activation and subsequent accumulation of Ca^{2+} in Schwann cells as a potential

pathomechanism in CMT1A has led to pre-clinical studies of P2X7 inhibitors. Nobbio et al. found that antagonizing P2X7 using pharmacological inhibitors and small-interfering RNA (siRNA) rescues the phenotype of CMT1A Schwann cells [100]. Sociali et al. then demonstrated improved myelination in DRG cultures in response to a P2X7 antagonist (A438079) [104]. In vivo studies in transgenic CMT1A rats also showed improvement in hind limb strength in response to treatment with A438079 and further supported the hypothesis that P2X7 inhibition promotes Schwann cell differentiation. While the authors observed an increase in myelinated axons, there was no change in CMAP amplitudes in treated animals. These studies also underscored a potential adverse impact of P2X7 inhibition, namely muscle weakness resulting from higher doses of A438079.

Reduction of oxidative stress has also been examined as a therapeutic strategy in CMT1A. In this regard, curcumin, which is safe and possesses both neuroprotective and antioxidant effects, is an appealing candidate treatment. Curcumin is also a known sarcoplasmic/endoplasmic reticulum calcium pump (SERCA) inhibitor and can therefore alleviate the accumulation of misfolded proteins in the ER, thereby reducing excessive UPR activation in Schwann cells [143–145]. To overcome the inefficient pharmacokinetic properties of curcumin, Caillaud et al. developed curcumin-cyclodextrin/cellulose nanocrystals (Nano-Cur) and examined their effects in transgenic CMT1A rats [146]. Treatment resulted in improvement in the clinical phenotype, as well as in electrophysiological findings (motor and sensory conduction velocities) and in myelin thickness. The authors also observed an associated reduction in markers of oxidative stress in both nerve and muscle, and in vitro proof of concept experiments confirmed a reduction in reactive oxygen species and improved mitochondrial membrane potential in CMT1A Schwann cells [146].

Another approach to minimizing impact of protein misfolding in CMT1A has been to activate the heat shock pathway, an intracellular stress response mediated by chaperones that facilitate protein folding and trafficking, thereby reducing protein aggregation [85, 147]. Specifically, the heat shock pathway can be activated through the inhibition of HSP90, a molecular chaperone protein, and HSP90 inhibitors have previously shown benefit in disorders related to protein misfolding [148]. Chittoor-Vinod et al. demonstrated that select HSP90 inhibitors enhance myelin synthesis in vitro and found that intraperitoneal treatment in C22 mice improved peak muscle force and slowed decline in rotarod performance [147]. Because HSP inhibitors can result in cellular toxicity, further study will be needed prior to their potential use in humans.

HSP90 also proved to be targetable in a recent pre-clinical study evaluating Histone deacetylase 6 (HDAC6) inhibition in CMT1A [149]. Histone deacetylase 6 (HDAC6) is

an enzyme that controls the acetylation of cytosolic proteins, including α -tubulin, and plays a role in microtubule stability and axonal transport [149]. HDAC6 inhibitors were previously found to improve the CMT2 phenotype in animal models via the acetylation of tubulin [150]. Ha et al. recently evaluated the effect of CKD-504, an HDAC6 inhibitor, on mesenchymal stem cell-derived Schwann cells from CMT1A patients and demonstrated reduced PMP22 protein expression and induction of Schwann cell differentiation in response to treatment [149]. In C22 mice, treatment resulted in the acetylation of α -tubulin and reduced PMP-22 protein in the sciatic nerve, with improved myelination, motor function, and electrophysiological features. HDAC6 also induced the acetylation, and thereby inhibition, of HSP90, which was hypothesized to increase folding and reduce the aggregation of excess protein. While the significant toxicity associated with HDAC6 inhibitors has limited their use in patients, CKD-504 is currently in Phase 1 clinical trials for use in Huntington's disease (NCT0371389) [149].

As previously discussed, alterations in both the metabolism and distribution of lipids have been observed in association with excess PMP-22, and studies have begun to examine whether ameliorating these lipid derangements can improve myelination in CMT1A. Fledrich et al. showed that substitution of phosphatidylcholine and phosphatidylethanolamine in the diet increased the number of myelinated axons in peripheral nerves (without changes in the thickness of the myelin sheaths), prevented axonal loss, and improved the clinical phenotype in CMT1A rats [105]. The clinical benefit did not persist beyond the treatment period, however. Zhou et al. also treated TrJ mice with advanced neuropathy with a lipid enriched, high fat diet and identified improvements in the maintenance of myelinated axons [112]. As mentioned, PMP-22 is also important for cholesterol metabolism in Schwann cells, and alterations in cholesterol trafficking have been observed in CMT1A. Future studies may explore whether cholesterol supplementation or manipulation of cholesterol transport can offer benefit in CMT1A.

Emerging Genetic Therapies in CMT1A

The recent success of genetic therapies, including antisense oligonucleotides (ASOs) and small-interfering RNA (siRNA)-based treatments, in other neuromuscular disorders has greatly informed the current approaches to the treatment of CMT1A and offered promise for a disease modifying treatment [17, 18, 151]. Genetic therapy studies in CMT1A began when Sahenk et al. introduced adeno-associated virus (AAV)-mediated neurotrophin 3 (NT-3) gene therapy in the TrJ mouse model [152]. NT-3 is an autocrine-derived factor expressed by Schwann cells that promotes both myelination and axonal regeneration [95, 152]. The authors demonstrated that rAAV1.NT-3 gene transfer into muscle allows for NT-3

secretion, with an increase in serum levels and associated improvement in clinical, pathological, and electrophysiological features in treated animals. Follow-up work by the same group revealed that AAV.NT-3 gene therapy in TrJ mice affects muscle enzyme metabolism and activates the mammalian target of rapamycin complex 1 (mTOR) pathway, resulting in an increase in muscle fiber size [96]. NT-3 may therefore have a synergistic effect on both nerve and muscle. Interestingly, the aforementioned effects of AAV.NT-3 were not observed in the WT animals, suggesting a specific predilection for pathological nerve fibers. While these results are encouraging, it is important to note the TrJ mouse strain, which harbors a naturally occurring point mutation in PMP-22, is not a model of CMT1A but rather better represents CMT1E. An ongoing Phase I/II clinical trial is evaluating the effect of AAV.NT-3 gene therapy delivered via a single intramuscular injection in humans with CMT1A (NCT03520751).

AAV vectors allow for stable expression, do not integrate into the host genome, and have low immunogenicity. Due to these favorable properties, they have served as the preferred delivery mechanism in neurologic disorders. There are barriers to the use of AAV-mediated gene therapies, however, including their potential for off-target effects as well as concerns regarding ectopic gene expression [153]. Targeted delivery to Schwann cells could help offset these adverse effects. To this end, Gautier et al. recently reported successful intraneural delivery of a recombinant adeno-associated viral vector serotype 9 (AAV2/9) expressing a small hairpin inhibitory RNA (shRNA) directed against PMP-22 mRNA, to transgenic CMT1A rats, and demonstrated efficient transduction [124]. Early treatment also prevented myelination defects, as well as motor and sensory impairments. Given that treatment induced a reduction of PMP-22 protein expression, without reducing PMP-22 mRNA, the authors postulate that the disruption occurred at the level of translation machinery rather than via direct targeting of mRNA [124]. Importantly, intraneural delivery did restrict biodistribution of the vector, suggesting that direct delivery to Schwann cells could help minimize off-target effects.

As mentioned, a particularly exciting recent development in the treatment of genetic neuromuscular disorders has been the use of antisense oligonucleotides (ASOs). ASOs are single-stranded synthetic nucleic acids that can target specific cell types and bind target mRNA, leading to its degradation [154]. Zhao et al. demonstrated that ASOs successfully suppress PMP-22 mRNA in the nerves of both the C22 mouse and the transgenic CMT1A rat models [19]. Treatment of C22 mice with weekly subcutaneous injections of the PMP-22 ASO after disease onset resulted in a dose-dependent reduction in PMP-22 mRNA. Furthermore, treatment improved motor function, electrophysiology (with motor conduction velocities approaching normal levels and

an increase in motor amplitudes) and pathological features, as evidenced by increased numbers of myelinated axons and reduced onion bulb formation. The authors also demonstrated a treatment-induced reduction in PMP-22 mRNA levels in Schwann cells from skin biopsies of CMT1A rats, suggesting that this could serve as a treatment-specific biomarker in future studies. The finding that ASOs can cross the blood nerve barrier to target Schwann cells and favorably affect PMP-22 gene expression and myelination opens an exciting new pathway for therapeutic development in CMT1A [19]. At the same time, important challenges remain. Specifically, the timing of ASO treatment, as well as mode of administration and dose, will all require further investigation [155]. Furthermore, concerns about off-target effects, and the known side effects of ASOs including thrombocytopenia, could outweigh the benefit of treatment for a slowly progressive neuropathy such as CMT1A [156]. Finally, as maintaining the right degree of PMP-22 expression is critical to nerve health, excessive suppression could result in an HNPP phenotype, which is associated with patient morbidity comparable to that of CMT1A [157, 158].

Another gene therapy approach that has shown promising results in pre-clinical studies of CMT1A is the use of small-interfering RNA (siRNA). siRNAs are small double-stranded RNAs that can selectively silence the expression of a targeted gene by degrading its mRNA [159]. Intraperitoneal injection of siRNAs reduced mutant PMP-22 expression, improved myelination, and alleviated the clinical phenotype in the Tr-J mouse model [160]. Boutary et al. recently tested i.v administration of siRNAs in the JP18 and JP18/JY13 mouse models, which carry one and two extra copies of *PMP22*, respectively [161]. To achieve successful delivery of siRNAs to Schwann cells, the authors used a nanoparticle-stabilized siRNA (siRNA PMP22-SQ NPs). Treatment resulted in a rapid and dramatic improvement of the clinical phenotype with improved limb strength and locomotor function, as well as normalization of CMAP amplitudes and sensory conduction velocities, with positive effects persisting for 3 weeks beyond the treatment period [161]. The clinical improvement was accompanied by normalization of sciatic nerve levels of transcription factors Krox20 and Sox10, as well as heavy neurofilament levels, consistent with the recovery of both the myelin and axons. Importantly, in the aforementioned studies, the expression of other myelin proteins was not impacted by siRNA PMP-22, underscoring the potential of this treatment to limit off-target effects [159, 160].

A recent study evaluated CRISPR-based gene editing in CMT1A. In contrast to other treatment strategies, this approach offers the potential for a single-dose therapy. In a proof-of-concept study, Lee and colleagues delivered CRISPR/Cas9 intraneurally to C22 mice in order to target the TATA-box of the P1 promoter in Schwann cells [162].

Treatment prior to disease onset resulted in downregulation of PMP-22 expression and improvement in both electrophysiological features and in myelination. As discussed by the authors, various challenges, including the potential for immunogenicity and off-target effects, will need to be closely examined in future studies.

While the recent advances in genetic therapies for CMT1A are encouraging, it is important to emphasize that we are still in the early stages of understanding the impacts of gene therapy in humans and that many obstacles, both expected and unanticipated, likely lie ahead. This is exemplified by the recent discovery that treatment of SMA mouse models with adeno-associated virus serotype 9 (AAV9)—SMN gene therapy—can result in toxic gain of function injury to motor neurons due to aggregation of the overexpressed protein [163].

Myelin Protein Zero-Associated Neuropathy: Epidemiology and Clinical Features

MPZ neuropathies account for 5% of all of CMT and 10% of all demyelinating forms of CMT [9, 13, 164]. While MPZ neuropathy is an AD disorder, *de novo* mutations are common, and the absence of a family history should not preclude consideration of this diagnosis [165]. To date, there have been over 200 disease-causing mutations identified in the MPZ gene, with 76 new mutations reported between 2005 and 2018 [24]. MPZ neuropathies span a wide phenotypic spectrum from severe infantile onset demyelinating neuropathy to milder adult-onset axonal forms [165]. This striking genotypic and phenotypic heterogeneity poses challenges both for accurately identifying patients with MPZ neuropathy and for designing effective clinical trials.

The nomenclature used to describe MPZ neuropathies has evolved over the years leading to some confusion. Traditionally, demyelinating neuropathy, with upper extremity CV < 38 m/s resulting from MPZ mutations, has been referred to as CMT1B and the axonal forms as CMT2I. Other descriptors have included “Dejerine-Sottas,” in reference to infantile onset neuropathy, and “congenital hypomyelination,” in reference to severe, early-onset neuropathies with pathological evidence of myelination failure [166–169]. Increasingly, MPZ neuropathy phenotypes are being classified by the patient’s age at presentation, the primary nerve pathology, and the specific genetic mutation [170].

The clinical features of most MPZ-associated neuropathies are similar to those seen with other forms of CMT, namely foot deformities, distal muscle weakness and atrophy, and length-dependent sensory loss. Additional features can include scoliosis, and hip dysplasia, which are more

common in patients with the infantile onset demyelinating forms [170]. In contrast, tonic pupils, dysphagia, and neuropathic pain are distinguishing features that occur more commonly with the axonal forms [171]. Hearing loss can also occur, with both the early and adult-onset forms of MPZ neuropathy [170]. Electrophysiological findings in MPZ neuropathy are diverse. Motor conduction velocities in the demyelinating, axonal, and intermediate ranges can be seen, and rare cases of partial conduction block have been reported, leading to suspicion for immune-mediated rather than hereditary nerve disease [172]. Similarly, nerve pathology can include findings of either dysmyelination or demyelination, as well as of primary axonal degeneration [173–179].

Genotype–phenotype correlation studies have identified three distinct phenotypic groups in MPZ neuropathy, including infantile, childhood, and adult-onset CMT [30, 33, 170, 180]. Patients in the infantile-onset group develop symptoms prior to 3 years of age and have severely slowed motor CVs (ulnar motor CV < 15 m/s) and more difficulty with ambulation than the other groups (19% wheelchair dependent in one series) [170]. The childhood-onset group demonstrates higher ulnar motor CVs (15–35 m/s) and presents similarly to patients with CMT1A with symptoms emerging in the second decade, and the adult-onset group is distinguished by axonal range CVs [165, 170]. Importantly, the majority of MPZ mutations consistently manifest with one of the three distinct clinical phenotypes [15, 24, 165, 171]. Examples include the His10Pro and Thr95Met mutations, which result in adult-onset neuropathy, versus Ser34del, which manifests with early-onset, demyelinating CMT [165]. Why individual MPZ mutations result in specific clinical phenotypes is not known [170]. The clinical progression of MPZ neuropathies is also highly variable. Early-onset, severe neuropathies tend to cause notable disability in childhood, with slower rates of progression beyond adolescence [181]. In contrast, select axonal forms can present in adulthood and progress rapidly leading to a loss of ambulation in later life [25, 181, 182].

While this review focuses on demyelinating forms of CMT, it is worth noting that the preponderance of recently discovered MPZ mutations is responsible for adult onset, axonal neuropathies; the prevalence of which likely continues to be underestimated [24]. Defining the pathomechanisms of axonal forms of MPZ neuropathy is especially important, given that MPZ is expressed exclusively in myelinating Schwann cells, and yet minimal pathological evidence of demyelination is observed in patients with axonal neuropathy related to MPZ [26, 183]. It has been hypothesized that the axonal injury may result from disruptions in the signaling pathways between the myelin and the axon, though the detailed nature of this disruption at the cellular level is not well understood [170, 177, 178].

MPZ Biology and Pathomechanisms

MPZ, also termed P0, is the major protein in peripheral nerve myelin and a member of the immunoglobulin (Ig) supergene family [26]. The protein plays an important role both in the formation of myelin and in the maintenance of myelin homeostasis and stability throughout adulthood [184]. The protein is encoded by the *MPZ* gene on chromosome 1q22-q23 and is only expressed in myelinating Schwann cells [185]. MPZ consists of three structural domains: a 124 amino acid immunoglobulin-like extracellular domain, a 26 amino acid transmembrane domain, and a 69 amino acid intracellular domain. The protein is synthesized in the ER of Schwann cells, trafficked through the Golgi compartment, and ultimately sorted into vesicles and incorporated into the myelin sheath [186–188]. Of the 248 amino acids encoded by *MPZ*, the first 29 comprise a signaling protein that targets MPZ to the myelin sheath and is cleaved prior to the protein's incorporation into myelin [26]. MPZ additionally undergoes post-translational modification by the addition of an N-linked oligosaccharide, as well as sulfate, acyl, and phosphate groups [189, 190]. While MPZ largely localizes to compact myelin, it is also found in the paranode and node of Ranvier, where it helps maintain nodal structure through interactions with neurofascins [191].

Once incorporated into the myelin sheath, MPZ behaves as a homophilic adhesion molecule, facilitating the compaction of myelin. Compaction is achieved when the extracellular MPZ domains on opposing myelin wraps form homotetramers that interact in-trans, thereby adhering the opposing myelin wraps to each other [165, 189, 192–194]. The critical role of MPZ in myelin compaction is evidenced by the presence of thin and uncompacted myelin, and severe neuropathy in *MPZ* knockout mice, as well as in transgenic mice containing extra copies of *MPZ* [195, 196].

MPZ neuropathy results from diverse mechanisms, including numerous gain of function and loss of function mechanisms [197, 198]. Some structural changes to the protein result in retention of MPZ in the ER, whereas other derangements allow the protein to successfully incorporate into the myelin sheath but disrupt interactions with the wild-type protein, thereby impairing myelin adhesion [199]. While early-onset demyelinating forms of MPZ neuropathy more commonly impede successful compaction, later onset forms tend to disrupt MPZ-mediated signal transduction and Schwann cell-axonal interactions. Importantly, the cellular mechanisms of MPZ do not reliably predict the clinical phenotype in MPZ neuropathy, as exemplified by the R198S mutation, which fully prevents myelin adhesion but causes a late-onset neuropathy [197, 198]. Given the varied gain of function mechanisms of MPZ neuropathy, it is increasingly being recognized that treatments will likely be diverse with focus on allele-specific gene silencing approaches [200].

MPZ Mutations Resulting in Altered Protein Structure and Functionality

Mutations in *MPZ* can alter normal function at varied intracellular locations. The majority of pathogenic mutations are located in the extracellular domain; however, both the extracellular and the cytoplasmic domains are necessary for the effective compaction of myelin [173, 174, 198, 201]. Particularly disruptive changes to MPZ include the addition of a charged amino acid, the alteration of a cysteine residue in the extracellular domain, the truncation of the cytoplasmic MPZ domain, and the alteration of an evolutionarily conserved amino acid [165]. Packing defects in the myelin intra-period line also result from several mutations in the extracellular domain [202]. Additionally, mutations can disrupt the post-translational modification of the protein. For example, increased glycosylation resulting from a second glycosylation site in the D23N mutant protein results in a severe, early-onset demyelinating neuropathy [203]. In contrast, mutations that prevent glycosylation of MPZ do not appear to interfere with myelination but may disrupt axon-Schwann cell interactions leading to the development of late-onset axonal neuropathies [177, 197, 204]. Lastly, mutations that alter MPZ's ability to interact with the node and paranode underlie select adult-onset forms of MPZ neuropathy [191].

As mentioned, mutations in the cytoplasmic domain of *MPZ* can also be disease causing, and the truncation of the cytoplasmic domain has specifically been shown to prevent myelin adhesion [198, 205]. The cytoplasmic domain is believed to contribute to myelin compaction through an adhesion-mediated signal transduction cascade that enables interactions with the cytoskeleton [165, 206–208]. Gaboreanu and colleagues specifically demonstrated phosphorylation of the cytoplasmic domain, which is mediated by PKC α , and the receptor for activated C kinase 1 (RACK1) is important in the regulation of MPZ-mediated adhesion [206]. Changes in the cytoplasmic domain additionally impact effective MPZ targeting at the pre-myelinating stage. Fratta and colleagues used knock-in mice with the nonsense Q215X mutation (a cause of congenital hypomyelinating neuropathy in humans) to demonstrate that eliminating the last 33 amino acids of the cytoplasmic domain results in altered trafficking of MPZ to non-myelin plasma membranes and alters radial axonal sorting by Schwann cells [200].

Depolarization changes in Schwann cells may also contribute to the pathogenesis of MPZ neuropathy. Sural nerve biopsies from a patient with the R69C mutation demonstrated a switch to the subtype 1.8 voltage-gated sodium channels at the demyelinating/remyelinating internodes [181]. Additionally, Moldovan and colleagues examined homozygous mice deficient in MPZ with severe,

demyelinating neuropathy and found abnormal potassium ion currents and ectopic Na(V)1.8 channels in unmyelinated nerve segments, which disrupted axon excitability [209]. Follow-up work examining a family harboring an *MPZ* frameshift mutation (Asp104ThrfsTer14) suggested that axonal depolarization resulting from abnormal voltage-gated sodium channels may precede axonal degeneration, mirroring the prior findings in mouse models [210].

Intracellular Changes in Response to MPZ Derangement

The Unfolded Protein Response

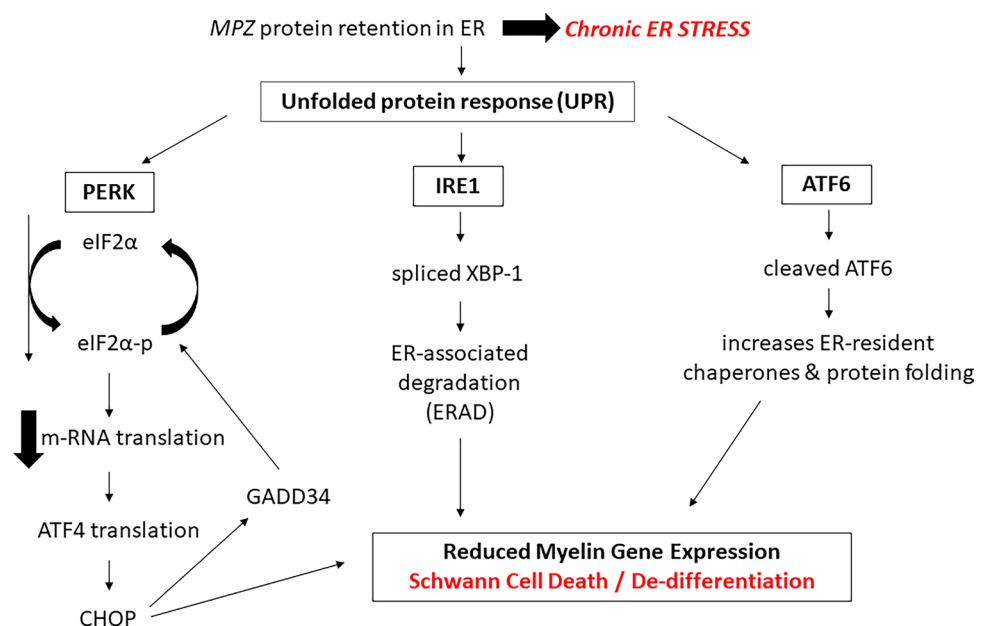
A major focus in the study of *MPZ* neuropathy pathogenesis has been that of the unfolded protein response (UPR), which has also been implicated in other forms of CMT, including PMP-22 and GJB1-associated neuropathies [199]. Because Schwann cells produce large amounts of protein, they are particularly vulnerable to potential endoplasmic reticulum stress resulting from protein misfolding [211–214]. The UPR serves as an adaptive mechanism employed by the cell to handle the accumulation of misfolded proteins and acts by upregulating transcription of chaperones, reducing translation of proteins, and increasing proteasomal protein degradation [215, 216]. While adaptive under normal circumstances, at excessively high levels of ER stress, the UPR can alter the phenotype of the cell in a way that impedes its normal function or potentially leads to apoptosis [215, 217].

In humans, three transducers mediate the UPR: inositol requiring enzyme (IRE1), activating transcription factor (ATF6), and protein kinase RNA-like endoplasmic reticulum

kinase (PERK), all of which are located in the ER membrane (Fig. 4). The UPR cascade is activated by BiP, an ER chaperone that in normal circumstances binds IRE1 and PERK but in the presence of misfolded protein dissociates from the transducers rendering them active [215]. Once activated, IRE1 promotes the activation of genes involved in ER-associated degradation (ERAD) through the spliced X box binding protein (XBP1) transcription factor [211, 218, 219]. ATF6 promotes ER-resident chaperones, thereby supporting folding within the ER [220–224]. The PERK arm of the UPR is particularly important to Schwann cell survival in the setting of increased ER stress [225, 226]. PERK phosphorylates the α subunit of eukaryotic initiation factor 2 alpha (eIF2 α) leading to a reduction in the translation of messenger RNAs. In addition, PERK increases the translation of activating transcription factor 4 (ATF4), which in turn upregulates the *CCAAT/enhancer-binding protein homologous gene* (*CHOP*) [227–230]. *CHOP* is a transcription factor associated with apoptosis related to ER stress and is a key regulator of cell death. Paradoxically *CHOP* also upregulates DNA damage-inducible protein 34 (*GADD34*). The *Gadd34* gene encodes a regulatory subunit of protein phosphatase 1 (PP1) holophosphatase, which dephosphorylates eIF2 α and thereby reactivates protein translation, enabling protein translation to resume [226]. Surprisingly, Musner and colleagues found that PERK haploinsufficiency actually improves myelin defects in vitro and in vivo, despite reduced levels of P-eIF2 α , suggesting that PERK has effects on neuropathy that are unrelated to the UPR [211, 231].

A large number of *MPZ* mutations activate the UPR in a dose-dependent fashion, resulting in Schwann cell dysfunction and ultimately in demyelination [212, 229, 232–234]. The most extensively studied mutations that result in the retention of misfolded *MPZ* in the ER and subsequent UPR

Fig. 4 Unfolded Protein Response



activation are R98C and S63del, both of which are found in the extracellular domain [144, 197, 199, 217]. In S63del Schwann cells, globally misfolded mutant protein triggers the canonical UPR by exposing a hydrophobic surface of MPZ and promoting BiP binding with downstream activation of CHOP [229]. Increased *CHOP* expression results in growth arrest, demyelination, and secondary Schwann cell death. This is distinct from the immediate CHOP-induced cell death seen in many other disorders and suggests a unique function of CHOP in Schwann cells [184, 217, 229].

It is important to emphasize that *MPZ* mutations that activate the UPR do so by varied mechanisms. While the PERK pathway appears to underlie nerve injury in S63del, it is the IRE and ATF6 arms of the UPR that are implicated in R98C [145]. This difference may contribute in part to the two neuropathies being pathologically distinct, with R98C primarily causing dysmyelination or hypomyelination, and S63del resulting primarily in demyelination [184]. It has been suggested that in addition to activating the UPR, S63del mutant protein also negatively impacts wild-type MPZ, causing it to be retained in the ER and reducing its levels in the myelin sheath [235].

The precise mechanism by which R98C impedes myelination is not well understood but is believed to involve an elevation in transcription factor C-Jun (a negative regulator that inhibits myelination) and a reduction in Krox-20. Importantly, UPR activity resulting from *MPZ* mutations does not clearly correlate with the clinical onset or severity of the neuropathy [232], with activation correlating with an infantile onset neuropathy with Arg98Cys but a childhood onset neuropathy with Ser63Del [229, 236]. It is also worth emphasizing that not all *MPZ* mutants retained in the ER actually activate the UPR in animal models, a phenomenon that may be related to the varying proteasomal capacity and ability to eliminate misfolded protein in Schwann cells [197].

Biological Models of MPZ Neuropathy

The two most commonly examined mouse models of MPZ have been the hemizygous S63del and heterozygous R98C transgenic mice [181, 184, 199, 217, 236–238]. Parallel MPZ neuropathy in humans, the R98C knock-in mice demonstrate a more severe and earlier onset demyelinating neuropathy, whereas the S63del animals present later and do not show the same degree of developmental hypomyelination. Phenotypically, S63del mice demonstrate motor impairment, uniformly slowed NCS velocities, pathological evidence of demyelination with onion bulb formation, and clinical progression with age [199]. The model does not manifest the axonal loss seen in human disease, perhaps owing to the limited lifespan and the reduced nerve length

in the animals [199, 239]. Both the heterozygous (R98C/+) and homozygous (R98C/R98C) mice demonstrate weakness, abnormal nerve conduction velocities, and pathologically abnormal myelin, with the homozygous animals being more severely affected [217]. Both models also demonstrate retention of mutant protein in the ER with a resulting increase in UPR activation and CHOP expression; however, while CHOP ablation fully rescues the motor phenotype in S63del mouse models, it does not improve neuropathy in R98C mice [199, 217, 229, 230, 236]. This underscores the observation that different arms of the UPR are likely involved in nerve injury in the two mutants, namely the PERK pathway in S63del, versus IRE1 and ATF6 in R98C. A model that does not involve activation of the UPR is the Q215X mouse model of congenital hypomyelination, which has been used to examine aberrant MPZ trafficking [200, 232].

Methods that evaluate the degree of activation in the three arms of the UPR pathway (i.e., determination of CHOP levels to gauge activity in the PERK pathway) are increasingly being employed to examine UPR-mediated treatments in animal models [240]. In addition, in vitro assays, such as those assessing XBP1 splicing as a measure of IRE1 activation, have been used to identify UPR-activating MPZ mutants and to evaluate the effect of pharmacological agents [145, 232].

Therapeutic Targets in MPZ Neuropathy (Table 3 and Fig. 3)

Targeting the UPR to Treat MPZ Neuropathy

Interventional studies targeting the UPR in animal models have led to important insights into MPZ neuropathy pathogenesis and have offered promise for future therapies. Specifically, CHOP ablation in S63del mice rescued the motor deficits and reduced active demyelination two-fold [229]. Prolonging eIF2a phosphorylation and further attenuating protein translation by manipulating the PERK arm of the UPR is hypothesized to reduce the translation of mutant MPZ and thereby enhance the delivery of wild-type protein to the myelin sheath [232]. Furthermore, genetic and pharmacological inhibition of GADD34 reduced mutant protein retention in the ER and ameliorated the clinical phenotype in S63del, even more effectively than CHOP ablation [230]. Salubrinal, a molecule that inhibits the dephosphorylation of eIF2 by Gadd34, also reduced the accumulation of mutant MPZ in the ER and improved myelination in S63del nerves [230, 241]. Finally, Das et al. showed Sephin1, a selective inhibitor of the Gadd34 holophosphatase, effectively prolongs eIF2a phosphorylation, and ameliorates neuropathy in Ser63del mice, as evidenced by clinical and pathological measures [242]. Selectively correcting protein homeostasis

Table 3 Treatment targets in MPZ-associated neuropathy

Target	Mutation/model	Intervention	Reference
UPR pathway	S63del	CHOP ablation	Sharifov (2013)
		GADD34 inhibition	D'Antonio (2013)
		Salubrinal	Boyce (2005), D'Antonio (2013)
		Sephin1	Das (2015)
	R98C	Curcumin derivatives	Patzko 2012
ERAD pathway	S63del	N-Acetyl-D-glucosamine (GlcNAc)	Volpi (2019)
Axonal neuregulin type III	S63del	Overexpression of Nrg1TIII	Scapin (2019)
		Suppression of Nrg1TIII inhibitor (TACE/ADAM17)	Scapin (2019)
NaV1.8 sodium channels	MPZ-deficient mice	NaV1.8 blocker	Rosberg (2016)

UPR unfolded protein response, *CHOP* CCAAT-enhancer-binding protein homologous protein, *GADD34* growth arrest and DNA damage-inducible protein, *SERCA* sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase, *ERAD* endoplasmic-reticulum-associated protein degradation, *Nrg1TIII* axonal neuregulin 1 type III, *TACE* tumor necrosis factor- α -converting enzyme, *ADAM17* A disintegrin and metalloprotease 17, *MPZ* myelin protein zero

and delaying recovery of protein translation may therefore offer an important therapeutic avenue for MPZ neuropathy, as well as other protein misfolding disorders.

Another approach to reducing UPR activation in MPZ neuropathy has been to employ sarcoplasmic/endoplasmic reticulum calcium pump (SERCA) inhibitors. SERCA inhibitors reduce ER stress and UPR activation by inhibiting calcium binding and disrupting calnexin function and have been shown to improve the phenotype of MPZ mutants in vitro [144]. Patzko and colleagues treated R98C mice with curcumin, a low affinity SERCA inhibitor, and found that phosphatidylcholine curcumin administration, designed to increase bioavailability, resulted in improvements in rotarod performance, CMAP amplitudes, and in the number of large diameter axons in the treated animals [145]. Treatment attenuated the IRE and ATF6 arms of the UPR, but did not alter the PERK pathway. Interestingly, treatment was also associated with changes in the NMJ, including an increase in the percentage of fully myelinated preterminal internodes and a decrease in the length of demyelinated segments approaching the NMJ [145, 240]. The authors hypothesized that the reduction in the “toxic gain of function” caused by mutant MPZ enabled Schwann cells to remain in a pro-myelinating state and suggested that improvement to haploinsufficiency could have a substantial clinical impact in MPZ neuropathy [145]. Despite these promising pre-clinical results, curcumin is not likely to be examined in clinical trials given its suboptimal pharmacokinetic properties [10].

The potential to manipulate the ER-associated degradation (ERAD) pathway to minimize the burden of misfolded protein in MPZ neuropathy was underscored by the work of Volpi and colleagues. ERAD facilitates the targeting of misfolded proteins to proteasomes for degradation, and the

authors showed that Schwann cell-specific ablation of the ERAD factor Derlin-2 in S63del nerves increased myelin defects and the UPR in vivo. In contrast, treatment with N-Acetyl-D-glucosamine (GlcNAc) (an ERAD enhancing metabolite) of S63del dorsal-root-ganglia (DRG) explants improved nerve myelination [243]. These findings suggest that the ERAD has a protective role in MPZ neuropathy and that variations in ERAD may, in part, explain the phenotypic variability seen in neuropathies related to UPR activation.

UPR-Independent Treatments of MPZ Neuropathy

A non-UPR-mediated therapeutic target previously examined in MPZ neuropathy is axonal neuregulin 1 type III (Nrg1TIII), which activates the signaling pathways that lead to the expression of myelination genes, as well as increases in cholesterol and fatty acids [244]. Both overexpressing Nrg1TIII and suppressing the Nrg1TIII inhibitor tumor necrosis factor- α -converting enzyme (TACE/ADAM17) improved the clinical phenotype of the S63del mouse model without increasing ER stress [244]. As mentioned previously, ectopic expression of NaV1.8 sodium channels has been identified on motor axons in animal models of MPZ neuropathy. Rosberg et al. therefore evaluated the effects of an oral NaV1.8 blocker and demonstrated improved membrane dysfunction and motor performance in mice deficient in MPZ [245]. Lastly, as discussed in the next section on the treatment of connexin 32-associated neuropathy, reducing cytokine-activated macrophages and low-grade inflammation in P0Het mice (a model mimicking a heterozygous P0 loss-of-function mutation in humans) using a colony-stimulating factor 1 (CSF-1) receptor kinase inhibitor led to improved preservation of myelin, increased muscle action

potential amplitudes, improved nerve conduction velocities, and improved muscle strength [246]. This suggests a potential role for immunomodulation of the secondary inflammatory response seen in some genetic demyelinating neuropathies.

Connexin 32-Associated Neuropathy: Epidemiology and Clinical Features

CMT1X, the most common form of X-linked CMT, is caused by mutations in *GJB1*, which encodes the gap junction protein connexin 32 (Cx32) [247]. CMT1X represents between 10 and 15% of CMT cases with a defined molecular diagnosis, and 5 to 10% of all CMT cases [9, 14, 248]. Over 400 mutations in *GJB1* have been linked to CMT1X, and they span across all domains of Cx32. Most mutations are missense variants and are believed to cause predominantly loss of function phenotypes [249]. Interestingly, several variants in the non-coding regions of *GJB1* have been demonstrated to cause CMT1X [250–252], at least in some cases due to abnormal splicing of *GJB1* [253]. Furthermore, copy number variations in *GJB1* have also been identified in patients with CMT1X [254, 255]. Therefore, care should be taken when interpreting results from commercial genetic testing, as non-coding variants as well as copy number variations may be missed. Of note, there is no specific correlation between phenotype and specific *GJB1* mutations [256].

As in other dominant X-linked diseases, male patients with CMT1X present with a more severe phenotype, and women are usually only mildly affected; however, moderately to severely affected female patients with CMT1X are seen in approximately one-third of cases as a consequence of skewed X-inactivation of the nonmutated allele [257]. Most men will have symptoms in childhood, though about 20% have a later age of onset [9]. Clinically, CMT1X has distinctive features when compared to other demyelinating CMT subtypes. A split hand syndrome (abductor pollicis brevis more wasted and weaker than the first dorsal interosseous) can often be observed, as well as marked atrophy of all compartments of the calf muscles. Asymmetrical (non-uniform) slowing of nerve conduction velocities, with conduction block and temporal dispersion, which are characteristic of true segmental demyelination and also seen in hereditary neuropathy with liability to pressure palsy (HNPP), CMT4J, HSAN1C, and acquired inflammatory neuropathies, may be found in patients with missense mutations in *GJB1*, leading to misdiagnosis as an inflammatory neuropathy and unnecessary immunosuppressive treatment [258, 259]. CMT1X is also a common cause of intermediate nerve conduction velocities, with men usually presenting motor nerve conduction velocities (MNCV) between 25 and 45 m/s and women usually having MNCV greater than 35 m/s [9].

Another unique feature of CMT1X, predominantly in men, is the occurrence of transient stroke-like episodes with MRI changes following stressors, such as infection or fever, travel to high altitude, and intensive exercise [260].

Biological Models of Connexin 32 Neuropathy

Based on the hypothesis that most cases of CMT1X are due to loss of function of connexin 32, several of the early studies addressing the biology of CMT1X used mice knockdown for *Gjb1* (*Gjb1*^{−/−} or Cx32 null mice) [261]. As genome editing technology advanced, several knock-in *Gjb1* mice models have been created including the R142W [262], T55I, R75W, and N175D [263], which allowed investigators to study the trafficking properties of these different Cx32 mutants. Other model systems used in mechanistic studies of CMT1X include *Xenopus* oocytes [264] and N2A cells [265], which are used to evaluate the expression level and biophysical parameters of mutant forms of Cx32 in regard to their ability to form functional gap junctions. HeLa cells co-expressing wild type and mutant Cx32 have been used to study trafficking and interactions between different mutant forms of Cx32 [266].

Connexin 32 Biology and Pathomechanisms

Connexins are a group of membrane-spanning proteins that interact to form gap junction channels, allowing for the passage of ions and small molecules between cellular membranes. In the peripheral nervous system, Cx32 is found in the paranodal myelin loops and Schmidt-Lanterman incisures of myelinating Schwann cells where they form hexameric hemichannels. The docking of two hemichannels forms intracellular gap junctions between folds of Schwann cell cytoplasm, allowing the transfer of ions and molecules across the span of this highly polarized cell.

Abrams et al. demonstrated in paired *Xenopus* oocytes expressing seven distinct CMT1X-associated Cx32 mutants (G12S, R15Q, R15W, S85C, H94Q, H94Y, and V139M) that all mutants resulted in reduced or no conductance across the resulting gap junctions, albeit through different biophysical mechanisms. The authors concluded that a large number of CMT1X are due to loss of function of Cx32 [264]. Using a similar approach in N2A cells, Wang and colleagues evaluated 22 CMT1X mutant Cx32 proteins for their ability to traffic to the cell membrane and form functional channels. Ten mutant Cx32 proteins either assembled dysfunctional junctional channels (Y65C, V95M, R107W, L156R, R164W, and G199R) or failed to form gap junctions (G12S, S182T, E208K, and Y211stop). Most mutant proteins were localized

in the cell membrane despite their impaired ability to form functional gap junctions. Interestingly, 12 CMT1X mutants (V13L, R15Q, R22Q, I30N, V35M, V63I, R75Q, Q80R, W133R, P158A, P172S, and N205S) did not affect the ability of Cx32 to form homotypic gap junctions, suggesting that other mechanisms besides impaired gap junction formation should play a role in CMT1X. Abrams et al. proposed a possible mechanism to explain this discrepancy when studying the S85C Cx32 mutant [267]. This mutant Cx32 protein forms functional cell–cell channels in paired *Xenopus* oocytes but have a higher open probability compared to wild-type Cx32. Open hemichannels may render Schwann cells exposed to increased influx of calcium and loss of ionic gradients and metabolites, which can be damaging to the cells. Interestingly, the same group demonstrated differences in the ability of a Cx32 mutant to form gap junctions and produce at least some degree of junctional coupling that may determine whether a CMT1X patient is at risk of presenting central nervous system (CNS) manifestations. By comparing 10 Cx32 mutations associated with CNS involvement with 4 “neuropathy-exclusive” mutations, Abrams et al. found that all 10 CNS mutations formed no morphological gap junction plaques or, if they did, produced little or no detectable junctional coupling. In contrast, all four neuropathy mutations formed gap junction plaques and produced levels of junctional coupling similar to those for wild-type Cx32 [268].

Mutant Cx32 proteins also differ in their trafficking and subcellular localization. Yum et al. investigated the distribution of several mutant Cx32 proteins in HeLa cells and demonstrated preferential subcellular localization including the endoplasmic reticulum (M34K, N205I, and Y211x), the Golgi apparatus without reaching the cell membrane (M34T, V38M, A40V, R75Q, R75P, R75W, and C217x), the Golgi apparatus but also forming rare small gap junction-like plaques (M34I, M34V, and V37M), or mainly on the cell membrane, forming gap junction-like plaques (V35M, I213V, R219C, R219H, R220G, R230C, R230L, R238H, L239I, and S281x) [269]. They confirmed their findings in cultured rat Schwann cells for some of the mutants. Similar differences in trafficking were also identified by Jeng et al. [262] and Matsuyama et al. [270]. Kyriakoudi and colleagues also demonstrated using HeLa cells co-expressing wild-type and different mutant Cx32 proteins, that Golgi-retained mutants hinder gap junction assembly by wild type Cx32. Confirming these findings, *in vivo* intraneural delivery of wild-type Cx32 in mice bearing a Golgi-retained mutant (R75W) did not traffic normally, while the same virally delivered protein was correctly localized in mice expressing an endoplasmic reticulum-retained mutant (T55I). This work suggests that patients with CMT1X may respond differently to gene replacement therapy depending on their Cx32 mutation [266]. It is important to mention that although useful as a research tool to catalogue the different Cx32 mutations,

impaired gap junction gating only explains a limited portion of the CMT1X pathogenesis and has not been correlated well with clinical disability. Therefore, care should be taken when using this parameter to predict disease severity or potential treatment response.

A possible second mechanism involved in the pathophysiology of CMT1X is the role of immune-mediated damage to Cx32 mutant peripheral nerves. Kobsar and colleagues identified an age-related increase in the number of macrophages in demyelinating nerves of Cx32-null mice [271] and were able to reduce endoneurial macrophages and both myelin and axonal degeneration by cross breeding Cx32-null mice with mice deficient for the recombination activating gene-1 (RAG-1), which lack mature T and B lymphocytes [272]. Groh et al. further demonstrated the connection between macrophages and peripheral nerve damage in CMT1X by cross breeding Cx32 null mice with monocyte chemoattractant protein-1 (MCP-1) knockout mice [273]. MCP-1 is a chemokine involved in the recruitment of macrophages to the peripheral nerves and has been shown to mediate macrophage-related neural damage in other models of inherited neuropathies [274]. The resulting double knockout mice still displayed increased endoneurial macrophages due to compensatory proliferation of resident macrophages. However, heterozygous deletion of MCP-1 led to reduced numbers of phagocytosing macrophages, transient improvement in myelination, and persistent improvement of axonal degeneration, with robust axonal sprouting lasting up to 12 months. This improvement in nerve pathology also translated into improved electrophysiological parameters, reduced muscle denervation and atrophy, and increased muscle strength. This study also implicated the MEK-ERK signaling pathway as mediating MCP-1 expression in Cx32-deficient Schwann cells. The authors concluded that preventing MCP-1 upregulation by inhibiting ERK phosphorylation may be a promising approach to treat CMT1X [273]. Groh et al. also explored the role of macrophage activation and low-grade inflammation in the dedifferentiation of Schwann cells, a typical feature of nerve fiber damage associated with several forms of demyelinating CMT. The authors determined that dedifferentiation of Cx32-deficient Schwann cells was strictly dependent on macrophage activation by the fibroblast-borne cytokine colony-stimulating factor-1 (CSF-1), as CSF1/Cx32 double knockout Schwann cells demonstrated improvement in myelin preservation and did not upregulate dedifferentiation markers NCAM and L1. Importantly, this effect of CSF-1 was independent of the ERK signaling pathway [275, 276]. Taken together, these results provided proof of concept for the role of a secondary immune-mediated damage leading to Schwann cell dedifferentiation as part of the pathophysiology of CMT1X.

Cx32 mutations have been shown to cause polyploidy and an increase in nuclear volume due to mitotic instability

causing centrosome overduplication [277]. This abnormal mitotic activity has been shown to be mediated through increased CamKII (Ca^{2+} /calmodulin-dependent protein kinase II) activity and to result in perturbation in cell division of cell lines from transgenic CMT1X mice [278]. Abnormal Schwann cell division has been hypothesized to cause defects in myelination in experimental models as well as in patient nerves. However, further work in patient cells is needed to confirm this mechanism is also present in the human disease.

Therapeutic Targets in Connexin 32-Associated Neuropathy (Table 4 and Fig. 3)

Gene Replacement Therapy for Connexin 32-Associated Neuropathy

As loss of function is considered to be the main disease mechanism in CMT1X, gene replacement therapy has been in the forefront of therapy development for Cx32-related CMT. Accordingly, Sargiannidou and colleagues first demonstrated delivery of *GJB1* under a Schwann cell-specific promoter (myelin protein zero promoter) by direct lentiviral intraneural injection in Cx32 null mice resulted in expression of the construct throughout the length of the sciatic nerve in up to 50% of Schwann cells starting 2 weeks after injection and remaining stable for up to 16 weeks as measured by eGFP expression. Cx32 expression was detected at the expected physiologic locations (non-compact myelin of the paranodal and Schmidt-Lanterman incisures) and resulted in the formation of gap junctions. Of note, a gene therapy trial in 2-month-old Cx32 null mice significantly improved myelination and reduced secondary inflammation at 6 months of age when compared to control, mock-treated animals [279]. Following up on these results, Kagiava and colleagues investigated the therapeutic effect of intrathecal delivery of the same lentiviral vector through a single lumbar injection in Cx32-null mice. A similar, widespread, and stable expression of Cx32 was observed in up to 50% of

Schwann cells in multiple lumbar spinal roots and peripheral nerves. Importantly, this study demonstrated not only pathological rescue, but also behavioral improvement, with treated mice showing significantly improved motor performance, quadriceps muscle contractility, and sciatic nerve motor conduction velocities. As observed for the Cx32 mice treated with the intraneural injection, treated mice also exhibited reduced numbers of demyelinated and remyelinated fibers and fewer inflammatory cells in lumbar motor roots, as well as in the femoral motor and sciatic nerves [280].

To answer whether different Cx32 mutants would respond differently to gene replacement therapy, a question was raised by the identification of differentially trafficked Cx32 mutants causing dominant-negative effects by direct interactions with WT Cx32 (266, 269); Kagiava and colleagues treated transgenic Cx32 KO mice expressing the T55I (ER-retained), R75W, or N175D (Golgi-retained) CMT1X mutations. Following intrathecal delivery of the *GJB1* gene, virally delivered wild-type (WT) Cx32 was observed in non-compact myelin of T55I KO mice, but only rarely in N175D KO or R75W KO mice, confirming the dominant-negative effects of the Golgi-retained mutants. *GJB1*-treated T55I mice also showed behavioral and pathological improvement with better motor performance, lower ratios of abnormally myelinated fibers, and a reduction of inflammatory cells in spinal roots and peripheral nerves compared with mock-treated littermates. For the Golgi-retained mutants, only partial (N175D) or no (R75W) improvement was observed. The authors concluded that certain CMT1X mutants may interfere with gene therapy for CMT1X, and further studies will be needed to determine the best strategy to treat patients who carry such mutations. Recently, Kagiava et al. moved their work closer to future human application by using adeno-associated virus (AAV) as their vector system. AAV are not known to cause human disease and have been the delivery system of choice for several FDA-approved gene replacement therapies and for ongoing clinical trials. Using AAV-9 to deliver a similar *GJB1*/Cx32 gene under the myelin protein zero (Mpz) promoter for targeted expression in Schwann cells, the authors observed widespread distribution in the peripheral nervous system, including lumbar

Table 4 Treatment strategies in *GJB1*/Cx32-associated neuropathies

Treatment	Mechanism	Model	Reference
<i>GJB1</i> /Cx32 gene delivery	Connexin 32 expression	Cx32 null mice	Sargiannidou (2015), Kagiava (2016), Kagiava (2019)
PLX5622	CSF-1 receptor kinase inhibitor	Cx32 null mice	Klein (2015)
KN 93	Ca^{2+} /calmodulin-dependent protein kinase II (CamKII)	Transgenic <i>GJB1</i> mice	Mones (2014)
AAV1.NT-3 gene therapy	NT-3 secretion	Cx32 null mice	Ozes (2021)

GLB1 Gap junction beta-1, *Cx32* connexin 32, *CSF-1* colony-stimulating factor 1, *AAV1* adeno-associated virus 1, *NT-3* neurotrophin-3

roots, sciatic, and femoral nerves after intrathecal lumbar injection. This gene replacement strategy was successful in rescuing the normal Cx32 expression in non-compact myelin and improved behavioral, neurophysiological, and pathological features of both pre- and post-onset-treated Cx32-null mice [281]. Of note, neurofilament light chain (NEFL), a biomarker of axonal degeneration, also improved in response to gene replacement therapy. These results were in keeping with a similar study using their lentiviral vector and suggest that gene replacement therapy may be beneficial even in patients with established disease [282].

Immune Regulation as a Treatment Strategy for Connexin 32-Associated Neuropathy

Cross breeding of Cx32-null mice with mice deficient for MCP1 or CSF-1 has demonstrated the role of cytokine-activated macrophages and low-grade inflammation in Schwann cell dedifferentiation and demyelination in CMT1X and provided a target for therapy development for connexin 32-associated neuropathy [273, 275, 276]. Klein et al. targeted this pathway by orally administering a CSF-1 receptor kinase inhibitor (PLX5622) to Cx32-null mice [246]. CSF-1 receptors are expressed by macrophages and activated by fibroblast-released CSF-1. PLX5622 led to a significant reduction in nerve macrophage numbers and fewer abnormal axons while increasing the number of axonal sprouts (an indication of axonal reinnervation). PLX5622 treatment also increased hindlimb grip strength and compound muscle action potential amplitudes in the intrinsic foot muscles. Interestingly, this approach did not change the number of abnormally myelinated axons [246].

Ca²⁺/Calmodulin-Dependent Protein Kinase II (CamKII) Inhibitor to Treat Mitotic Instability in Connexin 32-Associated Neuropathy

Transgenic mice expressing CMT1X-associated Cx32 mutants displayed evidence of mitotic instability, such as polyploidy, nuclear volume, and centrosome overduplication likely due to overexpression of CamKII. Mones et al. used a CamKII inhibitor, KN 93, to treat transgenic GJB1 mice carrying the G12S or S26L mutations [277]. KN 93 treatment resulted in slower progression of muscle weakness as determined by rotarod analysis, as well as improvement in mitotic instability in fibroblasts derived from the transgenic mice [278].

Adeno-Associated Virus/Neurotrophin-3 Gene Therapy for CMT1X

Similar to work done for CMT1A, Ozes and colleagues treated Cx32 null mice using a scAAV1.tMCK.NT-3 vector to induce expression of neurotrophin-3 (NT-3).

Intramuscular delivery of NT-3 in 3-month-old Cx32 null mice resulted in significant improvement in myelin thickness, nerve conduction velocity, and muscle fiber diameter, with normalized compound muscle action potential amplitudes, and no functional decline at 9 months of age [283]. An increase in the number of Schmidt-Lanterman incisures was also observed, although the functional meaning of this finding is uncertain.

Pathomechanisms and Emerging Treatments in Select Forms of AR Demyelinating CMT (Table 5 and Fig. 3)

The most prevalent of the recessively inherited demyelinating CMTs is CMT4C, which results from loss of function mutations in the *SH3TC2* gene on chromosome 5q32. Over 100 pathogenic mutations have been identified in *SH3TC2* to date [14, 284, 285]. In addition to the typical features seen with CMT, SH3TC2 neuropathy is distinguished by the presence of scoliosis and cranial neuropathies (manifesting as hearing loss, sluggish pupils, and tongue fasciculations) [285–289]. Nerve conduction studies reflect demyelinating neuropathy, and nerve biopsy reveals an increase in basal membranes around both myelinated and unmyelinated axons, as well as characteristic large cytoplasmic Schwann cell extensions [290–292]. The *Sh3tc2* $-/-$ mouse model recapitulates the clinical phenotype of progressive hypomyelinating and demyelinating polyneuropathy [293, 294].

The SH3TC2 protein contains two Src homology 3 (SH3) and 10 tetratricopeptide repeat (TPR) domains, and based on observations in the *Sh3tc2* $-/-$ mouse model, it is believed to serve as a scaffolding protein and to play a role in the formation of the nodes of Ranvier [153, 292, 293]. In normal circumstances, SH3TC2 is involved in the endocytic pathway; however, mutations in *Sh3tc2* can lead to the mis-targeting of the protein away from endosomes [153, 295].

Schiza et al. developed a lentiviral vector under control of the *MPZ* promoter to drive the expression of *SH3TC2* cDNA in Schwann cells [296]. Intrathecal injection of the vector into *Sh3tc2* $-/-$ mice at 3 weeks of age resulted in effective localization of SH3TC2 to the Schwann cell cytoplasm and resulted in partial improvement of motor function and motor conduction velocities, as well as the numbers of myelinated fibers in the treated animals [296]. Treatment also resulted in reduced blood levels of neurofilament light chain, a biomarker of axonal degeneration that was previously evaluated in CMT [297]. This study serves as an important proof of principle for viral gene replacement therapy in *SH3TC2* though further work will be needed to ensure safety, while also optimizing gene expression.

Genes associated with endo-lysosomal membrane trafficking have been implicated in several recessive forms

Table 5 Treatment strategies in AR demyelinating neuropathies

Gene	Treatment	Mechanism	Model	Reference
FIG4	Ca ²⁺ (Calcium) chelation	Reduction of abnormally elevated intracellular Ca ²⁺	Plt (Fig4 ^{-/-}) mice	Hu (2018)
	TRPML1 synthetic ligand	Reduction of abnormally elevated intra-lysosomal Ca ²⁺	Plt (Fig4 ^{-/-}) mice culture cells	Zou (2015)
	AAV9-mediated FIG4 gene delivery	Restore FIG4 expression	Plt (ig4 ^{-/-}) mice	Presa (2021)
MTMR	Niaspan	NRG1 type III inhibition (by activating TACE secretase)	<i>Mtmr2</i> -null mice	Bolino (2016)
	PIKfyve kinase inhibitor (YM201636)	Reduction of PtdIns(3,5)P2 phospholipid levels	<i>Mtmr2</i> -null Schwann cell/DRG neuron co-cultures	Vaccari (2011)
SH3TC2	lentiviral vector LV-Mpz.SH3TC2.myc	Increase expression of the human SH3TC2 cDNA	Sh3tc2 ^{-/-} mice	Schiza (2019)

FIG4 FIG4 phosphoinositide 5-phosphatase, *TRPML1* transient receptor potential cation channel, mucolipin subfamily, *AAV9* adeno-associated virus 9, *MTMR* myotubularin-related protein, *NRG1* neuregulin1, *TACE* tumor necrosis factor- α converting enzyme

of CMT. Most of these genes are phosphatases involved in phosphoinositide metabolism and include the myotubularin-related (MTMR) protein family (*MTMR2*, 13 and 5) and *FIG4* [298–301].

MTMR2, *MTMR13*, and *MTMR5/SBF1*, the genetic causes of CMT4B1, CMT4B2, and CMT4B3, respectively, are associated with abnormal myelin formation characterized by myelin outfoldings, and redundant loops of myelin lamellae, which can be seen in patients' nerve and skin biopsies. Interestingly, while *MTMR2* encodes for an active phosphate, *MTMR13* and *MTMR5* express inactive enzymes (also known as a pseudophosphatases). Nonetheless, both *MTMR2* and *MTMR13* have been demonstrated to interfere with PI 3-kinase-Akt signaling pathway activation, causing dys/demyelination [302]. Experimental work on the biology of the MTMR protein family has demonstrated that this group of phosphatases regulates the metabolism of phosphoinositides, signaling molecules that regulate membrane trafficking by controlling membrane dynamics and vesicle movement, tethering, and fusion. These cellular processes are key mediators of axonal transport and myelination in the nervous system.

A recent multicenter, retrospective study of patients with MTMR-related neuropathy characterized the phenotype associated with this group of neuropathies in a total of 50 patients, including 26 with CMT4B1 (*MTMR2*), 19 with CMT4B2 (*MTMR13*), and 5 with CMT4B3 (*MTMR5*). CMT4B1 patients demonstrated a significantly more severe phenotype compared to CMT4B2, with earlier onset, motor milestone delay, wheelchair use, and respiratory involvement. Vocal cord involvement is a feature of both subtypes, but glaucoma occurred in CMT4B2 only [298, 303]. CMT4B3 patients demonstrated a distinct phenotype, with a slowly progressive, pure demyelinating neuropathy with myelin outfoldings and, in some cases, a syndromic

neuropathy characterized by axonal degeneration, multiple cranial nerve involvement, intellectual disability, microcephaly, and dysmorphic features. Nerve conduction velocities were similarly slowed in all subtypes [303].

The advances in understanding the biology of MTMR phosphatases have opened new therapy development avenues for this group of autosomal recessive neuropathies. Vaccari and colleagues demonstrated that inhibition of the kinase PIKfyve (also known as PIP5K3) by small molecule kinase inhibitors rebalances the levels of PtdIns (3,5) P2 phospholipids, which have been demonstrated to be elevated in the absence of *MTMR2* activity. The use of such kinase inhibitors was able to significantly reduce the occurrence of myelin outfoldings in vitro, in a co-culture system using dorsal root ganglia-derived neurons and Schwann cells from *Mtmr2*-null mice [304]. It is important to note that this approach relies on the precise dosing of PIKfyve as reduction of PtdIns(3,5)P2 phospholipids may be detrimental and cause severe toxicity.

A second approach focuses on the neuregulin pathway, one of the main signaling pathways regulating Axon-Schwann cell interactions, Schwann cell development, and myelination [305]. Neuregulin 1 (NRG1) type III is expressed in the surface of axons and interacts with ErbB2/3 receptors in the Schwann cell membrane to activate the PI 3-kinase-Akt signaling pathway, which promotes myelination. Inhibition of the NRG1 type III signaling therefore should reduce the amount of abnormal myelin formed in MTMR-related neuropathies. For this purpose, Bolino et al. explored the use of tumor necrosis factor- α converting enzyme (TACE), a negative regulator of the NRG1 type III pathway, as a treatment strategy for MTMR-associated neuropathies. Using Niacin-Niaspan, an FDA-approved TACE activator, the authors demonstrated reduction in NRG1 type III signaling as well as a reduction in myelin outfoldings in

the same co-culture system used to validate PIKfyve kinase inhibitors [306].

Another autosomal recessive CMT gene, *FIG4*, also encodes for a phosphatase involved in phosphoinositide metabolism. *FIG4* was first identified as a disease-associated gene in the spontaneously occurring “pale-tremor” (plt) mutant mice. These mice present with severe tremor, abnormal gait, peripheral neuropathy, and diluted pigmentation and were found to carry a transposon insertion in intron 18 of the mouse *FIG4* gene, resulting in a loss-of-function allele. Loss of *FIG4* function reduces the intracellular concentration of phosphoinositol(3,5)P₂- [PtdIns(3,5)P₂], leading to impaired vacuole fission, formation of enlarged vacuoles, and reduced retrograde traffic to the late endosome [301]. The *FIG4* protein is a [PtdIns(3,5)P₂] phosphatase, which forms a complex with other enzymes that generate PI(3,5)P₂, including PIK-FYVE and VAC14. Loss of *FIG4* causes instability of this complex, resulting in the mislocalization of all three proteins and reduced levels of PI (3,5) P₂ [307].

Clinically, CMT4J is characterized by a heterogeneous phenotype with significant variability in age of onset and severity of symptoms, ranging from mild symptoms to wheelchair use. Unlike the typical length-dependent and symmetrical presentation of most CMT subtypes, CMT4J can present with both proximal and distal weakness as well as marked asymmetry in clinical and electrophysiological findings [308, 309]. Sensory motor demyelinating polyneuropathy was consistently found in all patients studied by Hu et al. [310] and was associated with non-uniform slowing of conduction velocities, conduction block, and temporal dispersion on nerve conduction studies. These features sometimes cause diagnostic error, with CMT4J patients being frequently misdiagnosed as having acquired inflammatory neuropathies or motor neuron disease. From a genetic perspective, affected individuals are compound heterozygotes carrying the missense allele *FIG4*^{I41T} in combination with a null allele. Lenk et al. demonstrated, in a mouse model of CMT4J expressing a *Fig4*^{I41T} cDNA transgene on *Fig4* null background, that *FIG4*^{I41T} is a hypomorphic allele encoding a protein that is unstable in vivo. Expression of *FIG4*^{I41T} protein at 10% of normal level was shown to be enough to rescue the mouse phenotype, suggesting that increasing the levels of *FIG4*^{I41T}, by increasing its production or stability, should be a goal of therapies for CMT4J [307].

This hypothesis was recently pursued by Presa et al., by treating pale tremor (plt) mice with a single-stranded AAV9 to deliver a codon-optimized human *FIG4* sequence. Compared to untreated plt mice, which have a median survival of approximately 5 weeks, mice treated at postnatal day 1 or 4 survived at least 1 year, with significant rescue of their clinical, electrophysiological, and pathological phenotypes. AAV9 treatment at postnatal day 7 or 11 still increased life span, albeit less than the earlier treatment, and the clinical

phenotype was only partially rescued. These results are in keeping with findings from other gene replacement therapies for neurodegenerative genetic disorders, where the earlier the treatment, the better the overall treatment response [311].

Another mechanism identified in the pathophysiology of CMT4J is abnormally reduced lysosomal calcium efflux resulting from impairment in the calcium channel TRPML1. PI(3,5)P₂ is an endogenous ligand of TRPML1, and its deficiency in CMT4J leads to deactivation of TRPML1. The consequent reduced efflux of lysosomal calcium leads to its accumulation inside the organelle, impairing lysosome fission and leading to the enlarged vacuoles seen in fibroblasts, neurons, and Schwann cells of patients with CMT4J [301, 308, 312]. This mechanism was explored by Zou et al. by treating plt mice with a synthetic ligand of TRPML1, ML-SA1, to activate this calcium channel and reduce the intralysosomal calcium level. This pharmacological intervention rescued abnormal lysosomal storage in plt mice culture cells and ex vivo DRGs [313].

Abnormal calcium accumulation was also observed in Schwann cells of plt mice, leading to overexpression of c-Jun, a negative regulator of myelination, and consequent Schwann cell de-differentiation and segmental demyelination. Of note, chelation of calcium using BAPTA reduced dedifferentiation and demyelination of Schwann cells in vitro and in vivo, providing another treatment strategy for *FIG4*-related CMT [310].

Looking Towards the Future: the Evolving Arena of Clinical Outcome Assessments and Biomarkers in Demyelinating CMT

As disease-modifying treatments for select forms of demyelinating CMT become increasingly plausible, it is critical to address the current barriers to successful clinical trials. Most forms of CMT progress slowly and can plateau, or even improve, over the course of the disease, posing a challenge in developing clinical outcome assessments sensitive enough to capture disease progression. Clinical trials evaluating ascorbic acid in CMT1A have also highlighted the limited responsiveness of the Charcot-Marie-Tooth Neuropathy Score (CMTNS), the main clinical outcome assessment in CMT, and revealed a positive placebo effect [131, 314]. The CMNTS was subsequently revised and modified using Rasch analysis, with a resulting increase in sensitivity [315, 316]; however, its use continues to require very large sample sizes to adequately assess therapeutic benefit [317]. The development of new instruments to assess progression in CMT has therefore become an active area of research. Given that the optimal time for the treatment in CMT is in infancy or early childhood, several early-life clinical outcome assessments (including the CMT pediatric, CMT infant scales, and

a pediatric CMT-specific quality of life outcome measure) have been developed [318–320].

Rather than using a single optimized measure of disease progression, clinical trials in CMT will need to employ a composite of rigorously examined and validated clinical outcomes assessments and biomarkers. Encouragingly, quantitative calf muscle fat accumulation on MRI has proven to be a highly sensitive measure of progression in patients with CMT1A, with a notable increase in fat fraction observed over a 12-month period [321, 322]. MRI is now being increasingly employed in natural history studies of CMT and will play an important role in clinical trials. It is worth emphasizing, however, that in assessing treatment response, MRI will still have to be used alongside optimized CMT-specific functional outcomes.

In regard to plasma biomarkers, Sandelius et al. have demonstrated elevations in the concentration of neurofilament light (NfL) chain (a marker of axonal damage) in patients with two forms of demyelinating CMT (CMT1A and CMT1X) [297]. Additionally, Wang et al. highlighted the TMPRSS5 protein (Transmembrane protease serine 5) as a potential Schwann cell-specific biomarker in patients with CMT1A, with a greater than twofold increase seen in the CMT1A patients as compared to controls [323].

Skin biopsy has also offered promise as a biomarker in CMT, both in regard to assessing neuropathy progression and as a method for examining gene-specific targets [324, 325]. As previously discussed, evaluation of PMP-22 in skin biopsy to test the effect of PMP-22 lowering therapies has proven effective in animal models but showed variable results in human studies as reviewed by Pantera et al. [55]. Interestingly, the authors demonstrate that employing digital methods of PMP-22 analysis and normalizing skin PMP-22 levels to Schwann cell-specific genes may offer more consistency going forward; however, the inter-patient variability in PMP-22 levels remains an ongoing challenge [55].

In summary, the biology underlying demyelinating forms of CMT is complex and varied, and unifying therapeutic approaches are unlikely to be feasible. Rather, treatments will need to be developed with attention not just to gene, but also to allele-specific pathomechanisms. While important challenges lie ahead, this is undoubtedly a very exciting time in CMT research, as years of biological investigation converge towards the development of novel and increasingly attainable disease modifying treatments for several demyelinating CMT subtypes.

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