

Review

Defects in Axonal Transport in Inherited Neuropathies

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Abstract. Axonal transport is a highly complex process essential for sustaining proper neuronal functioning. Disturbances can result in an altered neuronal homeostasis, aggregation of cargoes, and ultimately a dying-back degeneration of neurons. The impact of dysfunction in axonal transport is shown by genetic defects in key proteins causing a broad spectrum of neurodegenerative diseases, including inherited peripheral neuropathies. In this review, we provide an overview of the cytoskeletal components, molecular motors and adaptor proteins involved in axonal transport mechanisms and their implication in neuronal functioning. In addition, we discuss the involvement of axonal transport dysfunction in neurodegenerative diseases with a particular focus on inherited peripheral neuropathies. Lastly, we address some recent scientific advances most notably in therapeutic strategies employed in the area of axonal transport, patient-derived iPSC models, *in vivo* animal models, antisense-oligonucleotide treatments, and novel chemical compounds.

Keywords: Axonal transport, inherited peripheral neuropathies, Charcot-Marie-Tooth disease, genetics, neurodegeneration, cytoskeleton, molecular motors, cargoes, therapeutics

COMMON ABBREVIATIONS

AF actin filament
ALS amyotrophic lateral sclerosis
CMT Charcot-Marie-Tooth
dHMN distal hereditary motor neuropathy
ER endoplasmic reticulum

HDAC6 histone deacetylase 6
HSAN hereditary sensory and autonomic neuropathy
HSP hereditary spastic paraplegia
IPN inherited peripheral neuropathies
iPSC induced pluripotent stem cell
MIM mitochondrial inner membrane
MOM mitochondrial outer membrane
MT microtubule
MTA microtubule-targeting agents
NEFL neurofilament light
NEFM neurofilament medium
NEFH neurofilament heavy

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| NF | neurofilament |
| NMJ | neuromuscular junction |
| PS | Perry syndrome |
| PTM | post-translational modifications |
| RBP | RNA-binding protein |
| RNP | ribonucleoprotein particles |
| <i>SARM1</i> | Sterile Alpha and TIR Motif Containing 1 |
| sHSP | small heat shock protein |
| SMA | spinal muscular atrophy |
| SMALED | spinal muscular atrophy with lower limb predominance |

Axonal transport is a highly complex process allowing movement of molecules and organelles within neurons over tremendous distances towards presynaptic nerve termini (anterograde transport), and transferring material from the periphery back to the neuronal soma (retrograde transport). This intracellular trafficking is crucial for sustaining proper neuronal functioning as well as clearing misfolded proteins or damaged organelles to avoid accumulation of harmful aggregates. Neuronal homeostasis depends on an efficient axonal transport allowing neuronal growth (axonal outgrowth), repair and regeneration upon injury, endocytosis and exocytosis of large and small molecules. The complex morphology and length (which can reach over one meter) of neurons make them particularly vulnerable to changes in axonal trafficking. Transport of a molecule or organelle (cargo) within the axon implies its recognition and binding to motor proteins, followed by an ATP-dependent movement of the motors along the cytoskeleton, direction of the cargo to the correct destination and release of the cargo upon reaching its destination. Axonal transport is regulated at all of these stages in a number of different ways. Impaired axonal transport can result in an altered neuronal homeostasis, aggregation of cargoes, and ultimately a dying-back degeneration of neurons.

Here we review the cytoskeletal components, molecular motors and adaptor proteins involved in axonal transport mechanisms and their implication in neuronal functioning. Furthermore, we discuss how genetic mutations, associated with inherited peripheral neuropathies (IPN), impact on axonal transport. Finally, we focus on some of the underlying mechanisms of axonal transport disturbances causing neurodegeneration and highlight future therapeutic prospects.

CYTOSKELETAL COMPONENTS OF AXONAL TRANSPORT

Neurons become post-mitotic cells in early development and need to remain functional for a lifetime, requiring a solid structural cytoskeleton. The neuronal cytoskeleton consists of microtubules (MTs), intermediate filaments and actin filaments (AFs), each having their own intracellular distribution (Fig. 1).

The MTs originate from the centrosome and are the major component of the neuronal cytoskeleton. MTs consist of α - and β -tubulin polymers that form polarized tubular structures, which are subjected to a dynamic process of polymerization and depolymerization [1]. Parallel MTs form unipolar arrays with the ‘minus-ends’ orientated towards the soma and the ‘plus-ends’ towards the axon, directing motor proteins in antero- or retrograde direction respectively. The tubulin polymers are involved in spatial organization and cell shape maintenance, as well as in many features of cytoplasmic structure, including organization of specific signaling pathways [1]. Microtubule-associated proteins, motor proteins, post-translational tubulin modifications and plus-end tracking proteins regulate MT dynamics.

The major intermediate filaments of neurons are neurofilaments (NFs), which control axon diameter and thereby axonal conductance [2]. Mature NFs contain neurofilament light (NEFL), medium (NEFM) and heavy (NEFH) chains. However, NFs in the peripheral nervous system can also contain peripherin. NF monomers share a common structural organization: a central α -helical rod domain, flanked by a N-terminal head, and a C-terminal tail domain [2]. In addition to their function in maintenance of the axonal architecture, NFs are fundamental to maintain Schwann cell-axon interactions, NF complex assembly and axonal transport [3].

The AFs consist of polymers of globular actin units and have a growing end (plus-end) where actin monomers are attached, while monomers are dissociated at the minus end. AF dynamics sustain a balance between polymerization and depolymerization and provide a structured cytoskeletal network for cell support, shape and migration. Within the cell, the function of AFs is regulated by a range of actin-binding proteins such as profilins and formins.

Disturbance or alteration of cytoskeleton stability and dynamics occurs in various neurodegenerative diseases, including IPN, and can be caused by mutations in the cytoskeletal proteins themselves, in

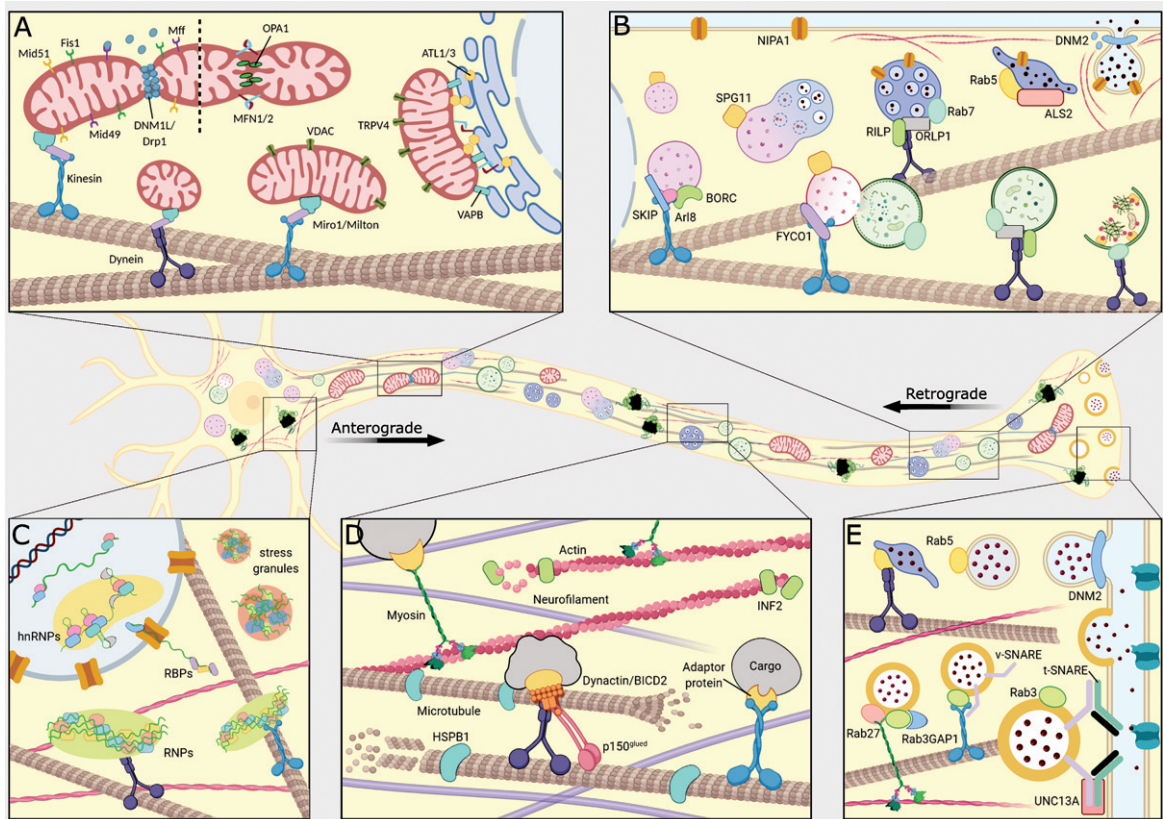


Fig. 1. Schematic overview of the peripheral nerve and the mechanisms directly involved in typical and pathogenic axonal transport in anterograde and retrograde direction. (A) Transport of mitochondria occurs along microtubules by dynein (purple) and kinesin (blue) motors. Mitochondria undergo a coordinated balance between mitochondrial fission and fusion. Mitochondria-ER tethering and mitochondrial Ca^{2+} flux are maintained to sustain proper axonal transport. (B) Formation of autophagosomes and endosomes with subsequent fusion with lysosomes. Anterograde transport of lysosomes and retrograde transport of endosomes and autophagosomes occurs along microtubules by kinesin and dynein motors, respectively. (C) mRNA are bound by RNA-binding proteins (RBPs) and heterogeneous nuclear ribonuclear proteins (hnRNPs), and subsequently formed into ribonucleotide particles (RNPs) capable of being transported by kinesin and dynein motors along the microtubules. Increased aggregation of RBPs causes formation of stress granules. (D) The neuronal cytoskeleton provides the tracks on which the molecular motors move to facilitate axonal transport. The dynein and kinesin motors move along the microtubules, whereas the myosin motors move along actin filaments. Both actin filaments and microtubules undergo dynamic polymerization and depolymerization mediated by adaptor proteins. (E) Synaptic vesicles are transported in anterograde direction by binding to kinesin motors, which move along microtubules. Fusion of synaptic vesicles at the nerve terminal is mediated by v-SNARE (purple) and t-SNARE (green) complexes. Here the t-SNARE complex is depicted as Syntaxin (light green) and SNAP25 (dark green).

their adaptor proteins or in other regulators. Mutations in *INF2*, encoding formin, cause autosomal dominant focal and segmental glomerulosclerosis (FSGS) as well as dominant intermediate Charcot-Marie-Tooth (CMT) type E [4, 5].

Mutations in tubulin genes are commonly referred to as *tubulinopathies*, such as mutations in β -tubulin known to cause polymicrogyria, congenital fibrosis of extraocular muscle (CFEOM) or malformation of cortical development. Mutations in *TUBB3* are not only associated with CFEOM, e.g. the *D417N* mutation can also cause a polyneuropathy by reducing kinesin localization to microtubule plus-ends and affecting the axonal transport. *TUBB3* mutations can

lead to microtubule overstabilization and increased depolymerization [6].

Mutations in *NEFL* were identified in several subtypes of axonal CMT (CMT2) neuropathies, with dominant mutations causing their aggregation and recessive mutations resulting in a loss-of-function [7–9]. *NEFL* mutations can target different domains in the protein affecting the NF assembly, e.g. the *Q333P* mutation leads to destabilization of coiled-coil interactions resulting in reduced self-interaction and dimerization of NFs [10]. A mutation located in the *NEFL* head domain, *P8R*, affects phosphorylation and destabilization of the NF complex resulting in NF aggregation [11]. Furthermore,

phosphorylation of the NEFL head domain not only regulates NF assembly and disassembly, but also its transport in the axon [11, 12]. Dominant mutations in *NEFH* can also cause CMT2 [7, 13]. All reported *NEFH* mutations are frameshifts leading to the translation of additional 3'UTR regions encoding a cryptic amyloidogenic element. This leads to toxic protein aggregation similar to mutational mechanisms in *NEFL* and *FUS*, which when recognized by the autophagic pathway in motor neurons, trigger caspase 3 activation resulting in apoptosis [7, 13].

Several small heat shock proteins (sHSP), HSPB1 and HSPB8 are associated with cytoskeletal abnormalities and neuromuscular dysfunction [14–16]. Dominant mutations in *HSPB1* are associated with CMT type 2F and distal hereditary motor neuropathy (dHMN) [16]. The *S135F* mutation results into a higher affinity of HSPB1 for α -tubulin and over-stabilization of MTs, and several HSPB1 mutants reduce α -tubulin acetylation, affecting axonal transport [16]. *HSPB1* mutations can also affect the assembly and transport of NFs [17]. Moreover, spinal motor neurons, differentiated from patient-derived induced pluripotent stem cells (iPSC), carrying *HSPB1* mutations showed a significant reduction of the mitochondrial mobility along the axons [18].

MOLECULAR MOTORS AND ADAPTORS IN AXONAL TRANSPORT

Neurons depend on efficient transport of cargoes, which is enforced by several different classes of molecular motor proteins (Fig. 1) [19]. Microtubule based molecular motors are grouped into the kinesins (for anterograde transport) and cytoplasmic dyneins (for retrograde transport) [19]. The kinesin family consists of 45 genes classified into 15 subfamilies of which the kinesin-1, kinesin-2 and kinesin-3 families contribute to axonal transport [20]. Kinesin motor domains homo- and heterodimerize, and bind additional kinesin light chains forming the kinesin motor complex [20]. The dynein family is much smaller, and the main component -dynein heavy chain- is encoded by only one gene (*DYNC1H1*). Dynein functions as a complex consisting of two dynein heavy chains that dimerize at their tail domains. Additional dynein intermediate chains and dynein light chains will bind to these tails by forming a cargo-binding domain [20].

Many axonal cargoes have multiple motor types that can bind simultaneously, even cargoes that move steadily in either antero- or retrograde direction [21,

22]. This shows that molecular motors do not function independently from each other when pulling an organelle in opposite directions. Rather they are co-dependent, as shown by impairment of minus-end motors resulting in severe suppression of plus-end motility [22]. This simultaneous application of opposite forces does not necessarily result in stalled cargoes. Instead, it likely provides additional support for transport coordination to overcome mechanical obstacles on MT tracks [21].

The third type of molecular motors are myosins, specifically involved in the transport over actin filaments [19]. Mammals have 40 myosin genes [19]. The most essential differences are found in the C-terminal globular tail domain, which recognizes various cargoes through direct interactions or via adaptor proteins [19]. For instance, Myosin Va is able to interact with kinesin heavy chains, with MTs through its tail domain and with NEFL through its head motor domain. Doing so, Myosin Va plays a crucial role in coupling microtubule- and actin-based transport mechanisms and regulates distribution of cargoes across the cytoskeletal network [23, 24].

Post-translational modification (PTM) of cytoskeletal components includes polyglutamylation, polyglycylation, detyrosination, acetylation, phosphorylation and palmitoylation [1]. For MTs, these preferentially occur on tubulin subunits already incorporated into microtubules. Molecular motors can recognize different PTMs as signature for their recruitment; e.g. polyglutamylation recruits the kinesin-3 family member KIF1A motor, whereas tyrosination recruits kinesin-1 family member KIF5 motor proteins [25, 26]. Additionally, protein kinases regulate axonal transport through direct phosphorylation of motors, adaptors and cargoes, or protein kinases can phosphorylate several factors involved in the regulation of microtubule stability [27].

Dynactin is the best known adaptor protein of the cytoplasmic dynein complex. Dynactin-dynein interaction expands the range of cargoes that dynein can move, and increases the dynein motor processivity. Independently of cytoplasmic dynein, dynactin can anchor microtubules at the centrosome. The bicaudal D homologue (BICD) proteins represent another group of activating adaptors. In mammals, there are two BICD proteins, BICD1 and BICD2, as well as two related proteins, BICDR1 and BICDR2. The BICD functioning is diverse; BICD2 mostly functions in a complex of one dynein and one dynactin, whereas BICDR1 can recruit two dynein dimers to

a single dynein, further enhancing the force and velocity of the motor complex [28–30]. BICD2 also interacts with the dynein heavy chain and dynein, enhancing the affinity of the dynein–dynein interaction. Another class of microtubule adaptors are the Hook proteins, which are involved in motor–microtubule interactions as well as in cargo binding [31]. However, mutations in Hook proteins have so far not been associated with IPN or other neurodegenerative diseases.

Where the cytoskeleton functions as the tracks, the molecular motors are equally important for proper axonal transport. Disturbances in these motor complexes and their adaptors are a recognized causal mechanism for IPN and related diseases. Dominant mutations in dynein heavy chain (*DYNC1H1*) are linked to several distinct neurodegenerative phenotypes, e.g. CMT type 2O, spinal muscular atrophy with lower limb predominance (SMALED), hereditary spastic paraplegia (HSP) and intellectual disability with neuronal migration defects (MRD13) [reviewed in [32]].

Several dynein adaptors and modulators are associated with neurological disease; dynein (*DCTN1*), Huntingtin (*HTT*), *LIS1*, *BICD1* and *BICD2*. Mutations in *DCTN1* can cause Perry Syndrome (PS) and dHMN, and susceptibility to develop amyotrophic lateral sclerosis (ALS) [33–35]. *DCTN1* mutations found in PS patients affect amino acid residues within or immediately adjacent to the p150^{glued} CAP-Gly GKNMG motif of dynein. A G59S variant in *DCTN1* causing HMN occurs centrally in the p150^{glued} CAP-Gly domain. However, both PS and HMN associated variants induce a modest decrease in MT binding. Cells transfected with *DCTN1* mutants have a dramatic redistribution of dynein and more p150^{glued} aggregates [34, 35]. It remains unclear why mutations localized in different part of the *DCTN1* sequence manifest such different disease phenotypes [34, 35].

BICD2 mutations cause a spectrum of phenotypes including SMALED, HSP, and distal myopathy [36–40]. *BICD2* joins dynein and dynein in a motor protein complex capable of processive movement. Modeling of SMALED-*BICD2* mutations in cells showed an increased retrograde transport, suggesting that an imbalance of anterograde and retrograde dynein motor complex motility could be the underlying pathomechanism for these mutations [41, 42]. In addition, *BICD2* has been implicated in Golgi functioning as the *BICD2* coiled-coil (CC) structure allows interaction with the small GTPase RAB6A

located at the Golgi apparatus and fragmentation of the Golgi apparatus has been shown in *BICD2*-patient fibroblasts [43].

Mutations in the other major group of molecular motors (kinesins) can also give rise to a spectrum of neurodegenerative diseases. Variants in *KIF1A* have been associated with HSP, hereditary sensory and autonomic neuropathy (HSAN) and complex phenotypes (combining HSP, HSAN and ataxia) [44, 45]. *KIF5A* variants can cause CMT2, HSP and ALS [46–49]. Genotype/phenotype correlations revealed that the site of *KIF5A* mutations determine the clinical phenotype. Mutations in the kinesin motor or neck domain cause CMT2 and HSP. Mutations affecting splicing (exon 27) at the C-terminus cause atypical ALS with an earlier onset and a slower disease progression [46–49]. *KIF5A* mutations in HSP alter processivity and directionality of kinesin by changing order–disorder transition of the neck linker, or affecting ATPase activity/microtubule gliding. Both mechanisms can alter MT-dependent transport and net anterograde transport of cargoes [50–52]. *KIF1B* mutations were first implicated in CMT2A in 2001, but as only few patients were identified, the association has been controversial [37, 53, 54]. Functional evidence for *KIF1B* mutations in neuropathy phenotypes suggests that different mechanisms may be at play. The initially reported Q98L mutation resides in the conserved ATP-binding site and significantly reduced ATPase activity and perinuclear accumulation of the mutant *KIF1B* protein. Only recently a novel CMT2-associated Y1087C mutation in *KIF1B* was identified. It specifically impairs *KIF1B* binding capacity and transport of Insulin-like growth factor 1 receptor (IGF1R) down the axons affecting IGF-I/IGF1R signaling, which is essential for neuronal survival and axonal development [54].

CARGOES IN AXONAL TRANSPORT

Synaptic vesicles, NFs, and cytosolic proteins are cargoes transported in anterograde fashion. Signaling endosomes, autophagosomes and proteins involved in injury signaling are transported towards the cell body. Lastly, mitochondria, a variety of endosomes, lysosomes and mRNA are transported in a bi-directional manner [31]. Depending on the cargo and its destination, specific regulatory proteins and motor complexes are recruited (Fig. 1). While cargoes make use of different transport pathways with their specific components, there is clear interdependence between

these pathways and many of them converge [55]. In addition to the cargoes discussed below, axons also provide possibilities to transport viruses [reviewed in [56]].

mRNA and cytosolic proteins

Axons constitute the connections between neurons and their targets allowing them to communicate and respond to environmental stimuli. A large and diverse pool of cytosolic proteins is transported slowly from the soma towards the axon [57]. With a couple of examples studied so far, current models suggest that these cytosolic proteins form spontaneously aggregated complexes that undergo ‘dynamic recruitment’ to allow short bursts of anterograde transport by hitching a ride on passing vesicles [58, 59]. However, the fast-acting mechanisms of the axon, required to promptly respond to external stimuli, could not depend solely on slow transport of proteins from the soma. As such processes in the distal axons rely on localized protein synthesis to spatially and temporally regulate protein content by localized mRNA translation. In addition, local translation allows for differential PTM variants of translated proteins according local requirements.

The mRNAs subjected to local translation bind to RNA-binding proteins (RBPs) before undergoing active transport by molecular motors. While RBPs are reported to bind to both anterograde and retrograde motor complexes, it is unclear whether these RBPs have bound mRNAs, which would allow for differentiation between the two supposed functions: (I) relocation of mRNAs within the axon or (II) delivery of RBPs to the cell body for reuse [60]. Binding of mRNAs to RBPs regulates PTMs and stress responses ensuring mRNA stability, translation efficiency, and localization to cytoplasmic granules [61]. mRNA association with RBPs also guarantees the formation of transport-competent ribonucleoprotein particles (RNPs), which are protein complexes commonly referred to as ‘RNA transport granules’. Specificity of RBP binding to mRNAs is dictated by sequences within the 5′ and 3′ UTR of mRNAs, which are recognized by RBPs. Although more rarely, RBP regulatory sequences can also be present in protein coding regions. RBPs can cooperate or compete for a regulatory outcome at more than one of these sites per mRNA and specific RBP binding sequences are often present in many different mRNAs. Interestingly, mRNAs encoding proteins with complementary functions were shown to bind

similar sets of RBPs leading to the concept of ‘RNA regulons’, which could be involved in subcellular compartment specific transport and translation.

Mutation in genes encoding RBPs are linked with several neuromuscular diseases such as ALS, SMA, multisystem proteinopathy (MSP) and frontotemporal lobar degeneration (FTLD). Well-known examples are TDP-43 mutations causative for ALS and FTLD. TDP-43 mutations affect its subcellular localization causing accumulation of RNP granules in the cell soma and proximal axons, they show disturbance in RNP complex transport and alter the axonal content of both mRNAs and miRNAs [62]. A major subtype of RBPs are the heterogeneous nuclear ribonuclear proteins (hnRNPs), wherein mutations are responsible for a number of cases of familial ALS and FTD [63]. Purice et al., (2018) reviewed the subset of disease-causing RBPs that are hnRNPs, namely TDP-43, FUS, hnRNPA1, hnRNPA2B1, matrin-3, and TIA1 [63]. While hnRNPs have an intrinsic tendency to aggregate, a common causal mechanism for these genes is the presence of mutations in the low complexity prion-like domain, which exacerbate the propensity to form self-seeding fibrils, resulting in accumulation of persistent stress granules [64, 65].

Mitochondrial transport

Neurons require functional mitochondria that need to travel long distances to provide support, such as adequate ATP-production, at specific sites of the neuron, like synaptic termini. Mutations affecting the balance between either division (known as fission), or collision and fusion of mitochondria, can cause alterations in the mitochondrial morphology. Subsequently, a more fragmented or dense mitochondrial network impacts axonal transport. The most prominent axonal CMT subtype (CMT2A) consists of mutations in *MFN2* affecting mitochondrial transport [66]. Together with the *MFN1* and *OPA1*, *MFN2* plays a major role in the mitochondrial fusion process. Mitochondrial fusion is a unique process involving two membranes, i.e. the mitochondrial outer membrane (MOM) and mitochondrial inner membrane (MIM), that requires rearrangement in a coordinated manner in order to maintain the organelle’s integrity [67]. Mitochondrial fission is mediated by the recruitment of the *DNM1L/Drp1* on the MOM [68, 69]. This happens through the interaction with the mitochondrial fission factor (Mff), mitochondrial dynamics protein *Mid49* and *Mid51*, and in a minor interaction with mitochondrial fission

1 (Fis1) [70–72]. Mutations in *GDAP1*, causative for several types of CMT, alter the regulation of mitochondrial fission activity dependent on the fission factors Drp1 and Fis1. Interestingly, the effect of *GDAP1* mutations is dependent on the mode of inheritance. Recessive mutations cause a reduction in the fission activity, whereas the dominant inherited mutations hamper mitochondrial fusion events [73]. Mitochondrial function and localization can be indirectly affected by mutations in the Miro/Milton complex, which mediate MT interaction, and by mutations in *NEFL*, which in turn alter the mitochondrial distribution [74]. Interestingly, CMT associated mutations in small heat shock proteins HSPB1 and HSPB8 indirectly affect mitochondrial function transport by altering cytoskeletal properties. Furthermore, mitochondrial functioning in neurons can also be affected by mutations causing loss of contacts between the mitochondria and the endoplasmic reticulum (ER) or ER network stability. As a consequence, the ER is hampered to initiate mitochondria to promote fission, e.g. mutations in *VAPB* induce the formation of abnormal ER-derived inclusions [75]. Mutations in *REEP1*, associated with HSP and dHMN, were shown to disrupt ER network and promote ER fragmentation [76]. Whereas, decrease of VAPB MOM protein, causes a perturbation of the uptake of Ca^{2+} by mitochondria, which is required to maintain an intracellular homeostasis as well as mitochondrial transport. Ca^{2+} is an important factor for various other functions such as cell signaling as well as regulating mitochondrial function and structure [77]. Therefore, mutations in Ca^{2+} -channels such *VDAC* or *TRPV4* lead to mitochondrial dysfunction in IPN [77].

Membrane-bound dynamin like GTPases known as atlastins (ATLs) mediate the formation of ER-mitochondria contact sites essential for calcium communication [78]. Mutations in the human isoforms *ATL1* and *ATL3* are associated with HSP and HSAN. Transmission electron microscopy studies reported higher ER-mitochondria contact sites upon expression of mutant *ATL3*, resulting in increased Ca^{2+} uptake into the mitochondria [78]. Moreover, aberrant calcium signaling affects mitochondrial trafficking through the Rho GTPases Miro1 and Miro2. Atlastin mutations demonstrate a reduced mobility of mitochondria in the cytoplasm together with altered mitochondrial localization, where the mitochondria are retained within the soma rather than distributed to the neuronal processes [78]. Furthermore, mutations in *SPTLC1* and *SPTLC2* produce atypical sphin-

golipids observed in HSAN type I. Alecu et al., (2017) demonstrated that these toxic deoxysphingolipids could localize into mitochondria disrupting the mitochondrial integrity [79].

Mutations in tRNA synthetases (e.g. *GARS*, *HARS* or *KARS*) also affect mitochondrial function. Mitochondrial dysfunction could contribute to neuromuscular junctions (NMJ) degeneration. E.g. a CMT2 type D mouse model expressing mutant *GARS* mutant displayed affected NMJs as well as muscle atrophy prior to synaptic degeneration [80].

Vesicular transport

Vesicular transport in axons occurs in different forms depending on the origin of intracellular components. Late endosomes and autophagosomes are subjected to retrograde transport to reach the lysosome, which mainly have a perinuclear localization. In contrast, synaptic vesicle precursors are produced in the soma and transported in anterograde direction towards the axon. These, mature synaptic vesicles are essential for proper neuronal growth, function and survival as they contain neurotransmitters, contribute to synapse formation and location, as well as help to sustain a balance between exo- and endocytosis [81, 82]. The transport of all vesicle types occurs through conserved mechanisms. Defects in the formation of endosomes, autophagosomes, lysosomes and synaptic vesicles, as well as impairments in their antero- and retrograde trafficking, may cause axonal degeneration [83, 84].

Rab GTPases regulate vesicular trafficking at different levels from vesicle formation, vesicle movement along actin and tubulin networks, to membrane fusion. Members of this protein family are involved in autophagy, lysosome and synaptic vesicle transport. Relevant for IPN and related neurodegenerative diseases is the small GTPase Rab5, which mediates transport and fusion of early endosomes, inducing neurite outgrowth and dendritic branching [85]. *ALS2* encodes a Guanine Nucleotide Exchange Factor for Rab5 and is involved in endosomal dynamics. Mutations in *ALS2*, associated with the onset of HSP, ALS and primary lateral sclerosis, produce truncated forms of alsin causing alterations in Rab5 and Rab7 signaling and Rab5-to-Rab7 conversion [86]. Mutations in *NIPA1* or *SPG11* similarly affect endosomal trafficking. A list of other HSP genes, divided by functionality has been reviewed in [87]. In addition to Rab5, Rab7 plays a crucial role in trafficking of late endosomes to lysosomes and is responsible for

bidirectional transport of autophagosomes as well as for autophagosome-lysosome fusion in all cells. Rab7 binds to effector proteins like FYCO1, responsible for plus-end, or to ORP1L and RILP, responsible for minus-end-directed transport [88].

Other classes of GTPases are involved in membrane trafficking and in vesicle formation in the endo-lysosome pathway. Dynamin2 (*DNM2*) polymers work by wrapping the neck of budding membranes, promoting membrane fission. Mutations in *DNM2* are associated with autosomal dominant centronuclear myopathy (ADCNM) and intermediate CMT [89]. *DNM2* contains actin binding sites suggesting that it may regulate actin dynamics during membrane tabulation [90].

An ensemble of kinesin-1, SKIP, Arl8, and subunits of the BLOC-one-related complex (BORC) direct anterograde transport of lysosomes into the axon. These eight BORC subunits consist of BLOS1, BLOS2, Snapin, KXD1, LOH12CR1 (myrlysin), C17orf59 (lyspersin), C10orf32 (diaskedin) and LOC729991 (MEF2BNB). Interference of the BORC function decreases lysosome transport in the axon, and its function is required for maintenance of axonal growth cone dynamics and autophagosome clearance [91].

The different functions of Rab GTPases correlated with the pathological conditions was reviewed by [86]. Nian et al., (2019) demonstrated that primary neurons from Rab^{-/-} mice showed a reduction in lysosomal trafficking together with the accumulation of autophagosomes, suggesting an altered autophagic vesicle transport [92]. Rab3 and Rab27 are important in synaptic vesicle exocytosis, while Rab5 is a key regulator of synaptic vesicles retrieval/endocytosis. Warburg Micro Syndrome (WARBM) and CMT2 type B neuropathy are associated with mutations in *RAB3GAP1* and *RAB7* respectively. Zebrafish *Rohon-Beard* spinal sensory neurons expressing *Rab7* mutations reported defects in neurite outgrowth and branching, and a marked decrease in the speed of *K157N* Rab7 containing vesicles, underscoring the importance of Rab7 in endosome transport [93]. Furthermore, mutations in *CHMP2B* cause a reduced Rab7-endosome recruitment and are linked to a FTD phenotype with partial overlap with ALS.

Another aspect is the fusion of synaptic vesicles at the nerve terminals. The neuronal v-SNAREs, vesicle Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptors, are essential for these fusion processes. Mutations in *VAMP2* encoding one of the v-SNAREs, cause aberrant synaptic vesicle

morphology and vesicle endocytosis often characterized by axial hypotonia [94]. Other mutations such as those in *UNC13A* can affect synaptic regulators and are known to be involved in neurodevelopmental disorders often in combination with involuntary movements [95].

RESEARCH AND THERAPEUTICS PROSPECTS

Axonal transport deficits are one of the most common and recurrent pathomechanisms in IPN and other neurodegenerative diseases (Table 1 and Fig. 2). For this reason, axonal transport studies are crucial, not only to understand its physiological role, but also to determine the effect of mutations that impair the network of axonal transport process and its regulation. Of note is that similar disturbances of axonal transport, or one of its key components, are also at play in other far more prevalent acquired disorders of the PNS [96]. Axonal transport in human peripheral nerves is difficult to investigate, as the tissue of interest is inaccessible. Therefore, animal models have been used especially for real-time monitoring of cargoes trafficking and axonal transport defects in order to develop novel therapeutic strategies. The wings of the fruit fly *Drosophila melanogaster* offer an *in vivo* model to study axonal cargo dynamics [97] and specific targeting of fluorescently-labelled organelles in the nematode *Caenorhabditis elegans* allow studying of axonal growth and synaptogenesis [98]. Furthermore, in transgenic *MFN2* zebrafish (*Danio rerio*) it is possible to genetically label mitochondria in motor neurons [99]. Other platforms for *in vivo* axonal transport studies compared to *ex vivo* tools have been reviewed by [100].

Anatomical, metabolic and physiological differences between small animal models and human complicate the translation of many therapies into clinical trials. However, iPSC technology offers the possibility to reprogram patient-derived cell lines into pluripotent cells before differentiation into a specific cell type relevant for the disease [101]. This provides new possibilities for IPN, allowing the generation of patient-derived motor and sensory neurons, potentially Schwann cells as well, without using peripheral nerve biopsies or post-mortem tissues. Nevertheless, further improvements are required to more closely model the *in vivo* situation to have a more representative environment and to take the necessary steps in optimization of co-cultures to study motor neuron-Schwann cell interactions.

Table 1
Overview of genes causative for IPN-related disorders involved in axonal transport

| Gene symbol | Protein name | Function | Mutational effects | Mode of inheritance | Disease associations | Refs |
|----------------|--|---|--|---------------------|--|---------------|
| <i>TUBB3</i> | Tubulin Beta 3 Class III | Structural component of neurofilaments | Impairment of tubulin heterodimer formation. | AD | CMT2 | [1, 6] |
| <i>NEFL</i> | Neurofilament Light | Structural component of neurofilaments | Reduced self-interaction and neurofilament dimerization. Neurofilament aggregation. | AD/AR | Congenital fibrosis of extraocular muscles 3 CMT1F CMT2E CMT2B5 | [2, 3, 7–12] |
| <i>NEFH</i> | Neurofilament Heavy | Structural component of neurofilaments | Translation of cryptic amyloidogenic element causing protein aggregation. Microtubule overstabilization. | AD | Nemaline rod myopathy CMT2CC | [2, 3, 7, 13] |
| <i>HSPB1</i> | Heat Shock Protein Family B (Small) Member 1 | Molecular chaperone for reducing protein aggregation and misfolding | Impaired mitochondrial transport. Cytoskeletal destabilization. | AD | CMT2F | [16–18, 114] |
| <i>HSPB8</i> | Heat Shock Protein Family B (Small) Member 8 | Molecular chaperone for reducing protein aggregation and misfolding | Reduced mitochondrial membrane potential. Impairment of aggregate degradation via autophagy. | AD | dHMN2B CMT2L | [14, 15] |
| <i>INFE2</i> | Inverted formin-2 | Stimulating actin filament growth and mitochondrial fission | Perturbation of actin filament dynamics and cytoskeletal networks. | AD | Focal and segmental glomerulosclerosis CMT intermediate type E | [4, 5] |
| <i>DYNC1H1</i> | Dynein Cytoplasmic 1 Heavy Chain 1 | Dynein subunit for retrograde axonal transport | Inhibition of microtubule gliding. | AD | CMT2O | [31, 32] |

Motor complexes
and adaptors

(Continued)

Table 1
(Continued)

| Gene symbol | Protein name | Function | Mutational effects | Mode of inheritance | Disease associations | Refs |
|----------------|--|--|--|---------------------|--|----------------------|
| <i>DCTN1</i> | Dynactin subunit 1 | Dynein adaptor protein for retrograde axonal transport | Compromised dynein processive movement activation. Altered localization of dynein. | AD | SMALED MRD13 HMN7B | [31, 33–35] |
| <i>BICD2</i> | Protein bicaudal D homolog 2 | Dynein adaptor protein for retrograde axonal transport | Reduced dynein-microtubule binding. Imbalance in anterograde and retrograde dynein motor complex motility. | AD | ALS susceptibility SMALED2A | [28–31, 36–43] |
| <i>KIF1A</i> | Kinesin-like protein KIF1A | Motor protein for anterograde axonal transport | Impaired microtubule binding. | AD/AR | SMALED2B Spastic paraparesis Distal myopathy HSN2C | [19, 20, 25, 44, 45] |
| <i>KIF1B</i> | Kinesin family member 1Bbeta isoform III | Motor protein for anterograde axonal transport | Impaired movement along microtubules. | AD | CMT2 with acrodystrophy SPG30 CMT2A1 | [19, 20, 37, 53, 54] |
| <i>KIF5A</i> | Kinesin heavy chain isoform 5A | Motor protein for anterograde axonal transport | Reduced ATPase activity and perinuclear localization. Impairment of binding capacity and transport of IGF1R. Altered processivity and directionality of kinesin dependent transport. | AD | CMT2 with pyramidal signs SPG10 ALS25 | [19, 20, 46–50, 52] |
| <i>HNRNPA1</i> | Heterogeneous nuclear ribonucleoprotein A1 | Packages mRNA into RNP particles | Increased fibrillization and self-aggregation. | AD | Myopathy (IBM) with Paget disease of Bone without Dementia (IBMPFD3) | [61, 63–65] |

mRNA and
proteins

| | | | | | | | | |
|--------------|---------------|--|---|--|-------------------------|---|---------------|--|
| Mitochondria | <i>ATL1</i> | Atlastin-1 | Formation of ER-mitochondria contact sites | Increased formation of cytoplasmic stress granules. | ALS20 | HMN (unpublished) | [78] | |
| | <i>ATL3</i> | Atlastin-3 | Formation of ER-mitochondria contact sites | Impairment of GTPase activity and dimer formation. Reduced ER-mitochondria fusion effect. Higher ER-mitochondria contact sites. | HSN1D SPG3A HSN1F | | [78] | |
| | <i>GDAPI</i> | Ganglioside-induced differentiation-associated protein 1 | Mediates mitochondrial fission | Increased Ca ²⁺ uptake into the mitochondria. Impairment of mitochondrial fusion (AD). | CMT2K | | [67, 73, 74] | |
| | <i>MFN2</i> | Mitofusin-2 | Mediates mitochondrial clustering and fusion | Reduction in mitochondrial fission activity (AR). Aberrant mitochondrial morphology and altered mitochondria-ER tethering. | CMT4A CMT2A2A | | [67, 99, 115] | |
| | <i>REEP1</i> | Receptor Expression Enhancing Protein 1 | Stabilization of ER tubules | Destabilization of ER tubules. ER fragmentation. | CMT2A2B HSP | | [76] | |
| | <i>SPTLC1</i> | Serine palmitoyltransferase 1 | Production of sphingolipids | Accumulation of synaptic vesicle proteins. | dHMN | Congenital axonal neuropathy with diaphragm palsy | [79] | |
| | <i>SPTLC2</i> | Serine palmitoyltransferase 2 | Production of sphingolipids | Production of toxic deoxysphingolipids that disrupt mitochondrial integrity. Production of toxic deoxysphingolipids that disrupt mitochondrial integrity. | HSANIC | | [79] | |
| | <i>TRPV4</i> | Transient receptor potential cation channel subfamily V member 4 | Non-selective calcium permeant cation channel | Abnormal TRPV4-regulated Ca ²⁺ influx. | HMN8 | | [77, 128-130] | |
| | | | | | | | | |

(Continued)

Table 1
(Continued)

| Gene symbol | Protein name | Function | Mutational effects | Mode of inheritance | Disease associations | Refs |
|--------------|--|--|--|---------------------|--|------------|
| | | | Higher basal intracellular Ca ²⁺ levels. | | SMA | |
| <i>VAPB</i> | Vesicle-associated membrane protein-associated protein B/C | Formation of membrane contact sites between ER and other organelles | Formation of intracellular aggregates not associated with membranes. | AD | SMALED CMT2C Proximal SMA | [75] |
| <i>DNM2</i> | Dynammin-2 | Production of microtubule bundles | Increased GTPase activity and oligomerization. Impaired autophagic degradation. Impaired DNM2 lipid binding and GTPase activity. | AD | CMT intermediate type B CMT2M Centronuclear myopathy | [89, 90] |
| <i>GAN</i> | Gigaxonin | Involved in crosstalk of the cytoskeletal architecture and E3 ligase | Inhibition of autophagosome synthesis and altered fusion to the lysosome. | AR | Lethal congenital contracture syndrome 5 GAN | [131, 132] |
| <i>RAB7</i> | Ras-related protein Rab-7 | Regulation of endo-lysosomal trafficking | Increased nucleotide exchange rates. | | CMT2B | [93] |
| <i>SPG11</i> | Spatacsin | Endosomal trafficking | Reduced hydrolysis of GTP. Alters Rab5 and Rab7 signaling. Alters Rab5-to-Rab7 conversion. | AR | CMT2X HSP ALS5 | [87, 133] |

AD - autosomal dominant; ALS - amyotrophic lateral sclerosis; AR - autosomal recessive; CMT - Charcot-Marie-Tooth; (d)HMN - (distal) hereditary motor neuropathy; GAN - giant axonal neuropathy; HSN - hereditary sensory and autonomic neuropathy; HSN - hereditary sensory neuropathy; HSP - hereditary spastic paraplegia; MRD13 - mental retardation, autosomal dominant 13; SMA - spinal muscular atrophy; SMALED - spinal muscular atrophy with lower limb predominance; SPG - spastic paraplegia.

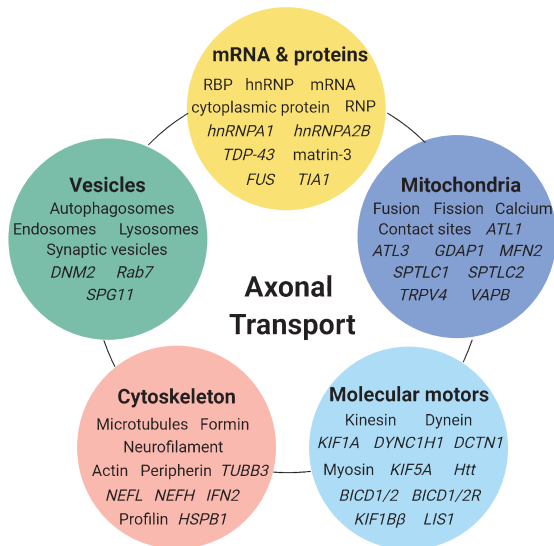


Fig. 2. Overview of the subcellular components and genes (illustrated by gene symbols) involved in axonal transport mechanisms and in which defects are associated with IPN and related neurodegenerative diseases.

Working with 2D-monolayers, microfluidic chambers provide the possibility to investigate retrograde and anterograde transport of organelles with live-cell imaging. Moreover, microfluidic chambers are a flexible tool to mimic the generation of neuromuscular junctions (NMJ), when motor neurons are co-cultured with iPSC-derived muscle cells. Nowadays, bioengineering and iPSC-research work together to develop functional 3D-models to properly reconstruct the immunological, biochemical and anatomical feature of specific organs. A 3D-printed heart-like structure was recently reported using personalized hydrogel, combined with patient-derived cells to print thick, vascularized and perfusable cardiac patches [102]. This suggest that we are not far from the generation of 3D models which could include iPSC-derived peripheral nerves, Schwann cells, blood vessels and myofibers, supported by 3D-scaffolds obtained with new biomaterials and methods of fabrication [103], being entirely supplied by a regulated oxygen transfer with flow pumps. The iPSC-derived models are therefore moving closer to a preclinical application, by providing a pre-screening platform for candidate drugs before testing them *in vivo*. However, animal models will still remain indispensable to investigate translational research in a whole organism [104, 105].

To date, treatment of IPN is limited to supportive measures that have a partial benefit in relieving the symptoms, such as neuropathic pain or gait impairment. However, no effective interventions exist targeting the causative pathway. Thanks to the understanding of genetic causes, together with the development of more appropriate cellular and animal models, axonal transport emerged as candidate drugable pathway to restore the neuronal function. This will apply to IPN, however many of these insights will also be relevant for the more common acquired diseases of the peripheral nervous system.

The first attempts in targeting axonal degeneration have come from the field of drug repurposing of small molecules. To reduce protein aggregation and improve axonal transport, chemical compounds target MTs through direct binding, PTMs, or chaperone upregulation, aiming to reach the right balance between highly stable and hyperdynamic MTs. In HSP patient-derived neurons, low doses of microtubule-targeting agents (MTA), such as taxol and vinblastine, increased the acetylated α -tubulin levels and restored peroxisome trafficking speeds and distance travelled, effectively improving the mutant phenotype [106]. Other MTA like Epothilone D improved microtubule density, axonal density and cognition in an AD mouse model [107]. However, these compounds, already approved as anti-cancer drugs, have been discontinued in clinical trials for neurodegenerative diseases due to neurotoxicity [108]. This suggests that new strategies are required to effectively intervene in the treatment of IPN.

Recent studies reported that histone deacetylase 6 (HDAC6) inhibitors such as ACY-1215 (Ricolinostat), currently in clinical trials for cancer, or ACY-1083 showing a higher selectivity towards HDAC6, are able to restore nerve function. They also affect microtubule dynamics by increasing the acetylation of α -tubulin, which re-established the transport function of MTs and offer neuroprotection [109]. Miro1 has been identified and shown to act directly as a novel target of HDAC6, in which Miro1 is deacetylated, at lysine 105, resulting in reduction of mitochondrial transport and outgrowth of the axonal cone. Therefore, HDAC6 inhibitors significantly correct anterograde transport of mitochondria, supporting the therapeutic use of this class of molecules for IPN [110].

Axonal transport involves specific kinase cascade activation, which is altered in neurodegenerative diseases [111]. In ALS, overactivation of p38 MAPK α causes excessive phosphorylation of molecular

motors preventing their movement along MTs and p38 MAPK α phosphorylation of NF subunits altering their transport and inducing bundling, effectively inhibiting retrograde transport. The use of p38 MAPK α inhibitors reverses these transport deficits in SOD1-G93A motor neurons, representing a novel therapeutic strategy in ALS. This represents a promising perspective in the treatment of AD, as well as in IPN [112, 113]. Failure in the protein quality control system can lead to deficits in axonal trafficking and aggregate clearance, which can drastically affect neuronal function. Therefore, upregulation of chaperones could be beneficial. Dual treatment using celastrol and arimocloamol increases the expression of a set of sHSPs (HSPA6, HSPA1A, DNAJB1, HO-1, HSPB1) in differentiated SH-SY5Y neuronal cells [114]. Despite the ability of celastrol in reducing the percentage of neuronal inclusions in the transgenic SOD1 mouse model of ALS, or in neurons expressing aggregation prone NEFL mutants, celastrol shows a motor neuron specific effect, with no effect in sensory neurons, limiting its use in IPN with sensory involvement [10]. As drugs can act on different levels of interactions (e.g. level of target, pathway, processes), co-administration of pharmaceutical active molecules should be considered to target the complexity and diversity of affected molecular pathways in IPN [115].

Despite the benefit of small molecules to rescue axonal degeneration, the risk for side effects has limited the use of these treatment strategies. However, a new class of molecules active on microtubule, molecular motors and autophagy [116], as well as antisense oligonucleotides (ASO) or gene therapy directly targeting the affected genes, have emerged and offer promising tools to treat IPN with axonal transport defects. ASO therapies have recently been developed and approved in the treatment of patients with spinal muscular atrophy (SMA) [117]. Similarly, ASO therapies could be applied to reduce PMP22 transcription levels in the CMT1A duplication [118]. Further advancements have been made in the delivery of vector-based gene therapies using adeno-associated viruses (AAV) in the treatment of SMA, consisting of delivery of a functional copy of the human SMN1 gene into motor neuron cells [119, 120]. Likewise, intrathecal injection of lentiviral vectors for Schwann cell-targeted expression has been used to restore the nodal architecture in demyelinating neuropathies. In *Sh3tc2*^{-/-} mice, a genetic model of CMT4C, or in the mutant *GJB1* mouse model for CMTX1, gene delivery respectively of

hSH3TC2 or WT Cx32, resulted in amelioration of motor performances together with a reduced myelin pathology [120, 121]. These few examples show that understanding dysfunctional genes operating in the axon, together with optimization of gene delivery methods, including vectors and administration routes, opens promising prospects to treat IPN and related axonopathies.

Deficits in axonal transport are inevitably linked with progressive axonal degeneration, a common feature of IPN and other neurodegenerative diseases. *SARM1* (Sterile Alpha and TIR Motif Containing 1) is a mediator of axonal degeneration and initiates cellular self-destruction. Deletion of *SARM1* or expression of dominant negative *SARM1* mutations, both impairing its activation, showed a reduction in axonal degeneration after axonal transection, the most rapid and aggressive trigger of axonal degeneration [122]. Furthermore, Turkiew et al., (2017) reported that *Sarm1*^{-/-} mice are resistant to distal axonal degeneration in a model of chemotherapy induced peripheral neuropathy and in high fat diet induced metabolic neuropathy [123]. AAV-mediated delivery of dominant negative *SARM1* in mice induces long-lasting axon protection following nerve transection. This approach may provide a new strategy to slow axon loss in chronic neurodegenerative diseases [124].

For neuropathies with toxic aggregations, therapeutic strategies are currently in development making use of gene replacement or silencing in a cell-specific manner. Recently, for CMT2A caused by dominant heterozygous mutations in *MFN2*, a combined therapy was tested on iPSC-derived spinal motor neurons which include the simultaneous use of RNA interference to silence the mutant allele and insertion of a mutagenized *MFN2* gene, resistant to shRNA activity, encoding for the native protein [125]. Next to therapeutic strategies, the identification of biomarkers for disease is relevant to diagnose and treat pre-symptomatic patients or to follow-up ongoing treatments. Currently, measuring NEFL levels in plasma is correlated with disease severity in multiple forms of CMT neuropathies [126], and also PFN2 and GAMT were identified as molecular determinants for CMT2 neuropathy, with a possible role of PFN2 in disease progression [127].

CONCLUSION

Axonal transport is a highly dynamic process involving the movement of different types of cargoes

(mRNA, proteins, mitochondria, lysosomes and synaptic vesicles) that are essential to sustain healthy neuronal functions. In this review, we have highlighted how deficits in cargo transportation and related factors affect axonal transport. The identification of numerous genetic causes for IPN-related disorders provides important insights into the underlying mechanisms of axonal degeneration. This knowledge allows the design of targeted therapeutic approaches, some of which have taken up a lead role and moved into clinical trials. Despite this, not all components and mechanisms of axonal transport have been unraveled, novel research strategies have emerged and will move towards patient-derived model systems (e.g. iPSC derived neurons in 2D and 3D-cultures) and *in vivo* animal models. These models will create new platforms to study and test therapeutic strategies for axonal degeneration.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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