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

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AF</td>
<td>actin filament</td>
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<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<td>Charcot-Marie-Tooth</td>
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<td>dHMN</td>
<td>distal hereditary motor neuropathy</td>
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<td>ER</td>
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<td>HDAC6</td>
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<td>HSAN</td>
<td>hereditary sensory and autonomic neuropathy</td>
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<td>HSP</td>
<td>hereditary spastic paraplegia</td>
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<td>IPN</td>
<td>inherited peripheral neuropathy</td>
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<td>iPSC</td>
<td>induced pluripotent stem cell</td>
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<td>mitochondrial inner membrane</td>
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<td>NEFL</td>
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<td>NEFH</td>
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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 nucleation end (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
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).

Mutations in the cytoskeletal proteins themselves, in 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 polyeuropathy 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 autophagy 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 CMT2F and distal hereditary motor neuropathy (dHMN) [16]. The SL35F mutation results into a higher affinity of HSPB1 for α-tubulin and overstabilization 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 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 (DYNCIH1). 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, adapters 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 dynactin, further enhancing the force and velocity of the motor complex [28–30]. BICD2 also interacts with the dynein heavy chain and dynactin, enhancing the affinity of the dynein–dynactin 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. CMT2O, 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; dynactin (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 p150Glu CAp-Gly GKNDG motif of dynactin. A G59S variant in DCTN1 causing HMN occurs centrally in the p150Glu 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 dynactin and more p150Glu CAp-Gly 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 dynactin 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 cargos [50–52]. KIF1Bβ 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 KIF1Bβ 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 KIF1Bβ protein. Only recently a novel CMT2-associated Y1087C mutation in KIF1Bβ was identified. It specifically impairs KIF1Bβ binding capacity and transport of insulin-like growth factor 1 receptor (IGF1R) down the axons affecting IGF-1/IGF1R signaling, which is essential for neuronal survival and axonal development [54].

CARGOES IN AXONAL TRANSPORT

Synaptic vesicles, NFs, and cytosolic proteins are cargos 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 bidirectional manner [31]. Depending on the cargo and its destination, specific regulatory proteins and motor complexes are recruited (Fig. 1). While cargos 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 ribonucleoproteins (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 fission 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 sphingolipids observed in HSAN type I. Alecu et al., (2017) demonstrated that these toxic deoxy sphingolipids 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 anterograde 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 sub-units 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 (lyspermsin), 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, Li et al., (2019) demonstrated that primary neurons from Rab7+/− mice showed a reduction in lysosomal trafficking together with the accumulation of autophagosomes, suggesting an altered autophagic vesicle transport [92], Rab3a and Rab27a are important in synaptic vesicle exocytosis, while Rab5a 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 neurodegenerative 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
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<td>Neurofilament Light</td>
<td>Structural component of neurofilaments</td>
<td>Reduced self-interaction and neurofilament dimerization. Neurofilament aggregation.</td>
<td>AD/AR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFH</td>
<td>Neurofilament Heavy</td>
<td>Structural component of neurofilaments</td>
<td>Translation of cryptic amyloidogenic element causing protein aggregation.</td>
<td>AD</td>
<td>CMT2E</td>
<td></td>
</tr>
<tr>
<td>HSPB1</td>
<td>Heat Shock Protein Family B (Small) Member 1</td>
<td>Molecular chaperone for reducing protein aggregation and misfolding</td>
<td>Microtubule overstabilization.</td>
<td>AD</td>
<td>CMT2F</td>
<td>[16–18, 114]</td>
</tr>
<tr>
<td>HSPB8</td>
<td>Heat Shock Protein Family B (Small) Member 8</td>
<td>Molecular chaperone for reducing protein aggregation and misfolding</td>
<td>Impaired mitochondrial transport. Cytoskeletal destabilization.</td>
<td>AD</td>
<td>CMT2L</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>INF2</td>
<td>Inverted formin-2</td>
<td>Stimulating actin filament growth and mitochondrial fission</td>
<td>Perturbation of actin filament dynamics and cytoskeletal networks.</td>
<td>AD</td>
<td>Focal and segmental glomerulosclerosis</td>
<td>[4, 5]</td>
</tr>
<tr>
<td><strong>Motor complexes and adaptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DYNC1H1</td>
<td>Dynein Cytoplasmic 1 Heavy Chain 1</td>
<td>Dynein subunit for retrograde axonal transport</td>
<td>Inhibition of microtubule gliding.</td>
<td>AD</td>
<td>CMT2O</td>
<td>[31, 32]</td>
</tr>
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<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>Function</th>
<th>Mutational effects</th>
<th>Mode of inheritance</th>
<th>Disease associations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DCTN1</strong></td>
<td>Dynactin subunit 1</td>
<td>Dynein adaptor protein for retrograde axonal transport</td>
<td>Compromised dynein processive movement activation.</td>
<td>AD</td>
<td>SMALED</td>
<td>[31, 33–35]</td>
</tr>
<tr>
<td><strong>BICD2</strong></td>
<td>Protein bicaudal D homolog 2</td>
<td>Dynein adaptor protein for retrograde axonal transport</td>
<td>Altered localization of dynactin.</td>
<td>AD</td>
<td>ALS susceptibility</td>
<td></td>
</tr>
<tr>
<td><strong>KIF1A</strong></td>
<td>Kinesin-like protein KIF1A</td>
<td>Motor protein for anterograde axonal transport</td>
<td>Impaired microtubule binding.</td>
<td>AD/AR</td>
<td>HSN2C</td>
<td>[19, 20, 25, 44, 45]</td>
</tr>
<tr>
<td><strong>KIF1Bβ</strong></td>
<td>Kinesin family member 1Bbeta isoform III</td>
<td>Motor protein for anterograde axonal transport</td>
<td>Impaired movement along microtubules.</td>
<td>AD</td>
<td>CMT2 with acrodystrophy</td>
<td></td>
</tr>
<tr>
<td><strong>KIF5A</strong></td>
<td>Kinesin heavy chain isoform 5A</td>
<td>Motor protein for anterograde axonal transport</td>
<td>Altered processivity and directionality of kinesin dependent transport.</td>
<td>AD</td>
<td>CMT2 with pyramidal signs</td>
<td>[19, 20, 46–50, 52]</td>
</tr>
<tr>
<td><strong>HNRNPA1</strong></td>
<td>Heterogeneous nuclear ribonucleoprotein A1</td>
<td>Packages mRNA into RNP particles</td>
<td>Increased fibrillization and self-aggregation.</td>
<td>AD</td>
<td>Myopathy (IBM) with Paget disease of Bone without Dementia (IBMPFD3)</td>
<td>[61, 63–65]</td>
</tr>
<tr>
<td>mRNA and proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- SMALED: Spinal Muscular Atrophy, Lower Motor Neuron, Dynactin
- MRD13: Myopathy, Neuropathy, Developmental Delay
- HMSN7B: Hereditary Motor and Sensory Neuropathy Type 7B
- ALS25, ALS20: Amyotrophic Lateral Sclerosis Types 25 and 20
- CMT2: Charcot-Marie-Tooth Disease Type 2
- HSN2C: Hereditary Sensory and Autonomic Neuropathy Type 2C
- SPG30: Spastic Paraplegia Type 30
- SPG10: Spastic Paraplegia Type 10
<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Impairment</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATL1</strong></td>
<td>Atlastin-1</td>
<td>Formation of ER-mitochondria contact sites</td>
<td>Impairment of GTPase activity and dimer formation. Reduced ER-mitochondria fusion effect.</td>
<td>AD</td>
<td>HSN1D</td>
<td>[78]</td>
</tr>
<tr>
<td><strong>ATL3</strong></td>
<td>Atlastin-3</td>
<td>Formation of ER-mitochondria contact sites</td>
<td>Higher ER-mitochondria contact sites. Increased Ca$^{2+}$ uptake into the mitochondria.</td>
<td>AD</td>
<td>HSN1F</td>
<td>[78]</td>
</tr>
<tr>
<td><strong>GDAP1</strong></td>
<td>Ganglioside-induced differentiation-associated protein 1</td>
<td>Mediates mitochondrial fission</td>
<td>Impairment of mitochondrial fusion (AD).</td>
<td>AD/AR</td>
<td>CMT2K</td>
<td>[67, 73, 74]</td>
</tr>
<tr>
<td><strong>MFN2</strong></td>
<td>Mitofusin-2</td>
<td>Mediates mitochondrial clustering and fusion</td>
<td>Aberrant mitochondrial morphology and altered mitochondria-ER tethering.</td>
<td>AD/AR</td>
<td>CMT2A2A</td>
<td>[67, 99, 115]</td>
</tr>
<tr>
<td><strong>REEP1</strong></td>
<td>Receptor Expression Enhancing Protein 1</td>
<td>Stabilization of ER tubules</td>
<td>Destabilization of ER tubules. ER fragmentation. Accumulation of synaptic vesicle proteins.</td>
<td>AD/AR</td>
<td>CMT2A2B HSP dHNM</td>
<td>[76]</td>
</tr>
<tr>
<td><strong>SPTLC1</strong></td>
<td>Serine palmitoyltransferase 1</td>
<td>Production of sphingolipids</td>
<td>Production of toxic deoxysphingolipids that disrupt mitochondrial integrity.</td>
<td>AD</td>
<td>HSAN1A</td>
<td>[79]</td>
</tr>
<tr>
<td><strong>SPTLC2</strong></td>
<td>Serine palmitoyltransferase 2</td>
<td>Production of sphingolipids</td>
<td>Production of toxic deoxysphingolipids that disrupt mitochondrial integrity.</td>
<td>AD</td>
<td>HSAN1C</td>
<td>[79]</td>
</tr>
<tr>
<td><strong>TRPV4</strong></td>
<td>Transient receptor potential cation channel subfamily V member 4</td>
<td>Non-selective calcium permeant cation channel</td>
<td>Abnormal TRPV4-regulated Ca$^{2+}$ influx. Higher basal intracellular Ca$^{2+}$ levels.</td>
<td>AD</td>
<td>HMN8</td>
<td>[77, 128–130]</td>
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</table>

SMA SMALED CMT2C

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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>VAPB</strong></td>
<td>Vesicle-associated membrane protein-associated protein B/C</td>
<td>Formation of membrane contact sites between ER and other organelles</td>
<td>Formation of intracellular aggregates not associated with membranes.</td>
<td>AD</td>
<td>Proximal SMA</td>
<td>[75]</td>
</tr>
<tr>
<td><strong>DNM2</strong></td>
<td>Dynamin-2</td>
<td>Production of microtubule bundles</td>
<td>Increased GTPase activity and oligomerization. Impaired autophagic degradation. Impaired DNM2 lipid binding and GTPase activity.</td>
<td>AD</td>
<td>CMT intermediate type B</td>
<td>[89, 90]</td>
</tr>
<tr>
<td><strong>GAN</strong></td>
<td>Gigaxonin</td>
<td>Involved in crosstalk of the cytoskeletal architecture and E3 ligase</td>
<td>Inhibition of autophagosome synthesis and altered fusion to the lysosome.</td>
<td>AR</td>
<td>GAN</td>
<td>[131, 132]</td>
</tr>
<tr>
<td><strong>RAB7</strong></td>
<td>Ras-related protein Rab-7</td>
<td>Regulation of endo-lysosomal trafficking</td>
<td>Increased nucleotide exchange rates. Reduced hydrolysis of GTP.</td>
<td>AR</td>
<td>CMT2B</td>
<td>[93]</td>
</tr>
<tr>
<td><strong>SPG11</strong></td>
<td>Spatacin</td>
<td>Endosomal trafficking</td>
<td>Alters Rab5 and Rab7 signaling. Alters Rab5-to-Rab7 conversion.</td>
<td>AR</td>
<td>CMT2X</td>
<td>[87, 133]</td>
</tr>
</tbody>
</table>

AD - autosomal dominant; ALS - amyotrophic lateral sclerosis; AR - autosomal recessive; CMT - Charcot-Marie-Tooth; (d)HMN - (distal) hereditary motor neuropathy; GAN - giant axonal neuropathy; HSAN - 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.
steps in optimization of co-cultures to study motor neuron-Schwann cell interactions.

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 anticancer 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 MAPKα causes excessive phosphorylation of molecular
motors preventing their movement along MTs and p38 MAPKα phosphorylation of NF subunits altering their transport and inducing bundling, effectively inhibiting retrograde transport. The use of p38 MAPKα inhibitors reverses these transport defects 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 arimoclomol increases the expression of a set of sHSPs (HSPA6, HSPA1A, DNAJB1, HOS1, 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 iPCS-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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