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Chapter

Targeting Axonal Transport: A New Therapeutic Avenue for ALS

Wenting Guo, Laura Fumagalli and Ludo Van Den Bosch

Abstract

Motor neurons have an extreme polarized morphology and heavily rely on efficient cargo transport along axons to maintain their neuronal connections and connections with muscles. Axonal transport deficits have been observed in almost all model systems of ALS. More and more studies have confirmed the close genetic and mechanistic linkage between axonal transport deficits with ALS pathogenesis. Moreover, several therapeutic approaches have been developed to target axonal transport deficits in ALS and showed promising effects in disease models. In this concise chapter, we summarize some major discoveries of axonal transport deficits in ALS pathogenesis and some related therapeutic strategies. We propose that targeting axonal transport may provide a potential therapeutic avenue for ALS.

Keywords: ALS, axonal transport, pathogenesis, therapeutics

1. Introduction

The unique morphological feature of neuronal cells compared with other cell types is their extreme polarity and the incredibly long axons [1]. Although the soma size ranges from 5 μm to 100 μm, axons can be up to 1 m long [1]. Axons keep the efficient communication between soma and axonal terminals [1]. This axonal communication is especially important to motor neuron as they not only need connections with each other but also far reach to muscles in order to control proper muscle contractions [2]. As for ALS, axonal transport defects are one of the most prevalent reported phenotypes from different model systems [2]. In addition, the classical "dying-back" hypothesis could explain the sequence of events during motor neuron degeneration in ALS [3]. The idea is that motor neurons lose their connection with muscle fibers and that the axon retracts back towards the soma, which ultimately results in cell death [3]. This theory is supported by the observation that motor neuron pathology begins at the terminal part of the axon and proceeds in a "dying-back" pattern [3]. In addition, the longest and largest neurites with the highest metabolic demand seem to be the most susceptible to this "dying-back" phenomenon [3].

Mechanistically, axonal transport process relies on three main elements: cargoes, microtubules, and motor proteins and their adaptors [1]. Axonal transport is tracked on microtubules which are polymers of α-tubulin and β-tubulins [1]. Microtubules determine the neuronal polarity with "plus" end at the axonal distal part and "minus" end at the soma part [1]. This polarity allows the directionality of the axonal transport [1]. Kinesin (mainly responsible for anterograde axonal transport, from soma to axonal terminal) and dynein (mainly responsible for retrograde

axonal transport, from axonal terminal to soma) are two types of ATP-dependent motor proteins responsible for carrying cargoes along the microtubules [1]. Adaptor proteins are responsible for the connection between motor proteins and cargoes [1]. Axonal transport maintains the efficient supply of cargoes including proteins, RNAs, lipids, and organelles from soma to terminals and is responsible to clear or recycle some misfolded proteins or aggregates under cell stress [1]. It is known that several ALS genes can directly cause axonal transport defects, and axonal transport also actively interacts with other major ALS pathological changes. Gene therapies and compounds that target axonal transport have shown beneficial effects in ALS animal models, and some have already been tested in clinical trials in the context of other diseases. Therefore, a better understanding of transport mechanisms and its role in the disease will open up a new therapeutic avenue for ALS.

2. Axonal transport defects in ALS pathogenesis

2.1 ALS mutations directly interfere with the axonal transport machinery

With the fact that about 10% of ALS cases are considered as "familial ALS" with clear genetic factors involved [4], several ALS gene mutations have been shown which could directly interfere with different aspects of the axonal transport machinery and eventually cause axonal transport defects.

ALS genes that are directly linked to kinesin- and dynein-mediated axonal transport have been uncovered by the discovery of ALS mutations in kinesin family member 5A (KIF5A) and dynactin subunit 1 (DCTN1) [5]. As a member of the kinesin family, KIF5A is mainly expressed in neuronal cells. In the year 2018, two independent large-scale genome-wide association and exome sequencing studies have found that mutations in KIF5A cause ALS [5, 6]. Most of the mutations are loss-of-function mutations that localized in the C-terminal region of the protein where cargoes bind. KIF5A mutations cause altered ATP activity and the dysfunction of kinesin-1 that eventually interfere with the anterograde transport of cargoes along the microtubules [6]. Moreover, for one of the most common ALS-causing genes called fused in sarcoma (FUS) it has been reported that its protein product functions as DNA-/RNA-binding protein that can bind or regulate mRNAs of several other motor proteins including KIF5C, KIF1B, and KIF3A [7], which are actively involved in regulating mitochondrial transport in neurons. Dynactin is a multi-subunit protein that binds and activates dynein by forming a dynein-dynactin motor complex that conveys cargoes in a retrograde transport [8]. The DCTN1 gene encodes dynactin subunit 1, which is responsible for binding microtubules and motor proteins. Heterozygous missense mutations in the DCTN1 gene have been suggested as risk factors for ALS in both sporadic and familial ALS patients [9].

The direct influence from ALS genes to microtubules has been described by the tubulin alpha 4a (TUBA4A) gene and spastic paraplegia 11 (SPG11) gene [10]. Exome-wide variant burden analysis revealed that mutations in TUBA4A associate with both sporadic and familial ALS cases [11, 12]. TUBA4A encodes the tubulin alpha 4A protein. Tubulins are the basic constituents of microtubules [11]. Mutations in TUBA4A destabilize the microtubule network, diminishing its re-polymerization capability that eventually disrupts the transport process [11]. Mutations in the SPG11 gene are the cause of autosomal recessive juvenile-onset ALS. Most of the mutations are loss-of-function mutations [10]. SPG11 encodes a protein called spatacsin, which co-localizes with the cytoskeleton in neurons [13]. Knockdown of SPG11 in mice showed a decreased acetylation level of α-tubulins [13]. With the fact that acetylation of α-tubulin facilitates the stabilization of the

microtubules and the binding of motor proteins to the microtubules, silencing SPG11 causes axon outgrowth defects and retrograde axonal retraction in cortical neurons of mice [13]. This is in line with the reduced axon plasticity in human iPSC-derived neurons from patients carrying SPG11 mutations [13]. These studies highlighted the importance of spatacsin in axon maintenance due to insufficient transport and cargo trafficking [13].

Except influencing motor proteins and microtubules, ALS genes can also interfere with axonal transport by affecting the cargoes. As the cargoes can participate into different mechanisms, the interplay between axonal transport and other ALS pathological mechanisms is also linked via cargo-specific transport. Rab proteins are a group of small GTPases that belong to the Ras superfamily [14]. Rabs are responsible for proper vesicle sorting, fission, docking, fusion, and transporting spatially and temporally by switching from an inactive guanosine 5'-diphosphate (GDP)-bound state to an active guanosine 5'-triphosphate [14]. Several Rabs have been reported to play a crucial role in driving neuronal transport in the central nervous system [14]. Mutations in the ALS2 gene cause juvenile-onset ALS. The protein product of the ALS2 gene specifically binds to Rab5 and functions as a guanine nucleotide exchange factor (GEF) for Rab5. The vacuolar protein sorting 9 (VPS9) domain of alsin mediates the activation of Rab5 through endosome and guanine-nucleotide exchanging reaction [14]. Most of the ALS2 mutations are loss-of-function mutations that cause the loss of the VPS9 domain and eventually fail in Rab5 activation [14]. Subsequently, Rab5-dependent endosome and AMPA receptor trafficking are hampered in neuronal culture [15, 16]. The reduction of GluR2-containing AMPA receptors at the synaptic surface in ALS2 knockout neurons results in vulnerability to glutamate toxicity [16]. In addition, more than 20 mutations in the OPTN gene have been described being the causative mutations of ALS [17]. Optineurin, the protein product of OPTN, regulates vesicle trafficking by forming a complex with myosin VI and Rab8 [17]. Myosins are a superfamily of motor proteins that move cargoes along microtubules, while Rab8 is a marker of recycling endosomes. The formation of the complex is Optineurin dependent [17]. This complex localizes at the Golgi apparatus and in cytoplasmic vesicles. It mediates Golgi organization, post-Golgi trafficking, exocytosis, and the basolateral delivery of membrane proteins [17]. In line with this, impaired axonal vesicle transport has been observed in a zebrafish model with optineurin loss. Furthermore, chromosome 9 open reading frame 72 (C9orf72), the most common genetic cause of ALS, is a GEF for Rab8 and is also associated with Rab1 [18, 19]. The knockdown of C9orf72 affects cellular trafficking from the cell membrane to Golgi. Overall, Rab-related transport processes are a good example of the cargo-induced impaired axonal transport in ALS.

2.2 Interplay between axonal transport defects with other ALS pathogenic mechanisms

Based on the genetic discoveries of ALS, intensive studies have proposed several major pathogenic mechanisms that contribute to motor neuron degeneration in ALS. Together with axonal transport defects, other dysfunctional mechanisms such as mitochondrial dysfunction, endoplasmic reticulum (ER) stress, neuroinflammation, excitotoxicity, and abnormal DNA/RNA metabolism have been identified [4]. Although it is still under debate which mechanism plays the vital role in initiating the motor neuron degenerative process, axonal transport defects have been reported to interplay with other mechanisms involved in disease progression.

With the most polarized morphological structure, motor neurons demand high-energy supply to maintain their normal function in controlling muscle

contractions [20]. Mitochondria play a pivotal role as the energy supply center in cells. Abnormal mitochondrial morphology and function are observed in postmortem tissues from ALS patients as well as in different ALS animal models [21]. Mitochondrial transport is important to clear the damaged mitochondria and maintain sufficient energy supply from the soma side to the distal side in motor neurons [21]. In line with this, fast motor neurons that have the highest needs of ATP, are more severely affected in ALS compared to slow motor neurons [22]. Therefore, the interplay between mitochondria quality control and transport is crucial for motor neuron survival. Mitochondrial movement along microtubules is controlled by a large complex containing kinesin, dynein, mitochondrial Rho (Miro), and milton [23]. Milton is an adaptor protein that connects Miro and motor proteins. Miro is a GTPase localized to the outer membrane of mitochondria. Miro is regulated by PINK/Parkin, which are genetic modifiers of FUS-induced neurodegeneration [24]. Axonal transport defects have been observed in ALS patient-derived motor neurons and FUS transgenic flies. Decreased expression of either PINK or Parkin is beneficial for reversing the locomotive defects and enhancing the survival of FUS transgenic flies [24]. In addition, overexpression of FUS also increased the ubiquitination of the Miro1 protein [24]. We have observed that mitochondria-associated ER membranes (MAM) are significantly decreased in motor neurons carrying FUS mutation [49]. As Miro1 tends to localize at MAM sites, the decrease of MAM might cause mitochondrial axonal transport reduction due to reduced Miro1-linkage [49]. Similarly, mitochondrial transport defects have also been reported for other ALS genes including vesicle-associated membrane protein-associated protein B/C (VAPB) and C9orf72 genes both in primary cultured neurons and transgenic flies [25]. ALS mutant VAPB interferes with anterograde mitochondrial axonal transport through disrupting Ca^{2+} homeostasis and affecting the amounts of tubulin associated with the Miro1/kinesin-1 complex [25, 26]. The C9orf72 repeat expansion can cause a severe disruption of mitochondrial transport but only a slight inhibition of vesicle transport. Although the exact mechanisms are not clear yet, evidence showed that the toxicity comes from the DPRs translated from the hexanucleotide repeats present in C9orf72 [26].

ER stress is a widely observed pathological change in different ALS models [27]. The ER is responsible for protein synthesis and quality control. Misfolded proteins are one of the earliest finding in models based on SOD1 mutations and later on in other ALS subtypes [27]. In normal conditions, the ER can identify the misfolded protein and trigger the unfolded protein response (UPR) to clear these proteins [27]. While under ER stress, the misfolded proteins will be accumulated without a proper UPR process [27]. It has been suggested that the ER stress can also cause an axonopathy and an irregular microtubule distribution [28]. This has been highlighted by the discovery that mutations in two major genes called PDLA1 and PDLA3 that code ER chaperons are linked with ALS [28]. The ER chaperones or protein disulfide isomerases (PDIs) play a pivotal role in the UPR process [27]. Expression of mutant PDIs in motor neurons affects dendrite outgrowth and causes motor defects in mice [26, 28].

TDP-43 aggregation has been identified as the most prevalent clinical pathological hallmark of ALS patients. In addition, the coding gene TARDBP is an ALS-causing gene. TDP-43 aggregation also widely occurs in other familial as well as sporadic ALS patients. Axonal transport defects have been observed in different model systems of ALS with TDP-43 aggregation [29, 30]. TDP-43 mutations impair mRNA transport in transgenic Drosophila, primary cultured mouse cortical neurons and stem cell-derived motor neurons from ALS patients [29, 30]. Impaired anterograde axonal transport of microtubule plus tip proteins has been

observed in primary cultured rat cortical neurons [29]. It has been suggested that cytoplasmic TDP-43 aggregation impairs the cytoskeletal integrity and results in transport deficits [29, 30]. While another study has shown that age-dependent organelle transport defects in iPSC-derived motor neurons from ALS patients carrying TARDBP mutations is independent from TDP-43 aggregation [31], thus, a clear interplay between TDP-43 aggregation and axonal transport still needs to be clarified. Recently, we found that arginine-rich dipeptide repeat proteins (DPRs), which are the pathological translational products from C9orf72 repeat expansion, can cause axonal transport defects in human stem cell-derived motor neurons [32]. We found that several components of the axonal transport machinery interact with arginine-rich DPRs both in vitro and in vivo. It has been proposed that arginine-rich DPRs might directly affect axonal transport through an inhibitory interaction with the microtubule-based transport machinery [32].

DNA damage has been recently proposed as an early pathological change in ALS patients [33]. With the fact that TDP-43 and FUS are DNA-/RNA-binding proteins, the mutations in these genes cause insufficient DNA damage repair and result in motor neuron degeneration [33]. Both DNA damage and distal axonal transport defects have been observed in ALS patients carrying FUS mutations [34, 35]. It has been proposed that DNA damage might play a role in axonal transport defects [34]. When DNA damage is induced in cultured motor neurons, axonal transport defects occur thereafter [34]. In line with this, improving the DNA damage repair process by inhibiting poly(ADP-ribose) glycohydrolase (PARG) shows a rescue of axonal transport defects [34]. Although the exact underling mechanism is not clear yet, it is very likely that DNA damage might induce universal transcriptional changes of some genes that produce proteins that are involved in axonal transport.

3. Therapeutic strategies targeting axonal transport in ALS

As summarized above, different molecular mechanisms underlie axonal transport deficits in ALS. Based on these evidences, therapeutic strategies to restore axonal transport deficits have started to emerge. Given the complexity of the axonal transport machinery and its regulatory mechanisms, multiple approaches have been devised to target the system at different levels.

3.1 Restoring the tracks: approaches to modulate microtubule dynamics

Microtubule-stabilizing agents are currently in clinical use as chemotherapeutic drugs [36]. Compounds that modulate microtubule stability have shown promising results also in the context of neurodegeneration. Epothilone D, for example, has shown beneficial effects in several models of Alzheimer's disease [37, 38]. Because of these findings, Epothilone D underwent a clinical phase 1 trial investigation in patients with mild Alzheimer's disease (NCT01492374, NCT01966666) [39]. Beneficial effects have been reported also for Parkinson's disease [40] and hereditary spastic paraplegia (HSP) [41]. Epothilone D was shown to protect the soma and distal axon of spinal motor neurons early in the disease course of the $SOD1^{G93A}$ model mouse of ALS [42]. However, this was not associated with improved motor performance or survival [42]. While another microtubule-stabilizing agent, Noscapine, was shown to restore axonal transport, to delay the onset of symptoms and to extend the survival of $SOD1^{G93A}$ mice [43].

Pharmacological agents that increase the level of microtubule acetylation have been also used to rescue axonal transport deficits. Acetylated microtubules are considered to be stable, long-lived microtubules [44]. Although the

mechanisms and functional consequences of microtubule acetylation is not fully understood [44], it has been suggested that acetylation of α-tubulin promotes the recruitment of molecular motors kinesin-1 and dynein [45, 46], indicating that boosting the level of microtubule acetylation might positively affect intracellular transport. Strong evidence has shown that inhibition of HDAC6, a major tubulin de-acetylating enzyme, stimulates intracellular transport of different cargoes in several models [45–50].

HDAC6 belongs to the histone deacetylases (HDACs) family, and, unlike the other HDACs, HDAC6 is mainly localized in the cytoplasm where it associates with microtubules and with the dynein-dynactin motor complex containing p150glued [51]. In line with these observations, HDAC6 has been implicated in the regulation of cytoskeletal stability, intracellular transport, and cell motility [51, 52]. The beneficial effect of HDAC6 inhibitors has been shown in a broad variety of neurodegenerative diseases. For example, the inhibition of HDAC6 by trichostatin (TSA) increases microtubule acetylation and rescues axonal transport deficit in primary neurons carrying the LRRK2 mutation, which is the most common genetic causes of Parkinson's disease. In addition, in vivo knockdown of HDAC6 and administration of TSA restore locomotor deficits caused by LRRK2 mutation in a Drosophila model [48]. Inhibition of HDAC6 by TSA or Tubastatin A (TubA) restores the levels of acetylated α-tubulin and corrects the axonal transport defects in a mutant HSPB1-induced Charcot-Marie-Tooth disease (CMT) mouse model [47, 53]. TSA also enhances tubulin acetylation and rescues microtubule-based transport deficits observed in Huntington's disease (HD) mutant cells [46]. However, despite the increased microtubule acetylation, the loss of HDAC6 did not rescue neurodegenerative phenotypes and deficits in motor coordination in a HD mouse model [54]. In contrast, genetic deletion of HDAC6 significantly slows disease progression and extends survival of the mutant $SOD1^{G93A}$ mouse model of ALS [55]. The therapeutic potential of HDAC6 inhibition in ALS has been further investigated in FUS iPSC-derived motor neurons [49]. Both TubA and ACY-738 HDAC6 inhibitors rescue the axonal transport deficit in ALS patient-derived motor neurons. This beneficial effect on intracellular transport was further confirmed using HDAC6 antisense oligonucleotides (ASOs) [49]. Furthermore, HDAC6 inhibition increases the acetylation level of α-tubulin in patient-derived motor neurons [49].

Overall, targeting microtubules might represent an interesting therapeutic target in ALS. In particular, modulating the acetylation levels of α-tubulin might be beneficial in restoring axonal transport deficits observed early in the disease course of ALS. HDAC6 inhibitors have shown promising results in the context of ALS; however additional studies are required. For instance, it has been shown that HDAC6 plays an important role in autophagy by promoting the clearance of protein aggregates [56] including mutant SOD1 [57–59]. In this regard, inhibitors of the deacetylation function of HDAC6 that leave the other functions unhampered need to be further validated in the available ALS disease models as they might represent an interesting therapeutic approach.

3.2 Restoring the motors: the role of kinases

Several kinases can directly modulate axonal transport through phosphorylation of motors, adapters, and cargoes [60]. Deregulation of axonal transport by protein kinases has been associated to ALS; therefore the possibility of targeting protein kinases has started to emerge as a novel therapeutic avenue.

An abnormal activation of p38 MAP kinase (MAPK) was reported in mutant SOD1 mice [61–63]. It has been shown that active p38 MAPK phosphorylates kinesin-1, leading to impaired translocation of kinesin-1 along axonal microtubules

and inhibition of fast axonal transport [62]. The p38 MAPK inhibitor, SB203580, completely inhibits mutant SOD1-induced apoptosis of motor neurons in vitro [61]. In addition, Semapimod, a p38 MAPK inhibitor potentially suitable for clinical purposes, protects motor neurons from degeneration in vivo, although it only mildly extends the survival of $SOD1^{G93A}$ mice [61]. Importantly, a more recent study has shown that p38 MAPK is directly responsible for $SOD1^{G93A}$-induced axonal transport deficits in motor neurons, further strengthening the link between p38 MAPK and axonal transport [63]. Both genetic and acute pharmacological inhibitions of p38 MAPK rescue axonal transport deficits in motor neurons of $SOD1^{G93A}$ mice both in vivo and in vitro [63]. However, long-term treatment with the p38 MAPKα inhibitor SB239063 (a potentially interesting compound given the ability to cross the blood-brain barrier) has shown significant toxic side effects and, probably because of that, failed to improve axonal transport and muscle function in $SOD1^{G93A}$ mice [63]. Therefore, additional investigation is required to evaluate and optimize the long-term effects of this approach.

Overactivation of GSK3β has been found in the brain and spinal cord of $SOD1^{G93A}$ mice as well as in spinal cord samples from sporadic ALS patients [64–67]. Inhibition of GSK3β was protective in $SOD1^{G93A}$ transgenic mice in some studies [67, 68], but these findings were not confirmed in later ones [69, 70]. Therefore, at present, the involvement of GSK3β in ALS remains controversial. Aberrant activation of cyclin-dependent kinase 5 (CDK5) has been reported in the spinal cord of mouse models of ALS [71–73]. Hyperactivation of CDK5 mis-regulates transport of several cargoes via the Lis1/Ndel1 complex, which directly regulates dynein activity [72]. Reduction of CDK5 activity in neurons from $SOD1^{G93A}$ mice by roscovitine rescues transport deficits [72]. Similarly, inhibition of CDK5 by overexpression of calpastatin improves motor axon survival, delays disease onset, and increases survival of $SOD1^{G93A}$ mice [73].

Overall, modulating kinase activation seems to be beneficial for the transport defects in ALS; however most of these studies focus on the $SOD1^{G93A}$ mouse model. It remains to be determined whether targeting kinases is beneficial also in the context of other ALS-causing mutations. In addition, many protein kinases have multiple targets and are involved in several cellular processes. Therefore a more detailed understanding of kinase signaling pathways is required to effectively implement this strategy.

4. Conclusions and perspectives

Axonal transport defects have been strongly linked with ALS pathogenesis. Different therapeutic strategies have been tested in ALS disease models, showing prospective results. However, a deeper understanding of the pathological mechanisms that are responsible for axonal transport deficit is required to properly target axonal transport in ALS. Most of the therapeutic strategies proposed have been mainly tested in the $SOD1^{G93A}$ transgenic mice, and it is still unknown whether they are beneficial in other familial ALS models. In addition, the use of iPSC-derived motor neurons could potentially help to validate whether these compounds might also be beneficial for the sporadic ALS cases.

Other cellular mechanisms have been shown to be altered in ALS. Therefore it is not easy to clarify whether altered axonal transport causes neuronal degeneration or whether neuronal dysfunction, due to other upstream mechanisms, ultimately leads to malfunctioning of axonal transport. However, the evidence that axonal transport is an early identifiable phenotype in several in vivo models suggests that targeting axonal transport needs to be addressed for an effective treatment.

Relatedly, studies are ongoing to improve the specificity and the ability of drugs to cross the blood–brain barrier. The advent of techniques such as gene therapy and antisense oligonucleotides might speed up the process of effectively targeting axonal transport in patients. The feasibility of gene therapy to ameliorate axonal transport deficits has been already successfully shown in ALS mice [58]. In addition, ASOs that specifically target HDAC6 have been already tested in iPSC-derived motor neurons of FUS patients showing positive effects on transport [49]. Therefore, these approaches might represent an interesting area for future research and might help to identify effective therapeutic strategies.

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