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Review

Axonal transport of neurofilaments in normal and disease states

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Abstract. Neurofilaments are among the most abundant organelles in neurones. They are synthesised in cell bodies and then transported into and through axons by a process termed 'slow axonal transport' at a rate that is distinct from that driven by conventional fast motors. Several recent studies have now demonstrated that this slow rate of transport is actually the consequence of conventional fast rates of movement that are interrupted by extended pausing. At any one time, most neurofilaments are thus stationary. Accumulations of neurofilaments are a pathological feature of several human neurodegenerative diseases suggesting that neurofilament transport is disrupted in disease states. Here, we review recent advances in our understanding of neurofilament transport in both normal and disease states. Increasing evidence suggests that phosphorylation of neurofilaments is a mechanism for regulating their transport properties, possibly by promoting their detachment from the motor(s). In some neurodegenerative diseases, signal transduction mechanisms involving neurofilament kinases and phosphatases may be perturbed leading to disruption of transport.

Key words. Neurofilaments; phosphorylation; amyotrophic lateral sclerosis; Alzheimer's disease; Parkinson's disease; axonal transport.

Introduction

Most, if not all proteins of neurones are synthesised in or close to the cell bodies, where ribosomes predominate. As with all cells, these proteins are then trafficked around the cell to their appropriate subcellular locations. However, for neurones, this can present a unique problem since axons and dendrites can be extremely long. For example, a human motor neurone axon can exceed a metre in length; in a whale, it might exceed 10 m in length. How do neurones move axonal proteins over such distances? The answer is: via molecular motors such as members of the kinesin and dynein superfamilies [for reviews see refs. 1, 2].

The cytoskeleton represents a particularly abundant set of neuronal proteins that has to be transported along the length of axons. It comprises microtubules and actin and their associated proteins, and neurofilaments, which are the major intermediate filament proteins of neurones. Neurofilament accumulation is a pathological feature of several human neurodegenerative diseases. Increasing lines of evidence suggest that these neurofilament accumulations are mechanistic in the neurodegenerative process and are not just some end-stage epiphenomenon.

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How therefore do neurofilaments accumulate? In this review, we describe several new findings that have cast light on the mechanisms of neurofilament transport in both normal and disease states.

Neurofilaments, the intermediate filaments of neurones

The intermediate filaments of neurones comprise the neurofilament triplet proteins, neurofilament light chain (NF-L), middle chain (NF-M), and heavy chain (NF-H), α-internexin and peripherin [for reviews see refs. 3, 4]. Neurofilaments are the major intermediate filaments in most terminally differentiated neurones whereas α-internexin is expressed more abundantly in the developing nervous system [5, 6], and peripherin is expressed mostly in autonomic nerves and sensory neurones [7–9].

In common with other members of the intermediate filament family of proteins, NF-L, NF-M, NF-H, α-internexin and peripherin share a common structural organisation which comprises a central alpha-helical rod domain that is flanked by amino-terminal head and carboxy-terminal tail domains. The central rod domains facilitate the formation of coiled-coil oligomers that can then assemble into filaments 10 nm in diameter; the amino-terminal head domains are believed to regulate the assembly properties of the filament [10, 11] and the carboxy-terminal tail domains of NF-M and NF-H (which are longer than those of NF-L, α-internexin or peripherin) form side arms that project from the filament and which appear to form interconnections between neurofilaments and between neurofilaments, microtubules and other organelles [12, 13].

One function of neurofilaments is to control axonal calibre and this is important since the speed of conductivity of an impulse down the axon is in part proportional to its calibre. Thus neurofilaments are particularly abundant in neurones with large-diameter axons such as those of motor neurones where fast impulse conduction velocities are crucial for proper functioning. Formal demonstration of the role of neurofilaments in calibre determination came first from analyses of a mutant quail in which the NF-L gene was disrupted [14, 15]. Later studies of transgenic mice lacking axonal neurofilaments confirmed this role [16].

Neurofilaments are phosphoproteins, and this phosphorylation is dynamic [for reviews see refs. 17, 18]. Neurofilaments are phosphorylated on their amino-terminal head domains and this is possibly a mechanism for regulating neurofilament assembly; such phosphorylation is known to regulate the assembly properties of other intermediate filament proteins [17–19]. In addition, the carboxy-terminal side arm domains of NF-M and NF-H, and the short carboxy-terminal domain of NF-L are also phosphorylated in vivo. Rat and bovine NF-L are phosphorylated on serine 473 by casein kinase II [20, 21] and, so far, 7 sites in rat NF-M and 12 sites in rat NF-H have been identified in vivo [22–24]. However, more sites will be identified, since NF-M and NF-H contain at least 10 and up to approximately 50 moles of phosphate, respectively [25].

Many of the sites that are phosphorylated in NF-M and NF-H side arms are serines within the motif Lys-Ser-Pro (KSP). NF-M contains clusters of these sites and NF-H harbours up to approximately 53 KSP repeats located within a multiphosphorylation repeat domain [23, 26, 27]. Most, if not all of the serines within this repeat domain are believed to be phosphorylated in axons, making NF-H one of the most phosphorylated proteins known. The increase in charge caused by such heavy phosphorylation is likely to influence the properties of the side arms and hence neurofilament function.

Axonal transport of neurofilaments – slow axonal transport arises by intermittent fast transport

Movement of the cytoskeleton into and through axons has been classically studied in vivo via metabolic labelling of proteins. In these experiments, ^{35}S-methionine is injected to label proteins in either the sciatic nerve or retinal ganglion cells of the optic nerve. After set periods of time, these nerves are removed, dissected into segments and the cytoskeleton purified. By analysing the distance travelled by radiolabelled cytoskeletal proteins at set times, their rate of transport can be calculated. Such studies reveal average rates of transport between 0.1–3 mm day^{-1}. Since movement through axons of organelles such as vesicles and mitochondria by kinesin and dynein family motors is in the region of 1 μm s^{-1}, this slower rate of cytoskeletal transport has been termed ‘slow axonal transport’ [28–30]. A major conundrum of slow axonal transport is that there are no known molecular motors that move at such slow speeds.

Several recent studies have provided some answers for this puzzle and all have involved monitoring neurofilament movement in real time either in living neurones or in in vitro systems. For the studies in living neurones, monitoring of movement was achieved by transfection of green fluorescent protein (GFP)-tagged neurofilament subunits [31–34]. Two of these studies utilised superior cervical ganglion cells in which the axonal neurofilament network is sparse, enabling movement to be observed through the neurofilament ‘gaps’ [32, 33]. These revealed that both GFP-NF-M and GFP-NF-H move at conventional fast rates of up to approximately 1 μm s^{-1} but that this movement is interrupted by prolonged pauses. At any one time, most neurofilaments are thus stationary (esti-

mated as >90%). Interestingly, movement of GFP-NF-M and -NF-H was seen in both anterograde and retrograde directions.

Neurofilament movement has also been assayed in real time both in exuded squid axoplasm [35] and along microtubules in vitro [36]. Again, both studies revealed that neurofilaments move at conventional fast rates of up to approximately 1 μm s^{-1} and bidirectional movement was detected along the microtubules in vitro. Thus the slow overall rate of movement of neurofilaments seen in the conventional in vivo assays appears to be the result of fast intermittent movement towards both the axon terminal and the cell body.

These fast rates of neurofilament transport are consistent with analyses of other intermediate filament proteins following GFP-tagging and transfection into non-neuronal mammalian cells [37–41] where fast rates of movement towards both the cell periphery and nucleus have been observed. Such rates are consistent with movement being via conventional motors such as members of the kinesin and dynein families. Indeed, recent evidence suggests that neurofilaments (and other intermediate filament proteins) can associate with kinesin and dynein/dynactin [31, 35, 36, 39, 42]. Kinesin and dynein families are extensive [for reviews see refs. 1, 2] and the precise motor species responsible for neurofilament transport is still not known. Movement is even possibly generated by more than one motor since kinesins are generally microtubule plus-end directed whereas dyneins are minus-end motors. Exchange between motors would explain the bidirectional movement of GFP-neurofilaments seen in transfected neurones and in the in vitro models [32, 33, 36].

The form in which neurofilaments are transported has been a topic of debate for many years [for reviews see refs. 28, 29]. In one (the subunit) model, neurofilaments are disassembled into subunits or small oligomers prior to transport, whereas in the alternative, polymer, model, neurofilaments are transported as intact filaments. Analyses of GFP-NF-M and GFP-NF-H in living superior cervical ganglion cells revealed movement of intact filaments, lending strong support for the polymer model. However, some researchers have reported movement of smaller GFP-NF-M particles, which suggests some degree of neurofilament disassembly prior to transport [31]. Movement of not clearly filamentous particles is also seen in some of the other assays of neurofilament transport [35, 36] and in studies of GFP-tagged vimentin where motile vimentin 'dots' and 'squiggles' can be observed [39]. Possibly both filamentous and non-filamentous neurofilaments are capable of undergoing transport, and the form observed depends upon the type and age of the neurone [43]. Whatever the precise form in which they are transported, some neurofilament disassembly possibly occurs prior to transport, since only a small proportion move at any one time. Thus, neurofilaments may be disassembled into shorter filaments and/or particles to permit disassociation from the rest of the neurofilament matrix and transport through the axon.

Neurofilament phosphorylation as a regulator of assembly and transport

The intermittent movement of neurofilaments through axons suggests the presence of a mechanism to control their attachment and release from the motor proteins. Many studies have demonstrated a correlation between a slowing in the overall speed of neurofilament transport through the axon and the degree of phosphorylation of NF-M/NF-H side arm domains [see for example refs. 44–52]. NF-M/NF-H side arm phosphorylation may therefore be a regulatory mechanism for controlling neurofilament attachment to the motor(s) and recent studies have lent support for this notion [36, 42]. Furthermore, there is evidence that NF-M and NF-H side arm phosphorylation alters the physical and biological properties of neurofilaments with heavily phosphorylated side arm domains inducing bundling of filaments that coincides with a reduction in motility [50, 53]. Thus, a reduction in the overall rate of neurofilament transport could be via increased NF-M/NF-H side arm phosphorylation and detachment of neurofilaments from the anterograde motor. Alternatively, phosphorylation may promote transfer of neurofilaments to the retrograde motor. Clearly, proper characterisation of the motors involved in neurofilament transport will enhance studies in this area.

Since some disassembly of neurofilaments may also be necessary for their movement through axons (see above), phosphorylation of neurofilament head domains might additionally regulate transport. NF-L, NF-M and peripherin (and probably also NF-H and α-internexin) are all phosphorylated on their head domains and this is known to control neurofilament assembly [54–62].

Neurofilament transport and neurodegenerative disease

Several lines of evidence suggest that abnormalities in neurofilament metabolism are part of the pathogenic process in certain human neurodegenerative diseases. First, mutations in NF-L are the causative genetic defect for some types of Charcot-Marie-Tooth disease type 2 [63, 64] and mutations in NF-H side arm domains are a risk factor for amyotrophic lateral sclerosis (ALS) [65–67]. Interestingly, these latter mutations affect phosphorylation sites within the NF-H side arm domain. Second, overexpression of neurofilament proteins (also including peripherin and α-internexin) in transgenic mice can provide models of ALS [68–71] and other neurode-

generative diseases [72, 73]. Third, transgenic mice expressing mutant copper/zinc superoxide dismutase-1 (SOD1), involved in familial forms of ALS, develop motor neurone disease, and crossing of mutant SOD1 transgenic models of ALS with transgenics in which expression of the individual neurofilament subunits has been altered can protect against the disease [74–76] although this is not always the case [77]. However, the mechanism of protection in these cases is not clear, since both overexpression and ablation of neurofilament subunits are protective. Finally, accumulations of neurofilament proteins are a pathology of several human neurodegenerative diseases. These include ALS, Alzheimer's disease, diabetic neuropathy and Lewy bodies in Parkinson's disease and some dementias (neurofilaments are a component of the Lewy body along with α-synuclein) [78–87].

The accumulations of neurofilaments seen in disease states suggests that their transport is somehow perturbed, and for some of the transgenic models of ALS, this is now known to be the case [88–90]. Interestingly, the slowing of neurofilament transport in the mice is an early pathological feature which precedes disease, demonstrating that neurofilament accumulation is not just an end-stage epiphenomenom [88–90]. What is not clear is what mechanisms lead to this slowing.

Since NF-M and NF-H side arm phosphorylation is believed to be a regulator of transport, one possibility is that side arm phosphorylation is altered in disease states. Analyses of neurofilament phosphorylation using mass spectrometry have not revealed any overall differences between ALS and control tissues [91] but this does not exclude the possibility that neurofilament phosphorylation is altered topographically in disease. NF-M and NF-H side arms are largely non-phosphorylated in cell bodies and proximal axons but become much more heavily phosphorylated in distal regions of axons [45, 92–96]. Many of the neurofilament accumulations seen in disease states are located within cell bodies/proximal axons but are labelled with antibodies that detect phosphorylated NF-M/NF-H side arms [80, 84, 85, 97–99]. Increased side arm phosphorylation in cell bodies might therefore disrupt neurofilament transport leading to accumulation. If this is indeed the case, then one can infer that the mechanisms regulating NF-M and NF-H side arm phosphorylation might be perturbed in some disease states. Several kinases have now been shown to phosphorylate NF-M and NF-H side arms. These include glycogen synthase kinase-3α and -3β, cyclin-dependent kinase-5 (cdk5/p35), p42/p44 mitogen-activated protein kinases (p42/p44 MAPKs) and members of the SAPK1 family [47, 100–113]. Recently, deregulated cdk5/p35 has been described in mutant SOD1 mice [114] and we have shown that glutamate, a proposed pathogenic mechanism for several neurodegenerative diseases, activates members of the MAPK/SAPK family and causes a slowing of neurofilament transport and increased cell body phosphorylation of neurofilaments [47]. Other studies have shown that SOD1 influences the activity of calcineurin, a neurofilament phosphatase [115, 116]. Thus, there is increasing evidence to link known pathogenic processes involved in neurodegenerative diseases with signal transduction cascades that regulate neurofilament phosphorylation.

A further possible route that might lead to neurofilament accumulation is damage to the motor proteins. Although the motors responsible for neurofilament movement have not been properly characterised, recent work has demonstrated that a mutation in the kinesin motor protein Kif1bβ is the cause of Charcot-Marie-Tooth disease type 2a [117]. Thus, there is now a precedent to demonstrate that damage to motor proteins can cause neurological disease. Defective axonal transport and, in particular, transport of neurofilaments and the cytoskeleton may therefore be mechanistic in nerve cell death in several human neurodegenerative diseases.

Future studies will be aimed at identifying the motor(s) responsible for neurofilament movement and the mechanisms that govern this movement. Since phosphorylation is clearly a mechanism that regulates neurofilament assembly and transport, identifying the full complement of phosphorylation sites in neurofilament proteins and the kinases and phosphatases that regulate this phosphorylation will be important. With this information, further, dissection of the signal transduction mechanisms that control normal neurofilament function, and also neurofilament dysfunction in disease states will be possible.

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