Neurofilament function and dysfunction: involvement in axonal growth and neuronal disease

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Neurofilaments make up the major intermediate filament system in mature neurons. Recent studies demonstrate that neurofilaments in vivo are obligate heteropolymers and are required for proper radial growth of axons. Furthermore, forced over-expression of neurofilament subunits in transgenic mice shows that abnormal accumulation and assembly of neurofilaments, similar to that commonly found in human motor neuron disease, can directly cause motor neuron dysfunction.

Introduction

Neurofilaments are the major intermediate (10 nm) filaments in many types of mature neurons. Assembled from three polypeptide subunits, NF-L (68 kDa), NF-M (95 kDa) and NF-H (115 kDa), neurofilaments are most abundant in large myelinated axons. On the basis of sequence homology and the exon-intron structure of the genes encoding each subunit, neurofilaments, α-internexin and nestin comprise the type IV intermediate filament family. [Other intermediate filament families include types I and II (keratins), type III (desmin, GFAP, vimentin) and type V (nuclear lamins).]

Given their abundant presence in the thin axonal processes (which for human motor neurons can extend more than one meter in length), structural roles for neurofilaments in establishing or maintaining the unusual asymmetry of neurons have long been postulated. Indeed, since the initial visualization of neurofilaments by silver staining in the early 19th century, mounting correlative evidence has pointed to the importance of neurofilaments in specifying the diameter (caliber) of large myelinated axons. Furthermore, abnormal accumulation of neurofilaments has been seen as a common early feature of many motor neuron disorders [2,3]. To this background, although much progress had been made in resolving the factors important for neurofilament assembly by analysis of filaments assembled in vitro, a series of recent efforts have uncovered some surprising insights regarding the assembly and organization of neurofilaments in vivo, and have identified O-linked glycosylation to be a post-translational modification that may affect assembly properties. Finally, use of transgenic mouse models has provided evidence directly linking neurofilament abnormalities to neurodegenerative processes, particularly in motor neurons.

Neurofilaments are obligate heteropolymers in vivo

Early experiments with purified neurofilament subunits had shown that only NF-L can self assemble into 10 nm filaments in vitro (e.g. [2,3]), findings that prompted the view that neurofilaments in vivo consist of a core filament assembled from NF-L with both NF-M and NF-H assembling onto the NF-L filament backbone. Further analysis of in vitro assembled filaments, using site-directed antibodies against the neurofilament subunits and immunoelectron microscopy, confirmed this view that all three neurofilament subunits are incorporated integrally into the filaments [4,5] and that the tail domains of NF-M and NF-H extend away from the filament surface [6]. Despite this, in vivo reassembly requires an unphysiologically low pH (typically 5.2) and produces only short filaments, features which have always left nagging doubts about the faithfulness of in vitro assembly.

To complement and extend the in vitro efforts, in vivo filament assembly properties have now been examined using DNA transfection and transgenic mice. To no one's surprise, initial efforts showed that all three neurofilament subunits can individually co-assemble with vimentin into cytoplasmic filament networks [7,9]. Competence for co-assembly requires both the head and rod domains, but most of the tail domains are dispensable. Exploitation of SW13 IF- cells, a line in which vimentin expression has been silenced, in which vimentin expression has been silenced, has shown most recently that, unlike type III intermediate filament subunits (such as vimentin), none of the neurofilament subunits is capable of de novo assembly in the absence of an endogenous cytoplasmic intermediate filament network [10**]. Instead, network assembly requires NF-L, and a substoichiometric amount of either NF-M or NF-H. Furthermore, deletion

Abbreviations

ALS—amyotrophic lateral sclerosis; CDK—cyclin-dependent kinase; NF—neurofilament.
of the head or tail domain of NF-M or substitution of the NF-H tail onto an NF-L subunit reveals that restoration of in vivo NF-L assembly competence requires an interaction provided by the NF-M or NF-H head domains [11**]. Similarly, assembly synergy between NF-L and NF-M requires interactions provided by both the NF-L head and rod, as full length NF-M can restore assembly competence to tailless, but not to headless, NF-L. In all cases, unlike the earlier vimentin-driven assembly experiments, neurofilament subunits show strong bundling characteristics, with the resultant networks characterized by swirls of filaments encircling the nucleus [10**,11**].

The inability of individual neurofilament subunits to self-assemble has also been tested in a true in vivo context, through the use of transgenic mice and transgenes whose promoter elements direct expression of NF-L or NF-M in oligodendrocytes [11**]. These cells, which myelinate central nervous system axons, do not normally express cytoplasmic intermediate filaments. Extended arrays of filaments were not observable in oligodendrocytes expressing only NF-L or only NF-M, but oligodendrocytes from animals carrying both NF-L and NF-M transgenes contained prominent, closely spaced bundles of 10 nm filaments.

These results collectively demonstrate that neurofilaments in vivo are obligate heteropolymers requiring NF-L and either NF-M or NF-H. They also unequivocally establish that both NF-M and NF-H directly participate in the assembly and organization of neurofilaments. Both the obligate heteropolymeric nature and the tendency for these filaments to bundle laterally in vivo resemble the properties of keratin filaments. An important distinction, however, is that keratin filaments are stoichiometric heterodimers requiring one Type I and one Type II keratin, whereas neurofilament heteropolymers can apparently accommodate a wide range of NF-subunit ratios. This is a point of some significance, because the NF subunit ratios do change dramatically during neuronal development (e.g. [12]). Whether the requirement for heteropolymerization acts at the dimer, tetramer or higher-order oligomer level has not yet been determined, although the results of crosslinking experiments [13] and of antibody labelling of filaments assembled in vitro [5] are most easily explained by NF-L/NF-M or NF-L/NF-H heterodimers.

Unlike NF-L, NF-M and NF-H, α-internexin is self-assembly competent

Although individual neurofilament subunits are self-assembly incompetent in vivo, expression of the type IV subunit α-internexin revealed that it is able to assemble into an extended filament network in the absence of other intermediate filament proteins [10**]. Further-

more, each neurofilament subunit can co-assemble into these α-internexin filaments. The biochemical basis for the obligate, heteropolymeric assembly of neurofilaments, and the homopolymeric assembly of the highly related α-internexin, is not known. However, comparison of neurofilament and α-internexin protein sequences reveals that the rod domain of α-internexin is organized into segments called 1a, 1b, and 2, similar to those in NF-L, whereas the rod 1 domains of NF-M and NF-H do not have obvious subdivisions. On the other hand, the head and tail domains of α-internexin contain sequences homologous to NF-M. Thus, the apparent 'hybrid' nature of α-internexin may underlie its competence for homopolymer assembly in vivo. (Assembly characteristics of nestin, the final type IV subunit, have not yet been examined.)

Neurofilaments become less dynamic as axons mature: implications for mechanisms of axonal transport

Despite the apparent stability of neurofilaments, with almost all subunits remaining cytoskeleton-associated after extraction of soluble contents of the cell, microinjection of fluorescently tagged NF-L into cultured dorsal root ganglia neurons has now demonstrated that neurofilaments are not static structures in vivo [14**]. On the contrary, after allowing 24 to 36 hours for labelled subunits to assemble into axonal neurofilaments, recovery of fluorescence after photobleaching was seen to occur with a half-time of 40 minutes. This rate, although slow in comparison with the rates for actin or tubulin dynamics, is still remarkably rapid when viewed in the context of the 'expected' static filament array and the very small pool of soluble subunits available for exchange. Furthermore, the rate of fluorescence recovery is faster in rapidly growing axons, a finding consistent with stabilization of filaments in maturing axons.

Concerning the mechanism of neurofilament transport through axons, the bulk filament array does not move, as photobleaching failed to reveal any net movement of the bleached spot before recovery [14**]. If these neurons have developed an organized, slow axonal transport machinery, this experiment clearly demonstrates that the transported component must comprise a small minority of the filament subunits. To examine the site(s) of subunit incorporation in axonal neurofilaments, biotinylated NF-L was microinjected, and immunoelectron microscopy used to track the subunits after assembly [14**]. Incorporation was seen all along the axon at numerous discrete sites which slowly grew to yield continuous arrays by 24 hours after injection. Collectively, these results indicate that neurofilament assembly in growing axons is a dynamic process involving lateral subunit exchange and segmental incorporation of new subunits [14**].
Head and tail domains of NF-L and NF-M are post-translationally modified by addition of O-linked sugars: implications for assembly

In addition to being post-translationally modified by phosphorylation (particularly on NF-H), both NF-L and NF-M have now been identified to be substrates for addition of O-linked N-acetylglucosamine moieties [15]. Such cytoplasmic glycosylation is found on many proteins and, at least in some cases, is highly dynamic in a manner similar to phosphorylation (see review by Hart [16]). Following purification of neurofilaments from rat spinal cord, glycosylation of NF-L and NF-M was shown by using galactosyltransferase to add radiolabelled galactose to O-linked monosaccharides. Sites of glycosylation were identified by proteolysis of the labelled filament subunits, purification of radiolabelled peptides and manual Edman sequencing. Identification of the sugar moieties was achieved by mass spectrometry. NF-L was glycosylated at two positions (Thr21 and Ser27), both of which lie in the head domain. NF-M was also modified in two positions, once in the head (Thr48) and once in the tail (Thr431).

The discovery of glycosylation of neurofilament subunits, particularly within the head domain, strongly suggests that this modification may alter assembly properties. As introduced earlier, mounting evidence supports the direct involvement of the amino-terminal head region of most intermediate filament subunits in assembly (this issue, Heins and Aebei, pp 25–33), including neurofilaments [7–9,10**,11**]. Moreover, the amino-terminal head domains of Type III, IV and V subunits are known to be substrates for phosphorylation that, at least for types III and V, inhibits assembly or causes disassembly of filaments both in vitro and in vivo (see review pp 25–33). In this context, it seems highly likely that reversible glycosylation of neurofilament subunits, particularly on the head domains, affects assembly propertics. In fact, initial evidence for this has emerged from in vitro reassembly experiments of purified NF-L: wild-type, unglycosylated NF-L derived by bacterial expression assembles in vitro into thicker filaments than does authentic NF-L purified from spinal cord [17*]; see also pp 25–33. It remains for future efforts to document the compartments in neurons (cell bodies and/or axons) in which subunits are glycosylated and to define how each modification ultimately affects network assembly and organization.

Neurofilaments are required for proper radial growth of axons

Although neurofilaments (and other intermediate filaments) are minor components during initial elongation of neurites, after the neurons have successfully attached to their target neurons neurofilament expression is markedly elevated, myelination begins, and the fully elongated axons increase in diameter by up to five-fold (volume increases by up to 25-fold!) (e.g. [18,19]). It is in this second growth phase, referred to as radial growth, that neurofilaments become the most abundant cytoskeletal element, often exceeding the number of microtubules by up to an order of magnitude. Earlier correlative evidence had shown a linear relationship between neurofilament number and cross-sectional area throughout normal radial growth [18] and during re-growth following axonal injury [19], observations that strongly suggested that neurofilaments are intrinsic determinants of radial growth. This point has now been unequivocally proven with the identification of a mutant Japanese quail (named quiver or Quv) which accumulates no neurofilaments in axons [20]. In the homozygous state, the recessive quiver mutation yields a mild generalized quivering. The mutation results in a very low NF-L mRNA level (<5% of normal [21**]) and no detectable NF-L [21**,22**]. Sequencing of the NF-L gene from the mutant line has identified the lesion responsible for the absence of neurofilaments to be a point mutation that introduces a premature termination at codon 114 of NF-L [21**]. The absence of neurofilaments blocks normal radial growth, with all three components of the normal trimodal distribution of axonal calibers showing significant reduction [23*]. The inhibition of radial growth is most pronounced for the largest caliber axons, which normally comprise about two thirds of myelinated axons in mature nerves. Furthermore, as expected, because axonal diameter itself has long been argued to be a primary determinant of conduction velocity, direct measurement of conduction velocity in the small, medium and largest diameter axons reveals an overall twofold diminution in velocity for all three size classes [23*]. The analysis of the quiver quail leaves no doubt that neurofilaments are one key element required for radial growth and that failure of radial growth results in generalized quivering stemming from reduction of conduction velocity.

The mechanism through which neurofilaments mediate increases in axonal size remains unsettled. The linear correlation between neurofilament number and axonal cross-sectional area initially suggested that the axon expanded or contracted so as to maintain a constant density of neurofilaments [19]. However, analysis of transgenic mice that express elevated levels of wild-type neurofilament subunits has disproven this simple view. Increasing NF-L levels lead to an increase in the number and density of neurofilaments and to an inhibition of radial growth [24]. Thus, while neurofilaments are required for normal radial growth of axons, determination of axonal diameter by neurofilaments is specified by other factors in addition to simple filament number. One reasonable postulate is that in mice expressing higher levels of NF-L, it is the ‘dilution’ of NF-M and NF-H subunits (and the corresponding dilution of their tail domains) that inhibits the normal neurofilament-stimulated radial growth. Strong support for the involvement of the highly phosphorylated NF-M and NF-H tail domains in mediating axonal organi
zation has emerged from examination of the Trembler mouse [25]. In this mouse strain, myelination fails as the result of a mutation in the gene encoding myelin basic protein. The absence of normal myelination triggers a cascade of events that results in decreased phosphorylation of NF-H and NF-M, an increase in neurofilament density and inhibition of radial growth [25].

Search for neurofilament kinases: phosphorylation of NF-H by neuronal cdc2-related kinases

The likelihood that phosphorylation of the NF-II tail domain plays a central role in modulating neurofilament organization within the axon has prompted an extensive search for the kinase(s) responsible. Following the initial observation that a cdc2-like, cyclin-dependent kinase (CDK) can phosphorylate a subset of Lys-Ser-Pro repeats in the tail of NF-H [26], a search for such a CDK kinase was undertaken by screening a rat brain cDNA library with a cloned cdc2 sequence. This approach identified a novel CDK, which in situ hybridization proved to be expressed in neurons [27**]. Furthermore, several groups have now biochemically isolated kinases exhibiting CDK-like properties from bovine brain [28*] and from rat spinal cord [29**] on the basis of the kinase's ability to phosphorylate peptides containing a cdc2 consensus phosphorylation site (Xaa-Ser/Thr-Pro-Xaa-Lys, where Xaa is any amino acid). In one case [29**], the purified CDK was shown to phosphorylate specifically the consensus motifs present in the carboxy-terminal tail of both NF-H and NF-M. Although neither kinase reacts with an antibody to a domain that is conserved in authentic cdc2 kinases, cross-reactivity is found with antibodies to amino and carboxyl regions of cdc2 kinases.

In addition, another kinase (named tau protein kinase II or TPKII) with a cdc2-like specificity (i.e., phosphorylation of serines or threonines next to a proline) was first identified on the basis of its ability to phosphorylate the microtubule-associated protein tau [30], but most recently has also been shown to phosphorylate the tail domain of NF-H [31**]. Furthermore, phosphorylation of NF-H by TPKII retards mobility of NF-H in vitro, thus establishing that TPKII is probably phosphorylating the Lys-Ser-Pro sequences in the NF-H tail. This in vitro phosphorylation reduces the ability of NF-H to bind to microtubules [31**].

Despite their promise, both the cdc2-like kinase [29**] and TPKII [31**] phosphorylate only a small subset of available Lys-Ser-Pro sites (approximately 10%), and neither kinase is yet proven to play a significant role in the phosphorylation of NF-H. Indeed, an appealing view is that neurons may express multiple CDKs [32]. The involvement of multiple kinases has received strong experimental support by the recent identification of several kinases that bind to an affinity matrix of recombinant NF-H [33**]. Of particular interest is one 115 kDa kinase (NAK115) which co-immunoprecipitates with NF-H both in vitro and in vivo. NAK115 selectively phosphorylates NF-H in comparison with NF-L, although whether NAK115 phosphorylates the NF-II Lys-Ser-Pro motif is not yet known.

Neurofilaments and motor neuron disease

In addition to supporting the growth and maintenance of axonal caliber in normal neurons, it has been suspected that neurofilaments play a role in the pathogenesis of several types of neurodegenerative diseases, including the motor neuron diseases amyotrophic lateral sclerosis (ALS), infantile spinal muscular atrophy and hereditary sensory motor neuropathy. The common clinical symptom of motor neuron disease is progressive failure of motor neurons. This in turn causes atrophy of the skeletal muscles synapsed to the dying neurons, ultimately culminating in paralysis and death.

A central question has been whether aberrant accumulations of neurofilaments are merely by-products of the pathogenic process or whether they are active participants in motor neuron dysfunction. Partial resolution of this issue came from two studies [34**,35**] in which forced over-expression of neurofilament subunits in transgenic mouse models was shown to cause selective dysfunction of motor neurons. In one report, mating of two lines of transgenic mice, each of which expressed wild-type mouse NF-L at about twice the normal level, resulted in mice accumulating four times the normal level of NF-L [34**]. In these mice, the increase in NF-L was accompanied by large accumulations of neurofilaments in the motor neuron cell bodies, as well as a small but significant number of proximal axonal swellings and degenerating motor axons. Furthermore, the skeletal muscles showed signs of denervation atrophy, indicative of motor neuron dysfunction. Most of these doubly transgenic mice died at three to four weeks of age, although a few survived and eventually recovered, concomitant with silencing of the transgene promoter in motor neurons.

In contrast to the early onset and the rapid time course of the motor neuron pathology in the NF-L transgenic mice, forced over-expression of human NF-H in mice has been reported to result in slower onset, progressive neuronopathy [35**]. By introducing the human NF-H gene (including its flanking regulatory elements), transgenic mice were produced that expressed a three to fourfold increase in NF-H mRNA levels in...
spinal cord. The resulting mice displayed limb weakness with an onset beginning at three to four months of age. Morphological and ultrastructural analysis revealed perikaryal accumulations of neurofilaments in motor and sensory neurons, axonal swellings, atrophic axons (shrunken axons with reduced neurofilament content) and denervation atrophy of skeletal muscles. However, degeneration of motor axons was not found. In light of the >150 differences in amino acids between murine and human NF-H, an unresolved ambiguity is whether the human NF-H functions in mice as a wild type or as a mutant subunit.

The morphological effects of over-producing neurofilament subunits in motor neurons bear most striking resemblance to those observed in rapidly progressing motor neuron diseases, as well as to the early stages of ALS. In these cases, severe accumulations of neurofilaments are accompanied by only a minor loss of motor neurons. In this context, in many human examples, a relatively low frequency of swollen cell bodies and a higher proportion of degenerating axons may simply reflect the slow progression of disease, which allows compromised neurons to initiate subsequent degeneration. Although for neither set of transgenic animals have direct measurements of motor neuron function yet been reported, the presence of moderate to severe muscle atrophy in these transgenic mice suggests that mis-accumulation of neurofilaments can impair motor neuron function even before widespread loss of motor neurons occurs.

In any event, these transgenic animal models have established one point of pathological significance for human motor neuron disease: primary changes in the cytoskeleton, and specifically in neurofilaments, are sufficient to produce most of the pathological changes encountered in neurodegenerative diseases such as ALS. The data further reinforce the suggestion that abnormal accumulation of neurofilaments is a central pathological intermediary leading to subsequent axonal swelling and degeneration. Clearly, one of the next challenges will be to determine the mechanism(s) by which neurofilamentous accumulation results in neuronal dysfunction and death.

Conclusions

In the past few years, significant progress has been made in elucidating the factors that regulate neurofilament assembly and dynamics. Filaments are now known to be obligate heteropolymers requiring NF-L and either NF-M or NF-H. Photobleaching experiments have clearly demonstrated that filaments within neurites are dynamic, not static, as had been inferred from their resistance to dilution-induced disassembly. Post-translation modifications by phosphorylation and glycoylation probably affect assembly properties, but precisely how must still be determined. What is now certain is that in the normal context neurofilaments are an intrinsic determinant of radial growth of axons. Mis-accumulation of neurofilaments, however, is sufficient to cause motor neuron disease, a finding sure to stimulate a search for mutations in neurofilament-encoding genes as a proximal cause of some instances of human disease.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


In contrast with in vitro assembly experiments that have found NF-L to be competent for filament assembly, by using DNA transfection to express headless, tail-less and wild-type NF-L, NF-M and NF-H in cells without endogenous cytoplasmic intermediate filaments, neurofilament assembly in this in vitro context was found to require heteropolymers of NF-L and NF-M or NF-H.


14. OKAIBE S, MIYASAKA H, HIROKAWA N: Dynamics of the Neuronal Intermediate Filaments. Cell 1993, 121:375-386. By photobleaching following microinjection of fluorescently labelled NF-L into cultured dorsal root ganglion neurons, neurofilaments in situ were found to turn over within about 40 minutes. Not only does this demonstrate the dynamic nature of filament assembly, but the rate of exchange was found to slow as the axon matures. Furthermore, by microinjecting bioxin-labelled NF-L and localizing the subunits at the electron microscope level, the authors showed that the collective experiments supported a model of neurofilament assembly combining segmental incorporation of new subunits and lateral subunit exchange.

15. DONG D, XI Z, CHERRY M, COYTER R, CLEVELAND D, HART G: Glycosylation of Mammalian Neurofilaments. J Biol Chem 1993, 268:16679-16687. Neurofilament subunits NF-L and NF-M were demonstrated to be post-translationally modified by addition of a monosaccharide (N-acetylgalosamine) to serine or threonine residues. Such cytoplasmic glycosylation is now recognized to be an abundant modification and is likely to be rapidly reversible. Two sites of glycosylation were mapped within the NF-L head domain as well as sites in the head and tail of NF-M.


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23. SAKAGUCHI T, OKADA M, KITAMURA T, KAWASAKI K: Reduced Diameter and Conduction Velocity of Myelinated Fibers in the Sciatic Nerve of a Neurofilament-Deficient Japanese Quail mutant quiver. The absence of neurofilaments was demonstrated to inhibit radial growth and to lower conduction velocity by a factor of two.


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28. LEW J, BEAUDETTE K, LIWIN CM, WANG JH: Purification and Characterization of a Novel Proline-Directed Kinase from Bovine Brain. J Biol Chem 1993, 267:13385-13390. A kinase isolated from bovine brain was shown to direct phosphorylation of residues that have adjacent prolines. Both this substrate specificity and the immunoreactivity to anti-cdc2 kinase antibodies indicate that this kinase is cdc2-related.


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33. XIAO J, MONTEIRO M: Identification and Characterization of a Novel (115kDa) Neurofilament-Associated Kinase. J Neurosci, in press. A 115kDa kinase (NAR-115) was isolated by its binding to an affinity column of recombinant NF-H. This kinase specifically phosphorylates native and recombinant NF-H and co-immunoprecipitates with NF-H in vivo.

34. XU Z, CORK L, GRIFFIN J, CLEVELAND D: Increased Expression of Neurofilament NF-L Produces Morphological Alterations that Resemble the Pathology of Human Motor Neuron Disease. Cell 1993, 73:23-33. By constructing transgenic mice that accumulate wild-type mouse NF-L to about four times the normal level, the authors showed that increasing the burden of completely wild-type NF-L is sufficient to cause massive accumulations of neurofilaments in motor neuron cell bodies, proximal axonal swelling, axonal degeneration and severe skeletal muscle atrophy arising from failure of the innervating motor neurons. As these are the pathologic hallmarks of human motor neuron disease (including ALS), these findings strongly support...
the view that neurofilament mis-accumulation is an active participant
in neuronal dysfunction and degeneration.

35. COTE F, COLLARD J, JULIEN J: Progressive Neuropathay
in Transgenic Mice Expressing the Human Neurofilament
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By constructing transgenic mice that accumulate human NF-H to a
level about twofold above that of the endogenous mouse NF-H, Cote
et al. demonstrated that an increased burden of NF-H causes a series
of abnormal accumulations of neurofilaments in the cell bodies and
proximal axons and a progressive skeletal muscle atrophy. These re-
results offer strong support for the notion that modest upregulation in
NF-H can result in progressive neurological defects reminiscent of
those found ALS.

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