

Neurofilaments and Neurofilament Proteins in Health and Disease

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SUMMARY

Neurofilaments (NFs) are unique among tissue-specific classes of intermediate filaments (IFs) in being heteropolymers composed of four subunits (NF-L [neurofilament light]; NF-M [neurofilament middle]; NF-H [neurofilament heavy]; and α -internexin or peripherin), each having different domain structures and functions. Here, we review how NFs provide structural support for the highly asymmetric geometries of neurons and, especially, for the marked radial expansion of myelinated axons crucial for effective nerve conduction velocity. NFs in axons extensively cross-bridge and interconnect with other non-IF components of the cytoskeleton, including microtubules, actin filaments, and other fibrous cytoskeletal elements, to establish a regionally specialized network that undergoes exceptionally slow local turnover and serves as a docking platform to organize other organelles and proteins. We also discuss how a small pool of oligomeric and short filamentous precursors in the slow phase of axonal transport maintains this network. A complex pattern of phosphorylation and dephosphorylation events on each subunit modulates filament assembly, turnover, and organization within the axonal cytoskeleton. Multiple factors, and especially turnover rate, determine the size of the network, which can vary substantially along the axon. NF gene mutations cause several neuroaxonal disorders characterized by disrupted subunit assembly and NF aggregation. Additional NF alterations are associated with varied neuropsychiatric disorders. New evidence that subunits of NFs exist within postsynaptic terminal boutons and influence neurotransmission suggests how NF proteins might contribute to normal synaptic function and neuropsychiatric disease states.



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1 INTRODUCTION

Neurofibrils in the cytoplasm of neurons were first visualized with silver stains a century ago by the neuroanatomists Ramón y Cajal, Golgi, and others, who also noted their striking abundance in axons. Decades later, electron microscopy analyses revealed that neurofibrils in axons are bundles of 10-nm-diameter filaments (Schmitt and Geren 1950), which were designated “neurofilaments” (NFs) based on their location exclusively in neurons. NFs are now known to be members of the family of intermediate filaments (IFs) defined as cell-selective or cell-specific 10-nm filaments that are “intermediate” in diameter between actin filaments (6 nm) and myosin filaments (15 nm) found in muscle cells (Ishikawa et al. 1968). Six major classes of IF proteins are known: class I (acidic keratins), class II (basic keratins), class III (vimentin, desmin, peripherin, and glial fibrillary acidic protein [GFAP]), class IV (NF-L or neurofilament light, NF-M or neurofilament middle, NF-H or neurofilament heavy, α -internexin), class V (nuclear lamins), and class VI (nestin) (Dahlstrand et al. 1992; Steinert et al. 1999; Michalczyk and Ziman 2005; Guerette et al. 2007) (for an introduction to IFs, see Herrmann and Aebi 2016).

NFs are relatively sparse and tortuous in dendrites and perikarya, whereas in axons they can be numerous, mainly straight and unbranched, and very long (118 μ m on average) (Burton and Wentz 1992). The very abundant NFs in large myelinated axons are highly organized into parallel arrays, within which individual NFs are kept a minimum distance from each other by side arms that project perpendicularly from the filament core (Fig. 1). After axons establish synaptic contacts, signals from myelinating glia (Sanchez et al. 2000) induce many axons to expand radially as much as 10-fold, during which the NF number increases commensurately. Microtubules (MTs) can also proliferate in expanding axons, but, in the largest axons, NFs outnumber MTs by as much as an order of magnitude. Unlike most other IF systems (Schnapp and Reese 1982), NFs establish extensive cross-bridges with each other and with actin filaments and MTs through several types of cross-bridging proteins, including actin (Frappier et al. 1992), spectrin (Frappier et al. 1987, 1991), bullous pemphigoid antigen (BPAG) (Yang et al. 1996, 1999), plectin (Svitkina et al. 1996), and MT-associated proteins such as τ (Miyata et al. 1986) and MAP 1 and MAP 2 (Leterrier et al. 1982; Miyata et al. 1986; Frappier et al. 1991; Ma et al. 2000).

The size and composition of the cytoskeleton along large axons can be markedly nonuniform along their length. The abundance of NFs in the axonal cytoskeleton of peripheral nerves decreases more than twofold at more distal levels (Schlaepfer and Bruce 1990a), whereas the NF

number in optic axons increases threefold proximally to distally (Nixon and Logvinenko 1986). Moreover, at specific local sites along axons, abrupt striking threefold to fourfold variations in NF number and phosphorylation state can be seen, such as at the junction between unmyelinated and myelinated portions of retinal ganglion cell axons (Nixon et al. 1994b), at initial segments of dorsal root axons, and at nodes of Ranvier (Fig. 2) (Hsieh et al. 1994). NFs within the node of Ranvier, for example, are fewer and more closely packed than in the adjacent myelinated internode of the same axon (Hsieh et al. 1994). Although signals from myelinating cells modulate local accumulation of NFs in axons, myelin itself does not regulate local NF density (Sanchez et al. 1996; Lunn et al. 2002).

The unique structure of NFs and their prominent involvement in the pathogenesis of multiple neurological diseases have long made NFs a focus of active research. In this review, we summarize the current understanding of NF structure and function in health and diseases and draw attention to important unanswered questions.

2 SUBUNIT COMPOSITION OF NFs

NFs are distinguished from other types of IFs by the complexity of their subunit structure and composition. The first neuronal IFs to be isolated were derived from bovine myelinated nerve fibers and initially assumed to have only one subunit of 58 kDa (Shelanski et al. 1971), although these filaments were later shown to be predominantly glial filaments containing the class III IF GFAP as their major subunit. Studies of axonal transport in 1975 identified three proteins—of \sim 200-, \sim 160-, and \sim 68-kDa apparent molecular size by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis—that displayed identical patterns of slow translocation along axons (“slow component” of transport) under various conditions (Hoffman and Lasek 1975). Biochemical studies later confirmed the hypothesis that these proteins were subunits of NFs (NF-H, NF-M, and NF-L) (Liem et al. 1978; Schlaepfer and Freeman 1978) and identified an associated protein— α -internexin—as an IF-binding protein (Pachter and Liem 1985). Recently, α -internexin was shown to be the fourth subunit of NFs in the adult central nervous system (CNS) based on criteria used earlier to establish the other three subunits as NF components, including a characteristic pattern of axonal transport and subunit stoichiometry within 10-nm filaments, as well as new genetic criteria (Yuan et al. 2006b). Similar criteria applied to peripherin, another NF-associated protein selectively located in peripheral neurons (Portier et al. 1983; Parysek and Goldman 1987; Aletta et al. 1988), have established this protein as the fourth subunit of NFs in the adult peripheral nervous system (PNS) (Fig. 3)

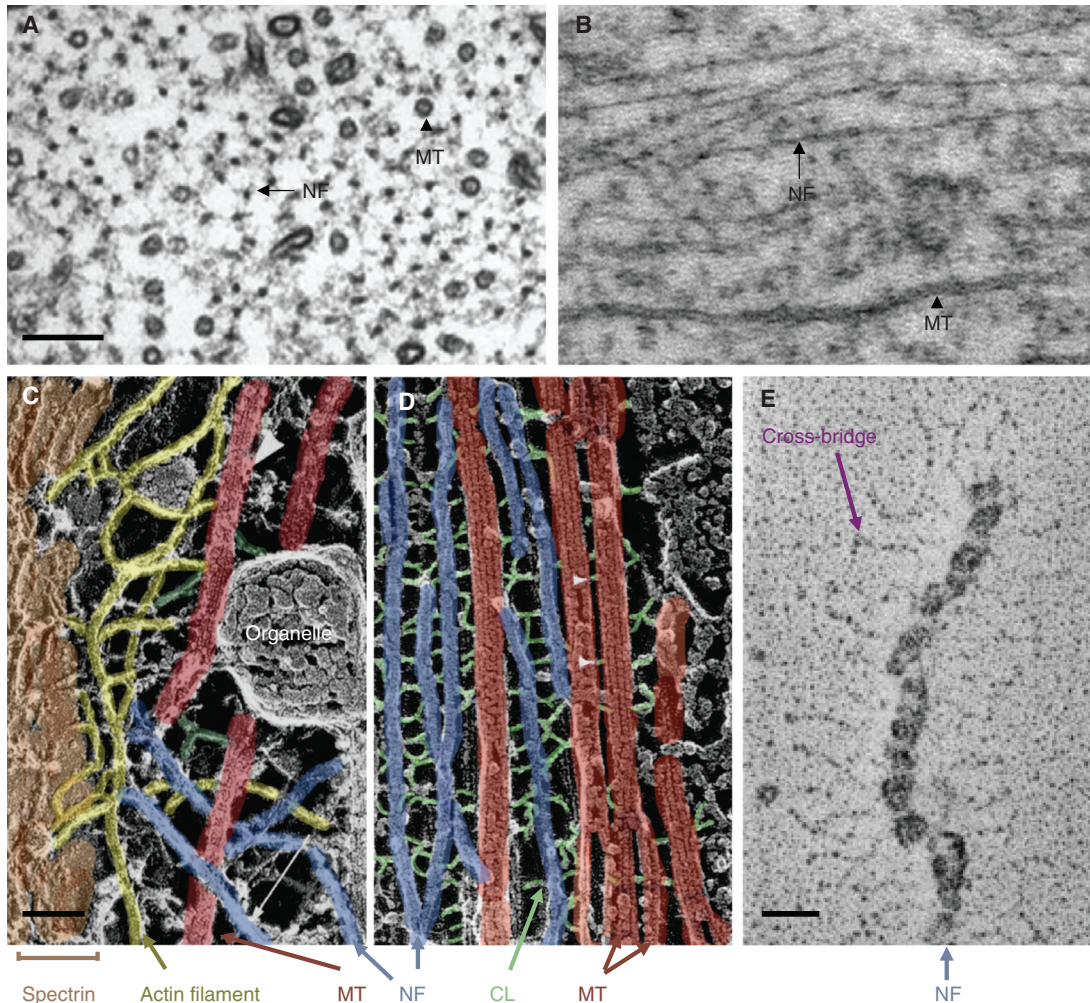


Figure 1. The neurofilament (NF) network in axons. Ultrastructural representation of NFs from mouse optic nerves in cross section (A) and longitudinal section (B). Arrows point to NFs, whereas arrowheads point to microtubules (MTs). Comparison of A and B illustrates bundles of parallel NFs and shows that individual NFs might be at a considerable distance from the nearest MT. Most NFs are regularly spaced from their neighbors as a result of the wispy projections of neurofilament middle (NF-M) and neurofilament heavy (NF-H) tails that emanate radially from the cores of some NFs in these mouse optic axons. These projections form cross-bridges between NFs, as visualized here in false-color images obtained by the quick-freeze deep-etching technique (C,D) (adapted, with permission, from Hirokawa 1982). The core cytoskeleton of axons typically comprises parallel arrays of NFs and MTs interconnected by cross-linker (CL) elements (D). Closer to the axolemma, the core cytoskeleton is cross-linked to the actin filament network and membrane skeleton by longer bridging elements (C). (E) Electron micrograph of an isolated NF prepared by rotary shadowing. (Adapted, with permission from Elsevier, from Hisanaga and Hirokawa 1988.) Scale bars, 100 nm (A,B); 50 nm (C,D); 20 nm (E).

(Yuan et al. 2012b). Three additional IF proteins—nestin, vimentin, and synemin—are present mainly in developing neurons and nearly disappear as neurons in the brain mature postnatally and express increasing amounts of the four subunits of NF. The progressive replacement of class III IF (vimentin) and class III/VI (nestin) filaments by heteropolymeric assemblies of class IV NF subunits as neurons mature and establish synaptic contacts is part of a developmental program thought to convert a more plastic cytoskeleton into a more permanent and rigid structure (Nixon

and Shea 1992). As the axon further matures, NF-L, NF-M, and α -internexin assemblies gradually acquire more NF-H subunits, and the progressive phosphorylation of NF-H and NF-M side arms promotes formation of a highly stable axonal NF cytoskeleton (Nixon and Shea 1992) extensively cross-linked to other stationary elements of the core and membrane cytoskeleton. This organization of the axonal cytoskeleton provides structural support and docking sites for reversible interactions with vesicular organelles (Steiner et al. 1987; Wagner et al. 2003; Rao et al. 2011) and molec-

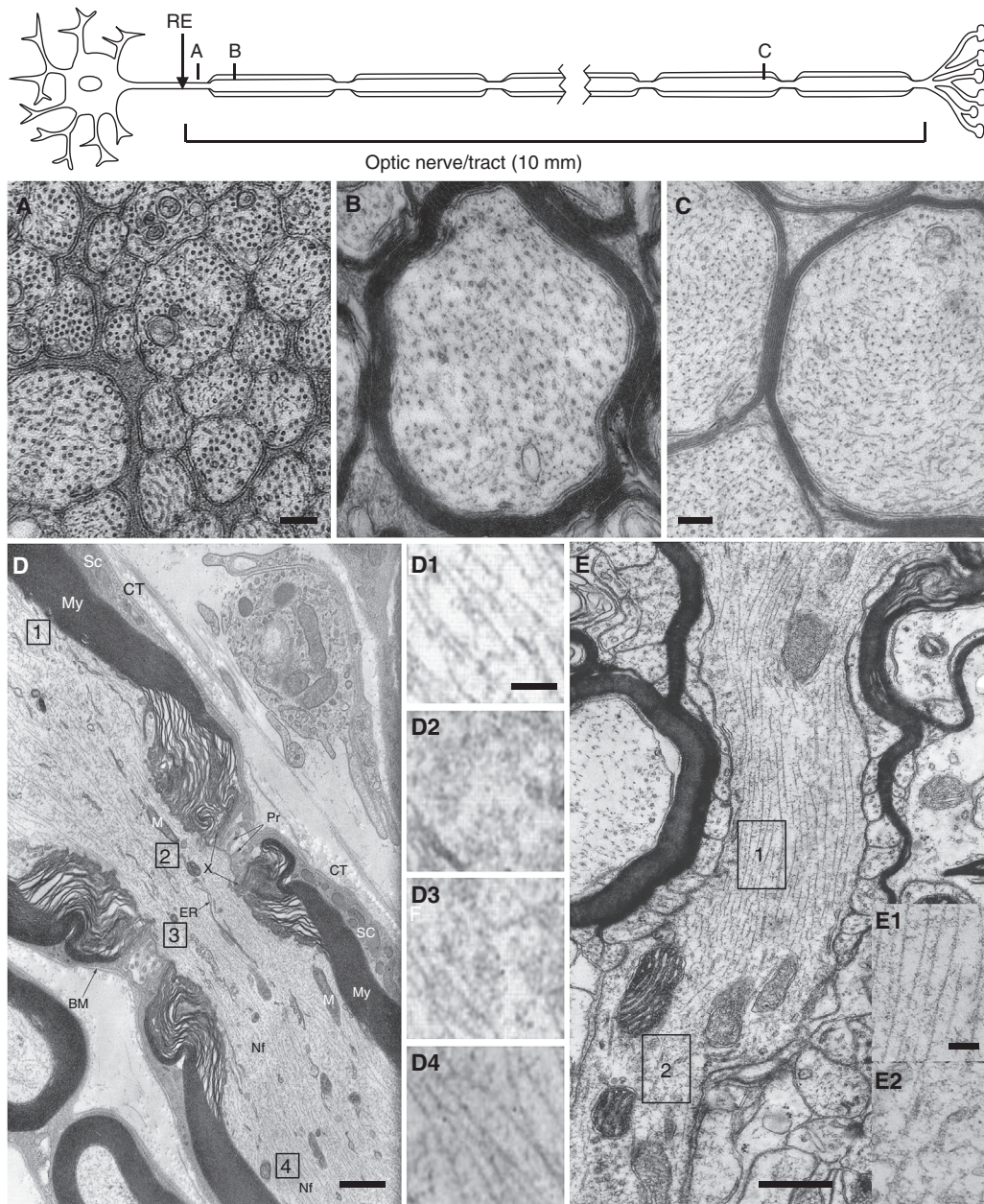


Figure 2. Nonuniform distribution of neurofilaments (NFs) along axons. (A–C) Electron micrographs of mouse optic axons magnified equally and viewed at three levels—50 μm (A), 2000 μm (B), and 7000 μm (C) from the eye—and summarized in cartoon form above. In these axons, NFs are sixfold more numerous distally than proximally. (D) Electron micrograph of a node of Ranvier in the long section showing the node of Ranvier flanked by internodal segments insulated by layers of compact myelin (My). (Adapted from Porter and Bonneville 1973.) Enlarged images of myelinated (1 and 4) and unmyelinated regions (2 and 3) are displayed in insets (D1–D4). (E) An electron micrograph of a different node of Ranvier in the long section showing how the myelin sheath terminates through widening rims of myelin loops and the ensheathed axon becomes naked in the region of the node of Ranvier (adapted, with permission, from Spacek 2004). Insets (E1 and E2) are enlarged images of myelinated (1) and unmyelinated (2) regions. The cytoskeleton organization of unmyelinated portions of the optic axons and node of Ranvier is strikingly different from that of the myelinated portions just a short distance away, reflecting the influence of oligodendroglial or Schwann cell signaling on NF organization. RE, retinal excavation. Scale bars, 200 nm (A,B,C); 300 nm (D); 100 nm (D1–D4); 600 nm (E); 100 nm (E1–E2).

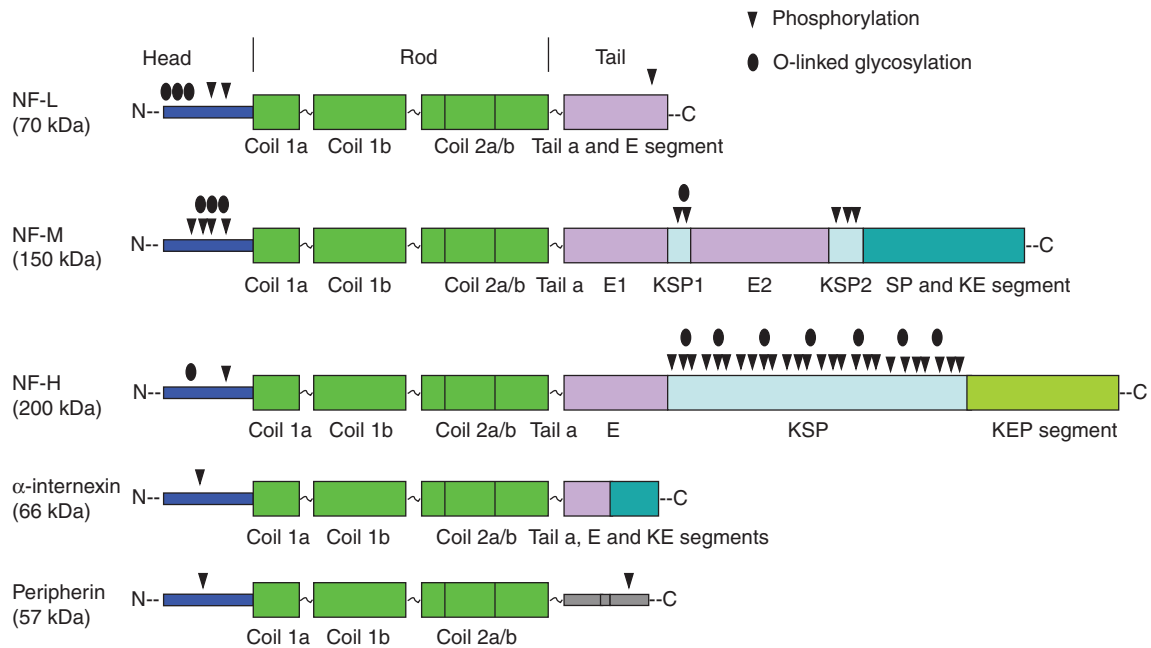


Figure 3. Domain structure and posttranslational modifications of neurofilament (NF) subunits. Neurofilament light (NF-L), neurofilament middle (NF-M), neurofilament heavy (NF-H), α -internexin, and peripherin are the subunits of NFs in the mature nervous system. All NF subunits include a conserved α -helical rod domain, comprising several coiled coils, and variable amino-terminal globular head regions and carboxy-terminal tail domains. NF-M and NF-H subunits are unique among intermediate filament (IF) proteins in having long carboxy-terminal domains with multiple lysine–serine–proline (KSP) repeats that are heavily phosphorylated. Phosphorylation and O-linked glycosylation sites on NF subunits are shown. E segment, glutamic-acid-rich segment; E1, glutamic-acid-rich segment 1; E2, glutamic-acid-rich segment 2; KE, lysine–glutamic acid; KEP, lysine–glutamic acid–proline; SP, serine–proline; KSP, lysine–serine–proline.

ular motors (Yabe et al. 1999; Shah et al. 2000; Rao et al. 2002a; Xia et al. 2003).

The molecular masses of NF-H, NF-M, NF-L, α -internexin, and peripherin predicted from the DNA sequences are 112.5, 102.5, 61.5, 55.4, and 53.7 kDa, respectively, in human and 117, 95.9, 61.5, 55.4, and 54.3 kDa, respectively, in mouse. These subunits migrate anomalously on SDS-containing gels because of an abundance of negatively charged amino acids (glutamic acids) in their sequences and posttranslational modifications such as phosphorylation and glycosylation. The commonly reported molecular masses on SDS-PAGE (polyacrylamide gel electrophoresis) are 200–220, 145–160, 68–70, 58–66, and 57–59 kDa for NF-H, NF-M, NF-L, α -internexin, and peripherin, respectively.

3 NF PROTEIN DOMAIN STRUCTURE AND ASSEMBLY

All NF subunits have a tripartite structure: a conserved central α -helical rod region (\sim 310 amino acids), a short variable head domain at the amino-terminal end (\sim 98 amino acids), and a tail of highly variable length at the

carboxy-terminal end (63–618 amino acids) (Fig. 3). The short head domain is rich in serine and threonine residues and contains consensus sites for O-linked glycosylation or phosphorylation. The central rod domain, shared with other members of the IF family, contains long stretches of hydrophobic heptad repeats, which favor the formation of α -helical coiled-coil dimers. The carboxy-terminal domains differ substantially in the lengths of long glutamic- and lysine-rich stretches, which mainly determine the sizes of the four NF subunits. Peripherin has a short tail that includes a single phosphotyrosine residue (Angelastro et al. 1998). The NF-L tail is also short and contains many glutamic acid residues (an “E segment”), whereas the NF-M and NF-H tails are much longer and contain multiple E segments. There are six to 15 repeats of a Lys–Ser–Pro (“KSP”) motif in the tail domain of NF-M, depending on the mammalian species, whereas the number of KSP repeats in NF-H ranges from 40 to 51 (Fig. 3). NF-H and NF-M also have one or a few shorter “SP” motifs lacking an amino-terminally adjacent lysine.

On their own, NF-M and NF-H appear to be assembly incompetent, but NF-L, peripherin, and α -internexin can form 10-nm homopolymeric filaments in vitro and hetero-

polymers with other NF subunits (Yuan et al. 2006b, 2012b). The subunit stoichiometry of NFs can vary in different neuronal populations and during the period of neuronal development—the four NF subunits NF-L, NF-M, NF-H, and α -internexin invariably compose CNS NFs, whereas peripherin, NF-L, NF-M, and NF-H compose NFs in the PNS (Yuan et al. 2006b, 2012b). The backbone of the filament is mainly formed by NF-L, plus peripherin or α -internexin, whereas NF-M and NF-H associate more peripherally (Leermakers and Zhulina 2010). The two largest subunits—NF-M and NF-H—are distinguished from the other NF subunits, and other IF proteins, by the exceptional lengths of their tail domains (Geisler et al. 1983; Franke 1987; Steinert and Roop 1988; Shaw 1991). These tail domains are localized peripherally along the filament core (Fig. 1) (Willard and Simon 1981; Sharp et al. 1982) and, especially when extensively phosphorylated, these domains project radially from the filament core to form side arms that maintain a minimal spacing between individual NFs and between NFs and MTs (Pant et al. 1978; Letierrier et al. 1982; Liem and Hutchison 1982; Julien and Mushynski 1983; Hirokawa et al. 1984; Nakagawa et al. 1995; Chen et al. 2000; Rao et al. 2002b, 2003). Finally, recent evidence indicates that α -internexin fine-tunes the NF network through increasing interfilament spacing (Kornreich et al. 2015).

Assembly of NF subunits into NFs does not require nucleotide binding or hydrolysis but is strongly dependent

on ionic strength, pH, and temperature (Angelides et al. 1989). The formation of NFs, like that of other IFs, is believed to begin when two monomers associate into polar coiled-coil dimers (45–50 nm in length), which is the basic and essential building block of NFs (Fig. 4). The mechanism that specifies heterodimeric interaction among NF proteins is believed to reside in the tail domains (Carpenter and Ip 1996). Two dimers then form staggered antiparallel tetramers. As a result of this antiparallel arrangement, NFs lack polarity, unlike the polar structure of MTs and actin filaments, which have two distinct ends. The lateral association of eight tetramers (a total of 32 monomers) results in the formation of cylindrical structures known as unit-length filaments (ULFs), with an approximate diameter of 16 nm. Gradual end-to-end annealing of these ULFs in the longitudinal direction results in filament elongation, which is followed by radial compaction to ultimately achieve the final long NF of diameter 10 nm (Fig. 4) (Fuchs and Weber 1994; Herrmann and Aebi 2004; Herrmann et al. 2007; Goldman et al. 2008) (for further assembly details, see Herrmann and Aebi 2016).

4 NF PROTEIN EXPRESSION

The NF-M subunit exists in all vertebrates, and all other NF proteins are believed to have originated by gene duplication from an ancestral NF-M-like protein. The most primitive

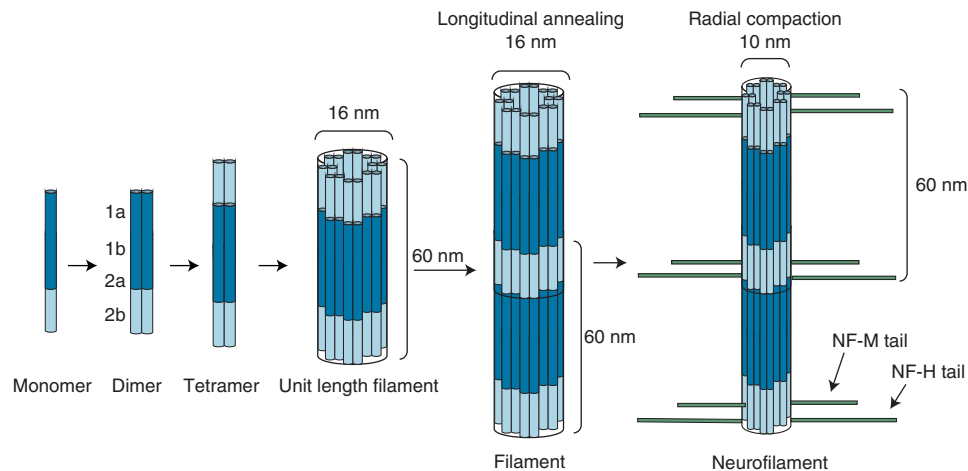


Figure 4. Neurofilament (NF) assembly. Different monomers of NFs form parallel coiled-coil heterodimers between subunit core domains. Two dimers then form staggered antiparallel tetramers through interactions between coil domains 1a, 1b, and 2a (for further details, see Herrmann and Aebi 2016). The lateral association of eight tetramers results in the formation of cylindrical structures known as unit-length filaments (ULFs), with an approximate diameter of 16 nm and length of 60 nm. Gradual end-to-end annealing of these ULFs in the longitudinal direction results in filament elongation, which is followed by radial compaction to ultimately achieve the mature long NF polymer of diameter 10 nm. Tail domains of neurofilament middle (NF-M) and neurofilament heavy (NF-H) radiate outward from the filament core because of the extensive negative charges arising from large numbers of glutamic acid and phosphorylated serine and threonine residues. Tail repulsion is not driven by the phosphorylation of the head domain of NF-H and NF-M.

living vertebrate is the lamprey, which has only a single NF-M-like subunit (NF-180) (Hall et al. 2000), but more-recent studies suggest that this subunit might need other NF proteins for filament assembly (Jin et al. 2011). In tiger salamander olfactory axons, NF-M is abundant, but NF-L and NF-H subunits are present at trace levels, resulting in formation of only rare NFs (Burton and Wentz 1992). The molecular subunit composition of NFs progressively changes during axon development. In the developing *Xenopus laevis* spinal cord, peripherin emerges at the earliest stages of neurite outgrowth. NF-M and α -internexin (or α -internexin-like *Xenopus* protein) appear later, as axons continue to elongate, and NF-L is expressed after axons contact muscle (Undamatla and Szaro 2001). In developing mouse myenteric neurons, peripherin, α -internexin, and NF-M emerge simultaneously before the appearance of NF-L and NF-H subunits, indicating a close relationship among peripherin, α -internexin, and NF-M (Fausone-Pellegrini et al. 1999). The expression of NF-H during postnatal development is delayed compared with that of NF-L and NF-M (Shaw and Weber 1982; Pachter and Liem 1984; Carden et al. 1987; Schlaepfer and Bruce 1990b; Giasson and Mushynski 1997a). Reduced NF expression is a general response to axonal injury in both PNS and CNS axons (Hoffman and Cleveland 1988; Hoffman et al. 1993). In normal regenerating axons, NF subunits reemerge in a temporal sequence reminiscent of that in developing neurons (Zhao and Szaro 1995).

5 POSTTRANSLATIONAL MODIFICATIONS OF NF PROTEINS

NF proteins are modified, after they are translated, by attachment of phosphate and O-linked *N*-acetylglucosamine (O-GlcNAc) to individual serine and threonine residues. In addition to these well-established modifications, NFs can also be modified to some extent by nitration, oxidation, and ubiquitination. NFs are among the most highly phosphorylated proteins in brain tissue. The phosphorylation status of NFs depends on a complex regulation between NF kinases and phosphatases (Nixon and Sihag 1991). All subunits are phosphorylated at their amino-terminal head domain, whereas only the high-molecular-mass subunits (NF-H and NF-M) are extensively phosphorylated along their carboxy-terminal tail domains (Fig. 3) (Nixon and Sihag 1991; Pant and Veeranna 1995; Sihag et al. 2007). NF head-domain phosphorylation occurs in the cell body, and phosphate turnover on this domain is relatively dynamic as NFs enter the axon (Sihag and Nixon 1991), suggesting roles in regulating initial events in NF subunit behavior. The more extensive phosphorylation of NF-M and NF-H tail domains is delayed until newly formed NF

assemblies have translocated into axons, and many of these added phosphate groups are slowly turned over (Nixon et al. 1994a, 1994b). Subunit phosphorylation is mediated mainly by second-messenger-dependent protein kinases (Nixon and Sihag 1991; Pant and Veeranna 1995; Grant and Pant 2000; Sihag et al. 2007) and is, therefore, subject to modulation by a vast array of second-messenger signals (McKnight 1991; Perrot et al. 2008).

Phosphorylation mediated by protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase type II (CAMK II) acting along the head domain plays a key role in regulating the assembly of NFs. Both PKA and PKC are able to phosphorylate NF-L at serine 55 and serine 23 (Sihag and Nixon 1991; Sihag et al. 1999), and a proline-directed threonine (Thr21) is phosphorylated by cyclin-dependent kinase 5 (cdk5), mitogen-activated protein kinase 3 (Erk1), and mitogen-activated protein kinase 1 (Erk2) (Sasaki et al. 2006). Serine residues 12, 27, 33, and 51 on NF-L phosphorylated by PKC promote disassembly (Gonda et al. 1990; Giasson et al. 1996). Similar proline-directed phosphorylation sites exist in the NF-M head, and PKA is implicated for at least one of these sites (serine 23) (Sihag et al. 1999). It is less clear, however, why there are more head-domain phosphorylation sites on NF-M than on other subunits, and what functions are served by the complex changes in phosphate topography on the NF-M head domain occurring as NF subunits are transported and phosphates are removed from certain sites (Sihag and Nixon 1991). Phosphorylation of the peripherin head domain induces filament fragmentation (Giasson and Mushynski 1998) and, in pheochromocytoma PC12 cells, is enhanced by nerve growth factor (NGF)-induced activation of PKA and PKC (Aletta et al. 1989). Similarly, phosphorylation of the α -internexin head domain by PKA influences its assembly and disassembly (Giasson and Mushynski 1998). Collectively, these findings point to assembly/disassembly being the major function of head phosphorylation. They also suggest that it is important to regulate the timing of subunit assembly to prevent pathological aggregation of NFs within the cell body, which disrupts their export into axons. Consistent with this idea, NF head domain phosphorylation also prevents phosphorylation of the tail domain within the cell body (Zheng et al. 2003). Beyond these roles, phosphorylation of the head domain of NF-L and NF-M alters the interactions of NFs with protein ligands, including protein 14-3-3 (Miao et al. 2013), the secretory regulator synapsin (Steiner et al. 1987), and brain spectrin (Frappier et al. 1987) and, not surprisingly, influences neurite outgrowth (Kesavapany et al. 2003).

The carboxy-terminal tail domains of NFs are phosphorylated on their entry into and transit along the axon

(Nixon and Sihag 1991). Most of this phosphorylation occurs at the KSP motif and the related smaller serine/threonine KSP repeats that vary in number across mammalian species (Barry et al. 2010). Human and mouse NF-H have 45 and 51 KSP repeats, respectively (Kesavapany et al. 2003). There are two major types of KSP repeats in the NF tail domain, KSPXK and KSPXXXK, in which X represents any amino acid.

The KSPXK type is the majority in human NF-H, whereas the KSPXXXK sequence comprises the majority of KSP motifs in rodents. This distinction is important because of the specificities of the protein kinases that regulate phosphorylation of these two motifs—cyclin-dependent kinases (CDKs) phosphorylate only KSPXK sites, and mitogen-activated protein kinases (MAPKs) preferentially phosphorylate KSPXXXK sites (Shetty et al. 1993; Veeranna et al. 1998; Kesavapany et al. 2003). The state of phosphorylation of the tail domains increases proximodistally along axons (Nixon et al. 1987), and rates of phosphate turnover on the different subunits vary along the axon (Nixon and Lewis 1986). The proline-directed kinases, CDKs, extracellular signal-regulated kinases (ERKs), Jun amino-terminal kinases (JNKs), p38 kinases, and glycogen synthase kinase 3 (GSK3) phosphorylate the tail domain of NFs (Sihag et al. 2007), and these kinases are influenced by signaling cascades responsive to stress, neurotransmitters, growth factors, and other receptor-mediated signaling alterations. The cross talk among kinases, and between kinases and phosphatases, is also important in regulating NF tail domain phosphorylation (Veeranna et al. 2011). Interestingly, myelination and synaptogenesis influence tail domain phosphorylation (de Waegh et al. 1992; Sanchez et al. 2000). As synaptic connections are established, bidirectional signaling from myelinating glial cells (Sanchez et al. 1996, 2000) initiates a marked radial expansion of axons supported by commensurate accumulation of NFs in axons, which is accompanied and possibly mediated by the increased phosphorylation of NF-M and NF-H tail domains (Sherman et al. 2012). Phosphates turn over while NFs are in the cell body and during their entry and transit along the axon, and only some of them are replaced by new phosphates, suggesting that the topography of phosphates on NFs changes in relation to functions served in different domains of the neuron (Nixon and Lewis 1986; Saito et al. 1995).

Less is known about NF phosphatases. The phosphatases associated with NFs dephosphorylate NF-H, causing a shift in its electrophoretic mobility, which might imply a change in conformation (Guru et al. 1991). The observation that okadaic acid, an inhibitor of protein phosphatases PP2A and PP1, enhances NF phosphorylation in the cell body and neurites suggested that these phosphatases are involved in regulating NF phosphorylation in several neu-

ronal cell types (Hall and Kosik 1993; Giasson et al. 1996). PP2A dephosphorylates cdk5 sites in NF-H (Veeranna et al. 1995). PP2A and PP1 associate with enriched preparations of NFs from bovine brain, and PP2A was implicated as the major phosphatase for NFs in this *in vitro* preparation, with PP1 playing a lesser role (Strack et al. 1997). Although the activity of NF kinases decreases during maturation and aging (Veeranna et al. 2011), this is more than offset as NF tail domains actually become progressively more phosphorylated over time, mainly because of the decreasing activities of protein phosphatases PP2A and PP1 (Veeranna et al. 2011).

6 NF PROTEIN DEGRADATION

NF proteins are substrates of multiple proteolytic systems, including calpains, the proteasome, and autophagy (Nixon and Marotta 1984; Hollenbeck and Bray 1987; Suzuki et al. 1988; Shaw et al. 2004; Song et al. 2012)—although the contributions of each system to turnover in different regions of the neuron under physiological conditions or in disease states are poorly understood. NF assembly confers significant proteolytic resistance to NF subunits because deletion of three NF subunits leads to degradation of the fourth subunit (Yuan et al. 2015c). Moreover, multiple *in vivo* pulse-labeling studies indicate that NFs are extraordinarily stable in axons (half-life of ~55 d) (Nixon and Logvinenko 1986; Yuan et al. 2009; Rao et al. 2012). By an independent method, Julien and colleagues also showed that NFs have extremely long half-lives in axons *in vivo* (Millecamps et al. 2007). In that study, NF-L expression in a conditional NF-L knockout mouse was acutely shut off and the fate of the preexisting NFs was monitored. Preexisting NFs in the axon remained stationary and displayed exceptionally slow turnover. The observed half-lives of between 4.5 and 8 mo are much longer than would be predicted by a loss through slow axonal transport.

NF-H and NF-M subunits are highly sensitive to proteases once they are dephosphorylated (Pant 1988), suggesting that phosphate groups on these subunits confer resistance to protease cleavage and, hence, stability to the NF cytoskeleton in axons. Indeed, eliminating the NF-H and NF-M tails substantially reduces stability of the NF cytoskeleton *in vivo* and increases the degradation of NF-L and the truncated, tailless NF-M and NF-H subunits (Rao et al. 2012). These data suggest that carboxy-terminal tails of NF-H and NF-M together impart stability to NFs.

It was earlier believed that NFs must be mainly degraded at the synapse if it is assumed that they are moving continuously in bulk into the nerve terminals by axonal transport (Roots 1983), although the metabolic burden on neurons, especially within the presynaptic terminal,

would need to be enormous in this scenario. Instead, later evidence that the NF network is stationary and turns over very slowly (Nixon 1998; Millecamps et al. 2007) explains how a large NF population in mature axons can be maintained by a small population of transported precursors at a much smaller cost of energy. Finally, the even smaller proportion of NF proteins that undergoes axonal transport but is not incorporated into the axonal cytoskeleton might ultimately reach the presynaptic terminal and be degraded at this site (Roots 1983; Hoffman et al. 1992).

7 AXONAL TRANSPORT OF NF PROTEINS

Axonal transport comprises both anterograde and retrograde traffic, the components of which can be subclassified into fast or slowly transported cargoes. NF proteins, as well as MT proteins, are transported in the slow transport component, which carries at varying slow velocities a wide assortment of nonvesicular protein cargoes. Membranous organelles are the main constituents of the fast transport component, and although the molecular mechanisms underlying their transport have become increasingly clear (Hirokawa et al. 2010; Zala et al. 2013), those regulating the slow transport of cytoskeletal and associated proteins have been more elusive, despite significant recent progress.

7.1 The Transport Forms of NF Proteins

Because methods were initially not available to visualize NF subunits or manipulate their levels individually, the assembly state of NF proteins during axonal transport was a subject of much debate during the 1980s and 1990s (Baas and Brown 1997; Hirokawa et al. 1997; Nixon 1998). With the application of newer *in vivo* genetic approaches and live-cell imaging techniques, evidence now supports both sides of the original controversy over whether NFs are translocated as assembled polymers or as individual subunits and oligomers. On the one hand, live-cell imaging methods revealed movements of individual short NFs (1.0–15.8 μm in length) along the relatively NF-poor growing axons of developing neurons transfected with green fluorescent protein (GFP)-labeled NF subunits (Roy et al. 2000; Wang et al. 2000; Yuan et al. 2009). On the other, fluorescent puncta representing nonfilamentous assemblies of GFP-labeled NF subunits (Yabe et al. 1999; Prahlad et al. 2000) have been observed in axons. Moreover, *in vivo* analyses of transport along myelinated axons of adult mice in which single or multiple NF genes are deleted showed that formation of complete NFs is not a requirement for the transport of NF subunits (Yuan et al. 2003, 2006b). These analyses established that the minimum requirement for NF transport is a heterodimer containing

either NF-M or α -internexin as obligatory subunits (Yuan et al. 2003).

Seemingly conflicting observations about the assembly state of transported NF subunits have been reconciled by photobleaching analyses of GFP-tagged NF-L in cultured primary neurons, which showed transport of both nonfilamentous NF subunit assemblies and short NF polymers in the same axon (Yuan et al. 2009). At proximal axon levels, nonfilamentous NF subunit assemblies are transported, whereas at distal levels of the same axon, predominantly short NFs are observed in transit. Collectively, the observations show that NF proteins exist in multiple assembly forms during axonal transport and suggest that nonfilamentous heterooligomers assemble into filaments as they are transported (Yuan et al. 2009). At a critical level of NF expression in these growing neurons, transported NF assemblies and short NFs establish a stationary network beginning at proximal axonal levels. Once axons have fully matured, *in vivo* pulse-radiolabeling studies have shown that NFs along the entire axonal length have been integrated into a stationary cytoskeletal network (Nixon and Logvinenko 1986). It is possible that NFs elongate within this structure, given that end-to-end annealing of short NFs to longer NFs has been seen in mouse cortical neurons in culture (Uchida et al. 2013).

7.2 Motors Mediating NF Protein Transport

The principal motor protein superfamilies (Miki et al. 2001; Brown and Bridgman 2004)—MT-based kinesins and dynein, and actin filament-based myosins—have often been considered candidate motors for the translocation of NFs at slow rates (Prahlad et al. 2000; Alami et al. 2009; Sunil et al. 2012). Kinesin-I has been proposed as an anterograde motor for NF based on its reported interactions with NF-H or NF-M subunits (Yabe et al. 2000; Jung et al. 2005) and on the inhibitory effect of antibodies against kinesin-I on NF transport (Yabe et al. 1999). NF-H is dispensable for NF transport (Rao et al. 2002b; Yuan et al. 2006b), however, implying that if kinesin-I is an NF motor, a different NF subunit is likely the mediator of this transport. Although this issue is not yet resolved, additional investigations in mice lacking kinesin-I A (an isoform of kinesin-I) (Xia et al. 2003) or in cultured neurons expressing mutant kinesin-I (Wang and Brown 2010) have supported the earlier findings. Because kinesin-I transports many different cargoes, including synaptic vesicles, synaptic membranes, mitochondria, lysosomes, and tubulin dimers (Hirokawa et al. 2010), it is conceivable that the NF transport and distribution changes are secondary effects arising from interference with the role of kinesin-I with respect to these other cargoes.

The dynein–dynactin complex is believed to be the motor mechanism for retrograde transport of NFs based on evidence that dynein interacts directly with the rod domain of the NF-M subunit in yeast two-hybrid assays (Wagner et al. 2004) and the dynein–dynactin complex copurifies with NFs (Shah et al. 2000). Also consistent with this notion, knockdown of dynein heavy chain expression using small interfering RNA (siRNA) significantly decreases retrograde NF transport (He et al. 2005; Uchida et al. 2009).

Myosin Va binds directly to NF-L, transports together with NFs in the slow phase of axonal transport, and influences the organization and density of NFs in axons (Rao et al. 2002b). Further transport analyses have suggested that myosin Va might support short-range NF movement by decreasing the duration of NF pauses (Jung et al. 2004; Francis et al. 2005; Alami et al. 2009). Despite these advances, interactions between candidate motors and NFs have not yet been fully characterized in live-cell imaging and *in vivo* studies, and important aspects of the molecular mechanisms regulating NF motility are still not well understood.

One model of transport posits that slow and fast rates are determined by differences in the amount of time the cargo spends pausing rather than by differences in transport velocity (Brown 2000; Li et al. 2012). In this model, the slow rate of NF transport would reflect long pauses alternating with rapid movement at a single velocity (Wang et al. 2000). However, an alternative view coming from computational modeling by Mitchell and Lee suggests that a single motor can carry an NF and that no cooperativity is required to produce the slow-transport velocity profile (Mitchell and Lee 2009). In contrast, cooperativity is required to produce the experimental velocity profiles for fast transport (Mitchell and Lee 2009), consistent with evidence that the average velocity of gliding MTs can transition from slow to fast, depending on the number of active motors engaged (Scharrel et al. 2014), and that larger organelle cargoes require multiple motors for fast transport (Kural et al. 2005). The enrichment of kinesin in fast axonal transport is consistent with the computational modeling of Mitchell and Lee (Elluru et al. 1995). Further investigation is therefore needed to address whether a single motor can explain both slow and fast anterograde NF transport or whether multiple motors, rather than a “fast motor,” are required to support the velocities of cargoes in fast transport.

7.3 Formation and Maintenance of the Axonal Cytoskeleton by Transported NF Precursors

Both *in vitro* (Trivedi et al. 2007; Yuan et al. 2009) and *in vivo* studies (Nixon and Logvinenko 1986; Millecamps

et al. 2007) have documented the existence of two populations of NFs—one undergoing slow-phase transport and a second stationary population comprising the NF network distributed nonuniformly along axons in patterns. Formation of the stable NF network, which has been visualized by time-lapse imaging of cultured neurons, is triggered in axons when NF expression reaches a critical threshold (Yuan et al. 2009), as occurs during normal brain development *in vivo* (Nixon and Shea 1992). *In vivo* pulse-labeling studies have shown that once newly synthesized NF proteins incorporate into the NF network, their distribution does not change for many months, even as the cytoskeletal network is very slowly turned over within the axon (Nixon and Logvinenko 1986; Nixon 1998; Millecamps et al. 2007) at a rate much slower than can be explained by the anterograde axonal transport velocity of NFs (Hoffman and Lasek 1975). For example, in conditional NF-L gene knockout mice, a third of the NF-L subunits are still present in axons 8 months after NF-L synthesis is shut off (Millecamps et al. 2007). These and other observations on mature neurons have shown that newly synthesized NF proteins undergoing transport are a small population compared with the size of the stationary population (Fig. 5) and, thus, correspond to the pool of precursors that maintains the large stable NF network along axons (Nixon and Logvinenko 1986; Hirokawa 1993; Yuan et al. 2009). Consistent with this concept, the transport rates and abundance of newly synthesized NF proteins are not invariable predictors of axonal NF content and radial growth, as proposed previously (Hoffman et al. 1984, 1985). For example, optic axons of mice lacking NF-L and NF-H have 90% fewer NFs and greatly reduced axon calibers, yet NF-M is transported at normal slow transport rates. Moreover, in mice lacking the carboxy-terminal tail domains of both NF-M and NF-H, NF number is reduced sixfold proximally and threefold distally along optic axons, but the rate of NF transport is unaltered (Rao et al. 2012). The minimal impact of NF transport rate on steady-state NF content in axons strongly implies that NF precursors undergoing transport comprise only a small pool in axons. Recent evidence suggests that axonal NF content can be dissociated from NF transport rate and indicates that the rate of incorporation of transported NF precursors into the metabolically stable cytoskeletal network is the major determinant of axonal NF content, enabling the generation of the striking local variations in NF number seen along axons (Yuan et al. 2015a).

The formation of a stationary NF cytoskeleton allows neurons, especially those with long axons, to conserve energy while maintaining a large NF network that can constitute as much as 15% of the total protein content of peripheral nerves (Yuan et al. 2012b). The local regulation

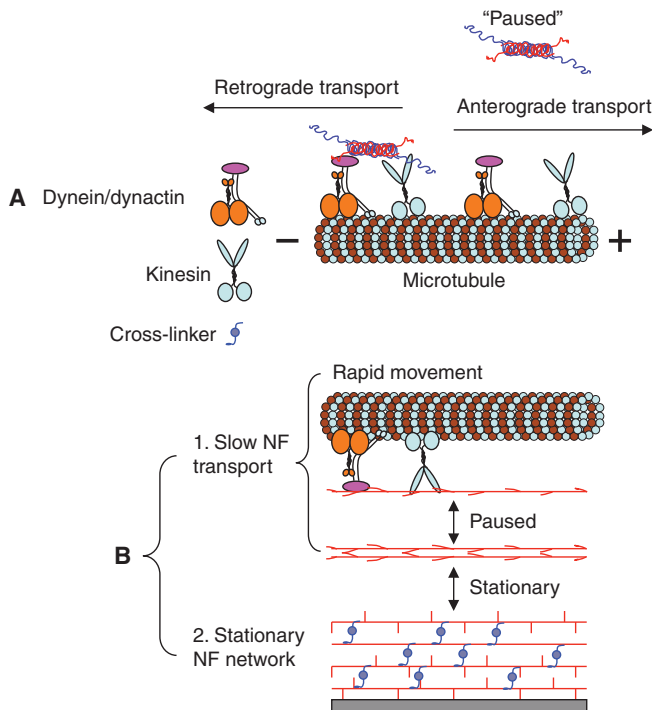


Figure 5. Formation of the axonal neurofilament (NF) network. (A) Transport of short NFs or NF heterooligomers (Yuan et al. 2003, 2009) by molecular motors (Prahlad et al. 2000; Shah et al. 2000) is bidirectional. Here, an NF assembly is depicted moving in the retrograde direction (toward the “minus end”) along a microtubule (MT), driven by dynein/dynactin. Another NF assembly is paused and was dissociated from kinesin in the anterograde direction (toward the “plus end”). (B) (1) Slow transport of NF proteins is the net rate achieved from the many intermittent, rapid motor-driven movements accompanied by “pauses,” possibly reflecting the reversible attachment of NFs (long filament with side arms) to a molecular motor moving on an MT. (2) Stationary NF networks can arise by a process in which NFs are prevented from reattaching to the available molecular motors, presumably through interactions with stationary axonal structures (e.g., MTs and the membrane skeleton), indicated by cross-linking proteins (e.g., BPAG [bullous pemphigoid antigen] and plectin).

of the rates of incorporation of NF precursors into the NF network (Lewis and Nixon 1988; Sanchez et al. 2000) enables the neuron to control the size of the NF cytoskeleton regionally along the axon in response to myelinating cells and other needs. An earlier proposal of NF transport, originally advanced by Lasek and colleagues (Lasek 1986), envisioned axons as containing a single population of NFs continuously moving within the slow phase of axonal transport and degraded as the wavefront reaches the synaptic terminal (Li et al. 2012). The model, however, did not explain features of the axonal cytoskeleton that are now well established, including the striking variation of NF content and discrete regional specialization of the cytoskeleton along axons (Fig. 2), extensive cross-linking among ele-

ments comprising the axonal cytoskeleton, the changing phosphate topography on NFs during transport, and the exceptionally long half-lives of axonal NF proteins. Moreover, given that motors carrying NFs probably move on MTs, translocation of the large bundles of extremely long NFs that are often not in proximity to a MT is challenging to explain. Beyond the energy required to power a continuous movement of the entire cytoskeleton, the large proteolytic capacity and energy that would be needed within presynaptic terminals to turn over all NF proteins entering this compartment are not well substantiated by current experimental evidence.

7.4 Phosphorylation of NF Proteins and Axonal Transport

A role for phosphorylation in the dynamic behaviors of NFs was first raised by observations that the average rate of bulk NF transport slows significantly when the NF-H subunit appears in abundance during axon maturation (Willard and Simon 1983) and becomes highly phosphorylated (Carden et al. 1987; Giasson and Mushynski 1997b; Veeranna et al. 1998). The carboxy-terminal regions of NF-H and NF-M protrude laterally from the filament backbone when phosphorylated to increase negative charges, and phosphorylation-dependent interactions of NFs with each other and with other cytoskeletal structures are thought to regulate formation of a cytoskeletal lattice that provides stability to mature axons (Grant and Pant 2000). Consistent with this relationship, overexpression of NF-H decreases NF transport rates in sciatic nerves in vivo (Collard et al. 1995; Marszalek et al. 1996), whereas NF-H deletion increases these rates (Zhu et al. 1998). Moreover, in cultured primary neurons in vitro, highly phosphorylated forms of NF-H are translocated more slowly than hypophosphorylated species (Jung et al. 2000a, 2000b), and NF transport accelerates when the phosphorylation state of overexpressed NF-H is lowered by mutagenesis of the KSP repeats (Ackerley et al. 2003). Thus, repetitive phosphorylation of the tails of NF-H and NF-M subunits is a predictable mechanism to regulate the NF transport rate. However, in vivo NF transport rates along optic axons are not altered in NF-H-null mice (Rao et al. 2002b). Similarly, abolishing tail domain phosphorylation on NF-H (Rao et al. 2002b; Yuan et al. 2006a) or from both NF-H and NF-M by eliminating the extensively phosphorylated carboxy-terminal tail domains of these subunits in mice does not alter NF transport rates in vivo (Rao et al. 2012). Taken together, these results suggest that, normally, NF-M and NF-H carboxy-terminal domain phosphorylation is not a key determinant of the NF transport rate. The current findings, however, are compatible with the possibility that abnormal states of NF-M and NF-H tail phos-

phorylation achieved by subunit overexpression, mutagenesis, or disease states could slow NF transport and influence disease pathogenesis.

8 FUNCTIONS OF NF PROTEINS

8.1 NFs in Axon Radial Growth and Nerve Conduction

NFs are essential for the radial growth and structural stability of myelinated axons and for achieving the optimal propagation speed (conduction velocity) of electrical impulses along axons (Friede and Samorajski 1970; Ohara et al. 1993; Eyer and Peterson 1994; Zhu et al. 1997; Yum et al. 2009). Loss of NFs from axons of the Japanese quail due to nonsense mutation of NF-L and from mice lacking NF-L severely inhibits axon radial growth and nerve conduction velocities (Sakaguchi et al. 1993; Zhu et al. 1997) and in the mouse model reduces regenerative properties of adult peripheral axons (Zhu et al. 1997). NF content in axons supports increased conduction velocity in large myelinated fibers by expanding axon caliber and modulating ion channels (Kriz et al. 2000). In spite of their preserved caliber, large myelinated axons in NF-H knockout mice showed a significant decrease in conduction velocity compared with those in wild-type controls (Kriz et al. 2000). Further investigation revealed that NF-H knockout mice exhibited a significant decrease in outward rectification in comparison with that of wild-type control animals, indicating that NF-H might have a specific role in modulating ion-channel function in large myelinated axons (Kriz et al. 2000). Specifically deleting NF-L or NF-M, and thus lowering NF numbers by 50%–90% (Elder et al. 1998a; Jacomy et al. 1999), severely inhibits axonal radial growth (Zhu et al. 1997; Jacomy et al. 1999). In contrast, NF-H deletion has little (Elder et al. 1998b) or no effect on radial growth (Rao et al. 1998; Zhu et al. 1998); however, eliminating the tail domains of *both* NF-H and NF-M markedly inhibits axonal caliber expansion and reduces interfilament distances (nearest-neighbor distances) (Garcia et al. 2003) by reducing the numbers of long cross-bridges between filaments (Rao et al. 2002b; Garcia et al. 2003; Rao et al. 2003).

8.2 Novel Roles for the Individual Subunits of NFs in Synapses

Synapses have long been considered to be degradative sites for NFs reaching terminals by axonal transport (Roots 1983). When NF proteins have been detected in synaptic fractions and bound to synaptic proteins *in vitro*, they have usually been considered to be axonal NF contaminants (Matus et al. 1980; Ehlers et al. 1998; Kim et al. 2002). Using multiple independent approaches, however, Yuan and col-

leagues recently provided definitive evidence both for the presence of NF proteins in synapses and for a role for these proteins in synaptic function (Yuan et al. 2015c). NF assemblies isolated from synapses are distinctive. Compared with those present in axons, synaptic NF assemblies have high proportions of α -internexin, and NF-M is present in a low state of phosphorylation. Moreover, when NF-M is deleted from mice, phosphorylation of NF-H and the proportion of NF-H relative to NF-L increase significantly in the synaptic pool compared with the total NF pool. This unique population of synaptic NF proteins is more abundant in the postsynaptic area compared with those in the presynaptic region and preterminal dendrite. The observation of 10-nm filaments in some synapses and the decoration of these filaments by immunogold antibodies against different NF proteins indicate that at least some synaptic NF subunits exist in polymerized form (Fig. 6).

Yuan and colleagues also showed that deletion of NF-M in mice, but not deletion of any other NF subunits, amplifies dopamine-D1-receptor-mediated motor responses to cocaine while redistributing postsynaptic D1-receptors from endosomes to the plasma membrane. In addition, NF-M colocalizes with D1 receptor in synaptic boutons and synaptosomal fractions, and coimmunoprecipitation of NF-M and endosomal markers also pulls down D1R, further indicating an *in vivo* interaction between NF-M and D1R-containing endosomal fractions. These findings are consistent with a model (Fig. 7) in which NF-M, within a synaptic cytoskeletal structure, anchors D1-receptor-containing endosomes within synapses, thereby establishing a reservoir of receptors available for rapid recycling to the synaptic plasma membrane following dopamine agonist stimulation. Without NF-M, recycling back to the surface is accentuated, favoring hypersensitivity to D1-receptor agonists. The D1 receptor has been shown to interact with the carboxy-terminal tail of NF-M (Kim et al. 2002), and it will be important for future studies to address the role of phosphorylation of this domain in regulating D1-receptor binding.

The complex heteropolymeric structure and dynamically changing phosphate topography of NF proteins has suggested that all NF proteins might serve additional biological roles beyond static structural support of the axon caliber. In fact, eliminating all NF proteins from the CNS profoundly disrupts synaptic plasticity and social memory without altering the structural integrity of synapses. Supporting this view, our further observations also indicate depressed induction of hippocampal long-term potentiation (LTP) is also NF subunit selective: Maintenance of LTP is deficient in NF-H-null mice, whereas basal neurotransmission and induction of LTP are normal in NF-M-null mice. Moreover, NF-L selectively influences dendritic spine

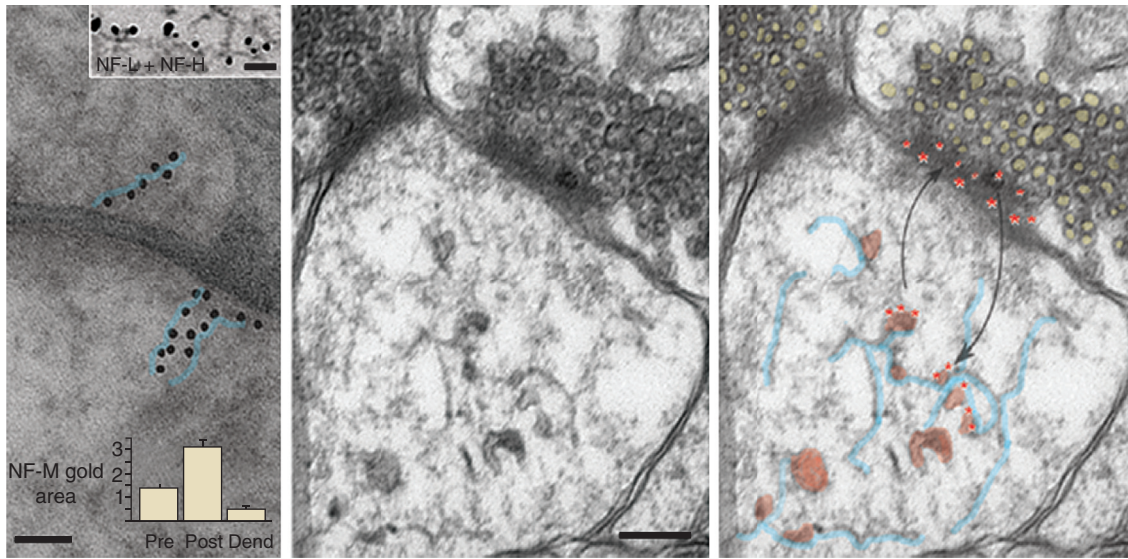


Figure 6. Functional neurofilament (NF) subunit assemblies in synapses. (*Left*) Immunogold-labeled antibodies against the neurofilament middle (NF-M) subunit decorating synaptic structures in a linear pattern (immunogold particles outlined in blue), suggesting the presence of short NFs and protofilament/protofibril or unit length filament assemblies. Scale bar, 60 nm. In the *upper inset*, a filament within a postsynaptic bouton is decorated by immunogold antibodies to both neurofilament light (NF-L; large gold dots) and neurofilament heavy (NF-H; small gold dots). Scale bar, 50 nm. Morphometric analysis indicates a higher density of immunogold labeling in postsynaptic boutons than in preterminal dendrites or presynaptic terminals (*graph inset*). (*Middle*) Ultrastructural image of a human synapse depicting membranous vesicles, many of which appear to be associated with a loose network of short 10-nm filaments in the postsynaptic region. Scale bar, 100 nm. (*Right*) Evidence supports a biological mechanism whereby D1 dopamine receptors internalized on endosomes from the postsynaptic surface (red asterisks) dock on synaptic NF subunit assemblies (outlined in blue), where they are readily available to recycle on endosomes to the surface in response to ligand stimulation. The cartoon overlay of the EM image is the hypothetical depiction of this process. (Adapted, with permission from Macmillan Publishers Ltd., from Yuan et al. 2015b.)

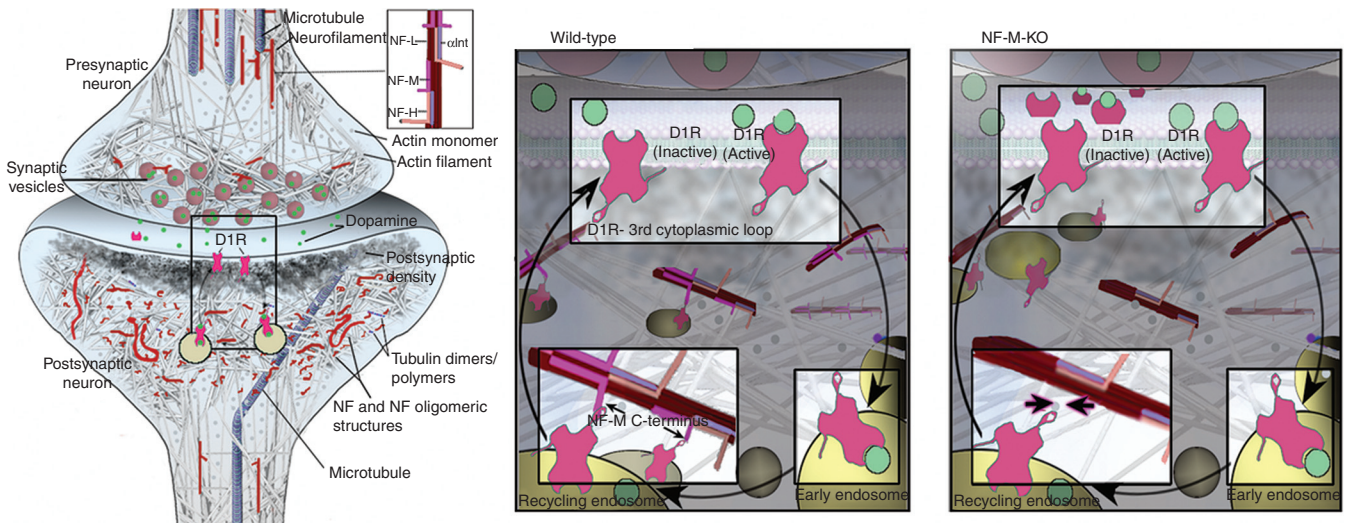


Figure 7. Model of D1-receptor (D1R)-containing endosomes anchored on Neurofilament middle (NF-M)-containing cytoskeletal assemblies. Based on collective findings on NF scaffolding functions and our D1R data on NF-subunit-null mice, we propose a model by which NF-M acts in synaptic terminals to anchor D1R-containing endosomes formed after agonist-induced internalization of membrane D1R. Retention of D1R in a readily available internal pool within the synapse would favor desensitization to D1R stimulation: In the absence of NF-M, the greater recycling back to the plasma membrane surface would favor hypersensitivity to D1R agonists, as observed in our *in vivo* studies. NF-L, neurofilament light; NF-H, neurofilament heavy; C-terminal, carboxyl terminal. (Adapted, with permission from Macmillan Publishers Ltd., from Yuan et al. 2015c.)

and glutamate receptor function because NF-L-null mice show not only abnormalities of these membranous protrusions but also dysfunction of hippocampus-dependent spatial memory, *N*-methyl-D-aspartate (NMDA) receptor protein expression, and synaptic plasticity (A Yuan, Veeranna, BS Basavarajappa, et al., unpubl.).

9 NF SUBUNIT-INTERACTING ORGANELLES AND PROTEINS

The NF network interacts with many proteins and organelles, including mitochondria, actin, tubulin, brain spectrin, kinases, phosphatases, molecular motors, receptors, proteases, and other degradative systems (Shaw et al. 2004; Song et al. 2012; Yuan et al. 2012a). MT organization in axons is strongly influenced by NFs. A negative regulatory influence of NFs on MT polymerization in axons was first suggested by the increased formation of MTs, despite normal tubulin levels, when axonal NF content was markedly reduced (Eyer and Peterson 1994; Fressinaud and Eyer 2014). An extensive analysis of all NF protein sequences subsequently revealed MT binding sites in the head domain of all NF subunit proteins, which inhibit MT polymerization (Bocquet et al. 2009). Moreover, peptide sequences of NF-L proteins containing these tubulin-binding sites selectively inhibit MT polymerization, cell proliferation, and tumor growth in rats (Berges et al. 2012). In addition, other manipulations that lead to depletion of axonal NFs, such as NF-L gene deletion, also increase MT number and tubulin levels (Zhu et al. 1997). Loss of NF-H and NF-M tails, or both NF-(H/M) tails, leads to an increase in total tubulin levels, as well as MT numbers in the axons (Rao et al. 1998; Zhu et al. 1998; Rao et al. 2003, 2012), suggesting the possibility of additional tubulin binding sites on NF-H/M tail domains that modulate MT dynamics in axons, as previously proposed (Hisanaga and Hirokawa 1990; Miyasaka et al. 1993). These results collectively suggest that dynamic interactions between NFs and MTs regulate the balance of these two fibrous elements so that both structural and transport functions of the axon are properly supported.

Apart from being structural elements in axons, NFs form cellular scaffolds for docking and organization of synaptic vesicles, endosomes, and the endoplasmic reticulum (ER) (Rao et al. 2002a; Balastik et al. 2008; Kim et al. 2011; Rao et al. 2011). Myosin Va in neurons binds to NF-L, and this interaction is essential for the normal distribution of ER and other organelles in the axon. Myosin V is also involved in recycling of endosomes at the synapse (Kim et al. 2002; Wang et al. 2008). It has been proposed that NFs act as “tracks” for the positioning of endosomes, lysosomes, ER, and other vesicles (Rao et al. 2011). NF-L also

binds mitochondria and might regulate their dynamics. For example, mutations of NF-L causing the peripheral neuropathy Charcot–Marie–Tooth disease retard movement of mitochondria in neurons (Gentil et al. 2012). Phosphorylated NFs bind to mitochondria that have high membrane potential, suggesting another possible mechanism by which NF–mitochondria interactions could be regulated (Wagner et al. 2003). Finally, peripherin, α -internexin, and vimentin bind to adaptor protein-3, which might modulate positioning, content, and distribution of a set of late-endosome and lysosomal membrane proteins (Styers et al. 2004).

10 NF PROTEINS IN DISEASE STATES

10.1 Primary NF Disorders

NF defects cause multiple familial neurodegenerative disorders and might be risk factors in other diseases. In disorders involving NF gene mutations, NF aggregation and transport failure leading to further NF accumulations are often the most prominent pathological manifestations of the disease and are frequently considered primary pathogenic factors leading to general axonal dysfunction and degeneration. In these disorders, the close link between defective subunit assembly and perikaryal aggregation of all subunits underscores other evidence that assembly competence is crucial for axonal transport of NF subunits. When this balance is altered by overexpressing one subunit in genetic mouse models or by other experimental manipulations (Gibb et al. 1998), the outcome is commonly NF protein aggregation in the cell body and/or proximal axon and degeneration that particularly affects long axons. Thus, motor neuron disease with these pathological characteristics develops in mice overexpressing mouse NF-L (Xu et al. 1993), mouse peripherin (Beaulieu et al. 1999), human NF-H (Cote et al. 1993), or human NF-M (Fig. 8) (Gama Sosa et al. 2003), or expressing mutant mouse NF-L (Lee et al. 1994). It has also been shown that the organization of the NF network can be altered by raising the concentration of NF-H microinjected into a single cultured dorsal root ganglion neuron. This is followed by fragmentation of the Golgi complex, accumulation of mitochondria within the proximal regions of neurites, peripheralization of the nucleus, significant decrease in neurite caliber, and later appearance of abnormally phosphorylated neurofilament heavy chain (pNF-H) (Straube-West et al. 1996).

More than 14 NF-L gene mutations are known to cause type 2E and 1F forms of Charcot–Marie–Tooth disease (CMT2E/CMT1F) (Fig. 9), the most common inherited peripheral nerve disorder (Mersyanova et al. 2000; De Jonghe et al. 2001; Georgiou et al. 2002; Yoshihara et al.

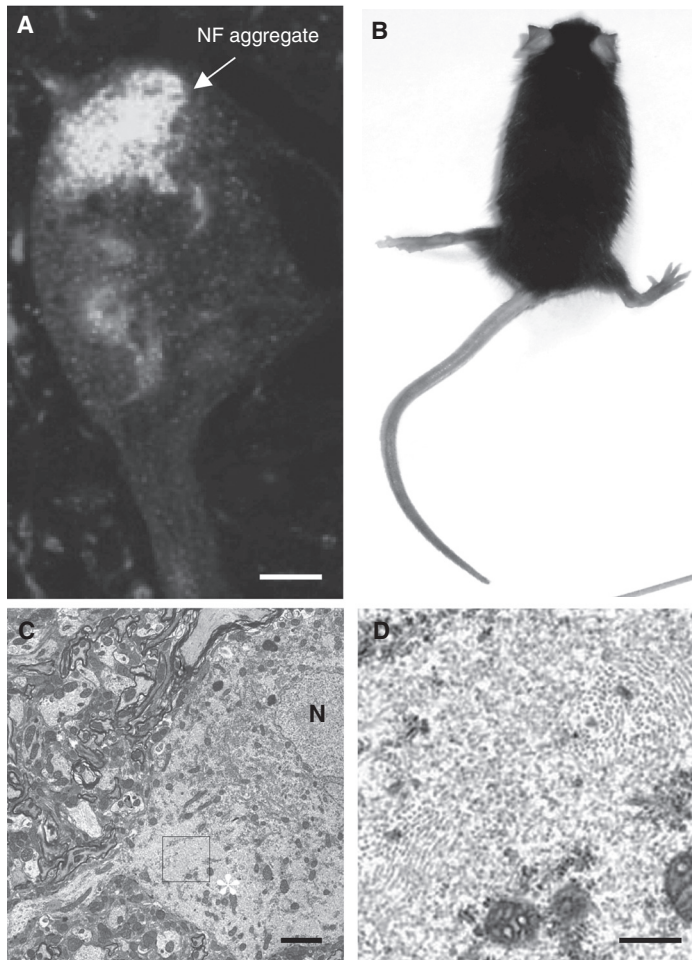


Figure 8. Abnormal neurofilament (NF) accumulation in neurological diseases. (A) Inclusions containing peripherin, neurofilament light (NF-L), neurofilament middle (NF-M), and neurofilament heavy (NF-H) (identified by specific antibodies) detected in cell bodies of the remaining motor nerve cells of a patient with the D141Y mutation of peripherin. (Adapted, with permission, from Leung et al. 2004, © John Wiley and Sons, Inc.) (B) A 7-month-old transgenic mouse expressing human NF-M and displaying hind-limb paralysis. Note the abnormal posture and the splaying of the hind limbs owing to muscle weakness and paralysis. Overexpression of NFs leads to motor neuron dysfunction. (C) Electron micrograph showing an abnormal motor neuron from the human NF-M transgenic mouse in B. (Adapted, with permission from Elsevier, from Gama Sosa et al. 2003.) In the cell body of the neuron, organelles are concentrated toward the nucleus (N), and large numbers of 10-nm filaments are present in the area marked with a white asterisk. The area outlined by the black box in C is enlarged in D. Scale bars, 5 μm (A,C); 1 μm (D).

2002; Jordanova et al. 2003; Choi et al. 2004; Zuchner et al. 2004). CMT2E/1F is typically inherited in an autosomal dominant manner, although a few families with autosomal recessive CMT2E/1F (caused by homozygous nonsense mutations of the NF-L gene) have been reported (Abe et al. 2009; Yum et al. 2009). Most NF-L gene mutations are missense, whereas other mutations of NF-L delete the gene encoding NF-L. These disease-causing NF-L mutations have been shown to disrupt both the assembly and axonal transport of NF proteins (Brownlees et al. 2002; Perez-Olle et al. 2002, 2005; Sasaki et al. 2006) and induce axonopathies characterized by reduced axon calibers and impaired nerve conduction.

Genetic alterations of other NF subunits can also be associated with neurological disease. Susceptibility to amyotrophic lateral sclerosis (ALS) is increased by certain peripherin mutations (Gros-Louis et al. 2004; Leung et al. 2004; Corrado et al. 2011) and by polymorphisms of the gene encoding NF-H (Figlewicz et al. 1994; Tomkins et al. 1998; Al-Chalabi et al. 1999). Point and deletion mutations in the NF-M rod domain have been linked, respectively, to

familial Parkinson's disease (Lavedan et al. 2002) and Alzheimer's disease (Fig. 9) (Wang et al. 2002). Although a mutation in the gene encoding α -internexin has not yet been identified, the accumulation of α -internexin is a neuropathological hallmark of NF protein aggregates in neurofilament inclusion body disease (NIBD), a form of frontotemporal dementia (Cairns et al. 2004). Transgenic mice overexpressing α -internexin develop progressive loss of neurons, together with deficits in motor coordination (Ching et al. 1999).

Loss of NF subunit function can induce neuronal abnormalities by reducing the NF content, and thus the calibers, of large axons, leading to atrophy of these fibers and decreased conduction velocities. Spontaneous mutant Japanese quail (*quv*), which lack NFs as the result of a premature translation terminator in the gene encoding NF-L (Ohara et al. 1993; Toyoshima et al. 2000), develop generalized ataxia and a characteristic quivering behavior (Yamasaki et al. 1991) associated with reduced conduction velocity (Sakaguchi et al. 1993). Deletion of NF-H (Elder et al. 1998b) and NF-M (Elder et al. 1999) in mice also

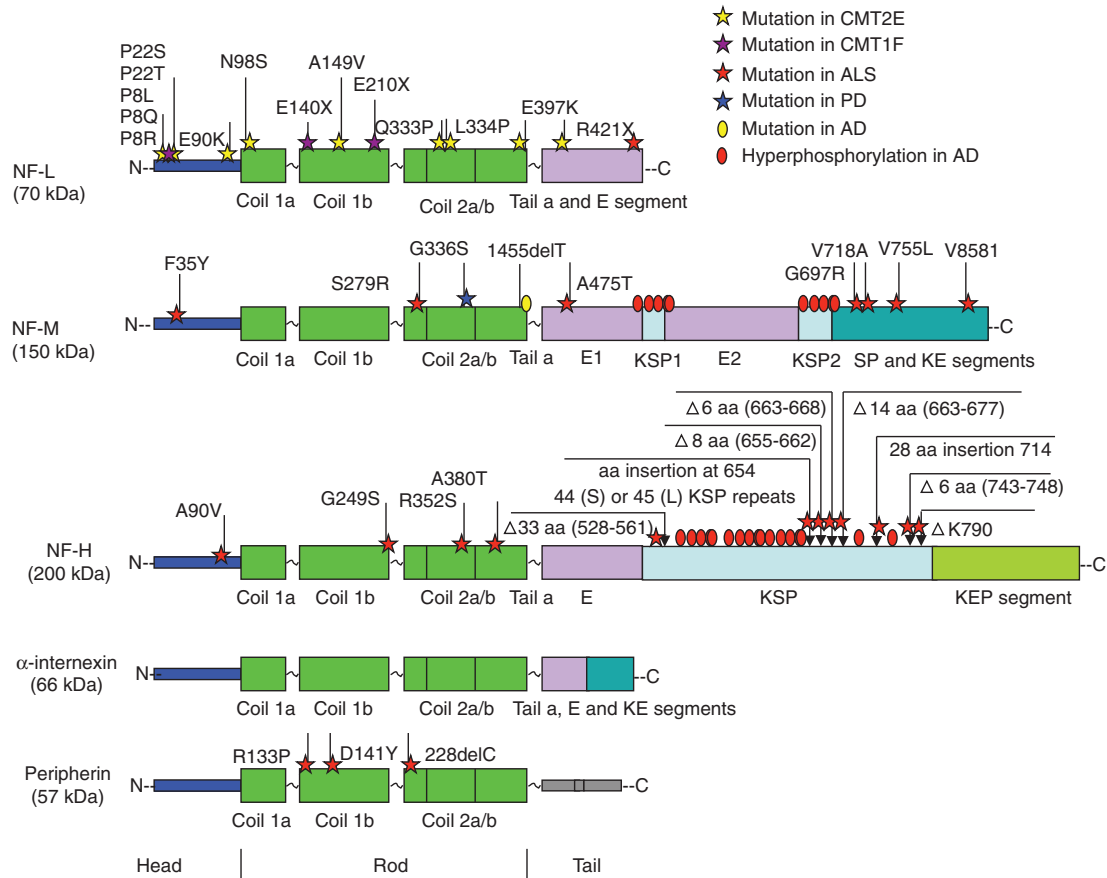


Figure 9. Neurofilament (NF) protein mutations and disease. The locations of mutations at sites on the different domains of NF subunits—neurofilament light (NF-L), neurofilament middle (NF-M), neurofilament heavy (NF-H), and peripherin—are shown in relation to their occurrence in neuropsychiatric diseases (Jordanova et al. 2003; Leung et al. 2004). No disease mutations of α -internexin have been reported in neuropsychiatric diseases. AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CMT, Charcot–Marie–Tooth disease; PD, Parkinson's disease; E segment, glutamic-acid-rich segment; E1, glutamic-acid-rich segment 1; E2, glutamic-acid-rich segment 2; KE, lysine–glutamic acid; KEP, lysine–glutamic acid–proline; SP, serine–proline; KSP, lysine–serine–proline.

reduces the diameter of large-caliber axons and induces age-related atrophy of motor axons.

Exposure to a variety of chemical agents, such as β,β' -iminodipropionitrile (IDPN), aluminum, lead, acrylamide, arsenic, carbon disulfide, and 2,5-hexanedione, can chemically modify NF subunits, leading to neurodegeneration characterized by accumulation of phosphorylated NFs (Llorens 2013). IDPN induces abnormal cross-linking of hyperphosphorylated NFs, probably mediated through the NF-H subunit (Zhu et al. 1998), resulting in NF aggregation and subsequent segregation from MTs (Eyer et al. 1989).

10.2 Secondary NF Disorders

Mutations other than those affecting NF genes can secondarily disrupt transport of NFs specifically and lead to general transport failure. These include mutations of

heat-shock 27-kDa protein 1 (HSPB1) in CMT2F (Evgrafov et al. 2004), gigaxonin in giant axonal neuropathy (Bomont et al. 2000), and superoxide dismutase superoxide dismutase 1 (SOD1) in ALS (Zhang et al. 1997). Interestingly, HSPB1 and SOD1 are both carried in slow axonal transport (Yuan 1997; Borchelt et al. 1998), in which they might play a role in NF transport and assembly (Ackerley et al. 2006) or in maintaining stability of the NF network (Menziés et al. 2002). Gigaxonin, an E3 ligase adaptor, can bind to microtubule-associated protein 1B (MAP1B) and control its degradation (Ding et al. 2002; Allen et al. 2005). Mutations of the gigaxonin gene impair NF degradation, resulting in an excess of NFs in the axons, impaired conduction of action potentials, and, eventually, degeneration of both motor and sensory neurons (Ganay et al. 2011; Mahammad et al. 2013). A deficiency of ubiquitin ligase tripartite motif containing 2 (TRIM2) (Balastik et al. 2008) or multisynthetase

complex p43 (Zhu et al. 2009) causes NF-L accumulation and disorganization, leading to neurodegeneration. Recently, it has been reported that CMT2B disease-causing RAB7A mutant proteins show an interaction with peripherin, suggesting that abnormal NF function could be a key intermediate in the pathogenesis of this disease (Cogli et al. 2013). More recently, a heterozygous missense mutation in the gene encoding the PKA type 1- β regulatory subunit (*PRKAR1B*) has been identified in a novel late-onset familial NF-related disorder presenting with dementia and/or parkinsonism, suggesting that an altered phosphorylation state of amino-terminal head and carboxy-terminal tail domains of NF subunits could be involved (Wong et al. 2014).

11 NF PROTEINS AS POTENTIAL MARKERS OF DISEASE AND INJURY

NF compaction, characterized by a high packing density of NFs near the site of traumatic injury to axons (Hall and Lee 1995), is believed to result from modifications of NF-M side arms that reduce the spacing between NFs (Hall and Lee 1995; Povlishock et al. 1997; Okonkwo et al. 1998). This phenomenon is thought to involve the related events of Ca^{2+} elevation and calpain activation (Shaw et al. 2004), as well as, possibly, other proteolytic modifications on NF-M that expose an epitope recognized by the RMO14 antibody, a commonly used marker of traumatic nerve injury (Hollenbeck and Bray 1987; Suzuki et al. 1988; Song et al. 2012).

Because NF proteins are enriched in axons and might be released from damaged or diseased axons in significant amounts into blood and cerebrospinal fluid (CSF), their elevated levels in either CSF or serum are often used as potential biomarkers of axonal injury, axonal loss, and neuronal death, although they have not been shown to have diagnostic utility for any given disease. Soluble pNF-H is undetectable in the sera of normal animals but is readily detectable in the sera of adult rats following various types of experimental spinal cord injury and traumatic brain injury (Shaw et al. 2005; Anderson et al. 2008). It has been suggested that pNF-H and NF-L could serve as biomarkers for monitoring axonal degeneration in ALS-model rodents and as potential biomarkers of disease progression in ALS patients (Brettschneider et al. 2006b; Boylan et al. 2009; Gaiottino et al. 2013). The presence of NF subunits in serum is also being actively investigated as a potential prognostic indicator in multiple sclerosis (Gresle et al. 2011; Teunissen and Khalil 2012) and to assess nerve damage in patients suffering from severe burns (Gatson et al. 2015) or spinal cord injury (Kuhle et al. 2015).

NF proteins are also being used to identify ongoing axonal degeneration in dementias. Higher than normal lev-

els in CSF of NF-L and NF-H have been reported in Alzheimer's disease, frontotemporal dementia, and vascular dementia (Sjogren et al. 2001; Brettschneider et al. 2006a; Petzold et al. 2007; Pijnenburg et al. 2007; Bjerke et al. 2011) and correlate with disease severity in a recent study of frontotemporal dementia (Scherling et al. 2014). A more recent study has suggested that the CSF NF-L concentration is increased by the early clinical stage of Alzheimer's disease and is associated with cognitive deterioration and structural changes over time (Zetterberg et al. 2016). Levels of CSF NF-H and NF-L might also have value in discriminating atypical parkinsonian syndromes from Parkinson's disease.

12 NF BIOLOGY—THE NEXT DECADE

Although much has been learned about the biology of NFs, fundamental questions remain unanswered, especially regarding the possible functions of NFs beyond the structural support role shared with other classes of IFs. Although neurons have unique structural needs because of their asymmetric geometries, it is not clearly evident from comparisons with other cells why these known structural needs necessarily require the complexity of subunit composition and regulation by phosphorylation that characterizes NFs. In the future, it is likely that insights into this question will emerge by a closer examination of interactions of the individual NF subunits, either within filaments or as oligomeric assemblies, with other proteins and organelles. There is a strong need to understand the significance of the interactions between specific NF subunits and neurotransmitter receptors *in vivo*. How broad is the synaptic role of NF subunits, and what are the implications of these roles for psychiatric disease, not just neurological disorders? What do the changes in NF structure and phosphorylation linked to NIBD, Parkinson's disease, and addictive states tell us about the neural mechanisms underlying these disorders? Even in the neurodegenerative diseases caused by NF mutation, is neuronal function disrupted by one common mechanism or multiple mechanisms, and do these involve a loss of function or gain of toxic function of NF proteins?

As these aspects of NF function are being explored, answers to additional questions about the fundamental biology of NF will also need to be pursued. Much additional insight is needed about NF protein turnover and how it is regulated. For example, are there functional roles for the abundant NF subunit fragments that are generated by proteolysis? How variable are the assembly forms of NF subunits and what is the functional significance of the variation in subunit composition of NFs seen in different neurons? Which molecular motors interact with which NF proteins to mediate slow NF transport and what are the signals to NFs to trigger their incorporation into the axonal

cytoskeleton? Forthcoming answers to these and other questions are likely to alter any preconception that we are presently close to fully understanding the scope of NF roles in neurons. These future insights should reward us with a better understanding of neural function and exciting new ideas on how to translate this information into disease therapies.

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