MBoC | REVIEW

Neurofilament Biophysics: From Structure to Biomechanics

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ABSTRACT **Neurofilaments (NFs) are multisubunit, neuron-specific intermediate filaments consisting of a 10-nm diameter filament "core" surrounded by a layer of long intrinsically disordered protein (IDP) "tails." NFs are thought to regulate axonal caliber during development and then stabilize the mature axon, with NF subunit misregulation, mutation, and aggregation featuring prominently in multiple neurological diseases. The field's understanding of NF structure, mechanics, and function has been deeply informed by a rich variety of biochemical, cell biological, and mouse genetic studies spanning more than four decades. These studies have contributed much to our collective understanding of NF function in axonal physiology and disease. In recent years, however, there has been a resurgence of interest in NF subunit proteins in two new contexts: as potential blood- and cerebrospinal fluid-based biomarkers of neuronal damage, and as model IDPs with intriguing properties. Here, we review established principles and more recent discoveries in NF structure and function. Where possible, we place these findings in the context of biophysics of NF assembly, interaction, and contributions to axonal mechanics.**

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INTRODUCTION

Neurofilaments (NFs) are intermediate filaments (IFs) specific to neurons and serve as a major cytoskeletal component of large-diameter axons. Although classically viewed as space-filling axonal structures (Figure 1A), NFs are also found in the cell body and at synapses (Zheng *et al.*, 2003; Yuan *et al.*, 2015b; Figure 1B). In vivo, NFs are obligate heteropolymers in that they are always composed of more than one type of subunit protein (Yuan *et al.*, 2017). In mammals, the five subunit proteins are Neurofilament-Light (NF-L), -Medium (NF-M), -Heavy (NF-H), α-internexin, and peripherin. These subunits assemble in a ratio that depends on species, developmental stage, neurodegenerative condition, age, localization in the neuron, and neuron type (Scott *et al.*, 1985; Kaplan *et al.*, 1990; Uchida *et al.*, 2004; Yuan *et al.*, 2006; Chinnakkaruppan *et al.*, 2009; Yuan *et al.*, 2012; Chen *et al.*, 2014).

The NF proteins share a general structure common to all IFs proteins: a central α-helical rod domain flanked by a short unstructured head domain and an unstructured tail domain of variable length (Figure 1C). Like other IF proteins, NF proteins assemble into filaments via their rod domains, which form coiled-coil dimers. These dimers assemble into tetramers, which associate into unit-length filaments and anneal end-to-end to form a long, 10-nm-diameter filament (Eldirany *et al.*, 2021).

Although the NF proteins have long been of interest due to their role in axonal structure, the head and especially the tail domains have attracted renewed attention in recent years as functional intrinsically disordered proteins (IDPs). Disordered proteins lack a stably folded structure and instead dynamically sample a relatively large space of conformations. In recent years, IDPs have been increasingly implicated in the assembly of many intracellular condensates and membraneless organelles, cell signaling, and other protein–protein interactions (Martin and Holehouse, 2020; Borcherds *et al.*, 2021; Morris *et al.*, 2021; Uversky, 2021; Bondos *et al.*, 2022). In NFs, these repetitive, highly charged sequences also serve as a dynamic and tunable cytoskeletal building block.

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Abbreviations used: ALS, amyotrophic lateral sclerosis; AFM, atomic force microscopy; CMT, Charcot-Marie-Tooth disease; IDP, intrinsically disordered protein; IF, intermediate filament; NF, neurofilament; NF-L, Neurofilament-Light; NF-M, Neurofilament-Medium; NF-H, Neurofilament-Heavy; PTM, posttranslational modification; SCFT, self-consistent field theory

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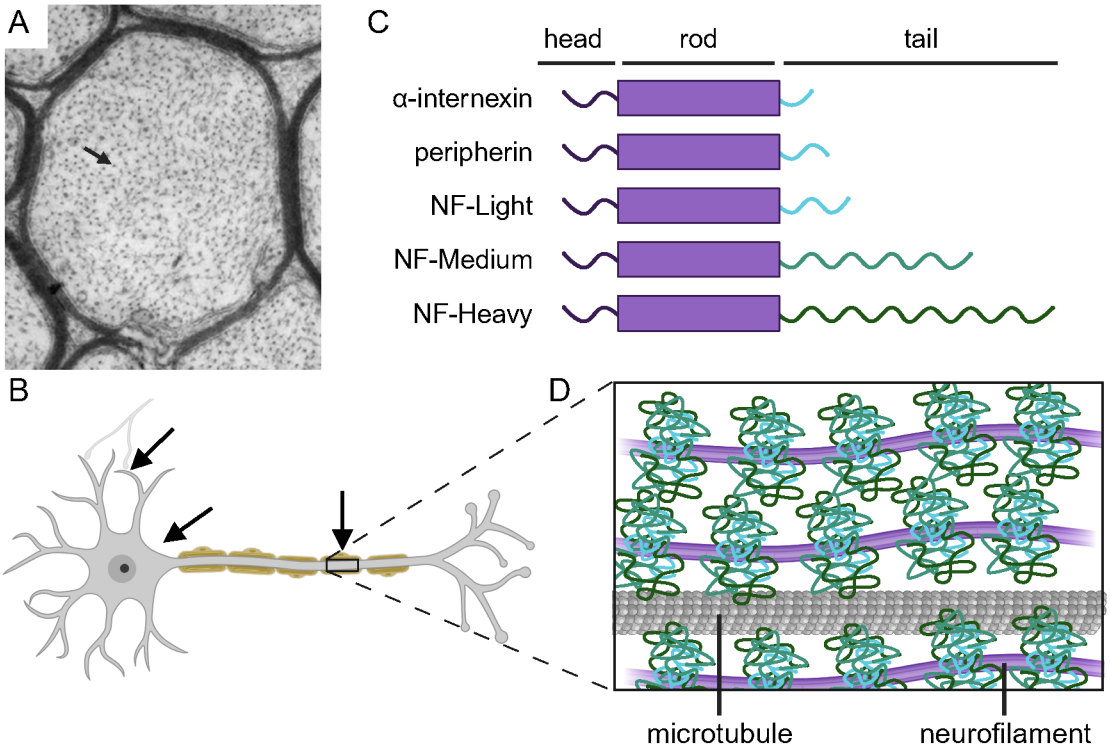

FIGURE 1: Localization and composition of axonal NFs. (A) Electron micrograph of a cross-section of distal region of mouse optic nerve, adapted from (Yuan *et al.*, 2015a). NF cores appear as distributed dark points (arrow). (B) NF localization within a neuron at synapses, in the soma, and in the axon (arrows). (C) Schematics of the NF subunit protein domains. (D) Diagram of assembled NFs filling the axon along with microtubules. Figure created with BioRender.com.

The disordered tail domains physically protrude from the filament core, forming a bottlebrush-like structure (Willard and Simon, 1981; Hisanaga and Hirokawa, 1988; Figure 1D). Because of their position at the periphery of the filament, the tail domains may govern inter-NF interactions depending on their posttranslational modifications (PTMs). The NF tail domains vary in phosphorylation capacity – the tail domains of NF-M and particularly NF-H are extensively phosphorylated in vivo, with the NF-H tail carrying ~50 phosphates (Yuan *et al.*, 2017). NF tail domain phosphorylation is regulated by a host of kinases and phosphatases (Veeranna *et al.*, 2011; Holmgren *et al.*, 2012; Lee *et al.*, 2014), and the degree of NF tail domain phosphorylation varies with age and with position along the length of the axon (Uchida *et al.*, 1999; Yuan *et al.*, 2015a). NFs can also undergo nitrosylation, glycosylation, and other PTMs, though the functions of these PTMs remain less well studied (Dong *et al.*, 1993; Snider and Omary, 2014; Petzold, 2022).

In recent years the NF proteins, especially NF-L, have become promising diagnostic biomarkers with the capacity to report on many neurodegenerative conditions (Lu *et al.*, 2015; Gaetani *et al.*, 2019; Lin *et al.*, 2019; Preische *et al.*, 2019; Rafii *et al.*, 2019; Lambertsen *et al.*, 2020; Zucchi *et al.*, 2020; Bittner *et al.*, 2021; Yuan and Nixon, 2021; Huehnchen *et al.*, 2022; Petzold, 2022). After axonal injury, the NF proteins are released from their canonical intracellular environment and end up in the blood or cerebrospinal fluid, where they can be measured as a proxy for neuronal damage. For example, a cohort of multiple sclerosis (MS) patients was found to have elevated serum NF-L relative to healthy controls (Disanto *et al.*, 2017). A recent meta-analysis of 31 studies further supported the diagnostic value of serum and plasma NF-L in MS and its subtypes and demonstrated predictive value for disease progression as quantified by the time to reach expanded disability status scale (EDSS) score > 4.0 (Ning and Wang, 2022). Nonetheless, diagnostic and prognostic applications of NF-L remain complicated, in part because baseline blood NF-L levels are affected by systemic factors such as body mass index, medication history, and especially age. Moreover, elevated NF-L is a somewhat nonspecific marker of neuronal damage and may reflect a variety of disease etiologies. Still, the relative stability of NF protein fragments enable their detection by single-molecule enzyme-linked immunosorbent assay. Patient autoantibodies recognizing NF proteins are also emerging biomarkers of neurodegeneration, and may complement the diagnostic value of the NF proteins themselves (Zmira *et al.*, 2020; Puentes *et al.*, 2021).

In addition to their translational potential, NFs represent an interesting biological system in which IDP conformational properties may directly contribute to whole-cell mechanics. Multiple excellent reviews have discussed other aspects of NF biology including axonal transport, assembly, mutations in neurodegenerative disease, and potential as biomarkers (Didonna and Opal, 2019; Bomont, 2021; Eldirany *et al.*, 2021; Falzone *et al.*, 2021; Stone *et al.*, 2021; Yuan and Nixon, 2021, 2023; Petzold, 2022; Phillips *et al.*, 2023; Zhou *et al.*, 2023). In this review, we focus primarily on NFs as IDPs and regulators of neuronal biomechanics, tying together a selection of studies that have shed light on these structural yet unstructured proteins and their functions in forming filaments, maintaining axon caliber, and contributing to axon biomechanics.

FILAMENT ASSEMBLY AND AGGREGATION

Filament assembly

While the NF protein rod domains form the central filamentous core, filament assembly is strongly regulated by the disordered

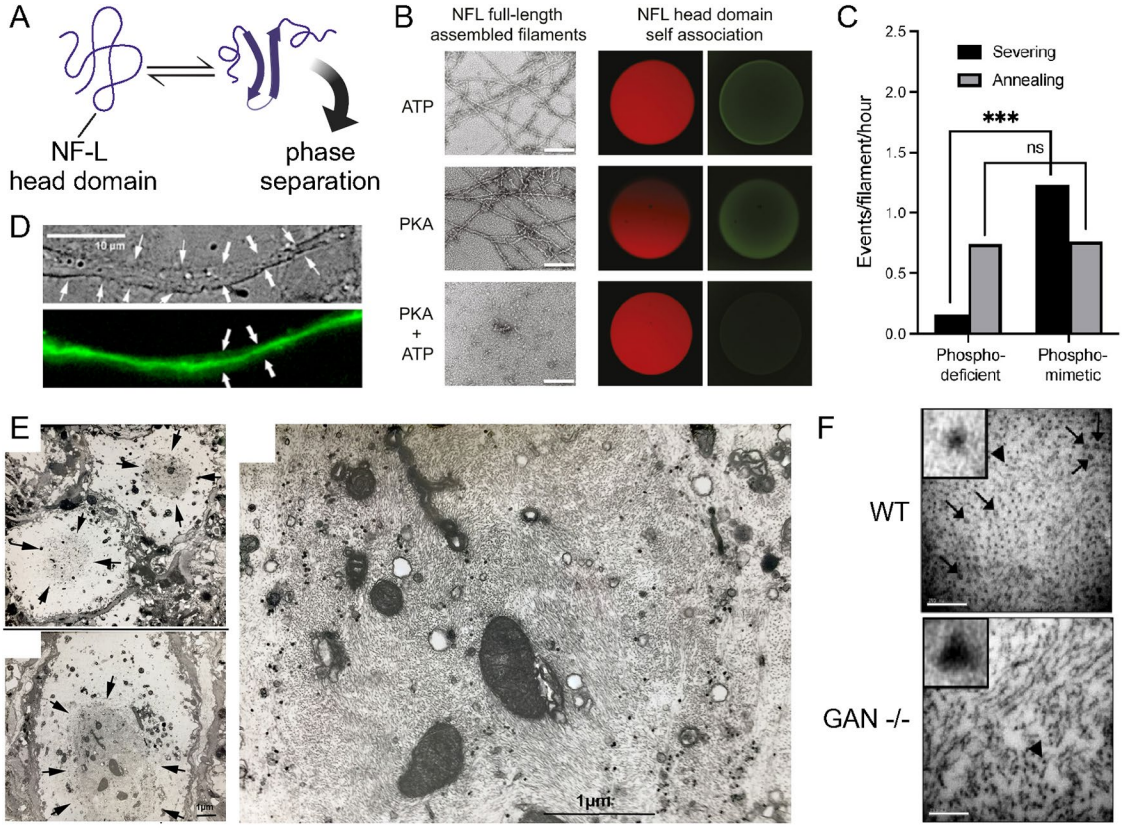

FIGURE 2: Regulation of NF assembly and bundled or aggregated NF ultrastructures. (A) The NF-L head domain takes on a transient cross-beta structure, enabling phase separation. (B) Filament assembly (left), as well as colocalization between mCherry-tagged (center) and GFP-tagged (right) NF-L head domains, are ablated after phosphorylation by protein kinase A (PKA). Reproduced from (Zhou *et al.*, 2021). (C) Head-domain phosphodeficient NF-L is less frequently severed than phosphomimetic NFL in cultured neurons. Reproduced from (Uchida *et al.*, 2023). (D) Phase-contrast (top) and fluorescence (bottom) micrographs of a neurite of a neuroblastoma cell expressing NFH-GFP fusion protein, reproduced from (Boumil *et al.*, 2018). Arrows indicate neurite size; NF "bundle" is visible as a central region of high NFH density within the neurite. (E) Left: Bundled NF ultrastructures (arrows) in regenerating lamprey neurons. Right: higher-magnification view of bundled NFs, showing dense filament packing and variety of orientations. Scale bars: 1 µm. Adapted from (Lee *et al.*, 2019). (F) Electron micrographs of axons from wild type (top) or giant axonal neuropathy model mice (bottom), showing altered NF orientation and size. Arrows indicate microtubules, arrowheads indicate single NFs shown in higher-magnification insets. Scale bars: 200 nm. Adapted from (Ganay *et al.*, 2011). Figure created with BioRender.com.

head domains. Analogously to other IFs such as vimentin (Herrmann *et al.*, 1996) and desmin (Sharma *et al.*, 2009), in vitro reconstitution shows that the head domain is required for NF-L filament assembly, as recombinant headless NF-L forms only short protofilaments when viewed by electron microscopy under standard filament assembly conditions (Heins *et al.*, 1993).

More recently, the molecular mechanism of NF-L head domain regulation has begun to be uncovered. Part of the head domain of NF-L can take on a transient beta strand-enriched conformation (Zhou *et al.*, 2021), which weakly binds other NF-L head domain proteins during the annealing stage of assembly (Figure 2A). This transient interaction is easily disrupted by phosphorylation (Figure 2B), and indeed, head domain phosphorylation has long been known to prevent reconstituted filament formation (Hisanaga *et al.*, 1990) and to control filament localization in neurons (Zheng *et al.*, 2003). Several NF-L head domain mutations associated with Charcot-Marie-Tooth disease (CMT) aberrantly strengthen the head domain self-interaction, preventing normal filament reconstitution in vitro and in SW13vim- cells (Sasaki *et al.*, 2006; Stone *et al.*, 2019; Zhou *et al.*, 2021, 2022). Interestingly, isolated NF-L head domains phase-separate in vitro into high-aspect-ratio or filamentous structures even without a rod domain, and CMT-associated mutations stabilize this structure even in multimolar levels of urea (Zhou *et al.*, 2021). Less clear is how this potentially amyloid-like phase separation functions within the filamentous geometry of NF assembly and annealing. Indeed, very recent work on the structure of vimentin has shown that the analogous disordered head domains form an amyloid-like fiber within the filament core (Eibauer *et al.*, 2024). It is possible that the distinct NF subunit head domains carry distinct phase separation properties, and it remains to be seen how these differences might regulate filament assembly.

In addition to filament formation, the head domain also regulates filament disassembly both in vitro and in cells (Hisanaga *et al.*, 1994; Giasson and Mushynski, 1998). Recent work has proposed that the cell regulates NF severing and transport by controlling phosphorylation of the NF protein head domains (Uchida *et al.*, 2023), as NF-L variants with phosphorylation-deficient head domains were severed significantly less frequently than phosphomimetic variants in cultured neurons (Figure 2C).

NF Bundles

Once formed, NFs are transported intermittently along microtubule tracks but spend long periods of time "paused" as part of a cytoskeletal ultrastructure filling the axon (Wang *et al.*, 2000; Yuan *et al.*, 2015a). This model arises from observations of bidirectional, intermittent NF transport from time-lapse imaging of fluorescently labeled NFs in cultured neurons as well as transgenic mice (Wang *et al.*, 2000; Yan *et al.*, 2007; Boyer *et al.*, 2022). The NFs stationary at a given time have sometimes been referred to as a "bundle" formed of closely apposed filaments (Yamada *et al.*, 1971; Hirokawa *et al.*, 1984; Uchida *et al.*, 1999; Chen *et al.*, 2000; Yabe *et al.*, 2001; Figure 2D). Centrally situated bundles have been documented in cultured cell lines and neurons, as well as in regenerating nerve tissue from lamprey (Figure 2E; Boumil *et al.*, 2018; Lee *et al.*, 2019).

Bundled NFs can be separated from nonbundled NFs by centrifuging tissue or cell homogenates over a sucrose cushion (Leterrier and Eyer, 1987; Leterrier *et al.*, 2009), enabling comparative studies. Bundled NFs are heavily phosphorylated at the NF-H tail domains, while the surrounding filaments are less phosphorylated (Kushkuley *et al.*, 2009; Boumil *et al.*, 2018). Studies in cultured cells have revealed that phosphorylation of the NF-H tail domain regulates the rate of NF bundling (Lee *et al.*, 2011, 2014; Vohnoutka *et al.*, 2017), and specifically that phosphorylation sites within the C-terminal ~190 amino acids of this tail domain are required for bundling (Chen *et al.*, 2000; Lee *et al.*, 2014).

NF bundles dissociate with the addition of EGTA (Kushkuley *et al.*, 2009), suggesting that multivalent cations are involved in crosslinking NFs into bundles. It has been proposed that crosslinking of tail-phosphorylated NFs prevents their transport by kinesin (Yabe *et al.*, 2000; Kushkuley *et al.*, 2009; Shea and Lee, 2011; Sunil *et al.*, 2012), while nonphosphorylated NFs associate with kinesin and are more readily transported along microtubule tracks (Hisanaga and Hirokawa, 1990; Shea and Lee, 2011), though NF bundles do collapse upon application of actin- or microtubule-depolymerizing drugs (Lee *et al.*, 2019).

Aggregation

NFs can also be observed as large intracellular aggregates which characterize many neurodegenerative conditions including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, CMT, and neurofilament inclusion disease (Trojanowski and Lee, 1998; Uchikado *et al.*, 2006; Rudrabhatla *et al.*, 2011; Didonna and Opal, 2019). The molecular mechanisms by which filaments aggregate are complex and not well understood. Although NF-binding chemical moieties such as 3,3′-iminodipropionitrile (IDPN) or aluminum ions can cause general filament aggregation and neurotoxicity (Kushkuley *et al.*, 2010; Grande-Aztatzi *et al.*, 2020), disease models with NF aggregates have pointed to other causes such as altered NF subunit composition, disease-associated mutations, and misregulated clearance pathways.

One pathway driving filament aggregation is a misbalance in NF subunit stoichiometry, which has been principally studied in the context of ALS. Patient spinal cord samples show decreased NF-L, α-internexin, and peripherin mRNA levels, while NF-M and NF-H are unaffected (Wong *et al.*, 2000). Similarly, ALS-associated mutant SOD1 may destabilize NF-L mRNA, leading to aggregates which can be rescued by restoring NF-L expression (Chen *et al.*, 2014). Several miRNAs involved in ALS also differentially regulate the NF proteins, altering subunit stoichiometry (Campos-Melo *et al.*, 2018; Hawley *et al.*, 2019).

Moreover, overexpression of any of the NF proteins in mouse models can lead to NF accumulation, though not necessarily neuron loss. Comparison of mouse models and their effects are presented elsewhere (Didonna and Opal, 2019; Table 2 in that publication). In order for subunit stoichiometry to support filament assembly, a minimum number of short-tailed NF proteins (α-internexin, peripherin, or especially NF-L) must be included. In reconstitution assays, NF-L/NF-M filaments become saturated at a ratio of 37.5 mol% NF-M, and NF-L/NF-H filaments at 25 mol% NF-H (Jones and Safinya, 2008). This requirement may reflect similar steric constraints to those found in synthetic bottlebrush polymer self-assembly, where in a "grafting-to" approach the side-chain density can be limited by the crowding of long polymer chains (Verduzco *et al.*, 2015; Li *et al.*, 2021).

Disease-causing mutations such as those found in CMT may also cause NF aggregation. In a few cases, frameshift variants cause translation of an amyloidogenic sequence usually obscured in the 3′ UTR, causing aggregation (Rebelo *et al.*, 2016). However, many more reports have focused on other CMT-associated NF-L mutants, showing that these form aggregates in cultured cells (Zhai *et al.*, 2007; Lee *et al.*, 2008; Zhao *et al.*, 2017; Sainio *et al.*, 2018; Feliciano *et al.*, 2021). However, these assays frequently employ transient and uncontrolled overexpression of NF-L in systems with no other NF subunits. More recent work has shown that a variety of CMT-associated NF-L variants, while indeed unable to form homopolymeric filaments, are still able to incorporate into heteropolymeric filaments when coexpressed with other NF proteins such as NF-M or peripherin (Stone *et al.*, 2019). The authors suggest that CMT NF-L variants in heteropolymers in vivo may not aggregate due to improper filament assembly but rather due to aberrant protein–protein interactions, pointing to the importance of distinguishing filamentous from nonfilamentous aggregates in model systems.

Aggregation may also reflect misregulated NF degradation. NFs can be degraded by the proteasome via gigaxonin (Johnson-Kerner *et al.*, 2015) or TRIM2 (Balastik *et al.*, 2008), macroautophagy (Rao *et al.*, 2023), and calpain-mediated degradation after neuronal injury (Stys and Jiang, 2002). Disrupted degradation, such as in neurons lacking gigaxonin, results in a great increase in NF protein quantity and subsequent aggregation (Ganay *et al.*, 2011; Israeli *et al.*, 2016), potentially because kinesin is not recruited for NF transport (Renganathan *et al.*, 2023).

Whether NF-containing aggregates are themselves pathogenic or are simply byproducts of disease progression remains an unsettled question. Mouse models perturbing NF gene expression often result in aggregates, but only sometimes cause neurodegeneration (Eyer *et al.*, 1998; Perrot and Julien, 2011). The effects of NF-containing aggregates may depend on their location (Beaulieu *et al.*, 2000) – aggregates in the axon may block axonal transport and disrupt organelle localization (Straube-West *et al.*, 1996; Pérez-Ollé *et al.*, 2005; Zhai *et al.*, 2007; Lee *et al.*, 2012; Israeli *et al.*, 2016), while aggregates in the cell body are not predictive of neuropathic phenotype in NF overexpression cell culture or mouse models (Beaulieu *et al.*, 2000; Perrot and Julien, 2011).

Bundles and Aggregates

A comparison of bundling and aggregation may be useful in considering the molecular mechanisms of these processes. Bundling and aggregation both seem to involve a lateral association of large quantities of NFs, rather than improper filament assembly (Carter *et al.*, 1996), though aggregated filaments may have larger core diameters than wild type NFs when visualized by TEM (Uchikado *et al.*, 2006; Ganay *et al.*, 2011). Aggregates may be spherical in nature,

while bundles have high aspect ratio. Aggregates also tend to show "swirling" patterns of various filament orientations while nonaggregated NFs are more aligned with the axon (Ganay *et al.*, 2011; Figure 2F), though single NFs in cultured neurons are observed to fold and bend, straightening out during transport (Fenn *et al.*, 2018).

Broadly, there remain fundamental unanswered questions around the molecular mechanisms of bundling and aggregation. For example, are NF "aggregates" physically crosslinked or entangled, or do they simply reflect local NF accumulations due to disrupted NF transport? How do inter-NF interactions control filament spacing, orientation, and aspect ratio, and how do these interactions relate to the usual dynamics of NF severing and annealing (Uchida *et al.*, 2013, 2023)? Structures described as bundles, filamentous aggregates, nonbundled filaments, and amorphous nonfilamentous aggregates are all observed in model systems, but can be difficult to distinguish by fluorescence microscopy alone. In this regard, mechanistic studies and quantification of NF spacing and orientation data from existing electron micrographs (Ganay *et al.*, 2011) may be useful.

Interfilament interactions and axon caliber

The disordered tail domains are widely thought to mediate NF-NF interactions. NFs are generally found at a regular nearest-neighbor spacing on the order of ~30–50 nm in vivo (Table 1), and there has been much interest in understanding whether and how the tail domains maintain this spacing, particularly the relative contributions of the NF-M and NF-H tails. Two prevailing and nonmutually exclusive mechanisms by which NF tail interactions govern spacing include transient inter-NF ionic crosslinks and entropic repulsion (Figure 3A). Both mechanisms are predicated on a model in which the intrinsically disordered NF protein tails protrude from the filament cores, forming a protein halo or brush around each filament that enables adjacent NFs to interact, and whose thickness determines inter-NF spacing.

Inter-NF crosslinks

Early evidence for NF crosslinks came from quick-freeze deep-etch electron microscopy of axonal cytoskeletons (Ellisman and Porter, 1980; Hirokawa, 1982), which revealed thin structures between adjacent filaments lost upon deleting the NF-M or NF-H tail domains (Chen *et al.*, 2000; Garcia *et al.*, 2003). These findings motivated the proposal that the NF tail domains directly participate in crosslinking of adjacent NFs, though inference of the strength or permanence of apparent crosslinks is complicated by the use of chemical fixation during sample preparation.

Divalent cations have also been implicated in this crosslinking mechanism. NF preparations purified from animal tissue form soft hydrogels in the presence of millimolar levels of magnesium or calcium ions. Some report that these hydrogels consist of both loose NFs and bundles of closely packed parallel NFs (Leterrier and Eyer, 1987; Leterrier *et al.*, 1996, 2009), while others do not report bundle formation (Yao *et al.*, 2010). This variation in structures suggests that there are multiple and potentially coexisting gel microstructures whose presence may depend on gelation conditions. Indeed, gelation is sensitive to many factors including copurifying proteins, ionic strength, phosphorylation state, and ATP concentration in the preparation (Leterrier and Eyer, 1987; Eyer and Leterrier, 1988; Leterrier *et al.*, 1996, 2009; Gou *et al.*, 1998). Importantly, NFs denatured into their subunit proteins and reconstituted into filamentous form are incapable of gelation, even with divalent cations (Leterrier and Eyer, 1987), implying that divalent cations are necessary but not sufficient for gelation. The gelation mechanism may depend on factors lost during reconstitution such as another crosslinking protein, tail domain protein conformation (Leterrier and Eyer, 1987), physical force, or inter-filament entanglements due to ~µm-length reconstituted filaments being significantly shorter than native NFs (up to hundreds of µm; Uchida *et al.*, 2023).

Instead of using intact filaments purified from tissue, NF hydrogels can also be formed by reconstitution of purified NF subunit proteins into filaments by dialysis, then ultracentrifugation with divalent cations (Jones and Safinya, 2008; Beck *et al.*, 2010b). This method forms hydrogels which, depending on salinity and protein stoichiometry, can take on birefringent nematic, isotropic, or opaque liquid crystalline phases (Deek *et al.*, 2013). In a nematic phase, long particles (NFs) are relatively aligned, as opposed to an isotropic phase in which filament orientation is random (Figure 3B). The opaque phase, which corresponds to crosslinked bundles of NFs, was only observed at very low ionic strengths. In the vicinity of physiological ionic strength the nematic phase is predominant, with NFs aligned and relatively evenly spaced (Deek *et al.*, 2013, 2016).

The spacing between filaments within these gels can be measured by small-angle x-ray scattering (SAXS), with or without molecular crowders to control osmotic pressure (Beck *et al.*, 2010b). Beyond a critical osmotic pressure threshold around 10 kPa, the filaments irreversibly compact together, with NF-NF spacing reduced from >60 to ~40 nm. The reconstituted filament system allows control over subunit composition, removal of proteins that copurify with tissue-derived NFs, and a detailed study of how subunit composition impacts NF-NF spacing. The results are complex, interdependent, and not always in agreement with other models (Table 1).

These data led to the "handshake" model of crosslinking between NF tails (Beck *et al.*, 2010b), which assigns an energetic contribution for pairwise residue interactions based on charge and hydrophobicity. This model has identified some regions of NF-M and NF-H tails that may enable hydrophobic and ionic crosslinks, respectively. Though the handshake model does not predict attractive interactions between NF-L tails, an in vitro study has shown that gold nanoparticles coated with NF-L tail constructs aggregate in the presence of divalent cations, suggesting an additional ionic crosslinking mechanism involving NF-L tails (Pregent *et al.*, 2015).

Entropic repulsion

The entropic repulsion model (Brown and Hoh, 1997) was inspired by atomic force microscopy (AFM) which revealed a region around native NFs from which other proteins are excluded (Figure 3C). The size of this region depends on the solution ionic strength, filament composition, phosphorylation state, and presence of divalent cations (Kumar and Hoh, 2004). In the entropic model, confinement would restrict the large set of tail domain conformations and is thus entropically unfavorable, resulting in an interfilament repulsive force. This "entropic brush" model is in analogy to polymer brushes, synthetic polymer systems where one side of each polymer (tail domain) is tethered to a surface (filament core) and the other end is free.

Quantitative analysis of axonal electron micrographs, together with Monte Carlo simulations under varying NF-NF pair potentials, show that purely repulsive interfilament interactions could recapitulate the observed NF spacings seen in vivo (Kumar *et al.*, 2002). In general, theoretical and computational approaches have proven particularly well suited to study protein conformations within NF brushes, which are difficult to access experimentally. Several groups have performed Monte Carlo simulations using models of NF tails coarse-grained at the amino acid level and grafted to a cylindrical core. These studies have revealed that at low salt and without tail

Platform	NF-NF Spacing or Brush Size (nm)[a]	Role of subunit in spacing or brush size			References
		α-internexin	NF-M	NF-H	
Mice	Spacing: ~45			Does not affect spacing	(Rao *et al.*, 2002)
Mice	Spacing: WT ~45 NF-M tail deleted ~39 NF-M/NF-H tails deleted ~30		Increases spacing		(Garcia *et al.*, 2003; Rao *et al.*, 2003)
Mice	Spacing: WT ~30 NF-M phospho-incompetent tail ~25 but distribution overlaps		NF-M phosphorylation does not affect spacing		(Garcia *et al.*, 2009)
Hydrogel SAXS			Promotes more nematic gel phases than NF-H	Promotes isotropic gel formation more than NF-M	(Jones and Safinya, 2008; Deek *et al.*, 2013)
Hydrogel SAXS	Spacing: 80 (low pressure) 40 (high pressure)			Increases spacing more than NF-M	(Beck *et al.*, 2010b)
Hydrogel SAXS	Spacing, α-internexin/ NF-L/ NF-M/NF-H quadruplet: 80 (low pressure) 25 (high pressure)	Increases spacing compared with NF-L, only if NF-M present			(Kornreich *et al.*, 2015)
Hydrogel SAXS				Increases spacing if NF-M is also present	(Deek *et al.*, 2016)
Hydrogel SAXS	Spacing: 80 (low pressure) 30 (high pressure)		NF-M phosphorylation decreases spacing	NF-H phosphorylation increases spacing	(Malka-Gibor *et al.*, 2017)
AFM imaging	Exclusion zone size: ~50–100		No exclusion zone when NF-M and NF-H removed	No exclusion zone when NF-M and NF-H removed	(Brown and Hoh, 1997)
Grafted recombinant NF-H tail				Expands upon phosphorylation	(Srinivasan *et al.*, 2014; Lei *et al.*, 2018)
SCFT	Brush height: ~40			Expands upon phosphorylation to join NF-M at the brush periphery	(Zhulina and Leermakers, 2007)
SCFT	Brush heights: ~45 within a range of stoichiometries, strongly dependent on phosphorylation level				(Zhulina and Leermakers, 2010)
SCFT	Brush heights: NF-L/NF-M/NF-H: ~45 α-internexin/ NF-L/NF-M/NF-H: ~45 α-internexin/NF-M/NF-H: ~40	Decreases brush size compared with NF-L due to less repulsion of NF-M or phospho-NF-H			(Leermakers and Zhulina, 2010)
Simulation				Expands upon phosphorylation, but still not as extended as NF-M	(Chang *et al.*, 2009)
Simulation	Brush height: ~60			Slightly more extended than NF-M at high ionic strength	(Jayanthi *et al.*, 2013; Lee *et al.*, 2013)

[a]Spacing and brush sizes for nonmouse studies are given for phosphorylated proteins, 100–200 mM solution ionic strength, and compositions including NF-L, NF-M, and NF-H tail proteins as well as α-internexin when noted.

TABLE 1: Effects of NF subunit proteins on inter-NF spacing and tail domain brush conformations.

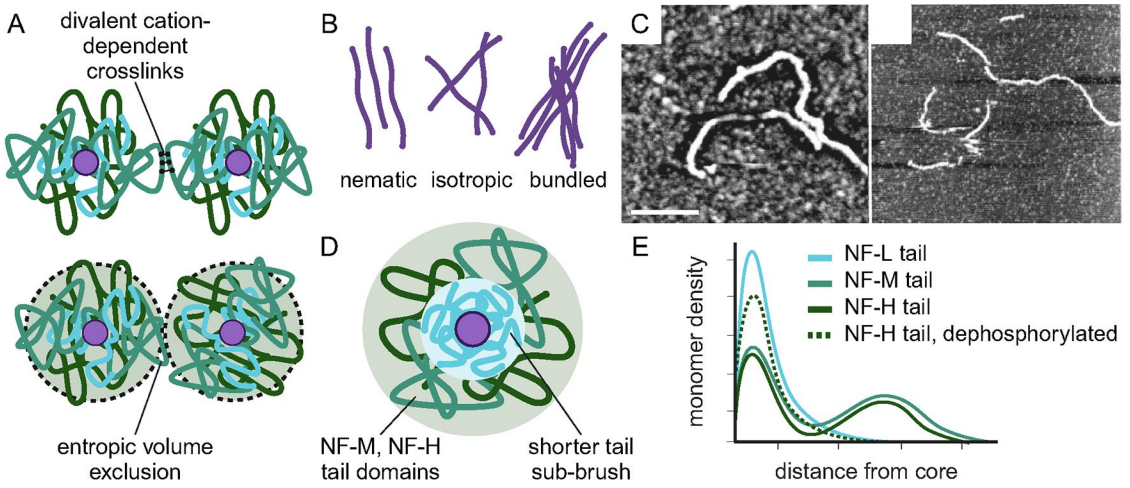

FIGURE 3: Inter-filament interaction models and NF tail domain conformations. (A) Diagrams of two models of inter-filament interaction: divalent cation based crosslinking and entropic volume exclusion. (B) Diagrams of structures in liquid crystal hydrogel phases from (Deek *et al.*, 2016). (C) Left: native NFs with excluded volume; right: NF-L only reconstituted NFs with no excluded volume. Scale bar: 500 nm. Reproduced from (Brown and Hoh, 1997), copyright 1997 American Chemical Society. (D) Schematic of NF tail brush structure, showing subbrush formed of shorter tailed proteins such as NF-L. (E) Example brush substructures and subunit localizations accessible by simulation or theoretical modeling. Results qualitatively adapted from (Zhulina and Leermakers, 2007) and (Lee *et al.*, 2013). Figure created with BioRender.com.

phosphorylation, the NF-L tail forms a subbrush layer near the filament core (Stevenson *et al.*, 2011; Figure 3D). The composition of this subbrush may govern the more extended conformations of the NF-M and phosphorylated NF-H tails (Leermakers and Zhulina, 2010). Filaments pushed together will sometimes repel, but can overlap especially at close packing and significant amounts of salt (Jayanthi *et al.*, 2013). At high ionic strength, simulations have found opposite effects of NF-H and NF-M (Chang *et al.*, 2009; Kim *et al.*, 2011; Lee *et al.*, 2013; Table 1).

Self-consistent field theory (SCFT) is also well suited to studying polymer brushes and has also been applied to the NF tails (Zhulina and Leermakers, 2009; Yokokura *et al.*, 2023). SCFT applies a mean-field approximation to a coarse-grained model of the NF tails, using the sequence-dependent charge and hydrophobicity within each protein chain to determine the location profiles of amino acid monomers making up the brush structure (Figure 3E). SCFT studies have aligned well with the computational observation that NF-L forms a subbrush (Leermakers and Zhulina, 2010), with the longer NF-M and NF-H tails protruding, especially when phosphorylated. However, in the SCFT model, NF-M tends to set a taller brush height than NF-H (Zhulina and Leermakers, 2007, 2009, 2010; Table 1). This model predicts that the NF-L subbrush electrostatically repels or attracts the NF-H tail depending on whether the latter is phosphorylated (Zhulina and Leermakers, 2009). Substituting α-internexin for NF-L reduces the repulsive force on NF-M and NF-H tails, leading to overall shorter brush heights (Leermakers and Zhulina, 2010; Table 1).

Grafting purified NF tail proteins to a solid support is a powerful reconstitution paradigm that allows assembly of NF tails in their physiological orientation and direct measurement of the resulting brush thickness by AFM. In one set of studies, NF-H tail domains were expressed in *Escherichia coli* and grafted to a functionalized substrate in an end-directed manner via an engineered cysteine, creating a brush of similar grafting density to a native neurofilament tail brush (Srinivasan *et al.*, 2014). At neutral pH, a purely NF-H tail brush takes on a collapsed conformation. These brushes can also be phosphorylated by purified kinases in vitro; upon phosphorylation, the NF-H tail brush expands significantly, though less dramatically at physiological ionic strength (Lei *et al.*, 2018). As expected for a polyelectrolyte brush, divalent cations collapse the brush at lower concentrations than monovalent cations (Lei *et al.*, 2018).

Other work has examined how the protein sequence determines the conformational ensembles taken by the tail domains. Using SAXS and time-resolved Förster resonance energy transfer (trFRET), Koren *et al.* (2023) showed that the C-terminal region of the NF-L tail may loop back to interact with more N-terminal segments of the sequence. Loops have also been noted to form in Monte Carlo simulations of NF-M and NF-H tails (Chang *et al.*, 2009). Molecular dynamics simulations showed that salt bridges within the NF-H tail can make small loops, which help the tail stay within the brush (Adiga and Brenner, 2010). These loops are disrupted upon phosphorylation enabling a dramatic expansion of the chain, though this work considered the context of a dilute untethered protein rather than a crowded brush. NF-H tail loops are also consistent with single-molecule force spectroscopy data (Aranda-Espinoza *et al.*, 2002), though in this technique it is challenging to ensure pulling of only single protein molecules without experimental controls such as covalent surface linking or recombinant fingerprint domains (Yang *et al.*, 2020).

Recent experimental work has also examined the conformation of a fragment of the NF-M tail which has blocks of positive and negative charge of various lengths (Bianchi *et al.*, 2020). The authors shuffled the charged residues to be more evenly spaced and found that the charge-shuffled protein takes on a more expanded conformation than the wild type. While it is not clear whether this sequence-conformation relationship would also hold for the full length NF-M tail in the context of a crowded brush, Monte Carlo simulations in the brush context support the idea that a positively charged block near the center of the NF-M tail remains pinned to the subbrush, with more C-terminal negatively charged blocks protruding further and contributing to brush height (Chang *et al.*, 2009; Jeong *et al.*, 2016). In that case, an evenly shuffled charge distribution could un-pin the central charged block and expand the protein.

Axon caliber

NFs are important in developing axons of large caliber, which is critical for conduction velocity. NFs in large-caliber axons can significantly outnumber microtubules (Hoffman *et al.*, 1984), and caliber is reduced in multiple axonal-NF-deficient mouse models (Eyer and Peterson, 1994; Zhu *et al.*, 1997) and in NF-L-nonsense quail (Yamasaki *et al.*, 1991; Ohara *et al.*, 1993). The relative expression levels of the NF proteins are important to support caliber increases, as various NF protein knockout and overexpression mouse models have resulted in decreased calibers while only simultaneous overexpression of NF-L and either NF-M or NF-H resulted in increased caliber (Marszalek *et al.*, 1996; Xu *et al.*, 1996; Meier *et al.*, 1999; Perrot and Julien, 2011). However, knockout mouse models used to study this effect can potentially produce compensatory changes in expression of other NF proteins or cytoskeletal elements such as microtubules, making interpretation challenging (Elder *et al.*, 1998; Rao *et al.*, 1998; Jacomy *et al.*, 1999; Yuan *et al.*, 2006).

Gene replacement mouse models have shown that deleting the NF-M tail domain results in reduced axonal caliber and more NF clustering, indicating the importance of this protein domain for caliber maintenance (Garcia *et al.*, 2003). However, replacement of the NF-M tail phosphorylation sites with nonphosphorylatable alanine residues produces effectively no change in spacing or caliber (Garcia *et al.*, 2009). Similar models have also shown that deleting the NF-H tail surprisingly does not affect caliber, inter-NF spacing, or NF transport rate, though NF-M tail phosphorylation was upregulated in response (Rao *et al.*, 2002, 2003).

NF spacing and axon caliber were originally thought to be directly related; however, genetically altered animal models have exhibited changes in axon caliber without changes in NF-NF spacing (Elder *et al.*, 1998; Barry *et al.*, 2012), as well as large changes in spacing without correspondingly large changes in caliber (Xu *et al.*, 1996). The amount of correctly assembled heteropolymeric NFs in the axon does seem to be an important factor in determining axon caliber. Myelin-dependent signaling via myelin-associated glycoprotein locally regulates NF phosphorylation in internodes and subsequent changes in NF transport rates (De Waegh *et al.*, 1992; Yin *et al.*, 1998; Monsma *et al.*, 2014). However, the mechanism connecting NF content and axon caliber growth remains unclear (De Waegh *et al.*, 1992; Yin *et al.*, 1998; Garcia *et al.*, 2003).

CELL MECHANICS AND CYTOSKELETON

NFs as a structural element in the axon

As some IFs are cell-type specific, IFs have been hypothesized to support cell-specific mechanical needs. NFs have been thought to mechanically support the thin and fragile axon (Kornreich *et al.*, 2016) as part of the larger axonal cytoskeleton (Figure 4A). This biomechanical function remains incompletely understood in the full complexity of a neuron. However, insights may be gained by examining the body of in vitro work on the mechanical properties of single NFs and NF-based materials.

Along with other IFs, single NFs are highly stretchable up to 3.4 times their original length in axial tension (Kreplak *et al.*, 2005). Upon stretching, single NFs also thin dramatically, which may be due to partial filament unraveling (Wagner *et al.*, 2007), or partial unfolding of the rod domain proteins as is the case for vimentin (Block *et al.*, 2018). NF-L-only filaments are less mechanically robust than native filaments (Brown *et al.*, 1998), but whether this is due to the filament reconstitution process or subunit composition itself is unclear. Single filaments can also be characterized by their persistence length L_p, which is a measure of filament stiffness. While L_p is >1 mm for microtubules and ~10 μm for F-actin, IFs tend to be much more flexible, with L_p ~ 100s of nanometers (Dalhaimer *et al.*, 2005; Wagner *et al.*, 2007). For NFs, L_p has been measured by AFM to be between 100–450 nm (Dalhaimer *et al.*, 2005; Beck *et al.*, 2010a). Notably, the mechanics of isolated NF bundles have not been studied.

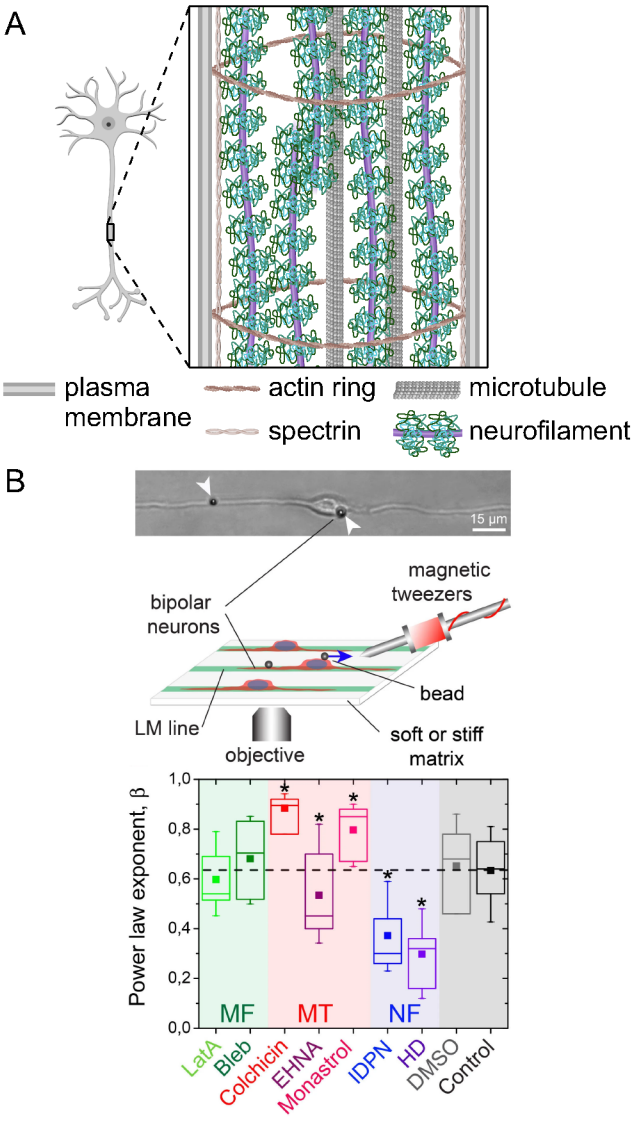

FIGURE 4: NFs in axon mechanics. (A) Simplified schematic of the axonal cytoskeleton including NFs, microtubules, and membrane-associated periodic scaffold including actin rings and spectrin. (B) Top: magnetic tweezers were used to pull a fibronectin-coated bead to measure cell mechanical properties at the neurite. Bottom: Neurite viscoelasticity decreases when treated with the compounds IDPN and 2,5-hexanedione (HD), which disrupt NF organization. Reproduced from (Grevesse *et al.*, 2015). Figure created with BioRender.com.

NF hydrogel mechanical properties have been more thoroughly characterized. Rheological studies have measured storage and loss moduli, which respectively reflect a material's ability to store and internally dissipate applied stress. Tissue-prepared native NF hydrogels are very soft with shear storage moduli of ~1-100 Pa. These gels also demonstrate strain stiffening up to 200 Pa, are capable of stress relaxation (Leterrier *et al.*, 1996), and can self-heal several times after mechanical disruption (Leterrier and Eyer, 1987).

Several studies have explored the mechanism by which NF hydrogels resist stress. The storage modulus of these gels is much greater than the loss modulus, reflecting a crosslinked solid hydrogel (Yao *et al.*, 2010). The hydrogel storage modulus depends directly on the concentration of Mg^{2+}, implicating divalent cations in the crosslinking mechanism. Yao *et al.* (2010) suggest that the hydrogel elasticity derives from the entropic penalty of stretching single NFs, which due to their low persistence length are semiflexible between crosslink sites. In this work and a related study (Lin *et al.*, 2010) the authors calculated mesh sizes on the order of a few 100 nanometers and did not observe bundling by optical microscopy, while other studies of similar NF preparations measured mesh sizes on the order of micrometers and correspondingly observed bundles by electron microscopy (Leterrier and Eyer, 1987; Leterrier *et al.*, 1996; Rammensee *et al.*, 2007). Interestingly, significant amounts of glycerol or sucrose are also required for gelation, suggesting the importance of crowding and osmotic pressure for NF-NF crosslinking (Leterrier *et al.*, 1996). Gelation is also slowed significantly and gels are significantly softer at physiological levels of monovalent salt, which is not present at high concentrations in the standard preparation buffer (Leterrier and Eyer, 1987; Rammensee *et al.*, 2007).

Reconstituted NF hydrogels, where filaments can be aligned, reconstituted, and controlled more precisely, generally exhibit much stiffer storage moduli of hundreds to several thousands of Pa (Deek *et al.*, 2013), though they can be softened somewhat with monovalent salt. Measuring the effect of osmotic pressure on filament spacing enables calculating the bulk modulus, another measure of the stiffness of a material (Malka-Gibor *et al.*, 2017). Phosphorylation of NF-H increased the hydrogel bulk modulus, suggesting that the effect of NF-H phosphorylation may be mechanical rather than structural (Malka-Gibor *et al.*, 2017). This suggestion has also arisen in Monte Carlo simulations (Kim *et al.*, 2011). However, the specific mechanism – for example, due to steric or osmotic swelling effects – remains unclear.

In living cells, of course, the picture is more complicated; only a few studies have quantified contributions of NFs to cell-scale mechanical properties. Direct measurements of axonal mechanics by magnetic tweezer creep tests have demonstrated that NFs generally contribute to stiffness of the cell body and promote axonal viscoelasticity, while microtubules promote elastic character in the axon (Grevesse *et al.*, 2015; Figure 4B). Another previous study indicated that NFs contribute significantly to neurite stiffness, though not as much as microtubules (Ouyang *et al.*, 2013). Notably, the effect of NFs in both studies was assessed by treating cells with chemical agents directed against IFs such as acrylamide and IDPN, which may have many off-target effects at moderate levels and are cytotoxic at higher levels.

Crosslinking proteins and cytoskeletal crosstalk

NF ultrastructure may be remodeled by crosslinking proteins. In a pulldown assay using the NF-L head domain as "bait" (Zhou *et al.*, 2021), many cytoskeleton-related proteins were enriched from mouse brain lysate including other NF proteins, actin, spectrin, tubulin, dynein, and kinesin. Dynein and kinesin are known to link NFs to the microtubule network, enabling NF transport (Shah *et al.*, 2000; Yabe *et al.*, 2000; Xia *et al.*, 2003; Wagner *et al.*, 2004; Francis *et al.*, 2005; He *et al.*, 2005; Kushkuley *et al.*, 2009; Uchida *et al.*, 2009). NFs are also a negative regulator of microtubule dynamics (Bocquet *et al.*, 2009; Yadav *et al.*, 2016), possibly in an aggregation-dependent manner (Kurup *et al.*, 2018). This finding might shed light on compensation between MTs and NFs in NF-deficient models. The contrast between the stable structural NF core and the dynamic, disordered outer layer stands in structural analogy to microtubules and their dynamic surrounding layer of C-terminal tails and often disordered MAPs (Bodakuntla *et al.*, 2019), which may serve as an alternative neuronal cytoskeleton in the absence of NFs (Prokop, 2020).

Other putative crosslinking proteins include BPAG1-n, a neuronal isoform of BPAG1 whose knockout causes perikaryal NF accumulation in mice (Yang *et al.*, 1996), and plectin, which binds other IFs at the rod domain (Potokar and Jorgačevski, 2021; Wiche, 2021). Plectin isoform P1c has been observed to colocalize with NFs in vivo (Potokar and Jorgačevski, 2021).

CONCLUSION

NFs are a complex, tunable, and dynamic structural system within the neuron, with properties enabled by a combination of ordered and disordered protein regions. The exciting IDP physics unfolding in the NF-L head domain invites comparison to other NF subunit head domains, as each subunit's head domain might differentially regulate filament formation. In cell mechanics, many mechanisms remain unknown, for example how NF abundance contributes to axon caliber; how divalent cations mediate NF bundling; the mechanism of NF aggregation; and whether the distinct subunit proteins, phosphorylation, and NF ultrastructure affect axon mechanical properties. Many of these mechanistic questions will benefit from a deeper understanding of the relevant IDP physics, especially of the NF tail domains.

Understanding the biophysical function of NFs and their disordered domains may facilitate aspects of biomarker development. Work exploring the prognostic value of different NF subunits or fragments and their phosphorylation levels, or accounting for aggregation kinetics in biofluids (Lu *et al.*, 2011; Adiutori *et al.*, 2018; Budelier *et al.*, 2022; Petzold, 2022), could be informed by a deeper understanding of the biophysics underlying NF protein stoichiometry, phosphorylation, and aggregation. For example, whereas NF-L is a somewhat nonspecific marker of neuronal damage, there could be value in developing more precise NF subunit-based biomarkers that reflect specific mechanisms of damage.

The disordered NF domains are also a unique model system for understanding IDP function more broadly. In particular, the extensive phosphorylation and high proline content in the NF-H tail domain is quite unusual within the proteome and poses an opportunity to explore multiphosphorylated and proline-dependent conformational ensembles, both of which are current frontiers within IDP research. Further, disordered NF domains demonstrate the effects of system geometry, with the head domains contributing to filamentous assembly and the tail domains tethered to the core in a cylindrical protein brush. A protein brush geometry is also found in the disordered proteins of the nuclear pore complex, enabling comparison of these to other dense IDP assemblies and condensates.

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