Neurofilaments at a glance

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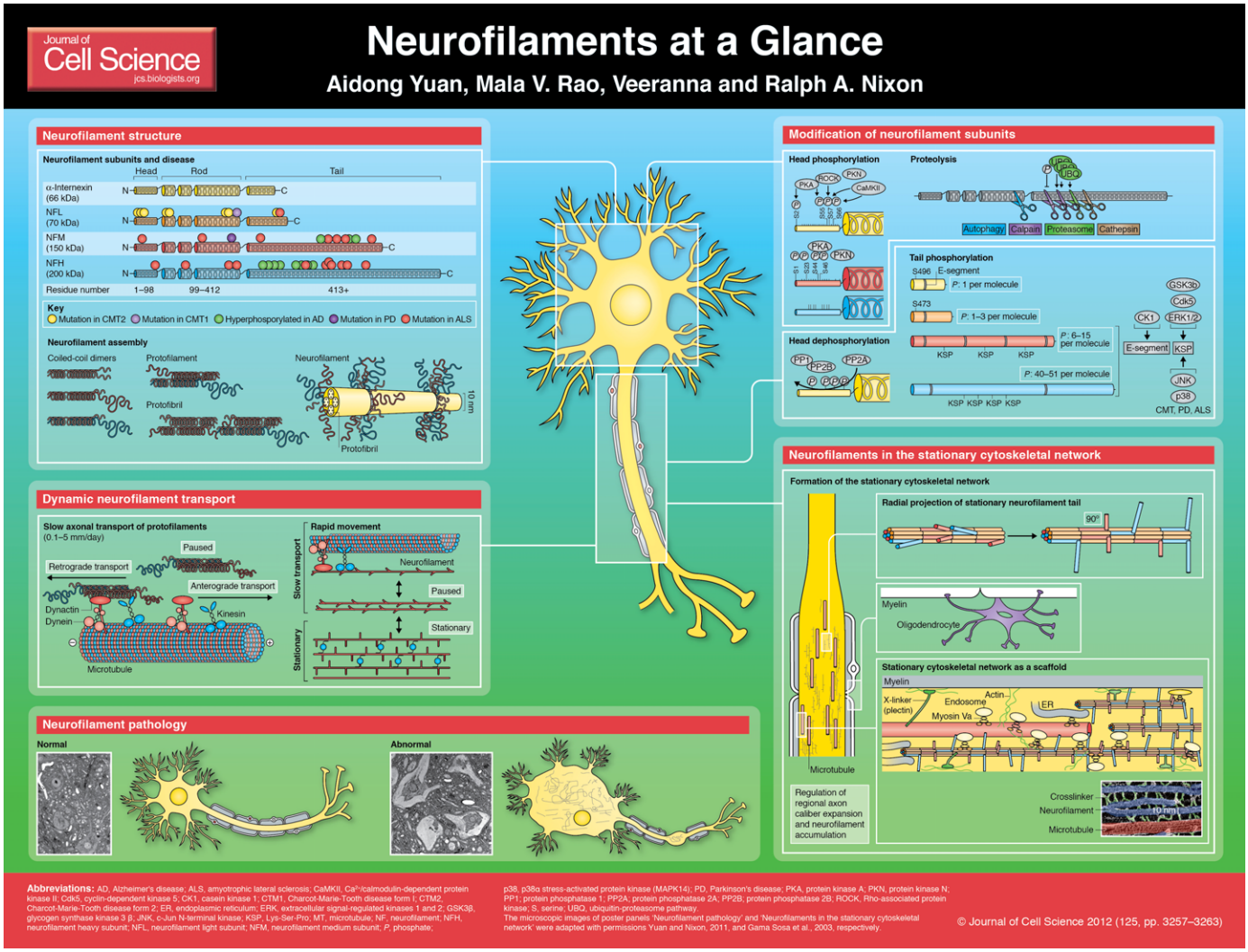
Neurofilaments (NFs) are intermediate filaments with a diameter of 10 nm, similar to that of neurons. Although they are present in perikarya and dendrites, neurofilaments are particularly abundant in axons, where they are essential for the radial growth of axons during development, the maintenance of axon caliber and the transmission of electrical impulses along axons, i.e. velocity of nerve conduction (Eyer and Peterson, 1994; Friede and Samorajski, 1970; Ohara et al., 1993; Yum et al., 2009; Zhu et al., 1997). In some pathological conditions, neurofilaments can accumulate in large numbers within cell bodies and proximal axons of affected neurons (Liu et al., 2009; Munoz et al., 1988). In patients with amyotrophic lateral sclerosis (ALS), these accumulations are a hallmark pathological lesion, but they are also prominent in sufferers of other neurological diseases, such as Charcot-Marie-Tooth (CMT) disease, neurofilament inclusion disease (NFID), giant axonal neuropathy (GAN), diabetic neuropathy, spinal muscular atrophy (SMA) and spastic paraplegia, and are present in those people that suffer from Alzheimer's disease (AD) and Parkinson's disease (PD) (Abe et al., 2009; Perrot and Eyer, 2009; Szaro and Strong, 2010). Transgenic mouse models support the idea that these aberrant NF accumulations contribute to the death of the affected neurons, rather than simply being by-products of the pathogenic process (Côté et al., 1993; Couillard-Després et al., 1998; Williamson et al., 1998). In this Cell Science at a Glance article, we review the current understanding of neurofilament functions in health and disease.

Neurofilament structure and function

Neurofilaments from the central nervous system (CNS) are heteropolymers that are composed of four subunits, namely neurofilament heavy, medium and light



(See poster insert)

polypeptides (NFH, NFM and NFL, respectively; also known as NEFH, NEFM and NEFL), as well as α-internexin (Int), whereas in the peripheral nervous system, neurofilaments are made up of NFH, NFM, NFL and peripherin (Beaulieu et al., 1999; Yan et al., 2007; Yuan et al., 2006b). Neurons may also express other intermediate filament proteins, including nestin, synemin, syncoilin and vimentin (Perrin et al., 2005). Mouse NFH, NFM and NFL subunits are unable to self-assemble into homopolymer filaments, although – at least in vitro – human NFL can do so (Carter et al., 1998). Among the notable properties of NFs are their exceptionally long half-lives (Millecamps et al., 2007; Nixon and Logvinenko, 1986; Yuan et al., 2009) and their elastic fibrous properties that enable them to maintain the markedly asymmetrical shape of neurons (Wagner et al., 2007). Neurofilaments are required for axon radial growth (Eyer and Peterson, 1994; Hoffman et al., 1987) and the NFL and NFM subunits are especially important (Elder et al., 1998; Ohara et al., 1993; Zhu et al., 1997). Although deletion of the phosphorylated tail domain of NFM inhibits radial growth of axons and reduces their conduction velocities (Garcia et al., 2003; Rao et al., 2003), mice expressing NFM subunits that lack phosphorylation at KSP (Lys-Ser-Pro) sites along the tail domain have normal axon calibers and conduction velocities (Garcia et al., 2009), indicating that the NFM tail domain but not tail phosphorylation is crucial for axon radial growth. Decreased axon caliber that is accompanied by reduced conduction velocity has been observed in mutant Japanese quail that lack NFs because of a nonsense mutation in the NFL gene (Sakaguchi et al., 1993), in transgenic mice expressing a NFH–β-galactosidase fusion protein that interferes with NF transport into axons (Perrot et al., 2007), and in knockout mice that lack *Nefl* or *Nefm* (Križ et al., 2000). However, normal axonal caliber but decreased conduction velocity is observed in *Nefh*-null mice, indicating that NFs have roles beyond being determinants of the physical dimensions of axons (Križ et al., 2000).

A number of specific roles have been identified for the protein domains in each NF subunit, which confer to the heteropolymer (see poster panel, NF assembly) the general properties of a scaffold for the docking and organization of different axoplasmic constituents (Balastik et al., 2008; Kim et al., 2011; Rao et al., 2011). NF subunits contain a globular head, an α-helical rod domain, and variable tail domains that differ in length and amino acid composition. Each neurofilament subunit contains a highly conserved 310 amino acid rod domain that is important for the co-assembly with other NF subunits to form filaments. The head domains of all NFs have a microtubule (MT) polymerization inhibitory domain that regulates the number of MTs in the axon (Bocquet et al., 2009). The rod domains have important roles in the polymerization of NF subunits into NFs, and serve as a binding site for the myosin Va motor protein, which modulates levels and local topography of specific vesicular organelles (ER, endosomes, synaptic vesicles) within the axoplasm (Rao et al., 2011). Loss of NFL or myosin Va partially depletes these organelles from axons (Rao et al., 2011). The C-terminal domains of both NFH and NFM form fine lateral extensions that increase the spacing between NFs, thereby maximizing their ability to occupy space during axon caliber expansion. The C-terminal domain of NFM is more important for radial growth of axons than that of NFH (Rao et al., 2003; Rao et al., 2002). Crossbridging between neurofilaments through the tail domains of NFH and NFM is believed to be influenced by the phosphorylation level of these tails (Eyer and Leterrier, 1988; Gou et al., 1998) and mediated by divalent cations (Kushkuley et al., 2009; Kushkuley et al., 2010).

Interestingly, a splice variant of the N-methyl-D-aspartate (NMDA) receptor subunit NR1 associates with NFL (Ehlers et al., 1998), and the dopamine D1 receptor selectively associates with the C-terminus of NFM (Kim et al., 2002), although the functional significance of these intriguing interactions is not yet clear. In humans, mutations in NFL are associated with Charcot-Marie-Tooth disease. These mutations affect the assembly of NFs in the neurons (Sasaki et al., 2006) and, upon that, can inhibit NF transport (Yates et al., 2009). Mutations of the NFM rod domain have occasionally been identified in early-onset PD (Lavedan et al., 2002). Mutations in the NFH gene have been identified in a small number of sporadic ALS patients (Al-Chalabi and Miller, 2003).

Neurofilament transport

Most neurofilament proteins are synthesized within the cell body and must travel long distances along axons to reach their sites of function. The mechanisms that underly axonal transport of neurofilament proteins are elusive, although recent genetic and live-cell imaging approaches have yielded general principles regarding the dynamic behaviors of neurofilaments (Roy et al., 2000; Wang et al., 2000; Yabe et al., 1999; Yuan et al., 2003; Yuan et al., 2006b; Yuan et al., 2009). For example, by transfecting green fluorescent protein (GFP)-labeled NF subunits into developing sympathetic neurons in culture, Brown and colleagues were able to directly visualize movements of individual short filaments (1.0–15.8 μm in length) along the relatively NF-poor growing axons (Trivedi et al., 2007; Wang et al., 2000). Studies in mice that lack one or more NF subunit genes have shown, however, that transport of NF subunits does not require the formation of complete neurofilaments (Yuan et al., 2003; Yuan et al., 2006b). For instance, we have demonstrated that the number of neurofilaments in optic axons of NFH-NFL double knockout mice is less than 10% of the usual number of neurofilaments, yet these mice have 50% of the usual level of NFM subunits, which move along axons at typical transport rates (Yuan et al., 2003).

Further studies with different combinations of NF subunit deletions have shown that the minimal requirement for axonal transport is the formation of heterodimers that involve specific NF subunits. NFM and α-internexin have been identified as the subunits that are crucial for the transport of dimers or NFs, as deleting both prevents transport of NFL and/or NFH, whereas deleting either NFH, NFL or both, only minimally alters NFM or α-internexin transport in optic axons (Yuan et al., 2006a; Yuan et al., 2003; Yuan et al., 2006b). The early presence of α-internexin in developing rat optic axons explains why NFM and NFL subunits are detectable in these axons before morphologically definable neurofilaments appear, further indicating its crucial role for neurofilament partnership and transport (Pachter and Liem, 1984). The state of assembly of neurofilament proteins during axonal transport became an active area of investigation and debate during the past three decades, with considerably indirect evidence being amassed to support movement of polymers or subunit or oligomer assemblies (Baas and Brown, 1997; Hirokawa et al., 1997). Movement of fluorescent puncta that represent non-filamentous assemblies of GFP-labeled NF subunits have been reported (Prahlad et al., 2000; Yabe et al., 1999); however, short neurofilaments have also been observed in other types of cultured cells and in the squid

giant axon, depending on the methods used (Ackerley et al., 2003; Galbraith et al., 1999; Roy et al., 2000; Yan and Brown, 2005). Transport of both non-filamentous NF oligomers and filaments has also been reported in neuroblastoma cells (Yabe et al., 2001), as well as movement of vimentin non-filamentous particles and filaments in spreading baby hamster kidney cells (Prahlad et al., 1998). Our recent photobleaching analyses of GFP-tagged NFL protein in cultured cortical neurons further reconcile these, apparently conflicting, observations by demonstrating that both non-filamentous NF subunit assemblies and short NF polymers can be transported in the same axon (Yuan et al., 2009). At proximal axonal levels in these neurons, the transport of subunit assemblies predominates, whereas the transport of short NFs predominates at distal levels of the same axon. Collectively, these observations establish that NF proteins might exist in multiple assembly forms during axonal transport and suggest that the transported NF subunits assemble into filaments during axonal transport (Yuan et al., 2009).

The molecular motors that regulate NF transport are believed to be the fast microtubule-based motors kinesin and dynein (Prahlad et al., 2000; Sunil et al., 2012), and microfilament-based motor myosin Va (Alami et al., 2009). Kinesin-I has been proposed to be an anterograde motor for NF because it interacts with the NFH or NFM subunits (Jung et al., 2005; Yabe et al., 2000) and because antibody against kinesin-I blocks NF transport (Yabe et al., 1999). However, later studies have found that NFH is dispensable for NF transport (Rao et al., 2002; Yuan et al., 2006a; Yuan et al., 2006b), implying that, if kinesin-I were the NF transport motor, it would have additional NF subunit partners. Studies from kinesin-IA (an isoform of kinesin-I) knockout mice (Xia et al., 2003) and analyses of the effect of kinesin-I mutation on NF transport in cultured neurons (Wang and Brown, 2010) suggest that it acts as an anterograde motor for NF transport. The dynein-dynactin complex is believed to be the retrograde motor for axonal transport of NF because the dynein–dynactin complex co-purifies with NF (Shah et al., 2000) and dynein interacts with the rod domain of the NFM subunit in yeast two hybrid assays (Wagner et al., 2004). Knockdown of expression of the dynein heavy chain using small interference RNA (siRNA) has been shown to significantly decrease retrograde NF transport (He et al., 2005; Uchida et al., 2009). Despite these advances, the interaction between NFs and motors has not yet been directly visualized by live-cell imaging in cultured neurons or in live animals.

The earliest in vivo pulse-labeling studies of neurofilament protein transport by Lasek and colleagues were initially interpreted to support the idea that the labeled neurofilaments that undergo transport constitute the entire NF cytoskeleton within axons, which, according to this model, is continuously moving (Lasek et al., 1984; Lasek et al., 1992). A different model, however, was proposed by the authors on the basis of studies of short and exceptionally long-term (6 months) pulse-labeling by using the mouse optic system (Nixon and Logvinenko, 1986). These studies supported the idea that neurofilaments (or oligomeric assemblies) that undergo slow transport in myelinated axons are a small precursor pool that maintains a large pre-existing fixed NF lattice. This lattice, in turn, exists within a complex stationary cytoskeletal network that is composed of the various cytoskeletal elements (NF, microtubules, actin filaments) that are visible in ultrastructural images of the cytoskeleton. Later studies added support to this general model (Millecamps et al., 2007; Yuan et al., 2009), including an in vivo study of NF turnover by Julien and colleagues, in which NFL expression was acutely shut off in a conditional NFL knockout mouse, followed by measuring the fate of the pre-existing neurofilaments (Millecamps et al., 2007). This study demonstrates that pre-existing neurofilaments in the axon remain stationary and display an exceptionally slow turnover (>2.5 months) – much longer than could be explained by loss through axonal transport. On the basis of this current evidence (Millecamps et al., 2007; Yuan et al., 2009), the neurofilament network is now viewed as a large stationary and metabolically stable structure that is assembled from transported elements (Yuan et al., 2009). These elements can either be short polymers or oligomers of NF subunits. The proportions of these different assembly forms might vary depending on cell type and developmental state (Nixon and Shea, 1992; Yabe et al., 2001). Regardless of the assembly form that neurofilament proteins take during transport (i.e. dimer, oligomer or short filament), they subsequently undergo additional steps of integration into a stationary neurofilament network, which involves regulatory events that still remain to be fully explored (Yuan et al., 2003; Yuan et al., 2009).

Neurofilament phosphorylation

Neurofilaments undergo various post-translational modifications, such as phosphorylation, glycosylation, nitration, oxidation and ubiquitylation (Perrot et al., 2008). The head domains of NFL, NFM, NFH and α-internexin are phosphorylated and glycosylated (Dong et al., 1996; Manser et al., 2008; Tanaka et al., 1993; Vosseller et al., 2006). Phosphorylation of the NF head domain is mediated by the second messenger dependent kinases protein kinase A (PKA) and C (PKC) (Sihag et al., 1988; Sihag and Nixon, 1989; Sihag and Nixon, 1990) and possibly also by Cam kinase II (Hashimoto et al., 2000). The occurrence of NF head phosphorylation in the cell body soon after subunit synthesis reflects its suggested role in maintaining the disassembled state of NFs, as head phosphorylation of NFL inhibits NF assembly (Hashimoto et al., 2000; Hisanaga et al., 1990; Sihag et al., 1999; Sihag and Nixon, 1991). Phosphorylation of the NF head domain is also known to modulate their interaction with fodrin, an important protein of the sub-axolemmal cytoskeleton (Frappier et al., 1987). However, as neurofilaments are transported along axons, many of the initially incorporated phosphate groups are removed (Nixon and Lewis, 1986). The assembly of neurofilaments prior to their entry into the axon and the rapid turnover of phosphates during their transit along the axon requires dephosphorylation of the head domain, which is effected by protein phosphatase 2A (PP2A) (Nixon and Lewis, 1986; Saito et al., 1995).

The C-terminal domains of NFM and NFH have multiple lysine-serine-proline (KSP) sites, the phosphorylation status of which is regulated by multiple protein kinases and phosphatases (Veeranna et al., 2011). Soon after the NFs enter and move along the axon they are extensively phosphorylated on the tail domains of NFM and NFH (Julien and Mushynski, 1983; Nixon and Lewis, 1986; Nixon et al., 1989; Nixon et al., 1987). Phosphorylation of the tail domain is specific for particular regions within the cell, with non-phosphorylated tails predominately found in the cell bodies, which become highly phosphorylated in mature axons (see poster panel Modification of neurofilaments: NF head phosphorylation, Tail phosphorylation). This developmentally regulated phosphorylation appears to depend on myelination and synaptogenesis (Carden et al., 1987;

Sánchez et al., 2000). Although the dendrites also harbor NFs, these filaments are, for unknown reasons, not highly phosphorylated (Lee et al., 1987; Sternberger and Sternberger, 1983). Most phosphorylation of the tail domain occurs on KSP repeats (Geisler et al., 1987; Jaffe et al., 1998; Lee et al., 1988; Xu et al., 1992), although non-SP sites are also phosphorylated. KSPxK motifs are phosphorylated by proline-directed cyclin-dependent kinase 5 (Cdk5) (Shetty et al., 1993; Sun et al., 1996; Veeranna et al., 1995). Both KSPxK and KSPxxxK sites are phosphorylated by mitogen-activated protein kinases (MAPKs), such as extracellular-signal-regulated kinases (Erks) (Veeranna et al., 1998), c-Jun N-terminal kinases (JNKs) (Giasson and Mushynski, 1997) and p38 (MAPK14) (Ackerley et al., 2004), which constitute signaling cascades that respond to growth factors (Li et al., 1999b; Pearson et al., 2001), Ca^{2+} influx (Li et al., 1999a), integrins (Li et al., 2001) and myelination (Nixon et al., 1994). Myelination has an effect on NF phosphorylation. This is evidenced by the observations that NF phosphorylation is decreased in dysmyelinating mutant Trembler mice (de Waegh et al., 1992) and at the initial segment of optic nerves and nodes of Ranvier (the gaps formed between the myelin sheaths generated by different cells) (Hsieh et al., 1994; Mata et al., 1992; Reles and Friede, 1991), as well as by the possible role of myelin associated glycoprotein (MAG) as a mediator of myelin signals that alter NF phosphorylation (Dashiell et al., 2002; Yin et al., 1998). Phosphorylation of the tail domain can regulate both the interactions between the NF domains themselves and with microtubules (Hisanaga and Hirokawa, 1989; Hisanaga et al., 1991). Tail phosphorylation is also the crucial modification that confers their exceptional proteolysis resistance to NFs (Pant, 1988). Although the activity of NF kinases decreases during maturation and aging (Veeranna et al., 2011), neurofilament tail domains become increasingly phosphorylated mainly owing to decreased activities of PP2A and protein phosphatase (PP1). However, PP1 has only a minor role in the regulation of NF phosphorylation compared with that of PP2A (Strack et al., 1997). Aberrant phosphorylation of NFs leads to their accumulation in cell bodies and has been observed in the brains of AD patients and those suffering from other neurodegenerative disorders (Rudrabhatla et al., 2011). The abnormal phosphorylation of NFs in AD patients has been attributed to a decrease in the levels of PP2A and PP1 (Gong et al., 2005; Gong et al., 1995; Gong et al., 1993), and to elevated levels of NF kinases, including Cdk5, ERK1 and ERK2 (Veeranna et al., 2004), and JNKs (Zhu et al., 2001). These observations are further supported by mass spectrometric analyses of NFM and NFH in brains of AD patients, in which phosphorylation of KSP repeats was increased approximately four- to eightfold compared with the phosphorylation of these sites in brains of control patients (previously documented) (see Rudrabhatla et al., 2011). Also in the brains of AD patients, Deng and colleagues observed a reciprocal relationship between O-GlcNAcylation and phosphorylation of NFM, in that a decreased O-GlcNAcylation of NF due to lower glucose uptake in AD patients is accompanied with increased KSP phosphorylation of NFM (Deng et al., 2008). The mechanisms underlying the topographic regulation of phosphorylation of NFs (phosphorylation in axons and cell bodies is modulated by different phosphatases and kinases associated with compartment-specific multimeric complexes), the identification of NF-interacting proteins and the role of phosphorylation in guiding those interactions remains a challenge for future investigations.

Perspectives

Although the studies reviewed here have increased our understanding of neurofilament biology and pathophysiology, many unanswered questions remain. NFM is important for axonal transport of NFs in vivo and for regulating their transport rates; however, it is not known which domain(s) of NFM regulate the transport kinetics of NFs. Another remaining question is how formation and stabilization of the stationary NF network are affected by the phosphorylation of NFM heads and tails. How do kinesin and dynein–dynactin interact with NFM for slow NF transport? The functional significance of the interactions between specific NF subunits and neurotransmitter receptors in vivo is also not entirely clear. Finally, how do NF mutations that have been linked to neurodegenerative diseases affect NF function? For example – as yet – little is known about the mechanisms that regulate protein turnover of NF subunits, and the relevance of NF turnover for aging and neurodegenerative diseases. With this in mind, a deeper understanding of neurofilament function and dysfunction in health and disease is clearly desired.

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