Neurofilaments: neurobiological foundations for biomarker applications


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Abbreviations

AD  Alzheimer’s Disease
ALS  Amyotrophic Lateral Sclerosis
APOE  Apolipoprotein E
ARSACS  Autosomal Recessive Spastic Ataxia of Charveloix-Saguenay
CMT  Charcot-Marie-Tooth Disease
CSF  Cerebrospinal fluid
D1R  Dopamine D1 receptor
FTD  Frontotemporal Dementia
GAN  Giant Axonal Neuropathy
IF  Intermediate Filaments
IPAD  Intramural Peri-Arterial Drainage Pathway
ISF  Interstitial fluid
LTP  Long term potentiation
MAPK  Mitogen-activated protein kinase
MS  Multiple Sclerosis
MVB  Multivesicular bodies
NF  Neurofilament
NfH  Neurofilament heavy chain
NFIR  Neurofilament subunit immunoreactivity
NfL  Neurofilament light chain
NfM  Neurofilament medium chain
NMDA  N-methyl-D-aspartate
PKN1  Protein kinase N1
PP1  Protein Phosphatase-1
PSD  Post Synaptic Density
SACS  Sacsin gene
SILK  Stable isotope labelling kinetic
SOD1  Superoxide dismutase 1
TBI  Traumatic Brain Injury
TDP-43  TAR DNA-binding protein 43
UPS  Ubiquitin Proteasome System
Abstract

Interest in neurofilaments (NF) has risen sharply in recent years with recognition of their potential as biomarkers of brain injury or neurodegeneration in cerebrospinal fluid (CSF) and blood. This is in the context of a growing appreciation for the complexity of the neurobiology of NF, new recognition of specialised roles for NF in synapses and a developing understanding of mechanisms responsible for their turnover. Here we will review neurobiology of NF proteins, describing current understanding of their structure and function, including recently discovered evidence for their roles in synapses. We will explore emerging understanding of the mechanisms of NF degradation and clearance and review new methods for future elucidation of the kinetics of their turnover in humans. Primary roles of NF in the pathogenesis of human diseases will be described. With this background, we then will review critically evidence supporting use of NF concentration measures as biomarkers of neurodegeneration. Finally, we will reflect on major discovery challenges for studies of the neurobiology of intermediate filaments with specific attention to identifying what needs to be learned for more precise use and confident interpretation of NF measures as biomarkers of neurodegeneration.
Neurofilaments are a family of neuronal intermediate filaments involved in both the growth and stability of axons, and, through incorporation into different supramolecular assemblies, also in synaptic organization and function in the central nervous system.

The fundamental importance of NF to axonal structure and function was first appreciated with serial discoveries of causal NF gene mutations for rare forms of Charcot–Marie–Tooth disease and amyotrophic lateral sclerosis (ALS).

Evidence that their normal intracellular assembly and turnover involves the ubiquitin-proteasomal pathway (and possibly also non-canonical autophagy pathways) came from studies of rare genetic diseases characterised by prominent accumulations of NF (Charcot-Marie-Tooth Disease Type 2R (mutations in the E3-ubiquitin ligase, TRIM2), Giant Axonal Neuropathy (mutations in gigaxonin, a regulator of proteasomal degradation of cytoskeletal proteins) and Autosomal Recessive Spastic Ataxia of Charveloix-Saguenay (ARSACS) (mutations in sacsin, a protein with potential roles in neurofilament assembly and turnover).

Increased concentrations of extra-neuronal NF peptides in cerebrospinal fluid (CSF) and blood can be quantified using ultra-sensitive immunoassays after peripheral nerve or brain injury or in association with clinical progression of several chronic neurodegenerative disorders including multiple sclerosis, Alzheimer’s disease, frontotemporal dementia, progressive supranuclear palsy and Huntington’s disease.

However, as the mechanisms and kinetics of NF protein release from axons and trafficking between brain and blood compartments are ill-defined, interpretations of increased CSF or blood NF concentrations in terms of the specific nature or extent of any associated neuronal dysfunction or injury or the rate of neurodegeneration should be made with caution.

Given the growing interest in using soluble NF proteins as biomarkers for clinical decision making, elucidating the identities of peptides detected by current assays and the mechanisms by which these are released from neurons are particularly urgent questions to be addressed.
Introduction

NF are a family of five highly phosphorylated intermediate filaments (Julien and Mushynski, 1983) that are distinguishable based on their relative apparent molecular masses on SDS-polyacrylamide gels. The largest of these is NF heavy chain (NfH), followed (in order of descending molecular weight) by the medium chain (NfM), the light chain (NfL), α-internexin and peripherin (Figure 1). NF contribute to growth and stability of axons in both central and peripheral nerves as well as to maintaining mitochondrial stability (Lariviere et al., 2015) and microtubule content (Bocquet et al., 2009). Roles for distinct NF isoforms in maintaining the structure and function of dendritic spines and in regulating glutamatergic and dopaminergic neurotransmission synapses have been discovered recently (Ratnam and Teichberg, 2005).

The fundamental importance of NF to neurons has been highlighted by molecular characterisation of diseases of the brain and peripheral nerves associated with abnormal NF structure and function. Mutations in the NEFL gene, which encodes NfL lead to peripheral neurodegeneration in Charcot-Marie-Tooth (CMT) disease types 2E and type 1F (Mersiyanova et al., 2000). Polymorphisms in NEFH, encoding NfH, are associated with ALS (Figlewicz et al., 1994) and mutations of NEFL are a cause CMT type 2 (Rebelo et al., 2016). NF dysfunction or aggregation also may play roles in the neuropathology of Alzheimer’s, Parkinson’s Disease and other neurodegenerative disorders (Khalil et al., 2018).

Studies in preclinical models have shown that NF turn over slowly in healthy neurons. Their expression is regulated by neuronal activity acting through developmentally regulated promoter regions (Yaworsky et al., 1997) involving transcription factors belonging to the POU family (Smith et al., 1997). Post-transcriptional regulation of NF mRNA stability also may contribute to determining levels of expression of NF protein (Schwartz et al., 1994; Schwartz et al., 1995). Additional insights into mechanisms for turnover of NF have come through studies of rare diseases arising from gigaxonin E3 ligase mutations causing Giant Axonal Neuropathy (GAN) (Bomont et al., 2000; Bomont, 2016) and those in TRIM2 (another E3 ligase) and sacsin (which includes both ubiquitin-like and chaperone domains) that are responsible, respectively for a form of CMT (Ylikallio et al., 2013) and for the cerebellar degeneration occurring in Autosomal Recessive Spastic Ataxia of Charveloi-
Saguenay (ARSACS)(Engert et al., 2000). Degradation of NF protein thus is controlled by combined activities of proteasomal and, possibly, autophagocytic mechanisms(Bomont, 2016). NF or its fragments also are released from neurons secondary to axonal damage or neurodegeneration, although the predominant peptide species released and the mechanisms responsible for the release have not been clearly characterised. Release may occur actively (for example, by means of exosomes(Faure et al., 2006; Lachenal et al., 2011)) or passively with loss of neuronal membrane integrity. NF in different supramolecular structures or with different isoforms may show differences in degradation rates(Nixon and Logvinenko, 1986; Millecamps et al., 2007). Studies of pathways for trafficking of other proteins(Szentistvanyi et al., 1984a) suggests that degraded NF proteins may enter the peripheral circulation via perivascular drainage along basement membranes of capillaries and arteries(Carare et al., 2008) to drain into cervical or lumbar lymph nodes and then into the blood.

Reliable, sensitive assays for measuring concentrations of NF in both CSF and plasma now are available(Kuhle et al., 2016a). This has enabled several recent studies assessing the potential utility of NF (particularly NfL) peptide concentrations in the CSF or plasma as clinical biomarkers of neuronal damage with disease progression (Kuhle et al., 2016a; Disanto et al., 2017; Khalil et al., 2018). For example, CSF and plasma NF protein concentrations are increased after stroke or traumatic brain injury (TBI)(Khalil et al., 2018). NF concentrations in both CSF and plasma are increased in some people with multiple sclerosis (MS)(Amor et al., 2014; Kuhle et al., 2016b; Disanto et al., 2017; Novakova et al., 2017; Barro et al., 2018; Hakansson et al., 2018; Piehl et al., 2018) and, considered as a group, those with increased NfL have greater brain and spinal cord atrophy over the following 2-5 years(Barro et al., 2018). Both serum and CSF concentrations are correlated with radiological(Kuhle et al., 2016b; Disanto et al., 2017; Novakova et al., 2017; Siller et al., 2018) and clinical measures of disease activity or progression(Disanto et al., 2017; Novakova et al., 2017; Barro et al., 2018; Hakansson et al., 2018; Piehl et al., 2018; Siller et al., 2018). Longitudinal measures in people with MS suggest that higher concentrations may reflect greater rates of neurodegeneration and more rapidly progressive disease. Evidence for treatment responsiveness further supports a causal link between disease activity and increased NF concentrations in CSF and plasma(Disanto et al., 2017; Piehl et al., 2018; Gafson et al., 2019; Kuhle et al., 2019). Increased CSF and plasma NF concentrations also are associated with primary neurodegenerative diseases including Alzheimer’s disease(Mattsson et al., 2016; Weston et al., 2017).
Whilst the concentration in CSF is approximately 20-50 fold greater than in plasma(Bergman et al., 2016), moderate to high correlation between concentrations measured in the two compartments have been reported(Gaiottino et al., 2013; Gisslen et al., 2016). Nevertheless, given that NF also can be released from peripheral nerves, depending on the pathological context, plasma and CSF measures are not necessarily correlated(Khalil et al., 2018). Longitudinal measures of CSF and serum concentration changes after brain injury(Shahim et al., 2016) or following the initiation of effective treatments(Khalil et al., 2018) suggest that turnover times in the blood and CSF compartments are similar. This is consistent with models positing that central and peripheral turnover are linked functionally. However, important questions regarding the neurobiology, mechanisms of turnover and kinetics in the brain and blood compartments remain. These will be highlighted as the current understanding of NF neurobiology in health and disease is reviewed in more detail below.

**Structure and assembly of NF**

NF are major cytoskeletal components in mature neurons. They are found in the cytoplasm of neurons within the peripheral (PNS) and central nervous system (CNS), most abundantly in axons, but also in cell bodies, dendrites and synapses(Yuan et al., 2015b). They are expressed more highly in large myelinated axons, where they are organised in parallel structures maintained by side arms projecting outwards from a filament core(Yuan et al., 2017). However, the relative abundance of NF proteins can widely differ along the course of even a single axon, e.g., amounts of NF are three-fold greater in regions of myelinated regions of axons that at the nodes of Ranvier(Nixon et al., 1994b)(Hsieh et al., 1994).

NF are hetero-polymers composed of NfL, NfM, NfH and either α-internexin(Yuan et al., 2006) or peripherin(Yuan et al., 2012b) in the CNS or PNS, respectively (Figure 2). Multiple types of post-translational modifications to NF occur (e.g., phosphorylation, ubiquitination, nitration and addition of O-linked N-acetylglicosamine)(Nixon and Sihag, 1991; Yuan et al., 2017). NfM and NfH can be phosphorylated extensively(Beck et al., 2012). The relative proportions of the NF protein components and their post-translational modifications change with development and vary between different types of neurons and neuronal functional states.
NfL is uniquely important amongst the NF. NfL is required for NF protein assembly and NfL knockout mice exhibit severe atrophy of peripheral myelinated axons (Zhu et al., 1997). The latter observation provided evidence that NF are necessary for the radial growth of large myelinated axons and associated fast nerve conduction (Kriz et al., 2000). α-internexin interacts with NfL in forming a backbone to which NfM and NfH attach.

The carboxyl terminals of NfH and NfM proteins form side-arm projections at the periphery of NF structures that contain multiple Lys–Ser–Pro (KSP) repeats which can be phosphorylated by proline-directed kinases, Erk1/2, Cdk5/p35, and JNK3 (Lee et al., 2014). Because phosphorylation of KSP repeats, especially in NfH (Julien and Mushynski, 1982, 1983), increase the negative charge of these projections (and, by inference, the spacing between NF in the axon), it was believed initially that NfH must play a major role in determining axonal caliber. However, surprisingly, targeted disruption of the NfH gene had little effect on the radial growth of myelinated axons (Rao et al., 1998; Zhu et al., 1998). In contrast, deletion of NfM gene (Jacomy et al., 1999) or deletion of the NfM carboxy-terminal tail domain substantially reduced the calibers of large myelinated axons (Rao et al., 2003). The exact NfM domain which modulates axon caliber remains to be elucidated. A mouse knock-in substitution of KSP repeats by KAP (NF-MS→A) repeats (which cannot be phosphorylated) in NfM demonstrated that phosphorylation of NfM KSP repeats does not determine axonal caliber (Garcia et al., 2009). The current model for their structural organisation proposes that the tail domains of NfM and NfH form side-arms that interconnect NF and link them to other cytoskeletal elements and organelles such as mitochondria and microtubules (Yuan et al., 2017). Phosphorylation of NF stabilises this structure by inhibiting NF proteolysis, increasing the half-life of the whole supramolecular assembly (Rao et al., 2012).

Most NF proteins are synthesized and assembled in the neuronal perikaryon and must be transported along axons for functional integration (Yuan et al., 2012a). It was proposed initially that, after their assembly in the perikaryon, NF are transported unidirectionally by slow transport mechanisms (0.1–1 mm/day) distally along the axon, where they ultimately are proteolytically degraded (Hoffman and Lasek, 1975). This classical model of NF transport and turnover in the axon was based on pulse chase radiolabelling techniques with low time resolution (Hoffman and Lasek, 1975). However, subsequent time-lapse microscopy of
fluorescently tagged NF proteins in growing axons of neurons in vitro revealed fast transport of tagged-NF at rates up to 2 μm/s that is interrupted by long pauses resulting in an average rate approximating the slow NF transport estimates (Roy et al., 2000; Wang et al., 2000; Li et al., 2012). Live imaging of immature or regenerating axons in vitro also discriminates particularly slow NF transport of a pool of labelled NF proteins that remains in axons for very long periods (Trivedi et al., 2007; Yuan et al., 2009). This pool reflects the initial stage of construction of a stable stationary NF network that maintains caliber sizes of large PNS and CNS fibers by integration of NF with other cytoskeletal elements (Nixon and Logvinenko, 1986; Yuan et al., 2017). These and more recent observations have contributed to a revised model positing that NF can move bi-directionally along microtubules in the axon via motors such as kinesin and dynein (Shea and Flanagan, 2001). Earlier observations, such as the directional reversibility and alternations between rapid movements and long pauses leading to a net slow movement of NF along axons, are well explained by this model. The precise kinetics also can be related to local axonal structure; pulse-escape fluorescence photoactivation recently demonstrated slowing of NF transport at nodes of Ranvier, where there is constriction of the axon (Walker et al., 2019).

Transgenic mouse studies have highlighted the importance of NF protein stoichiometry for correct NF assembly (Julien et al., 1987). Abnormal NF accumulation in the perikaryon of motor neurons can be induced by overexpressing any NF protein alone. Overexpression of NfH in mice led to formation of large perikaryal NF aggregates in spinal neurons and reduction of NF transported into axons (Cote et al., 1993). Maintaining a higher ratio of NfL to NfH and NfM is critical for the normal growth of axons and dendrites (Kong et al., 1998); NfL plays an essential and distinct role in NF assembly from those of NfM and NfH.

Additional observations further highlight the importance of maintaining proper intermediate filament protein stoichiometry in an axon. In the mouse, large perikaryal accumulations of NF due to human NfH transgene overexpression were associated with severe atrophy of peripheral axons, but did not cause substantial neuronal death (Cote et al., 1993). Even with normal stoichiometry of the major proteins, NF disorganization alone appears sufficient to lead to neuronal death; overexpression of peripherin (Beaulieu et al., 1999) or of a mutated NfL protein (Lee et al., 1994) in transgenic mice induced the formation of ALS-like NF aggregates and selective degeneration of spinal motor neurons. Interestingly, sequestration of peripherin in the perikaryon of motor neurons by NfH overexpression rescued the peripherin-
mediated death of motor neurons in transgenic mice (Beaulieu and Julien, 2003), suggesting that axonal NF aggregates (or spheroids) are more toxic than perikaryal NF aggregates, perhaps due to interference with axonal transport of organelles by the former.

**Genetic abnormalities in NF and disease**

Historically, the fundamental importance of NF to neuronal structure and function became apparent through the discovery of associations between NF gene mutations and disease (Table 1). Abnormal accumulation of NF and of the related intermediate filament, peripherin, is a pathological hallmark of ALS (Corbo and Hays, 1992). Several factors could be responsible for the abnormal NF accumulations observed, such as dysregulation of NF gene expression, NF mutations, defective axonal transport, abnormal post-translational modifications, and proteolysis. Degenerating neurons in ALS show a 70% decrease in levels of NfL, α-internexin and peripherin mRNA post-mortem (Campos-Melo et al., 2013). Modifications in the stability of these NF mRNA contribute to this; the (TAR) DNA-binding protein 43 (TDP-43) which forms cytoplasmic aggregates in ALS was found to bind and destabilize or sequester NfL mRNA (Strong et al., 2007; Volkening et al., 2009), a phenomenon that also could contribute to alterations of NF protein synthesis, consequent stoichiometry changes and aggregation of NF (Rosengren et al., 1996).

Evidence for a potentially causal pathogenic role of NF abnormalities in ALS came from the discovery of codon deletions or insertions in the lysine–serine–proline (KSP) repeat motifs of NfH in a small number of patients with sporadic ALS (Figlewicz et al., 1994; Tomkins et al., 1998). A frameshift deletion and an amino acid substitution in the peripherin gene also have been discovered in two sporadic ALS cases (Gros-Louis et al., 2004; Leung et al., 2004). However, other studies have failed to identify common polymorphisms or rare genetic variants of NF genes in association with sporadic and familial ALS (Rooke et al., 1996; Vechio et al., 1996) suggesting that NF gene mutations define an aetio logically distinct subtype of ALS.

However, pathogenic roles for NF in ALS may extend beyond associations with rare genetic coding variants: NF abnormalities in ALS also occur as a result of post-translational protein modifications. Phosphorylation changes can alter the axonal transport of NF, leading to their
accumulation in cell bodies and axons. Treatment of neurons with glutamate activates mitogen-activated protein kinase (MAPK), resulting in phosphorylation of NF and slowing of their axonal transport, potentially another mechanism by which glutamate might effect its excitotoxicity in ALS (Ackerley et al., 2000; Veyrat-Durebex et al., 2014). Additional observations link glutamate excitotoxicity to NF phosphorylation. Glutamate induces caspase cleavage and activation of protein kinase N1 (PKN1), a kinase targeting the NF head rod domain to disrupt NF organisation and axonal transport (Manser et al., 2008). The peptidyl-prolyl isomerase Pin1, which selectively binds to phosphorylated proline-directed serine/threonine residues in NfH, also may play a significant regulatory role in glutamate stress-induced NF phosphorylation. In ALS, Pin1 is co-localized with spinal cord neuronal inclusion bodies. Glutamate-stressed neurons exhibit increased phosphorylated NfH in perikaryal accumulations, which co-localize with Pin1 (Kesavapany et al., 2007). In addition, down-regulation of Pin1 by small interfering RNA reduces glutamate-induced NfH phosphorylation and neuronal apoptosis (Kesavapany et al., 2007). Abnormal accumulations of NF also can potentiate glutamate receptor mediated increases intracellular calcium levels and cell death in response to NMDA receptor agonism (Sanelli et al., 2007).

Indirect evidence suggests that additional post-translational modifications of NF may contribute to ALS. Advanced glycation end-products have been detected in NF aggregates of motor neurons in familial and sporadic ALS (Chou et al., 1998). This observation is of clinical interest given associations discovered between diabetes and ALS, although there is still uncertainty about the clinical significance of the abnormal glycosylation (Kioumourtzoglou et al., 2015; Mariosa et al., 2015).

Several causal mutations in the NfL gene have been linked to less common forms of Charcot–Marie–Tooth disease (CMT) (Jordanova et al., 2003; Zuchner et al., 2004) (Figure 3). Some of these mutations cause the axonal CMT type 2E, but other NfL mutations are associated with slow nerve conduction velocities and clinical presentations resembling demyelinating type CMT (type 1F). Two autosomal recessive NfL mutations also have been reported, leading to truncated NfL proteins and a severe early onset axonal form of CMT (Abe et al., 2009; Yum et al., 2009).

The P22S mutation in NfL was first discovered in a Slovenian (Mersiyanova et al., 2000) with an early onset form of type 2E CMT (axonal type) associated with axonal deformation and
swelling. The P22S mutation abolishes the Thr–Pro Proline-directed protein kinase (PDPK) consensus phosphorylation sequence by perturbing normal regulation of NF assembly through phosphorylation (Sasaki et al., 2006); the mutated NfL proteins do not self-assemble with NfM and NfH and cause NF aggregation in cultured cells (Perez-Olle et al., 2002; Perez-Olle et al., 2004). Similar NF aggregates caused by CMT-associated mutations in NfL (e.g., P22S, as well as P8R, Q333P) trap motor proteins and organelles in the cytoplasm, resulting in axonal transport defects (Zhai et al., 2007).

Transgenic mouse models based on mutations in CMT provide powerful tools for study of the disease pathology. A NfL mutant model that recapitulates the cellular neuropathology found in human axonal CMT was generated by a knock-in strategy replacing one mouse NfL allele with the N98S mutation in the rod region of NfL (Adebola et al., 2015), a mutation that has been described in sporadic cases of CMT with early age of onset (< 2 years). Consistent with the clinical presentation, mutant NFLN98S/+ mice were symptomatic at an early age. Tremor was observed at 1 month of age. The N98S mutation caused a severe reduction of NF in myelinated axons of the PNS and CNS, axonal hypotrophy and distal axonal loss in the PNS. Cellular immunopathology revealed abnormal NF aggregates in neuronal cell bodies and axons of the cerebellum and spinal cord from an early age. The mice exhibited hind limb clasping, a likely behavioural expression of the axonopathy. The NFLN98S mice thus provide a model with face validity for testing potential therapeutic strategies directed towards preventing or reversing neuropathic symptoms in humans. In contrast, knock-in of a different point mutation (P8R) that causes symptoms in humans with variable ages of onset was associated with weak phenotypes without NF accumulation. Together, these results suggest the hypothesis that phenotypic severity in mouse models of CMT caused by NfL mutations may be related to the extent of NF aggregation found in neurons and severity of the associated human disease.

A critical proof of principle for therapeutic targeting of mutant NF expression came from a transgenic mouse study with conditional mutant NfL P22S expression demonstrating that the pathology caused by mutant NfL can be reversed. This animal model was generated using a tetracycline-responsive gene system (tet-off) gene system that allowed suppression of mutant NFL P22S expression in mature neurons after administration of doxycycline (Dequen et al., 2010). The NFL P22S mice recapitulated the key features of CMT type 2E neuropathy: progressive development of an abnormal hind limb posture with motor deficits, hypertrophy
of muscle fibres and muscle denervation. Suppression of mutant NFL\textsuperscript{P22S} production after clinical disease onset reversed these pathological features. This important observation suggests that therapies able to abolish mutant NfL expression may be able to reverse pathology and disability even with established disease- the situation most commonly encountered in the clinic. However, this conclusion still needs to be translated to patients with recognition not just of species differences, but also that symptoms in the mouse model occurred very late, expression of mutant NfL was low and NF aggregates were not seen in motor neurons. Additional work is needed to determine whether neuronal function is restored if pathological NF aggregates are present and whether any functional recovery can be related directly to clearing of the aggregates. Another laboratory has generated a mouse with neuronal expression of E397K NfL mutant(Shen et al., 2011). In this mouse, symptoms of reduced locomotor activity and muscle atrophy were first observed first at 4 months, although NF inclusions were not seen in this model either.

**NF in synapses**

Although NF have been viewed traditionally as structural components primarily of axons, recent evidence has shown that distinctive assemblies of NF subunits also are integral components of synapses. For decades, synaptic terminals were associated only with degradation of NF transported distally along the axon. However, early observations supporting this concept(Roots, 1983), have not been confirmed. For example, recent proteomic analyses show that many synaptosomal proteins have half-lives of weeks to months(Heo et al., 2018), which is longer in some cases than the half-lives of NF in axons. Other observations of neurons in the intact, mature brain also proved difficult to reconcile with models of NF transport and distribution that mostly relied on *in vitro* observations made on embryonic neuronal axons which have few NF and reflect an early developmental or regenerative state(Nixon, 1998). Most notable are *in vivo* studies of mature brains showing that only a small pool of newly synthesized NF subunit precursors needs to be transported to maintain the large stationary NF network in myelinated axons because of the exceptionally slow turnover of this network(Yuan et al., 2017). In this model, the small amount of NF protein reaching terminals accords with evidence for the long half-lives of synaptic proteins(Heo et al., 2018)implying low synaptic proteolytic activity and similar rates of local turnover of a predominantly stationary NF network uniformly along axons(Nixon and Logvinenko, 1986). The role of NF as a critical determinant of axon
calibre - their accepted principal role in peripheral nerves - appears much less important for axons of CNS neurons. Despite the presence of abundant NF proteins in the CNS, intrinsic axons of the brain, even the larger corpus callosum axons, have a relatively low NF density and exhibit minimal volume reductions when their NF expression is suppressed genetically (Dyakin et al., 2010).

Direct evidence now has established unequivocally that synapses contain a unique pool of NF that has distinctive functional roles (Yuan et al., 2015a, b). NF assemblies isolated from brain synaptosomes are distinguishable both morphologically and biochemically from those in other parts of the neuron; NF subunits in synapses exist in unconventional assemblies and even likely in small hetero-oligomeric forms (Yuan et al., 2003). The latter are capable of axonal transport (Yuan et al., 2003). Electron microscopy combined with immunogold labelling has identified short, irregularly oriented and bent 10nm filaments that often are associated with the post synaptic density (PSD) or with vesicular organelles (Figure 4). Synaptic NF proteins are distinctive in both stoichiometry and states of phosphorylation and respond differently to genetic subunit perturbations than the larger NF protein pool in brain white matter (Yuan et al., 2015b). Changes in synaptic NfL phosphorylation associated with calcium/calmodulin-dependent protein kinase II activation during modulation of long-term potentiation (LTP) suggest a role for synaptic NF proteins to enable the latter (Hashimoto et al., 2000) and hint at a broader functional significance of the complex regulation of NF subunits by phosphorylation (Nixon and Sihag, 1991; Sihag et al., 2007).

Synaptic NF proteins have been found to be more abundant in the postsynaptic compartment than in adjacent dendritic areas or presynaptic terminals using quantitative immunogold analysis with electron microscopy (Yuan et al., 2015b). Immunocytochemical studies (Bragina and Conti, 2018 In Press) have confirmed NF subunit immunoreactivity (NFIR) in pre- and post-synaptic compartments and greater NFIR in GABAergic than in glutamatergic synapse (Bragina and Conti, 2018 In Press).

Recent findings that individual subunits serve unique roles in neurotransmission provide indirect, but compelling evidence for the functional importance of synaptic NF (Yuan et al., 2015b; Yuan and Nixon, 2016). N-methyl-D-aspartate (NMDA) receptors (Li and Tsien, 2009) are highly concentrated in postsynaptic membranes of glutamatergic synapses (Huntley et al., 1994). NfL has long been known to interact directly with the
cytoplasmic C-terminal domain of GluN1 through its rod domain (Ehlers et al., 1998; Ratnam and Teichberg, 2005). NfL co-expression with GluN1 and GRIN2B subunits in HEK293 cells increases the surface abundance of GluN1 (Ehlers et al., 1998) and blocks its ubiquitination (Ratnam and Teichberg, 2005) Both of these actions of NfL are expected to stabilize NMDA receptors within the neuronal plasma membrane. Consistent with this hypothesis, the abundance of synaptic GluN1 subunits is reduced and ubiquitin-dependent GluN1 subunit turnover is greater in NfL−/− than in wild-type mice (Yuan et al., 2018a). Binding of antibody that only recognizes poly-ubiquitin chains formed with the Lys-48 (K48) residue is greater in GluN1-rich postsynaptic membranes of the hippocampus; GluN1 interactions with NfL may inhibit their accessibility to the ubiquitin ligases known to initiate GluN1 degradation (Kato et al., 2005; Ratnam and Teichberg, 2005; Yuan et al., 2018b). Additionally, NfL binding to protein phosphatase-1 (PP1), a protein/serine/threonine phosphatase in the PSD (Terry-Lorenzo et al., 2000), suggests possible regulation of the phosphorylation states of NF subunits and NMDA GluN1 receptors in ways that may influence the cellular distribution of the receptor (Ehlers et al., 1998). These observations may be relevant for understanding the role for regulating the phosphorylation state of NfL with LTP and long-term depression (LTD) (Hashimoto et al., 2000).

Loss of surface GluN1 receptors and NMDAR hypofunction associated with NfL deletion could contribute to clinical presentation of psychiatric and neurodegenerative disorders including Alzheimer’s disease (Lin et al., 2014). NfL gene deletion in mice, which depresses GluN1 protein levels, both reduces dendritic spine number and length and leads to increased hippocampal glutamate levels as an adaptive response (van Elst et al., 2005; Homayoun and Moghaddam, 2007; de la Fuente-Sandoval et al., 2011; Merritt et al., 2016; Yuan et al., 2018b). Responses to NMDAR antagonists also are lost with NfL deletion, although effects on NMDA-independent motor activity are minimal. Multiple NMDAR-related behaviours such as pup retrieval, spatial and social memory, pre-pulse inhibition and night-time activity also are impaired. Importantly, similar NMDAR-related synaptic and behavioural deficits (albeit in milder forms than in NfL-null mice) are seen in NfL+/-mice that have 40-50% lower brain NfL levels than in the wild-type mice – a relative reduction within the range of NfL loss seen in some brain regions with schizophrenia (Kristiansen et al., 2006). Interestingly, NF genes map to chromosomal regions implicated in schizophrenia (Badner and...
Gershon, 2002; Lewis et al., 2003) and concentrations of NfL are reduced consistently in this disease(Kristiansen et al., 2006; Pennington et al., 2008).

Highlighting the differential roles of NF subunits in synaptic function, NfM colocalizes with the G-protein-coupled D1 receptor in synaptic boutons(Girault and Greengard, 2004) and the deletion of NfM but not any other NF subunit causes postsynaptic D1-receptors to redistribute from a reserve pool on endosomes to the synaptic plasma membrane, which significantly increases D1R-stimulated hippocampal LTP and greatly amplifies dopamine D1-receptor-mediated motor responses to cocaine(Yuan et al., 2015b). Furthermore, deletion of the NfM gene in mice enhances dopamine D1-receptor-mediated motor responses to cocaine(Yuan et al., 2015b). The lack of NfM leads to a redistribution of postsynaptic D1-receptors from endosomes to plasma membrane, implying that NfM is playing a role in the recycling of the D1-receptor. NfM deletion also inhibits the desensitization response to cocaine and amphetamine, while enhancing and prolonging ERK activation and ERK mediated NfM phosphorylation(Beatn-Johnson et al., 1992; Berhow et al., 1996), not unlike the effects of chronic exposure to drugs of abuse in humans(Beatn-Johnson et al., 1992). Notably, basal neurotransmission and induction of LTP are normal in NfM-null mice, distinguishing them from mice lacking NfH, which exhibit deficient LTP maintenance and NfL-null mice that display both deficient basal neurotransmission and LTP(Yuan et al., 2015b).

Furthermore, NfL is known to interact with the GluN1 subunit of the NMDA receptor. NfL deletion in mice reduces GluN1 protein levels and dendritic spines and elevates ubiquitin-dependent turnover of GluN1(Yuan et al., 2018a). Interestingly, interactions between dopamine D1 (D1R) and NMDA receptors are facilitated through NF subunit-assemblies(Yuan et al., 2015b; Yuan et al., 2018b). The motor stimulant effect of the NMDA antagonist phencyclidine is blocked by D1R antagonists and deletions of NfL and NfM, which regulate NMDAR and D1R, respectively and have opposing effects on D1R-dependent motor activity induced by NMDA inhibition(Yuan et al., 2018b). Thus, the known functional interdependence of these two distinct receptor complexes appears to depend on a synaptic scaffold containing assemblies of multiple NF subunits.

**Degradation and turnover of NF**
As outlined briefly in the overview, major insights into mechanisms of assembly and turnover of NF have come from the study of genotype-phenotype associations in three rare diseases neuropathologically characterised by prominent abnormal NF accumulation in axons: Autosomal Recessive Spastic Ataxia of Charveloix-Saguenay (ARSACS)(Engert et al., 2000), the early onset CMT Type 2R(Ylikallio et al., 2013) and Giant Axonal Neuropathy (GAN)(Bomont et al., 2000).

ARSACS is an early onset autosomal recessive CNS disorder caused by mutations in the gene encoding sacsin (SACS), a protein with both putative ubiquitin and chaperone functions. ARSACS is found world-wide and is the second most common inherited cause of ataxia(Engert et al., 2000). Patients present with a cerebellar ataxia and cognitive impairment that is associated with a variably severe motor-sensory neuropathy. On examination, thickening of the retinal nerve fibre layer can be seen(Bouchard et al., 1998). Accumulation and abnormal bundling of NF is the most prominent neuropathological finding in affected neurons, which include Purkinje cells(Lariviere et al., 2015).

Sacsin is not restricted to neurons, nor is its role limited to the degradation of intermediate filaments. Cellular neuropathology in other cell types provide additional insights into the other functions of intermediate filaments. Fibroblasts derived from ARSACS patients or SACS knockout fibroblasts show pathological intermediate filament structures, with vimentin filaments collapsed around or beside the nucleus, rather than radiating outwards to the plasma membrane(Duncan et al., 2017). This is accompanied by an altered distribution of organelles (including autophagosomes and lysosomes) and displacement of the nucleus(Duncan et al., 2017). The latter is associated with disruption of the LINC complex(Lee and Burke, 2018) connecting cytoplasmic intermediate filaments to the nuclear cytoskeleton (Gentil, Bouchard and Durham, unpublished data). Mitochondrial pathology also is seen, with increased mitochondrial lengths, reduced mitochondrial fission and impaired transport(Girard et al., 2012; Lariviere et al., 2015; Bradshaw et al., 2016). The mechanism underlying the mitochondrial abnormalities is not known, but could be related to roles for intermediate filaments in organizing mitochondria(Tradewell et al., 2009) or a more direct role of sacsin in regulating mitochondrial dynamics; loss of sacsin has been linked to compromised ability to recruit or retain the major fission protein Drp1 at the mitochondrial membrane(Bradshaw et al., 2016).
Sacsin has several functionally distinct domains: a C-terminal ubiquitin-like domain (Ubl), three SIRPT domains (SIRPT1 bearing homology with the ATP-binding domain of HSP90), a J-domain, and an N-terminal HEPN domain (Engert et al., 2000). The presence of the Ubl and domains with chaperone homology suggests that sacsin is involved in NF assembly and/or turnover. The Ubl domain has been shown to interact with a proteasomal component (Parfitt et al., 2009). In cell models, expression of the Ubl and J-domain peptides both inhibited normal assembly of NF, whereas SIRPT1 and HEPN domain peptides promoted NF protein assembly. In cultured SACS−/− motor neurons modelling the pathology in ARSACS, selective expression of both the SIRPT1 and J-domain peptides led to the clearance of NF bundles in a similar way to that seen with overexpression of heat shock proteins (Gentil et al., 2019). These data highlight multifunctional roles of sacsin as a key player in organising NF proteins and in regulating subunit levels, assembly, maturation of their supramolecular structures and turnover (Engert et al., 2000).

Nonetheless, mechanisms responsible for the turnover of NF still are incompletely understood. NF appear to undergo degradation all along axons by mechanisms regulated by their density and phosphorylation status (Nixon and Logvinenko, 1986). To investigate the turnover and axonal transport of NF quantitatively, Millecamps et al. (Millecamps et al., 2007) generated mice with the human NfL transgene under doxycycline control in the presence or absence of endogenous mouse NfL proteins. In these mouse models, although the human NfL mRNA expression was turned off after one week after administration of doxycycline, the human NfL proteins persisted with a half-life of approximately 3 weeks. The half-life was extended to months when an intermediate filament scaffold was present. These findings are broadly consistent with the half-lives of NF proteins estimated from the decay of [3H] proline radiolabeling proteins in mouse retinal ganglion cell neurons (Nixon and Logvinenko, 1986; Rao et al., 2012).

Studies with conditional NfL transgene suppression revealed that the turnover of NF proteins is slower in large-caliber axons of the PNS having a high content of NF: no substantial decrease of human NfL protein levels in sciatic nerve axons was detected even after 3 months of human NfL transgene mRNA suppression by doxycycline treatment of mice able to maintain a NF structural network despite the transgene suppression because of endogenous mouse NfL expression (Millecamps et al., 2007). NF proteins might last several months or
even years in large axons with dense NF networks. In conjunction with the observation that
the rate of human NfL transport is enhanced by an order of magnitude (10 mm/day) in
peripheral axons lacking a NF network, these results support the notion of a stationary NF
network in axons (Nixon and Logvinenko, 1986) that contributes to slowing both the turnover
of NF and its net unidirectional transport rate and the turnover of NF.

Although regulation of the formation and exceptional stability of the stationary axonal NF
network is incompletely understood, a higher state of phosphorylation is one crucial factor
that distinguishes stationary from moving NF (Lewis and Nixon, 1988; Nixon et al., 1994a),
possibly through causing their dissociation from the kinesin motor (Yabe et al., 2000) and
conferring proteolytic resistance.

Various proteases can contribute to NF proteolysis (Perrot et al., 2008). The calcium-activated
proteases have a high degree of substrate specificity for NF. Calpain is capable of a limited
proteolysis of NF. One provocative study, still to be replicated to our knowledge, proposed
that NF is cleared after transport to the synaptic terminal, at least in part through activities of
calcium-activated proteases such as calpain (Roots, 1983). Calpain proteolysis is one of the
key molecular processes in Wallerian degeneration (Wang et al., 2012), as well in growth
cone formation. Other non-specific proteases can trigger NF turnover and generate NF
peptides, too (Perrot et al., 2008). These include cathepsin D (Nixon and Marotta, 1984) and
caspases 6 and 8 (Shabanzadeh et al., 2015).

Important insights into degradation pathways more specific to NF have come from the study
of rare, inherited genetic diseases. Mutations in TRIM2 (tripartite motif containing 2) cause a
rare cause of an early-onset, recessive form of CMT Type 2R (Ylikallio et al., 2013). TRIM2
is an E3 ubiquitin ligase that binds and ubiquitinates NfL (Balastik et al., 2008). The
pathology shows swollen axons with abnormal aggregation of NF in myelinated fibres. CNS
neurodegeneration with tremor, ataxia and seizures are seen in a TRIM2 gene trap mouse
line (Balastik et al., 2008).

A central role for E3-ligase activity in the turnover of NF was discovered through studies of
GAN (Bomont et al., 2000), a fatal autosomal recessive neuropathy (Cavalier et al., 2000) in
which giant axons (up to 50 μm in diameter) filled with densely packed and disorganized NF
are found throughout the peripheral and central nervous system (Asbury et al., 1972). Starting
early in infancy, the disease is associated with progressive loss of motor and sensory function(Johnson-Kerner et al., 2014). CNS symptoms arise later from cerebellar dysfunction and cognitive impairments. In the most severe cases, GAN is fatal in young adulthood, usually before the third decade of life. The pathological aggregates of GAN, found both in neuronal and in non-neuronal tissues, include multiple subtypes of IF(Prineas et al., 1976). The NF accumulation in nerves within the so-called ‘giant axons’ identified in nerve biopsies of patients are most characteristic(Asbury et al., 1972). However, with the broad range of abnormal IF aggregates seen in patients (e.g., extending from desmin in muscles to GFAP in astrocytes, keratin in hair and vimentin in numerous cell types), GAN is considered as a unique disease of the IF network.

GAN is caused by mutations in the gigaxonin-encoding gene(Bomont et al., 2000), through a loss-of-function mechanism triggered by both non-sense-codon mediated mRNA decay and protein instability(Boizot et al., 2014). The decreased abundance of gigaxonin in human samples is considered an essential and sufficient diagnostic test to discriminate GAN from other similar neuropathies(Boizot et al., 2014). This is important clinically, as patients with GAN share similar clinical and histopathological features with several Charcot-Marie-Tooth neuropathies, including the presence of giant axons and NF bundles in nerve biopsies(Fabrizi et al., 2004; Ylikallio et al., 2013).

With its N-terminal BTB domain and C-terminal Kelch domain(Bomont et al., 2000), gigaxonin belongs to the large family of BTB-Kelch proteins. It is presumed to act in the ubiquitin proteasome system (UPS); proteasome inhibition reduces the clearance of NF in cells overexpressing gigaxonin(Mahammad et al., 2013). This appears to be mediated by interactions between the gigaxonin BTB domain and the Cul3 subunit of E3 ubiquitin ligase complexes(Purukawa et al., 2003). Gigaxonin may target its binding partners for ubiquitin-mediated degradation through their interactions with the Kelch domain(Johnson-Kerner et al., 2015b). Among putative partners identified by mass spectrometry approaches or double screening in yeast, IF are so far the only major biological targets for gigaxonin, as confirmed in cellular and animal models of the pathology(Johnson-Kerner et al., 2015a). Indeed, numerous types of IF are abnormally aggregated in disease, e.g., vimentin in primary fibroblasts from patients(Bomont and Koenig, 2003), peripherin and NfL in motor neuron-like cells derived from induced pluripotential stem cells(Johnson-Kerner et al., 2015a), and vimentin, NfL, NfM, NfH and α-internexin in two different GAN mouse models(Dequen et
Whilst the neurological phenotypes of these mice are mild in comparison to the human pathology, both GAN mouse models exhibit pronounced alterations of the abundance and spatial distribution of neuronal IFs throughout the peripheral and central nervous systems. The putative role of gigaxonin in regulating the steady state IF levels also has been demonstrated compellingly in vitro: lentiviral over-expression of Gigaxonin was sufficient to drive the clearance of multiple wild type IF (vimentin, peripherin and NfL) and IF bundles (including vimentin, NfL, NfM, NfH, peripherin and α-internexin) in GAN cells(Mahammad et al., 2013; Israeli et al., 2016). This effect is mediated by the interaction of gigaxonin with the central rod domain common to all IF types(Mahammad et al., 2013), supporting the clinical and mouse model data suggesting a key role of gigaxonin in controlling the degradation of the whole IF family(Bomont, 2016).

GAN highlights that gigaxonin appears to be the only E3 ligase able to target NF (and IF proteins more generally) for degradation. However, several questions remain to be answered. What form of IF (e.g., short IFs, single unit length filaments, mature filaments or multimeric forms) are targeted by gigaxonin? What specific ubiquitination chain type and degradative route is involved? Surprisingly, while a role for gigaxonin in controlling NfL, NfM and NfH abundance has been demonstrated in cells in vitro and in two distinct GAN mouse models(Dequen et al., 2008; Ganay et al., 2011), direct evidence for NF ubiquitination by gigaxonin remains to be discovered. Experimental challenges to addressing this are the multi-subunit nature of the gigaxonin-E3 ligase complex and the insolubility of gigaxonin when ectopically expressed; ubiquitin laddering of IF with gigaxonin expression has been extremely challenging, although reported once for peripherin in GAN dorsal root ganglion(Israeli et al., 2016).

The proteasome has been partially implicated in vimentin degradation and, as the observations above suggest, may contribute to NF turnover mediated by proteins such as TRIM2 or gigaxonin(Mahammad et al., 2013). However, recent findings also demonstrate a central role for the gigaxonin-E3 ligase in controlling the autophagy pathway through the ATG16L1 protein(Scrivo et al., 2019), presenting the interesting possibility that a non-canonical autophagy pathway also plays a role in NF turnover. Indeed, activation of autophagy is accompanied by reduced NF levels(Chen et al., 2013).

**Release and clearance of NF peptides and proteins**
While the nature of the NF species detected with current immunoassays has not been
determined because of the technical challenges posed by their low concentrations, it seems
likely that most or all of the NF detected in the CSF or plasma compartments represent
peptides generated from partial degradation of NF in the neuron. With injury to peripheral
nerves, these might be expected to arise from axons, but, major contributions from synaptic
NF are possible in the brain and spinal cord because of the relative abundance in the synaptic
compartment within the CNS.

The mechanisms for release of these NF peptides from neurons are not yet defined(Khalil et al., 2018) and we can do little more than speculate at this time. However, testable hypotheses regarding mechanisms of release can be made based on what is known about pathways for release of other neuronal peptides and proteins. Intracellular endosomal organelles known as multivesicular bodies (MVB) may play important roles in the release of peptides(Von Bartheld and Altick, 2011). This may occur through ‘back-fusion’ events and budding from the plasma membrane to generate microvesicles (100-2000nm diameter)(Kleijmeer et al., 2001; Murk et al., 2002) or through release of smaller endosomally-derived exosomes (30-140 nm)(Faure et al., 2006; Lachenal et al., 2011). The latter, if relevant, may make a smaller contribution, as the endosomal pathway is associated with non-ubiquitin degradation of proteins and the evidence from GAN cited above suggests indirectly that it does not play a major role. By contrast, neuronal MVB have been shown to contain protein aggregates that accumulate in Parkinson’s and Alzheimer’s disease, for example(Nixon et al., 2005). They are more abundant with neurodegenerative diseases and in aging(Nakadate et al., 2006), where they are associated with enhanced autophagy(Truant et al., 2008). Microvesicle production also can increase with higher intracellular [Ca\textsuperscript{2+}] (as with excitotoxic injury), cell stress or with inflammation(Sproviero et al., 2018). Upregulation of molecular chaperones rescues the NF phenotype in SACS knockout neurons(Engert et al., 2000) and in motor neurons expressing mutant NfL associated with CMT(Tradewell et al., 2009). Chaperones have the potential to increase a more mobile pool of NF proteins accessible to secretory mechanisms such MVB(Manek et al., 2018).

Pathways for degradation (e.g., proteasomes(Johnson-Kerner et al., 2015b),
autophagy(Nixon, 2006) or release into the extracellular space(Wang et al., 2006) for degradation by extracellular proteases) could be differentially important in the context of
healthy, injured or chronically damaged neurons. The varicosities or large spheroids which occur with neurodegeneration may modify this (Coleman, 2005) (Beirowski et al., 2010).

We speculate that the different mechanisms of release may have different kinetics and that they may lead to variable relative levels of different types of NF peptide fragments in blood or CSF. Quantitative interpretations of the relative concentrations of NF or their peptide fragments in either compartment (or between compartments) demands a better understanding of the mechanisms of these release pathways, as well as how peptides are transported from the parenchyma into the fluid compartments and between the CSF and blood.

The mechanisms by which NF traffic between parenchymal, CSF and blood compartments also are unknown. However, the apparently general pathways by which large molecules such as amyloid-β (Aβ) pass from the interstitial fluid (ISF) of the brain into the CSF and blood that are beginning to be elucidated suggest a tentative model for how NF species could be transported between compartments.

Soluble metabolites or peptides released from cells in most organs are absorbed directly into the blood or drain via lymphatic vessels to regional lymph nodes (Engelhardt et al., 2017). Lymphatic drainage may contribute to NF peptide distribution with peripheral nerve injury. However, the brain constitutes a specialized compartment both because of the selective permeability of the blood-brain barrier and because there are no conventional lymphatic vessels in the CNS. Large tracer molecules or small particles injected into the ISF of the brain drain to cervical lymph nodes along the walls of cerebral capillaries and arteries (Szentistvanyi et al., 1984b). This drainage occurs initially along basement membranes that surround capillary endothelial cells and then along the basement membranes between smooth muscle cells in the tunica media of intracerebral and leptomeningeal arteries (Carare et al., 2008). Together, this constitutes an Intramural Peri-Arterial drainage (IPAD) pathway (Albargothy et al., 2018) (Figures 5 and 6).

IPAD appears to provide a route for the drainage of soluble peptides and proteins from the extracellular spaces in the brain to cervical lymph nodes (Szentistvanyi et al., 1984b; Carare et al., 2008; Albargothy et al., 2018). With impaired IPAD, tracer labelled protein injected intracisternally accumulates around veins draining from the brain (Hawkes et al., 2011), the walls of which appear to provide a downstream drainage pathway (Iliff et al., 2012), although
the specific route for transport of molecules in the paravenous compartment to lymph nodes along veins has not been defined. Modelling studies suggest that the motive force for IPAD could be derived from waves of contraction of smooth muscle cells (vasomotion) in the walls of cerebral arteries and arterioles (Aldea et al., 2019). Any additional motive force along veins, if it is needed has not been characterized.

Clearance through this mechanism may change with aging or pathology if the functional capacity of IPAD changes in ways that could affect the kinetics of transport of NF. For example, age-related changes in artery walls (Hawkes et al., 2011) impair IPAD and appear to be a factor limiting elimination of Aβ from the ageing brain in the genesis of Alzheimer's disease (e.g., reflected by the accumulation of Aβ within the IPAD pathways in Cerebral Amyloid Angiopathy) (Keable et al., 2016) (Weller et al., 2015). Although it seems likely that neurofilament proteins are eliminated from the brain with interstitial fluid along IPAD pathways, no direct evidence is available, as yet.

Levels of proteins or peptides in the CSF cannot be assumed to reflect levels in the ISF directly. Although some reports have suggested that ISF and solutes from the brain drain directly into CSF, this conclusion is confounded in most cases by uncertainty because direct leakage of tracer from intracerebral injections into the CSF cannot be excluded. In better controlled studies, only 10-15% of tracer injected into cerebral hemispheres passes into the CSF (Szentistvanyi et al., 1984b; McIntee et al., 2016); 85% of the ISF passes to cervical lymph nodes via IPAD (Szentistvanyi et al., 1984b). As yet, there are no direct measurements of the proportion of NF released from the brain that reaches the CSF. It also is not known whether there is any regional neuroanatomical selectivity for this.

The subarachnoid space is one neuroanatomical region for which distinct mechanisms of clearance have been characterised. CSF drainage from the subarachnoid space into the lymphatic system occurs through mechanisms distinct from those of the IPAD pathway for the drainage of ISF. In experimental animals and in humans, drainage of CSF into lymphatic vessels of the nasal mucosa via the cribiform plate, appears to be a major lymphatic drainage pathway (Kida et al., 1993; de Leon et al., 2017), although other routes, including dural lymphatics, have been described (Kida et al., 1993; Aspelund et al., 2015; Louveau et al., 2015). The proportion of CSF that drains directly into the blood through the arachnoid granulations is uncertain.
Together, these observations raise cautions for quantitative inferences regarding particularly CNS neuronal pathology based on NF concentration measures in the CSF or serum. The relationships may be influenced by the rate of release of NF species from the injured or degenerating neuron, where it is occurring in the CNS and variation in the kinetics of clearance related to aging or direct effects of pathology on the clearance mechanisms themselves.

Changes either in rates of synthesis of NF proteins or differences in mechanisms and rates of peptide release could lead to differences in measured levels of NF or its peptide fragments in plasma or CSF. As far as we are aware, there are no data describing how turnover in any compartment might change with disease in people. Nor, as is described above, is anything specific known about mechanisms of release of NF or their peptides from injured neurons. This knowledge gap substantively limits quantitative interpretations of NF peptide levels in plasma or CSF.

One important step forward would be to generate data defining the dynamics of NF turnover in healthy people, with aging and in those with diseases associated with increased NF peptide concentrations in plasma or CSF. Although not yet applied to these problems, a promising approach for obtaining precise estimates of the kinetics of synthesis and elimination of NF in blood and CSF in healthy humans and those with disease is the Stable Isotope Labelling Kinetic (SILK) method (Bateman et al., 2006; Paterson et al., 2019). SILK can measure protein turnover rate and half-life minimally invasively in humans. In the past decade, SILK has been used to characterize the kinetics of turnover of pathological protein in a range of neurological disorders, e.g., amyloid beta (Aβ), apolipoprotein E (APOE) and tau in Alzheimer’s Disease and superoxide dismutase 1 (SOD1) in motor neuron disease (Mawuenyega et al., 2010; Basak et al., 2012; Crisp et al., 2015). The method relies on serial analyses of the body fluid or tissue of interest after a single period of infusion of stable isotope (e.g., 2H [deuterium] or 13C) -labelled amino acids. There then are incorporated into proteins. The subsequent enrichment and decay of enrichment in target proteins after this “pulse-labelling” is measured in the fluid compartment of interest over time by mass spectrometry (Bateman et al., 2006). Studies using SILK have demonstrated that structural proteins such as tau have a significantly longer half-life (approximately 20 days).
than do membrane proteins such as Aβ (approximately 10 hours) (Bateman et al., 2006; Sato et al., 2018).

Based on this observation and rodent data demonstrating very slow turnover of NF incorporated into filamentous structures in the axon (Nixon and Logvinenko, 1986), SILK NF studies may require the use of a SILK protocol specialised for use with very long half-life proteins. This does not promise to be a straightforward task. Protocols for very long-lived peptides face three major technical challenges: i. sampling of biofluids from participants may need to be performed over periods of several months; ii. the dilution of the incorporated tracer with non-labelled proteins synthesized over the study period leads to low tracer incorporation rate (for example, less than 1% tracer enrichment of the target protein is measured at the apex of the kinetic curve for tau protein) and, iii. a mass spectrometry assay that is sufficiently sensitive to detect the low fraction of tracer incorporated into NF present in biofluid has to be developed.

The last point is likely a major technical hurdle for the analysis of NF peptides because of their low concentrations in CSF (1-10 ng/ml range) and plasma (10-100 pg/ml range) (Petzold et al., 2006; Miyazawa et al., 2007; Zetterberg et al., 2016; Mattsson et al., 2017). Mass spectrometric (as opposed to the immunoglobulin capture assays described below) assays of NF in CSF or plasma have not been reported yet (although the first patients have now been recruited to investigate this); NF SILK in CSF will require a limit of detection below 10 pg/ml. However, this sensitivity could be achieved theoretically using immuno-purification (IP) combined with the latest generation of mass spectrometers operating in targeted MS mode (Gallien et al., 2012; Peterson et al., 2012; Gillette and Carr, 2013). Identifying antibodies that efficiently recover a representative range of NF peptides will be a prerequisite. Development of such a mass spectrometric assay would pay dividends by enabling further NF peptide characterization to test for differences in the nature of the species present in CSF or plasma with aging or diseases and characterising post-translational modifications.

**NF peptide concentrations as biomarkers of neurodegeneration**
Interest in NF as a soluble biomarker has risen dramatically in recent years as robust methods for detecting NfL or its constituent peptides in CSF and blood have allowed strong associations between elevated NfL peptides (albeit with currently unspecified structural characteristics) and nervous system injury and disease (Table 2)(Khalil et al., 2018). Concentrations of NfL peptides in the CSF can be measured reliably by enzyme-linked immunosorbent assay (ELISA) using antibodies directed against the mid-domain rod region of the protein(Khalil et al., 2018). For a long time, there was only one ELISA for NfL available on the market(Petzold et al., 2010), but now additional assays exist(Gaetani et al., 2018). However, the analytical sensitivity of the ELISA (around 25-50 ng/L) precludes its general use for measurement of NfL in serum

Advances in technology have enabled major extensions of the range applications possible. Semi-sensitive electrochemiluminescence detection was the first approach that allowed disease-related increases in plasma concentration to be measured in samples from patients with ALS(Gaiottino et al., 2013) or active MS(Kuhle et al., 2016a). A further major advance enabling new applications came in 2015, when the first ultrasensitive assay for NfL using single molecule array (SIMOA) technology to enhance the ELISA signal was described(Gisslen et al., 2016). This assay allowed concentrations in plasma to be measured reliably even in people without peripheral or central nervous system pathology. For the first time, correlations between CSF and serum levels of NfL could be demonstrated using this assay in patients with HIV encephalopathy(Gisslen et al., 2016). Paired CSF and serum measures showed similar dynamics following acute brain injury, suggesting relatively rapid transport between the compartments, both peaking around 40-70 days post-injury and normalizing within 6 months(Bergman et al., 2016). Increases in plasma NfL concentration in patients with Charcot-Marie-Tooth disease demonstrated sensitivity to NfL released from peripheral nerves(Sandelius et al., 2018), both highlighting the potential of plasma NfL concentration as a biomarker of injury with this disease and the potential uncertainties for the interpretation of plasma NfL in other contexts in which there could be combined central and peripheral nerve injury. Whether NF markers associated with central and peripheral nerve injury can be differentiated biochemically is an important topic for future research, e.g., through development of assays sensitive not just to the mid-domain, but also epitopes distributed more widely in the NfL protein.
CSF and serum/plasma NfL are increased in most acute and chronic CNS diseases characterised by neuronal damage (Table 2) and correlate with longitudinal imaging findings of neurodegeneration(Khalil et al., 2018). Increases in NfL concentrations in serum and CSF thus do not provide specific markers of disease. Serum or plasma NfL concentrations (either sample matrix works well) are moderately to strongly correlated with CSF concentration measures (correlation coefficients of 0.74 to 0.97) for diseases affecting the CNS primarily(Hakansson et al., 2018); CSF findings with a range of neurodegenerative diseases (increased NfL concentrations in Alzheimer’s Disease (AD), frontotemporal dementia (FTD), vascular dementia and atypical parkinsonian disorders) have been replicated in blood plasma or serum(Zetterberg, 2016). Recent data also show that serum NfL appears sensitive to neurodegeneration and clinical progression in pre-clinical AD(Weston et al., 2017; Preische et al., 2019; Weston et al., 2019) and in Huntington’s disease(Byrne et al., 2017)(Johnson et al., 2018) and correlates with other measures of disease progression in AD(Mattsson et al., 2017), FTD(Meeter et al., 2016) and progressive supranuclear palsy(Rojas et al., 2016; Donker Kaat et al., 2018). In people at risk of familial AD, increases in serum NfL have been detected even 10 years before the expected age of onset of symptoms(Weston et al., 2017). While serum NfL appeared insufficiently sensitive, increased CSF NfL levels were a risk factor for later mild cognitive impairment independent of increases in amyloid beta in a population-based cohort(Kern et al., 2018). Following traumatic brain injury, serum concentrations of NfL increase within days, reaching a maximum weeks following the injury with normalization after 6-12 months(Shahim et al., 2016; Shahim et al., 2017).

Both the potential utility and potential limitations of NfL as a biomarker for neurological diseases are illustrated by applications in MS. Increased levels of NfH and NfL were first described in the CSF of MS patients(Malmestrom et al., 2003; Norgren et al., 2004; Teunissen et al., 2009; Gunnarsson et al., 2011; Kuhle et al., 2013) in association with clinical relapses and proposed as biomarkers of acute inflammatory activity(Lycke et al., 1998). Moreover, it was recognized that CSF NF concentrations tend to be increased across all clinical stages of MS relative to healthy volunteer groups even in the absence of evidence for new, acute inflammatory activity evident as a clinical relapse or detected using MRI(Teunissen et al., 2009; Kuhle et al., 2011). However, the chronically increased levels detected across groups of MS patients are lower (3- to 5- fold) than those reported for some primary neurodegenerative diseases (e.g., FTD or ALS)(Gaiottino et al., 2013).
With the introduction of the more sensitive SIMOA technology, plasma NfL was been explored in several studies as a marker of otherwise occult acute disease activity, drug response or future disease progression(Khalil et al., 2018). Unlike other indirect and retrospective measures of neurodegeneration in MS used clinically now (e.g., MRI or magnetic resonance spectroscopy(De Stefano et al., 2007; Pini et al., 2016; Kalra, 2019)), NfL measurements potentially allow neurodegeneration to be assessed in near “real-time”. Furthermore, NfL should be sensitive to neuronal damage in the brain and spinal cord, the latter being a CNS compartment where quantitative MR-based imaging methods for assessment of neuronal damage are technically more difficult, less standardized and not yet able to be used routinely in the clinic(Disanto et al., 2017; Barro et al., 2018). While the evidence still is limited, serial plasma NfL concentration measurements also appear to be as sensitive as MRI for the assessment of treatment effects(Gasperini et al., 2019).

Current evidence supports use of NfL as a biomarker for inflammatory disease activity in MS for group or population-based analyses; a recent retrospective analysis suggests that plasma NfL concentration measures could act as an endpoint for future Phase 2 clinical studies(Sormani et al., 2019). Increases in plasma (or CSF) NfL also might provide a potential biomarker of sub-optimally controlled acute inflammatory activity in people at high risk who are being considered for a change in treatment but are without clinical evidence of a relapse or objective inflammatory changes on MRI. This type of information could become more important as evidence for continuing inflammatory activity is needed to stratify people with progressive forms of MS for treatment with new, highly active anti-inflammatory treatments (https://www.nice.org.uk/guidance/TA585).

However, there also are important limitations to the use of NfL concentrations in CSF or plasma for disease monitoring of individual patients(Berger and Stuve, 2019). The lower levels of NfL that are found in most people with MS outside of periods of acute inflammation still generally cannot be confidently interpreted as pathological if obtained as single time-point measurements(Kuhle et al., 2016b). There is an approximately 2.2% per year increase of concentration between the ages of 18 to 70 years(Disanto et al., 2017; Mattsson et al., 2017; Barro et al., 2018), but standardised, age-corrected, normative distributions of NfL in CSF and plasma are not available to define values from individual subjects as being pathological. Measures of NfL concentrations in CSF or plasma also do not distinguish the underlying pathology and cannot differentiate between neuronal damage arising from acute
or chronic inflammatory injury and other contributions to neurodegeneration (e.g.,
comorbidities of MS) (Marrie, 2016). Lack of knowledge of the kinetics of NfL peptide
turnover in the blood also precludes confidence in the relative timing of presumed
inflammatory events giving rise to NfL increases measured (Thelin et al., 2017). Finally, not
only is the precise nature of the peptide (and thus whether it might change with disease stage
or other factors) unknown, but the CNS pathology being assessed itself may be uncertain: is
the major release of NfL peptide with CNS injury due to synaptic damage or turnover or, as
with peripheral nerve injury, does it primarily reflect axonal damage?

Fundamental to addressing any of these questions will be to develop consensus amongst
analytical laboratories for a harmonised assay standard to allow uniform interpretation of
results between all laboratories. While the increasing diffusion of SIMOA is contributing to
this now, other assay platforms also are in development by a number of diagnostic medicine
companies. This continued commercial innovation may delay harmonisation of assays and
definition of the kinds of normative data that are needed for confident clinical use of
measures from individual patients.

Conclusions and future directions

Neurofilaments play fundamental roles in the neuronal development, organisation and
function in the central and peripheral nervous systems. Primary roles of NF in the
pathogenesis of ALS (Figlewicz et al., 1994; Tomkins et al., 1998; Gros-Louis et al., 2004;
Leung et al., 2004) and CMT (Mersiyanova et al., 2000; Rebelo et al., 2016) and secondary
pathogenic roles in other disorders have been discovered. Most striking to date amongst the
latter are disorders arising from impairments in normal mechanisms for NF degradation that
are associated with progressive and severe axonal pathology. However, fundamental
questions still remain concerning basic mechanisms regulating NF expression, assembly and
turnover.

The identification of functional roles for synaptic NF in modulation of excitatory
glutamatergic activity (Huntley et al., 1994; Ratnam and Teichberg, 2005; Yuan et al., 2018a)
has opened an entirely new range of investigations of NF neurobiology. The contributions of
the synaptic pool to neuronal dysfunction associated with schizophrenia-like behaviours in an
NfL/- mouse suggests the potential for abnormalities in synaptic NF to contribute to the genesis neuropsychiatric diseases more generally(Yuan et al., 2018a). Although the disease relevance still is speculative, the importance of the question and the novelty of this neurobiology make better understanding of the structurally unique synaptic NF, its organisation and its functions a priority.

However, understanding how NF are degraded and which and how degradation products are released from neurons is a particularly urgent agenda given the degree of clinical interest in using NF in CSF and serum as biomarkers. Physiological mechanisms for more dynamic control through post-translational modifications need to be better defined. For example, how is NF phosphorylation regulated relative to the targeting of NF for E3 ligase and protease activity? Exactly what forms of NF (short IFs, unit length filaments (ULF), tetramers or dimers) are the major substrates for degradation with normal turnover? Applications of NF measures for assessment of pathology demand some appreciation for whether these mechanisms of NF degradation and release are altered with disease or injury. We hypothesise that they may vary. For example, oxidative stress leads to increased protein carbonylation and degradation of carbonylated cytoskeletal proteins including NfM and NfH is largely mediated by calpains with involvement of proteasomes(Smerjac et al., 2018), suggesting a particular role for this degradation mechanism in the context of oxidative pathologies.

Technological advances in measurement of the low concentrations of NF (particularly NfL) in CSF or serum have allowed exploration of how levels increase with aging, brain injury and neurodegenerative diseases. These suggest that NF concentration measures in these compartments could be used as an index of peripheral or central nerve damage with trauma(Shahim et al., 2016) or neurodegeneration(Weston et al., 2017; Preische et al., 2019; Weston et al., 2019). Serial measures can be sensitive to sub-clinical disease activity in MS in ways that suggest the potential to monitor treatment responses(Lycke et al., 1998), potentially at an individual patient level with active disease. Particular clinical impact could arise with use of NfL measures as evidence to support therapeutic decisions regarding continuing disease activity when other clinical or imaging evidence is lacking.

However, at this point, increased NfL measures in CSF or serum are non-specific to disease aetiology. The molecular characteristics of the precise peptide species being measured are uncertain and the mechanisms of their release and trafficking from the parenchyma to CSF or
blood are speculative. Interpretation of plasma measures of NfL also can be uncertain when both central and peripheral nervous system injury is possible, such as after some forms of trauma. Can the release of NfL peptides and their levels in blood or CSF be related quantitatively to the degree of neuronal injury or does relationship vary substantially with the nature of the pathological insult? Are there disease- or stage-specific differences between the NF species detected? What is the time course of injury over which they are reporting? Do the differences seen with age or disease solely reflect neurodegenerative changes, or could they also reflect differences in transport or turnover? If medical decisions are to be made based on these measures, answering such questions will become important.

We believe that basic questions like these emphasise the need for some caution in interpretation of NF measures in serum or CSF from individual patients. At the same time, they also highlight why there is currently such excitement amongst those interested in the neurobiology of intermediate filaments. Moreover, with a growing range of new tools for characterising major aspects of their biology (e.g., SILK), it is timely to ask these questions. The strong foundations that have been laid for discovery, the availability of new tools and approaches and practical importance of developing confidence in understanding NF better, all highlight both the clinical promise for NfL as a biomarker and the great potential for future investigation of the neurobiology of NF and IF more generally.

Acknowledgments

This review was written following an open workshop concerning neurofilament biology and use as biomarker including all of the co-authors that was held at Imperial College London on 18 Nov 2018 (https://www.imperial.ac.uk/dementia-research-institute/seminars--events/past-events/neurofilaments-as-a-biomarker/). The workshop was initiated and planned by Prof. Paul Matthews and Prof. Henrik Zetterberg. Funding for the workshop was provided by the UK Dementia Research Institute at Imperial College and an educational grant provided by Biogen.
Investigator Funding

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Conflicts Of Interest:
HZ has served at scientific advisory boards for Roche Diagnostics, Samumed, CogRx and Wave, has given lectures in symposia sponsored by Biogen and Alzecure, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (all outside submitted work). PMM acknowledges consultancy fees from Adelphi Communications, Biogen, Celgene and Roche. He has received honoraria or speakers’ honoraria from Biogen, Novartis and Roche, and has received research or educational funds from Biogen, GlaxoSmithKline, Nodthera and Novartis. He is a paid member of the Scientific Advisory Board for Ipsen Pharmaceuticals. All of these, except the educational grant from Biogen described above, are outside of the scope of this review.
### Tables

<table>
<thead>
<tr>
<th>Neurofilament pathology</th>
<th>Proteins affected</th>
<th>Associated Diseases</th>
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<tr>
<td>Primary neurofilament gene mutations</td>
<td>NfH, Peripherin</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td></td>
<td>NfL, NfH</td>
<td>Charcot-Marie Tooth Disease</td>
</tr>
<tr>
<td>Mutations in genes involved in NF assembly, turnover and degradation</td>
<td>Sacsin</td>
<td>Autosomal Recessive Spastic Ataxia of Charveloix</td>
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<tr>
<td></td>
<td>TRIM2</td>
<td>Charcot-Marie Tooth Disease Type 2R</td>
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<tr>
<td></td>
<td>Gigaxonin</td>
<td>Giant Axonal Neuropathy</td>
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</tbody>
</table>

Table 1. Diseases associated with mutations in neurofilament genes or genes involved in proteins for neurofilament assembly, turnover and degradation.
<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>Key references</th>
</tr>
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<tbody>
<tr>
<td>• Peripheral neuropathies</td>
<td></td>
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<tr>
<td>o Charcot-Marie-Tooth Disease</td>
<td>(Sandelius et al., 2018)</td>
</tr>
<tr>
<td>o Guillain-Barré syndrome</td>
<td>(Petzold et al., 2006)</td>
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<td>o Chronic Inflammatory Demyelinating</td>
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<tr>
<td>Polyneuropathy</td>
<td>(van Lieverloo et al., 2019)</td>
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<tr>
<td>• Ageing</td>
<td></td>
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<tr>
<td>• Multiple Sclerosis</td>
<td></td>
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<tr>
<td>• Amyotrophic lateral sclerosis</td>
<td></td>
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<tr>
<td>• Dementia</td>
<td></td>
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<tr>
<td>o Pre-clinical Alzheimer’s Disease</td>
<td>(Weston et al., 2017; Preische et al., 2019; Weston et al., 2019)</td>
</tr>
<tr>
<td>o Alzheimer’s Disease</td>
<td>(Mattsson et al., 2017)</td>
</tr>
<tr>
<td>o Frontotemporal dementia</td>
<td>(Meeter et al., 2016)</td>
</tr>
<tr>
<td>o Atypical Parkinsonian disorders (e.g., progressive supranuclear palsy)</td>
<td>(Rojas et al., 2016; Donker Kaat et al., 2018)</td>
</tr>
<tr>
<td>• Stroke</td>
<td></td>
</tr>
<tr>
<td>o Subarachnoid haemorrhage</td>
<td>(Nylen et al., 2006; Zanier et al., 2011)</td>
</tr>
<tr>
<td>o Ischaemic stroke</td>
<td>(Gattringer et al., 2017)</td>
</tr>
<tr>
<td>• Traumatic brain injury</td>
<td>(Shahim et al., 2016; Shahim et al., 2017)</td>
</tr>
<tr>
<td>• Huntington’s Disease</td>
<td>(Byrne et al., 2017; Johnson et al., 2018)</td>
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<tr>
<td>• Neuropsychiatric conditions</td>
<td></td>
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<tr>
<td>Bipolar disorder</td>
<td>(Jakobsson et al., 2014)</td>
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<tr>
<td>• Spinal muscular Atrophy</td>
<td>(Darras et al., 2019)</td>
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Table 2. Major disorders reported to have associations with increased NfL concentration in blood or plasma
Figure Legends

Figure 1. Schematic representation of the structure of neuronal intermediate filament (IF) proteins. All IF proteins have a highly conserved central domain of 310 amino acid residues that is responsible for the formation of coiled-coil structures. Flanking this central rod domain are the amino- and carboxyl-terminal domains. These latter domains confer functional specificity to the different types of IF proteins. For example, the NfM and NfH carboxyl-terminal domains contain multiple repeats of phosphorylation sites KSP (Lys–Ser–Pro) that account for the unusual high content of phosphoserine residues for these proteins. The N- and carboxyl-terminal regions contain multiple α-linked glycosylation sites. Neurofilament proteins NfL, NfM and NfH are obligate heteropolymers. Although α-internexin or peripherin can form homopolymers in vitro, these IF proteins usually copolymerize with the neurofilament triplet proteins in vivo.

Fig. 2 Intermediate filaments are formed by the assembly of IF protein dimers. Two polypeptide chains form a coiled-coil dimer and two coiled-coil dimers form a 3-nm protofilament. These protofilaments associate in a staggered manner to form filaments of 10-nm in diameter (32 chains). The carboxy-terminal domains of NfM and NfH form side-arm projections at the filament periphery.

Figure 3.Mutations in the NEFL gene encoding NfL account for a small percentage of Charcot-Marie-Tooth disease. It is noteworthy that mutations have been detected in various regions of NfL. Some mutations have been shown to disrupt self-assembly of NfL into a filamentous network.

Figure 4. Functions of NF subunit assemblies in synapses. Left panel: Immunogold labelled antibodies against the NfM subunit decorating mouse brain synaptic structures in a linear pattern (immunogold particles outlined in blue) suggest the presence of short neurofilaments and protofilament/protofibrils. In the upper inset, a filament within a postsynaptic bouton is decorated by immunogold antibodies to both NfL (large gold dots) and NfH (small gold dots). Graphic inset: morphometric analyses indicate a higher density of immunogold labelling in postsynaptic boutons than in preterminal dendrites or presynaptic terminals (graph inset). Middle panel: Ultrastructural image of a human brain synapse.
illustrates membranous vesicles (tentatively identified as endosomes), most associated with short 10nm filaments in the post-synaptic region. Right panel: Evidence (Yuan et al., 2015a) supports a biological mechanism whereby D1 dopamine receptors internalized on endosomes from the postsynaptic surface dock on synaptic neurofilament subunit assemblies (outlined in blue) where they remain available to recycle from endosomes to the synaptic surface in response to ligand stimulation. In the absence of NfM, retention of D1R on the plasma membrane surface induces hypersensitivity to D1R agonists, as observed in vivo. Selective NfL deletion in mice induces an NMDAR hypofunction phenotype by lowering membrane surface levels of the GluN1 subunit. Evidence (Yuan et al., 2018a) supports a mechanism in which NfL binds GluN1 associated with NMDAR on postsynaptic terminals and stabilizes the receptor on the membrane by directly anchoring GluN1 and preventing access of the ubiquitin ligase that ubiquitinates GluN1 and targets it for degradation by the proteasome (UPS) leading to reduced NMDAR function. A key below the figure identifies the depicted cellular elements that are depicted.

**Figure 5. The IPAD pathway.** A length of cerebral artery in a mouse brain showing fluorescent amyloid protein (Aβ) co-localising (pink) with collagen IV in basement membranes between smooth muscle cells in the tunica media of the artery wall; this is part of the IPAD pathway (indicated by arrows) along which Aβ is draining out of the brain. The IPAD pathway for Aβ and, by inference, perhaps that for NF peptides, forms a spiral pattern along arterial walls (smooth muscle cells in the artery walls section illustrated are stained green. This figure is modified from an original figure reproduced as Fig 1d of Albargothy et al, 2018 (Albargothy et al., 2018).

**Figure 6. Fluid balance in the brain.** Entry and drainage of fluid into and from the brain is along basement membranes associated with the walls of arteries: i. CSF enters the surface of the brain along Pial-GliaI basement membranes on the outer aspects of cortical arteries; ii. CSF mixes with interstitial fluid (ISF); to then, iii. leave the brain along Intramural Peri-Arterial Drainage (IPAD) pathways.
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