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Neurofilaments: neurobiological foundations for biomarker applications

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Interest in neurofilaments has risen sharply in recent years with recognition of their potential as biomarkers of brain injury or neurodegeneration in CSF and blood. This is in the context of a growing appreciation for the complexity of the neurobiology of neurofilaments, new recognition of specialized roles for neurofilaments in synapses and a developing understanding of mechanisms responsible for their turnover. Here we will review the neurobiology of neurofilament 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 neurofilament degradation and clearance and review new methods for future elucidation of the kinetics of their turnover in humans. Primary roles of neurofilaments in the pathogenesis of human diseases will be described. With this background, we then will review critically evidence supporting use of neurofilament concentration measures as biomarkers of neuronal injury or degeneration. Finally, we will reflect on major 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 neurofilament measures as biomarkers of neurodegeneration.

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Abbreviations: ALS = amyotrophic lateral sclerosis; ARSACS = autosomal recessive spastic ataxia of Charvleoix-Saguenay; CMT = Charcot-Marie-Tooth disease; D1R = dopamine D1 receptor; GAN = giant axonal neuropathy; GluN1 = glutamate ionotropic receptor NMDA type subunit 1; IPAD =intramural peri-arterial drainage pathway; ISF = interstitial fluid; LTP = long term potentiation; NfH/L/M = neurofilament heavy/light/medium chain; NMDA = *N*-methyl-D-aspartate; SILK = stable isotope labelling kinetic

Introduction

Neurofilaments are assembled from a family of five 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 neurofilament heavy chain (NfH), followed (in order of descending molecular weight) by the medium chain (NfM), the light chain (NfL), α-internexin and peripherin (Fig. 1). Neurofilaments contribute to growth and stability of axons in both central and peripheral nerves as well as to maintaining mitochondrial stability (Gentil *et al.*, 2015) and microtubule content (Bocquet *et al.*, 2009). Roles for distinct neurofilament isoforms in maintaining the structure and function of dendritic spines and in regulating glutamatergic and dopaminergic neurotransmission synapses have also been discovered (Schwartz *et al.*, 1994, 1995).

The fundamental importance of neurofilaments to neurons has been highlighted by molecular characterization of diseases of the brain and peripheral nerves associated with abnormal neurofilament structure and function. Mutations in the *NEFL* gene, which encodes NfL, lead to peripheral neurodegeneration in Charcot-Marie-Tooth (CMT) disease types 2E (Mersiyanova *et al.*, 2000) and type 1F (Jordanova *et al.*, 2003) and G (Zuchner *et al.*, 2004). While polymorphisms in *NEFH*, encoding NfH, are associated with amyotrophic lateral sclerosis (ALS) (Figlewicz *et al.*, 1994), mutations in this gene also are a cause of CMT type 2 (Rebelo *et al.*, 2016). Neurofilament dysfunction or aggregation also may play roles in the neuropathology of Alzheimer's disease, Parkinson's disease and other neurodegenerative disorders (Khalil *et al.*, 2018).

Neurofilaments' turnover in healthy neurons is slow. Their expression is regulated by neuronal activity acting through developmentally regulated promoter regions (Yaworsky *et al.*, 1997). Post-transcriptional regulation of neurofilament mRNA stability also may contribute to determining levels of expression of neurofilament protein (Schwartz *et al.*, 1994, 1995). Additional insights into mechanisms for turnover of neurofilament have come through studies of rare diseases arising from gigaxonin E3 ligase mutations causing giant axonal neuropathy (GAN) (Bomont *et al.*, 2000) and mutations 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 Charlevoix-Saguenay (ARSACS) (Engert *et al.*, 2000). Study of these diseases has elucidated major pathways responsible for the degradation of neurofilament protein, which is a consequence of combined activities of proteasomal and, possibly, autophagocytic mechanisms (Bomont, 2016). Neurofilament or its fragments can be 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 characterized. Release may occur actively (e.g. by means of exosomes; Faure *et al.*, 2006; Lachenal *et al.*, 2011) or passively with loss of neuronal membrane integrity. Neurofilaments 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.*, 1984) suggest that degraded neurofilament proteins may enter the peripheral circulation via perivascular drainage along basement membranes of 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 neurofilaments in CSF have been available for many years (Norgren *et al.*, 2002). These provided an early foundation for exploration of the associations of increased CSF neurofilaments with neurological diseases (Rosengren *et al.*, 1996; Lycke *et al.*, 1998). Ultra-sensitive assays of neurofilaments in blood are now available routinely for clinical applications in many centres (Kuhle *et al.*, 2016*a*). This has enabled several studies assessing the potential utility of neurofilament (particularly NfL) peptide concentrations in the CSF or peripheral blood as clinical biomarkers of neuronal injury or neurodegeneration (Kuhle *et al.*, 2016*a*; Disanto *et al.*, 2017; Khalil *et al.*, 2018). For example, CSF and peripheral blood neurofilament protein concentrations are increased after stroke or traumatic brain injury (Khalil *et al.*, 2018) and are associated with ageing (Disanto *et al.*, 2017) and primary neurodegenerative diseases including Alzheimer's

Box 1 Summary points

- 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 CNS.
- The fundamental importance of neurofilaments to axonal structure and function was first appreciated with serial discoveries of causal neurofilament gene mutations for rare forms of CMT disease and ALS.
- Evidence that their normal intracellular assembly and turnover involves the ubiquitin-proteasomal pathway (and possibly also the autophagy pathway) came from studies of rare genetic diseases characterized by prominent accumulations of neurofilaments: CMT type 2R, GAN and ARSACS.
- Increased concentrations of extra-neuronal neurofilament peptides in CSF and blood now are measured routinely using ultra-sensitive immunoassays after peripheral nerve or brain injury or in association with clinical progression of several chronic neurodegenerative disorders and neuropathies including multiple sclerosis, Alzheimer's disease, ALS, Huntington's disease and CMT.
- However, as the mechanisms and kinetics of neurofilament protein release from neurons and trafficking between brain and blood compartments are ill-defined, interpretations of increased CSF or blood neurofilament 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 neurofilament 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.

disease (Mattsson *et al.*, 2016; Weston *et al.*, 2017). Neurofilament concentrations in both CSF and peripheral blood are increased in some individuals with multiple sclerosis (Amor *et al.*, 2014; Kuhle *et al.*, 2016*b*; Disanto *et al.*, 2017; Novakova *et al.*, 2017; Barro *et al.*, 2018; Hakansson *et al.*, 2018; Piehl *et al.*, 2018) and have potential roles as clinical biomarkers of disease activity, treatment responses or prediction of future disease progression and disability. Both peripheral blood and CSF concentrations are correlated with radiological (Kuhle *et al.*, 2016*b*; Disanto *et al.*, 2017; Novakova *et al.*, 2017; Siller *et al.*, 2018) and clinical measures of disease activity (Disanto *et al.*, 2017; Novakova *et al.*, 2017; Barro *et al.*, 2018; Hakansson *et al.*, 2018; Piehl *et al.*, 2018; Siller *et al.*, 2018). Evidence for treatment responsiveness further supports a causal link between disease activity and increased neurofilament concentrations in CSF and peripheral blood (Gunnarsson *et al.*, 2011; Disanto *et al.*, 2017; Piehl *et al.*, 2018; Gafson *et al.*, 2019; Kuhle *et al.*, 2019). NfL levels have a potential role in assessing prognosis in multiple sclerosis. For example, the predictive association between increased NfL and longer-term brain and spinal cord atrophy (Barro *et al.*, 2018) likely arises from the sensitivity of NfL to the neuroinflammatory neuronal injury and degeneration that provides a substrate for future disease progression (Matthews, 2019).

While the concentration in CSF is ~20–50-fold greater than in peripheral blood (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 neurofilaments can also be released from peripheral nerves, depending on the pathological context, peripheral blood and CSF measures should not necessarily be correlated (Bergman *et al.*, 2016). Longitudinal measures of CSF and peripheral blood concentration changes after brain injury (Shahim *et al.*, 2016) 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 neurofilament neurobiology in health and disease is reviewed in more detail below (Box 1).

Structure and assembly of neurofilaments

Neurofilaments are major cytoskeletal components in mature neurons. They are found in the cytoplasm of neurons within the peripheral nervous system (PNS) and CNS, most abundantly in axons, but also in cell bodies, dendrites and synapses (Yuan *et al.*, 2015*b*). They are expressed more highly in large myelinated axons, where they are organized in parallel structures maintained by side arms projecting outwards from a filament core (Yuan *et al.*, 2017). However, the relative abundance of neurofilament proteins can widely differ along the course of even a single axon, e.g. amounts of neurofilaments are 3-fold greater in myelinated regions of axons than at the nodes of Ranvier (Hsieh *et al.*, 1994; Nixon *et al.*, 1994).

Neurofilaments are hetero-polymers composed of core neurofilament proteins (NfL, α-internexin or peripherin)

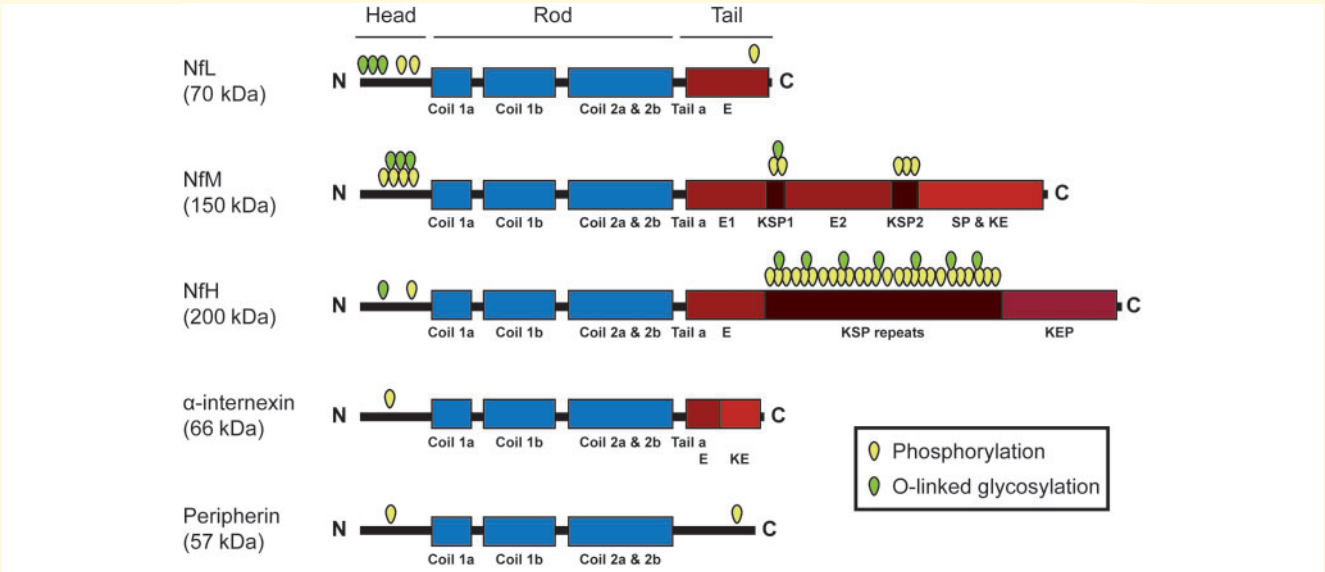

Figure 1 Schematic representation of the structure of neuronal intermediate filament proteins. All intermediate filament proteins have a highly conserved central domain of 310 amino acid residues that is responsible for the formation of coiled-coil structures. Flanking this central rod domain are the amino- and carboxyl-terminal domains. These latter domains confer functional specificity to the different types of intermediate filaments 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 C-terminal regions contain multiple *O*-linked glycosylation sites. Neurofilament proteins NfL, NfM and NfH are obligate heteropolymers. Although α-internexin or peripherin can form homopolymers *in vitro*, these intermediate filaments proteins usually co-polymerize with the neurofilament triplet proteins *in vivo*.

(Yuan *et al.*, 2006, 2012*b*) co-assembling with NfM and NfH (Fig. 2). α-Internexin interacts with NfL to form a backbone to which NfM and NfH attach. Multiple types of post-translational modifications to neurofilament occur (e.g. phosphorylation, ubiquitination, nitration and addition of *O*-linked *N*-acetylglucosamine) (Nixon and Sihag, 1991; Yuan *et al.*, 2017). NfM and NfH can be phosphorylated extensively (Fig. 1) (Beck *et al.*, 2012). The relative proportions of the neurofilament 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 among the neurofilaments as it is required for neurofilament protein assembly in some neuronal subtypes. NfL knockout mice exhibit severe atrophy of peripheral myelinated axons (Zhu *et al.*, 1997). The latter observation provides evidence that neurofilaments are necessary for the radial growth of large myelinated axons and associated fast nerve conduction (Kriz *et al.*, 2000).

The complexity of mechanisms by which neurofilament proteins play their roles in maintaining axonal organization have begun to be defined. Their phosphorylation appears to be a fundamental element. The carboxyl terminals of NfH and NfM proteins form side-arm projections at the periphery of neurofilament 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), increases the negative charge of these projections (and, by inference, the spacing between neurofilament in the axon), it was believed initially that NfH must play a major role in determining axonal calibre. However, surprisingly, targeted disruption of the *NEFH* gene had little effect on the radial growth of myelinated axons (Rao *et al.*, 1998; Zhu *et al.*, 1998). In contrast, deletion of *NEFM* gene (Jacomy *et al.*, 1999) or deletion of the NfM carboxy-terminal tail domain substantially reduced the calibres of large myelinated axons (Rao *et al.*, 2003). The exact NfM domain that modulates axon calibre remains to be elucidated. A mouse knock-in substitution of KSP repeats by KAP (NF-M$^{S\rightarrow A}$) repeats (which cannot be phosphorylated) in NfM demonstrated that phosphorylation of NfM KSP repeats does not determine axonal calibre (Garcia *et al.*, 2009). The current model for their structural organization proposes that the tail domains of NfM and NfH form side arms that interconnect neurofilaments and link them to other cytoskeletal elements and organelles such as mitochondria and microtubules (Yuan *et al.*, 2017). Phosphorylation of neurofilament stabilizes this structure by inhibiting neurofilament proteolysis and increasing the half-life of the whole supramolecular assembly (Rao *et al.*, 2012).

Most neurofilament proteins are synthesized and assembled in the neuronal perikaryon and must be transported along axons for functional integration into the axonal cytoskeleton (Yuan *et al.*, 2012*a*). The newly synthesized

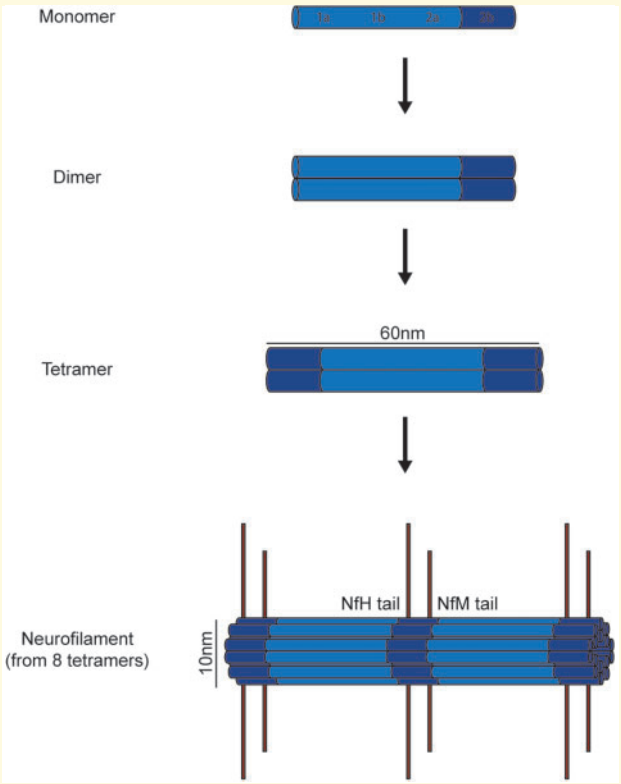

Figure 2 Intermediate filaments are formed by the assembly of intermediate filament protein dimers. Two polypeptide chains form a coiled-coil dimer and two coiled-coil dimers form a 3-nm protofilament. These protofilaments associate in a staggered manner to form filaments of 10-nm in diameter (32 chains).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

neurofilament population enters axons in the form of short filaments and possibly also smaller polymeric/oligomeric assemblies (Pachter and Liem, 1984). It was proposed initially that, after their assembly in the perikaryon, neurofilaments 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 neurofilament 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 neurofilament proteins in growing axons of neurons *in vitro* revealed fast transport of tagged-neurofilament at rates up to 2 μm/s that is interrupted by long pauses resulting in an average rate approximating the slow neurofilament transport estimates (Roy *et al.*, 2000; Wang *et al.*, 2000; Li *et al.*, 2012). Live imaging of immature or regenerating axons *in vitro* has also identified a pool of labelled neurofilaments that, after entering the axon, remains there for very long periods (Trivedi *et al.*, 2007; Yuan *et al.*, 2009). This pool reflects the initial stage of construction of a stable stationary neurofilament network that maintains calibre sizes of mature large PNS and CNS fibres by integration of neurofilaments 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 neurofilaments undergoing transport 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 the neurofilament population undergoing axonal transport, are well explained by this model. The precise kinetics can also be related to local axonal structure; pulse-escape fluorescence photoactivation recently demonstrated slowing of neurofilament transport at nodes of Ranvier, where there is constriction of the axon (Walker *et al.*, 2019).

Neurofilament protein stoichiometry is essential for appropriate neurofilament assembly and axonal integrity (Julien *et al.*, 1987). Abnormal neurofilament accumulation in the perikaryon of motor neurons can be induced by overexpressing any neurofilament protein alone. Overexpression of NfH in mice led to formation of large perikaryal neurofilament aggregates in spinal neurons and reduction of neurofilament 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 neurofilament assembly from those of NfM and NfH. Additional observations further emphasize the importance of maintaining proper intermediate filament protein stoichiometry in an axon. In the mouse, large perikaryal accumulations of neurofilaments due to human *NEFH* transgene overexpression were associated with severe atrophy of peripheral axons, but did not cause substantial neuronal death (Cote *et al.*, 1993). However, even with normal stoichiometry of the major proteins, neurofilament 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 neurofilament aggregates and selective degeneration of spinal motor neurons.

Neurofilament pathologies are commonly expressed as aggregates, but their functional significance appears to depend on their localization within the neuron. 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 neurofilament aggregates (or spheroids) are more toxic than perikaryal neurofilament aggregates, perhaps because of interference with axonal transport of organelles by the former.

Genetic abnormalities in neurofilaments and disease

Historically, the fundamental importance of neurofilament to neuronal structure and function became apparent through

Table 1 Diseases associated with mutations in neurofilament genes or genes involved in proteins for neurofilament assembly, turnover and degradation

Neurofilament pathology	Proteins affected	Associated diseases
Primary neurofilament gene mutations	NfH, peripherin	ALS
	NfL, NfH	CMT
Mutations in genes involved in NF assembly, turnover and degradation	Sacsin	ARSACS
	TRIM2	CMT type 2R
	Gigaxonin	GAN

the discovery of associations between neurofilament gene mutations and disease (Table 1). Abnormal accumulation of neurofilament and of the related intermediate filament, peripherin, is an early pathological hallmark of ALS (Corbo and Hays, 1992). Several factors could be responsible for the abnormal neurofilament accumulations observed, such as dysregulation of neurofilament gene expression, neurofilament mutations, defective axonal transport, abnormal post-translational modifications, and proteolysis. Degenerating neurons in ALS show a 70% decrease in levels of NfL (*NEFL*), α-internexin (*INA*) and peripherin (*PRPH*) mRNA post-mortem (Campos-Melo *et al.*, 2013). Modifications in the stability of the associated neurofilament 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 *NEFL* mRNA (Strong *et al.*, 2007; Volkening *et al.*, 2009), a phenomenon that also could contribute to alterations of neurofilament protein synthesis, consequent stoichiometry changes and aggregation of neurofilament (Rosengren *et al.*, 1996).

Evidence for a potentially causal pathogenic role of neurofilament abnormalities in ALS (Table 1) came from the discovery of codon deletions or insertions in the 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 (*PRPH*) 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 neurofilament genes in association with familial and sporadic ALS (Rooke *et al.*, 1996; Vechio *et al.*, 1996), suggesting either that neurofilament gene mutations define a rare subtype of ALS or that they make only a minor contribution to general ALS susceptibility.

Pathogenic roles for neurofilaments in ALS may extend beyond associations with rare genetic coding variants: neurofilament abnormalities in ALS also occur as a result of post-translational protein modifications. Phosphorylation changes can alter the axonal transport of neurofilaments, leading to their accumulation in cell bodies and axons. Treatment of neurons with glutamate activates mitogen-activated protein kinase (MAPK), resulting in phosphorylation of neurofilaments and slowing of their axonal transport, hence potentially defining another mechanism by which glutamate might affect its excitotoxicity in ALS (Ackerley *et al.*, 2000; Veyrat-Durebex *et al.*, 2014). Additional observations link glutamate excitotoxicity to neurofilament phosphorylation. Glutamate induces caspase cleavage and activation of protein kinase N1 (PKN1), a kinase targeting the neurofilament head rod domain to disrupt neurofilament organization 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 neurofilament 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, downregulation of PIN1 by small interfering RNA reduces glutamate-induced NfH phosphorylation and neuronal apoptosis (Kesavapany *et al.*, 2007). However, over-expression of human NfL alone also is associated with the potentiation of *N*-methyl-D-aspartate (NMDA)-dependent calcium entry and apoptosis (Sanelli *et al.*, 2007).

Indirect evidence suggests that additional post-translational modifications of neurofilaments may contribute to ALS. Advanced glycation end-products have been detected in neurofilament 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).

Mutations in *NEFH* have been associated with the type 2CC axonal form of CMT (Jacquier *et al.*, 2017) (Fig. 3). Causal mutations in the *NEFL* gene have been linked to several other (also less common) forms of CMT (Horga *et al.*, 2017). Some of these mutations cause the axonal CMT type 2E (Mersiyanova *et al.*, 2000), but other *NEFL* mutations are associated with slow nerve conduction velocities and clinical presentations resembling demyelinating type CMT (type 1F) (Jordanova *et al.*, 2003) or the type G intermediate form (Zuchner *et al.*, 2004). While the majority of *NEFL* mutations resulting in CMT are dominantly inherited, autosomal recessive *NEFL* mutations that lead to truncated NfL proteins and a severe early onset axonal form of CMT have also been reported (Abe *et al.*, 2009; Yum *et al.*, 2009; Sainio *et al.*, 2018).

The P22S mutation in NfL was first discovered in a Slovenian patient (Georgiou *et al.*, 2002) 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 within the head domain and perturbs normal regulation of neurofilament assembly through phosphorylation (Sasaki *et al.*, 2006); the mutated NfL proteins do not self-assemble with NfM and NfH and cause neurofilament aggregation in cultured cells (Perez-Olle *et al.*, 2002, 2004). Similar neurofilament aggregates caused by

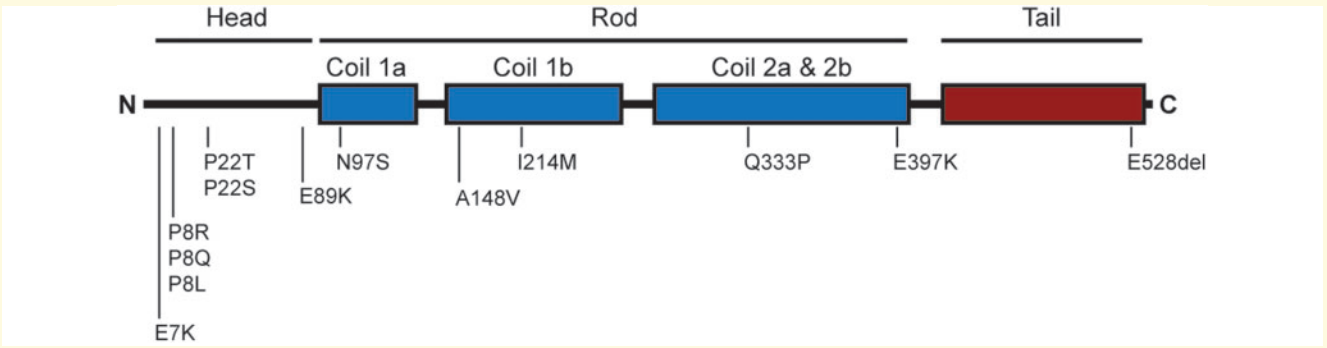

Figure 3 Mutations in the *NEFL* gene encoding NfL account for a small percentage of CMT disease. It is noteworthy that mutations have been detected in various regions of NfL. Some mutations have been shown to disrupt self-assembly of NfL into a filamentous network. The mutations highlighted here are not exhaustive (Horga *et al.*, 2017) and do not include recently identified recessive mutations.

CMT-associated mutations in NfL (e.g. P22S, as well as P8R, Q333P) trap motor proteins and organelles in the cytoplasm, contributing to axonal transport defects (Zhai *et al.*, 2007). Interestingly, mutations in the *NEFH* gene also have been recently identified in an axonal form of CMT (Rebelo *et al.*, 2016; Jacquier *et al.*, 2017).

Transgenic mouse models based on mutations in CMT provide powerful tools for study of the disease pathology. An 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 $NFL^{N98S/+}$ mice were symptomatic at an early age. Tremor was observed at 1 month of age. The N98S mutation caused a severe reduction of neurofilament in myelinated axons of the PNS and CNS, axonal hypotrophy and distal axonal loss in the PNS. Cellular immunopathology revealed abnormal neurofilament 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 NFL^{N98S} 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 neurofilament accumulation. Together, these results suggest the hypothesis that phenotypic severity in mouse models of CMT caused by NfL mutations may be related to both the extent of neurofilament aggregation found in neurons and clinical severity in corresponding human diseases.

A critical proof of principle that selective suppression of mutant neurofilament expression alone could not only slow the progression of, but also reverse disease-related pathology came from a transgenic mouse study with conditional mutant NfL^{P22S} expression. The model was generated using a tetracycline-responsive (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^{P22S} production after clinical disease onset reversed these pathological features. This important observation suggests that therapies able to abolish mutant NfL expression also may be able to reverse pathology and disability in the clinic. However, whether such dramatic results would be seen in patients still is uncertain given the species differences and also that symptoms in the mouse model occurred very late, expression of mutant NfL was low and neurofilament aggregates were not seen in motor neurons. Additional work is needed to determine whether neuronal function is restored if pathological neurofilament aggregates are present and whether any functional recovery can be related directly to clearing of the aggregates.

Neurofilaments in synapses

Although neurofilaments have been viewed traditionally as structural components primarily of axons and dendrites, recent evidence has shown that distinctive assemblies of neurofilament subunits also are integral components of synapses. For decades, synaptic terminals were associated only with degradation of neurofilaments 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 neurofilaments in axons. Other observations of neurons in the intact, mature brain also proved difficult to reconcile with models of neurofilament transport and distribution that mostly relied on *in vitro* observations made on embryonic neuronal axons, which have few neurofilaments 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 neurofilament subunit precursors needs to be transported to maintain the large stationary neurofilament network in myelinated axons because of the exceptionally slow turnover of this network (Yuan *et al.*, 2017). In this model, the small amount of neurofilament 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 neurofilament network uniformly along axons (Nixon and Logvinenko, 1986). The role of neurofilaments 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 neurofilament proteins in the CNS, intrinsic axons of the brain, even the larger corpus callosum axons, have a relatively low neurofilament density and exhibit minimal volume reductions when their neurofilament expression is suppressed genetically (Dyakin *et al.*, 2010).

Direct evidence has now established unequivocally that synapses contain a unique pool of neurofilament that has distinctive functional roles (Yuan *et al.*, 2015*a*, *b*). Neurofilament assemblies isolated from brain synaptosomes are distinguishable both morphologically and biochemically from those in other parts of the neuron; neurofilament 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 10-nm filaments that often are associated with the postsynaptic density (PSD) or with vesicular organelles (Fig. 4). Synaptic neurofilament proteins are distinctive in both stoichiometry and states of phosphorylation and respond differently to genetic subunit perturbations than the larger neurofilament protein pool in brain white matter (Yuan *et al.*, 2015*b*). 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 neurofilament proteins to enable the latter (Hashimoto *et al.*, 2000) and hint at a broader functional significance of the complex regulation of neurofilament subunits by phosphorylation (Nixon and Sihag, 1991; Sihag *et al.*, 2007).

Synaptic neurofilament 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.*, 2015*b*). Immunocytochemical studies (Bragina and Conti, 2018) have confirmed neurofilament subunit immunoreactivity (NFIR) in pre-and post-synaptic compartments and greater NFIR in GABAergic than in glutamatergic synapses (Bragina and Conti, 2018).

Recent findings that individual subunits serve unique roles in neurotransmission provide indirect, but compelling evidence for the functional importance of synaptic neurofilament (Yuan *et al.*, 2015*b*). 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 *NEFL*$^{-/-}$ than in wild-type mice (Yuan *et al.*, 2018*a*). Binding of antibody that only recognizes poly-ubiquitin chains formed with the Lys48 (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.*, 2018*b*). 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 neurofilament 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 presentations of psychiatric and neurodegenerative disorders including Alzheimer's disease (Lin *et al.*, 2014). *NEFL* 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.*, 2018*b*). Responses to NMDAR antagonists are also lost with *NEFL* deletion, although effects on NMDA-independent motor activity are minimal. Multiple NMDAR-related behaviours such as pup retrieval, spatial and social memory, prepulse inhibition and night-time activity are also impaired. Importantly, similar NMDAR-related synaptic and behavioural deficits (albeit in milder forms than in NfL-null mice) are seen in *NEFL*$^{+/-}$ 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, neurofilament 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).

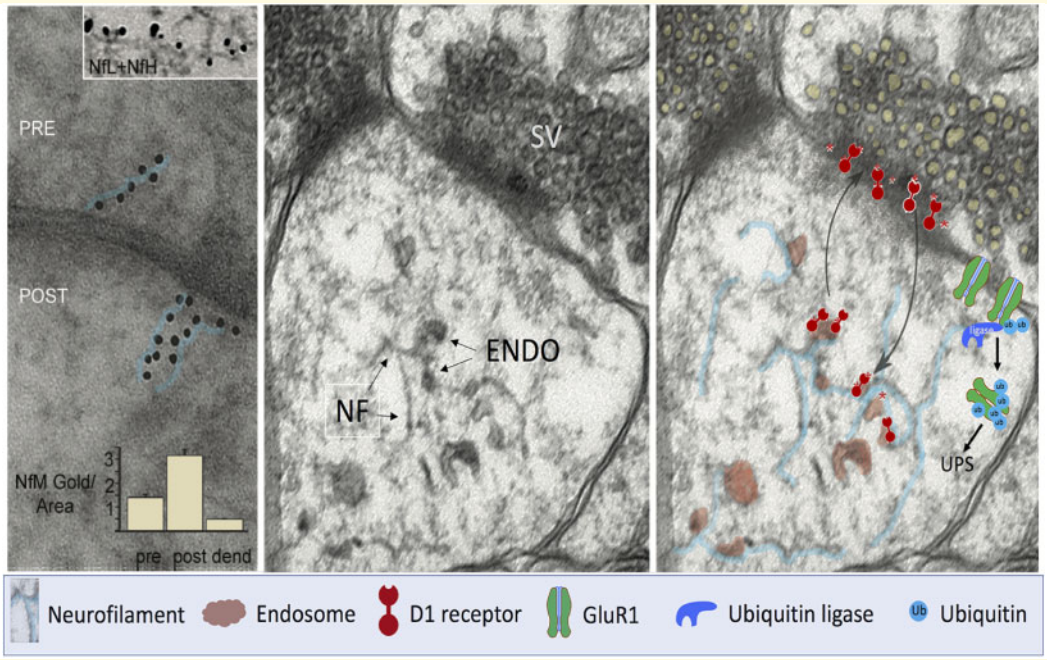

Figure 4 Functions of neurofilament subunit assemblies in synapses. *Left*: Immunogold labelled antibodies against the NfM subunit decorating mouse brain synaptic structures in a linear pattern (immunogold particles outlined in blue) suggest the presence of short neurofilaments and protofilament/protofibrils. In the *top 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. *Middle*: Ultrastructural image of a human brain synapse illustrates membranous vesicles [tentatively identified as endosomes (ENDO)], most associated with short 10-nm filaments in the postsynaptic region. *Right*: Evidence (Yuan *et al.*, 2015*a*) supports a biological mechanism whereby D1Rs 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.*, 2018*a*) 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. The key below the figure identifies the depicted cellular elements that are depicted. NF = neurofilament; POST = postsynaptic; PRE = presynaptic; SV = synaptic vesicles; UPS = ubiquitin proteasome system.

Different neurofilament subunits likely have distinct roles in synaptic function. NfM co-localizes with the G-protein-coupled dopamine D1 receptor (D1R) in synaptic boutons (Girault and Greengard, 2004) and the deletion of NfM but not any other neurofilament subunit causes postsynaptic D1Rs to redistribute from a reserve pool on endosomes to the synaptic plasma membrane, which significantly increases D1R-stimulated hippocampal LTP and greatly amplifies dopamine D1R-mediated motor responses to cocaine (Yuan *et al.*, 2015*b*). Furthermore, deletion of the *NEFM* gene in mice enhances D1R-mediated motor responses to cocaine (Yuan *et al.*, 2015*b*). The lack of NfM leads to a redistribution of postsynaptic D1Rs from endosomes to plasma membrane, implying that NfM is playing a role in the recycling of the D1R. *NEFM* deletion also inhibits the desensitization response to cocaine and amphetamine, while enhancing and prolonging ERK activation and ERK mediated NfM phosphorylation. 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.*, 2015*b*).

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.*, 2018*a*). Interactions between D1R and NMDA receptors are facilitated through neurofilament subunit assemblies (Yuan *et al.*, 2015*b*, 2018*b*). 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.*, 2018*b*). Thus, the known functional interdependence of these two distinct receptor complexes appears

to depend on a synaptic scaffold containing assemblies of multiple neurofilament subunits.

Degradation and turnover of neurofilaments

Mechanisms responsible for the turnover of neurofilaments are poorly understood. Neurofilaments 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 neurofilaments quantitatively, Millecamps *et al.* (2007) generated mice with the human *NEFL* transgene under doxycycline control in the presence or absence of endogenous mouse NfL proteins. In these mouse models, although the human *NEFL* mRNA expression was turned off 1 week after administration of doxycycline, the human NfL proteins persisted with a half-life of ~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 neurofilament proteins estimated from the decay of ^{3}H-proline radiolabelling proteins in mouse retinal ganglion cell neurons (Nixon and Logvinenko, 1986; Rao *et al.*, 2012).

Studies with conditional *NEFL* transgene suppression revealed that the turnover of neurofilament proteins is slower in large-calibre axons of the PNS having a high content of neurofilaments: human NfL protein levels expressed in transgenic mouse sciatic nerves were unchanged even after 3 months of suppression of the human *NEFL* transgene transcription by doxycycline treatment (Millecamps *et al.*, 2007). Neurofilament proteins might last several months or even years in large axons with dense neurofilament 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 neurofilament network, these results support the notion that a stationary neurofilament network in axons (Nixon and Logvinenko, 1986) contributes to slowing both the turnover of neurofilament and its net transport. The local neurofilament density thus is a key determinant of the half-life of neurofilament proteins in axons.

Phosphorylation of neurofilaments, which causes their dissociation from the kinesin motor for incorporation into stable cytoskeletal networks in axons (Yabe *et al.*, 2000), is one molecular mechanism by which this resistance to degradation is conferred (Yuan *et al.*, 2017); proteolysis of neurofilaments is increased in NF-(H/M)$^{tail\Delta}$ mice, in which the heavily phosphorylated carboxyl-terminal tail domains of NfH and NfL are deleted (Rao *et al.*, 2012). Extrapolation from these observations suggests that the degradation of neurofilaments may be regulated differentially in different types of neurons, during different developmental stages and depending on whether an axon is myelinated.

Various proteases can contribute to neurofilament proteolysis (Perrot *et al.*, 2008). The calcium-activated proteases have a high degree of substrate specificity for intermediate filaments. Calpain is capable of a limited proteolysis of neurofilaments. One provocative study, still to be replicated to our knowledge, proposed that neurofilament 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 as in growth cone formation. Other non-specific proteases can also trigger neurofilament turnover and generate neurofilament peptides (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 neurofilaments have come from the study of rare, inherited genetic diseases characterized by prominent abnormal neurofilament accumulation in axons (Table 1): ARSACS (Engert *et al.*, 2000), the early onset CMT type 2R (Ylikallio *et al.*, 2013) and 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). Accumulation and abnormal bundling of neurofilaments is the most prominent neuropathological finding in affected neurons, which include Purkinje cells (Lariviere *et al.*, 2015). 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). There is also altered distribution of organelles (including autophagosomes and lysosomes), displacement of the nucleus (Duncan *et al.*, 2017) and mitochondrial pathology (Girard *et al.*, 2012; Lariviere *et al.*, 2015; 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 neurofilament 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 neurofilament, whereas SIRPT1 and HEPN domain peptides promoted neurofilament 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 neurofilament 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 organizing neurofilament proteins and in regulating subunit levels,

assembly, maturation of their supramolecular structures and turnover (Engert *et al.*, 2000).

Mutations in *TRIM2* (tripartite motif containing 2) cause a rare 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 neurofilaments 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 neurofilaments 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 neurofilaments are found throughout the PNS and CNS (Asbury *et al.*, 1972). Begining early in infancy, the disease is associated with progressive loss of motor and sensory function (Kuhlenbaumer *et al.*, 1993; 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 intermediate filaments (Prineas *et al.*, 1976). The neurofilament 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 intermediate filaments 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 intermediate filaments network.

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) through interactions between its BTB domain and the Cul3 subunit of E3 ubiquitin ligase complexes (Furukawa *et al.*, 2003). Through interaction with the Kelch domain, gigaxonin is predicted to target its partners for ubiquitin-mediated degradation. Among putative partners identified by mass spectrometry approaches or double screening in yeast (Johnson-Kerner *et al.*, 2015*b*), intermediate filaments are so far the major biological targets for gigaxonin, as confirmed in cellular and animal models of the pathology. Indeed, numerous types of intermediate filaments 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 pluripotent stem cells (Johnson-Kerner *et al.*, 2015*a*), and vimentin, NfL, NfM, NfH and α-internexin in two different GAN mouse models (Dequen *et al.*, 2008; Ganay *et al.*, 2011). While 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 intermediate filaments throughout the PNS and CNS. The putative role of gigaxonin in regulating the steady state intermediate filament levels also has been demonstrated compellingly *in vitro*: lentiviral over-expression of gigaxonin was sufficient to drive the clearance of multiple wild-type intermediate filaments (vimentin, peripherin and NfL) and intermediate filaments bundles (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 intermediate filament 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 intermediate filament family (Bomont, 2016).

Gigaxonin appears to be the only E3 ligase able to target neurofilaments (and intermediate filament proteins more generally) for degradation. However, several questions remain to be answered. What forms of intermediate filament (e.g. short intermediate filaments, 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 neurofilament 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 intermediate filaments upon 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 (Mahammad *et al.*, 2013) and, as the observations above suggest, this may contribute to neurofilament turnover mediated by proteins such as TRIM2 or gigaxonin. 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 additional possibility that the autophagy pathway may also play a role in neurofilament turnover; activation of autophagy is accompanied by reduced neurofilament levels (Chen *et al.*, 2013).

Release and clearance of neurofilament peptides and proteins

While the nature of the neurofilament 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 neurofilament detected in the CSF or peripheral blood compartments are peptides generated from partial degradation of neurofilament in the

neuron. With injury to peripheral nerves, these might be expected to arise from axons, but, major contributions from synaptic neurofilaments 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 neurofilament 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 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 (100–2000 nm 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). By contrast, neuronal multivesicular bodies 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 ageing (Nakadate *et al.*, 2006), where they are associated with enhanced autophagy (Truant *et al.*, 2008). Microvesicle production can also increase with higher intracellular [Ca^{2+}] (as with excitotoxic injury), cell stress or with inflammation (Sproviero *et al.*, 2018). Upregulation of molecular chaperones rescues the neurofilament phenotype in *SACS* knockout neurons (Gentil *et al.*, 2019) 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 neurofilament proteins accessible to secretory mechanisms such multivesicular bodies (Manek *et al.*, 2018).

Pathways for degradation [e.g. proteasomes, 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 could lead to variable relative levels of different types of neurofilament peptide fragments in blood or CSF. Quantitative interpretations of the relative concentrations of neurofilament 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 neurofilament traffic between parenchymal, CSF and blood compartments also are unknown. However, the apparently general pathways by which large molecules such as amyloid-β pass from the interstitial fluid (ISF) of the brain into the CSF and blood suggest a tentative model for how neurofilament 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 neurofilament 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. Soluble tracers such as radioionodated serum albumin (RISA) injected into the ISF of the brain drain to cervical lymph nodes along the walls of cerebral arteries (Szentistvanyi *et al.*, 1984). This drainage occurs initially along basement membranes that surround capillaries 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) (Fig. 5).

IPAD might 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.*, 1984; Carare *et al.*, 2008; Albargothy *et al.*, 2018). With impaired IPAD, tracer labelled protein injected intracerebrally also 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 ageing or pathology. For example, age-related changes in artery walls (Hawkes *et al.*, 2011) impair IPAD and appear to be a factor limiting elimination of amyloid-β from the ageing brain in the genesis of Alzheimer's disease (e.g. reflected by the accumulation of amyloid-β within the IPAD pathways in cerebral amyloid angiopathy) (Weller *et al.*, 2015; Keable *et al.*, 2016).

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.*, 1984; McIntee *et al.*, 2016); 85% of the ISF passes to cervical lymph nodes via IPAD (Szentistvanyi *et al.*, 1984). As yet, there are no direct measurements of the proportion of neurofilament released from the brain that reaches the CSF. It also is not known whether there is any regional neuroanatomical selectivity for this.

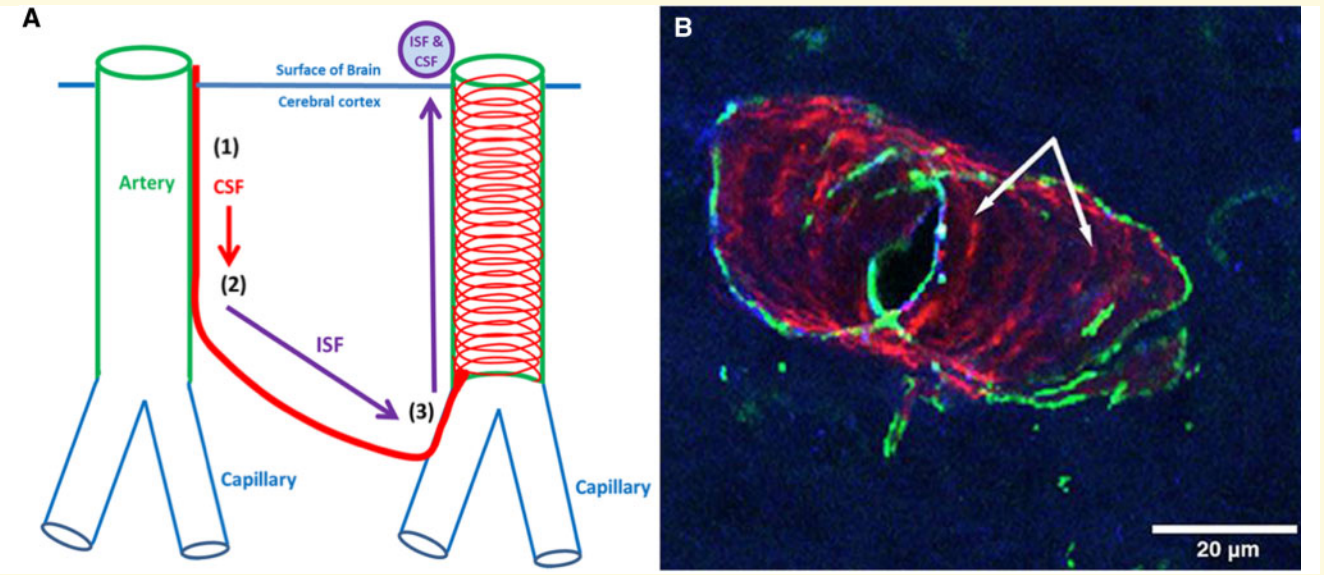

Figure 5 Fluid balance in the brain and the IPAD pathway. Entry and drainage of fluid into and from the brain is along basement membranes associated with the walls of arteries: (1) CSF enters the surface of the brain along pial-glial basement membranes on the outer aspects of cortical arteries; (2) CSF mixes with ISF; to then, (3) leave the brain along IPAD pathways. (**B**) A length of cerebral artery in a mouse brain showing fluorescent amyloid-β protein co-localizing (magenta) 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 amyloid-β is draining out of the brain. The IPAD pathway for amyloid-β and, by inference, perhaps that for neurofilament peptides, forms a spiral pattern along the artery wall (smooth muscle cells in the section of artery wall illustrated are stained green). This figure is modified from an original figure reproduced as Fig. 1d of Albargothy *et al.* (2018).

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 cribriform 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 inferences regarding the extent or severity of CNS neuronal pathology based on neurofilament concentration measures in CSF or peripheral blood. The relationships between neurofilament concentrations in CSF and peripheral blood may be influenced by the rate of release of neurofilament species from the injured or degenerating neuron, where it is occurring in the CNS and variation in the kinetics of clearance related to ageing or direct effects of pathology on the clearance mechanisms themselves.

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

Data defining the dynamics of neurofilament turnover in healthy individuals, with ageing and in those with diseases associated with increased neurofilament peptide concentrations in peripheral blood or CSF are needed. Although not yet applied to these problems, a promising approach for obtaining precise estimates of the kinetics of synthesis and elimination of neurofilament 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-β, APOE and tau in Alzheimer's disease and 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. ^{2}H (deuterium) or ^{13}C]-labelled amino acids. These 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 (~20 days) than do membrane proteins such as amyloid-β (~10 h) (Bateman *et al.*, 2006; Sato *et al.*, 2018).

Based on this observation and rodent data demonstrating very slow turnover of neurofilaments incorporated into filamentous structures in the axon (Nixon and Logvinenko, 1986), SILK neurofilament studies may require the use of a SILK protocol specialized 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; and (iii) a mass spectrometry assay that is sufficiently sensitive to detect the low fraction of tracer incorporated into neurofilament present in biofluid has to be developed.

The last point is likely a major technical hurdle for the analysis of neurofilament peptides because of their low concentrations in CSF (1–10 ng/ml range) and peripheral blood (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 neurofilaments in CSF or peripheral blood have not yet been reported; neurofilament 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 mass spectrometry mode (Gallien *et al.*, 2012; Peterson *et al.*, 2012; Gillette and Carr, 2013). Identifying antibodies that efficiently recover a representative range of neurofilament peptides will be a prerequisite. Development of such a mass spectrometric assay would pay dividends by enabling further neurofilament peptide characterization to test for differences in the nature of the species present in CSF or peripheral blood with ageing or diseases and characterizing post-translational modifications.

Table 2 Major disorders reported to have associations with increased NfL concentration in peripheral blood

Clinical condition	Key references
Ageing	Disanto *et al.*, 2017; Mattsson *et al.*, 2017; Barro *et al.*, 2018
Peripheral neuropathies	
CMT	Sandelius *et al.*, 2018
Guillain-Barré syndrome	Petzold *et al.*, 2006
Chronic inflammatory demyelinating polyneuropathy	van Lieverloo *et al.*, 2019
Multiple sclerosis	Lycke *et al.*, 1998; Malmestrom *et al.*, 2003; Norgren *et al.*, 2004; Teunissen *et al.*, 2009; Gunnarsson *et al.*, 2011; Kuhle *et al.*, 2016*b*, 2017
ALS	Lu *et al.*, 2015; Steinacker *et al.*, 2016
Dementia	
Preclinical Alzheimer's disease	Weston *et al.*, 2017, 2019; Preische *et al.*, 2019
Alzheimer's disease	Mattsson *et al.*, 2017
Frontotemporal dementia	Meeter *et al.*, 2016
Atypical parkinsonian disorders (e.g. progressive supranuclear palsy)	Rojas *et al.*, 2016; Donker Kaat *et al.*, 2018
Stroke	
Subarachnoid haemorrhage	Nylen *et al.*, 2006; Zanier *et al.*, 2011
Ischaemic stroke	Gattringer *et al.*, 2017
Traumatic brain injury	Shahim *et al.*, 2016, 2017
Huntington's disease	Byrne *et al.*, 2017; Johnson *et al.*, 2018
Neuropsychiatric conditions	
Bipolar disorder	Jakobsson *et al.*, 2014
Spinal muscular atrophy	Darras *et al.*, 2019

Evolution of NfL immunoassays

Interest in neurofilaments as a soluble biomarker of disease and its progression have risen dramatically in recent years, driven in part by evolution of the assay and the associated improvements in analytical sensitivity (Kuhle *et al.*, 2016*a*). Improved analytical methods for detecting NfL or its constituent peptides in CSF and blood have enabled strong associations to be demonstrated 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 (~25–50 ng/l) precludes its general use for measurement of NfL in peripheral blood.

Advances in technology have enabled major extensions of the range of applications possible. Semi-sensitive electrochemiluminescence detection was the first approach that allowed disease-related increases in peripheral blood concentration to be measured in samples from patients with ALS (Gaiottino *et al.*, 2013) or active multiple sclerosis (Kuhle *et al.*, 2016*a*). 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 peripheral blood to be measured reliably even in people without PNS or CNS pathology. For the first time, correlations between CSF and peripheral blood levels of NfL could be demonstrated using this assay in patients with HIV encephalopathy (Gisslen *et al.*,

2016). Paired CSF and peripheral blood measures showed similar dynamics following acute brain injury, suggesting relatively rapid trafficking between the compartments; concentrations of NfL reach a maximum around 40–70 days post-injury in both CSF and peripheral blood and normalize at similar rates within about 6 months (Bergman *et al.*, 2016). However, in conditions in which central and peripheral neuronal injury or degeneration could be found, specific interpretations of blood measures in terms of these distinct pathologies may be ambiguous. Given that α-internexin is CNS-specific intermediate filament, a blood-based assay could potentially help distinguish CNS-specific pathologyin cases where the disease is associated with altered levels of this specific protein (Shaw, 2015). Whether neurofilament 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.

Neurofilament peptide concentrations as biomarkers of brain injury or neurodegeneration

CSF and peripheral blood NfL are increased in most acute and chronic CNS diseases characterized by neuronal damage (Table 2) and correlate with longitudinal imaging findings of neurodegeneration (Khalil *et al.*, 2018). Here, we briefly review applications of the assay to neuroinflammatory injury and neurodegeneration with specific reference to diseases where this approach has been most extensively researched. 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 (Gaiottino *et al.*, 2013); CSF findings with a range of neurodegenerative diseases (increased NfL concentrations in Alzheimer's disease, frontotemporal dementia, vascular dementia and atypical parkinsonian disorders) have been replicated in peripheral blood (Zetterberg, 2016).

The potential utility of NfL as a biomarker of neuroinflammatory injury is well-illustrated by applications in multiple sclerosis, in which NfL assay results are being used as evidence routinely in some centres for continuing inflammatory disease in diagnosis, treatment monitoring or estimating prognosis if treatment is not changed. These assays also are increasingly integrated as secondary or exploratory measures in clinical trials (Sormani *et al.*, 2019). Increased levels of NfH and NfL were first described in the CSF of patients with multiple sclerosis (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 neurofilament concentrations tend to be increased across all clinical stages of multiple sclerosis (even in the absence of evidence for active inflammatory activity evident as a clinical relapse or detected using MRI) relative to healthy volunteer groups (Teunissen *et al.*, 2009; Kuhle *et al.*, 2011). The chronically increased levels detected with multiple sclerosis are 3–5-fold greater than those reported for some primary neurodegenerative diseases (e.g. frontotemporal dementia or ALS) (Gaiottino *et al.*, 2013). With the introduction of the more sensitive SIMOA technology, peripheral blood 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 multiple sclerosis 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 magnetic resonance-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). However, there also are important limitations to the use of NfL concentrations in CSF or peripheral blood for disease monitoring of individual patients with multiple sclerosis (Berger and Stuve, 2019). For example, the lower levels of NfL that are found in most patients with multiple sclerosis outside of periods of acute inflammation still generally cannot be confidently interpreted as pathological if obtained as single time-point measurements (Kuhle *et al.*, 2016*b*). While the evidence is still limited, serial peripheral blood NfL concentration measurements appear to be as sensitive as MRI for the assessment of treatment effects (Gasperini *et al.*, 2019). Increases in peripheral blood (or CSF) NfL also might provide a potential biomarker of suboptimally controlled acute inflammatory activity in individuals 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 individuals with progressive forms of multiple sclerosis for treatment with new, highly active anti-inflammatory treatments (https://www.nice.org.uk/guidance/TA585). An analogous application is emerging for the management of children with type I spinal muscular atrophy (SMA) who are being treated with nusinersin and in whom CSF NfL provides a measure of the effectiveness of treatment (Olsson *et al.*, 2019). Other conditions characterized by neuronal injury may also result in raised NfL measurements and therefore the clinical context or presentation must be borne in mind for their interpretation. For example, following hypoxic or traumatic brain injuries, NfL concentration increases within days, reaching a maximum weeks following the injury followed by

normalization of concentration within 6–12 months (Shahim *et al.*, 2016).

Neurofilaments are also increased in neurodegenerative diseases and neuropathies and may inform the clinical picture at different stages of disease. For example, increased serum NfL concentrations distinguished both symptomatic and presymptomatic causal gene mutation carriers from healthy controls in familial Alzheimer's disease (Weston *et al.*, 2017, 2019; Preische *et al.*, 2019) and Huntington's disease (Byrne *et al.*, 2017), but also correlate with longitudinal measures of neurodegeneration in sporadic Alzheimer's disease (Mattsson *et al.*, 2017), frontotemporal dementia (Meeter *et al.*, 2016), progressive supranuclear palsy (Donker Kaat *et al.*, 2018) and Huntington's disease (Johnson *et al.*, 2018). Peripheral neuropathies also are associated with increased blood NfL concentrations; NfL concentrations in peripheral blood are higher (almost doubled) in patients with CMT peripheral neuropathy compared with healthy controls and correlate with disease severity as measured using clinical rating scales (Sandelius *et al.*, 2018). However, just as with brain injury and neurodegeneration, the increased blood NfL must be contextualized for clinical interpretation of its significance with peripheral neuropathies. NfL biomarker concentrations do not differentiate between inflammatory and genetic peripheral neuropathies, for example.

There are other limitations to the use of neurofilament as a clinical biomarker. There is a ~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 standardized, age-corrected, normative distributions of NfL in CSF and peripheral blood are not available to define values from individual subjects as being pathological. Measures of NfL concentrations in CSF or peripheral blood 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. relevant comorbidities) (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 harmonized 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 harmonization 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, organization and function in the central and peripheral nervous systems. Primary roles of neurofilament 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 among the latter are disorders arising from impairments in normal mechanisms for neurofilament degradation that are associated with progressive and severe axonal pathology. However, fundamental questions still remain concerning basic mechanisms regulating neurofilament expression, assembly and turnover.

The identification of functional roles for synaptic neurofilament in modulation of excitatory glutamatergic activity (Huntley *et al.*, 1994; Ratnam and Teichberg, 2005; Yuan *et al.*, 2018*a*) has opened an entirely new range of investigations of neurofilament 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 neurofilament to contribute to the genesis of neuropsychiatric diseases more generally (Yuan *et al.*, 2018*a*). Although the disease relevance is still speculative, the importance of the question and the novelty of this neurobiology make better understanding of the structurally unique synaptic neurofilament, its organization and its functions a priority.

However, understanding how neurofilaments 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 neurofilaments in CSF and peripheral blood as biomarkers. Physiological mechanisms for more dynamic control through post-translational modifications need to be better defined. For example, how is neurofilament phosphorylation regulated relative to the targeting of neurofilament for E3 ligase and protease activity? Exactly what forms of neurofilament (short intermediate filaments, unit length filaments, tetramers or dimers) are the major substrates for degradation with normal turnover? Applications of neurofilament measures for assessment of pathology demand some appreciation for whether these mechanisms of neurofilament degradation and release are altered with disease or injury. We hypothesize 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 neurofilaments (particularly NfL) in CSF or peripheral blood have allowed exploration of how levels increase with ageing, brain injury and neurodegenerative diseases. These suggest that neurofilament concentration measures in these compartments have potential clinical utility as indices of peripheral or central nerve damage with trauma (Shahim *et al.*, 2016) or neurodegeneration (Weston *et al.*, 2017, 2019; Preische *et al.*, 2019). Serial measures can be sensitive to subclinical disease activity in multiple sclerosis 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 and treatment response when other clinical or imaging evidence is lacking. They may play a role in better estimating prognosis for patients or patient groups, as well.

However, at this point, increased NfL measures in CSF or peripheral blood 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 peripheral blood measures of NfL also can be uncertain when both CNS and PNS 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 neurofilament 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 emphasize the need for some caution in interpretation of neurofilament measures in peripheral blood or CSF from individual patients. At the same time, they also highlight why there is currently such excitement among those interested in the neurobiology of intermediate filaments. Moreover, with a growing range of new tools for characterizing 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 neurofilament better, all highlight both the clinical promise for NfL as a biomarker and the great potential for future investigation of the neurobiology of NfL and intermediate filaments more generally.

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Competing interests

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